## **Fundamentals of Microscopy**

Jeremy Sanderson<sup>1,2</sup>

<sup>1</sup>Bioimaging Facility Manager, MRC Harwell Institute, Mammalian Genetics Unit, Harwell Campus, Oxfordshire, UK

<sup>2</sup>Corresponding author: *j.sanderson@har.mrc.ac.uk* 

The light (or optical) microscope is the icon of science. The aphorism "seeing is believing" is often quoted in scientific papers involving microscopy. Unlike many scientific instruments, the light microscope will deliver an image however badly it is set up. Fluorescence microscopy is a widely used research tool across all disciplines of biological and biomedical science. Most universities and research institutions have microscopes, including confocal microscopes. This introductory paper in a series detailing advanced light microscopy techniques explains the foundations of both electron and light microscopy for biologists and life scientists working with the mouse. An explanation is given of how an image is formed. A description is given of how to set up a light microscope, whether it be a brightfield light microscope on the laboratory bench, a widefield fluorescence microscope, or a confocal microscope. These explanations are accompanied by operational protocols. A full explanation on how to set up and adjust a microscope according to the principles of Köhler illumination is given. The importance of Nyquist sampling is discussed. Guidelines are given on how to choose the best microscope to image the particular sample or slide preparation that you are working with. These are the basic principles of microscopy that a researcher must have an understanding of when operating core bioimaging facility instruments, in order to collect high-quality images. © 2020 The Authors.

**Basic Protocol 1:** Setting up Köhler illumination for a brightfield microscope **Basic Protocol 2:** Aligning the fluorescence bulb and setting up Köhler illumination for a widefield fluorescence microscope

Basic Protocol 3: Generic protocol for operating a confocal microscope

Keywords: confocal microscopy • fluorescence microscopy • image formation • Köhler illumination • light microscopy

### How to cite this article:

Sanderson, J. (2020). Fundamentals of microscopy. *Current Protocols in Mouse Biology*, *10*, e76. doi: 10.1002/cpm0.76

## **INTRODUCTION**

Sight is our primary sense; we are a visual species. In the last decade technological advances, together with the maturation of network sharing and the development of both the internet and local intranets, have resulted in vastly more published papers, data, and experimental research being produced (Chessel, 2017; Meijering, Carpenter, Peng, Hamprecht, & Olivo-Marin, 2016; Ortell, Switonski, & Delaney, 2019; Zaritsky, 2018). Data handling and its effective management may well be rapidly becoming the second language of science; nevertheless, our capacity to interpret results visually still predominates. The electromagnetic spectrum offers a rich resource to allow visible light,



Current Protocols in Mouse Biology e76, Volume 10 Published in Wiley Online Library (wileyonlinelibrary.com). doi: 10.1002/cpmo.76 © 2020 The Authors. This is an open access article under the terms

of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited. Sanderson

1 of 30

X-rays, and other forms of non-ionizing radiation to illuminate samples. In our case, these samples are whole mice or mouse tissues. Previous articles in this journal have covered X-ray computed tomography (X-ray CT), positron emission tomography (micro-PET), single-photon emission computed tomography (micro-SPECT), X-ray microtomography (micro-CT; Hsu et al., 2019), and magnetic resonance imaging (micro-MRI; Constantinesco, Choquet, Goetz, & Monassier, 2012). Advances in biomedical imaging are bringing together the integration of microscopy with other imaging modalities across the electromagnetic spectrum (Geyer, Mohun, & Weninger, 2009; Norris et al., 2013; Powell & Wilson, 2011; Tsien, 2003; Weissleder & Nahrendorf, 2015). Another very important imaging technique is ultrasound, used both in mice (Bou About et al., 2019; Phoon & Turnbull, 2016) and human diagnostic procedures (Lu et al., 2020; Suetens, 2009), but because this does not involve the electromagnetic spectrum, it is not covered here.

Unlike many scientific instruments, a light microscope will always deliver an image of some sort, however badly it is set up. Because images are now acquired and collected in digital, rather than analog, form, a microscope image is not merely a pretty picture but a dataset of photon intensity. As such it can—always provided it has been collected using a microscope that has been properly set up and adjusted—be used to validate or reject experimental hypotheses. This is the chief purpose of collecting image data using microscopes. The microscope is therefore used:

- to resolve fine detail in the structure of an object or sample
- to provide a magnified image of an object or part of an object
- as an analytical tool to measure length, angles, area, and/or thickness or to determine optical properties of the object, such as the refractive index, reflectance, polarization, or phase change.

As an image facility manager, the author continually finds that those who use microscopes may be unaware of how sophisticated these instruments are. Even a simple bench microscope requires proper alignment to set it up. It is easy to overlook crucial adjustment steps needed to operate a microscope effectively, whether an electron microscope or a light microscope. If a poorly adjusted microscope is used to collect images, these data cannot be used experimentally (Arena et al., 2017; Jost & Waters, 2019; Lee & Kitaoka, 2018), the result being both time and expense lost.

## The electromagnetic spectrum, waves, and the interaction of light with matter

An image is formed when light interacts with the matter comprising the object or sample being viewed. Therefore, it is worth reminding ourselves of the properties of the electromagnetic spectrum. The electromagnetic spectrum comprises the span of all electromagnetic radiation and extends from beyond very long wavelengths at low frequencies used for radio to very high frequency gamma radiation at the short wavelength end of the spectrum, encompassing wavelengths from thousands of kilometers down to a fraction of a nanometer (for further information, see the Nature Milestones in Light Microscopy, listed in the Internet Resources at the end of this paper). Although all electromagnetic waves travel at the speed of light, c (exactly 299,792,458 m/s), in a vacuum, they do so at a wide range of frequencies, wavelengths, and photon energies. A wave repeats regularly in space and time. Electromagnetic waves are described by spatial frequency f over time, by wavelength  $\lambda$ , or by photon energy E.

Frequency, f (sometimes the Greek letter nu, v), is defined as the number of occurrences of a repeating event per unit of time. The frequency of a wave (the SI derived unit is the Hertz, Hz) is the number of waves (e.g., measured at their crests) that pass a fixed point in a given time period: the cycles per second (the SI base unit of time is the second, s). Wavelength is the spatial period of a periodic wave—the distance over which the wave's shape repeats. It is the distance between consecutive corresponding points of the same

phase on the wave, such as two adjacent crests or two adjacent troughs. The amplitude of a wave is the height, or maximum displacement, of the wave crest above the undisturbed position. (It is not the distance between the top and bottom of a wave cycle.) Photon energy, *E*, is directly proportional to the electromagnetic frequency of the photon and is thus inversely proportional to its wavelength, as  $E = hc/\lambda$ , where h = Plank's constant (6.626  $\times 10^{-34}$  J·s) measured in Joule-seconds. The joule is the SI derived unit of energy; photon energies are also measured in electron-volts. For a given wavelength in nanometers, the photon energy in electron-volts is given by  $E_{eV} = 1239.841/\lambda$ . The common laser line 488 nm has a photon energy of 2.54 eV or  $4.07 \times 10^{-19}$  Joules and a frequency of  $6.143 \times 10^{14}$  Hz. As microscopists, all we need to remember is that because *h* and *c* are both constants, photon energy *E* changes inversely to wavelength  $\lambda$ . Thus, the shorter the wavelength, the higher the energy of the light or photon, and vice versa.

A photon is a quantum (discrete package) carrier of electromagnetic radiation energy. Light exists in particulate and wave form (Piazza et al., 2015), both states being necessary to explain all phenomena of light. A light wave does not propagate infinitely, but the wave trains (photons) are of distinct length and travel like a group of ripples from the light source. Light originates from the transitions of electrons that occur when atoms or molecules absorb energy by virtue of being at transiently elevated temperature, such as in filament lamps. This process is called incandescence. When the excited electron returns to the ground state, the energy used to excite the electron is given off, either as non-radiant energy or as light. The light emitted from an incandescent source is the result of spontaneous emission. A lamp filament emits photon wave trains  $\sim 3$  m long. The photons have the same frequency, but there are no phase relationships between the individual waves: the light is thus said to be incoherent. Also, the vibration direction of each wave is random: the light is unpolarized. On the other hand, coherent rays of light emanate from the *same* light source, have a constant phase relationship with respect to one another, and thus can interfere with each other. Lasers (by virtue of stimulated emission) produce coherent monochromatic light.

Spectroscopy is used to study how light interacts with matter and how waves of different frequencies give information about the composition, physical structure, and electronic structure of matter. The boundaries between radio waves, microwaves, infrared, visible light, ultraviolet, X-rays, and gamma rays are human definitions. In reality the different bands overlap in their properties: for example, red light resembles infrared radiation. Humans are able to discriminate the visible wavelengths from long-wavelength, low-energy red radiation at around 710 nm to short-wavelength, high-energy violet radiation at around 380 nm. Light and other forms of electromagnetic radiation are energy sources. It is easy to forget that light can perturb, permanently alter, or damage the microscope specimen. This is particularly so with live samples (Cole, 2014; Anonymous, 2013; Anonymous, 2018; Icha, Weber, Waters, & Norden, 2017; Tinevez et al., 2012). Minimizing the light flux for live-cell imaging is often referred to as "photon budgeting".

Light interacts with the sample under the microscope in one of six ways:

- Light may pass straight through matter and be partially absorbed. This is how coloured stains work.
- Light may be reflected or refracted by matter.
- Light may be scattered or diffracted (i.e., undergo a regular form of scattering) by matter.
- The light wave may undergo a phase change as a result of passing through matter.
- Light may be polarized by passing through, or reflecting off, matter.
- Light may excite matter to cause the emission of fluorescence.

## How an image is formed in the microscope

The wave nature of light allows us to explain both diffraction and refraction. Whenever waves pass through matter, their wavelength decreases. The proportion in which they do so relative to the speed of light in a vacuum is called the refractive index, which follows Snell's law. Refractive index is denoted by the letter n (sometimes the Greek letter eta,  $\eta$ , is erroneously used). The refractive index is defined as n = c/v, where c = the speed of light in a vacuum and v is the speed of light in matter. In practice, the speed of light in air (refractive index = 1.0003) is regarded as equivalent to c. Whenever electromagnetic waves exist in a medium with matter, their wavelength is decreased (Bradbury, 1984; Spencer, 1982).

Rays of light propagate in straight lines within homogeneous media. They reflect and refract at smooth interfaces between materials of different refractive indexes. A lens focuses or disperses rays of light by refraction. Dispersion is the phenomenon by which light is spread out according to its wavelength as it passes through material. For our purposes, lenses are usually represented simply as biconvex or plano-convex in shape. In reality microscope objectives and eyepieces are complex constructions of several lens elements designed to correct for optical aberrations such as spherical and chromatic aberration (for descriptions of the different classes of objective and the degree to which they are corrected for spherical and chromatic aberration, see Benham, 2002; LoBiondo, Abramowitz, & Friedman, 2011; Piston, 1998).

When the object being imaged is very large compared to the wavelength of light, such as when we use a camera or a single-lens magnifying glass, we can consider light as rays and use ray tracing and geometric optics to understand how the image is formed. We will not consider geometrical optics further here; for further information on the topic, the reader is advised to consult chapter 3 in Sanderson (2019) or chapters 29 and 30 in Franklin, Muir, Scott, Wilcocks, and Yates (2010) for further information. However, when the size of the object being studied is approximately the same size as the wavelength of the illuminating light, then the effects of diffraction (which always occurs) become much more noticeable. We must now consider light as waves, rather than rectilinear rays.

When light passes through, or reflects off, the surface of an object, it is diffracted by the structure of the specimen. Light passing through the specimen on-axis without diffracting is called the zero order and forms the bright background against which the image is seen. The light scattered by diffraction interferes constructively and destructively to form a series of orders of light—maxima and minima, respectively—that form a diffraction pattern in the back focal plane of the objective. This is seen in the left-hand diagram in Figure 1. Interference is an inherent property of all waves propagating from a coherent source. The classic experiment demonstrating this is Young's two-slit interference experiment (e.g., see the video listed in Internet Resources; Houston, 1921; Thomas, 2019; Young, 1802, 1804). The objective produces a Fourier transform of the object in the back focal plane, and at the primary image plane another Fourier transformation, equivalent to an inverse Fourier transform.

