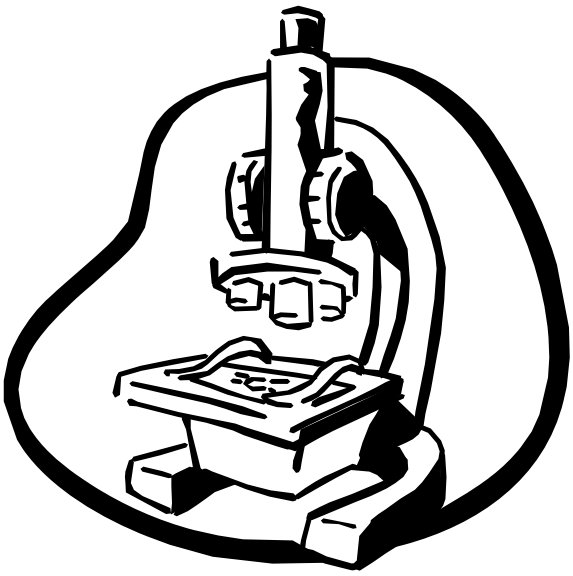
Biomedical & X-ray Physics
Kjell Carlsson

<u>Important:</u> Study the preparatory exercises carefully <u>before</u> the lab session starts!



Practical Light Microscopy

Laboratory instructions for course SK2500/01,

Physics of Biomedical Microscopy

by

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Introduction

In this laboratory exercise you will familiarize yourself with the light microscope. You will set up and use the microscope for different imaging techniques such as transmitted light, phase contrast, dark-field, and epi-fluorescence. In the text references are sometimes given to the compendium "Light Microscopy." It is a good idea to study this compendium in parallel with these instructions. In addition, you can look at:

http://micro.magnet.fsu.edu/primer/anatomy/kohler.html
http://www.microscopy-uk.org.uk/primer/index.htm

The first website is interactive, and lets you see the effects as you play around with the Koehler controls. In the text of these lab instructions two preparatory exercises (labeled with the symbol ▶) are given on pages 6 and 14. These should be studied carefully ***before*** the lab session. In this way you will learn much more when you are actually working at the microscope. Before work starts, the supervisor will discuss the preparatory exercises with the students. Carefully read the ***WARNING*** and ***Cautions*** in the text before you start working.

Text sections labeled with ● indicate tasks to be performed by the students. The symbol ➡ indicates questions to be answered during the lab work and/or lab results to be shown to the supervisor.

The lab exercise includes the following tasks:

1. The setting up of correct Koehler illumination in transmitted light microscopy.

2. Correct use of the transmitted light microscope when studying some "typical" specimens. Different settings of the illumination system (condenser *N.A.* and luminous field diaphragm) will be investigated, as well as oblique illumination, phase contrast and darkfield.

3. The setting up and use of an epi-fluorescence microscope. A wide range of different magnifications (including oil immersion objectives) will be used. Filter and beam splitter choice will be discussed, and fluorescent specimens will be studied.

Two different microscopes will be used. The practical embodiment of the different controls (diaphragm adjustments etc.) will differ slightly between these microscopes, but a practical microscopist is expected to handle such difficulties (just as you would expect a person with a driving license to be able to drive more than one brand of car!). The following microscopes will be used:

1. Zeiss Primo Star "student microscope": Will be used for transmitted light microscopy, phase contrast and darkfield.

2. Zeiss Universal "research microscope": Will be used for epi-fluorescence.

When using microscopes, it is important to understand the information that is marked on objectives and eyepieces. Otherwise the equipment may be incorrectly used, resulting in poor image quality. Examples of such information can be seen in Fig. 1.

