Counting tagged molecules one by one: Quantitative photoactivation and bleaching of photoactivatable fluorophores

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Determining the number of molecules in a given assembly, such as the number of proteins in a toxic aggregate, is often critical to understanding chemistry and function. Herein, we report a variation of a limitless method for counting photoactivatable fluorescent dyes in which single dye molecules are photoswitched to a fluorescent state, counted, and then irreversibly photobleached. We use this method to count the number of CAGE 552 covalently bound to the surface of 500 nm polystyrene beads. Activation of CAGE 552 was achieved with a 405 nm laser pulse. Once activated, the dye was excited with 532 nm light, and the fluorescence emission was collected with a CCD camera. The results from the fluorescence experiments were then compared to bulk fluorescence measurements to assess the error in counting. There are other ways of counting molecules, such as photobleaching and statistical analysis of reversible switchable chromophores. The method reported here provides a lower bound to the number of chromophores, with no upper limit to the number of molecules that can be quantified. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4929991>]

INTRODUCTION

Knowing the number of molecules in a given subensemble is often a critical quantity for understanding function. For example, the number of antibiotic polypeptides determines the size of pores formed in membranes, and, in amyloid aggregation, the number of proteins that nucleate into pre-fibrillar oligomers is needed to understand fiber formation. There exist many methods for determining numbers of molecules, including biochemical methods such as gel electrophoresis, crosslinking, and mass spectrometry.^{1–3} By counting photobleaching steps, fluorescence microscopy can also be used to determine the number of molecules.^{4–7} For example, if an aggregate consists of three fluorescent proteins, then under constant illumination, three steps should be observed in a time trace corresponding to a photobleaching event for each chromophore. Statistical modeling of the time traces has been used to count up to 30 molecules,⁸ but more typically about 7–10 molecules can be counted due to the dynamic range and inherent noise of single molecule spectroscopy.^{9–11}

Photoactivatable fluorophores provide a way to surmount the dynamic range limitation and can, in principle, be used to count arbitrarily large numbers of molecules.^{12–17} Two light sources are needed for photoactivatable dyes. The dyes are not fluorescent until the first light source causes some sort of chemical reaction, such as a *cis*-trans isomerization, charge transfer reaction, or bond cleavage. A second light source, usually longer in wavelength than the first, induces fluorescence emission of the new state. By keeping the intensity of the activation light source low, a very small subset of the fluorophores can be activated at one time. Thus, unlike an ensemble fluorescence measurement, photoactivatable dyes measure single

fluorophores even when there are thousands of other chromophores present that would create a background in typical fluorescence experiments. Photoactivatable dyes are used in new super-resolution fluorescent microscopies like photoactivation localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) in which the centroid of each individual molecule is carefully measured and localized in order to obtain images below the diffraction limit.^{18–21}

In some PALM studies, the number of photoactivations was used to determine size of clusters.^{22–24} PALM chromophores can be activated many times, and so statistical analysis is necessary to interpret overcounting as well as the photophysics of photoactivatable fluorophores including fluorescence intermittency (or blinking), which depends on the conditions of the experiment such as pH, excitation light intensity, and amount of oxygen present in the sample.^{25,26} Bustamante and coworkers recently used photoactivatable fluorescent proteins to quantitatively count proteins in a cell.^{27,28} In their work, they fluorescently labeled the bacterial flagellar motor protein FliM with Dendra2, a photoactivatable protein that undergoes a structural change at the chromophore-forming triad His-Tyr-Gly. Although the photoconversion of Dendra2 is irreversible, the activated fluorescent protein intrinsically blinks due to a long-lived dark state, leading to overcounting of fluorophores.^{29,30} To correct for overcounting, Bustamante and coworkers optimized the blinking tolerance time interval in multiple iterations. They counted 33 ± 1 proteins, which is the number of proteins established by electron microscopy. In another example, they counted up to 200 molecules in a single spot with a 6% bias.²⁷ Other statistical analysis methods, such as PC-PALM (pair correlation photoactivation localization microscopy), which utilize a pair correlation algorithm, have also been shown to account for overcounting from blinking.^{31–33}

Our counting scheme uses an irreversible, rather than a reversible, chromophore. The method is illustrated in

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FIG. 1. Photoactivation and detection cycle. The 405 nm and 532 nm diode lasers were not on simultaneously. In the fluorescence images, by comparing the images in the first column (before activation) and those in the second column (after activation), we can avoid overcounting fluorophores with long-lived fluorescence lifetimes.

