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Single-molecule measurements calibrate green fluorescent protein surface densities on transparent beads for use with 'knock-in' animals and other expression systems

Chi-Sung Chiu^a, Emil Kartalov^b, Marc Unger^b, Stephen Quake^b, Henry A. Lester^{a,*}

^a Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA ^b Division of Applied Physics, California Institute of Technology, 156-29 Caltech, Pasadena, CA 91125, USA

insion of Applieu Physics, California Institute of Technology, 150-25 Callecti, Pasadena, CA 91125, 051

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Abstract

Quantitative aspects of synaptic transmission can be studied by inserting green fluorescent protein (GFP) moieties into the genes encoding membrane proteins. To provide calibrations for measurements on synapses expressing such proteins, we developed methods to quantify histidine-tagged GFP molecules (His₆-GFP) bound to Ni-NTA moieties on transparent beads (80–120 µm diameter) over a density range comprising nearly four orders of magnitude (to 30 000 GFP/µm²). The procedures employ commonly available Hg lamps, fluorescent microscopes, and CCD cameras. Two independent routes are employed: (1) single-molecule fluorescence measurements are made at the lowest GFP densities, providing an absolute calibration for macroscopic signals at higher GFP densities; (2) known numbers of His₆-GFP molecules are coupled quantitatively to the beads. Each of the two independent routes provides linear data over the measured density range, and the two independent methods agree with root mean square (rms) deviation of 11–21% over this range. These satisfactory results are obtained on two separate microscope systems. The data can be corrected for bleaching rates, which are linear with light intensity and become appreciable at intensities $> \sim 1$ W/cm². If a suitable GFP-tagged protein can be chosen and incorporated into a 'knock-in' animal, the density of the protein can be measured with an absolute accuracy on the order of 20%. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: GFP (green fluorescent protein); Single-molecule imaging; Calibration; Photobleaching rate; pH sensitivity; Epi-fluorescent microscope; Histidine tag

1. Introduction

Several problems in synaptic transmission call for knowledge about the absolute surface density of receptors, channels, and transporters (Anglister et al., 1994; Lester et al., 1996; Nusser et al., 1998). In one potential route to such measurements, the gene for the membrane protein is replaced by a construct containing the protein fused to green fluorescent protein (GFP). If the GFP is maintained in a monomeric state and otherwise prevented from interacting with other chromophores, the fluorescence properties are independent of ionic strength, polarity of the solution, and other conditions that might be encountered in living cells (Tsien, 1998; Lippincott-Schwartz et al., 1999; Piston et al., 1999). Furthermore, the resolution of the fluorescence microscope (better than ~ 0.5 μ m) corresponds well (1) to the size of individual synapses and (2) to the distance neurotransmitter molecules are expected to diffuse during the time of chemical synaptic transmission (~ 3 μ m during ~ 10 ms).

Such 'knock-in' animals will yield useful data if one has methods for absolute quantification of the fluorescence protein density. For this purpose, we have chosen to couple GFP to the surface of transparent beads large enough ($\sim 90 \ \mu m$ average diameter) to present a functionally flat surface on the distance scale of μm . Such calibrated beads can be introduced into microscopic preparations as internal standards. Commercially available fluorescent beads are available from several

^{*} Corresponding author. Tel.: +1-626-3954946; fax: +1-626-5648709.

E-mail address: lester@caltech.edu (H.A. Lester).

sources with precise diameters, and the FocalCheck microspheres from Molecular Probes have dye on the surface only; but dye densities are neither controlled or specified.

This study addresses the challenge of calibrating GFP-coated beads. It would be inappropriate to rely solely on the macroscopic method of coupling known masses of GFP - and therefore known numbers of GFP molecules — to the beads. Such measurements could be distorted if an appreciable fraction of the coupled molecules do not fluoresce, for instance because of changes during purification or because of interactions with the bead surface. The most rigorous method for calibration employs recently developed procedures to measure the fluorescence of single GFP molecules at low surface densities under microscopes in common laboratory use (Unger et al., 1999). Although it is unlikely that densities this low would be generally interesting, the linearity of CCD detectors and the use of proper neutral-density filters allow one to extrapolate to much higher surface densities.

