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Photobleaching step analysis for robust determination of protein complex stoichiometries

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Abstract

The counting of discrete photobleaching steps in fluorescence microscopy is ideally suited to study protein complex stoichiometry in situ. The counting range of photobleaching step analysis has significantly improved with more sophisticated algorithms for step detection, albeit at an increasing computational cost and with the necessity for high data quality. Here, we address concerns regarding robustness, automation, and experimental validation, optimizing both data acquisition and analysis. To make full use of the potential of photobleaching step analysis, we evaluate various labelling strategies with respect to their molecular brightness, photostability, and photoblinking. The developed analysis algorithm focuses on automation and computational efficiency. Moreover, we validate the developed methods with experimental data acquired on DNA origami labeled with defined fluorophore numbers, demonstrating counting of up to 35 fluorophores. Finally, we show the power of the combination of optimized trace acquisition and automated data analysis by counting labeled nucleoporin 107 in nuclear pore complexes of intact U2OS cells. The successful in situ application promotes this framework as a new resource enabling cell biologists to robustly determine the stoichiometries of molecular assemblies at the single-molecule level in an automated fashion.

Introduction

The fundamental functions of living cells are carried out by protein assemblies at the molecular level. Precise quantitative knowledge on the composition of these protein complexes in the cellular environment is crucial to deepen our understanding of their cellular functions (Matthews, 2012). In many cases these protein assemblies contain not only a variety of different components, but also several copies of each component (Ahnert et al., 2015).

To investigate the stoichiometry of a particular protein of interest in a molecular assembly, fluorescence microscopy offers several advantages. It is highly specific, live-cell compatible, single-molecule sensitive, and therefore capable to resolve heterogeneities within ensembles in situ. In the last two decades, different fluorescence based molecular counting methods have been developed (Gruβmayer et al., 2019). Among them are methods relying on brightness calibration (Wu and Pollard, 2005), counting of photobleaching steps (Ulbrich and Isacoff, 2007), single-molecule localization microscopy (Jungmann et al., 2016; Lee et al., 2012; Puchner et al., 2013; Rollins et al., 2015), or photon antibunching (Grußmayer and Herten, 2017; Ta et al., 2010). To date, photobleaching step analysis (PBSA) and brightness estimation are most widely used in biological applications of molecular counting, due to their simplicity in data acquisition, and the relatively straight-forward interpretation (Arant and Ulbrich, 2014). PBSA has the advantage that counting of fluorophores requires no calibration and that it is relatively robust to variations in molecular brightness. Importantly, any molecular counting approach based on fluorescence microscopy requires additional calibration of the degree of labeling, i.e., the number of fluorophores attached per target to relate measured fluorophore numbers to the underlying number of target proteins (Gruβmayer et al., 2019; Hummert et al., 2021).

While the idea of counting photobleaching steps is straight-forward, numerous approaches exist for data analysis. Often the number of steps is classified by visual inspection (Dixon et al., 2015; Engel et al., 2009; Ulbrich and Isacoff, 2007), which is not only time-consuming but also highly subjective. The exclusion of traces which cannot reliably be classified upon visual inspection will inevitably lead to a biased estimate, since traces with a higher number of fluorophores tend to exhibit a higher complexity. More reliable is the determination of the unitary step height by pairwise frequency analysis (Leake et al., 2006). Thereby, however, differences in step height

over the field of view will broaden the measured emitter number distribution. Chung-Kennedy (Das et al., 2007) or median rank (Dixon et al., 2015) filters are often applied to photobleaching traces to improve step detection. Measuring ensemble photobleaching against density allows fast determination of mean fluorophore numbers but lacks single complex resolution (Liesche et al., 2015). Recently, molecular counting via photobleaching has attracted renewed attention due to the development of novel analysis modalities based on Bayesian statistics (Garry et al., 2020; Tsekouras et al., 2016) and machine learning (Xu et al., 2019).

However, these novel methods are demanding in terms of data quality, which in turn leads to new requirements regarding fluorophore properties. In the trade-off between signal-to-noise ratio (SNR) of the individual bleaching steps and the rate of photobleaching, bright and stable fluorophores are advantageous. Thus, buffer systems (Aitken et al., 2008; Vogelsang et al., 2008) to increase photostability and reduce complex photophysical behavior could help to improve data quality. This motivates an investigation into which labelling approaches and buffer systems are most suited to generate data compatible with automated and robust photobleaching step analysis at an increased counting range. Additionally, Bayesian methods are computationally costly and therefore limit the number of photobleaching traces that can be processed in a given experiment. Therefore, we see the necessity for an approach to bridge the gap between simple methods such as visual inspection and the novel Bayesian methods. Lastly, the experimental validation with standard samples is often not the focus of theoretical methods development although calibration samples are readily available (Schmied et al., 2014; Thevathasan et al., 2019).

Here, we address these hurdles to make photobleaching step analysis a more robust and thoroughly validated tool in the biophysics toolbox. We describe a comprehensive framework for PBSA that provides guidelines for the choice of fluorescent label and acquisition conditions as well as a new photobleaching trace analysis algorithm. The quickPBSA package was developed with a focus on automation and speed, providing the high throughput necessary to meet the demands for in situ protein counting. We then validate the method with molecular counting experiments on DNA origami carrying defined label numbers. Finally, we show that quickPBSA, in conjunction with optimized trace data acquisition, is well suited to characterize protein structures in cells by determining the copy number of Nucleoporin 107 (NUP107) in the nuclear pore complex (NPC).

Results

The reliability of automated photobleaching trace evaluation strongly depends on the quality of the input data, i.e., individual photobleaching traces. Historically, PBSA has mostly been performed using fluorescent proteins as labels (Arant and Ulbrich, 2014). However, fluorescent proteins tend to be less photostable than small organic fluorophores and are known to exhibit complex photophysics, complicating trace interpretation (Ha and Tinnefeld, 2012). To identify fluorophores suited for generating photobleaching traces with high SNR, we therefore compared the fluorescent proteins EGFP, mCherry and mNeonGreen, as well as the organic fluorophores tetramethylrhodamine (TMR) and silicon rhodamine (SiR) conjugated to the self-labeling protein tags SNAP-tag and HaloTag with respect to their photostability, brightness and propensity for photo-induced blinking. In addition, we tested to which degree photostabilizing buffers composed of reducing and oxidizing systems (ROXS) and an oxygen scavenger could be used to increase photostability, suppress photoblinking and thereby improve trace quality.

