Chapter 1

Fluorescence Microscopy as an Introduction to Cell Biology

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Introduction

The canonical animal and plant cell illustrations found in all biology textbooks form the basis upon which students envision cells. However, these simplified drawings rarely give the student an appreciation for the relative size, shape, and distribution of organelles within the cell. We use this lab to introduce students to the cell and to one of the basic tools of cell biology -- the fluorescence microscope. The fluorescent stains reveal structures within the cell dramatically, capturing the attention of the students. In addition, the availability of different colored fluorochromes allows the student to stain multiple structures simultaneously, and observe the distribution of structures relative to each other in the same cell. By including DNA stains, the students can also describe changes in the organization of organelles and the cytoskeleton as the cells undergo mitosis. Finally, we have the students examine transgenic tobacco hair cells expressing a modified green fluorescent protein (GFP) that has been targeted to the mitochondria. This part of the exercise demonstrates the use of GFP as a non-invasive probe for structures in living cells. While we use this lab for biology majors in their first biology course, it is also appropriate as an introductory lab for an upper-level cell biology course. In a follow up exercise, we treat cells with cytoskeletal inhibitors to reveal relationships between organization of the organelles and the cytoskeleton.

Materials

Materials for each pair of students:

- Bench liner (1)
- Fluorescence microscope (1)
- 100-mL plastic "PBS dipping" beakers (1-2)
- 1-L plastic "WASTE" beaker, lined with a small autoclave bag (1)
- Microscope slides (1 box; need 6-10 slides per day)
- 22-mm square cover slips (1 box; need 2-4 cover slips per day)
- Parafilm (1 box)
- Kimwipes (1 box)
- Nail Polish (1 bottle)
- Aluminum foil covered boxes (4) - we use old tip lid boxes
- P1000 micropipetter (1)
- P200 micropipetter (1)
- Yellow (P200/P20) tips (1 box)
- Blue (P1000) tips (1 box)
- Microfuge tube rack (1)
- Fine (#7S) forceps (2)
- Razor (1)
- Scissors (1 pair)
- Markers (2)
- 1-oz dropper bottles with distilled water (1)

General materials for entire class:

- Lens paper (1 pad)
- Latex gloves
- Nitrile gloves (for students with latex allergies)
- Autoclave bag for plant waste
- Squirt bottles (6), filled with distilled water
- Sharps containers
- Glass Waste containers

Optional equipment:

- Fluorescence microscope with trinocular camera mount
- Low light video or digital camera
- Monitor
- Color printer

Notes for the Instructor

This exercise requires preparation at least 4-6 weeks ahead of the lab. Plant cultures are started six weeks before the scheduled lab. The PtK_2 cell line is also started from frozen stocks approximately four to six weeks before the scheduled lab. PtK_2 cells are seeded onto cover slips one to four days ahead of the lab. See Appendix B for a schedule of cell culture duties. Stocks of all reagents can be prepared and aliquoted well in advance of the lab. Working solutions of stains and antibodies are prepared on the day of use. Staining of PtK_2 cells for microtubules requires approximately 2.5 hours of preparation time before the lab. After fixing and permeabilizing the cells, they are treated with an anti-tubulin antibody. We carry out these steps for the students; however, if time permits, the students could carry them out.

The lab can be somewhat labor-intensive and expensive depending on the number of samples examined and the number of stains used. However, the lab can be modified to the instructor's needs relatively easily and many of the stains are supplied in quantities sufficient to last for many years. For example, instead of each student performing stains of all three organelles, the class can be divided into three groups, each focusing on a different organelle. This reduces the number of cover slips needed, as well as reducing the amount of stain required.

One of the best commercial sources for fluorescent stains used in cell biology is Molecular Probes, Incorporated. Molecular Probes also publishes a handbook available on CD-ROM (Haugland 2001) or online *(http://www.probes.com/handbook/)* that describes various applications of stains and their properties.

> *Most of the fluorescent stains used in the lab are toxic. For all stains, read the Material Data Safety Sheets (MSDS) available from the supplier. Although the stain concentrations are low and the volumes used are small, students should be required to wear gloves. Lab coats and protective eyewear should also be considered. All stain waste should be collected and disposed of properly. Consult your institution's Safety Department for disposal procedures.*

Formaldehyde is used as fixative for the PtK_2 cells. Preparation of formaldehyde from paraformaldehyde is best, however, paraformaldehyde solid should be handled very carefully. Always weigh out and work with paraformaldehyde using a fume hood and wearing appropriate protective gear (gloves, mask, lab coat). In addition, the prill form of solid paraformaldehyde is preferable since it minimizes dust formation. A more expensive but safer alternative to weighing solid paraformaldehyde is to purchase EM grade 16% paraformaldehyde (e.g. from Electron

Microscopy Supplies). Fixation of cells is performed in the hood by a trained technician or by students who have received instructions on the proper handling and disposal of formaldehyde. Be sure to read MSDS information on handling paraformaldehyde. Fume hoods are also used by the students when applying nail polish to their slide. This reduces the levels of nail polish fumes in the lab.

The transgenic tobacco plants expressing GFP were obtained directly from the research lab that generated the plants and they are grown in the greenhouse facilities on campus. Because the plants are transgenic organisms, there are stricter regulations for growth and disposal of the plants. For example, each pot or tray has to be labeled with a "Transgenic" plant sign, the flowers must be bagged so they cannot pollinate regular tobacco, and the seed has to be collected for further use or autoclaved with the plant. The leftover plants, any soil, as well as the pots and tags that were in the soil must be sealed in bags and autoclaved before disposal. Consult NIH guidelines on Recombinant DNA and Gene Transfer *(http://www4.od.nih.gov/oba)* or your institution's Biosafety Committee for details on requirements for growth and disposal of transgenic plants.

