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Session History (max. 10)

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

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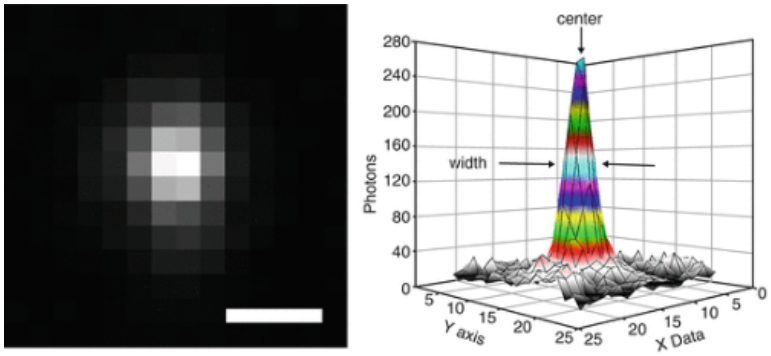
Fluorescence Imaging with One Nanometer Accuracy

Introduction

The microscope allows scientists to peer into small objects. With new organic dyes and fluorescent proteins, the microscope has improved our ability to extract biological information from selectively labeled proteins, DNA, and organelles. However, a physical property of light, called diffraction, has made it nearly impossible to see objects smaller than the diffraction limit, which is about 250 nm. However, much information smaller than this distance can be insightful. This can be accomplished without violating the diffraction limit using a technique called FIONA (Fluorescence Imaging with One Nanometer Accuracy). With FIONA, the position of a diffraction-limited spot can be located to within a few nanometers, on a time-scale longer than a millisecond. In this chapter, we discuss some background principles underlying FIONA and its applications.

Diffraction-Limited Spot

Modern biology relies on visible light-based imaging techniques, commonly using fluorescent dyes to label organelles, proteins, or nucleic acids. Fluorescence, as opposed to simple absorption, is typically used to specifically label a particular object and increase its contrast. Sensitivity is excellent, having reached the single-molecule level. Readily available choices of labels include organic dyes, quantum dots, and fluorescent proteins. Although they have endless stories of success in biology, they all have a critical limitation imposed by diffraction. No matter how small the fluorescent object is, diffraction blurs their images to a diffraction-limited size, about 250 nm. However, one oftentimes wants to measure changes smaller than this. For example, in the cell, a molecular motor called kinesin, "walks" with 8 nm center-of-mass movements. But, due to diffraction, we cannot see this. Nevertheless, there is a light-based technique that can reveal such small steps. It is called Fluorescence Imaging with One Nanometer Accuracy or FIONA. One simply takes the diffraction image of a single molecule, which looks like an isolated mountain, and determines its maximum (Fig. 1). Naturally, one can determine the center of a distribution much more accurately than the width, the latter being known as the resolution of the microscope.



Fluorescence Imaging with One Nanometer Accuracy, Fig. 1 An EMCCD image of a single fluorophore (*left*) and its point-spread function graph. Note that the

fluorophore looks much bigger than its actual size (around 20 nm) due to diffraction. Scale bar represents 400 (nm), and 1 pixel is around 107 (nm)

Accuracy of Finding the Center of Diffraction-Limited Spot

The width of a diffraction-limited spot image is $\approx \frac{1}{2} \cdot \frac{\lambda}{NA}$, which is around 200-300 nm, where λ is wavelength ≈ 550 nm (green color), and NA is numerical aperture of an objective, ≈ 1.4 (Yildiz et al. *2003*) (Fig. 1). Nevertheless, its position can be localized much more accurately than this. Just how well can the center be determined? This is what's called the standard error of the mean (s.e.m.). To first order, it is inversely proportional to the square-root of the total number of photons collected. The more photons collected, the clearer the image is. It is also related to the diffraction width - the wider the mountain, the more difficult it is to localize the mountain top. The s.e.m. is therefore proportional to the width/ $\sqrt{N}$. With w = 250 nm and 10,000 photons, the s.e.m. is about 2.5 nm. (More precisely, it's closer to a nanometer - see below.)

There are two other terms that come into how well a peak can be localized. One is the pixel size of the detector. If, the pixel size is much bigger than the entire mountain width, then the center can be moved around and you can't tell. Typically, to get good accuracy an effective pixel size of about 80-100 nm is required. An 8 × 8 arrangement of pixels (a CCD commonly records a 512 × 512 array) are then recorded and fit with a Gaussian function. (Technically, the diffraction-limited spot is an Airy function, which is a first-order Bessel function, but a Gaussian function generally does a good job of fitting the function.) The third term accounts for background noise that can come from various sources such as a noisy camera or autofluorescence. All of these effects are summarized nicely in a paper by the Webb group in 2002 (Thompson et al. *2002*) and shown originally in a paper by Yildiz et al. (*2003*) for an organic fluorophore.

