Journal of Visualized Experiments

Quantifying the Fraction of Photoactivated Fluorescent Proteins in Bulk and in Live Cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58588R1
Full Title:	Quantifying the Fraction of Photoactivated Fluorescent Proteins in Bulk and in Live Cells
Keywords:	Photoactivatable fluorescent proteins; photoactivation efficiency; ratiometric intensity-based ensemble studies
Corresponding Author:	Malte Renz Stanford University School of Medicine Stanford, CA UNITED STATES
Corresponding Author's Institution:	Stanford University School of Medicine
Corresponding Author E-Mail:	renzmalt@stanford.edu
Order of Authors:	Malte Renz
	Vanessa Chen
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	300 Pasteur Drive, Stanford University School of Medicine, Stanford, CA 94305

Stanford MEDICINE

Department of Obstetrics and Gynecology

August 26, 2018

Editor-in-Chief
JoVE, Journal of Visualized Experiments

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

TITLE:

Quantifying the Fraction of Photoactivated Fluorescent Proteins in Bulk and in Live Cells

AUTHORS & AFFILIATIONS:
Vanessa Chen[1], Malte Renz[1]
[1]Gynecologic Oncology Division, Stanford University School of Medicine, Stanford, CA, USA

Corresponding author:
Malte Renz (renzmalt@stanford.edu)
Tel: 650-724-0456

Email Address of Co-author:
Vanessa Chen (vanchen@nuevaschool.org)

KEYWORDS:
Photoactivatable fluorescent proteins, photoactivation efficiency, ratiometric intensity-based ensemble studies, photoconvertible fluorescent proteins, GFP, mCherry

SUMMARY:
Here, we present a protocol that involves genetically coupled spectrally distinct photoactivatable and fluorescent proteins. These fluorescent protein chimeras permit quantification of the PA-FP fraction that is photoactivated to be fluorescent, *i.e.,* the photoactivation efficiency. The protocol reveals that different modes of photoactivation yield different photoactivation efficiencies.

ABSTRACT:
Photoactivatable and -convertible fluorescent proteins (PA-FPs) have been used in fluorescence live-cell microscopy for analyzing the dynamics of cells and protein ensembles. Thus far, no method has been available to quantify in bulk and in live cells how many of the PA-FPs expressed are photoactivated to fluoresce.
Here, we present a protocol involving internal rulers, *i.e.,* genetically coupled spectrally distinct (photoactivatable) fluorescent proteins, to ratiometrically quantify the fraction of all PA-FPs expressed in a cell that are switched on to be fluorescent. Using this protocol, we show that different modes of photoactivation yielded different photoactivation efficiencies. Short high-power photoactivation with a confocal laser scanning microscope (CLSM) resulted in up to four times lower photoactivation efficiency than hundreds of low-level exposures applied by CLSM or a short pulse applied by widefield illumination. While the protocol has been exemplified here for (PA-)GFP and (PA-)Cherry, it can in principle be applied to any spectrally distinct photoactivatable or photoconvertible fluorescent protein pair and any experimental set-up.

INTRODUCTION:
In 2002, the first broadly applicable photoactivatable (PA-GFP[1]) and photoconvertible (Kaede[2]) fluorescent proteins were described. These optical highlighter fluorescent proteins change their spectral properties upon irradiation with UV-light, *i.e.,* they become bright (photoactivatable fluorescent proteins, *i.e.,* PA-FPs), or change their color (photoconvertible FPs). To date, several

reversible and irreversible photoactivatable and photoconvertible fluorescent proteins have been developed[3,4]. In ensemble or bulk studies, optical highlighters have been used to study the dynamics of entire cells or proteins, and the connectivity of subcellular compartments. Furthermore, optical highlighters enabled single-molecule based superresolution imaging techniques such as PALM[5] and FPALM[6].

Although the photo*chemical* processes during photoactivation or -conversion have been described for many optical highlighters and even crystallographic structures before and after photoactivation/ -conversion have been made available[7,8], the underlying photo*physical* mechanism of photoactivation and -conversion is not completely understood. Furthermore, thus far only crude estimates exist of the efficiency of photoactivation and -conversion, that is, the fraction of fluorescent proteins expressed that is actually photoconverted or photoactivated to be fluorescent. *In vitro* ensemble studies have been reported quantifying the shift in absorption spectra and the amount of native and activated protein in a gel[9-11].

