**Title**
Digital Microscopy



**ISBN**
978-0-470-51059-9

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**Publication Date**
2014-03-31

Peer reviewed

# DIGITAL MICROSCOPY

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**Keywords:** Microscopy, Confocal Microscopy, Fluorescence Microscopy, Automated Imaging

**Abstract:** Light microscopes can visualize structures down to 200 nm (or sometimes less) and are therefore the tool of choice to visualize cells, the basic unit of life. No longer do researchers peer through the microscope's eyepieces and document their findings through drawings or on film, rather, digital image acquisition and motorized control of the microscope play a key role.

Modern light microscopes capture data on digital detectors and are often fully automated, providing computer control of fluorescence excitation and detection and sample positioning. This makes it possible to automatically acquire multi-color three-dimensional time-lapse movies. Moreover, accurate, sensitive detectors enable quantification of microscope images and are a key component of various strategies to improve the spatial and temporal resolution of the microscope. Here, we review modern biological light microscopes, discuss their components, and describe how these tools are utilized experimentally.

## INTRODUCTION TO MODERN BIOLOGICAL LIGHT MICROSCOPY

The light microscope has been a powerful tool in biology for well over a century. Indeed, some of the first samples imaged with a microscope were biological: bacteria by van Leeuwenhoek and cork cells by Hooke. The capabilities of the light microscope have advanced considerably since this early work (see http://www.nature.com/milestones/milelight/timeline.html), and today light microscopy plays a critical role in virtually all aspects of the life sciences. For instance, patient biopsies are routinely examined using transmitted light microscopes, either by direct observation or by automated analysis of digital images obtained from a (sometimes robotic) microscope (digital pathology, (1)). Virtually all laboratories culturing cells or micro-organisms monitor their health and growth using light microscopes. Many cell biology laboratories routinely use fluorescence microscopes to image fixed or live cells, and many technological improvements of the last three decades have been geared towards improving the lateral (left/right) and axial (depth) resolution, lowering the amount of illuminating light needed for imaging, and developing better probes for such applications (many of these aspects will be discussed below). In biophysics and biochemistry, observation of reactions by microscopy has become common place, enabling reaction conditions much more closely mimicking the in vivo situation. Examples are the observation of the action of individual molecular motor proteins, such as myosin and RNA polymerase through optical microscopes, using methods that allow for nm resolution localization, sometimes while using the microscope to apply force to the motor in an optical trap (2), as well as the increasing use of microfluidic devices, enabling miniaturization of reactions, necessitating readouts with high spatial resolution such as microscopes (3).

A schematic of the major components of a modern research microscope is shown in Figure 1, and examples of modern research microscopes are shown in Figure 2. Light from the sample is collected and collimated by an objective lens and then focused onto a camera or detector by a

tube lens. The objective is typically movable to adjust the focal plane in the sample, although the sample can be movable as well. Most microscopes have both a transmitted light illumination path and an epi-fluorescence illumination path. The transmitted light path allows imaging of the sample by light transmitted through it where contrast is generated by absorption or scattering of light by the sample. The epi-fluorescence illumination path (’epi-’ indicates that the illumination and detection paths are the same) illuminates the sample with light chosen to excite fluorescence from fluorescent dyes in the sample. Fluorescence emission from these dye molecules can then be collected and imaged, giving information about the distribution of the dye molecules within the sample. Additional components of the microscope include a movable sample holder to allow imaging of different positions within the sample and a movable prism or mirror to direct the collected light to multiple detection paths, which typically include eyepieces in addition to the camera. Microscopes can also have multiple excitation paths for integration of additional microscopy modalities, such as confocal microscopes. A good general introduction to biological light microscopy is (4).

Samples for biological light microscopy are typically prepared on coverslips, 0.17 mm thick glass plates. Objectives for biological light microscopy are chosen to produce the best images from samples in contact with a coverslip. Live cells can be grown directly on the coverslip for imaging and petri dishes with coverslip bottoms can be purchased to simplify this. Other samples may be placed on microscope slides and then covered with a coverslip. Typically, the sample and the surrounding space is filled with a mounting medium designed to match the refractive index of the glass, stabilize the sample, and hold the sample and coverslip in place against the slide.

**The resolution limit of the light microscope**

Regardless of the imaging method - transmitted light or fluorescence, as well as more complicated methods - the resolving power of the microscope is fundamentally limited by

properties of the microscope objective. This resolution limit can be defined in a number of ways. Ernst Abbe, in 1873, defined the resolution limit of the microscope objective as the most finely spaced periodic grating that could be resolved with that objective using transmitted light illumination. Because the periodic grating diffracts the light incident on it, and collecting two diffracted beams is necessary to form an image of the sample, it is relatively easy to show that the resolution limit of the objective is determined by the largest angle it can collect. More formally, the smallest resolvable grating spacing is given by $d = \lambda / n \sin \theta$, where $\lambda$ is the illumination wavelength, n is the lowest refractive index between the objective and the sample, and $\theta$ is the largest angle that objective can collect. $n \sin \theta$ is defined as the Numerical Aperture or NA of the objective and is one of the critical parameters defining the performance of an objective lens. Modern objective lenses can collect very large angular ranges; for a high performance lens, it can be as large as 72 degrees, corresponding to an NA of 0.95 when working in air.

An alternative definition of resolution, by Rayleigh, considers how a point source of light is blurred by the microscope objective. Because the objective has a finite aperture, a point source of light will be blurred into an Airy disk. This blurred image is known as the point-spread-function of the objective, and the width of the Airy disk is given by $1.22 \lambda / NA$. The minimum distance that can be resolved is half that, $0.61 \lambda / NA$. For more information about the resolving power of the light microscope, see (4–6).

Because of the dependence of NA on refractive index, the highest NA objective lenses use fluid immersion. In a fluid immersion lens, the lens is designed to work with a layer of fluid between the objective and the sample. Most commonly, the fluid is an oil chosen to have the same refractive index as the glass that makes up the top lens of the objective and the coverslip on the sample. In this case, when oil is applied between the objective and the sample, the coverslip, oil, and objective top lens all function as a single optical element with no refraction occurring

between them. Since this oil has a refractive index of ~1.5, the use of an oil immersion lens results in a ~1.5-fold improvement in resolution. For a modern oil objective with 1.4 NA, the resolution limit is about 220 nm for green light (λ = 500 nm).

**Contrast in biological microscopy**

Even with the highest resolving power attainable, an object will only be visible if it differs from the background, i.e. if it has contrast. In biology, most objects are quite thin and have a refractive index close to that of water, causing them to be virtually transparent. Early breakthroughs in biological imaging resulted from the development and use of stains that provided contrast to objects of interest, such as the silver stain developed by Golgi, and used by Cajal and others, that selectively label sets of neurons. Stains are still widely used, and most histology and pathology studies will employ well-established tissue staining procedures to generate contrast.

Great effort has gone into the development of contrasting techniques that are compatible with live cells, employing physical principles other than the absorption of light. For instance, phase contrast microscopy, developed by Fritz Zernike in the 1930s and 1940s, uses the difference in refractive index between living material and its surroundings and changes this into a difference in amplitude, visible by eye or on a camera. Likewise, techniques such as Differential Interference Contrast (DIC), Hoffman modulation contrast, polarization microscopy, as well as many less widely used optical contrasting techniques, all seek to modify the image such that a physical property (such as refractive index, or birefringence in the case of polarization microscopy) is converted in an amplitude difference. We will not discuss these technologies in detail, largely since most developments in microscopy in the last two decades have made use of another contrasting technique: fluorescence.

**Fluorescence microscopy**

Modern biological light microscopy is dominated by the use of fluorescence microscopy. Introduction of fluorescent labels into the sample is an important contrast generation mechanism. Because most biological samples are transparent, and have relatively small refractive index differences, transmitted light imaging produces relatively little contrast. Some subcellular structures can be resolved by transmitted light microscopy, particularly if techniques such as phase contrast or differential interference contrast (4) are used. These techniques allow imaging refractive index differences in the sample, producing intensity contrast from otherwise transparent samples. However, these techniques only allow imaging of naturally occurring refractive index variation. Much of modern biology is concerned with understanding molecular mechanism, and these transmitted light microscopy tools do not provide a means of imaging specific molecules.

Imaging of specific molecules such as proteins, in a biological sample, is typically done by targeting fluorescent dyes to those molecules. This can be accomplished by generating antibodies that recognize the molecule of interest, and then labeling these antibodies with fluorescent dyes. When incubated with the sample, the antibodies will bind to the protein of interest. Washing away unbound antibody results in specific labeling of the molecule of interest. This process is called immunofluorescence and is widely used in biology. However, immunofluorescence requires that the antibodies be able to access the molecules in the sample being studied. Because cells are not permeable to antibodies, this approach cannot be used to label molecules in living cells. However, in many cases, imaging cells that have been fixed (killed and chemically crosslinked so that their contents are trapped in the locations they occupied when the cell was alive) gives sufficient information. Immunofluorescence imaging of fixed cells is often much easier than fluorescence imaging of live cells, and allows targeting of species, such as post-translationally modified proteins, that may be difficult to observe with live

cell imaging modalities. For these reasons, immunofluorescence of fixed cells is a widely used technique in biological microscopy. For more information, see (7–9).

Imaging of live cells was revolutionized by the discovery of the jellyfish green fluorescent protein (GFP) and that it fluoresces when expressed in other organisms (10, 11). GFP is a small protein that catalyzes the formation of a chromophore from three amino acids in the center of the protein. It requires no cofactors or other molecules to become fluorescent (except oxygen); this means it can be introduced into nearly any organism to label proteins. Labeling of a specific protein in an organism is typically done by genetic engineering to encode a fusion of GFP to the protein of interest. This can either be done in the genome of the organism or by introducing a foreign DNA segment that contains the GFP fusion protein. Identification of related proteins from other species and engineering of these molecules mean that they are no longer restricted to just the color green: fluorescent proteins ranging from the blue to the near infrared have been developed, enabling imaging of four or more colors simultaneously (12–14).