Both, the molecular brightness of a fluorescent label and its photostability contribute to the overall photon budget and thereby directly influence the SNR. Since the molecular brightness is a well-studied property of fluorophores it can be readily compared across different fluorophores based on reported values in the literature (Table S1). For organic fluorophores conjugated to protein tags, a strong influence of the tag on the fluorophore was observed and has to be taken into account when comparing the brightness of different fluorophores after conjugation to protein tags (Erdmann et al., 2019). In contrast, the photostability of a fluorophore can strongly depend on its environment and on the applied measurement conditions. For this reason, we performed systematic photobleaching measurements under comparable conditions with the selected fluorophores. For this, we expressed each label as fusion protein in U2OS or COS-7 cell lines (Figure S1) and imaged them after chemical fixation. We determined the photostability of each fluorophore in phosphate buffered saline (PBS, pH 7.4) and three photostabilizing buffers each containing methyl viologen and ascorbic acid as ROXS and an oxygen scavenger system consisting of either glucose oxidase and catalase (GodCat) (Harada et al., 1990), protocatechuate-3,4-dioxygenase (PCD) (Aitken et al., 2008) or sodium sulfite ($NaSO_3$) (Hartwich et al., 2018).

Upon high intensity illumination, we observed biexponential intensity decay patterns for all tested fluorophores (Figure S2). Such behavior has been reported before for both, organic fluorophore and fluorescent proteins (Bakker and Swain, 2019; Song et al., 1995). We therefore decided to use the time to reach half maximum ($t_{1/2}$) as a model-independent metric to compare conditions and fluorophores. Across all tested conditions, $t_{1/2}$ varied considerably covering three orders of magnitude (0.5-200 s). Overall, fluorescent proteins were less photostable than organic fluorophores and far-red organic fluorophores exhibited the highest photostability. Differences between fluorophores were less pronounced in conventional buffers without photostabilizing additives. Addition of ROXS and oxygen scavengers resulted in strongly increased photostability for organic fluorophores, particularly for the red-absorbing fluorophores ATTO 647N and SiR (Figure 1a). For ATTO 647N, photostability was improved 2-fold with ROXS buffer supplemented with $NaSO_3$ as oxygen scavenger. Replacing $NaSO_3$ with the enzymatic oxygen scavengers GodCat or PCD resulted in further improvements of ~10 and 20-fold respectively. Similar trends were observed for both TMR and SiR conjugated to SNAP-tag or HaloTag. Here, SiR conjugated to SNAP-tag in ROXS PCD showed the highest stability with a $t_{1/2}$ of 120 s (20-fold improvement over PBS), a 60-fold improvement over EGFP, but still 50% lower stability compared to ATTO 647N in the same buffer. In contrast, a decrease in photostability for EGFP and mCherry in the methyl viologen and ascorbic-acid-based ROXS buffer was observed for all three oxygen scavenging systems, possibly due to pH changes of the buffers during imaging (Swoboda et al., 2012). Interestingly, no such decrease in photostability was observed for mNeonGreen, which could be due to improved stability of the protein structure of mNeonGreen (Shaner et al., 2013).

In addition to the increased photostability of organic fluorophores, ROXS buffers were also reported to reduce emission intensity fluctuations on the millisecond to second timescale, also known as photoblinking, which complicates trace interpretation in PBSA (Vogelsang et al., 2008). For a quantitative characterization of photoblinking, we performed on- and off-rate measurements for a selection of fluorophores and buffer conditions using an image correlation approach which robustly determines photoblinking rates across a range of blinking regimes without requiring traces of individual fluorophores (Sehayek et al., 2019). For these measurements, we recorded image series on chemically fixed cells with the labels EGFP, SNAP-tag or HaloTag localized at the plasma membrane. These image series were then used to compute the temporal autocorrelation function (ACF) which we fitted with a three-state

fluorophore model (Figure S3). Of note, such a three-state fluorophore model does not necessarily reflect the underlying photophysical processes for the evaluated fluorophores, but rather serves to facilitate a quantitative comparison of fluorophores and conditions. The degree of photoblinking observed in this assay varied strongly between the tested fluorophores and was apparent both, by visual inspection of intensity traces from individual fluorophores (Figure 1c-f) and in the corresponding ACFs (Figure S4). Fitting the ACFs obtained from image correlation then allowed us to determine on- and off-rates for photoblinking across the different conditions (Figure S5).

In PBS, the on-time (t_{on}, $1/k_{off}$), as well as the equilibrium constant K (k_{on}/k_{off}) of EGFP and TMR or SiR conjugated to SNAP-tag were comparable (Figure 1b). In contrast, fluorophores conjugated to HaloTag exhibited a much lower degree of photoblinking evidenced by higher K and ton even without addition of ROXS or oxygen removal. For EGFP, the strong decrease in photostability described above prevented a photoblinking analysis in ROXS PCD. For TMR and SiR conjugated to both tags, we observed an increased on-fraction K upon switching to ROXS PCD buffer. Interestingly, the effect on the on-time was less consistent. While fluorophores conjugated to SNAP-tag showed increased ton in ROXS PCD as compared to PBS, fluorophores conjugated to HaloTag exhibited lower ton after ROXS addition and oxygen removal. In the case of TMR, this resulted in very similar photoblinking behavior in ROXS PCD for both tags. For SiR, conjugation to HaloTag resulted in ~3x larger K and t_{on} in ROXS PCD compared to SNAP-tag in the same buffer. It was recently reported that fluorophores conjugated to HaloTag are tightly associated to the protein surface, while fluorophores conjugated to SNAP-tag protrude away from the protein surface (Wilhelm et al., 2021). We speculate that this could result in differences in accessibility for soluble factor which could explain the different propensity for photoblinking and the different effect of ROXS components. Based on these observations, the trace quality and counting range of PBSA-based quantification can be improved by using self-labeling protein tags in combination with longer-wavelength organic fluorophores and ROXS buffers supplemented with enzymatic oxygen scavenger systems.

