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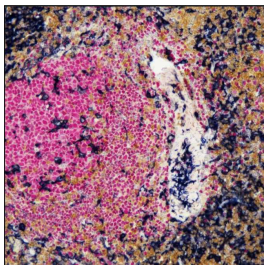
Multiplexed brightfield detection of CD3e, IBA-1, and Pax5 in formalin-fixed, paraffin-embedded lymph node tissues of pigs, cattle, and white-tailed deer



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We use this protocol and it's working

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Abstract

Simultaneous detection of CD3e (T cells; stained in brown), IBA-1 (macrophages/dendritic cells; stained in blue), and Pax5 (B cells; stained in red) in formalin-fixed, paraffin-embedded (FFPE) tracheobronchial lymph node tissues from pigs, cattle, and white-tailed deer.

Attachments

PDF

Wiarda_3plexIF_PigCo...

4.3MB

Guidelines

Assay-specific Information

· The general scheme of reagents used to detect each target (IBA-1, CD3e, Pax5) are listed in the table below:

Antigen retrieval	Protein block	Primary antibody	Secondary antibody	Chromogen/color
None	2.5% horse serum	Rabbit anti-IBA-1	Horse anti-rabbit IgG-AP	BCIP/NBT (blue)
Sodium citrate HIER	2.5% horse serum	Rabbit anti-CD3e	Horse anti-rabbit IgG-HRP	DAB (brown)
Sodium citrate HIER	2.5% goat serum	Rat anti-Pax5	Goat anti-rat IgG-AP	Vector Red (red)

· Primary antibodies are used atspecies-specific dilutions listed in the table below

Target	**Primary antibody**	**Pig**	**Cow**	**Deer**
IBA-1 (macrophages/dendritic cells)	Rabbit anti-IBA-1 (stock concentration 500 ug/mL)	0.5 ug/mL (1:1,000*) 1 hour RT	0.5 ug/mL (1:1,000*) 1 hour RT	0.5 ug/mL (1:1,000*) 1 hour RT
CD3e (T cells)	Rabbit anti-CD3e (stock concentration 400 ug/mL)	0.4 ug/mL (1:1,000*) overnight 4C	0.4 ug/mL (1:1,000*) overnight 4C	0.4 ug/mL (1:1,000*) overnight 4C
Pax5 (B cells)	Rat anti-Pax5	0.5 ug/mL	0.5 ug/mL	0.5 ug/mL

		(stock concentration 500 ug/mL)	(1:1,000*) 1 hour RT	(1:1,000*) 1 hour RT	(1:1,000*) 1 hour RT

*Dilution factors will need to be adjusted if stock antibody concentrations differ from those listed in the table

Assay Controls

· Controls will be required to ensure there is minimal/no cross-reactivity with off-target epitopes in the tissue and to ensure stain colors are specific to the intended antibody targets

· All controls should be run on serial sectioned recuts of the same tissue block

· IHC controls:

o No stain control

§ This slide receives only diluent in place of all primary antibodies. All other reagents are still applied to the slide.

o Single antibody stain controls

§ This slide receives only diluent in place of all primary antibodies except one. All other reagents are still applied to the slide.

Assay Variations

· Parameters may need to be further optimized for different tissues, targets, or species.

Materials

Equipment

- **Pipettes/pipette tips**
- **Dry weigh scale (0.1 gram increments required)**
- **Weigh boats**
- **Reagent weighing spatulas**
- **pH meter**
- **Graduated cylinders for liquid measurements (1 mL increments required)**
- **Glassware/plasticware for liquid reagents**
- **Drying oven (able to reach & hold 60°C)**
- **Fume hood**
- **Slide staining tray (e.g. Simport M920-2)**
- **EZ-Batch Slide Holder (Advanced Cell Diagnostics 321716)**
- **EZ-Batch Wash Tray (Advanced Cell Diagnostics 321717)**
- **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)**
- **5.5 Quart Digital Steamer (Hamilton Beach 37530Z)**
- **Brightfield microscope and/or slide imaging platform**