Student Outline

Goals

1. Familiarize yourself with the distribution, organization, and relative sizes of organelles within a cell.
2. Understand fluorescence microscopy and the principles behind the staining techniques.

Resources

For further reading on cell structure and fluorescence microscopy, consult standard cell biology textbooks (e.g. Alberts *et al.* 1998; Becker *et al.* 2000). In addition, a very nice web site with virtual microscope tutorials can be found at *http://www.micro.magnet.fsu.edu/primer/index.html.*

Introduction

Using regular *brightfield* microscopy, cellular features appear as differences in light intensity (light versus dark regions) or as different colors. However, because most unstained cells have little contrast, they appear transparent. Staining enhances details by adding color to the cell. Yet, most dyes are toxic to cells limiting their use to fixed (dead) cells. One technique that overcomes this limitation in living cells is *phase contrast* microscopy. Light passing through certain parts of a cell is refracted because differences in biochemical composition alter the refractive index of the cellular structure. The refracted light is out of phase with the unaltered light. The phase contrast microscope enhances the phase differences so that you see the structures as light versus dark regions. For example, the nucleolus appears as a very dark spot within the nucleus.

Another technique used to increase contrast is *fluorescence microscopy*. With regular light microscopy, when you look at a sample, in addition to the sample, you are also observing the background illuminating light. This light can make it difficult to see weakly stained cellular components. With fluorescence microscopy, the background light is eliminated so you see the fluorescently stained object contrasted against a black background. It is like looking at a dim light outside on a sunny day versus in a dark room. Fluorescent molecules absorb light of one particular wavelength and then emit at a longer (lower energy) wavelength. The fluorescence microscope

takes advantage of this difference, filtering out the excitation light, so only the emitted light is seen. For example, the fluorochrome Alexa Fluor 488 is excited by blue light (maximally at 495 nm), yet it emits in the green (520 nm) range. To examine structures stained with Alexa Fluor 488, cells are illuminated with a blue light. The cells give off a green fluorescence; in addition, blue light is reflected back from the sample. However, *dichroic* filters in the microscope block the blue light while allowing the green light to pass through. Then, as you look at the cells through the microscope, the Alexa Fluor 488 stained structures glow green against the black background.

Fluorescence microscopy is frequently used in cell biology because of its increased sensitivity and because of the ability to stain specific parts of a cell. However, there are not many fluorescent chemicals with innate high affinity for directly staining particular parts of the cell, so more often an *indirect* staining method is used. Here, a non-fluorescent molecule that binds specifically to some part of the cell is covalently bound to a fluorochrome. By itself, the fluorochrome would not stain the cell, but by "piggy-backing" on the specificity-conferring molecule, the fluorochrome indirectly labels cellular structures (Table 1).

Table 1. Functional groups of direct versus indirect fluorescent stains

Stain class	Stain	Fluorochrome	Specificity domain	Structure stained	Stain Color
Direct	MitoTracker Red	MitoTracker Red	MitoTracker Red	Mitochondria	Red
Direct	$DiOC_5(3)$	$DiOC_5(3)$	$DiOC_5(3)$	Endoplasmic Reticulum	Green
Direct	DAPI	DAPI	DAPI	DNA (Nuclei)	Blue
Direct	Hoechst 33342	Hoechst 33342	Hoechst 33342	DNA (Nuclei)	Blue
Indirect	BODIPY FL C_5-ceramide	BODIPY FL	ceramide	Golgi Apparatus	Green
Indirect	Alexa Fluor 488 phalloidin	Alexa Fluor 488	phalloidin	Actin (Microfilaments)	Green
Indirect	Texas Red anti-mouse 2° antibody	Texas Red	anti-mouse 2° antibody†	Tubulin (Microtubules)	Red

† Used in combination with mouse anti-tubulin 1° antibody

A specialized case of the indirect method is *indirect immunofluorescence*. In this technique, a cell is incubated with a *primary* antibody (1°ab) that reacts with a specific cellular antigen (*e.g.*, tubulin). Antibodies are particularly useful tools because they bind to antigens with high affinity and specificity. The primary antibody itself is not fluorescent. However, it can be detected by staining with a fluorochrome-conjugated *secondary antibody* (2°ab) that binds specifically to the primary antibody.

By coupling different detector molecules to different colored fluorophore, it is possible to co-stain for different structures and to distinguish the components by their color (Table 1). For example, in today's lab, you will stain microfilaments green, microtubules red, and DNA blue all in the same cells. The ability to co-stain structures allows one to examine their relative organization and to identify overlapping patterns of expression. Following is a description of the structures to be examined in this lab.

Actin: Microfilaments, composed of actin, will be stained using phalloidin that has been conjugated to the green fluorochrome, Alexa Fluor 488. Phallotoxins, originally isolated from poisonous mushrooms, bind relatively tightly to F-actin, the polymerized **F**ilamentous **actin** in microfilaments.

Tubulin: Microtubules (composed of tubulin) will be stained red by indirect immunofluorescence using a mouse anti-tubulin primary antibody and a Texas Red conjugated secondary antibody.

