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Immunofluorescent Staining of Mouse Intestinal Stem Cells

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Abstract

Immunofluorescent staining of organoids can be performed to visualize molecular markers of cell behavior. For example, cell proliferation marked by incorporation of nucleotide (EdU), or to observe markers of intestinal differentiation including paneth cells, goblet cells, or enterocytes (see Figure 1). In this protocol we detail a method to fix, permeabilize, stain and mount intestinal organoids for analysis by immunofluorescent confocal microscopy.

Materials and Reagents

1. 8 well chamber slides (Thermo Fisher Scientific, Lab-Tek™, catalog number: 154532)
2. Cover Glass, Rectangular #1 (24 × 50 mm, 0.12–0.16 mm) (Corning, catalog number: 2975-245)
3. Primary antibodies
 - a. Rabbit anti-KRT20 (1:200) (Cell Signaling Technologies, catalog number: 13063)
 - b. Rabbit anti-Lysozyme (1:200) (Dako, catalog number: EC 3.2.1.17)
 - c. Rabbit anti-Muc2 (1:200)/VHL Antibody (M-20) (Santa Cruz Biotechnology, catalog number: H-300, sc-1534)
 - d. Villin Antibody (C-19) (1:200) (Santa Cruz Biotechnology, catalog number: sc-7672)
4. Secondary antibodies:
 - a. Goat Anti-rabbit 568 (1:500) (Thermo Fisher Scientific, Molecular Probes, catalog number: 11036)

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11. Tris(hydroxymethyl)aminomethane (Sigma-Aldrich, catalog number: 252859)
12. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
13. PIPES (Sigma-Aldrich, catalog number: P6757)
14. Magnesium chloride (MgCl₂) (Sigma-Aldrich, catalog number: M8266)
15. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
16. TritonTMX-100 (Sigma-Aldrich, catalog number: X100)
17. TWEEN[®] 20 (Sigma-Aldrich, catalog number: P2287)
18. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2058)
19. Tris buffered saline (TBS) (see Recipes)
20. 10x PME buffer (see Recipes)
21. IF buffer (see Recipes)
22. Blocking solution (see Recipes)
23. Permeabilization solution (see Recipes)

Equipment

1. Benchtop Multi-Purpose Rotator (Thermo Fisher Scientific, model: 2309)
2. Leica Inverted Confocal SP8 equipped with a White Light Laser, a Leica HyD Detector and the Leica Application Suite software

Software

1. Leica Application Suite

Procedure

1. Plate 40 µl of the matrigel containing organoids into 1 well each of an 8-well chamber-slide. After letting harden in the incubator for 10–15 min, add 400 µl of media to the well and change at least every two days. If analyzing proliferation by EdU incorporation, add 10 µM EdU to the wells 6 h prior to fixation.
2. When ready for fixation, remove the media and add 300 µl freshly prepared 4% PFA in PME buffer (room temperature) for 20 min. For this step, and throughout the entire protocol, to change the solution in the well, carefully place a pipette tip into the side of the well and draw up the solution. Then carefully add the new solution down the side of the well. This is performed the same way when changing media (O'Rourke *et al.*, 2016).

3. Remove the fixative, wash once with IF buffer. Add 300 µl of Permeabilization Solution to the well to permeabilize for 20 min.
4. Remove the solution, wash once with IF Buffer and then add Blocking Solution for 30 min at room temperature.
5. Alternative step: To visualize enterocytes by alkaline phosphatase staining, wash the fixed cells twice with TBS and then incubate with 300 µl of the BCIP/NBT Substrate Kit Solution for 15 min in the dark (follow directions that accompany the kit to make the BCIP/NBT solution). The chambers are then washed twice with TBS and then imaged using bright field microscopy. An example is shown in Figure 2.
6. Alternative step: Prior to primary antibody incubation, the samples can be processed for EdU visualization via Click-It chemistry using the directions provided with the kit. At this point follow the Invitrogen Protocol step 4.3 and finish with 4.7. Then, if required, incubate your sample in 150 µl of primary antibody as in step 7. For an example of an EdU and Keratin 20 stained small intestinal organoid, see Figure 3. Be sure to protect the sample from light from this step forward.
7. Prepare the primary antibody in blocking solution and add 150 µl to each well. See Notes on Materials for antibodies we have validated. Let the chamber-slide sit overnight in a large, humidified chamber at 4 °C. Do not leave the chamber-slide lid on the chamber-slide as it can cause liquid to be pulled out of the chamber.
8. After overnight incubation, wash each well 3 times for 5 min each with 300 µl IF buffer. Place the chamber slide on a benchtop orbital shaker set to 60 RPM during each wash.
9. Prepare secondary antibody in Blocking Solution. For a list of secondary antibodies we have validated, see Note 4. Add 150 µl of the solution to the well and let sit at room temperature for 1 h, being sure to protect from light. Optionally, a phalloidin-conjugated fluorophore can be added to the secondary solution to visualize F-actin polymers.
10. Optional step: To visualize DNA/nuclei, prepare a solution of DAPI (1 µg/ml) in IF buffer and add 300 µl to each well for 5 min. Then proceed with washing (step 11).
11. After secondary incubation, wash each well 3 times with 300 µl IF buffer as in step 8.
12. Remove the IF buffer and detach the chambers using the removal kit that accompanies the chamber slides.

