3 Dimensional Cell Culture in Hollow Fiber Bioreactors

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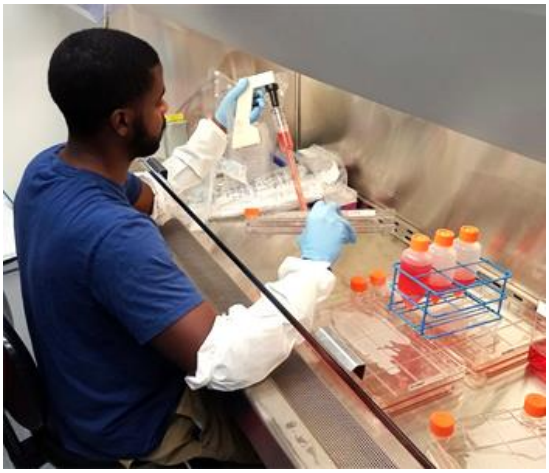
INTRODUCTION

The *in vitro* culture of mammalian cells in the biomedical research laboratory is a near ubiquitous technique, and a fundamental tool of biological research. The first attempt at culturing cells in the laboratory outside of an organism was performed by Harrison in 1907. By placing frog embryo nerve fibers onto a plasma clot, the first 3-dimensional culture method was created. Many new tools had to be developed along the way to make modern cell culture the common technique it is today. The plasma clot was replaced by glass dishes and then by plastic flasks. Dissociation enzymes to generate single cell suspensions from tissue became available in the late 1940's. The HELA cell line was created in the early 1950's providing a robust and hardy transformed cell line that could be used for research. Various cell culture mediums were developed such as RPMI, DMEM, Ham's etc. and the collection and processing of fetal bovine serum became standardized, a requirement to getting cells to grow in culture. Liquid nitrogen cell storage provided easy access to reproducible cell lines. The introduction of the laminar flow hood in the 1970's was a major advance, providing a suitable working environment for cell culture in any laboratory. Mammalian cell culture is now a huge market, valued at $12-$14 billion dollars worldwide and is practiced by high school students, university researchers on up to biopharmaceutical manufacturing scientists.

Despite the wide spread use of conventional cell culture techniques, whether it be in 2-D dimensional plastic flasks or low density suspension culture, it is becoming recognized that this is not the most physiologically relevant way to grow cells, or the best way to collect important biologics that they can produce. Cells grown on non-porous plastic dishes must be passaged or split before they reach confluence, and maintained in a sub-confluent manner. Nutrients are delivered from the top down in flask culture. When the cells pile up on top of one another the bottom layer doesn't receive oxygen and nutrients. An enzyme such as trypsin is used to dissociate the cells from the plastic before they reach confluence and the cells are seeded into new plastic flasks. This process is repeated every 2-5 days and disrupts the cells while generating a lot of plastic waste.

Sub-confluent growth on a non-porous surface is not a physiologically relevant way to grow cells. Some cells can also be cultured in suspension, under constant stirring or agitation, and at low density, densities 100 times lower or more than they grow *in vivo*. Again, these are non-physiologic conditions. The use of serum is also not physiologically relevant. Serum is plasma minus the clotting factors. The only time cells are exposed to serum rather than plasma is in a wound state, and the clotting factors have been activated. In point of fact, most cells are never exposed to serum or plasma *in vivo*. Interstitial fluid, which bathes cells, is different than serum. Lot-to-lot variations in serum composition and quality can also affect the relevance of cell culture results.

