Practical 1
Fluorescence Microscopy

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1 Purpose of the work

Your goal is to learn the basic imaging with widefield fluorescence microscope. The difference of multiband and single band filters is shown in practice. You will also get an idea of z-stack imaging.

2 Background

2.1 Fluorescence imaging

Fluorescence microscope is a light microscope used to study properties of organic or inorganic substances using the phenomena of fluorescence instead of, or in addition to, reflection and absorption.

In most cases, a component of interest in the specimen is specifically labeled with a fluorescent molecule called a fluorophore (such as GFP, Green Fluorescent Protein). The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a dichroic mirror. Typical components of a fluorescence microscope are the light source (Xenon or Mercury arc-discharge lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter (see Figure 3 to Figure 5 below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.
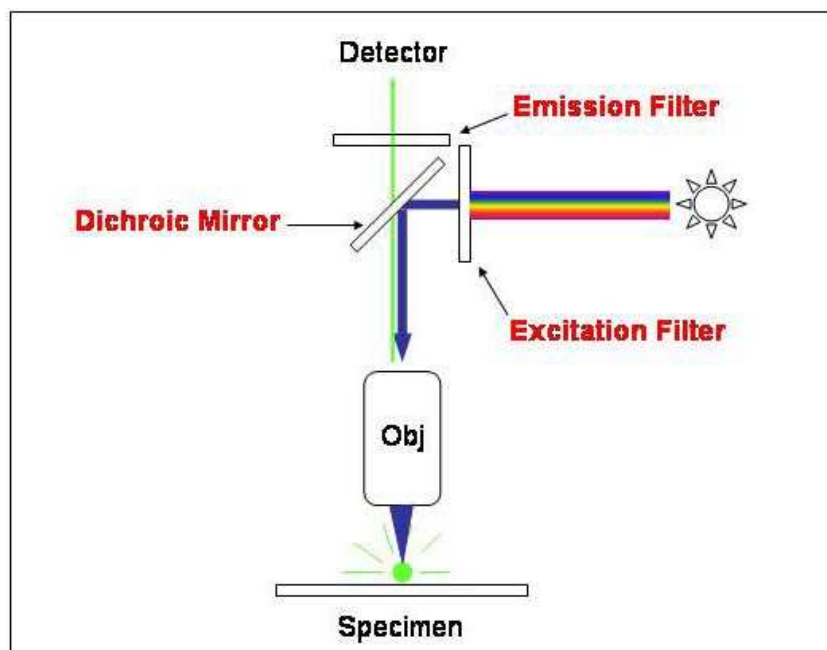

Figure 1. Fluorescence imaging principle *(Wikipedia:Fluorescence_microscopy)*

2.2 Fluorescent dyes

The Alexa Fluor family of fluorescent dyes is produced by Molecular Probes, a subsidiary of Invitrogen. Alexa Fluor dyes are typically used as cell and tissue labels in fluorescence microscopy and cell biology. The excitation and emission spectra of the Alexa Fluor series cover the visible spectrum and extend into the infrared. The individual members of the family are numbered according roughly to their excitation maxima (in nm). In this practical the samples have been stained with Alexa Fluor 488 and 594 (Figure 2).
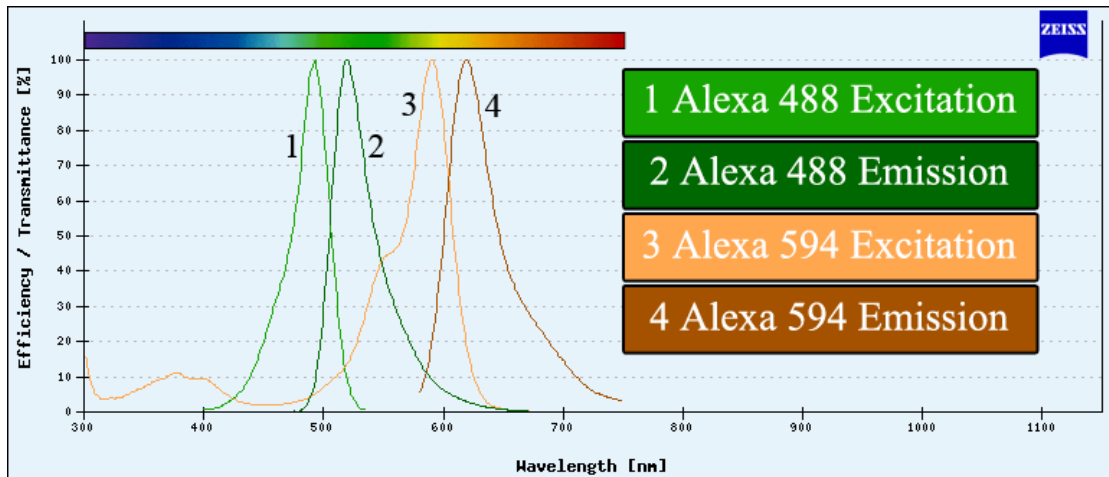