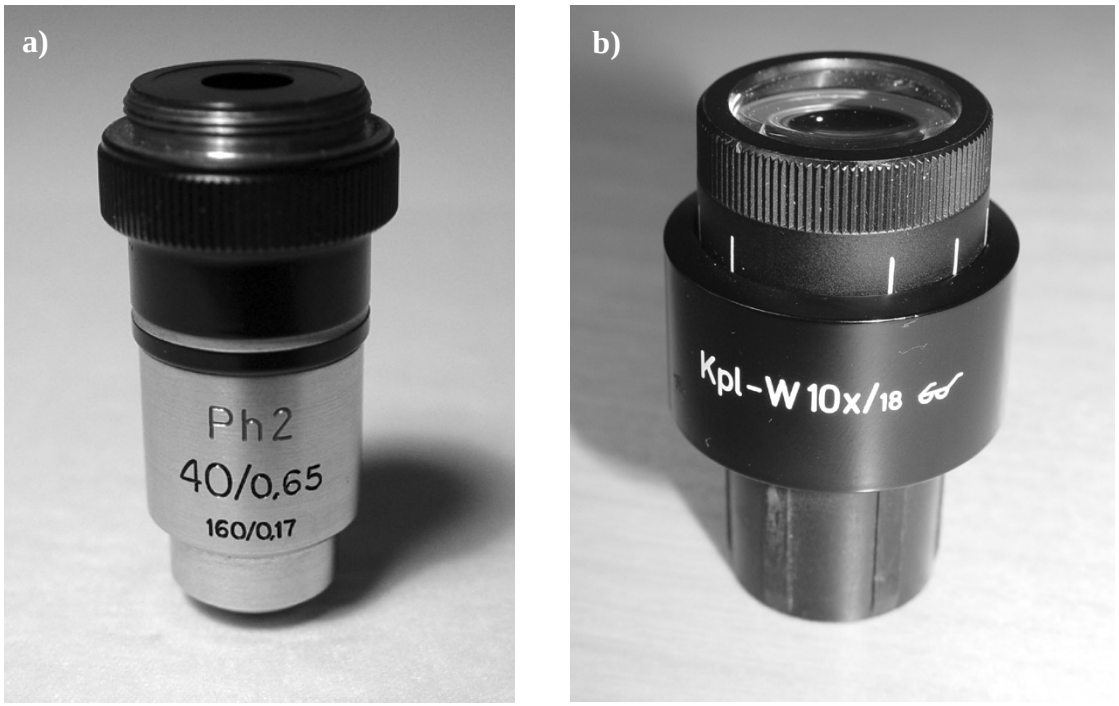


Fig. 1. Microscope objectives and eyepieces are marked with magnification and other data that are important for the user to know. See text for details.

Microscope ***objectives*** are always marked with (at least) magnification and numerical aperture. The marking 40/0.65, Fig. 1a, means that the magnification is 40 and the numerical aperture (*N.A.*) is 0.65. Oil immersion objectives are usually marked "Oil" (or Oel), and phase contrast objectives are marked "Ph" (the number 2 indicates the size of the phase ring). In addition "tube length"[♣] (usually 160 mm or ∞) and cover glass thickness[♠] are often indicated. The type of objective correction (against aberrations) is also usually marked. If it is not, you can assume that it is an achromat (i.e. cheapest type). "Plan" means flat-field corrected. "Fluo" means it has less chromatic aberration than an achromat, but the best color correction is obtained in an "Apochromat." Thus the marking "Plan-Apo" is found on objectives with the best aberration corrections (and highest price!).

Microscope ***eyepieces*** are marked with (at least) magnification and field-of-view[♦]. In Fig. 1b the marking 10x/18 means that the magnification is 10, and the field-of-view is 18 mm. The spectacle symbol indicates "high eyepoint," meaning that the exit pupil, see Fig.3, is located so far from the eyepiece that spectacles can be worn. Additional information like Pl(an), i.e. flat-field correction, and K (compensating for residual objective aberrations) can sometimes be found. W usually means wide-field (unnecessary information if you have the field-of-view number).

♣ Tube length refers to the distance to the image plane. Microscopes are designed to be used with 160 mm ***or*** ∞ objectives. Performance will be degraded if the wrong type is used. Modern microscopes are designed for ∞.

♠ Common values are 0.17 mm, 0 mm (i.e. no cover glass) and – (can be used with or without cover glass).

♦ The field-of-view number, e.g. 18 mm, is the diameter of the circular image you see in the eyepiece ***measured in the image plane of the objective***.

During the course of the lab exercise you will be asked about resolution, contrast and depth of field in the image you see through the eyepieces. To avoid confusion concerning these concepts, Fig. 2 may prove helpful.



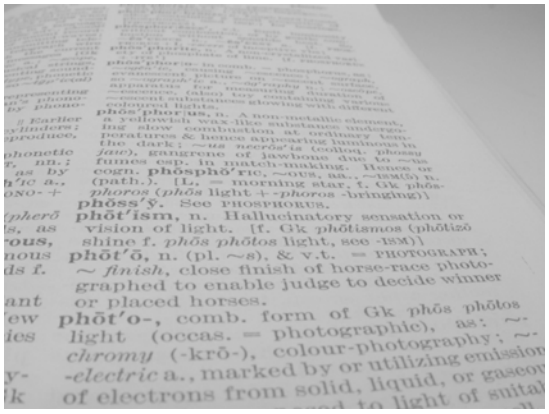
High resolution



Low resolution



High contrast



Low contrast



Large depth of field



Small depth of field

Fig. 2.Illustration of basic imaging concepts.

Exercise 1: Correct set-up of Koehler transmitted light illumination.

