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Jan 25, 2022

Multiplex Immunofluorescence on Fresh Frozen Tissue-V2 V.2

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dx.doi.org/10.17504/protocols.io.bs68nhhw

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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.

DOI

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Maya Brewer, Liz McDonough, Yuantee Zhu, Elizabeth Neumann, Mark De Caestecker, Danielle Gutierrez, Jeff Spraggins 2022. Multiplex Immunofluorescence on Fresh Frozen Tissue-V2. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.bs68nhhw>
Maya Brewer

Glycerol % content and fluorophore inactivation solution have changed.

HuBMAP, BIOMIC, MxIF, Immuofluorescence, Kidney, Imaging, Antibody

protocol ,

Mar 10, 2021

Jan 25, 2022

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MATERIALS

[☒ 1X PBS \(Phosphate-buffered saline \)](#) **Contributed by users**

[☒ Phosphate Buffered Saline](#) **Thermo Fisher**

Scientific Catalog #28374

[☒ Hydrophobic Barrier Pen](#) **Vector**

Laboratories Catalog #H-4000

[☒ Glycine](#) **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

[☒ Sodium Bicarbonate](#) **Sigma**

Aldrich Catalog #S5761

[☒ 30% Hydrogen Peroxide](#) **Sigma –**

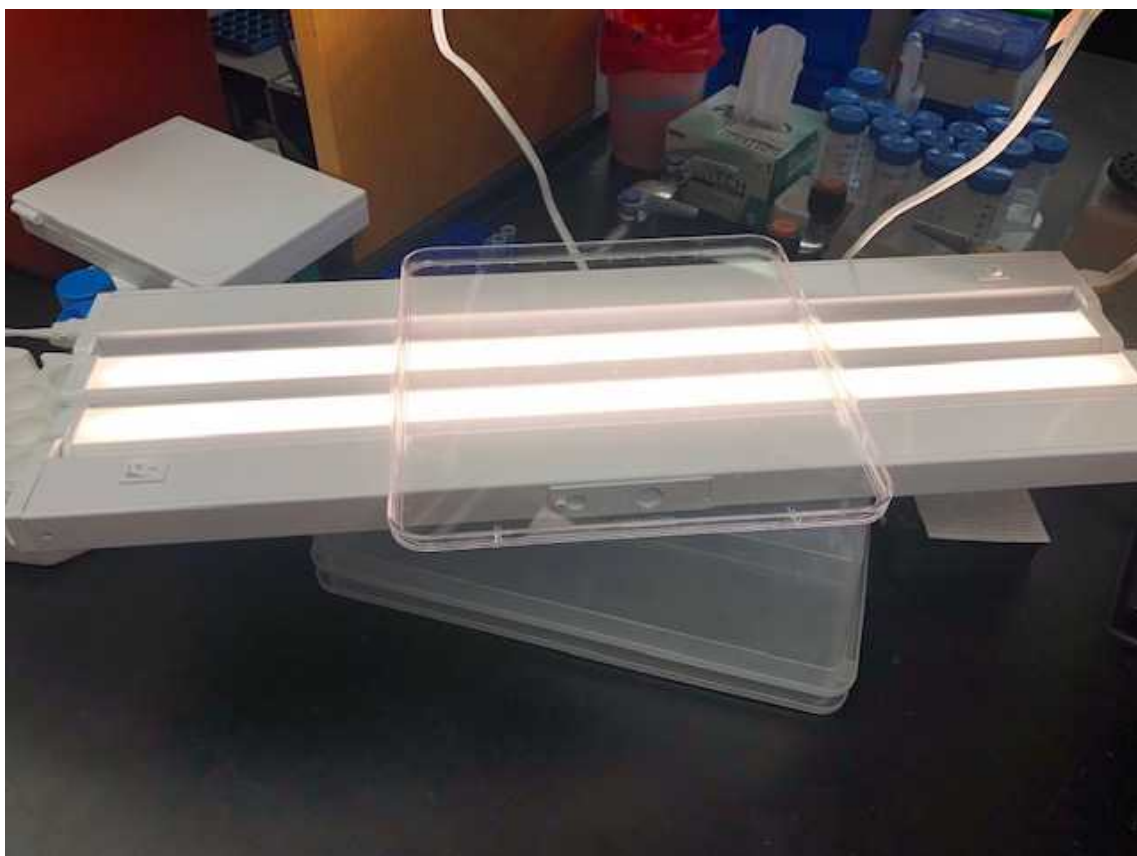
Aldrich Catalog #216763

[☒ Sodium Hydroxide Pellets](#) **Fisher**

Scientific Catalog #S318

Equipment:

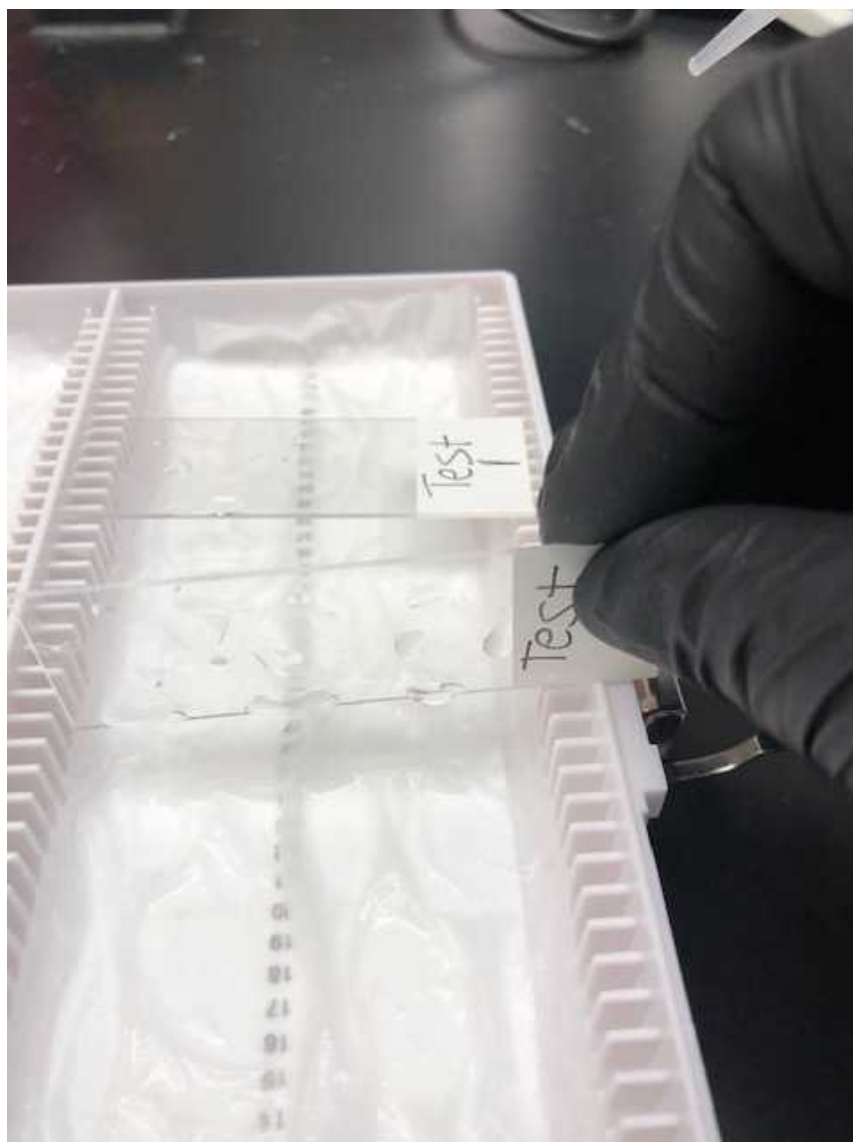
- Coplin Jar
- Moisture/Humidified Chamber
 - 100-Slide Storage Box (Fisher Scientific, 03-448-1)
 - Kimwipes, 8.4 in x 4.4 in (Fisher Scientific, 06-666)
 - ddH₂O in a wash bottle
- LED Cabinet Light (Sears, SPM11582738325)
- Square Cell Culture Plate OR any container with clear bottom



LED light system for fluorophore inactivation.

Immunofluorescence

- 1 Place frozen slides from the -80°C freezer directly into the formalin, and post-fix for 5 minutes.
- 2 Pour used formalin into the appropriate waste container, then fill jar with 1X PBS and wash sections for 5 minutes four times.
- 3 Using a hydrophobic pen, draw a large barrier around each section. **Do not allow pen to touch section.**
- 4 Place slides in humidified chamber and cover section in PBS by using a pipettor so that they do not dry.
 - 4.1 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.

- 5 Incubate sections for 5 minutes with 50 mM glycine (dilute stock in 1X PBS). This reduces autofluorescence by reducing free aldehyde groups.
- 6 Remove glycine, and wash sections in PBS for 5 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 ddH₂O to 1-part UBR.

