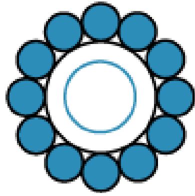


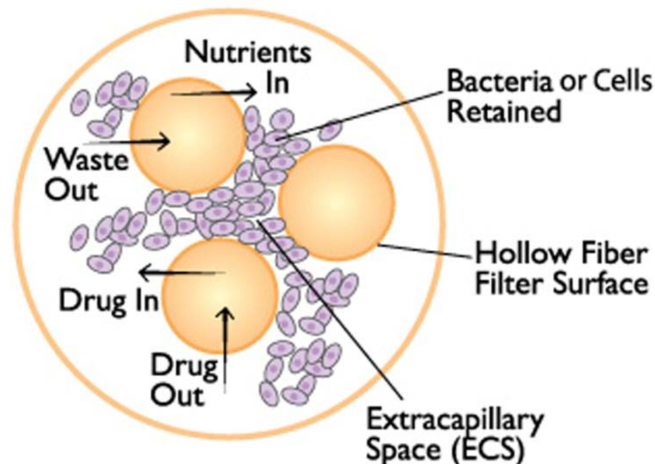
FiberCell Systems Inc.
a better way to grow cells

Production of Recombinant Proteins and Monoclonal Antibodies in Hollow Fiber Bioreactors

By John J. S. Cadwell

Introduction

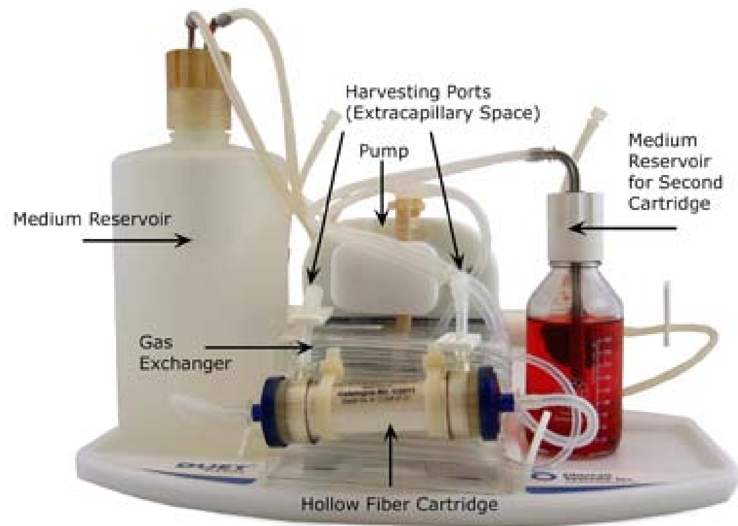
The production of secreted products from mammalian cells such as recombinant proteins and monoclonal antibodies is generally performed in standard flask, roller or spinner culture. The typical cycle of seeding cells at a very low density in an excess of medium and then harvesting (often quite aggressively) just before the point of medium exhaustion, is itself a very unnatural system. It is only recently understood how dramatically culture conditions can affect protein quality. Popular culture systems take cells that originally grew attached to a porous matrix at high densities with little variability in per cell nutrient and oxygen supply and adapt them to low density styrene-bound or amorphous suspension culture. While well-understood, robust and convenient, classical batch-style 2-D culture on non-porous supports or 3-D suspension culture in other devices are really not very biologically relevant models. Cell culture conditions can affect the quality of the antibody or protein produced.



The Hollow Fiber Bioreactor System

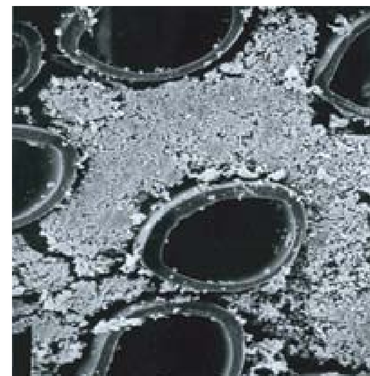
In order to more closely approximate *in vivo* cell growth conditions, Richard Knazek developed the hollow fiber bioreactor (HFBR) in 1972 (Science, October 1972). The HFBR system is a high-density continuous perfusion culture system. It consists of thousands of semi-permeable hollow fibers in a parallel array within a tubular housing or cartridge fitted with inlet and outlet ports. These 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 within the cartridge, but outside of

the hollow fibers in what is referred to as the extra capillary space (ECS). Culture media is pumped inside the hollow fibers allowing nutrients and cell products to diffuse both ways across the fiber walls. Once having passed through the cartridge, the culture medium is oxygenated and returned to the cartridge. There are three fundamental characteristics that differentiate hollow fiber cell culture from any other method.



