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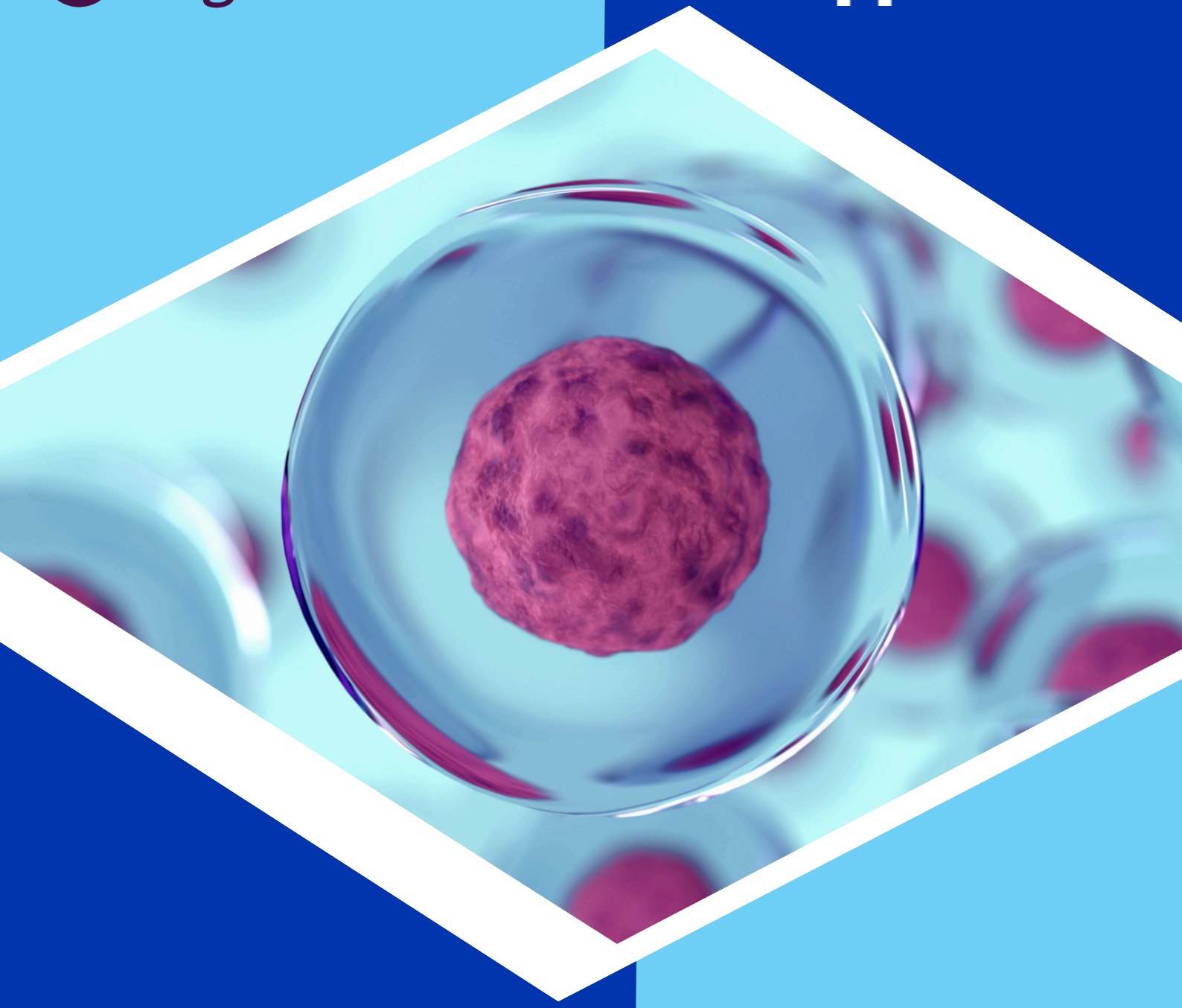

Bioprocess fundamentals

from cell lines to cell and gene therapies

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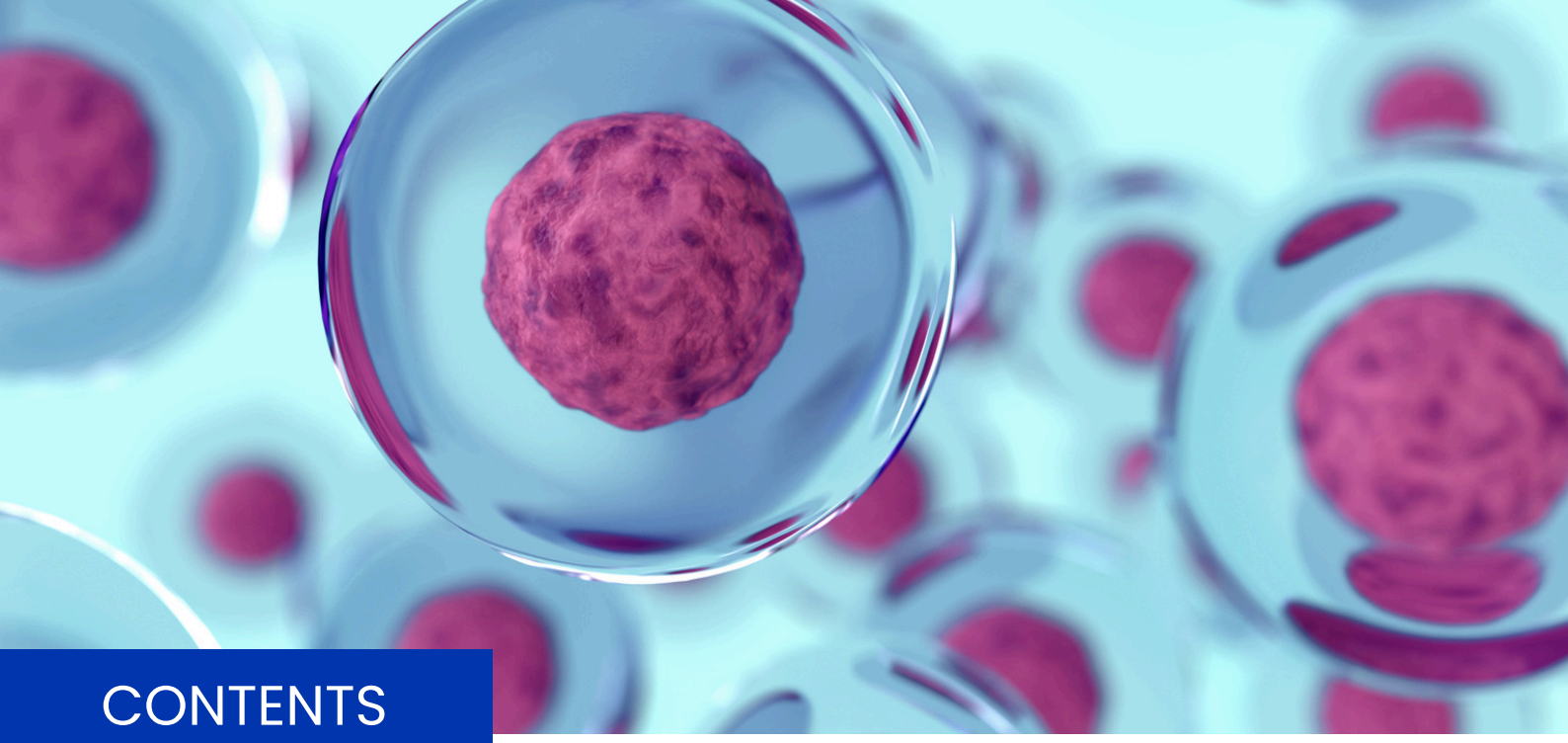

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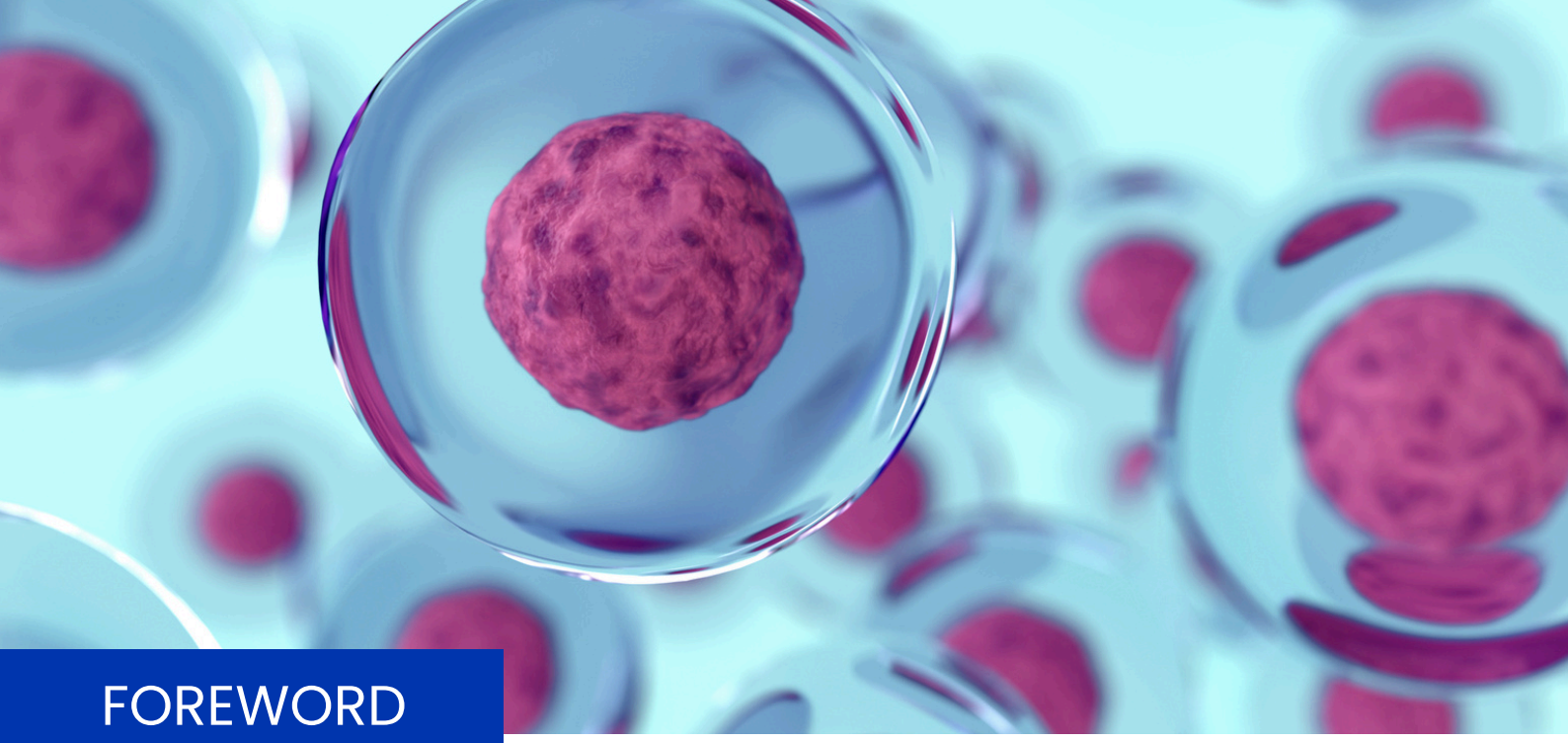

FOREWORD

We are delighted to present this eBook on bioprocessing, sponsored by Eppendorf.

Bioprocessing has become a cornerstone of many industries, from chemical compounds to modern food, from single antibodies to advanced cell and gene therapy applications. In all of these fields, bioprocessing offers scalable, efficient, and reproducible solutions for cultivating cells and producing therapeutic products. This eBook aims to provide foundational knowledge and practical insights to help readers navigate this transformative field.

Bioreactors have revolutionized the way we approach cell culture, enabling precise control over environmental conditions and facilitating the production of high-quality cells at scale. The curated content pieces in this eBook reflect the diverse and dynamic nature of bioprocessing. Whether you are seeking to resolve cultivation bottlenecks, optimize culture modes, or implement closed and automated systems for cell therapy manufacturing, this collection provides actionable insights and real-world examples.

We hope this eBook serves as a valuable resource for your bioprocessing journey, equipping you with the knowledge and tools to unlock the full potential of bioreactors in your work.

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WHITE PAPER No. 21

Bioreactors and Fermentors—Powerful Tools for Resolving Cultivation Bottlenecks

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Executive Summary

Shake flasks, cell culture dishes, and T-flasks are the first vessels that come to mind, when we think about cultivation systems for growing eukaryotic and prokaryotic cells in the lab. Bioreactors and fermentors are another alternative to consider if we need larger quantities of cells, increased efficiency of cultivation, or enhanced reproducibility. In this white paper we explain the key characteristics of stirred-tank bioreactors and which organisms are typically grown in them. Using specific examples, we demonstrate how bioreactors and fermentors can help to resolve cultivation bottlenecks.

Introduction

Many applications are well served by the cultivation of bacteria or yeast in shake flasks and cells in dishes or T-flasks. Bioreactors and fermentors, however, improve productivity and save work, time, and lab space for scientists,who

> need large quantities of cells, microbes, or of the products they express
> would like to improve the reproducibility of growth, the product formation or the product quality
> would like to systematically compare different growth conditions
> would like to increase the cultivation efficiency.

What are bioreactors and fermentors?

Broadly speaking, bioreactors and fermentors are culture systems to produce cells or organisms. They are used in various applications, including basic research and development, and the manufacturing of biopharmaceuticals, food and food additives, chemicals, and other products. A broad range of cell types and organisms can be cultivated in bioreactors and fermentors, including cells (like mammalian cell lines, insect cells, and stem cells), microorganisms (like bacteria, yeasts, and fungi), as well as plant cells and algae.

Bioreactor and *fermentor* are two words for basically the same thing. Scientists who cultivate bacteria, yeast, or fungi often use the term fermentor. The term bioreactor often relates to the cultivation of mammalian cells but is also generically used. If we talk about bioreactors in this

white paper we usually mean systems for the cultivation of microbes or mammalian cells.

Stirred-tank bioreactors

Though many types of bioreactors exist, we will focus on stirred-tank bioreactors. The name is accurately descriptive. Cultivation takes place in the bioreactor tank—often called a vessel—and the culture is mixed by stirring (instead of shaking, for example).

Stirred-tank bioreactors come in different sizes, for cultures of a few milliliters to thousands of liters, and are made of various materials—usually glass, plastic or stainless steel. The basic components and functioning of stirred-tank bioreactors are always the same.

A stirred-tank bioreactor system consists of several parts (Figure 1):

> A vessel, which is filled with medium in which cells are cultivated
> A head plate, to close the vessel
> Components, within or attached to the vessel or the head plate, to measure and adjust the culturing conditions, such as feed lines and sensors
> A control system comprising external components used to adjust the culturing conditions (for example pumps) and control software

Creating optimal cultivation conditions

Like incubators and shakers, bioreactors allow for the creation of optimal environmental conditions for the growth of cells or microbes. They differ, however, in how these are established.

Culture mixing

Instead of mixing by shaking, in a bioreactor the culture is stirred with an impeller. The impeller is mounted to the impeller shaft, which in turn is connected to a motor. In a bioreactor, not only are bacterial, yeast, and suspension cell cultures constantly mixed, but also cultures of adherent cells attached to a growth matrix.

Tempering

To obtain the right cultivation temperature, a bioreactor does not need to be placed inside a shaker or incubator but can remain on the lab bench. The temperature of the culture medium is continuously monitored with a temperature sensor. To regulate it, the vessel is placed in a thermowell, wrapped with a heating blanket or has a water jacket. Cooling is possible as well.

Establishing aerobic or anaerobic conditions

In a shaker or incubator, oxygen is transferred from the surrounding air to the culture medium. This process is more efficient in shake flasks than in static cultures, because shaking increases exposure of the liquid surface. In bioreactors, usually air or pure oxygen (coming for example from a compressed air cylinder) is introduced to the culture. With the use of spargers the gas/liquid interface can be increased and the oxygen supply maximized. Oxygen is important for culture growth, and the amount of oxygen dissolved in the medium (dissolved oxygen concentration, DO) is continuously measured with a DO sensor.

To keep DO at setpoint, a DO cascade is often set up in and executed by the bioprocess control software. Figure 2

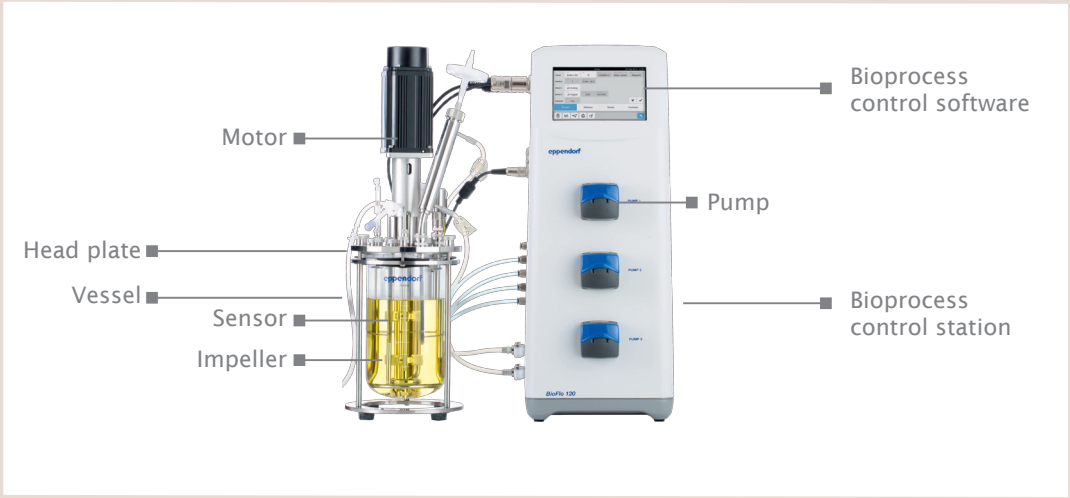

Figure 1: Stirred-tank bioreactor system consisting of bioprocess control station, vessel, and bioprocess control software. The BioFlo® 120 bioprocess control station is shown.

shows an example for a typical DO cascade. If DO drops below setpoint, first the agitation speed is gradually increased up to 1,200 rpm to increase oxygen transfer from the surrounding air. If this is not sufficient to keep DO at setpoint, the gas flow rate is increased up to three standard liters per minute (SLPM). As a final measure, the oxygen concentration in the gas mix is increased, shifting from gassing with air (containing 21% oxygen) toward gassing with pure oxygen. This is just an example. The minimum and maximum values of agitation, gas flow rate, and oxygen concentration can be optimized depending on the organism and process needs.
Anaerobic conditions can be established by gassing with N_2 or other anaerobic gases.

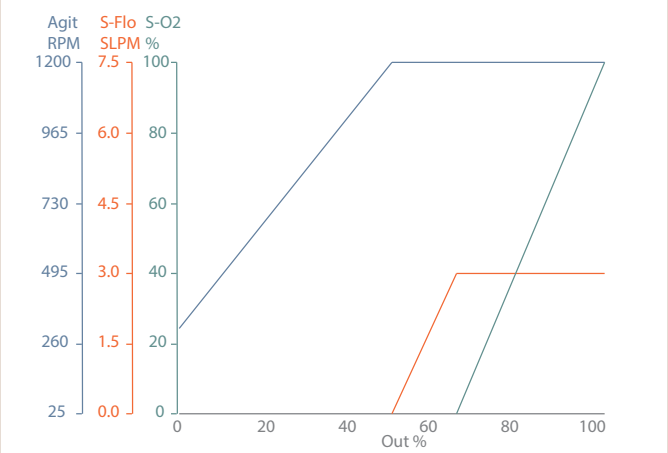

Figure 2: DO cascade. See text for details.

pH control
To regulate the pH of carbonate-buffered cell culture media, cell cultures in flasks or dishes are usually placed in CO_2 incubators. In bioreactors, the principle is the same; CO_2 is introduced to the culture from a compressed gas cylinder. In bioreactors, the medium pH is continuously measured using a pH sensor and CO_2 is added as needed. This is different from the situation in a CO_2 incubator, in which the CO_2 concentration in the internal incubator atmosphere is measured and controlled, rather than the medium pH. Bioreactors also differ in that a basic solution is often added to compensate for acidification during culture growth.

For microbial cultures in bioreactors, basic and acid solutions are commonly used for pH adjustment. This is different from cultures in shake flasks, where the culture pH is usually not controlled.

Control of parameters at setpoint
In bioreactors, different components and the control software play together to control pH, temperature, and dissolved oxygen at the desired setpoint. The parameters are constantly measured using pH, temperature, and DO sensors. The sensors transmit the information to the bioprocess control software which regulates the addition of CO_2 and liquid pH agents, the activity of tempering devices, agitation, and the gassing with air and/or O_2.

A typical bioprocess run

To maintain cultures, scientists usually keep them in cultivation systems within incubators. Bioreactors are usually used for a specific experiment or production run, which may last hours, days, or weeks, depending on the organism and application. A bioprocess run typically comprises the following steps (Figure 3).

1. **Preculture:** The medium in the bioreactor is inoculated with a preculture. Often, the preculture is grown in a shaker or incubator. Sometimes smaller bioreactors are used to grow precultures for the inoculation of larger bioreactors.
2. **Bioreactor preparation:** The bioreactor is prepared in parallel to inoculum preparation. Preparations include the sterilization of bioreactor, feed lines, and sensors; medium addition to the bioreactor; the connection of the bioreactor with the bioprocess control station; and the definition of process parameter setpoints in the bioprocess control software.
3. **Inoculation:** Once the bioreactor is prepared, the medium is inoculated.
4. **Cultivation period:** During the cultivation period, agitation, pH, temperature, and DO are typically monitored and controlled in real time via the bioprocess control software. In addition, scientists often take culture samples to analyze, for example, the biomass and the concentration of metabolites. Eventually researchers feed the culture by adding nutrient solutions.

   Cultures typically pass through four growth phases.
   - > In the lag phase, at the beginning of the culture, the organisms do not multiply or multiply only slowly, probably because they need to adapt to the new culture conditions.
   - > In the exponential growth phase, as the name says, the culture grows exponentially.
   - > In the stationary phase growth stops, because the nutrient concentration, the oxygen concentration, the accumulation of byproducts, or other factors become growth-limiting.
   - > The stationary growth phase is followed by the death phase, in which the viable cell density decreases.
5. **Culture harvest:** Scientists typically end the bioprocess run and harvest the culture when it enters the stationary growth phase.
6. **Downstream processing:** The culture broth is further processed.
7. **Bioreactor cleaning:** The bioreactor is sterilized to inactivate culture residues and cleaned.

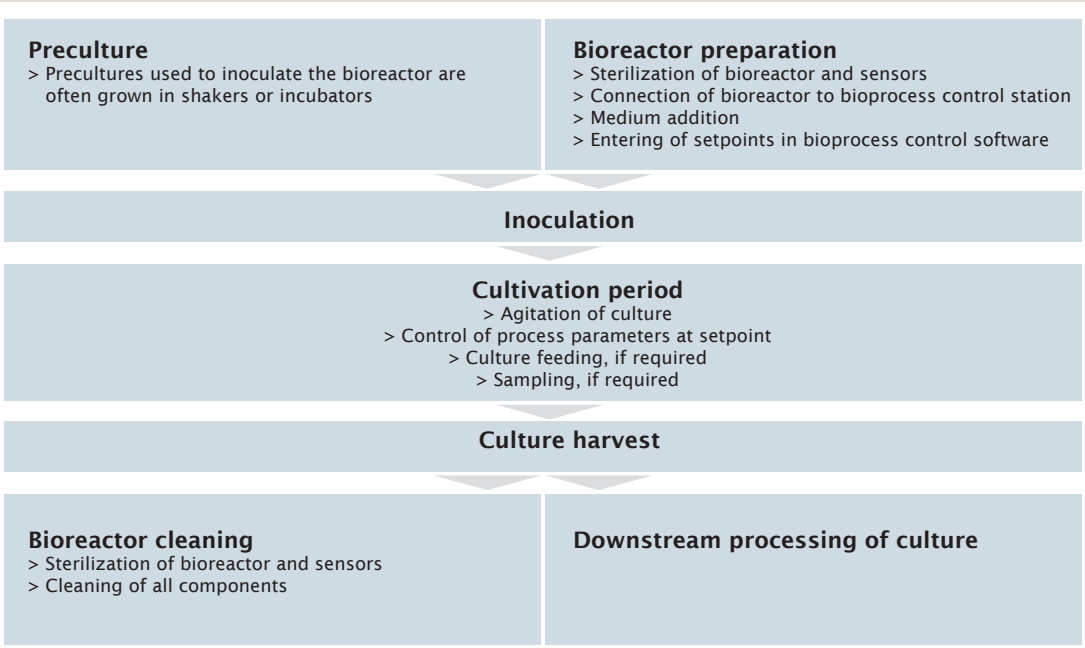

Figure 3: Series of events in a typical bioprocess run.

How bioreactors and fermentors help in resolving cultivation bottlenecks

Bioreactors may save work, time, and lab-space of scientists who need large quantities of cells, microbes, or of the products they express. Furthermore it can improve reproducibility of growth, product formation, and product quality and increase cultivation efficiency.

Culturing large amounts of cells and microbes

Sometimes researchers require large amounts of cells or of the product they produce. Large amounts of a recombinantly expressed protein may be needed for example for biochemical or structural characterization, for ongoing use as research tools, or for evaluating medical applications. Small molecules produced by microbes and intended for use as chemical building blocks, fuels or food and feed additives may be required in small quantities in the R&D phase, but usually large quantities are then needed for their commercial use. Large quantities of cells are needed for stem cell-based cell therapy and drug research applications.

Handle less vessels

Producing cells in a couple of T-flasks or preparing a few liters of a bacteria or yeast culture in shake flasks are feasible. But if dozens or even hundreds of flasks are needed to produce the required amount of biomass, lab space becomes limiting and the amount of manual work explodes. Stirred-tank bioreactors are scalable, meaning they allow increasing the size of the cultivation vessel instead of the number of vessels, so that the work and space requirements stay manageable.

As an example: In conventional cell culture consumables, CHO cells typically reach a density of 2-4 x 10^5 cells per cm^2 [1]. This corresponds to 3.5 – 7 x 10^7 cells per T-175 flask. In bioreactors we can easily reach cell densities of up to 1 x 10^7 cells per mL [2, 3]. This corresponds to 3 x 10^9 cells per bioreactor with a working volume of 250 mL. In this example, one comparably small bioreactor replaces 250 T-175 flasks.

For microbial cultures in shake flasks the situation is similar. Typically, Erlenmeyer flasks with capacities up to 5 L are used. In contrast, bioreactors with working volumes of hundreds and thousands of liters are available.

The availability of larger vessels is not the only advantage. In bioreactors, higher cell densities can be achieved, sometimes making an increase of the working volume unnecessary.

Achieve higher cell densities

At some point, the growth of cells and microbes in flasks and dishes reaches a stationary phase. The cell concentration does not increase further. In bioreactors, cultures reach a stationary phase as well (unless you perform a continuous bioprocess), but much higher culture densities can be achieved.

