Protocol

Efficient RNA and RNA-protein co-detection in 3D colonoids by whole-mount staining

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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Efficient RNA and RNA-protein co-detection in 3D colonoids by whole-mount staining

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SUMMARY
Here, we describe a protocol to visualize RNA oligos and proteins independently or together using a combination of fluorescence in situ hybridization (FISH) and immunofluorescence in human colonoids, expanding on previously published research. Whole-mount staining is used to preserve the colonoid structure and fix onto glass slides. We describe procedures for efficient plating, fixation, and preservation of the colonoids. This workflow can be adapted to 3D organoid models from other tissues or organisms. For complete details on the use and execution of this protocol, please refer to In et al. (2020).

BEFORE YOU BEGIN

Note: All human colonoid experiments are performed in accordance with and approved by the Institutional Review Board (IRB) at the University of New Mexico. All human colonoid/organoid experiments must be approved by an IRB at your research institution. For this protocol, colonoids are first grown in 24 well tissue culture plates with >200 colonoids per well.

This protocol builds on (Borrelli and Moor, 2020; Kersigo et al., 2018).

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Critical commercial assays |        |            |
| RNAscope wash buffer | ACD    | 310091     |
| RNAscope multiplex fluorescent reagent kit V2 | ACD    | 323100     |
| RNA-protein co-detection ancillary kit | ACD    | 323180     |
| RNAscope TSA buffer | ACD    | 322809     |
| RNAscope hydrogen peroxide and Protease Plus | ACD    | 322330     |
| RNAscope probe diluent | ACD    | 300041     |
| Opal 520 reagent pack; use at 1:1000 | Akoya Biosciences | FP1487001KT |
| Opal 570 reagent pack; use at 1:1000 | Akoya Biosciences | FP1488001KT |
| Opal 690 reagent pack; use at 1:1000 | Akoya Biosciences | FP1497001KT |
| Antibodies |        |            |
| Alexa Fluor secondary antibodies; use at 1:1000 | Thermo Fisher Scientific | A-11001, A-21207 |
| DAPI; use at 0.5 mg/mL | Thermo Fisher Scientific | D1306 |

(Continued on next page)
MATERIALS AND EQUIPMENT

All reagents used in this protocol should be molecular biology grade. All buffers and solutions should be made with nuclease-free ultra-pure water. Many solutions and reagents used in this protocol are RNAscope proprietary solutions.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| DMEM | Gibco | 11995073 |
| Double distilled water (ddH2O) | University of New Mexico Facilities | N/A |
| Ethanol | Decon Labs | 2701 |
| FluorSave mounting media | MilliporeSigma | 345789 |
| Matrigel | Coming | 356231 |
| Organoid Harvesting Solution | R&D Systems | 3700-100-01 |
| Paraformaldehyde (PFA) | Electron Microscopy Sciences | 15710 |
| Poly-D-lysine | R&D Systems | 3439-100-01 |
| PBS | Gibco | 10010023 |
| Sodium chloride | MilliporeSigma | 59888 |
| Sodium citrate | MilliporeSigma | 567446 |
| Triton X-100 | MilliporeSigma | X100-500ML |
| TrypLE Express | Gibco | 12604013 |
| UltraPure DNase/RNase-free water | Thermo Fisher Scientific | 10977015 |
| | | |
| Human colonoids; Experiments performed on passages <30 | University of New Mexico | N/A |

Saline sodium citrate buffer (SSC) 20×

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Sodium chloride | 3 M | 175.3 g |
| Sodium citrate | 300 mM | 88.2 g |
| ddH2O | N/A | ~950 mL |
| Total | N/A | 1 L |

Note: Adjust pH to 7.0. Sterilize by autoclaving or filtering and store at 4°C. Solution can be used within 6 months if kept sterile and stored at 4°C. Prepare 5× dilution on day of use.

