ONBI Practical 2: Bright Field

RM Parton revised 2014 - (adapted from a worksheet by Ken Sawin)

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Aims of the practical:

1) Familiarize yourself with the basic parts of the microscope.

2) Be aware of correct alignment and use of different components in the bright field light path and how they are adjusted to increase contrast

3) How to clean objective lenses safely.

Sample Preparation:

Test samples for examination will be provided. These will be either fixed pre-prepared slides or freshly mounted live material (onion epidermis or Drosophila embryos). There will be the opportunity to prepare microscope samples in other practical sessions.

Aim1: The basic parts of the microscope:

This practical is intended for standard wide field microscopes with a bright field condenser and ideally with different contrast enhancing components.

Familiarise yourself with the parts of a basic compound microscope using the diagram (Figures 1 and 2) as a guide. Before you can set up a microscope correctly you need to find the components that need to be adjusted.

Find the following components on your microscope:

The stage and specimen holder – There are two main forms of microscope: the Upright and the Inverted. With an upright you look down upon the specimen, with an invert you look up at the specimen from below. In both cases the bright field path is "transmitted", the illumination light from the lamp is collected by the condenser and passes through the specimen (transmitted) to the objective lens on the other the other side.

The objective lens and eyepiece - The object is viewed by two lenses, known as the objective lens and eyepiece. This arrangement gives two stages of magnification (hence "compound" microscope). In the first stage the objective lens forms an image of the specimen at the intermediate image plane. In the second this image is viewed by the eyepiece (and the eye). Identify the different objective lenses on the microscope.

The bright field condenser lens – Located either under (Upright microscope) or above (Inverted microscope) the microscope stage. When correctly set up, light from the source is focused onto the specimen by the condenser lens, to provide high intensity, even illumination so that the highest powers of the microscope can to be used to optimum effect. Identify the condenser and locate the adjustable components associated with it and what they do.

The condenser or aperture iris - This limits the angle of the light passing from the illumination source through the microscope. The result is reduction in illumination brightness at the specimen and increasing contrast at the expense of resolution due to the fact that resolution is dependent upon the angle of light passing through the microscope).

The field iris - This iris restricts the field of illumination at the specimen plane. This increases contrast in the view and reduces photo-damage outside the viewed region of the specimen.

Figure 1A – simple compound microscope (bright field path)

