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DETECTORS FOR FLUORESCENCE MICROSCOPY

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Abstract

The low light levels originating from living cells viewed in the fluorescence microscope place significant limitations on the spatial and temporal resolution which can be achieved. The development of intensified video cameras has enabled the detection, visualization and measurement of these low level signals. The performance characteristics of popular intensified video cameras are compared and guidelines are given for the selection of the appropriate detector for various experimental requirements. Intensified or cooled CCD cameras appear to be the most suitable device for quantitative imaging at low light levels in fluorescence microscopy.

Key Words: Low Light Imaging, Image Intensifiers, Photomultipliers, Resolution, Response Speed, Intensified Video Cameras.

Introduction

When fluorescent probes are illuminated in the presence of oxygen, bleaching and photodynamic damage result which are a function of the number of fluorescent molecules excited by the illuminating beam. Two approaches have been used to reduce bleaching: (1) removal of oxygen; (2) reduction of the intensity, or duration of the illumination. Although the addition of antioxidants to the mounting medium for fixed cells effectively reduces bleaching (Giloh and Sedat, 1982), this approach is unacceptable for studies on living preparations. Investigators are left only with reduction of excitation light intensity as a practical solution to the problem. The extent of bleaching is approximately proportional to the integral of the exciting light flux (Foskett, 1985; Plant et al, 1985; Wells et al, 1989), and the investigator must balance illumination intensity and duration to acquire the desired image with a minimum of damage to the preparation. With very low intensity excitation light the number of photons emitted by the fluorochrome is so limited that the resultant image is of poor quality. I will describe the general approach to measurements of fluorescence intensity and to the acquisition of low light level video images in microscopy.

Detector Properties

Photomultiplier Tubes as Detectors for Fluorescence Microscopy:

It is clear from decades of experience with photomultiplier tubes that they constitute an ideal photosensor for fluorescence microscopy when spatial resolution is not a factor. Photomultiplier tubes have a number of highly desirable characteristics: 1) stability, 2) low noise, 3) very large dynamic range (as much as 1 million fold), 4) sensitivity, 5) wide range of spectral response, 6) rapid response, 7) small physical size, 8) low cost. Whenever possible, it is highly desirable to make intensity measurements with a photomultiplier tube rather than an imaging detector. If measurements of a uniformly stained field are to be

made, there is no obvious value in employing an imaging detector. An important, and often overlooked, virtue of the photomultiplier tube stems from the modest data rate produced by the device. Instead of dealing with a quarter megabyte image every 33 ms, the data acquisition system is confronted by a single stream of voltage or current data at typical rates below 1 kHz. The combined use of a photomultiplier tube and a video camera enables monitoring specimen focus and position while the intensity data is acquired (Foskett, 1985). Photomultiplier tubes form the imaging detector in laser scan confocal microscopes and a variant of the photomultiplier tube, the image dissector tube, is experiencing renewed interest for use in high speed confocal microscopy (Goldstein, 1989). Finally the photomultiplier tube may be supplanted by the silicon avalanche photodiode, which when cooled becomes a high quantum efficiency, low noise, sensitive detector.

Light Fluxes and Imaging Detectors

A significant source of noise in low light level images is related to the quantal nature of photons. At $1x10^{-8}$ fc illuminance of the photocathode about 1300 photons impinge on a 1 cm^2 photosensor in a video frame. This corresponds to about 40,000 photons arriving at the faceplate per second. For a photocathode with a 20% quantum efficiency about 8,000 photons are detected and amplified. If the investigator is using an imaging system with a resolution of 512 x 512 pixels, each pixel will display a signal due to a photon once each 32.8 seconds. If the investigator were to confine his view to a single pixel it would show a true signal about once in every 1000 video frames. Since the detector also exhibits noise, the likelihood of the observed signal being real must be weighed against the possibility of it being due solely to random noise. The solution to this problem has generally been to sacrifice spatial and temporal resolution for the resultant improvement in signal detection ability (Bright et al, 1987; Spring and Lowy, 1988). Quantal noise can be reduced by improvements in detector quantum efficiency and detector noise can be reduced to insignificance in photon-counting imaging systems (Csorba, 1985; Tsuchiya et al, 1985, Wick, 1985).

