Dual staining for Ki67 protein and *MKI67* transcript detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

A protocol for staining of protein (Ki-67) and RNA (*MKI67*) in pig tissues

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Supporting Information

Starting specimens:

Starting samples = FFPE tissues cut to 4 micron thickness and adhered to positively-charged microscopy slides (e.g. SuperFrost Plus Slides; Fisher Scientific 12-550-15). It is crucial that tissues are adequately fixed to prevent tissue degradation but not over-fixed as to over-fragment RNA. Tissues no thicker than 0.5 centimeters should be freshly harvested and placed into 10% neutral-buffered formalin (NBF) or 4% paraformaldehyde (PFA) at a ratio of at least 20 volumes fixative per one volume tissue. Tissues should be fixed for between 16-30 hours at room temperature (RT), followed by immediate transfer to 70% ethanol and processing into FFPE tissue blocks. Fixation times should be optimized for individual tissues and experiments.

Equipment:

- Pipettes/pipette tips – volumes ranging between 2-1000 uL
- Drying oven (able to reach & hold 60℃)
- Fume hood
- Decloaking Chamber NxGen (Biocare Medical DC2012/DC2012-220V)
    - Can substitute with hot plate by using alternative target retrieval protocol; see Appendix B: Manual Target Retrieval from Advanced Cell Diagnostics [ACD] FFPE Sample Preparation and Pretreatment User Manual (Document No. 322452)
- Slide staining tray (e.g. Simport M920-2)
- HybEZ II Hybridization System with ACD EZ-Batch Slide System (ACD 321710/321720)
    - HybEZ oven (ACD 321710/321720)
    - Humidity control tray (ACD 310012)
    - HybEZ Humidifying Paper (ACD 310025)
    - EZ-Batch Wash Tray (ACD 321717)
    - EZ-Batch Slide Holder (ACD 321716)
- Tissue-Tek Vertical 24 slide rack (American Master Tech Scientific LWS2124)
- Tissue-Tek Staining Dishes (American Master Tech Scientific LWS20WH)
- Tissue-Tek Clearing Agent Dishes, xylene resistant (American Master Tech Scientific LWS20GR)

Reagents/Supplies:

For all reagents, refer to MSDS to determine appropriate precautions, personal protective equipment (PPE), and disposal methods before use

- Distilled water (obtained in-house)
- 0.05% PBS-Tween (PBS-T), pH 7.35 (made in-house)
- Xylenes (Macron Fine Chemicals 8668-16)
- 100% ethanol (Pharmco 111000200)
- 10% NBF (3.7% formaldehyde; Cancer Diagnostics, Inc. 111)
- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- RNAscope H2O2 & Protease Plus Reagents (ACD 322330)
    - Hydrogen Peroxide (ACD 322335)
    - Protease Plus (ACD 322331)

- RNA-Protein Co-Detection Ancillary Kit (ACD 323180)
    - Co-Detection Target Retrieval Reagents (ACD 323165/323166)
    - Co-Detection Antibody Diluent (ACD 33160)
    - Co-Detection Blocker (ACD 323170)
- RNAscope Wash Buffer Reagents (ACD 310091/320058)
- RNAscope Multiplex Fluorescent Detection Reagents v2 (ACD 323110)
    - Amp 1 (ACD 323101)
    - Amp 2 (ACD 323102)
    - Amp 3 (ACD 323103)
    - HRP-C1 (ACD 323104)
    - HRP blocker (ACD 323107)
    - DAPI (ACD 323108)
- RNAscope Multiplex TSA Buffer (ACD 322809)
- Opal 570 (Perkin Elmer FP1488A)
- Alexa Fluor 488 F(ab') fragment of goat anti-mouse IgG (Invitrogen A11017)
- Anti-Ki-67 antibody; mouse IgG1; clone B56; stock concentration 250 ug/mL (BD 550609)
- RNAscope Probes, Channel 1 (interchangeable with other channel 1 probes if desiring to target other transcripts)
    - *MKI67* (ACD 470841)
- ProLong Gold Antifade reagent (Invitrogen P36930)
- #1 thickness cover glass (Fisherbrand 12-545-F)

Protocol

Before starting the assay:

- Preheat a dry oven to 60℃
- Load slides for assay into vertical slide rack

Baking

- **Bake slides 30 min 60℃**
- *Optional stopping point: store slides in a dry place & use within 1 week*

While slides bake:

- Prepare 0.05% PBS-T (can store at RT up to 1 month)
- Prepare 1X Co-Detection Target Retrieval solution by adding 1 bottle (70 mL) Co-Detection Target Retrieval Reagent (10X stock concentration) to 630 mL distilled water (can store at 4℃ up to 1 month)

Immediately before deparaffinizing:

Add ~200 mL xylenes to each of two clearing agent dishes in a fume hood
Add ~200 mL 100% ethanol to each of two staining dishes in a fume hood

