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Circulating tumor cells: a review of non EpCAM-based approaches for cell enrichment and isolation

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Abstract

Background: Circulating tumor cells (CTCs) are biomarkers for non-invasively measuring the evolution of tumor genotypes during treatment and disease progression. Recent technical progress has made it possible to detect and characterize CTCs at the single-cell level in blood.

Content: Most current methods are based on epithelial cell adhesion molecule (EpCAM) detection, but numerous studies have demonstrated that EpCAM is not a universal marker for CTC detection since it fails to detect both carcinoma cells that undergo epithelial-mesenchymal transition (EMT), and CTCs of mesenchymal origin. Moreover, EpCAM expression has been found in patients with benign diseases. A large proportion of the current studies and reviews about CTCs describe EpCAM based methods, but there are evidences that not all tumor cells can be detected using this marker. Here we describe the most recent EpCAM-independent methods for enriching, isolating and characterizing CTCs, based on physical and biological characteristics, and point out their main advantages and disadvantages.

Summary: CTCs offer an opportunity to obtain key biological information required for the development of personalized medicine. However there is no universal marker of these cells. To strengthen the clinical utility of CTCs, it is important to improve existing technologies and develop new, non-EpCAM based systems to enrich and isolate CTCs.

Circulating tumor cells (CTCs) are defined as cells that originate in primary tumors, recurrences, or metastases. They circulate freely in peripheral blood, and have antigenic and genetic characteristics specific to the tumor of origin [1]. CTCs are important because the majority of deaths from cancer are linked to the development of disseminated metastases [2]. In the last few years, emerging data have challenged the traditional theory of sequential metastasis development [3] (Supplemental Figure 1). Several studies have pointed out that CTCs can be isolated in patients at relatively early stages of tumor growth [4, 5], even before the primary tumor mass is detected by conventional methods [6]. Furthermore, current high-resolution imaging technology is not sensitive enough to detect micro-metastases or early tumor cell dissemination, which are the key events in tumor progression (Supplemental Figure 1).

Because they can be obtained by non-invasive methods, CTCs can be used as therapeutic markers for monitoring treatment effectiveness in real-time, and for detecting recurrent disease. CTCs also have potential for evaluating drug resistance mechanisms, and may have utility in estimating the risk of metastatic relapse and progression. Unlike the characterization of primary tumors, which only provides a static view at the time of diagnosis, analyzing CTCs may improve understanding of the different steps involved in the metastatic cascade, from invasion of tumor cells into the blood stream to the formation of clinically-detectable metastases [7].

Although studying circulating tumor cells is a promising approach for better characterizing cancer, there are certain issues inherent to the nature of CTCs that should be considered. CTCs are rare events, which are present at very low concentrations in the blood (i.e. one tumor cell in the background of millions of blood cells) [8]. In addition, only a restricted number of CTCs has the ability to generate metastases [9] and consequently it is necessary to characterize them precisely to be able to distinguish

metastatic and non-metastatic CTCs. Numerous methods have been developed to isolate tumor cells, most of which are based on epithelial cell adhesion molecule (EpCAM) detection. Indeed, EpCAM is a conventional marker expressed by cancer cells of epithelial origin and has been then used for carcinoma cell isolation. However, as described below, EpCAM is not expressed by all CTCs and alternative approaches need to be considered. There are multiple recent useful reviews on CTC isolation methods [10-12] but none of them have exclusively focused on non-EpCAM based methods. The aim of this review is to provide an overview of the most recent EpCAM-independent methods for enriching, isolating and characterizing CTCs.

EpCAM is not a universal biomarker for isolating CTCs

A great deal of effort and resources has been invested into developing methods for detecting CTCs in peripheral blood. In the last decade, several methods have emerged for detecting and characterizing CTCs. However, these methods and consequently the biological characterization of CTCs are still technically challenging. The first step in the detection of CTCs was the discovery that EpCAM was expressed at variable degrees on epithelial derived carcinomas and related cancers but was absent in the peripheral blood cells [13]. This finding resulted in the investigation and development of different methods for enriching and isolating CTCs based on the EpCAM marker [14, 15] and led to the first and only automated EpCAM-based system (CellSearch[®]) currently approved for clinical use by the United States Food and Drug Administration for the detection of CTCs. CellSearch[®] is thus considered the gold standard for CTC detection methods [16, 17]. However, recent evidence has challenged the suitability of this method; EpCAM-positive circulating epithelial cells have been reported in patients with benign colon diseases [18] and are a potential source of false-positive findings. In addition,

carcinoma cells can undergo epithelial-mesenchymal transition (EMT) which results in decreased expression of epithelial markers, such as EpCAM and CK [19], and the appearance of mesenchymal markers. The loss of epithelial markers may therefore result in false-negative findings. In this context, the EpCAM marker is not suitable for isolating CTCs from carcinomas that have undergone EMT or those cancers with primary mesenchymal origin. Consequently EpCAM cannot be considered as a universal marker for CTC detection. This highlights the need to develop non-EpCAM based technologies for isolating and detecting CTCs.

Enrichment of circulating tumor cells: conventional methods

The major challenge for isolating and characterizing CTCs is their low concentration compared to the other cell types in the peripheral blood. Enrichment approaches take into consideration several parameters: capture efficiency/recovery rate, purity, cell viability, processing speed, blood sample capacity, sample pre-processing requirements, cost of consumables and equipment, repeatability and reliability. The optimal enrichment solution may require a compromise between these performance parameters and the intended downstream application. Current enrichment approaches include a wide range of technologies based on the different properties of CTCs that distinguish them from surrounding normal hematopoietic cells, including biological properties (cell surface protein expression, viability, invasive capacity) and physical properties (size, density, electric charges, deformability) (Figure 1).