Here, we present a protocol involving fluorescent protein chimeras to assess the fraction of photoactivated fluorescent proteins in bulk and in live cells. Whenever working with genetically encoded fluorescent proteins, the absolute amount of protein expressed varies from cell to cell and is unknown. If one cell expressing a PA-FP shows a brighter signal after photoactivation than another cell, it cannot be differentiated if this brighter signal is due to higher expression of the PA-FP or a more efficient photoactivation of the PA-FP. To standardize the expression level in cells, we introduce internal rulers of genetically coupled spectrally distinct fluorescent proteins. By coupling the genetic information of a photoactivatable fluorescent protein to a spectrally distinct always-on fluorescent proteins, internal rulers are created that will still be expressed to an unknown total amount but in a fixed and known relative amount of 1:1. This strategy allows the quantitative characterization of different UV-light photoactivation schemes, *i.e.,* the assessment of the relative amount of PA-FPs that can be photoactivated with different modes of photoactivation, and thereby permits to define photoactivation schemes that are more effective than others. Furthermore, this strategy allows in principle the assessment of the absolute quantification of the photoactivated PA-FP fraction. To this end, it is important to realize that the presented ensemble studies are intensity-based which makes the analysis more complex as laid out in this protocol. Parameters determining the measured fluorescent intensity, *i.e.,* different molecular brightness, absorbance and emission spectra and FRET effects, need to be considered when comparing fluorescence intensities of different fluorescent proteins.

The presented ratiometric intensity-based quantification of photoactivation efficiency is exemplified for PA-GFP and PA-Cherry in live cells, but is in principle broadly applicable and can be used for any photoactivatable fluorescent protein under any experimental condition.

PROTOCOL:

1. Plasmid Construction

1.1. Generate two-color fusion probes. Use a mammalian cell expression vector (see **Table of Materials**) in which mCherry1[12] and PA-mCherry1[13] have been inserted with the restriction sites

AgeI and *BsrGI.*

1.2. Order custom oligo-nucleotides to amplify the monomeric variants of eGFP and PA-eGFP containing the A206K mutation, *i.e.,* mEGFP and PA-mEGFP[14] without a stop codon as a *SalI-BamHI* fragment. Use the N-terminal primer 5'-AAT TAA CAG TCG ACG ATG GTG AGC AAG GGC GAG G 3' and the C-terminal primer 5'-AAT ATA TGG ATC CCG CTT GTA CAG CTC GTC CAT GC 3' and insert this *SalI-BamHI* fragment into the multiple cloning site of the expression vector. This will create the five amino acid linker RNPPV between the green and red fluorescent protein. We will refer to the fluorophore chimeras as GFP—Cherry, PA-GFP—Cherry, and GFP—PA-Cherry for the remainder of this article.

Note: The coupling of fluorophores and creation of internal rulers to assess photoactivation efficiency can be done with any spectrally distinct fluorophore pair, *e.g.* superfolder PA-GFP [15]/ PA-TagRFP [16], *etc.* Many of the photoactivatable fluorescent proteins are derived from GFP. Hence, the primer sequence provided above can be used for many fluorescent proteins to construct a fluorophore chimera in a mammalian cell expression vector.

2. Cell Culture and Transfection

2.1. Use either any standard cell line such as HeLa, NRK, Cos-7 or a specific cell line to be used for specific photoactivation experiments. Use DMEM (supplemented with 10% fetal bovine serum, and 2 mM glutamine) and trypsin *without* phenol red (see **Table of Materials**) to reduce background fluorescence.

2.2. Detach the cells of a confluent culture with trypsin, count the number of cells in cell suspension using a Neubauer chamber, and seed 5,000 – 10,000 cells per well. Alternatively, use 1 drop from a 2-mL pipette of a 10-mL cell suspension from a confluent cell culture grown in a T 25-cell culture flask or 3 drops from a 5-mL cell suspension from a confluent culture grown in a T 12.5-cell culture flask.

2.3. Grow cells in 8-well chambers with #1.0 cover glass (see **Table of Materials**) for fluorescence live-cell microscopy.