Long periods of image integration of a preparation illuminated with very low excitation intensities are often used in an attempt to minimize bleaching and still obtain acceptable image information. Modern video cameras often incorporate image integration directly onto the camera photoconductor or detector by cessation of readout for a predetermined period. This method of integration has the virtue of a reduction in the noise associated with image readout and digitization but results, in some detectors, in a loss of resolution. Prolonged image integration suffers both from photodynamic effects during the relatively long exposure period as well as decreased temporal resolution. It is better to increase the excitation light intensity and shorten the exposure time and thus the integration time such that the duration-intensity product and signal-to-noise are unchanged. This approach enables improved temporal and spatial resolution with sufficient measurement speed to accommodate the rapid changes which may occur in living cells.

Noise in Imaging Detectors

The noise of a low light level video camera is due both to thermal emissions from the photocathode and to subsequent amplifier stages (Csorba, 1985; Electro-Optics Handbook, 1974). Noise arising at a silicon photosensor is typically temperature dependent and can be reduced by cooling of the detector. Cooled CCD detectors have been used with great success in astronomy (Timothy, 1983) and biology (Arndt-Jovin et al, 1985). In these systems, cooling virtually eliminates noise arising from the photo-sensitive surface; noise is then primarily associated with readout of the stored charge (Aikens et al, 1988; Arndt-Jovin et al, 1985; Timothy, 1983). Although cooling of photomultiplier tubes is used routinely to reduce detector noise (dark current), lowering the temperature of image tubes is not a common practice.

As with photomultiplier tubes, signal-to-noise may be increased by image averaging with the resultant reduction in temporal resolution. The trade-off between spatial and temporal resolution is of prime consideration in the selection and use of low light level video systems. It is generally not sufficient for the biologist to merely ascertain the presence of a fluorescent signal; we wish to know considerably more. A low signal-to-noise does not permit measurements with confidence either of the intensity of the fluorescent signal or of its spatial distribution. At low photon fluxes, the only way to increase the information content of the video image is to accumulate images. Since detector noise is random, image averaging increases the signal-to-noise ratio by approximately the square root of the number of frames (Bright et al, 1987; Inoue, 1986). Image averaging may be done by means of a digital image processing system (Bright and Taylor, 1986; Bright et al, 1987; Inoue, 1986; Wick, 1985) or by integration of the light input on the face of the photodetector. Such integration is crucial for the successful employment of CCD cameras (Aikens et al, 1988; Arndt-Jovin et al, 1985) and may also be used on room temperature CCD cameras or image tube systems (so called "gated tubes"). In these gated devices light is allowed to fall on the face of the detector in the absence of any scanning beam or chip readout. The signal will be integrated on the tube photosensor (up to the charge storage capacity of the photocathode) and may then be read out when the tube is gated to the "on" state.

Spatial versus Temporal Resolution

As described above, low light level imaging involves a balance between spatial and temporal information. The accuracy of an illuminance measurement improves as the sample area and sampling time increase (Spring and Lowy, 1988). As an example of the

trade-off between spatial and temporal resolution, Spring and Lowy (1988) illuminated a sample at a low illuminance (10^{-6} foot candles), reduced the illuminance by 10% and evaluated the measurement precision of their system. Their requirement for acceptable performance was that this 10% perturbation should be detectable with 90% or better precision (equivalent to the measurement of a 0.01 pH unit change with a pH sensitive dye). When the sample window was a box 4 x 4 pixels in size, it took 128 frames of image integration to achieve the required precision. When the sample window size was increased to 20 x 20 pixels, 90% precision was achieved in 4 frames. A large window (100 x 100) pixels gave an accurate measurement in a single video frame. Increasing the size of the sample window improved measurement precision without sacrificing speed, but decreased spatial resolution. A small window required more samples resulting in high spatial but poor temporal resolution.

Spectral Sensitivity

Typical tube-type imaging detectors exhibit a multialkali spectral curve (S-20), with peak sensitivity at 440-500 nm. Many image tubes have glass or fiber optic input windows which do not transmit at wavelengths below 350 nm limiting their use at these wavelengths (Csorba, 1985; Electro-Optics Handbook, 1974). Note that it is possible to obtain image intensifiers with specialized photocathodes and input windows such that images may be obtained at 200-900 nm. Quantum efficiency of S-20 photosensors is in the 20% range, while the red-sensitive S-25 photocathode, developed primarily for night vision applications, is about 10-15% quantum efficient at its peak sensitivity (680-720 nm). Silicon photosensors, such as those in CCD cameras, exhibit broad spectral sensitivity and extremely high quantum efficiencies of 30-80%. It is difficult to take advantage of the high quantum efficiency without cooling the detector (Aikens et al, 1988; Timothy, 1983). Extended red photocathodes (S-25 and silicon) can be of value in some microscope applications, however the red and infra-red emissions from the light source must be reduced to prevent high background signals from reducing contrast.

Speed of Response

The speed of response of low light level tube-type cameras depends on photoconductive and capacitative effects at the photocathode (Electro-Optics Handbook, 1974). The response speed of video cameras is expressed as the lag of the image which is a percentage of the final response (Bookman, 1990; Csorba, 1985; Electro-Optics Handbook, 1974, Inoue, 1986). Since it is the object of the investigator to use the camera as a two dimensional photometer, it is necessary to ascertain the time at which the signal obtained is a valid indicator of the faceplate illuminance. In most image tube detectors, lag is an exponential process with time constants (1/e) in the 25-50 ms range. Lag is increased in most image tube cameras when the signal current is reduced as in low light level microscopy. This occurs because the scanning electron beam does not fully discharge the charge accumulated on the camera tube target. The manufacturer's lag curves for image tubes must be viewed with attention to the corresponding illuminance when choosing a detector for low light level video (Electro-Optics Handbook, 1974; Inoue, 1986). The lower the light flux falling on the photocathode, the slower the response of the tube; in dimly illuminated regions of the image the response may be an order of magnitude slower than in bright regions. This lag in detector response is a major shortcoming of the conventional intensified target cameras (SIT and ISIT). The advent of intensified CCD cameras in which lag is limited to the readout time of the array (typically 1 ms or less) has eliminated the response speed concerns of many investigators. Image intensifiers may also be used to rapidly "gate" the signal to the CCD camera. The intensifier may be made light sensitive for times as short as 5 nanoseconds, a very useful capability for time resolved measurements, as well as for increasing the operating range of the camera system at high light fluxes (Jovin and Arndt-Jovin, 1989).

Shading

Image tubes always exhibit some spatial non-uniformity in the photocathode sensitivity; solid state detectors (CCD) are generally more uniform in their responsivity. Additional non- uniformities in the video signal result from camera electronics involved in scanning the electron beam. Such "shading" is more substantial in low light level tube cameras because of distortion introduced by the image intensification stage (Bright and Taylor, 1986; Bright et al, 1987; Csorba, 1985; Spring and Smith, 1987; Tsay et al, 1990); elaborate gain correction maps may be required to achieve photometric accuracy over the entire image (Bright et al, 1987; Williams et al, 1985). Modern second generation image intensifier tubes exhibit far less shading or gain variation and may be used in conjunction with solid state detectors to produce a camera free of shading (Csorba, 1985; Timothy, 1983; Spring and Smith, 1987; Tsay et al, 1990).

Photometric Linearity

When a low light level camera is used as a photometer, it is assumed that the camera output is a linear function of the faceplate illuminance (Bright and Taylor, 1986; Inoue, 1986). In video camera nomenclature, the tube is said to have a gamma of 1. Graphs of image tube signal current versus faceplate illuminance are readily available (Electro-Optics Handbook, 1974; Inoue, 1986) and show a linear relationship over a wide range of input light intensities. These graphs do not always represent the operating characteristics which prevail during quantitative imaging. If the gain of the camera is fixed, as must be the case in photometric applications, the usable range of camera operation is greatly reduced. A typical curve for a newvicon tube camera exhibits a linear range of operation which

is limited to a 10 to 20 fold variation in input intensity when 8 bit digitization of the video signal is employed. An 8 bit digitizer divides the 750 mV signal into approximately 3 mV increments. If camera noise is substantially greater than 3 mV, the operating range of the camera is further reduced. The investigator is presented with the problem of adjusting the specimen illumination, or degree of staining, so that the resultant fluorescence intensity falls within the linear operating range of the camera (Bright and Taylor, 1986; Bright et al, 1987; Tsay et al, 1990). This constraint can severely limit the utility of some image-tube cameras and has led to the employment of CCD based systems because of their larger dynamic range (Aikens et al, 1988; Timothy, 1983). Cooled CCD cameras exhibit a wide dynamic range for two reasons: the large full well capacity of the diodes enables a great number of electrons to be collected without saturation, and cooling reduces detector noise to very low levels with the result that signals may be digitized with 12-16 bit resolution. The dynamic range of conventional low light cameras within a single scene ("the intrascene dynamic range") was evaluated by Tsay et al (1990) by digitizing the image of a gray scale step wedge. The intrascene dynamic range defines the range of intensities of an image over which the detector is capable of responding. In quantitative imaging with a video detector, the relevant intrascene dynamic range is that over which the camera produces a linear response proportional to object brightness. Most intensified cameras respond linearly to a maximum of a fifty-fold range of intensities within a given scene (Electro-Optics Handbook, 1974; Tsay et al, 1990).

