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Mark Jesus M. Magbanua
John W. Park *Editors*

Circulating Tumor Cells

Methods and Protocols

Humana Press

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Circulating Tumor Cells

Methods and Protocols

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Dedication

In memory of Teresita Mendoza Magbanua, and all the women and men we have lost to cancer.

Foreword

Treatment options for cancer patients are rapidly increasing, especially those targeting specific molecules in cancer cells. For targeted therapies the presence and distribution of the targets on the cancer cells will need to be known to assess the potential effectiveness of the drug. Availability of tumor material before administration of therapy is therefore essential. A biopsy from a metastatic site is however not always feasible and may not be representative of all other metastatic sites. Tumor cells from the primary tumor or metastatic sites, disseminated into the blood, may well provide a better representation of the tumor. These circulating tumor cells (CTCs) are however extremely rare and the number of CTCs may not be sufficient to provide a good representation of the tumor. The quest for these CTCs is ongoing for a long time as visualized in Fig. 1 by the number of research articles that appeared during the years of my lifetime. The first demonstration that CTCs could be reliably enumerated and that their presence was associated with poor clinical outcome for patients with metastatic breast cancer came in 2004 with the introduction of the CellSearch® system [1]. Subsequent studies showed a strong relationship between CTC load in blood and progression free and overall survival in both the metastatic and nonmetastatic setting in various types of carcinomas. The original results have been confirmed and validated in numerous studies since the original 2004 publications. These results increased the awareness that "*a liquid real-time biopsy*" for cancer patients was within reach and a large group of researchers from different fields of expertise are now engaged to improve the isolation of CTC and extract potential clinically relevant information from these CTCs.

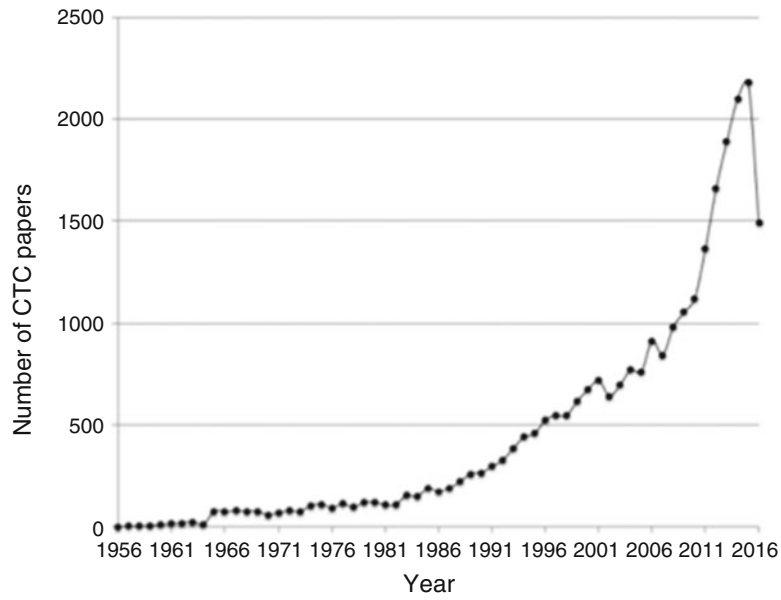

Fig. 1 27,280 publications that appeared from 1956 up to November 2016 with "Circulating Tumor Cells" in title, abstract, or as key word (Scopus search)

A glimpse of recently introduced technologies to isolate and characterize CTC is presented in the chapters of this book. CTCs are being isolated based on differences in immunophenotype and/or physical properties such as size, deformability, and dielectric properties from the other blood components. The challenge of all technologies is how to prove that the identified objects are indeed tumor cells and whether or not the information pertinent for therapy can be extracted. The difficulty in development of CTC assays is that one does not know how many tumor cells are present in a blood sample. Spiking of cells derived from tumor cell lines is useful to test the analytical performance of the system, but it is not a surrogate of tumor cells in cancer patients. Another hurdle is the extreme heterogeneity of the morphology of CTCs making it difficult what objects to assign as CTCs and which one not. Figure 2 illustrates the heterogeneity and relative frequency of CTC subsets observed in the blood of metastatic cancer patients with the CellSearch system. The number of CTCs reported greatly depends on where to draw the cut-off of what objects are considered CTCs. In metastatic prostate cancer patients the presence of all these CTC subsets was associated with poor clinical outcome [2]. Whether or not this observation holds up for other cancers and with other technologies considering EpCAM+ as well as EpCAM- CTC is still an open question. More important may well be whether or not information can be extracted from the CTC subsets that can predict response to a specific

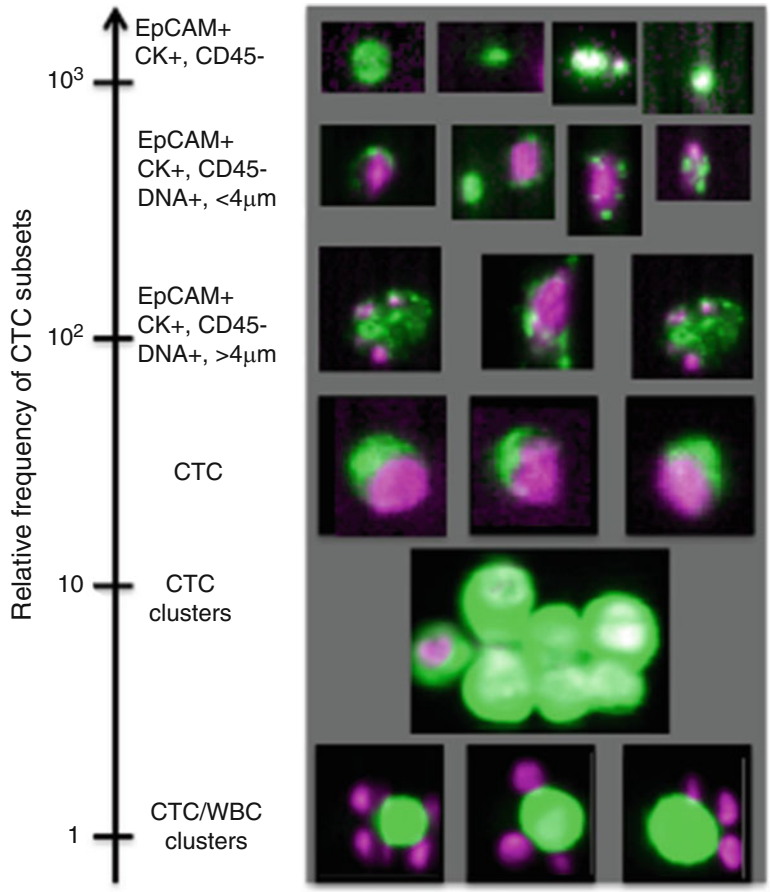

Fig. 2 Relative frequency of subsets of CTC (green=cytokeratin, purple =DNA) identified by CellSearch

treatment. A glance at the images in Fig. 2 is sufficient to deduce that the chance of extracting treatment-relevant information will be better for the intact tumor cells as compared to the CTCs undergoing apoptosis or the tumor-derived extracellular vesicles.

Although the preference will be to have tumor cells for a liquid biopsy, they may not be available in sufficient enough numbers. In these cases, the availability of tumor-derived extracellular vesicles and/or tumor-related proteins, DNA and RNA in plasma, is a good alternative. A concerted effort to validate different methods to extract treatment-relevant information from CTCs, plasma DNA and RNA, has been initiated in Europe by the CANCER-ID consortium (http://www.cancer-id.eu/). As part of this project the open-source imaging program baptized as ACCEPT is under development to enable comparison of CTCs identified with different platforms and quantify the number of treatment-related antigens [3]. The availability of tools that enable comparisons of the results obtained with different CTC platforms will hopefully expedite the enormous efforts ongoing worldwide to extract the information from CTCs and provide the information needed to guide therapy.

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Preface

Circulating tumor cells (CTCs) provide unique opportunities for real-time monitoring of disease progression and response to treatment. Molecular characterization of CTCs may offer clues to understand how cancers develop resistance to treatment and spread to distant organs. Formidable technical and technological challenges had to be overcome to reliably detect and characterize these extremely rare tumor cells in the blood. Efforts towards developing novel technologies with greater sensitivities to detect CTCs, including EPCAM-independent approaches, as well as techniques for characterization of these cells have significantly grown in the past few years. This book outlines different protocols for enrichment, detection, isolation, and molecular profiling of CTCs, contributed by key leaders in the field. Comprehensive and authoritative, our book will serve as a valuable resource for laboratory researchers and clinicians who are interested in pursuing CTC research.

San Francisco, CA, USA

Mark Jesus M. Magbanua
John W. Park

Acknowledgments

The editors thank Feng Hsiao and Tulasi Solanki for their assistance in reviewing manuscripts and for preparing this book for publication. We also thank all the contributors for their highly informative chapters, without which this book project would have not been possible. M.J. Magbanua acknowledges the Breast Cancer Research Foundation for funding support.

About the Cover

Fluorescence in situ hybridization analysis in circulating tumor cells from castration-resistant prostate cancer showing androgen receptor (*AR*) amplification with highly clustered AR gene signals (red) and normal copy number of the centromere X reference locus (green).

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Chapter 1

Microfluidic Capture and Multiplex Immunofluorescence of Circulating Tumor Cells to Identify Cancer of Origin

Chian-Hui Lai and Ying-Chih Chang

Abstract

Circulating tumor cells (CTCs) are an important biomarker and their analysis can be considered a form of "liquid biopsy." The purpose of this book chapter is to describe the use of the 4-channel CMx (cells captured in maximum) microfluidic chip, containing special micropatterns coated with an antibody-conjugated supported lipid bilayer (SLB) on its surface, to capture and isolate CTCs from the blood of cancer patients. Captured CTCs are subsequently released by an air foam to an immunofluorescence (IF) staining panel that enables further analysis, including the identification of the primary cancer source of the CTCs.

Key words Circulating tumor cells (CTCs), Supported lipid bilayer (SLB), Non-fouling, Surface coating, Micropatterns, Microfluidic, Air foam, Immunofluorescence (IF) panel

1 Introduction

Cancer is a serious health issue and a major cause of human death [1, 2]. It is urgent to develop a reliable and sensitive method for the early detection of cancer and the determination of its origin in the body. Circulating tumor cells (CTCs) are cells that are shed from a primary solid tumor and transferred into the blood stream, creating the possibility of establishment of distal, secondary tumors. Because CTCs have been recognized to be a multifunctional biomarker, CTCs enable a form of "liquid biopsy" from the blood of cancer patients [3]. CTCs can provide clinically significant information for diagnosis, prognosis, and cancer therapy treatment. However, the only FDA-approved system for CTC analysis, the CELLSEARCH® System, which uses magnetic beads with immuno-affinity, has been reported to lack sensitivity to CTCs with low epithelial cell adhesion molecule (EpCAM) expression [4]. The CMx (cells captured in maximum) platform was developed to enable the capture of in the order of 1 ~ 1,000 CTCs out of billions of blood cells per 2 mL of blood in symptomatic cancer

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patients [5]. The CMx platform can detect CTCs and circulating tumor microemboli (CTM) simultaneously and with the quantity more than other reported platforms. The presence of CTMs is strongly associated with a poor prognosis [6–8]. Previously, we have reported that CTC counts, isolated by CMx, can correlate with organ-specific metastasis [9]. Building upon the CMx's stability and efficiency in capturing CTCs, we created an immunofluorescence (IF) panel by processing six CMx chips in parallel with different immunostaining antibody pairs to analyze CTCs for the identification of their primary cancer source [10].

The CMx microfluidic platform is assembled with a special, 4-channel micropattern etched in a poly(methyl methacrylate) PMMA plate on the top and an oxygen-plasma-treated glass slide on the bottom. Among various micropatterns of channels previously addressed to create different flow disturbances and increase the capture rate of CTCs (Figs. 1 and 2), type F of a 4-channel PMMA micropattern (Fig. 1b) with expanded open entrances was chosen for having the best CTC capture efficiency. We refer to the microfluidic chip containing the type F micro-pattern as the CMx chip [5]. The interior of the CMx channels was coated with an anti-EpCAM-conjugated supported lipid bilayer (SLB) which is a biomimetic material with a unique, non-fouling behavior [11–13] that enables a high capture efficiency of CTCs while not capturing large amounts of either red or white blood cells. A layer-by-layer surface assembly method was used in the channels (Fig. 3). Only 2 mL of blood is needed for an analysis in a CMx device (Fig. 4). The CMx chip showed linearity in capture efficiency (Fig. 5), and featured a method for easily releasing captured, viable CTCs. The release

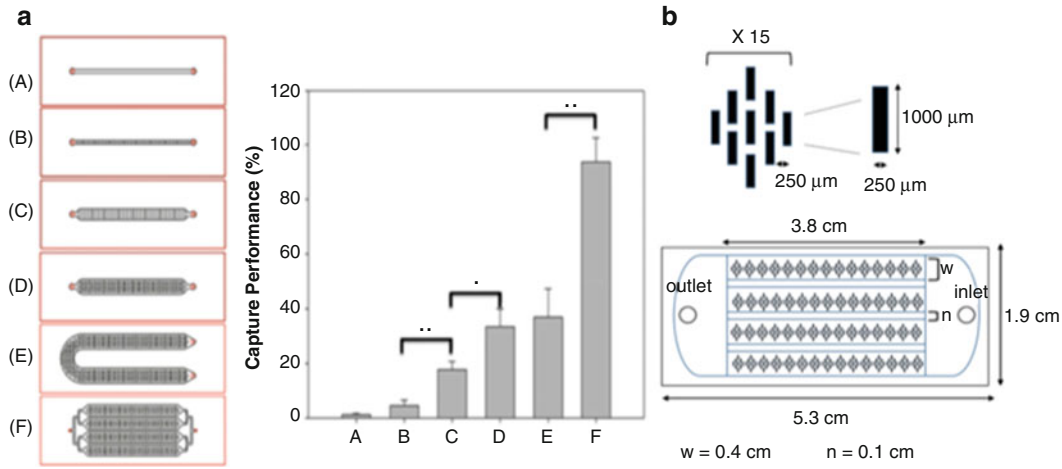

Fig. 1 (**a**) The micro-patterns for the PMMA chips and its corresponding capture efficiency; reprinted from ref. 5. (**b**) Pattern F type was chosen with slight variation of entrance design for the use of CMx chip. The micro-pattern details were shown as above

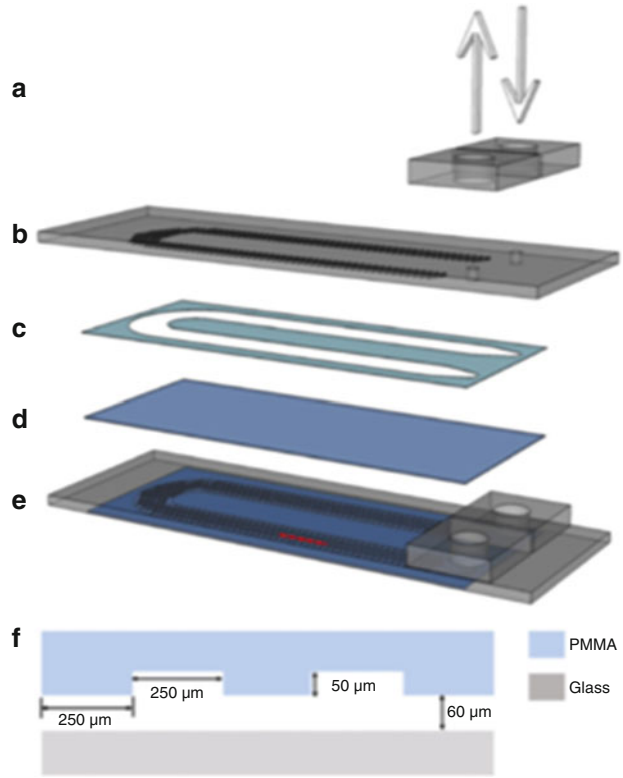

Fig. 2 An example of the assembly of a PMMA microfluidics device. Exploded view of the type E microfluidic chip: (**a**) PMMA adaptors, (**b**) a PMMA top plate, (**c**) a spacer of 3 M tape, (**d**) a bottom cover slide, and (**e**) a complete microfluidic device. (**f**) Dimension of micro-channel and microstructure. Reprinted with permission from ref. 16. Copyright AIP Publishing LLC

method uses an air foam (Figs. 6 and 7). To demonstrate the method of determining the cancer origin of CTCs in a blood sample, a 12 mL sample of blood was applied to six CMx chips for processing and evaluation in the IF staining panel. Depending on the prediction tree (Fig. 8), the IF panel can reflect the CTC characteristics and marker expression of specific cancer types, which provides the ability to identify CTC origin in clinical use, and also make CTC analysis an early cancer detection tool.

2 Materials

All solutions were prepared with ultrapure water (double-deionized water (dd-H_2O), 18 MΩ at 25 °C). Phosphate buffered saline (PBS) buffer (10 mM PBS + 150 mM NaCl, pH 7.4) solution was diluted by 10× with PBS (*see* **Note 1**).

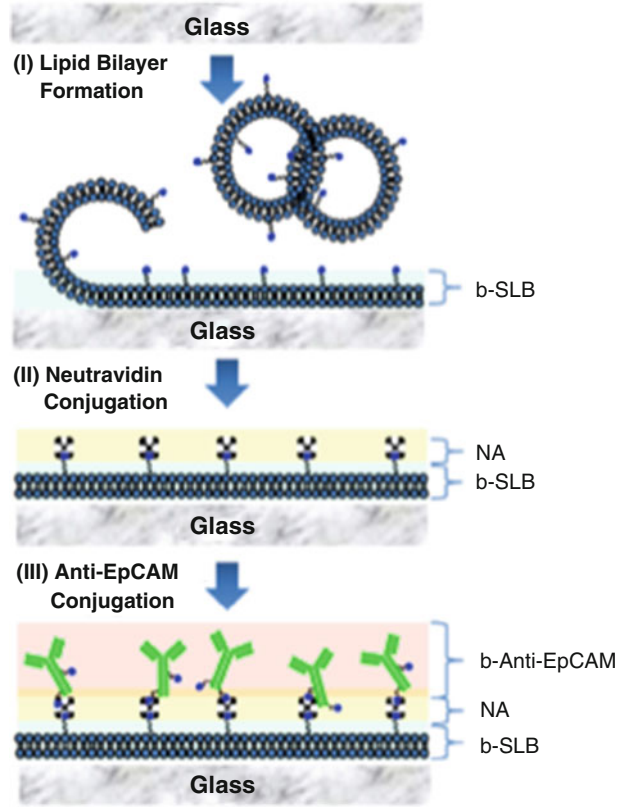

Fig. 3 Layer-by-layer molecular coating in the CMx channel. Reprinted with permission from ref. 16. Copyright AIP Publishing LLC

2.1 Equipment and Software

1. A-CO_2 laser cutting machine, V2000 Laser Scriber, 60 W (LTT Corp).
2. CorelDraw (Corel, Ottawa, Canada), used for the micro-trenched patterns and microstructure of PMMA slide, and 3M tape as well.
3. A sonicator.
4. An oxygen plasma system, AP-300 (Nordson March).
5. A syringe pump, PHD 2000 (Harvard Apparatus).
6. Nanodrop 1000 spectrophotometer (Thermo Scientific), used for determining protein concentration.
7. A LIPEX™ Extruder (Northern Lipids, Inc.), used for lipid preparation.
8. Fluorescence microscopy (cell images were taken with a Nikon-Ti Eclipse microscope at 100× magnification, and analyzed with NIS-Elements AR Analysis software, Nikon).

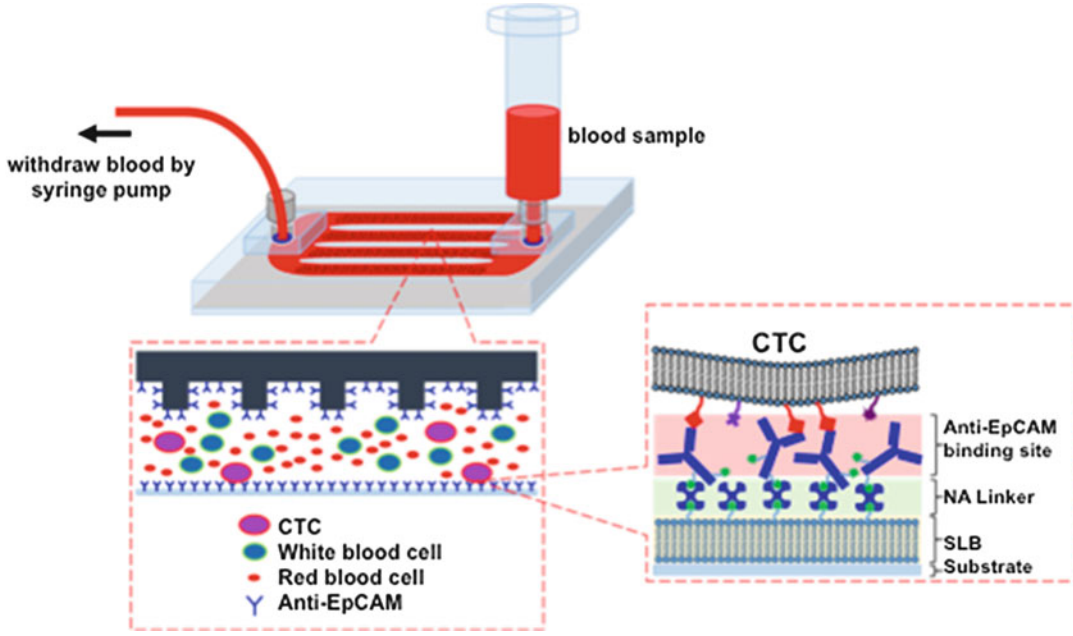

Fig. 4 Schematic of CTC capture on a CMx chip. A 2 mL blood sample is drawn from a syringe connected at the inlet of CMx chip and processed through the microfluidic channel on the CMx chip by syringe pump withdraw. CTCs can be captured by the anti-EpCAM antibody layer that is linked to the functional SLB coated on CMx inner channel. Reprinted from ref. 10

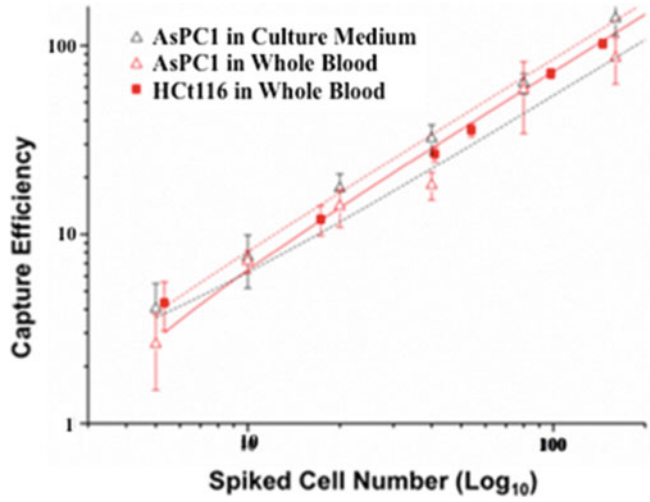

Fig. 5 The CTCs capture efficiency in spiking cell experiments of both cell culture medium and human whole blood (the spiked cell numbers are ranged from ~1 to ~1000 in 2 mL sample) showed linear relationship. Reprinted from ref. 5

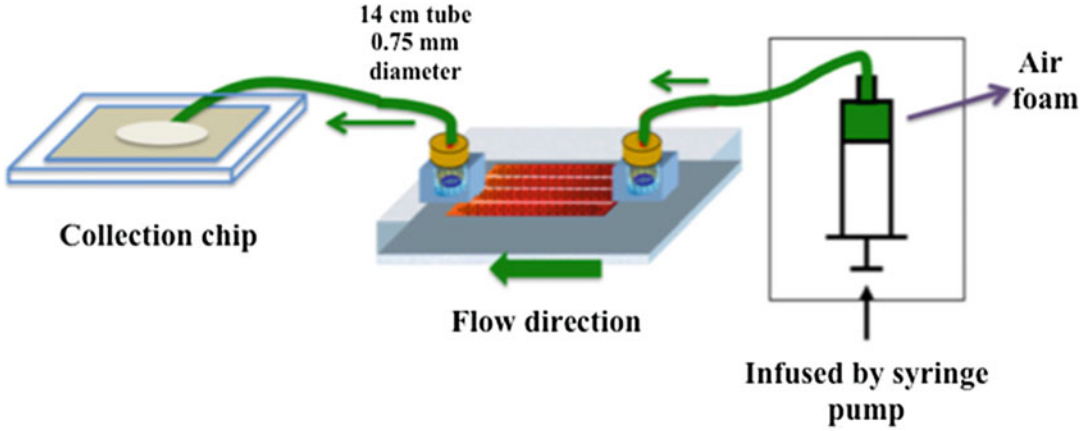

Fig. 6 Graphic illustration of release CTC by an air form went through CMx chip channels to collection chip. The collection chip with 2 μm pore size was used to concentrate the released cells from the air foam. Reprinted with permission from ref. 16. Copyright AIP Publishing LLC

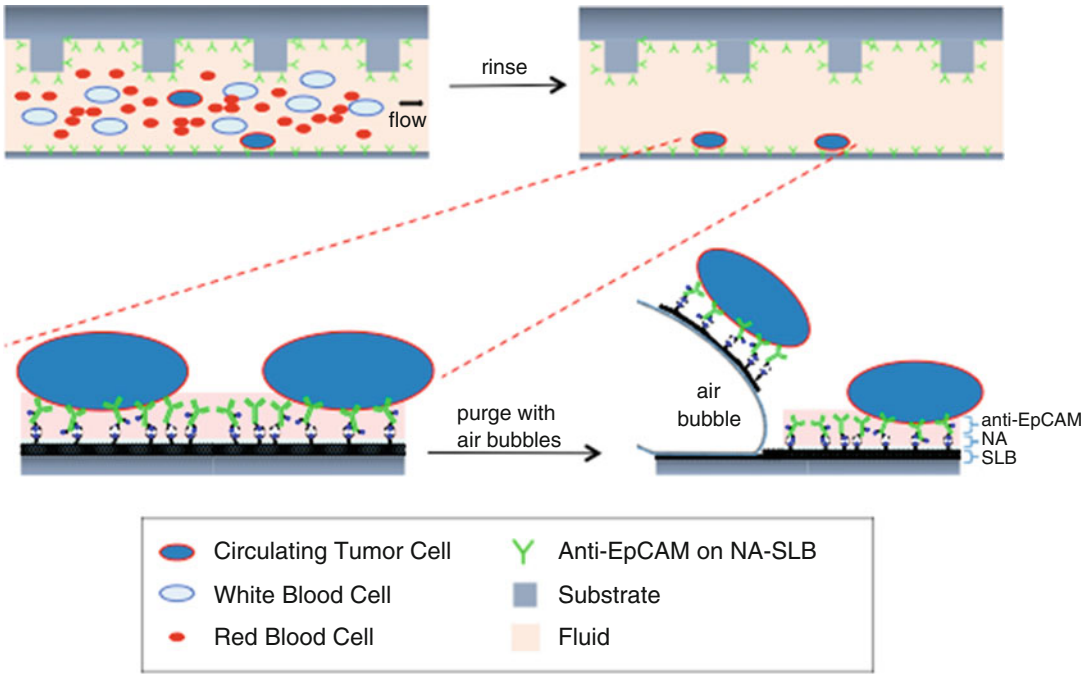

Fig. 7 CTCs release mechanism in the CMx platform. After a blood sample flowed through the microfluidic channels, the channels were then rinsed with PBS to remove excess red and white blood cells. Captured CTCs were then released by injecting an air foam into the channel, which disrupted the SLB coating without damaging the CTCs. Reprinted from ref. 10

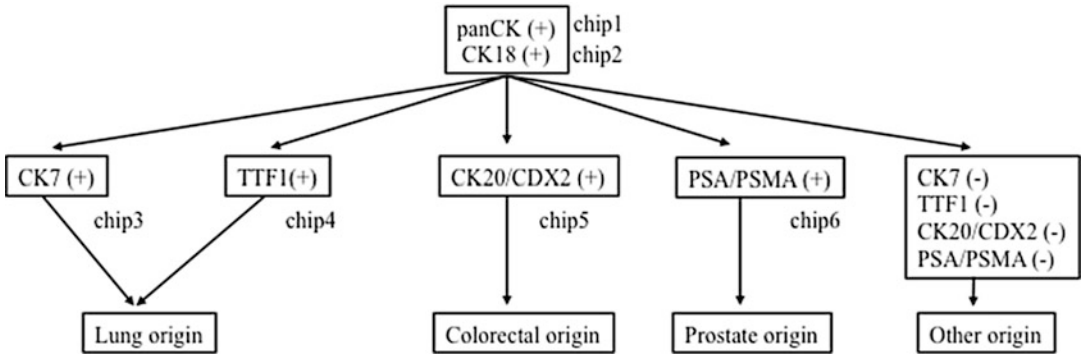

Fig. 8 Decision tree for the prediction of CTC tissue origin. CTCs from lung cancer origin could be identified in chip1/chip2/chip3/chip4. CTCs from colorectal cancer could be identified in chip1/chip2/chip5. CTCs from prostate cancer could be identified in chip1/chip2/chip6. Additionally, CTCs of tissue origins other than lung, colorectal, and prostate could be identified in chip1/chip2. Reprinted from ref. 10

2.2 Etching of PMMA Chip and Chip Base Materials (See Note 2)

1. 2-mm-thick poly(methyl methacrylate) (PMMA) slides used for the base of microfluidics chip (*see* **Note 3**).
2. A 63-μm-thick double-sided adhesive tape (8018PT; 3 M Corp) (*see* **Note 3**) [5].
3. PMMA chip adaptors and connectors (Upchurch Scientific).
4. A bottom cover slide (microscopy cover slide 24 × 60 mm, *see* **Note 4**).

2.3 Vesicle Lipid for Supported Lipid by Layer (SLB) on Chip

1. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids).
2. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-cap-biotinyl (b-PE, Avanti Polar Lipids).
3. 50 nm and 100 nm Nuclepore® track-etched polycarbonate membranes (Whatman Schleicher & Schuell).
4. PBS buffer.
5. 0.125 mM NeutrAvidin (NA) in PBS.
6. Degassed, dd-H_2O.

2.4 Biotinlyated Anti-EpCAM

1. Monoclonal mouse anti-human antibody EpAb4-1 (anti-EpCAMmAb) was generated as previously reported [11, 14].
2. Sulfosuccinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin, Pierce).
3. Dialysis membrane cassettes (10 KD molecular weight).

2.5 Processing of Blood Samples or Cancer Cell Spiking Experiments

1. Plastic syringe (5 mL).
2. Needles (19 G × 11/2).
3. Teflon tubes (14 cm of length, 0.75 mm inner diameter).
4. Two HPLC adaptors (Upchurch Scientific) to link the CMx chip to the Teflon tube.

5. PBS.
6. Human colorectal cancer cell line HCT116.
7. Accutase (Merck Millipore).
8. CellTracker green 5-chlormethylfluorescein diacetate (CMFDA, Life Technologies).
9. Dulbecco's modified eagle medium (DMEM, Gibco) for cell culture with 10% FBS.
10. An automated hemocytometer (Millipore).

2.6 Release Cell

1. Two plastic syringes (5 mL).
2. A three-way stopcock.
3. 5% bovine serum albumin (BSA) in PBS.
4. 2 μm pore size, 20 mm diameter collection membrane (polycarbonate membrane, Isopore™ Membrane Filters, *see* **Note 5**) on 10 mm diameter drilled hole of the PMMA plate.
5. 16% paraformaldehyde (PFA) in PBS.
6. Eppendorf tubes.

2.7 Immunofluorescence (IF)

1. 4% and 16% PFA in PBS.
2. 0.1% Triton X-100 (Sigma-Aldrich) in PBS.
3. 1% and 10% normal goat serum (NGS, Thermo Fisher Scientific) in PBS.
4. The primary antibodies used as cancer markers are as follows: rabbit-anti-human PanCK (Abcam), rabbit-anti-human CK18 (Abcam), rabbit-anti-human CK7 (Abcam), rabbit-anti-human TTF-1 (Abcam), rabbit-anti-human CK20 (Abcam), rabbit-anti-human CDX2 (Cell Marque), rabbit-anti-human PSA (Abcam), and rabbit-anti-human PSMA (Abcam). All antibodies were diluted in 1% NGS (dilution factor 1:200) at 4 °C and ready for use.
5. Goat-anti-rabbit-647 secondary antibody (dilution factor 1:500, Life Tech.) in 1% NGS for CTC staining.
6. Anti-CD45-fluorescein isothiocyanate (FITC) antibody (dilution factor 1:10, DAKO) in 1% NGS for white blood cell (WBC) staining.
7. Prolong Gold with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) as a mounting medium.
8. Microscope cover slides.
9. Swabs and Asahi KASEI wipers.
10. Tweezers.

3 Methods

3.1 Assembly of Chip

1. The PMMA chips were cut by CO_2 laser and etched with micropatterns as shown in Fig. 1 (*see* **Note 2**). The chips were all drilled with one inlet and one outlet hole. The CMx platform contains the type F microfluidic pattern. The microfluidic pattern is shown in Fig. 1b [15].
2. 3 M tape was cut to fit the corresponding PMMA or molding chip.
3. The bases of all of the chips were assembled in a sandwich method, with the following order: PMMA chip, 3 M tape, bottom cover glass slide. The inlet and outlet of the chip were both connected with PMMA adaptors (Fig. 2 shows one example of an assembled chip).

3.2 Lipid Vesicles for SBL Surface

1. 1.9 mL of POPC (25 mg/mL in chloroform) and 1.225 mL biotin-PE (10 mg/mL in chloroform) were added to a clean glass tube to form a mixture of lipid chloroform solution. The lipid mixture chloroform solution was initially dried either under reduced pressure or under nitrogen stream and subsequently dried in a vacuum chamber overnight to remove residual chloroform (*see* **Note 6**).
2. To the dried lipid mixture in the tube was added 10 mL of PBS, which was then sonicated at 50 °C until the solution became translucent.
3. Another 10 mL of filtered PBS was added to achieve the final concentration of 3 mg/mL of lipid mixture in PBS (*see* **Note 7**).
4. 20 mL of the lipid mixture in PBS was then extruded through Nuclepore® track-etched polycarbonate membranes (100-nm, followed by 50-nm, 20 times for each membrane) under 150 psi at 50 °C, using a LIPEX™ Extruder to generate homogenous population of unilamellar vesicles (*see* **Note 8**).
5. The biotin-containing lipid vesicles were stored at 4 °C.
6. The 3 mg/mL lipid vesicles were diluted 20 times by PBS to be a concentration of 0.15 mg/mL for ready usage on CMx surface coating.

3.3 Coating the CMx Chip

1. The assembled chip was treated with an oxygen plasma system for 20 s (*see* **Note 9**).
2. The chip was then filled with degassed dd-H_2O and was withdrawn by a suction pump for three times until no bubbles were inside of the chip (*see* **Note 10**).
3. The chip was put on a microsyringe pump (Harvard apparatus) in refill mode. The chip was washed three times with PBS (volume: 0.15 mL; rate: 9 mL/h).

4. An aliquot of the biotin-contained lipid vesicle was thawed in a 50 °C water bath.
5. The chip was then coated with 200 μL of the lipid vesicles at 50 °C (volume: 0.15 mL; rate: 1.5 mL/h), and was then incubated at room temperature for 20 min (*see* **Note 11**, Fig. 3).
6. The chip was washed with PBS three times (volume: 0.15 mL; rate: 6 mL/h) to remove excess uncoated lipid vesicles. At this step, the interiors of the chip channels were already coated with biotin-containing SLB.
7. 190 μL of 0.125 mM NA in PBS was introduced to the CMx chip (volume: 0.14 mL; rate:1.5 mL/h) at room temperature and then incubated at 4 °C overnight (*see* **Note 12**).
8. The chip was washed with PBS (volume: 0.15 mL; rate: 6 mL/h) to remove excess NA.
9. 190 μL of 0.03 mg/mL biotin-conjugated-anti-EpCAM antibody in PBS was introduced (volume: 0.15 mL; rate: 1.5 mL/h) and then incubated at room temperature for 1 h.
10. The chip was again washed with PBS (volume: 0.15 mL; rate: 6 mL/h) to remove excess, uncoated anti-EpCAM.
11. After PBS wash, the chips were ready for usage (*see* **Note 13**).

3.4 Biotinlyated Anti-EpCAM

1. The synthesis of biotin-conjugated anti-EpCAM was similar to our previously described protocol (*see* **Note 14**) [11].
2. Freshly prepared 130 μL of 10 mM (2.8 mg in 500 μL dd-H_2O) sulfo-NHS-LC-biotin was dissolved in dd-H_2O.
3. 2 mL EpAb4-1 antibody solution (2.0 mg/mL) in PBS buffer was then added to the above solution in **step 2** (*see* **Note 15**).
4. The mixture was allowed to react at room temperature for 1 h to form the biotin-conjugated anti-EpCAM antibody.
5. Excess biotin ligands were removed by dialysis in PBS buffer at 4 °C for 24 h with the buffer solution changed three times.
6. The final concentration of biotin-conjugated anti-EpCAM was determined with a Nanodrop 1000 spectrophotometer.
7. The biotin-conjugated anti-EpCAM antibody was kept in a −80 °C freezer for storage.

3.5 Capture Efficiency Test on Chip

1. The HCT116 cell line was gently detached with an Accutase solution from the cell culture dish (*see* **Note 16**). The reaction was stopped by adding 1000 μL of 10% FBS in DMEM. The cell culture dish was then gently washed with 10% FBS in DMEM and the cells were transferred to a tube containing CMFDA for green fluorescence staining at 37 °C for ~20 min (*see* **Note 17**).

2. The prestained cells were dispensed by pipette into a new Eppendorf tube and centrifuged at 300 × *g* for 5 min.
3. The supernatant was removed and 1000 μL of fresh DMEM was added to suspend HCT116 in cell culture medium.
4. The number of cells was measured by an automated hemocytometer. The desired concentration of cells (cell number/mL) was obtained by serial dilution of the detached cells in culture medium (*see* **Note 18**).
5. For the binding efficiency test the spiked cell numbers were in the range of 1–1000 (100 μL consisted of ~1–1000 labeled cells) in a total 2 mL of DMEM or whole blood from a healthy donor (Fig. 4).
6. For experiments using the CMx platform, the cancer cells were spiked into DMEM or whole blood experiments (for detail processing refer to the next section). The linearity of capture efficiency in DMEM and blood was yielded (*see* **Notes 19** and **20**; Fig. 5).

3.6 Chip Processing

1. Several anti-EpCAM coated CMx chips were placed on a microsyringe pump in refill mode (*see* **Notes 13** and **21**).
2. 2 mL of blood sample were pipetted into a 5 mL syringe that was linked to its corresponding labeled chip.
3. A general control experiment was performed in parallel by spiking ~200 HCT-116, prestained with CMFDA, into 2 mL DMEM in the chip for processing (*see* **Notes 17** and **22**).
4. The microsyringe pump was set up with the following parameters: volume: 2.0 mL; rate: 1.5 ~ 1.8 mL/h; refill mode. The samples were flowed through the chips. The opening of the syringe was covered with aluminum foil to prevent dust particles from entering.
5. When the process was completed, 200 μL of PBS was added with a pipette to resuspend the remaining cells in the syringe. The microsyringe pump was set up (volume: 0.2 mL; rate: 1.5 ~ 1.8 mL/h), and the process was repeated twice.
6. 500 μL of PBS was pipetted into the inlet of each chip to remove excess blood cells. The microsyringe pump was set up (volume: 0.5 mL; rate: 3 ~ 5 mL/h, *see* **Note 23**).
7. The chips were disconnected from the microsyringe pump and the inlet and outlet of CMx chip were carefully washed. The chips were ready for in-chip counting on a microscopy for capture efficiency of spiking CMFDA prestaining cell experiments (*see* **Note 19**).
8. The chips were then immediately injected with an air bubble foam to release CTCs.

3.7 Cell Releasing by an Air Foam [16]

1. ~5 mL of 5% BSA in PBS was prepared for each chip and filtered with a 0.22 μm filter before usage.
2. A 5 mL screw thread syringe was filled with 4 mL of 5% BSA and 2 mL of air (ratio of 5% BSA to air is 2:1).
3. The syringe with the 5% BSA was screwed onto the three-way stopcock and another empty 5 mL syringe was screwed on in the perpendicular direction. The syringes were pushed quickly eight times, alternating between the left and right syringes (*see* **Note 24**).
4. The foam-containing syringes were attached to the outlet end of the CMx chips (*see* **Note 25**).
5. A CMx chip and a foam-containing syringe were assembled together with an upright automatic microsyringe pump (Fig. 6, *see* **Note 26**).
6. The automatic syringe pump was started (rate: 9 mL/h, volume: 1.5 mL) to release CTCs by air form (*see* **Note 27**).
7. Once all of the samples had been collected into 1.5 mL Eppendorf tubes, the remaining liquid on the adaptor and inlet region of the chips were pipetted into the Eppendorf tubes (*see* **Note 28**).
8. The released sample was placed below an atomizer, and 70% ethanol was pipetted onto the port of the atomizer to de-bubble the collected foamy mixture.
9. After the de-bubbling process, the sample was then fixed in a final concentration of 4% PFA for 10 min on ice (*see* **Note 29**).
10. The sample was then gently transferred from the 1.5 mL Eppendorf tubes onto the collecting membrane for IF staining (*see* **Note 30**).

3.8 Immunofluorescence (IF) Staining Process

1. The collection membranes containing CTCs from the preceding step were washed with PBS three times to remove any PFA remaining on the membrane (*see* **Note 31**).
2. Permeabilization of the captured cells on membrane was done by adding ~200 μL 0.1% Triton X-100 for 10 min, and then washed with PBS three times.
3. The captured cells were treated with 10% NGS at room temperature for 1 h (*see* **Note 32**).
4. The primary antibodies (rabbit anti-human CK-20 for colon cancer patients) were diluted in 1% NGS, which were then added to the collection membranes and incubated at 4 °C overnight.
5. The collected cells on the membrane were then rinsed with PBS three times, with PBS staying on the membrane for 5 min per repeat.

6. The samples were incubated with Alexa Fluor-647 conjugate goat anti-rabbit IgG secondary antibody and FITC pre-conjugated mouse anti-human CD45 antibody at room temperature for 1 h each.
7. After incubation, the cells were rinsed three times with PBS and the collection membranes were then mounted onto glass slides with ProLong Gold Antifade Mountant with DAPI.
8. Images were taken with Nikon-Ti Eclipse microscope at 100-fold magnification.
9. The data was analyzed with NIS-Elements AR Analysis software (*see* **Note 33**).

3.9 Calculations and Criteria of Defined Efficiencies

1. Staining efficiency (E_s) [10] is defined as the efficiency of the cancer cell to be stained by the IF system (*see* **Note 34**).

$$E_s = \frac{N_s}{N_{cmfda}}$$

N_s represents number of cancer cells staining by IF on the collecting membrane, and N_{cmfda} represents the total number of cancer cells on the collecting membrane, on which the cell was prestained with CMFDA to determine the exact number of cells (*see* **Note 35**).

2. Capture efficiency (E_c) is defined as the efficiency of the CMx device to capture cancer cells:

$$E_c = \frac{N_c}{N_i}$$

N_c represents the number of cells captured on the channel surface of CMx chip and N_i represents number of cells initially spiked into the chip (*see* **Note 36**).

3. Release efficiency (E_r) is defined as the efficiency of an air foam to release captured cancer cells from CMx channels:

$$E_r = \frac{N_c - N_r}{N_c}$$

N_r represents the number of cells remaining on the CMx chip after releasing process (*see* **Note 37**).

4. Overall capture efficiency (E_o) is defined as the efficiency of released CTCs loaded and found on the collecting membrane.

$$E_o = \frac{N_m}{N_i}$$

N_m represents the number of cells found on the collection membrane (*see* **Note 38**).

3.10 IF Panel

1. A 12 mL sample of blood was drawn either from a patient or a healthy donor at room temperature.
2. 3 mL of Streck cell preservative reagent was added to the blood sample and gently mixed (*see* **Note 39**).
3. Six CMx chips were prepared for processing.
4. 2.5 mL of Streck cell preservative reagent mixed with the blood sample was pipetted into 5 mL syringe that was linked to its corresponding, labeled chip (*see* **Note 40**).
5. The CMx devices were processed as described in Subheading 3.6.
6. The CTCs from the six individual CMx chips were released from the CMx chips, as described in Subheading 3.7.
7. The IF staining process of the CTCs from each CMx chip was the same as described in Subheading 3.8, except for the primary antibody that was used. Chip1 was stained with anti-panCK, chip 2 with anti-CK18, chip 3 with anti-CK7, chip 4 with anti-TTF1, chip 5 with anti-CK20/CDX2, and chip 6 with anti-PSA/PSMA.
8. The data was analyzed with NIS-Elements AR Analysis software.
9. The decision tree was used to predict the origin tissue of the CTCs (Fig. 8, *see* **Note 41**) [10].

4 Notes

1. All PBS buffer solutions were adjusted to have pH = 7.4 and were filtered with a 0.22 μm filter before usage.
2. The chip surface contained special micropatterns that could be created either by laser etching or injection molding. The method to engrave the chip surface is not important. The chip's special micropatterns and SLB surface coating are more significant. A series of inner micropatterns on the PMMA chips with the same SLB surface modification, but with different flow paths, dimensions, and microstructures was prepared, and they were investigated for CTC capture efficiency as shown in Fig. 1a [5, 15]. Type F of the 4-channel PMMA micropatterns (Fig. 1b) was chosen because it could improve capture efficiency to more than 90% [5]. The CMx chips were 53 mm long, 19 mm wide, and were comprised of an O_2-plasma-treated glass slide bottom and a PMMA top plate with a 50 ~ 60-μm-deep line groove that was bounded by a 63-μm-thick, double-coated, acrylic adhesive 3 M tape.
3. The etched PMMA chips were cleaned by isopropanol (IPA), dd-H_2O, and 70% ethanol, and the double-sided adhesive

tapes (3M tape) were wiped with 70% ethanol after laser-cutting before the assembly of the CMx chip.

4. The bottom cover slide glasses were washed with cycles of IPA, 70% ethanol, natural detergent, and dd-H_2O before assembly of a CMx chip.
5. The 2 μm pore size membrane was attached by 3M tape to the drilled hole with 10 mm diameter on the PMMA plate, as a collection chip (Fig. 6). Due to the small pore size, all of the cells, including cancer cells and WBCs, stayed on the membrane.
6. Work with chloroform was performed in a fume hood while wearing a mask to avoid exposing chloroform to lab-mates and oneself.
7. The compositions of lipid vesicles for the SLB formation include 85% POPC and 15% b-PE in molar percentage.
8. The size of the POPC/b-PE lipid vesicles was determined to be ~50 nm by dynamic laser light scattering (Zetasizer Nano ZS, Malvern Instruments, Germany).
9. An oxygen plasma treatment was used on the CMx chip to make the inner surface more hydrophilic, which is important for SLB spreading and coating.
10. Degas of the chip is necessary because the CTCs on the SLB can be released by air foam.
11. The coating of the chip channel was formed as a layer-by-layer coating, as shown in Fig. 3 [11, 16].
12. This step can be used as a stopping point for storage. The chip surface coated with NA-SLB can be stored at 4 °C for 1–2 weeks.
13. Before processing a blood sample, make sure that there are no air bubbles inside the channel of the chips.
14. In our previous work it was shown that the use of EpAb4-1 in the CMx system can significantly improve CTC capture efficiency in comparison to when commercially available biotin-conjugated-anti-EpCAM is used in the CMx system [11]. Recently, we further developed a site-specific biotinylation method on Fc-domain of EpCAM antibody, which allow preferential alignment on inner sufrace of CMx. The aligned coating was shown to increase the capture efficiency of CTCs and microemboli by 1.6 and 3.0-fold from colorectal cancer patients, respectively (both $P < 0.05$) [17].
15. The molar ratio of antibody to sulfo-NHS-LC-biotin was 1 to 50.
16. Cells were gently harvested by using Accutase to ensure that cell surface antigen would not be degraded.
17. CMFDA reagent was used to prestain active cancer cell and thus resulted in green fluorescence.

18. To ensure the accurate spiking cell number in the cell capture efficiency experiments, three 100 μL prestained cell solutions were separately spiked into glass-bottomed wells (diameter: 6 mm, height: 5 mm). The exact spiking cell number was obtained by counting the average number of spiked cells from the three glass wells by microscopy.
19. The definition of capture efficiency was based on the captured cancer cells in CMx chip divided by the original spiking cell numbers. These cells were directly counted by microscopy since they already had green fluorescence from CMFDA prestaining.
20. As shown in Fig. 5a, the capture performance of the CMx chip, using both HCT116 and AsPC1 in culture medium and whole blood (2 mL), had linear regression in the cell range of 1–1000. The linear regression of each cell line: HCT116 in blood: $y = 0.7359x–0.90844$, $R2 = 0.99723$. Pancreatic cancer AsPC1 in medium: $y = 0.85148x–0.43378$, $R2 = 0.99626$. AsPC1 in blood: $y = 0.53265x + 0.9605$, $R2 = 0.93984$. Each point was the average of three repeated experiments [5].
21. The use of a specific chip number depended on further applications. We had reported for CTC counting, molecular analysis, CTC culture, storage as cryobank, and IF panel [5, 10].
22. The control experiment served as the quality control of individual periods of CMx platform processing.
23. The purity of the captured CTCs could be further improved by increasing the flow rate of the PBS buffer flushed into the chip. As the flow rate of PBS increased, the percentage of retained WBCs could be significantly decreased. In our previous study, the slightly increased shear stress could remove 55 ~ 80% of WBCs while retaining more than 90% of HCT116 cells. The shear stress required was ~4–8 dynes/cm^2 (corresponding to flow rates of ~5–10 mL/h) [5]. For the clinical samples, we chose a smaller flow rate of 3–5 mL/h to avoid any potential loss of CTCs.
24. A mass of air bubbles in the foam was used to detach cells bonded to the micro-channel surfaces. Mechanically shaking a syringe could produce an air foam. The resultant foam was then driven into the micro-channel for cell detachment by a syringe pump.
25. It is important to assemble air form release site from the outlet end of CMx chip to maximize release efficiency. In this way, the released CTCs would travel a shorter path, which would be beneficial for the released cell morphology and maintenance of cell activity for cell culture. We had reported that the released CTCs can be cultured in a cell culture dish [5].

26. The level of all syringes with bubbled 5% BSA should be the same (approximately at 5 ~ 6 mL) so that the same force can be applied evenly when the pump starts.
27. The foam-induced shear stress is much less than the physiological stress (15 dyn/cm^2) observed in human blood vessels; therefore, the release method should not damage cell viability [18].
28. The released air foam also can be connected directly to the collection chip such as Fig. 6.
29. The released cell solution was approximately 300 μL in volume. A 100 μL solution of 16% PFA (pH = 7.4) in PBS was added with gentle mixing via pipetting, and incubated for 10 min in the Eppendorf tube.
30. The sample should be loaded onto the center of the membrane.
31. Rinsing of collection membrane can be done by setting the collection chip upon a few pieces of Asahi KASEI wiper and then blotting the bottom to absorb all of the solution from the membrane.
32. 10% NGS functions as a blocking agent to avoid nonspecific binding.
33. CTCs are defined with triple immunostainings: primary antibody maker positive, CD45 negative and nuclear DAPI positive, as red+/green−/blue+. WBC are also defined with triple immunostainings: primary antibody maker negative, CD45 positive and nuclear DAPI positive, as red−/green+/blue+.
34. IF staining is a technique that uses the specific fluorescent antibodies to their antigen on the target cell surface or cytoplasm of the cell. This process allows the visualization of the distribution and location of the target marker in the sample.
35. In our previous study, the staining efficiency was as high as ~90–100% [10], except CK-20/CDX2 on HCT-116 cell line, which is known to have low CK20/CDX2 expression level [19, 20].
36. For the CMx chip assembly quality control, the capture efficiency should be higher than 80%.
37. In our previous study, the release efficiency was up to 99.7% [16].
38. With a well-designed microfluidic system and an experienced lab worker, the overall capture efficiency ($\boldsymbol{E_o}$) could approach capture efficiency ($\boldsymbol{E_c}$).
39. Streck cell preservative reagent could maintain cellular antigen expression for up to 7 days, eliminating adverse effects of time, storage, and transport conditions. Here we processed samples within 24 h.

40. The 0.5 mL additional volume in the processing sample was due to adding Streck cell preservative reagent.
41. In the previous report, cancer cell lines of lung (H1975), colorectal (DLD-1, HCT116), and prostate (PC3, DU145, LNCaP) were selected as cell-based models to establish the sensitivity and specificity for distinguishing CTCs from either lung, colorectal, or prostate cancer [10]. The IF panel had been successfully applied to 25 clinic samples.

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Chapter 2

Microfluidic Separation of Circulating Tumor Cells Based on Size and Deformability

Emily S. Park, Simon P. Duffy, and Hongshen Ma

Abstract

Circulating tumor cells (CTCs) have been implicated as the seeds of cancer metastasis and therefore have the potential to provide significant prognostic and diagnostic values. Here, we describe a procedure for separating CTCs from whole blood based on size and deformability using the microfluidic ratchet device. This device leverages the ratcheting motion of single cells created as they are deformed through funnel-shaped constrictions using oscillatory flow in order to divert cells based on differences in size and deformability. Subsequent methods for CTC identification and enumeration using immunofluorescence after separation are also described.

Key words Circulating tumor cells (CTCs), Deformability, Cell sorting, Microfluidic ratchet mechanism

1 Introduction

Circulating tumor cells (CTCs) are believed to play a key role in metastasis, or the spread of cancer that is responsible for >90% of all cancer-related deaths. During metastasis, CTCs are released from a primary tumor, and then travel through the bloodstream to spread the cancer to secondary sites. The isolation and enumeration of CTCs from blood presents a potentially rapid, minimally invasive, and longitudinal method to evaluate cancer disease status and treatment efficacy [1–4]. The primary challenges in CTC isolation and enumeration are their extreme rarity in circulation, where CTCs are found at frequencies as low as <1 cell per ml of whole blood. While these exceedingly rare cells have been isolated using immunoenrichment [5–8], such methods rely on the expression of epithelial surface antigens and may fail to enrich for CTCs with poor antigen expression [9–12]. An alternative antigen-independent CTC isolation strategy involves separation of CTCs from contaminating leukocytes on the basis of differences in their morphological characteristics. Among these, differences in size and

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deformability have been used to devise numerous alternative label-free CTC enrichment strategies [13].

In this protocol, we describe the procedure to first fabricate the microfluidic ratchet device and then to use it for isolating rare CTCs from whole blood based on cell size and deformability. The principle of the microfluidic ratchet device relies on the asymmetrical force required to deform single cells through funnel-shaped constrictions. When the opening of the constriction is smaller than the diameter of the cells, the force required to push the cell through the constriction along the direction of the taper is less than against the direction of the taper. Therefore, an oscillatory flow of an appropriate magnitude produces a ratcheting transport that is selective based on a combination of cell size and deformability (Fig. 1).

This approach provides two key advantages over cell separation using simple micropore filtration. First, oscillatory flow minimizes the contact time between cells and microstructures, and thereby enables whole blood to be processed directly without clogging and adsorption [14–16] (Fig. 1). Second, the ability to separate cells based on a combination of size and deformability is particularly relevant to the separation of CTCs from whole blood where CTCs may not be easily distinguishable from leukocytes based on size alone. Specifically, CTCs from patients with prostate and colorectal cancer have been found to be smaller than cultured cancer cells and have significant size overlap with leukocytes [17, 18].

The design of the microfluidic ratchet device consists of a 32 × 2048 matrix of 2D funnel constrictions connected with supporting microchannels for flow control. Each funnel constriction is approximately 30 μm thick with horizontal openings that are gradually reduced from the bottom row of the matrix, where they

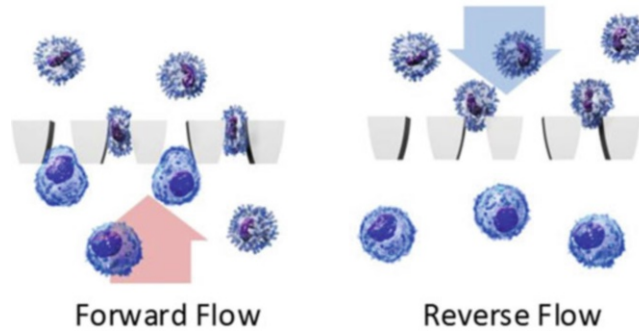

Fig. 1 The Microfluidic Ratchet Mechanism: Cells of an appropriate size and deformability are transported through the funnel microstructures in forward flow and are unable to return during reverse flow, thereby ratcheting through the constrictions with oscillatory flow. Larger and more rigid cells are completely blocked by funnel constrictions, while smaller cells oscillate through the constrictions unimpeded. (Fig. 1 is reproduced from ref. 17)

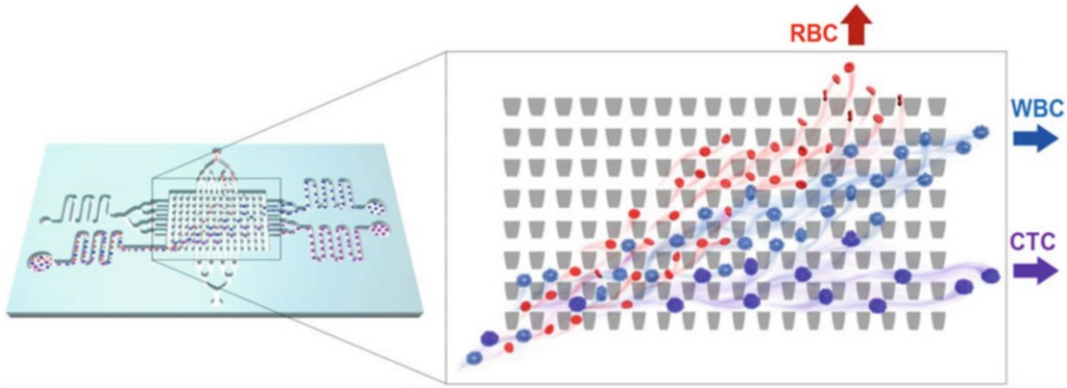

Fig. 2 Size and deformability-based cell separation using microfluidic device. The device consists of 32 × 2048 matrix of funnel constrictions with openings decreased from 18 to 2 μm, from the *bottom row* to the *top row*. The blood sample is introduced at the bottom-left of the funnel matrix, where the cells are propelled using a biased oscillatory flow in the vertical direction, as well as a constant flow in the vertical direction. These flows cause the cells to traverse through the funnel matrix in a zig-zag diagonal path until reaching a limiting funnel size. The limiting funnel size is greater for CTCs than leukocytes because of their limited deformability, enabling them to be diverted to a separate path. Highly deformable RBCs pass through the top of the funnel matrix

are 18 μm, to the top row of the matrix where they are only 2 μm. Detailed geometries of the device are provided in Park et al. [19]. The blood sample is infused into the funnel matrix at the bottom-left corner where the cells are pushed by a constant rightward flow simultaneously as a vertical oscillatory flow. Each cell traverses through the funnel matrix in a step-wise diagonal path until reaching a limiting funnel size, at which point the cell moves horizontally toward the outlet (Fig. 2). CTCs are the least deformable cells and reach their limiting funnel size relatively quickly. Leukocytes are more deformable and travel to a smaller funnel region. Finally, erythrocytes are extremely deformable and exit through the top row. Separated cells are immunostained using multiple fluorochrome-conjugated antibodies such as cytokeratin (CK), EpCAM, and CD45 as well as counterstained for DAPI for further identification and characterization. The CTCs are defined as DAPI+/CK+/CD45− whereas leukocytes are defined as DAPI+/CD45+.

2 Materials

2.1 Fabrication of Silicon Masters

1. Silicon wafer, 4 in. (Silicon Quest).
2. SU-8 8025 photoresist (Microchem).
3. SU-8 developer (Microchem).
4. Photomasks (*see* **Note 1**).

2.2 Fabrication of PDMS Devices

1. PDMS-Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI).
2. Hole punches, 0.5 mm (Harris, Redding, CA).
3. Standard glass slides (50 × 70 mm, Fisher Scientific).
4. Air plasma chamber (Model PDC-001, Harrick Plasma, Ithaca, NY).
5. Oven.

2.3 Instrumentation and Accessories for Microfluidic Device Operation

1. Fluid controlling actuators (MFCS-4C, Fluigent, Paris, France) (*see* **Note 2**).
2. Custom pressure controller (*see* **Note 3**) to supply pressure from 0 to 1 bar using manual pressure regulators (Omega, McMaster-Carr) (Fig. 3a).
3. Pressure board control software designed with a Visual C# (*see* **Note 4**) (Fig. 3b).
4. Pump for the positive pressure source (UN86KNDC-B, KNF Neuberger Inc., NJ, USA).
5. Masterflex Tygon lab tubing (i.d. 16 mm, Cole-Parmer Canada Inc., Montreal, QC), cat. no. 06-06409-14).
6. Tygon microbore tubing (i.d. 0.02 in., o.d. 0.06 in., Cole-Parmer Canada Inc., Montreal, QC, cat. no. 06-06409-14).
7. 23-gauge stainless-steel needles (i.d. 0.017 in., o.d. 0.025 in., New England Small Tubes, Litchfield, NH).
8. 15 ml Falcon conical tubes.
9. 1× PBS pH 7.4.
10. Running buffer: 0.2% Pluronic™ F-127 (Invitrogen) in 1× PBS.

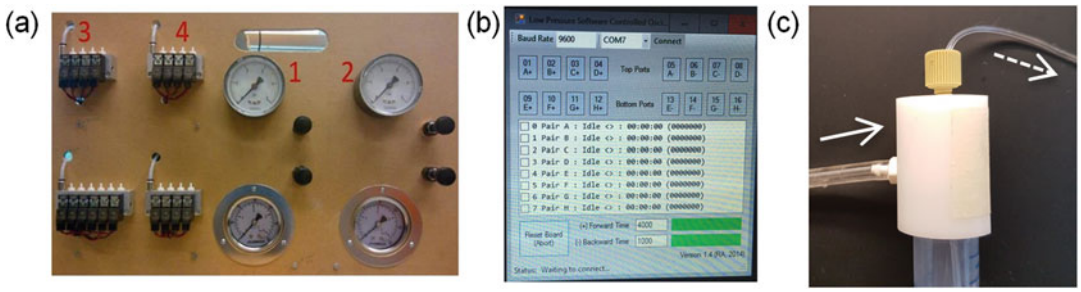

Fig. 3 (**a**) Custom-built pressure board supplying the air pressure. Pressure regulators for oscillation inlets 1 and 2 (1 and 2 in *red color*) and oscillation pressure manifolds along with solenoid valves (3 and 4 in *red color*) are installed and used for cell separation experiments. (**b**) Pressure board control software designed with a Visual C#, (**c**) A complete assembly of cap and tubing connected. Pressure is applied through a Masterflex Tygon lab tubing (*solid white arrow*), while the buffer/sample is exiting through a Tygon microbore tubing (*dotted white arrow*), in which a needle is inserted at the other end to connect on a device

2.4 Immunofluorescence

1. 1× PBS pH 7.4.
2. Formaldehyde solution (4% diluted from 16%, Alfa Aesar) (*see* **Note 5**).
3. Cell permeabilization buffer (0.025% Tween 20 in 1× PBS) (*see* **Note 6**).
4. Blocking buffer (3% bovine serum albumin in 1× PBS) (*see* **Note 7**).
5. Fluorochrome-conjugated antibodies: pan-keratin (cytokeratin, CK; C11) mAb—Alexa 488 (Cell Signaling, #4523), EpCAM (VU1D9) mAb—Alexa 594 (Cell Signaling, #7319), and CD45 mAb- APC (Biolegend, #304012) (*see* **Note 8**).
6. DAPI solution (Vector lab).
7. Shaker.

3 Methods

3.1 Fabrication of Silicon Masters

1. Draw the microfluidic device design in DraftSight software (*see* **Note 9**).
2. Submit the design to a photomask company for mask printing.
3. Use SU-8 negative photoresist and standard photolithography method (*see* **Note 10**) to prepare a silicon wafer to serve as the master mold for the ratchet microfluidic device.

3.2 Production of PDMS Devices

1. Place the silicon wafer on a 15 cm diameter Petri dish, and secure it in the center with the tape.
2. Mix PDMS base with PDMS curing agent at a weight ratio of 10:1.
3. Pour the PDMS mixture into the petri dish to a thickness of ~5 mm, and degas the PDMS in a vacuum chamber for 15 min to void air bubbles from the features (*see* **Note 11**).
4. Bake the petri dish at 65 °C in an oven for 2 h to cure the PDMS.
5. Gently peel off the PDMS layer from the silicon wafer, and use 0.5 mm punches to punch holes for the inlets and outlets. Cut and trim the PDMS layer.
6. Bond the PDMS layer on a standard glass slide using air plasma treatment for 90 sec. (*see* **Note 12**).
7. Bake devices at 65 °C in an oven for 10 min, and allow the devices to cool to 25 °C before using them.
8. Inspect the devices under the microscope for the proper bonding and check for debris that might contribute to channel blockage.

3.3 Operation of the Ratchet Microfluidic Device

1. Turn on the air pump and Fluigent pressure controller.
2. Prepare four 20 cm pieces of Masterflex Tygon lab tubing (ID 1.6 mm) and insert tubing into each of four assembly caps (sample cap, buffer cap, oscillation inlet 1, and inlet 2 caps), as illustrated in Fig. 3c. Connect the other end of the tubing to the appropriate pressure sources (*see* **Note 13**).
3. Prepare six lengths of Tygon microbore tubing (ID 0.02 in., OD 0.06 in.): four 20 cm pieces of Tygon microbore tubing for fluid transport (sample, buffer, oscillation inlets 1 and 2), and two 10 cm pieces Tygon microbore tubing for outlets (CTC and leukocytes outlets).
4. Insert needles (ID 0.017 in., OD 0.025 in., New England Small Tube) into each of the six lengths of Tygon microbore tubing, prepared in **step 3**.
5. Thread four 20 cm pieces of Tygon microbore tubing with a needle inserted from **step 3** (sample, buffer, oscillation inlets 1 and 2) through the filter and the cap. *See* Fig. 3c for the assembly of cap (*see* **Note 14**). Insert two 10 cm pieces Tygon microbore tubing (CTC and leukocytes outlets) on the outlets on the device as shown in Fig. 4.
6. Place ~10 ml of running buffer (0.2% Pluronic in 1× PBS) in a 15 ml of falcon conical tube for the oscillation inlet 1 and in a 15 ml of falcon conical tube for the buffer. Place ~1 ml of running buffer (0.2% Pluronic in 1× PBS) in a 15 ml of falcon conical tube for the oscillation inlet 2. Attach caps (prepared in **step 5**) on the top of a 15 ml of falcon conical tube with the tubing extended to the bottom of the conical tube).
7. Launch the Microfluidics Control System software and increase the flow pressure to 100 mbar for buffer. Allow the running buffer (0.2% Pluronic in 1× PBS) to fill the tubing and form a

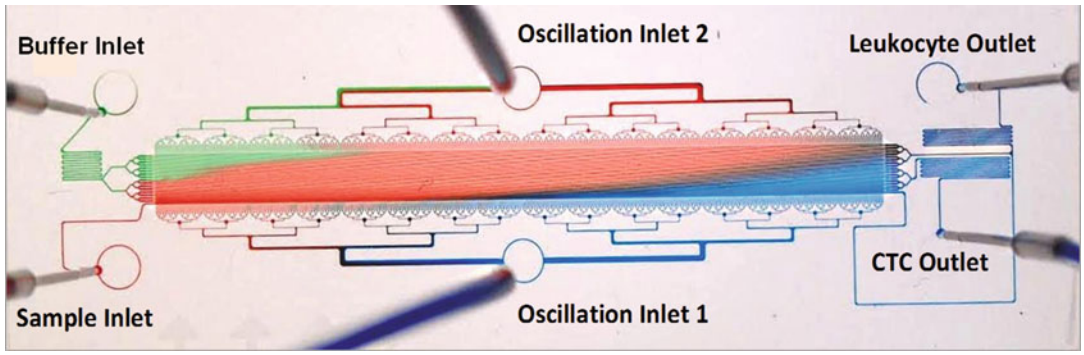

Fig. 4 Photograph of the microfluidic ratchet device infused with colored water to show the inlets and outlets as well as a diagonal flow pattern in the separation. The blood (*demonstrated in red colored water*) is introduced at the left bottom corner and travels in a diagonal direction while cells are separated in the funnel matrix

droplet at the end of the needle before inserting the needle on the device as shown in Fig. 4. Increase the flow pressure to 300 mbar to prime the device for at least 15 min.

8. Launch the Low Pressure Oscillation Controlling Software (custom-designed using a Visual C#, Fig. 3b). Set the period of the forward flow to 4000 ms and the period of the reverse flow to 1000 ms as shown in Fig. 3b.
9. Adjust the custom-designed pressure controller to set the forward flow pressure to 3 psi and the reverse flow pressure to 1 psi, and initiate the oscillatory flow by clicking the paring box on the software. Allow the running buffer (0.2% Pluronic in 1× PBS) to fill the tubing and insert needles to oscillation inlets 1 and 2 on the device as shown in Fig. 4 once the buffer has formed a droplet at the end of the needle.
10. Flow running buffer (0.2% Pluronic in 1× PBS) through the device for >15 min after all needles are connected except for the blood. Inspect the device to ensure that the device has filled completely and that there are no bubbles within the device.
11. Place a 15 ml conical tube at the each end of CTC and leukocytes outlets to collect cells (*see* **Note 15**).
12. Add 1 ml of whole blood to a 15 ml conical tube and close the cap. As described in **step 7**, flow blood through the tubing at 100 mbar until the sample forms a droplet at the end of the needle and insert the needle on the sample inlet on the device (Fig. 4). Increase the flow pressure to 300 mbar.
13. Verify that the blood flows along a diagonal path within the sorting region of the microfluidic device and that the red blood plasma flows to the outlets at the uppermost right corner of the sorting area, as illustrated in Fig. 4 (*see* **Note 16**).
14. After the entire blood sample has flowed through the sorting region of the device, decrease sample and buffer pressure to 0 mbar, and uncheck the pair in the pressure board software.
15. Remove the CTC outlet tube and proceed to immunofluorescence steps (Subheading 3.4).
16. Remove all other tubes and needles, and discard the device in the glass waste box, and anything else that has contacted the blood sample in the biohazard waste box.
17. Turn off the Fluigent and the air pressure pump.

3.4 Immunofluorescence

After microfluidic separation, collected cells should be in a 15 ml of falcon tube at room temperature.

1. Suspend the volume of collected cells, in the CTC outlet, with 5 ml of 1× PBS and gently agitate the tube.

2. Collect the cells by centrifuging the 15 ml falcon tube for 5 min at 300 × *g* with break-on at room temperature (*see* **Note 17**).
3. Using a sterile aspiration tip, carefully remove the supernatant by vacuum suction. Ensure that a small volume remains and that the cell pellet is not disturbed in this process.
4. Wash the cells again by resuspending them in 5 ml of 1× PBS (*see* **Note 18**), collecting the cells by centrifugation for 5 min at 300 × *g* at room temperature, and aspirating the supernatant (*see* **Note 19**).
5. For cell fixation, resuspend the cell pellet with 2 ml of 4% formaldehyde solution and agitate for 10 min on a shaker at room temperature.
6. Wash the sample twice by centrifuging for 5 min at 300 × *g* (room temperature), aspirating the supernatant and resuspending in 5 ml of 1× PBS.
7. Resuspend the pellet from the final wash (**step 6**) in 2 ml of cell permeabilization solution (0.025% Tween 20 in 1× PBS) and incubate for 10 min on the shaker.
8. Following permeabilization, wash the sample by centrifuging for 5 min at 300 × *g* (room temperature), aspirating the supernatant, and resuspending in 5 ml of 1× PBS.
9. Centrifuge again for 5 min at 300 × *g* (room temperature), aspirate the supernatant, and resuspend in 2 ml of blocking buffer (3% BSA in 1× PBS).
10. Allow cells to incubate for 1 h at room temperature on the shaker.
11. While blocking, prepare fluorochrome-conjugated antibodies diluted in 50 µl blocking buffer (*see* **Note 20**). Limit the exposure of these solutions to light.
12. Centrifuge the cell suspension after incubation with blocking buffer for 5 min at 300 × *g* at room temperature.
13. Aspirate the blocking buffer, leaving ~100 µl of blocking buffer. Resuspend and transfer the cell pellet into a PCR tube (0.5 ml).
14. Apply diluted fluorochrome-conjugated antibodies to the cell suspension. Add blocking buffer to the final volume of ~300 µl.
15. Wrap the specimen in aluminum foil and incubate for 16 h at 4 °C on the shaker.
16. Following staining, add 250 µl of 1× PBS and centrifuge cell suspension for 5 min at 300 × *g* at 4 °C.
17. Wash the sample twice by centrifuging for 5 min at 300 × *g* (room temperature), aspirating the supernatant and resuspending in 400 µl of 1× PBS.
18. Aspirate the supernatant, leaving ~80 µl of 1× PBS.

19. Add DAPI (1.5 μg/ml) to the cell suspension and mix well.
20. Distribute 40 μl cell suspension into each well on a 384-well plate (*see* **Note 21**).
21. Centrifuge the 384-well plate for 3 min at 300 × *g*.
22. Cells are ready to be scanned using fluorescent microscope. For best results, scan the sample in 1–2 h. Otherwise, the sample can be stored at 4 °C for 1–2 days.

4 Notes

1. We obtain the mask for microscale constrictions (less than 2 μm resolution) from Advance Reproductions (Andover, MA) and the mask for flow channels from CAD/Art Services (Brandon, OR).
2. The MFCS-4C system (Fluigent SA, Paris, France) is used to precisely control the pressure with a resolution of 30 mbar up to 1000 mbar.
3. A custom pressure controller is designed to supply pressures between 0 and 1 bar on four individual channels using manual pressure regulators (Omega, McMaster-Carr). Solenoid valves (Pneumadyne, McMaster-Carr) are activated by MOSFET switches and act as control valves to turn pressurize or vent each individual channel. These MOSFET switches are controlled using an MSP430 microcontroller (Texas Instruments) integrated on a custom-printed circuit board. The MSP430 is controlled over serial using a Visual C# user interface on a target PC and allows timing profiles to be adjusted programmatically.
4. A Visual C# user interface program is installed on a PC to turn on/off pressure control.
5. Use fresh and store opened vials at 4 °C in the dark. Dilute one in four in 1× PBS to make a 4% formaldehyde solution.
6. To prepare 10 ml of cell permeabilization buffer, add 2.5 μl of Tween 20 to 10 ml of 1× PBS, and mix well. You may use the vortex to mix it well, but this creates bubbles. Let all bubbles disappear before use.
7. To prepare 10 ml of blocking buffer, add 0.3 g of bovine serum albumin (BSA) to 10 ml of 1× PBS, and mix well. Placing the blocking buffer in 37 °C water bath for 3 min helps to dissolve BSA in PBS.
8. All fluorochrome-conjugated antibodies should be kept on ice and centrifuge briefly before use.

9. Refer to the manuscript, "Continuous Flow Deformability-Based Separation of Circulating Tumor Cells Using Microfluidic Ratchets" (ref. 17), for the specific design used for the ratchet microfluidic device.
10. Refer to the standard photolithography protocol (http://www.biomemsrc.org/resources/protocols-procedures) for a detailed procedure. Briefly, the photolithography process is composed of first fabricating the sorting region microstructures followed by the second layer of microstructure of supporting microchannels precisely aligned using the mask aligner (CANON PLA-501F). The masks contain the align markers for the easier patterning using the standard photolithography method. The specific microfluidic device fabrication protocol can be found in the experimental section from ref. 19.
11. Make sure there are no bubbles on the features on a silicon wafer.
12. Clean the standard microscope slides beforehand with acetone and isopropanol.
13. Connect sample and buffer tubing to the Fluigent pressure controller and forward and backward tubing to the customized pressure board. Fluigent pressure controller gives more accurate control of pressure.
14. Have enough tubing to reach the bottom of a 15 ml Falcon conical tube. The filter should be fairly tight against the cap.
15. Ensure that the other side of tubing is secured in a 15 ml conical tube using the small strip of parafilm.
16. Slightly modify the forward pressure if needed. For example, if the blood flow does not reach to the top right corner with a steeper slope, decrease the forward pressure. If the slope of blood flow is lower and some red blood cells are collected in the leukocytes outlet, increase the forward pressure (maximum 3.5 psi).
17. All centrifuge steps are performed with break-on unless otherwise specified.
18. Do not try to aspirate all the supernatant while you cannot see the pellet. Leave ~100 μl of the supernatant to save the pellet. This applies to all the centrifuging and aspirating steps.
19. If many red blood cells are present in the CTC outlet collection tube, which may interfere with the downstream immunofluorescence steps, perform red blood cell lysis using lysis buffer (Ammonium Chloride solution, StemCell Technology) before proceeding to the next step.
20. Optimal amount of each fluorochrome-conjugated antibody is shown in Table 1.

Table 1
Optimal concentration of antibodies to dilute in 50 μl blocking buffer

Antibody	Concentration (μg/ml)	Volume to add (μl)
pan-keratin (C11) mAb—Alexa 488	100	0.5
EpCAM (VU1D9) mAb—Alexa 594	100	1
CD45 mAb-APC	9	2

21. We found that cell density is quite suitable when the stained cells are distributed into two wells on a 384-well plate for 1 ml whole blood sample. However, if cell density is too high for scanning, add more PBS to dilute the stained cells and use as many wells as it is necessary to obtain the good cell density to scan.

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Chapter 3

A Novel Microfluidic Device for Isolation of Circulating Tumor Cells from Pancreatic Cancer Blood Samples

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Abstract

Enumeration of circulating tumor cells (CTCs) can provide valuable prognostic information to guide cancer treatment as well as help monitor disease progression. Analysis of these rare malignant cells has the potential to further our understanding of cancer metastasis by gaining insights into CTC characteristics and properties. Microfluidics presents a unique platform to isolate and study CTCs. In this chapter, we describe the detailed procedures for the fabrication and use of a microfluidic device to detect CTCs from the blood of pancreatic cancer patients.

Key words Microfluidics, Circulating tumor cells, Pancreatic cancer

1 Introduction

Cancer metastasis is responsible for the majority (>90%) of all cancer-related deaths. Blood-borne metastases arise after the dissemination of cancer cells into the bloodstream from a tumor site. Tumor cells that are identified in transit within the bloodstream are referred to as circulating tumor cells, or CTCs [1]. CTCs are present in rare numbers, estimated to be between 1 and 100 CTCs per billion normal blood cells in the circulation of patients with advanced disease [2]. Consequently, CTC isolation, identification, and characterization require extremely sensitive and specific analytical methods as well as novel technologies.

In this chapter, CTC sample analysis is demonstrated with a mixer-based microfluidic device. The device is patterned with herringbone (or chevron) structures to disrupt flow streamlines and induce chaotic mixing, maximizing collisions and interactions between target cells in the sample and functionalized device surfaces. The device design was inspired by several groups [3–5], and was geometrically optimized for enhanced capture efficiency and

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purity in our previous work [6]. The device consists of a polydimethylsiloxane (PDMS) layer on the top, engraved with the desired fluidic structure, permanently bonded to a standard microscope glass slide (as a bottom layer). Other amorphous materials, such as cyclic olefin copolymer (COC), can be used to create a similar microfluidic design.

