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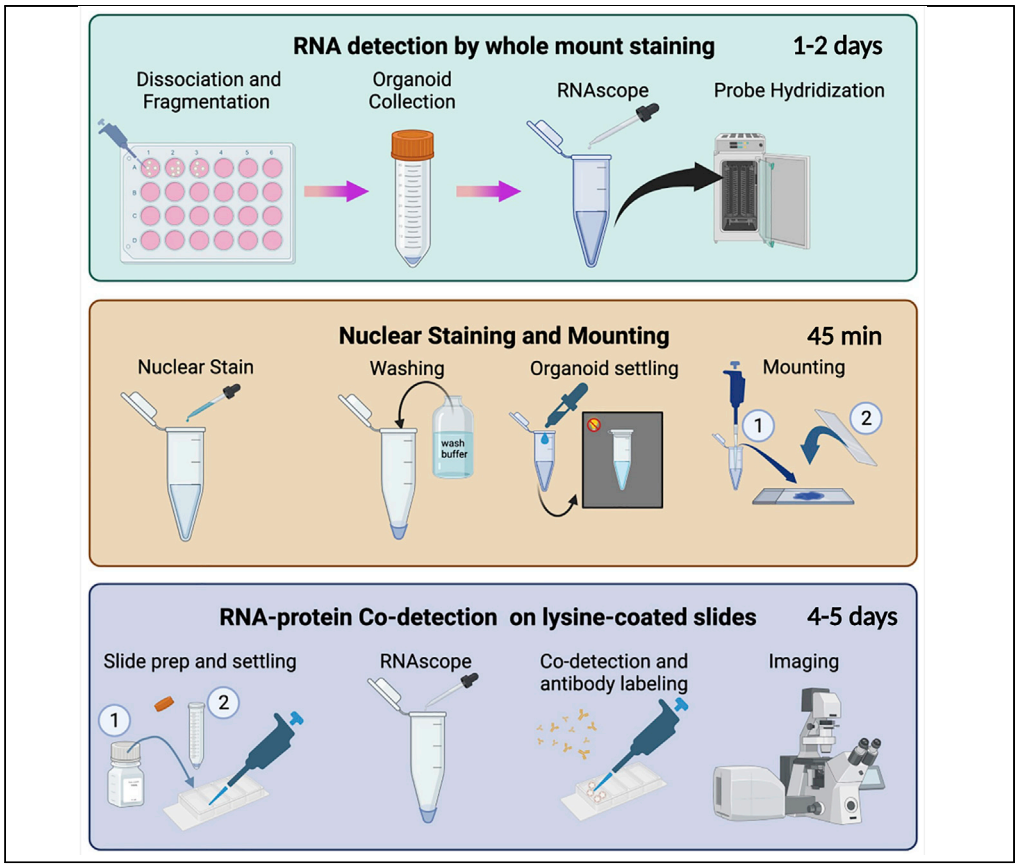
Efficient RNA and RNA-protein co-detection in 3D colonoids by whole-mount staining



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.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Whole-mount staining of 3D colonoids in suspension detect RNA oligos

Whole-mount staining by fixing colonoids onto lysine-coated slides

RNA-protein co-staining in colonoids using suspension or lysine-coated approach

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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 commerical 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*)

Continued		
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	Corning	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	S9888
Sodium citrate	MilliporeSigma	567446
Triton X-100	MilliporeSigma	X100-500ML
TrypLE Express	Gibco	12604013
UltraPure DNase/RNase-free water	Thermo Fisher Scientific	10977015
Experimental models: Cell lines		
Human colonoids; Experiments performed on passages <30	University of New Mexico	N/A

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
Other		
Chamber slides	MilliporeSigma	C86024
Coverslips	Thomas Scientific	64-0704
ImmEdge hydrophobic barrier PAP pen	Vector Labs	H-4000
Simport Scientific EasyDip slide staining jars	Fisher Scientific	22-038-495
HybEZ II hybridization system (oven)	ACD	N/A
HybEZ humidity control tray	ACD	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
ddH_2O	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
ddH_2O	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 1× 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 1× RNAscope wash buffer then gently triturate 10×.
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 1× RNAscope wash buffer.

