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Clinical Scale Cell-Surface Marker Independent, Acoustic Microfluidic Enrichment of Tumor Cells from Blood

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Abstract

Enumeration of circulating tumor cells (CTCs) predicts overall survival and treatment response in metastatic cancer, but as many commercialized assays isolate CTCs positive for epithelial cell markers alone, CTCs with little or no EpCAM expression stay undetected. Therefore, CTC enrichment and isolation by label-free methods based on biophysical rather than biochemical properties could provide a more representative spectrum of CTCs. Here, we report on a clinical scale automated acoustic microfluidic platform processing 5 mL erythrocyte depleted paraformaldehyde (PFA) fixed blood (diluted 1:2) at a flow rate of 75 >L/min, recovering 43/50 (87±2.3%) breast cancer cell line cells (MCF7), with 0.11% cancer cell purity and 162-fold enrichment in close to 2 hours based on intrinsic biophysical cell properties. Adjustments of the voltage-settings aimed at higher cancer cell purity in the central outlet provided 0.72% cancer cell purity and 1445-fold enrichment that resulted in 62±8.7% cancer cell recovery. Similar rates of cancer cell recovery, cancer-cell purity and fold-enrichment were seen with both prostate cancer (DU145, PC3) and breast cancer (MCF7) cell line cells. We identified eosinophil granulocytes as the predominant WBC contaminant (85%) in the enriched cancer cell fraction. Processing of viable cancer cells in erythrocyte depleted blood provided slightly reduced results as to fixed cells, (77% cancer cells in the enriched cancer cell fraction, with 0.2% WBC contamination). We

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†Author Contributions

These authors contributed equally to the current study.

The authors declare the following competing financial interest(s): Dr. Lilja holds patents for free PSA, intact PSA, and hK2 assays. P. Augustsson, C. Magnusson and T. Laurell are inventors on a patent licensed to Acousort AB, based on the reported method: Title: System and method to separate cells and/or particles; Inventors: P. Augustsson, C. Magnusson, C. Grenvall and T. Laurell; Publication info: EP2761291 and US14/374, 793. T. Laurell, H. Lilja, A. Lenshof, and P. Augustsson hold stock in AcouSort AB.

Supporting Information: Supporting Information Material and Methods (pdf)

Supporting Information Table 1–4 (pdf)

Supporting information Figure 1 (pdf)

demonstrate feasibility of enriching either PFA-fixed or viable cancer cells with a clinical scale acoustic microfluidic platform that can be adjusted to meet requirements for either high cancer cell recovery or higher purity, and can process 5 mL blood samples in close to 2 hours.

Graphical Abstract

INTRODUCTION

Circulating tumor cells (CTCs) are shed to the peripheral blood from both primary and metastatic tumor sites. They are implicated from experimental models to be present in the circulation of patients before metastases are detected^{1, 2} despite lack of evidence based on current commercially available techniques, such as the Veridex Cell-Search™ assay (Warren, NJ, USA). There is need to improve the molecular characterization of CTCs to better understand which functional properties of these disseminated cells are critical in the formation of distant metastatic lesions³. Further, there are urgent clinical needs for accurate and readily accessible automated methods to rapidly enrich and isolate CTCs from peripheral blood as a “liquid biopsy”, as CTC-enumeration has been shown to be an independent predictor of progression-free survival and overall survival in patients with metastatic cancer⁴.

Due to the low number of CTCs in the circulation, separation technologies must achieve high recovery of CTCs as well as reasonable cancer cell purity through efficient depletion of blood cells. Further, since CTCs are scarce, several milliliters of blood must be processed. Most commercially available or well-established techniques for CTC isolation achieve this by positive selection targeting epithelial cell surface markers such as the epithelial cell adhesion molecule (EpCAM) or cytokeratins (CKs)⁵; *e.g.*, the CellSearch assay⁶. However, it has become evident that some CTCs do not express these surface markers at detectable levels⁷. CTC isolation relying only on epithelial cell surface markers may therefore give an incomplete picture of the CTC population. These undetected cells might be cancer stem cells, mesenchymal cells or epithelial cells undergoing epithelial to mesenchymal transition (EMT)^{8, 9}. EMT is associated with cancer progression and metastasis¹⁰, although, other modalities may also be involved with tumor cell dissemination¹¹. It has been observed that multicellular clusters of tumor cells can break loose from the primary tumor site and be transported by the blood to distant sites of metastatic lesions, where they have been reported

to increase the metastatic potential compared to single cells¹². Cancer patients with detectable CTC clusters have been associated with a poor prognosis¹³; the cluster-chip is one proposed label-free method to capture CTC clusters¹⁴. Regardless of whether EMT, cell clusters, or cancer stem cells¹⁵ are the main cause of metastasis, alternative methods for isolation and characterization of CTCs from liquid biopsies are now being intensively pursued^{16–24}.

