SHORT COMMUNICATION

Nanometre localization of single ReAsH molecules

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Summary

ReAsH is a red-emitting dye that binds to the unique sequence Cys-Cys-Xaa-Xaa-Cys-Cys (where Xaa is a noncysteine amino acid) in the protein. We attached a single ReAsH to a calmodulin with an inserted tetracysteine motif and immobilized individual calmodulins to a glass surface at low density. Total internal reflection fluorescence microscopy was used to image individual ReAsH molecules. We determined the centre of the distribution of photons in the image of a single molecule in order to determine the position of the dye within 5 nm precision and with an image integration time of 0.5 s. The photostability of ReAsH was also characterized and observation times ranging from several seconds to over a minute were observed. We found that 2-mercaptoethanesulphonic acid increased the number of collected photons from ReAsH molecules by a factor of two. Individual ReAsH molecules were then moved via a nanometric stage in 25 or 40 nm steps, either at a constant rate or at a Poisson-distributed rate. Individual steps were clearly seen, indicating that the observation of translational motion on this scale, which is relevant for many biomolecular motors, is possible with ReAsH.

Introduction

Attaching fluorescent or other useful labels to specific proteins has become an important tool in the biological sciences. The genetic fusion of specific proteins to fluorescent proteins, such as green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been widely used in understanding gene expression, and the localization and fate of proteins in cells (Chalfie *et al.*, 1994; Tsien, 1998). However, GFP has disadvantages,

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for example, its large size (238 amino acids). This can cause problems such as hindering the folding of the native protein or changing the local environment around the protein.

A new method for labelling specific proteins with small dye molecules has recently been developed. Small membrane-permeant organic molecules, which bind a short motif of genetically encodable amino acids within a recombinant protein inside cells, have been developed (Griffin *et al.*, 1998). The motif consists of four cysteine amino acids in the sequence Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa is a noncysteine amino acid. FlAsH-EDT_2 is nonfluorescent, but FlAsH becomes fluorescent after binding the tetracysteine motif in the protein. Each atom of arsenic in FlAsH binds each pair of thiol groups of tetracysteines with considerable affinity and specificity. This FlAsH molecule has an emission peak at 528 nm.

Recently, a red-emitting variant of FlAsH called ReAsH has been developed (Adams *et al.*, 2002). The emission peak of the ReAsH molecule is 608 nm. Red-emitting fluorophores have advantages over green-emitting fluorophores like FlAsH in fluorescence microscopy because cellular absorption, scattering and autofluorescence decrease at longer wavelengths. ReAsH is also membrane-permeant and has very little fluorescence before binding to the tetracysteine motif.

Gaietta *et al.* (2002) carried out *in vivo* experiments using FlAsH and ReAsH. They distinguished older and younger recombinant connexin43 proteins in the gap junction in cells. Using optical and electron microscopy in cells, they also showed that older connexin43 proteins were removed from the centre of the gap junction, and new connexin43 proteins were incorporated at the periphery of the gap junction.