Turning from the data acquisition to the data analysis, we set out to develop a routine capable of estimating fluorophore number distributions directly from the experimental data (i.e. image stacks) with minimal user input. To that end, apart from the novel quickPBSA algorithm for trace interpretation, the framework includes modules for automated trace extraction from raw time-

lapse image stacks and filtering. The underlying principle of the framework is to perform a preliminary step detection for each trace and then refine the results iteratively. The final refinement step makes use of a Bayesian posterior from Pressé and coworkers (Tsekouras et al., 2016), thus incorporating prior knowledge about the photobleaching process. In contrast to the previously published approach, the Bayesian posterior is not used to detect step positions, but only to determine step significance and find simultaneous bleaching events. The full data analysis workflow is comprised of four major parts (Figure 2a) which are described in the following:

Trace Extraction

The first step is the identification of regions of interest (ROIs) and the automated extraction of photobleaching traces from image stacks. Here, the ROIs can be provided as pixel coordinates (for instance generated by a localization algorithm) or via a segmentation mask image (for instance generated by thresholding). As the photobleaching trace is extracted from the ROI, a ring-shaped region with variable offset from the ROI is used to extract a background bleaching trace (Figure S6). Other ROIs are automatically excluded from the background region. Especially for measurements in cells we found that background bleaching due to autofluorescence and out-of-focus fluorescence occurred on similar timescales as the fluorophore bleaching. Therefore, background subtraction proved to be essential to recover traces with discernible photobleaching steps. Additionally, background subtraction also facilitates identifying and excluding ROIs that are not fully bleached at the end of the measurement.

Preliminary Step Detection

After trace extraction, a preliminary step detection is performed on all extracted traces. This is accomplished by successively placing steps and evaluating each added step using the Schwarz Information Criterion (SIC) as first demonstrated by Kalafut and Visscher (Kalafut and Visscher, 2008). In our implementation of this algorithm, a user-defined threshold to ignore minor changes in the mean intensity reduces the detection of spurious steps rendering the preliminary step detection more robust.

Filtering

Traces are excluded based on the result of the preliminary step detection. The model selection in the quickPBSA algorithm critically depends on the correct detection of the last and the penultimate bleaching steps in each trace since the period where only one fluorophore is active is used to retrieve the properties of an individual fluorophore. Therefore, traces which are not fully bleached at the end of an acquisition or where the last step potentially corresponds to a double bleaching event need to be excluded. Assuming that the last two steps are correctly identified in most traces from the image stack, the distribution of single fluorophore signal means across all traces can be used to filter out traces. Using this information from the entire image stack we exclude traces where the single fluorophore or background signal are out of bounds.

quickPBSA algorithm

Ultimately, the result is refined by evaluating the entire trace according to the full marginal posterior from Pressé and coworkers (Tsekouras et al., 2016). This posterior incorporates the possibility of simultaneous bleaching events as well as a penalty for too many bleaching events and thus is a far more content-aware evaluation of step placement than the information criterion used in the preliminary step detection. The quickPBSA algorithm iteratively minimizes the negative logarithm of the posterior (-log(P), starting from the result of the preliminary step placement with all steps considered to be single events. Figure 2b shows a flowchart of the iterative optimization procedure, together with a simulated trace showing how the algorithm removes steps and optimizes step heights from the preliminary result. The iterative procedure is:

I. Find candidate positions for simultaneous steps. In the first iteration (double steps) these are all positions. For more fluorophores bleaching simultaneously, only the locations from the last iteration are considered. For example, triple steps are considered only where double steps yielded an improvement. If there are candidate positions, proceed with II, otherwise go to III.
II. Try all possible combinations with step heights up to the current occupancy. The result of this process is accepted and considered the new optimum if a lower value of -log(P) is found at any point. Step heights from 1 to the maximum step height are considered,

so that, for instance, [2,2,2] can be replaced by [1,3,2]. Return to step 1 to find new candidate positions.

III. Remove the least significant step found during preliminary step detection. The final two steps in a trace are always kept in place since they are required for posterior calculation. If there are only 3 steps left, proceed with step IV, else reset all steps to single occupancy and return to step 1.

IV. If no steps could be removed to yield an improved posterior, i.e. the current optimum contains the same number of steps as the preliminary result, the algorithm proceeds to add single fluorophore steps. This is accomplished by calculating -log(P) for additional positive or negative steps at all positions before the penultimate step. Repeat step IV until the last two added steps yielded no improved posterior or a specified maximum number of added steps is reached.

V. Return the step/event combination with the minimal value for -log(P) found at any point in I-IV.

In the quickPBSA algorithm, the evaluation of simultaneous step arrangements is computationally most expensive. Especially for traces with many steps in the preliminary result, the number of possible combinations is excessive. We therefore implemented several strategies to reduce the computational cost at this point, as detailed in the documentation of the software package.

We benchmarked the quickPBSA trace analysis algorithm using semi-synthetic data generated from experimental data. For this, we acquired experimental data from an in vitro sample with few fluorophores per diffraction-limited spot, namely immobilized DNA oligonucleotides labeled with four ATTO 647N fluorophores. We selected traces where the quickPBSA result could be confirmed by visual inspection, obtaining a set of traces with known ground truth. We then generated increasingly complex semi-synthetic traces with known ground truth by combining several of these traces (Figure 3a). Using this approach, benchmarking traces with fluorophore numbers up to 40 were generated and used to evaluate the accuracy of the quickPBSA trace analysis.

For each fluorophore number, approximately 100 semi-synthetic traces with 3,000 time-points per trace were included in the analysis (Figure S7). To compare the performance of the

algorithm with a state-of-the-art Bayesian algorithm, we analyzed the benchmarking dataset with the algorithm previously published by Pressé and coworkers (Tsekouras et al., 2016), hereafter called Pressé2016. Figure 3b shows the results of the benchmark dataset after the filtering step (Preliminary Only), including the quickPBSA refinement algorithm, and from the Pressé2016 algorithm. For the benchmarking dataset the result of the preliminary step detection starts to deviate systematically from the ground truth for fluorophore numbers beyond 20, most likely due to missed bleaching events which occurred in close temporal succession. The results after quickPBSA step refinement and from the Pressé2016 algorithm, on the other hand, show a slight overestimation. But in both algorithms the mean estimated fluorophore numbers never deviates by more than one fluorophore from the expected value. Additionally, the overestimation is likely caused by the selection of ground truth traces, since a simple ruler method shows an even larger overestimation for the benchmarking dataset (Figure S7). Overall, the results from the quickPBSA algorithm and Pressé2016 are very similar. A 2-sample t-test shows that the means obtained from the 2 algorithms never differ significantly ($p>0.01$, Figure S7).

