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Label-free microfluidic stem cell isolation technologies

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ABSTRACT

Stem cell research has great potential in clinical applications and regenerative medicine, which highlight the need for efficient stem cell isolation technologies. Enrichment of stem cells is challenging because of its sensitivity to the surrounding environment and its overlapping features with non-target cells. Current gold-standard isolation techniques depend on surface antigens that are exclusively expressed on stem cells, where surface immuno-labeling is followed by flow cytometry or magnetic sorting procedures. It is well-established that stem cells should be enriched with minimal alteration, therefore, isolation without immunological tagging in a label-free format is of great interest. Microfluidic technologies have demonstrated high level of control to precisely manipulate stem cells. Here, we review the recent emerging label-free microfluidic based cell isolation technologies that provide unbiased stem cell enrichment based on their size and deformability, adhesion, and electrical characteristics, and discuss their potential to achieve effective stem cell isolation for research and clinical applications.

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1. Introduction

The ability to isolate and grow stem cells in a laboratory setting [1,2] has led to advancements in stem cell therapy to understand and treat diseases that have challenged conventional treatment approaches [3,4]. Some of the most widely used applications of stem cell therapies have included bone marrow and umbilical cord transplantations for the purpose of repopulating the hematopoietic stem cell in the bone marrow of patients undergoing chemotherapy [3–5]. There are currently several clinical trials underway covering a broad spectrum of stem cell based clinical treatments for various disorders and diseases including lung cancer [6,7], neurological [8–10], cardiac [11–13], and kidney diseases [7,14]. Recently, stem cells have also been used in treating diseases such as Parkinson, Alzheimer's and multiple sclerosis. With their strong potential in clinical and research applications, there is a significant need for the development of stem cell isolation technologies that are efficient and associated with high throughput, high purity, and high recovery rates. Purity can be defined as the ratio of the target cells to the total number of enriched cells expressed in percentage terms, and recovery can be defined as the percentage of enriched target cell to their original number within the sample. Currently the use of regenerative therapies is looked at as one of the last options available for patients with poor prognosis [15]. However, the targeted patient population is expected to increase over time to include those at an early disease stage and ultimately move towards disease prevention. This trend will result in an exponential increase in the requirement for highly pure viable stem cell populations.

The primary steps involved in obtaining a pure population of transplantable stem cells include cell isolation, culture and enrichment as highlighted in Fig. 1 (a)–(c). The current gold standard techniques for stem cell separation are mainly Fluorescence Activated Cell Sorting (FACS) and Magnetic-Activated Cell Sorting (MACS) methods, Fig. 1 (b) and (c) [16–18]. FACS relies on an optical readout and often requires surface labeling, thereby altering the surface of these sensitive cell types. This technique achieves a throughput of roughly 10^7 cells/hour with a high recovery of over 90%, but cells are subject to high shear stresses that could be destructive [19,20]. An alternative to FACS is the use of magnetic bead-conjugated antibodies and isolation of stem cell populations based on MACS. MACS can achieve faster separation of approximately 10^{11} cells/hour. However, in both methods, the purity of stem cells is compromised by the use of external markers. The use of bound fluorescent markers and magnetic beads for FACS and MACS, respectively, are considered as contaminants which could potentially interfere with cell proliferation and differentiation

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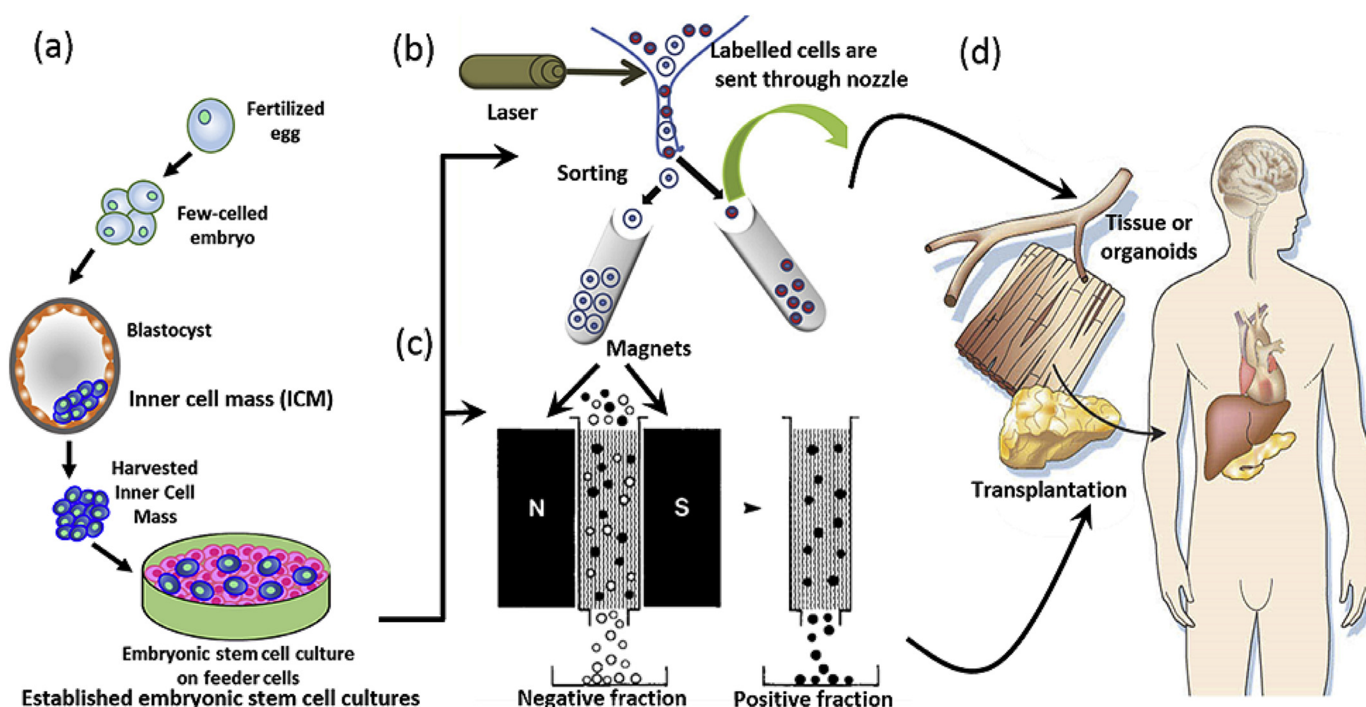

Fig. 1. Stem cell isolation, culture and enrichment techniques in regenerative medicine. (a) Stem cell culturing procedure, (b) and (c) Schematics demonstrating the sorting/enrichment principle using the FACS and MACS techniques, from Refs. [23] and [24], (d) Isolated cells can be used for tissue engineering/cell therapy needed for organ transplantation [22].

[19,20]. Schematic shown in Fig. 1 (d) represents the use of enriched stem cells for tissue and organ regeneration and transplantation [21,22].