Acoustophoresis, is a gentle^{19, 25, 26} and label-free CTC separation method, using ultrasonic standing wave technology in a microfluidic setting. It is well suited for automation and enables multiple cell handling unit operations, such as alignment, separation, and concentration of rare cells, to be incorporated on a single chip²⁷. To date, acoustophoresis has successfully been used to discriminate cells based primarily on size^{19, 28–32}, and to a lesser extent also on compressibility and density. While the size range of single CTCs can partly overlap that of white blood cells (WBCs), this drawback for size-specific acoustophoresis is partially mitigated by the fact that CTC clusters well exceed the size of any cells normally occurring in blood¹⁴. Furthermore, recent findings indicate the feasibility of cell type-specific separation based on the cell properties of mass-density and compressibility³³, which would add further dimensions to acoustophoresis. Based on these considerations, we find it well worth investigating the potential for acoustophoresis as an alternative way to isolate CTCs from blood.

In this work, we present an acoustic microfluidic platform for enrichment of tumor cells from red blood cell depleted (RBC⁻) blood that can process 5 mL blood in approximately 2 h. The system is largely automated and can therefore be operated by non-experts, and the time to wash the system and load a subsequent sample is less than 2 min. Using (RBC⁻)-blood from healthy donors spiked with prostate (DU145, PC3) or breast (MCF7) cancer cells as a model system to evaluate the performance of the platform, we identify eosinophil granulocytes as the major WBC sub-population contaminant in our enriched cancer cell fraction. We process clinically relevant volumes of blood (5 mL) spiked with 50 cultured cancer cells. We also show this platform's feasibility for processing viable cells, opening up a wider range of post-separation analysis and characterization of CTCs in future patient samples.

MATERIALS AND METHODS

Briefly, we conducted five main experiments. We evaluated the performance of our acoustophoretic platform in terms of the upper limiting cell concentration that can be processed. We identified contaminating cell types. We evaluated the separation performance of our platform with respect to high purity or high recovery. We evaluated the platform's clinical scale performance, using PFA-fixed samples with a volume and tumor cell concentration relevant to clinical practice: 5.0 mL (RBC⁻)-blood spiked with 50 cultured MCF7 cancer cells. Finally, we did a "proof of principle" assessment of performance for viable cancer cells.

System and Microfluidic Chip

An overview of the cell separation platform is shown in Figure 1A. A chip and the piezoelectric ultrasound actuators on its aluminum support are enclosed in an aluminum manifold (Figure 1B). The chip was fabricated in silicon and glass and is similar in design to what was first proposed by Petersson *et al.*³⁴, with acoustic pre-focusing of cells later added for better resolution¹⁹. The temperature of the chip is controlled in a closed loop configuration consisting of a resistive temperature detector and a Peltier element, which enables both heating and cooling. Liquid and cell flow is driven by pressurizing the attached containers (Figure 1C). All samples were processed at a flow rate of 75 $\mu\text{L}/\text{min}$. Additional information can be found in Supporting Information.

Cell Separation Principle

The mechanism of separating cancer cells from WBCs (Figure 1A) has previously been described¹⁹. Briefly, cells are introduced through a pre-focusing channel ($20 \times 0.30 \times 0.15 \text{ mm}^3$) where they are exposed to acoustic radiation forces at 5 MHz, acting transversely to the flow in two dimensions. Upon entering the separation channel ($30 \times 0.38 \times 0.15 \text{ mm}^3$), the cells are laminated in proximity of the channel sidewalls by introduction of cell-free medium through the central branch of a trifurcation inlet at the end of the pre-focusing channel. While flowing through the separation channel, cells are exposed to a 2-MHz, half-wavelength acoustic standing wave field that force them toward the center of the separation channel. Larger cells migrate towards the center at a higher rate than smaller cells. Consequently, at the end of the separation channel, the majority of the cancer cells can be collected through the central branch of a trifurcation outlet while the smaller WBCs exit through the side branches. System calibration is described in Supporting Information.

Cell Culture and Blood

Human prostate cancer cell lines DU145, PC3 and breast cancer cell line MCF7 were acquired from American Type Culture Collection, and grown according to the supplier's recommendations. Whole blood was acquired from healthy volunteers at the blood donor center at Skåne University Hospital (Lund, Sweden). The blood was collected in VacutainerTM tubes (BD Bioscience, San Jose, CA) containing ethylenediaminetetraacetic acid (EDTA). The blood was processed and used the same day it was collected. More detailed cell preparation can be found in Supporting Information.