2.4. Transfect cells 24 h after plating using commercial reagents (see **Table of Materials**) per distributer's protocol with the GFP—Cherry, PA-GFP—Cherry, and GFP—PA-Cherry chimeras.

2.5. Image cells after a total of 20 h post transfection to allow for protein expression, folding and maturation.

3. Imaging and Photoactivation

3.1. Image cells in a humidified and heated environmental chamber at 37 degrees Celsius. To buffer the cell media at physiological pH and render it CO_2-independent, add 20 mM HEPES, or use CO_2 gas set to 5% flow.

3.2. First, image cells expressing the GFP—Cherry construct. Set parameters that define time-integrated laser intensity per pixel in a confocal image, *i.e.,* pixel dwell time in microseconds, acousto-optical tunable filter (AOTF) transmission in percent, and digital zoom.

Note: Using a 60x objective and a digital zoom of 3x allows imaging of a cell in its entirety while providing sufficient magnification. Set pixel dwell time to 2-4 µs and AOTF transmission for the 488-nm and 561-nm laser such that images show a good signal-to-noise ratio without any bleaching and no pixels indicating fluorescence intensity saturation.

3.3. Image, using the set laser power, AOTF transmission, pixel dwell time and digital zoom, 15-20 cells expressing GFP—Cherry.

3.4. Then, image with the same set laser power, pixel dwell time, AOTF transmission and digital zoom cells expressing GFP—PA-Cherry and PA-GFP—Cherry. Search for expressing cells in the green channel or red channel, respectively. Avoid long exposure of the cells during the search for expressing cells in order to not bleach the fluorescent proteins.

3.5. Set up a mini-time series with one pre-activation image and three post-activation images. The post-activation images will help identify potential transient dark states due to the exposure to UV-light.

Note: In our hands, changes in detected fluorescence intensity due to transient dark states were <1% and could be neglected, but those dark states should be assessed for every experimental set-up.

3.6. To determine photoactivation efficiency, *i.e.,* the fraction of PA-FPs that is switched on to be fluorescent, in the specific photoactivation experiments that have already been established, apply the same photoactivation settings to 15-20 cells that are expressing the internal rulers introduced here and proceed with image analysis (section 4). If beginning to set up photoactivation experiments, find here a few different settings based on our experimental experience with PA-GFP and PA-Cherry; modify as needed.

3.6.1. For instantaneous photoactivation of PA-GFP and PA-Cherry using a confocal laser scanning microscope (CLSM), apply 90 µW of 405-nm laser light in 3 or 5 iterations, respectively.

Note: With our microscopic set-up, using a 38% AOTM transmission and a 2 µs pixel dwell time, we measured this 405-nm laser power in line-scan mode at the objective lens. With these settings, about 8% and 16% of the PA-GFP and PA-Cherry expressed can be photoactivated to be fluorescent. The entire titration series using CLSM for instantaneous photoactivation has been published previously[17].

3.6.2. If a higher fraction of photoactivated PA-GFP and PA-Cherry fluorophores is advantageous *e.g.* to achieve a higher signal-to-noise ratio, and photoactivation does not have to be immediate,

apply 40 µW of 405-nm laser light with a 2 µs pixel dwell time and 6% AOTF transmission for 450 iterations. Then, photoactivation will take up to 4 min as opposed to only 1-2 seconds, but photoactivation efficiency for PA-GFP will be 29% instead of 8%, allowing for a higher signal-to-noise ratio.

3.7. If a mosaic digital illumination system that contains micro mirror arrays in a spatial light modulator is available, 405-nm laser light can be used for widefield-photoactivation. This allows for efficient photoactivation within milliseconds.

Note: With 1.6 mW laser power as measured at the objective lens and an exposure time of 250 ms, 29% of PA-GFP can be photoactivated to be fluorescent.

3.8. Image with any set photoactivation parameters 15-20 cells expressing PA-GFP—Cherry and GFP—PA-Cherry, respectively.

3.9. Since pH, reactive oxygen species and other environmental factors may influence photoactivation efficiency, it may be important to assess photoactivation efficiencies of the PA-FPs in the respective subcellular micromilieu where the protein of interest is located. By coupling the fluorophore chimeras to the protein of interest, as the authors have done with the plasma membrane protein VSVG [18], photoactivation efficiency can be assessed in the specific subcellular compartment of interest.