Reagents/Supplies

- Distilled water (obtained in-house)
- Phosphate-buffered saline (PBS), pH 7.2-7.4 (made in-house)
- 0.05% PBS-Tween (PBS-T), pH 7.35 (made in-house)
- Xylenes (Macron Fine Chemicals 8668-16)
- 100% ethanol (Pharmco 111000200)
  - Dilute with distilled water to make 95%, 85%, and 70% concentrations
- Pro-Par Clearant (Anatech 510)
- Fixative
  - 10% NBF (Cancer Diagnostics, Inc. 111) or 4% PFA (Electron Microscopy Sciences 15713)
- ImmEdge Hydrophobic Barrier Pen (Vector H-4000)
- 1X Sodium Citrate Antigen Retrieval Solution, pH 6.0 (made in-house)
  - 10X sodium citrate solution: Dissolve 29.4 g sodium citrate dihydrate in 950 mL distilled water. Add water to total volume of 1L after solid has dissolved. Store at 4C up to one year.
  - 1X sodium citrate solution: 100 mL 10X sodium citrate solution + 900 mL distilled water. Adjust pH to 6.0. Store at 4C up to three months.
- BLOXALL Endogenous Blocking Solution (Vector SP-6000)

- 1% bovine serum albumin (BSA) in PBS (made in-house)
- 100 mM Tris-HCl with 0.1% Tween, pH 9.5 (made in-house)
- Rabbit anti-IBA-1 polyclonal antibody; stock concentration 500 ug/mL (Fujifilm Wako 019-19741)
  - Stock concentration can vary by antibody lot
- Rabbit anti-CD3e polyclonal antibody; stock concentration 0.4 g/mL (Dako A0452)
  - Stock concentration can vary by antibody lot
- Rat anti-Pax5 monoclonal antibody, clone 1H9; stock concentration 500 ug/mL (Invitrogen 14-9918-82)
- Horse anti-rabbit IgG-AP polymer (Vector MP-5401)
  - Normal Horse Serum 2.5%
  - Horse Anti-Rabbit IgG Polymer Reagent
- Horse anti-rabbit IgG-HRP polymer (Vector MP-6401)
  - Normal Horse Serum 2.5%
  - Horse Anti-Rabbit IgG Polymer Reagent
- Goat anti-rat IgG-AP polymer (Vector MP-5404)
  - Normal Goat Serum 2.5%
  - Goat Anti-Rat IgG Polymer Reagent
- BCIP/NBT AP substrate (Vector SK-5400)
  - BCIP/NBT Reagent 1
  - BCIP/NBT Reagent 2
  - BCIP/NBT Reagent 3
- DAB HRP substrate (Vector SK-4103)
  - DAB EqV Reagent 1 (Chromogen)
  - DAB EqV Reagent 2 (Diluent)
- Vector Red AP substrate (Vector SK-5105)
  - ImmPACT Red diluent
  - ImmPACT Red Reagent 1
  - ImmPACT Red Reagent 2
- Gill's Hematoxylin I (American Master Tech Scientific HXGHE1LT)
- VectaMount PT (Vector H-5600-60)
- #1 thickness coverglass (e.g. Corning 2975-245)

Safety warnings

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

Before start

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. 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. Fix tissues 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.

Baking

1 Before starting the assay:
- Preheat a dry oven to 60°C
- Load slides for assay into vertical slide rack

2 • **Bake slides 20 min 60°C** 20m

2.1 While slides bake:
- o Prepare 0.05% PBS-T (can store at RT up to 1 month)

2.2 Immediately before deparaffinizing:
- o Add ~200 mL xylenes to each of three clearing agent dishes in a fume hood
- o Add ~200 mL 100% ethanol to each of two staining dishes in a fume hood
- o Add ~200 mL 95% ethanol to a staining dish in a fume hood
- o Add ~200 mL 85% ethanol to a staining dish in a fume hood
- o Add ~200 mL 70% ethanol to a staining dish in a fume hood
- o Add ~200 mL distilled water to a staining dish in a fume hood
- o Add ~200 mL PBS-T to a staining dish

Deparaffinizing & Rehydrating 25m

3 25m
- o Submerge slide in fresh **xylenes 5 min RT**
- o Submerge slide in fresh **xylenes 5 min RT**
- o Submerge slide in fresh **xylenes 5 min RT**
- o Submerge slides in fresh **100% ethanol 1 min RT**
- o Submerge slides in fresh **100% ethanol 1 min RT**
- o Submerge slides in fresh **95% ethanol 1 min RT**
- o Submerge slides in fresh **85% ethanol 1 min RT**
- o Submerge slides in fresh **70% ethanol 1 min RT**
- o Submerge slides in fresh **distilled water 3 min RT**
- o Submerge slides in fresh **PBS-T for transport**

3.1 While slides deparaffinize/rehydrate:
- o Turn off dry oven
- o Prepare humidified slide staining tray by adding water to bottom & placing lid on top
- o Add ~200 mL PBS-T to each of three staining dishes
- o Prepare humidified slide staining tray by adding water to bottom & placing lid on top

