# Light Microscopy Digital Imaging



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## ABSTRACT

This unit presents an overview of digital imaging hardware used in light microscopy. CMOS, CCD, and EMCCDs are the primary sensors used. The strengths and weaknesses of each define the primary applications for these sensors. Sensor architecture and formats are also reviewed. Color camera design strategies and sensor window cleaning are also described in the unit. *Curr. Protoc. Cytom.* 46:2.3.1-2.3.10. © 2008 by John Wiley & Sons, Inc.

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## HISTORY OF MICROSCOPY IMAGE CAPTURE

Prior to the 1980s, still capture of microscope images required a 35-mm, or more rarely, a large-format film camera. If the proper conditions and exposure time were observed, film images recorded close to all of the resolution the microscope could offer within the captured field of view. Rarely, 16-mm movie cameras captured moving specimens, usually for educational purposes. By the late 1970s, vacuum tube (Fig. 2.3.1) video cameras were beginning to be used to capture monochrome images using videotape, with significantly greater control over the camera's gain and black level, enabling microscopists to match the dynamic range of the camera to the specimen contrast range. Frame grabbing boards, popularized following the advent of the personal computer, allowed digitization of single images and, later, image streams, which could be stored, copied, and printed without loss of the original image quality.

Even though solid state–type video sensors eclipsed tube-type cameras, both were limited by the 525 horizontal lines specified by the U.S. television standard that ensured compatibility with peripheral devices such as printers, video recorders, and monitors. As higher resolution, more sensitive sensors became available, more capable cameras in a variety of formats allowed imaging for a much wider range of applications.

## SOLID-STATE SENSORS

The solid-state sensors of today grew out of bubble memory research at Bell Labs in the 1950s. Most of these devices possess a matrix of light-sensitive units built up on a silicon monocrystalline substrate. Called pixels, these units independently change incident light to an electrical charge. The amplified charge passes to the analog-to-digital converter (ADC), where gray-level values are assigned proportional to the quantity of incident light. Most of these sensors are either complimentary metal oxide semiconductors (CMOS), charge-coupled devices (CCD), or electron multiplication charged-coupled devices (EMCCD), each with their own application-related strengths and weaknesses.

Lower cost CMOS sensors with a color mosaic filter perform well as photodocumentation cameras. Low power consumption allows powering, camera control, and image transfer to a personal computer via a USB port. High-pixel-count cameras, with the proper microscope adapter, record all of the image resolution in the field covered, while maintaining a sufficient refresh rate to allow focusing. As development continues, the application palette covered by CMOS sensors can only increase. The higher fill factor, quantum efficiency, and lower noise characteristics of CCD and EMCCD make them better-suited for low-light applications.

CCD's higher fill factor, integration with microlens arrays, and low-charge diffusion rates produce higher quantum efficiencies, greater full well capacity, and lower thermal noise (dark current). CCD pixels must transfer their charge thousands of times before serial digitization. CCDs achieve a 99.999% or greater transfer efficiency, preventing signal degradation during transfer. Some CMOS have a digitization transistor at each pixel,



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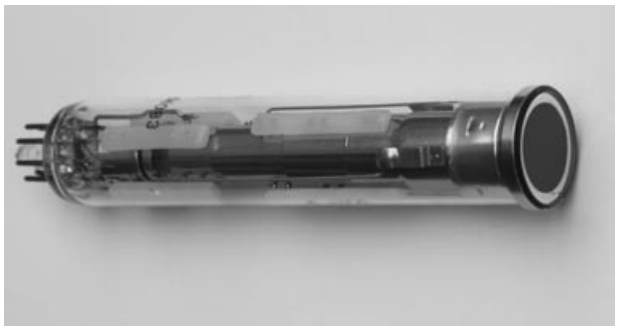

