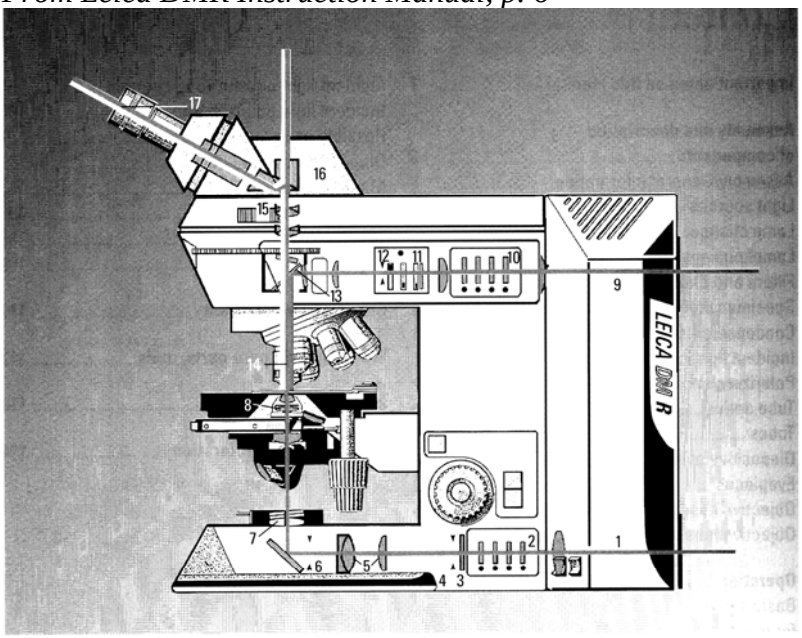
Basic microscopy for PEARL

Prepared as a guide for PEARL by Kathleen Rühland

Paleolimnological research at PEARL will undoubtedly require extensive use of a light microscope. Fortunately, PEARL has numerous high-end microscopes specifically configured for resolving the minute details of the microscopic paleoindicators that we have (or will) come to love. Unfortunately, these microscopes are all too often improperly used and therefore fail to meet their full potential. Mastering how to properly set up a light microscope is important, regardless of what magnification you are working with and which paleoindicator you are working with (diatoms, chrysophytes, cladocerans, chironomids, and even pollen). Proper alignment of the microscope's light path or illumination system is essential for obtaining crisp, clear, high resolution images of our microfossils. If this is not done then you might as well work on a teaching microscope and never reap the benefits of a state-of-the-art, fully equipped research microscope. The following pages hope to guide you through some microscope basics that will help improve your counting and identification work.

From Leica DMR Instruction Manual, p. 6



Transmitted light path*
1 Light source (lamphousing not illustrated), **2** Filter magazine*, 4-pos., **3** Diffusing screen, **4** Aperture diaphragm, **5** Imaging system of aperture diaphragm, **6** Field diaphragm, **7** Polarizer*, **8** Condenser

Incident light path*
9 Light source (lamphousing not illustrated), **10** Filter magazine*, 4-pos.
Diaphragm module with:
11 Aperture diaphragm* or filter and diffusing screen, **12** Field diaphragm, **13** Reflector or filter cube

Imagine light path
14 Objective, **15** Tube optics/Bertrand lens*, **16** Tube, **17** Eyepiece

Parts of the compound microscope (Leica DMR HC)

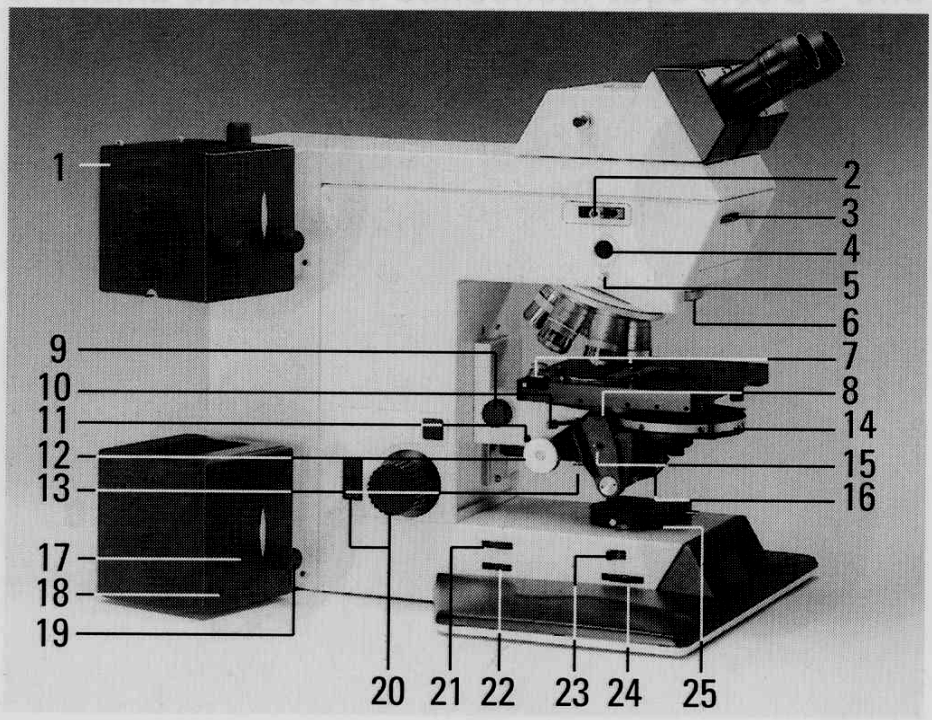
You will find that being familiar with some of the basic parts of the microscope, what they do and how light passes through them will make your life much easier, particularly when you are setting up Kohler illumination and/or when you are setting up the scope for differential interference contrast (DIC), an optical system that many of us use at PEARL (discussed in detail later).

1) Lamphousing (light source)
2) On/Off switch
3) Transmitted/incident light selector switch
4) Grey Field (GF) switch
5) Illumination control dial
6) Filter magazine
7) Objective lenses
8) Rotating nosepiece
9) Condenser lens
10) Prisms (analyzer, polarizer)
11) Objective-side prism turret
12) Condenser-side prism turret
13) Aperture diaphragm
14) Field diaphragm
15) Oculars (eyepieces)
16) Fine focus knob
17) Coarse focus knob
18) X-Y stage orientation
19) Vernier calipers
20) Centering screws

