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A SINGLE-CELL METABOLIC ASSAY FOR THE DETECTION OF CIRCULATING TUMOR CELLS

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Background

Cancer

Definition

According to World Health Organization (WHO reference) and Us National Cancer Institute (NCI reference), two of the most important authorities in medicine and oncology, respectively, cancer is a generic term for a large group of diseases that can affect any part of the body. Other synonyms are malignant tumors and neoplasms. The word tumor derives from the Latin verb "*tumeo*", to swell, because a swollen mass is usually the common sign of the presence of cancer in the body (except for some blood cancer). The tumoral mass is formed by abnormal cells that grow beyond their usual boundaries.
Normally, in the healthy condition, human cells grow and proliferate to form new cells as needed, they are shaped to specific functions – differentiated – useful to the respective organ, and when they grow old or become damaged, they die and are replaced by new cells. This ordered process is a consequence of cells acceptance of the organism signals of proliferation, differentiation and suppression. The disruption of this orderly process give rise to cancer, which can thus be seen as a non-observance of signals and instructions given by the organism to individual cells.
As a consequence, in cancer cells proliferate also when they are not needed and damaging other cells, then progressively acquire a phenotype more and more different from their original nature and refuse to die at the proper moment. In this way many cancers form solid tumors, which are masses of tissue. Cancers of the blood, such as leukemias, generally do not form solid tumors, but proliferating neoplastic cells spread into the bone marrow and blood stream. In both cases cancer cells can eventually invade nearby parts of the body and spread to other organs, generating what are called metastasis – which are in the end the major cause of death from cancer.
Tumors are defined as malignant when they show the above-mentioned characteristics of invasion of nearby tissues; if this features is absent, tumors are called benign. The only problem with benign tumors is that they may compress sensible organs, such as brain, otherwise they would be innocuous. When they are removed, they usually don't recur, while cancer often does.

Key facts and cost of cancer care

World Health Organization (WHO reference) also provides key facts about cancer, giving an overall idea about the burden of the disease, main types and correlation with known causes.

Cancer is a leading cause of death and its trend is expected to increase:

- "Cancers is a leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012" (Stewart and Wild 2014).
- "The number of new cases is expected to rise by about 70% over the next 2 decades."
- "It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next 2 decades" (Stewart and Wild 2014).

Cancer most common sites are well-defined. Among all, lung cancer is the big killer and its mortality at 5-years is over 90% (Cancer research UK). In women, breast cancer is the leading cause of death, with currently a good survival for early detected cancer (Cancer Research UK):

- "Among men, the 5 most common sites of cancer diagnosed in 2012 were lung, prostate, colorectum, stomach, and liver cancer."
- "Among women the 5 most common sites diagnosed were breast, colorectum, lung, cervix, and stomach cancer."

Currently, scientific community thinks that some cancers are due to known behavioral and dietary risks:

- "Around one third of cancer deaths are due to the 5 leading behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol use."
- "Tobacco use is the most important risk factor for cancer causing around 20% of global cancer deaths and around 70% of global lung cancer deaths."

Other cancer are caused by known infections:

- "Cancer causing viral infections such as HBV/HCV and HPV are responsible for up to 20% of cancer deaths in low- and middle-income countries" (De Martel et al. 2012).

Cancer geographic distribution highlight how the majority of cancer and cancer-related death is in developing countries, thus indicating that cancer is more of a global plague rather than a disease of the wealthy, developed world.

- "More than 60% of world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths" (Stewart and Wild 2014).

Cancer is heavily impacting national healthcare system balance, and whole society both from a social and economic perspective. According to Fernandez, (Luengo-Fernandez et al. 2013), cancer cost the EU €126 billion in 2009, including healthcare cost, productivity losses and informal care, with healthcare accounting for €51 billion (40%). Please note that, contrary to what often expected, direct costs are a minor part of the whole. Usually, productivity losses and informal care are underestimated due to their hidden nature, but their weight is comparable to direct costs. Across the EU, the healthcare costs of cancer were €102 per citizen, but with a high variation from €16 per person in Bulgaria to €184 per person in Luxembourg, showing the impact of different healthcare models. Productivity cost because of early death is €42.6 billion and lost working days €9.43 billion. Informal care cost €23.2 billion.
The cost per type of cancer mirrors cancer epidemiology, with lung cancer having the highest economic cost (€18.8 billion, 15% of overall cancer costs), followed by breast cancer (€15.0 billion, 12%), colorectal cancer (€13.1 billion, 10%), and prostate cancer (€8.43 billion, 7%).
In a US-review, total costs of cancer care have been estimated in $157 billion dollars in 2010. Forecasts contained in the same review predicted that costs of care will increase annually by 2% in the initial and last year of care, with a total cost in 2020 projected to be $174 billion (Mariotto et al. 2011).

The Hallmarks of cancer

In year 2000, Hanahan & Weinberg published a review (Hanahan and Weinberg 2000) listing six distinctive biological capabilities acquired during the multistep development of human tumors, and shared by most, if not all, types of cancer (Figure 1). These features were named “hallmarks of cancer”, and are described below.

Sustaining proliferative signaling

Normal cells cannot proliferate without a growth signal. In this aspect, the cell can be viewed as passive, “biological substance”, shaped by body signals. These signal are called mitogenic growth signals (GS) and are required to shift cells from a quiescent state into a proliferative condition.

Tumor cells independently generate their own growth signals, thereby reducing their dependence on stimulation from the organism.

Evading growth suppressors

Normal cells must be functional to the organism needs, and old or unneeded cells must accept death at the advantage of organ architecture. For this reason, within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. These suppressive signal can arrest proliferation by two distinct mechanisms:

- Cells are reversibly moved out of their proliferative G1/S/G2/M cell cycle into the quiescent (G_0) state, from which they may re-enter active cell cycle if needed and if appropriate extracellular signal are secreted.
- Cells are permanently forced out of their proliferative potential into postmitotic states, usually associated with acquisition of specific differentiation-associated traits.

Cancer cells evade these antiproliferative signals, enabling them to maintain proliferation and to enlarge tumoral mass.

Resisting cell death

Similarly to what said above, apoptosis – a latent program of programmed cell death – is triggered and activated when cells are not needed by the body.

Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer.

Enabling replicative immortality

There is an intrinsic mechanism inside mammalian cells limiting the number of replications they can endure. This program appears to be independent of the cell-to-cell signaling pathways described above.

When most types of cancer cells are explanted from the body and cultured in vitro, they don't show this replication limit. This suggests that cancer cells lose their replicative limit during in vivo cancer progression.

Inducing angiogenesis

All cells are strictly dependend by blood irroration, allowing them to capture oxygen and all necessary nutrients needed for their activity. For this reason almost all cells in a tissue reside within 100 μm of a capillary blood vessel. The growth of new blood vessels – the process of angiogenesis – is transitory and carefully regulated.

Cancer cells rapid growth requires a strong support of nutrients. For this reason, every tumoral mass must be sustained by a dense growth of new blood vessels if it wants to survive. Cancer cells progressively acquire the skills of inducing and maintaining angiogenesis.

Activating invasion and metastasis

During the course of cancer progression, there comes a time in which cancer cells acquire the ability of invading nearby tissues, and even travel to distant sites to colonize and form new masses. Tumoral masses formed in a site different from the primary tumor are called metastasis – from the Greek "distant location". Metastasis are the ultimate cause of death of most cancers.

The process of invasion and metastasis is a complex process, and its genetic and biochemical features are incompletely understood.

The present work is strongly related to this hallmark, which we will describe with more details than the others in the following chapter.

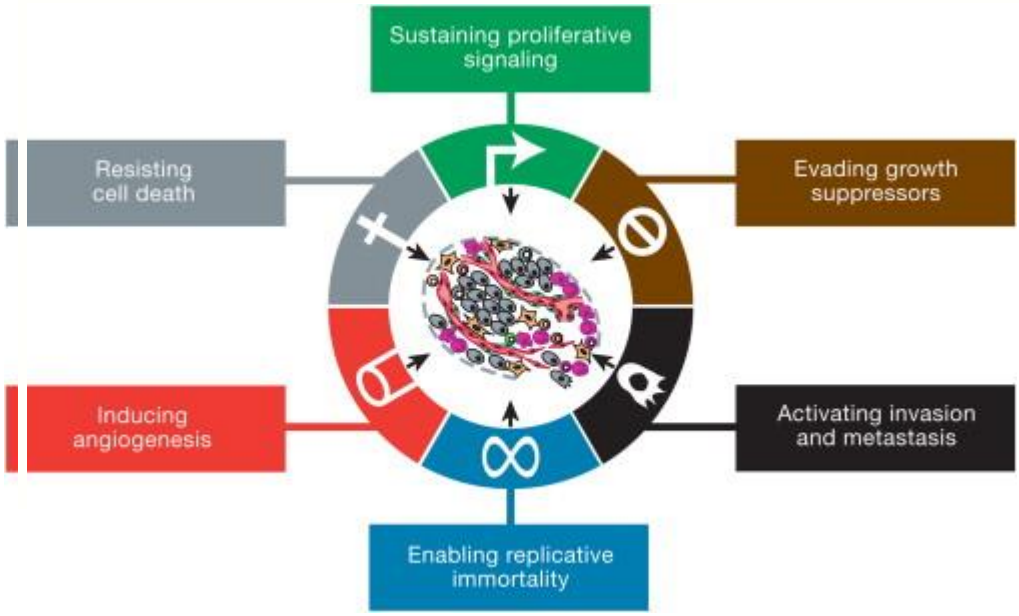

Figure 1 (Hanahan and Weinberg 2000) Summarizing schematics of Hallmarks of cancer described in the text above.

Invasion and Metastasis

Metastasis, as cited above, is the ultimate cause of death of most cancers patients, yet it remains the one of the most poorly understood step in cancer progression. During metastatic dissemination, a cancer cell from a primary tumor executes the following sequence of steps:

- It locally invades the surrounding tissue, breaking the basal membrane if of epithelial origin, and often acquiring a mesenchymal phenotype with an epithelial to mesenchymal transition (EMT)
- Secretes matrix metalloproteinases and similar enzymes to break the extracellular matrix and travel through it
- Enters the microvasculature of the lymph and blood systems (intravasation)

- Survives and travel through the bloodstream to microvessels of distant tissues
- Exits from the bloodstream (extravasation), surviving in the microenvironment of distant tissues
- Finally adapts to the distant environment of colonized tissues in order to proliferate and form a macroscopic secondary tumor (colonization).

Particularly, spread to the anatomically distant sites seems to occur almost entirely through the blood via the process of hematogenous dissemination. (Chaffer and Weinberg 2011). (Figure 2)

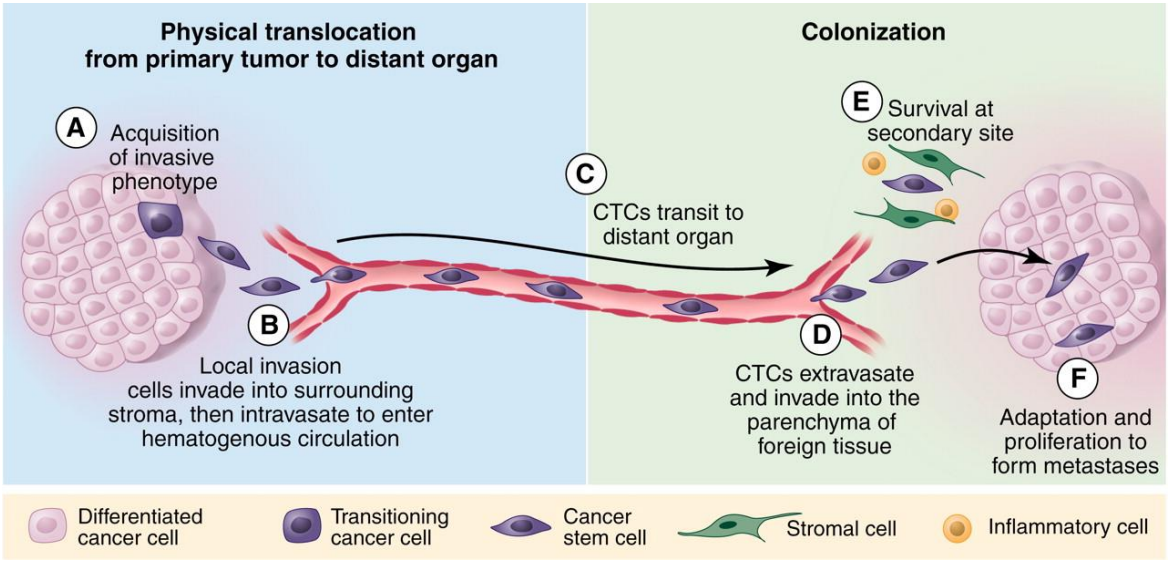

Figure 2 (Chaffer and Weinberg 2011) - The metastatic cascade. At the bottom, the legend for different cell types. Blue quadrant shows event in proximity of primary mass, while green quadrant shows events far from primary mass. In section A, cancer cells with acquired invasive phenotype invade nearby tissue and move through extracellular matrix aiming for a blood vessel. In section B, intravasation occurs – please note that between section B and D the cell is considered a circulating tumor cells, strictly connected with this thesis, and later described. In section C the cell travel together with the blood torrent to a distant site and extravasate D, invading distant stroma. Please note that it is far from trivial that a cell from e.g.: breast tissue can survive a e.g.: lung environment, having to evade innate immunity and surviving as single cells. In the last stage F, a new tumoral mass is formed and the process might start again.

The duration of this metastatic cascade ranges from weeks to years - sometimes metastatic tumors become apparent decades after a patient is considered cured.

Needless to be said, a better understanding of metastasis mechanisms and triggers would help to prevent most of cancer deaths. Even if with this knowledge might not fulfill tumoral mass eradication, at least its spread throughout the body could be heavily limited and other combined approached can be used at the same time.

The first useful piece of knowledge to understand metastasis would be the identification, between the heterogenous mix of cells in the primary tumour, of the cells responsible of invasion, and their characterization with respect to cells unable to carry on the whole process.

Our knowledge on this topic is still incomplete because there are currently some barriers to the study of metastasis:

1. Secondary tumours large enough to be detected are often **not biopsied** because patients are in fragile health, and being biopsy an invasive, painful and risky procedure, especially in specific organs (e.g.: lung, brain, common sites of metastasis)
2. The initial growth and progression of metastasis would be an extremely interesting topic to study, but single cells and even initial clusters are **too small** to show up in imaging scans, which have a resolution power of 0.5 mm at their best.
3. The attempts to stop metastatic spread in its initial phase is often not studied, because today's **clinical trials are not designed for this purpose**. Although potential compounds to stop or slow down metastatic spread are already available, trials tend to enroll patients with advanced disease and established metastatic tumours. Cancer progression in this groups is far from its early stage and these compounds cannot show their efficacy.

In the last years, an alternative approach to access metastatic cells and thus face above-mentioned problems 1 and 2 is emerging. This approach consists in isolating cancer cells present in patients' blood between the intravasation and extravasation step of the metastatic cascade. These so-called circulating tumor cells (CTCs) *de facto* constitute a "liquid biopsy", minimally invasive and serially repeateble. CTCs may unveil some

mechanisms of metastasis, since among them there should be some cells responsible of eventual colonization. Once isolated, genetic, molecular and functional analysis can be performed, and their characterization compared to that of the primary tumor's, highlighting key factors of invasive phenotype. (Bourzac 2014).

The aim of this work has been the implementation of a new method to detect and isolate CTCs, overcoming current limitations. For this reason, in the next chapter we will focus on CTCs definition and potential value in the clinic, describe current state of the art and its limitations.

Circulating Tumor Cells (CTCs)

Definition

CTCs are defined as neoplastic cells found in blood, supposedly coming from primary tumors and with at least a subgroup forming the *seeds* of future metastasis, as Zhang et al. proved (Zhang et al. 2013). CTCs are considered rare cells in the blood of cancer patients: they can be found in cancer patient – easier but not only in advanced metastatic cancer patients – with a frequency of 1-10 CTCs per mL of blood (Miller, Doyle, and Terstappen 2010).

Traditionally, CTCs were defined as cells with an intact nucleus, presence of cytokeratins indicating their epithelial origin and absence of CD45 membrane expression, excluding their hematopoietic origin (Racila et al. 1998). CTCs can be also found in clusters (Aceto 2014). More recently, the emergence of multiple other methods of detection enlarged the definition including CK negative cells, cells undergoing Epithelial to Mesenchymal transition (EMT), apoptotic CTCs and small cells morphologically similar to white blood cells. (Marrinucci et al. 2012; Yu et al. 2013). Importantly, cells undergoing EMT or with a mesenchymal phenotype seem to be more correlated with progression of disease. (Yu et al. 2013).

