

# INSTRUCTIONS FOR COURSE WORK 2: TRANSMITTED LIGHT MICROSCOPY AND RESOLUTION

**Location: Microscopy room B501b**  
**Instructor: Anne Vaahtokari (MIU)**

## 1. Purpose of the work

In this work, you will get familiar with an inverted epifluorescence microscope, and practice how to adjust Koehler illumination and acquire transmitted light microscopy images. In addition, you will learn what instrument and image acquisition settings are important for image analysis. For the summary session, you will need to calculate the camera resolution that matches the microscope optical resolution used and answer the questions found after each task.

## 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.

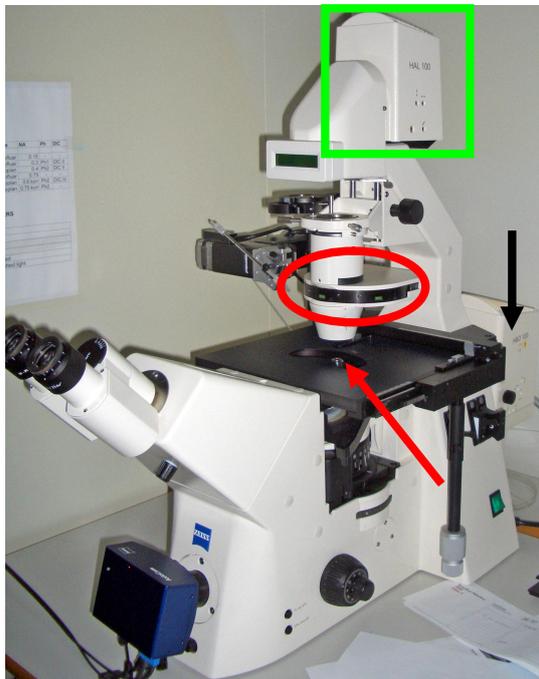


Figure 1. Inverted epifluorescence microscope. Red arrow: specimen plane. Red oval: condenser. Green square: transmitted light source. Black arrow: reflected light source (mercury bulb).

### 2.2 Transmitted (bright-field) light microscopy

Transmitted light microscopy is used for specimens that are stained, or have natural pigment so can they are able to absorb light. Koehler illumination ensures that illumination is homogeneous and devoid of disturbing scattered light. Thus, adjustment of Koehler illumination is essential every time transmitted light microscopy is done. Switching objectives requires readjustments.

### 3. Specimen and instrument/software used

Specimen: Autofluorescent pollen grains (Carolina)

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 upside down on the objective slide holder at the microscope stage. Turn the two adjustment wheels connected to the stage on the right-hand side 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.

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

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. In this microscope, the condenser DIC position is used for brigh-field imaging (other DIC optics is not in the light path). Move the condenser to position DIC.3-.4 by manually turning the black ring in the condenser (Fig. 7). You can see the selected condenser position in the window in the front of the condenser (Fig. 7, red circle).



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 decreased; 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, red circle).

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 Koehler illumination adjustment

Koehler illumination needs to be adjusted for optimal illumination. Proceed as follows: Make sure you are using 10x objective, condenser position DIC .3-.4, and that the specimen is in focus:

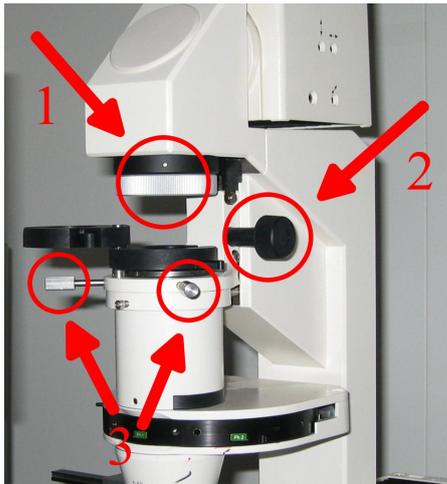
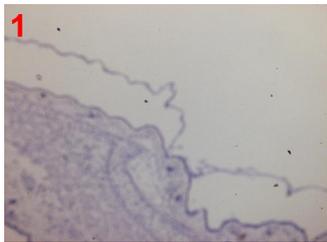
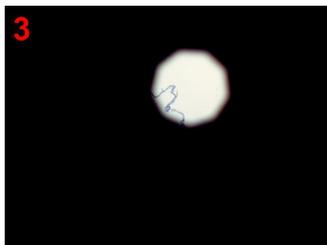
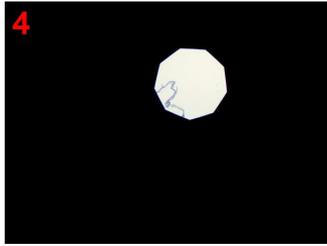


Figure 8. Adjusters for field diaphragm (1), condenser elevation (2) and condenser centering (3).

