Back-illuminated electron multiplying technology: The world's most sensitive CCD for ultra low-light microscopy

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ABSTRACT

The back-illuminated Electron Multiplying Charge Coupled Device (EMCCD) camera stands to be one the most revolutionary contributions ever to the burgeoning fields of low-light dynamic cellular microscopy and single molecule detection, combining extremely high photon conversion efficiency with the ability to eliminate the readout noise detection limit. Here, we present some preliminary measurements recorded by a very rapid frame rate version of this camera technology, incorporated into a spinning disk confocal microscopy set-up that is used for fast intracellular calcium flux measurements. The results presented demonstrate the united effects of: (a) EMCCD technology in amplifying the very weak signal from these fluorescently labelled cells above the readout noise detection limit, that they would otherwise be completely lost in; (b) back-thinned CCD technology in maximizing the signal/shot noise ratio from such weak photon fluxes. It has also been shown how this innovative development can offer significant signal improvements over that afforded by ICCD technology. Practically, this marked advancement in detector sensitivity affords benefits such as shorter exposure times (therefore faster frame rates), lower dye concentrations and reduced excitation powers and will remove some of the barriers that have been restricting the development of new innovative low-light microscopy techniques.

Keywords: CCD, EMCCD, Calcium Flux Microscopy, Single Molecule Detection, Intracellular Ion Signalling, Back-thinned CCD, Electron Multiplying CCD, Single Photon Sensitivity.

1. INTRODUCTION

Current trends towards single molecule measurements are heralding a new era of low concentration analysis, by which biological reactions and tests can be carried out with minute samples, which are normally insufficient for measurement purposes, and the behaviour and interactions of individual biomolecules can be tracked, even within their natural cellular environment. Furthermore, techniques such as intracellular ion signalling microscopy (e.g. Ca^{2+} flux microscopy) and 4D microscopy impose considerable demands on detection technology, which can fundamentally be filtered down to two key requirements sensitivity and speed! The camera must be sensitive in order to: (i) detect the weak signal of low dye concentrations and single molecules; (ii) cope with the lower photon fluxes afforded by shorter exposure times (complementing fast frame rates); (iii) detect the weaker photon fluxes afforded by reduced excitation powers (reduces photobleaching of dyes and photodamage to tissues, lengthening experimental lifetimes) (iv) overcome the significant readout noise detection limit of high speed readout rate. High frame rates are required to enable: (i) the study of dynamic interactions between single biomolecules; (ii) single molecule tracking; (iii) the study of the fundamental transient blinking effects of single molecules; (iv) recording of fast calcium flux processes, in accordance with the major temporal resolution requirements of intracellular ion signalling studies.

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To this end, optimal signal-to-noise (S/N) standards must be achieved at significantly lower input signal levels. An advanced CCD camera design, offering unsurpassed sensitivity performance, will be shown here to yield markedly improved S/N under ultra low-light conditions *at high speed operation*, which will uniquely facilitate demanding fast speed low-light microscopies such as single molecule detection and intracellular ion signalling microscopy. The detector makes use of a new CCD architecture[2-5] that unites the sensitivity of an ICCD, with the inherent advantages of a CCD. This technology is sold by E2V under the trade name 'L3Vision', and is covered by a patent (EP 0 866 501 A1). Presently three CCD formats are commercially available from Andor under the part numbers DV860, DV865, and DV887. The results presented here are based on the E2V CCD60 and CCD87 devices incorporated into the Andor Technology 'iXon' range of scientific cameras, sold under the trade name 'EMCCD' (Electron Multiplying CCD). Andor were the first commercial providers of digital scientific CCD cameras incorporating this new innovative technology and have reported the principle of operation of the all solid-state EMCCDs in detail elsewhere[6,7].

In the present paper we report, for the first time, the combined effect of two performance enhancing technologies in the one camera, *electron multiplication approach* in a *back-thinned sensor*, enabling by far the most sensitive detector available.

The new back-illuminated EMCCD (BV-EMCCD – 'BV' being a coding for back-illuminated, optimized for visible/near IR) is designed to optimize the two key parameters of detection sensitivity - **Detection Limit** and **Quantum Efficiency**:

- *Detection Limit* - In a sufficiently cooled CCD, the detection limit is defined by the readout noise[1]. In fact, this may be regarded as the single main weakness of CCDs. Scientific CCDs can achieve 2-4 electrons of readout noise but only at slow readout speeds. However, at the more practical speeds of 5 MHz pixel rates or above, this noise is typically 30 electrons or more. In applications where raw sensitivity is required, particularly at high readout rates, either Intensified CCDs (ICCDs) or Electron Bombardment CCDs (EBCCDs) have been favoured. But these detectors have their own drawbacks, such as resolution artefacts, high spurious noise, cross-talk, higher noise factor (which effectively increases the signal shot noise) and most importantly, severely restricted QE of the photocathode at the 'front-end' of light detection. They are also inherently complex and expensive devices. A more detailed comparison of these detector alternatives is given elsewhere[6,7]. Electron Multiplying CCD technology however, based on an all solid-state design[2-5], overcomes many of the ICCD/EBCCD drawbacks whilst offering amplification technology which can essentially render the readout noise negligible *at any readout speed*. That is to say, EMCCD technology (sometimes known as 'on-chip multiplication') affords an image sensor that is capable of detecting single photon events *without* an image intensifier, achievable by way of a unique electron multiplying structure built into the silicon, therefore avoiding the QE and resolution limitations of intensifier tubes. Gain can be increased to a degree, tuned easily though the software, where extremely weak signals may be detected above the readout noise of the camera, even under conditions of high readout noise**.**