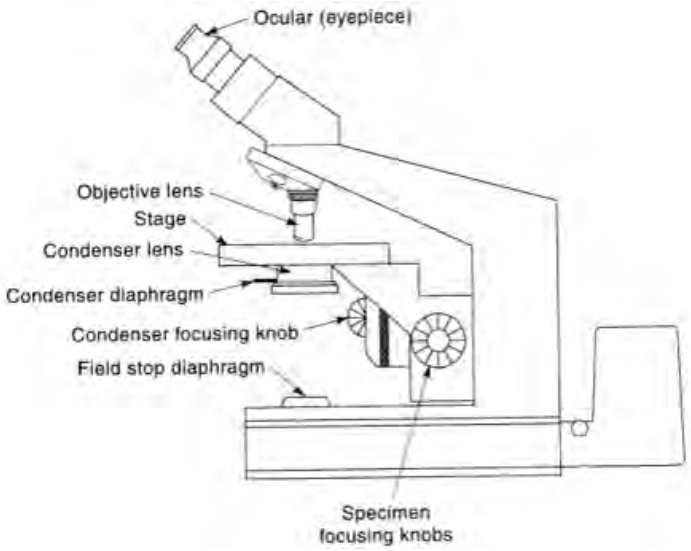
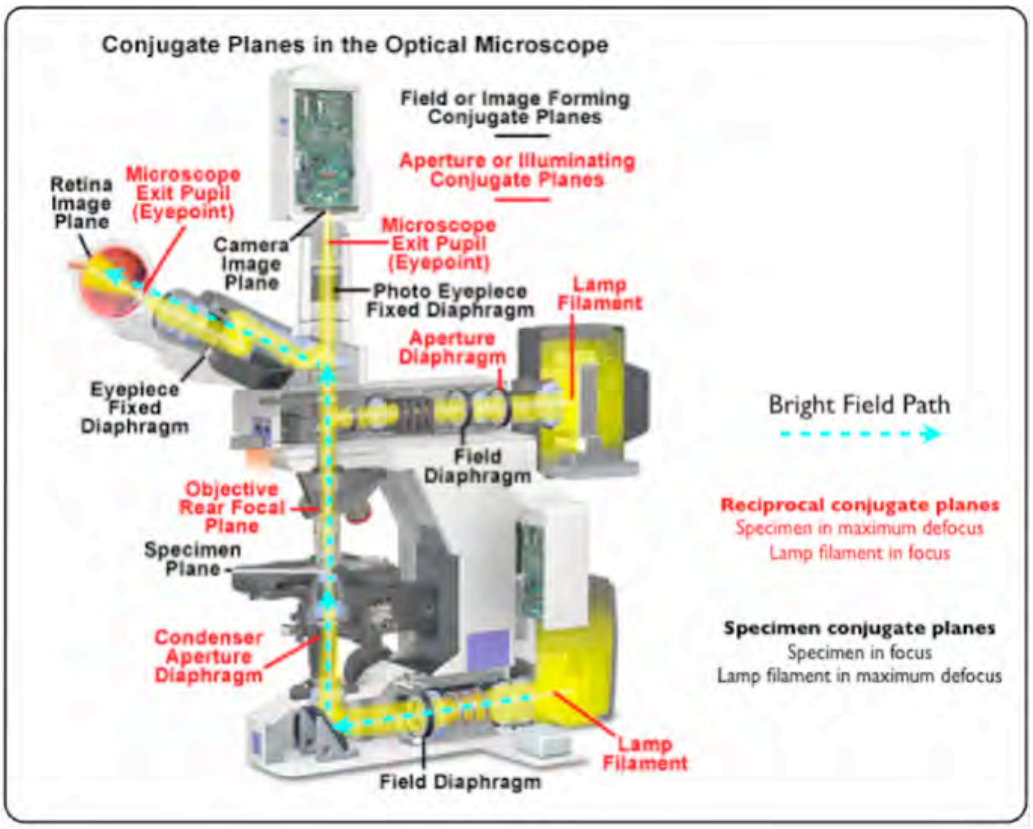


Figure 2 – Research Microscope (bright field path) http://www.olympusmicro.com/primer/



Aim2: Setting up Koehler Illumination and correct adjustment of the condenser for bright field microscopy:

The primary purpose of Koehler illumination is to ensure even illumination of the specimen field plane, without any "image" of the illumination source itself (e.g. a tungsten filament) projected onto the specimen. Nowadays we rarely use brightfield microscopy alone, but it is useful for demonstrating aspects of the microscope. Refer to Figure 3 for a diagram of the optical arrangement of components for Koehler illumination.

(We assume here that the tungsten lamp filament or other illumination source is itself centred, as in most modem microscopes this is fixed. Historically this was not the case, and more steps were involved in setting up Koehler illumination.)

1. With the condenser wheel set to “bright field or BF ” (i.e. with no DIC, phase or dark field optics in place), and the aperture diaphragm (NOT the field diaphragm) closed to more than 50%, to increase contrast, focus the objective on the **test sample**.

2. Now close down the field diaphragm, and focus the condenser (NOT the objective) such that the field diaphragm iris appears in sharp focus on the same plane as your sample (the specimen plane).

3. Centre the field diaphragm by adjusting the pair of knobs on either side of the condenser. (Beware condensers that have a third “locking” knob nearby.) Note that when you move these, the edges of the field diaphragm will move (because it is "upstream" of the condenser lens), whereas the image of the specimen does not move (because it is "downstream" of the condenser). Adjust these such that the field diaphragm is properly centred.

