3-D *in vitro* Models using Hollow Fiber Bioreactors

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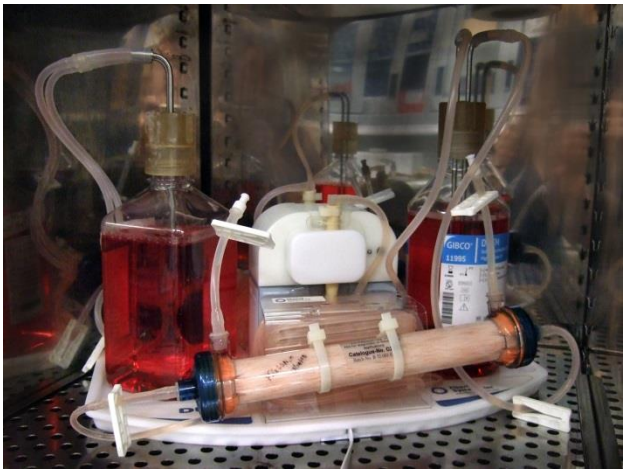
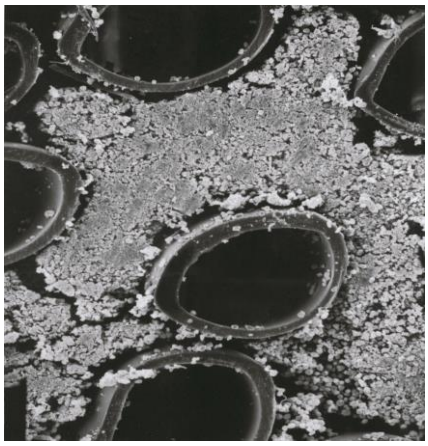


Historically, the scientific method had been based upon the reduction of complex systems to their simplest forms in order to understand their individual processes. In biology, a whole animal will be reduced to its constitutive organs, these organs to individual tissues, and then individual areas to single cell types. The isolation of a single cell type and the use of artificially created immortalized cell lines or disaggregated primary cells, and artificial environments for them are the foundation of modern cell culture.

Sixty years of culturing individual cell types on 2-dimensional glass or plastic surfaces using fetal bovine serum have shown it to be a powerful technique for studying cellular functions. However, cells do not grow on plastic in nature, are only exposed to serum in an activated state, and cell-matrix and cell-to-cell interactions not found in flasks play a key role in generating the 3-D biological organization we call multicellular "life."



Current cell based assays and 2-D *in vitro* models are a useful, time and cost-effective tool for drug discovery and basic cell biology. Static cell-based assays in plates, flasks or other 2-dimensional formats do not always completely mimic *in vivo* behavior. Hollow fiber bioreactors can recreate the *in vivo* circulatory system geometry and culture cells in a 3-D, physiologically relevant manner. They have been used to recreate many tissue/cell specific structures in a manner that allows data to be collected in meaningful ways.

EXAMPLES OF HF-BASED CELL ASSAYS

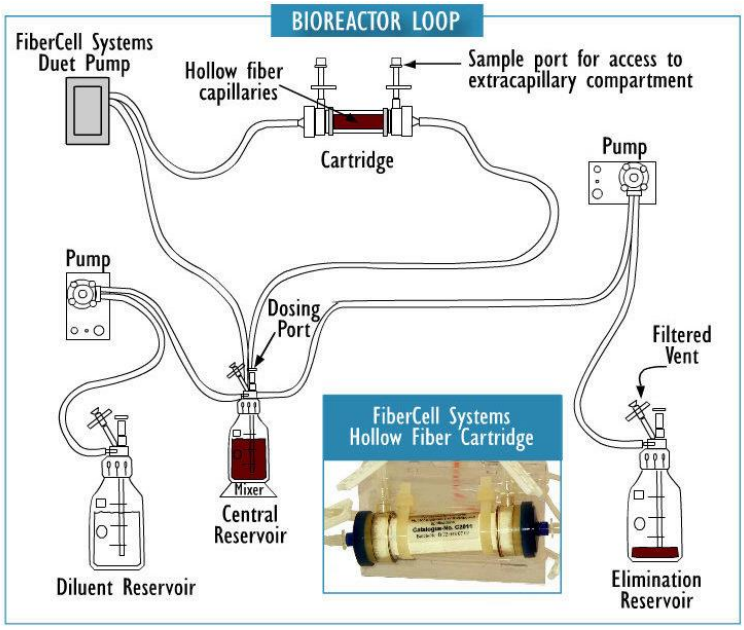
HF based assays are inherently more complex and costlier to design and set up than conventional cell-based assays. However, these assays can generate data that is not available in any other manner and can bridge an important gap between animal

