WHITEPAPER

Guide to Seeding, Expanding, and Harvesting Stem Cells

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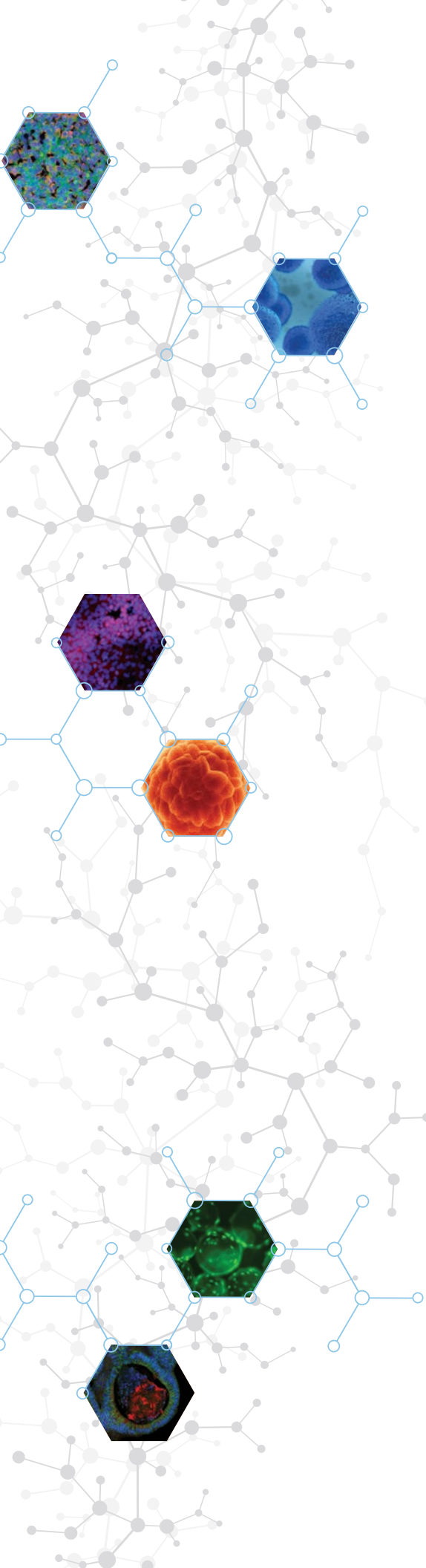

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The stem cell therapy revolution is well on its way. While progress towards realizing the promise of regenerative medicine is still largely limited to preclinical research or early clinical trials, the use of stem cells has already accelerated our understanding of human diseases and the search for effective treatments. This white paper covers important considerations for working with three different stem cell types: mesenchymal stem/stromal cells (MSCs), induced pluripotent stem cells (iPSCs), and neural stem cells (NSCs).

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Mesenchymal Stem/ Stromal Cells

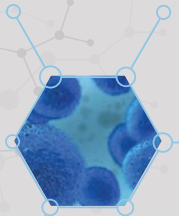
The International Society for Cellular Therapy defines MSCs based on three cellular properties:

1. Adherence to plastic
2. Expression and nonexpression of specific proteins
3. Potential to differentiate into the mesenchymal lineages of osteoblasts, adipocytes, and chondroblasts

Of the different types of stem cells that can be cultured *in vitro*, MSCs have shown the most promise as a therapeutic agent. For example, they secrete molecules that can quiet an overactive immune system.

Another reason MSCs have been so popular among stem cell researchers is that they're easy to obtain ethically. "Traditionally, mesenchymal stem cells would commonly come from bone marrow or adipose tissue," explains Tom Bongiorno, a Field Application Scientist for Corning. "More recently, we've seen a lot of work with MSCs that are coming from placental tissue or umbilical cord tissue."

These tissues are relatively easy to obtain and contain very young cells that haven't had time to accumulate mutations. However, Bongiorno cautions, "Even within those different tissue sources, there is a lot of donor variability with MSCs, compared to other stem cell types. So it's very important to do some testing on your specific cells, because while you can get general starting points and information from other MSC work, it doesn't necessarily translate perfectly to your cells."



