

Photobleaching step analysis for robust determination of protein complex stoichiometries

Johan Hummert, Klaus Yserentant, Theresa Fink, Jonas Euchner, Yin Ho, Stanimir Tashev, and Dirk-Peter Herten

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-09-0568

TITLE: Photobleaching step analysis for robust determination of protein complex stoichiometries

Dear Prof. Herten:

Your manuscript, entitled "Photobleaching step analysis for robust determination of protein complex stoichiometries" has been seen by two referees whose verbatim comments are enclosed. While the referees felt that your findings, in principle, would be of interest, both reviewers raised some important points that need to be addressed. There are also a number of key points that require additional explanation. We would be willing to consider a revised manuscript that satisfies the joint concerns of the referees. Therefore, we look forward to receiving your revised manuscript, together with a letter indicating the changes you've made and your responses to the referees.

Sincerely,

Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Herten,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In their manuscript entitled "Photobleaching step analysis for robust determination of protein complex stoichiometries" the authors present a platform for stepwise bleaching analysis that integrates a recently published Bayesian analysis procedure by Presse and coworkers into a data analysis platform that in addition performs trace extraction with background subtraction, preliminary step analysis and filtering. The authors also characterize the bleaching of various fluorophores attached to different target proteins with different approaches in an effort to characterize their usefulness for stepwise bleaching. The authors validate their analysis platform with DNA origami containing a known number of fluorophores and show results from manually selected nuclear pore complexes as a proof of concept.

Overall the manuscript is well written and structured. The presented analysis platform for stepwise bleaching is likely very useful for the community still working with this technique and should be published in some form. However, I have concerns about the level of novelty or conceptual originality that would justify the publication of this work in MBoC as a research article. The characterization of bleaching and proof of concept with nuclear pore complexes are well conducted and rigorously analyzed. However, there are some conceptual problems regarding the comparability of data sets and the way that individual nuclear pores are manually selected for the data analysis platform that is meant to promote automation. In case the editor decides for publication or in case the authors approach a journal where they could publish this e.g. as a "resource", we strongly suggest working on revisions to address our points summarized below.

Fig 1 / Fig. S1: This data aims to compare the quality of different fluorescent proteins and fluorophores at different buffer conditions for stepwise bleaching analysis. However, a problem with these data sets is that the authors used different proteins for different fluorophores. The majority of these tagged proteins is expressed at such high levels that a single molecule or stepwise bleaching analysis seems impossible. The bleaching curves obtained from bulk fluorescence are not useful to assess the photophysics (e.g. blinking and brightness during on-time) and quality for stepwise bleaching. Instead, the authors should rather focus on showing more step-wise bleaching curves of different fluorophores at different conditions (such as Fig. 1c-f) and show single-molecule quantification with a larger statistics (e.g. blinking, off- and on- times; reversibility of bleaching etc.) that actually compares the quality of fluorophores for step-wise bleaching.

Fig. 4. The authors use the nuclear pore complex as a proof of concept and run into the main limitation of stepwise bleaching, which is the limited resolution and therefore the overlap of pores and out of focus fluorescence. The authors therefore manually select NPCs that are spatially well separated as well as regions for the background correction. It seems unfortunate that a platform meant for automating data analysis is then performed on manually selected objects (and the authors criticize earlier any subjective manual selection as the main motivation for their work). Furthermore, this data makes a big case for PALM/STORM since NPCs have been extensively used as a calibration standard for counting molecules with PALM, which does not have the problem of spatial overlap and has no out-of-focus background signals. Could the authors automate this by e.g. running a PALM analysis on the first frames and only selecting NPCs with a small width/ circular PSF and lower background and then running quickPBBSA? Also, it would be interesting if the broad distribution in Fig. 4d is real or an artefact from the analysis. Could the authors e.g. correlate the initial intensity of a NPC with the number of observed steps to further elucidate this?

Minor comments:

The comment in the first sentence of the abstract..."posing minimal demands on the microscope" is misleading. The authors use the same microscope/cameras/lasers as for PALM/STORM - techniques that have big advantages of higher resolution and single molecule counting capability.

In this introduction the sentence that "no calibration measurements are necessary..." is misleading. As the authors show with DNA origami, not all fluorophores are detectable or not all sites are labeled, which needs a calibration measurement of the fraction of detectable fluorophores in order to estimate absolute molecule numbers.

Page 7 "a new algorithm for trace interpretation". It is not clear what that algorithm is and whether the authors refer to the algorithm by Presse and coworkers or their initial step extraction and filtering. Other groups routinely employ similar means to extract traces from raw data so it should be explained what the novel aspect of the author's approach is.

Figure 3c. The comparison of the full algorithm to the crude initial step detection is not particularly useful. It would be more interesting to see a comparison of the full algorithm with the algorithm up to and including step 3. It would also be interesting to perform an ANOVA or t-test to see if results are significantly different.

Methods, "Photobleaching step analysis":

The beginning states that first a thunderSTORM has to be performed before using the author's software platform. This seems to defeat the author's motivation of this work to integrate different stepwise bleaching algorithms in one easy to use platform. Can the authors include this analysis in their platform? Also, one could argue that the first steps could just be completely performed in thunderSTORM since the brightness and the background of each cluster can be tracked over time. Can the authors compare their approach to other existing ones?

The authors mention a reduction in computational time as a big advantage of their platform. However, it is not clear what the

durations are of the steps up to and including step 3 last. One could think that the increase in speed comes from restricting the parameter space during step 4, but this could affect the accuracy. The authors should provide a bit more information about the analysis time of different steps and, moreover, if/how parameter-space restriction affects the accuracy of results.

