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Quantitative Microscopy based on Single-Molecule Fluorescence

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Abstract

Quantitative microscopy is needed to understand reactions or phenomena carried out by biological molecules such as enzymes, receptors, and membrane-localized proteins. Counting the biomolecules of interest in single organelles or cellular compartments is critical in these approaches. In this brief perspective, we focus on the development of quantitative fluorescence microscopies that measure the precise copy numbers of proteins in cellular organelles or purified samples. We introduce recent improvements in quantitative microscopies to overcome undercounting or overcounting errors in certain conditions. We conclude by discussing biological applications.

Introduction

Quantitative biological measurements have become increasing important. To address this need, new biochemical and biophysical methods have been introduced, while conventional approaches have been significantly advanced. For example, mass spectrometry has become ever more sensitive, and traditional workhorses of the biochemistry lab, such as Western Blotting, has been greatly improved for ascertaining the relative amounts of proteins present. Despite these improvements, bulk methods suffer from two inherent limitations: 1) Bulk measurements can only provide information about the average value, but not information regarding the distribution about the average (i.e. heterogeneity); and 2) Bulk approaches usually require calibration and the use of a standard or internal reference, and thus are relative measurements and do not offer absolute quantification. These two limitations can be overcome using single-molecule counting, which often is based on fluorescence microscopy. As a result, this perspective article focuses on fluorescence imaging based approaches.

Fluorescence microscopy requires the use of an imaging agent, such as dye-tagged antibodies or the use of fluorescent proteins (e.g. green fluorescent protein, known as GFP)

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Conflict of Interests

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that co-express in cells with the proteins of interest. Conventional fluorescence microscopy, however, has a critical downside in accurately measuring the number of proteins of interest in small organelles (< 100 nm) because of the limit in optical resolution (> ~ 200 nm for lateral resolution and > ~ 500 nm for the z-direction). This resolution limit prevents conventional fluorescence microscopy from resolving individual molecules of interest for counting in cellular compartments. Although methods to calibrate the measured intensity in fluorescence microscopy are available (vide infra), these are difficult to be applied to quantify low-copy-number proteins due to the stochastic nature of fluorescence emission from a few molecules. Non-fluorescence based microscopy has also been attempted for counting molecules, such as the use of immuno-gold combined with electron microscopy. This method, in which antibodies labeled with gold are detected by electron microscopy, can measure nanometer-scale localization of proteins in cellular compartments; however, precise counting of proteins is difficult with this technique because the antibodies do not label all target proteins which cause inaccurate and large standard deviations in measurements.

Therefore, researchers have been motivated to develop a new technique to count protein copies and to overcome most of the limitations of conventional optical and biochemical methods. After single-molecule detection was realized [2, 3], it was soon recognized that this breakthrough would allow researchers to gain new perspectives on biomolecular mechanisms [4-11]. We focus here on quantitative microscopy based on single-molecule fluorescence that counts cellular materials.

Several quantitative microscopy methods have been developed to estimate protein copies in cellular compartments. The first approach used beads of known brightness that corresponded to a known copy number of fluorescent proteins after calibration [12]. It carefully estimated the number of several representative proteins in postsynaptic density. However, it only works well for large copy numbers of proteins, usually of more than a hundred, because of large standard deviations in measurement when low copy numbers are involved.

After discovery of localization by photobleaching [13], sequential photobleaching steps of single-molecule fluorophores were used to determine functional stoichiometry of proteins [8, 14] and to count the number of proteins [15]. But it is also limited by the difficulty of correlating the photobleaching step size with copy number, especially as the copy number increases, due to coincident photobleaching of single fluorophores, non-homogeneous brightness, and poor signal-to-noise [16*].

To address the above challenges in quantitative microscopy, we developed statistical deconvolution quantitative microscopy based on single-molecule fluorescence. This approach covered the range from a single to a few tens of copies of proteins [17*]. Soon after, researchers recognized that photoswitchable organic dyes [18] or photoactivable fluorescent proteins [19] were useful for localization microscopy with nanometer-scale precision to quantify protein numbers. However, a critical issue has been the blinking of fluorophores.

