

Practical work no. 2: FLUORESCENCE MICROSCOPY AND IMAGE ACQUISITION

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1. Purpose of the work

The purpose of this work is to learn how to use an epifluorescence microscope and different kinds of fluorescence filters.

2. Background

In wide-field epifluorescence microscope, light from either a xenon or mercury source is reflected from the specimen to the eyepiece or, in the case of image capture, to the camera. The path of reflected light beam is shown in figure 1.

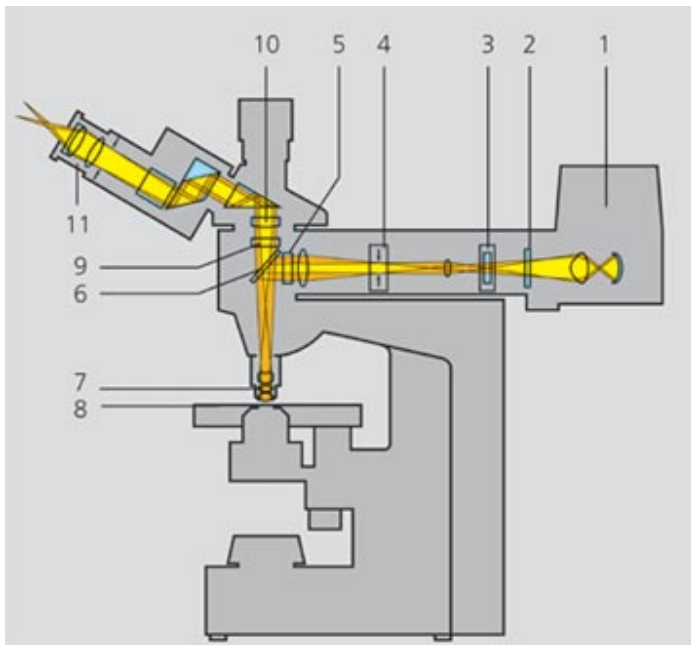


Figure. 1. The illustration on the left shows the beam path in the microscope with fluorescence equipment. The light travels from the powerful additional light source (1) to the exciter filter (5) via the heat-protection filter (2), the red-attenuation filter/barrier slider (3) and the luminous-field diaphragm (4). The exciter filter is integrated into the reflector slider which also contains the beam splitter (6). The dichroic beam splitter reflects the short-wave exciting light into the specimen (8) via the objective (7). The resulting emission is gathered by the objective (7) and transmitted by the dichroic beam splitter (6) because it exhibits longer wavelengths than the excitation light. The rays then also pass through the emission filter (9), where the remaining excitation light is filtered

out. Therefore, this filter is also called barrier filter. As usual, the tube lens (10) and the eyepiece (11) form the microscope image, which now only consists of fluorescence light. (<http://www.zeiss.com/c1256b5e0047ff3f/ContentsFrame/52c01f2588066339c1256c3e003669f0> -> beam path)

Fluorescence is the ability of certain molecules that usually contain rigid conjugated polyaromatic hydrocarbons or heterocycles to absorb light in shorter wavelengths and emit it in longer wavelengths. When such a molecule (i.e. fluorophore) absorbs a photon, an electron in the absorbing molecule is lifted to a higher energy level, and the molecule is therefore in an unstable excited state. Fluorescence occurs when the electron returns rapidly to its normal lower-energy orbital and the molecule releases part of the absorbed energy as longer-wavelength light emission.

Cellular proteins can mainly be labeled with fluorescent dyes in two ways: a) by using fluorescent dyes conjugated to antibodies that attach to the protein of interest or, b) by transfecting green fluorescent protein (GFP) or its variants fused to the protein of interest into the cell. To distinguish different proteins in a specimen, dyes with different fluorescence spectra are used, and their emission signals are separated by using proper filter sets in the microscope.

If you wish to analyse your images automatically, it is important to plan image analysis before capturing the image. Common problems concern background (there should be no difference in background intensities in different parts of the image ie. illumination correction should be made) and saturation (your objects of interest should have intensities within the measurable range ie. exposure time should be correct).

3. Instrument and software used

In this work we will use Zeiss Axioplan 2 microscope. The software used is Zeiss AxioVision 4.5 SP1. The camera in this microscope is Zeiss AxioCam HRc 14-bit color CCD camera. The objective used in this work is 20x, NA=0.5, Ph2.