Reviewer #2 (Remarks to the Author):

The manuscript by Hummert and colleagues presents a method to detect and quantitatively analyze photobleaching steps from fluorescence emitters as recorded in a wide-field fluorescence microscope. The authors developed novel code to employ automated analysis on a time-series of images. They validated the analysis procedure on well-characterized samples based on DNA origami and demonstrated applicability to cellular protein complexes. The manuscript is well structured, and based on a good data selection to convincingly demonstrate the analysis scheme. I have a few detailed remarks that the authors might want to address:

- 1) Page 3: Is it true that Bayesian methods need too much computational power for such analysis schemes? The amount of data is not that large; I would guess that the Bayesian algorithms are performing well within a reasonable amount of time. The interesting question is maybe rather how much better a Bayesian method could be; maybe not that much. Please add a comment on this.
- 2) Page 5: "...input data, i.e. individual photobleaching traces" - please add a clarification if a single photobleaching trace refers to a single PSF image or to an arbitrary region (what size). How is a trace defined if single-molecule spots are not spatially well separated? It would help to elaborate a bit more on these fundamentals in the beginning of the results section.
- 3) Page 5: The beginning of the second paragraph is a lot of repetition of the first paragraph and could be shortened.
- 4) Page 6: "... observed varying photobleaching decay patterns..." How much do the patterns deviate from a single exponential decay? Is a double exponential usually good enough to fit the decays? Is there any rational explanation for the observed deviation?
- 5) From Fig. 3 it appears that the length of step levels increases over time. This is expected for stochastic reasons. It would be interesting to see, if the step duration indeed exhibits a distribution as expected, or if this distribution might hint at unexpected photophysical effects or other influences. The presented data and the analysis scheme offers a great opportunity to have a quick look on this.
- 6) Page 13: The fluorophore number estimates were fitted to a normal distribution with a standard deviation much larger than expected. Do you have any idea what else can contribute to this broadening? E.g. are there experimental issues involved? How much bleaching is expected during initial searching and focusing procedures? Comparing origami results with NPC results, the relative standard deviation is surprisingly similar. This indicates that it is not the labeling procedure that determines the width. Along these lines: a labeling efficiency of 70% was stated several times. Could you provide some more information on this estimate (experimental method, reference, variation, accuracy). Some more discussion on the observed widths will be helpful.
- 7) Page 14: "the expected mean and width of the fluorophore number... were well reproduced." But as you just said a few lines earlier, this is not the case. The width deviates strongly from expectation.
- 8) An interesting and important application would be to derive the number of antibodies that are bound to a certain protein complex. Assuming that a single antibody carries 1 to ~5 fluorophores (according to the degree of labeling and including some randomness) the presented analysis scheme should be capable of determining if there is more than one antibody (or more than two etc.) bound. Maybe a short paragraph on the statistical power that this analysis could offer would make an interesting addition to the discussion section.

Overall, I find the manuscript of great interest, providing a well characterized analysis procedure that can easily be adapted by researchers in the field. I recommend accepting the manuscript for publication after minor revision.

Point-by-point response

Reviewer #1 (Remarks to the Author):

Comment 1.0: *In their manuscript entitled "Photobleaching step analysis for robust determination of protein complex stoichiometries" the authors present a platform for stepwise bleaching analysis that integrates a recently published Bayesian analysis procedure by Presse and coworkers into a data analysis platform that in addition performs trace extraction with background subtraction, preliminary step analysis and filtering. The authors also characterize the bleaching of various fluorophores attached to different target proteins with different approaches in an effort to characterize their usefulness for stepwise bleaching. The authors validate their analysis platform with DNA origami containing a known number of fluorophores and show results from manually selected nuclear pore complexes as a proof of concept.*

Overall the manuscript is well written and structured. The presented analysis platform for stepwise bleaching is likely very useful for the community still working with this technique and should be published in some form. However, I concerns about the level of novelty or conceptual originality that would justify the publication of this work in MBoC as a research article. The characterization of bleaching and proof of concept with nuclear pore complexes are well conducted and rigorously analyzed. However, there are some conceptual problems regarding the comparability of data sets and the way that individual nuclear pores are manually selected for the data analysis platform that is meant to promote automation. In case the editor decides for publication or in case the authors approach a journal where they could publish this e.g. as a "resource", we strongly suggest working on revisions to address our points summarized below.

Response 1.0: We thank Reviewer #1 for sharing our view that a comprehensive platform for photobleaching step analysis will be useful for the community. The raised concerns made us aware that we missed to clearly describe the novelty of our algorithm (response 1.5) and its benefits (automation and significantly reduced computational cost; response 1.8) compared to existing photobleaching analysis methods. We would like to stress that, in contrast to the reviewer's impression, the presented measurements on nuclear pore complexes were performed in an automated manner without manual selection as we will explain in more detail below (response 1.2).

Comment 1.1: *Fig 1 / Fig. S1: This data aims to compare the quality of different fluorescent proteins and fluorophores at different buffer conditions for stepwise bleaching analysis. However, a problem with these data sets is that the authors used different proteins for different fluorophores. The majority of these tagged proteins is expressed at such high levels that a single molecule or stepwise bleaching analysis seems impossible. The bleaching curves obtained from bulk fluorescence are not useful to assess the photophysics (e.g. blinking and brightness during on-time) and quality for stepwise bleaching. Instead, the authors should rather focus on showing more step-wise bleaching curves of different fluorophores at different conditions (such as Fig. 1c-f) and show single-molecule quantification with a larger statistics (e.g. blinking, off- and on- times; reversibility of bleaching etc.) that actually compares the quality of fluorophores for step-wise bleaching.*

Response 1.1: We thank Reviewer #1 for flagging the importance of fluorophore characterization for assessing the suitability of different fluorescent labels for photobleaching step analysis. However, we think that bulk photobleaching experiments are indeed well suited for assessing the photostability of labels and we think that for such measurements neither a high expression rate nor the conjugation to the target will influence the results of this characterization. To stress the importance of photostability (and molecular brightness) for recording photobleaching traces with high SNR we added the following statement to the manuscript:

"Both, the molecular brightness of a fluorescent label and its photostability contribute to the overall photon budget and thereby directly influence the SNR."

As suggested by the reviewer, we have furthermore included a new set of experiments to characterize label photophysics. Here, we used the image correlation approach published by Sehayek et al. in 2019 (doi: 10.1021/acsnano.9b06033) to measure the on- and off-rates for fluorophore transitions into dark states during photoblinking. In the revised manuscript, we report the respective on-times and the on/off equilibrium constants obtained from these measurements. The additional data are presented in fig. 1 and in the supporting information (figs. S3 – S5) and provide selection guidelines for suitable fluorophores. Since our approach for characterizing photoblinking is not based on the analysis of individual single-molecule intensity traces, we provide exemplary intensity traces obtained from different fluorophores and conditions in fig 1c-f to provide the readers with additional visual evidence for our findings.