In this perspective, we discuss ways to quantify the absolute number of proteins in single organelles or cellular compartments and introduce recent developments and improvements

of single-molecule-based, high-resolution microscopies and their potential impact in biological studies.

Single-Molecule-based Statistical Deconvolution Quantitative Microscopy

Previously, researchers introduced counting methods for proteins of interest tagged with GFP by using single-molecule photobleaching microscopy (SMPM). Simple sequential photobleaching steps were used for determining the functional stoichiometry of membrane-localized proteins [8, 9, 14] and for counting protein copies [15]. However, copy numbers of more than 10 proteins in a punctum can be difficult to count by the photobleaching steps because multiple photobleaching steps, depending on the laser power, can occur simultaneously, although analytical tools have been proposed to address this issue [16*, 20, 21].

One potential limitation with using GFP is that co-expression of GFP with the proteins of interest may perturb the trafficking and expression level of the native protein, depending on the concentration of the native protein [12]. For crowded environments, such as the synaptic vesicle, we found the number of the protein VAMP 2 was lowered when tagged with GFP [22, 23]. To address this issue, fluorescent antibodies may be used to target and label the proteins of interest. In this case, however, care must be taken to remove any artifacts caused by non-specific binding of antibodies to off-target molecules in the specimen, which would introduce ‘overcounting’ errors; incomplete labeling of target proteins by antibodies would introduce ‘undercounting’ errors. Furthermore, it was difficult to apply single-molecule photobleaching to dye-tagged antibodies because each antibody had a different copy number of dyes [24]. There was no direct way to correlate the number of bleached dyes with the number of antibodies present unless a single dye was labeled to a single antibody [25], which was a difficult feat to accomplish.

In order to overcome these issues, as shown in Figure 1, we developed single-molecule-based statistical deconvolution quantitative microscopy combined with a microfluidic chamber to count copies of native proteins in individually purified ~ 40-nm-diameter synaptic vesicles (Figure 1a/b). The detailed analytical procedures are shown in Figure 1c-g [26**]. Our approach had several advantages: 1) Statistical deconvolution of fully-labeled samples compared with single-molecule-intensity distribution allowed us to estimate precisely the copy number of a protein of interest [17*]. 2) Our method did not need to consider the number of fluorophores on a single antibody [24] because it did not rely on photobleaching of fluorophores [17*]. 3) The method minimized the effect of incomplete labeling of antibodies to the target proteins because epitopes (antibody binding sites) were well exposed to the solution. 4) The microfluidic device allowed us to measure more than 10,000 small organelles per experiment. 5) We could minimize potential artifacts introduced by nonspecific binding of fluorescent-dye-labeled antibodies to the glass because the antibody labeling step was before the injection of sample into the chamber and because we required co-localization of different color antibodies targeted to the same synaptic vesicle for analysis [22, 26**].

These merits allowed us to count a broad range in the number of proteins, from single protein to a few tens of copies of a protein, in the single organelles or cellular compartments. In the case of synaptic vesicles, for example, we found that synaptophysin and VAMP2 to be among the more abundant proteins, with an average number of around 13 and 10.5 copies per vesicle, respectively. The more interesting finding was that some proteins are highly monodisperse in copy number while others are not. The observation brings up an interesting question of whether neurodegenerative pathophysiological conditions, like Alzheimer's disease, may change the degree of polydispersity of some of the synaptic vesicle proteins and thus affect neuronal communication in the brain. In addition to using dye-tagged antibodies, we also tested synaptic vesicles that expressed GFP [23]. After photobleaching of ~ 10 copies of GFP down to a single molecule of GFP to generate the single-molecule calibration, we counted the GFP number using the statistical deconvolution method as described above. While we applied our statistical deconvolution quantitative microscopy so far only to count proteins on isolated organelles, with the development of a photobleached internal standard [23], this method may be extended for use to count the number of proteins in individual puncta often observed in fluorescence microscopy of intact cells.