**Figure 2.3.1** Vacuum tube ½-in. image sensor.

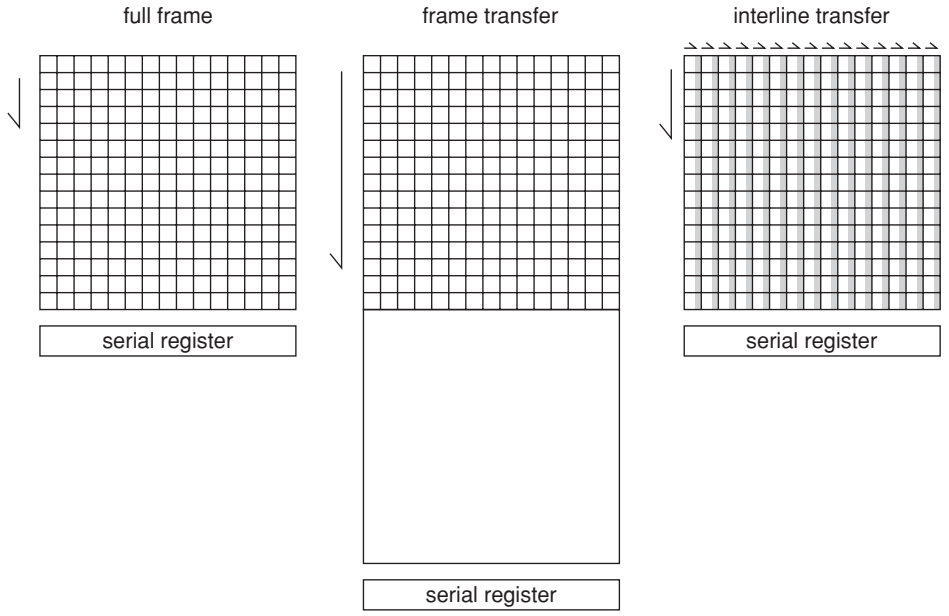


**Figure 2.3.2** Charge-coupled device architecture.

which only needs a single transfer. Variations in each of these transistors produces fixed pattern noise, which is often mitigated by downstream signal processing. Since the 1990s, CMOS technology has improved significantly, while maintaining low costs.

There are three different CCD architectures. Each provides different strategies for transferring the charge to the serial register prior to being read in the ADC.

The full-frame sensors (Fig. 2.3.2) have fill factors approaching 100%. Fill factor is the percentage of active area of the chip that can turn photons into electrons. After exposure, the sensor shifts all charges like foot soldiers in formation down one row. The transfer of all charges by one row puts the bottom row of charge packets into the serial register and leaves the top row empty of charge. Now only the serial register is read out in single file, digitizing each charge packet. Again the remaining charge matrix shifts down, repeating the process until all charges are read out. Pixels remain light sensitive during the shift process so the sensor must be dark during readout. Typically, a mechanical shutter in front of the sensor or at the light source is synchronized with the readout timing. A shutter near the sensor must have an aperture larger than the sensor's active area and may require many tens of milliseconds to open and close. Cameras with full-frame sensors and integrated shutters are often used in applications that require very long exposure, and readout times benefit from the maximized fill factor. The milliseconds required to operate the shutter are negligible in comparison to the several-second exposure.

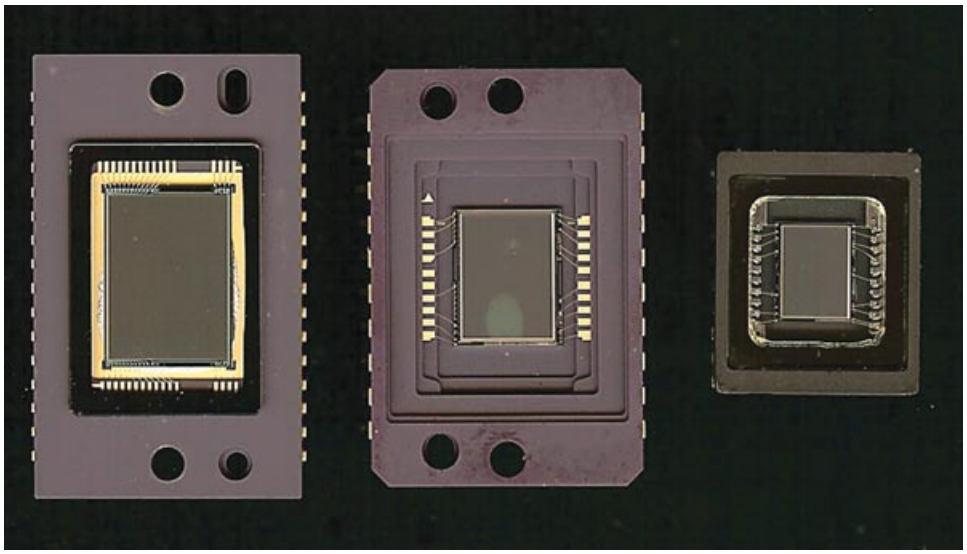

**Figure 2.3.3** Interline transfer sensors.

Frame-transfer sensors are divided into two halves. The active area, as in the full-frame sensor, collects photons, converting them into electrical charge. The other half is an opaque copy of the active area. At the end of the exposure in the active region, the entire charge matrix shifts to the masked portion of the CCD. During this shift to the mask, no readout occurs; as a result the shift takes a millisecond or less. The charge matrix under the masked region now transfers a row at a time to the serial register and is digitized. A second exposure may proceed even though the first image, now under the mask, is still being read out, so image pairs may be captured within a very short time interval.

Interline transfer sensors (Figs. 2.3.2 and 2.3.3) are the most common and possess masked readout pixel rows between each active row of pixels. Following exposure, the charge of all pixels shifts to the dark interline region simultaneously. Once in the interline pixels, the charge matrix shifts toward the serial register and is passed to the ADC for digitization. Under some conditions, the next exposure can begin while the masked interline region reads out.

