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# Thermoresponsive release of viable microfiltrated Circulating Tumor Cells (CTCs) for precision medicine applications†

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Stimulus responsive release of Circulating Tumor Cells (CTCs), with high recovery rates from their capture platform, is highly desirable for off-chip analyses. Here, we present a temperature responsive polymer coating method to achieve both release as well as culture of viable CTCs captured from patient blood samples.

The Circulating Tumor Cell (CTC) is an important biomarker in cancer management. Enumeration of CTCs has been proven of prognostic value in multiple cancer types including breast, prostate and colorectal cancer.[1–3] Recent studies, however, have revealed the limited clinical relevance of CTC enumeration alone in interventional trials.[4] Additionally, single CTC transcriptomic studies have revealed the wide heterogeneity of CTCs,[5] further indicating the need for in depth molecular and functional characterization of CTCs. Indeed, there have been increased efforts to implement molecular and genomic characterization of CTCs for informing clinical trial design, treatment selection, and ultimately, precision cancer management.[6] To fulfil such goals, a technology that not only allows for efficient CTC capture but also accommodates various downstream characterization of CTC both on-chip and off-chip, is highly desirable.

We have previously reported a very successful size-based strategy for CTC isolation,[7] and its variation employing slot-shaped pores for viable CTC capture and analysis.[8] These Parylene-C based microfilters not only capture and retain CTCs with high efficiency but also allow *in situ* analyses, which require extensive manipulation of CTCs. However, the strong retention of CTCs hinders those experiments that require that the captured cells be transferred onto other platforms. For example, microfluidic platforms, such as the Fluidigm™ single cell analysis system[5] or the DEPArray™ system[9] require suspended cells as input, and novel *ex vivo* culture methods require CTCs to be cultured on specialized surfaces, such as ultra-low attachment plates.[10] All of these platforms, which have great promise as a companion chemosensitivity test, are incompatible with many of the capture platforms, including our microfilters. Thus, a facile route to viably recover CTCs captured from the blood of cancer patients is highly desirable.

Several platforms that allow for viable CTC capture and release have been reported based on immobilized antibodies conjugated to a cleavable linker,[11] poly(*N*-iso-propylacrylamide) (PIPAAm),[12] or electroactive films.[13] However, these systems are affinity-based and can be potentially biased by the target antigen. For example, the most commonly targeted antigen for CTC capture, EpCAM, can be absent within certain CTC populations possessing mesenchymal phenotypes.[14] In contrast, label free, size-based isolation and release of CTCs can provide a method to study the CTC population in a potentially more comprehensive manner, such as the centrifuge-on-a-chip method reported by Mach *et al.*[15] Here, we demonstrated that by combining our filtration-based platform with the PIPAAm coating method, we could achieve an antigen-agnostic, efficient method to effectively capture and isolate viable CTCs from blood.

PIPAAm is a polymer that undergoes a reversible lower critical solution temperature (LCST) phase transition at a solution temperature of 32 °C.[16] Traditionally, this property of PIPAAm has been widely explored for tissue engineering applications. Typically, cells are cultured on PIPAAm coated surfaces at 37 °C when PIPAAm is hydrophobic. The cells can then be detached as a sheet when the culture temperature is shifted to below 32 °C, where the PIPAAm coated surface becomes hydrated.[17,18]

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In our formulation, epithelial cancer cells are bound to the Parylene C membrane by non-specific electrostatic interactions instead of extracellular matrix (ECM) mediated adhesion. This necessitated a modification of the conventional PIPAAm based release strategy. The epithelial cell capture is performed at room temperature (below 32 °C) and cell release is enabled by placing the filter in our culture media maintained at 37 °C. At this temperature, the PIPAAm polymer layer becomes hydrophobic, thereby releasing the electrostatically bound cells.