⁴We have validated the following secondary antibodies for use in this assay: Anti-rabbit 568 (1:500), anti-goat 594 (1:500) and anti-rat 488 (1:500).

13. Using a p200 pipette tip with the tip cut off (see Note 6), add a small amount of ProLong Gold antifade mounting medium over each specimen (~20–30 μ l each).
14. Place a cover slip over the specimen, avoiding bubbles. Use clear nail polish to seal the sides of the cover glass to the slide. Let harden overnight at room temperature in the dark. After imaging, the slide can be stored at 4 °C for at least 6 months with minimal loss of fluorescence.

Notes

¹During washes you will need to pipette liquid in and out of the chamber wells while carefully avoiding the organoids. A p1000 pipette tip with a tapered end can be helpful for this.

²We have had success with the Click-iT Edu Alexa Fluor 647 kit from Invitrogen, but note this kit is available with a wide range of fluorophores, which can be used to prevent overlap with any fluorescent protein that may be native to your cells. Note that this kit will also destroy F-actin, which prevents visualization of the actin cytoskeleton by Phalloidin conjugated fluorophores, as well as native GFP.

³We have validated the following primary antibodies for use in this assay: Rabbit anti-KRT20 (1:200), rabbit anti-Lysozyme (1:200), rabbit anti-Muc2 (1:200) and goat anti-Villin (1:200).

⁴We have validated the following secondary antibodies for use in this assay: Anti-rabbit 568 (1:500), anti-goat 594 (1:500) and anti-rat 488 (1:500).

⁵After the fixation and permeabilization steps, most of the Matrigel will have degraded, leaving the organoids to rest on the bottom of the well. This is true for small and large intestinal organoids grown for 4–6 days in culture prior to fixation, perhaps due to the production of intestinal proteases. We have noticed that shorter culture times, or different organoid cell types, can prevent this.

⁶When using the p200 to pipette the mounting medium over the cells, it is helpful to first use scissors to cut the tip at the tapered end to make it larger in diameter, which will avoid bubbles when pipetting the solution.

⁷We have had the best results imaging these samples using a confocal microscope. Specifically, we use a Leica Inverted Confocal SP8 equipped with a White Light Laser, a Leica HyD Detector and the Leica Application Suite software. This setup allows the excitation laser to be set to any wavelength between 470 and 670 nm, in addition to a 405 laser for DAPI excitation and the standard argon laser lines of 458, 476, 488, 496 and 514 nm. This means one can optimally excite and cleanly separate any fluorophores. The SP8 is also equipped with gating technology, which significantly eliminates autofluorescence.

⁸We have used the HC PL APO 20x .7NA IMM and the HCX PL APO 40x 1.1NA objectives for imaging these samples.

⁹For an example of an immunofluorescent image obtained using this microscope setup (see Figure 3).

⁶When using the p200 to pipette the mounting medium over the cells, it is helpful to first use scissors to cut the tip at the tapered end to make it larger in diameter, which will avoid bubbles when pipetting the solution.

Recipes

1. Tris buffered saline (TBS)
 - 25 mM Tris
 - 0.15M NaCl
 - Final pH of solution should be 7.2 to 7.5
2. 10x PME buffer
 - 500 mM PIPES
 - 25 mM MgCl₂
 - 50 mM EDTA
3. IF buffer
 - PBS containing:
 - a. 0.2% Triton X-10
 - b. 0.05% Tween
4. Blocking solution
 - IF Buffer containing:
 - 1% bovine serum albumin (BSA)
5. Permeabilization solution
 - PBS containing
 - 0.5% Triton X-100

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Figure 1. A schematic depicting a crypt-villus forming organoid, and visualization of Paneth cells by immunofluorescence staining

Left: Small intestinal organoids grow as crypt-villus structures that contain all of the multiple differentiated lineages of the intestine. Right: Immunofluorescent staining can be used to visualize individual cell types in the organoid. Here paneth cells are visualized by staining for lysozyme (“Lyso,” Green), which reveals Paneth cells located at crypt bases. F-Actin (Red) reveals crypt structure at the apical surface of the epithelium, and DAPI (Blue) reveals cell nuclei. Scale bar is 25 μ m.

Figure 2. A brightfield image of a small intestinal organoid showing strong alkaline phosphatase activity (indicating the presence of differentiated enterocytes)
Scale bar is 50 μm .

Figure 3. Immunofluorescent image of a small intestinal organoid showing markers of proliferation (EdU, Red), differentiation (Krt20, Green) and nuclei (blue)
Scale bar is 50 μ m.