When image intensifier gain is allowed to change, the linear operating range can be greatly extended (Electro- Optics Handbook, 1974; Inoue, 1986). Difficulties in gain stability and reproducibility have limited this approach in image tube low light level cameras (Bright and Taylor, 1986). Second generation image intensifiers exhibit linear input-output relations over a wide operating range (Csorba, 1985; Spring and Smith, 1987). The gain of these devices can be rapidly and reproducibly changed to extend the dynamic range of the imaging system (Csorba, 1985; Spring and Smith, 1987). The linear operating range of such a device is largely determined by the sensitivity of the video camera to which it is coupled rather than by the characteristics of the intensifier. Thus combining a high performance image intensifier with a CCD, operating at room temperature, results in a linear device with a relatively large dynamic range.

Geometric Distortion

The extent to which the output image truly represents the geometry of the original specimen varies among different low light level cameras. The distortion of some cameras is obvious to the observer upon imaging a test target (Bright and Taylor, 1986). Correction of geometric distortion in the image processor is a complex and computationally intensive task. The lowest distortion systems are based on solid state detectors with or without image intensification (Aikens et al, 1988; Spring and Smith, 1987). It is our experience that geometric distortion of 3% or more is readily detectable from observation of the image of a grid or linear target.

Performance Characteristics of Existing Low Light Level Cameras

Silicon intensified target cameras (SIT)

In this popular camera photoelectrons are emitted from a multialkali photocathode, accelerated and impacted onto a silicon target surface where they are read out by a scanning electron beam as in a vidicon tube. These image tube cameras are useful for moderately low light intensity inputs (to 10^{-5} fc); the rule of thumb being that the SIT cannot see what is not visible to the dark adapted eye. SIT cameras have a very wide dynamic range in the automatic gain configuration, high limiting resolution (700 TV lines), low lag (25 ms for 67% response) at high light levels (10^{-4} fc), moderate (50 ms) to severe (250 ms) lag at their low light limit (10^{-5} fc). The photocathode is of the multialkali family with peak sensitivity at about 440 nm. The SIT is a good choice for imaging applications where bleaching and photodynamic damage are not serious problems, and where precise control of camera gain is not needed. Applications such as immunofluorescence at multiple wavelengths can take advantage of this type of camera.

Intensified silicon target cameras (ISIT)

This camera tube contains a SIT fiber optically coupled to an image intensifier. The intensifier is of the "first generation" type (Csorba, 1985; Electro-Optics Handbook, 1974) and therefore exhibits both gain non-uniformity and considerable geometric distortion (Bright and Taylor, 1986; Bright et al, 1987). ISIT cameras are capable of photon-limited imaging at light levels as low as $5x10^{-8}$ fc. They have good limiting resolution (600 TV lines), moderate lag (25-50 ms) at 10^{-5} fc, and severe lag (50-250 ms) at 10^{-6} fc. The multialkali photocathode of the image intensifier has a peak sensitivity near 480 nm. ISIT cameras have poor signal to noise at low light levels and are best utilized in conjunction with image processors (Bright and Taylor, 1986; Bright et al, 1986). In the automatic gain mode ISIT cameras have an extraordinary operating range, however gain reproducibility and stability are poor under manual control (Bright and Taylor, 1986). The ISIT has been the low light level camera most frequently used in the fluorescence microscopy of thin living cells over a relatively wide range of wavelengths.