The microscope used for fluorescence imaging is typically an epi-fluorescence microscope, where the fluorescence excitation light is focused onto the sample using the same objective that is used to collect the fluorescence emission. A filter cube is used to separate the excitation and emission light. This consists of a dichroic mirror, which reflects the excitation light and transmits the emission light, and two interference filters, one which specifically passes the excitation wavelengths and one which specifically passes the emission wavelengths. Typically these filter cubes are placed in a movable turret so that the filter cube can be changed for observing different fluorescent dyes. Often, multiband dichroic mirrors are used, paired with excitation and emission filters in filter wheels; these allow rapid switching between different dyes (15). For simultaneous imaging of multiple colors, the sample can be illuminated with two excitation wavelengths simultaneously, and the emitted light split between two detectors with a dichroic mirror.

**Confocal Microscopy**

The simplest way to acquire an image of a fluorescence samples is to directly image the sample onto a camera. However, if the sample is thicker than the depth-of-field of the objective lens, out-of-focus parts of the sample will be excited by the cone of excitation light. The out-of-focus parts of the sample will be imaged out-of-focus onto the camera, resulting in a blurred, out-of-focus image being superimposed on the in-focus image. This is a serious problem when trying to produce three-dimensional images of thick samples. Multiple solutions to this problem have been developed. Probably the most common is the confocal microscope. First proposed by Marvin Minsky in 1957, the confocal microscope illuminates a single point in the sample, rather than the entire field of view. Light emitted from this point in the sample will then be focused to a point at the image plane of the microscope. Light emitted from the sample from regions that are not at the focal plane of the objective will be out of focus at the image plane, and imaged into a larger disk. This out-of-focus light can then be rejected by placing a pinhole in the image plane. This pinhole is placed conjugate to the sample, hence the name confocal microscope. Because this only allows a single point in the sample to be viewed, some mechanism of scanning this spot across the sample is needed. Originally, this was done by scanning the sample, but nearly all confocal microscopes now scan the spot across the sample (Figure 3; (6, 16)).

In this confocal laser-scanning microscope, the illumination spot is usually generated by imaging the tip of a single mode optical fiber onto the sample. This spot is scanned across the sample by two scanning mirrors which are placed conjugate to the back aperture of the objective lens. Changing the angle of the illumination beam at the back aperture changes the position of the spot at the focal plane. These scanning mirrors are used to descan the emitted light from the sample, so that it is always coincident with the detection pinhole, regardless of the point in the sample it originated from. A dichroic mirror is used to separate the emitted light from the excitation light. Because the same mirrors are used to scan the excitation beam and descan the

emission from the sample, the emitted light is guaranteed to be focused to the same point in the image plane, regardless of the position of the excitation spot. This allows the pinhole to be fixed.

Because the sample is imaged point by point, an imaging detector is no longer needed. Furthermore, to generate a high resolution image of the sample, many points must be imaged, which in turn means that the time spent imaging each point (the pixel dwell time) must be short. For example, a one megapixel image acquired with a 1 microsecond pixel dwell time would require one second to acquire. These two requirements dictate the use of high-speed point detectors, typically photomultiplier tubes, avalanche photodiodes, or hybrids of the two, for laser-scanning confocal microscopy. Movement of the mirrors and recording of the signal from the detectors is coordinated by the controlling computer. Once the scan area and pixel dwell time is set, this scanning process is handled without user intervention. The confocal microscope returns a multichannel image and can be treated similarly to any other imaging detector by the user.

Because the scanning process can be slow, and the detectors are relatively inefficient compared to high sensitivity CCD cameras, a second type of confocal microscope has also come into wide use for biological light microscopy. This is the spinning disk confocal microscope (17). A spinning disk confocal microscope illuminates the sample with many pinholes simultaneously. These pinholes are arranged in a spiral pattern (a Nipkow or Petran disk) so that the pinholes sweep across every point in the sample once and only once when the pattern is rotated around its axis. A typical disk pattern, used in instruments manufactured by the Yokogawa Electric Company, has 50 µm pinholes spaced 250 µm apart. The same pinholes are used for both excitation of the sample and detection of the in-focus light emitted by the sample, and a dichroic mirror is used to separate the excitation and emission light. By integrating the light collected through the pinholes during one rotation of the disk pattern, an image of the sample is produced that can be captured on a camera. Typically the disk rotation speed is

chosen to produce a full image of the sample in 1 millisecond. The spinning disk confocal microscope thus allows confocal images to be acquired rapidly using high sensitivity cameras. The disadvantage of spinning disk confocal compared to laser-scanning confocal is that it rejects less out-of-focus light because out-of-focus excited by one pinhole can be collected through adjacent pinholes. For thin samples the out-of-focus rejection for both confocal approaches is identical but for thick samples the laser-scanning confocal outperforms spinning disk confocal (18). Examples of both laser-scanning confocals and spinning disk confocals are shown in Figure 4.

**Microscope automation**

Both widefield and confocal microscopes can be automated and computer controlled. A modern high-end research microscope is likely to include a motorized sample stage, one or more motorized focus drives, a motorized objective changer, and motorized dichroic mirrors and filter wheels. The illumination sources will include computer-controlled shutters, and there may be additional peripherals such as pumps, fluidic valves, or robotics integrated with the microscope system. This automated hardware requires a computer system for control. This integration of automated hardware and computer control also enables a variety of experiments to be carried out automatically.

A wide range of experiments will typically be carried out on a system like this, but most feature some combination of the following experimental building blocks:

- Multi-channel imaging: this allows multiple different fluorescent dyes to be imaged on the microscope, and requires setting the filter wheels and dichroic mirror to the relevant position. In a laser based system it will also require selecting the correct excitation laser. It may also involve setting different light paths, for example if acquiring both fluorescence and transmitted light images of a sample. The camera settings will likely be different for each channel imaged as well.

- Time-lapse imaging: this simply involves repeating a set of image acquisitions at programmed times. Typically the time interval is constant, but occasionally it can vary, for example to follow a rapid initial process followed by a slow adaptation.
- Multiple focal planes (Z-stack imaging): acquiring images at multiple focal planes allows a three-dimensional image of the sample to be reconstructed. This is particularly useful on confocal microscopes. The objective, the sample, or both can be motorized, and often a mechanical Z-drive for long range motion is combined with a piezoelectric Z-drive for rapid short range motion.
- Multiple sample positions: by moving the sample on a motorized X-Y stage, multiple sample positions can be imaged. This can be done to acquire images of different regions of the sample, or can be used to acquire a large field of view by acquiring overlapping images and stitching them together into a single image.
- Automated focus: Most microscopes now include some kind of hardware autofocus. Typically this optically monitors the position of one face of the coverslip and uses feedback to maintain the coverslip at a constant height above the objective. Provided the sample does not move with respect to the coverslip, this guarantees that the sample will remain in focus.

Typically, this automated hardware communicates with the host computer over standard protocols - most often, USB or RS232.

**Biological Microscopy Experiments**

Biological light microscopy now encompasses a diverse range of experiments. These experiments run the gamut from single molecule and super-resolution experiments, where the whole field of view may be only a micron on a side, to imaging of whole organisms or tissues, where the field of view may be a centimeter across or larger. Time scales may vary similarly,

from imaging at hundreds of frames per second to time-lapse experiments that run for weeks. However, the hardware used to carry out these diverse experiments is by and large the same. Consequently, a state-of-the art microscope from one of the four major vendors (Leica, Nikon, Olympus, and Zeiss) can be used as the base for a wide range of experiments. For experiments that push these limits, specialized hardware will likely be added to the microscope, but it is rare for microscopes to be built completely from scratch for a dedicated purpose.

Laser-scanning confocal microscopes will typically be produced and assembled by a single vendor, but widefield systems are frequently assembled from components from multiple vendors. This allows components like cameras, stages, and filter wheels to be chosen to match the experimental requirements. More specialized equipment, like systems for spatially patterned illumination, can be installed as well. In the sections that follow, we review some of the components of a modern light microscope.

## LIGHT DETECTORS IN MICROSCOPY

As in other applications, light detectors in microscopy are used to measure photon flux during a predetermined time interval and relate this measurement to a spatial element in the image plane, thus producing an image. Two distinct strategies are used. In the first strategy, the sample is illuminated with a single spot, and the signal (reflection, absorption, fluorescence, etc.) is measured with a detector that measures light intensity at a single spatial element (like a photomultiplier tube), and an image is formed by moving the spot over the sample and correlating light flux measurements with positions. In the second strategy, light is measured in many spatial elements in parallel (i.e. on a two-dimensional grid as in a camera), and the image is directly projected onto the sensor. The choice between point detectors and cameras is dictated by the type of microscope. Those that require readout of a single point at each time instance (such as confocal and multiphoton microscopes) use point detectors, whereas microscopy techniques that form a real image use cameras.

**Point detectors**

Two types of point detectors are commonly used in microscopy: Photomultiplier tubes (PMTs) and Avalanche photodiodes (APDs). Remarkably, although the design of the PMT is over a century old, it is still an extremely useful photon flux measuring device due to its high speed, linearity, and sensitivity. PMTs make use of two principles discovered at the end of the 19th and beginning of the 20th centuries: the photoelectric effect, i.e. the ability of certain materials to absorb photons and reemit electrons, and secondary electron emission, in which electrons bombarding a solid object can cause the emission of 'extra', secondary electrons. The primary photo-sensing element in a PMT is the photocathode, which converts incoming photons into electrons. The efficiency of this conversion (the quantum efficiency, QE) depends on the photocathode material. In light microscopy applications, most often cesium activated gallium arsenide (GaAs) or cesium activated indium gallium arsenide (InGaAs) materials are used, where the latter has a higher QE in the infrared than the former. The QE of these photocathode materials is generally less than 30%, implying that more than 70% of the signal is lost at the photocathode. Electrons emitted by the photocathode are passed to the anode through a series of dynodes (intermediate nodes between cathode and anode), each at a higher voltage. Electrons arriving at a dynode will elicit more electrons through the secondary emission effect, and the amount of amplification at each dynode depends on the voltage differential between the nodes. This cascade of amplifications will result in a much larger number of electrons at the anode than the photocathode. The amplification can be as high as $10^8$ and can be controlled by changing the voltage difference between the dynodes. The current at the anode is converted into a voltage by an output amplifier and digitized by an analog to digital converter (ADC) before being recorded by the computer.