4. Image Analysis and Algorithm for Ratiometric Intensity-based Quantification of Photoactivation Efficiency

4.1. Image analysis can be done with the open source image processing platforms ImageJ or Fiji. Determine the background fluorescence intensity in non-transfected cells in the green (B_1) and red (B_2) channel. Avoid perinuclear or any areas showing increased auto-fluorescence.

4.2. To determine the fluorescence intensity in a transfected cell, outline the cell body with the freehand selections tool. Again, avoid perinuclear or any other areas showing auto-fluorescence.

4.3. Subtract the background from the measured fluorescence intensity in each channel.

$I_G = I_{Green\_measured} - B_1$

$I_R = I_{Red\_measured} - B_2$

4.4. Use the GFP—Cherry construct to calculate the red-to-green ratio (RtoGr) and correct for donor-quenching due to fluorescence resonance energy transfer (FRET). The FRET efficiency E was determined to be 0.3 in previous experiments for the GFP—Cherry construct using the same amino acid linker between the two fluorophores[18].

$RtoGr = (I_{Red\_measured} - B_2) / (I_{Green\_measured} - B_1)$

$RtoGr_{corr} = RtoGr * (1 - E)$

Note: In this intensity- based approach, donor quenching for mEGFP and PA-mGFP may be

different given possible distinct spectral properties which have not been characterized. The rate of FRET (kET) and the Foerster distance (R0) depend upon the quantum yield of the donor which has not been determined for mEGFP and PA-mGFP.

4.5. Use the GFP—PA-Cherry construct to assess the fraction of photoactivated PA-Cherry. Determine the expected fluorescence intensity of PA-Cherry by multiplying the measured unquenched green fluorescence intensity of the GFP—PA-Cherry construct *prior* to photoactivation with the corrected red-to-green-ratio ($RtoGr_{corr}$).

$I_{Red\_expected} = (I_{Green\_measured} - B_1) * RtoGr_{corr}$

Note: As indicated above, the molecular brightness of the always-on FP and the photoactivatable FP may be different. See Discussion for more information on how to account for these differences.

4.6. Calculate the PA-Cherry photoactivation efficiency as a fraction of the measured red fluorescence intensity *after* photoactivation and the expected fluorescence intensity in the red channel.

$(F_{PA\text{-}Cherry}) = (I_{Red\_measured} - B_2) / I_{Red\_expected}$

4.7. Use the PA-GFP—Cherry construct to assess the fraction of photoactivated PA-GFP. Determine the expected fluorescence intensity of PA-GFP by dividing the measured red fluorescence intensity of the PA-GFP—Cherry construct *prior* to photoactivation by the red-to-green-ratio (RtoGr). Here, the RtoGr does not need to be corrected for donor quenching, because GFP and PA-GFP are subject to donor quenching to the same amount.

$I_{Green\_expected} = (I_{Red\_measured} - B_2) / RtoGr$

4.8. Calculate the PA-GFP photoactivation efficiency as a fraction of the measured green fluorescence intensity *after* photoactivation and the expected fluorescence intensity in the green channel.

$(F_{PA\text{-}GFP}) = (I_{Green\_measured} - B_1) / I_{Green\_expected}$

REPRESENTATIVE RESULTS:

The protocol presented here shows the ratiometric quantification of the fraction of fluorescent proteins that are photoactivated to be fluorescent (**Figure 1**). This fraction differs depending upon the mode of photoactivation.

A typical result using short time high-power photoactivation with a confocal laser scanning microscope (CLSM) is shown in **Figure 2c**. After titrating the laser power as measured at the objective lens by pixel dwell time and ATOF transmission, the maximum photoactivation efficiency for PA-GFP was about 8% and for PA-Cherry about 16%. The comparably low photoactivation efficiency of PA-GFP and PA-Cherry may be explained by simultaneous photoactivation and -destruction when exposed to a continuous deterministic stream of photons by CLSM. The shorter fluorescence lifetimes and the different, right-shifted absorption spectra of the red PA-FPs may contribute to the higher photoactivation efficiency of PA-Cherry compared to PA-GFP.