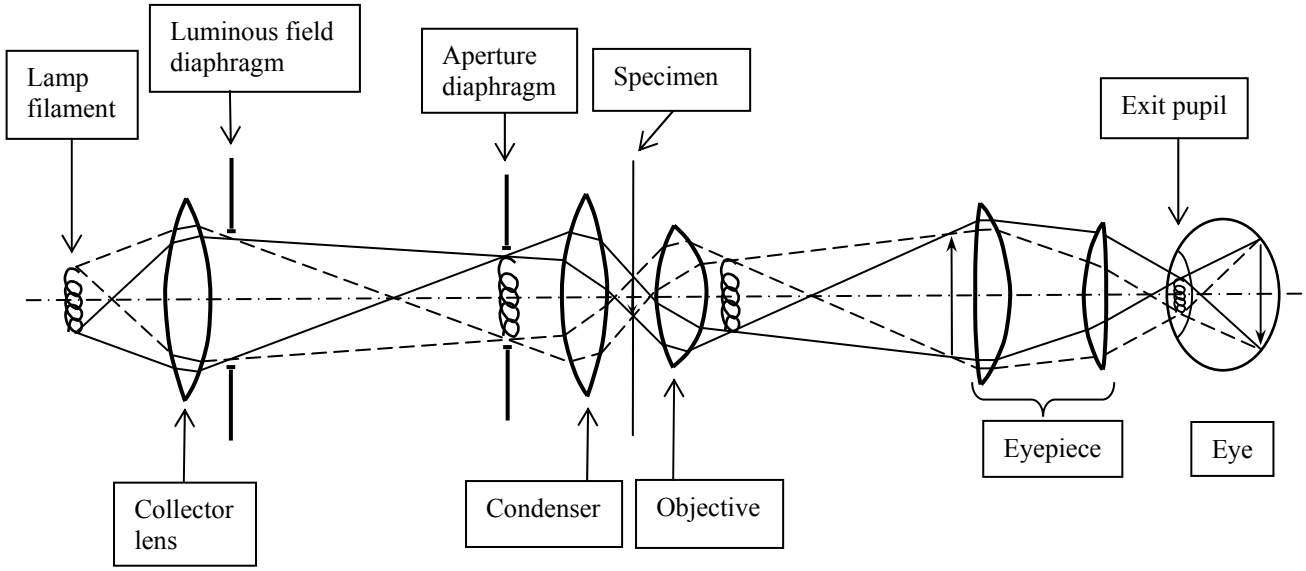


Fig. 3. Ray path in a microscope with Koehler illumination.

► **Preparatory exercise 1:** Study section 1.2, Illumination systems, in the compendium (you can skip the epi-illumination part for the time being). Looking at ***Fig. 3***, explain the functions of luminous-field and aperture diaphragms.

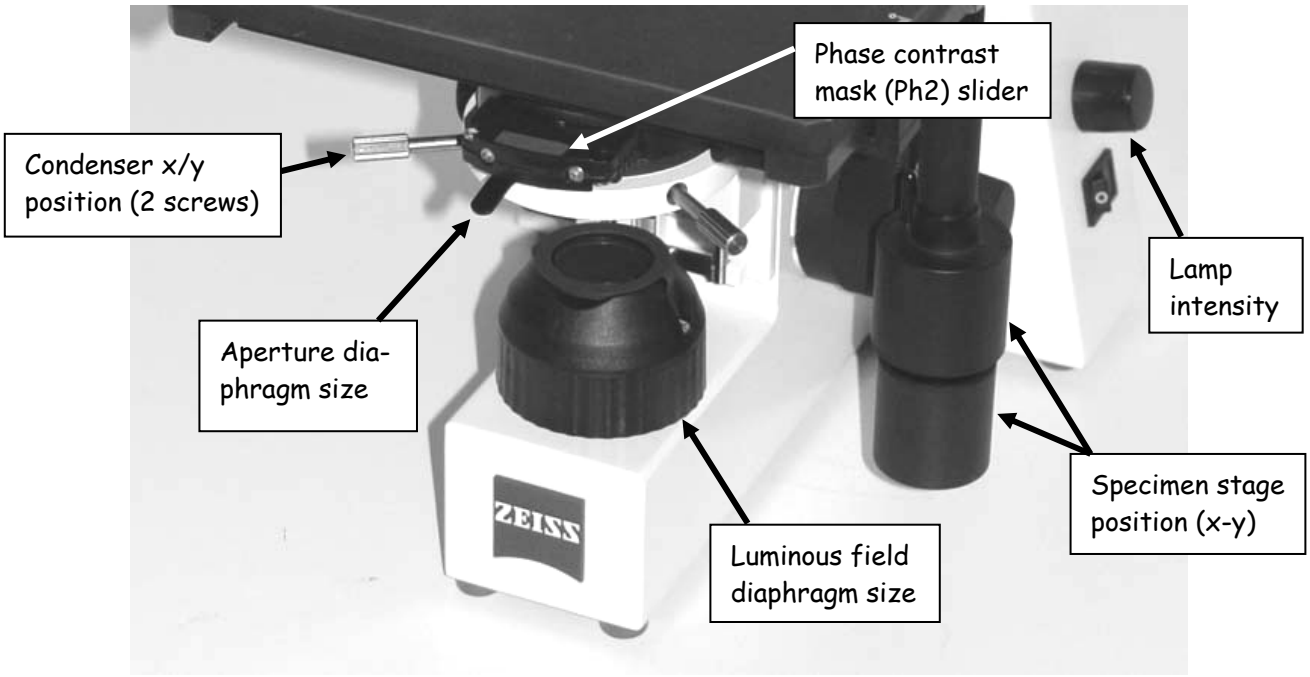


Fig. 4. Microscope controls. Not seen in the photograph is the condenser focus knob, which is found on the left side of the condenser.

You will now familiarize yourself with a small Zeiss "student" microscope (Primo Star). The position of the controls can be seen in Fig. 4.