Fig. 1. The sample is illuminated with a short activation pulse (405 nm) that is low enough in intensity that only a few fluorophores in the active area of the beam will be activated at the same time so that their fluorescent signals are not spatially overlapped. After the first laser pulse, the second laser source (532 nm) starts to irradiate the sample so that the activated chromophore fluoresces. The fluorescence is then localized and counted. The second laser continues to irradiate the sample until the activated fluorophores are irreversibly photobleached, after which the first light source is applied again to activate another set of fluorophores, which are subsequently counted. This procedure is followed until no more molecules are activated or until the data can be statistically extrapolated to final values. If the sample consists of aggregates of some sort, then one would expect that the final data would consist of spatially arranged clusters and the number of fluorophores in each cluster would be known. To avoid overcounting from blinking, we do not count signals that occur at the same spatial location between any two adjacent activation laser pulses.

In this work, we make benchmark measurements to determine the accuracy of our pulsed method for counting photoactivatable fluorophores. We covalently attach the organic photoactivatable dye, CAGE 552 (Abberior), to 0.5 μm polystyrene beads. CAGE 552 is initially non-fluorescent until it is photoactivated by 405 nm light, which induces the photolysis of the diazo group. The subsequent Wolff rearrangement of the ketene to the major product allows for absorption of the 532 nm light, and, consequently, fluorescence emission centered around 574 nm.³⁴ The photoactivation process is irreversible because covalent bonds are broken. Because the activated chromophore is irradiated until it permanently photobleaches, each fluorophore will only be counted once. Irradiation of the caged compound results in the fluorescent major product 75% of the time, while the non-fluorescent ketone forms 25% of the photolysis products.³⁴ To benchmark the accuracy of the counting, we calibrate the dye-loading on the beads with ensemble measurements. Silica and polymeric nanospheres containing fluorescent dyes have been used for markers in various biomedical assays.^{35,36} In previous photobleaching studies of nanospheres containing fluorescent dyes, fewer than 10 dyes could be counted.^{9–11} In this work, the upper counting range is limitless: We demonstrate by counting as many as 60 dyes on a single bead.

EXPERIMENTAL METHOD

Materials

CAGE 552 (1 mg, NHS ester) was purchased from Abberior (Göttingen, Germany), dissolved in anhydrous dimethyl sulfoxide (DMSO), flash-frozen, and kept at -20°C in the dark until use. Amine functionalized polystyrene beads in de-ionized (DI) water (0.53 μm , 1.2×10^{13} beads/g) were purchased from Bangs Laboratories, Inc. UltraPureTM 1M Tris-HCl, and pH 7.5 was obtained from Invitrogen. 3-aminopropyltriethoxysilane (APTES), ethanol, nitric acid, biotin *N*-hydroxysuccinimide ester, ethanolamine, and sodium borate were purchased from Sigma-Aldrich and used without further purification. In preparation of all aqueous solutions, deionized water (18 M Ω -cm) from a Millipore Milli-Q System was used. Glass coverslips (No. 1.5) and glass slides were purchased from Fisher Scientific and cleaned by sonication in Alconox solution, acetone, and 1M KOH solution sequentially.³⁷

Instrumentation

Single-molecule fluorescence imaging was performed on an Olympus IX-71 microscope. Fluorescence of the labeled beads were collected with an Olympus UPlanSApo 100x, 1.4-NA oil-immersion objective lens and imaged onto an electron multiplying CCD camera (Andor Ixon DU897E) after passing through a 605/70 bandpass filter (ET605/70, Chroma). A 405 nm diode-pumped laser (World Star Tech) was used for the photo-activation of CAGE 552. A 532 nm diode-pumped laser (Crystalaser) was used for excitation of activated form of the fluorophore. The switching on and off of the laser beams were controlled with mechanical shutters (SH05, Thorlabs). Quarter-wave plates (Thorlabs) were placed in the middle of the laser lines to convert the plane polarized light into circularly polarized light. They were combined on a dichroic mirror (z532/bcm, Chroma) and sent to the back port of the microscope which is set up for epifluorescence illumination. A piezoelectric objective lens positioner (Nano-F100, MadCity Labs) was used for correcting focal drift of a sample. Single-molecule fluorescence movies were acquired with Andor's Solis software and analyzed with home-built Matlab codes. For the ensemble experiments, fluorescence intensities of CAGE 552 solutions were measured with a plate reader (EnVision; Perkin Elmer) after photoactivation by a UV lamp for 10–15 min.

