How to Setup Köhler Illumination for Photomicrography

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Köhler illumination in microscopy is an important technique that all microscopists should know because it provides even lighting, optimum contrast and resolution for photomicrography. Early light microscopes were illuminated by the sun, sky-light, candle flame, gas lamps, mirrors or other primitive light sources that often resulted in uneven illumination. August Karl Johan Köhler (1866-1948) a German professor developed Köhler illumination. During Köhler's doctorate thesis on limpets he developed a microscope configuration to produce an even illuminated field which also reduced glare from the light source. This illumination method is widely used today with modern microscopes and is considered one of the most important principles in achieving the best optical resolution and image quality with a light microscope. This technique is described here in easy to follow steps.

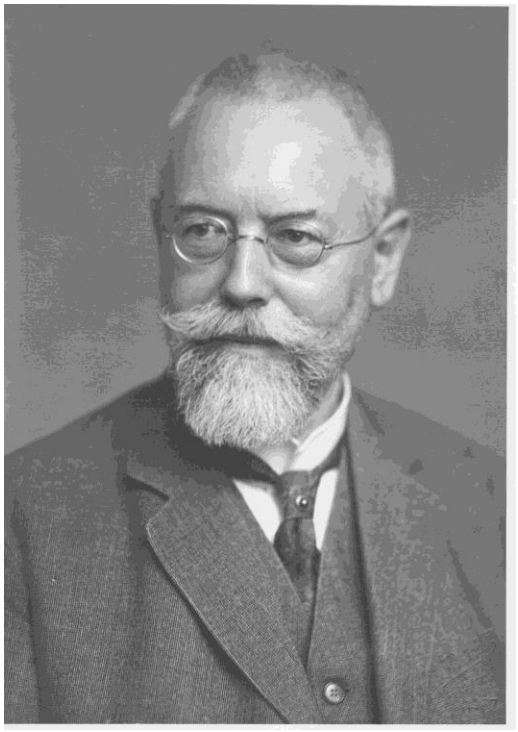

Fig. 1 August Köhler developed a method to provide even illumination and optimum contrast in light microscopy – picture from Wikipedia
(https://commons.wikimedia.org/wiki/File:August_K%C3%B6hler_(1866-1948)_(8527804902).jpg)

Some photomicrographs on the web, in text-books and science publications display uneven background lighting and/or poor contrast. Setting up your microscope for Köhler illumination (also spelled Koehler) will result in significantly better photos especially when combined with image processing (e.g. using Adobe Photoshop). The optimum microscope configuration depends on the specimen being viewed, its refractive index, mounting medium and light source (J.G. Delly, 1988, 2017, M.W. Davidson and T.J. Fellers, 2003). Today many lab microscopes use pre-centered tungsten halogen lamps, though LED (light emitting diode) lighting is growing in popularity. LED lighting often includes a

diffusion filter in the light path which helps make the small LED lights produce more even illumination. The basic principles of adjusting the microscope field diaphragm (diaphragm in front of the light source) and the condenser diaphragm are still useful. Older microscopes using a mirror and external light source also benefit from Köhler illumination though the tungsten light filament should be centered first.

In short, Köhler illumination ensures the light source is centered, provides evenly distributed light, and the condenser aperture is adjusted to provide the optimum contrast for the specimen under investigation. Köhler illumination should be performed for each objective 10, 20, 40, 60 and 100X for photomicrography. Köhler illumination should always be used whenever photomicrography is performed though it will also improve general microscope viewing. Low power objectives from 1X to 4X require the top lens of the condenser to be swung out of the view or the use of special condensers to produce even lighting. The Motic BA310 polarizing microscope uses a condenser that permits the top condenser lens to be swung out of the light path. It is possible to use a frosted glass filter (diffusion filter) to make some light sources more even especially when using low power objectives (4X or lower), but this also reduces the intensity of the light and does not ensure the optical path is centered.

Köhler Illumination

With Motic microscopes the light source on the BA310 microscopes uses a 6V 30W bulb with a tungsten filament that is pre-centered in the base. Microscopes from some manufacturers permit the bulb filament to be centered and others do not. I describe the steps to set up Köhler illumination with a Motic BA310 polarizing microscope but this procedure can be used with most light microscopes.