Comment 1.2: *Fig. 4. The authors use the nuclear pore complex as a proof of concept and run into the main limitation of stepwise bleaching, which is the limited resolution and therefore the overlap of pores and out of focus fluorescence. The authors therefore manually select NPCs that are spatially well separated as well as regions for the background correction. It seems unfortunate that a platform meant for automating data analysis is then performed on manually selected objects (and the authors criticize earlier any subjective manual selection as the main motivation for their work). Furthermore, this data makes a big case for PALM/STORM since NPCs have been extensively used as a calibration standard for counting molecules with PALM, which does not have the problem of spatial overlap and has no out-of-focus background signals. Could the authors automate this by e.g. running a PALM analysis on the first frames and only selecting NPCs with a small width/ circular PSF and lower background and then running quickPBSA? Also, it would be interesting if the broad distribution in Fig. 4d is real or an artefact from the analysis. Could the authors e.g. correlate the initial intensity of a NPC with the number of observed steps to further elucidate this?*

Response 1.2: We are grateful for the critical comment of Reviewer #1 as it indicates that we missed to clearly state that the selection of nuclear pore complexes is fully automated and is in fact already based on localization parameters as the reviewer suggested. We have edited the corresponding section of our manuscript to describe the selection process more clearly. The revised version of the manuscript now states:

" 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."

Regarding the second point raised by the reviewer: We compared the initial (t_0) intensity against the respective quickPBSA emitter number estimate in Figure R1 of this response (see below). Although there is a correlation, there is a larger variance in t_0 intensity, probably stemming from NPCs located in different focal planes or at different positions within the non-uniform illumination profile. We believe that the broadened emitter number distribution observed on NPCs is not caused by the sample, i.e. by heterogeneous composition of NPCs, but rather gives an estimate about the precision of emitter counting with quickPBSA.

Furthermore, a recent paper (Thevathasan *et al.* 2019, doi: 10.1038/s41592-019-0574-9) reported similar variances using PALM- and intensity-based counting of NUPs in NPCs suggesting that our PBSA-based counting approach achieves a comparable accuracy. To facilitate a direct comparison with data from this report, we included an analysis of the mean number of labeled NUP107-SNAP proteins per NPC per cell in fig. 5e of the revised manuscript.

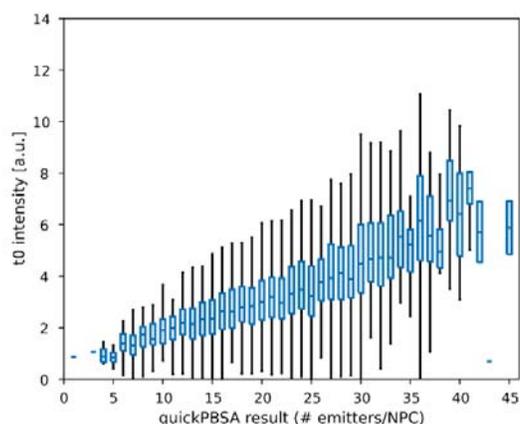


Figure R1: Comparison of intensity and quickPBSA emitter number estimate for NUP107-SNAP dataset shown in Figure 5 of our manuscript.

Note: When reviewing the NPC dataset, we removed one dataset from the manuscript that was prepared under different fixation conditions. Removal only insignificantly changed the distribution mean from 20.6 to 20.7 and is still within the standard error of mean (0.2). The updated dataset still contains 4000 individual NPCs from 32 cells and two independent experiments.

Minor comments

Comment 1.3: *The comment in the first sentence of the abstract... "posing minimal demands on the microscope" is misleading. The authors use the same microscope/cameras/lasers as*

for PALM/STORM - techniques that have big advantages of higher resolution and single molecule counting capability.

Response 1.3: We agree that the equipment used in this study to perform photobleaching step analysis is similar to what is typically used for PALM/STORM-type experiments. However, from our experience the laser powers required for high fidelity STORM experiments such as quantitative PALM/STORM are typically higher than the laser powers used in this study. To avoid misleading readers, we removed the second part of the first sentence in the abstract.

Comment 1.4: *In this introduction the sentence that "no calibration measurements are necessary..." is misleading. As the authors show with DNA origami, not all fluorophores are detectable or not all sites are labeled, which needs a calibration measurement of the fraction of detectable fluorophores in order to estimate absolute molecule numbers.*

Response 1.4: We thank Reviewer #1 for indicating the lack of clarity and edited the sentence to:

" PBSA has the advantage that counting of fluorophores requires no calibration and that it is relatively robust to variations in molecular brightness."

to make clear that calibration of labelling efficiency is still required.

Comment 1.5: *Page 7 "a new algorithm for trace interpretation". It is not clear what that algorithm is and whether the authors refer to the algorithm by Presse and coworkers or their initial step extraction and filtering. Other groups routinely employ similar means to extract traces from raw data so it should be explained what the novel aspect of the author's approach is.*

Response 1.5: We acknowledge the critical assessment of Reviewer #1 and agree that we were not sufficiently clear about the novel aspect of our algorithm. To mitigate this, we edited the description of the algorithm and moved the flowchart previously shown in the SI to fig 2 in the main text to stress the novelty of the iterative approach to maximize the Bayesian Posterior defined by Presse and coworkers. We also included an example trace in the same figure to clearly show how our iterative algorithm finds the optimal step height distribution. Trace extraction, background subtraction, and the Kalafut Vischer algorithm are not conceptually novel, and were only included in the quickPBSA software package to facilitate automated analysis of large datasets. However, using the Bayesian posterior not to determine step positions but only to determine significance and step heights for pre-detected steps is completely different from the approach taken in the 2016 paper from Pressé and coworkers, where every possible step position is evaluated with the Bayesian posterior. Exactly this difference is what causes the major runtime gain (see answer to points 1.8 and 2.1) and the significant improvement in computational cost compared to the previous approach by Pressé et al.

Comment 1.6: *Figure 3c. The comparison of the full algorithm to the crude initial step detection is not particularly useful. It would be more interesting to see a comparison of the full algorithm with the algorithm up to and including step 3. It would also be interesting to perform an ANOVA or t-test to see if results are significantly different.*

Response 1.6: We anticipate that this comment is related to the lack in clarity of the algorithm's description in the previous version of our manuscript. The comparison in question is already shown in figure 4 (formerly fig. 3). We edited the figure caption for fig 4c (previously 3c), which, together with the updated description of the algorithm, should improve clarity. Additionally, we now show the results of a t-test in fig 4c, supporting that improvements from step refinement are significant for origami with 20 and 35 binding sites.

Comment 1.7: *Methods, "Photobleaching step analysis": The beginning states that first a thunderSTORM has to be performed before using the author's software platform. This seems to defeat the author's motivation of this work to integrate different stepwise bleaching algorithms in one easy to use platform. Can the authors include this analysis in their platform? Also, one could argue that the first steps could just be completely performed in thunderSTORM since the brightness and the background of each cluster can be tracked over time. Can the authors compare their approach to other existing ones?*

Response 1.7: We would argue that not including the initial localization step makes the quickPBSA framework more flexible, since alternative ROI selection methods can be applied. Our aim is rather to provide an algorithm that is not purely for trace analysis, but which uses the information from the image stacks, for instance, via existing localization algorithms for selection of ROIs suitable for photobleaching step analysis. To illustrate the flexibility of quickPBSA to extract traces based on different inputs, we included an additional SI figure (Fig. S6) showing how traces are extracted based on sub-pixel localization or masks as input.

