

UNDERSTANDING CONJUGATE PLANES AND KÖHLER ILLUMINATION

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Keywords: conjugate planes, field, aperture, illumination, image-forming, eye iris diaphragm, Ramsden disc, eyepoint, rear focal plane, lamp filament, retina, specimen, Köhler illumination, orthoscopic, conoscopic, eyepiece, condenser, field-limiting, aperture-limiting.

Introduction

The development of advanced light microscopy techniques in recent years has been quite impressive. Cellular biologists are now able to visualize processes such as protein development in real time due to advanced techniques such as Fluorescence Recovery After Photobleaching (FRAP); they are no longer limited to indirect biochemical tests to determine sequences of biological events. However, despite the increasing sophistication of microscopy techniques, the fundamentals of microscope alignment are often neglected and misunderstood although they are vital to obtaining the best microscopy results. Correct alignment of microscope components ensures proper specimen illumination in order to achieve the best balance between image contrast and resolution.

In 1893, August Köhler developed a technique still used today for aligning microscope components that results in optimal specimen illumination. It is important to understand more than the procedural steps of Köhler Illumination; being familiar with the concepts behind these steps will foster the knowledge necessary to effectively improve image quality for challenging specimens. An understanding of the illumination and image-forming pathways is necessary in order to realize the full imaging potential of microscopes.

In a properly focused and aligned optical microscope, a review of the geometrical properties of the optical train demonstrates that there are two sets of principal conjugate focal planes that occur along the optical pathway through the microscope. One set consists of four field planes and is referred to as the field or image-forming conjugate set, while the other consists of four aperture planes and is referred to as the illumination conjugate set. Each plane within a set is said to be conjugate with the others in that set because they are simultaneously in focus and can be viewed superimposed upon one another when observing specimens through the microscope.

Presented in Figure 1 is a cutaway diagram of a modern microscope (a Nikon Eclipse E600), which illustrates the strategic location of optical components comprising

the two sets of conjugate planes in the optical pathways for both transmitted and incident (reflected or epi) illumination modes. Components that reside in the field set of conjugate planes are described in black text, while those comprising the aperture set are described in red text. Note that conjugate planes are illustrated for both observation and digital imaging (or photomicrography) modes. Table 1 lists the elements that make up each set of conjugate planes, including alternate nomenclature (listed in parentheses) that has often been employed and may be encountered in the literature. A minor difference exists in the relative location of the field and condenser apertures between the incident and transmitted modes of illumination, which will be explained later.

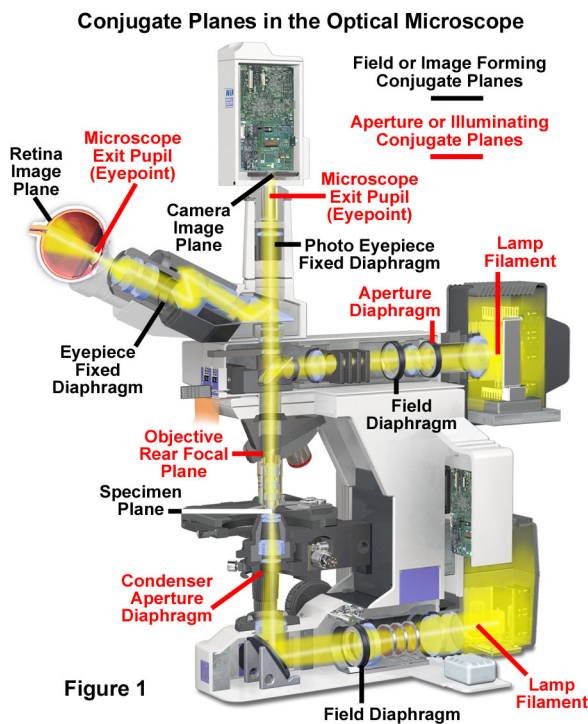


Figure 1. Microscope cut-away diagram illustrating the image-forming and aperture conjugate planes.