Optional: Colonoids can be stored in 5× saline sodium citrate (SSC) buffer at RT overnight. If stored in SSC buffer, wash colonoids twice with 1× 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 5× 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 1× RNAscope wash buffer then gently triturate 10×.
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 1× RNAscope wash buffer.
g. Remove wash buffer, add 2–3 drops of Multiplex FL v2 Amp 2, gently triturate 5× 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 1× RNAscope wash buffer then gently triturate 10×.
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 Multiplex FL v2 Amp 3, gently triturate 5× 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 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.

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 1× RNAscope wash buffer then gently triturate 10×.
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 1× 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 1× RNAscope wash buffer then gently triturate 10×.

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

Timing: 2 days

Timing: 2 h–overnight for step 9

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.

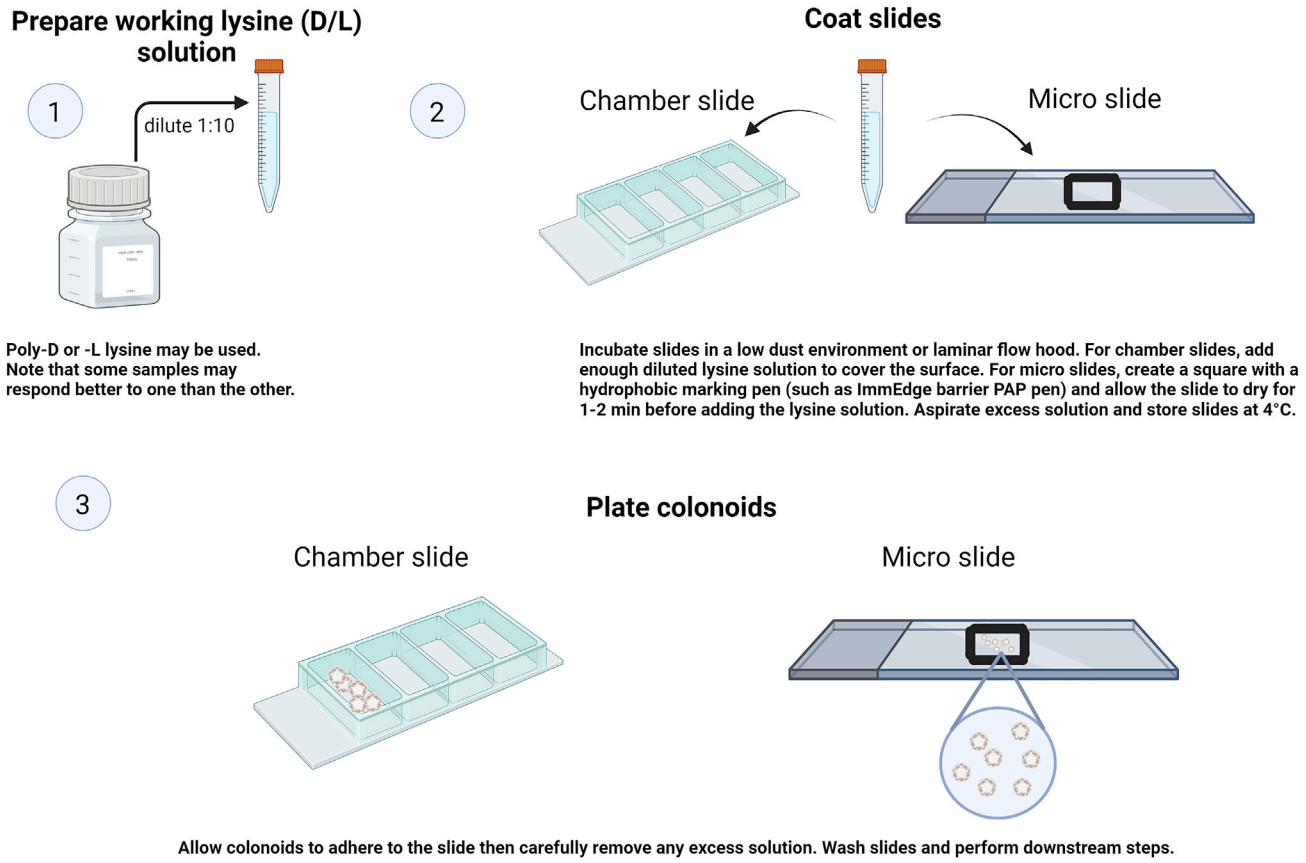

Figure 1. Lysine coating and whole-mount staining

Schematic illustrating lysine coating of slides showing 1) preparation of the lysine solution, 2) coating onto slides, and 3) plating of colonoids onto slides.