A precise nanometre localization using fluorescent probes was developed (Thompson *et al.*, 2002). This method was extended to fluorescence imaging with 1-nm accuracy, which has achieved better than 1.5 nm localization with subsecond temporal resolution (Yildiz *et al.*, 2003). Fluorescence imaging with 1 nm accuracy was applied to molecular motors – myosin
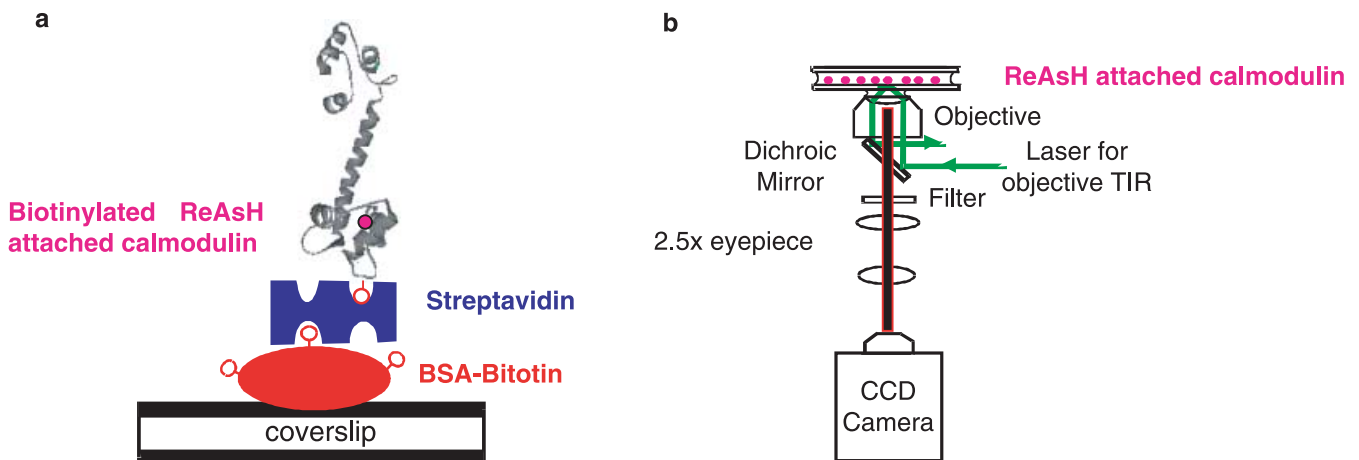
Fig. 1.   (a) Biotinlyated ReAsH calmodulins were immobilized to cover slip by Streptavidin and bovine serum albumin biotin. (b) Experimental set-up.

V and kinesin, step sizes were measured, and myosin V and kinesin were shown to walk hand-over-hand (Yildiz *et al.*, 2003, 2004).

Here we apply fluorescence imaging with one-nanometre accuracy to enable the nanometre localization of single ReAsH molecules using total internal reflection fluorescence microscopy. We can localize a single ReAsH molecule in two dimensions to 5 nm with subsecond temporal resolution and with a photostability that enables an observation time of > 1 min. We furthermore used a piezoelectric stage capable of moving in arbitrary increments, with 0.7 nm accuracy, to see clear individual steps with ReAsH. These results showed that ReAsH molecules can be used with fluorescence imaging with one-nanometre accuracy to measure step sizes relevant to biomolecular motors with *in vivo* experiments because ReAsH-EDT$_2$ is membrane-permeant, and also brightly fluorescent after it forms a fluorescent complex with a protein containing tetracysteine. We also found that 2-mercaptoethanesulphonic acid (MESNA) increased the photostablity of ReAsH molecules. and the photostability of ReAsH molecules was compared with that of eGFP and Cy3 DNA.

Materials and methods

Sample preparation

ReAsH-labelled calmodulins were formed by binding ReAsH-EDT$_2$ to calmodulin containing the amino acid sequence of the Cys-Cys-Xaa-Xaa-Cys-Cys. The calmodulin construct used was MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDPMADQLT **CCEQCC**FKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAEL QDMINEVDADGNGTIYFPEFLTMMARKMKDTDSEEEIREAFR VFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGD GQVNYEEFVQMMTAK.

The N-terminus of the protein has a large leader sequence that contains a hexahistidine affinity tag and an additional sequence

for protease cleavage. The native N-terminus would normally begin at MADQLT... The tetracysteine-ReAsH binding motif is located at residues 7–12. The protein was expressed from the pRSET vector in Bl21 (DE3) bacterial cells and purified via Ni-NTA affinity chromatography. Lumio™ Red (Invitrogen, Carlsbad, CA) was used as the commercial supplier of the ReAsH labelling reagent.