Table 1 Conjugate Focal Planes

| | |
|--|---|
| Aperture or Illuminating Conjugate Plane Set | Field or Image-Forming Conjugate Plane Set |
| Microscope Exit Pupil (Eye Iris Diaphragm) (Ramsden Disc) (Eyepoint) | Retina of the Eye Camera Image Plane |
| Condenser Aperture Diaphragm (Condenser Front Focal Plane) | Specimen Plane (Object Plane) |
| Lamp Filament | Field Diaphragm (Field Stop) (Köhler Diaphragm) |

In normal observation mode (using the eyepieces), the conjugate set of object or field planes can all be simultaneously viewed when the specimen is focused, an observation mode that is referred to as the orthoscopic mode. The corresponding image is known as the orthoscopic image. Observing the other conjugate set of aperture or diffraction planes requires the ability to focus on the rear aperture of the objective, which may be accomplished by using an eyepiece telescope in place of an ocular, or a built-in Bertrand lens on microscopes that are so equipped. This observation mode is termed the conoscopic, aperture, or diffraction mode and the image observed at the objective rear aperture is known as the conoscopic image. Although the terms orthoscopic and conoscopic are scattered widely throughout the literature, many microscopists favor using normal mode and aperture mode because the latter nomenclature more clearly relates to the operation of the microscope. Planes belonging to the pair of conjugate sets alternate in succession through the optical train from the light source filament to the final microscope image produced on the retina or the image plane of an electronic sensor. A thorough understanding of the relationships between these conjugate plane sets, and their location within the microscope, is essential in understanding image formation and carrying out correct adjustment of illumination. In addition, the location of principal conjugate planes is often a key factor in the proper placement of optical components such as phase plates, differential interference contrast (DIC) Wollaston prisms, polarizers, modulators, filters, or graticules.

The imaging and illumination ray paths through a microscope adjusted for Köhler illumination are presented in Figure 2, with the focal conjugates of each plane set indicated by crossover points of the ray traces. Illustrated diagrammatically in the figure is the reciprocal nature of

Conjugate Planes in the Optical Microscope

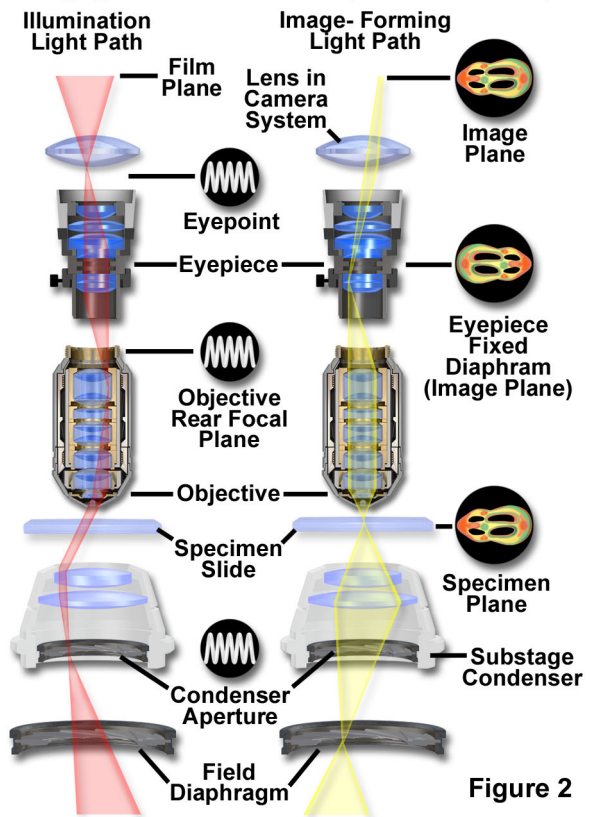


Figure 2. Relationship between the conjugate plane sets in the optical microscope. Image-forming conjugate planes reside at the field diaphragm, specimen slide, eyepiece fixed diaphragm, and retina of the eye. Aperture planes occur at the lamp filament, condenser aperture, the objective rear focal plane, and the eyepoint of the eyepiece.

the two sets of conjugate planes that occur in the microscope. The optical relationship between the conjugate plane sets is based upon the fact that, in the illuminating ray path (shown in red), the spherical wavefronts converge and are brought into focus onto the aperture planes, while in the imaging ray path (shown in yellow), the spherical waves converge into focused rays in the field planes. Light rays that are focused in one set of conjugate planes are nearly parallel when passing through the other set of conjugate planes. The reciprocal relationship between the two sets of conjugate planes determines how the two ray paths fundamentally interact in forming an image in the microscope, and it also has practical consequences for operation of the microscope.

