IMMUNOFLUORESCENCE ANTIBODY (IF) STAINING PROTOCOL

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ABSTRACT

This is the basic protocol for antibody staining of formalin fixed paraffin embedded (FFPE) tissue.

ATTACHMENTS

IF_Paraffin slides protocol.docx

GUIDELINES

Principle:

For antibody staining to be successful, most FFPE tissue requires antigen retrieval of some kind. Formalin fixation cross-links proteins during the course of fixation. Antigen retrieval unlinks the proteins and opens up the antigen sites so that the antibody will be able to bind to them.

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Protocol status: Working
We use this protocol and it's working

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MATERIALS

Specimen Preparation:

10% Neutral buffered formalin fixed tissue, or 4% paraformaldhyde fixed tissue, paraffin embedded sections cut at ~5-6 microns and mounted on charged slides.

Materials and Equipment:

1. Charged slides
2. Coverslips
3. Drying oven, 60 °C
4. Fume Hood
5. Gloves
6. Microtome
7. Staining racks
8. Timer

Reagents:

1. Xylene (green staining containers only!)
2. 100% Alcohol
3. 95% Alcohol
4. 80% Alcohol
5. Dapi nuclear stain
6. Background Sniper (Biocare Medical)
7. TRIS with tween 20X (Biolegend,[hydroxymethyl aminomethane])
8. Secondary antibody, typically one from Jackson Immunoresearch
9. 3% Hydrogen peroxide
10. True View/True Black Autofluorescent quenching media
11. Aqueous mounting media with or without DAPI

Pretreatment reagents:

1. Reveal Decloaker (Biocare Medical) preferred
2. Citrate buffer solution if you don’t use the Reveal
3. Specific case for Beta-amyloid antibody
4. 70% Formic acid treatment

Solutions to cover tissue:

	A	B
	Xylene	5 minutes
	Xylene	5 minutes
	Xylene	10 minutes
	100% Alcohol	3 - 5 minutes
	100% Alcohol	3 - 5 minutes
	95% Alcohol x 2	3 - 5 minutes
	80% Alcohol	3 - 5 minutes
	Filtered water	3 - 5 minutes

SAFETY WARNINGS

Precautions:
Personal Protection: Gloves, lab coat, goggles, fume hood, and use of universal precaution practices.
Chemical Wastes: Dispose of alcohols, dyes, and xylene in appropriate labeled waste containers as directed by the University of Minnesota Hazardous Chemical Waste Management Manual 5th Edition.
Hazards: Xylene = Flammable, Carcinogen, Skin irritant, Eosin & Alcohols = Flammable, Skin irritant
Hematoxylin = Skin irritant, Avoid strong oxidizers with all listed chemicals.

DAY 1: Deparaffinize tissue

45m

1 Either place slides on a slide warmer with temperature set to approx. 57 °C . Leave the slides on the warming plate until the paraffin looks melted on all of the slides (about 0- 00:15:00). 15m

2 Put slides in a 60 °C oven for about 00:30:00 . 30m

Note

Place slides vertically in the gray plastic slide holders & run the slides through the following solution containers located in the fume hood. Check that solutions cover tissue completely.

	A	B
	Xylene	5 minutes
	Xylene	5 minutes
	Xylene	10 minutes
	100% Alcohol	3 - 5 minutes
	100% Alcohol	3 - 5 minutes
	95% Alcohol x 2	3 - 5 minutes
	80% Alcohol	3 - 5 minutes
	Filtered water	3 - 5 minutes

DAY 1: Preferred Antigen retrieval steps

1h 45m

3 Fill the vegetable steamer with deionized water to the second line in the transparent corner section of the steamer.

4 Add capillary gap slides (same number as the number of slides you are staining) in the slide holder to wet the slides, or if you have a lot of slides, rinse the capillary gap slides separately before use.

4.1 Fill the plastic “boat” containers with approx. 20 mL of one of the 1X antigen retrieval solutions (Reveal or citrate buffer). Pick up a slide (rough side of the capillary gap slide facing the tissue section slide) and put the slides into one of the troughs in the boat container.

4.2 Push slides toward each other and allow the fluid to come up slowly between the two slides. Try not to get bubbles between the slides or this will obstruct the antigen retrieval process and give you uneven staining.

