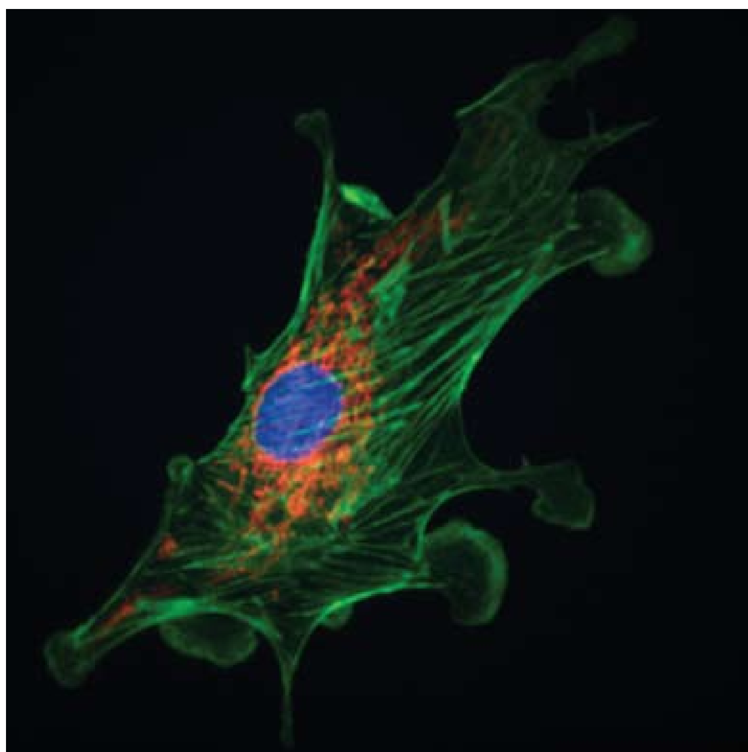
Advances in EMCCD Technology: Making Imaging Less Arbitrary

By Deepak Sharma, PhD, Senior Product Manager, Photometrics

Recent advances in EMCCD technology have solved the problem of non-standardized measurement units by using the photoelectron to standardize imaging experiments.

Over the past decade, EMCCD (Electron Multiplying Charge Coupled Device) cameras have revolutionized the world of life science imaging. Because they boost signal-to-noise by multiplying incoming light signals, EMCCD cameras have given researchers access to the entire gamut of low-light imaging applications—from whole animal *in vivo* imaging to super-resolution fluorescence visualization of sub-cellular structures—allowing researchers to see biological entities that were difficult to see before.

But there's a problem: EMCCD as well as CCD cameras report imaging data in Analog-to-Digital Units, which are arbitrary. These ADUs (called "grey scale units" in Europe) are completely dependent on the camera's gain settings, which vary between manufacturers and even camera to camera. In most cases, manufacturers do not measure or reveal these gain values at each setting.



Fibroblast cell triple-stained with DAPI, FITC-Actin and Mito-Tracker. The image was taken with the Evolve camera and Olympus DSU confocal. (Source: Graham Dellaire, PhD, Nuclear Structure and Cancer Laboratory, Dalhousie University, Halifax, Nova Scotia)

Because the measurement unit in scientific cameras is not standardized, researchers cannot reliably compare imaging data or reproduce their results between, or even within their own labs.

Imagine a world where researchers could reproduce their imaging experiments and more directly compare their data. Think of the scientific advances we could make if studies were more quantitative and verifiable. And consider the new insights we could derive from being able to integrate data from different experiments.

Recent advances in EMCCD technology have solved the measurement problem by using the photoelectron—an absolute unit—to standardize imaging experiments. This innovation is helping to standardize imaging experiments and advance emerging fields such as super-resolution microscopy.

Standardizing Imaging Results

EMCCDs work by generating a greater signal than was initially emitted by the incoming signal itself, thus producing sufficient amplification to detect even a single fluorophore.

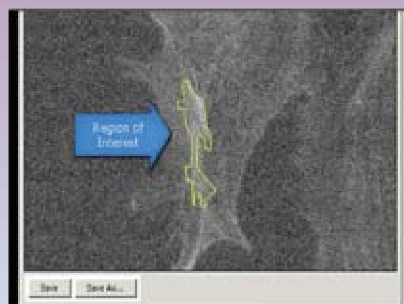
As with CCD cameras, incoming photons generate photoelectrons, which are then shifted through pixels and each pixel is measured sequentially as it is read out. In an EMCCD, however, incoming photons are sequentially moved into an extended register where high voltages are applied, and the electron undergoes impact ionization, multiplying one electron into many.

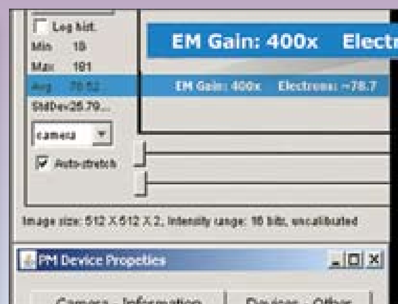
Until recently, EMCCD cameras counted photoelectrons by converting them into Analog-to-Digital Units (ADUs). This value, however, depends entirely on the gain settings of the camera, and because these settings are undefined by most camera manufacturers and easily manipulated by users, ADUs are not a reliable way in which to evaluate imaging experiments between cameras or between labs. For example, if imaged at different gain settings, a feature with a given quantum yield will appear to have different brightness levels and possess different ADU values. This lack of reproducibility confines the significance of the data to a single snapshot in time, limiting its contribution to future scientific discovery.