1. Cells are bound to a porous support much as they are *in vivo*, not a plastic dish, microcarrier, or other impermeable support.
2. The MWCO (Molecular Weight Cut Off) of the support matrix can be controlled.
3. Extremely high surface area to volume ratio (150 cm^2 or more per ml.)

Cells are bound to a porous support much as they are *in vivo*. There is no requirement to split cells. Cells in this perfusion system maintain viability and production-relevant metabolism in a post-confluent manner for extended periods of time, months or longer. For example, one hybridoma was reported to maintain efficacious productivity for over one year of culture. The more *in vivo* like growth conditions afforded by HFBRs result in significantly reduced apoptosis (1). The majority of cells which become necrotic will not release cytoplasmic proteins or DNA into the culture medium again resulting in a product which is cleaner and easier to purify from the bulk harvest.

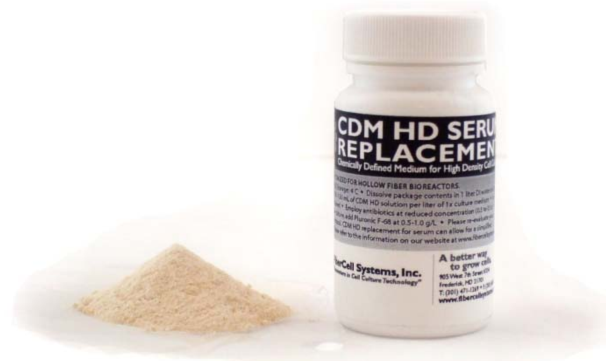


The MWCO of the fiber can be controlled. Desired products can be retained to significantly higher concentrations and the effects of cytokines can also be controlled. This is especially important for hybridoma culture in

which the inhibitory cytokine TGF beta can be selectively removed from the culture while the secreted antibody is retained.

Extremely high surface area to volume ratio. The small diameter of the fibers (200 μ) generates an extremely high surface area to volume ratio in the range of 100 - 200cm^2/ml of volume. Coupled with the high gross filtration rate of FiberCell® Systems polysulfone fibers the exchange of nutrients and waste products is very rapid. Cell densities of 1 - 2 X 10^8 or more are achieved, close to *in vivo* tissue-like densities. A 20 ml cartridge will support as many cells as a 2 L spinner flask or 20 - 40 roller bottles. High cell densities produce more protein per milliliter volume and also facilitate adaptation to lower serum concentrations or a simplified protein free serum replacement such as CDM-HD. The use of protein free mediums results in much cleaner harvests of products and simplified purification.

CDM-HD is a protein free, animal component free, chemically defined serum replacement that is optimized for cell culture at high densities, such as is found in HFBRs. It contains no surfactants, an excess of amino acids, significant amounts of free iron, an extra gram of glucose per liter and additional buffering capacity as part of its proprietary formulation. It is supplied as a dry powder and intended to be used as a serum replacement for most cell types.



The above features result in protein and antibody concentrations that can be 100 X higher than that found in flask or spinner culture with almost no contaminating proteins from either the cell culture medium or the cells themselves when used with CDM-HD or a serum free, protein free medium. The more *in vivo* like cell culture conditions can also result in improved protein folding and more uniform glycosylation patterns over time. Since it is a continuous perfusion system the amount of protein produced is determined as much by the length of time the culture is maintained as by such parameters as the clone's specific productivity or the size of the cartridge.

