“2-D, 3-D and Histocentric Bioreactors, Recapitulating the *in vivo* Microenvironment”

FiberCell Systems
A BETTER WAY TO GROW CELLS

3-D Hollow Fiber Bioreactors:
“A better way to grow cells”

By John J. S. Cadwell

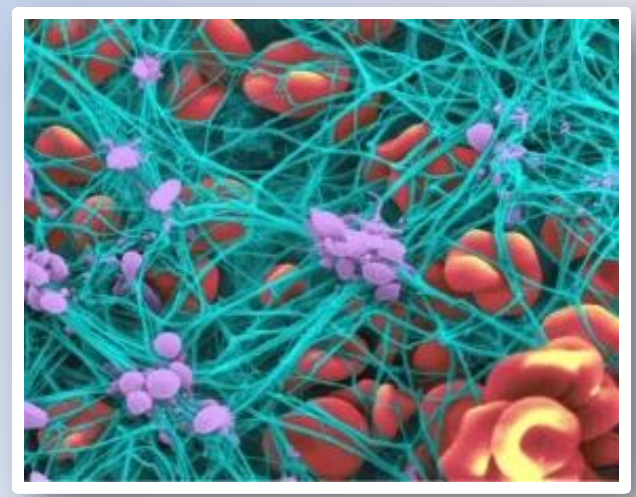
Milestones in Cell Culture

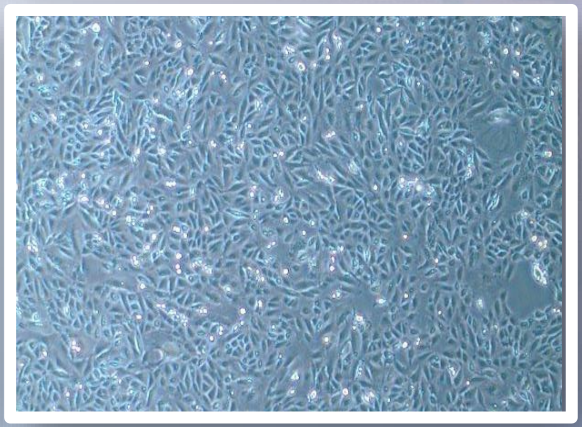
* 1665 Hooke first observes “cells”
* 1876 Charles Chamberland invents autoclave (Pasteur)
* 1907 Harrison frog neuronal explants on plasma clots
* 1935 Carrel and Lindberg develop Pyrex for organ perfusion pump
* 1951 HELA Cells (roller tube culture)
* Late 50’s Tissue dissociation enzymes from Worthington
* 1954 Connaught Labs makes polio vaccine in 5 liter Pyrex flasks
* 1958 Theordore Puck first uses fetal bovine serum
* Mid 1970’s Plastic is now more common than glass for cell culture
* Early 1970’s Laminar flow hoods
* 1978 Milstein Hybridoma Nobel Prize

Science October 1972 Hollow Fiber Bioreactors Richard Knazek

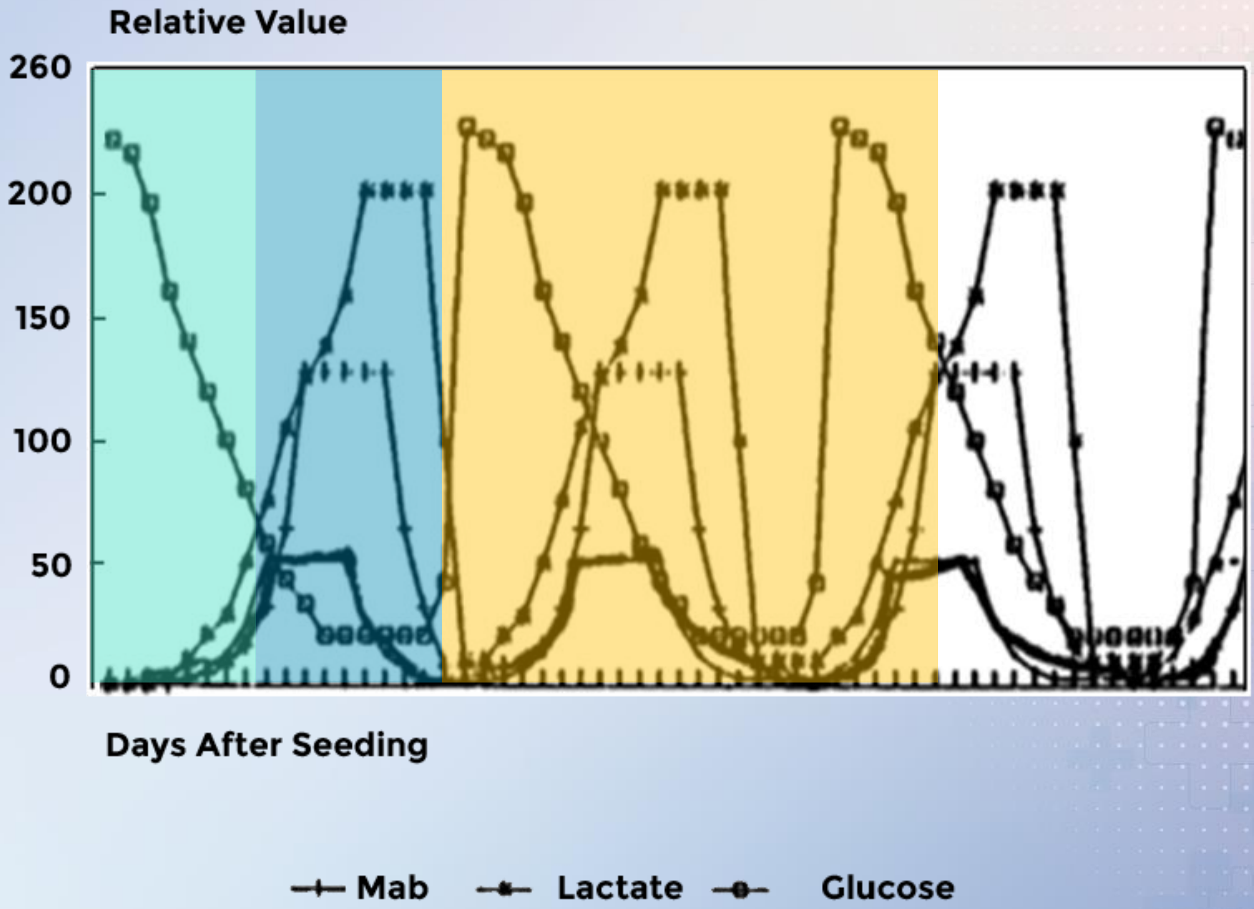

TIME
The Weekly Newsmagazine

Cell Culture Through the Ages





"Feast or Famine"

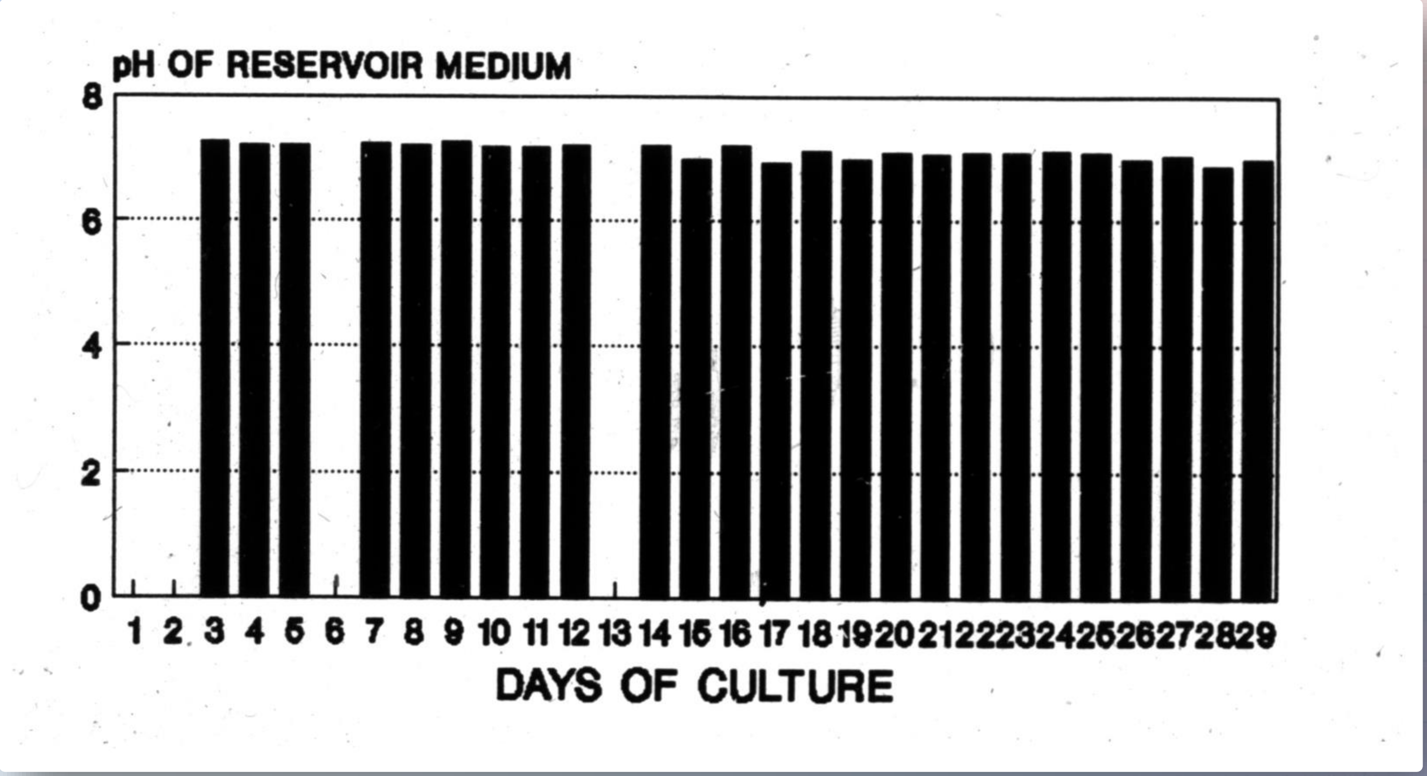


Cell Culture Options for Scale-up

- Roller Bottles
- Cell Factory
- Cell Cube
- Cell Culture Bags
- Spinner Flasks
- Bioreactors
- Microcarriers

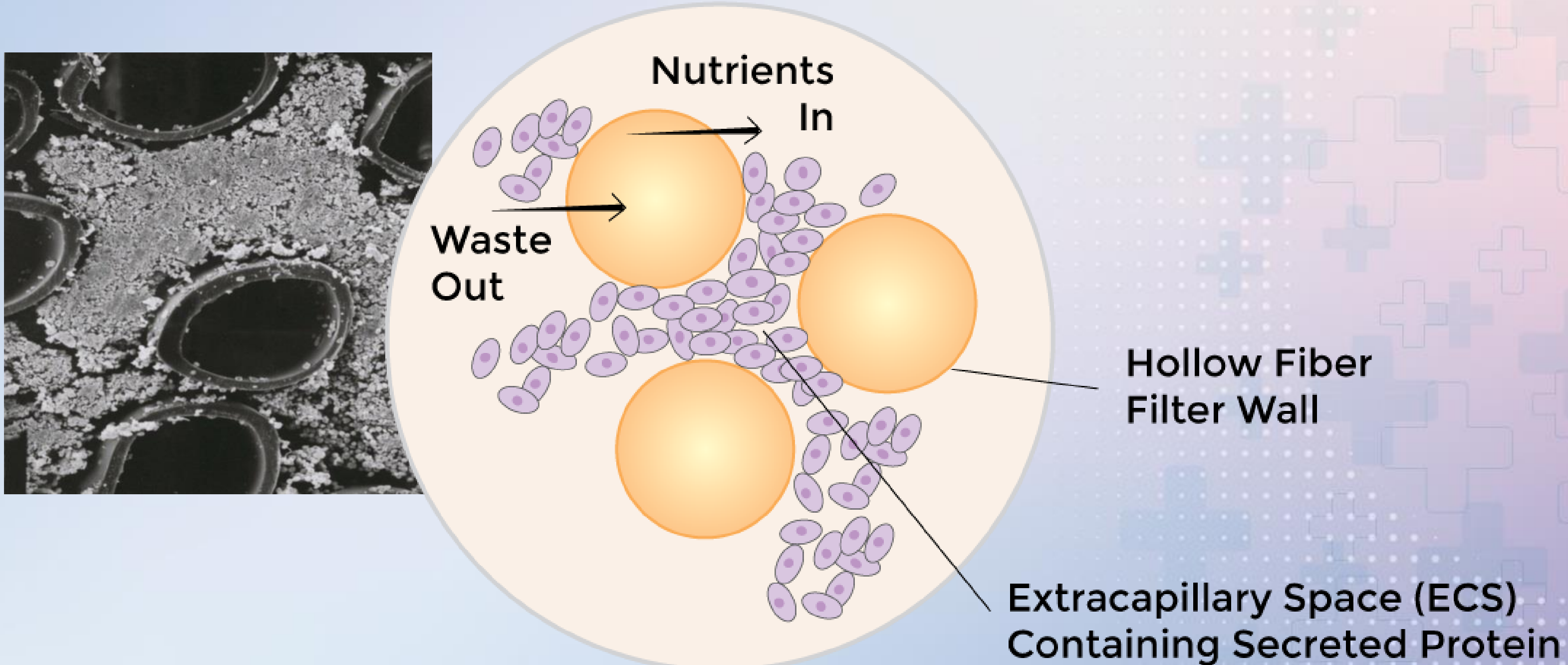
- Check the patents!

Hollow Fiber Culture of CHO Cells, pH Changes



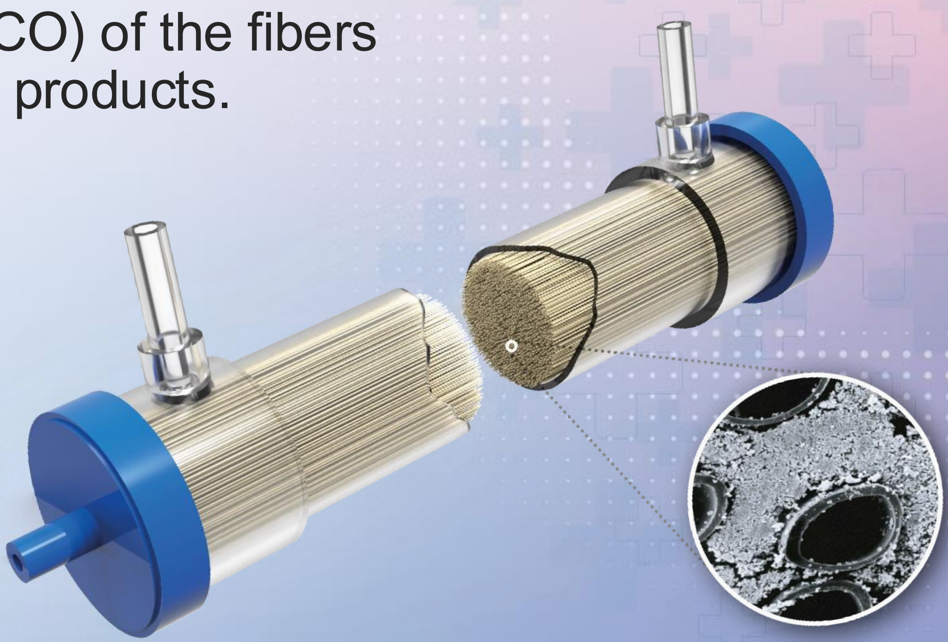
Hollow Fiber: How it Works

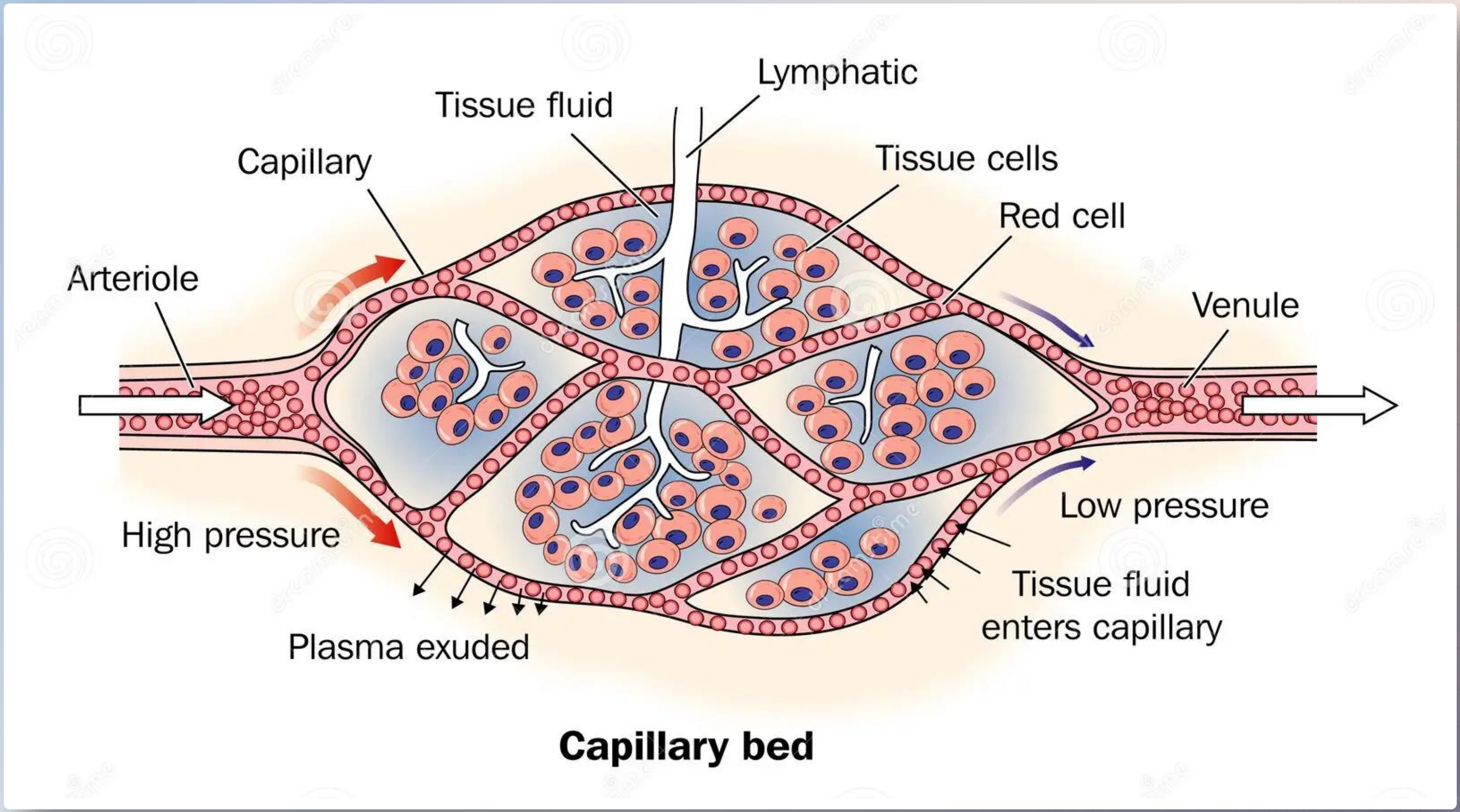
Hollow fiber was 3-D before 3-D was cool!