## SPECTRAL SENSITIVITY OF SENSORS

Quantum Efficiency (QE) quantifies the ability of a sensor to change photons to electrons. As Figure 2.3.4 shows, this efficiency is wavelength dependent. In the case of interline transfer CCDs and CMOS sensors, opaque components on the surface block some of the incident light from reaching the light-sensitive area. The portion of unblocked area is called fill factor and may only be 45% or less. Microlens arrays diffract light that would otherwise fall on opaque segments into the light-sensitive region. Although some fluorochrome emission wavelengths extend to 1000 nm, few biological imaging techniques require imaging <400 nm, where the QEs of most CCDs drop off rapidly. More common in nonbiological imaging, the short wavelength QE may be extended by camera manufacturers with the application of a phosphor over the sensor, which excites in the UV and emits at visible wavelengths where the CCD is naturally more sensitive. This phosphor, called Metachrome II or Lumogen, is largely transparent at visible wavelengths, causing only a nominal reduction of sensitivity at these wavelengths when applied to full-frame or frame-transfer sensors. The phosphor is applied directly to the active area of the CCD. Interline transfer sensors must have the microlens array removed to allow direct contact with the sensor's active area. As a result, the 450 to 800 nm QE is greatly reduced for phosphor-coated interline sensors.

## MATING DIGITAL CAMERAS TO MICROSCOPES

It is curious that a sensor format does not possess any dimensions that exemplify that measurement. A $^1/_2$-in. format sensor, for example, has no definable feature that is $^1/_2$-in. in size. This apparent mismatch originated with vacuum tube sensors. Geometric

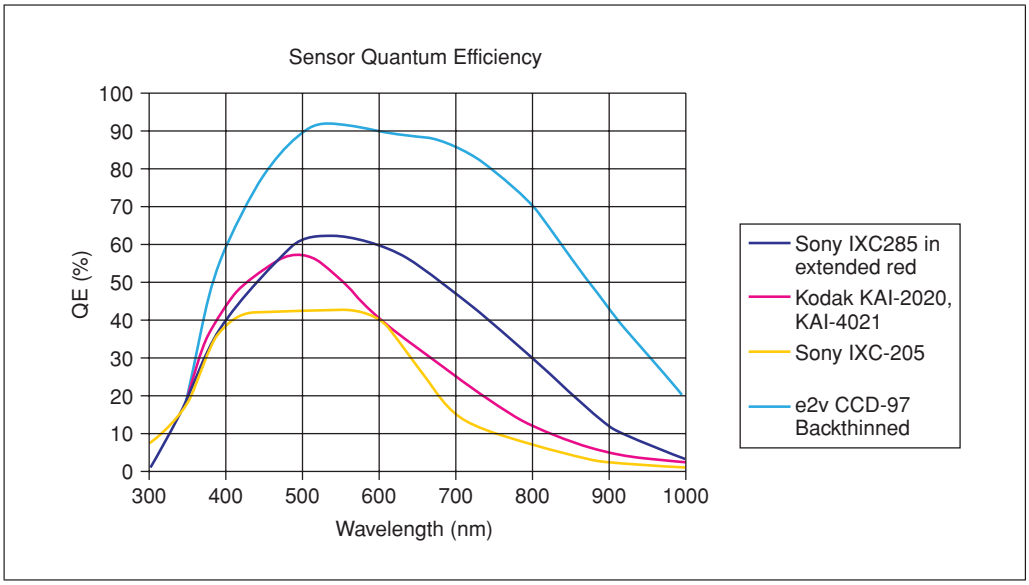


**Figure 2.3.4** Sensor quantum efficiency. See Table 2.3.1 for data. For color version of this figure, go to *http://www.currentprotocols.com*.

**Table 2.3.1** Sensor Quantum Efficiency[a]

| | Sony IXC285 in extended red | Kodak KAI-2020, KAI4021 | Sony IXC-205 | e2v CCD-97 backthinned |
|---|---|---|---|---|
| Wavelength (nm) | QE (%) | | | |
| 300 | 0 | 0 | 7 | 0 |
| 350 | 19 | 21 | 17 | 20 |
| 400 | 40 | 44 | 39 | 60 |
| 500 | 61 | 57 | 42 | 90 |
| 600 | 60 | 40 | 40 | 90 |
| 700 | 47 | 25 | 15 | 86 |
| 800 | 30 | 12 | 7 | 70 |
| 900 | 15 | 5 | 2 | 43 |
| 1000 | 3 | 2 | 1 | 20 |

[a]See Figure 2.3.4 for graphical representation of the above data.

distortion and phosphor defects naturally increased toward the outside of the tube's circular photosensitive area (Fig. 2.3.1). To minimize these effects the rectangular active area scanned was always well inside the tube's total circular area. Generally, the diagonal of the scanned area on the tube face was roughly 2/3 of the diameter of the tube. A 1-in. tube, for example, had an active area with a 0.63-in. (16 mm) diagonal. Since most images were displayed on 4:3 rectangular format monitors, the electron beam would scan a 4:3 aspect ratio rectangle inscribed within the tube face. As CCD solid state devices replaced vacuum tube pick up devices, manufacturers maintained the same naming convention, even for sensors that vary from the traditional 4:3 aspect ratio (Fig. 2.3.5). In recent years, the sensor manufacturers have dropped the format designation.

