Objective: The goal of this lab exercise is to perform fluorescence imaging of live cells expressing GFP-tagged proteins.

Introduction: Observation of live cells offers many advantages over examination of fixed (dead) specimens, but has unique challenges as well. The main advantage of using live cells is that dynamic processes can be observed, and the spatial and temporal regulation of the process examined directly. In addition, if the cell is living, possible artifacts of fixation and staining are avoided. However, stained cells, especially preparations prepared with multiple layers of fluorescent antibodies, have excellent signal-to-noise characteristics, making imaging easy, especially for proteins that are present in low abundance. In contrast, the fluorescent signal from a live cell expressing a fluorescent protein is usually much weaker. Finally, live cells are sensitive to light – the fluorophores are readily bleached and the cells can be damaged by shorter wavelengths of illumination.

In spite of the challenges of imaging live cells in the light microscope, fluorescence imaging has become relatively routine in recent years. A major step forward in live cell imaging was the discovery and cloning of the gene encoding green fluorescent protein (GFP) a naturally occurring fluorescent protein isolated from jelly fish Aequorea victoria (Chalfie et al., 1994). With this sequence, nearly any protein for which a gene sequence is available can be fluorescently tagged by creating a chimeric gene consisting of the GFP sequence in frame with the sequence of the gene of interest. GFP has been engineered to improve its spectral characteristics and color variants of GFP have also been generated. More recently other naturally occurring fluorescent proteins have been discovered, expanding the tool box of fluorescent proteins that can be used to tag genes and distinguish them in the light microscope. For additional information see: http://en.wikipedia.org/wiki/Green_fluorescent_protein.

Reagents:
Non-CO2 media
VALAP
Live cells that have been transiently transfected with GFP.
Live cells that have been transiently transfected with GFP-tubulin.
Live cells that constitutively express GFP-tubulin or GFP-actin.

Procedure:
A. Imaging cells transiently transfected with GFP.
1. Obtain a dish of cells that have been transfected with a vector containing the DNA sequence for Green fluorescent protein (GFP).
2. Take a clean class slide and label it. Now, prepare a miniature chamber for the cells as follows. Cut two small strips of parafilm, each ~2 cms long and about 1 cm wide. Place these on the slide, separated by ~2 cm (width of the coverslip). Press the parafilm with the forceps to stick it to the slide. Place a SMALL (~100ul) drop of warm, non-CO2 media in between the two strips.

3. Remove the coverslip from the dish using your fine forceps and blot the excess media using a kimwipe. Invert the coverslip, so that it rests on the two strips of parafilm. The space between the coverslip and the slide should be filled with non-CO2 media; if it is not, add a bit more, by pipetting media in from the side.

4. Carefully, remove excess media from the top surface and edges of the coverslip. Use the corner of a kimwipe, or a Q-tip.

5. Seal the coverslip to the slide so that the media does not evaporate. To do this, use VALAP. The VALAP is liquid at ~37 C, but at room temperature it hardens, making a seal.

6. When the VALAP hardens, carefully rinse the top surface of the coverslip with a little distilled water on a Q-tip.

Microscopy:

1. Begin by imaging the cells in phase contrast. Start with low magnification. Can you observe the nucleus? Organelles? Lamellipodia? Make a short movie of the live cells. Are the cells alive? How can you be sure? If the cells have died during the preparation process, obtain a new sample.

2. Switch to fluorescence. Use the shutter to acquire images of the cells. If you leave the shutter open and look with your eyes, the sample will bleach rapidly. Are all of the cells fluorescent? What is the location of the GFP?

3. Test various settings of Image J and the neutral density filters to obtain a good image of the cells. Begin with low magnification, and switch to higher magnification.

4. Set up Image J to acquire a short time lapse sequence of cells.

5. Collect sufficient data (images) to answer the following:
   - What percentage of cells express the transgene?
   - What is the range of expression level of the GFP-tagged protein? To do this, you will need to quantify the level of expression.
Part B. Imaging cells constitutively expressing GFP-tubulin

1. Obtain a dish of cells that express GFP-tubulin from the TA.
2. Make a preparation as in part A.
3. Image the cells using the same procedure used for the GFP expressing cells.

Part C. Imaging cells transiently transfected with a plasmid expressing GFP-tubulin

1. Obtain a coverslip of cells that have been transiently transfected with a plasmid vector encoding Tubulin-GFP.
2. Make a preparation as in part A.
3. Obtain images of these cells, and compare with the permanent cell line expressing GFP-tubulin and the cells transiently transfected with the GFP vector.
4. What is the location of the GFP-tubulin in the cells? Do all cells show fluorescent microtubules? Are any other structures fluorescent? Explain your observations.

Questions:

Compare what you observed in live cells with the images that you obtained of cells fixed and stained with antibodies to tubulin. How were they similar? Different? Summarize the advantages and disadvantages of each approach.

Transfection Protocol

For this lab, the cells were transfected for you. To do this, we used an instrument called a nucleofector. With this instrument, cells are subjected to a brief electric pulse while they are suspended in a special media in the presence of the plasmid containing the gene of interest. The DNA (or RNA) can enter the cell nucleus directly. After the pulse, the cells are plated in the usual manner.

Reference: