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Culturing cells for life: innovative approaches in macroscopic and microfluidic cultures, with an emphasis on stem cells

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In 2006, Whitesides, writing about microfluidics, said that microfluidics is in early adolescence and it is not yet clear how it will develop. Today, almost 20 years later, microfluidics became a fully developed, highly sophisticated, multidisciplinary field that had entirely honoured its early promise. Its strength stems from the knowledge and know-how, coming from multiple disciplines such as physics of fluids, engineering, and microfabrication in the beginning, followed, more recently, by cell biological research, in full bloom nowadays. In microfluidic devices, the environment of cells such as chemical and mechanical gradients can be reproduced, making biological studies even more compelling. The red thread of this review paper follows the new insights and discoveries in both traditional macro- and microfluidic cell culture brought into the cell biology field, especially in the culture of stem cells, filled with promise in the field of regenerative medicine. Microfluidic devices provide an environment that is much closer to that of *in vivo* cell culture than the conventional culture platforms, where large amounts of cells are cultured and the environment of individual cells cannot be distinguished. The convenience of live cell imaging, portability, and the integration of sensors to precisely control various parameters, has expanded cell biologists' arsenal. In addition, microfluidic devices, integrated with different functionalities, that is, the automated cell culture systems, will be discussed as well.

KEYWORDS

macroscopic culture, robotics, AI, microfluidic culture, stem cell culture, stem cell differentiation

1 Introduction

Stem cells are a special category of cells that have potential for tissue engineering and other cell-based therapies, because they can transform themselves into other types of functional cells. Until recently, scientists, mainly, worked with embryonic stem cells which are pluripotent, and non-embryonic, or with mesenchymal stem cells that are multipotent, that is, they only can differentiate into specific stem cells (Zhang and Austin, 2012; McKee and Chaudhry, 2017; Zhang et al., 2017; Aghlmandi et al., 2021).

Embryonic stem cells as well as induced pluripotent stem cells, are, especially, interesting for cell replacement therapy (Konagaya et al., 2015). It has been suggested that the dysregulation of stem cell properties may induce certain types of cancer (Reya et al.,

2001; Dalerba and Clarke, 2007; Ramalho-Santos and Willenbring, 2007). The large amounts of potent cells for cell-therapy applications are supplied mostly by traditional large-scale culture and, more recently, by automated culture systems. The choice to focus the discussion of cell culture on stem cells was dictated by the importance of regenerative medicine. Fuchs and Segre in their excellent review paper (Fuchs and Segre, 2000) called stem cells “A New Lease on Life”. The large-scale culture methods were significantly improved recently (Liu et al., 2010; Konagaya et al., 2015). To maintain induced pluripotent stem cells in an undifferentiated state for a long time, automated culture methods were developed (see also (Terstegge et al., 2007; Thomas et al., 2009; Hussain et al., 2013; Soares et al., 2014)). Even more recently, autonomous robotic AI systems were developed that allow the optimization of cell culture protocols (Sebastian, 2022). All these changes have transformed traditional macroscale culture into a most interesting technique, with a wide perspective. In the traditional cell culture of the past, the microenvironment of stem cells that determines their fate, could not be controlled but in the emerging robotic AI systems and in microfluidic cell culture, this becomes possible.

In this review, the recent applications of automated macro- and microfluidic cultures in cell biology are discussed, and their future perspectives are evaluated (Scheme 1). The comparison is not confrontational, and no conclusion on the superiority of the different cell culture methods is imposed onto the readers.

2 Macroscale cell culture traditional culture and robotics

Cell culturing is a basic experimental technique, a versatile tool in cell biology research. It is essential to cell biology, and tissue engineering, as cells can be cultured in large amounts. Mammalian cells are almost all, adherent, needing a surface, appropriate for attachment and proliferation. *In vitro*

experiments with adherent human cells, currently, are performed, by using a two-dimensional (2D) monolayer platform, where the cells are plated onto a polystyrene plate that is surface-treated, to stimulate cell binding. This model is called a two-dimensional (2D) monolayer platform.

Conventional (traditional) cell culture methods rely on Petri dishes, cell-culture shake flasks, microtiter plates, and bioreactors. Reagents, and cell suspensions have to be individually pipetted, a laborious process. The Petri dish was invented over a century ago by Petri, a technician in the laboratory of Koch, the well-known German bacteriologist who discovered the TB bacterium. Since then, the Petri dish became a staple in cell culturing (Zhang, 2004).

Conventional *in vitro* methods allow the growth of most cells in humidity, temperature, and CO₂-controlled incubators, and precise protocols have been established for different cell lines. However, these methods are quite expensive, because of the amounts of cells and reagents and the time they take up.

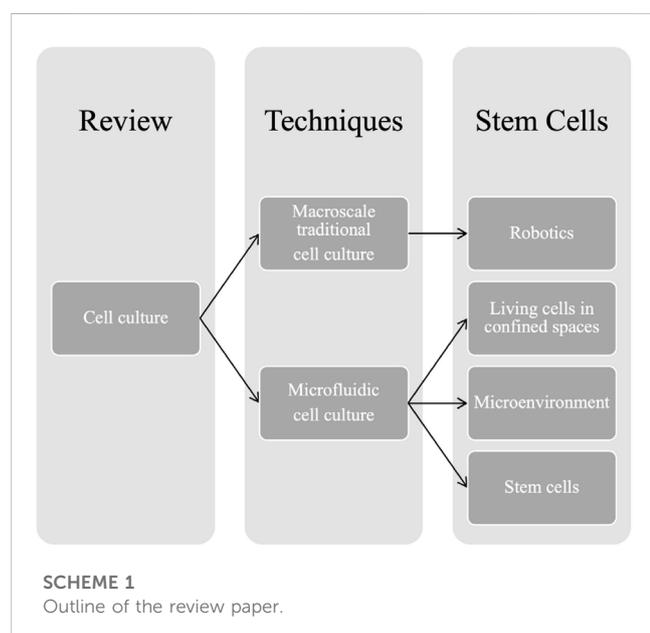
The macroscale environment is very different from that of the real biological systems, while three-dimensional platforms (3D) represent much better the natural context of cells in tissues. The 3D architecture (Zhang, 2004) represents much better a cell in its *in situ* environment. In the 3D platform, cells are in contact with each other through a soft extracellular material and not a rigid flat surface as in the case of the 2D culture.

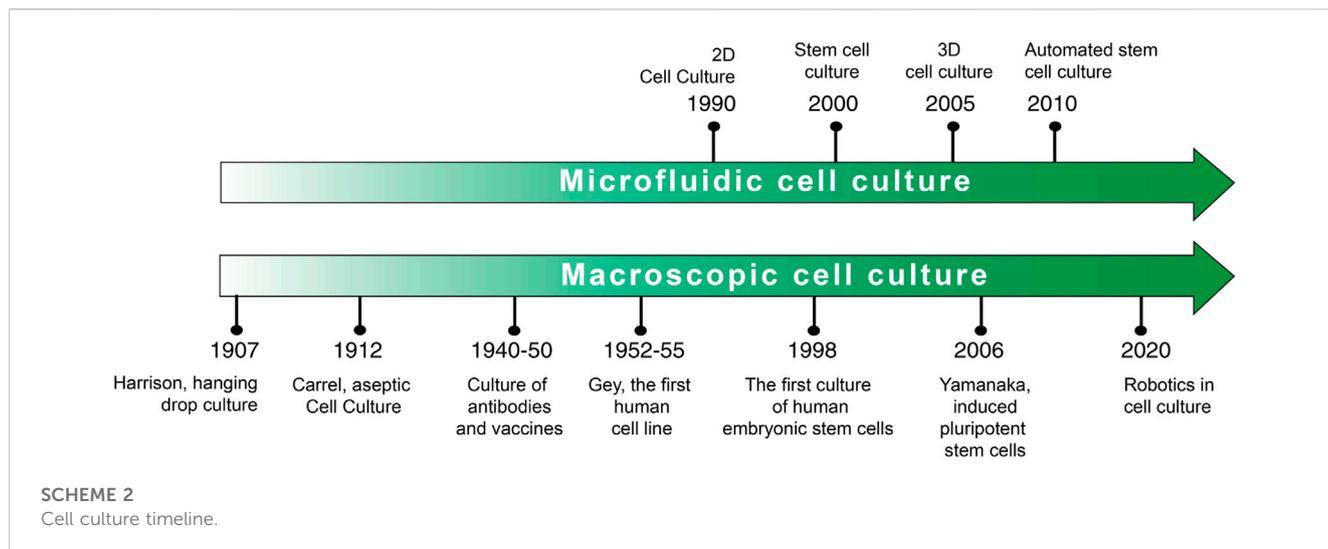
Even now, the most used culture platform, is the 2D macroscopic cell culture, by using Petri dishes or flasks. Although these platforms are less expensive than animal models and result in reproducible quantitative studies, the physiological relevance of the information retrieved from these *in vitro* studies is often debatable. The structure and physiology of living tissues, as well as the dynamic 3D environments *in vivo* (Huang et al., 2022) are not reproduced correctly in the 2D platforms but, in spite of all these disadvantages, 2D remains still the method of choice for research laboratories and the pharmaceutical industry while 3D culture, gradually, is gaining popularity.

Scheme 2 a timeline of the two most important cell culture techniques is shown below. It has to be emphasized that, presently, the so-called traditional cell culture is quite different from the way it was a century ago. Many recent improvements and refinements along the way have relieved some of the hurdles and opened new ways to culture cells.

Generally, 2D cell culture requires passaging of cells, until a confluence level of 70%–80% is reached. Then, they are split between flasks to provide them with enough space to grow effectively. The use of robots that can automate this process, eliminates, not only subjective judgment, but also automates processes such as harvesting and passaging. Interestingly, studies that compared the automated to the manual culture, have shown no statistical differences in cell count and viability of cells when harvested, demonstrating the significant advantage of automation to save time, without compromising efficacy. Additionally, these systems allow unattended operation over a long time period, as well as allowing for evaluation of variables, including, pH, nutrient or waste concentration, cell concentration, and viability (Chapman, 2003). The use of robotics in automated cell culture can reduce human intervention and error and increase the efficacy (Sebastian, 2022).

Kanda et al. (Kanda et al., 2022), recently, has demonstrated how to optimize the process by using robotics and an artificial





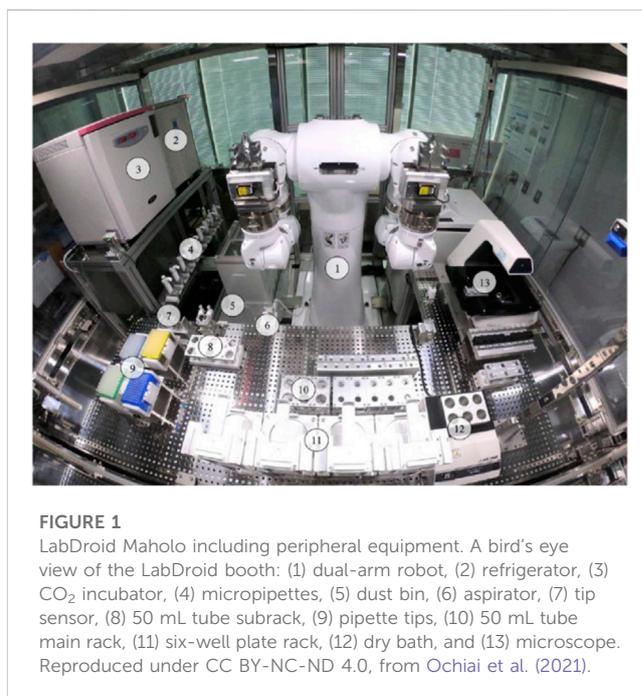
intelligence system, called LabDroid Maholo has been developed with an integrated imaging system and computer algorithms (Sasamata et al., 2021; Kanda et al., 2022) that have improved, significantly, the cell culture protocols. For example, the system was programmed to differentiate stem cells into retinal pigment epithelial cells and the level of cell differentiation was analyzed by using computer algorithms, and statistical analyses.

It has been found that combining robotics and artificial intelligence to optimize complex cell culture protocols is a viable way to use macroscale cell culture to differentiate stem cells for therapeutic purposes (Figure 1).

As mentioned previously, stem cells, having the ability to self-renew and differentiate in specialized cell types, are extremely important in regenerative medicine. They may be especially useful to study early human development (Imreh et al., 2006; van Noort et al., 2009). The use of stem cells for clinical applications requires high quality and quantity of cells.

Although robotic platforms such as LabDroid Maholo will need further improvements, it is an important step toward the reproducible manufacturing of cells, especially, for regenerative treatments. Other automated platforms are currently available, and they may incorporate biosafety cabinets and incubators as well (Crombie et al., 2017). Less expensive pipetting robots, for academic settings, have also been developed (Dettinger et al., 2022) and they can be used, without prior knowledge, to automate many experimental procedures in standard cell culture vessels. These studies are extremely important as stem cells hold enormous promise for fundamental biological studies and as well as for cell-based therapies.

These innovative approaches to macroscale cell culture show that this culture became again a viable technique, creating niches for applications in special fields such as, for example, stem cell studies. Most probably, in the future, new developments will emerge, and macroscale cell culture will continue to be used for different purposes, in parallel with new technologies such as microfluidic cell culture, microcarriers, and microencapsulation (McKee and Chaudhry, 2017).

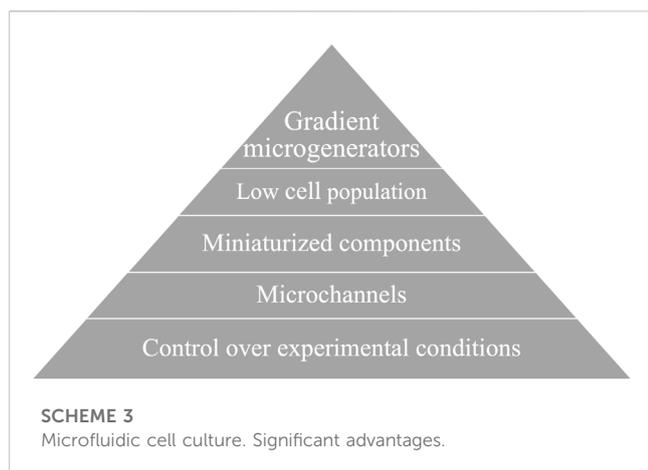


3 Microfluidic cell culture

3.1 Introduction

“Learning to think on an entirely different scale is a new and exciting challenge in microfluidics” (Beebe et al., 2002).