Value

Personalized/Precision medicine

Evidence-based occidental medicine usually provides treatment to patients based on the diagnosed pathology, regardless of individual differences (except for dose adjustment in the children and elders or in patient with organ failure e.g: kidney or liver failure). Although the observation that different patients can react differently to the same therapy is not new, it's not easy to predict which therapy will fit a certain patient in the best way. The advent of low-cost genomic brought also the idea that indications for tailored, personalized treatments could be obtained by analyzing genomic dataset.
This consolidating trend in medicine is called personalized, or precision medicine (the terms are used interchangeably, sometimes with slight differences). The supporters of precision medicine propose the customization of healthcare, with medical decisions, practices, and products being tailored to the individual patients. Such customization is based on diagnostic testing, that is the analysis of a patient's genetic content or other molecular or cellular data (Lu et al. 2014).
This model does not necessarily mean the creation of individual-tailored medical devices or drugs, but rather the possibility of classifying individuals into subgroup that share common features, like susceptibility to a certain disease, natural history of that disease and response to treatment.

As a consequence, clinicians might concentrate positive effects of therapies in those responsive patients, sparing side-effects to non-responders. There would be also an advantage in the economical perspective, in this era of sky-rocketing cost of targeted drugs, avoiding to waste such advanced treatments on non-responsive patients. Thus, precision medicine could improve the overall clinical and economic efficiency of treatments.

In order to understand better this concept, I'll present Imatinib emblematic case, which describes the context in which such concept of medicine arouse:

"In 1996, early clinical trials were designed for one of the first drugs aimed at a cancer-specific genetic mutation (Imatinib, a monoclonal antibody targeting BCR-ABL mutated tyrosin kinase in chronic myeloid leukaemia, suppressing its activity and consequently cancer cells growth). Patients who had been debilitated by the disease rapidly improve when given the medicine. (Bourzac 2014) This success comes at a price: in 2012, a year's worth of the therapy cost US$92,000 (Gravitz 2014). Unfortunately, many of those cancers

relapsed as they became resistant to the drug. In this scenario, it emerged the technological challenge of figuring out what mutation caused the disease and designing a drug to target it, but also the malignant potential of cancers rapidly developing drug resistances. Imatinib was then followed by two further drugs to combat the emerging drug resistance, but treating cancer by chasing mutation after mutation with drug after expensive drug is not a sustainable model, and not considering the fact that most of tumours show a plethora of mutations rather than a single one (Bourzac 2014).
Vulnerabilities such as the one that Imatinib capitalizes on are known as driver oncogenes, genetic changes that generate the proteins driving a cancer's growth. Understanding how to disable the common driver oncogenes should therefore enable the treatment of a large number of cancers (Gravitz 2014).
Such an approach means that oncologists were no longer limited to treating cancer on the basis of the organ in which it first appeared, but they were instead starting to classify tumors by their mutations and expression profile as opposed to their histology."

Exploiting this kind of stories, Baselga and his colleagues are designing clinical trials that group patients by genotype rather than by a cancer's organ of origin (Hyman et al. 2015). In this scenario, pharmaceutical companies, which are obviously sensitive to trends in drug treatments, perceived the efficiency of this strategy of studying genomic characteristics to guide treatments. For this reason, pharmaceutical companies has partnered with diagnostic companies to develop and produce a whole new category of diagnostics, called *companion diagnostics*[1] - and ensuring in this way diagnostic foundations for future targeted and effective treatments.
There are also other more subtle parameters beyond matching tumour or patient to drug, which new insights into patients characterization might generate. As an example, providing drugs at the right time or at the right dose might be a key factor for the success of a treatment. Considering targeted treatments, in order to monitor a tumour state and its vulnerabilities, repeated biopsies would be necessary, but they would be difficult, invasive and often impossible (in small or hard-to-reach lesions), and can be confounded by intra-tumor heterogeneity (Murtaza et al. 2013).
Researchers have therefore been working on non-invasive ways to monitor mutations.
In this context, the emergent concept of "liquid biopsy" is dominant, which consists in obtaining tumoral samples from venous blood, mainly of two kinds: CTCs and circulating DNA (Gravitz 2014). At the beginning there was uncertainty on whether such samples would have been relevant, but their clinical meaning has been widely assessed. For

[1] A *companion diagnostic* is a medical device, often an in vitro device, which provides information that is essential for the safe and effective use of a corresponding drug or biological product (Health n.d.)

example, Dawson and colleagues have found that changes in cell-free tumour DNA are detectable, on average, five months before any changes to the disease are seen in computed tomography (CT) or other scans (Dawson et al. 2013), and Cristofanilli and colleagues, confirmed by many others, have found that CTCs frequency in blood at three to four weeks is correlated with prognostic behavior, usually monitored by imaging at 6-8 weeks (de Bono et al. 2008; Cohen et al. 2008; Cristofanilli et al. 2004; Hou et al. 2012; Riethdorf et al. 2007).

CTCs role in precision medicine

Because dissemination mostly occurs through the blood, and CTCs have been proven to be competent for metastasis generation (Zhang et al. 2013), it follows that the potential clinical value of CTCs is clear:

"Early detection and treatment of metastatic spread are key for disease outcome, and CTCs offer the ability to target metastasis in real time" (Vicki Plaks, Charlotte D. Koopman et al. 2013)

The presence of CTCs correlates with increased metastatic burden, aggressive disease, and a decreased time to relapse (Chaffer and Weinberg 2011). Furthermore, these CTCs offer the prospect of understanding how cells are able to survive in the circulation and generate metastasis.

For these reasons and for the minimal invasiveness of the assay, CTCs might be monitored longitudinally in time and a clinician might be guided by their number and nature in the evaluation of the ongoing treatment efficacy and in his clinical decisions.
CTCs nature and biology though is not completely clear, and a deeper understanding of their behavior might help to identify subpopulation of CTCs with real competence to form metastasis, and thus refine the clinical meaning of the assay. For this reason researchers are currently trying to provide robust CTC isolation and single-cell "omics" techniques, comprising genomic, transcriptomic and proteomic (Vicki Plaks, Charlotte D. Koopman et al. 2013).

State of the art

State of the art in CTCs detection is summarized in Figure 3. We could divide the whole scenario in two categories: methods trying to exploit CTCs protein phenotype – named biological properties – or others – named physical properties. At present, the only FDA-approved CTCs detection device – Veridex CellSearch® – provides enumeration of CTCs with positive selection methods. Specifically, CTCs are first enriched via magnetic EpCAM labeling and then identified by eye-verification of a trained operator as DAPI-positive, EpCAM-positive, CK(8, 18, 19)-positive and CD45-negative.

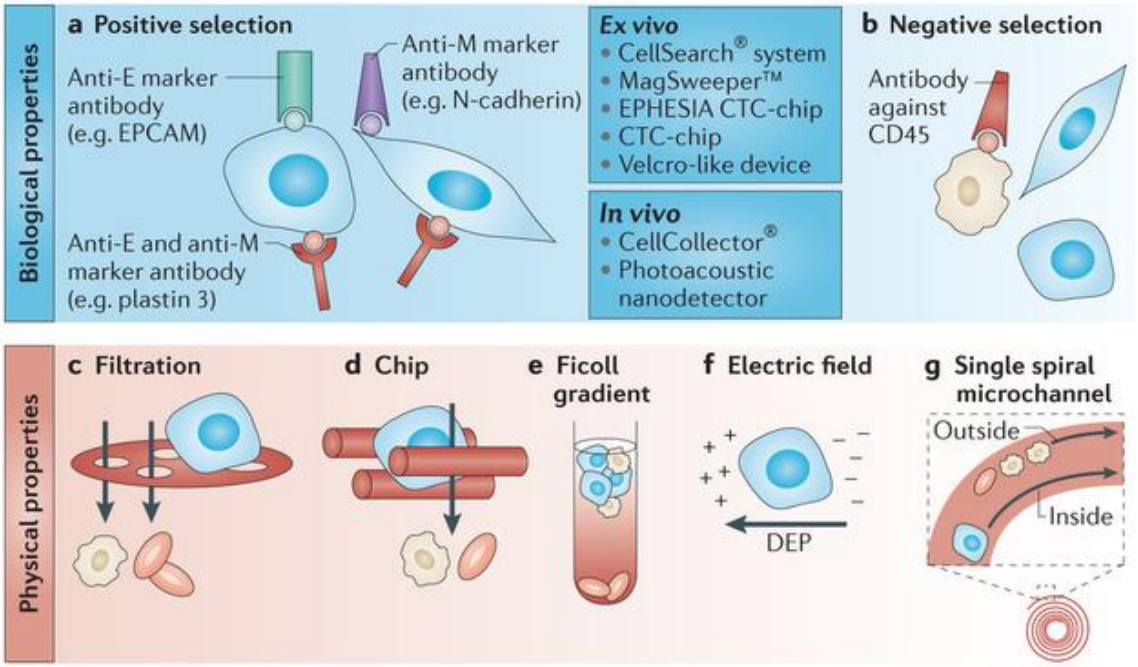

Figure 3 – State of the art of CTCs technologies (Alix-Panabières and Pantel 2014)

CellSearch® has received FDA approval for its use in breast, colorectal and prostate cancer. (Cellsearch® specifications) Other methods are using other combinations of antibody cocktails or improved methods of mixing and staining, based on the same concept. Alternative approaches depletes CD45-positive cells, enriching CTCs for further immunostaining procedures. The main drawbacks of these methods are:

- Cancer protein expression must be known *a priori*, and being cancer both a heterogenous and rapidly transformating disease this is a difficult task. E.g.: Cells undergoing EMT, shown to be highly significant for cancer progression, are missed by EpCAM-based strategies.

- Intracellular protein labeling often require fixation and permeabilization of cells, compromising cell viability and thus possibly modifying their original condition

- Good quality monoclonal antibodies production is expensive compared to other approaches not based on antibodies.

Physical approaches are trying to identify CTCs by some of their parameters as size (c, g), deformability (d), density (e), charges distribution (f). Generally, these approaches have a lower-cost and a higher throughput than antibody-based approach, leaving the cells alive and intact. None of these methods, though, has reached clinical significance, putting them a step below above-mentioned antibody-based approaches.

Limitations

A key limitation in the capture and analysis of CTCs is their extreme rarity with respect to the 5 x 10^9 erythrocytes and 1-10 x 10^6 leucocytes per mL of blood. Although red blood cells can be easily removed by osmotic cell lysis, leucocytes (white blood cells) share many of the physical, chemical and biological properties of CTCs, leading to high contamination levels in many CTC detection methods (Alix-Panabières and Pantel 2014). This intrinsic rarity makes extensive study of CTCs a cumbersome task, and doesn't help to solve open questions on CTCs biology and the role they play in metastatic disseminations, summarized in Figure 4.

Circulating carcinoma cells have diameters typically ranging from 20 to 30 μm, that are far too large to allow them to pass through capillaries (~8-μm diameter), such as those present in the capillary beds of the lungs. Shed CTC should thus be trapped in capillary beds just after be released from the primary tumor. The persistence of some CTCs type for far longer periods of time (with half-lives of 1 to 2.4 hours) suggests the possibility that **only exceptionally small or physically plastic CTCs** can pass through pulmonary microvasculature and thus circulate freely in the blood.

For the same reason, CTC clusters should pass even more hardly small vessels and thus should be underrepresented by a blood sample obtained by venous access.

Recent results on CTC concentration in blood from arterial access apparently confirm this view (Terai et al. 2015).

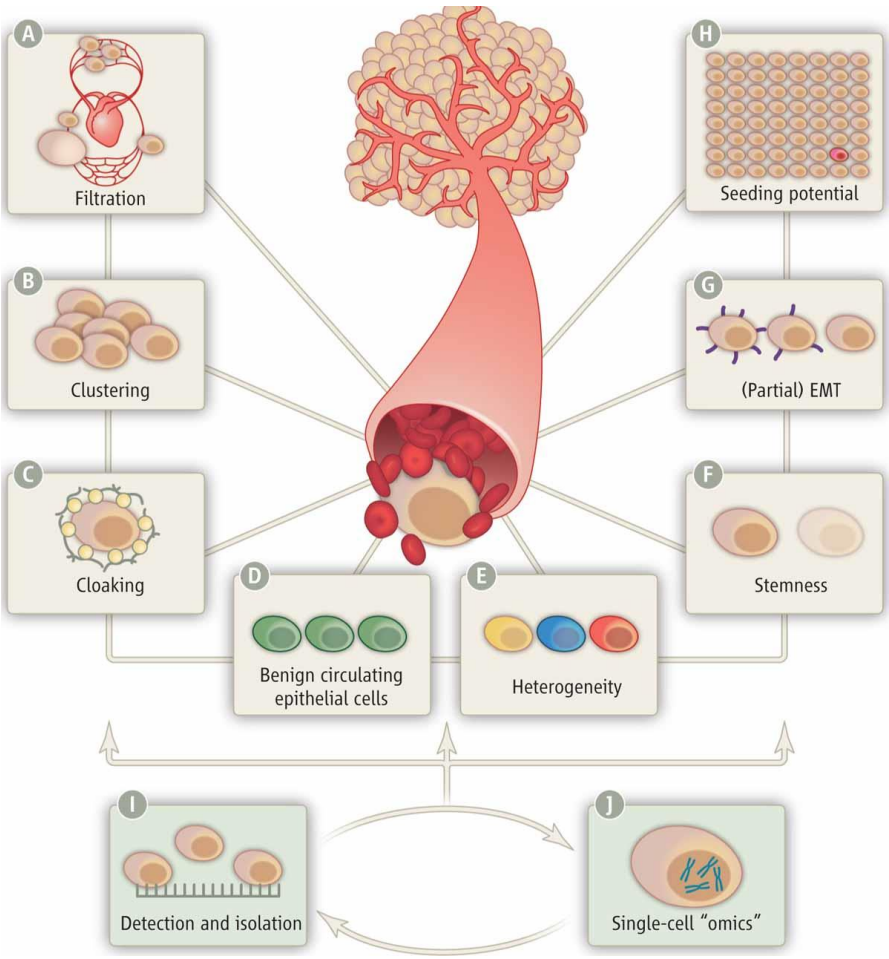

Figure 4 (Vicki Plaks, Charlotte D. Koopman 2013) – The picture summarizes open questions in CTCs detection. There are biophysical factors that may decrease CTC detection sensitivity, including trapping of A single CTCs and B clusters of CTCs in capillary bed; covering of CTCs by platelets or coagulation factors named "cloaking". Biological factors impairing precise CTC quantification are D the presence of benign epithelial cell sharing protein expression with CTCs, E cellular heterogeneity making it difficult to select a single labeling factor to detect them all and F the possible stemness and loss of differentiation phenotype of some CTCs or G EMT cells undergoing EMT transition and loss of epithelial phenotype. Moreover, real metastatic seeding potential of detected CTCs is still unclear H. Current research lines are trying to improve detection and isolation of CTCs I and working towards a robust single-cell 'omics' platform for sequencing isolated CTC genome and transcriptome, as well as proteomics J.

Platelet adhesion to CTC membrane is documented (Palumbo et al. 2005) and could impair detection in two different directions. It could both induce stickiness of CTCs to small vessel walls and hide cell-surface marker antigens preventing antibody based detection.

CTCs detection by antibodies shares also other complications: given the rapidly transformating nature of cancer and its high level of heterogeneity between single cells, the subpopulation targeted by selected antibodies could be unclear and time-changing, leading to confused diagnostics.
For example, we know that many cancer cells undergo EMT, achieving a phenotype for many aspects useful for dissemination – secretion of metalloproteinases for matrix destruction, higher mobility, elongated shape for intra and extravasation, basal membrane independent survival. Recent studies have demonstrated that the EMT can induce non-Cancer Stem Cells (CSC) to enter into a CSC-like state, and that drug-resistant cell subpopulations often exhibit a more mesenchymal phenotype. (Chaffer and Weinberg 2011). Needless to be said, the main target of a therapy should be the CSC cell subpopulation, which if left untouched, will regrow the tumor over and over again.

Not all CTCs may be clinically relevant. Some events tagged as CTC by current technologies can be detected in patients with benign conditions, such as inflammatory colon disease, while healthy patients not showing evidences of CTC presence. Curiously, in a mouse model of pancreatic cancer, CTCs were found in the blood before the appearance of a primary tumor. Thus, current CTC assays are limited in distinguishing between cancer cells, noncancerous tumor components, and benign cells (Vicki Plaks, Charlotte D. Koopman et al. 2013).