RNAscope wash buffer 1×

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| RNAscope 50× wash buffer | 1× | 60 mL |
| ddH2O | N/A | 2.94 L |
| Total | N/A | 3 L |

Note: Warm RNAscope 50× wash buffer to 40°C prior to dilution. 1× dilution can be used within 1 month and stored at room temperature.
STEP-BY-STEP METHOD DETAILS
RNA detection by whole-mount staining

- Timing: 1–2 days
- Timing: 2 h for step 1
- Timing: 1 h for step 2
- Timing: 1 h for step 3
- Timing: 30 min for step 4
- Timing: 45 min for step 5
- Timing: 45 min for step 6
- Timing: 2.5 h for step 7
- Timing: 2 h for step 8
- Timing: 2–4 h for step 9
- Timing: 45 min for step 10

This section provides step-by-step details to harvest colonoids (or other organoids) from Matrigel and perform RNA fluorescence in situ hybridization using a microfuge tube-based method in steps 1–8. We have included considerations to help prevent significant loss of colonoids throughout this procedure. Alternatively, steps 9–11 detail the lysine coated slide approach.

1. Harvest colonoids from Matrigel.

   **Note:** Aim to start with > 200 colonoids since some can be lost during staining.

   a. Remove growth media from wells of colonoids in the 24 well plate by vacuum aspiration.
   b. Add 1 mL cold Organoid Harvesting Solution to each well and mechanically dissociate Matrigel by scratching it off the bottom of the well with a P1000 pipette tip.
   c. Shake on orbital shaker (200 rpm for orbital shakers with a radius of 2.5 cm) for 45–60 min at 4°C.
   d. Gently triturate the colonoid suspension in the Organoid Harvesting Solution 10–20 times using a P1000 pipette (This should keep the colonoids intact and in the closed structure) to completely remove any remaining Matrigel.
   e. Transfer colonoids suspension to 15 mL conical tube.
   f. Add cold DMEM (4°C) for a final volume of 10 mL.
   g. Spin at 600 × g for 10 min at 4°C.
   h. Remove all supernatant. If Matrigel is not fully de-polymerized, add Organoid Harvesting Solution and repeat steps 4–7.
   i. Resuspend pellet in 200 μL 1× PBS and transfer to 1.5 mL microfuge tube.
   j. Allow colonoids to settle at bottom of tube for 15–20 min at room temperature (RT).

2. Cell Fixation and staining.

   a. Gently remove 1× PBS and add 300 μL of 4% PFA/PBS for 30 min.
   b. Remove the fixative and rinse colonoids twice in 500 μL 1× PBS. Spin for 15–20 s using a tabletop microfuge to settle colonoids at the bottom of the tube between each wash.
c. If continuing with the protocol on the same day, continue to step 5.

Optional: Dehydrate and store cells.

Note: Trituration is performed using a P200 pipette.

a. Remove 1× PBS and replace with 500 μL 50% ethanol, gently triturate 5×, incubate 5 min.
b. Replace supernatant with 500 μL 70% ethanol, gently triturate 5×, incubate 5 min.
c. Replace supernatant with 500 μL 100% ethanol, gently triturate 5×, incubate 5 min.
d. Replace supernatant with 500 μL 100% ethanol for storage, gently triturate 5×, incubate 10 min.
e. Store tubes at 4°C for use next day or –20°C for longer storage. Cells can be stored for several weeks at –20°C.

Optional: Rehydrate cells.

a. Remove the ethanol and replace with 500 μL 70% ethanol, gently triturate 5×, incubate 2 min.
b. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
c. Replace supernatant with 500 μL 50% ethanol, gently triturate 5×, incubate 2 min.
d. Spin for 15–20 s.
e. Replace supernatant with 500 μL 1× PBS, gently triturate 5×, incubate 10 min.

The following steps are amended from the RNAscope protocol (https://acdbio.com/rnascope-multiplex-fluorescent-v2-assay).

Note: Before you begin, prepare RNAscope probes. Warm probes at 40°C for 10 min, then cool to room temperature. If using C1 probe, no dilution is necessary. If using C2, C3, or C4 probes, dilute 1:50 with RNAscope probe diluent. Prepared probes can be used within 6 months if stored at 4°C.