Photomultiplier tubes can be operated in two fundamentally different modes. When the PMT operates in linear mode, the input signal varies linearly with the output signal over a range

determined by the PMT and associated electronics. In a second mode, called “Photon counting mode”, the gain of the PMT is so high that the single electron produced by the absorption of a single photon at the photocathode produces a measurable signal at the output. By applying a threshold, all noise (those signals of lower amplitude than that produced by a single electron) is eliminated and incidences of single photons can be counted. In this mode, a PMT can only operate at low light intensities (to avoid multiple photons being absorbed at the photocathode simultaneously, which cannot be distinguished from the absorption of a single photon) and must run at lower clock speeds.

The main shortcoming of the PMT is its low quantum efficiency, which has not been overcome even after almost a century of development of new photocathode materials. A completely different design of a sensitive single point detector is the Avalanche Photodiode (APD). APDs are made of semiconductor material (most often silicon for use in the visible and near infrared) and have a very high quantum efficiency (>95% in some parts of the spectrum). Absorption of light in the semiconductor generates photo-electrons, which are stored in a potential well in the device until they are read out. However, before storage, the photo-electrons are accelerated by a large voltage differential (typically ~100V in silicon) that causes the electrons to accelerate and elicit new electrons from the semiconductor material through a process called impact ionization. This results in an amplification of the signal (within the semiconductor material) of around 100-fold, although other designs with much higher gain are possible. The net result is a detector with high QE and appreciable gain. Although APDs are used in several commercial confocal microscopes and many home-built ones, they have not completely replaced PMTs. The main reason is that APDs tend to overheat if run for prolonged periods of time at or near full well capacity. Thus, APDs often contain a protection circuit that will shut off the device when such a situation is detected and can only be used again after a cool-down period.

A recently developed point detector combines aspects of both PMTs and APDs and is appropriately called a hybrid photo-detector. It consists of a photocathode mounted in a vacuum tube. The electrons emitted by the photocathode are accelerated through a voltage of several kilovolts after which they bombard an avalanche photodiode. The high energy electrons produce many secondary electrons when they bombard the APD, giving rise to a gain of ~1000-fold from electron bombardment; the APD provides an additional ~50-fold gain. Compared to PMTs and APDs they are substantially lower noise and provide very good photon counting capability but are susceptible to damage due to illumination with too much light (19).

**Area Detectors (Cameras)**

Two different digital camera architectures are widely used in microscopy: charge-coupled devices (CCDs) and complementary metal-oxide semiconductor (CMOS) detectors. The latter were until recently considered too noisy for use in scientific microscopy imaging, but recent designs have greatly reduced noise and are being marketed as sCMOS (scientific CMOS) cameras. Both types of detectors contain rows and columns of addressable image sensors (picture elements, or pixels) built from semiconductor materials (silicon for applications in the visible and near infrared). The main difference between CCD and CMOS lies in the presence of active electronics in the CMOS as opposed to the CCD sensor. A more appropriate name for CMOS devices is therefore “Active Pixel Sensor”. Both devices accumulate electrons in a potential well inside the pixel element for the duration of the exposure. The difference between the designs lies in the methods used to read out the accumulated charge and eventually convert this charge into a digital number to be used in the computer’s memory as a proxy for the number of photons arriving at the pixel during the exposure time.

CCDs contain rows of transparent electrodes layered on top of the chip. By changing the voltage of these electrodes in a specific pattern (this is driven by a clock; therefore this process is often referred to as “clocking” the chip) charge is moved from one pixel to the next. Charge

from the last row of the chip is moved into a serial readout register, from where it is clocked into a readout amplifier. Typically, there is a single readout amplifier on the chip, although some chip designs are more complicated and contain multiple readout amplifiers, for instance one for each quadrant of the chip. The readout amplifier transforms the charge into a voltage that is then sent to the output of the camera. The camera either transfers this analog data to the computer, where it is digitized by a frame grabber card, or it carries out the digitization on board and transmits the digitized data to the computer.

The speed with which the charge is moved from one pixel to the next (the clock speed) determines the time it takes to read out the chip. Charge transfer from pixel to pixel is highly efficient in current camera designs and in practice no longer contributes to noise in the final image. However, the readout amplifier is still a considerable source of noise. Readout noise increases with readout speed: the higher the readout speed, the less time is available for the measurement of charge in each pixel, and the higher the noise contribution of the readout amplifier (the higher the read noise). Readout speeds vary from about 1 to 30 MHz (pixels per second) in cameras currently used for microscopy. The resulting readout times depend on the number of rows and columns on the CCD, and range from ~10 ms to several seconds. By clocking two rows into the serial register without clocking the serial register, the content of two pixels is combined into a single pixel in the serial register. Likewise, two pixels in the serial register can be clocked into the readout amplifier before reading out the pixel. This process, called binning, combines the electrons from multiple pixels, resulting in higher signal at the cost of spatial resolution. The advantage of on chip binning over binning inside the computer memory is that on chip binning contributes only a single error from the readout amplifier as opposed to binning after readout.

If no precautions were taken to avoid light exposure during the readout period, extensive smearing of the image would occur. Several strategies are used by chip designers to avoid such

smearing. For instance, a physical shutter can be placed in front of the chip and closed before readout starts. However, physical shutters are slow and cause mechanical vibrations making this solution undesirable. A widely used approach involves rapid transfer into a frame transfer buffer. In such designs, the chip is twice as large as it appears to the end user with one half of the chip covered with an opaque mask. When exposure ends, the charge on the chip is clocked very quickly to the area under the mask from where it can be read out normally. There is still a slight risk of smearing. However, since the “frame transfer” is fast, this is often negligible. Another strategy (called “interline”) also involves doubling the size of the chip but with columns of covered pixels interspersed between columns of uncovered pixels. The charge from the uncovered pixels is clocked into the covered ones in a single cycle, avoiding any smearing. Obviously, this design reduces the photosensitive area of the chip by 50%. To overcome that loss, chip designers place microlenses over the chip that direct as much light as possible to the uncovered pixels.

As in point detectors, the quantum efficiency of cameras is an important parameter. Silicon can provide a high QE in many parts of the visible spectrum, however, the transparent electrodes used for clocking, active electronics in CMOS designs as well as other structures needed for the chip to function all lead to light loss. One clever trick to work around this light loss is to illuminate the photosensitive area from behind. To do so, a significant amount of the thickness of the chip needs to be removed in a process called “back-thinning”. Back-thinned chips can have a QE > 95% in some parts of the visible spectrum, whereas the best non back-thinned chips have a maximum QE of about 70%. Back-thinning is an expensive process and results in lower yield of chips produced, hence back-thinned chips are significantly more expensive than their front-illuminated counterparts. Currently, frame transfer designs are often combined with back-thinning, whereas interline designs are not. Current sCMOS chips are not back-thinned. Since

consumer grade CMOS chips can be back-thinned it seems likely that some progress in the QE of sCMOS chips can be made by back-thinning.

An important source of noise in low light images is the readout noise introduced by the readout amplifier. Although this noise can be reduced by clocking more slowly, a high frame rate is often desirable, hence various strategies to amplify the signal before it arrives at the readout amplifier have been devised. In microscopy, the most widely used strategy is on-chip electron multiplication (EM) gain. This is achieved by clocking the charge through a special EM gain register that has higher than normal voltage differential. Transferring charge between pixels at high voltage can result in impact ionization, eliciting new electrons from the chip material and thereby amplifying the signal. Although the gain at each transfer step is usually small (on the order of 1%), by transferring charge through a large number of pixels (typically on the order of 500), very high gain can be achieved. Moreover, the amplification can be modulated by changing the voltage differential between the pixels in the EM gain register. Current cameras have a built-in calibration of amplification versus voltage differential in the EM register, making it possible for the end user to employ a “linear EM gain”. The net result of EM gain is that the signal is amplified before being read out by the readout amplifier, so that a signal that previously would have been impossible to discriminate from readout noise now clearly stands out. This ability has made EM cameras the device of choice for applications involving low light detection at relatively high frame rates (up to ~60 frames per second). However, there is some cost associated with EM amplification. Since the amplification process itself is stochastic, the actual amount of amplification is Poisson distributed. Because photon shot noise (see below) has a similar distribution, one can equate the use of EM amplification as doubling photon shot noise, i.e. detecting half the amount of light, i.e. halving the QE. Other spurious noise sources on the chip, previously invisible, become an issue when using EM amplification. For instance, clock-

induced-charge can be amplified, resulting in occasional higher pixel values. Camera manufacturers use various schemes to reduce such artifacts.

An alternative camera technology capable of low read noise at high frame rates is provided by sCMOS detectors. Unlike the pixels on a CCD, each CMOS pixel contains its own charge to voltage converter. This charge-to-voltage converter and other electronics on each pixel are constructed from a few transistors (fabricated using complementary metal oxide semiconductor technology, hence the name CMOS). To read out the chip quickly, analog to digital converters (ADCs) are built into the chip itself, either one or two per column of pixels. When using two ADCs, the top half of each column is digitized by one ADC and the bottom half by another (this design can result in an image with slightly different pixel values in the top half versus bottom half of the image). Since it is difficult to construct all the charge to voltage converters and ADCs identically, their gain and offset characteristics vary slightly, resulting in fixed pattern noise in the image. In scientific CMOS (sCMOS) designs, the gain of each read-out amplifier is calibrated and corrected using electronics built into the camera itself (implemented using a field programmable gate array, FPGA). Some current sCMOS cameras have readout noise far lower than the best CCD cameras on the market, making them suitable for low light applications previously only accessible with EMCCD cameras.