Filters used:

- 1) Chroma 31000v2 (DAPI), for blue fluorescence (Fig. 2 a)
- 2) Chroma 41001 (FITC), for green fluorescence (Fig. 2 b)
- 3) Chroma 41002b (TRITC), for red fluorescence (Fig. 2 c)
- 5) Zeiss 25HE (DAPI/FITC/TRITC), for blue/green/red fluorescence (Fig. 2 d)

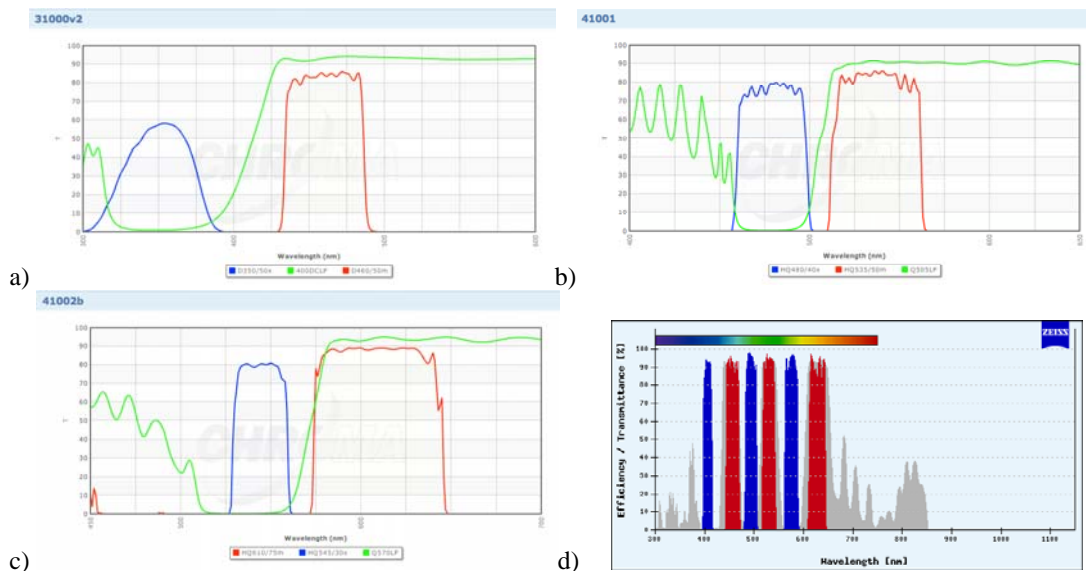


Figure. 2 Spectra of the fluorescence filters used in this work.

4. Sample description

U2OS osteosarcoma cells plated on coverslips were transfected with DsRed (red) and GFP (green). Hoechst 33258 was used for staining the nuclei (blue). Hoechst 33258 has very similar spectral properties as DAPI nuclear stain.

5. Task

The task is to take images in two different ways and then compare if there are differences between the final images. A) Capture separate images of the sample by DAPI, FITC and TRITC filters by using multi-channel acquisition settings in the software. B) Capture just one image using the triple filter DAPI/FITC/TRITC that will show all three expression patterns.

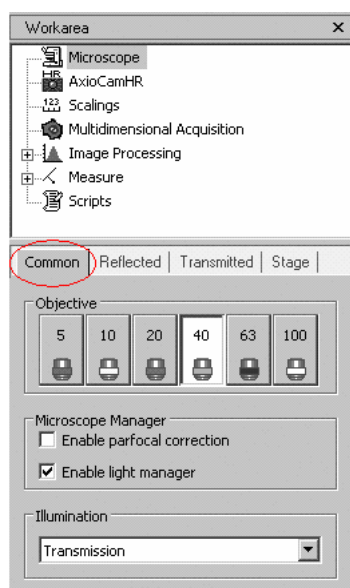
6. Instructions for practical work

6.1. Starting up

The microscope and mercury bulb are already on. The AxioVision software is open. Put the specimen at the sample holder on the stage. Before starting make sure that the light path is directed to oculars by pushing in the right rod and pushing out the left rod on the microscope (Fig. 3).



Figure 3. Rods controlling the oculars/camera light path.



Then, select a COMMON tab from the MICROSCOPE menu on the left (Fig. 4)

Figure 4. The COMMON menu.

6.2. Objectives

You can select an objective to achieve the right magnification in the COMMON tab (Fig. 4). Select a 20x objective by clicking on its button.

