

Work no. 1: Fluorescence imaging, oil objectives, and tissue sample

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1 Purpose of the work

Your goal is to learn the effect of oil as the medium between the objective and sample. A comparison in image quality is done between a dry objective and an oil objective. 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 phenomenon 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 below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen. (Wikipedia:Fluorescence microscopy)

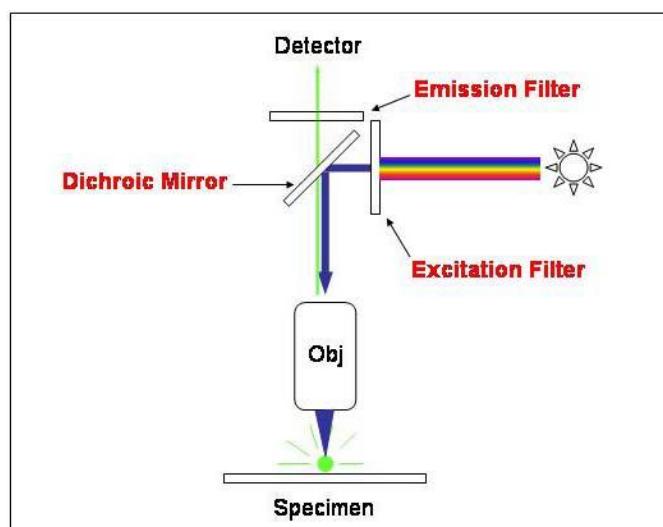


Figure 1: Fluorescence imaging principle (Wikipedia:Fluorescence microscopy)

2.2 Oil objectives

The main idea in using oil between the sample and objective is to bring the refraction index n of the medium between the objective and cover slip (glass) to the same value with the n of the cover slip. Whenever there is a change of medium (and a change of n), refraction and reflection will happen. By making the change as small as possible, more light will pass to the objective front lens. The difference is illustrated in Figure 2. The better light collection capability of oil objectives means that they have better resolution compared to dry objectives. This is because they are able to gather more of the diffracted light which carries information on the fine details of the specimen. Light collection ability is expressed as numerical aperture.

2.3 Numerical aperture (NA)

The numerical aperture of an objective follows the equation

$$NA = n \sin(\alpha),$$

where α is the half-angle of the maximum cone of light that can enter or exit the lens. The angle α is always bigger for oil objectives, since the difference of the refraction indexes of the media is small -thus the numerical aperture for oil objectives is greater.

The size of the finest detail that can be resolved is proportional to λ/NA , where λ is the wavelength of the emitted light. This is why numerical aperture is an important factor in microscopy.

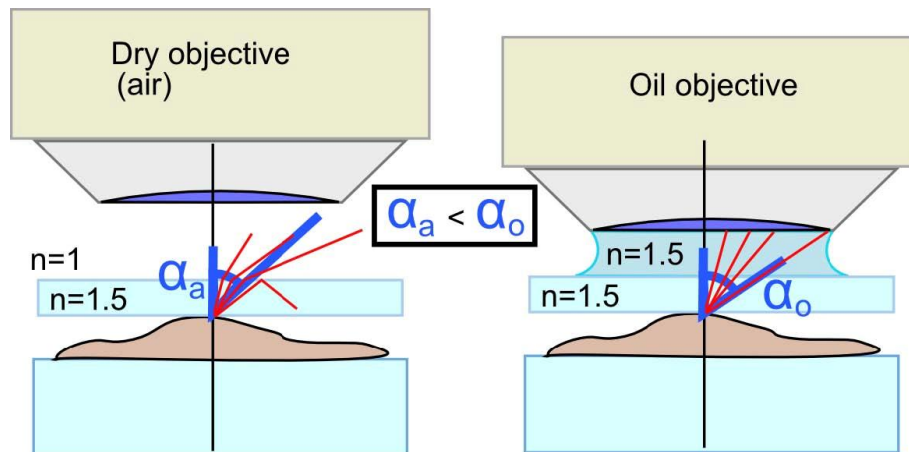


Figure 2: Dry objective and oil objective.

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 depthwise (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.

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 microscope is equipped with the following objectives:

Table 1: Objectives in Zeiss Axioplan microscope

Magnification	NA	Medium
10x	0.3	air
20x	0.5	air
40x	0.75	air
63x	1.25	oil
63x	1.25	oil
100x	1.3	oil

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 basic 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 (2/3 in. diagonal).

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.

The 'normal' sample shows lymphatic vessels in a mouse ear. Alexa 594-conjugated antibody (red) staining is used to show lymphatic vessels. Alexa 488-conjugated antibodies (green) are used to show cell nuclei as they bind to a factor located in the nucleus.

Stimulated (CA64) sample is transduced with a lymphatic vessel growth-increasing adenovirus. Alexa 594 (red) staining shows specifically the lymphatic vessels, while FITC (green) shows blood vessels by staining endothelial cells.

Choose one of these two samples to be used in the practice.

5 Your task

- Your task is to acquire images of the sample with and without oil as transfer medium.
- 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

Start out by placing the sample on the stage. The specimen (text side) must be facing up.

There are seven fluorescence filters in the microscope. Filter position can be changed by clicking the corresponding reflector in MICROSCOPE / REFLECTED (Figure 3(a)). 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 TRITC filter and if you have time, take the FITC images too.

There are six Plan-Neofluar objectives in the microscope. Objectives can be changed by clicking the corresponding icon in MICROSCOPE / COMMON (Figure 3(b)). In this practice you will use the 63x objective. You may want to search for an interesting area first with less magnification e.g. with 20x.

