Research Report

Single-Molecule Fluorescence Observed with Mercury Lamp Illumination

BioTechniques 27: 1008-1014 (November 1999)

M. Unger, E. Kartalov, C.-S. Chiu, H.A. Lester and S.R. Quake
California Institute of Technology, Pasadena, CA, USA

ABSTRACT

We demonstrate that it is possible to observe single fluorescent molecules using a standard fluorescence microscope with mercury lamp excitation and an inexpensive cooled charge-coupled device (CCD) camera. With this equipment, we have been able to observe single molecules of tetramethylrhodamine, rhodamine 6G, fluorescein isothiocyanate and green fluorescent protein. Immobilized molecules were observed both in air and in aqueous solution.

INTRODUCTION

Single-molecule imaging and spectroscopy are beginning to make important contributions to biology. These techniques have been used to observe the stepping of motor molecules, such as kinesin (20) and myosin (9) at the single molecule level, and also to make measurements correlating ATP hydrolysis and myosin force generation (8). Single-molecule experiments with the enzyme cholesterol oxidase have shown static and dynamic disorder in the turnover rates that are masked in bulk studies (10). Similarly, single-molecule fluorescence resonance energy transfer experiments have revealed complex catalytic dynamics of Staphylococcal nuclease (19) and co-localization of multiple ligands on a single receptor (15).

Green fluorescent protein (GFP) has proven useful to study static and dynamic aspects of proteins within living cells because the GFP sequence can be expressed as a fusion with another intact protein, often with few functional changes (1,18). Single molecules of GFP have been observed in an aqueous enviroment (3), and the use of GFP-fusions for the study of biomolecule dynamics is promising.

Because of the extremely low light levels involved, most single-molecule experiments have used techniques such as total internal reflection microscopy (3,4), laser confocal illumination (13) and laser-induced fluorescent excitation (4,11). The complexity and cost of these instruments has limited the general use of single-molecule techniques, especially in biology. Here, we show that single fluorescent molecules can be observed with an inexpensive charge-coupled device (CCD) camera, a commercial epi-fluorescence microscope and a mercury lamp for fluorescent excitation. Because many laboratories have access to this equipment, we hope these results encourage more applications of single-molecule imaging.

MATERIALS AND METHODS

Coverslips were cleaned by two different methods. For the "Chromerge" method, coverslips were sonicated in a surfactant solution (2% Micro-90; Cole-Parmer, Vernon Hills, IL, USA) for 20 min, washed for 3 min in a stream of deionized water, rinsed thoroughly with high-purity water, then immersed in a H_2SO_4/CrO_3 cleaning solution (Chromerge; VWR Scientific, West Chester, PA, USA) for 1 h. For the "RCA" method, coverslips and slides were sonicated in a surfactant solution (2% Micro-90) for 20 min, washed for 3 min in a stream of deionized water, rinsed thoroughly with high-purity water, then immersed in boiling RCA solution (6:4:1 high-purity H_2O, 30% NH_4OH, 30% H_2O_2) for 1.5 h. Both types of coverslips were rinsed and stored in high-purity water to avoid particulate contamination. High-purity water was 18.3 MΩ-cm and 0.2 μm filtered. Coverslips were blown dry with filtered nitrogen (MMCFA02 filter: 0.01 μm filtered, oil removed to 1 part per trillion of line input; Airmaze, Stow,

OH, USA) immediately before use.

Single molecules of tetramethylrhodamine isothiocyanate (TRITC) and rhodamine 6G (R6G) were imaged in air on the surface of glass coverslips cleaned with the Chromerge or the RCA method. The molecules were deposited by evaporation of a small volume of dilute solution of dye molecules in ethanol on a cleaned coverslip (typically 5 μL of 10^{-12} M in spectroscopic grade ethanol, yielding a coverage of approximately 1 molecule per 5×5 μm area).

Single molecules of streptavidin-tetramethylrhodamine conjugate (SA-TRITC; Pierce Chemical, Rockford, IL, USA) were imaged in aqueous solution while nonspecifically adsorbed to the surface of a Chromerge-cleaned glass coverslip. Ten microliters of 10^{-11} M SA-TRITC in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2) were placed between a cleaned glass coverslip and a glass slide.