Certain surface molecules are known to be present on stem cells, and are being used for characterization and isolation. In the hematopoietic stem cell population, CD34 and CD133 are common markers. Other stem cell lineages include several other markers such as CD146, Nestin, and PSA-NCAM (Anti-Polysialylated Neural Cell Adhesion Molecule), among others [25]. Despite a relatively wide selection of surface makers, expression levels are not stable, and depends on a variety of factors such as cytokines, disease status, growth factors, and culture conditions [26]. Therefore, the many unknowns relating to the magnitude of surface antigen expressions and the subsequent functions compromises the recovery rate and purity of cells separated using immunological methods. One of the primary objectives of enriched stem cells is likely to be in regenerative medicine, and hence it is important to minimize cell surface alterations and retain high purity rates. In addition, antibody based methods such as FACS rely on the use of expensive equipment and preparatory procedures such as incubation with antibodies and several washing steps.

The recent advances in stem cell based treatments are driving the need for stem cell isolation technologies beyond existing methods [27,28]. Label-free methods used in micro-engineered devices are an attractive alternative to antibody based techniques and is mainly based on physical parameters [29] such as shape, size, elasticity, adhesion and electrical identifiers utilized for cell separation [30–32]. Technological innovation in the fields of microfabrication and nanotechnology has enabled the development of such separation technologies. These scientific advances have helped propel the field of microfluidics.

Microfluidics was introduced two decades ago, and presented an attractive approach for engineering and miniaturizing bioassays with accurately controlling biological samples and their microenvironment, leading the way for potentially innovative cell biology studies and effective single cell isolation platforms, including stem cell applications [32,33]. Microfluidics is a set of miniaturized technologies that have the potential to achieve high throughputs with the required precision, sensitivity and selectivity [34,35]. Microfluidics has previously been used in stem cell research [35] with vast applications including isolation, co-cultures, regulating microenvironments for cell maintenance, cell differentiation, and high throughput screening [36]. To understand stem cell differentiation, microfluidic tools are being used to distinguish the role of various biochemical signals such as cytokines, growth factors and culture conditions within controlled environments [37]. Microenvironments play an important role in the behavior of stem cells as they promote cell maintenance to achieve differentiation or to remain in an undifferentiated state [38,39]. Microfluidic devices have been used to study microenvironments mainly from two perspectives; screening a wide range of conditions to achieve high throughput and reconstructing the physiological environment [40,41].

Microfluidic techniques for cell separation can be broadly classified into two categories, namely, active and passive microfluidics, based on physical parameters and types of external forces such as electric, magnetic, acoustic and optical forces, used for cell segregation. A comprehensive comparison of various microfluidic cell separation technologies have been carried out and discussed previously [42,43]. Microfluidic devices in these two categories can utilize both the biophysical and biochemical differences between cell types. Active microfluidic devices can be identified as those employing external fields and forces to induce cell movement. Microfluidic cell manipulation using electric fields commonly consists of variations to the dielectrophoretic technique [44,45]. A technique equivalent to dielectrophoresis, but using magnetic fields, is referred to as magnetophoresis [46]. This technique has been extensively used within microfluidic channels to separate a wide range of rare cells using magnetic labels [47,48]. Lately, acoustic microfluidics is being explored as another cell

manipulation method that provides good spatial control and cellular viability [49,50]. Passive microfluidic methods consist of a variety of methods relying on inertial forces, deterministic lateral displacement, pinched flow fractionation, filtration and cellular adhesion [43,51–53]. Raman based cell sorting is another approach that utilize Raman spectra of single cells for separation. Raman spectra of single cells represent an intrinsic biochemical image of cells that can be used to identify phenotypes and physiological states of cells. A recent review [54] discussed Raman activated cell sorting. The most common application among the various microfluidic cell sorting methods discussed above has been for CTC (Circulating Tumor Cell) isolation from blood samples [55–57]. Label-free isolation methods are also of interest to the CTC community as it offers an alternative approach to cell surface expression based isolation methods. As a result, label-free CTC isolation has moved beyond research labs into commercial products, and a few such companies are Vortex Biosciences, Apocell Inc., Clearbridge BioMedics, ScreenCell, among others. The label-free technologies used for CTCs should be applicable for stem cells, however the commercialization of microfluidics for stem cell isolation is currently lagging behind for two primary reasons. Firstly, markets prioritize cancer research over stem cells since the disease affects a large percentage of the population. Secondly, there are fewer research groups working at the interface of stem cells and engineering compared to cancer.

In order to produce viable stem cell sorting device alternatives, the technologies must meet user requirements of rapid sorting, high accuracies in term of recovery and purity, ability to sort various types based on origin and function, and a contamination free device for a reduced cost that is easily available to researchers and clinics [27]. Several review papers in the literature discussed separation of stem cells and microfluidic methods for cell isolation [28,29,34–36,43,44,78,80,88], which make a great resource for the reader. However, there are no reviews focused at the intersection of microfluidics, label-free, and stem cell isolation. We believe this is a very interesting crossing with great potential for efficient stem cell separation in the near future.

This article is an up-to-date review on the use of label-free microfluidic based stem cell isolation to provide enrichment based on size, deformability, adhesion and electrical characteristics. This review also covers an approach referred to as "cell rolling" which is based on transient cell surface adhesions to immobilized antibodies and selectins. However, it is important to distinguish that the cell rolling technique does not permanently bind tags to the cell surface, unlike methods such as FACS and MACS that alter cells, and hence we include this as a label-free method. The state-of-the-art along with the need for improvement in the field is highlighted to bring continued focus to this research area.

2. Electrical cell separation

Separation techniques based on the electrical properties of cells has gathered a lot of interest in the last 10 years due to the ability to enrich based on attributes such as membrane morphology, membrane conductivity, molecular composition and cell size [58,59]. Dielectrophoresis (DEP) is a powerful and commonly used cell separation technology by the microfluidics community since the manipulation forces are directly proportional to the third power of cell radius ($F_{DEP} \propto r^3$), thereby inducing significant forces on particles suspended in length scales typically used for microfluidics. However, this does not imply that DEP is solely a size dependent method, since the critical factor is the relative difference in polarizability of the cell with the surrounding medium. This depends on parameters such as membrane morphology, membrane thickness, membrane conductivity, solution conductivity and cell size [60,61]. DEP movement is observed in a biological cell when it is subject to strong non-uniform electric fields while suspended in a weak ionic medium. Depending on the direction of movement, it can be broadly classified as positive DEP when the particle moves towards high field regions and negative DEP when the particle is repelled into regions of low electric field strength. The transition from negative to positive DEP occurs at a specific frequency referred to as cross-over frequency at which cells undergo no observable movement. It has been reported [62] that the inherent electrophysiological properties help identify lineages of stem cells. Identification takes place by inducing movement in polarizable biological particles, and this procedure can also be used to purify cells into different sub-fractions. The DEP technique coupled with microfluidics has been successfully used to isolate viruses, bacteria and various mammalian cells including blood cancer cells [63–73]. DEP-field flow fractionation (DEP-FFF) has been used on collagenase-digested adipose tissue suspension to enrich cells containing stem/progenitor cell markers of NG2 and nestin by up to 14 fold [74]. The DEP-FFF system consisted of interdigitated microelectrodes on polyimide substrate manufactured using a commercial flex-circuit process. The entire instrument was setup to achieve a level of automation required for scaling up to enrich larger number of stem cells. DEP has been used by Stephens et al. and Talary et al. to enrich the $CD34^+$ stem cell population from peripheral blood by an average of five folds [75,76]. They found that cells were capable of colony formation and remained viable for two weeks in culture.