Illumination is perhaps the most critical factor in determining the overall performance of the optical microscope. The full aperture and field of the instrument is usually best achieved by adjusting the illumination system following the principles first introduced by August Köhler in the late nineteenth century. It is under the conditions of

Köhler illumination that the requirements are met for having two separate sets of conjugate focal planes, field planes and aperture planes, in precise physical locations in the microscope.

The basic requirements of Köhler illumination are very simple. A collector lens on the lamp housing is required to focus light emitted from the various points on the lamp filament at the front aperture of the condenser while completely filling the aperture. Simultaneously, the condenser must be focused to bring the two sets of conjugate focal planes (when the specimen is also focused) into specific locations along the optical axis of the microscope. Meeting these conditions will result in a bright, evenly illuminated specimen plane, even with an inherently uneven light source such as a tungsten-halogen lamp filament (the filament will not be in focus in the specimen plane). With the specimen and condenser in focus, the focal conjugates will be in the correct position so that resolution and contrast can be optimized by adjusting the field and condenser aperture diaphragms.

There is a simple five-step procedure that many microscopists follow in order to achieve Köhler illumination:

- 1) **Preparing the Binocular Head** - Before putting the specimen on the stage, turn on the illumination to a comfortable intensity level. Rotate a 10x objective into position. Rotate eyepiece diopter ring to "0" setting and adjust the interpupillary distance so that both right and left images from the right and left eyepieces merge into one.
- 2) **Focusing the Eyes** - Place specimen on the stage and using your coarse focus knob focus using the 10x objective. Adjust with fine focus on the smallest visible detail. Position the 40x objective and fine focus. Switch to a 4x objective and adjust the eyepiece diopters for best focus. Recheck the focus at 40x and select a working objective.
- 3) **Setting the Condenser Focus** - Using the field diaphragm control ring, close the field diaphragm to its smallest size. Bring the image of the field diaphragm into focus with the condenser focus knob. Position the field diaphragm image into the center of the field of view by using the condenser centering mechanism on your microscope.
- 4) **Centering the Illumination** (This step may not be required for pre-centered illumination systems) - Remove the diffuser filter from your microscope. Close the aperture diaphragm on the condenser. Use a neutral density filter as a mirror to observe the filament image on the underside of the condenser and focus the filament image by moving the lamp housing in or out until it becomes sharp. After the filament is in focus and centered, replace the diffuser.

- 5) **Adjusting the Condenser Diaphragm** - Remove one eyepiece and look through the eyepiece tube down toward the back of the objective. Adjust the aperture diaphragm so that it just inside the exit pupil of the objective.

By following these steps, the microscope will be in Köhler illumination and will produce the best possible images.

The concept that specific planes in the optical path of the microscope are conjugate indicates that they are equal. That is, whatever appears in focus in one plane of a conjugate set will appear in focus in all the other planes belonging to the same set. On the other hand, the reciprocal nature of the two sets of microscope conjugate planes requires that an object appearing in focus in one set of planes, will not be focused in the other set. The existence of two interrelated optical paths and two sets of image planes characterize Köhler illumination and is the foundation that allows the various adjustable diaphragms and aperture stops in the microscope to be used to control both the cone angle of illumination, and the size, brightness and uniformity of illumination of the field of view. The aperture planes may be considered aperture-limiting because the numerical aperture of the optical system can be controlled by fixed or adjustable (iris) diaphragms inserted at any of these positions. Components containing the set of aperture planes, which are not in focus with the specimen image, are presented (removed from their locations in the microscope) in Figure 3.

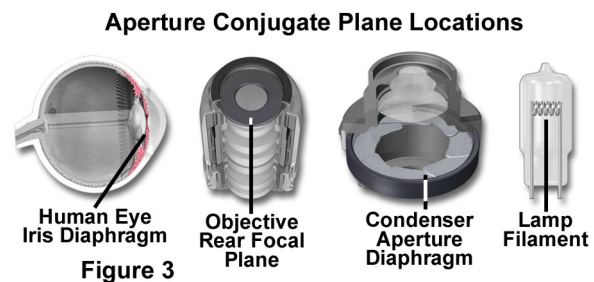


Figure 3. Aperture conjugate plane locations in actual microscope components.