4. Open the field diaphragm such that almost the entire field of view can be seen.

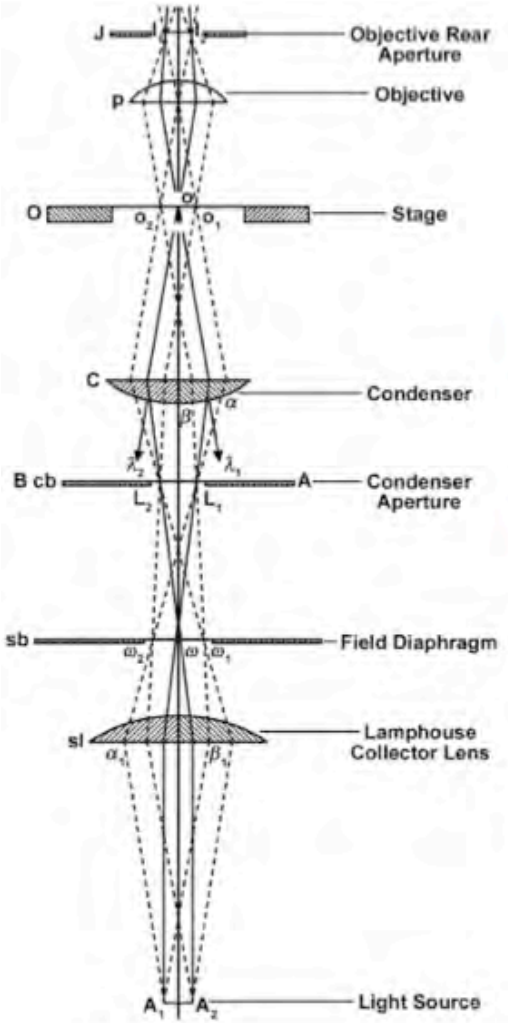
   This is now Koehler illumination. By focusing the condenser in this way (that is, adjusting the position of the condenser relative to the already focused specimen) you have brought 2 sets of conjugate planes into the special relationship in which each set of planes represents the optical transform of the other set (or, more simply, when one set is at best focus, the other set is at maximal "defocus"). See Fig 1 above.

5. Now experiment with opening and closing the APERTURE diaphragm (previously closed below 50%). Note that as the aperture diaphragm is opened, the contrast decreases, and the spatial resolution improves The increased resolution may actually be hard to see, as the brightness will increase quite a lot, therefore you may have to adjust the lamp intensity to see this. Conversely, when you close the aperture diaphragm, the contrast increases, while the spatial resolution decreases. Also note that whereas you could see the edges of the field diaphragm iris, when you're looking at the specimen you don't see the edges of the aperture diaphragm as you open and close it. Also, alter the objective focus above and below the plane of focus, to see how the contrast can change. Before phase-contrast and DIC were invented, this method was often used to get a higher contrast image of transparent objects.

6. Return to the properly adjusted Koehler illumination. Now remove one of the eyepieces and replace it with a **phase telescope** or insert a **Bertrand lens** below the objective if your microscope has one. Either of these will give you a clear view of the back focal plane of the objective, which is conjugate to the condenser aperture plane (This can also be done by **simply removing the eyepiece** but with a phase telescope/Bertrand lens the back focal plane is magnified and therefore easier to see). Note that the phase telescope itself can be adjusted for the best focus.

7. Looking down the telescope, adjust the aperture diaphragm—open and close it, and see that the edges of the iris open and close, and they should be in sharp focus. Do the same thing with the field diaphragm. Notice that if you change the diameter of the field diaphragm while looking down the phase telescope, you will not see the edges of the field diaphragm. Now repeat the same adjustments of the aperture diaphragm, but look down the eyepiece rather than the phase telescope and observe the same changes in contrast that you observed before. Hopefully by now you will have seen and appreciated the notion of conjugate planes and inverse relationship between the field planes and the aperture planes.