- *Quantum Efficiency* – Sensitivity is also dependent on QE, a measure of the light collection efficiency. In standard CCD devices, higher QE means that more incoming photons are converted to photoelectrons sufficient to overcome the noise detection limit. The higher signals afforded also reduce the relative signal shot noise. In a system such as the EMCCD, where the readout noise detection limit has essentially been removed, QE can be expected to have a direct bearing on the shot noise of the signal. Since the EMCCD does not require an intensifier tube, the full higher and broader QE curves of the CCDs can be harnessed. Indeed, even the first produced front-illuminated EMCCDs (FI-EMCCD) themselves have distinct QE advantages over ICCDs. Back-thinned CCDs however, are recognised as having the highest QEs of any detector (up to ~ 95% at max. compared to the ~ 45-50% max. QE of a typical front illuminated CCD).

Therefore, the BV-EMCCD, a device that combines both electron multiplying CCD technology and back-thinned CCD technology, can be expected to offer both single photon detection sensitivity (by virtue of having eliminated the readout noise) *AND* the ultimate in photon conversion efficiency (therefore improved shot noise). A back-illuminated EMCCD device can theoretically offer improvements of up to a factor of 2 (or greater at some wavelengths) in Signal/Shot Noise ratio over a front-illuminated EMCCD device.

This paper describes some basic ultra low-light experiments carried out with FI-EMCCD and BV-EMCCD cameras, principally to illustrate the effect of these technologies on fundamental Signal/Noise considerations. The cameras have in turn been incorporated into a spinning disk (Nipkow) confocal microscopy set-up, used routinely for performing rapid intracellular ion measurements (Ca^{2+} flux) on smooth muscle cells. The BV-EMCCD was also compared to a GenIII ICCD under demanding low-light conditions of low excitation power and very short exposure times. Furthermore, EMCCD technology has been demonstrated within a light tight imaging chamber, imaging very faint pinhole light sources with short exposure times, effectively simulating the signal that can be reasonably expected from a well optimized single molecule imaging experiment.

2. EXPERIMENTAL DESCRIPTION

EMCCD and ICCD Cameras

Two FI-EMCCDs were used in these experiments, an Andor iXon DV887 (FI) and an iXon DV860 (FI). The DV887 is a 5 MHz camera with a 512x512 frame transfer sensor, capable of delivering 17 full frames/sec (faster with sub-area/binning). The DV860 has a smaller 128x128 frame transfer sensor, capable of > 100 full frames/sec. The new BV-EMCCD that was employed, the iXon DV860 (BV), is a back-thinned version of the DV860. The ICCD camera used was a Stanford Photonics XR Mega 10 ICCD, containing a GenIII intensifier and 1280 x 1024 CCD sensor.

Intracellular Ion Measurements

An inverted Nikon TE300 Microscope with x40 and x100 objectives was combined with a spinning Nipkow disk arrangement (Visitech, UK - approx 1800 revs/sec building approx. 360 complete confocal images/second) and a Krypton/Argon Ion laser with excitation wavelength 488 nm. Cameras were easily interchanged via a side oriented c-mount adaptor. Freshly dispersed rabbit urethral and guinea-pig vas deferens smooth muscle cells were obtained as described previously[11] and were loaded with 10 uM of Fluo-4-AM dye (Molecular Probes, emission maximum at 516 nm) for 15 minutes at 35^{o}C. Throughout all measurements, the Krypton/Argon Ion excitation laser was kept at 'standby power', i.e. the minimum power to allow stable lasing. This was considered a very desirable experimental condition, minimizing photodamage and enabling prolonged lifetimes of the cells. When bound to free Ca^{2+}, the emission quantum yield of Fluo-4 increases significantly. Caffeine addition (10 uM for 5 seconds) was used to induce release of intracellular Ca^{2+} from the cellular calcium stores and resulted in a rapid build up (over 10's milliseconds) of Ca^{2+} levels within the cell, followed by a slower (100's milliseconds – seconds) decay of the intracellular Ca^{2+} concentration. These dynamics where readily analysed and displayed using quantitative processing functions with Andor's imaging software. Note that a banding pattern visible in the background of some images is due to the non-synchronized nature of the fast frame rate camera with the spinning Nipkow disk. For the effect to cancel, the frame rate of the camera would have to be divisible into and synchronized with the imaging rate of the spinning Nipkow disk.

Pinhole Imaging

The EMCCD cameras where mounted on top of an in-house constructed light tight imaging chamber, containing a focusable F-1.8 lens and a red LED (λmax. ~ 620nm) mounted below a partially sampling platform. Two layers of black card were placed on top of the sampling platform, the uppermost one containing an array of small pinholes. On top of this, a strong ND filter was placed, cutting the weak light down by a further x100. At short exposure times (10's to 100's milliseconds), light levels from the point sources were sufficiently weak that the signal was lost in the readout noise floor when EM gain is turned off.