CTCs may be shed from different locations of tumors, which are heterogeneous in nature, and even from metastases. Frequently there is a discrepancy in gene expression between primary tumors and CTCs, as well as heterogeneity within the CTC population. It has been shown, though, that CTCs represent the most updated data available for gene expression. In theory, it should be possible in future to identify the tissue of origin of CTCs by detecting organ-specific signatures. This would help to localize small metastatic lesions and to guide further diagnostic and therapeutic strategies(Vicki Plaks, Charlotte D. Koopman 2013).

Our metabolism-based approach

Given the state of the art when the project started in 2013, our research group perceived the need of a method overcoming said CTCs detection limitations. We can now say that, despite many advances in the fields and the emergence of many CTC detection companies providing new technologies, the same need is still not fulfilled, allowing our research to be fortunately still actual.

Back at the beginning of this project, not convinced by antibody labeling limitations and by weakly described physical properties of CTCs, we envisioned an unexplored, transversal approach, based on the abnormal metabolism of cancer cells. To better understand our approach, we will briefly describe established knowledge on cancer peculiar metabolism.

Reprogramming of energy metabolism - an emerging Hallmark of cancer

Following the cited 2000 review, in 2011 Hanahan and Weinberg published an updated edition of the first paper (Hanahan and Weinberg 2011), showing how conceptual progress occurred in the time gap between the two reviews has added two emerging hallmarks of

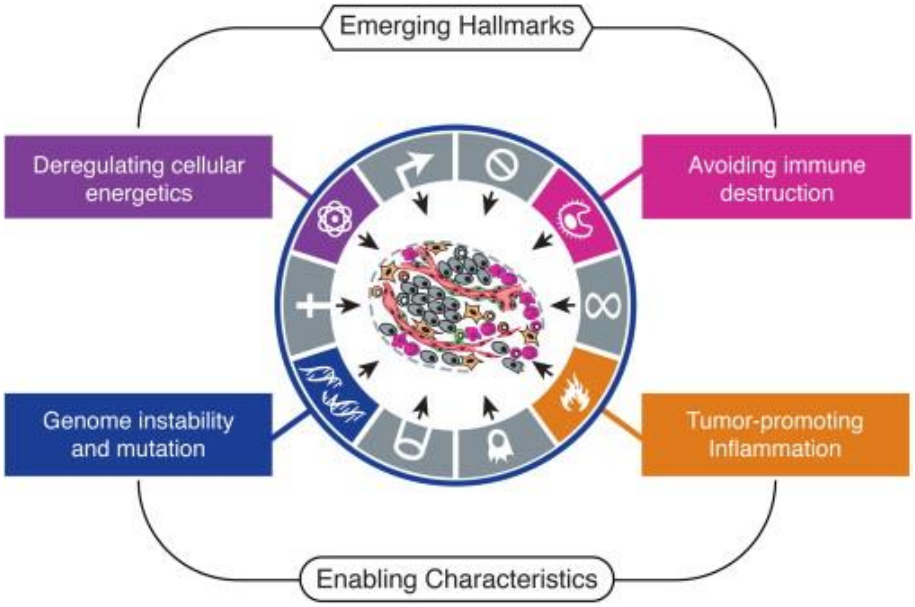

Figure 5 - (Hanahan & Weinberg, 2011) Emerging Hallmarks and Enabling Characteristics. Two emerging hallmarks was added to the seven original ones. The alteration of cellular metabolism (top-left) and the adaptation to elude immune system (top-right). Two features named enabling characteristics are also identified: genomic instability that speed up mutation and thus acquisition of hallmarks, and chronic inflammation easing tumor progression. In this work we will focus only on the deregulation of cellular energetics and its consequences.

the original list – one of which is **reprogramming of energy metabolism** - Figure 5. The uncontrolled cell proliferation that constitutes one of the foundations of neoplastic disease requires corresponding adjustments of energy metabolism in order to meet demanding hyperproliferation and abnormal cell growth.

Among all known deregulations, we selected two of them, trying to identify measurable alterations both widespread and specific to cancer cells, with the aim of founding the most general yet specific marker of neoplastic nature, in order to generate a highly sensitive and specific assay. The two selected features in this work are, primarily, the acidification of the extracellular medium, and in minor part, aerobic glycolysis, or Warburg effect – (the increased rate of glycolysis with lactate production even in the presence of oxygen).

The acidification of the extracellular medium

The most widespread metabolic feature of cancer cells is their ability to acidify the extracellular medium, by secreting protons (H^+) and acids (Cardone, Casavola, and Reshkin 2005; Montcourrier et al. 1997; Parkins et al. 1997; Webb et al. 2011). Initially, this phenomenon was considered as a consequence of Warburg effect, but it proved to be even more widespread between different cancer cell types and more precocious in tumor natural history. This reversed pH gradient is already apparent during the earliest step of neoplastic progression, and, notably, this ability increases with tumour aggressiveness. (Cardone et al. 2005).

More precisely, tumor cells have an imbalance between extracellular (pHe) and intracellular pH (pHi), with an alkaline pHi values (7.12–7.65 compared with 6.99–7.20 in normal tissues) and acidic pHe values (6.2–6.9 compared with 7.3–7.4).

The development and maintenance of this gradient was commonly considered based primarily on glycolytic lactate production and release. However, this hypothesis has been disrupted in the ‘90s by the observation that glycolysis-deficient tumor cells and cells lacking lactate dehydrogenase were still fully able to acidify their micro-environment (Newell et al. 1993; Yamagata et al. 1998). This indicates that there are other mechanisms that maintain pH dysregulation. Protein responsible of proton secretion have been identified in Na^+/H^+ exchanger NHE1, the Na^+-independent and Na^+-dependent HCO_3^-/Cl^-

exchangers and the H^+/lactate cotransporter (known as the monocarboxylate transporter, MCT) (Cardone et al. 2005).

Aerobic glycolysis, or Warburg effect

In the presence of oxygen, under aerobic conditions, normal cells process glucose first to pyruvate via glycolysis in the cytosol and then to carbon dioxide in the mitochondria; under anaerobic conditions, glycolysis is favored, with relatively little amount of pyruvate produced, in favor of larger amounts of lactate (Hanahan and Weinberg 2011).

Otto Warburg in the 1920s documented an anomalous characteristic of cancer cell energy metabolism (Warburg 1925): even in the presence of oxygen, cancer cells displayed a high rate of 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".

Even though it's not quite clear why cancer cells base their energy metabolism on glycolysis, given the low efficiency of this pathway compared to normal mitochondrial one (18-fold lower efficiency), altered energy metabolism proved to be as widespread in cancer cells as other traits accepted as hallmarks of cancer (Hanahan and Weinberg 2011).

The upregulation of glucose transporter GLUT1, and the consequent avidity of cancer cells for glucose is a phenomenon associated with aerobic glycolysis and already exploited in the clinical settings. ^{18}F-fluorodeoxyglucose Positron-Emission Tomography (FDG-PET) measures glucose uptake and is currently the gold standard for metastasis detection in virtually all solid tumors, proving that metabolism is a robust and transversal base for cancer diagnostics (Juweid and Cheson 2006).

Extracellular pH measurement - compartmentalization

The method described in this thesis aims at detecting CTCs based on their described aberrant metabolism: acidification of the extracellular medium and Warburg effect 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 or lactate levels in a sample of blood.

By enclosing each cell in a volume so little that is altered by the low amount of protons secreted by the single cell though, this in principle would be possible.

To achieve this, we split the macroscopic blood sample into picoliter aqueous droplets. In order to measure pH robustly, monodisperse water-in-oil emulsions was produced with microfluidics with established techniques (Chokkalingam et al. 2013; Mazutis et al. 2013a). In this way, we could measure the extracellular acidification (or lactate concentration) of individual cells, trying to identify CTCs with these parameters.
The explained concept is illustrated in Figure 6.

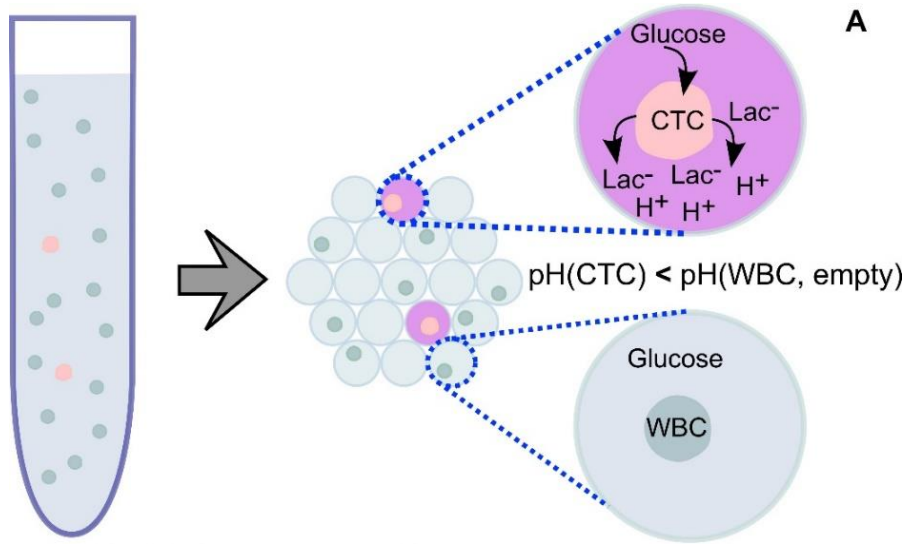

Figure 6 – the concept of compartmentalization – a cell suspension is divided in multiple um-sized picoliter droplets. Thanks to the small volume, concentrations of secreted molecule rapidly increase up to measurable level. Lactate and protons are symbolically shown secreted only by the cancer cells, which can thus be detected by the assay.

Materials and methods

Microfluidic circuit fabrication

25 µm thick layer of SU8-2025 was spun on silicon wafer, baked, exposed through transparency mask, baked again and developed according to manufacturer instructions (MicroChem corp.). Sylgard 184 (PDMS) prepolymer and crosslinking agent (Dow Corning) were mixed at a mass ratio of 10:1 (w/w); a mixture was poured onto a master, degassed and cured at 65°C for at least 2h. The replica was detached from master and reservoirs were bored using a blunt hypodermic needle. A PDMS replica was washed in soapy water and ethanol, and blow dried with nitrogen. A clean glass slide and a clean PDMS replica were treated with oxygen plasma and bonded. The device was silanized with 1% (Tridecafluoro-1,1,2,2-Tetrahydrooctyl)-1-Trichlorosilane (Sigma-Aldrich) in FC-40 (3M), fluorinated oil, which was introduced into microfluidic channels (enough to completely wet whole microfluidic network) and then the device was kept at 95°C for at least 30 min. To fabricate a reservoir for an emulsified sample a brass cone (10 mm in dia. and 5 mm tall; ~130 µL volume) was placed directly on silicon wafer and replicated together with photolithographically defined features.

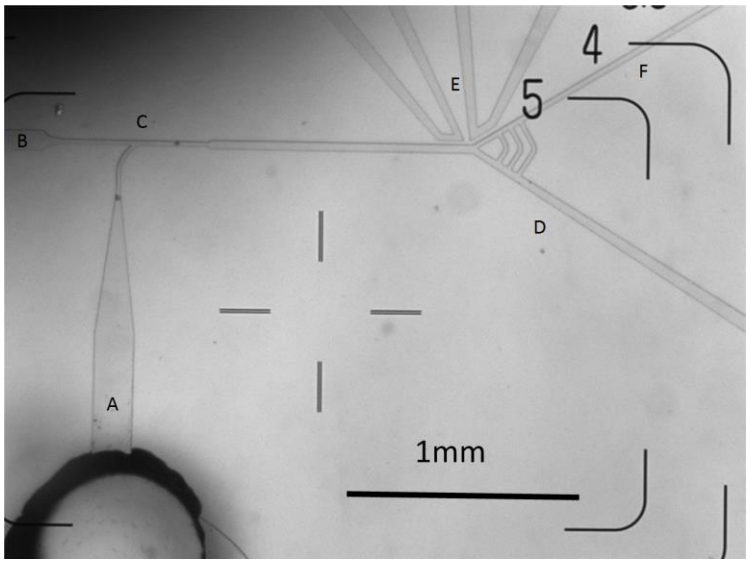

Figure 7A Picture of PDMS fabricated circuit. Sample is coming from A, oil is coming from the B. Droplets are formed at the T-junction C, and naturally go to the lower resistance arm D. When electrodes E are activated, droplets are deviated into F arm.

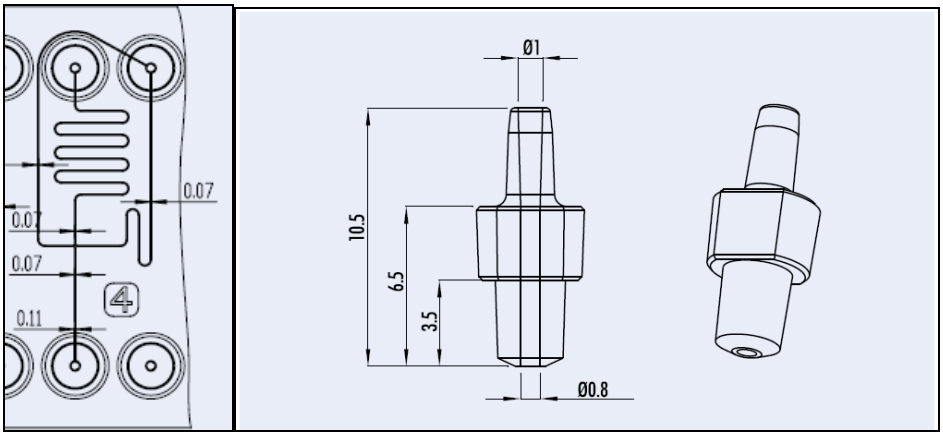

Figure 7B – Schematics of microfluidics ChipShop droplet generator circuit and connector. Quotes are expressed in mm.

Since late 2015, microfluidics circuits and connectors have been purchased at microfluidics ChipShop, with significant advantage on experimental quality. A sample circuit architecture is shown in Figure 7B.

Cells

A549 cells (human lung carcinoma, Hubrecht lab), MDA-MB 231, MDA-MB 453, MDA-MB 468 (breast cancer cell lines, Colombatti lab) TOV21G (Ovarian cancer cell lines, Colombatti lab), HT-29 (Colorectal cancer cell line, Colombatti lab) were cultured in DMEM (Dulbecco's Modified Eagle Medium, a culture medium) + 10% Fetal Bovine Serum + 1% Penicillin-Streptomycin, detached using 0.25% Trypsin-EDTA and re-suspended in the incubation buffer.

White blood cells are obtained by lysing blood with Beckton-Dickinson or Miltenyi lysis solution, according to manufacturer's protocol. Cells are then spun down at 300g for 10 min, and resuspended in the incubation medium.

Droplet production

Monodisperse droplets are generated in chips with 20 µm wide T-junction.Continuous phase: 2% (w/w) surfactant (Krytox–Jeffamine–Krytox A–B–A triblock copolymer) (Chokkalingam et al. 2013) in HFE-7500 (3M) Dispersed phase: cell suspension in HBSS or Joklik's modified EMEM (without Ca^{2+} and Mg^{2+}, to avoid cell adhesion), Optiprep 15%, pH-sensitive dye (2-8uM). Flowrates are set such as continuous phase flow is at least

2 times higher than a flowrate of a dispersed phase; a typical flowrate for disperse phase was 300 uL/h and 600 uL/h for continuous phase.

Lactate enzymatic assay

A three-channel architecture microfluidic circuit was used: one channel bringing the cell suspension (dispersed phase), one bringing the reagents of lactate assay (Cell Technology, Inc.) and one bringing continuous phase. Emulsification step was performed at 4°C to slow down cell metabolism to avoid lactate contamination of the whole solution by cancer cells. With this microfluidic device we could expose cells to the enzymes of the lactate assay only after encapsulation in the micro-droplets, to avoid unspecific activation prior the encapsulation. Images were taken after 15 min incubation at room temperature after emulsification.

Widefield fluorescence imaging

Lactate assay, pHRodo green experiments: An inverted epifluorescence microscope (Olympus IX81) was equipped with xCite 120Q lamp (Lumen Dynamics Group Inc.), resorufin and FITC filter sets (Semrock) and iXon 897 camera (Andor). An aliquot of processed sample was pipetted on a microscope glass slide and covered with a cover slip to prevent evaporation.