Determining the Upper Limiting Cell Concentration

A series of 0.2 mL samples with increasing concentrations of WBCs (1.5×10^5 to 9.0×10^6 cells/mL) was run through the acoustic separator using a constant concentration of spiked DU145 cells (2.5×10^5 cells/mL). Cells were paraformaldehyde (PFA) fixed and immunofluorescently labeled with CD45-APC and EpCAM-PE. Central and side outlet cell fractions were analyzed by flow cytometry.

Identification of Contaminating WBC Sub-populations

PFA fixed samples of 0.2 mL (RBC^-)-blood spiked with 10,000 DU145 cells were prepared and processed through the acoustic chip and analyzed by flow cytometry. WBCs were gated

as CD45⁺; to identify lymphocytes, we used a CD3 antibody that stains T-cells, which constitute the majority of the lymphocyte population. Among the CD66b⁺ cells, neutrophils were identified as highly positive for CD16, whereas eosinophils and basophils were negative or moderately positive for CD16; see Supporting Information (Tables S1 and S2). Cancer cells (DU145) were gated as EpCAM⁺ and CD45⁻.

Tuning Cancer Cell Purity and Recovery

A series of 38 PFA-fixed samples from 13 different healthy donors, each consisting of 0.5 mL (RBC⁻)-blood spiked with 50 cancer cells, either DU145, PC3 or MCF7 was processed. For each sample, a Petri dish with EpCAM stained cancer cells were placed under a microscope and a pipette was used to collect 50 single cells. The cancer cells were added to 0.5 mL CD45-labeled (RBC⁻)-blood and diluted with FACS buffer to a total sample volume of 1.0 mL. RBC⁻ samples containing DU145 (n=12), PC3 (n=8) or MCF7 (n=8) were run in 'high recovery' mode and ten RBC⁻ samples with DU145 were run in 'high purity' mode by lowering the actuator voltage. After separation, the collected central cell fraction was analyzed by flow cytometry for number of cancer cells and contaminating WBCs. In addition, 1.0 mL of the collected side waste fraction (total volume $\approx$ 4 mL) was analyzed for estimation of the contaminating central WBC fraction.

Clinical Scale Cancer Cell Enrichment from 5 mL Blood

To demonstrate the ability to process PFA-fixed samples of clinically relevant composition, cell samples (MCF7 and RBC⁻ blood) were prepared, processed and analyzed. 50 EpCAM-PE labeled MCF7 cells were spiked in 5 mL CD45-APC labeled (RBC⁻) blood (8 different healthy donors), and diluted with FACS buffer to a total sample volume of 10 mL. Samples were run in 'high recovery' mode (n=5) and in 'high purity' mode (n=3).

Effect of RBC lysing solution on cancer cells' acoustic properties

DU145 cells and blood was incubated with BD FACS lysing solution and analyzed by flow cytometry, for details see Supporting Information.

Separation of Viable Cells

Samples were prepared of 0.05 mL (RBC⁻) blood diluted 1:4 with FACS buffer to a total sample volume of 0.2 mL and spiked with approximately 10,000 DU145 cells. These experiments were repeated with three different healthy blood donors. The samples were processed at three different voltage amplitudes low = 5.3 V, intermediate = 5.6 V and high = 5.9 V. Non-viable tumor cells were excluded from statistical analysis as determined by forward and side-scatter position in the flow cytometry dot plots. N = 15.

Definitions of separation evaluation parameters

The system processes sample from an input sample container and separates cells to a central cancer cell outlet and a side WBC outlet. *Cancer cell recovery* is defined as the total number of cancer cells in the central outlet divided by the number of cancer cells that were spiked in the input sample. *The cancer cell purity* is defined as the number of cancer cells divided by the total number of all cell types in a container. *Enrichment* is defined as the purity in the

outlet container divided by the purity in the inlet container. *The central cancer cell fraction* is defined as the number of cancer cells in the central outlet container divided with the total number of cancer cells in both the central and side outlet containers. *The central WBC fraction* is likewise defined.

RESULTS

We developed and evaluated an acoustic micro-fluidic platform for enriching cancer cells from (RBC⁻)-blood, using clinically relevant sample volumes and cancer cell concentrations, targeting label-free CTC enrichment from 5 mL of blood. The acoustic microfluidic processing diverts the cells in the input sample into two output fractions based on each cell's acoustophoretic mobility, which correlates strongly with cell size. The larger cancer cells predominantly end up in the high-mobility central outlet fraction, while the vast majority of the smaller WBCs are collected from the low-mobility side outlets.