(1)

$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right)}$$

$\sigma_{\mu i}$: Standard error of the mean (i : x or y direction)

s_i : standard deviation of the Gaussian function in the i direction (typically 125 nm)

N: collected photon number, (typically 14,000 photons in ½ s.)

a: effective detector pixel size (typically 86 nm)

b: uncertainty (standard deviation) of the background noise (typically 10)

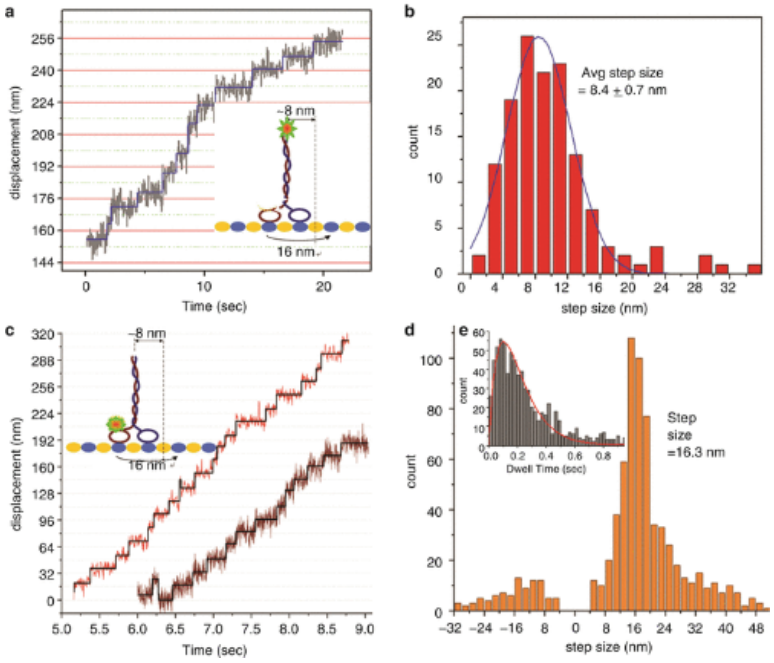
Using the typical values yields, σ_μ = 1.24 nm, in each direction, meaning that the position of a dye can be determined to this accuracy (Yildiz et al. *2003*). Photon noise only (first term, (1)) leads to 1.02 nm; pixelation due to the finite detector area (second term, (1)) increases σ_μ to 1.04 nm; and background noise (third term, (1)) increases σ_μ to 1.24 nm. This shows that photon noise is the dominant contributor to σ_μ . With the FIONA technique, particularly with quantum dots, it is possible to gather 20,000 or more photons during the detector exposure time, and therefore achieve 1 nm accuracy.

Applications of FIONA

FIONA has especially been applied to cytoplasmic motor proteins, i.e., to myosins that walk on the "roadway" called actin, or to kinesins and dynein, which walk in the (+) or (−) direction on microtubules, respectively. It has been applied in vitro, in vivo, and to living organism (summarized in Toprak et al. *2010*). FIONA has achieved the time resolution of as fast as 2 ms, which made it feasible for in vivo study (where the ATP concentration is very high, so motors walk rapidly). And due to the nature of total internal reflection fluorescence (TIRF) microscopy (see below), FIONA is suitable for single-molecule studies on surface-tethered proteins/nucleic acids or membrane proteins in a cell.

Figure 2 is an example, applied to kinesin with a quantum dot. (Many of these studies have also been done with regular fluorophores, which tend to be smaller than quantum dots.) Figure 2a shows the kinesin moving with a quantum dot attached to its neck region. As determined in the 1990s by the Block lab using optical trap measurements, kinesin moves with a center-of-mass motion of 8.3 nm (Svoboda et al. *1993*). A histogram shows a step size of 8.4 ± 0.7 nm (Fig. 2b) in excellent agreement with the optical trap data. To determine just how the kinesin takes a step, it was necessary to label one of the feet (also called a "head" or sometimes "hand"), as shown in Fig. 2c. If a kinesin "walked," i.e., moved in a hand-over-hand fashion, the step size would be 2 × 8.3 =16.6 nm. If it inchwormed along, all parts of the kinesin would move by 8.3 nm. From the step sizes and histogram, the step size is 16.3 nm (Fig. 2d), and clearly it walks hand-over-hand. In fact, one can also