Figure 1 shows a schematic of our experiment. ReAsH-labelled calmodulin was attached to the surface of a cover slip using bovine serum albumin–biotin–Streptavidin system. Calmodulin was first labelled with ReAsH and biotinylated by amine-reactive biotin. A 50 μL labelling reaction consisted of 0.1 mg mL^{-1} calmodulin, 1 mM triscarboxyethylphosphine, 1 mM 2-mercaptoethanol, 10 μM ReAsH-EDT$_2$ in 50 mM HEPES buffer (pH 7.5) and 140 mM NaCl. After 30 min incubation at 4 °C, the reaction mixture was buffer exchanged into 50 mM HEPES (pH 7.5) and 140 mM NaCl using a micro BioSpin 6 column (Bio-Rad, Hercules, CA) following the manufacturer's instructions. To the flow through from ReAsH labelling and buffer exchange, 100 μM biotin-NHS was added and allowed to react at 4 °C for 1 h. The reaction was buffer exchanged as described above.

Sample chambers were prepared as described in Snyder *et al.* (2004). After making the sample chamber, 100 μL of 1 mg mL^{-1} bovine serum albumin–biotin (A-8549; Sigma, St. Louis, MO) in 0.01 M phosphate-buffered saline (PBS, P-4417; Sigma) was added in the sample chamber, allowed to sit for 2–3 min and then washed with 100 μL PBS. One hundred microlitres of 0.5 mg mL^{-1} Streptavidin (S-888; Molecular Probe, Eugene, OR) in PBS was added, allowed to sit for 2–3 min and then washed with 100 μL of PBS. One hundred microlitres of 0.8–1.6 nM biotinylated ReAsH-calmodulin molecules in PBS was added, allowed to sit for 2–3 min and then washed with 100 μL of PBS. One hundred microlitres of imaging buffer was added. This imaging buffer consisted of 0.4% glucose, 1% 2-mercaptoethanesulphonic acid and 1% Gloxy in PBS. Gloxy was made by adding 20 mg

glucose oxidase (G-7016; Sigma), 40 μL catalase (Roche Diagnostics Corp., Indianapolis, IN) in PBS, centrifuging for 5 min and filtering twice with a 0.2 μm syringe filter.

Total internal reflection fluorescence microscopy

Objective-type total internal reflection fluorescence microscopy was used to excite ReAsH molecules and obtain images of single ReAsH molecules placed on a cover slip (Tokunaga *et al.*, 1997). An Olympus IX-70 epifluorescence microscope and an Olympus 60×1.45 NA oil immersion objective were used. 532 nm laser light (Nd:YAG, model # GCL-075-L, 75 mW; CrystaLaser, Reno, NV) was used to illuminate samples. The power at the sample was ~1–10 mW. The fluorescence was separated from scattered light by a 545 DRLP dichroic mirror and an HQ585/70 emission filter (Chroma, Rockingham, VT). Images were magnified by a $2.5 \times$ eyepiece (Olympus, Melville, NY) and obtained by a frame-transfer charged-coupled device (CCD) camera (MicroMAX 512 BFT, 512×512 pixels, 13 μm pixels, Roper Scientific, Tucson, AZ). A full frame was acquired in 0.5 s.

Nanometric stage stepping experiment

A nanometric stage (NanoH-70/invar, with 0.7 nm position accuracy, used in closed loop scanning mode; Mad City Labs, Madison, WI) was used to simulate steps in total internal reflection fluorescence microscopy. The nanometric stage was bolted to the mechanical stage of the microscope. To avoid drift in the microscope, we waited for 2 min after moving the mechanical stage in the microscope. The nanometric stage was moved unidirectionally in 25 or 40 nm increments. Experiments were performed in two modes: one was at a fixed step rate (such as one step every 4 s) and the other was at a Poisson-distributed step rate (average of one step every 4 s).

Data analysis

We fit the point-spread-function (PSF) to a two-dimensional Gaussian function defined as

$$F(x,y;z_0,A,x_0,y_0,\sigma_x,\sigma_y) = z_0 +$$

$$A \exp\left[-\frac{1}{2}\left[\left(\frac{x-x_0}{\sigma_x}\right)^2 + \left(\frac{y-y_0}{\sigma_y}\right)^2\right]\right] \tag{1}$$

where z_0 is a constant term due to background fluorescence or detector noise, A is the amplitude, x_0 and y_0 are coordinates of the centre, and σ_x, σ_y are standard deviations of the distribution in each direction.

