Microscopy and preparation of fluorescent cells and tissues

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Abstract

This report is an overview of the lab techniques used in the laboratory of Michael Davidson, concentrating on fluorescence microscopy as an investigative tool to explore the intricacies of cellular biology. These techniques include the staining of cells and tissues with fluorescent dyes, the preparation of fluorescent protein DNA through plasmid amplification and purification using the QIAGEN Endofree Maxi kit, and finally the procedures for digital imaging using widefield fluorescent microscopy coupled with digital image processing.

Introduction

Fluorescence microscopy is an invaluable technique that is widely used today, especially in the biological and medical sciences. Due to significant recent advances in the development of fluorescent dyes or probes (fluorophores) and fluorescent proteins, it is now possible to target specific cytoskeletal elements, such as β- and f-actin, tubulin, and vimentin, and cellular organelles, such as mitochondria, endoplasmic reticulum, Golgi bodies, lysosomes, and nuclei with highly accurate probes. The technical advances achieved through the enhancement, variability, and applicability of fluorescent dyes and proteins allows the principal tools, microscopes, to capture higher quality images as the microscopes themselves also improve. While the fluorescent dyes and proteins light up the cells and tissues when excited at an appropriate wavelength, microscopy enables us to view and study these cells and tissues.

Scientists and educational specialists in Michael Davidson's program at the NHMFL have banded together with researchers and industrial organizations from around the world to build collaborations having a targeted goal of producing a world-class web-based educational system dealing with topics related to optical microscopy. This effort has resulted in the construction of several websites that are now used on a daily basis by thousands of educational institutions from around the world, with applicability that spans all ages, from K-12 to post-graduate. Aside from the educational efforts, the program is also developing a research initiative that may result in the establishment of a "user-facility" for investigations in cell biology and related disciplines at the NHMFL. At the heart of this facility are the tissue culture laboratory, a live-cell imaging laboratory, and several laser scanning confocal microscopes.

Fluorophores provide the basis for staining. They are typically highly conjugated molecules that when excited at a specific wavelength of light, absorb this light, and reemit it at a longer wavelength (red shift). The higher quantum efficiency at which the fluorophore reemits light, the better a fluorescent dye it is. Synthetic fluorescent probes have been constructed to target and bind to specific biological macromolecules, a nucleic acid or a protein for example, or to localize within a specific organelle. They can also be used to monitor cell processes such as membrane fluidity, endocytosis, or exocytosis and are useful in chromosome analysis and genomic mapping. Successful fluorophores are specific for the structure or species to be observed, are used in conjunction with the appropriate light source emitting at the wavelength at which the fluorophore absorbs, and are subsequently imaged at the emission wavelength of the fluorophore. Some typical dyes that are used in Michael Davidson's lab are: SYTOX Green, Alexa Fluor 488, and Oregon Green 488 (green emission); SYTOX Orange, Alexa Fluor 568, and Texas Red (red emission); Hoechst 33342 and Alexa Fluor 350 (blue emission). When choosing a fluorescent dye factors such as quantum efficiency, structural specificity, and bleed through over time into the media are important to consider.

Upon choosing a fluorophore, it is possible to dye cells and tissues with the fluorescent stain and multiple stains may be used to target different structures in a given tissue or cell, so long as the fluorophores do not interfere with one another. The protocols for cell and tissue staining vary slightly due to the difference in manipulating adherent cells on coverslips and tissue cryosections pre-mounted onto microscope slides, but they both follow a procedure that is basically the same.

