

EMCCD Camera Technology Advances

New EMCCD Camera Offers 16-Bit Quantitative Stability for Ratiometric Imaging

Several years ago, Photometrics' introduction of the first electron-multiplying CCD (EMCCD) cameras designed for microscopy enabled life science researchers to detect ultra-low-light signals via on-chip multiplication gain. Since the advent of EMCCDs, camera manufacturers have focused their R&D efforts on areas that offer the most room for improvement of such devices, including bias and EM gain stability as well as sensor aging. Here we highlight the development of a new imaging platform that provides a greater capacity to control EMCCDs, thus allowing biological researchers to quantitatively measure low-light signals with extremely high fidelity. Advantages in high-speed measurement of a calcium ratiometric time-course application are demonstrated.

EMCCD Basics

Back-illuminated EMCCDs offer high quantum efficiency (QE), permitting the conversion of greater than 90% of incident photons to electrons. As well as extremely high QE, these detection devices have a unique structure in which electrons (generated by photons hitting the EMCCD) are accelerated through a series of pixels in order to cause impact-ionization events (fig. 1). The fundamental design of EMCCDs enables the generation of a greater number of electrons than were initially generated by the incoming signal itself, effectively providing amplification of low-light signals and allowing detection of signals as small as single fluorophores. This amplification technique does not lead to a loss in image resolution as is seen when using intensified or electron-bombardment cameras.

Timing Is Everything

Running an EMCCD is a complex task. Essentially, the key to unlocking the full potential of this detection technology is to control how the charge is moved from pixel to pixel. In order to move electrons from one pixel to the next, great care must be taken to ensure good charge-transfer efficiency (CTE). Voltage clocks linked to the pixels are used to control the movement of electrons. Precision timing of these clocks is critical to provide ultimate device performance.

Some camera manufacturers have gravitated towards lowering the operating temperature of the EMCCD in order to help control this process. The deep-cooling approach is valid, as the amount of voltage required to shift charge between pixels in the EMCCD electron-multiplication register is proportional to the

temperature at which the sensor is operated. For instance, it takes less voltage to amplify a given amount of charge at -80°C than it does to move the same amount of charge at -30°C . Therefore, lowering the camera temperature has allowed manufacturers to control the pixels' voltage clocks more easily.

To really tackle the issue of finely controlling the voltage clocks, however, the engineers at Photometrics employ patent-pending ACE (Advanced Clocking Enhancement) technology and an intelligent FPGA design. The resultant timing resolution is over 12x more precise than other EMCCD cameras. This precision voltage-clock timing is an integral element of a newly introduced EMCCD imaging platform that delivers significantly improved bias stability (measured by looking at the interframe variance of the detector with no incident light) and quantitative performance.

When we measured the interframe bias stability of the new QuantEM camera (Photometrics) and compared it to another EMCCD camera, the interframe average bias intensity for the other camera demonstrated a slope over time while the interframe average bias intensity for the QuantEM camera remained virtually unchanged (fig. 2).

In fact, the other EMCCD camera's drift is completely unpredictable and

(more troublingly) nonlinear. Until now, experiments for which frame-to-frame variances matter, such as time-course studies or ratiometric analysis over time, have been extremely difficult to quantify accurately using EMCCDs. The new EMCCD imaging platform eliminates this drift, yielding quantitative data that is stable over time.

Active Feedback and Device Regulation

Another crucial element of the new EMCCD camera design is an active-feedback system that monitors the timing of the pixel-to-pixel charge movements and allows the measurements of these charges to remain extremely precise. This patent-pending PAR (Photometrics Active Regulation) feedback system continually controls EM gain to a level unprecedented for EMCCD cameras and ensures there is no deviation from the accurate, quantitative, factory-set parameters.

Although cameras that allow users to change operating parameters (e.g. parallel shift times) are available, the draw-

