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Multiplex Immunofluorescence on Fresh Frozen Tissue V.1

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

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Abstract

Scope:
To describe the procedure for multiple cycles of immunofluorescence on human kidney tissue embedded in carboxymethylcellulose.

Expected Outcome:
Kidney tissue sections that have been tagged with antibodies for imaging microscopy.

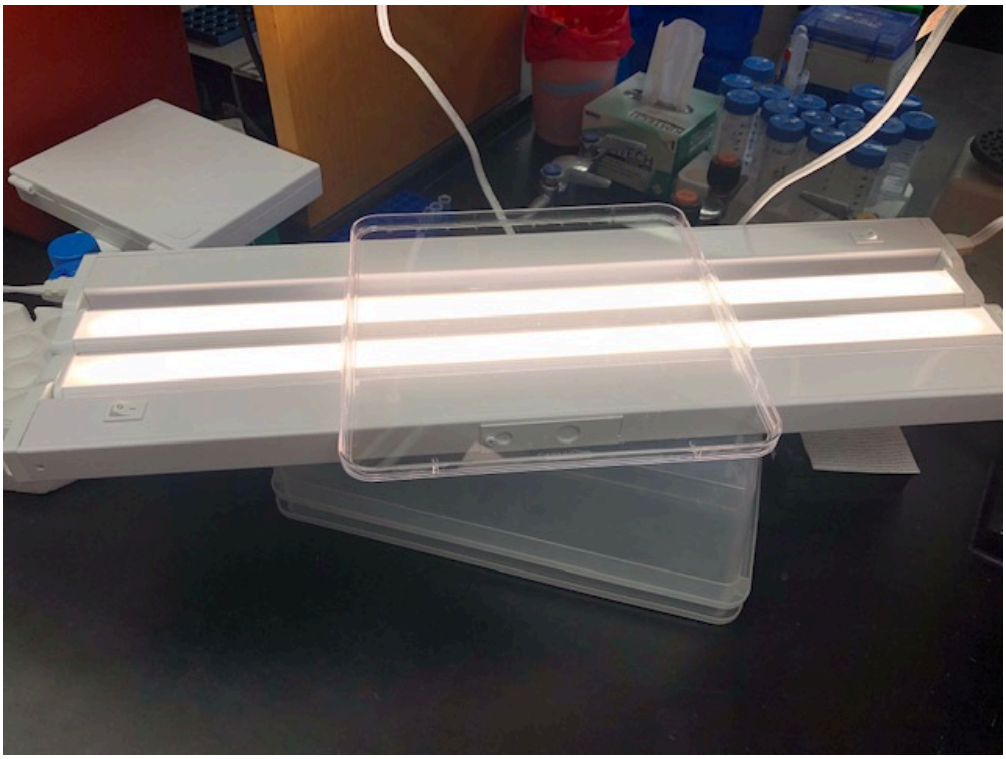
Materials

MATERIALS

- 1X PBS (Phosphate-buffered saline)
- Phosphate Buffered Saline **Thermo Fisher Scientific** **Catalog #**28374
- 1N NaOH
- Hydrophobic Barrier Pen **Vector Laboratories** **Catalog #**H-4000
- Glycine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**410225
- 10X Power Block Universal Blocking Solution **BioGenex** **Catalog #**HK085-5K
- Antibody Diluent Reagent Solution **Thermo Fisher Scientific** **Catalog #**003218
- Hoechst 33342 **Thermo Fisher Scientific** **Catalog #**62249
- 100% Glycerol **Fisher Scientific** **Catalog #**G33
- 30% Hydrogen Peroxide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**216763

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
- LED Cabinet Light (Sears, SPM11582738325)
- Square Cell Culture Plate OR any container with clear bottom



LED light system for fluorophore inactivation.

