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Large-Scale Production of Extracellular Vesicles from High-Density 3D Cell Cultures in Hollow Fiber Perfusion Bioreactors

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Abstract

Progress in the field of exosome research is hindered by the small cell numbers and low productivity of secreted exosomes in traditional 2-D cell culture. High-density cell cultures in hollow fiber bioreactors (HFBRs) allow continuous production of secreted biomolecules and extracellular vesicles (EVs), from large numbers of cells, over extended periods of time, at high concentrations in 3D culture. This protocol describes the collection of concentrated secreted exosomes while maintaining optimal cell mass and 3D perfusion culture conditions supporting high-density cell growth over several weeks or months of continuous production in a commercially available HFBR. The system is compact, closed, single-use, can be cGMP compliant, simple to use, and fits in a standard CO^2 incubator. Certain cells, such as mesenchymal stem cells (MSCs) and human amniotic fluid stem cells (HAFSCs), may exhibit reduced proliferation under these conditions, potentially influencing differentiation and phenotype over time. Representative results demonstrate typical EV yields and cell viability over weeks or longer of continuous production. This method provides reproducible, high-quality EV harvests suitable for both research and clinical applications.

Introduction

Extracellular vesicles are increasingly recognized for their roles in intercellular communication and therapeutic applications, including regenerative medicine. Traditional 2-D cell culture methods are labor-intensive and of limited yield, sometimes requiring hundreds of culture flasks to obtain sufficient EVs for experiments or animal studies[1,2].Clinical translation scale-up generally utilizes traditional stirred bioreactors using microcarriers or other supports. These systems are inherently non-physiologic, inefficient, and can have variable results. Hollow fiber bioreactors provide a three-dimensional, perfusion-based environment that supports high-density cell cultures within a compact footprint and on a scale potentially appropriate for clinical applications[3,4].

HFBRs offer a compact, high-density cell culture system enabling continuous production of concentrated EVs with reduced labor, time, and space compared to traditional 2-D culture[5].They enable high-density cell cultures in a compact space, supporting large-scale EV production in any laboratory. HFBRs culture large numbers of cells ($1X10^9$ to 1×10^{10}) in a small volume (20–60 mL), in a standard CO_2 incubator, and do not require any specialized equipment. These systems allow continuous, concentrated EV harvests over weeks or months while facilitating the use of reduced serum or chemically defined media. In an HFBR, cells are retained in the small volume of the extra capillary space (ECS) separated from the recirculating medium by a semi-permeable hollow fiber membrane of defined molecular weight cut-off (MWCO) (**Figure 1**). Small molecules such as glucose and lactate can easily cross the fibers, while larger elements such as antibodies or extracellular vesicles are retained and concentrated in the ECS, where they can be harvested.

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Three fundamental characteristics differentiate hollow-fiber cell culture from any other method: 1) the high surface area to volume ratio (200 cm^2/mL) permits *in vivo-like* cell densities, 1–2 × 10^8cells per mL. 2) The cells are bound to a porous support, not a non-porous plastic dish. Cell splitting is not required[3]. The molecular weight cut-off (MWCO) of the fiber can be controlled. Secreted products are retained and concentrated 10–100X compared to 2-D flask culture within the cartridge's ECS. Also, specific protein matrices, such as fibronectin or collagen, can be easily applied to the fiber surface without affecting flux across the fibers.

HFBR provides a 3D perfusion-based culture environment that is more *in vivo-like* than 2-D culture[6] and uniquely reduces cells' dependence on serum support and simplifies their medium requirements. This high-density culture environment may support differences in protein processing compared to 2-D culture, along with reported enhanced EV bioactivity[7,8,9].Hollow fiber bioreactors are "histocentric"; they attempt to mimic the tissue-like properties of *in vivo* cell physiology[10].The two key characteristics of a histocentric bioreactor are 1) high cell density, permitting the cells to generate their own specific microenvironment, and 2) splitting or passaging of cells is not required, allowing cell-to-cell interactions and 3D structures such as spheroids to develop organically over time. This method enables cell densities that are difficult to achieve with conventional 2-D culture systems and can facilitate the use of simplified, protein-free, and chemically defined media.

