A method for detecting circulating tumor cells based on measurement of single cell metabolism in droplet-based microfluidics

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Dedicated to Prof. G. Scoles on the occasion of his 80th birthday

Abstract: The number of circulating tumor cells (CTCs) in blood is strongly correlated with the progress of metastatic cancer. Current methods to detect CTCs are based on immunostaining or discrimination of physical properties. Here, we present a label-free method exploiting the abnormal metabolic behaviour of cancer cells. We demonstrate a single-cell analysis technique to measure the secretion of acid of individual, living tumor cells compartmentalized in microfluidically prepared, monodisperse, pL droplets. We can detect as few as 10 tumor cells in a background of 200,000 white blood cells and show proof-of-concept data on the detection of CTCs in the blood of metastatic patients.

Cancer is a generic term for a large group of diseases that can affect any part of the body. At some point during the development of most types of human cancer, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new colonies. A key step in the process of metastasis is the shedding of cells from primary tumors into the vasculature, which then circulate through the bloodstream and eventually re-penetrate vessel walls to form another tumor.[1] There is considerable evidence that these so-called circulating tumor cells (CTCs) are a key biomarker marking the progression of cancer metastasis and there is a direct correlation between survival times and number of CTCs in the peripheral blood.[1–6] A key limitation in the capture and analysis of CTCs is their extreme rarity relative to the 5 x 10^9 erythrocytes and 1-10 x 10^6 leukocytes per mL of blood. Although red blood cells can be easily removed by osmotic cell lysis, leukocytes (white blood cells) share many of the physical chemical and biological properties of CTCs, leading to high contamination levels in many CTC detection methods.[7] Biochemical techniques for detecting and counting CTCs exploit the presence of surface and cytoplasmic proteins (epithelial cell adhesion molecule, EpCAM, or HER2, EGFR, MUC1, CKs) that are not present on leukocytes.[8] Currently, the only clinically validated method (CellSearch®) is based on the enumeration of epithelial cells, which are separated from the blood by EpCAM-coated magnetic beads and identified with the use of fluorescently labeled antibodies against cytokeratin (8, 18, 19) and with a fluorescent nuclear stain.[9] Although CellSearch® and other immunostaining-based methods are able to detect EpCAM-positive CTCs reliably, not all CTCs may have epithelial surface markers due to a partial or complete epithelial-mesenchymal transition (EMT) that CTCs undergo when they escape from the primary tumor. As such, the EMT confers on epithelial cells precisely the set of traits that would empower them to disseminate from primary tumors and seed metastases.[10,11] Furthermore, Yu et al. have shown that the EMT is correlated to disease progression,[12] so these methods might be missing most relevant CTCs. There has been a large effort to develop alternative, low-cost, label-free techniques for the detection of CTCs based on physical properties such as the mechanical properties of CTCs, size selection, deformability, or electric charge. These techniques are reviewed elsewhere,[7] but the general conclusion is that although much promising progress has been made, the robustness in distinguishing between healthy cells and CTCs, and the isolation of live CTCs need to be improved further. We believe that cancer metabolism provides unique opportunities to achieve this requirement: an altered energy metabolism has been proven to be widespread in cancer cells and is one of hallmarks of cancer.[13,14,15-18] Otto Warburg first observed an anomalous characteristic of cancer cell metabolism in the 1920s:[19] even in the presence of oxygen, cancer cells limit their energy production largely to glycolysis, leading to massive secretion of lactate and acidification of the tumor environment, a phenomenon that has been termed the "Warburg effect" or "aerobic glycolysis".[20] Acidification of the medium has been proven to be independent from Warburg effect, appearing early in tumorigenesis and increasing with the acquisition of more aggressive and metastatic phenotypes.[18] These metabolic alterations have been known for over 50 years, but they have never been used to detect CTCs, as such cells are so rare that they do not noticeably alter the pH levels or lactate concentration in a sample of blood. The key technological breakthrough presented here lies in splitting the macroscopic (blood) sample into small (picoliter-nanoliter) aqueous droplets in oil (making a water-in-oil emulsion) using microfluidic technology[21]. Each droplet contains at most a single cell and all molecules secreted by this single cell are retained by the droplet.[22] The pH range of cancer extracellular environment is known to be 6.2–6.9 compared with 7.3–7.4 of normal tissue, and secretion rate of lactic acid by tumor cells is in the range of 10^{-16} mol/cell/sec, which is approx. 30-fold higher than the typical secretion rate of leukocytes.[23,24] Because of the small volume of the droplets, the concentrations of these secreted molecules rapidly increases up to measurable levels. CTCs are thus detected by pH measurements or lactate concentration changes in the extracellular compartment of individual cells, without the need for surface-antigen labelling (Figure

