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Single-Molecule Fluorescence Quantification with a Photobleached Internal Standard

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Abstract

In cellular and molecular biology, fluorophores are employed to aid in tracking and quantifying molecules involved in cellular function. We previously developed a sensitive single-molecule quantification technique to count the number of proteins and the variation of the protein number over the population of individual sub-cellular organelles. However, environmental effects on the fluorescent intensity of fluorophores can make it difficult to accurately quantify proteins using these sensitive techniques. In this paper, we demonstrate the use of photobleaching to extract an accurate single-molecule calibration intensity distribution from the sample directly to avoid any differences in environment that may alter the count. Using this technique, we were able to show that goat anti-mouse IgG antibody labeled with Alexa Fluor 488, an environmentally insensitive fluorophore, exhibited an average fluorescence equivalent to 4.6 single fluorophores. SynaptopHluorin vesicles, which contain the environmentally sensitive green fluorescent protein, exhibited an average of 4.4 single green fluorescent proteins per vesicle.

INTRODUCTION

Bio-analytical studies often employ fluorescent probes, such as dyes or fluorescent proteins, to observe and quantify bio-molecules that are essential to cellular function and physiological processes. The photophysical properties, like emission intensity, of fluorophores can be affected by a variety of environmental factors such as solvent polarity,^{1,2} pH,³⁻⁵ or temperature.^{6,7} To further the understanding of cellular dynamics, studies have employed environmentally sensitive fluorophores as biosensors within cellular systems. For instance, measurements of the acidification kinetics within the lumen of individual synaptic vesicles have been studied in synaptopHluorin (spH) vesicles which are synaptic vesicles that have been modified to express pH sensitive green fluorescent proteins (GFPs) on the luminal portion of the synaptobrevin 2 (VAMP 2) proteins.⁴

In our efforts to aid the development of an accurate and predictive model of cellular function, we developed a sensitive quantitative microscopy technique to determine the average number of proteins within sub-cellular compartments and to provide a distribution of the protein number over the entire population.⁸⁻¹⁰ To accurately quantify the protein number, it is essential to obtain an intensity distribution that is representative of a single fluorescent entity that is being used for quantification. However, the environment that some fluorophores are in, such as the GFP in spH vesicles, is not entirely known making it difficult to generate an environment in which a calibration sample, such as isolated GFPs, would accurately represent a single fluorophore. Any differences in environmental factors between the calibration sample and the sample of interest could alter the count, especially

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when one is trying to quantify fewer than 100 molecules. To overcome this potential drawback, we demonstrate how photobleaching can be used to obtain an intensity distribution representative of a single fluorophore directly from the sample of interest while maintaining the original environment, for instance photobleaching to a single GFP emitting within a spH vesicle. To demonstrate the counting capability of this technique we counted the number of Alexa Fluor 488 molecules, an environmentally insensitive fluorophore, on goat anti-mouse IgG antibodies and the number of GFP, an environmentally sensitive fluorophore, in spH vesicles.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich unless noted otherwise. Goat anti-mouse Alexa-488-(GAM-488) was obtained from Invitrogen.

SynaptopHluorin Vesicle Isolation

Whole brains were extracted from transgenic mice at 20-25 days old then frozen and stored at -80°C before use. SynaptopHluorin (spH) vesicles were isolated using a modified procedure from Hell *et. al.* as discussed in previous work.¹⁰ Briefly, 5-10 frozen brains were blended with liquid nitrogen to a fine powder. The fine powder was re-suspended with homogenization buffer (2mM EGTA, 0.3M sucrose, 50mM HEPES, pH 7.4) and then homogenized with a Teflon-glass homogenizer. A spH vesicle-rich supernatant was acquired by centrifuging the homogenate at 100,000 g for 28 min at 4°C with a MLA-80 rotor (Beckman-Coulter, Fullerton, CA) in a Beckman Coulter tabletop ultracentrifuge. The supernatant was loaded onto a 1.5 M/0.6 M sucrose gradient and centrifuged at 250,000 g for 72 min at 4°C to isolate the spH vesicles. Isolated spH vesicles were extracted from the 1.5 M/0.6 M sucrose interface, quickly frozen and stored at -80°C for up to 4 months. Before imaging, spH vesicles were exchanged into fresh phosphate buffered saline (PBS) using a P10 size exclusion column (Bio-Rad).

Sample Imaging and Photobleaching

Total internal reflection fluorescence (TIRF) microscopy was used to simultaneously image and photobleach the sample. For ease of imaging, an imaging chip consisting of 4-straight channels 1mm-wide and 300 μm -high were fabricated in polydimethylsiloxane (PDMS) and sealed irreversibly to a borosilicate glass coverslip as previously described.¹⁰ The sample was introduced to an imaging channel through gravity driven flow and allowed to adsorb to the glass surface then clean PBS buffer was flowed through to remove excess, non-adsorbed sample. Using a home-built TIRF microscope the sample was continually illuminated for 5 min to ensure complete photobleaching with a 488nm light from a 20 mW solid-state laser (Coherent). A stream of images was acquired with a Photon-Max EMCCD camera (Princeton Instruments) with an exposure time of 300 msec per image. We used a 100 $\times$, NA 1.45 Nikon objective and each 16 micron pixel of the EMCCD corresponds to 160nm in the sample.

