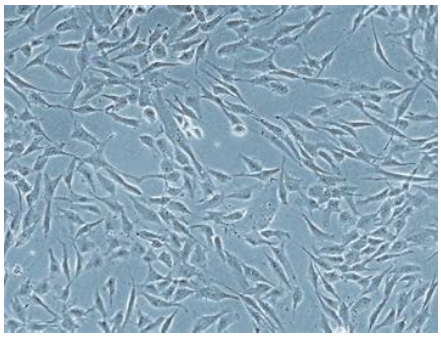
Hollow Fiber Bioreactor Protocol for Mesenchymal Stem Cells

INTRODUCTION

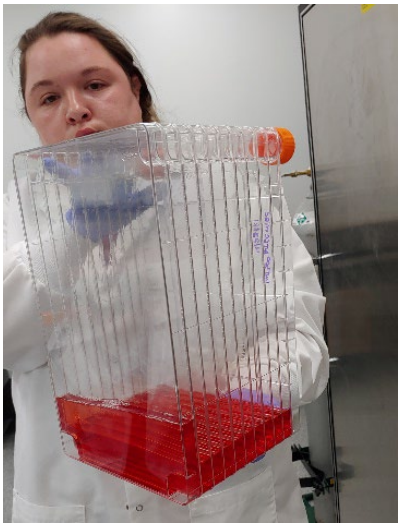


Mesenchymal stem cells are multipotent adult stem cells that are present in multiple tissues, including umbilical cord, bone marrow and adipose tissue. Mesenchymal stem cells can self-renew by dividing and can differentiate into bone, cartilage, muscle, adipocytes, and connective tissue. The International Society for Cellular Therapy (ISCT) has recommended the use of the name multipotent mesenchymal stromal cell (also abbreviated to MSC) for the *in vitro* cultured cells, restricting the term stem cell to designate the proposed in vivo precursors/stem cells.

Due to the diversity of characteristics displayed by hMSCs from different sources by various isolation methods, the ISCT recommended minimal criteria to define MSCs: (1) cell adhesion to a plastic surface, (2) specific positive and negative surface markers and (3) *in vitro* tri-lineage differentiation capability.

The unequivocal identification of MSCs *in vivo* has been hindered by their extremely low frequency in tissues and the lack of a distinct MSC-specific immunophenotype to enable their identification and isolation. Cultured human mesenchymal stromal cells do express a panel of cell surface markers such as CD73, CD105, CD90 and lack endothelial or hematopoietic cell markers (CD34, CD31 and CD45). Nevertheless, these are not homogeneously expressed throughout stromal cultures, vary with isolation protocols and passage number, and therefore are not necessarily representative of MSCs *in vivo*. The microenvironment for MSC *in vivo* is quite different from 2-D flask culture, though unfortunately 2-D flask culture is part of the working definition for MSC identification.

3-D ARTIFICIAL CAPILLARY HOLLOW FIBER BIOREACTORS VS. T-FLASKS

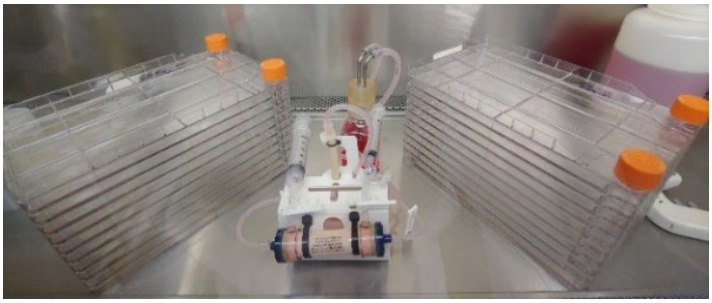


3-D hollow fiber artificial capillary bioreactors present a more *in vivo* like microenvironment to cells in culture. The tremendous surface-area-to volume-ratio allow cells to be cultured at *in vivo* like cell densities, 1-2X10^8/mL. Cells are bound to a porous support, not a non-porous 2-D plastic surface, so cells do not require splitting or passaging. The molecular weight cut-off (MWCO) of the fibers helps control the composition of the micro-environment and concentrates secreted autocrine factors and biological products. The issue with MSC relative to 3-D hollow fiber bioreactors is that part of their very definition is that MSC bind to 2-D plastic cell culture flask surfaces and proliferate. However, the number of passages that can be performed before the cells differentiate is limited, 12-16 passages at most. A 3-D hollow fiber bioreactor presents a more *in vivo* like environment to cultured cells of all types.