The microscope-to-digital camera adapter may contain optics allowing one of a variety of magnification values. While viewing a live digital image on a monitor, a lower magnification microscope adapter provides a field of view closer to that viewed through the eyepieces D. The 2/3-in. sensor, for example, has an 11-mm diagonal. This is the longest linear imaging dimension on the actual sensor. Most microscope eyepieces designate the magnification and the field of view index. An eyepiece might be inscribed with "10×/25,"

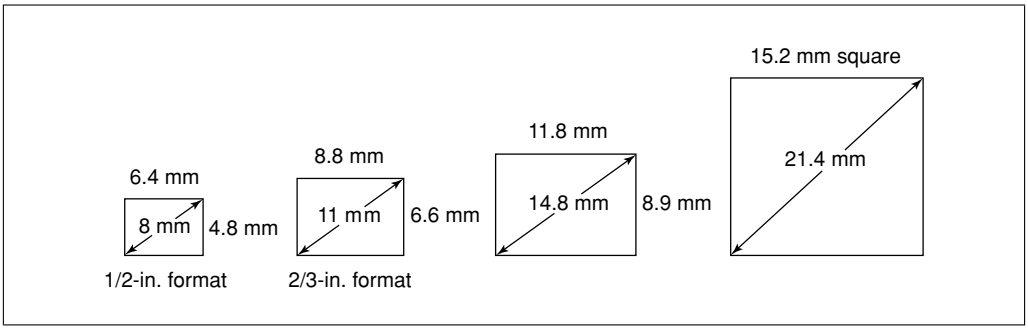


**Figure 2.3.5** CCD and CMOS sensor formats.

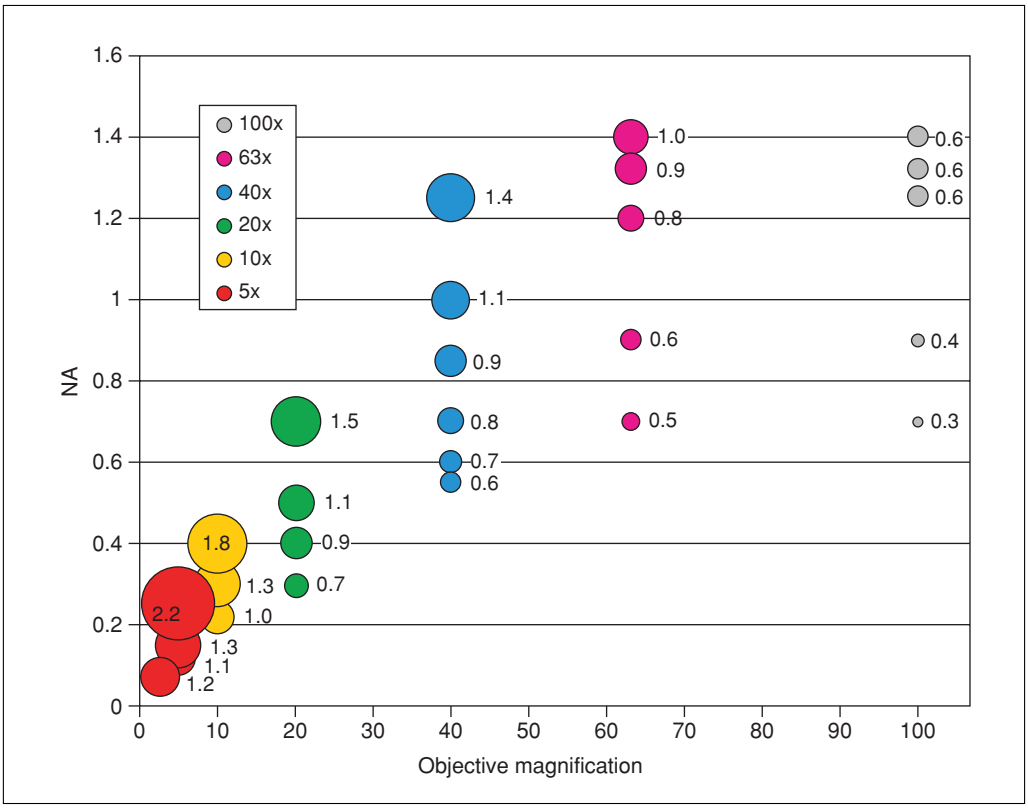


**Figure 2.3.6** Optimal microscope C-mount magnifications for 6 to 7 μm pixel cameras using 2.3 pixels/resolving element. Abbreviations: NA, numerical aperture. For color version of this figure, go to *http://www.currentprotocols.com*.