Caution! ***Avoid touching the specimen with the objective during focusing (it may be damaged). This should especially be observed at high magnifications, when the objective is very close to the specimen surface. Therefore, it is recommended that, before looking through the eyepieces, you raise the specimen stage toward the objective until the specimen surface is close to, but not touching, the objective. Focusing is then done by moving the stage downward, while looking through the eyepieces, until the image is in focus.***

- Turn on the microscope lamp.
- Make sure no phase contrast mask (marked Ph1 or Ph2) is inserted in the condenser.
- Put a specimen (e.g. fly's leg) provided by the supervisor onto the specimen stage. Make sure it rests firmly on the surface with the clamp in place.
- Move the condenser, by using its focusing knob, so that it is close to the uppermost position.
- Open up luminous field and aperture diaphragms maximally.
- Using a 10x objective, focus the microscope so that a sharp image of the specimen is seen through the eyepieces (adjust the lamp intensity for comfortable viewing).
- Reduce the size of the luminous field diaphragm until it becomes visible in the eyepieces (at this stage it is probably off-center and defocused). If the luminous field diaphragm cannot be seen, and the only effect of closing it down is that the light intensity becomes practically nil, it is strongly off-center. Try to open it a bit and adjust the condenser horizontal position (x/y adjustment) to increase light intensity. Then continue closing down and adjusting until the opening becomes visible.
- When a (defocused) bright luminous field aperture is seen through the eyepieces, focus the condenser until the image of the aperture becomes as sharp as possible (don't refocus the microscope!). Because of aberrations in the condenser lenses the image will not be perfectly sharp, and it will display colors at the aperture edges. When the image of the aperture is as sharp as possible, use the condenser x/y adjustment to center the aperture in the field of view (this can be done more accurately if the size of the aperture is adjusted so that it lies just inside the field of view seen through the eyepieces).
- Locate the exit pupil of the microscope (see Fig. 3) by holding a paper just above one of the eyepieces and increasing the lamp intensity to maximum. Move the paper up and down until you find the correct position.

➡ ***Approximately how far from the eyepiece is the exit pupil located? Approximately how large is it?***

In the simple microscope used in **Exercise 1** the lamp and collector lens cannot be adjusted. More advanced microscopes usually have this possibility. In such cases additional alignment steps are necessary so that a focused and centered image of the lamp is formed in the plane of the aperture diaphragm, see Fig. 3. As it is, the Koehler adjustments of the "student microscope" are now finished.

➡ ***Let your supervisor see the microscope, and discuss your results, before you proceed!***

Exercise 2: Correct use of the transmitted light microscope.

Basic experiments with diaphragms

Now the microscope is set up with Koehler illumination. The next task is to learn how to use the controls correctly when studying specimens. Both luminous field and aperture diaphragms need to be adjusted when using different objectives. In addition, the condenser may have to be refocused if specimens of different thickness are studied. In summary, the correct use of these controls ensures that a specimen area corresponding to the field of view is illuminated, and that the numerical aperture of the condenser matches that of the objective.

- Place a specimen provided by the supervisor onto the specimen stage of the microscope (fly's leg). Using the 10x objective, adjust the microscope focus so that a sharp image of the specimen is seen through the eyepieces (adjust the lamp intensity for comfortable viewing).

- Adjust the luminous field diaphragm and condenser focus correctly.

➡ ***What are the criteria for correct adjustment of the luminous field diaphragm?***

Now we come to a very important point concerning correct use of the microscope. Most novices, and even many rather experienced users, totally misunderstand (or disregard) the correct use of the aperture diaphragm.

- With the microscope settings as above, open up the aperture diaphragm maximally, and then close it down maximally. The first thing you will notice is that the light intensity varies as you change aperture setting. Many microscope users use the aperture diaphragm to get the desired light intensity. This use of the aperture is ***incorrect***!

- Open up the diaphragm maximally, and adjust the lamp intensity so that viewing is comfortable.

➡ ***How is image contrast? Can you see the different depth layers of the specimen without refocusing, i.e. do you have a large depth of field (compare Fig. 2)?***

- Close down the aperture to its minimum size. Readjust lamp intensity for comfortable viewing.

➡ ***Compare the contrast and depth of field with the previous case. Conclusions?***

- Dirt, dust etc. on the outer surfaces of the specimen is difficult to avoid completely. Find a specimen area where you can see some dirt or dust. Change the aperture diaphragm size and see what happens.

➡ ***How does the aperture size influence the visibility of such artifacts? <u>Discuss your findings with the supervisor before proceeding!</u>***

<u>Optimizing resolution in the microscope</u>

In this experiment we will investigate how the resolution is affected by the setting of the aperture diaphragm. To be able to see more clearly the smallest specimen details, we will use another microscope, the Zeiss Universal that will be used also in Exercise 3, which is equipped with both 10 x and 20x eyepieces. The diaphragm controls are similar on both microscopes - ask the supervisor if there are any problems.

- Insert a diatom specimen provided by the supervisor in the microscope. (Diatoms are algae with silica shells.) Using the 10x objective, and 10x eyepieces, adjust the microscope focus so that a sharp image of the specimen is seen (adjust the lamp intensity for comfortable viewing).

- This is a more difficult specimen to study than the previous one. The diatoms are rather small, and the contrast is low. Take your time, and look at different parts of the specimen. Use different settings of the aperture diaphragm.

