Culturing Cells in 2D and 3D

Astghik Karapetyan and Narine Sarvazyan

Contents

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What You will Learn in This Chapter and Associated Exercises

Students will learn about main types of cell cultures and the key ingredients of cell culture media. The concepts of osmolarity, pH, and sterility will be reviewed. The practical exercises will help students to gain basic knowledge of the main steps involved in media preparation, cell defrosting, and passaging.

6.1 Cell Culture

Cell culture is a combination of techniques, which enables cell isolation from an organism and subsequent maintenance of the cells in a favorable in vitro environment. There are two main ways to obtain isolated cells:

- Cells can be removed from a fresh animal or plant tissue by disaggregation that can be either enzymatic, mechanical, or both. These processes lead to disruption of the extracellular matrix, which holds cells together.
- Cells can be derived from previously established cell lines.

Primary cell cultures contain cells isolated from fresh human or animal tissue. *Passaging,* or *subculturing*, means detaching cells and transferring a small amount of them to a new flask or Petri dish with fresh culture media to facilitate further growth. *Secondary culture* is a term usually used to describe passaged primary cells. *Cell line* consists of cells, which have been continually passaged for a long time while preserving homogeneous genotypic and phenotypic characteristics. Cell lines can be *finite* and *continuous*. Cells of finite cell lines can divide, but only a limited number of times. Continuous cell lines can propagate indefinitely and are also called immortal, with immortality induced chemically, virally, or spontaneously.

There are two main types of cell cultures: adherent and suspension. For animal and human cells, suspension cultures involve mostly blood cells, although some other cell types derived from tumors can be also cultured in suspension. Adherent cells require attachment for growth and are called anchorage-dependent cells. When adherent cell cultures reach confluency (i.e., attached cells occupy most of the available surface—see Fig. 6.1), they need to be passaged. Below we will mostly discuss protocols used for adherent monolayer cultures. Most of them are also applicable to 3D cultures discussed at the end of this chapter.

6.2 Cell Culture Media

Media are a critical component of cell culture because they provide cells with the necessary nutrients, growth factors, and hormones. The basic constituents of the media include inorganic salts, amino acids, carbohydrates, vitamins, fatty acids, lipids, proteins, peptides, and trace elements. Media components also help to maintain pH and proper osmolarity of the cell environment. Different media formulations exist, each optimized for different types of cells or conditions. To chemically defined media formulations, a small amount of animal serum or chicken embryo extract is usually added to provide additional growth factors and hormones. The most commonly used serum is fetal bovine serum (FBS). Other types of serum are available,