Figure 3A – Koehler Illumination

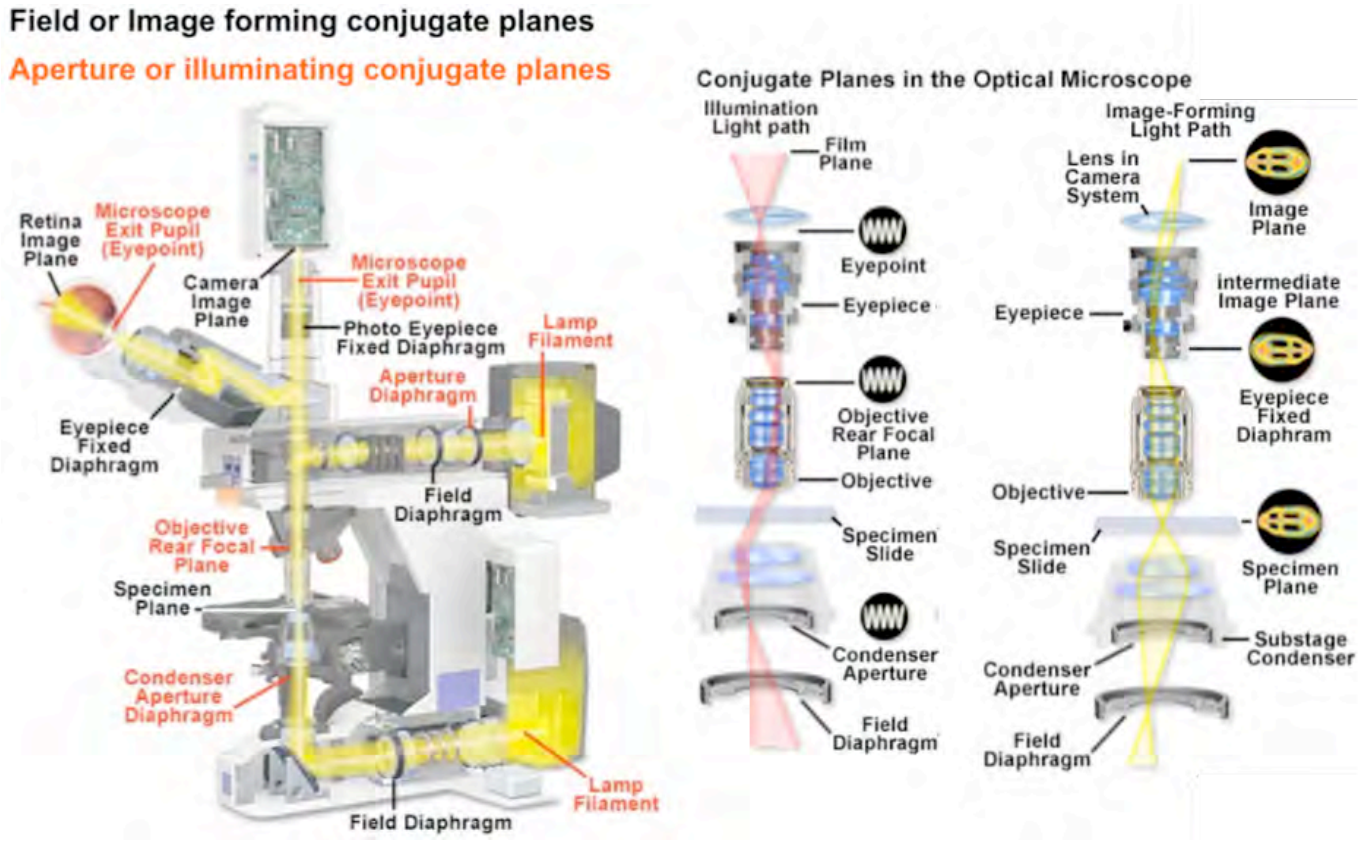


August Koehler
1866-1948

Adapted from "Fundamentals of light Microsocpy" Murphy and Davidson

Koehler: Illuminating the image plane with a maximally defocused image of the lamp each point from the lamp contributes equally to illuminating the image plane

Figure 3B – Koehler Illumination conjugate planes



http://www.microscopyu.com/articles/formulas/formulasconjugate.html

Setting up Koehler Illumination and correct adjustment of the condenser for bright field microscopy continued - contrast generation:

Phase contrast microscopy ***this assumes that your microscope has both phase condenser components and phase objectives (identified by Ph and a number e.g. Ph3).**

The second purpose of Koehler illumination is that phase-contrast and DIC, the two major methods generating contrast from "transparent" specimens with transmitted light, require that the condenser aperture plane is conjugate to a specific plane in the objective—the "back focal plane" (which is what you were looking at with the phase telescope/Bertrand lens).