3. RESULTS AND DISCUSSION

Low-light Intracellular Ion Microscopy

Initially, a large number of Fluo-4 loaded smooth muscle cells were imaged with FI-EMCCD and BV-EMCCD cameras, each of the Andor iXon DV860 format, housing a 128x128 EM sensor (capable of >100 full frames/sec.), under conditions of very low photon fluxes. In these initial experiments, caffeine induced calcium release was not carried out, and comparison of the combined signal from the unbound dye or background cellular Ca^{2+} levels was compared between cameras over a number of cells.

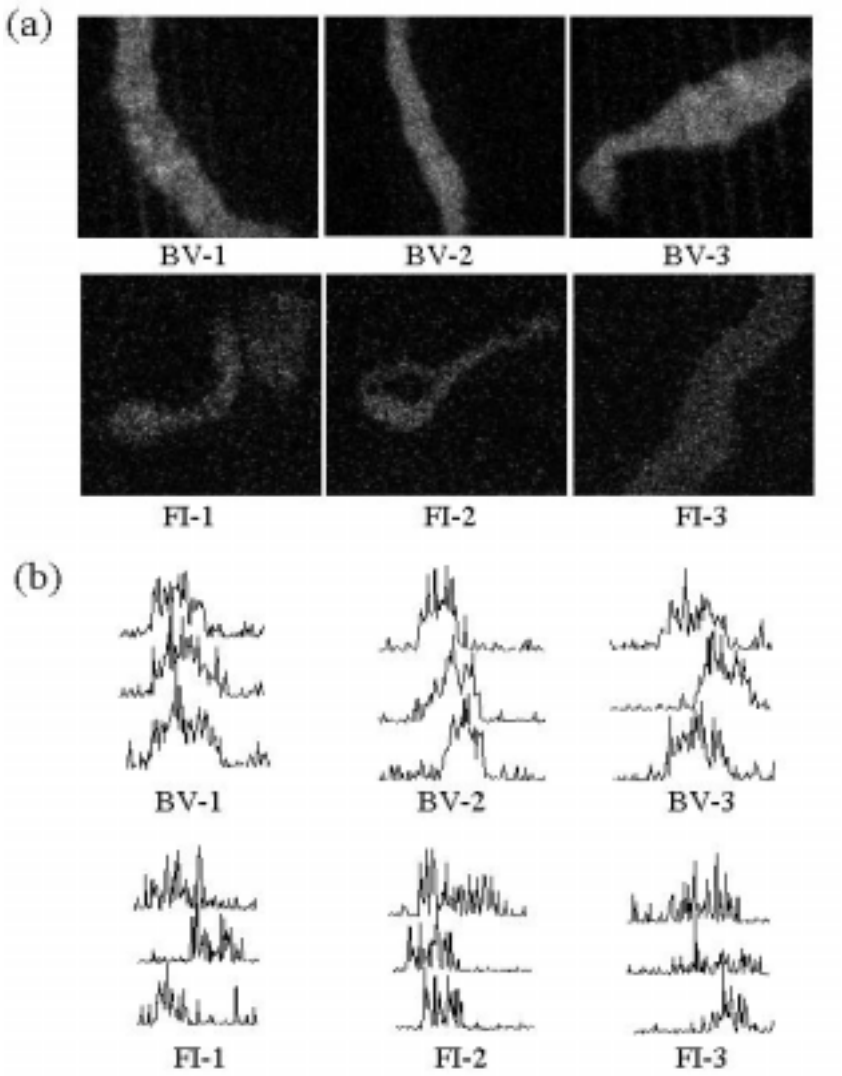

Figure 1: (a) Selected images from kinetic series recorded of Fluo-4 loaded smooth muscle cells with both FI- (FI-1, FI, 2 and FI-3) and BV- (BV-1, BV-2 and BV-3) EMCCDs; 17 ms exposure time per frame; high EM gain setting throughout. (b) Line intensity profiles for signal-to-noise visualization, derived by randomly drawing a line across the corresponding cell images of (a).

Figure 1(a) shows a selected series of images recorded. Each image represents a single frame from a 1500 frame kinetic series, recorded with minimum laser excitation power, using 17 ms exposure times (~ 60 frames/sec). The selected EM gain setting was approximately similar for each camera (~ x650), and was sufficient to render the readout noise negligible (single photon sensitive), enabling the signal/shot noise ratio to be viewed. It should be noted that when EM

gain was turned off, signal could not be detected under these conditions of excitation power, dye concentration and exposure time.

It is immediately apparent that contrast is better and signal is stronger for the BV-EMCCD images than for those recorded with the FI-EMCCD. At this emission wavelength (516nm), up to ~ x4.6 improvement in signal intensity and ~ x2.15 in signal/shot noise ratio is expected upon using the back-thinned technology. To further illustrate this, selected random line profiles are shown for each image in Fig. 1(b). These line profiles represent intensity variation across a line of pixels drawn randomly across the cells and give a reasonable visualization of relative signal intensity and noise sources. It should also be noted however that the intensity shows some variation across the cell due also to localisation of dye within the cell. Furthermore, although all cells were treated and loaded identically, one should also expect the intensity to differ slightly from cell to cell. However, by sampling a sufficient amount of cells the general qualitative difference between images recorded with each camera was readily recognizable. Furthermore, compared to the typical intensity of even single photon noise spikes in these profiles, the peak-to-peak readout noise is minuscule and can indeed be considered negligible.

