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Stem Cell Separation Technologies

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Abstract

Stem cell therapy and translational stem cell research require large-scale supply of stem cells at high purity and viability, thus leading to the development of stem cell separation technologies. This review covers key technologies being applied to stem cell separation, and also highlights exciting new approaches in this field. First, we will cover conventional separation methods that are commercially available and have been widely adapted. These methods include Fluorescence-activated cell sorting (FACS), Magnet-activated cell sorting (MACS), pre-plating, conditioned expansion media, density gradient centrifugation, field flow fractionation (FFF), and dielectrophoresis (DEP). Next, we will introduce emerging novel methods that are currently under development. These methods include improved aqueous two-phase system, systematic evolution of ligands by exponential enrichment (SELEX), and various types of microfluidic platforms. Finally, we will discuss the challenges and directions towards future breakthroughs for stem cell isolation. Advancing stem cell separation techniques will be essential for clinical and research applications of stem cells.

Introduction

The regenerative potential of stem cells residing in tissues, blood, and bone marrow has been firmly established. These stem cells have been applied with varying success to the regeneration of these tissue and organs over the last decade. For example, implantation of autologous stem cells has been applied to the clinical treatments of myocardial infarction and macular degeneration [1, 2]. These progresses have created increasing needs for stem cell isolation techniques that are more efficient, simplified, ready-to-use, and can be broadly applied to many tissue types to provide highly functional stem cells. Although there are many stem cell separation methods that are commercially available and have been widely used, their efficiency and specificity are still insufficient. This review aims to briefly describe the current status in stem cell separation techniques with an emphasis on the latest, most promising achievements in this field. First, a short review of commercially established separation methods will be presented. This will be followed by a list of exciting emerging technologies that have shown potential as more powerful approaches to isolate rare stem cells populations.

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Traditional Separation Methods

The isolation of pure stem cell populations from a heterogeneous suspension is a fundamental aspect of clinical application and basic research. As such, many stem cell separation techniques have been developed and are commercially available (Table 1). Cell separation techniques can be broadly classified into two categories: techniques based on physical parameters (size/density), and techniques based on affinity (chemical, electrical, or magnetic couplings) [3]. Techniques of the first category take advantage of the fact that stem cells frequently have distinct size and density from other cells from the same tissue. The simplest example is density gradient centrifugation, in which a density gradient is established longitudinally in a test tube and the cell sample is layered on the top. When subjected to a centrifugal force, cells move along the density gradient and accumulate at a position where density of the medium matches that of the cells [4]. This technique requires *a priori* knowledge of the density of target cell type. Solutions capable of generating a density gradient (e.g. Ficoll-paque™, Percoll™, and RosetteSep™) are used as media for stem cell separation.

Field flow fractionation (FFF) is a sorting method that does not require the tagging of stem cells. In FFF, cells are exposed to either inertial or non-inertial forces, which move cells, based on size and morphology, to collectors at different time intervals. Therefore, FFF can separate different cell types based solely on physical parameters [5, 6]. When a stem cell population in the starting population has a distinct size and density from the rest of the cell population, FFF is a highly efficient cell separation method due to its tagging-free nature. One example of FFF in stem cell separation was illustrated by Roda *et al.* [7]. The authors selectively isolated human mesenchymal stem cells from fetal membrane and amniotic membrane-derived epithelial cells by a non-equilibrium gravitational FFF.

Dielectrophoresis (DEP) sorts out cells based on the intrinsic electro-physical properties of cells, and thus it does not require antibody labeling. A cell is polarized and creates a dipole when being placed in an electric field [5]. When placed in a non-uniform electric field, a net force is placed on the cells causing motion in one direction – based on cell size, surface charge, nucleic acid content, etc. [5]. CD34⁺ hematopoietic stem cells from blood or bone marrow have been isolated by DEP [8, 9]. In addition to being label free, DEP can be used to directly concentrate cells during isolation – attributes not found in most other techniques. However, the difference in cell size and dielectrophoretic potential is frequently not enough to distinguish stem cells from non-stem cells and stem cells purified by DEP are often contaminated by unwanted cell [6].