Microfluidics is the science and technology of the controlled manipulation of low volumes of liquids (nanoliters or less), constrained to microchannels with, usually, rectangular cross-sections, and diameters around hundreds of micrometres. In such small channels, the behaviour of a liquid is significantly different from that at the macro scale as the flow gains completely new features at this scale. The effect that become dominant in



microfluidics includes laminar flow, and the mixing, mainly occurs by diffusion, at the interface between laminar layers, compared to the macrosystems, where turbulent flow determines fast convective processes. When proper mixing configurations are integrated with microfluidics, due to the small distances in the channels, mixing is completed rapidly.

Microfluidics involves the design of these devices and the study of the behaviour of fluids in the narrow microchannels. In addition to the channels, microfluidic devices contain also miniaturized components for fluid's manipulation, such as microvalves, micropumps, micromixers, and micro separators. The small length scales, associated with microfluidics, enable faster analysis, requiring lower amounts of, possibly, expensive reagents.

It has to be mentioned that microfluidics is often considered a new field but, actually, it was born in the 1990s when the seminal paper by Andreas Manz (Manz et al., 1990) was published. The paper envisioned, for the first time, an integrated platform for performing a multitude of analysis steps. Microfluidics, developed by the confluence of a number of concepts, techniques, and materials lends from previously existing fields, especially, analytical chemistry (microanalysis) and later on, material science, physics of fluids, and microelectronics.

As stressed by George Whitesides, an eminent personality in this field (Whitesides, 2006), "microfluidics can be considered as having four "parents" that contributed to building it up: molecular biology, molecular analysis, national security, and microelectronics". Gas-phase chromatography and capillary electrophoresis, allowing the separation of chemical compounds, by flowing very small amounts of a sample in capillaries, was developed already in the 1950s. These methods inspired researchers to improve the separation, by reducing even more the size of capillaries. However, the most important precursor of microfluidics is the microchip (the word "chip" used for "microfluidic chip" is borrowed from microelectronics). Although at the beginning of microfluidics, photolithography was used with mainly standard semiconductor materials, silicon, and glass, soon, due to lower cost and more convenient properties, they were displaced by polymers. The main drawback of Silicon microfluidic chips is their opacity, making it impossible to use optical detection. For this reason, microfluidics split from microelectronics and semiconductors technology and developed techniques that facilitated significantly the further development

of the field. Research for military purposes stimulated the effort in the development of microfluidics and molecular biology strongly contributed to microfluidics' birth and evolution. In the 1980s, the interest of scientists in sequencing nucleic acids as well as developing precision equipment for the PCR (polymerase chain reaction) technique, led to the development of machines capable of working with very small samples.

Today, microfluidic technology represents a distinct, well-consolidated research field, with well-defined boundaries and extensive applications in various domains such as synthesis of nanoparticles, point-of-care devices, biosensors, drug screening and delivery systems, wearable biosensors, devices for cell analysis, and cell sorting. Also, microfluidic systems can be used for point-of-care clinical applications, or even deployed in remote locations. The development of inkjet printheads, and DNA sequencing chips are only some of the multiple applications of microfluidics. In addition, Micro Total Analysis Systems (μ TAS) or lab-on-a-chip, may integrate complex analytical processes that commonly solicit a whole laboratory, in a small device, without off-chip sample preparation. They are also able to perform separations and detection with high resolution and sensitivity. The convergence of microfluidics and nanoparticle synthesis allowed the preparation of nanoparticles with a narrow size distribution, important for developing new functional materials (Badilescu and Packirisamy, 2012).

3.2 Culturing cells on the microscale

As Young and Beebe stressed in their excellent tutorial review paper, 'culturing cells in microscale environments requires knowledge of multiple disciplines, including physics, biochemistry, and engineering' (Paguirigan and Beebe, 2008; Young EW and Beebe D, 2010). They have shown that at the beginning of microfluidics, research was focused more on understanding the chemical and physical phenomena at the microscale, and only later on, started the study of complex structures such as cells.

Over the past decades, the potential of microfluidics to change cell biology research had become gradually a reality (Scheme 3). The similitude of the dimensions of microfluidic channels and the size of cells, and the ability to manipulate small volumes of fluid in micrometre-scale channels, together with many other important advantages of microfluidics such as flexibility, rapid energy dissipation, and a fast-prototyping fabrication for customized experimental design, make microfluidics suitable for cell culture and for cells' subsequent analysis (Tehranirokh et al., 2013; Mehling and Tay, 2014; Kerk et al., 2021). Microfluidics reduced cell populations to a few hundred cells, compared to macroscopic cell cultures that contain around 10^4 – 10^7 cells (Halldorsson et al., 2015). Microfluidics allows precise control over experimental conditions via custom-designed chip architectures, automation, and coupling to downstream analysis platforms (Yeon and Park, 2007). One of the key benefits of microfluidics is the ability to closely mimic the cell's natural microenvironment, by creating chemical gradients. The composition of the media can be optimized rapidly because of the small volumes of liquids involved.

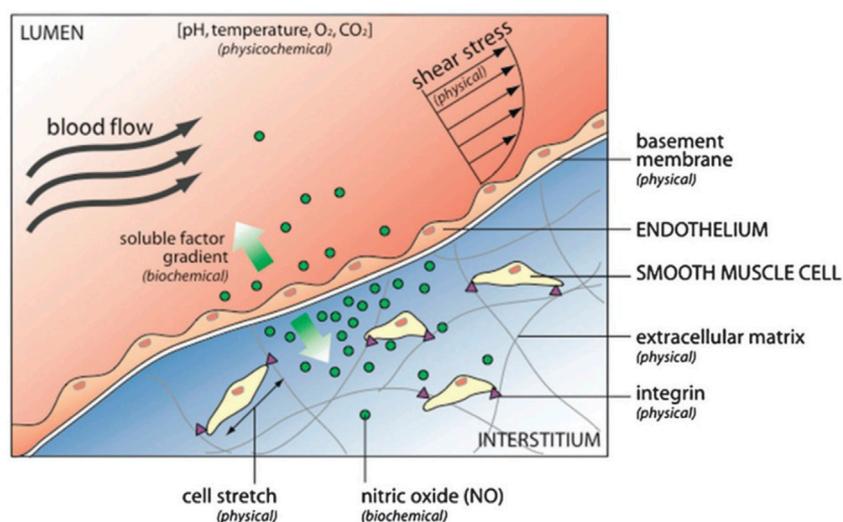


FIGURE 2

The cell microenvironment consists of physical, biochemical, and physicochemical factors. For example, the endothelium that lines blood vessels is exposed to hemodynamic shear stress (external physical force) that stimulates biochemical response releasing nitric oxide. Nitric oxide diffuses to neighboring smooth muscle cells, where it regulates cell contraction and relaxation. Reproduced from Young EW and Beebe D. (2010) with permission from the Royal Society of Chemistry.

Also, microfluidics allows the relatively easy patterning of molecules and cells and high levels of experimental automation. In microfluidics, the consumption of nutrients is faster than in traditional culture, because of the higher surface-to-volume ratio (Torino et al., 2018). For this reason, microfluidic culture often needs an integrated perfusion system.

