Lab Write-Up Week 9

Fluorescence Microscopy

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Questions

a. **Illumination light color without filters: why is the light this color? What wavelengths are present?**
    - The illumination light without the filters is visibly white. The Halogen lamp exhibits black body radiation, create a large bandwidth, peaking around 560nm a yellow-green light.

b. **Illumination light color with excitation filter: what wavelengths are present?**
    - With the Excitation filter, the light is visibly a deep green color. From The spectra the filter has a band pass between 500 and 540 nm, which only allows a narrow spectrum of green light through.

c. **What color illumination light is hitting the pink plastic slide?**
    - As the pink slide in in the optical path, the illumination light hitting the slide is the same as passes through the excitation filter; that is 500 – 540 nm green light.

d. **How much of that color illumination gets through the pink slide? Where is the light going?**
    - At the back of the pink slide, there is almost no illumination, so very little light is being transmitted by the slide. The light visible from the front side of the pink slide is not green, so very little of the light is being reflected. Therefore, nearly all the illumination light must be absorbed by the sample.

e. **What color light do you see on the pink plastic slide, right where the illumination hits the slide? Explain what you see.**
    - The light at the front of the pink plastic slide is yellow, whereas one might expect to see green light due to the illumination color. From the spectrum of the light, we can see the wavelengths of light being emitted by the plastic slide are not found in the illumination light. Therefore, the explanation for this phenomenon is that the dyes inside of the pink plastic fluoresce at the Excitation filter wavelengths.

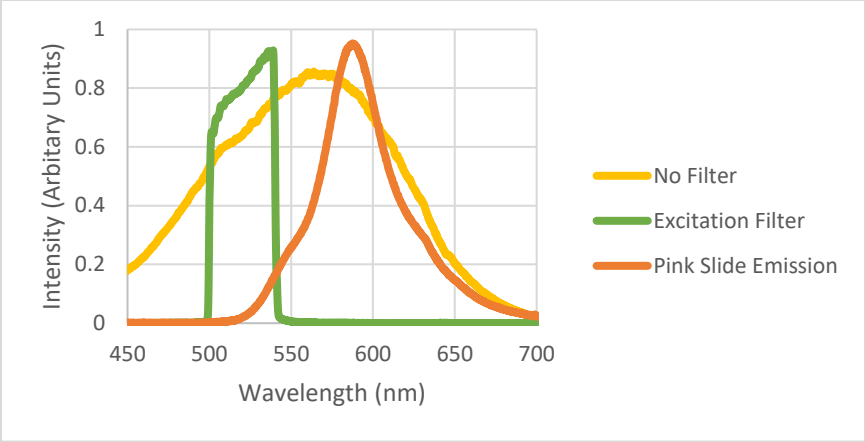

Figure 1. Spectrums of lamp light, Excitation filter and Slide emission. Emission from the slide has been shifted from the excitation light.

Filter Imaging

With only the emission filter on the tube lens, the fibers of the cloth can be clearly seen.

Figure 2. lens paper image with emissions filter on. Exposure 0.1ms.

When the excitation filter in placed into the optical path, the image is altered. The imaged becomes much darker, and gain must be turned up to image the paper with a reasonable exposure time. Whereas in Figure 2 is in brightfield, Figure 3 is dark where light is expected to pass through the paper unobstructed. Figure 4 shows that there is no crossover between excitation filter transmission and emission filter transmission, and as such all light passing through both filters should be obstructed. The fibers are still faintly visible if gain increased due to ambient light from the room reflecting off of the lens paper.

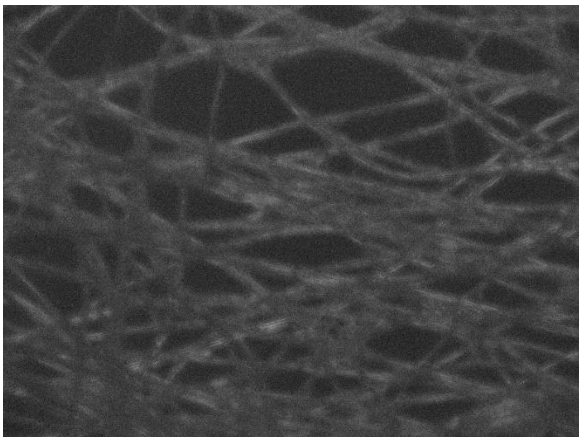

Figure 3. lens paper imaged with emission and excitation filter. Exposure 100ms. Gain 30db.