Fluorescent proteins offer another method to those wishing to visualize cellular mechanisms and structures. Green fluorescent protein (GFP), a protein isolated from *Aequorea Victoria* by Shimomura and co-workers in 1962, was cloned in 1994 by Martin Chalfie and Douglas Prasher, and has since paved the way to pursue exciting new research in live and cultured tissues and cells. Mutations have been made to the protein to change its color, to increase its compatibility with living systems, and to augment its quantum yield, among others. Two techniques, FRET (Fluorescence Resonance Energy Transfer) and FRAP (Fluorescence Recovery After Photobleaching), have become very important since the discovery of GFP. FRET allows the interactions between two proteins *in vivo* as there is an energy transfer from the excited donor protein (typically cyan fluorescent protein, CFP) to the acceptor protein (typically yellow fluorescent protein, YFP) which then begins to fluoresce. FRAP is based around the principle that the fluorescent protein or fluorescent dye can be photobleached (loss of fluorescence after bombardment with too much light) and the migration of still fluorescent molecules or proteins into the darkened, photobleached area can be monitored. One may measure the percent recovery of fluorescence and measure how fast the fluorescent molecules migrated to the photobleached area.

In summary, the discovery of green fluorescent protein in the early 1960s ultimately heralded a new era in cell biology by enabling investigators to apply molecular cloning methods, fusing the fluorophore moiety to a wide variety of protein and enzyme targets, in order to monitor cellular processes in living systems using optical microscopy and related methodology. When coupled to recent technical advances in widefield fluorescence and confocal microscopy, including ultra-fast low light level digital cameras and multitracking laser control systems, the green fluorescent protein and its color-shifted

genetic derivatives have demonstrated invaluable service in many thousands of live-cell imaging experiments.

When fluorescent proteins are desired for experimentation it important to be able to produce them in high quantities. One method to amplify proteins is to use bacteria. Once the gene sequence for a protein is known, the gene can be inserted into a mammalian expression vector and then transformed into bacteria, which will replicate the plasmid as they themselves replicate. Mammalian expression vectors are DNA molecules that contain genes for efficient expression of both the DNA and RNA transcripts in mammalian cells, antibiotic genes for selection, and bacterial replication genes. After a mammalian expression vector plasmid is amplified in bacteria, it must then be isolated and purified, a process for which commercial kits are now available. The DNA coding for the fluorescent protein is now ready for protein expression, in our case, in the lines of mammalian cells into which they are transfected. Part of the discussion in this paper will focus on the plasmid purification by use of the QIAGEN Endotoxin-free Plasmid Maxi kit which prepares up to 500 μg of plasmid DNA at a time.

In order to image fluorescent cells and tissues it is necessary to have the appropriate equipment. Typically still images are taken with either a wide field fluorescence microscope or a confocal laser scanning microscope (CLSM). Confocal laser scanning microscopes have innate advantages over wide field fluorescence microscopy. As a light source alternative to epi-fluorescence in wide-field fluorescence microscopes, CLSM's use lasers (typically with excitation wavelengths of 405, 488, 543, and 633 nanometers), which are able to pinpoint a specific plane in the specimen instead of directing light on the whole cell or tissue. The second critical difference between CLSMs and wide field fluorescence microscopes is the aperture, absent in conventional microscopes, which blocks out-of-focus light rendering a clearer image. There are several advantages to CLSM, among which are the minimization of out-of-focus blur, increased lateral resolution, increased signal-to-noise ratio, and direct non-invasive serial optical sectioning eliminating the need for extremely thin physically-sectioned specimens and opening up the possibility for high-resolution imaging of living species. Confocal laser scanning microscopes, high-resolution cameras, and computers have also made it possible for images to be taken over time, from which a video can be compiled. In special time-lapse dishes that are heated by electrodes to 37˚C to maintain living cells, the activity and interaction of cells or the targeted structures within them may be monitored. DIC (Differential Interference Contrast Imaging) produces contrast from refractive index differences within the sample allow transparent objects to be visualized. This is in contrast to light microscopy which uses light absorbance differences to visualize a specimen. DIC is useful to combine with fluorescence microscopy, especially in live cell imaging, so that the location of fluorescent structure may be viewed within the cell.