A major limitation of these size and density based methods is the lack of resolution, since the size and density difference between stem cells and non-stem cells is not absolute. Furthermore, these methods cannot be applied to the isolation of many other stem cells that has similar size and density as the surrounding non-stem cells. To overcome these limitations, affinity-based separation methods were developed, which uses antibodies to label surface markers that are specific to stem cells. These antibodies are linked to a tag that can be picked out by automated machines. In fluorescence-activated cell sorting (FACS), antibodies tagged with fluorescent dyes are attached to cells in mixed suspensions. The cells are then sorted individually based on fluorescence and light scattering. FACS can provide highly pure (95% or higher) cell populations. On the other hand, it requires expensive equipment and has a limited throughput ($\sim 10^7$ cells/hour) [10, 11]. Separation using antibody-coated magnetic beads is an alternative to FACS. Magnet-activated cell sorting (MACS) allows target cells to be processed in parallel, achieving faster separation ($\sim 10^{11}$ cells/hour) [10]. The purity of stem cell population using MACS is about 75%, and a major

concern is interference caused by the magnetic beads in the purified stem cell population [5, 12].

In contrast to labeling methods, static adhesion separation of stem cells, also known as pre-plating, is based on the phenomenon that stem cells, compared to the rest of the cell population, tend to adhere to culture plates and dishes. This method has been shown to be able to enrich mesenchymal stem cells from bone marrow and to isolate human adipose-derived stem cells from lipoaspirate (tissue removed during liposuction) [13–15]. However, since some non-stem cells also adhere to the culture plate, albeit less efficiently, stem cells isolated by pre-plating often contain un-wanted cell types. As a means of selectively expanding the stem cell population, a stem cell promoting media can be used [5]. An optimal media composition (e.g. glucose concentration, serum percentage, etc.) can substantially favor the proliferation of various stem cells over other cells. As a consequence, culturing the starting population under the selective media can effectively enrich a target stem cell population.

Emerging Methods

For the purpose of achieving high purity in stem cell separation, simplicity of the separation process and mass production of samples, new separation methods have been emerging in the field. This section focuses on the latest stem cell separation technologies. These new techniques are either an improved version of the existing method or based on novel rationale. Special features of each technology and examples of various stem cell types are listed in Table 2.

One novel stem cell separation method utilizes poly(N-isopropylacrylamide) (PNIPAAm), a temperature-sensitive polymer that is soluble in water at room temperature (20 °C), but precipitates from aqueous solutions at 32–35 °C. Based on this rationale, using PNIPAAm-conjugated antibodies that recognize stem cell-specific markers, stem cells can be captured and precipitated out of aqueous solution by switching temperature from 20 °C to 32–35 °C. These precipitated cells can be separated from the rest cells by centrifugation in a two-phase density gradient formed by polyethylene glycol (PEG) (upper phase) and dextran (lower phase). The resulting target cells form sediment bands at the interface of the two phases, while contaminating cells are pelleted at the bottom. This temperature-sensitive polymer approach in aqueous two-phase system is simple, fast, and suitable for large-scale separation [16]. However, when tested on separating CD34⁺ KG-1 cells with a 10% starting population in Jurkat cells, the KG-1 cell sediment band contained significant amount of contaminating Jurkat cells. Since partition of cells is affected by concentrations of NaCl added into the lower phase, this can be the main reason why non-target Jurkat cells were present in the target KG-1 cell band. Increasing NaCl concentration in the lower phase, Promoting sufficient amount of antibody conjugates bounded to cells, or repeating the aqueous two-phase separation technique a second time on purified cell mixture can improve purity. In addition, an improved system needs to be designed for high recovery rate and even higher purity, considering the very low number of stem cells from the starting population in real applications.