- 8 Dilute primary antibody to desired working concentration in Antibody Diluent Reagent during blocking step.
- 9 Add diluted antibody to section and incubate overnight at 4°C.
- 10 Remove solution, and wash sections with PBS for 5 minutes twice.
- 11 If primary antibody is directly conjugated with a fluorophore, skip to #16.
- 12 If using indirect immunofluorescence, dilute fluorophore-conjugated secondary antibody using Antibody Diluent Reagent.
- 13 Add antibody solution to sections and incubate for 60 minutes at room temperature.
- 14 Remove solution, and wash sections with PBS for 5 minutes three times.
- 15 Incubate sections in Hoechst 33342 (1:5,000 dilution of 20mM solution in 1X PBS) for 10 minutes.
- 16 Remove Hoechst 33342, and wash sections with PBS for 5 minutes twice.
- 17 Mount slides in 50% glycerol in PBS. Do not seal the coverslips as they will need to be removed later.

17.1 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.

- 18 Image

- 19 Store slides at 4°C in a moisture chamber.

De-Coverslipping

- 20 Remove coverslip from sections by incubating slides in a vertical staining jar filled with PBS for 30 minutes with slight agitation (i.e. plate rocker).
- 21 Slowly lift slide from vertical jar and allow coverslip to release from slide via gravity.
 - 21.1 If the coverslip does not release from the slide, return the slide to PBS for an additional 15 minutes.
- 22 Wash slide in PBS for 5 minutes three times to remove any residual glycerol.
 - 22.1 Place slides back in vertical staining jar full of PBS with slight agitation to wash.

Fluorophore Inactivation

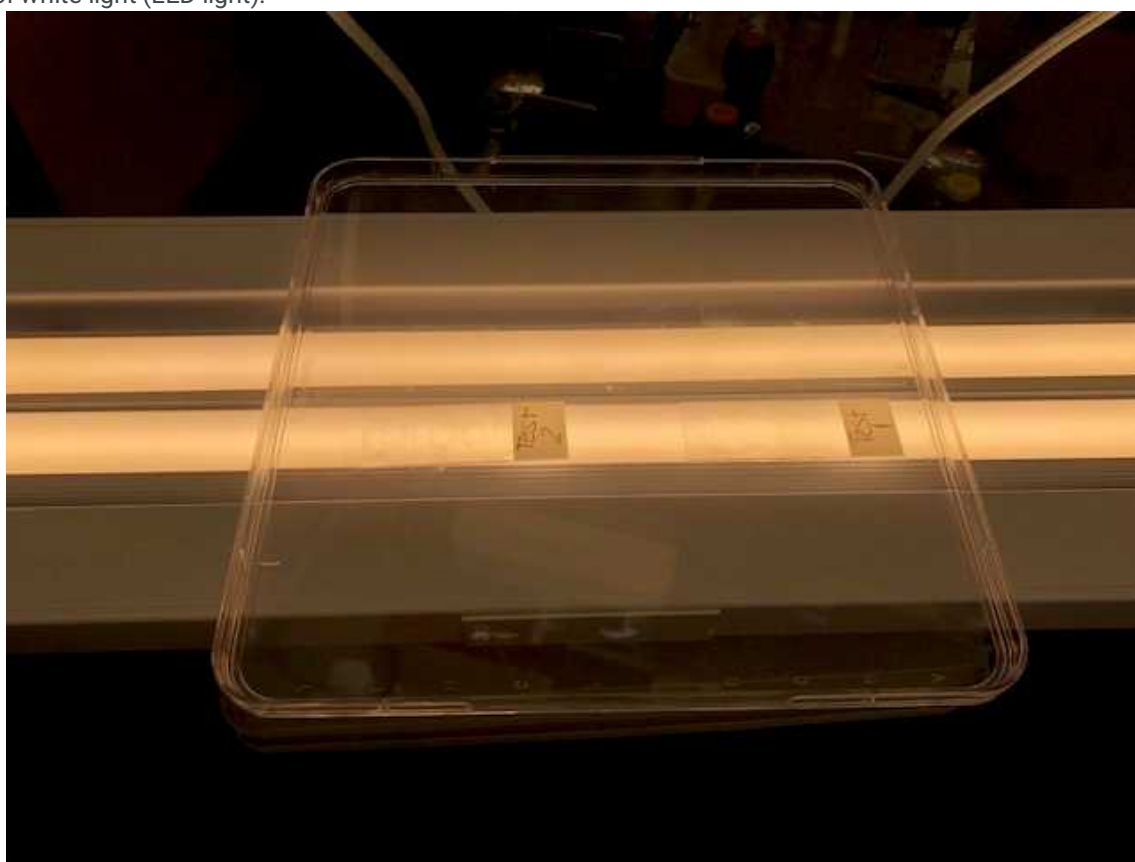
- 23 Prepare basic solution of 0.5 M sodium bicarbonate (NaHCO_3), pH 11.2 (acceptable range: 10.9-11.3).
 - 23.1 Mix 21 g of sodium bicarbonate with 500 ml ddH₂O thoroughly.
 - 23.2 Using sodium hydroxide pellets, bring the solution to a pH 11.2 (acceptable range: 10.9-11.3). Measure the pH each week it is used and adjust as needed.
 - 23.3 Solution can be stored at 4C for 6 months.
- 24 Prepare a solution of 0.1 M NaHCO_3 , 3% H_2O_2 in ddH₂O.