Single-Molecule Localization Microscopies (SMLM) for counting proteins

Localization microscopies are very powerful tools for visualizing and pinpointing the proteins of interest with nanometer-scale precision. They are especially useful for studying biological scaffold complexes or small organelles, whose sizes fall in the diffraction limit [18, 19, 27, 28, 29**]. This group of methods is based on the notion of photoswitchable single-molecule fluorescence and point-spread function localization: A single fluorophore will appear in an image as a blurred diffraction-limited spot, but the position of the fluorophore can be determined to within nanometers by performing centroid analysis, provided there aren't any neighboring fluorophores within the diffraction-limited spot. By sequentially turning "on" and "off" different subsets of fluorophores and by noting the position of each fluorophore, a super-resolution image can be generated. The resolution is not dictated by the diffraction limit, but by the number of photons collected for centroid analysis. Resolutions down to a few nanometers can be achieved, albeit 10-20 nm is more common. To estimate accurate protein compositions using stochastic optical reconstruction microscopy (STORM) or direct STORM, however, issues of undercounting, caused by incomplete labeling by antibodies, and overcounting, caused by blinking ('reversible transition from the fluorescent state into the non-fluorescent dark state') of dyes on antibodies [18, 30], need to be accounted for and corrected. Furthermore, multiple fluorescent dyes on individual antibodies generated low precision for counting of proteins of interest because of an increase in the standard deviation. Similarly, for photoactivated localization microscopy (PALM), it is necessary to consider overcounting issues caused by blinking of photoactivatable proteins in sparsely distributed molecules in the imaging plane [31**] and undercounting issues by a population of nonfluorescent proteins due to misfolding of fluorescent proteins [12] and fluorescence mixing effect due to high molecular density [32**].

The simplest approach to correct the blinking events was to assume that blinking contributes linearly to the number of proteins of interest, e.g. double or triple counting by two or three

blinks of the same molecules before the final photobleaching step. Later, researchers recognized that the counting error could be exponentially reduced by tolerance time (or 'dark time') defined by a time to distinguish individual molecules. The tolerance time is an empirical parameter for analysis that depends on the off-time of blinking of individual fluorophores, the species of fluorophores, the excitation light power, and acquisition frame rate [31**]. For instance, if the tolerance time is shorter than the off-time ('dwell time of dark state") of the real blinking events, it can generate overcounting errors because the algorithm does not have enough time to distinguish between two different molecules. In other words, not only tolerance time but also the activated molecular density would be critical factors for minimizing counting errors of the SMLMs. How can we deal with those issues?

To address this question, Lee and coworkers found an elegant way to optimize tolerance time and photoactivation time to control the number of active molecules using a photokinetic model [32**] although a model-independent approach was recently proposed [33]. After simulation of blinking events with a four-state model — nonactive ('nonfluorescent'), active ('fluorescent'), dark, and photobleached — Lee and coworkers applied rate constants obtained from the blinking model to correct for the contributions of individual blinking events to minimize the standard deviation (Figure 2a). The most important kinetic parameter was the transition rate from the dark state to the active state, which provided the right tolerance time to correct for overcounting error (Figure 2b). To reduce fluorescence mixing effect by too many activated molecules at the same time, which could lead to a molecular undercounting error, they found an optimal activation time ('Fermi activation', Figure 2c). Furthermore, if the tolerance time was rapidly increased to reduce overcounting errors, the undercounting error could be increased by missing real events. To optimize tolerance time without any biased analysis (Figure 2d) to reduce uncertainty (Figure 2e), an iterative approach was chosen to balance the overcounting and the undercounting errors caused by blinking and the fluorescence mixing effect, respectively. Finally they counted 33 molecules of FliM protein per bacterial flagellar motor with a very small standard deviation.

Compared with the previous photokinetic-modeling-dependent methods, a different approach was proposed to calibrate the contribution of mEos2 (monomeric photoactivable fluorescent protein of Eos2) blinking to quantify lipid binding sites on single vesicles in yeast [34*]. Extracting photokinetic parameters sometimes can be tricky because of the complexity of blinking behaviors and the variation in individual fluorophores. Puchner and coworkers prepared a single mEos2-fused, membrane localizing phosphatidylinositol 3-phosphate (PI3P)-sensitive PH domain of phospholipase C delta (PLCδ), which was randomly distributed in vesicle membrane of yeast. As expected, they observed a pronounced peak of distance distribution with a single mEos2 tagging the PH probe. This meant that blinking events potentially contributed to the distance distribution of the probes as an artificial clustering of proteins derived by blinking of fluorophores [35*]. To remove the artifact by blinking, they analyzed the same data with a pair-correlation function so they could generate a random distance distribution of the PH probes (Figure 2f/g). Finally, Puchner and coworkers reported that endocytosed vesicles in cytoplasm increase ~ 100-fold of PI3P binding sites in relation to their fusion [34*].