In the course of time, two major microfluidic technologies have been developed: continuous fluid flow in enclosed microchannels and droplet-based cell culture systems, involving, either immiscible droplets in enclosed microchannels, or digital microfluidic manipulation of droplets on planar surfaces (Ng et al., 2015). In digital microfluidics, discrete droplets are manipulated on a hydrophobic surface. The first lab-on-a-chip platform for complete mammalian cell culture was introduced by Wheeler (Barbulovic-Nad et al., 2010) and it was, at the time, the first microfluidic platform able to implement all the steps, required for mammalian cell culture, including passaging. The results obtained by using digital microfluidics were found to be similar to those obtained by macroscale cell culture experiments. An electrical potential is applied to an array of insulated electrodes to actuate the droplets. Cells that grow while suspended in a liquid culture medium may be further manipulated by the use of electrical, optical, or magnetic forces. To enhance suspension culture performance, mammalian and microbial cells are encapsulated in hydrogel beads as a scaffold to create a 3D environment (Mulas et al., 2020; Kerk et al., 2021).

Separating cells, positioning them in confined regions can be done by using cell patterning methods (Takayama et al., 1999). To perform patterned cell deposition, the authors used parallel liquid streams in capillary networks to pattern the substrate with adhesion promoters and inhibitors (cell non-adhesive molecules,

for example, polyethylene glycol), followed by depositing cells in patterns of parallel stripes. This method generates patterns inside the channels of microfluidic networks. Other techniques, such as microcontact printing of collagen patterns, were also used to permit cell attachment on PDMS substrates (de Silva et al., 2004; Huang et al., 2022).

Three-dimensional culture in digital microfluidics was first demonstrated by Fiddes (Fiddes et al., 2012). He cultured NIH-3T3 cells in hydrogel discs for 7 days, by pipetting the cell suspension and agarose onto the bottom plate. After assembling the top plate, the mixture was cooled to help the gelation of the agarose.

Cells can also be encapsulated, by using hydrogel materials such as collagen, gelatin, fibrin, alginate, and agarose (Wan, 2012). Droplet microfluidics technologies are able to encapsulate the cells into picoliter-sized droplets for growing and subsequent analysis.

A plethora of microfluidic devices, based on two-dimensional and three-dimensional cultures have been developed. As mentioned previously, the most important advantages of microscale technology are better spatial and temporal environmental control and the ease of patterning molecules and cells. At the microscale, complex 3D environments can be created, for example, mimicking microvasculature (Mannino et al., 2018), by incorporating endothelial cell monolayers into microchannels to generate blood vessels-on-a-chip. Endothelialized microfluidic technology has allowed the study of microvascular phenomena, impossible to investigate previously.

The first applications of microfluidics to cell culture and subsequent analysis were using devices made of silicon and glass because polystyrene, frequently used in macroscopic cell cultures, has limited use in microfluidics, due to the difficult mold fabrication and bonding. A number of challenges of PDMS-based microfluidic cell culture are summarized in

reference (Tanyeri et al., 2018). One of the most important requirements for PDMS-based devices is a special surface treatment to promote cell adhesion and proliferation on the hydrophobic surface of PDMS. Typically, the surface of PDMS is treated with oxygen plasma and then coated with fibronectin or collagen which are adhesion-promoting. It has been shown that surfaces need to be only moderately hydrophilic, in order to avoid the antifouling properties of highly hydrophilic surfaces that may preclude cell culture. Selective treatment of PDMS has been recommended with only the culture chambers made more hydrophilic.

Small molecules absorbed by the porous PDMS may have a negative effect on cell culture applications. To avoid this, PDMS can be coated with low-permeability materials such as parylene and wax (Tanyeri et al., 2018).

Progress in the area of biology-related microfluidic systems has been mostly in proof-of-principle demonstrations and it has been delayed because the behavior of living cells in confined spaces was difficult to understand. Cell culture techniques cannot be directly transferred from macroscale to microfluidic environments, without a deep understanding of the physics of the microscale. Some recent reports have also begun to reveal challenges with existing microfluidic methods, and have, in some cases, provided possible solutions that may lead to new directions in the field (Velve-Casquillas et al., 2010).

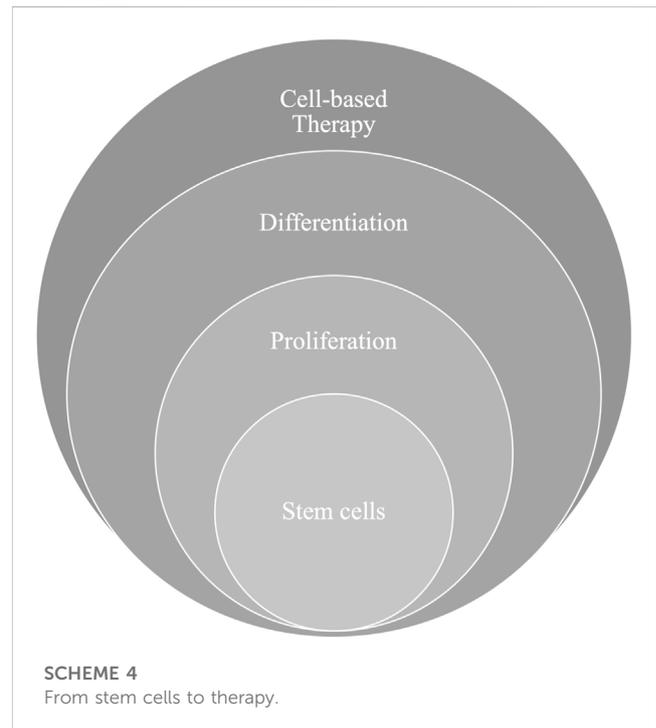
3.3 The cellular microenvironment gradient microgenerators

The most important benefit of using microfluidics for biology is the ability to tailor the cellular microenvironment. Unlike the traditional macroscale culture, where the parameters can be controlled only for a population of cells, at the microscale, the spatial and temporal gradients in the microenvironment of cells can be created and controlled.

The microenvironment of a cell is defined by chemical and mechanical parameters. The chemical environment is composed of soluble molecules around the cell, associated with the composition of the culture medium, and the mechanical environment is composed of the extracellular matrix (ECM), associated with the substrate composition.