Figure 2 shows a comparison between similar cells recorded with FI and BV-EMCCD cameras using only 11 ms exposure time (> 90 frames/sec). In this experiment, caffeine was used to induce calcium release into the cell during the course of the 1500 frame kinetic series. Figure 2(a) shows selected images from each camera, both before and after addition of caffeine to the cells. As was the case in figure 1, the improvement in signal intensity of the BV-EMCCD

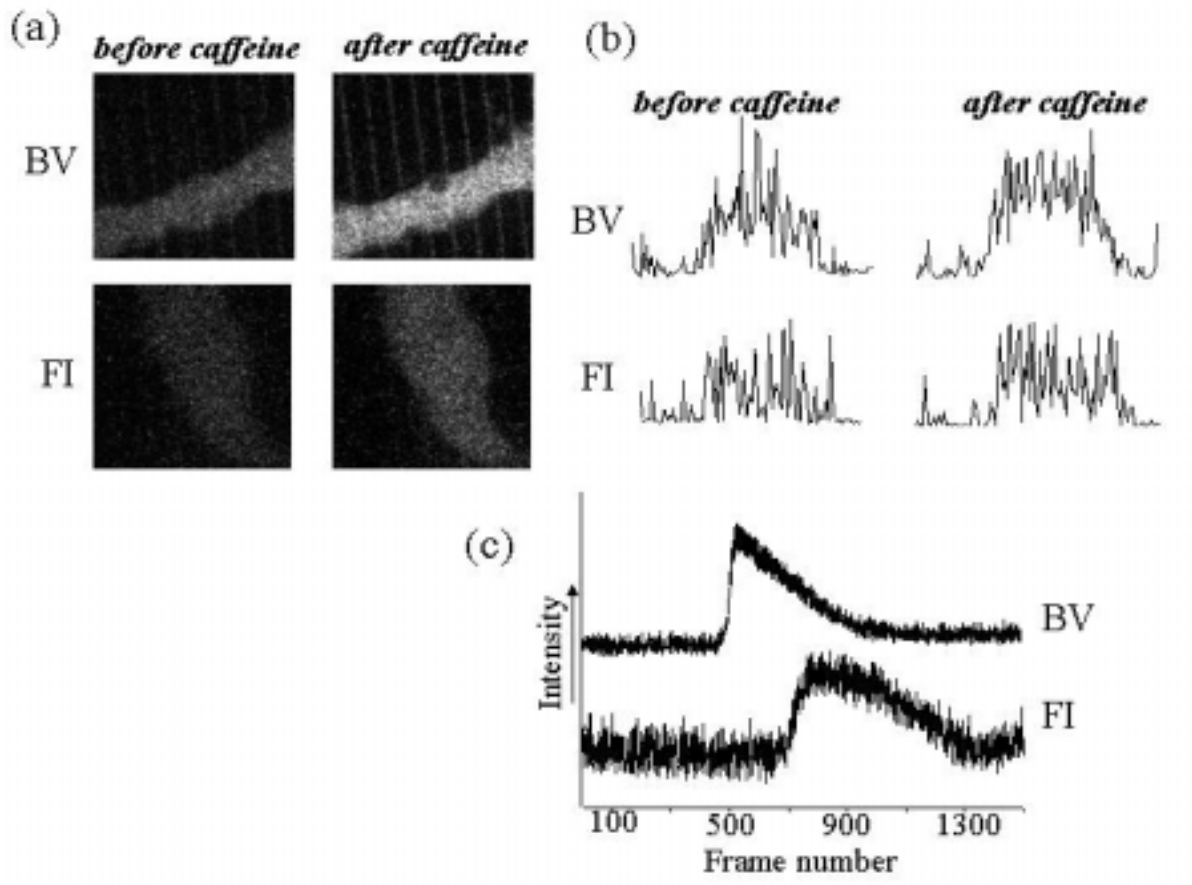

Figure 2: (a) Selected images from kinetic series, recorded with both FI- and BV-EMCCDs, of Fluo-4 loaded smooth muscle cells before and after addition of caffeine; 11 ms exposure time per frame; high EM gain setting throughout. (b) Line intensity profiles for signal-to-noise visualization, derived by randomly drawing a line across the corresponding cell images of (a). (c) Kinetic plots derived from ROI within cell, illustrating signal boost due to intracellular calcium release upon addition of caffeine.

images is readily apparent. The increase in emission upon release of Ca^{2+} into the cells is also shown. Again, random line intensity profiles derived from each image are representative of a significant increase in signal/shot noise ratio. Note that the signal from the FI camera after caffeine addition is closer in appearance to that from the BI camera before caffeine addition. Also shown in (c), are kinetic plots derived from each cell, calculated using the Region Of Interest (ROI) function within the Andor imaging software. The average signal from only the top 60% pixels were used, in order to exclude background pixels from the calculation. The point of caffeine injection and calcium build-up can readily be observed from the plots, followed by a slower decay. Furthermore, upon comparing the plots from FI and BV cameras, it is evident that more noise exists in the baseline of the FI plot, which may be assumed to arise from a greater relative contribution of shot noise to the signal.

