Tri-plex staining for IBA-1, *CD4,* and *CSF1R* detection in formalin-fixed, paraffin-embedded (FFPE) pig tissues

A protocol for staining of protein (IBA-1) and RNA (*CD4, CSF1R*) 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-C2 (ACD 323105)
    - HRP blocker (ACD 323107)
    - DAPI (ACD 323108)
- RNAscope Multiplex TSA Buffer (ACD 322809)
- Opal 690 (Perkin Elmer FP1497A)
- Opal 570 (Perkin Elmer FP1488A)
- Alexa Fluor 488 F(ab') fragment of goat anti-rabbit IgG (Invitrogen A11070)
- Anti-IBA1 rabbit polyclonal antibody (500ug/mL; Fujifilm Wako 019-19741)
- RNAscope Probe, Channel 1 (interchangeable with other channel 1 probe to detect different transcript)
    - *CSF1R* (ACD 1234601-C1)
- RNAscope Probe, Channel 2 (interchangeable with other channel 2 probe to detect different transcript)
    - CD4 (ACD 491891-C2)
- SSC Buffer 20X Concentrate (Sigma S6639-1L)
- 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 IBA-1 antibody to Co-Detection Antibody Diluent at a 1:1000 dilution (0.5 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 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 RT 1 hour**; 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 CD4-C2 probe to CSF1R-C1 probe at a dilution of 1:50. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting.

Probe Hybridization

- Decant slides (without removing slides from holder)
- Incubate with appropriate RNAscope **probe cocktail 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
- If performing optional stopping point (next step):
    - Prepare 5X SSC buffer by combining 60 mL 20X SSC with 180 mL distilled water
    - Add 240 mL 5X SSC buffer to staining dish
- If omitting optional stopping point (next step):
    - Place AMPs from RNAscope Multiplex Fluorescent Detection kit at RT for at least 30 min before use (should be RT when used)

Stopping Point (optional)

- Transfer slides from slide holder into slide rack
- Submerge slide rack in fresh **5X SSC buffer overnight at RT with lid**
- Decant slides and transfer into slide holder
- Submerge slide holder in fresh **1X wash buffer 2 min RT**
- Submerge slide holder in fresh **1X wash buffer 2 min RT**

The next day:
- Place AMPs from RNAscope Multiplex Fluorescent Detection kit at RT for at least 30 min before use (should be RT when used)
- Add ~200 mL 1X wash buffer to each of two wash trays

RNA Detection

- 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 Opal690 incubation:

- Prepare diluted Opal fluorophore by diluting Opal690 into Multiplex TSA Buffer at 1:1200 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 690 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**
- Decant slides (without removing slides from holder)
- Incubate with **HRP-C2 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 Opal570 incubation:

- Prepare diluted Opal fluorophore by diluting Opal570 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 last 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-rabbit 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

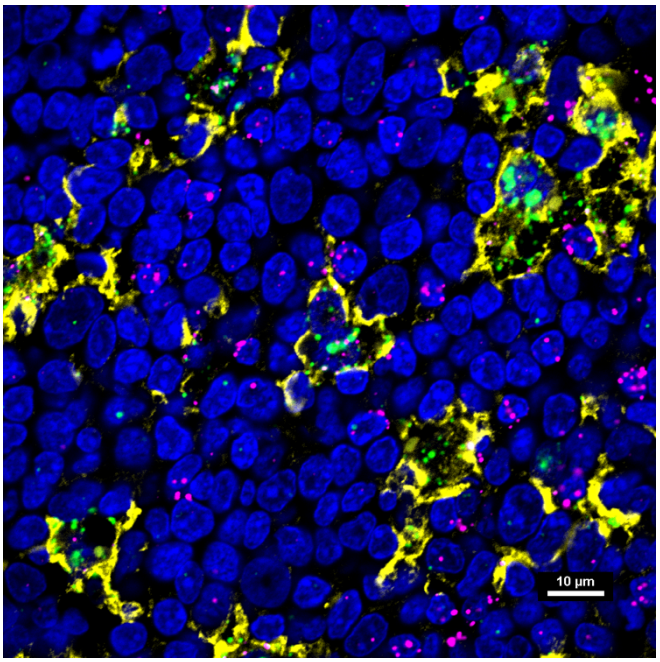
- One at a time, remove slides from slide holder and:
  - Apply **DAPI 30 sec RT**
  - Decant slide to remove DAPI
  - **Mount slides** by adding 2-4 drops of ProLong Gold antifade mounting media to each slide, followed by application of a cover glass. Remove bubbles from tissue by applying pressure 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

IBA-1 protein (yellow), *CSF1R* RNA (green), *CD4* RNA (magenta), and DAPI nuclei (blue) staining in ileum of an ~8-week-old, weaned pig. Tissue was fixed in 4% PFA ~24 hours. Frame shows Peyer's patch follicle.



Contributions

Jayne Wiarda: protocol creation, protocol writeup, data interpretation

Hannah Mazon: protocol execution, protocol writeup

Crystal Loving: financial support