Any cell type that can be cultured in flasks can be cultured in a hollow fiber bioreactor. Transformed cell lines will be proliferative[11]. The key parameter to monitor is the glucose uptake rate. Glucose monitoring provides a direct measure of metabolic activity, enabling optimization of harvest timing and control of cell density[12]. It is important to control cell mass within the cartridge so that the glucose uptake rate does not exceed the system's capacity to deliver oxygen and remove CO^2. This is done by simply removing cell mass during harvesting. MSCs and HAFSCs do not proliferate in the hollow fiber bioreactor but remain quiescent. These cells require expansion prior to seeding in the bioreactor. However, once the culture is established, EVs can be harvested continuously for weeks or months. The MSC phenotype does not change over 30 days of culture, except perhaps for CD105 expression, which decreases over this time. When MSCs from the bioreactor were subsequently plated in a flask with serum-containing medium, CD105 expression returned to initial levels[13].CD105 expression may serve as a marker for 2-D vs. 3D culture. Transient transfection techniques can also be used to make a quasi-stable transfectant[14].

Examples of the more *in-vivo* cell culture conditions and advantages of 3D hollow fiber cell culture include complete and uniform post-translational modifications over time[15,16], formation of villi by gastric epithelium for cryptosporidium culture[17], 3D liver[18], 3D blood-brain barrier model[19], plasmodium sporozoite formation[20], and bone marrow/stem cell co-cultivation[21].

In HFBR, EVs are highly concentrated, with reduced contamination from intracellular proteins and membrane fragments, as cell lysis is limited. Hollow fiber bioreactors are compatible with any medium, provided there are no high-molecular-weight components that do not cross the fibers. If required, these large molecules can be added directly to the bioreactor's ECS. High glucose media, such as DMEM, are preferred. Less complex, protein-free cell culture media can simplify purification by reducing protein load. Mesenchymal stem cells and human amniotic fluid stem cells have been cultured in standard basal media and FBS, as well as in several commercially available MSC media formulations. HFBR can produce gram quantities of exosomes using off-the-shelf cartridges. A hollow fiber bioreactor is a useful method for the large-scale production of exosomes under potential cGMP conditions and represents a paradigm shift in advancing both exosome research and clinical translation.

The successful use of a hollow fiber bioreactor for extracellular vesicle production is highly protocol-specific. Manipulation of the bioreactor, cell seeding density, harvesting frequency, and the medium used can all contribute to a successful bioreactor run. This protocol describes a standardized method for using a hollow fiber bioreactor to produce and collect extracellular vesicles from high-density cell cultures. The goal is to provide a reproducible approach for EV harvesting, including guidance on system setup, cell inoculation, culture maintenance, and harvesting procedures. The method is intended to support consistent EV production for research applications and to provide a framework that may be adapted for different cell types and experimental objectives.

Protocol

All procedures comply with standard Good Laboratory Practice (GLP) and Good Cell and Tissue Culture Practice guidelines.

NOTE: The cartridge is pre-assembled and gamma sterilized. It is double-bagged, and luer caps are on each connection, so it is completely sealed and will remain sterile outside the bags. See **Figure 2**for cartridge port identification. All steps were performed at room temperature in a laminar-flow hood.

1. Preparation and sterilization of the Reservoir Cap.

1. Remove the two short pieces of tubing from the double-bagged cartridge.
2. Attach the tubing to the hose barbs on the stainless-steel tubing of the reservoir cap.
3. Wrap the short tubing and the exposed ends of the stainless-steel tubing with aluminum foil to maintain sterility after

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autoclaving. Place the assembled components into an autoclave bag.

4. Autoclave using a dry cycle at 121 °C for 15–30 min. Allow the cap to cool to room temperature before use.

2. Attach the hollow fiber cartridge to the reservoir bottle

1. Transfer 50 mL of PBS from a 1 L bottle into a 50 mL conical tube for later use.
2. Remove the reservoir cap from the autoclave bag and remove protective foil from the long steel tubes.
3. Remove the cap from the PBS bottle and screw the reservoir cap in its place. Remove the cartridge from the inner sterile bag.
4. Remove the aluminum foil from one of the pieces of tubing on the top of the reservoir cap.
5. Remove the luer cap from one of the long pieces of cartridge tubing and attach it to the reservoir tubing on the reservoir cap. Use a little backspin to prevent kinking.
6. Repeat for the second tubing connection.
7. Gently squeeze the brown pump tubing to drive PBS through the system until all air bubbles are removed.

