

INSTRUCTIONS FOR COURSE WORK 4 (AxioVert)

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1. Purpose of the work

In this work, you will get familiar with an inverted epifluorescence microscope. Also, you will learn how to do phase contrast and DIC microscopy.

2. Background

2.1 Inverted microscope

An inverted microscope is usually used for observing living cells, as culture dishes and bottles do not physically fit under an upright microscope. In an inverted microscope, condenser is above the specimen plane, and the transmitted light source (halogen bulb) is above the condenser (see Fig. 1). In an inverted epifluorescence microscope, there is also a light source (usually a mercury bulb) for reflected light that enables detection of fluorescence signal from the specimen.

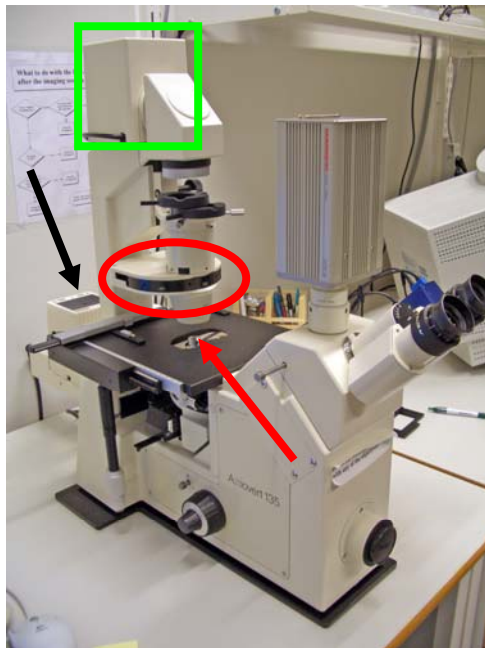


Figure 1. Inverted epifluorescence microscope. Red arrow: specimen plane. Red oval: condenser. Green square: transmitted light source. Black arrow: reflected light source (mercury bulb).

2.2 Contrasting methods

When unstained live cells are viewed with the microscope, they are often hard to see with transmitted (bright-field) light because they do not absorb light. To improve contrast in these kinds of specimens, there are two commonly used contrasting methods: phase contrast and differential interference contrast (DIC). Modulation contrasting method, developed by Robert Hoffman, is not discussed here as it is not commonly used.

2.3 Phase contrast

Phase contrast method utilises the fact that small variances in refractive indexes of different parts of the specimen cause slight phase shifts of light. As our visual system is not able to detect phase shifts, they are converted into differences in the intensity of light. This is achieved by placing a ring annulus at the front focal plane of the

condenser, and a phase plate at the back focal plane of the objective. Thus, for phase contrast microscopy, both a phase condenser and a matching phase objective are needed. Use of a green filter is beneficial as the optics is calculated for the wavelength of green light.

Phase contrast is suitable for thin (single cell layer) specimens that are scattered across the field of view. In thick specimens, phase shifts occurring in planes above and below the focus plane will affect the quality of the phase contrast image. Also, phase contrast method often creates haloes around borders (areas of high contrast). In addition, the use of the ring annulus decreases the working numerical aperture, which results in reduced resolution. Phase objectives should not be used for fluorescence microscopy, as the phase plate inside them absorbs some light and, thus, reduces the amount of light that will pass through the objective.

2.4 Differential interference contrast (DIC)

In DIC, the enhanced contrast is based on the method described by George Normarski. DIC optics consists of two polarizing filters, the one located in between the light source and specimen is called a polarizer, and the one in between the objective and eye/camera is called an analyzer. One modified Wollaston prism is in between the polarizer and specimen, and another similar prism is in between the specimen and analyzer.

The first pair of the polarizing filter and beam-splitting prism is used to convert slight differences in refractive indexes in different regions of the specimen into differences in light path lengths. Also, differences in the actual thickness of the specimen will be converted into different light path lengths. The second polarizing filter/prism pair, in turn, is used to convert the different light path lengths into differences in light intensity and color.

For DIC, the microscope has to be equipped with a polarizer and analyzer that can be inserted into the light path when needed. The first Wollaston prism is located in the condenser, and the second is often a removable one that is inserted into a slot in the nosepiece at the base of the objective.

Compared to phase contrast, DIC does not reduce the working numerical aperture, thus giving better resolution. Also, no haloes are created in DIC. The 3-D appearance and colors produced by DIC often enhance structures in thick specimens much better than phase contrast. However, DIC may not give as good images as phase contrast in thin specimens. Also, DIC cannot be done through most plastics, as they affect polarization of light.

3. Specimen and instrument/software used

Specimen: A374 (melanoma cell line) cells incubated in D-MEM cell culture medium (high glucose) with 10% fetal calf serum (FCS) at 37°C, 5% CO₂

Microscope: Zeiss AxioVert 200 inverted epifluorescent microscope

Camera: Zeiss AxioCam HRc color CCD camera, 14-bit

Software: Zeiss AxioVision, version 3.1

4. Instructions for practical work

4.1 Microscope use

Settings for the light path

1. Place the specimen on the microscope stage so that the part you want to view is over the objective. The microscope is already on.
2. Turn the knob on the left side of the microscope into VIS position for the oculars (Fig. 4, arrow 1).
3. Rotate the wheel so that a circle is displayed to direct the path of light to the oculars and front-mounted AxioCam camera (Fig. 1, arrow 2).