HFBR are Fundamentally Different in 3 Ways

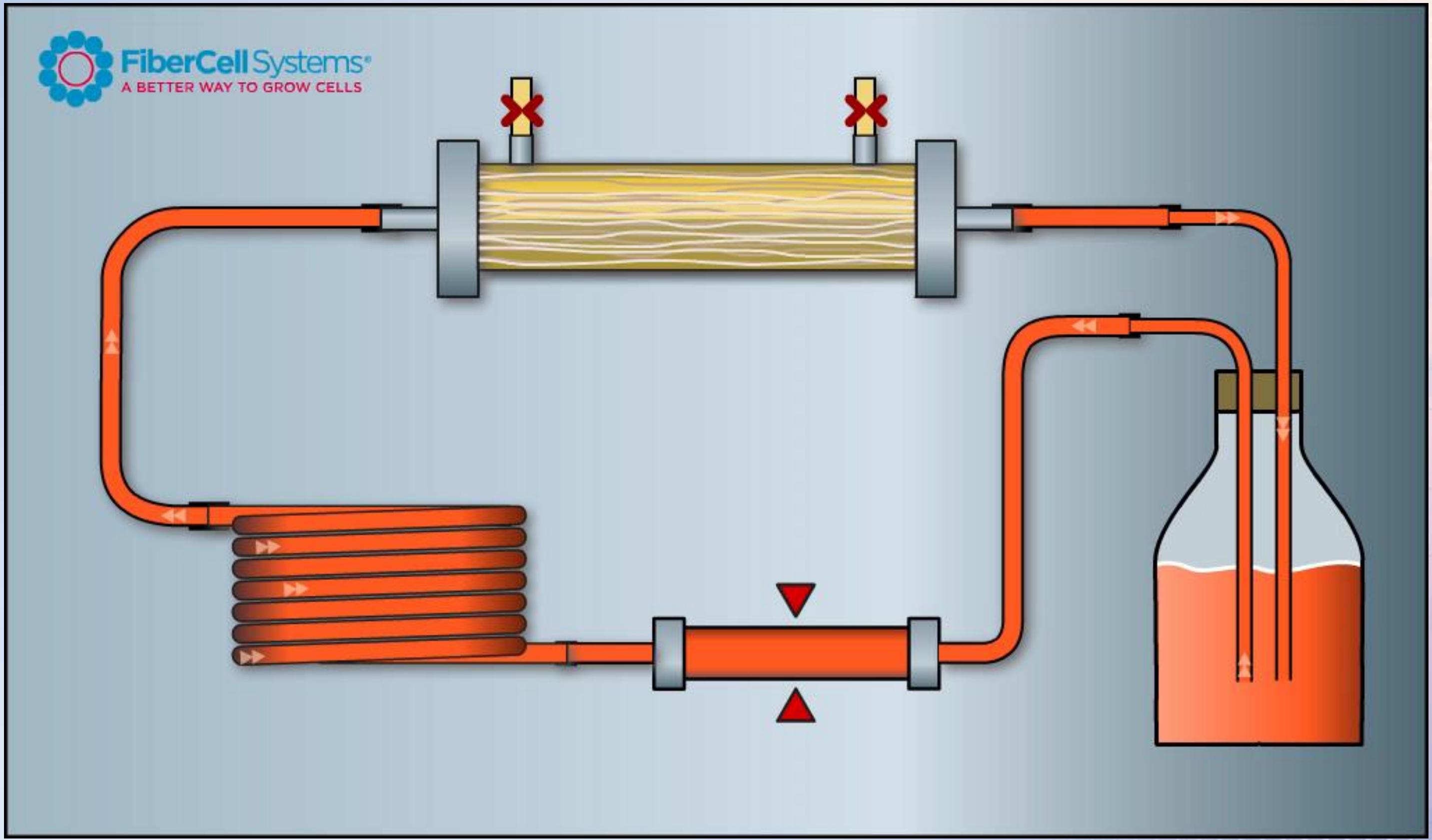
1. Extremely high surface area/volume permits high density cell culture.
2. Cells are bound to a porous support, not a non-porous 2-D flask.
3. The molecular weight cut off (MWCO) of the fibers retains and concentrates secreted products.





Histocentric Bioreactor: attempts to recapitulate the *in-vivo* microenvironment.

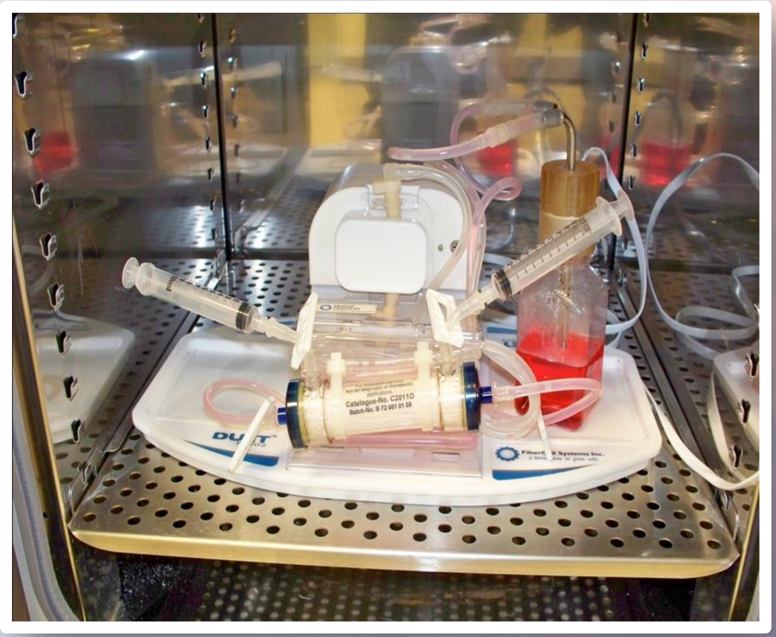
How many different medias are there in the human body?



- Positive pressure displacement pump
- Silicone tubing for gas exchange
- Closed, bio-safe system

In the Laboratory

- Fits in any standard sized incubator
- Gas controlled by incubator
- Temperature controlled by incubator
- Thin cord for power



Not very physiologic!

Glucose in Cell Culture: The Warburg Effect

The Warburg effect, also known as aerobic glycolysis, is a metabolic phenomenon observed in cancer cells where they convert glucose into lactate even in the presence of oxygen. (primary vs. transformed cells)

* Physiologic glucose: 80-90 mg/dl (.8 to .9 grams per liter)

- Cell culture medium 1-5 grams per liter
- Glucose has 4 calories per gram
- 100 kg person, 2,000 calories or 500 grams of glucose per day
- 5 grams per kilo of tissue
- Bioreactors for transformed cells consume 1 gram of glucose per $1X10^9$ cells (1 gram) per day

2-D vs. 3-D Cell Culture

2D Cell Culture:
Environment: Cells are grown on flat surfaces (e.g., Petri dishes), forming a monolayer.
Cell Interactions: Limited cell-cell and cell-matrix interactions, which can lead to altered cell behavior.
Morphology: Cells appear flat and stretched, often not reflecting their natural shape.
Gene Expression: Often differs from *in vivo* conditions, leading to less accurate biological responses.

3D Cell Culture:
Environment: Cells grow in a three-dimensional space, allowing for more natural interactions.
Cell Interactions: Enhanced cell-cell and cell-matrix interactions, mimicking *in vivo* conditions more closely.
Morphology: Cells can form spheroids or organoids, resembling natural tissue structures.
Gene Expression: More similar to *in vivo* models, providing better insights into cellular functions and drug responses.

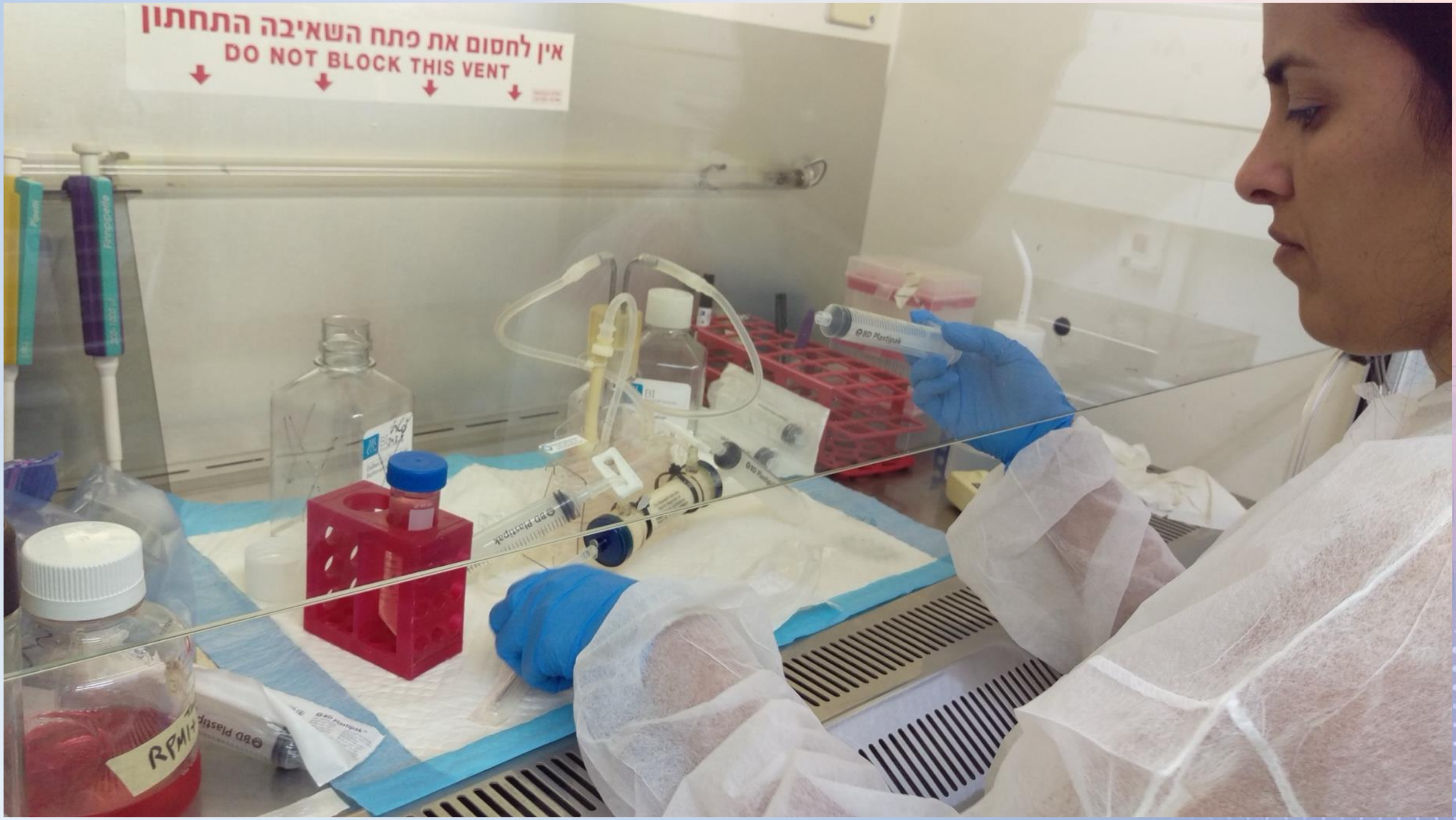
Types of 3-D cultures

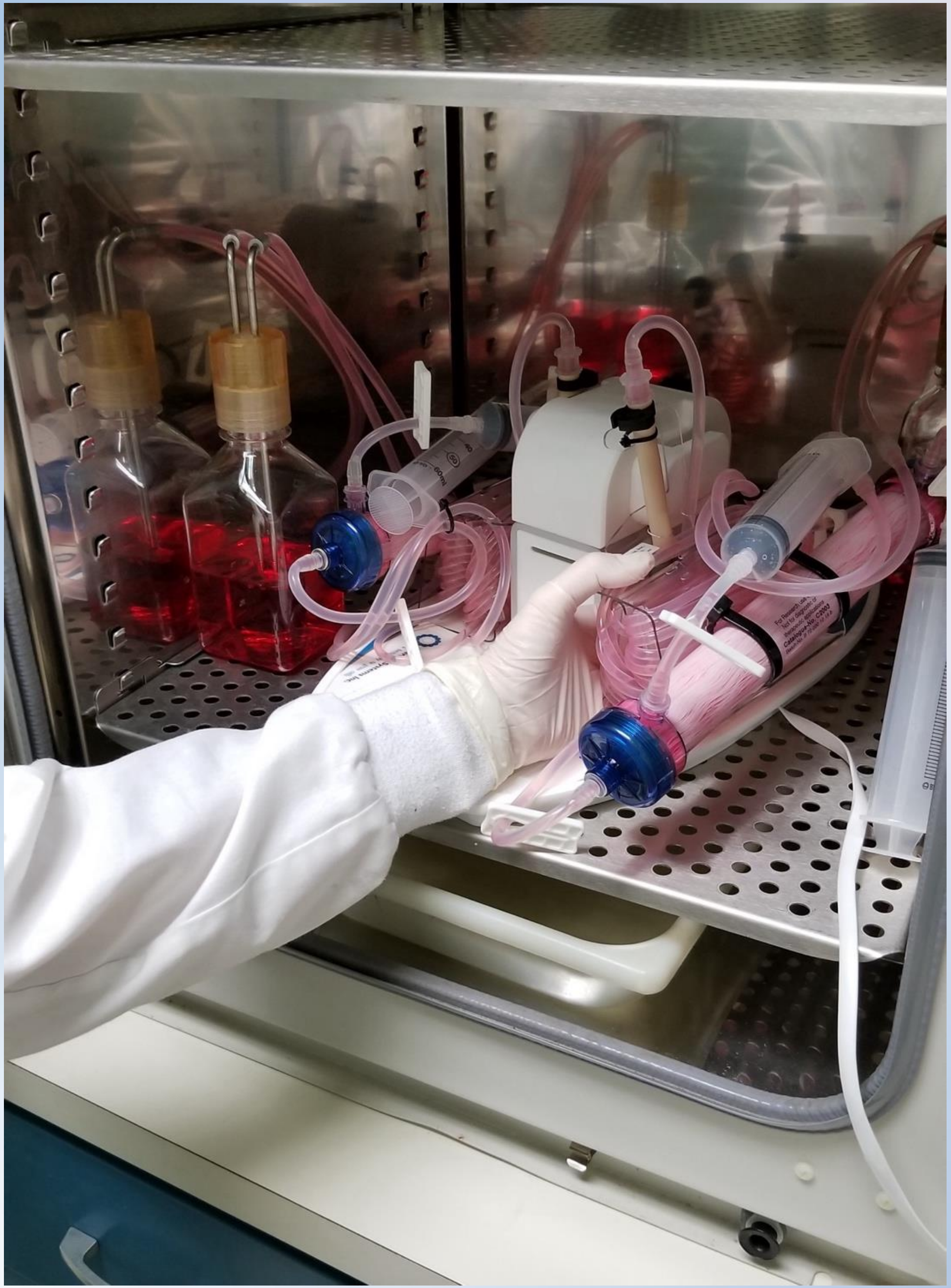
- Spheroids (one cell type, no scaffold)
- Organoids (multiple cell types, self-organize, scaffold)
- Bio-Printed Tissues
- Hydrogels
- Organ on a chip
- Hanging drop
- Transwell dishes
- Hollow fiber bioreactors

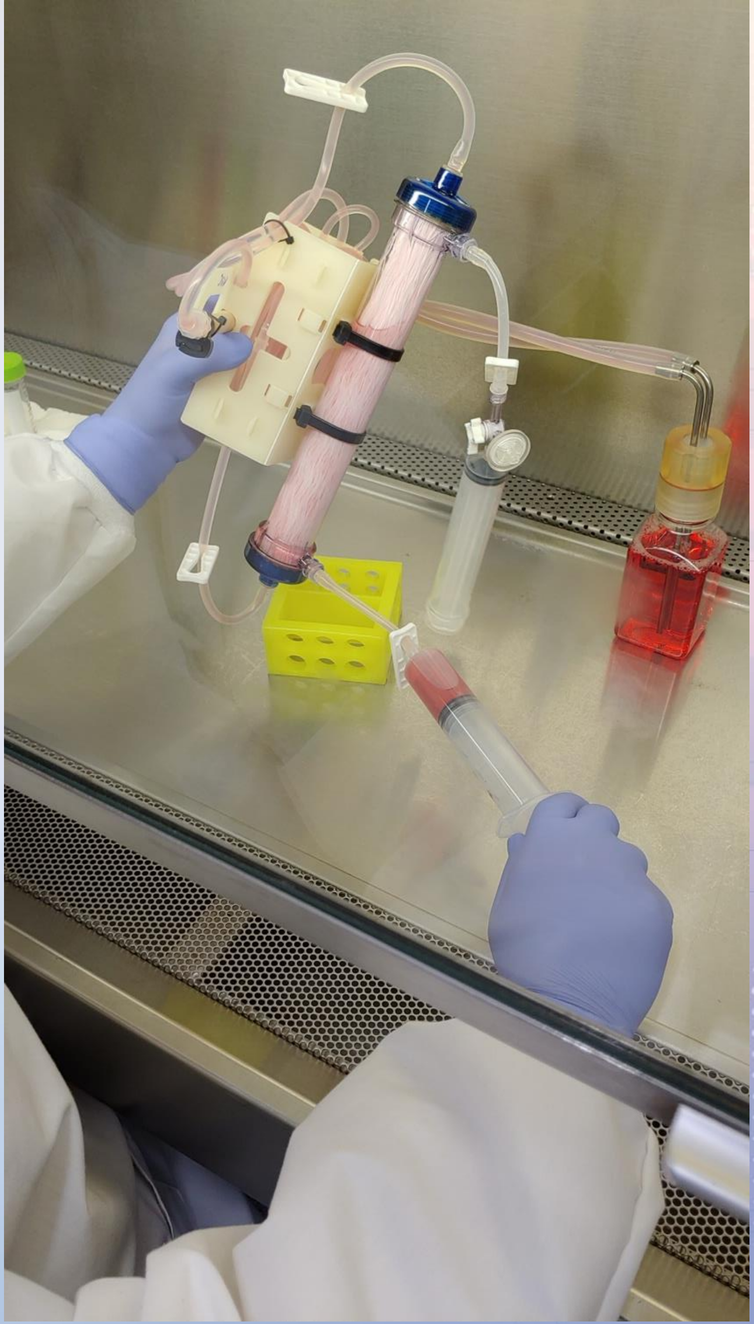
Working with the Cartridge

+ Moves easily into hood
+ Good sterile technique always a plus
+ Maintenance only 15 minutes per day
+ Harvest product and measure glucose consumption

Good Sterile Technique is Always a Plus!