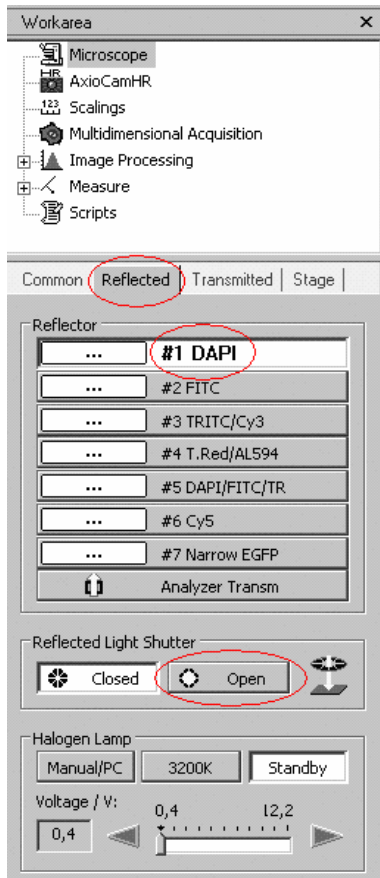


Figure 5. The REFLECTED menu.

6.3. Filters

You can select a filter on the REFLECTED menu (Fig. 5). Select a DAPI filter in order to visualize cell nuclei.

6.4. Reflective light shutter

For fluorescence imaging open the fluorescence shutter by selecting SHUTTER OPEN (Fig. 5).

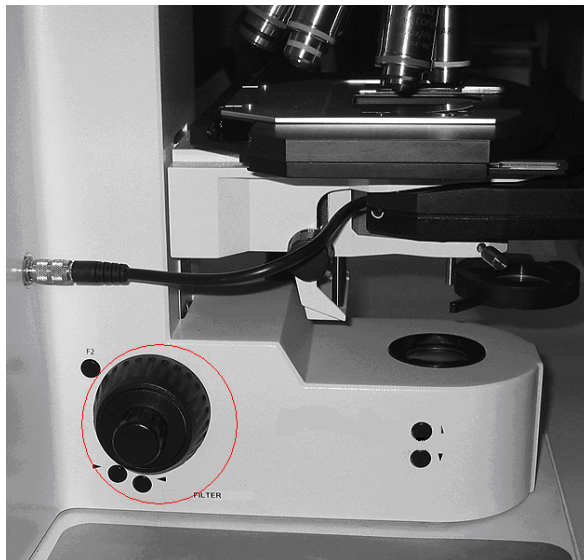


Figure 6. Focusing knobs.

6.5. Focusing

You can focus into the sample by using focusing knobs on both sides of the microscope, the outer wheel is for coarse adjustment and the inner wheel is for fine adjustment (Fig. 6). Turn the focusing knobs so that you can see blue nuclei.

6.6. Image acquisition

Redirect the light path to the camera by pulling out the right-hand rod, and pushing in the left-hand rod (Fig. 3). Enable continuous imaging by clicking the LIVE-button the main menu (Fig. 7).

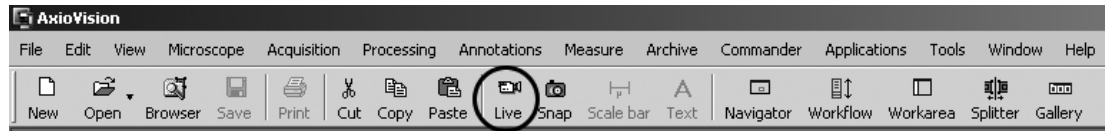


Figure 7. The main menu of the AxioVision software.

Set the camera settings: click AxioCamHR and choose FRAME (Fig. 8), select 1300x1030 for resolution, then select RGB camera.

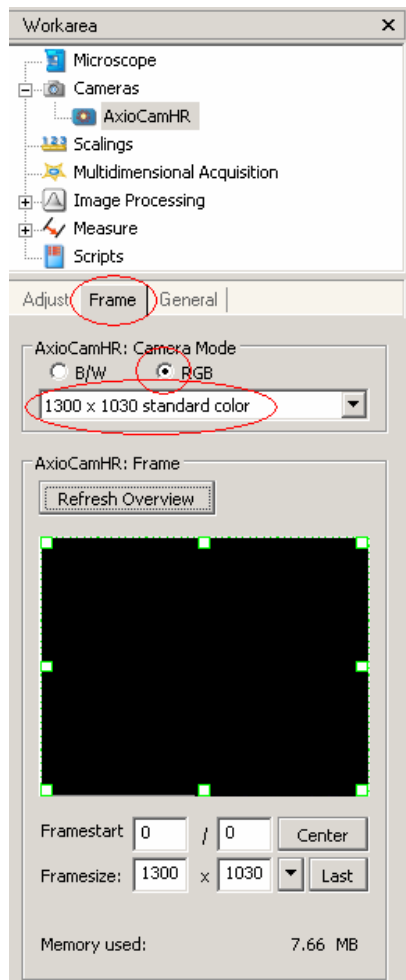


Figure 8. Camera settings.

