Perfusion and hollow fibre reactor

What is a perfusion bioreactor?

Perfusion, or upstream continuous bioprocessing, has been practiced since the 1980s towards maximizing facility utilization, expanding process flexibility and minimizing costs. Innovative technologies, sophisticated control logic systems, cell culture supplements and single-use assemblies have tremendously simplified implementation, establishing perfusion as the cornerstone of intensification and continuous processing.

Perfusion cell culture utilizes a cell retention device and continuous media exchange to achieve and maintain high cell densities and viabilities over extended periods of time, typically weeks. The cell retention device retains cells inside the bioreactor, while fresh media is added, and product of interest, waste products and spent (or depleted) media are continuously removed. Fresh media is provided at the same rate that product and spent media are removed from the bioreactor. Hollow fiber-based membrane filters acts as the most reliable and commonly used membrane type. Long term perfusion is just one application of prefusion and contrasts with shorter time-based perfusion applications, such as HPH that focus on intensifying fed patch processes over the course of days. Perfusion may also be used to intensify the seed train with reduced steps and faster overall time to production.

Early perfusion technologies, developed during the 1980s, frequently negatively impacted cell health and entailed complicated engineering scale-up. Despite promising advantages, adoption was not widespread. In addition, advances in cell line engineering, media composition, and bioreactor design generated multiple-fold increases in product titer from batch and fed-batch cell cultures, temporarily reducing the need for, and the impact of, perfusion cell culture approaches.

The recent pursuit of alternative manufacturing strategies that can boost facility capacity, throughput, and productivity with reduced costs and scale of operation has led to a renewed interest in perfusion technology and its benefits. Technology innovations, such as XCell ATF®, using novel alternating tangential flow filtration, have been instrumental in solving historical challenges and in shifting the paradigm towards perfusion as a key platform for modern upstream processing.

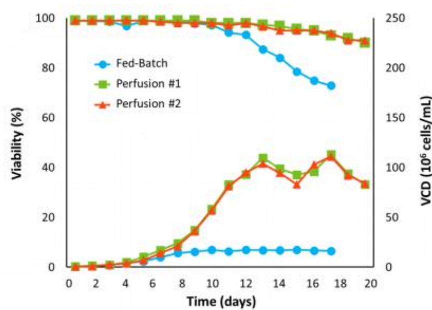
A **Hollow fiber bioreactor** is a 3-dimensional cell culturing system based on hollow fibers, which are small, semi-permeable capillary membranes arranged in parallel array with a typical molecular weight cut-off (MWCO) range of 10-30 kDa. These hollow fiber membranes are often bundled and housed within tubular polycarbonate shells to create hollow fiber bioreactor cartridges. Within the cartridges, which are also fitted with inlet and outlet ports, are two compartments: the intracapillary (IC) space within the hollow fibers, and the extracapillary (EC) space surrounding the hollow fibers.

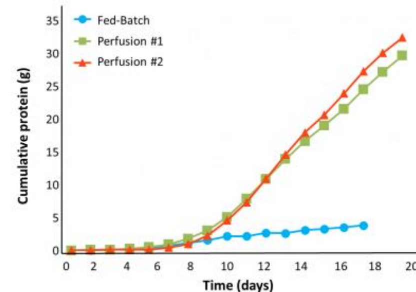
Cells are seeded into the EC space of the hollow fiber bioreactor and expand there. Cell culture medium is pumped through the IC space and delivers oxygen and nutrients to the cells via hollow fiber membrane perfusion. As the cells expand, their waste products and CO_2 also perfuse the hollow fiber membranes and are carried away by the pumping of medium through the IC space. As waste products build up due to increased cell mass, the rate of medium flow can also be increased so that cell growth is not inhibited by waste product toxicity.

Because thousands of hollow fibers may be packed into a single hollow fiber bioreactor, they increase the surface area of the cartridge considerably. As a result, cells can fill up the EC space to densities $>10^8$ cells/ml. However, the cartridge itself takes up a very small volume (oftentimes the volume of a 12-oz soda can). The fact that hollow fiber bioreactors are very small and yet enable incredibly high cell densities has led to their development for both research and commercial applications, including monoclonal antibody and influenza vaccine production. Likewise, because hollow fiber bioreactors use up significantly less medium and growth factors than traditional cell culture methods such as stirred-tank bioreactors, they offer a significant cost savings. Finally, hollow fiber bioreactors are sold as single-use disposables, resulting in significant time savings for laboratory staff and technicians.