We have compared this single-molecule fluorescence method to the macroscopic method, which we optimize by amino-acid analyses to measure the number of GFP molecules most accurately. We report both excellent linearity and excellent agreement between these two methods, providing known densities of GFP molecules over a range of nearly four orders of magnitude that span expected membrane densities of channels, receptors, and transporters. As a result, the beads provide a simple and versatile tool for absolute quantification of GFP densities.

2. Materials and methods

2.1. His₆-GFP/Ni-NTA beads preparation

We employed GFP37, a GFP mutant containing the S65T, V163A, I167T, and S175G mutations (Siemering et al., 1996; Grabner et al., 1998). 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.

The procedures for preparing histidine-tagged GFP (His₆-GFP), for cleaning slides, and for minimizing background fluorescence were described previously (Unger et al., 1999). The beads (Qiagen, Cat # 30210) are derivatized by the manufacturer with Ni-NTA at the surface only, at a density of ~4 × 10⁶ sites/µm². The beads' diameter (mean ± S.D.) and surface area are 87.6 ± 26.87 (range 45–160) µm and 26 370 ± 16 300 µm², respectively.

2.2. Amino acid analysis

To determine the absolute quantity of GFP in stock solutions, amino-acid analysis was performed. Aliquots of (His₆-GFP) stock solutions were placed in pyrolyzed hydrolysis tubes and precipitated with 80% ethanol overnight at -80° C, and washed with 80% ethanol. Hydrolysis was carried out in a vacuum chamber with vaporized 6 N HCl/1% β-mercaptoethanol at 110°C for 24 h. Subsequent free amino acid mixtures were dried in a speed-vac to eliminate any residual acid and resuspended to approximately 3 µg/100 µl in Na-S buffer (Beckman, Palo Alto, CA). Analysis was performed with a Beckman amino acid analyzer model 6300, where ion-exchange chromatography with a Na buffer system was used for separation. A control protein (β-lactoglobulin A from bovine milk, Sigma L-7880) was included in each sample batch and gave concentration results with 6-9% average error. Corrections were applied for conversion of Gln and Asn into Glu and Asp, respectively. Data for Cys, Trp, and Met were ignored because of possible underestimation.

The GFP concentration was 1.55 ± 0.04 mg/ml (mean \pm S.D., n = 3). The MW for His₆-GFP is 30 474, so that the concentration is 51.0 μ M = 3.07×10^{16} GFP/ml. The GFP stock solution is stored in 25 mM Tris–Cl, pH 8.0; 150 mM NaCl; 125 mM imidazole and 50% glycerol. For comparison with more readily performed protein analyses, we determined that the GFP protein concentrations determined with the Bradford method (Coomassie Plus reagent, Pierce) should be multiplied by 0.764 to yield the more accurate concentration determined from amino-acid analyses.

2.3. GFP dilutions

A total of 18 dilutions of GFP from the stock solution were made at one, two, five, ten intervals spanning the range from 5×10^{-3} to 1×10^{-8} in 1 ml in 1.5 ml Eppendorf tubes. The dilution solution contained 300 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1.5 mg/ml BSA. The Ni-NTA agarose beads, 30 µl of a 50% slurry from the commercial stock, were added to each tube. The GFP solutions were mixed with the beads overnight in 4°C. The density of GFP on the Ni-NTA beads was calculated from the dilution factors. the average bead surface area, and the number of beads in each tube (determined by counting duplicate 5 µl samples from each tube in a hemacytometer; mean + S.D. was 34 560 \pm 3200, n = 30). The total surface area on the beads in each tube was $\sim 9 \text{ cm}^2$. The highest expected surface density of GFP, produced by incubating the beads at 10^{-7} M His₆-GFP, is 60 000-70 000 GFP/ μ m² (or 37.8–40.8 Å between GFP molecules). This is $\sim 1.5\%$ of the full capacity of the beads, indicating that no correction need be applied for saturation of the Ni-NTA sites. Concentrations between 5×10^{-12} and 10^{-7} M are used for macroscopic measurements, and lower concentrations are appropriate for single GFP imaging.