Ernst Abbe, working for Carl Zeiss, showed (Abbe, 1873) how a microscope image is formed from light diffracted by the specimen. At least two diffraction maxima must enter the objective, form a diffraction pattern in the back focal plane of the objective, and proceed to interfere at the primary image plane. This is shown in Figure 1. At the limit of resolving power, this may be the zero order and one of the first orders entering either side of the aperture of the objective (oblique illumination). Abbe formulated an expression for the angular aperture of the objective, called the numerical aperture. This is the defining characteristic of the resolving power and light-gathering ability of the microscope objective, and is usually abbreviated to NA.



**Figure 1** A diagram of the ray paths when narrow axial illumination is used to form a diffraction pattern in the back focal plane of the objective. The rays proceed from the objective back focal plane to interfere and form an image in the primary image plane. From each of the three slits (A, B, and C) shown, the undeviated direct light is projected by the objective into its back focal plane as the zero-order maximum. Two maxima to either side of the zero-order maximum are accepted by the objective aperture (1 and 2, and –1 and –2) and form the diffraction pattern shown in the objective back focal plane. The direct background light forms from the undeviated zero order. If the numerical aperture of the lens is too small to accept the diffracted light, no detail will be resolved in the image. At least two diffracted orders, or the zero order plus one diffracted order, must be accepted to form an image showing resolved detail in the primary image plane. This is how a microscope image is formed. Top right, the importance of numerical aperture (NA =  $n \cdot \sin \alpha$ ). In air the maximum half-angle of aperture is 72°. Using a high-NA objective with immersion (oil, water) ensures the higher-order diffracted rays are not refracted out of the aperture of the objective and lost. For a value of  $\alpha = 72^\circ$ , the objective NA = 1.518 × 0.951 = 1.4. Bottom right, the diffraction pattern seen in the objective back focal plane

The information-gathering power of a microscope objective is limited by diffraction, and depends upon the angle of the cone of rays (diffracted orders of light) that can be accepted by the aperture of the objective. Refraction of light at the coverglass-air interface of the slide preparation (the object) determines the number of diffracted rays actually passing through the objective to be focused to an image at the primary image plane. To take account of this, the NA of the objective, which describes the information-gathering power of the objective mathematically (so that one objective can be compared arithmetically with another), is the product of the refractive index of the medium through which the objective is designed to operate (air, water, glycerol or oil) and the sine of *half* the aperture angle.

## $NA = n \cdot \sin \alpha$

The numerical aperture of the objective also determines the light-gathering power of the objective. An objective with a higher NA will gather a greater flux of light than a low-NA objective. This is often an important consideration with fluorescently stained samples, where the emitted signal may be weak.

The diffraction pattern is not normally seen in the back focal plane of the objective unless (1) a grating or periodic object (e.g., a diatom such as *Pleurosigma angulatum*) is used as a specimen and (2) a narrow axial beam of illumination (i.e., a coherent source) is used. This is achieved in practice by reducing the illuminating aperture of the condenser to a

minimum, which in practice is *never* the case: normally the condenser aperture is set to fill the objective aperture at the back focal plane of the objective. These two preconditions for viewing the diffraction pattern (which encodes the information forming the microscopical image) caused some difficulty in the widespread acceptance of Abbe's theory of image formation, and so he devised a series of elegant experiments to demonstrate this. Abbe's diffraction experiments remain didactically the best way to understand the diffraction theory of image formation in the microscope (see Evennett, 1996; Sanderson, 2019; also see the video listed in Internet Resources). Another difficulty is that Abbe's original 1873 paper promised a mathematical proof that was never forthcoming, for he died in 1905 without publishing the underlying mathematics. Horst Köhler, a later employee at Zeiss Oberkochen (and no relation to the Köhler who proposed the illumination adjustment for the microscope in 1893), later published the mathematical derivation of Abbe's theory of image formation (Köhler, 1981).

When the condenser is adjusted properly so that a solid cone of (broadly incoherent) light of high aperture is admitted into the objective, the diffraction maxima formed in the back focal plane of the objective overlap so that they cannot be separately distinguished. The resolving power, d, of a microscope is therefore given by:

$$d = \lambda / NA_{objective} + NA_{condenser}$$

so long as the aperture of the condenser is equal to, or smaller than, that of the objective.

When using a microscope, we use objectives with circular apertures instead of slits. The diffraction pattern resulting from rays passing from a point through a uniformly illuminated circular aperture consists of a central bright region, the Airy disc, surrounded by a series of much fainter concentric rings (Airy, 1835). This is the Airy pattern. A densitometric trace of the intensity of light across the diameter of the Airy pattern forms the distribution looking like the "normal distribution," seen in the upper right diagram shown in Figure 2. Simply put, all microscope specimens can be considered to be made up of a series of closely apposed points, rather as all substances are composed of atoms. The diameter of the Airy disc, and indeed the Airy pattern, depends upon (1) the numerical aperture of the microscope objective and (2) the wavelength of the illumination. The larger the objective NA, and also the shorter the wavelength, the smaller the diameter of the Airy disc will be. The resolution limit of a microscope objective is determined by how closely two Airy patterns, formed by two closely apposed points of fine detail in the specimen, overlap yet can still be perceived as distinct (Fig. 2). From his study of images of stars viewed with telescopes, John Strutt, Lord Rayleigh (Rayleigh, 1896), established an arbitrary criterion of resolution that in practice also holds good for microscope images. The resolution limit occurs when the first dark ring (minima) of one Airy pattern coincides with the central maximum of the second. At this point the contrast in the microscope image is sufficient for the two Airy discs to just be resolved as distinct entities in the image. The resolving power of the microscope is given by:

### $d = 0.61\lambda/\text{NA}$

This equation is applicable when using a fluorescence microscope to view self-luminous fluorescently stained specimens, and when (as in the design of a reflected-light fluorescence microscope) the objective is used as its own condenser. Rayleigh's criterion assumes incoherent illumination—as when observing stars. This holds true in microscopy for self-luminous points, but otherwise the illumination from a properly set condenser is broadly coherent (see Hammond, 2009), and thus it is better to regard image formation according to Abbe's criterion: namely that the limit of resolution is determined by the rays diffracted by the specimen that enter the objective to interfere and form an image.



**Figure 2** Formation of Airy discs and Rayleigh's resolution criterion. When a wave passes through a circular aperture of finite diameter, the interference maxima and minima form an Airy pattern comprising a central bright disc and alternating light and dark concentric circles. Viewed with a telescope, the image of a star, for example, is never a point, but a diffraction-limited point spread function—the Airy pattern. The same is true for microscope images. The intensity distribution of two Airy patterns in shown in elevation view (top right) and in plan view (bottom). Two closely apposed points (*a*) subtend an angle  $\alpha_{zom}$ . For small angles, sin *i* = (*d*/2)/*f* or *d*/2*f*, and therefore  $a/f = 1.22 \lambda/(d \text{ and } a = 1.22 \lambda/(f/d)$ . Substitute  $f/d = 2 \sin i$ , and since for small angles f/d = 2NA, then a = 0.61/NA.

The way in which an image is formed by (Fraunhofer) diffraction and interference of electromagnetic radiation also applies to transmission electron microscopes (Chescoe & Goodhew, 1990) as well as to the light microscopes that we discuss here. Scanning electron microscopes are different, forming images arising from electrons, X-rays, and cathodoluminescence scattered from the surface of the sample (Zhou, Apkarian, Wang, & Joy, 2006) by scanning an electron beam in raster fashion across the sample.

## The importance of resolving power

Four factors determine the resolution of fine detail in the image:

- The aperture half-angle,  $\alpha$ , of the microscope objective, where NA =  $n \cdot \sin \alpha$ . The larger the aperture angle, the smaller the detail resolved in the image. The sine of the aperture angle cannot be  $\geq 1$ , and is always less, because some working distance is required between the objective and the front of the slide preparation or sample to enable the objective to work.
- Wavelength,  $\lambda$ . Using shorter wavelength illumination results in smaller diffraction angles from each point in the object, so that a greater number of interference orders can be collected within the aperture of the objective. The finer details are encoded within the higher orders of diffracted light.
- The refractive index, n, of the medium or media between the front of the objective and the sample or slide preparation. A higher refractive index of the medium also reduces the diffraction angles. For objectives with numerical apertures > 1, oil, water, or glycerol is used as an immersion medium (effectively replacing air) between the front of the objective and the object being viewed (Visser & Oud, 1992; Besseling, Jose, & Blaaderen, 2015; Fouquet et al., 2015; Tejedo et al., 2019; see Fig. 1).



**Figure 3** Diagram showing how the condenser diaphragm is closed very slightly, at the expense of sacrificing some resolving power, in order to improve contrast when adjusting the microscope for Köhler illumination. Just how much the condenser aperture needs to be reduced is dependent upon the pellucidity of the specimen and the degree of correction and quality of the objective; the "80% rule" is merely a good rule of thumb. Experienced microscopists can judge the reduction needed from observing an image, although it is good practice also to view the back focal plane of the objective directly. The numerical aperture (NA) of the objective is designed by the manufacturer and is usually fixed (some objectives intended for dark ground have a variable-NA iris built into the objective back focal plane). The NA of the condenser is controlled by the condenser (illuminating aperture) diaphragm.

• The angle of the illumination. This is seldom altered from being on-axis, but resolving power in one azimuth can be increased by oblique illumination using large illumination angles up to the maximum aperture angle  $\alpha_{max}$ . Generally the condenser is opened to allow a full cone of light (omnidirectional oblique illumination rather than in one azimuth) to fill the back focal plane of the objective (for optimum contrast, the condenser aperture should be  $\approx 80\%$  that of the objective; see Fig. 3).

The limit that diffraction sets upon the information-gathering power of the microscope (which when using coherent illumination is  $d = \lambda/2$ NA) is sometimes referred to as the Abbe diffraction limit, or colloquially as the "Abbe limit." Compare this equation to the two given above, and you will see that all three are very similar. For explanations and proof of the mathematics underlying the Abbe limit, see Hammond, 2009. There are now means of circumventing this limit (Anonymous, 2009; Lambert & Waters, 2017; Schermelleh et al., 2019; Turkowyd, Virant, & Endesfelder, 2016) for so-called super-resolution microscopy.

## The parts of a microscope and the anatomy of the stand

A light microscope consists of two functionally distinct elements: the illumination part and the imaging part. In an ordinary bench microscope, otherwise called a brightfield microscope, the illumination part is arranged on one side of the object or slide preparation, and the imaging part on the other. In an inverted fluorescence microscope, or a reflected-light microscope, both the illumination and imaging parts of the microscope are physically located on the same side in relation to the position of the



**Figure 4** The various component parts found on a modern research microscope stand. On modern stands, the illumination is now provided by light-emitting diodes (LEDs) rather than incandescent lamps or, in the case of fluorescent light, mercury bulbs. An image of the illumination source is expanded to fill the front aperture of the condenser by the lamp collector lens. The area of the field of the illuminated field of view is controlled by the illuminated field diaphragm (IFD) normally situated in the base of the microscope stand, and the angle of the cone of light supplied by the condenser is controlled by the condenser diaphragm, otherwise called the illuminating aperture diaphragm. These two controls are required to set up Köhler illumination; the other controls are present either for contrast enhancement or to facilitate moving the sample and recording an image.

object or sample under observation. We will deal with this configuration later; for the moment, let us consider the transmitted-light microscope. The various parts that comprise a modern microscope are shown in Figure 4. Not all of these components may be found on your stand, but the essential features—the illuminated field diaphragm, condenser, condenser adjustment controls, condenser diaphragm, objectives, adjustable dioptre adjustment control(s), and eyepieces—ought to be present. Space precludes a detailed description here, but further details regarding the individual components found on research microscopes can be found in Salmon and Canman (1998) and Sanderson (2019).