The combination of an unlimited nutrient supply and the ability to de-bulk the culture through the cartridge ports allows the system to be maintained at relative equilibrium for several months or longer. This continuous

production over long periods of time, rather than the severely batch-style results from other systems provide several benefits, including 1) consistency in culture condition, 2) dramatically increased production per unit footprint and culture volume, 3) continuous or daily product harvest allowing timely and convenient stabilizing treatment or storage conditions, and 4) products that might be toxic or inhibitory to cells can be selectively removed from the culture.

In summary a HFBR can be a practical solution for protein and antibody production in the range of 10 mg on up to gram quantities of protein. This is especially significant in cases where the general sentiment is that mammalian cell-based approaches to protein production are to be avoided due to their relatively low expression levels.

Production of Large Quantities of Monoclonal Antibody using the FiberCell® Systems Inc. Bioreactor

Case Study 1

The FiberCell® Systems Bioreactor allows the production of about 50 - 200 mg of antibody per month using the medium sized reactor (FiberCell® Systems catalog number C2011 or C5011). The advantage of this system is antibody production can be performed in chemically defined protein free medium resulting in extremely clean antibody product with little or no contamination from fetal bovine serum, culture medium proteins, intracellular proteins or from mouse ascities fluid. The product also is harvested in a concentrated form 500 - 3000 µg/ml and production of the antibody can be accomplished in a standard CO_2 incubator.

Equipment

FiberCell® Systems Duet	(Cat # P3202)
FiberCell® Systems Reservoir Cap (sterilized) *fits standard plastic media bottle*	(Cat# A1006)
Hyclone DMEM high glucose	(Cat# SH30022.02)
FiberCell® Systems CDM-HD	(Cat# CDM-HD-1)
FiberCell® Systems Medium Cartridge	(Cat# C2011)
Hyclone Antibiotic/antimycotic soln	(Cat# SV30079.01)
20 ml syringes	
Alcohol wipes	
70% ethanol spray bottle	
Centrifuge tubes	
Sterile pipettes	

Set Up and Cartridge Inoculation

Set up, cartridge pre-culture and cell inoculation per the FiberCell® Systems Quick Start Guide (www.fibercellsystems.com). Cells from 4 T75 flasks (approximately $1X10^8$ viable cells) were inoculated in DMEM +1% antibiotics +5% FBS in cell innoculum only (this helps to establish the hybridomas in the

cartridge. No further serum was used) Medium changes were performed when the glucose level reached 2 grams per liter or less. Cartridge was run until the glucose uptake rate reached 1 g/day or more (about 1 week) then the following schedule was followed.

Monday – Bottle change and supernatant harvest. Exchange DMEM bottle with a new 1L bottle (with antibiotics and CDM-HD). Gently tip the apparatus to one side. Draw all the media from cartridge into a 20 ml syringe and put the harvested media into a centrifuge tube. Replace the syringe with a new sterile syringe. Draw fresh media into the bioreactor using vacuum pressure. Aspirate back and forth.

Wednesday - Bottle change and supernatant harvest. Exchange DMEM bottle with a new 1L bottle (with antibiotics and CDM-HD). Gently tip the apparatus to one side. Draw all the media from cartridge into a 20 ml syringe and put the harvested media into a centrifuge tube. Replace the syringe with a new sterile syringe. Draw in fresh media using vacuum pressure. Aspirate back and forth.

Friday - Bottle change, cell and supernatant harvest. Exchange DMEM bottle with a new 1L bottle (with antibiotics and CDM-HD). Gently tip the apparatus to one side. Draw all the media from cartridge into a 20 ml syringe and put the harvested media into a centrifuge tube. Replace the syringe with a new sterile syringe. Draw in fresh media using vacuum pressure. Aspirate back and forth. Gently tip the apparatus to one side and draw all the media containing a huge number of cells from cartridge into a 20 ml syringe. Replace the syringe with a new sterile syringe. Draw in fresh media using vacuum pressure. Aspirate back and forth. Gently tip the apparatus to one side and draw all the media containing cells from cartridge into a 20 ml syringe. The number of time you want to do this depends on the growth characteristics of your cells, usually twice is sufficient to reduce cell numbers to allow the cartridge to go unattended over the weekend, while still getting a good yield of antibody on Monday.