3. Filling the extra capillary space (ECS)

1. Draw 20 mL PBS into a sterile syringe using a large-gauge needle at least 1.5" in length; a blunt needle is preferred for safety.
2. Disinfect both side-port luer fittings with alcohol and allow them to dry.
3. Attach the PBS-filled syringe to one side port and an empty sterile syringe to the opposite port. Close both end ports and open both side ports.
4. Slowly inject PBS into the ECS while holding the cartridge at a slight angle to minimize air bubbles. The empty syringe on the opposite side port will fill with air.
5. If air is present in the ECS, open the right end port and withdraw fluid into the empty syringe until the ECS is filled with liquid (this pulls PBS from the reservoir bottle, through the fibers, and into the ECS).
6. Close both side port slide clamps, open both end port slide clamps, and verify flow by gently compressing the pump tubing with your fingers.
7. Leave the empty syringes attached to the side ports to serve as caps. Replace the syringes with new ones every time they have been out of the laminar flow hood.

4. Coating fibers and cell inoculation

NOTE: The procedures for coating fibers with a specific matrix protein and for inoculating cells are essentially the same. Excess liquid will flow through the fibers and into the reservoir, while the cells or coating material will remain on the outside of the fibers in the ECS. 1 × 10^8 cells for seeding are recommended. 100–500 µg of matrix protein in 20 mL of PBS is recommended for the 4,000 cm^2 HFBR. Matrix proteins bound to fiber surfaces do not affect MWCO

1. Prepare coating solution or cell suspension in a sterile 50 mL conical tube. 1 X 108 cells in conditioned medium from flask expansion or 100–500 ug matrix protein in PBS. Volume 20 mL.
2. Withdraw 20 mL of cells or coating solution into a sterile syringe using a large-gauge needle.
3. Attach the syringe containing the solution to one side port and an empty sterile syringe to the other side port.
4. Close both end ports and open both side ports.
5. Gently mix the solution by transferring fluid between the two syringes. Leave 1/2 of the solution in each syringe. Close both side ports.
6. Open the right end port and loosen the reservoir cap 1/4 turn.
7. Inject the solution from one syringe into the ECS. Close the attached side port.
8. Inject the solution from the other syringe. Close the attached side port. Open both end ports.
9. If inoculating cells, leave the cartridge in a horizontal position for 30 min at room temperature, then rotate 180 ° along the long axis and let it sit for an additional 30 min.
    1. If coating the fibers with matrix protein, leave overnight at room temperature.
10. Place the cartridge into the Duet pump system and initiate flow at 25.

5. Preparation for harvest (see **Figure 2**)

1. Attach an empty sterile syringe with a 3-way stopcock and sterile filter to the right side port.
2. Set the 3-way stopcock to allow venting through the sterile filter. Attach a second empty sterile syringe to the left side port.

6. Collection of conditioned medium (Harvest)

1. Open the right-side port and maintain sterile venting through the filter.

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2. Tilt the right end of the cartridge upwards and withdraw conditioned medium (product) into the left syringe. Completely drain the ECS contents into the left syringe.
3. Close the right-side port. Close the left side port, remove the syringe, and collect the harvest into a 50 mL conicaltube.
4. Replace the syringe on the left side port. Open the left side port. Open the right end port.

   Unscrew the reservoir cap 1/4 turn.
5. Tilt the left end of the cartridge upwards and fill the ECS by withdrawing on the syringe connected to the left side port. Fill the ECS completely until the liquid meniscus is in the middle of the side port tubing.
6. Close the right end port. Open the right-side port and set the 3-way stopcock to vent through the filter.
7. Open the left side port. Tilt the cartridge to the right and drain the ECS completely into the left syringe.
8. Close the left side port. Remove the syringe and collect the harvested ECS into the 50 mL conical tube.
9. Replace the syringe into the left side port. Close the right-side port. Open the right-end port
10. Tilt the left side of the cartridge upwards and fill the ECS by withdrawing on the left syringe. Fill the ECS completely until the liquid meniscus is in the middle of the side port tubing.
11. Close the left side port. Open the left end port. Tighten the reservoir cap.
12. Confirm system flow by manually compressing the pump tubing before returning the cartridge to the Duet pump.

7. Insert the bioreactor into the pump system.

1. Set the pump flow rate to a setting of 25 on the control box. The cartridge is easier to install while the pump is running.
2. Grasp the right edge of the flow path stand. Slide the pump tubing into the pump tubing.
3. Slide the base of the flow path into the slot on the pump base. Ensure that the rectangular register snaps into the cut-out on the flow path stand.
4. Place the reservoir bottle in the indentation on the side of the pump base.
5. The thin cord for power will fit in between the gasket on the incubator. The control box has a magnet for attaching it to the side of the incubator.

8. Monitor glucose levels

1. Using a 1 mL pipette, remove a 1 mL sample of medium from the reservoir bottle.
2. Measure glucose concentration using a compatible glucose meter by placing a small drop of sampled medium onto the meter test strip.