"THE DIFFERENCE BETWEEN ART AND SCIENCE IS MATHEMATICS." *EUCLID*

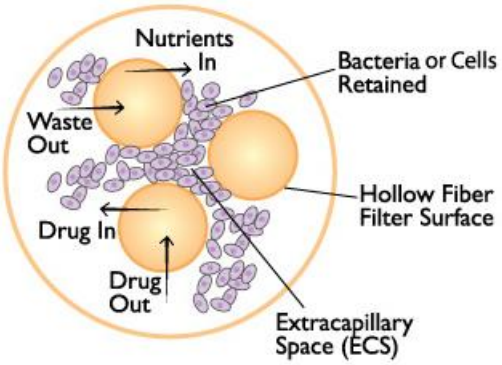


The evaluation of cells in flasks is rather imprecise. Simple observation of the cells under a microscope generally serves as the main method for determining the quality of the culture. Do the cells look nice and round, or do they appear crenellated? Do they appear abnormal in some other way? Direct measurement of some cell growth parameter, such as the glucose uptake rate (as performed in hollow fiber bioreactors) provides direct numerical determination of culture health. Not only is the culture of cells in plastic flasks non-physiologic, it is also imprecise, time and labor intensive. The splitting of 100 flasks can take many hours, and must be repeated every 3-4 days. Repetitive use syndrome or carpal tunnel syndrome is a common ailment for scientists who have performed cell culture for their careers.

In summary, the culture of mammalian cells on a non-porous 2-dimensional surface, subconfluently with constant passaging and usage of fetal bovine serum is not the most physiologically relevant, nor most efficient manner to culture mammalian cells. For precisely these reasons, along with more complex culture requirements such as cell co-cultivation there is currently tremendous interest in 3-dimensional cell culture systems.

In many ways, the hollow fiber bioreactor may be considered the original modern 3-dimensional cell culture system. Hollow fiber based cell culture was first developed by Richard Knazek at the NIH in 1972. He was searching for a way to culture adrenal tumor cells under in vivo like conditions to study hormone secretion in response to drug stimuli. His reports that response curves can be generated by assaying the

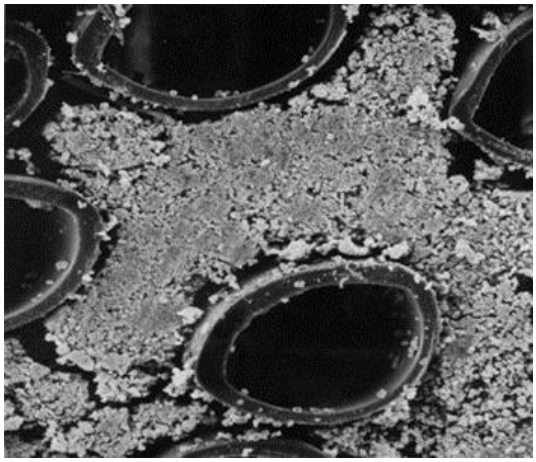
amount of hormone secreted in response to biochemical stimuli were the original dynamic *in vitro* cell-based assays (Knazek et al, Science October 1972). Hollow fiber bioreactors offer a method by which cells can be cultured at tissue-like densities over long periods of time. Hollow fibers act as "artificial capillaries" and act much as capillaries do in the human body.



Previously it had been thought that the benefits of hollow fiber bioreactors were restricted to simply higher secreted protein concentrations, the ability to reduce serum levels, or more easily adapt cultures to commercially available serum-free media. It is now understood that hollow fiber bioreactors represent a more *in vivo*-like method for culturing cells, and in 3 dimensions. There is no question that the conditions under which cells are cultured have a profound effect on their behavior, and cell culture conditions are fundamentally different in a hollow fiber bioreactor.

THE HF BIOREACTOR

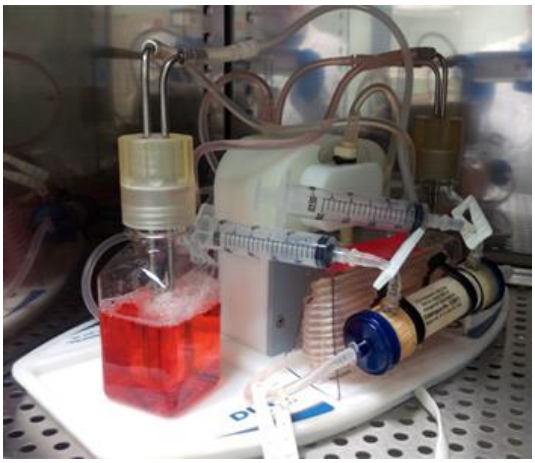
The hollow fiber (HF) bioreactor is a high-density continuous perfusion culture system, different from the non-porous plastic surfaces of e.g., flasks, microcarrier beads or roller bottles. A HF bioreactor consists of a cartridge containing thousands of semi-permeable hollow fibers in a parallel array within a tubular housing fitted with inlet and outlet ports. The fiber bundles are potted at each end so that any liquid entering the ends of the cartridge will necessarily flow through the interior of the fibers. Cells are generally seeded outside of the hollow fibers in what is referred to as the extra capillary space (ECS).