High throughput detection with SNARF-5F.

An inverted microscope (Olympus IX70) was used to analyze flowing droplets one by one. A laser (488 Argon-ion Cyonics) beam was expanded (2x) and focused down with a cylindrical lens crossing orthogonally the microfluidic channel. The fluorescence signal of excited SNARF-5F dye (Life Technologies) in droplets was collected with a 40x objective (Olympus LUCPlanFLN, 40x/0.60), split with dichroic filter (DLP555, Semrock) and detected through bandpass filters (579/34 630/38) by Photo Multiplier Tubes (PMTs) (H957-15, Hamamatsu). Signal went through a transimpedance amplifier with 1V/uA gain and was detected by the acquisition system (National Instruments cRIO-9024, analog input module NI9223) with a 10 μsec scan rate. The acquisition system was driven by

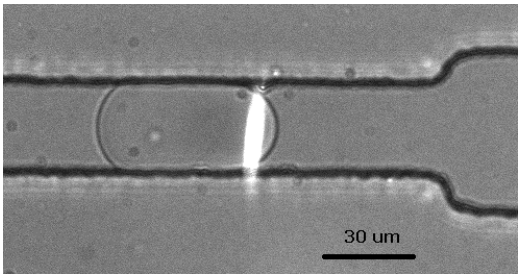

Figure 8 droplet crossing the excitation laser slit in the microfluidic circuit.

LABView custom software. The software detects all data-points of a droplet over a set threshold and computes in real time averaged values; it also provides trigger pulse for image capture on a camera. Liquids were pumped using neMESYS (Cetoni) low-pressure syringe pumps.

A549 quantitation

Cultured cells were washed with PBS, trypsinized and transferred into medium (typical concentration 500,000 to 1000,000 cell/mL). Cells were spun down and resuspended in Joklik's modified EMEM (pH 7.4). If lower concentration of A549s was required, cell suspension was diluted to ~ 1000 cell/mL in Joklik's modified EMEM. 100 µL of sample solution was obtained by mixing cell suspension, SNARF-5F stock (2 mM) and in Joklik's modified EMEM. Fraction of obtained solution was used to verify A549 concentration in counting chamber. 1 to 2M cell/ml suspension of WBC in Joklik's modified EMEM was used to prepare samples of A549 with WBCs. Samples were emulsified at flowrate of 300 µL/h (600 µL/h for oil), collected in cone reservoir and incubated for 20 minutes. Droplets were reinjected from cone device directly into a readout device (50 to 100 µL/h for droplets; 300 to 500 µL/h for spacer oil). Detected acid droplets were verified to be cancer cells with images acquired for droplets with reduced pH.

CD45 immuno-magnetic depletion and/or staining

We followed manifacturer's protocol for CD45+ depletion using Miltenyi human CD45 Microbeads, MidiMACS™ Separator, MultiStand and LD Column.

CD45 staining protocol: Anti-CD45 antibody (Alexa 488 – conjugated) (Life Technologies) was added 5uL/100uL of sample, incubated 20 min at 4°C and washed (sample centrifuged 300g x 5 min and resuspended in incubation buffer)

Patient protocol

2 mL of whole blood from metastatic cancer patients, taken with a venous puncture and collected in EDTA tubes was lysed with BD lysis RBC lysis solution, depleted of CD45+ fraction with CD45 magnetic beads and LD columns (Miltenyi), CD45-Alexa488 stained (BD – 4 °C incubation for 20 minutes) and resuspended in incubation medium and 15% Optiprep for a final volume of 50-70uL. Washing steps are performed with centrifugations at 300g x 5 minutes at room temperature.

Spike protocol

A549 cells were spiked into 1-2mL of whole blood from healthy donor and "patient protocol" was followed. In some experiments, cells were pre-stained with Calcein Violet AM (ThermoFischer) according to manifacturer's protocol.

Labview software

Custom LabVIEW software has been implemented on a cRIO 9024 modular system. Analog input module NI9223. Digital I/O module NI9401. Each voltage channel is acquired simultaneously using NI9223 Analog input module. Scan rate is adjustable, starting from one acquisition every 5 usec. A threshold value is set so that all fluorescence below threshold value is ignored. For each droplet, all data-points are averaged and number of data points, average value and peak value of channel is computed in real-time. Triggering conditions as set by the user activate camera and electrodes for cell isolation.

Results

Cell occupancy in droplets

By generating water in oil emulsion droplets from a cell suspension, the number of cells in each droplet depends on cell concentration and droplet size, following a Poisson distribution. For a droplet of approximately 40 um diameter, most droplets contain a single cell, as shown in Figure 9. A row of new-generated drops, most of which empty and with one drop showing single cell encapsulation is shown in Figure 10.

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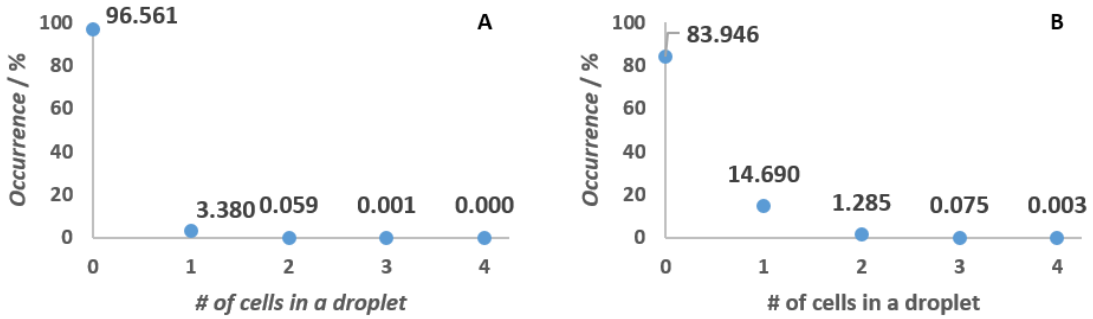


Figure 9 Distribution of cell occupancy in droplets. Distributions shown for emulsification of 1 000 000 cell/mL (A) and 5 000 000 cell/mL (B) in 35pL drops. By far the majority of droplets are empty, and more than 90% of droplets containing cells have only 1 cell per droplet.

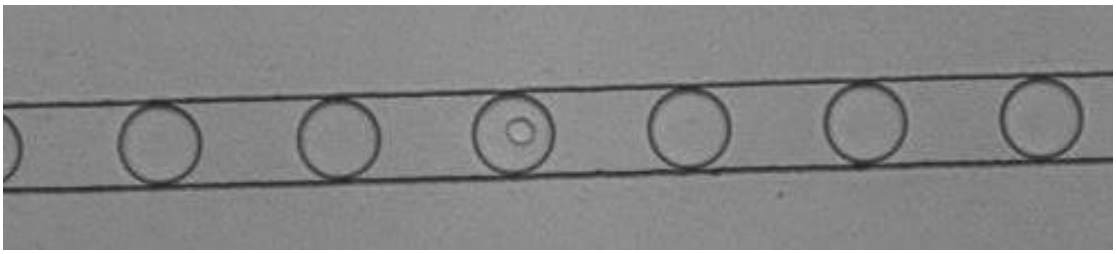


Figure 10 A single MDA-MB 231 encapsulated in a 50um diameter droplet.

Detection of lactate secretion via lactate enzymatic assay

The concept of CTC detection is represented in Figure 11, showing how compartmentalization in droplets from a usual undivided sample enables cell secretion measurements for each individual cell, thanks to a rapid increase in concentration due to small droplet volume. The drawing shows lactate and proton symbolically secreted only by the cancer cell. The results shown constitute the proof of concept that cancer cells can be discriminated by their abnormal lactate secretion. In this particular case, cancer cells are bigger because they come from a big-size cancer cell line, and thus easily identified with respect to smaller white blood cells. Only cancer cells display increase in fluorescence, quantified in the dot plot. Averages from different droplets populations are averaged in Figure 12.

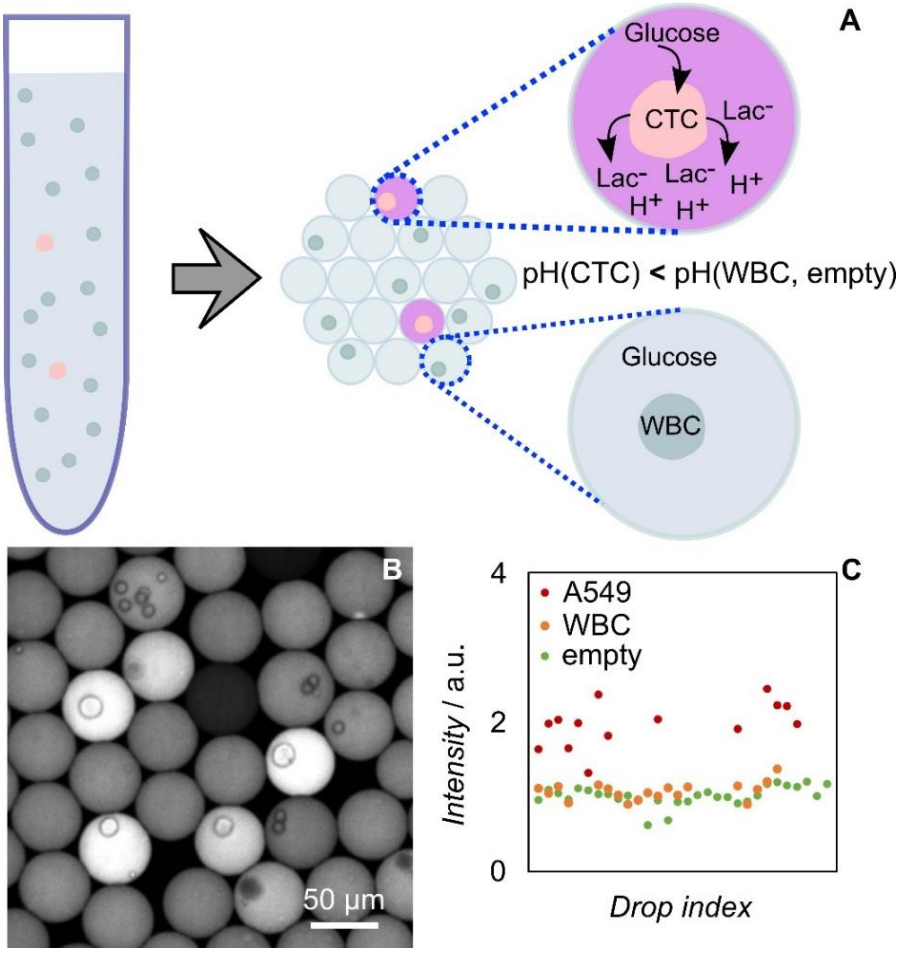


Figure 11 (A) CTC detection based on Warburg effect using compartmentalization in microdroplets. (B) Production of lactate by A549 cell in droplets. Only A549 containing

droplets (large cells) show increase in fluorescence. Please note that even clusters of white blood cells do not reach the level of lactate generated by A549, suggesting high specificity of the method. The picture has been brightness/contrast enhanced for clarity. (C) Dot plot is instead measuring raw picture average intensity per droplet, clearly showing two distinct populations: the red one formed by the cancer cells above a baseline formed by empty droplets and white blood cells containing droplets, showing the same values than empty ones.

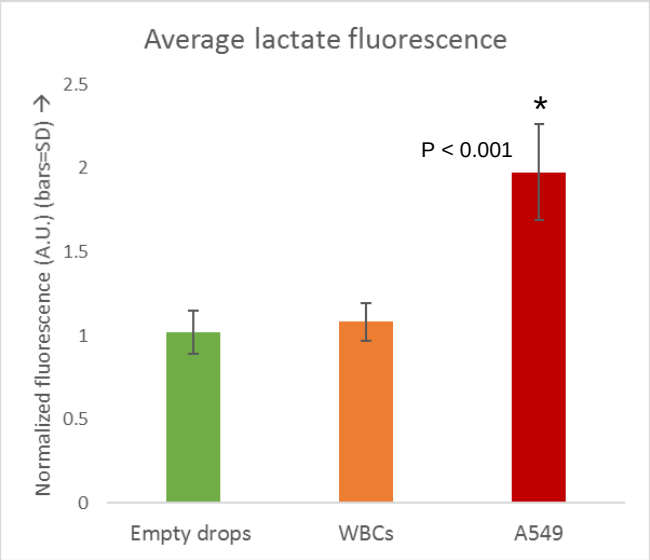


Figure 12 Averaged values of Figure 11C. A549 average is significantly higher than other populations.

Detection of medium acidification via pHRodo Green

In order to detect measure pH, we used pHRodo Green, which is a pH-sensitive dye that increases its fluorescence while pH value decreases.

A sample of A549s (and WBCs in a separate experiment) was resuspend in Joklik's modified EMEM; pHRodo Green was added to yield 5 µM final concentration and obtained mixture was emulsified and incubated at 37°C for 30 min. Comparison of empty droplets and droplets containing tumor or WBCs is shown in Figure 13. Results were consistent with the experiment with lactate secretion: cancer cells showed a significantly higher average acidity compared to both empty droplets and white blood cells, which in turn show values comparable to empty droplets.

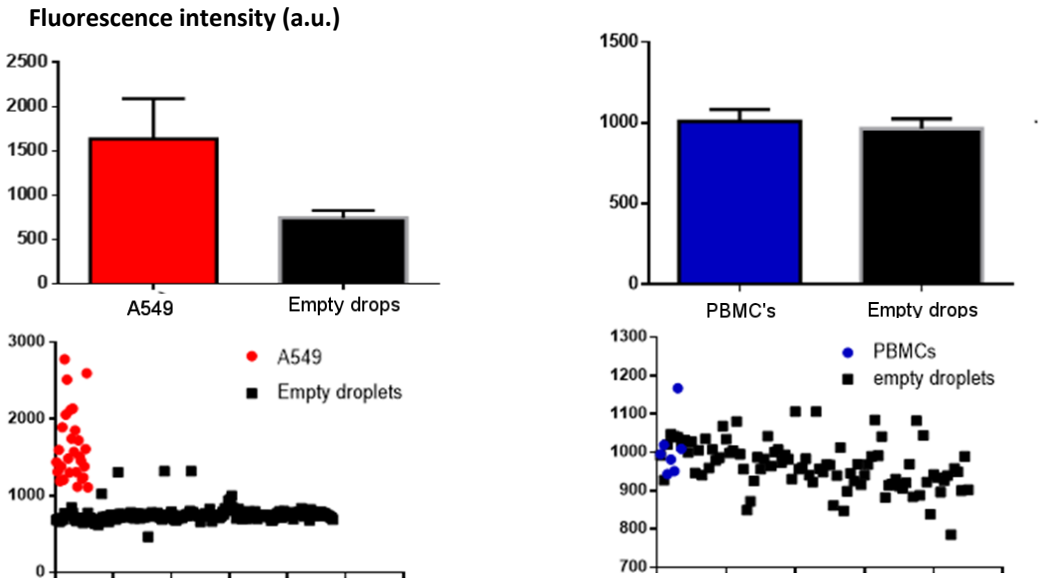


Figure 13 Detection of pH. pHrodo Green dye was used to indicate pH changes. Results are comparable to lactate assay and confirm the difference between cancer cells and white blood cells. Both averages and scatter plot are reported. The difference between A549 and empty drop is statistically significant with $P < 0.01$.

SNARF-5F calibration

pHRodo green dye was not suitable for high-precision measurement of pH, its detection being sensitive to dye concentration and possible differences in detection pathway or excitation conditions. In order to detect pH changes with higher accuracy, we changed it with SNARF-5F, a fluorescent pH-sensitive ratiometric dye with two emission peaks, at 580nm and 630nm. With lowering of the pH, the ratio between 580nm and 630nm emission peaks increases., as shown in Figure 14.

SNARF-5F ratiometric measurement is independent from dye concentration, light source variation and electrical noise, because it always has the internal reference of itself. We monitored SNARF-5F in the prototype system described in materials and methods, being comparable to a customized cytofluorimeter in which droplets, instead of cells, are flowing and detected. To calibrate the system, we prepared a set of solutions of Joklik's modified EMEM titrated to various pHs between 7.4 and 5. These solutions were emulsified and pH values (emission ratio) of obtained droplets detected similarly to A549

samples. Raw track examples can be found in Figure 14B, showing how the gap between red and blue track increases with pH decrease. Raw data appear in Table 1 and curve fitting in Figure 14C. We obtained a coefficient of variation (Standard deviation/average) close to 1% for 300.000 measurements, confirming the precision of the combination of this ratiometric dye with our system.