Hydrophobic Barrier 20m

4 20m

- **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 a hydrophobic barrier pen. Keep tissue area wet the whole time. Draw barrier and place slides into the EZ-Batch slide holder placed inside 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). Slides will remain locked in the EZ-Batch slide holder throughout the protocol until being transferred back to a vertical rack for counterstaining.
- Leave slides in slide staining tray

Tissue Quenching 15m

5 15m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **BLOXALL Endogenous Blocking Solution 10 min RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

5.1 While slides incubate with enzyme block:

- Discard deparaffinizing & rehydrating reagents
- Add ~200 mL PBS-T to each of two wash trays

Protein Blocking (horse serum) 30m

6 30m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **2.5% horse serum 30 min RT**
  - Serum is a component of the horse anti-rabbit IgG-AP polymer kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed

6.1 While slides incubate with serum:

- Discard tissue quenching reagents
- Prepare diluted anti-IBA-1 antibody by adding IBA-1 antibody stock to 1% BSA in PBS. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Mix diluted antibody well before use. Dilutions to use are species-specific, as listed in the Antibody Information section at the beginning of this document.

Primary Antibody (IBA-1) 1h 5m

7 1h 5m

- Decant slide holder and again place flat in slide staining tray
  - Do not wash with PBS-T between decanting serum and applying secondary antibody

- Incubate with **diluted IBA-1 antibody 1 hour RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

7.1 While slides incubate with primary antibody:
- Discard protein blocking reagents
- Add ~200 mL PBS-T to each of two wash trays

Secondary Antibody (anti-rabbit AP) 35m

8
- Decant slide holder and again place flat in slide staining tray
- Incubate with **horse anti-rabbit IgG-AP polymer 30 min RT**
  - Polymer is a component of the horse anti-rabbit IgG-HRP polymer kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

8.1 While slides incubate with secondary antibody:
- Discard primary antibody reagents
- Add ~200 mL PBS-T to each of two wash trays

8.2 Immediately before chromogen detection:
- Prepare diluted BCIP/NBT chromogen by adding two drops of Reagent 1, two drops of Reagent 2, and two drops of Reagent 3 to 5 mL prepared 100mM Tris-HCl in 0.1% Tween, pH 9.5. Total volume to use is dependent on tissue sizes. Make sure to mix reagents thoroughly. Store in the dark due to light sensitivity

Chromogenic Detection (BCIP/NBT) 25m

9 25m
- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted BCIP/NBT chromogen 20 min RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

9.1 While slides incubate with chromogen:
- Discard remaining secondary antibody reagents
- Add ~200 mL PBS-T to each of two wash trays

- Prepare steamer:
  - Fill the bottom reservoir of steamer to the 'Fill' line with tap water
  - Assemble the steamer, using both tiers 1 and 2 of steam bowls. Do not place the divider between steam bowls, as an extra tall compartment is needed
  - Pour 200 mL prepared 1X Sodium Citrate Buffer, pH 6.0 into staining dish and place in the steamer
  - Preheat the prepared steamer, programmed for 1 hour
    - Perform this step ~25 minutes before use so that retrieval solution can adequately heat to ~105-110°C

Heat-Induced Epitope Retrieval (HIER)/Antibody Stripping

20m

10 20m

- Leave slides in PBS-T at RT until steamer is preheated
- Transfer slides to vertical slide rack
  - Do quickly to avoid drying out slides or alternatively place vertical slide rack in a staining dish containing PBS-T and then transfer slides
- Once steamer has preheated, submerge slide rack in **preheated 1X Sodium Citrate Buffer 15 min**
  - Slides should be maintained at ~105-110°C for the duration of target retrieval. To ensure temperature is not reduced, open steamer lid, load slides, and shut steamer lid as quickly as possible.
- Remove slide rack from steamer
- Submerge slide rack in fresh **PBS-T 2 min RT**
- Submerge slide rack in fresh **PBS-T 2 min RT**

10.1 While slides incubate with sodium citrate:
- Discard chromogen detection reagents
- Add ~200 mL PBS-T to each of two staining dishes

Protein Blocking (horse serum)

30m

11 30m

- Transfer slides back to slide holder
  - Do quickly to avoid drying out slides or alternatively place slide holder in a wash tray containing PBS-T and then transfer slides
- Decant slide holder and again place flat in slide staining tray
- Incubate with **2.5% horse serum 30 min RT**
  - Serum is a component of the horse anti-rabbit IgG-HRP polymer kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed

11.1 While slides incubate with serum:
- Discard HIER reagents
- Turn off and clean steamer
- Prepare diluted anti-CD3e antibody by adding CD3e antibody stock to 1% BSA in

PBS. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Mix diluted antibody well before use. Dilutions to use are species-specific, as listed in the Antibody Information section at the beginning of this document.

