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MICROFLUIDIC: AN INNOVATIVE TOOL FOREFFICIENT CELL SORTING

Julien Autebert[a], Benoit Coudert[a], François-Clément Bidard[b], Jean-Yves Pierga[b], Stéphanie Descroix[a], Laurent Malaquin[a], and Jean-Louis Viovy[a,1]

[a] Institut Curie, Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Unité Mixte de Recherche 168, 75005 Paris, France;

[b]Institut Curie, Département d'Oncologie Médicale, 75005 Paris, France.

[1]To whom correspondence should be addressed. E-mail: jean-louis.viovy@curie.frmailto:.

Abstract

At first mostly dedicated to molecular analysis, microfluidic systems are rapidly expanding their range of applications towards cell biology, thanks to their ability to control the mechanical, biological and fluidic environment at the scale of the cells. A number of new concepts based on microfluidics were indeed proposed in the last ten years for cell sorting. For many of these concepts, progress remains to be done regarding automation, standardization, or throughput, but it is now clear that microfluidics will have a major contribution to the field, from fundamental research to point-of-care diagnosis. We present here an overview of cells sorting in microfluidics, with an emphasis on circulating tumor cells. Sorting principles are classified in two main categories, methods based on physical properties of the cells, such as size, deformability, electric or optical properties, and methods based on biomolecular properties, notably specific surface antigens. We document potential applications, discuss the main advantages and limitations of different approaches, and tentatively outline the main remaining challenges in this fast evolving field.

INTRODUCTION

Cells sorting, identification and countinghave been for decades a widely used and standard tool of clinical diagnosis. Routine applications essentially consist in enumerating the different populations of red and white blood cells. They rely on Coulter counter (based on the change of conductivity of a micropore upon its crossing by a cell), flow cytometry (based on the optical detection of cells encapsulated at high speed in droplets passing in front of a detector) or direct cytology (based on direct observation of cells spread on a slide, or centrifuged onto it). These tools have also been extensively used in research, e.g. for controlling and studying cell cultures. In recent years, however, the development of genomics and cell biology have expanded the type of questions and investigations involving cells sorting and identification, and also the number and complexity of criteria of interest. These new demands have fostered new technical challenges. Also, some domains such as oncology, stem cells research or infectious disease, have raised interest for the identification of “rare cells”, i.e. cells present as a very small subpopulation in a large amount of surrounding cells (e.g. blood cells). In this context, the direct screening of the whole cell population becomes impractical, although a regular increase in the throughput of flow cytometry continuously expands the range of applications of this technology. Thus, rare cells analysis most often involves a pre-sorting. Classical sorting methods involve centrifugation, density gradient(based on a physical property, the density), filtration (based on another physical property, size or deformability) or immunomagnetic selection (based on the presence of a specific antigen at the cell’s surface). Flow cytometry may also be used for cells sorting, but presents an insufficient sensitivity for rare cells and in sorting mode the throughput is seriously diminished (up to 70 000 events per second, see section 1). All these techniques, however, show limitations when confronted to extremely rare cells (such as circulating tumor cells) or when more complex criteria, such as genetic analysis of single cells, are involved.

With the expansion of microfluidics,cells sorting enters a new era: the previous decade has seen a progressive improvement of performance of devices based on a relatively limited number of principles. Microfluidics, in contrast, is currently in an emerging and highly creative mode, in which the number of new concepts, and concomitantly the number of publications increases drastically (see Fig. 1). A strong advantage of microfluidics in these applications is the possibility to structure space or flows at a scale commensurate with that of single cells. Through the “lab on chip” concept, microfluidics also allows high and user-friendly automation, reduction of sample treatment time on-chip (by integrating multiple steps in adjacent chambers), reagents consumption and chemical waste. Microfluidics is therefore of tremendous interest and many innovative systems were recently developed for mammalian cells sorting. The selection of a

sorting system first depends on choosing between a “label-free” method (i.e. methods that rely on an intrinsic property of the cell like size or deformability) and a biochemically-enhanced method. Depending on the type of target cell and the information available, one will choose one approach oranother, but in many envisioned applications, the situation is still evolving and the “right choice” is hard to make.

We hope, in this review, to provide the reader with some help in this direction. Wewill first give a quick overview of potential cells sorting devices in current biology, with an emphasis on rare cells (especially circulating tumor cells), and a glimpse on non-microfluidic existing methods. Then we will see in more details how cells can be sorted only on physical criteria (what is called “label-free” sorting). Finally, we will see that, even if many papers have been reported on“label-free sorting”, itstill lacks proofs of its potential clinical usefulness. Many microfluidics cells sorting methods, especially the ones with clinical applications are thus based on biochemical criteria such as antibody expression.

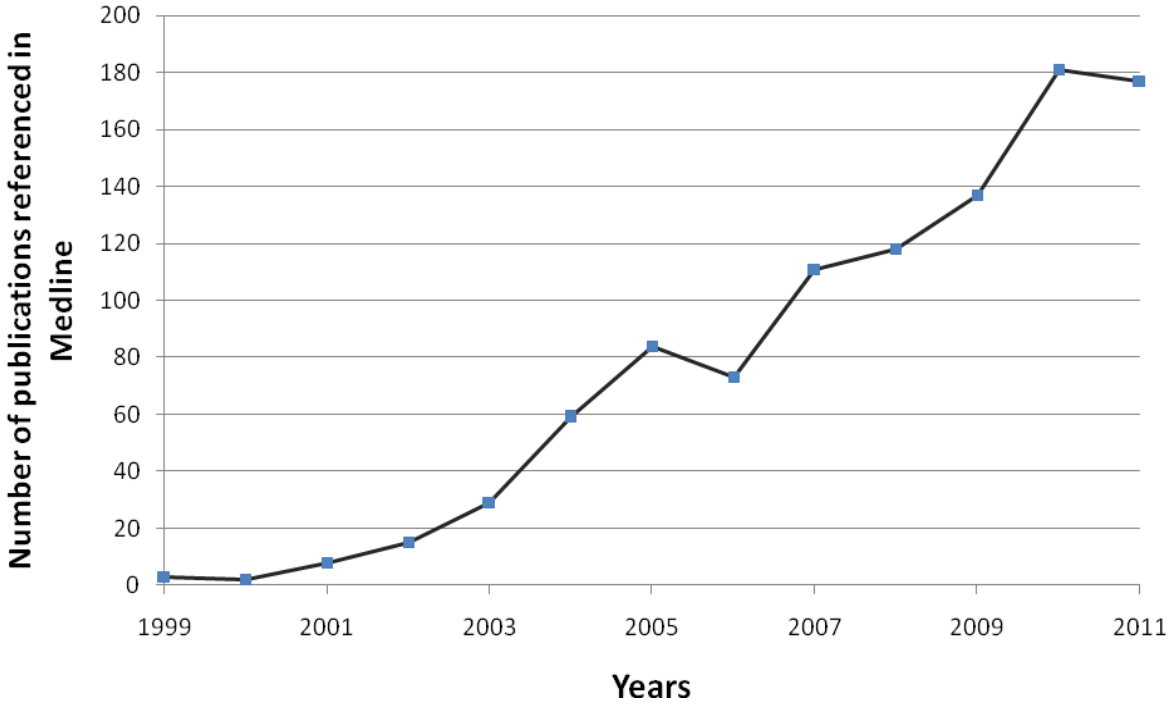

Fig 1. Number of publication referenced under “microfluidic cell sorting” each year, from 1999 to 2011 and indexed in Medline.

