Basics of 3D Cell Culture: Forming spheroids in the presence of extracellular matrix

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Introduction: What is 3D cell culture?

- A general definition of **three dimensional (3D) cell culture is the assembly of cells *in vitro* into a pattern that resembles the situation *in vivo*.**

- There are three types of 3D cell culture: Standard 3D culture (with extracellular, chemically and structurally relevant gels), organ-on-a-chip models (with engineered materials for scaffolding of specific geometry, microfluidics, biosensing, etc.), tissue bioengineering (for replacement of tissues *in vivo*)

- Good practice in 3D cell culture is to provide cells with the necessary chemical and mechanical environment for tissue differentiation, regardless of the purpose of such cultures.

Scaffolds for 3D cell culture

- Different scaffolds are used for 3D cell culture: Naturally produced extracellular matrix components (e.g., collagen I, laminin, fibronectin, vitronectin), chemically manufactured gels (e.g., self assembly of specific molecules), engineered surfaces (materials-based with given geometry to support cell culture).

- **However, in order to obtain physiologically relevant differentiation of the cells of interest it is essential that the scaffold provides the necessary chemical inducers (e.g., matrix proteins) or fosters the production of these chemical inducers by cells if appropriate.**

When to use spheroids…

- The goal of 3D cell culture is to mimic aspects of tissues *in vivo*. **Therefore, it only makes sense to produce spheroids if the tissue of interest is organized as such *in vivo***.
- Examples of tissues that adopt a spheroidal shape include tumors (although the nodules are often of an irregular shape) and acini/alveoli (like those observed in parts of the glandular tissues). It is essential to understand that, within the spheroids produced, the cells should adopt the organization observed *in vivo*.
- If the goal is to mimic the formation of spheroids, the environment can be derived from extracellular matrix (ECM) components that are placed in hydrogels or are forming gels.

Some applications of 3D cell culture

- Gene and protein expression studies
- Cell physiology studies (proliferation, signaling, survival,death, etc.) in a context that includes cell-cell and cell-extracellular matrix interactions properly organized in space
- Cell morphology and tissue architecture
- Disease mechanisms
- Drug discovery and drug response
- Design of engineered tools/sensors for applications in research and in the clinics

Important note: Use the 3D cell culture method that best produces the *in vivo* aspects of the tissue for which an *in vitro* model is needed. In other words, the method used depends on each scientific query.

MANAGING 3D CELL CULTURES in the presence of a gel

- Cell seeding
- Medium change
- Retrieval of multicellular structures from the gel
- Preparation for O.C.T. embedding

Medium used in 3D cell culture

- The goal of 3D cell culture is to induce and maintain a physiologically relevant environment for the cells to differentiate *in vitro* as they would *in vivo*.
- The serum that is commonly added to the cell culture medium is undefined, with variability in constituents and their concentrations. If serum is used, then it is important to remember that this aspect of the culture cannot be controlled and could produce variability in the results as well as introduce artifacts in the response of cells to their environment.
- When possible it is preferable to use serum free-medium with defined concentrations of additives (elements, growth factors, hormones) that provide survival and protection, and enable controlled proliferation. The lists of additives depending on the tissue type and protocols for additive preparation and storage are widely available.

Seeding cells in the presence of a gel

- Cells may be embedded within the gel (most common) or seeded on top of a thin gel coat with a drip of gel components in the medium.
- The exact method for seeding cells depends on the manufacturer's instructions for a particular type of gel.
- Not all cells will thrive in all types of gels.
- Cells that cannot secrete their own ECM will usually require chemical induction of differentiation and survival (i.e., with inclusion of certain components of the ECM) if the gel is not already made of ECM components.
- One of the most frequently used gel at the moment is that derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma that is rich in basement membrane components.

Movies included in this slide presentation show examples of cultures with EHS-derived gel.

Classical embbeding method

- Individual cells or multicellular structures are mixed with the liquid form of the gel before inducing the formation of the ‘solid’ form of the gel.
- Follow the manufacturer’s instruction for each type of gel
- Example for EHS-derived gel
  - Precoat the surface of the culture vessel with a small amount of liquid gel (10.4 µl/cm^2) and let it gelify in the cell culture incubator for 5 minutes (do not incubate longer than 20 minutes to avoid drying of the gel).
  - Spin down separately the amount of cells needed for each culture vessel and resuspend the pellet of cells in a tiny amount of medium (not more than 10% of the final volume of gel), then mix with the gel slowly (see table for quantity), preferably only once to avoid bubbles; add the mixture drop by drop to the culture vessel. Do this for each of the culture vessels.
  - Let the mixture form a gel in the cell culture incubator for 30 minutes (one hour maximum), then add carefully the culture medium (see ‘adding medium’ slide).