Single molecules of avidin-fluorescein isothiocyanate conjugate (A-FITC; Sigma, St. Louis, MO, USA) were imaged in aqueous solution while nonspecifically adsorbed to the surface of a Chromerge-cleaned coverslip. Ten microliters of 10^{-11} M A-FITC in sodium bicarbonate buffer (0.1 M, pH 8.9) were placed between a cleaned glass coverslip and a glass slide.

GFP 37 (5,16), a GFP mutant containing the S65T, V163A, I167T and S175G mutations, was studied. The S65T mutation increases the brightness and shifts the absorbance peak from 397 to 488 nm; the emission peak (at 509 nm) remains close to that of the wild-type (504 nm). The additional three mutations allow for more efficient GFP expression at 37°C.

A histidine-tagged GFP, His_6-GFP 37, was constructed. The pGFP37 vector (5) used by Grabner et al. was digested with *Pst*I and *Hin*dIII and was ligated into pQE32 (Qiagen, Valencia CA, USA), which contains the His_6 sequence, at corresponding restriction sites. As a result, 28 N-terminal residues, including His_6, were added upstream of the GFP start codon, and 3 additional C-terminal residues were added downstream of the GFP stop codon. SG13009 [pREP4] cells (Qiagen) carrying the GFP expression vector were grown in 25 mL medium (LB broth containing 100 μg/mL ampicillin and 25 μg/mL kanamycin) in a 37°C shaker overnight. Of this culture, 10 mL were then inoculated into 500 mL medium and grown at 37°C until it reached 0.6 OD (600 nm), then placed in isopropyl-β-D-thiogalactoside (IPTG) (1 mM) for 4 h. The cells were harvested by centrifugation, stored at -20°C, lysed (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 4 mL/g) and digested with lysozyme (1 mg/mL) for 30 min on ice. RNase A (10 μg/mL) and DNase I (5 μg/mL) were then added, followed by incubation on ice for 15 min and centrifugation (10 000× *g* for 30 min). The supernatant was mixed with 50% Ni-NTA agarose slurry (4 mL of sample per 1 mL of agarose slurry) at 4°C for 1 h and then was loaded into a column. The mixture was washed (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 mL per 1 mL agarose slurry) and eluted with 2 mL of 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole. The concentration of eluted GFP was determined by bicinchoninic acid reagents (Pierce Chemical), and the GFP was mixed with an equal volume of glycerol and stored at -20°C. The resulting solution was diluted to 10^{-8} M with TE buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM NaCl, pH 8.0) for imaging.

Single molecules of His_6-GFP 37 were imaged in aqueous solution on the surface of an RCA-cleaned glass coverslip. Ten microliters of GFP solution were incubated on the coverslip for 1 h. The coverslip was then washed thoroughly with TE buffer and mounted on a cleaned glass slide. Excess buffer volume between the coverslip and slide was reduced by applying mild pressure with a stream of dry nitrogen.

The microscope is a Model IX50 (Olympus America, Melville, NY, USA) with a 100 W mercury lamp and a standard TRITC filter set (D540/25, 565 DCLP, D605/55; Chroma Technology, Brattleboro, VT, USA). For imaging SA-FITC and GFP molecules, a custom filter set designed for use with the nucleic acid stain YOYO was used (D470/40, 500DCLP, D535/50; Chroma Technology). Two oil-immersion objectives were used in the course of this work: a UplanApo 100X 1.35 NA and a PlanApo 60X 1.4 NA (both from Olympus America). A low-fluorescence immersion oil was used (Cargille Laboratories, Cedar Grove, NJ, USA). With the 60× objective, illumination intensity at the sample was approximately 4.8×10^5 W/m^2 with the TRITC filter set and approximately 1.1×10^5 W/m^2 with the YOYO filter set.

Two CCD cameras were tested; the Model ST-7I (Santa Barbara Instrument Group, Santa Barbara, CA, USA) is a low-cost (ca. $3000), cooled CCD camera, while the Pentamax (Roper Scientific, Trenton, NJ, USA) is a higher-cost (ca. $30 000), image-intensified camera that was used for time-resolved images. Both cameras have sufficient sensitivity to image single fluorophores of TRITC in 0.1 s.