showing that it has a 25-mm field of view index. This is the size of the intermediate image plane projected into the eyepiece by the microscope objective and infinity tube lens. Using a 1× microscope-to-camera coupler, the light projects to the sensor as above. The digital image diagonal will be 11/25$^{th}$ of the visible image diameter observed in the eyepieces. Lower magnification camera adapters allow larger fields of view to be imaged by the sensor. If a sensor with an 11-mm diagonal connects to a microscope with a 0.7× magnification, the resultant digital field diagonal expands to 11 mm/0.7 = 15.7 mm. It sometimes seems desirable to electronically image as much of the field as is directly seen in the eyepieces. This is especially important if a single image needs to represent an entire organism, or for scanning for features visible at a lower resolution. Lowering the microscope coupler magnification spreads the sensor out over more of the sample and effectively lowers the sampling resolution of the final image. Although it is easy to compare the eyepiece field of view with the digital field of view, it is more difficult to determine the optimum coupler magnification in terms of resolution. Figure 2.3.6 shows the calculated mount magnifications. Using less than the optimal mount magnification is only relevant if image detail exists close to the resolution limit of the objective. Continual

swapping of couplers is generally unnecessary since microscopists can just go to the next higher magnification objective to see additional image detail. Using couplers at magnifications higher than those recommended below oversamples the image, and this often occurs with a 100× oil immersion objective.

Most digital cameras made for microscopy possess a C-mount threaded hole or bayonet Nikon 35-mm F-mount interface. When analog tube-type video cameras began to be used instead of 16-mm movie cameras, the cinematography lens mount (Cine mount or C mount) name was maintained. That is, the same C-mount threads in cameras receive microscope C-mount couplers that match the intended microscope. The 1-in. diameter thread has 32 threads per inch. The sensor is 17.526-mm deep inside the outside face of the C mount on a camera. Filters or filter wheels placed in front of the sensor on a camera may need more than the 17 mm allowed on a C-mount camera. In this case a Nikon F type 35-mm mount is used.

## COLOR IMAGING

One of several strategies allows a sensor or camera to distinguish color within a scene.

**Color Mosaic:** A repeating pattern of filters are evaporated over the pixels. Common patterns are red (R), green (G), blue (B) with a RGRG. . . repeating row followed by a GBGB. . . row.

Other color filter strategies include cyan, magenta, yellow, and cyan, magenta, and white.

The raw signal readout for each pixel only represents a single color. Post processing interpolates neighboring pixels, providing the other two color values.

The frame rate of a monochrome and color mosaic sensor is usually the same. The resolution loss is generally considered to be ∼30%, but varies with the color characteristics of the specimen. Images of uncolored objects, such as some embryos, experience little or no resolution loss compared to that monochrome sensor. Since the filters absorb most of the light that does not match their own color, mosaic sensors are not well-suited for fluorescence microscopy.

**Scanning Color Mosaic:** Using the same color mosaic sensor, this implementation more than compensates for the resolution loss by attachment of piezoelectric stepper motors. Oriented orthogonally, these motors move the sensor across the image in 1 to 1/3$^{rd}$ pixel steps, capturing image data at each step. The final image results from reassembly of the 4 to 16 exposures. The time between individual raw captures may be a few milliseconds, so this method requires stationary or slow moving specimens. The higher frame rate is available by turning off the higher resolution stepped capture. These color cameras are especially beneficial on compound microscopes with high numerical aperture, middle- and low-magnification objectives that produce high-resolution density images.

Each exposure employing mosaic color filter sensors, captures R, G, and B information simultaneously. The brightness of a 12-V, 100-W halogen-illuminated brightfield microscope may cause a user to leave the voltage control low. A microscope at this low color temperature, without a daylight conversion filter, produces a red light component an order of magnitude more intense than the blue light. Mosaic cameras under these conditions achieve neutral or white balance by amplifying the blue and green components, increasing noise and degrading the image. Set the light source voltage to 9 to 10.5 V and use a daylight conversion filter to approach proper color balance in the microscope before the light gets to the camera. Use neutral density filters if the optical image is too bright to the eyes.

**RGB Tunable Filter Color:** A solid-state RGB tunable filter can sequentially let the red, green, and blue components of the image fall on the sensor. Either as an add-on accessory or integrated into the camera, an RGB tunable filter is introduced between the sensor and the camera mount. Although the RGB tunable filter is related to those found in flat panel monitors, the entire RGB tunable filter changes to red, green, or blue filtration with a change in control voltage that is synchronized with the capture of three sequential images. The raw data are full resolution red, green, and blue images. The image seen and stored on the PC is a single composite of the R, G, and B images. Like the scanning color mosaic technology, multiple captures for each final image means objects in the field cannot be moving quickly. The time it takes to capture a composite is the sum of the three exposures plus 2 msec, as 1 msec each is needed to change to R to G and G to B. Like LCD monitors, an RGB tunable filter emits plane polarized light. Orientation of the preferred polarization direction of the RGB tunable filter with the analyzer in the microscope ensures efficient operation. Some camera-only optical systems employ the RGB tunable filter as the system analyzer. Many cameras using an RGB tunable

filter allow the filter to slide out of the optical path, allowing unfiltered light to incident the sensor for low-light imaging.