Simon et al. has demonstrated the use of a large interdigitated electrode array to improve the capacity of stem cell sorting [77]. Undifferentiated mouse NSPCs were utilized in this device. The cells were differentiated post-sorting to generate astrocytes from the astrocyte progenitors and the differentiated astrocytes were detected and quantified using the Glial fibrillary acidic protein that identify astrocytes. Initially, a high frequency of 1000 kHz is applied as a control on a cell suspension flowing through a microfluidic channel in order to trap almost all viable cells by DEP forces, Fig. 2 (a and b). The applied AC frequency is then lowered to 200 kHz, due to which a subpopulation of the trapped cells are released and collected at the outlet. The applied AC frequency is then further lowered to 80 kHz to release another subpopulation of the cells. The remaining cells are released by further lowering the frequency, and that way, the authors were able to process approximately 150,000 cells/h. In this manner, the astrocyte progenitors were enriched approximately 2 fold at a frequency range of 0–80 kHz. Testing cells with Trypan blue staining showed viability of 91.4%, 88.7% and 86.4% at low, medium and high frequencies respectively.

Continuous DEP cell separation was demonstrated for myotubes differentiated from C2C12 myoblasts, and for C2C12 myoblasts co-cultured with MRC-5 fibroblasts [78]. The study reported achieving cell separation purities of greater than 96% and these results were validated by means of flow cytometry and western blotting. The employed device consisted of angled electrodes on the top and bottom surfaces of the microfluidic channel to provide sufficient deflection of the C2C12 myoblasts into the center of the channel under negative DEP, Fig. 2 (c). The dielectrophoretic differences between the C2C12 myoblasts and MRC-5 fibroblasts were attributed to differences in membrane capacitance. Propidium iodide fluorescent DNA marker was used for assessing cellular viability; 96% C2C12 myoblasts and 65% myotubes were found to be viable after DEP separation.

Song et al. reported the use of a continuous-flow microfluidic DEP device to separate human mesenchymal stem cells (hMSCs) and their differentiated osteoblast progeny [79]. Their device also employs angled electrodes as shown in Fig. 3 (a). However the electrodes in this device are all on one plane, thereby minimizing

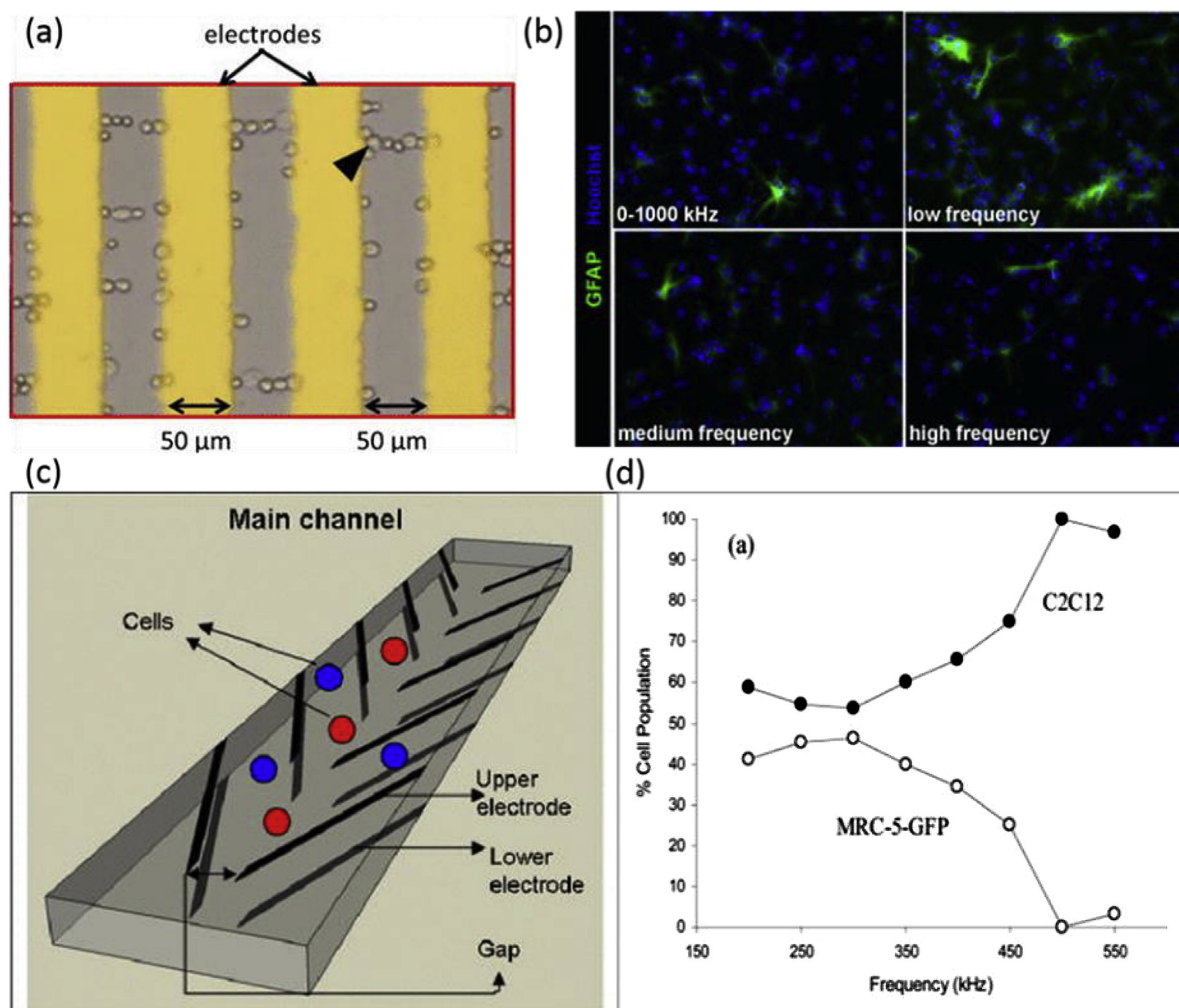

Fig. 2. DEP based stem cell separation. (a) Gold electrode array showing cells trapped by DEP force at the edges of the electrodes [77], (b) neural stem and progenitor cells sorted into low, medium and high frequency fractions, showing highest enrichment of labeled astrocyte progenitor cells in the low frequency fraction. The top left panel shows cells trapped at 1000 kHz as a control [77], (c) angled electrode array at the top and bottom surface of the DEP microfluidic chamber to create 3D funneling action [78], and (d) FACS data quantifying the level of DEP separation of C2C12 myoblasts from MRC-5-GFP fibroblasts at various frequencies [78].

alignment issues during the microfabrication process. Separation was carried out in the positive DEP regime where osteoblasts experienced stronger DEP forces thereby undergoing larger deflections following a zig–zag trajectory, whereas most of the hMSCs continued on a straight trajectory due to weaker DEP force (Fig. 3). A maximum recovery of 92% and purity of 84% was obtained for hMSCs at one outlet, whereas osteoblasts exhibited a recovery of 67% and a purity of 87% at the other outlet. Using Trypan blue test, separated osteoblasts and hMSCs showed more than 95% viability. The overlapping DEP behavior in a small subsection of the cell populations were attributed to the non-uniformity in cell sizes and dielectric property.

