PROVOST & WALLERT RESEARCH
Investigating the Biochemistry &
Cellular Physiology of NHE1
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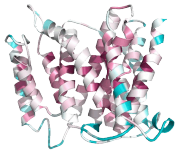
Immunoflourescence - Immuno-Stain

Protocol



Cover slip - Use #1 thickness cover glass, either 12 mm round cover slips (cat 1943-10012 – Belco through fisher) or fisherbrand 22x22 (cat 12-542-B).

IF the glass is not acid washed when purchased, clean glass for 4 hours to overnight by submersing cover slips in cleaning solution (fisher cat SC88-500). The solution can be reused several times, use a separate bottle to collect and hold all used material.

- ensure the cover slips are not stacked on one the other.
- use the Teflon cover slip rack for cleaning and washing.
- wash liberally with deionized water
- autoclave round cover slips in water in 100 ml beaker or in small beaker if slips are still on rack.
- autoclave square cover slips in Chex-all II sealing pouch (cat 024008). Keep flat.

Culture – Culture cells as according to experimental needs. Culture in 35 mm or 6 well plate. 1 square cover slip per well/dish 2 round cover slips per dish/well.

IMPORTANT POINTS

- Before starting and after fixing cells observe EACH cover slip. Look for intactness of cells. Focus on the shape and edges (margins) of the cells. Do they look dried out or hairy? If so than the cells are likely to have dried. If using round cover slips, identify the slip(s) to use for the rest of the labeling. Continue monitoring after digitonin/triton x-100.
- Cells, parafilm and media must all be at 37oC or the cells will round up!
- When rinsing, changing buffers or fixing cells, NEVER let the slip go dry. That is, only rinse or remove solution from 1 or 2 wells at a time. Even though moist, the cells will dehydrate and membranes become damaged. Carefully minimize the time the cells are out of a buffer/solution to seconds.
- "Incubation Chamber" For all incubations, cut a #1 Whatman filter paper to fit a 100 mm polystyrene dish, wet ½ of the filter paper with deionized water (the rest of the paper will become wet as the water wicks through the filter paper). Place on the bottom of the dish. Blot excess water from the plate with a kim wipe. Cover the moistened paper with a smaller sized square of parafilm. Mark the parafilm with a number to correspond with a cover slip. When incubating at 37^{o}C, seal the chamber with parafilm. When doing this, hold the chamber firmly against your body to avoid sudden movements if (when) the parafilm rips.
- Pay attention to the orientation. Know what side of the slip has the cells. This is vital when washing the cells after primary Ab incubation.
- See the end of the protocol for rules of selecting blocking serum and antibodies.

Processing Cells/Cover Slips ***(ALL media must be at 37^{o}C to work. Keep cells in incubator as much as possible and do not place cells on bench or the solutions will quickly get cold and cells will lift).***

DETAILED PROTOCOL

1) **Fix the cells** to the coverslips by adding 1.0 ml of freshly prepared 7.4% paraformaldehyde in PBS for 20 minutes at 37^{o}C.

2) **Wash the cells** 2 x 2 min. each with ~ 2 ml of warmed Na2 buffer in the dish. (***Prepare phaloidin while washing***)

3) **Permiabilize the cells** with 0.1% Triton x-100 (~1 ml at 37^{o}C) for 10 min.

3) **Wash the cells** 2 x 2 min. with ~ 2 ml of warmed PBS/0.1% BSA solution.

4) **Transfer the slips** to the incubation chamber. Use a fine tip forceps to pickup slips (protect tip by covering with 200 ul pipet tip when the forceps are not in use). Wick excess moisture with a small wedge of #1 Whatman filter paper. Quickly add 3 drops of blocking solution (5% normal serum/0.1% BSA/PBS) just about 100 ul. Incubate at RT for 30 min. Keep chamber covered when not manipulating slips.

5) **Primary Antibody** - Pick up each slip and remove blocking solution by wicking solution with filter paper. Only process one slip at a time. Use a Q-Tip placed onto a glass transfer pipet – aspirator to remove left over soln. from the parafilm. As soon as each slip is ready, incubate with (50 μl on the top of each coverslip) of the appropriate 1° Ab in 1% normal serum/BSA/PBS at 37°C for 1 hr. 1o Ab dilutions depend on which antibody you use. 1:1000 is not uncommon. Incubate in the sealed chamber.