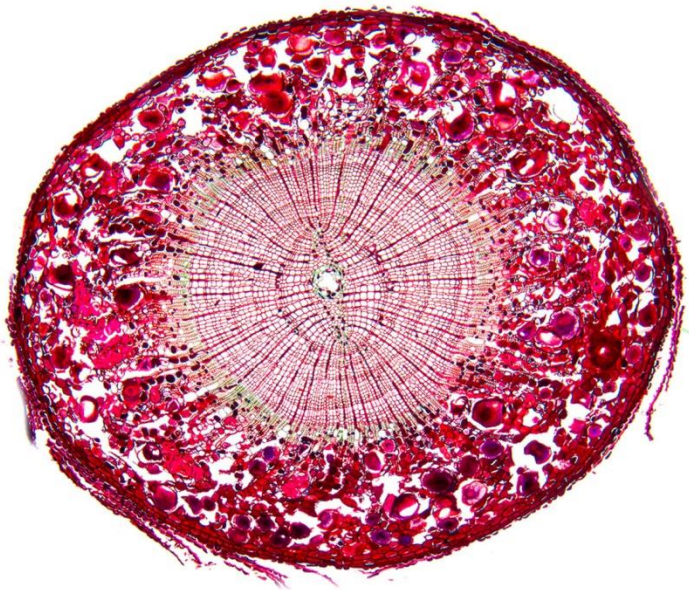

Fig. 2 Douglas fir (*Pinus taxifolia*) stem cross section photographed using Köhler illumination and bright field microscopy.