Culture media is circulated through the insides of the hollow fibers allowing nutrients, gases and waste products to diffuse both ways across the fiber walls. After passing through the cartridge, the culture medium is oxygenated and re-circulated to the cartridge. Hollow fiber bioreactors offer a unique environment for a more in vivo like cell cultivation and cell co-cultivation. They present a 3-D environment similar to the conditions found in the body, and support the continuous control of oxygenation levels, medium composition, and drug concentration. HF bioreactors are an effective means for the generation of a number of cellular products, from secreted proteins, antibodies, and exosomes to cells or conditioned medium. There are three

fundamental characteristics that differentiate hollow fiber cell culture from any other cell culture method:

- Cells are bound to a porous support, much as they are *in vivo*
- The molecular weight cut off of the hollow fiber filter can be controlled, anywhere from 5 kd to .1 micron
- A very high surface area to volume ratio (>150 cm^2 per mL)



CELLS BOUND TO A POROUS SUPPORT

Cells bound to a porous support have no requirement to split or passage the cells. Cultures in a HF bioreactor can maintain viability and productivity in a post-confluent manner for extended periods of time– months or longer. For example, one hybridoma cell line maintained productivity in a HF bioreactor based culture for over one year of continuous production. There are some cell types that do not seem to proliferate in the hollow fiber bioreactor. This seems to be specifically true of mesenchymal stem cells, although the data is very new. This is likely a result of the more in vivo like cell culture conditions, as MSC do not actively divide in situ. This lack of proliferation is actually an advantage, as the lack of division means that the cells will also not differentiate. MSC have been cultured in a hollow fiber bioreactor for 3 months of continuous culture. In a HF bioreactor the cells are not subject to shear. This is also a feature of the *in vivo* milieu. In a HF bioreactor the majority of cells that become necrotic do not become apoptotic and do not release significant cytoplasmic proteins or DNA into the product harvest. This provides a cleaner culture environment, more accurate culture parameter monitoring and a harvest that is simplified and easier to handle for downstream assays and purification. Also, no surfactant is required in the medium to protect the cells from this shear.

CONROLLED MOLECULAR WEIGHT CUT-OFF

Through the selection of fiber molecular weight cut-off (MWCO), secreted products can be retained within the ECS to concentrations up to 100 times higher than in standard cultures. The effects of cytokines on the cells can also be controlled. This is well-illustrated in the case of hybridoma culture where the inhibitory cytokine TGF-beta is selectively removed from the culture by diffusing through the fiber while secreted antibody is retained. Secreted recombinant proteins can also be selectively retained and concentrated while cytokines and other factors that facilitate cell-to-cell interactions can be concentrated as well. Small molecule drugs can easily exchange

across the fiber and rapidly reach equilibrium while larger bacteria and cells are retained.

HIGH SURFACE-TO-VOLUME RATIO/HIGH CELL DENSITIES

The small diameter of the fibers (200 microns) generates an extremely high surface-area-to-volume ratio of 150-200 cm^2/mL within the cartridge. Along with the high gross-filtration-rate polysulfone fibers, the exchange of nutrients and waste products is high enough to support cell densities of 1-2 x 10^8/mL, approaching *in vivo* tissue-like densities. A 20 mL cartridge will easily support the cell mass provided by a standard 2-liter suspension culture or 20-40 roller bottles. High cell densities produce more protein per reactor volume and facilitate adaptation to both lower serum concentrations and simplified serum-free medium, such as FiberCell System's CDM-HD (Chemically Defined Media for High Density) serum replacement. The high cell density found inside a hollow fiber bioreactor is different enough from standard cell cultures that the cell culture medium used can be simplified and optimized to take advantage of these unique culture conditions. Differences in medium formulation performance are mitigated as the cells auto-support with their own secreted factors. In fact, the more complex and sometimes more expensive a cell culture medium is, the less effective it is in a hollow fiber cell culture environment.