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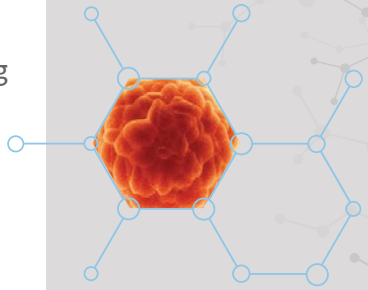
Low Seeding Density Translates to Fast Proliferation

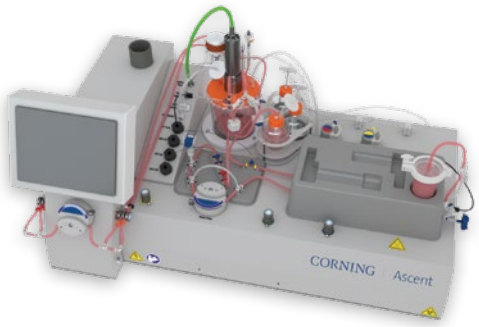
Despite the variability in culture methods, MSCs are among the less complicated stem cells to work with. "Roughly speaking, they're pretty similar to most other adherent cells," says Bongiorno. "Early-passage MSCs grow in colonies and are generally easy to culture because they can be passaged as single cells. They also require less frequent media exchanges and are less sensitive to precisely timed passaging at a given confluency level. Their seeding density is generally going to be very low compared to other cell types, perhaps in the range of 1,000 to 10,000 cells/cm^2. I've even seen it go down to about 100 cells/cm^2 if you're really trying to reduce the number of passages that are required." Reducing the number of passages also reduces labor requirements but does not affect the *in vitro* "age" of the cell, commonly expressed as the population doubling level.



Corning CellSTACK Vessel

Corning HYPERFlask Vessel

Corning HYPERStack Vessel



Corning Ascent FBR System

Indeed, a 2014 review of clinical trials that used MSCs found that plating densities ranged from 200 to 12,000 cells/cm^2, with the most common range being 3,000 to 6,000 cells/cm^2. A higher plating density was associated with reduced proliferation ability, likely due to contact inhibition and/or reduced nutrient availability per cell. The authors were concerned that a very low plating density could be unrealistic due to increased cost, so they suggested an evidence-based compromise of 1,000 cells/cm^2. They also suggested using Corning® CellSTACK® vessels as a way to decrease costs and increase safety when scaling up for clinical applications. It is important to note that MSCs also have very limited expansion life — a limited number of times they can be passaged or have population doublings in culture — before they senesce.

Since that research was published, Corning has introduced Corning HYPERFlask® and HYPERStack® vessels, and the Corning Ascent® Fixed Bed Reactor (FBR) system to support the process of scaling up. These systems also accommodate or provide automation, which simplifies scalability, improves control, lowers contamination risk, and reduces operator-introduced variability. Additionally, Corning polystyrene microcarriers and dissolvable microcarriers provide a growth surface for adherent cells in a suspension environment when using a stirred tank bioreactor.

Process and Material Choice Can Influence Growth

MSCs are defined by their ability to adhere to plastic. "Generally speaking, MSCs will attach to the large majority of surfaces, including TC-treated and Corning CellBIND® surfaces," according to Bongiorno. "In some cases, depending on the type of MSCs you're working with, they may require collagen or some other surface coating to attach." This goes back to the variability of MSCs. As Bongiorno explains, "MSCs from different tissue sources may behave differently, especially with regard to their degree of true stemness."

Whitney Wilson, also a Field Application Scientist for Corning, agrees. "Normally, mesenchymal stem cells don't need an extracellular matrix (ECM) to grow. But when you work inside a bioreactor — like the Ascent® FBR — you may benefit from coating. Collagen or fibronectin are commonly used as ECMs for expanding MSCs in bioreactors."