We also used the semi-synthetic traces to benchmark the runtime of the analyses in dependence on the number of fluorophores (Figure 3c). We observed that for up to 20 fluorophores, the quickPBSA total runtime was dominated by the preliminary step detection. For higher fluorophore numbers the runtime increases due to the quickPBSA refinement and increases further for higher fluorophore numbers. Nonetheless, the mean runtime remained below 10 s per trace (3,000 data points) for the entire benchmarking dataset containing traces with up to 40 fluorophores. In contrast, the runtime of the Pressé 2016 algorithm is over 30 minutes per trace for the entire benchmarking dataset. Thus, using the quickPBSA algorithm, we were able to analyze the dataset with comparable results and an over 100-fold lower computation time. The mean analysis times for all datasets included in this publication are below 3 minutes per trace even for complex experimental traces with up to 15,000 data points (Table 1).

To fully validate the developed framework with experimental data, we used DNA origami carrying a well-defined number of fluorophores. DNA origami with 9, 20, and 35 binding sites for labelling strands carrying a single ATTO 647N fluorophore (R09, R20, and R35) were sparsely immobilized on coverslips to ensure that stochastically overlapping origami structures

did not significantly influence the measurement (Figure 4a). The labelling efficiency specified by Gattaquant for the DNA origami is 70%, which we independently validated for the R20 origami using counting by photon statistics (Grußmayer and Herten, 2017) (Figure S8). Photobleaching traces from individual DNA origami structures were then extracted using the trace extraction module described above, using thunderSTORM (Ovesný et al., 2014) for ROI localization and removing origami with close nearest neighbors (Figure S6). Since the extracted traces exhibited only weak background bleaching, the background subtraction step for this sample mainly removed a constant offset caused by excitation bleed-through and read-out noise (Figure 4a, center).

Processing the background-corrected traces using the full quickPBSA algorithm resulted in good agreement between intensity and predicted fluorophore numbers over time (Figure 4a). The obtained fluorophore number distributions were symmetrical indicating no systematic deviation and an unbiased measurement error (Figure 4b). The means obtained from fitting a normal distribution to the quickPBSA fluorophore number estimates agreed with the expected values for a labelling efficiency of 70%. In contrast, the means of distributions obtained without quickPBSA refinement (Preliminary only), while similar for origami with 9 binding sites, exhibited a significant underestimation for origami with 20 and 35 binding sites (Figure 4c). This underestimation for larger fluorophore numbers is in line with the benchmarking results with semi-synthetic traces.

A full comparison of all parameters from the fits and a comparison to the mean and standard deviation of a binomial distribution with the known number of binding sites and the expected labelling efficiency is shown in Table 1. We observed that the measured distributions broadened with increasing fluorophore number, stronger than would be expected from the binomial distribution of label numbers alone. For instance, while broadening of the measured data was negligible for R09 origami, the standard deviation increased by a factor of 3 for R20 origami (Table 1). This suggests that experimental data contains additional sources of uncertainty which are not fully reproduced using semi-synthetic data and therefore highlights the importance of additional benchmarking with standardized samples.

To validate that the quickPBSA algorithm performed robustly upon variation of experimental parameters, we performed additional measurements with the R09 origami samples on a

different widefield microscope setup with homogeneous illumination power and a sCMOS instead of an emCCD camera for detection. As in the first experiment with the R09 origami, the expected mean and width of the fluorophore number distribution were well reproduced (Figure 4d, Table 1). A large field of view is advantageous for the acquisition of photobleaching data since overall measurement time is decreased and the potential impact of degrading buffer performance can be minimized.

We further explored the sensitivity of the quickPBSA algorithm to fluorophore properties by measuring a fluorophore number distribution for origami with 9 binding sites labelled with ATTO 565 (Y09). Here, the measured distribution showed a peak at 7.9 fluorophores, significantly above the expected mean fluorophore number of 6.3 (Figure 4e, Table 1). A likely explanation for this deviation is that ATTO 565 exhibited two distinct brightness states as frequently observed in individual photobleaching traces (Figure S9). If the last photobleaching step occurs from a lower brightness state in a significant number of traces, the mean signal of a single fluorophore is underestimated for these traces, leading to an overestimation of the total number of fluorophores. This again highlights how important label selection is in photobleaching experiments, even for organic fluorophores. Additionally, taking the complex photochemical behavior into account can extend the counting range of photobleaching analysis even further (Bryan et al., 2020).

While DNA origami samples are ideally suited for determining the accessible counting range of a novel method, the application to biological targets within the complex cellular environment is subject to additional challenges that are not captured in simplified in vitro experiments. Background (auto)fluorescence, density of structures and biological variation cannot readily be controlled in a biological sample and will impact data quality. To assess how the quickPBSA framework coped with a more complex in situ sample, we decided to determine the number of NUP107 protein copies contained in individual nuclear pore complexes. To minimize the influence of protein expression and labelling efficiency, we used a genome-edited U2OS cell line expressing NUP107-SNAP from its native genomic context (Li et al., 2018). Labelling of SNAP-tag conjugated NUP107 was performed with the corresponding silicon rhodamine substrate BG-SiR. From epifluorescence images of chemically fixed and labeled cells, it is immediately evident that fluorescent background is much more pronounced in situ than in the origami experiments described above (Figure 5a). Additionally, the high density of NPCs