Older microscopes, so-called finite-tubelength microscopes, focused the primary image formed by convergent rays at a finite distance. Inspection of the ray paths in Figure 5 should make this clear. These microscope objectives have a "160" symbol to denote this—160 mm being the mechanical tubelength, the distance between the shoulder of the objective (screwed into the nosepiece) and the rim of the eyepiece. The optical tubelength is the distance between the back focal plane of the objective and the primary image plane (the later usually situated by convention 10 mm below the rim of the eyepiece). Modern microscopes are generally "infinity corrected." That is, the image is formed at the front focal plane of the objective so that parallel image-forming rays emanate, and these are brought to a focus by a tube lens—effectively a well-separated element of the objective. The parallel ray path allows extra optical components (e.g., for fluorescence and



**Figure 5** The principles of Köhler illumination. Source-focus illumination (far left) shows how the bulb filament is imaged inconveniently into the image plane. Adding a lamp collector lens creates an imaging or field set (middle left) and an illuminating or aperture set (middle right) of conjugate planes whereby the image of the filament no longer resides in the image planes. The combined ray paths are shown on the far right. Conjugate planes are optically equivalent: an image formed or projected into one plane is seen at subsequent planes in the series. For simplicity, the ray diagram shown in this figure is that of an older finite-tubelength configuration, but Köhler illumination also holds good for the infinity-corrected microscope (see Sanderson, 2019, Figures 9.19 and 9.20).

differential interference contrast [DIC]) to be inserted into the ray path without the correction that the converging ray paths of the finite-tubelength microscope require.

In the transmitted-light microscope, the illuminated field diaphragm (normally built into the base of the microscope stand) lies below the illuminating aperture diaphragm (otherwise known as the condenser diaphragm) built into the condenser (see Figure 4). In a reflected-light microscope, the positions of these two diaphragms are interchanged. It is important to be aware of this when setting up and adjusting a microscope according to the principle of Köhler illumination.

## The purpose of Köhler illumination

Before the introduction of illumination by electric light bulbs with small filaments, early microscopes used cloudy sunlight, oil lamps, or gaslight to illuminate the object uniformly over the entire field of view. All these sources are diffuse and so could be imaged directly onto the specimen using a condenser lens. This manner of illumination is called "source-focused" or "critical" illumination (Fig. 5, far left-hand diagram). As Köhler found, the difficulty arises when the image of a filament bulb is projected into the specimen plane and is superimposed onto the image of the object when using this method of illumination. August Karl Johann Valentin Köhler's PhD project depended heavily upon photomicrography; an evenly lit uniform field of view was essential. Köhler rejected the use of a diffuser as being too wasteful of light, nor was it possible to place a hot or incandescent light source at the front focal plane of the condenser (to illuminate the

specimen with parallel beams of light). Inserting the light source at the front focal plane of the condenser would also prevent placing a diaphragm there to regulate the illumination aperture. If Köhler had placed his irregular light source at infinity, the filament structure would have ceased to be problematical, but it would have been too far away and too dim to be practical. He therefore chose the optical equivalent of placing the light source at infinity: introducing an extra lens into the optical train, the lamp collector lens. Using this lens, an image of the source is projected into the front focal plane of the condenser (Fig. 5). It is now possible to include a diaphragm for regulating the illuminating aperture at the front focal plane of the condenser.

## The function of the lamp collector lens

With a lamp collector lens in place, a small and irregular light source, which has a definite filament structure, now provides a uniform and intense patch of illumination of considerable area. To verify for yourself how this works, draw a small spot (a red spot works better than a black one) about 3-5 mm in diameter on a piece of paper. Place this on a desk and, standing up, take a magnifying glass of at least  $4 \times$  power. Lay the magnifying glass on the spot and raise the magnifying glass. You will see the red spot gradually fill the aperture of the magnifying glass, acting as a uniform secondary light source. For further explanation of Köhler illumination and an additional didactic experiment, see Evennett, 1983, under the Key References.

The microscope illumination should be uniform and controllable so that stray light and glare—which do not contribute in any way to the image but merely reduce visibility or contrast—are excluded. A well-designed illumination system should:

- provide uniform illumination at the specimen over an adjustable *area*, corresponding to the field of view; and
- fill the aperture of the objective with light over an adjustable *angle*, corresponding to the NA of the objective.

The diagrams illustrate another elegant feature of Köhler illumination. Figure 5, middle left, shows that each individual point on the object receives light from all parts of the filament admitted by the condenser diaphragm, also called the illuminating aperture diaphragm, and the same figure, middle right, shows that rays arising from each individual point on the filament pass through all parts of the object or specimen. The illuminating filament in the aperture set of conjugate planes does not disturb the image which lies in the field set of conjugate planes. Köhler illumination therefore provides a large and uniform, intense, structureless light source from a small, filament structure. Köhler illumination allows independent control of the illuminating and imaging ray paths, and stray non-image-forming light can (largely) be excluded from the microscope, leading to improved contrast.

For a stepwise procedure, refer to Basic Protocol 1: Setting up Köhler illumination for a brightfield microscope.

## The three principal factors: resolution, contrast, and fluorescence

A microscope must provide:

- 1. Resolving power to carry fine detail in the specimen to the image, with
- 2. Sufficient contrast to show differences between image features and the background, at
- 3. Sufficient magnification to present the resolved detail to the eye or digital detector.

Although the primary function of the microscope is to resolve fine detail, this cannot be achieved satisfactorily unless sufficient contrast is present in the image. It is more difficult to resolve details in a pellucid image arising from an unstained object than in one that



**Figure 6** Design of the fluorescence microscope. The Köhler illumination ray path is shown at top left, and the design of the fluorescence filter carousel, which permits quick and easy changing of filters, at top right. The modern fluorescence microscope has a reflected-light configuration, whether upright (bottom left) or inverted (bottom right) for live-cell imaging.

is stained. Resolving power and contrast are therefore linked (Stelzer, 1998). It is also possible to introduce contrast optically; this is a common means of viewing unstained tissues and cells by phase contrast and differential interference contrast (DIC). These topics are not discussed here, and the reader is referred to chapters 6 and 7, respectively, in Bradbury and Evennett (1996) for more detail. Of all the contrast-enhancing techniques, if properly applied, fluorescent stains, antibody-conjugated fluorescent markers, and endogenous fluorescent proteins give the highest contrast, with a dark background and high signal-to-noise ratio (SNR; for a review, see Webb & Brown, 2013). Fluorescence microscopes give the highest signal-to-noise ratio when the objective acts as its own condenser, so all modern fluorescence microscopes are of the reflected-light design (Fig. 6), whether in an upright or inverted configuration.

## The disadvantages of fluorescence

Fluorescent probes are very versatile indeed, but suffer from three inherent disadvantages that must be borne in mind when conducting any imaging experiment. First, because they

are self-luminous, fluorescent samples and slide preparations containing fluorescent cells will give a blurred image unless the sample is thin, or you are viewing a stained monolayer of cells. High-magnification objectives (because of their large numerical apertures) have extremely limited depth of field, yet relatively large depth of focus. Depth of field is the axial depth of the space on both sides of the object plane within which the object can be moved without detectable loss of sharpness in the image, and within which features of the object appear acceptably sharp in the image while the position of the image plane is maintained. The depth of field of a high-NA microscope objective is usually <1  $\mu$ m (see Table 7.5 of Sanderson, 2019). With very thick cells and tissues, fluorescent signal emanating from outside the depth of field of the objective will cause blurring in the image. So-called optical sectioning is used to remove blurring or otherwise prevent it from contributing to the image.

The second disadvantage of fluorophores is that they bleach. This is a consequence of how electron shuttle between atomic orbitals to give a fluorescent signal (Ishikawa-Ankerhold, Ankerhold, & Drummen, 2012; Litchman & Conchello, 2005). The best stratagem is to mount stained specimens in antifade mounting medium (Bogdanov, Kudryavtseva, & Lukyanov, 2012; Collins, 2006; Florijn, Slats, Tanke, & Raap, 1993; Longin, Souchier, Ffrench, & Bryon, 1993) and to minimize exposure to light. Keep the slide preparations and samples refrigerated when not being imaged. It is easy to illuminate the sample for too long, whether with high-energy light in widefield mode or with lasers in confocal mode, when focusing the sample, selecting a suitable field of view, or setting up imaging parameters.

The third disadvantage of fluorescent probes occurs when two or more are used together to stain different targets in a single specimen. If the excitation and emission spectral profiles of the fluorophores are close together, particularly the emission profiles, then one emission signal can be detected in the channel of the other fluorophore(s). This is called bleedthrough, cross-talk, or cross-emission (see Sanderson, 2019, for a discussion of these terms). To reduce the chances of bleedthrough, select (if possible) fluorophores whose emission spectral profiles are widely separated. Also, image the longest wavelength fluorophore first and, in particular, image the fluorophores sequentially one after the other, rather than simultaneously.

For a stepwise procedure, refer to Basic Protocol 2: How to align the fluorescence bulb and set up Köhler illumination for a widefield fluorescence microscope.

## **Optical sectioning**

There are various optical sectioning stratagems to reduce blurring. First and foremost is confocal microscopy. Here, rather than the entire field of view being seen at once (otherwise known as widefield microscopy), the sample is illuminated pointwise in a raster, and the signal is collected through a pinhole placed in front of the detector. Usually, a laser is used to provide sufficient illumination flux. The image is necessarily built up as a digital image on a monitor as the laser rasters across the sample. Such point illumination and point detection ensure that the specimen is illuminated only within the depth of field of the objective. Only the emitted signal is collected, and any blurred signal from above and below the plane of focus, outside the depth of field of the objective, is prevented from being detected. By altering the diameter of the detection pinhole, the thickness of the optical section collected may be changed; opening the pinhole creates a thicker section, and vice versa.

To create thin optical sections, and take best advantage of the improved resolving power of the confocal microscope over a non-confocal widefield configuration, a high-numerical-aperture objective should always be used. A popular objective is the  $63 \times NA$ 

1.4 plan-apochromat. Because the confocal microscope uses point illumination and point detection to optically section the sample and exclude fluorescence blurring from the image, the microscope stand does not have to be set up and adjusted for Köhler illumination before use. However, if the fluorescence image is going to be combined with a widefield image (brightfield, phase, or DIC), then it is necessary to ensure that the transmission illumination pathway is set up according to the principles of Köhler illumination. For those readers who wish to use the fluorescence microscope quantitatively, Waters and Wittmann (2014) Jonkman, Brown, & Cole (2014), and Jonkman, Brown, Wright, Anderson & North (2020) are good references.

Two other methods of optical sectioning are used (for an excellent review, see Conchello & Litchman, 2005). The first, sine-modulated illumination microscopy or structured illumination microscopy (Langhorst, Schaffer, & Goetze, 2009; Wilson, 2010), employs a special grating to focus a sinusoidal illumination pattern onto the in-focus plane of the specimen. An image is collected, and the pattern is shifted twice and two out-of-focus images are collected to enable the fluorescence blurring to be subtracted by computation. This technique necessarily uses special equipment and software (e.g., ApoTome, Carl Zeiss; Optigrid, Aurox).

The other means of optical sectioning is deconvolution (McNally, Karpova, Cooper, & Conchello, 1999), which does not require specific hardware or optical components. It is software based, and although it may require paid-for software for a particular branded microscope system, it is possible to use a freeware deconvolution package (e.g., ImageJ/Fiji; Sage et al., 2017; see also the Internet Resources at the end of this paper). Besides optical sectioning, deconvolution can be used to improve image quality per se, because all imaging systems are imperfect and images are only representations of the objects they are formed from. Lenses suffer from manufacturing defects, an objective only collects a small proportion of the light emanating from the specimen, and diffraction—the very means by which an image is formed-also limits image fidelity. For mathematical ease, let us consider that all objects are composed of "points," the smallest irreducible sub-resolution component. When viewed with a lens, these points are convolved by the transfer function of the microscope system/objective lens to form an image: the pointspread function (PSF). If, knowing the transfer function, we apply reverse mathematics deconvolution-to the PSF, a sharper image as a representation of the original object is the result. Microscope objectives can be characterized by their unique PSFs (Theer, Mongis, & Knop, 2014).