➡ ***Based on image contrast, which aperture setting would you prefer?***

- Some of the diatoms contain fine, grating-like structures that can just barely be seen with the 10x eyepieces. ***<u>Let the supervisor find a suitable diatom</u>***. It should have a line structure that is as dense as possible, but that can still be clearly seen. A large opening on the aperture diaphragm should be used when looking for such diatoms. Switch to the 20x eyepieces when a suitable diatom has been found. (When changing eyepieces, the microscope needs to be refocused slightly.)

- Looking at the line structure of the diatom, change the size of the aperture diaphragm (and, if necessary, also the lamp intensity for comfortable viewing).

➡ ***How is the visibility of small structures affected by the aperture setting, i.e. which setting gives optimum resolution? <u>Discuss your findings with the supervisor before proceeding!</u>***

According to the compendium "Light Microscopy," sect. 1.4, "Imaging properties (coherent imaging)," maximum resolution is obtained if $N.A._{cond}$ is at least as large as $N.A._{obj}$. $N.A._{cond}$ is adjusted by the aperture diaphragm, but how do we know when we have the correct setting? Sometimes there is a *N.A.* scale at the adjustment lever, but often this is not the case. Then the following procedure can be used (do this!):

- With the microscope set up as above (and diatom specimen in place), remove one of the eyepieces and look down the tube (hold your eye close to the opening of the tube). Adjust the lamp intensity for comfortable viewing. Close down the aperture diaphragm to a small opening. You should now see an image of the diaphragm. What you are looking at is the back focal plane of the objective, where a real image of the aperture diaphragm is formed, see Fig. 3. If you open up the aperture more and more you will come to a point where it disappears, and you will only see a circular bright opening whose size does not increase any more. At this point $N.A._{cond.} = N.A._{obj}$. The simple rule for obtaining maximum resolution would then be to open up the aperture so much that it is no longer visible in the back focal plane of the objective. ***In reality***, however, this is not optimal. First, if $N.A._{cond.} > N.A._{obj}$ we don't gain any resolution, but the large $N.A._{cond}$ means that oblique light rays may enter the objective and hit the inside of the microscope tube thereby producing stray light that reduces image contrast. Therefore, never use $N.A._{cond.} > N.A._{obj}$. Second, the optimum is usually to make $N.A._{cond}$ slightly smaller than $N.A._{obj}$ ($N.A._{cond} \approx 90\%$ of $N.A._{obj}$ is common). This means that, theoretically, we lose some resolution, but this is usually more than compensated for by the fact that stray light is reduced and contrast is improved. In practice, this means that when looking down the microscope tube with the eyepiece removed, you should open up the aperture until it nearly disappears. Do this, replace the eyepiece, and look at the specimen. The image should now look good concerning contrast and resolution. Open up the aperture fully. Note the reduction in contrast. Then close it fully. Note the increase in contrast and reduction in resolution.

How to create contrast in highly transparent specimens

Now return to the Zeiss Primo Star "student microscope" for the next experiment. In some cases specimens are stained, often with brightly colored substances, in order to improve contrast. Sometimes, however, this is impossible or inconvenient, and therefore completely unstained specimens have to be used. You will now look at such a specimen. You will manufacture it yourself by donating parts of your body. To make the donation as bloodless as possible, the following procedure is recommended (students who have fainted should now be carried out of the room before we proceed):

Gently scratch (absolutely no blood) the inside of your cheek with a plastic spoon or similar tool to collect a drop of saliva mixed with epithelial cells. Place this drop on a specimen glass. Dilute with <u>one</u> drop of clean water. Gently place a cover glass on top of the moist spot. Finished!

- Place the specimen on the microscope stage, and study it with a 10x objective. Especially observe how image contrast and resolution is affected by the size of the aperture diaphragm.

➡ ***Are you satisfied with the result, or do you feel that you are forced to make a trade-off between contrast and resolution?***

Now you are going to try a cheap and simple method for improving contrast in highly transparent specimens. It is called oblique illumination.

- Using the same specimen as above, open up the aperture diaphragm fully (i.e. disobey the rule $N.A._{cond.} < N.A._{obj}$). On the side of the condenser unit there is an elongated square opening into which you can slide a "darkfield mask". A mask that is inserted into this opening will be positioned in the plane of the aperture diaphragm. This is useful if you want to experiment with non-circular apertures, which is exactly what you will do now. While observing the specimen, insert the darkfield mask slowly until some interesting contrast pattern is seen (it should now be only partly inserted). Inserting the mask even further will result in dark-field illumination.

➡ ***Describe and explain the results to your supervisor***.

- Remove the darkfield mask from the condenser, and view the epithelial cells with the 10x objective. Use correct settings for the diaphragms and condenser focus. When looking at the back focal plane with eyepiece removed, you will observe an annular ring. This is the phase plate of the objective, which is of the phase contrast type (Fig. 14 in compendium). Replace the eyepiece and look at the specimen. Note the poor contrast. Insert phase contrast mask Ph1, and open up the aperture diaphragm fully. You are now using phase contrast imaging. Then use the 20x and 40x objectives together with the Ph2 mask. Especially with the 40x objective, you can note the amount of details you can see inside the cells when using phase contrast microscopy.

➡ ***Let your supervisor see the result, and explain what you see in the microscope. What do the different shades of gray in the image represent physically?***

Exercise 3: Fluorescence microscopy.