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

Go to camera settings in the AxioCamHR window (Figure 3(c)) and click the tab 'Frame'. Select black/white (B/W) camera mode with maximum resolution. When taking images through narrow bandwidth filters, the signal is stored

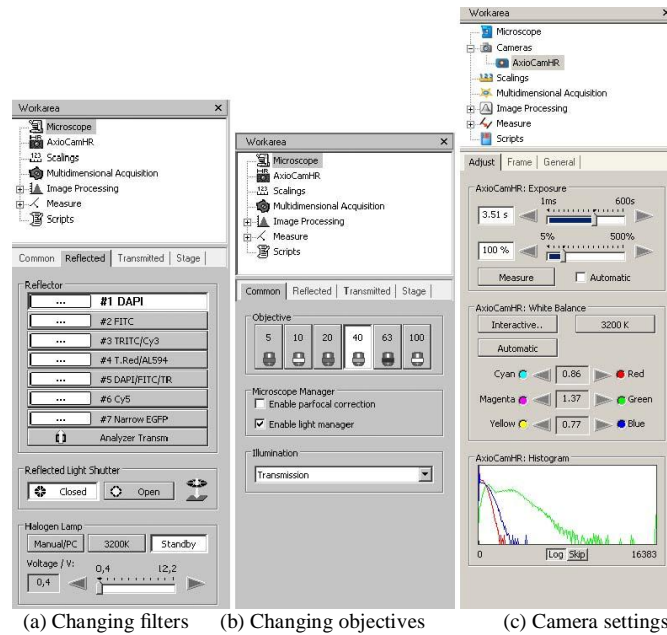


Figure 3: Microscope and camera settings. The actual collection of filters and objectives may differ.

in only one of the RGB channels (e.g. red channel through TRITC filter). The acquisition in RGB mode would result in two black channels (e.g. blue and green) and one containing the actual signal. In other words, the same data can be stored in only one-third of the memory in B/W mode. Memory needs may become a real issue especially in Z-stack imaging.

Enable real-time video by clicking the LIVE-button in the main window. It may be practical to drag the live-window to the secondary display and maximize it there.



Figure 4: Realtime video button (Live)

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 3(c)). 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). Snap images with 40x and 63x magnifications. Be sure to make notes on the exposure time used.

6.2.3 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.

6.3 Applying oil

Lower the stage with the lower button on the left front side of the microscope (down and up arrows). Be careful not to touch the focusing wheel while the stage is down, otherwise it will remain in the lowered position. Apply a drop of oil on the sample so that when you lift the stage back up using the upper button, the objective touches the oil. Refocus carefully, as the objective is now very close to the cover slip. Remember NOT to use any dry objectives after the oil has been applied!

After applying oil, snap an image of the same area as without oil. You may have to re-measure exposure time in both cases. Compare the images and exposure times. When comparing, check that intensity mapping curves and values are similar.

6.4 Z-stack image

Z-stacks are acquired by going to the menu MULTIDIMENSIONAL ACQUISITION/Z (Figure 5(a)). It is possible to define either the center point of the stack, or start/end points. Number of slices can be entered directly, or slice distances specified. OPTIMAL DISTANCE -button calculates the correct 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, since the deconvolution will take too much time and the file size will grow too large.

6.5 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, [Axiovision LE1](#), from Zeiss web site. [ImageJ2](#) , [the free image analysis program, can be fitted with a ZVI import plugin3](#) .

Save the Z-stack image as Zeiss native .ZVI file. The deconvolution software can read ZVI metadata.

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).

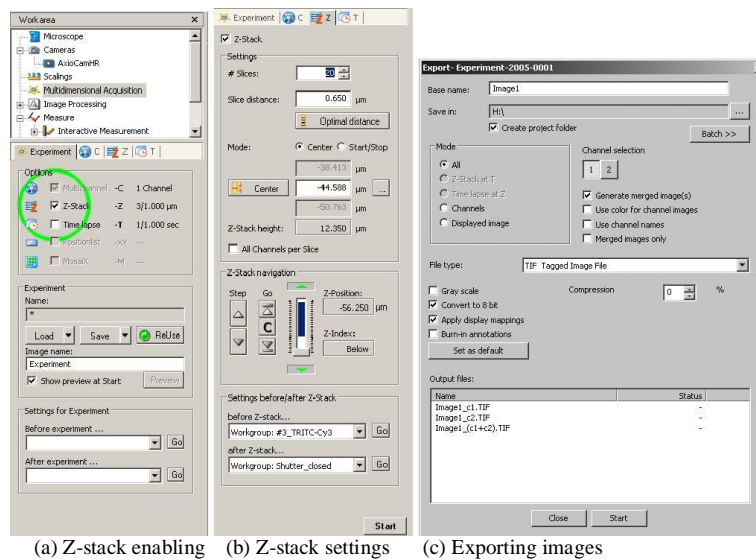


Figure 5: Z-stack settings

7 Problems

Think of these problems for later:

- Did oil give better imaging results?
- Compare the exposure time and display mappings between the images.
- The PC monitors often work in 6 to 8 bits colour-channel depth. The signal may be 12 to 16 bits in depth. Which problems arise? (Human vision senses roughly 7 bits but is flexible in adjusting to what is black and what is white.)

- 1) <http://www.zeiss.com/c12567be0045acf1/Contents-Frame/cbe917247da02a1cc1256e0000491172>
- 2) <http://rsb.info.nih.gov/ij/>
- 3) <http://rsb.info.nih.gov/ij/plugins/zvi-reader.html>