



Tech Report 202:

Immunofluorescent Techniques for Preparation of Cells Grown on Flexible Substrates

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Culturing Cells in a Mechanically Active Environment[™]
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INTRODUCTION

Immunofluorescent techniques are useful for visualizing proteins and other cellular structures in cells grown on flexible substrates such as in BioFlex® and Flex I® culture plates.

Cells are best used at near confluence, but not at super confluence, to give the best visualization. If you are using scanning confocal microscopy, the confluency level is less important. If you are using conventional fluorescence microscopy, then super confluent cells will give overlapping images which may make observation of a specific protein (i.e., cytoskeletal filament system) particularly difficult.

***NOTE:** The silicone rubber membranes have an autofluorescence when excited at 540-560 nm (in the red range) and emission collected at 580-600 nm. Therefore, a fluorescein tagged secondary antibody may be better for visualization than a rhodamine tagged secondary antibody.*

METHODS

1. Aspirate medium from the cells. Wash the cells carefully with warm culture media without serum, pH 7.2. Apply the wash medium down the sidewall of a well to avoid a direct fluid stream on the cells.
2. Fix the cells for 20 minutes in 3.7 % formaldehyde or paraformaldehyde in phosphate buffered saline (PBS), pH 7.2, or a fixative of your choice.
3. If you are studying an intracellular protein, permeabilize the cells with 0.1% Nonidet-P40 or 0.1% Triton X-100 in PBS for 5 minutes. Permeabilization can cause cell sheet lift-off from the rubber

membrane. If this occurs, reduce the concentration of detergent to match the adherence state of your cells.

4. Rinse gently three times with PBS for 5 minutes per wash.
5. Rinse once with 1% bovine serum albumin containing PBS for 5 minutes.
6. Dilute the primary antibody in PBS with 0.5% BSA. The primary antibody dilution should be from 1:50 to 1:250. A dilution of 1:100 is common. This condition must be worked out for each primary antibody used. It is recommended that a concentration curve be performed on test cells before your experiment is conducted to work out the appropriate antibody conditions.
7. Sample handling in flexible bottom culture dishes:
 - a. **Flex I® Culture Dish.** If you are working with a Flex I® culture dish substratum, then you may carefully lift out the entire rubber membrane by gently pushing up from below around the edge of the membrane until it is free from the plastic well. At this point, place the membrane inside a well of a 6-well culture dish or of a 100 mm diameter culture dish. You may trim the rubber sidewall material away from the perimeter of the well. If you are planning to use several different samples or antibody treatments, you may cut the Flex I® membrane into pie-shaped segments and treat each separately. One may be used for a second antibody only control.
 - b. **BioFlex® Culture Dish.** If you are using the BioFlex® culture plate, fix



- and stain target proteins with specific antibodies. After immunostaining, you can cut the membrane out of the plate using a sharp sterile blade, then mount the membrane on a glass slide and observe under a microscope.
8. From this point in the technique forward, you may select your method of antibody addition to the cells depending on the amount of antibody you have and the number of different antibodies you intend to use on any one flexible membrane.
 9. Flex I® dishes whose bottoms have been cut into pie-shaped wedges can be handled in the following way:
 - a. Place a disc of Parafilm or other hydrophobic film in a 100 mm diameter culture dish. Place a small piece of sponge soaked with water in the culture dish to prevent drying of your samples. Mark the sectors of the film as to the position of the membrane samples you will treat. Place 10-20 µl of first antibody dilution on the Parafilm at a specific location. Place your wedge of rubber membrane with cells facing toward the first antibody solution. Alternatively, you may place the antibody solution directly on the cells on the wedge of rubber membrane. You may then place a piece of Parafilm onto the cells or not as you choose. Incubate your specimens for 1 hour or more depending on the strength of reaction and your knowledge of your specific antibody. One hour to several hours for reaction of first antibody with your cells is usually sufficient for reaction.
 - b. Carefully, return your rubber membrane to the cells up position and wash the cells three times for 5 minutes each wash in 0.5 % BSA in PBS to remove excess, unreacted antibody.
 - c. Dilute the second antibody 1:100 to 1:500 depending on your knowledge of the antibody reaction with its substrate. Apply the second antibody under the same conditions as for the first antibody.
 - d. Rinse three times for 5 minutes each in 0.5 % BSA in PBS.
 10. BioFlex® Culture dishes whose well tops have been cut away to allow for better working room. can be treated in the following way:

You may apply antibody to the entire surface of the well if you are not antibody limited. If you would like to add several different antibodies to a single well to perform several different tests on one well, you may segregate sections of the membrane by adding discs with antibody applied in a small drop to a given section of the membrane. Cut a small piece of Parafilm, nylon mesh or use an electron microscope grid to contain a small drop, 10 µl, of first antibody solution on the cells. React the antibody solutions as above for the Flex I® samples and wash.
 11. A small glass coverslip with either PBS or a mounting medium of your choice that has low fluorescence quality may be used.
 12. Observe samples with the excitation and emission filters set for the given fluorescence.
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