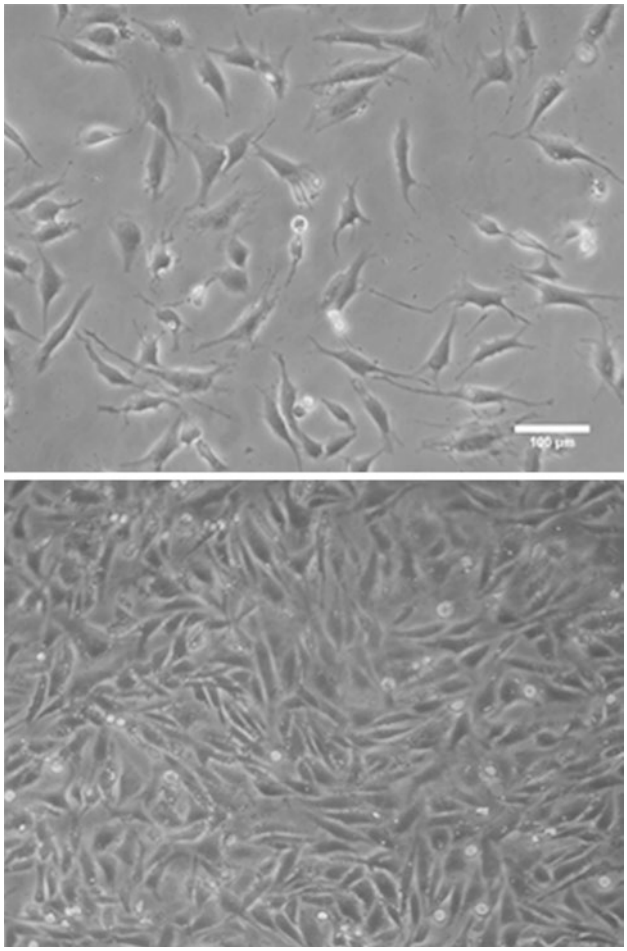


Fig. 6.1 Illustrative images of sparse (top) versus confluent (bottom) cell layers from primary culture of isolated bovine chondrocytes

including newborn calf serum and horse serum. Serum is a rich source of hormones and nutrients and is also able to bind and neutralize toxins. Disadvantages of serum include its high cost, batch-to-batch variations, and the fact that its composition cannot be precisely defined.

Three main types of cell culture media include basic media, reduced-serum media, and serum-free media. It is important for students to understand the key components of each media type as this can be critical for the success of their experiment. For example, it is perfectly fine to keep the sample in PBS for a short period of time, especially when they need to quickly check of cell morphology or behavior under the microscope. But if the goal is to observe cell contractions, then this medium will not work since it does not have physiological levels of extracellular calcium. On the other hand, having calcium in the media will significantly impair the digestion efficiency of collagenase. This is because lowering calcium concentration helps to dissociate cells by affecting adhesion molecules. A chart that summarizes the key ingredients in the main media types is shown in Table 6.1. More detailed information about different media types can be found online or in [1].

Table 6.1 Preferential use of basic salt solutions and cell culture media

Name	Key ingredients	Can be used for		
		Washing	Live cell imaging	Cell culture
Saline	NaCl	YES	NO	NO
PBS	NaCl + phosphate buffer	YES	NO	NO
Tyrode solution	NaCl, KCl, $MgCl_2$, $CaCl_2$, glucose, and phosphate	YES	YES	NO
Media	Essential salts, glucose, vitamins, amino acids, bicarbonate	Depends	NO[a]	YES
Media + serum	Media + proteins, hormones and growth factors	NO	NO	YES

[a]Unless w/o phenol red and pH adjusted with HEPES for CO_2 levels outside incubator

Basic cell culture media contain inorganic salts, amino acids, vitamins, and essential carbohydrates such as glucose. Following are the most commonly used basic medium formulations: MEM (minimum essential medium or Eagle's minimal essential medium), DMEM (Dulbecco's modified Eagle's medium), IMDM (the basic DMEM modified by Iscove), RPMI 1640 (Roswell Park Memorial Institute medium), McCoy's 5A, Opti-MEM medium, 199/190 medium, and HamF10/HamF12. Their exact chemical composition can be found on the manufacturer's website.

Reduced-serum media consists of the same basic media, which is enriched with nutrients and growth factors. The latter reduces the amount of serum to be added to basic media. Components of serum supplements usually include attachment factors such as fibronectin and laminin, growth hormones such as somatomedin, enzyme inhibitors, binding proteins, translocators, and trace elements.

Besides cell culture media, there are also washing and digestion media that are commonly used to release and wash cells during passaging:

Washing media Balanced salt solution (BSS) is a basic medium, which is used for washing tissues and cells. It contains salts of main physiological ions such as potassium, calcium, magnesium, chloride, and sodium. Commonly used BSS names in cell culture include the following:

- Hanks' balanced salt solution (HBSS)
- Dulbecco's phosphate buffered saline (PBS)
- TRIS-buffered saline (TBS)
- Tyrode's balanced salt solution (TBSS)

Digestion media For digestion of tissues, the proteolytic enzymes such as trypsin and collagenase are used to digest proteins of the extracellular matrix. Chelating agents such as EDTA or EGTA are commonly included to bind calcium ions that are involved in cell-cell adhesion.