Determining the Upper Limiting Cell Concentration

To optimize processing of clinical samples, we performed initial experiments to determine the maximum cell concentration that could be processed by acoustophoresis without compromising the cell separation performance. Cell separation accuracy was independent of the input cell concentrations up to $\sim 3 \times 10^6$ cells/mL. At higher cell concentrations, the contamination of WBC increased in the central outlet fraction. This cutoff concentration, termed 'critical cell concentration', corresponds to the lower range of an undiluted WBC population in typical patient samples (3×10^6 to 10×10^6) WBC/mL (Figure 2A). In the following experiments, all samples were diluted to ensure a WBC concentration well below this critical cell concentration.

Identification of Contaminating WBC Sub-populations

After separation, the enriched cancer cell fraction from the central outlet is contaminated by WBC, indicating partly overlapping distributions of acoustophoretic mobility for the two cell types. In the first set of experiments, the data showed that approximately $0.6\% \pm 0.2\%$ (mean $\pm$ SD) of all processed WBCs ended up contaminating the cancer cell fraction for separation settings generating a $91\% \pm 1.9\%$ recovery of cancer cells in the central outlet fraction (Figure 2B). Of the contaminating WBCs, $91\% \pm 8.5\%$ were granulocytes (Figure 2C). The major contaminant was identified as eosinophil granulocytes (Figure 2D), constituting $93.3\% \pm 1.8\%$ of the granulocytes and 85% of all the contaminants in the central fraction. The basophils were excluded as a major contamination candidate due to their low number and smaller size. Although eosinophil granulocytes were the major contaminant, only $26\% \pm 10.6\%$ of all eosinophils in the sample ended up in the cancer cell fraction.

Tuning Cancer Cell Purity and Recovery

To evaluate the recovery of cancer cells at low concentrations, 50 cancer cells were spiked in 0.5 mL (RBC⁻) blood and then processed. Results from the first sets of 28 samples showed that after separation, the enriched cancer cell fraction in the central outlet had collected an average of 38 DU145, 41 MCF7 or 41 PC3 cells, respectively. This constituted 77%

$\pm 11.4\%$ (mean $\pm$ SD) recovery for DU145, $81\% \pm 6.1\%$ for MCF7 and $81\% \pm 11.4\%$ for PC3. The average central outlet WBC fraction was $0.28\% \pm 0.13\%$ in the DU145 experiments, $0.20\% \pm 0.11\%$ for MCF7 and $0.22\% \pm 0.30\%$ for PC3 (Figure 3A). The absolute number of contaminating WBCs varied between 405 and 5414 cells for the 28 samples, resulting in a mean cancer cell purity in the central outlet fraction of $1.5\% \pm 0.34\%$ for DU145 cells, $2.7\% \pm 1.6\%$ for MCF7 cells and $4.9\% \pm 2.8\%$ for PC3 (Figure 3B). The cancer cell enrichment factor, ranged from 94-fold to 1652-fold enrichment, with an average of 340 ± 156 -fold cancer cell enrichment for DU145, 576 ± 413 -fold for MCF7 and 877 ± 560 -fold for PC3 (Figure 3C and Table S3).

Aiming at increased cancer cell purity, 0.5 mL (RBC⁻) blood samples spiked with 50 DU145 cells (n=10) were run at slightly lower acoustic amplitude. The cancer cell recovery dropped to an average of 29 cells ($58\% \pm 11.1\%$) and the contaminating WBCs in the central outlet fraction dropped to $0.03\% \pm 0.02\%$ (Figure 4A). In addition, the average purity in the cancer cell outlet increased by a factor of 10 ($14.7\% \pm 8.5\%$) (Figure 4B). Likewise, the cancer cell enrichment increased to an average of 2785 ± 1760 -fold (Figure 4C and Table S4).

