The CCDiode: An optimal detector for laser confocal microscopes

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ABSTRACT

The laser confocal microscope (LCM) is now an established research tool in biology and materials science. In biological applications, it is usually employed to detect the location of fluorescent marker molecules and, under these conditions, signal levels from bright areas are often <20 photons/pixel (from the specimen, assuming a standard 512x768, 1 sec. scan). Although this data rate limits the speed at which information can be derived from the specimen, saturation of the fluorophor, photobleaching of the dye, and phototoxicity prevent it being increased. Currently, most LCMs use photomultiplier tubes (PMT, QE = 1-30% @ 400-900 nm). By contrast, rear-illuminated, scientific charge-coupled devices (CCD) now routinely readout the signal from square sensors ~30 μm on a side with a QE of 80-90%, a noise of only ±3 e^-/pix and with no multiplicative noise. For this reason, in 1989, one of us (JJ) developed a rear-illuminated, single-channel Si sensor, called the Turbodiode, employing some of the sophisticated readout techniques used to measure charge in a scientific CCD. We are now extending this work to a device in which a single 36x36 μm sensor is read out through a low-noise FET charge amplifier with a reset circuit and then passed to a correlated, double-sampling digitizer (CSD). To maintain the desired ±3 e noise level at the relatively high data rate of 1 MHz, our new device utilizes 64 separate readout amplifier/digitizer systems, operating in sequence. The resulting detector is more compact, efficient and reliable than the PMT it replaces but as its sensitive area is smaller than that of a PMT, it will require auxiliary optics when used with any LCM having a large (mm) pinhole. As the signal light is parallel, a simple lens mounted axially and with the CCDiode at its focus would suffice. Future versions may use 3x3 or 5x5 arrays of sensors to "track" the confocal spot as it is deflected by inhomogeneities of the specimen, change its effective size or shape or detect system misalignment.

Keywords: Confocal microscope, photodetector, parallel readout CCD, low-noise CCD.

1. INTRODUCTION

1.1 Confocal laser-scanning microscope operation

The laser confocal microscope (CFM) is now an established research tool in biology[1] and is used increasingly in materials science. In its most common form, the instrument is a scanning-laser microscope with the detector mounted behind a spatial filter aperture which is placed conjugate to the "reflected" image of the focal spot. The purpose of the spatial filter is to insure that reflected or fluorescent light originating from other than the plane of focus is prevented from reaching the detector. This creates an image of an optical section.

1.2 Confocal microscope signal levels

In biological applications, the CFM is usually employed to detect the location of fluorescent marker molecules inside cells and, under these conditions, signal levels from bright areas are often <20 photons/pixel at the detector, (assuming a standard 512x768 raster scanned in 1 sec. for a 600 kHz pixel rate). Although this low data rate limits the speed at which information can be derived from the specimen, singlet-state saturation of the fluorophor prevents higher rates. Even with only 1 - 2 mW of laser power, the photon flux present in the focus of a high-NA objective is high enough to keep a substantial fraction of the fluorescent molecules present (~50%) in the singlet excited state, significantly reducing the effective dye concentration. Therefore, it is not possible to increase the data rate by increasing the input laser power. In

practice, photobleaching of the dye, and in studies of living cells phototoxicity, place even more stringent limits on the amount of laser power that can be used.

As the maximum rate of signal generation is fixed by saturation or specimen damage, the only way to increase the data rate is to improve the efficiency of the system used to count the signal. In early instruments, the efficiency of the optical system used to convey the excited light to the detector, and the elecronics then used to digitize it, was very low (~1%)[1]. However, in more recent instruments, transmission losses have been sharply reduced and digitization techniques improved. As a result, the photodetector is now the only component in which substantial efficiency improvements seem possible.

1.3 Photodetectors for the confocal microscope

Currently, most laser scanning instruments use a photomultiplier tube (PMT) and in fluorescence applications the average data rate may be 1-10x10^6 counts/sec. with a peak rate perhaps 10x higher. As long as it is kept at room temperature, the dark current of the PMT is negligible compared to this data rate, however, its quantum efficiency (QE) is quite low: 15-30% in the blue/green depending on the photocathode material but only 0.5-3% in the range of 700-800 nm. The low red sensitivity is particularly important because it has become evident that living cells seem to be far less sensitive to red/ near-IR light and hence there is increased interest in operating in this part of the spectrum. In addition, the charge multiplication process that occurs in the PMT produces multiplicative noise which reduces the effective QE by an additional 30%[2]. Finally, most PMTs are relatively large and delicate.

Single channel detectors based on Si have much higher QE but, as the noise current associated with reading out a PIN photodiode at a similar pixel rate is ~± 200 electrons/pixel, such devices are only useful in the confocal microscope when used for measuring high-level signals such as transmitted light or the light reflected from solid surfaces.