Cell SELEX (systematic evolution of ligands by exponential enrichment) uses RNA, ssDNA, or modified nucleic acids as aptamers to selectively capture target cells [5, 17]. To begin SELEX-based cell selection, libraries of aptamers (non-naturally presented oligonucleotides) are incubated with stem cell population and any unbound aptamers are removed. The bound aptamers are subsequently released from surfaces of the stem cells and are then further amplified by RT-PCR for incubation in following SELEX cycles (or aptamer evolution) [17]. These capture and release cycles will yield a population of

aptamers with high affinity to target stem cells. Mesenchymal stem cells from human and other species have been selected from bone marrow using the SELEX technique [18, 19]. The major advantage of SELEX is that aptamers for enriching target stem cells are experimentally evolved. This advantage overcomes the lack of *a priori* knowledge of the surface marker properties of many target stem cells [17]. In addition, SELEX does not use costly antibodies, and can capture non-protein markers on stem cells. As a screening technique, SELEX tests large amount of candidate aptamers [5]. However, SELEX requires lengthy processes of aptamer selection and amplification, and the throughput of SELEX is low. Finally, even after numerous rounds of aptamer evolution, the specificity is sometimes insufficient to collect high purity stem cells.

Microfluidics is an emerging field aims at miniaturization of devices and processes, and it has been applied to stem cell separation. Most of the methodologies discussed here have been adapted to microfluidic platform. Microfluidics consumes less sample and reagent. The whole process of sample injection, sorting, and collection is under continuous operation on a single chip. Some parts of the procedures can be automated and requires less operator handling [5, 6]. On the other hand, throughput is a potential concern for high-volume applications. Some researchers engineered creative ways to increase flow rates, increase single device surface area, or parallelize the microfluidic devices. Below are a few examples of cell separation techniques being miniaturized onto microfluidic platform.

For example, SELEX microfluidics was used to select suitable aptamers for a more efficient and rapid screening process [17]. Negative DEP (nDEP)-based microfluidics has been used to separate Stro-1⁺ human mesenchymal stem cells from bone marrow and endothelial progenitor cells (EPCs) from white blood cell samples [5, 19]. Droplet microfluidics encapsulates single cells in distinct field drops, and screens single droplets on a microfluidic platform to detect and collect rare progenitor cells from human periosteal tissue [20]. Label-free affinity-based microfluidic cell sorting devices does not require pre-labeling of cells with fluorescent dyes/magnetic beads but instead focus on immobilized antibodies. Antibodies have been immobilized onto the luminal surface of a parallel array of hollow fibers for the capturing of CD34⁺ cell lines (KG1a cells). Detachment of target cells was performed in fluid flow with a pre-defined shear stress. Microfluidic devices coated with antibodies have been demonstrated to selectively capture mouse adipose-derived stem cells from a heterogeneous suspension [21] and EPCs from human whole blood [22, 23]. In the EPC selection study, antibodies are immobilized on alginate hydrogel coating. After target cells were captured in antibody-gel complexes, a rinse of EDTA can dissolve the alginate hydrogel and collect EPCs at high purity [22]. The above mentioned promising applications showed the advantage of microfluidics in multiplying devices via parallel running, and simplifying the separation process, but great efforts should still be put to achieve higher purity, higher stem cell recovery, less operation time, and minimizing the use of costly antibodies in adhesion-based separation.

Although each tissue engineering and clinical case is unique there are a couple of benchmarks that can be generally applied to successfully use microfluidic across all engineering and clinical applications. The target cell populations must be reliably isolation without tags or labels that could potential interfere with post-separation analyses and/or adversely change cell function. Although no specific quantitative benchmark in terms of throughput, purity, efficiency, or total cell yield have been established, for a microfluidic platform to progress as a stem cell separation platform it much exceed state of the art conventional platforms: purity greater than 95%, throughput of at least 10¹¹ cells per hr., and efficiency of 95% or better. To date, no microfluidics platforms have achieved such benchmarks for large-scale separation. Several stem cell separation microfluidics platforms in the literature have been to meet and excess one or two benchmarks, but no platform to the

best of the authors' knowledge has separated stem cells at throughputs comparable to MACS without sacrificing efficiency, purity, or both. Additionally, there has been very little work on understanding the influence the separation process has on the function and phenotype of stem cells post-separation, so it is not known which technique is best suited specifically for separation of these sensitive cells. It should be noted that although most researchers continue to aim to achieve high purity isolation of cells from the starting heterogeneous cell suspension, ultra-high purity in the context of stem and progenitor cells might not always be desirable. The lack of supporting non-stem/progenitor cells may ultimately impact the viability and/or functional characteristics of the stem/progenitor cells in culture, since supporting cells mimic micro-cellular environment for stem cell culture.