Clinical Scale Cancer Cell Separation

To demonstrate sufficient throughput at maintained separation performance, cancer cells were enriched from 10-mL samples (5 mL (RBC⁻)-blood diluted with 5 mL FACS buffer and spiked with 50 MCF7 cells). In 'high recovery' mode the average cancer cell recovery was 43 cells ($87\% \pm 2.3\%$) with a central WBC fraction level of $0.57\% \pm 0.14\%$ (Figure 5A). In 'high purity' mode the average cancer cell recovery was $62\% \pm 8.7\%$ with a central WBC fraction level of $0.10\% \pm 0.07\%$. The cancer cell purity of the collected central fraction was $0.11\% \pm 0.05\%$ for 'high recovery' mode and $0.72\% \pm 0.69\%$ for 'high purity' mode (Figure 5B). Enrichment factor was 162 ± 55 for 'high recovery' mode and 1445 ± 1811 for 'high purity' mode (Figure 5C). Future clinical samples will be subjected to RBC lysing solution before acoustic separation. Therefore, we used DU145 cells spiked into blood to determine whether RBC lysing solution would importantly influence the acoustic properties on cancer cells and nucleated blood cells. After the incubation with RBC lysing solution, we lost $9.2\% \pm 5.2\%$ DU145 cells compared to $4.5\% \pm 3.8\%$ WBCs (Figure S1A). The loss of cells was higher after running the samples through the acoustic microfluidics platform (DU145 $19.4\% \pm 4.8\%$ and WBC $7.8\% \pm 2.7\%$, Figure S1B), but there was no notable effect on cell separation or cancer cell recovery ($76\% \pm 4.9\%$, Figure S1C) associated with the RBC treatment.

Separation of Viable Cells

Samples containing viable non-PFA-fixed cells were processed at three different acoustic amplitudes to determine central cancer cell and central WBC fraction. The lowest amplitude generated the lowest central WBC fraction (mean $0.2\% \pm 0.1\%$) but also the lowest central cancer cell fraction (mean $77\% \pm 5.4\%$). For intermediate amplitude, the mean central cancer cell fraction was $87\% \pm 6.6\%$ with a mean central WBC fraction level of $0.8\% \pm 0.4\%$. The highest amplitude generated a mean central cancer cell fraction of $95\% \pm 4.0\%$ with considerably increased mean central WBC fraction: $5.1\% \pm 2.4\%$ (Figure 6).

DISCUSSION

We have presented an acoustic cell separation platform for cancer cell isolation that is able to process clinically relevant sample volumes. Cell suspension with concentrations up to 3×10^6 cells/mL can be processed without compromising separation accuracy. Hence, the acoustic micro-fluidic platform is able to process 5 mL (RBC⁻) blood diluted 1:2 in close to 2 hours. This instrument was designed so that a non-expert operator can produce repeatable sample treatments similar to Fong *et al*³⁵.

Many cell separation techniques display a dependency in separation performance in relation to cell concentration of the sample. This is due to the hydrodynamic interaction between closely spaced cells. When processing blood samples, cell concentration becomes a rate-limiting factor since the clinical level exceeds the concentration where hydrodynamic interaction plays a role and sample dilution is consequently needed. For acoustic pre-focusing and separation, a simple theoretical estimate of this critical cell concentration can be made by calculating the concentration for which the pre-focused cells are perfectly lined up. Above this concentration, the cells become too closely packed to be treated as single cells in terms of their interaction with the surrounding liquid and become influenced by hydrodynamic interactions such that their acoustofluidic migration velocity towards the pressure node increases³⁶. Given the channel width $w = 375 \mu\text{m}$ and height $h = 150 \mu\text{m}$, two cells of mean diameter ($d = 10 \mu\text{m}$) would occupy a thin slab of liquid of volume $V = whd$, corresponding to a cell concentration of $3.6 \times 10^6/\text{mL}$ (Figure 2A *blue dashed line*). This simple model predicts the experimental observation that WBC concentrations above $3 \times 10^6/\text{mL}$ in the input sample leads to an increase in WBCs in the central outlet. Therefore, in order to achieve highest possible separation efficiency, the input sample needs to be diluted to a cell concentration below this limit.

We concluded that for acoustic cancer cell enrichment in healthy donor blood the major contaminant is eosinophil granulocytes. One likely reason for this is that they are relatively large and dense, yielding an acoustophoretic migration velocity partly overlapping with that of some of the cancer cells. Granulocytes have previously been reported to have higher acoustophoretic migration rate than monocytes and lymphocytes³¹. This finding has further support in recent measurements of the acoustic impedances and sizes of neutrophils, lymphocytes and monocytes at single-cell level using iso-acoustic focusing³³. Lymphocytes present less of a contamination problem, likely related to their smaller size and thus minimal overlap in distribution of acoustophoretic mobility versus cancer cells.

The effectiveness of using cancer cell lines in assessment of CTC separation techniques has been scrutinized and extensively discussed. Reports of CTCs being smaller in size than cultured cells³⁷ have been used as a counterargument. However, there are also contradicting reports finding CTCs to be larger than or about the same size as cultured cells³⁸. More recent reports also highlight the importance of capturing CTC clusters, as these are hypothesized to have a higher metastatic potential¹⁴ and poor prognosis¹³. Since CTC clusters are larger, they can be more easily isolated by acoustophoresis.