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Induced Pluripotent Stem Cells

The development of iPSCs in 2007 — achieved by reprogramming somatic cells into an embryonic-like pluripotent state through the forced expression of key transcription factors — has opened up endless possibilities for research and therapy.

Compared to MSCs, iPSCs are typically more challenging to grow in culture. As Wilson explains, "iPSCs have traditionally been grown in what's called cluster culture, so they grow as colonies. When scientists first started culturing these cells outside of the body, we originally did so on a feeder layer of mouse embryonic fibroblasts (MEFs). Those fibroblasts lay out an ECM that helps support colony growth."

However, this feeder layer becomes problematic if the cells are intended for therapeutic use. "Over the years we've developed several different ECMs that can support iPSC and human embryonic stem cell (hESC) growth without a feeder layer," says Wilson. "One of the most popular is Corning Matrigel® matrix," which is available in a hESC-qualified version. One thing to note is that Matrigel matrix isn't fully defined, which can lead to lot-to-lot variability and potential safety issues.

The two main alternatives are vitronectin and laminin. Corning Synthemax® vitronectin substrate is an animal-free, synthetic, vitronectin-based peptide substrate for stem cell culture. Corning recombinant human laminin 521 (rLaminin-521) is a biocompatible, full-length laminin. Both Synthemax and rLaminin-521 can be used to culture stem cells in colony or cluster formation and can be applied to Corning microcarriers for larger-scale stem cell expansion applications.

Seeding Density is Often Difficult to Define

The process of culturing iPSCs is "very nuanced," according to Wilson. In fact, many institutions have core facilities that provide stem cell culture as a service to affiliated research groups. In contrast to MSCs, says Wilson, "it's very hard to define a seeding density for iPSCs because the culture is usually done in a cluster or colony formation. You're often not doing a cell count. You're just counting clusters and seeding those. Typically, the way that people talk about passaging iPSCs is going from one well to three wells. When the colonies grow large enough that you can visually identify that they're starting to show signs of spontaneous differentiation, they're getting too close together" and need to be passaged.

"iPSCs can be grown as single cells," says Wilson. "However, when you grow them in that way, you put selective pressure on the cells that can lead to chromosomal aberrations and problems with the cell lines. So unless they're working in a bioreactor system like the Ascent FBR, people who are scaling up iPSCs typically try to keep them in cluster formation and avoid single cell suspensions."

Following Procedural Best Practices is Crucial

Wilson's priorities align with published protocols for the culture of human iPSCs for clinical use under current good manufacturing practice (cGMP) requirements. These feeder-free and xeno-free methods use Synthemax as an ECM on untreated T-flasks. Cell cultures are examined daily under a microscope to check for iPSC-like morphology, the presence of any differentiated cells, and cell culture confluence. Healthy ESC and iPSC cultures have a rapid doubling time of approximately 16 to 20 hours, which supports pluripotency and requires subculturing every 4 to 5 days. This corresponds to passaging when iPSCs have reached medium-to-large colony size or when the culture reaches approximately 60 to 75 percent confluence. The recommended cell seeding density ranges from 10,000 to 20,000 cells/cm^2. To ensure adequate nutrition, daily media changes are recommended.

Unsurprisingly, this procedure needs to be modified when an iPSC culture is being established from frozen stock. One cryogenic vial of iPSC should be appropriate for seeding a coated 75 cm^2 T-flask or 6-well plate. An initial 24- to 48-hour recovery period is to be expected with slower growth, followed by exponential growth and standard handling for iPSCs.

Culture conditions, including cell density and cell passaging methods, affect the genetic and epigenetic stability of iPSCs. Enzymatic passaging has been associated with increased genomic instability of iPSCs. Therefore, the recommendation is to use enzyme-free dissociation methods, preserve iPSC clusters when passaging or harvesting, and complete the procedure within about 30 minutes. Accutase® and Corning CellStripper® are cell detachment solutions that allow for the gentle detachment of adherent cells within minutes.