studies and human clinical trials. Large numbers of cells can be assayed and over a long period of time. Drug concentrations can be controlled in a dynamic fashion and both adsorption and elimination curves can be modeled. Multiple tests can be performed on the same cell population. Three dimensional cultures of multiple cell types can model complex processes such as virus infections in tissues, hematopoiesis, cancer cell propagation, cancer cell metastasis, the blood brain barrier and parasitic infections. A single cell type or a more complex co-cultivation model using two or more cell types can be used to recapitulate *in vivo* structures and physiology.

ASSAYS USING ONLY ONE CELL TYPE

The simplest type of 3-dimensional models using hollow fiber bioreactors (HFBR) consist of only one cell type, cultured in the ECS. HFBRs are the only method that can support cells at physiologic cell densities and demonstrate *in vivo* like cell physiology. Both adherent and suspension cells can be cultured in a HFBR. Adherent cells bound to a porous support do not require periodic splitting and can be maintained for extended periods of time. Suspension cells can also be supported for extended periods of time due to constant feeding and removal of metabolites.

The ability to add and remove drug is particularly important for dose fractionation pharmacodynamic studies where drugs are added to the system and then removed by dilution with drug free medium without disturbing the cells. Both drug absorption and elimination can be simulated. The small volume of the ECS and the high surface area ensures rapid equilibration of drug between the circulating medium and ECS of the cartridge. Viruses and virus infected cells are retained in the small volume of the extra-capillary space and cannot cross the fibers into the medium. The system is completely closed and provides an added biosafety component protecting laboratory personnel from exposure.



ANTIVIRAL PHARMACODYNAMICS

HFBR supports the establishment of a pharmacodynamic index (dose and schedule) for anti-viral agents. Drug can be administered in a time-dependent and concentration dependent manner in order to closely replicate human pharmacokinetics and pharmacodynamics.

HIV

The nucleoside analogue, 2', 3'-didehydro-3'deoxythymidine (d4T) was examined using hollow fiber infection models (HFIM) (Drusano et al, 2002). The HFIM system predicted that the minimum effective dose of d4T to treat patients infected with HIV was approximately 0.5 mg/kg/day administered twice a day.

INFLUENZA VIRUS

The current recommendation for treatment of influenza with oseltamivir is to take two 75 mg tablets twice a day. Researchers employed hollow fiber-based models to performed dose range and dose fractionation experiments in MDCK cells (McSharry et al, 2009b). The data showed that in the absence of drug the virus grew well in the HFIM system and that the pharmacodynamically-linked index for oseltamivir for the R292 strain of influenza A virus is the AUC/EC50/95 ratio. This means that the model indicates that at the appropriate dose, oseltamivir could be given once a day. The demonstration that adherent cells can be used to grow virus in the HFIM system opens this system up to the pharmacodynamic analysis of antiviral compounds for a wide variety of viruses (Brown et al, 2011).

ANTICANCER AGENTS

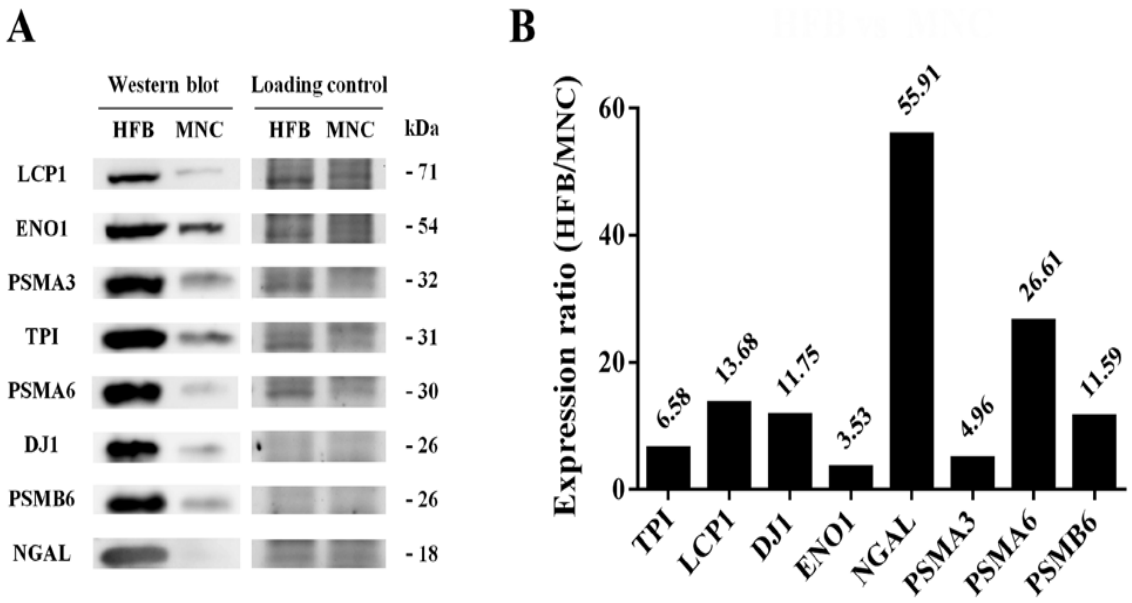
Another example of a 3-D cell-based assay using only one cell type in the ECS of the hollow fiber cartridge is for the analysis and characterization of anticancer agents. Anti-cancer agents also exhibit both time and concentration dependent efficacy.