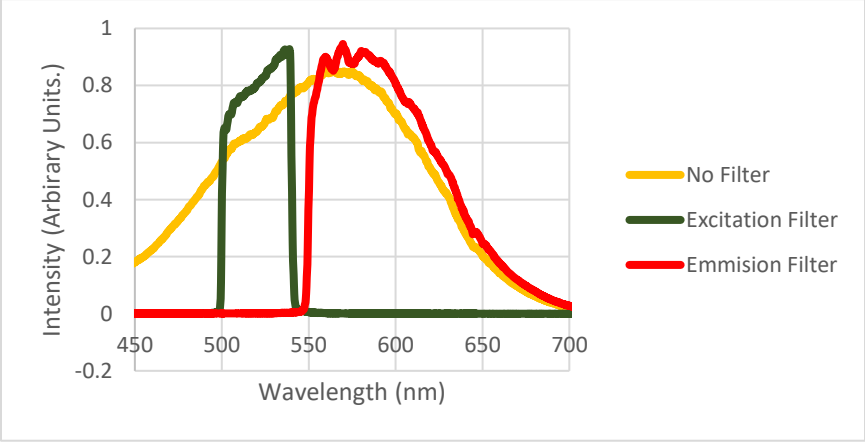

Figure 4. Excitation and Emission Filter spectra. There is no crossover between them.

If the VG-9 green color filter is used instead of the Excitation filter, the image again returns to be in brightfield. Although from view the illumination light with the eye, the green light from the excitation filter and from the color filter appear to be very similar, the images are vastly different.

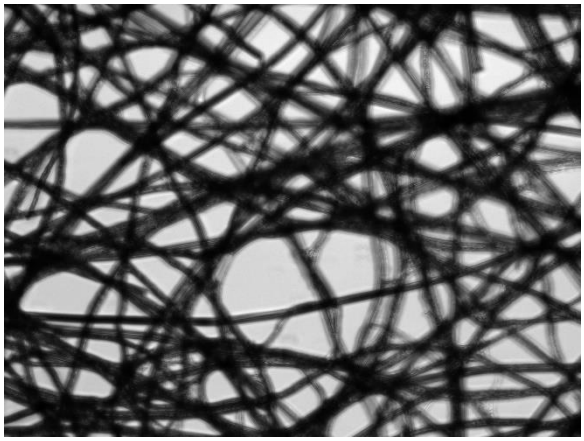

Figure 5. lens paper imaged with VG-9 color filter and emission filter. Exposure 2.2ms.

The cause of this difference is apparent from the spectra shown in Figure 6. Wavelengths of light between 550 to 625nm are transmitted by both filters, allowing such light to pass through both filters unobstructed causing brightfield imaging. As a significant amount of light is blocked by the filter pair, the exposure must be higher than in Figure 2, to have similar contrast. The exposure in Figure 5 is 22 times longer than that of Figure 2.

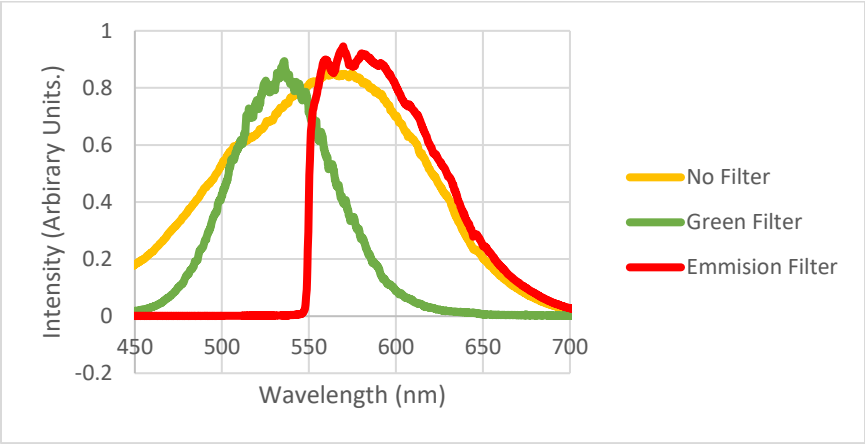

Figure 6. VG-9 Green Color and Emission Filter spectra. There is approx. 75nm crossover between wavelengths transmitted by both filters.