resulted in regions where it was no longer possible to identify individual NPCs. As for the in vitro samples, individual NPCs were localized with thunderSTORM (Ovesný et al., 2014). The trace extraction routine in the quickPBSA framework automatically excludes NPCs based on localization parameters such as width of the fitted Gaussian or nearest neighbor distance. Thus, only sufficiently isolated and diffraction-limited structures are considered for further analysis. (Figure S6, Figure S10). Despite this pre-filtering, raw traces from individual ROIs did not exhibit clear bleaching steps and the decay in the background region occurred on a similar timescale as the fluorescence signal of the ROI (Figure 5b). Background fluorescence can therefore be mainly attributed to out-of-focus fluorescence rather than autofluorescence. After subtraction of the background signal, photobleaching steps could be observed towards the end of photobleaching traces (Figure 5b). Despite the substantially lower SNR compared to the previously successfully evaluated traces recorded using ATTO 647N as fluorophore, we subjected the extracted traces to analysis with the quickPBSA algorithm (Figure 5b and 5c). The resulting fluorophore number distribution cumulated across 32 cells from two independent experiments (Figure S10) was well described by a normal distribution with a mean of 20.7±0.2 fluorophores per NPC and a standard deviation of 8.5±0.2 (Figure 5d). The mean fluorophore number per NPC per cell was 21.6±1.7 indicating that quickPBSA yielded robust estimates across the entire population of cells (Figure 5e). The width of the cumulated distribution is comparable to that of the distribution obtained from R35 DNA origami, indicating that background subtraction and spot pre-filtering successfully reduced the complexity of obtained traces and did not result in reduced precision. NUP107 has been reported previously to be present in NPCs at 32 copies per pore (Bui et al., 2013; Ori et al., 2013). Based on the mean fluorophore number of 20.7±0.2 per NPC, this translates into a labelling efficiency of ~65% for SNAP-tag labelling with BG-SiR which is in excellent agreement with recent reports (Thevathasan et al., 2019). This indicates that quickPBSA is able to correctly measure fluorophore numbers even for less bright fluorophore labels, in the complex environment of a eukaryotic cell and with comparable precision as localization microscopy-based methods (Thevathasan et al., 2019).

Discussion

The presented framework for photobleaching step analysis offers a robust, fast, and well-validated approach for molecular counting in situ. The evaluation of various fluorophores along

with different buffer conditions in respect to the precision of photobleaching step analysis may serve as practical guide for robust counting of proteins and other biomolecules. Additionally, it may serve as blueprint for extended screening of other fluorescent labels and experimental conditions. The high photostability and low degree of photoblinking of organic fluorophores imaged in ROXS buffer with enzymatic oxygen removal enabled generating high-quality input data for automated trace interpretation. Despite the lower brightness compared to HaloTag, the high photostability of SiR conjugated to SNAP-tag in ROXS PCD buffer was found to be suitable for PBSA protein counting. Clearly, fluorophore characteristics strongly depend on the specific environment, as is especially evident by the influence of protein tags on photoblinking of TMR and SiR and the effect of SNAP-tag conjugation on SiR brightness. Therefore, evaluating fluorophore properties should always be considered when using alternative labelling approaches. Fluorophores with improved molecular brightness and photostability (Grimm et al., 2015; Scott et al., 2018), as well as recently reported self-healing fluorophores (Henrikus et al., 2020; Velde et al., 2018) might allow to extend the accessible counting range of photobleaching step analysis. Approaches to improve the SNR during image acquisition such as confocalized detection or single-plane illumination could help to improve trace quality and thereby further extend the accessible counting range of quickPBSA (Mi et al., 2015).

On the analysis side we found it beneficial to make use of information from the entire field of view during trace selection, combining features from pairwise frequency methods with features from Bayesian approaches. In this spirit, the methodology for trace analysis is a combination and extension of two previous approaches to photobleaching step analysis. The combined method has only few user-defined parameters simplifying automation and improving robustness. The high degree of automation together with the more than 100-fold improved computational efficiency of the combined method provide a significantly increased throughput required for biological applications. We believe that the developed method of testing algorithms with semi-synthetic data will be highly useful, not only for benchmarking other PBSA algorithms, but also to generate training data for machine learning based approaches (Xu et al., 2019).

Using ATTO 647N-labelled structures with a known stoichiometry in vitro, we showed that quickPBSA yields highly accurate (<10% deviation across all samples) estimates of mean fluorophore numbers for structures containing up to 35 fluorophores. We furthermore demonstrated the robustness of the quickPBSA workflow by successfully analyzing data

acquired on different experimental setups. We also demonstrated that the complex photochemical behavior of fluorophores can skew fluorophore number estimates highlighting the importance of careful fluorophore characterization prior to experiments.

To show that quickPBSA performs well in biological applications, we determine the number of NUP107 protein copies in nuclear pore complexes of U2OS cells. At this point, the background subtraction and trace filtering modules of quickPBSA proved crucial to obtain traces from complex samples. Factoring in the expected labelling efficiency for SNAP-tag labelling, the previously reported number of 32 protein copies was well reproduced. This constitutes, to our knowledge, the highest stoichiometry successfully measured with photobleaching step analysis in a biological sample so far and demonstrates the robustness of the outlined approach in biological samples.

Future developments of alternative algorithms for PBSA to further improve the precision of fluorophore counting, such as novel Bayesian approaches (Bryan et al., 2020), will be of high interest to move beyond measuring mean complex stoichiometries and towards characterizing stoichiometry distributions across ensembles of individual complexes. At this point, however, advances in data analysis will need to go hand in hand with the development of novel labelling schemes with improved labelling efficiency to reduce the variation in fluorophore numbers caused merely by incomplete labelling of target proteins.

Overall, the combination of improved data acquisition and the novel analysis routines contained in the quickPBSA framework provide a reliable way to determine protein stoichiometries in cellulo and will enable the use of automated PBSA as routine tool for cell biology in future applications.

Figure 1: Fluorophore and image acquisition buffer selection for PBSA. **a**, Comparison of photostability and molecular brightness for different fluorescent proteins and organic fluorophores conjugated to protein tags. Symbols indicate mean t1/2 and molecular brightness under indicated condition. Color coding according to excitation wavelength used in this study: (blue: 488 nm, green: 561 nm, red: 640 nm). t1/2 was normalized to 1 kW/cm2 excitation power density. The molecular brightness of fluorophores was corrected for mismatches between excitation wavelength and absorption maximum (Table S1). Full dataset shown in Figure S2. **b**, Comparison of mean equilibrium constant K (kon/koff) between fluorescent and non-fluorescent state and mean on-time ton obtained from image correlation experiments for indicated conditions. Color coding as in a. See Figure S5 for full set of rate values. **c-f,** Representative single-fluorophore intensity traces for indicated fluorophores and buffer recorded via TIRF microscopy of surface immobilized ATTO 647N (c) or plasma membrane-localized EGFP and labelled HaloTag constructs in chemically fixed HeLa cells (d-f).

Figure 2: Framework concept and quickPBSA algorithm. **a**, The four parts of the framework as detailed in the text. **b**, Flowchart of the quickPBSA step refinement algorithm. **c**, Example to illustrate how a simulated trace propagates through the algorithm, starting from the result of the preliminary step detection. Note that step IV is skipped for the example trace, since steps were removed in step III.