Under certain operating conditions, cells may be irreversibly damaged as a result of exposure to DEP forces. As mentioned earlier, stem cells are susceptible to environmental stresses, and hence careful parameter optimization must be a strong consideration. Cell damage during DEP is mainly the result of excessive electric field stresses in the membrane or an outcome of cells being suspended in a non-physiological medium. In general, in order to minimize cell destruction, the possibility of cells being exposed to high strength fields should be avoided, especially in the frequency range immediately above and below the cross-over frequency where the electric field membrane stresses are high. DEP work reported previously for mammalian cells uses low conductivity buffers that are osmotically balanced. For clinical utility, most biologists are hesitant to suspend cells in DEP buffer for loss of cell viability. Previous studies provides sufficient evidence that cells retain their viability for up to 3 h after DEP processing in the conductivity adjusted DEP medium. The final choice of an appropriate conductivity adjusted suspending medium must be made based on the cell type, but in all cases it is important to suspend stem cells in a pH and osmolarity adjusted medium. Post DEP separation, cells can be re-suspended in a long term maintenance buffer such as cell culture growth medium.

DEP has been used to separate target cells from a single population of non-target cells as well as from multiple cell types. For populations of cell types with non-overlapping DEP cross-over frequencies, a single frequency that lies between the cross-over frequencies of the two populations can be utilized. However, for a single sample containing multiple cell types, it is more likely to have partially overlapping cross-over frequency distributions. In such cases, in order to improve purity it is advisable to use multiple

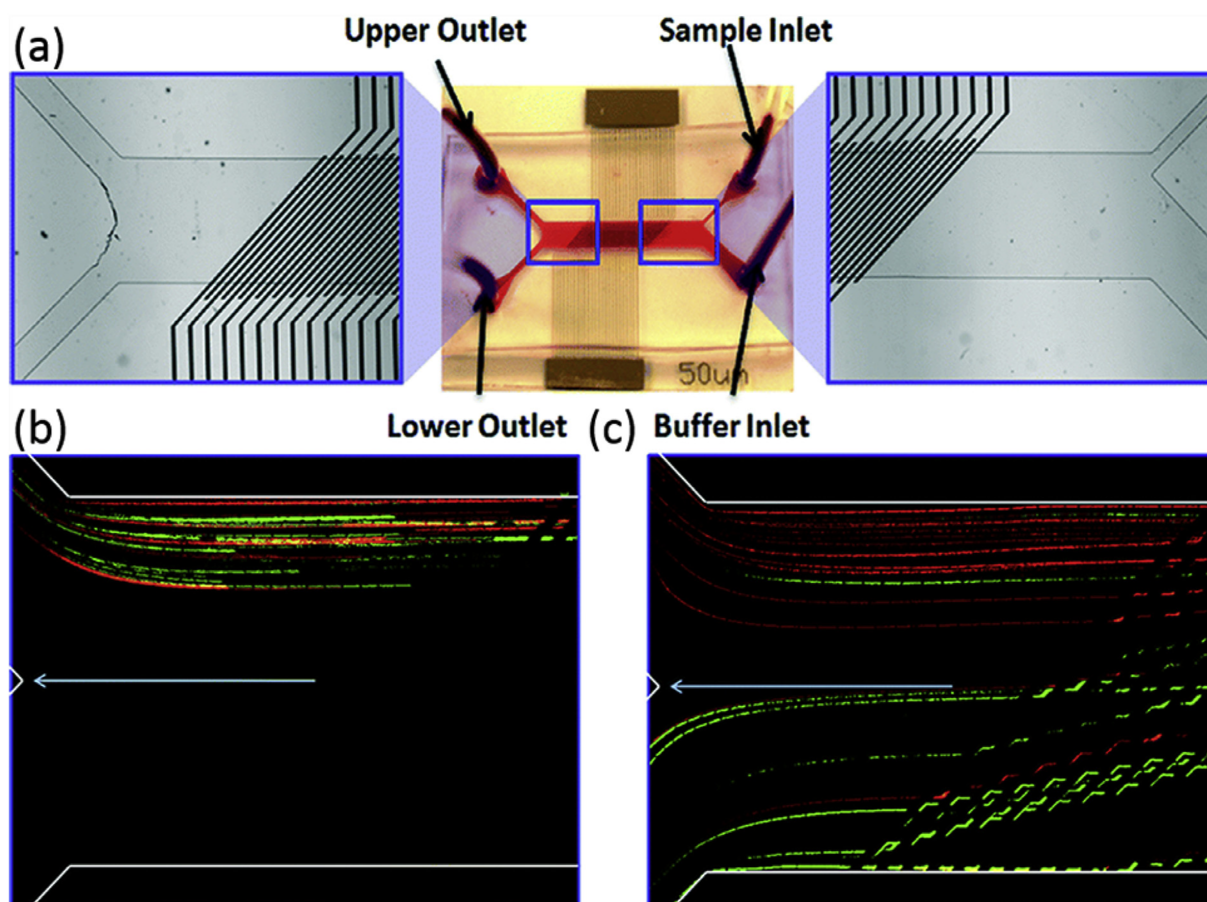

Fig. 3. Continuous DEP based separation of stem cells. (a) Microfluidic PDMS device bonded on a glass substrate with angled interdigitated electrodes, (b) Superimposed cell trajectories of hMSCs (in red) and osteoblasts (in green) under a higher flow rate with an alternating AC field of 7.2 V peak to peak at 3 MHz, and (c) alternating AC voltage increased to 15.4 V peak to peak at 3 MHz [79].

frequencies and electrode arrays to individually target each subpopulation. DEP based high throughput separation is generally achieved in a continuous separation mode rather than a trap and release system. However, attention must be given to flow velocity while in a continuous separation mode, in order to avoid compromising separation efficiency.

3. Passive size and deformability based separation

Passive size based separation is quite commonly used for cell enrichment and consists of a variety of technologies utilizing appropriate pore designs for filtration and hydrodynamic forces such as inertial forces and dean vortices [80]. The size of embryonic stem cell colonies and embryoid bodies has been reported to have a significant impact on lineage specific differentiation [81]. Proliferation, differentiation potential, and rate of cell death were dependent on the size of the embryoid bodies [82]. A simple and effective size based separation technique of embryoid bodies separation technique has been reported by Lillehoj et al. [83] as shown in Fig. 4(a). Three pillars with a precise spacing in the flow path were used to separate the embryoid bodies into three sizes ranging up to 300 μm with very high recovery rate. The critical design parameters of this device include the spacing between the pillars and the specific channel dimensions. Cell viability was confirmed higher than 92% using flow cytometry analysis in the control and sorted population by measuring cells negative to 7-AAD viability staining marker. Another interesting size-based separation method is to utilize fluid flow inertial forces. The use of dean flow-coupled inertial focusing for particle separation is becoming an increasingly common label-free technique for sorting cells [84,85]. This phenomenon is prevalent in curved channel geometries where the curvature creates a secondary cross-sectional flow referred to as Dean flow resulting in two counter-rotating vortices [86]. In curved microfluidic channels, along with lift forces, centrifugal effects due to dean flow act on particles to influence particle position. Using this technique, Nathamgari et al., demonstrated the isolation of single stem cells from a mixed population of cells and clusters from dissociated neurospheres [87], Fig. 4 (b). The differences in size between the single cells and clusters was exploited to focus the single cells near the inner wall of the curved microchannel while clusters equilibrated in the middle. A flow rate of 1 mL/min was optimized to yield high viabilities greater than 90% and maintain multipotency to differentiate into neurons and astrocytes. They were able to capture approximately 84% of the single cells from a mixture of cells and cluster of cells present in the sample.