To coat PIPAAm onto the slot filter, we first dissolved PIPAAm in butanol at 10% (w/v) concentration. The PIPAAm solution was then spin coated onto the slot filter at a top speed of 6000 rpm for one minute.[19] The coated filter was air-dried and stored at room temperature before use (Fig. 1A and B). We compared the pore sizes before and after coating by phase-contrast microscopy (Fig. S1†) and as anticipated, found a 7% decrease in pore length and 15% decrease in pore width after PIPAAm coating (Fig. S1†). The effect of this pore size decrease was enhancement in capture efficiency (Table 1), along with decrease in enrichment factor, with more erythrocytes and leukocytes seen post capture (Fig. S2A†). This is consistent with our previous report on slot filters with a 5 μm pore width.[8] However, this decrease in enrichment factor does not hamper the functionality of the PIPAAm coated slot filter for viable CTC capture and release, since the additional erythrocytes and leukocytes captured can be easily removed by a gentle wash of fresh media at day 1 (Fig. S2B†). This did not affect cell viability, proliferation or metabolic rate post capture (Fig. 2, 4 and Fig. S4†). To perform CTC capture, the coated filter was cut into 6 mm by 6 mm squares and fit into a filtration cassette (top acrylic piece: *H* 4 mm, *L* 30 mm, *W* 18 mm, bottom acrylic piece: *H* 8 mm, *L* 30 mm, *W* 18 mm) (Fig. 1). Methodologically, CTCs are captured onto PIPAAm coated slot filters at room temperature with PIPAAm coating in its hydrophilic state. Post capture, a mild reverse flow was applied to release cells trapped in the pores and then the filter was incubated in the release medium at 37 °C to induce the phase transition. The cells bound to filter are then released (Fig. 1).

To test the PIPAAm coated slot filters, we performed detailed analyses of tumor cell capture and release efficiency using ~1000 SKBr-3 cells labeled by Carboxyfluorescein Succinimidyl Ester (CFSE) spiked into 7.5 mL of healthy donor's blood as a model system (details in ESI† method).

**Table 1** Capture, release and retrieval efficiency of PIPAAm coated slot filters. Capture efficiency is calculated by dividing cell numbers captured on filter before release by cell numbers spiked into blood. Release efficiency is calculated by dividing cell numbers released from the filter by cell numbers captured on the filter before release. Retrieval efficiency is calculated by dividing cell numbers released from filter by cell numbers spiked into blood