Methods

Cell and Tissue Staining

The number of fluorescent dyes that can be used to stain a tissue or batch of cells depends upon the number of excitation wavelengths available with a given microscope,

whether wide field or confocal. Each fluorescent dye needs to correspond to the excitation wavelength of the laser or filter of the microscope so that the emission wavelength detected by the microscope is specific to the desired structure or organelle. For example, it would not be desirable to stain a tissue with Texas Red targeting for phalloidin and Alexa Fluor 488 targeting for wheat germ agglutinin because the two fluorophores would both emit light at a red wavelength and the structures would not be differentiable.

It is typical in Michael Davidson's lab to triple stain cells or tissues. The cell and tissue stain protocols were developed in this lab and follow the same basic steps, differing mainly in the manipulation of the specimen. The general procedure for any cell or tissue stain follows the following protocol: *fixation* of the specimen to preserve the existing form and structure of the constituent elements, *permeabilization* of the cell membrane permitting the stains to enter into the cytoplasm, *blocking* of non-specific binding in order to decrease background signal, a series of *staining* with the desired fluorophore followed by washing to rinse the specimen, and finally *mounting* of the cells or tissues onto glass slides with coverslips. More detail on cell and tissue staining can be found in the *The Handbook — A Guide to Fluorescent Probes and Labeling Technologies,* available from the Invitrogen-Molecular Probes website.

The following protocol describes tissue staining with three fluorophores (2 desired structures and a nuclear stain):

Thin sections of tissues, 8-16 μm in thickness, undergo staining and are prepared outside the lab by the commercial organization Zyagen. The staining is conducted primarily in glass slide holder jars that can hold up to 5 slides. Cells, by contrast, are grown in the in lab cell culture room on cover slips which are in 60 mL sterile petri dishes that hold 3 cover slips at a time, the staining for cells is done in the petri dishes.

The first step of fixation begins with the preparation of 3.7% paraformaldehyde in DPBSA (Dulbecco's phosphate buffered saline). Typical fixative agents also include acetone, methanol, ethanol, and glutaraldehyde. The volume of solution prepared depends on the number of slides stained at a given time. For each staining jar (holding up to 5 slides) 50 mL of solution is prepared or, depending on the size of the staining jar, enough to cover the tissues on the slides. The paraformaldehyde is heated on a hot plate under a hood until completely dissolved. While the solution is cooling to room temperature, the slides (stored in a -80˚C freezer) are also allowed to warm to room temperature in the staining jars. The jars are filled with fixer to just below the slide labels and fix for 30 minutes. They are next washed in DPBSA 3 times for 5 minutes each wash.

The tissues are permeabilized for 1 hour in 0.2% Triton X-100 (octyl phenol ethoxylate) in DPBSA. Tissues are commonly permeabilized with HCl, detergents (Triton or SDS), or Proteinase K. Detergents (hydrophobic and hydrophilic) permeabilize the membrane by extracting the lipids (hydrophobic) and allowing them to solubilize. After permeabilization the tissues are washed in 0.05% Triton in DPBSA 3 times for 5 minutes each. Washing with a lower concentration of detergent maintains permeability of the cell membrane while still allowing passage of relative large fluorophores, such as antibodies, lectins, and phalloidins.

Non-specific binding of proteins to reaction surfaces is accomplished with a solution of 1% BSA (bovine serum albumin) in 0.05% Triton/DPBSA. The tissues block for 2 hours. This maximizes the signal-to-noise ratio.

A humidity chamber (250 mL, 20 x 145 mm non-sterile Petri dish with sponge on the bottom) is prepared by adding approximately 5 mL of DPBSA to the bottom of the Petri dish. One chamber is prepared for 5 slides and prevents the drying out of the tissues during incubation with the fluorophore stain. Excess blocker is removed from the slides by gently wiping the back and bottom edges of the slides with a Kimwipe. The slides are placed in the humidity chamber and 500 µL of a mixture of proprietary fluorophores (e.g. Alexa Fluor 568 – phalloidin and Oregon Green – wheat germ agglutinin) are applied to the surface of each slide. The fluorophore stains may be applied simultaneously as they do not tend to interfere with one another. The humidity chambers are covered with aluminum foil to decrease photobleaching by exposure to incident light and set in the 37˚C incubator for 1 hour.