Deparaffinizing

- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slide in fresh **xylenes 5 min RT**
- Submerge slides in fresh **100% ethanol 5 min RT**
- Submerge slides in fresh **100% ethanol 5 min RT**
- **Air dry slides ~5 min** or until completely dry
- *Optional stopping point: store slides in a dry place & use within 24 hours*

While slides deparaffinize:

- Prepare decloaking chamber:

    - Pour 500 mL distilled water into central chamber
    - Pour 200 mL distilled water into left/right staining dishes
    - Pour 200 mL prepared Co-Detection Target Retrieval solution into middle staining dish
- Turn off dry oven
- Prepare HybEZ Oven:
    - Place humidifying paper within the humidity control tray & apply distilled water to fully wet paper
    - Place humidifying tray into HybEZ oven and clamp down the gasket to seal
    - Preheat oven to 40℃ for at least 30 minutes before use

Immediately before tissue quenching:

- Preheat the prepared decloaking chamber, programmed for 15 min at 95℃
    - Chamber will take exactly 15 min to preheat, and there will be a 2 min window to add slides before chamber pressurizes & locks

Tissue Quenching

- Unload slides from vertical slide rack and place on flat surface of bench top
- Incubate with **Hydrogen Peroxide 10 min RT**
    - Invert bottle immediately before use; apply drops to completely cover tissues; let incubate on bench top
- Decant slides and transfer to vertical slide rack
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**

While slides incubate with Hydrogen Peroxide:

- Discard deparaffinizing reagents
- Add ~200 mL distilled water to each of two staining dishes

Target Retrieval

- Leave slides in water at RT until decloaker is preheated (<5 min)
- Once decloaker has preheated, submerge slide rack in **preheated distilled water 10 sec** (left or right dishes in decloaker)
- Submerge slide rack in **preheated 1X Co-detection Target Retrieval solution 15 min 95℃**
    - Once slides are places in center staining dish of decloaker, close the decloaker (make sure pressure valve is in place to hold pressure when replacing lid) & wait for alarm to go off in 15 min
- Release decloaker chamber pressure valve & open chamber
- Submerge slide rack in **preheated distilled water 10 sec** (left or right dishes in decloaker)
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
- Submerge slide rack in fresh **distilled water, dunking 3-5 times**
- Submerge slide rack in fresh **PBS-T, dunking 3-5 times**
- Leave slides in PBS-T

While slides incubate in 1X target retrieval solution:

- Discard tissue quenching reagents
- Add ~200 mL distilled water to each of two staining dishes
- Add ~200 mL PBS-T to one staining dish
- Prepare humidified slide staining tray by adding water to bottom & placing lid on top
- Add Ki-67 antibody to Co-Detection Antibody Diluent at a 1:100 dilution (2.5 ug/mL). Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Hydrophobic Barrier

- **Apply hydrophobic barrier** around each tissue
    - One by one, unload slides from vertical rack submerged in PBS-T. Dry off only the area around the tissue where a barrier will be drawn with an immedge hydrophobic barrier pen. Keep tissue area wet the whole time. Draw barrier and place slide flat in the slide staining tray. Using a pipette, apply a small amount of PBS-T within the barrier (just enough to keep the tissue wet while drawing barriers on remaining slides)

Primary Antibody

- Decant slides and again place flat in slide staining tray
- Incubate with **diluted primary antibody overnight 4℃**; place lid back on slide staining tray during this time
- Remove slides from slide staining tray, decant, and transfer to vertical slide rack
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

While slides are incubating with primary antibody:

- Discard target retrieval reagents
- Add ~200 mL PBS-T to each of three staining dishes
- Add ~200 mL 10% NBF to one staining dish in a fume hood

Antibody cross-linking

- Submerge slide rack in **10% NBF 30 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

While slides are incubating with 10% NBF:

- Discard primary antibody reagents
- Add ~200 mL PBS-T to each of three staining dishes
- Prepare HybEZ Oven:
    - Place humidifying paper within the humidity control tray & apply distilled water to fully wet paper
    - Place humidifying tray into HybEZ oven and clamp down the gasket to seal
    - Preheat oven to 40℃ for at least 30 minutes before use

Protease

- Transfer slides into EZ-Batch Slide Holder, taking care not to let tissues dry out
- Incubate with **Protease Plus 15 min 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **distilled water, dunking 3-5 times**
- Submerge slide holder in fresh **distilled water, dunking 3-5 times**

While slides are incubating with protease:

- Empty the slide staining tray used for primary antibody incubations & put away
- Discard antibody cross-linking reagents

- Add ~200 mL distilled water to each of two wash trays
- Preheat RNAscope probes to 40℃ for 10 min before use; this can be done by placing them inside the HybEZ oven during protease incubation. Once preheated, add MKI67 probe undiluted on slides. Total volume to use is dependent on tissue sizes.