6.3 Osmolarity

Osmolarity is a colligative property in that it depends on the concentration of solute ions, but not on their respective identities. Solution osmolarity is expressed as Osm/L. The unit of osmolarity is osmole, which defines the number of moles of solutes that provides an osmotic pressure of the solution. For example, NaCl dissociates into Na^+ and Cl^- ions. This means that 1 mol NaCl becomes 2 osmoles. Therefore, the solution of 1 mol/L NaCl has osmolarity 2 Osm/L. In cell culturing, osmotic pressure of media is an important factor that can affect cell proliferation. In vivo osmolarity of extracellular space is one of the most tightly controlled physiological variables unless there are changes caused by aging or disease. The osmotic pressure of human plasma is about 290 mOsm/kg, which is considered to be optimal for human cell culture. It is highly advised to have an osmometer in a cell culture lab to measure the osmolarity of media. Alternatively, it can be estimated based on the concentration of individual media components.

6.4 Sterile Environment

One of the most important procedures in cell culturing is to keep cells from contamination with microorganisms, bacteria, viruses, or fungi. These may be done by sterilizing work surfaces, media, equipment, culture waste, as well as by keeping good personal hygiene. Sterilization may be implemented with heat, filtration, UV light, as well as 70% ethanol. Air surrounding cells and their media are kept clean by using a laminar hood or other types of cell culture hood. The cell culture hood is an enclosure into which sterile air is forced through a HEPA-like filter.

An essential piece of equipment, called an autoclave, sterilizes instruments and glass bottles used to prepare and store media. It uses pressurized steam to heat the material to be sterilized. Autoclaving effectively kills most microbes, spores, and viruses.

Filtration is used for media that contain thermally labile components. It employs filters with pores so small that microorganisms can't pass it. Most of the filters used for sterilization are made from porous cellulose acetate and have either 0.22 μm or 0.44 μm pores.

Below are some basic rules that can help to avoid cell culture contamination:

- The working surface and surrounding areas, as well as equipment, must be disinfected before and after each use. Ultraviolet (UV) light can be used for sterilization when left on for a significant amount of time. In order to prevent damage to skin and eyes, UV light must be turned OFF when actual work in cell culture hood is being done.
- Before and during the work, a person is required to wipe work surface and hands with 70% ethanol. Particular care has to be exerted in cases of any media spillage. Everything, including plates, dishes, pipettes, has to be wiped with ethanol before entering the workplace inside the hood.
- Media, reagents, solutions, plates, dishes, pipettes, tips, and flasks have to be sterilized.
- Avoid pouring reagents or solutions directly from the bottle. Instead, use pipettes to draw needed amounts.

- Don't leave the solutions uncovered in an open environment.
- Put the cap of bottles on the surface with the cap opening facing down.
- Wash hands before and after work.
- Wear personal protective equipment (PPE) such as gloves, laboratory coat, safety visor, overshoes, and head cap.
- Don't converse or sing during the preparation of solutions.
- Do the work as quickly as possible in order to avoid contamination.

Antibiotics are often added to the cell culture media in order to prevent contamination. However, their long-term usage can cause the formation of antibiotic-resistant cell strains. In addition, antibiotics can interfere with differentiation protocols or maintenance of stem cell cultures. Hence, for such cases, the use of antibiotics should be avoided or minimized as much as possible. On the other hand, most protocols to isolate cells for primary cell culture are not done in sterile conditions. Therefore, primary cell cultures almost always require the use of an antibiotic. The most common antibiotics used in cell culture are ampicillin, penicillin, streptomycin, and gentamicin.