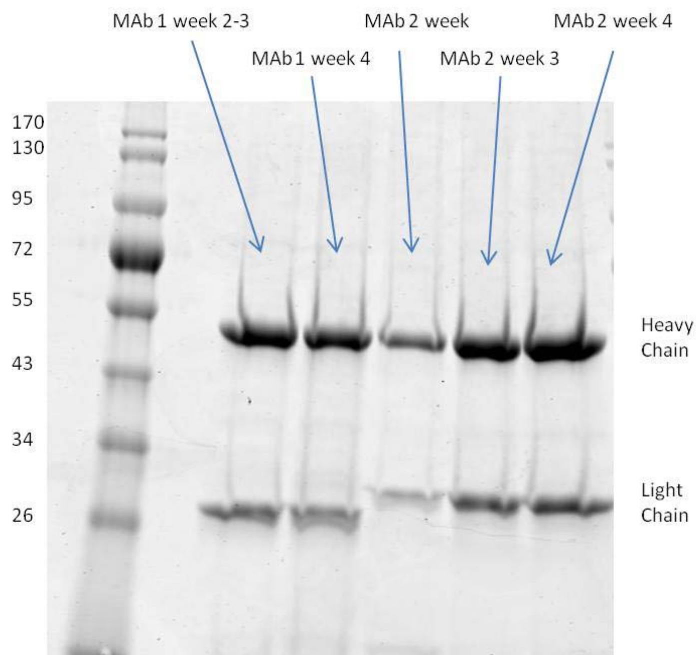
Supernatants – Spin at 1000 g to pellet cells, harvest supernatant, discard cell pellet. Pool supernatants from Monday, Wednesday and Friday harvests. Place into dialysis tubing and dialyze against 2 L PBS with 0.3M EDTA, repeat, then dialyze against PBS (or whatever buffer you wish). Yields are usually 12ml per harvest (36 ml week) at an antibody concentration of 1-3 mg/ml (total yield 36 - 108 mg/week) once the system is stabilized (usually day 14).

Cells - The supernatant and cells collected from the two washes on Friday does not contain significant antibody. However, you can place the cells pellet into sterile PBS and allow it to secrete overnight and you will get another 1 – 3 mg of antibody from those cells.

As an example, two mabs produced in DMEM + CDM-HD media were treated as above. The dialyzed supernatants had their protein concentration quantified and purity check on a 12 % SDS - PAGE gel.

Mab 1 – 2.6 mg/ml week two and three harvest	(40 ml)
Mab 1 – 3.2 mg/ml week four harvest	(20 ml)
Mab 2 – 0.8 mg/ml week two harvest	(25 ml)
Mab 2 – 3.2 mg/ml week three harvest	(20 ml)
Mab 2 – 3.0 mg/ml week four harvest	(25 ml)

Please note that the gel (below) represents the actual supernatant harvest that has simply been dialyzed. No purification has been performed; the antibody is harvested in a very clean state with little or no contaminating protein.



Data courtesy of Dr. Erin Bromage, U. Mass. Dartmouth

Case Study 2

Production of a rabbit monoclonal antibody utilizing the FiberCell® Systems C2011 cartridge. Medium used was RPMI/2%FBS/10% CDM-HD. Medium was changed every 3 - 4 days, when the glucose level was less than 50% of the starting glucose concentration or around 1.5 grams per liter. 16 mg of antibody in a volume of 140 ml of harvest. 6 L of medium were consumed.

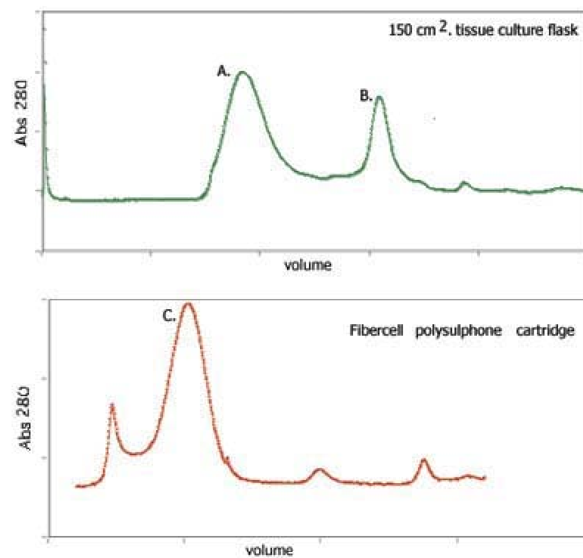
Day	[ng/ml]	[mg]
1	140581	2.11
2	213741	3.21
4	98588	1.48
7	149001	2.24
10	135789	2.04
14	265100	3.98
18	60841	0.91

Data courtesy of Tong Ming Fu and Daniel Freed, Merck and Co.