tell that each foot proceeds at the same rate. That is, the quantum dot doesn't hinder the foot it is attached to and it is not limping. (When a molecular motor is labeled with a significantly smaller conventional fluorophore, it also yields the same values.) A hand-over-hand model with only one head labeled should allow the 0 nm step to be indirectly detected. The total probability of the labeled foot moving, P(t), is a convolution of the labeled and unlabelled head stepping, and results in: $P(t)=k_1k_2\left(e^{-k1t}-e^{-k2t}\right)/(k_1-k_2)$, where k_1 is the rate of motion forward. With the rates being equal, ($k_1 = k_2 = k$), then $P(t)=tk^2e^{-kt}.\ k_1=k_2$ fits the data well, which can be seen in Fig. 2e. In contrast, if the 16.3 nm steps arise from a single process, then the dwell-time histogram would be expected to yield an exponential decay (the Poisson-distributed rate). In conclusion, one foot steps over the second foot, and then the second footsteps over the first, with equal rates.



Fluorescence Imaging with One Nanometer Accuracy, Fig. 2 (**a**) shows the motion of step sizes and its histogram and (**b**) of the center-of-mass motion of kinesin. Here the kinesin is labeled with a quantum dot near the C-terminus, near the cargo-binding domain. (**c**) and (**d**) show kinesin labeled on a foot; it walks with a step size of 16.3 nm. (**e**) is a histogram versus dwell time, showing that it fits very well to a form $P(t)=tk^2e^{-kt}$. Hence, kinesin walks in a hand-over-hand fashion. (**a**-**b** are adapted from Yardimci et al. *2008*; **c-e** are adapted from Toprak et al. *2009*)

Getting the Maximum Number of Photons for FIONA

So far, we have learned from (1) that to locate a fluorophore with the maximum accuracy, one needs to

1. Extract as many photons as possible from the fluorescent dye
2. Send the photons to a detector with minimal loss
3. And reduce the background noise

The success of the FIONA is attributed to ways of achieving (1) through (3).

Getting the maximum number of photons emitted before the fluorophore photobleaches is important since this determines the accuracy of localization. Also, it is important that the fluorophores do not blink since when they are in the off state, one cannot locate them. With very good fluorophores, we can get over one million photons (=1/e value). For example, Cy3, CF633, Atto647N all emit at this level, although the exact value depends on what they are attached to. (Anecdotally, we have found that dyes when attached to the ends of DNA are more photostable than when attached to proteins.) In general, this requires adding deoxygenation agents (oxygen scavenger system), e.g., glucose oxidase/catalase (Yildiz et al. *2003*) or the PCA/PCD system (Aitken et al. *2008*), or a reducing and oxidizing system

(ROXS) (Vogelsang et al. *2008*). For blinking, β-mercaptoethanol (BME) (Toprak et al. *2009*), or Dithiothreitol (Yildiz et al. *2003*; Toprak et al. *2009*) or 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Rasnik et al. *2006*) works well.

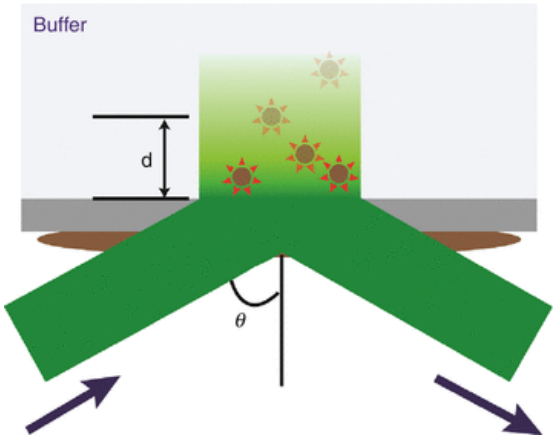
The above reagents are for in vitro FIONA work. For in vivo FIONA, we are left with fluorescent proteins (FPs). We have found the most photostable FP is eGFP. Unfortunately, eGFP is still not stable enough to see a single one; instead, you must look at a group of eGFP's. Here, the bunch must act together, as a giant fluorophore, and should move together (Kural et al. *2005*, *2009*). Another alternative is looking at absorption - looking at single melanosomes, dark pigment granules, for example (Kural et al. *2007*).