Either the deconvolved image can be created using algorithms—based on the properties of the imaging system—to calculate a theoretical PSF, or a measured PSF (Cole, Jinadasa, & Brown, 2011; Parton & Davis, 2006) can be calculated from the image formed by a sub-resolution fluorescent bead under conditions close to those of the actual experiment. In software, deconvolution (Lee, Wee, & Brown, 2014; for a review, see Biggs, 2010) is usually performed by computing the spatial Fourier transform of the image and PSF. In the absence of noise, the Fourier transform of the image,  $F_{image}$ , is defined as  $F_{image} = F_{object} \times F_{PSF}$ .

Applying the inverse Fourier transform to  $F_{\text{image}}$  will recover the deconvolved image. It is worth remembering that aggressive deconvolution can give rise to artifactual spots in the image, in which case the deconvolution algorithms have to be re-run with different parameters. Also, deconvolution works less well when imaging thick specimens (Murray, 2011; Swedlow, Hu, Andrews, Roos, & Murray, 2002), and some authorities (Boutet de Monvel, Le Calvez, & Ulfendahl, 2001; Ikoma, Broxton, Kudo, & Wetzstein, 2018; Laasmaa, Vendelin, & Peterson, 2011; Pawley, 2006) rightly advocate that deconvolution is required to properly process confocal images.

## The confocal microscope

Confocal microscopes are classed by the method of scanning employed (Dean, 1998). The earliest microscopes employed stage scanning, keeping the illumination static. This method has the advantage of reducing artifacts at the edge of the field of view and of using the central, best-corrected portion of the objective. It is ideal for very large samples, limited only by the translation range of the stage. However, the stage-scanning design requires high mechanical precision and, crucially, it is much slower than beam scanning. Single-beam laser-scanning confocal microscopes employ mirror galvanometers to raster scan a single laser beam across the sample. An acousto-optical tuneable filter (AOTF) is used that can switch between lasers very quickly (Fig. 7) and, crucially, the pinhole situated in front of the photomultiplier tube (PMT) detector can be adjusted to alter the thickness of the optical section collected. This is the most common type of optical sectioning microscope, and its operation is described in the following protocol. However, the singlebeam laser-scanning confocal, though perfectly adequate for many fixed samples, is too slow and delivers far too high a light flux onto the sample to be ideal for imaging living samples or very delicately stained fixed samples. One approach to speeding up the raster scan is to use resonance scanning (Callamaras & Parker, 1999; Hendriks et al., 2011; Leybaert, De Meyer, Mabilde, & Sanderson, 2005) or non-laser so-called "white-light" systems such as aperture correlation microscopes. These white-light systems have the advantage that they can be added to an existing widefield fluorescence system relatively cheaply to upgrade it to a system with optical sectioning capability. Nevertheless, the most common confocal designs are the single-beam scanning-laser confocal, followed by the Yokogawa spinning-disc system design.

The spinning-disc microscope, along with hybrid designs such as swept-field (Castellano-Muñoz, Peng, Salles, & Ricci, 2012) and slit-scanning confocals (Elliott, 2019), collects images much faster. The Nipkow spinning-disc design illuminates the sample not with a rastered single laser beam, but with thousands of points of light across the entire field of view simultaneously. This means that a charge-coupled device (CCD) or complementary metal-oxide semiconductor (CMOS) camera can be used to collect the image, with the advantages of much lower light fluxes, greater dynamic range (more gray levels in the image), better quantum efficiency, and a much better signal-to-noise ratio. The quantum efficiency of a PMT-the efficiency with which analog photons are converted into a photoelectron signal—is  $\sim 15\%$ , whereas that of a scientific CCD or CMOS camera is 80%-90%. The Yokogawa spinning disc uses a dual disc system with microlenses to increase the sensitivity of image acquisition. The fixed pinholes of the spinning disc present a disadvantage: the optical section thickness cannot be adjusted, and must be optimized for a single objective. Also, the fixed pinhole design results in cross-talk, giving rise to background haze and reduced signal-to-noise ratio. Further details on the spinning disc can be found in Murphy and Davidson (2013), Oreopoulos, Berman, and Browne (2014), and Sanderson (2019).

For a stepwise procedure, refer to Basic Protocol 3: Generic protocol for operating a confocal microscope.

## Choosing the best microscope for your imaging

Because fluorescence blurring obscures image details, necessitating optical sectioning, which microscope platform you should choose to acquire images depends upon the thickness of your sample. A flow chart with guidance on microscope choice is given in Figure 8. Essentially, very thin samples and cell monolayers grown on coverslips (<3  $\mu$ m thick) are best imaged with fluorescence widefield microscopes. The advantages of the widefield microscope are that the illumination does not bleach the fluorophores as readily and that the CCD/CMOS camera has a much higher quantum efficiency than a PMT or



**Figure 7** Design of the confocal microscope. (**A**) The essential components of the single beamscanning confocal microscope, showing the illumination pinhole, the fluorescent specimen, and the detection pinhole, all in conjugate focus with one another. The scanning galvanometer mirrors that raster the laser beam across the sample are also shown. (**B**) The same configuration shown in more detail. A primary beam-splitter allows illumination of selected wavelengths from a continuous-wave laser to pass, and be rastered over the sample by the slow (*y*) and fast (*x*) galvanometer mirrors that impart angular scans orthogonal to one another. The laser is focused by the scan lens and the objective onto the sample. The emitted signal proceeds back up the microscope. In the short time the mirrors have hardly moved, so the signal is descanned into the detectors. The chromatic beam splitter sends the signal into the appropriate fluorescence detection channel. The detection pinhole(s) prevents the out-of-focus light from reaching the detector and blurring the image. An optional non-confocal transmission detector is shown.



Figure 8 Flow chart to help choose which type of microscope to use.

avalanche photodiode (APD) used on the confocal microscope. For samples between 3 and 30  $\mu$ m in size, the confocal microscope will, providing that the sample is well stained, remove the out-of-focus blur and give a good image or *z*-stack. A *z*-stack comprises multiple images taken at different lateral (x-y) focal planes to provide a composite image with a greater depth of field. For specimens larger than 30  $\mu$ m, where features cannot be seen in the widefield image due to blurring, the multi-photon microscope (Helmchen & Denk, 2005; Ustione & Piston, 2011) is the best choice. The alternative is to prepare thinner specimens from which images can be acquired on the confocal microscope. The limits given are not hard and fast; sometimes specimens up to 10  $\mu$ m thick can be viewed

with the fluorescence widefield microscope, depending upon the nature of the sample, the staining, and the details that must be recorded. Lightsheet microscopes are good for light-sensitive preparations and small living samples (Chatterjee, Pratiwi, Wu, Chen, & Chen, 2018; Elisa et al., 2017; Liu, Xiao, Li, Liu, & Chen, 2019). A future paper in *Current Protocols in Mouse Biology* will cover super-resolution microscopy, but Combs and Shroff (2017) provides a useful guide to the various fluorescence imaging techniques, discussing the tradeoffs between resolution, signal-to-noise, and speed of acquisition.

BASIC PROTOCOL 1

# SETTING UP KÖHLER ILLUMINATION FOR A BRIGHTFIELD MICROSCOPE

There are four steps to follow in order successfully to adjust a microscope so that it gives a good image. These are:

- Focus the specimen upon the microscope stage
- Illuminate the specimen using the condenser and illuminated field diaphragm control
- Adjust the angle of the cone of illumination using the condenser control
- Fine-tune the eyepiece dioptre adjustments to suit your eyes for relaxed viewing.

## Focus the specimen

- 1. Open the illuminated field diaphragm and the condenser diaphragm fully.
- 2. Turn on the light source and swing in a  $10 \times$  objective.

*This will have sufficient working distance (the clearance between the front of the objective and the slide) not to hit the slide while initial adjustments are being made.* 

- 3. When using the microscope, your eyes should be relaxed. Before using the microscope, first look into the distance—i.e., at "infinity"—and then check that the interocular distance, the distance between the two eyepieces via the folding (or sliding) bridge on the binocular head, is set correctly for you to see one circle of light instead of two.
- 4. Raise the condenser to the top of its travel path.
- 5. Place a slide on the stage, checking that the coverslip is uppermost (or on an inverted microscope, downmost), facing towards the objective—we all forget this at one time or another!
- 6. Use the coarse focus control to reduce the distance between slide and objective to a minimum, closer than the focal point of the objective.

While doing this, look side-on at the gap between the objective and slide, to ensure that they do not collide. Remind yourself which way to turn the coarse focus to focus away from the objective. Looking down the eyepieces, you should see the image of the specimen, as a blur, very much out of focus.

7. Now, while looking into the eyepiece(s), increase the distance between slide and objective using the coarse focus control, and stop when the image of the specimen comes into focus. Adjust as necessary with the fine focus control. This sets the specimen in correct relation to the objective.

## Set the height of the condenser: Illuminate the specimen using the illuminated field diaphragm control

The correct height of the condenser must now be set.

- 8. Raise the condenser up to its top limit, or nearly so, ensuring that it does not contact the lower face of the microscope slide.
- 9. Close down the illuminated field diaphragm (IFD) almost to a pinhole, or at least to its smallest extent.

Sanderson 18 of 30 This diaphragm is usually situated in the base of the microscope, underneath the condenser and substage assembly. If the lamp is an external one, the field diaphragm will be the iris built into the lamp at the front, after the lamp collector lens. Using the condenser height control, rack the condenser down slowly until the image of this diaphragm is sharply in focus at the specimen plane, superimposed upon the image of the object.

10. Open up the field diaphragm until its image almost reaches the edge of the field of view.

This way, it is easy to operate the two centring screws (usually set at 120° to one another, one on either side of the condenser) such that the narrow penumbral shadow just inside the field of view, and surrounding the illuminated part of the image of the specimen, can be made of parallel width. The adjusting screws act against a sprung pin to enable the condenser to move laterally and be centred.

11. Open the diaphragm further by a small amount until the image of the diaphragm lies *just* outside the field of view.

Do not open it too much, as otherwise stray light will reduce contrast in the image. The IFD should confine the area illuminated to that under observation by a particular objective lens. (For contrast enhancement methods such as phase contrast and darkfield, which employ their own annular diaphragms in the condenser, the IFD must be left fully open.)

### Adjust the condenser control

The correct height of the condenser has now been set. The condenser diaphragm can now be adjusted so that the aperture of the variable cone of light supplied by the condenser can be correctly matched to the (generally) fixed numerical aperture of the objective.

12. Remove an eyepiece and look into the microscope body tube to inspect the back focal plane of the objective, which should be seen as a disc of light at the base of the tube.

The rays of light are brought to a focus in the back focal plane of the objective, where a (usually fixed) diaphragm is located. This aperture diaphragm of the objective limits the effective numerical aperture of the objective.

In addition to removing an eyepiece, the back focal plane of the objective may be observed by other means, such as inserting a phase-contrast centringce telescope or (very rarely) screwing a low-power objective to the bottom of the drawtube (if fitted). These methods provide a magnified image of the back focal plane of the objective for inspection. If a magnification changer is included, this often includes a supplementary lens system—a Bertrand lens—for the same purpose. A final method is to place an inverted eyepiece over the exit pupil of the eyepiece to view the exit pupil of the microscope.

13. Adjust the condenser aperture diaphragm until the image of this iris is just a little smaller than the diameter of the disc of light ( $\sim$ 80% diameter), which represents the full aperture of the objective (Fig. 3). Replace the eyepiece.