9. Calculation of Glucose Uptake Rate

NOTE: Glucose uptake rate is calculated by subtracting the glucose measurement 24 h later from the initial glucose measurement. Since the rate depends on the reservoir volume, multiply the drop in glucose concentration by the reservoir volume in liters. For example, if there are 250 mL in the reservoir bottle, multiply by 0.25 (25%).(Glucose1-Glucose2) X (reservoir bottle in liters). 250 mL is 0.25 L. If more than one day has elapsed, divide this by the number of days. For example: Starting glucose 4.5 g/L; measure glucose 24 h later: 2.7 g/L; reservoir bottle: 250 mL. 4.5-2.7=1.8 g. 1.8X.25 (reservoir bottle is 0.25 L), 0.45 g of glucose consumed. The glucose uptake rate is 450 mg/day.

10. Viability Check

1. Collect a small sample of cells utilizing the harvest protocol.
2. Plate cells into a T 25 culture flask with fresh 10% serum containing medium. Visually evaluate attachment and viability.

11. Cartridge daily maintenance and the first week of culture.

NOTE: During the first week of culture, proliferative cells will be expanding to their target rate of 1–2 grams of glucose consumed per day.

1. Day 0: Inoculate cells, determine glucose starting concentration from media formulation. Starting reservoir volume: 125 mL.
2. Day 1-2: Measure glucose each day. When the starting glucose is 50% depleted, replace the 125 mL reservoir with 250 mL fresh media, and reduce the serum to 7.5%.
3. Day3-4: Measure glucose. When the starting glucose is 50% depleted, replace the 250 mL reservoir with 500 mL fresh media, and reduce the serum to 5%.
4. Day 4-5: Measure glucose. When the starting glucose is 50% depleted, replace 500 mL with 1L of fresh medium. Reduce the serum to 2.5% and switch to chemically defined media if desired.
5. Day 6+: Measure glucose, harvest ECS. Control cell density by harvesting to keep glucose uptake between 1 and 2 grams per day.
6. For non-proliferative cells, start with 125 mL in the reservoir bottle, and change the medium every 3 days.

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7. If there is significant glucose consumption, increase the reservoir to 250 mL. Remove the serum from the ECS on days 3-4 and replace it with basal medium.

Results

Following this protocol, a 4,000 cm^2 cartridge seeded with proliferating cell lines typically consumes 1–2 g of glucose per day. This translates to 1 L of medium every 2–3 days when using a high-glucose medium such as DMEM. Cells reach maximum density within one week after seeding. Harvesting should commence after 5–7 days or when the glucose uptake rate reaches 1 g per day and should then be performed every day, if possible. During this first week of culture, the serum-containing medium in the ECS can be replaced with serum-free basal medium while maintaining serum in the circulating medium, if serum is required. Once the glucose uptake rate reaches 1 g/day, you can easily switch to reduced-serum media or other chemically defined media. MSCs and HAFSCs consume glucose at a much lower rate than transformed cell lines, such as CHO, 293, or hybridomas. Formation of spheroids is typical of MSC and HAFSC, along with other cell types (**Figure 3**). Daily glucose monitoring provides a reliable measure of culture health and metabolic activity. Standard, commercially available xeno-free media have been successfully used with MSC and HAFSC. Harvest volumes are about 20 mL. 24 h intervals between harvests are optimal as EV concentrations reach a maximum after 24 h (**Figure 4**). Typical exosome concentrations in harvested medium range from 1–5 × 10^{12} particles/mL, depending upon cell type, cell seeding numbers, and other factors, and can result in visible pellets after centrifugation (**Figure 5**). Large numbers of cells can be seeded and should be based on cell number, not culture area (**Figure 6**).

The results demonstrate that EV production correlates with metabolic activity as measured by glucose consumption, providing a practical parameter for monitoring and optimizing culture performance. Daily harvesting at 24 h intervals maximizes EV concentration while maintaining culture stability. These findings support the use of glucose monitoring and controlled harvesting frequency as key parameters for reproducible EV production in hollow fiber bioreactors.