Using low laser power and hundreds of iterations, a higher photoactivation efficiency can be achieved. 450 iterations of UV-light delivered by a CLSM over a total of 4 min yielded a photoactivation efficiency of 29% for PA-GFP (**Figure 3c**). The higher photoactivation efficiency with repetitive exposure to UV-light photons may suggest a multi-step photoactivation process. Alternatively, the applied UV-light is strong enough to photoactivate but not enough to photodestruct which leads cumulative over time to a higher fraction of photoactivated fluorescent proteins.

With widefield illumination, the fluorophores are stochastically and repetitively exposed to 405-nm photons. Here, exposure for only 250 ms yielded a 29% photoactivation efficiency for PA-GFP.

FIGURE AND TABLE LEGENDS:

Figure 1: Concept of how to determine photoactivation efficiency in bulk and in live cells. By coupling spectrally distinct fluorescent proteins, internal rulers are created which allow for the ratiometric intensity-based assessment of photoactivation efficiency. Measured intensities of PA-Cherry and PA-GFP were related to expected intensities. Expected intensities were derived from determining the fluorescence intensity of the always-on fluorescent proteins in the GFP—Cherry, GFP—PA-Cherry or PA-GFP—Cherry chimeras prior to photoactivation. Figure modified from Renz and Wunder 2017[17].

Figure 2: Bulk photoactivation of PA-GFP—Cherry (a) and GFP—PA-Cherry (b) as instantaneously and completely as possible using a confocal laser-scanning microscope in live cells. 8% of PA-GFP expressed was photoactivated with a pixel dwell time of 2 µs and an AOTF transmission of 38% which result in laser power of 90 µW, as measured at the objective lens and 3 iterations (c). Increasing the 405-nm laser power did not increase photoactivation efficiency. Figure modified from Renz and Wunder 2017[17].

Figure 3: Iterative low-power photoactivation with a confocal laser-scanning microscope (a) and short high-power widefield illumination (b) yield higher photoactivation efficiencies. 29% of PA-GFP was photoactivated with a pixel dwell time of 2 µs and an AOTF transmission of 6%, which result in laser power of 40 µW, as measured at the objective lens and 450 iterations (c). Figure modified from Renz and Wunder 2017[17].

DISCUSSION:

So far, no method existed to determine in bulk the fraction of PA-FPs expressed in live cells that is photoactivated to be fluorescent. The presented protocol can be used for any spectrally distinct fluorescent protein pair. While exemplified here for the irreversible PA-FPs PA-GFP and PA-Cherry, this approach is in principle applicable to photoconvertible proteins as well. The spectrally distinct fluorescent protein, however, must be selected carefully to minimize spectral overlap given that photoconvertible fluorescent proteins shift their absorbance and emission spectra, *e.g.* from green to red fluorescence.

As outlined above, it is important to state that the presented approach is ratiometric and intensity-based. It can be used to standardize the unknown expression level in cells and define

relative differences in photoactivation efficiency by different modes of photoactivation. The protocol can also be used to assess the absolute fraction of photoactivated PA-FPs. Then, different spectral properties of different FPs need to be taken into account.

The molecular brightness (MB) is the product of quantum yield (QY), extinction coefficient (EC) and percent absorbance at the given excitation wavelength relative to the absorbance peak. For Cherry[12] and PA-Cherry[13], respective values of QY and EC have been published. The percent absorbance at the given excitation wavelength of 543 nm relative to absorbance peak is 0.5 and 0.7, respectively.

MB_{Cherry} = 0.22 * 72,000 * 0.5 = 7,920

$MB_{PA\text{-}Cherry}$ = 0.46 * 18,000 * 0.7 = 5,796

Thus, the lower molecular brightness of PA-Cherry compared to Cherry can be taken into account by dividing $I_{Red\_expected}$ by 1.37 (derived from 7,920/5,796).

However, it is unknown under which photoactivation conditions the published molecular brightness of PA-Cherry has been determined. This is important, since we show here that the mode of photoactivation changes the measured fraction of photoactivated PA-FPs. Furthermore, for the monomeric versions comprising the A206K mutation. *i.e.,* mEGFP and PA-mEGFP, no molecular brightness has been published.