1 BIOLOGICAL AND TECHNICAL CHALLENGES

Handling cells in biological sample is challenging, moreover when the cells of interest are rare (such as CTC or fetal cells). We briefly describe here what we consider as biological and technical challenges, along with the non-microfluidic methods that are used extensively.

1.1 BIOLOGICAL ISSUES

1.1.1 BLOODCELLS

Blood is a specialized fluid that delivers necessary substances such as nutrients and oxygen to the cells and tissues and transports metabolic products away from those same cells and tissues. It contains a huge amount of information because it is draining every single part of the body. Whole blood analysis is of prime interest in diagnosis, therapeutics and research. However, it is a very complex fluid, constituted at 55% of plasma, 44% of erythrocytes (red blood cells, RBCs) and less than 1% of leukocytes (white blood cells, WBCs). A milliliter of blood contains billions of RBCs and millions of WBCs. Selecting a specific subpopulation of blood cells can be a technical challenge. Blood can be technically difficult to handle too, as the use of anticoagulant or fixatives can alter cell viability. Objectives of blood cells subpopulation capture and existing solutions have been described by Toner and Irimia[1].

1.1.2 CIRCULATING TUMOR CELLS

More than 90 % of cancer deaths are due to metastases. It is expected that most distant metastases are created by cells escaped from the primary cancer, and transiently circulating in the blood as "circulating tumor cells" or CTC. Several clinical studies reported that CTC detection in patients with localized primary tumor was indeed correlated with the onset of later metastatic relapse[2–4]. CTC levels in the blood stream were also correlated in several tumor types with the survival of patients with overt metastases[5–7].In addition to this predictive power, CTCs can also be used as a "liquid biopsy", in order to perform a longitudinal follow-up of patients, a molecular characterization of the circulating tumor cells, and prescribe the most efficient treatment on the basis of this molecular characterization[8,9]. This is, however, a very challenging task from an analytical point of view, since clinically relevant concentrations of CTCs in blood of patients can range from less than 1 CTC per 10 ml to several hundreds of CTCs per ml. The technology currently considered as "state of the art", i.e. the Veridex CellSearch(R) (Raritan, NJ), is based on an automated immunomagnetic sorting (i.e. an immunoselective enrichment using magnetic antibodies-coated beads). It typically finds CTCs in only 60% of metastatic patients, and in30% of non-metastatic patients who developed later on metastases. These rates may vary according to tumor type. Moreover, this technique only gives simultaneous access to a limited number of biomarkers. Thus, considerable efforts are currently dedicated to the development of new tools able to improve the sensitivity of CTCsdetection, as well as the CTCsmolecular typing. The rareness of CTCs makes their separation a technical

challenge, as described in a recent review by Jaap den Toonder[10]. As CTCs are so few, large fluid volumes must be handled, and cell integrity of the CTCs must be maintained throughthe whole selection process. Another problem arises from the variations of size, deformability and molecular characteristics of what is currently called “CTCs”. Indeed, a recent consortium[11]agreed that the “circulating tumor cells” denomination might have to evolve to meet the diversity of cells observed by research groups over the world. Finally, as CTCs might be used in clinics to guide therapeutics, numerous difficulties come from the sample conservation and the need for a highly automated and reproducible process fitting the regulatory requirements of “in vitro diagnostics” regulations[12]. In terms of requirements, any device dedicated to rare cells sorting should allow the highestcapture rate (most available methods are over 50%) or be ableto capture one cell in a few millions,but moreover require an excellent specificity (more than 99.9% rejection of unwanted cells) is required to be very selectiveand obtain a very pure sample. Additionally, the device should be able to process classic blood samples tubes(7.5 ml) in a time frame of a few hours. In terms of sorting throughput, the device should at least be able to sort 10 000 cells per second to cope with the 50 millions of white blood cells present in blood (after exclusion of red blood cellby lysis or density gradient separation).

1.1.3 Circulating Endothelial Cells and Circulating Endothelial Progenitors

An increasing number of circulating endothelial cells (CECs) and progenitors (CEPs) have been reported in various pathological conditions that involve tissue ischemia, including acute heart attack, cerebrovascular accident or cancer[13]. When a tumor reaches a size of 1 to 2 mm^2, angiogenesis becomes necessary to promote growth, as blood supply is a prerequisite for cells proliferation[14]. Indeed, it has been recently shown that the comparison of CECs levels at baselineand after therapeutics, as well as the CECs quantity variation may predict objective response rate and progression-free survivalin metastatic colorectal cancer[15–17]. However, CECs are not easy to enumerate for multiple reasons. First, as CTCs, the sorting assays have to deal with the low number of CECs in peripheral blood, ranging from 0 to 20 per ml in healthy donors. Second, traumatically detached CECs from the vessels during puncture can compromise the significance of the analysis. Last but not least, there is no specific surface markers of CECs and current assays rely on multiple markers (such as CD146 or VEGFR-2) which might be expressed by other cells[18]. Current methods use systems such as flow cytometry with four channels to count CECs specifically[19]. Finally, circulating endothelial progenitors (CEP), that originate from the bone marrow and contribute to the angiogenesis, might also potentially be an interesting biomarker for cancer metastases, as VEGFR2 positive CEP correlates with metastatic disease[20], but these cells are more sparse than CECs.

1.1.4 Fetal cells

Due to the risks of fetal loss associated with prenatal diagnosis invasive procedures (biopsy of chorial villosities, puncture of amniotic fluid or fetal blood), the search of non-invasive prenatal diagnosis for genetic and chromosomal diseases raises strong interest. The presence of fetal cells in woman's blood during pregnancy is known since the end of the XIX century, but the first scientific proof available came from the discovery of chromosome Y carrying lymphocytes in the blood of a mother carrying a boy[21]. During pregnancy, red blood cells pass through the placental barrier but mostly all of them are not nucleated and thus not suitable for diagnosis. However, some fetal erythroblast, lymphocytes and stem cells might be found in the mother's blood[22]. Using a triple density gradient and MACS (see section 1.2), Gänshirt et al.[23]showed that the quantity of fetal nucleated red blood cells increases during gestation, ranging from 100 (week 6) to 1000 cells (term) per 40 mL of maternal blood.