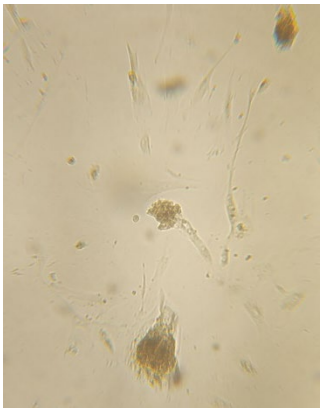
SYSTEM	CULTURE SURFACE CM^2	SUPERNATANT VOL	SURFACE/VOLUME RATIO	HARVESTING
Hollow Fiber	4000	20	200 cm2/mL	At least once per day for weeks
T Flask	175	35	5 cm2/mL	Single Batch

MSC IN 3-D CULTURE

MSC culture in hollow fiber bioreactors behave differently, and perhaps more like they do *in vivo*. Based upon data collected to date, MSC cultured in a 3-D hollow fiber bioreactor do not proliferate. It has not been possible to identify reliably MSC in their *in vivo* environment, but it is likely that the "normal" state of MSC is to be non-proliferative *in vivo* in the absence of specific stimuli. The 3-D environment created by a hollow fiber bioreactor recapitulates the *in vivo* situation, and therefore MSC do not proliferate in this *in vivo* like environment. The bad news is that MSC do not proliferate in 3-D culture, this means you require a lot of cells to seed the reactor. The good news is that MSC do not proliferate in 3-D culture. If MSC are not proliferating, they are not differentiating. MSC have been cultured in the hollow fiber bioreactor for 30-100 days with continuous production of extracellular vesicles, and no change in MSC phenotype or EV characteristics.



OPERATING GUIDELINES FOR THE FIBERCELL C2011, C2018 AND C2025D CARTRIDGES



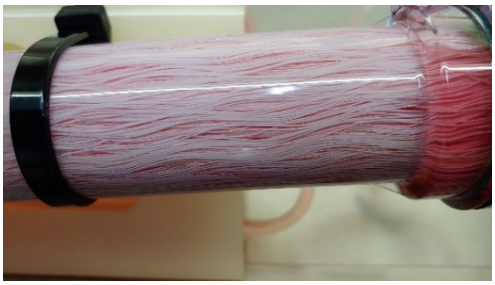
The following should be considered a set of guidelines, and not a formal protocol for the culture of MSC in a hollow fiber bioreactor.

1. MSC seem to require quick attachment as they appear flat and spread out in 2-D flask culture but can form spheroids when seeded into a hollow fiber bioreactor. For this reason, we coat the fibers with fibronectin. The protocol for this is laid out below.
2. The surface area of the C2011 is 4,000 cm^2. We have seeded up to 1x10^9 cells into this cartridge, which represents approximately 6 m^2 of area, or the size of ten Corning 10 stacks (6,200 cm^2). This means that MSC not only bind to the fibers but can also bind to each other. A minimum of 1X10^8 cells should be seeded into the C2011, and a minimum of 1X10^7 into the C2025D. Up to 1X10^9 MSC can be seeded into a C2011.
3. Various specialty xeno-free MSC mediums have been used. It may still be necessary to coat the fibers with fibronectin. We have not tried any of the specialty attachment factor solutions. The most commonly used medium is DMEM with 10% FBS. Another commonly used medium is DMEM/10% human platelet lysate sometimes with the addition of 5% CDM-

HD. One of the advantages of the hollow fiber bioreactor with a 20 kD MWCO filter fiber is that serum can be used in the circulating medium, and not in the extra-capillary space with the cells. The growth factors in serum that support the cells can cross the fibers, but the endogenous exosomes found in serum, along with high molecular weight proteins, like immunoglobulins, cannot cross the fiber, and will not contaminate the MSC derived exosomes harvested from the Extra-Capillary Space (ECS).

4. Glucose consumption is much lower than that of transformed cells lines grown in the hollow fiber bioreactor. $1X10^9$ 293T cells, CHO cells, or hybridomas will generally consume 1 gram of glucose per day. MSC may consume 10% of that, and glucose consumption may be difficult to assay.
5. There is evidence that MSC may primarily consume glutamine and produce ammonia under some circumstances. This may be related to the total amount of glucose present in the medium. Low concentrations of glucose (1 gram per liter) may result in more glutamine metabolism, higher concentrations of glucose (4.5 grams per liter) may result in more glucose being consumed.
6. Do not expect to be able to harvest large numbers of cells from the ECS of the hollow fiber bioreactor. MSC adhere very tightly to the fibers. They can form spheroids, and these too bind very tightly and cannot be removed even when using trypsin. Small numbers of cells can be harvested, and a viability check performed by placing these cells into a T25 flask with fresh serum containing medium to look for attachment and proliferation (MSC collected from the ECS will attach and proliferate when placed into a flask).