In this ratiometric intensity-based approach, the molecular brightness of the PA-FPs and the always-on FP counterparts in a first approximation have been considered identical. We decided on this approach, since (i) for some FPs no molecular brightness has been reported, and (ii) 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. Furthermore, (iii) for a comparative analysis the knowledge of the molecular brightness is not necessary; it is only needed for the intensity-based determination of the absolute fraction of photoactivated PA-FPs which can be calculated as shown above.

Our approach involving fluorescent protein chimeras as internal rulers shows that different exposure to UV-light yields different photoactivation efficiencies. Thereby, it defines options as how to photoactivate a larger PA-FP fraction and achieve a better signal-to-noise ratio. Furthermore, it opens up opportunities to differentially photoactivate different PA-FPs in the same cell given their differential response to UV-light or to differentially photoactivate the same PA-FP in different subcellular compartments by exposing it differently to UV-light. In summary, our protocol will help further the quantitative understanding of cellular processes using PA-FPs in live-cell microscopy.

ACKNOWLEDGMENTS:

We would like to thank the Dorigo laboratory and the Neuroscience Imaging Service at Stanford University School of Medicine for providing equipment and space for this project.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Patterson, G. H. & Lippincott-Schwartz, J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science.* **297** (5588), 1873-1877, (2002).

2 Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America.* **99** (20), 12651-12656, (2002).

3 Renz, M. & Lippincott-Schwartz, J. in *The Fluorescent Protein Revolution Series in Cellular and Clinical Imaging* eds R. N. Day & M. W. Davidson) Ch. 9, 201-228 (CRC Press, 2014).

4 Shcherbakova, D. M., Sengupta, P., Lippincott-Schwartz, J. & Verkhusha, V. V. Photocontrollable fluorescent proteins for superresolution imaging. *Annual Review of Biophysics.* **43** 303-329, (2014).

5 Betzig, E. *et al.* Imaging intracellular fluorescent proteins at nanometer resolution. *Science.* **313** (5793), 1642-1645, (2006).

6 Hess, S. T., Girirajan, T. P. & Mason, M. D. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophysical Journal.* **91** (11), 4258-4272, (2006).

7 Henderson, J. N. *et al.* Structure and mechanism of the photoactivatable green fluorescent protein. *Journal of the American Chemical Society.* **131** (12), 4176-4177, (2009).

8 Subach, F. V. *et al.* Photoactivation mechanism of PAmCherry based on crystal structures of the protein in the dark and fluorescent states. *Proceedings of the National Academy of Sciences of the United States of America.* **106** (50), 21097-21102, (2009).

9 Wiedenmann, J. *et al.* EosFP, a fluorescent marker protein with UV-inducible green-to-red fluorescence conversion. *Proceedings of the National Academy of Sciences of the United States of America.* **101** (45), 15905-15910, (2004).

10 Habuchi, S., Tsutsui, H., Kochaniak, A. B., Miyawaki, A. & van Oijen, A. M. mKikGR, a monomeric photoswitchable fluorescent protein. *PLoS One.* **3** (12), e3944, (2008).

11 McKinney, S. A., Murphy, C. S., Hazelwood, K. L., Davidson, M. W. & Looger, L. L. A bright and photostable photoconvertible fluorescent protein. *Nature Methods.* **6** (2), 131-133, (2009).

12 Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature Biotechnology.* **22** (12), 1567-1572, (2004).

13 Subach, F. V. *et al.* Photoactivatable mCherry for high-resolution two-color fluorescence microscopy. *Nature Methods.* **6** (2), 153-159, (2009).

14 Zacharias, D. A., Violin, J. D., Newton, A. C. & Tsien, R. Y. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science.* **296** (5569), 913-916, (2002).

15 Slocum, J. D. & Webb, L. J. A Double Decarboxylation in Superfolder Green Fluorescent Protein Leads to High Contrast Photoactivation. *Journal of Physical Chemistry Letters.* **8** (13), 2862-2868, (2017).

16 Subach, F. V., Patterson, G. H., Renz, M., Lippincott-Schwartz, J. & Verkhusha, V. V. Bright monomeric photoactivatable red fluorescent protein for two-color super-resolution

sptPALM of live cells. *Journal of the American Chemical Society.* **132** (18), 6481-6491, (2010).