Image intensifiers

Image intensifiers have been used by biologists for many years in conjunction with film and video

cameras. These devices and their operating characteristics have been described in detail in a number of recent publications (Csorba, 1985; Reynolds and Taylor, 1980; Spring and Smith, 1987; Wick, 1985). These devices amplify the light falling on their photocathode and produce an output image on a phosphor screen. Modern intensifiers (so-called second or third generation devices) employ microchannel plates as electron multipliers and do not introduce geometric distortion or gain inhomogeneities into the output image. They are linear devices with a very large dynamic range in the automatic gain mode; gain may be readily and reproducibly altered under manual control. Image intensifiers are available which approach the theoretical limit for photon detection and resolution (Csorba, 1985; Spring and Smith, 1987). The response speed of these devices is usually much faster (μsec) than any biological requirements and is primarily determined by the properties of the intensifier output phosphor. Spatial resolution of image intensifiers is adequate for the requirements of typical image processors (Spring and Smith, 1987). A 50-fold range of intensities is faithfully reproduced within a given scene by these devices. Most low light level cameras employ an image intensifier as the first stage. Incident photons cause the emission of photoelectrons which impact the microchannel plate capillaries. The large potential difference across the microchannel plate (typically 800-1000 V) leads to electron multiplication. The emitted electron cloud impacts onto a phosphor screen which converts the electrons back to visible light. The hexagonal packing pattern of the glass capillaries in the microchannel plate leads to the fixed pattern noise ("chicken wire") commonly observed with these devices at high light fluxes. This pattern develops because the microchannel plate gain is slightly lower at the juncture of the glass hexagons because of the absence of open capillaries in this region.

Proximity Focused Intensifiers

These small, light weight intensifiers are used in weapons, night vision devices, and some low light level cameras for light microscopy. These intensifiers are very compact and the electron cloud is "focused" by the close proximity of the microchannel plate to the photocathode and anode. These tubes are also available with two microchannel plate intensifiers coupled together to achieve higher gain. In general proximity focused tube intensifiers do not have the high gain, signal-to-noise and resolution exhibited by the two stage intensifiers (so called "inverter tube"). Proximity focused intensifiers have virtually no shading or geometric distortion; lag with standard phosphors (P-20 or 1052) is also less than 1 msec. An additional feature of the proximity focused tube is its ability to be rapidly gated by reversal of the photocathode voltage.

Two Stage Intensifiers

The two stage tube employs an accelerating and electrostatic focusing stage before the microchannel plate to achieve higher gain and reduced ion feedback compared to the proximity focused tube. The voltage between the photocathode and microchannel plate is 500-1000 V, much higher than in the proximity focused intensifier. Microchannel plate and anode voltages are similar to those in the proximity tube intensifier. Shading and geometric distortion are more evident in the two stage tube because of the long electron path and electrostatic focusing system. Careful internal design and specifications have lead to very high quality two stage intensifiers well suited to quantitative light microscopy (Spring and Smith, 1987). Gating, while possible in a two stage intensifier involves higher voltages, larger capacitative transients, and slower time response than in the proximity focused tube.

Coupling The Intensifier to the Video Camera

Modular intensifiers coupled by relay lens optics to the detector of choice have been used for quantitative microscopic applications for many years (Reynolds and Taylor, 1980; Spring and Smith, 1987). There are two choices for coupling an image intensifier to a video camera-relay lenses and fiber optics-both methods are used by individual investigators and manufacturers. The efficiency of fiber optic coupling is much higher (20-50%) compared to relay lenses (6-12%) with resultant improvements in signal to noise at very low light levels. The advent of high quality, tapered fiber optics permits the matching of the size of the intensifier output window to the input window of the camera. This is particularly important when a CCD camera is used as the second stage because of the small size of the light sensitive area of most CCD chips. The potential difficulties with fiber optic connections include failure of the optical couplant, the need for obtaining a CCD chip without a front window, and the mating of the fiber optic plug to the CCD. Finally fiber optic coupling is generally permanent and precludes easy alteration in the camera used as the second stage detector behind the intensifier.

Cooled Solid-state Cameras (CCD)

These devices exhibit high resolution, extraordinary intrascene dynamic range (several thousand fold), broad spectral sensitivity, very high quantum efficiency, and the capability of on- chip integration of the light input. Their principle disadvantages are associated with the requirement for slow readout (to reduce readout noise) and the relatively high noise floor (Aikens et al, 1988; Timothy, 1983). The result is a device which is best utilized as a slow-scan image integrator rather than a real-time low light level video camera. Since solid-state detectors exhibit no lag, blooming, geometric distortion, or shading they are far superior to image tubes. Some investigators have succeeded in employing cooled CCD cameras for imaging fluorescence signals from living cells (Jovin and Arndt-Jovin, 1989). There has also been a steady

improvement in the design of the chips, readout electronics, and image storage technology which may make these cameras even more useful in the future.