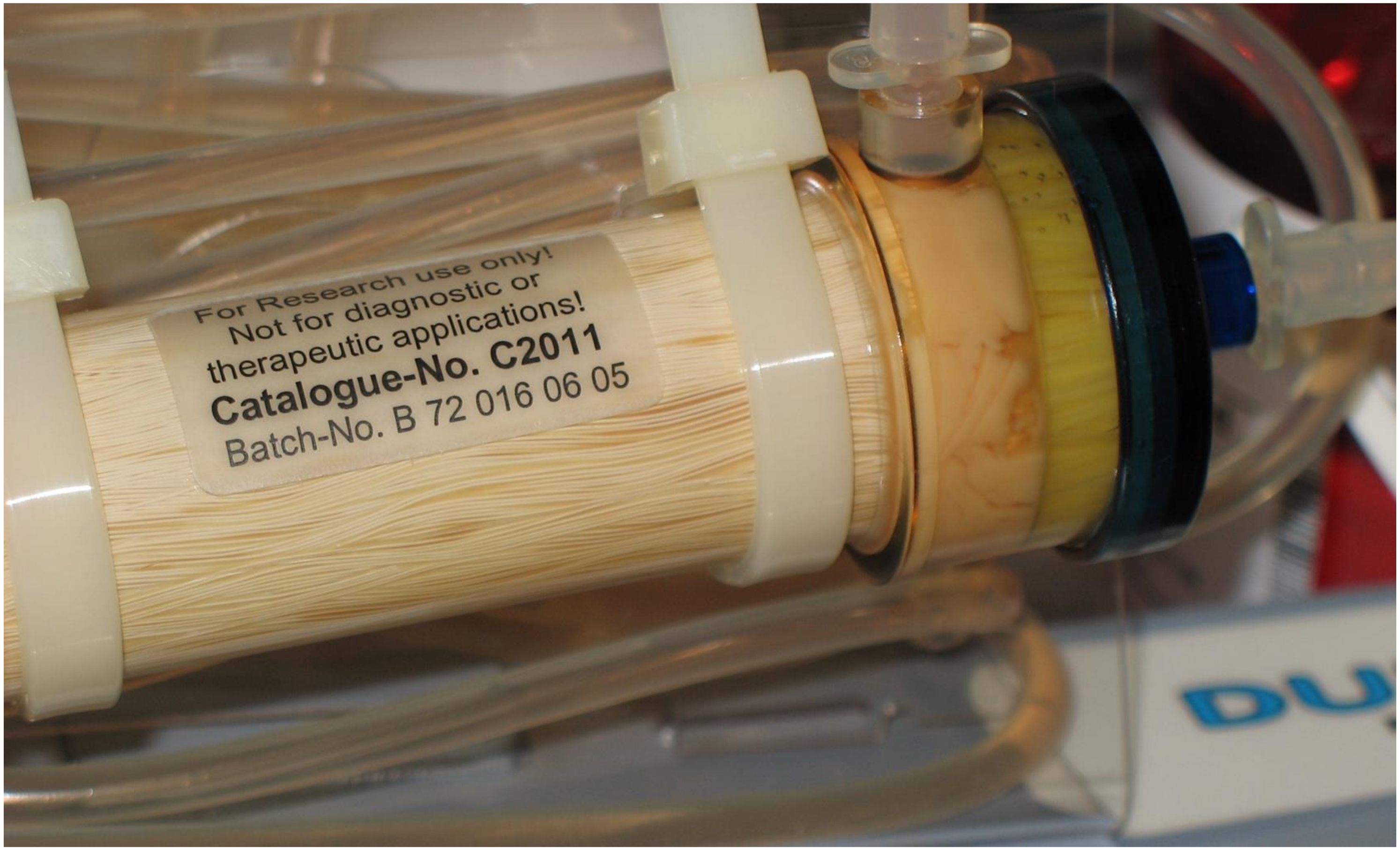






HF Applications

- Monoclonal antibody production
- Recombinant protein production
- Conditioned medium
- Exosome production
- Endothelial cell culture under shear stress
- Cell co-cultivation
- in vitro toxicology



Best for collecting secreted products.

Advantages of Hollow Fiber Cell Culture

+ Concentrated product
+ Uniform and complete post-translational modifications
+ Low apoptosis, less contamination with intracellular proteins and DNA
+ Consistency of production over many months
+ Protein-free medium (CDM-HD)
+ Histocentricity

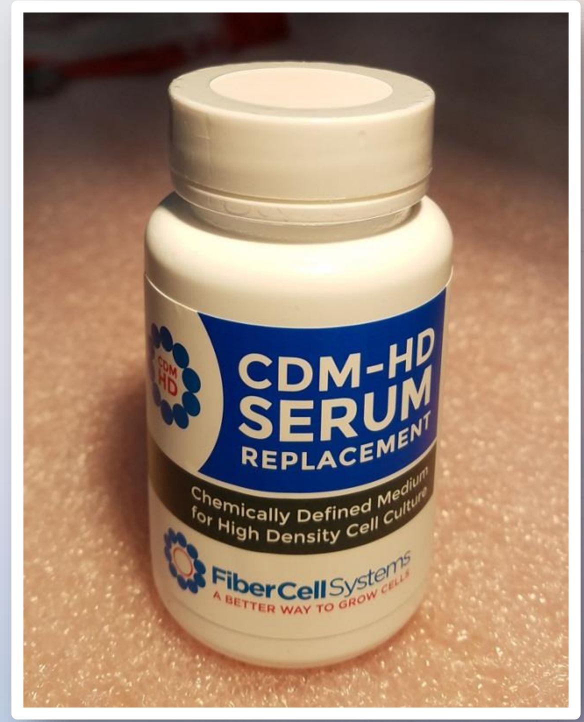
Principles of Histocentric Bioreactors

Histocentric Bioreactors recreate the *in vivo* microenvironment while allowing cell physiology to develop over time. 2-D, 3-D and now 4-D cell culture.

- Biological Mimicry
- Controlled Environment
- Dynamic Culture
- Cell Derived Microenvironment
- Compatibility With Various Cell Types
- Long Term Culture

CDM-HD Serum Replacement

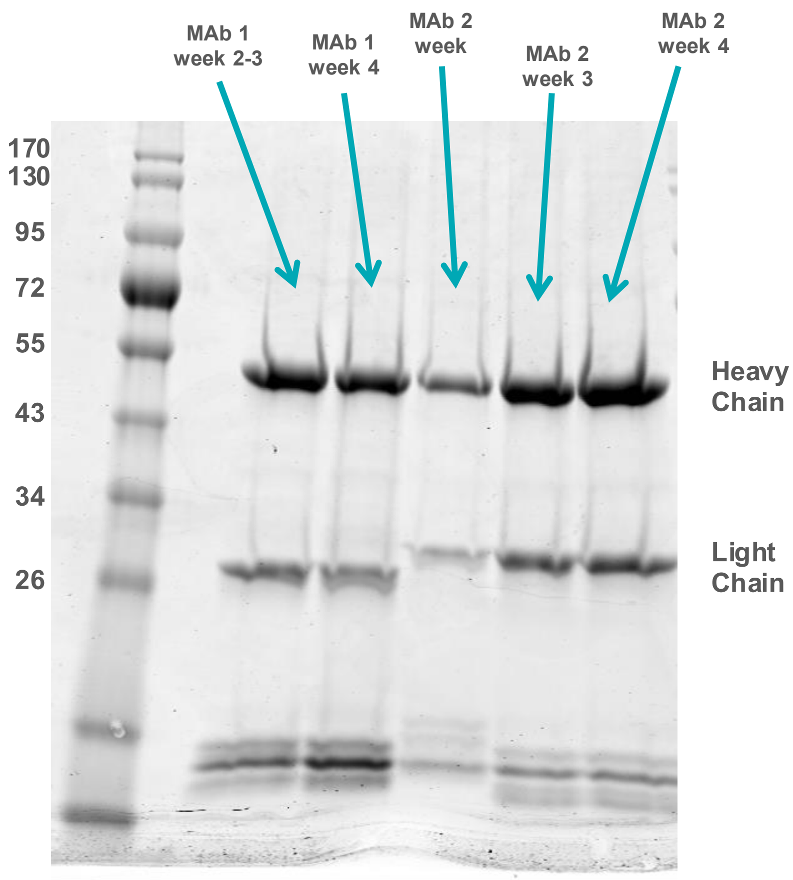
- Optimized and simplified for HFBR
- Contains no surfactants
- Chemically defined, protein-free
- cGMP compliant
- Lot-to-lot consistency
- Ship at ambient, store at 4˚C
- Does not support cells at low density



Mab Production using CDM-HD

Mab 1:
- 168 mg in 60 mL volume, 2.8 mg/mL.
- 9 L of medium consumed, 3 weeks culture.

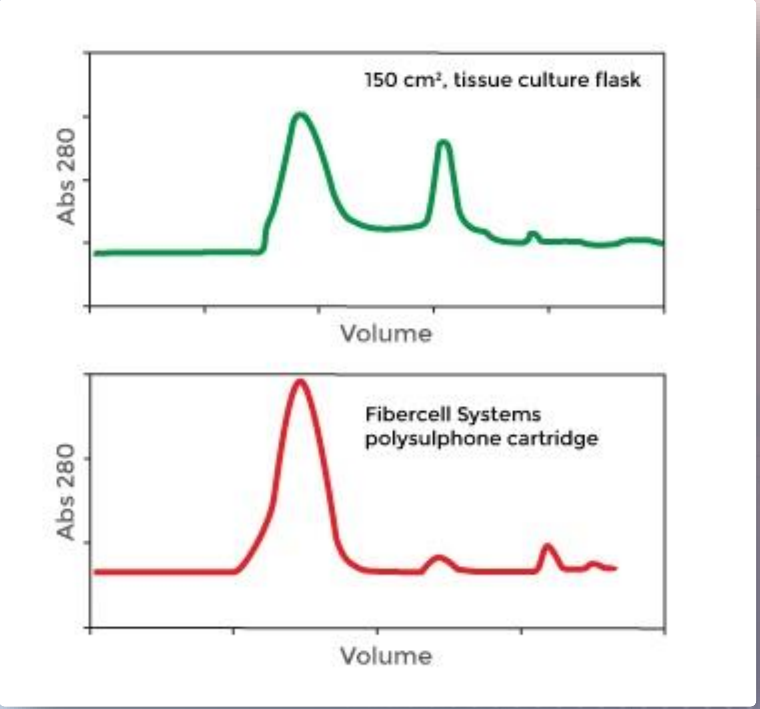
Mab 2:
- 159 mg in 70 mL volume, 2.3 mg/mL.
- 11 L of medium consumed, 3weeks culture.



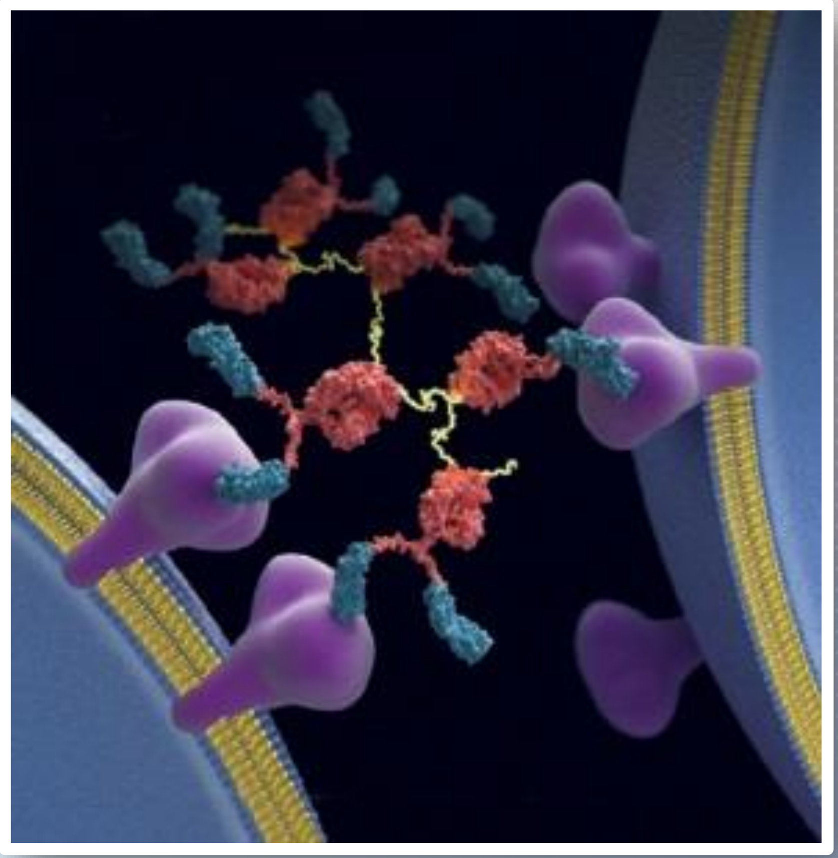
- TGF Beta diffuses out
- Mab trapped in ECS
- Easily adapt to SFM/CDM-HD
- Lower endotoxin
- .5 to 5 mg/mL conc.
- 5-100 mg per harvest
- Continuous production for over 6 months

Recombinant Protein Production

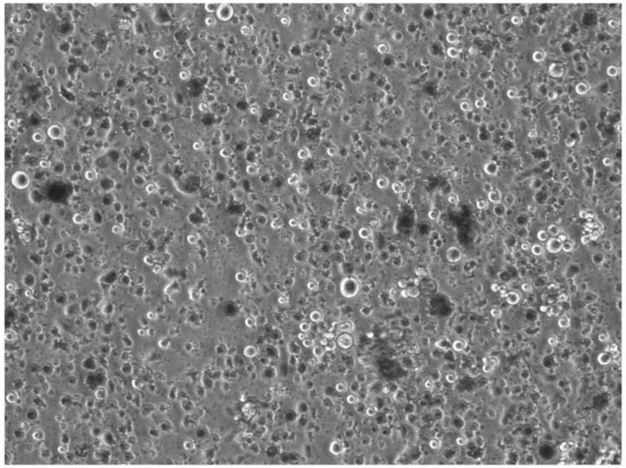
+ Both suspension and adherent cell types
+ 100x+ higher concentration
+ Easily adapt to SFM
+ Can provide improved protein folding

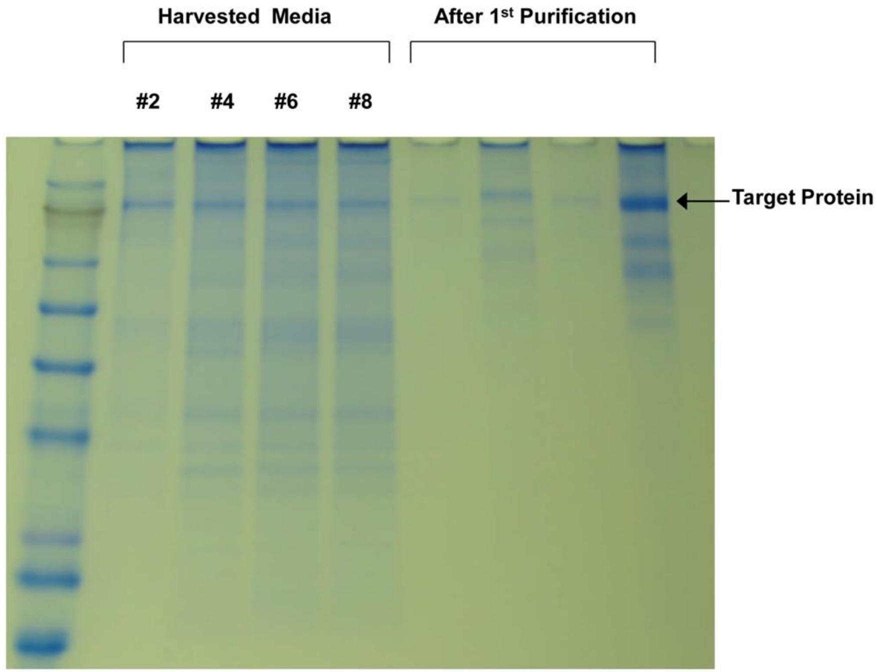


Journal of Biological Chemistry, Sept 2007



Raw Harvests from DG44 CHO Cell Line



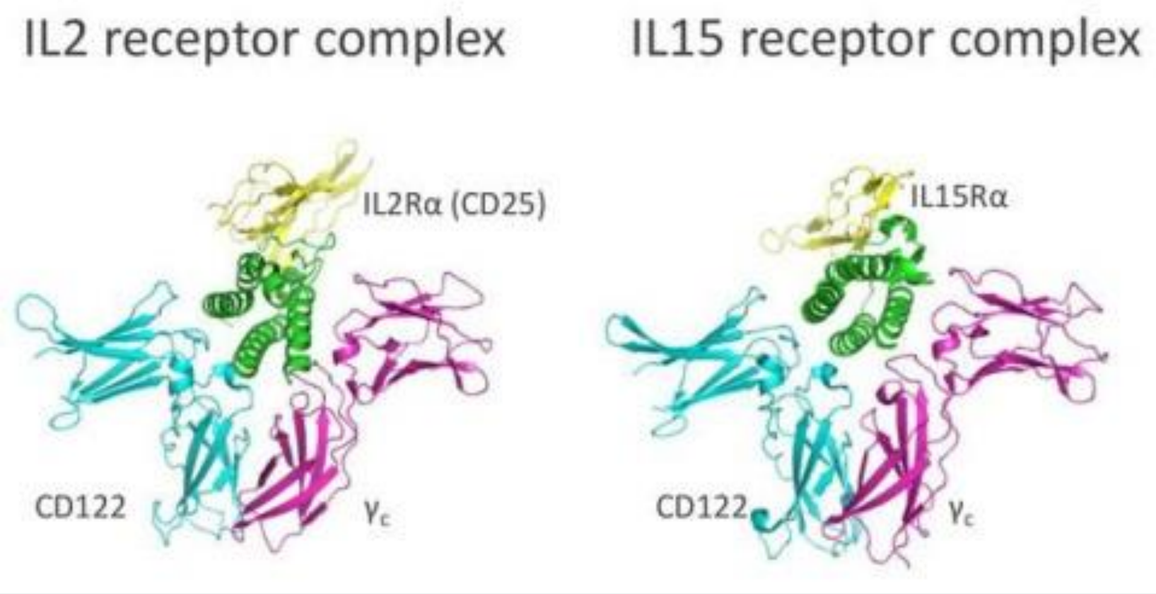


Difficult to Express Proteins

- Highly Glycosylated
- Large and/or Unstable
- Low Titers
- BITE and TRIKE, structures not found in nature