Figure 3 Benchmark with semi-synthetic traces. **a**, Semi-synthetic traces for benchmarking are generated by combining manually classified traces. **b**, Deviation from ground truth for semi-synthetic traces analyzed without quickPBSA step refinement, including step refinement, and with the algorithm from Pressé and coworkers (Tsekouras et al., 2016). **c**, Runtime per trace for the three algorithms. Mean (line) and standard deviation (shaded region) are shown.

Figure 4 Validation with DNA origami samples **a,** Representative image and traces from the origami experiment with 20 binding sites for ATTO 647N. Scale bar: 10 µm. **b,** Fluorophore number distributions for origami with 9, 20, and 35 binding sites. The histograms are modeled with a Gaussian to extract means and standard deviations (results and sample sizes in Table 1). Vertical dashed lines and areas shaded in grey indicate the expected mean and standard deviation obtained from binomial distributions. **c,** Fit results from b compared to the expected mean of the label number distribution, which is a binomial distribution with a labelling efficiency of 70%. Error bars and shaded region show the standard deviation. The quickPBSA result differs significantly from the result without quickPBSA refinement for 20 and 35 binding sites (two sample t-test, ***: $p<0.001$, n.s.: not significant) **d,** Measured label number distribution of origami with 9 binding sites for ATTO 647N on a different microscope setup with a larger field of view

and sCMOS detector. **e,** Measured label number distribution for origami with 9 binding sites for ATTO 565.

Figure 5 Protein counting of NUP107 in U2OS cells. **a,** Representative image of U2OS cell stably expressing NUP107-SNAP-tag labeled with BG-SiR. Scale bar: 5 µm. **b,** Traces extracted from the segmented ROI and background regions and evaluated difference trace from example trace b. **c,** Evaluated background-corrected trace extracted from ROI c (scaled to overlap). **d,** Measured fluorophore number distribution and Gaussian model fit (black line, mean 20.7±0.2, SD 8.5±0.2). 4000 traces from 32 cells, 2 independent experiments. **e**, Mean Fluorophore number per NPC per cell. Bars show mean±SD across cells (21.6±1.7).

Table 1 Mean and standard deviations extracted from Gaussian modelling of measured emitter number distributions from DNA origami experiments. Standard errors in brackets as extracted from least squares fitting. The expected values are calculated assuming a 70% labelling efficiency. *Measurement on alternative microscope setup with sCMOS detector. SD: Standard deviation. Sample size: number of traces; measurements; independent experiments.

Sample	Mean	Mean expected	SD	SD expected	Sample size	Runtime/ Trace [s]
R09	6.7 (0.1)	6.3	1.9 (0.1)	1.4	197;13;1	25
R20	14.2 (0.2)	14	6.1 (0.2)	2.1	636;43;2	53
R35	22.6 (0.5)	24.5	8.6 (0.5)	2.7	499;25;2	168
R09*	6.0 (0.1)	6.3	2.2 (0.1)	1.4	1,667;5;1	19
Y09	7.9 (0.1)	6.3	1.6 (0.1)	1.4	853;12;1	88

METHODS

Preparation of DNA *in vitro* samples

Custom brightness DNA origami with 9,20 or 35 nominal binding sites labeled with ATTO 647N or ATTO 565 at approximate labelling efficiencies of 70% (Gattaquant DNA Nanotechnologies, Germany) were dissolved in 0.5xTBE buffer supplemented with 11 mM $MgCl_2$ and stored at -20°C until use. DNA oligonucleotides labeled with 4 ATTO 647N (tetraATTO 647N) as previously described (Liesche et al., 2015) (biomers.net, Germany) were dissolved in phosphate buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4, Sigma Aldrich, Germany) and stored at -20°C until use. Both, DNA origami and DNA oligonucleotides were immobilized in 8-well LabTek (Nunc/Thermo Fisher, US) chambered coverslips via biotin-streptavidin linkage as previously described (Grußmayer and Herten, 2017). Prepared samples were kept in PBS (DNA oligonucleotides) or PBS supplemented with 20 mM $MgCl_2$ (DNA origami) unless stated otherwise.

Cell Culture

COS-7, U2OS and HeLa cells (all ATCC) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with GlutaMax and 1 mM sodium pyruvate (all Gibco Technologies, US). Cells were grown at 37°C, 5% CO_2 in humidified atmosphere and routinely subcultured every 3 days or upon reaching 80% confluency. Cultures were kept in culture for up to 30 passages and were not routinely tested for Mycoplasma contaminations. For widefield imaging, cells were seeded into 8-well LabTek chambered coverslips. Prior to seeding cells, LabTeks coverslips were cleaned with 0.1 M hydrofluoric acid for 2x30 s and extensively washed with PBS. Transfection of COS-7 cells was performed with TransIT-X2 transfection reagent (Mirus Bio, US) according to manufacturer's instructions 24 hours after cell seeding and at least 22 hours before fixation. Fixation of cells was performed with 3.7% (w/v) pre-warmed paraformaldehyde (PFA, EM Grade, Electron Microscopy Sciences, US) freshly diluted in PBS for 20 minutes at room temperature. All samples were washed repeatedly with PBS after fixation and imaged directly after or kept in PBS at 4°C until being imaged.

Preparation of cells expressing SNAP-tag or HaloTag

HeLa cells stably expressing *Escherichia coli (E. coli)* glutamine synthetase GlnA-HaloTag (Finan et al., 2015) were a gift of Florian Salopiata (DKFZ Heidelberg). U2OS cells stably

expressing NUP107-SNAP-tag were a gift of Jan Ellenberg (EMBL Heidelberg) (Otsuka and Ellenberg, 2017). Both cell lines were labeled with corresponding tag substrates directly prior to fixation. GlnA-HaloTag expressing cells were labeled with tetramethyl rhodamine (TMR) HaloTag ligand (HTL-TMR, Promega, US) or silicon rhodamine HaloTag ligand (SiR-HTL, Spirochrome, Switzerland) at 100 nM in growth medium for 120 min at 37°C. NUP107-SNAP-tag expressing cells were labeled with benzylguanine-functionalized TMR (BG-TMR, NEB, US) or SiR (BG-SiR, Spirochrome) at 200 nM in growth medium for 120 min at 37°C. After labelling, cells were washed repeatedly with growth medium and fixed as described above.