6.5 Temperature

As mentioned above, for the optimal growth of most of the mammalian cells, a temperature of 36–37 °C is needed. For short-term handling of the cells, including washing, re-plating, imaging, and dye loading, room temperatures are perfectly suitable. Placing cells in a refrigerator for a short term (10–30 min) is not detrimental. Overheating cells to temperatures above 50 °C leads to irreversible cell damage. If cells are frozen at temperatures below −4 °C without specific precautions, ice crystals formed within the cells rupture cell membrane leading to cell death.

6.6 Concept of pH

pH is one of the most important physicochemical factors for maintaining cell growth. pH is a measure of hydrogen ion concentration in solution and used to specify acidity and basicity of the solution under appropriate temperature. The equation, which defines pH, is: $pH = -\log [H^+]$, where $[H^+]$ is a concentration of hydrogen ions. For example, if the concentration of hydrogen ions in solution is 0.00001 M, the pH of the solution will be equal to 5.

$$\mathrm{pH} = -\log\left[0.00001\right] = -\log\left[1\times10^{-5}\right] = -\left(-5\right) = 5$$

pH value can range from 0 to 14. Solutions with a high concentration of hydrogen ions have a low value of pH, and solutions with a low concentration of hydrogen ions have a high value of pH. Acidic solutions have a pH lower than 7, while basic solutions have pH higher than 7. Solutions with a pH of 7 are considered neutral.

The physiological pH falls within a narrow range of 7.2–7.5. The most commonly used buffer for maintaining a stable pH of solutions outside of CO_2 incubator is

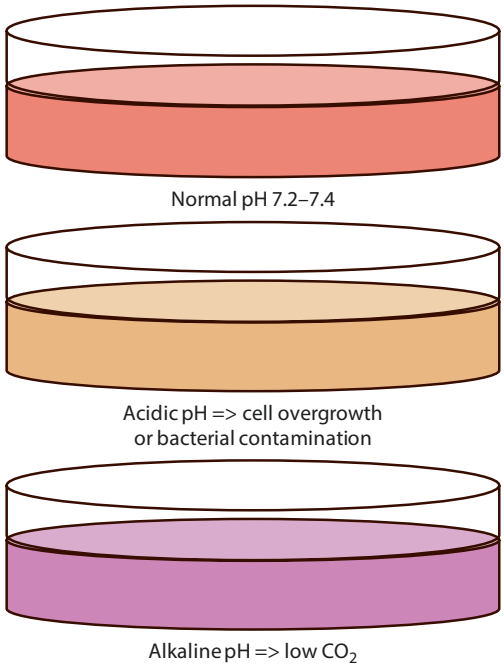


Fig. 6.2 Visual changes in the color of cell culture medium containing phenol red allow making conclusions about pH

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). It can be made as a 0.5M–1M stock, pH of which is brought to a range of 7.2–7.4 using concentrated HCl solution. It can be then added to the media to achieve the final 10–20 mM concentration.

Any cell culture lab must own a sensitive pH-meter to ensure pH measurements of prepared media. Many media formulations include an indicator called phenol red. It allows a user to constantly visually monitor the pH status of the media by its color (Fig. 6.2). If the medium color turns yellow (acidic pH), it is likely an indicator of cell overgrowth or bacterial contamination. If the medium color turns purple (alkaline pH), this usually indicates a lack of CO_2. The latter can be a result of incubator dysfunction or due to simply having a cell culture dish for too long.

6.7 Cell Culture Incubator

One of the required pieces of equipment for cell culturing is cell incubator, which provides an appropriate environment for cell growth. It must have forced-air/CO_2 circulation and temperature control within ±0.2 °C. Three main environmental conditions provided by cell incubators include physiological temperature of 36–37 °C, 85–95% humidity, and 5% carbon dioxide. These conditions mimic the environment to which cells are exposed while inside the body. High amount of gaseous carbon dioxide within the cell incubator leads to acidification of the media due to the conversion of CO_2, once it is dissolved, into carbonic acid. To counteract this effect, most media formulations require the addition of 2 g/L sodium bicarbonate. To pre-

vent significant pH changes due to taking cells in and out of the incubator as well as metabolic activity of the cells that leads to CO_2 production, the above-mentioned HEPES or other biologically compatible buffers can be also included in cell culture media.