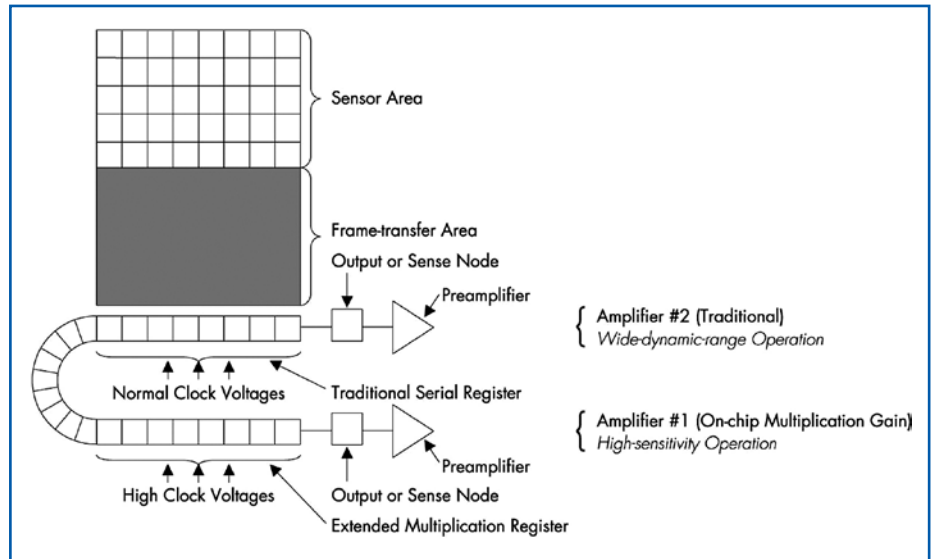


Fig. 1: Electron-multiplying CCD (EMCCD) cameras move charge through the pixels of a special serial register with high clock voltages in order to amplify signal via an impact-ionization process.

back of these cameras is that changing such parameters has numerous effects on the electronics and stability of the imaging device in relation to clocks and

voltages. These effects can produce variations in the measurements taken, causing users to unintentionally alter the quantitative nature of their camera. It