Stem and progenitor cells are thought to be continuously capable of tissue regeneration in human bodies. One such example is found in adrenal cortical tissue regeneration using stem cells from the adrenal cortex [88], and transplantation of progenitor cells from a biopsy can restore adrenal functionality. However, previously, most researchers have relied on markers that label cholesterol with Nile Red. The population of cells that have been isolated with a lower intensity of Nile Red was found to be useful for adrenal cortical regeneration. Hur et al. utilized the biophysical changes of

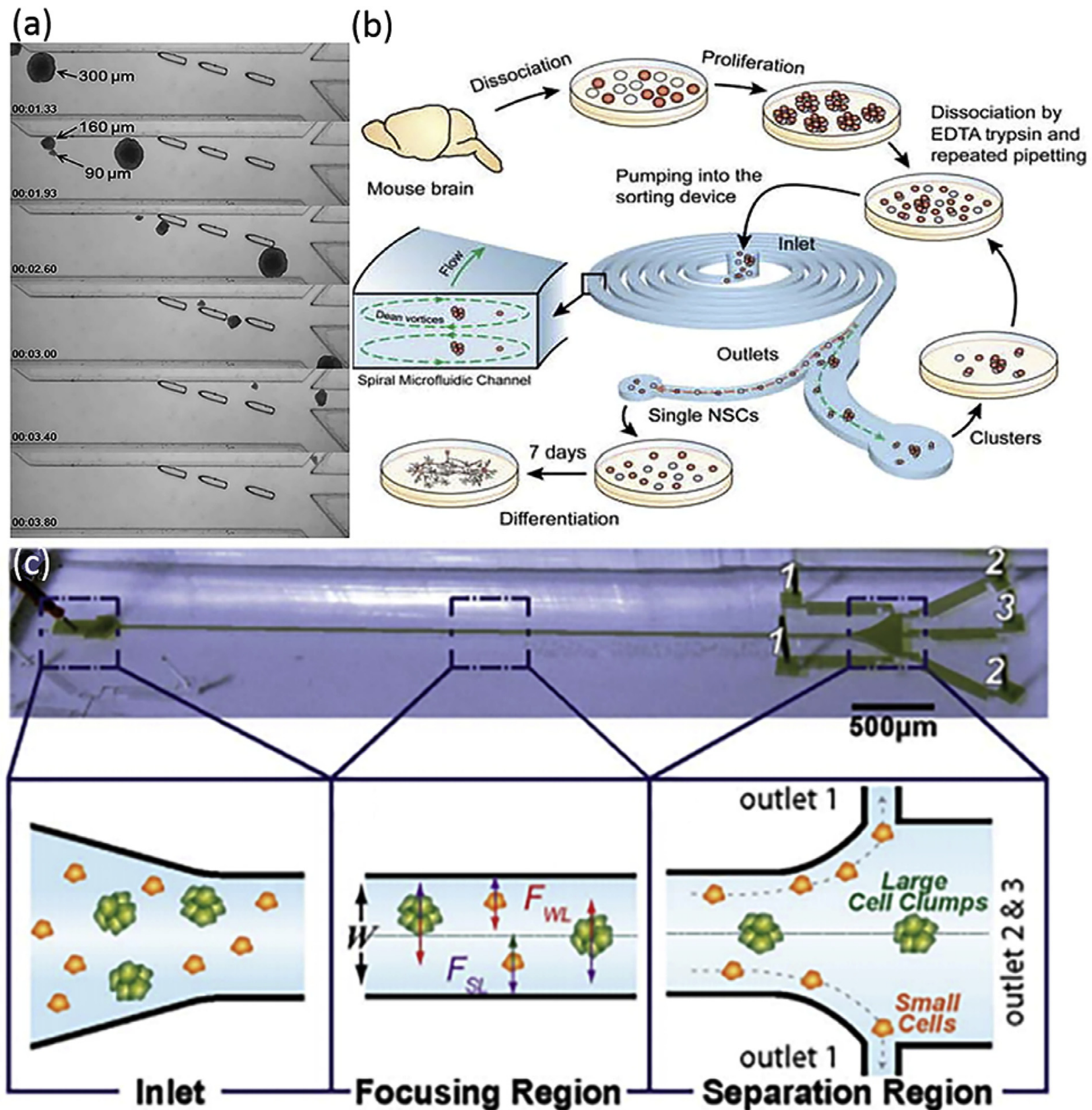

Fig. 4. Size based microfluidic separation of stem cells. (a) Consecutive video frames demonstrating the separation of mouse embryoid bodies 90, 160 and 300 μm in size [83], (b) Dissociated neurospheres from mice pumped through the inlet of inertial microfluidic device and collected at the outlet for culturing [87], and (c) The microfluidic device used for isolation of progenitor cells and lower part showing the inertial focusing of cells based on diameter variation [89].

cell–cell adhesion associated with varying degrees of differentiation of cells from the adrenal cortex to achieve enrichment of the progenitor cells [89], Fig. 4(c). The differentiated cells with higher cholesterol content remained in multicellular clusters whereas the less differentiated progenitor cells with lower cholesterol content could be dissociated as single cells. Inertial lift forces were used to create translocation of flowing cells across the width of the channel and position the larger cell clumps closer to the center of the channel, while smaller and stiffer cells were closer to the channel walls. A throughput of 24,000 cells/min was achieved when the device was operated at 60 μL/min and more than 70% cells were viable for 24 h post processing. Viability tests were performed using a live/dead assay kit (Calcein AM and Ethidium homodimer-2). Jung et al. designed and optimized a microfluidic network to hydrodynamically separate mesenchymal stem cells derived from human bone marrow [90]. Their device was operated at 30 μL/min, and was able to separate cells into three subpopulations; small, medium, and large cells. They processed 10^4 cell/min and achieved around 86% recovery rates, more than 90% viability (trypan blue), complete purity of small cell sub-population, and less than 90% purity rate for the other two sub-populations. The authors recommended that the effect of cell deformation should be studied further and considered in optimizing the microfluidic chip for better recovery and purity rates of the separation.