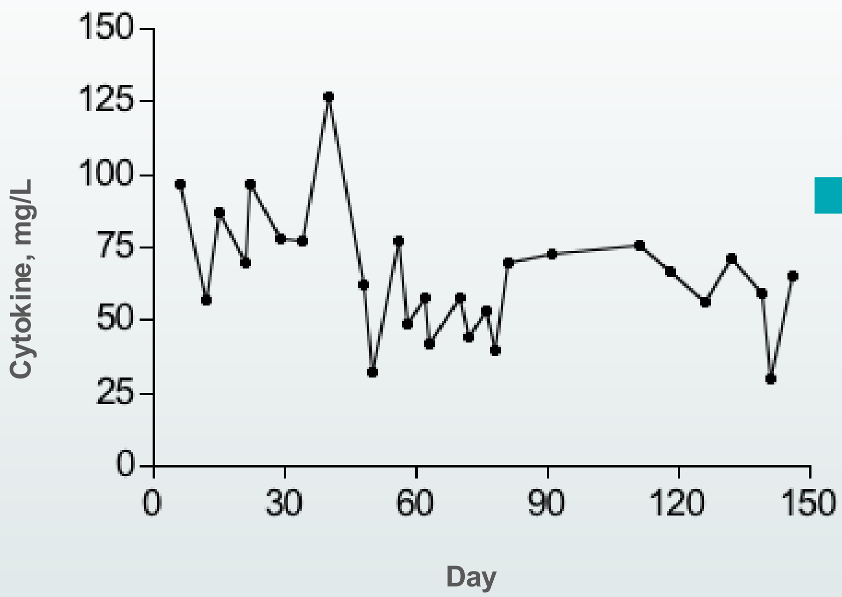
(Mammalian expression provides better solubilty, longer serum ½ life, better antigenicity and functionality)

IL15 RC is a Difficult to Express Protein

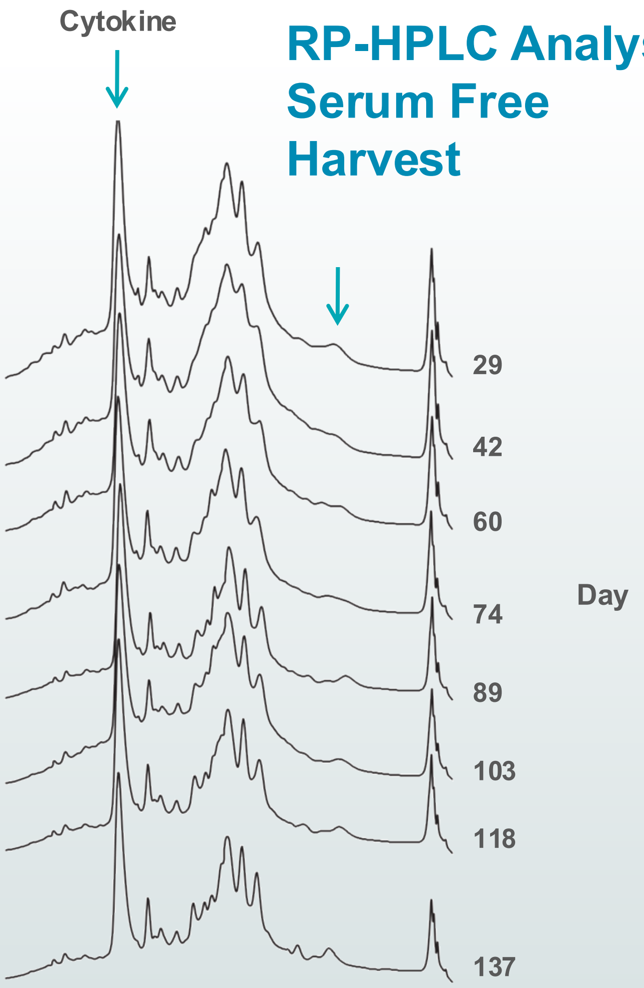


Stable IL15 RC Production in HFBR Over 5 Months

HFBR Production - mg/L

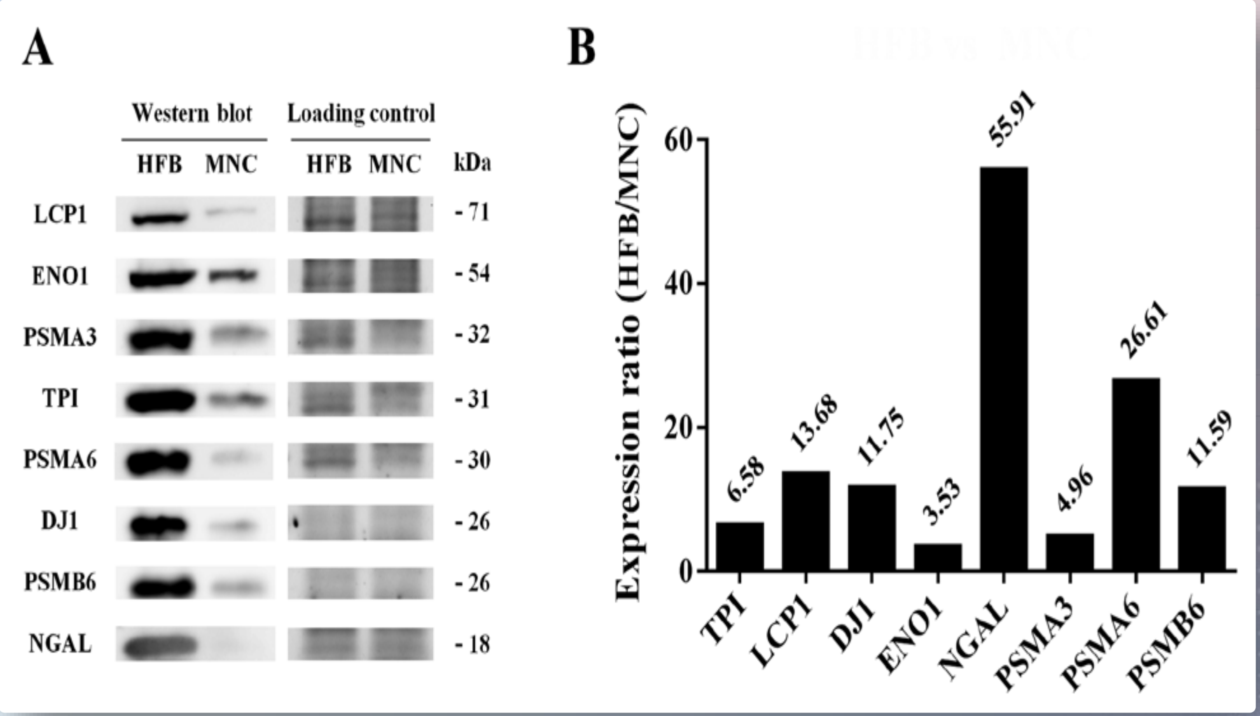


RP-HPLC Analysis, Serum Free Harvest

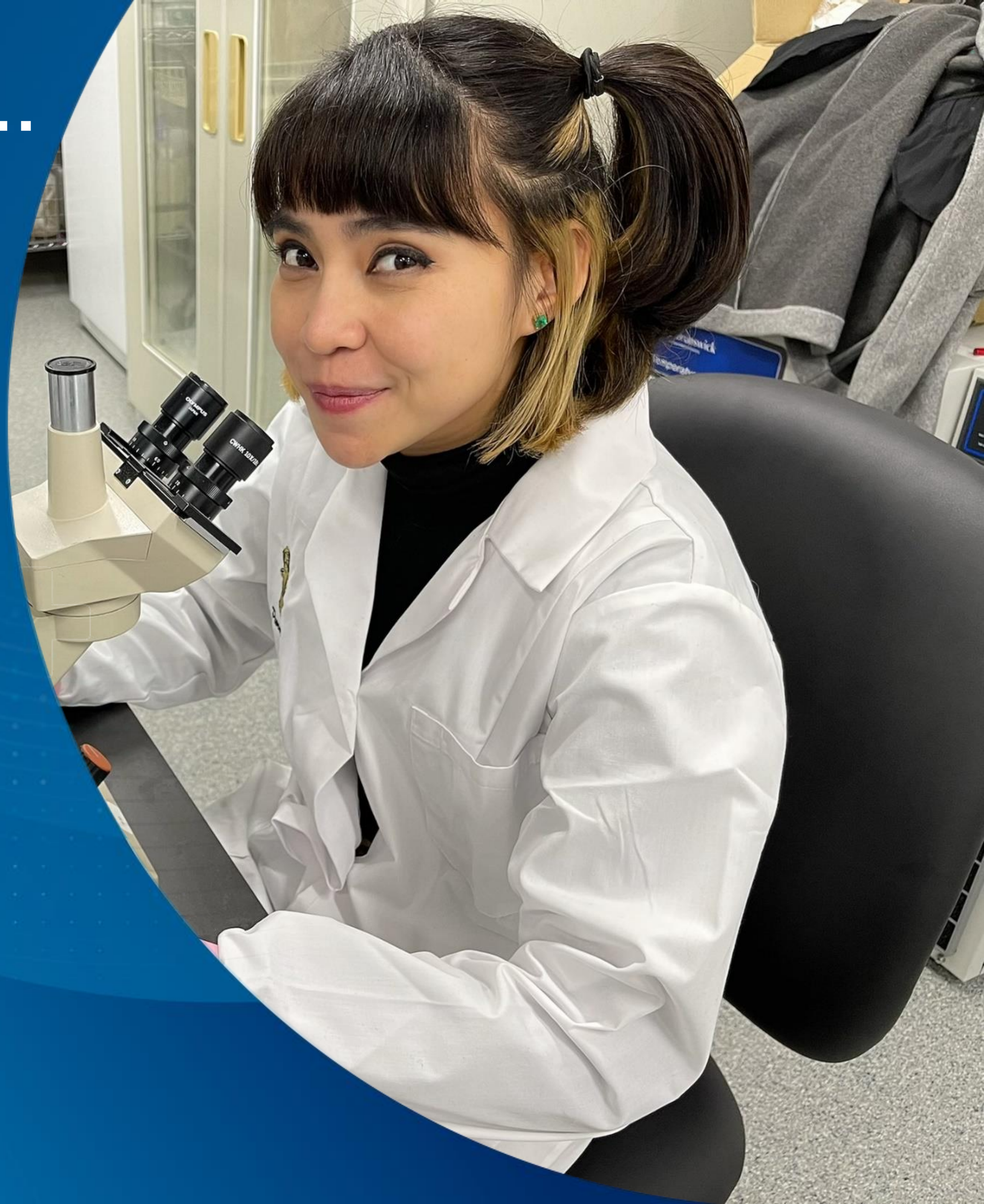


Secretome Analysis for Cancer Biomarker Discovery

Comparison of Flask vs. HFBR

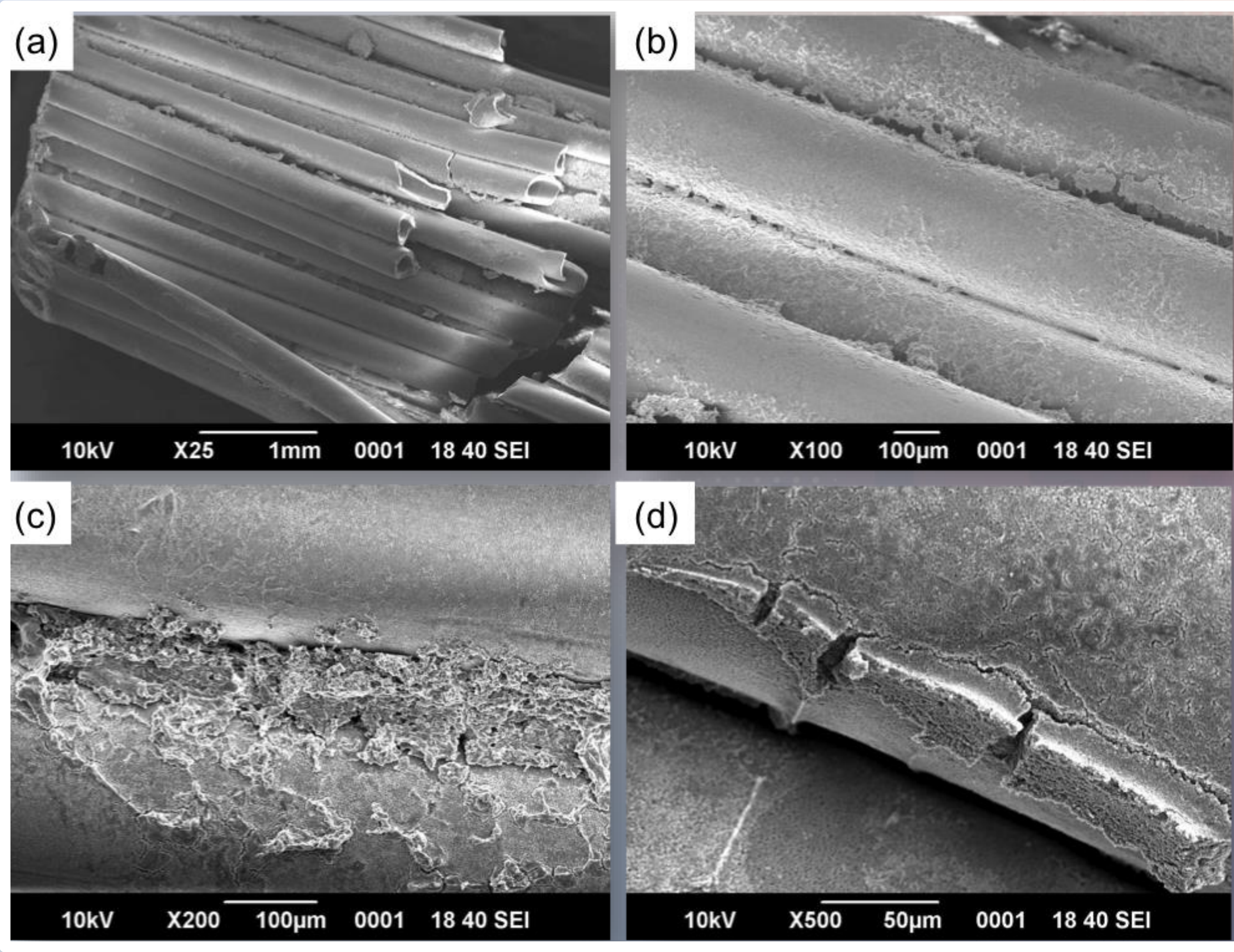


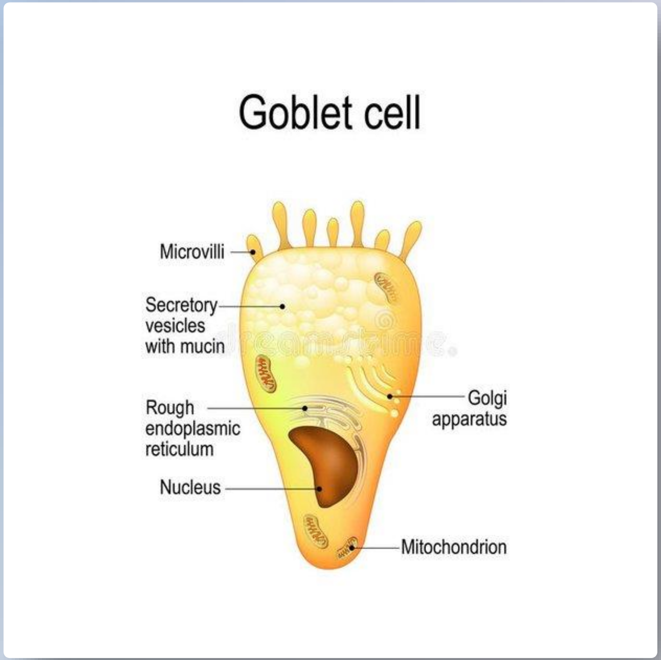
3-D Models……



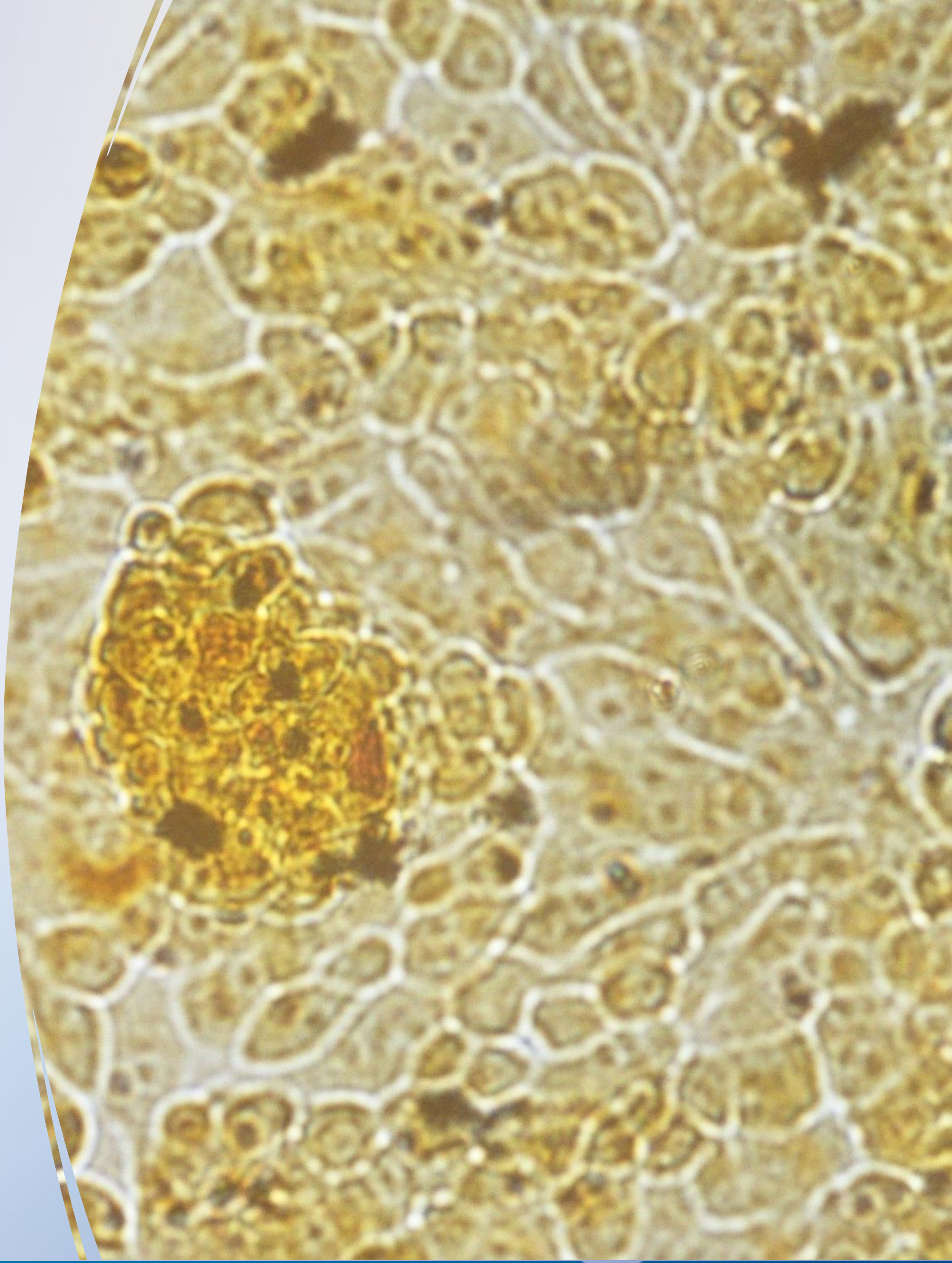
Bone Marrow Stroma/HSC Co-Culture

3-D CD 34 HSC engrafted more rapidly in nude mice compared to 2-D produced HSC.