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Neural Stem Cells

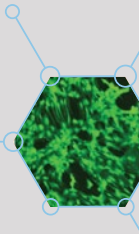
Among their many potential benefits, NSCs provide an unprecedented opportunity to model the pathogenesis of central nervous system diseases and explore applications in regenerative medicine. This work relies on the use of iPSCs or ESCs.

As Wilson explains, "Neural stem cells are very few and far between in the body. They're also very difficult to isolate because they reside within the brain in an area called the subventricular zone (SVZ). So unless someone's already getting shunt surgery, it's pretty much impossible to get neural stem cells ethically. This is why working with iPSCs is so powerful. You can expand those cells — to our knowledge indefinitely — and get a really large starting population to then differentiate into neural stem cells."

NSCs are among the most challenging cells to work with in the lab. "Think about where NSCs normally exist in the body," says Wilson. "Everything in the body is 3D, but the brain is an especially complex 3D environment. Neural stem cells really don't like 2D platforms, to be honest. So we have to make them extra, extra happy to get them to attach. Often that requires adding an extra positive charge — to a tissue culture treated plate or a CellBIND surface plate — using something like Corning PureCoat™ amine or Corning Poly-D-Lysine coated surface. Then on top of that, we have to add laminin."

High Seeding Density Helps Prevent Differentiation

"NSCs need a very high seeding density. Otherwise, they will spontaneously differentiate. Neural stem cells typically are seeded between 10,000 to 100,000 cells/cm^2," Wilson says. "For MSCs, typically you passage them when they're between 75 to 80 percent confluent. For neural stem cells, they really like to be very close together so you typically let them grow to 95 to 100 percent confluence before you passage them." NSC also have very limited expansion life — a limited number of times they can be passaged or have population doublings in culture — before they differentiate.



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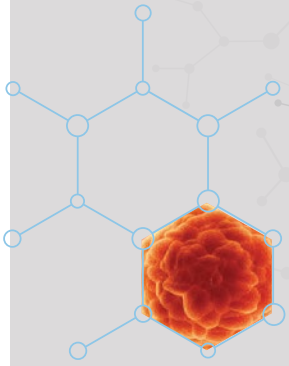

A 2020 publication in Frontiers in Cell and Developmental Biology reviews the many different protocols used for the induction and differentiation of NSCs from iPSCs and ESCs *in vitro*. The protocols are mainly based on creating specific conditions for stem cell cultivation and adding specific molecules that drive their differentiation. The different protocols result in a wide range of neural cell types, including oligodendrocytes and astroglia.

Briefly, neural induction of pluripotent stem cells may be conducted in 2D conditions (monolayer and coculture with stromal cells) or in 3D conditions (embryoid bodies). Further generation or cultivation of NSCs can be achieved in 2D conditions (e.g., monoculture or neural rosettes) or in 3D conditions (e.g., serum-free culture of embryoid bodies, neurospheres, or brain organoids). Most of the protocols produce cell populations that aren't capable of self-renewal. There are protocols to generate NSCs from hESC under serum-free, feeder-free conditions.

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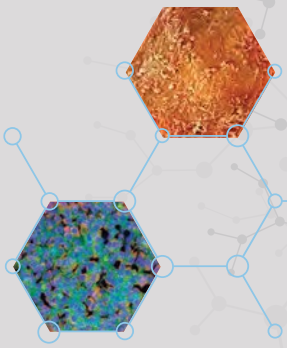
The Path Forward

Turning the enormous potential of stem cell research into reality is a daunting challenge. But the work of researchers around the world brings that reality closer every day. Occasionally, breakthroughs will occur, such as the discovery of MSCs or the development of induced pluripotent stem cells. More often, there will be relentless trial and error and the testing of optimistic hunches that may become hypotheses.

Exploration is ongoing, which promises to lead to innovations that accelerate the work, such as automated handling of more samples, greater volumes, or faster processing. Other innovations such as synthetic, xeno-free reagents will allow stem cells to save lives and improve health.

Curious to see this process of seeding, expanding, and harvesting outlined visually? Review our infographic, Stem Cell Therapy Production, which details the important considerations at each stage in the process.



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