Table 1. *Quantities of EHS-derived gel and medium advised for use with 3D Embedding method*

Container Type	Surface Area	EHS-gel coat	Number of S1 cells	Number of T4-2 cells	EHS-gel embedding	Culture medium
35 mm dish	9.62 cm^2	100 µl	830,000	415,000	1 ml	1.5 ml
60 mm dish	28.27 cm^2	280 µl	$2.5x10^6$	$1.25x10^6$	3 ml	4.5 ml
4-well plate (1 well)	2.01 cm^2	20 µl	250,000	125,000	300 µl	500 µl
12-well plate (1 well)	3.80 cm^2	40 µl	473,000	236,500	570 µl	950 µl
6-well plate (1 well)	9.08 cm^2	90 µl	$1.1x10^6$	550,000	1.4 ml	2.3 ml

Note: Examples of cell numbers are for slow proliferating non-neoplastic breast epithelial cells (HMT-3522 S1) and rapidly proliferating, mildly invasive breast ductal carcinoma type cells (HMT 3522 T4-2 derived from S1 cells). The seeding cell numbers should be adapted to the cell lines or primary cells used based on their behavior in 2D culture.

Classical embbeding method

Precoat the culture vessel with EHS gel (10.4 µl/cm^2)

watch video #1

Place vessel in cell culture incubator for 5 minutes (maximum 20 minutes)

Centrifuge the amount of cells needed for each culture vessel

watch video #2

Resuspend cells in tiny amount of medium (~10% final volume of gel)

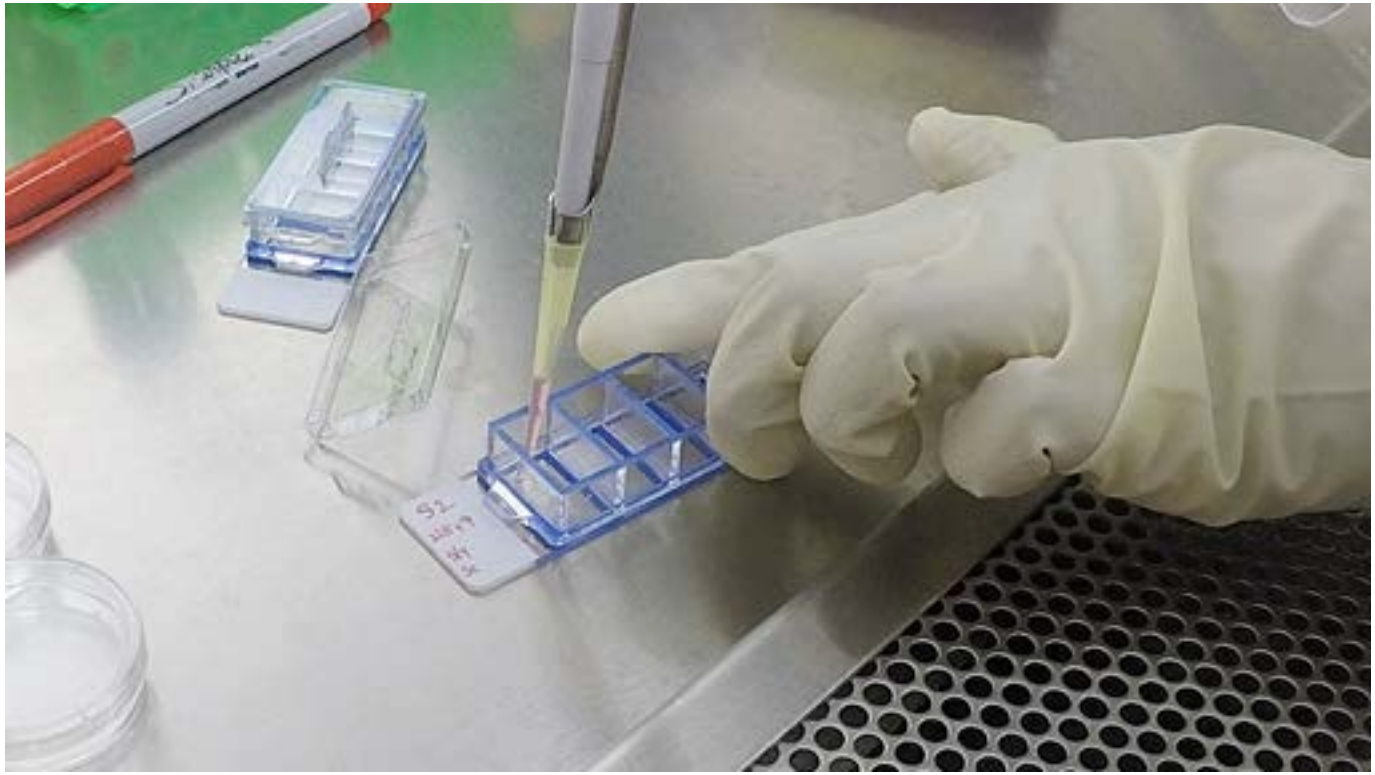
Mix the cells slowly with the gel once (avoid bubbles)