2.4. Microscopes

Two microscopes were used in the experiments. An Olympus IX50 inverted microscope was equipped with a manually shuttered 100 W Hg lamp (HBO 103W/2, Osram), a custom filter set (peak/band at half max) (D470/40, 500DCLP, D535/50, Chroma), and a PlanApo 60X 1.4 NA oil immersion objective (Olympus). The CCD camera was the ST-7I from Santa Barbara Instrument Group, equipped with a chip containing 765 × 510 pixels, each 9 × 9 μ m. The Olympus microscope was equipped with a field stop corresponding to an octagon of 4385 μ m². The optical power, measured through the objective (S20MM meter set at 490 nm; Thorlabs) for no filter, 0.7 OD, 1.0 OD, and 2.0 OD neutral density filters was 22.8 ± 0.1, 4.33 ± 0.07, 1.32 ± 0.02, and 0.16 W/cm², respectively.

A Nikon Eclipse TE300 inverted microscope was also used, equipped with a 100 W Hg lamp (HBO 103W/2, Osram). The excitation filter, dichroic mirror, and emission filters were 493/17, Cat # 86006, and 530/40 nm. The microscope has a PlanApo 60X 1.4 NA oil immersion objective (Nikon). The CCD camera was the Hamamatsu ORCA II (model C4742-98) with a chip containing 1280 × 1024 pixels, each $6.7 \times 6.7 \mu m$. The field stop was set at 4861 μm^2 . The measured optical power for no filter and a 0.9 OD neutral density filter was 9.8 \pm 0.1 and 1.28 \pm 0.02 W/cm², respectively.

For both microscopes, we measured the absorbance spectra of the (nominal) neutral density filters to determine the actual absorbance A at 470–490 nm. In the calibrations of Figs. 2 and 3, the measured counts/ μ m² were multiplied by 10^A, then corrected for bleaching as described in Section 3, to give the macroscopic counts/ μ m².

2.5. Macroscopic measurements

The beads with bound His₆-GFP were imaged on a glass cover slip. Single images were acquired for each of ten beads at each dilution. We measured beads whose diameters $(80-120 \ \mu\text{m})$ lay within -10 and +30% of the average. For the Olympus microscope, the lamp shutter was opened manually for 3.5 s, and the image was acquired for 1 s during this interval. For the Nikon microscope, an electrically operated lamp shutter opened only during the 1 s image acquisition episodes.

Counts/pixel were averaged over a 150×150 pixel area that was flattened against the cover slip by placing an additional cover slip on top of the beads (equivalent results were obtained in some experiments with a $31 \times$

31 pixel area). The measured area corresponds to a 22.5 or 16.8 μ m square for the Olympus or Nikon, respectively. Analysis employed CCDOPS 1.04 (Santa Barbara Instrument Group) for the Olympus microscope, or Metamorph 4.1 (Universal Imaging) for the Nikon.

The fluorescence intensity of the GFP dilutions stored at 4°C was quite stable for 1 week. After 25 days, the beads exposed to 5×10^{-9} M (3960 GFP/ μ m²) gave no detectable loss of fluorescence intensity; those exposed to 5×10^{-10} M (341 GFP/ μ m²) displayed a reduction of 16%.