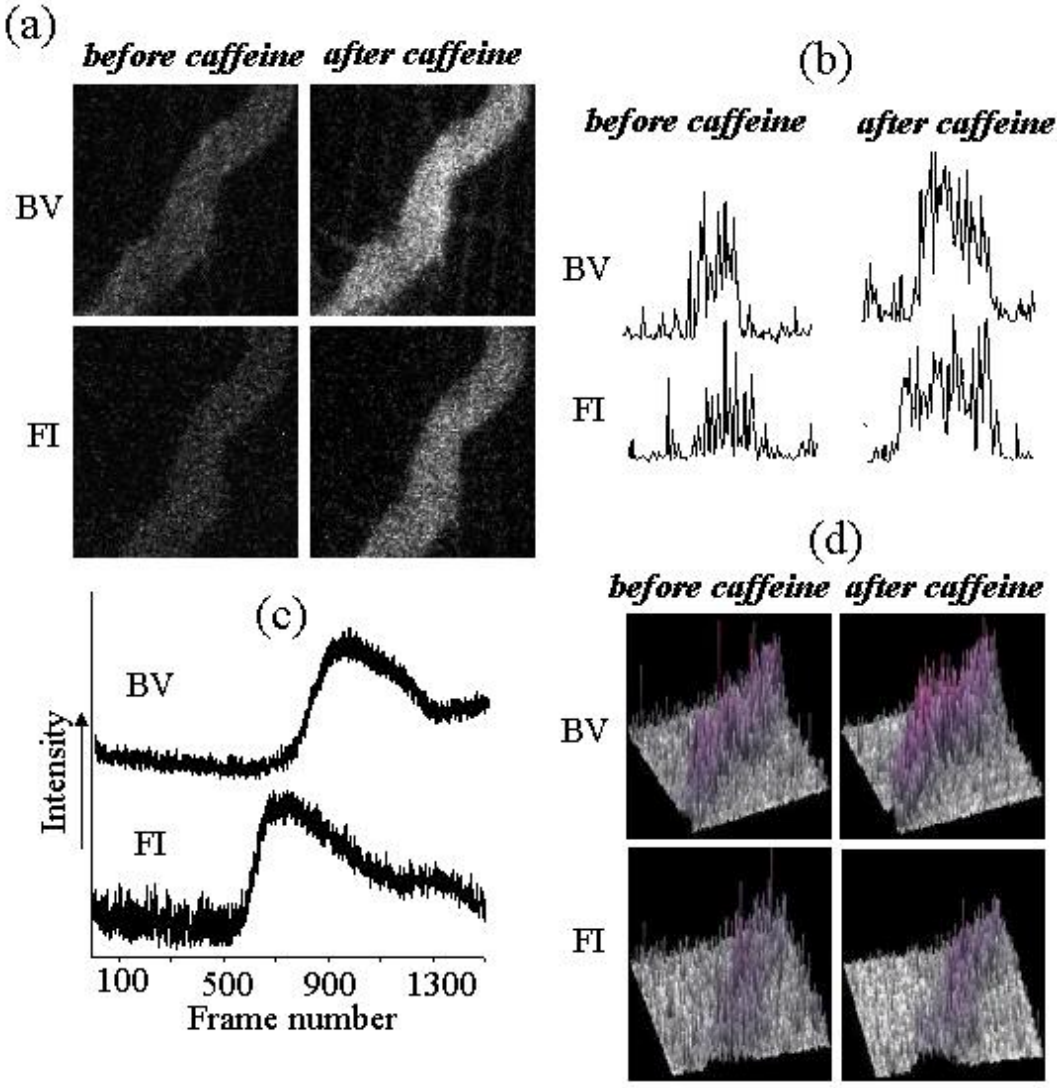

Figure 3: (a) Selected images from kinetic series, recorded with both FI- and BV-EMCCDs, *of the same Fluo-4 loaded smooth muscle cell*, before and after addition of caffeine; 17 ms exposure time per frame; high EM gain setting throughout. (b) Line intensity profiles for signal-to-noise visualization, derived by drawing a line across *the same* cross-section of the corresponding cell images of (a). (c) Kinetic plots derived from ROI within cell, illustrating signal boost due to intracellular calcium release upon addition of caffeine. (d) Series of 3D surface plots derived directly from each of the images in 3(a).

Figure 3 presents a series of data derived from kinetic series recorded with each camera in turn imaging a very weak signal from *the same cell*. The FI camera series was recorded first, followed by that from the BV camera, in order to ensure that any difference in signal intensity could be attributed only to camera performance and not to cell degradation or dye photobleaching. It is obvious from the representative selected images from each camera, before and after caffeine addition, shown in 3(a), that a stronger signal intensity is evident from the BV camera. In this case, line intensity profiles in 3(b) were derived from a very similar cross section across the cell in selected images. Again, the improvement in BV-EMCCD signal/shot noise ratio is immediately recognizable. As for figure 2 above, kinetic plots are provided for each cell, shown in figure 3(c), each clearly showing the increase in Fluo-4 emission intensity as caffeine is added. Again it is clear, particularly from the baseline before addition of caffeine, that the greater shot noise contribution from the lower QE, FI camera results in a noisier plot, despite the ROI pixel averaging used to derive these plots. Finally, by way of a further visualization of signal/noise ratio, 3(d) shows a series of 3D surface plots derived directly from each of the images in 3(a). It is clear that the lowest quality signal is that recorded by the FI camera before addition of caffeine to the cell. Addition of caffeine results in an improved surface plot that comes close in appearance to that recorded by the BV camera *before* caffeine addition. As expected, the highest quality signal/noise is evident from the BV camera, after addition of caffeine.