1.2 NON-MICROFLUIDIC EXISTING METHODS

Among all the non-microfluidic methods developed for cells separation, a few stand out in terms of applications and performance. We provide here ashort overview of these methods, since this is not the main topic of this review. We nevertheless provide references to additional more exhaustive sources, in the hope they will help the interested reader to go beyond the limited information provided here.

1.2.1 MEMBRANES FILTRATION

Membranes are used to capture cells using size and deformability as discriminating parameters. They can be made of pure nylon fibers (CellMicroSieves by Biodesign of NY), or polycarbonate (Isolation by Size of Epithelial Tumor cells - ISET[24]). ISET is composed of a polycarbonate membrane with calibrated 8µmdiameter, cylindrical pores. Theoretically, by flushing the blood through this membrane, red blood cells should pass through easily, white blood cells should deform to enter the pores and epithelial tumor cells,which are supposed to be more rigid and bigger, won'tpass through.Membrane-based devices show interesting results regarding CTCs capture as theydon't rely on antigen expression, but unfortunately they are subject to clogging and cells recovery on the membrane can be difficult. Also, the need to process each membrane individually for identification makes the overall process cumbersome.

1.2.2 CENTRIFUGATION-BASED SORTING

Centrifugation is widely used for the separation of blood constituents. The method relies on the difference of density among the different cell types in blood. Typical centrifugation can occur on raw blood or on blood layered on a ficoll preparation[25]. After centrifugation, red blood cells lay on the bottom of the tube while nucleated cells form a ring just over it, with plasma on top. The advantage of ficoll is that it creates an artificial barrier between red blood cells and nucleated cells, avoiding most of the contamination, and it has already been successfully

applied to CTCs sorting[26]. However, centrifugation may apply on cells stresses that modify their phenotype. The recovery of cells from the “ring” is a delicate process requiring experienced hands, and some variants using microgels were developed for some applications (see e.g. BD diagnostics). An interesting alternative to conventional centrifugation was proposed by Stem Cells Research: RosetteSep^{TM}. In this approach, antibodies complexes aggregate leucocytes and red blood cells, creating large objects that sediment rapidly and in principle leaves only non-hematopoietic cellsin the supernatant. This enrichment method is used as a first sample processing step for different further applications such as the Epispot technology[27].

1.2.3 Fluorescence Activated Cell Sorting

The use of a flow cytometer coupled to a sorting device shows excellent sorting performance. Each cellis individually put into a droplet using a nozzle and passesin the beam of one or multiple laser beams at high speed[28]. Cameras then detect the fluorescence emitted by the cell (if stained) as well as forward and side scatter signals.Depending on sorting criteria, each droplet is electrically charged and then sorted usingelectrostatic deflection[29].Current state of the art devices typically use up to 7 lasers, can sort 6 different types of cell and manageup to 70.000 sorting decisions per second (MoFlo Astrios^{TM}, Beckman Coulter). A serious limitation to the use of these systems is their price and complexity. They may be subject to cross-contamination, clogging in the nozzle and high reagent consumption. Additionally, these high end systems remain too limited regarding their throughput for direct separation of CTCs from whole blood as it would take hundreds of hours to sort the billions of red blood cells present in a sample tube. Lysis or pre-treatment of the sample can be performed to decrease the sample analysis time down to a few hours or minutes by removing the red blood cells, but with an associated risk to reduce the capture yield.

1.2.4 Magnetic Activated Cell Sorting

Magnetic activated cell sorting uses magnetic particles coated with antibody to target specific cell membrane antigens[30]. Magnetic particles can be directly coated with the antibody or indirectly via, for example, a biotin/streptavidin couple. After their coating, particles are mixed and incubated with the cells. The cell/particle couple (or cell/particles aggregate) can be retrieved specifically by using a magnet while unwanted cells are washed away. The first commercially developed system (MACS by Miltenyi) used high magnetic gradient columns[31].While this method showed interesting performance (10^5-fold enrichment, 20 to 40 mL of blood processed in a few hours) especially regarding sorting of CTCs[32],but the purification efficiency necessary for CTCs is still too low. By using a mechanical magnetic sweeper[33] (a magnet that moves slowly into the sample), enrichment rate was increased from a 10^5-fold enrichment to a 10^8-fold one. To furtherimprove this enrichment rate and to develop

the first commercially available system for CTCs detection, the Cellsearch® system (Veridex) was introduced. In a tube containing fixatives, the epithelial cancer cells are labeled using nanoparticles coated with anti-EpCAM antibodies. In a second step, the sample is flushed into a cartridge where a magnet aligns the magnetic nanoparticles on the surface. Imaging can then be performed with a low-resolution scanner. Manual counting is the final step in which an operator counts the cells based on size and staining criteria. As the first automated, reproducible and standardized system sensitive enough to observe CTCs, it showed convincing results regarding correlation of the number of CTCs in blood and patient's disease progression and it is now considered as the current standard for CTCs detection in patients' blood sample[34].

2 « LABEL FREE » PHYSICAL SORTING IN MICROCHANNELS

Cells intrinsic properties available for sorting are numerous. Obviously, size is the one that first comes to mind but is not necessarily the most used due to the high polydispersity of cells size. As an example, cancer cellssorting from white blood cells in blood based on an unique size criterion is complex as circulating tumor cells and white blood cells size range overlap. Those difficulties led to creative devices based both on size and deformability, or size and shape. Others less obvious cells properties can be used for label-free sorting such as deformability, shape, density, magnetic susceptibility, compressibility, polarizability or refractive index.Two types of devices can be distinguished in the "label-free sorting" section: the ones called "passive" devices that are based on microchip channels geometry and hydrodynamic forces and the ones called "active" devices that rely on an external force field. In 2010, Gosset et al.[35] presented an interesting review on label-free microfluidic cells sorting. We will focus here on major contribution in the field, along with an overview of the innovations from the last two years.