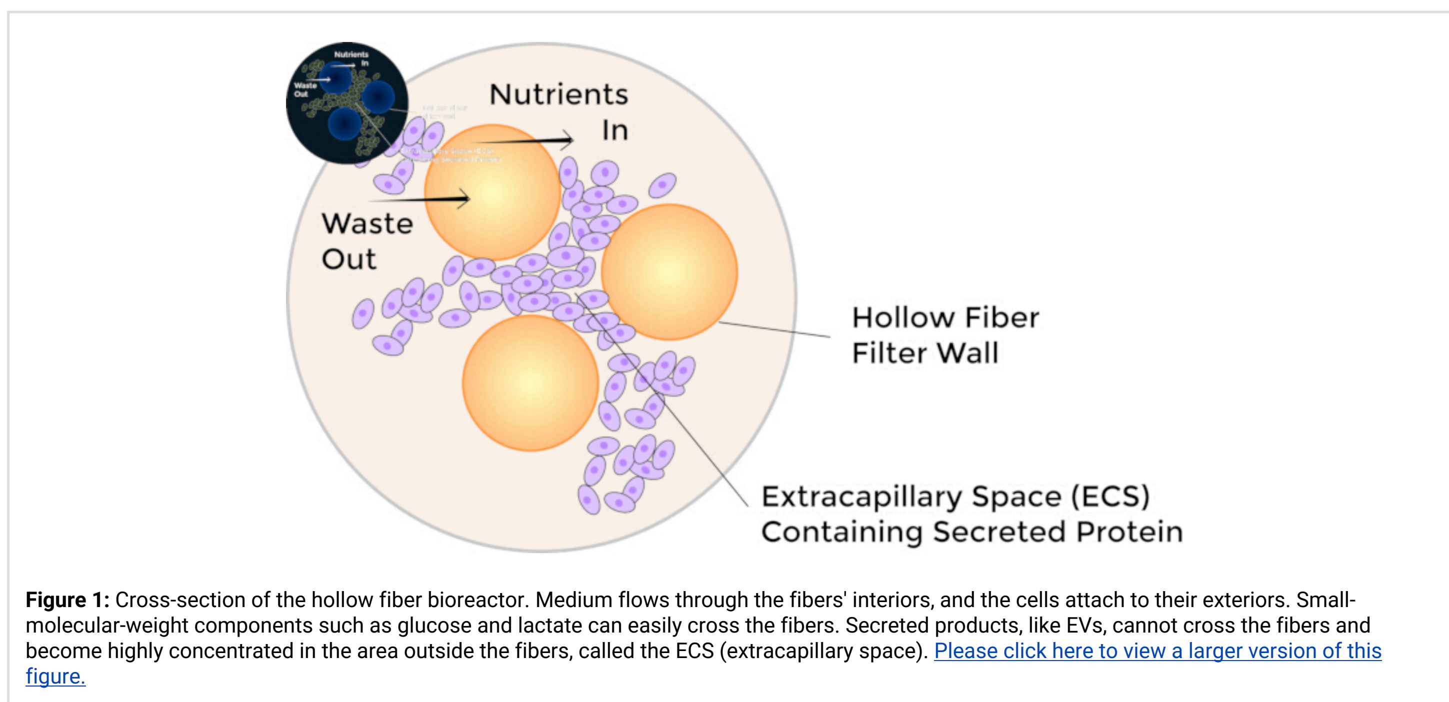

Figure 1: Cross-section of the hollow fiber bioreactor. Medium flows through the fibers' interiors, and the cells attach to their exteriors. Small-molecular-weight components such as glucose and lactate can easily cross the fibers. Secreted products, like EVs, cannot cross the fibers and become highly concentrated in the area outside the fibers, called the ECS (extracapillary space). Please click here to view a larger version of this figure.

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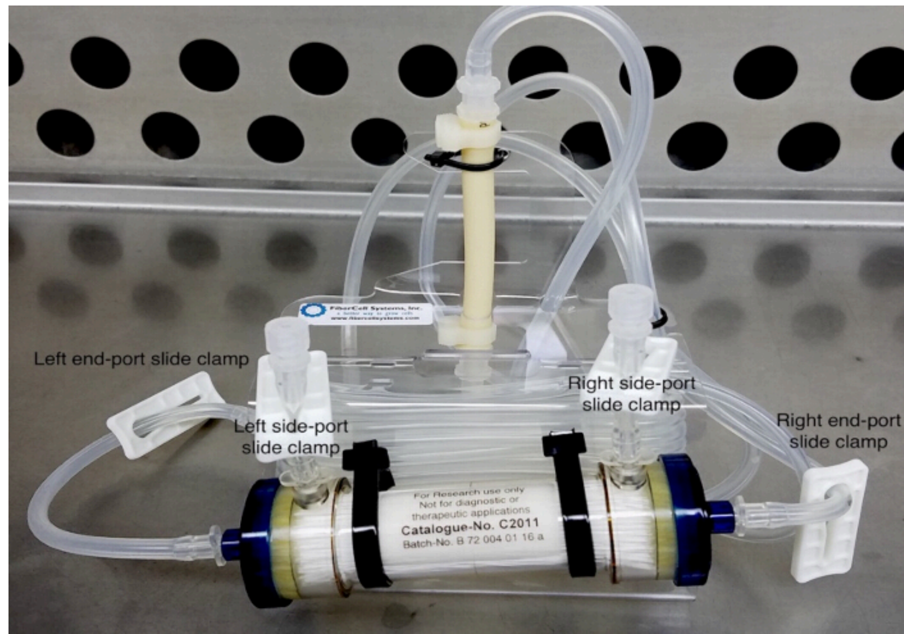