Operation of a HF bioreactor begins by seeding a prepared cartridge with either suspension or harvested adherent cells. The hollow fiber module is connected to an external reservoir and the medium recirculated from the reservoir through the cartridge. The FiberCell Duet pump provides high rates of flow (80-160 mL/minute) using a positive pressure displacement pump mechanism designed specifically for this purpose. A piston compresses a short piece of pump tubing and a one-way check valve on either side produces flow, in the same manner as the human heart. This frictionless pumping mechanism can generate high flow rates without wear on peristaltic pump tubing. Gas exchange is provided via a loop of gas permeable silicone tubing prior to the medium entering the bioreactor. The medium is constantly re-circulated providing a supply of oxygen and nutrients as well as removal of CO^2 and waste products. The glucose concentration is monitored and the medium in the reservoir replaced when the concentration is 50% of the initial level. The glucose uptake rate, i.e. the amount of glucose consumed in a 24-hour period is a direct indication of the health of the cartridge. As long as the rate remains stable, or is increasing the cells are growing well. It has been determined empirically that a glucose uptake rate of 1 gram per day corresponds to approximately 1x10^9 cells.

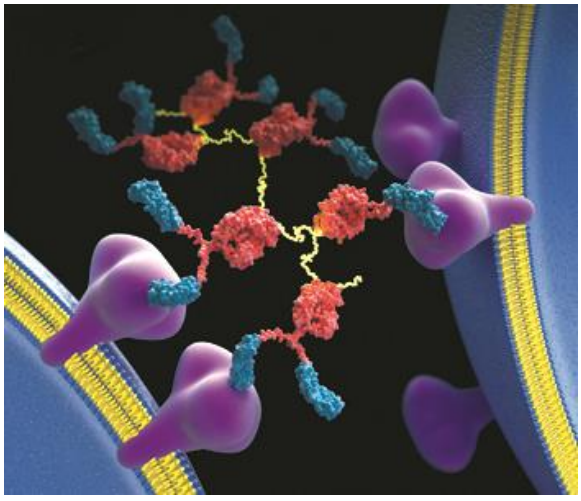
Several cartridges sizes and fiber types are available, including polysulfone (PS) and PVDF fibers. The molecular weight cut-off (MWCO) in the PS fibers includes 5 KD or 20 KD and the pore size of PVDF is 0.1 micron. The PVDF fiber is of particular interest as various protein matrices, antibodies or growth factors can be easily bound to its surface. Cartridges come pre-sterilized, assembled and ready-to-use and are intended for a single use. Several characteristics of hollow fiber cell culture are as follows:

- Reduction in apoptosis
- Consistency of culture over long periods of time
- More *in vivo*-like growth conditions resulting in improved cell physiology
- Facilitation of the use of serum-free, protein-free and chemically defined media formulations

REDUCED APOPTOSIS

Reduced proteolysis and contamination by host cell proteins and DNA is a characteristic of the HF bioreactor. During extremely long culture periods no degradation of secreted products is observed, even when using serum-free and protein-free media. This is a result of a significant reduction in apoptosis. The cells die, but do not lyse and go through the typical process of releasing proteolytic enzymes, intracellular proteins and DNA. This and the absence of significant shear forces inside the HF cartridge, results in dramatically reduced proteolysis and cleaner harvests, especially when using CDM-HD.