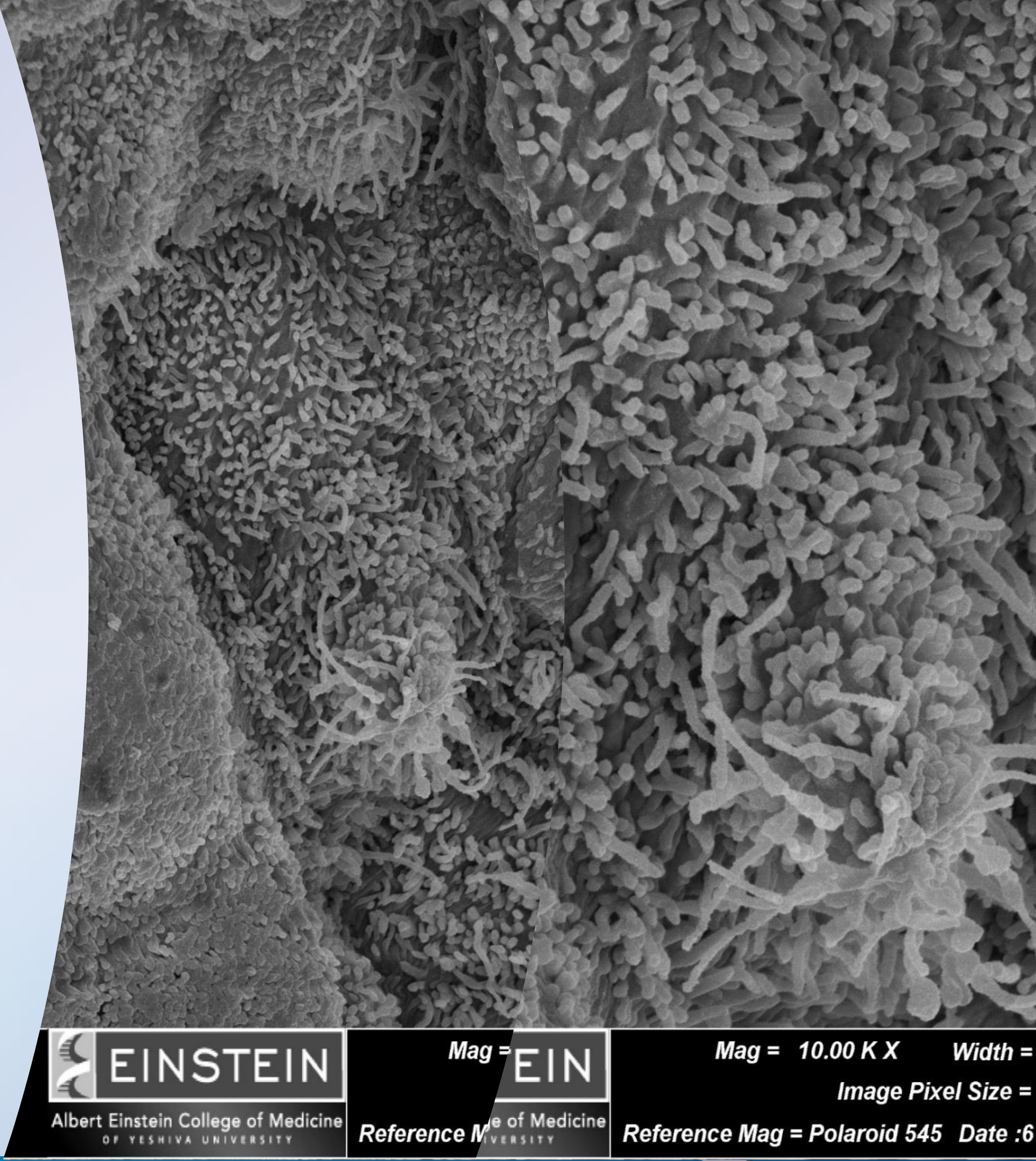


Mixed culture of 85% HCT-8 human intestinal epithelial cells and 15% LS174T goblet cells. Stained with bismark brown to show patches of goblet cells.

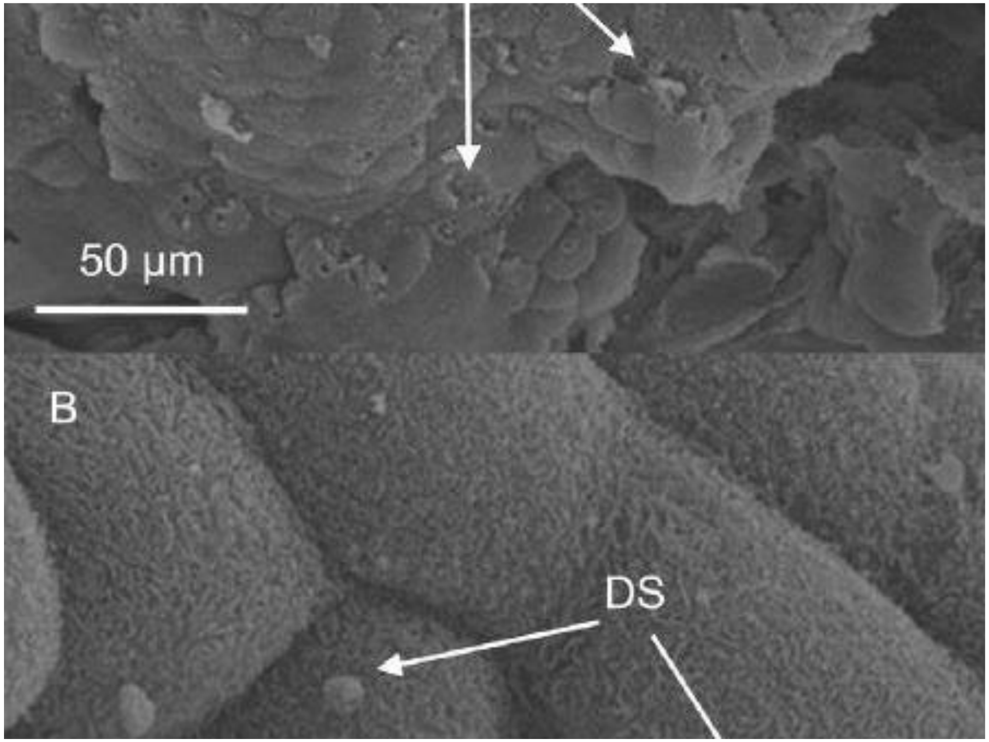


Cryptosporidium

Intestinal Epithelial cells cultured in 3-D perfusion hollow fiber bioreactor demonstrating polarity and microvilli.



Cryptosporidium Culture in an Artificial Gut Model

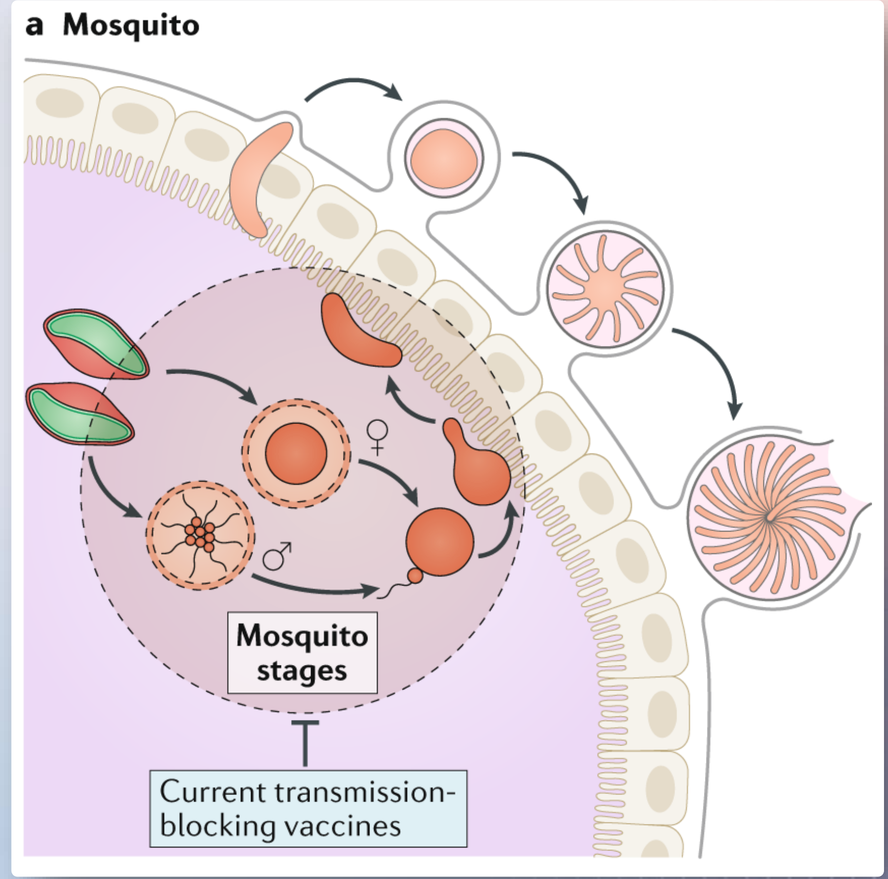


DS: Development stage.

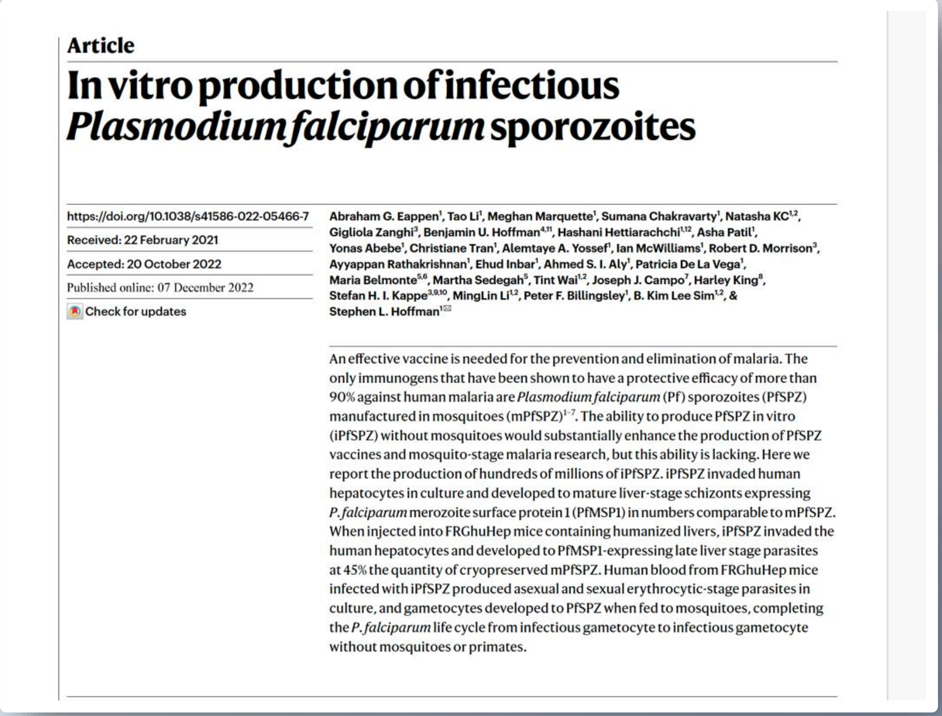
Intracellular but extra-cytoplasmic stage (meront), the enlarged parasitophorous vesicle filled with 8 merozoites which are released when the meront bursts

Malaria Vaccine

Malaria parasites exhibit a complex lifecycle, requiring extensive asexual replication in the liver and blood of the vertebrate host, and in the haemocoel of the insect vector. Yet, they must also undergo a single round of sexual reproduction, which occurs in the vector's midgut upon uptake of a blood meal.



Malaria sporozoites are produced in nature in mosquitoes. In December 2022, a team from Sanaria Inc., a malaria vaccine company, published their groundbreaking working reporting that they could produce infectious *Plasmodium falciparum* (Pf) malaria sporozoites (SPZ) *in vitro* (i). The goal is to use these iPfSPZ in a vaccine.



Article

In vitro production of infectious *Plasmodium falciparum* sporozoites

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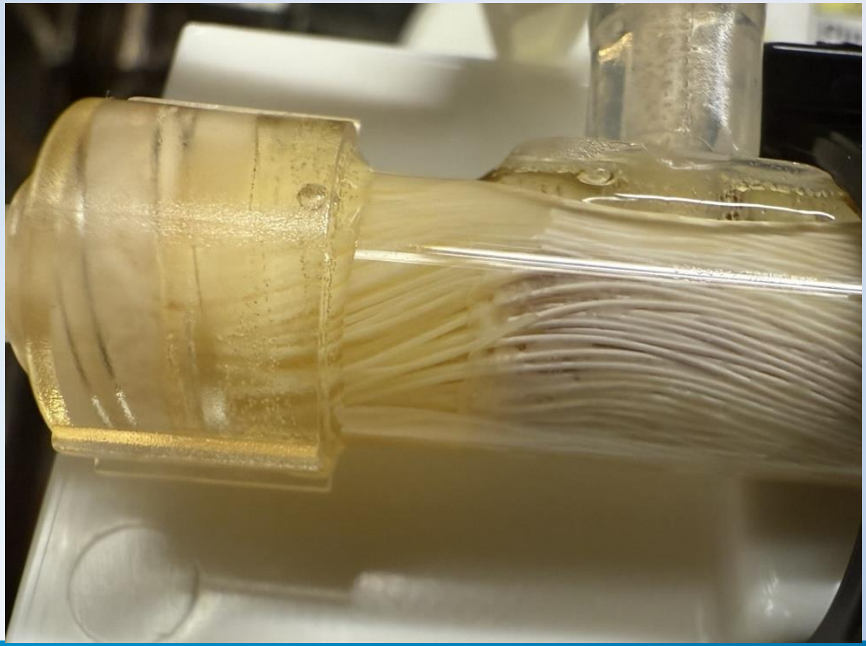
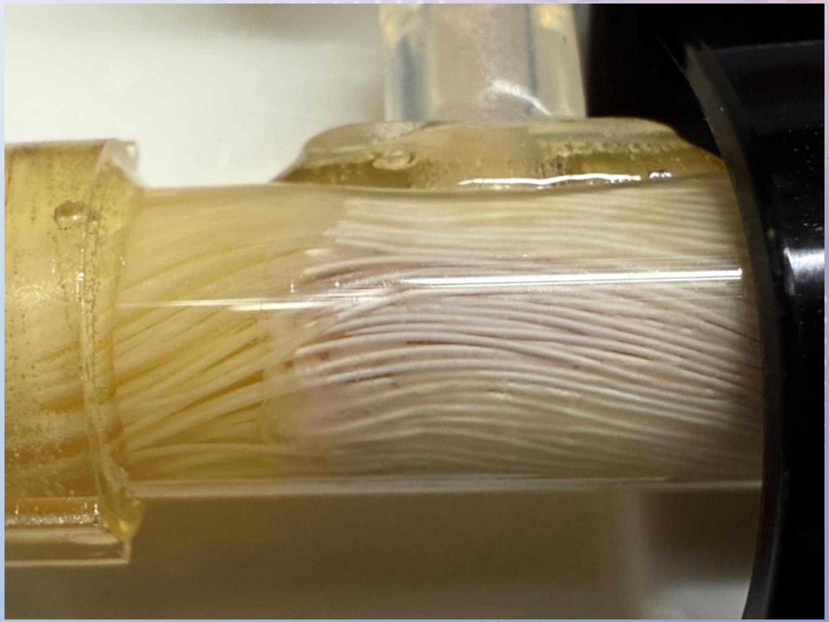
An effective vaccine is needed for the prevention and elimination of malaria. The only immunogens that have been shown to have a protective efficacy of more than 90% against human malaria are *Plasmodium falciparum* (Pf) sporozoites (PfSPZ) manufactured in mosquitoes (mPfSPZ)[1–7]. The ability to produce PfSPZ in vitro (iPfSPZ) without mosquitoes would substantially enhance the production of PfSPZ vaccines and mosquito-stage malaria research, but this ability is lacking. Here we report the production of hundreds of millions of iPfSPZ. iPfSPZ invaded human hepatocytes in culture and developed to mature liver-stage schizonts expressing *P. falciparum* merozoite surface protein 1 (PfMSP1) in numbers comparable to mPfSPZ. When injected into FRGhuHep mice containing humanized livers, iPfSPZ invaded the human hepatocytes and developed to PfMSP1-expressing late liver stage parasites at 45% the quantity of cryopreserved mPfSPZ. Human blood from FRGhuHep mice infected with iPfSPZ produced asexual and sexual erythrocytic-stage parasites in culture, and gametocytes developed to PfSPZ when fed to mosquitoes, completing the *P. falciparum* life cycle from infectious gametocyte to infectious gametocyte without mosquitoes or primates.

Source: www.nature.com

Primary lung tissue inoculated as a single cell suspension and supported by CDM HD.