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

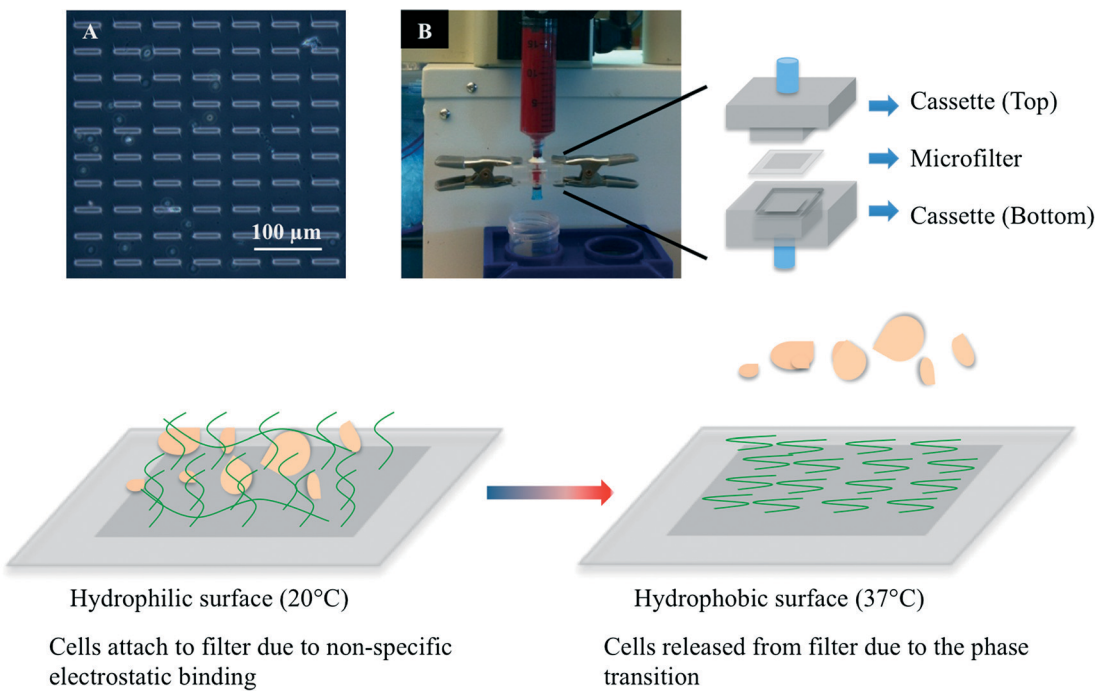


**Fig. 1** PIPAAm coated slot filters to capture and release circulating tumor cells from blood. (Top panel) (A) Microscopic view (400× magnification) of the slot filter post PIPAAm coating; (B) filtration set-up with syringe pump to capture CTCs from blood using PIPAAm coated slot filters, the PIPAAm coated slot filter is sandwiched between the top cassette and the bottom cassette. (Bottom panel) Scheme of using temperature change to release captured CTCs from PIPAAm coated slot filters.

 

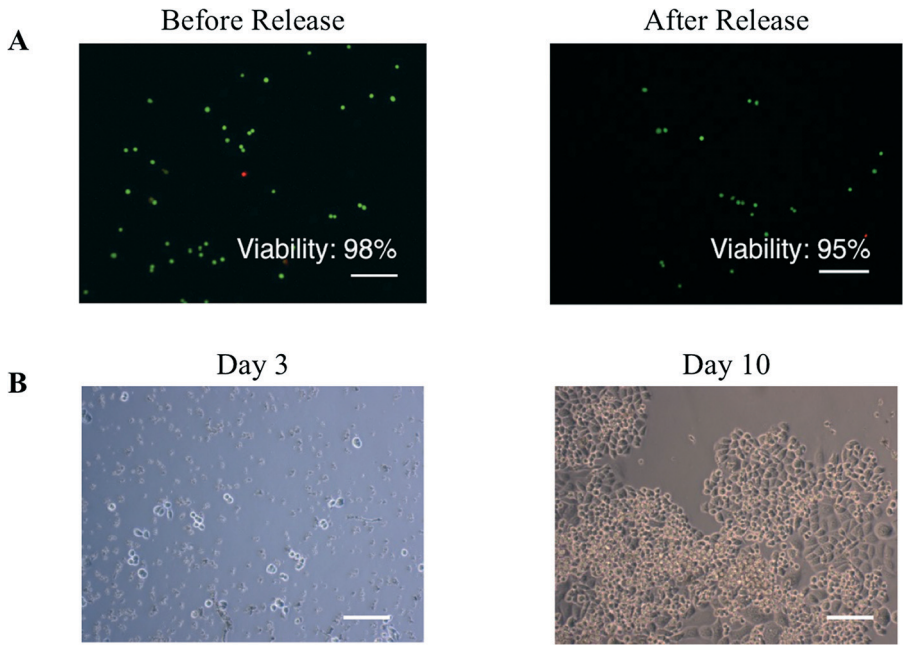


**Fig. 2** SKBr-3 breast cancer cells captured and released from blood remained viable and expanded in culture rapidly. (A) SKBr-3 cells released from the filter was tested with a live–dead assay. 95% (540 out of 567 cells counted) of the cells remained viable (green) following PIPAAm-filter release. Dead cells are labelled in red. (B) Cells released from PIPAAm coated slot filter remained viable and expanded in culture. Scale bar: 100 μm.

Healthy donors' blood samples in this study were obtained under a protocol approved by the University of Miami (Miami, FL, USA) IRB (20150020) following an informed consent. Results from 3 replicates are shown in Table S1.† The coating method did not hamper the capture efficiency of the filter itself. Overall, we achieved capture, release and retrieval efficiency averages of 94% ± 9%, 82% ± 5% and 77% ± 5% respectively (Table 1, summarized from data in Tables S1 and S2†). The release and retrieval efficiency was much higher than those of non-coated filter (7% ± 1% release efficiency and 6% ± 1% retrieval efficiency) (Tables 1 and S3†).