3. RNAscope hydrogen peroxide treatment.

Note: Trituration is performed using a P200 pipette.

a. Remove 1× PBS, add 5 drops RNAscope hydrogen peroxide. Gently resuspend colonoids by triturating 5× then incubate 10 min.
b. Remove RNAscope hydrogen peroxide solution, add 500 μL distilled water. Gently triturate colonoids 10×.
c. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
d. Repeat wash with distilled water and allow colonoids to settle to the bottom of the tube.

Note: As an alternative to hydrogen peroxide, cells can be permeabilized using 0.5% Triton X-100/PBS for 20 min.

4. RNAscope protease plus treatment.

a. Remove distilled water, add 2–4 drops of RNAscope Protease Plus. Gently triturate 5× then incubate 10 min.
b. Remove RNAscope Protease Plus, add 500 μL 1× PBS. Gently resuspend and triturate colonoids 10×.
c. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
d. Repeat wash steps c – d with 1× PBS.

5. Probe hybridization.
a. Remove 1x PBS, add 2 drops (100 µL) of target RNAscope probe and gently triturate to resuspend colonoids in probe solution.
b. Place tube in the HybEZ humidity control tray.
c. Close tray and insert in HybEZ oven for 2 h at 40°C.
d. Remove tube from oven every 30 min and gently triturate to resuspend colonoids in probe solution, then place tube back into HybEZ oven.
e. After 2 h, remove probe solution, add 500 µL 1x RNAscope wash buffer then gently triturate 10x.
f. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
g. Repeat wash steps e–f with 500 µL 1x RNAscope wash buffer.

Optional: Colonoids can be stored in 5x saline sodium citrate (SSC) buffer at RT overnight. If stored in SSC buffer, wash colonoids twice with 1x RNAscope wash buffer before proceeding to the next step.

6. Signal amplification (AMP) hybridization.

Note: AMP 1–3 reagents are provided in the RNAscope multiplex fluorescent reagent kit V2.

a. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 1, gently triturate 5x to suspend colonoids in solution.
b. Place tube in the HybEZ humidity control tray.
c. Close tray and insert in HybEZ oven for 30 min at 40°C.
d. Remove Amp 1 solution, add 500 µL 1x RNAscope wash buffer then gently triturate 10x.
e. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
f. Repeat wash steps d–e with 500 µL 1x RNAscope wash buffer.
g. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 2, gently triturate 5x to suspend colonoids in solution.
h. Place tube in the HybEZ humidity control tray.
i. Close tray and insert in HybEZ oven for 30 min at 40°C.
j. Remove Amp 2 solution, add 500 µL 1x RNAscope wash buffer then gently triturate 10x.
k. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
l. Repeat wash steps j–k with 500 µL 1x RNAscope wash buffer.
m. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 3, gently triturate 5x to suspend colonoids in solution.
n. Place tube in the HybEZ humidity control tray.
o. Close tray and insert in HybEZ oven for 15 min at 40°C.
p. Remove Amp 3 solution, add 500 µL 1x RNAscope wash buffer then gently triturate 10x.
q. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
r. Repeat wash steps p–q with 500 µL 1x RNAscope wash buffer.

7. Signal detection – develop HRP channels (C1, C2 or C3).

a. Remove wash buffer, add 2–3 drops of RNAscope Multiplex FL v2 HRP-C1 and gently triturate to resuspend colonoids.
b. Place tube in the HybEZ humidity control tray.
c. Close tray and insert in HybEZ oven for 15 min at 40°C.
d. Remove HRP C1 solution, add 500 µL 1x RNAscope wash buffer then gently triturate 10x.
e. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
f. Repeat wash steps d–e with 500 µL 1x RNAscope wash buffer.
g. Remove wash buffer, add 200 µL fluorophore (Opal 520, Opal 570, or Opal 620 diluted 1:1,000 in TSA buffer), gently resuspend colonoids.
h. Place tube in the HybEZ humidity control tray.
i. Close tray and insert in HybEZ oven for 30 min at 40°C.
j. Remove supernatant, add 500 µL 1x RNAscope wash buffer then gently triturate 10x.
k. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
l. Repeat wash steps j – k with 500 μL 1× RNAscope wash buffer.
m. Remove wash buffer, add 2–3 drops of RNAscope Multiplex FL v2 HRP blocker then gently triturate to resuspend colonoids.
n. Place tube in the HybEZ humidity control tray.
o. Close tray and insert in HybEZ oven for 15 min at 40°C.
p. Remove HRP blocker, add 500 μL 1× RNAscope wash buffer then gently triturate 10×.
q. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
r. Repeat wash steps p – q with 500 μL 1× RNAscope wash buffer.