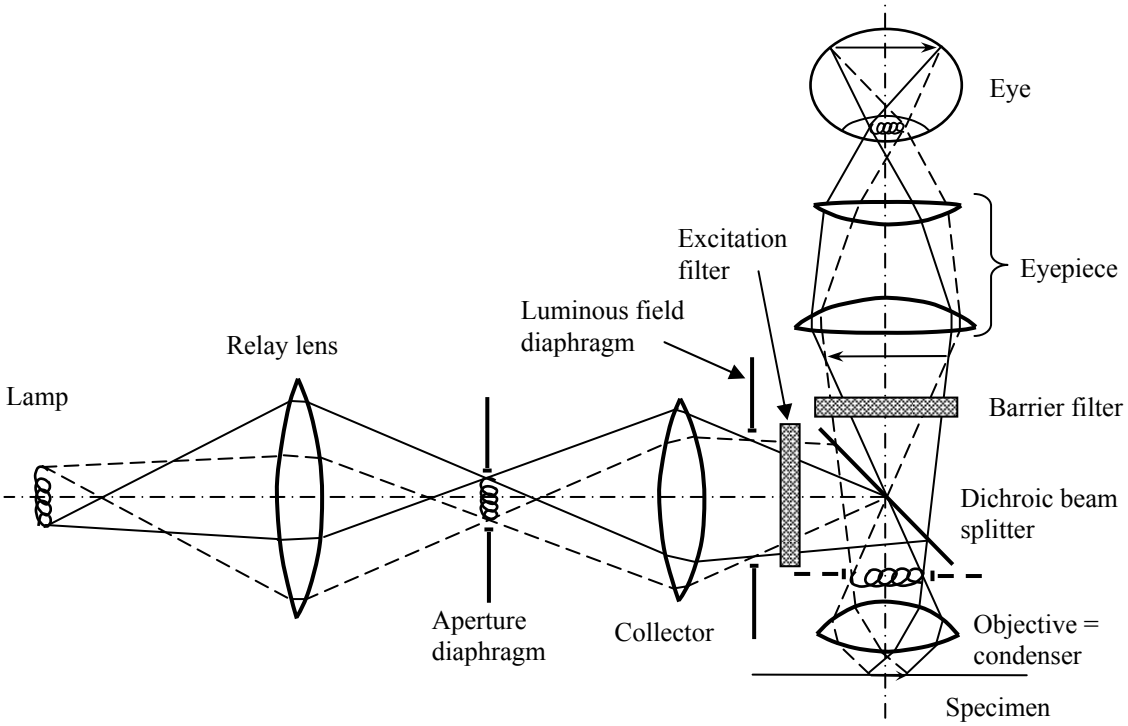


*Fig. 5. Ray path in a fluorescence microscope with Koehler **epi**-illumination.*

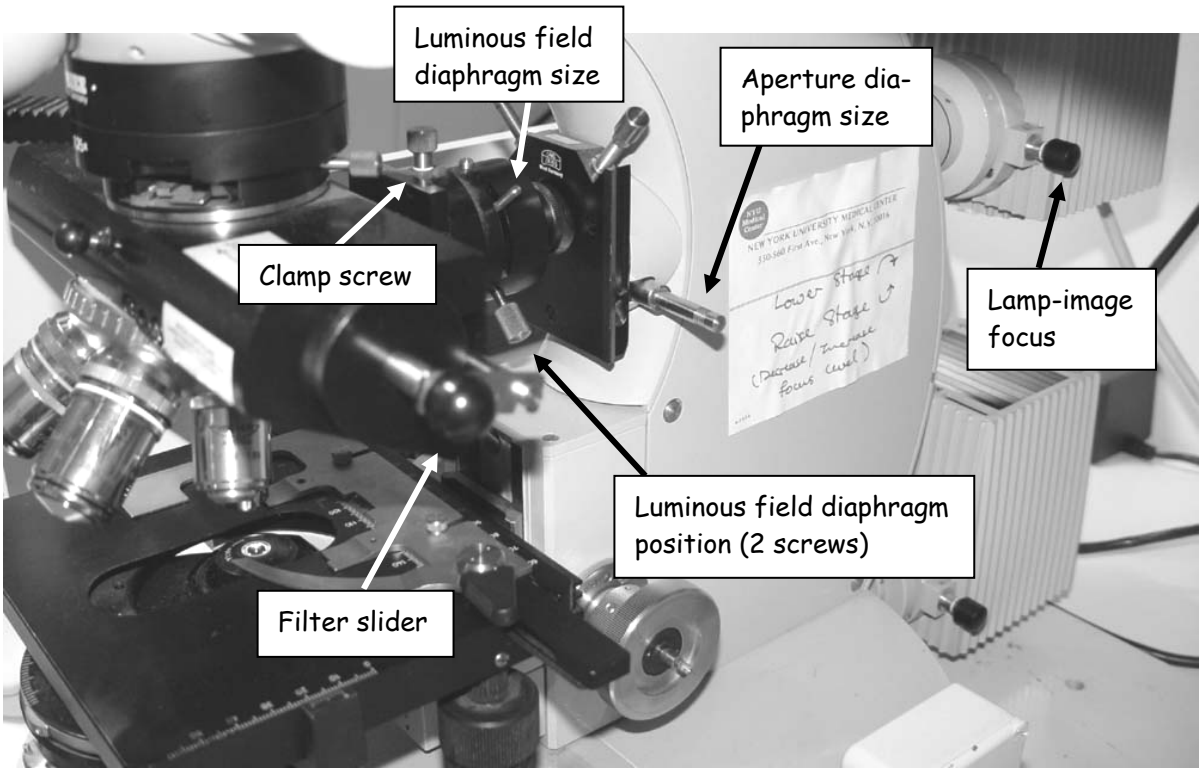


Fig. 6. Zeiss Universal epi-fluorescence microscope.