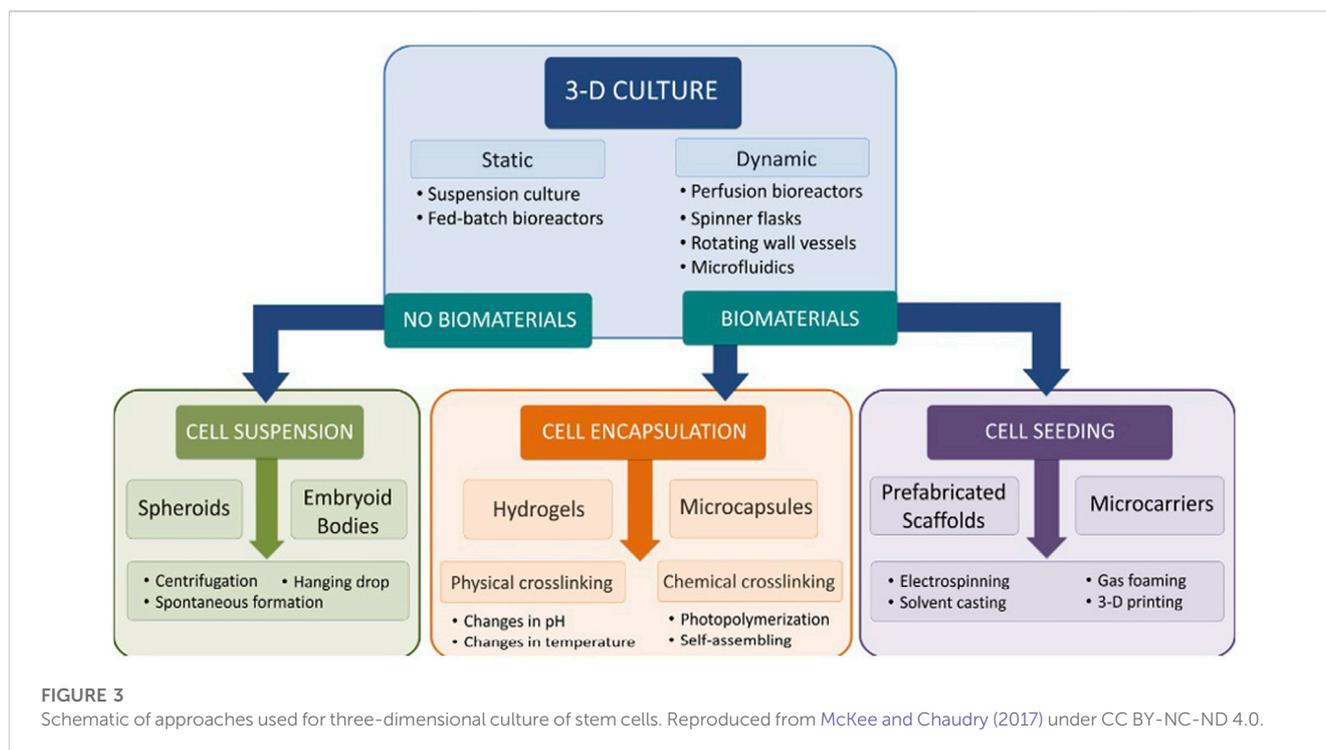
Cells reside in a milieu composed of soluble factors, cell-matrix interactions, and cell-cell contacts, living within an environment with specific physicochemical properties (pH, oxygen tension, temperature, and osmolality) (Figure 2).

Microfluidics has the ability, not only to locally address parameters of the cell environment but also to change them automatically. Natural stimuli that happen in biological processes can act by producing a chemical gradient. Concentration gradients using hydrogel from collagen or agars to create concentration gradients were found unstable (Velve-Casquillas et al., 2010) and other macroscopic methods, based on chambers, separated by membranes, generated unstable gradients as well (Zigmond, 1977). Precisely defined chemical gradients in microfluidic devices have been successfully generated by microscale gradient generators based on single-layer PDMS.



The simplest microgenerator is based on the laminar flow in a T-sensor and the shape of the gradient depends on the flow rate. However, in this device, cells may be subjected to mechanical stress that may damage them. In addition, long experiments require large volumes of often expensive reagents. The gradients are created by diffusion at the interface of two streams, then, flowed over cells to expose them to the gradients (Paguirigan and Beebe, 2008). In addition to the flow-based gradient generators, static gradient devices can be integrated more easily, and even for long experiments, minimal reagents are required (Young EW and Beebe D, 2010).

The cell microenvironment results from biochemical and physical factors and has an important effect on the growth and development of neighbouring tissues. For example, the local microenvironment of stem cells, called the stem cell niche, regulates their survival, self-renewal, and differentiation (Discher et al., 2009). Most cells in the body are attached to the surrounding extracellular matrix (ECM) *via* cell surface integrins, responsible for transducing mechanical signals from the adhesion sites to the cytoskeletal machinery. It has been shown that the mechanical properties of cells are essential to the mechanisms by which cells sense forces and transduce them into chemical signals (Janmey and McCulloch, 2007). Migration, proliferation, and differentiation of cells are all driven by chemical signals. Since gradients have an important role in many processes, recent studies are incorporating them into their assays to understand better the effect of gradients on cells. Microfluidics is expected to play a significant role in the implementation of such gradient assays, because it can establish stable gradients (Keenan T and Folch, 2008). Some recent studies examine the combinatorial effects of soluble factor signaling and cell-matrix interactions on cell behavior.



3.4 Stem cells in microfluidics

Stem cells are unspecialized pluripotent cells able to produce any type of cell in the human body. They can migrate to the disease-affected area, and play a pivotal role in wound healing, repair, and growth. They are highly self-regulating, but the fate of each stem cell is governed by the chemical and mechanical stimuli a stem cell receives during the period of growth and development. They are sensitive to shear stress caused by the flow of the medium, and morphology of the substrate/extracellular matrix.

As mentioned previously, until recently, scientists, primarily, worked with two kinds of stem cells from animals and humans: embryonic stem cells, which are pluripotent, and very attractive for therapeutical applications, and non-embryonic or “adult” stem cells (mesenchymal) that are multipotent, that is, they only differentiate into a limited number of stem cells (Burdick and Vunjak-Novakovic, 2009; Velve-Casquillas et al., 2010; Zhang and Austin, 2012; Tehranirokh et al., 2013; Sugiura et al., 2016; Luni et al., 2022) In 1981, scientists derived embryonic stem cells from early mouse embryos, and later, they discovered a method to derive stem cells from human embryos and grow them in the laboratory. These cells are called human embryonic stem cells. Stem cells have the capacity to both self-renew and give rise to descendants which can commit themselves in a number of distinct directions. Stem cell behavior is extremely sensitive to environmental cues that can be manipulated in microfluidic cell culture (Scheme 4).

Analyses of stem cells can be done in a much expanded way in a microfluidic device than in a conventional tissue culture dish. Conventional tissue culture dishes have been used to test

different culture conditions of stem cell growth and differentiation. However, this approach requires significant amounts of stem cells, which reports the average response of the population and it is very difficult to precisely control the cell number. It is also expensive and labour-intensive to maintain hundreds of dishes over a long time.

Microfluidics offers a new way to perform high-throughput screening, using a much lower amounts of starting cells, and the dynamic adjustment of culture conditions (James et al., 2012; Jensen and Teng, 2020).

Figure 3 shows other approaches used for the three-dimensional culture of stem cells and Figure 4 shows the stem cell niche.

3D stem cells culture systems are, either scaffold-based, or scaffold-free systems. Among the scaffold-free systems, the most used is the hanging drop culture where individual cells aggregate under the effect of their own gravity to form 3D spheres. This method is simple and low cost and allows the co-culturing of different types of cells but scale-up is difficult.