One example: When our application engineers cultivated *E. coli* in a complex medium in a shake flask, the culture entered the stationary growth phase overnight and typically had an optical density at 600 nm (OD_{600}) of around 12. When they cultivated *E. coli* in a bioreactor, after 12 hours the culture had also entered the stationary phase but reached an OD_{600} of 240 [2].

How did they manage? Using bioreactors allowed our engineers to lessen some of the growth-limiting factors in microbial cell cultures: They improved the supply with nutrients and oxygen, as well as the temperature control [2].

Fast-growing aerobic cultures consume lots of oxygen. If, at a high cell density, the culture needs more oxygen than is transferred to the medium, growth is impaired. In bioreactors, air or pure oxygen can be supplied by gassing, which is more efficient than shaking or agitation alone.

Growing cultures produce heat. To keep the temperature at setpoint, high-density bacterial cultures often do not need to be heated up any more, but cooled down. Bioreactors facilitate culture cooling, in contrast to conventional shakers.

If nutrients become limiting, growth stops. Cultures in bioreactors can quite easily and automatically fed by adding feed solutions using the system's integrated pumps. In bioprocessing we distinguish different process modes: In a batch process the culture grows in the initially supplied batch of medium. In a fed-batch process the culture is fed to keep the concentration of nutrients constant. In a continuous process the culture medium is continuously exchanged.

This is just one example. Dependent on the cell line or microbial strain other parameters may be critical for culture growth and/or product formation, for example the medium pH, metabolite concentrations, redox potential, and mechanical forces. Bioreactors are valuable tools to optimize cultivation conditions.

Comparing growth conditions

Parallel bioprocess systems are available to control more than one vessel (Figure 4). They often have small working volumes which helps saving resources. Parallel systems have the advantage that cultivation parameters can be

controlled independently in each bioreactor. This saves time and ensures maximum reproducibility between runs. If for example you would like to compare protein expression at eight different temperatures, in a parallel bioreactor system you can perform the experiments in parallel. This is more convenient than using a conventional shaker or a single bioreactor, where you would either need to perform one experiment after the other or would need several shakers or bioreactors.

The possibility of comparing multiple process conditions in parallel make bioreactors well suited to systematically analyze the influence of several parameters on the culture outcome (e.g., culture growth, product formation, byproduct formation). In this way, researchers can gain comprehensive process understanding, which in turn allows optimization of culture conditions to achieve the best possible results.

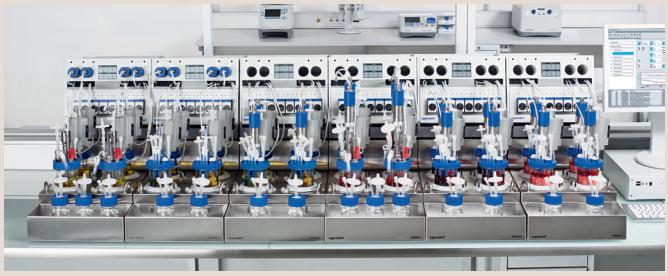

Figure 4: DASbox Mini Bioreactors System allows parallel operatition of up to 24 bioreactors.

Increasing reproducibility

In bioreactors, process parameters like pH, temperature, and dissolved oxygen can be constantly measured using sensors. The sensors transmit the information to the bioprocess control software, which regulates the action of actuators, like pumps, tempering devices, and gassing devices, to keep the parameters at setpoint. The software also continuously saves process values, making it possible to analyze them later. Monitoring, control, and recording of process values help increasing the reproducibility of culture growth, product formation, product characteristics, and more.

Let's take a simple example. You recombinantly express a protein. Let's assume the protein has the tendency to aggregate and aggregation strongly depends on the temperature. Let's further assume that you found the optimal temperature profile for growth and expression phase to balance growth, protein expression, and aggregation. You may employ that temperature profile in a shaker; however, it is prone to error. Unwanted and unnoticed temperature fluctuations may be caused by repeated opening of the shaker by other users who add or remove flasks. In a bioreactor the temperature of the culture medium is continuously monitored and adjusted as needed. As a result, the temperature profile is maintained more reliably, leading to more reproducible results. Furthermore, the temperature sensor data is recorded. Eventually, temperature deviations are detected, making it possible to identify the source of error.

And the situation may be much more complex. Besides the temperature, process parameters including the medium pH, DO, metabolite concentrations, mechanical forces, and medium composition may influence culture growth, product yield, protein glycosylation patterns, byproduct formation, and more. Process parameters influence cell viability, cell behavior, and differentiation. This is especially important if the cell itself is the product of interest, for example if cells are used for cell therapy applications.

In a bioprocess, temperature, pH, and DO are routinely monitored and controlled. Advanced bioprocess control software allows the integration of additional sensors, for example to monitor biomass and metabolites, as well as the setup of tailored process control strategies.

Home sweet home - A bioreactor for every organism

Eukaryotic cells, like mammalian cell lines, stem cells, insect cells, and plant cells, and microbial organisms like bacteria, yeasts, fungi, and algae can all be cultivated in bioreactors.

However, the optimal growth conditions differ and therefore differ the optimal bioreactor design as well as setpoints of agitation, temperature, DO, pH, and other parameters. Are you cultivating mammalian cells or microorganisms? This is the first important question when setting up a process, and will influence some basic decisions regarding certain bioreactor accessories. Dependent on the cell line or strain, parameters will then need to be finetuned.

Bioreactor accessories

Although generalized, much of the following applies for many microbial strains and cells:

Many of the microbial strains which are routinely used in industrial bioprocess applications (e.g *E. coli*, *C. glutamicum*, and *S.cerevisiae*, *P. pastoris*) grow much faster than commonly used mammalian cell lines (e.g. CHO and HEK293). For example, the growth rate of *E. coli* is in the range of 1/few hours or less; the growth rate of a CHO cell line is closer to 1/day. This has important implications for the design of the bioprocess system.

Oxygen demand

The oxygen demand of fast-growing, aerobic microbial cultures is high. The bioprocess system needs to be capable of high gas flow rates to supply enough air and/or oxygen to keep DO at setpoint. To maximize the dissolved oxygen concentration in microbial cultures air/oxygen are usually introduced to the culture medium through submerged gassing, either through a dip tube with an open end or a porous sparger. The pores of the gassing device determine the size and number of the gas bubbles and therefore the surface available for gas exchange with the medium. The oxygen demand of mammalian cell cultures is lower. Often it is not required to maximize the gas/medium interface through sparging, but air/oxygen supply to the bioreactor headspace is enough. An important advantage of headspace gassing is the avoidance of shear force-causing air bubbles which may damage sensitive mammalian cells.

Culture mixing

Besides gassing, agitation is a critical parameter to keep DO at setpoint. Rushton-type impellers mix the culture more efficiently than pitched-blade or marine impellers, but also cause higher shear forces. Therefore the former are commonly used for microbial cultures, whereas the latter are commonly utilized for mammalian cell cultures.

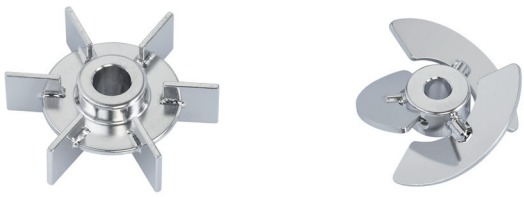

Figure 5: Two impeller types. Rushton-type impellers are commonly used for microbial bioprocesses. Pitched-blade impellers are commonly used for mammalian cell cultures.

Sterility

Because of their slower growth, the contamination risk in mammalian cell cultures is much higher than in microbial processes. The material and connection of feed lines are two factors to consider to ensure sterility. To minimize the contamination risks in cell culture bioprocesses, many scientists prefer feed lines made of autoclavable material (instead of feed lines which need to be chemically cleaned) and which can be safely connected by welding [5].

Setpoints

The above paragraph provides some general recommendations regarding the bioreactor accessories for microbial and cell culture applications. But this is not the whole story. Suitable setpoints for temperature, pH, DO, agitation, and strategies to control them differ between organisms, cell lines, and strains, and may even be different for a single strain used in different applications. Setpoints and control strategies need to be optimized on a case by case basis. Methods described in literature can serve as a starting point for further optimization [5, 6]

Cultivation of adherent cells in stirred-tank bioreactors

Many mammalian cell types need to attach to a growth surface to survive and multiply. Expansion of adherent cells in stirred-tank bioreactors sounds counterintuitive at first, but is feasible if an attachment matrix is provided. The cells attach to the matrix, which is kept in suspension by gentle agitation.
Various matrices are in common usage.

Microcarriers

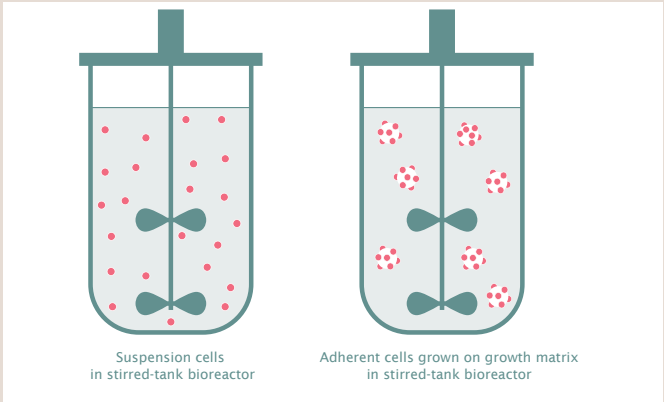

Figure 6: Schematic representation of suspension cells and adherent cells grown in stirred-tank bioreactors.

Microcarriers are spherical particles which provide a growth surface for adherent cells. Microcarriers typically have a diameter of 100 – 300 µm. They can be made of different materials, like glass, DEAE-Dextran, polystyrene, and alginate. There are also coated versions, whose core material is covered with peptides, proteins or protein mixtures like fibronectin, collagen or Matrigel®. The core material of non-coated microcarriers is not functionalized by the manufacturer, but may, however, bind proteins once the carrier is in contact with serum-containing culture medium. To start a cell culture process on microcarriers, they are typically added to the culture medium at a density recommended by the manufacturer and subsequently the bioreactor is inoculated with a single cell suspension. To support cell attachment to the microcarrier, the culture is only periodically agitated during the first few hours after inoculation. Once the cells have attached to the carriers the culture is continuously agitated to keep the carriers in suspension. For downstream processing, the microcarriers can be harvested and the cells detached, for example using trypsin.

Vero cells and mesenchymal stem cells are only two examples of adherent cells, which have been cultivated in stirred-tank bioreactors on microcarriers [7, 8].

Fibra-Cel® disks

Like microcarriers, Fibra-Cel disks provide a growth support for adherent cells. Fibra-Cel disks are made of a meshwork of polyester and polypropylene, which is electrostatically pretreated to support cell attachment. In contrast to many microcarrier types, Fibra-Cel provides a three-dimensional growth surface with a high surface-to-volume-ratio, and protects cells from damaging shear forces, thus increasing the total biomass which can be maintained in the bioreactor. Fibra-Cel disks have a diameter of 6 mm. They provide a growth matrix in packed-bed bioreactors, and in principle can also be used free-floating in shake flasks or disposable bags. Fibra-Cel is predominantly used for cell culture processes for the production of secreted products, like recombinant proteins and viruses. They have been used for example for the cultivation of Vero cells [7].

Cell-only aggregates

Instead of growing on a matrix, cells can grow in stirred-tank bioreactors as cell-only aggregates. This has been described, for example, for human induced pluripotent stem cells and cells differentiated thereof [9, 10], and certain tumor cell lines [11]. For expansion as cell-only aggregates, bioreactor cultures are usually inoculated with a single-cell suspension. Cell expansion leads to the formation and growth of aggregates. The attachment of cells to each other is influenced by the agitation speed and impeller type, among other factors. Stem cell-derived cell spheroids, neurospheres for example, can reach a remarkable degree of maturation and are promising model systems in basic research and drug screening applications [12]. The absence of a synthetic matrix may simplifies downstream processing, for example for cell therapy applications.

Summary

The advantages of cell cultivation in stirred-tank bioreactors, like simplified scalability and improved process control are not limited to suspension cells. By providing a growth matrix or cultivating as cell-only aggregates, adherent cells can be expanded in stirred-tank bioreactors, too.

Conclusion

Stirred-tank bioreactors simplify the cultivation of large amounts of cells compared to conventional culture systems using shakers and incubators. Bioprocess control software facilitates the precise monitoring and control of critical process parameters. Cultivating cells and microbes in bioreactors therefore can save the scientist work, time, and lab-space and improve the reproducibility and efficiency of cell growth and product formation.

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Ordering information

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DASbox® Mini Bioreactor System, for cell culture applications, max. 5 sL/h gassing, 4-fold system	76DX04CC
BioFlo® 120, advanced	please inquire
Fibra-Cel® Disks, 250 g	M1292-9988

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APPLICATION NOTE No. 456

A Beginner's Guide to CHO Culture: Bioprocess Modes – Batch, Fed-Batch, and Perfusion

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Abstract

In this application note, we explain the differences between batch, fed-batch, and perfusion cell culture bioprocesses using Chinese Hamster Ovary (CHO) cells at bench scale. We explain the importance of tracking specific parameters to achieve inoculation of bioreactors at high viability and how we analyze our samples and adjust the bioreactor conditions throughout the runs. We discuss what our data obtained from sampling means in terms of growth rate, production yields and bioreactor feeding or perfusion rates. The principles in this beginner's guide to CHO culture may be applicable to other suspension cell culture processes at smaller or larger scales.

Introduction

Recombinant protein manufacturing in CHO cells represents more than 70 % of the entire biopharmaceutical industry [1]. In fact, human monoclonal antibodies (hmAbs) produced in CHO cells have played a major role in the therapeutic markets for decades. Certainly, one of the first human-mouse chimeric mAbs obtaining FDA approval was Rituximab, Roche's Non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and rheumatoid arthritis treatment. Since that approval in 1997, there have been scores of chimeric, humanized, and human mAbs that have gained approval and entered the clinic. And there promises to be more, including recent FDA approval of several mAbs for the treatment of COVID-19.

In the development of any pharmaceutical production process, including those involving hmAbs produced in CHO cells, decisions regarding the best process parameters and methods are made based on various factors, such as media costs, process runtime, cell growth and viability as well as product yield and product quality. During process development, bioprocess scientists take these factors into consideration when choosing between a batch, fed-batch or perfusion bioprocess mode [2-8]. The process mode influences the availability of nutrients and the concentration of byproducts and therefore cell growth, viability, and product formation.

Cell growth curves

A culture's growth curve can typically be divided into several distinct phases (Figure 1). During the initial lag phase, growth is slow while cells are adapting to their new environment inside the bioreactor. During the exponential growth (or log) phase, the cell division continues at a constant rate. Once nutrients are depleted and by-products accumulate, cell growth starts to slow down and the culture enters the stationary growth phase. Typically, harvesting of the culture and product occurs at this time. The culture

then enters the death phase which is usually characterized by a steep decline in viable cell density. The duration of the exponential and stationary phases differ in batch, fed-batch, and perfusion bioprocesses (Figure 1), as explained in more detail below.

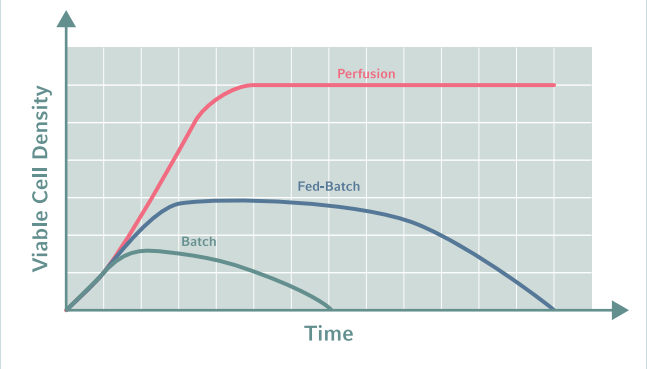

Fig. 1: Schematic representation of cell growth curves in batch, fed-batch, and perfusion bioprocesses.

Batch bioprocess

In a cell culture batch run, mammalian cells are inoculated into a fixed working volume of media in a bioreactor (Figure 2). Batch cultures are typically run at the maximum working volume of the vessel. Throughout the duration of the run, nutrients are gradually depleted and toxic byproducts accumulate inside the vessel. At some point the cells will stop growing and the proportion of viable cells will decline, because nutrients are consumed and toxic metabolites get concentrated. Major advantages of a batch culture are the ease of operation and process setup, and a lowered risk of contamination comparatively to other processes.

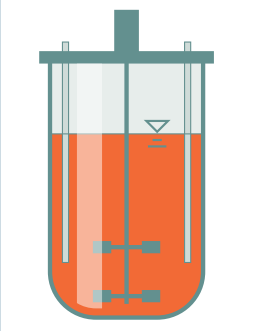

Fig. 2: Batch process. In a batch process the culture grows in the initially supplied batch of medium.

However, cell densities and production yields that can be achieved in batch cultures are much lower than those in fed–batch and perfusion processes. Batch cultures are a great starting point for beginners in the field looking to optimize culture conditions in the early stages of experimental development and design. A batch process can run for a duration of around 7 days.

Fed-batch bioprocess

A fed-batch culture is a modified version of a batch culture. Here the culture is fed to keep the concentration of nutrients constant (Figure 3). High substrate concentrations can be achieved, resulting in higher cell densities and product titers, and prolonged cell viability, but toxic metabolites still accumulate over time.

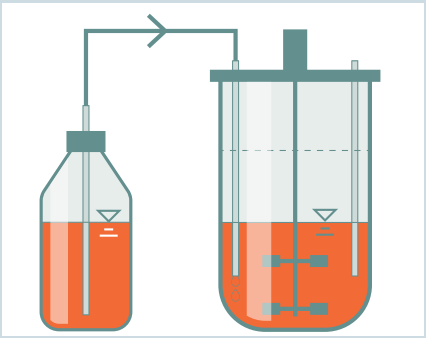

Fig. 3: In a fed-batch process the culture is fed to keep the concentration of nutrients constant.

The bioreactor is inoculated at a lower working volume, which is often the minimum working volume of the vessel and is grown under batch conditions for a short time until certain criteria have been met to trigger the start of feeding. Typically, feeding is started when initial glucose has been depleted or there is a significant buildup of harmful byproducts, such as ammonia (> 3 mmol/L), inside the bioreactor. Once either or both of those criteria have been met, nutrients are added to the bioreactor via a prepared feed media bottle in specifically calculated increments every day until the feed bottle has been emptied and the maximum working volume has been met.

Media addition calculations are based on end or maximum working volume. Typically, in our lab, a fed-batch bioreactor is fed daily at 3 to 5 % of the total vessel working volume desired at the end of the run. It is important to calculate this parameter properly so that the volume of medium added to the culture is not too low, which can lead to a buildup of toxic byproducts in the vessel. Alternatively, adding too much medium to the bioreactor can cause the culture to be too diluted and affect overall cell growth and production. In addition to feeding fresh medium to your vessel, a glucose bolus feed might also be necessary in the exponential growth phase if your feed medium addition is not supplementing enough glucose to keep up with the demand.

After the bioreactor has reached the maximum working volume and the feed bottle has been emptied, the culture will

continue until viability and cell growth decline, signifying the end of the run. With the addition of fresh nutrients and dilution of toxic byproducts, fed-batch cultures can often double the cell growth and production yields compared to a batch run and can run for 2-3 weeks.

Perfusion bioprocess

In a perfusion process, a continuous medium exchange occurs. Like in fed-batch cultures, fresh medium is continuously added to the bioreactor to replace depleted nutrients throughout the run. However, in perfusion processes, used medium is also collected with the harvested product at the same rate that fresh medium is being added to the bioreactor (Figure 4). The addition of fresh medium and removal of spent medium at the same rate will allow the culture volume in the bioreactor to stay constant; therefore, the maximum working volume of the vessel does not limit the amount of fresh medium that can be added to the culture for the duration of the run. The rate of this media exchange process is referred to as vessel volume per day or VVD.

For suspension cultures like CHO, a cell retention device is needed to retain the cells inside the bioreactor when removing used media and product in perfusion processes. Hollow fiber filters using alternating tangential flow (ATF) are successfully used for the retention of suspension cells, and achieved great success in supporting high density cell culture when combined with existing stirred-tank bioreactors. The ATF perfusion device allows media and small molecules to pass through the hollow fiber filter column and to be collected in the harvest (waste) bottle while retaining cells inside the bioreactor. Simultaneously, fresh media is circulated through the growing culture, supplying critical nutrients to achieve high cell densities [9].

In a tangential flow filtration-based perfusion process, the liquid flows passes the pores of hollow fiber filters tangentially, rather than being forced through them orthogonally, thus reducing the likelihood of clogging. ATF devices use the same principle of tangential flow but reverses the direction of flow regularly to further minimize fouling. ATF perfusion is suitable for perfusion processes with suspension cells [9].

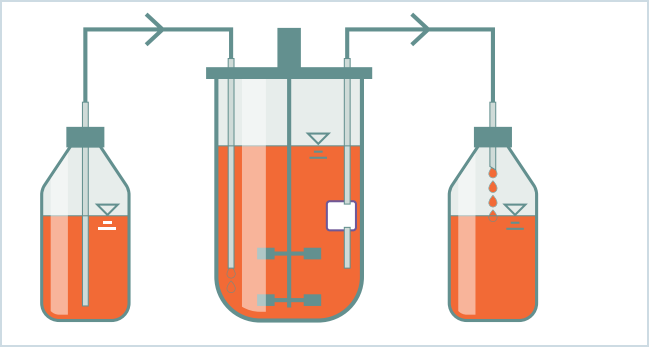

Fig. 4: In a perfusion process the culture medium is continuously exchanged while the cells are retained in the bioreactor.

The perfusion media exchange process is started once either glucose has been consumed, typically when concentration drops below 3 g/L in our lab, or there is a significant buildup of toxic byproducts, such as ammonia (> 3 mmol/L), inside the bioreactor.

A perfusion process can last three or more weeks, depending on the maximum VVD chosen to cap the medium consumption rate.

In this application note we describe how we set up a CHO cell culture bioprocess in batch, fed-batch, and perfusion mode, respectively. We compare the metabolic profiles and performance indicators like cell growth, product formation, and process costs.

Material and Methods

Cell line and medium composition

All experiments used a suspension CHO cell line from TPG Biologics, Inc., expressing a biosimilar hmAb. The cells were cultivated in Dynamis AGT Medium for the batch run. For the fed-batch and perfusion runs, cells were cultivated using CD-FortiCHO Medium (Thermo Fisher Scientific®). Both media were supplemented with 8 mM L-glutamine and 1 % Gibco® Anti-Clumping Agent and 1 % Antibiotic-Antimycotic (Thermo Fisher Scientific®). The feed media for both the fed-batch and perfusion runs were prepared with CD EfficientFeed C AGT. For the fed-batch, the media was supplemented the same as the initial culturing media.

For the perfusion run, the culture media was prepared as described above with one modification: the glutamine concentration in the perfusion media was changed to 2 mM. The glutamine feeding concentration was decreased to reduce ammonia production during the run.

Inoculum preparation

Optimizing culture parameters at the shake flask stage is key to preparing ideal inoculum with reproducible results. Flask culture with high viability (90 % or higher) is a sign of healthy cells. Cells exhibiting high viability before inoculation are less likely to result in a prolonged lag in culture when growing them in the bioreactor. Keeping track of this information can help quickly detect changes in viability or cell growth from flask to flask (Figure 5).

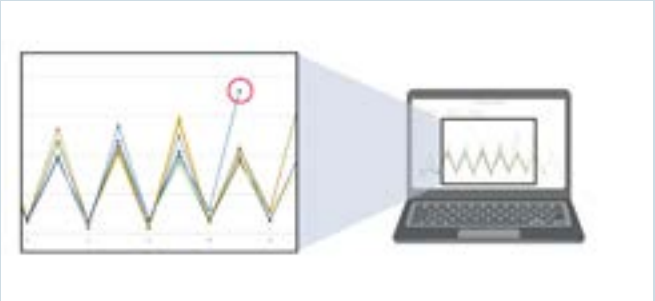

Fig. 5: Tracking cell growth and viability helps quickly detect and eliminate outliers amongst flask cultures during inoculum preparation.

Interested in more information on how to increase the reproducibility of your cell culture bioprocess?

Download our ebook!
www.eppendorf.group/ebook-reproducibility

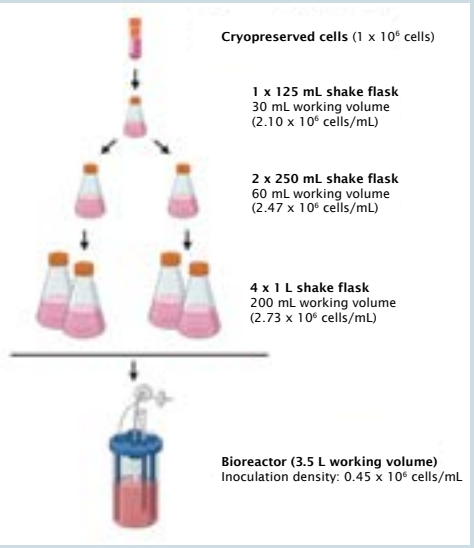

Fig. 6: Cell expansion for the inoculation of ATF perfusion run

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The bioreactor inoculum for each experiment was prepared by cultivating the cells in single-use baffled polycarbonate shake flasks (Corning®) in a New Brunswick S41i CO_2 Incubator Shaker (Eppendorf) set at 125 rpm and 8 % CO_2 with passive humidification. Cells from a cryopreserved stock vial were inoculated at a density of 0.3 x 10^6 cells/mL in a 125 mL flask with a 20 % fill volume. After one week of passaging every other day, the culture volume was scaled-up by increasing the flask size from 125 mL to 250 mL, and finally 1 L, while keeping the cell density, percentage fill, and all other parameters constant. Using this method, each bioreactor was inoculated with cells that were at approximately the same passage and duration of culture post-thaw. Each experiment had an inoculation density ranging from 0.3 x 10^6 cells/mL to 0.5 x 10^6 cells/mL. An example of the flask culture expansion process is shown for the ATF perfusion run in Figure 6.

Bioreactor control system

For this study, a BioFlo® 320 bioreactor control system was used (Figure 7). The BioFlo 320 was set up based on the operating manual [10].

Eppendorf BioBLU® Single-Use Bioreactors were used for all processes in this study. The cell culture BioBLU bioreactors are equipped with a pitched-blade impeller

Fig. 7: BioFlo 320 bioreactor control system

and non-invasive sensors for the measurement of DO, temperature, and optical measurement of pH that drastically reduce contamination risks.