**Color Filter Wheel:** A filter wheel with red, green, and blue positions can also capture images sequentially, producing a color composite. This stepper motor-driven wheel may be designed into the camera or mounted as a software driven accessory between the transmitted light source and the microscope. Either filter wheel benefits from a fourth open position. In integrated designs the open position enables low-light imaging to the unobstructed sensor, and the light-source-mounted wheel allows white light viewing through the eyepieces.

## CAMERA AND SENSOR CHARACTERISTICS

**Analog gain:** Signal amplification prior to digitization. It may be up to a 30:1 ratio and determines the number of photoelectrons needed to achieve a gray level.

**Backthinned:** A sensor that is imaged on the opposite side of the sensor increasing the fill factor. This side is thinned by etching to get the photosensitive side close to the surface.

**Binning:** A process in which four or more pixels are grouped together on readout to achieve a higher signal-to-noise image at the expense of resolution. It is frequently used with 6- and 7-μm pixel cameras used in live-cell imaging.

**Blooming:** The growth of a bright area on an image that affects more pixels than the ones receiving too much light. When pixels receive their full well capacity between exposures the camera output indicates those pixels at maximum brightness. If an exposure allows a quantity of light that exceeds the full well capacity, those photons continue to convert to electrons. Those extra electrons may spill into adjacent unsaturated pixels enlarging the apparent saturated region of the sensor and therefore the image. Depending on the sensor, unprotected overflow sends the excess charge down the path of least resistance. Often this is down a row of pixels saturating the entire row. Antiblooming features in many CCDs drain off the oversaturated pixels, reducing the spread of saturated areas.

**Dark Current:** The signal accumulated in each pixel due to thermal excitation with no incident photons. It is quantified in values of electrons per pixel per second. The square root of the dark current gives the thermal noise or dark noise. Depending on the sensor architecture and manufacturer, the dark current decreases by half for every 7°C drop in sensor temperature. Cooled sensors with lower dark current allow for overall lower noise, even during long exposures required for low light samples. Carefully cap the camera and save dark images at 1, 10, and 100 msec, and 1, 10, and 100 sec. Progressive increases in signal level with exposure time should represent thermal noise.

**EMCCD:** A specialized CCD sensor that has a light amplification section under the pixel region. Since this amplification occurs before the read amplifier, sensitivity is increased two or three orders of magnitude without any increase in read noise. EMCCD cameras fit well into low-light applications that require a moderate to high frame rate.

**EM Gain:** The maximum amount of gain multiplication prior to readout in an EM camera.

**EM noise (or excess noise factor):** The noise caused by the probabilistic nature of the electron multiplication process in EMCCDs.

**Frame Rate:** The refresh rate of the camera. This is measured in the number of frames that can be read out or displayed per second.

**Read Noise:** The variation in apparent signal as a result of the digitization process. It is measured in electrons. Slower digitization speeds produce lower read noise values.

**Read Out Frequency:** This is the speed of digitization measured in Megahertz (MHz).

**Latent Charge:** A portion of the electrical charge that is not transferred out of CCD pixels during the transfer process, resulting in lower intensity values for these pixels. It more commonly occurs in deeply cooled sensors.

**Pixel Size:** Pixel-to-pixel linear spacing.

**Shot Noise:** The sampling uncertainty due to Poisson nature of the detected light itself. It is approximated by the square root of the signal. The only way to minimize this uncertainty is to collect as much signal as allowed without saturating pixels important to the image. In standard, non-EMCCD cameras this noise value dominates, except in photon-starved signal collection.

**Full Well Capacity:** The number of electrons required to fully charge a pixel. Larger pixels typically allow larger full well capacity and offer increased signal-to-noise ratios. The full well capacity is one of the most important factors in light quantification applications.

## MODES OF IMAGE CAPTURE

Photodocumentation is the collection of single images representative of the view in the eyepieces, generally in the context of

bright-field microscopy. Often accomplished with a higher-pixel-count color camera, the images are usually 24 bit (8 bits of 3 colors each).

Low-light imaging involves collection with very little signal available at the sensor. Fluorescence microscopy, the most common low light application, usually employs a cooled monochrome camera. Multiple fluorochrome emissions are often captured sequentially with a change in fluorescence filters between each capture. Simultaneous capture of multiple emissions is possible using external dichromatic filter devices such as the DualCam, QuadView, and DualView. Capture software provides each image with the appropriate emission hue and overlays the images into a single-color image composite.

Streaming is the collection of sequential images as fast as the camera can provide them to the PC. Longer time exposures required on low light specimens can greatly exceed the readout time of a frame, slowing the frame rate. In this case, EMCCD cameras can multiply the electron charge before readout in order to maintain a high frame rate.