Note: If using more than one probe, repeat step 9 using the appropriate channel (C2 or C3) corresponding to the probe.

8. Stain nuclei and mount slides.
   a. Remove wash buffer from tube, add 2–3 drops of DAPI (concentration of 0.5 μg/mL) or other nuclear stain.
   b. Incubate for 5 min.
   c. Add 500 μL 1× RNAscope wash buffer then gently triturate 5×.
   d. Spin for 15–20 s using a microfuge to settle colonoids at the bottom of the tube.
   e. Repeat wash step with 500 μL 1× RNAscope wash buffer.
   f. Allow colonoids to naturally settle at the bottom of the tube for approximately 20–30 min. Place tube away from light.
   g. Carefully remove all wash buffer from tube.
   h. Add 40–50 μL of mounting media then gently triturate 5× to evenly resuspend colonoids. Avoid formation of air bubbles.
   i. Carefully draw up all colonoids in mounting media solution with a pipette and transfer onto a glass slide.
   j. Carefully place a glass coverslip over the mounting media to uniformly spread the colonoids on the slide. Avoid trapping air bubbles.
   k. Dry slides overnight in the dark at RT.
l. Store slides at 4°C until imaging.

Note: Slides should be imaged within one week to avoid signal fading.

**RNA whole-mount staining on lysine-coated slides**

గ Timing: 2 days

గ Timing: 2 h–overnight for step 9

గ Timing: 1–2 h for step 10

This section details the procedure to lysine coat chamber slides or glass micro slides. This is an alternative to the microfuge based method detailed in steps 1–8.

9. Lysine coating of slides.

Note: This step should be performed in a low-dust environment. Coating the slides with poly-D-lysine or poly-L-lysine will provide an appropriate surface for the organoids to attach to prevent sample loss. Coverslips should be handled with forceps designed for coverslips. CAUTION: Coverslips will shatter if not handled with care. Coated slides can be stored at 4°C for up to one year.
Note: Refer to Figure 1 for a schematic representation of lysine coating and colonoid plating onto slides.

- Warm poly-D/L-lysine solution to RT before use.
- Dilute lysine solution 1:10 with distilled water.
- Pipette enough solution to cover the surface of the cover slip or culture surface on the slide. If coating a slide, it is recommended to create a culture surface area by making a 1”x1” square with a hydrophobic PAP pen.
- Incubate for 10 min.
- Aspirate the solution and rinse the slides using UltraPure DNase/RNase-free water.
- Allow the slides to dry for at least 2 h or overnight in a laminar flow hood or in a low-dust environment.
- Store lysine-coated slides at 4°C until use.

10. Cell fixation on lysine coated slides.
- Harvest colonoids from Matrigel (described in step 1).
- Add 200–300 μL of 4% PFA/PBS to colonoids and transfer entire volume onto lysine coated slide.
- Allow colonoids to fix onto lysine coated slides for 1–2 h at RT.
- Carefully remove fixative solution and incubate slide for 10–15 min at 37°C to ensure colonoids are well adhered to the slide.
- For wash steps, place slides in slide staining jars. Rinse slides twice for 2 min with 1 x PBS to remove residual fixative.
RNA staining of colonoids

- Timing: 6 h

- Timing: 45 min for step 11

- Timing: 45 min for step 12

- Timing: 2.5 h for step 13

- Timing: 2 h for step 14

- Timing: 2–4 h for step 15

- Timing: 45 min for step 16

Note: Since colonoids have been fixed onto lysine coated slides, wash steps are different. Keep slides immersed in wash buffer for 2 min at low speed on a rocking shaker. Always perform at least two washes. Reagents can be removed from the colonoids within the hydrophobic barrier by gently wicking with dust free lab wipers.

