1.2. Immunofluorescence staining on unfixed cryosections

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A. OBJECTIVE

This procedure can be used to define the localization of proteins in cryosections of hearts from animal models.

B. CAUTIONS

- The quality of sections cut from each sample should first be confirmed by hematoxylin & eosin staining to assure the absence of ice-crystal damage or other abnormalities resulting from technical problems with the section.
- Once sections have been cut and slides are stored in a -80° C freezer, slides can not be thawed and refrozen at any point prior to conducting experiments.
- Once the procedure has been started and the slides have been equilibrated in buffer, at no time during the procedure should the sections be allowed to dry out. Ensure that the sections are completely and evenly covered with solution during all incubation steps.
- Sections stained with primary antibody only and secondary antibody only are necessary controls for accurate interpretation of experiments.
- When troubleshooting new antibodies, the optimal dilutions and fixation should be determined empirically on positive and negative control samples, if available, prior to conducting the protocol on experimental samples and interpreting any data. Too high or low concentrations of antibody may lead to false positive or false negative results. If no negative control sample is available (i.e. a knockout mouse for the protein under investigation), results should be interpreted with caution, particularly for polyclonal antibodies that may cross-react with proteins homologous with the protein of interest.
- Great caution should be taken in using and interpreting mouse monoclonal antibodies on mouse tissues, particularly on dystrophic striated muscles. Cardiac tissue contains a large amount of endogenous mouse IgG to which the secondary antibody will bind. This amount of IgG is amplified in dystrophic heart due to the ongoing and prior myocardial damage. In fact, anti-mouse IgG staining can be used as an assay to detect myocyte damage in dystrophic hearts. Serum proteins such as IgG accumulate in myocytes with damaged membranes and also in areas of fibrosis. Although mouse-on-mouse kits may be useful for some antibodies with very high affinity, interpretation of data should be done very cautiously in dystrophic muscles to avoid false positives, particularly if no negative control for the antibody is available. The normal pattern of staining may be able to be confirmed on heart sections from another closely related species (rat or rabbit) to compare with the positive control mouse section after mouse-on-mouse blocking to avoid false negative results.
- All samples to be compared must be processed together and imaged using identical settings on a widefield epifluorescence or confocal microscope in order to correctly interpret data.

C. MATERIALS

- Primary antibodies (Before you start, double check to make sure the primary antibody is compatible with the species of the tissue of interest and the secondary antibody is compatible with primary antibody).
- Fluorophore conjugated secondary antibodies against the species of the primary antibodies
- Shaker or rocker
- Pipetmen
- Pipette tips
- microfuge
- Pipet lids, slide staining dishes, or coplin jars for washes
- Tupperware or pyrex dishes and saran wrap
- 1X PBS or KPBSG (recipes below)
- 1% gelatin in 1X PBS or KPBS (recipes below)
- Kimwipes
- vectashield
- DAPI stock solution: 1 mg/ml in dH_2O
- vortex
- Cover slips (24 x 50mm)
- Nail polish (optional)
- Slide boxes or folders

RECIPES:

Hartman's stock for KPBS (This is a 1M solution - adjust amounts if not using anhydrous salts)

143.2 g dibasic potassium phosphate -dissolve in 750 ml dH_2O at 45°
24.2 g monobasic potassium phosphate- dissolve in 150 ml dH_2O; then add dissolved dibasic to monobasic
dH20 to 1 liter

KPBS

20 ml Hartman's stock solution
9.35 g NaCl
dH2O to 1 liter

10% Gelatin Stock

- add approximately 30-40 ml of KPBS to 50 ml Falcon Tube
- Slowly pour 5g of gelatin (Bloom 75) so that it does not clump; mix
- incubate at 65° or microwave very carefully to dissolve

KPBSG

20 ml Hartman's stock solution
9.35 g NaCl
20 ml 10% gelatin in KPBS (Bloom 75)
dH2O to 1 liter

1% gelatin in KPBS

1 ml 10% gelatin stock
9 ml KPBS

D. METHODS

1. Keep your slides with 8 µm cryosections frozen at -80°C until you are ready to begin the antibody work.

2. Bring the slides to room temperature, label them with initials, date and experimental information, and circle each section with a hydrophobic "PAP" pen. Allow the hydrophobic circle to dry for a few minutes.