Signal-to-noise ratios (S/N) were computed using two different definitions. The first (following the point source flux convention in astronomy) is the total signal of a fluorophore divided by the total noise in the same area. The second definition (peak-pixel S/N) is the peak per-pixel signal of the fluorophore divided by the root-mean-square (RMS) variation in background.

To measure the S/N ratio with the astronomy convention, image processing software was used to determine the average intensity (per pixel) of a box of pixels around fluorescent spots. Average background (per pixel) and RMS variation were determined using 5×5 boxes on non-spot areas within the vignetted area. Using an $n \times n$ box of pixels, the total signal of the fluorophore is $n^2 \times$ (average – background). The total noise is added in quadrature and therefore totals $(\sqrt{n^2}) \times$ (RMS variance) = $n \times$ RMS variance. The box size was 5×5 for the TRITC images and 3×3 for the GFP images. All values quoted are the average of 10 or more boxes.

RESULTS

Figure 1 shows images of TRITC molecules deposited from ethanol taken with the ST-7I, with an image acquisition time of 1 second. The number density of molecules on the surface scales directly with concentration: Figure 1a is

deposited from 10^{-11} M solution and exhibits approximately 10× as many molecules as 10^{-12} M (Figure 1b); the control (ethanol only) is shown in Figure 1c. Minor variations occur because of differences in how well the droplet spreads out over the coverslip before evaporation; the expected and experimentally observed numbers of molecules generally agree to within a factor of 2. Molecules are found to exhibit the expected spectral dependence—for instance, TRITC (bulk: max Ex 547, max Em 572) fluoresces strongly using a TRITC filter set (Ex 530–550 and Em 580–630), but exhibits no observable fluorescence with a YOYO filter set (Ex 450–490 and Em 510–560).

The molecular fluorescence emission is quantized—molecules exhibit rapid photobleaching, rather than a gradual decrease in fluorescence. Molecules also exhibit "blinking" behavior, as previously observed in single-molecule spectroscopic studies (6,12,21): before photobleaching, the molecule's fluorescence flutters on and off. Figure 2 shows examples of this blinking and bleaching behavior, with a sequence of cropped images and traces of the integrated spot fluorescence (data acquired with the Pentamax camera).

Single molecules of R6G in air were also observed, as well as FITC-labeled avidin and TRITC-labeled streptavidin molecules in aqueous solution. These fluorophores all have similar properties [bulk extinction coefficient ε ca. 80 000 $M^{-1}cm^{-1}$, quantum efficiency (17) ϕ ca. 0.9 in ethanol or ca. 0.5 in water]. Images of TRITC in air gave a S/N of 46 (total fluorophore signal divided by total noise) and a peak-pixel S/N of 54. The strength of the signal suggested that even the less expensive ST-7I would have the sensitivity to image single molecules of the various GFPs [wild-type (14) GFP ε ca. 7000–15 000, ϕ ca. 0.72–0.85; S65T mutant (7) GFP ε = 39 200, ϕ = 0.68].

This hypothesis proved correct; it is possible to image single molecules of GFP. Figure 3a shows an image of GFP nonspecifically adsorbed onto coverslips and observed in aqueous solution with a YOYO filter set with an image acquisition time of 1 s on the ST-7I. Under similar conditions (2), His_6-GFP was not found to form dimers. As with