Performing the entire trace extraction via thunderSTORM as suggested by the reviewer is, in our eyes, computationally substantially less efficient than performing trace extraction with quickPBSA (on average 21 s for a 15.000 frame image stack with quickPBSA). In addition, vanishing localization due to complete photobleaching would prohibit extraction of intensity traces beyond the last frame where a trace contains active emitters. However, precisely this information beyond the last photobleaching event is crucial for initial step detection and would therefore require additional processing of the thunderSTORM result.

Comment 1.8: *The authors mention a reduction in computational time as a big advantage of their platform. However, it is not clear what the durations are of the steps up to and including step 3 last. One could think that the increase in speed comes from restricting the parameter space during step 4, but this could affect the accuracy. The authors should provide a bit more information about the analysis time of different steps and, moreover, if/how parameter-space restriction affects the accuracy of results.*

Response 1.8: Since trace extraction and filtering requires negligible runtime (see response 1.7) and alternative algorithms usually do not contain modules for this purpose, the relevant comparison is the one for the runtime of step detection during trace analysis. We now show a

detailed comparison of runtimes required and precision achieved with the quickPBSA algorithm, the initial step detection algorithm by Kalafut and Visscher and the Bayesian algorithm by Pressé and coworkers in Fig. 3 of the revised manuscript. This comparison shows that quickPBSA achieves a 100-fold speed up with no significant loss in accuracy as compared to the approach by Pressé et al. In our framework, runtime is dominated by the Kalafut Vischer implementation for up to ~20 fluorophores. Only at >20 fluorophores the refinement step starts to contribute significantly. The gain in runtime mainly comes from the restriction of the parameter space (i.e., not finding step positions with the posterior), which as we show does not result in decreased accuracy.

Reviewer #2 (Remarks to the Author):

Comment 2.0 *The manuscript by Hummert and colleagues presents a method to detect and quantitatively analyze photobleaching steps from fluorescence emitters as recorded in a wide-field fluorescence microscope. The authors developed novel code to employ automated analysis on a time-series of images. They validated the analysis procedure on well-characterized samples based on DNA origami and demonstrated applicability to cellular protein complexes. The manuscript is well structured, and based on a good data selection to convincingly demonstrate the analysis scheme. I have a few detailed remarks that the authors might want to address:*

Response 2.0: We very much appreciate the positive assessment of Reviewer #2.

Comment 2.1 *Page 3: Is it true that Bayesian methods need too much computational power for such analysis schemes? The amount of data is not that large; I would guess that the Bayesian algorithms are performing well within a reasonable amount of time. The interesting question is maybe rather how much better a Bayesian method could be; maybe not that much. Please add a comment on this.*

Response 2.1: We thank Reviewer #2 for this interesting question. The admittedly large benchmarking dataset (~4.000 traces) was run on high-performance computing nodes (40 cores) with a total runtime of ~10 min (~400 Traces/min) for quickPBSA and a runtime of ~60 h (~1 Trace/min) for the algorithm by Pressé et al ("Presse2016"). We would argue that biological experiments requiring controls and replicates as well as accounting for cell-to-cell variation, typically produce large datasets where the achieved difference in runtime is meaningful.

Comment 2.2 *Page 5: "...input data, i.e. individual photobleaching traces" - please add a clarification if a single photobleaching trace refers to a single PSF image or to an arbitrary region (what size). How is a trace defined if single-molecule spots are not spatially well separated? It would help to elaborate a bit more on these fundamentals in the beginning of the results section.*

Response 2.2: We thank Reviewer #2 for indicating the lack in clarity. We edited the description of the trace extraction and added a supplementary figure (Fig. S6) to show how traces are generated. Also, overlapping ROIs are removed from further analysis by filtered as stated in the description and shown in Fig. S6. However, at this point in the manuscript, a trace is just the decay of fluorescence intensity from an arbitrary region within an image.

Comment 2.3 *Page 5: The beginning of the second paragraph is a lot of repetition of the first paragraph and could be shortened.*

Response 2.3: We thank the reviewer for this suggestion which should help to improve the readability of our manuscript. Following the reviewer's suggestion, we rewrote and shortened the first paragraphs of the results section by ~25%.

Comment 2.4 *Page 6: "... observed varying photobleaching decay patterns..." How much do the patterns deviate from a single exponential decay? Is a double exponential usually good enough to fit the decays? Is there any rational explanation for the observed deviation?*

Response 2.4: As suggested by the reviewer, a biexponential fits well for all dye/conditions included in our dataset. To reflect on this, we now specify that the intensity decays followed a biexponential decay as has previously been reported by others (e.g., Diaspro *et al.* 2006, Handbook of Confocal Microscopy; Song *et al.* 1995, doi: 10.1016/S0006-3495(95)80442-X; Bakker *et al.* 2019, doi: 10.1038/s41598-019-50921-7). The updated sentence in the manuscript reads:

"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)."

Comment 2.5 *From Fig. 3 it appears that the length of step levels increases over time. This is expected for stochastic reasons. It would be interesting to see, if the step duration indeed exhibits a distribution as expected, or if this distribution might hint at unexpected photophysical effects or other influences. The presented data and the analysis scheme offers a great opportunity to have a quick look on this.*

Response 2.5: We thank Reviewer #2 for this very interesting suggestion. As shown in the figure below, the distribution of step distances shows a biexponential decay as it was observed in the stability measurements. However, this seems a very lengthy way of measuring essentially the same information as in the fluorescence decay, so we did not include this analysis in the manuscript.

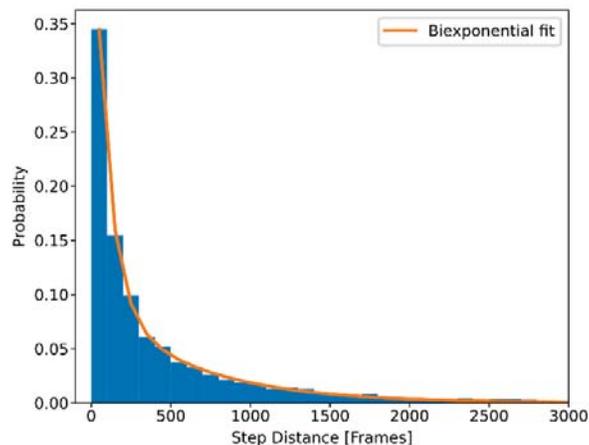


Figure R2: Distribution of distances between steps for the dataset obtained with the R20 origami.