Figure 2. Excitation and emission spectra of the dyes used in this practical

2.3 Filters

Filters used in this work are called FITC (Figure 3), TRITC (Figure 4), and DAPI/FITC/TRITC triple filter (Figure 5) according to the traditional fluorochromes that were earlier commonly used for green and red fluorescence. In the figures, the blue (1) curve shows the excitation i.e. the wavelengths that illuminate the sample. The red (2) curve shows the emission i.e. the wavelengths that are shown to the viewer.
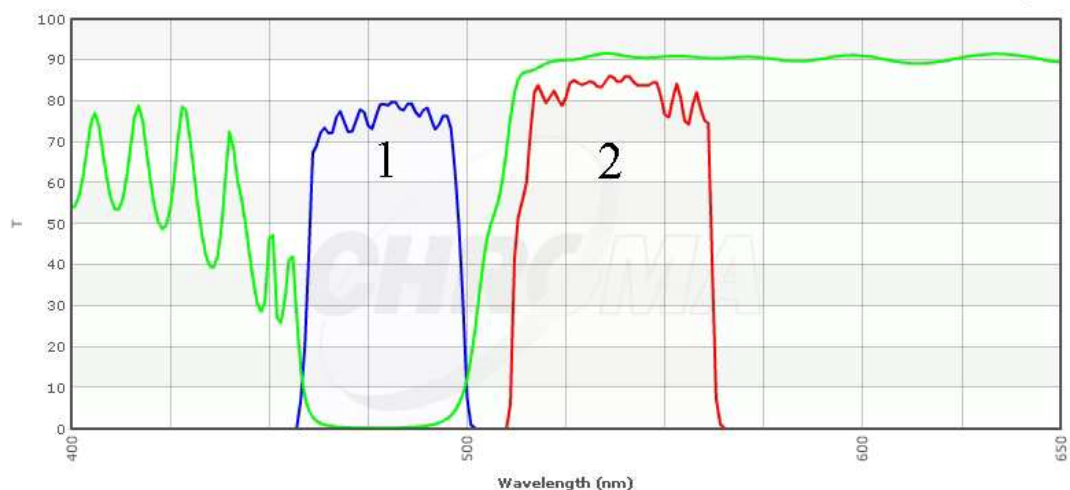

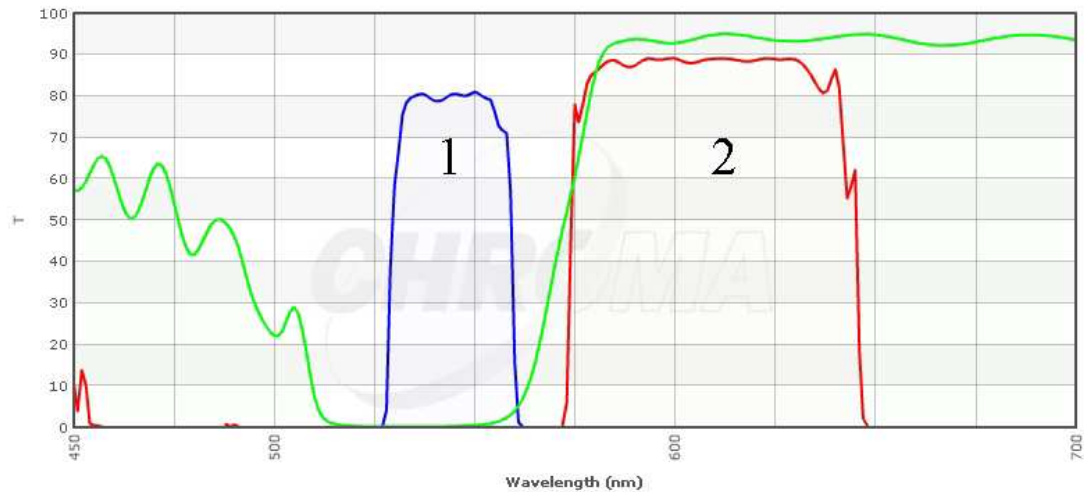**Figure 3. FITC filter spectrum. 1 = excitation band, 2 = emission band**