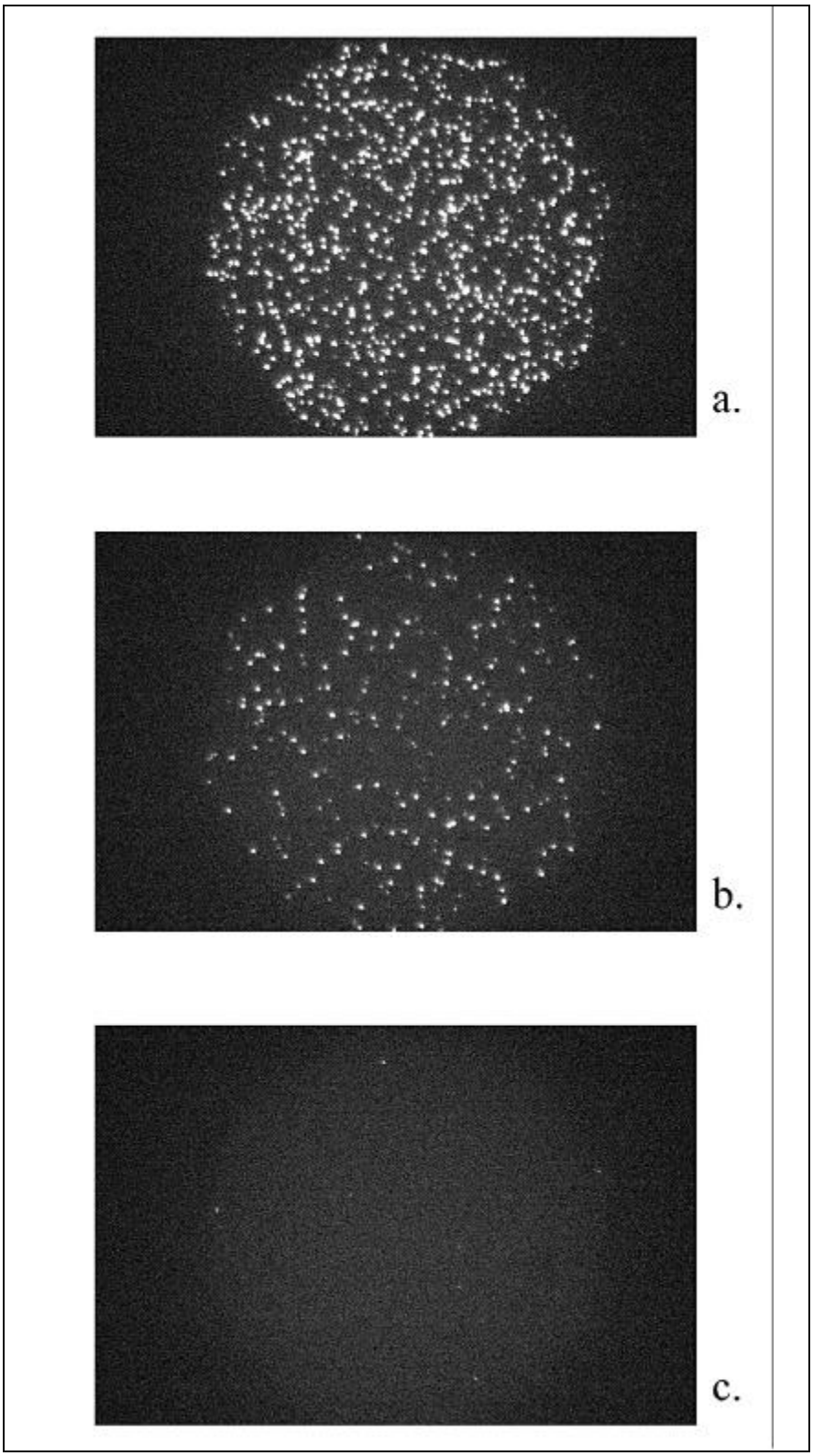

Figure 1. Single-molecule fluorescence. One-second exposures were taken with the ST-7I camera, vignetted to a circle with the field iris diaphragm. (a) TRITC molecules adsorbed on a glass coverslip from 5 μL 10^{-11} M solution in ethanol. (b) TRITC adsorbed from 5 μL 10^{-12} M solution. (c) Control (5 μL pure ethanol). Field of view is 115 × 75 μm; illumination intensity is 4.8×10^5 W/m^2. The images are acquired without binning and are displayed with a grayscale range of 0 counts (black) to 100 counts (white). In panel b, the S/N is 46, and the peak-pixel S/N is 54. The RMS noise is 5.9 counts per pixel.

the other fluorophores observed in solution, the presence of the large number of Raman scattering water molecules, etc., increases the background noise level, but single molecules are still readily observed with S/N of 9 (peak-pixel S/N of 8). The number of observed molecules scales appropriately with concentration. The spectral dependence is also correct; as expected, single molecules of GFP do not fluoresce with the TRITC filter set. Successive images taken in the same spot (Figure 3, a–c) demonstrate that molecules of GFP also exhibit quantized photobleaching.