From Leica DMR Instruction Manual p. 69

Fig. 48*

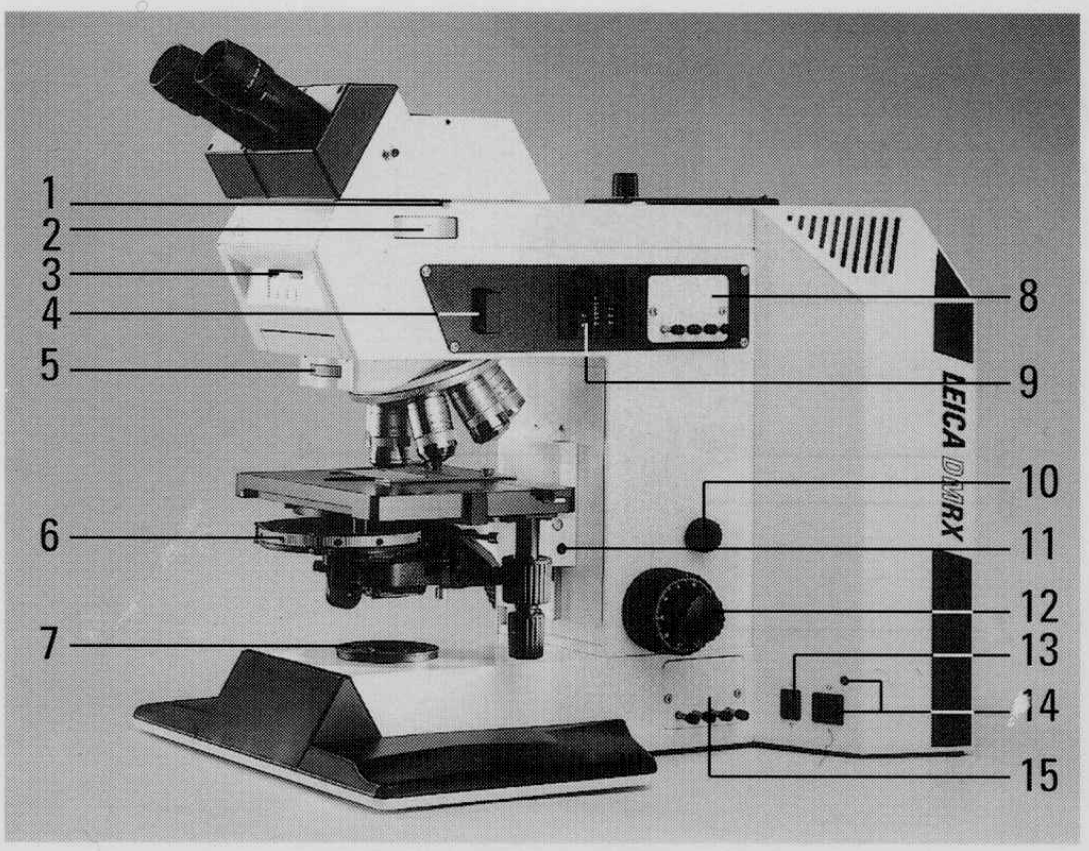
1* Lamphousing 106 z for reflected light, **2*** Analyser, **3*** Rotatable reflector turret, **4*** Window for incident light lamp adjustment, **5*** Clamp screw for nosepiece change, **6*** ∞ Turret for objective side Wollaston prisms, **7*** Knurled knob for adjusting the object holder, **8** Stage rotation clamp, **9*** Stage clamp, **10** Centering keys for condenser disc, **11** Fixing screw for condenser holder, **12** Condenser height adjustment, **13** Adjustable upper stop of condenser, **14** Condenser disc, **15** Lever for condenser top, **16** Condenser centering screws (hidden, cf 27.1 and 27.5), **17, 18** Centering screws for lamp holder, **19** Collector adjustment, **20** Focusing, **21** Aperture diaphragm, **22** Field diaphragm, **23** Grey (neutral density) filter, **24** Illumination intensity control (12 V 100 W lamp), **25*** IC/P polarizer



From Leica DMR Instruction Manual p. 53

Fig. 42

1 Tube clamp screw, **2** Bertrand lens* in/out, cf Fig. 50, **3** Reflector/filter system turret*, **4** Incident light polarizer*, **5** IC objective prism disc*, **6** Condenser disc*, **7** Coverring for base of stand, **8** Filter magazine*, **9** Incident light diaphragm module* cf Fig. 23, **10** Stage adaption*, **11** Place to keep centering keys* (interchangeable stage only), **12** Mechanical coarse and fine focusing, **13** Transmitted/ incident light selector switch, **14** Mains switch with pilot lamp* (not for motor focus), **15** Filter magazine* for transmitted light



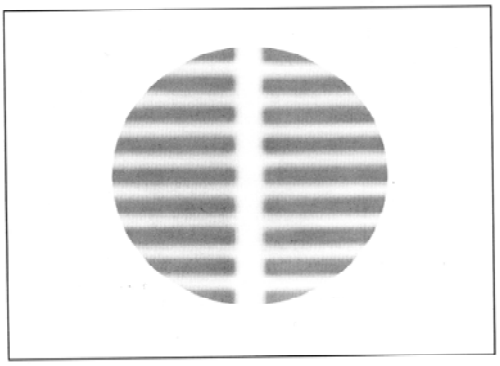
1) **Lamphousing**- holds halogen lamps that are 12 V 100 W (we have used both OSRAM and Philips).
2) **Lightbulbs** -

If a lamp burns out and you need to change it, you should refer to the manual (p. 62 and 90-92) to make sure that you are replacing the right bulb (other scopes in the lab have a different voltage and wattage).

Don't touch the new bulb with your fingers as the oil from fingers can heat up the bulb and cause it to explode or at the very least, burn out much more quickly (the easiest way to do this is to keep it in the little plastic bag that it comes in and simply make an opening in the bag).

Centre and focus the light filament (p. 68 & 92 of manual).

Fig. 47a Lamphousing 106
Reflection of the lamp filament, greatly schematized: in reality the reflection is extremely low in contrast. In incident light the bright overlap area is wider and less defined.

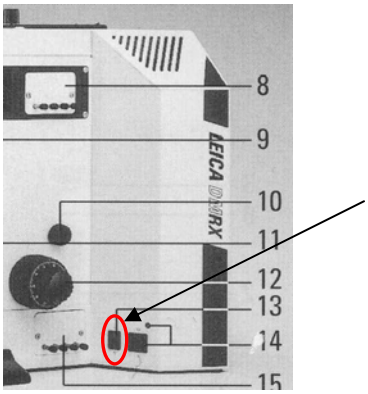


From the Leica DMR Instruction Manual p. 68

A microscope lightbulb gets surprisingly hot!
For this reason take extra precautions to remove items near the lamphousing…particularly the microscope cover as well as books and papers.

A word on lightbulbs!

Before you change lightbulb PLEASE check whether you may have accidentally pressed the "transmitted/incident light selector switch"…this is located right beside the on/off switch of the scope (see p. 53, Fig. 42 #13 in Leica DMRB scope manual). 9.5 times out of ten the lightbulb does not need to be changed because the wrong switch was pressed.

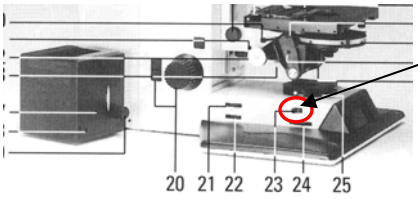


This switch is NOT the on/off switch, but because of its location it is often pressed down by mistake. When you press this button, the transmitted light no longer travels through scope and everything is dark. When this happens, have a quick look at the lamphousing to see if it is dark or if there is light coming through it. Now press this switch and check again. If by pressing this switch light now emits from the lamphousing, you do NOT have to change a lightbulb.

In general, with regular usage, a lightbulb should last several years!

From the Leica DMR Instruction Manual p. 53

When you press **the transmitted/incident light switch**, you will see that the light in the lamphousing turns off. Simply press this button again and look at the lamphousing…if the light goes back on then you are saved the trouble of changing a lightbulb. If the light does not go on…and the scope is also turned on…check to see whether or not the Grey (neutral density) filter is ON. It should be OFF. This switch is on the left-hand side of the scope.



This is the Grey (neutral density) Filter (GF). It should be in the **OFF** position.

From the Leica DMR Instruction Manual p. 69

If you have checked both and there is still no light, then you likely need to change the lightbulb.