Note: Refer to Figure 1 for a schematic representation of lysine coating and colonoid plating onto slides.

a. Warm poly-D/L-lysine solution to RT before use.
b. Dilute lysine solution 1:10 with distilled water.
c. 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.
d. Incubate for 10 min.
e. Aspirate the solution and rinse the slides using UltraPure DNase/RNase-free water.
f. Allow the slides to dry for at least 2 h or overnight in a laminar flow hood or in a low-dust environment.
g. Store lysine-coated slides at 4°C until use.

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

Optional: Colonoids fixed onto lysine coated slides can be stored in 5× saline sodium citrate (SSC) buffer at RT overnight. If stored in SSC buffer, wash slides twice with 1× 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.
j. Close tray and insert in HybEZ oven for 15 min at 40°C.
k. Place slide in slide staining jar and wash with 1× RNAscope wash buffer.
l. Repeat wash step with 1× RNAscope wash buffer.

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

Timing: 30–45 min for step 18

Timing: 35 min for step 19

Timing: overnight for step 20

Timing: 45 min for step 21

Timing: 45 min for step 22

Timing: 5 h for step 23

Timing: 45 min for step 24

Timing: 30 min for step 25

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 for 2 min 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,

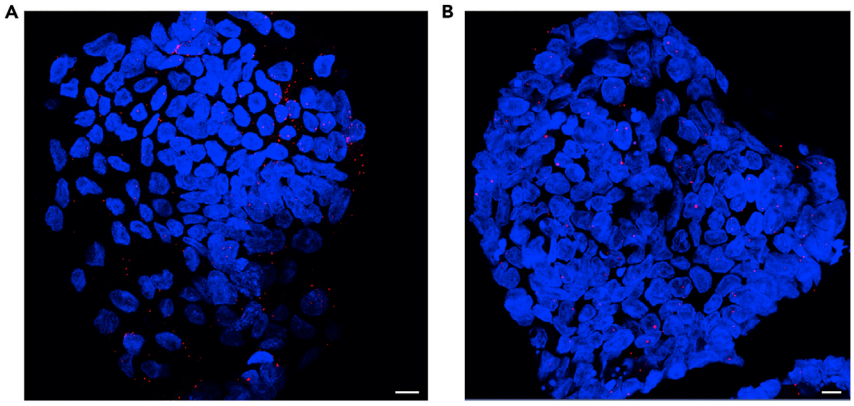

Figure 2. RNAscope staining in whole-mount colonoids using tube method
(A) Representative confocal image of colonoid stained for *WNT2B*, red.
(B) Representative confocal image of colonoid stained for *TPH1*, red. Scale bars = 10 μm.

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.

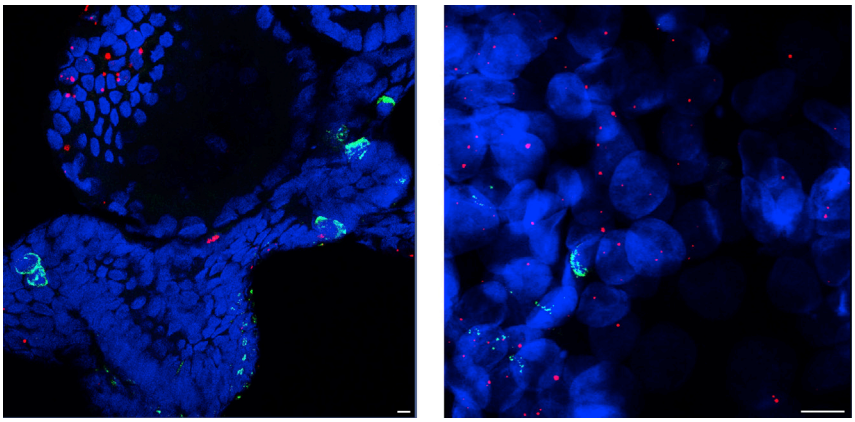

Figure 3. RNA-protein co-staining in whole-mount colonoids using lysine-coated slide method
Representative confocal images of colonoids co-stained for *WNT2B* (oligos, red) and CHGA (antibody, green). Scale bars = 10 μm.

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.