Figure 4. TRITC filter spectrum, 1 = excitation band, 2 = emission band
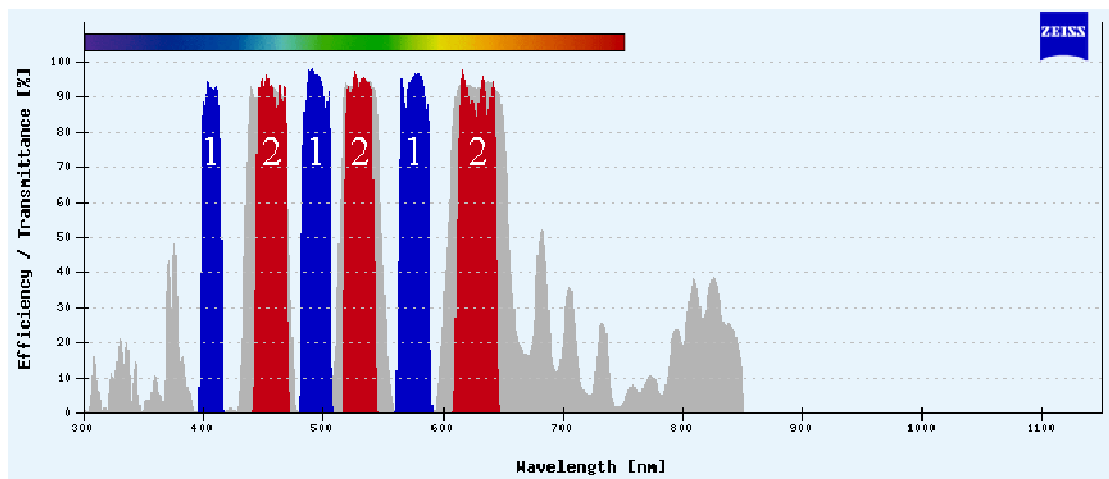

Figure 5. Triple filter spectrum, 1 = excitation band, 2 = emission band

2.4 Tissue imaging

The difficulty with tissues is that they are often so thick that some light is always emitted from above and below the focal plane, which means that the resulting image will have a background signal (color). The background decreases the contrast of the signal and may make image analysis more difficult. Contrast in this context is the difference of intensities between an object and its background.

2.5 Z-stack

A Z-stack is a series of images taken of a specimen at different focal planes. Stacks are useful when the objects of interest in the sample are at different levels in depth (Z-axis direction). The stack can be used to determine the 3D-structure of the sample, or the stack can be merged (or deconvolved) to one image with information gathered from all the in-focus areas. Z-stacks are also powerful in removing unwanted background signal.

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3 Instruments/software used

3.1 Microscope

The microscope used is a Zeiss Axioplan 2 upright microscope. It can be used for both reflected light imaging (fluorescence) and transmitted light imaging (halogen light source). The images are to be taken with the 63x (NA=1.25) oil objective.

3.2 Camera

The camera in the microscope is a Zeiss AxioCam HRc color CCD camera. It has 14 bits of color depth and it is capable of taking 5 images / second at full resolution.

The CCD pixel count is 1388 x 1040 (= 1.4 megapixels) and pixel size is 6.45 μm x 6.45 μm, making the sensor-size equal to 8.9 mm x 6.7 mm.

3.3 Software

The software used is Zeiss AxioVision 4.5 SP1, which offers multidimensional acquisition methods such as time-lapse, Z-stack, and multichannel. It has a few measurement and analysis functions too.