The opportunity was taken also to perform a cursory comparison of the BV-EMCCD to a GenIII ICCD in this low-light calcium flux approach. A detailed comparison was difficult however, as the pixel size of the ICCD sensor was smaller (6.7 um ^{2}) than that of the EMCCD sensors, ICCDs are known to exhibit markedly more cross-talk than EMCCDs (resulting in an uncontrolled 'artificial smoothing') and, since gating ability of the ICCD was inactivated, it had to be operated with significant sub-binning in order to achieve exposure times (and frame rates) close to that afforded by the smaller sensor of the iXon DV860. Although, the 4x1 vertically binned 'superpixel' employed by the ICCD in this comparison, served to make the effective pixel area closer to that of the EMCCD. Figure 4(a) shows a series of images taken from a kinetic series recorded by each camera, at 9 ms exposure time per frame (enabling ~ 110frames per second). Several image series where recorded first with the ICCD, then with the BV- EMCCD on the same field of cells. Whereas the field of view is obviously more extensive for the larger sensor of the ICCD, the contrast of the BV-EMCCD images appears to be superior. This is to be expected given the markedly restricted QE of ICCDs compared to the unrestrained QE of the BV-EMCCD. Indeed, at the 516nm $\lambda_{max.}$ of Fluo-4 dye, the BV-CCD should have > x4 QE than a GenIII filmed intensifier. Figure 4(b) shows a series of line intensity profiles taken from each image, confirming the overall superior signal/shot noise quality of the BV-EMCCD images.
A more in-depth comparison of EMCCD vs. ICCD technology has been given elsewhere[7].

Pinhole Imaging

In order to provide more controlled depiction of the signal-to-noise improvement offered by EMCCD technology in ultra low-light imaging, a simple experiment was carried out whereby a very weak LED light through pinholes (substantially filtered from the source by black card and a strong ND filter) was imaged at short exposure times.
The effect of turning up gain in such an experiment can be easily observed in Figure 5. This particular series of frames was captured with an iXon DV887 (FI), which contains a frame transfer 512x512 CCD87 from E2V, using 58ms exposure times (enabling 17 frames/sec) and shows images corresponding to a variety of increasing gain settings. For better visualization of signal-to-noise, a line intensity profile through the bottom pinhole spot is shown also. At Gain 0 the signal from the spots is completely lost within the readout noise of the camera. However, application of even 5x or 10x gain results in the signal beginning to amplify beyond this detection limit. As gain is further increased, readout noise becomes less and less of a factor, with the emphasis being more on signal shot noise considerations. The shot noise limitation of the signal can be readily observed – the 'spiky' profile of the intensity line at high gains illustrates that although the signal is clearly visible above noise floor we are still nevertheless dealing with a very low photon flux during a given short exposure. At this wavelength (~ 620nm), the BV-EMCCD has a x2.6 greater QE and would be expected to yield a x1.6 higher Signal/Shot Noise ratio. It is interesting to note also the smaller spurious spikes being amplified above the readout noise, particularly visible under the highest x500 gain setting. These are Clock Induced Charge spikes and are single electron noise events that arise as a result of charge transfer between pixels during CCD readout[7]. The fact that we can clearly observe these spikes indicates that the camera is at a gain setting where it can be