DISCUSSION

Since optical microscopy has limited resolution, extremely dilute solutions are required to visualize single molecules. Allowing a low surface tension solution to evaporate deposits the molecules on a surface. One microliter of a 10^{-6} M solution spread over a 1 cm^2 area gives a mean intermolecular separation of 12.9 nm on the surface, a distance far too small to resolve with optical microscopy. Each factor of 10 dilution increases the average intermolecular separation by a factor of $10^{1/2}$ = 3.16. A 10^{-12} M solution gives a mean intermolecular separation of 12.9 μm, easily resolved with an optical microscope. Assuming molecules are distributed randomly across the surface, one expects a Poisson distribution of intermolecular distances; with further dilution, the chance of molecules overlapping decreases. If molecules are focused to appear to be 0.5 μm in diameter, and the average intermolecular separation is 5 μm, the chance of two molecules overlapping (i.e., center-to-center distance of 0.5 μm or less) is 1%.

Molecules sufficiently separated by dilution can be examined with fluorescence microscopy. An intense light source is required to excite the fluorophores (in this case, a mercury lamp), and a reasonably sensitive detector is required to capture the emitted fluorescent photons (a cooled CCD camera).

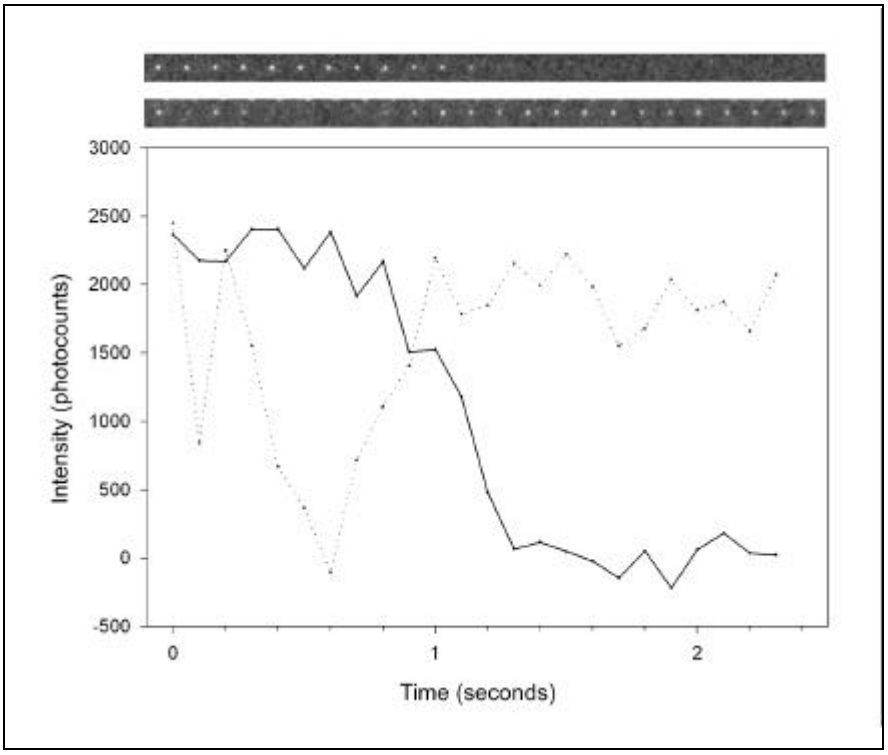

Figure 2. Blinking and bleaching of single molecules. Exposures of 0.1 s were taken with the Pentamax camera, and cropped images are shown in sequence. Below, the trace of the integrated spot intensities are shown vs. time. Top sequence and solid line: irreversible photobleaching. Bottom sequence and dotted line: blinking behavior. In these images the S/N is 37, and the peak-pixel S/N is 25. The RMS noise is 11.3 photocounts per pixel, which corresponds to half the size of the dots indicating the data points.