Troubleshooting

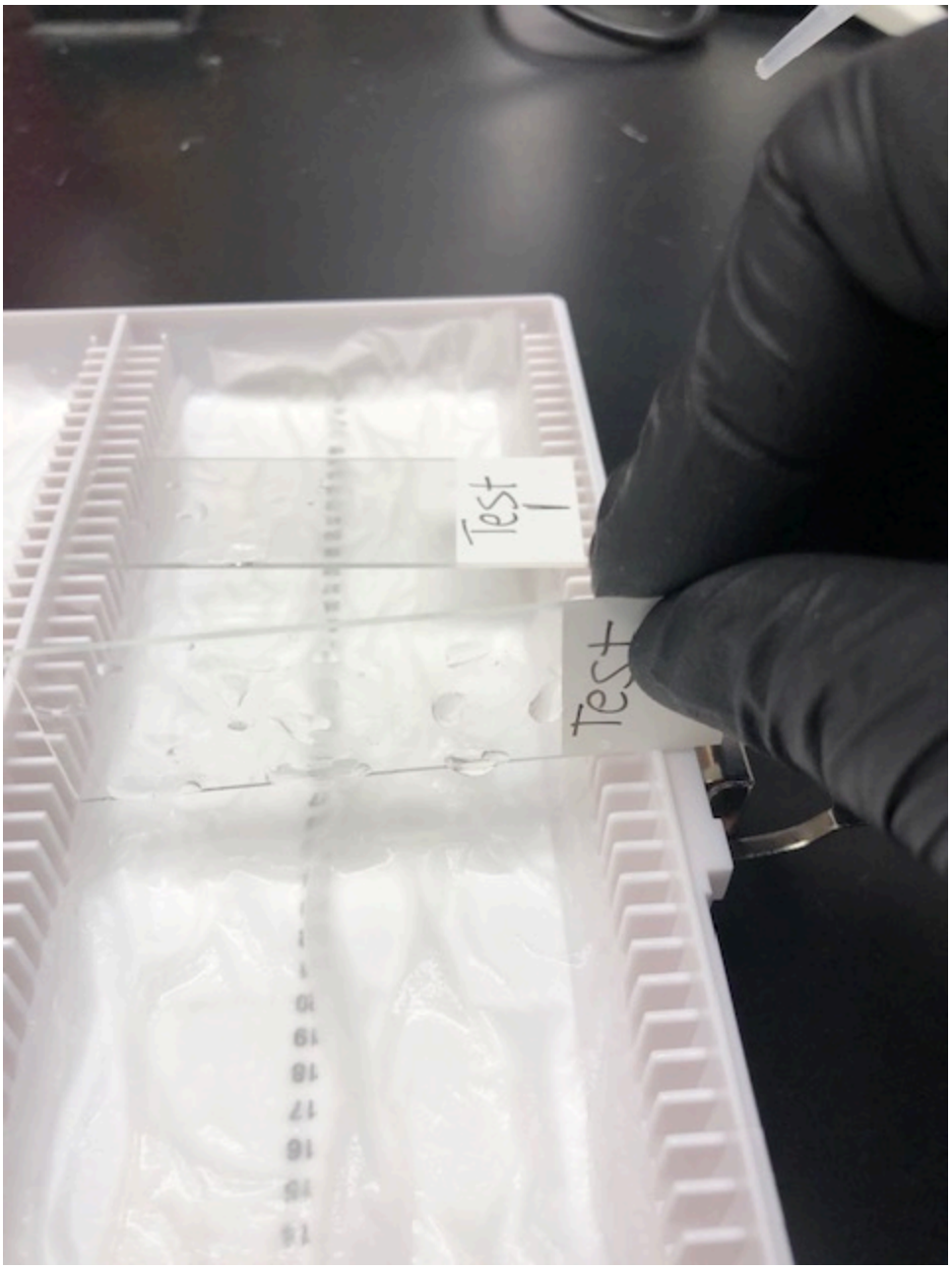
Immunofluorescence

1 If sections are frozen, allow them to equilibrate to room temperature (~15 minutes). Place slides in a humidified chamber. They will remain in the chamber throughout this protocol.

2 While slide is dry, using a hydrophobic pen, draw a large barrier around the section. **Do not allow pen to touch the section.**

3 Post-fix sections in 10% formalin for 5 minutes.

4 Remove fixative by tilting the slide, allowing the solution to flow from the section into the humidified chamber.



Removing solutions from slides in humidified chamber.

5 Wash sections in 1X PBS for 5 minutes three times. For this, tip solution off the slide into the chamber, add PBS to the slide using a pipettor, tip again and repeat.

6 Incubate sections for 5 minutes with 50 mM glycine (dilute stock in 1X PBS). This reduces autofluorescence by reducing free aldehyde groups.

7 Remove glycine, and wash sections in PBS for 5 minutes twice.

8 Incubate sections with 3% hydrogen peroxide (dilute 30% H_2O_2 in 1X PBS) at room temperature for 10 minutes to further reduce autofluorescence. This reduces non-specific fluorescence signals

9 Remove hydrogen peroxide, and wash sections in PBS for 5 minutes four times.

10 Block sections for 60 minutes with 1X Universal Blocking Reagent (UBR) at room temperature.

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

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

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

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

14 If primary antibody is directly conjugated with a fluorophore, skip to #16.

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

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

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

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

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

20 Mount slides in 70% glycerol in PBS. Do not seal the coverslips as they will need to be removed later.

20.1 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.