2.1 PASSIVE DEVICES

2.1.1 HYDRODYNAMIC SORTING

Hydrodynamic sorting relies on the behavior of particles in a laminar flow at low Reynolds number (i.e. the particle's center will follow a stream line). Based on this principle, sorting by size can be performed usingmicrochannelbifurcation, pinched flow fractionation or the so-called "biomimetic" behavior. Briefly, when a microchannel splits into two channels, depending on where the particle is initially located, it follows different stream lines. In the case of bifurcations, the flow velocity difference between the two bifurcations determines the particle behavior. This effect, known as Zweifach–Fung effect, is described by Yang et al[36] in a device developed to perform plasma separation from blood (see Fig 2).Pinched flow fractionation acts differently on the particle: the pinching flow force pushes the particle to the wall of the channel, moving it to a new stream line by steric exclusion. The distance of this streamline from the wall directly dependson the particle size and a widening of the channel allow to sort particles.Finally, biomimetic devices are based on the exploitation of cells behavior observed in vessels. For instance, red blood cells are concentrated on the center of a blood vessel while white blood cells are marginalized near the vessels walls. From a physical point of view, in contrast to the previously mentioned methods that seem more suitable in a dilute suspension of particles, this biomimetic method is a "crowding" effect specific of steric effects in concentrated sheared suspensions.

Regarding performance, 20 to 50 fold particles concentration rates were achieved using hydrodynamic filtration[37], as well as two-step carrier-medium exchange[38] allowing washing or treatment of cells. Using the same technique, Migita et al.[39] managed to synchronize cell cycle (based on size) to study transfection efficiency on human hepatocellular liver carcinoma cells. Hydrodynamic sorting, coupled to dielectrophoresis was used to sort epithelial cancer cells from red and white blood cells with 95% efficiency at a flow rate of 126 µl per minute[40]. By using pinched-flow fractionation, Yamada et al.[41] first achieved continuous size separation of 15 and 30um particles, which was then enhanced by asymmetrical collection channels[42]. Since then, this technique has been used for detection of single nucleotide polymorphisms, using selectively hybridized polystyrene microspheres[43], and of circulating tumor cells[44]. Interestingly, this rare cells sorting using pinched flow fractionation was achieved by combining this technique with inertial effects. Results on epithelial cells spiked into whole blood show 80% cell recovery with a throughput of 10^8 cells per minute[44]. The flow rate of this device is around 400ul per minute, thus making it one of the only hydrodynamic-oriented devices ready for rare cells sorting.On a 10 times diluted blood, operating time would require a few hours to pass a 7.5ml blood tube. Biomimetic devices are still not much exploited, but show interesting results, especially regarding whole blood filtration. Shevkoplyas et al.[45] demonstrated the possibility to sort white blood cells from blood by margination with a 10 fold enrichment rate. Recently, a biomimetic microfluidic device made for nucleated cells population enrichment starting from a sample of whole, unprocessed blood was presented[46]. This device is based on successive triangular channel expansions that mimic the postcapillaries venules expansions in human body. The current device extracts 90% of the sample nucleated cells with 45 fold enrichment in concentration, at the rate of 5nl per second.

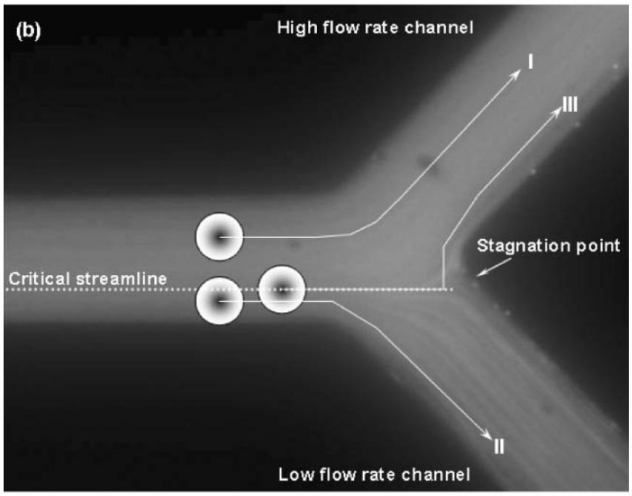

Fig 2. Representation of the critical streamline in the Zweifach–Fung effect. Particle with a center above the critical streamline will preferentially go to the high flow rate channel. Ref: [36]with permission of The Royal Society of Chemistry

2.1.2 DETERMINISTIC LATERAL DISPLACEMENT

Microfluidic systems typical dimensions range from nanometer to millimeter while most systems described are in the micrometer range. In those dimensions, the Reynolds number is lower than 1, with laminar flows and mostly no inertial effects. Reynolds number R_e is a dimensionless parameter representing the inertial to viscous forces ratio in a flow ($R_e = \frac{\rho U H}{\mu}$ where ρ is the fluid density, H is the channel dimension, U is the mean flow velocity and μ is the fluid viscosity). At this low Reynolds number, a particle entering a streamline will have a deterministic behavior (i.e. will follow that stream line), superposed with its intrinsic Brownian motion. By adding an obstacle in the course of this particle, the behavior will change depending on the size of the particle. Below a given size (called critical size), the particle will follow the main stream line around the obstacle with no displacement perpendicular to the stream line. When the particle is bigger than the critical size, it will collide into the obstacle and this lateral displacement is responsible for a stream line switch. This method is called"Deterministic Lateral Displacement" (see Fig 3). By tuning the sizeof obstacles, the gap between them and the shift in the array, particles from different sizes can be separated laterally[47].Deterministic lateral displacement was used to sort cells from whole blood[48], parasites[49] andfetal nucleated red blood cells[50].This technique is very sensitive, as observed by Huang[47] with a 10nm resolution. Results showed very good efficiency by removing 99.99% of unwanted red blood cells at aflow rateof 0.35ml/h[50].This technique is very accuratefor hard spherical particlesbut it is much more complicated to model the required space and shift of the obstacles when it comes to soft or non-spherical biological samples. This problem can be solved efficiently by tuning the cell orientation along with the obstacle array dimensions[51]. Moreover, recent work[49] showed that this method can be used for separation of non-spherical objects and in particular long-shaped parasites by tweaking the device depth parameter, and thus modifying the behavior of the non-spherical particles.The deterministic lateral displacement method showed to be efficient mainly for rigid microspheres. When applied to biological samples, sorting of cells with very different shape and size (such as red blood cells and white blood cells) was also demonstrated but it showed to be poorly sensitive to small difference in cell sizes. Moreover, microfabrication of pillars can be tricky if the height to diameter ratio exceed 5, thus leading to either big pillars of a "small" channel of a few tenths of microns. In both cases, flow rate will remain relatively low compared to what would be necessary for rare cells sorting. Finally, the separation zone needs to be long enough for efficient sorting, thus leading to relatively big and inefficient cell sorting devices.