However, sCMOS cameras bring their own peculiarities. For instance, since the readout amplifiers are all different, their noise characteristics are also different and not necessarily normally distributed. Therefore, the average readout noise is not a useful parameter and the manufacturer should also provide the median readout noise and preferably the actual noise per pixel. Another issue is the so-called shutter mode. CCD chips expose all pixels simultaneously. This is not necessarily the case for sCMOS cameras. Most CMOS devices use so called “rolling shutter” mode in which exposure starts in the top row and then proceeds (“rolls”) down the chip so that the bottom of the chip starts exposing later than the top row. The time delay between the

top and the bottom row exposures can be as much as 10 milliseconds. Readout follows the same pattern so that all pixels are exposed for the same amount of time but at slightly different time points. Rolling shutter works fine for most types of microscopy imaging but can cause problems for fast moving objects and poses interesting problems for hardware synchronization with other parts of the microscope. Some sCMOS cameras can operate in “global shutter” mode (which is the same mode as used in CCD cameras). However, the ability to do so requires an extra transistor on each pixel, incurring light loss and lower QE than chips that do not support global shutter mode. Despite these peculiarities, current sCMOS cameras provide very large sensors (> 2k x 2k pixels) with attractive pixel size (6.5 μm square) that can have very low average readout noise (~1 electron/pixel) and can operate at very high speeds (100 fps full chip).

**Choosing a camera**

When selecting a camera for a particular microscope, several factors should be considered:

- *Pixel size and number*. The physical size of a chip is the product of the pixel size and pixel number. The field of view of the microscope limits the maximum useful size of a camera. Conversely, a small physical size results in a smaller than possible field of view. It is possible to adjust the field of view to the chip size using extra optics in the camera adapter. However, such optics can result in light loss and undesired optical aberrations. The standard microscopy camera mount (C-mount) is a 25 mm diameter thread, limiting the image size at the sensor plane to about 21 mm in diameter. This is well matched to the field of view the microscope produces, typically 21-25 mm. In principle, pixels could be made very small so that many millions of pixels would fit in this area. However, it is useless to use pixels that are much smaller than the optical resolution of the microscope. As described above, the resolution limit of the microscope is a function of the wavelength of the light used and the numerical aperture of the objective lens, and is

about 220 nm for green light and a 1.4 NA lens. If a 100x objective lens (and 1x tube lens) is used, 220 nm in the object plane corresponds to 22 µm in the camera sensor plane. What size pixel is needed to capture all information in such a system? The answer to that question is given by the Nyquist-Shannon sampling theorem which states that to faithfully reproduce an analog signal, digital samples need to be taken at at least twice the rate of the highest frequency contained in the signal, implying that the pixel size needs to be at most half the size of the smallest resolvable element, in this case at most 11 µm. For a 40x 1.4 NA objective, the smallest resolvable element in the camera sensor plane is 8.8 µm, and pixels should be no larger than 4.4 µm to capture all available information. Widely used chip sizes are 512 x 512 pixels of 16 µm square for an 8.2 mm square chip size (in frame-transfer EM cameras, these will often need extra magnification to fulfill the Nyquist-Shannon criterion) and 1360 x 1024 of 6.45 µm square pixels for a chip 8.8 x 6.6mm in size (the Sony interline chip ICX285). Current sCMOS cameras have about 2000 x 2000 pixels of 6.45 µm square for a chip size of about 12.9x12.9mm, and image clipping can be an issue with such large sensors.

- *Sensitivity and full well capacity.* The sensitivity of a camera is ultimately a function of its quantum efficiency, i.e. what fraction of the photons hitting the detector are converted into the measured signal? In general, the higher the quantum efficiency, the better. Pixels can accumulate only a certain amount of charge before they overflow. In general, the larger the size of the pixel, the higher its “full well capacity” (i.e. how many electrons can the pixel contain?). Various camera settings (such as the clock speed) can influence the full well capacity. For most fluorescence microscopy applications, in which low amounts of light are detected, it is uncommon to need a very high full well capacity.
- *Noise*. Obviously, it is advantageous for a light sensor to add little noise to the measured signal. It should be realized, however, that the signal itself is noisy due to photon shot noise. Light particles (photons) are discrete entities and are emitted by a light source at

random intervals. This causes the arrival of photons at a detector to follow a Poisson distribution. The standard deviation of shot noise is equivalent to the square root of the average photon flux. As a result, the signal/noise ratio for a signal that only contains photon shot noise equals N/sqrt(N) (where N is the number of photons) which reduces to the square root of the signal (N). Therefore, for an image with no noise other than shot noise, doubling the signal-to-noise ratio requires increasing the amount of light collected by fourfold. Cameras can add three main types of noise to photon shot noise:

*Readout noise:* Noise contributed by the readout electronics. This is a constant added to every pixel (note that this constant is the same for every pixel in a CCD sensor whereas it can be different from pixel to pixel in a sCMOS).

*Dark current:* When a camera is not exposing, pixels can sometimes still accumulate charge. Some pixels do so more than other, resulting in “hot pixels”. Dark current is reduced by cooling and - as long as relatively short exposure times are used - is usually not an issue in biological imaging with cooled cameras.

*Varying pixel response.* Even though CCD and CMOS sensors are very linear, not every pixel will respond equally to the same photon flux. This non-uniform pixel response can be measured by illuminating the sensor homogeneously (fine pitched LCD screens such as those in current cellular phones are great for this purpose). Pixel response is often wavelength dependent and the image generated by even illumination will often look different at different wavelengths. Global effects, such as those caused by back-thinning, can be seen in the flatfield image, as well as pixel-specific effects (some pixels appear to have a much lower quantum efficiency than others). As long as these effects are linear, they can be corrected for using the flatfield illuminated image as a reference.

- *Speed.* In fluorescent imaging, exposure times often are in the 0.1 - 1.0 second range. For single snapshots or single channel time-lapse imaging, readout speed of the camera is not a great concern, since the large delay between taking images allows complete

readout from the camera even at very low clock speeds. However, when multiple channels are imaged at each time point, Z-stacks are taken, or when fast moving objects are imaged, camera readout speed becomes an important issue. Readout speed is specified as the frequency at which pixels are recorded and is most often specified in MHz. For CCD cameras, readout noise increases with readout speed, and scientific grade cameras will let the user choose between high speed and higher read noise or low speed and lower read noise. Depending on the chip design, the maximum readout speed is 10-30 MHz. A CCD chip of 512x512 pixels running at 10 MHz can theoretically produce images at 38 fps (26.2 ms per image). In practice the performance is somewhat lower, producing images at 30 fps (33 ms per image). The frame rate can be somewhat increased by only reading out a subregion, however, this increase in speed does not scale linearly with subregion size. Due to their different design, sCMOS cameras are capable of much higher frame rates; the current crop of sCMOS cameras can be read out at 100 fps even with a chip size exceeding 2000 x 2000 pixels. Moreover, readout speed scales linearly with the number of rows that are read out and speeds of up to 20,000 frames per second are possible for images containing only a few rows of pixel data. The large chip size combined with high frame rate results in a very high data transfer rate that can exceed the maximum possible transfer rate for current camera-PC interfaces, such as Camera Link. Also, the computer and the software running on the computer will need to keep up with data rates that can approach or exceed 1 GB/s.

All scientific grade camera manufacturers have datasheets available for their cameras that specify all these factors. As a first pass in camera selection, it is therefore useful to decide the camera parameters that are needed (pixel size, pixel number, QE, noise and speed characteristics) and then to pre-select a number of cameras based on these parameters and price. At this point, it is also important to consider the software application that will be used to

control the microscope setup and evaluate if the software will work with the camera and how well it does so. Even though many physical parameters of cameras can be measured it is still important to test the camera, preferably on the same microscope that it will eventually be used.

## DIGITAL CONTROL OF MICROSCOPE HARDWARE

Motorization of microscope components enables complete software control of image acquisition, and not only provides easy access to techniques such as multi-channel imaging, Z-stacks, and time lapse imaging, but also opens the door to more advanced techniques such as improved resolution using structured illumination. We will discuss here several of the most important motorized microscope components as well as the issues involved in combining many of such components into a functional microscope workstation

### Motorized XY and Z stages

Although virtually any type of computer controllable positioning device can be used in microscopy, the field has converged on the use of stepper and piezoelectric motors. Most often, the sample carrier is attached to stepper motors that provide XY movement. For Z movement, either the sample carrier or objective (or both) can be motorized. Motors used to be an add-on device that was attached to an existing microscope (for instance, by attaching a motor to the focusing knob). Current microscopes often have built-in motors, reducing the footprint of the microscope and making it easier for the microscopist to operate the equipment. Speed and precision are important for microscope stages. To improve the precision with which a stage returns to a given position, linear encoders are used. The stage controller reads out the position of the encoder and adjusts the position of the motor to minimize error. Although linear encoders add considerably to cost, they provide the extra benefit of the stage controller remembering its position after being switched off and back on again. Stepper motors have a minimum step size, which is especially important for Z stages. It is not uncommon to acquire Z-stacks with 100 nm intervals and for such cases the stepper motor should be geared such that it provides steps of

no more than 25 or 50 nm. To accurately and quickly move between positions, acceleration, top speed and deceleration need to be finely tuned, a task accomplished by the stage controller. Piezoelectric motors can move equipment faster and more accurately, but have only a limited range of motion (<500 µm). These motors can be placed between the nose piece and objective (such that the motor only moves a single objective) or within the sample carrier. The motor controller receives input from a joystick (for XY movement) and a dial (or focus knob on the computer) translating these signals into expected movement. In addition, software running on the computer communicates with the controller, most often through a serial (RS232) or USB interface.