6) **Wash the coverslip** 3 x, 10 min. each with PBS containing 0.1% BSA.

- Tape down parafilm flat on bench and number spots for coverslips. Make certain the parafilm is flat. Small bumps or "hills" will interfere with your ability to form a bubble.
- Create three rows of 250 ul of warmed PBS per cover slip.
- Wick the antibody soln from the coverslip using filter paper or a Q-Tip covered glass transfer pipet – aspirator (use the Q-Tip to hold the slip when grasping the coverslip with forceps).
- Carefully drop (float) the coverslip on top, cell side down, onto the bubble of PBS. Keep the cover slip from sinking. If the slip insists on sinking, turn the slip upside right (cell side up) at the bottom of the bubble.
- When removing the cover slip, do so with a motion drawing back the cell such that the surface tension of the bubble pulls the liquid from the cover slip.
- Replace the slip directly onto the next wash bubble without wicking residual soln.
- *See the immunolabeling youtube video for a demonstration of this technique.*

7) **Secondary Antibody** - Remove the slip from the last BSA/PBS bubble and place cell side up in the appropriate spot in the incubation chamber. Incubate cells in 2° Ab for 1 hr at 37°C. The 2° Ab is prepared in 0.1% BSA/PBS. Use dye conjugated anti-mouse antibody if possible for specificity, diluted 1:2000 in 0.1% BSA in PBS. Re-moisten the filter paper at the bottom of the incubation chamber if needed.

8) **Wash each slip** 3 X, 10 min. each with PBS containing 0.1% BSA as described in step 6.

9) **Mount the coverslip** Transfer one or two drops of ProLong Gold antifade reagent (Invitrogen P36934) mounting agent to a small (enough for 12 coverslips) into a small microfuge tube. Warm the tube to above room temp. Centrifuge the tube at 12,000 x g for five minutes to remove bubbles.

- Transfer a small amount of the media to the slide, just about the size of the circle shown to the right -> "O". This is typically 3 to 5 ul. This can be accomplished by dipping a 20 μl pipet tip into the antifade and letting the media drop onto the slide.
- Place the slip cell side down, in a forward motion with the front edge of the slip dipping into the middle of the mounting medium. Wick excess (the liquid flowing out from under the cover slips and spilling one the sides of the cover slip) using a think pie shaped piece of filter paper. Let the water wick into the tip of the paper, don't push the slip around. This will cause sheering of the cells.
  - ALTERNATIVELY the soln can be aspirated if care is used not to remove too much of the mounting medium or pushing the coverslip.
- If using Prolong antifade, allow the solution to cure overnight. Do not use nail polish until after one day.

10) **Clean the coverslip** after curing/drying the sealing mount. Do this by applying a moistened kimwipe. Use just the corner of a bent kimwipe. Do not rub, but instead, allow the weight of the kimwipe to apply the pressure. Simply remove salts in a circular motion with the tip of a bent or folded kimwipe.

Solutions:

7.4 % paraformaldehyde solution:

- Make a 10% paraformaldehyde solution, dissolve 1.0 g pararaformaldehyde with 10 mL Millipore water in a 50 ml beaker with a stir bar. Warm the solution and add 1 M NaOH dropwise to dissolve and clear the soln. Adjust
- Place 1.0 mL 10X PBS in a fresh falcon tube, add 7.4 mL of the 10% paraformaldehyde solution to the tube. Check pH and adjust to 7.2. THEN QS to 10 mL using Millipore water.

PBS-BSA blocking solution 0.1% BSA (Fatty acid free bovine serum albumin) - 100 mg BSA in 100 ml 1X PBS

Digitonin (Alternative Solubilizing Solution**)** (F.W. 1229.3) (Sigma cat #:D-1407) Store at -4oC. Re-freeze unused samples. May need to test different lots of digitonin for minimal conc needed to permiablilze cells – use ERK Ab. May need to heat soln to 100oC for about five min. to get compound into solution. This may need to be repeated after thawing samples.