Methods based on physical properties

Cytological analyses have revealed that CTCs exhibit a greater nuclear to cytoplasm ratio, are larger in size, and have different nuclear morphology compared to normal

cells [20]. These cytological alterations result in the differences of their mechanical properties, providing CTCs with several capabilities. The cytoskeletal stiffness of CTCs is dynamically modified. This flexibility may facilitate their invasion to distal sites from the primary tumor, and may confer their resistance to damage from fluid shear stress within the blood vessels during the metastatic process [21]. These modifications in the stiffness alter the conservation of the membrane structure, which in turn affects their surface charge and electrical properties [21]. Various approaches have been used to exploit the differences in physical properties between tumor cells and blood cells as a means of enriching and separating CTCs from blood samples (Figure 1).

Density gradient centrifugation is a conventional approach for separating blood components based on differences in their sedimentation coefficients. As whole blood is deposited in the liquid gradient and subjected to centrifugation, cells will distribute along the gradient depending on their density (Figure 1, Table 1). Erythrocytes or polymorphonuclear leukocytes migrate to the bottom, while mononuclear leukocytes and CTCs remain at the top as a buffy coat [22]. Percoll, Ficoll-Hypaque® (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) and OncoQuick® (Greiner Bio-One, Kremmünster, Austria) are the most commonly used density gradient media in pre-clinical and clinical research. Ficoll-HyPaque®, formed by the copolymerization of sucrose and epichlorohydrin, is mainly used in biology laboratories to recover peripheral blood mononuclear cells. Despite its long history of use in laboratories, there are some pitfalls associated to this technique, such as the possible loss of tumor cells that migrate either to the plasma fraction or to the bottom of the gradient due to the formation of aggregates [22]. It has been suggested that this cell loss may be due to the cytotoxicity of the density medium [23]. Alternative to Ficoll, there is Percoll density

(GE Healthcare Life Sciences) gradient medium made of a colloidal silica particle suspension. The main advantages over Ficoll include reduced toxicity and a wider density gradient range [23]. There are certain discrepancies in the literature regarding the use of Percoll since some studies report a high purity rate [24] while others report low isolation efficiencies compared to Ficoll [25]. A third density system named OncoQuick® is composed of a 50 mL tube with a porous barrier inserted above a separation medium. Cells are separated and pass through the barrier depending on their different buoyancy densities during centrifugation. CTCs, together with the lymphocytes, will remain above the porous barrier, making them easily accessible for subsequent collection. OncoQuick® has mildly higher reported recovery rate compared to Ficoll density gradient, 87% and 84%, respectively [26]. Moreover the mononuclear cell depletion using the OncoQuick® system is significantly higher compared to Ficoll; this facilitates processing higher sample volumes, which is beneficial for CTC characterization [26, 27]. However, during the isolation process, CTCs migrate into the plasma fraction and are frequently lost [29]. Overall major advantages of all the density centrifugation methods are that they are inexpensive and reliable (Table 1). However, the disadvantages include the loss of large CTCs and CTC aggregates that fall to the bottom [29], as well as the fact that leukocytes cannot easily be eliminated, resulting in very low purity. It is therefore necessary to combine centrifugation with another enrichment method.

Microfiltration enrichment methods process circulating cells through an array of microscale constrictions in order to capture target cells based on their size or a combination of size/deformability. There are multiple different microfiltration devices, some are available on the market and others remain currently prototypes (Figure 2).

Membrane microfilters are composed of a semipermeable membrane with a 2D array of micropores. A membrane with a pore size diameter of 8 μ m has been demonstrated to be optimal for CTC retention [30]. The typical configuration used for microfiltration is dead-end filtration (Figure 2A), in which the blood flow is perpendicular to the membrane. The main limitation of this strategy is that the layer of cells retained on the membrane can reduce the efficiency of recovery due to the build-up of filtration resistance [32] (Table 1). To overcome this issue, Zheng et al. created a 3D membrane microfilter consisting of two pored layers (Figure 2B), between which CTCs are retained [32]. In contrast to conventional microfiltration devices, this system reduces the tension stress on the cell plasma membrane and demonstrates a high recovery rate (86% with a theoretically fast throughput of 3.75 mL/min) [32].

Another system based on a 2D membrane slot filter (Figure 2C) was proposed by Lu et al., in which the forces exerted on the cells are reduced, reaching viability of 90% with a high recovery [33]. The bead-packed filtration device consists of a chamber where uniform beads measuring 45 μ m in diameter and non-uniform beads (with diameters ranging from 15 to 100 μ m) are packed and act as the filtration element (Figure 2D) that retains CTCs and allows red and white blood cells to pass through [33]. Studies performed by Lin *et al.* reported a low recovery rate (between 21% and 40%) in contrast to filtration performed using membrane systems [34].

There are systems available that make it possible to enrich and isolate CTCs in a single step. For example, ScreenCell[®] technology (ScreenCell, Sarcelles, France), is an innovative single-use and low-cost device. It is based on a filter that isolates and sorts tumor cells by size. There are three different types of device, depending on the downstream analysis: ScreenCell[®] Cyto (molecular techniques that require fixed cells), ScreenCell[®] CC (cell culture) and the ScreenCell[®] MB (RNA or DNA analysis) [35,

36]. The main advantages of this system are its low-cost, small format and ease of use. Another platform in development is the parylene-C slot microfilter that measures telomerase activity from captured, viable CTCs. It has 90% recovery rate [36]. The 90% of the cells recovered are viable and yield 200-fold sample enrichment [36]. In contrast to ScreenCell, parylene-C only detects viable CTCs and can be re-used.