By fitting a PSF to a two-dimensional Gaussian function, we can determine the position of the centre and its uncertainty, or the standard error of the mean. The standard error of the mean (σ_μ) was derived by Thompson *et al.* (2002) in two-dimensional localization:

$$\sigma_\mu^2 = \frac{\sigma^2}{N} + \frac{a^2/12}{N} + \frac{8\pi\sigma^4 b^2}{a^2 N^2} \tag{2}$$

where N is the number of collected photons, a is the effective pixel size of the imaging detector, b is the standard deviation of the background and σ is the standard deviation of the PSF. The first term comes from photon noise, the second term comes from the finite pixel size of the detector, and the last term comes from the background noise. The equation shows that as the number of collected photons increases, the uncertainty in the position of the centre decreases.

Data were analysed using IDL (Research Systems Inc., Boulder, CO) programs written by our laboratory. Images of a dye in successive frames of a movie of stepping were fitted to a two-dimensional Gaussian function in order to determine the centre of the image in each frame. By plotting the position of a single ReAsH molecule as a function of time, we found that steps become visible. Sigmaplot 8.0 (SPSS Inc., Chicago, IL) was used to visualize single frames of individuals PSFs and fit them to two-dimensional Gaussian functions.

Results and discussion

Figure 2(a) shows the PSF of a single ReAsH molecule taken with an integration time of 0.5 s. The inset shows the number of photons collected from a ReAsH molecule per 0.5 s image. That molecule lasted 107 s and photobleached in one step, which demonstrates that it is a single ReAsH molecule. The red solid line represents the curve-fit to a two-dimensional Gaussian function ($R^2 = 0.93$). Figure 2(b) shows the residual from the curve-fit ($\chi_r^2 = 1.21$). We performed autocorrelation analyses and found that the residual graph did not show any significant structure. The reason why $\chi_r^2 > 1$ is that the real PSF has Airy rings; thus there is a small error in the approximation of the real PSF to a two-dimensional Gaussian function. From R^2, χ_r^2 and autocorrelation analyses, we concluded that a two-dimensional Gaussian function is a good approximation to the shape of PSF of a single ReAsH molecule. As the power of the laser increased, fits became better. When 1.0 mW of laser power was used, $R^2 = 0.75$. When 3.1 mW of laser power was used, $R^2 = 0.92$.

The total number of photons collected in this PSF was 4850. The maximum photons per pixel (I_0) considering background was 321. The full width at half maximum of the PSF is 287 and 283 nm in x and y direction, respectively. The ratio of semi-minor to semi-major axis was 0.99. The standard deviation of the background (b in Eq. 2) was 9. The signal-to-noise ratio (SNR) of this pixel was 16 from the following equation:
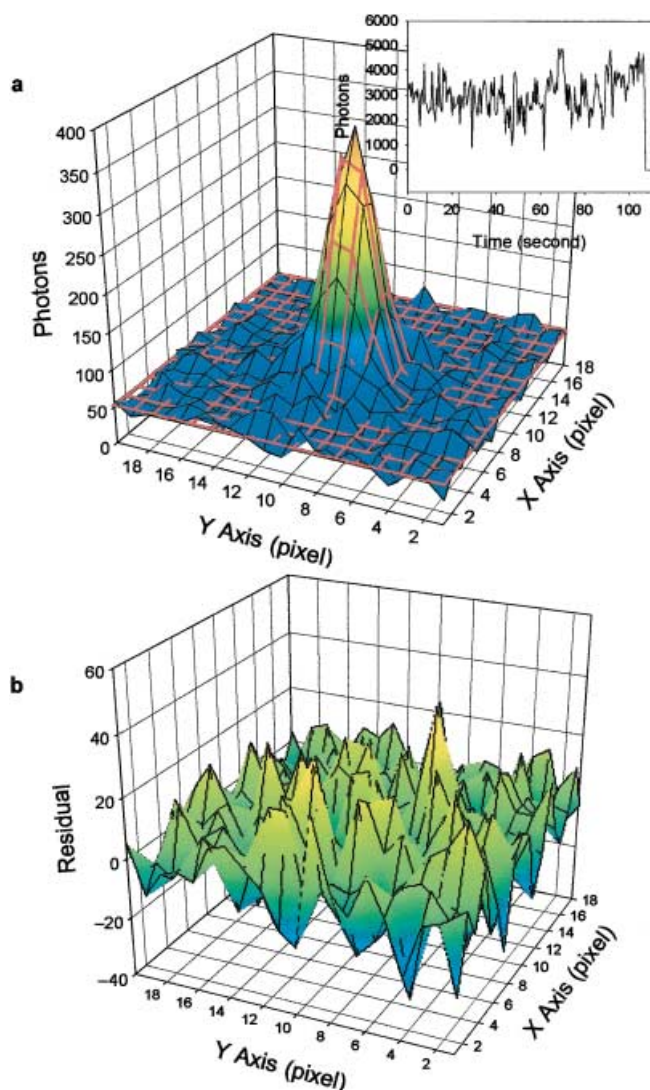
$$SNR = I_0 / \sqrt{I_0 + b^2} \tag{3}$$