2.6. Single GFP calibration procedures

Single GFP measurements were taken from the beads incubated with the most dilute GFP samples, which produced the lowest surface density of GFP. CCDOPS 4.03 or Metamorph 4.1 we used to sum counts from a 5×5 pixel area chosen to center on a fluorescent spot. Background fluorescence, determined at neighboring pixels, was subtracted. The signal to noise (S/N) ratio was determined as described previously (Unger et al., 1999). We collected approximately 200 single GFP images and a Gaussian was fitted to the histogram of fluorescence counts. These single-GFP counts provided the calibrations for the macroscopic measurements at the higher surface densities that were produced by incubating the beads with more concentrated GFP solutions.

2.7. pH sensitivity

The pH ranges for this experiment are 9.0-5.5 with 0.5 U increments. For pH 7.5-9.0, buffers were prepared using 50 mM Tris-Cl plus 300 mM NaCl and 1.5 mg/ml BSA. For pH 5.5-7.0, buffers were prepared using 50 mM Na H₂PO₄ versus NaOH plus 300 mM NaCl and 1.5 mg/ml BSA.

The experiments employed a stock solution, 10 ml, containing 300 µl GFP-beads at 5×10^{-9} M (3960 GFP/µm²). Aliquots (0.5 ml) were placed in 1.5 ml Eppendorf tubes and were adjusted to the desired pH by washing 2 times (centrifuged at ~ $300 \times g$, 1 min) with the buffers at various pH values. The beads were then resuspended with the appropriate buffer and were stored at 4°C for 2–6 h until the imaging session.

3. Results

3.1. Single-molecule GFP Images

Fig. 1 presents images of single His_6 -GFP molecules. The GFP fluorescent spots were not observed for blank beads, increased in density roughly in proportion to the increased expected density and were observed only when the GFP specific filters were used. We observed all-or-none bleaching and occasionally blinking (Unger et al., 1999). These observations support our interpretation that we observed fluorescence from single GFP molecules in the beads.

Two imaging systems were used for both the singlemolecule and macroscopic measurements. For the Olympus IX50 microscope, the distribution of singlemolecule GFP fluorescence data were fitted to a single Gaussian with a peak, which also corresponds to the average fluorescence, at 376.4 ± 2.6 counts/s (Fig. 2A). The rms was 10.0 ± 1.0 and the S/N was 9.3, in the same range as the previous observations (Unger et al., 1999). For the Nikon TE300 microscope, the peak (average) single-molecule GFP fluorescence was 174.77 ± 3.41 counts/s (Fig. 3A). The rms was $3.62 \pm$ 0.42 and the S/N was 9.6.

To estimate the total photon emitted rate from a single GFP, the detection efficiency the Olympus micro-scope was estimated,

$$\eta_{\text{total}} = \eta_{\text{NA}} * T_{\text{obj}} * T_{\text{bs}} * T_{\text{TL}} * T_{\text{cw}} * Q_{\text{CCD}}$$
$$= 0.30 * 0.85 * 0.68 * 0.90 * 0.95 * 0.3$$

Here, $\eta_{\rm NA}$ designates the collection efficiency of the objective lens due to the limited solid angle, $T_{\rm obj}$ the transmittance of the objective lens, $T_{\rm bs}$ the transmittance of the dichroic beam splitter and of the emission band-pass filter, $T_{\rm TL}$ designates the transmittance of the tube lens, $T_{\rm cw}$ the transmittance of the camera window,

and Q_{CCD} the detection quantum efficiency of the CCD sensor (Kubitscheck et al., 2000).

Therefore the detection efficiency is 4.44%. On the other hand, the photoelectron-to-digital unit-conversion factor is 2.3 e⁻/count. We estimate that the average photon emission rate for a single GFP is 1.95×10^4 photons/s.

For the Nikon microscope, $T_{\rm bs}$ is 0.55 and the Hamamatsu CCD camera has a $Q_{\rm CCD}$ of 0.45 at 509 nm, so that the detection efficiency is 5.40%. The photoelectron-to-digital unit-conversion factor is 2.4 e⁻/count (at gain = 1). Therefore the estimated photon emission rate from a single GFP is 7.8×10^3 photons/s, or about 40% of the estimate for the Olympus microscope. This ratio is due mostly to the ratio of incident light intensity ratio for the two microscopes (9.8 vs. 22.8 W/cm²).