Future Directions and Challenges

Much progress has been made over the past decade in rare stem cell isolation and enrichment. Among the many stem cell separation methods, affinity-based approaches are so far most efficient and reliable, due to the high specificity of antibodies that recognize stem cell surface markers. FACS can achieve an impressive >95% purity, while MACS is portable. With growing knowledge on better stem cell markers, and the generation of more specific aptamers by SELEX, affinity-based techniques will still be very powerful in the future for stem cell separation. In parallel to pursuing higher purity, other important challenges should also be met, such as less perturbation to stem cells, rapidness, and cost effectiveness. As a method to isolate stem cells in an automated, miniaturized, multiplex, and portable fashion, microfluidics offer exciting solutions to these challenges. Although the purity of stem cell separation by microfluidic devices needs to be substantially improved, merging conventional technologies onto microfluidic platform will be extremely beneficial. Last but not the least, achieving stem cell separation in large scale is a major challenge for tissue engineering and its clinical application. Most separation methods have room to increase throughput. Among them, FFF, DEP, and density gradient centrifugation show great potential in large scale production. Future improvements on stem cell isolation will play important roles in the development of clinical stem cell therapy and translational biological research.

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Highlights

- Stem cell isolation methods that provide functional, viable stem cells are needed.
- Traditional stem cell separation platforms and their limitations.
- Emerging stem cell separation methods improve and complement traditional protocols.
- Several challenges still remain in stem cell isolation.

Table 1

Conventional Stem Cell Separation Technologies.

Method	Advantage	Disadvantage	Examples	Reference
Density gradient centrifugation	Large processing quantity, easy scaling up, facile,	Low purity, more heterogeneity output	Ficoll-paque; Percoll	[23, 24]
Density gradient centrifugation-negative selection	Large processing quantity, easy scaling up, facile, high purity	Only applied in whole blood	RosetteSep (Stem Cell Technology)	[25]
Pre-plating	Large processing quantity, easy scaling up, facile,	Low purity, more heterogeneity output	Human embryonic stem cells; human adipose-derived stem cells	[13–15]
Conditioned expansion media	Large processing quantity, easy scaling up, facile,	Low purity, more heterogeneity output	Human mesenchymal stem cells from bone marrow	[26, 27]
Dielectrophoresis (DEP)	Label free on stem cells, sort cells based on viability, sort cells directly without cell dilution in fluid	Cross reactivity cause low purity	Live adipose derived stem cells	[8, 9, 28]
Field flow fractionation (FFF)	Label free on stem cells,	Can only separate stem cells based on size and density differences	Human mesenchymal stem cells from fetal membrane	[7, 29]
Flow cytometry (FACS)	Powerful, high precision, high sensitivity, high resolution	Requires well-trained personal for operation, costly, requires a special apparatus, not for large-scale processing	Human Embryonic Stem Cell-Derived Neural Cell Populations, human hematopoietic stem and progenitor cells	[30–32]
Magnetic Activated Cell Sorting (MACS)	Simpler than FACS	Target stem cells contains magnetic particles	Column free EasySep (Stem Cell Technology); MACS (Miltenyi)	[13, 29, 30, 32, 33]

Table 2

Emerging Methods for Stem Cell Separation

Method	Advantages	Disadvantages	Examples+	Reference
Aqueous two-phase system using temperature responsive polymer	Simple, scalable, antibodies reusable,	Low purity, low recovery rate	CD34+ KG1 cells	[16]
SELEX	Select stem cells without any molecular information, capture protein and non-protein targets, reduce in vitro testing cost,	The screening process for aptamer identification is not efficient, low specificity, low processing quantity	Mesenchymal stem cells from human and other species	[18, 19]
Microfluidics	Label free on cells, miniaturization, several processes on one chip, automation, less operator handling, mass processing	Not very high purity and high recovery rate in some cases, operation time not short	Negative DEP microfluidics, droplet microfluidics, cell affinity chromatography based microfluidics	[5, 20–22, 34]