Bioreactors and process parameters

For the batch culture, a BioBLU 10c was used at a working volume of 10 L. The batch culture was agitated at 126 rpm. A BioBLU 3c was used for the fed-batch culture with a starting working volume of 1.5 L and an ending working volume of 3 L with an agitation of 100 rpm (Table 1).
For the perfusion run, a BioBLU 5c Single-Use Bioreactor equipped with a pitched-blade impeller was used with a working volume of 3.5 L and an agitation of 100 rpm. For medium harvest, the bioreactor was equipped with a dip tube already installed inside the vessel with an AseptiQuik® connector (Colder Products Company). The dip tube was

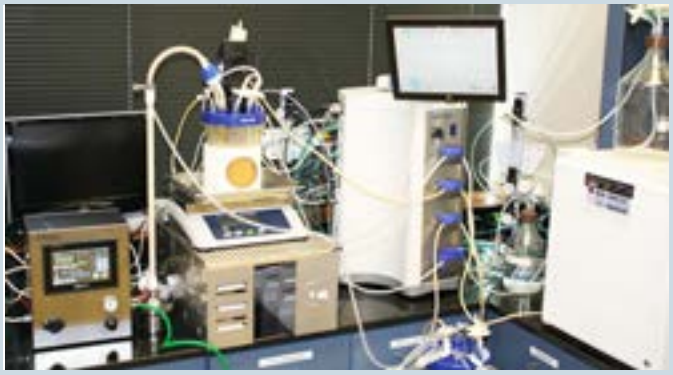

Fig. 8: Connection of the BioFlo 320 bioreactor control station with ATF cell retention device

Attention: In case of a rupture of the ATF membrane the pressure inside the vessel can increase.
> To avoid rupture of the vessel, maintain an unrestricted air vent
> This can be done by installing a pressure relief valve on the head plate of the bioreactor
> It needs to be ensured that there is no filter between safety valve and vessel to enable an unrestricted air vent

then connected to a XCell® ATF 2 (Repligen®) via silicone tubing and a CPC AseptiQuik fitting (3/8" hose barb fitting). The filter had a pore size of 0.2 µm. The filter setup was connected to an ATF controller (C24 Controller, Repligen). The ATF circulation rate was set to 1 L/min [11].

Because a temperature shift to 32 °C is common practice

Table 1: Process parameters for each experiment in this study

Parameter	Batch process	Fed-batch process	Perfusion process
Bioreactor	BioBLU 10c Single-Use Bioreactor	BioBLU 3c Single-Use Bioreactor	BioBLU 5c Single-Use Bioreactor with ATF dip tube
Bioreactor control system		BioFlo 320	
Process duration	7 days	12 days	14 days
Starting working volume	10 L	1.5 L	3.5 L
Ending working volume	10 L	3 L	3.5 L
Inoculation density	0.5 x 10^6 cells/mL	0.3 x 10^6 cells/mL	0.45 x 10^6 cells/mL
Agitation	126 rpm	100 rpm	100 rpm
Temperature	37 °C	37 °C; shift to 32 °C at day 3	37 °C; shift to 32 °C at day 7
DO sensor		polarographic sensor	
DO setpoint		50 %	
pH sensor	potentiometric sensor	optical sensor	optical sensor
pH setpoint	pH 7.0 (deadband = pH 0.2) via a cascade to CO_2 (acid) and 0.45 M sodium bicarbonate (base)		
Gassing strategy	3-gas auto mixing, 0.05 SLPM - 1 SLPM	3-gas auto mixing; 0.05 SLPM - 1 SLPM	3-gas auto mixing; 0.05 SLPM - 5 SLPM
Feeding strategy	n/a	3 % of total volume per day	0.2 VVD - 1.7 VVD
Glucose bolus feed target	n/a	> 3 g/L	> 3 g/L
ATF circulation rate	n/a	n/a	1 L/min; 1.2 L/min from day 12 to prevent clogging

for increasing CHO cell protein production, the temperature shift was utilized for increased hmAb production in some of the culture processes: In the fed-batch run, the temperature was decreased to 32 °C on initiation of feeding; in the ATF perfusion process, the temperature was shifted to 32 °C on day 7 [11].

Feeding and perfusion control

For the fed-batch run, the bioreactor was fed 3 % of the total volume per day. The feed media was prepared as described previously in the media composition section.

The glucose concentration target for the fed-batch and perfusion runs was to keep glucose levels 3 g/L or above. When the glucose concentration dropped below 3 g/L, the culture was fed by pumping in the appropriate amount of 200 g/L sterile glucose (bolus feed) into the culture twice daily. This was in addition to replenishing the glucose throughout the perfusion and fed-batch processes via media feeding.

The perfusion rate was between 0.2 VVD and 1.7 VVD. The rationale behind perfusion rate adjustment was to keep the ammonia concentration in the bioreactor < 4 mM. The perfusion rate was adjusted based on ammonia level at the time of sampling.

ATF perfusion device setup

The ATF device was turned on a few hours prior to inoculation to allow the media to circulate through the filter at the setpoint used for the run (1 L/min), as recommended by the manufacturer. This is an important step to properly wet the filter by media circulation and allow for any air bubbles to gradually work themselves out of the filter prior to inoculation. The ATF circulation of media and cells were started five hours before it was needed to initiate perfusion so that the cells could adjust to the alternating tangential flow-related stress, as recommended by the manufacturer. The connection of the ATF to the BioFlo 320 controller is shown in Figure 8.

More details on this specific perfusion process can be found in our publication titled, "Comparing Culture Methods in Monoclonal Antibody Production" [11]. Additionally, a description of a small scale ATF perfusion run using the DASGIP® Parallel Bioreactor System can be found in our Application Note 410 [9].

Sensor calibrations

The BioFlo 320 controller supports a range of Mettler Toledo® dissolved oxygen (DO) sensor technologies: polarographic (either analog or digital ISM®) or optical. Based on sensor type, calibrations will vary. For an in-depth look into DO sensor calibrations, please see "A Guide to Calibration on the BioFlo 120 and BioFlo 320: Dissolved Oxygen Sensors" [12].

In addition to using the calibration guide for reference, the BioFlo 320 also includes an Auto Calibration feature for DO sensors that will automatically calibrate the sensor once it has stabilized in pre-culture conditions. Auto Calibration also allows for process reproducibility between users which is extremely important for scale-up processes.

In this study, DO was measured using a polarographic sensor (Mettler Toledo) for all experiments and was controlled at 50 % by sparging air and/or oxygen.

For the batch culture, a potentiometric (gel-filled) pH sensor was used. Fed-batch and perfusion cultures used an optical pH sensor that is compatible with the BioBLU bioreactors. For all experiments, the pH was automatically controlled at 7.0 (deadband = 0.1 or 0.2) via a cascade to CO_2 (acid) and 0.45 M sodium bicarbonate (base).

The potentiometric pH sensor is calibrated outside of the vessel prior to sterilization. It is calibrated based on a 2-point calibration method on the bioprocess controller. For cell culture, a pH 7.00 buffer is used to set ZERO, and a pH 10.00 buffer to set SPAN. The sensor is then sterilized in a steam sterilizer (autoclave) pouch and inserted aseptically into the vessel in a laminar airflow cabinet (hood). For the optical pH sensor compatible with the BioBLU bioreactors, follow the instructions from the user manual.

Pump calibrations

Pump calibrations are an important step to any process. Calibrating with the correct tubing that is used in a process will help with pump rate accuracy throughout the run. Additionally, making sure that the tubing length and inner diameter (ID) for a particular run is mimicked in the calibration process to make sure the pumps are calibrated at user parameters.

This is especially important in processes like fed-batch and perfusions which rely heavily on pumps to deliver nutrients or remove wastes at a specific rate. For example, if an addition pump is not properly calibrated, this can lead to over pumping nutrients, and potentially, overflowing a vessel causing culture dilution. Alternatively, it can also lead to under feeding or under removal of wastes that can lead to culture lag or premature death.

The pump's operating minimum and maximum range should also be taken into consideration when choosing the right pump for each task in a specific process. Pump calibration processes vary between controllers. The

controller's operator manual should be consulted to ensure proper pump calibrations.

Aseptic connection of addition and harvest bottles

At the bioreactor stage, it is extremely important to connect addition and harvest bottles aseptically. Maintaining aseptic conditions can be done in several different ways. For example, a laminar airflow cabinet can be used to make aseptic connections, however, it is not always practical to make connections inside a hood.

One way to keep aseptic connections outside the hood is by using a tube welder. We use the SCD®-II Sterile Tubing Welder from Terumo (Figure 9). The Terumo welder reaches temperatures up to 300 °C to maintain sterility while welding. The tube welder requires the use of a special type of tubing made of weldable material like C-Flex. We like to prepare our bottles with silicone tubing (for our pump heads) and add a reducer to a piece of C-Flex tubing, usually 4 to 8 inches in length, for welding. An example of the silicone to C-Flex tubing set up can be seen in Figure 9.

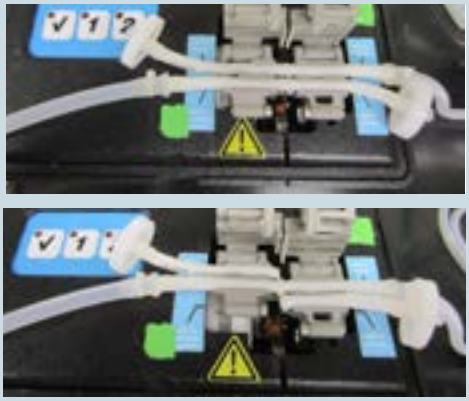

Fig. 9: Tube welding. Silicone tubing is extended with weldable connectors made of C-Flex® via straight connectors. A: Before welding B: After welding

CPC AseptiQuik Connectors from Colder Products Company (CPC) are an easy alternative to investing in a tube welder (Figure 10). We used these connectors in our ATF run featured in this beginner's guide for the ATF filter connection to our bioreactor.

These connectors allow the user to make sterile connections to the bioreactor without the added risk of contamination, via a sterile boundary membrane on each side half of the connector. AseptiQuik Connectors come in a variety of hose barb sizes and other types of connection ends.

When using an AseptiQuik connector, we integrate one side of the connector onto our tubing assemblies and autoclave them. We also integrate a connector onto our bioreactors. Depending on using either glass or single-use bioreactors, this is either assembled in a way to connect it to our single-use bioreactors after autoclave inside the hood or directly onto our glass bioreactor prior to autoclave. Once the AseptiQuiks are sterilized, we are able to connect them together. For first time users, I highly recommend visiting CPC's website. They have great resources, including how-to videos, that will guide you through the process step by step for every type of connector they offer.

Sampling and analytics

For each experiment, the bioreactor was sampled twice daily, one in the morning and one in the evening, to check offline values such as cell density, viability, glucose, ammonia (NH_3), lactate, and hmAb concentration. To collect the highest quality sample from the growing culture in the bioreactors, a sterile 5 mL syringe was connected to the sample port Luer Lock and a dead volume of 4 mL was removed. After the dead volume was removed, a second 4 mL sample as taken using a new syringe to provide a fresh, viable sample for analytics [13]. It is important to collect enough dead volume from the sampling line to ensure the best sample is taken from the bioreactor that accurately depicts the growing culture.

The cell density and viability were measured via the trypan blue exclusion method using a Vi-Cell® XR Viability Analyzer from Beckman-Coulter®. The in-vessel pH values read on the controllers were checked offline using an Orion Star® 8211 pH-meter (Thermo Fisher Scientific) to verify that the pH sensor values were accurate. By checking the offline pH value, the pH was able to be restandardized, if necessary, to prevent any discrepancies between online and offline measurements. An offline pH check is important to minimize any sensor drift that can happen throughout an experiment. Glucose, ammonia, lactate, and hmAb were measured using a Cedex® Bio Analyzer (Roche Diagnostics®) [13]. Measuring these analytics twice daily helps keep track of ammonia build up and glucose levels, which are especially important to monitor in processes such as fed-batches and perfusions.

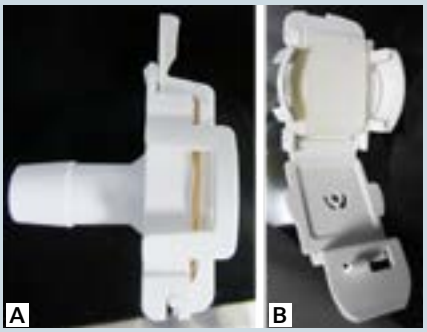

Fig. 10: AseptiQuik genderless connector. **A.** The connector closed and ready for autoclave. **B.** The sterile boundary membrane of the AseptiQuik connector after opening the tab.

Results

Batch Run

For the batch run, the bioreactor was inoculated at 0.5 x 10^6 cells/mL. The culture in the BioBLU 10c reached a peak density of 14.1 x 10^6 cells/mL on day 5 (Figure 11A).

The metabolic profile and antibody production for the batch run is shown in Figure 11B. The culture reached a peak antibody production of 195 mg/L on day 6. Ammonia gradually increased throughout the run until it reached toxic levels of 12 mmol/L on day 6. The lactate concentration remained under 2 g/L for the duration of the run.

Fed-batch Run

For the fed-batch run, the bioreactor was inoculated at 0.3 x10^6 cells/mL. The maximum ammonia level was targeted for 3 mmol/L and monitored daily. Feeding was started on day 3, when those levels reached close to 3 mmol/L. The bioreactor was fed 3 % of the total volume per day until the feed bottle was empty. The fed-batch culture reached a peak density over 20 x 10^6 cells/mL on day 9 (Figure 12A).

The metabolic profile and antibody production for the fed-batch is shown in Figure 12B. Peak antibody concentration was 1550 mg/L on day 15. Ammonia was over 6 mmol/L for the first few days of feeding. With culture feeding it got diluted and remained low until feeding was complete.

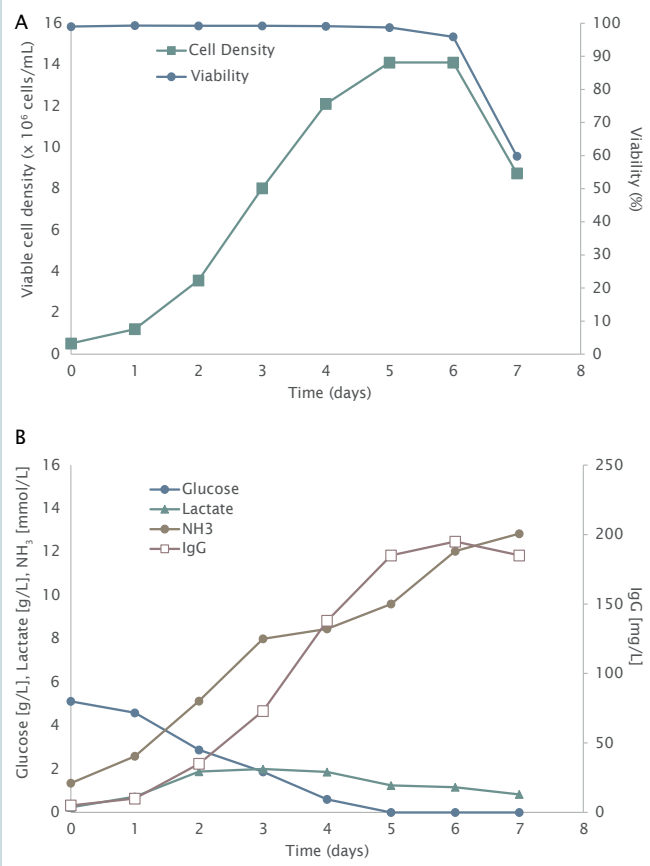

Fig. 11: CHO cell bioprocess in batch mode
A. Viable cell density and viability.
B. Metabolic profile (concentrations of glucose, lactate, and NH_3) and IgG concentration.

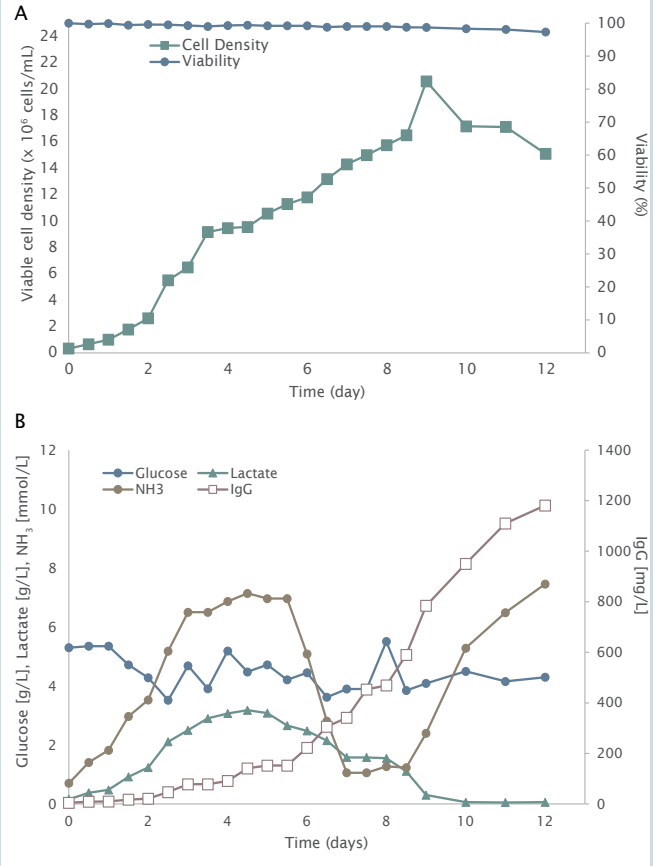

Fig. 12: CHO cell bioprocess in fed-batch mode
A. Viable cell density and viability.
B. Metabolic profile (concentrations of glucose, lactate, and NH_3) and IgG concentration.

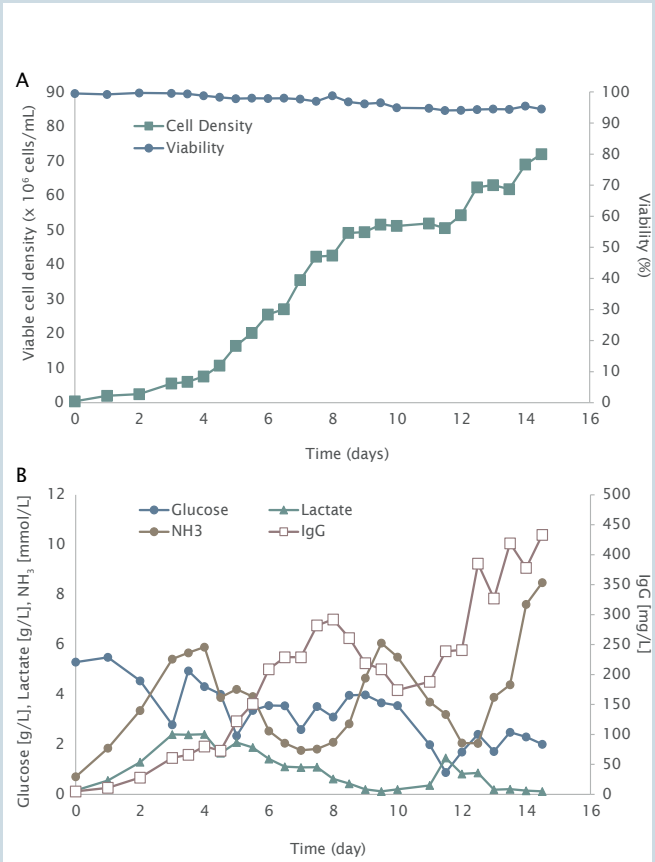

Fig. 13: CHO cell bioprocess in perfusion mode
A. Viable cell density and viability.
B. Metabolic profile (concentrations of glucose, lactate, and NH_3) and IgG concentration.

Ammonia increased to toxic levels by day 15 when the culture entered the decline phase. Lactate concentration remained under 3 g/L for the duration of the run.

ATF Perfusion

The culture was inoculated at a density of 0.45 x 10^6 cells/mL. On day 12, the culture reached a peak cell density of 72 x 10^6 cells/mL. At that time point, 90 % of the cells were viable (Figure 13A).

The ammonium concentration target was < 4 mM and the glucose concentration > 3 g/L in the course of the run. On day 3 the ammonia concentration exceeded 3 mM and perfusion was started at 0.5 vessel volumes per day (VVD). Based on the ammonia concentrations determined offline, the perfusion rate was gradually increased up to 1.7 VVD. The ammonia concentration stayed below 6 mmol/L until day 3 when perfusion was started. Ammonia reached over 8 mmol/L at day 14. The lactate concentration remained below 2.5 g/L for the duration of the run. When the glucose concentration dropped below 3 g/L, the culture was fed by pumping in the appropriate amount of 200 g/L sterile glucose into the culture twice daily, in addition to replenishing the glucose throughout the perfusion process (Figure 13B).

The IgG concentrations in the bioreactor increased up to approximately 433 mg/L. As expected, IgG production steadily increased following the cell growth profile (Figure 13B).

Discussion and Conclusion

Shown in Figure 14, we compared the growth curves from our batch, fed-batch and perfusion runs. Our batch culture ran for a total of 7 days and reached a peak density of 14.1 x 10^6 cells/mL on day 5. The fed-batch culture ran for 12 days and reached a peak density of 21 x 10^6 cells/mL on day 9. Our perfusion culture ran the longest and reached the highest density of all bioprocess modes by day 15. A batch culture takes significantly less run time than a fed-batch or perfusion and is the easiest process to execute. A batch culture is a great way for a beginner to start their optimization process without added complexity or equipment costs. Using this process, it is an easy way to test different parameters like inoculation densities, varying media compositions or gassing strategies, especially at small scale. However, culture growth and yield production are limited in batch processes. A fed-batch has increased yield and process time with a higher complexity but can be overall more cost efficient than running batch cultures. As you see in Table 2, we achieved a significantly higher IgG yield in the fed-batch run than in the batch process. Fed-batch processes are great for those looking to achieve higher cell growth and yield productions in comparison to batch runs. Fed-batch cultures also do not require additional equipment costs to run.

Perfusion can achieve higher cell density and yields than a batch or a fed-batch culture. In our example, we obtained almost trice the amount of IgG per bioreactor

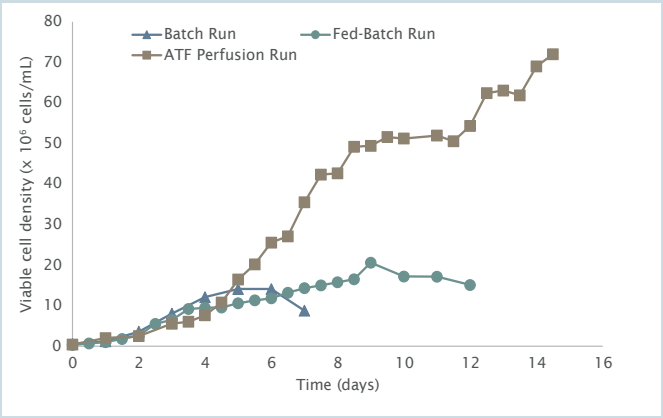

Fig. 14: Growth curve comparison for batch, fed-batch and perfusion runs

volume compared to the fed-batch run. However, perfusion processes are also associated with higher equipment costs and media consumption.

Since higher cell densities can be achieved with perfusion processes, using smaller bioreactors and optimizing process parameters could potentially reduce space requirements and investment costs while still achieving the highest production yields over batch or fed-batch processes. Perfusion cultures can add many layers of challenges into its processes and often requires additional in-process optimization to fine-tune process parameters. However, once established, high cell culture growth and production yields are achievable [14].

Overall, the choice between processes depends on many factors such as experimental needs and budget. We hope that this application note will help our beginner cell culture scientists confidently choose their bioprocess mode that is well suited to their needs.

Table 2: Run comparison of each bioprocess mode

Run type	Typical duration	Media consumption/ ending working volume [L]	Equipment costs	Process complexity	Peak cell density	Total hmAB produced [g]	Total hmAB/ ending working volume [g/L]
Batch	~ 1 week	1	costs for bioreactor control system and bioreactor	Low	14 x 10^6 cells/mL	1.95 g	0.195 g/L
Fed-batch	~ 2 weeks	1	costs for bioreactor control system and bioreactor	Medium	21 x 10^6 cells/mL	4.5 g	1.5 g/L
ATF perfusion	~ 2 to 4 weeks	14.4	costs for bioreactor control system and bioreactor; additional costs for hollow fiber filters and ATF perfusin control device	High	72 x 10^6 cells/mL	11.5 g	3.3 g/L

ADDITIONAL RESOURCE

Interested to know how it works with E. coli fermentation instead of CHO Cells?

Download this free resource to learn more!

Literature

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Ordering information

Description	Order no.
BioFlo® 320, base control station, with water connection	1379963011
BioBLU® 3c Single-Use Bioreactor, cell culture, macrosparger, 1 pitched-blade impeller, optical pH, sterile	1386000300
BioBLU® 10c Single-Use Bioreactor, cell culture, macrosparger, 1 pitched-blade impeller, optical pH, sterile	1386141000
BioBLU 5c Single-Use Bioreactor, cell culture, macrosparger, 1 pitched-blade impeller, optical pH, sterile with ATF dip tube	inquire

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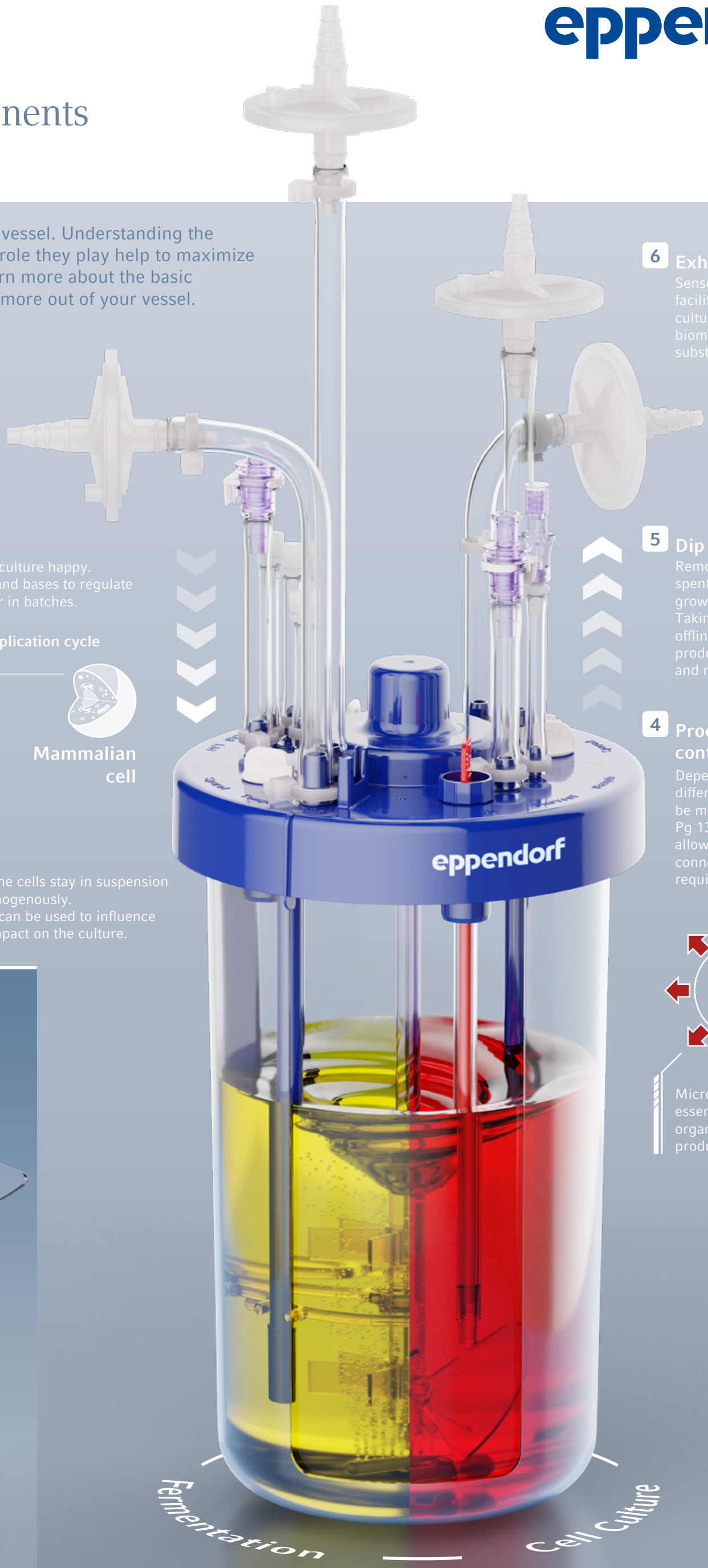
Stay Informed

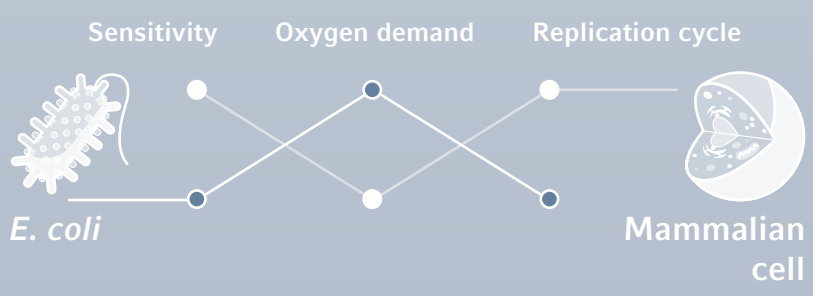
The Basic Components of a Bioreactor

Bioreactors are more than a just a vessel. Understanding the individual components and which role they play help to maximize the efficiency of your process. Learn more about the basic components of a bioreactor to get more out of your vessel.