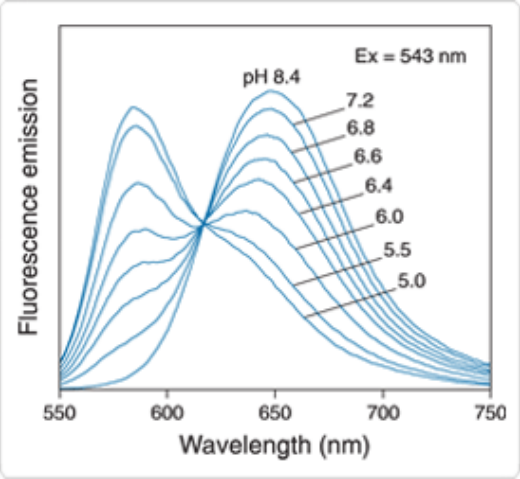


Figure 14 Fluorescence emission spectra of SNARF-4F 5-(and 6-)carboxylic acid showing the pH-dependent spectral shift that is characteristic of this and other SNARF pH indicators. (Anon n.d.)

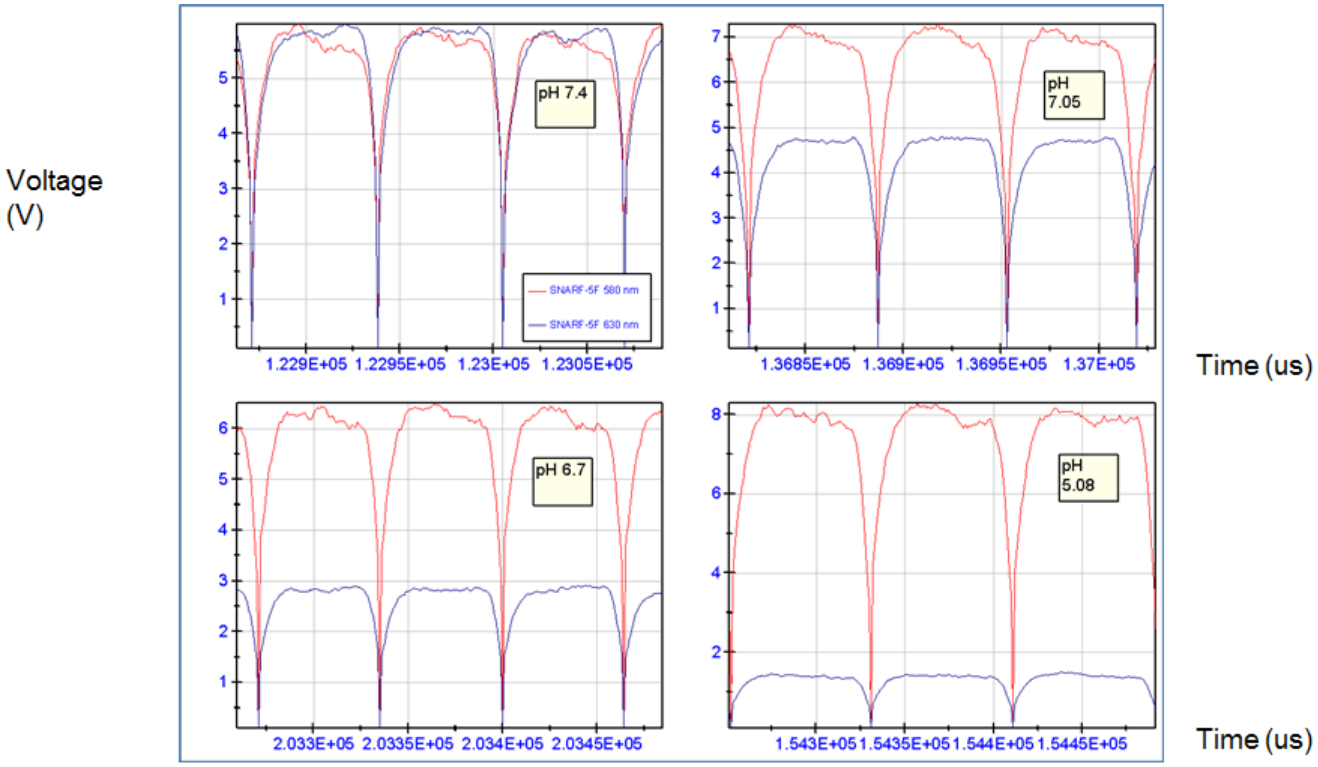


Figure 14B Red and blue tracks are respectively 580nm and 630nm fluorescence channels. With lowering of the pH, 580/630nm ratio increases.

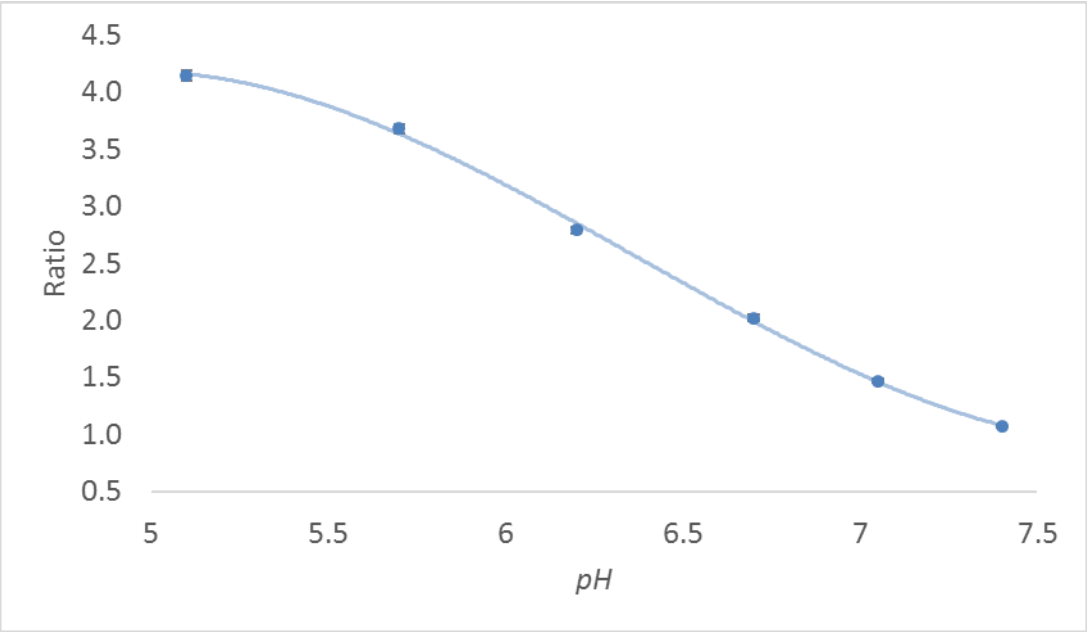


Figure 14C Calibration of SNARF-5F response. Ratio between fluorescence intensity at 580 nm and intensity at 630 nm. Standard deviation is smaller than dot shown and was not clearly visible.

pH	Ratio average	SD	Range	N	CV (%)
7.4	1.07	0.015	0.13	46000	**1.40**
7.05	1.47	0.0187	0.156	111000	**1.27**
6.7	2.02	0.026	0.219	119000	**1.29**
6.2	2.79	0.03	0.223	70000	**1.08**
5.7	3.68	0.0378	0.316	315000	**1.03**
5.1	4.15	0.0424	0.344	316000	**1.02**

Table 1 – Raw parameters of calibration. Please note relatively low Coefficient of Variation (Standard deviation/average).

Extracellular acidification, evaluation of various cell lines

After calibration, we tested our system with cancer cell lines, in the same method described above for pHRodo Green. The results were consistent, and with much higher throughput and number of droplet evaluated in short time (1000 drops/second). We tested several cell lines, obtaining comparable results: all cell lines showed an acid population raising above empty droplet baseline (horizontal distribution at the bottom). We tested cell lines from breast cancer (MDA-MB-231, MDA-MB-453, MCF-7), colorectal cancer (HT-29, TN420), ovarian cancer (TOV21G), lung cancer (A549), and glioblastoma – brain cancer (U231).

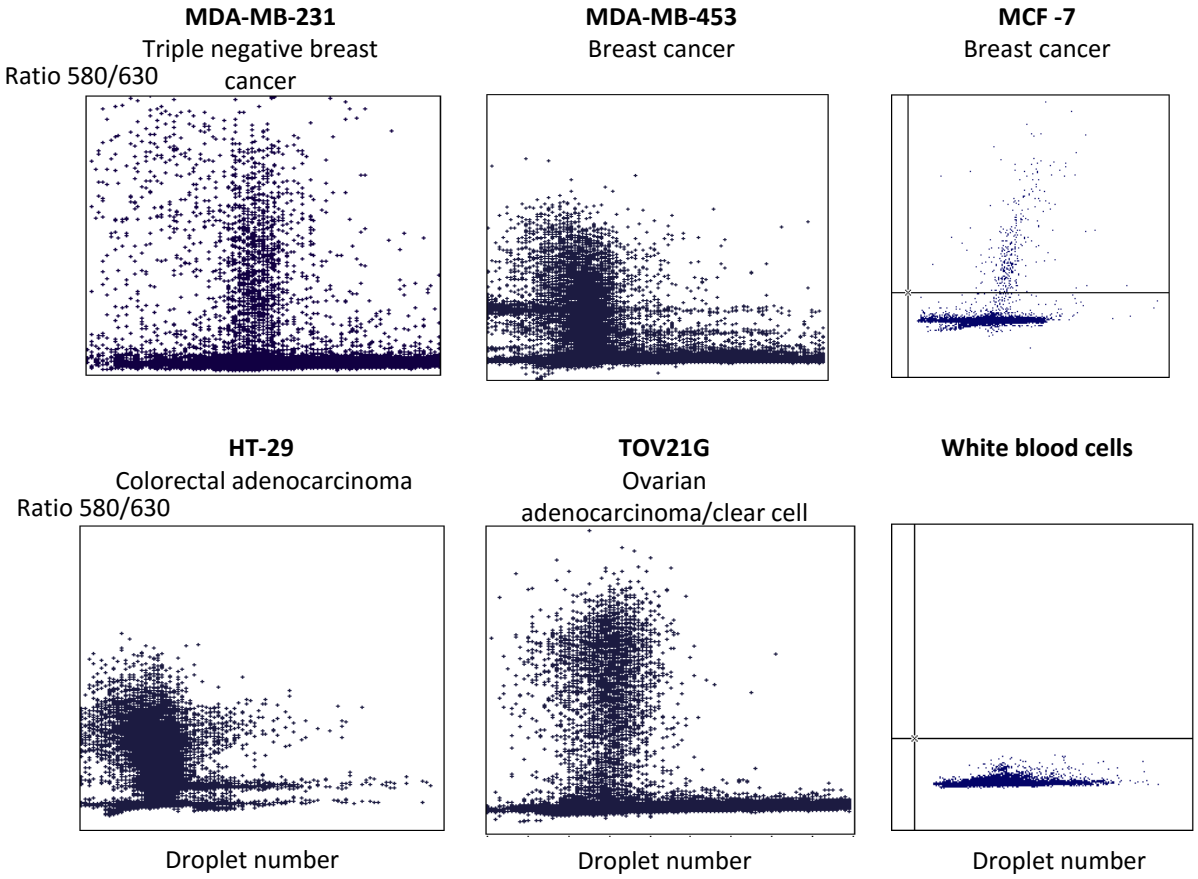


Figure 15 Dot plots obtained analyzing several cancer cell lines with the prototype and SNARF-5F. Y-axis Ratio (Acidity), X-axis (droplet width – to obtain a two –dimensional distribution). WBC dot plot is given for comparison in bottom right corner. Please note the ubiquitous presence in cancer cell lines of an acid population raising above empty droplet baseline (horizontal distribution at the bottom). White blood cells do not show this population.

White blood cells dot plot is shown in the bottom right corner as negative control, lacking acid population. The cell lines tested were notable both EpCAM(+) and (-), with several degrees of epithelial and mesenchymal phenotype, demonstrating the transversal validity of the method, Figure 15.

Evaluation of proton secretion dynamics

We evaluated how the above shown acid population was affected by incubation time. All samples were processed similarly to protocol described above. After droplet generation, the same sample was measured at different incubation time, ranging from less than 2 minutes to 90 minutes. The cancer population showed higher values of average acidity with higher incubation times, reaching a plateau phase after 10 minutes, as shown in Figure 16A.

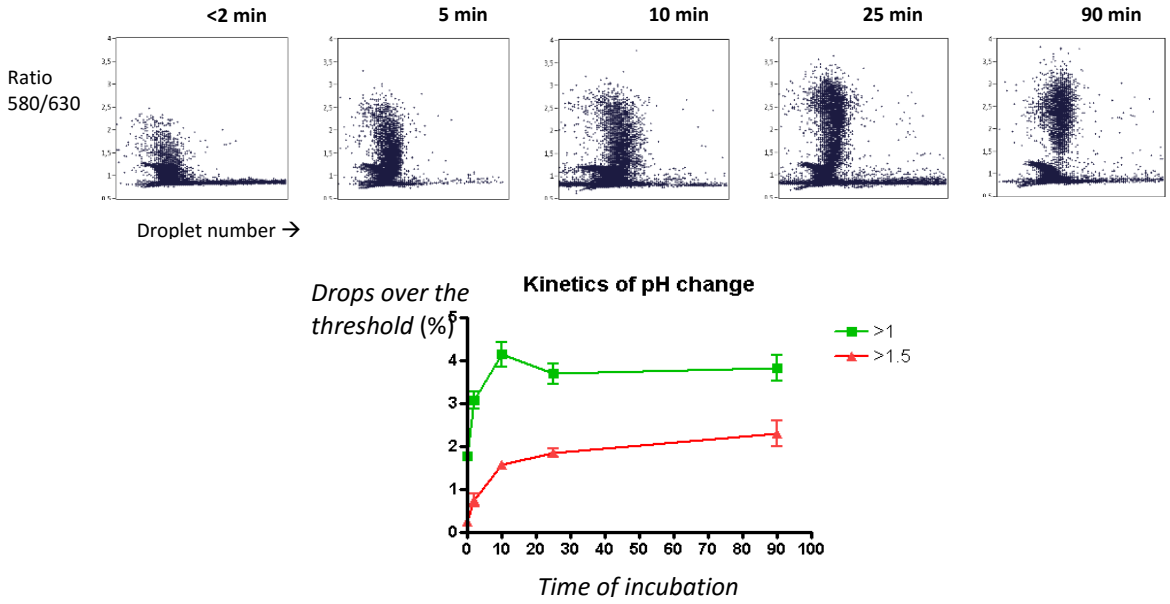


Figure 16A (Top panels) Dot plots showing a population of droplets becoming increasingly acid over time. (Bottom panel) The fraction of droplets reaching a threshold value (>1 – green trace; >1.5 – red trace) increases with time, reaching a plateau phase after 10 min.

We repeated the same experiment using white blood cells instead of cancer cells. WBCs didn't show such an increase in acidity. We can observe a slight emergence of a population from baseline at 25 min, suggesting WBCs do acidify the droplet, but a much slower rate, Figure 16B.

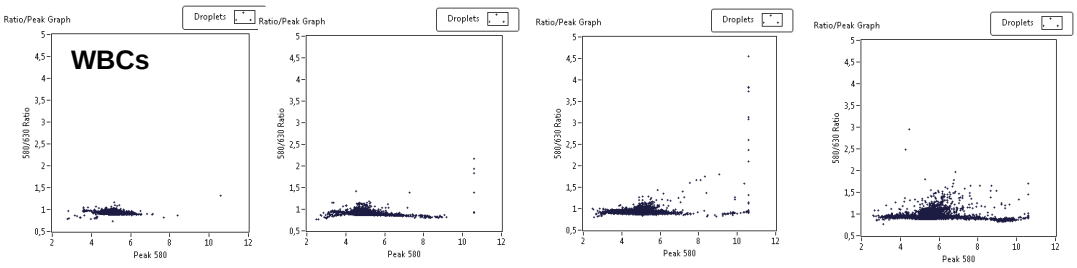


Figure 16B WBCs tested for different incubation times. Panels are showing from left to right <2 min, 5 min, 10 min and 25 min of incubation. The acidic population is not comparable to cancer cells. There is a slight emergence of a population from baseline at 25 min, suggesting WBCs do acidify the droplet, but a much slower rate

Confirmation of positive events by antibody labeling and pictures

To confirm true positivity of observed acid events, we followed several different strategies. We stained MCF-7 EpCAM(+) cancer cells with EpCAM and verified the correlation of low pH and EpCAM (+), as shown in Figure 17. Dot plots are visible in Figure 18. This technique enabled a precise quantification of sensitivity and specificity, because we could see also if cancer droplets overlapped with baseline values. Frequency distribution of pH at different incubation time of cancer cells vs WBCs is shown in Figure 18. At 30 minutes of incubation, we measure a sensitivity of 60% and a specificity of 99.99%.