- Digitonin stock = 50 mg digitonin (Sigma cat #: D-1407)/ml dH_2O.
- To make the stock, digitonin is added to the water, heated in a heating block to 100°C, cooled to RT and filtered with a syringe filter (0.22 mm).
- Working solution – 6.2 µl / 10 ml PBS

0.1% BSA (Fatty acid free bovine serum albumin) - 100 mg BSA in 100 ml 1X PBS

5% Normal Serum (normal goat or donkey serum) – 100 µl normal serum + 1.9 ml 0.1% BSA in PBS.

1% Normal Serum – 500 µl of 5% normal serum diluted with 2 ml of 0.1% BSA in PBS.

Na2 Buffer

1L water	0.0294g $CaCl_2$ (1mM) (dessicator)	9.532g HEPES (20mM)
16.948g NaCl (145mM)	1.802g Glucose (5mM)	pH to 7.58 with 1M NaOH
0.448g KCl (3mM)	0.406g $MgCl_2$ (1mM)	Dilute to 2L

NOTES (from Jacksonimmuno.com)

Blocking with Normal Serum - As a general rule, it is most effective to block tissue or cells with 5% normal serum from the same host species as the labeled secondary or tertiary antibody. The IgG in serum should occupy sticky sites on the tissue or cells to prevent non-specific binding of the labeled IgG antibody. Never block the tissue or cells with normal serum from the same host species as the primary antibody. For example, if a primary antibody is made in mouse and normal mouse serum were used for blocking, the mouse IgG would bind to the sticky sites and be recognized by labeled anti-mouse IgG. A higher background would result.

Affinity-Purified Antibodies - Affinity-purified antibodies are isolated from antisera by immunoaffinity chromatography using antigens coupled to agarose beads. A proprietary elution process is used to dissociate antibodies from the antigen. Unconjugated affinity-purified antibodies are supplied sterile filtered in phosphate buffer without stabilizers or preservatives. Conjugated affinity-purified antibodies are freeze-dried in phosphate buffer with stabilizers and sodium azide, with the exception of horseradish peroxidase conjugates, which do not contain a preservative. **Caution:** Never block with normal serum or IgG from the host species of the primary antibody when using a labeled secondary antibody. If immunoglobulins in the normal serum bind to the specimen of interest, they will be recognized by the labeled secondary antibody, resulting in higher background.Bovine serum albumin (BSA) and dry milk, both commonly used for blocking, may contain bovine IgG. With the exception of Bovine anti-Goat IgG, many secondary antibodies such as anti-bovine, anti-goat, and anti-sheep will react strongly with bovine IgG. Therefore, use of BSA or dry milk for blocking or diluting these antibodies may significantly increase background and/or reduce antibody titer. For blocking, use normal serum (5% v/v) from the host species of the secondary antibody.

***Select the host species of the secondary antibody*. -** Selection of the host species for a secondary antibody involves many considerations, including but not limited to: 1) Antibodies from some host species may not be adsorbed against cross-reacting species of interest. Chose a host species with the required adsorptions. 2) Host species compatibility. Some host species may not be compatible with other species in multiple-labeling experiments. In general, all secondary antibodies should come from the same host species for multiple labeling. 3) Binding to Protein A and Protein G. Rabbit antibodies bind well to Protein A and G, but goat and donkey antibodies bind better to Protein G. 4) Personal preference or experience. In our experience there appears to be no species specific difference in the quality of secondary antibodies.

Storage:

1° Unconjugated antibodies - Store at 2-8°C under sterile conditions. Prepare working dilution fresh each day.

2° Conjugated with Fluorophores - Store freeze-dried powder at 2-8°C. When ready to use, rehydrate with indicated volume of d. water and centrifuge if not clear. Product is stable for about 6 weeks at 2-8°C as an undiluted liquid. Prepare working dilution fresh each day. For extended storage after rehydration, add an equal volume of glycerol (ACS grade or better) for a final concentration of 50%, and store at -20°C as a liquid. Note: after the addition of glycerol, the concentration of protein and buffer salts is one-half of the original. Alternatively, aliquot and freeze the product at -70°C or below in the absence of glycerol. Avoid repeated freezing and thawing. ***Expiration date:*** one year from date of rehydration. However, the expiration date may be extended if the product is stored according to the recommendation and the test results are acceptable for its intended use.