To test the viability of the cells released from the filter, we spiked ~1000 SK-Br-3 cells into 7.5 mL of healthy donor's blood, captured and released them from PIPAAm coated slot filter using the method described above. A Live-Dead® assay (Life Technologies, Grand Island, NY) was performed to evaluate the cell viability before spike into blood and after release. The pre-spike viability was 98% (592 out of 602 cells counted) and the viability of cells captured and released from blood was 95% (540 out 567 cells counted) (Fig. 2A). We also cultured, in parallel, the cells released from blood in McCoy's 5A culture medium (Gibco, Life Technologies, Grand Island, NY). Images were taken at day 3 and day 10. As shown in Fig. 2B, cells released from the filter remained viable and expanded rapidly in culture establishing their viability post cell capture from blood and release from the filter.

The ultimate application of this technology is to capture patient-derived CTCs from blood and release them with minimal impact on their viability. The released CTCs can then be subject to optimal culture surfaces/conditions, which would have been impossible on cells retained on filter. To demonstrate the feasibility of this application, we used conditionally reprogrammed cells (CRCs)[20] as a model system. The CRC approach enables the establishment of continuous primary cell cultures from all epithelial tissues and could potentially be applied for CTCs culture establishment. Using the CRC method, a tumor cell line was established from a non-small cell **L**ung carcinoma that had **M**etastasized **T**o the **S**pine and termed LMTS. This line was stably labeled with green fluorescent protein (GFP) and termed LMTS-GFP. Although Parylene C can be favorable for culturing certain immortalized cell line,[21,22] it was not the optimal surface to culture LMTS-GFP cells (Fig. 3A). Thus, in order to retrieve LMTS-GFP cells from blood and expand them in culture, we tested the PIPAAm coated slot filter for viable cell release, post capture. First, we tested the capture, release and retrieval efficiency of LMTS-GFP cells on the PIPAAm coated filters. ~1000 LMTS-GFP cells were spiked into 7.5 mL of healthy donor's blood and the capture and release experiment was performed in triplicate. As indicated in Table 1, efficiencies of capture, release and retrieval produced averages values of 87% ± 10%, 79% ± 14% and 69% ± 12%, respectively. With the confirmative results showing high efficiency retrieval of LMTS-GFP cells from blood, we then tested the culture of these cells retrieved from blood. We spiked ~1000 LMTS-GFP cells into 3 mL of healthy donor's blood. The blood was then diluted to 6 mL volume using PBS. Post capture, the released cells (Fig. 3C) were then cultured in a 48 well plate (Greiner Bio-One, Monroe, NC) in irradiated J2 conditioned F+Y media prepared as