During the experiments, we observed some acid dots even in white blood cells samples. Sometimes raw tracks showed a strange pattern, and we wanted to verify the true cellular nature of these events. We implemented a triggered camera set to capture images of acidic droplets. In this way we could discriminate between artifacts/junk Figure 20A and true events in Figure 19.

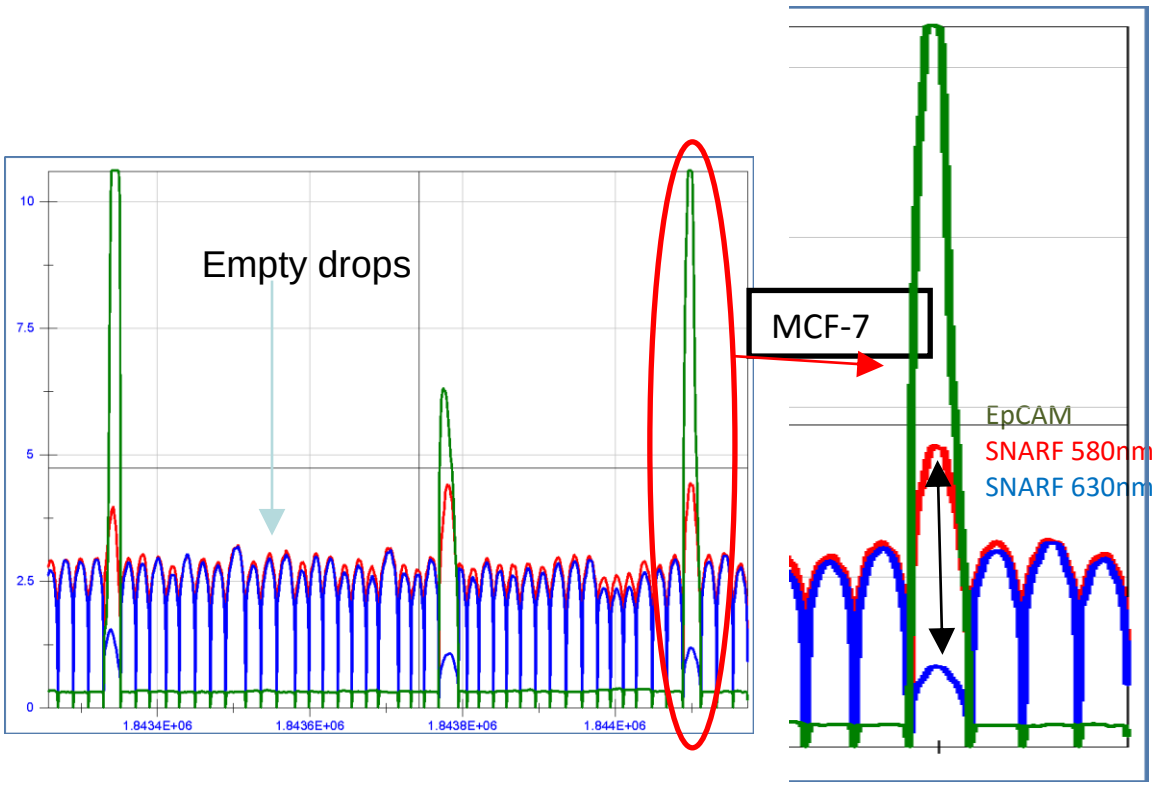


Figure 17 Raw track showing co-presence of antibody labeling and acidification of the droplet

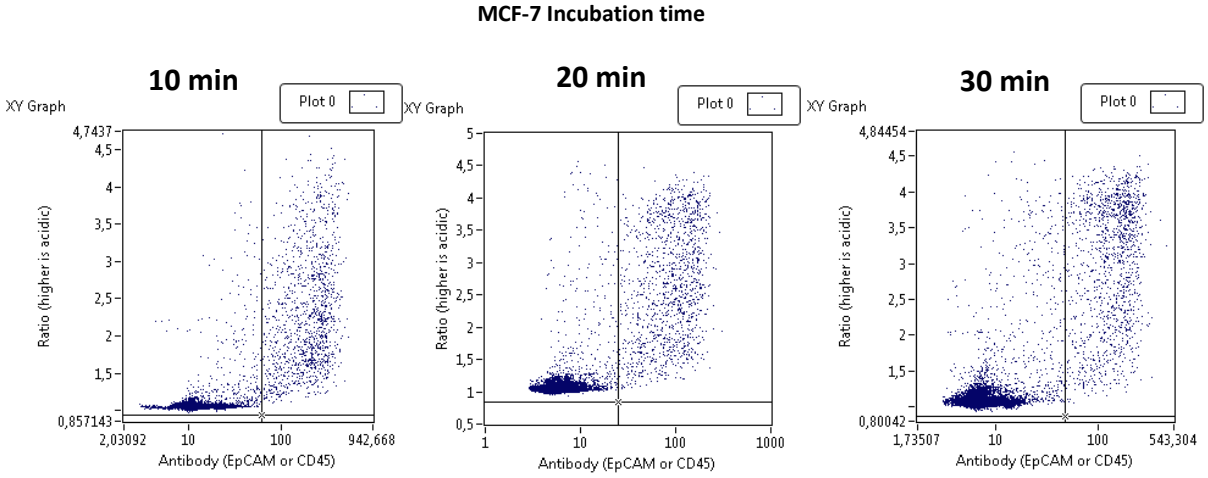


Figure 18 – Dot plots showing EpCAM labeling on X Axis and acidity on Y axis. The EpCAM(+) population is clearly distinguishable and becomes increasingly acid with time. Please note that an EpCAM(-) population can be observed even in MCF-7 EpCAM(+) cell line, proving the lower sensitivity of antibody labeling compared to our method.

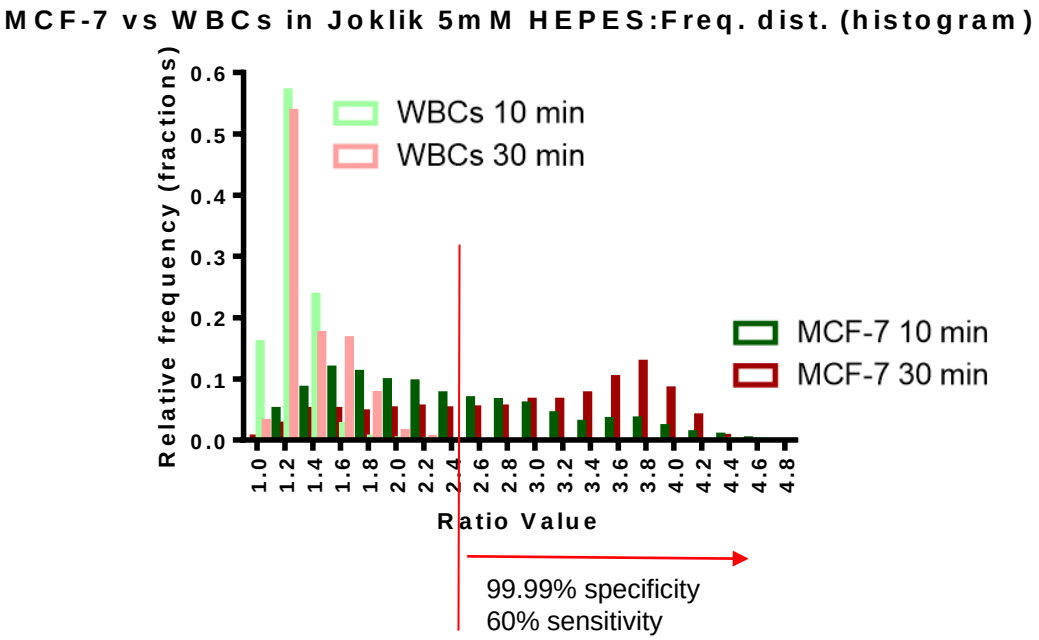


Figure 19 Frequency distribution of WBCs and MCF-7. The red line shows an arbitrary threshold over which we reached 60% sensitivity with 99.99% specificity.

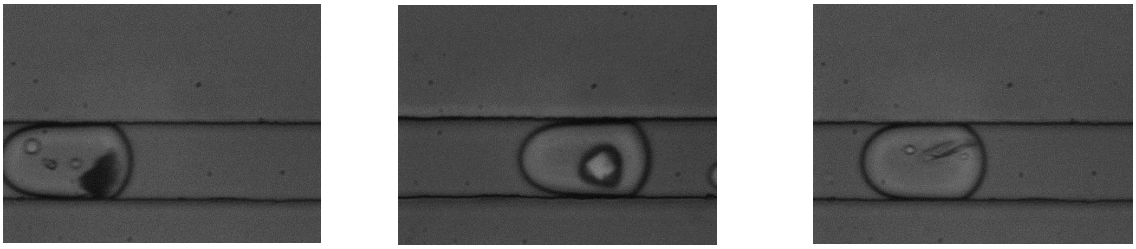


Figure 20A Triggered camera captured picture of acidic events revealing artifacts. Since our project addresses a rare cell problem, specificity is key, thus it is mandatory to verify false positives.

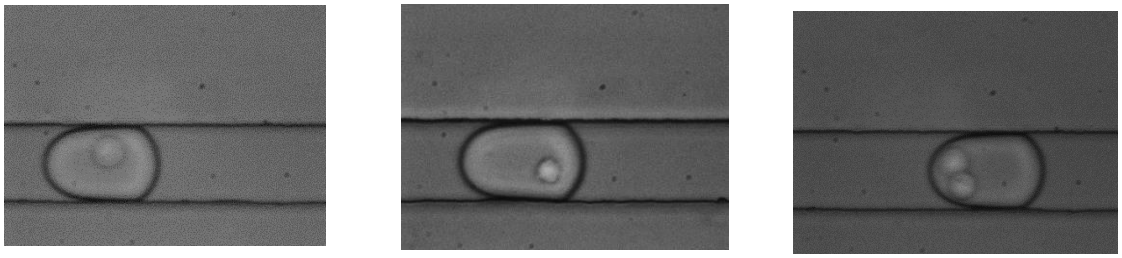
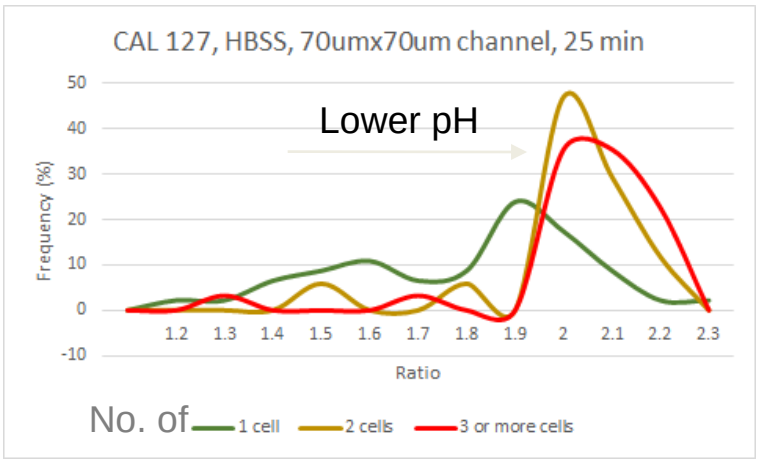


Figure 20B True events, showing clearly cells contained in droplets. Please note that doublets and clusters can also be detected.

Picture characterization

The implementation of camera revealed that sometimes a doublet or a cluster was enclosed in a droplet. We measured differences in acidity in these groups, showed in Figure 21. We observed that more cells produced more acid, and more importantly that single cells distribution suggested the existence of multiple subpopulations, which according to us would be worth to investigate further.



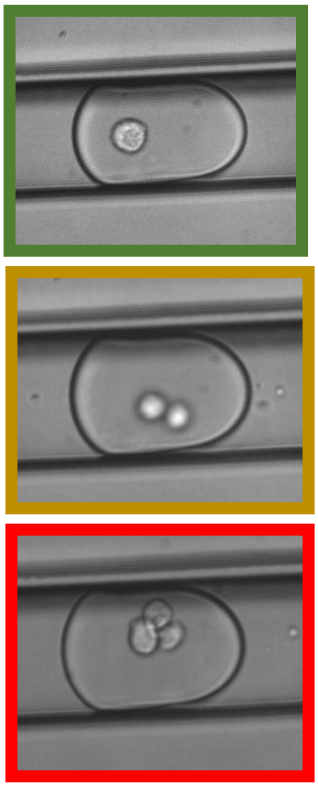


Figure 21 Frequency distribution of acidity measured separately for single-cells, doublets or triplets and clusters. The average acidity is increasing with the number of cancer cells enclosed. Please note that single cell frequency distribution has a two peaks distribution, suggestina cellular heterogeneity inside the population, maybe due to cell cycle differences or other reasons.

Spike experiment – ex vivo model

In order to simulate ex vivo real patient samples, we spiked known numbers of cancer cells in blood samples from healthy donors. We pre-stained spiked cells with Calcein Violet AM, which is a dye staining only viable cells, in order to distinguish them from WBCs – Figure 22. We could thus demonstrate that by gating for acidity we retrieved mostly cancer cells.

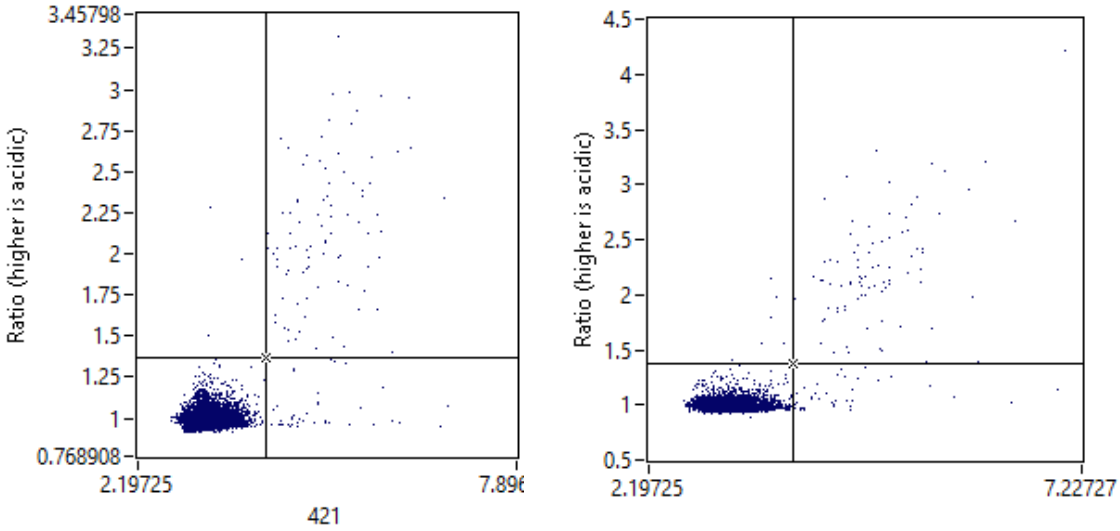


Figure 22 Dot plots of pre-stained FaDu spiked 1:50 in WBCs suspension from lysed blood. 421nm X-Axis displays Calcein Violet AM. Top-right quadrant contains Acid-producing cancer cells. Please note: bottom-right quadrant overestimate false negatives. We checked them by picture and they are mostly true negative, being empty droplets. Thus, the sensitivity of the method is over 90%. Top-left quadrant indicates false positives. They are mostly empty droplets. Sometimes, white blood cells cluster might reach those values.

We tried to go lower and lower with number of spiked cells. We could detect cancer cells averaging 60% recovery efficiency, with a good correlation between numbers of cells spiked and recovered, Figure 23.