Blood samples were first prepared by using density gradient centrifugation. Ficoll-based centrifugation methods separate blood components into different layers based on their density, size, and mass, in order to extract cells of interest while reducing the amount of nontarget cells. Typically, erythrocytes (i.e., red blood cells), polymorph nuclear leukocytes (PMNLs), and platelets are separated in the pellet while mononuclear cells (MNCs), including tumor cells, are kept in the interphase often referred to as the buffy coat. Ficoll is a density gradient medium used to separate the low-density buffy coat cells from the bottom pellet. Other similar methods have been developed for the extraction of rare cell populations from whole blood, such as OncoQuick® (Greiner Bio-One, Monroe, NC) and RosetteSep™ (StemCell Technologies, Vancouver, Canada) enrichment systems.

Following blood preparation, the final sample suspension is introduced into a microfluidic device with immobilized epithelial cell adhesion molecule (EpCAM) antibodies. At the end of the capture experiment, a detection antibody mixture is introduced, after cell fixation and permeabilization, to visualize marker expression or lack thereof. CTC enumeration is then performed, based on the FDA (Food and Drug Administration)-approved method for defining a CTC: an EpCAM-antibody-captured cell that has confirmed positive expression for cytokeratins (CK), positive staining with 4′,6-diamidino-2-phenylindole (DAPI), and negative for CD45 (i.e., $EpCAM^{+}CK^{+}DAPI^{+}CD45^{-}$) [1, 7]. This marker combination effectively confirms the cell to be of an epithelial origin and excludes blood cell contamination. An automated sample stage with a microscope as well as fluorescence quantification and counting software is used to help minimize operator's manual intervention and increase enumeration reliability and reproducibility.

The protocol detailed in this chapter has been used to process and enumerate CTCs from whole blood samples of patients with advanced pancreatic cancer who are under active palliative treatment. The sensitivity of our methods and techniques is highlighted by our ability to consistently detect low number CTCs in blood from patients with pancreatic cancer, which are otherwise difficult to detect using the FDA-approved method (CellSearch®) [7].

2 Materials and Equipment

2.1 Materials for Device Design and Mold Preparation

1. CAD software (e.g., AutoCAD).
2. Photomask with microfluidic designs, either chrome photomask or high resolution transparency photomask (≥2500 dpi) (*see* **Note 1**).
3. One blank 100-mm diameter silicon wafer (*see* **Note 2**).
4. Teflon tweezers, for handling silicon wafers.
5. Acetone (Fisher Scentific, Hampton, NH) (*see* **Note 3**).
6. Three Pyrex® 3140 dishes, 125 × 65 mm (Corning Inc., Corning, NY).
7. Isopropanol (IPA), in a wash bottle (Fisher Scientific, Hampton, NH).
8. Piranha etch solution (H_2SO_4 and H_2O_2) (*see* **Note 4**).
9. Buffered oxide etch (BOE) 6:1 solution (Mallinckrodt Baker, Inc., Phillipsburg, NJ).
10. Deionized (DI) water, in a wash bottle.
11. A commercial furnace capable of reaching 125 °C.
12. Hexamethyldisilazane (HMDS), with a hot plate to heat/evaporate HMDS.
13. A commercial spin coater (*see* **Note 5**).
14. SU-8 negative photoresist (MicroChem, Newtown, MA) (*see* **Note 6**).
15. SU-8 developer (MicroChem, Newtown, MA).
16. Cotton swabs.
17. Mask aligner and UV exposure equipment (*see* **Note 7**).
18. Filtered, pressurized inert gas pistol (e.g., air or nitrogen).
19. Desiccator connected to a vacuum line.
20. Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma-Aldrich, St. Louis, MO).
21. Small weigh boat, or a small vial.

2.2 Materials for Device Fabrication

1. Sylgard 184 kit: silicone elastomer base and curing agent (Dow Corning, Midland, MI).
2. Weight boat.
3. Scale.
4. Wooden stirrers (*see* **Note 8**).
5. Large petri dish (>100 mm), or tin foil (*see* **Note 9**).
6. Desiccator connected to a vacuum line with compatible polymer tubing.

7. A commercial oven capable of 70 °C.
8. Razor blade or scalpel.
9. Polymethylmethacrylate (PMMA) sheet, or any other clean plastic sheet.
10. Micropuncher.
11. Glass microscope slides, 75 mm × 25 mm (Fisher Scientific, Hampton, NH).
12. Pyrex® 3140 dish, 125 mm × 65 mm (Corning Inc., Corning, NY).
13. Detergent.
14. Ionized water.
15. Benchtop ultrasonic cleaner.
16. Acetone, in a wash bottle.
17. DI water, in a wash bottle.
18. Ethanol, in a wash bottle.
19. Inert gas pistol (e.g., air or nitrogen).
20. Corona discharger (*see* **Note 10**).
21. Parafilm M™ wrapping film (Fisher Scientific, Hampton, NH).
22. 35-mm petri dishes (Corning Inc., Corning, NY).

2.3 Materials for Device Functionalization

1. Female luer-to-barb adapters (IDEX Health & Science, Oak Harbor, WA).
2. Tubing: 0.0625-in. outer diameter, 0.008-in. inner diameter (IDEX Health & Science, Oak Harbor, WA) (*see* **Note 11**).
3. Sterile 3-mL Luer-Lock syringes (Becton Dickinson, Franklin Lakes, NJ).
4. Anhydrous ethanol.
5. Dulbecco's phosphate buffered saline (DPBS) free of Ca^{2+}/Mg^{2+} ions (Fisher Scientific, Hampton, NH).
6. Bovine serum albumin (BSA) (MP Biomedicals, Solon, OH) (*see* **Note 12**).
7. Two commercial programmable syringe pumps with infuse function (*see* **Note 13**).
8. Vacuum line with compatible polymer tubing.
9. Precision vacuum regulator (0–30″ Hg).
10. One truncated 1-mL pipette tip.
11. Avidin (Invitrogen, Carlsbad, CA).
12. Biotinylated anti-EpCAM (anti-human CD326, clone 1B7, eBioscience, San Diego, CA) (*see* **Note 14**).

2.4 Materials for Sample Processing

1. Ficoll-Paque centrifugation media (GE Healthcare Life Sciences, Marlborough, MA) (*see* **Note 15**).
2. Alcohol, 100% (Thomas Scientific, Swedesboro, NJ).
3. Cotton swabs.
4. Sterile 5-mL Luer-Lock syringe with needle (Becton Dickinson, Franklin Lakes, NJ).
5. Sterile 50-mL centrifuge tubes (Corning Inc., Corning, NY).
6. Sterile 10-mL disposable serological pipettes (Fisher Scientific, Hampton, NH).
7. Serological pipette controller (Fisher Scientific, Hampton, NH).
8. Centrifuge with swing-out rotor.
9. Two commercial programmable syringe pumps with infuse function (*see* **Note 13**).
10. DPBS free of Ca^{2+}/Mg^{2+} ions (Fisher Scientific, Hampton, NH).
11. Fetal bovine serum (FBS) (Fisher Scientific, Hampton, NH).
12. Sterile 1-mL syringes (Becton Dickinson, Franklin Lakes, NJ).
13. Polytetrafluoroethylene (PTFE) micromagnetic stirrer bar, 5 mm length × 2 mm diameter (Cowie, Wilmington, DE).
14. Basic magnetic stirrer (Fisher Scientific, Hampton, NH).
15. Female luer-to-barb adapters (IDEX Health & Science, Oak Harbor, WA).
16. Tubing: 0.0625-inch outer diameter, 0.008-in. inner diameter (IDEX Health & Science, Oak Harbor, WA) (*see* **Note 11**).
17. Sterile 2-mL microcentrifuge tubes.
18. Sterile 3-mL Luer-Lock syringes (Becton Dickinson, Franklin Lakes, NJ).
19. Paraformaldehyde (PFA), 96%, extra pure (Acros Organics, Morris Plains, NJ).
20. Triton X-100, 98%, molecular biology grade (Amersham Biosciences, Sweden).
21. Anti-cytokeratin-FITC (Cytokeratin conjugated with fluorescein isothiocyanate, clone CAM 5.2, BD Biosciences, San Jose, CA) (*see* **Note 14**).
22. Anti-CD45-PE (CD45 conjugated with phycoerythrin, clone HI30, BD Biosciences, San Jose, CA) (*see* **Note 14**).
23. DAPI (4′,6-diamidino-2-phenylindole, Invitrogen, Carlsbad, CA) (*see* **Note 16**).

2.5 Materials for CTC Detection

1. Fluorescence microscopy setup: inverted IX71 Olympus microscope, 10× and 20× objectives, xenon lamp, fluorescent filters to match the dye-conjugated antibodies (*see* **Note 17**), computer with CellSens software (Olympus, Center Valley, PA) or another software; optional: automatic or semiautomatic stage (Prior, Rockland, MA).

3 Methods

3.1 Design and Mold Preparation

1. Design and draw the microfluidic architecture in CAD software (e.g., AutoCAD).
2. Submit the CAD file to a commercial supplier for a photomask order (*see* **Note 1**).
3. Place a silicon wafer in an acetone bath inside a polyethylene beaker for 5 min to remove organic materials. Occasional stirring is recommended.
4. Wash the silicon wafer with IPA for 20 s using a wash bottle.
5. Using Teflon tweezer, immerse the wafer substrate into a Pyrex® dish with piranha wet etch (H_2SO_4 and H_2O_2) for 5 min. Wash the wafer with running DI water for 2 min. Transfer the wafer into a different Pyrex® dish with BOE and leave it in for 20 s. Rinse the wafer thoroughly for 30 s with a DI water wash bottle. Use an inert gas pistol to make the wafer surface dry.
6. Bake the wafer at 125 °C in the oven for 10 min.
7. Place the wafer in the HMDS hot plate and release HMDS for 30 s. Incubate the wafer within the HMDS for 2 min. Remove the wafer off the hot plate and wait at least 2 min to let the wafer cool off before moving on to the next step. Alternatively, you may treat the wafer with HMDS via evaporation in a Pyrex® dish.
8. Place the wafer on a headway spinner and dispense 4 mL of SU-8 photoresist on the top of the wafer.
9. Spin the wafer at 500 revolutions per minute (RPM) for 10 s, with an acceleration of 100 RPM per second.
10. Increase the spinner speed to 2250 RPM ($\sim 288 \times g$) for 30 s, with an acceleration of 300 RPM per second.
11. Clean the underside and the edges of the wafer with a cotton swab wetted with SU-8 developer to remove excess SU-8 photoresist buildup. This step is to avoid contaminating the hot plate and mask aligner equipment with photoresist. Dedicated spin coaters have edge bead removal (EBR) and can be programmed to perform this step automatically after spin coating (*see* **Note 18**).

12. Soft bake: place the wafer on the hot plate at 65 °C for 3 min. Remove the wafer from the hot plate and allow it to cool down to room temperature. Place the wafer on a hot plate, at 95 °C for 6 min (*see* **Note 19**).
13. Calculate the exposure time based on the equipment's power setting and an exposure dose of 160 mJ/cm^2 (*see* **Note 20**). Set the wavelength of the exposure to 365 nm.
14. The photoresist-coated wafer is inserted into the mask aligner for UV exposure.
15. Post exposure bake (PEB): place the wafer on a hot plate at 95 °C for 6 min. The design pattern will become visible on the layer surface about 1 min into the PEB. If no visible latent image is seen during or after PEB, there was insufficient energy exposure, heating, or both.
16. Repeat **steps 8–13** to coat a second layer of photoresist. Use the MA6 alignment machine to align the second-layer photomask with the exposed first layer feature. The success of alignment is determined by overlapping of alignment marks in the photomask and alignment marks in the first layer. After alignment, UV exposure is applied to the wafer.
17. Repeat **step 15**.
18. Submerge the wafer in SU-8 developer in a Pyrex® dish. Leave the wafer in the developer for 6–9 min (*see* **Note 21**). Wash the developed wafer with running SU-8 developer for 20 s, using a wash bottle. Rinse the resist layer with IPA for another 20 s.
19. Dry the wafer surface with a filtered, pressurized inert gas pistol. During the drying, white marks indicate an incomplete development. If the developing time is significantly longer or shorter than the indicated time, the problem may have occurred during the SU-8 spin coating or energy exposure steps.
20. The resolution of the master's feature sizes can be measured using a profilometer (*see* **Note 22**).
21. Place the master in a desiccator next to a small weigh boat (or a small vial) containing 2–3 drops of trichloro (1H,1H,2H,2H-perfluorooctyl) silane. Connect the desiccator to a vacuum line and evacuate the air from the desiccator. Incubate the master in the desiccator for at least 30 min to allow the silane to evaporate and form a monolayer on the surface of the master. Silanizing the master is important for preventing the PDMS from adhering to the master during PDMS device fabrication. The master is now ready to be used for PDMS device fabrication.

3.2 Device Fabrication

1. Fill a weigh boat with 10 parts of Sylgard 184 monomer base and 1 part of the curing agent, by weight (*see* **Note 23**).

2. Use a wooden stirrer to mix the two components thoroughly, for at least 3 min. Small bubbles will form in the mixture and can be used as guides to determine the extent of mixing. Ensure that the mixture is homogeneous, since incomplete mixing may affect the mechanical properties of the cured PDMS.
3. Place the master in a petri dish and pour the polymer mixture to the desired thickness. Alternatively, you can also encompass the master using tin foil to form a tin foil boat. In either case, you should ensure that the bottom-side of the silicon wafer is flat; an uneven surface may cause the wafer to break more easily when cutting PDMS out of it, in later steps.
4. Place the petri dish in a desiccator connected to a vacuum line and turn on the vacuum. Incubate the master in the vacuum for 20–30 min, or until most bubbles have disappeared or risen from the surface of the master. Degassing time will vary depending on the amount of PDMS and the width of the container. Turn off the vacuum and vent the desiccator. This step can be repeated to fully remove all bubbles from the PDMS mixture.
5. Place the master layered with PDMS in an oven at 70 °C, and cure for at least 1 h (*see* **Note 24**).
6. Take the master out of the oven and allow it to cool down to room temperature. Cut out the desired PDMS pieces using a razor blade, or scalpel, and place the PDMS on a clean plastic sheet with the features facing down. The PDMS piece should be cut to be the same size as a microscope glass slide (3 in. × 1 in.). Be careful not to apply too much pressure on the master, as it may break.
7. Use a micro-puncher to create holes in the PDMS pieces to serve as inlets and outlets; the holes should be punched at each end of the fluidic channels. Ensure that the punched holes are equal or slightly smaller than the outer diameter of the tubing you use in Subheading 3.3.
8. Place the PDMS pieces and standard microscope glass slides in a glass dish (e.g., Pyrex®) with a solution of detergent and ionized water. Place the container in an ultrasonic cleaning bath for at least 30 min to wash off any debris on the PDMS microfluidic features and glass slides.
9. Rinse the glass slides thoroughly with DI water to remove any remaining detergent. Use an acetone wash bottle to rinse the slides for 10 s, followed by a DI water rinse for 30 s. Rinse off the glass slide with ethanol for 10 s and dry it off with an inert gas pistol. Place the prepared microscope glass slides in a covered hood to avoid dust particles and other debris from settling on their surface.

10. Rinse a PDMS piece with DI water for at least 1 min, ensuring that the detergent has been washed off completely. Rinse off the PDMS piece with ethanol for 10 s using a wash bottle, focusing on the feature side. Dry off the PDMS piece with an inert gas pistol and place it inside a covered hood, to avoid debris from falling on the feature side of the PDMS pieces.
11. Treat the feature side of a PDMS piece and one side of a microscope glass slide with a corona discharger for 5 min (each). Align the corona-treated PDMS piece, feature side down, against the corona-treated microscope glass slide and apply pressure evenly from one end to the other, to ensure proper bonding and to avoid bubbles between the glass and the PDMS. You may also treat the PDMS–glass interface around the device to further enhance the bonding at the edges (*see* **Note 25**).
12. Place the device in an oven at 70 °C for 1 h to strengthen the permanent PDMS–glass bond. The microfluidic device and its design is shown in Fig. 1.
13. Protect the inlet and outlet of the PDMS device from debris by covering them with Parafilm. Store the PDMS devices in 35-mm petri dishes until use. Alternatively, continue to the next step without covering the device if it is to be used at the time. Starting the steps in Subheading 3.3, immediately after the PDMS–glass bonding will make the initial device fill easier, since bubbles will not form as often in recently treated hydrophilic PDMS channels.
14. Repeat **steps 10–13** with other PDMS pieces.

3.3 Device Functionalization

1. Attach luer-to-barb adapters to 10 in. of tubing. Insert the adapter–tubing complex to the ends of three sterile 3-mL Luer-lock syringes.
2. Fill one sterile 3-mL Luer-lock syringe with anhydrous ethanol, another sterile 3-mL syringe with DPBS, and a third sterile 3-mL syringe with 2% BSA in DPBS. When a syringe is loaded on the pump, you should expel air bubbles within the syringe and tubing prior to inserting the tubing into a device's inlet hole.
3. Microchannels are easily filled with ethanol due to its hydrophilicity. Load the pump with the syringe filled with anhydrous ethanol. Place a few drops of ethanol at the inlet hole of a prepared device, and the ethanol solution should fill most of the device up via capillary forces. Insert the tubing into the inlet hole, and wash the microchannels with three ethanol washing steps at a high flow rate (900 μL at 2 μL/s) (*see* **Note 26**). Repeat the three washing steps but with DPBS buffer this time (900 μL at 2 μL/s) (*see* **Note 27**).

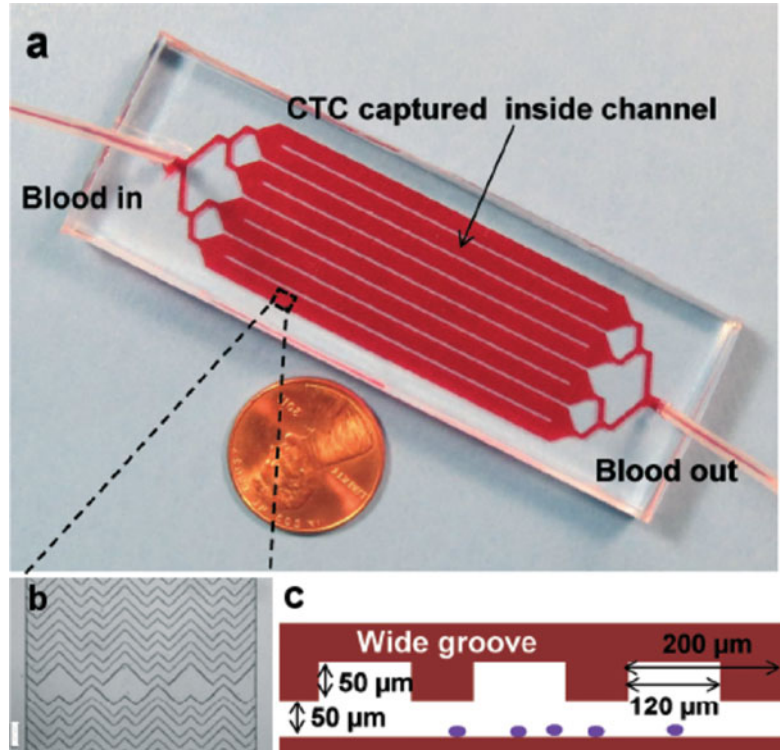

Fig. 1 (**a**) The microfluidic chip, consisting of a single inlet that bifurcates into eight parallel channels that bifurcate into an outlet (each channel is 2.1 mm wide and 50 mm long). (**b**) Micrograph (4× magnification in bright field) of the staggered chevron grooves within a channel, showing their asymmetry and periodicity; scale bar = 200 μm. (**c**) Cross-sectional view of the grooves within the microchannels, with a channel depth of 50 μm and a groove depth of 50 μm; the groove pitch is 200 μm and the groove width is 120 μm. Adapted with permission from [6]

4. At this point in time, you should make sure there are no bubbles housed within the microdevice. If there are no bubbles, move on to the next step. If there are bubbles, you can try removing them via extra washing steps or by tapping the top of the PDMS piece lightly during a washing step. If bubbles still persist, you may use higher flow rates (i.e., 10 μL/s) or use manual force to push the fluid in the syringe into the device. However, be cautious since a higher flow rate may cause device debonding if irreversible bonding between the PDMS piece and the glass slide is not strong enough.
5. A 1-mL pipette tip, with 2 cm of the tip cut off (truncated), is inserted inside the end of the tubing which is connected to a filter flask. The flask is connected to a pressure gauge and a precision vacuum regulator (0–30″ Hg) that is then connected to a vacuum line. This setup, depicted in Fig. 2 below, is used to

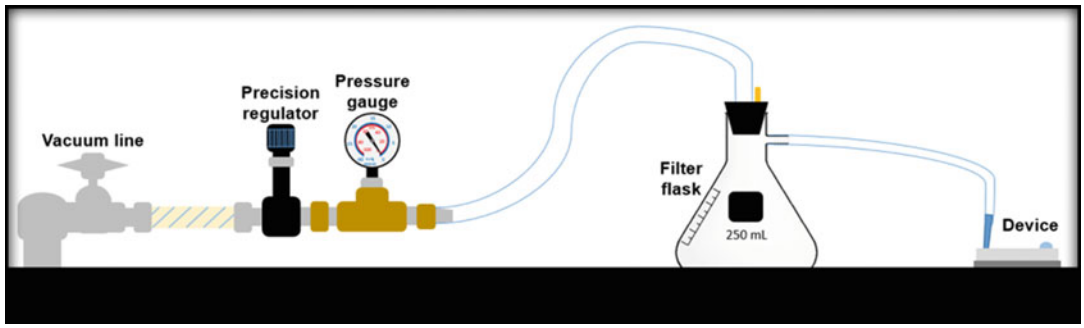

Fig. 2 Vacuum-enabled reagent introduction setup. The vacuum line is directly connected to a precision vacuum regulator and pressure gauge, which are then connected to a filter flask that is attached to tubing with a truncated pipette tip at the end. The pipette tip is pressed up against the microfluidic device inlet/outlet to withdraw the reagent solution into the device

introduce low volume reagents (100 μL) into the microfluidic devices.

6. Use a pipette to remove excess liquid from the inlet hole, leaving enough liquid for fluid continuity to avoid the introduction of bubbles into the device. Place a large droplet (100 μL) of avidin solution (1 mg/mL) at the device inlet. Place the truncated syringe tip attached to the vacuum line at the device outlet to begin withdrawing the avidin solution into the microchannels. Ensure that the vacuum pressure is very low (close to 0 mmHg) during this step. Incubate the device at room temperature for 15 min (*see* **Note 28**).
7. After the avidin incubation is completed, rinse the device with three DPBS washes at a medium flow rate (900 μL at 1.5 μL/s).
8. Incubate the device with a 100-μL solution of 20 μg/mL biotinylated anti-EpCAM and 1% BSA in DPBS at room temperature for 15 min.
9. Rinse the device with three medium-flow rate washes (900 μL at 1.5 μL/s) with a solution of 1% BSA in DPBS. Incubate the BSA solution in the device for at least 20 min. The device is now ready for sample introduction. If the sample is not ready to be processed, the device can be stored at room temperature. If the device needs to be stored longer than 2 h, it should be stored at 4 °C to preserve antibody immobilization.

3.4 Sample Processing

1. Invert the Ficoll-Paque bottle several times to ensure a homogeneous mix. Snap off the propylene cap to expose the rubber stopper. Disinfect the rubber stopper with alcohol on a cotton swab and allow it to dry. Puncture the rubber stopper with the needle of a 5-mL syringe, and transfer 10 mL of the Ficoll-Paque media to a sterile 50-mL centrifuge tube.

2. Invert the vacutainer several times and remove the cap to draw out the 10-mL patient blood sample (*see* **Note 29**), using a sterile 10-mL serological pipette, and transfer it to a sterile 50-mL centrifuge tube; keep the serological pipette in the vacutainer after use.
3. With a new sterile 10-mL serological pipette, add 10 mL (or equivalent volume to the blood sample) of Ca^{2+}/Mg^{2+}-free DPBS to the blood sample in the 50-mL tube; keep the serological pipette in the DPBS container. Use 2 mL of the diluting DPBS solution to rinse out any remaining blood from the walls of the vacutainer and the cap, and transfer it to the blood sample in the 50-mL tube. Use the same serological pipette (blood-treated) from the vacutainer to mix the blood and buffer via pipetting.
4. Very slowly and carefully layer the diluted blood sample on top of the Ficoll-Paque media; make sure to not mix the blood and the Ficoll-Paque solution. The Ficoll media and diluted blood should be separate layers, as shown in Fig. 3. If you're using a pipette controller with a speed setting, use the gravity (G) setting. Use 2 mL of the diluting DPBS solution to rinse out any remaining blood from the walls of the 50-mL tube (that contained the diluted blood) as well as from the serological pipette, and add it on top of the blood sample already layered on top of the Ficoll-Paque solution.
5. Centrifuge the tube at 800 × *g* for 30 min at 18 °C (*see* **Note 30**) with slow acceleration and no breaks. Make sure to balance the rotor with a 50-mL centrifuge tube filled with the same volume of water, or with a second blood sample processed in parallel.
6. After centrifugation, the 50-mL sample tube should be layered as it is depicted in Fig. 4. Withdraw the upper layer containing

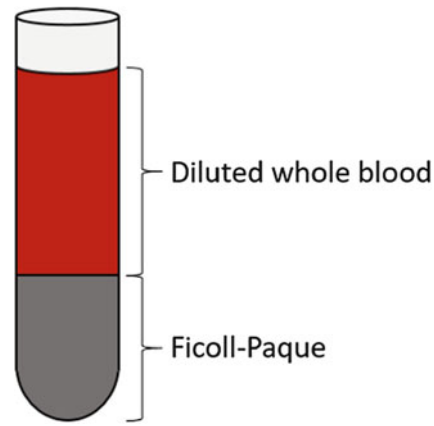

Fig. 3 Diluted blood sample layered over the Ficoll-Paque solution

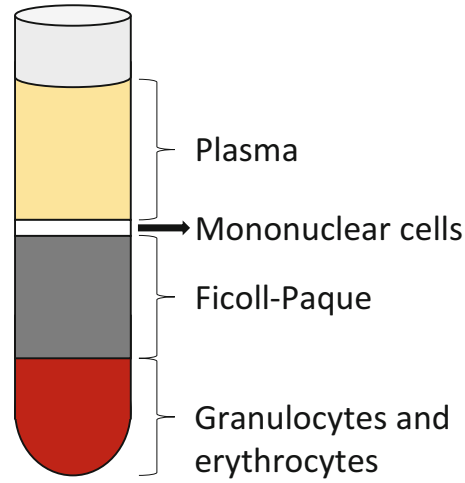

Fig. 4 Blood sample separated into gradients via centrifugation. The bottom-most layer is the hematocrit, composed mainly of red blood cells. Above the hematocrit lies the Ficoll-Paque solution, which serves to separate the hematocrit from the desired mononuclear cell layer. Above the mononuclear cells are plasma

plasma and platelets, using the blood-treated serological pipette, and transfer to a different tube for storage and analysis, or dispose of it properly (*see* **Note 31**). Leave some of the upper plasma layer above the undisturbed layer of mononuclear cells (*see* **Note 32**).

7. Using the blood-treated serological pipette, transfer the layer of mononuclear cells to a new sterile, BSA-treated 50-mL centrifuge tube. You can withdraw some of the Ficoll-Paque layer, leaving some of the layer above the bottom hematocrit (*see* **Note 33**).
8. Using the diluting DPBS solution, fill up the sample tube to the 50-mL mark. Use pipetting to homogenize the sample, then cap the centrifuge tube. Centrifuge at 300 × *g* for 10 min at 18 °C (max acceleration and breaks). Make sure to balance the rotor.
9. Discard the supernatant (*see* **Note 31**). Add 30 mL of 2% FBS in Ca^{2+}/Mg^{2+}-free DPBS. Use pipetting to ensure the sample is thoroughly mixed and the cell pellet has been fully resuspended. Cap the tube and centrifuge it at 300 × *g* for 10 min at 18 °C (max acceleration and breaks). Make sure to balance the rotor.
10. Discard the supernatant (*see* **Note 31**). Resuspend the cell pellet in 1 mL of 2% BSA in DPBS (equivalent to 100 μL of buffer for every 1 mL of whole blood prepared) and transfer it to a sterile 2-mL microcentrifuge tube (*see* **Note 34**). Make

sure the 2-mL centrifuge tube has a relatively flat bottom, to facilitate sample withdrawal in the following step.

11. To avoid cell settling in the 1-mL sample syringe, a micromagnetic stirring bar is placed inside. A magnetic stir-plate is placed next to the syringe pump, so that the end of the syringe is above the plate. The mixing within the syringe keeps the cells in suspension while the sample is pumped through the device.
12. Use the 1-mL sample syringe to withdraw the final cell suspension from the 2-mL microcentrifuge tube. Connect a luer-to-barb adapter attached via tubing to the syringe, and secure the syringe on the pump. Place the magnetic stir-plate below the tip of the sample syringe; you may need to raise the height of the stir-plate to move it closer to the syringe tip.
13. Set your syringe pump to inject 800 μL (equivalent to 8 mL of whole blood, *see* **Note 35**) at 1 μL/s (equivalent to 3.6 mL/h). Insert the tubing from the sample syringe into the inlet hole of the microdevice (*see* **Note 26**). Start your pump to introduce the sample into the antibody-functionalized device. Collect the outlet stream by directing it to a microcentrifuge tube via tubing; the outlet stream solution can then be analyzed separately, reprocessed in another device, or treated as waste (*see* **Note 31**).
14. Wash the device with three high-flow rate DPBS washes (900 μL at 2 μL/s). You should treat all outlet streams, following this washing step, as biohazardous waste (*see* **Note 31**).
15. Fill a sterile 3-mL syringe with a solution of 4% PFA in DPBS, and attach a luer-to-barb adapter and tubing. Introduce 300 μL of the 4% PFA solution at 1 μL/s and incubate for 10 min. After the incubation time, wash the device with three high-flow rate DPBS washes (900 μL at 2 μL/s).
16. Fill a sterile 3-mL syringe with a solution of 0.2% Triton X-100 in DPBS, and attach a luer-to-barb adapter and tubing. Introduce 300 μL of the 0.2% Triton X-100 solution at 1 μL/s and incubate for 10 min. After the incubation time, wash the device with three high-flow rate washes (900 μL at 2 μL/s) using 2% BSA in DPBS solution. Incubate the BSA solution in the device for at least 20 min.
17. Using sterile pipette tips in a covered hood, create a fluorescent antibody cocktail by mixing 10 μL of 12.5 μg/mL anti-CD45-PE, 10 μL of 25 μg/mL anti-CK-FITC, and 80 μL of 300 nM DAPI.
18. Introduce the 100-μL fluorescent antibody solution into the device using the vacuum line (refer to **steps 5** and **6** from Subheading 3.3). Incubate at room temperature for 30 min, shielded from light, and rinse out the device with three high-flow rate DPBS washes (900 μL at 2 μL/s).

3.5 CTC Detection

1. Turn on the computer and fluorescence light source (*see* **Note 36**). Place the microdevice in the glass microscope slide slot.
2. Use the bright field channel, with 4× magnification, to inspect the microchannels and bonding of the PDMS to the glass. Use the automatic scanning function to take an overview image of the entire device's microchannel area (*see* **Note 37**). This bright field overview image will serve as a map to guide the operator through the device's fluidic boundaries.
3. Switch to the FITC fluorescent channel and use the automatic scanning function to image the entire device's microchannel area with 10× magnification.
4. Repeat **step 3** using the DAPI and CY3 fluorescent channels.
5. Using the imaging software (*see* **Note 38**), the DAPI, FITC, and CY3 fluorescent overview images are overlaid. The images are ready to be analyzed.
6. During analysis of the overlaid fluorescent channels, the FDA-approved definition of a CTC is used. The cell must be DAPI-positive, CK-positive (FITC-green), and CD45-negative (PE-red) (*see* **Note 39**) as shown in Fig. 5. Cell's morphological features such as cell size and shape, preserved cell nucleus, entire nucleus housed within cytoplasm, and increased nuclear to cytoplasmic (N/C) ratio are also considered.

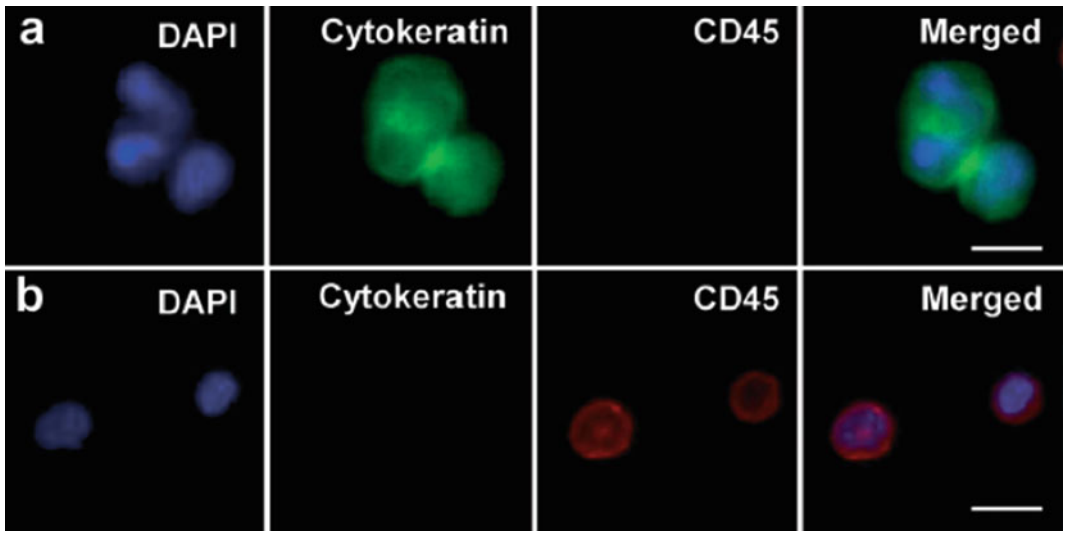

Fig. 5 Fluorescent microscope images (40× magnification) of CTCs captured from patient blood samples; separate microscope channels (DAPI, FITC, and CY3, respectively) as well as the merged image. (**a**) Representative image of three CTCs (DAPI+, Cytokeratin+, and CD45−). (**b**) Images of nonspecifically captured white blood cells (DAPI+, Cytokeratin−, and CD45+). Scale bar = 10 μm. Reproduced with permission from [6]

4 Notes

1. Turnaround time for transparency printing service is normally 3–5 business days (including the shipping time by a professional printing shop), while a chrome photomask may take a week or two. Either one of these options may work, depending on the smallest feature size of the microfluidic design.
2. It is not recommended to use test quality wafers since their deformities are often severe enough to disrupt silicon master fabrication.
3. Waste disposal regulations should be followed with all chemicals, bio-reagents, and sharps; always protect yourself by wearing personal protective equipment (PPE).
4. Piranha solutions are used to remove organic residues from substrates; the solution is highly corrosive and a powerful oxidizer. Acid piranha is a 3:1 mixture of concentrated sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2), respectively. When preparing, always add the peroxide to the acid very slowly. Whenever handling piranha, only use glass containers. Prepare only as much as is needed, do not store any piranha solution. Piranha solution must be handled on a solvent bench with good ventilation. Proper protection clothes (nonflammable), thick rubber gloves, and helmet must be used during preparation. Piranha solution waste should be disposed of properly in a glass container; they should not be disposed of before cooling down to room temperature. Heated piranha solution in the waste container may also cause an explosion. *Caution:* if the H_2O_2 concentration is at 50% or greater, an explosion could occur.
5. A headway photoresist spinner was used.
6. SU-8 2035 photoresist is recommended. However, other photoresists with different viscosities will work as well (e.g., 2025, 2050, and 2075). The steps in Subheading 3.1 are specific to using SU-8 2035 photoresist for generating a two-layer silicon wafer master, in which each layer is 50 μm thick.
7. The Karl Suss MA6 is used. The SU-8 photoresist is in hard contact with the photomask. The photomask should be cleaned prior to this step to ensure there are no dust particles or other artifacts that may lead to an imperfect contact.
8. Alternatively, you can use plastic silverware or a pipet tip to stir the PDMS mixture. Plastic silverware is useful for quickly and easily mixing the PDMS mixture. However, they should be thoroughly cleaned if they are reused, as PDMS can cure at room temperature and may contaminate your next batch.

9. Another type of container may be used; wider containers may be better since they expose a larger surface of the contained fluid to air, which would aid in degassing the PDMS. Alternatively, you may use tin foil to wrap the silicon master and enclose the PDMS prepolymer solution.
10. The BD-10AS high frequency generator (Electro-Technic Products, Chicago, IL) was used. This lightweight handheld high frequency generator is meant for intermittent use, not to be operated for more than 10 min at a time. Alternatively, an oxygen plasma chamber or a UV ozone machine may be used under proper conditions. Please follow all safety regulations and read equipment instructions prior to use; high voltage equipment may cause harm to operator if used improperly.
11. FEP (Fluorinated Ethylene Propylene) tubing is mostly used for low-pressure microfluidics since it exhibits desired properties such as biocompatibility, flexibility, optical clarity, and resistance to most chemicals. Other tubing, such as fluorinated polymer tubing may also be used.
12. Chromatopur™ bovine albumin, low IgG, immunoassay grade was used. It is recommended that all pipette tips, centrifuge tubes, syringes, micromagnetic stir bars, and devices should be treated or rinsed with a solution of 2% BSA in DPBS prior to use with whole blood or the final sample suspension. BSA solutions should be aliquoted and stored at −20 °C; they may be stored at 4 °C for a limited length of time. Do not use BSA solution that has become turbid, as it will cause issues during Subheading 3.3.
13. KDS Legato (KD Scientific, Holliston, MA) and PHD Ultra (Harvard Apparatus, Holliston, MA) syringe pump series were used. These pumps have dual-syringe capability, allowing for 2 devices to be functionalized or used at one time.
14. Antibody clone selection is very important in CTC analysis. The clones used in our study have been previously shown to consistently work in clinical and translational studies.
15. It is recommended to use Ficoll-Paque PREMIUM of 1.077 g/mL (± 0.001 g/mL).
16. It is recommended to use the dilactate salt form of DAPI as it is more water soluble than the dihydrochloride salt. Use caution when using DAPI since it is a known mutagen and should be handled with care. The dye must be disposed of safely and in accordance with the applicable local regulations.
17. There is a range of options for fluorescent dye and filter selection. For CTC applications, FITC- and PE-conjugated antibodies are most commonly used. It is important to ensure that there is minimal overlap in fluorescent excitation and emission

Table 1
Fluorescent filter selection used. All filters were purchased from Chroma Technology Corporation (Bellows Falls, VT)

Fluorophore	DAPI	FITC	CY3
Excitation (EX)	AT350/50×	HQ480/40×	ET545/25×
Beam splitter (BS)	T400lp	Q505lp	T565lpxr
Emission (EM)	ET460/50 m	ET525/30 m	ET605/70 m
Set catalog #	49000	49011+q505lp	49004

between dyes and filters used. The filter selection is shown below in Table 1.

18. There is a buildup of photoresist at the wafer edges during spinning. By removing any edge bead, the photomask can be placed into contact with the silicon wafer, resulting in the best resolution using contact lithography.
19. Soft bake includes 65 °C and 95 °C heating step. The 65 °C heating step helps smoothen the photoresist layer and promote uniformity. The 95 °C heating step increases solvent evaporation and hardens the photoresist. If the soft bake is too short, it will make the photoresist soft and sticky. The photomask can be contaminated during contact exposure and the photoresist layer can be destroyed. After the soft bake of the SU-8 is complete, the wafer can be kept in a box away from light and then be exposed, baked, and developed within a month. Therefore, the time before exposure is not a critical parameter. However, it is recommended to continue to the next step rather than waiting.
20. This energy exposure dose is specific to a photoresist layer thickness of 50 μm. This is one of the most important parameters to achieve the desired dimensions of the design. If the exposure time is too short, the photoresist may not be cross-linked over its entire depth, therefore it will lift off during the following development step. If the exposure time is too long, the width of the channels may be increased.
21. This development time is specific to a photoresist layer thickness of 50 μm.
22. The Dektak 150 surface profilometer was used.
23. It is recommended to use unpowdered gloves in this and all following steps to avoid contamination of the PDMS.
24. The curing process will vary, highly dependent on the amount of PDMS, thickness, and surface area exposed. PDMS can be cured between 25 and 150 °C, per the manufacturer. Curing at higher temperatures requires less time, while using lower

temperatures requires longer time. Leaving the PDMS to cure overnight is acceptable, as excess time will not affect the results.

25. PDMS is hydrophobic with a low-energy surface that is nonreactive. Exposing PDMS to corona discharge makes the PDMS surface temporarily hydrophilic and reactive. This allows for irreversible bonding of PDMS to glass, silicon, or another PDMS piece that was also treated with corona discharge.
26. When inserting tubing to the inlet hole of a device, ensure that all air has been expelled from the syringe, the luer-to-barb connection, and from within the tubing itself. This will prevent unwanted introduction of bubbles into the microdevice.
27. Each device wash is approximately 3 times the device volume (100 μL ×3). For this device, we utilize three different levels of flow rates. A low flow rate is 1 μL/s, a medium flow rate is 1.5 μL/s, and a high flow rate is 2 μL/s. These flow rate recommendations are highly device-specific.
28. During this incubation period, you should begin preparing the blood sample if it is available (move onto Subheading 3.4). Anytime the device is kept at room temperature, ensure the inlet and outlet are kept wet to avoid air introduction into the device due to fluid evaporation.
29. The blood sample should be as fresh as possible and free of clots. Delay in processing blood samples can result in loss of cell viability, lower cell recovery, and higher contamination of red blood cells. If a blood sample can not be processed immediately, it should be stored at room temperature rather than in the fridge (4 °C) to prevent cell loss [7]. An anticoagulant can be added to the blood sample to ensure it is free of clots, though most vacutainers are coated with an anticoagulant.
30. It is recommended to use a centrifugation temperature of 18 °C by the manufacturer. However, if temperature control is not available for centrifugation, room temperature will work as well.
31. Dispose of biomedical waste properly. Treat any materials contaminated with blood with bleach for at least 20 min, and rinse with water. You should also treat excess blood sample or sample supernatant with bleach in this manner. Bleach (sodium hypochlorite) can be a hazardous chemical when not handled properly; use proper workplace controls and practices as well as PPE when handling bleach.
32. You should take extreme caution to avoid disturbing the layer of mononuclear cells. It is also possible to withdraw the layer of mononuclear cells without first removing the upper plasma layer. As an alternative to **step 6** (Subheading 3.4), you can transfer the plasma and mononuclear layers together. Drawing excess plasma layer causes unnecessary contamination by

platelets and plasma proteins; however, these would be washed away in the following centrifuge washing steps.

33. The desired mononuclear layer is theoretically separate from the Ficoll-Paque solution below it. However, in practice, some mononuclear cells will be suspended within the Ficoll-Paque solution. Therefore, it is suggested to withdraw as much of the upper layer of the Ficoll-Paque layer without extracting the hematocrit layer below. Keep in mind that drawing excess Ficoll-Paque increases granulocyte contamination.
34. There will be 50–150 μL of remaining liquid sample at the bottom of the tube when you discard the supernatant. You can use a standard micropipette to measure and take this into account when creating your final 1-mL cell sample. It is advised to treat the 2-mL microcentrifuge tube with 5% BSA solution (in DPBS) for at least 1 h prior to use, in order to reduce unwanted cell losses.
35. Although 10 mL of the whole blood sample was treated, only 8 mL is introduced into the device. Keep in mind that it is not possible to introduce all of the prepared sample volume within the syringe due to physical constraints; the micro stir bar will block the piston from pushing all of the fluid inside the syringe out. Furthermore, there is some dead volume within the syringe, the luer-to-barb adapter, and the tubing.
36. Some light sources, such as xenon and mercury lamps, require 5–10 min to stabilize their arcs after the burner is ignited.
37. If automated or semiautomated scanning hardware and software is not available, manual scanning and inspection can be performed. Individual cell images should be taken and analyzed for marker expression, or lack thereof. The same CTC enumeration guidelines from **step 6** (Subheading 3.5) should be used.
38. Image editing software such as Adobe Photoshop® (Adobe, San Jose, California) has also been used to overlay fluorescent images.
39. A threshold is typically used to determine whether a specific cell is considered to be positive or negative for the expression of a given marker. This threshold was obtained from previously quantified CTCs.

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Chapter 4

Microfluidic-Based Enrichment and Retrieval of Circulating Tumor Cells for RT-PCR Analysis

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Abstract

Molecular analysis of circulating tumor cells (CTCs) is hindered by low sensitivity and high level of background leukocytes of currently available CTC enrichment technologies. We have developed a novel device to enrich and retrieve CTCs from blood samples by using a microfluidic chip. The Celsee PREP100 device captures CTCs with high sensitivity and allows the captured CTCs to be retrieved for molecular analysis. It uses the microfluidic chip which has approximately 56,320 capture chambers. Based on differences in cell size and deformability, each chamber ensures that small blood escape while larger CTCs of varying sizes are trapped and isolated in the chambers. In this report, we used the Celsee PREP100 to capture cancer cells spiked into normal donor blood samples. We were able to show that the device can capture as low as 10 cells with high reproducibility. The captured CTCs were retrieved from the microfluidic chip. The cell recovery rate of this back-flow procedure is 100% and the level of remaining background leukocytes is very low (about 300–400 cells). RNA from the retrieved cells are extracted and converted to cDNA, and gene expression analysis of selected cancer markers can be carried out by using RT-PCR assays. The sensitive and easy-to-use Celsee PREP100 system represents a promising technology for capturing and molecular characterization of CTCs.

Key words Microfluidic chip, Size filtration, Breast cancer, CTCs, Cell retrieval, RT-PCR

1 Introduction

Circulating tumor cells (CTCs) have been demonstrated to be present in breast, prostate, and colon cancer patients' blood and bone marrow [1–3]. CTCs are rare in patients, as few as one cell per 100 million or 1 billion blood cells. However, molecular characterization of CTCs may provide a greater understanding of the disease metastases, identify aggressive tumors and enable therapeutic selection and monitoring of disease for patients undergoing treatment [4, 5]. A variety of technologies have been developed to improve detection and capture of CTCs from peripheral blood, which include immune-magnetic bead separation using monoclonal

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antibodies targeting cell-surface antigens for positive or negative selection, cell sorting using flow cytometry, filtration based size separation, density gradient centrifugation, microfluidic devices, and fast-scan imaging [6–10]. For example, CellSearch™ was the first CTC technology that demonstrated its clinical validity in predicting progression-free survival and overall survival of metastatic cancer patients based on CTC enumeration [3–6].

It is of great interest to go beyond cell enumeration and further characterize the CTCs by assessing molecular markers on or within CTCs to gain insight into mechanisms of metastasis and best treatment modalities for patients [1–3, 11, 12]. For example, significant progress has been made in breast cancer care, including effective hormonal therapy, chemotherapy therapies, and targeted therapies against estrogen receptor (ER) and HER-2. It becomes critically important to determine which patients are most likely to benefit from specific therapies. Established clinical, pathologic features and biomarkers status are routinely used to guide treatment options. To detect such molecular markers using a minimally invasive blood test for CTCs has a great potential for use in clinical practice to guide therapy choice for patients. However, despite advances in CTC technologies, the low frequency of CTCs in cancer patients and the extensive background leukocytes have limited the synergism of biomarkers and CTC echnologies [11, 12].

We have developed a novel microfluidic device, Celsee PREP100 that uses a size and deformability based capturing mechanism of CTCs. The microfluidic chip has a parallel network of fluidic channels which contain about 56,000 capture chambers [13, 14]. The chip fabrication begins with a silicon master device containing microfeatures. The microfeatures make up a fluidic network (75 μm deep), leading to individual cell trapping chambers (20 μm × 25 μm × 30 μm) with pore size of 10 μm × 8 μm. Each chamber ensures smaller blood cells such as red blood cells and most of the leukocytes escape while larger cancer cells get trapped and isolated in the chamber. The manufacturing process uses standard photolithography and deep reactive ion etching for microfabrication. From the master device, a soft elastomeric negative mold is created by pouring and curing against the silicon master. The final microsubstrate is created by hot embossing a plastic plate made of cyclic olefin polymer (COP) against the elastomeric negative mold. A thin plastic laminate containing pressure-sensitive adhesive is then laminated against the COP microsubstrate to create the final microfluidic chip. The chip is placed on Celsee PREP100 device for CTC capturing.

We found that the device captured cells in a range of 10–2000 or more with high reproducibility. Using breast cancer cells spiked into normal donor blood, we determined that the capturing efficiency of the cells was greater than 80% [13]. Because the device captures cells using a label-free mechanism, the device provides an

open plan for the investigator to use a variety of antibodies to identify and characterize CTCs upon capturing [13, 14]. The captured CTCs can also be retrieved from the microfluidic chip for nucleic acid extraction and molecular analysis. The device can capture as low as 10 cells with high reproducibility using a back-flow procedure. The cell recovery rate of the procedure is 100% and the level of remaining background leukocytes is very low (about 300–400 cells). RNA from the retrieved cells are extracted and converted to cDNA, and gene expression analysis of cancer markers can be carried out by using RT-PCR assays.

2 Materials

2.1 Consumables

1. General reagent cartridge: The reagent cartridge has 10 chambers filled with the reagents required to run an assay. The user is required to fill three of these chambers, as outlined in Reagent Preparation, before processing a sample. Store at 4 °C. Do not freeze. Discard the cartridge prior to a run if the cartridge has leaked.
2. Microfluidic Slide: The Celsee microfluidic slide is Celsee Diagnostic's proprietary microfluidic CTC capture and analysis technology. The slide has one laminated side and the other side contains the inlet and outlet holes. Store the slides at room temperature (20–25 °C).
3. Manifold: The disposable Celsee PREP manifold holds the Celsee PREP microfluidic slide. Each manifold has a slide slot and an inlet and outlet port. The U-shaped cutout at one end of the manifold designates the outlet direction.
4. Inlet funnel: The inlet reservoir is a funnel through which blood and other reagents enter the manifold. Attach the inlet funnel firmly to the inlet port of the manifold.
5. Waste jar: The Celsee PREP100 waste jar is disposable and is optimized for one sample run.
6. Top cover: The top cover ensures that the microfluidic slide is held firmly in the manifold during CTC enrichment.
7. Vacuum syringe: The 10 mL syringe provided with the Celsee PREP100 is for pulling liquids into the waste jar. Attach the syringe adapter to the taller port of the waste jar.
8. Priming Genie: The Priming Genie is used to eliminate air from the microfluidic slides prior to adding blood.
9. BD Vacutainer® K2 EDTA tube (Becton, Dickinson and Company, Product Number 366643).
10. SepMate-15 tube (STEMCELL Technologies, Inc., Catalog # 85415).

2.2 Reagents

The following reagents are provided separately from the reagent cartridge:

1. Priming buffer.
2. Dilution buffer.
3. Fixing buffer.
4. Antibodies and nuclear stain.
5. RosetteSep Human CD45 Depletion Cocktail (STEMCELL Technologies, Inc., Catalog # 15122/15162).
6. Lymphoprep (STEMCELL Technologies, Inc, Catalog #07801).
7. AllPrep DNA/RNA Mini Kit (Qiagen, Catalog # 80204).
8. High Capacity cDNA Reverse Transcription Kits (Applied Biosystems. Catalog # 4368814).

2.3 Reagents to Prepare Before a Sample Run

1. Pretreatment buffer: To make 5 mL of pretreatment buffer, add 500 μL of the fixing buffer to 4.5 mL of the dilution buffer provided. Use 4 mL of pretreatment buffer for every 4 mL of blood. Make fresh pretreatment buffer before every run. Discard any leftover pretreatment buffer.
2. Primary antibody cocktail (1 reaction): Make 1 mL of the primary antibody cocktail in an Eppendorf tube by pipetting 5 μL of antibody 1A and 5 μL of antibody 1B into 1 mL of dilution buffer. Transfer this solution to the chamber labeled "primary antibody" on the reagent cartridge (*see* **Note 1**).
3. Secondary antibody cocktail (1 reaction): Make 1 mL of the secondary antibody cocktail in an Eppendorf tube by pipetting 1.5 μL of antibody 2A and 1 μL of antibody 2B into 1 mL of dilution buffer. Transfer this solution to the chamber labeled "secondary antibody" on the reagent cartridge.
4. Counterstain solution: Make a 1:10 dilution of the counterstain solution by pipetting 10 μL of stock solution to 100 μL of dilution buffer. Counterstain solution should be stored at 4 °C. Discard after 2 weeks. Add 4 μL of counterstain solution to the chamber labeled "counterstain" on the reagent cartridge.

3 Methods

3.1 Microfluidic Slide Setup on Celsee PREP100

1. Place the manifold onto the PREP100, locating it onto the pins.
2. Place the microfluidic slide onto the manifold ensuring the labels are facing up. The small holes visible on the underside of the microfluidic slide should be aligned with the black O-rings on the manifold.

3. Carefully place the top cover onto the microfluidic slide, locating the holes in the top cover with the pins on the PREP100.
4. Close both clamps onto the top cover to make a seal (Fig. 1).

3.2 Priming Microfluidic Slide to Remove Air Bubbles from the Microfluidic Slide

1. Priming buffer is rapidly passed through the microfluidic slide in both directions using the Priming Genie (Fig. 2) (*see* **Note 2**).
2. Ensure that the Priming Genie is plugged in. Clear out any liquid present in the tubing of the priming genie by turning it on in the "Forward" direction for 30 s into a paper towel.
3. Place an inlet funnel on the inlet port on the manifold
4. Add 8 mL priming buffer into the inlet funnel. Connect the inlet cover onto the top of the inlet funnel and the outlet of the Priming Genie to the outlet of the manifold.

Fig. 1 Microfluidic slide setup on Celsee PREP100

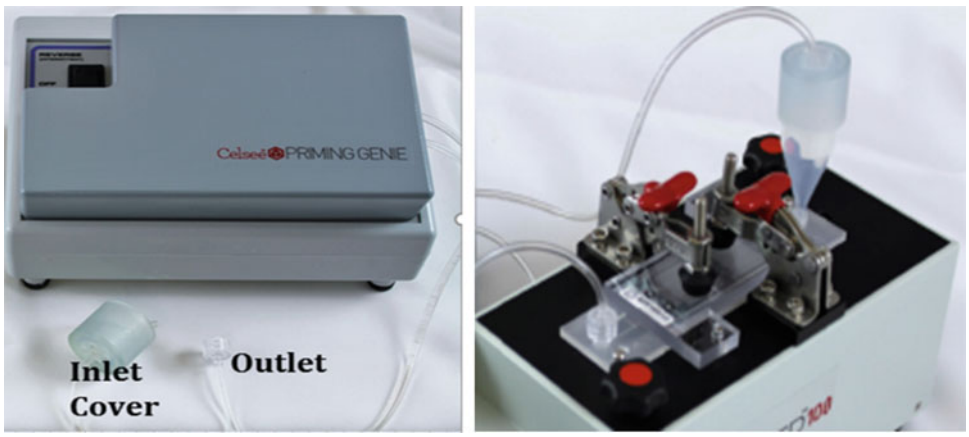

Fig. 2 Priming microfluidic slide to remove air bubbles from the microfluidic slide

5. Start the priming pump in the Forward direction for 60 s. You will start to see bubbles coming out of the outlet tube.
6. Reverse the priming pump for 30 s.
7. Forward pump for another 30 s.
8. Reverse pump for another 30 s.
9. Forward pump for another 30 s.
10. Reverse pump for another 60 s.
11. Remove the inlet cover from the inlet funnel and outlet tubing from the manifold. Pipette out most of the remaining priming buffer from the inlet funnel leaving approximately 2 mL at the bottom of the inlet funnel.

3.3 Processing Blood Through the Microfluidic Slide for Immunostaining of Cells

1. Collect venous blood specimen into a BD Vacutainer K2 EDTA tube. The specimen should be stored at ambient temperature and processed within 24 h of the collection.
2. Transfer 4 mL of blood to a conical tube and add 4 mL of pretreatment buffer. Incubate for 10 min on a rocker at a low speed.
3. Remove the Priming Genie fittings and attach the end of the outlet tubing provided on the waste jar to the outlet port of the manifold.
4. Push the 10 mL syringe plunger to the zero mark before attaching it to the longer port on the lid of the waste jar.
5. Add the blood suspension to the inlet funnel.
6. Pull the plunger on the syringe attached to the waste jar to the 10 mL mark and hold it there until approximately 100 μL remains in the inlet funnel. Remove excess fluid in the inlet funnel by using a pipette. Do not plunge the pipette into the manifold inlet port. Make sure there is always some fluid in the inlet funnel, otherwise air bubbles will enter the microfluidic chip and adversely affect the outcome of the assay. Use the syringe restraint to hold the plunger in its place (Fig. 3).
7. Remove the syringe from its adapter and immediately close the pinch valve on the outlet tube of the waste jar. Push the syringe plunger to the zero mark and reattach the syringe to the waste jar.
8. Add 4 mL of wash buffer from chamber 1 of reagent cartridge to the inlet funnel and open the pinch valve on the waste jar.
9. Fix the cells with 1 mL of fixing buffer from chamber 2 of the reagent cartridge and incubate at room temperature for 10 min.
10. Wash with 2 mL wash buffer from chamber 3 of the reagent cartridge.

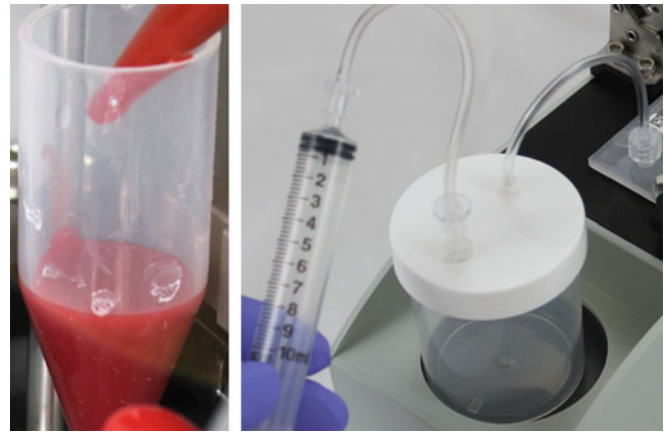

Fig. 3 Processing blood through the microfluidic slide

11. Permeabilize with 1 mL permeabilizing buffer from chamber 4 of the reagent cartridge for 10 min.
12. Wash with 2 mL wash buffer from chamber 5 of the reagent cartridge.
13. Block with 1 mL of blocking buffer from chamber 6 of the reagent cartridge for 20 min.
14. Next, add 1 mL primary antibody cocktail from the reagent cartridge and incubate for 45 min.
15. Wash with 4 mL wash buffer from chamber 5 of the reagent cartridge.
16. Add the secondary antibody cocktail from the reagent cartridge and incubate in dark for 30 min.
17. Add 4 mL of the counterstain solution from the reagent cartridge and incubate for 5 min
18. Observe captured cells on the slide under fluorescent microscope (*see* **Note 3**).

3.4 Processing Blood Through the Microfluidic Slide for Cell Enrichment and Retrieval

1. Collect venous blood specimen into a BD Vacutainer® K2 EDTA tube. The specimen should be processed within 24 h of the collection.
2. Transfer 4 mL of blood to a 15 mL conical tube.
3. Add RosetteSep Human CD45 Depletion Cocktail (STEMCELL Technologies) at 50 μL/mL of whole blood (For 4 mL of whole blood, add 200 μL of cocktail). Mix well.
4. Incubate for 20 min at room temperature using a slow rocker.
5. Dilute sample with an equal volume of PBS and mix gently (e.g., for 4 mL PBS for 4 mL whole blood).

6. Add 4 mL density gradient medium, Lymphoprep (STEMCELL Technologies) to SepMate-15 tube (STEMCELL Technologies) by carefully pipetting it through the central hole of the SepMate-15 tube insert.
7. Keeping the SepMate-15 tube vertical, add the diluted blood sample above by pipetting it down the side of the tube.
8. Centrifuge 1200 × *g* for 20 min at room temperature.
9. Pour off the top layer into a new 15 mL conical tube.
10. Add the sample to the disposable inlet funnel of PREP100.
11. The use of RosetteSep™ and SepMate™ in this protocol is optimized for use with the Celsee PREP100 system.
12. To start the run, set the supplied 10 mL syringe to zero, attach it to its adapter and then pull the plunger to the 10 mL mark. Once liquid starts to flow, the syringe stopper can be used to hold the plunger in place.
13. If the flow starts to slow down, decouple the syringe from the waste jar and remove the syringe stopper, being careful not to push the plunger in while it is still attached.
14. Stop the fluid flow when approximately 3–5 mm of fluid remains just above the straight section of the inlet funnel.
15. Add 2 mL of wash buffer at the inlet and draw the buffer through the microfluidic slide by pulling out the syringe plunger to the 5 mL mark.
16. Replace the used inlet funnel with a new inlet funnel. On the outlet side, remove the connector and replace it with a priming reservoir.
17. Add 1.0 mL of wash buffer to the priming reservoir.
18. Pull the plunger of the 60 mL syringe to the 60 mL mark and attach it to the outlet reservoir.
19. Push the plunger to the 50 mL mark. After 3 s pull the plunger back to the 60 mL mark. Hold for 3 s, then push back to 50 mL (Fig. 4).
20. Continue with this pulsing procedure until most of the wash buffer has been passed through the microfluidic slide to the inlet, leaving 3–5 mm of fluid in the priming reservoir. Caution: Always ensure that some buffer remains in the outlet reservoir.
21. Using a pipette, remove most of the fluid (containing cells) from the inlet funnel, leaving 3–5 mm of fluid above the straight section of the inlet funnel and place into a separate tube.
22. Repeat **steps 17–21** with an additional 1 mL of wash buffer.
23. Centrifuge at 1200 × *g* for 10 min at room temperature, gently remove the supernatant (*see* **Note 4**).

Fig. 4 Pulsing procedure to backflow cells from the microfluidic slide for cell enrichment and retrieval

24. Lyse cells, extract RNA, convert RNA to cDNA, and use 5 μL cDNA for PCR (*see* **Note 5**).

4 Notes

1. The antibodies and buffers are provided in kits of 8 or 20 reactions each. Store all reagents at 4 °C, in the dark. Do not freeze. Return the antibodies and buffers to storage immediately after use.
2. To clean the Priming Genie after use, rinse the tubes attached to the priming genie using DI water by dipping both the ends into a beaker with DI water and pressing either the forward or backward button for 30 s. After 30 s, clear out any liquid present in the tubing by turning the priming genie on for an additional 30 s into a paper towel and air-dry the tubing.
3. Cell counting: cells that are round and bright green (FITC filter) with large nucleus and not stained with red (Texas red filter) are counted (CK+/CD45−/DAPI+) as CTCs.
4. The procedure in Subheading 3.4 results in enriched cells that are depleted of leukocytes and are ready for lysis and nucleic acid extraction for downstream applications.
5. RNA extraction is carried out by using AllPrep DNA/RNA Mini Kit (Qiagen). The AllPrep DNA/RNA Mini Kit purifies genomic DNA and total RNA simultaneously from a single sample. Lysate from cells is first passed through an AllPrep DNA spin column to isolate DNA, then through an RNeasy® spin column to isolate RNA.
6. Conversion RNA to cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems).

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Chapter 5

Microscale Laminar Vortices for High-Purity Extraction and Release of Circulating Tumor Cells

Soojung Claire Hur, James Che, and Dino Di Carlo

Abstract

Circulating tumor cells (CTCs) are disseminated tumor cells that reflect the tumors of origin and can provide a liquid biopsy that would potentially enable noninvasive tumor profiling, treatment monitoring, and identification of targeted treatments. Accurate and rapid purification of CTCs holds great potential to improve cancer care but the task remains technically challenging. Microfluidic isolation of CTCs within microscale vortices enables high-throughput and size-based purification of rare CTCs from bodily fluids. Collected cells are highly pure, viable, and easily accessible, allowing seamless integration with various downstream applications. Here, we describe how to fabricate the High-Throughput Vortex Chip (Vortex-HT) and to process diluted whole blood for CTC collection. Lastly, immunostaining and imaging protocols for CTC classification and corresponding CTC image galleries are reported.

Key words CTC purification, CTC enumeration, High-throughput size-based cell separation, Microscale laminar vortices

1 Introduction

Circulating tumor cells (CTCs) are cancerous cells that are derived from a tumor and found in patients' circulatory systems. The relatively short life span of CTCs (clearance within 24 h [1]) makes the cells ideal candidates for identifying cellular biomarkers associated with recent genetic or phenotypic changes progressing in the tumor [2]. CTC counts in patients have been reported to correlate with overall survival and/or metastatic relapse and it was suggested as a surrogate predictive marker for cancer prognosis and first-line treatment efficacy [3–6]. There is also significant interest in the mutational profile and phenotype of CTCs, which may provide useful information in prescribing the most efficacious targeted treatment. In spite of their significant importance and utility in clinical and research applications [7, 8], the extremely low frequency of CTCs in blood (~10 CTCs in 1 mL of blood with

Mark Jesus M. Magbanua and John W. Park (eds.), *Circulating Tumor Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1634, DOI 10.1007/978-1-4939-7144-2_5,

millions of white blood cells) makes it difficult to implement accurate and routine CTC enumeration and molecular analyses.

The majority of current state-of-the-art techniques available for CTC classification and enumeration utilize affinity-based strategies, requiring knowledge and availability of predetermined CTC-specific biomolecular markers [9]. The most commonly implemented marker for CTC-capture is epithelial cell adhesion molecule (EpCAM) while cytokeratin staining is used as a complementary marker for increased specificity in discrimination [10, 11]. Evolving understanding of the molecular signatures of CTCs as well as substantial heterogeneity [12] causes difficulties to isolate comprehensive CTC populations using affinity-based methods. In addition, affinity-based approaches often have off-target binding, leading to low purity [13].

Attempts to address these shortcomings have led to development of numerous innovative CTC isolation technologies based on alternative approaches, such as size-based capture or negative depletion of blood cells [7, 9, 12, 14]. Although newly developed techniques can be used to acquire cells with low or no EpCAM and undergoing Epithelial-to-Mesenchymal (EMT) transitions [15], challenges remain in terms of purity, processing time and simplicity, cost, and the ability to perform downstream assays on cells in a seamless workflow. Thus, further high-throughput CTC enumeration and enrichment techniques are still being developed.

The Vortex technology passively and rapidly purifies larger CTCs in a high-throughput manner and releases them into a small volume. It was first demonstrated with a simple microfluidic device with geometries that generated microscale vortices at high flow speed (>1 m/s) (*see* Fig. 1a) [16, 17]. Because the cell-trapping phenomenon highly depends on cell sizes as well as channel dimensions, the range of cell size to be purified can be fine-tuned to further enhance trapping efficiencies or to broaden size distributions of collected CTCs, representing greater diversity [18]. Inherently high operational flow rates enable rapid processing of large volumes of clinical specimens for target cell enrichment with a low frequency of background blood cells which may interfere with downstream assays. The technology allows collecting CTCs from 8 mL of patient bloods within a roughly 20 min period, and purified CTCs can be collected off-chip in a relatively small volume (<200 μL).

The feasibility as a high-throughput rare cell purification technology has been evaluated using blood collected from breast, lung, and prostate cancer patients [18–20] as well as pleural fluids obtained from patients across a wide spectrum of illness and disease severity [21] (*see* Fig. 2). Notably, the High-Throughput Vortex Chip (Vortex-HT) found 85% of stage IV patients with CTC levels above healthy thresholds in contrast to 15% positive detection

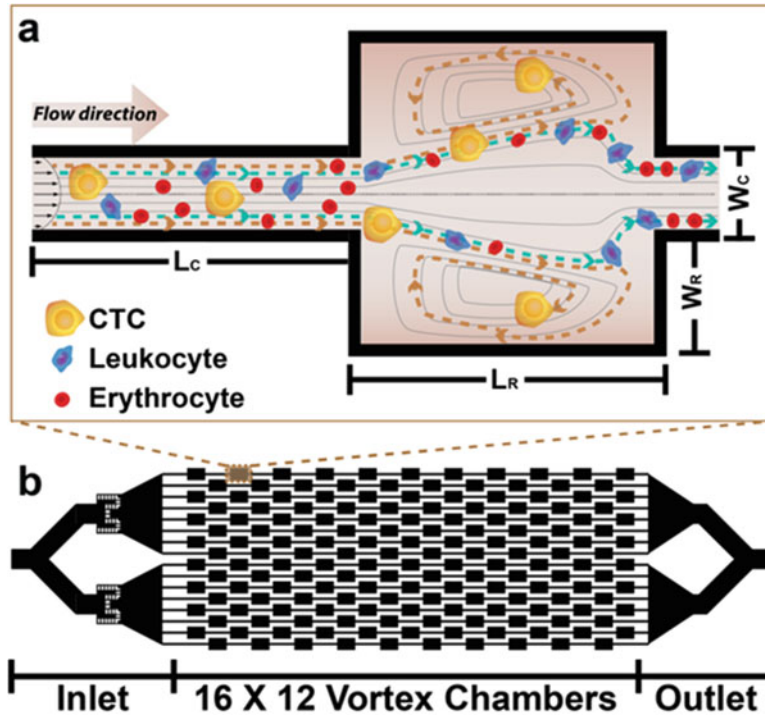


Fig. 1 (**a**) The schematic describes the size-based CTC trapping mechanism of the Vortex-HT. Large cells can be selectively trapped in microvortices due to a combination of size-dependent inertial lift forces and other fluid dynamic factors. The cells can be released from the chip into a desired container simply by lowering the solution injection speed. Highly pure CTC collection can be achieved by incorporating a device flushing step between the cell trapping and the releasing step. (**b**) The Vortex-HT consists of 16 parallel straight channels, individually comprising 12 cell-trapping chambers in series

obtained using the FDA-approved CellSearch system for 13 cancer patient samples tested [20]. Highly pure and viable CTCs collected off-chip are readily available for assays developed to enhance diagnostic accuracy, including immunostaining, cytological analysis, RT-PCR [21], genotyping [22], CTC culturing for pharmacological screening [23], and single-cell proteomics [24]. In addition, flexibility in sample collection containers, including conventional well plates, slide glasses, and/or microcentrifuge tubes, allows for integration of the Vortex technology with established workflows for subsequent assays without cell loss. Here, we describe how to fabricate the high-throughput prototype of the Vortex-HT using polydimethylsiloxane (PDMS) and detailed sample processing steps for isolation of CTCs from diluted blood. We also provide suggested guidelines to enumerate and classify collected CTCs among a background of white blood cells (WBCs).

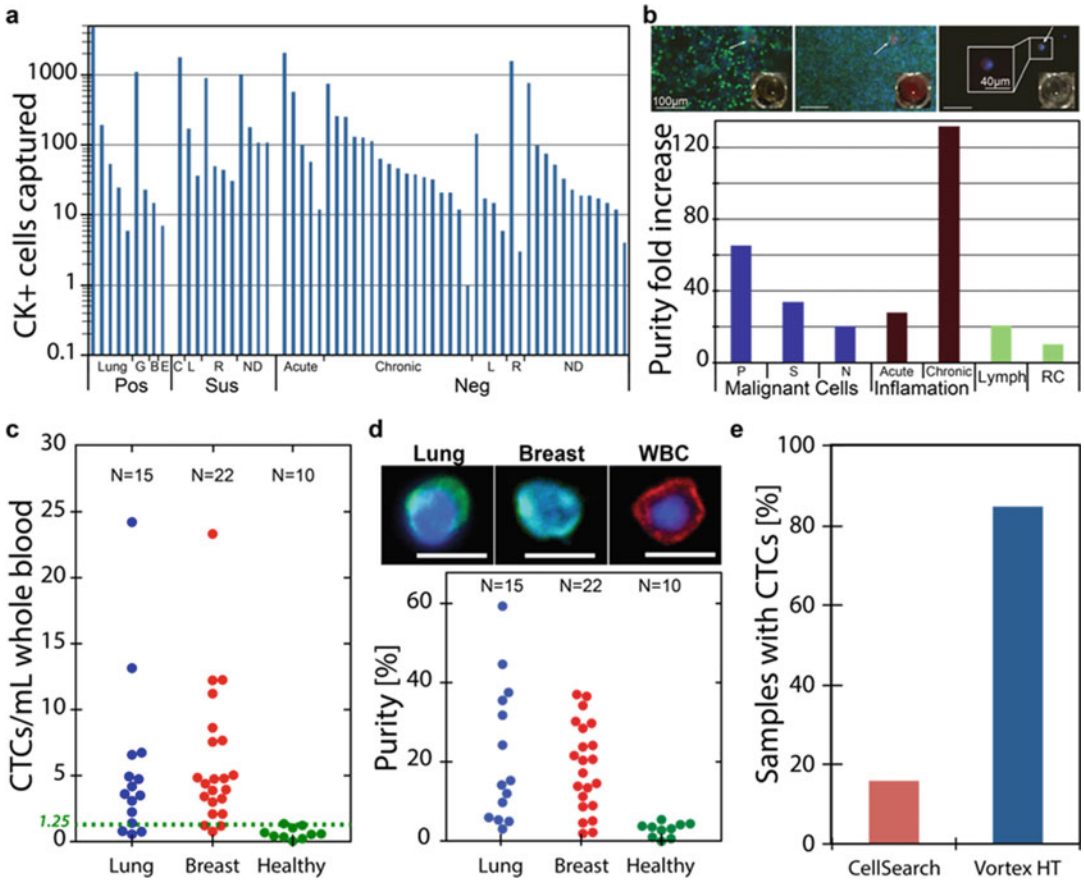


Fig. 2 The Vortex cell trapping technology has been used to purify rare atypical cells and CTCs from various clinical specimens. (**a**) Number of CK+ CTC-like cells purified from 115 patient pleural fluids and (**b**) corresponding purity of those collections. *Inset*: representative images showing the purity of solutions collected for downstream analyses. Plots were generated from data reported in [21]. Complete summary of patient conditions plotted in (**a** and **b**): [Pos or P] = positive for malignancy, [Sus or S] = suspicious for malignancy, [Neg or N] = negative for malignancy, G = gastric cancer, B = breast cancer, E = esophageal, [C, CI, or Chronic] = chronic inflammation, Acute = acute inflammation, [L or Lymph] = lymphocytosis, [R or RC] = reactive changes, and ND = not determined. (**c**) The number of CTCs collected from diluted whole blood of cancer patients using the Vortex-HT and (**d**) the purity of collected samples and representative microscopic images of immunolabeled CTCs and WBC. *Green dashed line* (marked as 1.25) is the healthy threshold. *N* denotes number of samples tested for each cancer type. (**e**) Vortex-HT extracted a higher number of CTC-positive samples (85%) in contrast to the CellSearch system (15%). (**c**–**e**) were plotted based on data reported in [20]

2 Materials

2.1 Chemical Reagents for General Usage

1. Virkon-S or equivalent.
2. 70% ethanol.
3. Bleach.
4. Deionized or distilled water.

2.2 Consumables

1. De-identified blood sample collected in an EDTA-coated BD™ tube and stored at room temperature (RT) for less than 4 h.
2. Waste containers for normal, biohazardous, and paraformaldehyde waste.
3. Plastic 20 mL sterile syringes with luer lock.
4. 50 mL conical centrifuge tubes.
5. 1.5 mL microcentrifuge tubes.
6. PEEK tubing with 1/32 in. OD.
7. 25 ga Luer stubs.
8. Sterile 96-well plates.
9. Sterile 0.2 μm syringe filter.
10. Microscope slides (3 in. × 1 in.).
11. Paper towels, Kimwipes® and/or absorbent bench underpads.
12. Long filter pipette tips (p10, p200, p1000, p5000).
13. 5 mL sterile serological pipette and sterile disposable aspirating pipettes.
14. Tin Foil.
15. Laboratory Tapes.
16. Parafilm®.

2.3 Lithography Facility, Software, and Reagents

1. Clean room with class 1000 or above.
2. Spinner capable of up to 4 in. substrates.
3. Mask aligner (Karl Suss MA5 or equivalent).
4. Mechanical profilometer (Dektak 6M, Veeco or equivalent).
5. Plasma cleaner (Technics Micro-RIE or equivalent).
6. Computer aided design software (AutoCAD or equivalent).
7. The photomask with 2D projections of the Vortex-HT.
8. Aqueous TMAH developer.
9. Negative photoresist: KMPR 1050 (Microchem).
10. Sylgard 184 Elastomer kit (Dow Corning).
11. 4-in. silicon wafer.
12. Desiccator with a leveled plate.
13. Vacuum pump or in-house vacuum line.

2.4 Equipment

1. Two manual PHD Syringe pumps (Harvard Apparatus # 702001 or equivalent) or two programmable PHD Ultra Pump Syringe pumps (Harvard Apparatus # 703006).

2. An inverted microscope with imaging capability for bright-field, DAPI (Ex/Em: 360/460), FITC (Ex/Em: 480/535), and TRITC (Ex/Em: 545/620).
3. Optional: High speed camera with > 3000 fps capability (Phantom v611 or equivalent).
4. BSL II Biosafety cabinet.
5. Fume hood.
6. Desiccator.
7. Oven or hot plate.
8. Pipetter (p200, p1000).
9. Pipet-aid.
10. Timer.
11. Pin vise set A (Syneo).

2.5 Cell Fixation and Staining Reagents

1. Sterile PBS (PBS 1×, pH 7.2 without calcium and magnesium).
2. 4% paraformaldehyde.
3. 0.4% Triton X-100.
4. 1 mg/mL DAPI prepared at in sterile water and filtered through a 0.2 μm filter.
5. 10% goat serum that has been filtered through a 0.2 μm filter.
6. Recommended antibodies: anti-human CD45 (BD cat#555483), anti-human cytokeratin (CK3-6H5, Miltenyi Biotec, cat#130-080-101), anti-human cytokeratin (AE1/AE3, eBioscience, cat#53-9003-82), anti-human cytokeratin (CAM5.2, BD, cat#347653).

3 Methods

3.1 Vortex-HT Fabrication

3.1.1 Replica Mold Fabrication

1. Design the 2D projection of Vortex-HT chip geometry using an appropriate software (Fig. 1b and **Note 1**).
2. Print the photomask containing the 2D projection of the Vortex-HT on transparency films with resolution greater than 20,000 dpi or 10 μm (*see* **Note 2**).
3. Transfer the 2D projection of the Vortex-HT to a negative photoresist spin-coated on a 4-in. silicon wafer by following standard lithographic techniques and according to the KMPR datasheet (*see* **Note 3**).

3.1.2 Polymer Casting and PDMS Chip Creation

1. Rigorously mix a cross-linker and a base from Sylgard 184 Elastomer kit in a ratio of 1:10. Pour the mixture onto the KMPR mold and degas in a desiccator connected with a

vacuum for 30 min to remove air-bubbles. Completely degassed polydimethylsiloxane (PDMS) mixture over the mold should be cured by placing in an oven at 65 °C for 21 h (or 150 °C hot plate for 2 h) to create inverted patterns of the mold in the cured PDMS.

2. Carefully peel off the cured PDMS from the reusable mold and punch two inlets for the blood sample and PBS buffer and an outlet, using a pin vise with the cutting edge diameter of 0.76 mm, adequate for tightly holding PEEK tubing whose outer diameter is 1/32 in.
3. Create an enclosed microfluidic Vortex-HT by bonding O_2 plasma-treated PDMS replica with a 3″ × 1″ glass slide. O_2 plasma treatment is conducted for 10 s in an oxygen-plasma cleaner operating at a radio frequency power and oxygen partial pressure of 80 W and 500 mTorr, respectively.

3.2 Fluidic Control Setup

3.2.1 Syringe Pumps

1. Place two syringe pumps near the microscope where tubing from syringes on the pump can be reach the inlet of the Vortex-HT chip (*see* **Note 4**).
2. Select the syringe type and size and ensure the unit for "Infuse Rate" is in mL/min.

3.2.2 Microfluidic Device and Tubing

1. Flush all tubing and luer stubs before the experiment with sterile and filtered PBS once. Make sure the fluid flows well and straight through the tubing without any visible kinks.
2. Secure the examined device on the microscope stage (*see* **Note 5**). Insert a PEEK tubing for the outlet port in the device, with care not to pierce the surrounding PDMS, and direct it to the waste container.