Time lapse is a collection of a sequence of images that are separated by a time interval. A wide variety of cameras may be used in streaming and time lapse depending on the light availability of the specimen and the desired temporal resolution.

Photometry is the collection of light for the purpose of quantifying light intensities. Ratiometric techniques allow quantification of ion concentrations. A camera with wide and linear dynamic range assists in photometric techniques.

## MICROSCOPE OPTIMIZATION FOR DIGITAL IMAGING

There are a few microscope considerations beyond selection of the proper C-mount adapter. For instance, always store a picture of a stage micrometer for each objective magnification. Even photos not intended for geometric measurement may require knowledge of scale in the future.

Careful use of the illumination field and aperture diaphragm is critical for low-light imaging fluorescence because optics may back reflect and autofluoresce, causing stray light to degrade the image. Background, especially, can be reduced by centering and restricting the illumination area to that just outside of the sensor's capture area with field diaphragm. The only danger in setting this too tightly is that in a multiple wavelength capture, changing the microscope fluorescence filter cube can change the relative position of that diaphragm to the field of view. The field diaphragm tightly restricted to just outside the sensor capture region with one filter cube may cross the field with another cube, so check that this diaphragm will allow the entire FOV to be captured by the camera at all filter cube positions. The illumination aperture diaphragm should match or be just below the aperture of the objective. This can be set by viewing a nonfading specimen with the aperture diaphragm fully closed. As the diaphragm is opened, stop at the smallest opening that does not produce a brightness increase in fluorescence. During all but the brightest fluorescence capture, flip out the high numerical aperture condenser top elements. They often fluoresce brightly under UV excitation and add to the background.

Adjust objective correction collars in fluorescence mode only. Find a dark area immediately adjacent to a fluorescing region and adjust the collar to maximum darkness in the darker area. In fluorescence, avoid phase contrast objectives that reduce excitation and emission light and all but eliminate features that match the phase ring's special frequency. Since the emission brightness increases with the numerical aperture (NA) to the fourth power and decreases with the square of the magnification, maximizing NA will yield bright images that require shorter or less exposure. Figure 2.3.7 shows the fluorescence brightness comparison of objectives considering only NA and magnification.

Microscope fluorescence filter cubes employ an exciter filter and dichromatic beam splitter for illumination and the same dichromatic and barrier filter for emission. As researchers attempt to image cells with less and less fluorescence emission, the relative quantity of system auto fluorescence and out-of-bandpass light increases. Even though these filters may exceed out-of-band blocking by $10^6$, some direct illumination at unintended wavelengths may arrive at the sensor and degrade or obscure the intended image. For UV, blue, or green excitation, additional blocking of red and IR wavelengths with a red suppression filter in the illumination path may improve image quality. Some excitation filter stacks now include this as the first filter. Removable IR blocking filters often are needed as a camera accessory as well. Figure 2.3.8A exemplifies an extreme condition of IR that has leaked through the excitation and emission filters. The red suppression filter is added