Labeling procedure for the polystyrene beads

1.2 ml of 0.5 μm polystyrene beads (10% wt.) were washed by centrifugation at 6000 rpm for 5 min with 1 ml DI water 3 times and divided into five of 200 μl aliquots. One of these aliquots was used for quantification after being dried. 50 mM sodium borate buffer (pH 8.5) was added to the other bead suspensions followed by addition of CAGE 552 solutions containing 10-, 20-, 30-, and 40-fold molar excess of the dye over the number of beads. The reaction mixtures were gently vortexed at room temperature for 5 h in the dark. Following the labeling reaction, 1 μl of 10 mM biotin *N*-hydroxysuccinimide ester was added to the labeled bead

suspension to biotinylate the beads. To quench the reactions, 1 μ l of 100 mM ethanolamine solution was added to each tube. The labeled beads were washed six times with 200 μ l of DI water and kept from light in a 4 °C refrigerator until the measurement. The DI water was collected from all six washes in order to quantify the amount of unreacted dyes and thereby determine the average number of dyes bound to the beads.

Preparing a sample chamber containing the labeled beads

Since the imaging data on each spot were acquired over several minutes, we immobilized the beads onto the coverslip. The bead positions were also used to correct for drift of the microscope stage. To immobilize the biotinylated beads, 30 μ l of biotin-BSA solution (2 mg/ml) was injected to each sample chamber and incubated for 10 min. Then, the sample chambers were washed with 2 ml of Tris buffer (pH 7.5), filled with streptavidin solution (1 mg/ml), and washed with the buffer again. The labeled bead samples, ranging in dye-to-bead ratios of 10:1, 20:1, 30:1, and 40:1, were added to the streptavidin-coated sample chambers. After 10 min of incubation, the sample chamber was flushed with buffer and sealed with epoxy. All the processes involving labeled samples were done under dim red light to minimize photoactivation of the dye before the counting experiments.

The beads are also used to correct for drift of the microscope stage and to illustrate the counting methodology. The positions of the beads were determined by fitting images of each bead with a 2-D Gaussian as measured by transmitted white light images. The average size of the beads was ~ 0.5 μ m, in agreement with the manufacturer. Drift was corrected every 5 min. A cutoff filter (RG610, Chroma) was used to prevent the white light from activating the CAGE 552 fluorophores.

Measuring the number of fluorescence activations

The sample chambers containing the labeled beads were transferred to the microscope. Due to the spherical shape of the beads and their average size, the focal plane did not encompass the entire bead. When a fluorophore is out of focus, its fluorescence image has a different shape from a well-focused point spread function depending on the distance and orientation of the molecule.^{38,39} We used a similar algorithm to detect single molecules on focus for our measurement, because imaging a single molecule at less than ± 250 nm off from its true *z*-position does not deform a shape of the image. Thus, we set the distance between the sample and the objective to focus on the bead equator. To achieve the distance, we detected the inner surface of the coverslip first, and from that position, we moved up the objective positioner (Mad City Labs, Nano-F100) by 250 nm, which is the average radius of the beads. A central area of the CCD camera (300 $\times$ 300 pixels) was chosen for collecting data, since the illumination from the lasers was very uniform on the area.