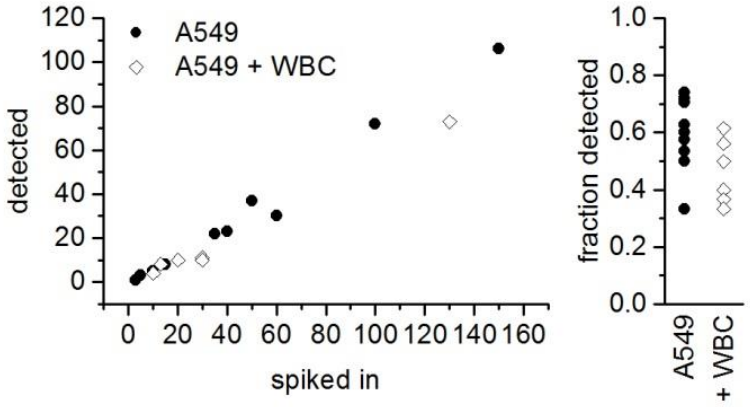


Figure 23 Correlation between numbers of cells spiked and numbers of cells recovered. Black dots indicate cancer cells alone, while white dots indicate cancer cells mixed with WBCs. In the right section of the picture fraction detected by the analysis.

Figure 24 shows a nice example of how, by fine-tuning incubation time, three distinct populations can be observed (empty droplets, white blood cells and cancer cells). Those three populations corresponded to cell concentration spiked. We verified positive values with picture, shown below the figure. Every dot over the threshold shown in the dot plot correspond to a picture of a cancer cell.

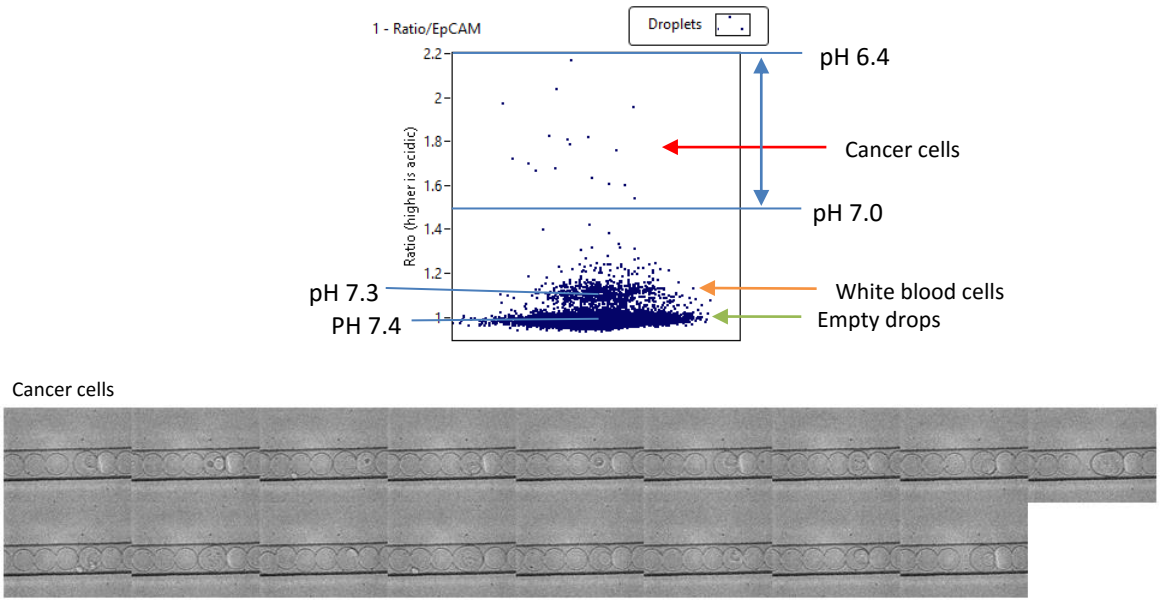


Figure 24 Dot plot of a spike experiment. Three distinct population can be observed: empty droplets, white blood cells and cancer cells, respectively with 7.4, 7.3 and 6.7 pH median. Picture of acid events demonstrated cancer cells present inside. Droplet detected is always in the same position, which is the drop right at the left of the bright laser slit.

As said above, we observed a partial recovery of spiked cells. Please note that this is not due to low sensitivity of the assay, as demonstrated by Calcein Violet AM and EpCAM experiments, but to low efficiency of encapsulation and cell loss in circuitry.

In order to cross-check this aspects, we took random pictures of generated droplets and counted the actual cells present in it compared to the original value of cell suspension.

With 50.000 cells/mL for example, we observed 20% of cells actually going into droplets. In spiked experiments though, spike recovery was measured at 60%. We speculate that, given much higher amount of WBCs than cancer cells, statistically the loss of WBCs was higher with respect to rare spiked cells.

Patients samples – Depleted

We analyzed blood samples from metastatic cancer patients, after red blood cells lysis, CD45 positive cells magnetic depletion and CD45 immunostaining. Healthy donors showed essentially no CD45(-) acid(+) population (only 1 doubt event in 1 donor over 4). The patients instead (4 patients – 2 lung, colorectal and breast cancer) displayed a variable number of CD45(-)acid(+) population of drops (Table 2). Drops in patients showed a pH value comparable to the positive control (spikes with cancer cell lines) – Figure 25. The number of cells detected in patients was in the range of 2-16/mL, thus consistent with known concentrations of CTCs. Please note that patients selected were highly metastatic, in order to maximize CTCs number. We did not confirmed that positive events were CTCs, so the exact number might be confused by the presence of false positives, and will be discussed in the discussion section. The presented results are the cleanest in a much larger dataset of experiments performed (N>20). Unfortunately, the majority of experiments was aborted or gave messy readouts due to clogging of microfluidics and breaking of droplets. For this reason, we consider these data as highly preliminar and to be confirmed with a dedicated industrial-level device overcoming existing limitations in microfluidics, as discussed in the discussion section.

CD45(-)Acid(+) cells/10^6drops	Cancer patient	Healthy donor
Lung	9	0
Lung	20	1
Breast	32	0
Pancreas	5	0

Table 3 The table reports patient statistics compared to healthy donors. Data are obtained with the "patient protocol" explained in the methods section. Data reported show CD45(-) Acid(+) cells number normalized for 10^6drops, corresponding approximately to the total 50uL sample (2mL whole blood). Number of droplets measured range from 700.000 to 1.500.000.

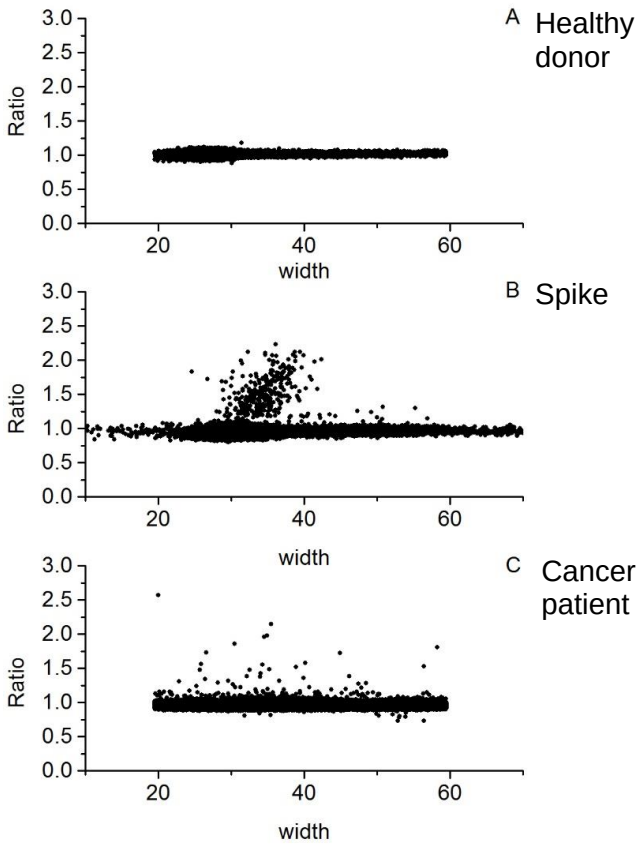


Figure 25 Healthy donor vs spike vs colorectal cancer patient. Plots are showing the CD45(-) population, after magnetic depletion and immunostaining. We excluded all droplets showing CD45 positivity. Please note that in the patient there is a population of drops ranging pH values comparable with the cancer cell line spiked in the positive control.

In patients, the size of CD45(-)/acid(+) cells sometimes was not compatible with white blood cells. We also performed experiments labeling with EpCAM and noticed one case of EpCAM positive cell in a breast cancer patient. These experiments did not have the CD45 possibility of control, so we do not know whether it was an unspecific labeling.

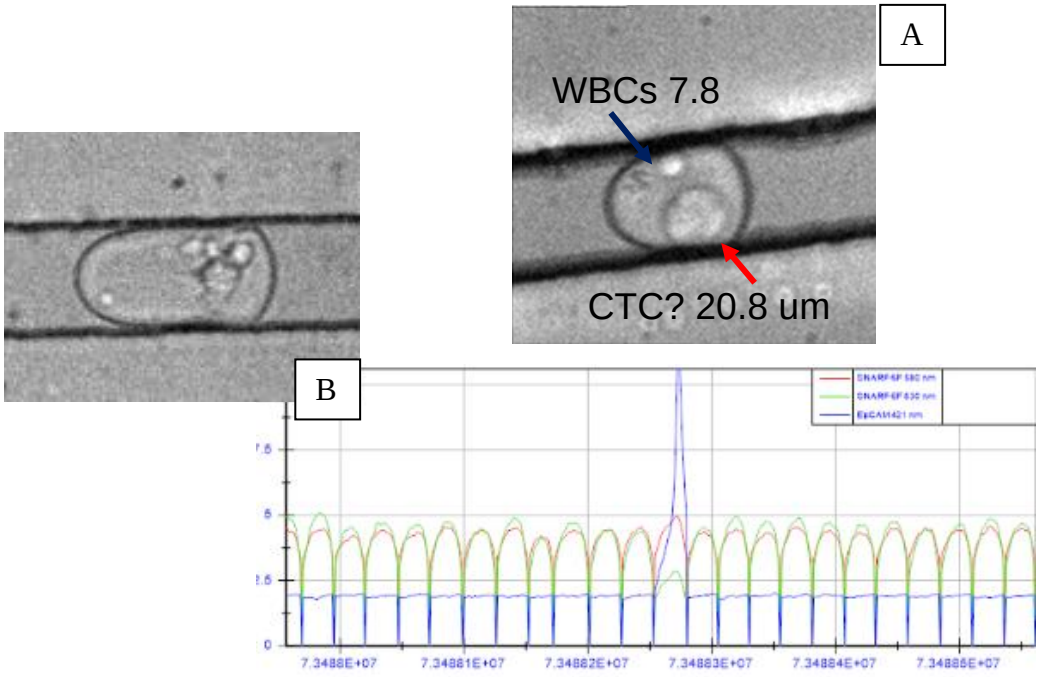


Figure 26 Picture A is showing a positive droplet from a metastatic lung cancer patient, including a big cell with a size not compatible with a white blood cell. Picture B is showing a positive droplet from a metastatic breast cancer patient, with raw track showing EpCAM positivity and high acidity. Several cells are detected in the droplet.

Patient samples - Undepleted

By depleting sample spiked with low number of cells, we observed that we couldn't detect cells anymore, most likely due to high number of passages for magnetic staining, depletions, and washings. For this reason, we decided to avoid the depletion passage in order to avoid underestimation of CTCs. Our throughput, though didn't allow us to process undepleted lysed blood samples quickly enough.

For this reason, we tried to increase flow rates by purchasing polymer devices with Luer connectors, as described in materials and methods section. We managed to increase flow-rates of 4 to 8-fold, as shown in table 3. The total experimental duration with these flow-rates would have been 3 hours for a sample of 2 mL (including 30 minutes RBC lysis).

	Emulsification step	Reinjection step
Old 30x22um circuit	500uL/hour	500uL/hour
New 35x48um circuit	2mL/hour	2mL/hour
New 70x70um circuit	4mL/hour	2mL/hour

Table 3 – Max flow rates achieved with polymer circuits.

Unfortunately, by processing lysed blood, the higher number of cells induces the formation of a lot of clusters, and a consequent increase in false positives, leading to a messy readout.

Between both depleted and undepleted samples, we tested many patients and controls (20 to 30), but frequent problems in microfluidics led to inconclusive results.

We concluded that our technology is not ready to collect clinical data without improvements discussed in the next section.

Epithelial vs Mesenchymal phenotype

After founding out that the device was unable to collect clinical data efficiently, we focused on measuring if there was a quantitative difference in acid production between epithelial and mesenchymal phenotype. We selected MCF-7 as an emblematic epithelial phenotype and MDA-MB-231 as a more mesenchymal, invasive phenotype. Notably, MCF-7 is a tumorigenic, low glycolysis cell line, while MDA-MB-231 is a metastatic, heavily glycolytic cell line (Hart et al. 2015).

Our results indicate that MDA-MB-231 are acidifying more extensively the droplets, as shown in Figure 27.

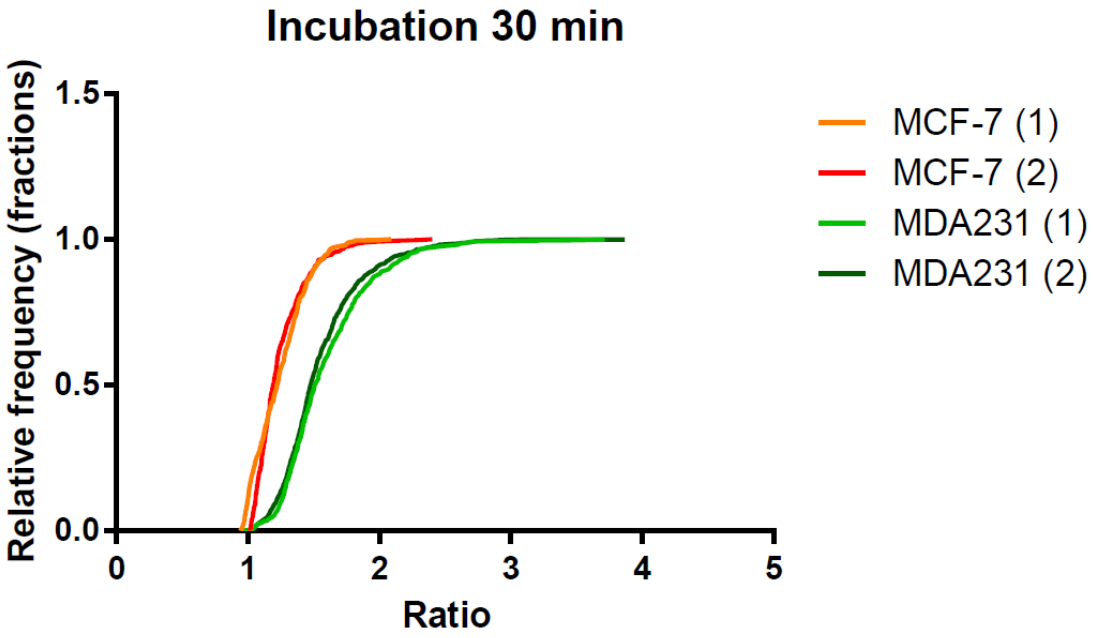


Figure 27 – Cumulative frequency of droplets. Two replicates per cell line, showing high coherence between replicates and a significative difference between MCF-7 and MDA231.

MDA-MB-231 proved to be consistently acidifying droplets at a higher degree both at 30 minutes and 60 minutes of incubation, with two averaged replicates shown in Figure 28.

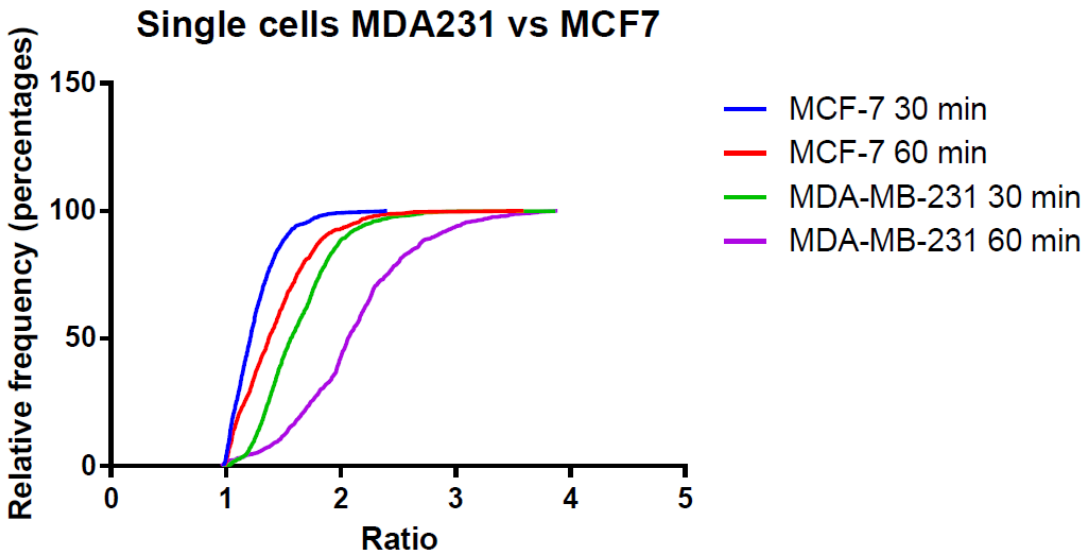


Figure 28 – Single-cell containing droplet with MDA-MB-231 vs MCF-7 at 30 and 60 minutes of incubation. Please note that MDA-MB-231 show a higher degree of acidification even at 30 minutes compared to MCF-7 at 60 minutes.