3.3 Processing Blood Samples

3.3.1 PBS Syringe Preparation

1. Fill in a 20 mL plastic syringe with sterile and filtered PBS. Remove all air bubbles from the syringe. Attach the PBS filled syringe to a luer stub connected to PEEK tubing (i.e., the tubing-luer stub assembly). Place the tubing end in the waste container and slowly flush a small volume of PBS from the syringe through the tubing to ensure that no air bubbles remain in the tubing-luer stub assembly.
2. Place the PBS syringe with the tubing assembly on the PBS syringe pump and clamp down the syringe. Secure the plunger straightly in place.
3. Connect the tubing from the PBS syringe to the first inlet of the PDMS Vortex-HT while being careful not to pierce the PDMS.

3.3.2 Blood Syringe Preparation

1. Place the blood tube in the biosafety cabinet (*see* **Notes 6** and **7**). Gently invert the blood tube several times to homogenize the blood. Dilute 2 mL of whole blood with 18 mL of filtered PBS (10× dilution) in a 50 mL conical tube. Gently invert the

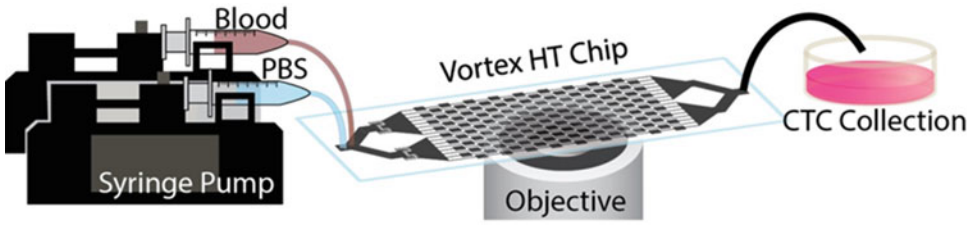


Fig. 3 The schematic of the fluidic control setup. The diluted blood sample and the flushing PBS buffer are injected sequentially into the PDMS Vortex-HT PDMS using two syringe pumps. The purification process can be visualized and monitored using an inverted microscope. Upon completion, purified CTCs can be released from the Vortex HT into a well in a 96-well plate or other containers depending on types of downstream analyses to be performed

50 mL tube several times to uniformly mix the blood with the diluent.

2. Remove the plunger from the Blood syringe. Carefully pour the 10× diluted blood while holding the syringe upright and blocking the nozzle with one finger to prevent leakage. Gently insert the plunger into the syringe and invert the syringe to make air bubbles float towards the syringe nozzle. Slowly release the finger blocking the nozzle and cover the nozzle with a paper towel. With a paper towel still covering the nozzle, carefully and slowly push the plunger so that the blood reaches the nozzle as air bubbles exit the syringe. Make sure to remove all bubbles in the syringe that may be stuck to the plunger in the syringe by gently tapping the sides of the syringe.
3. Assemble a PEEK tubing to a luer stub and connect the tubing-luer stub assembly to the blood syringe. Place the tubing end in the waste container and slowly flush a small volume of diluted blood from the syringe through the tubing to ensure that no air bubbles remain in the tubing-luer stub assembly.
4. Place the Blood syringe with the tubing assembly in the Blood syringe pump and clamp down the syringe. Secure the plunger straightly in place.
5. Connect the tubing to the second inlet of the PDMS Vortex HT device while being careful not to pierce the PDMS. The final setup should look like Fig. 3.

3.3.3 Sample Processing Flow Sequence

CTC-purification using the Vortex-HT requires injecting the sample solution and the washing buffer at various flow rates, optimized for each step. The end of outlet tube has to be timely moved among the waste, recycling and sample collection containers, corresponding to each step.

1. Prime the Vortex-HT: Inject the PBS at 8 mL/min for 30 s to establish stable cell-trapping vortices in chambers prior to the

blood injection. The outlet tube should be directed into the waste container.

2. Cell Trapping: Simultaneously inject the 10× diluted blood and PBS into the device at 7 and 1 mL/min, respectively, to trap CTCs from the blood in microscale vortices (*see* **Note 8**). The outlet tube should be removed from the waste container and placed into the recycling container at the start of this step if you plan to process the flow-through solution one more time (multiple cycles). Otherwise, the outlet tubing can remain directed toward the waste container.
3. Wash: Stop the blood syringe pump and simultaneously increase the PBS syringe infusion to 8 mL/min into the device for at least 30 s to remove blood cells that are not stably trapped in microscale vortices from the device. The outlet tube should be removed from the recycling container and placed into the waste container (*see* **Note 9**).
4. Release: Cells are released by quickly lowering the flow rate to dissipate the vortices (*see* **Note 10**). Simultaneously, the outlet tube should be removed from the waste container and guided to a collection well in a 96-well plate (*see* **Note 11**).
5. Final Flush: Inject briefly the PBS solution at a fast flow rate to ensure all CTCs released from the device are flushed from the tubing and collected in the well. The outlet tube should remain in the collection well in a 96-well plate (*see* **Note 12**).
6. Two syringe pumps controlling the PBS syringe and the blood syringe can be controlled manually or programmed individually to enable synchronized solution injections. The flow injection sequence, including flow rates and durations for each step, is summarized in the following Table 1.

Table 1
Syringe pump setup parameters

		Infuse rate [mL/min]		
Process step	**Duration [s]**	**PBS syringe**	**Blood syringe**	**Outlet tubing position**
Prime	>30	8	STOP	Waste
Cell trapping	Variable	1	7	Waste or recycling
Wash	>30	8	STOP	Waste
Release	5–10	STOP	Rapid pull-back of the plunger	Well plate
Final flush	1–2	8 or fast forward	STOP	Well plate

3.3.4 Serial Processing

Serial processing indicates reprocessing of the flowed-through diluted blood solutions (*see* **Note 13**). If serial processing is desired, steps in Subheadings 3.3.1 through 3.3.3 can be repeated using the diluted blood samples collected in the recycling container as the sample.

3.3.5 Cleanup

1. Wash all the tubing-luer stub assemblies by injecting successively 3–5 mL of: 1× PBS, 10% bleach, deionized or distilled water, 70% ethanol, and deionized or distilled water (*see* **Note 14**).
2. Disassemble luer stubs from tubing and submerge the luer stubs in 10% bleach for 10 min and rinse them with 70% ethanol and deionized or distilled water sequentially. Spray and wipe down all surfaces with Virkon-S or 10% bleach then with 70% ethanol. Throw away all disposables (syringes and PDMS devices) in appropriate biohazard waste bins. Dispose of the blood waste and remaining blood accordingly by following the local EH&S regulations.

3.4 Immunolabeling (Optional, See Note 15)

3.4.1 Purified CTC Fixation

1. Preliminary wash: Fill all the wells to be stained to the top (avoid overfilling, resulting in a convex meniscus) with 1× filtered PBS and centrifuge at up to 800 RCF at room temperature for 3 min. Aspirate 200 μL of PBS from the well using a P200 tip pressed against the wall without touching the bottom of the well. This will leave about 150 μL of liquid in each well (*see* **Note 16**).
2. Fixation using 4% PFA under a fume hood: Add 200 μL of 4% PFA to each well and incubate the well plate for 10 min at RT. Wash samples 4 times with 1× filtered PBS (*see* **Note 17**).

3.4.2 Staining Control Cell Addition

1. White blood cells or cancer cell lines prepared beforehand by following the CTC fixation protocol (Subheading 3.4.1) should be added to two separate wells in the plate as positive controls for staining (*see* **Note 18**).
2. Centrifuge the well plate and visually inspect whether the wells contain enough number of control cells prior to the permeabilization step.

3.4.3 Permeabilization, Blocking, and Staining

1. Add 200 μL of 0.4% Triton X-100 into each well and incubate for 7 min at room temperature. Wash samples three times with 1× filtered PBS.
2. Add 200 μL of 10% goat serum filtered through a 0.2 μm filter into each well and incubate for 30 min at room temperature. Wash samples three times with 1× filtered PBS.
3. Add 200 μL of staining master mix into each well and incubate in the dark for 40 min at 37 °C. Wash samples three times with 1× filtered PBS. Components of the master mix are listed in Table 2.

Table 2
Immunostaining antibody mixture compositions

Reagents/antibodies	Host	Fluorophore	Volume [μL]
Anti-human cytokeratin	Mouse	FITC	5
Anti-human cytokeratin	Mouse	FITC	5
Anti-human cytokeratin	Mouse	FITC	5
Anti-human CD45	Mouse	PE	10
DAPI		Dapi	1
10% goat serum (filtered)			174

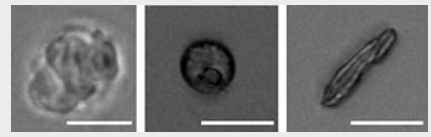
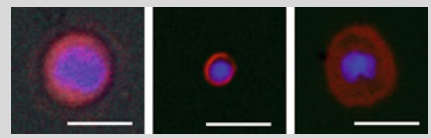
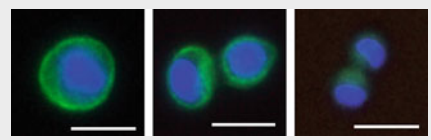
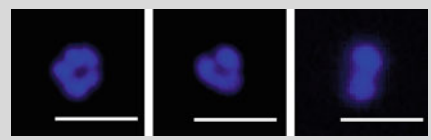
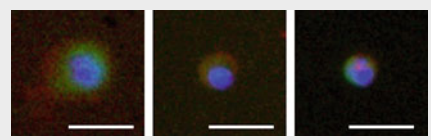
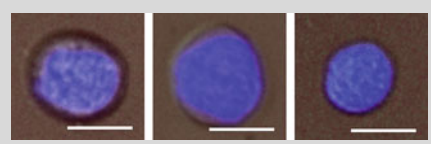
3.5 Classification and Enumeration of CTCs (Optional, See Note 15)

The following procedure is recommended to differentiate between CTCs and WBCs purified using the Vortex-HT, following the staining procedure in Subheading 3.4 with CK-FITC, CD45-TRITC, and DAPI using the listed antibodies (*see* Table 3 and **Note 19**). Only DAPI-positive cells should be considered for the CTC classification in the described procedure. Each DAPI positive cell should be evaluated for each characteristic in the order they are described. If not definitive, the cell should be evaluated with the subsequent characteristic (*see* **Note 20**). More sample images and descriptions for classification categories have been reported in [20].

4 Notes

1. The chip is composed of 196 cell-trapping reservoirs, arranged as 16 parallel channels, consisting of 12 reservoirs in series. Upstream of the 16 parallel channels, two pillared filter regions with 50-micron gaps bifurcated from the solution inlet individually to prevent the parallel channels from clogging by debris. Each channel is comprised of a single rectangular channel segment (W_C = 40 μm and L_C = 1 mm), followed by 12 reservoirs (W_R = 230 μm and L_R = 720 μm) spaced 1 mm apart. The center-to-center distance between two adjacent parallel channels should be at least 455 μm for irreversible bonding between PDMS and the slide glass to ensure no leakage during operation at extreme flow rates. Outflowing solutions from 16 channels are merged and exit through a single outlet of the chip.
2. Alternatively, a Cr-mask can be fabricated and used as the photomask if higher resolution is desired.
3. The rotational speed for the spin-coating should be adjusted to create a 70–75 μm-thick KMPR layer. The suggested fabrication recipe consists the following:

Table 3
CTC classification sequence

Order	Characteristic	Cell type	Sample images
1	Objects whose morphologies have a jagged shape, dark fill, or dark outline in bright-field	Debris	
2	Cells expressing DAPI+/CD45+/CK−	WBC	
3	Cells expressing DAPI+/CK+/CD45−	CTC	
4	Cells with lobed nucleus	WBC	
5	Cells expressing DAPI+/CD45+/CK+	WBC	
6	Cells not expressing any markers but whose Nucleus < 9 μm	WBC	
7	Cells not expressing any markers but whose Nucleus > 9 μm and N/C[a] < 0.8	WBC	
8	Cells not expressing any markers but whose Nucleus > 9 μm and N/C[a] > 0.8	CTC	

[a] *N/C* nuclear-to-cytoplasmic ratio. Scale bars represent 20 μm

(a) Spin-coating speed: 368 × *g* for 30 s with ramp rate of 28 × *g* for 10 s

(b) Soft bake: 15 s at 100 °C

(c) Exposure: 7 s exposure at 30 mW/cm^2 with hard-contact mode

(d) Post exposure bake: 4 min at 100 °C

(e) Developing: 10 min in an aqueous TMAH developer

4. Two syringe pumps can be placed on an elevated platform to match the height of the Vortex-HT device on the microscope's objective stage.
5. Inspect a new Vortex-HT PDMS device to make sure there are no fabrication defects or debris blocking the flow path.
6. Always wear personal protective equipment, including safety goggles, face shield, closed-toe shoes, lab coat, and gloves while handling biohazardous samples.
7. Keep all caps closed when tubes are not in use to minimize dust or bacterial contamination into blood samples and potential blood coagulation. Prepare blood dilution right before each run.
8. The cell trapping duration can be varied depending on the total volume of diluted blood in the blood syringe. The duration, t, can be calculated by

$$t\,[\text{min}] = \frac{(\text{total diluted volume loaded in the syringe} - 0.5)\,[\text{mL}]}{7\,[\text{mL/min}]}$$

9. This step ensures collection of pure population of CTCs. Purified and orbiting CTCs can be visualized if Vortex-HT is placed on the microscope equipped with a high-speed camera recording at 3000–10,000 fps.
10. Rapid dissipation of vortices can be achieved by (1) pulling the blood sample syringe plunger backward rapidly, and (2) stopping the pump controlling the PBS syringe. The back end of the plungers should not be secured into the holsters on the syringe pumps, if present; the adapters should be removed if possible. If programmable syringe pumps are used, the blood syringe should be set to withdraw at 25 mL/min for 5 s.
11. This step should be carefully timed so that the released cells are not lost into the waste container, and excess wash fluid is not collected in the well plate.
12. The final volume should fill an entire single well.
13. For optimal results, it is recommended to perform two cycles of sample processing per run (i.e., 2 cycles per 2 mL of undiluted blood). The same device can be used up to 3 runs when 2 cycles

per run are performed (corresponding to 6 mL of whole blood).

14. Collect bleach and ethanol wastes in separate containers. The use of deionized or distilled water as a final washing step will prevent the formation of crystalized salt clogs in the tubing during storage.
15. Subheadings 3.4 and 3.5 can be omitted if the downstream applications to be implemented for the purified CTCs do not require immunolabeling for enumeration and classification.
16. Hereafter in this manuscript, the steps of adding 200 μL of PBS, centrifuging, and aspirating 200 μL of PBS in this fashion are referred to as the standard washing step unless specified otherwise.
17. If desired, the samples can then be stored at 4 °C for future use. For storage, each well should be filled with 1× filtered PBS and the plate should be wrapped with Parafilm to minimize evaporation.
18. A cancer cell line highly expressing CK, such as HCT116 or MCF7 cells, is preferred.
19. It is recommended to image the entire well, containing purified CTCs and control cells using a 10× objective on an inverted microscope equipped with an image stitching capability.
20. Expression level thresholds of CK- and CD45-positive cells should be determined using those of the staining control cells (WBCs and cancer cell lines) added to each plate and stained simultaneously with CTC samples.

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Chapter 6

Separable Bilayer Microfiltration Device for Label-Free Enrichment of Viable Circulating Tumor Cells

Sijie Hao, Merisa Nisic, Hongzhang He, Yu-Chong Tai, and Si-Yang Zheng

Abstract

Analysis of rare circulating tumor cells enriched from metastatic cancer patients yields critical information on disease progression, therapy response, and the mechanism of cancer metastasis. Here we describe in detail a label-free enrichment process of circulating tumor cells based on its unique physical properties (size and deformability). Viable circulating tumor cells can be successfully enriched and analyzed, or easily released for further characterization due to the novel separable two-layer design.

Key words Circulating tumor cells, Viable CTC isolation, Cancer metastasis, CTC in vitro culture, Microfabrication

1 Introduction

Cancer metastasis accounts for 90% of cancer-related deaths, which are the second leading cause of death in the USA [1]. Circulating tumor cells (CTCs) are rare cancer cells that are shed from the primary tumor into the circulatory systems. Researchers have correlated the patient CTC counts with disease progression and treatment response [2–4]. Accumulating evidence indicates that CTCs in the peripheral blood seed and colonize the distant organ, forming metastasis [5, 6]. Its cancerous origin can inform the disease status of primary tumor and secondary micrometastasis. More importantly, CTCs carry the mechanistic information that orchestrate the lethal but elusive cancer metastasis process [7]. To elucidate CTC biology is therefore critical not only to advance our understanding of cancer metastasis but to develop strategies and methods for early diagnosis and targeted therapies. However, CTCs extreme rarity hinders efforts to interrogate them at molecular level. One milliliter of blood normally contains a few millions of leukocytes and billions of erythrocytes. The estimated CTC count in 7.5 mL of blood ranges from single digits to a few hundreds even in the case of patients of the most advanced stage of cancer.

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As a result, remarkable progress has been made in the CTCs enrichment technologies which isolate cancer cells of epithelial origin from normal blood cells. Among other technologies, immumo-based methods isolate CTCs by screening CTCs-containing blood by antibody-primed structures binding epithelial cells expressing epithelial surface antigens [8, 9]. More often than not, malignant cancer cells mask their epithelial cell identity through the epithelial-to-mesenchymal Transition (EMT) process [10, 11]. CTCs that undergo EMT downregulates their epithelial cell markers. Thus, the bias of immuno-based methods prevents the isolation of this important subpopulation of CTCs. To address this problem, isolation methods exploring the unique physical properties of CTCs have been invented, including size, density, stiffness, and electrical property-based isolation [12, 13]. Size-based isolation methods are particularly attractive as cancer cells are characteristically larger and less deformable than most normal blood cells. However, most sized-based isolation methods are limited by CTC viability, sample volume, processing time, and compatibility with downstream analysis. The separable bilayer (SB) filter is categorized as a size-based CTC isolation method, but its novel geometry and bilayer design significantly increases the sample processing efficiency and preserves CTC viability [14]. Figure 1a illustrates the size-based filtration mechanism. Cancer cells of large size are wedged in the gap defined by the top and bottom layers. Leukocytes of smaller size and greater deformability pass through the gap and the small openings on the bottom layer.

The SB filter is made from polymer parylene-C. This biocompatible material not only better preserves the viability of the cells, but can achieve in situ cell culture on the same device that isolates the CTCs. The device is composed of a 2-by-2 big array of patches (Fig. 1b). Each patch is an 8 by 8 array of the elemental units (Fig. 1c). Each elemental unit is 530 μm by 530 μm in dimension. An element unit is separated from its neighbors by 50 μm (Fig. 1d). The top and bottom layers adhere to each other at the 50 μm borders. To efficiently isolate CTCs, we define the critical gap size between the top and bottom layers (5.5 μm in the current device). Large openings of 40 μm diameter are created on the top layer. This design allows greater freedom for isolated tumor cells to expand, migrate, and proliferate in situ on the device. The bottom layer contains the 8 μm diameter openings arranged hexagonally to maximize the flow rate. A cross-sectional view in Fig. 1f summarizes the geometrical design parameters. Further, the bilayer membrane structure can be separated after CTC capture for easy cell release. Collectively, these modifications have significantly enhanced our ability to enrich and characterize viable CTCs.

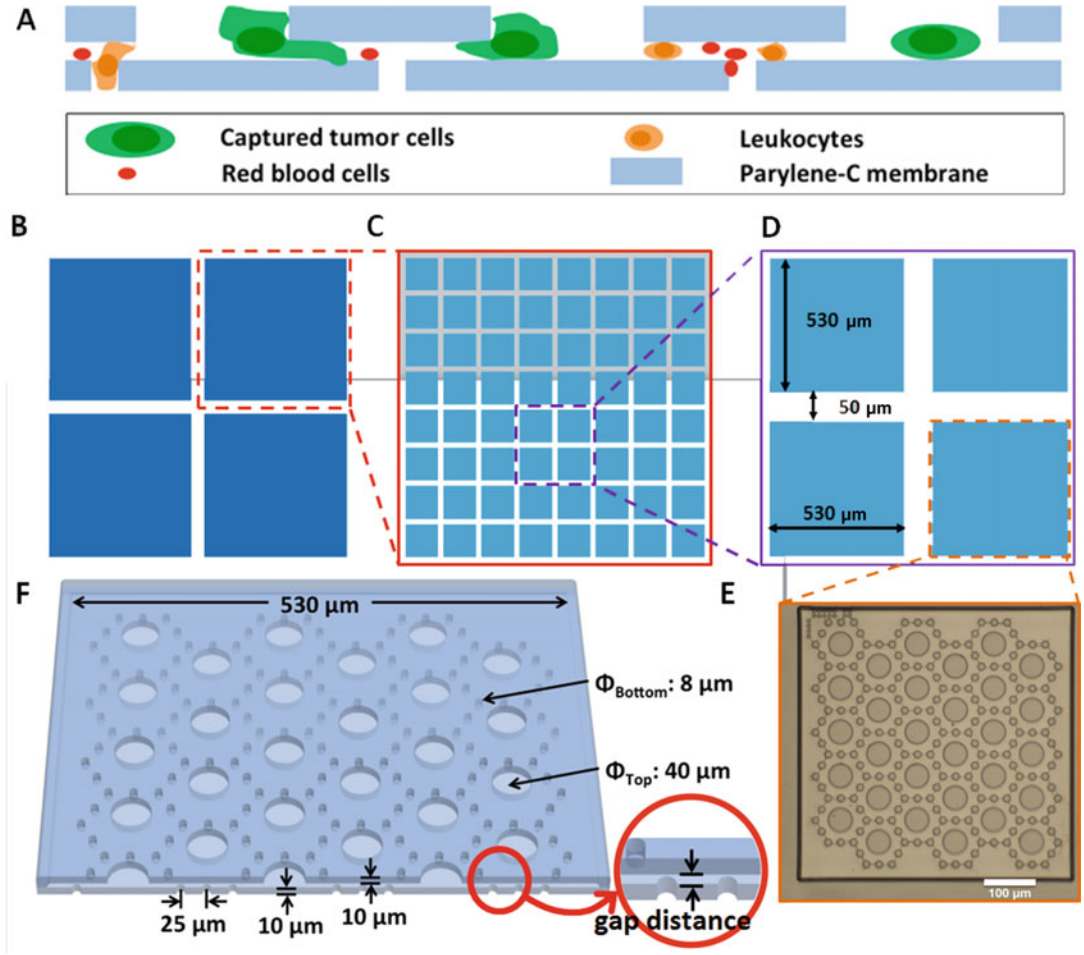

Fig. 1 Device design. (**a**) Each device has four large patches; (**b**) Each large patch is a 8-by-8 array of a single unit; (**c**) Geometry of single units and their arrangement; (**d**) Microscopic picture of top view of a single unit showing large pores on top parylene-C layer and small pores on bottom parylene-C layer; (**e**) Cross-sectional view of a single unit model with key geometrical parameters labeled. (**f**) Cartoon of device cross-sectional view showing tumor cells are captured along the edges of the large top parylene-C pores

2 Materials

2.1 Collection of Mouse Blood

1. Heparin-coated blood collection tubes (1.5 mL).
2. One milliliter syringe and 22 gauge needle.

2.2 Device Assembly

1. One separable bilayer microfiltration device (SB filter).
2. One pair of tweezers with flattened tip.
3. One 5 mL plastic Luer-Lok syringe.
4. Two PDMS (polydimethylsiloxane) gaskets of 1 mm thickness, 7 mm ID, and 20 mm OD.

5. One plastic housing (top and bottom) with a luer adapter.
6. One pair of metal clamp with rubber cushion.
7. One flow-through collection tube with a Luer-Lok adapter.
8. One collection pedestal.
9. Isopropyl alcohol (IPA, analytical grade).
10. One 12-well plate.

2.3 Isolation of Circulating Tumor Cells

1. Phosphate-buffered saline (1× PBS, pH 7.4).
2. One milliliter sterile serological pipette.
3. Pipette controller.

2.4 Detection of Circulating Tumor Cells

1. 10% buffered formalin.
2. Phosphate-buffered saline (1× PBS, pH 7.4).
3. Goat serum blocking solution: Dilute goat serum in PBS to the working concentration of 5% w/w.
4. One microgram per milliliter of DAPI (4′,6-diamidino-2-phenylindole).
5. Monoclonal anti-mouse CD45 antibody conjugated to Alexa Fluor®488. Dilute mouse monoclonal anti-CD45 antibody to 1 μg/mL by adding 0.5 μL of antibodies to 49 μL of 5% goat serum blocking solution.
6. Monoclonal anti-mouse EpCAM antibody conjugated to Alexa Fluor®594. Dilute mouse monoclonal anti-EpCAM antibody to 1 μg/mL by adding 0.5 μL of antibodies to 49 μL of 5% goat serum blocking solution.
7. One pair of tweezers with flattened tip.
8. One microscope glass slide.
9. One microscope sample coverslip, No. 1.5.
10. One 100 mm petri dish.
11. Two 0.2 μm syringe filters.
12. Microscope sample mounting media.
13. Fluorescence microscope equipped with DAPI, FITC and Texas Red channel.

2.5 Culture of Circulating Tumor Cells After Isolation

1. One six-well plate.
2. Cell culture media (e.g., DMEM supplemented with 10% fetal bovine serum).
3. Penicillin–streptomycin (10,000 U/mL)
4. Two pairs of tweezers with flattened tip.
5. One marking paint brush.

3 Methods

3.1 Collection of Mouse Blood

1. Collect peripheral blood from mice in heparin-coated tubes via cardiac puncture. Proceed with CTC isolation within 1 h (*see* **Note 1**).

3.2 Device Assembly

Perform the following procedures in a biological safety cabinet. Use aseptic techniques when appropriate.

1. Add 1 mL of IPA in one well of a 12-well plate (*see* **Note 2**).
2. Immerse one SB filter into IPA solution using flattened tip tweezers (*see* **Note 3**). The SB filter is maintained in IPA solution overnight for disinfection.
3. Hold the bottom plastic housing (Luer-Lok adapter side facing down). Place one gasket onto the bottom plastic housing. Align the gasket opening with the Luer-Lok adapter outlet (Fig. 2a).
4. Transfer the SB filter onto the gasket (*see* **Note 4**). Cover the gasket opening with the central portion of the SB filter (*see* **Note 5**). Allow IPA to dry for 30 s.

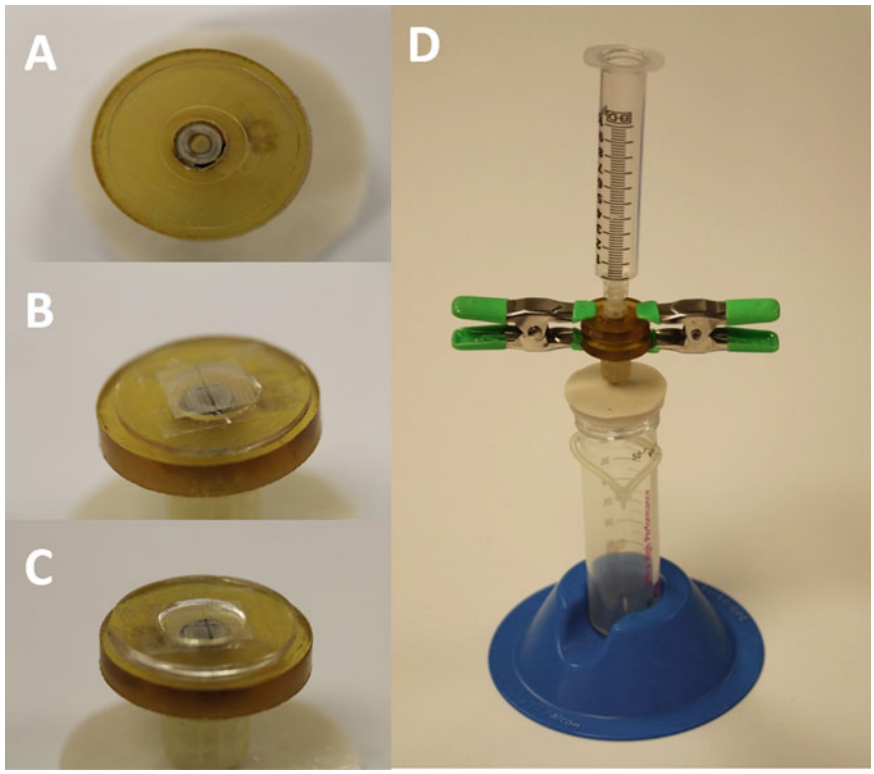

Fig. 2 Device assembly. (**a**–**c**) Illustration of assembling sequence. (**d**) Filtration setup including device housing

5. Place the other gasket on top of the SB filter and align with the bottom gasket (*see* **Note 6**). Press down on the top gasket to eliminate air bubbles between the gaskets (*see* **Note 7**).
6. Place the top plastic housing onto the top gasket (Luer-Lok adapter side facing up). Align the gasket opening with the Luer-Lok adapter outlet (Fig. 2b).
7. Secure the sandwiched device with a pair of clamps (Fig. 2c). Evenly position two clamps on the circular edge of the plastic housing (*see* **Note 8**).
8. Sit the flow-through collection tube on a tube pedestal. Connect the tube to the bottom plastic housing via the luer adapter.
9. Remove the plunger from the 5 mL syringe barrel. Connect the syringe barrel to the top plastic housing via the luer adapter (*see* **Note 9**; Fig. 2d). Proceed with circulating tumor cells isolation immediately (*see* **Note 10**).

3.3 Isolation of Circulating Tumor Cells

1. Aspirate 1 mL of PBS using 1 mL sterile serological pipette. Hold the pipette controller using one hand.
2. Open the cap of the blood collection tube and hold the tube using the other hand.
3. Position the pipette inside the syringe barrel. Lower the pipette tip to the 1 mL marker line. Release the PBS against the wall into the syringe barrel (*see* **Note 11**).
4. Aspirate all of the blood sample using the same pipette. Note the volume of the blood sample.
5. Position the pipette inside the syringe barrel. Lower the pipette tip to the 1 mL marker line. Slowly release the blood sample against the wall once the PBS volume is below 0.2 mL marker line (*see* **Note 12**).
6. Aspirate the same volume of PBS as the blood sample using a fresh 1 mL pipette.
7. Position the pipette inside the syringe barrel. Lower the pipette tip to the 1 mL marker line. Release the PBS in drops against the wall once the blood volume reaches the bottom (*see* **Note 13**). Wait 30 s after PBS reaches the bottom.
8. Disconnect the syringe barrel from the sandwiched device.
9. Make sure that there is no visible fluid accumulated at the top plastic housing inlet or left over on the flow-through collection tube outlet. Disconnect the sandwiched device from the flow-through collection tube.
10. Apply pressure on the plastic housing to stabilize the device using one hand. Unclamp the sandwiched device using the other hand.

11. Insert the flattened tweezer tip between the top plastic housing and the top gasket. Make a full circle around the gasket. Pry open the top plastic housing using the tweezers (*see* **Note 14**). Immediately proceed with circulating tumor cells detection (Subheading 3.4) or culture after live isolation (Subheading 3.5).

3.4 Detection of Circulating Tumor Cells

1. Transfer the SB filter together with gasket pairs from the bottom plastic housing to a clean 100 mm petri dish.
2. Fix the cells by applying 100 μL 10% formalin to cover the exposed area of the SB filter (*see* **Note 15**). Fix for 20 min at room temperature.
3. Pass the 5% goat serum blocking solution through the 0.2 μm syringe filter.
4. Aspirate 5 mL of 1× PBS in a 10 mL syringe. Connect the syringe to a 0.2 μm syringe filter.
5. Dab the gasket–filter sandwich on the petri dish to remove formalin solution.
6. Apply one drop of filtered PBS to wash the formalin off the filter. Dab the gasket–filter sandwich on the petri dish to remove PBS.
7. Apply 100 μL of DAPI (1 μg/mL) onto the SB filter and incubate for 10 min at room temperature.
8. Separate the gaskets using tweezers. Transfer the SB filter to a clean microscope slide.
9. Block the antigens by applying 50 μL of 5% goat serum to cover the filter. Remove the air bubbles trapped under the SB filter and incubate for 1 h at room temperature (*see* **Note 16**).
10. Dilute mouse monoclonal anti-EpCAM antibody and anti-CD45 antibody with 5% goat serum blocking solution (*see* **Note 17**).
11. Dab the gasket–filter sandwich on the petri dish to remove blocking buffer. Apply 50 μL of monoclonal antibodies to the filter and incubate for 1 h at room temperature (*see* **Note 18**).
12. Rinse off the antibodies from the filter using filtered PBS. Dry excessive PBS solution from the microscope slide. Repeat this step three times (*see* **Note 19**).
13. Apply 30 μL of mounting media to the SB filter. Set a clean coverslip on top. Use tweezers to squeeze out any large air bubbles. Cure the mounting media at room temperature overnight before imaging (*see* **Note 20**).

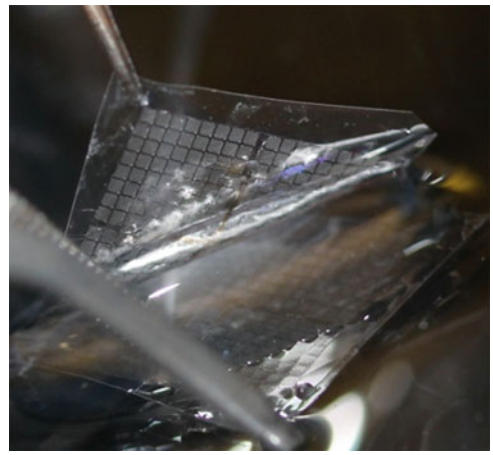

Fig. 3 Images of a fabricated device with magnified views of the separable structures

3.5 Culture of Circulating Tumor Cells After Live Isolation

1. Transfer the SB filter together with its gasket pair to one six-well plate.
2. Insert the tweezers tips between the gaskets and carefully separate them without damaging the SB filter.
3. Transfer the SB filter to a different well. Immediately apply 500 μL of cell culture media to immerse the filter.
4. Grasp the top layer of the SB filter using one pair of tweezers. Hold down the bottom layer of the SB filter against the plate bottom.
5. Separate the two layers by peeling off the top layer using tweezers (Fig. 3).
6. Wet the paint brush with cell culture media. Brush the cells on the top layer into the well. Rinse off the residual cells using 200 μL of cell culture media.
7. Lift the bottom layer out of the media. Brush the cells on the bottom layer into the same well. Rinse off the residual cells using 200 μL of cell culture media.
8. Rinse off the residual cells on the paint brush into the same well using 500 μL of cell culture media. Add more cell culture media for a final volume of 2.5 mL (*see* **Note 21**).
9. Transfer the six-well plate into the incubator for cell culture.

4 Notes

1. Coat the blood collection syringe needle and barrel with heparin solution to prevent blood coagulation during cardiac puncture.

2. During filtration, air bubbles trapped in the device gap disturb the gravity flow. Due to low surface tension, IPA wets the entire filter surface to eliminate air bubbles.
3. The SB filter is fabricated in the form of thin films. Microfabrication tweezers with flattened tips are used to handle the filters with caution. Tweezers of sharp tip might damage the microstructure of the SB filter. This might result in low isolation efficiency.
4. The SB filter has a separable two-layer structure. Use extra caution when removing the filter from the well. To maintain the intact two-layer structure, allow enough space in the well to handle the filter using tweezers.
5. Failure to completely cover the gasket opening results in blood bypassing the SB filter, lowering the isolation efficiency.
6. Misalignment might disrupt the gravity flow, thus slowing down the filtration speed.
7. Although the thin film SB filter is sandwiched between the gaskets, the two gaskets should adhere to each other spontaneously due to the hydrophobic nature of PDMS.
8. During filtration, uneven positioning of clamps might result in blood leakage from the sandwiched device.
9. Finger-tighten the Luer-Lok to prevent fluid leakage during filtration.
10. Delay between device assembly and isolation might lead to complete evaporation of IPA in the filter gap. Air bubbles between the gaps disrupt the gravity flow.
11. Fluid flow should start immediately. The PBS flow clears the residual IPA from the SB filter. Air bubbles trapped in the filter gap might prevent the initiation of gravity flow. In the situation of no fluid flow, tap the device assembly together with the tube pedestal on the hood surface to initiate flow. Do not release the fluid directly at the syringe outlet. This might damage the filter structure.
12. Early release of the blood sample into the PBS dilutes the blood. This might change the viscosity of the blood sample, thus lowering the isolation efficiency. Delay in releasing the blood sample might introduce air bubbles into the filter gap. Air bubbles discontinue the flow. In the situation of paused flow, tap the device assembly together with the tube pedestal on the hood surface to resume the flow.
13. This step washes the SB filter with the same volume of PBS as the blood sample. This minimizes the residual erythrocytes and leukocytes trapped on the filter. Larger volume of PBS wash might rinse off the circulating tumor cells trapped on the filter.

Although the erythrocytes generally do not interfere with the downstream immunocytochemistry staining, it serves as a visible marker correlated with the number of leukocytes left on the filter. Try to rinse off all visible erythrocytes in the barrel.

14. Do not insert tweezers tip between the gaskets. This might damage the two-layer SB filter, thus interfering with the downstream application.
15. Apply more formalin solution to cover the exposed SB filter if the solution leaks out between the bottom gasket and the petri dish.
16. Put the petri dish on a leveled bench to prevent solution running off the filter. Usually 50 μL is enough to cover the entire surface of the SB filter.
17. Follow the manufacturers' recommended dilution of each antibody.
18. Keep the sample in the dark during incubation of fluorophore-conjugated antibodies. Maintain humidity to prevent drying out.
19. Use extra caution. Avoid dropping the PBS solution directly on the SB filter. This might rinse off the isolated cells on the filter.
20. Dissolve the mounting media by soaking in PBS overnight if there are lots of bubbles or if you want to release the filter.
21. Check the SB filter for residual cells under microscope. Repeat **steps 6–8** when necessary.

Acknowledgment

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Chapter 7

Microfilter-Based Capture and Release of Viable Circulating Tumor Cells

Siddarth Rawal, Zheng Ao, Ram H. Datar, and Ashutosh Agarwal

Abstract

Microfilters with slot-pore geometry can be used for size-based capture of circulating tumor cells (CTC) from the blood of cancer patients. The slot pore geometry reduces the shear stress that the cells would typically experience during filtration process and allows the cells to remain viable. The microfilter provides a platform capable of high CTC capture efficiency; however, the release of these cells from the filter following capture is nontrivial, possibly due to the strong nonspecific electrostatic adhesion of CTC to the microfilter surface. Techniques such as reverse flow or cell scraping result in recovery of only a small percentage of captured cells. We describe, in detail, a protocol for novel application of thermo-responsive polymer poly (N-iso-propylacrylamide) (PIPAAm) to release viable CTCs from microfilters with slot pores. Following fabrication of the microfilter, a coating of PIPAAm is applied to the surface to exploit its thermoresponsive interfacial properties to release the cells. Typically, cancer patient's blood is filtered at room temperature (below 32 °C) when PIPAAm is hydrophilic. Thereafter, the filter is placed in either culture medium or a buffer maintained at 37 °C, which renders PIPAAm hydrophobic, allowing subsequent release of the electrostatically bound cells with high efficiency. Using this method, viable CTC captured directly from cancer patients' blood can be subjected to downstream off-chip culture, analyses, and characterization.

Key words Circulating tumor cell, CTC culture, Viable CTC, Microfilter, Live cell capture, Precision medicine, Personalized medicine, Translational medicine, Liquid biopsy

1 Introduction

Metastatic spread of cancer is the cause of 90% of human cancer deaths [1]. One of the most important determinants of prognosis and management of cancer is the absence or presence of metastatic dissemination of tumor cells at the time of initial presentation and during treatment [2–4]. This early spread of tumor cells to lymph nodes or bone marrow is referred to as "disseminated tumor cells" (DTC), or when in the peripheral blood, as "circulating tumor cells" (CTC). CTC form the basis for the development of distant metastases. It has also been well established that CTC can be present even in patients who have undergone complete surgical resection of the primary tumor, and implicated for the later

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development of overt metastases in these patients. While bioptic tissue sampling is invasive and often difficult to repeat, CTC analysis requires a simple blood draw and thus provides an easy-to-repeat, real-time "liquid biopsy" approach [5].

Here, we demonstrate the use of a Parylene C membrane microfilter with slot pore geometry for the capture of viable CTC which exploit the size differences between larger epithelial tumor cells and the smaller hematopoietic cells [6, 7] in patient's peripheral blood. We have previously reported on the *round pore enumeration filter* and compared it with a FDA-cleared CellSearch™ platform, where the microfilter-based capture was shown to be superior in CTC capture efficiency for cancer patient blood sample [8, 9]. The round-pore microfilter was developed specifically to capture and perform CTC enumeration and on-chip CTC characterization and post-capture analysis [10]. However, a limitation of the round-pore architecture microfilter is requirement to use a mild formaldehyde-based fixation prior to filtration, which preserves the cells morphology and allows them to withstand the shear stress and pressure experienced by the CTC during the filtration process. While this fixation enables immunofluorescent detection critical for CTC enumeration, it negates the ability to capture viable CTC for functional molecular characterization or propagation via culture. To circumvent this limitation to capture viable CTC, a *slot pore filter* that obviates the necessity to fix cells prior to filtration has been developed (Fig. 1). The slot pore geometry (6 μm

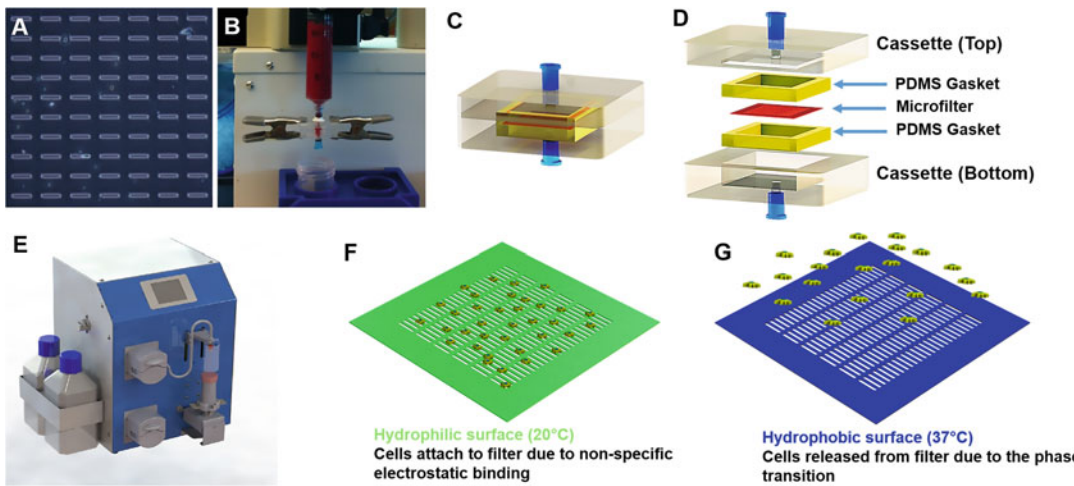


Fig. 1 PIPAAm-coated slot filters to capture and release circulating tumor cells from blood. (**a**) Microscopic view (400× magnification) of the slot filter post PIPAAm coating; (**b**) Manual filtration setup with syringe pump to capture CTCs from blood using PIPAAm-coated slot filter; (**c**, **d**) Schematic of the manual filtration cassette: the PIPAAm-coated slot filter is sandwiched between the top cassette and the bottom cassette with two PDMS pieces to seal the cassette; (**e**) Automated CTC capture device; (**f**, **g**) Scheme of using temperature change to release captured CTCs from PIPAAm-coated slot filter

Table 1
Capture, release, and retrieval efficiency of PIPAAm-coated slot filter

Cell line	Filter	Capture efficiency, %	Release efficiency, %	Retrieval efficiency, %
SKBr-3	Noncoated	89 ± 3	7 ± 1	6 ± 1
	PIPPAm-coated	94 ± 9	82 ± 5	77 ± 5
LMTS-GFP	PIPPAm-coated	87 ± 10	79 ± 14	69 ± 12

Capture efficiency is calculated by dividing cell numbers captures on filter before release by cell numbers spiked into blood. Release efficiency is calculated by dividing cell numbers released from filter by cell numbers captured on filter before release. Retrieval Efficiency is calculated by dividing cell numbers released from silter by cell numbers spiked into blood

width × 40 μm length slot pores) allows CTC to be captured on individual slots while only partially occluding the pore, and thus permits free passage for most hematopoietic cells without causing an increase in pressure across the filter surface that would otherwise lead to cell lysis [11, 12]. Both the slot pore and round-pore filter can be used interchangeably in the same filtration cartridge, where a top and bottom acrylic pieces with luers sandwich the filter between two Polydimethylsiloxane (PDMS) gaskets to provide a leak proof seal [9, 11]. As an alternative an automated device has developed to handle the filtration and viable CTC capture to provide a standardized, efficient, and precise process while eliminating any user-to-user variability. While the slot pore filter exhibits a high capture efficiency (Table 1), the electro-physical properties of the Parylene C membrane surface result in strong nonspecific binding of the captured CTC via electrostatic interactions [11]. While methods such as reverse flow or cell scraping have been explored and tested, these resulted in cell damage and cell death. Effective release of viable CTC from the filter remains nontrivial. Hence, we postulated the use of PIPAAm to formulate a release strategy [11]. PIPAAm is a polymer that undergoes a reversible lower critical solution temperature (LCST) phase transition at a solution temperature of 32 °C [13]. Traditionally, this property of PIPAAm has been utilized in tissue engineering applications, where cells are cultured on PIPAAm-coated surfaces at 37 °C while PIPAAm is in a hydrophobic state. Once cultured, the cells can be removed as a whole sheet simply by shifting the culture temperature to below 32 °C, hydrating the PIPAAm-coated surface [13, 14]. We exploited this property in a reverse fashion, where the filtration is carried out at room temperature (below 32 °C) when PIPAAm is hydrophilic. Following filtration, the filter is simply placed into culture medium maintained at 37 °C, which enables release of electrostatically bound cells through conversion of the PIPAAm polymer layer to a hydrophobic state cell [11] (Fig. 1).

Effective and efficient methods are desirable for the capture of the rare CTC. Currently, only a few antigen-agnostic technologies

exist [15–18] for the capture and release of viable CTC, critical for robust downstream molecular and functional analysis [19, 20]. Majority of these microfabricated devices are coupled to microfluidic platforms and thus have an intrinsic limitation that the amount of blood that can be processed is small, ranging from 2 to 4 mL, which makes capture of rare CTC that much harder. Additionally, these microfluidic methods require longer time periods to process samples. Although the temperature responsive method as well as other methods have been successfully implemented to achieve viable CTC capture and release [21–23], one key potential drawback shared by these technologies reported is that they all are antigen-dependent immuno-affinity CTC capture assays. Antigen-based CTC capture, as shown previously, may lead to biased CTC analysis given the heterogeneity of antigen expression across CTCs [6, 9]. For example, many affinity-based technologies employ antibody that binds EpCAM for CTC capture. However, CTC have been shown to express variable levels of EpCAM, leading to omission of EpCAM-low and EpCAM-negative CTC by these technologies. Also, additional limitations may arise when CTCs of interest are non-epithelial in origin such as CTCs in melanoma and sarcoma patients. Thus, an antigen-independent technology that allows for viable CTC capture and release is highly desirable. A major significance of the technique lies in the fact that while several platforms commonly used in CTC capture and analyses are limited in molecular and cellular analyses as a fixative is necessary for processing the sample, or because the CTCs are immobilized on platform. The slot-pore microfilter with the PIPAAm coating provides an alternative, label-free platform that allows efficient capture and release of viable CTC from whole blood, and permits further characterization of viable CTC, including single-cell phenotypic and genomic analysis. It also opens avenues for short-term ex vivo CTC propagation useful in developing chemo-sensitivity assays for individual patients (thus helping personalized patient-specific therapeutic approach), and can also help with long-term CTC culture. Importantly, the microfilter capture device is purely size based and the release strategy is agnostic to the presence of specific surface markers.

CTC has been well validated as a prognostic biomarker in various cancer settings, including breast, prostate, and colorectal cancer [2–4]. However, recent advances in CTC field have indicated that the mere enumeration of these rare cells has limited clinical utility, as shown in interventional clinical trials [24]. Recent studies reveal that CTC have the potential to be an excellent companion diagnostic biomarker for metastasis crucial for cancer management and treatment [2–4]. Thus, there is an emerging need for technologies that allow for molecular and functional characterization of CTC. Molecular and cellular analyses of CTC provide valuable information for cancer prognosis and may help drive precision medicine [15]. The viability of the cells released from the

capture filter is a key development toward the ability to culture patient CTC for drug sensitivity and screening tests. It has been long known that CTC tend to differ from the primary tumor and thus the necessity to treat both accordingly. As an example, Schneeweiss et al. reported that in metastatic breast cancer, antigen profiles of metastatic tissue and primary tumor differ in up to 20% of patients. Reassessment of predictive markers, including human epidermal growth factor receptor 2 (HER2) expression, might help to optimize MBC treatment. Recent studies have shown that breast cancer CTC can switch between HER2+ and HER2− status in culture, necessitating the need to interrogate cancer molecular profiles along treatment courses. Thus, "liquid biopsy" of cancer may hold crucial clinical significance as companion diagnostics [25]. Therefore, further advancements in this area will provide clinicians much needed insight to drive cancer management toward precision medicine and better treatment plans.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Microfilter PIPAAm Coating

1. Microfilter Membrane—custom slot geometry microfilter can be purchased from Circulogix Inc., FL, USA.
2. Plastic microscope slides.
3. Polyimide Tape also known as Kapton Tape.
4. Straight edge scissors.
5. Spin Coater.
6. Poly(*N*-iso-propylacrylamide) (PIPAAm) 10% w/v: Dissolve 1 g PIPAAm in 10 mL of 100% Butanol. Vortex till the solution is clear.

2.2 Filtration

1. PIPAAm-coated microfilters.
2. Filtration cartridge—custom cartridge can be purchased from Circulogix Inc., FL, USA.
3. Syringe Pump.
4. Hank's Balanced Salt Solution (HBSS; 1×).
5. Culture Media: Use media of choice based on type of cancer cells that are being captured on filter.
6. Forceps.
7. 6-well tissue culture plate.

3 Methods

The process of capturing viable CTC from whole blood and releasing them from the microfilter is relatively easy and a straightforward process. However, a few important points worth mentioning are that maintain a sterile condition though the whole process especially if the cells are being captured for culture. And carry out all procedures at room temperature unless otherwise specified as the process involves exploiting PIPAAm's temperature responsive interfacial properties. Time frame from collection of blood sample to filtration should be minimized as much as possible. Ideally, the sample should be processed within the hour after collection if possible but no more than 2–12 h (*see* **Note 1**).

3.1 Coating the Microfilter with Poly (N-iso-propylacrylamide) (PIPAAm)

1. Weigh out PIPAAm to prepare a 10% w/v solution in butanol. Mix using a vortex until the solution is clear.
2. Cut plastic microscope slides into roughly 12 mm × 12 mm squares either with a pair of scissors or sharp blade. Alternatively use a guillotine.
3. Using a sharp pair of straight-edge scissors, cut the slot pore microfilter wafer into 8 mm × 8 mm squares (*see* **Note 2**).
4. Using a polyimide film tape secure the cut filters onto the plastic squares that were cut in step 3.1.2. Apply the film only to the edge and the corner of the filter so that at least 7 mm × 7 mm of filter area is un-covered by the tape (Fig. 2).
5. Place the microfilter that is secured on the plastic square, onto the vacuum chuck of the spin-coater.
6. Program the spin-coater to the following recipe: Start, 500 rpm for 10 s, 6000 rpm for 60 s, 500 rpm for 10 s, Stop.
7. Dispense enough 10% w/v PIPAAm solution (prepared in **step 3.1.1**) to completely cover the microfilter surface using a standard plastic transfer pipette and start the spin coater.
8. Remove the PIPAAm-coated microfilter from the spin-coater after the coating process is completed. Leave the filter attached to the plastic square during storage. The coated filter can be stored at room temperature for up to 3 months (*see* **Note 3**).

3.2 Assembly of Filtration Cassette with PIPAAm-Coated Microfilter

1. Rehydrate PIPAAm-coated microfilter at room temperature by placing the microfilter still secured on the plastic square into a petri dish. Add 1× PBS until the microfilter is fully submerged and let it stand for 5 min.
2. After the hydration step, release the filter from the plastic square by cutting the polyimide film tape using a pair of scissor (*see* **Note 4**).

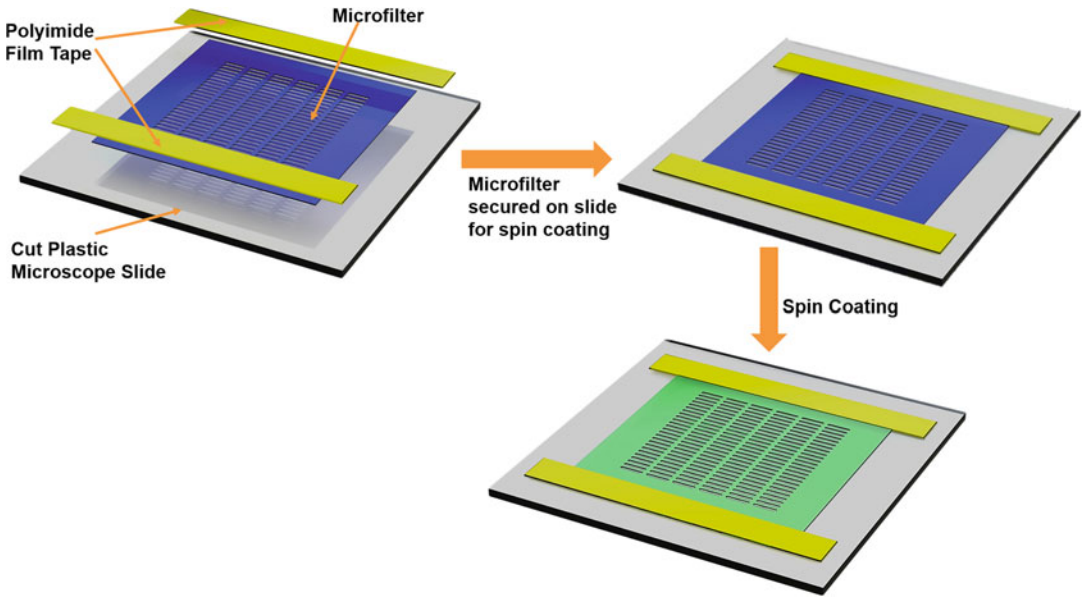


Fig. 2 Representative illustration of the microfilter setup for PIPAAm coating. 8 mm × 8 mm microfilter is positioned onto the 12 mm × 12 mm plastic square cut from a plastic microscope slide. Polyimide tape (*yellow*) is used to secure the microfilter in place to spin coat the PIPAAm. (*Blue* colored microfilter represents native uncoated surface of filter. *Green* colored microfilter represents spin-coated PIPAAm filter)

3. Assemble the microfilter into the filtration cassette, by sandwiching the microfilter between the top and bottom acrylic pieces along with the two Polydimethylsiloxane (PDMS) pieces acting as a gasket/seal. Clamp the acrylic cassette with clips to secure the filter and provide a tight seal (Fig. 2).

3.3 Filtration of Blood Sample with Capture and Release of CTC from the Microfilter

1. As each syringe pump brand has its own user interface, refer to the user manual of the pump and program the syringe pump to flow at a rate of 75 mL/h and set the volume to 20 mL.
2. Warm 3 mL of cell culture media (prepared by adding in the desired amount of Fetal Bovine Serum based on the need for the cells that will be captured and 1% Penicillin and Streptomycin) to 37 °C (*see* **Note 5**).
3. Add 7.5 mL of commercially available Hank's Balanced Salt Solution (HBSS) to the 7.5 mL of blood sample and aspirate this diluted blood into a 25 mL syringe (*see* **Note 6**).
4. Engage the filtration cassette with the syringe and place it onto the syringe pump. Place a 50 mL tube at bottom to collect the flow through and start the pump (*see* **Note 7**).
5. After filtration is complete, disengage the filtration cassette taking note which side is the top that has the cells caught on

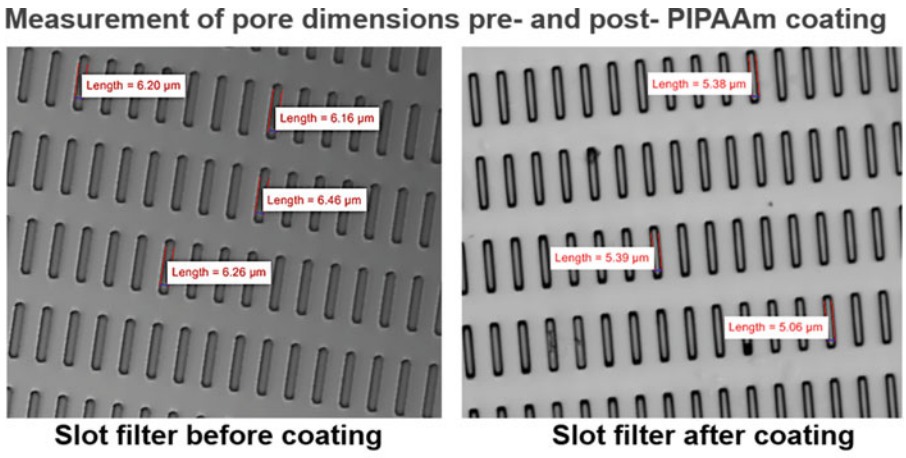
the PIPAAm-coated surface. Aspirate 1 mL of the warm culture media into a new syringe.

6. Re-engage the filtration cassette with the syringe containing the media, but this time engage the bottom end of the cassette so that the PIPAAm-coated surface of the filter is facing away from the syringe (*see* **Note 8**).
7. Place the syringe back onto the syringe pump and hold a small petri dish or 6-well plate right under the filtration cassette. Set the flow rate to 100 mL/h and start the pump.
8. Pass all the media through the filter and collect the flow-through in the petri dish. Wait for all the media to pass through and then stop the pump and remove the syringe and filtration cassette from the pump.
9. Disengage the filtration cassette from the syringe and open it to retrieve the filter from the cassette. Place the filter with the PIPAAm surface facing down into the same petri dish used to collect the reverse flow when releasing the cells from the pores. Add the rest of the warm media and place the petri dish into a culture incubator set at 37 °C (*see* **Note 9**).
10. After 30 min all the cells should be released from the filter. However, you may leave the filter within the petri dish in the incubator for 24 h to ensure all the cells have been released (*see* **Notes 10** and **11**).
11. After 24 h in culture, carefully remove the microfilter using forceps. The filter can be discarded at this time if it was not already discarded earlier and left in the media to ensure the cells have been released from it.
12. Gently pipette the culture medium up and down using a 1000 μL pipette, to resuspend the erythrocytes and peripheral blood mononuclear cell (PBMC) that may have been caught on the filter and released into the plate with the CTC. Perform this action carefully and gently to avoid detaching the loosely attached cancer cells. Carefully remove all the medium with the resuspended erythrocytes and PBMC while leaving behind the attached cancer cells and substitute with fresh medium pre-warmed to 37 °C (*see* **Note 12**).

4 Notes

1. Blood to be filtered for CTC capture should be collected in an EDTA tube to prevent coagulation. Ideally, the sample should be processed within 2–12 h if possible. However, the sample processed within a 24-h period does yield live cells but for the most part these cells have been subject to capture and analysis for telomerase activity.

2. These squares will serve as a holder for the filters during the spin-coating process. The squares will sit on the vacuum chuck and hold the filter in place as the PIPAAm is spin coated on the filter surface.
3. The initial step of coating the filter with PIPAAm is critical, as the basis for the technique of releasing the cells from the filter is based on exploiting PIPAAm's temperature responsive interfacial properties. To ensure the filter has been coated effectively, measure the size of a pore (width and length of a slot pore) on the filter under a microscope prior to coating and then again measure a pore size post coating. The pore size should be decreased by 1 μm on both the width and the length (Fig. 3).
4. Take note of which side of the filter has the PIPAAm coating. If it helps, cut the edge of the same corners every time, such as the left top corner, to keep track of the orientation of the filter.
5. The media choice will be based on the patient's cancer type or else if this is a model system experiment where cultured cells are to be spiked into healthy donor blood, use the media the cells



Filter	Average Length	Average Width	Area (Length X Width)
Non-coated	38.08±0.82	6.35±0.34	241.68
PIPAAm-coated	35.31±0.79	5.37±0.35	189.76
Decrease Percentage	7%	15%	21%

Fig. 3 Measurement of pore parameters pre- and post-PIPAAm coating. (**a**) Slot filter pre and (**b**) post-PIPAAm coating was imaged using Nikon Eclipse Ti-E microscope. (**c**) Pore Length was found to be decreased by 7.3 ± 2.1% after PIPAAm coating and pore width was found to be decreased by 15.3 ± 5.6% after PIPAAm coating

are normally cultured in. If unsure, please refer to culturing guide/method provided by the company the cells were purchased from. The identification of culture media, or modification of commercially available media, that will provide the optimal environment for these CTC to grow is a challenge in itself and multiple different media may need to be tested to identify what is optimal.

6. Adjust the volume of HBSS so that the blood sample is diluted with equal volume of 1:1 with HBSS. E.g. if 10 mL of blood is used, then mix with equal volume of 10 mL HBSS to achieve the 1:1 dilution. This will reduce the viscosity and allow for the filtration process to run smoothly. There will be certain instances when the viscosity is extremely high or large clots are present, in which case the syringe pump will not be able to force the blood through the filter. It is advised in these instances to stop the syringe pump, disengage the cassette from the syringe, open the top part of cassette, but leave the filter still attached to the bottom part. Large blood clots will be easily visible on the filter. Gently pipette 1 mL of HBSS onto the filter while holding the cassette at a slight angle to allow the clot to wash off. Once the clot has been removed, close the cassette, re-engage it to the syringe, and continue filtration.
7. All the filtration steps are to be carried out at normal room temperature (20–25 °C) including the later step for reverse flow of media to release any captured cells from the pores.
8. This step ensures that the cells embedded into the slot pores during filtration will be released through the application of a gentle reverse flow.
9. One of the limiting factors to establish a culture from patient CTC will lie in the number of CTC that are present in the blood sample. Low numbers will likely make the ability to expand the cells extremely difficult if not impossible. However, sequestering a sample with low cell counts into smaller culture areas may help, such as in a single well of a 96-well plate instead of a 6-well plate. If this method is chosen, then less volume of media will need to be used to compensate for the 96 well's volume size or alternatively the media with the captured cells can be equally distributed in to a few 96 wells.
10. In our experience usually after 30 min all the cells have been released from the filter. To ensure that the release is complete, one may gently shake the filter in the media using forceps to assure complete release of cells from the membrane. If in doubt, the filter can be left within the media in the incubator at 37 °C overnight as a fail-safe precaution to ensure complete release of the cells.

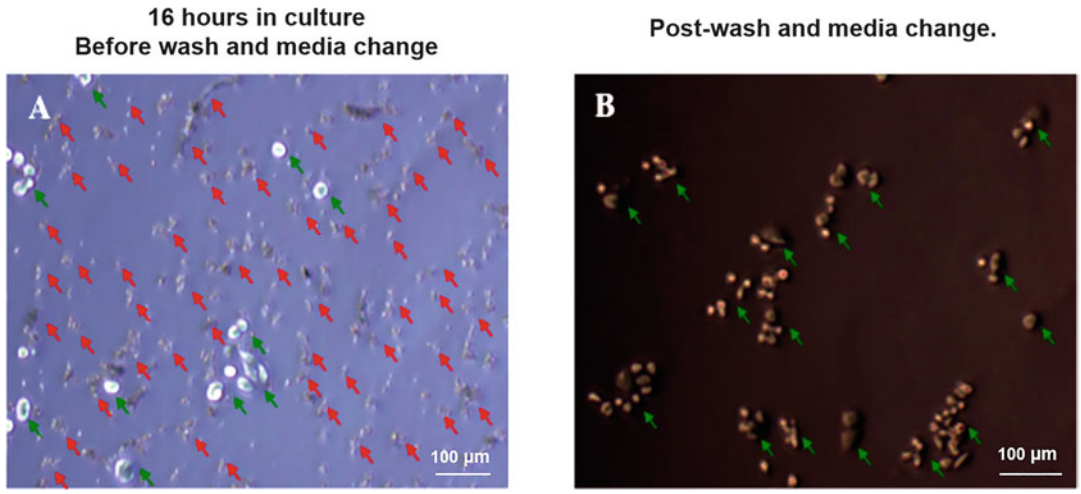


Fig. 4 Contaminating erythrocytes and leukocytes were removed by gentle washes. ~1000 SKBr-3 cells were retrieved from blood by PIPAAm-coated slot filter and were plated on a 48-well plate. (**a**) At 16 h in culture, SKBr-3 tumor cells adhered to culture plate (*green arrows*), whereas apoptotic erythrocytes and leukocytes were also settling at the bottom of the plate (*red arrows*). (**b**) Post-wash, non-adherent cells were removed, leaving adherent tumor cells on the plate (*green arrows*)

11. Alternatively, the released cells can be collected into a microcentrifuge tube to perform further downstream analyses such as PCR, ELISA, Western Blot to name a few examples. Also, the filter may be placed directly into the microcentrifuge tube and the relevant reagents add to it.
12. After the 24-h incubation period the cancer cells should have attached to the 6-well surface. Blood cells that may have been captured on the filter and introduced into the media along with the cancer cells, will sediment to the bottom of the well but will not have attached to the surface. This allows us to remove these contaminating cells while retaining the cancer cells through gentle pipetting. After performing this step once check under a microscope if suspended blood cells as seen in the media. If so, repeat the pipetting step once more to remove all unwanted cells (Fig. 4).

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Disclosures

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Chapter 8

Fourier Ptychographic Microscopy for Rapid, High-Resolution Imaging of Circulating Tumor Cells Enriched by Microfiltration

Anthony Williams, Jaebum Chung, Changhuei Yang, and Richard J. Cote

Abstract

Examining the hematogenous compartment for evidence of metastasis has increased significantly within the oncology research community in recent years, due to the development of technologies aimed at the enrichment of circulating tumor cells (CTCs), the subpopulation of primary tumor cells that gain access to the circulatory system and are responsible for colonization at distant sites. In contrast to other technologies, filtration-based CTC enrichment, which exploits differences in size between larger tumor cells and surrounding smaller, non-tumor blood cells, has the potential to improve CTC characterization through isolation of tumor cell populations with greater molecular heterogeneity. However, microscopic analysis of uneven filtration surfaces containing CTCs is laborious, time-consuming, and inconsistent, preventing widespread use of filtration-based enrichment technologies. Here, integrated with a microfiltration-based CTC and rare cell enrichment device we have previously described, we present a protocol for Fourier Ptychographic Microscopy (FPM), a method that, unlike many automated imaging platforms, produces high-speed, high-resolution images that can be digitally refocused, allowing users to observe objects of interest present on multiple focal planes within the same image frame. The development of a cost-effective and high-throughput CTC analysis system for filtration-based enrichment technologies could have profound clinical implications for improved CTC detection and analysis.

Key words Circulating tumor cells, Fourier ptychographic microscopy, Filtration-based CTC enrichment, Single-cell imaging, Metastasis

1 Introduction

Metastatic disease accounts for 90% of cancer-related mortality, and is the most important determinant in clinical management of patients with cancer. As a result, circulating tumor cells (CTCs) in peripheral blood have emerged in recent years as a valuable biomarker with strong potential to improve prognosis and diagnosis of recurrence. Assaying for CTCs requires only a simple, minimally invasive blood draw, providing a unique opportunity for repeated

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sampling in patients to monitor both metastatic disease and therapeutic response in real time. Thus, enumeration of CTCs with respect to progression-free survival, overall survival, and therapeutic response has been widely reported in a number of solid tumor malignancies [1–6].

Currently, isolation of CTCs by density gradient centrifugation [7, 8], indirect detection of CTCs by RT-PCR [9–11], and affinity-based capture of CTCs using cell surface markers specifically expressed by malignant cells [1–6, 12] are the strategies most commonly used to identify and isolate CTCs. However, current strategies for CTC analysis each have limitations [13, 14]. In addition, size-based isolation of CTCs from whole blood has been attempted since the 1960s [15], and has been revisited more recently. Utilizing the well-known characteristic that malignant cells are larger than surrounding normal blood cells, CTCs are isolated by using microfilters fabricated with a defined pore size which allow for the passage of smaller blood cells to pass while capturing larger CTCs [15–22]. Where the sensitivity and efficiency of affinity-based CTC enrichment strategies rely primarily on tissue- and/or tumor-specific cell surface biomarkers with the potential for highly variable inter tumor expression, size-based enrichment technologies are "antigen expression-agnostic," allowing analysis of CTCs in tumor types with low or no target antigen expression.

Among others [16–20], our group has developed a novel membrane microfilter device for the size-based isolation of CTCs in blood [21, 22]. Our technology provides the opportunity to perform molecular characterization of CTCs beyond their enumeration, a critical step toward a better understanding of the mechanisms involved in their release, hematogenous spread, and colonization of tissues at distant sites from the tumor origin. Our microfilters for CTC capture and analysis are fabricated using a precisely defined, stepwise photolithography process as previously described [21]. Although based on similar principles for CTC enrichment, the fundamental differences between our technology and other size-based technologies are (1) the material from which the filters are manufactured (i.e., parylene-C vs. polycarbonate, and biocompatible polymers) and (2) the manner in which the pores are deposited onto the membrane, where pores are evenly and specifically dispersed on our device rather than randomly dispersed [21]. Since our original description of this technique [22], we have developed a fully automated system for blood sample delivery through the microfilter, providing an improved user-friendly device with enhanced intra- and inter-user consistency. Briefly, the individual microfilters are secured within a housing cassette that assures an airtight seal to prevent sample leakage. The house cassette is then mounted into the automated fluid delivery device, and samples are passed through the microfilter with vacuum negative

Table 1
Commercially available microfilter devices for CTC enrichment

Device	Company	References	Description
ISET	Rarecells (Paris, France)	[16, 18, 19]	Semiautomated device drawing samples across ten filtration areas in parallel
CellSieve	Creatv Microtech (Potomac, MD)	[25, 26]	Semiautomated device and housing cassette with 13 mm filtration area
Nucleopore	GE Life Sciences (Pittsburgh, PA)	[15]	Track-etched microfilter and housing cassette, used for CTC enrichment as well as other applications
faCTChecker	Circulogix, Inc. (Miami, FL)	[21–23]	Fully automated sample mixing and fluid delivery across disposable filter-slide cartridges; highly compatible for downstream automated immunolabeling and other molecular analyses

Commonly used microfilter devices for CTC enrichment, as well as their manufacturers, are listed. The faCTChecker by Circulogix, Inc. (in bold print) is the CTC enrichment platform upon which FPM for CTC imaging was developed.

pressure under precise regulation. When a blood sample is received, it is briefly fixed in 1% neutral buffered formalin for 10 min, and then subjected to microfiltration for sized-based CTC enrichment. While this is a method that we have developed and validated, it is important to note that any commercially available microfilter can be used upstream of imaging by FPM, provided they are optically transparent and equipped with a similar housing cassette to prevent cell loss during sample passage. A list of some commercially available microfilters used for CTC enrichment is provided in Table 1.

Identification and characterization of CTCs by IHC is carried out using standard protocols used in clinical cytology and histology. Due to the brief fixation of cells, antigen retrieval is not required for antigen unmasking. Briefly, a cocktail of primary antibodies raised in different animals against high and low molecular weight cytokeratins (CK) and CD45 are used for positive identification of CTCs and discrimination of non-tumor blood cells, respectively. Secondary antibodies raised in animals corresponding to the primary antibodies in the cocktail are conjugated to alkaline phosphatase (AP) and horseradish peroxidase (HRP), and used for signal amplification. 3′,3′-diaminobenzidine (DAB) reactive with HRP forms a brown precipitate reporting anti-CD45 reactivity and Warp Red reactive with AP, forms a red precipitate reporting anti-Pan CK reactivity. Microfilters are finally dehydrated and counterstained with hematoxylin for nuclear visualization. CTCs are identified as large (typically 15–40 μm diameter), nucleated, CK+/CD45− events with morphological criteria consistent with malignancy.

With the potential to overcome the limitations accompanying other platforms, size-based CTC enrichment strategies possess technical limitations of their own. The most significant of these

limitations is that following sample processing, the surface of the microfilters becomes uneven with short, micro-scale modulations. Because captured cells are randomly dispersed throughout the microfilter, often times multiple CTCs are present on different focal planes. This technical limitation requires that the user must constantly change focus while viewing and imaging cells of interest on the microfilter, making sample analysis labor-intensive, time-consuming, and inefficient [23].

To address this challenge, we have previously described the successful adaptation of Fourier Ptychographic Microscopy (FPM) for the molecular characterization of CTCs captured by our microfilter device. FPM is a coherent imaging system able to obtain the high-resolution complex field information of a sample over a wide field of view. It captures a series of low-resolution intensity images under varied angular plane wave illumination, and uses a phase retrieval algorithm to synthesize the images in the Fourier domain [24]. It effectively increases the NA of the microscope by the illumination NA defined by the maximum illumination angle. With the complex field information, we can easily propagate the field to different focal depths computationally in postprocessing such that CTCs in different focal planes in different sample regions can be all brought into focus, eliminating the need for a user to manually focus during imaging.

The FPM setup consists of the following components: a conventional microscope with a low NA and a wide field-of-view objective lens, a square LED array for illumination, and a monochrome camera attached to a computer for image capturing and processing (Fig. 1). As implemented in [23], the objective lens is a 2× magnification, 0.08 NA objective lens. The camera's pixel density is high enough to satisfy the Nyquist requirement for the given NA of the objective lens, which is in this case 5.5 μm pixel pitch. The sensor should have a high dynamic range, e.g., 16-bit well-depth. The LEDs have bandwidth of less than 10 nm, which can be achieved by selecting LEDs with the appropriate center wavelength and inserting a 10 nm bandpass filter in the microscope's optical path. The LEDs are each 100 μm in diameter and arranged in a 15 by 15 square matrix with 4 mm pitch. The matrix acts as an angularly varying illumination source for the sample. The LED pitch and the placement of the matrix with respect to the sample plane are defined by the Fourier spectrum overlapping requirement as described elsewhere [24]. Different LED pitch and matrix placement distances need to be used for a different objective lens appropriate for its NA. The LEDs are individually addressable by a computer to synchronize the camera's exposure with an LED's on-state. For RGB image acquisition, three sets of LEDs (red, green, and blue) are required.

Using FPM to image tumor cells enriched by our microfilter in model systems, we demonstrated a nearly perfect correlation

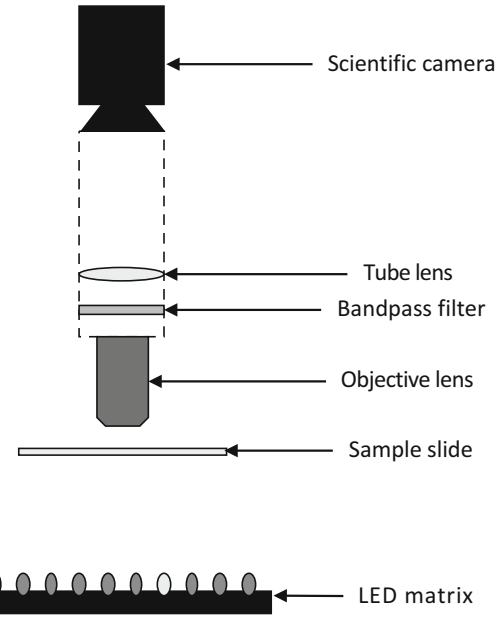

Fig. 1 Typical FPM setup. Conventional microscope consisting of a scientific camera, a tube lens, a bandpass filter with 10 nm bandwidth centered at the desired wavelength, and a wide-field, low NA objective lens. Instead of a conventional condenser for illumination, the microscope is fitted with an LED matrix to provide varied illumination angles to the sample

($R^2 = 0.99932$) when comparing detection of microfilter-captured tumor cells by FPM to standard microscopy [23]. Herein, we present a detailed protocol for the use of FPM integrated with our novel device for fast, high-quality imaging of CTCs enriched by microfiltration.

2 Materials

2.1 CTC Enrichment by Microfiltration

1. 10% Neutral Buffered Formalin (NBF).
2. Hank's Buffered Salt Solution (HBSS).
3. 8 μm round pore microfilters + housing cassette.

2.2 Post-enrichment Molecular Characterization by Immunohisto-chemistry (IHC)

1. 100% ethanol (histology grade).
2. 90% and 70% ethanol (dilution prepared from histology grade ethanol using deionized H_2O).
3. 0.03% H_2O_2 in methanol (histology grade).
4. Prepare blocking buffer: 5% normal serum (from the same animal secondary antibodies used are raised in), 1% bovine serum albumin (BSA), and 0.3% Triton X-100 in 1× tris

buffered saline (TBS). Store blocking buffer at 4 °C until ready for use.

5. DAB and Warp Red chromogens, used as specified by commercial manufacturer of choice.

2.3 Fourier Ptychographic Microscopy (FPM) Setup

1. 2×, 0.08 NA objective lens, or any low NA objective lens with a wide field of view.
2. Conventional microscope with a tube lens and a rotating filter mount.
3. Monochrome scientific camera with 5.5 μm pixel pitch and big sensor size for capturing the wide field of view afforded by the objective lens.
4. 100 μm monochromatic LEDs arranged in a 15 by 15 square matrix with 4 mm pitch.
5. Individually addressable LED driver.
6. 10 nm bandpass filter for desired LED center wavelengths.
7. A computer capable of interfacing with the camera to acquire images and the LED driver to turn on individual LEDs.
8. Programming software such as MATLAB or C++ for reconstructing high-resolution FPM images.

3 Methods

3.1 Blood Collection and CTC Capture by Microfiltration

1. Collect patient blood samples by standard phlebotomy technique into anti-coagulant tubes (*see* **Note 1**). *When using the faCTChecker device (Circulogix Inc., Miami FL), load blood sample into the system as directed by the manufacturer and proceed directly to Subheading 3.2 following completion of automated mixing and sample processing.*
2. Immediately following receipt of blood, dilute samples 1:1 in HBSS and 10% NBF to a final concentration of 1% (*see* **Note 2**), e.g., 8 mL whole blood should be diluted with 6.4 mL HBSS and 1.6 mL 10% NBF for a final volume of 16 mL at a final concentration of 1% NBF.
3. Place diluted blood sample on a rocker for 10 min at room temperature to sufficiently mix all components.
4. Raise the sample into a syringe, mount onto the inlet port of the microfilter housing cassette (given your chosen CTC microfiltration enrichment method), and pass the sample across the microfilter at low, constant speed.
5. Rinse the microfilter by passing an equal mixture volume HBSS across the microfilter, as described in **step 5**.

3.2 Post-enrichment IHC for CTC Identification and Molecular Characterization

1. Wash microfilters for 10 min in 1× TBS.
2. Rehydrate microfilters with washes in decreasing concentrations of alcohol: 100%, 90%, and 70% for 6 min each.
3. Wash microfilters in 0.03 H_2O_2 + methanol for 20 min.
4. Wash filters with deionized H_2O for 5 min.
5. To block nonspecific primary antibody reactivity, incubate microfilters in blocking buffer for 30 min (*see* **Note 3**).
6. Incubate microfilters with anti-human Pan-CK/CD45 primary antibody cocktail overnight (12–18 h) at room temperature (*see* **Note 4**).
7. Wash microfilter in 1× TBS for 10 min (*see* **Note 5**).
8. Incubate microfilters with secondary antibody cocktail containing both goat anti-rabbit AP and goat anti-mouse HRP activity for 30 min.
9. Wash microfilters in 1× TBS for 10 min.
10. Incubate microfilters in DAB (reactive with HRP) for 5 min.
11. Wash microfilters briefly in deionized H_2O.
12. Incubate microfilters in Warp Red (reactive with HRP) for 7 min (*see* **Note 6**).
13. Wash microfilters briefly in deionized H_2O.
14. Incubate microfilters in CAT Hematoxylin for 3 min.
15. Wash microfilters briefly in deionized H_2O.
16. Incubate microfilters in Tacha's Bluing Reagent for 3 min.
17. Wash briefly in deionized H_2O.
18. Dehydrate microfilters with washes in increasing concentrations of alcohol: 70%, 90%, and 100%, 6 min for each wash.
19. Incubate microfilters in xylene for 5 min. Add a coverslip using an aqueous mounting medium.