The highlighted passive size based separation technologies operate on cells suspended in physiologically complete buffers, and at flow velocities which induce minimal shear stresses, thereby making hydrodynamic separation a competitive and minimally disruptive technology. On the other hand, electrical separation techniques rely on suspending cells in buffers of low ionic strength

which are not supportive to long term cell viability. However, the efficiency of the passive size based separation might be dependent on the cell concentration in the sample, thus the end user might need to dilute samples to achieve higher efficiencies. Lillehoj et al. [83] observed more clogging during the separation of embryos compared to bead experiments because of heterogeneous population of embryos even at low cell concentrations. Another important factor is cell deformability, which cannot be neglected when using the mechanical trap concepts, or the dean-flow devices and inertial microfluidics. Achieving efficient cell separation based on size depends on both cell size and deformability. Therefore, both factors need to be studied and optimizations need to be performed on the design and the flow rates to enhance recovery and purity rates of stem cell separation. Mechanical traps offer high resolution in distinguishing cells based on their size, but suffer low throughput since high flow rates introduce undesirable stresses on stem cells that could be destructive. On the other hand, hydrodynamic flow based separation can be performed at higher flow rates and it does offer unmatched throughput when compared to all other microfluidic methods. However, it has a lower resolution in separating dissimilar cells with slight differences in size. Several studies demonstrated efficient hydrodynamic based cell separation using dissimilar cells with obvious difference in their size.

4. Adhesion based cell separation

Cell-substrate interface is an active field of research for controlling cell microenvironment and an important aspect in tissue engineering, yet it can also be utilized for cell separation based on dynamic interaction between both. Cell surface interactions are mainly modulated by engineering the adhesion substrate, and the applied fluid shear stresses in microfluidic devices where fluid flow can be controlled and as a result of that shear based separation can be performed precisely. Adhesion based separation is becoming popular because of its simplicity and ease of operation for label-free isolation of a specific cell type from a mixed cell population [91]. Stem cells typically grow in clusters, but cell separation techniques rely on dissociating clusters into single cell suspensions. However, it is advantageous to work with clusters to maintain their differentiation potential [92,93]. Differences in adhesion strength based on technology named μSHEAR (micro–stem cell high-efficiency adhesion-based recovery) to purify stem cells was demonstrated by Singh et al. [94] as shown in Fig. 5 (a). Sufficient shear stresses of 85–125 dyn cm^{-2} were generated using a laminar flow within a microfluidic channel to entirely detach human induced pluripotent stem cell (hiPSC) colonies within 14 min, Fig. 5 (b and c), during which the fibroblasts remain attached. The extracted cells had a purity of 99% and greater than 80% survived with normal transcriptional profiles and differentiation potential. This paper clearly demonstrates that differences in adhesion strength can be exploited to purify undifferentiated hPSCs from other cell types in an efficient label-free process. A recent study used a microfluidic device to apply shear stress and capture circulating cancer stem cells based on their adhesiveness to the channel that is coated with basement membrane extracts [95]. The study showed that adhesiveness of cancer stem cells is related to their mobility and resistance to chemotherapies. Therefore, there are open questions remaining about the relation of several adhesion ligands with the different cell types and their binding strength, as well as their role in cell–cell and cell–surface interactions as they could alter cellular crosstalk between cells and modify the extracellular environment. As a result, achieving separation with high specificity and selectivity of unaltered stem cells could represent a challenge using adhesion based separation [91]. Careful optimization of operating conditions need to be considered along with engineering new substrates that represent selective adhesiveness to specific cell types [96] to improve these stem cell separation technologies.

Traditionally, feeder layers consisting of fibroblasts are co-cultured with embryonic stem cells to provide the physiological conditions necessary for stem cell growth [97,98]. More recently, a feeder layer-free culture method consisting of chemically derived hydrogels has been developed for stem cell-culture [99]. However, the feeder layer method is still used as it is suitable in maintaining embryonic stem cells in an undifferentiated state [100]. Hence, the development of a viable approach for co-culture of embryonic stem cells with feeder cells is of importance in stem cell tissue engineering. Chen et al. demonstrated the use of a microfluidic device using a porous polydimethylsiloxane (PDMS) membrane [101], shown in Fig. 5 (d). The device was used to demonstrate membrane separated co-culture of mouse embryonic stem (mES) cells and

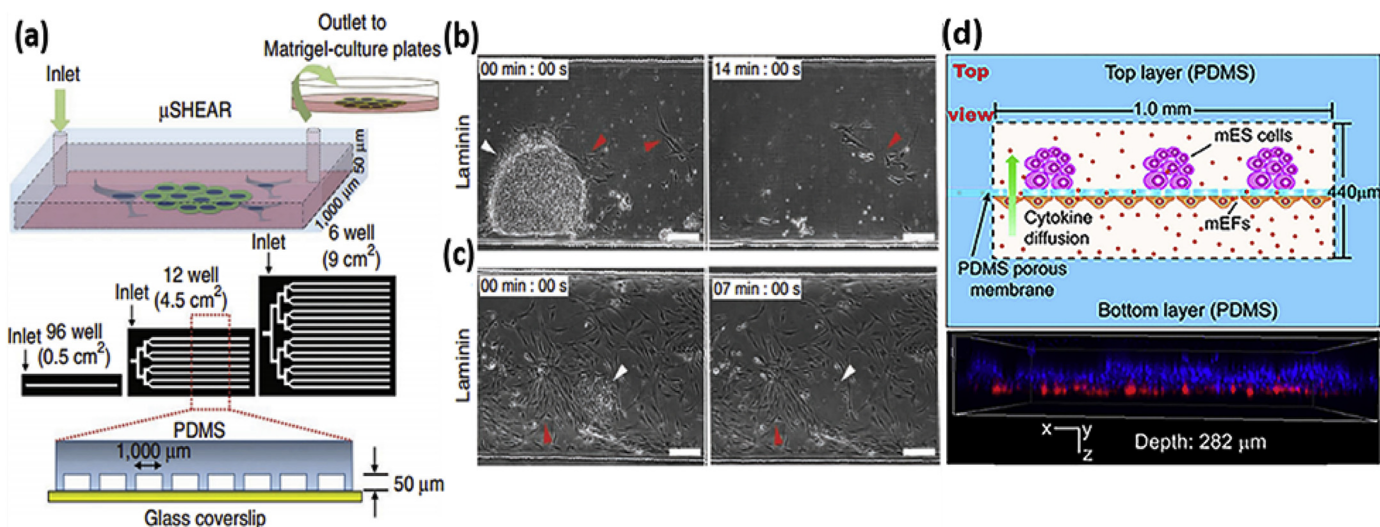

Fig. 5. Adhesion based isolation of stem cells in a microfluidic device. (a) Schematic of μSHEAR device, (b,c) Selective isolation of hiPSCs when co-cultured with IMR90 cells at low (b) and high (c) density [94]. The white arrowheads indicate a hiPSC colony that is detached by flow. The red arrowheads indicate IMR90 fibroblasts, and (d) porous membrane PDMS microfluidic co-culture device and the bottom figure shows a confocal image of the co-cultured mES cells stained with Hoechst 33342 and feeder layer stained with RFP-mEFs [101].

mouse embryonic fibroblasts (mEFs). The mES cells in the top layer of the chamber were detached after several days of co-culture using trypsin. Characterization of mES cells were carried out using flow cytometry and 89.2% was the reported purity of the extracted sample. They showed that mEFs can grow for several days inside the chip with 97% viability (live/dead assay kit Calcein AM/EthD-1). Conventional co-culture methods yields a significantly lower purity of 40% for mES cells [101], and hence the feeder-separated co-culture system is clearly advantageous in making stem cell separation easier afterwards with higher recovery and purity rates.

