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Single-Molecule High-Resolution Imaging with Photobleaching (SHRImP)

Synonyms

NALMS (nanometer-localized multiple single-molecule fluorescence microscopy)

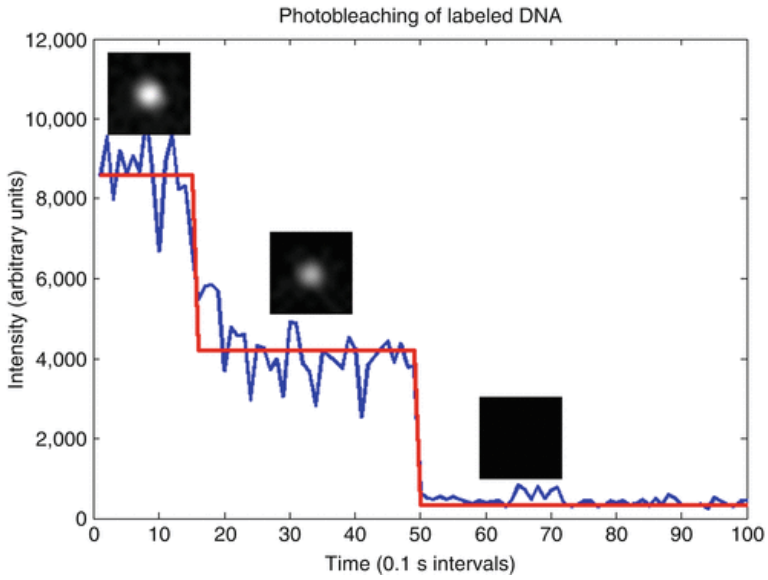
Definition

Single-molecule high-resolution imaging with photobleaching (SHRImP) is a single-molecule technique that uses the quantal photobleaching of two fluorophores to measure distances between 10 and 100 nm with nanometer precision.

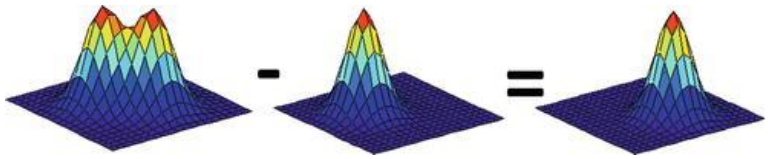
Basic Characteristics

Before the development of single-molecule imaging techniques, a gap existed in the ability of fluorescence microscopy to measure distances in biological systems. Distances smaller than 10 nm could be probed with fluorescence resonant energy transfer (FRET) and distances larger than ≈250 nm could be resolved using conventional microscopy. The SHRImP technique and its cousin, NALMS, bridge this gap by circumventing the Rayleigh criterion. In conventional light microscopy, the Rayleigh criterion limits the minimum resolvable level of detail in an image. According to diffraction theory, a point source of light - no matter how small the "point source" is - has a finite width of λ/(2 * numerical aperture) or approximately 250 nm. The Rayleigh criterion says that two point sources of light - two fluorophores, for example - are resolvable when the maximum of one source coincides with the first diffraction minimum of the other. For a 1.45 NA visible-light objective, this means that the smallest resolvable feature in an image is separated by approximately 250 nm. The SHRImP technique combines single-molecule localization with photobleaching to resolve distances smaller than this.

To measure distances using SHRImP, two identical fluorophores are attached to the structure of interest and excited by a light source. An EMCCD camera or other low-noise imaging device is used to record the emitted fluorescence over time. After prolonged excitation, the fluorophores will photobleach, that is, cease to emit light (Fig. 1). This process is the result of irreversible chemical damage or covalent modification to the fluorophores. SHRImP and NALMS use the sequential, stochastic photobleaching of the two or more fluorophores used as probes to localize each fluorophore individually. After the first fluorophore photobleaches, the FIONA (fluorescence imaging with one nanometer accuracy) technique can be used to locate the center of the point spread function of the single remaining fluorophore. This is accomplished by collecting enough photons from a single dye so that, when fit with a Gaussian function, the mean of the emission peak can be located very precisely. (See Fluorescence Imaging with one-nanometer accuracy (FIONA) section for more details.) By subtracting the image of the final remaining fluorophore from images containing the fluorescence of the two dyes (before either photobleached), the point spread function of the first photobleached dye can be reconstructed. FIONA can then be applied to localize this dye (Fig. 2). From the positions of the two SHRImP fluorophores, the dyes' separation can be computed.



Single-Molecule High-Resolution Imaging with Photobleaching (SHRImP), Fig. 1
Sequential photobleaching of a doubly labeled DNA oligomer



Single-Molecule High-Resolution Imaging with Photobleaching (SHRImP), Fig. 2
Application of the SHRImP algorithm

The SHRImP technique, however, does require that the fluorophores be stationary over the time course of the entire measurement. They must be immobilized to obtain reliable distance measurements since the two fluorophores are localized at different times. Another technique, called single-molecule high-resolution colocalization (SHREC) (Churchman et al. *2005*; Toprak et al. *2010*), overcomes this problem by using two fluorophores or quantum dots that emit at spectrally different wavelengths. Each emission is collected simultaneously, and therefore distance changes that are shorter than the photobleaching time (but slower than the integration time) can be measured. However, chromatic aberration must be carefully accounted for with these techniques. Another caveat of SHRImP (and SHREC) has to do with collecting high quality data. Although the application of SHRImP (and SHREC) only requires that the fluorophores be easily resolved as bright puncta against a uniform background, maximum precision is obtained by maximizing the signal-to-noise ratio of the fluorophores over the background. In practice, this optimization often requires imaging bright, photostable dyes, with total internal reflection microscopy (TIRFM).

The application of SHRImP allows greater precision than could be obtained by fitting the emission of two fluorophores with a sum of Gaussian functions. For separations between 10 and 100 nm, the overlap between the two point spread functions of single fluorophores is significant. A fit with a sum of Gaussians would involve six fitting parameters. An attempt to simultaneously fit this many parameters to a single fluorescent spot would yield a poor fit. However, the point spread function of a single dye can be fit using the FIONA technique with nanometer precision. The precision that can be obtained by SHRImP is limited by the same factors that limit the precision with which a single spot can be localized (photon shot noise, background noise, and finite pixel size of the detector) but is also affected by experimental conditions that may change over time such as drift of the microscope stage, or sample and temporal fluctuations in the emitted fluorescence of the dyes. The precision that can be obtained with SHRImP depends on the choice of fluorophores, but for bright organic dyes such as Cy3 with a lifetime of 10^6 photons, 8-10 nm precision can be obtained.

SHRImP has been applied to a variety of systems using several fluorescent labelling techniques. It has been used to measure the distance between the labeled ends of DNA oligomers labelled with Cy3 dyes (Gordon et al. *2004*; Qu et al. *2004*). It has also been applied to measure the inter-head separation of myosin VI dimers tagged with eGFP (Balci et al. *2005*), and the clustering of Cy5 labelled viral receptors in cell membranes (Bakker et al. *2007*). The use of photobleaching to resolve and localize many individual fluorescent probes has been extended beyond the SHRImP with super-resolution techniques such as PALM and STORM, which only localize a relatively small number of fluorophores at one time and form a composite image of many sequentially localized fluorophores with sub-diffraction limit detail (Toprak et al. *2010*).

Cross-References

Fluorescence Imaging with One Nanometer Accuracy

Photoactivated Localization Microscopy (PALM)

Single-Molecule High-Resolution Colocalization (SHREC)

Stochastic Optical Reconstruction Microscopy

Total Internal Reflection Fluorescence Microscopy for Single-Molecule Studies

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