Imaging buffers

Samples were imaged either in PBS or in buffers containing different reducing and oxidizing systems (ROXS). ROXS buffers were prepared from a base solution (50 mM phosphate buffer, 13.5 mM KCl, 0.685 M NaCl and 10 mM $MgCl_2$, 12.5% (v/v) glycerol, pH 7.4, all Sigma-Aldrich) which was degassed by flowing Argon through buffers for at least 20 minutes before mixing or addition of buffers to samples. 1 mM paraquat dichloride and 1 mM ascorbic acid were added as reducing/oxidizing agents. Oxygen was depleted from the buffer by addition of 10 mM $NaSO_3$, 50 nM protocatechuate-3,4-deoxygenase (>3 U/mg) and 2.5 mM of protocatechuic acid (ROXS PCD) or 0.66 M D-Glucose, 5000 U catalase, and 40-80 U glucose oxidase (ROXS GodCat). The ROXS GodCat buffer was additionally supplemented with 1 mM Tris(2-carboxyethyl)phosphine. All buffer components were obtained from Sigma Aldrich (Germany).

Widefield fluorescence microscopy

If not stated otherwise, single-molecule fluorescence microscopy was performed on a custom-built inverted microscope (Nikon Eclipse Ti, Nikon, Japan) with epi-fluorescence and total internal reflection fluorescence (TIRF) illumination. The microscope setup included an autofocus system (PFS2) and a 100x 1.49 NA oil immersion objective (Apo TIRF, both Nikon). Images were recorded using a back-illuminated emCCD camera (iXon Ultra 897, Andor, UK) at 96 nm pixel size in the sample plane. A fiber-coupled multi laser engine (MLE-LFA, TOPTICA Photonics, Germany) equipped with 488, 561 and 640 nm laser lines was used for illumination. The excitation light was filtered by a quadband notch filter. A quadband dichroic mirror separated the emission and excitation beam paths. Emitted signal was further filtered using 525/50 nm, 605/70 nm and 690/70 nm bandpass filters (all AHF Analysetechnik, Germany) mounted in a motorized filter wheel (FW102C, Thorlabs, US) placed before the camera. All

microscope components were controlled using μManager (Edelstein et al., 2014). Exposure times, electron multiplying gain and illumination intensities were optimized for each sample individually to ensure maximum signals at the start of measurements while avoiding saturation of individual pixels.

Widefield fluorescence microscopy with extended field of view and homogeneous illumination

Single-molecule trace acquisition with improved throughput was performed on a custom-built widefield fluorescence microscope built around an inverted Axiovert 200 stand (Zeiss, Germany). A 647 nm fiber laser with Gaussian-shaped emission profile (MPB Communications, Canada) was expanded to 6.0 mm and converted into a flat-top beam using a beamshaper (πShaper, AdlOptica Optical Systems GmbH, Germany) and further expanded to a diameter of 47 mm. The expanded beam was then guided into the microscope stand and focused on the backfocal plane of a 100x 1.49 NA oil immersion objective (Apo TIRF, Nikon, Japan) objective. The variation in irradiance was below 15% across the entire illuminated area. Emitted signal was collected through the same objective, separated from excitation light using a quad-band dichroic filter (R405/488/561/635 Semrock, US) and further filtered using a 405/488/532/635 nm notch filter (Semrock) and a 700/50 nm bandpass filter (Chroma, US). Images were projected onto a back-illuminated sCMOS camera with a 130x130μm field of view (Prime95B, Photometrics, UK). Samples were placed on a motorized stage (MS2000) and kept in focus using an autofocus system (CRISP, both Applied Scientific Instrumentation, US). Camera and laser were synchronized using an Arduino Mega microcontroller board. All microscope components were controlled using μManager.

Fluorophore stability measurements

The photostability of different fluorophores and the influence of ROXS buffers on the blinking and photostability of fluorophores was evaluated by recording time-lapse data from samples labeled with the corresponding fluorophore upon high intensity excitation. The stability of ATTO 647N was evaluated using DNA oligonucleotides labeled with ATTO 647N immobilized as described above. TMR and SiR substrates for SNAP-tag and HaloTag were evaluated in fixed cells using cell lines expressing NUP107-SNAP-tag or GlnA-HaloTag as described above. The stability of EGFP, mCherry and mNeonGreen was evaluated in COS-7 cells transiently expressing H2A-EGFP-HaloTag (kind gift of Richard Wombacher), TOMM20-mCherry-HaloTag

(Werther et al., 2020) or TOMM20-mNeonGreen (Allele Biotechnology, US) fixed 24 hours after transfection. For each fluorophore, the stability in PBS pH 7.4 and the $NaSO_3$, ROXS PCD and ROXS GodCat buffer systems was tested with buffer compositions as described above. Prior to imaging, samples were washed once with PBS pH 7.4 and sample chambers were filled with the respective buffer and sealed with Parafilm to minimize gas exchange during experiments.

Bleaching curves were acquired on an epifluorescence setup for all buffer-fluorophore combinations described above. EGFP and mNeonGreen labeled structure were bleached at 0.58 kW/cm^2 average irradiance, mCherry and TMR labeled samples were bleached at 0.84 kW/cm^2 average irradiance and ATTO 647N and SiR labeled samples were bleached at 2.42 kW/cm^2 average irradiance. Image series were acquired with constant illumination until samples were fully bleached and the signal reached a plateau.

All data was background corrected by subtracting a constant offset from acquired image series to account for camera offset and excitation light bleedthrough. Offsets were manually determined for each sample and were found to be well reproducible within one condition, but variable across conditions. Bleach curves were then extracted from image series by extracting the frame-wise average intensity within a masked region. Masks were obtained from a Gaussian-filtered average projection of the first 10 images in the series and local thresholding following the Bernsen method. Mask segmentation and intensity extraction was performed using custom-written code in Fiji/ImageJ. Bleach curves were normalized to the maximum intensity in the respective trace and the raw half bleach time ($t_{1/2,raw}$) defined as the time at which the intensity traces had decayed to <50% of the maximum intensity was extracted using custom-written Matlab code. To facilitate comparison between fluorophores excited at different wavelengths, $t_{1/2,raw}$ were normalized against the applied illumination power density (IPD) to obtain the excitation power-corrected $t_{1/2} = t_{1/2,raw}/IPD$ at an illumination power density of 1 kW/cm^2.