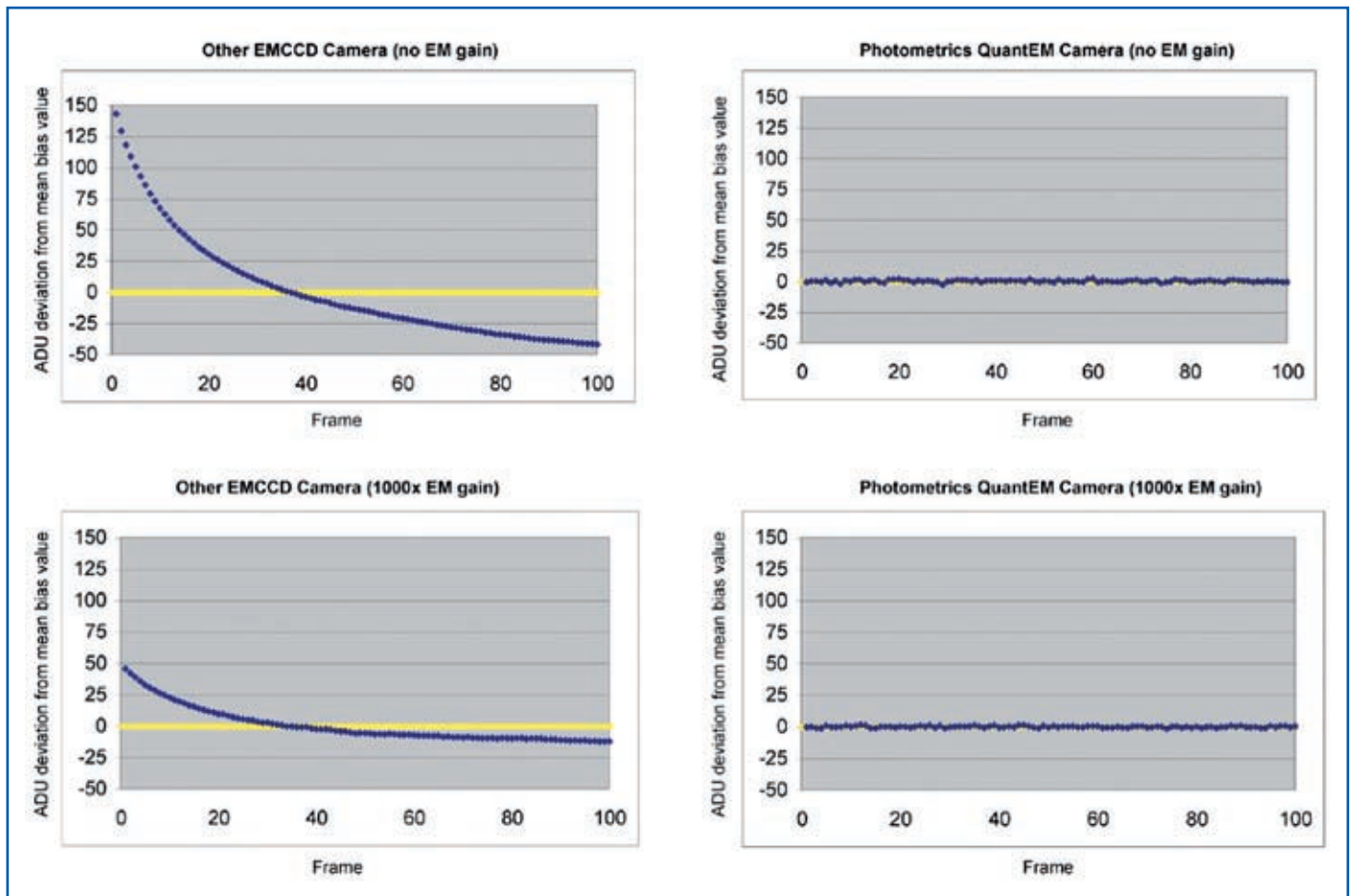


Fig. 2: Analog-to-digital unit (ADU) deviation from the sequence's mean bias (dark image) value over a 100-frame series for the new Photometrics QuantEM vs. another EMCCD camera. One hundred 70 ms bias frames were taken at equivalent analog gain settings both with and without 1000x EM gain and the average bias intensity value was measured. Each frame's deviation from the sequence's mean value was plotted against the frame number. The other camera exhibits a drift in this value over a sequence of frames; the QuantEM camera shows no drift characteristic.

Camera	Hot pixels (2e- above image mean, N = 100)	Hot pixels (5e- above image mean, N = 100)
Photometrics QuantEM (-30°C cooling)	0.39% of total pixels	0.01% of total pixels
Other EMCCD camera (-80°C cooling)	1.23% of total pixels	0.22% of total pixels

Table 1: Comparison of electron noise above the image mean for two EMCCD cameras. Experiments were conducted with 10 MHz readout in frame-transfer mode using 10 ms exposures at 200x EM gain (the maximum real gain achievable by the other EMCCD camera, probably due to aging of the sensor). One hundred images were acquired with each camera and the analysis was performed on every image. A mean value was obtained for each camera's sequence of images. Operating parameters were equivalent.

should also be noted that, unlike PAR, these operating parameters cannot be used to stabilize EM gain.

The active-feedback device regulation of the QuantEM imaging platform has resulted in unprecedented camera stability as well as interframe variations that are significantly smaller than any other EMCCD camera available for bioscience research. The new camera's high-fidelity, 16-bit measurements provide excellent quantitative accuracy and precision for applications such as total internal reflection fluorescence (TIRF) microscopy and fluorescence resonance energy transfer (FRET) imaging.

Clock-Induced Charge

Clock-induced charge (CIC, or spurious charge) occurs when an electron is inadvertently generated without being induced by an incident photon. Clock-induced charge is exactly what its name suggests – charge induced by clock voltages. Even though “CIC appears to be almost independent of temperature,” [1] some camera manufacturers nonetheless try to rely on deep cooling to suppress this unwanted charge. The new QuantEM imaging platform uses ACE technology to minimize the generation of clock-induced charge.

A simple yet effective method of measuring the generation of spurious hot pixels (sometimes referred to as dark background events) is to acquire dark images with EM gain on and determine the number of hot pixels anomalously generated. Of course, when measuring dark background events, it is important to clearly define what constitutes an event. Some camera manufacturers will quote a number of events per pixel without defining an event threshold, making direct comparison to their published numbers impossible. Therefore, we ran our own

measurements on the number of hot pixels for one of these other EMCCD cameras as well for the Photometrics QuantEM camera (table 1). We defined dark-background-event thresholds as being at least 2 or 5 electrons above the image mean.

This minimization of spurious events vastly improves image quality for applications such as widefield confocal microscopy and live-cell fluorescence imaging.

Overcoming Aging Instability

EMCCD aging manifests itself in the form of reduced EM gain output. Basically, the impact-ionization process described earlier becomes less effective as the EMCCD gets older. Extensive studies have revealed that the drop-off in EM gain due to aging follows an exponential-like decay curve. To date, this effect has served to compromise the utility of EMCCDs for quantitative imaging over extended time periods.

The QuantEM camera overcomes the aforementioned age-related deterioration by recalibrating the voltage applied to the electron-multiplication register so that it will continue to yield the amounts of EM gain requested by the user. The new EMCCD camera's intelligent FPGA facilitates this automated recalibration process. PAR and ACE technologies ensure the device provides uncompromised EM gain stability while minimizing detector noise. Thus, camera measurements remain consistent and quantitative even as the sensor ages.