You may say to yourself, how hard could it possibly be to change a lightbulb? Well, you will be surprised to know that most people who use a microscope do not know how to properly change the lightbulb. This is likely because 1) when used properly, a microscope lightbulb should last for many many years so you will likely not have much experience, 2) not all microscopes use the same type of lightbulb…the volts, wattage and the type of light (e.g., halogen, mercury etc.) differs from scope to scope, 3) unlike a regular lightbulb, the microscope uses a very bright, powerful, and very hot little bulb which means that if you get a little bit of oil from your fingers on it while changing it, it can potentially blow up or burn out quickly, 4) there is a chance that once you have put in a new lightbulb, you will need to center the filament.

The best thing to do when the scope you are using blows a bulb is to deal with it right away (i.e. don't abandon the scope to let someone *else* sort it out). Ask the lab technician for a new lightbulb and ask a senior PEARLite for assistance. Always refer to the lab manual as the manual will tell you which type of bulb is required and how to go about inserting the bulb and centering it.

3) Illumination Control Dial

In general, the illumination dial **does not need to go above 6** or so. If you find that you cannot see enough at this illumination, stop and check that your microscope is properly set up (*SEE SETTING UP KÖHLER ILLUMINATION*). Working at such high illumination is not good for 1) the lightbulb…burns out more quickly, and more importantly 2) your eyes. Microscope lightbulbs function at very high temperatures so be aware of this….keep the area around the lamphousing clear of clutter (books and the microscope cover)….it does not take long for things to melt or even burn!

4) Filter Magazine for transmitted light:

We have the following filters on the newer model Leicas:

BG 20 - Blue filter - highlights red light - corrects light for photography and improves image
DLF - Daylight filter
N4 - Grey Filter (25% permeability)

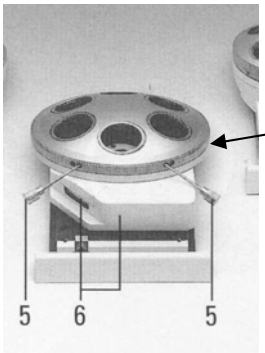
Generally, you would not use all of these filters at the same time. The DLF is a good one to use as it can greatly improve the image....do some tests and see. If using Brightfield, you may need to use more filters.

The N4 filter cuts down the light coming through substantially and is generally not used for our purposes with the exception being for brightfield work (especially at 40X)...here the light is very intense and the N4 will cut this down to a workable level.

5) Objective Lenses and Oil immersion objectives

The Leica microscopes we use at PEARL have a suite of objective lenses that are attached to a rotating nosepiece.

When you are swinging the objectives into position, **NEVER** use the objectives themselves to do this as you will over time loosen the objective out of its socket and it will fall off (has happened a few times…very costly$$) or you can misalign the objective.

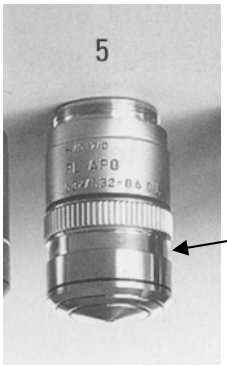


The proper way to change between objectives is to use the gridded disk that houses all of the objective lenses (actually called Objective nosepiece …see p. 45 of scope manual).

From the Leica DMR Instruction Manual p. 45

ONLY use oil on the **100X objective**. If unsure, check the labelling scribed on the objective....generally oil objectives have a black line inscribed at the bottom rim and it would say “oil” or “oel”

DO NOT GET OIL ON A NON-OIL IMMERSION OBJECTIVE LENS....it is extremely difficult and sometimes impossible to get the oil off and may mean buying a new lens (very expensive!)



Another little **tidbit of information** that you might one day find useful: the 100X oil objective lens has a built in **spring** that gives the objective flexibility when you bring it down into the oil on the coverslip. This is a good thing as it helps prevent you from crashing the objective lens through the slide and doing serious damage to your sample and more importantly, to the objective lens.

From the Leical DMR Instruction Manual p. 47

However, there are times when you can inadvertently lock the spring into the “up” position. When this happens it will be virtually impossible to be able to focus onto the specimen with the 100X lens. You may (like others before you) spend the next few hours trying to figure out what is happening. As well, at some point during your investigation the spring may suddenly release and crash through your sample etc.

Bottom line: swing the 100X objective out of the way (where it is safe). Check that the spring is not locked in the up position.

Why we use oil:

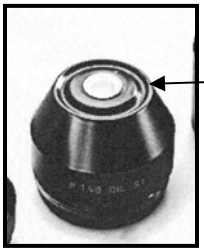
(Photo from www.cargille.com)

Light bends when it passes from glass to air or from air to glass because air and glass have different refractive indices. The bending of light as it passes through the glass slide to the air and then to the glass lens decreases the resolving power. At high magnification (1000X) it can prevent a clear image from being viewed. This decrease in resolution can be prevented by putting ***immersion oil*** between the slide and the lens because immersion oil has the same refractive index as glass. As a result, there is no refraction of light when it passes from glass to oil and vice versa.

The condenser also increases the resolving power of the microscope. When using the oil-immersion lens, the condenser (located beneath the stage) should be raised to a position very close to the stage for maximum resolution.

6) Condenser Lens

The Leica microscope we use at PEARL is equipped with an oil immersion condenser top lens. This is not a common feature on most microscopes but it does allow us to attain a higher numerical aperture (increased resolving power)….for more on this, refer to p. 7 of the excellent book put out by Kodak called " Photography through the microscope". A good way to make sure that the condenser top is made to take oil is to see if there is a well around the lens that will hold the oil in place. Below is a photo of what an oil immersion condenser top lens looks like.
What this means is that you can place two to three drops of oil on the condenser lens. NEVER put oil on a lens (objective or condenser) that is not made to receive oil.



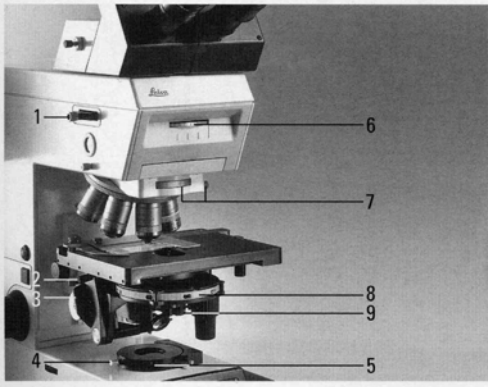
Note the well surrounding the lens on an oil condenser top lens. As well, the fact that it is made to receive oil will likely be inscribed at the bottom of the lens.

From Leica DMR Instruction Manual p. 22

7) Prisms: Analyzer, polarizer and objective side prism turret, condenser prism turret

Many of us at PEARL use diatoms or chrysophytes as our paleoindicators of choice. As many species are only a few micrometers in length, being able to resolve the minute details and ornamentation of these microfossils can be quite challenging. For this reason the Leica microscopes are equipped with specialized optics called *differential interference contrast* (DIC). DIC is an optical microscopy illumination technique that enables specimens that have a refractive index similar to their surroundings to be visually differentiated (i.e., enhances contrast). This is a relatively complex lighting system but fortunately for us, is functionally easy to set up.

Fig. 60 Controls for ICT transmitted light interference contrast
1 Analyser, cf Fig. 30, **2** Stage rotation clamp screw, **3** Condenser top lever, **4** Polarizer rotation clamp, **5** Polarizer index adjustment (cf Fig. 28), **6** Incident light reflector turret, **7** Objective-side prism turret with fine adjustment, **8** 8-position disc for condenser-side Wollaston prisms, **9** Mount for λ or λ/4 compensator (hidden, cf Fig. 27.6)



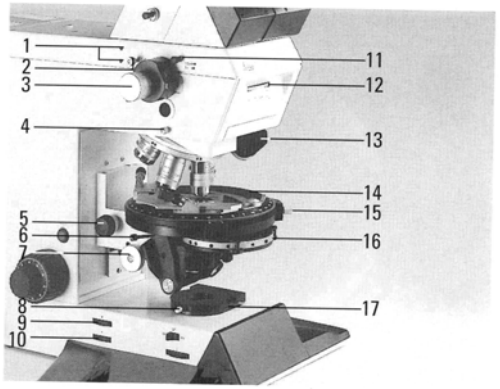
Figs. 54 and 60 *in Leica DMR Instruction Mmanual*…Important components for DIC; Fig. 30 # 5&6 (analyzer), Fig. 28 (polarizer), and Fig. 60 #7 (objective side prism turret).