Dr. Akhil Srivastava, U. Missouri





Meat and Milk Production

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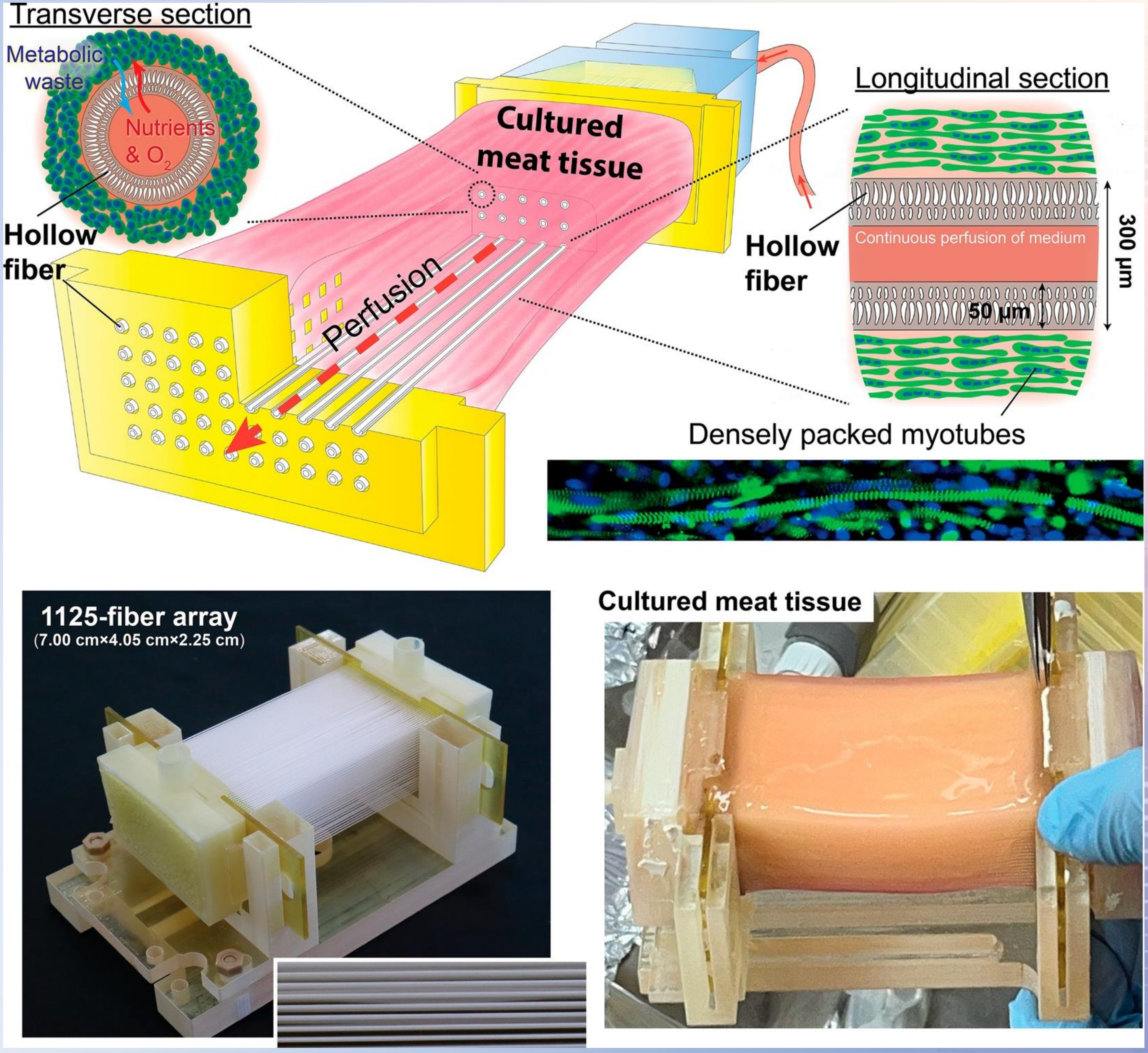

Trends in Biotechnology
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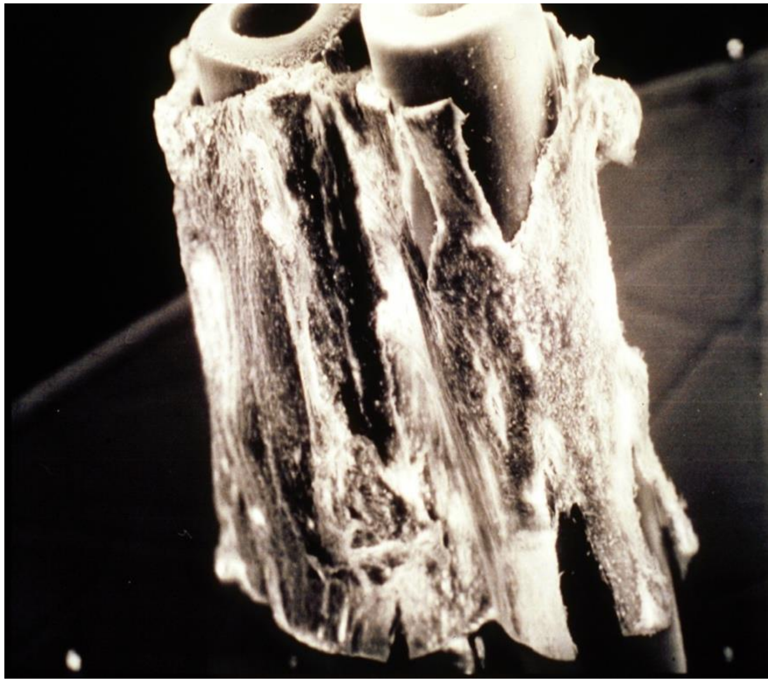
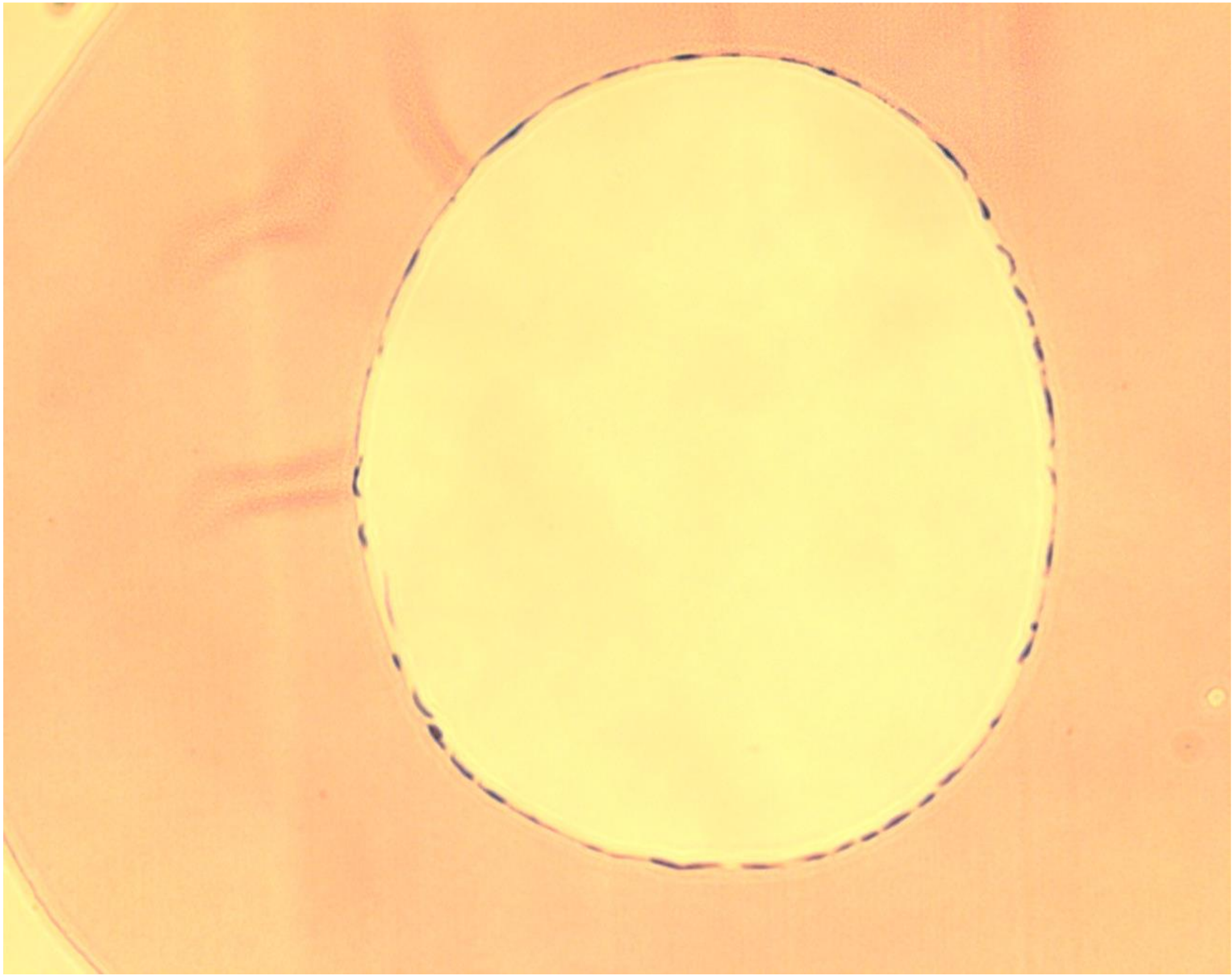
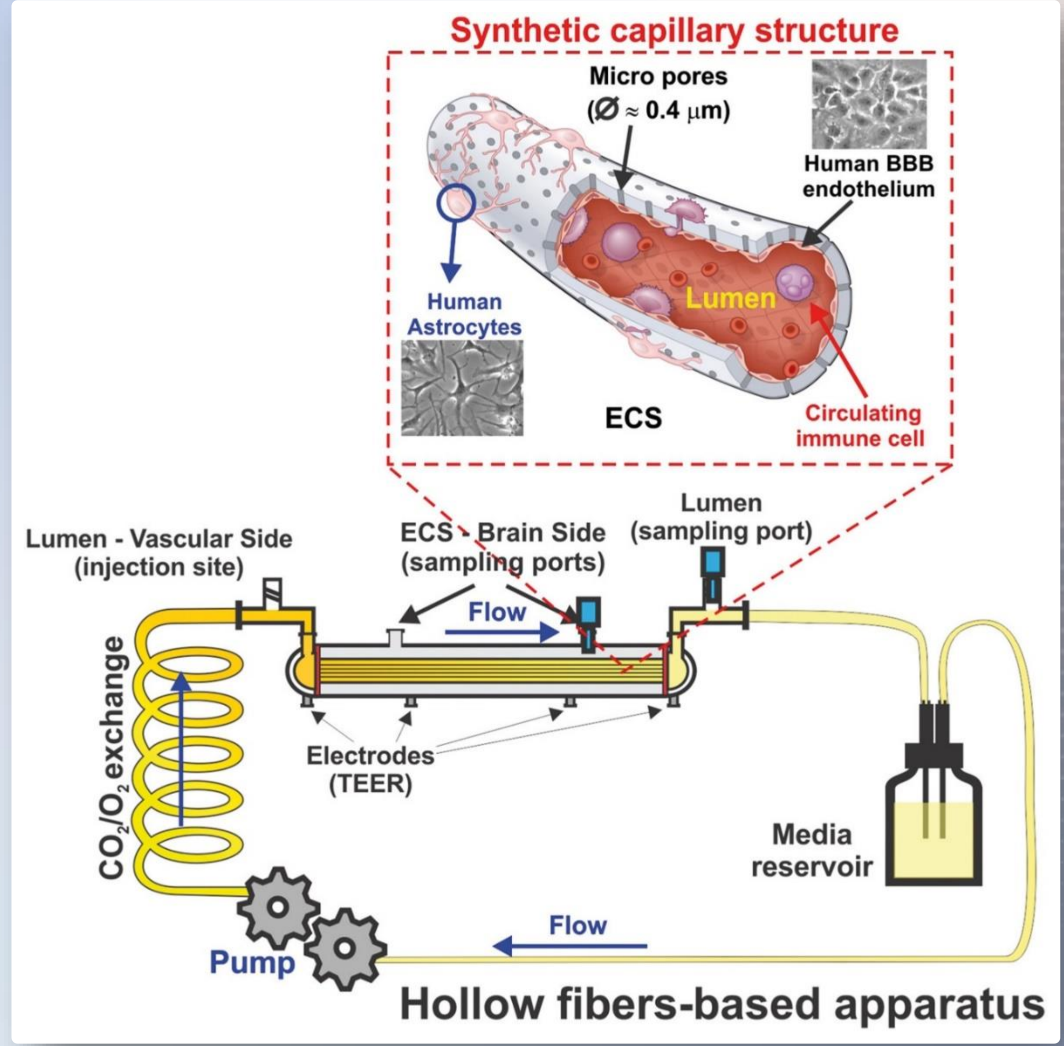
Asymmetric Cell Co-cultivation

+ Co-cultivation of endothelial cells (inside) and vascular smooth muscle (outside)
+ Brain endothelial and astroglial cells to form in vitro blood brain barrier

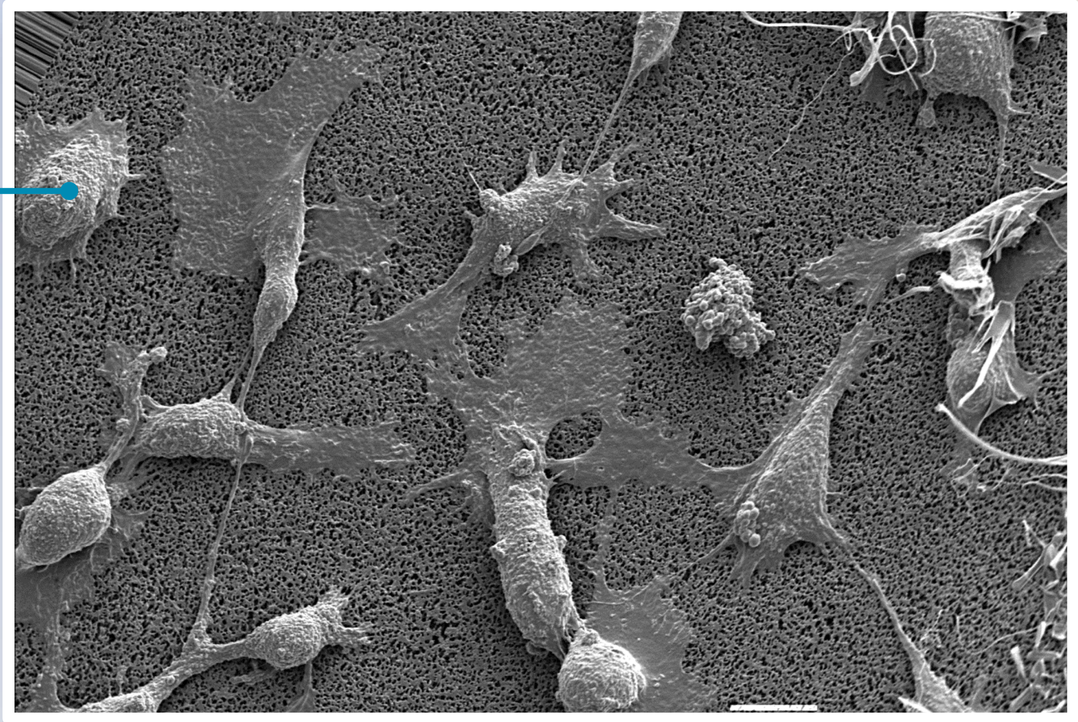




Blood-Brain Barrier Model

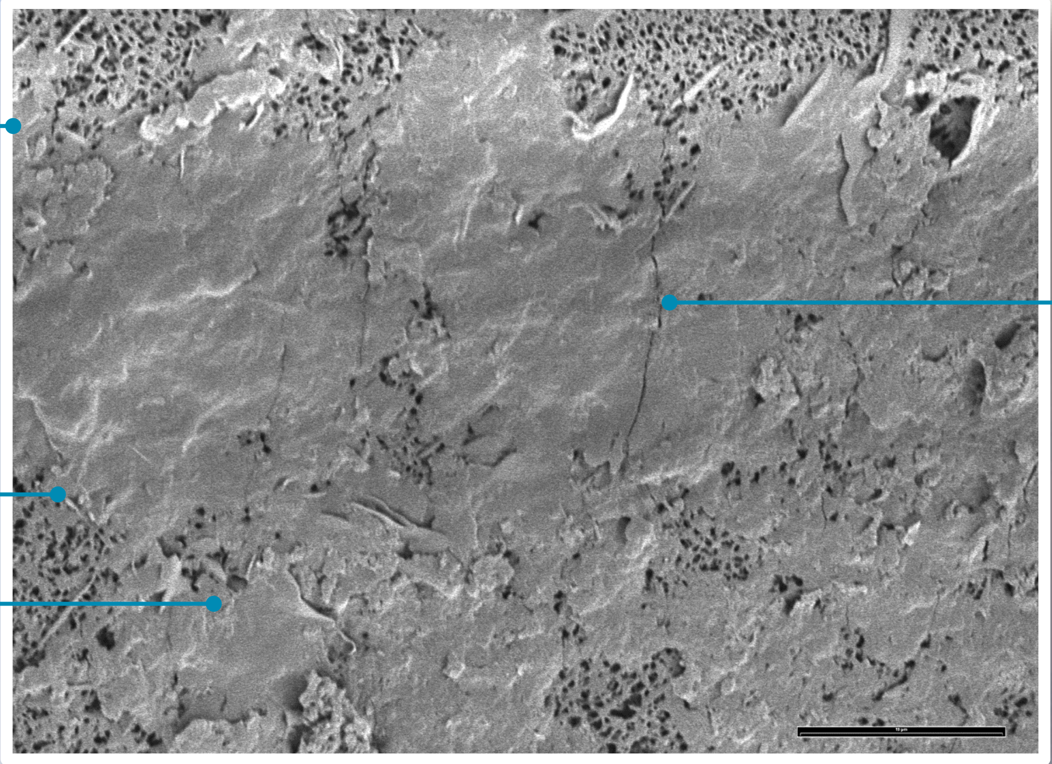


Pores of fiber



Endothelial cells on the inside on a fiber- these have been adhered to the wall then subjected to very low shear force overnight followed by a few hours at 5 dynes/cm^2.

While the majority of the cells here are still bulky it is possible to see then begin to flatted down onto the wall of the fiber and really stretch out.



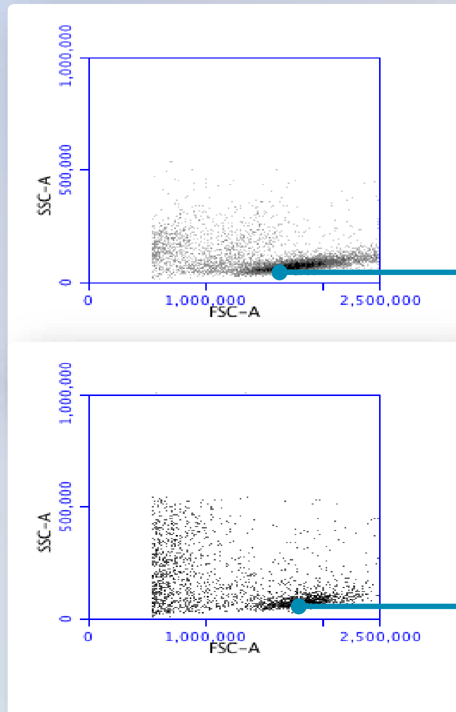
Flattened endothelial cells on inside of fiber. These endothelial cells had been adhered to the inside of the fiber and subjected to minimal shear force over night followed by a minimum of 5 hours at 10 dynes/cm^2. This was based on results we saw when testing the application of a different system that can apply shear force to channels lined with endothelial cells while visible under a microscope.