Set the exposure time: select CAMERA menu, click AxioCamHR, select ADJUST. Then press MEASURE, and the software estimates suitable time of exposure (Fig. 9).

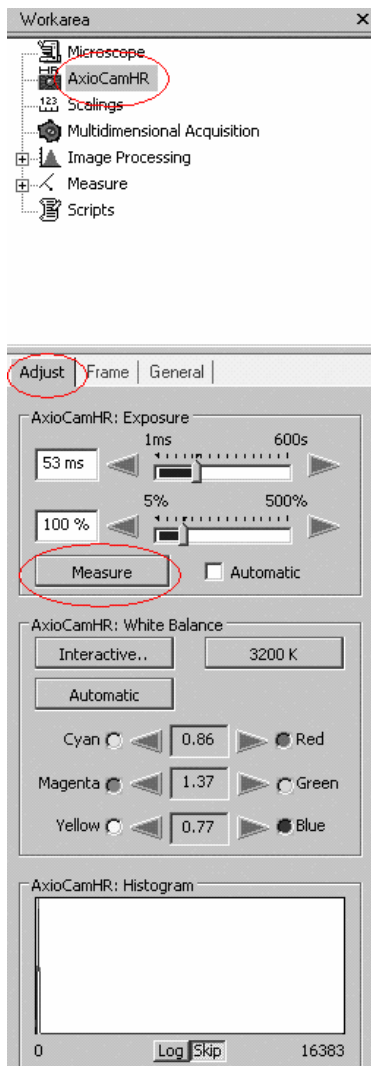


Figure 9. Adjusting the exposure time.

Make sure that the image is not overexposed, ie. it is not saturated. Click the “French flag” in the LIVE menu (Fig. 10) and lower the exposure time so that there are no red spots in the image.

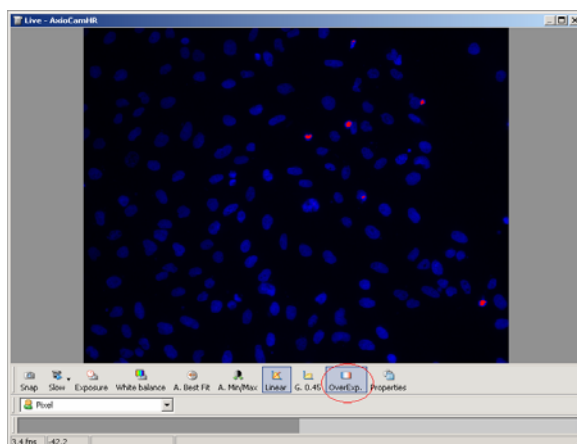


Figure 10. The “French flag” for saturation.

A single image is acquired by clicking the SNAP button (Fig. 11). Do this now to see if the image is in focus. You do not need to save the image.

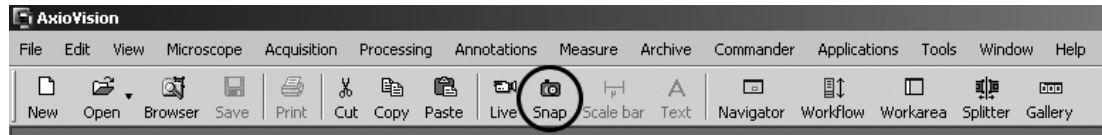


Figure 11. The SNAP button in the main menu.

6.7. Image capture

Now, we can perform the first part of the task. Because the specimen has been labeled with three different fluorescent dyes, we can separate their signal by using filters that are specific for certain wavelengths. Three images, each showing one of these dyes, can be captured sequentially, this is called multi-channel imaging. For multi-channel capture, choose MULTIDIMENSIONAL ACQUISITION and C for color channel settings (Fig. 12).

