PROTOCOL

How to 3D culture human mesenchymal stem cells — Seeding hMSCs on P3D Scaffolds

Application:
This protocol is for seeding and 3D culturing stem cells on P3D Scaffolds (Ø12 mm) in a 24-well plate. The procedure provides guidelines on how to create three-dimensional multicellular in vitro tissue constructs, while it is based on the use of human mesenchymal stem cells (hMSCs) that are differentiated into osteoblasts to create a 3D bone cell culture. For more protocols, please visit our Resources Platform.

Materials

➢ P3D Scaffolds (supplied sterile)
➢ Pipette
➢ Cell culture incubator
➢ Cell culture flask
➢ Cryopreserved hMSCs
➢ Hemocytometer
➢ Growth medium for instance Human Mesenchymal Stem Cell Growth Medium (MSCGM™)
➢ Trypsin/EDTA
➢ TNS
➢ Salt solution
➢ Non-adherence 24 well plates (if another plate size is used, see "Recommended seeding densities and volume")
➢ Maintenance medium (MEM medium with 1% P/S and 10% FBS) or PBS
➢ Osteogenic medium (maintenance medium with 10mM β-glycerol phosphate, 10nM dexamethasone, 10nM calcitriol and 250nM ascorbic acid)
➢ Collagen dye (Sirius Red)
➢ Spectrophotometer
**Flowchart**

1. Prepare cell suspension
2. Count cells and calculate the cell concentration
3. Add sterilized or disinfected P3D Scaffolds to each well in a 24-well plate
4. Seed 0.5mL 10^5 hMSCs on the 24-well plate with P3D Scaffolds

*Figure 1* Overview of the workflow for handling P3D Scaffolds to create a 3D mode tissue model. Prepare the cell suspension and calculate the cell density, then, before seeding the cells onto the 24-well plate, add one sterile P3D Scaffold to each well.

**Notes before starting and general advice on material handling**

- All handling of The P3D Scaffolds products should be performed using gloves, according to the standard aseptic methods.
- The scaffolds are supplied sterile by dry heat sterilization and remains sterile until opened.
**Procedure**

**Preparation of scaffolds**
1. Place one P3D scaffold in each well of a 24-well plate. We recommend using low-adherence well plates.
2. **Optional:** Wash the P3D Scaffolds in PBS x3 for one minute to remove any particles.
   Note: The material and structure of the P3D Scaffold are not compromised during sterilization or disinfection, but loose particles may occur.

![Figure 1: Place the P3D Scaffolds in a 24-well plate, preferably low-adherence.](image)

**Preparing cell suspension**
The procedure uses cryopreserved hMSCs which need to be thaw properly for preserving the cell culture health. The work is performed in a Biological Safety Cabinet.

1. Heat up the growth medium to 37°C in water/bead bath.
2. Prepare the appropriate number of flasks with the amount of warm medium and check the number of cells in the cryopreserved vial. The recommended cell seeding density for hMSCs is 5,000-6,000 cells/cm². It is recommended to use 0.2 mL of media per cm², however, the media volume varies by flask size. Use for formulas to find the number of flasks and volume of media.
   \[
   \text{Number of flasks} = \frac{\text{number of cell/ cell seeding density } / \text{ flask area in cm}^2}{\text{Amount of media}} = (\text{flask area in cm}^2) * 0.2 \text{ mL}
   \]
3. Incubate the media-containing flasks for 30 minutes before adding cells.
4. Remove the cell vial from liquid nitrogen and lower it in water/bead bath for approximately 1.5 minutes or until the sides of the vial are thawed.
5. Firstly add 5 mL of growth media and then pipette the cell solution to a sterile centrifuge tube and centrifuge at 500 g for five minutes.
6. Discard the supernatant and add the volume (mL) of media which correspond to the number of flasks to the cell pellet (Note: use 1mL for each flask) and resuspend the suspension. Example: Six flasks require 6 mL of media, then add 6mL to the pellet.
7. Add 1mL of cell suspension to each prepared flask and tilt the flasks gently.
8. Incubate the flasks at 37 °C and change culture media two to three times per week. This allows the cells to enter the growth phase.

Conduct the passaging of hMSCs to proliferate the cells until confluency of 90%.

9. Clean the space and material before use.
10. Remove the cell culture flask from the incubator. Examine the cell culture under a microscope and check the confluency. A confluency of ~90% should be obtained. Confluency is estimated by the ratio between the surface area occupied cell and the surface area not occupied cells.
11. Discard the cell media without touching the bottom of the flask.
12. Wash the flasks with salt solution. The solution should cover the surface. Discard the salt solution.
13. Add trypsin/EDTA to the flask. The volume is calculated by the following formula:

\[ \text{Volume} = \frac{\text{the recommended volume of flask surface area is 0.5 mL/10 cm}^2}{\text{flask surface area is 0.5 mL/10 cm}^2} \]

14. Tilt the flask gently and place it in the incubator for five minutes.
15. Check under the microscope to see if the cells are released from the bottom. If not, place the flask in the incubator for five minutes, otherwise continue.
16. To neutralize the activity of trypsin/EDTA, add TNS to the flasks. The volume is the same as calculated for trypsin/EDTA.
17. Transfer the cell suspension into a centrifuge tube by pipetting, and centrifuge at 600g for five minutes.
18. Discard the supernatant – do not disturb the pellet.
19. Count the cells by a hemocytometer. Resuspend the cell pellet in 1-5 mL of culture medium. For quantify viability add 1 μL of trypan blue and 10 μL of cell suspension to a tube and resuspend. Pipette 10 μL of the solution and place it into one hemocytometer chamber. Carefully place a coverslip on top and place the hemocytometer under a microscope. Count the cells using a cell counter. Calculate the number of cells \((n_{\text{cells}})\) in the suspension by the formula:
20. Replate the cells into flasks or culture dishes. Calculate the number using the following formula:

\[
\text{Number of flask} = \frac{\text{Total number of cells}}{\text{cell seeding density}} \times \frac{1}{\text{Flask surface area (a)}}
\]