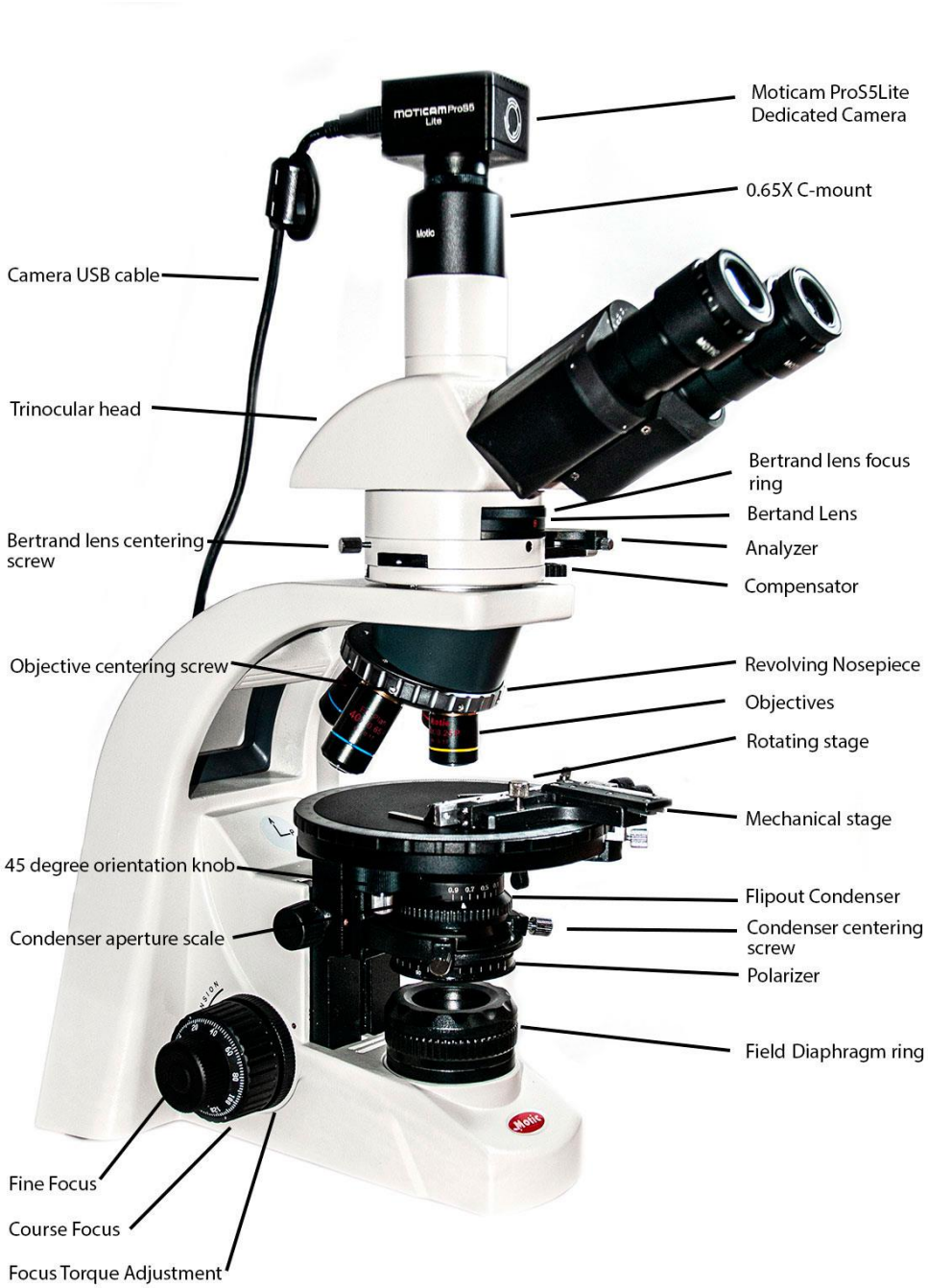

Fig. 3. Motic BA 310 Polarizing microscope. Note the field diaphragm ring is on the base as a component of the light source. The condenser offers a flip out feature which removes the top lens and provides a wider light source for use with a 4X objective. The condenser has its own built in diaphragm.

Procedure for Köhler Illumination for a microscope with a pre-centered tungsten filament

1. First choose a prepared microscope slide for setting up Köhler illumination. I recommend using a commercially prepared permanent slide of animal tissue or plant section. The section should be 10 microns or less in thickness and stained. Start with a 10x objective and place the slide on the stage below the objective.

2. Turn on the light source and adjust the intensity so it is comfortable for your eyes and focus on the specimen. Adjust the interpupillary distance of the eyepieces first if using a binocular microscope. The eyepieces, if they have diopter adjustment, should be set to 0 to start – then using your dominant eye adjust the diopter setting so the image is sharp after you focused on the specimen (you can also adjust the diopter focus on the eyepiece reticle or scale if your microscope has one). Both the eyepiece reticle and specimen should appear in focus. Then adjust the diopter setting on the other eyepiece so the specimen is in focus when viewing with both eyes. If you wear eyeglasses leave your glasses off unless you have significant astigmatism.

3. Close the field diaphragm in the base of the microscope directly in front of the light source until you see the field diaphragm in the eyepieces (Fig. 4A). Focus the condenser so the field diaphragm edges come into focus (Fig. 4B), use the condenser centering controls to adjust the field diaphragm so it appears in the center of your view (Fig. 4C and D), then open the diaphragm so the edges are just out of the field of view (Fig. 5). These steps center the light source illumination (see below).

