Evolve EMCCD for Fluorescence Photoactivation Localization Microscopy (FPALM)

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Classical methods in fluorescence microscopy involve a very wide range of approaches. However, up until fairly recently, all have been subject a common limitation: the smallest resolvable details are determined by the diffraction limit for the wavelength of light being used. It was previously thought that visualization of spatial localizations beyond this resolution limit would be impossible using light microscopy. Until recently, the only recourse to achieve higher spatial resolution was to resort to electron microscopy or scanned probe microscopy.

Fluorescence Photoactivation Localization Microscopy (FPALM)

Compelling and innovative approaches have been introduced that leverage new technology such as photoactivatable fluorescent proteins and break the conventional resolution limit of light microscopy. The appearance of methods such as FPALM, PALM, and STORM provide greater spatial resolution than ever before thought possible; such technologies also benefit heavily from the advantages provided by the latest generation of EMCCD camera technologies incorporated into the Evolve EMCCD. Eric Betzig, Xiaowei Zhuang of the Howard Hughes Medical Institute, Harvard and Sam Hess of the University of Maine have contributed to the development of the FPALM (2006), STORM (2006) and PALM techniques (2006).

FPALM and the related techniques of PALM and STORM (stochastic optical reconstruction microscopy) leverage the unique properties of photoactivatable chromophores. FPALM typically uses a conventional high-numerical aperture immersion lens and conventional widefield illumination. PALM frequently uses a TIRF illumination scheme (Axelrod, 1981) that restricts depth of field to a shallow region just above the coverslip interface; this measure may permit greater contrast but limits depth to which structures can be imaged. FPALM and related methods are suitable for imaging a wide variety of samples to a lateral resolution on the order of tens of nanometers.

PALMIRA (PALM + Independently Running Acquisition) has been developed by the group of Alexander Eggner and Stefan Hell (2007). PALMIRA is an extension of the photoactivation approach that improves the rate at which data can be acquired by a factor of 100.

These methods are based around the statistical probability of switching individual fluorochrome molecules on with a flash of light. The basic idea is to label structures of interest with photoswitchable fluorophores (in a dark state) that can be activated by a flash of light at a particular wavelength.

Because this activation phenomenon is stochastic, a distribution of well separated molecules is 'switched on' and the centroid of the single molecule localization can be calculated to well below the resolution limit. The optical magnification is an important consideration, and these techniques demand sufficient magnification to adequately sample a diffraction limited spot across two or more pixels at the camera plane (Figure 1). If necessary, a magnifying telescope in the detection path in front of the camera can be used to provide additional magnification.

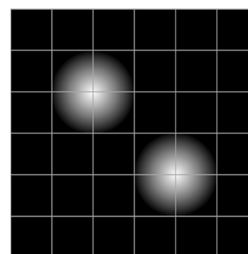

Figure 1. Diagram of gaussian photon distributions from point spread function of single fluorescent molecules overlayed on a pixel array. For FPALM imaging, the point spread function diameter should be equivalent to at least 2 pixels to permit precise molecular localization.

For precise localization, no more than one molecule can be localized within each diffraction limited area, per frame. When this is the case, the centroid of the Gaussian spot resulting from the diffraction point-spread-function can be taken to indicate the physical location of the single molecule yielding the signal (Figure 2). In other words, a very bright point source arising from the sample is unlikely to contribute only to a single pixel without having some proportional influence on the brightness of surrounding pixels. By triangulating the centroid from this Gaussian brightness distribution, which is spread over several pixels on the camera, the molecular location can be recorded in computer space to very high spatial precision.

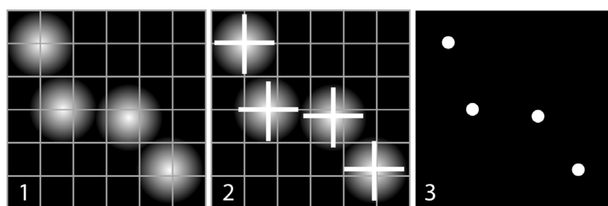

Figure 2. Determination of molecular locations and computer rendering of super-resolution image. (1) the single molecule point spread functions is used to determine (2) the centroid locations of the single molecules by triangulating the centroid from the photon distribution across multiple pixels. (3) the single molecule locations are computer rendered at super-resolution, the precision is determined by the statistics of the photon counts from the raw exposure.

Calculating the precision with which a molecule is localized requires knowing the number of photons detected from the molecule. For this reason, the gain (mapping of number of electrons to a brightness level) must be known in order to determine precision of localization (Gould, et al., 2009). Pixel brightness values in the offset subtracted images obtained by the camera are proportional to the number of detected photons; however, to make meaningful comparisons the brightness values must be calibrated to absolute units. This was formerly an involved task, however the Photometrics Evolve makes this much easier through thoughtful internal calibration measures.

As EMCCD chips age, the gain amplification characteristics change, and so it is of great importance to be able to reliably calibrate the gain amplification at regular intervals. The Evolve incorporates an innovative and convenient built-in light source to permit this calibration relative to a physical standard integral to the camera without the necessity of removing the camera from the microscope.

It is also important to linearize the gain mapping such that the investigator knows how much amplification is being applied (e.g. 20x gain amplifies the signal 20x, 100x gain amplifies the signal 100x). On some previous generations of EMCCD this gain mapping was non-linear so that the gain setting had no intuitive relation to the actual signal amplification. The Evolve linearizes the gain mapping with over 700 sample points all the way from 1x to 1000x.