Perfusion achieves 100-130 million VCD compared to 13 million for Fed-Batch. Perfusion culture viability is maintained above 90% over a period of 17 days. Fed-Batch viability begins to drop after day 9 to approximately 70% at days 14-15. Higher VCD and viability of perfusion cultures translates to an increase in total product yield. Perfusion process yields approximately 30-35 g. Fed-Batch process achieves 3 g in approximately 15

days.





Higher cell density, higher viability

- Unoptimized perfusion process achieves up to 10X VCD over optimized Fed-Batch
- Perfusion enables higher cell growth rates and healthier cultures

Higher productivity

- Higher cell density and viability of perfusion culture enables 10X yield increase over Fed-Batch

1. Monolayer Culture:

Monolayer cultures are essential for anchorage dependent cells. Scaling up of such cultures is based on increasing the available surface area by using plates, spirals, ceramics and micro-carriers (most effective). The various culture vessels used are briefly described below.

Roux Bottle:

It is commonly used in laboratory, and is kept stationary so that only a portion of its internal surface is available for cell anchorage . Each bottle provides Ca. 175- 200 cm^2 surface area for cell attachment and occupies 750-1000 cm^3 space.

Roller Bottle:

This vessel permits a limited scale up as it is rocked or preferably, rolled so that its entire internal surface is available for anchorage. Several modifications of roller bottle further enhance the available surface,

e.g., (i) Spira-Cel (spiral polystyrene cartridge),

(ii) glass tube (roller bottle packed with a parallel cluster of small glass tubes separated by silicone spacer rings), and

(iii) extended surface area roller bottle (the bottle surface is corrugated enhancing the surface by a factor of two), etc.

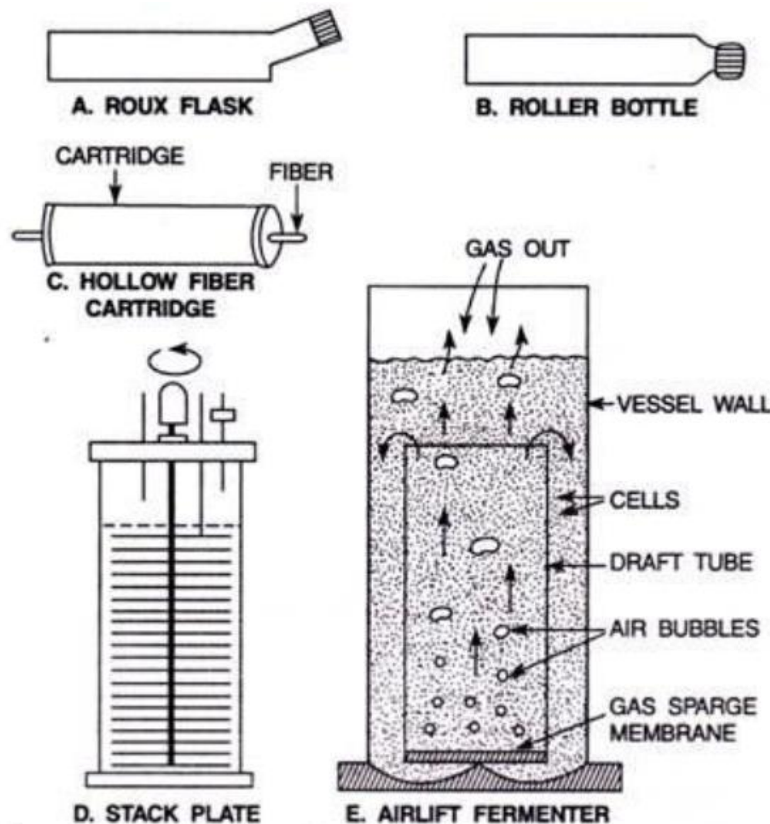


FIG. 5.3. A schematic representation of some of the various types of culture vessels used for scaling up of animal cell cultures. A. Roux flask, B. Roller bottle, C. Hollow fiber cartridge, D. Stack plate vessel, and E. Airlift fermentor.