1.4 Description of CCDiode

On the other hand, scientific-grade charge-coupled devices (CCD) now routinely read out the signal from arrays of square sensors, each about 20 to 30 µm on a side, with a measurement noise of only ± 3 e^- /pixel[3] and do so without generating any multiplicative noise. For this reason, in 1989, one of us (JJ) developed a rear-illuminated, single-channel Si sensor, called the Turbodiode, employing some of the sophisticated readout techniques used to measure charge in a scientific CCD[4]. We have now extended this work to a device in which a single sensor 36x36 µm in size is read out through a low-noise FET charge amplifier with a reset circuit and then passed to a correlated, double-sampling digitizer[5]..

Although the noise level of such an arrangement can be kept as low as 3 e^-/pixel at a readout rate of 50k pixel/sec, it rises with the square root of the readout speed and this poses a problem for use in the laser-confocal microscope where sampling rates of 10^6 pixels/sec. or even 10^7 pixels/sec. are not uncommon. To overcome this limitation, our new devices utilize either 16 or 64 separate readout amplifier/digitizer systems, operating in parallel. In this way, the bandwidth of each amplifier can be 16-64x less than would be necessary than if only one were used and the 3 e^-/pixel noise rate can be maintained even beyond a count rate of 10^6 pixels/sec.

2. RESULTS

2.1. Prototype 1:

Parallel CCD readout requires quite sophisticated digital circuitry to distribute sequential charge packets from the sensor to the appropriate amplifier. Our first effort to test the CCDiode concept involved the fabrication of a device that represented a small modification to a previous 64x64, rear-illuminated device having 64 parallel readout channels and designed to detect changes in the shape of the image of a reference star (with ±3 e-/pixel noise @ 2,000 frames/sec) as a means of regulating the adjustment of an adaptive telescope.[6] For set-up purposes, this sensor also incorporated a traditional, horizontal shift register leading to a separate, single-channel readout amplifier. The modification involved the addition of a

single 36x36 μm sensor to the left side of this horizontal transfer register. In operation, charge is shifted out of the sensor at up to 1 MHz, filling the horizontal register. When full, the 64 charge packets are shifted through the 64x64 sensor area to the 64 readout amplifiers/CDS systems which sample-and-hold the CDS signal level for subsequent sequential digitization by one or two ADCs. At the time of writing, this device has been fabricated and is now being mounted for testing.

2.2. Prototype 2:

Though Prototype 1 had the advantage of being easily fabricated by making a small change to an established design, it had several practical disadvantages: it provided 64 parallel readout channels where 16 would have sufficed and it contains the original 64x64 sensor array which was no longer needed. As both of these features might be expected to reduce the yield of acceptable devices, we have now designed Prototype 2 which employs the same rear-illuminated sensor area but only a 16x0 distribution array and 16 readout amplifiers/CDS systems. (Fig. 1). This system is now being fabricated.

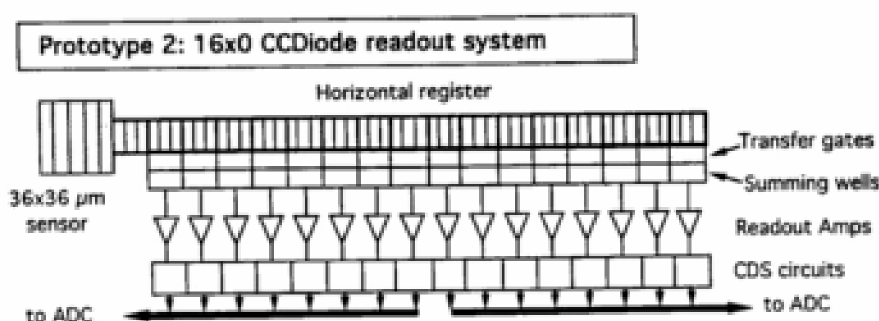

Figure 1.

2.3. Auxiliary optics

Although the sensitive area of the CCDiode is much smaller than that of the normal PMT, it is still of the same order as the size of a pinhole optimized for a variety of commonly used microscope objective lenses and placed in the intermediate image plane of a CFM[7]. However, the CCDiode will require auxiliary optics if it must be placed in a final image plane (after an ocular) where the appropriate size for a pinhole is several mm in diameter. As the light striking the detector in such a confocal system is almost parallel, a simple lens mounted axially and with the CCDiode at its focus would suffice. Both the lens and the sensor must be antireflection coated for the detected wavelengths.

3. DISCUSSION

3.1. The CCDiode compared to other suitable photodetectors.

Although the PMT has been the detector most commonly used in laser-scanning confocal microscopy, both PIN photodiodes and avalanche photodiodes (APD) have also be employed. Of these, only the latter is really suitable for the low signal levels that characterize fluorescence, laser-scanning confocal microscopy. More recently, however, the development of the Hybrid-PMT has produced a second serious challenger. These two devices are discussed below.