21 Image

22 Store slides at 4˚C in a moisture chamber.

De-Coverslipping

23 Remove coverslip from sections by incubating slides in a vertical staining jar filled with PBS for 15 minutes with slight agitation (i.e. plate rocker).

24 Slowly lift slide from vertical jar and allow coverslip to release from slide via gravity.

25 Wash slide in PBS for 5 minutes three times to remove any residual glycerol.

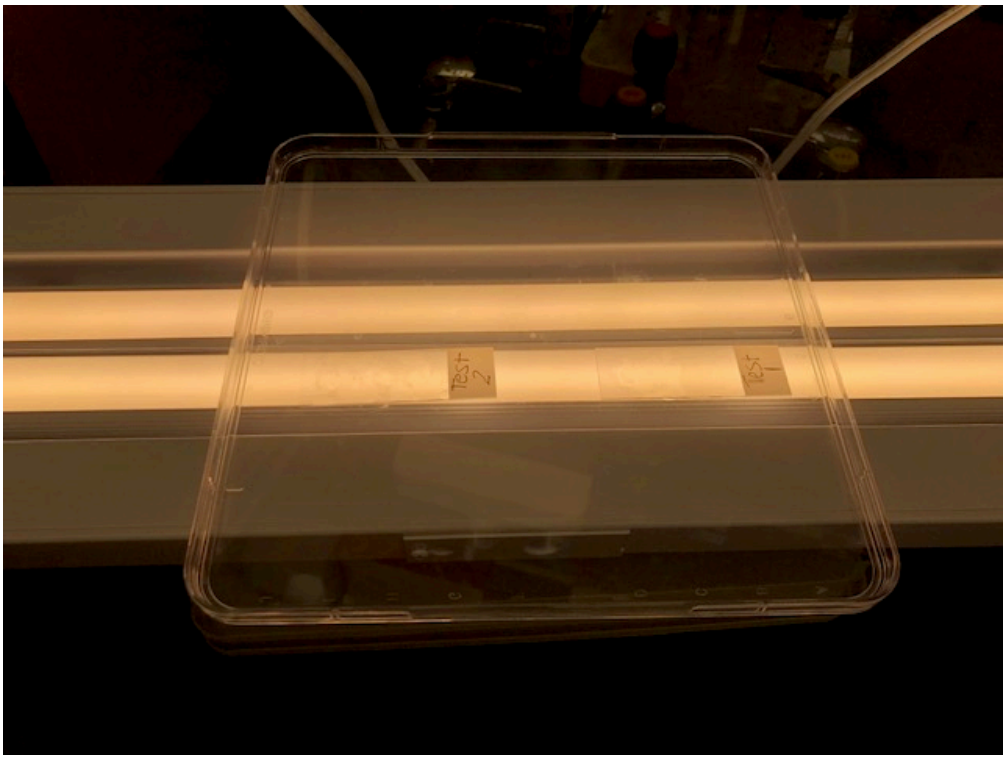
25.1 Place slides back in vertical staining jar full of PBS with slight agitation to wash.

Fluorophore I

26

Make a solution of 4.5% hydrogen peroxide and 24 mM sodium hydroxide made up in PBS.

27 Add bleaching solution to each section and incubate at room temperature for 90-120 minutes in the presence of white light (LED light).



LED light system for fluorophore inactivation.

27.1 For this, place slides on a plastic surface on top of the LED light. We often use multiple plates for this.

27.2 Halfway through this incubation, the solution may be removed by pipette and replaced with fresh solution to ensure complete inactivation occurs from the LED light.

28 Remove solution and wash sections in PBS for 5 minutes four times.

28.1 After inactivating the fluorophores, slides are mounted in 70% glycerol and imaged to confirm complete fluorophore inactivation, followed by the removal of the coverslip, and three 5 minutes washes in PBS. **Hoechst stain will not bleach** and is necessary for image registration later.

Subsequent Immunofluorescence Cycles

29 Dilute fluorophore-conjugated primary antibodies using Antibody Diluent Reagent.

30 Add diluted antibodies to each section and incubate overnight at 4˚C.

31 Remove antibody solution and wash sections with PBS for 5 minutes twice.

32 Incubate sections in Hoechst (1:10,000 dilution in 1X PBS) for 5 minutes (this may not be necessary, but does no harm).

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

34 Mount slides in 70% glycerol in PBS.

35 Image

36 Store slides at 4˚C in a moisture chamber.

37 **Sections II-IV are repeated for each remaining cycle.**