In terms of time resolution, one needs to get out enough photons to enable sub-diffraction-limited localization. One can turn up the excitation light so the photons are emitted more rapidly, thereby increasing the time resolution in the experiment. This works fine. On the other hand, with a finite number of photons emitted, the maximum lifetime becomes less. Also, we find that above a certain light intensity, the fluorophores tend to bleach more rapidly.

Ways to Reduce Background

Background noise is significantly reduced by adopting total internal reflection fluorescence microscopy (TIRFM). With this microscopy scheme, incoming laser light whose incident angle is larger than a critical angle impinges on the water interface and gets reflected back away from the interface. However, an evanescent field (which dies off exponentially as you move away from the interface) is generated in the aqueous medium upon reflection of the laser beam, which enters the water through a glass coverslip/slide. Consequently, only fluorophores near the glass-water interface are excited. The 1/e depth varies with the incident excitation light, generally about 100 nm. (The index of refraction of water, n_{water} = 1.33; the index of refraction of glass, n_{glass} = 1.518.) Other regions above this criterion are essentially not excited (see Fig. 3), thereby reducing the background as compared to epi-fluorescence microscopy.

$$\text{Penetration Depth } d = \frac{\lambda_0}{4\pi\sqrt{n_{\text{glass}}^2 \sin^2\theta - n_{\text{water}}^2}} = 118\,(\text{nm}) \text{ for } \lambda_0 = 532\,(\text{nm}), \theta = 65^\circ \tag{2}$$



Fluorescence Imaging with One Nanometer Accuracy, Fig. 3 Schematic of TIR. The laser impinges on the water(buffer)-glass interface with angle θ, which is over the critical angle. An evanescent field is generated, and its intensity exponentially decays away from the interface. Fluorescent dyes within penetration depth d are excited. The indexes of refraction are all the same for the cover glass (*gray*), the index-matching immersion oil (*brown*), and objective lenses (not drawn in this figure)

The background can be further reduced by proper selection of dichroic mirror and emission/excitation filters. The dichroic mirror and emission filter selection is such that reflected-laser and Raman-scattered light should not be allowed to enter the detector, yet emission signal from excited fluorophores is sent to the detector as much as possible. A notch filter is sometimes inserted in the emission beam path to cut off any reflected or scattered laser light. Putting an excitation filter in front of the dichroic mirror also helps clean up unwanted laser light wavelength and blocks the off-axis light, therefore reducing the background even further.

In addition to the proper dichroic mirror and filter sets, employing an objective with a high numerical aperture (NA) and a high-performance detector allows the maximum amount of photons to be registered for final FIONA data analysis. We now have high NA objectives (generally 1.40-1.49, even 1.65 is available) and a high-performance electron multiplying charge-coupled device (EMCCD) camera with almost 100% quantum efficiency and minimal-added camera noise. Note that the minimum NA of the objective to get TIR is 1.37, which corresponds to the index of refraction of cells. This means that a water immersion objective cannot be used with TIR. We strongly recommend investing money to purchase objectives with high NA. (Caution: An objective with 1.65 NA requires special immersion oil and fairly expensive sapphire coverslips.)

Once fluorophore image file data are gathered, the center of the fluorophore is found by a two-dimensional Gaussian fitting using custom (Matlab, IDL, SigmaPlot, and so on) programming code. Some codes are available free of charge through selvin@illinois.edu.

FIONA Variants

FIONA has several variants. One example is single-molecule high-resolution imaging with photobleaching (SHRImP) (Gordon et al. *2004*), also called, Nanometer-localized multiple single-molecule fluorescence microscopy (NALMS) (Qu et al. *2004*). It uses photobleaching to break the diffraction limit for two or more dyes located very closely. For example, when there are two dyes with close proximity, their signals are not resolved. However, when one of the dyes is photobleached, the position of the remaining dye can be determined accurately by FIONA. By then, subtracting the image of the remaining dye from that of the previous un-photobleached two dyes, the photobleached dye image can be inferred and its position is determined by FIONA. The resolution is then simply the difference between the two positions. With SHRImP, a resolution of 8-10 nm has been achieved (Gordon et al. *2004*; Qu et al. *2004*). The second example is single-molecule high-resolution colocalization (SHREC) (Churchman et al. *2005*). While SHRImP is a technique for two fluorophores with the same color, two different fluorophores, with distinct emission spectra, are used in SHREC. Each emission spectrum is separated and imaged onto the different portion of the same detector. FIONA can then be used to find their positions.