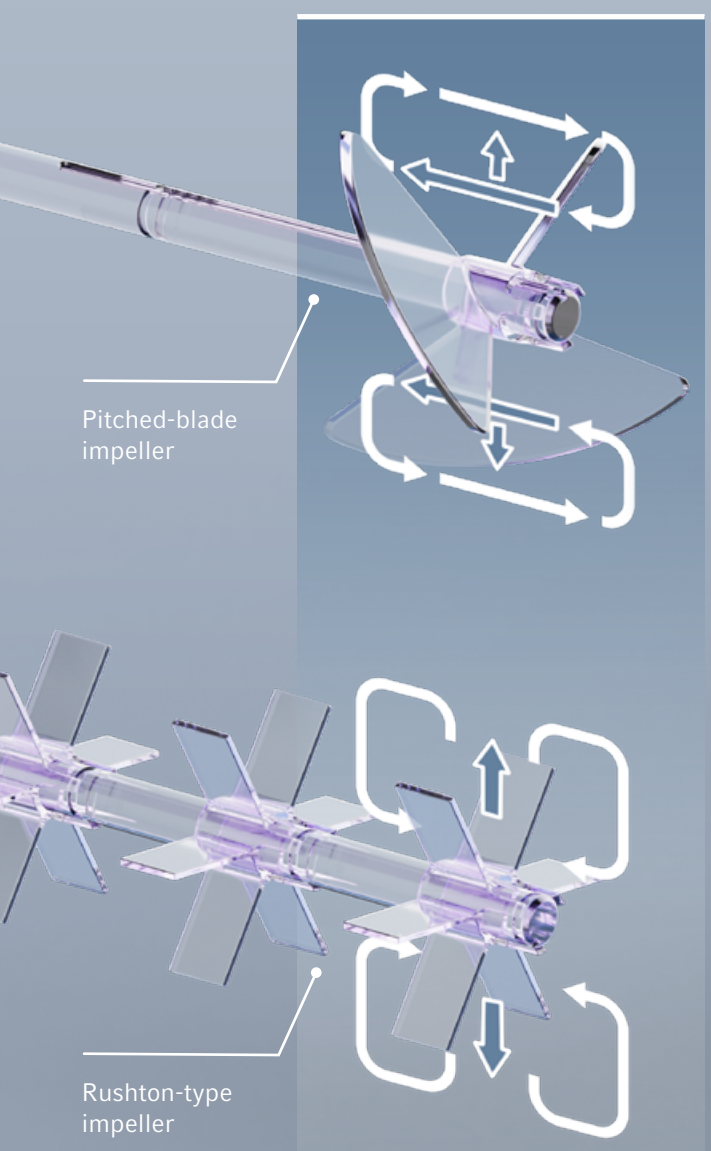
1 Feed lines

Feeding is the essential step to keep your culture happy. Nutrients, carbon sources, but also acids and bases to regulate the pH value can be added continuously or in batches.



2 Agitation system

Proper mixing is essential to ensure that the cells stay in suspension and that the culture medium is mixed homogenously.
The selection of different impeller shapes can be used to influence the medium flow, but also have a direct impact on the culture.

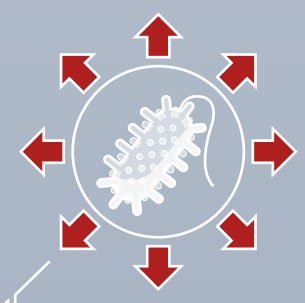


3 Gas control

Gas control is important to ensure optimal cell growth and product formation. Overlay or submerged gassing strategies can be chosen to influence the dissolved oxygen (DO) concentration, an important parameter for optimal cell growth.
Carbon dioxide (CO_2) sparging is used to control the pH values in cell culture processes.

4 Process control elements

Depending on the process, different parameters need to be monitored and controlled. Pg 13.5 ports in the headplate allow inserting sensors or connections that may be required for the process.



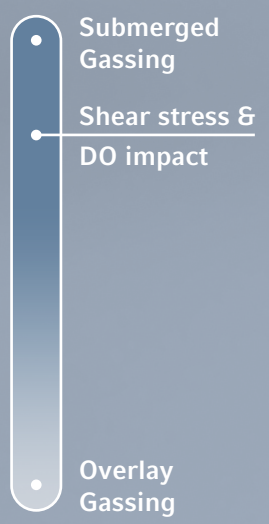
Microbiology: Cooling is essential! Depending on the organism, 1 L of culture can produce **100 W** of energy.

5 Dip tube

Removal of waste products in spent medium prolongs cell growth and product formation. Taking samples allows for offline analysis of cells, product formation, and medium composition.

6 Exhaust gas

Sensors for exhaust gas facilitate insights into critical culture parameters, such as biomass development and substrate consumption.



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APPLICATION NOTE No. 499

A Beginner's Guide to Mesenchymal Stem Cell Culture with the SciVario® twin Bioprocess Control Station

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Abstract

This publication is intended to provide a straightforward workflow to help users to culture human mesenchymal stem cells (MSCs) using Eppendorf stirred-tank bioreactors. We describe simplified workflow steps using the Eppendorf SciVario twin control station, including the preparation of MSCs for inoculation, expansion of MSCs in T-flasks and shake flasks, and MSC culture in bioreactors for cell production. To demonstrate the ease of use to beginners, we chose a bench-scale BioBLU® 1c Single-Use Bioreactor as the main vessel for this guide. The principles discussed in this document provide a foundational approach for scaling up MSC cultures, addressing the increasing demands for MSC-based cell therapy.

Content

Introduction

Stem cell-based therapy is at the forefront of cell and gene therapy (CGT), offering transformative potential in treating human diseases through personalized and regenerative medicine [1]. Mesenchymal stem cells (MSCs) are particularly prominent due to their accessibility from various tissue sources, such as bone marrow, adipose tissue, and umbilical cords, as well as their ability to differentiate into a wide range of tissue types [2]. MSCs play crucial therapeutic roles, including reducing inflammation through cytokine release, aiding tissue repair via growth factors, modulating immune responses, and functioning as structural cells in tissues like bone [2]. Their safety and efficacy have led to over 1,100 clinical trials, with Prochymal® being the first market-approved MSC product for allogeneic stem cell therapy [3].

While in vitro expansion of MSCs is well-established, transitioning these cells to stirred-tank bioreactor cultures requires substrates for attachment due to their adherent nature [4]. Microcarriers, typically 100-300 µm in size, are used to facilitate the transition from 2D to 3D suspension culture [5]. This adaptation from conventional suspension cultures, such as those used for CHO cells, necessitates specific medium exchange processes to separate the cell-microcarrier complex from the liquid before removal. This guide demonstrates an accessible MSC-microcarrier culture process in a stirred-tank bioreactor, enabled by the user-friendly SciVario twin controller.

The SciVario twin is a versatile bench-scale bioprocess controller capable of independently operating two bioreactors, making it ideal for cell culture and fermentation applications in R&D. Supporting both glass and BioBLU Single-Use Bioreactors, it offers a modular, customizable design that can be easily upgraded to meet evolving needs. Advanced process monitoring and control are achieved through the DASware® control 6 software, with the BioNsight® cloud solution allowing remote access and connectivity with other labs for data analytics. Its intuitive user interface simplifies process setup, guided by intelligent workflows, thus requiring no complex training.

Material

Cell lines, medium and microcarriers

MSCs for bioprocessing can be primary cells isolated from human body such as muscle, umbilical cord, bone marrow, or differentiated from induced pluripotent stem cells (iPSCs). Many commercial sources can provide cell source and medium for MSCs. Commercial sources, such as the American Type Culture Collection (ATCC), are available to obtain cryopreserved MSC vials, typically as 1-2 million cells per vial.

MSC culture medium originally started with DMEM supplemented with fetal bovine serum (FBS) in the early stages of development. In the CGT context, animal sourced reagents like FBS are discouraged for human use; in addition, chemically defined medium is preferred for better control of batch-to-batch consistency [6]. Accordingly, specialized MSC culture medium has been developed, using clinical grade growth factors to replace serum. MSC medium can be acquired from commercialized sources like the ATCC. Because MSCs are adherent cells, MSC cultures require a surface to attach to. Microcarriers have been favored for adherent cell cultures in bioreactors. Microcarriers are 100-300 µm beads made of biocompatible materials, including glass, polystyrene, dextran, and biological polymers like collagen [5]. Their structure and surfaces can be engineered for enhanced coverage per unit volume for maximized cell adhesion. Cell-laden microcarriers are mixed in stirred tank bioreactors as suspension culture. We found that the collagen-coated microcarriers performed better for supporting MSC cultures than non-coated polystyrene microcarriers (see Eppendorf Application Note 334, source 13).

As our application was primarily a proof-of-concept study, we had used DMEM plus 10% fetal bovine serum (FBS) in the beginning and transitioned to MSC specialized medium such as ATCC's MSC Basal Medium supplemented with IGF, EGF, 7% FBS and glutamine. We used collagen-coated polystyrene microcarriers.

In the following sections, we describe the basic steps of MSC bioprocessing from flask cultures to bioreactor runs.

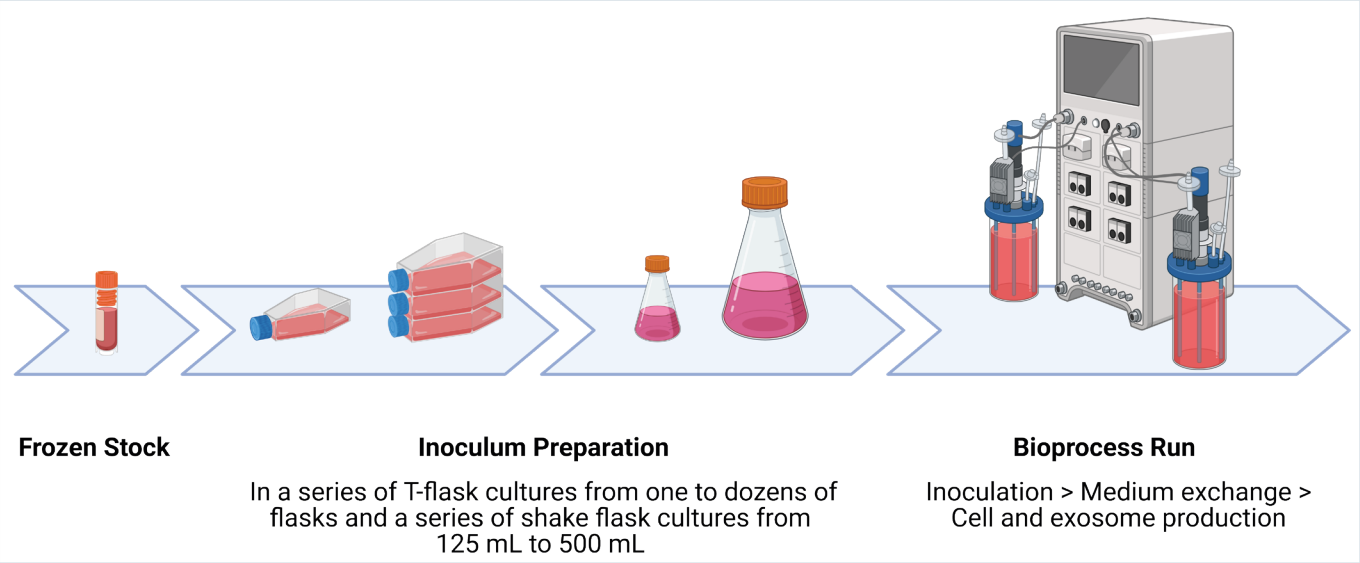

Fig. 1: MSC initial culture and bioreactor inoculum preparation workflow. Image created with BioRender.com

1. MSC initial culture and bioreactor inoculum preparation

From our experience, for the inoculation of a one liter bioreactor culture an inoculum of at least 200 million cells is needed based on an inoculation density of 0.2 million cells per milliliter. Compared to a 1 million cell cryovial stock, the 200-fold expansion needs to be realized in pre-culture before the bioreactor run. As illustrated in Figure 1, the MSC pre-culture expansion involves 2D T-flask culture and 3D shake flask culture.

1.1. 2D T-flask culture

MSCs are expanded from cryo vials in T-flasks, progressing to larger culture volumes. The initial culture is illustrated in Figure 1. T-flask culture is typical of all adherent cells with a seeding density of 1×10^4 and 5×10^4 cells/mL for routine subculture [7]. We passaged MSC T-flask cultures at split ratios of 1:3 to 1:5 upon confluency. T-flask cultures allow for visual examination of cell confluency, therefore, are useful tools for estimating cell saturation density, e.g., the maximum number of cells attainable per unit surface area (cm^2) or per unit volume (mL) of culture [7].

1.2. 3D Shake flask culture with microcarriers

Because of the limited surface area of T-flasks, ultimate yields are limited. Therefore, 3D microcarrier cultures in shake flasks are used as the next step for further expansion of MSCs. Microcarrier cultures benefit from a significantly larger surface area to volume ratio compared to monolayer culture. For example, a series of microcarrier cultures can proceed from 125 mL to 500 mL shake flasks and generate hundreds of millions of cells in one flask, compared to handling dozens of T-flasks for the same total yield.

The maximum MSC yield can be calculated in theory based on the total growth area of the substrate, and MSC surface density at confluency. For example, based on our T-175 cultures of MSCs, we determined a saturation density of 50,000 cells/cm^2 for the MSC line we used. Based on that, we can calculate the theoretical maximum cell densities for different microcarrier types given the same total surface area (Table 1).

Microcarriers are typically provided as dry powder. Preparation of microcarriers for cell culture involves washing with PBS, sterilization by autoclaving, and conditioning with cell culture medium. The most critical step of microcarrier cell culture is the initial cell adhesion, controlled by the cell and microcarrier mixing ratio and the mixing process.

Table 1: Example for MSC and microcarrier mixing calculation.

	SoloHill microcarriers	Cytodex 3 microcarriers	References & Notes
Material	Polystyrene (collagen-coated)	Dextran (collagen-coated)	
Area (cm^2) per g	360	2700	Product brochure
Size (µm)	125-212	120-180	Product brochure
Microcarrier loading density (g/L)	15	15	Examples based on published literature [8]
Total surface area (for 1 L of culture)	5400	40,500	
Cell seeding density by area (per cm^2)	3000	3000	Examples based on published literature [8]
Cell seeding density by volume ($\times 10^6$ per mL)	0.016	0.122	
MSCs maximum density by area (per cm^2)	50,000	50,000	Internal 2D T-175 studies; will depend on MSC source and culture medium
MSCs maximum density by volume ($\times 10^6$ per mL)	0.27	2.0	

1.2.1. MSC and microcarrier mixing ratio

In our workflows, microcarriers are loaded at a density between 10-20 g/L in bioreactors. With the manufacturer specs of the microcarrier, one can calculate the total surface area; based on the expected total cell numbers at full confluence. For each cell and microcarrier combination, initial experiments are needed to determine the optimal mixing ratio of cells and microcarriers upon cell seeding. Too few cells per microcarrier would result in too long lag times for cell expansion, or failure of cell growth especially for sensitive stem cells. Too many cells per microcarrier may not allow for much further cell growth, thus defeating the purpose of cell expansion.

1.2.2 MSC and microcarrier adhesion

Agitation is used to facilitate cell and microcarrier mixing; however, the speed of agitation needs to be carefully controlled to allow for efficient cell adhesion onto the microcarrier. If the agitation is too fast it may delay cell attachment to the microcarrier, resulting in fewer viable cells upon seeding. If it is too slow, the agitation may result in insufficient mixing and incomplete coverage. Shear stress from agitation also impacts MSC expansion and quality [9]. It has been found that intermittent fast agitation with steady agitation at a lower speed has the best outcome compared to a continuous agitation [10]. The initial cell adhesion step for each cell and microcarrier combination needs optimization to determine the appropriate cell-to-microcarrier ratio and agitation schedule.

For a beginner, success in shake flasks is fundamental before going into bioreactor. An example of MSC microcarrier culture in shake flasks is shown in the Eppendorf Application Note 259 "A Novel Method for the Expansion of Mesenchymal Stem Cells using a New Brunswick S41i CO_2 Incubator Shaker" (Figure 2) [11].

1.3. Inoculum preparation

The transition from flask cultures involves dissociating MSCs from seed train substrate (T-flasks or microcarrier in shake flasks), re-mixing and seeding MSCs onto new microcarriers, and introducing them to the bioreactor. The latter two processes can also be carried out directly in a bioreactor with optimized initial adhesion parameters.

We have tried several ways and found bead-to-bead transfer as an effective method for propagating MSC microcarrier cultures in bioreactors. Here we describe this method in comparison to the conventional method of mixing dissociated cells with microcarriers for the inoculum preparation.

1.3.1. Simplified cell inoculation with bead-to-bead transfer

The bead-to-bead transfer method uses a mixture of cell-populated microcarriers and the addition of fresh microcarriers to promote spontaneous cell migration to the fresh microcarriers, effectively subculturing MSCs without enzymatic dissociation [12]. This can avoid the laborious task of enzymatically detaching and re-seeding the cells.

In Application Note 334, we described the bead-to-bead transfer method for MSC and microcarrier cultures in BioBLU 5c Single-Use Bioreactors [13]. Essentially, an inoculum was generated in shake flask microcarrier cultures (e.g., 15-day culture). For bioreactor inoculation, the cell-laden microcarriers were mixed with fresh microcarriers

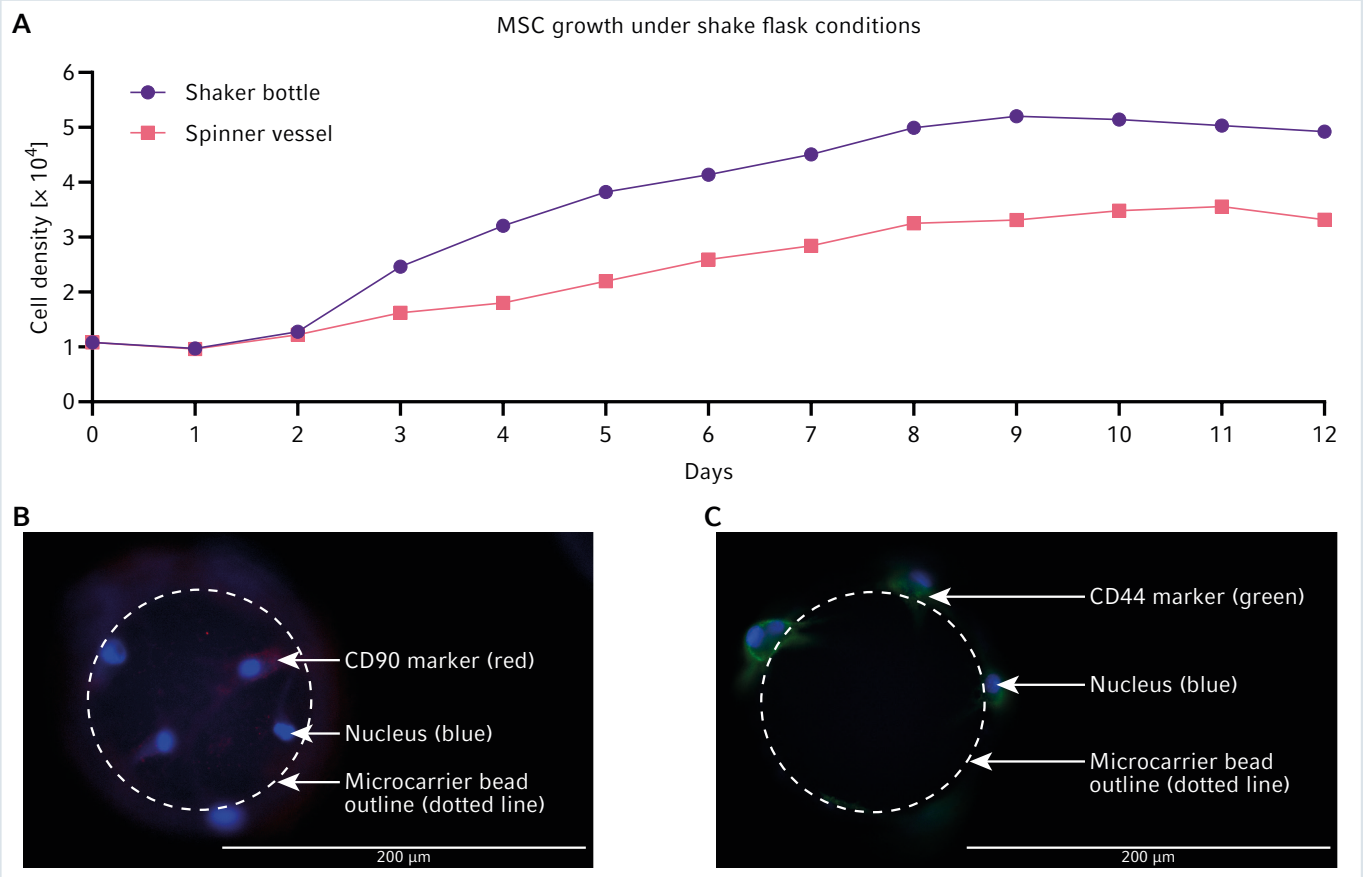

Fig. 2: Adipose-derived mesenchymal stem cells (AdMSC) cultured on microcarriers either in shake flasks or spinner flasks. Mixing of shake flask cultures was achieved by incubation in an incubator with shaking capabilities (Eppendorf New Brunswick S41i CO_2 Incubator Shaker). Mixing of the spinner flask culture was achieved by placing it on a magnetic stirrer in a static incubator. For more information see [11]. **(A)** Growth curves of the shake flask culture (purple) compared to the spinner flask (red) culture. **(B)** AdMSCs positive for CD90 stem cell marker. **(C)** AdMSCs positive for CD44 stem cell marker.

to reach a target microcarrier loading density (e.g., 17 g/L of microcarrier loading density and 0.02×10^6 cells/mL cell seeding density) and introduced into the bioreactor. Intermittent agitation can be introduced to promote cell migration between the beads. This method enabled serial subculture of MSCs without using proteolytic enzymes.

1.3.2. Traditional cell inoculation – Enzymatically released MSCs to mix with fresh microcarriers

Alternatively, one can continue to use the traditional method of inoculation by mixing dissociated cells with fresh microcarriers. Alternative enzymes to trypsin, such as dispase or accutase, should be tested for more gentle enzymatic dissociation of cells from the substrate to minimize cell loss [14]. Upon cell dissociation, the enzymes must be thoroughly deactivated and washed off the cell suspension to avoid residual activity. The dissociated cells are then mixed with fresh microcarriers at an optimized ratio, as discussed in the earlier section, to prepare the inoculum. We have found that co-incubation of cells and microcarriers prior to bioreactor inoculation for at least 2 hours is helpful for the cell adhesion.

2. Bioreactor and Control Station Setup

2.1. Bioreactor setup

BioBLU Single-Use Bioreactors with working volumes ranging from 0.1 to 40 liters are supported by the SciVario

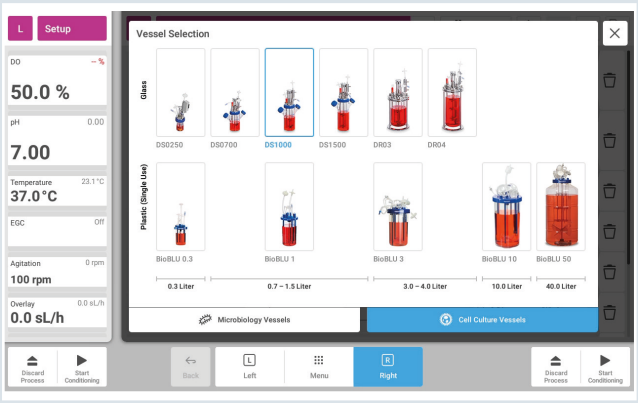

Fig. 3: Bioreactor setup screen of the SciVario twin bioprocess controller.

twin control station (Figure 3). The wide range supports easy scale-up of upstream bioprocess with only one controller, providing flexibility for R&D and process development.

Polarographic sensors for dissolved oxygen (DO) and electrochemical ones for pH are used. Prior to the vessel preparation, the ISM gel-filled pH sensors (Mettler Toledo) need to be calibrated, by connecting them to the SciVario twin controller. The pH sensors are automatically detected by the software of the controller. The calibration can be performed by following the dialogue window on the controller, using buffer solutions of pH 7 and pH 4 as "zero" and "span", respectively. Afterwards, the sensors are disconnected and sterilized in autoclavable pouches. The autoclaved pH and DO sensors are inserted into the vessels in a biosafety cabinet via the spare Pg13.5 ports.