Another quite simple method, easier to scale-up than the hanging drop culture is the rotating bioreactor culture, a dynamic culture, where the cell suspension is continuously stirred, distributing uniformly the nutrients and oxygen. This method maximizes the contact between the cells and the spheroids can be formed easily. However, stem cells are sensitive and may be easily damaged by the shear stress generated by the continuous stirring. Scaffold-based culture systems may use natural, or artificial (synthetic) materials as scaffolds. In this regard, it has to be mentioned that both natural and synthetic hydrogels are most widely used scaffold materials. Other natural materials are collagen, fibrin, gelatin, and composite hydrogels with good biocompatibility and

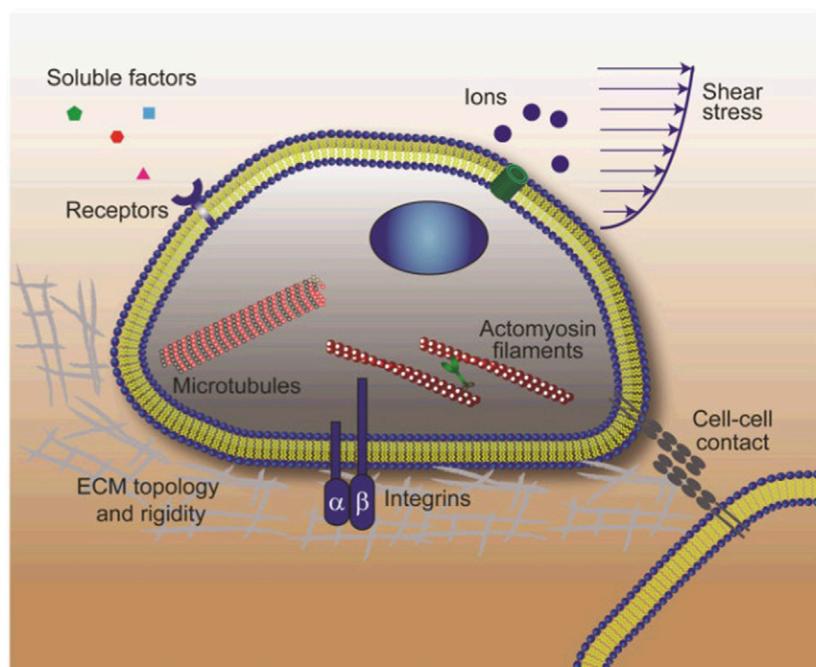


FIGURE 4

Schematic showing the stem cell niche where interactions between stem cells and their local microenvironment regulate stem cell fate. The stem cell niche is a complex dynamically regulated 3-D microenvironment comprising soluble biochemical and insoluble biomechanical cues, adhesive signals as well as signals arising from direct cell-cell contacts. Soluble biochemical cues include small ions, growth factors, cytokines, etc. Insoluble biophysical signals consist of matrix rigidity and topology, fluid shear-stress, and other mechanical forces exerted by adjacent cells or owing to tissue growth and loading. Stem cells sense and respond to these biophysical stimuli through different mechanosensory components including heterodimeric integrins, mechanosensitive ion channels, cytoskeleton structures (actin microfilaments, microtubules, and intermediate filaments) and cell-cell contacts. Reproduced from Sun Y et al. (2012), with permission from Wiley Periodicals, Inc.

mechanical properties. Synthetic hydrogels scaffold materials have a better-defined composition but poor biocompatibility (Scheme 5).

Because of the need for stem cells in cell-based therapies, automated cell culture techniques became extremely important. One of the main requirements in culturing stem cells is a controlled proliferation and differentiation (Ertl et al., 2014; Zhang et al., 2017; Aghlmandi et al., 2021). There are now important research efforts focused on microfluidic studies of stem cells, not only on culture, but also on tissue engineering and organ models.

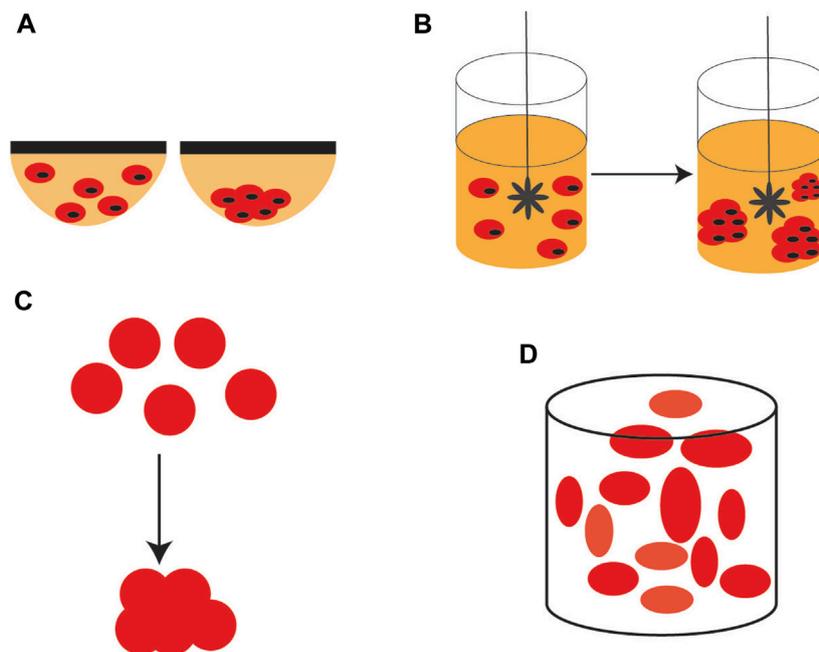
Microenvironmental control for stem cells is extremely important as they are highly sensitive. Microfluidic perfusion culture allows the production of small-volume cultures, with precisely controlled microenvironments and is applicable to high-throughput cellular environment screening. Microfluidic technology has great potential to improve stem cell (SC) cultures, whose promise in cell-based therapies is limited by the inability to precisely control their behavior in culture. Compared to traditional culture tools, microfluidic platforms provide much greater control over the cell microenvironment and rapid optimization of media composition, using relatively small numbers of cells. By controlling fluidic properties like convection, diffusion, and reaction, microfluidics can tune the microenvironment around stem cells in a variety of ways. Microfluidics can probe important biological processes like differentiation and

evolution in more biologically relevant conditions than conventional tissue culture dishes.

3.5 Microfluidic culture of stem cells. Examples

Recent work on the microfluidic culture of different stem cells is summarized below (see also Table 1).

- It has been shown that microfluidics can create dynamic microenvironments that can accelerate the growth rate of some stem cells such as hiPSCs. To investigate the growth under perfusion, a microfluidic perfusion culture system, using a microchamber array chip, was developed (Yoshimitsu et al., 2014). The authors found that both fibronectin and laminin were beneficial for culturing the cells in PDMS microfluidic devices. Interestingly, the authors found that, under pressure-driven perfusion culture conditions, the cells growth faster than under static culture conditions. The system was used to study the self-renewal and differentiation cultures of hiPSCs, and it was found that their state can be controlled by using the perfusion system.
- A fully automated, highly integrated cell culture system, with the capacity to screen 96 independent culture chambers and maintain cell viability for a long time was developed by



SCHEME 5

3D stem cell culture systems. (A) hanging drop culture (B) rotating bioreactor culture (C) formation of scaffolds, and (D) self-assembling scaffolds.