Mitochondria: MitoTracker Red will be used to stain mitochondria. Sequestration of this red dye is dependent on a membrane potential; thus, the stain can also reveal information about the health of the mitochondria. The membrane potential is rapidly lost if the mitochondria are no longer able to generate an ion gradient, e.g. by damage to the membrane, inhibition of the electron transport system, uncoupling, etc.

Endoplasmic reticulum (ER): The hydrophobic side chains of the lipophilic $DiOC_5(3)$ allow it to localize preferentially in the membranes of the ER; however, this stain also accumulates in mitochondria, especially after prolonged incubation.

Golgi apparatus: The glycolipid ceramide also associates with membranes however it accumulates preferentially in the Golgi, serving as a convenient marker for this organelle. Ceramide itself is not fluorescent so we will be using an indirect stain, BODIPY FL C_5-ceramide, in which the green fluorochrome BODIPY FL has been conjugated to ceramide.

DNA/nuclei: DAPI is a small water-soluble fluorescent molecule with extreme avidity and specificity for DNA, preferentially binding to the A:T pairs of DNA. DAPI will also reveal cells undergoing mitosis. During mitosis, the DNA condenses into brightly staining figures. During the rest of the cell cycle, when, the DNA is not so tightly packaged, the nucleus appears as a diffuse blue oval. Like DAPI, Hoechst 33342 is a DNA specific dye. Hoechst is more cell-permeable than DAPI, so it is a useful dye for staining live cells.

Green fluorescent protein tags: Most natural and synthetic fluorochromes are complex molecules and coupling of these chemicals to other molecules is only possible in the test tube. This limitation presents problems when one wants to use fluorescent probes in live cells. Vital stains are useful, but there are relatively few compounds that can stain a cell without some side effects, limiting the duration of exposure to the vital stain. More recently, a molecular genetic approach has been taken to generate fluorescent molecules from within the cell. This is possible because the fluorochrome in this case is a protein - the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. GFP is part of the natural luminescent system of the jellyfish. GFP absorbs in the blue range, maximally at 396 nm and 475 nm, and it emits at 508 nm, in the green range of the visible spectrum. GFP is useful chiefly for two reasons: (1) GFP is a protein and is encoded by a single gene. The jellyfish GFP gene has been cloned and genetic engineering can then be used to splice the GFP gene to genes from other organisms. (2) Generally, engineered GFP proteins are non-toxic, so GFP expression can be examined in living cells. Changes in the protein expression or distribution can be monitored as the cell/organism is perturbed experimentally, or as the organism develops. Researchers have now made a number of "green" organisms, including mice, frogs, flies, fish, nematodes, plants, yeast, and bacteria. These GFP transgenic organisms are proving to be very useful in working out mechanisms of cell and developmental biology.

In lab, you will examine tobacco plants that have been engineered to express the GFP protein. In one plant, normal cytosolic GFP is expressed. In the other plant, the GFP gene was altered so it now expresses a slightly longer protein, with a 29 amino acid long extension at the amino terminus (the beginning of the protein) of GFP (Kohler *et al.* 1997). This short but specific sequence addition is sufficient to generate a dramatic difference in the cellular distribution of the protein. The extension is an example of a transit peptide signal that directs the import of the newly synthesized protein into a specific organelle. Distinct sorting signals direct proteins to the mitochondrion, the chloroplast, the endoplasmic reticulum, the nucleus, and the lysosome.

Methods

Overview and hazards

For this lab, you will prepare six slides:

- SLIDE #1: Fixed PtK_2 cells triple-stained for actin, tubulin, and DNA.
- SLIDES #2-4: Living PtK_2 cells stained with a vital stains specific for organelles (Golgi, ER, and mitochondria).
- SLIDES #5-6: Transgenic GFP expressing tobacco cells.

You will also need to sketch and describe what you see. For all drawings, show the nucleus as a point of reference. Look at cells in different sections of the slide (e.g. near the edge, in the center of the slide). This will give you a more representative view.

> ***Hazards****: Wear gloves for all staining procedures. Nitrile gloves are available for individuals with latex allergies. Many of the reagents that you will use today are either toxic or potentially harmful. Avoid contact with skin, mouth, or eyes. Glass cover slips are fragile. Clean up all the glass fragments immediately. Dispose of all slides into the designated glass waste boxes. Do not put any glass into the regular waste.*

A) Triple stain - actin, tubulin, DNA

For this cover slip, you will be using three different fluorochromes simultaneously to stain the cytoskeleton and DNA of PtK_2 cells. PtK_2 cells are epithelial-like cells derived from the kidney of the marsupial rat kangaroo. Prior to lab, the cells were fixed in 4% formaldehyde and extracted with detergent to permeabilize the cells. The detergent treatment allows the antibodies to penetrate the cell membranes. The cells were also incubated with a mouse (1°) antibody directed against tubulin. You will treat the cover slip with a fluorescent secondary (2°) antibody (Texas Red conjugated anti-mouse antibody). This staining solution also contains Alexa Fluor 488 phalloidin, which will bind to and stain the F-actin green. Finally, the cover slips will be mounted in a drop of DAPI to stain the DNA in the cell blue.

1. If you have not already done so, turn on the power supply for the mercury arc lamp. The green "BURNER ON" LED should light up. If not, turn off the power supply and wait a few minutes before trying to light the lamp again. Once you have turned on the arc lamp, do not turn it off again until the end of the lab period. Also, please make sure the shutter is in the closed (PULLED OUT) position. The shutter is located on the top right hand side of the microscope - it is labeled SHUTTER.