21. Add 1 mL of cell suspension to each flask.

22. Calculate the amount of cell media for culturing the cells:

\[
\text{Volume of culture media} = 0.2 \text{ mL} \times \text{flask surface area in cm}^2
\]

23. Add the media to each flask.

24. Change the culture media two to three times per week. Before changing the media, wash the flasks with salt solution.

**Cell density**

Use the following formula for obtaining the correct cell density for seeding onto the P3D Scaffolds. The cell density should be around \( 1.9 \times 10^4 - 1.9 \times 10^6 \) cells/mL for seeding cells in a 24 well plate and the cell density for the culture flask is \( 5 \times 10^4 - 6 \times 10^4 \) cell/mL (\( 5 \times 10^3 - 6 \times 10^3 \) cell/cm\(^2\)):

\[
\frac{\text{density}_{\text{flask}}}{\text{density}_{\text{plate}}} \times 100 \text{ mL} = V
\]

The volume (V) is the media volume that is required to obtain the recommended cell density for cell plating onto 24 P3D Scaffolds in 24-well plate.

**Plating cells**

1. If not done already, add one sterilized or disinfected P3D Scaffolds to each well of a 24-well plate.
2. Add 500μL culture media to each well and let the scaffold soak. The scaffold must be completely submerged in medium.
3. Incubate the plate in a humidified incubator for at least 30 minutes, at 37°C and 5% CO\(_2\).
4. Seed \( 10^4 - 10^6 \) cell/mL hMSCs in 500 μL maintenance medium to each well, on top of the scaffolds (see Fig. 1). Place the cells in the middle of the well and do not touch the scaffold.
5. Replace the medium after 24 and 48 hours with maintenance medium.
6. To differentiate the hMSCs to osteoblast, replace the maintenance medium with an osteogenic medium after 48h.
7. Observe the implants 24 and 48h post seeding to observe the cell status.
8. Change the medium twice weekly.
9. The organoids are now ready for analysis and further experiments.

Optional step: Before analysis and further experiments, the option of quantifying the bone formation, and thereby presence of collagen type II, could be conducted by the following procedure:

The kit used for the procedure is Sirius Red/Fast Green Collagen Staining Kit (Catalog # 9046).
1. For example, P3D Scaffolds from six wells can be transferred to another plate.
2. Remove the culture media from the six wells and wash them with 1xPBS two times.
3. Add 0.5 mL of Kahle fixative or enough to submerge the Scaffolds and incubate for 10 minutes at room temperature (RT).
   - Kahle fixative recipe
     - 60 mL distilled water
     - 28 mL 96% ethanol
     - 10 mL 37% formaldehyde
     - 2 mL glacial acetic acid
4. Remove Kahle fixative and wash with 1x PBS.
5. Add 200-300 μL Dye Solution (90461), enough to completely submerge the scaffolds, and incubate at RT for 30 minutes.
6. Carefully remove the Dye Solution (90461) by pipetting.
7. Rinse the stained scaffolds with 0.5 mL distilled water. Repeat until the water runs clean.
8. Add 1 mL Dye Extraction Buffer (90462) in each well (only the wells used for this procedure) and gently mix by pipetting until the color is eluted from the scaffolds. Incubate for 20 minutes at a rocking table at RT.
9. Transfer 3 x 200 μL from each well to a 96-well plate.
10. Collect the eluted dye solution and read the OD values at 540 nm and 605 nm with a spectrophotometer.
Calculate the amount of collagen. Firstly, correct the OD 540 value by subtracting the contribution of Fast Green at 540 nm, which is 29.1% of the OD 605 value and divide with the color equivalence (OD values/μg protein) for collagen at OD 540 that is 0.0378. Use the following formula:

\[
\text{Collagen (μg section)} = \frac{\text{OD 450 value} - (\text{OD 605 value} \times 0.291)}{0.0378}
\]

Secondly, find the amount of non-collagenous proteins by dividing the OD 605 value with the color equivalence (OD values/μg protein) for non-collagenous proteins at OD 650 that is 0.00204.

\[
\text{Non collagenous proteins (μg section)} = \frac{\text{OD 650 value}}{0.00204}
\]

**Further Experiments**

Further experiments could include seeding of other cell lines, such as cancer cell lines, onto the P3D Scaffold for investigating tumor biology, metastases to bone, and converting the organoid to a tumoroid. Once a tumoroid line is established, this can be used to test several drugs. For example, you can hereby study the treatment of the imbalance in the bone remodeling process that occurs due to osteoporosis and metastasis to bone. However, only the imagination limits the usage of P3D Scaffolds in bone research.

### Recommended seeding densities and volumes

<table>
<thead>
<tr>
<th>Plate size</th>
<th>Min. cell density (cells/well)</th>
<th>Max cell density (cells/well)</th>
<th>Seeding volume (μL) for growth media</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>95,000</td>
<td>9,500,000</td>
<td>1,000 to 3,000</td>
</tr>
<tr>
<td>12 well</td>
<td>38,000</td>
<td>3,800,000</td>
<td>1,000 to 2,000</td>
</tr>
<tr>
<td>24 well</td>
<td>19,000</td>
<td>1,900,000</td>
<td>500 to 1,000</td>
</tr>
<tr>
<td>48 well</td>
<td>9500</td>
<td>950,000</td>
<td>200 to 400</td>
</tr>
</tbody>
</table>
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