Mark Kirstein reported on the use of the hollow fiber model for anticancer drug evaluations. In this case gemcitabine was examined in the anchorage dependent MDA-MB-231 breast cancer cell line (Kirstein et al, 2006). More accurate results are obtained for a few reasons. One, because the multi -layer, 3-dimensional organization of these continually perfused cells more closely reflects the in vivo structure of the tumor, they have an increased relevance for assays of anti-cancer agents. Another is that the cell division rates in static cultures are artificially high, and this can render them more sensitive to some chemotherapeutic agents then their natural counterpart (Kirstein et al, 2008).

CANCER BIOMARKERS

Circulating cancer biomarkers represent an important area for cancer diagnostics as well as indicators for monitoring the efficacy of therapy. 2-D, monolayer culture of primary cancer cells, especially in the presence of serum cannot usefully identify potential biomarkers secreted in low amounts compared to the protein concentration of serum. Cholangiocarcinoma, derived from the bile duct, occurs at a high incidence in Northeast Thailand. Early diagnosis is hampered by the lack of reliable circulating

biomarkers. Srisomsap et al (International Journal of Oncology, 2017) cultured primary cancer cells in a 5kd MWCO hollow fiber bioreactor using a protein free medium and compared the secretome with cells cultured in 2-D flasks that were expanded in the presence of serum and collected the secretome from a 24-hour collection using basal medium without serum. The high cell density of the HFBR allows cells to easily adapt to the protein free medium. The HFBR would also concentrate secreted proteins making identification easier. The authors also found a significant reduction in cell lysis in the HFBR. More of the protein was specifically secreted by the cells, and not released intracellular protein. Analysis showed a significant difference in the protein secretion profile between the two culture methods. If the secretome were merely being concentrated by the HFBR then the levels of all proteins would be the same. However, one protein in particular, NGAL, was shown to be 57-fold higher from the HFBR when compared to flask culture. This protein was found to be elevated in patients with cholangiocarcinoma and it being developed as a clinical biomarker for this cancer.



ASSAYS USING MORE THAN ONE CELL TYPE

HF cell culture permits the recapitulation of natural structures containing more than one cell type in a defined, controlled, and more biomimetic environment. There are two types of cell co-cultivations that can be performed in a hollow fiber bioreactor. They are, one or more cell types:

1. on both the inside and on the outside of the fibers (Davis, 2007)
2. on only one side of the fibers (typically on the ECS) (FCS Application, 2012)

3-D MODELS WITH ENDOTHELIAL CELLS

3-Dimensional cell-based assays utilizing co-culture of multiple cell types in hollow fiber bioreactors can recapitulate structure of higher complexity than those with a single cell type. Asymetric cellular co-cultivation is the use of HFBRs to culture endothelial cells on the insides of the fibers and culturing a different cell type on the outside (see Figure 3). Endothelial cells comprise the inner lining of all blood vessels. The endothelium should be considered a discrete organ, rather than a simple cell type. When cultured on the insides of a hollow fiber (the intracapillary space) and exposed to shear stress from medium flow, endothelial cells behave much differently (more *in vivo*-like) than they do in static, flask culture. Under shear, endothelial cells form monolayer, stop dividing, form tight junctions, and express more genes than when cultured under static conditions in flasks. Differences in shear stress throughout the body may explain different functions of endothelial cells in those areas.