2. Cut a three-inch square piece of Parafilm and tape it flat to the bench. Label the tape. Fill up the beakers labeled PBS with 1XPBS (**P**hosphate **B**uffered **S**aline solution).
3. Obtain a tube of "triple" stain from your TA. The tube will contain a mixture of Alexa Fluor 488 phalloidin and the Texas Red secondary antibody. Pipet the solution onto the piece of Parafilm, trying not to create air bubbles. Cells sitting under bubbles will not stain well.
4. The PtK_2 cells have been grown on one side of a glass cover slip, so pay attention to the orientation of the cover slip. The cells will be on the top side of the cover slip, *facing up* in the petri dish. Using a pair of jeweler's forceps, remove a cover slip from the petri dish with fixed PtK_2 cells. Dip the cover slip in PBS a couple of times to rinse it. If you drop the cover slip, you will not know which side has the cells (you have a 50-50 chance of guessing the right side).
5. Touch the edge of the cover slip to a Kimwipe briefly (no more than 2-3 seconds) to remove excess liquid. Gently place the cover slip *cell side down* onto the drop of staining solution. Place a foil-covered box over the cover slip and stain the cells for at least 45 minutes. While the cells are incubating with the triple stain, proceed to section B - the organelle stains.

Triple stain - continued

6. Wipe a microslide clean with a Kimwipe and lay it down on the bench. Label the white edge with your initials and the slide number (*e.g.*, CS#1, TRIPLE).
7. Just before the staining is finished, put 20 μl of DAPI stain onto the center of your labeled slide. Do not put the DAPI on the cover slip.
8. When the staining period is done, pipet 200 μl PBS onto the Parafilm, just adjacent to the cover slip. This will raise the cover slip somewhat. Carefully, slide your forceps under the cover slip and pick up the cover slip.
9. During the wash steps, remain aware of which side of the cover slip the cells are on. Dip the cover slip repeatedly for about 30 seconds in the PBS beaker.
10. Drain the PBS off the cover slip by briefly touching the edge of the cover slip to a Kimwipe. Don't let the cells dry out.
11. Mount the cover slip onto the slide, lowering the cover slip gradually so that the DAPI stain spreads under the cover slip without creating bubbles. Carefully slide out the forceps. Gently, use a Kimwipe to blot off any excess liquid.
12. Using a Kimwipe, soak up the staining solution on the Parafilm and place the wipe and Parafilm into the marked STAIN WASTE container.
13. Seal the cover slip with nail polish and dry the nail polish in the hood. When the nail polish has dried (it is no longer tacky), rinse off the cover slip with deionized water. Gently blot the top of the slide dry with a Kimwipe. Thoroughly dry the underside of the slide with a Kimwipe.
14. Observe the cells with the fluorescence microscope. Focus on the cells using the 20X objective and phase contrast setting. Without changing the *course* focus knob, switch to the 40X objective and turn the condenser dial to the appropriate phase contrast setting. Adjust the focus using the *fine* focusing knob. Only use the fine focus knob after shifting to 40X. Once the cells are in focus, turn off the visible light source (the switch is located on the back of the right-hand side of the microscope). You will not be able to see the fluorescent signal very well if the visible light is left on. The filters to be used are summarized in Table 2.

Table 2. Filter cubes to be used for the triple stain

Filter	Stain	Structure	Color
NIB	Alexa Fluor 488 phalloidin	Actin	Green
WIG	Texas Red 2° antibody	Tubulin	Red
WU	DAPI	DNA	Blue

15. Turn the filter cube to the "NIB" position to examine the Alexa Fluor 488 phalloidin stain. Open the shutter - you should see a blue light coming out of the objective. Examine the cells briefly and look at the microfilament staining pattern.
16. Next, rotate the filter cube turret to the "WIG" position to examine the Texas Red fluorochrome. This will reveal the microtubule pattern. Try not to move the stage when switching from NIB to WIG. This allows you to examine the different cytoskeletal structures in the same set of cells. Look at the microtubule staining pattern
17. Finally, switch to the “WU” cube to examine the DAPI staining pattern. Scan the slide quickly using the 20X objective to identify mitotic cells. During interphase, the nuclei will appear as diffuse blue ovals. During mitosis, the DNA (chromosomes) will be condensed into discrete figures. Look at the DNA staining pattern.

    For the triple stain slide:

    a. Describe and sketch the pattern of the cytoskeleton (microfilaments and microtubules) in interphase cells. Also, describe their distribution and organization relative to each other and relative to the nucleus.
    b. Look at several fields and try to find cells at different stages of mitosis (prophase, metaphase, anaphase, and telophase). Identify the stage of mitosis and then sketch and describe the distribution of the cytoskeleton relative to the chromosomes. Compare the appearance of the cytoskeleton in mitotic cells versus interphase cells.

B) Organelle stain - Endoplasmic Reticulum, Golgi, and Mitochondria

Live PtK_2 cells will be stained with $DiOC_5(3)$, which stains the Endoplasmic Reticulum (ER), BODIPY FL C_5-Ceramide, which stains the Golgi apparatus, and with MitoTracker Red, which stains mitochondria. Cover slips with cells will be arranged in Petri dishes with the cells facing up. Be sure to keep track of which side of the cover slip has the cells.

