Immunofluorescence of Organoids Embedded in Cultrex® Basement Membrane Extract

Organoid progenitor cells are derived from embryonic, adult or induced pluripotent stem cells, and they present many similarities when compared with their tissues of origin. Cultrex Basement Membrane Extract (BME) is commonly used to provide the extracellular matrix environment required to form organoids from their respective progenitor cells. Even so, conserving intact organoids embedded in Cultrex BME to analyze the expression of markers by immunostaining represents a current challenge. This protocol describes a method to preserve organoids intact (and potentially other types of 3-D cultures) in order to be immunostained and visualized.

Protocol for the Immunofluorescence of Organoids Embedded in Cultrex BME

Set-up of Organoid-Basement Membrane Extract Domes

To optimize culture vessel space and to make manipulation of organoids easier, we recommend culturing organoids embedded in Cultrex BME as domes arranged in wells of a 6-well plate; this may be scaled for larger area plates if needed. Each dome should contain 50 µL of resuspended organoids in BME. In Figure 1, the placement of 6 domes in each well of a 6-well plate is shown. Once finished, the plate is placed in a tissue culture incubator at 37 °C to polymerize the BME. After the BME has polymerized, three milliliters of organoid culture medium are added to each well.

Organoid Culture of Mouse Small Intestine and Mouse Colon Organoids

1. Mouse Small Intestine (mSI) Organoid Culture.
   a. Organoids were grown from murine small intestine progenitor cells embedded in Cultrex BME* and placed in 6-well plates as shown in Figure 1.

* Cultrex Reduced Growth Factor (RGF) BME, Type 2 (Catalog # 3533-005-02) is recommended for robust organoid cultures and Cultrex RGF BME, Type R1(Catalog # 3433-005-R1) is recommended for difficult to grow organoid cultures.
b. Organoids were cultured in mSI organoid culture medium for at least 3 passages.

i. Recipe for mSI organoid culture medium:

Note: The majority of reagents used in this protocol were sourced from the Bio-Techne brands of R&D Systems and Tocris Bioscience.

- 50% of the final volume was completed with L Wnt-3a-conditioned medium
- 1X N21-MAX Media Supplement
- 1X N-2 Media Supplement
- 2 mM Glutamine
- 10 mM HEPES
- 10 mM Nicotinamide
- 1mM N-Acetylcysteine
- 10 nM [Leu15]-Gastrin I Human
- 1 mg/mL HA-R-Spondin1-Fc (produced using Cultrex HA-R-Spondin1-Fc 293T Cells, Catalog # 3710-001-01)
- 100 ng/mL Recombinant Mouse Noggin
- 500 nM A83-01
- 7.5 µg/mL Human Insulin
- 10 µM SB 202190
- 10 µg/mL Human Transferrin
- 50 ng/mL Recombinant Mouse EGF
- Advanced DMEM/F-12 Cell Culture Medium was added to complete to final volume.

For differentiation experiments, mSI organoids were passaged and cultured in differentiation medium for several weeks. The composition of this medium was identical to mSI organoid culture medium with two exceptions:

- It did not contain L Wnt-3a-conditioned medium
- DAPT (a Notch inhibitor) was added fresh at every medium change with a final concentration of 5 µM. The difference in volume was adjusted by adding Advanced DMEM/F-12 Cell Culture Medium.

2. Mouse Colon (mCOL) Organoid Culture

a. Organoids were grown from murine colon progenitor cells embedded in Cultrex BME* and placed in 6-well plates as shown in Figure 1.

*Cultrex Reduced Growth Factor (RGF) BME, Type 2 (Catalog # 3533-005-02) is recommended for robust organoid cultures and Cultrex RGF BME, Type R1 (Catalog # 3433-005-R1) is recommended for difficult to grow organoid cultures.

b. Organoids were cultured in mCOL organoid culture medium for at least 3 passages.

i. Recipe for mCOL organoid culture medium:

Note: The majority of reagents used in this protocol were sourced from the Bio-Techne brands of R&D Systems and Tocris Bioscience.

- 50% of the final volume was completed with L Wnt-3a-conditioned medium
- 1X N21-MAX Media Supplement
- 1X N-2 Media Supplement
- 2 mM Glutamine
- 10 mM HEPES
- 1mM N-Acetylcysteine
- 1 mg/mL HA-R-Spondin1-Fc (produced using Cultrex HA-R-Spondin1-Fc 293T Cells, Catalog # 3710-001-01)
- 100 ng/mL Recombinant Mouse Noggin
- 50 ng/mL Recombinant Mouse EGF
- Advanced DMEM/F-12 Cell Culture Medium was added to complete to final volume.
Organoid Harvesting and Passaging

1. Organoids were harvested to be passaged and expanded using Cultrex® Organoid Harvesting Solution (R&D Systems, Catalog # 3700-100-01).

2. Culture or differentiation medium was discarded, and the wells were washed with 5 mL of cold (2–8 °C) PBS and incubated for 30 to 60 minutes with 5 mL of cold (2–8 °C) Cultrex Organoid Harvesting Solution.
   a. During this time, the plates were placed inside a container with ice or in a cold room with gentle shaking in order to achieve matrix depolymerization.

3. Once the matrix was dissolved and the organoids were released, the solution was transferred to a conical centrifuge tube, and organoids were mechanically disrupted by passing them through a 20 gauge needle attached to a syringe.