Comment 2.6 *Page 13: The fluorophore number estimates were fitted to a normal distribution with a standard deviation much larger than expected. Do you have any idea what else can contribute to this broadening? E.g. are there experimental issues involved? How much bleaching is expected during initial searching and focusing procedures? Comparing origami results with NPC results, the relative standard deviation is surprisingly similar. This indicates that it is not the labeling procedure that determines the width. Along these lines: a labeling efficiency of 70% was stated several times. Could you provide some more information on this estimate (experimental method, reference, variation, accuracy). Some more discussion on the observed widths will be helpful.*

Response 2.6: The question regarding the width of the distribution was also raised by Reviewer #1 (please see Comment 1.2). As stated in Response 1.2, we assume that additional sources of uncertainty in the experimental system are not reflected in our semi-synthetic dataset calling for additional benchmarking with counting standards (please check Response 1.2 for the resp. changes in the revised manuscript). We don't think that photobleaching during preparation of measurements (searching, focusing) plays a significant role since we limited the light exposure during this step in three ways: 1) We reduced the excitation intensity by >90%, 2) we made use of a hardware autofocus which allows us to focus on a distant part of the sample and maintain focus stability throughout measurements and 3) we acquired overview scans of the sample for selection of nuclei for measurements (~0.05 s exposure during search vs. 1.000 s during acquisition of photobleaching data).

Regarding the labelling efficiency of the DNA origami: We stated that this is specified by the manufacturer. Additionally, we have independently verified the labelling efficiency using our own counting approach, CoPS (Counting by Photon Statistics), for the R20 origami and included the results of this measurement in the SI (Fig. S8).

Comment 2.7 Page 14: *"the expected mean and width of the fluorophore number... were well reproduced." But as you just said a few lines earlier, this is not the case. The width deviates strongly from expectation.*

Response 2.7: We thank Reviewer #2 for spotting this inconsistency. The sentence was supposed to refer only to the R9 origami where the width is well reproduced. We changed the sentence in the revised manuscript accordingly to:

"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)."

Comment 2.8 *An interesting and important application would be to derive the number of antibodies that are bound to a certain protein complex. Assuming that a single antibody carries 1 to ~5 fluorophores (according to the degree of labeling and including some randomness) the presented analysis scheme should be capable of determining if there is more than one antibody (or more than two etc.) bound. Maybe a short paragraph on the statistical power that this analysis could offer would make an interesting addition to the discussion section.*

Response 2.8: We thank Reviewer #2 for raising this very interesting point. Indeed, we have characterized and discussed the label number distribution of fluorescently labelled antibodies (with a defined DOL) in previous experiments (Grüßmayer *et al.* 2014, doi: 10.1002/cphc.201300840).

Comment 2.9: *Overall, I find the manuscript of great interest, providing a well characterized analysis procedure that can easily be adapted by researchers in the field. I recommend accepting the manuscript for publication after minor revision.*

Response 2.9: Again, we thank Reviewer #2 for the very positive assessment and the helpful comments.

RE: Manuscript #E20-09-0568R

TITLE: "Photobleaching step analysis for robust determination of protein complex stoichiometries"

Dear Prof. Herten:

Thank you for submitting your revised manuscript. While the reviewers agree that it is much improved, there remain a few minor points that need to be addressed. We look forward to receiving your revised manuscript and a letter indicating your response to the referees in the near future. If you are able to address these points, I anticipate that there should be no need for further review.

Sincerely,
Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Herten,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In their revised manuscript entitled "Photobleaching step analysis for robust determination of protein complex stoichiometries" the authors addressed some of our concerns. However, we have some concerns regarding the new analysis they performed to characterize the photophysical properties of fluorophores. After these and a few other points are addressed, we believe that this manuscript is suitable for publication in MBoC and will be a useful resource.

Comment 1.1: The authors calculate an image autocorrelation across time and fit this data to an autocorrelation function derived from a known photophysical model to extract photobleaching rates. One of the challenges of this method (outlined by Sehayek et al 2019) is that the model needs to be pre-defined and assumes a 3 state model with one active state, one dark state, and one bleach state. Dyes and fluorescent proteins have different photophysical properties and often more than one dark state. Even the same dye/protein can exhibit different photophysical properties when placed in different environments including having a different number of dark states. Applying a 3 state model to different fluorophores leads to inaccurate kinetic rate constant estimation. This means that the rate constants calculated in Figure S3 and S5 are most likely inaccurate. Furthermore, later in the response, the authors justify they use a bi-exponential decay function to fit the observed bleaching curves. The bi-exponential decay function is derived from a 2 dark state model which is different from the model that is used to calculate rate constants.

Another concern is that the model used in Sehayek et al 2019 incorporates additive Gaussian noise which is incorrect since the camera noise of an EMCCD camera is multiplicative and is characterized by a Poisson distribution. Since the data is fit to an autocorrelation of the intensity measurements over time, the model is inaccurately estimating the true intensity to calculate rate constants. This also leads to inaccurate estimations of the rate constant. Though the deviation between the true and estimated rate constants can be tapered over at high photon count regimes where Poisson noise can be approximated as Gaussian, the deviation will be most profound at lower photon counts which is similar to the emission intensities of most fluorescent proteins and sparsely distributed dyes.

On a broader note, it is challenging to explicitly characterize the photophysical properties of different fluorophores or the even same fluorophore in different environments due to the differences in the number of dark states. Since photophysical characterization is not directly required for the quickPBSA algorithm, the authors should consider leaving figures S3 and S5 out of the paper. The autocorrelation data displayed in figure S4 is sufficient to show changes in photophysical properties among different fluorophores. If the authors want to include rate constant estimations in figures S3 and S5, then the authors should at least fit their autocorrelation function to a kinetic model with at least 2 dark states to make it consistent with the rest of the paper. If the authors choose the second route, these papers provide good models to choose from (DOI: 10.1214/19-AOAS1240, <https://doi.org/10.1093/bioinformatics/btab136>)

Comment 1.2: The authors have clarified that nuclear pores were not selected manually and were instead automatically filtered, which helps to promote the ease-of-use and usefulness of their software package. However, the authors have not directly demonstrated that quickPBSA can achieve a lower variance than estimating the stoichiometry from e.g. intensity information at t_0 , as claimed in Response 1.2 and in Figure R1, from which an uncertainty in the number is missing. Would the authors be able to address this point and demonstrate a more narrow number distribution of quickPBSA compared to other methods? The authors should also explicitly cite the width of the distribution as the accuracy but also state that this is comparable to previous PALM-based counting approaches.