This other system also allowed us to discover that endothelial cells will detach and roll away after alignment if a bubble passes over them so we eliminate all bubbles from the system to preserve the endothelial layer.

CLL cells actively migrate into the extra-vascular space

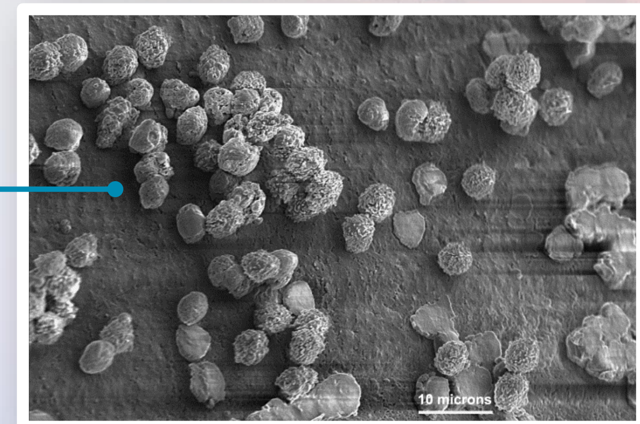
Circulating Compartment

Extra-vascular space (migrated compartment)



Cardiff CLL Research Group

CLL cells



Scanning electron micrograph of the outside of a hollow fibre after circulation of CLL cells around the system

Exosome Cell Culture Conditions Affect Composition

Laminar Flow Alters EV Composition in HUVECs: A Study of Culture Medium Optimization and Molecular Profiling of Vesicle Cargo

Arefeh Kardani, Jan Hemmer, Britta Diesel, Vida Mashayekhi, Annika Schomisch, Marcus Koch, Claudia Fecher-Trost, Markus R Meyer, Nicole Ludwig, Shusruto Rishik, Andreas Keller, Jessica Hoppstädter, Gregor Fuhrmann, and Alexandra K. Kiemer**

Endothelial cells (ECs) experience shear stress associated with blood flow. Such shear stress regulates endothelial function by altering cell physiology. Since most cell culture protocols and media compositions are designed for static cultures and experiments with ECs are predominantly conducted under these non-physiological conditions, a model for culturing ECs under flow conditions is developed, which more closely mimics their physiological environment. This approach also enables the isolation of EVs while minimizing FCS-derived contaminants. In this study, a comprehensive assessment of how physiologically relevant cultivation conditions influence the vesicle composition and function of ECs is provided. A detailed investigation is conducted for the effect of different cell culture media on morphology and marker expression of human umbilical cord endothelial cells (HUVECs) and EVs, and optimize the conditions to culture ECs under flow, tailoring them specifically to facilitate the efficient isolation of EVs using a hollow-fiber system model. These EVs are then characterized and compared to those isolated from traditional static culture conditions. Overall, this study presents a model on isolating EC-derived EVs under conditions that closely mimic physiological environments, and characterization at their proteome, gene expression, and microRNA profile.

1. Introduction

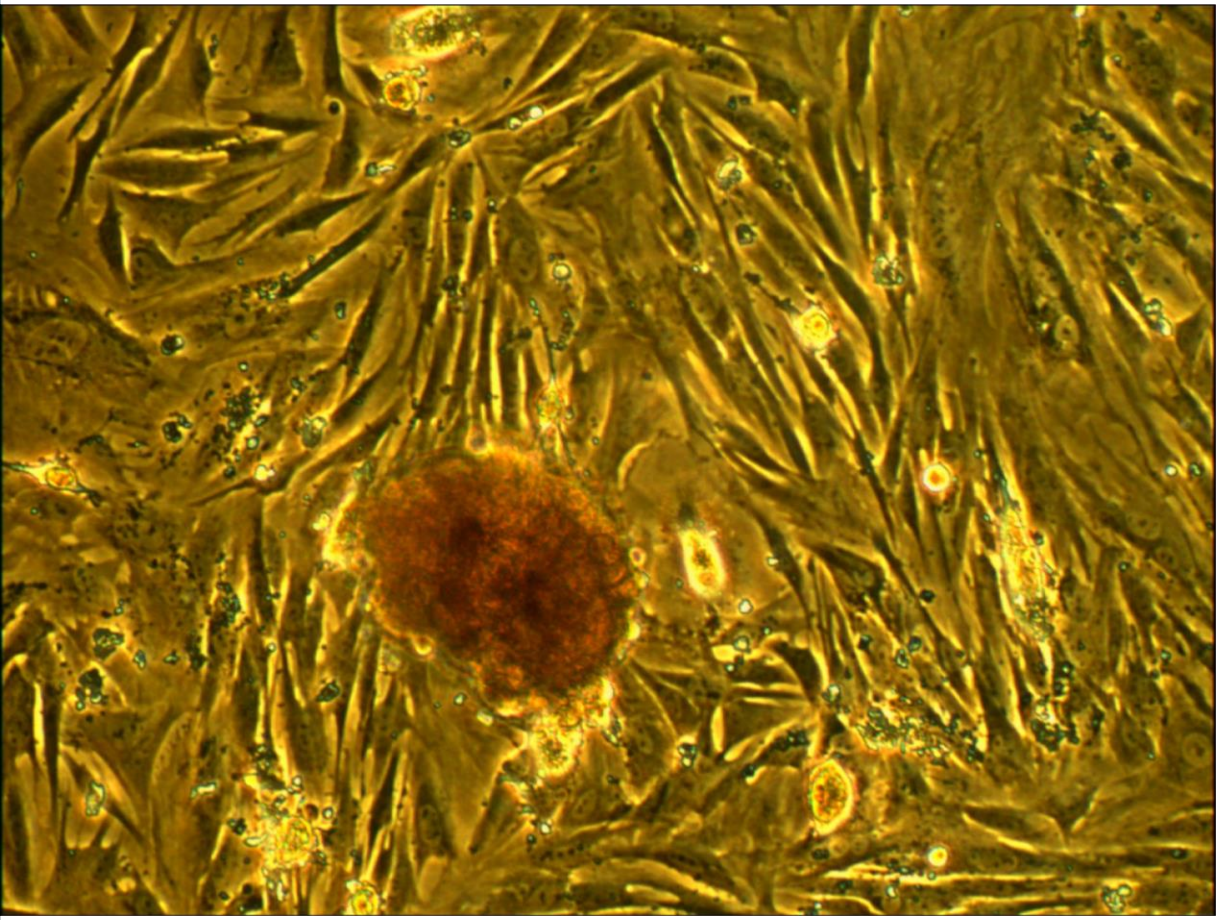
Extacellular vesicles (EVs) are nanosized membrane-bound structures released by almost all types of cells into their external environment. Eukaryotic EVs are usually classified into three main categories, based on their size and mode of production.[1] Microvesicles are formed by the outward budding of membrane vesicles from the cell surface.[2] Exosomes originate from the endocytic pathway through the 'outward' budding of the late endosomal membrane. Initially, they accumulate in structures known as multivesicular bodies (MVBs), which later fuse with the plasma membrane, releasing their contents as exosomes into the extracellular space.[3] The third major type of eukaryotic EVs called apoptotic bodies are produced from cells undergoing programmed cell death by outward budding from the surface of apoptotic cell.[4]

Pulsatile Perfusion of Placenta

1) Flush with PBS
2) Digest with collagenase for one hour
3) Collect cells
4) Seed into hollow fiber bioreactor

Placental Co-Culture

1) Remove non-adherent cells after 3 days
2) Nodules form in 5-7 days
3) New population of non-adherent cells collected



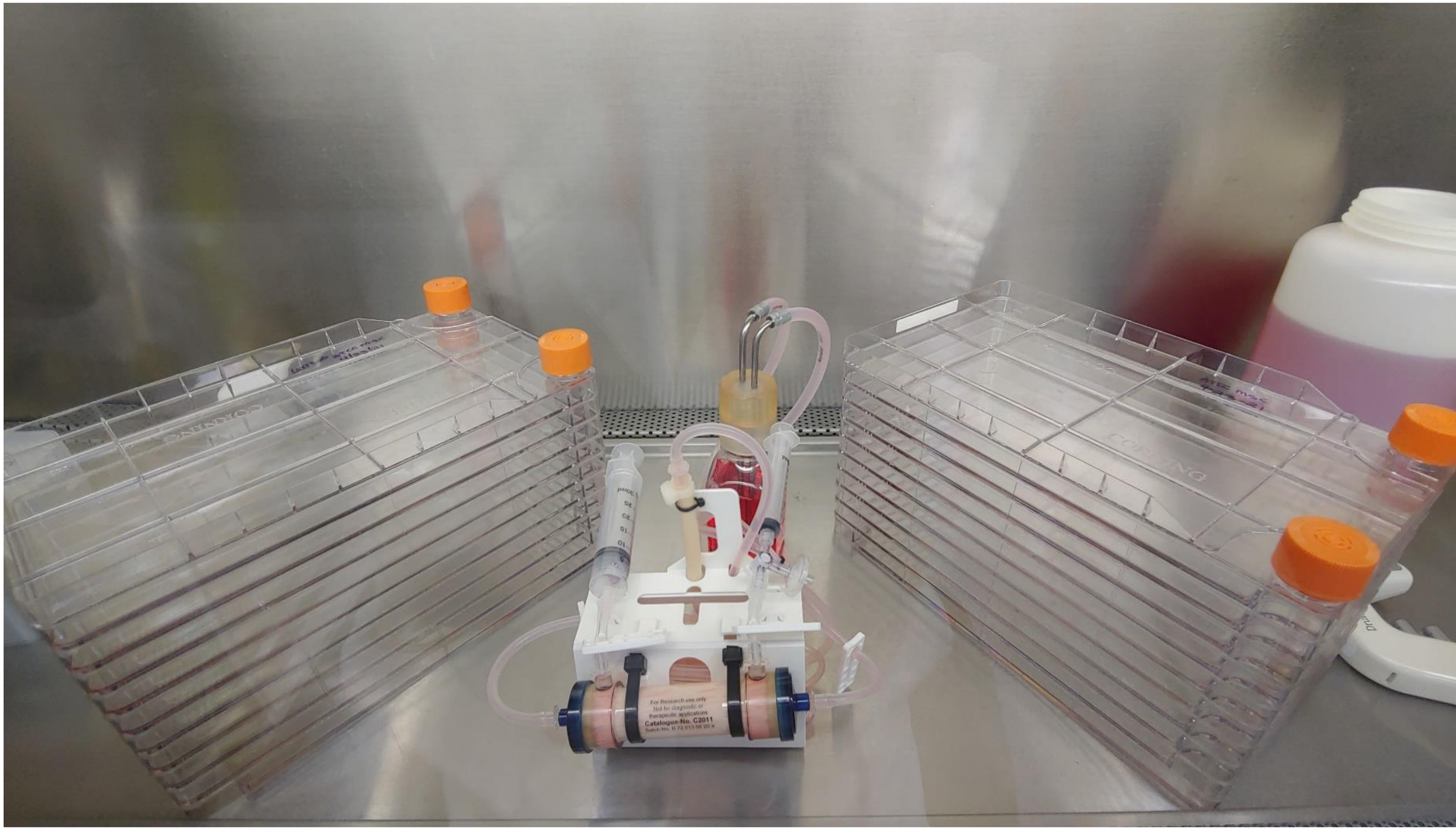
Harvest vs. Flask

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%

Plastic Waste Generated by $1x10^9$ Cells

MSCs are plastic adherent fibroblastic cells with the "trilineage potential" of osteogenic, chondrogenic and adipogenic differentiation capabilities. Furthermore, they express the cell surface markers CD73, CD90, and CD105, and do not express haematopoietic and endothelial antigens (CD14 or CD11b, CD19 or CD79α, CD34, CD45, HLA-DR)

Dominici M, Le Blanc K, Mueller I, et al. et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy*. 2006;8(4):315–317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Catalogue-No. C2011

Scalability of Production and Bio-Activity of Amniotic Fluid Stem Cell Extracellular Vesicles from 3-D Hollow Fiber Bioreactor and 2-D Culture.

FiberCell Systems
www.fibercellsystems.com/exosomes

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Introduction

EV clinical translation is constrained by limitations in scale-up of EVs production. Hollow fiber bioreactors (HFBR) support the culture of large numbers of cells, at high densities, producing significant numbers of EVs at high concentration. The high cell densities present in a HFBR can facilitate the use of xeno-free/chemically defined mediums, such as CDM-HD. Here we compare production, potency, identity and therapeutic potential of EVs collected from cells grown in culture dishes (2-D) vs. a HFBR (3-D).

Methods

Human clonal Amniotic Fluid Stem Cells, hAFSC, were derived from consented donor's amniotic fluid. 1×10^8 hAFSC were seeded in 2-D petri dishes (145 cm^2), and 9.2×10^8 hAFSC were seeded into a 20 kD MWCO HFBR (FiberCell Systems C2011, 20 kD, 4,000 cm^2) with fibronectin coating; both cultured in Chang's medium with 20% FBS. At confluence in the petri dish the medium was replaced with basal medium, starved for 48 hr and EVs collected. After three days the medium in the ECS of the HFBR was replaced with Chang's medium alone, without 20% FBS, complete Chang's with 20% FBS remained in the central reservoir. The ECS was flushed with basal Chang's over the next 3 days and then harvesting of EVs every day was initiate. After two weeks of production serum in the reservoir was reduced stepwise to 5% and 5% CDM-HD introduced. After one more week serum was completely removed and replaced with 10% CDM-HD. The final weeks of EV production were produced using chemically defined medium, CDM-HD alone. Glucose consumption was monitored on a daily basis. 2-D EVs and 3-D EVs were compared by Nanosight, potency assay and by WBand therapeutic effect (*in vivo* injections in an animal model of chronic kidney disease, Alport Syndrome).

Results

Control: 2-D EVs, Volume: 40mL, 3.07×10^9 EV/mL, Total EV: 2×10^{11}

Figure 1: Daily Glucose Consumption - C2011 Hollow Fiber Bioreactor Culturing historical hAFSC

Day 17: 15% FBS
Day 19: 10% FBS
Day 21: 7.5% FBS, 2.5% CDM-HD
Day 22: 5% FBS, 5% CDM-HD
Day 26: 5% FBS, 10% CDM-HD
Day 34: 10% CDM-HD

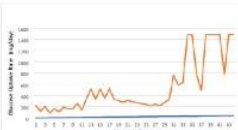


Figure 2: Daily EV Production - C2011 Hollow Fiber Bioreactor Culturing historical hAFSC

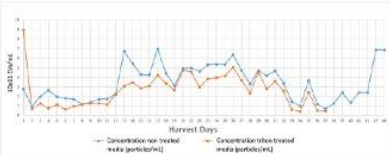
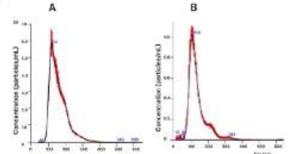


Figure 3 - hAFSC-EV size. Nanosight analysis hEVs derived from 2-D (A) and 3-D (B) average size is 113 nm mode.



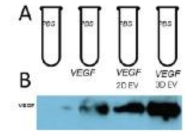


Figure 4: VEGF/VEGFR1 Co-immunoprecipitation. Immunoblot of VEGF (24 KDa, monomer), after co-IP with VEGFR1 in PBS+VEGF (100ng/mL) exposed-EVs from 2-D EVs (A) and 3-D EVs (B). Both EVs trap VEGF in a similar way. The weak band detected in the PBS/VEGF (second band) represents a VEGF carryover due to incomplete removal of VEGF during washing.

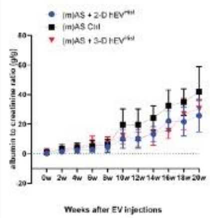
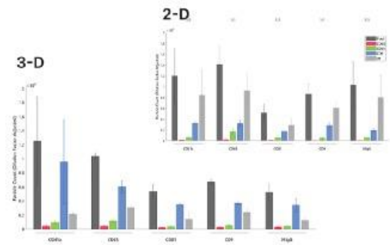


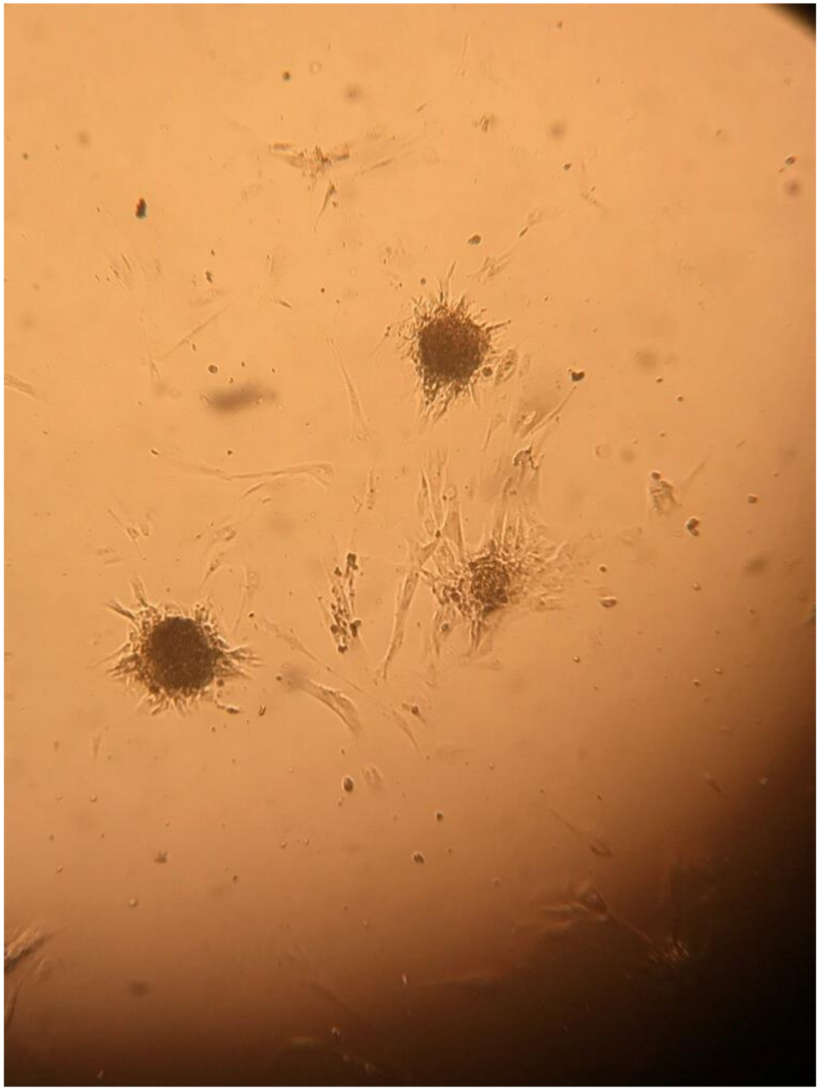
Figure 5: EVs ameliorate renal dysfunction. 2-D EVs and 3-D EVs, reduce proteinuria (measured by ELISA) in treated mice vs untreated mice. AS injected with 3-D: n=6 mice/time point; AS injected with 2-D EVs: n=15 mice/time point; AS non-injected n=18 mice/time point. Mice were 10wks old (week 0) at injection and urine collected every 2wks. WT and non-injected control mice were strain- and age-matched. *p<0.05**; p< 0.01; Mice are still in the study but the protective role of hEVs is evident after injection.