Filtration allows for rapid CTC enrichment from large volumes of blood in minutes, with minimal processing. Recovery rates are around 90%, but further processing is required for certain downstream applications, as the final purity is typically around 10% or less. The main disadvantages associated with filtration are: the heterogeneity in CTC size, cluster formation, the possibility of membrane clogging, difficulties in the detachment of cells retained in the filter, as well as the background signal on the filters after immunostaining for CTC detection.

Microfluidics includes several separation methods, which makes it possible to manipulate very small volumes of biological fluids. The past decade has seen many new technologies proposed for biological cell sorting and analysis on microchips. Arrays with pillars of varying geometries have been used to fractionate cells in blood and capture tumor cells [37]. Similarly, crescent-shaped trap arrays with a fixed 5 μm gap width within microfluidic chambers have been used to enrich CTCs from whole blood without pre-processing [38]. Parsortix system (Angle, Guildford, United Kingdom) (Figure 3A) is microfluidic technology that captures CTCs based on their less deformable nature and larger size compared to other blood components. With this system, it has been reported a higher number and purity of isolated CTCs in patient samples than with the Cell Search. Moreover, the processing time of 7.5 mL of whole blood is 2 h in contrast to the 4 h reported for the Cell Search (Cell Search, Jansen

Diagnostic, Raritan, NJ, USA). It is worth to remark that with the second version of Parsortix 10 mL can be processed in 2.5 h. The main drawback of this technique is the difficulty of eliminating all leukocytes due to size overlap with CTCs [39].

In addition to the previous devices described above, ClearCell® FX (Clearbridge Biomedics, Singapore) recovers viable cells in small sample volumes and in a short period of time (e.g. 1 mL of blood in 10 minutes) (Figure 3B) [40]. ClearCell® FX does not require pre-processing of the blood; this decreases the possibility of losing cells of interest (Table 1). This system takes advantage of the inertial and centrifugal forces causing the smaller red and white blood cells to flow along the channel's outer wall and the larger CTCs to flow along the inner wall, recovering both fractions in different channels of the system. Unfortunately, CTCs of different sizes may escape through the white/red cell channels, and certain white blood cells can be captured in the CTC fraction.

To limit CTC loss to white and red cell channels, CTC-iChip technology (D.A. Harber, Massachusetts General Hospital Cancer Center; M. Toner, Harvard Medical School; Boston, MA) was developed (Figure 3C). CTC-iChip technology combines continuous deterministic lateral displacement for size-based separation of red blood cells/platelets from tumor cells obtained from whole blood, inertial focusing for precise positioning of cells in a microchannel, and microfluidic magnetophoresis for immunomagnetic depletion of white blood cells. This is a fast system (it can process 8 mL of blood per hour) allowing the recovery of any viable cancer cell types according available for their characterization. Unfortunately, in the deterministic lateral displacement step, small CTCs are lost, and undesired large cells and aggregates pass on to the next step, due to particle deformability and can limit the use of this device [41].

Carefully applied microfluidic approaches are capable of achieving both excellent purity of more than 80% and high recovery rates with little disturbance to the CTCs. However, these advantages come at the expense of lower throughput requiring either reduced sample volumes or prolonged periods of time to process samples (e.g., several hours to process a full tube of blood).

Dielectrophoresis (DEP) has been initially described by Pohl as “the translational motion of neutral matter caused by polarization effects in a non-uniform electric field” [42]. To move a particle by dielectrophoresis, the particle needs to be polarizable once an electrical field is applied [43]. This phenomenon has inspired new approaches for the separation of cells based on their electrical properties. Because the DEP force is inversely proportional to the length scale [44], micro-scale chambers named microchips have been developed for isolating rare cell events. These microchips integrate arrays of electrodes to generate a non-uniform alternating current field characteristic of the DEP technology.

Interdigitated gold electrodes have been used to separate cancer cells from blood cells [45]. Tumor cells were attracted towards the electric field generated by the electrodes by means of positive DEP, while other cells were flushed away. When the electric field was turned off, the cell initially retained were released and recovered with an approximate rate of 95%. Moon *et al.* created a system with a DEP module integrated into a size-based hydrodynamic step, used as the enrichment stage to remove excess blood cells [46]. The first commercial instrument based on DEP field flow fractionation was the ApoStream™ system (ApoCell Inc., Houston, TX) (Figure 4A). To use this methodology, an initial enrichment step is required. Recovery rate is over 70% and the viability more than 97%; however, the purity obtained is less than 1%,

although this can be significantly improved with additional enrichment stages at the risk of reduced recovery rate [47]. The DEPArray™ technology (Silicon Biosystems, Bologna, Italy) combines the ability to manipulate individual cells using dielectrophoretic technology with high quality image-based cell selection (Figure 4B). The most attractive characteristics of this technology are the single cell resolution, high fidelity recovery, cell viability and, in the most recent version, the possibility of isolating individual cells from paraffin embedded samples [48].

Despite the many advantages presented by DEP-based enrichment methods, there are also some limitations, such as low sample volumes that are processed in a non-continuous manner (Table1). Furthermore, the dielectric characteristics of cells can gradually change due to ion leakage; this requires the isolation to be completed within a short period of time after the sample processing starts [49]. In addition, the electric conductivity of the medium used must be low, which is not achievable for all samples studied.