Comment 1.4. Our original concern was maybe not as clear. What we meant is that while PBSA may have a certain accuracy for counting detectable fluorescent proteins or dyes, not all fluorescent proteins may be detectable (e.g. only 60% of mEos2 proteins in a sample are detectable) or not all labeling sites may be occupied with a dye. This is not a problem of PBSA but any counting method in general. For instance, the authors show that they do detect fewer molecules on DNA origamis as they expect. It should therefore be clearly stated somewhere in the manuscript that PBSA can only count detectable fluorophores and that calibration measurements may be required to determine the labeling efficiency and the true number of proteins.

Minor suggestions:

Heading of Fig. 1 e and f are identical but should probably be different conditions.

Fig. 3 b and c (Presse 2016) We suggest making the y-axes range for all plots the same to allow a better visual comparison.

Fig. S6 please include scale bars.

Fig. S6 do the authors use a specific scheme for determining the background ROI? It seems that it is a ring located 1 pixel from the center ROI with a thickness of 2 pixels, but this is not mentioned specifically in the text.

Reviewer #2 (Remarks to the Author):

Hummert et al. have carefully revised their manuscript on photobleaching step analysis. The manuscript has been improved a lot and selected issues have been clarified in the manuscript and supporting information. In my opinion, the manuscript is well suited for publication in *Molecular Biology of the Cell*.

Rebuttal letter

Reviewer #1 (Remarks to the Author)

Comment R1.1: In their revised manuscript entitled "Photobleaching step analysis for robust determination of protein complex stoichiometries" the authors addressed some of our concerns. However, we have some concerns regarding the new analysis they performed to characterize the photophysical properties of fluorophores. After these and a few other points are addressed, we believe that this manuscript is suitable for publication in MBoC and will be a useful resource.

Response R1.1: We thank reviewer 1 for their positive feedback on our revised manuscript and their suggestions on how to further improve the manuscript. We are happy to hear that they deem our work suitable for publication in MBoC after final revisions.

Comment R1.2: Comment 1.1: The authors calculate an image autocorrelation across time and fit this data to an autocorrelation function derived from a known photophysical model to extract photobleaching rates. One of the challenges of this method (outlined by Sehayek et al 2019) is that the model needs to be pre-defined and assumes a 3 state model with one active state, one dark state, and one bleach state. Dyes and fluorescent proteins have different photophysical properties and often more than one dark state. Even the same dye/protein can exhibit different photophysical properties when placed in different environments including having a different number of dark states. Applying a 3 state model to different fluorophores leads to inaccurate kinetic rate constant estimation. This means that the rate constants calculated in Figure S3 and S5 are most likely inaccurate. Furthermore, later in the response, the authors justify they use a bi-exponential decay function to fit the observed bleaching curves. The bi-exponential decay function is derived from a 2 dark state model which is different from the model that is used to calculate rate constants. Another concern is that the model used in Sehayek et al 2019 incorporates additive Gaussian noise which is incorrect since the camera noise of an EMCCD camera is multiplicative and is characterized by a Poisson distribution. Since the data is fit to an autocorrelation of the intensity measurements over time, the model is inaccurately estimating the true intensity to calculate rate constants. This also leads to inaccurate estimations

of the rate constant. Though the deviation between the true and estimated rate constants can be tapered over at high photon count regimes where Poisson noise can be approximated as Gaussian, the deviation will be most profound at lower photon counts which is similar to the emission intensities of most fluorescent proteins and sparsely distributed dyes. On a broader note, it is challenging to explicitly characterize the photophysical properties of different fluorophores or the even same fluorophore in different environments due to the differences in the number of dark states. Since photophysical characterization is not directly required for the quickPBSA algorithm, the authors should consider leaving figures S3 and S5 out of the paper. The autocorrelation data displayed in figure S4 is sufficient to show changes in photophysical properties among different fluorophores. If the authors want to include rate constant estimations in figures S3 and S5, then the authors should at least fit their autocorrelation function to a kinetic model with at least 2 dark states to make it consistent with the rest of the paper. If the authors choose the second route, these papers provide good models to choose from (DOI: 10.1214/19-AOAS1240, <https://doi.org/10.1093/bioinformatics/btab136>).

Response R1.2: We agree with the reviewer that the three state model proposed by Sehayek et al. is not necessarily the correct model for all fluorophores in question. We also agree with the reviewer that the kinetic rates obtained from fitting a three-state model to ACFs do not necessarily reflect true kinetic rates due to a multitude of possible photophysical reactions for different fluorophores and conditions. However, we obtained good fits for this model across different conditions (see Fig. S3 for examples) and we therefore believe that the rates obtained are well-suited for a relative comparison of different fluorophores and imaging conditions. To stress this, we added the following sentence to the manuscript:

"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."

Further, we would like to note that the employed three-state model is consistent with a biexponential intensity decay upon prolonged illumination as it was observed in the photobleaching experiments. To demonstrate this, we numerically solved the rate equations for the three-state model used in the

autocorrelation analysis with and without photoblinking (Figure R1). In the case with photoblinking (Fig. R1b), the fluorescent on-state clearly exhibits a biexponential decay. We therefore disagree with the reviewers claim that the autocorrelation and bleaching analysis in the manuscript are inconsistent.

Finally, we would like to refer to the original publication by Sehayek et al. which shows that their correlation-based analysis is able to retrieve rate constants consistent with single-molecule analysis and the influence of chemical agents on photoblinking could clearly be quantified. This was accomplished despite possibly using an incorrect noise model, meaning that this does not seem to have significant influence.

For the reasons listed above, we decided to keep Figs. S3 & S5 as supplementary figures to support our findings presented in Fig. 1b.