With the Excitation filter put back into place, the system is focused on a piece of lens paper that is covered in dye from a pink highlighter. Whereas a large exposure and gain was required to view the cloth with no dye in Figure 3, with the dye the cloth can be seen with a relatively low exposure of 60ms and no gain. This is due to the dye from the highlighter being fluorescent and emitting in wavelengths transmittable through the emission filter.

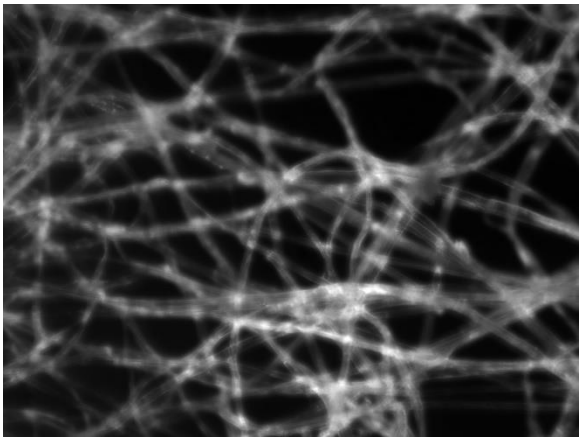

Figure 7. lens paper with pink, fluorescent highlighter dye imaged with emission and excitation filter. Exposure 60ms.

Beads

A ThermoFisher bead slide was imaged with the previously used fluorescence filters. The bead samples are either a plastic or glass material with a dye to make them more visible. The beads fluoresce faintly, and a large exposure time and gain is required to be able to see them.

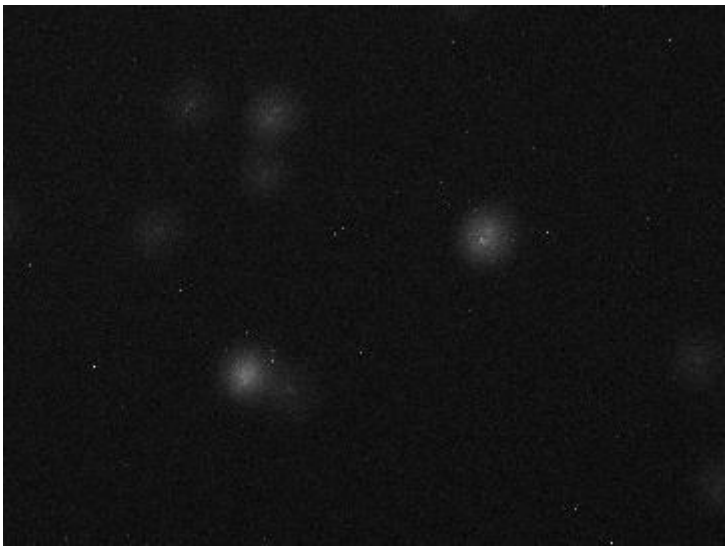

Figure 8. 4um bead samples. Exposure 2000ms. Gain 25db. BFP Diameter 7.5mm Objective NA = 0.15.

Closing the BFP aperture down to 6.5 mm diameter makes the bead samples appear dimmer in the image.