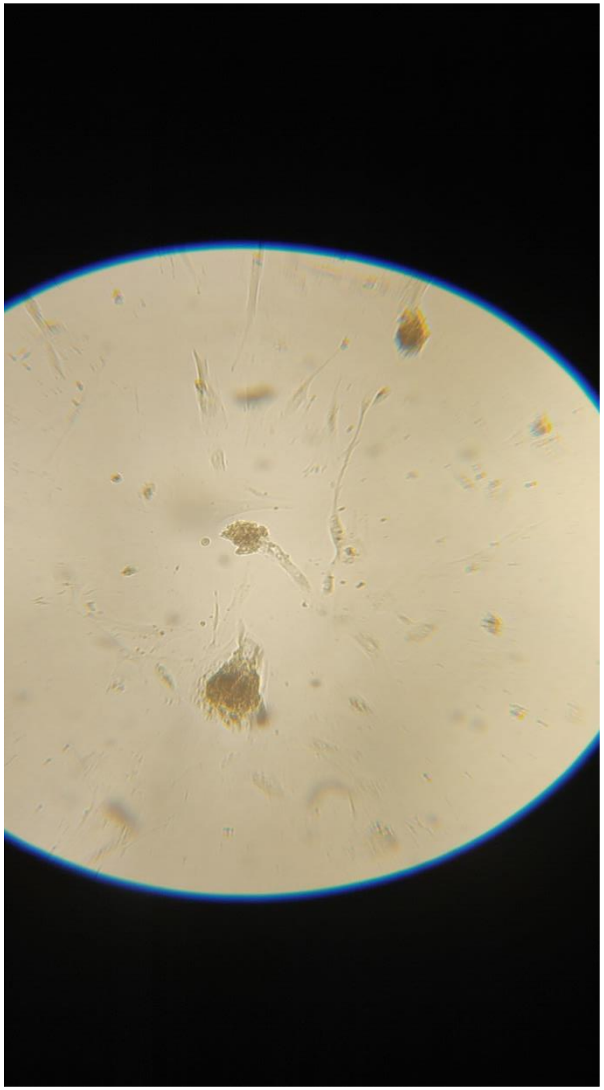
Fig 6: 3-D hEVs present similar tetraspanin profile of 2-D hEVs as evaluated by Exoview



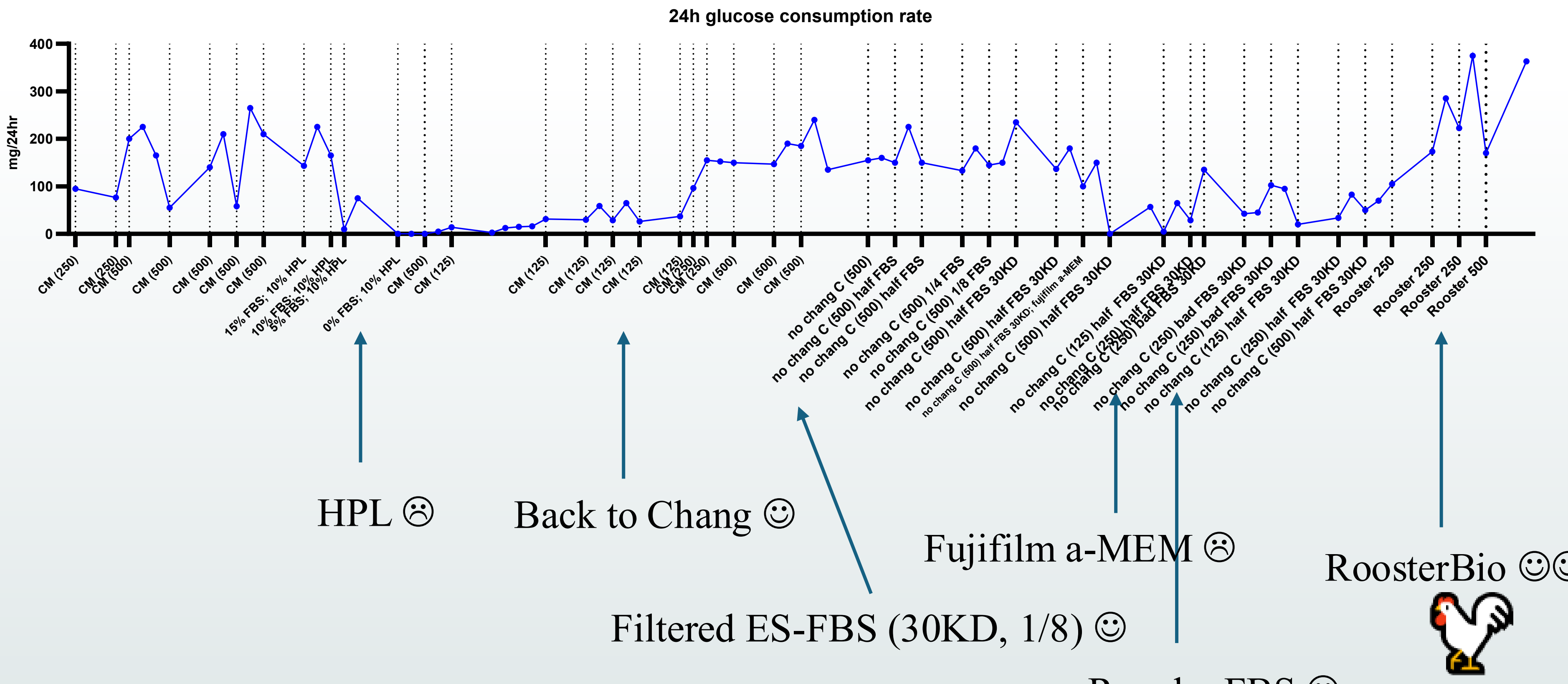
Discussion and Conclusion

3-D EVs had comparable properties and bio-activity relative to 2-D EVs, but the HFBR produced 100x more concentrated EVs per mL. Each daily harvest produced more than 1×10^{12} EVs, 1×10^{14} would be an estimated human dose. The adaptation of these cells to a chemically defined medium and the demonstrated production range represents a significant step towards enabling therapeutic applications of hAFSC for treating kidney diseases in humans. The C2018 hollow fiber bioreactor module represents an additional 5x scale-up from the data presented here. The HFBR is a closed system that can be cGMP compliant. In conclusion, the HFBR can produce sufficient numbers of EV to support pre-clinical and clinical applications of EVs with at least similar properties to EVs produced by conventional 2-D methods.





110 Days Continuous Production of EVs from HAFSC



Data courtesy of Dr. Laura Perin and Paolo Neviani,
Children's Hospital, Los Angeles.

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Introduction

Various methods have been applied to the transfer of genes into mesenchymal stem cells (MSC). The generation of stable MSC transfectants is hampered by the limited number of passages MSC can undergo before they start to differentiate and difficulty in performing at clinical scale. Current data suggests that mesenchymal stem cells, when seeded into a 3-D hollow fiber bioreactor (HFBR) show little proliferation and may be maintained in culture and produce EVs continuously for extended periods of time at high concentrations. Transient transfection under these conditions could result in usefully stable MSC transfectants. To this end a retroviral transfection of bone marrow MSC using an HFBR was performed.

Methods

Bone marrow MSC from ATCC were expanded to $5X10^7$ cells using DMEM/10% FBS and 10 T300 flasks. Retrovirus encoding for green fluorescent protein and Nanoluc luciferase protein was produced in culture. A FiberCell Systems C2025D 20 kD MWCO polysufone cartridge with 450 cm^2 of area and a 2.8 mL volume was seeded as follows. $5X10^7$ cells in a volume of 5 mL was attached to one side-port and $4.5X10^8$ retrovirus in a volume of 4 mL was attached to the opposite side port. Equal volume of cell/virus mixture was flushed into each syringe and then injected into the ECS of the cartridge with the excess volume flowing through the fibers into the medium reservoir, concentrating the cells and the virus together. After 24 hours the ECS was drained, and the medium replaced with basal DMEM (no serum). Harvests were performed at 24-hour intervals for four weeks.

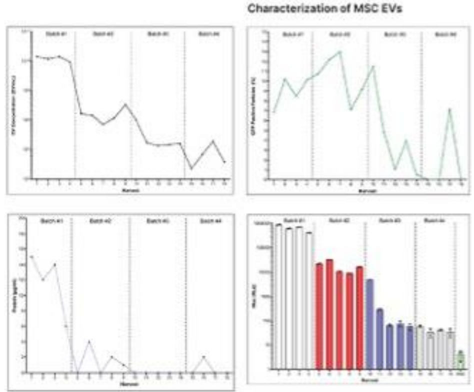
Results

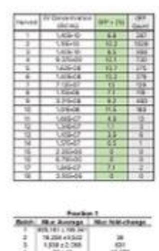
1. Isolated EVs from the first 16 days of harvest collection show strong Nluc signal and approximately 5-12% of all detected particles in those EV samples were GFP positive, indicating release of the GFP-Nanoluc fusion reporter proteins via EVs by MSC transfectants.
2. The particle counts of EVs produced by palmGRET MSC declined from $1.4X10^{10}$ particles/mL in the first harvest to $3.9X10^6$ particles/mL measured in the last harvest (day 27).
EV production was drastically reduced after 2 weeks of MSC transduction in HFBR.
3. It was not really possible to directly determine transduction efficiency under these conditions.

Discussion

A hollow fiber bioreactor can reduce the volume required to perform transductions by 100X, and utilizes a closed, cGMP compatible format. Overall, these promising preliminary data warrant further optimization and refinement of the transduction protocol, particularly by modifying the viral titer, selection strategy, and length of the experiment. Cell viability assays will also be performed to determine whether EV concentration declined after 14 days as a result of decreased cell viability or if other factors are at play. Increasing scale by a factor of 100x or more is possible using existing hollow fiber systems.

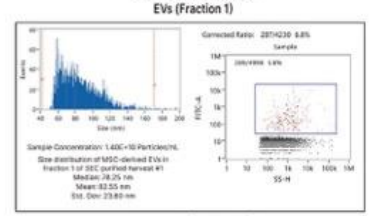
Characterization of MSC EVs



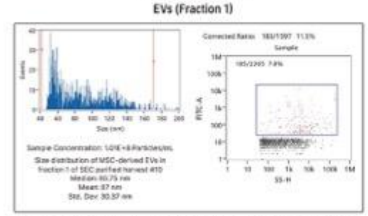


- Nanoluciferase activity measured in MSC EVs significantly decreased over time
- The particle count of EVs produced by palmGRET MSCs declined from 1.4E+10 particles/mL in the first harvest to 3.9E+06 particles/mL measured in the last harvest (day 27).

Harvest #1 (02.08.2022)
EVs (Fraction 1)



Harvest #1 (02.08.2022)
EVs (Fraction 1)



Here we determined the # of particles and the % of GFP+ particles in fraction 1 of harvest #1 of MSCs using NanoFCM

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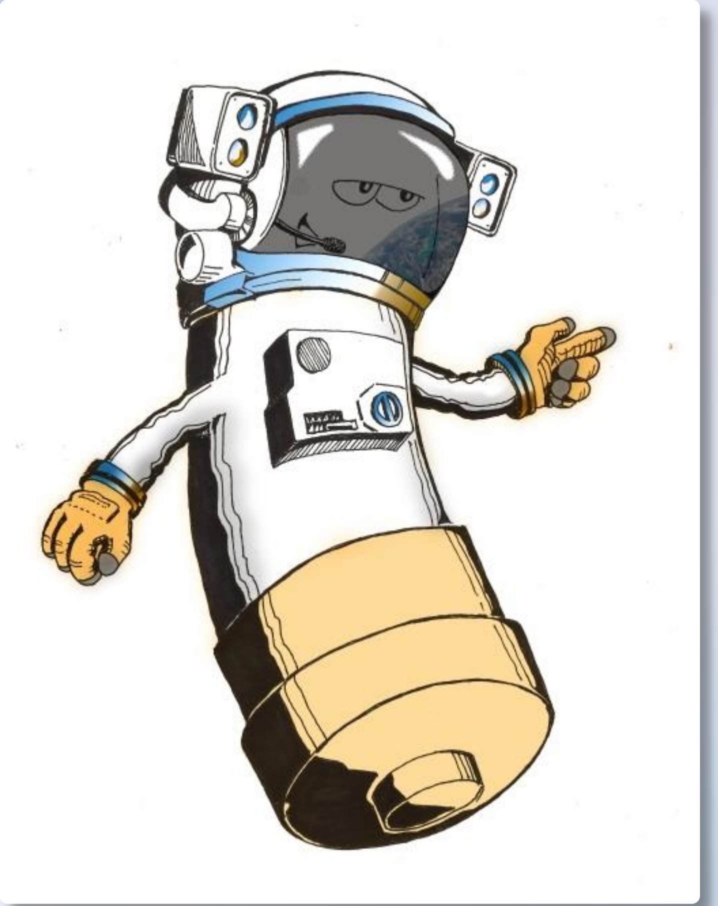
HISTOCENTRIC BIOREACTORS

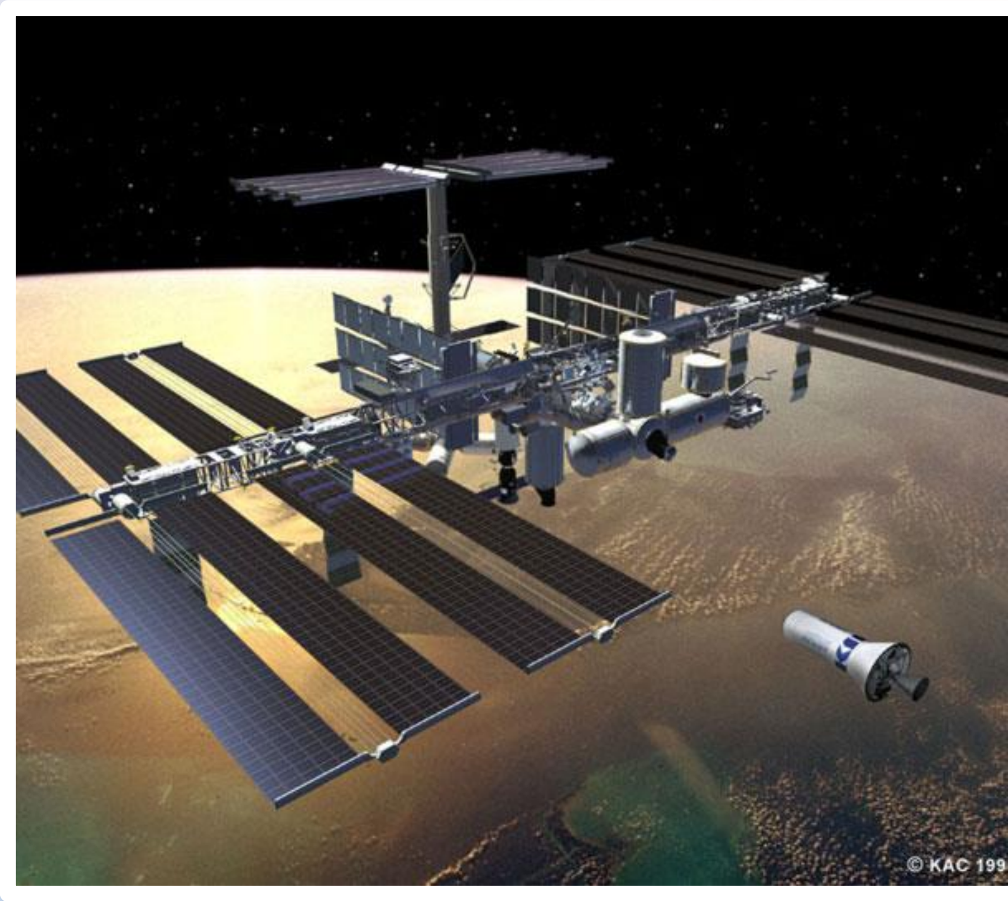
- The most *in vivo* method for culturing cells over long periods of time. Media and cells work together to generate their specific microenvironment.
- Hollow fiber was 3-D before the importance of 3-D culture was recognized
- Can be the only way to get two different cell types, in close enough proximity, at high enough density, for long enough time to observe interactions between the cells.
- 4-D culture, enough time for cells to self-organize, structures to form, and for cell-to-cell interactions to develop.

Summary

- Hollow fiber bioreactors are the method of choice for the culture of 10^9 to 10^{11} cells
- Can produce gram quantities of exosomes
- Concentration of 100x higher than with conventional methods
- The most *in vivo* method for culturing cells over long periods of time
- Suitable for cGMP production
- Permits use of FBS without endogenous EV contamination
- Enhanced bioactivity
- Saves time, space, purification costs

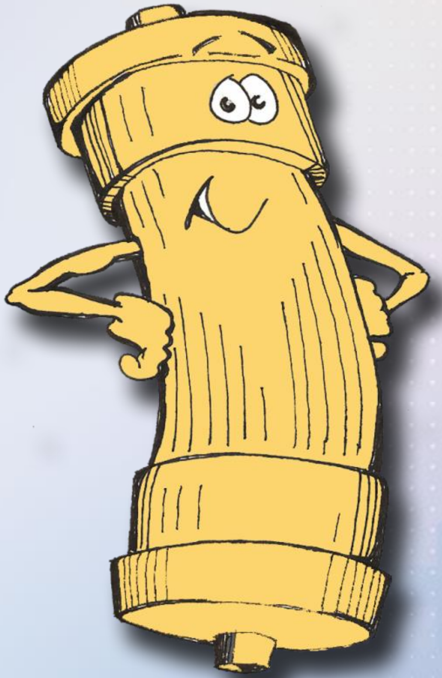
FiberCell Systems HFBR in Space






"...The evolution of cell culture"

Thank you.



Applications