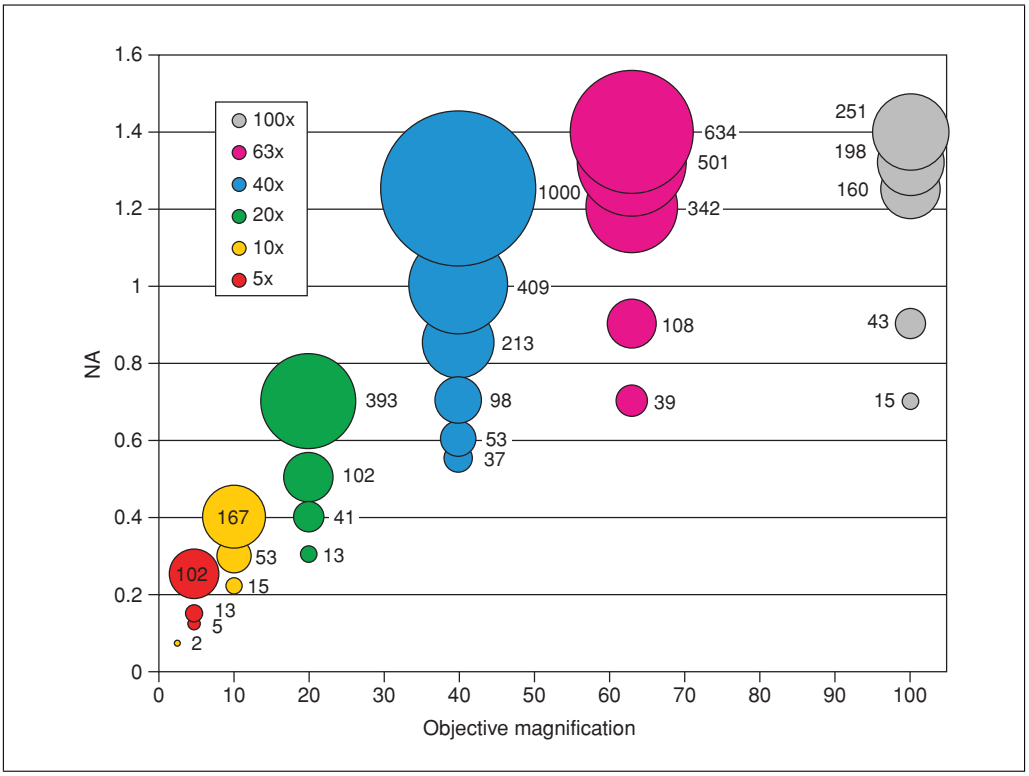


**Figure 2.3.7** Relative fluorescence image brightness comparison of objective lenses. Objective lens magnification and NA effects of fluorescence brightness. Abbreviations: NA, numerical aperture. For color version of this figure, go to *http://www.currentprotocols.com*.

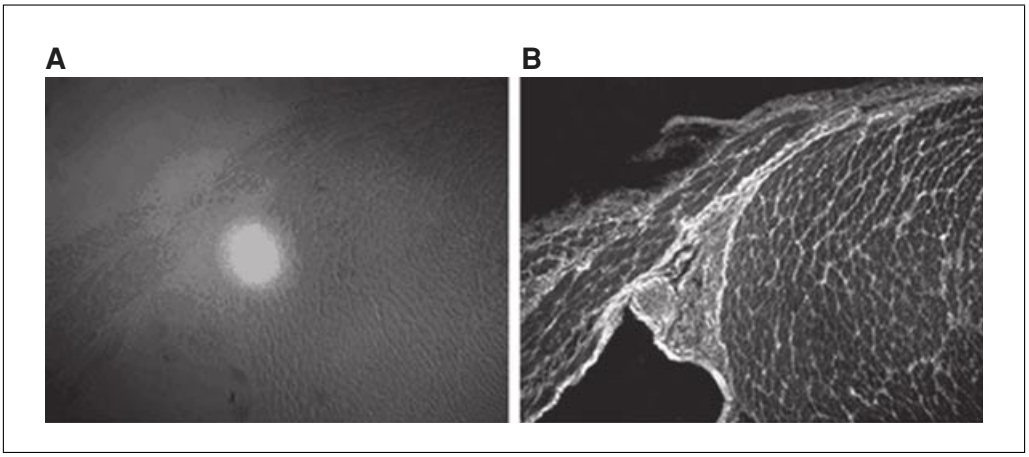


**Figure 2.3.8** Tissue section images showing IR leakage without IR blocking (**A**) and with IR blocking filter (**B**).

in Figure 2.3.8B, revealing the only fluorescence emission.

The only difference between the two images is the addition of the red suppression filter (shown in Fig. 2.3.8B) and the recalculation of the exposure time.

## CARE AND MAINTENANCE

Unless operating in harsh environments, most camera care consists of keeping the image window clean. Virtually all camera sensors possess an image window made of an optical flat with antireflection coating. Keeping this flat clean requires the same care used for coated optical surfaces. This is especially important because this flat, or coverslip, is immediately adjacent to the CCD. Particles on the window will appear to be in the image plane, especially with higher magnification, higher numerical aperture objectives. Although ammonia-based cleaners should be avoided, abrasion rather than chemical attack commonly causes the most harm. Do not clean the window unless material that diminishes

image quality exists on the window. Contaminants visible on the image reveal their location in the microscope by rotating when the optical component possessing the contaminant is rotated. In this case, if the contaminant rotates, then that component is the location. Though easily accomplished with condenser caps and objective lenses, localizing contaminants on the C-mount adapter requires that the camera be held while the adapter is rotated. Unlike particles in the microscope, particles on the sensor or sensor window will not rotate with camera rotation, also revealing their location. As C-mount microscope adapters are unscrewed and remounted, metal particles released from those threads tend to find their way to the sensor window. Clean threads with cotton swabs or fabric. Do so in a manner that allows dislodged metal shavings to fall out of, rather than into the mount. Gently remove particulate matter with compressed micro-filtered air. Compressed gas often contains lubricants that are intended to stay in the can but often find their way out. Test compressed gas by spraying on a clean glass slide. Although labeled "compressed gas," these cans will emit a stream of very cold ($-30^\circ$ to $-70^\circ$C) liquid if the orientation is off of vertical. Windows or other camera components rarely survive instantaneous thermal contraction caused by such a cold liquid. If a residue remains after the liquid evaporates, avoid use of that product for cleaning of microscopes or cameras. If particles remain after a few puffs of gas, a soft artist brush may dislodge them. If not, slightly moisten lens paper with a small amount of distilled water and swipe the window gently, followed by additional gentle dry swipes to pull up the excess water. Oils require organic solvents. If so, sparingly use naptha or ethanol without letting any excess drip into the camera body. If cotton swabs are used to roll up or drag the wet lens tissue across the flat, ensure that it is the type that has no dissolvable glues typically used to attach cotton to the stick.

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