In one study, bovine aortic endothelial cells were cultured on the insides of the fibers, and vascular smooth muscle on the outsides of the fibers in the ECS. Changing the flow rate on the endothelial cells changed the shear stress they were exposed to. Changes in shear stress modulated g-protein formation and endothelin receptor expression in the vascular smooth muscle, even though there was no physical contact between the two cells types (Redmond, Cahill and Sitzman, 1995).

BLOOD/BRAIN BARRIER MODEL

There are many *in vitro* approaches to the modeling BBB physical and biochemical behavior, but most fail to represent its natural three-dimensional nature, and do not support the associated exposure of endothelial cells to shear as *in vivo*. To answer this challenge, Janigro developed a new, dynamic, *in vitro* BBB model (NDIV-BBB) designed to allow for extensive pharmacological, morphological and physiological studies (Stanness et al, 1999). This dynamic HF-based model of the BBB allows for longitudinal studies of the effects of flow and co-culture in a controlled environment. Brain microvascular endothelial cells are seeded onto the insides of the fibers, where they are subjected to physiological shear stress, and astroglial cells are seeded onto the outsides of the fibers.

The DIV-BBB allows reproduction of multiple functional properties and physiological responses observed at the BBB in situ, particularly drug transport and TEER (trans-endothelial electrical resistance. Furthermore it allows for the inclusion of other relevant cell types, including pericytes and neurons.

However, its design does not allow for direct visualization of the intraluminal compartment to assess morphological and/or phenotypic changes of the endothelium. In contrast to conventional static BBB models (e.g., Transwell), this model is not designed for a high-throughput pharmaceutical study, and the technical skills/time required to establish the system is significantly higher.

In perhaps the ultimate embodiment of state-of-the-art hollow fiber based cell assays, Dr Chris Pepper et al have developed a model for primary investigations and assaying for cancer metastasis (Walsby et al, 2014. A dynamic *in vitro* model was developed in which circulating leukemic lymphocytes (CLL) cells experience shear forces equivalent to those in capillary beds and are flowed through hollow fibers lined with endothelial cells. CLL cells treated in this way increased their expression of CD62L, CXCR4, CD49d and CD5 and migrated through the endothelium into the extra capillary space. The degree of migration observed strongly correlated with CD49d expression and treatment with the CD49d blocking antibody. Taken together these data provide evidence for a novel, dynamic and reproducible *in vitro* model of lymphocyte migration and cancer metastasis.

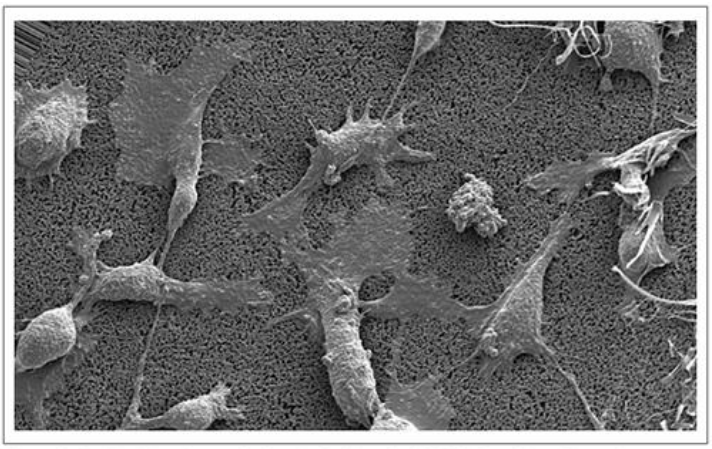
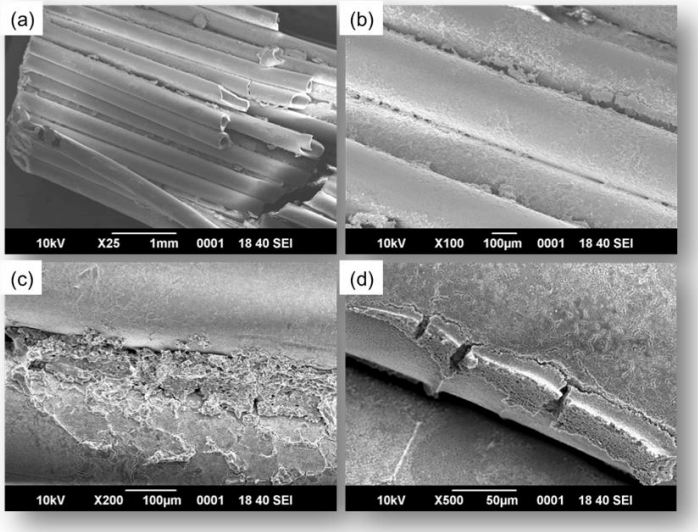


Figure 4: Endothelial cells attached to the inside of a PVDF hollow fiber without shear stress. Note the round shape of the cells.