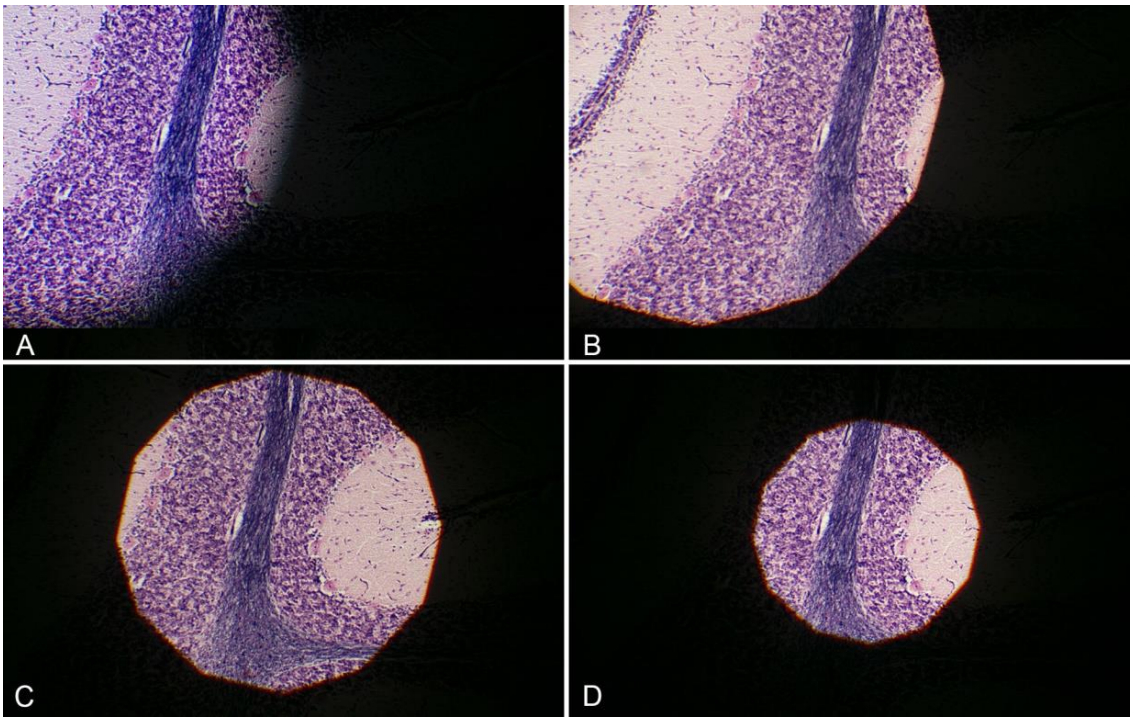

Fig. 4. Above shows cerebellum (brain) tissue from a prepared histology slide. A) shows the edge of the field diaphragm B) field diaphragm in focus C) field diaphragm centered D) field diaphragm made smaller and centered. If your microscopes eyepiece has a cross hair, you can position the diaphragm opening directly over the cross.

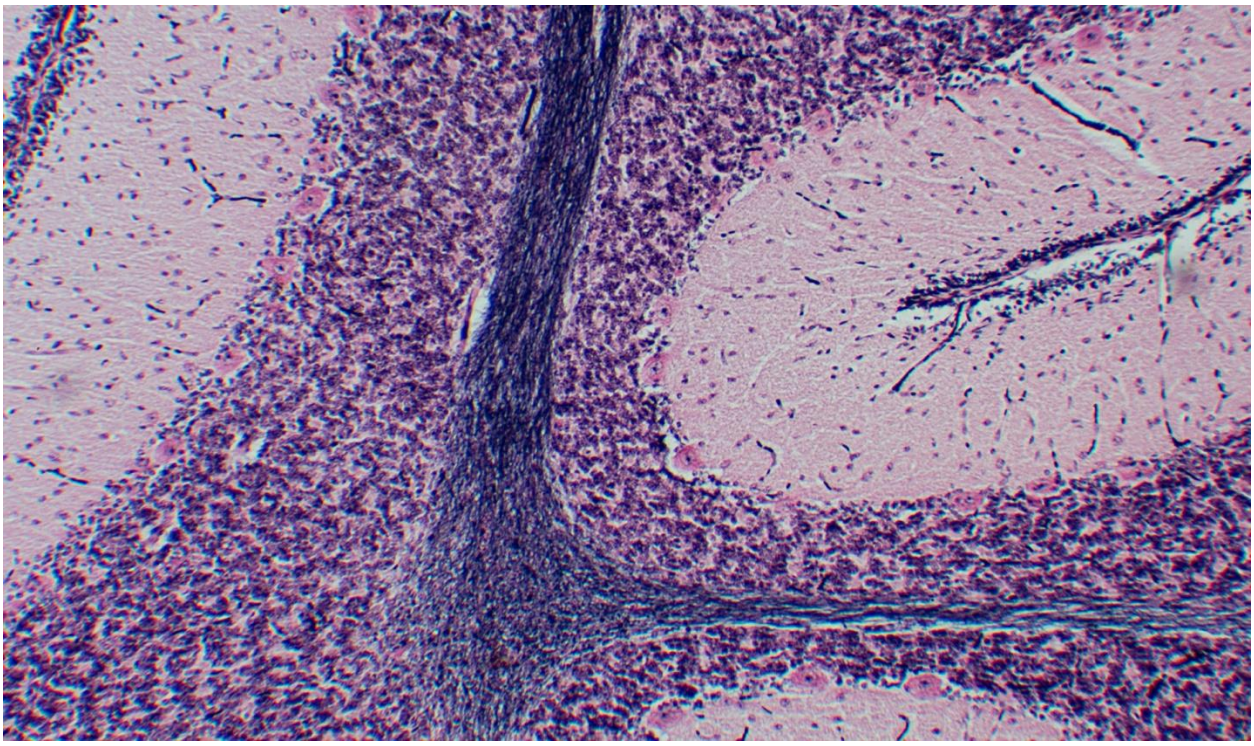

Fig. 5. After centering and opening the field diaphragm the image should have even illumination as you centered the optical axis. Cerebellum tissue stained with hematoxylin and eosin**.** 100X.