The following example (calculated for rhodamine) illustrates the numbers involved. At an illumination intensity of 4.8×10^5 W/m^2, the photon flux is 1.3×10^{24} photons/m^2·s. Rhodamine has a bulk molar absorptivity $\varepsilon = 80\,000$ cm^{-1} mol^{-1}L, which (including a geometric factor) converts to a molecular absorption cross section $\sigma_a = 4.0 \times 10^{-20}$ m^2. Multiplying the photon flux by the cross section gives approximately 52 000 absorptions per second. With a quantum efficiency of 0.9, approximately 46 000 photons per second will be emitted. A 1.4 N.A. objective captures photons from a half-angle of 67.5°, corresponding to collection of 31% of the total photons emitted, so approximately 14 400 photons per second should be collected by the objective and imaged onto the CCD. At this wavelength, the quantum efficiency of the CCD is approximately 40%, so approximately 5800 photo-electrons per second per fluorophore are produced; hardware conversion to "counts" occurs at a ratio of 2.3 photoelectrons per count, so approximately 2500 counts are expected per second per rhodamine molecule. The CCD registers approximately 1750– 2750 counts per second per rhodamine molecule. At the same time, background (from light leakage, background fluorescence and electronic noise) constitutes approximately 28 counts, with a RMS variation of approximately 5.9 counts.