BONE MARROW MODEL

Another example of cell co-cultivation utilizes stromal cell/suspension cell interactions on the same side of the fibers, in the ECS. The suspension cells can be sampled over time to collect data and more vigorous harvesting conditions can also provide samples of the stromal cells. A low molecular weight cut-off fiber, 5 kd, can be used to concentrate secreted factors to enhance cell-to-cell interactions. Dr. Mayasari Lim at University Hospital, Hong Kong reports on the co-cultivation of a human bone marrow stromal cell line with a leukemic T cell line (Usuludin, Cao and Lim, 2012). When cultured in the HF cartridge the T cells underwent a 4000-fold expansion, in line with what occurs *in vivo*.

A second bone marrow model was established to recreate the bone marrow microenvironment for hematopoiesis. A human stromal cell line (HS-5) was employed as a co-cultured stromal support of lineage-cell depleted human cord

blood cells (Xue et al, 2014).

Results showed that the performance of the HFBR in supporting total cell and CD34+ progenitor cell expansion was comparable to that of 2-D cultures, while cells harvested from the HFBR had a higher clonogenic ability and engrafted more rapidly into nude mice. The findings demonstrate the feasibility of utilizing an HFBR for creating a complex cell matrix architecture, which provides *in vitro* mimicry of the bone marrow supporting large-scale expansion of HSCs.

3-DIMENSIONAL PLACENTA CO-CULTIVATION MODEL

Current protocols for the collection of mesenchymal stem cells from placenta entail a perfusion step to remove cord blood followed by a digestion with collagenase or other enzyme. Typically, these cells are then placed into T flasks for culture over a period of several days. During this time the medium is changed on a regular basis. The medium changes aid in flushing out the non-adherent cells and debris and removes them from the plastic adherent cells. The adherent cells are mesenchymal stem cells derived from the placenta.

Instead of flasks a hollow fiber bioreactor was used to recreate an *in vitro* placenta and collect potential stem cells in a continuous process.

The umbilical vein of a normal, fresh, human placenta was cannulated, and a solution of PBS was perfused for one hour. Blood forming hematopoietic stem cells and other components were collected from the perfusate. This also reduced the red cell burden. After one hour at room temperature the solution was changed to PBS containing one gram per liter of collagenase (Worthington Biochemicals, Lakewood, New Jersey). This was perfused for 2 hours at room temperature. The placenta was digitally disaggregated, and the cells were collected, washed once in cell culture medium and then the red cells removed using ACK lysing buffer. Cells were washed once more in cell culture medium. $5x10^8$ cells were seeded into a 5kd MWCO polysulfone cartridge and the culture initiated with 100 mL of DMEM/F12 + 10% FBS and 2% pen/strep at a flow rate of 60 mL/minute.

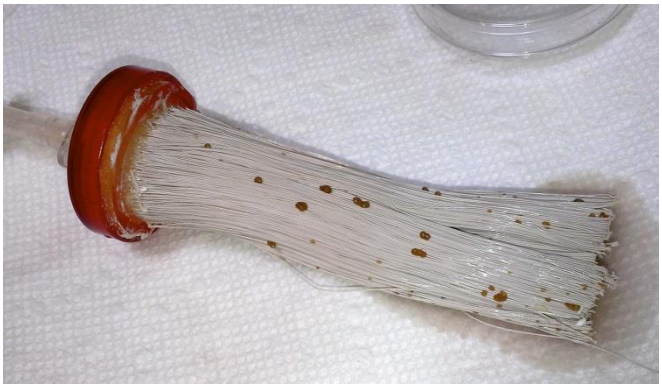
On day 2, day 3 and day 5 the ECS (extracapillary space) was washed with cell culture medium to remove any non-adherent cells. Starting with day 10 the ECS was harvested and plated into 6 well dishes twice a week. During this time red nodules formed on the surface of the fibers, and a new population of suspension cells was collected from the ECS and analyzed by flow cytometry. The cartridge remained viable for over three months of continuous culture. The culture was terminated at that time.