The separation efficiency in terms of WBC depletion for a given level of central cancer cell fraction is better than in our previous study of this method¹⁹. This is primarily a consequence of the implementation of a longer acoustic pre-focusing channel that defines identical spatial positions for all cells as they enter into the separation zone. Other contributing factors may be the more stable flow compared to syringe-driven flows and the automated and reproducible system priming prior to each run.

Variation in separation outcome depends on several factors. The use of different blood donors explains the varying number of WBCs in the input samples. Donors with high numbers of eosinophils generally generate higher contamination levels and lower cancer cell purities. The variation in separation performance for repeated samples may also be caused by flow instabilities due to cell aggregates or contaminating fibers. In addition, cell counting using flow cytometry introduces uncertainties in sample analysis, which is especially critical at low cell numbers. Also, approximately 40 μL of the sample will remain inside the system after stopping the flow at the end of each run, corresponding in the 1 mL sized samples to an average loss of 2 cancer cells out of the total 50 spiked cancer cells.

We show that it is possible to process and separate both viable and fixed cells in the acoustic setup and we find that the separation performance is unaffected by the number of spiked cancer cells for the investigated range from 10 mL^{-1} to $2.5 \times 10^5\text{ mL}^{-1}$. Fixation leads to decreased acoustic migration velocities for both WBC and cancer cells. However, for viable cells the overlap in acoustic migration velocity is larger than for fixed cells, which leads to higher WBC contamination levels. Nevertheless, the prospect of processing viable cells opens up possibilities of post separation culturing and performing functional characterization and potential *ex vivo* drug screening of isolated CTCs - a considerable step toward the goal of achieving personalized medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. He W, Wang H, Hartmann LC, Cheng JX, Low PS. Proc Natl Acad Sci U S A. 2007; 104:11760–11765. [PubMed: 17601776]
2. Pantel K, Speicher MR. Oncogene. 2016; 35:1216–1224. [PubMed: 26050619]
3. Danila DC, Anand A, Schultz N, Heller G, Wan M, Sung CC, Dai C, Khanin R, Fleisher M, Lilja H, Scher HI. Eur Urol. 2014; 65:1191–1197. [PubMed: 23954088]
4. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. N Engl J Med. 2004; 351:781–791. [PubMed: 15317891]