The components for DIC are mirror image pairs: a polarizer and Wollaston prism below the specimen and another Wollaston prism and polarizer above the specimen. Polarizers and Analyzers are terms used interchangeably.

All four of these components must be in place in order for Nomarski DIC to function….two on the objective side (above) and two on the condenser side (below).

Our scopes have prisms set into the turrets for both 100X oil and for 40X…but not for any other

Fig. 54 Controls on polarized light microscope
1 Centration* of Bertrand lens, **2** Bertrand lens* on/off, focusing, **3** Analyser, **4** Objective nosepiece clamp screw, **5** Stage clamp, **6** Centration of PH light rings and ICT prisms, **7** Condenser height adjustment, **8** Polarizer rotation clamp, **9** Aperture diaphragm, **10** Field diaphragm, **11** Tube lens 1x/1.6x*, **12** Quadruple* turret for incident light techniques, **13** Compensator slot (tube slot), **14** 45° clickstop (hidden), **15** Stage rotation clamp, **16** Condenser disc, **17** Index adjustment of transmitted light polarizer



From the Leica Instruction Manual p. 77

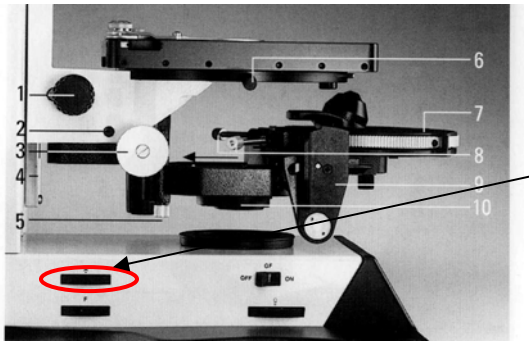
Analyzer (IC/P): two sides to this - one with the lambda sign (λ) is called a full wave compensator and allows all colours in the light spectrum to be used (known as colour contrast) - the other side (P) this whole-wave compensator is inactive and is limited to the red part of the colour spectrum.
Polarizer (ICT/P): at base of scope (Figure 30). Rotatable by 360º. Works together with the analyzer to control the direction of the light (see below under crossing the polarizers or Nichols)
Objective-side prism turret: the newer Leica scopes have a fine adjustment knob on the right side of the turret. Use this adjustment to fine tune the contrast of the image, together with the aperature diaphragm.

Condenser prism turret: slots in turret for prisms...we have two: one for 40X and 1 for 100X objective power. Always make sure that the setting here matches the objective being used (i.e 100X objective and 100 prism).

8) Diaphragms: aperture and field

Aperture diaphragm: this determines resolution, depth of field and contrast (i.e. the numerical aperture and character of the image). This is a cone of light that you can control *within* the field of view. You can narrow the cone to focus more on the subject or you can open the cone which will allow more light to scatter.

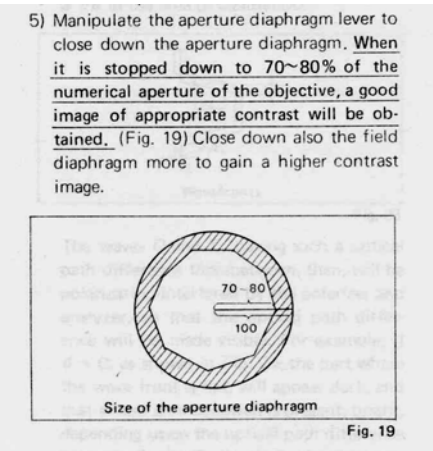
NEVER USE THE APERTURE DIAPHRAGM MERELY TO CONTROL BRIGHTNESS OF ILLUMINATION!!! Use the brightness knob or neutral density filters for this.



Aperture diaphragm: markded with an "A" above the Field diaphragm

From the Leica DMR Instruction Manual p. 19

Opening the aperture to much (higher numbers) will decrease the contrast (things will look washed out) but closing it too much will result in too much contrast and a loss of detail. To achieve the best resolution requires a compromise between the two.


5) Manipulate the aperture diaphragm lever to close down the aperture diaphragm. When it is stopped down to 70~80% of the numerical aperture of the objective, a good image of appropriate contrast will be obtained. (Fig. 19) Close down also the field diaphragm more to gain a higher contrast image.

From Nikon Optiphot Instruction Manual Fig. 19

Rule of thumb: when you remove the eyepiece (either one…it does not matter) you should adjust the aperture to be about 70-80% of the radius of the field of view. For 100X oil immersion objective lens this translates into a setting of about **3 on the aperture dial.** Because this number is now known for our scopes, we do not need to remove the eyepiece. This varies for different objective powers.

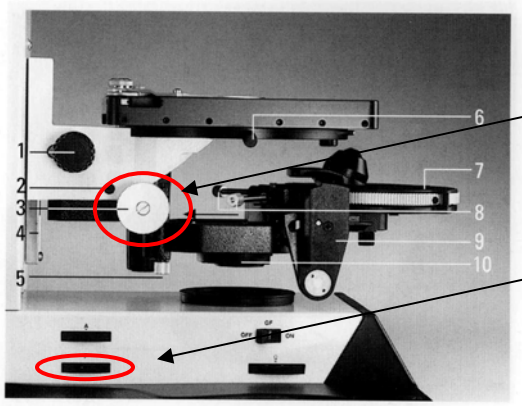
It is best to critically evaluate the specimen (e.g. diatom) while adjusting the aperture diaphragm until the optimum compromise position is found between depth of field and resolution.

Now you may need to make some minor adjustments to give the image "oomph". You can start with the aperture diaphragm wide open and then close it down slowly until the image gets "oomph" (see p. 34-35 in the book called "***Photography through the Microscope***" in the blue binder). If the diaphragm is open too much the image will lack contrast and depth of field; close it down too much and diffraction lines surround the now too contrasty image. The best compromise setting is at about 70-80% (i.e. ~3 aperture).

Field Diaphragm: The field diaphragm protects the specimen from heat and prevents the light not required for the image formation from entering the object. The diaphragm is therefore opened just far enough for the observable field of view to be fully visible. A change of magnification therefore always calls for adjustment of the field diaphragm.

The field diaphragm should be clearly focused through slight adjustments of the condenser top lens via the silver knob on the left hand side of the scope.