3.2. Macroscopic measurements and calibration of the beads

The single-molecule measurements were then employed to calibrate beads that had higher densities of GFP, produced by incubating the beads with higher [His₆-GFP]. In an important confirmation of the procedures, we noted that the macroscopic fluorescence intensity increased linearly with the expected surface density, over the entire measured range of three orders of magnitude (60–60 000 GFP/ μ m²) for the Olympus microscope (Fig. 2B) or four orders of magnitude (3–30 000 GFP/ μ m²) for the Nikon (Fig. 3B) microscope.



Fig. 1. Images of single histidine-tagged green fluorescent protein (His₆-GFP) molecules. The Nikon microscope was used to image the surface of beads at increasing densities in an area of 150×150 pixels ($16.75 \times 16.75 \mu m$). Panel A shows that the blank bead does not have the typical 'GFP points'. Panels B–F show images of the beads after incubation with solutions of increasing concentrations (10^{-12} , 2.5×10^{-12} , 5×10^{-12} , 10^{-11} , and 2.5×10^{-11} M, respectively). In panel B, the arrowheads point to the 38 images accepted as arising from GFP molecules.



The measurements of GFP mass also provided an absolute His₆-GFP concentration in the solutions used to incubate the beads. The [His₆-GFP] used for the macroscopic measurements were greater than the K_{d} for binding to the Ni-NTA groups ($\sim 1.5 \times 10^{-12}$ M) but less than 1.5% of the concentration of Ni-NTA groups in the bead slurry. Therefore virtually all the His₆-GFP was absorbed onto the beads, allowing a straightforward calculation of the His₆-GFP surface density on the beads. The macroscopic measurements agree with these expected densities, over the entire measured range for both microscopes. The rms deviation from the expected fluorescence intensity is 11% (Fig. 2C) and 25% (Fig. 3C) for the Olympus and Nikon microscopes, respectively; and the latter rms deviation decreases to 18% if one omits the point at the lowest surface density, 3 GFP/µm². Two additional full experiments gave rms deviations of 17 and 21%.

Some beads smaller than 70 μ m diameter or larger than 120 μ m diameter gave fluorescence intensities 30% higher or lower, respectively, than the values for the intermediate diameters (80–120 μ m) that we used for quantitative studies. We have eliminated obvious optical reasons for these differences and suspect that the cause is variations among the density of Ni-NTA sites.

3.3. Bleaching and blinking

We measured photobleaching of the fluorescence signal over a 140-fold range of incident light intensities (Fig. 4). Exponentials were fit to the decline of fluorescence intensity, and the rate constant for the unfiltered Olympus lamp (22.8 W/cm²) was 0.10 s⁻¹. At lower intensities, the bleaching rate decreased linearly with the illuminating light intensity, with a slope of 4.7×10^{-3} /s (W/cm²)⁻¹ (Fig. 4B). This rate of dependence of the rate constants on light intensity agrees with previous measurements (Peterman et al., 1999; Kubitscheck et al., 2000).

Fig. 2. Calibrations of beads. These results were obtained using the Olympus microscope at an incident beam intensity of 22.8 W/cm². (A) Histogram of fluorescence intensity distributions, fitted to a Gaussian with an average value of 376.4 ± 2.6 counts/s (n = 178). (B) The X axes are the $[His_6-GFP]$ in the incubation solutions, and the resulting expected density of green fluorescent protein (GFP) molecules on the beads. The expected density assumes that binding to the beads is quantitative and that all bound His₆-GFP molecules are active, with the average fluorescence as in panel A. The Y value is the average fluorescence intensity, in counts/µm². Results for beads carrying the highest six densities were multiplied by a 'photobleaching corrective factor' < 1 to correct for reduced bleaching due to the use of neutral density filters on the excitation beam (see text). The correction factor for the 2.0 OD filter (*) is 0.781, for the 1.0 OD filter (†) 0.803, and for the 0.7 OD filter (‡) 0.825. Standard errors of the observations are smaller than the symbol size. (C) Measured intensity is compared with the expected intensity, computed as expected density × average fluorescence counts per GFP.