► **Preparatory exercise 2:** Study sections 1.2 (epi-illumination part) and 1.5.1. (Fluorescence labeling) in the compendium. Looking at Fig. 5, explain the functions of luminous-field and aperture diaphragms. Also explain the functions of the excitation and barrier filters, and the dichroic beam splitter.

This exercise will be carried out on a Zeiss Universal epi-fluorescence microscope. In addition to epi-fluorescence, this microscope also allows transmitted-light imaging of the specimen with a standard Koehler illumination system. The choice of epi-fluorescence filters is simplified by the fact that excitation filter, dichroic beam splitter and barrier filter are mounted together in a filter slider (Fig. 6). As a consequence, all filters are changed simultaneously which removes the risk of "bad" combinations. Essentially what the operator has to decide is whether the fluorophore is such that "blue excitation/green emission" or "green excitation/red emission" is most appropriate. With the filter combinations used in this microscope, "blue excitation/green emission" means that the wavelength band 460-500 nm will be used for excitation[*], and wavelengths longer than 530 nm will be visible in the eyepieces. "Green excitation/red emission" means that the 546 nm line of Hg is used for excitation, and wavelengths longer than 590 nm will be seen in the eyepieces.

Setting up the microscope for Koehler epi-illumination

- Turn on the mercury high-pressure lamp that will be used for illumination. It takes a minute or two before the lamp warms up and becomes bright. In the meantime you can make sure that the 10x eyepieces are installed (not the 20x).

WARNING! ***High-pressure lamps are potentially dangerous, and, furthermore, can be damaged by improper operation and handling (the lamps are expensive as well). If the lamp explodes (happens extremely seldom) immediately leave the room! If the lamp is turned off (e.g. accidentally), it cannot be restarted until it has cooled, which takes several minutes. Never remove the lamp from its housing.***

Hint! ***Fluorescent specimens typically emit rather weak light. It is therefore a good idea to dim the lights in the room to reduce the level of stray light.***

We will now set up correct Koehler epi-fluorescence illumination of the specimen. To find the position of the different controls for focusing and diaphragm adjustment, please refer to Fig. 6.

- We will start by focusing the lamp correctly. Open up luminous field and aperture diaphragms maximally. Remove the microscope objective, leaving just an empty opening. Put a piece of white paper on the specimen stage and raise the stage as far as it will go without touching any of the objectives. Select a filter slider position with blue excitation. You will now see a large circular area of blue light on the paper.

[*] Looking at the Hg spectrum, Fig. 12 in the compendium, one can see that no strong emission lines are present in this wavelength range. Excitation is therefore provided only by the rather weak, continuous spectrum of the lamp. This may seem stupid, but the wavelength choice is optimized for a certain fluorophore (FITC).

Rotate the lamp-image focusing knob (see Fig. 6) until you get as sharp an image as possible of the light source (plasma arc). Note that the image of the light source will never look perfectly sharp no matter how the focus control is adjusted. This is due to aberrations in the relay lens and collector (Fig. 5).

➡ ***Referring to Fig. 5, which of the lenses are you moving when focusing the light source, and in which plane should the light source ideally be focused?***

- The image of the plasma arc should be located in the center of the blue circular area. This can be adjusted with controls on the lamp housing, but we will assume that a qualified person has already done this. If things don't look good, tell the supervisor to fix it.

- Manufacture a simple fluorescent test specimen by drawing a line with a fluorescent pen on a specimen glass (don't bother cleaning the glass, we need some specks of dust to focus on). Place the specimen on the specimen stage, and select blue excitation light. Open up luminous field and aperture diaphragms maximally. Using a 10x objective, look through the eyepieces and focus the microscope on some dust on the specimen glass (the dust must be on the same side of the glass as the fluorescent line). Close down the luminous field diaphragm until it becomes visible in the field of view of the microscope. If all is well, the aperture opening should appear in focus and be centered within the field of view. Assuming it is not, loosen the focus clamp screw (see Fig. 6) and slide the tube until it is in focus, then lock it again with the clamp screw. Then center the diaphragm using the two positioning screws. The illumination should now be reasonably uniform over the whole field of view, and when changing the aperture diaphragm size only the light intensity should change. The Koehler set-up is now finished, but before you take out the specimen try green illumination light. What happens?

➡ ***Show your supervisor the result before proceeding!***

Depth of field and contrast in fluorescence microscopy

- Insert a specimen provided by the supervisor (feathers with red dye). Using a 10x objective, and green excitation, focus the microscope so you get a sharp image. Adjust the diaphragms until you are satisfied with the illumination (don't touch the lamp focus). You should now see a bright orange-red image of the feathers. When you looked at specimens in transmitted light, you noticed that the depth of field, as well as the image contrast, varied dramatically depending on the size of the aperture diaphragm. Try varying the size of the aperture diaphragm now, and see what happens.