Surface topography can also be explored for selective separation of different stem cell types as shown by Chen et al. [102]. They cultured a mixture of hESCs and NIH/3T3 fibroblasts on strips of nanorough surfaces separated by smooth regions and found hESCs and NIH/3T3 cells segregated based on their preferential choice for rough and smooth surface. Quantitatively, they observed that 87% of hESCs preferred smooth glass surface where as 97% of cell on nanorough regions were NIH/3T3 fibroblasts.

5. Rolling based cell separation

Rolling cell adhesion is an important mechanism involved in our immune system response, where leukocytes roll on vascular surfaces in a multistep process to reach the site of infection. Leukocyte rolling occur by transient and dynamic interactions made between endothelial cells on the blood vessel surface and leukocyte surface through microvillus-like membrane protrusions. Cell rolling is a physiological phenomenon that occur with several cell types such as lymphocytes, stem cells, and cancer cells, where it depend on transient and continuous adhesive interaction between cells and their extracellular matrix [103]. In general, cell rolling represent the first step in a multi-step cascade of signaling where cell switch from flow to decelerating rolling by tethering with the substrate, until arrest and adhesion are achieved. A comprehensive review of cell rolling is described by McEver et al. [103]. Transmembrane glycoprotein receptors called selectins expressed on the surfaces of leukocytes were primarily responsible for tethering and rolling interactions. Examples of *in vivo* cell rolling include lymphocytes and leukocytes trafficking to lymph nodes and during inflammation [103], cancer cells during metastasis [104], and stem cell homing mechanisms [105].

Cell rolling is controlled by parameters such as deformation and receptor-ligand binding kinetics that modulates the adhesion process resulting in a reduction of cell velocity that can be exploited for cell separation, hence can be utilized for label-free cell separation techniques. Microfluidic channels were coated with appropriate cell surface marker molecules to modulate the interaction of target cells by which different cell types experience varying adhesive forces depending on the different expression levels of surface marker molecules. A mathematical model of cell interactions with surfaces mediated by receptors was first proposed by Hammer et al. in 1987 and was referred to as the “point attachment model” [106]. In their later work, Hammer et al. showed interactions of selectin molecules coated on slides with human hematopoietic stem and progenitor cell (HSPC) from adult bone marrow (ABM) and fetal liver. Selectins are cell adhesion proteins and *in vivo* these molecules exhibit a significant role in inflammation and control the rolling of neutrophils in response to certain stimuli. The interaction experiments were conducted in a flow chamber having parallel plates separated by a distance of 180 μm, more like a microfluidic device [107]. Later, using a similar approach, others also demonstrated rolling-based isolation of $CD34^+$ cell from blood and bone marrow samples using antibody and selectin coated surfaces [108–113].

Yamaoka et al. showed cell isolation based on cell rolling where transient interactions between cell surface and immobilized antibodies are made and broken when cells are flown through a microfluidic device. They demonstrated the separation of $CD34^+$ cell from $CD34^-$ cells in bone marrow by using anti CD34 antibody immobilized on the surface of their inclined cell separation column [108], Fig. 6 (a and b). They also quantified the interaction dynamics using a high speed CCD camera for different cell types by showing differences in rolling velocities for KG-1a which are $CD34^+$ cells and HL-60 which are $CD34^-$ cells [109]. They optimized antibody immobilization conditions such as temperature and time, column inclination, and operational flow rates to obtain a high performance cell separation efficiency. By using the antibody immobilized separation column they observed nonspecific cell binding because of multivalent cell interactions. More recently, Mahara et al. designed an amphiphilic phospholipid copolymer as antibody immobilizing modifier [110] to minimize non-specific binding. With this new surface modifier for antibody immobilization, less cells adhered on the surface while achieving higher number of rolling cells as shown in Fig. 6 (c).

King et al. showed that by using a combination of antibody and selectin molecules for surface immobilization, Fig. 6 (d), the capture purity increased from 5.1% to 36% depending on the type of selectin molecule used [111]. The same group have shown capture of $CD34^+$ HSPCs directly from the circulating blood *in vivo* by implanting a selectin coated device into the artery of rats, and achieved sevenfold increase in the capture purity of HSPCs compared with whole blood capture [112]. Another study demonstrated the use of acidic pH environment can enhance adhesion of $CD34^+$ HSPCs compared to $CD34^-$ mononuclear cells on L-selectin coated micro-tubes by mimicking the extracellular microenvironment [113]. The conformational change in orientation of L–selectin thereby increased cell adhesion because of decreased rolling velocity of 22.14 ± 1.87 μm/s at pH 6.6 compared to 31.24 ± 3.23 μm/s at pH 7.4, Fig. 6 (e) and (f). However, there were no data reporting on cell viability after the rolling-based separation experiments.

Similar to other methods relying on surface markers, variability in recovery for the cell rolling method can result from biological heterogeneity within cell types, where some cells express denser surface markers than others. Moreover, the purity of separation will be dependent on the entire population of cells including the non-target cells which might possess affinity to selectins or the used surface antibodies. In addition, the question of altering the cell state using this label-free approach arising from transient interactions is not well understood. More studies need to be directed toward a better understanding whether rolling stem cells on selectins/antibodies coated surfaces would alter their physiological state or not.

While cells roll on surfaces, there is a fine balance between cell-surface interaction and fluid drag forces on cells. Microfluidic flow is known to operate within the laminar regime, thus cells carried by the flow need to settle by gravitational forces before interacting with the coated surface. This results in throughput limitations, where long and shallow channels need to be used (i.e. high fluidic resistance) in order to achieve cell–surface interaction needed for rolling.

Other mechanisms explained before involve separation forces that can be tuned, such as the DEP induced forces and the fluidic inertial forces that compete with fluid drag forces in order to separate cells. For example, DEP forces can be changed based on the applied voltage magnitude and frequency, and the dean flow effect can be altered by modifying the curved channel geometry, or varying the used flow rate. However, cell rolling is a biophysical property of the transient interaction between the cell surface and the coated substrate that are generally fixed and cannot be altered. Perhaps cell rolling can be altered by patterning surfaces and