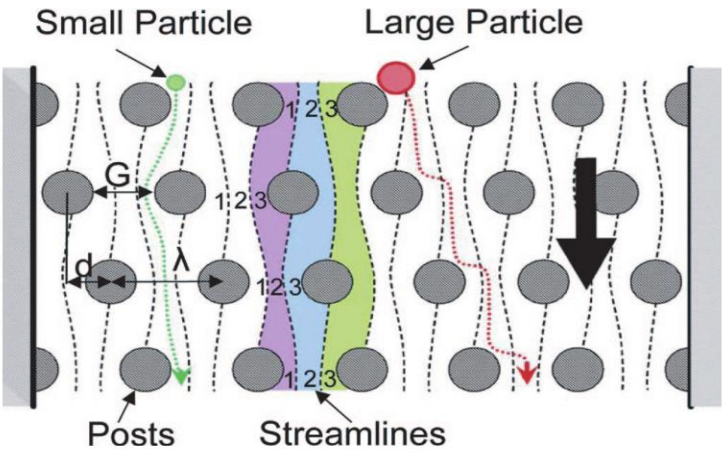

Fig 3. Separation by deterministic lateral displacement:An array of microposts with a row shift fraction of one-third is used to sort small particles (green dotted line) from large ones (red dotted line). Depending on particle's size, it will follow either one of the three streamlines (purple, blue or green). Dashed lines represent the critical streamline delimiting the particles' paths. Ref: [48]. Copyright (2006) National Academy of Sciences, USA.

2.1.3 INERTIAL SEPARATION

Inertia is generally neglected when designing microfluidic devices; flow can be considered laminar as turbulences appears for $R_e > 2000$ [52]. For intermediate Reynolds numbers (i.e. R_e ranging from 1 to 100),however, inertial effects can become significant and particles will no longer follow stream lines.In a rectangular straight channel, particles will move to preferential equilibrium points depending on their size due to a competition between the shear gradient lift and the wall effect lift.Taking advantage of the asymmetric nature of microchannel cross-section to modulate the shear rate, those equilibrium points can be tuned by modifying the channel geometry. Furthermore, using curved microchannel, the number and the position of equilibrium points can be tuned. One example of this phenomenon is the spiral channel[53]: in this kind of device, the curvature induces a force called Dean Drag force that will drag particles perpendicularly to the flow, thus enriching some flow lines at the expense of others. First used on red blood cells[54],inertial-based separationoffers an efficient size-based separation capable of isolating particles and cells ranging from 590nm[55] to 20um[56].The process can be highly-parallelized to ensure a high flow rate as described by Hur et al.[57]Bacteria separation from 20x diluted whole blood was achieved at a 15uL/min flow rate with a purity of 99.87%and a recovery rate of 62%[58]. Recent applications of inertial migration to cancer cells in diluted blood is promising[56], [59], [60] although preliminary results show that a high hematocrit (i.e. concentration of red blood cells) along with the cell softness (compared to rigid particles) tends to reduce the migration effect. A 5.4 fold enrichment of epithelial cancer cells spiked into diluted blood was observed with 96% recovery by using both cell size and deformability as

distinguishing markers[59]. Using Dean Flow Fractionation on a spiral device, Bhagat et al.[60] managed to remove 99% of RBCs and leukocytes with 90% tumor cells recovery in 20% hematocrit blood spiked with MCF7 breast cancer cells. Those results, presented at the MicroTAS 2012 conference, show that this sorting can be applied to sorting of epithelial cell lines from blood with a 4ml per hour maximum flow rate.Analyses of patient samples, along with staining validation have still to be performed. Finally, a new microfluidic technique based on the selectivity of phase partitioning and high-speed focusing capabilities of the inertial effects in flow was developed for continuous label-free sorting of particles and potentially of cells[61]. By fine-tuning the polarization between the two phases, one can create an electrostatic field that drives the particles into the most energetically favorable phase. Additional inertial focusing forces will then direct the particles towards the channel walls, resulting in an important migration and simplified sorting. While showing good results on polystyrene and polymethylmethacrylate particles, the device should be applicable to cells sorting based on their surface charge andsize. However, while the 80µl/min throughput is appealing, the potential interest of surface charge based sorting still hasto be demonstrated in a clinical situation.

2.1.4 FILTERS

As cells size is an easily observable criterion, filtration-based cell sorting is one of the most common microfluidic sorting methods. The development and standardization of microfabrication protocols allows for a very precise control of the channel geometry, a high resolution (typically micrometer) and a high reproducibility of the design.As an example, the ISET filtration system, made of a porous membrane, gives interesting results even ifthe recovery rate remains low, mainly due to the size dispersion and low density of the membrane pores. Since pores size and geometry are precisely controlled by microfabrication, microfluidic devices recover tumor cells with higher efficiency via filtration[62].Different types of filtration have been reported by Ji et al.[63] for whole blood filtration and describe mostly all types of filtration developed: weir-type, pillars, cross-flow and membrane (see Fig 4).In weir-type, pillars filtrations and membranes, clogging may occur as the flow is perpendicular to the obstacle. This problem can be overcome by the use of cross-flow[64] or by modifying the pillar geometry so that cells will be able to pass-by without clogging[65].Additionally, weir-type filtration does not promote high throughput while cross-flow shows some excellent flow rate up to167 µLper minute[66]. Interestingly, only few of the cells sorting devices using filtration relyonly on size, but more on combining size and deformability, as those two parameters have to be taken into account when choosing pores size for any filtration type.Such filtering methods have shown excellent performance in terms of sorting efficiency. More than 97% of leukocytes were depleted from whole blood using a diffusive filter at a flow rate of 5 ml per minute. This work was supplemented with an interesting theoretical modelisation[67] where the system was

described as a sum of discrete elements, leading to analysis simplification. Discrete variations in the diffuser width ensure uniform volumetric flowthrough the filter.

Fetal cells were isolated from cord blood using rows of pillars with decreasing spaces[68] , with a relatively low throughput of 0.35 mL/h, thus not suitable for a potential prenatal diagnosis where fetal cells can be as rare as a few hundreds per milliliter. Whole blood was separated into white blood cells and red blood cells with platelets using two overlapping membrane of 2.5 and 3.3 µm pore size, with 99.7% efficiency[69]. In a similar way, hematopoietic stem cells were trapped on a membrane with optimized pore size, showing a very good viability rate[70]. CTC were captured using micropillars[65], [71] or using a membrane-based device[62], [72], [73]. All of the above use size and deformability criterions and show efficiency over 80%. Finally, recent work on mammalian cell sorting (based on deformability) showed interesting results[66] regarding enrichment capacity of filters at high flow rate (over 160 µl per minute). Modelisation and study of a counter-flow unit that concentrates cell while avoiding clogging[74] show a good example of how to manage a classical filter problem.