Extraction of Initial and Final Intensity Distributions

Images of particles were analyzed using an image-processing program generated in Matlab.^{9,10} The program selects fluorescent puncta to be analyzed using a set of predetermined criteria. For these studies, criteria included the amplitude of the fluorescent signal, which was required to exceed a signal-to-noise cutoff and the width of the fluorescent spot, which had to be at the limit of resolution of the microscope system. The integrated intensities of acceptable fluorescent puncta were used to generate an intensity

distribution ($\{F_i\}$). To quantify fluorophores during photobleaching in the sample, initially the image-processing program was applied to the first image of the photobleaching series to identify all acceptable puncta and generate an initial intensity distribution ($\{F_i\}$). The image processing program was then used on all the images in the photobleaching series with all the criteria thresholds set to ensure that no potential puncta were rejected prematurely due to things such as a decrease in signal-to-noise during bleaching.

A second program was used to extract the final fluorescent intensities (F_f) corresponding to a single fluorophore in the sample from photobleaching tracks (Fig. 1) for each punctum found in the first frame. The program generated photobleaching tracks for each punctum by comparing the centroid of the initial punctum to those found within the subsequent images. If a punctum was found with a centroid within a designated acceptance distance (d_{ac}), the track was assumed to be continuing. Once a punctum was no longer observed for a specified number of consecutive frames (frame cutoff, n_{co}) the photobleaching track was terminated and F_f was outputted. The program required a designated number of blinks (n_b) (Fig. 1 inset) to occur during the photobleaching track and the frame from which F_f was obtained must be preceded and followed by a frame in which the punctum was observed to ensure that F_f represented a single fluorophore emitting during the entire exposure of the frame.

Data Analysis

The average number was simply determined by dividing the average intensity of the unbleached sample ($\bar{F}_i$) by the average final intensity ($\bar{F}_f$). Previously we have shown that single fluorophores exhibit an intrinsic lognormal distribution in fluorescence intensity when imaged in TIRF microscopy.⁸ By fitting the histogram of the intensities with a lognormal distribution and comparing the multiplicative standard deviations,¹¹ or relative widths (σ^*), of the initial intensity distribution (σ_i^*) and final intensity distribution (σ_f^*) we can determine if the variability in the number of fluorophores over the sample population was significant. When σ_i^* and σ_f^* were not equal, a distribution of fluorophores within the sample was found using a previously demonstrated deconvolution technique.⁸⁻¹⁰ To determine the distribution in the number of fluorophores, $\{F_i\}$ was fit with a series of calibration distributions generated from the final intensity distribution ($\{F_f\}$).

RESULTS AND DISCUSSION

Optimization of Intensity Extraction from Photobleaching Track

To verify that F_f obtained from the photobleaching track correlated to the fluorescence of a single fluorophore, there were two types of user controlled parameters which had to be optimized in the photobleach tracking program: (1) those that help to ensure that the fluorescence was not from multiple fluorophores that bleach simultaneously or the sample did not leave the imaging region before complete bleaching occurred and (2) those that ensure that the fluorescence was from a punctum that correlates to the original punctum. The parameters that contribute to accurately extracting F_f were the frame cutoff (n_{co}), the number of blinks (n_b), and the required number of frames with a usable punctum (n_{up}). If n_{co} was set too low, the track may have been terminated early and the corresponding intensity had a higher risk of correlating to two fluorophores blinking resulting in a seemingly lower final average fluorophore count. For our samples we found that n_{co} of 10 frames was sufficient. We found that varying n_{co} between 6 and 18 frames showed variations in the final average fluorophore count that all fell within two times the standard deviation of the mean found for n_{co} of 10 frames, which suggests there is no significant difference over this cutoff range. However, we did find that n_{co} set below 6 frames showed a dramatic difference

(outside the recommended standard deviation of the mean) in the average number of fluorophores.

The required number of frames (n_{up}) was the number of frames in which the punctum was considered part of the tail of the photobleaching track and was a suitable punctum from which to extract F_f (i.e. had a frame preceding and following in which the punctum was present). The larger n_{up} was set the longer the resulting tail lengths of acceptable photobleaching tracks were, therefore increasing the potential of extracting the proper single intensity. Additionally, the potential for an intensity corresponding to two fluorophores blinking simultaneously was decreased the larger n_b was set. We found that we only required 1 blink and 1 acceptable frame for our samples. Though the number of eliminated puncta increased as n_b and n_{up} were increased, resulting in a decreased size of $\{F_f\}$ and $\{F_f\}$, the average number of fluorophores found remained within a single standard deviation of the mean number of fluorophores found with only a single blink and a single acceptable frame.

