Dear Editor-in-Chief,

Thank you again for the invitation and for the in-depth review of our methods protocol ‘How to quantify the fraction of photoactivated fluorescent proteins in bulk and in live cells’ to *JoVE,* *Journal of Visualized Experiments*.

Please see attached the revised manuscript with the changes highlighted in grey and the point-by-point answers to the reviewers’ questions.

Thank you so much for providing us the opportunity to show our method through your unique format of visualization. We really look forward to working with you and the *JoVE* team.

Sincerely,



Malte Renz, MD, PhD

Fellow

Division of Gynecologic Oncology

Stanford University School of Medicine

**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- *done*  
2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

- *figures are appropriately modified and cited*  
3. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.  
4. Please provide an email address for each author.

*- email address for both authors on file*  
5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

*- changed*  
6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

*- done*  
7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*- done*  
8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Clontech, Thermo Fisher Scientific, LabTek, etc.

*- done*  
9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*- done*  
10. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

*- done*  
11. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Please move the discussion about the protocol to the Discussion.

*- done*  
12. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

*- done as much as possible*  
13. 2.2: Please specify the culture media and conditions. Please describe how to detach cells, etc. Please break up into sub-steps.

*- trypsinization is a very common step in cell culture, nothing specific to our approach*  
14. 2.3: Please describe how to transfect cells.

*- according to distributors protocol, nothing specific*  
15. Please ensure that the other computational steps of the protocol are done in a graphical user interface with explicit user input commands: File | Save | etc. Please note that calculations are not appropriate for filming.

*- we will work on a graphic interface for the filming session*  
16. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*- done*  
17. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

*- done*  
18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

*- revised*  
19. References: Please do not abbreviate journal titles.

*- we used the endnote file from JoVe*  
20. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

*- done*  
  
**Reviewers' comments:**  
  
Please note that the reviewers raised some significant concerns regarding your method and your manuscript, in particular regarding calibration to the mGFP1—mCherry construct. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.  
  
  
Reviewer #1:  
  
Manuscript Summary:  
The present manuscript by V. Chen and M. Renz describes a protocol for quantification of photoactivation efficiency of photoactivatable fluorescent proteins (PA-FPs) in live mammalian cells. The protocol is based on the method recently published by one of the authors:  
Renz M, Wunder C. Internal rulers to assess fluorescent protein photoactivation efficiency. Cytometry A. 2018 Apr;93(4):411-419.  
The problem of quantitative assessment of PA-FP photoconversion is indeed of immediate interest. Unfortunately, I regret to say that the suggested method is completely wrong for the following reasons.  
  
Major Concerns:  
1. The protocol utilizes fusions of two spectrally distinct FPs as "internal rulers". Three constructs are used:  
mGFP1—PA-mCherry1  
PA-GFP—mCherry  
mGFP1—mCherry  
  
The control fusion mGFP1—mCherry ("always ON") is used to determine the maximal "expected intensities"; photoactivation efficiencies of PA-GFP and PA-mCherry1 are estimated relatively to these values. In other words, PA-GFP is compared to mGFP1 and PA-mCherry1 is compared to mCherry. This comparison is made in a way when fluorescence images are taken under the same microscope settings (in green channel for PA-GFP and mGFP1 or in red channel for PA-mCherry1 and mCherry). This way is correct ONLY for probes with identical spectral characteristics. In contrast, here authors use FPs with different fluorescence quantum yields, extinction coefficients, maxima and shapes of excitation and emission spectra:  
photoactivated PA-mCherry1:  
absorbance max 564 nm, emission max 595 nm, QY 0.46, EC 18000 M-1cm-1  
mCherry:  
absorbance max 587 nm, emission max 610 nm, QY 0.22, EC 72000 M-1cm-1.  
  
The manuscript (as well as the original paper in Cytometry A) contains no exact information and reference on identity of the GFP used ("mGFP1" - this abbreviation is not clear for me). However, PA-GFP possesses quite unusual red-shifted spectra with excitation max 504 nm and emission max 517 nm (QY 0.79, EC 17000 M-1cm-1), which differs significantly from most common GFP variants. Thus, PA-mCherry1 and PA-GFP can not be compared with mCherry and mGFP1, respectively, by simple measurements of fluorescence intensities under the same microscope settings.  
  
There is the following statement in the manuscript (p. 6, lines 207-211):  
"In this ratiometric intensity-based approach, the molecular brightness of the PA-FPs and the always-on FP counterparts are considered identical. We decided on this approach, since for some FPs no molecular brightness has been reported, and it is thus far unclear in how far different modes of photoactivation may affect the molecular brightness of the PA-FPs reported in the literature."  
  
As I showed above, molecular brightness of the PA-FPs and the always-on FP counterparts are NOT identical. Moreover, spectral shifts between them result in yet another source of mistakes during excitation and detection at some fixed wavelengths. While some values could be unreported, it is definitely not the reason to consider them "identical".  
  
To conclude, the main advantage of the protocol - a simple way to determine percentage of the photoactivated FP - is fully incorrect. Of course, comparison of different photoactivation regimes (short high-power versus many low-level exposures, etc) is appropriate; however, it could be done even without "internal rulers".