In theory, the aperture of the condenser should equal that of the objective. However, in this case stray light refracted from the extreme edges of the objective lens elements would cause an appreciable loss in contrast. It is worse, however, to close down the condenser aperture diaphragm too far: this will cause serious degradation in image quality, and loss of resolving power. The condenser diaphragm is not be used to control brightness in the image; rather, use the rheostat control on the lamp transformer. Closing down the condenser aperture diaphragm from its optimum position will increase contrast, and may sometimes be the only method of introducing sufficient contrast to visualize the image at all, but this contrast is gained at the expense of resolution of fine details and good image quality. The rule that the condenser diaphragm is set open at  $\sim$ 80% of the numerical aperture of the objective is for guidance and good practice; by all means break it if you know what you are doing! Closing the condenser diaphragm too far will cause the

image gradually to become a less faithful representation of the object: outlines of all features will appear thickened, and adjacent features will merge. Decreasing the aperture of the condenser will also increase its depth of field, and bring into focus dust and other contamination from the surfaces of the specimen preparation normally invisible in the properly adjusted microscope.

When changing to another objective of different magnification, this objective will have a different field of view (requiring a change in the diameter of the illuminated field) or numerical aperture (requiring a different illuminating aperture), and both diaphragms need to be adjusted. With objectives of  $<10 \times$  magnification, the field of view may not be fully illuminated even with the field diaphragm fully open. In this case, the top lens of the condenser should be either swung out or unscrewed from the condenser assembly. Do not defocus the condenser to enlarge the illuminated area, as uneven illumination and a loss of resolving power and image quality will result.

## Adjust the eyepieces

This is done to avoid eye strain under conditions of prolonged visual use (e.g., when the image is viewed directly by eye, and not projected onto a computer monitor) for comfortable viewing.

One or both eyepiece tubes on the microscope binocular head may have adjustable socalled dioptre controls capable of adjusting the tubelength of the microscope. If only one eyepiece tube of the binocular has a variable control, set up Köhler illumination with the fixed eyepiece and adjust the variable control for the other eye until the image is in focus while the eyes are relaxed. Try not to close the opposing eye while focusing, nor to screw the eyes up, but rather relax them—you should be aiming to "look through" the microscope to infinity. Fatigued eyes result if the eyepieces are not correctly adjusted for relaxed viewing of an image at infinity, which is how the microscope is designed to be used.

If the binocular has two adjustable focusing controls, first focus the microscope using a high-magnification objective ( $40\times$ ; a "high-dry" objective—a non-immersion one with high aperture, but ideally *without* a correction collar). Change to the  $10\times$  objective (or preferably a lower-magnification objective, if it is part of a parfocal set), and adjust the eyepiece focusing adjustments separately, *without* refocusing the objective, so that the specimen is in focus.

Now the contrast-enhancement methods (e.g., phase contrast and DIC) may be adjusted if provision has been made for these techniques in the configuration of the microscope. For practical details of how to do this, see Salmon and Canman (1998), and for an excellent and concise explanation of the theory underlying these optical contrast-enhancing techniques, see Bradbury and Evennett (1996).

BASIC PROTOCOL 2

## ALIGNING THE FLUORESCENCE BULB AND SETTING UP KÖHLER ILLUMINATION FOR A WIDEFIELD FLUORESCENCE MICROSCOPE

When the entire field of view is seen at once, either with the eye or with a digital camera, the term "widefield" is used to make the distinction between this configuration and that of point illumination and point detection in confocal optical sectioning, in which a unitary detector such as a PMT or APD is used to acquire the image. It is necessary to set up a non-confocal fluorescence microscope for Köhler illumination, although this is often overlooked. To do so, refer to the ray path in Figure 6, and proceed as follows.

1. First determine what illumination source is provided on the microscope stand for fluorescence microscopy.

It will either be an older mercury bulb illuminator in a lamphouse, a metal-halide illuminator, or light-emitting diode (LED) illumination. The latter two are pre-centred, and the light is supplied to the microscope stand via a fiber-optic cable. These sources do not

Sanderson

20 of 30

require centring, in which case turn a (green) filter cube on axis and continue at step 8. The older lamphouse illuminator using a 50- or 100-watt mercury bulb will require centring, particularly after replacement of the bulb, which lasts 2-300 hr.

Some microscopes (e.g., from Olympus) have a special device for lamp alignment that screws into the objective nosepiece. It projects an image of the epi-illumination arc and electrodes onto a small diffusion screen within the barrel of the device. If this alignment tool is not used, select the lowest-magnification objective or, if this is  $>10\times$ , remove one objective, and rotate the nosepiece so that the open position is centred on the microscope axis.

- 2. Place a piece of reasonably stiff white paper on the stage of the microscope (a small filter paper clipped in place under the slide holder on the stage is ideal).
- 3. Turn a filter cube into position. If a green filter cube is available, use this, since the eye is most sensitive to green light.
- 4. Turn on the power source, ignite the lamp, and wait 5-10 min for it to achieve maximum brightness.
- 5. Close down the illuminated field diaphragm and fully open the condenser diaphragm (if fitted).

If the light is too bright, either insert a neutral-density filter or double the thickness of the white paper.

- 6. On the diffusion glass screen or on the white paper on the stage, the image of the arc between the tips of the two electrodes should be visible. Use the lamp focusing knob, acting on the collimator lens in the lamphouse, to obtain an in-focus image of the lamp arc and electrodes. The focus point will be sharpest at the minimum size of the image of the lamp arc and electrodes.
- 7. Use the lamp *x-y* adjustment controls to approximately centre the real image of the arc and electrode tips. There will also be a mirror at the rear of the lamphouse. Use the separate adjustment controls to focus and centre the mirror image of the arc and electrode tips. Further use the controls to bring the real image and mirror image of the arc and electrodes side by side, and arrange them so they are *just* overlapping. Arranged thus, the images from the arc lamp will evenly fill the field of view when defocused and spread out.
- 8. Use the condenser collimator control to spread the image of the arc and electrodes so that the photon flux is spread evenly in the back focal plane of the objective. Remove the paper and replace the objective.
- 9. Place a slide carrying a well-stained and evenly stained fluorescent section onto the stage.

A stained section of kidney or liver is ideal, because these tissues are relatively dense and therefore stain uniformly and evenly across their entire extent. A section of lung is not ideal.

10. Move the slide laterally in the x-y plane to check the evenness of the fluorescence illumination.

Look for apparent changes in intensity of the fluorescent stain in the image as the slide is moved: there should be none. Correct with the lamphouse controls; if necessary, repeat the lamp centring exercise from step 5.

11. Check the focus of the specimen and close down the field diaphragm until an edge comes into the field of view. Use the *x*-*y* adjustment screws for the field diaphragm to centre the field diaphragm image within the field of view.

12. Close down the illuminated field diaphragm until only the region of interest is illuminated.

This prevents photobleaching of areas outside the region of interest and reduces the amount of scattered fluorescent light in the image from outside the region of interest. Bear in mind that on a reflected-light-configured stand, the condenser diaphragm is located closer to the lamphouse and the illuminated field diaphragm closer to the objective (see Figs. 4 and 6).

13. If the condenser diaphragm is fitted (because the objective acts as its own condenser and contrast is generally high, it is not always present), remove an eyepiece and close the diaphragm very slightly to prevent non-image-forming glare from reducing contrast in the image.

This usually can be more than the 80% of the back focal plane suggested for Köhler illumination in brightfield microscopy.

When using an objective of a different magnification, or an oil-immersion objective, adjust the size and, if needs be, the centring of the illuminated field diaphragm (and adjust the condenser diaphragm if fitted).

To prevent photobleaching, be sure to close the epi-illumination shutter when not viewing by eye or acquiring images with the camera.

BASIC PROTOCOL 3

## GENERIC PROTOCOL FOR OPERATING A CONFOCAL MICROSCOPE

Just as different cars have the same generic controls, yet differ widely, the same is true for confocal microscopes built and offered for sale by different manufacturers. This protocol describes setting and adjusting the generic controls for a single beam-scanning laser confocal. Further details may be found in Hibbs (2004) and in Protocol 18.1 of Sanderson (2019).

- 1. First, plan your experiment and decide whether the confocal microscope is the most suitable instrument for acquiring the images you want. Refer to the references on experimental planning cited above, and to the section on choosing the best microscope platform (Fig. 8).
- 2. Use the microscope in non-laser, non-confocal (widefield) mode to focus on the sample and locate a suitable field of view for the cells or tissues of choice.

Ensure that any slide preparation is viewed with the coverslip (which should be no. 1/2 and 0.17 mm thick) facing towards the objective. Try to minimize the exposure of the sample to fluorescent light.

3. Switch the microscope into confocal mode via the software.

This will activate a shutter in front of the eyepieces so that no laser light can reach your eyes via the binocular tube head. It should not be necessary to switch back to visible mode unless the focal plane is altered and sight of the specimen is lost. If this occurs, switch back over and use a low-magnification objective to re-secure the field of view. Do not increase the detector gain in an attempt to locate the focal plane, as this will quite likely bleach the sample; return to visible mode to regain focus. Bleaching can also occur if a high zoom setting is used and the laser is projected into a very small area.

4. Select the appropriate laser lines and filter combinations into the PMT detectors for the fluorophores with which the sample preparation is stained.

Usually, each fluorophore is directed into a separate channel (or track), each with its own dedicated PMT, so that each can be operated independently and sequentially to minimize cross-excitation and bleedthrough. It is best to image the fluorophore with the longest-emission wavelength first.

Sanderson

22 of 30

- 5. Select one track (usually for the brightest or most abundant fluorophore). Set some gain on the PMT so that some level of signal is seen. Select the fast scan button or a raster speed of  $\sim$ 400 Hz, so that a signal can be seen in real time and can be focused while scanning.
- 6. Set the pinhole(s) value.

The pinhole(s) in front of the PMTs must be set to determine the correct optical section thickness. This is usually 1 Airy unit (1 AU), a normalized value that depends upon the numerical aperture of the objective and wavelength of the illumination. At 1 AU only the central intensity maximum of the PSF reaches the detector, which is the ideal. Because the NA is constant between tracks, and the laser wavelength changes, the pinhole may have to be greater or lesser than 1 AU to ensure that the thickness of the optical sections collected in all channels is equivalent. If one fluorophore is much weaker, or photobleaches more rapidly, it makes sense to assign the larger pinhole to this channel and adjust the pinholes in front of the other channels accordingly.

7. Select one active channel, and on fast scan, first set the black level and then set the signal gain to ensure that the signal fills the dynamic range of the PMT.

This will usually be 8 bit (256 gray levels), but 12 bit (4096 gray levels) can be used where the image must supply quantitative information (see Arena et al., 2017; Jonkman et al., 2014). There should be no oversaturation and a minimum of undersaturation. It is essential to use a false-colour look-up table to set these limits, as our eyes can discriminate no more than about 60 gray levels.

8. With all channels for each fluorophore set the to the correct PMT offset and gain levels, all channels can be activated and a multi-colour single image collected.

Normally frame-by-frame mode is used, but line-by-line acquisition is better for live samples that move, to minimize blur. Line-by-line scanning should also be used if you wish to see each fluorophore of a multi-colour experiment at once.

Image quality can be improved by optimizing the pixel size to the resolving power of the objective. This is called Nyquist sampling, and is explained below in the section on image acquisition. Essentially, at least two pixels must be collected per smallest resolvable distance. This determines an optimum zoom setting for each objective.

Image quality can also be improved by collecting a series of scans ("averaging") to improve the signal-to-noise ratio and/or by reducing the scan speed to increase the pixel dwell time on each sample point, which allows more emitted fluorescence signal to be collected. Take care, particularly when reducing the scan speed, not to bleach the sample unduly.

- 9. To collect a *z*-stack, open the appropriate dialog box, select one channel, and manually focus through the sample to set upper and lower limits for the *z*-stack. In most software, it is possible to perform a fast *x*-*z* scan to help set these limits.
- 10. Using the software macro button, set the Nyquist sampling limit for the z-stack.