**Light Sources**

From the perspective of computer control, a distinction can be made between light sources that can be directly modulated and those that need an additional device to do so. For instance, the traditional light source for transmitted light is a halogen bulb. The brightness of a halogen lamp can be directly controlled by changing the voltage applied to the lamp. However, the response of the bulb to a voltage change takes several seconds, which is why it is highly desirable to place a mechanical shutter in front of the lamp. Switching times of mechanical shutters depend on their size and range from about 1 to 25 ms. LED light sources are rapidly replacing halogen bulbs for the purpose of transmitted light illumination, mainly because their intensity can be modulated extremely rapidly (at sub-ms time scales) and they cost considerably less than a halogen lamp house, voltage supply and shutter. However, LEDs emitting white light often contain phosphors converting the spectrum of the underlying LEDs. When the microscope is properly configured for Koehler illumination, the epi-fluorescence light source will illuminate these phosphors resulting in an undesired transmitted light background of the fluorescence image. Therefore, it is advisable to use a green LED (not containing phosphors) unless color transmitted light imaging is needed.

The traditional light source for epi-fluorescence microscopy is the xenon or mercury arc lamp. These suffer from short bulb lifetimes (as little as 200 hours for some mercury lamps). More recently, these have been replaced by similar light sources with a longer lifetime, such as metal-halide lamps. As these sources often produce substantial amounts of heat and vibration (due to cooling fans), it is desirable to place them away from the microscope. This is typically done by coupling the output of the light source into a liquid light guide that is connected to the microscope. A liquid light guide is simply a flexible liquid-filled tube that transmits light via total internal reflection, similar to an optical fiber. It has the added benefit of scrambling spatial non-uniformities in the light source, producing even illumination. None of these light sources can be switched on and off rapidly (in fact, most of them need to stay on for at least 30 minutes before switching them back off to avoid sharply reduced life time) and therefore a shutter somewhere in the light path is essential. These lamps are broadband sources, and therefore bandpass filters are required to define the illumination wavelengths for specific dye molecules. For many of these sources the emission spectrum extends into the UV, and this UV light must be filtered for live cell imaging to reduce light-induced cell damage.

A few years ago, light emitting diodes (LEDs) became sufficiently bright for use in fluorescence microscopy, and ever since a slow transition to the use of LEDs as light source is taking place. Since the energy efficiency of LEDs is much higher than that of the previously discussed light sources they produce less heat and need less cooling. Nevertheless, cooling may still require a fan and LEDs are therefore often coupled to the microscope with a liquid light guide to avoid transmitting vibrations to the microscope. Multiple LEDs with different emission wavelengths can be combined in a single unit to provide white light with roughly constant intensity over the visual spectrum. Such light sources can be treated similarly to traditional fluorescence light sources, with excitation wavelengths selected using filters. Alternatively, each LED in such a unit can be individually controlled making it possible to switch excitation wavelengths at high speed.

Additional benefits of LEDs are their lack of harmful UV light and the ability to rapidly switch the LED on and off. This allows for tight coupling between camera exposure and illumination, reducing phototoxic effects. It should also be noted that the lifetime of LEDs (measured in the tens of thousands of hours) is much longer than that of older light sources and that they lack toxic mercury present in most alternative light sources.

Lasers are used as light sources in applications where a bright, collimated light source is needed such as confocal microscopy and total internal reflection microscopy (TIRFM). Although lasers can theoretically also be used in transmitted light and epi-fluorescence applications, the coherence of the laser light results in undesirable speckle patterns in the plane of focus (moreover, the cost of lasers is almost always much higher than the cost of alternative light sources). Many different types of lasers exist. From the standpoint of computer control, the most important property of a laser is whether or not its output intensity can be directly controlled (by either an analog voltage and/or a digital signal). Some laser types, such as diode lasers, can be directly modulated at very high frequency and are therefore easy to interface with a computer. Lasers that cannot be directly controlled include gas lasers (such as argon and krypton lasers) and many optically pumped solid-state lasers. Shutters can be used to control output of such lasers, providing switching times down to ~ 1ms (these shutters can be as small as the laser beam and therefore have much faster switching times than shutters used with epi-fluorescent light sources). Even faster switching as well as control over intensity can be obtained using an Acousto-Optical Tunable Filter (AOTF). This consists of a piezoelectric device that transduces acoustical signals onto a crystal. The sound waves cause alternating patterns of higher and lower refractive index in the crystal that act as a grating. This grating causes diffraction of the laser light passing through the crystal. Both the intensity of diffraction and selectivity for specific laser lines can easily be changed. AOTFs switch at microsecond time-scales, making them wonderful devices for fast changes in laser illumination. A downside is that they cannot be

completely switched off; the maximum extinction is about 5-6 orders of magnitude, but this rarely poses a problem. AOTF controllers usually take both digital input (to switch a laser line on or off) and analog inputs (which changes the intensity of the specific laser line, although not in a linear fashion).

**Projection of images into microscopes: spatial light modulators (SLMs)**

Many interesting applications rely on the projection of images into the focal plane. For instance, improved resolution can be obtained using structured illumination (20, 21). Localized projection of light can be used for photo-bleaching of dyes within a cell. The observer can then deduce information about the dynamics of the fluorescent molecule by measuring the rate of recurrence of fluorescence. Similar information can be obtained using photo-conversion or photo-activation of dyes, which usually occurs at much lower (more benign) power levels and results in a more easily detectable positive rather than negative signal (22, 23). Molecules that change activity upon illumination with light make it possible to locally affect function in living systems, an ability that can only be fully exploited by full temporal and spatial control of light in the microscope object plane. For example, light-controlled ion channels (which change the flow of ions across membranes upon illumination with light) can turn neuronal activity on and off, and light-gated protein interactions can be used to translocate fusion proteins within the cell upon irradiation with specific wavelengths of light (24, 25). Devices that can project patterned illumination are generally referred to as “Spatial Light Modulators” (SLMs). The following types of SLMs are used in microscopy:

- Galvanometers (Galvos): These are mirrors mounted on a galvanometer that can rotate at very high frequency. Galvo scanners can direct laser light to arbitrary positions in the image plane and thus provide patterned illumination. The same system is used in confocal scanning microscopes, which are usually easily adopted for photo-bleaching and photo-activation experiments. An interesting new alternative is provided by

microelectromechanical (MEMS) mirrors directly mounted on semiconductor material. These allow two-axis tilting, are smaller, and consume less power than galvanometers.

- Digital Micromirror Devices (DMDs): These are a type of SLM used in many computer projectors. A DMD consists of rows and columns of small mirrors mounted on semiconductor material. The mirrors can rotate between two positions (for instance +/-10 degrees), and are under computer control. By orienting the DMD and incident light path such that the light reaches the object plane in the microscope only when the mirrors are switched in one position, patterns can be projected onto the sample. In many cases, the DMD presents itself to the computer as a display screen. Switching times can be extremely fast, such that a different intensities at each position can be produced by rapidly toggling between the on and off position.
- Liquid crystal based SLMs: These can control the intensity and/or phase of light. They can be designed for use with reflected light (where the liquid crystals are mounted on reflective silicon) or for transmitted light. The ability to control both phase and amplitude makes it possible to position the SLM in a plane conjugate with the back-focal plane of the objective (rather than the object plane). A real image can be projected onto the sample by calculating the Fourier transform of the desired image and placing this on the SLM. The advantage of this approach over a DMD or amplitude only SLM is that the light efficiency is much higher, i.e. a much higher fraction of the light will reach the object plane. Moreover, patterns can be projected in three rather than two dimensions. One application of this technology is the generation of many optical traps simultaneously (26).

**Software**

Obviously, software is essential to synchronize the multitude of digital and motorized microscope components. Many laboratories used to write their own code, either using compiled programming languages or environments such as Matlab or Labview. This is still the case for

groups devising new microscopy technologies; however, most other groups rely on software written by others. A large number of commercial software packages exist and at least one free and open source microscope software project (μManager) has gained considerable traction (27, 28). The major microscope companies (Zeiss, Leica, Olympus and Nikon) all sell their own software products, either developed in-house or in association with external software development companies. An important consideration when assembling a system is whether the software supports all desired components. Software from a specific microscope company tends to work only with that vendor's equipment. Currently, MetaMorph (Molecular Devices) and μManager (http://micro-manager.org) support the widest range of equipment and μManager makes it possible to extend hardware support by writing just the code needed to interface with a device.

The most important task of the software is to grab images from the camera, present these on the screen and save them to disk. The camera exposure needs to be tightly synchronized with other components. For instance, a shutter may need to be opened just before the exposure starts and closed immediately afterwards. To obtain multi-channel images, the appropriate filters need to be moved in place (and the correct light source selected) before taking each image. Z-stacks and multi-position series require the software to move the appropriate stages to the correct position before taking images. Synchronization between the various devices can be difficult, especially with components that were not designed with integration in mind (for instance, many non-scientific grade cameras have varying delays between a request for exposure and the actual start of the exposure). Also, the various standard image acquisition modalities need to be presented to the user in an understandable fashion, yet allow for as much flexibility as possible in image acquisition strategy selection. For complicated experiments, it is desirable that the software provides some kind of scripting environment.

**High speed triggering**

In many cases, software-based synchronization between the multiple devices in a microscope system is considerably slower than is possible using hardware synchronization. Reasons for this difference are found in the use of computer operating systems with unpredictable response times (i.e. a non-real-time OS), slow communication between the computer and device (often through a RS232 serial interface with transmit times of one character per ms or slower), slow interpretation by the device of commands received from the computer, and non-optimal software on the computer. Additionally, most cameras can be run faster when acquiring a stream of images than when snapping a single image. If the hardware can switch rapidly enough that the camera need not pause between exposures, the camera can be run in this fast streaming mode. Many devices have the ability to use hardware-based, TTL (transistor-transistor logic) triggers. For instance, most scientific grade cameras can be put in “external trigger mode”, in which an exposure or series of exposures will start after receiving a TTL pulse. Other devices, such as a stage controller, can be pre-loaded with a sequence of states (positions in the case of a stage) which the device will switch between on receipt of a TTL trigger.

When using hardware synchronization, a single device takes the role of “master clock” and provides triggers to each of the devices at appropriate time points. Such a master controller can be implemented using a programmable micro-controller. The difficulty lies in interfacing a hardware-triggered microscope setup with a computer software program. For seamless operation, the software needs to be aware of the hardware synchronization wiring, needs to know the expected duration of device movements (alternatively, the devices can send a TTL signal when they finish moving), send appropriate sequences to each of the devices, and interpret the resulting images correctly (i.e., associate them with the correct channel, z- position, etc.). Such synchronization is relatively straight-forward to implement for a specific microscope, using software designed for that specific system. It is much harder to design a generalized system that can facilitate full hardware synchronization yet keep operation simple for the user.