Although it showed the way to reduce miscounting of the number of proteins, the SMLMs combined with stochastic switching were still not suitable to count with excellent precision and accuracy the number of proteins in densely clustered regions that have more than 100 protein copies. The variation of environment-dependent photophysics of fluorophores, e.g. blinking rate and number of photons emitted from individual fluorophores [30, 36], made the counting difficult. To overcome this limitation, a simple and robust quantitative super-resolution method called quantitative points accumulation in nanoscale topography (qPAINT) was developed [37**]. Instead of the stochastic switching, qPAINT achieved transient binding of free floating dye-labeled short DNA sequences (‘imager strand’) to complementary target DNA sequences (‘docking strand’) tagged onto secondary antibody which can be complexed with primary antibodies targeted to proteins of interest (Figure 3a). The binding rate of imager strands to a docking strand was proportionally increased as a function of the concentration of imager stand because of the second-order binding reaction (Figure 3b). So, Jungmann and coworkers could quantify the number of target strands on the proteins of interest after conversion of the binding rate of imager strands. A potential use of the method was to detect many, discrete single molecules of the nuclear pore complex protein (Figure 3c-d) and an even more densely packed molecular complex with more than 100 protein copies in synaptic active zone (Figure 3e-f). Because qPAINT was independent of the blinking kinetics of fluorophores and photobleaching, it avoided typical undercounting errors caused by already photobleached dyes or incomplete labeling of antibodies as well as overcounting errors caused by blinking artifacts. Major barriers for the qPAINT method are still dependent on stoichiometric labeling of protein targets and efficiency of target labeling as well as the accessibility of probe strand to the target DNA because of the secondary structure in the single strand target DNA under certain conditions.

SMLM is suitable not only for protein counting but also for RNA or DNA counting

The counting method of RNA or DNA called Fluorescence In Situ Hybridization, or FISH, is more straightforward than counting methods for proteins because of complementary interaction between synthetic oligonucleotides and target mRNA sequences [10]. In the original demonstration of FISH, conventional epi-fluorescence microscopy was used to detect single mRNAs using multiple dye-labeled synthetic oligonucleotides to compensate for the detection sensitivity of a normal CCD camera. The signal was then optically deconvolved to pick out a single mRNA. The downside to this approach was the labeling of a single oligonucleotide with the multiple dyes (> 5) and the non-specific binding of the synthetic sequence to off-target mRNA molecules. After the development of single-dye labeling at 3’ termini of short oligonucleotides, an improved approach was proposed [38]. Forty-eight different single-dye-labeled oligonucleotides, to reduce off-target or non-binding effects, were used to detect a coding region of GFP; the 3’ untranslated region also was targeted with 4 oligonucleotides labeled with different colored dyes. By combining the two independent signals, the investigators counted the mRNAs which had both colors located in a same region. Using the advanced FISH, researchers have addressed the old question of the correlation between cell volume and RNA or DNA copy number [39]: The RNA copy

number was dependent on the cell volume but not on the cell-cycle stage; as expected, the DNA copy number was volume-independent.

Influence of precise protein counting on biology and future directions

Many scientific findings are still qualitative even though quantitative approaches are strongly recommended to understand biochemical reactions. Quantitative microscopy allows us to show the heterogeneous distribution of biological materials inside individual cells or organelles which is important for understanding biological phenomena. In this perspective, we discussed recent improvements in calibration methods for SMLMs and new proposals for the precise and accurate counting methods. Despite these advances, several points should be addressed in near future.

First, it is necessary to improve the labeling of a specific target with a known stoichiometry of dyes to the protein of interest. Currently, the tagging strategies available include the six-peptide sequence for labeling with a FlAsH dye (‘FlAsH tag’) inserted into a specific domain of proteins or a small SNAP/CLIP tag on C- or N- termini of proteins of interest combined with cell-permeable dyes for live-cell imaging [29]. However, the photophysical properties of FlAsH and SNAP/CLIP dyes were not rigorously tested for quantitative microscopy.