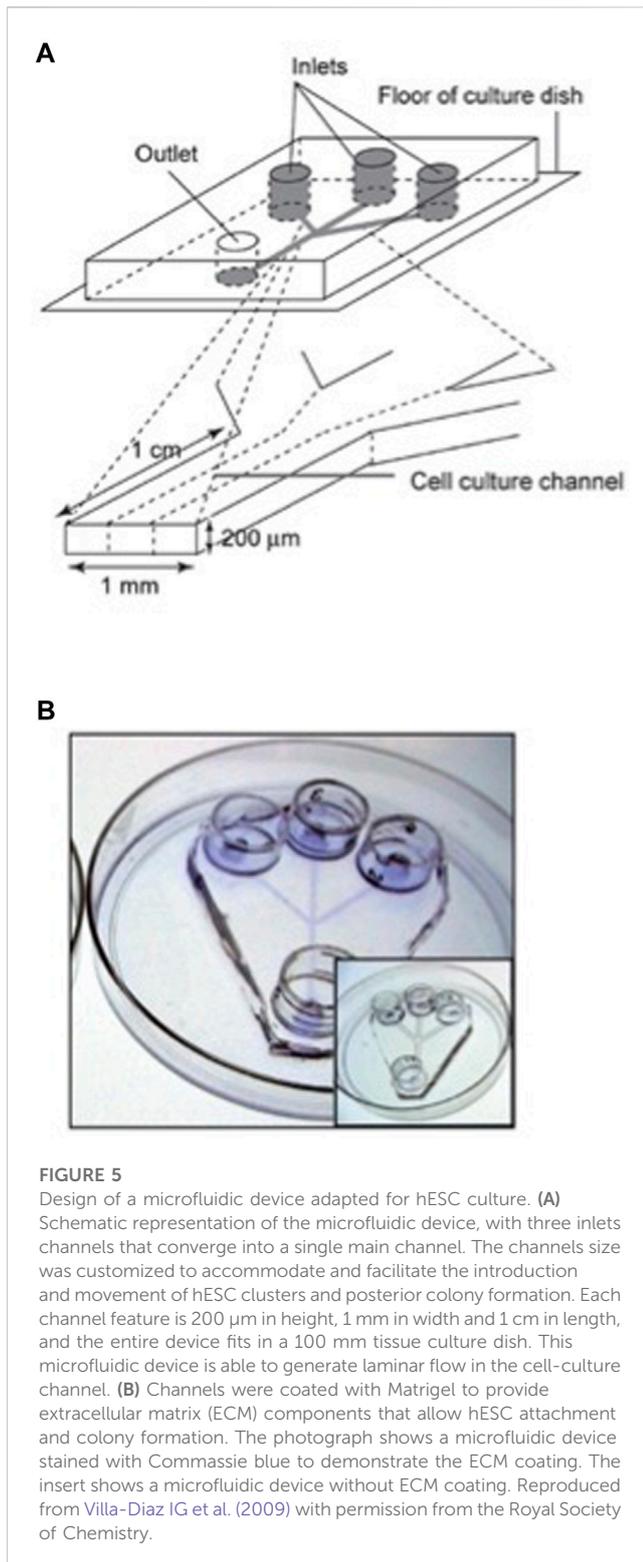
Gomez-Sjöberg et al. The device was able to establish different parameters in each chamber and change them separately. The authors worked with human mesenchymal stem cells in each chamber and studied the effect of inoculation number on their proliferation, differentiation, and motility, over several weeks (Gómez-Sjöberg et al., 2007).

- A prototype microfluidic system for the long-term culture and differentiation of Human mesenchymal stem cells (MSCs), composed mainly of seeding and waste reservoirs and stem cell culture areas, was developed by Wu et al. (Wu et al., 2009). The main advantage of this complex system is the capacity to maintain a constant pH, an important parameter that may determine the direction of the differentiation process. Depending of the need of tissue lineages for cell therapy, in this microfluidic system, the differentiation process can be oriented and studied by different methods. In this system, the cells can be cultured for several weeks.

A long-time culture and proliferation of hematopoietic stem cells (HSC) have been carried out by Lecault et al. in a programmable microfluidic platform, containing thousands of small-scale (nanoliter scale) chambers (Lecault et al., 2011). The control of proliferation of HSC on this device can be done at the single cell level. It is interesting to mention that in this device, cells are not trapped mechanically but gravity is used to immobilize them, without any perturbation. Cells are not disturbed either by the programmable medium exchanges and no nutrient limitation has been observed in long-time experiments. After culture, cells are recovered in bulk, by inverting and flushing the device. The authors found growth rates similar to those in standard macrocultures. They

anticipate that this device will be especially useful for culture optimization and cell characterization.

- Chemical gradients have been established in microfluidics devices by either laminar flow or controlled diffusion. For example, the proliferation and differentiation of human neural stem cells (hNSCs) from the developing cerebral cortex were optimized by culturing them in a gradient-generating microfluidics platform (Chung et al., 2005). The cells were cultured more than a week, exposed constantly to a gradient of a growth factor mixture. Time-lapse microscopy and immunocytochemistry were used to monitor the proliferation and differentiation of neural stem cells into astrocytes. The authors found that both proliferation and differentiation varied with the concentration of the growth factor. The device is made up of a gradient chamber and two control chambers. The microchannel network that produces the gradient (“Christmas tree”) generates a combinatorial mixture of soluble factors, utilizing laminar flow and diffusive mixing. Interestingly, the authors found in this work that, while proliferation is proportional to the concentration of growth factors, astrocyte differentiation was found inversely proportional to concentration of growth factors but this result has not been discussed. This microfluidic platform should be useful in the future for studies of a variety of cultured stem cells.
- In addition to growth factors, oxygen and temperature gradients have also been established in microfluidics devices. By using diffusion to localize oxygen delivery the authors could modulate the intracellular reactive oxygen species. It was found that embryos can adapt to the effects of temperature steps by increasing their development rate to compensate for the



fluctuating environment (Lucchetta et al., 2005; Lo et al., 2010). These two papers focus on creation of different gradients in microfluidic devices but are not specifically stem cell studies, therefore, will not be discussed in this section.

- An innovative microfluidic cell culture device for efficient hepatic differentiation of mesenchymal stromal cells (MSCs) has been designed and fabricated by Yen et al. (Ju et al., 2008).

The proliferation and hepatic differentiation of mouse MSCs have been studied in a device with an unusually large culture chamber (culture area 400 mm²), allowing a uniform flow distribution, creating an optimal environment for the hepatic differentiation, and accelerating it, compared with a conventional culture dish. A point of interest of this work is the presence of a continuous flow, supplying nutrients for the differentiation and removing the unhealthy cells. At the same time, the effect of fluid shear stress on the hepatic differentiation has been investigated. Also, the authors used a two-step hepatic differentiation protocol with growth factors that support hepatogenesis. Microscopic observations allowed real-time observation of cell morphology. The authors are confident that the cells differentiated in their microfluidic device will be suitable for cell therapy.

Individual human embryonic stem cell (hESC) colonies in dynamic or static conditions have been studied in a microfluidic culture system, allowing to treat in a different way desired parts of a colony (Villa-Diaz et al., 2009). Figure 5 shows a microfluidic device adapted for hESC culture. The authors found that both static and dynamic cultures for 96 h results in the same properties of hESC. They have demonstrated that, neither adhesion, nor proliferation and self-renewal of hESCs are affected by a dynamical culture at flow rates up to 1.5 mL/h. Portions of hESC colonies can be targeted, This may allow the study of propagation signals from one end to the other end of a colony. This experimental platform is useful in drug screening and studies of spatially regulated signaling, and differentiation of hESCs. The human embryonic stem cells may be differentiated into different lineages.

Table 1 summarizes the above microfluidic stem cell experiments, including a critical appraisal.

It is difficult to compare the experiments described in the text and summarized in the table. They are all different, with various designs and proliferation and differentiation conditions. The general trend seems to be automation, allowing any change, simultaneously, in the experimental conditions in thousands of culture chambers, during the long-time experiments. It has to be mentioned that microfluidic culture is convenient for selecting the best scaffold materials for differentiation of stem cells. The results of the experiments described in this paper show that microfluidic culture of stem cells is still in the stage of data collection on the way differentiation of stem cells happens in confined spaces. In addition, culture scale up in microfluidic devices, necessary for cell therapy, will need further advances in design of devices and changes in the methods. However, each work, discussed here, brings important novelties that do contribute to the progress of one of the most important research fields today, microfluidic stem cell culture.