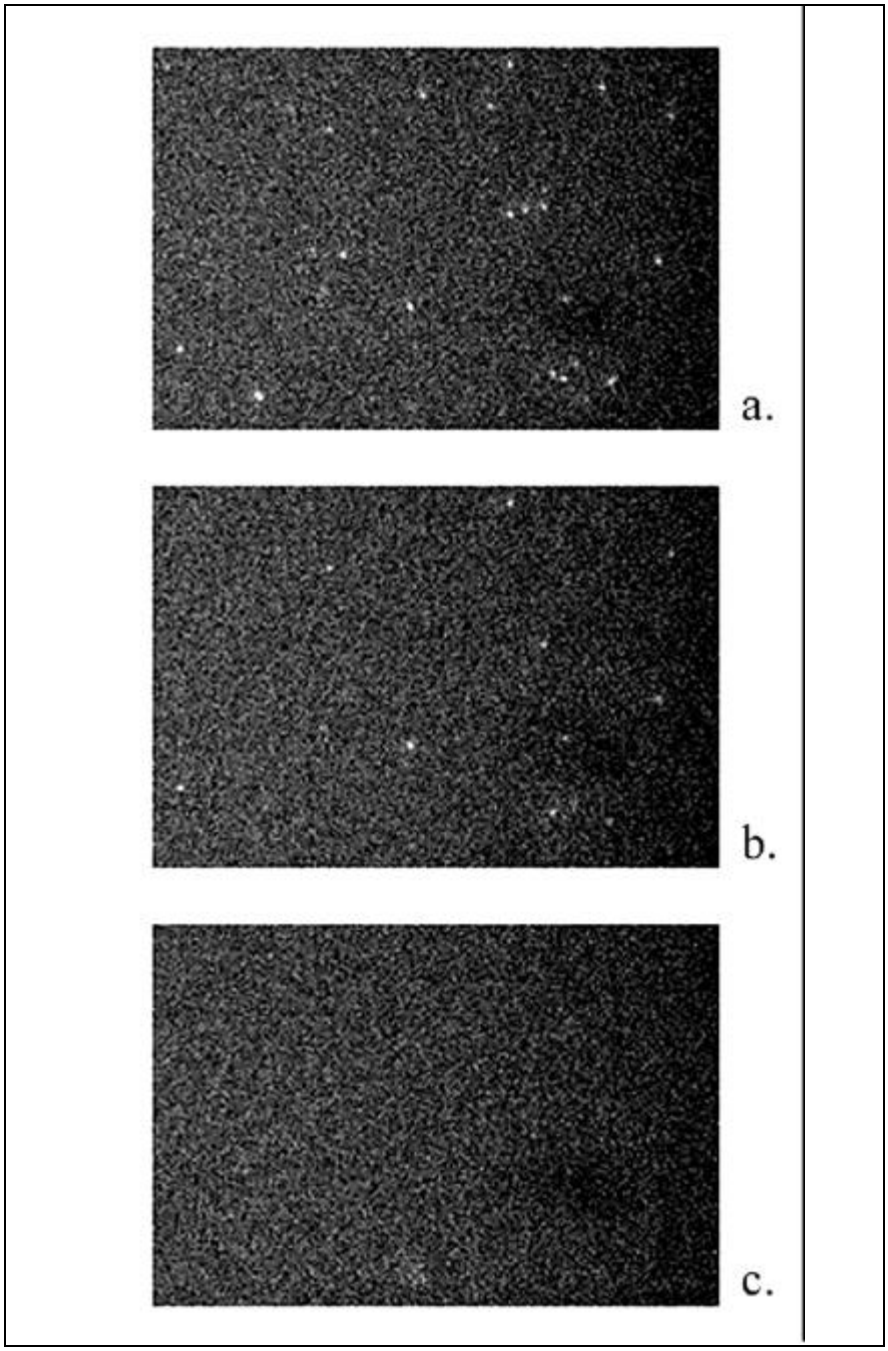

Figure 3. Single molecules of GFP and quantized photobleaching. (a) GFP molecules nonspecifically adsorbed on a glass coverslip from 10 µL 10^{-8} M solution in TE buffer, imaged in TE buffer. (b and c) Same area, after 2 and 4 s additional exposure to excitation light. One-second integrations were taken with the ST-7I camera. Field of view is 30×20 µm; illumination intensity is approximately 1.1×10^5 W/m^2. The images were acquired without binning and are displayed with a grayscale range of 50 counts (black) to 120 counts (white). In panel a, the S/N is 9, and the peak-pixel S/N is 8. Compared to Figure 1, S/N is lower because the absorption cross-section and quantum efficiency of GFP is lower than TRITC; the background is also higher, since the fluorescence of glass is higher at shorter wavelengths. The RMS noise is 6.6 counts per pixel.

Reducing the background noise is a crucial element in the protocol. Advances in the quality of dielectric filters over the past few years have allowed the use of off-the-shelf commercial filter sets (Chroma Laboratories). In spite of using a broad-band excitation source, these filters reduce the leakage of light so that the background shot noise is well below the signal level of a single fluorescent molecule. It is also well known that glass coverslips contribute to background fluorescence. While some workers use quartz coverslips, we found that the intrinsic fluorescence of well-cleaned glass coverslips is relatively low.

Another serious source of noise is the fluorescence of particulate contaminants. Small particles of dust and micro-droplets of oil fluoresce brightly at about the same level as single molecules. This source of noise can be eliminated by careful cleaning of the coverslips with detergent and either chromic acid (Chromerge) or a hydrogen peroxide per base bath (RCA solution). Storing the cleaned coverslips in high-purity filtered water ensures that they remain clean.

Single fluorophores may be distin-

guished from particulate contamination by several distinctive signatures: spectral dependence, concentration dependence, quantized emission and blinking. Particulate contaminants are found to have a broad-spectrum fluorescence and therefore are visible in multiple filter sets. Single fluorophores are only visible in the appropriate filter set. Particulate contaminants generally bleach gradually from exposure to exposure; single fluorophores generally bleach in one step.

While both cameras gave similar quality pictures, the Pentamax has an important performance distinction. The ST-7 can take images with exposure times as short as 100 milliseconds, but there is an approximate 15 s delay as each image is downloaded to the computer. This delay may be reduced to approximately 2 s by 3×3 binning the image (at the expense of image resolution), but in either case the ST-7 is really useful only for taking "snapshot" images or for studying slowly varying behavior. The Pentamax can take approximately 15 images per second continuously and is thus suitable for studying phenomena that vary more rapidly in time. This bandwidth advantage is reflected in the higher cost of the Pentamax.

We have demonstrated that it is possible to image single fluorophores with a commercial fluorescence microscope, a mercury lamp and an inexpensive CCD camera. Single molecules of fluorescent dyes, fluorescent conjugates and GFP have been imaged variously in air and aqueous solution. Given the demonstrated power of fluorescent conjugates and GFP fusion proteins to shed light on the distribution and dynamics of molecules and biomolecules, we expect that the ability to perform single-molecule studies using simple, commercially available equipment will prove useful in many laboratories.

ACKNOWLEDGMENTS

The S65T, V163A, I167T, S175G GFP mutant was kindly provided by Dr. Kurt Beam, Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, CO, USA. This work was partially supported by the National Institutes of Health Grant Nos. NS-11756 and DA-9121.