Figure 2: Photo of cartridge with ports and clamps identified. One-way check valves on the flow path generate a recirculation flow of medium. Please click here to view a larger version of this figure.

Figure 3: Mesenchymal stem cells, Human Amniotic Fluid Stem Cells, Cardiac progenitors, and some other cell types will form spheroids when seeded into the cartridge in sufficient numbers. Please click here to view a larger version of this figure.

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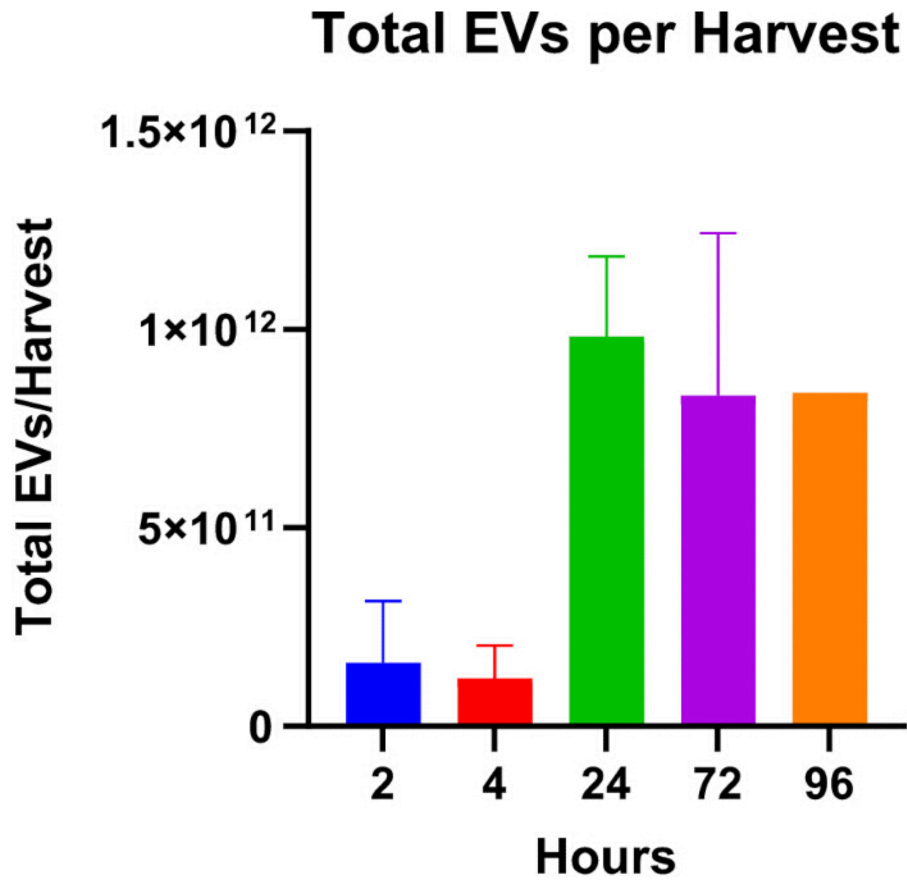

Figure 4: Measurement of EV concentrations after different time interval harvests. After 24 h, the EV concentration reaches a maximum concentration. Optimum harvesting is after 24 h. (Data courtesy of Paolo Neviani, EV core, Children's Hospital of Los Angeles). Please click here to view a larger version of this figure.