- *Alternative:* Sections can be postfixed using paraformaldehyde (4%, preferably EM grade) or 100% acetone. These postfix options may improve binding or the signal to noise ratio for some primary antibodies and should be tested when optimizing a new antibody. Post-fixing should be carried out for **5 - 10 minutes** at room temperature. Acetone fixing should occur prior to circling the sections on slides labeled with pencil, since it will dissolve the hydrophobic barrier and ink.

3. Immerse slides in containers already filled with a small amount of KPBS (PBS made with potassium phosphate instead of sodium phosphate -see recipe above) to just cover the samples. Leave the samples immersed for **5 min** at room temperature to equilibrate the tissues with the buffer.
(Glass slide staining dishes or p200 pipet tip lids, which hold 4 slides, can be used as containers for all buffer washing steps) (**DO NOT LET SLIDES DRY FROM THIS POINT FORWARD!**)

- *Alternative:* Either KPBS (potassium phosphate buffered saline) or PBS (sodium phosphate buffered saline) can be used as the buffer for all steps. In a direct comparison when optimizing this protocol, KPBS was found to give somewhat clearer staining than PBS, but either is acceptable for use with most antibodies.

4. Block slides with KPBS + 1% gelatin for **15 min** at room temperature by applying solution directly to the slides within the hydrophobic circles to completely cover the sections.

- *Alternative:* Using normal goat serum as a non-specific blocking agent instead of gelatin throughout all steps is an alternative, but gelatin is a cheaper option and can be used for all species of antibodies. The serum used as a non-specific blocking agent should be the species in which the secondary antibody was raised (i.e. goat for a goat-anti-rabbit IgG) and care should be taken to substitute correctly when using different species of antibodies (for instance a goat primary antibody).

5. While incubating slides in block, prepare the primary antibody solution. Dilute your primary antibody of choice in KPBSG + 1% normal goat serum.

- *Notes:* At least one no-primary and one no-secondary antibody control section should be included with each batch of stains. You can use as little as a total volume of 50 µl per section if you carefully apply the solution within each hydrophobic circle. Your dilutions will vary depending on the antibody used, but a rough estimate is 10 times the concentration used for a western blot {**not sure if you mean to times more concentrated or 10 times more diluted. Need clarification}**. A combination of primary antibodies can be used for co-localization experiments, but each antibody must be raised in a different species (i.e. rat and rabbit; rabbit and goat, etc.).

6. Rinse the slides for **5 min** by transferring to the pipet tip lids with KPBS + 0.2% gelatin (KPBSG) and shaking slowly on a rocker (**See Figure** 1). All washing steps should be done to allow continuous and gentle movement of buffer over the slides. Use of positively charged slides will prevent the sections from floating off the slides.

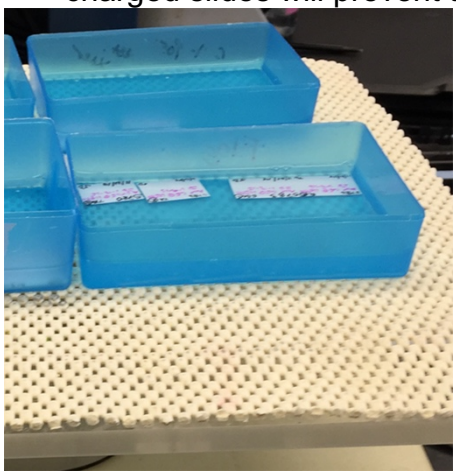

Figure 1.

7. Primary antibody binding. Remove the slides from the wash one at a time and wipe the bottom of the slide dry with a paper towel. Then, carefully tap the edge of the slide on the paper towel to remove most of the liquid from the front of the slide. Then, use the corner of a Kimwipe (which are lint-free - never use a tissue since lint will be introduced to your section), wick up the remaining liquid from the front of the section. Never touch the section directly, but try to remove as much of the excess buffer as possible to avoid dilution of the antibody solution.

Place the slide in a glass pyrex dish or tupperware container with a wet paper towels in the bottom of the dish (a moist chamber). Use a platform (such as the insert from a pipet tip box, pipet tip lids, or 5 ml pipets taped together) along the bottom of the dish to keep your slides off of the wet paper towels. Apply at least 50 µl of primary antibody solution (made in step 5) carefully with a p200 tip to each section encircled in hydrophobic marker (**See Figure 2**). Ensure that the tip does not touch the tissue section and the antibody covers the entire section and is evenly spread. Repeat for each slide. To prevent slides from drying out, only handle 1-4 slides at a time depending on your adeptness with the procedure and the humidity in the room. When all slides have been incubated with antibody, cover the dish with saran wrap to prevent the samples from drying and incubate for **2 hr** at room temperature.