Intensified CCD Cameras

The combination of an image intensifier and solid-state detector results in a fast, sensitive camera without many of the problems of the image tube systems (Csorba, 1985; Spring and Smith, 1987). As the resolution and sensitivity of room temperature CCD cameras have improved (along with a significant decrease in price), the popularity of the intensified CCD camera has grown rapidly. The major advantage of the intensified CCD compared to the SIT and ISIT is in the response time (lag). There is virtually no lag in an intensified CCD; the slowest step is the transfer of charge from the chip which requires about 1 msec (Bookman, 1990). CCD cameras do not exhibit shading, geometric distortion, or substantial gain inhomogeneities. The resolution of these cameras exceeds the requirements of a 512 x 512 image processor, and their sensitivity equals that of a newvicon camera. The addition of an image intensifier to such a camera yields sensitivity comparable to the SIT or ISIT, depending on the intensifier-camera combination. Another solid-state detector, the charge injection device (CID) has been less widely used in intensified cameras in biology. CID cameras have the virtue of a random access to regions of interest but tend to be rather insensitive because of their detector design.

Intensified CCD cameras in which the intensifier is coupled by tapered fiber optics to the CCD have also become more readily available. These cameras lack the flexibility of the modular intensifier-camera combination, but have the virtue of compactness and efficiency in the coupling of the two stages. Because of the small size of the active area of most CCD chips, the fiber optic connection between the intensifier and the chip must be tapered. Most intensifiers have an output window of 18 mm or larger in diameter, while typical CCD chips are 7 x 9 mm. A fiber optic taper of more than 2.5/1 is generally not advisable because of distortion and light loss introduced by the fiber optics.

Position-sensitive detectors

When the photon flux is very low, an image intensifier assembly may be used to detect and amplify individual photons. The resultant electron stream impacts onto a surface equipped with coordinate readout electronics. Such a photon-counting imaging system has been developed for light microscopy (Tsuchiya et al, 1985; Wick, 1985). The speed of the camera is limited by the coordinate readout electronics to typically about 10,000 counts per second; complete images require many seconds or minutes to accumulate. Some of the newest devices have faster readout rates of up to 10^6 counts per second. As many as five microchannel plates may be combined in the image intensifier to increase the amplitude of the electrical pulse derived from the photoelectron and ensure discrimination of the signal from the noise of the system. These devices achieve the ultimate in detector sensitivity and dynamic range. Their poor temporal resolution limits their applicability in biology to experimental circumstances in which the available light flux is below the limits of conventional intensified cameras (e.g. bioluminescence).

Future Developments

It seems clear that the solid-state camera (CCD, CID, etc.) will dominate the low light level detector market in the future. The main emphasis in these detectors seems to be in the areas of increased resolution in space and time. Inexpensive CCD cameras with 800 diodes per horizontal line are already available; 1000 diodes per line are in production and even higher resolution chips in development. When a 1000 x 1000 diode CCD is used as a detector, each image contains 1 megabyte of data. If the CCD is cooled, the image may contain 14-16 bits of information. Such massive data sets become an impediment to image storage and retrieval; future use of these devices may be dependent on improvements in digital image storage technology.

The demands of robotics and industrial process inspection have driven development of CCD cameras with electronic shutters for stop action imaging. These devices are useful for studies involving rapidly moving fluorescent objects, such as tagged red blood cells in studies of the microvasculature. Rapid readout cameras with framing rates of up to 1000 per second are commercially available, but expensive. The industrial pressure to develop such devices for manufacturing should result in low cost, high speed cameras for biological applications.

Image intensifier technology is mature, and one would expect little in the way of new developments in these devices. The big improvements may come from redesigning the coupling between the intensifier and detector stages. Direct coupling of intensifiers to CCD chips without reconversion of the electron stream to light by a phosphor has been described. Conversion phosphors applied to intensifier front windows may enable imaging at wavelengths well outside the visible spectrum. Finally the high speed gating capabilities of proximity focused intensifiers has not been exploited by light microscopists and may constitute an important adjunct to measurements at high light fluxes.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.