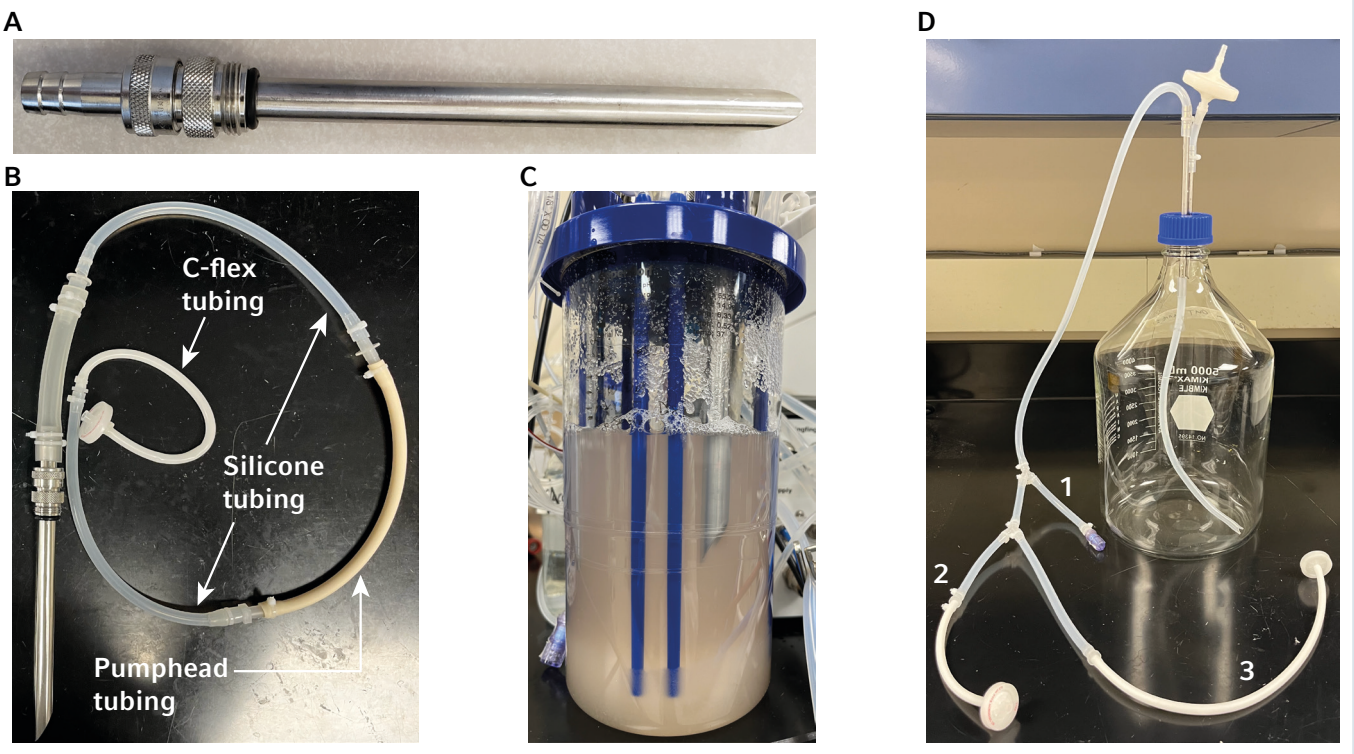

Fig. 4: Medium removal dip tube and perfusion out bottle assembly. **(A)** The medium removal dip tube is equipped with **(B)** tube extensions which include C-flex tubing for welding, silicone tubing, and pumphead tubing for the perfusion out pump on the controller. The tubing assembly is autoclaved before being inserted into the **(C)** bioreactor through an available Pg13.5 port. Prior to perfusion, the opposite end of the medium removal dip tube is welded to a **(D)** collection bottle. The tubing extension features a series of Y-junctions for (1) sampling the perfusate via a needleless port (2) C-flex tubing for welded connection to the bioreactor, and (3) C-flex tubing for an optional welded connection for additional medium removal.

Tube extensions, consisting of a pump head tubing (Feed Line Set for SciVario twin double pump drawer, C-Flex®, I. D. 0.5 mm, Eppendorf, Order No.: 7600 252 012) with the Luer connector at one end capped, are prepared, autoclaved and connected to the BioBLU 1c Single-Use Bioreactor via the Luer lock in a biosafety cabinet. Two overlay lines are used for base addition (0.45 M sodium bicarbonate) and anti-foam addition (0.1%, Antifoam-C Emulsion, Merck®, Cat. No. A8011-600ML), respectively. Two submerged lines are used for inoculum, fresh medium addition and/or glucose bolus feed (200 g/L). The third submerged line is the designated "Harvest" line.

The Medium removal dip tube (Figure 4) is prepared for the manual medium exchange step. In order not to disturb the microcarrier settlement during the process of medium removal, the dip tube opening should be positioned in the middle of the BioBLU Single-Use Bioreactor. The medium removal dip tube can be an open pipe (Length 8.25 inch/209.55 mm, Eppendorf, Order No.: M1287-9085 or Length 9.25 inch/234.95 mm, Order No.: M1287-9083) that fits in one of the standard Pg13.5 open ports with a compression fitting (Eppendorf, Order No.: M1287-5030).

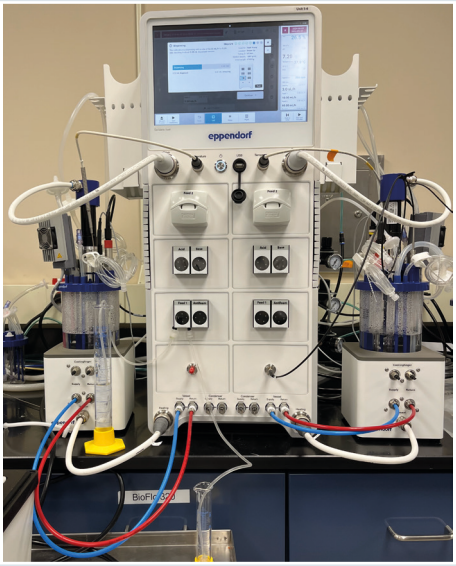

Fig. 5: SciVario twin bioprocess controller (middle) controlling two BioBLU Single-Use Bioreactors placed in the temperature control blocks

The bioreactor assembly can be installed in a biosafety cabinet, filled with PBS or culture medium at the minimum working volume (e.g., ~320 mL for BioBLU 1c), brought out of the biosafety cabinet and connected to the SciVario twin control station. BioBLU 1c Single-Use Bioreactors can sit in the temperature control block that enables electric heating and water-based cooling (Figure 5). Additional connections include the gas lines (submerse and overlay), sensors (temperature/RTD, pH and DO) and pump head tubing. For bottle connections (base, anti-foam, glucose, fresh medium addition, spent medium removal), the pump head tubing is mounted to the corresponding pump. Care is needed to prevent the Luer lock from accidently disconnecting, and to ensure that the flow direction is correct (clockwise "CW" as the default).

The liquid addition bottles of base, anti-foam and glucose are welded to the vessel assembly with a tube welder (Terumo). The fresh medium addition and spent medium bottles can be welded at a later point right before medium exchange. It is recommended to have multiple Y-junctions attached to the bottles (Figure 4D) to allow for aseptic sampling as well as liquid transfer to other bottles.

2.2. SciVario twin bioprocess controller setup

2.2.1. Process states and template

The SciVario twin bioprocess controller has an intuitive user-interface that guides the user step-by-step throughout the equipment use; once the user is familiar with the basics, no additional training is necessary. A key concept is the process state, as shown in Figure 6, of "Setup", "Conditioning" or "Running". These Process States follow the typical workflow of a bioreactor run, permitting certain control parameters to be changed while locking the process parameters during an actual run, thus ensuring process consistency and traceability.

In the "Setup" process, the first step is to choose a template, such as the default "Cell Culture fed-batch (concentration based)". The default template has been tested during its development, therefore, is a reliable process for standard cell cultures. Since the default template contains all the process parameters for a successful run, a user can easily build custom templates with modified parameters. User-built templates can be saved and re-used for the next time, ensuring process consistency.

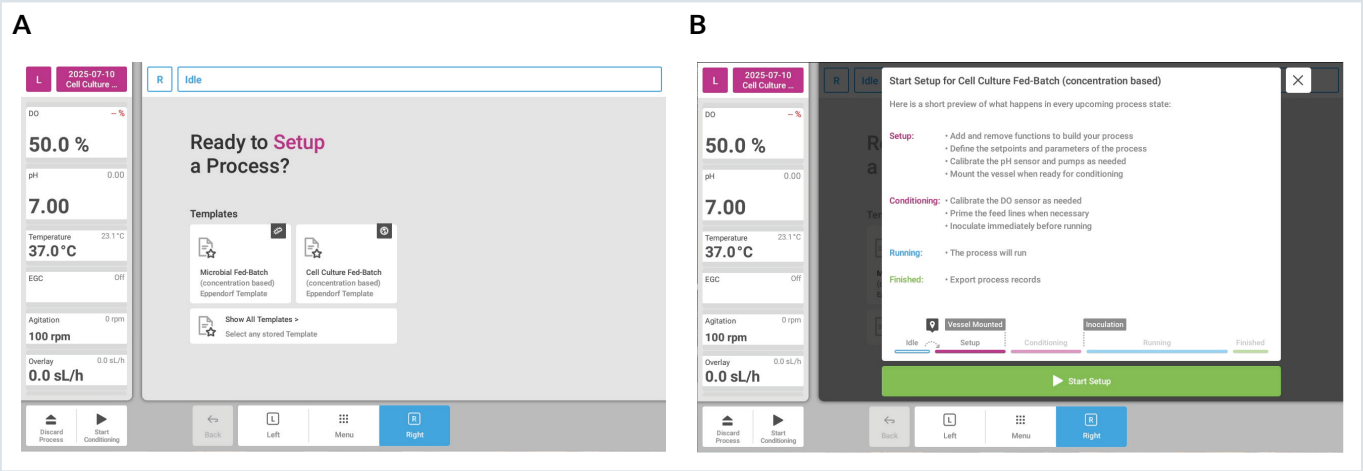

Fig. 6: SciVario twin bioprocess controller setup screen.

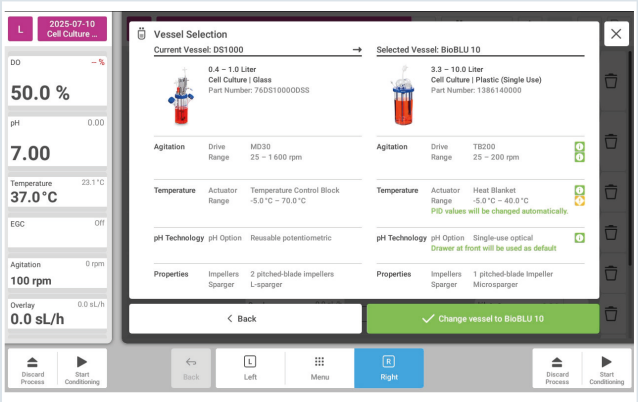

Fig. 7: SciVario twin bioreactor selection screen

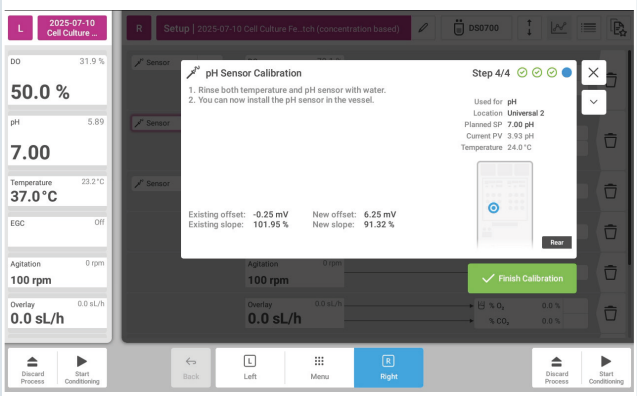

Fig. 8: SciVario twin bioprocess controller pH sensor calibration screen.

2.2.2. Vessel selection

The SciVario twin bioprocess controller user interface (UI) provides a well-arranged layout of all the vessels that are supported (Figure 7). In addition to the vessel type, the UI also provides key parameters of each vessel, including agitation, heating, and pH options. These features allow for easy comparison of vessel types and scale-up options.

2.2.3. pH and pump calibration

Before calibration, the correct sensor type needs to be selected from the dropdown menu. The selection is made easy by the straightforward visual guide of the sensor port layout. Similarly, the pumps may need to be assigned, in addition to the default assignment such as the base. Figure 8 shows the pH sensor calibration screen.

Calibrations of the pH sensor and the pumps need to be carried out during setup. The SciVario twin bioprocess controller provides dialog popups to guide the user through the process step-by-step, without needing specialized training. For the optical pH sensor, the values accompanying the single-use bioreactor can be inputted into the controller.

Fig. 9: SciVario twin bioprocess controller overview screen.

2.2.4. Agitation, temperature sensor, exhaust condenser

Additional functions other than sensors and pumps to be equipped include an agitation motor, temperature sensor, Peltier exhaust condenser, and other user-defined functions such as an anti-foam sensor. The SciVario twin bioprocess controller provides a process overview at the main page that lists all the key functions at one glance. This view shows each key function as a row, and each associated feature of the function as a column (e.g., sensor, setpoints, control parameters, cascades, and actuators) (Figure 9). Therefore, a user can go through each row systemically to check on each function, ensuring setup completeness and accuracy.

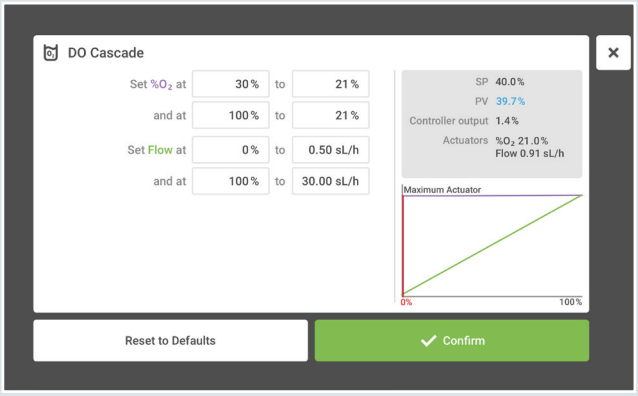

Fig. 10: Exemplary DO cascade for MSC culture controlled by the SciVario twin bioprocess controller as described in the Eppendorf Application Note 435.

2.2.5. Gas sparge and overlay control

The SciVario twin bioprocess controller provides an advanced and powerful mass flow controller module for bench-scale bioreactors, with a wide range of gas flow rates. Currently, the SciVario twin can support 4 gases (air, O_2, CO_2, N_2) in "Submerged" at 0.1 to 1200 sL/h for air, O_2, as well as 0.1 to 12 sL/h for CO_2, N_2, and in "Overlay" at 0.1 to 12 sL/h for all 4 gases. This means that with SciVario twin, a user can run a bioprocess from small scale (0.1 L) to 40 L on a single controller as opposed to two different ones in the past.

2.2.6. Template setup for MSC

MSCs are particularly sensitive to oxygen levels and shear stress [9, 14]. Therefore, the default template of "Cell Culture Fed-Batch (concentration-based)" needs to be modified for MSC bioreactor culture. The following changes from the default values are needed:

> DO.SP as "40". Under "DO Controller", "Proportional P" set as "0.1", "Integral I" set as "3.6/h"
> pH.SET as "7.6". Under "pH Controller", activate "deadband" of 0.1 and "automatically reset the integral memory if the process crosses the deadband or the set point"
> Once the Peltier condenser is attached to the vessel, activate "EGC" function
> All default "Feed" flow rates changed to 0

The modified template can then be saved by "Create Template Based on Current Settings" with a new "Template Name" and used in future.

3. Bioreactor conditioning with the SciVario twin bioprocess controller

3.1. Medium conditioning

The bioreactor is filled with MSC complete medium, maintaining a working volume, pH set point and agitation speed, for example, as shown in Figure 12. The medium in the bioreactor is conditioned for 24 hours prior to inoculation.

3.2. DO calibration and cascade.

DO sensor calibration can only be performed in the "Conditioning" state. For polarographic DO sensors, it is important to polarize the sensor for at least 6 hours by

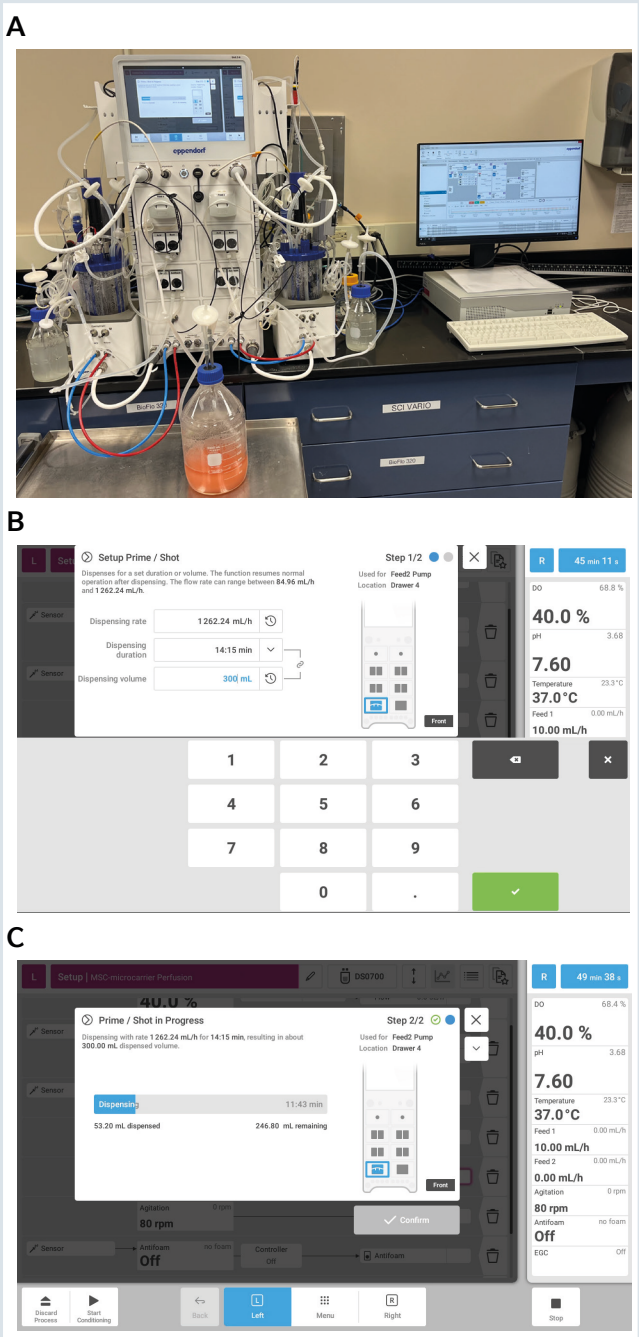

Fig. 11: Inoculation with SciVario twin bioprocess controller. **(A)** Inoculum bottle connection to both bioreactors and inoculum transfer in parallel. **(B/C)** The use of the "Prime/Shot" function to transfer the inoculum **(B)** to the bioreactor **(C)** with controlled timing.

keeping it connected to the controller prior to calibration. The DO Cascade default setting needs to be changed. For example, in our Application note 435, the DO cascade for a MSC culture with the SciVario twin bioprocess controller was set up in the following way [16]: Set O_2% to 21 when DO.OUT = 30%, and O_2% to 21% when DO.OUT = 100% (Figure 10).

3.3. Bioreactor Inoculation

After conditioning, once the bioreactor reaches the setpoints of the process parameters, it is ready for inoculation.

Figure 11 shows an example of an inoculation setup. The two bioreactors shared one inoculum. The inoculum bottle was welded to the two vessels via a Y-junction. It was found that efficient and smooth transfer into the bioreactor is key to post-inoculation cell viability. A maximum flow speed of the big pump can be used for simultaneous inoculum transfer into two bioreactors in less than 15 minutes. The use of the "Prime/Shot" function with the pump is an efficient method to transfer the inoculum to the bioreactor with controlled timing.

3.4. DASware® control software connection and BioNsight® cloud

It is at the SciVario twin control station's "Conditioning" stage that the DASware control software can be connected. Integration with DASware control allows for SCADA (Supervisory Control and Data Acquisition) control for remote process monitoring. A detailed procedure can be found in DASware control 6 Operation Manual. Cloud-based monitoring functions by the BioNsight cloud solution are also integrated in the DASware control software, further enhancing the possibility of process monitoring and analytics.

4. Bioreactor run

In this step, the SciVario twin bioprocess controller is in the process state of "Running". In-process adjustments can be made as needed. For example, in-process pH adjustment allows for off-line pH measurement validation.

4.1. Process parameters for MSC culture in bioreactors

As a sensitive cell line, MSC culture needs control of key parameters, such as pH, DO and gassing. The SciVario twin bioprocess controller provides the precision of bioprocess control in an easy-to-use manner. Figure 12 provides an examplary table of process parameters for MSC culture in BioBLU 1c, as reported in our Application Note 435 (Figure 12).

4.2. Manual medium exchange

Medium exchange is needed after five days of batch culture of MSCs in the bioreactor. As described in Application Note 435, the medium removal dip tube along with a compression fitting adapter is inserted in a spare Pg13.5 port allowing for medium exchange without disturbing the cell culture. Medium exchange starts with 10%-25% total volume every two days and then daily as required [16].

To do manual medium exchange, the agitation and gas flow are stopped for 5-10 minutes. Once the microcarriers settle at the bottom of the BioBLU 1c Single-Use Bioreactor, the old medium is removed using the medium removal dip tube. Subsequently, agitation and gas flow resume, and fresh medium is added using one feeding port of the vessel. The "Prime/Shot" function with the big pump on the SciVario twin bioprocess controller is an efficient method for liquid transfer from and to the bioreactor at a maximum speed of 6,000 mL/h (~100 mL/min).

Table 1: Process parameters and setpoints of the first and second experiments.

	First Experiment	Second Experiment
Parameters	**Setpoints**	
Starting volume	700 mL	
Ending volume	1 L	
Initial agitation	80 rpm (0.2 tip speed)	
Temperature	37 °C	
Inoculation density	3 x 10^4 cell/mL	10.4 x 10^4 cells/ mL
Cell culture medium	DMEM/F12 medium	ATCC complete medium
DO Setpoint	40% (P=0.1; I=3.6/h)	
pH Setpoint	7.2 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)	7.6 (deadband = 0.1), cascade to CO_2 (acid) cascade to 0.45 M sodium bicarbonate (base)
Overlay N_2 gas flow	0.20 SLPM	0.25 SLPM
Gassing range	0.1 SLPH-30 SLPH	
Gassing cascade	Set O_2 % at 30 % controller output to 21 % and at 100 % controller output to 21 %. Set flow at 0 % controller output to 0.1 SLPH, and at 100 % controller output to 30 SLPH.	

Fig. 12: Exemplary process parameters for MSC culture controlled by the SciVario twin bioprocess controller as described in the Eppendorf Application Note 435 [16].

4.3. Glucose bolus and metabolite monitoring

As regular bioprocess monitoring, daily samples are taken for metabolite measurement, i.e. glucose, lactate and ammonium. As cells expand in number, it is expected to have glucose concentration decrease, and lactate and ammonium concentrations build up. To prevent toxic levels of lactate and ammonium, medium exchange may need to be adjusted for ratio and frequency; for example, to limit ammonium to less than 4 mM [16].

Additionally, it is important to monitor glucose consumption to supplement glucose as needed. Glucose bolus addition can be easily achieved with the "Prime/Shot" function associated with the assigned glucose addition pump on the SciVario twin bioprocess controller.

Results

From our experiences, MSC cultures with non-porous microcarriers often peak at around 5 × 10^5 cells/mL. MSC marker expression is used for cell phenotype validation. Finally, functional validation of MSCs involves trilineage differentiation (adipogenic, chondrogenic, osteogenic) of the cell product. The following figures provide some examples of MSC results from bioreactor cultures using Eppordorf equipment.

MSC Growth Curve

An example of a MSC culture growth curve under control of the SciVario twin is shown below (Figure 13) [16].

3D MSC-microcarrier morphology

An example of MSC morphology during microcarrier culture controlled by the SciVario twin bioprocess controller is shown below (Figure 14) [16].

MSC marker expression

MSCs during or after bioreactor cultures can be characterized for their marker expression for quality control. Below is an example showing the cells were positive for CD90 and CD29 and negative for CD11b and CD34, maintaining their cell phenotype (Figure 15) [16].

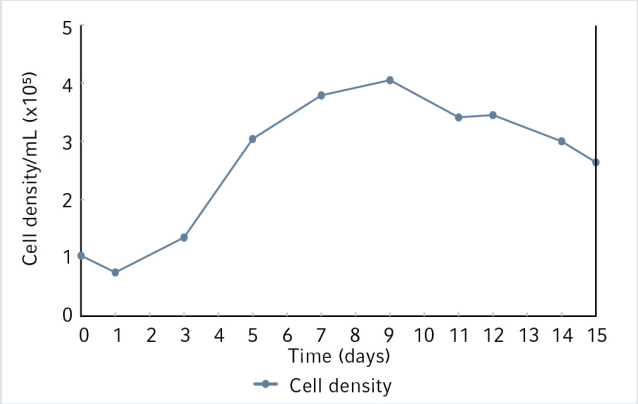

Fig. 13: Example of a MSC growth curve over a culture period of 15 days controlled by the SciVario twin bioprocess controller as described in the Eppendorf Application Note 435.

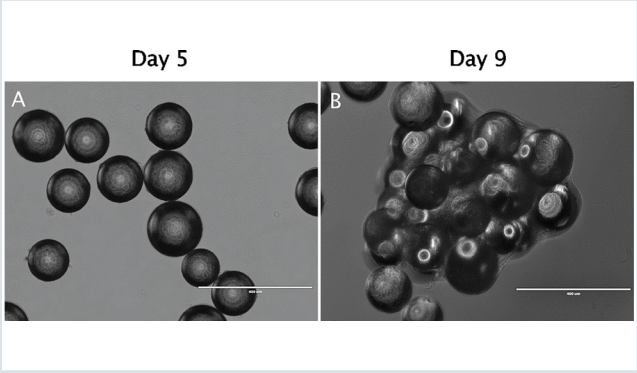

Fig. 14: Bright-field images (10x magnification) of human iPSC-derived MSC (small light circles) on collagen-coated microcarriers after (large dark spheres) **(A)** 5 days and **(B)** 9 days of culture as described in Application Note 435.