Interestingly, the same pattern appeared consistently also considering droplets containing two cells – Figure 29 – even though in absolute terms doublets (triplets and clusters) were more acid than singlets – Figure 30 – similarly to results shown before.

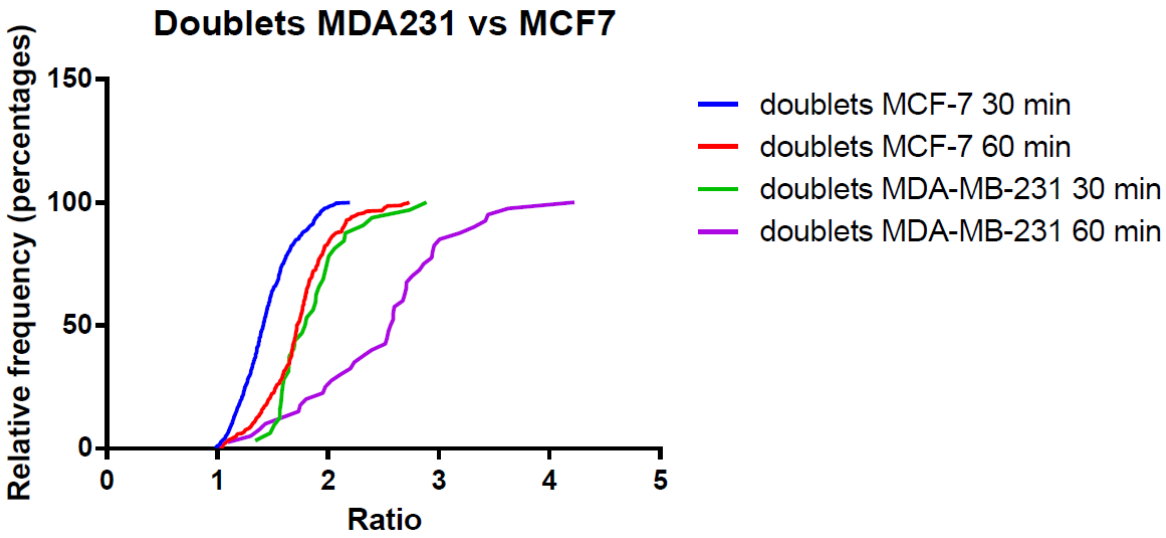


Figure 29 – Droplet containing two cells (doublets) were measured one by one thanks to camera implementation, and their frequency displayed the same pattern of single cells.

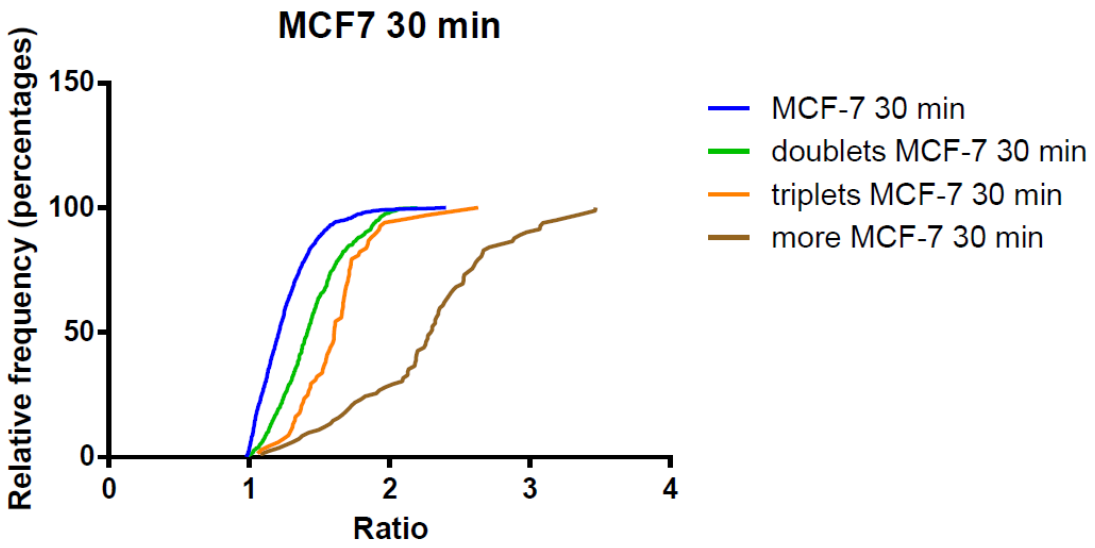


Figure 30 – MCF-7 singlets, doublets, triplets and clusters frequency distribution. As similarly shown before, acidification is proportional to number of cells.

These results suggest that the sensitivity of our method increases with more aggressive and metastatic phenotypes. This fits particularly well our task, which is of finding metastatic cells in the blood – CTCs.

Discussion

Our method has several advantages over state of the art, but experimental practice induces us to admit it is still crippled by some technical limitations. We will discuss these points, together with future steps needed to achieve clinical validation, and which impact might in the end this project have.

Advantages

Transversal validity

The method has been confirmed for all tested cancer cell lines, covering epithelial and mesenchymal phenotypes, showing transversal validity. This ensures a good sensitivity and a broad range of applications not limited by epithelial phenotype or *a priori* knowledge of protein biomarkers.

Metabolism already used in the clinic

Cancer peculiar metabolic properties are already recognized and of widespread use in the clinical routine: cancer avidity for glucose is exploited in positron-emission tomography scan (PET-scan, Schrevens et al. 2004). Since our method is based on a strongly correlated metabolic effect, it's brought closer to the clinic than the other physical properties-based methods – which need to demonstrate their clinical relevance and be accepted by the – usually conservative – clinical world.

Low-cost

The method has lower-cost reagents (Oil, medium, pH-sensitive dye) compared to monoclonal antibodies. In some of our experiment we used antibodies, but this was required to deplete sample volume, being the lab setting unable to process big volumes of sample. With an eventual industrial parallelization of circuitry in order to increase flow-rates, the method won't require any antibody labeling, and could process tens of mL of blood quickly enough for a routine laboratory practice.

Automated counting

The majority of emerging methods for CTCs detection doesn't provide automated counting, but only enrichment (size-based filters, CTC-iCHIP, spiral channel). Counting is then performed by immunostaining. Epic sciences apparently provides a semi-automated

counting system, but always based on massive immunostaining. Our system allows semi-automated counting, and is at this stage comparable with CellSearch architecture, requiring picture verification by human eye.

Viable cell isolation

Our system can provide viable cell isolation. Cells are label-free, untouched and alive in their own medium. They are exposed to less shear-stress compared to size-based filtration, and to comparable stress of other emergent technologies. Although our system for CTC isolation is not stable enough for such a rare cell problem, the proof of concept that dielectrophoretic isolation of droplets is possible is widely present in literature (Mazutis et al. 2013a; Sciambi and Abate 2014; Shields, Reyes, and López 2015).

Limitations and future steps

Confirmation of CTCs nature.

Although we have demonstrated that our method is capable of detecting cancer cells from a cell line in a white blood cells mix, and the pattern detected in patients is comparable to the one of the cell lines, two points are still not confirmed. If detected droplets are actually containing CTCs, and if the number of CTCs detected is clinically meaningful. In order to do this, droplet isolation would be essential. After isolating detected cells, they could either be matched with current definition for CTCs (immunostaining for e.g.: DAPI, EpCAM, CK, CD45) or be revealed as neoplastic by detecting known mutations present in primary cancer. Since complex tumor genome can be inferred by CTCs (Heitzer et al. 2013), the detection of cancer-specific mutations (e.g.: estrogen receptor in breast cancer) would be confirmative even if absent in the primary tumor, because of intrinsic limits of solid biopsies, overlooking disease progression and tumor heterogeneity (Diaz and Bardelli 2014).

Microfluidics

The main limitation we met in our study has been in microfluidics. In order to provide a reliable CTC counting, we identified technical issue that need to be addressed.

Cells encapsulated as first and as last undergo different incubation times, and at the same time, droplet measured as first and as last, undergo different incubation times. Although statistically the difference is balanced because encapsulation and measurements steps are

independent and droplet are mixed in between, implementing a method to keep produced droplet at constant 4ºC temperature would stop metabolism and enhance the quality of the experiment. Keeping both syringes and tubing at 4ºC would also have the advantage of reducing cell adhesion and possibly reduce cell loss in syringe and tubing.

Tubing seems to be the main cause of cells loss and clumping, resulting in low yield of encapsulation, obstruction of circuits and false positive generation. Shorter tubing might help, as well as smaller diameter, causing cells to move faster, and with higher pressure. Personally, I think that a method to completely get rid of tubing by directly connecting the syringe with the circuit would be the best option.

Circuit obstruction seems to be a very frequent problem in microfluidics. Despite our attempts to obtain a clean sample by strainers and filters, frequently experiments are burdened by clogging of the circuit. Thus, additional filtering steps are needed and parallel circuitry would be probably solving the problem, because even if a circuit would be clogged, the flow would be redirected to other circuits, not affecting the overall results.

Our system demonstrated to be able to detect cell clusters, which is an advantage as other important papers claim (Aceto 2014). There is the open question, though, whether the clusters we see are real clusters or are due to cell clumping in the tube/syringe.

There would also be the necessity of increasing the throughput of our system. Although our throughput is acceptable in terms of lab tests, in order to process larger volumes of blood and shorten the execution time for the assay, an increase of 4-5 folds would be needed. Currently, CellSearch® sample volume is of 7.5 mL and this increase in throughput would lead to processing time for a similar sample of 1-2 hours, compatibly with diagnostic procedures. According to our opinion, this would be most likely achievable by simple parallelization of circuitry. High-pressure, screwed connections might also benefit, because they would allow for higher flow-rates without possible spillovers.

In order to stabilize the whole system for diagnostic procedures, our opinion is that a closed reservoir to collect droplets inside the circuits would be desirable. Right now droplets are collected inside an external reservoir, which expose them to air and dust, increasing the risk for droplet evaporation and circuit clogging.

Our experience with cell sorting was somewhat oscillating. Although we have enough data to support the hypothesis of our sorting mechanism, the stability and robustness of sorting is insufficient. We are trying to redesign sorting circuit according to apparently successful papers (Mazutis et al. 2013b), but we cannot conclude that the technology is ready to work without interruptions for the whole duration of the assay. While our sorting technique works for positive sorted droplets, its main problem is that empty or negative droplets stochastically go into sorted channels, leading to downstream contamination and reduction of isolation purity.

The main problem which hampered our biological verification of detected putative CTCs is that sorted droplets are not trivial to collect and stain.

Potentially, we could use microfluidics for generation of droplets only, leaving detection and sorting to more stable devices. We are working to implement a method to screen generated droplets with FACS (Fluorescence Activated Cell Sorters) – in order to do that, either we need to adapt an existing FACS device to run with oil and droplets, or we need to produce double emulsions, which are soluble and can run into standard FACS (Bernath et al. 2004; Zinchenko et al. 2014). Commercial chips for the production of double emulsion are already available, for example at Microfluidic ChipShop or Dolomite, and should ease the management of this step.

Specificity

Since CTC detection is a rare cell problem, even the smallest amount of false positive could make the assay inconclusive. We observed artifacts and cell clustering identified as acid events, but these should be easily excluded by electronic filtering or automated image processing. In some of our experiments, we detected rare cells showing acidification of the droplet in healthy controls. At the same time, we detected empty drops showing acidification of their content. The reason behind it is unclear. This means that further work is needed to clarify whether observed acid cells are truly acidifying the environment or they are a random event corresponding to acid empty droplets. If they are a true event, that nature of the cell would need to be investigated, to understand if there is a way to discriminate it from actual CTCs or whether they might be clinically meaningful for other pathological conditions. The causes of potential decrease of the specificity of the method (e.g.: inflammations, infections) must also be investigated in dedicated clinical trials and a

cut-off value must be established to discriminate between different pathological conditions.

For these reasons, additional parameters to cross-correlate to correctly identify the real CTC might be needed. This parameter could be for example EpCAM or CD45, leading to a results where acid positivity is linked to antibody positivity to give a more complex and possibly more robust readout.

Clinical meaning

While our technology might look promising, no clinical meaning is still associated with our assay. This would be obtainable only with a dedicated clinical trial to assess sensitivity and specificity of the method for given conditions. These initial trials are relatively simple and can be performed quickly enough (2-3 months) once the technology has been stabilized. More detailed trials, linking CTC level to overall survival or progression free survival would require more time (3-5 years), although they could be simplified in first instance for example by associating CTC level to imaging outcome, that is now correlating with progression of disease, shortening required time to 6 months. Finally, most important trials, assessing the clinical utility, might be more cumbersome: for example, they would require randomized controlled architecture where we investigate the potential of the method to predict the most appropriate therapy.

Impact

The impact of CTCs diagnostics does not have a uniform consensus in the scientific community, especially in the clinical world. Despite rising evidence in CTCs evidence (Joosse, Gorges, and Pantel 2015) and amount of CTC companies pursuing the objective of delivering better CTCs devices, the clinical world is divided in supporters and detractors, due to many potential advantages, but also unsolved questions and problems (Hong and Zu 2013). Personally, we believe that the current unavailability of a low-cost, standardized device robustly detecting CTCs is a critical factor in leaving questions unsolved.

The current only FDA-approved device for CTCs testing, CellSearch®, is time-intense, cost-prohibitive and above all operator-dependent, a big defect for a diagnostic device,

leading to variability and lack of standardization of results (Miller et al. 2010; Tibbe, Miller, and Terstappen 2007).

Needless to be said, if the technology is not ready to test CTCs, questions can't be directly addressed and solved.

For this reason, we think that if we manage to implement our method in a robust diagnostic device, keeping the cost per analysis low enough, we would contribute to solve current tangles.

We already described in the introduction the potential advantages of CTCs isolation for the understanding of their biology and targeted therapy, so we focus here on CTCs enumeration, which according to us is currently underestimated with respect to the above-mentioned isolation.

In our personal view, we don't think that CTCs enumeration will directly solve cancer problem, but that, meeting diagnostic requirements of robustness and cost, it would be an excellent tool to drive the clinicians in a series of decisions that nowadays is taken in the dark – especially in therapy administration and follow-up. From the beginning of a treatment up to the first evaluation with imaging, there are several weeks in which the clinicians often don't have a clue of what's happening: biopsies are not repeatable due to their invasive nature, imaging isn't sensitive enough and current blood biomarkers often are not specific enough to support protocol changes. CTCs enumeration instead, being a specific blood-based assay, if kept at low cost could be used *serially* to monitor patients' status on a weekly, or even more frequent basis. Thus, unseen oscillations in patients' conditions might emerge, relapses and recurrences might be detected before current limits, and progression of disease might be addressed by rapid changing or adjustment of therapy protocols. Furthermore, differences in drug cocktails, doses, or administration methods might emerge. For example, the effect of different administration regimes of the same drug, metronomic vs standard chemotherapy (Gasparini 2001; Hanahan, Bergers, and Bergsland 2000), could be better understood by time-resolved CTCs enumeration.

Conclusion

In conclusion, we demonstrated that a single-cell metabolic analysis is possible and can discriminate cancer cell lines from white blood cells. We implemented the method in a semi-automated high-throughput prototype, paving the way to the first metabolism-based platform for CTCs detection. We gave the proof of concept that the detection is possible in cancer patients, although further confirmation of putative CTCs nature and their clinical meaning still needs to be assessed. Although our results are very promising, the real CTC detection potential and its clinical value must be rigorously confirmed, and in order to do this the mentioned technical issues need to be solved.

During the course of our PhD we acquired skills in several fields of knowledge – physics (optics, microfluidics), engineering (signal acquisition, signal processing), software programming (prototype software, data analysis) – combining them with our previous expertises – medicine, cellular biology – coherently with our idea of research and development, and with the multidisciplinary character of our mentor prof. G. Scoles and our PhD school.

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