The slides are then placed in the staining jar (now covered with aluminum foil) and washed with 0.05% Triton in DPBSA 3 times for 5 minutes each. If a nuclear stain is desired, the slides are then washed in Hank's BSS (buffered saline solution) twice for 5 minutes each. While there are multiple nuclear stains available commercially Hoechst 33342 is often used in Davidson's lab. A solution of 5 µL Hoechst per 150 µL Hanks is to a total volume of 50 mL per staining jar, applied to the slides, and incubated for 30 minutes. The tissues are then washed again in Hank's BSS twice for 5 minutes each and are allowed to dry overnight. Gelvatol (a viscous solution of polyvinyl alcohol and glycerol) is the mounting media applied to the slides and coverslips are placed over the slide. When staining tissues Cytoseal is used instead of Gelvatol and is applied to the slide and the coverslip covered in the cells is placed face down on the slide (2 coverslips per slide).

Plasmid Amplification and Purification

QIAGEN Endofree Plasmid Maxi kits were used to amplify and purify the plasmid DNA coding for various fluorescent proteins (e.g. EGFP-actin, EYFP-Endoplasmic Reticulum, ECFP-Peroxisomes, mEos-Golgi, dEos-tubulin). The vectors were ordered commercially from Invitrogen or prepared by Scott Olenych in the molecular cloning lab at FSU. They were transformed into commercially competent[1] endotoxin-free DH5-alpha bacteria, a strain of *E. coli*, by adding 100 ng to 2 µg of DNA to 50 to 100 µL of bacteria and heat shocking at 37˚C for 90 seconds. Recovery LB media (luria broth) was added and the bacteria was incubated for 60-90 minutes at 37˚C. The bacteria were then streaked on to an agar plate in the presence of antibiotic (kanamycin) and were allowed to grow up in the 37˚C incubator for 16-24 hours before being put in a 4˚C refrigerator, if not proceeding directly to the starter culture.

[1] To be competent means that the bacteria were chemically treated to be better recipients of the plasmid to that they would take up the DNA when heat shocked.

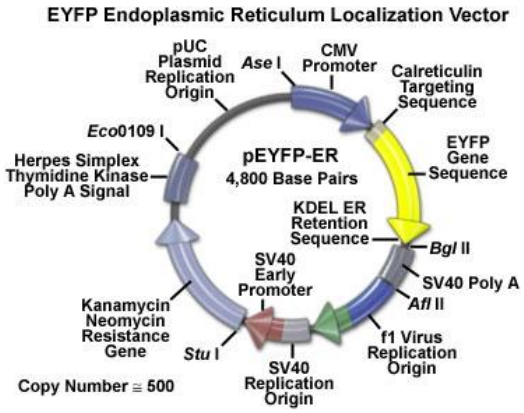

Figure 1: Vector coding for the EYFP gene sequence and kanamycin and neomycin resistence gene.