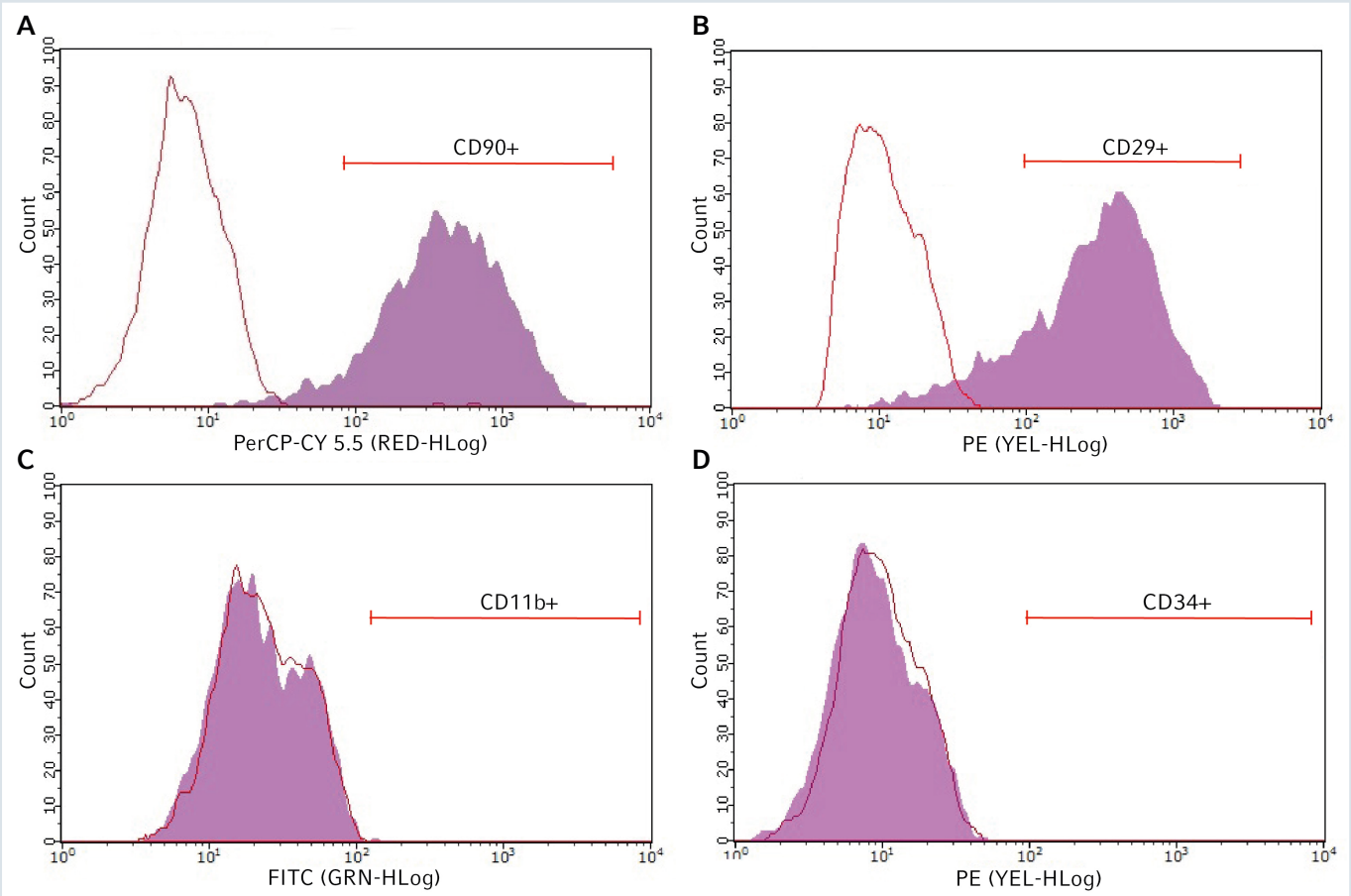

Fig. 15: Immunophenotyping of human iPSC-derived MSC as described in the Eppendorf Application Note 435. Filled areas depict the cells that were positive for the MSC markers CD90, CD29, and negative for the hematopoietic markers CD34 and CD11b.

MSC differentiation assays

After the bioreactor culture, MSCs can be characterized for their differentiation potential for further quality control. For example, MSCs can be dissociated from the microcarriers and seeded into 24 well plates that contain either adipocyte or osteocyte differentiation medium. The plates can be stained with Oil Red O or Alizarin Red S staining solutions, respectively. Below is an example of MSCs differentiated into either adipocytes or osteocytes successfully [11].

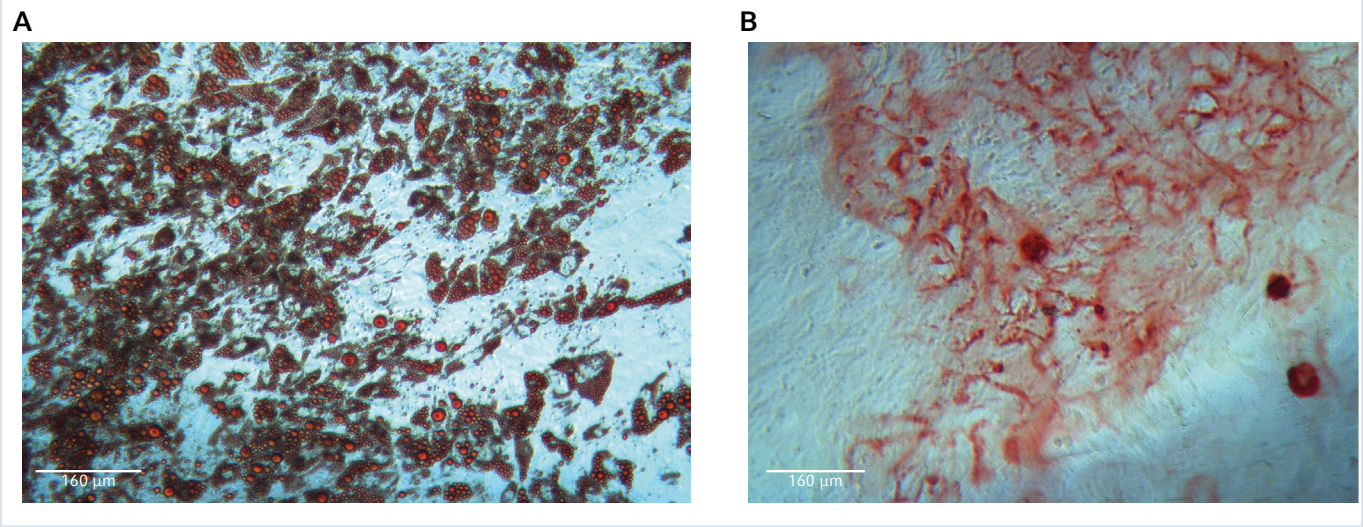

Fig. 16: Differentiation assays for adipose-derived MSCs expanded on microcarriers as describes in Eppendorf Application Note 259. After dissociation from the microcarriers, the cells were seeded into 24 well plates that contain either adipocyte or osteocyte differentiation medium. **(A)** Adipogenic differentiation formed lipid droplets as indicated by Oil red O positive staining. **(B)** Osteogenic differentiation caused calcium mineralization of extracellular matrix as indicated by Alizarin Red S positive staining.

Conclusion

This beginner's guide walks through the basic steps of MSC culture, starting from an initial cryopreserved cell stock, progressing to expansion in conventional flask cultures, and scaling up production in stirred-tank bioreactors. It details the critical transition from 2D to 3D microcarrier cultures, supported by the Eppendorf S41i shaker incubator, offering successful examples and strategies like bead-to-bead transfer to facilitate laborious working steps. 3D MSC production is made accessible with the Eppendorf SciVario twin control station, which simplifies medium exchange and controls critical process parameters such as dissolved oxygen and gassing. The examples shown here demonstrate high yields and consistent stem cell marker expression in cultures carried out in a BioBLU Single-Use Bioreactor, showcasing the power of the SciVario twin and BioBLU Single-Use Bioreactors for R&D and technology transfer approaches. A beginner in MSC bioprocessing can confidently use this guide to tailor their approach based on specific cell sources and microcarrier types to their bioprocessing needs.

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Ordering information

Description	Order no.
SciVario® twin bioprocess controller	7600 100 001
BioBLU® 1c Single-Use Bioreactor, cell culture, open pipe, 2 pitched-blade impellers, no pH, X-ray, 4 pieces	1386 111 100
New Brunswick S41i, 170 L, CO_2 incubator shaker with inner shelf and touch screen control, stackable	Inquire*
*Inquire the part number for your country	

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REVIEW ARTICLE

Microcarrier-based stem cell bioprocessing: GMP-grade culture challenges and future trends for regenerative medicine

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ABSTRACT

Recently, stem cell-based therapies have been proposed as an alternative for the treatment of many diseases. Stem cells (SCs) are well known for their capacity to preserve themselves, proliferate, and differentiate into multiple lineages. These characteristics allow stem cells to be a viable option for the treatment of diverse diseases. Traditional methodologies based on 2-dimensional culture techniques (T-flasks and Petri dishes) are simple and well standardized; however, they present disadvantages that limit the production of the cell yield required for regenerative medicine applications. Lately, microcarrier (MC)-based culture techniques have emerged as an attractive platform for expanding stem cells in suspension systems. Although the use of stem cell expansion on MCs has recently shown significant increase, their implementation for medical purposes is been hampered by bottlenecks in upstream and downstream processing. Therefore, there is an urgent need in the development of bioprocesses that simplify stem cell cultures under xeno-free conditions and detachment from MCs without diminishing their pluripotency and viability. A critical analysis of the factors that impact the up and downstream bioprocessing on MC-based stem cell cultures is presented in this review. This analysis aims to raise the awareness of the current drawbacks that limit MC-based stem cell bioprocessing in regenerative medicine and propose alternatives to overcome them.

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KEYWORDS
Microcarrier; stem cell bioprocessing; upstream; downstream; harvesting; cell detachment; regenerative medicine

Introduction

Regenerative medicine is a novel area positioned as a promising route for treating patients with diverse incurable diseases [1]. This therapy proposes the incorporation of biomaterials, growth factors, and stem cells (SCs) [2] to repair diseased and damaged cells, as well as to replace dead cells [3]. Most importantly, SCs are well known for their particular characteristics of proliferation, self-renewal, and differentiation potential [4]. These properties have suited SCs into an encouraging alternative to treat multiple chronic diseases such as amyotrophic lateral sclerosis [5–9], Parkinson [10–12], Alzheimer [13–15], myocardial infarction [16], liver failure [17], type I diabetes [18], spinal cord injuries [19], Stargardt's macular dystrophy [20], and advanced dry age-related macular degeneration [20]. Regenerative medicine based on cell therapy has achieved significant progress so far, and accelerated growth is anticipated for the next decades. It is expected to treat millions of patients, representing tens of billions of dollars in the US market [21]. For this reason, the use of SC for regenerative medicine and tissue engineering applications holds excellent potential [22], not only attractive for its therapeutic potential but also economic.

Although the therapeutic and financial possibilities of regenerative medicine are encouraging, there are various drawbacks concerning the number (Table 1), quality, and safety of SCs that still needs to be addressed to reach their maximum potential. In this context, Good Manufacturing Practice (GMP) guidelines establish the conditions to ensure the quality and biosafety of cells for therapeutic use [23]. However, the obtention of a high number of cells is not considered. Moreover, traditional two-dimensional (2 D) culture methods, including T-flasks and Petri dishes, exhibit several limitations in the scalability and the control of culture parameters, which are difficult for the obtention of high cell yields. Therefore, three-dimensional (3 D)-based culture methods emerge as the most viable option for SC expansion. Specifically, microcarrier (MC)-based 3 D culture systems have attracted attention for the expansion of SCs [24–26], as they mimic the *in vivo* cell environment [27], allowing cell-cell communication and their interaction with the extracellular matrix (ECM)

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Table 1. Number of cells/dose required for the treatment of different diseases.

Disease	Cell type	Cell number	Reference
Myocardial infarction	Cardiomyocytes	1×10^9	[16]
Liver failure	Hepatocytes	1×10^{10}	[17]
Type I diabetes	Insulin-producing beta cells	1×10^9	[18]
Spinal cord injury	MSCs	4×10^8	[19]
Parkinson	Dopaminergic neurons	1×10^5	[144]
Stargardt's macular dystrophy	Retinal pigmented epithelial cells	$0.5–2 \times 10^5$	[145]
Advanced dry age-related macular degeneration	Retinal pigmented epithelial cells	$0.5–2 \times 10^5$	[145]

compounds, nutrients, and gases [28]. MCs are microparticles that increase the surface area in a cell culture system for the attachment and growth of anchorage-dependent cells, reducing space, bioprocess steps, contamination risk, and cost of the whole process. They are relatively inexpensive (price/m^2), which makes their implementation affordable. Additionally, owing to their density (1.01–1.1 g/mL), these particles are ideal to be combined with stirred systems, resulting in a homogeneous cell culture environment that can be easily scaled-up. Moreover, this allows the continuous monitoring and control of the culture parameters (pH, temperature, gas concentration, nutrients, and waste) [29], as well as the obtention of high cell yields with high viability and pluripotency.

The before-mentioned advantages highlight MC-based SC culture as a promising candidate to lead the bioprocessing strategy of SC for regenerative medicine purposes. In this sense, the present review critically analyses each of the factors that directly impact the up and downstream bioprocessing on MC-based SC culture, with an ultimate aim to raise awareness of the current drawbacks and proposes new approaches to overcome them.

Microcarrier-based stem cell bioprocessing

SC bioprocessing, like any other bioprocess, can be divided into upstream and downstream processing. In general terms, upstream processing refers to any step of the bioprocess production, including the assessment of materials and production stages. Regarding SC, the upstream bioprocess includes selecting the cell type source, reagents required for the isolation (if applicable), culture techniques, and the culture conditions. Specifically, for MC-based SC, this last culture step is the core of the upstream, where the selection of MC, culture systems, media, and supplements are vital factors. Therefore, this step represents the main barrier in the upstream MC-based SC process, since the translation of the laboratory-developed culture techniques into clinical procedures continues to be a challenge, as presented in the next section.

On the other hand, downstream processing involves the successive steps of the process, such as harvesting, preservation, and transportation of SCs [30]. The control in these steps is critical for the quality and safety of expanded cells since small deviations during the process can result in variations regarding cell yield, viability, and pluripotency [31]. In the particular case of MC-based SC bioprocess, the core step is harvesting, as it depends on the MC selection, as discussed in the corresponding section. The main steps in the upstream and downstream bioprocessing of SC are summarized in Figure 1.

As previously described, the use of SCs is a promising alternative for the treatment of multiple diseases and health conditions [32]. However, its implementation is complex, and multiple problems arise during the bioprocess. In the following sections of this review, a critical analysis of the technical and regulatory elements involved in the bioprocessing of MC-based GMP-grade SCs, as well as its challenges and trends will be discussed. Figure 2 synthesizes the main elements that must be considered in the planning and development of an MC-based SC bioprocessing, which will be addressed in the following sections.

Upstream processing challenges and trends in microcarrier-based stem cell culture systems

Over the years, a great variety of MCs have been developed. Nowadays, MC production is carried out at the industrial level, following a well-standardized process and quality control parameters. The implementation of different natural and synthetic materials to form and coat MCs has evolved into a wide range of options [33–43]. Moreover, characteristics such as size, porosity, shape and surface modifications are crucial factors that impact cell attachment and growth. Furthermore, the selected culture system and its characteristics such as type, material, size, and shape will also have an impact on the number and quality of the cultured SCs. Finally, the selection of the culture reagents, such as media and supplements. Each of these upstream features will be further examined in this section.

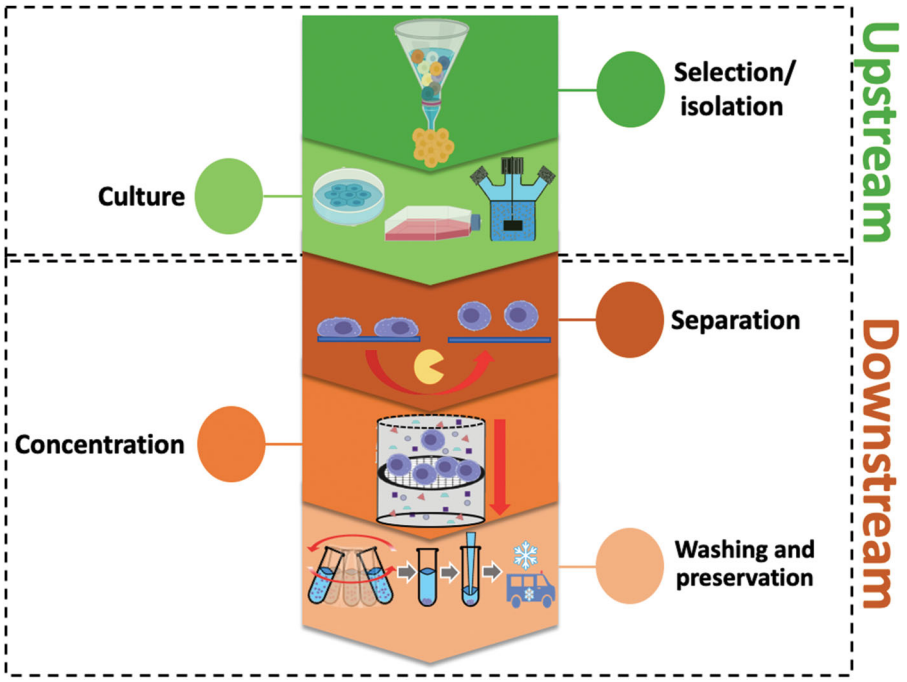

Figure 1. Representation of the main stages of stem cell (SC) bioprocessing. The upstream step includes the selection of cell type and reagents for isolation and culture. The downstream step involves the harvesting (separation and concentration of cells), preservation, and transportation.

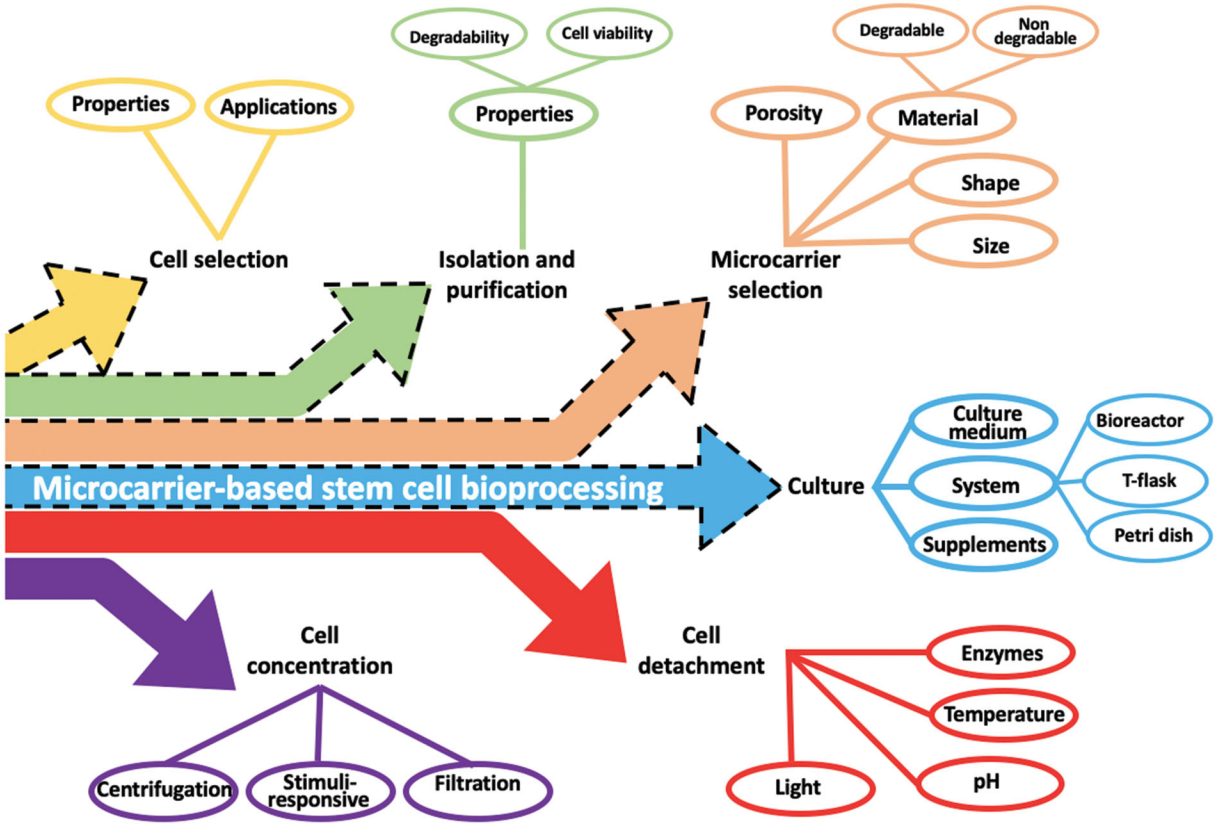

Figure 2. Graphical diagram of the main elements that must be considered in the planning and developing of a microcarrier (MC)-based stem cell (SC) upstream (contoured arrows) and downstream (not contoured arrows) bioprocess.

Microcarrier selection

MC selection is an essential parameter since their physical and chemical characteristics affect cell culture behavior [44]. MC without surface treatment and/or coating exhibit limited adhesion capacity [45]. This can be improved by modifying its surface by adding functional groups, ECM or polymeric coating to enhance cell attachment and cell survival [46]. For instance, Hillex (Solohill, Ann Arbor, MI, USA) are based MCs covered with a cationic amine to improve cell attraction [47]. Synthemax II (Corning, Corning, NY, USA) MCs are covered with Synthemax II (a synthetic layer that increases cell attachment and cell yield) [48]. Cytodex 1 (General Electric, Chicago, IL, USA) MCs are dextran beads with a positive charge conferred by the inclusion of diethylaminoethyl (DEAE) groups, which improve cell adhesion [49]. Similarly, MCs have been produced and covered with ECM molecules [50] and growth factors [46], showing encouraging results. Unfortunately, these MCs have only been tested at laboratory scale and are not yet commercially available.

Another factor to consider is that most of the current coating materials of the MCs are xenogeneic, such as Matrigel (Corning, Corning, NY, USA), collagen, and laminin. Thus, this practice limits its implementation in clinical-grade SCs culture by the presence of potentially dangerous compounds [51]. Fortunately, nowadays, there are recombinant ECM analogs such as vitronectin [52], fibronectin III [53], fibrillin-1 [53], laminin LN511 [54] that have been used effectively and can solve this problem. The inclusion of these compounds represents a not fully explored opportunity area to overcome cell adhesion problems by including ECM proteins on the MC surface to improve cell attachment conceding long-term expansion of undifferentiated cells [55]. Furthermore, the inclusion of growth factors on the MC surface represents a novel alternative to develop therapeutic bioactive MCs that can support both SC proliferation and growth factors delivery.

Size and porosity are other crucial characteristics of MCs, as they define their surface area and sedimentation rate, which can significantly affect the expansion and yield of cells. Even when cell attachment is not influenced by MC size [55], this parameter can affect the cell aggregation morphology. It has been observed that large MCs ($\sim$190 μm) result in open aggregate structures with fine cell layers, which are ideal for fluidized and packed systems. On the other hand, smaller MCs ($\sim$60 μm) generate more compact aggregates, and tiny MCs ($\sim$10 μm) cause very dense cell aggregates, so their use is recommended for stirred cultures. These reported results open a possibility that can be further explored to maximize the number of different SCs when using MCs by testing different sizes in the manufacturing of MCs.

Regarding porosity, MCs can be produced either as non-porous or porous particles. Microporous MC (where pores represent less than 60% of the total area) increase surface area, cell adhesion, and growth [56]. These MCs are especially convenient to improve cell retention in perfusion systems. Similarly, the cells cultured on microporous MC can easily take from the medium the necessary factors for survival and proliferation. In contrast, macroporous MC (where pores are present between 60 and 90% of the total area) significantly reduces the cell attachment surface. Moreover, due to the cell size being around 10 μm, these MCs allow the cells to enter and grow inside their structure, which improves the diffusion of factors and gases [57]. Therefore, the main advantages of macroporous MC are protection from shear stress when stirred or perfusion systems are used [58] and improvement of the microenvironment, enhancing the survival and growth of cells when serum-free culture media are employed [59].

Likewise, the shape of the MC is a parameter strongly involved in sedimentation velocity. Low sedimentation velocities ($<$30 cm/min) hinder circulation and mixing, which results in a deficient nutrient and gas supply, diminishing cell viability and yield. Chen et al. [55] evaluated the effect of the MC shape on cell culture, determining that it affects cell-MC aggregate morphology when culturing embryonic SCs (ESCs). This work has indicated that cylindrical MCs generate compact cell-MC aggregates, whereas spherical shape MCs induce more open aggregate configurations with thinner cell layers. Most importantly, cell expansion and pluripotency markers were not decreased by the shape of the MC. Figure 3 graphically synthesizes the different characteristics according to size, porosity, shape, and surface modifications that should be considered for the MC selection.

Even though multiple studies have overcome the problem of low adherence and cell growth by using different materials, altering the size, shape, and porosity, or by covering MCs with different materials, most of them employ xenogeneic materials. Thus, preventing their implementation for clinical purposes. In this regard, one of the main challenges in this area is to replace the conventional xenogeneic elements with the current and emerging contaminant-free ones. The xeno-free commercially produced MCs are listed in Table 2, where their main characteristics are summarized. As previously mentioned, the selection of MC is a crucial point for an effective MC-based SC process, but

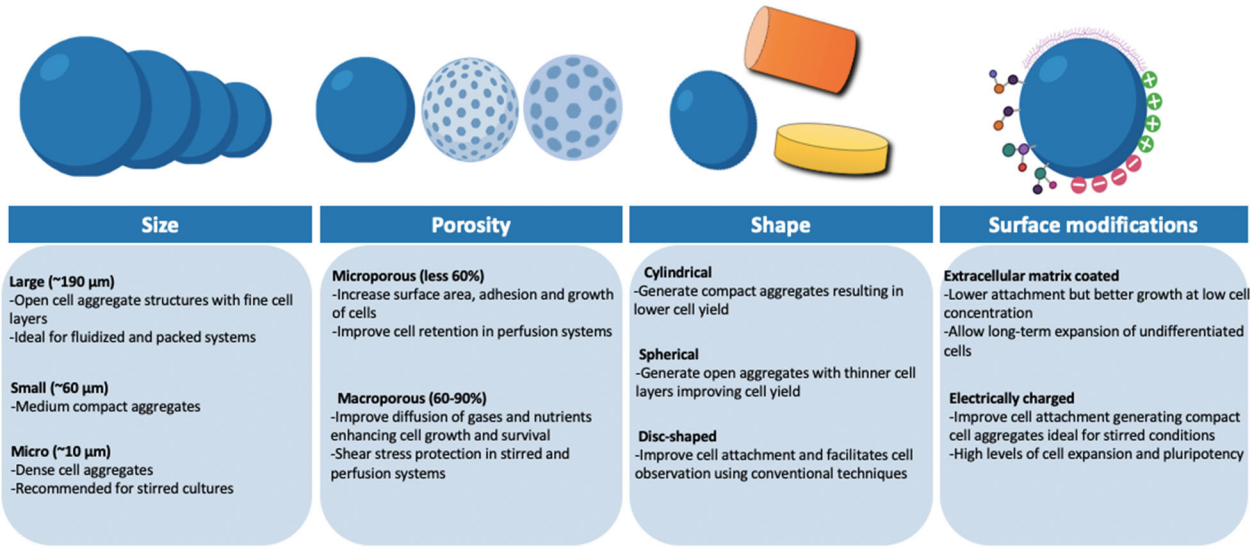

Figure 3. Graphical representation and main properties of the different physical and chemical characteristics of microcarriers (MCs) that should be considered while designing the upstream bioprocess strategy of a MC-based stem cell (SC) culture: size (large, small and micro), porosity (non-porous, microporous, and macroporous), shape (spherical, cylindrical, and disk shaped), and surface modifications (extracellular matrix (ECM) coating, chemical modification, positive or negative electrical surface charge).

other elements such as the selection of the culture system must be considered for optimal SC processing. An interesting approach was accomplished by Cytiva/GE Healthcare, comparing 23 important characteristics of commercial and in-house MCs [60]. However, these parameters depend on other factors such as the cultured cell type and cannot be generalized.