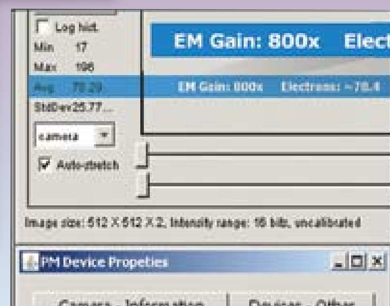
Absolute Units

The only way to achieve reproducible, quantitative imaging data is to measure that data in absolute units—in this case, the photoelectron.

If a camera's gain settings are available, researchers can manually back-calculate from ADUs to photoelectrons—a long, complicated and painstaking







The Evolve EMCCD camera allows the user to manually input EM Gain settings. While the ADU count rises significantly between 400× and 800× EM Gain, the electron count does not increase, giving the researcher an absolute measurement in real time.

process that requires careful characterization of the camera. Few biologists take the time or have the know-how to make these calculations.

The Evolve camera's Quant-View feature uses the camera's gain settings to automatically back-calculate pixel values to the number of photoelectrons, presenting brightness level in terms of photoelectrons in real time. By standardizing the unit of measurement, Quant-View technology allows researchers to compare images taken at different gain settings, even on different cameras—in absolute terms.

According to Sidney L. Shaw, Assistant Professor, Department of Physics, Indiana University, "The Evolve camera finally gets rid of arbitrary gray levels in favor of photoelectron counts, a meaningful standard that scientists can use for comparing their imaging systems and their image-based data."

Aging Instability

As EMCCD chips age, their gain amplification characteristics change and EM gain output is reduced. Thus a camera that had a 100x EM gain may not have the same gain in a matter of weeks, which will affect the accuracy and reproducibility of imaging data.

Knowing the camera's gain settings is critical to any truly quantitative application. Because of EMCCD chip deterioration, it is crucial to re-calibrate the camera at regular intervals. In the context of EMCCD cameras, this involves calibrating the gain so that a given brightness on the image corresponds to a reliable measurement of the "real-world" value representing the number of photons that a pixel has registered.

Previous cameras would 'calibrate' by altering electronic gain settings to closely match what the user requested. The Evolve camera takes the calibration one step further—it measures the real gain setting applied by the camera and uses the real number to back calculate the image data to the photoelectron number. For example, if a user dials in 100X EM gain, the calibration routine will 'tweak' the camera's electronic settings may actually provide only 99.2×.

While other cameras settle for the "as close as possible" approach, the Evolve camera features Rapid-Cal, which measures the real gain being applied and corrects the data in real time, and uses this calibration to accurately quantify data in photoelectrons, without having to remove the camera from the microscope.

Benefits of EMCCD for Super-Resolution Microscopy

Until recently, all fluorescence microscopy methods had been limited in resolution by the diffraction limit for the wavelength of light. This meant nothing below a quarter of a micrometer could be resolved, yet many cellular substructures are much smaller.

Now, new "super-resolution fluorescence microscopy" techniques have broken this barrier, allowing researchers to see structures only tens of nanometers apart. These technologies benefit from advances in EMCCD technology to record and quantify imaging experiments.

Fluorescence Photoactivation Localization Microscopy (FPALM) leverages the unique properties of photoactivatable chromophores, fluorescent labels that can be genetically encoded. In FPALM, structures of interest are labeled using photoswitchable fluorophores that can be activated by a flash of light at a particular wavelength. The molecule then emits a photon at a different but specific wavelength one nanosecond later, which an EMCCD camera records.

The activation process produces a distribution of well-separated "switched on" molecules, the centroids of which are triangulated from the photon distribution. After the initial round of molecules photobleach, another flash of light at the proper wavelength activates another round of molecules for imaging. This process is repeated until an EMCCD assembles an image of the structure of interest with very high structural resolution.

Spurious noise events, characterized by the appearance of a single bright pixel, are a phenomenon common to EMCCD cameras. Because FPALM looks at single molecules, spurious noise events can complicate FPALM imaging. The Evolve uses an on-the-fly noise reduction algorithm (Background Event Reduction Technology, or BERT) that can be applied to significantly reduce spurious events, which are generated by dark current or the CCD voltage clocks.

Because the Evolve converts incoming light signals into a photoelectron count in real time, the mean number of photoelectrons contributing to a given pixel can be presented on the fly without arduous secondary calibrations and calculations. This absolute unit brightness calculation leads to more meaningful comparisons, thus accelerating FPALM research.

Summary

Current advances in EMCCD technology have replaced an arbitrary unit of measurement—the ADU—with the photoelectron in an effort to standardize imaging data and make imaging experiments reproducible between scientists.

Because it enables accurate and reproducible quantitation of images in absolute units, and because of the quantitative calibration feature, the Evolve EMCCD can be leveraged towards advances in super-resolution microscopy. ■