1. Switch the condenser to a phase setting that matches the objective being used, marked on the objective as Ph-1 or similar. Note that (usually) there is no condenser aperture diaphragm to open or close, as the aperture diameter is now fixed by the fixed-size phase-annulus inside the condenser (Otherwise select the appropriate aperture diaphragm - too far closed and everything will be dark). If

possible, take the condenser off of the microscope and study the different possibilities that can exist in the aperture plane.

2. Assuming the phase contrast is already well-adjusted - look down the eyepieces at the **sample slide**. (Setting up good phase requires that the phase ring in the objective and in the condenser are correctly aligned.) Now you will see a great deal of contrast, against a hopefully uniform grey background. Note that the cell is likely to produce both areas that are darker than the background and also areas that are lighter than the background, especially the so-called "phase halo" around particularly thick or refractile objects, such as yeast. The reasons for the phase halo are quite complex, but it is useful to know that DIC does not generate such a strong halo (but is much more expensive)

3. You already have had some explanation of how phase-contrast works. To appreciate it more, take out one eyepiece and insert the phase telescope to look at the back focal plane of the objective. You should see two rings—a bright ring inside a somewhat larger dark ring. The bright ring represents the hollow cone light produced by the phase annulus in the condenser. It is matched in size with the darker phase ring in the objective. Remember the dark phase ring in the objective actually does two things. First, it has introduces a phase shift of 90° (one-quarter wavelength) to the light that passes through it (which is nearly all zero-order, non-diffracted light). Second, it is also darkened, to reduce the intensity of the zero-order light which passes through it. The purpose of this is that only a relatively small amount of incoming light is actually diffracted by the specimen. Therefore, if the interference between non-diffracted light and the light diffracted by the specimen ("higher-order" light) is to be visualised, it is important to reduce the total intensity of the non-diffracted light. This is also why the background in phase contrast appears to be grey, even though you may be putting a lot of light onto the specimen. You will notice if you switch back to the brightfield position on the condenser, that in phase-contrast, most of the incident illuminating light does not go into the final image.

4. Now, looking at the back focal plane of the objective, switch to the bright-field position on the condenser (if you haven't already done so). The bright ring of light should now be replaced by a broader circle of light. Then switch back to the phase-contrast position on the condenser.

5. Now see how different adjustments to the system affect the quality of the phase-contrast. First, with the edges of the field diaphragm in view, reposition the condenser using its positioning knobs, and see the effect of this, both at the back focal plane and on the image. Then adjust the position of the phase annulus within a properly adjusted condenser. (On older microscopes, this is easily accessed by the user and therefore often put out of adjustment - on most modem microscopes it is more difficult to put it out of alignment). Again observe the differences both on the back focal plane through the phase telescope and on the image of the specimen itself, through the eyepiece. You should see that as the bright ring no longer superimposes on the dark ring, the quality of the phase contrast is significantly altered.

Darkfield microscopy

If the microscope has a darkfield position in the condenser it can be interesting to see how this works. If the microscope does not, you can sometimes approximate darkfield by using the condenser phase annulus for a high-power objective (e.g., Ph 3) in combination with a low-power low NA objective (e.g. 10X or 20X Ph 1).

1. Look at the sample slide, and judge whether that's a useful image or not. Compare how the cells/tissue looks relative to air bubbles. Switch back to phase-contrast and see what details are different.

DIC microscopy (also know as Normarski)

The requirements for DIC are: polarizer, analyzer (the pair of polarising filters before and after the objective , respectively), Wollaston (in the condenser) and Wollaston (at the back of the objective). DIC is the most complicated of all transmitted light methods and the components are the most expensive. Although the principles of DIC are nearly the same on all microscopes, different manufacturers have engineered the components differently. Therefore we won't do a lot of playing around with DIC but rather will just observe the effects of bias retardation, etc. If possible, take out both Wollaston prisms (rotate the turret of the condenser to bright field and remove the prism at the objective) so that you have only the polarisers present (i.e., polariser and analyser). For good DIC the two polarisers should be “crossed” i.e they should be rotated 90 degrees with respect to each other so very little light gets through.