To verify that the final punctum correlates to the original punctum, there were two parameters, acceptance distance (d_{ac}) and maximum distance (d_{max}), which both compare the position of the center of the final punctum to the center of the original punctum. Our samples were allowed to adsorb to the glass surface and no further amount of immobilization was done. Small variations in the samples location between frames may occur due to drift in the microscope and diffusion on the surface and these two parameters account for these potential changes during the course of the photobleaching process. Acceptance distance (d_{ac}) was used when generating the photobleaching track and could be set larger than d_{max} , which compared the position one last time before outputting F_f to ensure that the photobleaching track was not prematurely terminated due to excessive movement over the time course of the photobleaching process. We found that when d_{ac} and d_{max} were set too low, the observed average fluorophore count was lower than the actual average count due to movement of the sample over the length of time needed to bleach the larger number of fluorophores. As d_{ac} and d_{max} were set larger, we found that the final average count did not change but the number of final puncta decreased because the program found multiple potential puncta during the tracking process and rejected the original punctum. For our samples, we found that d_{ac} of 2 pixels and d_{max} of 1 pixel were optimum.

Quantifying Fluorescence Intensity in Single Molecules and Single Organelles

To demonstrate the capability of using photobleaching to complement our previously developed quantitative microscopy technique, we counted the fluorophores in two types of samples: (1) single Alexa Fluor 488 (Alexa-488) on labeled goat anti-mouse (GAM-488) antibodies, and (2) GFP in synaptic vesicles from transgenic mice expressing synaptophysin (spH). The fluorescence of Alexa-488 dye molecules are environmentally insensitive and the average number of fluorophores per GAM-488 was previously determined by Invitrogen making it suitable to verify that F_f extracted from the photobleaching track properly represented a single fluorophore. The reported degree of labeling for the particular lot of GAM-488 was an average of 5 fluorophores. We found that average fluorescence of the labeled antibodies was the equivalent of 4.6 ± 0.3 (stand. err.) single Alexa-488s ($n=12$ data sets). Comparison of σ^* for $\{F_f\}$ and $\{F_f\}$ showed a similarity¹⁰ suggesting that the distribution in fluorescence intensity from the antibodies due to variability in the number of Alexa-488 over the antibody population was negligible. This result was important since it indicates that these antibodies could be used to obtain not just the average, but also the distribution of the number of bound antibodies in quantitative fluorescence experiments.¹⁰

Often, green fluorescent protein (GFP) are used as a reporter of expression in cellular and molecular biology and, unlike Alexa Fluor 488, GFP are sensitive to the surrounding

environments, specifically to pH. In the case of spH vesicles, the GFP are expressed within the lumen of the synaptic vesicle where the exact nature of the environment that GFP is expressed is unknown making spH vesicles an excellent example of the types of samples where this technique will be beneficial. Differences between σ_i^* (0.62 ± 0.03) and σ_f^* (0.46 ± 0.03) suggest that the variability in the number of GFP over the population of spH vesicles was a significant contributor to $\{F_j\}$. Figure 2B shows the variation in the number of GFP over the population of single spH vesicles found from the deconvolution of $\{F_j\}$ with $\{F_i\}$. The distribution demonstrated a similar polydispersed nature that we observed previously when quantifying the number of synaptobrevin 2 (VAMP 2) proteins in synaptic vesicles isolated from rat brains.¹⁰ However, the average number of GFPs we found in spH vesicles is 4.4 ± 0.4 which is approximately 60% lower than the average number of VAMP 2 found in rat wild type synaptic vesicles. Previous characterization of this spH vesicles showed that the expression of VAMP 2 was found to be nearly the same concentration between mouse wild type synapses and synapses containing spH vesicles.¹² Therefore, there are two potential possibilities that could contribute to the difference in the number of GFPs (assuming a single GFP is expressed per VAMP 2): (1) the difference in the level of VAMP 2 expressed between rats and mice vary, or (2) not every VAMP 2 within a single synaptic vesicle expresses a GFP.

CONCLUSION

Using photobleaching to extract single fluorophore intensities, we were able to demonstrate how quantitative microscopy could be used to determine the average number of fluorophores and the variation in that number for samples where environmental effects on the photophysical properties of the fluorophore make it difficult to obtain an accurate single intensity for calibration. In cellular and molecular biology, an increasing number of fluorophores are being expressed in a variety of biological systems as biosensors to help develop a better understanding of the biological processes. We feel this technique will help in the characterization of biological systems expressing a wide variety of environmental sensitive fluorophores that before could not easily be characterized.

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Figure 1.

(A) Photobleaching track of GFP in a single synaptopHluorin vesicle. The initial intensity, F_i , was from the first frame of the photobleaching track. (B) Photobleaching track tail enlargement. Blinks are denoted by (*). The photobleaching track was terminated after the punctum was no longer observed for a designated number of frames ($n_{c0} = 10$ frames). Once the track was terminated, the final intensity, F_f , was extracted from the photobleaching track. (C) Initial and final intensity distributions ($\{F_i\}$ and $\{F_f\}$) for all acceptable vesicles in a single photobleaching series. (D) Semi-log cumulative probability plots of $\{F_i\}$ and $\{F_f\}$ showing the conservation of the distribution shape during bleaching.

Figure 2.
(A) Intensity distribution of spH vesicles with the best fit of single GFP found from photobleaching. (B) Variation in the number of GFP expressed in spH vesicles.