Traditionally incubators for mammalian cell cultures are connected to 95% air / 5% carbon dioxide tanks. Thus, inside them, the concentration of oxygen is close to its atmospheric concentration of ~20%. This is equivalent to 150 mmHg partial pressure of oxygen (760 mmHg atmospheric pressure multiplied by 20%). Since it has been shown that many cell types, particularly stem cells, are better maintained when oxygen tension is at 2.5–5%, many labs are switching to gas tanks with lower oxygen concentrations [2].

Recent advances in device miniaturization enable visual monitoring of cells inside incubator via wireless access. An example of such a device is CytoSmart Lux2. It is a compact inverted microscope for brightfield live-cell imaging. The device can fit inside a standard incubator and can be programmed to acquire and send to the user's computer magnified pictures of cell culture at specified time points. The images are then automatically uploaded to cloud storage where they can be analyzed for confluency, cell division, differentiation, or other morphological changes.

6.8 Cell Storage, Defrosting, and Passaging

Since continuous cell lines are passaged many times, the risk of their genetic instability increases. Therefore, it is very important to keep the stock of these cells in cryogenic storage. Freezing temperatures are not suitable for this purpose, because at regular freezer temperatures (from −20 to −80 °C) the cell viability decreases. For long-term storage of frozen cells, Dewar containers filled with liquid nitrogen are used. They enable maintaining temperatures of −196 °C.

Defrosting cryopreserved cells without significant loss of viability requires quick and careful work. Below are detailed steps for successful thawing (Fig. 6.3):

- Remove the cryovial containing the frozen cells from liquid nitrogen storage and immediately place it into a 37 °C water bath. It is recommended to cautiously dip the vial in the water bath without exposing the threads or the top lip of the vial to the water to prevent contamination.
- Quickly thaw the cells (< 1 min) by gently swirling the vial in the 37 °C water bath until there is just a small bit of ice left in the vial.
- Transfer the vial into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.
- Transfer the thawed cells dropwise into the centrifuge tube containing the desired amount of pre-warmed complete growth medium appropriate for the selected cell line.
- Centrifuge the cell suspension at approximately 200 g for 5–10 min. The actual centrifugation speed and duration vary depending on the cell type.
- After the centrifugation, check the clarity of supernatant and the visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.
- Gently resuspend the cells in complete growth medium and transfer them into the appropriate culture vessel and into the recommended culture environment.

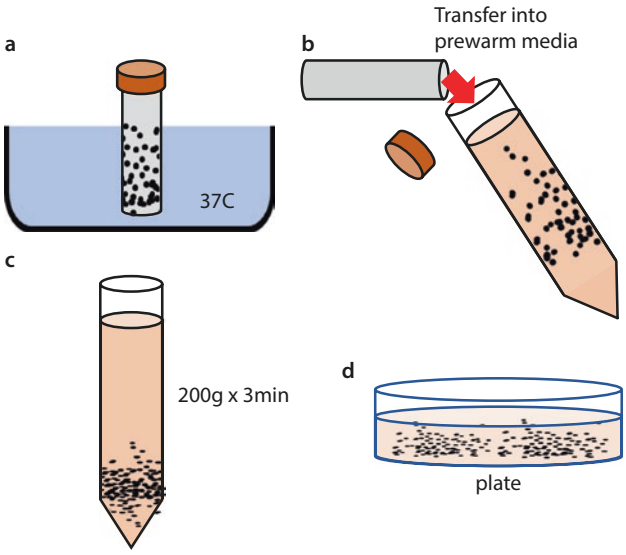


Fig. 6.3 Steps to cryopreserve cells. **a** Warm sample in 37 °C waterbath, **b** transfer cells into a centrifuge tube, **c** centrifuge at 200 g for 3–5 min, **d** plate cells

Note: The appropriate flask size depends on the number of cells frozen in the cryovial, and the culture environment varies based on the cell and media type.