PROTOCOL FOR COATING FIBERS WITH FIBRONECTIN:



1. Set up the C2011 or C2025 20 kD MWCO PS cartridge with PBS per the Quick Start Guide. Allow PBS to circulate for at least 24 hours in the incubator. The polysulfone fibers are co-extruded with polyvinylpyrrolidone, an FDA approved blood expansion product. This makes the fibers hydrophilic so they can be stored and shipped dry, and wet out easily. The removal of the PVP renders the fibers slightly hydrophobic. In this state the fibers will bind protein to its surface. Fibronectin will not cross the fiber, but bind to the outer surface of the fiber and facilitate cell attachment.
2. Prepare a solution of 20 mL of PBS containing between .5 and 1 mg of fibronectin (higher amount preferred) and place into a 20 mL syringe. Follow the video instructions for cell inoculation. Briefly, place the loaded syringe onto one side port, place an empty syringe on the other side port. Close the end ports. Swish the fibronectin solution back and forth between the syringes 4-5 times. At the final swish, leave 1/2 of the fibronectin solution in each syringe. Open the right end port. Close one of the side ports and ultrafilter the fibronectin solution through the fibers and into the reservoir bottle. Close that side port and open the opposite side port and repeat. The excess PBS will pass through the fibers into the reservoir bottle while the fibronectin will attach to the outer surface of the fibers. Ensure that

both side port slide clamps are closed, and that both end port slide clamps are open. Leave at room temperature in the hood overnight.
3. Proceed with the rest of the preculture according to the Quick Start Guide.

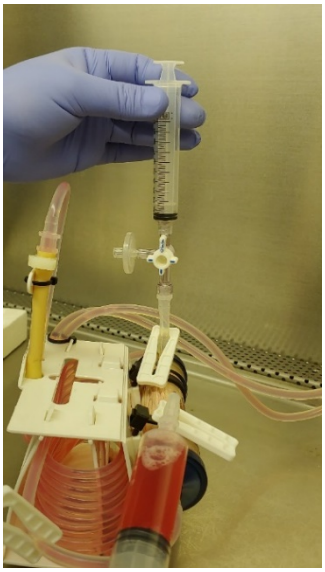
PROTOCOL FOR HIGH CONCENTRATION HARVEST OF EXTRACELLULAR VESICLES OR OTHER SECRETED PRODUCTS FROM THE EXTRA-CAPILLARY SPACE.

There are several different ways to harvest from the hollow fiber bioreactor cartridge. If the cell numbers are too high, it necessary to remove a portion of the cells using the "high glucose rate harvest method."

https://www.youtube.com/watch?v=elbvGep8Js4

If the glucose uptake rate indicates that cell removal is not required, the "low glucose rate harvest" method should be used. This will generally be the case with MSC culture.

https://www.youtube.com/watch?v=n3NxIG-7AOI&t=31s



Both of these methods are designed to protect the extra-capillary space from air inside the hood from entering the cartridge, a potential source of contamination. To harvest the most concentrated EVs from the bioreactor cartridge but protect the ECS from potentially non-sterile air a 3-way stop-cock and .2 um sterile syringe filter are used as shown. Attach an empty syringe to the right side-port. Switch the 3-way stop-cock so that the port to the filter is open. Tilt the cartridge up and drain the ECS into the opposite syringe. Refill the ECS by placing fresh medium into a syringe and introducing it into the ECS in the same way. Close the side port slide clamps and turn the stop-cock so that the filter port is closed and the closed end is closest to the side-port.

LOOKING TO THE FUTURE

Hollow fiber bioreactors provide a more *in vivo* like cell culture environment than standard 2-D culture, and this has a profound effect on the behavior of MSC *in vitro.* The precise definition of exactly what an MSC is, and how it behaves is under constant revision as new data is collected. The differences between 2-D and 3-D culture are demonstrated clearly with MSC and represent an important distinction for regenerative therapeutics and clinical applications. This is an evolving field and FiberCell Systems will continue to work with our end users to better understand the culture of various cell types and the production of extracellular vesicles from these cells.

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