Probe Hybridization

- Decant slides (without removing slides from holder)
- Incubate with appropriate RNAscope **probe 2 hours 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

While slides are incubating with probes:

- Discard protease reagents
- Prepare 1X wash buffer by adding 1 bottle (60 mL) Wash Buffer (10X stock concentration) to 2.94 L distilled water
    - If 10X Wash Buffer solution has a precipitant formed, preheat bottle at 37℃ for 1 hour before adding to distilled water
    - Will have to prepare another batch of 1X wash buffer later in protocol, after first batch runs out. Alternatively, prepare both batches at once (120 mL 1X Wash Buffer + 5.88 L distilled water)
    - Store at RT up to one month
- Add ~200 mL 1X wash buffer to each of two wash trays
- Place AMPs from RNAscope Multiplex Fluorescent Detection kit at RT for at least 30 min before use (should be RT when used)

RNA Detection

- Decant slides (without removing slides from holder)
- Incubate with **AMP1 30 min 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP2 30 min 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Decant slides (without removing slides from holder)
- Incubate with **AMP3 15 min 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

- Decant slides (without removing slides from holder)
- Incubate with **HRP-C1 15 min 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray within HybEZ oven
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

Immediately before Opal 570 incubation:

- Prepare diluted Opal fluorophore by diluting Opal 570 into Multiplex TSA Buffer at 1:750 dilution. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Store in the dark due to light sensitivity.

- Decant slides (without removing slides from holder)
- Incubate with **diluted Opal 570 30 min 40℃**
    - Pipette well to mix immediately before use; pipette appropriate volumes to completely cover tissues & transfer slide holder to humidifying tray left on bench top
- Remove slide holder from humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Incubate with **HRP blocker 15 min 40℃**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray left on bench top
- Remove slide holder from HybEZ oven/humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

During each incubation:

- Discard reagents from previous incubation step
- Add ~200 mL 1X wash buffer to each of two wash trays

While slides are incubating with HRP blocker:

- Add ~200 mL 1X wash buffer to each of two wash trays
- Add ~200 mL PBS-T to one wash tray

Immediately before protein detection:

- Prepare diluted secondary antibody by diluting Alexa Fluor 488 F(ab') fragment of goat anti-mouse IgG into Co-detection Antibody Diluent at a 1:1000 dilution. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Store in the dark due to light sensitivity.

Protein Detection

- Decant slides (without removing slides from holder)
- Incubate with **diluted secondary antibody 1 hour RT**
    - Invert bottle immediately before use; apply drops to completely cover tissues & transfer slide holder to humidifying tray on bench top
- Remove slide holder from humidifying tray & decant (without removing slides from holder)
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

While slides are incubating with secondary antibody:

- Discard tissue blocking reagents
- Add ~200 mL 1X wash buffer to each of two wash trays
- Add ~200 mL PBS-T to one wash tray (if you only have two wash trays, complete this step after one PBS-T incubation after secondary antibody incubation)
- Turn off HybEZ oven

Nuclei Staining and Coverslipping

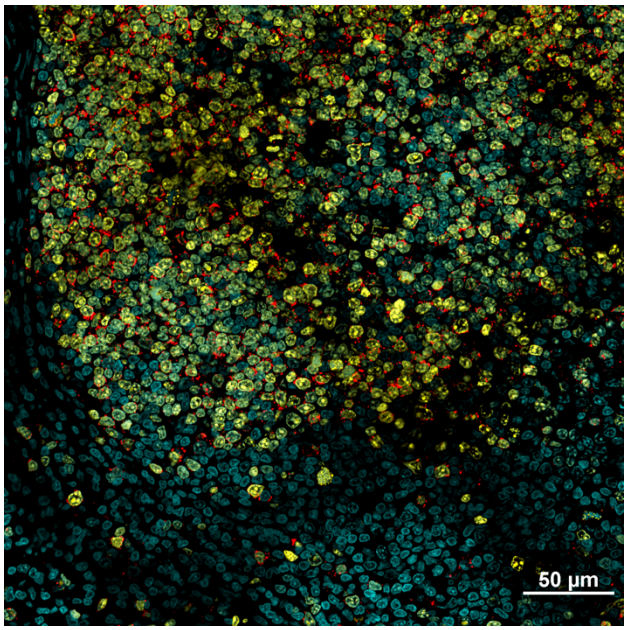
- One at a time, remove slides from slide holder and:
  - Apply **DAPI 30 sec RT**
  - Decant slide to remove DAPI
  - **Mount slides** by adding 2-4 drops of ProLong Gold antifade mounting media to each slide, followed by application of a cover glass. Remove bubbles from tissue by applying pressure to cover glass
- Place slides flat in a dry, dark space to air dry at RT 30 min
- Store at 4℃ and image within two weeks

While slides are air drying:

- Discard protein detection reagents

Results

Ki-67 protein (yellow), *MKI67* RNA (red), and DAPI nuclei (cyan) staining in ileum of an ~8-week-old, weaned pig. Tissue was fixed in 4% PFA ~24 hours. Frame shows Peyer's patch follicle and interfollicular zone. Staining mainly localizes to follicles.



Contributions

Jayne Wiarda: protocol creation, protocol writeup, data interpretation

Hannah Mazon: protocol execution, protocol writeup

Crystal Loving: financial support