Multitray Unit:

A standard unit has 10 chambers stacked on each other, which have interconnecting channels; this enables the various operations to be carried out in one go for all the chambers.

Each chamber has a surface area of 600 cm^2 and the total volume of the unit is 12.5 1. This polystyrene unit is disposable and gives good results similar to plastic flasks.

Synthetic Hollow Fibre Cartridge:

The fibres enclosed in a sealed cartridge provide a large surface area for cell attachment on the outside surface of fibres. The capillary fibres are made up of acrylic polymer, are 350 µm in diameter with 75 µm thick walls. The medium is pumped in through the fiber; it perfuses through the fiber walls and becomes available to the cells. The surface area available is very high (upto 30 cm^2/ml of medium volume). The system is mainly used for suspension cells, but is also suitable for cell anchorage if polysulphone type fiber is used.

Opticell Culture System:

It consists of a cylindrical ceramic cartridge in which 1 mm^2 channels run through the length of the unit, and perfusion loop to a reservoir is provided for environmental (medium, gas, etc.) control. It gives about 40 cm^2 surface area/ml of medium. It is suitable for virus, cell surface antigen and monoclonal antibody production, and for both suspension and monolayer cell cultures.

Plastic Film:

Teflon (fluoroethylenepropylene copolymer) is biologically inert and highly permeable to gas. Teflon bags (5 x 30 cm) filled with cells and medium (2-10 mm deep) serve as good culture vessels; cells attach to the inside surface of bags. Alternatively, teflon tubes are wrapped round a reel with a spacer and the medium is pumped through the tube; cells grow on the inside surface of tube (a culture vessel called stericell is available).

Heli-Cell vessels:

These vessels are packed with polystyrene ribbons (3 mm x 5-10 mm x 100 µm) that are twisted in helical shape. The medium is pumped through the vessel, the helical shape of ribbons ensuring good circulation; the cells adhere to the ribbon surfaces.

All the culture vessels, in addition to the increased surface area due to the vessel design, allow further scaling up by the use of multiple units of the vessels. In contrast, the following three culture systems allow scaling up in a single unit by increasing the vessel volume. In addition, they make the monolayer culture system considerably similar to suspension cultures.

Bead Bed Reactors:

These reactors are packed with 3-5 mm glass beads (which provide the surface for cell attachment) and the medium is pumped either up or down the bead column. Use of 5 mm beads gives better cell yields than that of 3 mm beads.

Heterogeneous Reactors:

These reactors contain circular glass or stainless steel plates stacked 5-7 mm apart and fitted to a central shaft . Either an airlift pump is used for mixing or the shaft is rotated either vertically or horizontally. The chief disadvantage of the system is very low ratio of surface area to medium volume (1-2 cm^2/ml).

Microcarrier cultures:

These systems use 90-300 pm dia particles as substrate for cell attachment. Initially, Dextran beads (Sephadex A-50) were used by Van Wezel in 1967; these were not entirely satisfactory due to the unsuitable charge of beads and possibly due to toxic effects.

The microcarriers available for use at present range from Dextran, polystyrene, polyacrolein, glass, poly- acrylamide, silica, DEAE sephadex, cellulose, gelatin to collagen; the specific gravity of microcarriers ranges from 1.02 to 1.05. Microcarriers greatly increase the surface area for cell attachment per unit medium volume, which can be upto 90,000 cm^2/ L depending on the size and density of the beads.

Microcarrier cultures are initiated by harvesting cells from 3 L of a logarithmic phase (log phase) culture and inoculating them in 1 L of fresh medium to which 2-3 g/L of microcarriers is then added. The culture is stirred at 15-25 rpm (revolutions per minute) for 3-8 hours. During this period, cells attach to microcarrier beads and later grow as a monolayer.
The volume of culture is slowly increased to 3 1 and stirring is enhanced to the normal rates (20-100 rpm). As the cells grow, the beads become heavier and need to be agitated at higher speeds. The medium needs to be changed every 3 days. Samples of beads can be drawn for observations on cell morphology, growth and number.