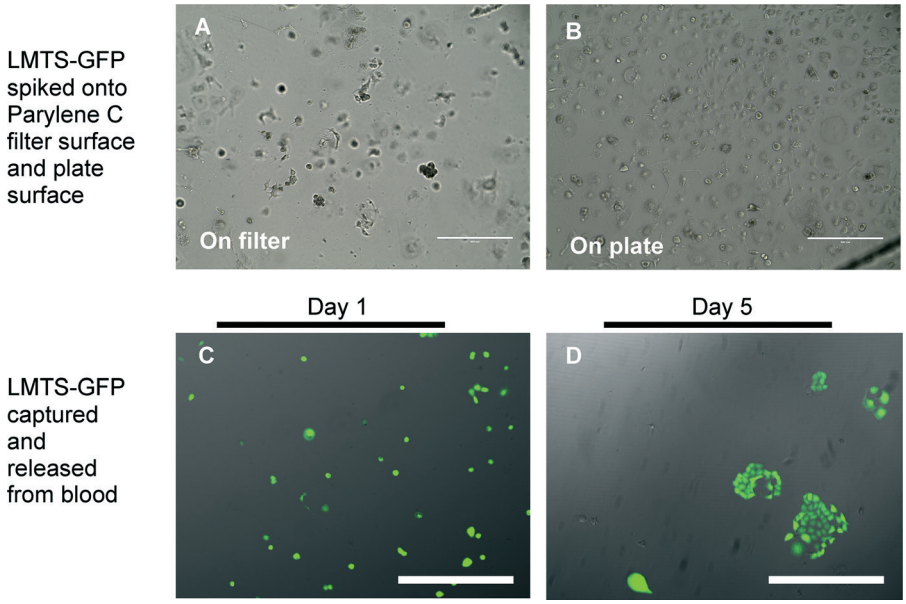


**Fig. 3** Capture and release of LMTS-GFP CRCs using PIPAAm coated slot filters. (Top panel) Comparison of LMTS-GFP cells cultured on Parylene C surfaces (A) and on culture plates (B). Parylene C surfaces do not support the growth of LMTS-GFP CRCs. Scale Bar 400 μm. (Bottom panel) LMTS-GFP cells were spiked into healthy donor's blood and retrieved by the PIPAAm coated slot filter. Images were taken under phase contrast microscopy and the FITC channel and merged for display. Images were taken at day 1 (C) and day 5 (D). Scale bar: 100 μm.

described.[20,23] As shown in Fig. 3D, released cells remained viable at day 5 and expanded in culture.

In order to confirm that the process of retrieving tumor cells from blood does not alter the cells' proliferation rate, metabolism and biochemical properties, we analyzed the tumor cells retrieved from blood by MTT and EdU assays as shown Fig. 4. For the EdU assay, the modified thymidine analog EdU (Life Technologies, Grand Island, NY) was integrated