Methods based on biological properties

Antibody-based CTC isolation takes advantage of highly specific affinity reactions between capture antibodies and the target antigens present on the cells of interest. CTCs can be captured directly (positive selection) or indirectly (negative selection). Various antigens have been used to detect or isolate CTCs. The most commonly used antibody is EpCAM as it is expressed in all epithelial cells but is absent from blood cells [13, 50]. However, the universality of EpCAM may be reduced when carcinoma cells have undergone the EMT process or when detecting tumor cells of mesenchymal origin.

Results from our laboratory have revealed the presence of EpCAM-expressing and non-expressing CTCs after the injection of either EpCAM expressing or non-

expressing tumoral cells in mouse paratibias (Supplemental Figure 2). Several organ- or tumor-specific markers, such as CEA, EGFR, PSA, HER-2, MUC-1, EphB4, IGF-1R, cadherin-11 and CSV have also been reported for antibody-based isolation of CTCs (Supplemental Table 1).

Immunoaffinity-based CTC isolation is based on antibody-conjugated magnetic nanoparticles or microbeads that often bind to a specific surface antigen [51, 52]. After antigen-antibody interaction, the sample is exposed to a non-uniform magnetic field to capture labeled cells. This method can attain high recovery and purity rates, with single-step detection and isolation of CTCs [51, 53]. The performance of the immunomagnetic method depends directly on both the expression and specificity of the target antigen, as well as on the binding quality of the associated antibody, the efficiency of the immunomagnetic labeling process and magnetic particles, and the separation mechanism designed to isolate labeled cells. A “cocktail” of antibodies targeting multiple antigens can also be used to partially overcome the lack of specificity of current tumor markers [51, 54]. Another approach is negative isolation of CTCs by first lysing erythrocytes and using specific markers to magnetically deplete leukocytes. CD45 is the most frequently used marker for leukocyte depletion. The RosetteSep[®] (STEMCELL Tech, Cambridge, United Kingdom), is a CTC negative selection system based on a mixture of antibodies that specifically crosslink red blood cells to each other and to white blood cells, forming cell rosettes consisting of multiple red and white blood cells. Due to the higher density of these clusters, they can effectively be separated from CTCs by a single centrifugation step. Negative selection methods are completely independent with regard to CTC phenotype, so they are not biased by a particular CTC marker. Negative selection also leaves CTCs untouched, which may result in higher viability. To achieve an acceptable degree of CTC purity, this separation method

requires a very high specificity to remove all the leukocytes and needs to avoid non-specific CTC binding. The binding between primary antibody and magnetic particles can be a direct (single-step) or indirect (two-step) method. The latter is composed of secondary antibodies that are already bound to magnetic particles and can specifically bind to an epitope on the primary antibody, potentially reaching higher labeling efficiency. This indirect approach shows a 15-fold increase in labeling efficiency compared to direct methods [55].

Regarding the use of the magnetic separation procedure to recover labeled cells, there are many different alternatives. In the batch separation approach, the whole labeled sample is subjected to a magnetic field at once, resulting in the migration of labeled cells to the regions of higher magnetic frequency [56]. The EasySep™ system (STEMCELL Tech, Cambridge, United Kingdom), MojoSort™ (Biolegend, San Diego, CA) and Dynabeads® (ThermoFisher, Waltham, Massachusetts) are based on this principle. Variations of these systems have been developed to increase the processed volume. Thus, continuous-flow separation can be used in which the sample is continuously fed through the separation module. This module can have an activated filter to capture and retain the labeled cells, like the commercially available MACS® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and MagniSort™ (eBioscience, San Diego, CA). Alternatively, the magnetophoresis mode can be used to selectively manipulate the direction of labeled cells within the flow and collect them at designated outputs [57, 58]. Reported recovery rates using these magnetic enrichment systems have shown significant variations (10–90%) [59–60]. This variation can be explained because the magnetic gradient generated by the separation structure can only attract labeled cells within a limited distance. The MagSweeper® system is a proposed (Figure 5A) solution [61] that uses a robotic arm equipped with a magnetic rod that binds labeled cells. This

was initially demonstrated for the recovery EpCAM-positive cells but can be adapted for other CTC markers. Recovery rates of 60% using this device have been reported [54].

Micro-scale separation devices have been also developed. Isolation efficiency in an immunomagnetic microfluidic chip is mainly governed by an equilibrium between hydrodynamic and magnetic forces acting on the labeled cells [62]. Hoshino *et al.* described immunomagnetic capture system for CTCs, based on a microchannel on top of a stack of permanent magnets (Figure 5B). As the sample flows into the microchannel, the magnetic gradient attracts the labeled cells. Recovery rates around 86% have been attained with this system [63].