1. Wipe a microslide clean with a Kimwipe and label the white edge with your initials, the slide number, and the organelle (*e.g.*, CS #2 ER).
2. Cut three 3-inch squares of Parafilm. Tape the Parafilm flat on the bench and label the tape with the corresponding stain name.
3. Pipet 300 µL of stain onto the piece of Parafilm.
4. Using a pair of forceps, remove a cover slip from the Petri dish with live cells, making sure to remember that the cells are facing upward.
5. Dip the cover slip 2-3 times in PBS. Blot off the excess PBS by briefly touching the edge of the cover slip to a Kimwipe.
6. Put the cover slip cell side facing down onto the drop of stain. Cover the slide with a foil-lined box and stain the cells for 15 minutes. Do not stain the cells with the ceramide for more then 20 minutes. Over-exposure to ceramide will kill the cells.
7. Rinses:

- *Ceramide stained cells only*. Set up a fresh piece of Parafilm with 300 µL PBS. After staining the cells in ceramide for 15 minutes, rinse the cells in PBS by dipping the cover slip 3-4 times in PBS. Then invert the cover slip onto the drop of fresh PBS and incubate for an additional 10 minutes. Rinse the cells by dipping the cover slip in the beaker of PBS for about 30 seconds. Briefly touch the edge of the cover slip to a Kimwipe to remove excess PBS. Do not let the cover slip dry out.
- *MitoTracker Red and $DiOC_5(3)$:* After staining, rinse the cells in PBS by dipping the cover slip in PBS for about 30 seconds. Briefly, touch the edge of the cover slip to a Kimwipe to remove excess PBS. Do not let the cover slip dry out. Mount the cover slip onto a drop of PBS on a slide.

8. Put a drop (20 µL) of PBS onto a clean, labeled slide. Using forceps, place one edge of the cover slip square against the slide, and gradually, lower the cover slip so that the PBS spreads under the cover slip. Try to avoid trapping air bubbles as you lower the cover slip. Carefully slide out the forceps. Using the corner of a Kimwipe, blot off any excess PBS from the surface of the cover slip.
9. Seal around the edges of the cover slip with a continuous narrow (2-4 mm wide) band of nail polish. The seal will prevent the cells from drying out while you are examining them under the microscope. Note: a good seal will not form if there is still some PBS where you apply the nail polish. Place the slide in the hood and allow the nail polish to dry completely. This will take several minutes.
10. When the nail polish is dry, rinse the cover slip with distilled water. There are bottles of distilled water by the sink. This step removes excess PBS from the slide. Dry the slide with a Kimwipe, being careful not to press down too hard on the cover slip. Thoroughly dry the underside of the slide with a Kimwipe.
    If you skip this step, salt crystals will form on the cover slip as the PBS evaporates. The crystals will obscure the image. If you see a Christmas tree pattern on your slide, it is most likely salt crystals. Just rinse and dry the slide again.
11. Observe the cells. Start out with the 20X objective using the appropriate phase contrast setting (Ph1). Change to the 40X objective without changing the *course* focus knob and turn the phase condenser to the Ph2 setting. Adjust the focus using the fine focusing knob. The objectives are parfocal. Only use the fine focus knob to focus after shifting from 20X to 40X. At 20X, the field of view is approximately 1 mm in diameter. At 40X, the field of view is approximately 0.5 mm (= 500 µm).

Describe the range of cell sizes and shapes. Draw a cell(s) showing the nucleus and any other structures you see. Can you see any other organelles? Also, be sure to include a scale bar for your drawing.

Turn off the base illuminator visible light source (the switch is located on the back right hand side of the microscope). You will not be able to see the fluorescent signal if the visible light is left on. The filters to be used are summarized in Table 3.

Table 3. Filter cubes to be used for the triple stain

Filter	Stain	Organelle	Color
NIB	BODIPY FL Ceramide	Golgi	Green
NIB	$DiOC_5(3)$	ER	Green
WIG	MitoTracker Red	Mitochondria	Red
WU	Hoechst 32242	DNA	Blue

12. To examine the Golgi (BODIPY FL-ceramide) and ER ($DiOC_5(3)$) stains, turn the filter cube dial to the "NIB" position. Open the shutter by pushing in the shutter knob. Focus using the fine focus knobs, if necessary. Sketch and describe the distribution of the Golgi, and ER (relative to the nucleus).
13. Examine the mitochondria (MitoTracker Red) stain using the WIG filter. Sketch and describe the distribution of the mitochondria (relative to the nucleus).
14. The DNA specific dye Hoechst 33342 was included with the organelle stains so the cells will also be stained for DNA. After examining the organelles in interphase cells, switch to the "WU" cube to examine the Hoechst staining pattern. Scan the slide for mitotic figures. In interphase cells, the nuclei will appear as diffuse blue ovals. During mitosis, the DNA (chromosomes) will be condensed into discrete figures. It is easier to scan the slide for mitotic figures using the 20X objective.

Look at several fields and try to find cells at different stages of mitosis (prophase, metaphase, anaphase, and telophase). Sketch one or more of these cells and describe the distribution of the Golgi, ER, and mitochondria. Does the shape and size of the cell change during mitosis? Is the organization/distribution of the organelles changed?

Photography:
If you would like a photograph of your stained cells, you can take your slides over to the fluorescence microscopes that are outfitted with low-light CCD cameras. The Lab Director or TA will take one picture for each lab partner.

C) GFP proteins in transgenic tobacco

For the final two slides, examine tobacco cells that express different GFP constructs. One plant will express cytosolic GFP protein while the other will express GFP protein that is targeted to the mitochondria. The two different strains are labeled A and B. Prepare slides of both strains.