The μManager software therefore implements a simplified approach to hardware synchronization in which the camera (running freely as a video camera) is the master clock driving acquisition. Devices can signal to the software that they are capable of executing a sequence, i.e. change state or position upon receiving a trigger. When the μManager software detects that the user wants to execute an acquisition that can be fully run using hardware triggering it will do so. Acquisition speeds can increase an order of magnitude without extra effort for the user. The downside of this approach is that it can only be used with devices that change state very rapidly, such as diode lasers or AOTF devices, since the TTL signal is generated at the same time as the exposure start. In practice, piezoelectric Z stages can also be used in this approach since they complete most of their movement within a few milliseconds.

**Data storage, file formats and Metadata**

Once acquired, images need to be recorded on digital media for later analysis and display. Data quantity can be enormous: multi-dimensional acquisitions lead to large numbers of images, each a few MB in size. In fact, the latest sCMOS cameras can produce images at a rate of close to 1 GB/s (~ 5 million pixels per image, 2 bytes per pixel, 100 images per second), filling up 1 TB of storage space in about 17 minutes (if the computer hardware and software can sustain that data rate). In addition to the pixel data themselves, the software is aware of a large volume of metadata, such as image dimension, magnification (i.e. pixel size in the object plane), many properties of all devices present in the microscope system, and any other information about the experiment that the user is willing to provide.

Many different file formats and methods to store such data within existing file formats exist. The first factor to consider when storing pixel data is whether or not the pixel data themselves can be faithfully restored. This will be the case when no compression or a “lossless” form of compression is used, however, many image file formats, including the JPEG format, use “lossy” compression algorithms that provide a large reduction in data size by focusing on the ability to

retain the visual appearance of the image rather than the underlying pixel data. Such lossy compression will render the data useless for almost all further analysis, and lossless compression is almost always advisable. Most software packages use their own file formats to save pixel and metadata, which used to make exchange of microscopy image data between software packages cumbersome. To deal with this problem, a group at the University of Wisconsin, Madison has developed a software library, BioFormats (http://loci.wisc.edu/software/bio-formats), capable of reading most microscopy file types. This code is used in a number of software packages, including ImageJ (http://rsb.info.nih.gov/ij/). Not only does BioFormats read pixel data, it also attempts to read as many metadata as possible and converts these to the OME (Open Microscopy Environment) - XML. OME-XML is a proposed standard for microscopy metadata. The image file format read by most applications is TIFF (tagged image file format). TIFF files contain tags that provide information about the pixel data (i.e., TIFF stores metadata in tags). TIFF can store uncompressed pixel data in many different data types (i.e, as bytes, shorts, floats, etc.), color and monochrome, and it can store stacks of images.

## EXAMPLE SCIENTIFIC MICROSCOPES

Here we provide some practical examples of how microscopy systems can be designed and constructed for specific purposes. We do not attempt to provide a comprehensive survey of microscope configurations used for biological imaging, but instead focus on high end, state-of-the-art configurations that push the limits of what can be done with current hardware.

### High Speed Spinning Disk Confocal Microscope

Our first example is a spinning disk confocal system designed for high-speed, low-light imaging of biological samples. Both authors have both constructed systems similar to this and they are primarily used for acquiring multi-channel fluorescence images of tagged proteins in single cells (for examples, see (14, 29)).

As the Yokogawa spinning disk confocal is capable of scanning the entire field of view in 1 ms, these instruments are a natural choice for high speed confocal imaging of biological samples. In the systems described here, the cameras have been chosen to maximize sensitivity. High sensitivity allows imaging of dim, low abundance proteins. It also allows minimizing the exposure time needed for imaging more abundant proteins, maximizing the possible acquisition speed. For these systems, a back-thinned EMCCD was chosen as it has very high quantum efficiency (~90% in the visible) and low effective read noise (<1 $e^-$), due to the electron multiplication gain. These cameras provide the highest signal-to-noise images for very low intensity signals. While there are many back-thinned EMCCD cameras on the market, these use only a few underlying sensors. The most common are from e2v - a 512 x 512 pixel sensor with 16 µm pixels (ccd97, used in the Photometrics Evolve and Andor iXon DU897), and a 1024 x 1024 sensor with 13 µm pixels. A third sensor is made by Hamamatsu (used in their ImageEM camera) and has properties very similar to the 512 x 512 pixel e2v sensor. All of these sensors have similar sensitivity and noise performance; the main differences are in size, speed, and cost.

For this application we have chosen to use the 512x512 pixel e2v ccd97 sensor, primarily because of its high speed. At the time that these microscopes were built, cameras based on this sensor were capable of recording data at 30 frames per second (fps), while newer models can record at 60 fps. By comparison, the 1024 x 1024 sensor can only record at 9 frames per second. To record diffraction-limited images on the ccd97 sensor requires magnifying the sample such that one pixel on the sensor corresponds to have the diffraction limit at the sample. For an oil objective with a numerical aperture of 1.4, this means that one pixel should correspond to a distance of < 100 nm in the sample. The pixel size referred to the sample is related to the camera pixel size by the objective magnification and so achieving diffraction-limited images with the 16 µm pixels of this sensor would require an objective magnification of at

least 160x. Because the maximum magnification of commercial objectives are 100x, we replace the 1x projection lens of the spinning disk confocal scanhead with a longer focal length lens to achieve this higher magnification. In our implementation, the magnification gives a pixel size, referred to the sample, of 91 nm. The main disadvantage to the 512 x 512 sensor is its relatively small field of view. At this magnification, the camera is only capable of imaging a 47 μm square region of the sample. However, this small field of view is a necessary trade off to obtain high speed imaging with an EMCCD camera. With newer sCMOS cameras, this tradeoff is relaxed, at the cost of lower sensitivity.

As described thus far, this microscope is capable of acquiring confocal images at the full speed the camera is capable of. However, acquiring multiple focal planes or multiple fluorescence wavelengths at the same speed requires additional hardware. Conventionally, synchronization of camera acquisition with changing filters, changing focal planes, and so on, is carried out by the control software of the microscope. The control software would first issue commands to the microscope hardware to ensure the filters and stages are in the correct position, and then signal the camera to acquire an image. However, the communication overhead associated with this scheme significantly reduces the acquisition speed of the system, even if the hardware itself is fast. To maximize the speed of multi-color 3D imaging on this system, we first eliminated any slow moving parts, and control as much of the microscope as possible in hardware, eliminating the software communication overhead. The result is a microscope capable of acquiring multi-color Z-stack images at 30 fps.

To maximize the hardware speed, we use a piezoelectric Z-stage to control focus. Such devices have settling times of ~10 msec following small (~1 μm) steps. Because the Z-resolution of a 100x / 1.4 NA objective is ~700 nm, we typically acquire 250 nm steps in Z to ensure Nyquist sampling. This also ensures that any movement during the settling period is smaller than the Z-resolution. We typically use a motorized XY stage with an integrated piezoelectric Z focus. This

allows any objective to be used with the stage (as opposed to piezoelectric objective focus units, which would only motorize a single objective).

Rapid switching of the fluorescence channel is somewhat more complicated. Changing the fluorescence channel usually requires changing both the excitation and detection wavelengths. In the spinning disk confocal, illumination is provided by lasers. These can be switched rapidly, either by using directly modulated lasers or by using an acousto-optical tunable filter (AOTF) to control which laser lines are transmitted. Both AOTFs and directly modulated lasers can be controlled extremely quickly; typical switching times are in the microsecond range. Changing the detection wavelength typically involves movement of different interference filters into the detection path. Even with fast filter wheels, these movements are typically slow, around 50-100 ms. To avoid this slow filter movement, we give up the ability to change detection wavelengths between channels and instead use a single multi-pass emission filter. This filter blocks all four laser lines we use to excite, but passes fluorescence from the four classes of molecules they excite. This eliminates the need to change the emission filter, but does result in crosstalk: if a laser excites more than one dye in the sample, both dyes will be detected. For example, if a 405 nm laser, typically used to excite the blue fluorescent dye DAPI, also excites the green fluorescent protein (GFP), then the GFP will be imaged in the 405 nm channel, which is nominally the DAPI channel. The use of a multi-pass emission filter dramatically speeds up imaging at the cost of some potential crosstalk. This crosstalk potentially results in ambiguity about which dye a given fluorescent signal results from, although this ambiguity can largely be minimized with careful matching of dyes to filters.

To operate this hardware at the full speed the camera is capable of requires a programmable controller. This can either be a simple microcontroller such as an Arduino board or a more complicated system like a National Instruments digital I/O board. The camera is programmed to acquire a sequence of images, and sends a TTL trigger signal to the controller at the beginning

of the exposure of each frame. The controller, in turn, sends signals to the AOTF and the Z-stage to set them to the correct state for each frame. Typically, the light source (AOTF) receives one TTL for each wavelength (to turn it on or off) and the Z stage receives an analog voltage corresponding to the desired position. The controller is preprogrammed by the host computer with the sequence of wavelengths and Z-positions to acquire, and advances through the sequence as each camera trigger is received. Controlling the acquisition through hardware in this manner eliminates software overhead and the speed at which the microscope can operate is limited only by the speed of the hardware.

**High Speed Light Sheet Microscope**

Our second example is another high speed microscope, but one designed for imaging much larger samples (30, 31). This microscope is a light sheet or selective plane illumination microscope (32, 33). It uses an objective to scan a laser beam through the sample at right angles to the objective imaging the sample. The laser beam scanned through the sample generates a light sheet that illuminates a single plane in the sample. This ensures that there is no out-of-focus light produced, but requires synchronizing the position of the light sheet and the focus of the imaging objective (Figure 5). Because of the unusual geometry, with objectives at right angles to each other needing access to the sample, the sample is typically mounted in a capillary tube, rather than on a slide.