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1A). To establish the validity of our approach, we emulsified a suspension of tumor cells from a cancer cell line (lung - A549) in 35 pL droplets in the presence of culture medium and a lactic acid assay mixture (Sigma). The number of cells in each droplet followed a Poisson distribution ensuring >90% single cell encapsulation (NB, this means that most droplets will be empty; see Figure S1) and we demonstrated the production of lactate by A549 in drops (data not shown). We then mixed A549 cells with white blood cells (WBCs, Figure 1B), as these will be the primary background in blood samples taken from patients, and observed a clear ~ 2-fold intensity difference between droplets containing a cancer cell and empty or WBC containing drops (Figure 1C). There is some spread in the fluorescence, most likely due to a difference in lactate secretion rates between individual cells. Subsequently, in order to simplify the assay, we measured lactate secretion indirectly, by monitoring the pH of the droplet using a pH-sensitive dye (pHrodo Green) and obtained similar results (i.e. A549-containing droplets showed a clear drop in pH; see Figure S2). To screen droplets with higher throughput in a semi-automated way, we engineered an inverted microscope (Figure 2), so that each droplet can be analyzed using laser-induced fluorescence at approximately 1 kHz.[25,26] We used a ratiometric dye (Snarf-5F, free carboxylic acid, Figure S3, S4) to increase the precision of the pH measurement. For each droplet the ratio of emitted fluorescence at 580 and 630 nm is calculated in real time. In the presence of a cell secreting lactate the pH inside a droplet reduces below 7.4 and as a result an increase in 580/630 ratio above 1 is observed (Figure 2A). Real time analysis of each droplet enables us to capture images of a subset of droplets with increased 580/630 fluorescence ratios, thus providing an additional verification. The assay consists of three steps: a sample emulsification, incubation and a readout. To facilitate subsequent reinjection, droplets were generated, collected and incubated in a device with a cone-shaped chamber. After incubation all drops are injected in another device where each droplet is interrogated and fluorescence ratio is determined (see Figure S3 and S4 for calibration data). Using the developed method, we investigated secretion in various cancer cell lines. The secretion of lactate leads to a rapid increase in the concentration of acid in cell-containing droplets. Even after short incubation times (<2 minutes) a population of acidified droplets appears. This population increases further, approaching saturation after 10-20 minutes. Therefore, all our experiments were carried out using incubation times of at least 10 minutes (see Figure S5). To demonstrate that this is a general method for detection of cancer cells, we tested several other cancer cell lines, both EpCAM(+) and (-) including ovarian TOV21G, breast MDA-MB 453, glioblastoma U231, colorectal HT-29, breast MCF-7 and MDA-MB-231 - and found that all show acidification of droplets (see Figure S6). We used the A549 cell line and WBC to simulate clinical samples and to investigate analytical figures of merit of the developed method. Experiments were then repeated using larger numbers of cells. Figure 3A shows data points for 2M droplets of an emulsified A549 suspension, and we see a clear fraction of cell-containing droplets with a reduced pH, against a large background of empty droplets of unchanged pH. Figures 3B shows data points for 2M droplets produced from a sample of WBCs from a healthy donor showing no acid-positive droplets. Figure 3C shows data points for 2M droplets of the same sample with A549 cells spiked in, leading to a distinct population of acidified droplets. These figures clearly demonstrate that our method is capable of distinguishing healthy cells from metabolically active A549 (see Figure S9 for representative images). Figure S7 shows data on a similar experiment where all tumour cells were stained using Calcein Violet AM – a viability staining dye not affecting cell behaviour (cell viability during this assay was confirmed separately (se Figure S12) – prior to mixing. Most acid-positive droplets were also positive in the Calcein channel, confirming the excellent selectivity of the assay. We detected only rare acid-positive Calcein-negative drops, and by visualizing them we found out they were clusters of 10+ WBCs or junk artefacts.

CTCs are extremely rare cells and the detection of these cells requires an assay with high sensitivity and specificity. To quantify both, we emulsified mixtures of A549 tumor cells with WBCs in ratios ranging from as few as 10:200,000 to 130:200,000 A549:WBC (total samples sizes containing 1M/mL WBCs). Figure 3D shows the number of tumor cells detected vs. number of tumor cells spiked in. Our method is capable of detecting A549's even at the lowest dilutions tested, with average detection rates for all experiments in the range of 60%. We note that at low cell count, deviations between expected and recovered cell numbers might be due to variations in actual cells compartmentalized, and losses due to adhesion to tubing or syringe. Importantly, none of the low pH droplets contained WBCs (as confirmed by analysing the video images). With the method now firmly established, we tested samples based on blood of healthy donors as well as cancer patients with confirmed metastatic disease. To be able to process a large amount of blood, we depleted lysed blood of CD45+ cells (see SI). Prior to encapsulation, we stained WBC using a fluorescent antibody for CD45. Figures 4 and S10 clearly show that in the CD45(-) fraction no positive droplets are observed in samples derived from the blood of healthy volunteers, whereas numerous positive droplets are detected when either A549s are spiked into the healthy donor sample, or the sample of a metastatic colorectal cancer patient is analyzed. These positive droplets contain cells that acidify the microenvironment but are negative for CD45, providing a strong indication that these cells are most likely tumor cells. Further experiments on 4 other cancer patients (see Table S1) showed droplets that were acid positive/CD45-, and pictures of these droplets shows sometimes cells of comparable size to WBCs, sometimes of clearly different morphology compared to WBC (See Figure S11)).