Apart from differentiation and proliferation, stem cell migration behavior gained high importance due to their application in regenerative medicines. Stem cell migration is a fundamental process that occurs during embryogenesis, organogenesis, immune response, and most importantly, cancer progression (Pijuan Marquilles et al., 2019). Various microfluidic models have been reported to model the cell migration that occurs during the above processes, neural embryogenesis models (Kurosaka and Kashina, 2008; Lee et al., 2014; Bae et al., 2016), and immune response (Vesperini et al., 2021). In the traditional migration assay or wound healing assay, the cells are cultured on a plate, and once confluence is achieved, a scratch is made, removing a section on the cells, creating a cell-free zone, then, the migration of the cells is measured. However, in a microfluidic platform, in addition to the various advantages already mentioned, the

TABLE 1 Summary of the microfluidic stem cell culture discussed in this paper.

Stem cells	Experimental conditions	Significant advantages	Applications and References
hiPSCs (human induced pluripotent)	Pression-driven intermittent perfusion culture	Important: Control of the growth rate, initial culture in static conditions for adhesion of cells on microchamber surface (24 h)	Promising for drug screening Ref. 60
	Microchamber array (8 × 8) chip		
	ECM: fibronectin and laminin		
	Defined conditions for differentiation		
hMSCs (human mesenchymal)	96 independent culture chambers (60 nL each), imaged with time-lapse microscopy	Capacity to maintain cell viability for weeks, Fully automated, arbitrary culture media formulation in each chamber, able to screen the effect of different mixtures of reagents on cells, over long-term culture	In quantitative cell culture technology Ref. 61
hMSCs (human mesenchymal)	Components: culture area, micropumps and microgates, seeding reservoirs, waste reservoirs	Able to maintain a constant pH, differentiation can be oriented, different methods are used for assessing the differentiation	Cell therapy Ref. 62
	Automated system		Only small-scale proliferation
HSC (hematopoietic)	Automated medium exchange, cell capture based on gravity, recovery in bulk, large no of chambers for long-term culture, small volume of chambers	Fully automated individual culture conditions, each chamber imaged with time-lapse microscopy, growth rates replicate standard macrocultures	Attractive for the analysis of rare cell types or minority subpopulations
			Applications anticipated in drug-response screens and culture optimization
			Ref. 63
hNSC (human neural)	Proliferation and differentiation into astrocytes monitored by time-lapse microscopy and immunocytochemistry	Optimizes proliferation and differentiation conditions using a gradient-generating device (growth factor mixture)	Gradient-generating microfluidic platforms are useful for basic and applied studies on cultured stem cells. Ref. 64
		Long culture time under constant fluid flow	
		Differentiation decreases with the concentration of growth factors	
		Graded responses	
MSCs (mesenchymal stromal)	Large culture chamber with uniform flow, two steps differentiation protocol, 3–4 weeks pH maintained at 7.3	Good differentiation efficiency, higher level of hepatocyte marker gene expression than in static cultures	Large number of hepatocytes for cell therapy due to a large culture surface area and uniform flow distribution. Ref. 67
	Homogeneous cell seeding	Observation of the cellular morphology during differentiation	
hESC (human embryonic)	hESC clusters of 100–150 um were used to establish colonies. Facilitates the movements of hESC clusters	Wide channels (1 mm) to accommodate hESC clusters, differentiation.shear stress did not affect cell adhesion, dynamic flow culture did not induce hESC	May be useful in drug screening and studies of migration and differentiation of hESCs. Ref. 68
	compatible with live-cell imaging, allows collection of specific areas of a colony		
	channels coated with Matrigel for adhesion		

device allows single-cell level tracking (Yazdanpanah et al., 2023), the creation of drug gradients similar to *in vivo* situations, and the compartmentalization of cells and molecules. In a microfluidic device, the entire channel is cultured with cells and later the cells from the desired area are removed by flushing liquids via specific channels. Here, the cells remain intact and are not injured as in the case of the conventional scratch assays.

Stem cell migration studies have gained further importance with the development of regenerative therapy, where stem cells are directly introduced into the body and they must traverse considerable distances to reach the desired location. By installing diffusion barriers, *in vivo*-like environments were recaptured on a multichannel microfluidic device to find out drug gradients and evaluate the effect of Substance P, a stem cell mobilizer, on the migration of human bone marrow-derived mesenchymal stem cells (BM-MSCs) (Kim et al., 2023).

4 Conclusion and outlook

This paper reviews recent advancements in both macro-scale and microfluidic culture. It shows that microfluidics is attractive in many aspects, especially reconstructing the physiological environment, and isolating rare stem cells. As in microfluidics, many factors, physiologically relevant, can be controlled precisely, microfluidic devices are well-suited to biological experiments and provide an ideal platform for the study of stem cells, especially, their behavior in cultures. Compared to traditional culture tools, microfluidic platforms provide much better control over the cell microenvironment and the composition of the media can be optimized rapidly. The macroscopic culture platforms, in spite of the recent improvements, do not have yet the capability to create concentration and other type of gradients. However, in our opinion,

there are issues that need to be solved before microfluidics can be widely applied to the resolution of fundamental biological problems, rather than simply to be a proof of concepts. It is important to make microfluidics devices easy to use by biologists in general and, particularly, by stem cell biologists. To help to solve this issue it would be important to standardize and commercialize the microfluidics technologies developed in academic labs. It will be necessary to be able to scale up microfluidic devices in order to meet the requirements, regarding large-scale applications. Also, it would be useful to train biologists who do not have experience in fluid physics or microfabrication as well as engineers who may not have a deep knowledge in the biology of stem cells.

As Paguirigan and Beebe (Paguirigan and Beebe, 2008) have shown, there is a “disconnect” between the engineers, who design and fabricate the devices, and the biologists who will use them. It seems to the authors of this review paper that, this disconnect still exists, regarding language (terminology), mindset, etc., and will have to be attenuated for a better collaboration.

Regarding the materials to make microfluidics devices for biology experiments, especially, stem cell research, PDMS which is a common choice today, will have to be, at least partially replaced, by more biocompatible, cell-friendly materials.

Absorption of molecules in PDMS can change the local chemical concentration and could reduce the reproducibility of experiments. Therefore, it is important to look in the future for more biocompatible materials for stem cell research, especially, if cells are exposed to PDMS for longer duration. Also, highly integrated systems are needed to address a specific question in stem cell biology from different aspects. Nevertheless, we believe microfluidics will become in the near future a powerful tool for both fundamental understanding and medical applications of stem cells.

The effect of microgravity on differentiation and cell growth in stem cells and cancer stem cells has been studied recently by (Grimm et al., 2020). The results of this study are extremely important for stem cell research.

A number of authors advanced the idea that microfluidic culture systems have the potential to become a substitute for macroscale techniques but, there is still much work to be done (Halldorsson et al., 2015; Tanyeri et al., 2018; Coluccio et al., 2019). Most

importantly, high reproducibility requirements and precise culture protocols have to be developed for the microscale. Due to all the recent improvements, we believe that macroscopic cell cultures will continue to be useful for culturing large amounts of cells required by biomedical applications.

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