We performed single molecule detection and quantification with photoactivatable fluorophores in a similar way as PALM super-resolution imaging (Fig. 1). Initially, there were few fluorescent spots, which were expected since the dye molecules are currently caged and non-fluorescent. Then, a 405 nm

laser pulse was given to the sample to uncage a small population of the dyes, allowing for excitation and, subsequently, fluorescence emission. The power of the 405 nm laser was controlled to make the average number of activated molecules within a diffraction-limited area less than 0.5, unless otherwise stated. Within a few seconds under 532 nm laser irradiation, the fluorescent spots became dark due to photobleaching and we waited for 20–30 s between activations for the majority of the dyes in the imaging area to photobleach out. We then repeated these steps until we could not detect any more fluorescence activations in the imaging area (~ 40 –120 cycles). We then quantified the number of the dye molecules by counting the number of photoactivation events in the activation and detection cycles.

RESULT AND DISCUSSION

Counting method and proof of principle

Figs. 2(a)–2(e) are a series of images that illustrate two full activation and counting cycles. Shown in Fig. 2(a) is

FIG. 2. Transmitted white light and fluorescence images of dye-labeled beads. (a) Transmitted light image of the beads on an imaging is (b) fluorescence image on the same area before an activation pulse was given. Almost no fluorescent spots were detected. (c) Fluorescence image on the same area after the activation. The arrows indicate the positions of the beads that have activated dyes on their surface. (d) Fluorescence image taken 10 s after the activation pulse arrived. All the activated fluorophores were photobleached and are now dark. Fluorescence images (e) before and (f) after an activation pulse are compared. Following the photobleaching of the fluorophores, another activation pulse illuminates the sample, creating three newly activated chromophores. In the next iteration of the photoactivation and detection cycle, the fluorophores that did not bleach out (shown with arrows) are not counted. Scale bars are at 5 μ m.

FIG. 3. Fluorescence lifetime of CAGE 552. Fluorescence of CAGE 552 with 532 nm excitation was measured at every 500 ms after activation (black square). The solid line is a fit by a single exponential with a time constant of 1.71 ± 0.07 s.

an image of a collection of beads that have an average of 31.5 fluorophores per bead, as measured from ensemble fluorescence measurements described previously. The image was taken with white light illumination, which is possible with $0.5 \mu\text{m}$ beads. Fig. 2(b) shows the fluorescence image of the same area, before activation of the dye molecules by 405 nm light. This image is representative of the background noise level inherent to the microscope. In Fig. 2(c), a fluorescent image is shown that is collected 100 ms after the 405 nm activation pulse was applied. Bright spots were observed at some of the locations of the beads, due to fluorescence from an activated dye on the bead. The positions of all the fluorescent spots above a certain threshold are determined by fitting to a 2D Gaussian. Each of these spots is recorded as a count. After 5 s of irradiation by 532 nm light, the fluorescent spots are now deactivated due to irreversible photobleaching as shown in Fig. 2(d). The fluorescent lifetime of the activated dye is 1.71 ± 0.07 s, as determined by ensemble measurements shown in Fig. 3. Thus, there is a 99.7% certainty that the activated dyes are irreversibly photobleached after 10 s of irradiation. The next activation pulse at 405 nm is then applied and the positions of the new fluorescent spots measured and counts added. If a count appears with the same spatial position in the image prior to activation (Fig. 2(e)), then it is not counted because in all probability it is a fluorophore from the previous activation cycle that has not fully photobleached. The activation, counting, and photobleaching steps are applied until there are no more observable photoactivations or the count rate has slowed enough that the final results can be extrapolated. Shown in Fig. 4 is a plot of the final count after 100 activations for the sample discussed above. Notice that for some beads, more than 40 fluorophores were counted, which exceeds methods that use photobleaching, thus illustrating the efficacy of a method that utilizes activation rather than bleaching, and thereby reducing the background.

We note that precise positioning is not the purpose of the measurement, unlike PALM or STORM microscopies.

FIG. 4. 3-D histogram of the counts on the beads (for 40:1 dye to bead sample) above the transmitted light image of the beads. The height of the bars corresponds to the counts on the positions.

The primary purpose is to simply observe a fluorescing dye, and so one does not need high signal-to-noise fluorescence spots. Our method benefits from high signal-to-noise which improves the counting statistics (discussed below) and would enable clusters of dyes to be counted at distances smaller than the diffraction limit. We also note that neither white light imaging nor the positions of the clusters need to be known *a priori*. White light is used here for illustration purposes, but identification of clusters comes about from the measurement, as computed by statistical significance below. For the 10:1 dye to bead sample, we counted 3.4 ± 2.2 dye molecules. Likewise, we counted 5.7 ± 1.5 , 8.4 ± 3.7 , and 13.6 ± 4.2 dye molecules for the 20:1, 30:1, and 40:1 dye to bead samples, respectively (Fig. 5).