4. Finally, the condenser should be moved up so it is close to the microscope slide (1-3 mm), but not touching it. Close the condenser aperture until you can see the diaphragm blades. Again the condenser aperture blades should be centered with the condenser centering controls. To reduce glare and provide optimum contrast the blades should be visible around the edge of the eyepiece ocular tube and allow about 60 to 90% of the light through – the exact size of the opening depends on the specimen. To do this take one eyepiece out and view the condenser aperture by looking through the eyepiece ocular Fig. 6 (a phase telescope can help you see the blades more easily). The condenser diaphragm should be visible around the edges of the ocular tube and permit 60-90% of the light to pass through. After doing these steps and putting the eyepiece back in you have achieved Köhler illumination. The condenser aperture size can be tweaked by viewing the specimen and adjusting it slightly to achieve optimum contrast of the specimen. Experienced microscopists often do this step while looking at the specimen rather viewing the condenser blades through the ocular tube without an eyepiece. If you change the objective repeat these steps before photomicrography. If you are causally examining microscope slides it is not necessary to optimize each objective for Köhler illumination so long as the light source is centered and provides even illumination.

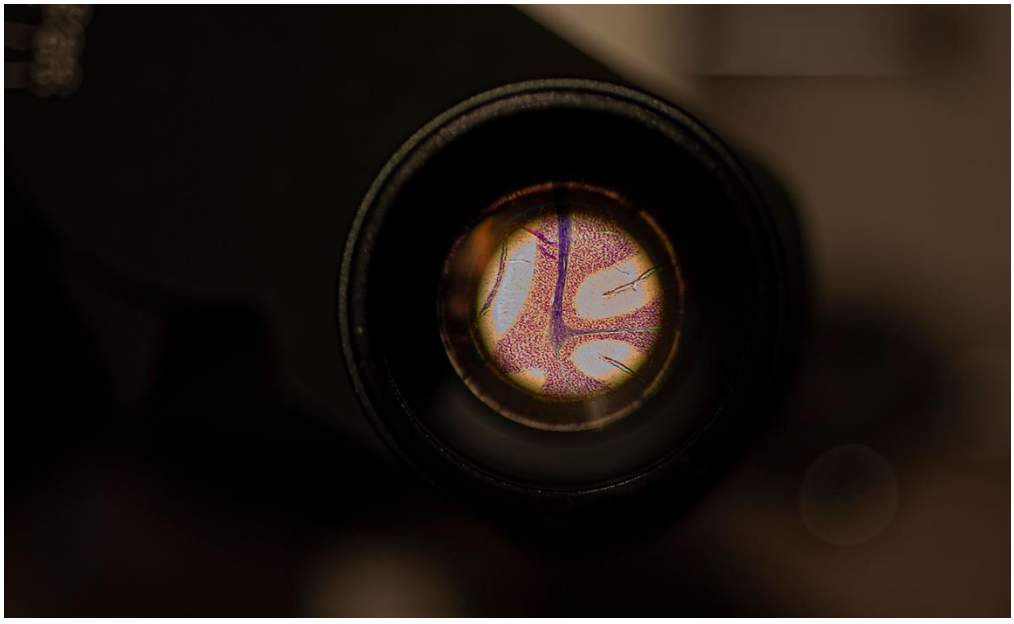

Fig. 6. Above image shows the eyepiece ocular with an eyepiece removed. Look down the ocular tube and adjust the condenser aperture blades so that they allow 60-90% of the field of view to appear as shown in Fig. 7). You can use a phase contrast telescope to view the blades more easily.