3.3 Image Acquisition and Microfilter Scanning Using FPM Setup

The following is a procedure for one color. For RGB acquisition, repeat the procedure for red (~630 nm), green (~532 nm), and blue (470 nm) LEDs.

1. Insert 10 nm bandpass filter with the desired center wavelength in the conventional microscope's filter mount.
2. Place the LED matrix 80 mm away from the sample plane to provide varied angular illumination.
3. Turn on one LED.
4. Capture an image with appropriate exposure (*see* **Note 7**).
5. Repeat **steps 3–4** for all LEDs on the matrix.
6. Break the captured sample region into small tiles (e.g., 128 by 128 pixels) to allow for the images of CTCs having different

depths in different tiles to all be brought into sharp focus after reconstruction (*see* **Note 8**).

7. Synthesize the captured images corresponding to each tile into a high-resolution, complex field image using FPM's phase retrieval algorithm.
8. Digitally refocus each tile on appropriate focal plane to bring CTC images into sharp focus.

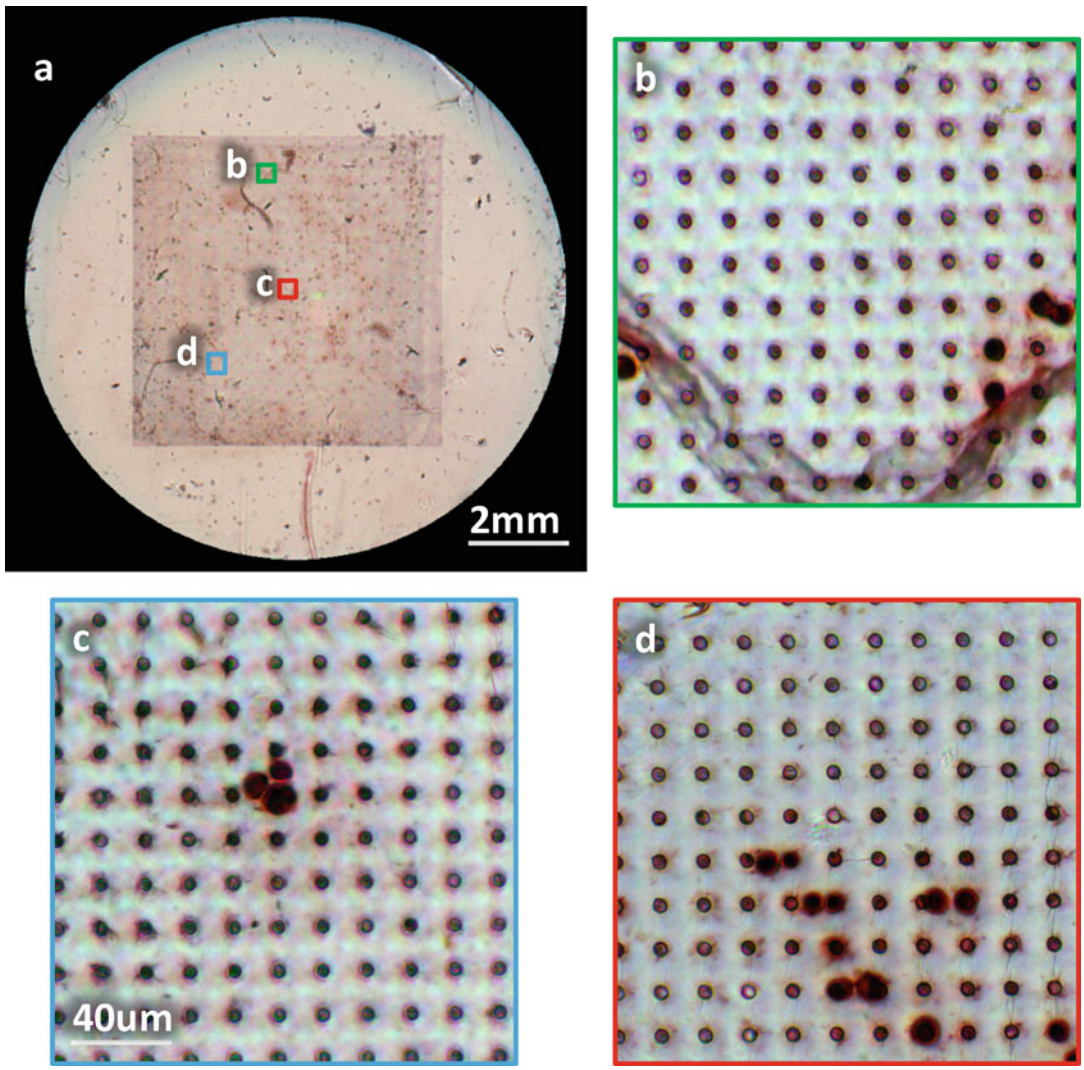

Fig. 2 Wide field-of-view image of tumor cells enriched by microfiltration. Our FPM setup permits a 13 mm diameter field of view using the 2× objective lens at the resolution equivalent to that of a 20× objective lens. (**a**) Wide field-of-view image. (**b**–**d**) Magnified view of different regions in the field of view demonstrating focused, high resolution afforded by FPM

9. Synthesize the tiles of reconstructed images to generate a wide-field, high-resolution image of the sample, sharply in focus throughout the field of view (Fig. 2).

4 Notes

1. If blood collection is being conducted on site at the institution processing the samples for CTC enrichment, EDTA tubes are recommended for use. Samples can be stored at room temperature for up to 4 h before microfiltration when collected into EDTA tubes. However, if samples need to be shipped overnight to a secondary site for microfiltration, CellSave Preservative Tubes (Janssen Diagnostics, Raritan NJ) are recommended for use. Blood collected in CellSave Preservative Tubes can be processed for microfiltration up to 48 h following sample collection with no observed loss of cellular integrity.
2. 1× PBS can also be used to dilute samples, but we have noted that the use of PBS can compromise cellular integrity and result in diminished morphologic quality when imaging CTCs.
3. Proceed directly to **step 6**, do not wash the samples prior to incubation in the primary antibody cocktail. Washing the samples after blocking could result in increased nonspecific binding of primary antibodies. However, if you are using a primary antibody against a protein of low abundance, or have observed that a weak signal from your primary antibody of interest in past trials, washing at this step could help increase signal intensity.
4. While our protocol calls for primary antibody incubation overnight at room temperature, some antigens show better reactivity if overnight incubation is conducted at 4 °C.
5. Although PBS is typically used as a wash buffer in IHC protocols, it is important that TBS be used as the wash buffer throughout this protocol. The use of PBS contributes a nonspecific phosphate substrate to the alkaline phosphatase-linked secondary antibody, leading to high background signal during analysis.
6. Other chromogens may be used if desired, taking into account appropriate contrast in colors to maximize reporter visibility.
7. For illumination angles that result in low image signal, the exposure time needs to be increased to capture an adequate amount of photons. For angles that allow for a lot of photons to be captured by the camera, the exposure should be reduced to prevent saturated pixels in the captured images. All captured

images should be normalized by their exposure times prior to reconstruction for accurate convergence.

8. Prior to FPM acquisition process, the LED illuminance for different angular illumination and different areas in the sample region should be characterized by placing an opal diffuser on the sample plane and capturing images under different illumination angles. The captured images for each tile of sample should be normalized by the illuminance value of the corresponding tile area and illumination angle.

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Chapter 9

Automated Microfluidic Filtration and Immunocytochemistry Detection System for Capture and Enumeration of Circulating Tumor Cells and Other Rare Cell Populations in Blood

Michael Pugia, Mark Jesus M. Magbanua, and John W. Park

Abstract

Isolation by size using a filter membrane offers an antigen-independent method for capturing rare cells present in blood of cancer patients. Multiple cell types, including circulating tumor cells (CTCs), captured on the filter membrane can be simultaneously identified via immunocytochemistry (ICC) analysis of specific cellular biomarkers. Here, we describe an automated microfluidic filtration method combined with a liquid handling system for sequential ICC assays to detect and enumerate non-hematologic rare cells in blood.

Key words Immunocytochemistry (ICC), Filtration, Circulating tumor cells (CTC), Circulating mesenchymal cells (CMC), Circulating endothelial cells (CEC), Cancer stem cells (CSC), Epithelial cell adhesion molecule (EPCAM), Cytokeratins (CK)

1 Introduction

Current methods that have been developed to quantify levels of CTCs in the blood rely on the expression of epithelial cell adhesion molecule (EPCAM) [1–3]. Recent studies, however, have identified CTCs that have downregulated expression of EPCAM, e.g., cells undergoing epithelial-mesenchymal transition or EMT [4, 5]. Selection by size using filter membranes provides an antigen-independent approach that can obviate the reliance on cellular markers for enrichment and capture of circulating rare cells [6, 7]. Additionally, cells captured on filter membranes can be subjected to cytomorphological and immunocytochemical analysis for cell phenotyping, identification, and classification. We recently tested an automated filtration-based approach coupled to a sequential ICC staining system to capture and enumerate non-hematologic rare cells populations, including CTCs, circulating mesenchymal cells (CMCs), circulating endothelial cells (CECs), and putative cancer

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stem cells (CSCs), in blood of cancer patients [8]. Details of the methods are described in the following protocol.

2 Materials

2.1 Reagents

1. Phosphate buffered solution (1× PBS): dilute 10× PBS pH 7.4 in ultrapure deionized water to make a working concentration (1×) with 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4. Sterilize 1× PBS by passing through a 0.2 μm filter.
2. Phosphate buffered solution-fibrin (PBS-fibrin): Dissolve 10 mg of fibrinogen in 100 mL of 1× PBS to make a solution of 0.1 mg/mL fibrinogen. Dissolve 2.4 mg of trypsin (>10,000 BAEE units/mg) in 83 mL of 1× PBS to make a trypsin solution. Dissolve 416 mg of benzamidine in 83 mL of 1× PBS to make a benzamidine solution. Add 83 mL of PBS 10× pH 7.4 to 750 mL of ultrapure deionized water, and heat in a water bath at 37 °C for 15 min. Remove the PBS solution from the water bath, and add 83 mL of the fibrinogen solution and 83 mL of the trypsin solution. Heat the resulting solution in the water bath at 37 °C for an additional 5 min. Cool the solution to room temperature, and add 83 mL of the benzamidine solution. Sterilize PBS-fibrin solution by passing through a 0.2 μm filter.
3. Blood collection tube containing potassium ethylenediamine tetraacetic acid (K3EDTA) and paraformaldehyde (0.45 mL Transfix®).

2.2 Antibodies and ICC Reagents

1. Fixative solution: 4% formaldehyde in PBS.
2. Permeabilization buffer: Dissolve 400 μL of 10% Triton™ X-100 in 19.6 mL of 1× PBS to make 0.2% solution.
3. Blocking solution: casein-based blocking solution (e.g., The Blocking Solution, Candor Bioscience).
4. Phosphate buffered solution-Tween (PBS-T): Add 0.5 mL of Tween®-20 to 100 mL of 10× PBS pH 7.4 and dilute to 1 L in ultrapure deionized water. Filter the resulting solution through a 0.2 μm sterile filter.
5. 1 mg/mL of 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst: Dissolve (DAPI) or Hoechst in ultrapure deionized water to make a 1 mg/mL solution. Divide the resulting solution into aliquots of 0.5 mL. Freeze and protect from light. Thaw the solution before each use.
6. 1,4-Diazabicyclo [2.2.2] octane (DABCO) cover medium: Add 0.25 mg of DABCO to 9 mL of glycerol and 1 mL of 10× PBS pH 7.4 and gently mix until dissolved. Divide the

solution into aliquots of 0.5 mL and freeze and protect from light. Thaw the solution before each use.

7. Tyramide-Alexa 488: Prepare as directed in the Anti-species Alexa Tyramide Signal Amplification (TSA™) kit (Life Technologies) (*see* **Notes 1** and **2**).
8. Dylight™ (ThermoFisher) conjugated antibodies: Prepare conjugated antibodies at 1.0 mg/mL by standard coupling chemistry allowing coupling via an amide bond with Dylight N-hydroxysuccinimide esters (Siemens Healthcare Diagnostics). Store materials in PBS pH 7.4 with 1% bovine serum albumin (BSA), and 0.1% NaN_3.
9. Primary antibody solutions: Add antibodies to the required volume of the blocking solution in a 15 mL tube. The concentrations of the antibody solutions required for each assay are shown in Table 1. The assays consist of antibodies made against cellular markers like cytokeratin (CK) and EPCAM to identify CTCs, CD45 to identify white blood cells (WBC), vimentin (VIM) to identify CMCs, CD144 to identify CECs, and Piwi-like protein 2 (PIWIL2) and TPBG/5T4 to identify putative CSCs from breast and lung cancer patients, respectively (*see* **Note 2**).
10. Secondary antibody solutions: These include horseradish peroxidase (HRP)-conjugated rabbit anti-goat and goat anti-rabbit antibodies to detect primary antibodies against PIWIL2 and TPBG/5T4, respectively. Dilute the secondary antibodies in the blocking solution in a 1:100 ratio.

Table 1
Antibodies and fluorescent labels

Marker	Clone	Conc. (μg/mL)	Fluorescent probe	Emission color	Excitation filter	Emission filter
			DAPI	Blue	360/40	470/40
CK8/18	UCD/PR10.11	10	DyLight550	Orange-red	546/12	600/40
CK19	A53-B/A2	10	DyLight550	Orange-red	546/12	600/40
PanCK	AE1/AE3	10	DyLight550	Orange-red	546/12	600/40
EpCAM	VU1D9	20	DyLight550	Orange-red	546/12	600/40
Vimentin	V9	15	DyLight550	Orange-red	546/12	600/40
CD45	9.5	15	DyLight650	Violet	620/60	700/75
CD144	FB	40	DyLight488	Green	480/40	527/30
PIWIL2	K-18 (goat pAb)	15	TSA Alexa 488	Green	480/40	527/30
TPBG/5T4	EPR5530 (rabbit mAb)	15	TSA Alexa 488	Green	480/40	527/30

Reproduced from Magbanua et al. [8]
pAB polyclonal antibody, *mAb* monoclonal antibody

Table 2
Cancer cell lines used as controls

Line	Source	Product number	Medium	Assay-relevant properties
SKBR3	ATCC Human breast cancer line	HTB-30	IMDM + 15% FBS	CK+, VIM−, CD144−, TPBG/5T4−, PIWIL2−, CD144−
NCI H226	ATCC Human lung cancer line	CRL-5826	RPMI-1640 + 10% FBS	CK+, VIM+, TPBG/5T4−, PIWIL2−, CD144−
NCI H2228	ATCC Human lung cancer line	CRL-5826	RPMI-1640 + 10% FBS	CK+, VIM+, CD144−, TPBG/5T4+, PIWIL2−, CD144−
MDA-MB-231	ATCC Human breast cancer line	HTB-26	Leibovitz's L-15 Medium	CK+, VIM−, TPBG/5T4−, PIWIL2−, CD144−
MDA-MB-231 PIWIL2[a]	ATCC Human breast cancer line	See below	Leibovitz's L-15 Medium	CK+, VIM−, TPBG/5T4−, PIWIL2+, CD144−

ATCC American type culture collection, *FBS* fetal bovine serum
[a]PIWIL2 transfected MDA-MB-231 was provided by Dr. Jian-Xin Gao, Shanghai Jiao Tong University School of Medicine, Shanghai, China

2.3 Control Cells

1. Cancer cell lines (American Type Culture Collection, ATCC) (*see* Table 2).
2. Hank's balanced saline solution (HBSS).
3. Fetal bovine serum (FBS).
4. 0.4% Trypan blue stain.
5. 2% formaldehyde HBSS: Add 0.625 mL of 16% formaldehyde to 4.75 mL HBSS.
6. 75 cm^2 culture flask.

2.4 Equipment

1. Micromanipulation system with inverted microscope adapter (Eppendorf, TransferMan NK2 or similar) with microinjector (e.g., Eppendorf, CellTram Vario, or similar).
2. Hamilton STARlet™ automatic pipette robot (Hamilton Company, Reno Nevada) for automation of the cell isolation and ICC (*see* Fig. 1 and **Note 3**).
3. A circular filter membrane with pore size of 8 μm and a diameter of 25 mm (Whatman™ Nucleopore™, GE Healthcare).
4. Micro-machined plastic support that serves as a microscope slide (*see* Figs. 1 and 2, and **Notes 3**).
5. Fluorescence microscope (e.g., Leica DM5000, Leica Microsystems GmbH).

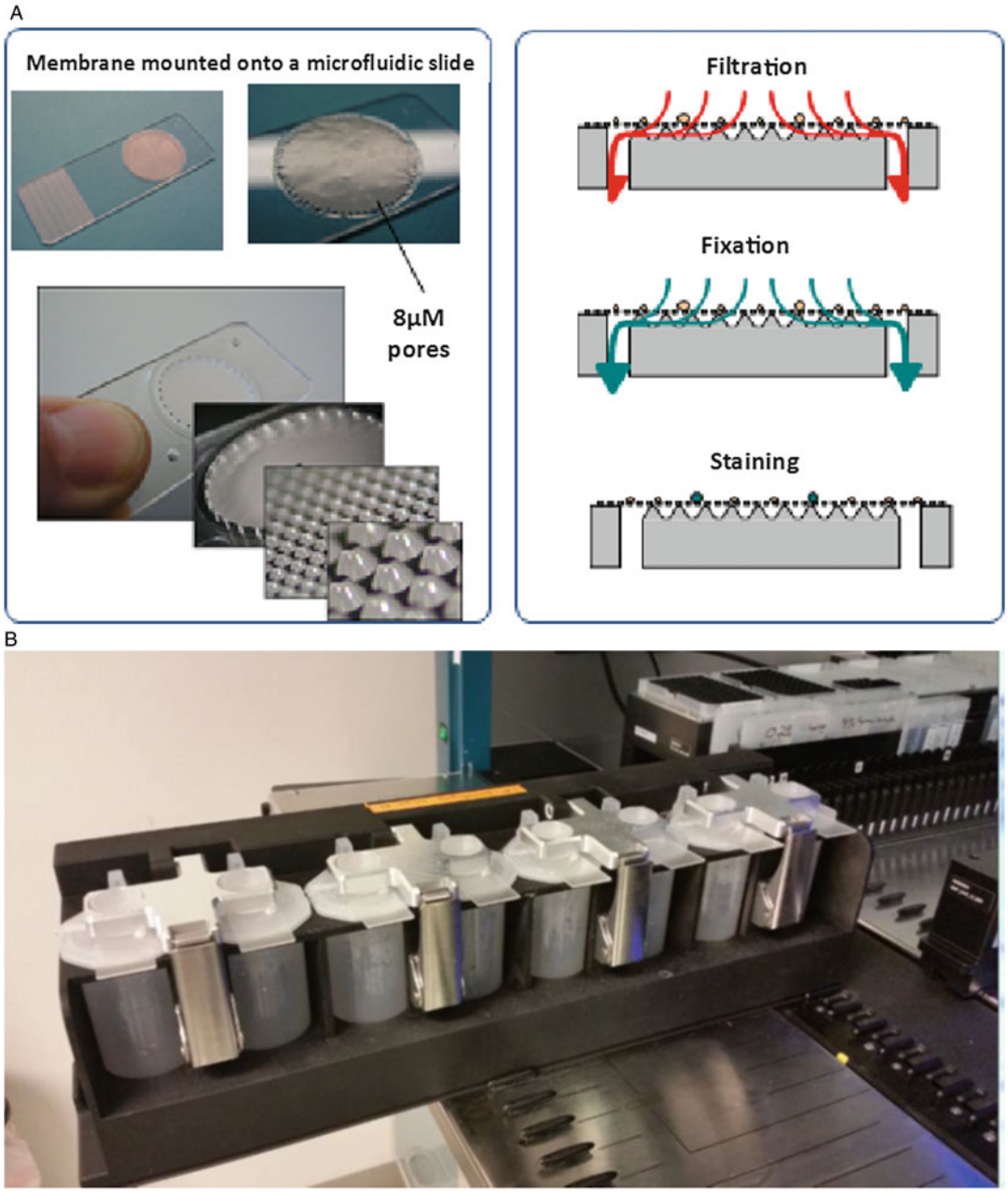

Fig. 1 The filtration system. (**a**) Design of the microfluidic filter device and the schematic overview of the processing steps for capture and detection of circulating rare cells. The filter membrane is mounted onto microscope plastic slide with microfluidic structures. The top of the membrane is exposed to the pipetting system of the robot, while the underside of the membrane is welded on the outer ring of the plastic support and is contact with the microfluidic structures. The structure consists of microfluidic posts, which allow for controlled filtration across a pressure range of negative 10–30 mbar as well as efficient washing of cells during the isolation and staining process (reproduced from Magbanua et al. 2015 [8]). (**b**) The disposable microscope plastic slide is integrated into a waste container with a top well for holding 5 mL of liquid and a bottom waste container capable of holding 110 mL of waste liquid. The waste container encloses around the top and bottom of the slide when mounting onto the filtration unit. The waste container connects directly to the vacuum from the underside of the slide

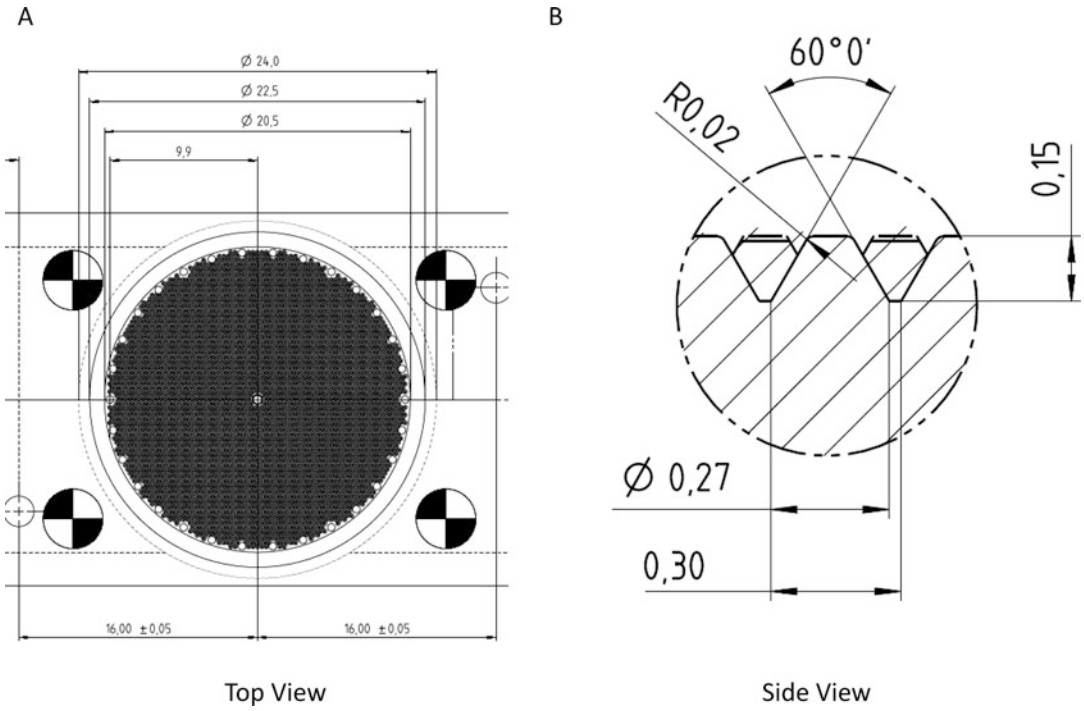

Fig. 2 Details of the microfluidic device. (**a**) Top view dimensions of the field of microfluidic posts that reside under membrane in the plastic base. The post field has a diameter of 20.5 mm. The membrane is welded to the plastic base in a ring at 20.5–24.0 mm. A ring of through holes resides at a ring diameter of 19.6 mm so that liquid can be drained in the waste container. (**b**) Side view dimensions of a microfluidic post that the membrane is placed onto. The height is 0.15 mm and the width is 0.30 mm (reproduced from Magbanua et al. [8])

3 Method

3.1 Blood Collection Procedure

1. Collect approximately 6–9 mL of blood into collection tube. Invert the tube ten times immediately after blood draw. Figure 3 shows a flowchart illustrating the entire process.
2. Store blood samples (for up to 9 days) at ambient laboratory temperature until further processing (*see* **Note 4**).

3.2 Cell Control Preparation

1. Harvest cancer cells by removing media from a flask of cells grown to ~80% confluency (*see* Table 2).
2. Add 5 mL of trypsin solution and place in an incubator at 37 °C for 5 min at time until cells are free floating.
3. Add 5 mL of media and transfer contents of flask to centrifuge tube and spin at 125 × *g* for 5 min.

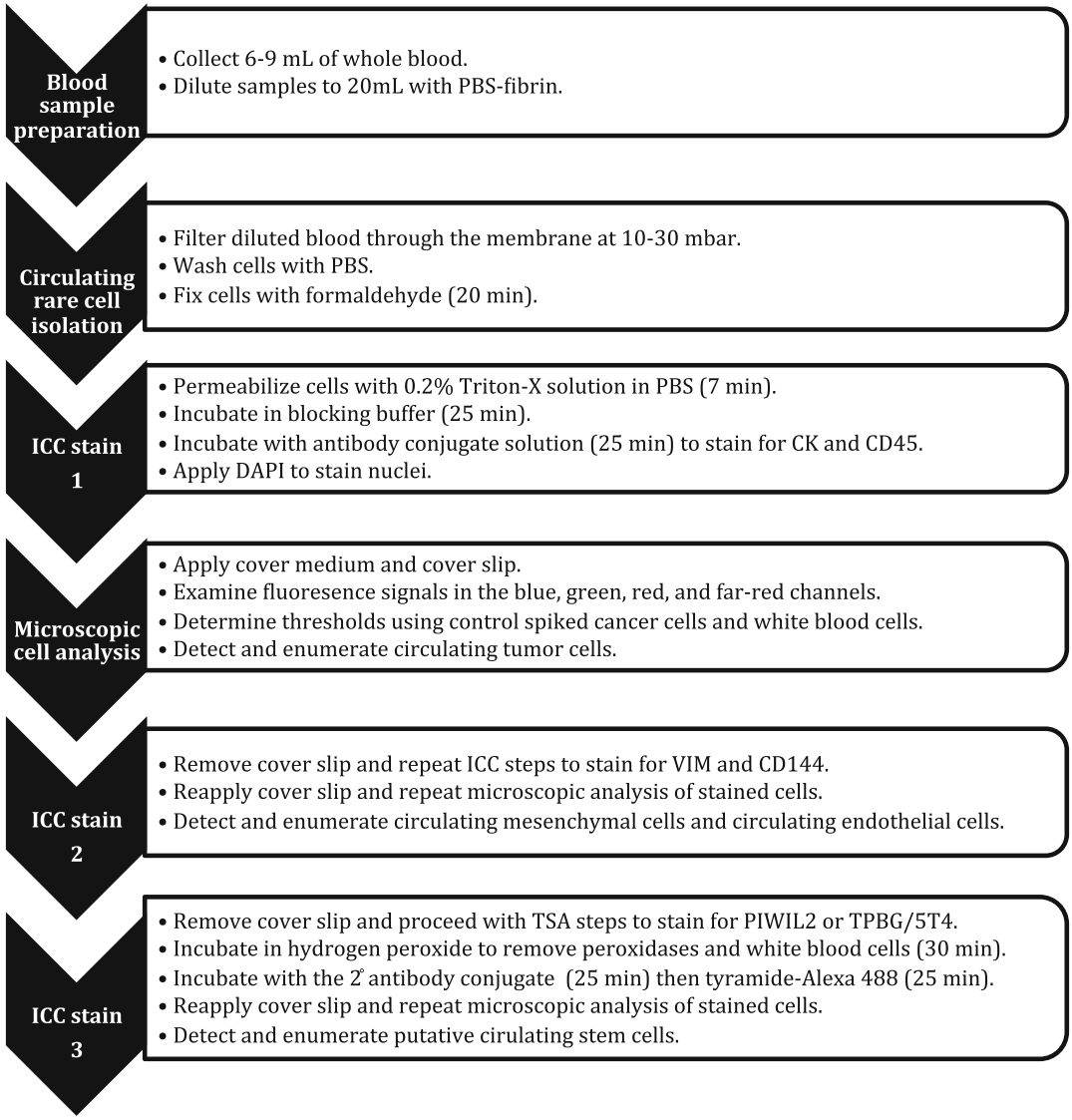

Fig. 3 Sample preparation and staining methods. Flowchart illustrating the blood sample preparation, isolation, and the staining procedures (reproduced from Magbanua et al. [8])

4. Decant the supernatant and resuspend pellet in 10 mL HBSS, then centrifuge again at 125 × *g* for 5 min, and decant the supernatant.
5. Resuspend cell pellet in 1.0 mL of 2% formaldehyde HBSS and incubate at 2–8 °C overnight for 24 h (*see* **Note 5**).
6. Centrifuge at 956 × *g* for 5 min in a microfuge, decant and wash twice with 1 mL PBS.
7. Resuspend cells in 0.5 mL PBS, 2 μL of FBS.

8. Mix 10 μL of cells with 30 μL of trypan blue solution (0.4%) and determine cell concentration (cells/mL) using a hemocytometer. Store at 2–8 °C until further processing.
9. To make positive controls, fixed cells in media were collected into a capillary attached to a micromanipulator with microinjector (Eppendorf transfer man NK2 attachment fitted to a Leica DM IL LED Invert microscope). Cells in the capillary were placed into a clean blood collection tube using the microinjector to achieve a concentration of 5–300 cells per tube.
10. Add healthy blood into the collection tube and invert tubes 23 times to mix.
11. Leave at room temperature for at least 1 h before filtering.

3.3 Cell Isolation by Filtration

The following are the automated steps for circulating rare cell isolation performed by the Hamilton STARlet™ robotic system (*see* Fig. 3 and **Notes 3** and **6**):

1. Transfer blood samples into a 50 mL tube and dilute to 20 mL with PBS-fibrin (*see* **Note 7**).
2. Keep samples at room temperature and process within 2 h of dilution.
3. Pipette 700 μL of 100% isopropyl alcohol onto the microscope slide to pre-wet the membrane and filter to remove extra liquid.
4. Pipette 1 mL of PBS onto the slide and filter to remove extra liquid.
5. Pipette blood sample in 1 mL fractions and filter after each addition until the total 20 mL sample has been filtered through the slide.
6. Wash membrane by filtering through 1 mL of PBS and repeat nine times.
7. Pipette and filter through 1 mL of fixative solution.
8. Pipette an additional 1 mL of fixative solution and incubate the membrane for 20 min before filtering.
9. Pipette and filter through 1 mL of PBS twice to wash the membrane.

3.4 Immunocytochemistry (ICC) Procedure

The following are the automated ICC staining and restaining steps performed using the Hamilton STARlet™ robotic system (*see* Fig. 3 and **Note 3**):

1. Permeabilize cells caught on the membrane by filtering through 1 mL of permeabilization buffer.
2. Pipette an additional 1 mL of permeabilization buffer and incubate the membrane for 7 min before filtering.
3. Wash membrane by filtering through 1 mL of PBS five times.

4. For the TSA protocol, pipette 900 μL of 3% hydrogen peroxide in PBS to block endogenous peroxidase activity. Incubate for 30 minutes and wash as in **step 3**. For staining using non-TSA protocol, go directly to **step 5**.
5. Add 1 mL of blocking solution to the membrane and incubate for 25 min before filtering.
6. Wash membrane twice with 1 mL of PBS-T.
7. Add 260 μL of the antibody solutions (fluorescently labeled CK panel and CD45) and incubate for 25 min (*see* Table 1 and **Notes 8** and **9**). For TSA protocol, use 300 μL of antibody solution.
8. Wash membrane five times with 1 mL of PBS-T. For staining using non-TSA protocol, proceed to **step 13**, otherwise go to **step 9**.
9. Add 260 μL of a 10 μg/mL solution of the HRP-conjugated secondary antibody. Incubate for 25 min before filtering.
10. Wash membrane as in **step 8**.
11. Add 260 μL of tyramide-Alexa 488 and incubate for 25 min before filtering.
12. Wash membrane as in **step 8**.
13. Add 500 μL of DAPI to the membrane and incubate for 1 min.
14. Wash the membrane twice with 1 mL of PBS-T and proceed to Subheading 3.5.
15. Wash the membrane with 1 mL of PBS to remove DABCO cover medium.
16. Proceed to **step 3** and follow the TSA steps to stain cells with antibodies against PIWIL2 and TPBG/5T4.

3.5 Fluorescence Microscopy to Detect and Image Cells

1. Once ICC is completed, remove the microscope slide (with the filter membrane containing the isolated and stained cells) from the filtration unit. Dry the bottoms of the slides using a clean Kimwipe.
2. Add 23 μL drop of DABCO cover medium on the membrane, and carefully place a 22 mm diameter cover slip.
3. Mount the slide onto the fluorescence microscope, and identify CTCs and WBCs based on appropriate CK and CD45 signals (Table 3). The representative images are shown in Fig. 4a (*see* **Notes 10** and **11**).
4. Once fluorescence microscopy detection of CTCs and WBCs is completed, carefully remove the cover slip with tweezers, and the remount slide onto the filtration unit in the Hamilton STARlet™ robot.

Table 3
Biomarkers and immunophenotypes of circulating rare cells

	First stain			Second stain		Third stain	
Cell population	**DAPI**	**CK**	**CD45**	**VIM**	**CD144**	**PIWIL2**	**TPBG/5T4**
Circulating tumor cell (CTC)	Pos	Pos	Neg				
White blood cell (WBC)	Pos	Neg	Pos				
Circulating mesenchymal cells (CMC)	Pos	Neg	Neg	Pos	Neg		
Circulating endothelial cells (CEC)	Pos	Neg	Neg	pos	Pos		
Putative circulating breast stem cell (B-CSC)	Pos	Pos/neg	Neg	Pos/neg	Neg	Pos	
Putative circulating lung stem cells (L-CSC)	Pos	Pos/neg	Neg	Pos/neg	Neg	NA	Pos

Reproduced from Magbanua et al. [8]

5. Wash the membrane with 1 mL of PBS to remove DABCO cover medium.
6. Proceed to **step 3** of Subheading 3.4 and follow the non-TSA protocol to stain cells with fluorescently labeled antibodies against VIM and CD144.
7. After staining is completed, proceed to **steps 1** and **2** in this section.
8. Mount the slide onto the fluorescence microscope, and identify CMCs (VIM-positive) and CECs (CD144-positive) (Table 3). The representative images are shown in Fig. 4b (*see* **Note 10**).
9. After fluorescence microscopy detection of CMCs and CECs is done, remove cover slip and wash membrane as described in **steps 4–5**.
10. Repeat staining procedure starting **step 3** of Subheading 3.4 and follow the TSA protocol to stain cells with fluorescently labeled antibodies against PIWIL2 and TPBG/5T4.
11. After staining is completed, dry the slide and add DABCO cover medium as in **steps 1** and **2**. Identify putative circulating stem cells (CSC) (Table 3). CSCs will display positive signals for PIWIL2 (breast) or TPBG/5T4 (lung). The representative images are shown in Fig. 4c (*see* **Note 12**).

4 Notes

1. The TSA assay involves an indirect immunoassay approach where a HRP-conjugated secondary antibody allows covalent attachment of fluorescent (e.g., Alexa 488) or chromogenic dyes to protein tyrosine groups to amplify signals.

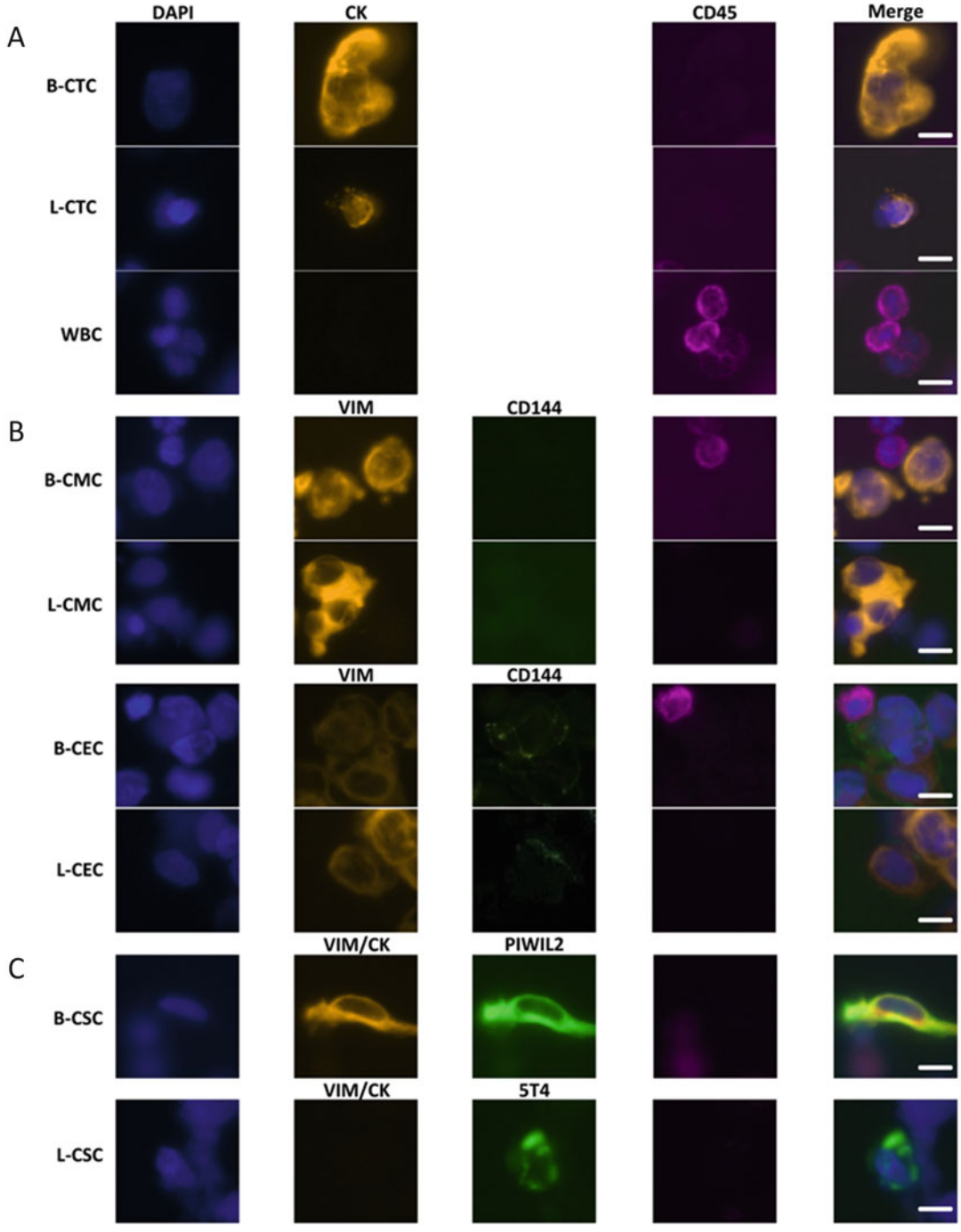

Fig. 4 Immunocytochemistry staining of circulating rare cells from blood of cancer patients. Representative images of (**a**) circulating tumor cells (CTC), white blood cells (WBC), (**b**) circulating mesenchymal cells (CMC), circulating endothelial cells (CEC), and (**c**) putative circulating stem cells (CSC) in metastatic breast (B) and lung (L) cancer patients. The scale bar represents 8 μM (reproduced from Magbanua et al. [8])

2. Tyramide-Alexa 488 is used to amplify signals from PIWIL2 and TPBG/5T4 because they are expressed at low levels. NeutrAvidin Dylight 488 can be used in place of Alexa 488 for signal amplification.
3. Circulating rare cell isolation and ICC steps were performed using the Hamilton STARlet™ robotic system with eight separate pipette channels (*see* Fig. 1). The robot is specially equipped with a filtration unit that can hold eight microfluidic slides each with mounted filter membranes. The robot controls the application of uniform negative pressure to each sample being filtered as it pulls blood and liquids through the filter membrane into a waste container below. The robot also controls the timing and the rates at which liquids are being delivered onto the membrane via a 1 mL pipette tip.
4. We recommend shipping blood specimens in insulated boxes equipped with controlled temperature packs (10–25 °C) and a digital temperature recorder. Blood samples should be processed within 9 days. To filter samples over 5 days old, vacuum pressure was decreased from typically around −30 to −100 mBar.
5. Recovery was dependent on fixation time for cancer cell lines spiked into blood. Spike-in experiments using cells incubated for 24 h (in the fixative) had better recovery compared to experiments using cells that were only incubated for 2 h prior to filtration.
6. The filtration process enriches for circulating rare cells but also retains approximately 10,000–20,000 WBCs.
7. Most clinical samples contain adequate fibrin and do not require the addition of PBS-fibrin solution, which can be replaced with PBS only without loss of recovery.
8. Fluorescently labeled antibodies to EPCAM can be added to the CK panel to identify EPCAM-positive CTCs. Choose a fluorescent stain that can be detected in the orange-red channel.
9. Fluorescently labeled antibodies to CD144 can be added to distinguish CEC (CD144-positive) from WBC (CD144-negative). Select a fluorescent stain that can be detected in the far-red channel.
10. The fluorescence signal threshold was defined as the point at which the signal was detectable over the noise of the background signal on the filtration matrix. The thresholds were set using known cell type controls (*see* Table 2). Normal blood was used as negative (non-tumor) controls. Fluorescence signals from WBCs and CECs were used to assign the threshold for positive CD45 and CD144 staining, respectively. Fluorescence

signals from cultured cell controls were also examined to assign thresholds for positive signals for all other markers (CK, VIM, CD144, PIWIL2, and TPBG/5T4). Imaging exposure times and gains were adjusted based on positive and negative signals from cultured cells.

11. WBCs can also be trapped on the membrane, but are excluded from further analyses, e.g., enumeration. Red blood cells are not likely to be captured as they may have lysed or are small enough to pass through the membrane.
12. The results of the three staining processes can be combined, as the cell positions on the filter membrane are recorded so staining patterns for all markers can be analyzed and compared for each specific cell.

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Chapter 10

Filter-Adapted Fluorescent In Situ Hybridization (FA-FISH) for Filtration-Enriched Circulating Tumor Cells

Marianne Oulhen, Emma Pailler, Vincent Faugeroux, and Françoise Farace

Abstract

Circulating tumor cells (CTCs) may represent an easily accessible source of tumor material to assess genetic aberrations such as gene-rearrangements or gene-amplifications and screen cancer patients eligible for targeted therapies. As the number of CTCs is a critical parameter to identify such biomarkers, we developed fluorescent in situ hybridization (FISH) for CTCs enriched on filters (filter-adapted-FISH, FA-FISH). Here, we describe the FA-FISH protocol, the combination of immunofluorescent staining (DAPI/CD45) and FA-FISH techniques, as well as the semi-automated microscopy method that we developed to improve the feasibility and reliability of FISH analyses in filtration-enriched CTC.

Key words Circulating tumor cells, FISH, FA-FISH, ISET, Filtration-enrichment

1 Introduction

Fluorescent in situ hybridization (FISH) is a key technique in routine clinical practice to detect predictive biomarkers and screen cancer patients eligible for targeted therapies. Gene aberrations such as gene-rearrangements or gene-amplifications can be identified by FISH in primary tumors or metastatic biopsies. However, in the case of biopsy samples, material availability in terms of both quantity and quality is often insufficient for patients with advanced/metastatic cancers such as non-small-cell lung cancer (NSCLC) or prostate cancer (mPCa). The molecular analysis of circulating tumor cells (CTCs) is an attractive option to assess predictive biomarkers. CTC methods commonly rely on two steps, a first enrichment step based on either CTC phenotypic or physical properties, and a secondary step for the detection of phenotypic or genetic biomarkers. Our and other groups have reported

[$] Marianne Oulhen and Emma Pailler contributed equally to this work.

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that higher numbers of CTCs are captured in cancers such as metastatic NSCLC and mPCa using a filtration system such as ISET (isolation by size of epithelial tumor cells) compared to the CellSearch [1–3]. As the number of CTCs is a critical parameter to identify molecular biomarkers, we developed FISH for CTCs enriched on filters (FA-FISH). Using FA-FISH, we reported the detection of *anaplastic lymphoma kinase* (*ALK*)-rearrangement in ISET-enriched CTCs from *ALK*-rearranged NSCLC patients [4] as well *c-ros oncogene 1* (*ROS1*)-rearrangement in CTCs from *ROS1*-rearranged NSCLC patients [5], and *ETS-related gene* (*ERG*)-rearrangement in CTCs from mPCa patients [6].

The FISH technique is based on the hybridization property of a fluorochrome conjugated-nucleotide probe on a target DNA (deoxyribonucleic acid) sequence. It was developed in the early 1980s to detect the presence or absence of target DNA sequences and precisely locate them on chromosomes. The three critical steps of FISH that need to be particularly controlled are cell-fixation, protein digestion, and DNA denaturation. Here, we describe the specific FA-FISH conditions established to detect gene-rearrangements or gene-amplifications in CTCs enriched by ISET filtration. We also describe the combined immunofluorescent staining (DAPI/CD45) and FA-FISH technique as well as the semi-automated microscopy method we established to improve the feasibility and reliability of molecular biomarker detection by FISH in filtration-enriched CTCs [7].

2 Materials

2.1 Blood Sample Collection and ISET Filtration

1. Ethylenediaminetetraacetic acid (EDTA) tubes.
2. ISET device (Rarecells).
3. ISET block containing filter (RareCells): ISET filters are composed of ten spots. Each spot corresponds to the filtration of 1 mL of blood.
4. Heating stage.
5. Fume hood.
6. Formaldehyde 37%.
7. Sodium hydroxide 30% (NaOH).
8. ISET buffer reagent (RareCells): dilute the products A, B, and C in 490 mL of sterile water. Add 10 mL of formaldehyde 37% under the fume hood and adjust the pH at 7.2 precisely with NaOH.
9. pH meter.
10. Dulbecco's phosphate buffered saline (DPBS) 10×, to dilute at 1× in sterile water.

11. Silica gel.
12. Aluminum foil.

2.2 FA-FISH

1. Hybridizer.
2. Hybridizer humidity control strips.
3. Fume hood.
4. Heating stage.
5. Vortex mixer.
6. Centrifuge.
7. Water bath.
8. High-temperature resistant plastic jars.
9. High-temperature resistant adhesive tape.
10. Rubber cement (Talens).
11. SuperFrost Plus slides.
12. Circular coverslips Ø12 mm.
13. Coverslips 22 × 22 mm.
14. Methanol.
15. Acetic acid.
16. Ethanol.
17. Sterile water.
18. DPBS 10×, to dilute at 1× in sterile water.
19. Hydrochloric acid (HCl) 1 N to dilute at 0.1 N in sterile water (store at room temperature). Extemporaneously, prepare HCl 0.01 N in sterile water and heat this solution at 37 °C in the water bath.
20. Pepsin from porcine gastric mucosa to dilute at 10% in sterile water (store aliquots at −20 °C). Just before the **item 7** of FA-FISH method, add 100 µL of pepsin 10% in the HCl 0.01 N solution at 37 °C.
21. Magnesium chloride ($MgCl_2$) 1 M: dilute 100 g in 500 mL of sterile water (store at 4 °C).
22. Formaldehyde 37%.
23. Formaldehyde solution: 57.6 mL of sterile water, 7 mL of PBS 1×, 3.5 mL of $MgCl_2$, 1.9 mL of formaldehyde 37% (under the fume hood).
24. Stringency Buffer 20× (4 °C) to dilute at 1× in sterile water.
25. Wash Buffer 20× (4 °C) to dilute at 1× in sterile water.
26. Mounting Medium for Fluorescence Vectashield® containing 4′,6-diamidino-2-phenylindole (DAPI) (4 °C).
27. FISH probe kits.

2.3 Immunofluorescent Staining

1. High-temperature resistant adhesive tape.
2. High-temperature resistant plastic jars.
3. Water bath.
4. Dark humid chamber.
5. Circular coverslips Ø12 mm.
6. Coverslips 22 × 22 mm.
7. Sterile water.
8. Tris Buffered Saline (TBS, powder): dilute 1 sachet in 1 L of sterile water to prepare TBS 1×.
9. Target Retrieval Solution pH 9 10× to dilute at 1× in TBS 1×; heat this solution at 98 °C in the water bath for approximately 30 min before the experiment.
10. Normal mouse serum (aliquots at −20 °C) to dilute extemporaneously at 5% in TBS 1×.
11. Tween 20 to dilute at 0.05% in TBS 1×.
12. DAPI: stock at 1 mg/mL (−20 °C) to dilute extemporaneously at 1:20,000 in TBS 1×.
13. Antibody: allophycocyanin (APC)-conjugated anti-CD45 (clone HI30).
14. Ibidi mounting medium.

2.4 Scanning and Image Analysis of Combined Immunofluorescent Staining and FA-FISH

1. Ariol scanning system (Leica Biosystems Richmond Inc., Richmond, IL, USA) including a Leica DM6000 B microscope with multibay stages (MB 8). Single interference filter sets for blue (DAPI), green (FITC), red (Texas Red), and dark red (Cy5) filters are used.
2. Ariol Review application 4.0.1.5 (Leica Biosystems Richmond Inc.).

3 Methods

3.1 Blood Sample Collection and ISET Filtration

CTC enrichment by the ISET filtration system is carried out according to the manufacturer's protocol.

1. Collect the blood (generally 10 mL) in EDTA tubes. Gently homogenize the blood by inverting the tubes ten times.
2. Immediately after blood collection, dilute blood samples at 1:10 in the ISET buffer reagent and incubate for 10 min at room temperature. Gently homogenize the sample every 2 min.
3. Lock the ISET block on the ISET device.
4. Distribute PBS 1× in each compartment of the ISET block. Press "Start' and adjust the pressure as recommended by the

manufacturer. Open the tap and verify that the block is sealed perfectly (no PBS leakage). Close the tap.

5. Distribute 10 mL of diluted blood in each individual one-spot compartment and/or 50 mL of diluted blood in the five-spot compartment and open the tap. Each spot corresponds to 1 mL of blood.
6. After processing, filters are dried, wrapped in an aluminum foil, and stored frozen in plastic bag containing a silica gel desiccant at −20 °C until use.

3.2 FA-FISH

1. Thaw the filters and immobilize individual spots on glass slides using the high-temperature resistant adhesive tape (*see* **Note 1**).
2. Rehydrate the filters in PBS 1× for 5 min.
3. Incubate the filters in the methanol:acetic acid (9:1) solution for 30 min under the fume hood to fix the cells.
4. Wash the filters in PBS 1× for 5 min.
5. Incubate the filters in the pepsin 10% HCl 0.01 N solution for 6 min.
6. Wash the filters in PBS 1× for 5 min.
7. Incubate the filters in the formaldehyde solution under the fume hood for 2 min.
8. Dehydrated the filters in successive baths containing increasing concentrations of ethanol for 2 min: 70, 85, 100%.
9. Dry the filters on the heating stage at 37 °C for 10 min to evaporate ethanol.
10. Add 10 μL of FISH probes on the spots at 37 °C and put a circular coverslip Ø12 mm.
11. Seal the samples with the rubber cement (*see* **Note 2**).
12. Put the filters in the hybridizer and run the program for co-denaturation and hybridization.
13. The day after, stop the hybridizer and remove the rubber cement from the slides (*see* **Note 3**).
14. Wash the filters in stringency buffer 1× at room temperature for 7 min.
15. Wash the filters in stringency buffer 1× at 65 °C for 5 min.
16. Wash the filters in wash buffer 1× at room temperature for 5 min.
17. Dehydrate the filters in successive baths containing increasing concentrations of ethanol for 2 min in each bath: 70, 85, 100%.
18. Dry the filters on the heating stage at 37 °C for 10 min to evaporate ethanol.
19. Add 10 μL of mounting medium for fluorescence Vectashield containing DAPI and put a coverslip 22 × 22 mm.

20. Seal the samples with the rubber cement.
21. Store the filters at 4 °C before manual analysis.

3.3 Combination of Immunofluorescent Staining and FA-FISH

The experimental process is shown in Fig. 1.

3.3.1 Immunofluorescent Staining

1. Thaw the filters and immobilize individual spots on glass slides using the high-temperature resistant adhesive tape (*see* **Note 1**).
2. Make a snick on each spot for precise $DAPI^+/CD45^-$ cell relocation for FA-FISH scanning (cf. Subheading 3.3.4).
3. Rehydrate the filters in TBS 1× for 5 min.
4. Incubate the filters in the target retrieval solution pH 9 1× at 98 °C for 5 min (*see* **Note 4**).
5. Wash the filters in TBS 1× for 5 min.
6. Add 100 µL of normal mouse serum 5%-TBS 1× on each spot, put a circular coverslip Ø12 mm and incubate for 30 min in the dark humid chamber at room temperature.
7. Wash the filters in TBS 1× for 5 min.
8. Add 100 µL of CD45-APC antibody diluted in TBS 1× on each spot, put a circular coverslip Ø12 mm, and incubate overnight in the dark humid chamber at 4 °C.
9. The day after, wash two times the filters in TBS 1×–Tween 0.05% for 5 min.
10. Wash the filters in TBS 1× for 5 min.
11. Add 100 µL of DAPI at 1:20,000 on each spot, put a circular coverslip Ø12 mm and incubate in the dark humid chamber at room temperature for 15 min.
12. Wash two times the filters in TBS 1×–Tween 0.05% for 5 min.
13. Wash the filters in TBS 1× for 5 min.
14. Add 10 µL of Ibidi mounting medium for fluorescence on each spot and put a coverslip 22 × 22 mm.
15. Seal the samples with the rubber cement.
16. Store the filters at 4 °C before scanning.

3.3.2 Scanning and Image Analysis of Immunofluorescent Staining

1. Define the scanning area (the ISET spot including the snick) at 5× magnification in the DAPI channel.
2. Set gain at maximum.
3. Calibrate exposure time and focus for DAPI and Cy5 channels at 20× magnification (*see* **Note 5**).
4. Calibrate offset as high as possible in the DAPI channel to individualize at best the nuclei.
5. Start the scan. Select and locate $DAPI^+/CD45^-$ cells.

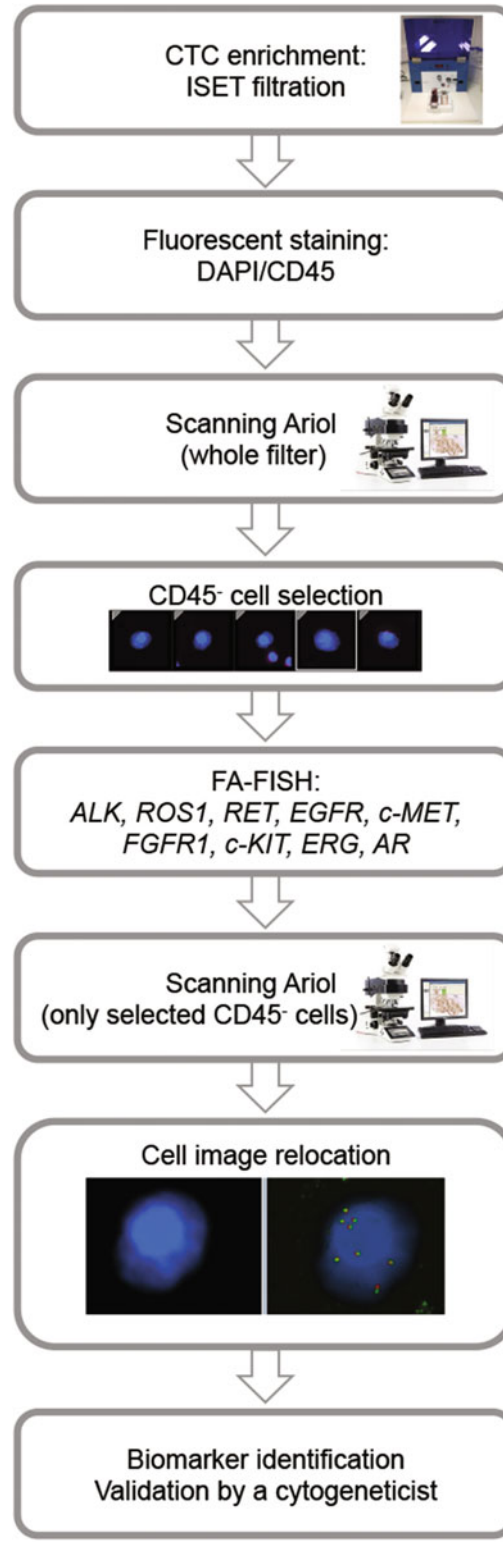

Fig. 1 Schematic workflow for detection of molecular biomarkers in filtration-enriched CTCs by combined fluorescent staining and fluorescent in situ hybridization (FISH). Reprinted with permission from [7]

3.3.3 Combined Immunofluorescent Staining and FA-FISH

1. Wash the filters in TBS 1×–Tween 0.05% and TBS 1× for 5 min.
2. Follow the protocol described in Subheading 3.2 from **step 3**.

3.3.4 Scanning and Image Analysis of Combined Immunofluorescent Staining and FA-FISH

1. Scanning and image analysis of fluorescent staining is carried out as described above.
2. Start FA-FISH scanning by a prescan at 5× magnification of the whole ISET spot in DAPI channel to precisely relocate cells.
3. Calibrate DAPI channel at 10× magnification for adjustment of focus.
4. Calibrate DAPI, FITC, and Texas Red (for green and red FISH signals) channels at 63× magnification (immersion objective) in three different regions of the ISET spot: focus, offset, exposure times.
5. FISH capture is performed at ×63 magnification in the small regions containing the preselected $DAPI^+/CD45^-$ cells. Optimal FISH scanning parameters should be established for each scanner. For the Ariol, the optimal FISH scanning parameters are: 30 zstacks spaced 0.6 μm apart and a multi exposure protocol to optimize the analysis of FISH signal intensity, size, and thickness [7].
6. Analyze FISH images in $DAPI^+/CD45^-$ cells.

4 Notes

1. Make sure that the spots are very well immobilized on the glass slides. If the spots move during the combined immunofluorescence and FA-FISH experiment, the relocation will not be possible.
2. If the sample is not correctly sealed, the FISH probes can evaporate during the co-denaturation and hybridization steps.
3. Pay attention not to remove the coverslip with the rubber cement. This will damage cells. The coverslip will leave in the next wash bath.
4. This step is useful to fix the cells to the filters and to avoid cell loss during the combined fluorescent staining and FA-FISH process.
5. Using only one parameter (i.e., exposure time for adjusting fluorochrome exposure) allowed comparison of settings between scans done at different times or by different users.

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Chapter 11

Negative Enrichment and Isolation of Circulating Tumor Cells for Whole Genome Amplification

Nisha Kanwar and Susan J. Done

Abstract

Circulating tumor cells (CTCs) are a rare population of cells found in the peripheral blood of patients with many types of cancer such as breast, prostate, colon, and lung cancers. Higher numbers of these cells in blood are associated with a poorer prognosis of patients. Genomic profiling of CTCs would help characterize markers specific for the identification of these cells in blood, and also define genomic alterations that give these cells a metastatic advantage over other cells in the primary tumor. Here, we describe an immunomagnetic method to enrich CTCs from the blood of patients with breast cancer, followed by single-cell laser capture microdissection to isolate single CTCs. Whole genome amplification of isolated CTCs allows for many downstream applications to be performed to aide in their characterization, such as whole genome or exome sequencing, Single Nucleotide Polymorphism (SNP) and copy number analysis, and targeted sequencing or quantitative Polymerase Chain Reaction (qPCR) for genomic analyses.

Key words Circulating tumor cells, Rare tumor cell enrichment, Immunomagnetic cell sorting, Laser capture microdissection, WGA

1 Introduction

There have been significant advances in early screening and targeted therapies to reduce deaths from cancer; however, metastasis remains the leading cause of mortality among patients. In metastasis, a select few cells acquire the ability to invade tissues that surround the primary tumor, thus allowing them to break free and enter the circulation via intravasation of the blood or lymphatic systems. Once in circulation, this "occult" process goes undetected. Some CTCs travel to distant organs, are able to survive chemotherapy and radiation, extravasate, reinitiate aberrant cell division, and propagate secondary tumors at new sites. The presence of circulating tumor cells in blood is associated with therapeutic resistance, metastasis, and worse outcome in cancer. By profiling the genomes of CTCs, we could identify novel genomic alterations specific to these cells, which may be utilized in the development of specific

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markers for CTCs in blood. Genes within these regions could provide novel avenues to target CTCs and block dissemination.

Tumor cells in circulation are rare, with approximately 1 cell in 10^9 nucleated blood cells [1]. A major obstacle in characterizing CTCs is obtaining a sufficient number of CTCs, without contaminating white blood cells. The "ideal" CTC marker would be one that is expressed exclusively in tumor cells, not in any subset of hematopoietic cells, and not repressed during the period of circulation when the cells are isolated. CTCs are currently isolated based on the expression of known epithelial or hematopoietic markers (Cytokeratin, EPCAM, CD45), or based on prior knowledge of the primary tumor or cancer type (EGFR, HER2, Prostate Specific Antigen (PSA), etc.). Enrichment is usually a prerequisite to any isolation protocol, and improves the detection of cells by at least 10,000-fold. Several methods of enrichment of CTCs have been described—immunomagnetic bead separation; density centrifugation, size-based exclusion, flow cytometric separation, and more recently microfluidic devices such as the CTC-chip and Herringbone chip that are covered with antibodies for tumor cell capture, and the DEPArray where CTCs are sorted into "cages" based on dielectrophoresis on the basis of their electric charge [2–6]. There have also been developments such as the CellCollector and leukopheresis which involve in vivo capture of CTCs from a device attached directly to a patients peripheral arm vein, which allows for enrichment of higher numbers of cells (median of 7500 versus 10 s–100 s), from much larger volumes of blood (1.5 L compared to 10 mL), within a smaller time frame for processing of the cells while they are still viable (30 min compared to a few hours or days) [7, 8]. With the rapid development of more standardized and sensitive methods to isolate CTCs, the clinical utility of these cells is likely to become more feasible.

CTCs are either positively selected with epithelial markers (Cytokeratin or EPCAM), or negatively selected by depleting white blood cells with the pan-leukocyte marker CD45. Here, we describe the enrichment of CTCs from peripheral blood using immunomagnetic depletion of white blood cells (CD45+ fraction). Magnetic beads conjugated to CD45 antibodies bind to the white blood cell (WBC) fraction of blood, which is selectively retained when passed through a magnetic column. The unlabeled fraction flows through the column for collection and is stained with a pan-cytokeratin antibody to detect CTCs of epithelial origin in breast cancer. Positively stained single CTCs are isolated using laser capture microdissection, and pooled together for Whole Genome Amplification (WGA) of single cells. Amplified DNA obtained from this protocol can then be analyzed using a high-resolution whole genome copy number platform or processed for whole genome/exome or targeted deep sequencing [9].

2 Materials

2.1 Immunomagnetic Cell Sorting

1. Red blood cell (RBC) lysis buffer: 0.6 g Ethylenediaminetetraacetic Acid (EDTA), 1 g potassium bicarbonate, 8.2 g ammonium chloride dissolved in 1 L water, pH 7.4.
2. autoMACS® Rinsing Solution: purchased from Miltenyi Biotec.
3. BSA stock solution: 1× Phosphate Buffered Saline (PBS) with 10% bovine serum albumin (BSA).
4. Cell Separation Buffer: 1:20 BSA stock solution to autoMACS® Rinsing solution.
5. Whole Blood CD45 MicroBeads, human (Miltenyi Biotec).
6. 70% Ethanol.
7. ISOTON® II Diluent (Beckman Coulter, Inc.).
8. Running Buffer: 1× PBS with 0.5% BSA and 2 mM EDTA.
9. autoMACS® Separator (Miltenyi Biotech).
10. autoMACS® Columns (Miltenyi Biotech).

2.2 Staining

1. Polyethylene terephthalate (PET) membrane framed slides.
2. Heat chamber.
3. Anhydrous acetone.
4. 1× PBS, pH 7.5.
5. Primary antibody: mouse anti-human cytokeratin clone AE1/AE3.
6. Vectastain ABC-GO Kit (Vector Laboratories).
7. Glucose Oxidase Substrate Kit I, Nitro-Blue Tetrazolium (NBT) (Vector Laboratories).
8. 50 mM Tris–HCl, pH 9.5.
9. Hydrophobic barrier pen or nail polish.

2.3 Laser Capture Microdissection

1. Leica ASLMD6500 system (Leica Microsystems).
2. DNase/RNase-free water.

2.4 Whole Genome Amplification

1. GenomePlex Single Cell Whole Genome Amplification Kit-WGA4 (Sigma-Aldrich).
2. GenElute PCR clean-up kit (Sigma-Aldrich).

2.5 Ligation

1. DNase/RNase-free water.
2. 10× T4 DNA ligase buffer.
3. DNA Polymerase, Large (Klenow) fragment.
4. T4 Polynucleotide Kinase 10 U/μL.

5. 2.5 mM deoxy nucleotide triphosphate (dNTP) mix.
6. T4 DNA ligase 5 U/μL.

3 Methods

3.1 Immunomagnetic Cell Sorting

Carry out all procedures at room temperature unless otherwise specified (*see* **Note 1**).

1. Collect 15–20 mL of peripheral blood in EDTA tubes from patient (*see* **Note 2**).
2. Transfer blood to 50 mL conical tubes (not more than 3 mL per tube). Aliquot 3 mL blood into as many 50 mL tubes as required based on total volume collected from patient.
3. Top up each 50 mL tube to 35 mL of RBC lysis buffer. Lyse RBCs for 15 min in 20 mL lysis buffer, centrifuge at 423 × *g* for 10 min.
4. Resuspend the pellet in 10 mL RBC lysis buffer and centrifuge at 423 × *g* for 10 min.
5. Resuspend the pellet in 3 mL Cell Separation buffer.
6. Add 50 μL/mL × 3 = 150 μL CD45 beads to each tube.
7. Mix well, incubate for 15 min at 2–8 °C.
8. Wash cells by adding 2 mL × 3 = 6 mL of Cell Separation buffer per tube, centrifuge (without the brake) at 445 × *g* for 10 min (*see* **Note 3**).
9. Aspirate the supernatant.
10. Resuspend in 1 mL Cell Separation buffer for each tube.
11. Proceed to magnetic separation (Fig. 1).
12. Fill 4× 1 L glass bottles with each of the following solutions, the waste bottle is empty for the collection of flow-through:
    (a) Running buffer.
    (b) Cell Separation buffer.
    (c) Cleaning solution: 70% EtOH.
    (d) Rinse buffer: ISOTON®.
    (e) Waste.
13. Turn on power for the autoMACS® Separator.
14. Replace solutions in bottles if required (each bottle should have at least 200 mL). Replace Running buffer bottle with 1 L separation buffer. Make sure waste is empty.
15. Run program.
    (a) Select "Options"—"Column exchange"—"continue." Once it beeps, Unscrew door, and replace separation column 1. Select "continue."

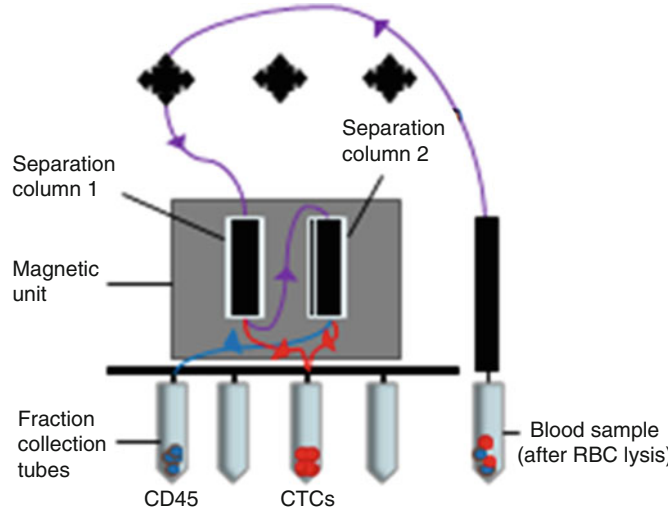

Fig. 1 autoMACS® Separator Flow, lysed blood sample is taken up into separation column 1 for cell sorting, unlabeled CTCs are eluted while CD45+ fraction is taken up into separation column 2 for further sorting. Unlabeled residual CTCs are again eluted, and CD45+ fraction is also eluted

(b) When done, remove waste collection tubes, empty, and replace with sample collection tubes in slots.

16. Place sample tube at uptake port (ensure mixed well).
17. Select "Separation" from the main menu and use up/down arrows to select program **posseld2**. Touch OK.
18. When program is complete, touch OK, remove your fractions. Keep negative fraction, this is where the unlabeled CTCs are. The positive fraction containing CD45+ WBCs can be discarded (*see* **Note 4**).
19. Replace with waste collect tubes. Select "QRinse." Then replace with new sample collection tubes and sample tube and continue from **step 15**.
20. Shut down.
    (a) Replace original blue line buffer.
    (b) Fill up ISOTON® or EtOH.
    (c) Select "Sleep."
    (d) Empty Waste bottle, wipe ports with alcohol swab.

3.2 Staining

The Vectastain ABC-GO Kit is used for detection of epithelial cells (CTCs from breast cancer) with biotinylated secondary mouse IgG antibody that reacts with avidin-biotinylated glucose oxidase enzyme. Glucose oxidase is absent from mammalian cells, thus this method of detection is used to abolish nonspecific staining of WBCs seen with phosphatase or peroxidase detection systems [10].

From the Vectastain ABC-GO Kit, prepare blocking buffer by adding 150 μL of blocking serum to 10 mL of 1× PBS. Prepare secondary antibody by adding 50 μL to 10 mL of 1× PBS. Prepare glucose oxidase reagent by adding 100 μL of avidin DH to 10 mL of 1× PBS, followed by 100 μL of biotinylated glucose oxidase H, mix immediately, and allow standing for about 30 min before use. Prepare the glucose substrate from the Glucose oxidase substrate kit I, NBT by adding 100 μL of reagents 1, 2, and 3 to 50 mM Tris–HCl, and mix well and keep in the dark till use. Epithelial cells (CTCs) will stain purple/blue in color with the NBT substrate (Fig. 2).

1. Cytospin cells onto PET membrane slides.
2. Dry slides in chamber at 37 °C for 20 min.
3. Fix slides with ice-cold acetone for 20 min.
4. Wash twice with 1× PBS.
5. If need to store, place in a slide box at 4 °C overnight.
6. Prepare a humid chamber with moist paper towels. Keep slides moist at all times.
7. If necessary, trace a waterproof barrier around area on slide with cells (using nail polish or hydrophobic pen).
8. Wash slides with 1× PBS for 5 min.
9. Incubate slides in blocking serum for 20 min.
10. Wash three times with 1× PBS.

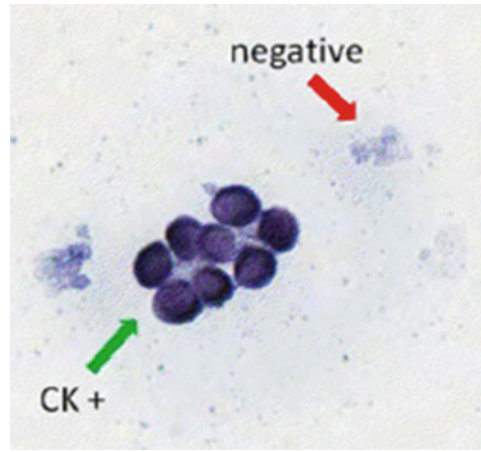

Fig. 2 Positively stained MCF7 breast cancer cells that were spiked and subsequently enriched from 5 mL of human blood using the autoMACS® protocol described above, and stained using the Vectastain ABC-GO Kit with glucose oxidase substrate NBT. Cytokeratin-positive epithelial cells (CK+) stain purple/blue compared to WBCs and other nonspecific entities (negative)

11. Add primary antibody—leave for 1–2 h at room temperature (1:100 dilution in PBS).
12. Wash slides three times with 1× PBS .
13. Add secondary antibody—leave for 30 min to 1 h at room temperature (1:200 dilution in PBS).
14. Wash slides ×3 with 1× PBS.
15. Incubate slides with Vectastain ABC-GO reagent for 30 min at room temperature.
16. Wash slides three times with 1× PBS.
17. Incubate slides with prepared glucose oxidase substrate solution for 15 min in the dark at room temperature.
18. Wash (stop reaction) with 1× PBS.
19. Wash with tap water.

3.3 Laser Capture Microdissection

CTCs stained positively with the pan-cytokeratin antibody are isolated using the Leica ASLMD6500 system. The PET membrane is cut by the laser around individual cells selected for isolation. The cut section is collected into the cap of a microfuge tube containing 4.5 μL of water. CTCs can be pooled per patient or processed as single cells for subsequent whole genome amplification procedures.

3.4 Whole Genome Amplification

The GenomePlex Single Cell Whole Genome Amplification Kit—WGA4 is used for amplification of genomic DNA from CTCs pooled together per patient (*see* **Note 5**). Prepare working Lysis and Fragmentation Buffer solution by adding 2 μL of Proteinase K solution to 32 μL of 10× Single Cell Lysis and Fragmentation buffer, vortex well. Following WGA, amplified DNA is purified using the GenElute PCR clean-up kit. The average fragment size generated by WGA is determined by gel electrophoresis (approximately 500 bp). The average yield of amplified DNA using this protocol from a starting point of 3–20 single CTCs is approximately 2 μg.

3.4.1 PCR programs

SIG50:

50 °C for 1 h .

99°C for 4 min.

SIGMA1:

16 °C for 20 min.

24 °C for 20 min.

37 °C for 20 min.

75 °C for 5 min.

4 °C hold.

SIG95:

95 °C for 2 min.

SIGMA2:

95 °C for 3 min.
25 cycles of: 94 °C for 30 s.
65 °C for 5 min.
4 °C hold.

1. Wash cells off microfuge tube cap using the 4.5 μL water (ensure proper washing of cells off the area by repetitive pipetting) (*see* **Note 6**).
2. Add 0.5 μL of prepared working Lysis and Fragmentation buffer. Mix well.
3. Run *SIG50* program, set timer for 1 h .
4. Cool tubes on ice immediately (timing is crucial) (*see* **Note 7**). Spin down once cooled.
5. Add 1 μL single cell library preparation buffer.
6. Add 0.5 μL of Library stabilization solution.
7. Mix well.
8. Run *SIG95* for 2 min and then cool on ice. Spin down once cooled.
9. Add 1 μL of Library Preparation Enzyme. Mix well.
10. Run *SIGMA1*.
11. Make master mix (scale by #*n* tubes).
    (a) 7.5 μL amplification master mix.
    (b) 48.5 μL water.
    (c) 5 μL WGA DNA polymerase.
12. Add 30.5 μL master mix to each tube, mix.
13. Run *SIGMA2*.
14. Store samples at −20 °C or purify and run on 1% agarose gel (Fig. 3).

3.5 Ligation

We use 800 ng of DNA per sample for a ligation reaction to increase the fragment length for copy number analysis with downstream genome platforms such as the Affymetrix Genome-Wide Human SNP 6.0 array, targeted copy number analysis using the Nanostring platform or whole genome sequencing. Prepare a dilution of DNA and water (800 ng of DNA in 16 μL of water) for each sample.

1. Prepare the following reaction:
   (a) 16 μL DNA + water (800 ng).
   (b) 2 μL 10× T4 DNA ligase buffer.

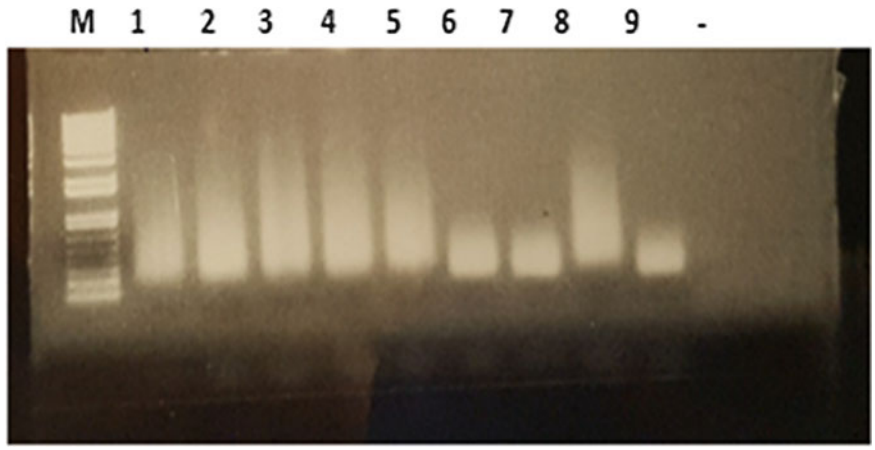

Fig. 3 Gel Electrophoresis of purified WGA pooled CTC samples (1–9), 1 Kb ladder (M) and negative control (−) with no DNA in amplification protocol. Good quality WGA samples appear as DNA smears with fragments of varying sizes. Some samples do not amplify as well due to poor quality of starting cells or too few cells, amplified DNA appears to be mostly consisting of shorter fragments of approximately 300–400 bp in size (samples 6, 7, and 9)

(c) 1 μL Large (Klenow) fragment.

(d) 1 μL T4 Polynucleotide Kinase (PNK).

2. Incubate for 30 min at room temperature.
3. Prepare a master mix (scale by #*n* tubes).
   (a) 1 μL 10× T4 DNA ligase buffer.
   (b) 1 μL 2.5 mM dNTP Mix.
   (c) 1 μL T4 DNA ligase.
4. Add the 20 μL reaction prepared in **step 1** to 3 μL of master mix from **step 3** for a total of 23 μL reaction.
5. Incubate for 16 h at 30 °C.
6. Incubate (stop reaction) for 10 min at 90 °C.
7. Store tubes at 4 °C short term or −20 °C long term (*see* **Note 8**).

4 Notes

1. For CTC enrichment, it is optimal for the cells to maintain their integrity to prevent degradation of DNA. For this purpose, all solutions must be made up in 1 L glass bottles and precooled to 4 °C before beginning the separation protocol.
2. When drawing blood from patients, discard the first 5 mL of draw to avoid collection of contaminating skin cells in the blood. This avoids amplification of "normal" skin cells which would confound the DNA profiles of pooled single isolated CTCs.

3. Centrifugation without the brake ensures a gentle slowdown after the spin, so as not to disrupt the binding of immunomagnetic beads to WBCs.
4. It is a good practice to keep both labeled (CD45+ WBCs) and unlabeled (CTCs) fractions that are eluted after cell sorting. The labeled fraction could also be cytospun onto slides and stained for cytokeratins to estimate the number of CTCs per sample that are lost due to technical processing. Such estimates could be reported for cell sorting technique sensitivity, and compared with other techniques or optimized in the future.
5. For WGA, a modified version of the manufacturer's protocol is used where all regent volumes are halved. This protocol was adapted after extensive comparison of the Sigma WGA4 kit with various other single-cell WGA kits, as well as optimization of the protocol for low CTC counts (between 3 and 20 cells).
6. During repetitive pipetting, avoid creation of air bubbles, as you are working with a very small volume of liquid. The introduction of air bubbles will lead to loss of volume or cells, resulting in failure of amplification of DNA material.
7. Prepare for the cool down step with an ice bucket and ice ready to use once the 2 min incubation is compete.
8. If long-term storage is intended, DNA may be stored at −80 °C.