Fig. 2. (a) The PSF of a single ReAsH molecule with two-dimensional Gaussian fit (red solid line) shows an excellent fit ($R^2 = 0.93$ and $\chi_r^2 = 1.21$). The difference between the Airy function and the Gaussian function causes $\chi_r^2 > 1$. (Inset) Photons per 0.5 s image. This molecule lasted 107 s. (b) The residual graph between the Gaussian fit and the PSF. We performed autocorrelation analyses and found that the residual graph did not show any significant structure.

From Eq. (2), the standard error of the mean was 2.40 nm for the *x*-direction and 2.35 nm for the *y*-direction. The Gaussian fitting gave a standard error of the mean of 2.50 and 2.46 nm in each direction, which was in good agreement with theoretical values. We fit hundreds of PSFs of ReAsH molecules to two-dimensional Gaussian functions. They fit very well, and their standard errors of the mean were normally below 5 nm.

Yildiz *et al.* (2003) used Cy3 DNA to achieve 1.3 nm standard error of the mean (σ_μ). They collected 14 200 photons from Cy3 DNA. Eq. (2) showed that the standard error of the mean (σ_μ) is inversely proportional to the square root of the collected

photons. As ReAsH produces fewer photons, the standard error of the mean (σ_μ) becomes larger. The maximum number of photons per pixel (I_0) of Cy3 DNA was 1134, and the SNR was 32. These differences are caused by the low production of photons of ReAsH compared with Cy3 DNA. However, ReAsH is much easier in labelling target proteins in *in vivo* experiments than Cy3. Therefore, ReAsH can be more easily used *in vivo* experiments than Cy3.

It has been shown that if spherical aberrations are present in the microscope, the PSF can have unusual shapes, for example, asymmetric shapes by single molecule orientation (Bartko & Dickson, 1999a,b). Under these conditions, the centre of a PSF does not correspond to the position of a dye. However, our analysis of PSFs of ReAsH molecules showed that PSFs of ReAsH molecules were symmetric and fit well to two-dimensional Gaussian functions. We concluded that spherical aberrations in our system were not significant, and the molecular orientation of the single ReAsH molecule did not limit our ability to localize ReAsH molecules.