Add the mixture drop by drop onto the surface of precoated culture vessel

Place gel containting cells in the cell culture incubator for 30 (maximum 60) minutes

Add carefully the cell culture medium on the top of the gel.

Video 1: Coating Culture Vessels with EHS-derived Gel



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Video 2: Adding Cells with the 3D Embedding Culture Method

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Drip method

- Not all gels can be used with the drip method! (e.g., not for collagen I)

Example for EHS-derived gel:

- Precoat the surface of the cell culture vessel with a layer of liquid gel (52 µl/cm^2 unless otherwise specified [see table 2]; let the gel form in the cell culture incubator for 20 minutes;
- Centrifuge the amount of cells needed per culture vessel multiplied by the number of vessels used + 1 (*X+1 method*);
- Resuspend cells in half the total volume of medium needed, and drip cells in half the total volume for each culture vessel. Do not shake the vessel; let the cells settle down for a few minutes;
- Add 10% of EHS-derived liquid gel to the other half of the total volume of medium needed (it will be 5% in the final volume). Add this mixture drop-by-drop throughout the surface of the culture vessels.
- The EHS drip is only done at the time of cell seeding. There is no need to do the drip again when changing the medium.

Table 2. *Quantities of EHS-derived gel and medium advised for use with 3D Drip method*

Container Type	Surface Area	EHS-derived gel coat	Number of S1 cells	Number of T4-2 cells	Cell culture medium (medium + cells)	10% EHS-derived gel in medium (drip)
35 mm dish	9.62 cm^2	500 µl	400,000	200,000	650 µl	650 µl (585 µl medium + 65 µl gel)
60 mm dish	28.27 cm^2	1.5 ml	$1.2x10^6$	600,000	1.9 ml	1.9 ml (1710 µl medium + 190 µl gel)
4-well plate (1 well)	2.01 cm^2	105 µl	84,000	42,000	150 µl	150 µl (135 µl medium+15 µl gel)
4-well chamber slide (1 well)	1.44 cm^2	60 µl	50,000	25,000	200 µl	200 µl (180 µl medium + 20 µl gel)
12-well plate (1 well)	3.80 cm^2	200 µl	158,000	79,000	250 µl	250 µl (225 µl medium + 25 µl gel)
6-well plate (1 well)	9.08 cm^2	470 µl	380,000	190,000	600 µl	600 µl (540 µl medium + 60 µl gel)

Note: Examples of cell numbers are for slow proliferating non-neoplastic breast epithelial cells (HMT-3522 S1) and rapidly proliferating, mildly invasive breast ductal carcinoma type cells (HMT 3522 T4-2 derived from S1 cells). The numbers should be adapted to the cell lines or primary cells used based on their behavior in 2D culture.