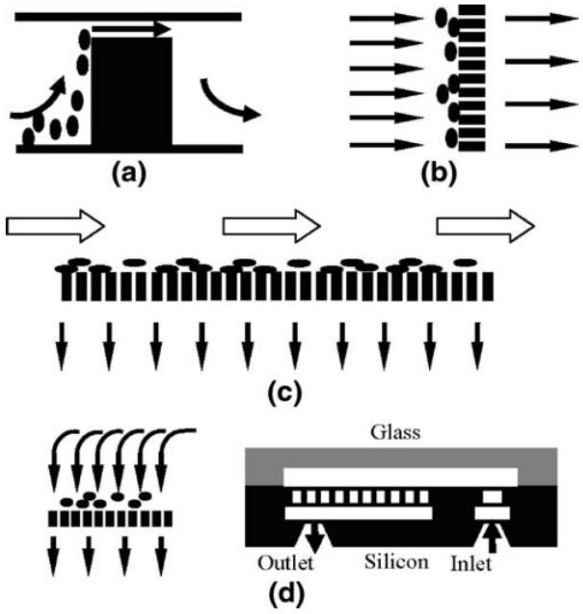

Fig 4. Different types of cell filtration devices. **(a)** Weir-type filters **(b)** Pillar **(c)** Cross-flow and **(d)** Membrane. Ref: [63]with kind permission from Springer Scienceand Business Media.

2.2 ACTIVE SORTING DEVICES

2.2.1 ACOUSTOPHORESIS

Acoustophoresis devices are based on particle migration in a sound field. Exposing cells or microparticles to an acoustic field in a fluid creates an acoustic radiation force that acts on the cells. This force arises from the differences in density and compressibility of the cells and

particles, compared to the surrounding fluid medium (called contrast factor). As cells have specific sizes, densities and compressibilities, the acoustic force can vary by orders of magnitude, and sometimes even change sign. In a standing wave, particles with positive and negative contrast factors migrate respectively to pressure nodes and pressure anti-nodes[75].This principle was applied successfully to red blood cells sorting in a microfluidic device[76], but it could not be used with most of mammalian cells, sincetheir contrast factor is of the same sign. More recently, an innovative method was proposed by Kumar et al[77]. The separation is achievedwithin a rectangular chamber by applying both a resonant ultrasonic field and a laminar flow field propagating in orthogonal direction. While the laminar flow transports the cells suspension along the chamber, the ultrasonic field causes the suspended cells to migrate. As previously mentioned, the cells migration rate is related to their size and physical properties.Devices using first, second and even third resonant modes[78], [79]have shown their ability to enrich particles based on size by a first separation step with the fundamental mode and enrich them further with the second and third modes. Recently, this method was applied with nice separation capacities (100% efficiency and 90% purity) of 10µm and 5µm microspheres[80]. Even if these proofs of concept obtained with particles seem promising, till now their interest for clinical application has never been proven

2.2.2 DIELECTROPHORESIS

Dielectrophoresis is related to the movement of a polarizable particle (such as cell) in a spatially non-uniform electric field.This process is applicable to living cells that behave as a dipole (with respect to the boundary conditions) and thus can migrate into non-uniform electric field [81].The migrationdirection of a cell is determined by (i) its dielectric properties (ii) the conductivity and permittivity of membranes and (iii)its morphology and structural architecture[82].This mechanism can be used to sort cells moving in a channel: a dielectrophoretic force is usually applied perpendicularly to the cell direction to induce different trajectories depending of the cell polarizability. This technique was applied to bacteria sorting from blood[83], [84], live and dead yeast cells[85], [86], breast cancer cells[40] and murine tumor cells[86]. However, this technology still presents various limitations for cells sorting. First, media suitable to sustain living cells are generally highly conductive, and the field strength necessary to move or trap cells may induce modification of cells phenotypes (caused by the electrical field itself or by subsequent Joule heating phenomena). Secondly, the dielectric properties of cells may vary significantly depending on their stagein the cell cycle, or environment. This variation may overcome the global dielectrophoretic specificity of the cells of interest. Finally, the forces that arise from such method areusually weak as compared to the hydrodynamic forces induced by the liquid flow. If the flow rate is too high, the time of interaction between the cell and the field might be too short to induce a significant displacement of the cell and thus an effective separation.

Even if label-free sorting methods showed encouraging results, they have not been widely applied to patient samples and are therefore not yet suitable for clinical applications. However, non-microfluidic methods such as the ISET filter (see part 1) have shown their ability to capture cells missed by classical methods based on antibody–based capture. In the contextof CTCs evaluation, they might show strengths capturing cells that have been through the epithelia-mesenchymal transition.

3 MICROFLUIDIC BIOCHEMICAL SORTING

3.1 FLUORESCENCE SORTING

While conventional flow cytometry (see section 1.2.4) is still a reference for fluorescence activated cell sorting (FACS), microfluidic-based FACS (µFACS) started to develop in 1999[87], capable of 80 to 96 fold enrichment of particles and bacteria. This first osmotic-flow driven device was slow (10–20 cells per second) but some pressure-drivenµFACS devices could attain 12000 cells per second[88].Fluorescence cells sorting can rely ondifferent physical concepts: on optical forces[89]but these kinds of system can be complex and expensive, on sol–gel transition of thermoreversible gelation polymer[90]however the transition time reduces the throughput to 20 cells per second for 90% purity, or on hydrodynamic gating valves[91]. An innovative device using a pulsed laser to create a small air bubble that will “push” cells of interest into another channel depending on fluorescence signal was recently developed[92] (see fig 5).The switching time of the laser-triggered bubble is below 30µs. The device can thus sort 20 000 mammalian cells per second with 37% purity in enrichment mode, and >90% purity in high purity mode at 1500 cells per second. While the flow line switching method is innovative, this device and most of the µFACS devices are limitedby a rather low purity at high sorting ratesin blood samples (a minimum rate of 10 000 cells per second would be necessary). Additionally, this system requiresa significantdilution (10 to 100 times) of blood samples in order to sort blood cells efficiently. While optical detection can be very efficient (below 0.1 ms detection with high speed cameras), the limiting parameter is still the speed of the actuation methods used to direct cells in different paths. Electric field induced forces showed to be very efficient for conventional FACS machines but still they have to be integrated efficiently into microfluidic systems. Finally, fluorescence based sorting methods usually require pre-staining of the cell, meaning an increased processing time and potential cell viability loss. However, these approacheshave proven their efficiency in sorting cell by intra-cytoplasmic (or nucleus) immunostaining while adhesion and magnetic beads based methods can only sort upon external membrane antigens.