The suspension cells collected from the 3-D environment were phenotypically undefined, showing only a maximum staining of CD105 of 43% (both an endothelial and MSC marker). However, when these cells were plated in T75 flasks with 10% FBS

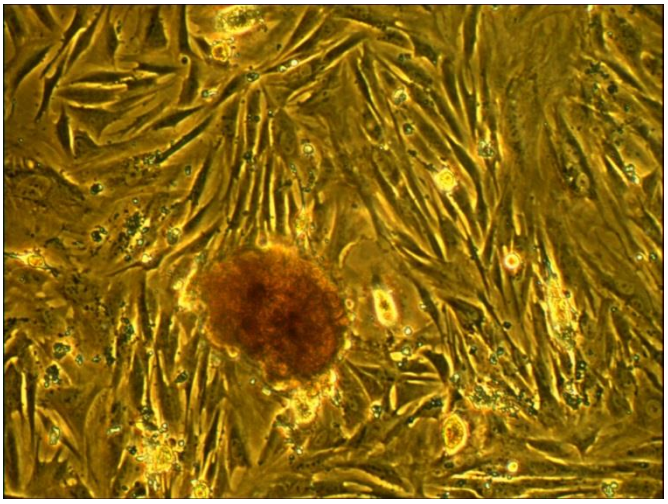
the attached and divided and had the appearance of MSC, complete with erythroid bodies. Flow cytometry showed these former suspension cells were now 99% MSC positive. This demonstrates the profound effect 3-D culture can have on cell phenotype and physiology.

Phenotype	ECS Harvest	Flask
CD 45	4%	1%
CD 34	0%	0%
CD 133/2	2%	0%
CD 31	3%	48%
CD 13	6%	83%
CD 105	43%	99%
CD 73	18%	99%
CD 90	5%	96%
CD 14	23%	4%
NANOG	0%	0%

CRYPTOSPORIDIUM CULTURE



Diarrheal disease is a leading cause of pediatric death in economically low resource countries. Cryptosporidium spp. are the second largest member of this group and the only member for which no treatment exists. One of the handicaps to developing a therapy is the lack of a reproducible, long- term culture method allowing *in vitro* drug screening beyond 48 h. Hollow fiber bioreactors can provide an environment that mimics the gut by delivering nutrients and oxygen from the basal layer upwards while allowing separate redox and nutrient control of the ECS for parasite development. Using this technique, oocyst production was maintained for >6 months, producing approximately $1x10^{8}$ oocysts mL^{1} day^{1}, compared with 48 h with a yield of $1X10^{6}$ - oocysts mL^{1} in two-dimensional cultures. Oocysts, after 4 and 20 weeks in culture, produced a chronic infection in a TCR-a-deficient mouse model. In vivo infectivity of



oocysts was confirmed using oocysts from a 6-week culture in a dexamethasone immunosuppressed mouse model.

The presence of two distinct compartments, ECS and ICS create a high oxygen side and a low oxygen side. Host cells are supported with oxygen and nutrients from the basal layer, while a low oxygen, nutrient rich environment develops in the ECS where the parasite is present, thus mimicking the situation in situ. The development of such a system provides the added advantage of allowing the development of a parasite-specific growth medium, which is not possible in current two-dimensional (2D) cultures where emphasis is directed to maintaining host cell growth to support the parasite burden. Drug can be administered in a time-course dependent manner mimicking human dosage profiles.

HFBRs provide several unique features:

1. A large surface area for metabolite and gas exchange, which are needed for efficient growth of host cells
2. A biphasic environment providing an oxygen rich nutrient supply to the basal layer of the host cells, while creating an anaerobic nutrient rich supply to the apical side mimicking the gut.
3. High numbers of *in vitro* cultured C. parvum oocysts for biochemical and molecular studies.
4. Study of the host-parasite relationship in a long-term *in vitro* infection.
5. Produce *in vitro* preclinical pharmacokinetic data, providing a unique method for drug selection.
6. Can be used for analysis and preparative isolation of Cryptosporidium growth factors.

SUMMARY

Hollow fiber cell culture uniquely provides a three-dimensional biomimetic cell culture environment. Recapitulation of the in vivo microenvironment can recreate tissue-like structures and support co-cultivation of two or more cell types. The resulting structures permit the culture of cells and organisms not supported in 2-D flask culture. The cell numbers are high enough to enable elucidation of receptor pathways and cell signaling. Long term cultures allow for cell to cell interactions to fully develop, and to expose the cells and other organisms to physiological time course dependent drug

dosage profiles. 3-D cultures in hollow fiber bioreactors represent a next step in *in vitro* tissue modelling and permits complex, in vivo models otherwise not possible.

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