A colony was selected from the selection plate using a sterile rod. To make a starter culture this colony was swirled around in a test tube that contained 5 mL LB (Luria Bertrani or luria broth) media (previously autoclaved). The bacteria were allowed to grow to reach log phase. Next, 25 µL of kanamycin was added to the starter culture, which was incubated in a water bath of 37˚C for 8 hours. A 1/500 to 1/1000 dilution of the starter culture was added to a 500 mL Erlenmeyer flask containing 100 mL of autoclaved LB media and 500 µL kanamycin (per 100 mL media) and incubated in the water bath overnight. The solution appeared turbid after bacterial growth. The media/bacteria mixture was then transferred to 300 mL centrifuge tubes, balanced with MilliQ water to have the same mass, and then placed in the centrifuge to spin at 6000 x g for 15 minutes at 4˚C. The pellet of bacteria which formed at the bottom of the centrifuge tube was resuspended in 10 mL Buffer P1, a non-detergent solution which allows the bacteria to be suspended freely so that the clumps are broken up. In order to access the interior of the bacteria it was necessary to destroy the cell membrane (lysis) by adding 10 mL Buffer P2, an alkaline solution of NaOH and SDS (sodium dodecyl sulfate). SDS solubilizes the phospholipids and protein components of the cell membrane to lyse and release the contents of the cell. NaOH denatures the chromosomal and plasmid DNA and proteins. It was necessary to neutralize this alkaline solution in the next step by adding 10 mL of chilled Buffer P3 (acidic potassium acetate). There was a reaction that precipitates KDS (potassium dodecyl sulfate) and causes the denatured proteins, chromosomal DNA, and cellular debris to be trapped in salt-detergent complexes that appear as a fluff white precipitate. The plasmid DNA is smaller than the chromosomal DNA and is covalently closed so that it renatures correctly and remains soluble in the now neutral solution. The plasmid DNA in solution was separated from the precipitate through filtration with the QIAfilter Maxi Cartridge. The cell lysate was filtered into a 50 mL tube to which 2.5 mL of Buffer ER are added in order to remove endotoxins, lipopolysaccharides (LPS) that are a component of the cell membranes of Gram-negative bacteria and are released when bacterial cells die or are broken down. The purification of the plasmid DNA in the lysate continued in the next step of anion-exchange column chromatography. The gravity flow column provided by the QIAGEN kit can hold up to 30 mL of solution and is a column of silica beads of 100 µm diameter coated with a hydrophilic surface coating to which positively-charged DEAE (diethylaminoethanol) is attached. The DEAE attracts and ionically binds the negatively-charged DNA. DNA is bound to or elutes from the column depending on the salt

concentration of the buffer that runs through the column. The column was first equilibrated with 10 mL of Buffer QBT, then washed twice with 30 mL Buffer QC, a medium salt solution that causes protein, RNA, and carbohydrates to elute, and finally the addition of 15 mL of Buffer QN, a high salt solution, allowed the plasmid DNA to elute into 50 mL centrifuge tubes. The DNA in the eluent is precipitated out by 10.5 mL of isopropanol (0.7 volumes), in which it is insoluble, and centrifuged at 15000 x g for 30 minutes at 4˚C. The supernatant was decanted off and the DNA pellet was washed and precipitated out again in 5 mL of 70% ethanol, in which it is even more insoluble. After centrifugation for 10 minutes under the same conditions, the supernatant was decanted and a white DNA pellet was left to be dried in a desiccator and resuspended in Buffer TE (Tris and EDTA). Once completely dissolved, the plasmid DNA could be used to transfect cells which could then be imaged by confocal laser scanning microscopy.

Digital Imaging using the Nikon Eclipse 80i

Images of tissues were taken using the digital QImaging Retiga EXi camera coupled to the Nikon Eclipse 80i wide field fluorescence microscope. Thicker tissue samples (16 μm) were photographed at a magnification of 10x, thinner tissue samples (8 μm) were photographed at magnifications of 10x and 20x, and cells were photographed at magnifications of 40x, 60x, and 100x. The digital imaging software was set up to run through Adobe Photoshop 6.0 or 7.0. In order to take a picture of a triple-stained specimen for each different channel (excitation wavelength or color) gray-scale images were taken at three different intensities, creating different "layers" in Photoshop. At the lowest intensity bright structures are clear and in focus and the dimmest structures are not visible. At the highest intensity bright structures are "blown out" and dimmest structures are visible. While photographing the specimens, especially the cells, it is important to keep the exposure time short in order to decrease photobleaching of the sample. After all nine images (three channels of three layers each) have been taken, Photoshop was used to assign pseudocolors corresponding to the emission wavelength of the fluorophore recorded by the microscope and imaging system.