Figure 4. Adjustment of the light path to a correct port. Arrow 1: ocular/camera selection. Arrow 2: selection of the front/side port. Red circle: focusing knobs.

4. Keep the knob below the oculars turned so that the white line points to the eye (Fig. 2, red arrow).



Figure 5. Ocular light path adjustment.

5. The lower button on the lower right of the microscope is used to activate/deactivate the halogen (HAL) light pathway (Fig. 6). Make sure that the HAL light pathway is active.



Figure 6. Red arrow: on/off button for transmitted light. Red circle: Transmitted light intensity adjustment.

Other microscope settings

6. There are following objectives in the microscope:

Position	Type	Magn.	NA	Oil/Dry	Phase/DIC
1	Plan-Neofluar	5x	0.15	Dry	-
2	Plan-Neofluar	10x	0.3	Dry	Ph1/DIC
3	LD Achroplan	20x	0.4 korr	Dry	Ph2/DIC
4	Plan-Neofluar	40x	0.75	Dry	-
5	LD Achroplan	40x	0.6 korr	Dry	Ph2/DIC
6	LD Achroplan	63x	0.75 korr	Dry	Ph2

Table 1. Axiovert 200 objectives

The objectives are changed by manually turning the objective revolver. You can see the chosen objective in the little lcd display on top of the condenser. Select 10x objective.

7. Bright field imaging is usually used for stained specimen but we will use it now first. Move the condenser to position DIC.3-4 (Fig. 7) by rotating manually the condenser wheel. Adjustment of the Koehler illumination has already been done.



Figure 7. The position of the condenser.

8. Look briefly through the oculars while keeping your eyes at a safe distance (about 10 cm) from the oculars - the light may be too intense for your eyes. If needed, adjust the light intensity by pressing the toggle bar in the front of the microscope, below the camera (Fig. 6, red circle). When you press the left side, light intensity is lowered; when right side is pressed, light intensity is increased.

9. Focus on the specimen by using the focusing knobs on both sides of the microscope. The outer wheel is for coarse adjustment and the inner wheel is for fine adjustment (Fig. 4). It is probably not so easy to focus into cells as they are not stained.

10. Make sure that the oculars are correctly adjusted for your eyes. If you have normal vision, the zero mark on the sides of the oculars should match the white dot. If you wear eyeglasses but want to use the microscope without them, you must adjust the oculars according to your vision. First look at your specimen with your right eye closed (through the left ocular). Slowly turn the eyepiece scale ring until the image is

optimally in focus. Repeat with the other eye. The scale on the oculars is the diopter scale.

4.2 Image acquisition

11. Pull the left push rod out so that the light will go to the camera. Push the right push rod in so that no light goes to the oculars. Enable real-time video by clicking the LIVE-button in software (Fig. 8).

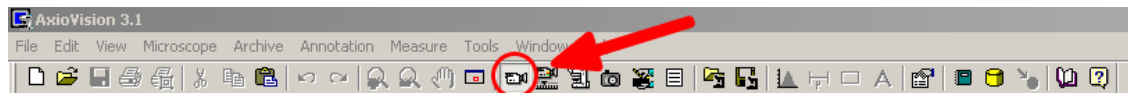


Figure 8. LIVE-mode button in AxioVision software

12. Focusing is done by using the focusing knobs on both sides of the camera. When the oculars are correctly adjusted (see 4.1. 10), the image is in focus when viewed either through the oculars or camera without further focusing.

13. Click CAMERA and select ADJUST. The time of exposure can be set manually, either with a slider or by entering the numeric value. By pressing Measurement, the software automatically estimates the suitable time of exposure for the current image.

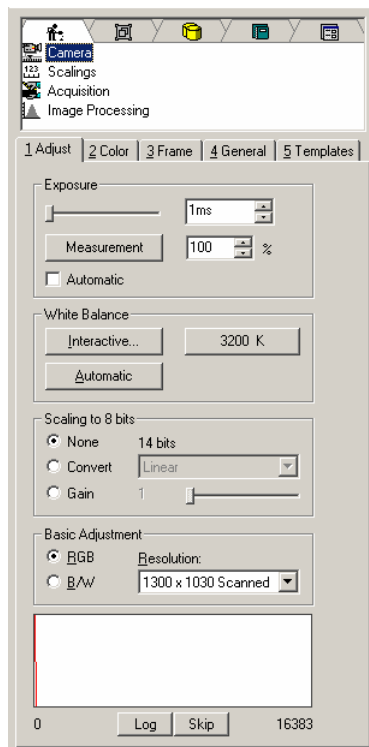
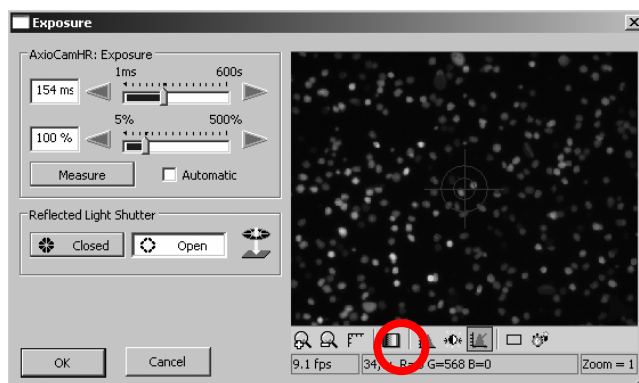


Figure 9 (on the left). Camera settings in AxioVision software.