4 Information on the sample

Tissue samples are whole mounts of mouse outer ear. Stimulated (CA64) sample is transduced with a lymphatic vessel growth-increasing adenovirus. Alexa 594 (red) staining shows specifically the lymphatic vessels, while Alexa 488 (green) shows blood vessels by staining endothelial cells.
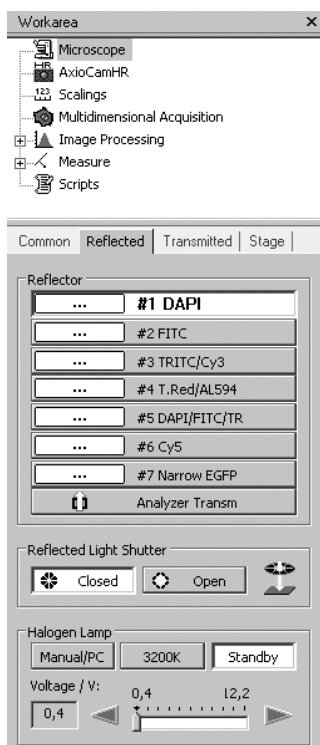
5 Your task

Figure 6. Filter selection

- Your task is to acquire images of the sample with multiband and single band filters.

- Do a quality comparison between the images

- Take a Z-stack image for later use in the Deconvolution practice

6 Practical instructions

6.1 Basic use of the microscope

There are seven fluorescence filters in the microscope. Filter position can be changed by clicking the corresponding reflector in MICROSCOPE / REFLECTED (Figure 6). The sample is stained with fluorophores suitable for FITC and TRITC filters. FITC filter is used for green wavelength fluorescence, such as Alexa 488. TRITC filter passes red wavelengths, such as Alexa 594. In the same window you can also OPEN or CLOSE the reflected light shutter (Fluorescence). Start with the #5 DAPI/FITC/TRITC filter.

There are focusing knobs on both sides of the microscope. The outer wheel is for coarse adjustment and the inner

wheel is for fine adjustment. The actual speed of the stage depends on the magnification of the objective.

XY movement is done by rotating the two co-axial vertical controllers on the right side of the sample stage.

6.2 Image acquisition and adjustments

6.2.1 Setting up the camera

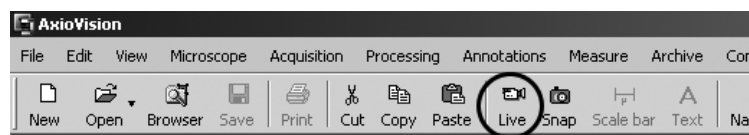Enable real-time video by clicking the LIVE-button in the main window.

Figure 8. Live button

A single image can be acquired by clicking the snap button next to the live button.

6.2.2 Shutter speed and image histogram

Click AxioCamHR menu and select ADJUST tab (Figure 7). There are two ways of adjusting the time of exposure:

- The time of exposure can be set manually, either with a slider or by entering the numeric value.

- Press MEASURE. The software estimates the suitable time of exposure and uses the same one for all images.

From the histogram at the bottom of the screen, you can estimate how well the sensor dynamic range is covered with the current settings. The more area covered in the histogram the more information is gathered. By making the exposure time longer the histogram values are shifted to the right, since the overall intensity of the image gets brighter.

Check that the pixel values are not packed in either end of the histogram (over/underexposure).

- Focus and view the sample through oculars

- Snap an image using the triple filter. Be sure to make notes on the exposure time used

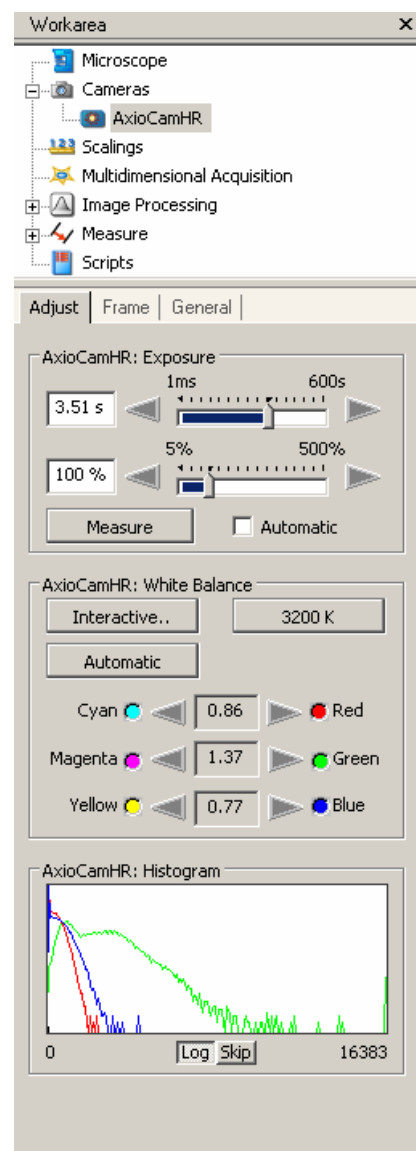- **Compare the view in oculars and the snapped image**