The exponential fits showed that the fluorescence intensity declined to a nonzero steady-state value (16-24% for the Olympus microscope using no filter and 0.7 OD filter, respectively, and 21% for the Nikon microscope). This nonzero value is too large to be explained by spontaneous recovery (which we estimated at $\sim 0.6\%$ /min) but is consistent with the observations that fluorescence of both wild type GFP and some red-shifted GFP mutants, including the S65T fluorophore used in the experiments, can be reactivated by absorbance of an additional photon (Dickson et al., 1997; Creemers et al., 2000; Kubitscheck et al., 2000). We also observed blinking of GFP fluorescence in the single-molecule fluorescence experiments (data not shown).

In conjunction with the calculated photon emission rate, these time constants indicate that an average GFP molecule bleaches after emitting $1.4-1.8 \times 10^5$ photons, in good agreement with other recent measurements (Peterman et al., 1999; Kubitscheck et al., 2000).

For exposures of a few seconds, the photobleaching correction amounted to at most ~ 21% of the intensities for the Olympus microscope and ~ 3% for the Nikon microscope. For the presentation of Figs. 2 and 3, we chose to accept the actual data taken at the highest light intensities, both the single-molecule fluorescence measurements and the macroscopic measurements, even though these measurements are distorted by bleaching. We then reduced those measurements taken with neutral-density filters in the incident beam by appropriate factors based on the linear dependence of photobleaching rate on incident intensity (Fig. 4B).

3.4. Sources of random and systematic error

There are possible errors both from sample preparation and from imaging. The possible errors for GFP bead preparation could come from pipette error (1– 3%), amino-acid analysis (10%), and number of beads transferred by pipetting (10%). We summed these errors in quadrature to estimate the total random error at \pm 14.5%. This total estimated random error accounts

Fig. 3. Calibrations of beads. These results were obtained using the Nikon microscope at an incident beam intensity of 9.8 W/cm². (A) histogram of fluorescence intensity distributions, fitted to a Gaussian with an average value of 174.8 ± 3.4 counts/s (n = 239). (B) The X axes are the [His₆-GFP] in the incubation solutions, and the resulting expected density of green fluorescent protein (GFP) molecules on the beads. The Y value is the average fluorescence intensity, in counts/ μ m². Results for beads carrying the highest two densities (*) were multiplied by a 'photobleaching corrective factor', 0.97, to correct for reduced bleaching due to the use of a 0.9 OD neutral density filter on the excitation beam (see text). (C) Measured intensity is compared with the expected intensity, computed as expected density × average fluorescence counts per GFP.



Fig. 4. Bleaching of histadine-tagged green fluorescent protein (His₆-GFP) fluorescence on beads. (A) Bleaching during cumulative exposure at various light intensities. The data were fitted to single exponential relaxations with time constants plotted in B and with nonzero infinite-time values given in the text. Data at 9.8 W/cm² were taken with the Nikon microscope; others with the Olympus microscope. The beads were generated by exposure to His₆-GFP at 10^{-9} - 10^{-7} M. (B) Rate constant for bleaching vs. intensity. The line has a slope of 4.7×10^{-3} /s (W/cm²)⁻¹.

for the rms deviations of 11-21%, which summarize the data over the entire range of Fig. 2C and most of the range of Fig. 3C. Evidently there are no major unexplained sources of random or systematic error.