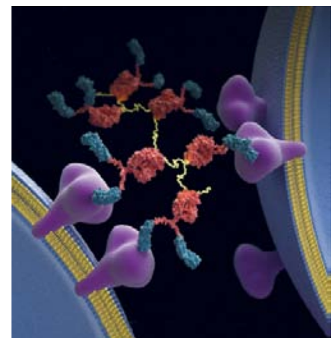
Production of Large Quantities of Recombinant Proteins from Mammalian Cells using the FiberCell® Systems Inc. Bioreactor

Case Study 1

473 mg of purified recombinant protein from a CHO cell line was harvested from the C2018 20kd MWCO cartridge. Medium was DMEM with 2% FBS, each harvest was 70 ml in volume, total harvest volume was 4.8 liters for an



average concentration of approximately 100 µ/ml/day. The protein was a very complex hexamerized IgG consisting of 6 IgG1 subunits held together with three IgA tails. The Fv region was modified to contain CD4 receptor. (2) The cartridge consumed an average of 2 liters of medium per day over a 60 day period of production. The protein is pictured below.



Most interesting was the comparison of protein produced using T-flasks vs. the hollow fiber cartridge. When produced in flasks approximately 40% of the protein was secreted as an un-folded monomeric subunit. Placing the

exact same cells into the hollow fiber cartridge resulted in nearly 95% of the protein being produced as a properly folded hexamer. Better cell culture conditions resulted in better protein expression fidelity.

Case Study 2

276 mg of purified recombinant IgG1 from a 293T cell line was harvested from the C2011 20kd MWCO cartridge. Medium was a serum free formulation similar to CDM-HD. Each harvest was 20 ml in volume; total harvest volume was 900 ml for an average concentration of nearly 300 µg/day/ml. The cartridge consumed 1 liter of medium every two days and the culture was maintained for a total of 50 days. The expressed protein was very clean and required minimal purification.

Conclusion

HFBRs from FiberCell® Systems represent the ideal method for the production of milligram to gram quantities of monoclonal antibodies and recombinant proteins. The harvested product is concentrated and free of contaminating proteins, DNA, RNA and proteases. The use of CDM-HD renders the medium used both economical and chemically defined/protein free. The harvested product is concentrated and free of contaminating proteins, DNA, RNA and proteases. Cultures can be maintained for long periods of time meaning that scalability of the system is determined by length of culture, not new equipment.

These advantages of these same characteristics i.e. increased concentration of secreted products and freedom from exogenous proteins also apply to secretome analysis from cancer cells for the study of biomarkers (3), protein production from S2 cells, the generation of conditioned medium for stem cell culture support and primary stem cell culture. Primary human placental derived mesenchymal stem cells have been supported for over 3 months of culture (FiberCell® Systems, data not shown.) Features particularly supporting stem cell culture are the high culture density, low turbulence, low shear, constancy of the nutritional environment, and the fact that matrix materials such as hydrogels may be infused within the cartridge during culture. Other advantages here are that the surface of the fibers may be functionalized in many different ways, with receptor-specific ligands such as antibodies to specifically select particular cell types or extracellular matrix factors to provide a more in *vivo* like surface for cell attachment.

While improvements in HFBR materials are inspiring a renewed interest in their application in such established demands as the production of protein biologicals, advancements in biotechnology are creating a need for some of the unique features they provide. Currently available HFBRs from FiberCell®

Systems offer the advantages from their use to any laboratory and are the method of choice for the production of larger quantities of monoclonal antibodies and recombinant proteins. The advancement of higher capacity HFBRs to a production scale unit would represent a potential paradigm shift in recombinant protein bio-manufacturing.

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