**Basic Protocol 4: IMMUNOFLUORESCENCE STAINING OF HUMAN INTESTINAL ORGANOIDS**

This protocol describes a way to perform immunofluorescence staining compatible with fluorescence and confocal microscopy. For this purpose, organoids must first be released from the BME without disrupting their 3D architecture. Subsequent fixation, permeabilization, blocking, staining, and washes are performed with the organoids in suspension. This method can be applied to organoids that have been subjected to various experimental conditions.

Materials

* Established organoid culture (see Basic Protocol [2](https://currentprotocols.onlinelibrary.wiley.com/doi/full/10.1002/cpim.106#cpim106-prot-0002))
* Fetal bovine serum (FBS, Sigma-Aldrich, cat. no. F7524)
* Cell Recovery Solution (Corning, cat. no. 354253)
* 4% (v/v) PFA
* **Permeabilization solution**

Phosphate-buffered saline (PBS)

0.5% (v/v) Triton X-100

2% (v/v) donkey serum (Bio-Rad, C06SB)

Prepare fresh before use

* **Blocking solution**

Phosphate-buffered saline (PBS)

0.1% (v/v) Tween-20

2% (v/v) donkey serum

Prepare fresh before use

* primary antibody
* PBS
* Alexa Fluor 488 anti-mouse secondary antibody
* ProLong Gold Antifade Mounting solution with DAPI (Thermo Fisher Scientific, cat. no. P36934)

**Release organoids**

1. Remove and discard medium from one well containing organoids.

*Starting with small organoids will help keep their structure intact. Starting with large and cystic organoids is more likely to result in disruption.*

2. With sterile scissors, cut the opening of a 1000-μl pipette tip 2-3 mm from its end. Coat the tip and a 1.5-ml microcentrifuge tube with FBS by pipetting 1 ml up and down once.

*Cutting the tip is required to avoid disrupting the organoid structure while pipetting. Coating with FBS minimizes adhesion of organoids to plastic surfaces.*

3. Using the coated pipette tip, pipette 1 ml Cell Recovery Solution to the well, collect the organoids, and transfer to the coated microcentrifuge tube.

*Repeat steps 1-3 for each well of cells to be stained (e.g., for different experimental conditions).*

4. Incubate on ice for 20-30 min, inverting regularly to prevent clumping and heterogeneous BME dissociation.

*Cell Recovery Solution dissolves the BME, freeing the organoids without disrupting their structure. Alternatively, the culture can be preincubated with dispase for 30 min at 37°C. Efficient dissolution of BME is key, as remaining BME can result in poor staining and higher background signal.*

5. Monitor BME dissociation under a microscope, stopping when BME is sufficiently dissolved.

6. Let organoids settle by gravity to the bottom of the tube and remove the supernatant.

Fix and permeabilize organoids

7. Add 1 ml of 4% formaldehyde and incubate 16 hr at 4°C (or 2 hr at room temperature) with constant rolling of the tube to ensure homogeneous fixation. Let organoids settle by gravity and remove supernatant.

*CAUTION: Formaldehyde is toxic. Avoid contact with skin and dispose of formaldehyde according to institutional and governmental safety rules.*

8. Add 1 ml permeabilization solution and incubate with constant rolling for 30 min at 4°C. Let organoids settle by gravity and remove supernatant.

*Permeabilization time and temperature may need to be optimized for particular staining methods.*

**Stain organoids**

9. Add 1 ml blocking solution and incubate 15 min at room temperature under constant rolling. Let organoids settle by gravity and remove supernatant.

10. Add 200 µl of 500 ng/ml mouse anti–human KI67 primary antibody in blocking solution and incubate for 16 hr at 4°C under constant rolling. Let organoids settle by gravity and remove supernatant.

*If using a different primary antibody, the final concentration must be optimized.*

11. Resuspend pellet in 1 ml PBS and incubate 10 min at room temperature with constant rolling. Let organoids settle by gravity and remove supernatant. Repeat wash two more times.

12. Add 200 µl of 4 µg/ml Alexa Fluor 488 donkey anti-mouse secondary antibody, 10 nM phalloidin-Atto 647N, and 2 µg/ml DAPI in blocking solution and incubate 1-2 hr in the dark at room temperature with constant rolling.

*From this point, samples must be kept in the dark to protect the fluorophores from bleaching.*

*If using a different secondary antibody, the final concentration must be optimized.*

13. Wash three times as in step 11.

14. Wash once with Milli-Q-purified water to prevent crystal formation.

15. Cut the tip of a low-retention 300-μl tip 2-3 mm from its end and then coat the tip with FBS by pipetting 200 μl up and down once.

*The FBS coating and low-binding tips minimize the number of organoids lost due to adhesion to the plastic surface in the next step.*

16a. *For imaging in 96-well plates*: Use the coated tip to resuspend organoids in 150-200 μl PBS and transfer them to one well of a 96-well black glass-bottom plate. For optimal results, image samples immediately after staining (Fig. [**4**](https://currentprotocols.onlinelibrary.wiley.com/doi/full/10.1002/cpim.106#cpim106-fig-0004)).

Confocal immunofluorescence images of human intestinal organoids cultured under expansion conditions. DAPI, nuclei; KI67, proliferation marker; phalloidin, F-actin staining. Scale bar, 0.1 mm.

16b. *For imaging on microscope slides*: Use the coated tip to resuspend organoids in 50 μl ProLong Gold mounting solution and transfer them to a microscope slide. Apply Vaseline at the edges of the slide so that the coverslip does not disrupt organoid structure after mounting. Seal the slide using nail polish.

*Slides can be stored at 4°C, maintaining the fluorescence signal for months.*

**PB Immunocytochemistry:**

CRC cells were grown on chamber slides (BD Biosciences) for 24hr, fixed in 4% paraformaldehyde for 15min at room temperature followed by permeabilization using 0.1% Triton X 100 for 10min. Cells were washed with PBS and blocking was done in 5% (w/v) bovine serum albumin for 1hr at room temperature. After blocking, the fixed material was incubated overnight with β-catenin primary antibody (BD biosciences) at 40C. Cells were washed with PBS for 5 min followed by incubation with Alexa fluor-488 conjugated secondary antibody (ThermoFisher scientific) for 1hr at room temperature. Cells were then mounted using Vectashield mounting medium with DAPI and visualized with LSM 700 confocal laser scanning microscope (Carl Zeiss) or Leica confocal microscope at 63X magnification.