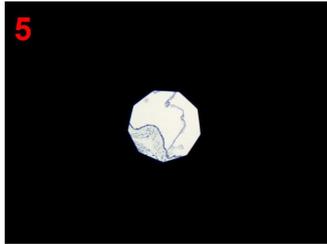
- Close luminous field diaphragm until its edges can be seen through oculars (Fig. 8, arrow 1).



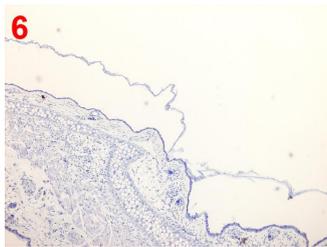
- Focus the image of diaphragm edges by moving the condenser up or down (Fig. 8, arrow 2).



- Use centering screws to move the diaphragm image to the center of the field of view (Fig. 8, arrows 3).



- Open the luminous field diaphragm until its edges are outside the field of view (Fig. 8, arrow 1).



- Remove one ocular by pulling it out and close the aperture diaphragm until it is about 80% of the diameter of the objective pupil. The aperture diaphragm is adjusted by moving left/right the black level above the condenser position window in the front of the condenser. Replace the ocular. Another way of adjusting the diaphragm size is simply to look at your specimen while opening/closing the diaphragm. If the aperture is too closed, the resolution is not optimal. If the aperture is too open, the contrast is suboptimal.

**QUESTION FOR THE SUMMARY:** If the adjustment of Koehler illumination is not properly done, how can that affect analysis of images taken, e.g. if you need to count particles in the image?

#### 4.3 Image acquisition

1. Turning the knob on the left side of the microscope into FP (front port) position will direct the light to the camera (Fig. 4, arrow 1). Turning the same knob into VIS position will direct the light path to the oculars. Enable real-time video by clicking the LIVE-button in software (Fig. 8).



Figure 8. LIVE-mode button in AxioVision software

2. 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.

3. 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.

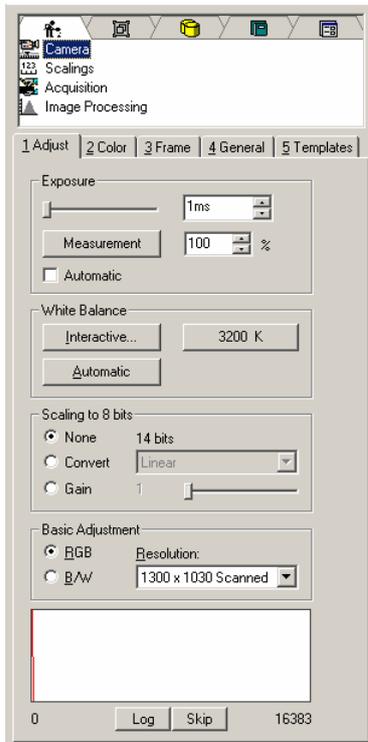
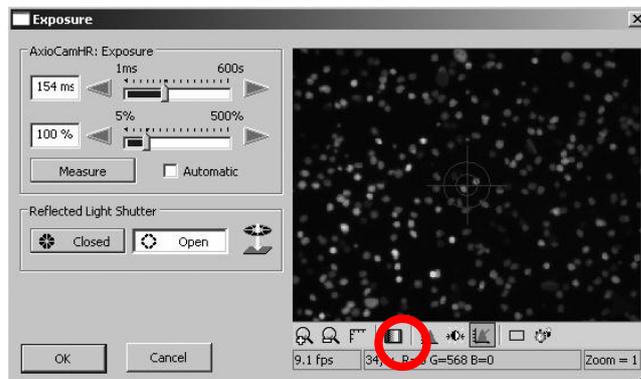


Figure 9 (on the left). Camera settings in AxioVision software.

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).

4. 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?

5. Take images of the same field of view using three different resolutions (click CAMERA and select the "Frame" tab):

1. 1300 x 1030 standard color (1300 pixels along X-axis, 1030 pixels along Y-axis)
2. 3900 x 3090 scanned color (3900 pixels along X-axis, 3090 pixels along Y-axis)
3. 432 x 342 3x3 binning (432 pixels along X-axis, 342 pixels along Y-axis)

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.

**QUESTIONS FOR THE SUMMARY:** What happens to the exposure time when you change the frame size? How about the total image acquisition time? When you compare the images, which one looks the best? Zoom each image and compare the image quality again (use the magnifying glass+ icon located below the image for zooming in, magnifying glass- icon for zooming out).

### **TASKS FOR THE SUMMARY**

1. Using the accompanying Zeiss “How to...” guide, calculate the optimal resolution for the optics you just used ( $d \approx \lambda/2NA$ ). For halogen light, you can use the mid-range wavelength of 550 nm.

2. Calculate the optimal camera resolution  $R(x)$  and  $R(y)$ . You will need:

$T_v = 0,63$  (camera adapter reduction factor)

$X = 8,9\text{mm}$  (camera chip size in x direction)

$Y = 6,7\text{mm}$  (camera chip size in y direction)

3. Which of the camera resolutions used fulfill this criterion?