Fig. 12 Assembly of condenser holder* and specimen stage* **1** Stage clamp, **2** Drill hole for clamping the condenser holder (3 mm hexagonal screwdriver), **3** Condenser height adjustment, **4** Dovetail guide, **5** Adjustable upper stop of condenser, **6** Stage rotation clamp (no. 1187 and 1189), **7** Universal condenser with disc, **8** Centering screws for light rings/ IC prisms, **9** Lever for condenser top, **10** Condenser holder (with slot for whole- and quarter-wave compensators)



Condenser height adjustment knob

Field Diaphragm dial

From Leica DMR Instruction Manual p. 19

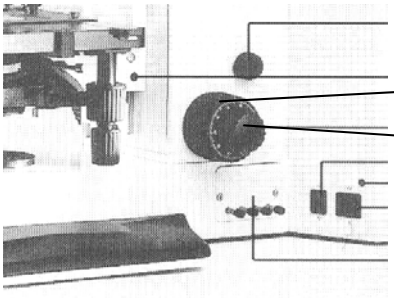
9) Adjusting the eyepieces:

-If you do not have an astigmatism, then you do not need to wear glasses when using a microscope as it can compensate in the same way that your glasses do.
-If you do have an astigmatism, you can wear your glasses while counting if the scope has oculars with a high eyepoint (most research scopes have this type of eyepiece). To use the scope with your glasses just remove or fold down the eye shades around the ocular so that you can get your glasses close enough to the lens.
-Also remember that many of us have eyes that are not identical, you can use the diopter adjustment on the eyepieces to compensate for this and will help reduce eye strain.

To adjust the eyepieces: Also see p. 31 in the *Photography through the Microscope* book.

1) defocus specimen
2) focus the graticule (one eye lens only has this) by adjusting the eyelens
3) focus the specimen using the fine focus knob by looking only through the eyepiece with the graticule
4) then close your eye and focus the specimen by adjusting the other eyepiece only (diopter adjustment collar on the eyepiece)…i.e., not with the fine adjustment knob.

10) Fine and Coarse Focus Knobs



Large dial = coarse focus. Use this when initially setting up your slide.

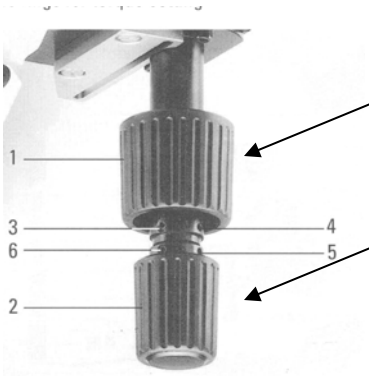
Smaller dial on inside = fine focus knob. Use this most often when fine adjustments are made.

From the Leica DMR Instruction Manual p. 53

12) X-Y stage orientation

y-adjustment knob

x-adjustment knob

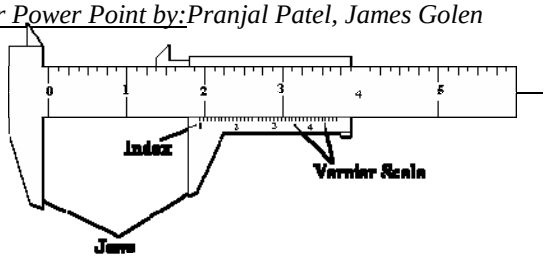


From the Leica DMR Instruction Manual p. 54

13) Vernier Scale

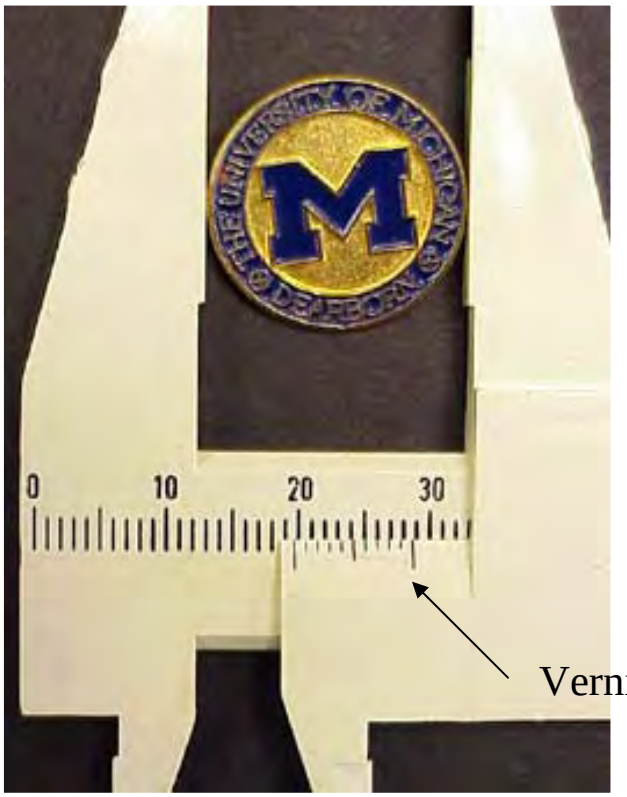
Along the right-hand sides of the stage, the Leica microscopes have scales known as Vernier scales. These scales are very useful for re-locating the exact position on your microscope slide. This may be important, particularly when you are new to counting microfossils, as it allows you to find your place back and resume counting the following day. It basically works as two sliding rulers.

The following figures were taken from a presentation found on the web:
Written and produced by:Linda Jones, Mary Lustig, Minnie Martin Mary Millar, Robin Odom
Edited for Power Point by:Pranjal Patel, James Golen



The location on your slide can be determined by reading the number on the main scale that is on or just to the left of the index.

In this case, the index is just to the left of the 20mm mark. So 19mm is our first measurement.



The decimal place is found by matching up a division on the vernier scale which lines up with a division on the main scale. Here we can see that the 7th vernier division aligns with the 26mm division of the main scale. Therefore, the location of this pin equals 19.7. If you take both the X and Y co-ordinates you will be able to find where you stopped your count.

14) Centering Screws

There are two small knobs located under the condenser turret at the front of the condenser assembly. These are set at 45º and are used to center the condenser (field diaphragm). You will become very familiar with these knobs when you set up ***Koehler illumination.***

Setting up Köhler (Koehler) illumination: all magnifications

Refer to *Photography through the Microscope* (Kodak publication) pp.28-38 for excellent and simple explanations and helpful visuals.

For a fun and interactive tutorial on Köhler illumination visit:

http://www.microscopyu.com/tutorials/java/kohler/index.html

The best resolution is achieved when all elements of the microscope are in perfect alignment and the diaphragms are adjusted to the best aperture (this is referred to as ***Köhler illumination***). This pertains to both brightfield and DIC microscopy and for all magnifications. On more simple microscopes, you may not be able to adjust the alignment of the different parts …but on the Leica scopes it is possible to align and focus the condenser to achieve *Köhler Illumination,* first described by August Köhler in the late 1800s. The fact that we are able to make these fine adjustments should not be viewed as a disadvantage as it truly is a benefit.

The key to using the microscopes we have to their full potential is mastering how to set them up properly. One of the basic steps to achieve this is to perfectly align the light path. This is important regardless of what magnification you are working with, whether you are using brightfield or DIC optics, or whether you are counting/identifying diatoms, cladocerans, chironomids or even pollen. If this is not done then you might as well work on a teaching microscope and never reap the benefits of a high end, fully equipped microscope.

Each time you sit down at the scope you must set up your light path perfectly (or at least nearly so) and this is referred to as setting up ***Köhler illumination***. This provides the highest intensity of even illumination of your light source.

It is virtually guaranteed that the first few weeks you sit down at the scope you will spend 60 – 80% of your scope session setting up Köhler illumination. But don't panic about this…by the time you are actually counting your indicators, setting up Köhler illumination will be second nature and you will forget that you are even doing this.

Basically, your goal is to center the light source and limit it to the field of view that you are working in. Therefore you will need to make slight adjustments each time you change magnification.

Unlike many other scopes, on the Leica scopes that we have, you can easily set up Köhler using the 100X objective….therefore you need not start at lower magnifications if you are counting diatoms, cysts and scales etc.