Statistical analyses

The above counting scheme uses a simple intensity cutoff for whether a fluorescent spot should be considered as a count

FIG. 5. Distribution of the counts from each sample was fitted with a Poisson distribution. The means of the counts in 10:1 (a), 20:1 (b), 30:1 (c), and 40:1 (d) dye to bead ratio samples were 3.4 ± 2.2 , 5.7 ± 1.5 , 8.4 ± 3.7 , and 13.6 ± 4.2 , respectively.

FIG. 6. Counting efficiency changes upon setting different thresholds. When the threshold level dropped from 25 to 20, the counts increased more rapidly than when it dropped from 30 to 25, which implies that to set a threshold level at 20 would be less accurate because the background counts are no longer non-zero.

and not a noise event. In this section, we consider a few simple methods for improving the counting accuracy.

Results from a fixed threshold

In the simplest analysis, a count was recorded for pixels whose intensities were above a certain threshold. The threshold level is necessary to discriminate against noise. Shown in Fig. 6 are the counting results for three threshold levels for four samples of beads labeled with increasing numbers of dyes. For each of the four samples, ensemble measurements were performed to calculate the average number of chromophores per bead, as described above (Fig. 7, squares). At a threshold

FIG. 7. Average number of dye molecules determined by different methods. The blue squares show the number of fluorophores for a given sample obtained using ensemble fluorescence measurements while the red circles are the number of fluorophores counted using single-molecule counting. The numbers from the fluorescence intensity measurement are more than two times larger than the numbers from the single-molecule counting.

of 30 (arbitrary units), which has the highest discrimination against noise, about 37% of the beads are counted (e.g., 11.7 out of 31.5 counts for the 40:1 dye to bead sample). Moreover, none of these counts are from noise (see “background” column of plot), which can be measured since we know the positions of the beads from the transmitted light images. At a threshold of 25, about 43% of the beads are counted, again with no background counts. However, at a threshold of 20, the background counts are now non-zero, meaning that they contribute to the counts in the dye-containing samples. Thus, at a value that gives zero background counts, a flat threshold provides a counting accuracy of 43%. Of course, some molecules will lie below the threshold and not be counted. As a result, a fixed threshold with no background provides a lower limit to the true number of dyes.

Results from an intensity profile analysis that gives probabilities

The difference between the measured and known number of dyes for the threshold method is largely due to variation in the fluorescence intensity of the dyes from molecule to molecule. Many factors will contribute to their brightness, including their local dielectric constant and orientation with respect to the laser field. In our sample, the fluorescence intensities of the single fluorophores are often not much greater than the dark counts, which is illustrated in Fig. 8(b) (solid line). Shown in Fig. 8(a) is a histogram in which we randomly chose 200 areas of 32 pixels where no beads were located. For each area, the average pixel intensity was calculated. Some of the intensities are below zero because a baseline was subtracted from the same pixels in the absence of an activation event, which accounts for variations in the excitation laser profile. The background distribution is fitted to a normal distribution. Shown in Fig. 8(b) is another histogram (shown in dashed lines) but generated solely from 32 pixels surrounding each of the beads, so that it will contain signals from both the dyes and background. We want to subtract the two distributions, to obtain the distribution of intensities of the dyes (Fig. 8(b), dotted). To do so, we assume that at intensities below 0, there are no contributions from fluorescence of the activated dye and scale the background intensity over the intensity range of -20 to 0 . Subtracting the normalized intensity distributions produces the distribution of the fluorescence intensity from an activated dye. The dye distribution is not symmetric about zero, with a tail to very high intensities, which is expected.

The dye intensity distribution enables us to predict the probability that a given fluorescence signal originates from a dye or background, as given by the ratio of the distributions shown in Fig. 8(c). Using this plot, all the measured intensities can be converted to the probabilities that there is an activation of the dye.