11. RNAscope hydrogen peroxide treatment.
   a. Remove 1x PBS, add 5 drops RNAscope hydrogen peroxide onto the colonoids. Incubate 10 min.
   b. Remove RNAscope hydrogen peroxide solution, place slide in slide staining jar with distilled water.
   c. Repeat wash with distilled water.

Note: As an alternative to hydrogen peroxide, cells can be permeabilized using 0.5% Triton X-100/PBS for 20 min.

12. RNAscope protease plus treatment.
   a. Remove distilled water, add 2–4 drops of RNAscope Protease Plus onto the colonoids. Incubate 10 min.
   b. Remove RNAscope Protease Plus, place slide in slide staining jar with 1x PBS.
   c. Repeat wash step with 1x PBS.

13. Probe hybridization.
   a. Remove 1x PBS, add 2 drops (100 μL) of target RNAscope probe directly onto the colonoids within the hydrophobic barrier.
   b. Place slide in the HybEZ humidity control tray.
   c. Close tray and insert in HybEZ oven for 2 h at 40°C.
   d. After 2 h, remove probe solution, transfer slide to slide staining jar and wash with 1x RNAscope wash buffer.
   e. Repeat wash step with 1x RNAscope wash buffer.

Optional: Colonoids fixed onto lysine coated slides can be stored in 5x saline sodium citrate (SSC) buffer at RT overnight. If stored in SSC buffer, wash slides twice with 1x RNAscope wash buffer before proceeding to the next step.

14. Signal amplification (AMP) hybridization.

Note: AMP 1–3 reagents are provided in the RNAscope multiplex fluorescent reagent kit V2.
a. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 1, then place slide in the HybEZ humidity control tray.
b. Close tray and insert in HybEZ oven for 30 min at 40°C.
c. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
d. Repeat wash step with 1× RNAscope wash buffer.
e. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 2, then place slide in the HybEZ humidity control tray.
f. Close tray and insert in HybEZ oven for 30 min at 40°C.
g. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
h. Repeat wash step with 1× RNAscope wash buffer.
i. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 3, then place slide in the HybEZ humidity control tray.

15. Signal detection – develop HRP channels (C1, C2 or C3).
   a. Remove wash buffer, add 2–3 drops of RNAscope Multiplex FL v2 HRP-C1, then place slide in the HybEZ humidity control tray.
   b. Close tray and insert in HybEZ oven for 15 min at 40°C.
   c. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
   d. Repeat wash step with 1× RNAscope wash buffer.
   e. Remove wash buffer, add 200 µL fluorophore (Opal 520, Opal 570, or Opal 620 diluted 1:1,000 in TSA buffer) directly onto the colonoids within the hydrophobic barrier.
   f. Place slide in the HybEZ humidity control tray.
   g. Close tray and insert in HybEZ oven for 30 min at 40°C.
   h. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
   i. Repeat wash step with 1× RNAscope wash buffer.
   j. Remove wash buffer, add 2–3 drops of RNAscope Multiplex FL v2 HRP blocker, then place slide in the HybEZ humidity control tray.
   k. Close tray and insert in HybEZ oven for 15 min at 40°C.
   l. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
   m. Repeat wash step with 1× RNAscope wash buffer.

Note: If using more than one probe, repeat step 15 using the appropriate channel (C2 or C3) corresponding to the probe.

16. Stain nuclei and mount slides.
   a. Remove wash buffer, add 2–3 drops of DAPI (concentration of 0.5 µg/mL) or other nuclear stain for 5 min.
   b. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
   c. Repeat wash step with 1× RNAscope wash buffer.
   d. Carefully wick all residual wash buffer.
   e. Add 40–50 µL of mounting media directly within the hydrophobic barrier.
   f. Carefully place a glass coverslip over the mounting media to uniformly spread the colonoids on the slide. Avoid trapping air bubbles.
   g. Dry slides overnight in the dark at RT.
   h. Store slides at 4°C until imaging.

RNA-protein Co-Detection protocol

© Timing: 2–3 days

© Timing: 4 h for step 17
This section details the procedure to combine RNA fluorescence in situ hybridization and protein immunofluorescence co-detection on the same colonoid samples. Successful application of RNA-protein co-detection optimizes the number of readouts on colonoids or organoids.