The second approach is to insert a photoactivable fluorescent protein into the gene using genome editing technologies, such as the CRISPR-Cas9 system [40, 41, 42] and the zinc-finger nuclease [43, 44]. This approach can greatly benefit the counting of proteins with low copy numbers. For instance, it has been estimated that ~ 26 copies of dynamin can be recruited to clathrin-mediated endocytic vesicles in live cells [44]. In addition, Leonetti and colleagues have successfully tagged GFP onto endogenous human proteins using the CRISPR-Cas9 system [45].

Most of the current single-molecule counting microscopies have been concentrated on static information, that is, counting molecules on fixed samples. One obvious and important direction to improve these counting techniques is to expand their reach to measure dynamic events. However, dynamic molecular interactions in live cells can occur even within milliseconds [29**]. Counting single molecules at such fast rates will put severe demands on both the brightness of the probe and the sensitivity of the microscope. To this end, a small-sized semiconducting polymer dot [46], which has a high brightness and low bleaching rate, together with improved sCMOS technology that offer high quantum efficiency, low noise, and fast frame rates [47*], may help to improve the image acquisition speeds of current single-molecule counting microscopies. The ability to observe time-dependent redistribution of protein copy numbers with good time resolution down to milliseconds would be an important advance. Integration of the obtained quantitative measurements with the appropriate cellular and mathematical modeling, will surely provide deep insight into biological processes in many different types of biological samples [48*].

Concluding remarks

Over the past two decades, researchers have had many breakthroughs in overcoming the diffraction limit and developing quantitative microscopies with nanometer-scale spatial precision. Beyond the breaking of optical resolution, we and others have tackled the development of new quantitative microscopy methods that correct undercounting or overcounting errors so that the precise counting of proteins and nucleotides can be achieved in cellular compartments. Our statistical deconvolution algorithm and other approaches using single-molecule localization microscopy help to measure the precise protein copies in many complex samples and conditions. This perspective article also discussed how to resolve blinking issues of fluorophores using photokinetic modeling, pair-correlation analysis, qPAINT, and advanced FISH. Finally, we discussed future directions such as the counting and mapping of biomolecules in a time-dependent manner to understand dynamic events in cells.

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* of special interest

** of outstanding interest

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Highlights

- Single-molecule based statistical deconvolution quantitative microscopy
- Single-molecule localization microscopy: Overcoming under- or over-counting errors
- Principle of qPAINT method to count the number of proteins of interest
- Future directions for quantitative microscopy based on single-molecule fluorescence