Drip method

Precoat the culture vessel with EHS gel (52 µl/cm^2)

Place vessels in the cell culture incubator for 20 minutes

Centrifuge the amount of cells needed for all culture vessels (X+1 rule)

Resuspend cells in half the total volume of medium needed

watch video #3

Drip cells onto the surface of each culture vessel and let cells settle down for a few minutes

Mix the EHS gel with the remaining half of the total medium needed at 10% v/v concentration

Add the mixture (half the total volume of medium per vessel) drop-by-drop throughout the surface of each culture vessel

watch video #4

Place culture vessels in the cell culture incubator

Video 3: Adding Cells with the 3D drip Culture Method

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Video 4: Adding the EHS-gel Derived Drip to the 3D Drip Cell Culture

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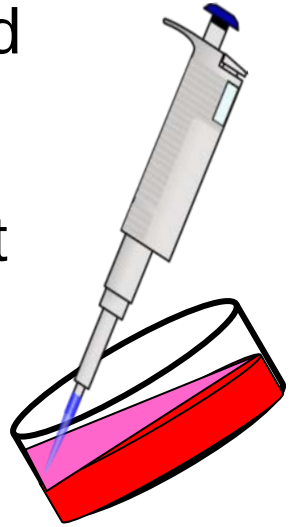
Removing and adding medium in the presence of a gel

- As usual the medium including additives (if used instead of serum) needs to be prepared the day of use (i.e., additives are added to the volume of medium needed for the experiment(s) or for the medium change that day).
- Make sure that the tip of the aspirating pipette does not touch the gel or the cells (if drip culture). To do so, tilt slightly the culture vessel and aspirate from the inner side of the vessel wall.
- Add the medium slowly against the inner wall on one side of each culture vessel.



watch video #5

Video 5: Adding Medium to a 3D Cell Culture Vessel Containing a Gel

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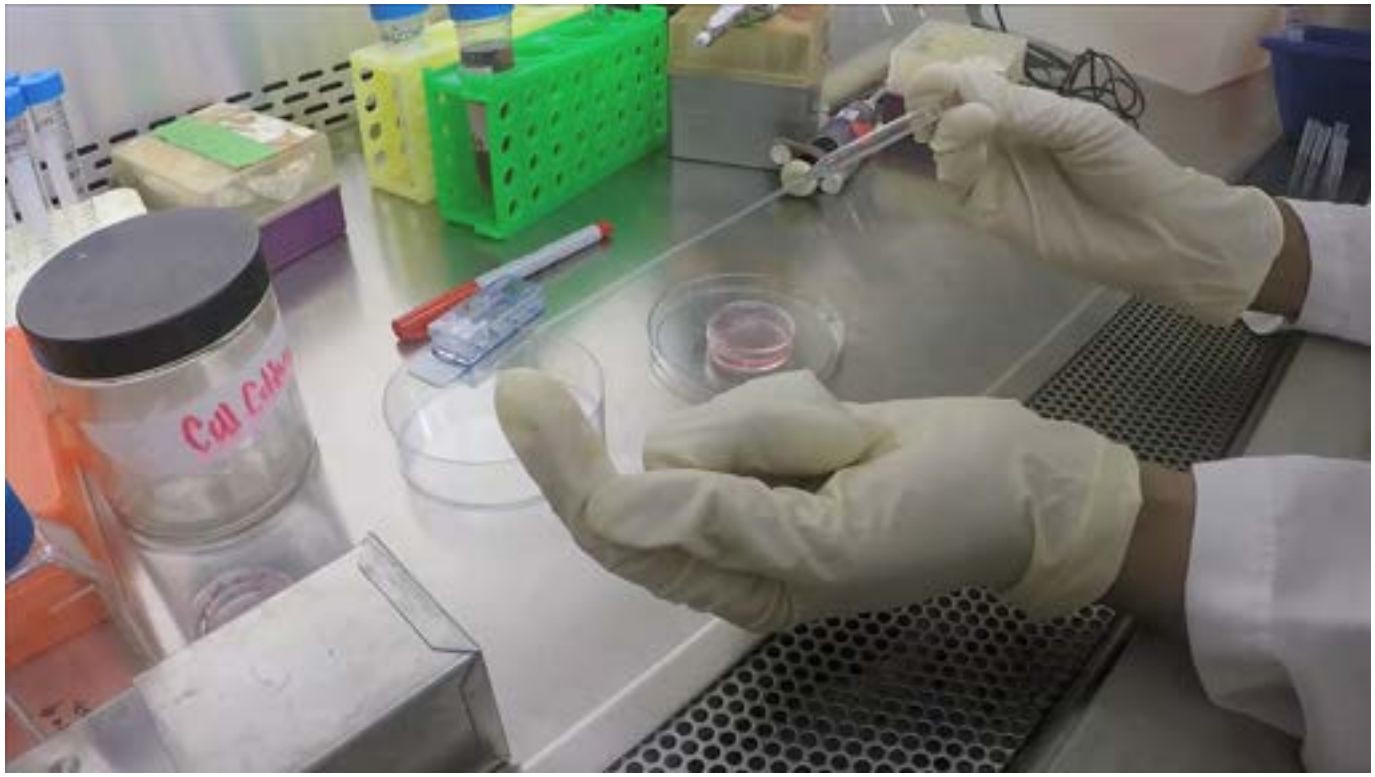
More on removing the medium…