1. Label two slides with your initials and the plant code letter (A or B). Make a circle in the center of each with a marker. Put two to three drops of water into the circled region of the slide.
2. Tear off a leaf from the stem at the base of the petiole (Fig. 1). Be sure to include part of the petiole because it is covered with many hair cells that you will want to examine.

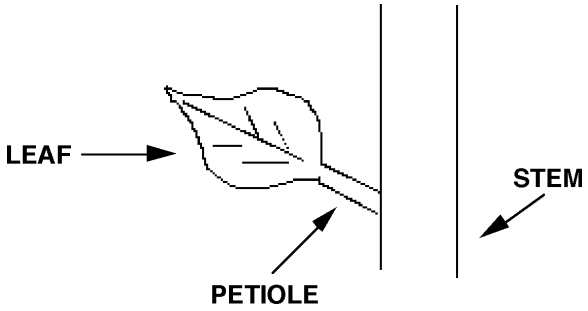

Figure 1. Example of plant stem with attached petiole and leaf. Hair cells can be obtained from the petiole and stem.

3. Put some hair cells onto a slide:
    - *Method 1*: Using a pair of fine forceps, pluck off at least a dozen hairs from the petiole or the leaf. Try grabbing at the base of the hair. Transfer the hairs to the drop of water.
    - *Method 2*: Using the corner of a razor blade, gently shave off the white hair cells from the leaf or the petiole. If done correctly, the cells will stick to the blade. Repeat this process until you can see hairs on the blade. Then, rub or tap the razor blade into the water on the slide.
4. Put a cover slip on the slide and blot off excess water with a Kimwipe. Seal the cover slip with nail polish, if desired.
5. Place the slide onto the stage of the microscope and begin scanning with the 20X objective. You will be looking for thin hair-like structures (Fig. 2). Some may be very short while others may be long. Once you have found a group of cells to observe, switch to the 40X objective.

Figure 2. Example of tobacco plant hair cells. The tip of the hair cell will often be green under visible light and bright red under fluorescent light because of an abundance of chloroplasts. Nucleus (n).

6. Use the "NIB" filter cube to observe the green GFP fluorescence. One caveat: in cells expressing the non-localized form of GFP, GFP is present in both the cytoplasm and the nucleoplasm, yet it is not in the vacuole. In cells with large vacuoles, this gives the impression that the GFP is localized to the nucleus. Unless you are red-green color blind, while looking at the green fluorescence from GFP, you will also observe red auto-fluorescence from chlorophyll in chloroplasts. Chlorophyll absorbs in the blue (430-460 nm) range, similar to the range that excites the GFP fluorescence.

Describe the GFP expression pattern in tobacco strains A and B. Which plant expresses the mitochondrial localized GFP?

Compare the number, size, and distribution of the mitochondria and chloroplasts. Also, describe any movement of the mitochondria and chloroplasts. Are they stationary?

Compare the appearance and distribution of the GFP-tagged plant mitochondria to the mitochondria in the PtK_2 cells (stained by MitoTracker Red).

Cleaning Up

Because the plants are genetically engineered, all plant waste goes into the labeled Biohazard bag.
Discard slides of plant cells into the labeled sharps container boxes.
Put used slides and cover slips into the labeled "GLASS" waste boxes.
Discard leftover stains and used pipet tips into the labeled "STAIN" waste containers at your bench.
Pour the PBS in the beakers at your bench down the sink. Rinse the beakers with deionized water and return them to your bench.

Acknowledgements

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Appendix A: Preparator's Guide

Schedule - ahead of time

- 6-8 weeks before lab: start growing tobacco plants.
- 4-5 weeks before lab: start PtK_2 cultures.
- 2 weeks before lab: sterilize cover slips. Prepare and test stain stock solutions.
- Day before or day of lab: dry down phalloidin in tubes.
- Day of lab: (1) Prepare cover slips for triple stain - fix, permeabilize, and incubate the cover slips with anti-tubulin primary antibody. (2) Resuspend dried-down phalloidin with the secondary antibody solution. (3) Prepare and aliquot working solutions of the vital stains. (4) Put 1 µg/mL DAPI aliquots into a rack.

Cell culture materials

- Complete media (DMEM + 10% serum). To 500 mL DMEM, add 5 mL penicillin/streptomycin mix, 10 mL L-glutamine, and 50 mL Fetal Bovine Serum. Mix and store at 4°C.
- Cover slips: autoclave 18-mm square cover slips in glass dishes. Using sterile technique and working in a tissue culture hood, transfer five sterile cover slips per 100-mm sterile, plastic petri dish.

Cell culture schedule

- Passage cells every three to four days. We usually passage cells on Monday and Thursday. For a three-day cycle, use 1,000,000 cells per T-75 flask; for a four-day cycle, use 800,000 cells per flask.
- Put cells onto cover slips two to four days before the lab. Use 12,000-15,000 cells per cover slip if plating cells four days before lab. Use 20,000-25,000 cells per cover slip if plating cells two days before the lab.