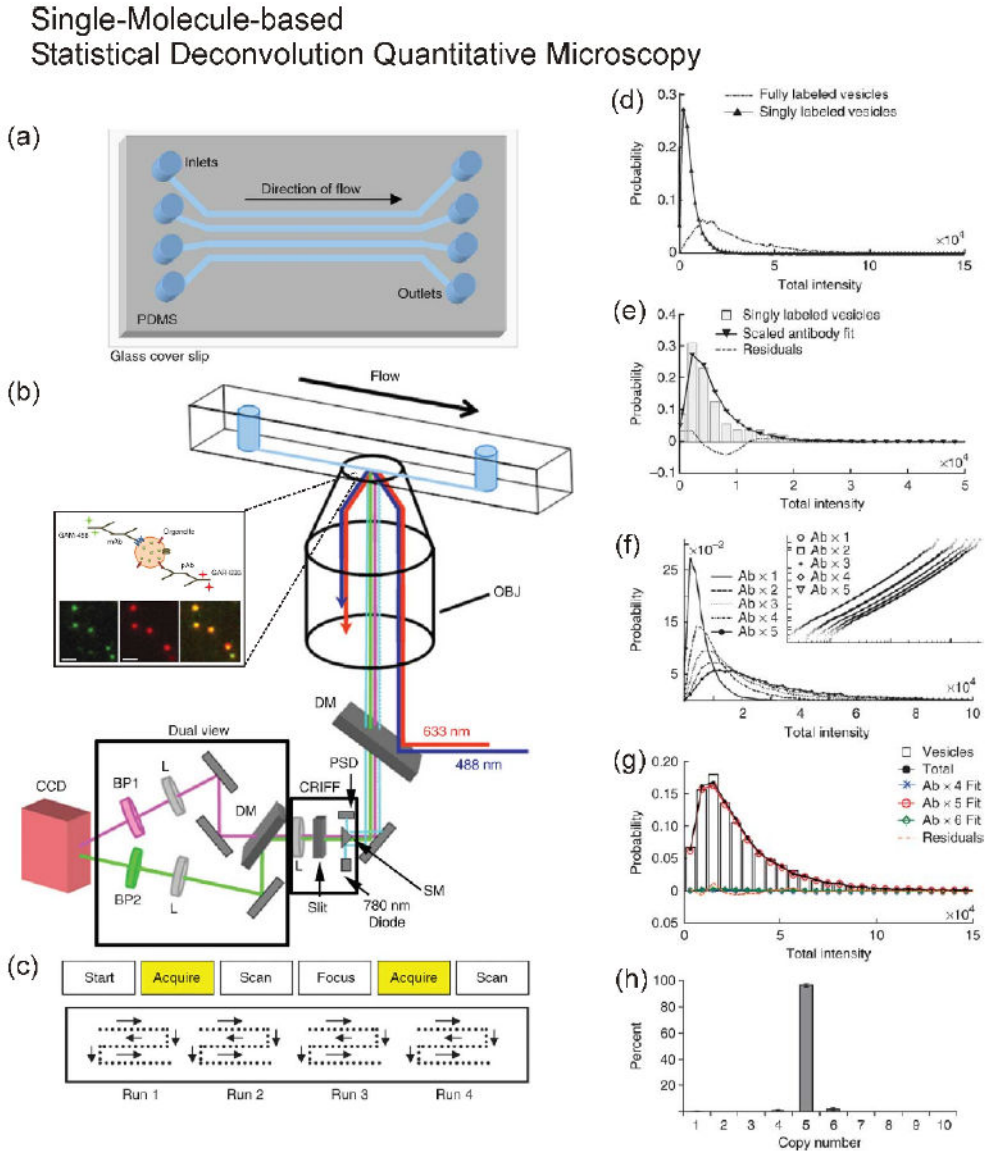

Figure 1.

Schematic diagram of single-molecule-based statistical deconvolution quantitative microscopy combined with a microfluidic chip and an example of statistical deconvolution. (a) Microfluidic chip, (b) Total-internal-reflection-fluorescence (TIRF) microscopy, and (c) automatic scanning patterns for collecting images. The expansion of inside of the microfluidic chip in (b) shows synaptic vesicles labeled with primary and dye-tagged secondary antibodies in the imaging plane using TIRF microscopy. (d-h) The detailed procedures of counting of a fully labeled sample using single-antibody distribution. (d) Comparison of synaptic vesicles singly or fully labeled with primary and secondary antibodies. (e) Fitting of single-labeled synaptic vesicles. (f) Plotting of multiplication of the scaled single-antibody distribution to generate calibration curves for fitting. (g) A plot showing the fit for the protein SV2 on synaptic vesicles. (h) Results of fitting six different SV2 data sets. Reprinted with permission from Nature Protocol [26].