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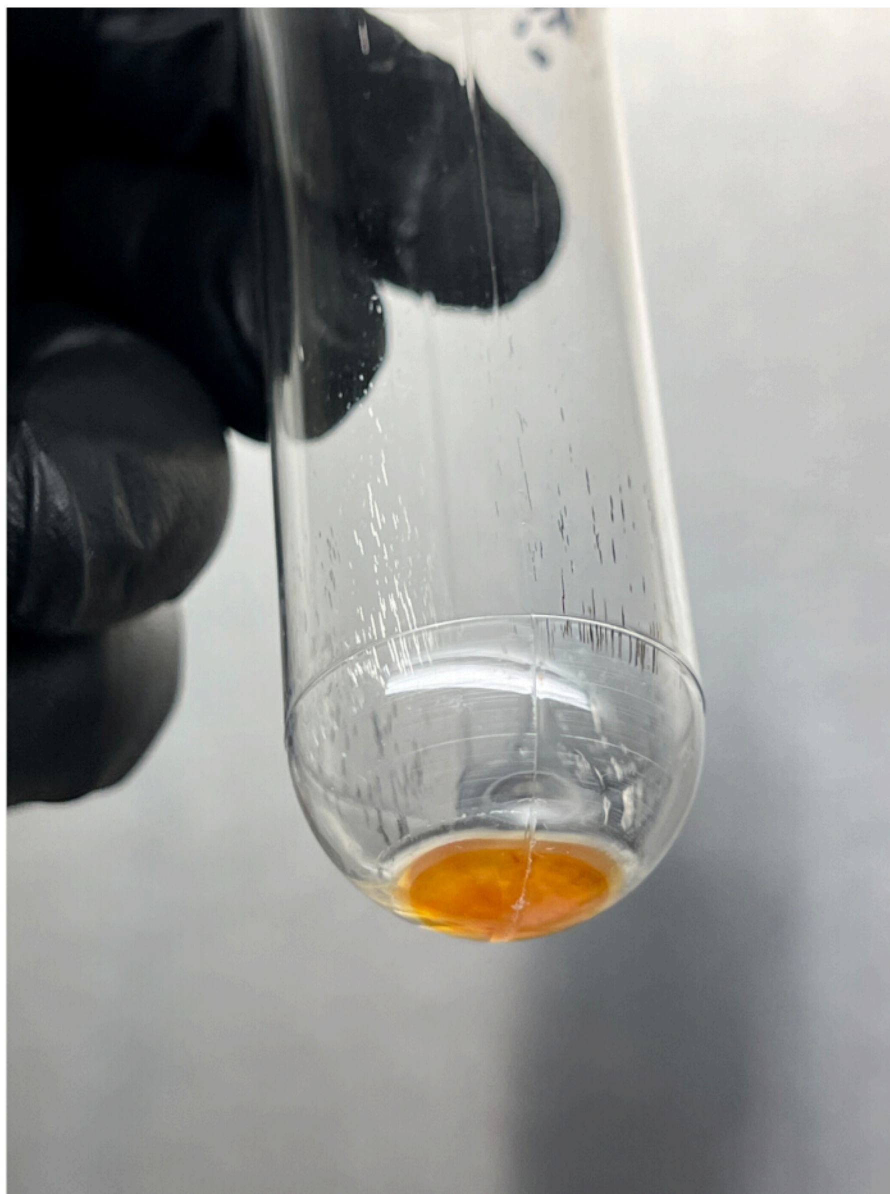

Figure 5: One week's harvest of EVs from DIFI cells cultured in the C2011 hollow fiber bioreactor. EVs isolated by differential centrifugation. (data courtesy Jeff Franklin, Vanderbilt University)[18,19]. Please click here to view a larger version of this figure.

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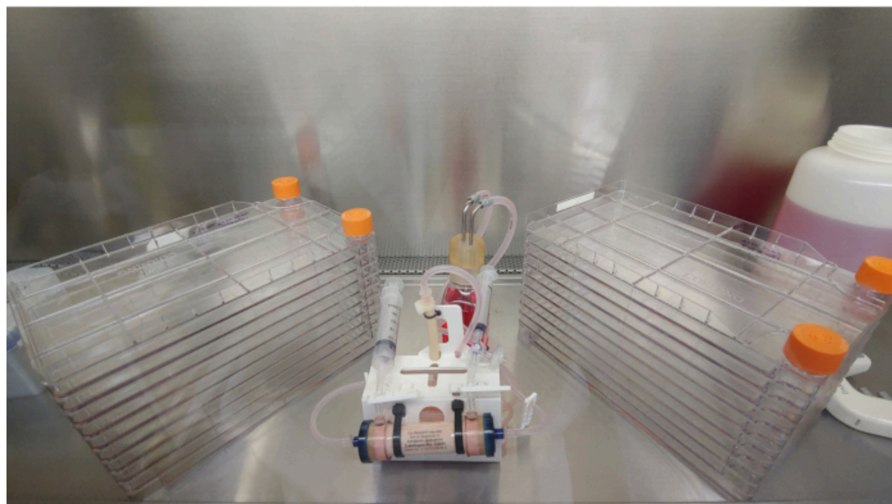

Figure 6: A surprisingly large number of cells can be seeded into the hollow fiber bioreactor. MSCs occupy a lot of area in 2-D culture; when grown as spheroids in 3D culture, they occupy less space. Please click here to view a larger version of this figure.