Figure 7. Camera settings
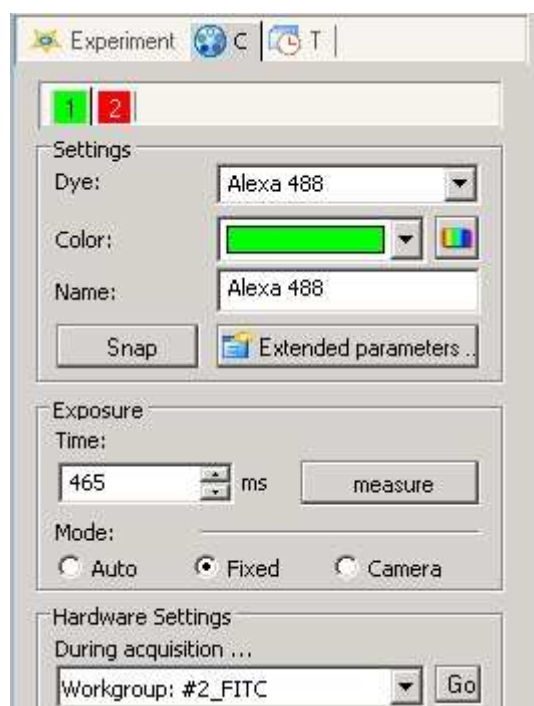
6.3 Multichannel acquisition

Figure 9. Multidimensional acquisition

- Choose MULTIDIMENSIONAL ACQUISITION/C in the Workarea menu

- Set the exposure time for FITC filter by clicking MEASURE in the window (Figure 9). Repeat using TRITC filter (the red tab)

- Acquire the image with the START button at the bottom

- **Compare the differences between the triple filter image and multidimensional image. Make notes on the exposure times and quality**

6.4 Post-imaging adjustments

Axiocam camera detects 16384 (14 bits) intensity levels of each color channel. However, monitors can display only 256 (8 bits) levels (or often less). The way how 16384 levels are mapped to 256 can be adjusted. Click the image with the right mouse button and select PROPERTIES (or press ALT+ENTER). Then select DISPLAY (on the left). The black line with five handles describes how the original intensity values are displayed on the monitor. The curve can be modified by sliders or mouse. There are also some buttons for predefined adjustments. Play around with the controls to see the effects. You can always return to the default mapping by pressing the Linear - preset.

6.5 Z-stack image

Taking images in color mode single dye at a time is a waste of space. Every color image contains the red, green and blue 'sub' images. If the whole image only contains green color, the red and blue 'sub' images are black, using three times the space compared to a grayscale image. Also the convolution done in a later practical is three times faster without the empty color channels. Therefore, before doing the stack, switch to black and white mode of the camera:

- In the working area, go to CAMERA / AXIOCAM HR (Figure 7)

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- Choose the FRAME tab and change the camera to B/W mode
- Go back to MULTIDIMENSIONAL ACQUISITION/C and find new exposure times

Z-stacks are acquired by going to the menu MULTIDIMENSIONAL ACQUISITION/Z (Figure 10).

- Press the OPTIMAL DISTANCE -button to calculate the correct slice distance according to the Nyquist theory

Since numerical aperture and signal wavelength used determine the smallest detected difference, the program can calculate the optimal sampling frequency or sample width. This is important when processing the stack with deconvolution software. Note that the correct dye (determines the dominant wavelength λ) should be selected in MULTIDIMENSIONAL ACQUISITION/C for the optimal distance to be calculated correctly.