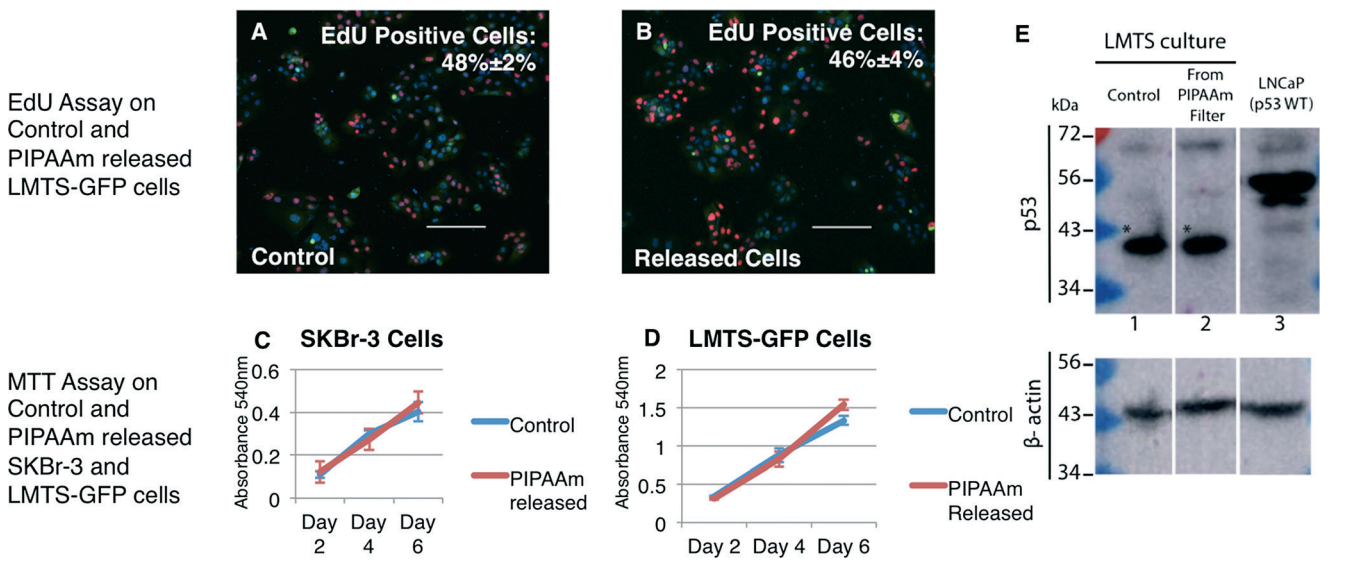


**Fig. 4** Measurement of proliferation rates and metabolism rates of tumor cells before and after PIPAAm coated slot filter capture and release proliferation rate of the LMTS-GFP cells was measured using an EdU assay. All cells were labeled with Hoechst (blue) and express GFP (green), and newly proliferated cells were labeled with EdU (red). Comparable proliferation rates were observed between LMTS-GFP cells plated as controls (A) and spiked in LMTS-GFP cells retrieved from blood by PIPAAm coated slot filters (B). Metabolism rate and growth curves of SKBr-3 cells and LMTS-GFP cells were measured using an MTT assay. No significant differences in metabolism rate and proliferation rate were seen between released cells from PIPAAm coated slot filter and control cells for either (C) SKBr-3 cells or (D) the LMTS-GFP CRC line. We also probed the captured and released LMTS-GFP cells for mutated p53 protein as seen in control cells. (E) Extracts from parental control (lane 1) and LMTS-GFP cells cultured following release from the PIPAAm filter (lane 2) were probed by immunoblot for the p53 tumor suppressor protein. The LMTS line harbors an E298* truncation mutation with a predicted molecular weight of approximately 43 kDa (asterisk). LNCaP cells (lane 3) were run as a control for wild type p53. β-Actin was used as a loading control as seen on bottom panel of E.

 

into newly synthesized DNA and then labeled with Alexa Fluor 594® dye. Cells were incubated with EdU for 2 hours and then fixed for labeling. We then enumerated the total number of cells (Hoechst+ cells) and newly proliferated cells (Hoeschst+, EdU+ cells) and calculated the percentage of EdU + cells. For SKBr-3 cells, EdU+ cells constituted 29% ± 6% of the control cell population and 32% ± 8% of the PIPAAm released cells population, with no significant difference between groups ($p$ value = 0.44) (Fig. S4†). For LMTS-GFP cells, EdU+ cells constituted 48% ± 2% of the control cell population (Fig. 4A) and 46% ± 4% of the PIPAAm released cells population (Fig. 4B), with no significant difference between groups ($p$ value = 0.38). To test the growth curve and metabolism rate of the PIPAAm released cells, we performed MTT assay (Life Technologies, Grand Island, NY) on released SKBr-3 cells and LMTS-GFP cells. As seen in Fig. 4C and D, the growth curves and metabolism rates remained the same for both SKBr-3 and LMTS-GFP cells pre- and post-filter capture and release. To verify that the biochemical markers of LMTS-GFP cells were not altered, we probed for the p53 E298* truncation mutation harbored by LMTS-GFP cell line in cells we spiked into blood and retrieved using PIPAAm coated slot filters. As seen in Fig. 4E, LMTS-GFP cells retrieved from blood by PIPAAm coated slot filters contain the same truncated p53 tumor suppressor protein as seen in control LMTS cells.

To test the applicability of our strategy for human patient samples, we tested this technology with 4 metastatic breast cancer patient samples. Briefly, we collected 15 mL of peripheral blood by venipuncture from each patient under a protocol approved by the University of Miami (Miami, FL, USA) IRB (20130312) following an informed consent. Blood samples were split evenly into 2 tubes of 7.5 mL blood for each patient. One tube of blood was processed using our round-pore microfilter, which has been demonstrated previously to capture greater number of CTCs as compared with CellSearch® in the same cohort of patients.[24] The other tube was processed using the PIPAAm coated slot filter. Post-release, the release medium was spun onto a slide for CTC enumeration. Both the microfilter and the slide with released cells were then subjected to immunofluorescence staining for pan-cytokeratin and CD45, markers for CTCs identification. As shown in Fig. S5,† the CTC enumeration using PIPAAm coated slot filter or round-pore filter in parallel samples were comparable. Thus, with the PIPAAm coating, we showed the ability to recover CTCs captured from human patient samples with a high retrieval rate.