The system described here uses tip/tilt mirrors to scan the laser beam that generates the light sheet. These are controlled by analog signals to raster the beam in the X direction to generate the light sheet, and scan the beam more slowly in the Z direction to sweep the light sheet through the sample. The analog ramps that produce these X and Z scans are generated in hardware so that there is no software overhead in producing and translating the light sheet. The imaging objective is mounted on a fast piezoelectric Z-stage, allowing it to be translated to so that the region illuminated by the light sheet is always in focus. The light sheet has a thickness

of ~4 μm, and the imaging objective has is either a 16x / 0.8 NA or 20x / 1.0 NA, so the illumination thickness is large compared to the depth-of-field of the objective.

On this microscope, images are acquired with a 4 MP sCMOS camera (a Hamamatsu Flash 4.0). This camera has 6.5 μm pixels, giving an effective pixel size in the sample of 325 or 406 nm/pixel, This is larger than the diffraction limited resolution of the objectives (250 - 300 nm) which means that the pixel size, not the objective NA, is limiting the resolution of this microscope. However, it is capable of imaging large fields of view (up to 800 x 800 μm). As this microscope has been designed for imaging the zebrafish brain, which is about this size, this is a reasonable design trade off. To achieve the full resolution the objective is capable of would require a pixel size about half that of the Flash 4.0. Sensitive, low noise cameras with this acquisition area and smaller pixels do not exist. Furthermore, such a camera would generate data at a prohibitive rate. The camera used here produces 850 MB of data per second, when operated at full speed; a camera with half the pixel size would generate data at four times the rate. The resulting data stream, 3.4 GB per second, would be very challenging to record and process.

**Localization-based Super-resolution Microscopy**

A major recent advance in microscopy has been the development of super-resolution techniques, defined as those that surpass the conventional resolution limit of 0.61 λ / NA. Super-resolution methods are too diverse to describe in detail here (see (34–37) for reviews), but we will focus on one particular family of methods: those that use localization of single molecules. Initially published under the names STORM (38), PALM (39), and FPALM (40), these methods share two key features to produce a super-resolution image: first, that certain fluorescent molecules can be switched between a fluorescent and non-fluorescent state, and second, that although the image of a single fluorescent molecule will be blurred by the

microscope, the location of that molecule can be measured to much higher precision than the resolution limit.

These techniques work by first labeling a sample with a photoswitchable fluorophore. This can either be a photoconvertible fluorescent protein, which can be switched between fluorescent and non-fluorescent states by illumination at specific (typically UV) wavelengths of light; a fluorescent dye chemically caged so that it is non-fluorescent until UV illumination removes the chemical caging group, or conventional fluorescent dyes driven into a dark state by optical or chemical means. The sample is prepared such that all the molecules in it are in the dark, non-fluorescent state. A small fraction of the molecules are then switched to the fluorescent state, either by specific illumination or by thermal recovery. The fraction of molecules switched to the fluorescent state is very small, so that the images of the individual molecules do not overlap. The molecules are then imaged until they bleach. The image of each molecule can then be fit to determine its centroid, which represents the location of that molecule in the image. The precision with which the centroid can be determined depends on the number of photons collected from the molecule, but is much higher than the resolution limit of the microscope. This process is then repeated tens of thousands of times until typically a million or more single molecules have been imaged and located. The super-resolution image is then constructed by generating an image where each localized molecule is represented by a point or a Gaussian with width comparable to the localization precision. The resulting image typically has a resolution of 20-30 nm, about 10-fold greater than the diffraction limit.

This type of imaging requires little specialized hardware. Typically, laser illumination is used to excite a thin sheet in the sample by illuminating at an oblique angle. High power (~100 mW) lasers are required to bleach the molecules rapidly so that many individual molecules can be recorded in a short time frame, and highly sensitive EMCCD or sCMOS cameras are used to maximize the signal-to-noise ratio and the acquisition speed. The only specialized hardware

required is that some additional optics must be added for 3D imaging. Because the point-spread function of the microscope objective is largely symmetric in the axial (focus) direction, it is difficult to tell whether an observed molecule is above or below to focal plane. To break this symmetry, a controlled aberration is added to the point-spread function. Most simply, this can be done by placing a cylindrical lens in the detection path, adding a small amount of astigmatism to the point-spread function (41). Fitting the image of each molecule then allows determination of its position in Z as well as in X and Y.

This type of imaging uses relatively simple hardware and achieves super-resolution imaging by a combination of carefully chosen sample chemistry (to allow the dye molecules to be switched on and off) and computation (to measure their position from the images). It is representative of other forces driving modern microscopy: not only do hardware improvements lead to development of new techniques, but so do improvements in sample preparation, fluorescent dyes, and computational tools.

**CONCLUSIONS**

Over the last twenty years, biological light microscopy has become fundamentally digital. Essentially all microscopes now record images with digital detectors, and many microscopes involve computer control as an integral part of their operations. Microscope objectives have been operating at the physical limit of resolution for many years; in recent years performance improvements in microscopes have been driven by cameras with higher sensitivity and higher speed and improved hardware integration and automation. Other improvements in microscope performance, such as super-resolution techniques, have been made possible by the tight integration of computer controlled hardware with the microscope optics (as in structured illumination microscopy (20)) or by intensive computational post-processing of the images (as in single molecule localization methods such as STORM and (F)PALM ((37, 42)).

We expect that this integration of hardware and software will continue to grow in future microscope systems. Automation of the microscope hardware will increase, improving the speed and ease with which multi-dimensional data can be collected. Image analysis and image acquisition will become more tightly integrated, allowing microscopes to make decisions about acquisition parameters based on the images collected. This can be used, for instance, to acquire images of cells with particular properties (43) or reduce data size by eliminating uninformative regions of the image (44). Despite this, it is likely that data rates will continue to grow and that storage and management of image data and metadata will become increasingly challenging. Digital imaging has revolutionized biological light microscopy and will play an increasingly large role in microscopy in the future.

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**FIGURE CAPTIONS**

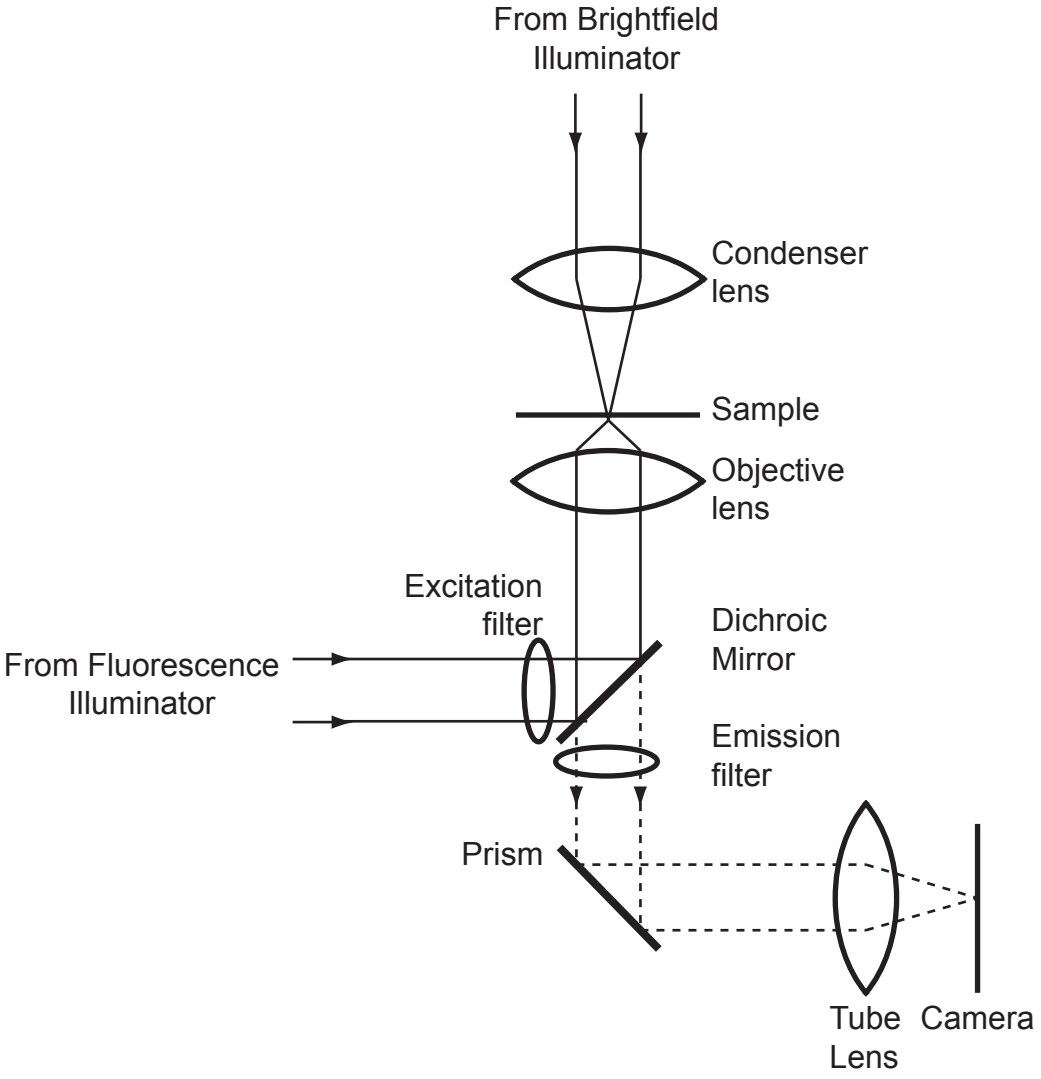
Figure 1: A schematic drawing of an inverted epi-fluorescence microscope. “Inverted” means that the objective lens images the sample from below; epi-fluorescence means that the objective lens is used to both illuminate the sample with excitation light and collect emitted light from the sample. A tube lens focuses the collected light onto the camera.The excitation filter selects the wavelength band that excites fluorescence in the sample; the emission filter selects the band of fluorescent wavelengths that are detected from the sample. The dichroic mirror reflects the excitation light and transmits the emission light. These three components are often combined into a single filter cube; alternatively, each one can be placed in a separate motorized wheel. The prism can be rotated to direct light to different optical paths, such as different cameras, or to the eyepieces. The condenser lens illuminates the sample for transmitted light (brightfield) imaging.