Photostability of ReAsH molecules with MESNA

We used oxygen-scavenging chemicals (glucose oxidase and catalase) to increase the photostability of ReAsH. We used MESNA as a reducing agent. We randomly chose 100 individual ReAsH molecules that photobleached within 250 s and analysed their photostability. Figure 3(a) shows the histogram of the number of photons that a single ReAsH molecule produced before photobleaching without MESNA. The average number of photons collected from a single ReAsH molecule in the absence of MESNA was 45 900. Figure 3(b) shows the histogram of the number of photons that a single ReAsH molecule produced with MESNA. The average number of photons of a single ReAsH molecule with MESNA was 102 200. In the presence of MESNA, 95% of ReAsH molecules produced > 10 000 photons; 49% of ReAsH molecules produced > 50 000 photons. Because the typical collection efficiency of microscopy is 1–10% (Ha, 1996), ReAsH molecules produce millions of photons before they photobleach. Figure 3(c) shows the histogram of time of ReAsH molecules before photobleaching without MESNA. The average time before photobleaching was 10 s. Figure 3(d) shows the histogram of time of ReAsH molecules before photobleaching with MESNA. The average of time was 39 s; 42% of ReAsH molecules lasted > 30 s and 22% of ReAsH molecules lasted > 1 min. The comparison with the data obtained without MESNA showed that MESNA makes ReAsH molecules photostable.

We chose 99 eGFP molecules in experiments that followed our procedures. The average number of photons collected from a single eGFP molecule was 23 440. The average observation time of eGFP before photobleaching was 9 s. These results showed that ReAsH molecules produce more photons than eGFP molecules, and that ReAsH molecules are more photostable than eGFP.
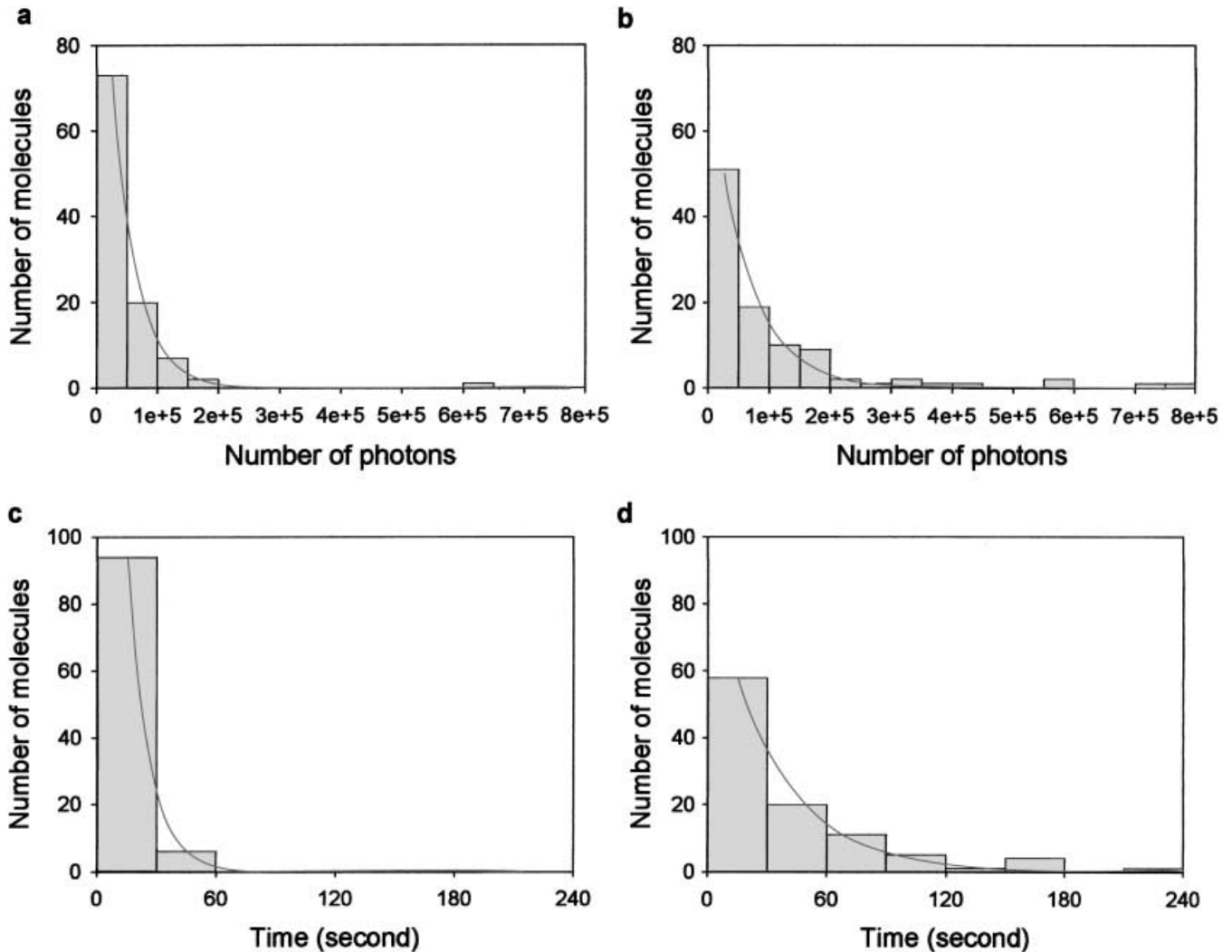
Fig. 3. (a, b) Histogram of number of photons that single ReAsH molecules produce (a) without and (b) with MESNA. MESNA increases the average number of photons that ReAsH molecules produce. (c, d) Histogram of time before photobleaching of single ReAsH molecules (c) without and (d) with MESNA. MESNA increases the photostability of ReAsH molecules. One exponential decay functions fit all histograms well.