Unlike the fixed threshold method, which simply yields a count for the dye, the above ratio provides a probability that this count is real. For a series of activation pulses, therefore, it is necessary to compound the probabilities of each of the activation pulses to generate a probability distribution of dye counts. For example, analyzing the same data set as for the

FIG. 8. Analysis of counting data without using a threshold. (a) Distribution of dark counts measured with the shutter off from the 40:1 dye to bead sample. (b) Distribution of detected fluorescence intensities on each bead can be separated into that of background intensities and that of activated single molecule fluorescence intensities. (c) Each of the single molecule fluorescence intensities detected in each measurement on each bead can be converted to the probability that there is an activation of a dye molecule. (d) Using the intensity probability distribution, the probability distributions of having a certain number of dyes on each bead were calculated. (e) Overall probability distribution is acquired from averaging the probability distributions. (f) The counts obtained using the thresholding method (blue circles) to the intensity profile analysis (red triangles) are compared to the ensemble measurement (black squares). The threshold method severely undercounts the number of dyes, while the counts from the intensity ratio analysis are considerably closer to the control experiment.

fixed threshold analysis above gives Fig. 8(d), which plots the probabilities that for each bead to contain a fixed number of dyes. For the most part, each bead has the same distribution. For each bead, the maximum probability is about 25% for N counts, 19% for $N \pm 1$, and 11% for $N \pm 2$. Averaging these distributions produces Fig. 8(e) gives the probabilities for the ensemble, whose values are plotted in Fig. 8(f) for each of the four samples. For the sample with the 40:1 dye to bead ratio, this intensity ratio analysis puts the number of dyes on each bead at 21.0 ± 4.0 , as compared to the 13.6 for the ensemble average with the threshold value method and thus considerably closer to the control experiments of 31.5.

Correcting for under counting by simultaneous activations

The analyses above assume that each fluorescence spot corresponds to a single activated dye, at extremely low laser fluences that are true. However, low fluences also require extended amounts of data collection time. In the above experiments, the activation laser was set so that there was about a 33% for any individual dye to be activated. (Hence, 6 of the 18 locations of beads marked with arrows in Figs. 2(a) and 2(c) are activated.) That means that there is about a 10% chance for two dyes to be activated on the same bead. In the methods above, such occurrences will result in a single count, resulting in an overall undercounting of the dyes per bead. Each of the above experiments took 3 h to collect. If half the laser fluence was used, data collection would have taken 6 h and there would be 3% double activations. Alternatively, undercounting can be corrected statistically by simply adding in the number of counts due to double activations. Thus, for the 40:1 dye to bead ratio sample, the number of counts should be increased by 1.4 and 2.1 for the threshold and ratio methods, respectively, giving total counts of 15.0 and 23.1, respectively.

Another way to correct for double activations is to look for photobleaching steps, like the traditional way of counting single molecules is done. That method is difficult due to signal-to-noise noise and potential blinking. Thus, we used the statistical method above.

CONCLUSIONS

We have shown that we can count to very high numbers using irreversibly activated photoswitchable dyes. Because the method has no background from other chromophores, there is no upper limit, in principle, to the number of molecules that can be counted. Several statistical analysis methods were reported and discussed, and the results were compared to controls using ensemble measurements. The most sophisticated method counted 23.1 out of 32.5 dyes per bead, on average. Thus, the counting efficiency is about 72%. Not all dyes are expected to be photoactivatable due to their environment or poor orientations for either absorbing the photoactivation or fluorescence photons. Oxidation in the sample or preparation steps will also increase the number of inactivatable dyes. Nonetheless, the data can be analyzed to

provide a lower limit to the number of fluorophores. Having a lower limit is a very different result from statistical analyses of PALM measurements, in which each chromophore is activated 10 or more times. Our method is much simpler in that we do not need to know the kinetic rates of emission, blinking, and photobleaching, and it is applicable to small molecules whose photophysics might vary from sample to sample. While we used several simple methods for counting, the counting efficiency might be improved with more sophisticated data analyses, such as pattern recognition,⁴⁰ that would raise the lower limit. Even so, we can already calculate the probability of an aggregate at a particular location. Interestingly, it follows that the optimal dyes for irreversible counting will not be the same as for typical single molecule experiments, because our method works best with dyes that have short lifetimes and blinking is not so important, whereas most single molecule experiments that sample trajectories require long lifetimes with little or no blinking.