Use of microcarriers permits the handling of monolayer systems as suspension cultures. However, cells do not grow to the same degree as they do in stationary cultures. Harvesting of

cells from microcarrier beads in rather simple.Stirring is stopped, the medium is drained off, the beads are washed in buffer, treated with trypsin or some other suitable enzyme, the culture is shaken at 75-125 rpm for 20-30 min, stirring is stopped for 2 min and the supernatant is poured and collected.

Alternatively, the beads may be dissolved where possible, e.g., gelatin beads are dissolved by trypsin, collagen-coated beads are treated with collagenase and dextranase is used for dextran beads; these treatments leave the cells free, which are collected.

Scaling up of microcarrier cultures can be done either by increasing the concentration of beads or by enlarging the culture vessel. When high microcarrier concentrations are used, medium perfusion becomes necessary, and efficient filters must be used to allow medium withdrawal without cells and microcarriers. The oxygen supply is problematic; it can be based on surface aeration, increased perfusion rate of fully aerated medium, and sparging into the filter compartment.

2. Suspension Cultures:

In scaling up, both chemical (O_2, pH, medium constituents and removal of wastes) and physical (the configuration of bioreactor and power supplied to the reactor) factors have to be optimised for good results. The medium must be suitably stirred to keep the cells in suspension and to make the culture homogeneous; it becomes increasingly difficult with the scaling up.

Various types of stirrers range from simple magnetic stirrers, flat blade turbine impellers, to marine impellers, to those using pneumatic energy, e.g., airlift fermenter, and those using hydraulic energy, e.g., medium perfusion. Improved mixing can be obtained by changing the design of stirrer paddle or by using multiple impellers.

The objective of stirring is to achieve good mixing without causing damage to the cells. Vibro-mixer achieves stirring by vertical reciprocating motion of 0.1-S mm at a frequency of 50 cycles/sec of a mixing disc fixed horizontally to the agitator shaft. These stirrers cause random mixing, less foaming and lower shear forces.

It is important to supply sufficient O_2 without damaging the cells. Mean 0_2 utilization rate by cells is about 6 mg $O_2/10^6$ cells/hour. But O_2 is only sparingly soluble in culture medium; the oxygen transfer rate (OTR) from gas phase into medium is about 17 μg /cm^2/hr.

Therefore, surface aeration can support about 50 x 10^6 cells in a 1 L culture vessel. When the medium depth in a culture is above 5 mm (especially, above 5 cm), aeration with a mixture of $C0_2$ and air becomes necessary to maintain adequate gas exchange.

Efficient aeration is achieved by bubbling air through the medium (sparging), but this may damage animal cells due to the high surface energy of the bubble and on the cell membrane. The damage can be reduced by using larger bubbles, lower gassing rates and by adding non-nutritional supplements like Pluronic F-6B (polyglycol), sodium carboxymethyl cellulose and polyvinylphyrrolidone (these protect cells from damage due to shear forces and bubbles, respectively). Silicone tubing (highly gas permeable) can be arranged inside the culture vessel (2-5 cm tubing of 30 m length for a 1000 L culture) and air is passed though the tube; however it is inconvenient to use.

Aeration may be achieved by medium perfusion, in which medium is continuously taken from culture vessel, passed through an oxygenation chamber and returned to the culture. The cells are removed from the medium taken for perfusion so that the medium can be suitably altered, e.g., for pH control. Perfusion is used with glass bead and, more particularly, with micro carrier systems.

Where considered safe and desirable, O_2 supply in the culture vessel can be enhanced from the normal 21% to a higher value and the air pressure can be increased by 1 atmosphere. This increases the O_2 solubility and diffusion rates in the medium, but there is a risk of O_2 toxicity.

The reactors used for large scale suspension cultures are of 3 main types:

(1) Stirred tank bioreactors,

(2) Continuous flow reactors, and

(3) Airlift fermenters.

Stirred Tank Bioreactors:

These are glass (smaller vessels) or stainless steel (larger volumes) vessels of 1-1,000 1 or even 8,000 1 (Namalva cells grown for interferon; but in practice their maximum size is 20 1 since larger vessels are difficult to handle, autoclave and to agitate the culture effectively).