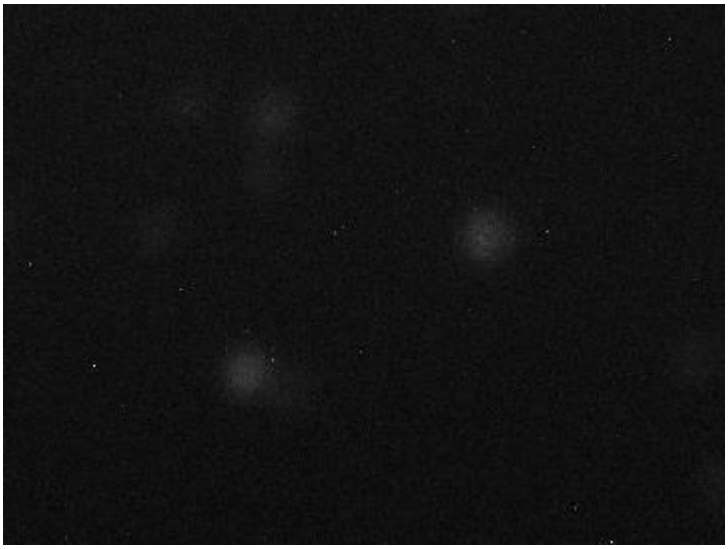

Figure 9. 4um bead samples. Exposure 2000ms. Gain 25db. BFP diameter 6.5mm. Objective NA = 0.12.

The reason for this is that when we close the objective BFP, we reduce the collection efficiency. For fluorescence the light emitted is incoherent, and the Condenser NA is equal to the Objective NA.

From this the collection efficiency of both images can be determined:

When BFP diam = 7.5mm: $C.E. = \frac{(NA_{obj}+NA_{cond})^2}{4} = \frac{(2*0.15)^2}{4} = 0.0225$

When BFP diam = 6mm: $C.E. = \frac{(NA_{obj}+NA_{cond})^2}{4} = \frac{(2*0.12)^2}{4} = 0.0144$

From the calculated collection efficiencies. Using the 7.5mm diam BFP gives a Collection Efficiency that is 1.56 times greater than with a 6mm diam BFP. Taking the max brightness of a bead in both images, and subtracting away the background, we can see the Figure 8 is 1.58 times brighter than Figure 9; which corresponds to the calculated Collection Efficiency. The reduced NA also increases the point spread function, which will reduce the intensity per pixel, which explains why the measured brightness ratio is greater than the calculated C.E. ratio.

Histology slides.

Various medical samples were imaged with fluorescence in order examine the benefits over brightfield imaging.

Human Prostate Gland

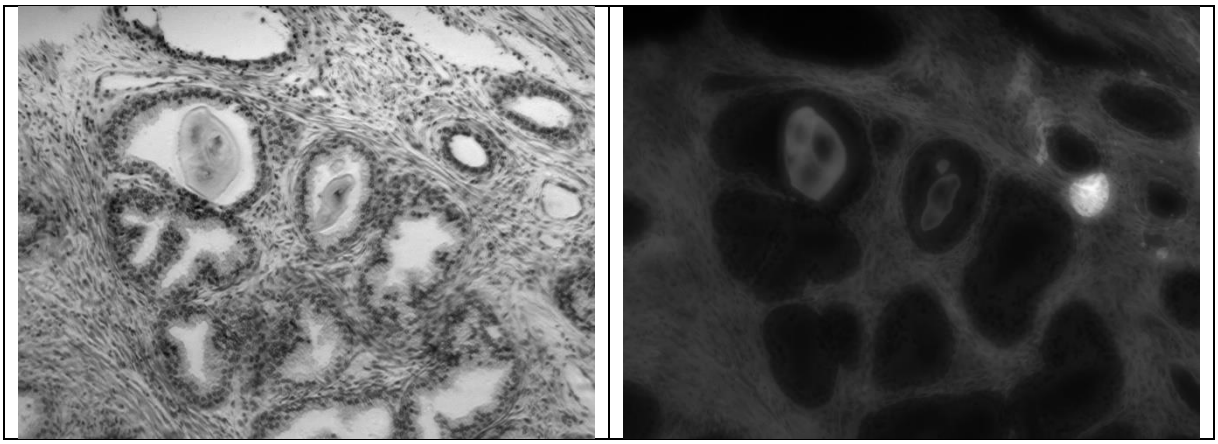

Figure 10. (Left): Brightfield Exposure 2ms. (Right): Fluorescence Imaging Exposure 1025ms. Objective BFP diameter for both images is 12mm.