5. Gabriel MT, Calleja LR, Chalopin A, Ory B, Heymann D. Clin Chem. 2016; 62:571–581. [PubMed: 26896446]
6. Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss TJ, Ciobanu N, Tallman MS, Kennedy MJ, Davidson NE, Sweet D, et al. Blood. 1993; 82:2605–2610. [PubMed: 8219214]
7. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD, Stanger BZ. Cell. 2012; 148:349–361. [PubMed: 22265420]
8. Nauseef JT, Henry MD. Nat Rev Urol. 2011; 8:428–439. [PubMed: 21691304]
9. Polyak K, Weinberg RA. Nat Rev Cancer. 2009; 9:265–273. [PubMed: 19262571]
10. Kalluri R, Weinberg RA. J Clin Invest. 2009; 119:1420–1428. [PubMed: 19487818]
11. Fischer KR, Durrans A, Lee S, Sheng JT, Li FH, Wong STC, Choi HJ, El Rayes T, Ryu SH, Troeger J, Schwabe RF, Vandat LT, Atorki NK, Mittal V, Gao DH. Nature. 2015; 527:472–476. [PubMed: 26560033]
12. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, Yu M, Pely A, Engstrom A, Zhu HL, Brannigan BW, Kapur R, Stott SL, Shioda T, Ramaswamy S, Ting DT, Lin CP, Toner M, Haber DA, Maheswaran S. Cell. 2014; 158:1110–1122. [PubMed: 25171411]
13. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, Priest LJ, Greystoke A, Zhou C, Morris K, Ward T, Blackhall FH, Dive C. J Clin Oncol. 2012; 30:525–532. [PubMed: 22253462]
14. Sarioglu AF, Aceto N, Kojic N, Donaldson MC, Zeinali M, Hamza B, Engstrom A, Zhu H, Sundaresan TK, Miyamoto DT, Luo X, Bardia A, Wittner BS, Ramaswamy S, Shioda T, Ting DT, Stott SL, Kapur R, Maheswaran S, Haber DA, Toner M. Nat Methods. 2015; 12:685–691. [PubMed: 25984697]
15. Malanchi I, Santamaria-Martinez A, Susanto E, Peng H, Lehr HA, Delaloye JF, Huelsken J. Nature. 2012; 481:85–95.
16. Antfolk M, Laurell T. Anal Chim Acta. 2017; 965:9–35. [PubMed: 28366216]
17. Jin C, McFaul SM, Duffy SP, Deng XY, Tavassoli P, Black PC, Ma HS. Lab Chip. 2014; 14:32–44. [PubMed: 23963515]
18. Karabacak NM, Spuhler PS, Fachin F, Lim EJ, Pai V, Ozkumur E, Martel JM, Kojic N, Smith K, Chen PI, Yang J, Hwang H, Morgan B, Trautwein J, Barber TA, Stott SL, Maheswaran S, Kapur R, Haber DA, Toner M. Nat protoc. 2014; 9:694–710. [PubMed: 24577360]
19. Augustsson P, Magnusson C, Nordin M, Lilja H, Laurell T. Anal Chem. 2012; 84:7954–7962. [PubMed: 22897670]
20. Xu L, Mao XY, Imrali A, Syed F, Mutsvangwa K, Berney D, Cathcart P, Hines J, Shamash J, Lu YJ. Plos One. 2015; 10:e0138032. [PubMed: 26397728]
21. Moon HS, Kwon K, Kim SI, Han H, Sohn J, Lee S, Jung HI. Lab Chip. 2011; 11:1118–1125. [PubMed: 21298159]
22. Park ES, Jin C, Guo Q, Ang RR, Duffy SP, Matthews K, Azad A, Abdi H, Todenhofer T, Bazov J, Chi KN, Black PC, Ma HS. Small. 2016; 12:1909–1919. [PubMed: 26917414]
23. Li P, Mao ZM, Peng ZL, Zhou LL, Chen YC, Huang PH, Truica CI, Drabick JJ, El-Deiry WS, Dao M, Suresh S, Huang TJ. Proc Natl Acad Sci USA. 2015; 112:4970–4975. [PubMed: 25848039]
24. Fan, ZH. Circulating tumor cells : isolation and analysis. Wiley; 2016.
25. Burguillos MA, Magnusson C, Nordin M, Lenshof A, Augustsson P, Hansson MJ, Elmer E, Lilja H, Brundin P, Laurell T, Deierborg T. Plos One. 2013; 8:e64233. [PubMed: 23724038]
26. Wiklund M. Lab Chip. 2012; 12:2018–2028. [PubMed: 22562376]
27. Antfolk M, Magnusson C, Augustsson P, Lilja H, Laurell T. Anal Chem. 2015; 87:9322–9328. [PubMed: 26309066]
28. Ding X, Peng Z, Lin SCS, Geri M, Li S, Li P, Chen Y, Dao M, Suresh S, Huang TJ. Proc Natl Acad Sci USA. 2014; 111:12992–12997. [PubMed: 25157150]
29. Petersson F, Aberg L, Sward-Nilsson AM, Laurell T. Anal Chem. 2007; 79:5117–5123. [PubMed: 17569501]
30. Thevoz P, Adams JD, Shea H, Bruus H, Soh HT. Anal Chem. 2010; 82:3094–3098. [PubMed: 20199060]

31. Grenvall C, Magnusson C, Lilja H, Laurell T. *Anal Chem.* 2015; 87:5596–5604. [PubMed: 25909882]
32. Dykes J, Lenshof A, Astrand-Grundstrom IB, Laurell T, Scheduling S. *Plos One.* 2011; 6:e23074. [PubMed: 21857996]
33. Augustsson P, Karlsen JT, Su HW, Bruus H, Voldman J. *Nat Commun.* 2016; 7:11556. [PubMed: 27180912]
34. Petersson F, Nilsson A, Jonsson H, Laurell T. *Anal Chem.* 2005; 77:1216–1221. [PubMed: 15732899]
35. Fong EJ, Huang C, Hamilton J, Benett WJ, Bora M, Burklund A, Metz TR, Shusteff M. *J Vis Exp.* 2015; 105:e5305.
36. Ley MWH, Bruus H. *Lab Chip.* 2016; 16:1178–1188. [PubMed: 26948344]
37. Park S, Ang RR, Duffy SP, Bazov J, Chi KN, Black PC, Ma HS. *Plos One.* 2014; 9:e85264. [PubMed: 24416373]
38. Lin HK, Zheng S, Williams AJ, Balic M, Groshen S, Scher HI, Fleisher M, Stadler W, Datar RH, Tai YC, Cote RJ. *Clin Cancer Res.* 2010; 16:5011–5018. [PubMed: 20876796]
39. Deshmukh S, Brzozka Z, Laurell T, Augustsson P. *Lab Chip.* 2014; 14:3394–3400. [PubMed: 25007385]

Figure 1.

