Immunofluorescence on Fresh Frozen Kidney Tissue - Validation of Antibodies V.1

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We use this protocol and it's working

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ABSTRACT

We describe the procedure for immunofluorescence on human kidney tissue embedded in carboxymethylcellulose or low melting point gelatin, and the subsequent validation criteria used for the antibodies based on their colocalization and/or localization with other segment specific antibodies.

We describe: a) primary antibody detection by indirect immunofluorescence using species-specific fluorescently conjugated secondary antibodies with minimal cross reactivity to other species antibodies being tested; b) antibody detection using commercially available or in-house fluorescently conjugated primary antibodies; c) our protocol for detecting biotinylated antibodies and lectins using streptavidin or neutravidin conjugated fluorophores after blocking endogenous biotins; and d) a sequential staining protocol that allows co-labeling of unconjugated and conjugated antibodies from the same species that minimizes binding of the secondary antibody to the fluorescently conjugated primary antibody. Finally, after image acquisition and image analysis using QuPath, we describe the steps we take to initially assess antibody staining (including evaluation of fluorescently conjugated primary antibodies, and steps that are taken if initial staining is negative), and the subsequent validation criteria we use for primary antibodies that show appropriate staining (i.e.: correct cellular segments, subcellular localization, kidney region etc.) that is based on co-localization with other antibodies, cell markers, and/or structures in the human kidney.

MATERIALS

Reagents:
10% Buffered Formalin (Fisher Scientific, SF100-4)
10X Phosphate Buffered Saline (Thermo Fisher Scientific, #28374)
Hydrophobic Barrier Pen (Vector Labs, H-4000)
Avidin/Biotin Blocking Kit (Vector Labs, SP-2001)
10X Power Block Universal Blocking Solution (BioGenex, HK085-5K)
Antibody Diluent Reagent Solution (Thermo Fisher Scientific, 003218)
Double Distilled Water to dilute PBS to 1X
Hoechst 33342 (Thermo Fisher Scientific, 62249)
100% Glycerol (Fisher Scientific, G33)
Coverslips
Serum for Additional Block (Example: Normal Rabbit Serum, Sigma-Aldrich, NS01L-1ML)
Secondary Antibody with Minimal Cross Reactivity

Equipment:
- Moisture/Humidified Chamber
  - 100-Slide Storage Box (Fisher Scientific, 03-448-1)
  - Kimwipes, 8.4 in x 4.4 in (Fisher Scientific, 06-666)
  - ddH_2O in a wash bottle

Immunofluorescence

1 Fresh frozen slides were prepared using the following protocols:

Freezing and embedding: dx.doi.org/10.17504/protocols.io.br4fm8tn

Sectioning: dx.doi.org/10.17504/protocols.io.bt8inrue

In-house fluorescent conjugation of antibodies: dx.doi.org/10.17504/protocols.io.kxygx34rdg8j/v1

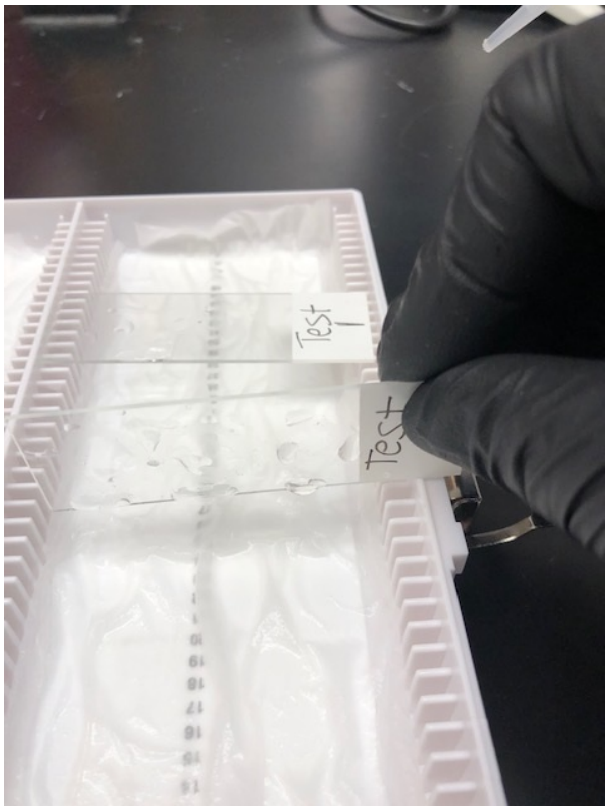
2 Place frozen slides from the -80˚C freezer directly into the formalin, and post-fix for 5 minutes.

3 Pour used formalin into the appropriate waste container, then fill jar with 1X PBS and wash sections for 5 minutes four times.