Passaging (splitting) cell culture and plating on cover slips

- Use standard sterile technique for culturing the cells. Sterilize the tissue culture hood surfaces with 70% ethanol. Warm the complete media to 37°C. Thaw a 5-10 mL aliquot of 1X trypsin/EDTA.
- Remove flasks from the tissue culture incubator and check for contamination. Discard any contaminated flasks. Put uncontaminated flasks in the tissue culture hood. For each flask, remove the media to a waste beaker and rinse the cells with 10 mL of sterile PBS. Remove the PBS rinse to the waste beaker. Add 5 mL trypsin/EDTA to cover the cells. Pipet the trypsin/EDTA over the cells a few times. Remove 4.5 mL of the trypsin/EDTA solution. Cap the flask. Place cells at 37°C for 1 minute (or leave the flask at room temperature for several minutes) and then check the cells under an inverted phase contrast microscope. Check to see that the cells round up. Rap the flask against your hand to detach the cells from the flask. After rapping the flask, most (>90%) of the cells should be floating free of the flask. In the tissue culture hood, add 10 mL complete media to the flask to inactivate the trypsin (the serum in the media inactivated the trypsin). Pipet the media up and down over the surface of the flask about a dozen times. Transfer the cell suspension to a 15-mL conical centrifuge tube. Check the flask. Most of the cells (>90%) should have been removed. If there are still many cells attached to the flask, scrape off the remaining ones with a cell scraper and add them to the tube of cells.
- Centrifuge the cell suspension at 1,300rpm (400 x g) for 5 minutes. Pipet off most of the supernatant and resuspend the cell pellet in 2 mL fresh media. Pipet the cells up and down about a dozen times until they form a suspension with no visible clumps. Take a 0.1 mL aliquot of the suspension and count the cell density using a hemacytometer. For setting up new flasks, add the appropriate number of cells (from 800,000 to 1,000,000) to 12 mL of complete media in a new T-75 flask.
- For setting up cells on cover slips, add media as needed to obtain the desired cell density (typically, 45,000 – 60,000 cells per mL). Plate 350 µL of the cell suspension per cover slip. Spread the cells around the cover slip but do not push the liquid too near the edge of the cover slip. After the cells are plated, incubate the dish for at least 3-4 hours to overnight (the media will not dry up) in a humidified 37°C incubator until the cells adhere to the cover slip. Check for adherence of the cells and add 10 mL complete media to each dish. Make sure the cover slips are completely covered with media. Incubate the cells at 37°C.

Triple stain - 1° antibody staining step

- Note: the day before the lab, pipet 7 µL Alexa Fluor 488 phalloidin each into 1.5-mL microfuge tubes and let the phalloidin dry down overnight in the tube. Alternatively, on the day of the lab, the phalloidin can be dried down in the tube using a Speed-Vac for about 5 to 10 minutes.
- For a lab section with 24 students, working in pairs, minimally, you will need three dishes (15 cover slips) per day. *Wear gloves* for all steps. Paraformaldehyde is toxic. When working with paraformaldehyde, always *use a fume hood*; wear gloves, safety glasses, and a lab coat.
  1) In a fume hood, prepare a fresh dilution of 4% paraformaldehyde. In a 50-mL tube with 26 mL deionized water, add 10 mL 16% paraformaldehyde with and 4 mL 10X PBS. Mix.
  2) Rinse the cover slips in the petri dishes three times with 1X PBS.
  3) In the fume hood, pour off the PBS and immediately add 10 mL of 4% paraformaldehyde. Fix the cells for 5 minutes at room temperature.
  4) After 5 minutes, transfer the paraformaldehyde into a paraformaldehyde waste container. Rinse the cells quickly with PBS and then wash the dishes three times with PBS, 1-2 min per wash.
  5) Pour off the final PBS wash and add 10 mL PBT (PBS plus 0.5% Triton X-100)**.** After 10 minutes, aspirate off the PBT and rinse the dishes three times with PBS, 1-2 minutes per wash.
  6) Dilute the anti-beta tubulin stock 1:50 in PBS. Prepare enough for 100-120 µL per cover slip. For example, add 40 µL of the stock antibody solution to 2 mL PBS. This provides enough diluted antibody for 15 cover slips at 120 µL per cover slip.
  7) Tape down strips of Parafilm to a bench or piece of plastic. Pipet 120 µL spots of diluted anti-tubulin onto the Parafilm and place a cover slip cell side down onto each drop of antibody. Stain the cells for 1 hour at room temperature. If desired, place the cover slips in a humid chamber during the 1-hour incubation.
  8) While the cover slips are incubating, prepare a 1:150 dilution of the Texas Red anti-mouse antibody stock (1 mg/mL). Prepare enough for 150 µL per cover slip. For example, add 17 µL stock to 2.5 mL PBS for fifteen cover slips. Aliquot 150 µL of the 1:150 diluted antibody each into the microfuge tubes with evaporated Alexa Fluor 488 phalloidin. Mix and store at 4°C until use.
  9) After one hour, rinse the cover slips with PBS three times for 5 to 10 minutes each. The cover slips are now ready for the students to use and the dishes can be moved to the lab room.

Dilutions of Vital Stains to Working Concentrations

- MitoTracker Red (0.05 µg/mL final) + Hoechst (2 µg/mL final). Dilute from 2 to 3 µl MitoTracker Red stock into 10 mL PBS. Add 20 µL 1 mg/mL Hoechst; mix. Aliquot 0.5 mL each into 1.5-mL tubes.
- $DiOC_5(3)$ (0.5 µg/mL final)+ Hoechst (2 µg/mL final). Dilute 5 µL $DiOC_5(3)$ stock into 10 mL PBS. Add 20 µL 1 mg/mL Hoechst; mix. Aliquot 0.5 mL each into 1.5-mL tubes.
- BODIPYFL Ceramide:BSA (0.5mM ceramide; 0.5mM BSA) + Hoechst (2 µg/mL final). Dilute 3.5 µL Ceramide-BSA into 10 mL PBS. Add 20 µL 1 mg/mL Hoechst; mix. Aliquot 0.5 mL each into 1.5-mL tubes.