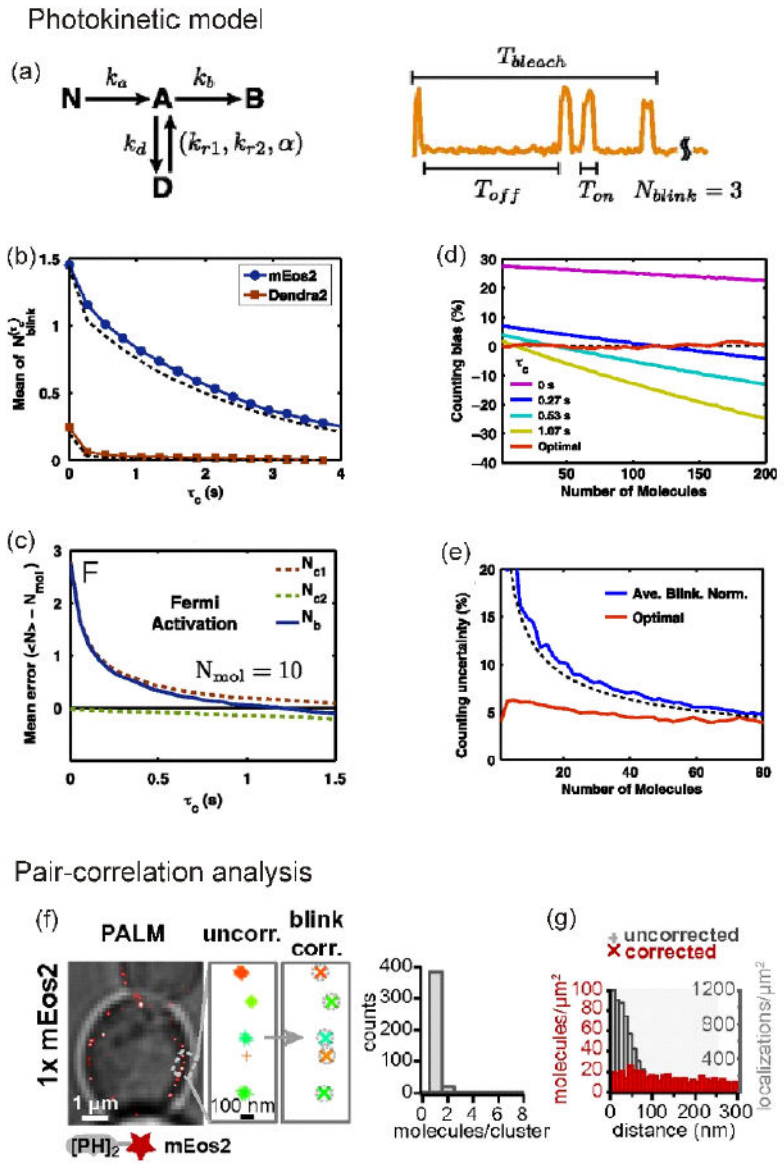

Figure 2.

Counting of proteins by a PALM-based photokinetic model and pair-correlation function. (a) Photokinetic model of blinking of photoactivable fluorescent protein (PAFP). Nonactive form, N, of PAFP is converted to the active form, A, with rate *k*a, upon 405-nm laser illumination. In the presence of a 561-nm laser, the molecule emits light and can either go to dark, D, or to bleach, B, states with rates k_d and k_b, respectively. The molecule recovers from D to A state with two rates k_{r1} (fast) and k_{r2} (slow); α is the ratio between the contributions of the fast and slow rates. The recovery rates are dependent on the T_{off}. (b) Correlation between the number of blinking of PAFP (N) and tolerance time (τ_c). (c) Fermi activation for optimization of the number of active molecules. (d) Counting bias by tolerance time. (e) Counting uncertainty in the case of iterative way versus a simple average blinking normalization method. Reprinted from [32]. (f) Pair-correlation analysis to remove artificial

clustering effect caused by blinking in a small region. Single mEos2-fused PI3P-sensitive PH probe (PH_2-mEOs2). (g) After correction, the blinking molecules do not have colocalization artifacts (red bars) compared with before correction (gray bars). Reprinted from [34].