FIONA is also exploited in two similar high-resolution techniques: photoactivated localization microscopy (PALM) (Betzig et al. *2006*) and stochastic optical reconstruction microscopy (STORM) (Rust et al. *2006*). In these schemes, a high density of fluorophores is used, but only a small percentage of the fluorophores - less than one per diffraction-limited spot - are turned on by a pulse of light. FIONA can then be applied to each of the "on" fluorophores, and a high-resolution image is built up by pulsing many times and applying FIONA repeatedly (reviewed in Huang et al. *2009*).

Summary

FIONA is a technique that can locate the center position of a fluorescent dye with nanometer accuracy and temporal resolution of up to ~2 ms. The technique is based on the theoretical study that the position of a fluorophore can be determined more accurately than the diffraction limit as long as we collect enough photons. For this purpose, oxygen scavenger systems to decrease photobleaching and reagents to suppress blinking have to be optimized. TIRF is also used to reduce background level by limiting illumination depth of a sample. With additional optimization such as using an objective with high NA, proper dichroic mirror/filters, and a high quantum efficiency detector, FIONA opened a way to see what has not been seen with traditional optics.

Cross-References

Photoactivated Localization Microscopy (PALM)

Single Fluorophore Blinking

Single Fluorophores Photobleaching

Single-Molecule High-Resolution Colocalization (SHREC)

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

Stochastic Optical Reconstruction Microscopy

Total Internal Reflection Fluorescence Microscopy for Single-Molecule Studies

References

Aitken CE, Marshall RA, Puglisi JD. An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. Biophys J. 2008;94:1826-35.

Betzig E, Patterson GH, Sougrat R, Lindwasswer OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF. Imaging intracellular fluorescent proteins at a nanometer resolution. Science. 2006;313:1642-5.

Churchman LS, Ökten Z, Rock RS, Dawson JF, Spudich JA. Single moleculre high-resolution colocalization of Cy3 and Cy5 attached to macromolecules measures intramolecular distances through time. Proc Natl Acad Sci. 2005;102:1419-23.

Gordon MP, Ha T, Selvin PR. Single-molecule high-resolution imaging with photobleaching. Proc Natl Acad Sci. 2004;101:6462-5.

Huang B, Bates M, Zhuang X. Super-resolution fluorescence microscopy. Annu Rev Biochem. 2009;78:993-1016.

Kural C, Kim H, Syed S, Goshima G, Gelfand VI, Selvin PR. Kinesin and Dynein move a peroxisome in vivo: a tug-of-war or coordinate movement? Science. 2005;308:1469-72.

Kural C, Serpinskaya AS, Chou YH, Goldman RD, Gelfand VI, Selvin PR. Tracking melanosomes inside a cell to study molecular motors and their interactions. Proc Natl Acad Sci. 2007;104:5378-82.

Kural C, Nonet ML, Selvin PR. FIONA on Caenorhabditis elegans. Biochemistry. 2009;48:4663-5.

Qu X, Wu D, Mets L, Scherer NF. Nanometer-localized multiple single-molecule fluorescence microscopy. Proc Natl Acad Sci. 2004;101:11298-303.

Rasnik I, McKinney SA, Ha T. Nonblinking and long-lasting single-molecule fluorescence imaging. Nat Meth. 2006;3:891-3.

Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Meth. 2006;3:793-5.

Svoboda K, Schmidt CF, Schnapp BJ, Block SM. Direct observation of Kinesin stepping by optical trapping interferometry. Nature. 1993;365:721-7.

Thompson RE, Larson DR, Webb WW. Precise nanometer localization analysis for individual fluorescent probes. Biophys J. 2002;82:2775-83.

Toprak E, Yildiz A, Hoffman M, Rosenfeld SS, Selvin PR. Why kinesin is so processive. Proc Natl Acad Sci. 2009;106:12717-22.

Toprak E, Kural C, Selvin PR. Super-accuracy and super-resolution: getting around the diffraction limit. Meth Enzymol. 2010;475:1-26.

Vogelsang J, Kasper R, Steinhauer C, Person B, Heilemann M, Sauer M, Tinnefeld P. A reducing and oxidizing system minimizes photobleaching and blinking of fluorescent dyes. Angew Chem Int Ed. 2008;47:5465-9.

Yardimci H, van Duffelen M, Mao Y, Rosenfeld SS, Selvin PR. The mitotic kinesin CENP-E is a processive transport motor. Proc Natl Acad Sci. 2008;105:6016-21.

Yildiz A, Forkey JN, McKinney SA, Ha T, Goldman YE, Selvin PR. Myosin V walks hand-over-hand: single fluorophore imaging with 1.5-nm localization. Science. 2003;300:2061-5.