Measurement of step sizes produced by nanometric stage

In order to demonstrate the suitability of ReAsH molecules for the measurement of step sizes of biomolecular motors, we used ReAsH molecules to measure the step sizes artificially generated by a piezoelectric stage.

We programmed the stage to take steps of 25 and 40 nm, a step size relevant to the motion of biomolecular motors. We moved the stage at a constant stepping rate and a variable Poisson-distributed stepping rate. The Poisson-distributed stepping rate is relevant to molecular motors because the motions of molecular motors are stochastic, dependent on the diffusive binding of ATP to the active site (Hua *et al.*, 1997).

Figure 4 shows the nanometric stage stepping movement of single ReAsH molecules including histograms of step sizes, means and the standard deviations. Figure 4(a,b) represents

the 40 nm stepping movement of single ReAsH molecules at a constant stepping rate and a Poisson-distributed stepping rate, with an average of one step every 4 s. Figure 4(c,d) represents the 25 nm stepping movement of single ReAsH molecules at a constant stepping rate and a Poisson-distributed stepping rate, with an average of one step every 3 s. The clear determination of stepping, and of long observation time before photobleaching (for instance 144 s for blue curve in Fig. 4a) suggests that it should be possible to measure the stepping of biomolecular motors using ReAsH.

Means of the measured step sizes are in good agreement with the programmed step size: 39.7 nm for a constant stepping rate with 40 nm stepping, 39.3 nm for a Poisson-distributed stepping rate with 40 nm stepping, 25.3 nm for a constant stepping rate with 25 nm stepping, and 24.9 nm for a Poisson-distributed stepping rate with 25 nm stepping. The standard deviations of