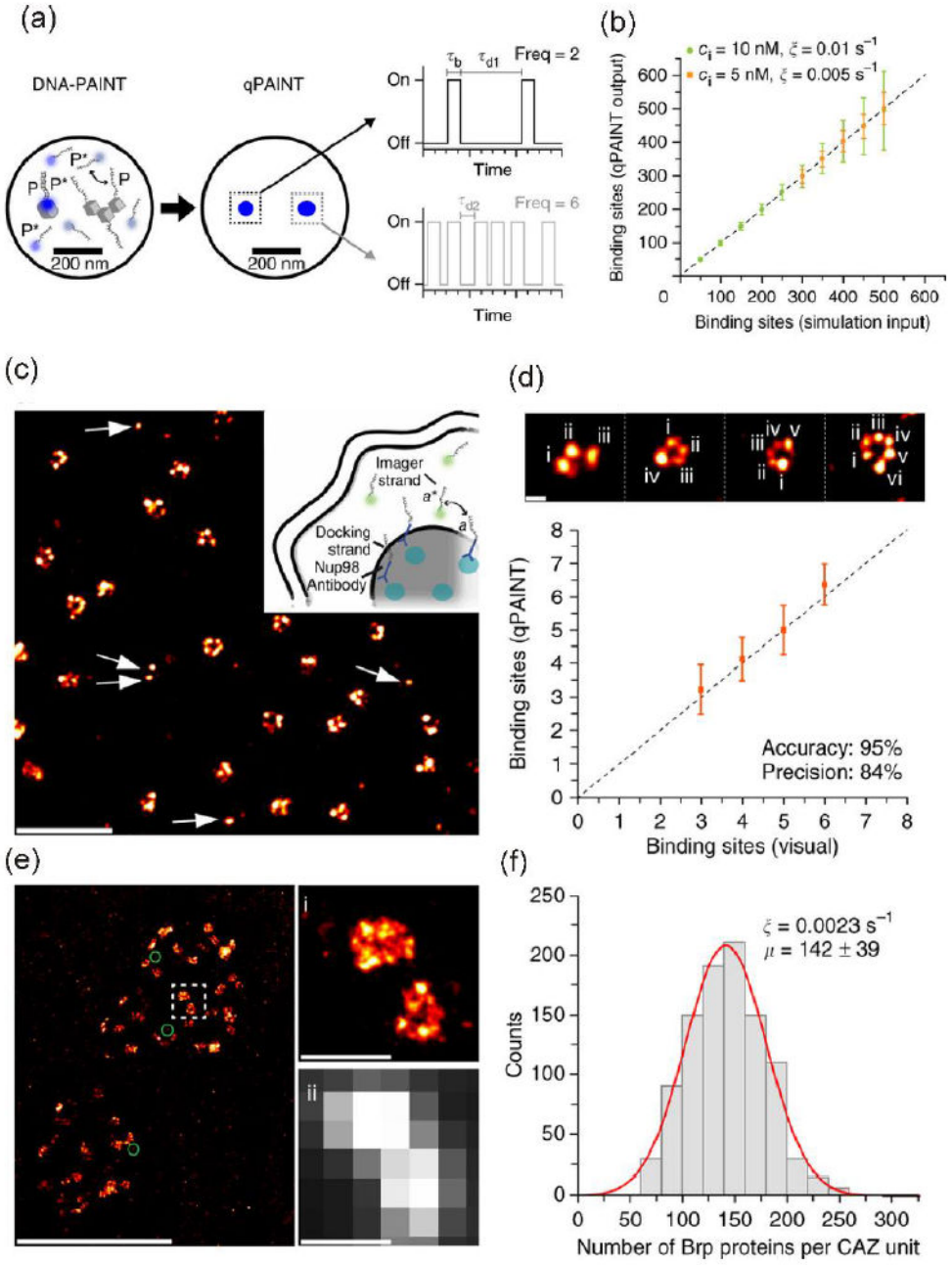

Figure 3.

qPAINT. (a-b) Principle of qPAINT and simulated results. Imager and docking strands are P* and P, respectively. Standardization of the rate of binding ('influx rate' or ξ) of imager strand to a single docking strand attached to target. $\xi = (k_{on} \times c_i) = \tau_d^{-1}$where k_{on}, c_i and τ_d are rate constant, known concentration of dye-labeled imager strand (P*), and dark time before binding, respectively. Then the rate (ξ) can be converted to a real number of binding sites, e.g. binding sites = $\tau_d/\tau_d^* = (\xi \times \tau_d^*)^{-1}$ where τ_d^* is the actual average dark time of the imager strand to real sample. For example, if ξ^* and τ_d^* are 0.005 s^{-1} and τ_d^* = 1 s, the actual binding sites would be 200. (c-f) Examples with qPAINT method. (c and d) qPAINT

quantification with nucleoporin Nup98 (anchored to the inner ring of the nuclear pore complex). (e and f) qPAINT quantification with Bruchpilot proteins (Brp) molecules which are components of the cytomatrix at the synaptic active zone (CAZ). The average Brp number is 142 ± 39 (mean ± s.d.). Reprinted from Nature Methods under their permission [37].