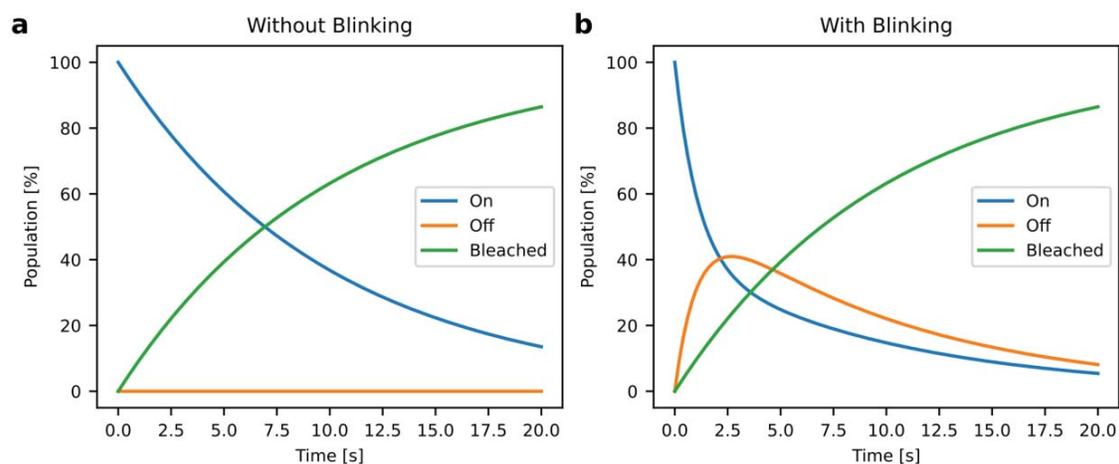


Figure R1: Numerically solved rate equations for the model depicted in Figure S3. **a**, without blinking, t_{on} 0 s, t_{off} 0 s, t_{pb} 10 s. **b**, with blinking, t_{on} 3 s, t_{off} 2s, t_{pb} 10 s.

Comment R1.3: Comment 1.2: The authors have clarified that nuclear pores were not selected manually and were instead automatically filtered, which helps to promote the ease-of-use and usefulness of their software package. However, the authors have not directly demonstrated that quickPBSA can achieve a lower variance than estimating the stoichiometry from e.g. intensity information at t_0 , as claimed in Response 1.2 and in Figure R1, from which an uncertainty in the number is missing. Would the authors be able to address this point and demonstrate a more narrow number distribution of quickPBSA compared to

other methods? The authors should also explicitly cite the width of the distribution as the accuracy but also state that this is comparable to previous PALM-based counting approaches.

Response R1.3: We thank reviewer 1 for pointing out the usefulness of quickPBSA for future users.

Unfortunately, a post-acquisition comparison of our stoichiometry measurement using quickPBSA with an approach based on the t0 intensity on the same dataset is not possible, since this would require a brightness standard to relate measured intensities for individual NPCs to fluorophore numbers.

To emulate an intensity-based stoichiometry estimate, we performed a reanalysis of the data NUP107/NPC data shown in Fig. 5 of our manuscript where we divided the t0 intensity by the mean last step height across all traces (Fig. R2). This analysis shows that the intensity-based stoichiometry estimate features a tail towards higher emitter numbers not observed in the corresponding distribution of quickPBSA emitter number estimates. We attribute this tail in the intensity-based analysis to differences in NPC position relative to the focal plane and spatial inhomogeneities in the illumination of the sample. In contrast to PBSA-based counting, intensity-based counting approaches are highly sensitive to such hard to control sources of variation. As mentioned above and in the introduction of the manuscript, intensity- and SMLM-based approaches both require calibration standards, which are not required for PBSA-based counting. We consider this a significant advantage of PBSA.

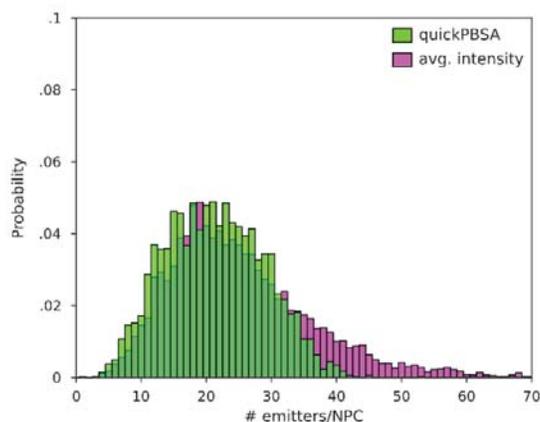


Figure R2: Comparison of quickPBSA and intensity-based emitter number estimates relating to our analysis of NUP107-SNAP copies/NPC shown in Fig. 5 of the original manuscript.

To facilitate a comparison with SMLM-based counting (and potentially other approaches in the future), we provided the cell-wise mean emitter number per NPC in the previous revision. The cell-to-cell variation achieved with quickPBSA (7.9%) is on par with the SMLM data shown in Thevathasan et al. (7.8% cell-to-cell variation). To further emphasize that quickPBSA yields results comparable to other state-of-the-art methods based on SMLM, we now explicitly refer to recent results by Thevathasan et al. in the results section of our manuscript:

"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)."

Finally, we would like to point out that we had already specified the center and widths of the emitter number distribution across all NPCs from all cells as well as the cell-wise aggregates in both, the caption of Fig. 5 and the main text of our revised manuscript:

*"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)."*

Comment R1.4: Comment 1.4. Our original concern was maybe not as clear. What we meant is that while PBSA may have a certain accuracy for counting detectable fluorescent proteins or dyes, not all fluorescent proteins may be detectable (e.g. only 60% of mEos2 proteins in a sample are detectable) or not all labeling sites may be occupied with a dye. This is not a problem of PBSA but any counting method in general. For instance, the authors show that they do detect fewer molecules on DNA origamis as they expect. It should therefore be clearly stated somewhere in the manuscript that PBSA can only count detectable fluorophores and that calibration measurements may be required to determine the labeling efficiency and the true number of proteins.

Response R1.4: We agree with reviewer 1 in that the labeling efficiency is highly relevant for molecular counting techniques based on fluorescence and

fluorescence microscopy in general. We discussed this issue in two recent reviews (Grußmayer et al. 2019, Hummert et al. 2021) and have stressed the importance of this topic in the context of our current manuscript by adding the following sentence to the introduction:

“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 (Grußmayer et al. 2019, Hummert et al. 2021).”

Minor suggestions:

Comment R1.5: Heading of Fig. 1 e and f are identical but should probably be different conditions.