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Chapter 12

Capture and Genetic Analysis of Circulating Tumor Cells Using a Magnetic Separation Device (Magnetic Sifter)

Chin Chun Ooi, Seung-min Park, Dawson J. Wong, Sanjiv S. Gambhir, and Shan X. Wang

Abstract

Circulating tumor cells (CTCs) are currently widely studied for their potential application as part of a liquid biopsy. These cells are shed from the primary tumor into the circulation, and are postulated to provide insight into the molecular makeup of the actual tumor in a minimally invasive manner. However, they are extremely rare in blood, with typical concentrations of 1–100 in a milliliter of blood; hence, a need exists for a rapid and high-purity method for isolating CTCs from whole blood. Here, we describe the application of a microfabricated magnetic sifter toward isolation of CTCs from whole blood at volumetric flow rates of 10 mL/h, along with the use of a PDMS-based nanowell system for single-cell gene expression profiling. This method allows rapid isolation of CTCs and subsequent integration with downstream genetic profiling methods for clinical applications such as targeted therapy, therapy monitoring, or further biological studies.

Key words Magnetic cell separation, Circulating tumor cell, Liquid biopsy, Genetic analysis, Magnetic sifter, Nanowell array

1 Introduction

Circulating tumor cells (CTCs) are of great interest in oncology because of their potential as a diagnostic and/or prognostic biomarker as part of a comprehensive liquid biopsy. Being readily accessible in the bloodstream, isolated CTCs can be purified and analyzed for their genetic signatures in diseases like non-small cell lung cancer (NSCLC), where targeted therapies exist for specific mutations such as the epidermal growth factor receptor (EGFR) exon 19 deletion [1]. However, CTCs are extremely rare, and hence, require high-throughput and high-purity isolation methods to ensure sufficient cells for analysis. Numerous methods have been published for CTC isolation, ranging from bulk methods like size selection, density centrifugation to microfluidic-based methods such as the inertial-based iChip [2] and cell surface marker-based selection methods like the MagSweeper [3].

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We previously reported the application of a microfabricated magnetic sifter, comprising ~4000 pores in a single 7 × 7 mm^2 Si chip, sputtered with a thin layer of paramagnetic film ($Ni_{81}Fe_{19}$), to achieve rapid (10 mL/h) and high-purity isolation of CTCs directly from whole patient blood in NSCLC, as illustrated in Fig. 1a, b [4]. When an external magnetic field is applied, magnetically labeled cells are held against hydrodynamic flow at the sides of the pores due to strong magnetic field gradients generated by the paramagnetic layer

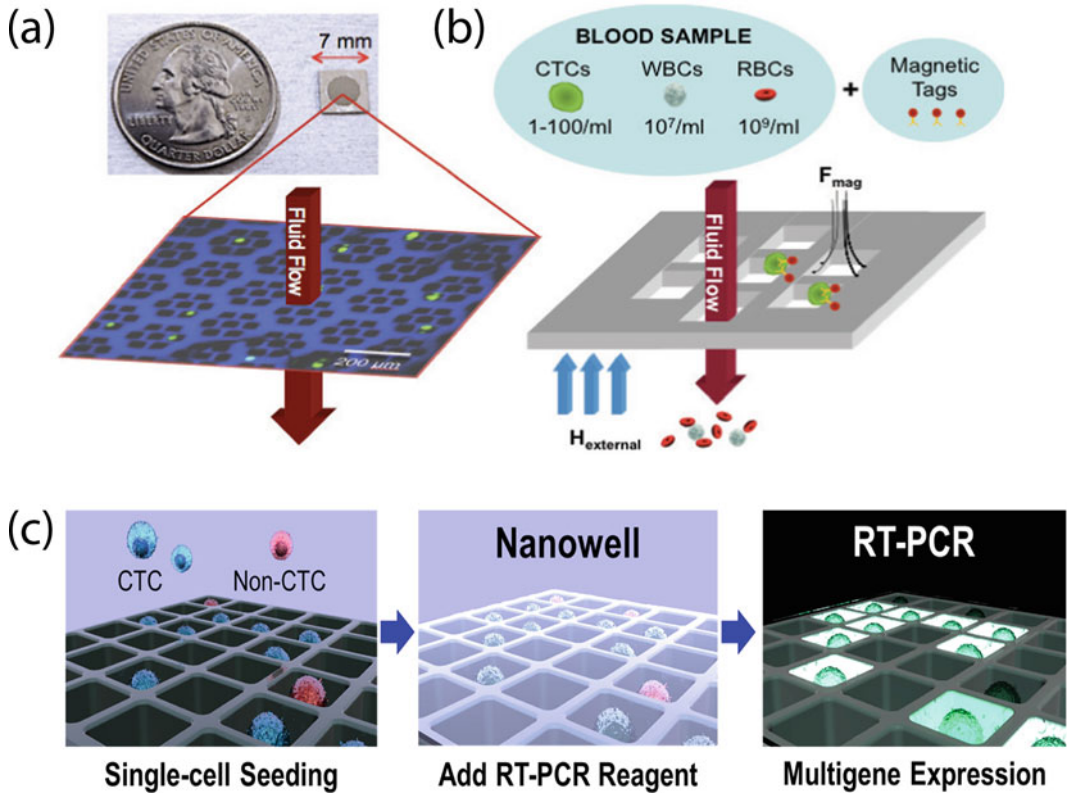

Fig. 1 Operating principle of the magnetic sifter and nanowell device for circulating tumor cell isolation and genetic analysis. (**a**) Images show a single magnetic sifter chip along with a magnified optical microscope image of a section of the 40 × 40 μm^2 pores that make up the sifter. The chip is artificially colored *blue* for clarity while the *green circles* are spiked H-1650 lung cancer cells that have been captured by the sifter. (**b**) In the typical operation of the sifter, a whole blood sample is incubated with magnetic nanoparticles before the entire mixture is pumped through the sifter in the presence of an external magnetic field. The circulating tumor cells (CTCs) with their bound magnetic nanoparticles are captured at the pores due to strong field gradients from the magnetic film on the chip, while unlabeled blood cells pass through the chip. Removal of the permanent magnet releases these captured cells from the magnetic sifter for further downstream analysis. (**c**) A mixture comprising CTCs and other blood cells are loaded onto the nanowell into individual 20 × 20 × 50 μm^3 chambers for single-cell RT-PCR of a panel of genes. Poisson statistics ensure that the majority of the wells contain maximally one cell, hence permitting single-cell gene expression analysis. Images (**a**) and (**b**) are reproduced from Earhart et al. [4] with permission from RSC Publishing, while image (**c**) is reproduced from Park et al. [5] with permission from PNAS

on the sifter. However, when the external magnetic field is removed, the paramagnetic layer is no longer magnetized, and any previously captured cells can be easily washed out by passing a buffer through the sifter. Specificity of this capture process is ensured via the use of magnetic nanoparticles and antibodies to CTC cell surface markers like *Ep*ithelial *C*ell *A*dhesion *M*olecule (EpCAM).

This process has three main advantages. First, the strong field gradients generated by the array of pores in the sifter allow for high-efficiency capture even at relatively fast volumetric flow rates. In previous work, we demonstrated >90% capture efficiency of NSCLC cells (NCI-H1650 cell line) spiked into whole blood at flow rates of 10 mL/h [4]. Second, the specificity to a magnetic signal of our capture mechanism ensures high enrichment purity. Unlike other transduction mechanisms, magnetism has very minimal background signal in most biological systems, with human cells having negligible magnetic content. In fact, prior experiments with six NSCLC patient blood samples indicate a 5–6 log enrichment factor, with an average of 17.7% of all cells imaged on the magnetic sifter post-capture identified as CTCs [4]. This is extremely beneficial to downstream analysis, as extraneous background signals from blood cells are significantly reduced. Last but not least, the paramagnetic layer will not retain its magnetization upon removal of the external magnetic field, hence facilitating retrieval of captured CTCs from the sifter by simply passing buffer through the sifter in the absence of an external magnetic field. Previous experiments indicate an average release efficiency of 92.7% of captured tumor cells [4]. Taken together, these advantages make the magnetic sifter a very compelling method for the isolation of CTCs for downstream analysis.

After processing with the magnetic sifter, the enriched CTCs are concentrated in a single solution. This final cell suspension can be analyzed as a single bulk sample for genetic signatures, or further concentrated into a small volume for integration with various other genetic analysis platforms, such as Fluidigm's C1 system or Silicon Biosystems' DEPArray.

A nanowell platform has previously been demonstrated for single-cell genetic analysis, and is easily fabricated with standard microfluidic methods, while allowing rapid, parallel sorting of a bulk cell suspension into single-cell reaction chambers for single-cell gene expression analysis [6, 7]. The nanowell device consists of 25,600 single-cell reaction wells of size $20 \times 20 \times 50\ \mu m^3$, and utilizes Poisson statistics to ensure maximally one cell per well if small cell numbers are loaded onto it. Hence, it is an extremely versatile and economical method for targeted single-cell gene expression analysis, and we choose to describe here the integration of this nanowell platform with the magnetic sifter to demonstrate the ability for genetic analysis of isolated CTCs [5, 8].

2 Materials

Materials required are listed under two separate categories, namely, CTC isolation with the magnetic sifter, and genetic analysis with the nanowell system. Additionally, these materials are further subdivided into the consumables and reusable sections for greater clarity.

2.1 Magnetic Isolation

2.1.1 Reusable Essentials

1. Programmable syringe pump (New Era Pump Systems Inc., Model NE-1000).
2. Laser-cut acrylic holders with screws and O-rings.
3. Transfer pipette (1000, 200, and 10 μL) with pipette tips.
4. NdFeB rare earth permanent magnets (*see* **Note 1**).

2.1.2 Consumables

1. Magnetic sifter chips (12-μm Permalloy film on a Si backbone).
2. Fluidic connectors: Vinyl tubing and syringes.
3. K_2-ethylenediaminetetraacetic acid (EDTA) blood collection tubes.
4. Working buffer: Calcium and magnesium-free phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), 2 mM EDTA, 0.2% Pluronic F-68 solution (*see* **Note 2**).
5. Antibodies: Monoclonal biotinylated anti-EpCAM antibodies (clone 9C4 from Biolegend, Inc., San Diego, CA).
6. Magnetic nanoparticles: MAG999 streptavidin ferrofluid (R&D Systems, Minneapolis, MN).

2.2 Nanowell Gene Analysis

2.2.1 Reusable Essentials

1. Centrifuge.
2. Thermal cycler.
3. Fluorescence microscope.
4. Transfer pipette (1000, 200, and 10 μL) with pipette tips.

2.2.2 Consumables

1. Nanowell devices with PDMS gasket on glass slide.
2. Adhesive PCR plate seals.
3. K_2-EDTA blood collection tubes.
4. Hemolysis buffer: Ultrapure distilled water, 154 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA solution.
5. PCR primers and probes: ddPCR probes (Bio-Rad, Hercules, CA).
6. Reverse transcription—polymerase chain reaction (RT-PCR) enzyme and buffer: CellsDirect One-Step qRT-PCR kits (to 50 μL of 2× reaction mix, add 3 μL of hydrolysis probes for each gene of interest, 3 μL of Superscript® III RT Platinum® Taq Mix, 3 μL of 50 mM $MgSO_4$, and sufficient diethylpyrocarbonate (DEPC) water to make the solution up to 100 μL) (*see* **Note 3**) (Thermo Fisher Scientific, Waltham, MA).

7. Deoxyribonuclease I (DNase I) enzyme and buffer: Reagents are included in CellsDirect One-Step qRT-PCR kits (Thermo Fisher Scientific, Waltham, MA).
8. Mineral oil (W.S. Dodge Oil, Maywood, CA).

3 Methods

As this protocol is intended primarily for interested biologists and clinician-investigators, details on the fabrication process for the magnetic sifter chip and the nanowell device are not included. Interested readers are directed to Earhart et al. [4] and Park et al. [5] for details. Additionally, it should be noted that both devices are fabricated with standard microelectromechanical systems (MEMS) and microfluidic fabrication techniques; hence, both sets of devices are readily available by contract order from commercial foundries as well.

3.1 Magnetic Isolation

3.1.1 Incubation

1. Pipette out 2 mL of whole patient blood from EDTA tubes and mix it with 2 mL of working buffer, along with 10 μL of 50 μg/mL biotinylated anti-EpCAM antibody solution (final concentration 125 ng/mL). Keep the mixture at 4 °C under constant rotation for 1 h (*see* **Note 4**).
2. Add an additional 20 μL of magnetic nanoparticle solution to the 4 mL blood mixture, and incubate the mixture at 4 °C for an additional hour.

3.1.2 Sifter Processing

1. Load the sifter into the acrylic holders, and connect it via tubing to a syringe pump as per Fig. 2a, b. Place the sifter directly over the NdFeB permanent magnet and secure the holder with tape if necessary (*see* **Note 5**).
2. Prime the entire device with an initial 250 μL of working buffer, and set the syringe pump to maintain a flow rate of 10 mL/h.
3. Gradually add the blood sample to the inlet well with a pipette as the mixture gets drawn through the magnetic sifter. Keep the blood sample on ice as much as possible while working (*see* **Note 6**).
4. After processing all the blood through the sifter, pump an additional 1 mL of working buffer through the sifter to ensure all remnant blood is thoroughly washed through the chip.
5. Remove the holder from the permanent magnet with caution, and elute the cells from the chip with additional working buffer. We typically elute by passing 500 μL of working buffer through the chip repeatedly away from the external permanent magnet (*see* **Note 7**).

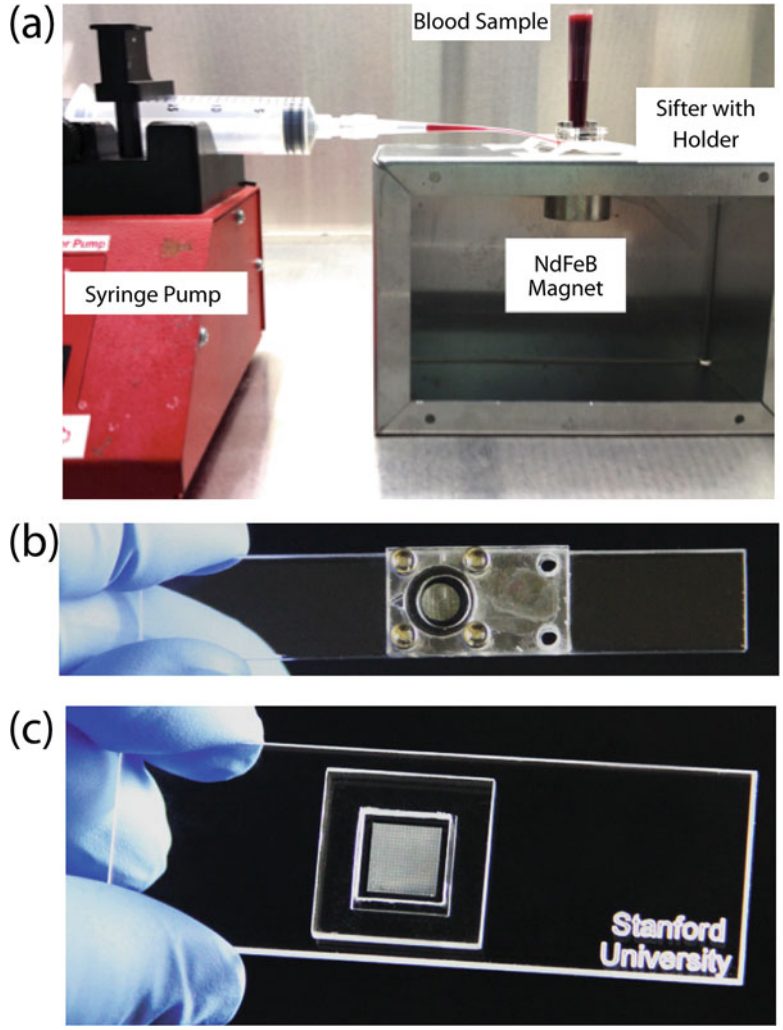

Fig. 2 Optical images of the actual magnetic sifter and nanowell device. (**a**) A sample setup is presented. The magnetic sifter is connected to a syringe pump to maintain a flow rate of 10 mL/h while blood is added to the system via an inlet well on the top of the sifter. The sifter is placed above the NdFeB magnet to ensure a strong perpendicular field is constantly applied during operation. (**b**) A sample sifter holder is presented. The acrylic holder provides a watertight seal for the sifter to the rest of the tubing and inlet sample well. A hole is cut through the top and bottom of the acrylic holder to maintain a press fit with the rest of the fluidic connectors, while O-rings are used to ensure a proper seal around the sifter. (**c**) The PDMS nanowell device is bonded to a glass slide after fabrication from a wafer mold, and a gasket is placed on the top of the device to help contain the fluid volume when centrifuging. This gasket is removed after the cells are seeded with the RT-PCR mix. The planar nature of the nanowell device facilitates microscope imaging after thermal cycling is complete. Image (**a**) is reproduced from Earhart et al. [4] with permission from RSC Publishing, while images (**b**) and (**c**) are reproduced from Park et al. [5] with permission from PNAS

3.1.3 Sifter Disposal and Cleanup

1. The remainder of the blood sample and any blood-containing volumes should be disposed of in a 10% bleach solution, while any solid waste such as the syringes and connective tubing should be disposed of as regular biohazards.

2. The sifter holders can be reused and only need to be replaced when physically damaged. They can be cleansed with a combination of water and 10% bleach solution before being left to air-dry. This applies to the O-rings and screws as well.

3.2 Nanowell Gene Analysis

1. Add 12.5 mL of hemolysis buffer to the eluted cell suspension and incubate at room temperature for 10 min (*see* **Note 8**).

3.2.1 Hemolysis

2. Centrifuge at 750 × *g* for 5 min and resuspend in 15 mL of PBS, and repeat the wash to ensure complete removal of the hemolysis buffer (*see* **Note 9**).
3. Centrifuge at 750 × *g* for 5 min and quantify the amount of PBS left in the tube.

3.2.2 DNAse I Digestion

1. Resuspend with 0.3 U/μL DNase I for 5 min at room temperature to remove any noncellular DNA present in the solution. 10× DNase I buffer should be used to make up the remaining solution.
2. Add EDTA solution subsequently to ensure a final concentration of 5 mM EDTA to inhibit the enzyme.

3.2.3 Nanowell Loading

1. Pipette the DNase I-treated cell suspension onto the top of a nanowell within the accompanying gasket and centrifuge at 1620 × g for 10 min. The nanowell and the gasket should be supported on a glass slide for easy handling as per Fig. 2c (*see* **Note 10**).
2. Identification can be done at this time if cells were stained with fluorescence markers by imaging the array under a fluorescence microscope with filter cubes corresponding to cell stains added previously.
3. Dry the sample at 70 °C for 10 min.
4. Add prepared RT-PCR mix to the nanowell and centrifuge the nanowell device for 10 min at 1620 × g.
5. Fully cover the array with a small piece of adhesive PCR sealant film and centrifuge at 1620 × g for another 10 min to seal the top completely.
6. Place a piece of cover glass onto the top of the nanowell and secure it with a piece of adhesive tape before creating a complete seal around the edges of the nanowell device with mineral oil to prevent reagent evaporation during thermal cycling (*see* **Note 11**).

3.2.4 Thermal Cycling

1. Load the sealed nanowell with its glass slide support into a thermal cycler.
2. Typical cycles used are as follows: (a) Denaturation and reverse transcription: 50 °C for 45 min, (b) Annealing and extension: 10 cycles of 95 °C for 60 s and 65 °C for 90 s, (c) Amplification: 35 cycles of 90 °C for 60 s and 60 °C for 90 s (*see* **Note 12**).

3.2.5 Image Acquisition

1. Acquire fluorescence images with a microscope equipped with the appropriate filter cubes across the whole array (e.g., a fluorescein isothiocyanate (FITC) filter cube if a 6-carboxy fluorescein (FAM) probe is used) (*see* **Note 13**).
2. Images at each nanowell location can then be analyzed for a positive or negative gene expression signal for each gene investigated.

4 Notes

1. 1-inch cube magnets were typically used as they can provide a sufficiently strong field (> 2000 Oe) to ensure the sifter and paramagnetic nanoparticles (150 nm in diameter) are fully saturated during operation. Other configurations can be used but the magnetic fields should be measured to ensure complete saturation. Different magnetic particles may also have variable saturating fields.
2. The working buffer should not be kept for prolonged periods of time, and should be syringe-filtered prior to use. Since this buffer does not contain sodium azide or other preservatives, it is susceptible to contamination and degradation.
3. The PCR reaction mix is absolutely critical for successful RT-PCR to occur, especially since most mixes as sold are not initially intended for multiplexing more than two genes. This reaction mix should be re-optimized for each combination of probes, but the formula introduced here can serve as a good starting point for optimization. Depending on the relative abundances of the genes of interest, the more abundant genes may deplete the reaction mix before the less abundant genes can amplify to a sufficient quantity for positive identification.
4. A no-wash protocol is applied here, whereby cell losses can be minimized from extraneous washing and centrifugation steps. However, the no-wash protocol's labeling efficiency is sensitive to the choice of incubation conditions. Hence, the incubation times and concentrations should be independently optimized if different antibodies and nanoparticles are used.
5. Check the magnet to ensure the magnet is oriented such that either the north or south pole is directly facing the sifter holder. This will ensure a perpendicular field through the magnetic sifter. Additionally, do not over tighten the holder when the chip is inserted, as the Si backbone can be damaged by the stresses and torques induced. Chips should also be checked at this time under a microscope to ensure integrity of the individual pores and cleanliness of the sifter surface.

6. Ensure there are no breaks in the addition of blood or buffer to the magnetic sifter during the entire isolation process. Maintaining a continuous flow of fluid through the chip is critical for ensuring there are no sudden pressure jumps and consequently, changes in flow velocity, through the chip during operation.
7. The amount of buffer can be adjusted to match any volume required for downstream processing. We typically use 500 μL as a convenient volume for elution and integration with the nanowell process. Additionally, the sifter surface can be checked under the microscope for thoroughness of the wash process, with its planar nature making scanning of the chip under a microscope extremely convenient.
8. Ensure hemolysis buffer is brought to room temperature before the start of incubation and keep the mixture in constant agitation during the incubation.
9. An additional incubation step with fluorophore-conjugated antibodies can be performed at this step. This will permit staining of the captured cells for cell surface markers such as CD45 for leukocyte identification. The cells can be imaged in the nanowells prior to lysis and RT-PCR if required.
10. Typically, volumes of 100 μL or less are loaded onto the nanowell surface. This will ensure the volumes stay on the chip and within the gasket during centrifugation.
11. The mineral oil is essential to ensure a complete seal around the chip. Due to the small volumes in each reaction well in the nanowell device, any evaporation that occurs will result in a significant change in the reagent concentrations, and potential desiccation of the individual reaction wells.
12. Cycle times and durations need to be tested for individual genes and probes of interest. Additionally, it is recommended that cell lines of similar biology to the cancer of interest be tested as a means of ascertaining the likely gene expression levels and to verify the appropriateness of these parameters and the number of amplification cycles for thermal cycling.
13. Multiple images may need to be acquired to cover the full array, and these images can be stitched together subsequently. A magnification of 10× or greater is recommended to ensure sufficient pixels cover each reaction well in the nanowell device for analysis.

Acknowledgment

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Chapter 13

RareCyte® CTC Analysis Step 1: AccuCyte® Sample Preparation for the Comprehensive Recovery of Nucleated Cells from Whole Blood

Arturo B. Ramirez, Lance U'Ren, Daniel E. Campton, David Stewart, Joshua J. Nordberg, Jackie L. Stilwell, and Eric P. Kaldjian

Abstract

The RareCyte platform addresses important technology limitations of current circulating tumor cell (CTC) collection methods, and expands CTC interrogation to include advanced phenotypic characterization and single-cell molecular analysis. In this respect, it represents the "next generation" of cell-based liquid biopsy technologies. In order to identify and analyze CTCs, RareCyte has developed an integrated sample preparation, imaging and individual cell retrieval process. The first step in the process, AccuCyte®, allows the separation, collection, and transfer to a slide the nucleated cell fraction of the blood that contains CTCs. Separation and collection are based on cell density—rather than size or surface molecular expression—and are performed within a closed system, without wash or lysis steps, enabling high CTC recovery. Here, we describe our technique for nucleated cell collection from a blood sample, and the spreading of these nucleated cells onto glass slides permitting immunofluorescent staining, cell identification, and individual cell picking described in subsequent chapters. In addition to collection of rare cells such as CTCs, AccuCyte also collects cells of the circulating immune system onto archivable slides as well as plasma from the same sample.

Key words Density-based separation, Circulating tumor cells, Microscope slide immobilization, Non-biomarker-dependent isolation, Immunofluorescent staining

1 Introduction

The evaluation of circulating tumor cells in blood is a rare cell problem. This is sometimes referred to as finding a "needle in a haystack." In this metaphor, the "needles" are the CTCs, and the "hay" is usually considered to be the white blood cells that can be present in more than a million-fold excess. Another way to consider the problem is as a "needle in scattered hay within a barn." In this variation on the metaphor, the "needles" and the "hay" remain as before, but the barn represents the volume that is made up of plasma and red blood cells.

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The sample preparation step, called AccuCyte, effectively collects the scattered "hay" within the barn into a small volume "haystack," containing white blood cells (WBCs) and CTCs, by removing plasma and red blood cells (RBCs). The entire "haystack" is processed to microscope slides and stained using immunofluorescence reagents on commercial automated immunohistochemistry (IHC) instruments. The "needles" (CTCs) within the "haystack" are identified in the second step by scanning and image analysis using the CyteFinder® instrument (in effect a highly sensitive "metal detector"). In the third step, individual CTCs are physically retrieved from the slides for single-cell molecular analysis [1] by CytePicker® (that can be thought of as a sensitive "magnet" that isolates an individual needle without disturbing the haystack), which is built into CyteFinder, completing the integrated process.

AccuCyte® allows comprehensive collection of nucleated cells from a blood sample with spike-in recoveries typically above 90% [2]. Because circulating tumor cells (CTCs) are rare and can be heterogeneous with respect to size, morphology, and expression of markers that characterize them, separating them out from other cells in the blood can create unique challenges. Typically, CTC platforms isolate these cells based on size, protein expression, or other physical characteristics (reviewed in [3]). Although there are advantages to each of these technologies, there is also the possibility of losing CTCs. For example, EpCAM is a cell surface marker commonly used to isolate CTCs and although EpCAM is generally present, its expression can be low enough so that some cells with low or no EpCAM expression are missed [4]. Another example is CTC capture based on size using filters [5–7]. This approach will not collect CTCs that are the same size or smaller than leukocytes if leukocyte exclusion is effective. The AccuCyte platform collects cells based on the first principle of density, which is similar for all nucleated blood cells, thereby minimizing the loss of CTCs with non-prototypical characteristics.

The AccuCyte kit includes a Separation Tube that has within it an internal float of the same density as leukocytes, and an Accucyte Collector® through which the cells are removed from the Separation Tube. These are precision engineered to collect all nucleated cells from a blood sample (Fig. 1). A blood sample (7.5 mL standard volume) is added to the Separation Tube and centrifuged, separating the plasma above from RBC fraction below. The float "floats" on the RBCs beneath it, and nucleated blood cells (the "buffy coat") localize to a thin circumferential band between the float and the tube wall since their density matches that of the float (Fig. 2a, b). Next, a small instrument (called CyteSealer®) applies a metal ring around the Separation Tube that seals the tube to the float to create a barrier between the buffy coat and red blood cell fraction below (Fig. 2c). The CyteSealer ensures collection of only the fraction of cells located on the float that are above the seal.

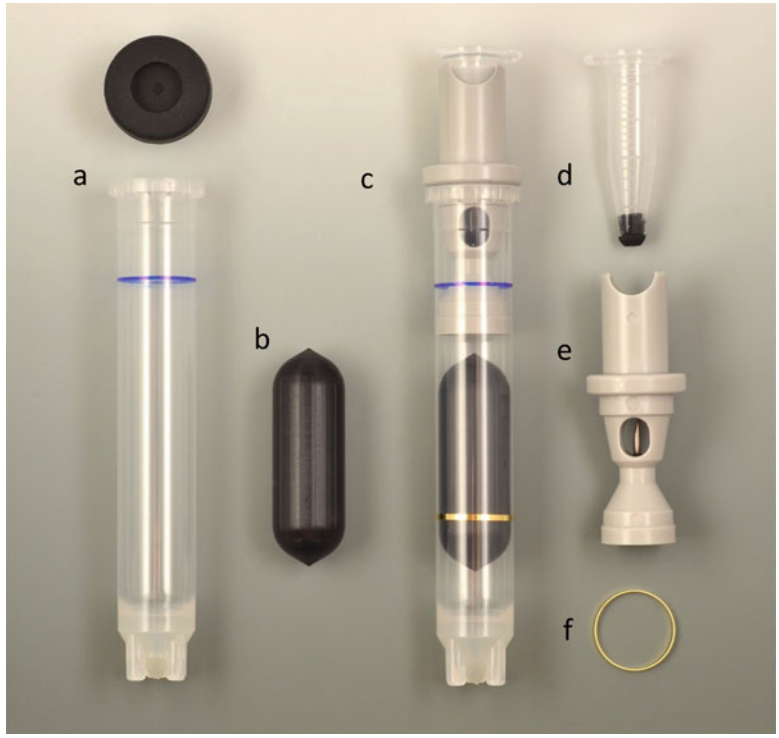

Fig. 1 Pictured are the components required for blood preparation using the AccuCyte kit including (**a**) the Separation Tube with cap, (**b**) the float, (**c**) the entire assembled system, and (**d**) the Isolation Tube where the nucleated cells are contained at the end of the final centrifugation. Also pictured below the Isolation Tube is (**e**) the AccuCyte Collector and (**f**) the ring that is applied by the CyteSealer

After the removal of the plasma (which itself may be saved for analysis from the sample), the cells above the sealing ring are isolated by adding an inert displacement fluid of heavier density than the cells to be collected, and inserting the AccuCyte Collector with Isolation Tube into the Separation Tube. This allows collection of the nucleated cells and platelets in a second centrifugation step in which the heavy density isolation fluid displaces the buoyant cells from the float through the AccuCyte Collector directly into the Isolation Tube within it (Fig. 2d). Lastly, a Transfer Fluid is mixed with the cells collected into the Isolation Tube and cells are spread via a simple manual device (the CyteSpreader®) onto microscope slides forming a monolayer smear (Fig. 2e). After the slides have dried they can be fixed and stained in an automated stainer (such as Ventana DISCOVERY, Leica BOND, or Dako Autostainer) typically using a nuclear stain and antibodies to cytokeratin, EpCAM, and CD45; manual staining is also possible. Slides may be archived frozen for later analysis. The AccuCyte method is a simple, fast, reproducible, and high recovery method of collecting nucleated blood cells and immobilizing them to a microscope slide for further characterization. A video of the AccuCyte process is here: https://www.youtube.com/watch?v=EciioR5zLFU.

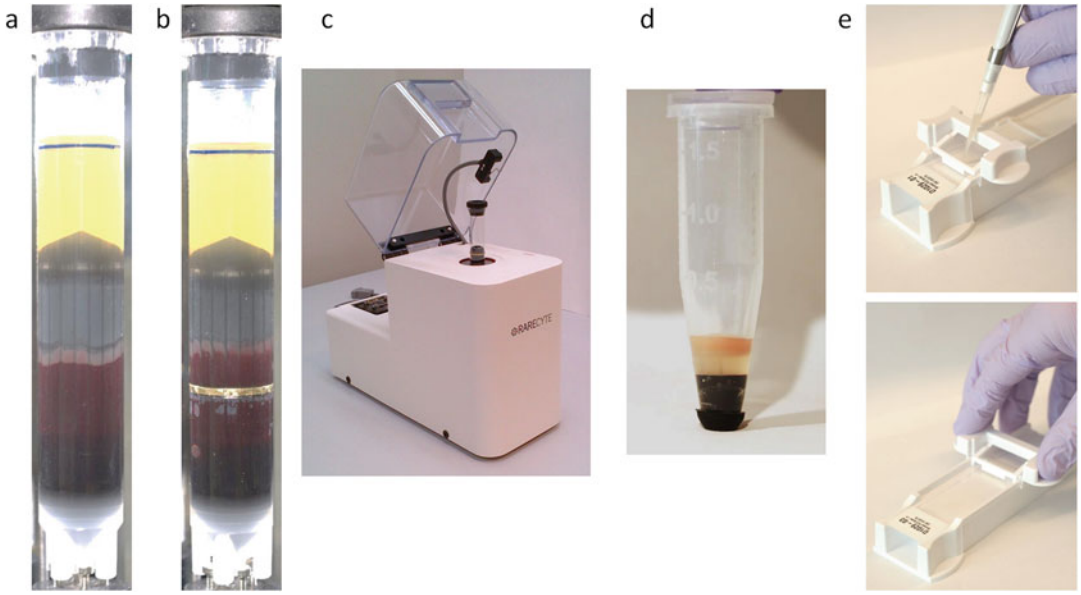

Fig. 2 Steps in the AccuCyte process from isolating nucleated cells from blood to spreading the cells onto glass slides are pictured in these panels. Panel (**a**) shows the Separation Tube containing blood after the first centrifugation. A thin band of nucleated cells (the "buffy coat"), appearing *white*, can be seen on the float above the RBC layer and below the plasma. In panel (**b**) the CyteSealer has been used to apply a ring around the separation tube creating a barrier between the buffy and the RBCs below. Panel (**c**) shows CyteSealer Instrument. Panel (**d**) is an image of the nucleated cells in an Isolation Tube collected by using Isolation Fluid and the AccuCyte Collector as described in Subheading 3. Once these cells are collected into the Isolation tube, Transfer Fluid is added to the tube, mixed and cells are spread onto glass slides using an AccuCyte Spreader Tray and Spreader Blade as pictured in panel (**e**) so that cells are evenly distributed across the slide in a monolayer

2 Materials

All materials required throughout this protocol can be purchased directly from RareCyte. Store all reagents at proper temperature and protect from light if indicated on the product label. Do not use materials after the expiration date printed on the product label. Refer to the Safety Data Sheet on proper handling and disposal of any reagents.

2.1 Separation of Whole Blood into Constituent Layers

1. 7.5 mL of blood collected into a RareCyte blood collection tube.
2. Beckman Coulter Allegra X-15R centrifuge.
3. Beckman Coulter swinging bucket rotor SX4750.
4. Beckman Coulter Aerosolve Canister/biocertified covers.
5. RareCyte Centrifuge Adapter.
6. RareCyte Bucket Adapter.

7. RareCyte AccuCyte Separation Tube and float with cap (Fig. 1a, b).
8. 10 mL serological pipette.
9. Electronic pipette.

2.2 Buffy Coat Isolation and Transfer to Slides

1. RareCyte CyteSealer.
2. RareCyte CyteSeal Insertion Tool.
3. RareCyte Centrifuge Adapter.
4. Beckman Coulter Allegra X-15R centrifuge.
5. Beckman Coulter swinging bucket rotor SX4750.
6. RareCyte Centrifuge Bucket Adapters.
7. RareCyte AccuCyte Spreader Base Tray with eight individual bases.
8. Coplin jars that hold eight slides.
9. RareCyte CyteSeal Height Gauge.
10. RareCyte CyteSeal Ring (Fig. 1f).
11. RareCyte AccuCyte Collector (Fig. 1e).
12. RareCyte AccuCyte Waste Tube.
13. RareCyte AccuCyte Isolation Tube (Fig. 1d).
14. RareCyte AccuCyte Locking Ring.
15. RareCyte AccuCyte Isolation Tube Sheath.
16. VWR Superfrost® Plus glass slides.
17. Standard-bore P200 and P1000 pipet tips.
18. 10 mL disposable plastic serological pipettes.
19. RareCyte AccuCyte Spreader Blade.
20. RareCyte Transfer Fluid.
21. RareCyte Isolation Fluid (store working stock at room temperature up to 1 week, long-term storage at 4 °C).
22. RareCyte Displacement Fluid.
23. RareCyte CyteMount Media.

3 Methods

Carry out all procedures in a biosafety cabinet, wearing the appropriate personal protective equipment, and handling all materials that are in contact with blood or blood components as biohazardous and possibly infectious. Do not modify or dilute any of the fluids or components of the AccuCyte kit. One kit is intended for use with one blood sample which may be 2–7.5 mL. RareCyte blood collection tubes, which stabilize whole blood and all of its

components up to 72 h after blood collection, are recommended. For live cell applications, EDTA anticoagulated blood may be used if processed within 4–6 h. AccuCyte kits are not typically provided sterile but can be sterilized upon request.

3.1 Separation of Whole Blood into Its Constitutive Layers

1. Invert blood collection tube containing sample eight times to ensure proper mixing of blood constituents.
2. Transfer 7.5 mL of blood to AccuCyte Separation Tube (float is already inside tube). Cap the tube. Place the tube in a centrifuge adapter and insert the centrifuge adapter into a bucket adapter. Attach the Aerosolve cover to the Bucket.
3. If running an odd number of tubes, make sure to have an appropriately weighted balance tube in the opposite position of the centrifuge rotor.
4. Centrifuge sample with the following set points: 3000 × RCF for 25 min at room temperature; ramp up: MAX; ramp down: MAX.
5. After centrifuge has stopped spinning, take centrifuge adapter out of bucket adapter and take tube out of centrifuge adapter. Proceed to buffy coat isolation and transfer to slides (*see* **Note 1**).

3.2 Buffy Coat Isolation and Transfer to Slides

1. Insert CyteSeal ring into CyteSealer device with the ring insertion tool. Make sure ring is flush with the collet and in a horizontal position. Place tube into CyteSealer, making sure that the 4 feet at the bottom of the tube lock into place into the four holes in the red plastic tube holder inside the CyteSealer. This will allow the position of the ring on the float to be controlled by twisting the tube clockwise to move the tube down and counter-clockwise to move the tube up (*see* **Note 2**). Use the height gauge to set clamp height so that the ring is positioned at the proper location on the float. To determine the optimum position of the ring on the float, place the height gauge on the collet and twist the tube clockwise or counter-clockwise until the tip of the float is just under the horizontal line at the top of the height gauge. Close lid of CyteSealer device to activate clamp mechanism. Wait for green light, indicating the collet has returned to its home position. Open lid and remove tube.
2. Remove tube from the CyteSealer. Working inside a biosafety cabinet, remove and dispose of tube cap. Remove plasma using a serological pipette. One does not have to worry about pipetting up any buffy coat components, since they are safely lodged in the narrow space between the inner wall of the tube and the outside of the float, so the user can pipette as much plasma as possible off the top of the float (a small amount of plasma will

remain at the top of the float). This plasma is highly depleted of cellular material and can be used directly to analyze proteins, cell-free DNA, or cell-free RNA. If desired, plasma may be frozen at −80 °C for long-term storage.

3. Add Displacement Fluid to reach the blue line on the tube. This amount of Displacement Fluid ensures a small excess that is required. Any extra volume will be retained on the top of the AccuCyte Collector and will not affect CTC recovery (*see* **Note 3**).
4. Insert AccuCyte Collector with Waste Tube in place, into the Separation Tube. A moderate amount of force is required to fully insert the collector all the way into the tube. Push AccuCyte Collector until the top of the tube is flush with the widest part of the AccuCyte Collector. Excess Displacement Fluid will flow into space around top of AccuCyte Collector. Attach locking clip to the tube and collector apparatus. The small opening on the locking clip faces up (locks around the AccuCyte Collector) and the large opening faces down (locks around the tube).
5. Remove Waste tube and discard it. Insert the Isolation Tube by pressing it into the AccuCyte Collector until the tube reaches the bottom of the needle and no part of the needle can be seen through the window in the AccuCyte Collector. Open the lid of the Isolation Tube and pipette 160 μL of Isolation Fluid.
6. Do not rotate the Isolation Tube during insertion or subsequent removal from the AccuCyte Collector.
7. Place entire tube assembly into Centrifuge Adapter. Place assembly into Centrifuge Bucket Adapter. Attach the Areosolve cover to the Bucket. Centrifuge sample with the following set points: 1000 × RCF for 20 min at room temperature; ramp up: SLOW; ramp down: MAX.
8. After centrifugation is complete, remove Centrifuge Adapter and tube assembly from centrifuge bucket. While holding the centrifuge tube and the centrifuge adapter in place with one hand, remove the Isolation Tube from the AccuCyte Collector with the other hand without removing the centrifugation tube from the Centrifuge Adapter. The best place to hold the centrifugation tube is by the AccuCyte Collector. Do not twist the Isolation Tube when removing it from the AccuCyte Collector, pull it straight up (*see* **Note 4**).
9. Add 700 μL of Transfer Fluid to the sample in the Isolation Tube. Mix thoroughly by pipetting up and down with a P1000 tip. Use the force of the liquid coming out of the pipette tip to fully dislodge any cells sticking to the side of the Isolation Tube and to mix the cell suspension. Do not use the pipette tip to dislodge the cells stuck to the Isolation Tube because this may

cause cells to get stuck on the tip. To confirm that there are no cells adhered to the Isolation Tube, cap the tube and turn upside down. If cells are still adhered, there will be a white ring of cells visible at the height of where the buffy coat was originally placed in the Isolation tube. If cells are still observed, continue to pipette up and down until this ring disappears.

10. Incubate sample mixture at room temperature for 10 min. While waiting, label eight SuperFrost Plus slides and place them into eight bases on a Spreader Base Tray with frosted end toward thumb holds and facing up. Place Spreader Blade onto the tracks of the first base, away from where the frosted part of the slide is. The plastic blade should be closest to the frosted end of the slide.
11. Pipette 95 μL of the sample from **step 8** onto the center of the slide so that it contacts the spreader blade. Carefully slide the blade in one direction, away from the frosted end, until it rises up and clears the end of the slide. Do not press downward on the thumb holds. Direct the blade slowly but deliberately. Remove Spreader Blade from the first Spreader Base and place it on the next Spreader Base. Repeat **step 10** with all of the remaining slides. The last slide may have slightly more or less than 95 μL, pipette all remaining sample onto the last slide.
12. Allow 30 min for slides to dry on Spreader Tray Base in a chemical fume hood with blower on. If samples will be stained immediately, fix the slides in 10% formalin for 1 h for automated stainers or 15 min for manual staining. If slides will be stained in less than 72 h, store slides at 4 °C prior to fixation. If slides will not be stained for more than 72 h, store dried slides at −20 °C prior to fixation.

4 Notes

1. After centrifuging sample, the user can leave tube without clamping for up to 2 h without any deleterious effects on sample recovery. Longer wait times are not recommended. After the sample has been centrifuged, do not hold the tube where the buffy coat is localized. The plastic tube is somewhat pliable and applying force to the walls of the tube where the buffy is located may disrupt the buffy coat layering, causing decreased recovery of CTCs. Instead, hold the tube by the cap or the bottom where the 4 ft are. This plastic is hard and will not disrupt the sample layering.
2. Placement of the ring on the float depends on the height of the float inside the tube after centrifugation, which in turn is determined by the volume of blood used and the hematocrit of the patient. This cannot be determined a priori. When

running multiple tubes of the same patient from blood collected at the same time, adjusting of the height of the ring will not be necessary, although checking the height gauge each time to make sure the ring will have the correct position on the tube is recommended. One can place the ring slightly higher on the float without detrimental effects on the recovery of CTCs (unless the ring is set so high that CTCs are trapped under it). In the event that the ring is placed lower than what the height gauge indicates (i.e., the tip of the float is above the horizontal part of the height gauge), the collet will clamp the ring onto the part of the float where the teardrop shape begins and the float will be pushed up in the tube, disrupting the buffy coat layering and causing sample loss.

3. Usually, 4 mL is sufficient to reach the blue line. More or less may be required depending on volume of blood used and the hematocrit of the sample.
4. If one does not want to dry and fix the cells onto a microscope slide, live cells can be isolated and stained in suspension using the AccuCyte isolation platform. To collect live cells, substitute the RareCyte blood collection tube with a standard EDTA blood collection tube. A float with slightly lower density made for EDTA blood samples should be used in this case. Once the buffy coat is isolated, add 500 μL of PBS to resuspend the buffy coat layer, similar to that done in **step 9** of buffy coat isolation and transfer to slides. Accurate identification of live CTCs will depend on the availability of adequate surface markers that can be stained with fluorescently labeled antibodies. Cells can be stained by traditional solution antibody staining after they have been collected into the Isolation Tube. Fluorescently conjugated antibodies can be added at this step (after adding 500 μL of PBS) to identify and differentiate CTCs of interest from white blood cells. Stained cells are then placed on a specialized chamber slide, called a CyteSlide for imaging and live cell retrieval.

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Chapter 14

RareCyte® CTC Analysis Step 2: Detection of Circulating Tumor Cells by CyteFinder® Automated Scanning and Semiautomated Image Analysis

Jeffrey L. Werbin, Joshua J. Nordberg, Jay Tzucker, Paulina Varshavskaya, Jackie L. Stilwell, and Eric P. Kaldjian

Abstract

The RareCyte CyteFinder instrument is an automated scanner that allows rapid identification of circulating tumor cells (CTCs) on microscope slides prepared by the AccuCyte process (*see* Chapter 13) and stained by immunofluorescence. Here, we present the workflow for CyteFinder scanning, analysis, and CyteMapper scan review which includes CTC confirmation and report generation.

Key words Circulating tumor cells, Fluorescence scanning, Image analysis, Scoring algorithm

1 Introduction

In the previous chapter (Chapter 13), the AccuCyte® sample preparation method is described as collecting scattered nucleated cells "hay" into a small volume "haystack" (containing WBCs and CTCs) by removing plasma and RBCs. The entire "haystack" is spread onto microscope slides and is stained by immunofluorescence on commercial automated IHC instruments, using antibodies to CD45, EpCAM, and cytokeratins, as well as DAPI, a nuclear dye. This chapter presents the protocol for identifying the "needles" (CTCs) within the "haystack" by scanning and image analysis using the CyteFinder instrument [1, 2].

The CyteFinder® instrument is a high-speed scanning fluorescence microscope that is capable of imaging at multiple resolutions. A standard 4-color AccuCyte slide smear can be imaged by the CyteFinder in around 12 min. The accurate motorized stage and precise sample slide holder permits revisiting of CTCs found in a previously scanned and archived sample in order to obtain a high-resolution image, or to retrieve an identified CTC using the CytePicker module (*see* Chapter 15).

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Once a scan is completed the RareCyte software suite automatically analyzes the sample to look for CTCs. The software initially finds all "objects" on a slide that stain with the epithelial markers (cytokeratin, EpCAM), but not the white blood cell marker (CD45), and then generates a prioritization score to indicate how likely these candidate objects are "real" CTCs. This scheme minimizes the chance that a real CTC is overlooked and significantly reduces reviewing time. The results of this analysis are then presented to the user for review in the CyteMapper® software, a user interface designed to streamline the review process.

Although the protocol here describes identification of circulating epithelial cancer cells, a similar approach may be taken to identify non-epithelial tumors cells (such as circulating melanoma cells) by substituting appropriate antibody reagents for EpCAM and cytokeratins. In addition, lineage-specific markers, drug targets, and other biomarkers may also be interrogated by using only a single fluorescence channel for the epithelial marker(s) and using the "empty" channel for the biomarker of interest. Furthermore, although this chapter describes 4-channel analysis, the CyteFinder is designed to support up to six fluorescent channels to allow greater biomarker flexibility in CTC phenotypic analysis.

2 Materials

1. Sample produced by the AccuCyte CTC kit (*see* Chapter 13).
2. RareCyte CyteFinder.
3. Desktop workstation with RareCyte software.
   (a) RareCyte Instrument Controller (RCIC).
   (b) Barcodes Application.
   (c) Dashboard Application.
   (d) CyteFinder Application.
   (e) Analyzer Application.
   (f) CyteMapper Application.

3 Methods

The following is a standard workflow for scanning and analyzing a slide produced by the AccuCyte CTC kit.

3.1 Scanning Slide

1. Turn on computer and CyteFinder scanner, start RCIC, and start Dashboard.
2. Open Barcodes Application. Enter the appropriate test name for your sample (it cannot contain spaces, underscores, or

special characters) (*see* **Note 1**). Select the "Favorite" from the pull-down menu for "4D" protocol. This protocol specifies that a scan is to be preformed of an AccuCyte slide with images recorded in the DAPI channel (DAPI), FITC Channel (Cytokeratin), TRITC channel (CD45), and Cy5 channel (EpCAM). It tells the analysis software that CTCs should be found using either Cytokeratin or EpCAM as positive markers and CD45 as a negative marker. Click the **Do It** button and then the **CopyToClipboard** button.

3. Start the CyteFinder Application. Click **New Test** on the Main Function Bar.
4. Create and enter a unique User ID for yourself, then click **Continue** on the Main Function Bar (*see* **Note 1**). A Door is Now Open window will appear and the scanner door will automatically open.
5. Load the sample slide(s). Check to make sure the door path is clear and then click **Close Door**. The scanner door will automatically return to its closed position.
6. The **Barcodes** sub-tab under the **Manual** Tab will then be automatically selected. For each sample, copy the barcode from the **Barcodes Application** (using the Copy to Clipboard button) and paste into the CyteFinder's barcode input fields (using a right-click or Ctrl-V) (*see* **step 2** above). If scanning a single slide, enter the barcode in field **A** (see the above) of the CyteFinder Application. If scanning dual slides, use fields **A** and **B**. The slide listed in field **A** will be scanned first. If scanning dual slides make sure that each slide has its own unique barcode. After pasting the first barcode repeat the steps in Subheading 3.1, **step 2**.
7. Designate each slide's position in the slide holder (**Right** or **Left**) using the respective drop-down menu. The **Status** area for the slides will be automatically updated by the software.
8. Click **Continue** on the Main Function Bar. Note: all barcode entries must be done before leaving this screen.
9. Follow the directions on the **Prompt Bar**. The **Patient Tab** should automatically be selected. If not, when prompted, select the **Patient Tab** and enter Patient ID(s). If a second barcode was entered in the **Barcodes Tab**, the Slide B Patient ID entry field will also be available (*see* **Note 1**). When finished, click **Continue** on the Main Function Bar.
10. The **Test Tab** should automatically be selected next. If not, when directed by the **Prompt Bar**, select the **Test Tab**. Enter Test Notes (if desired).
11. The test parameters will autofill, depending on what was designated during barcode generation in the **Barcodes**

Application. Verify that the test setup is correct by viewing all fields currently displayed on the Test Tab. If a test parameter is incorrect, you may click **Abort Test** and begin again.

12. Click **Continue.** This will initiate the scan starting with the Pre-Focus step for the first slide.
13. After Pre-Focus is competed the scanner will enter the Active Scanning step. During this stage a progress indicator will display the current status of the scan.
14. Once all scheduled scans are completed the user will be prompted with a **Test Complete** dialog. Click Ok.
15. Click the **Open Door** button and retrieve the samples.
16. Click the **Close Door** button.

3.2 Automated Analysis

1. The Dashboard application handles starting analysis and image processing of the scans once the scan is complete.
2. Once a scan is started a line will appear in Dashboard with identification information (Serial #, Test Type, Test Date).
3. Expand the section by double clicking on the line. This will show all files associated with this scan and their status.
4. Once the analysis is complete. Select the line with the file type "Analysis" and click **Review** to open this Analysis file in the CyteMapper reviewing software.

3.3 Manually Reviewing Sample with CyteMapper

CyteMapper is a feature-rich tool for examining, reviewing, and producing reports from RareCyte scans. Below is an overview of how to use the review software to enable precise and high-speed reviewing.

1. When prompted enter your unique User ID.
2. The **Sort Setup** dialog window gives you the ability to sort the found potential CTCs by a variety of criteria (*see* **Note 2**). We recommend using the default settings. Sort by "Score (max)" descending [–] and use a "Score–Minimum" cutoff of 15 (*see* **Note 3**).
3. The reviewer is now presented with Glyphs that contain all found objects in preview mode. Glyphs may contain multiple objects. In this mode the goal is to quickly select the glyphs containing objects that are likely CTCs or merit a closer look. To facilitate the review we recommend that you click the zoom button and then click the multi-color button below it to set a color palette and contrast settings to your preferences (*see* **Note 4**).
4. Click on each Glyph that should be reviewed in further detail. Once selected it will have a **blue** border.
5. When a page has been reviewed click the **Accept This Page (as Previewed)** button in the lower right corner. Note that you

can go back to the previous page and all previewed but not selected objects will have a green border.

6. Once all pages have been previewed click the **Mode** button in the upper right once to switch to **Review** mode. In review mode each Glyph object is represented by a merged color image from all channels and grayscale images for each individual channel.
7. Each Glyph contains at least one object. Glyph Borders will be marked by a dashed white rectangle and objects will be indicated by a drawn circle and a red Object Id #. The color of the circle indicates whether the object has been marked as a Cell/CTC (white), indeterminate object (blue), or the default state of not a cell (red).
8. If an object meets all criteria of a CTC (Fig. 1) the reviewer should mark it as a CTC by clicking on the object circle (*see* **Note 5**). Note the left click action can be changed by the pull-down menu on the middle right side. The default recommended setting is **Toggle Cell/Not Cell**.
9. Once all objects on a page have been reviewed click the **Accept This Page (As Reviewed)** button and repeat the process until all selected objects have been reviewed.
10. To finish the review close the window by clicking the red X in the upper right corner of the window.

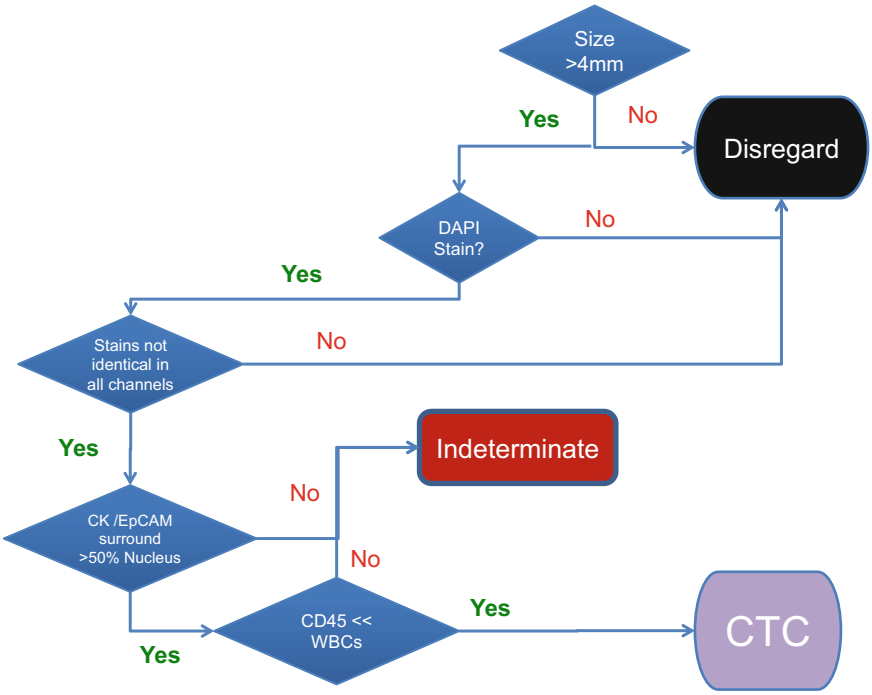

Fig. 1 A decision tree is pictured to define steps to verify that an object identified by the CyteMapper software is truly a CTC

11. When prompted click **Save and Generate** to save the results of this review and generate a PDF report of the review.

4 Notes

1. Some input fields restrict the types of characters you may type. Most invalid entries will result in an error message; however, some (i.e., spaces " " and underscores "_") may be converted to dashes "–". Restrict your entry to the following character types: (a) Letters "A" through "Z" (upper-case and/or lower-case), (b) Numeric digits "0" "9" (however, some fields may not allow a digit as the first character in a field), and (c) Dashes "–."
2. The CyteMapper software allows for Glyphs to be sorted by a number of criteria. For the initial review we generally recommend sorting by "Score (max)" descending but when looking at a previously completed review it can be helpful to sort by other things such as "Cell Count" which would place glyphs with cells at the beginning of the review. The sorting also supports nested sorting so you can sort first by "Cell Count" and then by "Score (max)" ascending which results in low scoring CTCs being displayed first followed by high scoring CTCs followed by low scoring Not CTCs followed by High Scoring Not CTCs.
3. The score for each object is an estimate of how likely it is that this object is a CTC. It is calculated from nearly 400 features measured from each object that captures information including membrane, nuclear, and cytoplasmic features. Our standard recommended score cutoff is 15. We believe that this provides a good balance between complete enumeration and reviewing speed. We have found that less than 1% of CTCs have a score of less than 15 and ~89% of non-CTC objects have scores of less than 15. Setting a cutoff of 15 lets you reduce review time by an order of magnitude while missing minimal CTCs. CTCs with scores of less than 15 tend to be edge cases that just barely meet the CTC criteria (*see* **Note 5**). In order to facilitate efficient manual reviewing we let you set the "Score–Minimum" to meet your specific needs. Refer to Table 1 to assist in making these trade-off decisions. (These data were generated from more than 750 slides from 120 clinical patients samples containing nearly 30,000 reviewer confirmed CTCs. Approximately 1.3% of all candidate objects were CTCs.)
4. The color palette that CyteMapper uses to colorize images can be customized to accommodate each user's needs and preferences. By default it assigns color based on the emission wavelength of the stains: DAPI in blue, FITC in Green, TRITC in

Table 1
Scoring performance

Score cutoff	0	5	10	15	20	30	50
Percent of CTCs at or above cutoff (mean CTCs per slide)	100% (38.3)	99.7% (38.2)	99.3% (38.0)	99.1% (37.9)	98.7% (37.8)	97.8% (37.4)	95.3% (36.5)
Percent of other objects at or above cutoff (mean non-CTCs per slide)	100% (2850)	18.4% (526)	13.0% (370)	10.8% (309)	9.4% (267)	7.2% (206)	4.0% (115)
Mean total objects per slide at or above cutoff	2888	564	408	347	305	243	151

yellow, and Cy5 in Red. These colors can be reassigned based on a reviewer's preferences/needs. The **Composite Channel Setup** dialog can be accessed by clicking on the button just below the **Zoom** button in preview mode or in review mode the button above the column of interest. Once a new channel configuration is set the configuration can be saved for future use by going to the pull-down menus at the top of the window clicking **Configure** -> **Save Chan Display Config**. A dialog will appear, enter a name for this configuration, and click **OK**. To load a saved Configuration go to the pull-down menu clicking **Configure** -> **Load Chan Display Config** and select a saved color palette. In preview mode some users find it useful to not display the CD45 channel since this is a negative selection channel.

5. The criteria for the CTC determination are outlined in Fig. 1. Overall size of CTC should be 4 μm or larger. The nuclear stain should define a structure of any size that is at least 50% contained within the cytoplasmic stain (cytokeratin, CK) or membrane stain (EpCAM). Staining features should follow the following criteria: (a) nuclear, CK, and EpCAM staining patterns must not be identical, (b) CK and/or EpCAM staining must be present, (c) CK and/or EpCAM must surround >50% of nucleus and the CK staining may be granular/globular, and (d) CD45 must be absent or noticeably lower compared to surrounding WBCs (weak autofluorescence may be present). If an object has a nucleus but does not meet the CK/EpCAM staining criteria above, then it is not a CTC.

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Chapter 15

RareCyte® CTC Analysis Step 3: Using the CytePicker® Module for Individual Cell Retrieval and Subsequent Whole Genome Amplification of Circulating Tumor Cells for Genomic Analysis

Jackie L. Stilwell, Paulina Varshavskaya, Jeffrey L. Werbin, Joshua J. Nordberg, Arturo B. Ramirez, Steve Quarre, Jay Tzucker, Jennifer Chow, Brennan Enright, and Eric P. Kaldjian

Abstract

The CytePicker module built into the RareCyte CyteFinder instrument allows researchers to easily retrieve individual cells from microscope slides for genomic analyses, including array CGH, targeted sequencing, and next-generation sequencing. Here, we describe the semiautomated retrieval of CTCs from the blood processed by AccuCyte (*see* Chapter 13) and amplification of genomic DNA so that molecular analysis can be performed.

Key words Circulating tumor cells, Single-cell analysis, Cell heterogeneity, Whole genome amplification, Single-cell retrieval, Cell picking

1 Introduction

The ability to analyze the genome of individual cells is becoming increasingly important as researchers attempt to understand molecular heterogeneity among cells (reviewed in [1]) including circulating tumor cells (CTCs) [2]. The CytePicker® module integrated with the CyteFinder® instrument enables isolation and downstream molecular analysis of individual rare cells. The CytePicker module uses a durable needle with ceramic tip to mechanically retrieve adherent cells from microscope slides after scanning by CyteFinder. This is accomplished by highly automated and precise three-dimensional calibration and hydraulic fluid flow control. Cells are collected into the needle tip and then can be deposited into RareCyte's imaging PCR tubes for visual confirmation of retrieval prior to further processing. Sophisticated software controls the operation

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of the CytePicker needle; minimal user technical skill is therefore required for single-cell retrieval, a process that takes 2–3 min. An instructional video of single-cell retrieval using the CytePicker can be found at https://www.youtube.com/watch?v=NSz18a53qSY.

Whole genome amplification (WGA) may be performed on isolated cells to permit subsequent molecular analysis. Since WGA may yield low coverage or have high sequence error rates, it is important to select an appropriate method for a particular application [3–5]. We use PicoPLEX® WGA (Rubicon Genomics) because it is compatible with our method of processing and staining cells (which includes formalin fixation) and provides adequate results for the typical molecular analyses we perform, including the detection of copy number variation by aCGH, targeted sequencing and whole genome or exome NGS [6, 7]. In order to evaluate genomic coverage by WGA we have adopted the quality control method of Leung and coworkers [3]. Individual PCR reactions using primers that target eight specific chromosomes are used to amplify the WGA product; the total number of positive reactions as measured by the presence of a specific PCR product correlates directly with extent of genome coverage.

2 Materials

Many of the materials required throughout this protocol can be purchased directly from RareCyte. Store all reagents at proper temperature as indicated on the product label. Do not use materials after the expiration date printed on the product label. Refer to the Safety Data Sheet for proper handling and disposal of any reagents.

2.1 Attach or Replace Needle

1. Sample produced by the AccuCyte® CTC kit (*see* Chapter 13).
2. RareCyte CyteFinder with CytePicker Module.
3. Desktop workstation with RareCyte software (*see* Chapter 14).
4. Needle Insertion Tool (Fig. 1a).
5. Needle Insertion Tool Cap.
6. RareCyte CytePicker needle 40 or 25 μm bore diameter. Always handle with Needle Insertion Tool.
7. UltraPure water.
8. Needle Priming tool: syringe, filter, tubing, and metal Luer fitting (Fig. 1b).

2.2 Calibrate CytePicker

1. Calibration Slide—clean SuperFrost Plus slide with finger print on top surface (*see* **Note 1**).
2. One RareCyte 24-well PCR tube holder.
3. PCR tubes.

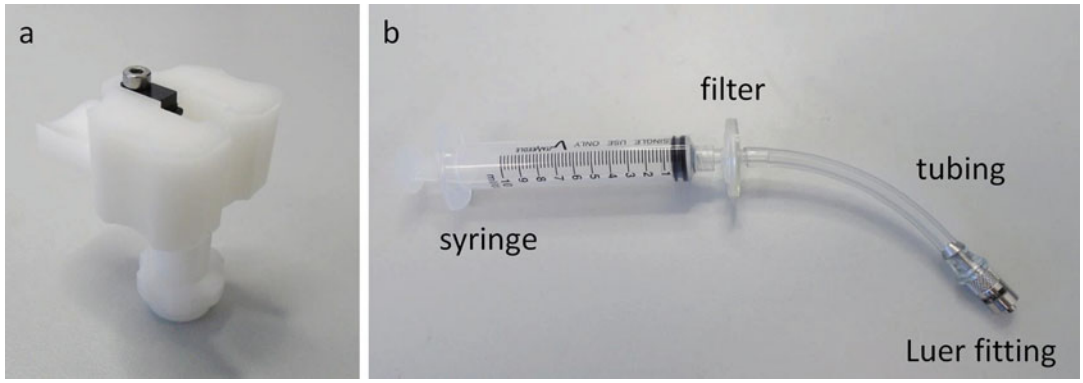

Fig. 1 (**a**) RareCyte Needle Insertion Tool. This is used to safely attach and remove the RareCyte CytePicker needle. (**b**) Needle Priming tool and its components including a 10 mL syringe, filter, plastic tubing, and a metal Luer fitting. This is used to flush and prime the RareCyte needle with UltraPure water before retrieving cells

4. RareCyte Picking Fluid.
5. PCR tube filled with 150 µL UltraPure Water.
6. PCR tube filled with 150 µL RareCyte Picking Fluid.

2.3 Deposit Cell in Imaging PCR Tube

1. RareCyte Imaging PCR tubes for individual cell deposition.

2.4 Perform WGA and Quality Control (QC) PCR

1. PCR tubes.
2. PicoPLEX WGA kit (Rubicon Genomics).
3. Thermal Cycler.
4. PCR primers for quality control (*see* **Note 2**).
5. PCR master mix (i.e., SYBR® FAST Universal qPCR kit, Kapa Biosystems).
6. E-Gel® 2% agarose gels (Thermo Fisher Scientific).
7. 100 bp DNA ladder.

3 Methods

3.1 Attach or Replace Needle

1. Open the **Imager Application** if not opened already.
2. In Imager **Picker Manual Controls** window, click the **2 µL Pump Dispense** (down) button repeatedly until the pump slider bar reaches the bottom. If there is currently a needle attached to the system move the x-y stage so that the needle is directly above a slide or CyteSlide in order to prevent dispensing liquid onto stage or optical components.
3. Click **Change Needle** in the Imager Application. The door will open automatically.

4. Remove old needle if present. Attach the Needle Insertion Tool (Fig. 1a) to the needle and twist it 180° clockwise to release the needle. Carefully remove the needle from the Needle Insertion Tool and dispose of the old needle in an appropriate biohazard sharps disposal bin.
5. Detach the syringe from the filter of the Needle Priming Tool (Fig. 1b). Draw up 5 mL UltraPure water into the syringe. Reattach the filter end to the syringe. Flush the filter with 0.5–1 mL UltraPure water.
6. Using the Needle Insertion Tool, attach a clean CytePicker Needle to the Luer fitting of the Needle Priming Tool. Remove the Needle Insertion Tool Cap.
7. Expel approximately 30 μL water through the needle. Check to make sure that the fluid stream is straight. Check to make sure a droplet forms vertically on the tip of the needle when the fluid stream is stopped and that the droplet does not move up the side of the needle. You may flick the needle to release bubbles. Tap the bottom of the priming tool on the benchtop to release any bubbles if present.
8. Attach the Needle Insertion Tool Cap to the tip end of the needle. Remove the needle from the Luer fitting of the Needle Priming Tool while depressing the syringe plunger to ensure the top of the needle is filled with fluid. Visually inspect the Luer fitting to ensure no bubbles are present.
9. Use the Needle Insertion Tool to attach the CytePicker needle to the Luer fitting on the CytePicker. Turn Needle Insertion Tool slightly clockwise until it catches the Luer fitting and then turn counterclockwise until finger tight (approximately ½ turn). Wait 10 s for any excess liquid to eject from the needle tip and collect in the Needle Insertion Tool Cap. Pull down on the Needle Insertion Tool to release it from the needle and remove. Gently wipe any excess liquid from the tip of the needle with a laboratory wipe.

3.2 Calibrate CytePicker

1. In the Imager Application, click the **Close Door** button, then click the **Load Slides** button. The door will close, then will open automatically and the slide holder tray will extend forward automatically for safer and more convenient slide manipulation.
2. Place the Calibration Slide in the left slide holder. Place the RareCyte 24-well PCR tube holder rack in the right slide holder. Load the PCR tube rack with the empty imaging PCR tubes, as well as the UltraPure water tube, and the RareCyte Picking Fluid tube (*see* **Note 3**).
3. In Imager, the locations of all tubes can be saved in the **Point List Window**. Click **View** and select **Show Point List**

Window. Depending on the software version you have, either: (1) Click the **+** button in the Point List Window and select **Add Named Point**, or (2) Click the **Add** button in the Point List Window. You can change the point name directly in its **Name** field. Depending on the software version you have, either: (1) Click the **gear** icon and select **Toggle Persistent**, or (2) Right-click on the point and select **Toggle Persistent**. This will italicize the point in the list and make it a default; it will appear in the list every time Imager is opened.

4. In the Imager **Main Window**, select the proper slide types for each slide holder. SuperFrost Plus Slide and Tube Holder respectively.
5. Drive the stage to a position over the calibration slide by double-clicking on its location in the Point List Window. Under the **Picker** tab, click **Calibration**. The Imager user interface will become disabled during calibration, and the **Picker Status Log** window will show the calibration progress. When complete, the interface will become enabled again.
6. In order to confirm the calibration procedure was successful, ensure that: (1) The message "Calibration at 10× Finished" appears in the Picker Status Log window and (2) the red circle appears in the center of the **Live Viewer** window corresponding to the bore of the picking needle. If needed, right-click and drag the red needle circle to be precisely positioned over the needle tip bore.
7. Click the **Top** button on the Picker Manual Controls window.
8. In the Point List Window, navigate to the **Needle Filling** location—the location of the PCR tube filled with RareCyte Picking Fluid, by double-clicking on it. In the Picker tab on the Main Window, click **Fill Needle**. Wait for the process to complete.
9. Drive the stage to a position over the calibration slide by clicking on its location in the Point List Window. Click **Load Slides** and extend the slide holder to remove the calibration slide and insert the sample slide in its place. Sample slide should not have a coverslip and sample side of the slide should be facing up. Click the **Lock Door** button to close the door.

3.3 Pick Cell

1. Place the sample slide in the left slide holder and the RareCyte 24-well PCR tube rack in the right slide holder (*see* **Note 4**). Load the PCR tube rack with the empty PCR tubes for sample dispensing, as well as the UltraPure water tube, and the RareCyte Picking Fluid tube.
2. Load a list of positions of previously identified cells of interest: from the Imager Application tool bar, click **File**, then **Open**. Navigate to C:\rcpnl\YOUR_FILE\, then select both the .rcpnl

and a reviewed .csv (hold Control on keyboard to select multiple items). A reviewed .csv will contain user initials in the file name. Choose the left slide holder position and the orientation (Flipped for picking—check) and click **OK**. The CytePicker software takes into account the flipping of the slide for determining location of previously identified cells.

3. If the Point List Window is not visible, click View and select Show Point List Window. Double-click on any point in the Point List Window to navigate to the coordinates of interest.
4. View cells using the **Cell Viewing Channels Window**. Use the **small camera** icon next to the channel of interest to take a single-channel picture. Use the **large camera** icon at the top of the window to take multi-channel images (all "checked" channels). Use the circular arrows button at the top to continuously acquire the last channel used.
5. Fine-tune the position of the cell of interest on the slide by clicking on the cell of interest to center it inside the circle that represents the needle bore. The stage will move such that the cell is under the red needle circle. Adjust by left-clicking on the cell as necessary. It is important: to NOT drag (right-click) the red circle to align it with the cell, always left-click on the cell to automatically center it under the circle (*see* **Note 5**).
6. **Wiggle Radius**, **Overdrive**, **Backoff**, and **Wet Volume** can be adjusted, but the default values have been optimized for retrieval of cells from SuperFrost Plus slides using 25 μm needles (*see* **Note 6**).
7. In the Picker tab of **Imager**, click **Pick Cell**. When complete, if the cell has disappeared from the view, click **Top** in the Picker Manual Control window and proceed to Subheading 3.4.
8. If the cell remains, repeat **steps 5**–7 (above).

3.4 Deposit Cell in Imaging PCR Tube

1. Confirm that the needle is in **Top** position. Drive the stage to a position over an Imaging PCR tube by clicking on its location in the **Point List** window. Activate continuous acquire in the Blue/ND2 channel and find the center of the imaging PCR tube and focus until you see the X at the bottom of the tube. Move the stage such that the red circle aligns with the center of the X. Activate continuous acquire in the UV/BV421 and Blue/A488 Cell Viewing Channel for target cell confirmation after Deposit Cell routine is completed.
2. In **Picker** tab of Imager, click **Deposit Cell**. Visually confirm target cell is visible inside the tube (*see* **Note 7**). The cell may be out of focus until it has a chance to settle to the bottom of the droplet, so adjust the focus as necessary.

3. If the cell does not appear, choose a different Imaging PCR tube and repeat **steps 1** and **2** up to three times. If the target cell still does not appear, choose a different Imaging PCR tube, change the deposit settings to **Dispense** 1000 nL, **Wait** 0 ms, **Redraw** 0 nL, and repeat **steps 1** and **2**. It is unlikely that you will be able to visualize the cell in a large volume (*see* **Note 8**).
4. If the target cell is visible in the droplet in the PCR Tube, click Top and then click Load Slides. Remove and cap the PCR Tube for downstream processing.
5. Complete the Rinsing the Needle section of this manual, then return to the Subheading 3.3 to process additional samples.

3.5 Rinsing the Needle

1. Go to the Point List window and select the position that contains the UltraPure Water tube. In the Picker Manual Control window, click **1 mm Above Slide** to lower the needle into the water. Click the **Dispense 2 µL** button several times to purge the fluid in the needle.
2. Click **Top** to bring the needle up.
3. Open the Point List window and click on the position for the Picking Fluid tube. In the Picker tab on the main window, click **Fill Needle**. Wait 10–15 s for operation to complete—Imager user interface will be grayed-out while operation is in progress. Confirm that the needle has returned to the **Top** position.
4. Return to Subheading 3.3 to pick the next cell.
5. Either freeze cells at −80 or −20 °C or go directly to Subheading 3.6 (*see* **Note 9**).

3.6 Perform WGA and QC PCR

1. If cells have been frozen directly after picking in 1 or 2 µL volume (2.5 µL max volume), add Cell Extraction Buffer up to a final volume of 5 µL to prepare Cell Sample.
2. Use PicoPLEX WGA kit to amplify the DNA from individual cells according to manufacturer's instructions (*see* **Note 10**). Include positive control as described by manufacturer. Also use a No Template Control. Add Extraction Cocktail and Pre-Amp Cocktail Mixes to the side walls of the PCR tube walls. Centrifuge to collect volume at the bottom of the PCR tube. Do not touch or mix PCR tube contents by pipetting during Cell Extraction and Pre Amp steps.
3. Use 14 cycles for amplification. Some cell types may require up to 16 cycles (See note in PicoPLEX Protocol Step #10). Monitoring WGA amplification using SYBR Green is not necessary (PicoPLEX Protocol Step #8), instead run 3 µL of final WGA reaction on a 2% EX e-gel to visualize DNA smear (Fig. 2). You may see cell-to-cell differences in the amount of WGA; likely due to the health/cell cycle of the cell.

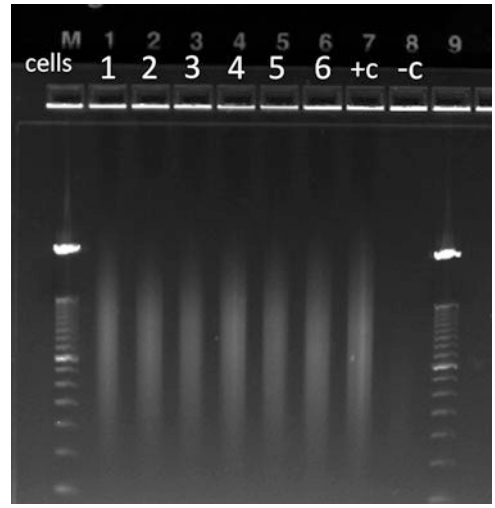

Fig. 2 A 2% agarose gel obtained with PicoPLEX WGA products from six different individual retrieved CTCs from an AccuCyte slide. These cells were positive for a nuclear stain, cytokeratin, and EpCAM, and also negative for CD45. The DNA smear represents the amplified DNA from these cells (labeled cell 1–6) and ranges in size from approximately 200–1000 bp with an average size of 500 bp. The two outer lanes contain a 100 bp DNA ladder and +c and −c represent positive and negative controls respectively. In this case the positive control is a single tissue culture cell retrieved with the CytePicker

4. QC WGA products by method of Leung and coworkers [3]. This QC uses a PCR panel of 22 primer pairs that target each chromosome independently. We use a subset recommended by paper's authors to an 8 primer pair subset to chromosomes 4, 7, 9, 12, 14, 17, 19, and 22. Add 1 μL of PCR product to each specific chromosome PCR into a new PCR tube and perform PCR as recommended in Leung et al. [3] (*see* **Note 11**).
5. When QC PCR reactions are complete, evaluate by loading approximately 3 μL on a 2% agarose gel, image and evaluate the number of positive PCR reactions (Fig. 3). Higher percentage of positive reactions correlates with better genomic coverage of the WGA reaction (*see* **Note 12**).

4 Notes

1. Ensure that the bottom glass surface of the calibration slides is clean so that the system does not calibrate to the bottom of the slide. This can result in difficulty finding the needle tip.
2. Primer sequences can be found in Table 1 and are from Leung et al. [3].

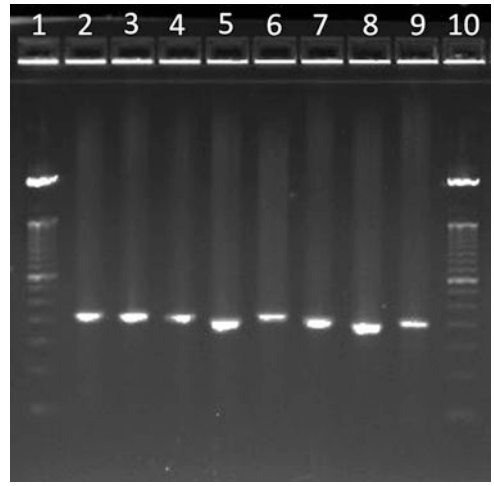

Fig. 3 A 2% agarose gel obtained using quality control PCR products representative of regions from eight different chromosomes. The PCR products are of different lengths and are all close to 200 bp. *Lanes 1* and *10* contain a 100 bp DNA ladder. *Lanes 2–9* represent PCR products from chromosomes 4, 7, 9, 12, 14, 17, 19, and 22 respectively

Table 1
PCR primer sequences for QC PCR described in Leung et al. [3]

Name	Chromosome	Orientation	Primer sequences (5′ to 3′)	Product size (bp)
chr4f	4	Forward	GAGCATCTCTTGGCTCTGCT	210
chr4r	4	Reverse	TTGGGAAAGCACAGATCCTT	
chr7f	7	Forward	CTTCCTGCCATTCCACAAGT	210
chr7r	7	Reverse	CCCACTTTCATGCCTCTGAT	
chr9f	9	Forward	CTGTGGAGCAGCTGTTTCTG	204
chr9r	9	Reverse	GAATTCACAAAGCCCCAAGA	
chr12f	12	Forward	ATCATGGAAATGCAGCCTCT	192
chr12r	12	Reverse	AGAACCCAGCTGGAATGATG	
chr14f	14	Forward	AGCCAAGACGTACCCTCTCA	208
chr14r	14	reverse	TGCTTTACACCAATCCCACA	
chr17f	17	forward	TCCTGGGCTAGCCTTTTACA	199
chr17r	17	Reverse	ATCGCTTGAGCACTGAAGGT	
chr19f	19	Forward	TACTCAAAGCTGGCAGCAGA	194
chr19r	19	Reverse	GAGCATGCCCAGGATACCTA	
chr22f	22	Forward	CTAGGATCCCGTGAAGGTCA	202
chr22r	22	Reverse	AGGTAAGGGGACTCCTTGGT	

3. It is best if one detaches the lids of the regular PCR tubes and does not attach the lids of the imaging PCR tubes so that the tube lids do not take up space on the 24-well PCR tube holder.
4. CytePicker may only be used on a sample slide without a coverslip. The sample slide must be soaked in PBS overnight to remove coverslip. The slide should be kept in a Coplin jar filled with PBS until it is ready to be loaded onto the scanner. The BOTTOM (non-sample) side of the slide should be wiped with a laboratory wipe to remove as much PBS as possible before being loaded onto the CyteFinder. The sample must be loaded with the smear side facing UP (this is opposite of how a slide is loaded when scanning).
5. If the desired cell is close to an unwanted cell, the bore of the needle (represented by the red circle) can be positioned with the desired cell slightly off center and the red circle placed between the cell of interest and the unwanted cell. The bore of the needle should separate both cells and when the cell is aspirated, often only the cell of interest is isolated. Be careful not to position the red circle too close to the nucleus of the cell of interest, as the needle edge could cut the nucleus, leaving a portion of it on the slide and losing genetic material.
6. It is possible to set these parameters to values that could damage the system; these values should be changed only if you have been trained.
7. It is easiest to find the cell if one looks carefully at the Live Viewer window as the deposit cell routine is operating. An object that is fluorescent in the blue and green channels should come out of the tip of the needle and float away as it moves in the volume of dispensed picking fluid. To find it, look in the general direction where the cell floated out of focus and move the objective in Z toward the more negative numbers (indicating focus plane is farther away from the objective). It should be able to find the cell and focus on it, allowing visualization of both a nuclear stain in blue and a cytoplasmic stain in green. If a cell is close to the edge of the tube, it may be hard to focus it and a reflection may be observed. Sometimes, this effect is reduced if one waits for the cell to fall down to the bottom surface of the imaging tube.
8. To save images of the cell before and after the picking routine, check the record images box under the picker tab and provide a destination directory and a base name for the files to be saved.
9. Cells should not be washed after picking to avoid loss.
10. Single-cell WGA reactions are highly sensitive to PCR carry-over contamination. Therefore, WGA should be performed in a designated area, preferably in a PCR setup laminar flow hood that is in a separate room and using pipets that have never

pipetted amplified DNA using low binding filtered pipet tips. All upstream processes should be performed exercising extreme caution to not contaminate tube, lid, and sample with extraneous DNA. Use DNAZap™ (ThermoFisher cat#AM9890) to treat racks, and work area before and after setting up each WGA reaction. We also expose the work area to 15 min of UV exposure before and after amplification reactions are set up. Change gloves frequently to ensure no carryover contamination from freezer handles, PCR machine buttons, centrifuge, or other surfaces. Final volume of PicoPLEX WGA reaction is 75 μL. Before beginning the protocol, ensure a 75 μL volume is entirely within the heat-block using the tube and thermal cycler you will use for the WGA.