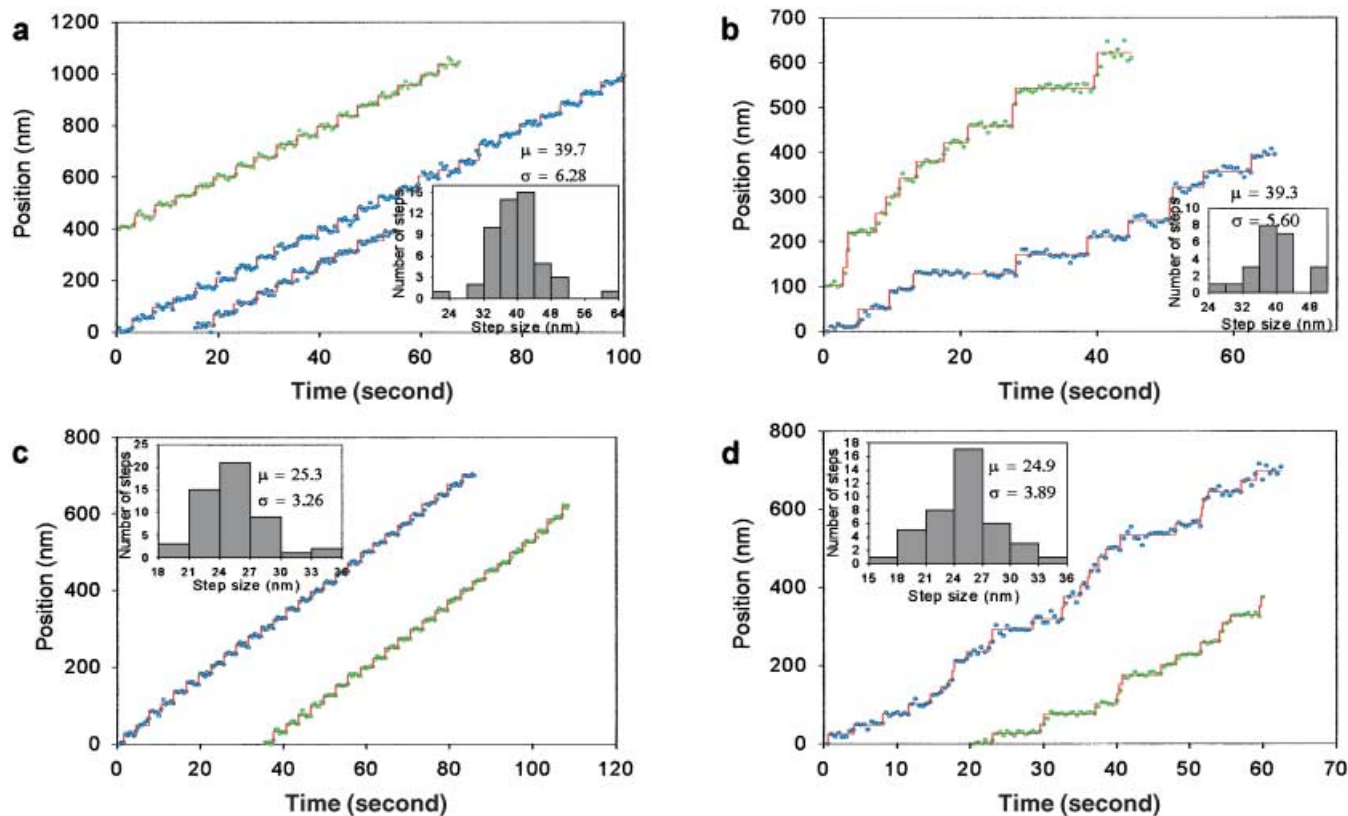
Fig. 4. Nanometric stage stepping of ReAsH calmodulin – (a, b) 40 nm nanometric stage stepping including histogram of step sizes; (c, d) 25 nm stepping including histogram of step sizes; (a, c) constant stepping rate; (b, d) Poisson-distributed stepping rate. The lower plot in (b) shows missing points from 20 to 22 s, which represent the blinking of ReAsH–calmodulin.

measured step size were 6.28 and 5.60 nm at 40 nm step and 3.26 and 3.89 nm at 25 nm step. These standard deviations of measured step sizes were satisfactory considering the error of localization of ReAsH (5 nm). We raised the power of the laser to measure the step size of ReAsH at 25 nm. High laser power gave a better standard deviation because high laser power makes ReAsH produce more photons and gives better localization, which decreased the standard deviations of measured step sizes. The lower plot in Fig. 4(b) shows missing points from 20 to 22 s, which represent the blinking of a ReAsH–calmodulin complex.

Conclusions

This study has shown that ReAsH molecules can be localized within 5 nm by determining the centre of distribution of photons. Therefore, it is possible to measure the step sizes of biomolecular motors using ReAsH molecules. ReAsH molecules may allow studies of the movement of specific proteins inside the cell. MESNA increased the photostablity of ReAsH molecules.

Acknowledgements

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