- Extreme care is required while removing the medium from a cell culture done in the presence of a gel as it is easy to puncture the gel or aspirate parts, or even the entire gel, depending on its texture.
- Although you may use a pasteur pipette, it is safer to use a yellow tip to aspirate the medium. Make sure that it does not touch the gel or the cells by tilting the culture vessel at a safe angle and aspirating from the region where the medium accumulates.
- Aspirate the medium from the inner wall of the culture dish or at the level of a corner if you are using a chambered slide.  **watch video #6**

Video 6: Removing Medium from a 3D Cell Culture Vessel Containing a Gel



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Dislodging cells from 3D cultures made with a gel

- Aspirate the medium gently without touching the surface of the gel or cells. Make sure that the medium is completely removed by tilting at a bigger angle as medium is being removed.

- For gels requiring an enzyme to dissolve them (e.g., EHS-derived gel, collagen I), add the dislodging enzyme directly onto the gel and proceed according to the manufacturer's instructions.

- With dispase (for EHS-derived gel), incubate at 37^0C. After 45 min check if nodules/spheroids are floating. Incubate for another 15 minutes if necessary. Once nodules/spheroids are floating it is ready.

Note: There is another method for EHS-derived gel, based on cold temperature (on ice) and addition of EDTA; it is not our preferred method because of the thermic shock and the fact that it will disrupt cell-cell interactions that need to be kept intact for many experiments.

Dislodging cells from 3D cultures made with a gel (cont.)

- Collect the mixture nodules/spheroids (avoid taking the remaining gel if not dissolved towards the bottom of the culture vessel) and spin down for 5 minutes at usual speed for collecting cells.
- Remove the supernatant carefully and wash the nodules/spheroids with fresh medium three times (spin down after adding each wash but do not pipet up and down to resuspend the pellet otherwise cells get stuck to the tip wall). It is important to remove the enzyme solution as much as possible.
- Proceed with the experiments planned after a fourth wash in PBS if appropriate (e.g., if cells will be lyzed), then add the lysis buffer… or add trypsin as usual if cells need to be reseeded or separated for any reason.

All steps should be performed under ‘sterile’ conditions until cell pellets are transferred to the bench to add the lysis buffer.

Dislodging cells from 3D cultures made with EHS-derived gel

Add dispase directly onto the gel

watch video #7

Incubate at 37^{0}C for 45 minutes; check if nodules/spheroids are floating. Incubate for another 15 minutes if necessary.

Collect the mixture of nodules/spheroids (avoid taking remaining gel) and centrifuge for 5 minutes at usual speed for cells.

Remove the supernatant carefully and wash the nodules/spheroids with fresh medium three times (do not resuspended the pellet after each wash)

Wash the nodules/spheroids with PBS before adding the lysis buffer

OR

Add trypsin to separate cells before reseeding cells or freezing cells, etc.

Video 7: Dislodging Cells with Dispase from Culture with EHS-derived Gel

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Using 3D cultures for staining purposes

- The drip culture and thin embedding culture (sandwich method) can be used for direct immunostaining and observation under the microscope.
- Thin and thick embedding cultures can be prepared for paraffin embedding or cryopreservation. The blocks can then be sectionned with the appropriate method before immunolabeling. Paraffin embedding is commonly preceeded with treatment with a fixing solution. Whereas, cell cultures embedded in cryo-O.C.T. compound and cryopreserved are not fixed. Only the cryosections will be fixed at the time of use. This process allows the scientist to choose the fixing method depending on the staining.