- Take a Z-stack image so that the Z range covers a structure of a lymphatic vessel. Try not to take more than 10 slices, otherwise the deconvolution will take too much time and the file size will grow too large.
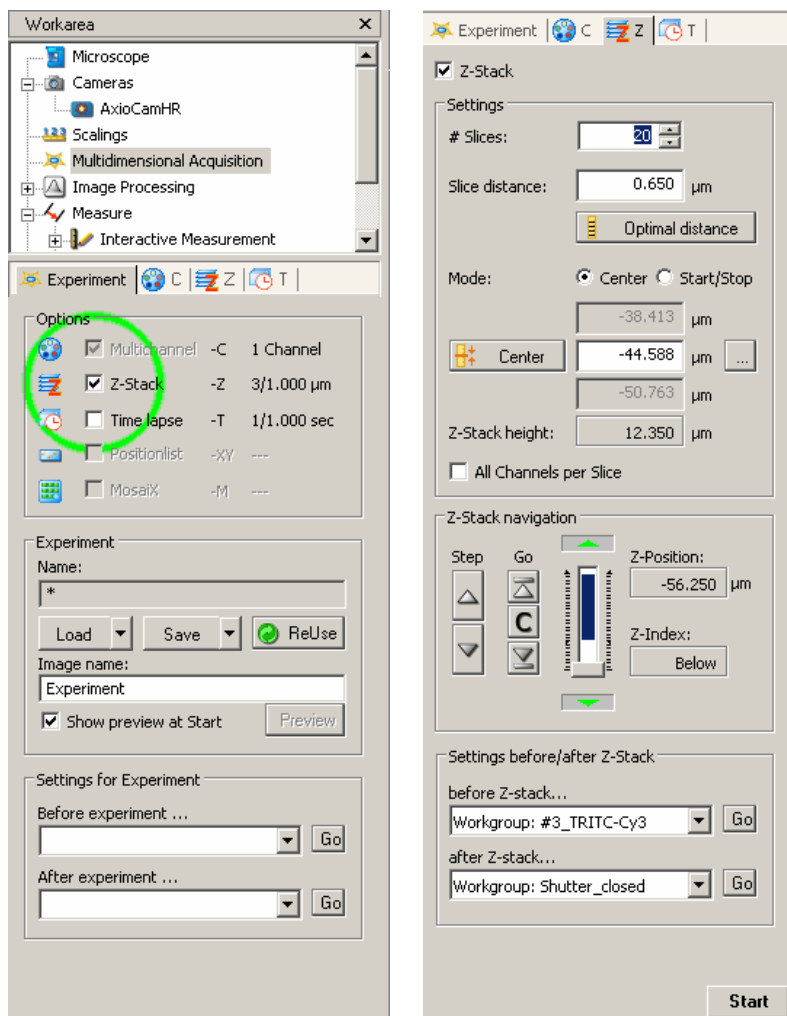

Figure 10. Z-stack acquisition settings

6.6 Save the results

Single images are saved using FILE / SAVE AS. Use the Zeiss native .ZVI format to save the images to be sure all the metadata is saved with the image. You can download a free viewer, http://www.zeiss.com/c12567be0045acf1/Contents-Frame/cbe917247da02a1cc1256e0000491172 , from Zeiss web site. ImageJ (http://rsb.info.nih.gov/ij/), the free image analysis program, can also read ZVI images.

- Save the Z-stack image as Zeiss native .ZVI file

You can also save TIFFs by using FILE / EXPORT. Note that the display mappings are saved on the exported image if the checkbox is selected. In scientific publications, linear changes of the intensity values (e.g. brightness/contrast) need not be reported. However, non-linear modifications have to be mentioned (e.g. γ-curve). If the file is exported for image analysis, no adjustments should be done to the data.

7 Questions for summary

Prepare to answer these questions at the summary meeting:

- Compare the excitation spectrum of Alexa Fluor 488 (Figure 2) with the exciter spectra of the filters used (Figure 3 to Figure 5). Which one best matches Alexa Fluor 488 excitation spectrum? How about emission? Make the same comparisons for Alexa Fluor 594. How does the spectrum bandwidth affect the image?

- The PC monitors often work in 6 to 8 bits color-channel depth. The camera signal is 14 bits in color depth. Which problems arise? (Human vision senses roughly 7 bits, but is flexible in adjusting to what is black and what is white.)

- Why is it important to save image files in the acquisition software native format?