These are closed systems with fixed volumes and are usually agitated with motor-driven stirrers with considerable variation' in design details, e.g., water jacket in place of heater type

temperature control, curved bottom for better mixing at low speeds, mirror internal finishes to reduce cell damage, etc. Many heteroploid cell lines can be grown in such vessels.

The needs for research biochemical from cells are met from 2-50 1 reactors, while large scale reactors are mainly used for growing hybridoma cells for the production of monoclonal antibodies although their yields from cultured cells is only 1-2% of those obtained by passaging the cells through peritoneal cavity of mice.

Continuous-Flow Cultures:

These culture systems are either of chemostat or turbidostat type. In both the types, cultures begin as a batch culture. In a chemostat type, inoculated cells grow to the maximum density when some nutrient, e.g., a vitamin, becomes growth limiting. Fresh medium is added after 24-48 hours of growth, at a constant rate (usually lower than the maximum growth rate of culture) and at an equal rate the culture is withdrawn.

When the rate of growth equals the rate of cell withdrawal, the cultures are in a 'steady state', and both the cell density and medium composition remain constant. One of the constituents of the medium is used at a lower concentration to make it growth-limiting. However, chemostat is the least efficient or controllable at the cell's maximum growth rate hence the steady-state growth rates in them are much lower than the maximum.

In contrast, in a turbidostat cells grow to achieve a predecided density (measured as turbidity using a photoelectric cell). At this point, a fixed volume of culture is withdrawn and the same volume of fresh normal (not having a growth-limiting factor) medium is added; this lowers the cell density or turbidity of the culture. Cells keep growing, and once the culture reaches the preset density the fixed volume of culture is replaced by fresh medium. This system works really well when the growth rate of the culture is close to the maximum for the cell line.

The continuous-flow cultures provide a continuous source of cells, and are suitable for product generation, e.g., for the production of viruses and interferons. It is often necessary to use a two- stage system in which the first stage supports cell growth, while the second stage promotes product generation.

Airlift Fermenters:

Cultures in such vessels are both aerated and agitated by air (5% CO_2 in air) bubbles introduced at the bottom of vessels . The vessel has an inner draft tube through which the air bubbles and the aerated medium rise since aerated medium is lighter than non- aerated one; this results in mixing of the culture as well as aeration.

The air bubbles lift to the top of the medium and the air passes out through an outlet. The cells and the medium that lift out of the draft tube move down outside the tube and are recalculated. O_2 supply is quite efficient but scaling up presents certain problems. Fermenters of 2-90 L are commercially available, but 20,000 L fermenters are being used by biotechnology industries.

3. Immobilized Cultures:

Cultures based on immobilized cells offer the following several advantages: (1) higher cell densities (50-200 x 10^{-6} cells/ml), (2) stability and longevity of cultures, (3) suitability for both suspension and monolayer cultures, (4) protection of the cells from shear forces due to medium flow (in case of many systems), and (5) less dependence of cells at higher densities on external supply of growth factors, which saves culture cost. There are the following two basic approaches to cell immobilization: (1) immurement

(2) entrapment.

Immurement Cultures:

In such cultures, cells are confined within a medium permeable barrier. Hollow fibers packed in a cartridge are one such system. The medium is circulated through the fiber, while cells in suspension are present in the cartridge outside the fiber.

This is extremely effective for scales upto 1 1 and gives cell densities of 1-2 X 10^8cells/ml; sophisticated units can yield upto 40 g monoclonal antibodies/month. Membranes permitting medium and gas diffusion are also used to develop bioreactors of this type; both small scale and large scale versions of membrane bioreactors are available commercially.

The cells may be encapsulated in a polymeric matrix by adsorption, covalent bonding, cross- linking or entrapment; the materials used as matrix are gelatin, polylysine, alginate and agarose. This approach (1) effectively protects cells from mechanical damage in large fermenters, and (2) allows production of hormones, antibodies, immunochemicals and enzymes over much longer periods than is possible in suspension cultures. (3) The medium diffuses freely into the matrix and into the cells, while cell products move out into the medium.

For production of larger molecules like monoclonal antibodies, agarose in a suspension of paraffin oil is preferable to alginate since the latter does not allow “diffusion of such products out of the alginate beads. Reactors of upto 3 1 are available commercially.