Because resolving power axially in z is about three times poorer than that laterally in x-y, this step is crucial to collecting a high-quality z-stack.

When collecting a very large z-stack, you may need to alter laser power or PMT gain values to maintain signal intensity throughout the stack. The z-stack may be collapsed to provide a two-dimensional maximum intensity projection of in-focus information from throughout the stack for publication. However, most journals will also accept three-dimensional movie datasets as supplementary information.

It is also worth noting that if a non-confocal transmission detector is fitted to the microscope, it is possible to collect a phase-contrast or DIC morphological image upon which the confocal fluorescence image can be overlaid.

Further operational details and checklists are given in chapter 18 of Sanderson (2019).

### COMMENTARY

### **Background Information**

Confocal microscopy is a very stable technology: commercial turn-key systems have been available since 1986. Nevertheless, improvements and technological advances have been made. These are discussed in Elliott (2019) and include the ribbon-scanning confocal approach (Watson et al., 2017), white-light supercontinuum lasers (Chiu, Su, Reichelt, & Amos, 2012), and photomultiplier tubes with better quantum efficiency, such as GaAsP photon-counting PMTs. An improvement in resolution has been implemented by the re-scan confocal microscope (De Luca, Breedijk, Hoebe, Stallinga, & Manders, 2017; Gregor & Enderlein, 2019). This principle has been combined with deconvolution to give improved signal-to-noise ratios. The Airyscan technology (Zeiss; Barlow, Mostaço-Guidolin, Osei, Booth, & Hackett, 2020; Korobchevskaya, Lagerholm, Colin-York, & Fritzsche, 2017) combines the resolving power advantages of imaging with a small pinhole (0.2 AU) combined with the collection efficiency of a large pinhole (1.25 AU) projected onto a 32-element GaAsP detector shaped like an insect eye. No light is rejected by the closed pinhole, but is collected by the off-axis GaAsP detector elements.

### Summary of fundamental principles

1. A microscope is used to resolve fine detail that the eye cannot see unaided.

2. The most important characteristics are resolution, contrast, and magnification—in that order.

3. A microscope must provide (a) resolving power to carry fine detail in the specimen to the image, (b) contrast to show differences between image features and the background, and (c) magnification to present sufficient detail to the eye or detector.

4. An image is only a representation of the object: it suffers from diffraction and aberrations.

5. The higher the numerical aperture of an objective, the more fine detail is resolved in the image.

6. The shorter the focal length of a lens or objective, the greater the magnification.

7. We can see  ${\sim}0.2$  mm unaided, the LM resolution limit is 0.2  $\mu$  m, and the EM limit is 0.2 nm.

8. Light exists as both particles and waves. For very small objects, diffraction dominates in image formation. 9. Both diffraction and interference, manifestations of the same phenomenon, form an image.

10. All images can be regarded as being composed of a series of "points," rather as all materials are made of atoms. Due to diffraction, a point is imaged by the microscope as a slightly blurred disc. The larger the objective numerical aperture, the smaller the so-called Airy disc that is formed.

11. Numerical aperture  $= n \cdot \sin \alpha$ , where *n* is the refractive index of the medium between the slide and the objective front lens, and  $\alpha$  the angle, measured from the optical axis, of the most widely diffracted ray accepted by the objective.

12. Resolving power is related to numerical aperture and the wavelength ( $\lambda$ ) of illumination by  $d = 0.61\lambda/NA$ .

13. An objective must accept as many rays as possible from each point in an object, reassembling these rays from each point in the specimen at corresponding points in the image, in such a way that the distance travelled by all the rays from each object point to its corresponding image point is the same—so that they all arrive "in phase."

14. The objective must therefore be well corrected for aberrations, principally spherical and chromatic aberration.

15. Chromatic and spherical aberration is minimized by the manufacturer in the class of objective used: achromat; semi-apochromat, or "fluorite"; and apochromat (most highly corrected). Spherical aberration may result if attention is not paid to the correct coverslip thickness or if a medium of the wrong refractive index is used with the objective.

16. Köhler illumination permits the use of nonuniform light sources, providing separate adjustment of area of the field of view illuminated and the angle of illumination—filling the back focal plane with light for resolution of fine details in the image.

17. Fluorophores are self-luminous. They offer very high contrast, are versatile, and are available as conjugated dyes and also as endogenous fluorescent proteins.

18. The disadvantages of using fluorophores are threefold: bleaching, blurring, and bleedthrough.

19. Optical sectioning—principally confocal microscopy—can circumvent blurring to produce sharp images.

20. Use a widefield fluorescence microscope for thin sections or adherent cell

layers, a confocal for thicker sections, and a two-photon microscope for very thick tissues. Use lightsheet microscopy for sensitive samples.

### **Critical Parameters**

### Image acquisition

In most cases, the research microscope you use will be fitted with an appropriately designed digital camera or PMT/APD. These are usually very straightforward to operate through the software package that forms part of the microscope digital imaging system. Usually, the digital camera will be matched to the resolving power of the microscope system and its objectives (for details, see DeRose & Doppler, 2018; Hinsch, 2007; Joubert & Sharma, 2011) so that the smallest resolvable detail in the sample is recorded by a minimum of two pixels and preferably three to five pixels in the image. This is known as the Nyquist (or Nyquist-Shannon) sampling criterion (Pawley, 2006; see also the reference link included in the Internet Resources at the end of this paper).

When a continuous wave signal is collected from an analog system (the microscope) and presented as a digital output, it must be sampled from a continuous stream of data to form a discretely sampled dataset. The photon flux from the microscope field of view is divided into a raster of geometrically square Cartesian subunits called pixels (picture elements), and the light intensity that falls on each pixel is stored as a number. Each pixel is therefore a sampling point of the original signal.

In order to record the image adequately, it is necessary to know the resolving power of the microscope and to sample the image so that (1) no high-frequency fine detail is lost and (2) nor is spurious information encoded into the digital image by the sampling process (Hazelwood, Olenych, Griffin, Cathcart, & Davidson, 2007; Pawley, 2006). Both these events will arise through undersampling, which leads to aliasing. An aliased signal provides a poor representation of the analog signal. When aliasing occurs, the original analog signal cannot be correctly reconstructed by the digital dataset. Continuous signals of differing frequency become indistinguishable from one another (aliases of one another) when sampled. Given that undersampling causes loss of true information and addition of false information into the digital image, why not routinely oversample? Certainly, oversampling will reduce shot noise (so-called Poisson noise), but

it necessitates smaller pixels. If the pixels sampling the image are too small, the signal transferred will be dimmer, and the contrast of small features will be lost in the noise of the system. Also, oversampling will cause increased bleaching and is likely to lead to phototoxicity of the specimen. Those readers who wish to know more are directed to chapter 31 in Sanderson (2019) and chapter 4 in Pawley (2006).

### **Other practicalities**

This paper explains the theory of microscopy and describes protocols for setting up and adjusting research microscopes. To conclude, it is worth mentioning a few practical points. When focusing the microscope, looking sideways on, and bring the objective carefully towards the specimen by eye to within the point of focus. This way, when viewing down the eyepieces, the object is brought into focus by increasing the distance between the objective and specimen, thus preventing any contact between the two. Take particular care when using an immersion objective with a short working distance. Finally, dust is the enemy of microscopists. Try not to let dust fall onto the faceplate of a digital detector. Also, keep microscopes covered when not in use, and clean objectives carefully after use with recommended cleaning fluids. More information can be found in Salmon and Canman (1998) and in Appendix 4 of Sanderson (2019).

### Acknowledgments

My thanks are due to Gareth Clarke, MRC Harwell Institute, for his considerable help with the figures published in this paper.

### **Literature Cited**

- Abbe, E. (1873). Beiträge zur Theorie des Mikroskops und der mikroskopischen Wahrnehmung. Archiv für Mikroskopische Anatomie, 9, 413–468. doi: 10.1007/BF02956173.
- Anonymous. (2018). Editorial: Phototoxicity revisited. *Nature Methods*, 15(10), 751.
- Anonymous. (2013). Editorial: Artifacts of light. *Nature Methods*, 10(12), 1135.
- Anonymous. (2009). Editorial: Beyond the diffraction limit. *Nature Photonics*, *3*(7), 361.
- Bou About, G., Thiebault, E., Wattenhofer-Donzé, M., Jacobs, H., Guimond, A., Sorg, T., ... Herault, Y. (2019). Ultrasound-guided approaches to improve orthotopic mouse xenograft models for hepatocellular carcinoma. *Current Protocols* in Mouse Biology, 9, e62.
- Airy, G. B. (1835). On the diffraction of an objectglass with circular aperture. *Transactions of*

the Cambridge Philosophical Society, 5, 283–291.

- Arena, E. T., Rueden, C. T., Hiner, M. C., Wang, S., Yuan, M., & Eliceiri, K. W. (2017). Quantitating the cell: Turning images into numbers with ImageJ. *Wiley Interdisciplinary Reviews: Developmental Biology*, 6(2), e260. doi: 10. 1002/wdev.260.
- Barlow, A. M., Mostaço-Guidolin, L. B., Osei, E. T., Booth, S., & Hackett, T. L. (2020). Super resolution measurement of collagen fibers in biological samples: Validation of a commercial solution for multiphoton microscopy. *PLoS One*, *15*(2), e0229278. doi: 10.1371/journal. pone.0229278.
- Benham, G. S. (2002). Practical aspects of objective lens selection for confocal and multiphoton digital imaging techniques. *Methods in Cell Biology*, 70, 245–299.
- Besseling, T. H., Jose, J., & Blaaderen, A. V. (2015). Methods to calibrate and scale axial distances in confocal microscopy as a function of refractive index. *Journal of Microscopy*, 257(2), 142–150.
- Biggs, D. S. C. (2010). 3D deconvolution microscopy. *Current Protocols in Cytometry*, 52, 12.19.1–12.19.20. doi: 10.1002/0471142956. cy1219s52.
- Bogdanov, A. M., Kudryavtseva, E. I., & Lukyanov, K. A. (2012). Anti-fading media for live cell GFP imaging. *Plos One*, 7(12), e53004. doi: 10.1371/journal.pone.0053004.
- Boutet de Monvel, J., Le Calvez, S., & Ulfendahl, M. (2001). Image restoration for confocal microscopy: Improving the limits of deconvolution, with application to the visualization of the mammalian hearing organ. *Biophysical Journal*, 80(5), 2455–2470. doi: 10.1016/ S0006-3495(01)76214-5.
- Bradbury, S. (1984). An introduction to the optical microscope. Oxford: OUP.
- Bradbury, S., & Evennett, P. J. (1996). Contrast techniques in light microscopy (RMS Handbook No. 34, Garland Science). Boca Raton, FL: CRC Press.
- Callamaras, N., & Parker, I. (1999). Construction of a confocal microscope for real-time *x-y* and *x-z* imaging. *Cell Calcium*, 26(6), 271–279. doi: 10.1054/ceca.1999.0085.
- Castellano-Muñoz, M., Peng, A. W., Salles, F. T., & Ricci, A. J. (2012). Swept field laser confocal microscopy for enhanced spatial and temporal resolution in live-cell imaging. *Microscopy and Microanalysis*, 18(4), 753–760. doi: 10.1017/ S1431927612000542.
- Chatterjee, K., Pratiwi, F. W., Wu, F. C. M., Chen, P., & Chen, B. C. (2018). Recent progress in light sheet microscopy for biological applications. *Applied Spectroscopy*, 72(8), 1137–1169. doi: 10.1177/0003702818778851.
- Chescoe, D., & Goodhew, P. J. (1990). The operation of transmission and scanning electron microscopes. Oxford: Oxford Science Publications.