Discussion

Hollow fiber bioreactors are unable to reasonably manufacture proteins or antibodies in true commercial quantities. However, they have a unique ability to culture relatively large numbers of cells at high density and secrete a concentrated EV product at a clinically relevant scale. It is only in the past few years that the true advantages of a histocentric bioreactor have been recognized. The high-density perfusion culture conditions result in concentrated EVs, using cGMP-compliant medium, in quantities that may be suitable for research and potential clinical applications. A single harvest from a 4,000 cm^2 may contain 5×10^{12} to 1×10^{14} EVs in a volume of 10–20 mL. These yields are within the range reported in studies evaluating EV dosing[22].

Daily monitoring of glucose uptake rate is an important parameter for assessing culture performance and is a direct indicator of cell metabolism, cell number, and health. Generally, higher glucose media are preferred, such as DMEM, which has a starting glucose concentration of 4.5 g/L. The medium should be changed to fresh medium when the glucose is 50% consumed. Certain cells, such as MSCs, seem to consume glucose and produce lactate at glucose concentrations above 3 g/L, then switch to consuming glutamine and producing ammonia at concentrations below 3 g/ L. Complete metabolic profiling of MSCs in 3D culture has not yet been performed.

The most critical parameter for a successful bioreactor culture is the cell inoculation number. A minimum inoculation cell number for a 4,000 cm^2C2011 bioreactor is 1×10^8 cells. For non-proliferating cells such as MSC or HAFSC, a greater number is preferred, up to 1×10^9 total cells. The reservoir volume should be proportional to the number of cells in the cartridge. 1×10^8 cells should use a reservoir no larger than 125 ml. 1×10^9 cells should have a reservoir no larger than 1 L. A reservoir that is too large can send cells into the lag phase. This is characterized by no glucose consumption by the cells, even though they are viable as determined by Trypan Blue staining. In this case, reduce the reservoir volume or seed more cells. Multiple cell inoculations may be required to get sufficient cell numbers for an effective bioreactor culture. Simply seed more cells on top of the cells already in the cartridge. When evaluating bioreactor performance, note the cell inoculum size. A 10X increase in cell seeding can result in 10X greater EV production.

Modifications to the protocol may include changes in media formulations, surface coatings, cell numbers, harvest volume, harvest frequency, or syringe size, depending on the cell type and desired yield. Continuous culture enables easy, real-time condition optimization, as changes can be made during the culture and results obtained in real time[21]. Troubleshooting should focus on maintaining sterility and monitoring metabolic activity. Ensure there are no blockages or kinks in the tubing, especially around the slide clamps. Bacterial contamination will result in rapid glucose consumption and cloudiness in the reservoir bottle. Fungal contamination is indicated by a fluffy white accumulation at the left end cap of the cartridge, blocking flow. Use of the hollow fiber bioreactor system requires skillful handling and strict adherence to protocols. Critical steps include seeding the correct number of cells, maintaining sterility during harvest, and carefully monitoring glucose consumption to prevent overgrowth or nutrient depletion. Most cells will attach to the fibers, sometimes depending on the fibronectin-coated protein matrix, which facilitates rapid cell

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attachment. Some cells, such as MSCs, HAFSCs, and cardiac progenitors, remain attached to fibers and form 3D spheroids.

The HFBR system supports high-density, chemically defined, and xeno-free cultures of most cell types, facilitating large-scale EV production[23]. Daily glucose monitoring provides a reliable metric for bioreactor performance. The closed, perfusion-based system is compatible with cGMP standards, and scale-up is possible using larger cartridges or even multiple cartridges. Gram-scale EV production may be possible under optimized conditions. The hollow fiber bioreactor provides a reproducible platform for sustained EV production and collection under controlled conditions. The protocol outlined here emphasizes practical considerations, including system setup, aseptic handling, and metabolic monitoring, to support consistent operation and reliable outcomes. Further studies may be required to evaluate EV characteristics and functional properties under different culture conditions and to compare performance with alternative production systems.

This method is broadly applicable for research and clinical EV production, including regenerative medicine and therapeutic applications.

Disclosures

The author is the president and CEO of FiberCell Systems Inc.

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