Primary Antibody (CD3e)

18h

12 18h

- Decant slide holder and again place flat in slide staining tray
  - Do not wash with PBS-T between decanting serum and applying secondary antibody
- Incubate with **diluted CD3e antibody overnight at 4C**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

12.1 While slides incubate with primary antibody:
- Discard protein blocking reagents and leftover antibody dilution reagents

12.2 The next day:
- Add ~200 mL PBS-T to each of two wash trays

Secondary Antibody (anti-rabbit HRP)

35m

13 35m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **horse anti-rabbit IgG-HRP polymer 30 min RT**
  - Polymer is a component of the horse anti-rabbit IgG-HRP polymer kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

13.1 While slides incubate with secondary antibody:
- Discard primary antibody reagents
- Add ~200 mL PBS-T to each of two wash trays

13.2 Immediately before chromogen detection:
- Prepare diluted DAB chromogen by adding equal volumes of DAB Reagent 1 (Chromogen) and DAB Reagent 2 (Diluent) together. Total volume to use is dependent on tissue sizes. Make sure to mix reagents thoroughly. Store in the dark due to light sensitivity

Chromogenic Detection (DAB)

15m

14 15m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted DAB chromogen 10 min RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

14.1 While slides incubate with chromogen:
- Discard remaining secondary antibody reagents
- Add ~200 mL PBS-T to each of two wash trays

Protein Blocking (goat serum)

30m

15 30m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **2.5% goat serum 30 min RT**
  - Serum is a component of the goat anti-rat IgG-AP polymer kit
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slides and transfer to vertical slide rack

15.1 While slides incubate with serum:
- Discard chromogen detection reagents
- Prepare diluted anti-Pax5 antibody by adding Pax5 antibody stock to 1% BSA in PBS. Total volume to use is dependent on tissue sizes. Make sure to mix reagents before pipetting. Mix diluted antibody well before use. Dilutions to use are species-specific, as listed in the Antibody Information section at the beginning of this document.

Primary Antibody (Pax5)

1h 5m

16 1h 5m

- Decant slide holder and again place flat in slide staining tray
  - Do not wash with PBS-T between decanting serum and applying secondary antibody
- Incubate with **diluted Pax5 antibody 1 hour RT**
  - Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

16.1 While slides incubate with primary antibody:
- Discard protein blocking reagents and leftover antibody dilution reagents

o Add ~200 mL PBS-T to each of two wash trays

Secondary Antibody (anti-rat AP) 35m

17 35m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **goat anti-rat IgG-AP polymer 30 min RT**
  - o Polymer is a component of the goat anti-rat IgG-AP polymer kit
  - o Apply to completely cover tissues; let incubate in slide staining tray with lid closed
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

17.1 While slides incubate with secondary antibody:
o Discard primary antibody reagents
o Add ~200 mL PBS-T to each of two wash trays

17.2 Immediately before chromogen detection:
o Prepare diluted Vector Red chromogen by adding 2 drops of Reagent 1 and two drops of Reagent 2 to 5 mL Diluent. Total volume to use is dependent on tissue sizes. Make sure to mix reagents thoroughly. Store in the dark due to light sensitivity

Chromogenic Detection (Vector Red) 35m

18 35m

- Decant slide holder and again place flat in slide staining tray
- Incubate with **diluted Vector Red chromogen 30 min RT**
- Decant slide holder and transfer to wash trays for PBS-T washes
- Submerge slide holder in fresh **PBS-T 2 min RT**
- Submerge slide holder in fresh **PBS-T 2 min RT**

18.1 While slides incubate with chromogen:
o Discard secondary antibody reagents
o Add ~200 mL PBS-T to each of two wash trays
o Add ~200 mL hematoxylin to one staining dish
o Add ~200 mL distilled water to each of three staining dishes

Counterstaining 2m

19 2m

- Transfer slides to vertical slide rack
  - o Do quickly to avoid drying out slides or alternatively place vertical slide rack in a staining dish containing PBS-T and then transfer slides
- Submerge slide rack in **hematoxylin 1 min RT**
- 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 **distilled water, dunking 3-5 times**
  - Water should no longer appear purple in the third water dish used

Mounting

45m

20

- **Air dry slides RT 20-30 min**
- In a fume hood, **mount slides** by adding 2-4 drops of VectaMount 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 overnight
- Assess staining with a bright-field microscope

45m

Protocol references

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