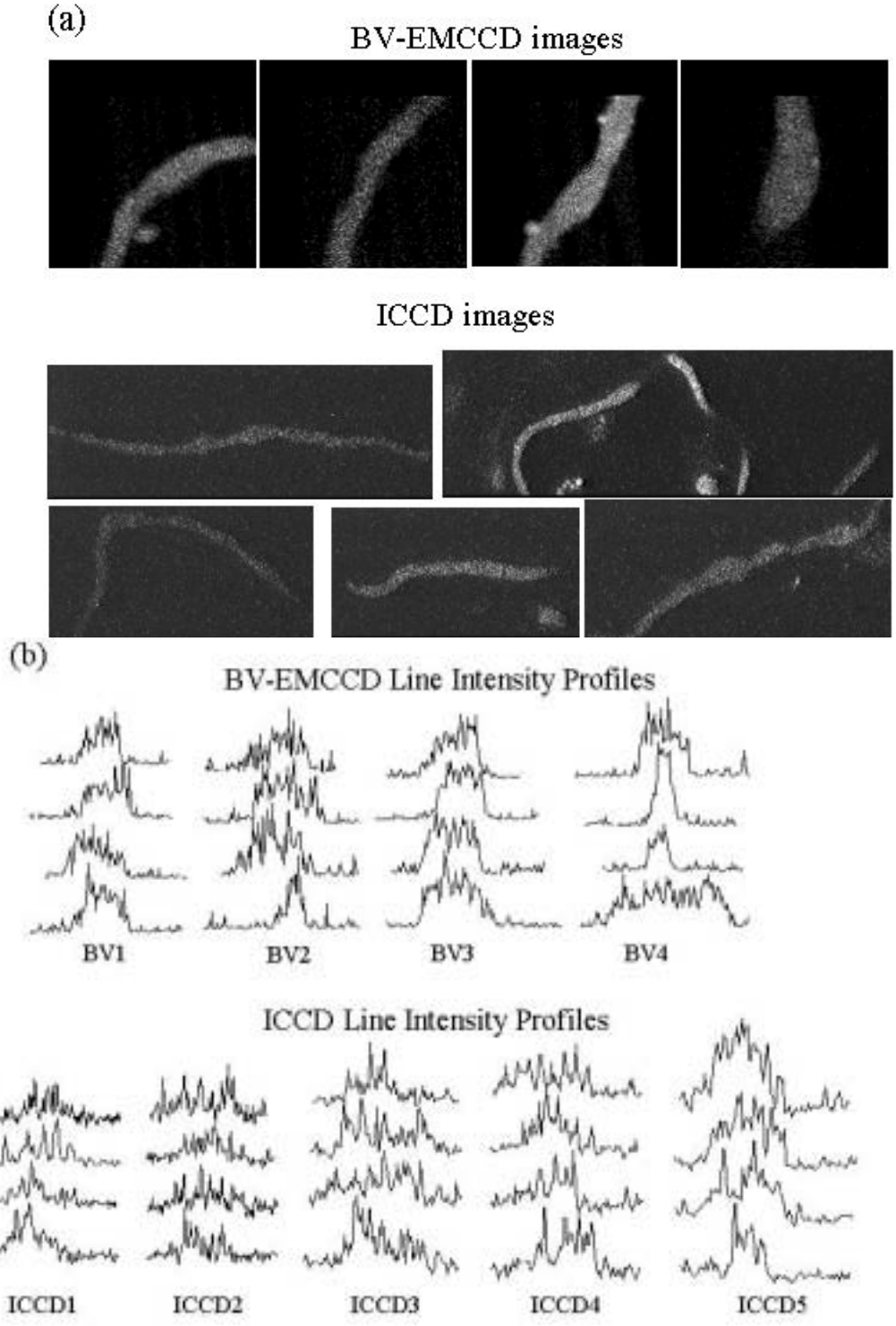

Figure 4: (a) Selected images from kinetic series, recorded with both BV-EMCCDs and ICCD cameras, of Fluo-4 loaded smooth muscle cells; 9 ms exposure time per frame; high EM and ICCD gain setting throughout. (b) Line intensity profiles for signal-to-noise visualization, derived by randomly drawing a line across the corresponding cell images of (a). Note that due to cross-talk between pixels in the ICCD images (50% of charge destined for a given pixel leaks to neighbouring pixels), the EMCCD images would have to be artificially smoothed to be more comparative.

considered to be single photon sensitive, since clearly single photoelectrons will also be observed. It is also interesting to note that this experiment serves to illustrate the use of EMCCD technology for single molecule detection (SMD), since these images are similar in nature, and signal level, to that expected from a well-optimized SMD set-up (for example using total internal reflection fluorescence microscopy)[8-10]. This would indeed be a well-optimized SMD image however as it would be unlikely to have excluded single photon background events from a TIRFM experiment to the extent that we have managed here.