Human Artery and Vein Cross Section

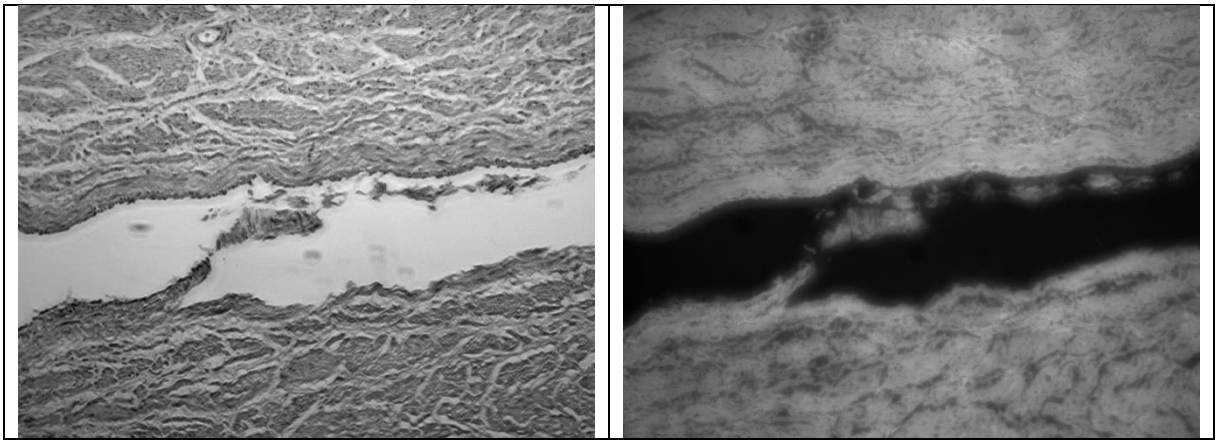

Figure 11. (Left): Brightfield Exposure 2ms. (Right): Fluorescence Imaging Exposure 2255ms. Objective BFP diameter for both images is 12mm.

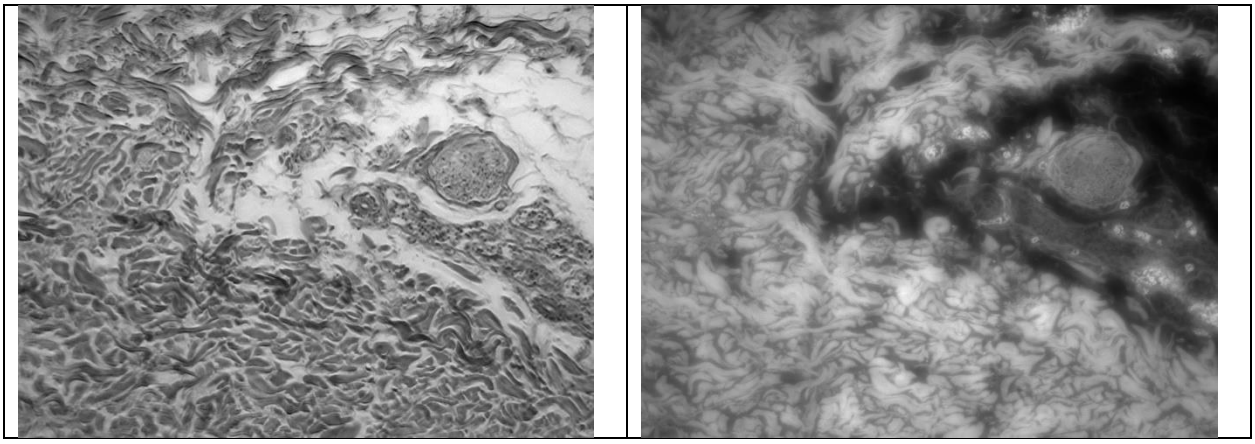

Figure 12. (Left): Brightfield Exposure 1.5ms. (Right): Fluorescence Imaging Exposure 600ms. Objective BFP diameter for both images is 12mm.

Using fluorescence imaging on histology slides brings out different features in the image compared to brightfield. Brightfield shows the differing depths of tissue better, whereas fluorescence shows empty space, such as the artery, better than in brightfield.