The major uncorrected source of systematic errors may be fluorescence from the antipodal bead surface. To estimate this contribution, we focused the objective about 1 bead diameter from the bead surface and measured about 6% of the fluorescence intensity of the bead surface. No corrections were made.

3.5. Salt and pH sensitivity

We found no detectable change in the fluorescence of

the beads over the range of NaCl concentrations from nominally zero to 500 mM and for artificial cerebrospinal fluid (ACSF; pH 7.4) (data not shown). In studies of pH dependence, GFP37 fluorescence was greatest at pH 7.5–8.5 (Fig. 5). Fluorescence intensity decreased by ~15% at pH 9.0, 25–30% at pH 6.5–7.0, 40% at pH 6.0, and ~75% at pH 5.5. This pH sensitivity profile agrees with previous measurements for EGFP (Patterson et al., 1997).

4. Discussion

4.1. Overview of GFP calibrations

The major result of this study is a set of procedures for calibrating transparent beads with surface densities of His₆-GFP. These procedures yield results that are internally consistent in two ways. First, fluorescence intensities are linearly proportional to the amount of GFP coupled to the beads, over nearly 4 orders of magnitude. Second, the absolute calibration (GFP/ μ m²) based on single-molecule fluorescence agrees, with an rms deviation of 11-21%, with the absolute calibration based on the total mass of GFP quantitatively absorbed onto the beads. This second consistency implies that at least ~ 85% of the expressed His₆-GFP molecules are active as fluorescent molecules with the characteristics noted in the single-molecule measurements. Because the rms deviation agrees with expected uncertainties of 14.5% over the range from 3 to 60 000 GFP/ μ m², we believe that there are no major unexplained sources of systematic or random error. The absolute measurements can be made with roughly the same confidence as the relative measurements.



Fig. 5. pH sensitivity of fluorescence from His₆-GFP beads.

As a result of these internal consistencies, researchers who follow these procedures for GFP purification and coupling, and who use the particular GFP mutant employed here, can now have confidence that the beads have an absolute density of GFP that is calculated in a straightforward way from the mass of the GFP and the area of the beads. The utility of the beads is shown further by the fact that two different microscope systems gave internally consistent results. The method is also directly applicable to confocal and two-photon microscopes. Although the method was specifically designed for planar membranes and was calibrated with the flat surface of a bead, we expect the technique to apply with unchanged characteristics for GFP distributions within the vertical resolution of standard, confocal, or 2-photon microscopy, roughly 0.5 µm. We are now exploring methods for extending these measurements and calibrations to thicker tissues, which would scatter both incident and emitted light.

4.2. Incident light intensity

Most studies reported to date have used laser-based microscopes to visualize single GFP molecules, and the incident power ranged between 2 and 8 kW/cm². This allows observation of single GFP molecules within 10–100 ms (Dickson et al., 1997; Kubitscheck et al., 2000). However, we used standard 100 W Hg lamps, which produce intensities in the range of 10-23 W/cm². As a result, 0.5–1 s was required to observe single GFP molecules.

The advantage of using such lower incident intensities is that casual imaging, for instance to find optimal areas, leads to minimal bleaching. Nonetheless we advocate the use of neutral-density filters where possible for preliminary observations; for instance, densities > $1000/\mu m^2$ can readily be imaged with incident intensities < 4 W/cm², which results from the use of an 0.7 OD neutral density filter on the microscopes used here. At these intensities, the time constant for bleaching is ~ 30 s; and in fact, the data of Fig. 4 allow an estimate of the intensity from the rate of bleaching. In all cases, we advocate the use of an electronic shutter to minimize exposure times. However, bleaching would be minimal with a two-photon microscope.