Entrapment Cultures:

In this approach, cells are held within an open matrix through which the medium flows freely. An example is the Opticell, in which the cells are entrapped within the porous ceramic walls of the unit.

Opticell units of upto 210 m^2 surface area are available, which can yield upto 50 g monoclonal antibodies per day. The cells can also be enmeshed in cellulose fibres, e.g., DEAE, TLC, QAE, and TEAE. These fibers are autoclaved and washed as prescribed and added in a spinner/stirred bioreactor at a concentration of 3 g/1.

Porous macrocarriers:

Porous macrocarriers are small (170 pm-6,000 pm) beads of gelatin, collagen, glass or cellulose, which have a network of interconnecting pores. These provide a tremendous enhancement in surface area/volume ratio, permit efficient diffusion of medium and product, are suitable for scaling up, and are equally useful for suspension and monolayer cultures.These can be arranged as fixed bed or fluidized bed reactors or used in stirred bioreactors. It is expected that future developments will make the immobilized cell systems the most dominant production systems.

Adherent Cell Culture	Suspension Cell Culture
Appropriate for most cell types, including primary cultures	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g., hematopoietic)
Requires periodic passaging, but allows easy visual inspection under inverted microscope	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth
Cells are dissociated enzymatically (e.g., TrypLE™ Express, trypsin) or mechanically	Does not require enzymatic or mechanical dissociation
Growth is limited by surface area, which may limit product yields	Growth is limited by concentration of cells in the medium, which allows easy scale-up
Requires tissue-culture treated vessel	Can be maintained in culture vessels that are not tissue-culture treated, but requires agitation (i.e., shaking or stirring) for adequate gas exchange
Used for cytology, harvesting products continuously, and many research applications	Used for bulk protein production, batch harvesting, and many research applications

The Rotary Cell Culture System (RCCS):It is a device designed to grow three-dimensional cell clusters in microgravity.

NASA BIOREACTOR:

The NASA Bioreactor was developed by NASA to simulate the weightless environment of space by putting cells in a growth medium that constantly rotates and keeps the cells in endless free-fall.Culturing cells means putting some small number into nutrient media in a dish or a tube and letting them grow. However, this kind of approach does not provide the culture environment that supports tissue assemblies. Without a proper 3-D assembly, epithelial cells (the basic cells that differentiate tissue into specific organ functions) lack the proper clues for growing into the variety of cells that make up a particular tissue.

In a rotating Bioreactor, the cells can be fooled into thinking they are in a body. With a plastic lattice to help direct their growth, cells can be encouraged to grow in predefined shapes.

NUNC CELL FACTORY:

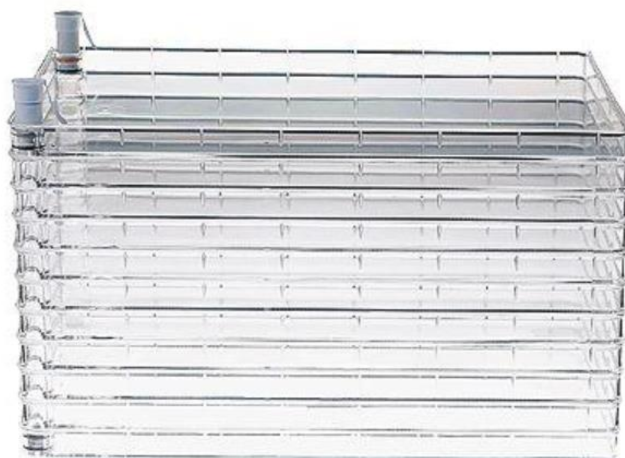
• Closed system with sterile fluid path

• Store or transport cells

• Excellent imaging properties

• Freeze and thaw directly in OptiCell

The Nunc OptiCell cell culture system has very stable growth conditions, as O_2 and CO_2 are efficiently diffused through the thin film. OptiCell products have low space requirements (compared to tissue culture flasks) and a low media consumption because of the large growth area-to-volume ratio. Besides growth, imaging, transport and storage of cell cultures, OptiCell cell culture system can also be used for biomagnetic cell separation and has proven itself as excellent for hybridoma antibody production and transfection studies.