As a ground-breaking measure in quantitative EMCCD technology, the Evolve permits 'on the fly' presentation of brightness levels in terms of absolute units of electrons. In other words, because of the sophisticated built-in calibration and quantitative considerations, the mean number of photo-electrons contributing to a given pixel can be presented on the fly without arduous secondary calibrations and calculations. This functionality is very useful for determining precision in FPALM imaging.

Spurious noise events may degrade the signal to noise ratio and complicate the determination of signal due to single molecules in FPALM imaging. Spurious noise is characterized by the appearance of single bright pixels and is a phenomenon associated with gain amplification. Under controlled circumstances, spurious noise events can be discriminated from bright pixels that originate from the actual image data (Figure 3).

The Photometrics Evolve provides additional benefit to super-resolution microscopy by incorporating an on-the-fly noise reduction algorithm (background event reduction technology or BERT) that can be optionally applied to greatly attenuate spurious charge speckling in each frame. The parameters of this data enhancing operation can be adjusted interactively to reflect noise events that clearly fall outside the limitations of the optics and into the realm of noise artifact.

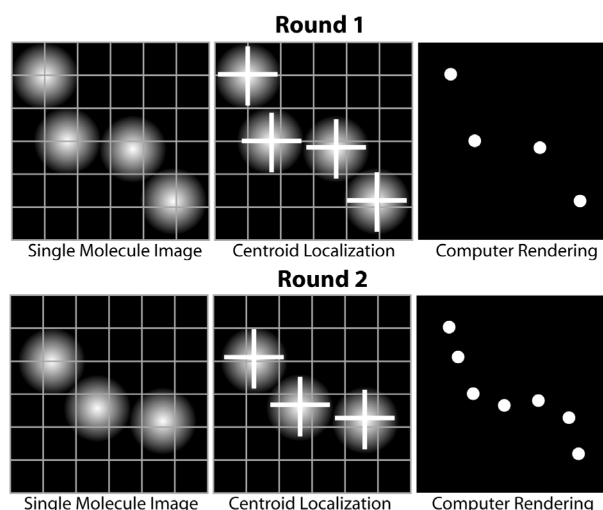

Figure 3. Spurious noise appears as individual bright pixels whereas the signal from Gaussian point spread functions is spread over multiple pixels (center diagram). BERT technology removes the noise and permits precise reconstruction of the molecular localizations in the rendered image.

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 in turn until an image of the structure of interest with very high structural resolution is assembled by combining the data from each round of activation. FPALM rendering generates an FPALM image by plotting the positions of localized molecules as two-dimensional Gaussian spots of width proportional to the calculated localization precision and integrated intensity proportional to the number of detected photons (Figure 4).

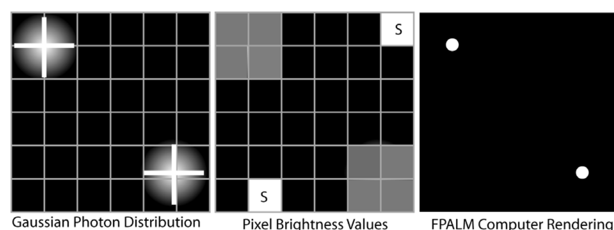

Figure 4. Successive rounds of photoactivation and single molecule imaging, centroid determination, and additive computer rendering of the molecular localizations builds up a more complete dataset. Individual molecular localizations are added to the rendering round after round until the complete structure can be determined.

A major consideration for FPALM imaging is that the sample must not move significantly during the acquisition time in order to allow confident determination of the point spread functions resulting from single molecules. Also, the molecules making up the structure of interest should not diffuse significantly during the time a frame is acquired as this would likewise cause blurring or streaking of the Gaussian signal distribution.

The development of genetically encoded fluorescent proteins that can be used in living cells to provide a marker for genetic expression has revolutionized biology. The fluorescent labels used for FPALM may be genetically encoded, and such genetically coded probes fall into 3 main groups (Lukyanov, et al., 2005). The first group includes fluorescent proteins such as PAGFP, and PSCFP as well as PAmRFP1. These proteins have an irreversible change from a neutral form to a bright anionic form with differential excitation characteristics from the neutral form. The second group is capable of an irreversible change of the chromophore from a green fluorescent to a red fluorescent state and consists of fluorescent proteins from the Anthozoan corals. Such fluorescent proteins include the tetrameric Kaede and EosFP proteins as well as the monomeric Dendra protein. The third group includes reversible proteins such as kindling red fluorescent protein (KFP1) and the Dronpa family of proteins.

Summary: Evolve Advantages for FPALM Superresolution

Photoactivation super-resolution imaging methods are single molecule imaging approaches and the benefits of the very high quantum efficiency and state-of-the-art low-noise optimizations of the Photometrics Evolve can be leveraged towards further advances of this approach. Precise molecular localization requires accurate photometric measurement of the number of photons and the quantitative calibration provisions of the Evolve permit access to such measurements with a level of convenience and control that is unparalleled by competing technologies. The Evolve EMCCD technology has reached a high state of refinement and permits accurate and repeatable quantitation of absolute signal levels.

Customer Assurance

In the interest of fueling rapid innovation and discovery, Photometrics Customer Assurance package has been developed to further assist researchers in meeting their research goals. The Performance Assurance Package provides training, rapid response to equipment maintenance, and loaner equipment in the event of service for the life of a research grant. This package provides maximum return on technology investment though tailored training on advanced operational use of imaging instrumentation in order to maximize data and also provides priority response in the event of instrument downtime.

For more information on the Performance Assurance Package for Photometrics cameras, please visit www.photomet.com/support.

To learn more about the ground-breaking high-performance EMCCD cameras from Photometrics, please visit www.evolve-emccd.com.

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