Total Internal Reflective Fluorescence Microscopy, Laser Coupling and Taking Images

Abstract

Total Internal Reflective Florescent Microscopy, or TIRFM, is used to get high resolution images of surfaces. The TIRFM is a specific type of optical microscope used to create florescence so that the surfaces illuminated emit their own light.

This project includes working hands on with a 200 milliwatt green laser, working with an Olympus IX71 Total Internal Reflective Florescent Microscope, and capturing images with a CCD camera.

To work hands on with this equipment, the physics behind total internal reflection, the safety procedures of working with a laser, and the inner workings of the microscope itself had to be learned or reviewed. The difficulties encountered were practical learning experiences in a lab when dealing with new equipment.

Introduction

This project was chosen because of the ability to work in a lab setting with high quality equipment. It also provided experience in working in a lab with equipment that takes theory and turns it into a practical application.

This project provided the opportunity to work with a wide range of optical equipment including but not limited to a 200 milliwatt green laser, an Olympus IX71
TIRFM, and a CCD camera.

The goals of this project were: to integrate a laser into the Olympus IX71 microscope; to learn the inner workings of the microscope itself; to capture images using a CCD camera attached to the Olympus IX71; and to raise the objective lens package and table.

Before any hands on work could begin, the theory behind TIRFM was examined. The TIRFM microscope uses optical objective lenses and eyepieces. It differs from a typical optical microscope in how the sample is illuminated. Instead of being illuminated from a backlight, the atoms in the surface of what is being imaged are caused to fluoresce, emitting their own light.

This is done through total internal reflection. A beam of light is directed through the objective lens, at an angle so that total internal reflection will occur at the interface. Oil of the same optical density is then placed upon the top of the objective lens. The beam of light travels through the objective lens, through the oil, and through the glass in the slide, until it reaches the interface at the top of the slide. It is totally reflected at this surface, and creates an evanescent wave that extends a short distance of only around 200 nanometers into the sample. This is the penetration depth of excitation. This evanescent wave excited the valence electrons, and the sample fluoresces.

Because the objective lens is doing double duty, as both an imaging lens, and an illuminating lens, and the only light from the sample is the fluorescence, a filter is used to remove the light from the source. This filter is between the objective lens and the eyepiece, and can be changed based on what wavelength of light you are using to excite the surface of your sample.
After the theory was understood, and the schematics of the microscope were reviewed, plans were started to begin work on the hands on section of the project. For the hands on project, the first goal was to couple a 200milliwatt green laser to the microscope to act as the illumination source. Typical safety precautions were used at all times when working with the laser. Safety goggles of the correct wavelength were worn, and shielding in the lab were erected to protect others.

An unused port was chosen, and two mirrors were set up, so that the laser beam could be adjusted for height, and moved back and forth so that it could be directed along an edge of the objective lens to get total internal reflectance. The laser was sighted in using a small pen laser. After the mirrors were adjusted with the pen laser, a second check of all of the shielding was made, and the safety glasses were worn. Polarized films
were placed in front of the laser to limit the output until its alignment could be checked. The objective lens and eyepiece of the microscope were covered to prevent the laser light from being scattered around the room, and the CCD camera was removed to protect it from saturation.

The laser and power supply were plugged in, the shutter was closed, and the laser was turned on and allowed to warm up. After the laser reached its operating temperature, it was switched on. The laser has a secondary safety feature requiring that the switch has to be toggled in order to begin lasing. This keeps the laser from being turned on accidentally when it is in standby mode by bumping the switch. The switch was toggled to begin lasing, and the shutter was opened slightly.

Instead of a collimated beam, the beam was highly dispersed. Working with the laser, the cause of this dispersion were found to be caused by three factors. The first factor was the shutter being partially open. The partial blocking of the lasers aperture caused a high level of dispersion in the beam. The second cause was found to be dispersion caused by the polarized film.

When the shutter was opened completely, and the polarized film was removed, a third cause for dispersion was found. The beam coming from the laser to the first mirror was now collimated, however, after hitting the mirror, the beam dispersed around the lab. Upon inspection of the mirror, a small fingerprint was found where the laser was striking the mirror. Alcohol was used to clean both of the mirrors, and the mirrors were allowed to dry.
After the mirrors were dry, the laser was again turned on and allowed to warm up. The laser was turned onto lasing, and a collimated beam went from the laser, was reflected from the two mirrors, and went into the access port on the microscope. Upon inspection no light was found at either the objective lens or the eyepiece. Some fine adjustments were made, but no light was emitted.

The laser was shut down, and schematics were reviewed. The schematics showed that the port being used had an optical filter in place before objective lens. To use this port, this filter would have to be removed or changed, so this port was scratched as an accessible port for coupling the laser to the microscope, and this goal was moved to a later date.

Without the laser illuminating the sample, a second approach was looked at. This was to use the mercury lamp that was already hooked up on a rear port. Because this illuminating source was already hooked up, the microscope could be used to get some images. The CCD camera was replaced, and the microscope was turned on.

A sample of Raccoon Uterus Fibroblasts was prepared. Oil with an n number of 1.5 was used between the slide and the objective lens. A blue source and eyepiece filter was used to illuminate the sample. When the sample was illuminated, the brilliant florescent colors showed the surface of the specimen in incredible detail.

Because three dimensional images were being taken, depth of focus came into play when working with this microscope. The microscope had a high level of resolution with a 100x magnifying objective lens, and therefore had a sensitive depth of focus. The focus could be changed to focus on specific parts of the specimen. This was evident in the images captured with the CCD camera.
The CCD camera is black and white. The image quality gives resolution of 16 micrometers per pixel, and a 512 by 512 pixel image. It just shows that it is receiving light or not. It does not show the incredible color from the illuminated sample. To show color, the images from the CCD cameras are artificially colored. Color filters in front of the CCD camera allow the color reaching the camera to be a known value, and then these images are artificially colored.

Images taken from the microscope that show the difference in the depth of focus. The focus on this microscope was 150 to 200 micrometers different, but the different portions of the specimen are in focus.
Conclusions

The overall experience of working in Professor La Rosa’s lab was beneficial. I enjoyed the time with Rodolfo, Christoph, and Shauhua. It was also a learning experience. In all I had to learn a lot about the equipment that was being used, and the theory about how it was working. I didn’t complete all of my goals, but I learned a great deal in the process of trying to work through them.

What I learned: The workings of an optical microscope; Total internal reflection, the evanescent wave that is produced, and how this relates to penetration depth of the excitation; Fluorescence, the excitation of atoms, and their reemitted light; The proper use of optical filters in a system; Diffraction resulting from apertures, and polarized films; Safety precautions when working with lasers; The importance of mirror cleanliness; The uses of optical oil; Depth of focus; and how to work with others when sharing an optical bench for working space.

If I was to do the lab over again, there are a couple of things that I would do differently. The first would be spending more time going over the schematics of the microscope, so that I would not make a mistake such as trying to use a port that had a filter. The second would be better time management. With a lot of pressure from outside sources I was not focused enough on getting through the goals while I was working in the lab, and could have accomplished more with the amount of time I spent, if I had worked more efficiently.
Sources

Hecht, Eugene, Optics, 4th Edition


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