4.3 Put the “boats” into the steamer and set for 00:35:00 . This will allow the steamer to come up to temperature and the slides will steam for about 00:30:00 . 1h 5m

5 Remove clear basket from the steamer and allow slides to cool for about 00:20:00 to room temperature. 20m

6 Transfer the “boats” of slides to the inner steamer container and rinse with running water for about 10-00:15:00 . Unpeel the slides from each other and transfer the slides to the gray slide holder for a final rinse (~ 00:05:00). 20m

Note

Alternative microwave method:

- If you don’t have the vegetable steamer you can do antigen retrieval in the microwave by putting the slides in a plastic coplin jar filled with citrate buffer. Microwave slides/solution for 00:05:00 . on high power (~700 watts). Make sure slides are still covered with retrieval solution or add fresh solution and repeat microwaving. This process can be repeated 2-3 times.
- Let slides slowly cool in coplin jar to room temperature for approx. 00:20:00 .

DAY 1: Staining steps 2h 3m

7 Dilute or use prediluted TRIS (1x concentration) for 00:10:00 , approx 250 µL - 300 µL per slide. 10m

8 3% Hydrogen peroxide made in 1X TRIS for 10-00:20:00 . 20m

9 Rinse slides in 1X TRIS and add to slides for 00:05:00 . 5m

10 100% Background Sniper for nearly 00:13:00 . Do not go longer than 00:15:00 . (alternative: 1h 28m normal goat serum made in 1X TRIS or PBS can be substituted for 01:00:00) or 1% sodium borohydride made in 1X TRIS soln to decrease autofluoresence.

11 Make up antibody solution or a cocktail of multiple antibodies at desired concentration in a diluent of 5% Sniper in 1X TRIS solution. Using approx. 100 µL per slide, make up enough antibody to cover all slides.

12 Cover the slides with either a glass coverslip (24 x 60 mm) or parafilm and put in a 4 °C refrigerator Overnight .

DAY 2:

2h 35m 20s

13 Take slides out of the refrigerator and warm up to room temperature about 15- 00:20:00 . Remove the 25m cover slips and cover the slides with 1X TRIS solution for a few minutes. Rinse off the antibody solution with 1X TRIS 00:05:00 x 2. At this stage you will need to use the black staining box to keep the reagents from light exposure.

14 Secondary reagent/antibody for 1- 02:00:00 . 2h

Apply the secondary reagent appropriate to the antibody, i.e., if your antibody was raised in a rabbit, you will apply a fluorescent antibody such as Jackson goat anti-rabbit 488 (yellow in tube, excites to green in IF light) using about 250 µL - 300 µL per slide or enough of the reagent to totally cover the tissue.

14.1 If your antibody was raised in some other animal, you will have to find a secondary to that animal, i.e., donkey anti-mouse, goat anti-rat, etc. Usually a dilution of 1:300 to 1:500 in a diluent of 5% Sniper made in 1X TRIS is a good starting concentration to use.

Note

Often a cocktail of 2 secondary antibodies is used for dual chromogen staining.
In this case, be careful to combine both chromogens in correct ratios with the diluent. If the secondary has glycerol added (which is often the case to keep the solution from freezing), you will have to double the amount of antibody used. Jackson ImmunoResearch sells a host of secondary antibodies. Colors 594 excites red, 488 excites green…

15 Rinse slides with 1X TRIS; 5 min x 3. 15m

15.1 Rinse slides with 1X TRIS for 00:05:00 (1/3). 5m

15.2 Rinse slides with 1X TRIS for 00:05:00 (2/3). 5m

15.3 Rinse slides with 1X TRIS for 00:05:00 (3/3). 5m

16 If you do not want to use the solutions to block autofluorescence, mount slides in dim light conditions with Prolong gold with DAPI. The dapi will stain the nuclear elements blue. Cover slides in a slide box or tin foil to dry overnight at room temperature to set the mounting media.

17 Blocking Autofluorescence (Optional step) with Vector TrueView.

17.1 In IHC 4 °C frig, TrueView kit on top shelf.

17.2 Add equal volumes of reagents in order, mix A with B and mix together ~ 00:00:10 . 10s

17.3 Add C and mix again ~ 00:00:10 . Calculate approx. 100/slide. 10s

17.4 Apply to tissue and incubate for 2- 00:05:00 . 5m

17.5 Tissue will stain dark blue. Rinse with 1X Tris.

17.6 Stain with DAPI separately as in the following steps:

17.7 DAPI aliquots are in the -20 °C freezer and should be diluted as follow: 10m

- 2.1 µL concentrated DAPI in 100 µL 1X Tris.
- Dilute the above solution 1:1000 in 1X Tris for the working solution of 1X DAPI.
- Stain with the working solution of DAPI for 00:10:00 .
- Rinse in 1X TRIS a few times and coverslip with VectaShield Vibrance.
- After coverslipping, cover slides and allow media to harden in the dark (Room temperature).