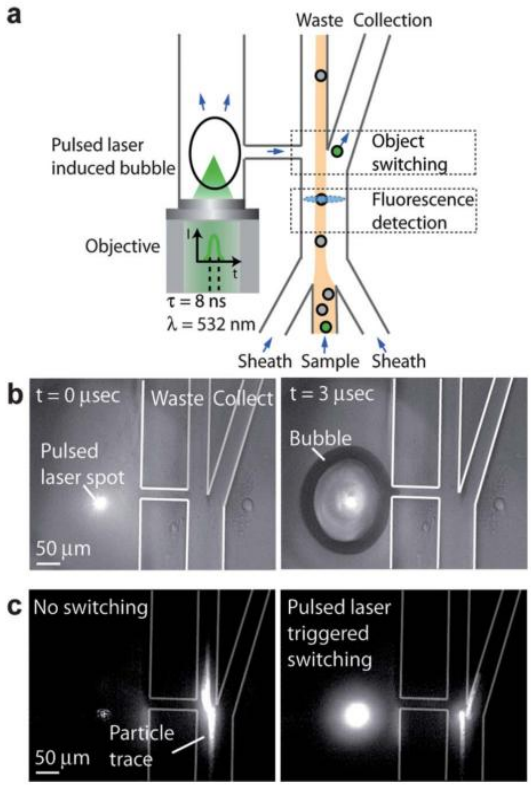

Fig 5. A pulsed laser, triggered by cells fluorescence detection, creates a bubble in a parallel channel, pushing the target cell in the collection outlet. Ref: [92]withpermission of The Royal Society of Chemistry.

3.2 IMMUNOMAGNETIC SEPARATION

Immunomagnetic cells separation, as described in section 1.2.4, is based on the interaction between antibodies grafted on magnetic beads and antigens on the cell membrane. By coupling this technique with microfluidic, one can get the best of two worlds. An important aspect of the immunomagnetic methods is that different kinds of antibody-coated magnetic particles are available commercially. These particles are generally expensive, and using microfluidic can reduce costs significantly by reducing the quantity of reagent and beads required for each experiment. Additionally, non-grafted magnetic particles are also commercially available and can easily be grafted with new antibodies. Different approaches are used to perform cells capture. In 1998, Chalmers et al.[93] presented two prototypes that could sort cells labeled with magnetic beads. While the first device, using a magnetic dipole, can extract labeled cells from non-labeled cells, the second device is more interesting as it allows a more accurate sorting depending on the size of the magnetic particle attached to the cell. Basically, the magnetic force applied on the particle depends on the size of the particle, meaning that particles of different diameters will undergo different deviations in a magnetic field.By grafting magnetic particles

with antibodies directed against C. albicans fungi surface antigens, whole blood cleaning was achieved at a 20ml/h flow rate[94]. The microfluidic device is simply inserted inside an electromagnet that generates a permanent magnetic field, cleaning fungi out of the blood. Application of immunomagnetic separation to rare mammalian cells is still a technical challenge. Cells can be sorted using an angle-oriented permanent magnet that deflects beads in flow[95]. The use of magnetic beads directed against EpCAM[96] or CD45[97] antibodies, retained on the bottom of a microfluidic device with a magnet underneath, allow for capture but with poor observation capacities due to cells and beads aggregates. This problem could be overcome with the Ephesia technique[98] by self-assembling antibody-coated magnetic beads into columns inside a microchannel, creating a capture array that acts as a sieve (see Fig 6). Cells captured on the columns can then be observed easily. Recent improvement of the chip's design allows for a higher throughput while maintaining flow velocity homogeneity[99]. With a 2ml/hour flow rate, this device is suitable for rare cancer cell sorting directly from blood and its optical performance is appealing to perform further cell analysis on chip. Moreover, the potential use of multiple antigens by mixing beads is very attractive making this device as the only microfluidic tools that meets the challenge of multi-target capture.

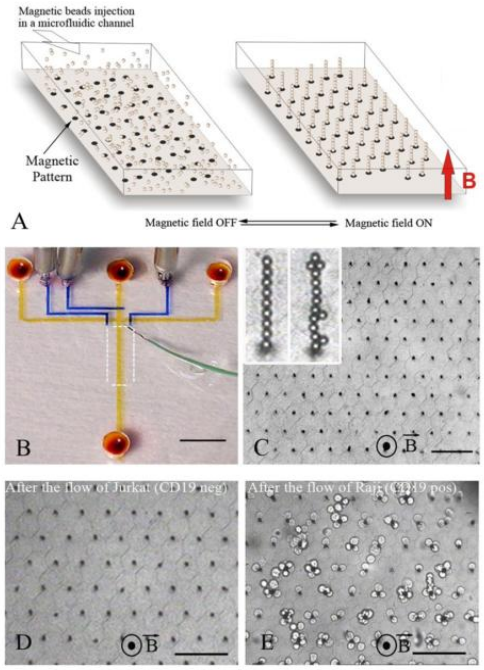

Fig 6. A, B and C. Beads solution is injected inside the chip via microchannels. Columns of those magnetic beads are then assembled inside the microchamber by applying a magnetic field. D. Cells are flown inside the chip and capture specifically on the antibody-coated beads. Ref: [98]Copyright (2006) National Academy of Sciences, USA.

3.3 ADHESION-BASED

Microfluidic adhesion based methods usethe biochemical properties of cells membrane to capture them specifically. As for immunomagnetic sorting, those devices rely mostly on antibody/antigen interactions to promote the adhesion. Microfluidic devices coated with antibodies dedicated to specific cells capture are numerous. We will focus here on the most recent work.

Most recent adhesion antibody-based systems are designed to capture rare cells, especially CTCs, maybe because surface antigens are easily accessible and the capture is very specific. However, for certain cells such as Circulating Endothelial Cells, no specific antigen have been found yet (as described previously). The first reported device was presented by Nagrath et al.[100], and described as the “CTC-chip”. This silicon microchip uses an array of 78000 microposts coated with anti-EpCAM antibodies. Laminar flows inside the device promote the adhesion of cells to post while shear forces applied on the captured cells remain smaller than the adhesion forces. This first system claimed capture rateson patient samples way higher than the “gold-standard” method (Cellsearch by Veridex) but these results were tempered later on. However, the system was capable of sorting CTCs from whole blood in a few hours very efficiently. Unfortunately, the silicon opacity and size of the micropost made cells imaging difficult. With a successful application to clinical samples, this device was the first to show the potential application of microfluidic to the CTCs field. Its ability to treat directly whole blood, along with its high flow rate of 1 to 2 ml per hour, was the key to success. The use of columns was studied in more details later on by modeling the specific capture of prostate cancer CTCs[101]. By adding a shift in the columns array, smaller cells of diameter under 15um (red and white blood cells) are supposed to have a lower chance (10% collision likelihood compared to 90% for CTC) of colliding into the columns. Targeting the same prostate-specific membrane antigen[102] (PSMA), Dharmasiri et al.[103]were able to capture prostate cancer CTCs using anti-PSMAaptamers that were immobilized onto the surface of a capture bed trappedwithin a PMMA microchip. Interestingly, cells were subsequently released intact from the affinity surface using trypsin followed by counting individual cells using a contact conductivity sensor integrated into the chip.