11. Approximately 1 μL of the WGA reaction product is mixed into a 2× PCR reaction mix (SYBR® FAST Universal qPCR kit), 0.5 μM of each primer pair (Table 1) and water for each of eight chromosomes (4, 7, 9, 12, 14, 17, 19, and 22) to a final volume of 20 μL in a standard PCR tube. Reactions are placed into a thermal cycler. Both a negative control and a positive DNA control (approximately 1 ng/μL genomic DNA) are also performed. Thermal cycling conditions are as follows: (1) incubation at 95 °C for 3 min and (2) 36 cycles of 95 °C for 30 s and 60 °C for 45 s. Samples are held at 4 °C until they are analyzed by gel electrophoresis. After PCR, the presence of the appropriately sized amplicon is confirmed by loading approximately 3 μL of the reaction onto a 2% agarose gel containing SYBR® safe (Invitrogen) and comparing its migration to a 100 bp DNA ladder.
12. Typically when quality WGA is performed using tissue culture cells we see all eight PCR QC products, however with circulating tumor cells this can vary depending on the condition of the cell. If the cell is undergoing apoptosis or necrosis the genome could be partially degraded, decreasing the coverage of the WGA reaction. A good positive control to ensure that the WGA and QC PCRs are working properly is a single, healthy tissue culture cell that has been picked from a slide using the methods outlined above.

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Chapter 16

Enumeration, Dielectrophoretic Capture, and Molecular Analysis of Circulating Tumor Cells

Stephanie S. Yee and Erica L. Carpenter

Abstract

The identification of therapeutically targetable mutations in circulating tumor cells (CTCs) from cancer patient blood is increasingly used to personalize patient care. Here, we describe a novel approach for the enumeration, capture, and molecular analysis of CTCs from blood using an FDA-approved CTC enrichment and enumeration platform followed by dielectrophoretic capture and next-generation sequencing.

Key words Circulating tumor cell, Liquid biopsy, Next-generation sequencing, Personalized medicine, Whole genome amplification

1 Introduction

The molecular characterization of circulating tumor cells (CTCs) can be used to noninvasively diagnose and monitor patients suffering from cancer. The identification of therapeutically targetable mutations in CTCs is dependent on the successful enrichment, capture, and sequencing of extremely rare CTCs from patient whole blood. While the CellSearch platform is the gold standard for CTC enrichment and enumeration [1–4], this system when performed alone does not deplete a sufficient quantity of white blood cells for reliable detection of therapeutically relevant mutations in rare CTCs by next-generation sequencing (NGS). In addition, the amount of DNA available from rare CTCs is generally too low for NGS and requires whole genome amplification (WGA) [5–10]. To address these issues, we developed a novel workflow to seamlessly integrate the CTC enrichment and enumeration capabilities of the CellSearch, the CTC dielectrophoretic capture and purification capabilities of the DEPArray, WGA, and NGS of CTCs.

Mark Jesus M. Magbanua and John W. Park (eds.), *Circulating Tumor Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1634, DOI 10.1007/978-1-4939-7144-2_16,

2 Materials

2.1 CTC Enumeration

1. CellSearch CellTracks AutoPrep System, the only FDA-approved platform for detecting circulating tumor cells.
2. CellSearch Analyzer II.
3. Blood draw tube: CellSave tube.
4. CellSearch Circulating Epithelial Cell Kit.
5. CellSearch Epithelial Cell Control Kit.

2.2 CTC Enrichment

1. CellSearch CellTracks AutoPrep System.
2. Blood draw tube: BD Vacutainer® EDTA or Streck Cell-Free DNA BCT® tube (*see* **Notes 1** and **2**).
3. CellSearch Profile Kit.

2.3 Preparation of Enriched Sample for CTC Purification and Capture

1. Ultrasonic water bath (*see* **Note 3**).
2. Swinging-bucket Rotor Centrifuge for 1.5 mL tubes.
3. Pipettes: P20, P100, P200, and P1000 (*see* **Note 4**).
4. Filtered pipette tips: 0.5–20 μL, 2–100 μL, 2–200 μL, and 50–1000 μL (*see* **Note 5**).
5. 1.5 mL low protein binding tubes (*see* **Note 6**).
6. Complete cell culture media with 1% BSA: RPMI-1640, 10% fetal bovine serum, 1% L-Glutamine, 1% Penicillin-Streptomycin, 1% bovine serum albumin (BSA).
7. Staining reagents: NucBlue Live ReadyProbes Reagent, monoclonal antibodies HER2-PE and EpCAM-AlexaFluor647 (*see* **Note 7**), and CD45-AlexaFluor488.
8. Cell counter.

2.4 CTC Purification and Capture

1. DEPArray™ System, an automated instrument that can identify, quantify, and recover individual rare cells. These instructions are specific to Version 1 of the machine but can be adapted for other versions.
2. Fixed rotor micro-centrifuge for 1.5 mL tubes.
3. 200 μL tube centrifuge adapters.
4. 30 cm plate.
5. DEPArray cartridge.
6. DNA decontaminant.
7. 0.2 mL PCR microtubes with cap.
8. 200 μL Gel Loading Tips, Round.
9. PBS.

2.5 Whole Genome Amplification and Quality Control

1. PCR hood.
2. Thermal cycler.
3. Micro-centrifuge for 1.5 mL tubes.
4. Mini-centrifuge.
5. UV lamp.
6. Ice bucket.
7. Thermoconductive tube holder.
8. −20 °C freezer.
9. DNA decontaminant.
10. 70% ethanol.
11. REPLI-g Single Cell Kit (Qiagen).
12. Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies).
13. Ampli1 QC Kit (Silicon Biosystems).

2.6 TruSeq Amplicon - Cancer Panel: Library Preparation and Sequencing

1. Illumina TruSeq Amplicon - Cancer Panel.
2. Agilent Genomic DNA TapeStation.
3. 2200 TapeStation (Agilent).
4. Illumina sequencer.

3 Methods

3.1 CTC Enumeration

1. Collect two tubes of patient blood for a total of 15 mL: 7.5 mL of blood into a CellSave tube (Tube 1) and 7.5 mL of blood into a BD Vacutainer® EDTA or Streck Cell-Free DNA BCT® tube (Tube 2). Set EDTA or DNA BCT® Tube (Tube 2) aside at room temperature for use in Subheading 3.2.
2. If CellSave tube (Tube 1) will not be processed immediately, it can be stored at room temperature and processed up to 96 h after blood draw.
3. To process Tube 1, use CellSearch Circulating Epithelial Cell Kit on the CellSearch CellTracks AutoPrep System for enrichment of cells of epithelial origin in whole blood according to the manufacturer's protocol (https://www.cellsearchruo.com/sites/default/files/docs/products/CELLSEARCH-Epithelial-Cell-Kit-PI-631-50-007-1.pdf).
4. As a control for Tube 1, use CellSearch Epithelial Cell Control Kit on the CellSearch CellTracks AutoPrep System according to the manufacturer's protocol (https://www.cellsearchruo.com/sites/default/files/docs/products/CELLSEARCH-Epithelial-Cell-Control-Kit-PI-631-50-005-1.pdf).

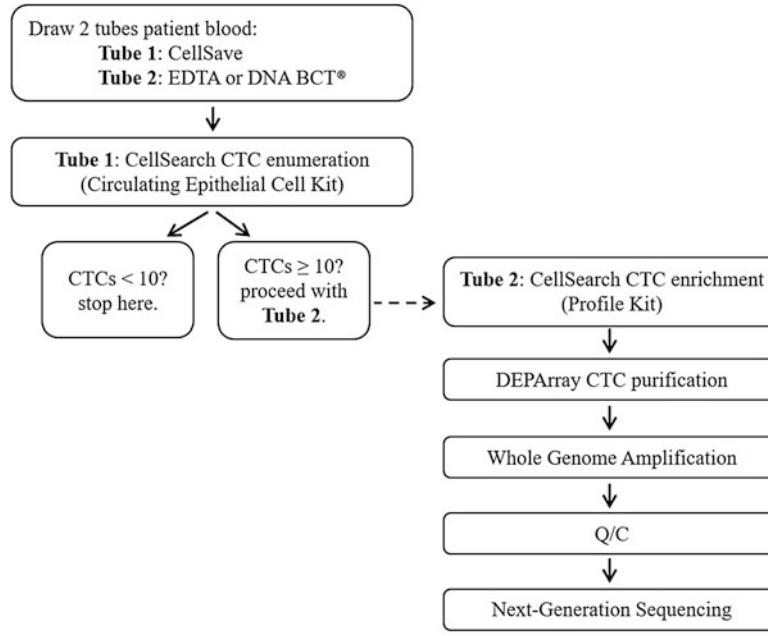

Fig. 1 Workflows used for enumeration, dielectrophoretic capture, and molecular analysis of circulating tumor cells (CTCs). The CellSearch is first used for CTC enrichment and enumeration (*black arrows*). If there are a sufficient number of CTCs from one 7.5 mL sample of whole blood, the CellSearch is used for CTC enrichment and the DEPArray used for CTC purification and capture (*dotted arrow*)

5. If there are ten or more CTCs from Tube 1, proceed with CTC isolation using the EDTA or Cell-Free DNA BCT® blood tube (Tube 2) and CellSearch Epithelial Cell Profile Kit (Fig. 1).

3.2 CTC Enrichment

The DEPArray cartridge used in Subheading 3.4 has a capacity of only 40,000 cells. Given that a 7.5 mL tube of blood contains billions of cells, pre-enrichment of the sample on the CellSearch is required prior to staining and processing on the DEPArray. Many fixatives, such as the fixative contained in the CellSave tube used in Subheading 3.1 for enumeration (above), can induce nucleic acid cross-linking thereby damaging DNA [11], can increase WGA error rate [7], or can be incompatible with commercially available WGA approaches, such as Qiagen REPLI-g used in this workflow. Therefore, the CellSearch-enumerated and enriched cells from Subheading 3.1 above cannot be used for any of the steps listed below. Instead, a second tube of blood, drawn in either an EDTA or Cell-Free DNA BCT® blood tube (*see* **Note 2**) is required for CTC enrichment, prior to WGA and NGS.

1. Using the EDTA or DNA BCT® tube of blood, process EDTA tubes within 24 h, and DNA BCT® tubes within 72 h of blood draw.
2. Use CellSearch Epithelial Cell Profile Kit on the CellSearch CellTracks AutoPrep System for enrichment of cells of epithelial origin in whole blood according to the manufacturer's protocol (https://www.cellsearchruo.com/sites/default/files/docs/products/CELLSEARCH-Profile-Kit-PI-7900004.pdf).

3.3 Preparation of Enriched Sample for CTC Purification and Capture

Perform all staining and washing steps in low protein binding tubes to minimize cell loss.

1. Using a 1 mL pipette tip, transfer the sample from the 15 mL CellSearch AutoPrep tube (included in CellSearch Profile Kit) to a 1.5 mL low protein binding tube.
2. Wash the CellSearch AutoPrep tube with two aliquots of 1.5 mL of cell culture media.
3. Centrifuge the sample in the low protein binding tube at 3000 × *g* for 5 min.
4. Resuspend cell pellet in 100 μL complete cell culture media with 1% BSA.
5. Stain cells with 4 μL NucBlue Live ReadyProbes Reagent, monoclonal antibodies 5 μL HER2-PE and 5 μL EpCAM-AlexaFluor647 to detect cells of interest (HER2+/EpCAM+/CD45-negative) (*see* **Note 7**), and 5 μL CD45-AlexaFluor488 to distinguish white blood cells.
6. Incubate for 20 min at room temperature in the dark.
7. Add 400 μL complete cell culture media with 1% BSA.
8. Remove 10 μL of sample to count cell density.
9. While counting, add 1 mL complete cell culture media with 1% BSA to the sample tube (bringing the total volume up to 1.5 mL in the 1.5 mL low protein binding tube).
10. Centrifuge the sample in the low protein binding tube at 3000 × *g* for 5 min.
11. Resuspend cell pellet: For <40,000 cells, resuspend cells in 13 μL (for DEPArray version 1.3 cartridges) of complete cell culture media with 1% BSA. For >40,000 cells, resuspend in an appropriately larger volume and take out 13 μL (for DEPArray version 1.3 cartridges) to load in the DEPArray cartridge; the number of cells in 13 μL should not be greater than 40,000 cells. Any remaining cells in excess of 40,000 can be processed in a second DEPArray run if desired.

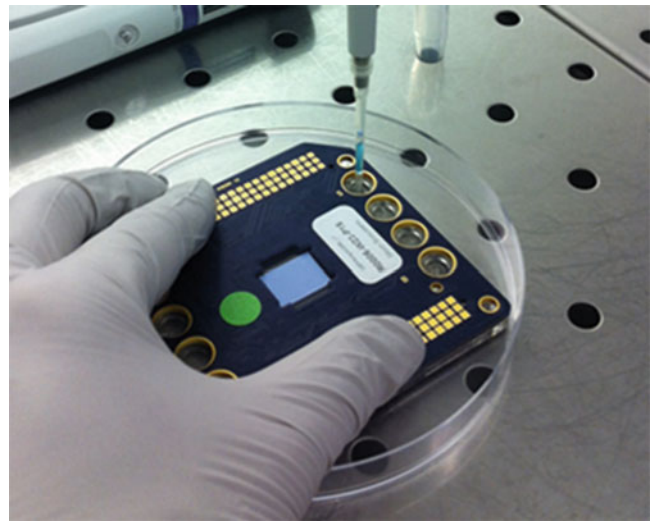

Fig. 2 Injecting sample into the "S" (for sample) port on the DEPArray cartridge

3.4 CTC Purification and Capture

1. Place DEPArray cartridge inside a new, sterile 30 cm plate (Fig. 2).
2. Using a 1000 μL Eppendorf pipette tip, load 830 μL of the cell culture medium into the "B" (for buffer) port on the DEPArray cartridge.
3. Observe DEPArray cartridge at eye level to inspect for air bubbles along the buffer tunnel.
4. Using a 20 μL Eppendorf pipette tip, load 13 μL of the sample into the "S" (for sample) port on the DEPArray cartridge (Fig. 2).
5. Observe DEPArray cartridge at eye level to inspect for air bubbles along the sample tunnel.
6. After the cartridge is placed in the DEPArray apparatus, non-uniform electrical fields are applied to isolate single cells (see the manufacturer's website for the detailed rare cell purification protocol: www.siliconbiosystems.com).
7. Prior to recovery, visually inspect all cells in the Cell Browser (Fig. 3).
8. Capture cell pools (*see* **Note 8**) into 0.2 mL microtubes.
9. Centrifuge the microtubes at 300 rcf in a fixed rotor microcentrifuge at 4 °C for 30 s for one cell, or 10 min for two or more cells per microtube (*see* **Note 9**).
10. Using 200 μL gel-loading tips, carefully remove the following volumes of supernatant without disturbing the cell pellet: 20 μL for 2–20 cells, or 95 μL for 21 or more cells (*see* **Note 10**).

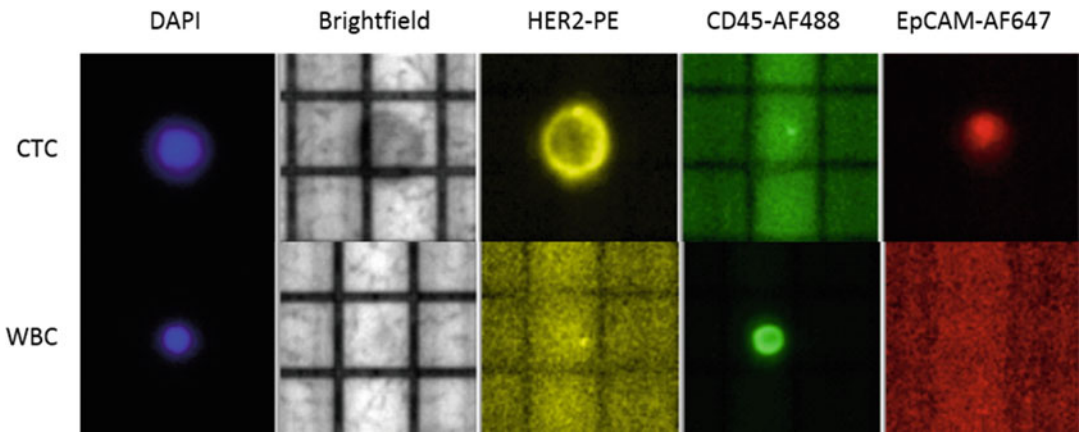

Fig. 3 DEPArray-isolated single cells from a DNA BCT® tube containing normal donor blood spiked with MDA-MB-453 breast cancer cell line cells

11. Add 100 μL of PBS to each microtube without disturbing the cell pellet.
12. Centrifuge the microtubes at 300 rcf in a fixed rotor micro-centrifuge at 4 °C for 15 min (*see* **Note 9**).
13. Remove the supernatant from each microtube, leaving the cells in approximately 5 μL of volume.
14. Immediately flash freeze the microtubes on dry ice and store at −80 °C until further processing.
15. Use DNA decontaminant to clean the removable rack and DEPArray system.

3.5 Whole Genome Amplification and Quality Control

All WGA and Q/C steps should be performed in separate dedicated clean rooms. Each clean room should be equipped with a PCR hood, thermal cycler, micro-centrifuge for 1.5 mL tubes, mini-centrifuge, UV lamp, ice bucket, thermoconductive tube holder, and −20 °C freezer.

1. Put on single-use disposable gown, new foot covers, and fresh gloves prior to entering the clean room area each time.
2. Prior to conducting WGA, wipe down all surfaces with DNA decontaminant followed by 70% ethanol.
3. Turn on benchtop UV lamp and UV lamp in PCR hood for 20–30 min. Place ice bucket and cooled thermoconductive tube holder under the benchtop lamp for maximum UV effect.
4. Turn off UV lamps and conduct WGA using the REPLI-g Single Cell Kit according to the manufacturer's protocol (https://www.qiagen.com/us/resources/resourcedetail?id=38faca1c-64b0-4281-aab3-aa8324bbd181&lang=en) (*see* **Note 11**).

5. Quantify DNA concentration in the WGA product of captured cells using Picogreen direct fluorescent staining according to the manufacturer's protocol and as previously described [7].
6. In a separate dedicated clean room to prevent carryover of amplified DNA, conduct multiplex PCR of four control genes using the Ampli1 QC Kit according to the manufacturer's protocol and as previously described [7].
7. Immediately following each WGA or Q/C experiment, open the thermocycler lid and turn on UV light for 20–30 min to disinfect the wells.

3.6 TruSeq Amplicon - Cancer Panel: Library Preparation and Sequencing

1. Use the Illumina TruSeq Amplicon—Cancer Panel (TSACP, FC-130-1008; Illumina) to capture mutational hotspots and surrounding exonic regions for 50 therapeutically relevant cancer-associated genes.
2. Use the Agilent Genomic DNA TapeStation (Agilent Technologies) to assess DNA quality according to the manufacturer's protocol and as previously described [10].
3. Load DNA into a 2200 TapeStation (Agilent Technologies).
4. Follow the Illumina guide to prepare the sequencing libraries.
5. Always use a no template sample as a negative control.
6. Sequence libraries on an Illumina sequencer using 2 × 185 base pair reads.
7. Analyze sequencing data using a clinically validated bioinformatics pipeline such as previously described [10, 12].

4 Notes

1. EDTA tubes need to be processed within 24 h of blood draw to minimize cell degradation. DNA BCT® tubes, on the other hand, enhance cell stability and viability 3 days or longer [10, 13, 14], thus providing the opportunity to batch clinical samples.
2. DNA BCT® tubes contain a proprietary preservative that is compatible with REPLI-g WGA and NGS [10].
3. The ultrasonic water bath is used to degas the buffer and media prior to transferring to the cartridge.
4. The DEPArray cartridges were designed to perfectly fit Eppendorf tips. Using the Eppendorf products prevents introducing bubbles to the cartridge.
   (a) P20 pipet (Light Grey operating button) (Item 3120000097).
   (b) P100 pipet (Yellow operating button) (Item 3120000046).

(c) P200 pipet (Yellow operating button) (Item 3120000054).

(d) P1000 pipet (Blue operating button) (Item 3120000062).

5. The following epTIPS LoRetention dualfilter pipette tips are recommended for use with the Eppendorf pipettes:
   (a) 0.5–20 μL (Item # 022493002).
   (b) 2–100 μL (Item # 022493006).
   (c) 2–200 μL (Item 022493014).
   (d) 50–1000 μL (Item 022493008).
6. Low protein binding tubes are recommended to reduce cell loss.
7. User should select monoclonal antibodies based on the cells of interest.
8. Cells can be captured in pools of up to 665 CTCs.
9. To reduce cell loss during supernatant removal, place the microtubes in the micro-centrifuge with the tube caps in the same orientation with respect to the center of the micro-centrifuge.
10. To reduce chances of sample contamination, perform supernatant removal in a biosafety cabinet.
11. WGA product can be used as input for a number of different variant detection approaches, including next-generation sequencing, digital PCR, and Sanger sequencing.

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Chapter 17

Enumeration of Circulating Tumor Cells and Disseminated Tumor Cells in Blood and Bone Marrow by Immunomagnetic Enrichment and Flow Cytometry (IE/FC)

Mark Jesus M. Magbanua, Tulasi I. Solanki, Andrea D. Ordonez, Feng Hsiao, and John W. Park

Abstract

Enumerating circulating tumor cells (CTCs) in blood and disseminated tumor cells (DTCs) in bone marrow has shown to be clinically useful, as elevated numbers of these cells predict poor clinical outcomes. Accurate detection and quantification is, however, difficult and technically challenging because CTCs and DTCs are extremely rare. We have developed a novel quantitative detection method for enumeration of CTCs and DTCs. Our approach consists of two steps: (1) EPCAM-based immunomagnetic enrichment followed by (2) flow cytometry (IE/FC). The assay takes approximately 2 h to complete. In addition to tumor cell enumeration, IE/FC offers opportunities for direct isolation of highly pure tumor cells for downstream molecular characterization.

Key words Circulating tumor cells, Disseminated tumor cells, Immunomagnetic enrichment, Flow cytometry, Enumeration

1 Introduction

Enumeration studies using the CellSearch system have shown that the presence of circulating tumor cells (CTCs) in the blood of cancer patients correlates with increased risk of progression and reduced overall survival [1]. To date, the CellSearch system is the only platform that has received a US Food and Drug administration (FDA) clearance for enumeration of CTCs in patients with metastatic disease [2–4]. Another component of "liquid biopsy" is the DTCs found in the bone marrow of cancer patients. Like CTCs, studies have shown that presence of DTCs portends poor survival [5]. A major limitation of CellSearch is that the assay is not optimized for detection and enumeration of DTCs. To address this concern, we have developed a novel method for quantitative detection of both CTCs and DTCs. Our protocol, referred to as IE/FC,

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entails two major steps: (1) EPCAM-based immunomagnetic enrichment followed by (2) flow cytometric analysis to detect EPCAM-positive cells. Head-to-head comparison of CellSearch and IE/FC has shown high concordance of CTC counts with similar prognostic value [6]. Another advantage of IE/FC over CellSearch is that our assay can be utilized for high-purity isolation of tumor cells from blood via fluorescence-activated cell sorting (FACS) [7–10]. Also, our previous studies have shown that tumor cells isolated using our method are amenable for high-content genomic analyses [9, 10].

Detection of CTCs and DTCs is challenging since they are present in very low concentrations. The initial step of our assay uses magnetic separation to concentrate tumor cells while removing most of the blood cells in the background. Iron beads (ferrofluid nanoparticles) coated with a monoclonal antibody against EPCAM are added to whole blood or bone marrow, and the sample is subjected to a magnetic field to capture EPCAM-positive cells. Another round of magnetic separation is performed to further reduce the levels of contaminating blood cells. Since the resulting admixture (tumor-enriched sample) still contains blood cells in addition to possible CTCs/DTCs , a second step using flow cytometry is performed to distinguish tumor cells from normal blood. Prior to flow cytometric analysis, the tumor-enriched sample is stained with a nucleic acid-specific dye along with fluorochrome-conjugated monoclonal antibodies against (1) EPCAM (distinct from the monoclonal antibody used for IE) to detect CTC/DTCs, and (2) CD45 to detect cells of hematopoietic lineage. The FC step allows for the accurate counting of CTCs and DTCs, defined as nucleated cells that are EPCAM-positive, and CD45-negative. The procedure takes approximately 2 h from tumor enrichment to cell enumeration.

2 Materials

2.1 Blood and Bone Marrow Collection

1. EDTA tube, collection tubes containing ethylenediaminetetraacetic acid (EDTA) (10 ml, 16 × 100 mm) (*see* **Note 1**).
2. Cell strainer (70 μm mesh size) for bone marrow sample.

2.2 Immunomagnetic Enrichment

2.2.1 Equipment and Consumables

1. Large and small magnetic separator (*see* Fig. 1).
2. Peristaltic pump and aspiration setup.
3. 50 ml conical tubes.
4. Round-bottom tubes (12 × 75 mm).
5. 7 ml glass tubes (13 × 100 mm).
6. Vortex mixer.
7. 10, 5, and 2 ml non-pyrogenic serological pipettes.
8. P20, P200, P1000 pipettes.
9. P20, P200, P1000 pipet tips.

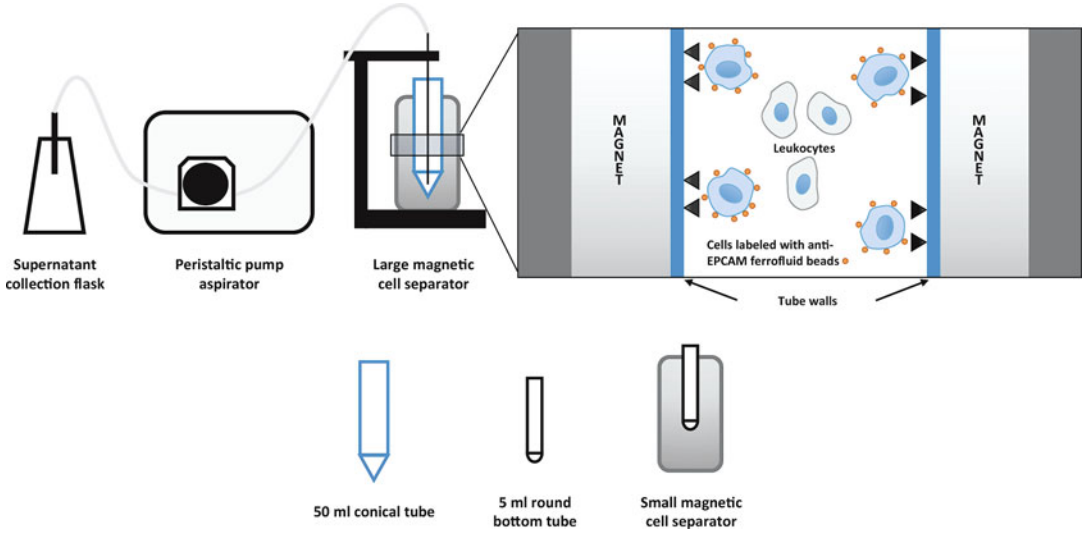

Fig. 1 Schematic diagram of the immunomagnetic enrichment platform. A sample in a 50 ml conical tube is first diluted with the blocking buffer, then ferrofluids and the EPCAM stains are added. Following incubation, the sample is placed into the large magnetic cell separator to capture cells that are labeled with ferrofluid beads. For smaller input volumes (5 ml or less), the sample can be transferred into round-bottom tubes, and a small magnetic cell separator can be used instead. Next, a peristaltic pump system is used to aspirate the unbound cells (supernatant). In this setup, the aspirator needle is attached to a fluidics line connected to the peristaltic pump used to control the flow rate. After aspiration, the cells (CTCs/DTCs plus normal blood cells) are then resuspended in the blocking buffer, and the sample is subjected to another round of enrichment using the small magnetic cell separator. The supernatant is once again aspirated, followed by the addition of the blocking buffer, the nucleic acid dye and the CD45 (leukocyte-specific) cocktail. After an incubation period in the dark, the sample is ready for flow cytometry. [Modified from Methods, Volume 64, Issue 2, Mark J Magbanua & John W Park, "Isolation of circulating tumor cells by immunomagnetic enrichment and fluorescence-activated cell sorting (IE/FACS) for molecular profiling", 114–118, 2013, with permission from Elsevier]

2.2.2 Reagents, Buffers, and Antibodies

Store stock and working aliquots at 4 °C. Except for the phosphate buffered saline (PBS) buffer, all reagents and antibodies are special ordered from BD Biosciences.

1. Blocking buffer: 1× casein buffer for flow cytometry.
2. Ferrofluid: anti-EPCAM (MJ37) conjugated to iron beads, 0.5 mg/ml.
3. EPCAM stain: Phycoerythrin (PE)-conjugated anti-EPCAM (EBA-1) monoclonal antibody (5 μg/ml).
4. Nuclear and leukocyte-specific stain cocktail: Prepare cocktail by mixing equal volumes of thioflavin (0.006 μg/μl) and PerCP-Cy5.5-conjugated anti-CD45 (2D1) monoclonal antibody (0.2 μg/μl).
5. 1× PBS buffer. Store at room temperature.

2.3 Enumeration by Flow Cytometry

1. Absolute counting tubes (e.g., Trucount tubes, BD Biosciences #340334).

2. 5 ml flow cytometry tubes with cell strainer cap (35 μm mesh size).
3. BD FACSCalibur™ or similar flow cytometer.
4. BD CellQuest™ software or similar flow cytometry data acquisition and analysis program.
5. Vortex mixer.

3 Methods

Carry out all procedures at room temperature in a biosafety cabinet. Wear proper personal protective equipment. All reagents must be brought to room temperature prior to use.

3.1 Immunomagnetic Enrichment of CTCs from Peripheral Blood

To maximize cell capture yield, the steps outlined below should be done continuously without significant delays.

1. Using a serological pipette, transfer 10 ml of whole blood from the EDTA tube to a 50 ml conical tube.
2. Dilute the sample by adding 5 ml of the blocking buffer (*see* **Note 2**).
3. Add 240 μl of ferrofluid and 400 μl of EPCAM stain (*see* **Note 3**). Mix by swirling.
4. Incubate the sample for 15 min at room temperature (*see* **Note 4**).
5. Place the mixture in the large magnetic separator for 45 min (*see* **Note 5**).
6. Aspirate the supernatant (containing the unbound cells) while the tube is still in the magnet (*see* **Notes 6** and **7**). After aspiration, remove the tube from the magnetic separator.
7. Use a serological pipette to resuspend the bound cells with 2 ml of blocking buffer (*see* **Note 8**). Briefly vortex the tube before proceeding to the next step.
8. Transfer the entire volume of the blocking buffer containing the resuspended cells into a 12 × 75 mm round-bottom tube.
9. Place the tube in the small magnetic separator for 5 min.
10. Aspirate the supernatant as in **step 6**.
11. Resuspend the bound cells into 150 μl of the blocking buffer following the technique presented in **step 7** and **Note 8**. Mix briefly using a vortex mixer.
12. Add 20 μl of the nuclear and leukocyte-specific stain cocktail (*see* **Note 3**). Flick the tube gently to mix and incubate for 15 min in the dark.
13. Following the 15 min incubation period, add 350 μl of 1× PBS to the sample.

3.2 Immunomagnetic Enrichment of DTCs from Bone Marrow

The steps involved in immunomagnetic enrichment of DTCs are similar to that of CTCs but with modifications as outlined below:

1. Place a cell strainer onto a 50 ml conical tube and moisten the filter with 100 μl blocking buffer. Using a serological pipette, pass 5 ml of bone marrow from the EDTA tube through the cell strainer. Wash the cell strainer with 2.4 ml of the blocking buffer, and transfer 6 ml of the diluted bone marrow into a 7 ml glass tube.
2. Add 96 μl of ferrofluid and 160 μl of EPCAM stain to the sample. Mix by inverting the tube.
3. Incubate the sample for 15 min at room temperature (*see* **Note 4**). Place the mixture in the small magnetic separator for 15 min (*see* **Note 5**).
4. Follow **steps 6**, **7**, **9–13** in Subheading 3.1. Skip **step 8** as the sample is already in a tube of appropriate size for the small magnetic separator.

3.3 Enumeration by Flow Cytometry

1. Immunomagnetically enriched samples are then subjected to flow cytometric analysis using the BD FACSCalibur machine. Set up the instrument following manufacturer's instructions. *See* **Note 9** and Figs. 2 and 3 for gating strategies.
2. Prior to starting enumeration, make sure to check the levels of the sheath fluid and waste compartments of the machine.
3. Flick the tube to mix the sample well. If large particles are visible, filter the sample using a 5 ml flow cytometry tube with a cell strainer cap.
4. Transfer the tumor-enriched sample (approximately 520μl) from the round-bottom tube into an absolute counting tube.
5. Vortex the sample briefly before placing it in the machine to dissolve the counting beads and resuspend the cells at the bottom of the tube.
6. Load tube into the machine. Using the CellQuest software, collect 35,000 beads to obtain the absolute count of CTCs or DTCs in the sample (*see* **Note 10**).

4 Notes

1. Leave the collection tubes containing blood and bone marrow samples at ambient temperature prior to processing.
2. The amount of reagents used for immunomagnetic enrichment was calculated based on 10 ml and 5 ml inputs of blood and bone marrow, respectively. Adjust proportionally for other input volumes.
3. Fluorochrome-conjugated antibodies should be protected from direct light by wrapping the tubes containing working aliquots with aluminum foil.

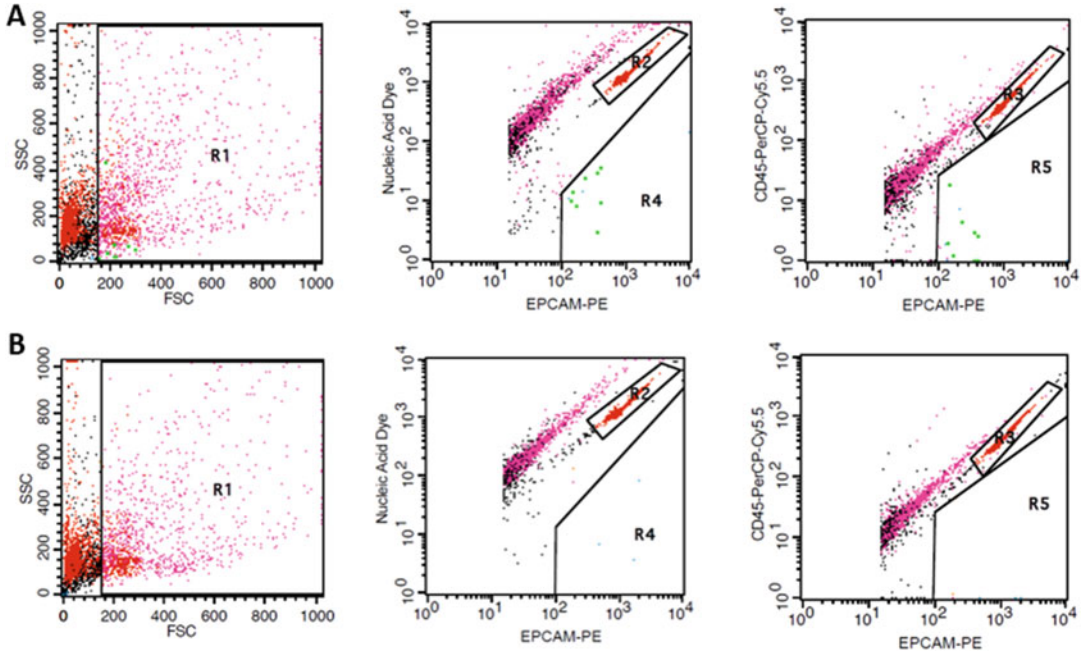

Fig. 2 Flow cytometric analysis of tumor-enriched blood samples from breast cancer patients to detect circulating tumor cells (CTCs). Representative flow cytometric results from patients (**a**) with detectable CTCs and (**b**) with no detectable CTCs. Gate R1 in the forward scatter (FSC) vs. side scatter (SSC) plot is used to exclude debris present in the sample. Gates R2 and R3 show the BD Trucount beads. The *green dots* in gate R4 (EPCAM-PE vs. Nucleic Acid Dye plot) and in gate R5 (EpCAM-PE vs. CD45-PerCP-Cy5.5 plot) indicate CTCs detected in the sample. CTCs are defined as nucleated, EPCAM-positive, and CD45-negative cells, and must be present in gates R1, R4, and R5. Forward scatter (FSC) vs. side scatter (SSC) are plotted on a linear scale while fluorescence intensities for EPCAM-PE, Nucleic Acid Dye, CD45-PerCP-Cy5.5 are plotted on a log scale

4. To ensure proper mixing of reagents in the sample, mix by swirling (or inverting the tube if processing bone marrow) the tube at halfway point, and at the end of the incubation period.
5. During this step, cells decorated with the ferrofluid beads are physically pulled to the walls of the tube by the magnet (*see* Fig. 1). To maximize cell capture yield, it is important to leave the tube undisturbed during this incubation step.
6. Before aspiration, turn on the peristaltic pump in order to stabilize the flow rate to about 1 ml per 30 s. Adjust the flow rate by aspirating 1× PBS while fine-tuning the speed setting of the peristaltic pump. Running 1× PBS through the system also flushes the fluidics line to avoid contamination from any prior use.
7. Aspirate the supernatant at a rate of about 1 ml per 30 s. Avoid touching the sides of the tube when inserting the aspiration needle, so as not to disturb the cells that are magnetically attached to the walls.
8. This washing step is associated with the biggest potential loss of target cells. It is important to wash the sides of the tube

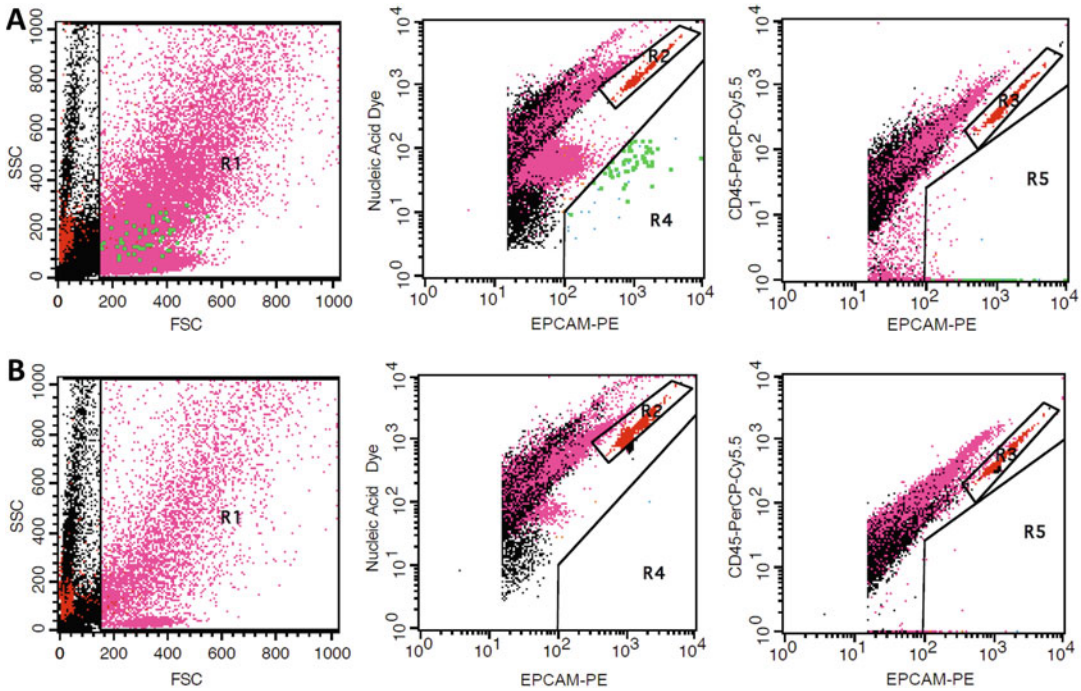

Fig. 3 Flow cytometric analysis of tumor-enriched bone marrow samples from breast cancer patients to detect disseminated tumor cells (DTCs). Representative flow cytometric results from patients (**a**) with detectable DTCs and (**b**) with no detectable DTCs. Gate R1 in the forward scatter (FSC) vs. side scatter (SSC) plot is used to exclude debris present in the sample. Gates R2 and R3 show the BD Trucount beads. The *green dots* in gate R4 (EPCAM-PE vs. Nucleic Acid Dye plot) and in gate R5 (EpCAM-PE vs. CD45-PerCP-Cy5.5 plot) indicate DTCs detected in the sample. DTCs are defined as nucleated, EPCAM-positive, and CD45-negative cells, and must be present in gates R1, R4, and R5. Forward scatter (FSC) vs. side scatter (SSC) are plotted on a linear scale while fluorescence intensities for EPCAM-PE, Nucleic Acid Dye, CD45-PerCP-Cy5.5 are plotted on a log scale

properly to collect as many cells to the bottom of the tube as possible. This can be accomplished by maintaining a constant and low dispensing rate when adding the blocking buffer, while simultaneously rotating the tube. Allow the buffer to drain to the bottom and then draw the buffer back into the pipette and repeat the whole process.

9. During flow cytometric analysis, CTCs or DTCs are identified based on light scattering properties and fluorescence patterns that are displayed real-time in a series of two-dimensional scatter plots. The optimized gating strategies to distinguish CTCs and DTCs from hematopoietic cells are shown in Figs. 2 and 3, respectively. The gate for forward and side scatter, shown as R1, is used for initial selection of target events and to exclude debris. Each event is also interrogated for fluorescence signal intensities of the EPCAM and CD45 (leukocyte-specific) stains, as well as the nucleic acid dye. The R4 gate selects for nucleated cells (nucleic acid dye-positive) that are EPCAM-positive, while the R5 gate selects for

EpCAM-positive and CD45-negative cells. CTCs and DTCs must be present within gates R1, R4, and R5, and are defined as EpCAM-positive, CD45-negative and nucleated cells.

10. Absolute counting tubes are used to determine the number of CTCs or DTCs in a sample. If using BD Trucount tubes, count only up to 35,000 of the total number of beads (approximately 48,000 depending on the lot) to avoid large debris/residue at the bottom of the tube that can potentially clog the machine and severely affect accuracy of cell counts, and perhaps, even result in sample loss. Also, make sure to use the bead count from the current lot. To calculate the total number of CTCs or DTCs, first divide the number of beads counted (i.e., 35,000) by the total number of beads (based on the current lot) in the Trucount tube, and then multiple the resulting ratio by the number of initial CTC/DTC counts. Divide the total of CTCs or DTCs by the volume of blood or bone marrow inputs, respectively, to obtain tumor cells per ml.

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Chapter 18

Flow Cytometric Detection of Circulating Tumor Cells Using a Candidate Stem Cell Marker, p75 Neurotrophin Receptor (p75NTR)

Tomoyuki Okumura, Tetsuji Yamaguchi, Toru Watanabe, Takuya Nagata, and Yutaka Shimada

Abstract

The most widely studied detection for circulating tumor cells (CTCs) in peripheral blood of cancer patients has been based on immunomagnetic enrichment using antibodies against epithelial cell adhesion molecule (EpCAM), which is overexpressed in epithelial cells. A neurotrophin receptor p75 (p75NTR) is expressed in a candidate stem cell fraction in esophageal squamous cell carcinoma (ESCC), which shows significantly higher colony formation, enhanced tumor formation in mice, along with strong expression of epithelial mesenchymal transition-related genes. Here, we describe a method to detect CTCs in ESCC based on the combined expression of EpCAM and p75NTR using flow cytometry, demonstrating the feasibility of expression analysis of multiple cell surface markers in viable cells.

Key words Esophageal cancer, Circulating tumor cells, Cancer stem cells, Flow cytometry, p75NTR

1 Introduction

Despite advances in its diagnosis and multimodal therapies, the prognosis for esophageal squamous cell carcinoma (ESCC) patients remains poor, due to high incidences of metastasis [1, 2]. In recent years, reports have demonstrated circulating tumor cells (CTCs) as an early detection marker for cancer metastasis [3]. The most widely studied CTC detection method is based on immunomagnetic enrichment using antibodies against epithelial cell adhesion molecule (EpCAM), which is broadly expressed in the most of epithelial cells and carcinomas [4, 5], in combination with 4′,6-diamidino-2-phenylindole (DAPI) staining, which requires fixation of the cells [6]. On the other hand, recent studies have provided evidence of cancer stem cells (CSCs) that represent a small number of cancer cells with stem cell properties, such as self-renewal, tumorigenicity, and drug resistance [7, 8]. Thereafter, reports also

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suggested the clinical significance of circulating tumor stem cells (CTSCs) which represent a subpopulation of CTCs with CSC properties [9]. Compared with CTCs, CTSCs might be a more accurate prognostic factor because CSCs are responsible for metastasis through processes such as epithelial mesenchymal transition, invasion into vessels, circulation, and tumor initiation in the metastatic sites [10–12].

A neurotrophin receptor p75 (p75NTR) is expressed in a candidate stem cell fraction in esophageal squamous cell carcinoma (ESCC), which shows significantly higher colony formation, enhanced tumor formation in mice, along with stronger expression of epithelial mesenchymal transition-related genes [13–15].

We have established a protocol for flow-cytometric detection of p75NTR-positive candidate CTSCs [16]. Our accuracy assay using ESCC cell lines (KYSE series), which express both EpCAM and p75NTR, demonstrated successful detection of the EpCAM+p75NTR+ cancer cells) when mixed with peripheral blood samples of healthy volunteers, with a slope of 0.71 (95% CI, 0.68–0.74) and 0.71 (95% CI, 0.67–0.74) in regression analysis, respectively (Fig. 1). Then investigation using peripheral blood samples obtained from 23 ESCC patients (Fig. 2) demonstrated that EpCAM+p75NTR+, but not EpCAM+p75NTR− CTC counts, correlated with clinically diagnosed distant metastasis and pathological venous invasion in surgically resected primary ESCC tumors, suggesting the highly invasive and metastatic potential of CTCs with p75NTR expression. In addition, malignant cytology was microscopically confirmed in isolated EpCAM+p75NTR+ cells with immunocytochemical double staining (Fig. 3).

Here, we describe a detailed protocol to detect a candidate CTSC fraction in ESCC based on the combined expression of EpCAM and p75NTR using flow cytometry.

2 Materials

2.1 Blood Sample Preparation

1. Disposable syringes 10 mL.
2. 22G needle, 18G needle.
3. 3 mL ethylene-diamine-tetra-acetic acid (EDTA)-2K Vacutainer Tubes 5 mL tube containing anticoagulant with EDTA.
4. Phosphate buffered saline (PBS): 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM NaH_2PO_4, pH 7.4.
5. Fetal bovine serum (FBS).
6. 15 mL centrifuge tube.
7. LymphoPrep™ (Nycomed AS, Oslo, Norway).
8. A swing-out rotor centrifuge (800 × *g*).
9. Pasteur pipette.

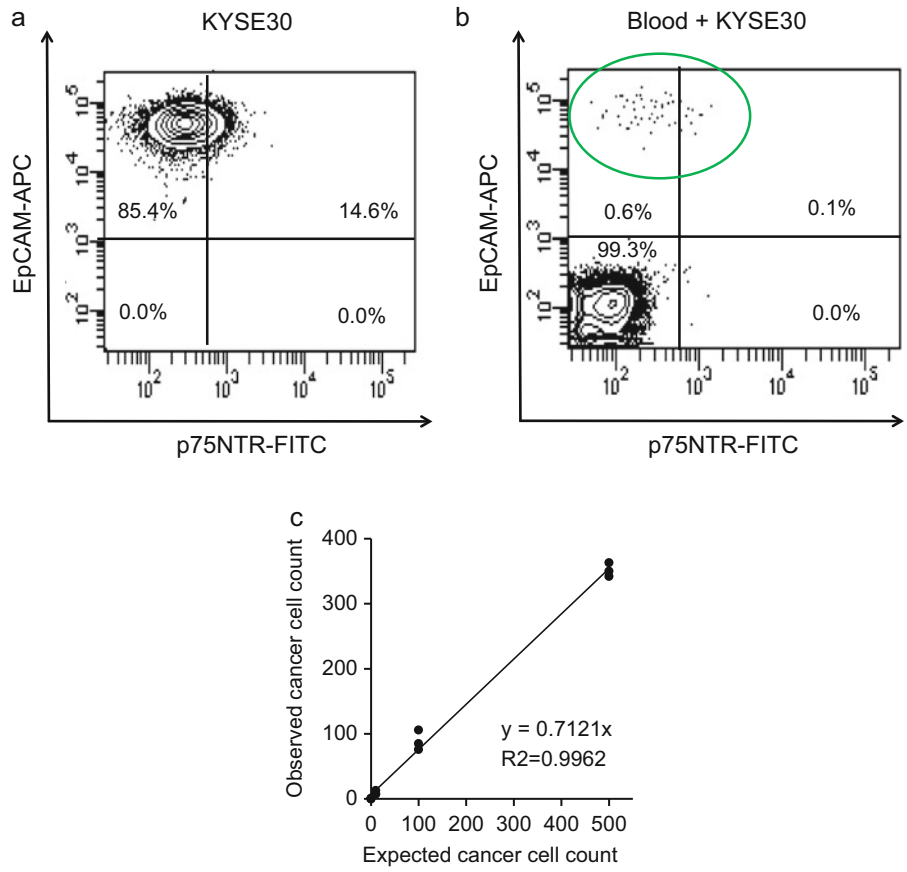

Fig. 1 Cells of an ESCC cell line (KYSE30) costained with anti-EpCAM-APC and anti-p75NTR-FITC were analyzed by two-color flow cytometry (**a**). Mononuclear cells from 3 mL peripheral blood from a healthy control, in which 100 of KYSE30 cells were mixed and costained with anti-EpCAM-APC and anti-p75NTR-FITC, were analyzed by two-color flow cytometry (**b**). Percentages of the cells in each quadrant are shown. Representative result of the number of spiked KYSE30 cells plotted against the observed number of cells using flow cytometry is shown (**c**). (Reproduced from ref. 16.)

2.2 Flow Cytometry by Fluorescence-Activated Cell Sorting (FACS)

1. Human monoclonal EpCAM-APC (clone: HEA125, MACS Miltenyi Biotec, Cologne, Germany).
2. Fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse antihuman p75 neurotrophic receptor (p75NTR, clone ME20.4-1. Miltenyi Biotec Inc. CA, USA).
3. Isotype-matched APC/FITC-conjugated antibodies (clone IS5-21F5, Miltenyi Biotec).
4. 7-aminoactinomycin D.
5. 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap.
6. Fluorescence-activated cell sorting (FACS) Cant II flow cytometer (BD Biosciences).

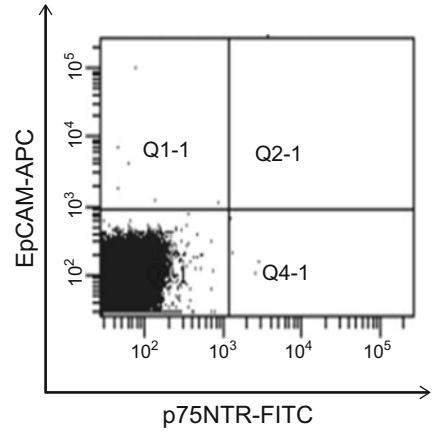

Fig. 2 Mononuclear cells from 3 mL peripheral blood of an ESCC patient were costained with anti-EpCAM-APC and anti-p75NTR-FITC, and analyzed by two-color flow cytometry. Quadrant markers were set according to isotype-matched controls. (Reproduced from ref. 16.)

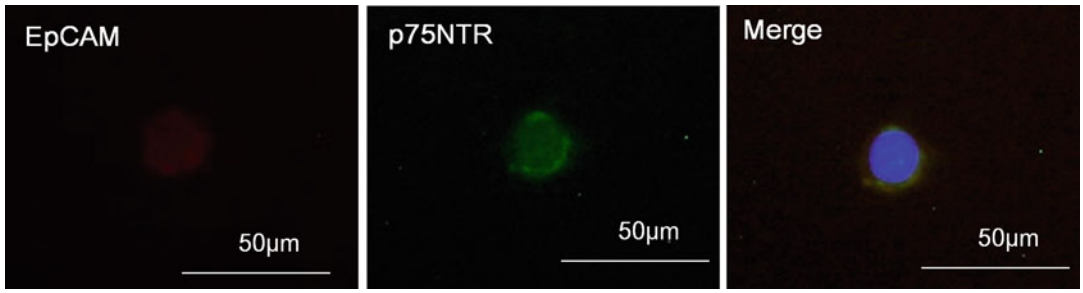

Fig. 3 Image of a representative EpCAM+ and p75NTR+ cell sorted from 3 mL peripheral blood in a patient with ESCC using flow cytometer. The images show an overlay of DAPI (*Blue*), p75NTR (*Green*), and EpCAM (*Red*). (Reproduced from ref. 16.)

7. FACS Aria II flow cytometer (BD Biosciences).
8. BD FACS Diva software (BD Biosciences).

2.3 Cytological Examination of the Sorted Cells

1. Cytospin chamber.
2. Transmitted light microscope equipped with a UV light source and filters.

2.4 Accuracy Assay

1. ESCC cell lines (KYSE30 and/or KYSE140).
2. Cell culture plates.
3. Ham's F12/RPMI-1640 cell culture medium.
4. Fetal calf serum.
5. 0.25% trypsin-ethylenediaminetetraacetic acid.

3 Methods

3.1 Blood Sample Preparation

1. Peripheral blood samples (6 mL) were extracted from a median cubital vein, collected in two of 3 mL ethylene-diamine-tetra-acetic acid (EDTA)-2K Vacutainer Tubes. Specimens were maintained at 4 °C and processed within 4 h (*see* **Note 1**).
2. Blood samples were transferred to 5 mL tube containing anti-coagulant with EDTA (*see* **Note 2**), and were diluted by the addition of an equal volume (3 mL) of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS).
3. 6 mL of the diluted blood samples were subsequently overlaid on a 4 mL Lymphoprep™ placed in a 15 mL centrifuge tube.
4. The tubes were spun at 800 × *g* for 20 min at room temperature in a swing-out rotor centrifuge (*see* **Note 3**).
5. After spinning, mononuclear cells were removed from the distinct band at the sample/medium interface using a Pasteur pipette into 5 mL tube without disturbing the upper layer.
6. Mononuclear cells were diluted in 2 mL PBS containing 2% FBS and were subsequently pelleted by spinning) for 5 min at 250 × *g*.

3.2 Fluorescence-Activated Cell Sorting (FACS)

1. Pelleted cells were diluted in 200 μL PBS containing 2% FBS.
2. Human monoclonal EpCAM-APC (dilution 1:100) and p75NTR-FITC (dilution 1:50) were added to be appropriate dilution. Isotype-matched APC/FITC-conjugated antibodies (dilution 1:50) were used as negative control.
3. After staining at 4 °C for 30 min, cells were diluted in 1 mL of PBS containing 2% FBS and pelleted by spinning at 250 × *g* for 5 min.

   Then the cells were resuspended in 1 mL of PBS containing 2% FBS.
4. Add two drops of 7-aminoactinomycin D (7-AAD) dye to the cell suspension. Vortex briefly. Then incubate for 10 min at room temperature.
5. Samples were analyzed on the FACS Cant II flow cytometer using BD FACS Diva software (*see* **Note 4**).
6. Viable cells were sorted on a FACS Aria II flow cytometer into a 5 mL tube containing 1 mL PBS containing 2% FBS.

3.3 Cytological Examination of the Sorted Cells

1. Sorted cells were washed twice with 1 mL PBS containing 2% FBS and diluted in 200 μL of cold PBS containing 2% FBS.
2. Slides and filters were placed into slots in a cytospin chamber with the cardboard filters facing the center.

3. For each sample, 200 μL was added to the appropriate wells of the cytospin, lids were applied, and they were spun at 250 × *g* for 5 min.
4. Filters were removed taking care not to disturb the smears on the slides.
5. Slides were examined under the microscope to check cell adherence, morphology, and monolayer formation.
6. Slides were dried overnight in a desiccator and evaluated using a transmitted light microscope equipped with a UV light source and filters.

3.4 Accuracy Assay

1. ESCC cell lines (KYSE30 and KYSE140) were cultured in Ham's F12/RPMI-1640 with 2% fetal calf serum. Cultures were maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide in air.
2. Cultured cells were washed once with PBS.
3. Cells were dissociated from the culture plates using 0.25% trypsin-ethylenediaminetetraacetic acid.
4. Cells were washed with 1 mL PBS containing 2% FBS and centrifuged at 250 × *g* for 5 min.
5. Pelleted cells were resuspended in 200 μL PBS containing 2% FBS.
6. The cells were stained and analyzed using a flow cytometer as shown in Subheading 3.2.
7. For the accuracy assay, 0, 10, 100, and 500 of KYSE cells were mixed with 3 mL each of peripheral blood samples obtained from the healthy volunteers.
8. The samples were prepared as shown in Subheading 3.1.
9. The cells were stained and analyzed using a flow cytometer, as shown in Subheading 3.2.

4 Notes

1. In patients who receive surgery, blood samples can be collected from the arterial pressure line under general anesthesia prior to surgery.
2. Be careful to avoid mixing the blood and separation fluid. Cap the tube to prevent the formation of aerosols.
3. Negative gate (quadrant markers) were set according to control staining using isotype-matched APC/FITC-conjugated antibodies, so that 99.9% of the each control immunofluorescence stain were included in the negative quadrant.

4. In the event of few cells being available, 100 μL of cold PBS containing 2% FBS was first placed in each cytospin, which was then spun at 250 × *g* for 5 min to prewet the filter, allowing more cells to reach the slide. Correct alignment of the filter/slide interface was ensured.

Acknowledgment

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Chapter 19

Multispectral Imaging Analysis of Circulating Tumor Cells in Negatively Enriched Peripheral Blood Samples

Brandon Miller, Maryam Lustberg, Thomas A. Summers, and Jeffrey J. Chalmers

Abstract

A variety of biomarkers are present on cells in peripheral blood of patients with a variety of disorders, including solid tumor malignancies. While rare, characterization of these cells for specific protein levels with the advanced technology proposed, will lead to future validation studies of blood samples as "liquid biopsies" for the evaluation of disease status and therapeutic response. While circulating tumor cells (CTCs) have been isolated in the blood samples of patients with solid tumors, the exact role of CTCs as clinically useful predictive markers is still debated. Current commercial technology has significant bias in that a positive selection technology is used that preassumes specific cell surface markers (such as EpCAM) are present on CTCs. However, CTCs with low EpCAM expression have been experimentally demonstrated to be more likely to be missed by this method. In contrast, this application uses a previously developed, technology that performs a purely negative enrichment methodology on peripheral blood, yielding highly enriched blood samples that contain CTCs as well as other, undefined cell types. The focus of this contribution is the use of multispectral imaging of epifluorescent, microscopic images of these enriched cells in order to help develop clinically relevant liquid biopsies from peripheral blood samples.

Key words Multispectral imaging, Circulating tumor cells, Circulating tumor cells, EpCAM

1 Introduction

A major tool in medical, biological, and biotechnology research and analysis is microscopy. Beyond simple bright field and dark field analysis, a large number of stains, such as the well-known Wright-Giemsa stain, are used to enhance subcellular structure. Even finer resolution, such as down to a few molecules, requires fluorescence.

While more than four different fluorescent dyes can be used to characterize cells on specially configured systems, most routine laboratory epifluorescence microscopes are equipped to differentiate up to only four different fluorescent signals. This limit of four colors is predominately the result of the limitations of commonly used, biologically compatible, fluorescent dyes available and the

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limits of excitation and emission filters. This confines the application of microscopy to different biological questions where multiple colors or multiparameter characterization is needed. One such area is regarding characterization of rare circulating cells in the blood of patients with malignancies, including circulating tumor cells (CTCs).

Micrometastatic spread of cancer tumors, including breast cancer, occurs early in the disease course, and emerging evidence suggests that circulating tumor cells (CTCs) play an active role in this process [1, 2]. Thus, CTCs isolated from peripheral blood samples of patients with solid tumor malignancies may provide additional tools for earlier diagnosis and serve as a source of clinically relevant biomarkers for targeted therapies [3, 4]. CTCs can change phenotype with disease progression, relative to phenotype of the original tumor, thus providing new molecular treatment options beyond those linked to the solid tumor [5]. Thus, multiparameter phenotypic characterization of CTCs has promising potential to elucidate new therapeutic targets and further improve current prognostic capabilities.

An example of the importance of accurate and comprehensive phenotypic characterization of CTCs can be further explained by these changes in phenotype that can occur with respect to the cancer marker HER2/neu. Recently, it has been reported that up to 30% of women lacking HER2/neu overexpression in the primary tumor have HER2/neu-positive CTCs [6] and that HER2/neu status changes during therapy [7]. These findings suggest that women with HER2/neu negative primary or metastatic tumors may be candidates for treatment with the HER2/neu-targeting drug trastuzumab (Herceptin). This is particularly important in treating women with aggressive triple negative tumors, i.e., women who are negative for HER2/neu expression in the primary tumor but may have HER2/neu expression in CTCs, which currently lack effective molecular drug targets, limiting drug treatment options to only chemotherapy.

A variety of technologies exists to isolate and characterize CTCs from the peripheral blood of cancer patients. A majority of these technologies/methodologies uses some form of positive selection followed by microscopic or molecular (i.e., RT-PCR) analysis. The most well known is the CellSearch system that positively recovers CTCs by selecting for a cell surface marker, EpCAM, followed by four-color, epifluorescence microscopy analysis [8].

To be considered a CTC by the CellSearch technology, a cell must, in addition to being positively selected by binding with an anti-EpCAM antibody, have the following: a nuclei; cytokeratin 8, 18, 19; lack expression of the pan-hematopoietic marker CD45; and morphology consistent with a tumor cell as determined by a trained operator. These markers are determined microscopically using antibodies conjugated to common dyes typically used for

flow cytometry: FITC (fluorescein isothiocyanate), PE (phycoerythrin), and APC (allophycocyanin). These dyes can be problematic in microscopy with respect to broad emission spectrum, photobleaching, and overlap with cellular autofluorescence. In addition, it is becoming increasingly evident that unusual, and potentially cancer-derived cells exist in the blood of cancer patients that do not fit this limited definition of a CTC [9–11]. However, to conclusively characterize these cells, additional markers are needed beyond the four parameters per cell typically used. Thus, better methodology for multiparameter characterization is needed.

To overcome the challenges and limitations of traditional four-color, epifluorescence microscopy analysis is the addition of a liquid crystal tunable filter in place of the traditional bandpass emission filter, and computer software that can not only control the filter, but also record emission intensity as a function of wavelength. Such approaches have been traditionally used in a number of spectroscopic instruments in analytical chemistry laboratories but are slow to be applied to microscopy. One exception is the commercial Nuance Multispectral Imaging System (Perkin Elmer, Waltham, MA), which is designed and marketed for imaging fluorescently stained tissue sections.

The focus of this chapter is on the use of the liquid crystal tunable filter combined with spectral unmixing software on four color stained peripheral blood of normal and breast cancer patients that has been enriched through a purely negative depletion technology. By enriching for the rare cells by using our previously presented, negative depletion methodology [12], which is not biased to only potential CTCs that express EpCAM, other cells are identified that are potential CTCs. A framework for the analysis will be described, and used to characterize cells and illustrate the limitations of traditional microscopy. In addition, the results will be compared with a typical flow cytometry analysis and both the strengths and weaknesses of this approach will be discussed.

1.1 Multispectral Imaging

Multispectral imaging allows a detector to record the intensity of emitted light across a range of wavelengths in 5–10 nm intervals. This combination of intensity and wavelength can be used to recreate the emission spectrum of the sample. And since every distinct fluorochrome has a unique emission spectrum, it is possible to determine the individual contribution of each fluorochrome in a sample using spectral unmixing techniques. In multispectral imaging, the number of fluorochromes is limited only by the ability to resolve two similar spectra. Figure 1 presents the emission spectra of seven common fluorochromes. These spectra are distinct, and the separation between the peak intensities of these fluorochromes varies between 33 and 59 nm, making it possible to separate them using unmixing techniques. In addition to separating distinct fluorochromes, it is also possible to separate a fluorochrome from

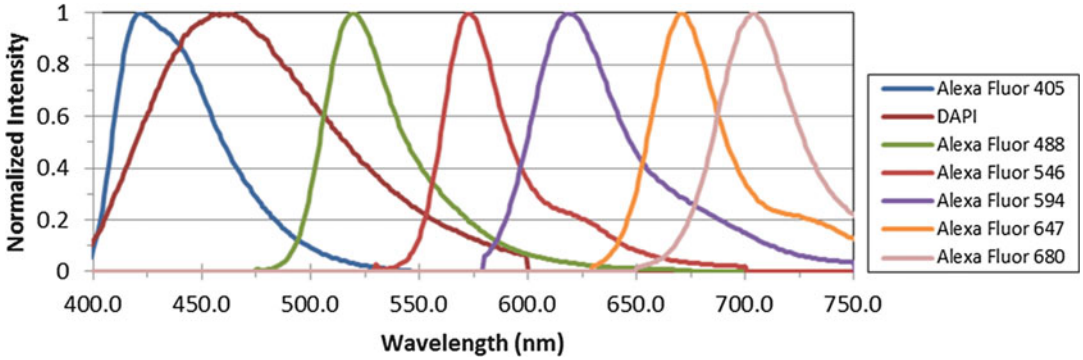

Fig. 1 Emission spectra of seven common fluorescent dyes (Life Technologies Web page, Grand Island, NY)

background spectra or autofluorescence. While different cells types may have different levels of autofluorescence, the much broader autofluorescence spectrum is always distinct from the well-defined spectra of the fluorochromes. The fluorochrome and autofluorescence spectra are determined by imaging single-color and unstained control slides. Since spectra are additive, the single-color control slides will contain spectra from the fluorochrome, autofluorescence, and background. The unstained control slide will contain spectra from only autofluorescence and background, allowing for the calculation of the fluorochrome spectra through simple subtraction.

The emission intensity from the slide is converted by the camera to a digital signal. For example, the Nuance system uses a 12-bit camera that relays the intensity as a value between 0 and 4095 for every image pixel. Single cells are identified using image processing software that detects cells based on the presence of the nuclear dye DAPI. After determining the cell region, it is possible to quantitatively determine the average intensity of each fluorochrome for every cell, which corresponds to the amount of fluorochrome present in the cell and therefore the amount of protein present. Even though this intensity is not related to absolute concentration, it will still allow for quantitative distinctions when determining a cell's phenotype.

In the simplest form, spectral unmixing can be accomplished by inversion of a system of linear equations of the form:

$$\mathbf{Ax} = \mathbf{y} \tag{1}$$

where $\mathbf{x}$ is a column vector of unknown fluorochrome concentrations, $\mathbf{y}$ is a vector of measured intensity at each wavelength, and $\mathbf{A}$ is a matrix specifying the unit intensities of known fluorochrome spectra at each wavelength [13]. This equation is solved for $\mathbf{x}$ at each pixel. When the number of wavelengths is larger than the number of fluorochromes, i.e., the system is overdetermined, a solution minimizing the least-square error can be found by solving:

$$\mathbf{A}^{\mathrm{T}}\mathbf{A}\mathbf{x} = \mathbf{A}^{\mathrm{T}}\mathbf{y} \tag{2}$$

where $\mathbf{A}^{\mathrm{T}}$ is the transposed matrix of $\mathbf{A}$ [13]. This algorithm can determine the contribution of each fluorochrome in a sample even when the spectra overlap. This key difference compared to traditional techniques allows for more than four fluorochromes to be imaged simultaneously as long as each fluorochrome has a unique and distinct spectrum.

Multispectral imaging is widely used in time-lapse imaging of living samples as well as in tissue imaging to remove the autofluorescence signal and allow for multiple fluorochrome detection with a single excitation wavelength [14–19]. However, it has not been used as a means to perform multiparameter analysis of single cells on slides, likely due to the difficulty associated with designing multi-color staining protocols.

1.2 Liquid Crystal Tunable Filters

Liquid crystals are organic liquids that exhibit properties of crystalline materials. They require anisotropic molecules, such as the rod-shaped 5CB molecule shown in Fig. 2a [20]. The molecules will align along their long axes in a common direction and, since the molecules exhibit dielectric anisotropy, this orientation can be changed by applying an external electric field. This orientation will in turn affect other properties of the liquid crystal, including the birefringence Δn. Birefringence is an optical property where the index of refraction for a material depends on the direction that light propagates through the material and the polarization of the light.

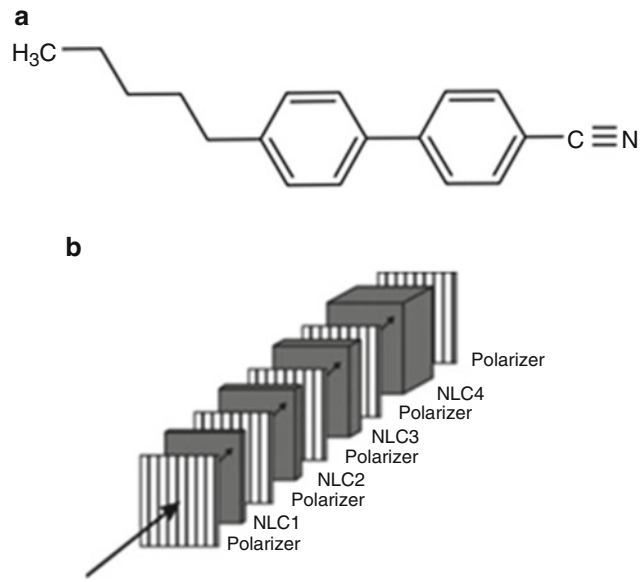

Fig. 2 The well-known liquid crystal molecule 5CB (4-cyano-4′-pentylbiphenyl), (**a**), and a of Lyot-Ohman filter consisting of four liquid crystal cells sandwiched between five linear polarizers (Adapted from [20]) (**b**)

This creates a relative phase shift, or retardation, between the components projected along the optical axis and perpendicular to it. The retardation R is given by Eq. 3:

$$R = d\Delta n \tag{3}$$

where d is the thickness of the liquid crystal. The propagation speed between the two components also differs, resulting in a phase delay Γ given by Eq. 4 [21]

$$\Gamma = \frac{2\pi R}{\lambda} \tag{4}$$

where λ is the wavelength of light. The transmission T through a one-stage liquid crystal filter is dependent on Γ and is given by Eq. 5

$$T = \frac{1}{2}\cos^2\left(\frac{\Gamma}{2}\right) \tag{5}$$

Liquid crystal tunable filters are based on the Lyot-Ohman filter, where several liquid crystals are sandwiched between linear polarizers. A four-stage Lyot filter is shown in Fig. 2b. In this case, the transmission through multiple liquid crystals is proportional to the product of the transmission through each individual crystal.

Since the liquid crystals are affected by an external electric field, the primary wavelength can be tuned to any value within a given range. In practice, a liquid crystal tunable filter will use ten or more stages, resulting in a sharper peak at the desired wavelength and nearly zero transmission at all other wavelengths. An example of a ten-stage filter incorporating a doubling sequence of retardation is shown in Fig. 3. Since the light must pass through a polarizing

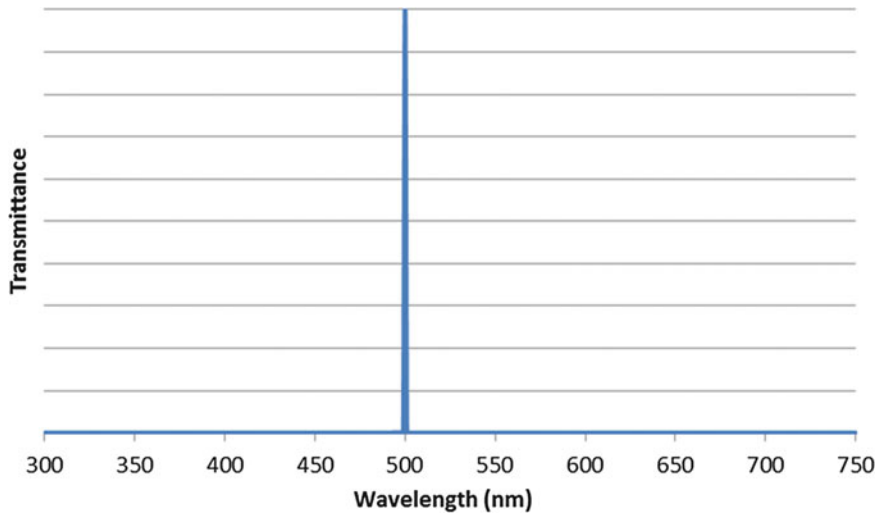

Fig. 3 Transmission vs. wavelength plot of a combined ten-stage filter

filter, the maximum transmission is 50%, which requires a camera with high sensitivity to detect the resulting signal. However, since the transmission is reduced and the camera exposure can be independently controlled, the camera will not become saturated, preserving quantitative spectral resolution.

1.3 Spatial Resolution

The multispectral imaging system is based on traditional epifluorescence microscopy, where the spatial resolution is limited by aberration and diffraction. A standard microscope objective is used to focus the excitation light on the specimen and collect the emitted light. A well-designed objective will correct for aberration, resulting in a diffraction-limited system. In typical fluorescence microscopy, the wavelength varies from ultraviolet through visible light, approximately 350–750 nm. Numerical apertures vary with the quality of the objective, but values of up to 1.40 are available for some high-magnification, oil immersion objectives. This results in a maximum theoretical resolution of approximately 200 nm. The multispectral imaging system is used in this study with air objectives ($n = 1$) and a numerical aperture of 0.50–0.85, results in a spatial resolution of approximately 0.5 μm. In practice, this resolution is only achieved using samples that give bright signals. However, when compared to typical cellular dimensions, the resolution is adequate. Red blood cells range in size from approximately 6–8 μm, while white blood cells are larger, typically 8–20 μm.

2 Materials

1. Cancer cell lines: To develop control spectra, breast cancer cell lines BT474, MCF7, and MDA-MB-231 were procured from ATCC (Manassas, VA).
2. Normal peripheral blood: Source leukocytes were obtained through an IRB approved purchase from the American Red Cross.
3. Peripheral blood samples from patients with no known diagnosis of breast cancer: Peripheral blood was collected from 19 individuals with suspected breast abnormality based on abnormal imaging or physical examination before undergoing diagnostic procedures at the Walter Reed National Military Medical Center Comprehensive Breast Care Center. The protocol was approved by the Cancer Institutional Review Board of the Walter Reed Army Medical Center (IRB net #354344), initiated in January 2011 and informed, written consent was obtained from all donors.
4. Peripheral blood samples from patients with metastatic breast cancer: Metastatic breast cancer patients diagnosed with either triple negative (TNBC) or estrogen positive (ER^+) tumors, who were older than 18 years of age, with two or less prior

Table 1
List of antibodies used for immunofluorescence staining

Target	Antibody clone, host species	Fluorescent dye	Secondary antibody	Secondary fluorescent dye
Nucleus	–	DAPI	–	–
Cytokeratin 8, 18, 19	CK3-6H5, mouse	FITC	–	–
Cytokeratin 8, 18, 19	CK3-6H5, mouse	AF488, custom-conjugated	–	–
CD45	HI30, mouse	–	Anti-mouse	AF594
CD45	HI30, mouse	AF594, custom-conjugated	–	–
EpCAM	Polyclonal, rabbit	–	Anti-rabbit	AF647

lines of systemic therapy were enrolled in an Institutional Review Board (IRB)-approved protocol. All patients gave their informed consent to participate in the study.

5. Nikon 80i epifluorescence microscope and custom filter sets: An epifluorescence microscope was used for obtaining images. For traditional epifluorescence analysis, the following excitation, emission filters were used: Blue 377/50, 447/60; Green 475/50, 536/40; Red 562/40, 625/40; and Far Red 620/60, 700/75. The following excitation, emission filters were used with the Nuance system: Blue 377/50 409LP; Green 475/50, 510LP; Orange 535/50, 572LP; Red 562/40 593LP; and Far Red 620/60, 700/75.
6. Nuance Multispectral Imaging System: A Nuance Multispectral Imaging System (Perkin Elmer, Waltham, MA) was used for spectral deconvolution. The system consists of a liquid crystal tunable filter, a CCD camera, and specific software to perform the spectral deconvolution.
7. Immunofluorescence staining. Table 1 lists the antibody clones used and the dyes to which they were conjugated. After a final wash, slides were mounted using ProLong Gold antifade reagent with DAPI.

3 Methods

3.1 Cell Preparation

1. Cancer cells were grown to mid-log phase in DMEM, supplemented with 10% FBS and nonessential amino acids, at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were harvested by washing the adherent cells with PBS and subsequently by incubating with Accutase for 5 min at 37 °C to detach the cells from the culture flask. Accutase was then

neutralized with the culture medium before pelleting the cells at 350 × *g* for 5 min.

2. Source leukocytes were typically subjected to a double lysis to remove the red blood cells, since the lower lysis efficiency could be offset by using more sample. The red cell lysis procedure was previously published [22].
3. Blood samples from cancer patients were collected after several standard blood tubes were collected for routine chemotherapy labs, either prior to initiation of a new line of systemic therapy, after one cycle of therapy, or at progression. Samples of 7–18 mL of peripheral blood were collected in Vacutainer tubes for CTC enumeration and processed within 4 h of blood draw.
4. Patient samples were negatively enriched for CD45 minus cell using previously published methodology [12].