## Conclusions

Circulating Tumor Cells (CTCs) have been established as an important biomarker in cancer and they may play promising roles in cancer management. Molecular and cellular analyses of CTCs can reveal valuable information for cancer prognosis and may help drive precision medicine. Several platforms commonly used in CTC capture and analyses have limitations in allowing for molecular and cellular analyses of CTCs since the cells captured are either fixed or immobilized. Further, affinity-based systems, which allow for viable CTCs capture and release, can potentially be biased by the choice of target antigen. In contrast, by combining our antigen-agnostic filtration based platform with the versatile PIPAAm coating method, we can achieve capture and release of viable CTCs from blood at a high efficiency. This will lay the foundation for the in depth characterization of viable CTCs, including single cell phenotypic and genomic analysis as well as *ex vivo* CTCs culture. Based on our proof-of-principle demonstration with human patient samples, future work is under way to employ this technology for clinical applications.

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## Notes and references

1 M. Cristofanilli, G. T. Budd, M. J. Ellis, A. Stopeck, J. Matera, M. C. Miller, J. M. Reuben, G. V. Doyle, W. J. Allard, L. W. Terstappen and D. F. Hayes, *N. Engl. J. Med.*, 2004, **351**, 781–791.

2 J. S. de Bono, H. I. Scher, R. B. Montgomery, C. Parker, M. C. Miller, H. Tissing, G. V. Doyle, L. W. Terstappen, K. J. Pienta and D. Raghavan, *Clin. Cancer Res.*, 2008, **14**, 6302–6309.

3 S. J. Cohen, C. J. Punt, N. Iannotti, B. H. Saidman, K. D. Sabbath, N. Y. Gabrail, J. Picus, M. A. Morse, E. Mitchell, M. C. Miller, G. V. Doyle, H. Tissing, L. W. Terstappen and N. J. Meropol, *Ann. Oncol.*, 2009, **20**, 1223–1229.

4 J. B. Smerage, W. E. Barlow, G. N. Hortobagyi, E. P. Winer, B. Leyland-Jones, G. Srkalovic, S. Tejwani, A. F. Schott, M. A. O'Rourke, D. L. Lew, G. V. Doyle, J. R. Gralow, R. B. Livingston and D. F. Hayes, *J. Clin. Oncol.*, 2014, **32**, 3483–3489.

5 A. A. Powell, A. H. Talasaz, H. Zhang, M. A. Coram, A. Reddy, G. Deng, M. L. Telli, R. H. Advani, R. W. Carlson, J. A. Mollick, S. Sheth, A. W. Kurian, J. M. Ford, F. E. Stockdale, S. R. Quake, R. F. Pease, M. N. Mindrinos, G. Bhanot, S. H. Dairkee, R. W. Davis and S. S. Jeffrey, *PLoS One*, 2012, **7**, e33788.