Response R1.5: We thank the reviewer for spotting this. We swapped Figs 1e and 1f and replaced Fig. 1e with a representative trace showing SiR conjugated to HaloTag and imaged in PBS to highlight the influence of acquisition buffers on trace quality. The updated figure is shown below:

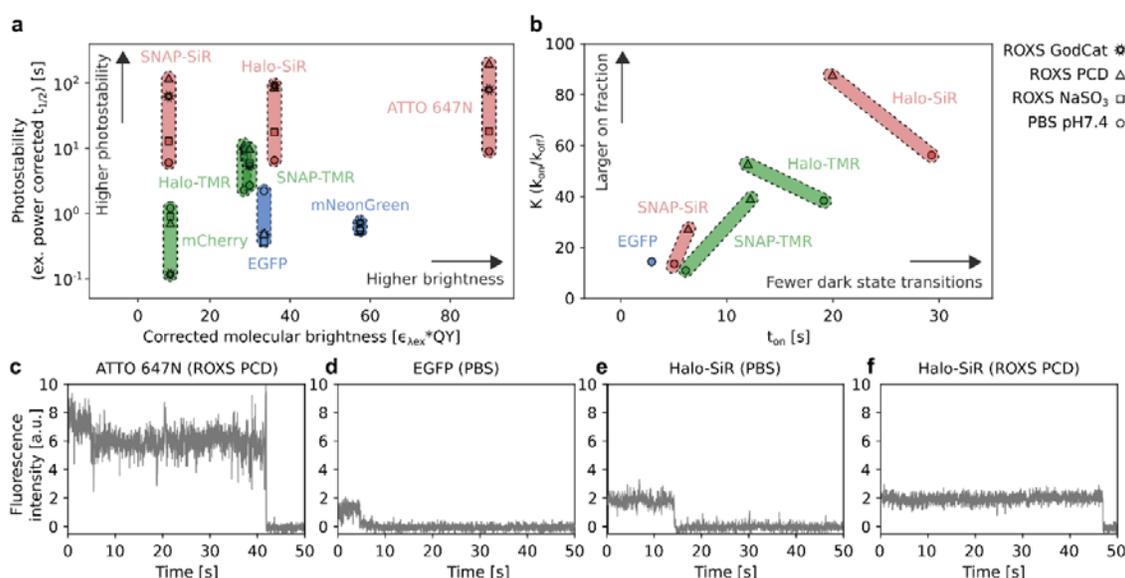


Figure 1 of main manuscript after updates to e,f).

Comment R1.6: Fig. 3 b and c (Presse 2016) We suggest making the y-axes range for all plots the same to allow a better visual comparison.

Response R1.5: We thank reviewer 1 for their thorough inspection of Fig. 3 and have adjusted the axis ranges or axis labels as shown below:

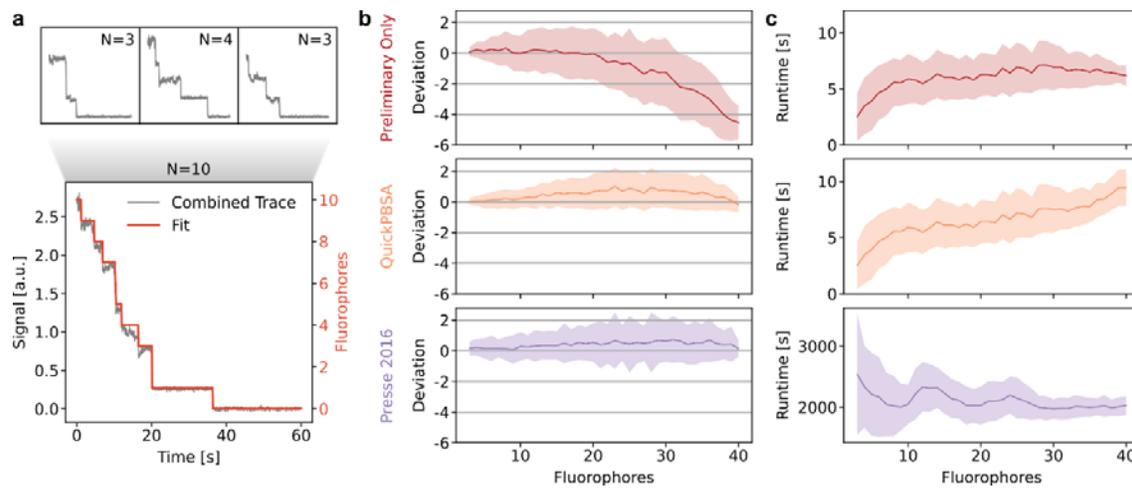


Figure 3 of main manuscript after updates to b) and c).

Comment R1.6: Fig. S6 please include scale bars.

Response R1.6: Since Fig. S6 shows a schematic illustration of how ROIs for trace extraction are determined based on varying inputs (localization coordinates or mask images), we believe that addition of a scale bar is neither necessary nor does it help the reader. To emphasize that the image shown in Fig. S6 is not primary data, we updated the caption of Fig. S6 as follows:

“Trace extraction routines included in the quickPBSA package shown schematically on artificial data. **a**, Extraction based on localization coordinates. Diffraction limited spots are excluded based on nearest neighbor distance and localization parameters such as width. **b**, Extraction based on a mask image. Overlap with other ROIs is excluded from background ROIs. ROIs can be excluded based on the ROI area.”

Comment R1.7: Fig. S6 do the authors use a specific scheme for determining the background ROI? It seems that it is a ring located 1 pixel from the center ROI with a thickness of 2 pixels, but this is not mentioned specifically in the text.

Response R1.7: After carefully inspecting our manuscript, we came to the conclusion that we would like to refrain from adding further information on how

the background ROIs are generated since details are already mentioned in several sections of the manuscript:

1. **Results, section Trace extraction:** *“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.”*
2. **Methods, section Photobleaching step analysis:** *“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.”*
3. **Documentation of quickPBSA software package:** *“ r_{peak} is the radius (in pixels) of the area around the localization from which the trace is extracted. r_{bg1} and r_{bg2} define a ring around the localization from which the background for background correction is extracted. min_dist is the minimum distance from one localization to the next. Localizations which are spaced less than min_dist apart are not considered in the trace extraction.”*

Reviewer #2 (Remarks to the Author)

Comment R2.1: Hummert et al. have carefully revised their manuscript on photobleaching step analysis. The manuscript has been improved a lot and selected issues have been clarified in the manuscript and supporting information. In my opinion, the manuscript is well suited for publication in Molecular Biology of the Cell.

Response R2.1: We thank reviewer 2 for their positive feedback on our revised manuscript.

RE: Manuscript #E20-09-0568RR

TITLE: "Photobleaching step analysis for robust determination of protein complex stoichiometries"

Dear Prof. Herten:

Thank you for revising your manuscript in response to the referees' recommendations. I have read the revised manuscript carefully along with your responses to the referees and it is clear that you have satisfactorily addressed each of their major concerns. I am pleased to accept your manuscript for publication in *Molecular Biology of the Cell*. Congratulations.

Sincerely,
Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Herten:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
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