Biology 446-1
Laboratory Exercise 9
Immunofluorescence

Objective: To gain experience in the carrying out the procedures of immunofluorescence and in labeling and visualizing DNA and some proteins to determine their subcellular localization by immunofluorescence.

Background

While some proteins are distributed ubiquitously in the cell, many others have localization signal(s) to target to specific subcellular compartments. Proteins can also localize to specific subcellular compartments or structures by interacting with other proteins. Often their functions are dictated by where they are or in what complex they are. In fact, a great number of proteins exert their functions only when they are in the right place at the right time. An example of this is the storage of a relatively large amount of splicing factors in specific nuclear bodies where active splicing is not taken place. Thus, mapping the distribution of a protein can often provide information about its functional properties.

One way of detecting the localization of a protein of interest is to tag it with a fluorescent protein. Fluorescent proteins have been widely used in recent years and many vectors for constructing fluorescent protein tags are commercially available. (We used GFP in lab 8 for the transfection exercise). Most of the fluorescent protein variants do not localize to a specific place in the cell and their toxicity is not an issue in most application. However, fluorescent proteins can not be used to detect endogenous proteins. Since vector borne fluorescent protein-tagged proteins are almost always over expressed and the expression level of a protein often dictates its function, immunofluorescence can not be replaced by fluorescent protein tagging and it is still the most desirable way for mapping protein distributions in the cell.

To use the method of immunofluorescence, one needs to have a good antibody against the protein(s) of interest. These antibody directed to the proteins of interest are called primary antibodies. One can conjugate fluorescent dyes to the primary antibodies and detect the fluorescence signal directly from the fluorescence labeled primary antibody. However, it will save time and resources if a secondary antibody against the invariable region of the primary antibody is conjugate with the fluorescent dyes, This is because the secondary antibody can be used in many experiments regardless of what is the protein of interest, as long as the primary antibody is raised in the same source. The secondary antibody can also be conjugated to biotin if fluorescent dye conjugated biotin interacting molecules, such as streptavidin, are to be used.

The immunofluorescence procedure includes growing cells on a cover slip, fixing and permeablizing the cells, incubate the cells with antibodies and visualize the signals using a fluorescence microscope. Over many years the sensitivity of image capturing has been greatly improved and fluorescence microscopy has been developed as the leading method for mapping the localization of different biomolecules in cells and tissues. For this lab exercise, We will determine the distribution of β-tubulin and focal adhesion kinase. We will also use DAPI to stain

DNA so that we will be able to "see" the cell nucleus. We will use a Nikon TE2000 U fluorescence microscope to visualize the fluorescence signals.

Protocol

Lab Day 1: grow cell on cover slip.

HeLa cells will be grown by the instructor well before the first lab day.

1. Sterilize the biosafty hood and put three 35 mm tissue culture plate in the hood.
2. Place a cover slip in each of the plate and leave the plate open with the cover slip exposed and sterilize the cover slips for 5 minute by UV. (This step is not required if the cover slip are already sterile.)
3. Warm up the DMEM media, trypsin solution, and the wash solution in a water bath equilibrated at 37°C. Wash the outside of the cap and the bottle with 70% EtOH before putting them in a biosafty hood. **DO NOT HEAT TRYPSIN and NEVER OPEN A BOTTLE OUTSIDE THE HOOD.**
4. Take a preexisting culture growing in a 100 mm culture plate to the hood and use an aspirator to aspirate the old media from the plate.
5. Add 4 ml HBSS without Ca^{+2} and Mg^{+2} and aspirate the HBSS after briefly swirling the plate.
6. Add 3 ml (or 0.2 for a 35 mm plate) Trypsin-EDTA solution and wait for ~2 minutes for the cell to detach. You can gently tap the side of the plate to help the cells to detach. Do not trypsinize the cells for too long, since this would lyse the cells. Check the cells occasionally and stop the trypsinization as soon as the cells detach.
7. While the cells the being trypsinized, add 1.8 ml of DMEM to each of the three 35 mm plate containing a cover slip.
8. Pipette up-and-down the trypsinizing cells 2-3 times to separate clumps of cells.
9. (If the cell is close to confluent before trypsinization, plate the cells in 1:4 dilution. This means that you will have enough cells to plate four 100 mm plates (0.85 ml detached cells for a plate). The surface area of a 100 mm plate is about 8 times that of a 35 mm plate. Hence 125 μl of the cells is enough for a 35 mm plate at 1:4 dilution. That is true if we are going to use the cells 24 hours later. Since the next lab day is at least three days later, we will make a series of dilutions.)
10. Add 125, 40, and 10 μl of the detached cell suspension to your first, second, and third plate, respectively.
11. Incubate the 35 mm plates with HeLa cells in a CO_2 incubator.

Lab Day 2: fluorescence labeling:

1. Exam all three of your plates under the inverted microscope and choose the best plate to perform the immunofluorescence exercise. Choose a plate with a big number of cells but not more than 70% confluent. Transfer the cover slip from the tissue culture dish onto the humid chamber.
2. Wash twice with PBS then fix in 3.5% paraformaldehyde for 7 minutes.
3. Aspirate paraformaldehyde and incubate with 300 μl KBX for 5 minutes.

4. Aspirate off KBX and wash three times with KB and incubate in the last KB wash for 5 minutes.
5. Dilute 1 μl of each of the two primary/1° antibodies in a single microcentrifuge tube containing 300 μl KB. Aspirate off the KB wash from the cover glass and incubate with the 1° antibody solution for 30-45 minutes. For this exercise, the 1° antibodies are rabbit anti human β-tubulin and mouse anti human annexin II.
6. Aspirate off 1° antibody and wash with and incubate in KBX for 5 minutes.
7. Wash twice with KB. Then Incubate in KB for 5 minutes
8. Dilute the secondary/2° antibodies in 300 μl KB. Aspirate off the KB wash from the cover glass and incubate with 2° Antibody for 45 minutes. For this exercise, the 2° antibodies are biotin conjugated Donkey Anti-mouse IgG (use 1 μl) and FITC conjugated Goat Anti-Rabbit IgG (use 1 μl).
9. Aspirate off 2° Antibody and wash 3 times with KB with 5 minutes incubation in the last KB wash.
10. Dilute 1 μl of rhodamine Red conjugated streptavidin and 1 μl of DAPI in 300 μl KB. Aspirate the KB wash from the cover glass and incubate it in the streptavidin and DAPI solution for 15 minutes.
11. Aspirate off residue and incubate 5 minutes in KB.
12. Mount the cover slip onto a slide with 10 μl mounting medium. Seal the cover slip with nail polish. Let the slide sit in a dark place for a while to dry the nail polish.
13. Store the slide in a refrigerator until next lab day.

Lab Day 3: Visualization:

Analyze the slide using the fluorescence microscope. You will take snapshots of the slides with different filters.

Report: You will need to merge the images with different colors in one picture.

10X KB:

0.1 M Tris pH 7.5
1.5 M NaCl
1.0% BSA
Store at 4 °C

KBX:

KB plus 0.2% Triton X-100

To make 3.5% paraformaldehyde solution (by the TA):

Weigh 3.5 gram of paraformaldehyde and add 90 ml of H_2O
Add 200 μl of 2N NaOH and dissolve with slight heat.
Add 10 ml of 10 X PBS.
pH to 6.6-6.9.