After cells are defrosted and cultured for several days, they reach a state of confluency. For adherent cultures, this means that cells occupy most of the available surfaces. Afterward, the proliferation of the cells dramatically decreases. In order to keep cells alive and growing, they must be split (this process is also called passaging or subculturing). Techniques for cell passaging differ depending on the cell type. Most commonly, it involves the addition of media containing trypsin to detach cells, followed by cell centrifugation, media replacement, and cell count. Afterward, cells are plated using an attachment surface that is about 5–10 times larger than the original surface (Fig. 6.4).

The content of the above sections is a compilation of useful information about 2D cell culture techniques from multiple freely available sources [3–5]. Students are strongly encouraged to read the full text of these articles.

6.9 3D Cultures

Cells grown in a 3D environment have different morphology compared to those grown in 2D on plastic or glass surfaces. They have also shown to differentiate and be affected by the drugs differently. Therefore, an increasing number of researchers are conducting experiments using 3D culture approaches. There are three main ways by which this is currently done (Fig. 6.5).

The first one is an *aggregation* of cells to form 3D spheroids, which can be *gravity- or stirring based*. When stem cells are used as starting material, these spheroids are called embryoid bodies (abbreviated as EBs). 3D cell spheroids can be created by using a "hanging drop" approach or by seeding cells in special cell culture plates with multiple miniwells having a round or conical bottom (an example being Sphericalplate 5D or similar designs). The hanging drop approach is laborious, but it does not

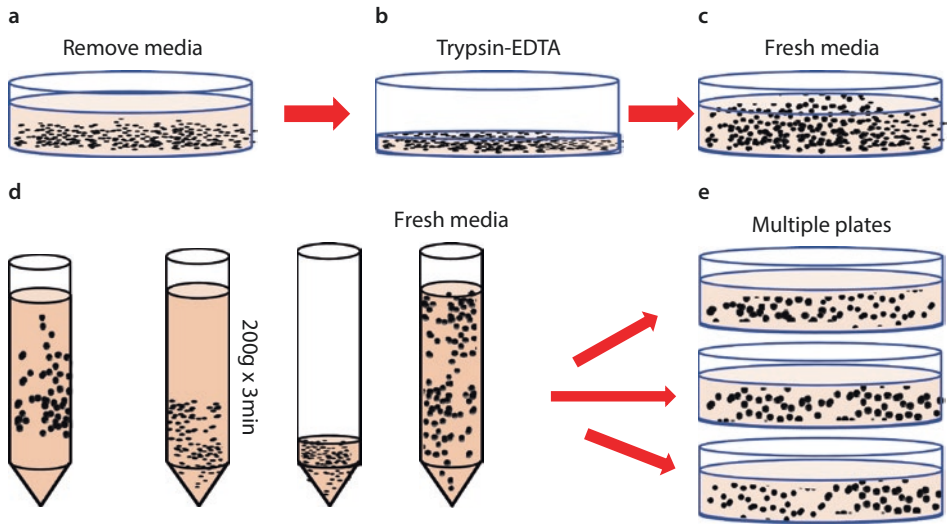
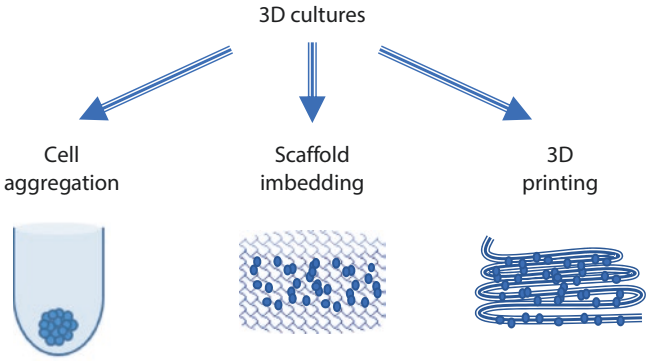