Our method is applicable to some interesting scientific problems. For example, many proteins aggregate into amyloid fibers through a nucleation process that involves an oligomeric state. One of the critical parameters for understanding this phenomenon is knowing how many proteins are involved in the oligomer, because the number is necessary to analyze the aggregation kinetics. Another example is pore-forming peptides, such as antibiotics, in which the number of peptides associated will dictate the size of the membrane-spanning pore. In cases like these, knowing the number of proteins involved, even without any structural information, is a very important step to solving the scientific problem. And many of these amyloid and antibiotics are polypeptides and thus small chromophores for counting would be necessary. The methodology reported here should help in exploring the scientific questions for which standard photobleaching or switchable proteins is not applicable.

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¹M. M. Garner and A. Revzin, *Nucleic Acids Res.* **9**(13), 3047–3060 (1981).

²V. C. Lai, R. Guan, M. L. Wood, S. K. Lo, M. F. Yuen, and C. L. Lai, *J. Clin. Microbiol.* **37**(1), 161–164 (1999).

³Y. Oda, K. Huang, F. R. Cross, D. Cowburn, and B. T. Chait, *Proc. Natl. Acad. Sci. U. S. A.* **96**(12), 6591–6596 (1999).

⁴P. D. Simonson, H. A. Deberg, P. Ge, J. K. Alexander, O. Jeyifous, W. N. Green, and P. R. Selvin, *Biophys. J.* **99**(10), L81–L83 (2010).

⁵M. H. Ulbrich and E. Y. Isacoff, *Nat. Methods* **4**(4), 319–321 (2007).

⁶W. Ji, P. Xu, Z. Li, J. Lu, L. Liu, Y. Zhan, Y. Chen, B. Hille, T. Xu, and L. Chen, *Proc. Natl. Acad. Sci. U. S. A.* **105**(36), 13668–13673 (2008).

⁷H. Ding, P. T. Wong, E. L. Lee, A. Gafni, and D. G. Steel, *Biophys. J.* **97**(3), 912–921 (2009).

⁸T. C. Messina, H. Kim, J. T. Giurleo, and D. S. Talaga, *J. Phys. Chem. B* **110**(33), 16366–16376 (2006).

⁹D. Casanova, D. Giaume, M. Moreau, J. L. Martin, T. Gacoin, J. P. Boilot, and A. Alexandrou, *J. Am. Chem. Soc.* **129**(42), 12592–12593 (2007).

¹⁰F. Delport, A. Deres, J. Hotta, J. Pollet, B. Verbruggen, B. Sels, J. Hofkens, and J. Lammertyn, *Langmuir* **26**(3), 1594–1597 (2010).

¹¹A. Bumb, S. K. Sarkar, X. S. Wu, M. W. Brechbiel, and K. C. Neuman, *Biomed. Opt. Express* **2**(10), 2761–2769 (2011).

¹²K. R. Gee, E. S. Weinberg, and D. J. Kozlowski, *Bioorg. Med. Chem. Lett.* **11**(16), 2181–2183 (2001).