Image Processing using Adobe Photoshop

Before images captured by the microscopes are used on the internet or for publication it is necessary to process the images. Adobe Photoshop was used to adjust the layers and create an image that has all the detail that is visible under the microscope. Layer by layer, beginning with the dimmest, the intensity of "blown out" structures was lowered using the eraser tool at 50% flow and 20% transparency. This allows the fine detail to show through after all the layers are stacked on top of each other. Images can be processed on Photoshop by way of adjustment of opacity of the layers and utilization of tools such as burning as well. It is important to note that image processing does not falsify data or lose information, but, on the contrary, the final image shows the detail of the structures as would be visible under the microscope. The difference before and after processing is visible in Figure 2 below.

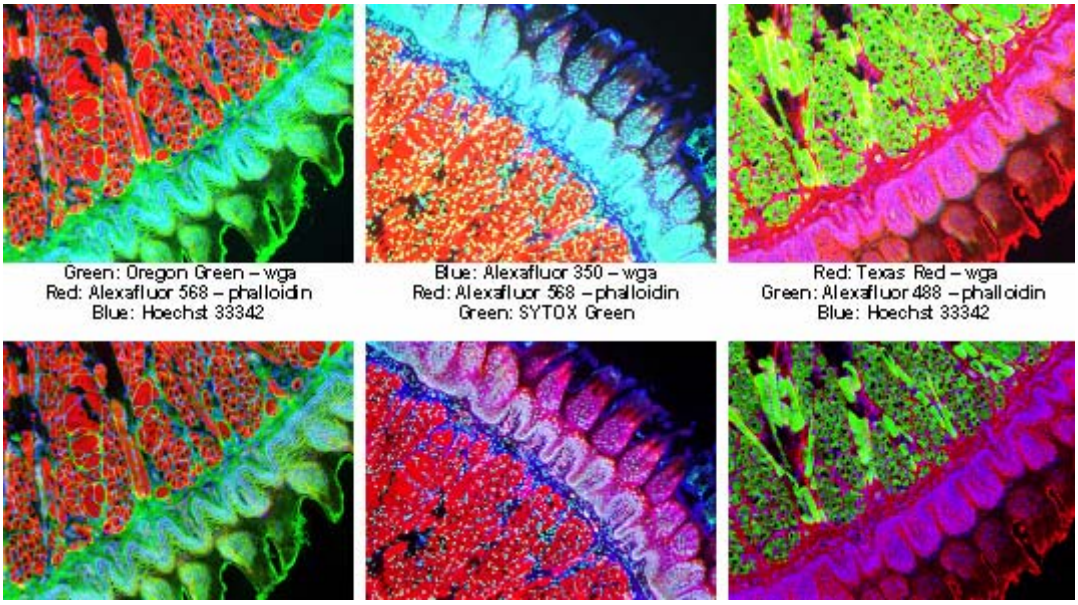

Figure 2: Before (top) and after (bottom) image processing. Much finer detail can be seen in the bottom images post-processing. The tissues shown are of 16 µm sections of rat tongue with three different triple stains. Wga is wheat germ agglutinin and phalloidin conjugated to f-actin. Hoechst 33342 and SYTOX Green are nuclear stains.

Summary

Fluorescence microscopy is an important technique to observe the fine details of cells, tissues, proteins, cellular organelles, structures, and processes. It is a sector in which microbiology, cell science, specimen preparation, microscopy and digital imaging technology, and graphic processing all converge. This report is only a glimpse of the techniques and research involved in this area. In general, it is important to have good technique in preparing the samples, whether staining with fluorescent probes or transfecting cells for fluorescent proteins, so that the best images can be acquired through fluorescence microscopy and the subsequent digital processing.

Resources

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