Fig. 6.4 Subculture procedure. **a** Remove media, **b** incubate with Trypsin-EDTA, **c** neutralize by adding fresh media, **d** collect and centrifuge, **e** resuspend in fresh media and plate using 5–10 times larger surface

Fig. 6.5 Main ways to culture cells in 3D



require any specialized equipment as droplets of solutions containing anywhere from 200 to 400 cells are simply pipetted on the lid of the standard cell culture place and then flipped during subsequent culturing. The miniwell approach enables upscaling the number of spheroids to thousands in a single plate, improves their homogeneity, and makes it easy to perform media exchange. The formed spheroids can then be even used as individual blocks for 3D printing applications. 3D cell aggregates can also be formed when certain types of suspended cells are stirred within cell culture flask for extended periods. The shape and size of such aggregates are more variable than 3D spheroids formed by hanging drop or miniwell approaches (Fig. 6.6).

The second way to culture cells in 3D is to seed on them on different types of meshes and/or to combine such scaffold with hydrogel embedding. All the criteria—fiber diameter, orientation, porosity, degradation rate, and mechanical properties—can be adjusted to create the most suitable 3D culture platform. Such meshes can be 3D printed using PCL polycaprolactone, electrospun from PLLA (poly(L-lactide))

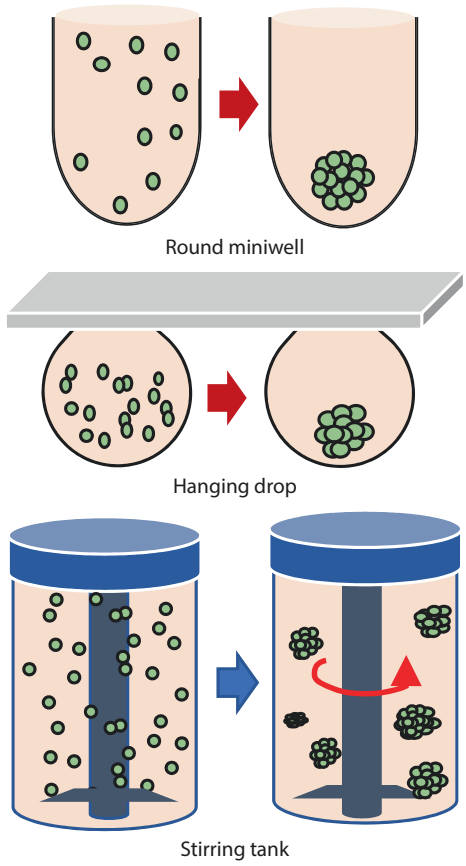


Fig. 6.6 Main methods for self-assembly of 3D cell spheroids

or other polymers. Numerous companies including Bellamesh, Mimetix, The Electrospinning Company, and many others can make scaffolds based on user's needs. Different mesh sizes and structures have been shown to dramatically affect cell behavior and morphology so screening of several mesh types is needed to optimize a particular mesh to the application of an individual user. 3D cancer models, cartilage and bone engineering, and vascularization studies can all benefit from using 3D versus 2D cell culture systems.

The third way to culture cells in 3D is to use *bioprinting*, which will be covered in more detail in ▶ Chap. 10.

When 3D tissues are small (less than 200 microns), they can be kept in cell culture dishes and cultured similarly to 2D cultures. For larger 3D constructs, it is better to switch to perfusion-based culturing or use bioincubators (discussed in ▶ Chap. 11). Doing so greatly increases the access of the cells within such constructs to the nutrients. And since combining a pump and a cell incubator is often a cumbersome endeavor, new devices such as TEB500 Cell Culture Bioreactor recently came to the market. The latter has a functionality of a CO_2–O_2 incubator but also incorporates an integrated double peristaltic pumping system (EBERS Medical, Zaragoza, Spain).