Finally, CTCs can be recovered using adhesion-based methods that exploit the ability of CTCs to bind to a surface whose biochemical and topographical properties have been modified without the need to label the cells. In static adhesion-based assays, the sample is first incubated on the capture surface. Non-adherent, supposedly non-target cells are washed off, leaving the CTCs attached to the surface. On the basis of this approach, cell adhesion matrix (CAM) has been used to detect and isolate the most invasive CTCs from patients with metastatic and local carcinomas of different origins [64, 65]. Microfluidic adhesion-based devices consist of microchannels coated with an antibody against CTCs. Their design determines both the efficiency of the cell binding, and the recovery rate by influencing the flow rate [66, 67]. Among these devices, OnQChip™ (On-Q-ity, Waltham, MA) and the CEE™ chip (Biocept Laboratories, San Diego, CA) are two commercialized microfluidic devices that have incorporated 3D structures (microposts) to increase the effective surface, thus promoting cell adhesion (Figure 5C). The first combines antibody affinity and size selection for the capture of CTCs and the second is based in immunoaffinity. In this field, Hughes *et al.* have

developed, for instance, a microfluidic system based on the binding of E-selectin, a molecule present in the endothelium on to which CTCs adhere prior to their extravasation [68]. This approach has attained high flow rates compared to the other adhesion-based methods (4.8 mL/h) and approximately 50% of capture efficiency. Interestingly, this device demonstrated a higher efficiency based on the number of CTCs isolated, compared to the CellSearch® system [68].

Concluding remarks

We have highlighted the non-EpCAM-based methods for CTC enrichment/isolation. The major advantage of these techniques is that they can enrich for CTCs that do not have EpCAM expression. However, many challenges associated with current methodologies must be faced, such as the need to improve purity and recovery rates, throughput, cell viability after recovery, and enrichment rates.

It would be beneficial to identify properties exclusive to CTCs, which may take the form of a single “master” marker or a combination of antibodies able to recognize all the CTCs present in the sample. Moreover, it would be desirable that those properties or markers were able to distinguish between metastatic and non-metastatic CTCs. Unfortunately, current knowledge does not make it possible to clearly identify and classify CTCs. This information is of most importance in clinics, for the prognosis of the disease, treatment decisions or the effectiveness of the treatment applied.

Despite the numerous methods for isolating CTCs described in the literature, some are still at the proof of concept stage with evidences only in cultured cells. The main drawback is that cell lines do not reflect effectively CTCs in a natural biological fluid, especially in terms of heterogeneity [69]. It would be interesting to develop new cell lines that exhibit the genomic and transcriptomic heterogeneity of cancer cell lines.

398 Recently, Alix-Panabières *et al.* [70] and Haber *et al.* [71] have reported the isolation of
399 CTCs and their growth in culture for the establishment as a cell line to examine tumor
400 heterogeneity. Another important point is the necessary sample volume required for
401 CTC isolation. In most cases, the inability to process whole blood is due to high cell
402 concentration or the necessity for reducing sample volume due to the device's capacity.
403 A frequently proposed solution is the dilution of samples; however, this is not ideal
404 since dilution reduces the probability of CTC capture and the prolonged enrichment
405 time compromises cell viability. In addition, the biological characteristics of the cells
406 can be altered by the composition of the dilution buffer.

407 When using immunologically-based enrichment methods, the wide range of
408 phenotypes presented by CTCs make it necessary to use specific cocktails for cell
409 surface epithelial and mesenchymal markers, that not cross-react with other blood cells
410 [72]. Yokobori *et al.* described Plastin 3 as a good alternative for avoiding the use of
411 large cocktails of antibodies, because this marker is not downregulated in CTCs during
412 their EMT and is not expressed in blood cells [73]. Although positive selection is very
413 specific and a high purity can be obtained, the presence of some uncharacterized CTCs
414 in each individual blood sample should be taken into consideration. This can be avoided
415 by negative selection, in which the blood sample is depleted of leukocytes using
416 antibodies against CD45 and other leukocyte antigens (not expressed on carcinomas or
417 other solid tumors). However, cytokeratin⁺ and CD45⁺ sub-cell populations have been
418 described and may be related to various artifacts such as cell doublets or non-specific
419 antibody bindings [74] or circulating cancer-associated macrophage-like cells [75]. The
420 role of EMT in tumor cell dissemination stimulates the development of technologies
421 based on the depletion of normal CD45⁺ hematopoietic cells to limit loss of CTCs with

high phenotypic plasticity. However, it should be noted that not all CD45⁺ cells in the blood are tumor cells (e.g., circulating endothelial cells) [11].

In the last decade, the strong interest of CTCs has accelerated the development of numerous isolation technologies based on EpCAM independent methods. Technologies based on physical approaches (density gradient centrifugation, microfiltration, microfluidics, dielectrophoresis) or biological properties of CTCs (e.g. membranous markers) have been demonstrated. However, further improvements in pre-enrichment steps will improve the capture and characterization of these cells.

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FIGURE AND TABLE LEGENDS

Figure 1. Methods for CTC isolation from whole blood. 1) Methods based on biological properties: immunoaffinity-based techniques target specific markers to selectively enrich CTCs or leukocyte depletion. 2) Physical properties such as size, deformability, density and electrical properties can also be used to separate CTCs from blood cells.

Figure 2. Microfiltration devices for CTC enrichment. A) Dead end filtration; B) 3D membrane microfilter. The smaller cells can easily traverse the gap while the large cells (e.g., tumor cells) will be trapped. Two types of force are exerted in the trapped cell such that force is caused by hydrodynamic pressure from the top and supporting force from the bottom membrane; C) 2D membrane slot filter design; D) Bead pack based filtration. The microchannel entrance is blocked by packing large sized beads. Different bead sizes were used to implement a blood/plasma separator at the inlet of the microchannel. When whole blood was dropped into the inlet of the microchannel, the structure allowed for the capillary flow of blood through the hetero-packed beads. During this movement of blood, the red blood cells pass through small pores while large cells such as CTCs are blocked from flowing into the channel.