Figure 2: Examples of widefield fluorescence microscopes. An upright microscope (Nikon AZ100) is shown on the left and an inverted microscope (Nikon Ti) is shown on the right. Components of the two microscopes are labeled. The AZ100 is a zoom microscope designed for low magnification imaging in both brightfield and fluorescence. The Ti is a fully motorized inverted microscope for both brightfield and fluorescence imaging. The AZ100 does not have a camera or fluorescence illuminator attached to it and the positions where these would mount are labeled. The fluorescence illuminator on the Ti is connected by means of a liquid light guide. The point at which the light guide connects to the microscope is labeled; the actual light source is located away from the microscope.

Figure 3: A schematic of a laser-scanning confocal microscope. The objective and tube lens are the same as in an ordinary microscope. A scan lens generates a conjugate back focal plane of the objective, where scan mirrors are placed such that rotation of the scan mirror leads to translation of the focal spot in the image plane. Illumination is produced by collimating the output of a fiber optic or pinhole and detection is through a pinhole in a conjugate image plane. Because the scan mirror affects both excitation and emission light identically the focal spot is always conjugate to the pinhole regardless of the mirror position. The pinhole blocks out-of-focus light, thereby ensuring that only in focus information is recorded.

Figure 4: Examples of confocal microscopes. A laser-scanning confocal (Nikon C1si) mounted on an upright microscope (left) and a spinning disk confocal (Yokogawa CSU-22) mounted on an inverted microscope (right). In both cases the laser sources are connected to the confocal by optical fiber and are not seen in the image. The laser scanning confocal is coupled to its detectors by optical fiber as well; the spinning disk confocal images onto a camera located off the left edge of the image. An emission filter wheel is located between the spinning disk confocal and the camera. The plastic box surrounding the spinning disk confocal microscope provides temperature control for imaging live samples.

Figure 5: A schematic of the light sheet microscope described in the text. A laser beam illuminates the sample from either the left or the right side. It is focused and scanned vertically to generate a light sheet illuminating a single plane in the sample. A detection objective at right angles to the illumination objective records light emitted from the illuminated region. This arrangement ensures that only the imaged field of view is illuminated. Translation of the imaging objective and the light sheet are synchronized to acquire volumetric data. The sample is mounted in an agarose cylinder and can be rotated if necessary.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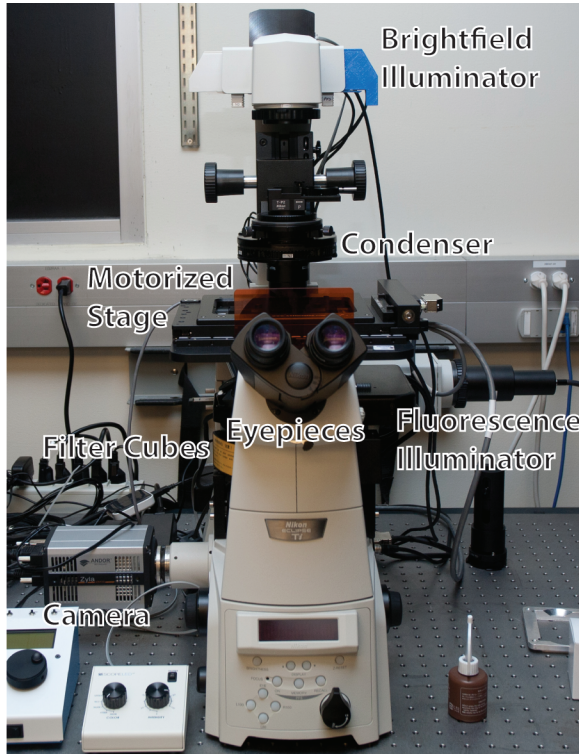
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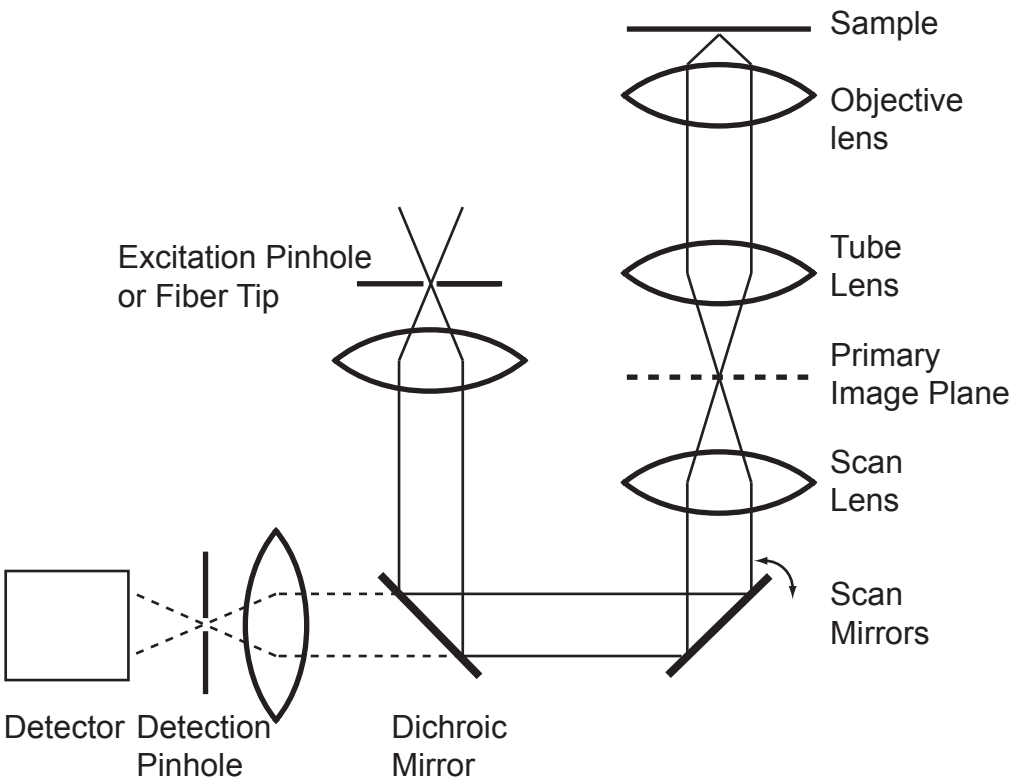
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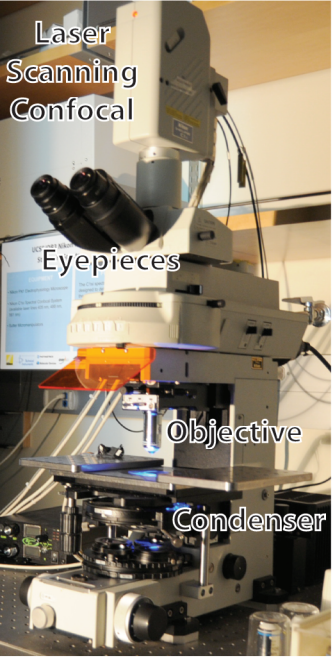
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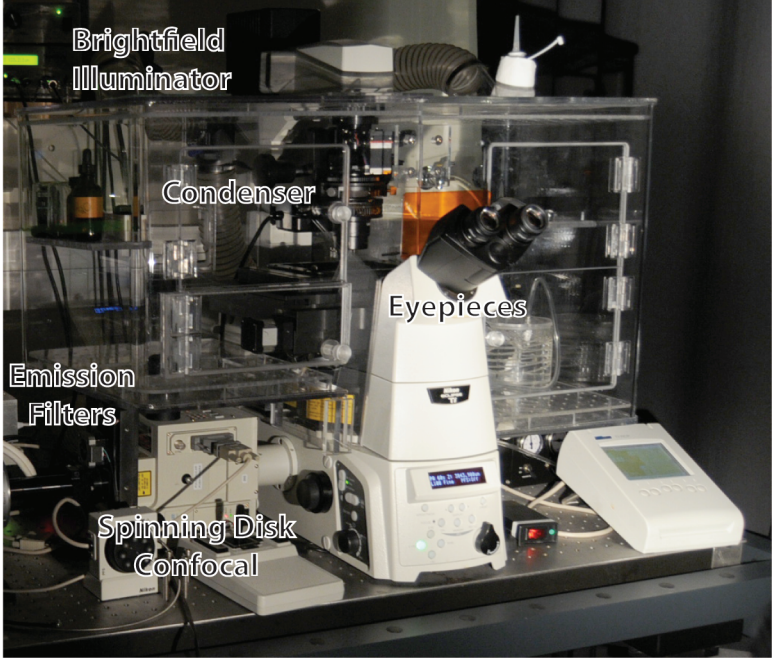
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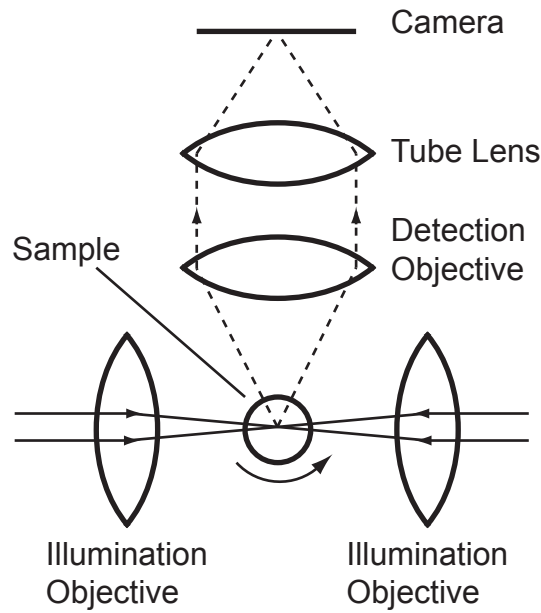
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