1) Turn in the 20X or 40X objective lens – focus specimen
2) Close field diaphragm
3) Slightly narrow aperture diaphragm
4) Swing in condenser top lens
5) Switch the condenser disc turret to H (Heindfeldt or Hoegfeldt = Brightfield in English) or leave it on DIC
6) Sharply focus the edge of the field diaphragm by carefully and slowly adjusting the condenser height adjustment knob (silver knob on the left side that moves the condenser unit up and down)
7) Center the image of the field diaphragm using the two centering screws (pins) located under the turret and pointing toward the front of the scope. N.B. this part of the process is what will take time to master. Trust me, with practice it will become second nature. It is usually easier if you close the field diaphragm down quite a bit, especially if it is *WAY* off center.

8) *If for some reason, the center of the field diaphragm is totally out in space and you cannot for the life of you find the light source, switch to the lowest objective on your scope (usually 10X).* ***Be sure to clean off any oil on lenses*** *if you were working at 100X. By switching to a lower magnification you are able to look at a much larger view and you should be able to locate the center of the field diaphragm and see the light.*

9) Open the field diaphragm to just inside the field of view. Adjust centering if necessary.

10) Make sure that your specimen (diatom etc.) and the edge of the field diaphragm are both sharply in focus. When working it may be a good idea to keep the opening of the field diaphragm in view so that you can keep tabs on it…it may fade out of focus after time and may wander from center…an important element for photomicrography. The field diaphragm keeps all light not required for imaging away from the specimen so that contrast can be enhanced. For this reason it is best to open the field diaphragm only enough to just illuminate the viewed or photographed object field.
11) A change in magnification usually requires a slight adjustment of the field diaphragm, focusing and centering. This should be second nature to you and you will find it unsatisfactory to work on a scope that is poorly set up. If this is the case, you have learned well!

Setting up Köhler Illumination: Condensed version:

1) **Place slide on the stage and bring to focus** . You can do this for any of the objectives (10X to 100X). If the microscope is way out of alignment, you should start with a lower magnification such as the 20X objective lens. You may need to make some initial rough adjustments by opening up and starting to focus the field diaphragm as well as opening the aperture diaphragm to provide enough light for you to focus on your microsfossils.

2) **Narrow the Field Diaphragm**. This will allow you to see how far off center you are at this point.

3) **Focus the Condenser**. Turn the silver focus knob (left side of scope) on the condenser until the edge of the Field Diaphragm is sharp.

4) **Using the two centering screws** bring the field diaphragm into the center of the field of view. Open the Field Diaphragm to the edge of the field and fine-tune your centering.

5) **Adjust the Aperture Diaphram**. The setting will change for different magnifications. From fully open, stop down until the image has “oomph”. Or remove an ocular (not recommended) and look at the back of the objective. The aperture should be about 70-80% of the field to give you the best compromise between too contrasty and too flat. This translates to an aperture setting of ~3 for 100X oil.

Note: It will likely be necessary to finely adjust the alignment each time you start a microscope session and each time you switch slides or objectives.

Setting up Differential Interference Contrast (DIC)

Differential Interference Contrast (DIC) is an optical technique that is excellent for rendering contrast in specimen samples. It is a beam-shearing interference system that enhances the depth of focus and gives the specimen a three-dimensional appearance.

You don't need to **SET UP** differential interference contrast (DIC) *per se* but you do need to know what is involved and which parts of the microscope need to be engaged to attain DIC. For example, if you are counting diatoms or chrysophytes and you are sharing your scope with someone who works on cladocerans both of you will need to know how to change the scope to accommodate both types of illuminations. This will eliminate confusion, poor scope set up and inevitably will avoid you missing elements of your count that just was not visible without DIC in place. This should take you all of 30 sec.

For a more informative explanation of differential interference contrast (DIC) and how it works an excellent site to look at is:

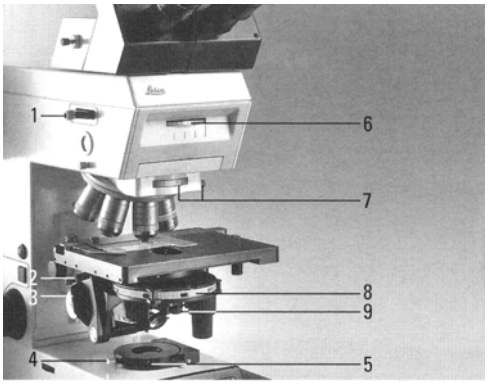
http://micro.magnet.fsu.edu/primer/techniques/dic/dicconfiguration.html

For a fun and informative interactive tutorial:

http://micro.magnet.fsu.edu/primer/virtual/dic/index.html

Our scopes have DIC prisms set for both 40X and 100X. Adjust the condenser-side turret to correspond to the objective used (40 or 100 (could also be labelled "oil")).

DIC (differential interference contrast) works through a series of prisms (analyzer, polarizer, and objective-side prism, condenser prism etc).



From the Leica DMR Instruction Manual p. 86

Components for interference contrast (DIC)

1) Analyser
5) Polarizer index adjustment
6) Incident light reflector turret
7) Objective-side prism turret
8) Turret for condenser-side Wollaston prisms

Light passes through the diatom and the slide and because they have different refractive indices, the light wave is altered or interfered with. This results in the image appearing three-dimensional.

This altered wave passes through the polarizer and then a prism. The prism splits the light into two paths and then to the analyzer.

When the analyzer and polarizer are perfectly crossed (90º) the two light waves interfere with each other maximally....the greatest contrast but they kind of cancel each other out....therefore we get blackness or “extinction”.

You can test this by looking at a diatom under DIC when the polarizer index adjustment is set to exactly 90º and the analyzer is exactly at 0º...you may need to adjust the objective-side prism to the middle position. You should see almost full darkness at this setting.

Move the polarizer to about 110º.....this seems to result in the nicest image here. You can tweak a little with this setting as well as with the objective-side prism and aperture diaphragm to give the nicest image.

How to set up DIC:

1) Place the analyzer, polarizer and objective-side prism into the optical path
2) Swing in the DIC objective to be used to the optical path. Set the position of the condenser turret to the power of the objective.
3) Turn the condenser disk to D for DIC for some of the Leica scopes (BFC lab and some of the older models)
4) Set Köhler illumination

How to set up Brightfield:

1) Remove the analyzer, polarizer and objective-side prism D from the optical path
2) Turn the objective-side turret to BF for Brightfield
3) Swing in the objective to be used to the optical path.
4) Turn the condenser disk to H for Brightfield (Hoegfeldt or Heindfeldt)
5) Set up Köhler illumination

A word on scope cleaning and regular maintenance practices

PLEASE READ THIS:

Keeping the microscopes clean is an easy enough task when it is part of your counting routine.....if you let it go it becomes a major problem. Not only does it drastically reduce your ability to clearly see what is on your slide but if not dealt with for a long period of time, it becomes a very frustrating and very costly undertaking to fix it. As you know, microscopy is an essential skill for what we do so it is in your interest to become familiar with both basic microscope skills and microscope maintenance. Believe me this will make your lives easier in the long run.

There is nothing more frustrating than not being able to use a microscope because it so filthy it is like looking through a greasy window. The microscopes that we have in the lab are state-of-the-art and as such have a great potential for crisp and clear, high resolution images.....this potential quickly deteriorates when the scopes are not set up properly and are not cleaned regularly.