Figure 3. Microfluidic devices for isolating CTCs. A) Parsortix (Angle). The patented microfluidic technology inside a cassette captures CTCs based on their less deformability and larger size compared to other blood components; Left diagram (plan view) and right diagram (cross section to see in details the device). B) ClearCell[®] FX (Clearbridge Biomedics). The inertial and centrifugal forces transport the smaller red

and white blood cells along the channel's outer wall and the larger CTCs along the inner wall recovering both fractions in different channels of the system; C) CTCi-chip technology, combines continuous deterministic lateral displacement (DLD) for size-based separation of blood cells, inertial focusing for precise positioning of cells in a microchannel and microfluidic magnetoforesis for immunomagnetic depletion of white blood cells.

Figure 4. Dielectrophoretic based approaches. A) ApoStream™ from ApoCell (adapted from [47]); B) DepArray™ technology from Silicon Biosystems (<http://www.siliconbiosystems.com/depparray-system>).

Figure 5. Antibody-based CTC isolation approaches. A) MagSweeper (figure adapted from [61]). Magnetic beads were coated with an antibody targeting surface markers and mixed into blood samples to bind cancer cells, which are captured with the magnetic rod. After several washings, the cells are extracted using a magnetic source. B) Microchip –based immunomagnetic assay. The sample is pumped in continuously through the microchannel, causing non-captured blood cells to exit the chip, whereas CTCs are retained due to the magnetic force. C) Diagram representation of OnCCChip™ (On-Q-ity) and CEE™ (Biocept) devices. These cell enrichment technologies exploit the placement of posts and flow rates through mathematical modeling to enhance isolation, and capture CTCs within a microfluidic channel.

747 **Table 1. Major advantages and disadvantages of CTCs enrichment methods.**

Method	Advantages	Disadvantages
Density gradient centrifugation	Inexpensive Reliable	Loss of large CTCs and cell aggregates Low purity Additional enrichment techniques required
Microfiltration	Rapid processing of large volumes High efficiency	Low purity Membrane clogging Different size of CTCs Difficulties to detach CTCs from the filter
Microfluidics	Excellent purity High capture rates Little cell disturbance	Long-time consuming Sample pre-processing requirement to reduce volume
Dielectrophoresis	Single cell isolation High cell viability High efficiency	Limited volume Low purity in some devices Cell electrical properties can be affected during the procedure Large number of parameters must be controlled simultaneously
Immunoaffinity based methods	High recovery High purity rates High cell viability using negative selection	Lack of cancer specific markers Heterogeneous expression of markers in cells Problems with the antibody affinity or specificity