**Day 1**

17. Harvesting and fixation of colonoids.
   a. Harvest colonoids in organoid harvesting solution (described in step 1).
   b. Resuspend in 4% PFA/PBS then transfer onto lysine coated slides and fix for 1–2 h at RT.
   c. Remove fixative solution and incubate slide for 10–15 min at 37°C to ensure colonoids are well adhered to the slide.
   d. Place slides into slide staining jars, then rinse slides twice with 1× PBS to remove residual fixative.

18. RNAscope hydrogen peroxide treatment.
   a. Add 3–5 drops of hydrogen peroxide to completely cover the colonoids and incubate 10 min.
   b. Place slides into slide staining jars, then rinse slides twice using 1× PBS.

   **Note:** As an alternative to hydrogen peroxide, colonoids can be permeabilized using 0.5% Triton X-100/PBS for 20 min.

19. Co-detection target retrieval.

   **Note:** Co-detection reagents including co-detection target retrieval solution and co-detection antibody diluent are provided in the RNA-protein co-detection ancillary kit.

   a. Prepare 1× co-detection target retrieval solution by diluting the 10× co-detection target retrieval solution in 70°C distilled water.
   b. Add 1× co-detection target retrieval to colonoids on lysine-coated slides and allow solution to boil at 100°C for 15 min in a steamer.
   c. Place slides into slide staining jars, then rinse slides twice with distilled water for 2 min at RT.

20. Primary antibody.

   a. Add primary antibody diluted in co-detection antibody diluent to colonoids, use enough solution to cover the colonoids. We recommend 200 μL.
   b. Place slides in humidity control chamber and incubate overnight at 4°C.

   **Note:** Do not counter stain slides with DAPI or other nuclear stain at this point. Nuclear stain will be used after secondary antibody hybridization.
Day 2

21. Post primary fixation.
   a. Place slides into slide staining jars, then rinse slides twice in 0.1% PBS-Tween-20 (PBS-T) for 2 min.
   b. Remove slides from slide staining jars, lay flat and add 4% PFA/PBS to colonoids for 30 min.
   c. Place slides into slide staining jars, then rinse slides in 0.1% PBS-T for 2 min, repeat wash step twice with 0.1% PBS-T.

22. RNAscope protease plus treatment.
   a. Add 3–5 drops of RNAscope protease plus to completely cover colonoids.
   b. Place slides in prewarmed HybEZ humidity control tray and incubate for 30 min at 40°C.
   c. Place slides into slide staining jars, then rinse slides 3–5 times with distilled water at RT.

23. Perform RNA staining.
   a. Proceed to RNA staining steps outlined in steps 11–15.

   Note: Do not counter stain slides with DAPI or other nuclear stain after the HRP blocker step. Nuclear stain will be used after secondary antibody hybridization.

24. Secondary antibody hybridization using fluorophore conjugated antibody.
   a. Add fluorophore-conjugated secondary antibody diluted in co-detection antibody diluent to completely cover colonoids. We recommend 200 µL.
   b. Incubate slides for 30 min, keep covered and away from light.
   c. Place slides into slide staining jars, then rinse slides twice with 0.1% PBS-T for 2 min with gentle agitation.

25. Counterstain and mount slides.
   a. Remove 0.1% PBS-T wash buffer then add 2–3 drops of DAPI or other nuclear stain.
   b. Incubate for 5 min.
   c. Place slides into slide staining jars, then rinse slides twice with 1× PBS.
   d. Carefully remove residual PBS from slides. Use lint-free wipes to wick residual buffer.
   e. Add 40–50 µL of mounting media.
   f. Carefully place a glass coverslip over the mounting media on the slide. Gently press to avoid air bubbles.
   g. Dry slides overnight in the dark at RT.
   h. Store slides at 4°C until imaging.

   Note: Slides should be imaged within one week to avoid signal fading.