Because our lab uses oil it is even more important to clean all lenses when finished for the day. Oil will trap dust and over time becomes a grimy and difficult surface to clean. PLEASE USE LENS PAPER. Lens paper is relatively inexpensive and is designed for this specific purpose. All too often the scopes at PEARL were very close to having oil seep into the inside of the condenser lens (lens you raise up on the under side of your slide). Once oil gets onto any lens not meant to receive oil, that lens is pretty much garbage (particularly on the more sensitive internal lenses). To replace the condenser lens (which has been done in the past) is shockingly expensive so my take on this is ...don't be afraid to use lens paper. It may be more expensive than Kimwipes but not as expensive as replacing a lens.

For someone who uses the scope regularly, you should notice a big difference when you clean your oculars (eyepieces) with lens paper. Also keep in mind that we need to clean our lenses constantly (as opposed to non-oil microscopes) and over time could wear down or scratch the lenses. A regular light cleaning (as opposed to a deep cleaning when things get too out of hand) is a better solution. Besides we have lens paper so why not use it.

Bottom line, if you want to make your counting and identification sessions more efficient and less frustrating, start by cleaning the oculars, then the lenses that require oil (100X objective and condenser top lens). AND also regularly clean the entire condenser as oil tends to spill over the sides and once it gets inside (where it is screwed into place) the condenser is ruined. IF you are unfamiliar with the parts that I have just mentioned it might be a good idea to look these up in the Leica manuals or ask someone.

Remember, if you are unsure or if things don't seem right don't be afraid to ask for help!

<u>Additional information</u>:
Lenses which are exposed to the outside on the scope usually have an additional coating or are made of a glass type which is less susceptible to scratching. For this reason it is very important not to try and clean the back of an eyepiece (or any other internal/shielded part of a microscope) because it is very prone to scratching. Therefore, it is very important not to unnecessarily remove the eyepiece etc. as it is surprising how quickly dust and particles can get into these parts of the scope and really affect the clarity of your

scope. Many coatings on the internal lenses are chosen purely for their optical properties and are very soft.

Dust is the most likely thing to scratch the lenses while you are cleaning the scope. It is not unusual for a scope that has not been used for several weeks to be inordinately dusty…even with the scope covers on. To avoid scratching the lenses of such a dusty scope, softly blow off excessive dust. Now use a WET piece of LENS PAPER. Usually we use a very dilute solution of ethanol to clean our lenses…alcohol will dry quickly and avoid streaking. Dab some ethanol onto the lens paper, take special care to wipe in one direction only and only use that part of the lens paper for a single wipe. Wiping in a circular motion is the worst thing you can do as you will be merely grinding the accumulated dust into the lenses and scratching them.

Make sure that you do not inadvertently get oil on the lenses….for this reason you should clean the oculars first before you proceed to clean the objective and condenser lenses.

For a quick lesson on scope cleaning look at this very short video:

http://www.rvc.ac.uk/review/Dermatology/Tests/Microscopy1.htm

Photomicrography (*not* microphotograpy!)

Successful photomicrography starts at the microscope as the recorded image is no better than the image produced within the scope. Correct illumination and microscope alignment are the most important aspects of critical microscopy and photomicrography.

All of our microscopes are now equipped with a digital camera system and accompanying software (Northern Elite). The camera unit also includes a zoom that allows you to zoom in on small specimens or parts of a specimen without sacrificing resolving power.

The following has been prepared by Kate Laird as she is undoubtedly PEARL's leading expert on photomicrography.

Enter computer system under PEARL user!

Northern Elite – Digital Camera Photos and Image Database

(see Image Database information below for setting up a database – the database will allow you to 'tag' information directly to photos, display photos at a chosen magnification, ie. 1500x, etc.. and has search functions)

Digital Camera:
1) Make sure camera is in the "on" position before booting up computer (Note: cameras should be routinely turned off – especially if computer is left on and cover is on the scope).

2) Make sure the microscope is set up properly (see Microscope Set Up)! This is vital for capturing good images. The camera can only take photos that are as good as the scope images (can't make the scope images better than they are).

3) Pull out camera stop (or more technically the beamsplitter rod) all the way on the microscope.
4) Use **digital focus** for taking photos – allows cropping of diatom at onset. (Can also take photo of whole field of view and then crop)

5) **Right mouse button** brings up options:
a) put on **Full Chip** for entire field of view of scope.
b) **Focus options (which is under Options Dialog)**: Grayscale 8 bits, Maintain aspect ratio, and Snap photo on closing should be checked. Full screen viewing is optional and depends on your preference. (Grayscale 8 is important for the photo settings, i.e. brightest of background, as well as for previewing the images in the image database program. Note: that Grayscale 16 does not work for the above and does not significantly improve resolution).

6) Need to refocus image on the screen because the scope and camera are not at the same focus level. (Focus slowly since the camera image has a time delay).

Things to adjust at time of taking photo: Note: the follow settings are only given as guidelines – settings can varying between slides and diatoms taking photos of. Note also that these guidelines are based on the scope being set up in Polar Illumination DIC (P up on analyzer) not in whole wave compensator (i.e. lambda up on analyzer).

1) Exposure for 1.6 zoom set around 70 millisec. (high exposures leads to glare). Time of exposure must be re-adjusted downward for lower zooms.
2) Gain (brightness) - optimum setting at 30%, and up to just below the offset setting.
3) Offset (contrast)- optimum setting around 40%
4) DIC setting – best adjustment is outlined in the microscope set up. However, sometimes need to decrease the contrast by adjusting the objective side prism to the brighter side of the range in order to get a good photo, this is especially true for heavily silicified diatoms. (Note: above settings will be different for brightfield).
5) Polarizer – set at approx. 110 degrees, otherwise lose sharpness of the image.
6) Light level on microscope – setting around 7.5 – 9.0.
7) The Blue filter often provides the best resolution in the photos.

Taking the photo:
1) For pennates **orient the diatom of interest either vertically or horizontal** (i.e. not on an angle) by carefully rotating the camera.
2) Holding down the **left mouse button – drag a box around the diatom** of interest.
3) To capture this image – first click **on Set ROI** (region of interest) (click right mouse button to bring up ROI option). Then close (right mouse button again) (closing takes the photos – assuming snap photo on closing is checked under Focus Options (as it should be as a default).
4) The other option is to take a photo of the whole field of view, then crop by dragging a box around the diatom (still needs to be oriented either vertically or horizontally) and then click the Crop function.

Touch-ups of photos:
1) Can use **equalize** and **autoequalize** option (if you don't like the result use the undo function under Edit) to "brighten" or "darken" the photo.

2) Save Image to directory structure. Please see below under image database on tagging information to your photo.

Calibration: There is a calibration factor already calculated for all lenses (i.e. 10, 20, 40, 100x) and for each lense at a particular zoom (e.g. 1.0, 1.25, 1.6).
1) For measuring and placing on micron bars **first select the appropriate calibration**. For example if you are on 100x and 1.6 zoom use the 100x,1.6zoom calibration. To do this first make sure that the **Tool Window** under View is checked. There are 5 ruler

options here, for choosing your calibration go into the **first ruler bar** and select the correct calibration.
2) To **measure distance between two points**: Then use the **"line" ruler** (3rd ruler bar) to measure between two points by holding down the left mouse button and drawing a line across the region of interest.
3) To place a **micron bar** (which is relative to the photo – sorry no option for placing say a 10micron bar) use the **2nd ruler bar**, drag the bar to the position you want and then stamp it. (Note once you stamp it – you cannot move it! Note also that to see the micron bar for placement you need to be on Normal view (Under view..zoom). As well, the micron bar sometimes can be initially just to the right of view and has to be dragged in).