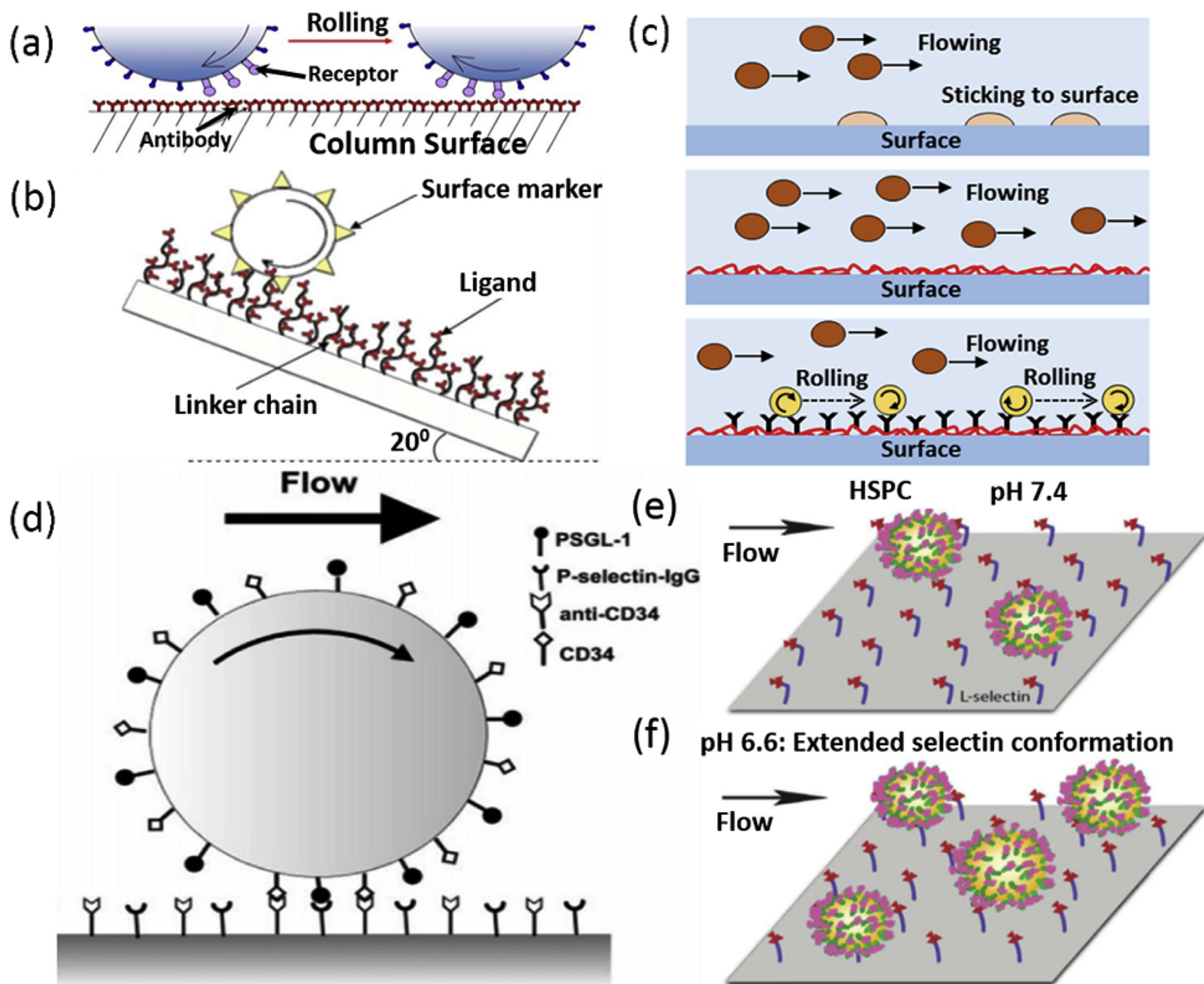

Fig. 6. Stem cell separation methods based on cell rolling. (a) Cell rolling on the antibody-immobilized solid surface [108], (b) cell rolling on a 20° inclined surface for optimum interaction [109], (c) cell motion effects on an unmodified and modified surface with amphiphilic phospholipid copolymer, (d) Interaction of a cell with P-selectin-IgG and antibody anti-CD34 when the cell rolls slowly [111], (e) and (f) increased HSPC adhesion to immobilized L-selectin molecules with acidic pH condition, pH 6.6 compared to pH 7.4 [113].

modifying them using surface chemistry, yet cell surface expression of markers would represent the limiting factor. Thereby the throughput of cell rolling based separation is expected to be lower than other competing technologies.

In general, cell rolling-based separation is a new and promising bio-inspired method for stem cell separation, thus more studies are expected to be performed in this area. We envision this technique could potentially be integrated with one of the previously explained techniques to represent a first step, or second step, in a 2-step label-free stem cell microfluidic separation technique.

6. Conclusion

The potential of microfluidics for stem cell isolation has been demonstrated, but there is still need for several improvements on existing devices. The label-free microfluidic isolation techniques discussed in this review has not reached the large-scale throughput required to process the number of cells needed in stem cell therapeutics. Using existing microfluidic devices, it can take several hours to process few milliliters of sample containing stem cells. However, throughput can be enhanced by parallelization of microfluidic processing techniques. We believe designing devices that combine two or more of the concepts discussed in this review could potentially bring a solution for achieving high throughput label-free stem cell separation with high recovery and purity rates. Another challenge hindering the wide adoption of microfluidics for commercial stem cell isolation is the low throughput manufacturing processes (i.e. photolithography and soft lithography) which do not lend itself to the production of disposable cartridges. With the utilization of microinjection molding and hot embossing, rapid microfluidic device manufacture can be realized in a cost effective manner.

Stem cells are being widely used for regenerative medicine, disease modeling, drug discovery and tissue/organ engineering, thereby driving the need for efficient stem cell isolation technologies. The two most commonly used cell isolation techniques, namely FACS and MACS, are reliant on labeling with monoclonal antibodies. The effect of antibody interference on cellular

function is not clear, and hence such techniques for therapeutics should be dealt with caution. New technologies such as microfluidics have proved to be viable alternatives in single cell separation, offering simplicity and reduced capital and operation costs. Stem cells are sensitive and often require enrichment without altering their native undifferentiated state, therefore, label-free separation is critically important for stem cells compared to other cell types.

Several label-free microfluidic methods to separate stem cells based on size, adhesion, surface receptor expression, and electrical characteristics have been proposed by scientists in the field, and we have attempted to summarize and discuss their findings in this review article. Electrical separation techniques discussed here primarily utilize DEP relying on physical characteristics of cell types such as size, membrane morphology and ionic conductivity differences. DEP forces work best when cells are close to the working electrodes, thus separation channels are limited to shallow heights that limit throughput. The requirement of resuspending cells in a specific medium increases processing time, and strong electrical fields could be damaging to stem cells. Despite these limitations, DEP has been widely adopted for cell separation both in commercial and academic settings, for its precise and reproducible results while maintaining cell viability. Size and adhesion based separation techniques discussed here rely on hydrodynamic interactions within microfluidic channels. Therefore, microfluidic chips need to be optimized according to the range of sizes of target stem cells and the non-target cells, and fluid flow needs to be precisely controlled. Size based separation could entail low purity rates when non-target cells have similar sizes of the target stem cells. Cell deformability also needs to be taken into account when considering a size based stem cell separator. Clogging could represent another challenge when working with high cell concentrations. Adhesion based separation of stem cells may impose high shear stresses that could damage cells or alter their native states. Hence, operational parameters need to be optimized for maximizing cell viability. This method has the potential to be used in a high throughput configuration given that the non-target cells are significantly different in terms of their adhesion to the substrate. The label-free method for stem cell purification based on cell rolling is underexplored, but is well suited for continuous flow separation. This technique is heavily reliant on cell–substrate interactions and for improvements in purity/recovery, more testing is needed using various cell types and surface markers. Techniques such as microfluidic mixing or by incorporating external forces such as DEP, enhanced cell–surface interactions can be achieved.

This article attempts to highlight potential technologies and the need for increased research at the interface of stem cells and engineering to bring label-free, non-destructive and minimally invasive stem cell sorting technologies to the clinics and hospitals. The proposed approaches have not matured to the level needed for stem cell therapeutics, and hence there is immense opportunity for technology optimization. The potential for lab-on-chip devices for stem cell isolation could be realized sooner by merging various techniques discussed here to yield higher purity and throughput.

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