Figure 10 (below). Image window tool bar. Under/overexposure tool is marked with a red circle.



In the image window there is a "French flag" button in the tool bar at the bottom (Fig. 10). This will show under/overexposed areas in blue/red pseudocolor. Pressing the button again removes the pseudocolor. Use this tool and determine the optimal exposure time. Remember also to check in the histogram (bottom in Fig. 9) that your image has good contrast (min. and max. intensity values far apart). Write down the exposure time.

14. Correct white balance settings are essential when taking images with transmitted light. The color of halogen lamp depends on voltage. The color turns from red to blue when voltage is increased. Human eye compensates for the color variation but the camera should be adjusted properly. There are three options (Fig. 9):

- Interactive... = you have to click on a spot in live image that will then represent white color
- 3200K = white balance will be adjusted according to color temperature 3200K
- Automatic = software estimates suitable white balance for the image

Try all these options. Which one works best?

15. Acquire a single image by clicking the snap-button, which is in the main tool bar three icons to the left from the live-button (Fig. 8). The new image will open in a new window.

16. Save the image (FILE/SAVE AS in the main menu) into your USB stick in ZVI format, which is specific for Zeiss AxioVision software. In addition to the image itself, also the imaging parameters will be stored. However, only some other imaging software will be able to open ZVI files such as the free AxioVision LE that can be downloaded from the Zeiss web site. For more information, see MIU web site (www.miu.helsinki.fi/links). Also save the image in 8-bit TIFF file format. If you use TIFF, only the image is saved, not the image acquisition parameters. However, almost any other imaging software (e.g. Photoshop) will be able to open a TIFF file. Also, your image data will remain unaltered.

4.3 Phase-contrast imaging

The 10x objective in the microscope is suitable for phase-contrast imaging. Rotate the condenser so that the matching condenser position (Ph1) is shown in the front (Fig. 7). Acquire an image as explained earlier, and save it in TIFF and ZVI formats.

4.4 DIC imaging

1. The 10x objective is also suitable for DIC imaging. First turn the polarizer into to the light path (Fig. 11).
2. Push the analyzer located on the left side of the microscope to the right (Fig. 12).
3. Rotate the condenser to the position DIC .3-.4 (Fig. 7).
4. The angle of the DIC-slider is adjusted with the little knob (Fig. 8). This affects the pseudo3D-effect that the DIC imaging creates.
5. Above the polarizer, there is a slider that is used for adjusting the angle between the polarizing plane of the polarizer and analyzer. Move the slider while looking at the DIC image.

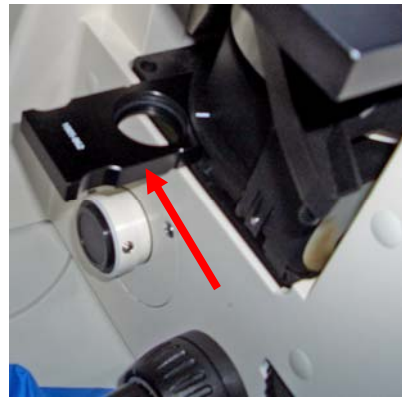


Figure 11 (left). The polarizer for DIC imaging.
Figure 12 (right). The analyzer for DIC imaging.

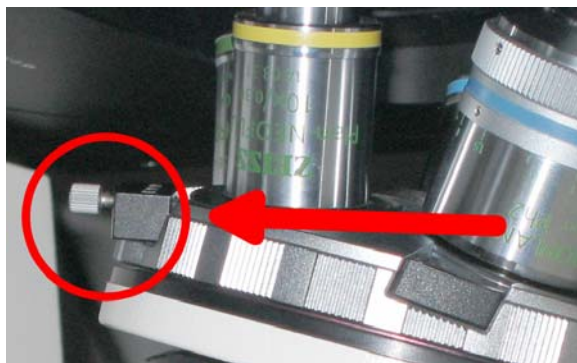


Figure 13. The knob for adjusting the angle of DIC-slider.

6. As a last step, adjust the iris diaphragm using the slider above the DIC condenser (Fig. 7). First, fully open it and then, if needed, close it as much as necessary for enhanced contrast.
7. After you have optimized all settings, acquire an image and save it (TIFF and ZVI).
8. When you are done with DIC imaging, remember to turn the polarizer away from the light path and pull the analyzer back to the left.

5. Questions

Compare bright-field, phase-contrast, and DIC images. Which one is the best? What differences are there? What is the advantage/disadvantage of each imaging technique?