REFERENCES

1. **Conn, P.M.** 1999. Methods in Enzymology, Vol. 302. Green Fluorescent Protein. Academic Press, New York.
2. **De Angelis, D.A., G. Miesenböck, B.V. Zemelman and J.E. Rothman.** 1998. PRIM: proximity imaging of green fluorescent protein-tagged polypeptides. Proc. Natl. Acad. Sci. USA *95*:12312-12316.
3. **Dickson, R., D. Norris, Y. Tzeng and W. Moerner.** 1996. Three-dimensional imaging of single molecules solvated in pores of poly(acrylamide) gels. Science *274*:966-968.
4. **Funatsu, T., Y. Harada, M. Tokunaga, K. Saito and T. Yanagida.** 1995. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. Nature *374*:555-559.
5. **Grabner, M., R.T. Dirksen and K.G. Beam.** 1998. Tagging with green fluorescent protein reveals a distinct subcellular distribution of L-type and non-L-type Ca^{2+} channels expressed in dysgenic myotubes. Proc. Natl. Acad. Sci. USA *95*:1903-1908.
6. **Ha, T., T. Enderle, D. Chemla, P. Selvin and S. Weiss.** 1996. Single molecule dynamics studied by polarization modulation. Phys. Rev. Lett. *77*:3979-3982.
7. **Heim, R., Cubitt, A.B. and R.Y. Tsien.** 1995. Improved green fluorescence. Nature *373*: 663-664.
8. **Ishijima, A., H. Kojima, T. Funatsu, M. Tokunaga, H. Higuchi, H. Tanaka and T. Yanagida.** 1998. Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin. Cell *92*:161-171.
9. **Iwane, A.H., T. Funatsu, Y. Harada, M. Tokunaga, O. Ohara, S. Morimoto and T. Yanagida.** 1997. Single molecular assay of individual ATP turnover by a myosin-GFP fusion protein expressed in vitro. FEBS Lett. *407*:235-238.
10. **Lu, H.P., L.Y. Xun and X.S. Xie.** 1998. Single-molecule enzymatic dynamics. Science *282*:1877-1882.
11. **Macklin, J., J. Trautman, T. Harris and L. Brus.** 1996. Imaging and time-resolved spectroscopy of single molecules at an interface. Science *272*:255-258.
12. **Moerner, W.** 1997. Polymer luminescence—those blinking single molecules. Science *277*: 1059-1060.
13. **Nie, S., D. Chiu and R. Zare.** 1994. Probing individual molecules with confocal fluorescence microscopy. Science *266*:1018-1021.
14. **Ormo, M., A.B. Cubitt, K. Kallio, L.A. Gross, R.Y. Tsien and S.J. Remington.** 1995. Crystal structure of the Aequorea Victoria green fluorescent protein. Trends Biochem. Sci. *20*:448-455.
15. **Schutz, G.J., W. Trabesinger and T. Schmidt.** 1998. Direct observation of ligand colocalization on individual receptor molecules. Biophys. J. *74*:2223-2226.
16. **Siemering, K.R., R. Golbik, R. Sever and J. Haseloff.** 1996. Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. *6*:1653-1663.
17. **Soper, S.A., H.L. Nutter, R.A. Keller, L.M. Davis and E.B. Shera.** 1993. The photophysical constants of several fluorescent dyes pertaining to ultrasensitive fluorescence spectroscopy. Photochem. Photobiol. *57*:972-977.
18. **Sullivan, K.F. and S.A. Kay.** 1998, Green Fluorescent Proteins. Academic Press, New York.
19. **Ting, A.Y., T.J. Ha, J. Liang, W.B. Caldwell, A.A. Deniz, D.S. Chemla, P.G. Schultz and S. Weiss.** 1999. Single-molecule fluorescence spectroscopy of enzyme conformational dynamics and cleavage mechanism. Proc. Natl. Acad. Sci. USA *96*:893-898.
20. **Vale, R.D., T. Funatsu, D.W. Pierce, L. Romberg, Y. Harada and T. Yanagida.** 1996. Direct observation of single kinesin molecules moving along microtubules. Nature *380*:451-453.
21. **Xie, X. and R. Dunn.** 1994. Probing single-molecule dynamics. Science *265*:361-364.

Received 11 February 1999; accepted 13 July 1999.

Address correspondence to:

Stephen R. Quake
Department of Applied Physics
M.S. 128-95
California Institute of Technology
Pasadena, CA 91125, USA