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BIOL 4140 Fall 2020

Protocol

Fixing and Staining of Cells for Fluorescence

In this lab you will have the opportunity to visualize fluorescently labeled cellular structures and organelles.

** BE AWARE: Paraformaldehyde is a hazardous chemical! Wear gloves and use caution when working with.**

Materials

Nonsterile

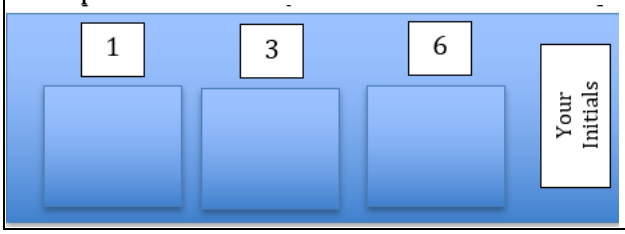
Item	**Amount**	**What it's for**
6-well plate of cells on glass coverslips, in D10	1 plate	Your cells. They should cover 30 to 50% of the glass and still be growing
Micro-tip forceps (or 2 pipette tips)	1	Picking up coverslips
1X PBS	40 mL	Rinsing between every step
4% formaldehyde in PBS	6 mL (1 mL per well)	Fixing the cells so they hold their shape
0.1% Triton X-100 in PBS	6 mL (1 mL per well)	Opening holes in the membrane so the stain can get in
2% Texas-Red phalloidin in PBS	40 µL per coverslip	Staining actin red. **Ask the instructor for it when you're ready**
DAPI mounting stain	250 µL total (40 µL per slide)	Staining nuclei blue and sticking the coverslip to the slide
Glass microscope slide	1 or more	Mounting the coverslips
EVOS microscope	Shared	Imaging in phase contrast, red, and blue
Pipettes (P1000 and P200)	As needed	Adding and removing liquid

Used in the steps but missing from this list:

Item	**What it's for**
Parafilm	Holding the 40 µL stain drops
Foil	Keeping light off the stain during the 20 min incubation
Kimwipes	Blotting extra liquid off the slide
"Used liquids" container	Collecting waste liquid

Procedure

Step	Original (word for word)	What to do
1. Look first	**1:** View your cells in each well and take notes on what you observe. You may only choose to fix and stain wells that have surviving cells attached, but make note or take images using phase contrast of the cells prior to fixing.	Check all 6 wells under the scope and take phase-contrast pictures. Skip any well with no cells stuck to the glass.
2. Rinse	**2:** Using a **P1000 pipet**, remove the solution from each well of the 6-well plate. Collect the discarded media in a "used liquids" container. **3:** Carefully add **1ml of 1xPBS to each well**. Use a P1000 pipet to deliver the media to the side of the well. Be gentle with the cells so that they will not come off of the plate	Remove the liquid from each well (put it in the used liquids container) and add 1 mL of PBS to the side of the well.
3. Fix, 15 min	**4:** Remove the PBS and then **add 1mL of the fixation solution (PBS + 4% formaldehyde)** to each well. Incubate the platen, with the fixation solution at room temperature, at your bench, for 15 minutes.	Remove the PBS, add 1 mL of formaldehyde, and wait 15 min.
4. Rinse	**5:** Remove the fixative solution and rinse each well with **1ml of 1xPBS.**	Remove the formaldehyde and add 1 mL of PBS.
5. Open the cells, 15 min	**6: Remove the PBS** and then add **1mL** of permeabilization solution **(0.1% Triton X100 in PBS**). Incubate in the permeabilization solution for 15 minutes at room temp, at your bench.	Remove the PBS, add 1 mL of Triton, and wait 15 min.
6. Rinse, then stop	**7:** Remove the permeabilization solution and then add 1ml of 1xPBS. Pause here and call me over.	Remove the Triton, add 1 mL of PBS, and **call the instructor**.
7. Stain the actin, 20 min	**8:** In this step you will make drops of your staining solution on Parafilm. The staining solution contains 2% Texas-Red Phalloidin in 1xPBS. Pipette one 40µl drop of staining solution for each coverslip you wish to stain. Using a sharp forceps remove the coverslips from the plate and place them, cell-side down, on top of the drop of stain solution. Never drop the slide down, start with the cover slip at an angle and slowly lay it down so that you do not add bubbles. **9:** Incubate your coverslips in the stain solution, on your bench, at room temp, for 20 minutes. Cover with foil to protect the fluorophore from the light.	Put one 40 µL drop of red stain on Parafilm for each coverslip. Pick up each coverslip with forceps and lay it on its drop **cells down**, lowering it slowly at an angle so no bubbles form. Cover with foil and wait 20 min.

Step	Original (word for word)	What to do
8. Wash off extra stain	**10:** After this incubation, place the coverslip back into the multiwall plate, cell-side up, in the 1xPBS solution. **11:** Remove the PBS and rinse with 1ml fresh 1xPBS three times to remove any unbound stain. **12:** Leave the cells in PBS until ready to mount onto slides.	Put the coverslips back in the plate **cells up**, rinse 3 times with 1 mL of PBS, and leave them in PBS until you mount.
9. Mount	**13:** Place 40µl of DAPI-mounting stain onto the center of your glass microscope slide. Using your forceps, remove the coverslip from the 6-well plate and invert, cell-side down, onto the drop of mounting stain on the glass slide. Press gently to remove bubbles and spread stain. *You can fit as many coverslips on the slide as you can. Below is an example: 	Put a 40 µL drop of DAPI on a glass slide and lay the coverslip on it **cells down**. Press gently to push out bubbles and write your initials on the slide.
10. Clean up the slide	**14:** Wait 5 minutes and dab with a small kimwipe to remove excess solution. If cystals form on the top of your coverslips, dampen a kimwipe with water from the DI water tap and gently drop this water onto the coverslip.	Wait 5 min, then blot the extra liquid with a Kimwipe. If crystals form, dab them with a little DI water.
11. Image fast	**15:** Image under EVOS microscope (phase-contrast, texas-red and DAPI). Photograph and note observations. Everyone will be waiting to use the EVOS, so unfortunately, you cannot spend a lot of time looking. Get an image in focus quickly and snap your pictures.	On the EVOS, take a picture of each coverslip in phase contrast, red (actin), and blue (nuclei). Focus quickly, snap, and move on.

Experiment Template-6well cluster

Table 1: Analysis of freezing conditions by fluorescence microscopy of actin filaments.

	Never Frozen	Frozen & Thawed	Frozen & Thawed
D10	Subcultured in D10	Slow cool in D10	Fast cool in D10

FM (10%DMS0)	Subcultured with 10%DMSO	Slow cool in FM	Fast cool in FM

Pointers:

- It is particularly important that you use caution with the chemical agents used today. The fixatives and stains all have the potential to cause health related illness. Use gloves. Recap solutions and cover petri dish immediately. Spills or exposures should be cleaned immediately and reported to Dr. Johnson or Madalyn.
- Be gentle with your cells. Remember when washing and applying stains to pipette gently and onto the side of the dish before tilting to wash over cells, not directly onto the cells.
- Do not leave cells exposed without a solution. They will dehydrate and ruin your results.
- High quality images are important. Please be sure your images are in focus, etc.

Thought Questions:

- Predict the appearance of the actin cytoskeleton in wells 1, 3, and 6.
- Other than imaging on a fluorescent microscope, what other tools could be used to evaluate cellular fluorescence? To quantify it? To isolate cells that fluoresce?
- How could antibodies be used with fluorophores to highlight specific cell structures?
- What are potential applications of this procedure?