Session I

Demonstration

The instructor demonstrates media preparation, passaging, counting, and storing cells using a commercial cell line. Alternatively, fibroblast or endothelial cells obtained during the week 4 demo can be kept in culture and used for these experiments. The concept of 3D culture by making spheroids using hanging drop can also be shown and discussed.

Homework

Each team selects from literature three different protocols that can be used to culture cells from their organ of choice. A table comparing major differences and similarities in these three culturing conditions is compiled to be discussed in the class.

Session II

Team Exercises

Each team is given one plate of cells to passage using their own media. The latter has to be prepared from media powder to which appropriate amounts of bicarbonate have to be added. This has to be followed by filtering and the addition of serum and antibiotics. Treated coverslips from week 3 can be used to plate excess cells on different adhesion surfaces.

Homework

Each team prepares a detailed protocol that describes steps involved in making culture media, passaging the cells, and monitoring their proliferation on different adhesion surfaces.

Sample Protocol

Cell passaging

1. Warm to room temperature all necessary solutions including trypsin, culture media, and PBS (phosphate-buffered saline).
2. Under the laminar hood, carefully remove media from cell plate using sterile pipette tips connected to a pipettor or a vacuum line.
3. Add a small volume of PBS to the dish, gently swirl it around, and carefully remove it using pipette or aspirator.
4. Add 0.05% of trypsin (the volume of added trypsin depends on the plate surface) and place the cell plate into the incubator for about 1 min. Place cell plate under a phase-contrast microscope and check cell appearance. If they look round, then they have started to detach from the plate surface. It is important to visually confirm that cells are properly lifted from the plate before neutralization.
5. To neutralize the impact of trypsin, add the same volume of media. Resuspend the cells in the media and transfer the cell suspension into a 15 or 50 mL tube. To calculate the number of cells, mix 10 μL of cell suspension with the same volume of Trypan blue dye and transfer it to the hemocytometer for cell count (see ► Sect. 4.5 of ► Chap. 4).

6. Centrifuge cell suspension for 5 min at 1500 rpm.
7. Remove the supernatant and carefully break the pellet by gentle triturating (pipetting back and forth) to separate cells from each other.
8. Add more media and carefully pipette cell suspension into a new plate with growth media (the volume of cell suspension depends on the number of cells). Then place the plate into an incubator for 24 h, followed by daily or bi-daily media change.

Take-Home Message/Lessons Learned

After reading this chapter and performing the requested assignments and exercises, students should be able to:

- Understand differences between different cell culture types
- Make cell culture media from commercial powder formulations
- Adjust media pH, check its osmolarity, and sterilize it
- Perform basic steps to passage cells in sterile conditions
- Understand the main principles to culture cells in 3D

Self-Check Questions

Q.6.1. Cell cultures cannot be classified as either _____
A. Suspension or adherent
B. Finite or continuous
C. Plant or animal
D. Synthetic or natural

Q.6.2. Ion present in ALL types of cell culture and cell washing media is
A. Potassium
B. Sodium
C. Calcium
D. Magnesium

Q.6.3. Cell culture media typically includes all these components, EXCEPT
A. Buffer
B. Nutrients
C. Antibodies
D. Antibiotics

Q.6.4. In typical cell culture, this is NOT a controlled parameter
A. Temperature
B. Carbon dioxide levels
C. Humidity
D. Nitrogen levels

Q.6.5. Choose the correct statement about the pH of culture media.

A. pH is not affected by levels of carbon dioxide within an incubator.
B. pH is affected by incubator humidity.
C. pH must be between 7.2 and 7.4 pH units.
D. pH is not affected by the presence of a buffer.

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