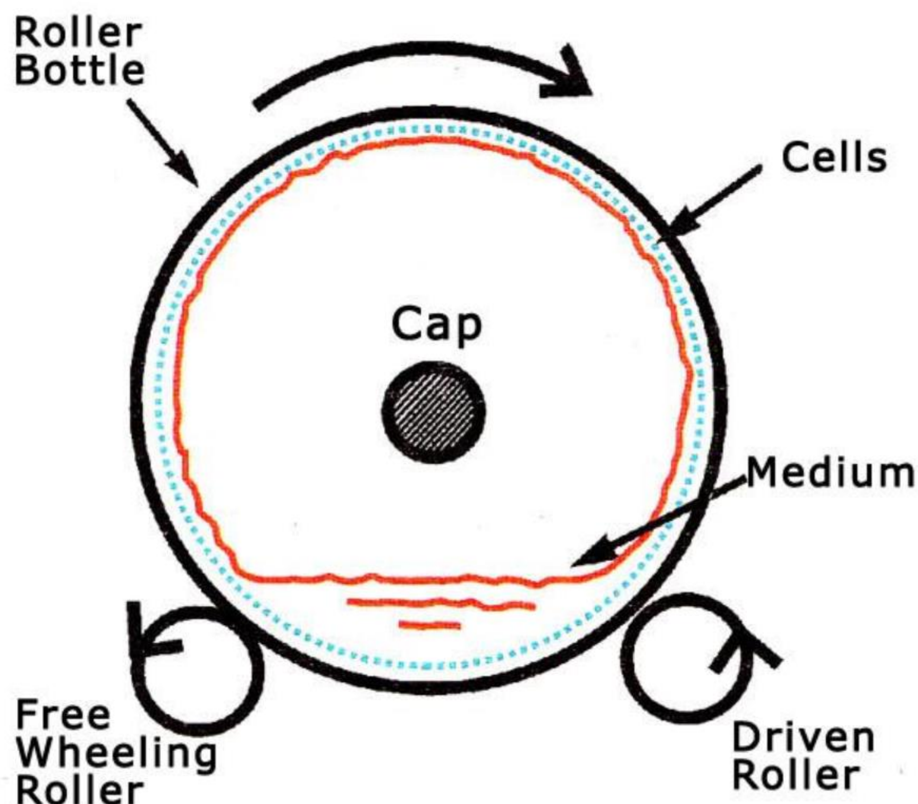
Nunc Cell Factory

In batch cultures, mainly Roller Bottles with Micro Carrier Beads (for adherent cells) and spinner flasks (for suspension cultures) are used in Scale-up of animal cell culture process.

Roller Bottles:

The Roller bottles provide total curved surface area of the micro carrier beads for growth. The continuous rotation of the bottles in the CO2 incubators helps to provide medium to the entire cell monolayer in culture.The roller bottles are well attached inside a specialized CO2 incubators. The attachments rotate the bottles along the long axis which helps to expose the entire cell monolayer to the medium during the one full rotation. This system has the advantage over the static monolayer culture: (a) it provides increase in the surface area, (b) provides constant gentle agitation of the medium, (c) provides increased ratio of surface area of medium to its volume, which allows gas exchange at an increased rate through the thin film of the medium over the cells. Typically, a surface area of 750-1500 cm2 with 200-500 ml medium will yield 1-2x108cells.

DIAGRAM SHOWING THE ROLLER BOTTLE CELL CULTURE



Micro Carrier Beads:

Micro carrier beads are small spherical particles with diameter 90-300 micrometers, made up of dextran or glass. Micro Carrier beads, increase the number of adherent cells per flask. These dextran or glass-based beads come in a range of densities and sizes. The cells grow at a very high density which rapidly exhausts the medium and therefore the medium has to be replaced for the optimum cell growth. At the recommended concentration when the microcarriers are suspended they provide 0.24 m2 area for every 100 ml of culture flask.

Spinner cultures:

The spinner flask, was originally developed to provide the gentle stirring of microcarriers but are now used for scaling up the production of suspension cells. The flat surface glass flask is fitted with a Teflon paddle that continuously turns and agitates the medium. This stirring of the medium improves gas exchange in the cells in culture. The spinner flask used at commercial scale consists of one or more side arms for taking out samples and decantation as well.

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