- Chessel, A. (2017). An overview of data science uses in bioimage informatics. *Methods*, 115, 110–118. doi: 10.1016/j.ymeth.2016.12.014.
- Chiu, L. D., Su, L., Reichelt, S., & Amos, W. B. (2012). Use of a white light supercontinuum laser for confocal interference-reflection microscopy. *Journal of Microscopy*, 246(2), 153–159. doi: 10.1111/j.1365-2818.2012.0360 3.x.
- Cole, R. (2014). Live-cell imaging: The cell's perspective. *Cell Adhesion and Migration*, 8(5), 452–459.
- Cole, R. W., Jinadasa, T., & Brown, C. M. (2011). Measuring and interpreting point spread functions to determine confocal microscope resolution and ensure quality control. *Nature Protocols*, 6(12), 1929–1941. doi: 10.1038/nprot. 2011.407.
- Collins, T. J. (2006). Mounting media and antifade reagents. *Microscopy Today*, 14, 34–39. doi: 10. 1017/S1551929500055176.
- Combs, C. A., & Shroff, H. (2017). Fluorescence microscopy: A concise guide to current imaging methods. *Current Protocols in Neuroscience*, 79, 2.1.1–2.1.25. doi: 10.1002/cpns.29.
- Conchello, J. A., & Litchman, J. W. (2005). Optical sectioning microscopy. *Nature Methods*, 2(12), 920–931. doi: 10.1038/nmeth815.
- Constantinesco, A., Choquet, P., Goetz, C., & Monassier, L. (2012). PET, SPECT, CT, and MRI in mouse cardiac phentotyping: An overview. *Current Protocols in Mouse Biology*, 2, 129–144. doi: 10.1002/9780470942390. mo110225.
- Dean, P. N. (1998). Confocal microscopy: Principles and practices. *Current Protocols in Cytometry*, 5, 2.8.1–2.8.12. doi: 10.1002/0471142956. cy0208s05.
- De Luca, G., Breedijk, R., Hoebe, R., Stallinga, S., & Manders, E. (2017). Re-scan confocal microscopy (RCM) improves the resolution of confocal microscopy and increases the sensitivity. *Methods and Applications in Fluorescence*, 5(1), 015002. doi: 10.1088/2050-6120/5/ 1/015002.
- DeRose, J. A., & Doppler, M. (2018). Guidelines for understanding magnification in the modern digital microscope era. *Microscopy Today*, 26, 20–31. doi: 10.1017/S1551929518000688.
- Elliott, A. D. (2019). Confocal microscopy: Principles and modern practices. *Current Protocols in Cytometry*, 92, e68.
- Elisa, Z., Toon, B., De Smedt, S. C., Katrien, R., Kristiaan, N., & Kevin, B. (2017). Technical implementations of light sheet microscopy. *Microscopy Research and Technique*, 81(9), 941– 958. doi: 10.1002/jemt.22981.
- Evennett, P. (1983). Köhler Illumination: A simple interpretation. *Proceedings Royal Microscopical Society*, 28(4), 189–192. Retrieved from http://www.microscopist.co.uk/ essential-techniques/kohler-illumination/.

- Evennett, P. J. (1996). The Ernst Abbe lecture: Ernst Abbe and the development of the modern microscope. *Proceedings of the Royal Microscopical Society*, *31*(4), 283–292.
- Florijn, R. J., Slats, J., Tanke, H. J., & Raap, A. K. (1993). Analysis of antifading reagents for fluorescence microscopy. *Cytometry*, 19(2), 177– 182. doi: 10.1002/cyto.990190213.
- Fouquet, C., Gilles, J. F., Heck, N., Dos Santos, M., Schwartzmann, R., Cannaya, V., ... Bolte, S. (2015). Improving axial resolution in confocal microscopy with new high refractive index mounting media. *PLoS One*, 10(3), e0121096. doi: 10.1371/journal.pone.0121096.
- Franklin, K., Muir, P., Scott, T., Wilcocks, L., & Yates, P. (2010). *Introduction to biological physics for the health and life sciences*. Chichester: John Wiley & Sons.
- Geyer, S. H., Mohun, T. J., & Weninger, W. J. (2009). Visualizing vertebrate embryos with episcopic 3D imaging techniques. *Scientific World Journal*, *9*, 1423–1437. doi: 10.1100/tsw. 2009.154.
- Gregor, I., & Enderlein, J. (2019). Image scanning microscopy. *Current Opinion in Chemical Biology*, 51, 74–83. doi: 10.1016/j.cbpa.2019.05. 011.
- Hammond, C. (2009). *The basics of crystallography and diffraction* (3rd. ed.). Oxford: OUP.
- Hazelwood, K. L., Olenych, S. G., Griffin, J. D., Cathcart, J. A., & Davidson, M. W. (2007). Entering the portal: Understanding the digital image recorded through a microscope. In S. L. Shorte & F. Frischknecht (Eds.), *Imaging cellular and molecular biological functions*, 3–43. New York, NY: Springer.
- Hibbs, A. R. (2004). *Confocal microscopy for biologists*. New York: Kluwer Academic/Plenum Publishers.
- Helmchen, F., & Denk, W. (2005). Deep tissue two-photon microscopy. *Nature Methods*, 2(12), 932–940. doi: 10.1038/nmeth818.
- Hendriks, B. H. W., Bierhoff, W. C. J., Horikx, J. J. L., Desjardins, A. E., Hezemans, C. A., 't Hooft, G. W., ... Mihajlovic, N. (2011). High-resolution resonant and nonresonant fiber-scanning confocal microscope. *Journal* of Biomedical Optics, 16(2), 026007. doi: 10.1117/1.3534781.
- Hinsch, J. (2007). Mating cameras to microscopes. *Methods in Cell Biology*, 81, 55–61. doi: 10. 1016/S0091-679X(06)81004-6.
- Houston, R. A. (1921). Young's interference experiment. *Nature*, *107*(2687), 268. doi: 10.1038/107268c0.
- Hsu, C. W., Kalaga, S., Akoma, U., Rasmussen, T. L., Christiansen, A. E., & Dickinson, M. E. (2019). High resolution imaging of mouse embryos and neonates with X-ray micro-computed tomography. *Current Protocols in Mouse Biol*ogy, 9, e63. doi: 10.1002/cpmo.63.
- Icha, J., Weber, M., Waters, J. C., & Norden, C. (2017). Phototoxicity in live fluorescence mi-

croscopy, and how to avoid it. *Bioessays*, *39*, 1700003. doi: 10.1002/bies.201700003.

- Ikoma, H., Broxton, M., Kudo, T., & Wetzstein, G. (2018). A convex 3D deconvolution algorithm for low photon count fluorescence imaging. *Scientific Reports*, 8(1), 11489. doi: 10. 1038/s41598-018-29768-x.
- Ishikawa-Ankerhold, H. C., Ankerhold, R., & Drummen, G. P. C. (2012). Advanced fluorescence microscopy techniques—FRAP, FLIP, FLAP, FRET and FLIM. *Molecules*, 17(4), 4047–4132. doi: 10.3390/molecules17044047.
- Jonkman, J., Brown, C. M., & Cole, R. W. (2014). Quantitative confocal microscopy: Beyond a pretty picture. *Methods in Cell Biology*, 123, 113–134.
- Jonkman, J., Brown, C. M., Wright, D. M., Anderson, K. I. & North, A. J. (2020). Tutorial: a guidance for quantitative confocal microscopy. *Nature Protocols*, 15, 1585–1611. doi: 10.1038/ s41596-020-0313-9.
- Jost, A. P. T., & Waters, J. C. (2019). Designing a rigorous microscopy experiment: Validating methods and avoiding bias. *Journal of Cell Biology*, 218(5), 1452–1466. doi: 10.1083/jcb. 201812109.
- Joubert, J., & Sharma, D. (2011). Light microscopy digital imaging. *Current Protocols in Cytometry*, 58, 2.3.1–2.3.11. doi: 10.1002/0471142956. cy0203s58.
- Köhler, A. (1893). Ein neues Beleuchtungsverfahren für mikrophotographische Zwecke. Zeitschrift für wissenschaftliche Mikroskopie und für mikroskopische Technik, 10(4), 433– 440.
- Köhler, H. (1981). On Abbe's theory of image formation in the microscope. *Optica Acta*, 28(12), 1691–1701. doi: 10.1080/713820514.
- Korobchevskaya, K., Lagerholm, B., Colin-York, H., & Fritzsche, M. (2017). Exploring the potential of Airyscan microscopy for live cell imaging. *Photonics*, 4(3), 41. doi: 10.3390/ photonics4030041.
- Laasmaa, M., Vendelin, M., & Peterson, P. (2011). Application of regularized Richardson-Lucy algorithm for deconvolution of confocal microscopy images. *Journal of Microscopy*, 243(2), 124–140. doi: 10.1111/j.1365-2818.2011.03486.x.
- Lambert, T. J., & Waters, J. C. (2017). Navigating challenges in the application of superresolution microscopy. *Journal of Cell Biology*, 216(1), 53–63. doi: 10.1083/jcb.201610011.
- Langhorst, M. F., Schaffer, J., & Goetze, B. (2009). Structure brings clarity: Structured illumination microscopy in cell biology. *Biotechnol*ogy Journal, 4(6), 858–865. doi: 10.1002/biot. 200900025.
- Lee, J. S., Wee, T. L., & Brown, C. M. (2014). Calibration of wide-field deconvolution microscopy for quantitative fluorescence imaging. *Journal* of *Biomolecular Techniques: JBT*, 25(1), 31–40. doi: 10.7171/jbt.14-2501-002.

- Lee, J. Y., & Kitaoka, M. (2018). A beginner's guide to rigor and reproducibility in fluorescence imaging experiments. *Molecular Biology* of the Cell, 29(13), 1519–1525. doi: 10.1091/ mbc.E17-05-0276.
- Leybaert, L., De Meyer, A., Mabilde, C., & Sanderson, M. J. (2005). A simple and practical method to acquire geometrically correct images with resonant scanning-based line scanning in a custom-built video-rate laser scanning microscope. *Journal of Microscopy*, 219(3), 133–140. doi: 10.1111/j.1365-2818.2005.01502.x.
- Lichtman, J. W., & Conchello, J. A. (2005). Fluorescence microscopy. *Nature Methods*, 2(12), 910–919. doi: 10.1038/nmeth817.
- Liu, A., Xiao, W., Li, R., Liu, L., & Chen, L. (2019). Comparison of optical projection tomography and light-sheet fluorescence microscopy. *Journal of Microscopy*, 275(1), 3–10. doi: 10.1111/ jmi.12796.
- LoBiondo, J., Abramowitz, M., & Friedman, M. M. (2011). Microscope objectives. *Current Pro*tocols in Cytometry, 58, 2.2.1–2.2.15. doi: 10. 1002/0471142956.cy0202s58.
- Longin, A., Souchier, C., Ffrench, M., & Bryon, P. A. (1993). Comparison of anti-fading agents used in fluorescence microscopy: Image analysis and laser confocal microscopy study. *Journal of Histochemistry & Cytochemistry*, 41(12), 1833–1840.
- Lu, G. J., Chou, L. D., Malounda, D., Patel, A. K., Welsbie, D. S., Chao, D. L., ... Shapiro, M. G. (2020). Genetically encodable contrast agents for optical coherence tomography. ACS Nano, Feb 10. doi: 10.1021/acsnano.9b08432.
- McNally, J. G., Karpova, T., Cooper, J., & Conchello, J. A. (1999). Three-dimensional imaging by deconvolution Microscopy. *Methods*, *19*(3), 373–385. doi: 10.1006/meth.1999. 0873.
- Meijering, E., Carpenter, A. E., Peng, H., Hamprecht, F. A., & Olivo-Marin, J. C. (2016). Imagining the future of bioimage analysis. *Nature Biotechnology*, 34/12, 1250–1255. doi: 10. 1038/nbt.3722.
- Murphy, D. B., & Davidson, M. W. (2013). Fundamentals of light microscopy and electronic imaging (2nd ed.). Oxford and New Jersey: Wiley-Blackwell.
- Murray, J. M. (2011). Methods for imaging thick specimens: Confocal microscopy, deconvolution, and structured illumination. *Cold Spring Harbor Protocols*, 2011, pdb.top066936. doi: 10.1101/pdb.top066936.
- Norris, F. C., Wong, M. D., Greene, N. D. E., Scambler, P. J., Weaver, T., Weninger, W. J., ... Lythgoe, M. F. (2013). A coming of age: Advanced imaging technologies for characterising the developing mouse. *Trends in Genetics*, 29(12), 700–711. doi: 10.1016/j.tig.2013.08.004.
- Oreopoulos, J., Berman, R., & Browne, M. (2014). Spinning-disk confocal microscopy: Present technology and future trends. Quantita-

tive imaging in cell biology. *Methods in Cell Biology*, *123*, 153–175.