Difficulty in establishing Köhler illumination can occur when high numerical aperture (and high magnification) objectives are employed. In some cases, the field diaphragm cannot be sufficiently closed to a diameter that can be imaged in the viewfield, or the condenser does not produce a diaphragm image that is small enough to be observed (especially at 60x and 100x). Changing to a lower magnification can alleviate this problem. Using slides that are too thick will also cause difficulty in

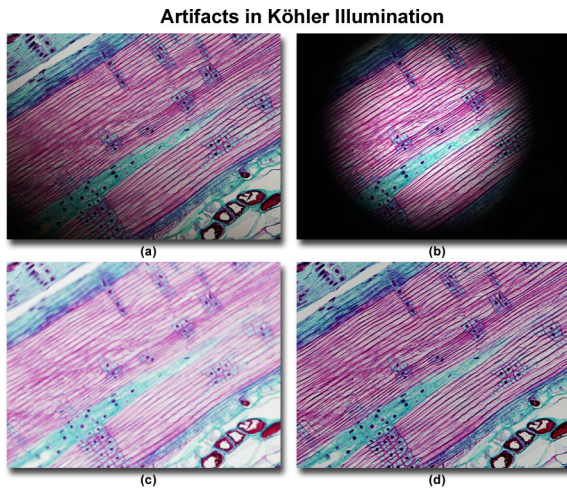


Figure 4

Figure 4. Artifacts in Köhler illumination. a) Incorrectly aligned condenser; b) Condenser position too low; c) Condenser aperture diaphragm too wide; and d) Condenser aperture diaphragm closed too far.

imaging the field diaphragm. In addition, low power objectives (less than 10x) are not designed to be used with Köhler illumination, and are usually accompanied by equally low numerical aperture condensers to enable even illumination across the viewfield.

Illustrated in Figure 4 are several digital images captured with a transmitted light microscope that has been incorrectly adjusted for proper Köhler illumination. The specimen is a multiply stained thin section of pine tree stem imaged in brightfield mode. Figure 4(a) shows how the specimen appears in the viewfield when the condenser is not coincident with the microscope optical axis. In this case, the condenser is significantly off-center and a vignette appears in the lower left-hand corner of the image. When the condenser does not focus an image of the lamp filament directly onto the condenser aperture plane, the field diaphragm appears out of focus (Figure 4(b)) and the intensity of illumination is uneven across the viewfield. The image in Figure 4(b) contains a bright spot in the center due to the unfocused lamp filament. Opening the condenser aperture diaphragm too wide results in loss of contrast and washed-out images (Figure 4(c)), while closing the aperture opening size too far (Figure 4(d)) produces diffraction artifacts and very dark images.

Poor images that initially appear to result from improper configuration of the microscope illumination system often have another source. For example, partial blocking of the viewfield can result from an incorrectly aligned condenser (see Figure 4(a)), but can also be a problem when an auxiliary slider (such as a Nomarski prism, polarizer, or neutral density filter) is not completely removed from the optical pathway. Likewise, images that appear

to be too bright and low in contrast (Figure 4(c)) can result from excessive lamp voltage or failing to use the appropriate neutral density filter. Dark images (Figure 4(d)) arise from the opposite effects: very low lamp voltages or too much neutral density. Improper configuration of digital or traditional film cameras also results in digital images or photomicrographs that appear to be suffering from artifacts due to microscope (illumination) alignment problems. If image problems occur, it is wise to remove all accessories from the optical train and re-align the microscope according to the steps listed above before proceeding.

Establishing Köhler illumination is important for practically all of the common imaging modes in optical microscopy, including brightfield, darkfield, differential interference contrast (DIC), and polarized light. Several other contrast-enhancing techniques, such as oblique illumination and phase contrast, have specialized condensers that do not utilize or require centerable aperture diaphragms. In the latter cases, it is always preferable to pre-align the microscope using Köhler techniques prior to inserting the oblique or phase condenser systems. Epifluorescence also benefits significantly from Köhler illumination, which is established using the arc-discharge lamp centering screws, the collector lens system, and the vertical illuminator field and aperture diaphragms. In reflected light, the objective serves the dual purpose of image-forming lens system and condenser, so alignment of this component is not necessary.

The best image quality can be achieved in the microscope only with a thorough understanding of the central role played by the two sets of reciprocal conjugate focal planes. For optimum results, the microscopist must be familiar with their interrelationships and where they are located in the microscope when the conditions required for Köhler illumination are met. With this understanding, the primary optical components of the microscope, as well as any accessories that might be added, can be utilized in a manner that exploits the reciprocal nature of the conjugate plane sets to maximum benefit.

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