EXPECTED OUTCOMES

Although whole-mount staining allows for observation through the whole colonoid, it is technically challenging and the many steps involved often result in loss of intact colonoids. Using this detailed protocol, it is expected that the researcher should successfully and reproducibly achieve whole-mount staining of colonoids for RNA FISH (presented here using the RNAscope method) (Figure 2) and co-detection of RNA and protein using RNAscope and immunofluorescence on the same sample (Figure 3) without great loss of colonoid numbers. We have found that >80% of starting colonoid numbers are maintained in our protocol. Quantitative analysis can be performed to measure expression of both RNA and protein.

LIMITATIONS

Whole-mount staining of organoids is technically challenging and time consuming compared to staining of tissues or cells fixed onto slides. Transferring colonoids between tubes and onto slides can lead to significant sample loss, making any quantitative analysis difficult. To avoid this problem,
we recommend using FBS or BSA coated tubes and pipette tips. This will prevent colonoids from sticking to tubes or the inside of pipette tips.

**TROUBLESHOOTING**

**Problem 1**
Colonoids are lost during the staining process due to the number of wash steps.

**Potential solution**
Start with at least 200+ colonoids and allow enough time for colonoids to settle at the bottom of the tube by gravity or briefly centrifuge to allow colonoids to settle at the bottom of the tube. Use FBS or BSA coated tubes and pipette tips to help prevent loss of colonoids. Alternatively, fix colonoids onto lysine coated slides.

**Problem 2**
Colonoids clump together during mounting (steps 10 or 22) making imaging of individual colonoids difficult.
Potential solution
Make certain that the colonoids are freely suspended in mounting media by gently triturating with a P1000 pipette before transferring onto slides. Avoid vigorous pipetting which may breakdown large colonoids or create air bubbles in the mounting media.

Problem 3
Excess hydrogen peroxide solution during RNAscope (step 5) leads to significant loss of colonoids.

Potential solution
As an alternative to hydrogen peroxide, colonoids can be permeabilized using 0.5% Triton X-100/ PBS for 20 min.

Problem 4
Poor protein detection during RNA-protein co-detection may occur due to numerous washes during the RNAscope steps following primary antibody hybridization.

Potential solution
Use double the concentration of primary antibody that would be used for standard immunofluorescence and incubate the sample with primary antibody for at least 16 h (overnight) at 4°C in the permeabilization/blocking solution. We have found this reduces poor protein detection during the RNA-protein co-detect assay.

Problem 5
Background autofluorescence from excess lysine precipitates on the slide (step 11) may impede proper signal detection.

Potential solution
Reduce lysine solution dilution to an optimal dilution that will allow colonoids/organoids to adhere to slides but with minimal precipitation. Additional rinses with UltraPure DNase/RNase-free water can also reduce background autofluorescence caused by lysine coating.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julie G. In (jgin@salud.unm.edu).

Materials availability
All non-proprietary reagents will be available upon request with a completed Materials Transfer Agreement. Colonoids will be available to IRB approved researchers with a completed Materials Transfer Agreement.

Data and code availability
This study did not generate any large data sets.

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AUTHOR CONTRIBUTIONS
Conceptualization, J.G.I.; methodology, R.A., A.S.P., and J.G.I.; resources, J.G.I.; investigation, R.A. and A.S.P.; writing – original draft, R.A. and A.S.P.; writing – review & editing, R.A. and J.G.I.; visualization, R.A. and J.G.I.; supervision, J.G.I.; funding acquisition, A.S.P. and J.G.I.
DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES

Borrelli, C., and Moor, A.E. (2020). Single-molecule RNA FISH in whole-mount organoids. Methods Mol. Biol. 2171, 237–247.

In, J.G., Yin, J., Atanga, R., Doucet, M., Cole, R.N., DeVine, L., Donowitz, M., Zachos, N.C., Blutt, S.E., Estes, M.K., et al. (2020). Epithelial WNT2B and desert hedgehog are necessary for human colonoid regeneration after bacterial cytotoxin injury. iScience 23, 101618.

Kersigo, J., Pan, N., Lederman, J.D., Chatterjee, S., Abel, T., Pavlinkova, G., Silos-Santiago, I., and Fritzsch, B. (2018). A RNAscope whole mount approach that can be combined with immunofluorescence to quantify differential distribution of mRNA. Cell Tissue Res. 374, 251–262.