CULTURE CONSISTENCY



In a HF bioreactor, the cells are bound to a porous support, not a non-porous plastic surface. Cell division rate and generation number is reduced, cultures do not require splitting, passage number is irrelevant, and cells grow in multiple layers in a "post-confluent" fashion. Cultures can be maintained for many months and up to a year or longer. Culture conditions remain highly consistent during this time as does cell physiology. In an example of this, a CHO cell line was transfected to produce a hexeramized IgG, six IgG subunits held together by 3 IgA tails— a very large and complex protein. When cultured in flasks 40% of the protein was improperly expressed as a monomeric subunit. When these exact cells were harvested from the flasks and seeded into a HF bioreactor, 95% of the protein was expressed as a properly folded hexamer.

IN VIVO-LIKE GROWTH CONDITIONS

In vivo, most animal cells grow in 3-D at very high density under tightly defined and highly controlled conditions. There is very little variation in oxygen tension, pH, glucose levels etc. These parameters can have a wide range in flasks and other culture devices. The 2-D cell culture systems used so far have several drawbacks: the morphology, proliferation, metabolism and expression profiles of cells grown in 2-D systems are very different to cells in living tissues. In a HF bioreactor it has been shown that consistency of culture conditions has a direct effect on cell physiology and product quality.

PROTEIN-FREE, CHEMICALLY DEFINED CULTURE MEDIUM

Besides providing many positive factors, animal serum has inherent problems including foaming, risk of contamination with viruses, mycoplasma and other adventitious agents along with variability in both production and cell-based assay performance. There are a number of non-culture related limitations as well; including high and variable cost and that high protein concentration that can interfere with culture analysis. Cells *in vivo* are exposed to serum only during a wound state, and many cells then respond by particular activations. HF bioreactor culture characteristics, particularly the very high cell density, allow for a reduction in serum concentration and facilitate adaptation to commercially available serum-free media. This has been taken one step further with the introduction of CDM-HD; a commercially available serum replacement. CDM-HD is a protein-free, chemically defined (CD) serum replacement that is simplified and optimized for the cell culture conditions found high-density cell culture. CDM-HD is in fact more than just a serum replacement. CDM-HD does not support culture well in low density spinner flasks or flask culture, it requires the cells to be at high cell densities in order to support cells. The use of CDM-HD results in cleaner product harvests and more simplified purification as well as a more defined culture environment compared to the presence of fetal bovine serum. CDM-HD also provides lot-to-lot consistency, shipping at ambient temperature, 3-year stability when stored at 4 degrees, and significantly reduced cost compared to serum. A significant consideration in the development of any cell based therapeutic product is the potential lack of serum to support large scale manufacturing. CDM-HD eliminates this concern.

CDM-HD is a direct manifestation of the different cell culture conditions found in a hollow fiber bioreactor and its optimization specifically for those conditions. Culture conditions are uniquely more *in vivo*-like in a hollow fiber bioreactor.

HF bioreactors provide many particular (and some unique) culture characteristics due to a number of physical and ambient chemical conditions provided by the system, including:

- Perfused medium flow and porous support permits long-term culture
- Selectable MWCO of fibers concentrates secreted products up to 100 times
- High cell density permits use of CDM-HD. Lag phase is eliminated
- Post-translational modifications are consistent and uniform over time
- Apoptosis reduced, less contamination with host cell proteins and DNA along with lysozyme
- Cell splitting eliminated, passage number is irrelevant
- Selectable MWCO of fibers concentrates interactive cytokines
- Shear stress on endothelial cells is required for proper physiology
- Long-term high-density culture on porous support facilitates development of cell-to-cell interactions over time
- Cell co-cultivation for physiologically relevant models

One of the primary goals of the *in vitro* culture of cells is to as closely as possible recapitulate the *in vivo* environment. Cell culture conditions have been shown to have a profound effect on the quantity and quality of secreted products from mammalian cells. Biologically relevant models of *in vivo* processes are also dependent upon mimicking the *in vivo* environment as closely as possible. The 3-D and more *in vivo* like cell culture conditions present in a hollow fiber bioreactor have been clearly demonstrated. Over the course of the next few months a series of short articles will be presented to highlight the advantages hollow fiber bioreactors can provide for the following topics:

- Monoclonal antibody production
- Recombinant protein production
- Exosome collection
- Cell co-cultivation
- Pharmacokinetics and pharmacodynamics

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