Table 4. Summary of stains and dilutions

Stain	Stock	Dilution
MitoTracker Red	0.5 µg/µl (5000X)	2 µL in 10 mL PBS
$DiOC_5(3)$	1 mg/mL (2000X)	5 µL in 10 mL PBS
BODIPY FL Ceramide:BSA	0.5mM (3000X)	3.5 µL in 10 mL PBS
DAPI	0.1 mg/mL (100X)	0.1 mL in 10 mL PBS
Hoechst 33342	1 mg/mL (500X)	20 µL in 10 mL PBS
Alexa Fluor 488 phalloidin	200 units/mL	7 µL
Anti beta-tubulin	50 µg/mL (50X)	1:50
TexasRed anti-mouse IgG	1 mg/mL (150X)	1:150

Appendix B: Materials

Cell culture materials

- Ten times concentrated solution of Phosphate Buffered Saline (10X PBS), pH 6.8. To 8L deionized (biology grade) water, add 800 g NaCl (1.37 M final), 144 g Na_2HPO_4 (0.1 M final), 24 g KH_2PO_4 (0.018 M final), and 20 g KCl (0.027 M final). Adjust pH to 6.8 and bring up to 10L with deionized water.
- Sterile 1X Phosphate Buffered Saline (1X PBS), pH 7.2 for cell culture. Dilute the 10X PBS stock with deionized water. Adjust pH if necessary. Autoclave in 100 mL aliquots for cell culture. For rinsing cover slips after staining procedures, the 1X PBS does not have to be sterile.
- 1X Trypsin/EDTA (Invitrogen #25300-054)
- Dulbecco's Modified Eagles medium (DMEM; Sigma #D5546)
- Penicillin/streptomycin solution (Invitrogen #15070-063)
- L-glutamine (Invitrogen #25030-081)
- Fetal Bovine Serum (Invitrogen #10082-147)
- PTK_2 (*Potorous tridactylis* kidney epithelial) cells (ATCC # CCL-56)
- Petri dishes: 12/day
- 18-mm square cover slips: 60/day
- T-75 cell culture flasks
- Hemacytometer
- 10-mL pipets, sterile, individually wrapped
- 5-mL pipets, sterile, individually wrapped
- 2-mL pipets, sterile, individually wrapped
- 15-mL conical centrifuge tubes, sterile
- 50-mL conical centrifuge tubes, sterile

Stains

1) MitoTracker Red (Molecular Probes # B-22650). Resuspend 50-µg aliquot in 100 µL DMSO; store desiccated in small (10 µL) aliquots at -20°C.
2) $DiOC_5(3)$ (Molecular Probes # D-272). Prepare a 1 mg/mL stock solution in DMSO; store desiccated in small aliquots at -20°C.
3) BODIPY FL C_5-Ceramide, complexed with BSA (Molecular Probes #B-22650). Resuspend 5 mg ceramide:BSA complex in 150 µL sterile deionized water (= 0.5 mM ceramide; 0.5 mM BSA); store desiccated in small (10 µL) aliquots at -20°C.
4) DAPI (Molecular Probes # D-1306). Prepare a 0.1 mg/mL stock solution in sterile deionized water. Store aliquots at -20°C. Prepare a 1 µg/mL working solution in PBS by diluting 100 µL 0.1 mg/mL stock in 10 mL PBS. Aliquot 1 µg/mL DAPI and store at 4°C.
5) Hoechst 33342 (Molecular Probes # H-1399). Prepare a 1 mg/mL stock solution in sterile deionized water. Store desiccated in aliquots at -20°C.
6) Alexa Fluor 488 phalloidin (Molecular Probes #A-12379). Resuspend 300 Units of Alexa Fluor 488 phalloidin in 1.5 mL methanol. Store desiccated at -20°C. To use: aliquot 7 µL phalloidin per tube and air dry.
7) Mouse monoclonal anti-tubulin antibody (Chemicon # MAB3408). Resuspend 50-µg lyophilized antibody in 1 mL sterile deionized water. Store in 45 µL aliquots at 4°C.
8) Donkey anti-mouse IgG, Texas Red (Jackson ImmunoResearch # 715-075-151). Resuspend 0.5 mg lyophilized antibody in 0.45 mL sterile deionized water. Store at 4°C.

Miscellaneous Materials

- 16% paraformaldehyde (Electron Microscopy Sciences # RT 15710-S)
- Triton X-100 (Sigma #X100)
- 1.5-mL microfuge tubes (blue, green, yellow, amber, red, purple, and clear)
- One-liter "waste" beakers for student stations. Beakers are lined with small autoclave bags.
- 100 mL beakers for PBS rinses
- Cover slips, 22-mm square
- Dropper bottles (for distilled water)
- Forceps, #7S
- Glass waste boxes
- Kimwipes
- Labeling tape
- Latex and nitrile gloves (different sizes)
- Markers
- Parafilm
- Pipet tips (for P200 and P1000 micropipetters)
- P200 and P1000 micropipetters
- Scissors
- Sharps containers
- Waste container for liquid formaldehyde waste
- Waste container for solid formaldehyde waste (lined with plastic bag)
- Optional: aspirator and vacuum trap