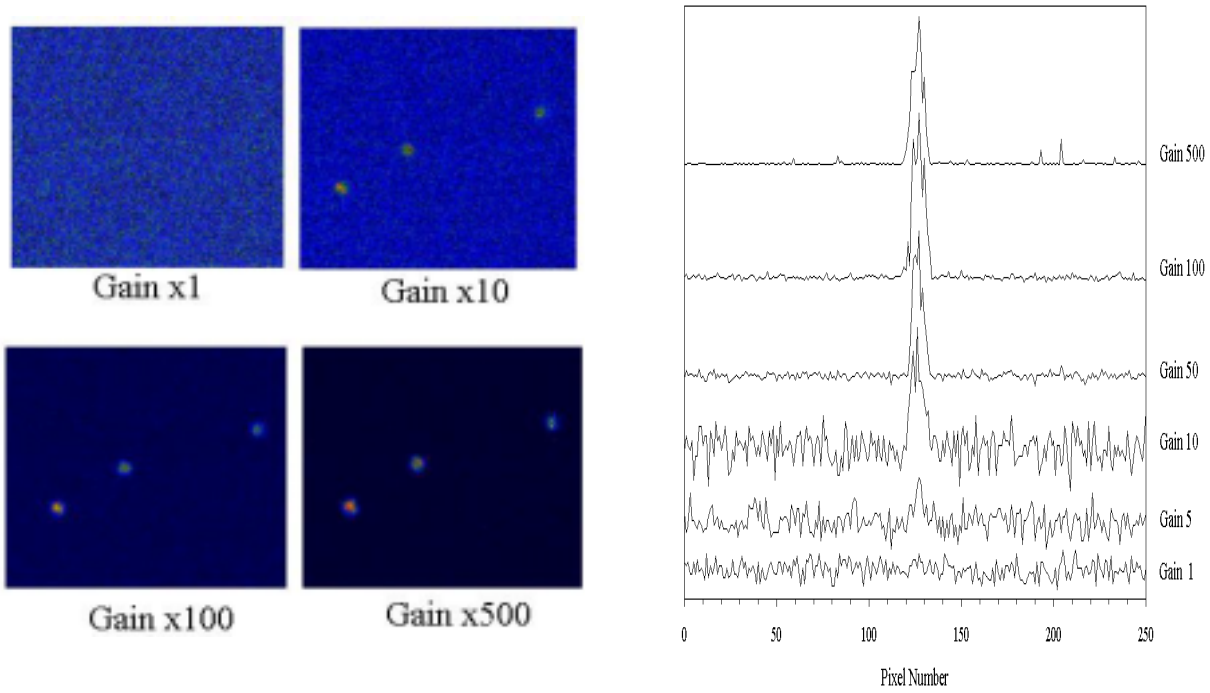

Figure 5: Frames captured with an iXon DV887 (FI), containing a frame-transfer 512x512 FI-EMCCD sensor, using 58ms exposure times (enabling 17 frames/sec), and showing images corresponding to a variety of increasing gain settings (only a sub-area of the images are shown). For better visualization of signal-to-noise, a line intensity profile through the bottom pinhole spot is shown also.

4. CONCLUSIONS

EMCCD technology (or on-chip multiplication) is being heralded as the future of high performance imaging and many future developments in CCD technology are likely to be focused in this area. The most immediate and prolific development in this area has been Andor's launch of a back-illuminated EMCCD device, combining extremely high photon conversion efficiency with the ability to eliminate the readout noise detection limit, yielding the most sensitive detector in the world, by a large margin. The results presented have demonstrated the combined effects of: (a) EMCCD technology in amplifying the signal from very weak sources (fluorophores within cells) above the readout noise floor that they would otherwise be completely immersed in; (b) back-thinned CCD technology in maximizing the signal/shot noise ratio from such weak photon fluxes. It has also been shown how this innovative development can offer significant signal improvements over that afforded by ICCD technology.

In terms of the spinning disk confocal microscopy set-up used for the intracellular calcium flux application that was demonstrated in these experiments, the extraordinary sensitivity of back-thinned EMCCD technology holds a number of distinct advantages. Ion flux changes are highly dynamic and fast frame rates are required in order to provide the necessary temporal resolution. Since short exposure times are employed in these measurements, very low photon levels are available in any given frame. Successful imaging requires not only amplification of these weak signals above the camera's inherent readout noise levels, which are increased at high-speed operation, but also requires that very few available photons are wasted. Use of a highly sensitive camera also permits good signal resolution with relatively low concentrations of indicator dye, thus reducing calcium buffering by the dye. Increased sensitivity has further important advantages since it makes it possible to reduce the excitation power, limiting both photobleaching of the indicator and photodamage to the tissue. This can greatly lengthen the experimental period over which living cells can be imaged. However, these benefits extend also to a large number of other of low-light dynamic techniques and applications, such as single molecule microscopy, 4D cellular microscopy, cell motility studies etc.

It should also be noted that use of back-thinned EMCCD technology can make the difference between seeing a signal and not if the signal is so extraordinarily weak that if during a given exposure, even a FI-EMCCD can detect only a few single photon events from the area of the emitting signal. In such an instance, such as in extremely weak single molecule detection, the extra photons (say a factor of 2 increase) converted and detected by the BV-EMCCD, may make the difference between confidently confirming the presence of the signal or not.

REFERENCES

1. J. R. Janesick, *Scientific Charge-Coupled Devices*, SPIE Press, Bellingham, Washington, 2001.

2. P. Jerram et al, "*The LLLCCD: Low Light Imaging without the need for an Intensifier*", SPIE **Vol 4306**, 2001.

3. C. D. Mackay et al, "*Sub-Electron Read Noise at MHz Pixel Rates*", http://www.marconitech.com/ccds/l3vision/technology.php.

4. S. H. Spencer and N. J. Catlett, "*Low Light Level Solid State TV Imaging*", March 2000, http://www.marconitech.com/ccds/l3vision/technology.php

5. E. J. Harris et al, "*Evaluation of a Novel CCD Camera for Dose Reduction in Digital Radiography*", http://www.marconitech.com/ccds/l3vision/technology.php

6. D. J. Denvir and Colin G. Coates, *"Electron Multiplying CCD Technology: Application to Ultrasensitive Detection of Biomolecules"*, January 2001, Photonics West, Biomedical Optics (BioS)

7. D. J. Denvir and Emer Conroy, *"Electron Multiplying CCD Technology: The New ICCD"*, July 2002, SPIE Annual Meeting, *in print.*

8. W. P. Ambrose et al, "*Single Molecule Fluorescence Spectroscopy at Ambient Temperature*", Chem. Rev. **Vol 99**, pp2929-2956, 1999.

9. R. M. Dickson et al, "*On/off Blinking and Switching Behaviour of Single Molecules of Green Fluorescent Protein*", Nature **Vol. 388**, pp355-358, 24 July 1997.

10. T. A. Byassee, W. C. W. Chan, and S. Nie, "*Probing Single Molecules in Single Living Cells*", Anal. Chem. **Vol. 72**, pp5606-5611, 2000.

11. G. P. Sergeant, M. A. Hollywood, K. D. McCloskey, K. D. Thornbury, and N. G. McHale, *"Specialised Pacemaking Cells in the Rabbit Urethra"*, J. Physiol. **Vol 526**, pp359-366, 2000.