If you have a de Sénarmont-type DIC (rotatable polarizer), rotate this and observe the effects on the brightness of the image. Otherwise, or in addition, slide the analyser in and out of position to observe the effects on the brightness of the image.

1. Now rotate the condenser to the appropriate DIC position to introduce the condenser Wollaston (the condenser wollaston should be matched to the objective used), and put in the objective Wollaston (this may be specific for the objective used or a single wollaston for all objectives). Observe the changes in the image.

2. Change the bias retardation ("shadow") by adjusting either the objective Wollaston (e.g. Zeiss) or the angle of the polarizer if you have de Sénarmont-type DIC (e.g. Nikon). Notice that as the bias changes from positive to negative (i.e., on either side of zero, you change the direction of the apparent shadow). You should aim for an even grey background with the features of interest well contrasted in the specimen.

Aim3: Cleaning components of the microscope

Always keep fresh lens tissue by the microscope. **ALWAYS**. This will encourage people to use it regularly; this is a lot cheaper than replacing objectives. Lens tissue is very cheap, and good value for money. Kimwipes, paper towels, toilet paper, must **NOT** be used **EVER**.

Know the difference between the oil and non-oil objectives on your microscope. If you are

aware of this, and also of the fact that working distances for high NA "dry" objectives (e.g., 40X/0.9 NA) can be quite short, you are very unlikely to get oil onto a dry objective.

Wiping the objective: This is especially important on inverted microscopes, since excess oil can leak down (by gravity) into the objective itself, through the gap used for the spring-loading. I usually take a piece of fresh lens tissue and fold it over twice, so that it has 4 layers. Then I wipe the region around the glass very gently, to soak up oil. The idea of the four layers is that it prevents any oils from your fingers from getting through. Then I use a fresh piece (folding again) and gently touch the front lens to remove any oil from there. The main goal is to avoid contacting the lens with the same region of tissue twice, since the tissue could contain small bits of dust that could scratch the lens.

Cleaning the objective front lens: The idea is to use a fairly powerful solvent, but to minimize the time that the lens is exposed to solvent by wicking it away even as it is applied. The reason is that many solvents can react with the cement that holds the lenses together, and eventually ruin the objective, so there is a compromise between using a strong solvent and minimizing its exposure to the lens. If the solvent is highly volatile, this can help to minimize exposure time. I use chloroform, but you can also use acetone or isopropanol. Isopropanol is less harsh but might not remove all contaminants and is less volatile, so will stay on the lens longer.

Take the objective off of the microscope and put it upright on the bench. Examine it using an inverted eyepiece as a magnifying glass. Alternatively, some people use a dissection scope. **If the objective front lens is covered in media, cells or agar then it should first be cleaned with water, since the use of solvents will fix the crud onto the objective, after which you may never get it off.**

Assuming that you want to get rid of just oil and dust, have a bottle of acetone nearby. Take a piece of lens tissue and hold it over the objective from lens. Using a rubber bulb and a glass pasteur-pipette, drop one or two drops of ether onto the lens tissue above the front lens. As you are doing this, wick it away by drawing the lens tissue across the front lens. Look to see if all of the oil is gone. If not, repeat the procedure, with either fresh lens tissue or a region of tissue that hasn't yet been used.

Cleaning the eyepieces: make a "fresh brush" by licking the ends of lens tissue and wrapping it around a stick, and licking and sealing the end, and then tearing it off to make virgin fibres that can be used to touch the glass. One the eyepieces, the major culprit is usually dust and also a bit of oil from your eyelashes. Quite often this can be cleaned with just a bit of water. You could squirt a little on the brush, and gently wipe it around. It is always better to wet the brush lightly rather than squirt a lot of liquid onto the glass and then try to wipe it all off, since it could seep into unwanted places. Mascara is very easy to get on and very hard to clean of eyepieces. If you are using a microscope **try not to** wear mascara.

Do not do apply these cleaning methods to anything that is coated with something sensitive, such as a fluorescence interference filter! Antireflection coatings should tolerate water— but many interference filters will be ruined by water. If you do need to clean them then talk to the manufacture about how best to clean them. The best solution is not to get fingerprints on them in the first place.