4 Using a hydrophobic pen, draw a large barrier around each section. **Do not allow pen to touch section.**

5 Place slides in humidified chamber and cover section in PBS by using a pipettor so that they do not dry.

5.1 To remove solutions from sections that are in the humidified chamber, tip the solution off of the slide into the chamber.



Removing solutions from slides in humidified chamber.

6 If using primary or secondary antibody conjugated to biotin:

6.1 Block sections with avidin solution for 15 minutes.

6.2 Wash sections with PBS for 3 minutes twice.

6.3 Block sections in biotin solution for 15 minutes.

6.4 Wash sections with PBS for 3 minutes twice.

7 Block sections for 30 minutes with 1X Universal Blocking Reagent (UBR) at room temperature.

7.1 Dilute 10X blocking reagent to 1X using 9-parts ddH_2O to 1-part UBR.

8 Dilute primary antibody to desired working concentration in Antibody Diluent Reagent during blocking step.

8.1 Multiple primary antibodies can be diluted together if validating antibody via localization. Antibodies from the same species can be diluted together as long as they are directly conjugated.

8.2 If validating an unconjugated and conjugated antibody of the same species on one section, only dilute unconjugated antibody at this time. Antibodies from other species can be diluted in this step.

9 Add diluted antibody to section and incubate overnight at 4˚C.

10 Remove solution, and wash sections with PBS for 3 minutes three times.

11 If using indirect immunofluorescence, dilute fluorophore-conjugated secondary antibody using Antibody Diluent Reagent.

11.1 If validating antibody directly conjugated to a fluorophore, use a secondary antibody with different wavelength fluorophore.

12 Add antibody solution to sections and incubate for 60 minutes at room temperature.

13 Remove solution, and wash sections with PBS for 5 minutes three times.

14 If validating an unconjugated and conjugated antibody of the same species on one section:

14.1 Following the removal of the secondary antibody diluent, wash sections with PBS for 5 minutes three times.

14.2 Using the serum of the primary antibody host species, dilute in PBS at 1:200 (may be dependent on serum used). This will block any non-specific binding of the secondary antibody to the directly-conjugated antibody of the same species.

14.3 Add diluted serum solution to section and block for 1 hour at room temperature.

14.4 Remove solution and wash section with PBS for 5 minutes three times.

14.5 Dilute directly-conjugated primary in Antibody Diluent Reagent and incubate at room temperature for 1 hour.

14.6 Remove solution and wash with PBS for 3 minutes three times.

15 If biotin-conjugated primary or secondary antibody was used:

15.1 Dilute fluorophore-conjugated Neutravidin in PBS at 1:250 for 30 minutes

15.2 Wash sections with PBS for 3 minutes three times.

16 Incubate sections in Hoechst 33342 (1:5,000 dilution of 20mM solution in 1X PBS) for 10 minutes.

17 Remove Hoechst 33342, and wash sections with PBS for 5 minutes twice.

18 Mount slides in 50% glycerol in PBS. Do not seal the coverslips as they will need to be removed later.

18.1 Because slides are not sealed, they must be kept horizontal to prevent the coverslip from falling off, and in the humidified chamber to keep them from drying out.

19 Image (Zeiss AxioScan was used for all imaging)

20 Slides can be store at 4˚C in a moisture chamber.

Validation Criteria

21 Initial staining showing anticipated localization of segment or cell state markers in the human kidney. Fluorescently conjugated primary antibodies are also initially evaluated by comparing staining with the conjugated fluorophore with indirect immunofluorescence against the primary antibody using a spectrally distinct fluorophore.

22 If the fluorescently conjugated primary antibody does not stain:

22.1 The ratio of dye to antibody is estimated by measuring the wavelength of serial dilutions of the conjugated antibody, as described in antibody conjugation protocol (dx.doi.org/10.17504/protocols.io.kxygx34rdg8j/v1). If the ratio of dye to antibody is > 2, then labeling is repeated after addressing the successful purification of the antibody, followed by using different ratio of dye to antibody, or decreasing the incubation time of the labeling reaction as described in the antibody labeling protocol (dx.doi.org/10.17504/protocols.io.kxygx34rdg8j/v1)

23 Subsequent validation based on colocalization with other antibodies, markers, and/or structures:

23.1 Enhanced-the same staining as an independent antibody against a different epitope on the same protein. Score=3

Enhanced-overlapping staining with an antibody for the same cell type, structure, or cell state. Score=2

Supported-correct cell type, subcellular localization, or cell state compared with other antibody data. Score=1

Uncertain-no staining, incorrect cell type, subcellular localization, or cell state: not validated. Score=0