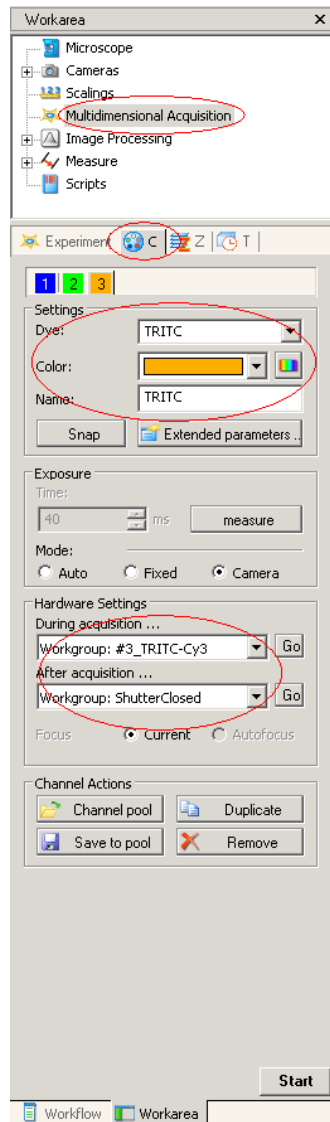


Figure 12. Multidimensional acquisition menu.

- 6.7.1 Use the following SETTINGS for imaging blue nuclei
- 6.7.2 Select the dye (DAPI) on the list, color for the channel will be selected according to the dye (blue for DAPI), but it can also be changed manually
- 6.7.3 In HARDWARE SETTINGS, select the filter that you need for the channel in DURING ACQUISITION... first, select the DAPI filter
- 6.7.4 By clicking MEASURE the software estimates the suitable time of exposure and the preview live window pops up
- 6.7.5 Add more channels by clicking DUPLICATE: we will still need FITC (for green fluorescence) and TRITC (for red fluorescence), and set hardware settings for them
- 6.7.6 Finally, select SHUTTER CLOSED from AFTER ACQUISITION to prevent photobleaching
- 6.7.7 Use START to initialize multi-channel capture
- 6.7.8 Save the images

6.8. Saving and exporting images

Click FILE / SAVE AS... to save your images. When you save the images as .zvi files, all the information about the objectives, filters etc. is available for you later. Use FILE / EXPORT, to export the images as 8-bit .tiff files (Fig. 13)

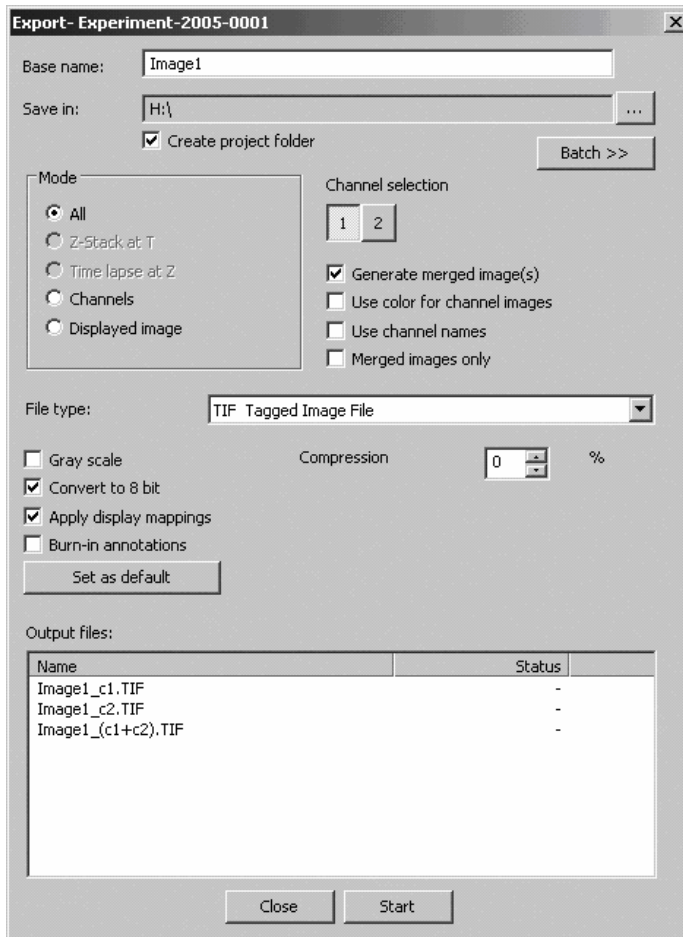


Figure 13. Exporting images.

6.9. Triple-filter imaging

In order to perform the second part of the task, use the triple filter (DAPI-FITC-TRITC). Select the channel like in D, use MEASURE (see 6.6. for Image acquisition) to measure exposure time, click SNAP (see figure 11). Compare the intensity from an image captured from same area as in 6.7. and an image captured from unexposed area. Save the image.

Compare the images for each channel. Are they identical?