3.2 Four-Color Immunofluorescence Staining of Cytospins

1. Mix 125 μL sample and 75 μL 70% EtOH in a microcentrifuge tube.
2. Prepare cytospin assembly by mounting a slide and funnel in a cytospin clip.
3. Add the sample cell suspension to the funnel.
4. Cytospin at 113 × *g* for 5 min, discard funnel.
5. Allow slide to dry at room temperature.
6. Add 200 μL normal serum blocking solution to permeabilize/block.
7. Incubate at room temperature for 30 min.
8. Add 200 μL primary antibodies in antibody diluent to slides. 1° Abs: CK-AF488—1:100
   CD45-AF594 (clone HI30, mouse, Invitrogen/BD Biosciences)—1:100
   EpCAM primary (polyclonal)—1:200 (*see* **Note 1**).
9. Incubate at room temperature for 1 h, in the dark.
10. Wash the cells three times with PBST for 5 min.
11. Add 200 μL secondary antibody in PBST to slides. 2° Ab: anti-rabbit AF647 secondary—1:400.
12. Incubate at room temperature for 1 h, in the dark.
13. Wash the cells three times with PBST for 5 min.
14. Add 10 μL Antifade Reagent with DAPI to mount and coverslip.
15. Incubate at room temperature overnight, in the dark.
16. Seal coverslip with nail polish.
17. Store slides at 4 °C, in the dark.

3.3 Spectral Library Creation

1. A spectral spectral library, which is used by the multispectral imaging software to unmix an image, needs to be created by acquiring multispectral images of single-color stained slides and unstained slides. The unstained slides were used to obtain autofluorescence and background spectra, while the single-color stained slides contained spectral contributions from the single fluorescent dye present as well as autofluorescence and background.
2. For a four-color experiment, the spectral library contained a total of five spectra, i.e., the four fluorescent dyes and background/autofluorescence. Figure 4 presents an example.

3.4 Image Acquisition and Analysis

1. Since the analysis of rare cells relies on examining large populations of cells, it is necessary to scan and image entire slides (*see* **Note 2**). Images are acquired with the Nuance software and processed using macros that performed unmixing using the previously obtained spectra library (Fig. 4).
2. Figure 5 presents a comparison of traditional epifluorescence and spectrally unmixed images in leukocytes and MCF7. Note the false-positive EpCAM signal present in the traditional epifluorescence images of leukocytes is correctly negative in the spectrally unmixed images. To further understand the power of spectral deconvolution, a plot of intensity vs. wavelength showing the combined spectra of selected cells is also included in Fig. 5. Each color corresponds to a single cell, circled at right. Excitation (dark gray) and emission filters (light gray) are shown under the plot. The vertical gray lines represent the edge wavelength for each of the four longpass emission filters. For clarity, each spectra is clipped near the edge of the next filter.
3. It is important that even with the spectral deconvolution approach, the choice of proper filters is very important. Figure 6 presents comparison of spectral unmixing results using different filter sets using cells from a patient without known diagnosis of malignancy. Analysis was performed in duplicate using two filter sets, A and B. As observed in Fig. 5, the traditional epifluorescence images in Fig. 6 using both filter set A and filter set B suggest that most of the cells are positive for EpCAM; however, upon unmixing, the images using both filter sets remove all this positive EpCAM signal. However, note the blue spectral from both filter sets A and B. The wider (lower cut-off) band pass for the emission filter allows enough of the CD45-AF594 signal to "bleed through" to make a cell appear to be positive for EpCAM-AF647 for images taken using filter set A, but not for filter set B.
4. The results shown in this chapter used only four fluorescent dyes to illustrate the performance of the spectral unmixing

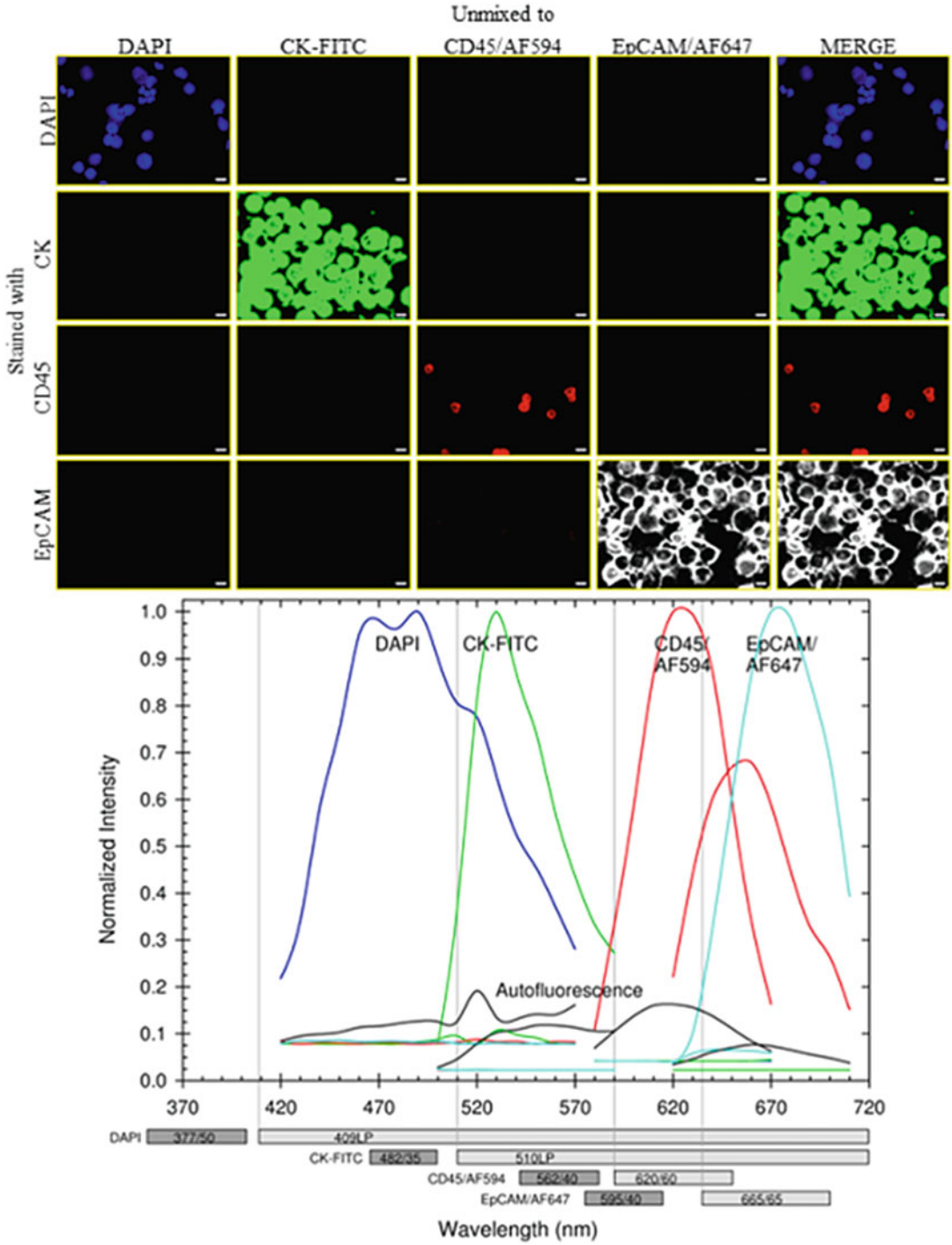

Fig. 4 Control samples and spectral library for undiagnosed patient data set. Samples containing normal peripheral blood leukocytes (*row 3*), breast cancer cell line MCF7 (*rows 2* and *4*), or a mixture of leukocytes and MCF7 (*row 1*) are presented. Each row represents a control sample stained with a single marker: DAPI, anti-cytokeratin-FITC, anti-CD45-AF594, and anti-EpCAM-AF647. *Columns 1–4* are the resulting spectrally unmixed images. Since each row is stained with a single color, the unmixing algorithm only produces images on the diagonal. *Bottom*, plot of intensity vs. wavelength showing the spectral library used for unmixing the undiagnosed patient data set (patients 1–19). Each color corresponds to one of the four markers or autofluorescence. Excitation (*dark gray*) and emission filters (*light gray*) are shown under the plot. The *vertical gray lines* represent the edge wavelength for each of the four longpass emission filters. Scale bars, 10 μm

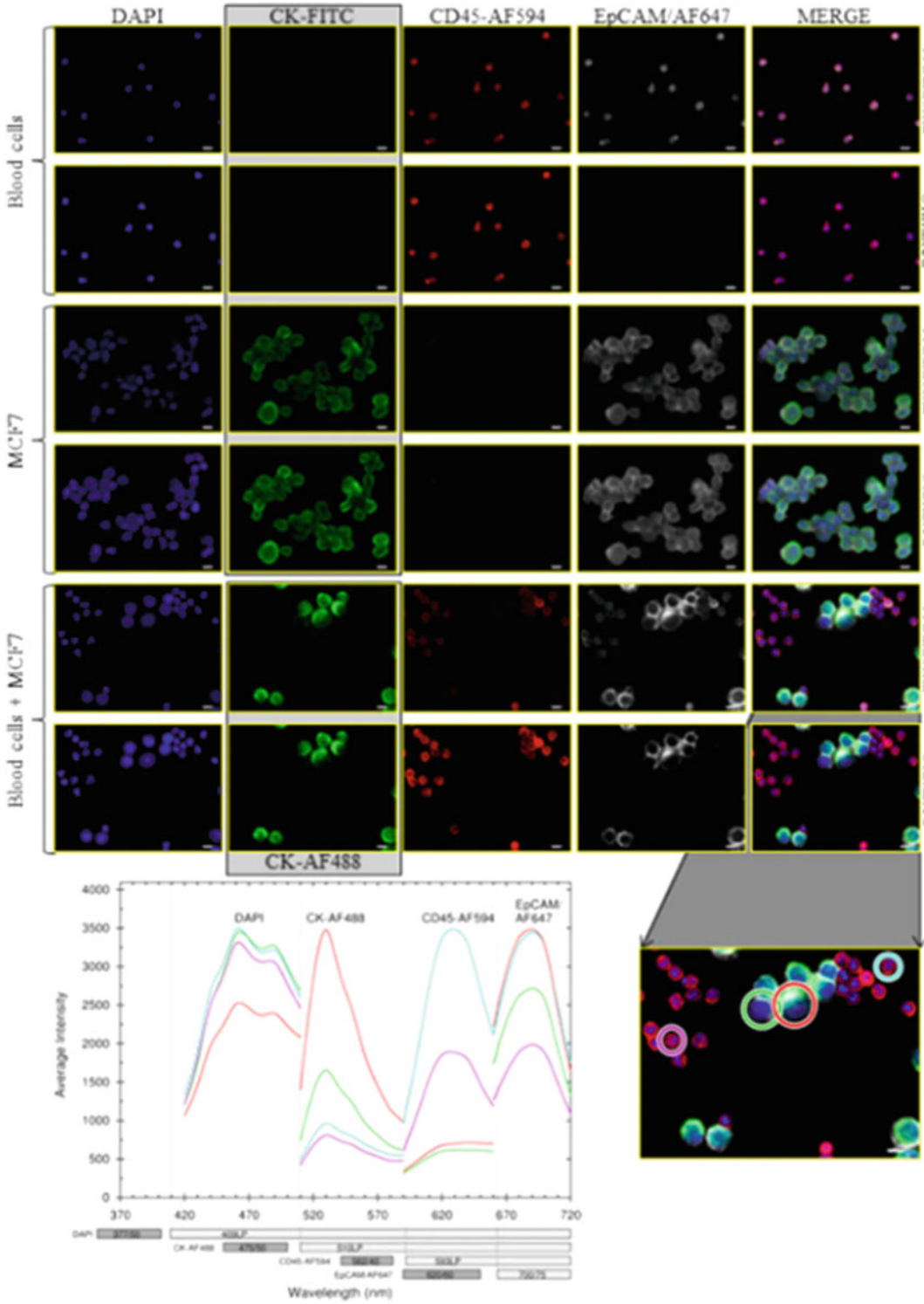

Fig. 5 Comparison of traditional epifluorescence and spectrally unmixed images in leukocytes and MCF7. Normal peripheral blood leukocytes (*rows 1* and *2*), breast cancer cell line MCF7 (*rows 3* and *4*), and a mixture of leukocytes and MCF7 (*rows 5* and *6*) are presented. Each pair of rows contains traditional epifluorescence

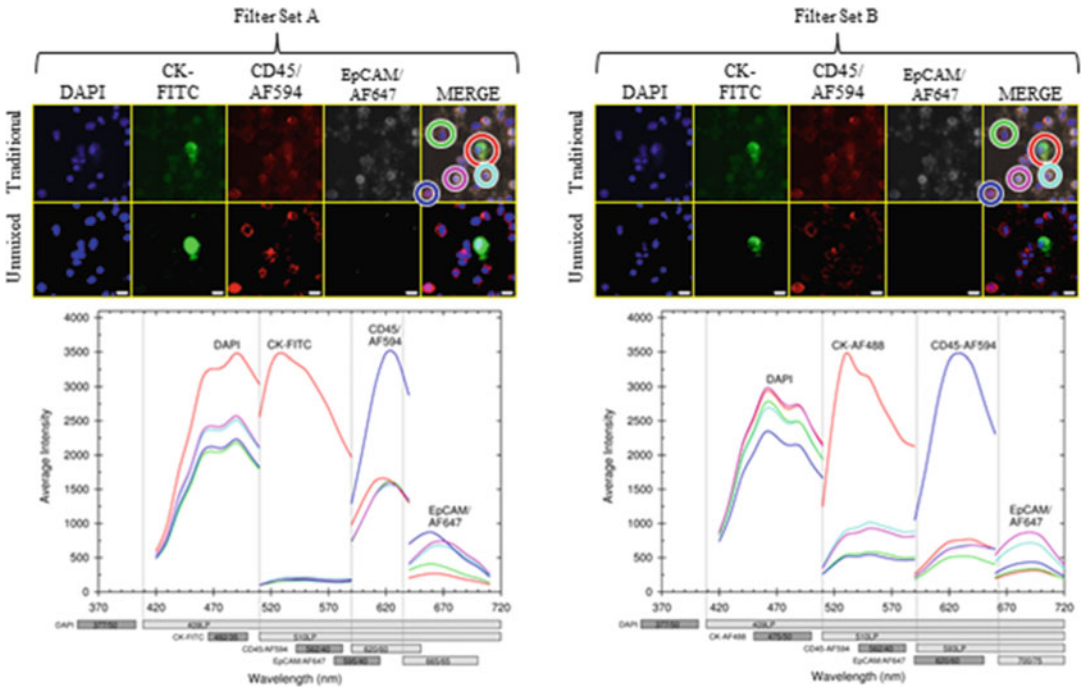

Fig. 6 Comparison of spectral unmixing results using different filter sets. Cells from a patient without known diagnosis of malignancy are presented. Analysis was performed in duplicate using two filter sets, A and B. The *top row* contains traditional epifluorescence images and the *bottom row* contains spectrally unmixed images at the same location. *Columns 1* through *4* are stained with DAPI, anti-cytokeratin-FITC, anti-CD45-AF594, and anti-EpCAM-AF647, respectively. *Bottom*, plot of intensity vs. wavelength showing the combined spectra of selected cells. Each color corresponds to a single cell, circled above. Excitation (*dark gray*) and emission filters (*light gray*) are shown under the plot. The *vertical gray lines* represent the starting wavelength for each of the four emission filters. For clarity, each spectra is clipped near the start of the next filter. Scale bars, 10 μm

Fig. 5 (continued) (*rows 1*, *3*, and *5*) and spectrally unmixed (*rows 2*, *4*, and *6*) images at the same location. *Columns 1–4* are stained with DAPI, anti-cytokeratin-FITC (*rows 1–4*), anti-CD45-AF594, and anti-EpCAM-AF647, respectively. *Rows 5* and *6* are stained with anti-cytokeratin-AF488. Note the false-positive EpCAM signal present in the traditional epifluorescence images of leukocytes is correctly negative in the spectrally unmixed images. *Bottom*, plot of intensity vs. wavelength showing the combined spectra of selected cells. Each color corresponds to a single cell, *circled* at *right*. Excitation (*dark gray*) and emission filters (*light gray*) are shown under the plot. The *vertical gray lines* represent the edge wavelength for each of the four longpass emission filters. For clarity, each spectra is clipped near the edge of the next filter. Scale bars, 10 μm

technique. However, the power of this technique is in its ability to move beyond the typical four-color staining of traditional epifluorescence microscopy and image more markers simultaneously, thus allowing for more complete phenotypic characterization of rare cells, such as circulating tumor cells.

4 Notes

1. While FITC is used in traditional microscopy and flow cytometry, in addition to typical significant signal as a result of photobleaching, use of the extended time needed to create the images for the spectral deconvolution system is problematic. Figure 7 demonstrates the rapid photobleaching of FITC (i.e., loss of signal intensity) and Fig. 8 demonstrates that AF488 has a very similar spectral profile to FITC. For this reason, we use AF488 in place of FITC.
2. The actual scanning of each full slide with the Nuance system is time-consuming and requires significant operator involvement. To facilitate this process and decrease the operator involvement, a semiautomated scanning system can be implemented. We implemented a Zaber (Vancouver, BC) XY stage combined/controlled with the LabVIEW (National Instruments, Austin, TX) programming language to automate the microscope stage/image acquisition step.

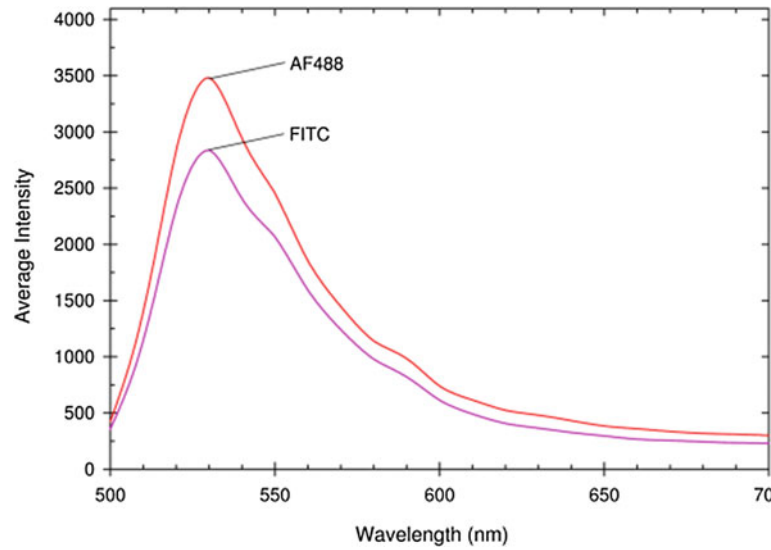

Fig. 7 Comparison of FITC and AF488 emission spectra. Plot of intensity vs. wavelength for breast cancer cell line MCF7 stained with either anti-cytokeratin-FITC or anti-cytokeratin-AF488. The same primary antibody was used in each case

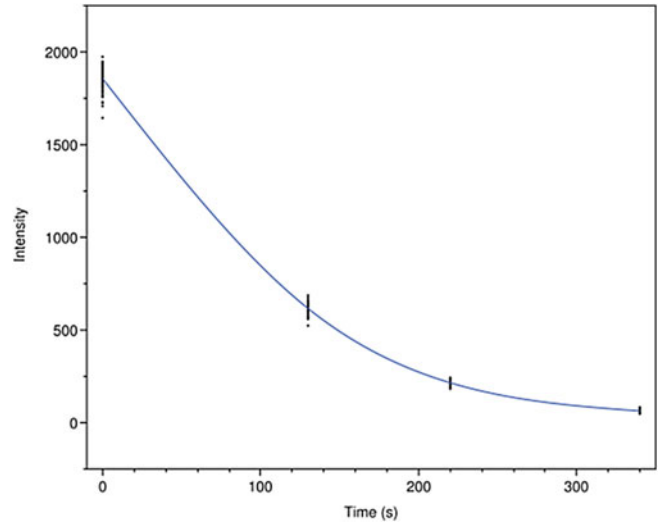

Fig. 8 Photobleaching of FITC. Plot of intensity vs. time for $n = 77$ MCF7 cells stained with anti-cytokeratin-FITC. Each *dot* represents a single cell

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Chapter 20

Fiber-Optic Array Scanning Technology (FAST) for Detection and Molecular Characterization of Circulating Tumor Cells

Zheng Ao and Xiaohe Liu

Abstract

Circulating tumor cell (CTC) as an important component in "liquid biopsy" holds crucial clinical relevance in cancer prognosis, treatment efficiency evaluation, prediction and potentially early detection. Here, we present a Fiber-optic Array Scanning Technology (FAST) that enables antigen-agnostic, size-agnostic detection of CTC. By immunofluorescence staining detection of a combination of a panel of markers, FAST technology can be applied to detect rare CTC in non-small cell lung cancer (NSCLC) setting with high sensitivity and specificity. In combination with Automated Digital Microscopy (ADM) platform, companion markers on CTC such as Vimentin and Programmed death-ligand 1 (PD-L1) can also be analyzed to further characterize these CTCs. FAST data output is also compatible with downstream single cell picking platforms. Single cell can be isolated post ADM confirmation and used for "actionable" genetic mutations analysis.

Key words Circulating tumor cells, Single cell analysis, Non-small cell lung cancer

1 Introduction

"Liquid biopsy" of cancer has been a hot-bed for cancer biomarker research since it provides unparalleled advantage of being minimal-invasive, re-samplable and has the capability of being monitored in real time. Circulating Tumor Cell (CTC) is an important component of "Liquid Biopsy." Since first reported by Ashworth in 1869, there has not been extensive research on these rare cells in cancer patients' peripheral blood till the past decade, primarily due to technical hurdles to enrich these rare cells. Finding these rare cells in peripheral blood, where their number varies from 1 to a few hundreds per milliliter of blood, from millions of leukocytes and billions of erythrocytes, is like finding a "needle in a haystack." Recent technology advances have enabled us to enrich and characterize them primarily based on their unique antigen expression profile [1] or their physical properties (size, deformability, etc.) [2]. However, these technologies have their limitations. Cancer

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cells have been known to undergo Epithelial-Mesenchymal-Transition (EMT) during the metastatic process. During EMT, epithelial cells will shed off epithelial phenotypes, including down-regulation of epithelial antigen—Epithelial cell adhesion molecule (EpCAM) [3], which has been widely adopted as the target molecule for antibody based CTC capture [1]. Meanwhile, other researchers have identified small CTCs that are ~4 μm in diameter [4], and studies on CTC clusters have found un-fixed tumor cells can transverse 5 μm channel by deformation [5]. These evidences indicate that both antigen and size-based methodology can lead to negligence of certain CTC subpopulations. Here, we describe a method that examines all nucleated cells in blood without leaving any subtypes behind using a Fiber-optic Array Scanning Technology (FAST) [6–11], (Fig. 1a). This immunocytochemical assay can rapidly detect rare cells without the need for pre-enrichment, the detected cells are subsequently analyzed in an automated digital microscope (ADM) at high resolution. ADM also allows for multiplex immunocytochemical marker analysis for potential companion diagnostics applications. In addition, the coordinates of identified rare cells can then be utilized for rapid location of the cell on our

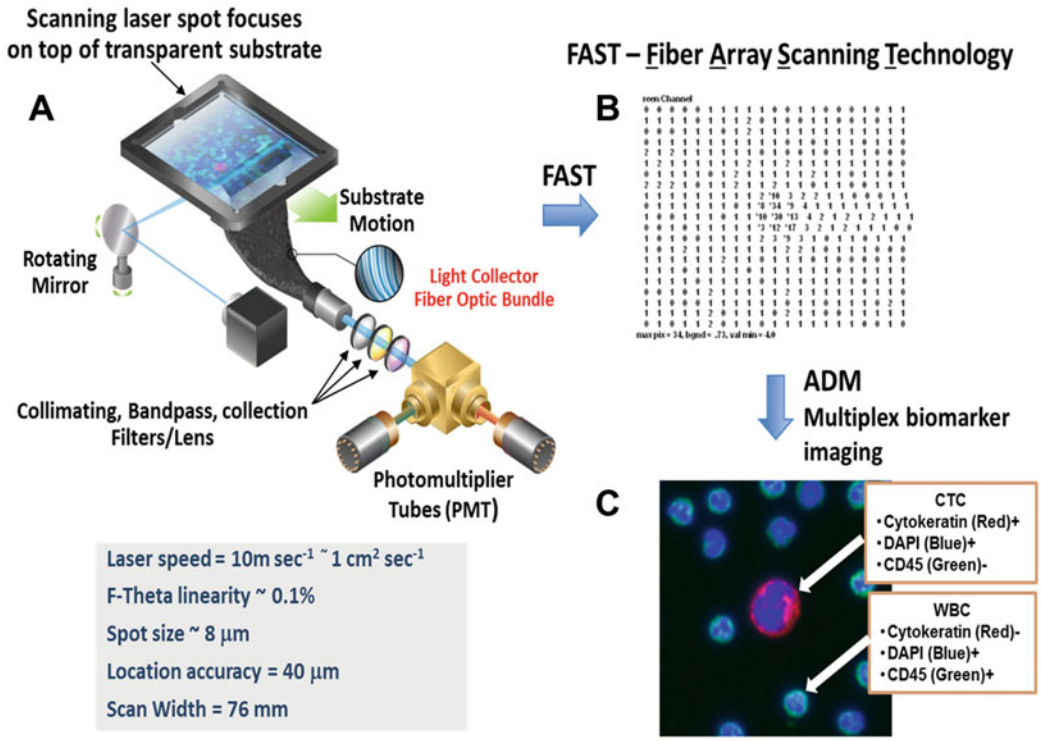

Fig. 1 (**a**) Schematic of Fiber-optic Array Scanning Technology. (**b**) Pixel map representing a target cell detected by FAST. (**c**) After ADM, targets detected by FAST were confirmed as CTC or not CTC by 20× imaging

downstream cell picking platform where automated single cell isolation and downstream genomic analysis takes place.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Blood Sample Preparation

1. Ammonium-Chloride-Potassium (ACK) lysing buffer: Prepare 10× ACK lysis buffer as follows: 1.55 M NH_4Cl, 100 mM $KHCO_3$, 1 mM Ethylenediaminetetraacetic acid (EDTA). Weigh 41.45 g of NH_4Cl, 5.00 g of $KHCO_3$, 0.15 g of EDTA, add in 450 mL of water. Mix and adjust pH to 7.4. Make up to 500 mL with water, store at 4 °C. Before use, make 1× ACK lysis buffer. For up to two tubes of blood received (~20 mL), take 10 mL of 10× ACK lysis of buffer, add in 90 mL of water, adjust pH to 7.4, store at room temperature for same-day use.
2. 1× Phosphate-buffered saline (PBS): Prepare 1× PBS by adding 4 L of water to 500 mL of 10X PBS. Adjust pH to 7.4, and then add water to make the volume up to 5 L.
3. Glass slide: Paint with hydrophobic pen (Vector Laboratories) around the edge of the active area on the slide. Pretreat glass slide to remove protective layer as follows: take the 64 cm^2 glass slides (Paul Marienfeld GmbH & Co., KG, Bad Mergentheim), submerge the slides under water in the glass bin for 2 min, then move the glass slides to another glass bin of water for two more min. Then treat the slides with methanol for 15 s to fully remove the protective layer (green) (*see* **Note 1**). Then transfer the slides to a clean bin of water for another 2 min. The slides can then be stored in 1× PBS and ready for use.
4. Prechill acetone (500 mL) at −20 °C.
5. 2% PFA: Dilute 16% Paraformaldehyde (PFA) (Thermo Scientific) 1:7 with 1× PBS.

2.2 Immunofluorescence Staining

1. 20% human serum: Dilute human serum 1:4 with 1× PBS, aliquot into 50 mL cornical tubes and store at −20 °C, prewarm 6 mL of serum per slide at 37 °C water bath before staining steps.
2. 4′,6-diamidino-2-phenylindole (DAPI): Add 1 mL of water into one DAPI vial (Thermo Fisher) to make a 10 mg/mL stock solution. On the day of staining, dilute DAPI stock solution 1:10,000 with 1× PBS for staining.

3. Fluorescence mounting media: Prepare mounting media, 20 mM Tris pH 8.0, 0.5% n-propyl gallate, and 90% glycerol. Weigh 500 mg n-propyl gallate, dissolve in 5 mL water, then add in 2 mL of 1 M Tris buffer, adjust volume to 10 mL, add in 90 mL of glycerol and mix thoroughly. Aliquot into 5 mL per tube and store at −20 °C. Pre-warm the mounting media at 37 °C before use.
4. Primary antibody cocktail: for immunofluorescence staining, mix the antibody cocktail during blocking step. Dilute all antibodies in 20% human serum. An example for the cocktail is as follows: primary antibody: monoclonal mouse IgG1 anti pan-cytokeratin (CK) antibodies for cytokeratin classes 1, 4, 5, 6, 8, 10, 13, 18, and 19 (C2562, Sigma and RCK108, DAKO), monoclonal mouse IgG1 anti-MUC1, EGFR, HER2 and Plastin-3, mouse IgG2a anti-human CD45 antibody (MCA87, AbD Serotec, Raleigh, NC) directly conjugated with Qdot 800 (Invitrogen custom conjugation). In addition, other companion tumor biomarkers can also be added into the primary antibody cocktail such as monoclonal rabbit anti-human Vimentin (EPR3776, Abcam) or monoclonal mouse IgG2b anti-human PD-L1 (329701, BioLegend).
5. Secondary antibody cocktail: biotin-goat anti-mouse IgG1 (A10519, Life technologies), and goat anti-rabbit (A21244, Life Technologies) if labeling Vimentin or anti-mouse IgG2b (A21242, Life Technologies) if labeling PD-L1 antibodies conjugated with Alexa 594.
6. Tertiary antibody: Streptavidin Alexa-555 conjugate (S-32355, Invitrogen).

3 Methods

Carry out all the procedures at room temperature unless otherwise specified.

3.1 Blood Sample Preparation

1. Collect 7.5 mL of blood sample.
2. Add in ACK lysis buffer into the blood at 5:1 ratio (*see* **Note 2**). Rock the sample on a two-axis rocker for 5 min.
3. Centrifuge sample at 300 rcf for 5 min to pellet the remaining leukocytes and rare cells.
4. Confirm that white blood cells (WBCs) have pelleted and aspirate the supernatant.
5. Resuspend cell pellet in 25 mL PBS (*see* **Note 3**).
6. Spin sample at 300 rcf for 5 min.
7. Aspirate the supernatant.

8. Resuspend all cells from each patient in 1 mL PBS (*see* **Note 4**).
9. Count cells on a hemocytometer.
10. Adjust cell suspension to a maximum of 13e6 cells/mL.
11. Remove prepared glass slides from PBS and place in a staining tray. Dry around the active area, carefully, using a Kim-Wipe.
12. Label slides by etching sample ID and date of sample preparation.
13. Add 2 mL of cell suspension per slide (up to 26e6 cells per slide).
14. Incubate for 40 min, in the dark, in a 37 °C incubator for cells to attach (*see* **Note 5**).

3.2 Immunofluorescence Labeling of Tumor Biomarkers

1. Decant cell suspension by tipping slide and pressing edge to a towel.
2. Add 2 mL of 2% PFA in 1× PBS to active plate area (*see* **Note 6**). Incubate for 10 min at room temperature.
3. Decant solution and place plate in a 1× PBS bath for 3 min.
4. Transfer plates to a prechilled acetone bath. Incubate for 5 min at −20 °C (*see* **Note 7**).
5. Transfer slides to a 1× PBS bath for 3 min.
6. Remove from bath, decant excess solution, and wick edges with a towel to remove additional liquid.
7. Add 2 mL of 20% human serum. Distribute evenly onto surface. Incubate at room temp for 30 min.
8. Mix antibodies during incubation.
9. Decant solution from slides and wick off excess.
10. Add 1 mL of primary antibody to patient samples. Incubate 60 min, in the dark, at 37 °C.
11. Decant solution and place in 1× PBS bath at room temperature for 3 min.
12. Transfer to a clean 1× PBS bath at room temperature for 3 min.
13. Decant solution and wick off excess.
14. Dispense 1 mL of secondary antibody and incubate 60 min, in the dark, at 37 °C.
15. Decant solution and place in 1× PBS bath at room temperature for 3 min.
16. Transfer to a clean 1× PBS bath for 3 min.
17. Decant solution and wick off excess.
18. Dispense 1 mL of tertiary antibody and incubate 60 min, in the dark, at 37 °C.
19. Decant solution and place in PBS bath at room temperature for 3 min.

20. Transfer to a clean 1× PBS bath at room temperature for 3 min.
21. Decant solution and wick off excess.
22. Dispense 2 mL of pre-diluted DAPI and incubate in the dark, at room temperature, for 10 min.
23. Decant solution and place in 1× PBS bath at room temperature for 3 min.
24. Dip plate into ddH_2O, briefly (~10 s).
25. Coverslip the slide (*see* **Note 8**).

3.3 Fiber-Optic Array Scanning Technology for Candidate CTC Coordinate Identification

1. Use nail polish to seal the edges of the coverslip on the FAST slide.
2. Place four fiduciary markers on the top of the nail polish, aligned with the printed fiduciary mark along the edge of the cover slip, on the four corners of the slide.
3. Turn on the FAST scanner and allow the scanner to warm up for 15 min.
4. Open software on the FAST computer.
5. Place the slide onto the FAST scanner.
6. Initiate the scan in the FAST software.
7. After the scanning is done (about 1 min per slide up to 26e6 cells), save the file onto the server. An example of the generated pixel map can be found in Fig. 1b.
8. Found CTC candidates using "RawV2" software. Use the "Find Cells" function to find coordinates that meet the setting criteria of potential CTCs.

3.4 Automated Digital Microscope (ADM) Imaging for CTC Confirmation

1. Turn on microscope and open the software "RawV2" for ADM.
2. Open the FAST file that was saved on the server on the last step.
3. Calibrate the microscope to read the coordinates of potential targets for automated imaging: Register the fiduciary marks on the FAST scan image, zoom in on the scanned image to locate the fiduciary marks. In the "calibrate" mode, click on the center of each fiduciary mark in the sequence of "upper left," "lower left," and "upper right" corners.
4. Load the slide onto the microscope stage. Find centers of the fiduciary marks under 20× bright field, in the same sequence as in finding the marks on the FAST scanned image. Once the center of each mark is located under the microscope, use "Get x,y" function to get the coordinates of each fiduciary mark (Fig. 2). And then click "Apply."

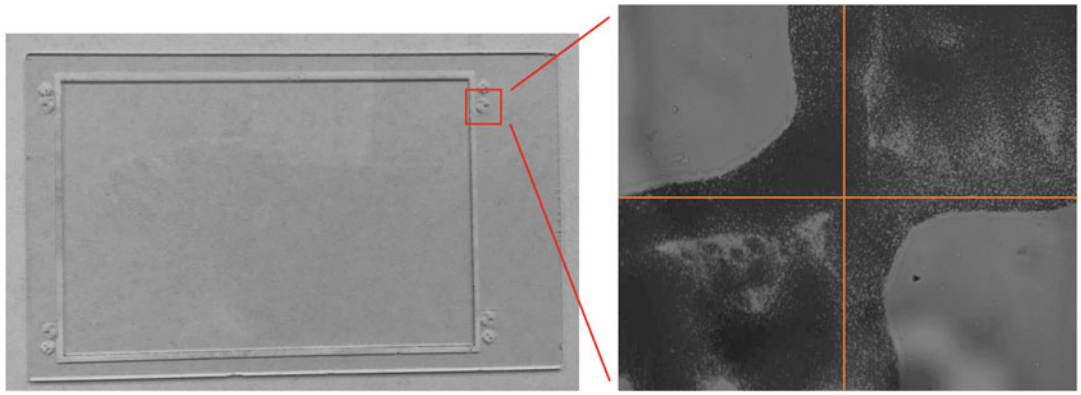

Fig. 2 Fiduciary markers (in *red box*) are placed onto the FAST slide and used for calibration of coordinates on ADM and cell picking platform

5. In the Camera tab, adjust exposure time for each channel (*see* **Note 9**).
6. Click "Capture," to capture a 3-channel image. Confirm the exposure time for DAPI, TRITC, and Q-dot 800 is appropriate.
7. Use "autofocus" function to determine the off-set between the center of true focus and the focus the software determines. Enter that value into the software to off-set the error in Z-axis.
8. Click "Start" to sequentially image all the potential target coordinates on the slide.
9. Review all images to determine which ones are "true positive" CTCs, by the criteria of cytokeratin (or tumor markers such as MUC1, HER2, EGFR, or plastin-3) positive intact cells with DAPI stained nucleus inside cytoplasm, at least 4 μm in size and negative for CD45 staining (Figs. 1c and 3a).
10. If additional biomarkers are stained on CTC for grading, such as Vimentin or PD-L1, all "true positives" need to be reimaged in the other channels. This can be done by labeling all "true positives" for retake. Then choose to take multiple images in Alexa-fluor 647, Alexa-fluor 700, Alex-fluor 750 or Qdot 705 channel in addition to the three channels that are used for CTC identification (DAPI, TRITC and Qdot 800). (Fig. 3b) (*see* **Note 10**).

3.5 Single Cell Isolation and Sanger Sequencing of CTCs Detected by FAST

1. Generate the pick target transfer list using the "Compute Statistics" function in the RawV2 software. Format the X.Y coordinates of the detected CTCs into an Excel worksheet and save the formatted pick list table into "comma separated values" (.csv) format.
2. Submerge the sample slide into 1× PBS for 30 min until the coverslip on the slide completely detaches (*see* **Note 11**).

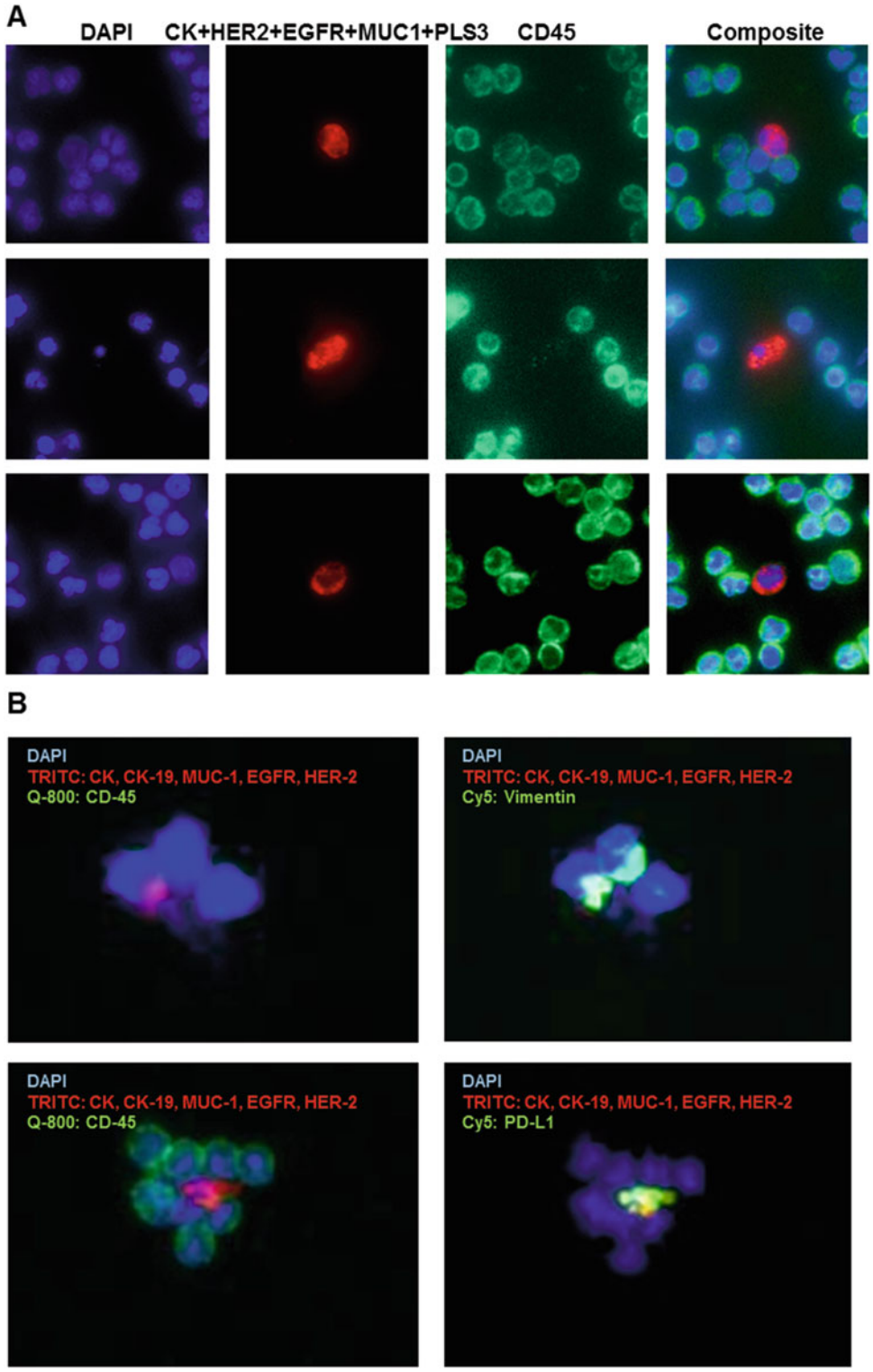

Fig. 3 (**a**) CTC can be identified as DAPI+, tumor marker+, CD45– cells with intact morphology and size > 4 μm. (**b**) Companion biomarkers can be analyzed on CTCs such as Vimentin and PD-L1

3. Place the uncoverslipped slide in a customized plate holder and transfer it to the ALS CellCelector platform.
4. Apply 1.5 mL of 1× PBS onto the active area of the slide using a 1 mL pipette to hydrate the sample.
5. Open the CellCelector software with the built-in function interface for pick list coordinates transfer. Import the ".csv" transfer list file.
6. Calibrate the coordinates four fiduciary marks by aligning them in the center of the field of view under 20× bright field. Click on the center of each mark to get the coordinates.
7. Click on each pick target to make sure that after calibration, they are centered in the field of view using both 20× bright field and 20× TRITC image settings. If not, use the "calibrate" function to re-center the target.
8. Place a 96-well PCR plate on the picking platform with 5 μL of cell extraction buffer preloaded into each well.
9. Calibrate the pick-up position and deposit height using the built-in functions in the software.
10. Mount a 30 μm diameter capillary onto the CellCelector. Sterilize the picking capillary by aspirating and dispensing 70% ethanol with the capillary.
11. Initiate the picking program (*see* **Note 12**).
12. Perform Whole-Genome Amplification (WGA) on the single cell deposited.
13. Examine the WGA product by electrophoresis (Fig. 4d) (*see* **Note 13**).
14. Use the WGA product for targeted PCR for genes of interest.
15. Confirm gene amplicon is again by electrophoresis (Fig. 4e).
16. If desired amplification is confirmed, clean up the product.
17. Measure the concentration of the purified product.
18. Adjust the concentration of the amplicon to 10 ng/uL. Prepare the sequencing reaction according to Table 1.
19. Sequence the target amplicon. An example of the result can be found in Fig. 4f.

4 Notes

1. Check if the protective layer (green) is fully removed from the slide, and if not, treat with methanol for an additional 10 s.
2. Use 1× ACK buffer to rinse the pipette used for transferring the blood and the collection tube(s) to ensure all cells were collected into the lysis tube.

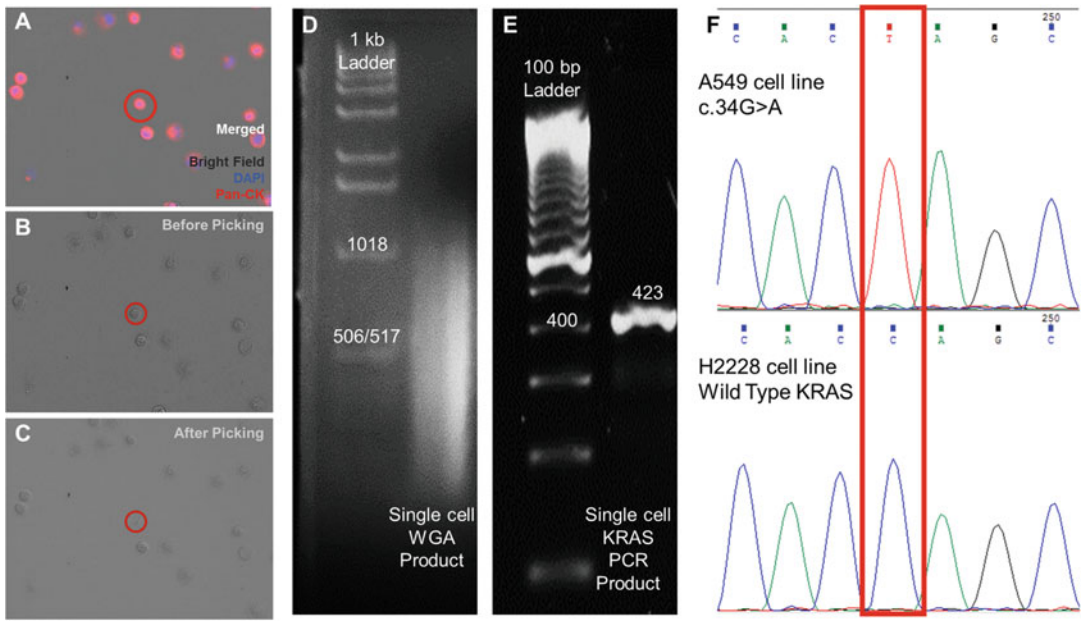

Fig. 4 (**a**) Merged image of immunofluorescently stained human A549 cell line to be picked. (**b**) A549 target cell in 20× bright field before picking. (**c**) A549 target cell in 20× after picking. (**d**) After whole genome amplification, the product is analyzed by electrophoresis. (**e**) KRAS gene is amplified by PCR, and the amplicon is analyzed by electrophoresis. (**f**) Sanger sequencing results indicate KRAS point mutation c.34 G>A detected in positive control cell line A549 but not in H2228 cell line as expected

Table 1
Sequencing reaction preparation

Reagents	Volume
PCR product (10 ng/μL)	10 μL
Custom primer (forward or reverse)	2 μL
Total volume	12 μL

3. Flick the tube gently until the entire pellet is resuspended into 1× PBS.
4. If more than one tube is used for blood lysis, use 1 mL of 1× PBS to first resuspend the pellet in one tube and then transfer the cell suspension (1 mL) to the next tube to resuspend the next pellet in the same 1 mL of 1× PBS; thus, all PBMCs from the same patient will be resuspended in the same 1 mL of 1× PBS.
5. Check the plate for cell suspension distribution after 3 min incubation. Make sure the cell suspension is dispensed evenly on the plate, and if not, re-level the shelf in the incubator until

the cell suspension can be distributed well on the plate without areas drying.

6. Gently add the 2% PFA onto the plate using a 1000 μL pipette. Adding the 2% PFA from too high above the plate may result in cells lifting up from the plate. Carefully roll plate to distribute 2% PFA evenly, and use a pipette tip to push solution all the way to the edges and corners.
7. Five slides can be treated with acetone in each glass tray. If more than five slides are being treated, prechilled acetone can be used for up to three batches of slides sequentially. After acetone treatment, slides can be air-dried and stored at −80 °C. If slides are stored, re-treat slide with prechilled acetone for 5 min when bringing out the slides from −80 °C before proceeding to **step 5** (Subheading 3.2).
8. Coverslip the slide with FluorSave mounting medium (345789, Millipore) if the endpoint is CTC enumeration. Coverslip the slide with the house-made fluorescence mounting media (*see* Subheading 2) if the endpoints is to uncoverslip and pick single cells for Sanger Sequencing.
9. Adjust the exposure time for each channel as follows: adjust DAPI exposure so that about 30% of leukocyte nuclei are partially saturated. Switch to Q-dot 800 channel (CD45) and adjust exposure so that 10% of leukocytes show saturated signal around their circumference and the signal is also visible in the internal region of the cell (i.e., where view angle is perpendicular to the cell membrane).
10. For quantification of companion biomarker, a moderate expressor for each sample was used as positive control, and leukocytes from the same sample were used as negative control. The expression level was determined by averaging the intensity of the pixels in the region where the marker protein was expressed. Then CTCs with an expression level between the 16th and 68th quantiles of the positive control distribution were scored 2, while the CTCs expressing higher levels were scored 3. CTCs expression level lower than the 68th quantile was scored as 0. The percent population was scored linearly, as less than 10% of population of CTCs expressing the marker was scored as 0, and 90% to 100% of the CTC population expressing the marker was scored as 10.
11. If the coverslip does not fully detach after PBS treatment, gently lift up the coverslip using forceps. Try avoiding sliding of the coverslip on the sample while lifting.
12. Set the picking parameters in the CellCelector program as follows: Pre-pick buffer volume: 1.5 μL (PCR grade water in buffer tank); aspiration volume: 1.5 μL; aspiration speed: 1%; scraping distance: 40 μm; scraping speed 2%. After each pick,

check the pre- and post-pick image to ensure the cell was successfully picked without picking up neighboring cells (Fig. 4a–c).

13. The majority of the WGA product should be at a length of between 200 bp and 1 kb.

Acknowledgment

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Chapter 21

A Noninvasive and Real-Time Method for Circulating Tumor Cell Detection by In Vivo Flow Cytometry

Xunbin Wei, Jian Zhou, Xi Zhu, Xinrong Yang, Ping Yang, and Qiyan Wang

Abstract

The quantification of circulating tumor cells (CTCs) has been considered a potentially powerful tool in cancer diagnosis and prognosis, as CTCs have been shown to appear very early in cancer development. Great efforts have been made to develop methods that were less invasive and more sensitive to detect CTCs earlier. There is growing evidence that CTC clusters have greater metastatic potential than single CTCs. Therefore, the detection of CTC clusters is also important. This chapter is aimed to introduce a noninvasive technique for CTCs detection named in vivo flow cytometry (IVFC), which has been demonstrated to be capable of monitoring CTCs dynamics continuously. Furthermore, IVFC could be helpful for CTC cluster enumeration.

Key words In vivo flow cytometry, Circulating tumor cells, Cancer metastasis, Fluorescence detection, Photoacoustic detection, Circulating tumor cell clusters

1 Introduction

1.1 Metastasis

Metastatic diseases are still the main cause of cancer-related death despite some achievements in cancer therapy [1]. Metastasis is a multistage process in which tumor cells spread to other tissues or organs. It starts with the invasion by primary tumor cells and followed by their intravasation into tumor vasculature. The tumor cells then enter and survive in the circulation as single circulating tumor cells (single CTCs) or circulating tumor cell clusters (CTC clusters). Circulatory pattern and different structures of capillary walls can affect where circulating tumor cells travel and stay [2]. Circulating tumor cells (CTCs) usually get arrested in the first set of capillaries they encounter downstream from where they enter into the blood. After being arrested in capillaries at distant sites, CTCs extravasate the endothelial layer and basement membrane, then colonize to form the metastases (Fig. 1). However, the majority of CTCs die in bloodstream while a tiny minority of CTCs can successfully form metastases [2, 3].

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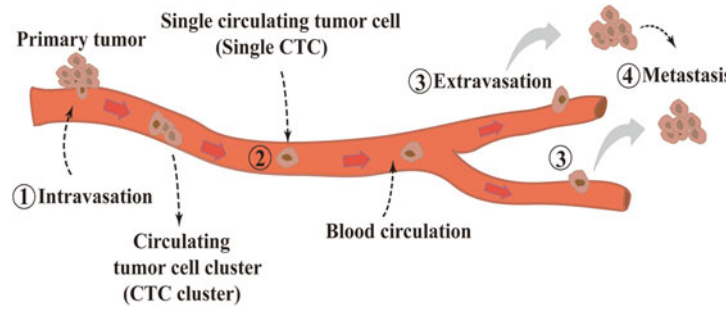

Fig. 1 The process of metastasis. ① Intravasation: primary tumor cells invade surrounding tissues and intravasate into the blood circulation. ② After entering the blood vessel, tumor cells circulate as single cells (single CTCs) or cell clusters (CTC clusters). ③ CTCs extravasate to distant organs. ④ After extravasation, CTCs seed, proliferate and colonize to form metastases

1.2 CTC Detection

The CTC was first reported by Ashworth in 1869 [4]. CTCs have been detected in the majority of cancers of epithelial origin, including breast, liver, colon, prostate, and pancreas as well as in cancers of non-epithelial origin, such as glioblastoma multiforme and melanoma [5–8]. Due to the important role in cancer metastasis, CTCs have been considered to be responsible for cancer progression [9, 10]. The detection of CTCs has been considered to provide a powerful tool for assessing the risk of relapse, monitoring treatment, and guiding anti-cancer therapy [11–13].

CTCs can appear in patient's blood at the early stages of tumor development. CTCs are very rare events at the level of 1 CTC in 7.5 ml of blood [14], which means 1 CTC mixed with 10^{10} blood cells, thus making high-sensitivity and high-specificity prerequisites for CTCs detection technique.

In general, conventional methods for CTCs detection usually include two steps: enrichment and detection. Enrichment is based on the various properties of CTCs, including size, density, cell surface protein expression, etc. These methods can be categorized into three types: immune-mediated methods, cytometry-based methods, and RT-PCR-based methods.

1.2.1 Immune-Mediated Methods

ISET (Isolation by Size of Epithelial Tumor Cells) is a size-based method, which utilizes the larger size of CTCs (>8 μm) compared with small leukocytes [15, 16]. 8 μm pores filters are used in this method to remove peripheral blood leukocytes. The enriched cells then can be characterized by immune-labeling. However, its sensitivity is under suspicion for lack of evidence that CTCs are bigger than leukocytes all the time.

The immunological assays use antibodies directed toward either tumor-specific antigens (such as EpCAM or cytokeratins) or common leukocyte antigen CD45. Among existing EpCAM-

based methods, CellSearch® system [17, 18], the only FDA-approved immunological assay for CTC detection, is widely concerned over the past 10 years. CTCs can be enriched by ferrofluid nanoparticles coated with antibodies directed against EpCAM. The enriched cells are then fixed and stained with DAPI, CK, and CD45. The CK+/DAPI+/CD45– cells are then counted as CTCS by the CellSpotter analyzer (Veridex).

Recently, a microfluidic platform named CTC-chip has been developed. This chip consists of 78,000 anti-EpCAM antibodies coated microposts, where EpCAM-positive cells are captured. Once captured, the cells can be detected by a camera to recognize their morphology, viability, and tumor markers [19].

However, it is important to note that not all types of tumor have been found to express EpCAM, which may result in false-negative selection [20]. In addition, false-positive selection could also occur due to the expression of the same antigens on non-tumor cells [21].

1.2.2 Cytometry-Based Methods

Cytometry-based methods use monoclonal antibodies to directly target different epithelium-specific antigens (CK, EpCAM, etc.) to isolate and count cells. After being labeled, the cells are scanned by different methods to identify the rare CTCs, e.g., fiber-optic array scanning technology (FAST) [22], the laser scanning cytometer (LSC) [23], and flow cytometry (FCM) [24]. By using FCM technology, many researchers start to study the mechanism of CTCs by examining the expression of CD44, EGFR, ALDH1, CD47, MET, and Heparanase [25, 26]. A disadvantage of flow cytometry technology is that cancer cells can easily settle and/or clump throughout the process [27]. As experiments typically require very large numbers of isolated CTCs, even high-speed sorters need to run for long durations. This is not only time-consuming and expensive, but also may affect the viability of cells. Consequently, the cells sorted from such long runs may no longer be usable for further characterization [28].

1.2.3 RT-PCR-Based Methods

Reverse transcription PCR (RT-PCR) can be used for detecting CTCs. CTCs have been reported to express tumor-specific genes, e.g., CK19 [29], MUC1 [30]. Therefore, RT-PCR can be used to amplify target mRNAs in CTCs by designing oligonucleotide primers specific for the gene of interest. The sensitivity of RT-PCR-based methods is higher than immune-mediated methods [31]. However both false-positive results and false-negative results can occur. It is also important to note that there is no single marker that will reliably identify CTCs [32, 33]. Alternatively, organ-specific markers such as HER2, transcription termination factor 1 (TTF-1), and CD56 may help to identify CTCs, which provide advantages to the detection of metastasis in early stage [34].

All of these in-vitro methods require taking blood samples. However, CTCs are rare cells in the circulation. Consequently, it is hard to know when to extract blood while CTCs could be missed by using these methodsv.

1.3 In Vivo Flow Cytometry

In order to overcome the limitations in conventional methods, Lin's group developed an in vivo technique for CTC detection, called in vivo flow cytometry (IVFC) [35], which combined the concept of standard flow cytometry and confocal detection. IVFC has been proved that it could be used to reproducibly quantify the number of CTCs [36]. The first generation of IVFC [35] was based on the principle of fluorescence excitation. Some other types of IVFC devices were successful developed afterward. This section is going to introduce two types of IVFC and their applications.

1.3.1 Fluorescence-Based IVFC

Cells of interest are needed to be labeled before using this type of IVFC to detect CTCs. The basic principle of fluorescence-based IVFC is similar to conventional FCM, except that a natural blood vessel in a living object is used to replace the single flowing sheath in conventional flow cytometer. When a fluorescently labeled cell passes through a slit of laser beam focused across a blood vessel, it can be excited to emit fluorescence. This signal then can be detected by a PMT (Fig. 2). Thus, biological information can be obtained for a long period of time without blood drawing. Details of how to use fluorescence-based IVFC to monitor CTCs will be provided in Subheading 3.

1.3.2 Photoacoustic Flow Cytometry

Photoacoustic flow cytometry (PAFC) is a promising method to detect CTCs based on photoacoustic effect. Due to different light absorption rates between cells of interest and background, the intensity of photoacoustic effect differs greatly. Therefore, some specific cancers cells, e.g., melanoma cells, can be detected in vivo

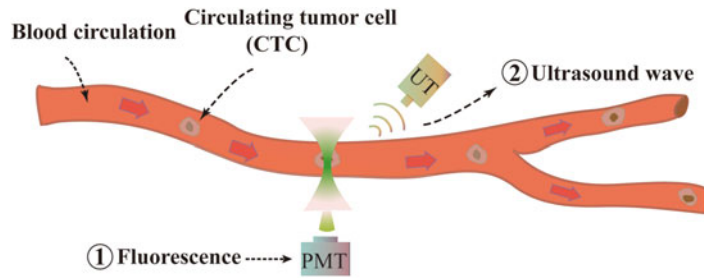

Fig. 2 Basic principle of IVFC and PAFC. A laser slit is focused across a blood vessel. ① Fluorescence-based IVFC: when a fluorescently labeled cell passes through the laser slit, a burst of fluorescence will be generated and recorded by a PMT. ② PAFC: when a cell with higher light absorption rate passes through a pulsed laser beam across a vessel, higher intensity ultrasound waves will be produced and detected by an ultrasonic transducer (UT)

[37]. PAFC was first developed by Zharov et al. [38]. It has been applied for more kinds of detection such as detecting white blood cells and other cancer cells.

When a laser irradiates biological tissue, the tissue absorbs energy and thermoelastic expansion is induced by a slight temperature rise [39]. Then a sound or stress wave is produced, which is called photoacoustic effect. This is a process of light energy conversion to heat energy. Based on this principle, when cells possessing higher light absorption rate flow through a pulsed laser beam put on the vessel, they will produce higher intensity ultrasound waves compared with the blood background. The ultrasound waves can be detected by a piezoelectric transducer (Fig. 2). By this principle, in vivo detection of melanoma CTCs can be realized. B16F10 is one kind of melanoma cells, which contain melanin for photoacoustic signal detection.

1.3.3 The Fluorescence-Based IVFC May Be Helpful for CTC Cluster Detection

In addition to be observed as single cells, CTCs have been recognized as clustered cells in both mouse models and blood samples from cancer patients. CTC clusters were reported as early as 1950s [40] and were later shown to range from 2 to 50 tumor cells that break off from primary tumors [41–44]. It was found out that CTC clusters showed higher metastasis potential than single CTCs when injected intravenously into mice [42]. A study from Dive's group has demonstrated that CTC clusters had a survival advantage over single CTCs, while their appearance was associated with poor clinical outcomes in small-cell lung cancer (SCLC) patients by using CellSearch® system [45]. Haber's group has established a mouse model to verify the significant source of CTC clusters. When injected a 1:1 mixture of MDA-MB-231-LM2 (LM2)-GFP cells and MDA-MB-231-LM2 (LM2)-mCherry cells into the mammary fat pad of immunodeficient (NSG) mice, 91% of CTC clusters were found out to be dual positives for GFP and mCherry. This implied that CTC clusters could be the aggregation of cells within the primary tumor rather than the proliferation of single cells in the vasculature. After that, they created another orthotopic mouse model by injecting LM2-GFP cells into one side of mammary fat pad and LM2-mCherry cells into the other side. It was found out that 96% of CTC clusters were single positive for either GFP or mCherry. This result demonstrated that the overwhelming majority of CTC clusters were derived from primary tumors [5].

Microfluidic devices are widely used in the detection of CTC clusters. Ordinary microfluidic devices that were designed to isolate single CTCs can also be used in CTC clusters isolation. However, CTC clusters may be lost given the different flow conditions for single CTCs capture. Sarioglu and his partners [46] have reported a Cluster-Chip, which took advantage of the stable equilibrium of clustered cells. They demonstrated this chip could realize the

specific and label-free isolation of CTC clusters from patients with various types of cancer. It allowed further analyses of the biological properties of CTC clusters and their roles in cancer progression and metastasis.

Nevertheless, in vitro methods are not capable of real-time detection. One as yet unpublished study from Wei's group indicates that fluorescence-based IVFC may be helpful to monitor CTC clusters. Fluorescence signal of CTC clusters displays as multiple peaks while that of single CTCs displays as single peak. Besides, signal of CTC clusters has a wider time duration than single CTCs. Utilizing the features of CTC clusters, Wei's group shows the mean proportion of CTC clusters increased to 27.7% and 36.7% in the late stage of liver cancer and prostate cancer, respectively. Those results suggest that the role of CTC clusters in metastasis may be more important than previously believed.

2 Materials

2.1 Circulating Hepatocellular Carcinoma Cells (Control)

1. A human hepatocellular carcinoma cell line HCCLM3.
2. High-glucose DMEM medium.
3. Fetal bovine serum (FBS).
4. pEGFP-C1 plasmid.
5. Lipofectin reagent.
6. G418.
7. Balb/c nude mice (male, 6-week-old, 20 ± 2 g).
8. Pentobarbital sodium salt.
9. 70% alcohol.
10. Betadine scrub.
11. 7–0 suture.
12. Glycerine (*see* **Note 1**).

2.2 Circulating Melanoma Cells (Control)

1. A mouse melanoma cell line B16F10.
2. High-glucose DMEM medium.
3. Fetal bovine serum (FBS).
4. Phosphate-buffered saline (PBS).
5. Balb/c mice (male, 6-week-old, 20 ± 2 g).
6. Balb/c nude mice (male, 6-week-old, 20 ± 2 g).
7. Pentobarbital sodium salt.
8. Glycerine.

2.3 Fluorescence-Based IVFC

1. A 488 nm laser.
2. A Light Emitting Diode (LED): 535 ± 15 nm.
3. A 40 × Objective: NA = 0.6.
4. Two dichroic mirrors: R25/T75; R510 nm/T488 nm.
5. Two bandpass Filters: 510 ± 10 nm; 530 ± 10 nm.
6. A CCD.
7. A Cylindrical Lens.
8. Two mechanical Slits.
9. A PMT.
10. Three achromatic lens.
11. A current amplifier.
12. A data acquisition card: 5 KHz.
13. A three dimensional adjusting sample stage.

2.4 PAFC

1. A pulsed laser: wavelength of 1064 nm, pulse width of 8 ns, the energy of single pulse is 60 μJ, the diameter is 1 mm.
2. A function generator.
3. Three reflectors.
4. Three concave lens: each focal length is 150 mm.
5. A cylindrical lens: focal length is 150 mm.
6. A convex lens: focal length is 150 mm.
7. A high pass beam splitter: 800 nm.
8. A 40 × Objective: NA = 0.6.
9. An LED light: wavelength of 524 nm.
10. A CCD camera: an 800 nm high pass filter is put in CCD camera.
11. A water immersion ultrasound transducer: center frequency is of 10 MHz, segment size is of 3 mm.
12. A pre-amplifier.
13. A date acquisition (DAQ) card: 200 MHz.
14. An image acquisition card.
15. A computer: the DAQ card and image acquisition card are in host.
16. A three dimensional adjusting sample stage.

3 Methods

Fluorescence-based IVFC can be single-color, two-color, or multi-color. Here, we take single-color IVFC as an example to demonstrate how it works.

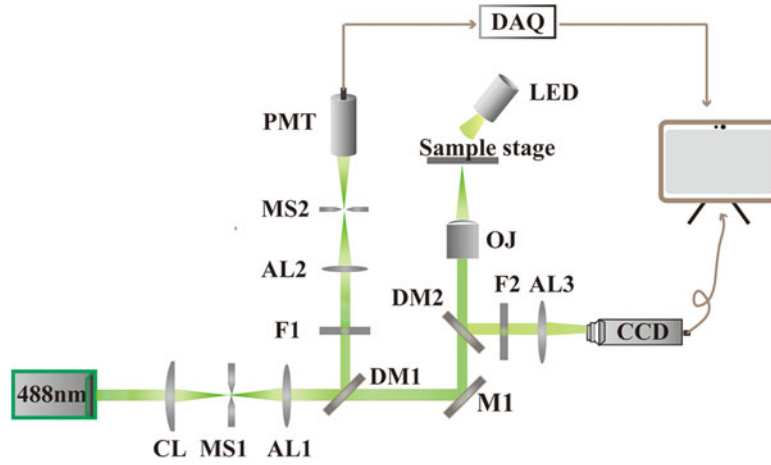

Fig. 3 A schematic of a fluorescence-based IVFC setup. *CL*, cylindrical lens; *MS1–MS2*, mechanical slit; *AL1–AL3*, achromatic lens; *DM1, DM2*, dichroic mirrors; *F1, F2*, band pass filters; *PMT*, photomultiplier tube; *CCD*, charge-coupled device; *M1*, mirrors; *DAQ*, data acquisition; *PMT*, photomultiplier tube

3.1 Experimental Setup

3.1.1 Fluorescence-Based IVFC

1. The fluorescence-based IVFC setup that is designed for detecting GFP^+ cells is shown in Fig. 3.
2. Imaging system: The sample (e.g., a mouse ear (*see* **Note 1**)) is positioned on the sample stage. The blood vessels are visualized by microscope objective onto a CCD camera using a 530 nm LED trans-illumination system (*see* **Note 2**).
3. Fluorescence excitation system: Fluorescence excitation is provided by a 488 nm laser. Light from the laser is shaped to a slit by a cylindrical lens (CL) and a mechanical slit (MS1). The laser slit is imaged across a blood vessel by a microscope objective lens (OL), The approximate length and width of the slit-shaped laser beam at the focal plane are 72 μm and 5 μm, respectively.
4. Fluorescence detection system: The fluorescence emitted from a fluorescently labeled cell when it passes through the laser slit is collected by the same objective (OL), reflected by a beam splitter DM1, then detected with a PMT, behind the confocal slit (MS2) and a bandpass filter (F1).
5. Data acquisition and processing: The signal is sampled at a rate of 5 KHz with a data acquisition card (DAQ), stored on a computer, and analyzed by Measure Foundry along with homemade software coded by MATLAB.

3.1.2 PAFC

The setup of PAFC includes three parts as shown in Fig. 4: illumination part, excitation part, and detection part. Here, we take the single-color PAFC as an example to demonstrate how it works.

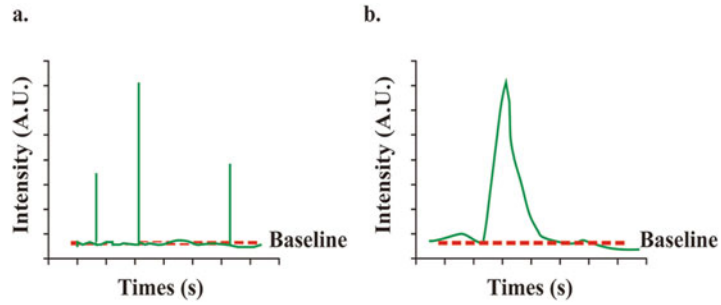

Fig. 4 Framework of photoacoustic flow cytometer includes three parts: (1) illumination part (LED light, objective (OB), beam splitter (BS), reflector (R), CCD Camera), (2) excitation part (pulsed laser, two reflectors, three convex lens (CL1), cylindrical lens (CL2), concave lens (CL3), beam splitters, objective), (3) detection part (ultrasonic transducer (UT), pre-amplifier, data-acquisition card (DAQ Card), computer). Laser and DAQ card are synchronously triggered by function generator

1. Fix the laser in an appropriate location (*see* **Note 3**) and the laser light is pumped.
2. The laser light is reshaped by optical elements including two reflectors, three convex lens (CL1), a cylindrical lens (CL2), a concave lens (CL3), a beam splitter, and an objective (*see* **Notes 4–8**).
3. A sample stage is above the objective while an LED is to light the sample for imaging. A reflector is to reflect the image of the cover glass to the CCD camera. An image acquisition card is used to acquire images from the CCD camera (*see* **Notes 9** and **10**).
4. Signals are detected by an ultrasound transducer, amplified by a pre-amplifier and recorded by a DAQ card (*see* **Note 11**).
5. A function generator is used to synchronously trigger the laser and DAQ card.

3.2 Monitoring of Circulating Hepatocellular Carcinoma Cells by Fluorescence-Based IVFC

3.2.1 Cell Transfection

1. Human HCC cell lines with high metastatic potential (HCCLM3) [47] are grown in high-glucose DMEM medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2.
2. Transfect HCCLM3 cells with linearized pEGFP-C1 using Lipofectin reagent.
3. Select stably transfected populations by culturing the cells in the same media as **step 1** described supplemented with 600 μg/ml G418 at 37 °C and 5% CO_2.
4. GFP-positive cells are isolated by fluorescence-activated cell sorting (FACS).

5. Culture HCCLM3-GFP cells in the same medium supplemented with 600 μg/ml G418 at 37 °C and 5% CO_2.

3.2.2 Subcutaneous (s.c.) Metastatic Tumor Model

1. When cells are 70–80% confluent, 3–4 h before harvesting, replace the medium with fresh medium to remove dead and detached cells.
2. Anesthesia is induced by intraperitoneal injection of pentobarbital sodium salt (1%, 0.1 ml/g mouse weight).
3. The anesthetized mice receive 5×10^6 HCCLM3-GFP cells of passage 0 or 1 by subcutaneous injection on the back of the mice.

3.2.3 Orthotopic Metastatic Tumor Model

1. Orthotopic tumor implantation is performed under aseptic conditions.
2. Remove tumor tissue from maternal tumor (s.c. tumor or orthotopic tumor) and cut it into small cubes about $1 \times 1 \times 1$ mm.
3. The mice are anesthetized with pentobarbital sodium salt (1%, 0.1 ml/g mice weight). The upper abdomen is sterilized with 70% alcohol and betadine scrub before opening up.
4. Make a small abdominal incision to expose left lateral lobe of the liver. Implant a $1 \times 1 \times 1$ mm cube of HCCLM3-GFP tumor.
5. The abdominal wall and the skin are closed with a 7–0 suture.

3.2.4 Detection

1. The IVFC measurement is performed for at least 1 h once per week for each mouse starting from tumor implantation till its death.
2. The mouse is anesthetized and positioned on the sample stage with its ear adhered to a microscope slide by glycerine (*see* **Note 12**).
3. Turn on the LED. Select an artery of 50–70 μm with the guidance of the CCD camera (*see* **Note 13**).
4. Turn on the laser and start Measure Foundry. The monitoring can begin with preparations completed.
5. Process the recorded fluorescence signals (*see* Fig. 5) with software coded in-house using MATLAB to eliminate noise and identify the signal peak.

3.3 Monitoring of Circulating Melanoma Cells by PAFC

1. Mouse B16F10 cell lines with high metastatic potential are grown in high-glucose DMEM medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2.
2. Cells are detached and resuspended in PBS.

3.3.1 Cell Preparation

3. Count cells with cell counting plate.
4. The cells are diluted to 10^7/ml with PBS.

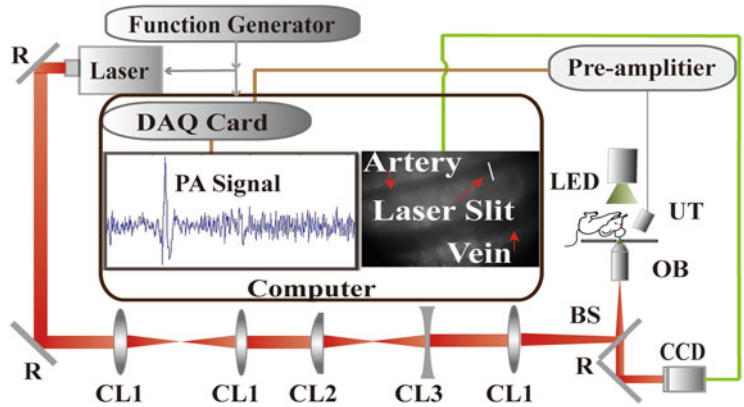

Fig. 5 (**a**) Typical data trace of fluorescence signals acquired by fluorescence-based IVFC. (**b**) A single peak fluorescence signal trace

3.3.2 In Vitro Detection

1. Turn on the PAFC system and set up the parameters (function generator: square wave, 5000 Hz, 5 V, rising edge trigger). Put the prepared cells in the syringe. Fix the syringe in the electric injection pump and connect it to a glass capillary.
2. Put the glass capillary on the sample stage. Make sure that the long dimension of the laser slit can cover the glass capillary.
3. Set up low speed of injection pump to mimic blood speed.
4. Put ultrasound transducer near the detection part (*see* **Note 14**). Couple the transducer and tube with water (*see* **Note 15**).
5. Start the syringe pump. The monitoring can begin with preparations completed. Photoacoustic signals are recorded and stored.

3.3.3 CTCs Detection After Tail Intravenous Injection

1. Turn on the PAFC system and set up the parameters (function generator: square wave, 5000 Hz, 5 V, rising edge trigger).
2. The mouse is anesthetized and positioned on the sample stage with its ear adhered to a microscope slide by glycerine.
3. Turn on the LED. Select an artery of 50–70 μm with the guidance of the CCD camera. Make the long dimension of the laser slit cover the selected vessel.
4. Put ultrasound transducer near selected artery and couple it with the mouse ear by ultrasound gel (*see* **Notes 14** and **15**).
5. Record the signals before injection as control. The detection is 60 min duration.
6. Take 200 μl of prepared cells and inject them by tail vein injection (*see* **Note 16**).
7. Detect signals immediately after injection. The detection is 60 min duration.

3.3.4 CTCs Detection After Subcutaneous Injection

1. The PAFC measurement is performed for at least 10 min once per week for each mouse starting from subcutaneous injection till its death. Record the signals before injection as control.
2. The mice are anesthetized with pentobarbital sodium salt (1%, 0.1 ml/g mice weight).
3. Inject 200 ml of prepared cells by subcutaneous injection in the armpit.
4. Turn on the PAFC system and set up the parameters (function generator: square wave, 5000 Hz, 5 V, rising edge trigger).
5. The mouse is anesthetized and positioned on the sample stage with its ear adhered to a microscope slide by glycerine.
6. Turn on the LED. Select an artery of 50–70 μm with the guidance of the CCD camera.
7. Put ultrasound transducer near selected artery and couple it with the mouse ear by ultrasound gel (*see* **Note 17**).
8. When laser spot is focused on the selected vessels, record the signal data. The detection is 10 min duration.
9. Process the recorded photoacoustic signals with software coded in-house using MATLAB to eliminate noise and identify the signal peak.

3.3.5 PA Signal Processing

1. Find obvious signals of high amplitude (*see* Fig. 6a).
2. Average and denoise the signals to decrease electronic noise (*see* Fig. 6b).
3. Make sure that the location of the signal appears in each pulse period (*see* Fig. 6c).
4. Transform the signals from time domain to frequency domain by fast Fourier transform (FFT) (*see* Fig. 6d).

4 Notes

1. Most experiments for IVFC detection of circulating cells are performed in thin, relatively transparent mouse ear.
2. 530 nm LED provides good contrast between blood vessels and surrounding tissues.
3. The height of the laser decides the height of the following lens.
4. The two reflectors are to keep the laser beam to the horizontal direction.
5. The two concave lens are to collimate the laser beam. The distance of the two lens is 300 mm.

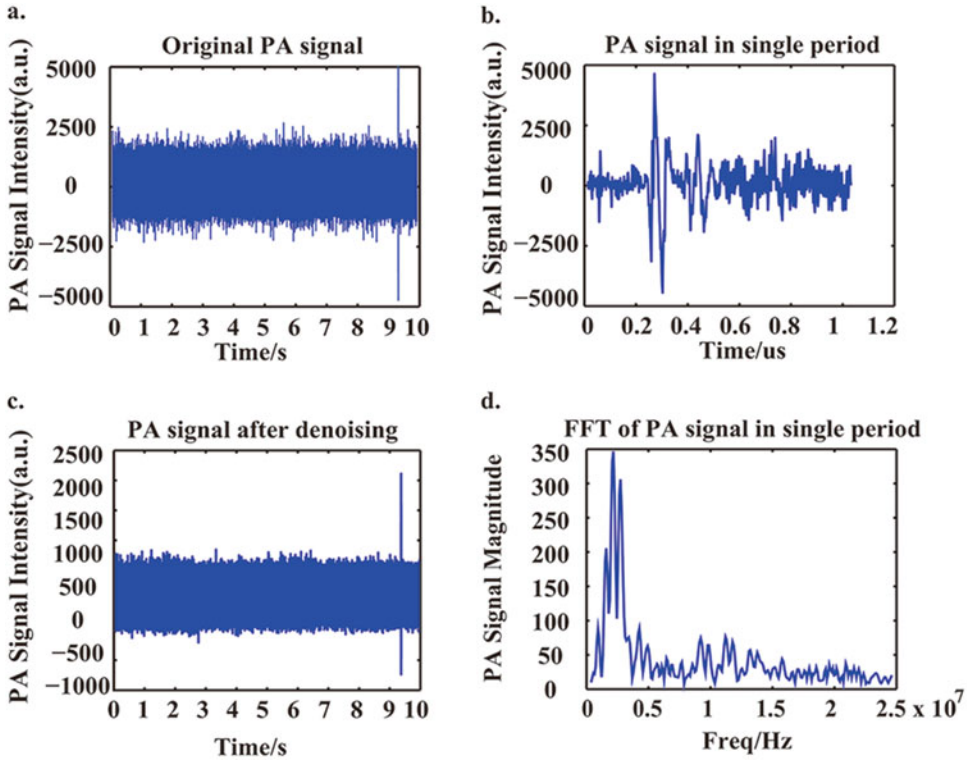

Fig. 6 PA signals detected from melanoma in vivo. (**a**) Original PA signals from melanoma cells in vivo. (**b**) Processed results of PA signals by averaging ten times and denoising algorithm with Matlab software. (**c**) PA signals in single period. (**d**) Signals in single period transformed to frequency domain by fast Fourier transform

6. The cylindrical lens are to reshape the laser beam to a slit shape. The distance between the cylindrical lens and the convex lens is 300 mm.
7. The convex lens and the concave lens are for laser beam expander. The distance between the convex lens and the concave lens is about 150 mm. The size of laser spot can be adjusted by changing the distance between the two lenses.
8. The beam splitter is to reflect the laser beam of 1064 nm to the objective. The distance between the concave lens and objective is 150 mm.
9. The reflector reflects the image to the CCD camera. While sometimes in the image the laser spot cannot be observed, the location of CCD camera and the angle of the reflector should be adjusted at the same time to make the laser spot show in the CCD image.
10. A piece of cover glass is put on the sample stage. The laser spot focused on the cover glass can also be seen.

11. The DAQ card has a software to record and display signals on computer screen edited with Matlab or Labview.
12. Glycerine is used as an optical clearing agent for enhancing photonic transference.
13. Make sure that the long dimension of the laser slit can cover the full width of the vessel, to avoid missing detection of cells.
14. The location of ultrasound transducer is very important to get high amplitude of signals. The transducer should be very close to the detection part of the sample. The direction of the transducer is usually to the focused laser spot.
15. The coupling agent is chosen depending on sound impendence of the sample. Water, glycerine, ultrasound gel can be used.
16. The concentration of cells is about 10^6/ml or less.
17. The typical detection positions in the animal are either the mouse ear or the mesenteric vasculature.