4.3. Single GFP images

For incubations with [His₆-GFP] lower than $\sim 2.5 \times 10^{-12}$ M, there were fewer single-molecule spots than expected from quantitative coupling of [His₆-GFP] to the beads. The ratio of observed molecules to expected molecules was 9% at 5×10^{-13} M, 25% at 10^{-12} M, and 80% at 2.5×10^{-12} M. These data suggest that the dissociation constant for binding of His₆-GFP to the Ni-NTA groups on the beads is $\sim 1.5 \times 10^{-12}$ M,

somewhat higher than the usual value of $\sim 10^{-13}$ M. This difference may be explained by the presence of 1.5 mg/ml BSA in the solutions.

We believe that most of the imaged GFP molecules are monomers, because only a single Gaussian peak was observed in GFP calibrations. In addition, the GFP homodimer dissociation constant was estimated to be 100 μ M, which is much higher than the working concentration (Phillips, 1997). We do not expect that fluorescence resonance energy transfer (FRET) will distort the data for surface densities in excess of those used in the study, because FRET becomes appreciable at distances less than the average intermolecular distance of ~ 5.9 nm at our highest calibrated density (De Angelis et al., 1998).

4.4. Macroscopic measurements

For beads of diameter far from the average (either <70 or $>120 \ \mu\text{m}$), the measured intensity was sometimes 30% greater or lower than average value. We have not systematically explored the basis for these variations; but we advocate the use of beads with average diameters near the average value ($\sim90 \ \mu\text{m}$; 60-80% of the beads qualify).

A microcapillary system that mimics cell thickness in cultured cells was developed to monitor GFP in the $1-10 \mu$ M range (Hack et al., 2000). This is an appropriate approach for cytosolic proteins that are evenly distributed and present in the indicated concentration range. The present approach is likely to be most useful for quantifying membrane proteins, especially for proteins localized at high densities such as at synapses. The lower limit of utility will depend on background fluorescence from other cellular proteins.

4.5. Uncertainties in measurements of membrane protein density

We have not explored polarization phenomena for the bound His_6 -GFP (Moerner et al., 1999; Peterman et al., 1999). It may be assumed that a GFP tag on the cytoplasmic or extracellular portion of membrane protein is roughly as mobile as the His_6 -GFP tethered to the beads. Therefore corrections due to fluorescence polarization are likely to be minimal. Another source of uncertainty, at roughly the 5% level, would be introduced by FRET between GFP moieties in a multimeric membrane protein, and by changes in FRET with protein conformation (Siegel and Isacoff, 1997).

4.6. Overall recommendations

Beads calibrated as described here are likely be most useful when measured at slightly alkaline pH, for durations less than 1 s at incident light intensities < 10 W/cm^2 . These procedures are expected to provide absolute calibrations for membrane proteins fused to GFP and expressed at densities between 3 and 60 000/ μ m², which encompasses the known densities of receptors, channels, and transporters in neuronal and non-neuronal membranes.

The procedures described here could also be used with the broad range of GFP mutants and homologs that have been described with shifted peak absorbance and fluorescence (Delagrave et al., 1995; Heim and Tsien, 1996; Matz et al., 1999) and environmentally sensitive fluorescence (Miesenbock et al., 1998). Because we have not fully explored the procedures for obtaining active His₆-GFP for mutants other than the specific one utilized here, we recommend that beads be calibrated using the single-molecule method or, better still, with both the single-molecule and the mass measurement methods. Because each microscope and filter arrangement has its own spectral properties, we believe that it would be inaccurate to state a conversion factor from the present His₆-GFP to each other GFP mutant. It would be more appropriate to generate calibrated beads for each fluorescent protein.

Once a calibrated batch of beads is available, the user need conduct only macroscopic measurements on the membrane of interest and then normalize these measurements to the calibrated beads nearby in the same microscopic field. Although CCD cameras are linear, we advocate choosing beads with roughly the same GFP density as the membrane.

Independent biological experiments must be performed to assure that a protein-GFP fusion is expressed, sorted, and inserted into the membrane with wild-type characteristics. If such a construct can be chosen and incorporated into a 'knock-in' animal, the densities of membrane proteins can be measured with an absolute accuracy on the order of 20%.

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