Culture system selection

Due to the reduced amount of SCs usually obtained from natural sources, one of the main challenges for a GMP-grade SC culture is the selection of the optimal expansion system. This system must expand SCs in their undifferentiated state using xeno-free culture media, growth factors, and supplements. 3 D bioreactors represent a viable option to overcome static culture limitations since they provide a scalable system in which operational factors can be monitored and controlled [61].

In this context, the first choice regarding the culture system implies the type of bioreactor, for which the final application of the expanded cells must be considered. For instance, stirred bioreactors are relatively simple culture systems that consist of a vessel with an impeller that stirs the content inside to provide a homogeneous solution [62]. These culture systems have been successfully tested for the expansion of different types of SCs under xeno-free conditions, including mesenchymal SCs (MSCs) [63], induced pluripotent SCs (iPSCs) [64,65], and ESCs [25]. Nevertheless, the shear stress generated by these systems can potentially damage shear-sensitive cells [66]. Fortunately, this problem can be minimized by controlling the shape and speed of impeller agitation [67].

Another important parameter that must be monitored and controlled in this bioreactor system is oxygen saturation. SCs cultured under diverse O_2 concentrations could show significant differences regarding proliferation, viability, and differentiation capacity of the cells [68]. Whereas low oxygen saturation (~5%) for the culture of iPSCs and ESCs is suggested for some authors [69,70], significant metabolic changes from aerobic to anaerobic routes of cells have been observed under hypoxia conditions [71]. These circumstances lead to a high lactate concentration and pH shift of the medium from neutral to acid [72]. In this regard, higher oxygen saturations were tested by the Alves' group as an attempt to solve low oxygen inconveniences. They achieved higher metabolism and cell growth rates when an oxygen saturation of 30% was implemented in the culture of hESCs. This process improvement was accomplished by using a perfusion system, which resulted in a cell yield 4 times higher than that observed with 5% oxygen saturation [73].

Other bioreactors such as single-use bioreactors and rotary bioreactors have been also suggested as suitable alternatives for the expansion of SCs. Single-use bioreactors consist of a sterile disposable bag incorporated into a closed system in which GMP-requirements can be easily accomplished. These systems are especially suitable for the culture of hematopoietic SCs (HSCs)

Table 2. Xeno-free commercially available microcarriers (MCs).

Brand	Tradename	Material	Description	Size (μm)	Area (cm^2/g)	Porosity	Density (g/cm^3)	Reference
Corning	Untreated	Polystyrene	Surface untreated	125–212	360	Microporous	1.026	[48]
	Enhanced attachment		Surface treated to increase cell attachment, cell yield and *via*bility					[48]
	CellBIND®							[146]
	Low concentration Synthemax™ II		Synthemax II surface treated to increase cell attachment, cell yield, and *via*bility					[48]
	High concentration Synthemax™ II							[48]
	Positive Charged		Surface positive charged to enhance cell attachment and expansion	160–200	515		1.09	[48]
Solohill	Hillex		Surface modification with a cationic amine to improve cell attraction	160–200	515		1.10	[147]
	Plastic		Cross-linked polystyrene	90–212	360–480		1.02	[147]
	Plastic Plus		Cationic charge beads to enhance cell attraction and attachment	125–212	360			[147]
	Star-plus		Cross-linked polystyrene					[147]
Cytodex 1	GE	Dextran	Dextran beads with positive-charged DEAE groups	180	4400		1.06	[49]
Cytodex 3	GE		Dextran beads coated with denatured porcine-skin collagen	175	2700		1.04	[148]
Cytopore 1	GE	Cotton-cellulose	Cross-linked cotton cellulose with positive-charged DEAE groups	200–280	11,000		1.03	[149]
Cytopore 2	GE							[149]
Macroporous microspheres	Fujifilm	Human collagen I	An injectable microsphere	100–400	10,000	Macroporous	–	[150]

due to the low shear stress and low oxygen saturation (2–9%) needed for the culture of these cells. However, it should be considered that the culture of SCs in low oxygen environment will imply the reduction of aeration, thus CO_2 will accumulate, changing the pH of the medium [74]. This problem could be solved by implementing pH sensors and replacing the culture medium when required or by adapting automated systems such as the one offered by Cytiva brand which consists of a single-use mixing platform with intelligent and automated pH adjustment system [75]. Although these systems are scalable and solve the mentioned problem, their high cost limits their full implementation [76].

On the other hand, rotary bioreactors mix the culture media at the same angular rate as the inner cartridge showing remarkable outcomes: reducing shear stress, improving mass transfer, and controlling oxygenation [77]. Nevertheless, it must be considered that these bioreactors are hard to scale-up, and the size of the vessels is limited, which significantly reduces the number of cells produced.

Jointly as the bioreactor type selection, its design is fundamental for the expansion of clinical-grade SCs and it must be fully compatible with GMP regulations. Thus, the bioreactor vessel characteristics such as material, size, and shape are key parameters that impact cell production and, eventually, the bioprocess costs. The materials and design of the bioreactor should facilitate the cleansing and production steps, thus preventing any possibility of contamination. In this regard, some bioreactor systems have been developed to use disposable vessels as a novel strategy to avoid the decontamination process and reduce contamination risks [78]. This strategy solves the before-mentioned inconvenience in bioreactor systems; however, the increased cost for not-reusable vessels must be considered.

It is essential to mention that all these systems described above can be combined with MCs, allowing them to merge the characteristics of the system with them. In this sense, the future trend could focus on exploring these bioreactor systems with different MCs characteristics for a specific SC type to maximize cell yield maintaining its viability and pluripotency.

Culture media and supplements

Culture media, growth factors, cytokines, and other supplements needed for SC maintenance and expansion are critical elements in the upstream bioprocess. The inclusion of xenogeneic or undefined compounds in the culture media formulations and the

incomprehension of multiple molecular and cellular mechanisms implicated in cell functions are the main problems to address [23,29]. Xenogeneic components in the culture media and supplements are potentially dangerous since they can include pathogenic agents that can be transmitted to the cultured cells, preventing its use for medical applications. For instance, fetal bovine serum (FBS), a xenogeneic complex mixture of low and high molecular weight biomolecules [79,80], is traditionally included in culture media formulations to promote cell growth. However, it is known that their production has significant batch-to-batch variations in its content, altering the lot-to-lot concentration of biomolecules and the reproducibility of the process [27]. Although its addition in culture media formulation is commonly accepted, it must be avoided entirely in GMP-grade SC culture since it can contain undesired factors, like: endotoxins, mycoplasma, viral contaminants, and prion proteins [81].

SC culture formulations are frequently supplemented with cytokines and growth factors that promote cell proliferation in an undifferentiated state. These supplements are commonly derived from xenogeneic origin, therefore their use in SCs culture for clinical purposes is also hampered. Fortunately, the development of xeno-free materials and the application for SC culture have been reported [82–84]. For instance, in a comparative study carried out in 2007 by Rajala et al. [26], nine xeno-free culture media were tested to expand hESC. Although none of the examined culture media was able to maintain the undifferentiated growth of the cells, this work gave rise to the following successful attempts. Lindroos et al. [85] showed the effectiveness of a commercially available formulation (StemPro MSC SFM basal culture medium, Life Technologies, Paisley, UK) for the expansion of human adipose SCs, reaching higher cell proliferation rates than conventional serum-containing media, maintaining a differentiation potential and surface marker expression. Swistowski et al. [86] reported using a serum-free StemPro hESC SFM defined medium for the successful expansion of hESC, followed by their differentiation to neural SCs, and their further induction and maturation into dopaminergic neurons without losing their functional ability. Since then, multiple publications of SCs cultured under xeno-free conditions have been reported [86–89]. Reports of the use of xeno-free culture media have demonstrated the expansion capacity of cells and their ability to preserve their differentiation potential throughout the culture process [90].

In this topic, one of the future perspectives, besides recombinant factors and cytokines, is the inclusion of plant-derived compounds that have demonstrated proliferation [91] and differentiation [92–94] potential. Although these compounds are not fully characterized, their inclusion in the culture media could significantly reduce the bioprocessing costs, making it worth the value of its further investigation.

Downstream processing challenges and trends in microcarrier-based stem cell culture systems

Following the SC bioprocess, after the expansion of the cells, they must be separated from the MCs and concentrated both in the undifferentiated and differentiated state. Although these operations are not a challenging task at a laboratory scale, they become very complicated in the industrial-scale process. Thus, current downstream process efforts have been focused on the search for novel options to supply methodologies that can be applied at the industrial level.

Anchorage-depended cells such as SCs are the most challenging type of cells to separate from the surface when cultivated. Contrary to the conventional suspension-based systems, cell harvesting from the MC surface is a challenging but essential procedure to maintain their properties. The harvesting process implicates two steps, cell detachment from the MCs surface and the final recovery of the detached cells or cell concentration [95]. It is important to mention that several authors have reported the possibility of cell differentiation on MCs [64,96–101], however, these processes will not be covered in this study. The following sections of this article will address in a general way the two crucial phases in the downstream processing of MC-based SC culture; detachment and concentration of cells.

Cell detachment

The detachment of the cells from MCs is an important step that directly influences the viability of the cells. Different detachment strategies have been used over time, which are fully compatible with MC-based bioprocessing. Among them, methodologies based on proteolytic enzymes are the most used. Traditionally, pancreas-derived bovine and porcine trypsin are widely used enzymes for cell detachment. Despite the effectiveness of these enzymes, their animal-based origin precludes their use for medical settings, and xeno-free alternatives must be explored. Recombinant trypsin-like enzymes such as TrypLE (Invitrogen, Carlsbad, CA, USA) [102] and TrypZean (Sigma-Aldrich Corp., St Louis, MO, USA) [103] are now commercially available and seem to be the solution to trypsin inconveniences. Although these laboratory-produced enzymes are expressed in

different organisms, they have demonstrated similar SC detachment activity than the trypsin enzyme in different comparative studies. For instance, Carvalho et al. [104] showed no significant difference between animal-derived and recombinant enzymes for adipose-derived SCs.

Similarly, Tsuji et al. [105] investigated the effect of incubation times of four enzymes in MSC expression markers. They found that multiple cell surface expression markers were decreased by trypsin at 30 min, while TrypLE did not affect any of them. Nevertheless, Ojima et al. [106] demonstrated that MSC overexposure (60 min) to TrypLE enzyme significantly reduces the pluripotency expression markers. The enzyme incubation time is an important parameter to achieve cell detachment without affecting cell integrity and viability successfully. While long enzymatic incubation times have been related to cell damage and cell death [105], short periods hinder the separation of the cells due to an incomplete breakdown of protein bonds.

Consequently, the exposure time of the cells to the enzyme must be carefully standardized to prevent a decrease in the cell viability and its properties. In an attempt to overcome this time inconvenience, Nienow et al. [95] applied the trypsin enzyme combined with agitation diminishing the enzyme-incubation time and the overexposure of cells to enzymatic activity.

Despite the wide use of xeno-free enzymes for cell harvesting, their effects on cell viability, potency, spontaneous differentiation, and differentiation potential still limit their full implementation [105]. Therefore, other alternatives have been explored to face this inconvenience. Stimulus-responsive materials have emerged as a novel alternative to overcome the drawbacks of enzymatic detachment. These materials can react with external stimulation, such as changes in temperature, pH, or electrolytic concentration [107], generating modification in their dimension/size, structure, solubility, or intermolecular interactions [108]. For instance, thermo-sensitive MCs have shown promising results to overcome enzymatic detachment disadvantages. These MCs are obtained when a thermo-sensitive material is used to fabricate or cover their surfaces, allowing cell detachment by temperature [109].

One of the first attempts to include thermo-sensitive materials such as PNiPAAm on MC systems was accomplished by Tamura et al. [110] when they used this polymer to cover chloromethylated polystyrene MCs for the culture of Chinese hamster ovary cells. The detachment of the cells was achieved by decreasing the temperature from 37 °C to 20 °C. Similarly, SC-routed thermo-responsive MCs were explored by Yang et al. [111] when Cytodex 3 MCs were covered with PNiPAAm polymer and used for the culture of MSCs, allowing cell adherence, spread, and growth successfully. The cell detachment was carried out by decreasing the incubation temperature below 32 °C, in which more than 82% of MSCs were detached from MCs. Later, Kim et al. [112] published the use of PNiPAAm MCs for the culture of human MSCs (hMSCs). The detached hMSCs can form 3 D aggregates with higher clonogenic properties compared to enzymatically detached hMSCs. Years later, the same research group showed the feasibility of scale-up formation of hMSCs aggregates in a bioreactor utilizing thermo-responsive PNiPPAm-covered MCs [113]. More recently, Nguyen et al. [114] reported the culture of fibroblast cells and MSCs on MCs fabricated using PNiPAAm polymer conjugated with polycaprolactone in an attempt to improve PNiPPAm characteristics, allowing the adherence and growth of both fibroblast and MSCs. Cell detachment of 70% of the cells was achieved by decreasing the temperature to 30 °C with 85% viability. Despite the multiple reports of the efficacy of the PNiPPAm thermo-responsive polymer as an enzyme-free detachment strategy, commercially temperature-responsive MCs are not yet commercially available.

Another attractive proposal for MC development was accomplished by the Fujifilm brand (Tilburg, North Brabant, Netherlands). This company has launched a revolutionary injectable MC (now commercially available) that can be utilized for either cell growth or cell delivery to patients without the necessity of harvesting [115]. This MC is biocompatible, fully biodegradable, and suitable for dynamic expansion systems. Similarly, the Steve Oh research group has made important advances on the development of biodegradable polycaprolactone-based MCs which can be applied in the body of both undifferentiated and differentiated cells [116–120]. These important advances have allowed them to found the Singcell company which seeks to transfer the knowledge and techniques of the laboratory to scalable processes in the culture of MSC and iPSC for preclinical studies, clinical trials, and commercial launches [121].

These examples demonstrate the potential of this technology and leave the door open to translate successful technologies in other areas to MC-based bioprocessing. For instance, polymers that can react to light or electric fields have been implemented in drug delivery research [122–124] and could be a promising alternative to improve cell detachment from MCs. In this sense, the current trend is the search for new materials that simplify this procedure and reduce or ultimately, eliminate the use of enzymes. Novel materials emerging

for drug delivery systems, nanoparticles, and tissue engineering applications could be extrapolated to MC fabrication. Similarly, multi-responsive systems (which combine two or more stimuli-response materials) could help to increase the separation of the cells when stimuli are applied [125–127].

Cell concentration

After cell detachment, a mechanical force or a physical barrier to recover the detached cells from the MCs is required [128]. For this stage, biotechnology and pharmaceutical industries have successfully used large-scale centrifuges like disk stacks [129] for the separation of bioproducts such as antibodies, proteins, and other high-value molecules. Unfortunately, this methodology is not appropriate for the processing of SCs, which are highly sensitive to shear stress forces generated during this procedure; therefore, other techniques need to be explored. From this perspective, continuous fluidized bed centrifuges have emerged as an effective strategy to solve these inconveniences. These centrifuges stabilize centrifugal and fluid flow forces to capture cells in a chamber, diminishing the shear stress [130].

Another successful alternative for the separation of the cells from MCs is the implementation of filtration methodologies, which are quite effective due to size differences between an MC (100–300 μm) and the cells embedded in them (10–20 μm). Nevertheless, its large-scale implementation is challenging. Despite the differences between these techniques, the final goal is the same: the effective separation of cells from MCs, maintaining cell integrity, cell viability, and pluripotency capacity.

In spite of the ongoing progress observed in the development of SC separation equipment when large-scale culture systems are implemented, future trends seem to point toward the development of MCs fabricated using novel exogenous stimuli-responsive materials. These materials could facilitate this process by reacting to an external stimulus such as: temperature, light (such as ultraviolet, visible or near-infrared light), electric charge, ultrasound, and magnetic field [131]. Among these innovative possibilities, the incorporation of magnetic nanoparticles has highlighted considerable interest due to their outstanding properties: magnetic, biocompatibility, and biodegradability [132]. The combination of MCs with magnetic particles has already been reported. This strategy showed high efficiency of MCs separation, simplifying the cell concentration [133]. For instance, some patent applications propose interesting ideas such as the use of magnetic beads covered with a dextran-based surface for cell separation *via* specific surface affinity interactions such as antibody-mediated separation [134] or the use of dextran-coated magnetic particles in which the cells can be separated by exposing the particles to dextranases [135]. However, it must be carefully analyzed since reports suggest that the use of magnetic fields could trigger the differentiation of SCs if time and intensity are inadequate [136,137]. As in the previous section, a new alternative is the fabrication of MCs utilizing multi-responsive materials, which are already tested with promising results in applications such as drug delivery systems [138–140], thus could simplify the process by replacing the use of enzymes.

Another concern that has been pointed out as one of the bottlenecks for the clinical implementation of the cultured cells, is the separation of the differentiated cells from undifferentiated ones. Different studies have demonstrated tumorigenic properties of SCs such as ESCs and iPSCs in both *in vitro* and *in vivo* models [141,142]. In 2009, Schriebl *et al*. estimated the required steps to purify 99.99999999% of the differentiated cells when employing highly selective magnetic activated cell sorting (MACS). Their results indicated that 31 steps would be required to achieve less than 10^{-1} cell per 10^{9} cells, demonstrating the latent need for other strategies to accomplish this task [143].

Conclusions and perspectives

In recent years, the use of stem cells (SCs) as a tool in regenerative medicine has gained interest. Both the scientific and medical community have focused their efforts on the use of SCs as a treatment for diverse medical conditions. It is expected that SC-based therapies are able to satisfy the growing demand for SCs according to the number, safety, and clinical characteristics of cells. However, conventional SC-culture techniques hinder its full implementation due to lack of process scalability and the utilization of xenogeneic materials. In this context, advances made in microcarrier (MC)-based development of bioprocesses for SC expansion under xeno-free conditions represent a promising route to comply with the exponential demand for Good Manufacturing Practice (GMP)-grade SCs for regenerative medicine. Thus, the commercial availability of xeno-free materials, including MCs, culture media, growth factors, proteolytic enzymes, and the extracellular matrix (ECM), make more feasible this process. Nevertheless, there are still unexplored possibilities to design and develop the “perfect” MC that would facilitate the upstream and downstream SC

processing to enhance their clinical implementation. Therefore, there is a huge opportunity area in the MC-based technology that could bring great benefits in both clinical and economic areas.

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REVIEW

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Culturing human pluripotent stem cells for regenerative medicine

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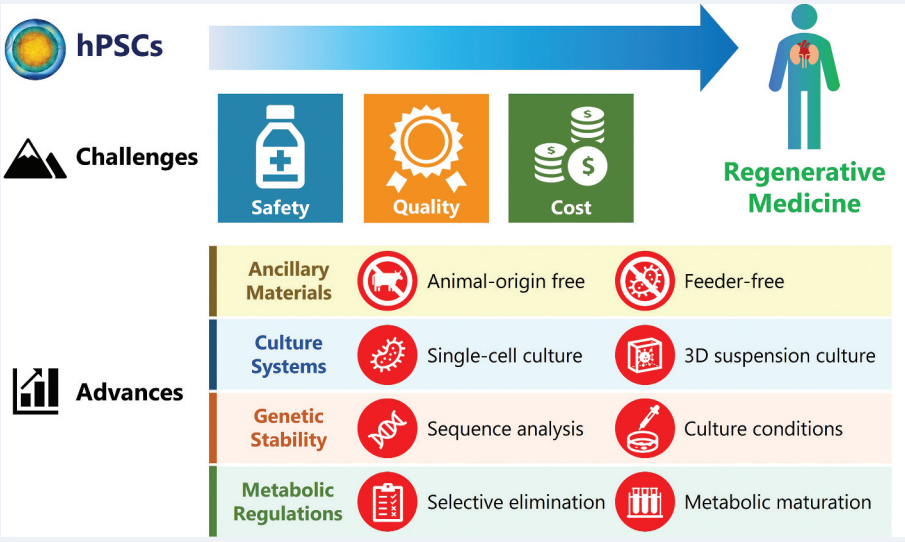
ABSTRACT

Introduction: The development of human pluripotent stem cell (hPSC) culture protocols has led to the establishment of directed differentiation induction methods, resulting in their application in regenerative medicine. Cell therapy products derived from hPSCs have been transplanted into patients, and promising results have been observed in some ongoing clinical trials.

Area covered: This review provides an overview of the challenges associated with the culture of hPSCs for clinical applications and the development of culture technologies designed to address these challenges. We also review future cell culture strategies for large-scale manufacturing to enhance patient access.

Expert opinion: Despite the great potential of hPSCs, difficulties such as safety, quality control, and cost management continue to pose obstacles to their product development and clinical translation. A substantial contribution of these issues lies in the cell culture process. Therefore, selecting the appropriate ancillary materials (AMs) and integrating effective culture methods in standard operating procedures (SOPs) from the early stages of clinical development are essential for success. Moreover, incorporating an automated scaling process is imperative to ensure the commercial feasibility of hPSC-based products.



Human pluripotent stem cells (hPSCs) show great potential as a valuable resource for regenerative medicine. However, three significant obstacles must be overcome: safety, quality, and costs. Thankfully, recent progress in hPSC culture techniques has effectively tackled these challenges, opening up exciting possibilities for realizing hPSC-based regenerative medicine.

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1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), have the ability to unlimitedly self-renew and differentiate into cells derived from the three germ layers [1,2]. Human PSCs can be directed to differentiate into specific cell lineages by introducing targeted growth factors and small molecules. One advancement in this area is the successful production of various functionally differentiated cell types, now used as alternative cell sources for disease modeling, drug discovery, and cell-based therapy [3,4].

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Article highlights

- The culture system for human pluripotent stem cells (hPSCs) has been developed to address the challenges of cell therapy production and meet regulatory requirements.
- Eliminating feeder cells and any components of animal origin from the culture is crucial to establish a defined and safer culture system for clinical applications.
- The single-cell culture method is essential for achieving higher proliferation and establishing a standardized culture process.
- Establishing an optimal cultural environment and implementing appropriate quality control methods are essential to address the genetic alteration of hPSCs in culture.
- The culture method that utilizes hPSC-specific metabolism can efficiently differentiate large quantities of hPSCs.

Clinical trials using hPSC-derived cells have been initiated recently for various diseases, such as age-related macular degeneration (AMD), Parkinson's disease (PD), spinal cord injury, and heart failure (Table 1) [4,24–26]. Technology to produce quality cells is essential to the success of cell therapy. For example, there are two approaches to manufacturing allogeneic versus autologous cell transplantation products. In the allogeneic process, producing a large and stable product from homogeneous raw materials and delivering it to many patients is important. Scale-up allows the production of standardized products for treatment, which is expected to ensure consistent treatment among many patients.

On the other hand, autologous transplantation is a personalized medicine approach. Cells and tissues taken from a patient at a hospital are cultured and then returned to the same patient at the same hospital. The aim is to provide the best possible treatment for the individual patient. The cell manufacturing process includes so-called upstream and downstream processes. Upstream processes include cell line establishment, cell amplification, differentiation induction. Downstream processes include cell isolation, purification, aliquoting, freezing, and packaging. Establishing these consistent process technologies is critical for ensuring the safety and efficacy of cell therapy. Cell culture is the most widely used of these processes, and improvements in cell culture technology will contribute to improved therapeutic efficacy and safety.

Feeder cells and serum-derived components were previously used for the early stages of hPSC culture. However, current methods employ a combination of extracellular

Table 1. Cell lines and culture systems for pluripotent stem cell therapy clinical trials.

ID number	Year	Cell line	Derived cell type	Indication	Culture medium	Scaffold	Reference
NCT01217008	2010	ESC (H7)	Oligodendrocyte progenitor cells	Spinal cord injury	Serum-free (KOSR)	Feeder cells (MEF)	Carpenter et al. [5]; Nistor et al. [6]
NCT01345006	2011	ESC (MA09)	Retinal pigmented epithelial cells	Stargardt's macular dystrophy	Serum-free (KOSR)	Feeder cells (MEF)	Schwartz et al. [7]
UMIN000011929	2013	iPSC (autologous, episomal)	Retinal pigmented epithelial cell sheet	Exudative AMD	XF medium (PrimeES)	Feeder cells (autologous)	Mandai et al. [8]; Kamao et al. [9]
NCT02286089	2014	ESC	Retinal pigmented epithelial cells	Advanced dry AMD	Serum-free (KOSR)	Feeder cells (human fibroblast)	Idelson et al. [10]
NCT02239354	2014	ESC (CyT49)	Pancreatic beta-cell precursors	Type 1 diabetes mellitus	XF medium (XF-KOSR)	Feeder-free (human serum)	Schulz et al. [11]
NCT02057900	2014	ESC (I6)	Severe heart failure	Cardiac progenitors	XF medium (Nutristem)	Feeder cells (human fibroblast)	Menasche et al. [12]
NCT02923375	2016	iPSC (episomal)	Mesenchymal stem cells	Steroid-resistant Acute GVHD	XF medium (E8)	feeder-free (vitronectin)	Bloor et al. [13]
NCT03119636	2017	ESC (Q-CTS-hESC−1)	Neural precursor cells	Parkinson's disease	XF medium (E8)	feeder-free (vitronectin)	Wang et al. [14]
UMIN000033564	2018	iPSC (QHJI01s04, episomal)	Dopaminergic progenitors	Parkinson's disease	AOF medium (StemFit)	Feeder-free (Laminin511E8)	Doi et al. [15]
NCT04106167	2019	iPSC (allogeneic, episomal)	Natural killer cells	Cancer	XF medium (FRM/FMM)	feeder-free (vitronectin)	Valamehr et al. [16]
UMIN000036539 jRCTa050190084	2019	iPSC (YZWJs524, episomal)	Corneal epithelial cell sheet	Limbal stem-cell deficiency	AOF medium (StemFit)	Feeder-free (Laminin511E8)	Hayashi et al. [17]
UMIN000035074 jRCTa031190228	2020	iPSC (YZWJs513, episomal)	Neural stem/progenitor cells	Spinal cord injury	AOF medium (StemFit)	Feeder-free (Laminin511E8)	Itakura et al. [18]; Sugai et al. [19]
jRCTa050190104	2020	iPSC (QHJI01s04, episomal)	Cartilage	Articular cartilage damage	AOF medium (StemFit)	Feeder-free (Laminin511E8)	Yamashita et al. [20]
NCT04945018	2021	iPSC (QHJI14s04, episomal)	Cardiomyocyte spheroids	Heart failure	AOF medium (StemFit)	Feeder-free (Laminin511E8)	Tohyama et al. [21]
NCT04696328 UMIN00032989	2021	iPSC (QHJI14s04, episomal)	Cardiomyocytes sheet	Ischemic cardiomyopathy	AOF medium (StemFit)	Feeder-free (Laminin511E8)	Miyagawa et al. [22]; Kawamura et al. [23]

ESC: embryonic stem cell; KOSR: knockout serum replacement; MEF: mouse embryonic fibroblast; iPSC: induced pluripotent stem cell; AMD: age-related macular degeneration; XF: xeno-free; GVHD: graft versus host disease; AOF: animal-origin-free.

matrices (ECMs) and defined xeno-free (XF) and animal-origin-free (AOF) medium under feeder-free conditions to improve safety, consistency, and efficiency. Ancillary materials (AMs) have been crucial for successful pre-clinical and clinical research with hPSCs. AMs are substances that contact cell and gene therapy products during processing but are not part of the final formulation, such as culture media, growth factors, cytokines, buffered solutions, and coated dishes [27]. In addition, for a more efficient and cost-effective process, hPSC lines used in clinical applications must be thoroughly characterized and deemed safe through well-designed quality control procedures. Therefore, several clinical-grade hPSC lines have been established under current good manufacturing practice (cGMP)-compliant conditions for use in these trials [28–31]. Various cultivation techniques have also been developed for more efficient and cost-effective processes, including the use of the ROCK inhibitor [32], single-cell culture [33], weekend-free feeding schemes, efficient passaging methods, 3D suspension cultures [34], and automation processes. These approaches also apply to creating cell lines for clinical purposes.