4. Fragmented organoids were centrifuged for 5 minutes at 500 × g at 2–8 °C. The supernatant was discarded.

5. The cell pellet was washed with 5–10 mL of cold (2-8 °C) 1X PBS and centrifuged once more, and the supernatant was discarded.

6. After a final centrifugation, PBS was discarded, and organoids were resuspended in Cultrex BME for re-plating.

Fixing of Organoids, OCT Embedding, and Cryosectioning

1. Remove organoid culture media and wash each well of a 6-well plate with 5 mL of 1X PBS at room temperature.

2. Fix structures in Cultrex BME with 5 mL of 2% paraformaldehyde (PFA) + 0.1% glutaraldehyde (GA) in 1X PBS for 30 minutes at room temperature.
   **Note:** Occasionally PFA may cause depolymerization of Cultrex BME. Addition of glutaraldehyde solves this issue, however, glutaraldehyde may produce higher autofluorescence, so it is important that the quenching step is optimized for each particular protocol.

3. Wash 3 times with 5 mL of 1X PBS for 10 minutes each wash to remove fixing solution.

4. Carefully, take the organoid-BME domes with a scoop or spatula and place them in a 50 mL conical tube containing 20% sucrose in 1X PBS and leave the tube at 2–8 °C overnight or until the domes fall to the bottom of the tube (it may take up to 3 days).

5. Remove the domes from the sucrose solution and place in an embedding mold containing Optimal Cutting Temperature (OCT) compound. Try to remove as much of the sucrose as possible. Place several domes per mold. We recommend embedding at least 6 organoid-BME domes in each block.

6. Snap freeze and store at ≤ -70 °C.

7. Using a cryotome, cut the organoid block into cryosections. We recommend a 10 μm-thickness for organoid cryosections.

Immunostaining

1. Wash the slides once with 1X PBS for 15 seconds to remove OCT.
   **Optional:** Use a hydrophobic marker to delimit the area around the organoids.

2. Quench aldehyde groups produced after fixing steps by incubating slides with a 10 mM solution of NaBH₄ in 1X PBS twice for 5 minutes at room temperature each time. If auto-fluorescence is detected, increase the quenching washes.
   **Note:** A 15 minutes incubation done twice with 0.2 M Glycine solution in 1X PBS can be used to quench the samples instead.

3. Wash 3 times with 1X PBS for 10 minutes each wash.

4. Permeabilize tissues with 0.15% Triton + 1X PBS for 15 minutes at room temperature.

5. Wash 3 times with 1X PBS for 10 minutes each wash.

6. Block slides with 3% BSA + 1X PBS (Blocking Solution) or 10% FBS + 1X PBS for 1–2 hours at room temperature.

7. Dilute the primary antibody in blocking solution to the desired final concentration.
concentration. Tap slides on the side to remove the blocking solution and, without washing, add 200–400 µL of primary antibody to the organoids. Spread throughout the slide.

8. Incubate with primary antibody overnight at 2–8 °C in a humidified chamber.

9. Wash slides twice with 1X PBS for 15 seconds each wash.

10. Wash 3 times with 1X PBS for 10 minutes each wash.

11. Dilute secondary antibodies in blocking solution to the desired final concentration. Add 200–400 µL of secondary antibodies to the organoids.

12. Incubate at room temperature for 1.5–2 hours in a humidified chamber.

13. Wash 3 times with 1X PBS for 10 minutes each wash.

14. To counterstain, use a dilution of DAPI (Tocris; Catalog #5748) or Hoechst 33342 (Tocris; Catalog # 5117) for nuclear staining or Phalloidin to stain actin. All three counterstain compounds are diluted in 1X PBS.

15. Wash once with 1X PBS for 10 minutes.

16. Rinse in distilled water, remove as much water as possible and mount using Fluor Mounting Medium (R&D Systems, Catalog # 4866-20).

17. Wait until the slides are dry and use an epi-fluorescence or a confocal microscope to visualize your samples.

Data Examples

Organoid Lumen Preservation

Both mouse small intestine and colon organoids develop a hollow lumen, as do other organoids, and it can be challenging to preserve these structures intact after fixing and cryosectioning steps. By following the protocol provided it is possible to maintain the anatomy of organoids as seen in Figure 3 and Figure 4.

Figure 3. Differentiation of Mouse Small Intestine Organoids. A) Mouse small intestine organoids usually grow as spherical hollow structures. B) Upon differentiation, crypt- and villi-like structures start to form. Immunofluorescence against E-Cadherin (green) shows staining of tight-junctions. Nuclei were stained with DAPI (blue). Scale bar: 50 µm.

Figure 4. Mouse Colon Organoids. Representative images of mouse colon organoids after 5 days in culture and immunostained with an anti-E-Cadherin fluorescent antibody (red). Nuclei were stained with DAPI (blue). Scale bar: 50 µm.
Expression of Intestinal Markers.

Using this protocol, it is also possible to analyze a variety of markers. In Figure 5, cells positive for Ki-67 are an indication of proliferating cells within crypt-like structures in mouse small intestine organoids. Figure 6 shows how mouse colon organoids produce and secrete Mucin 2 (MUC2), a mucus forming glycoprotein secreted by goblet cells in the intestinal epithelium.

References