Overview of the acoustophoretic microchip and the pressure-driven setup. (A) Schematic of the separation principle in the chip and the external fluidic components of the acoustophoresis cell-separation platform. The sample reservoirs are individually pressurized using a computer-controlled multichannel pneumatic terminal. An aqueous suspension of cells (represented by red and blue circles and lines) from the sample input tube enters the chip through the pre-focusing channel. After passing through the separation channel, the cells are collected in two test tubes. Schematic reprinted and adapted from ³⁹ - Published by The Royal Society of Chemistry. (B) Photograph of the chip and aluminum holder prior to assembly, showing the temperature sensor and the piezoelectric actuator for the sound generation. (C) The sample test tube holder designed for fast and reproducible sample loading. Tubes are pressure sealed by a spring-loaded bar.

Figure 2.

Evaluation of the acoustic platform performance. (A) Central cancer cell and WBC fractions versus total cell concentration. A series of samples with increasing concentrations of WBCs (1.5×10^5 to $9.0 \times 10^6 \text{ mL}^{-1}$) was run through the acoustic separation chip using a constant concentration of spiked DU145 cells ($2.5 \times 10^5 \text{ mL}^{-1}$). The blue dashed line indicates the estimated theoretical critical cell concentration for our acoustophoretic platform (*see Discussion*). Identification of contaminating WBC subpopulations. (B) Black circles show central cancer cell fraction (DU145) and red squares show central WBC fraction after cell separation in the acoustic microchip. (C) For WBCs contaminating the central outlet, percentage distribution of leukocyte sub-populations: granulocytes, monocytes and lymphocytes. (D) For granulocytes contaminating the central outlet, percentage distribution of sub-populations: neutrophils and eosinophils. Horizontal

Figure 3.

High cancer cell recovery. Acoustophoretic separation for 1.0-mL sample volumes. 0.5 mL (RBC –) blood was spiked with 50 cancer cells, diluted to a total sample volume of 1 mL. The blood samples originated from nine different healthy donors. Horizontal lines indicate mean values. (A) Black and grey circles (show percentage cancer cell recovery (DU145, MCF7 and PC3) in the central outlet compared to input. The red squares show percentage of the central WBC fraction compared to all detected WBCs in either central or side outlets. (B) Black circles (DU145), grey circles (MCF7) and black/grey circles (PC3) show cancer cell purity in the cell fraction collected from the central outlet. (C) Black circles (DU145), grey circles (MCF7) and black/grey circles (PC3) show cancer cell enrichment in the central cell fraction relative to the input sample.

Figure 4.

High cancer cell purity. Acoustophoretic separation for 0.5 mL RBC-lysed blood that was spiked with 50 cancer cells (DU145), diluted to a total sample volume of 1 mL. Samples were processed at reduced mean voltage for lower WBC contamination. The blood samples originated from four different healthy donors. Horizontal lines indicate mean values. (A) Black circles show percentage cancer cell recovery in the central outlet compared to input. The red squares show percentage of the central WBC fraction compared to all detected WBCs in either central or side outlets. (B) Black circles show cancer cell purity in the central cell outlet. (C) Black circles show cancer cell enrichment in the central outlet cell fraction relative to the input sample.

Figure 5.

Processing of 10-mL sample volumes. Acoustophoretic separation was performed using 5.0 mL (RBC-) blood spiked with 50 MCF7 cells, diluted to a total sample volume of 10 mL. The blood samples originated from eight healthy donors. Horizontal lines indicate mean values. (A) Black circles show percentage cancer cell recovery in the central outlet compared to input. The red squares show percentage of the central WBC fraction compared to all detected WBCs at the outlets. (B) Circles show cancer cell purity in the central outlet cell fraction. (C) Circles show cancer cell enrichment in the central outlet fraction relative to the input sample.

Figure 6.

Acoustic cell separation of viable cells. 0.2 mL samples containing 0.05 mL (RBC-) blood and 10,000 viable DU145 cells were processed. The samples were processed at three different voltages: low = 5.3 V, intermediate= 5.6 V, and high = 5.9V. Black symbols indicate the central DU145 fraction compared to all detected cancer cells in both central and side fractions. Red symbols indicate central WBC fraction. Horizontal lines indicate mean values. N = 15.