- ¹³G. H. Patterson and J. Lippincott-Schwartz, *Science* **297**(5588), 1873–1877 (2002).
- ¹⁴M. Bates, T. R. Blosser, and X. Zhuang, *Phys. Rev. Lett.* **94**(10), 108101 (2005).
- ¹⁵M. Heilemann, E. Margeat, R. Kasper, M. Sauer, and P. Tinnefeld, *J. Am. Chem. Soc.* **127**(11), 3801–3806 (2005).
- ¹⁶S. J. Lord, N. R. Conley, H. L. Lee, R. Samuel, N. Liu, R. J. Twieg, and W. E. Moerner, *J. Am. Chem. Soc.* **130**(29), 9204–9205 (2008).
- ¹⁷J. C. Vaughan, S. Jia, and X. Zhuang, *Nat. Methods* **9**(12), 1181–1184 (2012).
- ¹⁸E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, and H. F. Hess, *Science* **313**(5793), 1642–1645 (2006).
- ¹⁹S. T. Hess, T. P. Girirajan, and M. D. Mason, *Biophys. J.* **91**(11), 4258–4272 (2006).
- ²⁰B. Huang, W. Wang, M. Bates, and X. Zhuang, *Science* **319**(5864), 810–813 (2008).
- ²¹H. L. D. Lee, S. J. Lord, S. Iwanaga, K. Zhan, H. X. Xie, J. C. Williams, H. Wang, G. R. Bowman, E. D. Goley, L. Shapiro, R. J. Twieg, J. H. Rao, and W. E. Moerner, *J. Am. Chem. Soc.* **132**(43), 15099–15101 (2010).
- ²²D. Greenfield, A. L. McEvoy, H. Shroff, G. E. Crooks, N. S. Wingreen, E. Betzig, and J. Liphardt, *PLoS Biol.* **7**(6), e1000137 (2009).
- ²³D. Baddeley, I. D. Jayasinghe, L. Lam, S. Rossberger, M. B. Cannell, and C. Soeller, *Proc. Natl. Acad. Sci. U. S. A.* **106**(52), 22275–22280 (2009).
- ²⁴B. F. Lillemeier, M. A. Mortelmaier, M. B. Forstner, J. B. Huppa, J. T. Groves, and M. M. Davis, *Nat. Immunol.* **11**(1), 90–96 (2010).
- ²⁵R. M. Dickson, A. B. Cubitt, R. Y. Tsien, and W. E. Moerner, *Nature* **388**(6640), 355–358 (1997).
- ²⁶T. Ha and P. Tinnefeld, *Annu. Rev. Phys. Chem.* **63**, 595–617 (2012).
- ²⁷S. H. Lee, J. Y. Shin, A. Lee, and C. Bustamante, *Proc. Natl. Acad. Sci. U. S. A.* **109**(43), 17436–17441 (2012).
- ²⁸G. C. Rollins, J. Y. Shin, C. Bustamante, and S. Presse, *Proc. Natl. Acad. Sci. U. S. A.* **112**, E110 (2014).
- ²⁹N. C. Shaner, G. H. Patterson, and M. W. Davidson, *J. Cell Sci.* **120**(24), 4247–4260 (2007).
- ³⁰V. Adam, K. Nienhaus, D. Bourgeois, and G. U. Nienhaus, *Biochemistry* **48**(22), 4905–4915 (2009).
- ³¹P. Sengupta, T. Jovanovic-Talisman, D. Skoko, M. Renz, S. L. Veatch, and J. Lippincott-Schwartz, *Nat. Methods* **8**(11), 969–975 (2011).
- ³²P. Sengupta, T. Jovanovic-Talisman, and J. Lippincott-Schwartz, *Nat. Protoc.* **8**(2), 345–354 (2013).
- ³³S. L. Veatch, B. B. Machta, S. A. Shelby, E. N. Chiang, D. A. Holowka, and B. A. Baird, *PLoS ONE* **7**(2), e31457 (2012).
- ³⁴V. N. Belov, C. A. Wurm, V. P. Boyarskiy, S. Jakobs, and S. W. Hell, *Angew. Chem., Int. Ed. Engl.* **49**(20), 3520–3523 (2010).
- ³⁵L. Wang, K. M. Wang, S. Santra, X. J. Zhao, L. R. Hilliard, J. E. Smith, J. R. Wu, and W. H. Tan, *Anal. Chem.* **78**(3), 646–654 (2006).
- ³⁶D. K. Chatterjee, M. K. Gnanasammandhan, and Y. Zhang, *Small* **6**(24), 2781–2795 (2010).
- ³⁷P. R. Selvin and T. Ha, *Single-Molecule Techniques: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, c2008, Cold Spring Harbor, NY, 2008).
- ³⁸A. P. Bartko and R. M. Dickson, *J. Phys. Chem. B* **103**(16), 3053–3056 (1999).
- ³⁹E. Toprak, J. Enderlein, S. Syed, S. A. McKinney, R. G. Petschek, T. Ha, Y. E. Goldman, and P. R. Selvin, *Proc. Natl. Acad. Sci. U. S. A.* **103**(17), 6495–6499 (2006).
- ⁴⁰J. C. Olivo-Marin, *Pattern Recognit.* **35**(9), 1989–1996 (2002).