Cell seeding, assembly, and media change protocol for NANOSTACKS™

Introduction

This protocol provides comprehensive instructions for cell seeding, assembly, and media change using NANOSTACKS™, a specialized cell culture platform designed for organ function modeling, ensuring optimal cell adhesion and growth. The porous membrane enhances nutrient and gas exchange in cell models.

Materials and equipment

- NANOSTACKS™ (sterile, provided by Revivocell)
- 24-well plates
- Class II safety cabinet
- Complete media
- Sterile forceps
- Hemocytometer
- Sterile stripettes
- Sterile pipette tips
- 1X Dulbecco's Phosphate-Buffered Saline (DPBS)
- CO_2 incubator
- Inverted microscope

Preparation of NANOSTACKS™

1. Spray 70% ethanol on the NANOSTACKS™ package and place it in a Class II safety cabinet.
2. Carefully open the package and use sterile tweezers to grasp each stack using one of the four raised edges (see Figure 1 below), then place it into the wells of the 24-well plates. **Ensure that each NANOSTACK™ is properly positioned with the concavity facing up.**

Measuring cell density

1. Pipette 15 to 20 µl of the cell suspension between the hemocytometer and cover glass using a P20 pipette.
2. Count the cells in all four quarter squares and divide by four to determine the mean number of cells per square.

Cell seeding on NANOSTACKS™

1. To seed the cells on NANOSTACKS™, add 75 µl of cell suspension (25,000 – 75,000 cells per NANOSTACK™ depending on the cell type) on top of each NANOSTACK™ membrane area (Figure 1), making sure that the droplet of cell suspension is within the membrane area.

   For example, optimal seeding densities for the following cells are estimated as follows:

   - For SH-SY5Y cells, add 6500 cells per NANOSTACK™.
   - For differentiated HepaRG cells, add 72000 cells per NANOSTACK™.
   - For primary human hepatocytes, add 50000 cells per NANOSTACK™.

   Optimize seeding density for each cell type and experimental conditions.

2. Add 1 ml of PBS to empty wells to prevent evaporation of the cell suspension droplets.
3. Place the 24-well plates in a 37°C incubator for 2 hours to allow the cells to adhere to the membrane.
4. Gently add 925 µl of complete medium to each well to fully cover the NANOSTACKS™, taking care not to disturb the cells on the NANOSTACKS™.

   After adding the medium, check each stack to see if there are any air bubbles trapped below the membrane. If there any air bubbles, gently twist the stack with a plastic pipette tip to eliminate the bubble.

5. Place the plates back in the 37°C incubator overnight.
6. Inspect the cell plating efficiency (PE) under a microscope. The membranes of the NANOSTACKS™ are transparent, and cells are visible using a standard inverted cell culture microscope.

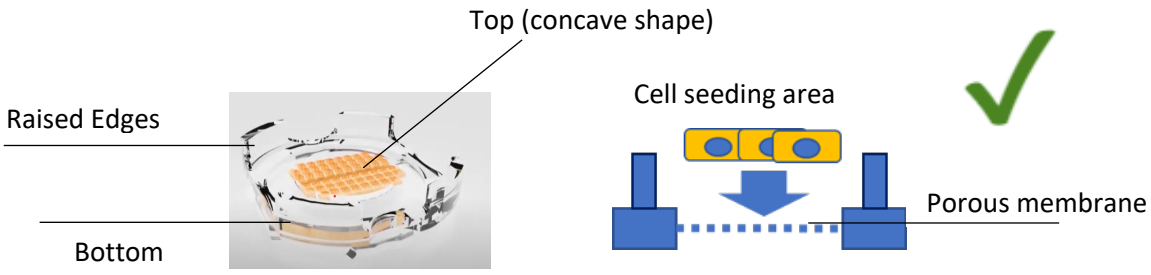
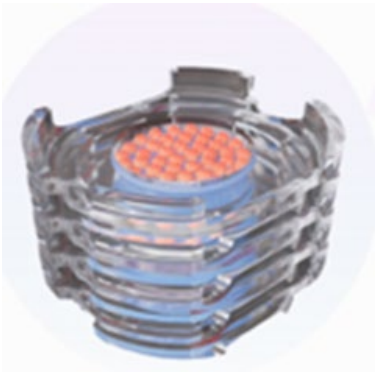


Figure 1. *Correct position of NANOSTACKS in well-plates and seeding area.*

Assembly of multiple NANOSTACKS™ in wells

Following successful cell seeding, **up to four NANOSTACKS™** can be stacked on top of each other to create organ models with multiple cell-cell interactions.



1. Add the required medium to each well (1 ml to 1.5 ml, depending on the number of NANOSTACKS™ used; 1.5 ml will cover 4 NANOSTACKS™).
2. To stack multiple NANOSTACKS™ in wells, use sterile forceps to gently position them on top of each other with the raised lips up (Figure 1). Ensure that each NANOSTACK™ is properly positioned with the concavity facing up (see Figure 1).

    - **If any bubbles form while stacking the NANOSTACKS™, remove the affected stacks and gently tilt or angle them to submerge their sides whilst aligning them with the underlying stacks.**

Media change

1. Open the lid of the well plate inside the cell culture hood.
2. Remove or add the media using a standard pipette from the side of the NANOSTACKS™. **Do not touch the filter membranes to avoid damage.**
3. Add a minimum of 1 ml of media onto each NANOSTACK™
4. Securely close the well plate lid after media change.
5. Return the well plate to the CO_2 incubator for further incubation.

Note: Maintain aseptic technique throughout the procedure to prevent contamination.