Ratiometric Cellular Calcium Ion Imaging

One of the most stringent tests that can be conducted by camera end-users is a ratiometric analysis of a stream of frames. Using this type of analysis, one frame is simply divided by the next (e.g.,

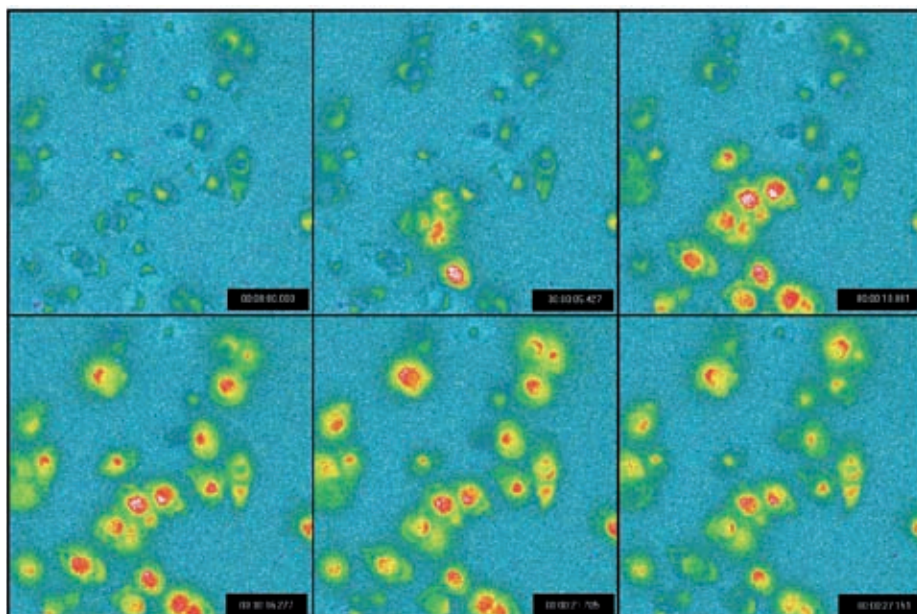


Fig. 3: Time course of calcium ion measurements in living THP1 cells (a monocyte-derived stable cell line). Cells were loaded with Fura and then 48 sequential images were acquired with excitation at 340 nm and 380 nm over a span of 46.2 sec (24 images at each emission wavelength). Next, the corresponding 340 nm and 380 nm time-point images were ratioed and pseudocolored. Six of the resultant 24 ratiometric images are shown here. For both excitation wavelengths, the camera was run at 10 MHz and gain state 3. For the 340 nm excitation, 100 ms exposures were taken with the EM gain set at 250x. For the 380 nm excitation, 50 ms exposures were taken with the EM gain set at 350x.

frame 1 is divided by frame 2). Any drift between the frames will prevent the signal from being properly measured.

Ratiometric analysis is commonly utilized for experiments such as Fura calcium measurements, in which cells are loaded with Fura dye and the emission of the dye is measured sequentially at two excitation wavelengths (340 nm and 380 nm). The 340-nm image is divided by the 380-nm image and the results are displayed as ratiometric pseudocolored images.

Such an analysis was recently performed at the lab of Simon Watkins at the University of Pittsburgh with a QuantEM camera. Cells were loaded with Fura for approximately 20 min and then the calcium response was triggered mechanically using a microinjection device to touch the cells [2]. Sequential images were acquired at 340 nm and 380 nm and the ratiometric results were displayed in pseudocolor (fig. 3). The absence of interframe variance demonstrated by the camera permits researchers to perform ratiometric analysis without seeing interframe-variance drift mixed together with calcium-signal response. Furthermore, owing to the camera's exceptional sensitivity, extremely short exposure times are possible, thereby allowing more rapid data acquisition and enabling greater temporal resolution in experiments.

Immediate Utility

The ability of the Photometrics QuantEM camera to provide unprecedented bias and EM gain stability over time enables life science researchers to conduct accurate ratiometric analysis in time-course experiments, acquire reproducible data during long-term studies, and capture streaming data for multidimensional time-lapse investigations. This new EM-CCD imaging platform's utility extends to a broad range of quantitative applications, including ratiometric cellular ion imaging (e.g., calcium and pH), TIRF microscopy, FRET imaging, fluorescence recovery after photobleaching (FRAP), widefield confocal microscopy, live-cell fluorescence imaging, and numerous multiprobe experiments.

References:

- [1] Dark Signal and Clock-Induced Charge in L3Vision CCD Sensors (e2v technologies limited 2003). Low-Light Technical Note 4.
- [2] Watkins S.C., et al., *Immunity* 23(3), 309-18 (2005)

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