A key parameter of the antibody-based capture is the understanding of the interactions between the cell membrane antigen and the antibody coated surface. As the cell will remain in the flow until the end of the experiment, it is important to find the maximal flow rate at which cells won’t detach due to the shear forces. Based on theoretical and experimental results, a model was presented recently[104]: depending on the applied shear flow rate, three dynamic states of cell motion have been identified: (i) free motion, (ii) rolling adhesion, and (iii) firm adhesion. Cell dynamics, cell-receptor density and surface-ligand density are investigated, showing a good correlation between the simple physical model and the experimental results.Additionally, the removal of non-specifically capture cells by using a higher flow rate

after the capture step showed to increase the purity of the captured cells[105]. However, before any type of interaction, the cell must come close to the surface, but this is not necessarily easy to achieve in an obstacle-free channel (i.e. with no columns). To increase the probability that cells interact with the surface, chaotic micromixers[106] and "herringbone-like"[107] mixers that createmicrovortices were developed. Another solution relies on the use of a semi-permeable membrane, coated with the antibodies, that will divert flow lines to ensure maximal cells interaction with the membrane[108].

We found only one report of negative enrichment (i.e. enrichment by depletion of non-target cells) using antibody-coated microchip[109]. The potential risk of losing some target cells remains low (<1%).

CONCLUSION

We hope we could convince the reader that, in a few years only, microfluidics has considerably increased the panel of tools and concepts for cells sorting. We fear, however, that readers will also leave this review somewhat frustrated by the absence of a simple take-home message. This is indeed difficult, because the interest of microfluidics for cells sorting is rather recent. The field is thus still in a "creative-diverging" phase, in which many concepts are emerging, but most of them did not live enough to mature into practically used integrated systems, and be validated (or rejected) by experimental evidence. Indeed, a lot of the methods described above are still at the proof of concept level, demonstrated on particles or cell lines, rarely yet on clinical samples. Additionally, many of these methods are still rather limited in throughput. There seems to be a difficulty in crossing limitation in the throughput achievable by microfluidic devices, lying around the processing of 2 or 3 ml of raw blood per hour. This is probably insufficient for CTCs, especially for diagnosis in an early stage, in which CTCs levels lower than 1/ml are expected. Another difficulty is the variety and instability of biomarkers available. We already mentioned that for either endothelial circulating cells or fetal cells, there is currently no reliable specific surface antigen. For CTCs, numerous potential biomarkers exist, but numerous controversies too. For instance, among the general family of epithelial cancers (which comprise the most common breast, lung, prostate) targeting specific epithelial biomarkers (and in particular the most use one, EPCAM), are expressed very differently by CTCs depending of the type of cancer and, for instance, weakly expressed in lung cancer. For this reason the relative apparent sensitivity of biochemical or physical methods varies widely. A recent study, for instance, compared the ISET size sorting and the CellSearch EPCAM based magnetic sorting. They did about equally well for breast cancer, but ISET performed much better for lung cancer[110]. This would argue in favor of size or deformability based sorting, which should be less sensitive to these differences in expression, but the hypothesis underlying this sorting, e.g. the fact that cancer cells are larger and less deformable than other blood cells, is not water-tight either.

Some cancers, such as the “small-cells lung cancer”, are known to systematically involve small cancer cells, and our own studies on breast cancer CTCs microfluidic capture reveal that real CTCs display a much larger size distribution (and a smaller average size) than cell lines.

Depending on the application, it is probable that there will not be a single “all purpose” microfluidic method for sorting cells, and that several technologies will find their own “niche”. As a general trend, however, one may assess that progress in three main directions will be needed. First the current limitation in throughput around 3 ml/hour remains a worry especially for CTCs, hampering use in early stages of cancer. Second, the variety of the cells to capture, on the level of biomarkers, appeals for more work. On the technological side, one may expect that methods able to involve multiple criteria in a single sorting process will have a significant advantage for clinics, whereas research programs may accept more specialized methods on a project-per-project basis.This also raises strong challenges on the biochemical side, since even if we develop systems able to capture cells according to a multiplicity of antigens, technologists will require the input of biologists regarding the right antibodies to use. So far, however, the question of relevant biomarkers remains largely open on the biological side. It is more and more clear that even in a single patient at a single time, a cancer is not a homogeneous population, even within a primary tumor[111]. In the last years, for instance, it was demonstrated that a minor subpopulation of cells from a tumor, called “cancer stem cells” or “cancer progenitor cells”, carries all the proliferative and dissemination power of the cancer[112]. If this is true, these cancer stem cells are the relevant ones to search in the blood, and it is not clear at present, if they will even be detected by the antibodies currently used to select “all round” cancer cells. Another related issue is that of the epithelial-mesenchymal transition model, suggesting that in an epithelial cancer, the cells that are most prone to circulate in the blood are those which have lost their epithelial character[113], and thus the use of epithelial markers such as EPCAM should not be favored. This opinion, however, seems contradicted by the relatively good performance of the CellSearch® system in many cancer types. The third still unmet challenge is ease of use. Typically, current microfluidic cell sorting systems, even those commercialized, are closer to laboratory instruments than real clinical ones. They have a low throughput, and are far too labor-intensive for routine clinical use. To solve this challenge will certainly require the involvement of large diagnosis companies, but some technologies will be more easily “industrializable” than others. Overall, the field is currently very open, and one can expect in the years to come still new concepts to appear, in parallel with the progressive maturation of existing ones, such as variations on the “post-array” or “microfabricated filters” general families.

ACKNOWLEDGMENTS

We thank F. Farace (Institut Gustave Roussy) and L.N. Claverie (Institut Curie) for help in finding the most suitable articles. This work was supported in part by ANR Nanosciences programme (Project MICAD, ANR-08-BIOT-015-02), INCA-DHOS project (IDF 2009-1-RT-9) and FP7 European project CAMINEMS (FP7 NMP project 228980) and FP7 European project DIATOOLS.

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