This work provides the first proof-of-concept indication that cancer cell metabolism, and more specifically, acidification of the extracellular microenvironment, can be used to identify and count rare tumor cells and CTCs. Further work is needed to confirm that these cells are indeed CTCs and if so, their cancer-specific proteins and genetic mutations must be profiled; to clarify how they can impact in clinical routine, positive events need to be isolated, while clinical parameters as sensitivity, specificity, predictive values, must be established by dedicated clinical trials.

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FDB, MT, AP, WTSH and GC filed a patent application based on this work.

Keywords: Cancer • Circulating Tumor Cells • Microfluidics • metabolism • Picodroplets

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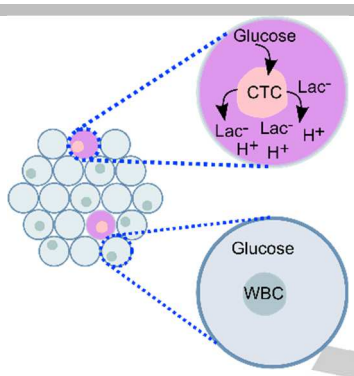
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

An altered metabolism is one of cancer's hallmarks. Differences between healthy and cancerous cell metabolism, hidden in bulk, are apparent in tiny (pL) droplets containing individual cells. Within minutes cancerous cells generate a pH change in their microenvironment. By measuring the pH in each individual droplet CTC detection in patient samples is possible.



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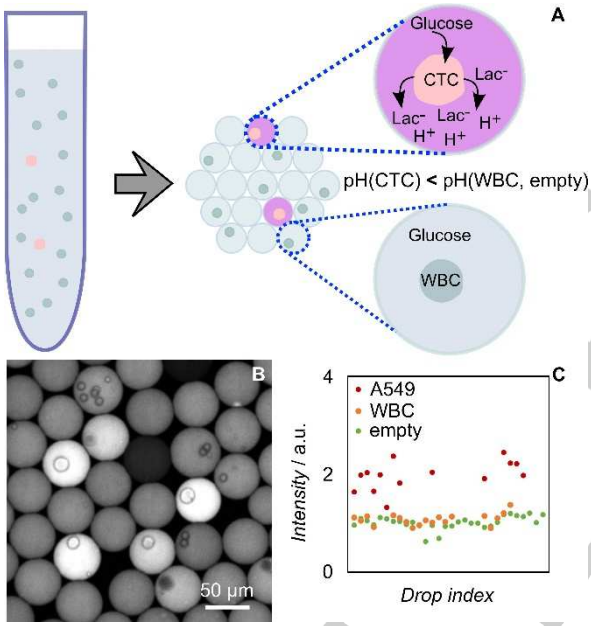

Figure 1. (A) CTC detection based on Warburg effect using compartmentalization in microdroplets. (B) A production of lactate by A549 cell in droplets. Only A549 containing droplets (large cells) show increase in fluorescence. Note that even with white blood cells clusters (smaller cells), drops do not show an increase in fluorescence. Pictures has been brightness/contrast enhanced. (C) A quantification of fluorescence in single drops based on raw pictures.

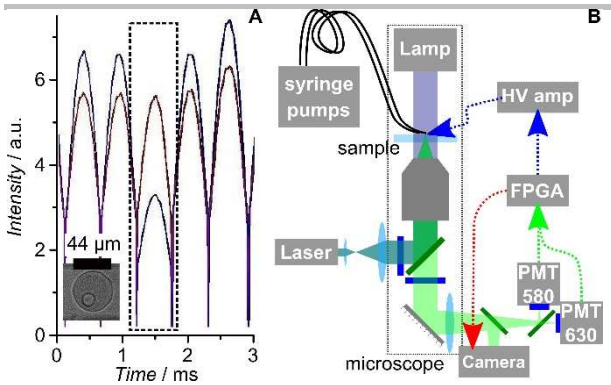

Figure 2. Detection of CTC using dual emission SNARF 5F dye. (A) Fragment of a raw data trace. Inset shows micrograph of a detected CTC. (B) Schematic of experimental setup.