Fluorophore photoblinking characterization

For characterization of fluorophore blinking, an EGFP-HaloTag-SNAP-tag fusion protein targeted to the plasma membrane via fusion to the N-myristoylation sequence MGCIKSKRKDNLNDDE was stably expressed in HeLa cells. Cells expressing this construct were grown in LabTek chambered coverslips and labeled with TMR or SiR for HaloTag or

SNAP-tag as described above. After labeling, cells were fixed in pre-warmed PBS supplemented with 4% PFA and 0.05% glutaraldehyde for 30 min at room temperature. Cells were then repeatedly washed in PBS and imaged directly after or kept in PBS at 4°C until being imaged. For recording of image time series, samples were prepared in either PBS or ROXS PCD buffer and individual cells were randomly selected for imaging. 2.000 frame image stacks were acquired with TIRF at an illumination power density of 50 W/cm^2 and with an exposure time of 25 ms on the alternative setup with homogenized illumination. Images were correlated following the approach developed in (Sehayek et al. 2019) using Matlab code provided by the authors available under https://github.com/ssehayek/blink-project.git. ACFs were computed using the first 1.000 frames of each image stack and a maximum lag time of 600 frames. The initial decay (lag times 2-125 frames) of obtained ACFs containing information about photoblinking were fitted to a 3-state model (*equalBleach*) developed by Sehayek et al.

Counting by Photon Statistics (CoPS) measurements

CoPS measurements were performed on a custom-built confocal microscope constructed around an inverted microscope stand (Axiovert 100, Zeiss, Germany). Linearly polarized light emitted by a ps-pulsed laser diode emitting at 640 nm (LDH P-C-640B, PicoQuant, Germany) operated at a repetition rate of 20 MHz diode was circularized using a quarter wave plate and coupled into a single-mode polarization maintaining fiber (Schäfter Kirchhof, Germany). The excitation light was directed towards a 100x NA 1.45 objective (Alpha Plan-Fluar, Zeiss) using a dichroic mirror (z532/640, CHROMA). Light emitted by the sample was collected by the same objective and passed through the dichroic filter. Scattered excitation light was removed using a quad-band notch filter (488/532/631-640 nm, AHF Analysetechnik). Remaining emitted light was then spatially filtered with a pinhole (100 µm diameter) placed in the focal plane between two achromatic doublet lenses (f=75 mm, Thorlabs). All remaining light was split into four equal paths using three 50:50 beamsplitters (Thorlabs) and focused on four APD detectors (SPCM AQR-13, Perkin-Elmer, US) using achromatic doublet lenses (f=200 mm, Thorlabs). 685/70 nm bandpass filters were place in front of each APD. Signals detected by the APDs were processed using a HydraHarp400 multichannel time-correlated single photon counting system and the SymPhoTime 64 software platform (both PicoQuant). The position of objective and sample were controlled by a one-axis piezo scanner (P-721 PIFOC) and a two-axis piezo stage (7332CD, both Physik Instrumente, Germany). The two-axis piezo stage was also used for stage scanning during image acquisition.

DNA origami samples were prepared for CoPS measurements as described above and imaged ca. 30 minutes after sealing of LabTek sample chambers. Measurements were initiated by acquisition of an overview scan to identify immobilized, diffraction-limited signals from individual DNA origami. TCSPC data was then recorded by focusing on individual origami with 10 µW excitation as measured before the objective.

CoPS coincidence data was analyzed by computing coincident photon histograms from the first 1×10^7 laser cycles (=0.5 s) of acquired TCSPC data for each origami. The histogram is then modelled with the analytical equation for coincidence probabilities (Ta et al., 2010) using the python scipy optimization model (Levenberg–Marquardt algorithm). Traces with a modelled molecular brightness of $<5\times10^{-3}$ were excluded.

Photobleaching step analysis

Data acquisition: Data for photobleaching step analysis was acquired in ROXS PCD buffer for all samples. DNA Origami data was acquired with 2.4 kW/cm2 average laser power at 640 nm for R09, R20, R35 and with 0.84 kW/cm2 average laser power at 561 nm for Y09 origami. NUP107 data was acquired with 1.2 kW/cm2 average laser power at 640 nm. Exposure times were 50 or 200 ms for all measurements.

Data analysis: The first 5 images from the measured image sequence were averaged and used to locate fluorophore clusters. The localization was performed with Fiji 1.52p (Schindelin et al., 2012; Schneider et al., 2012) using the plugin thunderSTORM (Ovesný et al., 2014). Trace extraction was done with the trace extraction submodule of the quickPBSA package, as detailed in the package documentation. In short, the average signal from circular regions around the localizations was extracted with typical diameters of 950 nm for the in vitro samples and 150 nm for the NUP107 experiment. For background correction, the average signal from ring-shaped regions was subtracted (inner diameter 1.7 µm for origami, 0.6 µm for NUP107, outer diameter 2.0 µm for origami, 0.9 µm for NUP107). Regions around neighboring localizations were excluded from the background region. Additionally, ROIs with nearest neighbors at a distance below 950 nm for DNA origami and 475 nm for NUP107 were excluded.

Photobleaching step analysis was performed using the quickPBSA package, as detailed in the main text and in the documentation of the quickPBSA package. Typically, the threshold parameter for preliminary step detection was set at 0.03 and maxiter at 200. Other analysis parameters were kept at their default values, except for the *mult_threshold* parameter in step refinement, which was typically set to 1.5 to decrease runtime.

Semi-synthetic datasets were generated by manual annotation of traces obtained from tetraATTO 647N DNA oligonucleotides measured in $NaSO_3$ ROXS buffer. The analysis according to (Tsekouras et al., 2016) was performed with the python code from https://github.com/lavrys/Photobleach. The analysis of the benchmarking dataset was performed on the University of Birmingham's High Performance Computing Service BlueBEAR (Intel CascadeLake, 40 cores). All other analyses were carried out on a workstation with an 8-core CPU @3.4 GHz (Intel(R) Core(TM) i7-3770) and 12 GB DDR3 memory.

Code availability

The quickPBSA package, example data and documentation are available under: https://github.com/JohnDieSchere/quickpbsa

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