Numerous clinical-grade hPSC lines have been established under cGMP-compliant conditions for use in these trials [28,29]. The human pluripotent stem cell (hPSC) lines used in clinical applications must be thoroughly characterized and deemed safe through well-designed quality control procedures and obtained with informed consent [35]. The delivery strategies for introducing reprogramming factors into cells to generate iPSCs have been scrutinized due to their significant impact on the efficacy and safety of clinical applications [36]. To avoid the quality and safety issues that can arise from the random insertion of transgenes, non-integrative strategies, such as Sendai virus [37], episomal vectors [38], or mRNA methods [39], are commonly employed. Various reprogramming techniques yield equivalent results and do not influence the gene expression profile of the resulting hPSCs [40].

Notwithstanding these advances, cell therapy with hPSC-derived products is still immature compared to long-established medical systems. There are three major challenges to the clinical application of hPSC-derived products for their production. Safety concerns are the first challenge. Due to the nature of hPSCs, residual undifferentiated cells and the acquisition of genetic mutations can pose a risk for potential teratoma formation and tumorigenesis [41]. Therefore, it is necessary to eliminate such mutations from the cell culture process. Furthermore, the manufacturing process of cell products involves a complex procedure that requires various raw materials. Therefore, there are concerns regarding the contamination arising from these materials and processes, which could potentially cause harm to patients.

The second challenge is quality control. Unlike conventional pharmaceuticals, producing living cell products requires evaluating and managing numerous proprietary critical quality attributes (CQAs). Notably, the plasticity of hPSCs makes it difficult to reproduce a uniform cell population, making it essential to follow the cGMP: (i) establish and validate standard operating procedures (SOPs) for the entire manufacturing process, (ii) define the specification and their criteria for release, and (iii) describe the methodology of quality control [42].

The third challenge is cost. While the primary advantage of iPSC-based therapy is autologous transplantation derived from the patient's cells, the current cost of cell manufacturing is exorbitant due to the complex cell processing procedure. Significant resources are invested in personnel, GMP facilities, materials, and equipment for manufacturing and quality control, resulting in a high cost of cell therapy products.

This review provides an overview of the cell culture methods used in various clinical trials involving hPSCs. We specifically focus on the advances and challenges encountered during the cell manufacturing process and the potential solutions that have arisen with the requirements for culture systems (Chapter 2), the recent development of culture systems (Chapter 3: feeder- and animal-origin-free culture systems, Chapter 4: single-cell culture systems), understanding of the genetic stability in culture (Chapter 5), culture methodologies employed in cellular metabolism (Chapter 6), and perspectives (Chapter 7).

2. Human PSC culture systems requirements for clinical applications

The most important factor in the application of hPSCs in regenerative medicine is their safety. To ensure the safety of cells, AMs, such as the media, additive factors, and culture equipment used in the cell and gene therapy manufacturing process, must be safe. While several standards and guidelines have been established for cell and gene therapy products, the use of AMs has only been addressed indirectly. There are some controversies surrounding the terminologies used to describe AMs. For instance, 'GMP grade' is often used as a marketing term, implying that the material is suitable for drug manufacturing. In contrast, GMP is a process rather than a material grade [43]. Similarly, 'clinical-grade' or 'USP/EP/BP/JP grade' are commonly used, but their definitions vary depending on the case. In this paper, we use the terms according to the definitions outlined in Table 2 [43,46–48].

ISO20399:2022 sets out the requirements and recommendations for both suppliers and users of AMs to ensure the safety and performance of manufactured cell products [43]. Moreover, there are variations in the emphasis placed on relevant guidelines and regulations among different countries [49]. For example, USP1043 guides the development of appropriate qualification programs for AMs used in cell, gene, and tissue-engineered products in the US [46]. In contrast, the EU EP5.2.12 focuses on materials extracted from biological sources and/or produced by recombinant DNA technology and addresses risk assessment, manufacturing, and quality control [50]. Japan has established the Standards for Biological Ingredients (SBIs) to ensure the safety of cell transplants and other products. These guidelines regulate using raw materials, animals, plants, and microorganisms and their products in pharmaceuticals, cosmetics, and foods. For example, fetal bovine serum (FBS) used in cell culture must follow these guidelines. Medical applications of hPSCs must also comply with these guidelines to ensure product quality and safety [51].

Table 2. Glossary of terms for ancillary materials.

Term	Definition	Alternative terms or synonyms
Ancillary material (AM)	The materials that come in contact with the cell or tissue product during manufacturing are not intended to be part of the final product. For example, AMs include culture media, growth factors, cytokines, serum, and biological material-coated dishes but not scaffolds or feeder cells.	Raw materials, Reagents
Animal-origin-free (AOF)	'Animal' refers to all animals, including humans. This term signifies that the AM does not contain any raw materials or materials isolated from animal sources in its product and process. Suppose this term extends to secondary or further downstream materials. In that case, it indicates that no animal-derived components were used to produce the AM's raw materials and that all recombinant proteins were produced in nonanimal cell lines.	Animal-derived component-free (ADCF), Animal-component-free (ACF)
Clinical-grade	AM suppliers use this term to indicate that the AM is suitable for manufacturing clinical products, as it is manufactured with high-quality materials and a process characterized by extensive documentation. Similarly, 'GMP compliant' refers to AMs manufactured under the guidelines of Good Manufacturing Practices (GMP), which require manufacturers to take the necessary steps to ensure that AMs are traceable, safe, pure, and effective.	GMP-compliant, Pharmacopeial-grade, API-grade
Defined	This term indicates that the AM does not contain undefined components such as serum, plasma, platelet lysate, or tissue extracts as ingredients. Although ingredients are defined by their origin and purity, this does not imply that all components are pure. For instance, numerous 'defined media' incorporate BSA or HSA with a purity exceeding 95%, leaving 5% of the composition unknown. 'Chemically defined' means that a chemical formula specifies all raw materials and that the AM does not contain proteins, polypeptides, or other ingredients with complex structures. However, interpreting these terms may vary case-by-case [44,45].	Chemically defined
Research use only (RUO)	This term indicates that the AMs are intended for research purposes only. They should not be used for manufacturing cell and gene therapy products unless properly qualified.	Lab-grade
Serum-free	This term indicates that the AM does not contain serum or plasma as primary ingredients. However, the AM may still contain ingredients derived from serum or plasma, such as albumin, transferrin, lipids, and platelet lysate.	Defined
Xeno-free (XF)	This term indicates that the AM contains no ingredients derived from non-human animals. However, it may contain human-derived components such as human serum, human platelet lysate, or human serum albumin.	

3. Feeder-free and animal-origin-free culture systems

Feeder cells are utilized to establish and maintain hPSCs, especially hESCs [1]. Feeder cells are believed to provide the essential elements for the growth of hESCs. The use of coculture systems with feeder cells can be traced back to the cultivation of mouse ES cells (mESCs). This practice was based on the capability of mESCs to differentiate into all three germ layers and gametes. Feeder cells were employed during the establishment of the first hiPSCs [2]. However, the risk of xenogeneic contaminants and the challenges regarding performance consistency are hurdles that must be overcome to address the safety, quality, and cost issues of clinical applications of hPSCs from feeder cell-based culture.

An obvious solution to this problem was feeder-free culture. Developing feeder-free culture methods requires a substrate (coating material) to replace feeder cells. Matrigel is a commonly used cell culture matrix primarily composed of basement membrane ECM proteins such as laminin, collagen IV, entactin, and heparan sulfate proteoglycan perlecan [52]. Furthermore, defined ECM molecules have been developed to support the pluripotency and proliferation of hPSCs. These include laminin [52], vitronectin [53], and SyntheMax, which is a synthetic peptide-acrylate surface [54]. Of the various laminin isoforms, laminin 511 [55,56] and laminin 521 [57] are particularly well suited for culturing hPSCs. Since full-length laminins are large heterotrimeric proteins, their large-scale production as recombinant proteins can be challenging. Consequently, an active fragment of the laminin 511 molecule called LN511-E8 was developed. This fragment has been shown to outperform the original full-length protein [58].

To establish the first hESCs, fetal bovine serum (FBS) was used with feeder cell culture [1]. Although human serum has been shown to support hESC self-renewal and xeno-free culture has been achieved [59], developing a serum-free medium was necessary to address the issue of undefined culture conditions. Under feeder-free conditions, serum-free culture has been achieved using Knockout Serum Replacement (KOSR) [60], N2 and B−27 supplements [44], and albumin [61]. Albumin is the most abundant protein in plasma and performs multiple functions that support cell culture [62]. Although albumin is not strictly necessary in hPSC medium [45], its combination with LN511-E8 improves cell viability. It allows for a higher split ratio in a single-cell manner, demonstrating the advantages of using albumin to enhance cell culture performance [63].

Serum albumin comprises a diverse range of proteins, not only major albumin proteins but also a variety of unidentified proteins. The significant variation in protein composition between lots can result in lot-to-lot variation that substantially impacts cell culture outcomes [64]. A xeno-free system that utilizes human-derived proteins and peptides, such as human serum albumin, reduces the risk of zoonotic infections and immune reactions. However, it still exhibits the same disadvantages associated with variations in protein composition as a serum-free system. Recombinant proteins have facilitated progress toward a defined and safer culture system by removing animal-derived components [65]. However, if animal-derived cell lines such as HEK293 are used to produce the recombinant protein, the potential infectious risks associated with the expression system must be addressed. Utilizing an animal-origin-free culture system consisting solely of chemically defined small-molecule compounds and recombinant

proteins expressed by nonanimal expression systems is crucial to meet clinical application safety and quality requirements.

4. Single-cell culture systems

When we pass human ES cells cultured on feeder cells, enzymes are used to detach the cells, which are then scraped off using a scraper or similar tool. The cells are pipetted several times to form small clumps of large colonies and then seeded onto fresh feeder cells. The problem with this passaging method is that it is difficult to make the same size clamps every time and that the size of the clamps varies from operator to operator. These problems indicate that it is challenging to prepare cells of the same quality and condition each time when culturing cells for clinical application. The significance of single-cell culture is that everyone can make single cells in the same way, and the number of cells can be measured so that the same number of cells can be seeded every time. These characteristics are necessary to establish a reproducible and robust SOP for producing cells suitable for clinical use. The optimal cell number and density, i.e. the local cellular microenvironments, are crucial to differentiation efficiency. The paracrine secretion of soluble factors and the contacts between cells can influence differentiation fates [66]. Therefore, precise control of the seeding density is one of the critical success factors in manufacturing the final hPSC-derived product.

Furthermore, the single-cell cloning step is essential for hPSC manipulation. hPSCs are known to have different properties, such as differentiation ability in each clone. The fact that the properties of hiPSCs differ from clone to clone suggests that the somatic cell population from which they were reprogrammed may be heterogeneous. Alternatively, it could be that the characteristics of the cell changed during reprogramming. Hence, cloning must be performed at some point after reprogramming the somatic cells. Likewise, performing single-cell cloning after gene editing with CRISPR/Cas9 enables the isolation of hPSCs for disease modeling and establishing clinical cell lines. As cloning efficiency is directly related to CRISPR/Cas9 experimental performance, improving the productivity of single-cell culture must be considered.

A challenge in hPSC single-cell culture is the tendency of hPSCs to undergo anoikis, a form of programmed cell death that occurs when cells detach from the surrounding ECM [67]. It has been reported that high-density feeder layers facilitate the successful single-cell culture of hESCs using an enzymatic dissociation method [33]. Additionally, Watanabe et al. [32] found that Rho-kinase inhibitors (Y −27632) enable the single-cell culture of hESCs on feeder cells and feeder-free culture conditions. Y−27632 is now commonly used in hPSC culture to improve culture quality, and new compounds have been found to prevent anoikis of dissociated hPSCs and enhance single-cell cloning productivity [68].

In the traditional clump passage method, the split ratio was approximately 1:3 to 1:5. While this was sufficient for research use of hPSCs, a more efficient passaging method was deemed necessary for future clinical applications, as larger-scale culture is expected. A single-cell culture system allows for further scale-up using a split ratio of 1:100 or higher [33]. Since hPSCs sense increased culture density via contact inhibition and the cell density and proliferation rate of hPSCs are inversely proportional [69], seeding at a lower cell density can achieve a more significant expansion rate with improved culture methodologies [63,70].

5. Genetic stability in culture

Human iPSCs undergo continuous genetic changes and evolution during long-term culture, contributing to the cells' proliferative potential. These changes have been shown to predominate in cell populations in culture. In general, regions of the long arms of chromosomes 1, 17, 20, and X and the short arm of chromosome 12 are often gained, while regions of chromosomes 10 and 18 are often lost [71–74]. Recent whole-genome sequencing has also revealed repetitive mutations in the TP53 gene that are not affected by common karyotypic changes [75]. In a region of frequent genomic amplification on the long arm of chromosome 20, analysis of genes located in minimally amplified regions revealed that BCL2L1, which regulates apoptosis, is the driver gene that confers a growth advantage to cells with amplification [76].

Similarly, dominant-negative mutations in TP53 May confer a survival advantage by limiting apoptosis. The possible involvement of NANOG on chromosome 12p has also been suggested [72]. In cell therapy, genomic instability of hiPSCs has been associated with abnormal tissue formation and residual undifferentiated cells in xenograft tumors and is considered a significant safety risk. It can also lead to genetic mutations well known to be associated with cancer and is considered a nonnegligible risk factor when present in cells used in regenerative medicine. However, assessing the consequences of specific mutations in hiPSCs in a given application is often difficult. Cancer development typically involves multiple mutations that accumulate over time. While a single cancer-associated genetic alteration may not lead to tumorigenesis, it is certainly more likely that a cell will acquire enough mutations over time to become malignant. From this perspective, the risk associated with a single genetic mutation may be context-dependent, but there is currently no consensus on assessing this risk. Yamamoto et al. [77] showed transplantation of differentiated cells followed by NGS evaluation of SNVs/del related to cancer genes listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) database or Shibata's list and in vivo tumors. No correlation was found between SNVs/del and abnormal tissue formation in the in vivo tumorigenicity study, indicating that the WGS test is not a quantitative risk predictor.

On the other hand, a positive correlation has been confirmed between the presence of a copy number greater than 3 and abnormal tissue formation after transplantation, with a prediction rate of 86%. In particular, it has been suggested that amplification of the 14q32.33 or 17q12 locus is involved. This CNV changes over time help assess the genetic instability of cells. In vitro CNV screening can reduce the cost and time of

iPSC-derived product development and QC and provide efficient source cells [77]. Mutations may be inherent in the original cells or may arise during the reprogramming, cloning, maintenance culture, and differentiation culture processes when iPSCs are manufactured into cell therapy products. Appropriate donor selection is essential for the former, and for the latter, culture methods and appropriate quality control (QC) are essential to control the possibility of mutations. For donor selection and QC, karyotyping is an essential part of the process. On the other hand, whole-genome analysis and other genetic and disease marker analyses are not mandatory but may be reported on a 'for information' basis if collected voluntarily. However, these data are difficult to interpret clinically and can be confusing. In addition, regulators do not currently require this information. As a quality indicator of the process, The Global Alliance for iPSC Therapies (GAiT) recommends that whole-exome sequencing be performed at the highest level of coverage and depth (ideally more significant than 50 × average coverage) [78].

Reducing the likelihood of genetic mutations is essential in the culture of cell therapy products. Reducing the number of passages and the duration of culture from thawing to final product formation effectively decreases the likelihood of mutations. The cultural environment and media composition are also important factors to consider. One trigger for DNA damage and genomic alterations is the acidification of the medium, which is related to culture density. The accumulation of lactate, which causes a decrease in extracellular pH, inhibits glycolysis and triggers G0/G1 cell cycle arrest [79,80]. On the other hand, hypoxia has been reported to inhibit base pairing changes associated with oxidative damage, resulting in a reduced mutation rate [81].

Since mutant cells tend to survive by inhibiting apoptosis, the possibility of inhibiting the appearance of mutant cells by culture methods without Rho kinase inhibitors was investigated. However, the Rho kinase inhibitor Y−27632 did not affect the mutation rate [81]. In a recent study, Neumayer et al. [82] demonstrated that improvements in the culture process affect the genetic stability of GMP-compliant autologous iPSC-based cell therapy products for dystrophic epidermolysis bullosa. Compared to previous results, their findings indicated that the integration of iPSC reprogramming and genetic correction into a single manufacturing process, which reduced the culture time, the mutation load, and clonal bottlenecks, significantly improved the karyotypic stability of the iPSC line [83].

The acquisition of genetic alterations in cultured hPSCs is an unavoidable problem and a challenge for the various applications of PSCs. However, it can be overcome by recognizing the problem and choosing the right way to address it.

6. Metabolic regulation of hPSCs

There is inherent heterogeneity and variation among individual cells in the process of propagation and differentiation of hPSC-derived cells. This poses a significant challenge for quality control when considering the clinical application of hPSC-derived cell products, as residual undifferentiated cells are a significant safety concern due to the nature of hPSCs. Additionally, it has been reported that hPSCs exhibit differences in differentiation propensity and resistance among various cell lines [84], and there is concern about strain-dependent yield differences when applying the same protocol to multiple strains. Various cytokines, growth factors, small-molecule compounds, and matrix proteins have been utilized to enhance the induction efficiency of differentiation protocols to address clonal variations. Additionally, efforts have been made to purify and isolate differentiated cells.

A method using cell lineage-specific surface antigens was developed to eliminate undifferentiated iPSCs and purify differentiated cells. CD30 was identified as a marker in hPSCs but not in differentiated cardiomyocytes. The residual iPSCs were eliminated with brentuximab vedotin, an anti-CD30 antibody with an antimitotic agent approved for treating CD30-positive lymphomas [85]. Regarding the isolation method of different cell types, anti-Chorin has been used to select dopaminergic neurons [86], and CD200(-), ITGB4(+), and SSET4(+) selection have been employed to isolate corneal epithelial cells [87]. Cell sorting is a highly effective technique for isolating specific cell populations; however, concerns regarding cost, time, and safety may arise due to the potential for antibody contamination in using antibodies during cell sorting [88,89].

Here, we present a discussion with a specific focus on cell metabolism. While much research on refining differentiation methods has concentrated on functional components such as growth factors, cytokines, and small-molecule compounds, recent attention has also been given to the nutritional aspect of the culture medium. Since each cell type has distinctive metabolic characteristics that maintain its tissue function, an in-depth analysis of cellular metabolism enables the reproduction of the culture conditions of each cell type to more closely replicate the tissue functions observed in vivo [90]. This information can be utilized to establish optimal expansion and differentiation culture protocols, especially for PSCs in a unique metabolic state. Purity can be enhanced by taking advantage of the metabolic distinctions between undifferentiated and differentiated cells [91]. One example of a cardiomyocyte differentiation protocol involves removing glucose and glutamine from the culture medium and adding lactate, an initiator metabolite of the mitochondrial TCA cycle. This change in the culture medium can help eliminate undifferentiated cells, facilitate the differentiation of cardiomyocytes, and promote metabolic maturation. This approach has led to the successful purification and culture of cardiomyocytes on a large scale [92,93].

Moreover, cellular metabolism is closely related to the epigenome. Many mediators of epigenome modification require metabolites, such as S-adenosylmethionine (SAM) for DNA and histone methylation and acetyl-CoA for protein acetylation. Metabolic pathways that supply substrates, cofactors, and coenzymes for these epigenetically related enzymes are directly involved in epigenome-mediated cellular regulation [94]. Human PSCs have a high requirement for S-adenosylmethionine (SAM). While mouse cells rely on threonine for one-carbon metabolism, the methionine pathway supposes it in human cells. Short-term methionine-deprived culture decreases H3K4me3 levels with decreasing intracellular SAM

concentration and suppresses the expression of transcription factors involved in the maintenance of pluripotency, such as NANOG [95]. Induction of differentiation in methionine-depleted hPSCs results in enhanced differentiation via histone modification changes [96]. Various cellular responses to methionine deprivation are also mediated by intracellular zinc dynamics, and culture of zinc removal from the medium contributes to increased differentiation efficiency. When endoderm differentiation was performed in several cell lines under zinc-depleted conditions, the marked propensity that originally varied between cells was overcome, and uniformly efficient endoderm cell differentiation was achieved. Moreover, differentiation efficiency was further enhanced when pancreatic cell differentiation was combined with undifferentiated methionine-depleted culture, and mature pancreatic beta cells with glucose-responsive insulin-secreting ability could be induced [97]. In conclusion, the culture method utilizing hPSC-specific metabolism efficiently differentiates large amounts of PSCs.

7. Future perspectives

Human iPSCs hold the potential for ideal personalized medicine through autologous cell transplantation since anyone can derive them. However, the challenges of high manufacturing costs and difficulties in establishing a stable supply chain are evident from recent experience with autologous CAR-T production [98]. Thus, the future of autologous transplantation of iPSC-derived cells is likely distant. More realistically, some organizations have established HLA-matched iPSC banks. However, even this approach requires a large number of donors and resources to generate a sufficient number of HLA homozygous lines [28,29,31,99,100]. Therefore, efforts are underway to commercialize universal allogeneic transplantation, which can treat multiple individuals from a single source. Host rejection, mainly due to the T-cell immune response, is the biggest obstacle to achieving allogeneic transplantation. Recent studies have developed methods to overcome T-cell-mediated immune responses by knocking out the β2 microglobulin (B2M) gene and the class II transactivator (CIITA) gene [101]. The B2M gene is important for presenting HLA class I molecules on the cell membrane surface. HLA class I molecules recognize nonself by presenting antigens to CD8 + T cells, triggering an immune response. This molecule's lack of cell surface expression allows donor cells to escape attack by the recipient's CD8+ T cells. On the other hand, donor cells that do not express HLA class I molecules activate NK cells responsible for innate immunity ('missing self') and are eliminated [102]. To suppress this 'missing self' response, donor cells have been reported to express HLA-E-modified genes or CD47, factors that suppress NK cell activation [103,104]. In addition, other techniques to capture antibodies with over-expressed CD64 have been reported, indicating the potential of overcoming immune rejection against minor antigens [105]. Ensuring the safety of these techniques is an important issue, but several clinical trials are underway, and the results are expected to clarify this issue.

The next step in solving the immune response problem to achieve cost-effective allogeneic transplantation is scalability and proper quality control in manufacturing. Human clinical cell therapy requires 10^8-10^{10} clinical-grade stem cells cultured under cGMP conditions, while commercial-scale manufacturing requires approximately 10^{11} -10^{14} cells [106]. Recent advances in culture systems have proposed robust, controllable, and scalable platforms. For efficient cell production, suspension culture-based methods that do not require microcarriers have been used to simplify downstream processes [107,108]. Challenges to overcome in this method include uncontrolled spheroid aggregation and degradation of cell quality due to shear stress, which leads to cell growth arrest, cell apoptosis, and heterogeneity in cell purity and quality [109]. Cohen et al. [110] recently showed that iPSCs had been encapsulated in hollow alginate microcapsules internally coated with ECM components to mimic epiblast stage development in vitro. This culture system achieved 277-fold growth in 6.5 days on a 10 L scale. Adherent culture is sometimes preferred over floating culture because it is more stable. This is a reasonable option, especially when producing cell therapy products that require a small number of cells, such as those for the retina and cornea [111]. In addition, automation is underway to achieve commercial-scale production and efficiency [112].

From a quality control perspective, the best approach for the industrial scale-up of hiPSCs is to design the manufacturing process according to the principles of Quality by Design (QbD). Advanced process analytical techniques (PAT) are essential to achieve QbD. Routine measurement of key nutrients, such as glucose, and metabolites, such as lactate, during culture, is important. Additionally, controlling process parameters is also crucial [113]. Recently, Chilmonczyk et al. [114] developed a PAT that combines a dynamic sampling platform (DSP) and an electrospray ionization mass spectrometer to detect trace components of cell quality in the culture medium in real-time. They claim that these real-time spectral data's principal component analysis (PCA) can be used to monitor the state of cells (confluence, differentiation, etc.). For many of these PATs, the mechanism linking the analysis results to the cell status is still unresolved, and demonstrating the validity of the causal relationship is a future challenge. However, it is expected to be solved with the advancement of analysis techniques with artificial intelligence.

Such technological advances are expected to promote allogeneic cell therapy and solve cell therapy product immune rejection, cost, and supply chain problems.

8. Expert opinion

Following the protein therapy market, the cell and gene therapy market is poised for substantial growth as a new therapeutic strategy. This growth is driven by advances in adaptive cellular therapies such as CAR-T cells [115] and the development of regenerative medicine for various tissues [116]. Human pluripotent stem cells (hPSCs) are gaining attention as starting materials because of their unique properties. As the fledgling industry progresses toward commercialization, supply chain management is a considerable challenge for cell and gene therapy.

The selection of ancillary materials (AMs) is a critical factor affecting the above issues in a cell culture system. To ensure safer and higher-quality cell production, serum-free, xeno-free, and animal-origin-free technologies have been developed alongside feeder-free culture systems. While research-

grade materials are commonly used in basic science research, clinical applications require more clinically relevant materials suitable for GMP-compliant culture systems. However, changing materials may require a major overhaul of the culture system. This overhaul is due to the impact of the culture medium and extracellular matrix (ECM) on cell characterization, as changes in pluripotency and differentiation require further optimization of the culture protocol. For example, even small changes in the refolding or purification process of a recombinant protein in one of the culture medium components could impact the biological activity of this protein [117,118], which may ultimately affect the culture outcome. Considering the overall project cost, including quality control (QC), process development (PD), and validation, is essential. Switching systems or materials during a clinical trial may result in substantial project delays and increased costs [119]. Designing a system from the earliest stage to anticipate commercial-scale production is necessary to avoid these issues. In particular, implementing an efficient and scalable culture system and process automation, developing low-cost selection methodologies (e.g. metabolic approach), and refining AM compositions and packages adapted to the culture systems are ways to achieve cost-efficient manufacturing [120].

Many of these challenges have been recognized in the production of approved biologics such as antibodies, hormones, cytokines, and vaccines. After years of research and development, commercial-scale production has been achieved, resulting in a thriving industry [121]. This established technology can be applied to commercial cell and gene therapy production. By modifying the culture process to accommodate the unique properties of hPSCs and engineering hPSC cell lines, it will be possible to advance clinical trials and commercial production of hPSC-derived products. This progress is expected to drive the growth of the cell and gene therapy market.

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