Note: that if you plan to use and make plates in the image database and want a micron bar on individual photos then for stamping you must make sure that the long axis of the rectangle around the diatom is in the x dimension. This is particularly relevant for centrics where you would typically put a square around it as opposed to a rectangle. The reason this is important is that when you choose photos for display in the image database they will be displayed all in the same orientation – long axis in the x. See below on image database for more details on calibration.
Image Database:

Setting up your own image database:

1) **First set up Project Folders under Image Databases** using windows New Folder option. In order for the image database to work the folders for your project must be in two layers (not 1 or 3, etc.. but 2). I would suggest for calibration datasets that your first folder level is the region of samples-name of analyst (for example British Columbia-Laird), and then lake names or codes as the second level. For core work the first folder should have the lake name, core number and type, and analyst name (e.g. Maggie GC1 – Laird) and then the levels analyzed (e.g. 0-0.5 cm). GC = gravity core, PC = piston core, FC = freeze core.
2) In order to set up your own database that is separate from other users on the computer/scope system you **need to create what is called a mdb file**. To do this go into ImageDB via Northern Elite, select Database in the top menu bar, then database name. Type in a name for the mdb file you wish to create (e.g. KLaird), then select Open. You will get a message that says "This file does not exist. Create the file? – Reply Yes. You now have an mdb file which is the memory of your database.
3) At this point your database that you just created will be selected and will be the one that the database program will be adding to. To open your database at a later point, again go into Database menu bar of ImageDB, select database name, and then select your database (e.g. Laird.mdb) and say Open.
4) If you do not **remember to select YOUR database at the start of a session**– all images that you add will be entered into whatever database was last used (opened).

Saving images to database: First need the **user window toolbar open**. Next click **Add Image**, and under this click **Save** (Click yes to replacing). This brings up a template for input that already has file information under "Main information". Input other data into

the "Full Information" template. Information put into this template is then tagged with your image. This information is extremely important for future references and will be needed if the photos are to be inputted into the "MASTER" image database.

1) To save input time on subsequent photos from the same slide/sample use the **Read History option**. **Alt H** will bring up the options – check the appropriate ones that are similar for your photos.
2) Once all appropriate fields are filled in click OK. Click Yes to "Do you want to annotate this image". This allows you to view this information in Elite (Under File … Annotate Image) without having to be in the database.

Searching and viewing photos in the database:
1) A **Quick Search** is located below the image preview
2) **Full Search under Image**, where you can search any field. Also you do not need to put in the complete name: For example, Steph under Genus and par under Species will bring up all of the following categories: Stephanodiscus parvus, S. cf. parvus, S. aff. parvus, etc. (Although for searching you do not need the full name – PLEASE put the full name (i.e. full genus, species, variety, etc.) in the database – this will save any future headaches on deciphering different peoples abbreviated names!).
3) There is also the option to **search taxa with particular dimensions** – width, length, straie count (assuming you fill this information in when saving a photo). e.g. Navicula, L= 8-10, W=5-6, str.=15-18.
4) You can view the "tagged information" of an image under **Image… Image Info**.
5) **Selecting images**: You can select all images by highlighting all of them from the search using the mouse or selectively highlight by holding down the CTR key, while clicking with the mouse.
6) **Set magnification** (under Image) you wish to view them at
7) Click **open images**
8) **Scale Bars**: If you want a scale bar that runs along each of the rows– click calibration bar before opening images. (Note: make sure that Image DB Calibration is selected under 1st ruler bar. See Calibration under Digital Camera for further details).
If you want a relative **micron stamp** then choose the correct calibration first (**see Calibration under Digital Camera**). The **calibrations for the image database are different from within Elite and are according to the magnification (i.e. 1000x, 1500x, 2000x, etc..).**
9) To change the font of the labels go into the tool bar 'Aa'.

Bringing images and composite plates into other graphics programs: (e.g.CorelDraw, PhotoShop). Make sure the pixel dimensions are the same in Elite and the graphic program. In Elite you can get this information under View…Image Info (dimensions in pixels). In Abode Photoshop its under Size..Photo Size, has an option of dimensions in pixels. In Corel Draw its under Edit..Properties..Size. In these two programs the photos are brought in at the same size.

Printing: To print a plate at a magnification of say 1500x, the tif plate file must first be saved at 2.5 times the magnification wanted or at 3750x. This is a scaling factor between

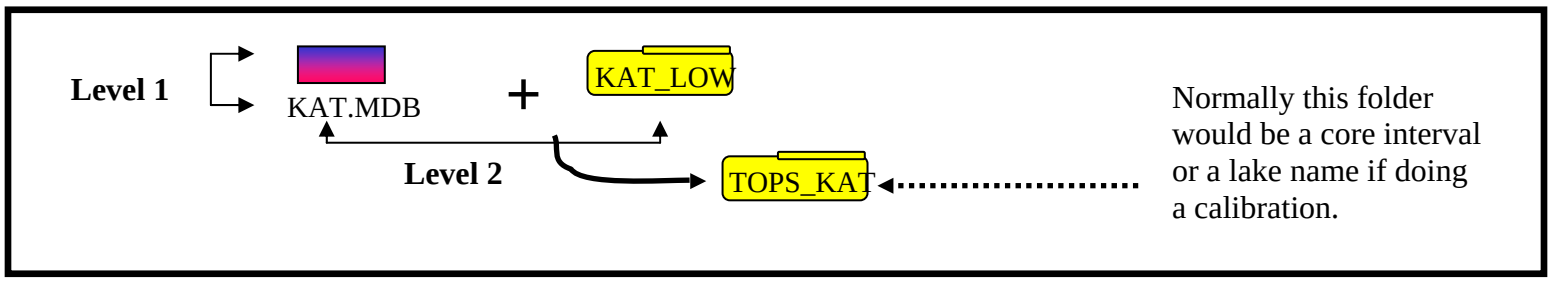
computer and printer – where a 2.5 x reduction occurs. Print at final magnification of 1000x, save tif plate at 2500x; 1500x save at 3750; 2000x save at 5000x, etc…..

Saving your image database onto a CD etc.

The way the image database must be set up to link images to the database options:

C:\New database name\MDB file + folder of project\folder of core sections etc.

e.g. C:\Image Databases\Kat.mdb + KAT_LOW\TOPS_KAT



You cannot have more than one MDB file in the same place…instead make a new folder
There must be levels of folders…any more or any less and the database won't function

C:\New database_2\MDB file_2 + folder of project

WHEN SAVING YOUR IMAGE DATABASE ONTO CD ETC.

1) You must copy the entire folder from the root directory : i.e. C:\Image Database
and then everything should still be linked. It is important that you do not change the pathways of the setup of all of the folders AND that the MDB file is also copied in the order that it was initially set up. If not then you will be left with a series of images….no database.

2) Once copied to CD – right click on folder – properties – uncheck the "read-only" box, otherwise nothing will function. If you do this at the root folder, all other subfolders should uncheck as well.

To change the name of an image already saved in the image database (let's say you changed your mind as to what this taxon is):

1) open Eclipse
2) open image of interest – SAVE HERE FIRST with new name…BEFORE YOU ADD TO DB. You will be asked if you want to overwrite the existing file…this is what you want so that you won't end up with two of the same images with two different identifications.
3) Now add image to DB (icon)
4) Save…again put in the new name…overwrite an existing file if there is one …make sure you are in the SAME directory as original image
5) Press Save
6) Annotate – read annotation – change the name of the diatom…all other information should be the same as when you originally took the picture

7) Now you should have only one name associated with that image.
8) If both old and new image names are still showing up in the Dbeditor, click on images…the one that is actually attached to the database will show up on the screen…the other will not. Right click on the image that does not show up on screen and delete this image.