- Ortell, K. K., Switonski, P. M., & Delaney, J. R. (2019). FairSubset: A tool to choose representative subsets of data for use with replicates or groups of different sample sizes. *Journal of Biological Methods*, 6(3), e118. doi: 10.14440/jbm. 2019.299.
- Parton, R. M., & Davis, I. (2006). Lifting the fog: Image restoration by deconvolution. In J. L. Celis (Ed.), *Cell biology: A laboratory handbook* (3rd ed., Vol. 3, pp. 187–200). San Diego: Academic Press.
- Pawley, J. B. (2006). Points, pixels and gray levels: Digitizing image data, chapter 4. In J. B. Pawley (Ed.), *Handbook of biology confocal microscopy* (3rd ed., pp. 59–79). New York, NY: Springer.
- Phoon, C. K. L., & Turnbull, D. H. (2016). Cardiovascular imaging in mice. *Current Protocols in Mouse Biology*, 6, 15–13. doi: 10.1002/ 9780470942390.mo150122.
- Piazza, L., Lummen, T. T. A., Quiñonez, E., Murooka, Y., Reed, B. W., Barwick, B., & Carbone, F. (2015). Simultaneous observation of the quantization and the interference pattern of a plasmonic near-field. *Nature Communications*, *6*, 6407. doi: 10.1038/ncomms7407.
- Piston, D. W. (1998). Choosing objective lenses: The importance of numerical aperture and magnification in digital optical microscopy. *The Biological Bulletin*, 195(1), 1–4. doi: 10.2307/ 1542768.
- Powell, K. A., & Wilson, D. (2011). 3-dimensional imaging modalities for phenotyping genetically engineered mice. *Veterinary Pathology*, 49, 106–115. doi: 10.1177/03009858114298 14.
- Rayleigh, L. (1896). XV. On the theory of optical images, with special reference to the microscope. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*, Series 5, 42, 167–195. doi: 10.1080/ 14786449608620902.
- Sage, D., Donati, L., Soulez, F., Fortun, D., Schmit, G., Seitz, A., ... Unser, M. (2017). DeconvolutionLab2: An open-source software for deconvolution microscopy. *Methods*, 115, 28–41. and http://bigwww.epfl.ch/deconvolution/. doi: 10.1016/j.ymeth.2016.12.015.
- Salmon, E. D., & Canman, J. C. (1998). Proper alignment and adjustment of the light microscope. *Current Protocols in Cell Biology*, 00, 4.1.1–4.1.26. doi: 10.1002/0471143030. cb0401s00.
- Sanderson, J. B. (2019). Understanding light microscopy. Chichester: John Wiley & Sons.
- Schermelleh, L., Ferrand, A., Huser, T., Eggeling, C., Sauer, M., Biehlmaier, O., & Drummen, G. P. C. (2019). Super-resolution microscopy demystified. *Nature Cell Biology*, 21(1), 72–84. doi: 10.1038/s41556-018-025 1-8.

- Spencer, M. (1982). Fundamentals of light microscopy (IUPAB Biophysics Series). Cambridge: CUP.
- Stelzer, E. H. K. (1998). Contrast, resolution, pixelation, dynamic range, and signal-to-noise ratio. *Journal of Microscopy*, 189(1), 15–24. doi: 10.1046/j.1365-2818.1998.00290.x.
- Suetens, P. (2009). Fundamentals of medical imaging (2nd ed.). Cambridge: CUP.
- Swedlow, J. R., Hu, K., Andrews, P. D., Roos, D. S., & Murray, J. M. (2002). Measuring tubulin content in Toxoplasma gondii: A comparison of laser-scanning confocal and wide-field fluorescence microscopy. *Proceedings of the National Academy of Sciences*, 99(4), 2014–2019. doi: 10.1073/pnas.022554999.
- Tejedo, M. I. A., Cervantes, J. C. M. N., Roldán, A. S. J., Rodriguez, M., Vega, A. G., & Piazza, V. (2019). 3,3'-thiodipropanol as a versatile refractive index-matching mounting medium for fluorescence microscopy. *Biomedical Optics Express*, 10(3), 1136–1150. doi: 10.1364/BOE.10. 001136.
- Theer, P., Mongis, C., & Knop, M. (2014). PSFj: Know your fluorescence microscope. *Nature Methods*, *11*(10), 981–982. doi: 10.1038/nmeth. 3102.
- Thomas, J. I. (2019). The classical double slit interference experiment: A new geometrical approach. *American Journal of Optics and Photonics*, 7(1), 1–9. doi: 10.11648/j.ajop. 20190701.11.
- Tinevez, J. Y., Dragavon, J., Baba-Aissa, L., Roux, P., Perret, E., Canivet, A., ... Shorte, S. (2012). A quantitative method for measuring phototoxicity of a live cell imaging microscope. *Methods in Enzymology*, 506, 291–309. doi: 10.1016/ B978-0-12-391856-7.00039-1.
- Tsien, R. (2003). Imagining imaging's future. *Nature Reviews Molecular Cell Biology*, 4, SS16– SS21. Retrieved from https://www.nature. com/focus/cellbioimaging/content/full/ nrm1196.html.
- Turkowyd, B., Virant, D., & Endesfelder, U. (2016). From single molecules to life: Microscopy at the nanoscale. *Analytical and Bioanalytical Chemistry*, 408(25), 6885–6911. doi: 10.1007/ s00216-016-9781-8.
- Ustione, A., & Piston, D. W. (2011). A simple introduction to multiphoton microscopy. *Journal* of Microscopy, 243(3), 221–226. doi: 10.1111/ j.1365-2818.2011.03532.x.
- Visser, T. D., & Oud, J. L. (1992). Volume measurements in three-dimensional microscopy. *Scanning*, *16*, 198–200. doi: 10.1002/ sca.4950160403.
- Waters, J. C., & Wittmann, T. (eds.) (2014). Quantitative imaging in cell biology. Methods in Cell Biology (Vol. 123). San Diego, CA: Academic Press.
- Watson, A. M., Rose, A. H., Gibson, G. A., Gardner, C. L., Sun, C., Reed, D. S., ... Watkins, S. C. (2017). Ribbon scanning confocal for high-speed high-resolution volume imaging of

brain. *Plos One*, *12*(7), e0180486. doi: 10.1371/ journal.pone.0180486.

- Webb, D. J., & Brown, C. M. (2013). Epifluorescence microscopy. *Methods in Molecular Biology*, *931*, 29–59. doi: 10.1007/ 978-1-62703-056-4\_2.
- Weissleder, R., & Nahrendorf, M. (2015). Advancing biomedical imaging. *Proceedings of the National Academy of Sciences*, 112(47), 14424– 14428. doi: 10.1073/pnas.1508524112.
- Wilson, T. (2010). Spinning-disk microscopy systems. *Cold Spring Harbor Protocols*, 2010(11), pdb.top88. doi: 10.1101/pdb.top88.
- Young, T. (1802). The Bakerian lecture: On the theory of light and colours. *Philosophical Transactions of the Royal Society*, 92, 12–48.
- Young, T. (1804). I. The Bakerian lecture. Experiments and calculations relative to physical optics. *Philosophical Transactions of the Royal Society*, 94, 1–16.
- Zaritsky, A. (2018). Sharing and reusing cell image data. *Molecular Biology of the Cell*, 29(11), 1274–1280. doi: 10.1091/mbc.E17-10-0606.
- Zhou, W., Apkarian, R., Wang, Z. L., & Joy, D. (2006). Fundamentals of scanning electron microscopy (SEM). In W. Zhou & Z. L. Wang (Eds.), *Scanning microscopy for nanotechnology*. New York, NY: Springer.

### **Key References**

Evennett, P. (1983). See above.

- This excellent description of Köhler's method for aligning the microscope gives a concise explanation of how and why this procedure is central to good microscopy and why this illumination method is still used today. The author is a superb didactic teacher who has taught many professional microscopists. Unless Köhler's illumination alignment method is used, it is not possible to set up a widefield light microscope properly. Much time is then wasted collecting poor-quality images.
- Litchman, J. W., & Conchello, J.-A. (2005). Fluorescence microscopy. *Nature Methods*, 2(12), 910–919.
- This one paper gives all you need to know as an introduction to fluorescence. It was published back to back with an excellent paper (pages 920–931) by the same authors on optical sectioning.
- Amos, W. B., & White, J. G. (2003). How the confocal laser scanning microscope entered biological research. *Biology of the Cell*, 95, 335–342. doi: 10.1016/S0248-4900(03)00078-9.
- Almost every research institution worldwide has a single beam-scanning confocal microscope. This paper is a fascinating first-hand account of how this practical instrument was developed.
- Cranfill, P. J., Sell, B. R., Baird, M. A., Allen, J. R., Lavagnino, Z., Martijn de Gruiter, H., ... Piston, D. W. (2017). Quantitative assessment of fluorescent proteins. *Nature Methods*, 13(7), 557– 562. doi: 10.1038/nmeth.3891.
- A comprehensive review of fluorescent proteins, helpful in selecting the optimum fluorescent

protein for your experiment, written by a team with much experience in this field.

- Vermot, J. (2008). Fast fluorescence microscopy for imaging the dynamics of embryonic development. *HFSP Journal*, 2(3), 143–155. doi: 10. 2976/1.2907579.
- An excellent introduction to the principles of fast-frame fluorescence recording and live-cell imaging, where temporal resolution (speed of acquisition), spatial resolution, and the signalto-noise ratio (otherwise known as the "iron triangle") all have to be balanced.

#### **Internet Resources**

https://svi.nl/DoingDeconvolution

http://cismm.web.unc.edu/software/

http://bigwww.epfl.ch/deconvolution/

http://www.deconvolve.net/

https://sites.google.com/site/ptadrous/

Several discussions of deconvolution.

http://www.microscopist.co.uk/ essential-techniques/kohler-illumination/

Explanation of Köhler illumination.

https://svi.nl/NyquistRate

Explanation of Nyquist sampling.

http://www.microscopist.co.uk/wp-content/ uploads/2017/04/Points-Pixels-Gray-Levels. pdf or http://bit.ly/3bTOoZz

- Another explanation of Nyquist sampling, from chapter 4 of Pawley (2006).
- https://www.nature.com/milestones/milephotons/ timeline.html

Nature Milestones: Photons (2010).

https://www.nature.com/milestones/milelight/ timeline.html

Nature Milestone: Light Microscopy (2018).

- https://opentextbc.ca/physicstestbook2/chapter/ youngs-double-slit-experiment/ or http://bit. ly/2SLwgth
- Description of Young's two-slit interference experiment.

https://www.ibiology.org/online-biology-courses/ microscopy-series/

General microscopy videos.

#### Theory of microscope image formation

- http://www.youtube.com/watch?v=fAuo7NIS97U or https://goo.gl/5iHJVf
- http://www.youtube.com/watch?v=rPAJ2Vs9A\_I or https://goo.gl/KtwMz6

http://www.youtube.com/watch?v=CbPoi9bie1I or https://goo.gl/A55RFH

- http://www.youtube.com/watch?v= wZ369WKiJdQ or https://goo.gl/hLWoJo
- A series of four YouTube videos demonstrating the theory of microscope image formation.

Sanderson

30 of 30