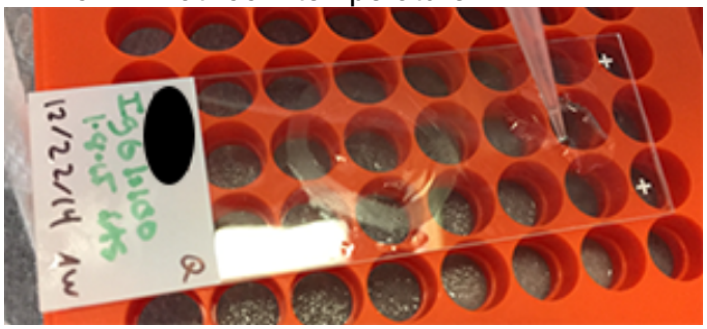

Figure 2.

8. Shake off the primary solution into the sink and rinse the slides **3 times for 5 min** in KPBSG in a container on a shaker. The buffer can be carefully poured out of plastic containers between each wash and the slides will remain in the container due to surface tension.

9. During the washes, prepare the fluorochrome-labeled secondary antibody solution. Dilute the 2° Ab in KPBSG + 1% normal goat serum.

- *Note*: The secondary antibody must be raised against the species of the primary antibody and must be fluorochrome labeled. Red and Green (555 and 488, respectfully) emitting Alexa or DyLight dyes are common fluorophore-labeled secondary antibodies. Ensure that the secondary antibody is reactive against the specific subtype of IgG of the primary antibody and that each secondary is labeled with a different color. Co-staining for co-localization of 2 or 3 proteins can be carried out for confocal imaging with 3 different fluorophore-labeled secondary antibodies against the 2 or 3 different species of primary antibodies. Anti-mouse IgG can be used in this step for co-localization of damaged myocardium in conjunction with localization of another protein. Care must be taken to carefully plan co-localization experiments to ensure secondary antibodies do not bind each other (i.e. goat-anti-rabbit and rabbit-anti-goat).

9. Secondary antibody binding. Repeat the process for removing excess buffer from slides as described in Step 7. Place the slides in the pyrex dish as described above and add just enough of your 2° Ab solution to cover each section. Cover the dish with saran wrap and incubate for **1 hr** at room temp.

10. Shake off the primary solution into the sink and rinse the slides **3 times for 5 min** in KPBSG in a container on a shaker.

11. Repeat the process for drying slides as described in Step 7.

12. Add ~35 µl of Vectashield mounting medium to your samples and carefully place a coverslip on the slide without introducing bubbles. The mounting medium will not harden and prevents rapid photobleaching of your fluorescent signal. For DAPI counterstaining: add 2µl of DAPI stock solution per ml of vectastain, vortex to mix well, and add mixture directly to slides. DAPI signal will appear immediately, but gains intensity overnight.

- *Alternative:* Prolong Gold + DAPI can be used for a permanent mount, but in direct comparisons has been observed to reduce signal for some antibodies. The cover slip on Vectashield mounted slides can also be sealed with nail polish to prevent sliding.

E. EVALUATION AND INTERPRETATION OF RESULTS

- View slides on an epifluorescence widefield microscope with the mercury lamp turned on and the correct barrier filter (green, red, blue, or triple-band) to confirm staining. Image all slides under identical conditions on widefield epifluorescence or confocal microscope on sections stained at the same time. Slides can be stored in a frost free -20°C freezer for over a year.
- If primary-only or secondary-only slides show a significant signal, then no data should be collected from the experimental samples and fixation and antibody dilutions should undergo further troubleshooting.
- If the negative control shows a signal, then no data should be collected and a more specific antibody should be identified before proceeding.

F. REFERENCES

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2. Heydemann A, Demonbreun A, Hadhazy M, Earley JU, McNally EM. (2007) Nuclear sequestration of delta-sarcoglycan disrupts the nuclear localization of lamin A/C and emerin in cardiomyocytes. *Hum Mol Genet*, 16:355-63.