6 C. Alix-Panabières and K. Pantel, *Nat. Rev. Cancer*, 2014, **14**, 623–631.
7 S. Zheng, H. Lin, J. Q. Liu, M. Balic, R. Datar, R. J. Cote and Y. C. Tai, *J. Chromatogr. A*, 2007, **1162**, 154–161.
8 T. Xu, B. Lu, Y. C. Tai and A. Goldkorn, *Cancer Res.*, 2010, **70**, 6420–6426.
9 D. J. Peeters, B. de Laere, G. G. van den Eynden, S. J. Van Laere, F. Rothe, M. Ignatiadis, A. M. Sieuwerts, D. Lambrechts, A. Rutten, P. A. van Dam, P. Pauwels, M. Peeters, P. B. Vermeulen and L. Y. Dirix, *Br. J. Cancer*, 2013, **108**, 1358–1367.
10 M. Yu, A. Bardia, N. Aceto, F. Bersani, M. W. Madden, M. C. Donaldson, R. Desai, H. Zhu, V. Comaills, Z. Zheng, B. S. Wittner, P. Stojanov, E. Brachtel, D. Sgroi, R. Kapur, T. Shioda, D. T. Ting, S. Ramaswamy, G. Getz, A. J. Iafrate, C. Benes, M. Toner, S. Maheswaran and D. A. Haber, *Science*, 2014, **345**, 216–220.
11 Y. Deng, Y. Zhang, S. Sun, Z. Wang, M. Wang, B. Yu, D. M. Czajkowsky, B. Liu, Y. Li, W. Wei and Q. Shi, *Sci. Rep.*, 2014, **4**, 7499.
12 S. Hou, H. Zhao, L. Zhao, Q. Shen, K. S. Wei, D. Y. Suh, A. Nakao, M. A. Garcia, M. Song, T. Lee, B. Xiong, S. C. Luo, H. R. Tseng and H. H. Yu, *Adv. Mater.*, 2013, **25**, 1547–1551.
13 Y. Xiao, H. Zhou, N. Xuan, M. Cheng, Y. Rao, Y. Luo, B. Wang and R. Tang, *ACS Appl. Mater. Interfaces*, 2014, **6**, 20804–20811.
14 M. Yu, A. Bardia, B. S. Wittner, S. L. Stott, M. E. Smas, D. T. Ting, S. J. Isakoff, J. C. Ciciliano, M. N. Wells, A. M. Shah, K. F. Concannon, M. C. Donaldson, L. V. Sequist, E. Brachtel, D. Sgroi, J. Baselga, S. Ramaswamy, M. Toner, D. A. Haber and S. Maheswaran, *Science*, 2013, **339**, 580–584.
15 A. J. Mach, J. H. Kim, A. Arshi, S. C. Hur and D. Di Carlo, *Lab Chip*, 2011, **11**, 2827–2834.
16 T. Okano, Y. H. Bae, H. Jacobs and S. W. Kim, *J. Controlled Release*, 1990, **11**, 255–265.
17 N. Yamada, T. Okano, H. Sakai, F. Karikusa, Y. Sawasaki and Y. Sakurai, *Makromol. Chem., Rapid Commun.*, 1990, **11**, 571–576.
18 Y. Kumashiro, M. Yamato and T. Okano, *Ann. Biomed. Eng.*, 2010, **38**, 1977–1988.
19 A. Agarwal, J. A. Goss, A. Cho, M. L. McCain and K. K. Parker, *Lab Chip*, 2013, **13**, 3599–3608.
20 X. Liu, V. Ory, S. Chapman, H. Yuan, C. Albanese, B. Kallakury, O. A. Timofeeva, C. Nealon, A. Dakic, V. Simic, B. R. Haddad, J. S. Rhim, A. Dritschilo, A. Riegel, A. McBride and R. Schlegel, *Am. J. Pathol.*, 2012, **180**, 599–607.
21 T. Y. Chang, V. G. Yadav, S. De Leo, A. Mohedas, B. Rajalingam, C.-L. Chen, S. Selvarasah, M. R. Dokmeci and A. Khademhosseini, *Langmuir*, 2007, **23**, 11718–11725.
22 M. Kaminska, W. Okroj, W. F. Szymanski, W. Szymanski, W. F. Jakubowski, P. Jakubowski, W. F. Komorowski, A. Komorowski, P. F. Nosal, H. Nosal, A. F. Szymanowski, M. Szymanowski, H. F. Gazicki-Lipman, H. Gazicki-Lipman, M. F. Jerczynska, Z. Jerczynska, H. F. Pawlowska, B. Pawlowska, Z. F. Walkowiak and B. Walkowiak, *Acta Bioeng. Biomech.*, 2009, **11**, 19–25.
23 N. Palechor-Ceron, F. A. Suprynowicz, G. Upadhyay, A. Dakic, T. Minas, V. Simic, M. Johnson, C. Albanese, R. Schlegel and X. Liu, *Am. J. Pathol.*, 2013, **183**, 1862–1870.
24 H. K. Lin, S. Zheng, A. J. Williams, M. Balic, S. Groshen, H. I. Scher, M. Fleisher, W. Stadler, R. H. Datar, Y. C. Tai and R. J. Cote, *Clin. Cancer Res.*, 2010, **16**, 5011–5018.

 