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Chapter 22

EpCAM-Independent Enrichment and Detection of Viable Circulating Tumor Cells Using the EPISPOT Assay

Alexandra Soler, Laure Cayrefourcq, Martine Mazel, and Catherine Alix-Panabières

Abstract

Identification and characterization of circulating tumor cells (CTCs) in peripheral blood can provide information on the direction and the efficacy of treatments. Current techniques such as CellSearch® are limited in differentiating between apoptotic and viable CTCs. In contrast, the fluorescent EPISPOT assay allows for the identification of viable cells by detecting proteins secreted/released/shed by functional single epithelial cancer cells. In addition, as CTCs are rare events, it is required to combine the EPISPOT assay with an enrichment step. In this article, the EPISPOT assay, as well as two technologies for enrichment of viable CTCs, RosetteSep™ and Parsortix™ techniques, will be presented and discussed in detail.

Key words Viable circulating tumor cells, EPISPOT assay, Solid cancers, Functional test, Circulating tumor cell enrichment

1 Introduction

The enumeration and characterization of circulating tumor cells (CTCs) in the peripheral blood and disseminated tumor cells (DTCs) in bone marrow may provide important prognostic information and might help to monitor efficacy of therapy [1].

Research on CTCs as new biomarkers has received great attention over the past decade. In particular, the capture and analysis of CTCs as "liquid biopsies" provides the possibility to avoid invasive tissue biopsies, with obvious implications in cancer diagnostics.

As CTCs occur at very low concentrations in the background of millions of normal leukocytes, a first step of enrichment is usually required [1]. Increasing the concentration of CTCs makes the subsequent CTC detection step easier to enable functional CTC studies; important points for keeping the CTCs intact and viable are: (1) to avoid any fixation and permeabilization of CTCs (i.e., use of tubes without fixatives), (2) to perform the blood analysis in a short time (<24 h), (3) not to perform intra-cellular proteins

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staining, and (4) to prepare CTCs under appropriate culture conditions.

Being a detection method, the EPISPOT assay can be combined, in principle, to any kind of CTC enrichment step. It includes a large panel of technologies based on the different properties of CTCs that distinguish them from the surrounding normal hematopoietic cells: physical (size, density, electric charges, and deformability) and/or biological properties (surface protein expression, viability, and invasion capacity).

Focusing on immunological assays, CTCs can be enriched via a positive or a negative selection using the expression of surface proteins such as Epithelial Cell Adhesion Molecule (EpCAM) (plus Epidermal Growth Factor Receptor (EGFR), HER2, Mucin-1 (MUC1)), or CD45, respectively. Positive selection requires that we have to make an assumption upfront on protein expression on CTCs. However, tumor heterogeneity is a hallmark of cancer; thus, it is very difficult to predict the spectrum of suitable capture antigens expressed on CTCs in an individual patient. Thus, during the last years, many groups focused more on the depletion of leukocytes, which shows two advantages: no bias for CTCs enrichment based on specific protein markers expressed at the cell membrane and no CTC stimulation or activation by the attachment of antibodies on membrane proteins. Concerning the physical properties for CTC isolation, size, deformability, electric charges, and density of CTCs remain suitable attributes to enrich CTCs. However, for subsequent functional assays and as already mentioned before , it is vital that tumor cells are neither fixed nor permeabilized and that they can be removed easily from the device used from enrichment without any damage. As an example, some filtration devices that require fixation of cells are not suitable for enrichment of viable CTCs.

The goal of this book chapter is to describe the principle and protocol of technologies for CTC enrichment before performing the fluorescent EPISPOT assay as well as those of the EPISPOT assay itself.

2 Materials for Enrichment and Detection of Viable CTCs

All the following experiments must be performed under sterile conditions at room temperature (RT) and all materials must be stored at 4 °C (unless indicated otherwise).

2.1 CTC Enrichment by the RosetteSep™ System (STEMCELL Technologies)

1. RosetteSep™, Human Circulating Epithelial Tumor Cell Enrichment Cocktail, Stem Cell Technologies (product No.: # 15167).
2. Lymphocytes Separation Medium, Eurobio (product No.: CH5H5L01-01).

3. Dulbecco's Phosphate-Buffered Saline 1× (D-PBS).
4. DMSO (Dimethyl Sulfoxide).
5. Decomplemented Fetal Bovine Serum (FBS).
6. Sterile tubes: 50 mL and 15 mL.
7. Blood samples in EDTA tube.

2.2 CTC Enrichment by Parsortix™ System (ANGLE)

1. Ethanol absolute .
2. D-PBS 1×.
3. Sterile tubes: 50 mL and 500 μL.
4. Blood samples in EDTA tube.
5. Cell Separation Cassette (ANGLE North America).
6. PARSORTER PR 1 (ANGLE North America).

2.3 Fluorescent EPISPOT Assay

1. MultiScreen-IP, 0.45 μm, clear, nonsterile (Product No.: MAIPN4550 Millipore).
2. Ethanol 70%.
3. D-PBS 1×.
4. D-PBS 1× with 5% BSA (Bovine Serum Albumine): Mix 5 g of BSA + 100 mL of D-PBS 1×.
5. D-PBS 1× with 0.5% BSA.
6. D-PBS 1×/0.1% tween20 (stock 4 °C cold room): Mix 5 mL of Tween 20 + 5 L of D-PBS. Use for plate washing with the washer.
7. RPMI 1640 medium with L-glutamin 1%.
8. Decomplemented FBS.
9. Plate washer.
10. CO_2 incubator at 37 °C.

3 Protocols of Technologies for Enrichment and Detection of Viable CTCs

3.1 CTC Enrichment by the RosetteSep™ System (STEMCELL Technologies)

Enrichment by protein expression is possible via the RosetteSep™ system from StemCell™ Technologies. It is a fast and easy immunodensity procedure for the isolation of untouched CTCs directly from whole blood where no columns or magnets are necessary. It is just needed to incubate , spin, and collect purified cells. This depletion of leucocytes is also called negative immunoselection.

By crosslinking unwanted cells namely leucocytes (neutrophils, eosinophils, basophils, lymphocytes, monocytes) to red blood cells by tetrameric complexes of antibodies present in the sample (inducing the formation of rosettes), target cells are purified during standard density gradient centrifugation (Fig. 1).

This protocol is designed in Fig. 2.

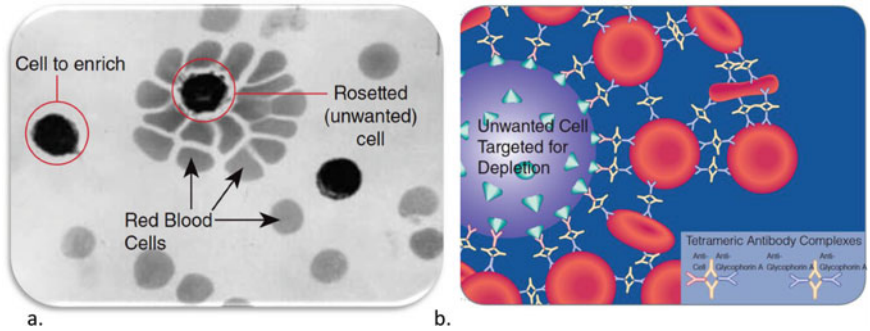

Fig. 1 General principle of immunorosette by the RosetteSep™ kit [9]. (**a**) Microscope picture of a blood sample after addition of the RosetteSep™ cocktail. (**b**) Illustration showing network of unwanted cell and red blood cells formed by RosetteSep™ tetrameric antibody complexes

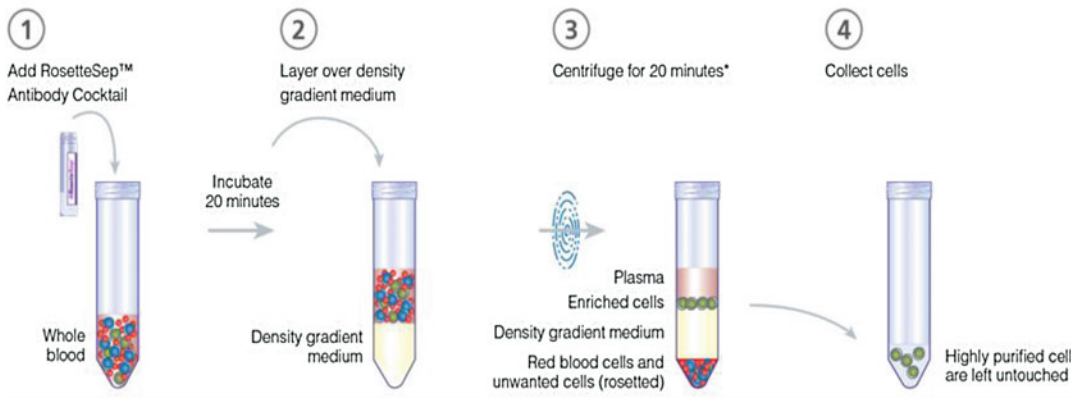

Fig. 2 Typical RosetteSep™ Protocol [9]

1. Blood samples are collected in EDTA tubes (*see* **Notes 1–3**) (Fig. 2).
2. Add RosetteSep™ Human Circulating Epithelial Tumor Cell Enrichment Cocktail at 50 µL/mL of whole blood (*see* **Note 4**).
   (a) Turn gently the tube without vortexing.
   (b) Incubate at RT for at least 20 min under slow rotation.
3. Dilute sample with an equal amount of D-PBS with 2% FBS.
   (a) Rinse lid and inner surface of tube carefully with the diluent.
   (b) Pipette up and down gently the mix.
4. Carefully transfer diluted sample on the top of Ficoll-Phase (Lymphocyte Separation medium) (Fig. 3).
   (a) Used Ficoll volume depends on blood volume (*see* Table 1).
5. Centrifuge the sample during 20 min at 1200 × *g* at RT (brake off).

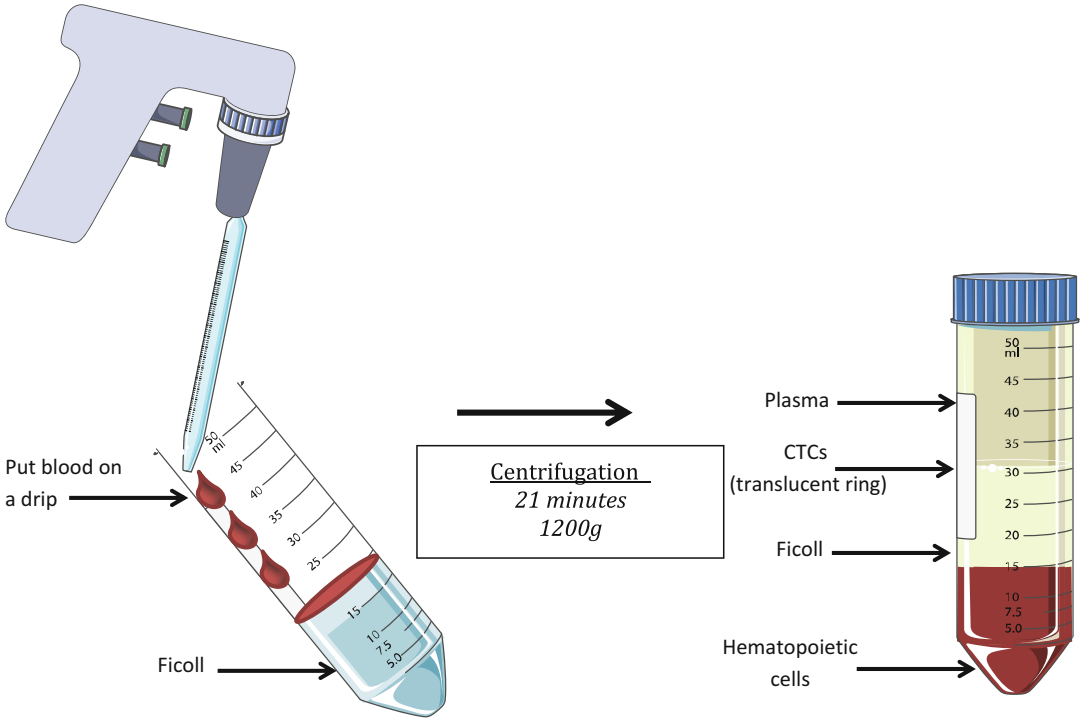

Fig. 3 Ficoll step advices for the RosetteSep™ enrichment. On the left, diluted blood is slowly let drop by drop on the Ficoll avoiding a "blood wave". After centrifugation, CTCs are in the transparent ring located at the interface between the plasma and the Ficoll (on the right)

Table 1
Recommended volume and tube for Ficoll step

Whole blood (ml)	PBS+2% FBS (ml)	Ficoll-Paque (ml)	Tube size (ml)
1	1	1.5	5
2	2	3	14
3	3	3	14
4	4	4	14
5	5	15	50
10	10	15	50
15	15	15	50

6. Remove totally the plasma phase including the enriched cells containing Ficoll-plasma interphase carefully and transfer into new 50 mL Falcon tube.
7. First wash: fill up Falcon tube with D-PBS with 2% FBS.

(a) Centrifuge during 10 min at 1200 g at room temperature.
(b) Discard the supernatant (*see* **Note 5**).

8. Second wash: fill up Falcon with D-PBS with 2% FBS.
   (a) Centrifuge during 10 min at 1200 × *g* at room temperature.
   (b) Discard the supernatant (*see* **Note 5**).
9. Dissolve the cell pellet in a final volume of 1.2 mL RPMI 1640 medium (+L-Glu, +IST, +FBS) in the case of using 12 wells (100 μL/well).
   (a) Ready for loading onto EPISPOT plate (day 2).

3.2 CTC Enrichment by Parsortix™ System (ANGLE)

Another way to enrich CTCs is to use the Parsortix™ system from ANGLE. The system is based on a micro-fluidic technology in the form of a disposable cassette to capture and then harvest CTCs from whole blood. The cassette captures CTCs based on their less deformable nature and larger size compared to other blood components.

The disposable cassette of the Parsortix™ system contains the cell separation gallery (Fig. 4). This gallery, arranged in staircase, is suitable for capturing CTCs in the blood. Cell sorting is then carried out via a specially designed system by the company ANGLE North America, the Parsorter, which by inducing a flow will allow CTCs to be retained in the cassette, while the blood cells are carrying their way.

Blood is integrated into the cassette through an inlet port, while unwanted cells exit through an outlet port disposed at the other end of the cassette. Between the two ports, the microfluidic circuit allows the sample to pass through the cartridge by trapping

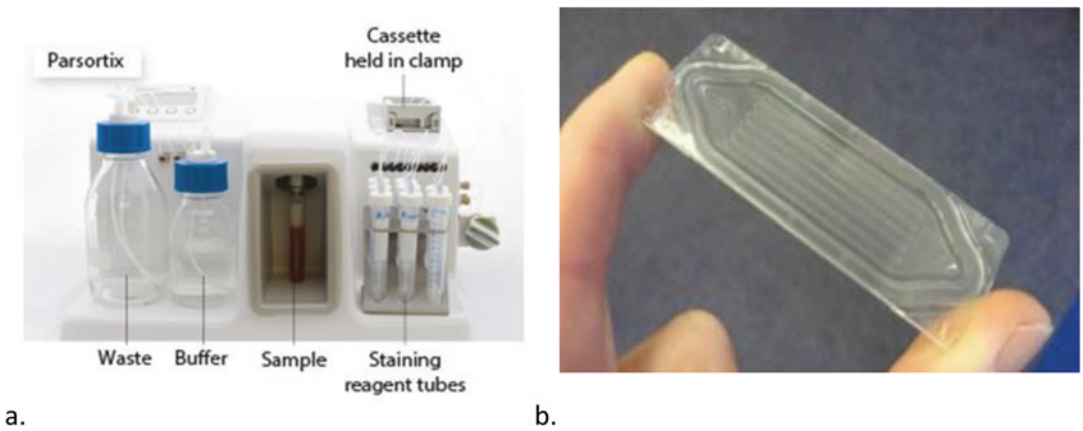

Fig. 4 How the Parsortix™ system works [10]. (**a**) Parsortix™ system. (**b**) Disposable Cassette. The disposable cassette is placed in a clamp, and the Parsortix™ system then automatically processes the patient blood sample

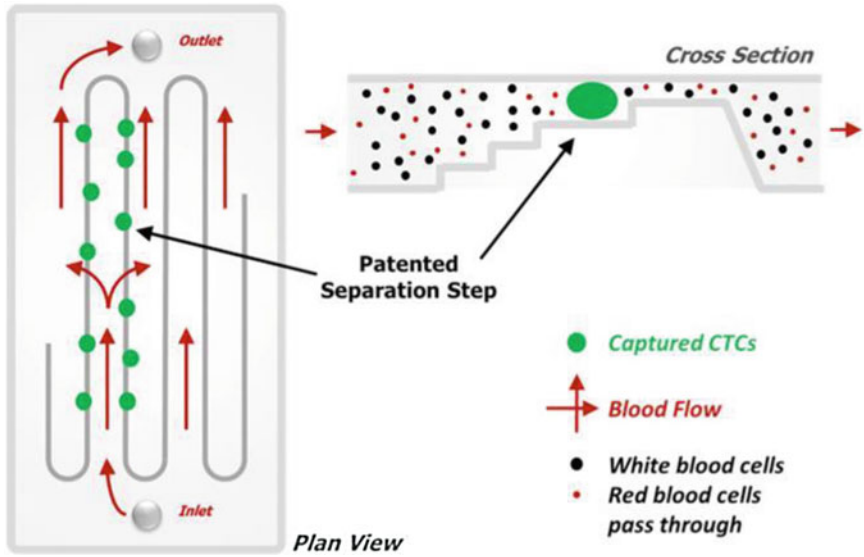

Fig. 5 Microfluidic system of the cassette [10]. CTCs are trapped in the cassette (last level of the steps) and only hematopoietic cells pass through the system

the CTCs in the stands (Fig. 5). Cell separation can be done, according to the tape with a spacing of 6.5 or 10 μm [2].

CTCs are then recovered by inducing a reverse flow, this step is called "harvest."

3.2.1 Priming

1. Select protocol PX2_PF and press [Run] then [Start] with the cleaning cassette.
2. On prompt insert a new separation cassette (*see* **Note 6**).
3. Press [OK] to start the priming process.
4. When the process is finished (after 15 min), press [OK] then [Continue].

3.2.2 Blood Separation

1. Select protocol PX2_S99F and press [Run] then [Start].
2. At prompt "Rinse vacutainer," partially remove the 50 mL tube and press [OK] to start rinse, collect the fluid in the tube.
3. Remove the tube, avoid flicking fluid off the tip of the line.
4. At prompt "Attach vacutainer" invert the sample several times to resuspend the blood cells, immediately mount onto the machine, and press [OK] (*see* **Note 7**).
5. At prompt "Start?" press [OK] to start the separation (*see* **Note 8**).
6. After approximately 30 min, resuspend the settled blood cells by tapping the vacutainer while it sits in the inclined position (*see* **Note 9**).
7. When the process is finished (after about 2 h for a 10 mL sample) press [OK] then [Continue].

3.2.3 Cell Harvest

1. Select protocol PX2_CT2 and press [Run] then [Start].
2. At prompt "Insert cleaning cassette," remove the separation cassette and insert the cleaning cassette, press [OK].
3. At prompt, "Empty rgt tubes," ensure reagent tubes are empty and press [OK].
4. When finished (after about 20 min) press [OK] then [Continue].
5. Remove cleaning cassette and reinsert separation cassette.
6. Select protocol PX2_H and press [Run] then [Start].
7. When prompted, rotate the harvest valve anticlockwise to the position "HAR" and press [OK].
8. At prompt "Start," remove the harvest line from de harvest waste tube and clean it with an alcohol wipe, wait until dry.
9. Place a collection vessel (e.g., an Eppendorf tube) beneath the harvest line.
10. Press [OK] to start the harvest .
11. 200 μL will flow through the line. If required, press [YES] to collect a further 1 mL or [NO] if not.
12. On prompt, rotate the harvest valve clockwise to the position "SEP" and press [OK]. Return harvest line to the position.
13. Press [OK] then [Continue].

3.3 Fluorescent EPISPOT Assay

Among all CTCs, both apoptotic and viable CTCs can be present in the peripheral blood of cancer patients. However, only viable CTCs can contribute to metastatic progression; thus, it is crucial to detect and characterize this specific subpopulation of CTCs. An easy way to detect non-apoptotic CTCs is to introduce apoptosis markers ($M30(^{+})Bcl\text{-}2(^{-})$) into immunological CTC assays such as the CellSearch® system. However, higher levels of CTC apoptosis were surprisingly associated with worse prognosis, while higher CTC-Bcl-2 levels correlated with better outcomes in breast cancer. Thus, the clinical relevance of introducing apoptotic markers into CTC assays is still under investigation.

The only existing functional assay detecting viable CTCs at the single-cell level is the EPISPOT assay (EPithelial ImmunoSPOT assay). It has been used on hundreds of patients with different tumor types [3] and allows the detection of viable clinically relevant CTCs.

The fluorescent EPISPOT assay is based on an in vitro short-term culture of CTCs. Its principle is simple and described in this chapter. Briefly, the nitrocellulose membranes of the ELISPOT plates are coated with an antibody against a specific protein marker. Then, cells are seeded in each well and cultured for 48 h. During this incubation step, specific secreted proteins are directly captured

on the antibody-coated membrane. Next, cells are washed off and a second antibody conjugated with a fluorochrome detects secreted specific protein markers. Immunospots are counted by video camera imaging and computer-assisted analysis (Fluorescent microscope (ZEISS) KS ELISPOT—Axio or ImmunoSpot_ Series 6 Ultimate Analyzer—C.T.L. Europe Gmbh with the Image Acquisition software, the BioSPot and ImmunoSpot Analysis software and the CellCounting software): one immunospot corresponds to the protein fingerprint of one viable cell. This assay is quantitative (the immunospots are counted) and qualitative (the proteins studied are well defined in the context of solid tumors, allowing the phenotypic characterization of the CTCs).

Dual fluorescent EPISPOT assay has been developed to target CTCs through the simultaneous secretion of two different proteins, allowing the detection of CTCs secreting Cytokeratin 19 (CK19)/Vascular endothelial growth factor (VEGF) in colon cancer, CK19/EGFR in head and neck cancer, CK19/HER2 in breast cancer. Moreover, the optimization of a multiple fluorescent EPISPOT assay is now in progress (Fig. 6).

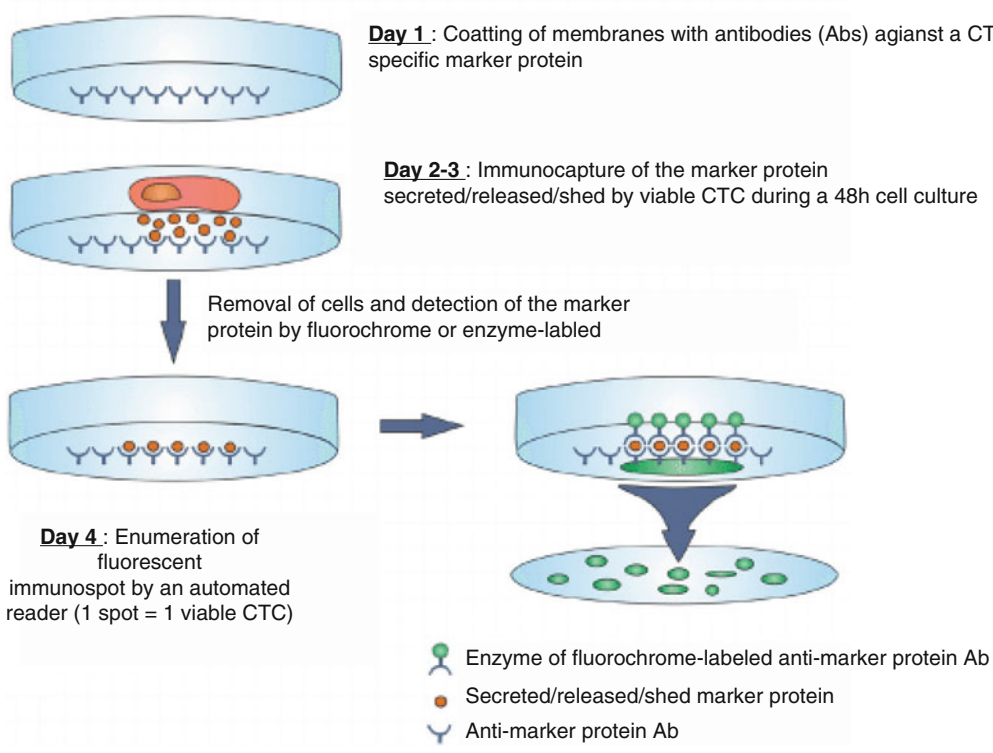

Fig. 6 Main schema of EPISPOT assay [4]

Day 1

Preparation of EPISPOT plate

1. Activate membranes of the 96-well plate with 25 μL 70% Ethanol (stored at 4 °C) in each well for changing membrane from hydrophobic to hydrophilic condition.
2. Incubation for 2 min at room temperature.
3. Wash each well three times with D-PBS.
4. Coat each well with 100 μL of antibody (diluted in D-PBS) that is specific for analyzed protein.
   (a) Appropriate antibody concentration depends on the antibody used.
5. Seal plate with an adhesive film.
6. Incubate at 4 °C overnight (until 5 days).

Day 2

Blocking of unspecific binding sites on membranes

7. Wash the plate once with D-PBS.
8. Add 100 μL of D-PBS with 5% BSA to each well.
9. Incubate at 37 °C for at least 2 h.

Preparation of cell lines for positive controls

10. Preheat cell media and D-PBS to 37 °C.
11. Wash cells in cell culture flask two times with D-PBS to get rid of the FBS.
12. Remove cells from the flask surface using Trypsin for adherent cells.
    (a) Add 1 mL Trypsin to a T25 flask, incubate at 37 °C until cells detach.
    (b) Add about 1 mL of FBS and 5 mL of cell media to inhibit the action of trypsin (*see* **Note 10**).
    (c) Transfer cells in to a 50 mL Falcon tube.
13. Determine the cell number using a Kowa slide counting chamber.
14. Prepare a solution of 40,000 cells/mL (*see* **Note 11**).
15. Ready for loading onto EPISPOT plate.

Loading cells on EPISPOT plate

16. After membrane blocking, wash the EPISPOT plate 1× with PBS using a plate washer.
    (a) If cells are not ready for loading after D-PBS wash add 100 μL of cell medium to each well to avoid dry out of membranes and store the EPISPOT plate at 37 °C.

17. Prepare cells for controls: 100 µL/well (=4000 cells per well).
18. Split enriched patient cells onto 12 wells for RosetteSep™ enrichment (to ensure the cell number loaded onto a membrane remains under 10,000, which is a requirement for reliable detection) and directly from the Parsortix in 1 well for the first 200 µL and dispatch the further 1 mL in 5 more wells at 200 µL per well (*see* **Note 12**).
19. Incubate plate at 37° (5%CO_2) for 24–48 h (without sealing plate).

Day 3

Addition of the fluorescence labeled antibody for the detection of secreted proteins

20. Wash plate six times with D-PBS/0.1% Tween 20 to remove cells.
21. Wash plate three times with D-PBS*.
22. Add 100 µL/well of secondary fluorescent antibody (diluted in D-PBS/BSA 5%) which is specific for analyzed protein but binds to another epitope than the coating antibody.
    (a) Appropriate antibody concentration depends on the antibody used.
23. Seal the plate with an adhesive film to avoid the evaporation of the D-PBS during the night.
24. Incubate the plate at 4 °C overnight as least.

Day 4

Detection of EPISPOT immunospots

25. Wash the plate 3× with D-PBS /0.1% Tween.
26. Wash the plate 3× with D-PBS (*see* **Note 13**).
27. Take off the underdrain of the plate and rinse membranes from underneath with tap water.
28. Let membranes dry completely for ~30 min at RT (*see* **Note 14**).
29. Transfer bottom of membranes on an adhesive film with the ELI-Puncher.
30. Observe immunospots on membranes with an inverse fluorescence microscope. Fluorescent immunospots were counted by video camera imaging and computer-assisted analysis (KS ELISPOT-Axio Imager M1; Carl Zeiss Vision/ ImmunoSpot_ Series 6 Ultimate Analyzer—C.T.L. Europe Gmbh with the Image Acquisition software, the BioSPot and ImmunoSpot Analysis software and the CellCounting software) (*see* **Notes 15**, **16** and **17**).
31. ONE fluorescent immunospot corresponds to ONE functional cell (Fig. 7).

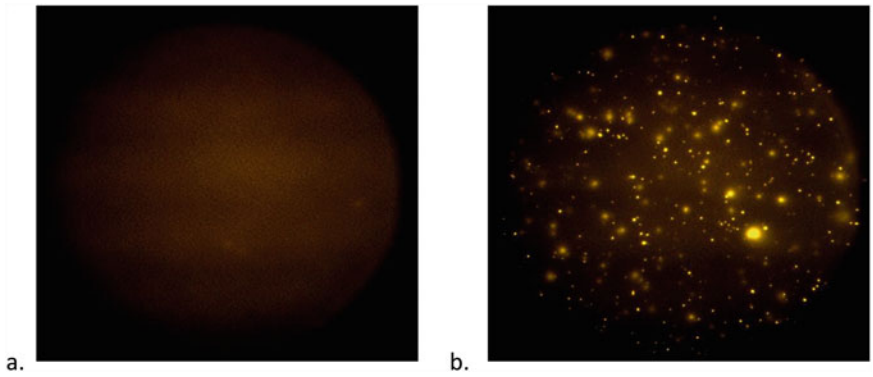

Fig. 7 Results obtained with the fluorescent CK19-EPISPOT assay (**a**) Membrane corresponding to the negative control: a well without any cells leading to a membrane without any immunospots. (**b**) Membrane with the presence of CK19-immunospots: a well with the CTC-MCC-41 cell line. CK19-releasing cells are detected using Alexa Fluor 555 conjugated anti-CK19 antibody. Each spot corresponds to a CK19-releasing viable tumor cell

3.4 Application of the EPISPOT Assay in Clinical Trials

In this chapter, we discuss the clinical relevance of detecting viable CTCs using the EPISPOT assay as well as present the clinical trials in progress where viable CTCs are evaluated as a new biomarker for treatment management.

For breast cancer, CK19, HER2, cathepsin D, and MUC-1 have been used as marker proteins, and the associated clinical data have shown that patients with CK19-releasing cells had an unfavorable outcome [4, 5]. For prostate cancer, prostate-specific antigen has been used for CTC detection and Fibroblast Growth Factor FGF2 as stem cell growth factor for further characterization [6]. For colon cancer, CK19 has been used for CTC detection [7], and our analysis showed that a considerable portion of viable CTCs detectable by EPISPOT are trapped in the liver as the first filter organ in colon cancer patients. Our clinical data demonstrated that patients with localized colon cancer and high numbers of CTCs have an unfavorable outcome. By adding drugs in the EPISPOT assay, the number and intensity of the immunospots can be decreased or inhibited when tumor cells are sensitive to these drugs, demonstrating their efficiency during short-time CTC culture [3]. This assay might be then considered an "*oncogram*" and might help to improve the clinical management of individual cancer patients.

Ongoing clinical trials in which the fluoro-EPISPOT assay is used to detect viable CTCs are [8]:

- *COLOSPOT study*: CTCs as predictor of response to chemotherapy and anti angiogenic therapy in metastatic colorectal cancer;
- *CIRCUTEC study*: CTCs as early response predictor in head and neck cancer patients receiving an anti-human epidermal growth factor receptor;

- *CTC-SCAN* study: CTCs as biomarker for minimal residual disease in high-risk prostate cancer; Prognostic relevance of viable CTCs in patients with localized prostate cancer;
- *MELANOSPOT study* : Prognostic relevance of viable CTCs in melanoma.

4 Notes

1. Do not manipulate blood of patients without protection;
2. If plasma has to be kept, replace it by D-PBS: centrifuge the EDTA tube 6 min at 1200 × *g*. Discard the plasma and replace it with the same volume of D-PBS;
3. To enrich rare tumor cells, rinse the tube with PBS correctly to not miss any CTCs;
4. After several in-house tests, the volume of RosetteSep tetrameric antibodies can be 20 μL (not less);
5. At the end of the RosetteSep procedure after washes steps, discard the PBS by turning quickly the tube instead of using a pipette: it allows not to touch the cell pellet containing the rare CTCs;
6. In the Parsortix system, the "Cleaning cassette" is a specific cassette always designated for cleaning steps. Each time, check carefully that the arrow is up and that the holes are on the left; otherwise the flow will be inverted;
7. In the Parsortix system, during the sorting, be careful not to put any blood on the top of the tube containing blood sample. This may block the pipe of the Parsortix to pump blood;
8. In the Parsortix, check if the blood flows correctly through the pipe;
9. In the Parsortix, if the blood does not flow very well, a succession of "click click" may be heard;
10. To prepare cancer cells (adherent cell lines) for the positive control of the EPISPOT assay: if cells cannot be unstick easily with the trypsin at 37 °C, you can also pat the flask to help them to detach;
11. For the EPISPOT assay, cell concentration can be adapt according to the protein we want to study and the cancer cell line used in that context: it can be from 1000 to 10,000 cells per well;
12. Make sure to transfer the entire volume of the patient sample prepared via RosetteSep™ or Parsortix™ systems to the EPISPOT assay;

13. Each time you wash plates in the EPISPOT procedure, pat the EPISPOT 96-well plate to eliminate the excess of D-PBS;
14. During the EPISPOT procedure, never let the membranes dry otherwise the experiment would be lost; the membranes have to be dried only at the last step;
15. In addition, never put any new drops on the membranes at the end of the experiments when they are dried: the experiment would be lost;
16. Be careful to use all fluorochrome-conjugated antibodies and fluorochromes themselves protected from light;
17. During the EPISPOT assay, do not shake the CO_2 incubator where the EPISPOT plates are; otherwise, immunospot formation will be perturbed due to cell movements.

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Chapter 23

Utilizing Matrigel Transwell Invasion Assay to Detect and Enumerate Circulating Tumor Cells

Xingtong Liu and Xiangwei Wu

Abstract

Metastasis is the cause of 90% of human cancer deaths. Circulating tumor cells (CTCs) in the peripheral blood and/or lymphatic vessels are cells shed from primary tumors and considered to be precursors of metastasis. Study of CTCs allows the serial monitoring of tumor progression and may provide predictive and prognostic biomarkers in clinic. Current CTC isolation and detection technologies encounter several challenges, including: heterogeneity of CTCs, low cell viability and/or high rate of contamination post-isolation, and the inability to distinguish viable/invasive from nonviable/nonfunctional CTCs, all of which can limit in vitro and in vivo characterization of CTCs. Here, we describe a new method to detect and enumerate of CTCs based on their invasive property.

Key words Circulating tumor cell, Functional CTC isolation, Invasion, Matrigel invasion assay

1 Introduction

CTCs are emerging as important biomarker for monitoring cancer treatment response and predicting clinical outcome [1]. However, the extra low number of CTCs in cancer patients (estimated as low as one CTC in 10^6–10^7 leukocytes of the peripheral blood) and heterogeneity of CTCs render the isolation and detection of CTCs a challenging task [2, 3]. Current CTC detection and isolation methods include cell surface marker-dependent procedures such as Veridex's CellSearch system, and filtration-based systems as represented by ScreenCell. The CellSearch system utilizes ferrofluids coupled to monoclonal antibodies against EpCAM to enrich for CTCs, and fluorescence-conjugated anti-cytokeratin monoclonal antibodies to detect. CellSearch is currently the only platform that has been cleared by the U.S. Food and Drug administration for disease prognosis and monitoring the effectiveness of treatment for patients with metastatic breast, colorectal or prostate cancer [4–6]. ScreenCell uses a filtration-based device to isolate CTCs from human whole blood based on cell size [7, 8]. However, cell-to-cell heterogeneity and

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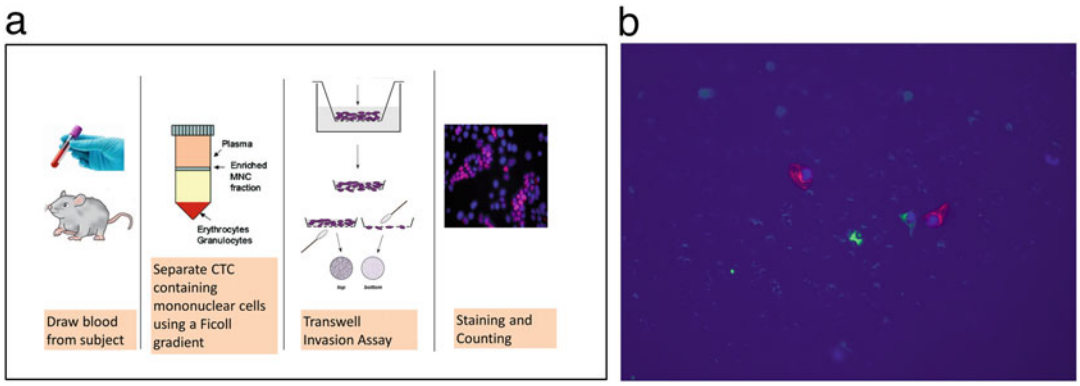

Fig. 1 (**a**) Schematic presentation of CTC isolation based on invasive phenotype. (**b**) Representative fluorescence microscopy image of CTCs. Invasive CTCs were stained with PE conjugated anti-pan-cytokeratin antibody (*red*) and blood cells were stained with CD45 antibody and FITC-conjugated secondary antibody (*green*). Nuclei were counter-stained with DAPI (*blue*)

epithelial-to-mesenchymal properties of CTCs may lead to variation in cell size and the expression of epithelial markers. Thus, these methods cannot detect all types of CTCs. Meanwhile, some CTCs may be derived from mechanical shedding rather than active invasion, and some CTCs may lose their viability or functionality and thus become irrelevant to cancer metastasis. Therefore, CTC counts generated by these methods may not be accurate in predicting cancer outcome and treatment response.

The formation of metastasis from a primary tumor involves multiple processes, including the local invasion and migration of tumor cells and their entrance into the lymphatic system or blood circulation (intravasation), survival in circulation, extravasation into distant organs and for distant metastasis [9–12]. Since CTCs enter circulation likely through migration and invasion, and maintain these properties for extravasation, it is reasonable to hypothesize that viable and functional CTCs would be invasive, and can be detected in vitro using an invasion assay.

In this chapter, we describe a CTC detection, enumeration and isolation method based on their invasive property [13]. Briefly, CTCs along with background peripheral blood mononuclear cells (PBMCs) will be separated from whole blood by gradient centrifugation and then subjected to functional invasion assay using Matrigel-coated Transwell insert. Invasive CTCs will be identified by monoclonal anti-pan-cytokeratin antibody and can be further used for in vitro and in vivo characterization (Fig. 1).

2 Materials

2.1 Tissue Culture Supplies and Equipment

1. 24-well plates for cell culture.
2. 15 and 50 mL conical centrifuge tubes.

3. 6.5 mm Transwell® with 8.0 μm Pore Polycarbonate Membrane Insert, Sterile.
4. Standard fluorescent microscope system with phase-contrast, blue, green, and red channels (Olympus BX51 with SPOT software 5.1).
5. Forceps for sterile handling of the Transwell inserts.
6. Cell culture incubator at 37 °C with 5% CO_2.

2.2 Reagents

1. Phosphate-buffered saline (PBS): 1× Dulbecco's PBS (pH 7.4) with 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4 in ddH_2O.
2. Ficoll-Paque PLUS ($\rho = 1.077 \pm 0.001$ g/mL), sterile, with a low (<0.12 EU/mL) endotoxin activity. Store at room temperature until open, then store at 4 °C.
3. FBS: Research grade Fetal Bovine Serum, triple 0.1 μm pore size filtered. Aliquot and store at −20 °C.
4. Antibiotics for growth medium: 104 U/mL of penicillin and 104 μg/mL streptomycin. Aliquot and store at −20 °C.
5. Growth medium: RPMI 1640 with 10% FBS, 1% penicillin/streptomycin. Store at 4 °C.
6. Matrigel matrix. Aliquot and store at −20 °C. Thaw Matrigel matrix by submerging the vial in ice in a 4 °C refrigerator overnight.
7. Heparin: 10 mg/mL of heparin sodium salt in 1× Dulbecco's PBS.
8. Antibodies for microscopy: For positive identification of CTCs use anti-pan cytokeratin antibody [C-11] (Phycoerythrin) (Abcam, ab52460), anti-cytokeratin 8 + 18 + 19 antibody [2A4] (Abcam, ab41825). For exclusion of hematopoietic lineage cells, human CD45 APC-conjugated antibody (R&D systems, FAB1430A) or mouse CD45 APC-conjugated Antibody (R&D systems, FAB114A) is used. For secondary antibody: Goat anti-mouse IgG (H + L) Secondary Antibody, Alexa Fluor® 594 conjugate (Thermo Scientific), is used when necessary.
9. BD Vacutainer® 10.0 mL sodium heparin tubes are recommended for human patient blood collection.
10. Slide mounting medium with DAPI.
11. Buffer 1: 1× Dulbecco's PBS (pH 7.4) with 2% Fetal Bovine Serum.

3 Methods

3.1 Preparation of Matrigel-Coated Transwell Insert

1. Thaw Matrigel Matrix aliquot on ice at 4 °C overnight. Once thawed, swirl vial to ensure that material is evenly dispersed and always keep Matrigel chilled on wet ice.
2. Mix Matrigel Matrix (final concentration of 1 mg/mL) with serum-free cold RPMI-1640 medium. Gently swirling to mix the solution, and place the tube on ice.
3. Under the hood, use a sterile pipet to carefully add 0.1 mL of diluted Matrigel Matrix to each 24-well, 6.5 mm diameter, 8 mm pore-size Transwell insert. Minimize contact of the Matrigel Matrix with the sidewalls of the Transwell insert (*see* **Note 1**).
4. Incubate the plates with the coated Transwell inserts at 37 °C for at least 1 h. Carefully remove the remaining liquid from the Transwell insert without disturbing the layer of Matrigel Matrix on the membrane just before use.

3.2 Isolation of Mononuclear Cell Layer

1. Blood collection from human/mouse: For patient sample, venous blood should be drawn from a cancer patient using three BD Vacutainer® 10.0 mL green capped, sodium heparin tubes. Blood samples collected in the clinic should be transported to the lab as soon as possible. For mouse samples, blood should be drawn using a 1 mL syringe and a 22-gauge needle and transferred to a heparin solution in a 15 mL conical tube (*see* **Notes 2** and **3**) (Fig. 1).
2. Dilute blood with 3× the volume of buffer 1.
3. Carefully layer 4 mL of diluted blood suspension over 4 mL of Ficoll-Paque layer in a 15 mL conical tube. Make sure the layer is undisturbed (*see* **Note 4**).
4. Centrifuge at 400 × *g* for 30 min at room temperature without brake (*see* **Notes 5** and **6**).
5. Remove most of the upper layer leaving the PBMC layer including the CTCs undisturbed at the interphase.
6. Carefully transfer the PBMC layer to a new 15 mL conical tube.
7. Washing the mononuclear cell layer with serum-free RPMI-1640 medium and centrifuge at 500 × *g* for 5 min at room temperature.
8. Resuspend the pellet with 300 μL of serum-free RPMI-1640 medium, after which the cell suspension containing CTCs and PBMCs is ready for Transwell Invasion Assay (*see* **Note 7**).

3.3 Transwell Invasion Assay and Immunofluorescence Staining

1. Gently pipet the cell suspension on the top of the Matrigel Matrix.
2. Fill the lower chamber of Transwell with 750 μL RPMI-1640 medium containing 10% FBS (*see* **Note 8**).
3. Incubate the invasion chambers in a tissue culture incubator at 37 °C for 72 h.
4. Remove medium and any noninvading cells from the upper side of insert membrane with cotton swab. Use gentle but firm pressure while moving the tip of the swab over the top surface of the membrane.
5. Discard the used swab and repeat **step 5** using a clean swab.
6. Rinse the Transwell membrane by pipetting PBS to the bottom of the well plate until it reaches the membrane, and then add PBS to the top of the membrane.
7. Fix and permeabilize cells with 100% cold methanol for 10 min at −20 °C.
8. Rinse Transwell insert with PBS three times, each for 5 min.
9. Cut the membrane of Transwell insert using a scalpel and put the lower side of membrane up in the 24-well plates.
10. Rinse Transwell membrane with PBS and incubate with 5% normal goat serum/PBS blocking solution for at least 30 min at room temperature.
11. Incubate Transwell membrane with anti-pan-cytokeratin (1:100) antibody overnight at 4 °C (*see* **Note 9**).
12. Repeat **step 8** to rinse Transwell membrane.
13. Incubate Transwell membrane with fluorescent-conjugated secondary antibody at 1:500 dilution for 1 h at room temperature.
14. Repeat **step 8** to rinse Transwell membrane.
15. Mount Transwell membrane on glass slide using 20–30 μL of DAPI-containing mounting medium and gently put a cover slip over the membrane without generating any air bubbles.

4 Notes

1. A 100 μL coating of 1 mg/mL Matrigel creates a gel whose thickness at least 1 cell diameters, a sufficient barrier for cell invasion.
2. For optimal CTC recovery and viability, blood should be handled as soon as possible, preferably within 24 h after sampling.

3. Heparin and acid–citrate–dextrose are better anti-coagulants for collecting blood samples for CTC isolation and detection compared to EDTA.
4. For optimal recovery of CTCs and PBMCs it is crucial to keep a good interface between blood and Ficoll.
5. 18–20 °C is the optimal temperature for Ficoll separation.
6. The absence of acceleration and brake is strictly required for separation of PBMCs over Ficoll-Paque gradient.
7. The cell pellet is loosely attached to the tube, and pouring could result in cell loss.
8. RPMI-1640 medium supplemented with 10% Fetal bovine serum is a useful standard chemoattractant for CTC invasion.
9. Skip **step 13** and **14** when fluorophore-conjugated primary antibody is used.

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Chapter 24

Circulating Tumor Cells: Markers and Methodologies for Enrichment and Detection

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Abstract

Cancer is a leading cause of disease worldwide; however, nowadays many points of its initiation processes are unknown. In this chapter, we are focusing on the role of liquid biopsies in cancer detection and progression. CTCs are one of the main components of liquid biopsies, they represent a subset of tumor cells that have acquired the ability to disseminate from the primary tumor and intravasate to the circulatory system. The greatest challenge in the detection of CTCs is their rarity in the blood. Human blood consists of white blood cells ($5–10 \times 10^6$/mL), red blood cells ($5–9 \times 10^9$/mL), and platelets ($2.5–4 \times 10^8$/mL); very few CTCs will be present even in patients with known metastatic disease, with often less than one CTC per mL of blood. CTCs are found in frequencies on the order of 1–10 CTCs per mL of whole blood in patients with metastatic disease, and it is reduced in half for non-metastatic stages. Therefore, accurate methodologies for their capture and analysis are really important. The main aim of the present chapter is to describe different methodologies for CTCs capturing and analysis.

Key words CTCs, EGFR, EMT, EpCAM

1 Introduction

Detection of circulating tumor cells (CTCs) in peripheral blood can serve as a "liquid biopsy" approach and as a source of valuable tumor markers. Early detection and characterization of CTCs have a prominent role as a prognostic and predictive factor in several types of solid tumors, especially in breast cancer (BC) evolution [1]. However, CTCs are rare (estimated at one CTC per billion normal blood cells in the circulation of patients with advanced cancer), and thus their detection, enumeration and molecular characterization are very challenging. Currently, there are limitations to current CTC-isolating methods, but CTCs have been detected in a majority of epithelial cancers, including those from breast, prostate, lung, and colon [2, 3]. Therefore, detection, isolation, and enumeration of CTCs from cancer

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patients has become an important modality in clinical management of patients with cancer, especially in BC [4].

2 Cellular Markers

2.1 Epithelial Cell Adhesion Molecule (EpCAM)

Some of the systems of CTCs capture are based on using antibodies against cell surface antigens. Epithelial cell adhesion molecule is most commonly used because its expression is virtually universal (albeit at variable levels) in cells of epithelial origin and is absent in blood cells. Conjugation of antibodies against EpCAM to magnetic beads, followed by purification of captured cells through a magnetic field, has been used to enrich CTCs from the blood of patients with breast, prostate, and colon cancers. And commercial platforms like CellSearch® system (Veridex) are based on this feature, characterizing CTCs as the subset of EpCAM-captured cells that are confirmed as both positive for cytokeratins and negative for CD45 [1, 2].

However, considering phenotypic heterogeneity and potential invasion-associated phenotypic plasticity of CTCs, such as epithelial-to-mesenchymal transition (EMT) which results in downregulation of epithelial proteins (including EpCAM), conventional EpCAM-based capturing techniques might miss CTC subpopulations with a more mesenchymal phenotype [5].

2.2 Cytokeratin (CK)

Cytokeratins belong to a large multigene family of proteins of intermediate filaments of the cytoskeleton that is specifically expressed in epithelial cells. Broad-spectrum anti-cytokeratin antibodies have been developed to recognize a single epitope that is common to most cytokeratins (CK7, 8, 18, and 19) and are included in CTCs isolation kits (e.g., MACS® MicroBeads [Miltenyi]). Cytokeratins also are used in CTC detection and characterization after different enrichment procedures. Individual cytokeratin proteins can be downregulated in epithelial tumors such as BC after epithelial to mesenchymal transition; however, detection of several CK proteins at the same time reduces false-negative results produced by this process [6, 7].

2.3 Cell-Surface Vimentin (VIM)

Some of the phenotypic changes in CTCs are associated with or are at least partially a consequence of the epithelial-mesenchymal transition phenomenon. The process of EMT involves the formation of metastatic cancer cells that gain the expression of mesenchymal markers such as vimentin or Slug and lose epithelial markers including EpCAM [1, 2]. This event can lead to the utilization of cell-surface vimentin as a marker for detecting mesenchymal CTCs from some cancer like sarcoma tumors [4].

2.4 Epidermal Growth Factor Receptor (EGFR)

EGFR signaling is important in normal epithelial development and in tumor cell proliferation, motility, survival and metastasis, and it is known to be overexpressed in BCs, especially in triple negative BC cases. Interestingly, it has been demonstrated that EGFR inhibition suppresses EMT and consequently decreases cell migration and invasion ability. Several studies have also reported that high EGFR expression induced EMT, with subsequent transcription factors (Twist, Snail and Slug) induction [1]. Furthermore, several authors have correlated EGFR expression with a poor prognosis in BC patients [8]. Expression of EGFR has been demonstrated in 18–25% of BCs and has been correlated with a higher risk of relapse and death from BC. Substantial increases in EGFR expression have been demonstrated in de novo endocrine-resistant Estrogen Receptor (ER)-positive disease, whereas a more modest increase in the expression of EGFR or ligands has been detected in acquired endocrine resistance of ER-positive disease. The kinetics of the EGFR(+) and EGFR(−) subpopulations of CTCs revealed that at the end of the third treatment course, CTC counts showed an increasing trend that mainly concerned the EGFR(−)/(CK)(+) subpopulation, whereas the number of EGFR(+)/CK(+) CTCs was further decreased. However, these observations seem to indicate that the EGFR(−)/CK(+) subpopulation of CTCs, which increases during treatment, could represent gefitinib-resistant cells. Gefitinib is a signal of transduction inhibitor of the EGFR tyrosine kinase, which has demonstrated antitumor activity against chemo- and hormone-resistant BC cell lines [9].

EGFR expression on CTCs has been evaluated as a marker of clinical outcome in patients treated with cetuximab with or without irinotecan, as third-line treatment in advanced colorectal cancer. But no statistically significant difference was observed in clinical outcome between EGFR-positive and EGFR-negative patients [10].

A summary of the main markers in CTCs isolation is grouped in Table 1.

2.4.1 Combination of CK and EGFR

In our previous study, we have performed an analysis of CK and EGFR expression on CTCs; the study aimed to determine the correlation between the expression of these EMT markers and the upregulation of EGFR expression in CTCs negative for CK. Finally, it was studied if CK-CTCs with EMT features could be used as indicators to evaluate the prognosis of operable BC patients. The results suggest that CK-negative CTCs with high EGFR expression induced EMT, and this phenotypic transition could involve the EGFR-mediated activation of VIM and the subsequent VIM-activated Slug gene expression. It was found that the activation of EGFR upregulated VIM and Slug mesenchymal markers and downregulated pan-CK epithelial markers. These results suggest that the activation of EGFR signaling by its ligand and the presence of TGFβ1 induces EMT and subsequently inhibits CK expression.

Table 1
Markers in CTCs

Marker	Cancer (e.g.)	Application/Clinical Relevance	Reference
EpCAM	Epithelial tumors	Detection of epithelial CTCs. Used for positive enrichment of epithelial CTCs	[18]
Cytokeratins (CK7, CK8, CK9, CK19, CK20. . ..)	Epithelial tumors	Detection of epithelial CTCs/Prognosis of disease	[18]
E.g. CK19	mBC	Associated with shorter OS	[24]
	BC	Associated with relapse and reduced PFS	[25]
CK20	mCRC	Associated with shorter OS	[26]
E-Cadherin	PCa	Associated with biochemical recurrence	[27]
	mBC	Detection of epithelial CTCs	[28]
Vimentin	mCRPC	Detection of EMT CTCs/associated with reduced OS	[29, 30]
	BC	Detection of EMT CTCs	[1, 31]
N-Cadherin	CRPC and mBC	Detection of EMT CTCs	[28]
O-Cadherin	CRPC and mBC	Detection of EMT CTCs	[28]
Slug	BC	Detection of EMT CTCs	[1]
Twist	BC	Detection of EMT CTCs	[31]
CD45	All tumors	To deplete leukocytes	[18]
Her2	mBC	Predicting response to Lapatinib therapy	[32]
	mPCa	Determination of HER-2 status in differents stages of PCa	[33]
ER	BC	Analysis of ER/PR status between CTCs, and primary/metastatic tumors	[34, 35]
PR	BC	Analysis of ER/PR status between CTCs, and primary/metastatic tumors	[34]
Mammaglobin	mBC	Associated with clinical response to treatment	[24]
	BC	Associated with relapse and reduced PFS	[25]
AR	mCRPC	Monitoring response to Enzalutamide and Abiraterone	[36]
AR-V7	mCRPC	Associated with resistance to Enzalutamide and Abiraterone	[37]
CD44	BC	Detection CTCs with stem cell-like phenotype	[18]
CD24	BC	Detection CTCs with stem cell-like phenotype	[18]
ALDH1	BC	Detection CTCs with stem cell-like phenotype	[18]
	mCRC	Associated shorter OS and PFS	[38]
	NSCLC	Associated with progressive metastatic disease	[39]
CD271	mMelanoma	Detection CTCs with stem cell-like phenotype	[40]
CD49f	BC	Detection of EMT CTCs	[41]

(continued)

Table 1
(continued)

Marker	Cancer (e.g.)	Application/Clinical Relevance	Reference
CD133	BC	Analysis of CD133 status in CTCs with clinicopathological characteristics and response to therapy	[42]
	PCa	Associated with biochemical recurrence	[27]
CEA	mCRC	Monitoring response to cryosurgery	[43]
	BC	Associated with relapse and reduced PFS	[25]
CA-125	Ovary cancer	Detection of CTCs	[44]
Mucin-1	Ovary cancer	Detection of CTCs	[44]
	mPancreatic cancer	Associated with shorter OS	[45]
PD-L1	mCRC and mPCa	Associated with shorter OS and PFS, respectively	[29]
	NSCLC	Associated with response to anti-PD-L1 therapy	[46]
	mBC	Demonstration of frequent expression of PD-L1 on BC CTCs	[47]
Ki-67	mCRPC	Associated with reduced OS	[30]
EGFR	NSCLC	Associated with early recurrence	[22]
EGFR mutations	NSCLC	Analysis of EGFR mutations with NGS	[48]
KRAS mutations	CRC	Study of corcondance between CTCs and tumor mutation status	[49]
BRAF mutations	CRC	Study of corcondance between CTCs and tumor mutation status	[49]
	Melanoma	Analysis of mutation status in CTCs compared with primary/metastatic tumors	[50]
KIT mutations	Melanoma	Analysis of mutation status in CTCs compared with primary/metastatic tumors	[50]
PIK3CA	mCRC	Associated shorter OS and PFS	[38]
	NSCLC	Associated with progressive metastatic disease	[39]
PIK3CA mutations	mBC	Determination of the mutational status between CTCs and matched primary tumors	[51]
TP53 mutations	mBC	Analysis of mutation status in CTCs compared with tumors	[52]
TMPRSS2-ERG	CRPC	Analysis of TMPRSS2-ERG as a biomarker of sensitivity to Abiraterone	[53]
PTEN	CRPC	Determination of PTEN status in CTCs compared with metastatic tumors	[54]
OTHERS:			
MCAM	BC	Detection of EpCAM-negative CTCs	[41, 55]
	mMelanoma	Detection of CTCs	[40]

(continued)

Table 1
(continued)

Marker	Cancer (e.g.)	Application/Clinical Relevance	Reference
HMW-MAA	mMelanoma	Detection of CTCs	[40, 50]
PSMA	mPCa	Determination of PSMA status in CTCs compared with primary tumors	[56]
PLS3	CRC	Associated with shorter PFS and OS	[57]
	BC	Associated with shorter PFS and OS	[58]
AKT-2	mCRC NSCLC	Associated shorter OS and PFS Associated with progressive metastatic disease	[38] [39]
MRP1	mCRC	Associated with resistance to Irinotecan therapy and with shorter PFS	[59]
ABCB5	mMelanoma	Detection of CTCs with stem-like phenotype	[40]
ERCC1	Ovary cancer mNSCLC	Associated with resistant to platinum and with shorter PFS and OS Associated with response to platinum and with shorter PFS	[44] [60]
Survivin	mCRC	Associated with shorter OS; Associated with progression	[26]
CA9	Renal carcinoma	Detection of CTCs	[61]
CD147	Renal carcinoma	Detection of CTCs	[61]
miRNA-21	mBC	Detection of CTCs	[62]

m metastatic, *BC* breast cancer, *CRC* colorectal cancer, *PCa* prostate cancer, *CRPC* castration resistant prostate cancer, *NSCLC* non-small cell lung cancer, *OS* overall survival, *PFS* progression-free survival

So, our findings suggest that the simultaneous detection of EGFR, EMT antigens (VIM and Slug), and CK in CTCs by enrichment methods may contribute to the better detection of CTC subpopulations and improve prognostic or predictive information during systemic therapy in patients with operable BC [1].

2.5 Other Cell Proteins Using in CTC Study

Some studies report the use of other markers in EpCAM-low or -negative cells, such as cell surface proteins (Trop2, CD49f, CD146, c-Met, CD44, CD47, AQP5, ADAM8, TEM8) as well as extracellular matrix (ECM) molecules (laminin, collagen I, HA) which were tested on BC cell lines with different EpCAM expression levels. Each of these markers has a different role; for example, CD49f has been considered stem cell marker in breast and other solid tumors. Trop2, a cell surface glycoprotein, was implemented since it had been shown to be overexpressed in a majority of tumors and to account for proliferation and invasion of tumor cells. Some studies reported that CTCs possessing metastasis-initiating properties express CD44, c-Met and CD47. Hyaluronic acid (HA) is one of

the major components of the ECM and serves as a ligand for CD44 and RHAMM (receptors for HA-mediated motility) affecting diverse cellular processes (adhesion, migration, invasion) [5].

3 Methods for Capturing CTCs

Since CTCs have proven potential in helping understand disease and therapeutic outcomes, new modalities that can be used to isolate, detect and enumerate CTCs from blood are being developed. We can mainly divide the methodologies in two groups based on physical or biological properties of CTCs. Due to their rarity, CTCs have to be enriched prior to their detection. Techniques for enrichment of CTCs are based on biological and/or physical properties that distinguish CTCs from normal blood cells. Affinity-based enrichment is by far the most commonly employed strategy to separate CTCs from blood cells. Affinity techniques take advantage of distinctive antigens expressed either by CTCs but not blood cells (e.g., EpCAM), or by blood cells but not CTCs (e.g., CD45). The most common strategy for affinity-based enrichment is immunomagnetic separation, which uses magnetic beads functionalized with antibodies that bind to CTCs (positive enrichment) or blood cells (negative enrichment) [11]. To date, the only technique for enumerating CTCs from patients in the clinic is the CellSearch® technology that has been cleared by FDA for CTC test for metastatic BC, metastatic prostate cancer and metastatic colorectal cancer [4]. This methodology is based on biological properties of CTCs using EpCAM antibody.

Positive selection is one of the most common technologies used for CTCs isolation. This methodology has developed a captured method based on the use of anti-epithelial antibody expression (EpCAM or cytokeratin). Among the most common systems for CTCs isolation we remark the MACS® Miltenyi system or CellSearch®. CellSearch® technology relies upon immunomagnetic particles bound to EpCAM antibodies based on positive selection [12]. However for some cancers like renal cancer, the EpCAM selection did not show great accuracy due to the downregulation of EpCAM expression during EMT, so new technologies based on physicochemical properties distinct from peripheral blood mononuclear cells (PBMC), such as size, density, or electrophoretic properties, were performed to avoid the heterogeneity presence in CTCs [13]. The ISET (isolation by size of epithelial tumor cells) did not involve immune-labeling of cell surface markers, and it is based on the different size of CTCs filtered in membranes with 8 or 7.5 μm average size of pore (Rarecells and ScreenCell systems) [14].

Recent publications have designed a 3D detection system of CTCs consisting in three-dimensional microfilter devices that can enrich viable CTCs from blood. This device provides a highly

valuable tool for assessing and characterizing viable enriched CTCs in both research and clinical settings [15]. There are also indirect selection methods for CTCs based on its molecular characteristics, which can detect "tumor-specific"s markers to be analyzed.

3.1 Isolating Based on Physical Properties

Physical isolation can help distinguish CTCs from normal cells present in blood (leukocytes, endothelial cells, hematopoietic stem cells, and mesenchymal stem cells) without biomarker labeling. Among the physical properties the strategies for isolation are focused on differences in properties between CTCs and blood cells:

1. Size exclusion: Selection is based on the different size of tumor cells (larger than other normal constituent blood cells) compared to sizes of blood cells. However, caution is needed as the method of measurement that varies among samples depending on preparation steps can result in different measured values, even within the same cell population. It is reported that there is a significant difference when cell size (diameter) is measured on a 2D surface by microscopy versus derived from cell volume measurement in suspension by flow cytometry.
2. Deformability: Several studies have demonstrated that metastatic cells (from both cell lines as well as body fluids) are often more deformable than cells of lower aggressive potential, and the deformability increases with increasing metastatic potential. But similar to the size exclusion method, the measurement of cell deformability is affected by the method of choice as mentioned above as well as the cell handling (e.g., culture media, ice/room temperature, dry/wet, fixed/unfixed) and the heterogeneity of cell mechanical properties (e.g., different regions of cells, cell cycle, cell differentiation, and aging).
3. Electrical properties (Surface charge): It is known that most mammalian cells have a net negative surface charge under physiological conditions, but due to the existence of several macromolecules with polarizable particles (like proteins, peptides, and nucleic acids), cells can have complicated dielectric properties. Several studies have demonstrated that cancer cells have a more negative surface charge, or zeta-potential, compared to white blood cells (WBCs). Furthermore, these cells were shown to have higher unit membrane capacitance and lower cytoplasm conductivity compared with leukocytes [16–18].

We have summarized the most common physical methodologies that utilize size and density of the cells (Table 2).

It is known that among physical properties, there are several strategies for capturing CTCs, such as centrifugation, microfiltration, microfluidics and dielectrophoresis. Among all the methodologies previously detailed in Table 2, we selected several of them to go in a deeper detail.

Table 2
CTC isolation methods based on physical properties

				References
PHYSICAL PROPERTIES	In vitro	Size	*Isolation by Size of Epithelial tumor cells (ISET®)*	[63]
			Microporous membrane filter (ScreenCell)	[17]
			Selective size amplification (SSA) in conjunction with multi obstacle architecture (MOA)	[64]
			Dean Flow Fractionation (DFF)	[65]
		Density	*Density Medium Centrifugation (Ficoll-Paque®)*	[66]
		Surface charge	*Dielectrophoretic field-flow fractionation (depFFF) ApoStream®*	[67]
		Size and density	*Porous barrier for size-based separation and density-based centrifugation (Onco-Quick®)*	[68, 69]
		Size and deformability	*Vortex chip*	[70]
			Microfluid vortex capture	[71]
			3D microfilter device	[15]
	In vivo	Size and density	*Leukapheresis*	[72, 73]

3.1.1 ScreenCell

ScreenCell® technologies are developed in order to isolate CTCs from blood by size selection on a microporous membrane filter which allows later cell selection and characterization. The filtration membrane allows passage of nucleated blood cells but retains CTCs. It is based on microfiltration technologies that have been demonstrated to produce the greatest potential for achieving high-throughput continuous processing of large volumes of blood [17]. There are three configurations of the ScreenCell® device:

1. ScreenCell® Cyto is used for cell enumeration and cytology, and it isolates rare, fixed, tumor cells with a high recovery rate. Cells are well preserved morphologically. Immunocytochemistry and FISH assays can be performed directly on the filter. It has been proven to obtain relevant results in EpCAM-negative CTCs from patient samples for enumeration, immunocytochemistry, and genetic analysis.
2. ScreenCell® CC is used for the culturing of CTCs, and it allows the isolation of live cells able to grow in culture.
3. ScreenCell® MB is used for molecular analysis; it is useful for personalized medicine because it allows the isolation of DNA for genotyping analysis from blood of patients. DNA isolated from CTCs captured with the device can be subjected to whole genome amplification, Sanger sequencing, genotyping, etc. This allows the discovery of many hotspot mutations with relevance in therapy, prognosis, evolution, etc.

This methodology has several advantages: First, tumor cells isolated onto the filter can be analyzed using all relevant cellular

and molecular biological techniques pertinent to the identification and characterization of CTCs and their potential genetic abnormalities. Second, the isolation of living cells allows further tissue culture experiments. Lastly, the isolation of cells can be achieved without using an antibody-based assay. This opens a wide range of isolation spectrum of tumor cells, including cells of non-epithelial origin [1, 19, 20].

3.1.2 Isolation by Size of Epithelial Tumor Cells (ISET)

ISET device enables the isolation of CTCs and circulating tumor microemboli (CTM) as intact cells, without a previous immune-based selection, from all types of cancer (except CLL). Lymphocytes are the smallest cells in the body; they have a very compact nucleus and a minimal cytoplasm. The vast majority of lymphocytes and neutrophils are thus eliminated by filtration. Larger cells are enriched by ISET and can be analyzed by cytomorphology and immuno-molecular characterization. Each spot on the filter shows the large cells isolated from 1 mL of blood.

For a summary of ISET procedure *see* Fig. 1. (Figure collected from http://www.rarecells.com/ctc-isolation.html).

3.2 Isolation Based on Biological Properties

Biological properties-based technologies use specific markers, which are expressed by tumor cells or blood cells but not for both, that are detected by antibodies. Strategies for protein-based isolation of CTCs can be divided into three categories:

1. Positive selection methods: Designed to capture tumor cells present in liquid biopsy samples, antibodies bind to specific markers (e.g., epithelial markers) that are expressed on normal epithelial cells and epithelial tumors but absent on the other cells present in blood. The most of these technologies are based on two phases, a first step of CTC enrichment followed by a detection and enumeration procedure. As it has been said above, EpCAM and the cytokeratin family of proteins are the most frequently used molecules for positive enrichment and

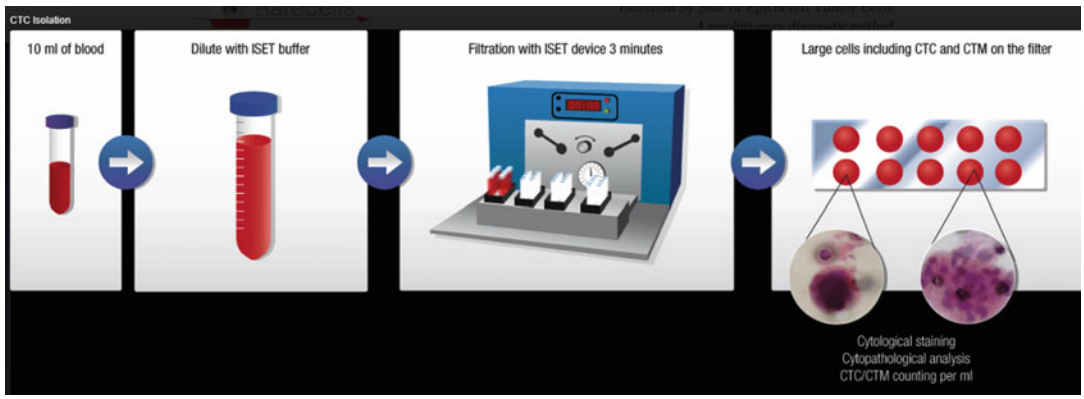

Fig. 1 ISET procedure

isolation of CTCs. Antibodies against these molecules have been implemented in semiautomated platforms mainly coupled to magnetic beads (CellSearch®, AdnaTest®, MagSweeper), microfluidic devices (CTC-chip, Herringbone chip), or a combination of these methods (CTC iChip®, IsoFlux). Detection, enumeration, and characterization steps are usually based on immunocytochemistry for CK, CD45, and DAPI, but other techniques have been described such as qPCR for tumor-specific panel of markers with the disadvantage of the loss of CTC enumeration (AdnaTest®). Moreover, in the last few years, a positive selection method has emerged that allows live cells isolation (MagSweeper™), and even an in vivo method for CTC isolation directly from antecubital vein of cancer patients, which processes a great blood volume and therefore recovers a large amount of CTCs, has been developed (CellCollector®) [18].

2. Negative selection methods: Depletion procedures are used to remove white blood cells and other components of blood based on cell surface marker that are unique to non-CTCs. For negative enrichment CTC methods, CD45 represents the gold standard marker that allows for the selection of leucocytes present in blood. Similar to positive selection, the usage of immunomagnetic beads against specific biomarker (CD45 and CD66b) is a strategy commonly utilized in many commercial kits. The other technique used in negative selection of CTCs is the RossetteSep method that combines density gradient centrifugation with an antibody enrichment process using tetrameric antibody complexes to CD45 and CD66b on WBCs and glycophorin A on red blood cells [16].

Leukapheresis, a clinical technique in which white blood cells are separated from the blood and returning the remainder to the circulation, has been used in CTC isolation, obtaining a median of 7500 CTCs per patient with the disadvantages such as invasiveness and the high cost of the procedure [18].

3. Selection-free methods: Nonselection approaches include among others RT-PCR analysis, droplet digital PCR (ddPCR), and high-throughput microscopy. After lysis of erythrocytes, Epic Sciences platform, which is based on high-throughput imaging, plate on slides nucleated cells that are submitted under immunofluorescence staining, and they are analyzed using fiber optic array scanning technology [16].

Main methodologies of CTC isolation using molecular markers are described in the following table (Table 3), and the methods are grouped into positive, negative, and free selection.

3.2.1 *MACS® Miltenyi Tumor Isolation System*

MACS Miltenyi isolation system includes an initial step of density gradient centrifugation followed by a CTC capture using a

Table 3
CTC isolation methods based on biological properties

			CTC enrichment	CTC detection	Notes	References
BIOLOGICAL PROPERTIES POSITIVE SELECTION	In vitro	*CellSearch® system*	Anti-EpCAM immunomagnetic beads	Immunofluorescence analysis for CK, CD45, and DAPI	FDA approved in advanced breast, prostate and colorectal cancer Semiautomatic procedure	[74–77]
		Adnatest® system	Anti-EpCAM and MUC1 immunomagnetic beads	Immunomagnetic beads cocktail optimized for each tumor type and RT-PCR for panels of tumor specific markers	Cell lysis prevents morphological analysis and count the number of cells	[78, 79]
		MagSweeper™	Anti-EpCAM immunomagnetic beads enriched using magnetic rod	Immunofluorescence analysis for CK, CD45 and DAPI and RT-PCR for disease-specific markers	High purity live cells can be isolated	[80]
		CTC-Chip and Herringbone-Chip	Anti-EpCAM functionalized microposts and chip structure	Immunocytochemical analysis or RT-PCR	Herringbone structure induces microvortex increasing the efficiency of capture Many CTC-specific antigens can be used for cell capture Live cells can be isolated	[81–83]
		EPHESIA CTC-Chip	Columns of functionalized superparamagnetic beads combined with microfluidics	High-resolution confocal 3D Immunocytochemical analysis	Many CTC-specific antigens can be used for cell capture	[84]
		IsoFlux	Anti-EpCAM magnetic beads and microfluidics	Immunocytochemical analysis	Many CTC-specific antigens can be used for cell capture	[85]
		Velcro-like device	Anti-EpCAM-coated biotin-P-polymer brushes and nanostructure	Immunocytochemical analysis or RT-PCR Functional assays	Cell capturing, and stimulated cell release based on thermally responsive polymer brushes Live cells can be isolated	[86]
		GEDI microdevice	Immunocapture and microfluidics	Immunofluorescence analysis for PSMA, CD45, DAPI	Allows functional assays in situ	[87]

NEGATIVE SELECTION	In vivo	*DEPArray®*	Dielectrophoretic field and microfluidic	Immunofluorescence analysis, PCR and sequencing methods	Requires CD45 depletion and manual staining Different antigens can be used for immunofluorescence analysis	[88]
		Cell Collector®	Anti-EpCAM antibodies functionalized nanodetector	Immunofluorescence analysis or RT-PCR Functional assays	Could filter 1,5 L of blood in 30 min Live cells can be isolated	[89]
		White blood cells (WBCs) depletion kits	Anti-CD45 immunomagnetic beads Lysis red blood cells	Immunofluorescence analysis or RT-PCR	High risk of loss of rare CTCs caught in massive movement of WBCs	[90, 91]
		CTC-iChip	Magnetic beads capture combined with microfluidic inertial focusing	Immunofluorescence analysis, RT-PCR, FISH	Antigen-dependent and -independent capture Unfixed cells can be isolated	[92]
		RosetteSep method	Tetrameric antibody complexes anti- CD2, CD16, CD19, CD36, CD38, CD45, CD66b and glycophorin A	Immunofluorescence analysis or RT-PCR	Immunoselection during gradient centrifugation	[93–95]
SELECTION FREE METHODS		*Epic Sciences*	Immunofluorescence staining analyzed by fiber-optic array scanning technology (FAST) faster than automated digital microscopy (ADM)		Needs red blood cell lysis	[96, 97]
		AccuCyte® – CyteFinder®	Immunofluorescence staining for EpCAM, EGFR and CD45 and digital scanning microscope. Single-cell retrieval device PCR and sequencing methods		Requires an automatic density-based cell separation Automatic procedure	[98]

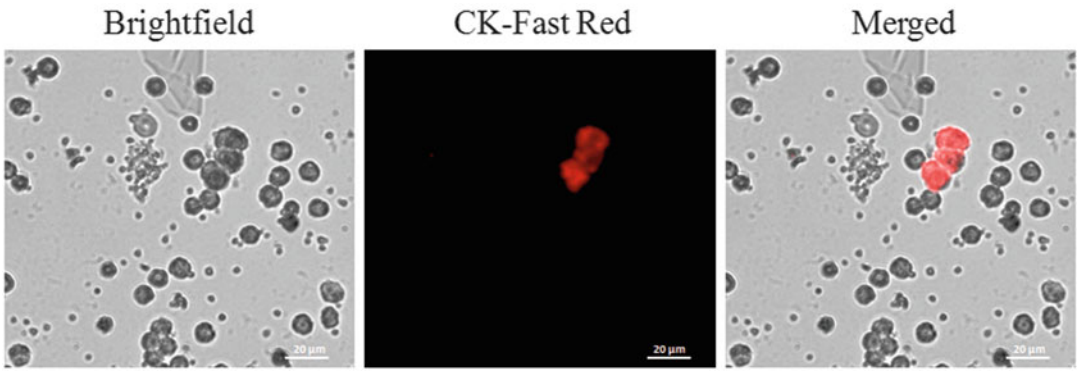

Fig. 2 Detection of CK+ circulating tumor cells (red staining) in a BC patient

magnetic separation with immunomagnetic beads against cytokeratin. This technique allows enumeration of CTCs by chromogenic staining and CTC characterization by immunofluorescence (Fig. 2) and PCR methods. Several authors published consistent results that show correlation between presence of CK+ CTCs in samples from BC patients and poor prognosis [21] and early relapse in Non-Small Cell Lung Cancer patients [22].

3.2.2 Microchip Platforms

Novel microchip platforms are based on CTC-capture which capture cells based on antigen expression. For example, the CTC-iChip uses continuous deterministic lateral displacement for size-based separation of WBCs and tumor cells from whole blood, inertial focusing for precise positioning of these cells in a micro-channel, and then microfluidic magnetophoresis for immunomagnetic isolation of CTCs. It is currently composed of two separate chips (CTC-iChip1 and CTC-iChip2) used serially; each chip is produced with different manufacturing procedures [23].

3.2.3 AdnaTest® (Qiagen)

The AdnaTest works in a two-step process. First, the CTCs in the blood sample are enriched in a process that uses antibody-coated magnetic beads. Each AdnaTest Select has a combination of antibodies that bind with high specificity and affinity to epitopes or antigens on the relevant cancer cells. After magnetic separation, the enriched cells are lysed and purified several times to make the relevant tumor cell information available in the form of mRNA. Highly sensitive RT-PCR is used to find tumor-associated expression patterns. The mRNA strands are reverse transcribed into cDNA. Subsequently, several associated tumor markers are amplified via multiplex PCR so that their expression patterns can be analyzed.

3.3 Other Methodologies

Functional assays have been developed with the aim to study CTCs properties, such as migration or invasion, which are closely related to dissemination and metastasis formation processes. We summarize some in vitro and in vivo functional assays in Table 4.

Table 4
CTC detection method based on functional properties

			Procedure	Notes	References
FUNCTIONAL PROPERTIES	In vitro	*EPISPOT*	CTCs are seeded on nitrocellulose membranes coated with an antibody against a specific protein marker (CK19, Her2, Cathepsin-D, MUC-1, PSA, VEGF, FGF-2, EGFR) and cultured for 48 h. Obtained immunospots are counted by video camera imaging and computer-assisted analysis. One immunospot corresponds to the protein fingerprint of one viable marker protein-secreting cell.	Must be combined with negative selection or size selection methods (usually *RosetteSep system*) Multiple fluorescence EPISPOT are in progress	[99]
		Vita-Assay®	Culture plates with cell adhesion matrix (CAM) that mimics the tumor microenvironment	Green and red fluorescent versions of Vita-Assay allow for direct visualization of CAM-uptake by cancer cells	[100]
	In vivo	*Transplantation of patient-derived CTCs in immunodeficient mice*	Blood samples depleted of hematopoietic cells are transplanted into the femoral medullar cavity of immunocompromised mice to evaluate the capacity of CTCs to initiate metastasis. Flow cytometry analysis for CD44, CD47, and MET identifies metastasis initiator cells	Require a depletion of hematopoietic cells	[101]

4 Conclusions

There is accumulating information regarding the development of novel technologies for CTC detection and their prognostic relevance and their use in therapeutic response monitoring. They are a very useful informative tool, and as a result of their rarity, CTCs have to be enriched for prior to their detection. A variety of techniques have been used for enrichment (cell surface antigen, physical and size properties of tumor cells) and detection (immunocytochemistry, CellSearch®system, microchips platforms) of CTCs. Although the enrichment process is useful, it is not totally effective and does not result in pure populations of tumor cells; for that reason detection methodologies are constantly in development. However, each detection system presents several drawbacks; for example, immunocytochemistry can miss cells not expressing the intended target antigens, and CellSearch®system is biased toward CTCs with high EpCAM expression, which emerging evidence suggests that CTCs are heterogeneous in this regard.

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