3.2. Avalanche photodiodes (APDs)

Though attractive for their relatively high QE and single-photon counting ability, the APD seems to have an inherent disability as a fluorescence detector for a CFM. The onset of avalanche breakdown in the depletion region is critically dependent on the temperature of this small region (0.1 mm diam. x 0.03 mm thick) . If the device is operated at high gain (10^7- 10^8) and a reverse bias of ~200 v, pulse rates of 10^7 counts/sec. generate heat in the depletion region at a rate of tens of mW, causing changes in its temperature that effectively make APD sensitivity dependent on the intensity of the previous signal. While such performance may be acceptable when detecting small changes in a relatively large constant signal level, it is not acceptable for use in the CFM where the signal level may vary over a range of 100:1 in an irregular manner.

Although this hysteresis effect can be avoided by operating the APD at a more moderate gain (~x200, or almost enough to raise the signal from a single electron above the noise level of the head amplifier), doing so has the unfortunate effect of greatly reducing the effective QE because, under these bias conditions, the most common gain for a newly-excited photoelectron is zero[2].

3.3. Hybrid-PMTs

In operation, the hybrid-PMT accelerates electrons from a photocathode onto the sensitive region of a photodiode. On impact, the energy picked up from the acceleration field is converted into electron-hole pairs. As the number of e^-/h+ pairs is subject only to Fano Factor noise, each photoelectron generates almost the same signal in the diode, thereby eliminating multiplicative noise: published curves indicate that such devices can discriminate multi-photon pulses produced by 13 photoelectron from those produced by 14.[8] In addition, these devices seem to be relatively small and sturdy. If coupled with a GaAs photocathode, with QE of ~30% over a fairly wide bandwidth, such a detector would have lower noise than the CCDiode that we have developed but would have only 33% of the QE.

4. FUTURE DEVELOPMENTS

Although the performance of the hybrid-PMTs recommend them to replace the standard PMT in the confocal microscope, even if they are optically enhanced[9], it seems unlikely that they will demonstrate a QE as high as that of a thinned, rear-illuminated CCD (~90%). In addition, whereas the hybrid-PMT is generally a single channel device, the CCD is not and there are a number of ways that this feature could be put to good use in CFM applications.

The flexibility of the charge-coupling circuitry makes possible other ways to utilize the 64 readout amplifiers. For instance, they could be used to readout an array of 3x3 sensors (at a somewhat lower refresh rate) as a quadrant detector to measure the optical (mis)alignment of the microscope. In fact, by searching for the pixel with the most signal, one might be able to “track” the center of the confocal signal spot in order to compensate for the small lateral beam deflections caused by optical homogeneities in the specimen. Such tracking has been suggested as the only way to perform confocal imaging using transmitted light.[10] Alternatively, one could use a 5x5 sensor array, such as that shown in Fig. 2, and change its sampling pattern to effectively vary the pinhole size electronically. This could be done by sampling only the central pixel, or by pooling the central 9 pixels or all 25 pixels. Given suitable digitizing electronics, one could even arrange to collect data at two, or even three, different effective pinhole sizes separately and simultaneously. This could be a very important ability because it is often unclear what size the pinhole "should" be: smaller pinholes give better resolution but larger ones collect more signal and the "best" size, (defined in terms of most information about the specimen for the fewest photons emitted by it), is not always clear. Being able to choose the proper size “after the fact”, by actually looking at the images produced would greatly simplify this process. Even better, it should be possible to use all 3 measurements to improve the quality of the data used as the input to sophisticated 3D-deconvolution techniques[11].

5. ACKNOWLEDGMENTS

This work was supported in part by Grant DIR 90-17534 from NSF to JP for the development of improved photosensors for the CFM.

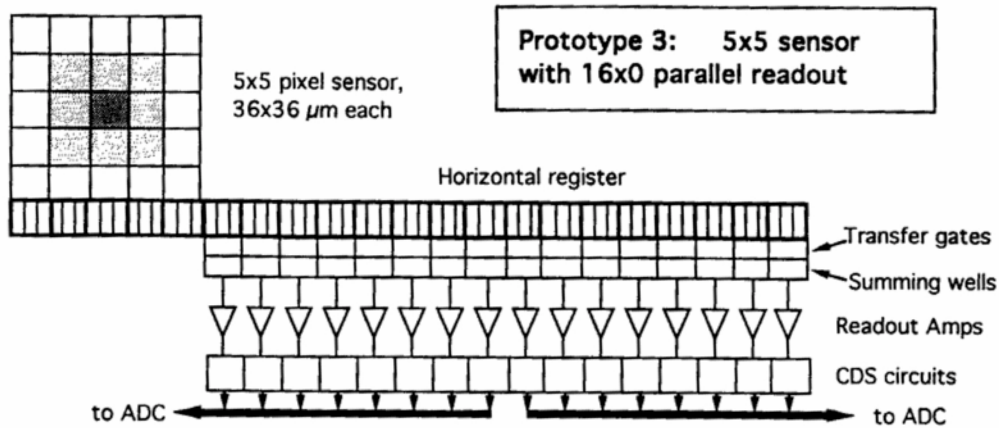

Figure 2.

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