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Figure 1

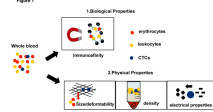


Figure 2

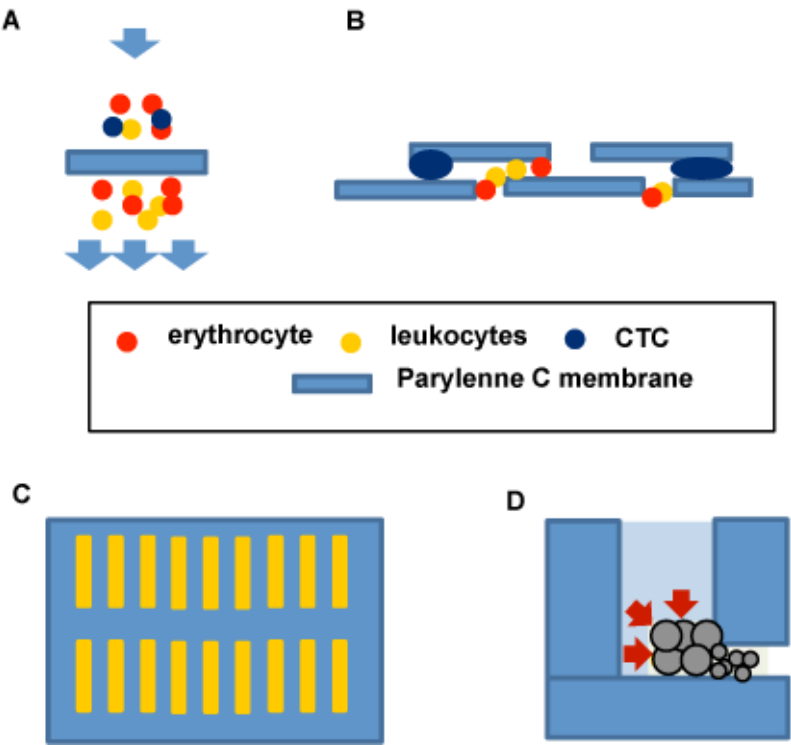
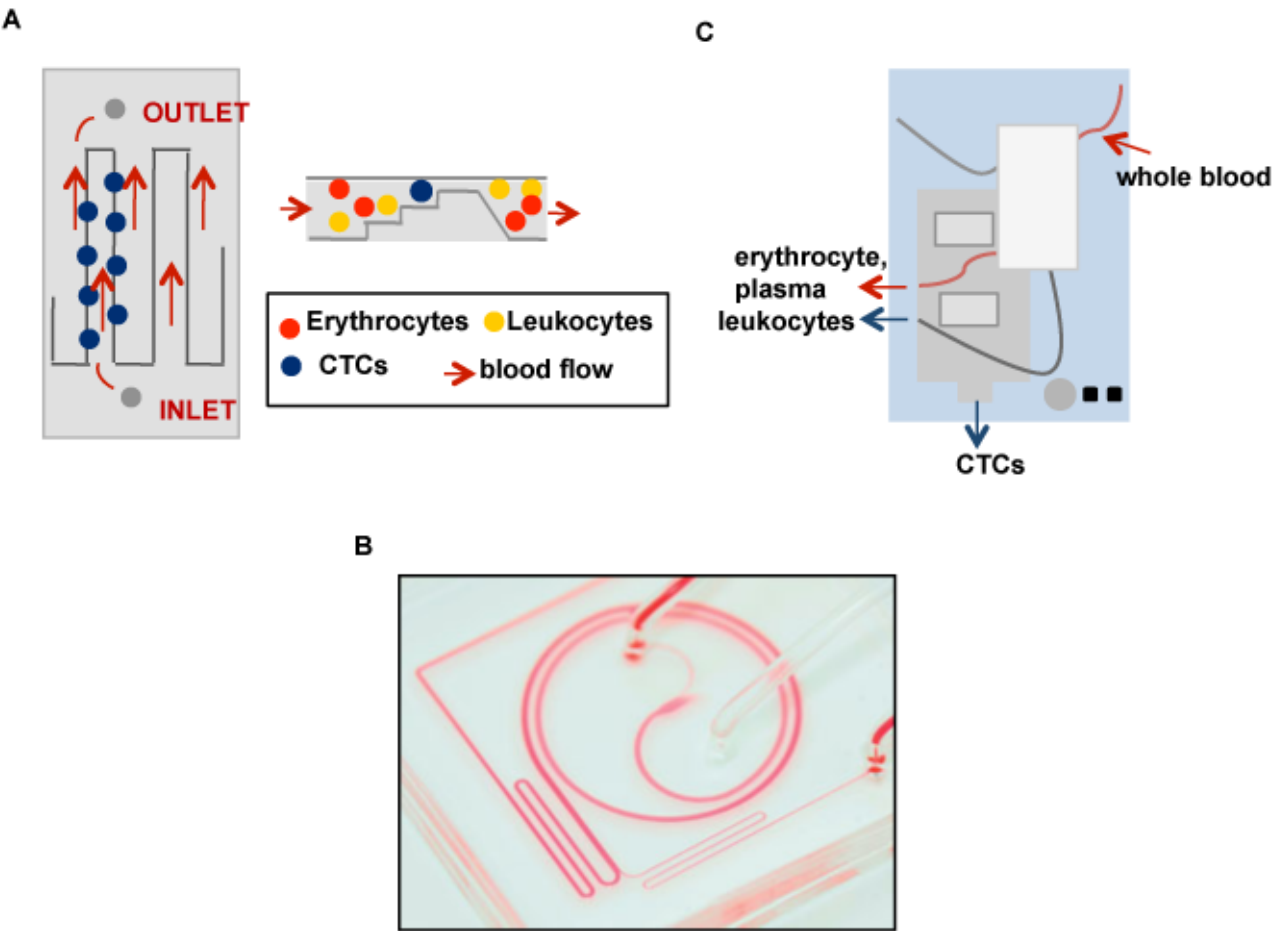
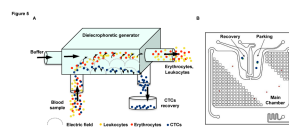
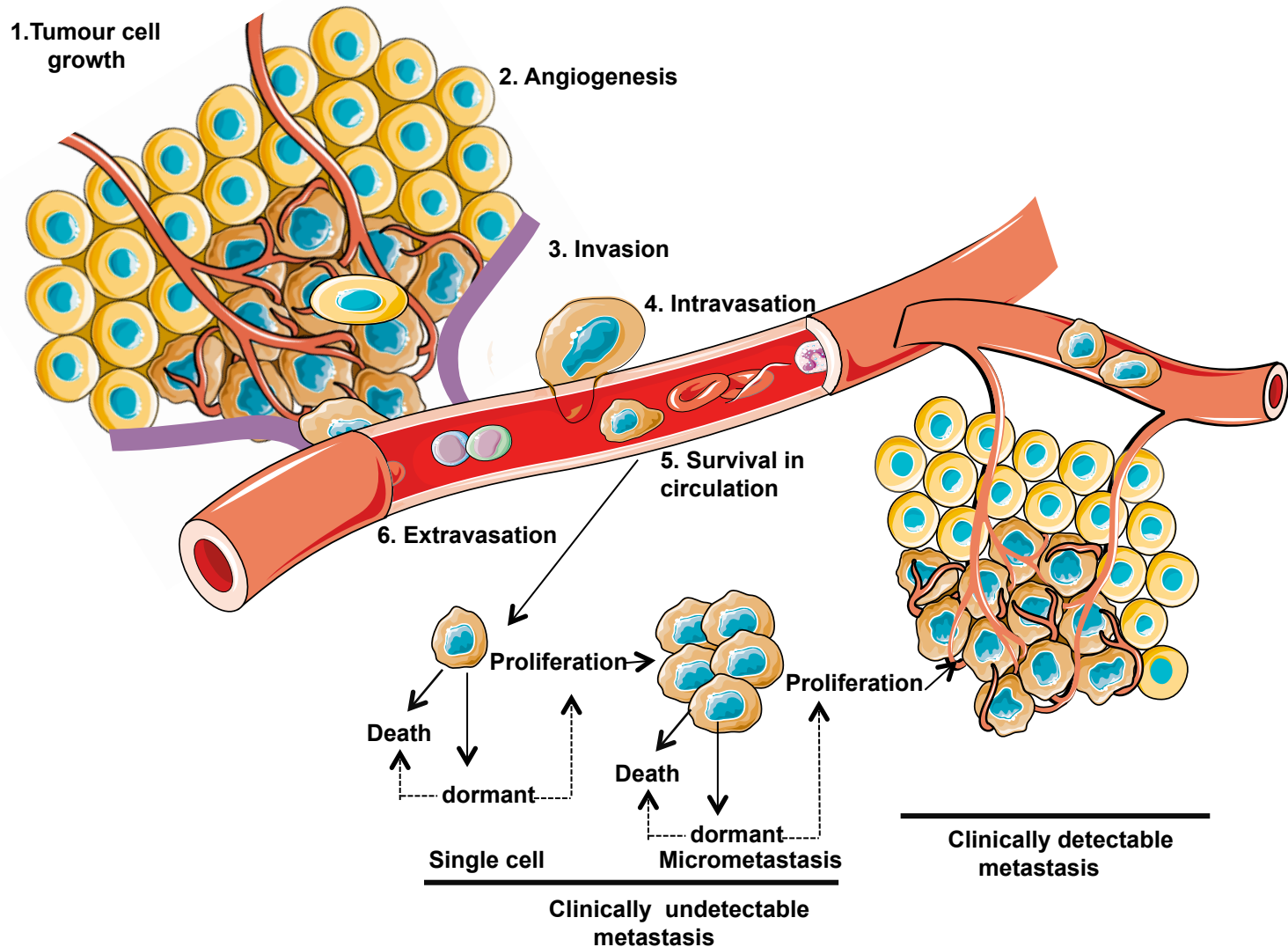