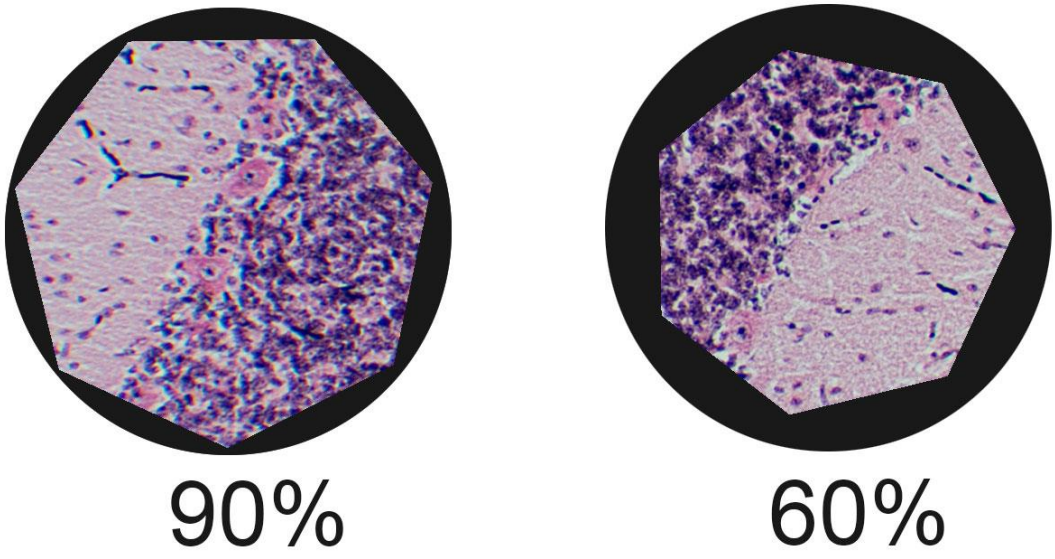

Fig. 7. Condenser aperture blades as seen when looking down the ocular tube. After centering the condenser aperture blades they should be adjusted to allow about 60-90% of the light through as shown above. The diameter of the condenser aperture will vary with different specimens. This step reduces glare and improves image contrast for photomicrography.

Follow the steps above for Köhler illumination before performing photomicrography. For casual microscope inspection the most important feature is to have even illumination that is optically centered. Köhler illuminations works for bright field, dark field, phase contrast, and DIC (Differential Interference Contrast) microscopy. Wide-field epi-fluorescence microscopy also uses Köhler illumination but it is achieved differently.

When using a digital camera and software where the image is viewed simultaneously on a computer LCD (liquid crystal display), you can optimize the white balance, zoom into the image and focus on enlarged regions viewed on screen to achieve better focus during photomicrography with the software. Post image processing of the image (e.g. Photoshop) can also improve image contrast, texture, white balance, fix uneven illumination, clean up background debris and remove dust spots produced by the camera's sensor. However, it is important not to alter the biological integrity of the specimen using digital image processing.

External Light Sources, Epifluorescence microscopy, and Confocal Microscopes

Using Köhler illumination with an external light source and a microscope with a mirror to reflect the light requires centering the lamp on the optical axis then performing the steps described above. For a full description on how to center the lamp filament see "J.G. Delly, 1988". Epifluorescence microscopes also use Köhler illumination by focusing a xenon or mercury filament onto the rear objective aperture (K. R. Spring et al. describes the steps). Confocal microscopes use a form of critical illumination see "A. Nolte et al., 2006" for a description of lighting for confocal microscopes.

Summary

Köhler illumination developed by August Köhler is a method of producing even illumination with a light microscope which minimizes flare and provides optimum contrast. The method involves centering the field diaphragm and condenser diaphragm, and then adjusting contrast with the condenser diaphragm for photomicrography. Köhler illumination also improves general viewing of specimens and can be achieved in a few minutes. Online instructional videos show steps to produce Köhler illumination with different microscopes. The reason for centering the light source is to provide even illumination. The reason for modifying the condenser aperture diaphragm opening is to control the numerical aperture of the system, the resolving power, depth of field and minimize flare. For maximum resolution the condenser aperture should be wide open, but in practice this often results in flare and loss of contrast. Köhler illumination produces even lighting, comprises resolution slightly, but optimizes contrast and is therefore a technique that every microscopist and photomicrographer should master.

References & Links

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K. R. Spring, M. Parry-Hill, T.J. Fellers and M.W. Davidson Focus and Alignment of Mercury and Xenon Arc Lamps – Nikon Microscopy U. https://www.microscopyu.com/tutorials/arclamp

Microscope Alignment and Köhler illumination.
http://zeiss-campus.magnet.fsu.edu/tutorials/basics/microscopealignment/indexflash.html

What is Köhler illumination, why is it important, and how to do it?
https://rsscience.com/kohler-illumination/

Critical Illumination – Wikipedia
https://en.wikipedia.org/wiki/Critical_illumination

Instructional Videos

Koehler illumination by Motic Europe – setup and alignment **(**ö German = oe English**)**
https://www.youtube.com/watch?v=dYP0uBxE0AA&t=2s

Setting up a compound microscope for Köhler Illumination (Motic BA 310)
https://www.youtube.com/watch?v=3EMoZrTIavk