24.1 As an example, to make up 10 ml of inactivation solution, add 2 ml of 0.5 M NaHCO₃ and 7 ml ddH₂O.

24.2 Solution is aliquoted into five tubes (1.8 ml per tube).

24.3 200 µl of hydrogen peroxide is added to each tube prior to the solution being added to the section. **Solution must be made fresh and added to sections immediately after the addition of H₂O₂. This reaction is time sensitive.**

25 Add inactivating solution to each section and incubate at room temperature for 15 minutes in the presence of white light (LED light).



LED light system for fluorophore inactivation.

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

25.2 Repeat this process with fresh solution four more times.

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

26.1 After inactivating the fluorophores, slides are mounted in 50% 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

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

28 Add diluted antibodies to each section and incubate overnight at 4°C.

29 Remove antibody solution and wash sections with PBS for 5 minutes three times.

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

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

32 Mount slides in 50% glycerol in PBS.

33 Image

34 Store slides at 4°C in a moisture chamber.

35 Sections II-IV are repeated for each remaining cycle.

Quality Controls

- 36 Controls: When testing new antibodies, extra sections are used for controls to determine the autofluorescence and specificity of each antibody.
- 36.1 Negative control: Used to observe autofluorescence of the tissue. No primary or secondary antibody is added to the section.
- 36.2 Isotype control: Checks for any non-specific interactions of the primary antibody. A non-immune antibody of the same species and isotype (i.e. mouse IgG1) of the primary antibody being tested is incubated at the same concentration. A secondary antibody is added.
- 36.3 Positive control: Most antibodies used have targets that are found in kidney tissue in high amounts. For the immune cell markers used within this project, the antibodies are first tested in spleen tissue to confirm they work. They are then validated in kidney tissue.

Antibody Validation

- 37 All antibody validation is done on the same tissue block. When that block is no longer available, all antibodies must be validated on the new block.
- 38 Working dilutions are determined by the recommendations from the company. A series of dilutions are tested based on these recommendations.
- 38.1 If there are no references from the company, antibody dilutions range from 2-20 µg/mL depending on the concentration of the stock.
- 39 Directly conjugated antibodies are validated in the same steps as those that are unconjugated. A secondary antibody in a different fluorophore from the direct conjugate is added to confirm the conjugation.
- 39.1 The working dilution for directly conjugated antibodies is determined based on the specificity, intensity (secondary vs. direct conjugate), and signal: noise ratio.
- 39.2 Directly conjugated antibodies are tested for the ability to withstand one freeze/thaw cycle. This is completed by freezing a small aliquot of the conjugated antibody at -20C. This aliquot is thawed and stained on a section adjacent to a section stained with an aliquot of the antibody that has not been frozen.
- 39.3 All antibodies conjugated in the lab are validated via quantifying the moles of dye: moles of protein (acceptable range: 0.7-2). See Antibody Purification and Labeling Protocol for details.

- 40 Each antibody is evaluated and validated using other antibodies to determine the sensitivity and selectivity. They are then placed in specific groups based on the imaging results.

40.1 Enhanced (orthogonal): Co-labeling with a different antibody to the same antigen.

40.2 Approved: Different antigens, yet the cell expression is the same.

40.3 Supported: Localization is the right cell type, but there is not a second antibody for validation.

- 41 New lots of antibodies are validated by staining the old lot while simultaneously staining the new lot using a series of dilutions including the optimized dilution of the old lot.

Multiplexing Qc

- 42 Prior to multiplexing, each cycle is stained independently and imaged.

- 43 The antibodies are compared to when they were previously stained alone to ensure the specificity and intensity remains the same.

- 44 A practice run of MxIF must be successfully completed prior to starting an experiment.