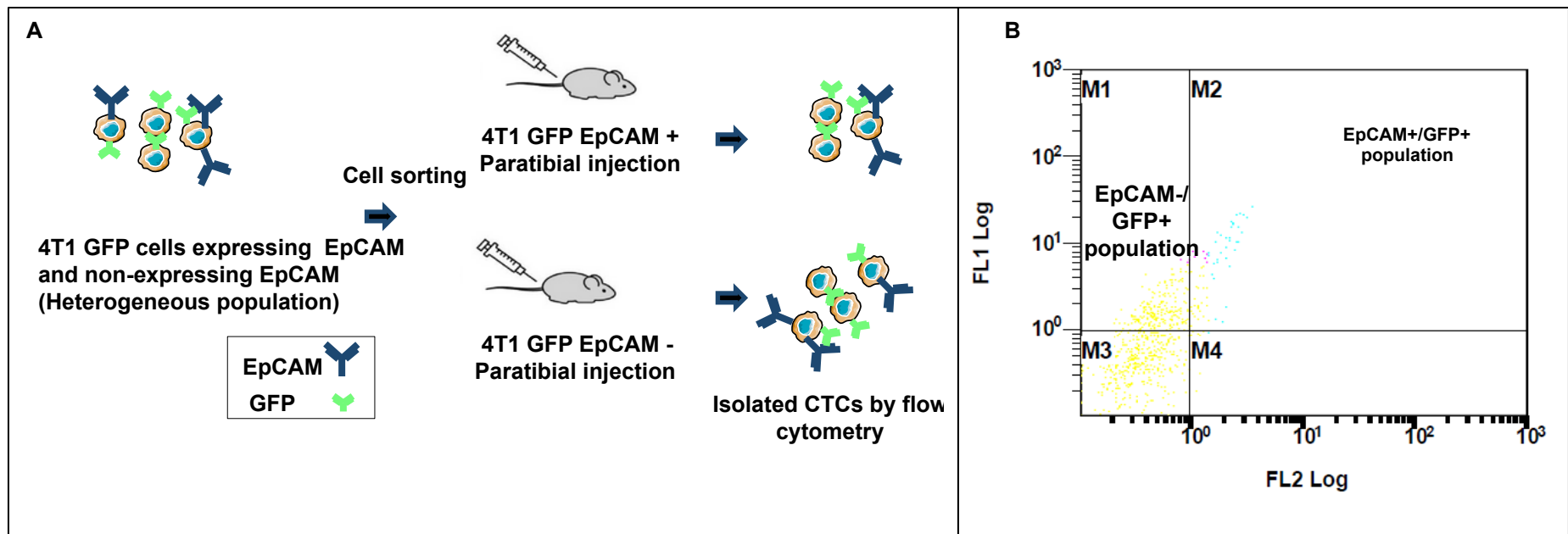
Figure 3







Supplemental Figure 1. Overview of the key molecular events in metastasis. During the metastatic process, cancer cells proceed through a series of limiting steps to form a secondary tumor. In the initial stages, tumour growth (1) is associated with neoangiogenesis (2). Cancer cells detach from the primary tumor mass, invade adjacent tissues (3) and then enter the lymphatic or circulatory systems (4), which transport them to distant sites (5) from where they extravasate (6) and enter the surrounding microenvironment. At this point, specific factors determine whether the cells will proliferate to form a clinically detectable metastasis or if they are to remain dormant as single cells or micrometastases.



Supplemental Figure 2. Flow-through experiment with paratibial injection of 4T1 cells in Nude mice. A) 4T1 GFP cells were sorted by EpCAM expression. We inoculated 1.5 million of 4T1GFP EpCAM⁺ or EpCAM⁻ in paratibias site of 8 NUDE mice respectively. After 25 days we collected blood from animals and we analyzed using flow-cytometry the presence of CTCs in each mouse as, well as EpCAM expression; B) Cytometry plot showing the coexistence of EpCAM⁺/GFP⁺ and EpCAM⁻/GFP⁺ CTCs in blood after 25 days of the injection.