➡ ***The intensity will vary, but what about depth of field and contrast? Explain the difference compared with transmitted light microscopy!***

As you have seen in the last experiment the aperture diaphragm can be used to vary the intensity of the illuminating light. There is no need to think about matching the numerical apertures of the illumination and imaging optics. This is quite fortunate, because the light intensity from high-pressure lamps cannot easily be varied. ***This is a fundamental difference compared with transmitted light microscopy***. In transmitted light microscopy (with tungsten light) the aperture diaphragm is used for controlling $N.A._{cond}$, and the lamp current is used for controlling the intensity. In epi-fluorescence, the aperture diaphragm is used for controlling light intensity, and the light source has a constant output. $N.A._{cond}$ is irrelevant in this case, because it does not affect the imaging properties.

Use of oil immersion objectives

So far you have been working only with dry objectives. Now you will get the opportunity to work with high-magnification oil immersion objectives.

➡ ***Why is oil immersion used at all?***

Working with high-magnification objectives is much more difficult. There are several reasons for this:

1. The working distance (i.e. the distance between objective and specimen) is usually very short. It is therefore easy to damage or move the specimen by touching it with the objective.

2. The depth of field is very small, and therefore it is difficult to find the correct focus position.

3. When moving the specimen stage to look at different parts of the specimen, the movement must be much more precise because of the high magnification.

4. Oil immersion is often used. The oil must be applied correctly, and afterwards specimens and objectives must be cleaned properly.

Caution #1! ***After an oil immersion objective has been used, there is still oil on the specimen surface. If the specimen is then to be studied with a dry objective, the oil must be removed. Otherwise the dry objective will not work properly (poor image quality), and, even worse, oil may be smeared on the front surface of the dry objective. If a dry objective is accidentally smeared with oil it must be thoroughly cleaned before it is used again. To clean immersion oil from a glass surface (specimen or objective), first wipe gently with a dry lens tissue, and then with a tissue (or Q-tip) moistened with alcohol.***

Caution #2! ***When working with immersion oil everything, including people, easily get smeared down with the substance. Try to avoid this! One easily gets oil on the fingers. Wipe them off using paper tissue (not your handkerchief!) before touching anything else. Avoid getting oil (even the trace amounts on fingers that have been wiped off) into your mouth, eyes, nose etc. Even extremely small amounts of oil may cause eye irritation. After the exercise, wash your hands carefully with soap and water!***

Before using a high-magnification objective, the specimen should always be studied at lower magnifications first. Typically one would use 10x, 40x, and 100x in succession. Let's try this with a simple specimen!

- Insert a specimen provided by the supervisor (feathers with red dye). Using a 10x objective, focus the microscope so you get a sharp image. As previously, adjust diaphragms to obtain a satisfactory image. We will now change to a 40/1.0 oil immersion objective. Swing the objective turret to an intermediate position between the 10x and 40x objectives. Apply ***one*** drop of immersion oil on the specimen surface. Squeeze the oil bottle very gently. It is essential that no air bubbles get trapped in the drop of oil. Before swinging the 40x into position, make sure it is in the upper locked position (ask the supervisor to demonstrate). After it is in position, release it and let it gently dip into the oil. When looking through the eyepieces, the plane of focus should correspond rather closely to what you saw with the 10x objective. The specimen area seen through the eyepieces is now much smaller than it was with the 10x objective.

➡ ***When you look through the eyepieces you see a circular field of view. What is the diameter of this field of view in the specimen plane when using 10x, 40x and 100x objectives? Ask the supervisor to explain about "tube factor."***

- Now that you have changed to 40x objective, set the luminous field diaphragm size correctly. Note that a surprisingly small correction is needed (if any at all!). This is a big difference compared with transmitted-light microscopy.

➡ ***Explain why there is hardly any need to adjust the luminous field diaphragm.***

- Finally, switch to the 100/1.3 objective. Probably the previous drop of oil will be sufficient also for this objective, otherwise apply an additional ***small*** amount of oil. The 100x objective has an extremely small working distance (approx. 90 μm), and therefore you must be careful not to damage the specimen (the objectives are spring-loaded as a precaution against damage, but you must be careful nevertheless). With this objective you have only about 0.3 μm depth of field. The specimen structures are distributed over a much larger thickness than this, and therefore most of the specimen structures are out of focus. This out-of-focus blur will be superimposed on the sharp in-focus image, which can therefore be difficult to see. The conclusion therefore, is that specimens to be studied at high magnification should be very thin, preferably less than 1 μm. The depth of field for some objectives are given in the table on next page.

Objective	***Depth of field (μm)***
10/0.32	7
40/0.6	1.4
40/1.0	0.6
100/1.3	0.3

To avoid the problem with out of focus blur one can use a confocal scanning microscope. This kind of microscope will only image the in-focus parts of a specimen, and it is therefore suitable for studying thick specimens. In lab. sessions 3 & 4 you will get the opportunity to work with confocal microscopy.