*- We thank the reviewer for this comment and clarified the advantage of our internal rulers to standardize expression levels and detect relative differences in photoactivation between different schemes of photoactivation. We also clarified what is needed to permit an absolute quantification of the photoactivated fraction using our approach efficiency (page 2, line 12-28; page 5 line 28-31, and page 6 line 1-24)*.  
  
2. A short linker between FPs is used that results in efficient FRET between them. Authors use 30% FRET value, measured for mGFP1—mCherry in an earlier work. Again, this FRET efficiency is probably incorrect for the pairs mGFP1—PA-mCherry1 and PA-GFP—mCherry because of the abovementioned differences in spectral characteristics, most of which directly affect FRET efficiency. Obvious ways to decrease or even fully eliminate undesirable FRET are (i) a longer linker, and (ii) 2A peptide between FPs.

*- We clarified the potential different FRET effects based on spectral properties of the fluorophores (page 5 lines 28-31).*  
3. No control experiments related to possible photobleaching of the second (non-activatable) FP during 405-nm photoactivation and further imaging are suggested. Photobleaching of the non-activatable FP can affect FRET.

*-* *We are currently characterizing quantum yield and extinction coefficient for different PA-FPs as well as FRET efficiencies between always-on FPs and PA-FPs. Manuscript is in preparation. The results go beyond the scope of this protocol.*  
  
Minor Concerns:  
4. Section 2.1: authors recommend using DMEM without phenol red for microscopy. In fact, DMEM (even without phenol red) is a poor medium for GFP imaging because of high levels of photobleaching and phototoxicity. Media formulated for fluorescence imaging (e.g., from Thermo Fisher Scientific or Evrogen) should be recommended.  
  
*- Thank you for this comment.*  
  
  
Reviewer #2:  
  
Manuscript Summary:  
This manuscript describes a protocol for measuring the fraction of photoactivated protein in live cells using a second fluorescent protein as an internal standard. While potentially useful, the protocol lacks some important details that will be necessary for other researchers to put it into practice.  
  
Major Concerns:  
1. The authors state in lines 207-221 that the molecular brightness for mCherry variants is assumed to be identical. However, the primary literature indicates that this is far from being true: mCherry has an extinction coefficient of 72,000 and a quantum yield of 0.22 (Shaner et al, Nat Biotech 2004), while PA-mCherry1 has an extinction coefficient of 18,000 and a quantum yield of 0.46 (Chozinski et al, FEBS 2014). This gives ~2-fold difference in molecular brightness between these two variants, which should be included in the analysis. The molecular brightness also affects the FRET efficiency, which is used for the correction in line 182.

*- We thank the reviewer for this comment and clarified the advantage of our internal rulers to standardize expression levels and detect relative differences in photoactivation between different schemes of photoactivation. We also clarified what is needed to permit an absolute quantification of the photoactivated fraction using our approach. And we clarified the effect of the quantum yield on the FRET efficiency (page 2, line 12-28; page 5 line 28-31, and page 6 line 1-24).*  
  
2. The protocol should include more detail about measuring light intensity. The model and brand of the light intensity meter and the particular sensor that was used should be stated. The authors should clarify whether the entire line (of line scanning mode) falls on the chip of the intensity meter sensor. If not, were there any calculations involved? Light intensity should be expressed as power per unit area. Is there a reason power was measured in line-scanning mode rather than with the laser fixed in one position? Is the light output of the AOTF linear?

*- A Thorlabs power meter was used. The entire line falls on the chip. For further calculations of light intensity and number of photons per μm2, please refer to the original publication in Cytometry Part A.*

3. The authors do not sufficiently describe the microscope itself in the protocol. What dichroic mirrors were used? What emission windows? Was there no need for bleedthrough corrections? Were the red and green images obtained sequentially or simultaneously, line-by-line? All of these details will have some bearing on the results obtained and need to be included in the protocol.

*- For details on the microscope settings please refer back to the original publication in Cytometry Part A*. *Details on dichroics used were omitted in the protocol since they are not essential part of the protocol.*

Minor Concerns:  
1. References to the original publications for the fluorescent proteins used in this protocol should be provided.

- *done (page 2 line 35 and 38)*

2. The authors should clarify exactly which fluorescent proteins they are using. Published and well-known fluorescent proteins are mCherry, mGFP, and PA-GFP. The manuscript refers to mCherry1, mGFP1, and PA-mGFP. Are these the same proteins as the published ones? If so, the authors should use the original names of these FPs in the manuscript. If these are mutants of the published FPs, the authors should describe the changes relative to published FPs in the manuscript.

*- clarified (page 2 line 35 and 38)*

3. In step 3.5 (line 120), the authors state that "post-activation images will help identify potential transient dark states due to the exposure to UV-light," but then these post-activation images are not mentioned in any other part of the protocol. The authors should describe how these images are to be used and specifically how to analyze them for the stated purpose.

*- clarified (page 4 line 8-10)*