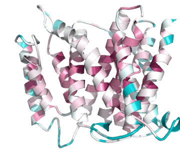
PROVOST & WALLERT RESEARCH

Investigating the Biochemistry & Cellular Physiology of NHE1

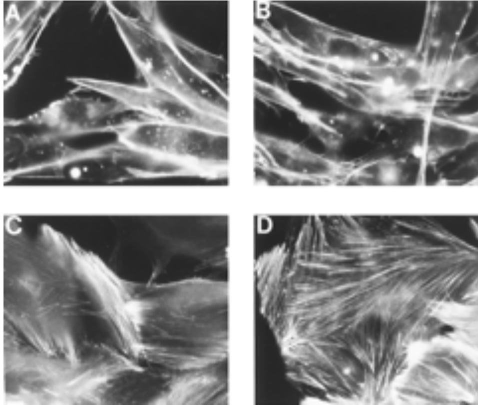
EST. 1998

Stress Fiber Assay
Protocol



INTRODUCTION

Stress fibers are a specific cytoskeletal organization of actin monomers. These fibers are involved in cell shape a structural functions of the cell. Actin monomers form long polymers, which attach to the plasma membrane at focal adhesions. Contraction of the actin stress fibers allows the cell to exert tension on the substratum, an important part of controlling morphogenesis. Formation of stress fibers and focal adhesion complexes are a key regulatory event in cell growth and cell movement such as migration and invasion.



When conducting the experiments it is crucial that you think about what you are planning to do! Not just the technical aspects of the experiments but what KIND of data are you going to get. If you are just looking for a picture of stress fibers vs actually counting the number of cells that are forming the fibers it will be very important spend the time getting the right kind of pictures. Just getting a picture does you no good if you also need to determine the percent of cells that are actually displaying strong organized fibers. Take several pictures for each slide/condition. Make certain they are centered in the screen. Use the example to the left to make that decision. When counting you need to count several fields from each slide.

For EACH field, count 1) the total number of cells; 2) the number of cells that display stress fibers and if necessary, 3) the number of cells that are showing weaker formation. The figure above has a control in A, weak formation in C and stronger stress fiber formation in D. Look at the following papers for examples:

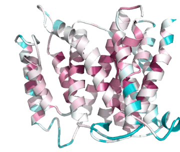
- Kam and Exton, Mol Cell Biol. 2001 Jun; 21(12): 4055-66
- Tominaga T, Ishizaki T, Narumiya S, Barber DL. EMBO J. 1998 Aug 17; 17(16): 4712-22

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).

Culture – Culture cells as according to experimental needs. Experiments looking at stress fibers - seed at a low density 20% (depending on cell type). Culture in 35 mm dish or 6 well plate (1 square cover slip per well/dish 2 round cover slips per dish/well).

IMPORTANT POINTS

- Before starting and after each step of the assay observe EACH cover slip. Monitor the cover slip for the entire experiment. 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.
- Cells, parafilm and media must all be at 37°C 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***.
- Pay attention to the orientation. Know what side of the slip has the cells. This is vital when washing the cells.
- **When processing cells/cover slips** ***ALL media must be at 37°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.***



SOLUTIONS

Prepare and warm in advance

1. **7.4 % paraformaldehyde solution**:

    a. 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 the paraformaldehyde. Continue until solution is clear.
    b. 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.

2. **1X PBS** (200 ml; pH 7.3)

    a. Dilute 20 ml of 10X PBS to 200 ml with dH_2O. Check the pH. It should be **close to 7.3.**

3. **1X PBS/ 0.1% BSA Blocking Solution**
    a. 100 mg BSA (fatty acid free bovine serum albumin) in 100 ml 1X PBS.

4. **Phaloidin-Dye Preparation**:
    a. ***IF using phaloidin from Sigma.***
        i. Stock soln = 0.1 mg of Sigma FITC phaloidin dissolved into 417 µl DMSO.
        ii. Dilute 1:200 for use. Calculations above are for Invitrogen Phaloidin

    b. *IF using invitrogen's phaloidin*:
        i. Dissolve toxin in 1.5 ml methanol – should be 6.6 µM
        ii. Aliquot 30 µl into microfuge tubes.
        iii. Cap each tube and label with phaloidin-488 (or whatever dye is conjugated to the toxin).
        iv. ***To use, during step 3 (see below) dissolve in 30 µl into 1.2 ml PBS and vortex. Incubate for 30 min. Use 100 µl per slide. This should be enough for 12 coverslips.***

PREPARING THE INCUBATION CHAMBER

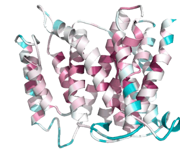
1. Cut a #1 Whatman filter paper to fit a 100 mm polystyrene dish and place on the bottom of the dish.

2. Drop some deionized water onto the middle of the filter paper (the rest of the paper will become wet as the water wicks through the filter paper).

3. Blot excess water from the plate with a kimwipe.

4. Cover the moistened paper with a smaller sized square of parafilm.

5. Mark the parafilm with a number to correspond with a cover slip.



PROTOCOL

1. **(Optional Step)** Transfer 1.0 ml of media from the dish with the coverslip into a new 35 mm dish, THEN transfer the coverslips to the new dish. This to help focus on the cells on top of the coverslip and not those cells on the dish underneath the coverslip.

2. **Fix the cells** to the coverslips by adding 1.0 ml of freshly prepared 7.4% paraformaldehyde in PBS for 20 minutes at 37°C.

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

4. **Permiabilize the cells** with ~1 ml at 37°C of 0.1% Triton x-100 for 10 min.

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

6. **Phalloidin Staining** – Place the coverslips onto a sheet of parafilm in the incubation chamber cell side up. Carefully pipette 100 µl of Invitrogen-Phaloidin onto each coverslip and incubate at 37°C for 40 min.

7. **Rinse the cells** 3 x 10 min. with 37°C PBS. This can be done in the dish or using the Parafilm-Drop Technique.

    a. Parafilm Drop Technique - 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 soln from the coverslip using filter paper. 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.*

8. **Mount the cover slip**. Warm the tube of ProLong Gold Antifade Reagent (Invitrogen P36934) to above room temp. Transfer one or two drops ProLong Gold antifade reagent (enough for 12 coverslips) into a small microfuge tube. Centrifuge the tube at 12,000 x g for five minutes to remove bubbles.
    a. Transfer a small amount of the antifade reagent to a microslide, just about the size of the circle shown to the right -> "o". This is typically 3 to 5 µl. This can be accomplished by dipping a 20 µl pipet tip into the antifade and letting the media drop onto the slide.
    b. 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 to prevent bubbles from forming.
    c. 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.
        i. ALTERNATIVELY the soln can be aspirated if care is used not to remove too much of the mounting medium or pushing the coverslip.
    d. Allow the solution to cure overnight in the dark. (Do not use nail polish to seal the coverslip!)

9. **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.