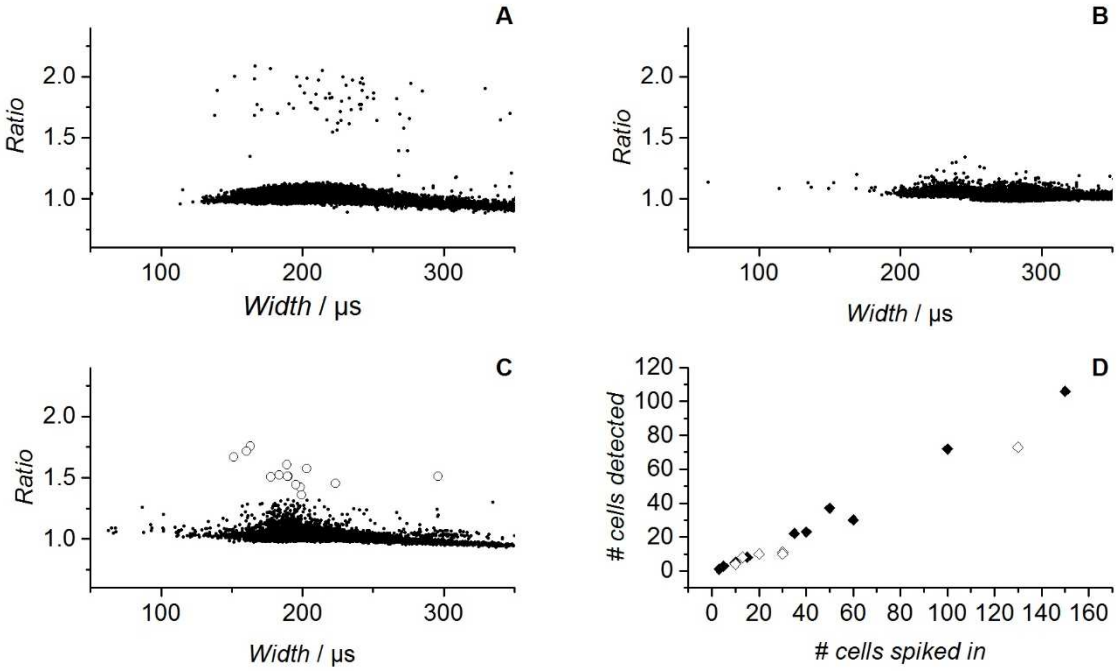

Figure 3. Detection of A549 cells. (A) Response of A549s alone in Joklik medium (pH 7.2). The dots with elevated ratio correspond to droplets with cancer cells. The majority of droplets don't show significant change in ratio – these are empty droplets always present after emulsification. (B) Response of isolated WBC alone in the medium. (C) Mixture of A549 and WBCs in the medium. Open circles represent acidic droplets containing A549 cells. (D) The recovery of A549 cells spiked in. Solid diamonds represent recoveries observed with A549s only in the buffer and open diamonds represent A549s detected in the presence of WBC.

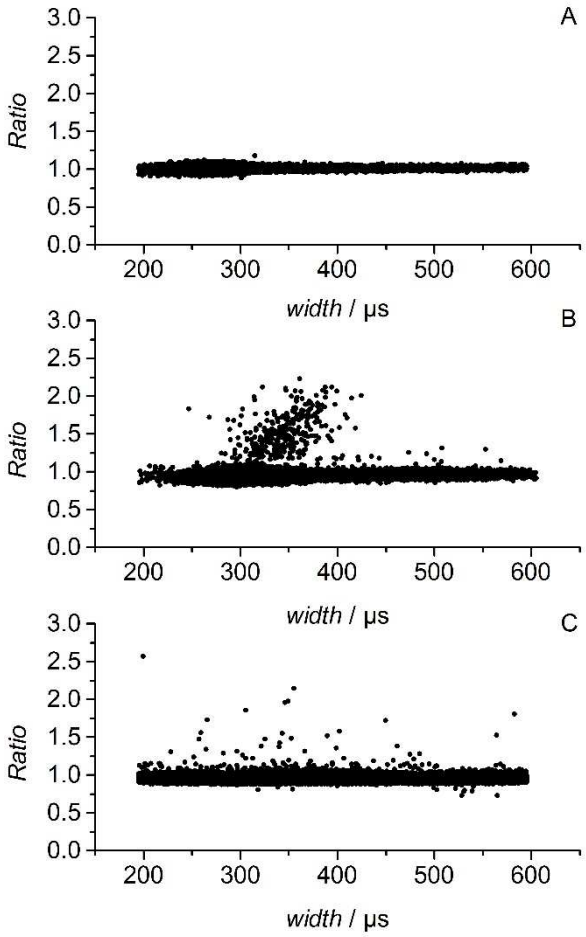

Figure 4. CTC detection in clinical samples. (A) Healthy volunteer sample. (B) Healthy volunteer sample with spiked tumor cells. (C) Sample from metastatic colorectal cancer patient.