17 Renz, M. & Wunder, C. Internal rulers to assess fluorescent protein photoactivation efficiency. *Cytometry A.* 10.1002/cyto.a.23319, (2017).

18 Renz, M., Daniels, B. R., Vamosi, G., Arias, I. M. & Lippincott-Schwartz, J. Plasticity of the asialoglycoprotein receptor deciphered by ensemble FRET imaging and single-molecule counting PALM imaging. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (44), E2989-2997, (2012).

Figure 1

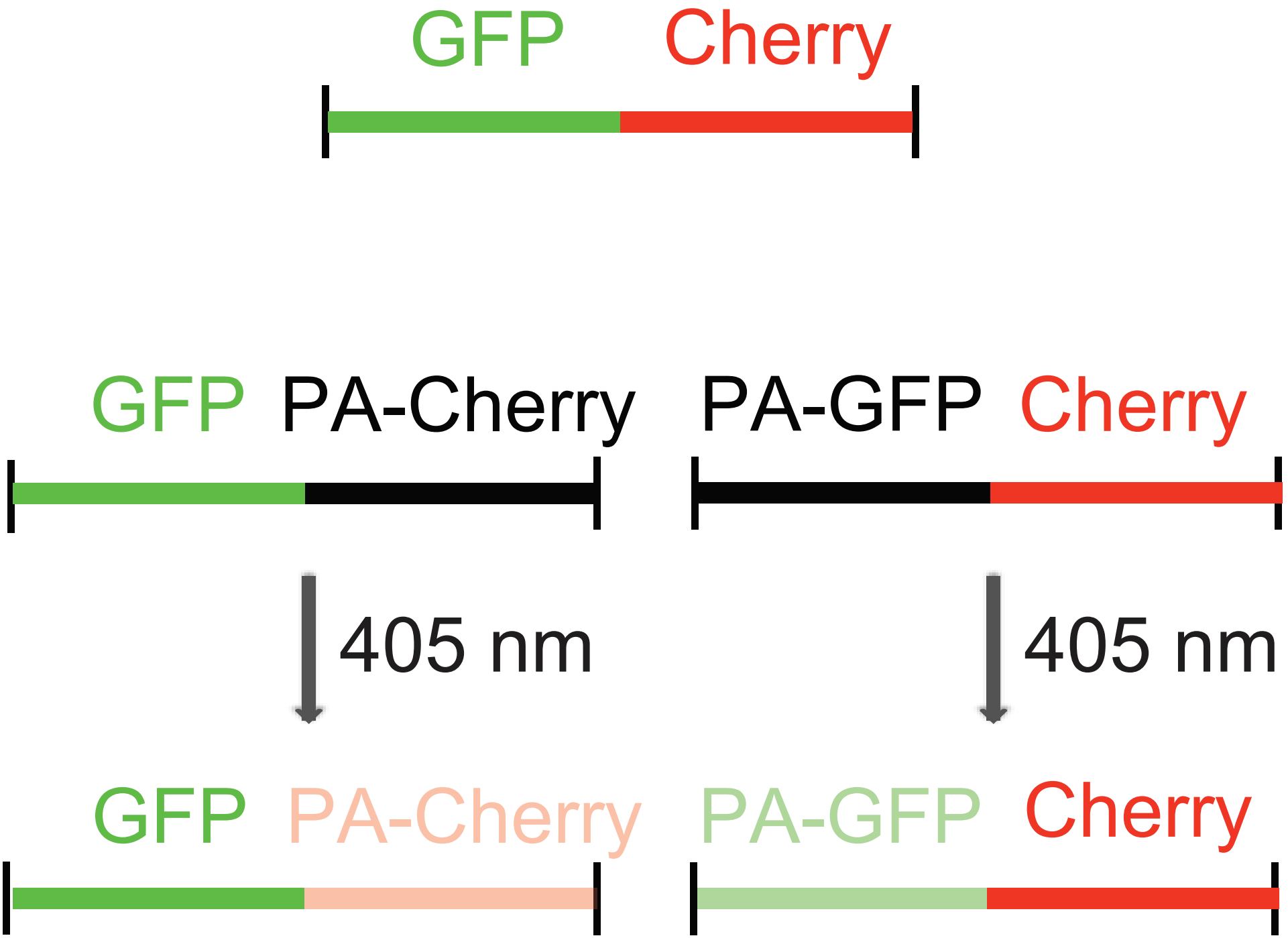


Figure 2

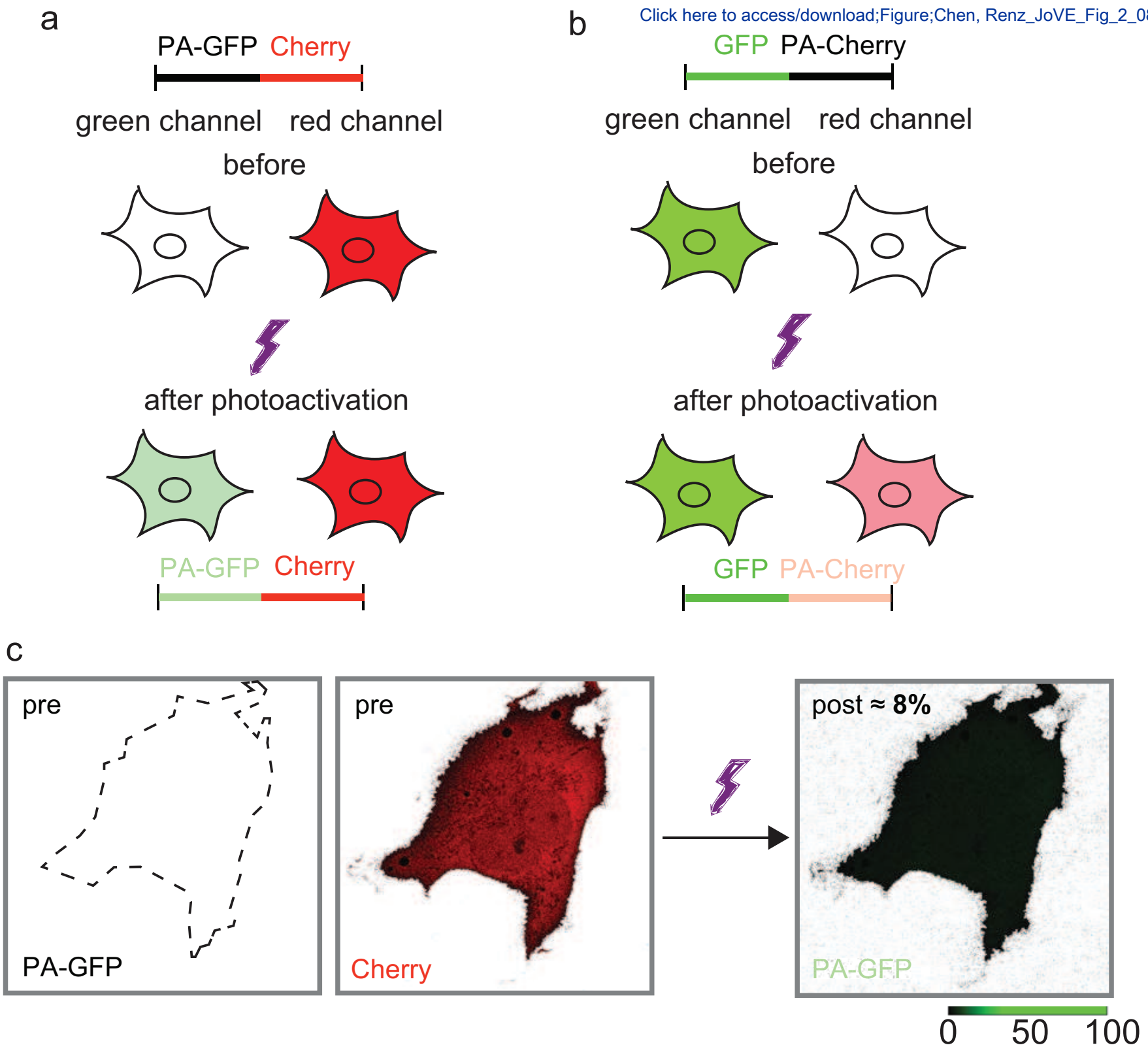


Figure 3

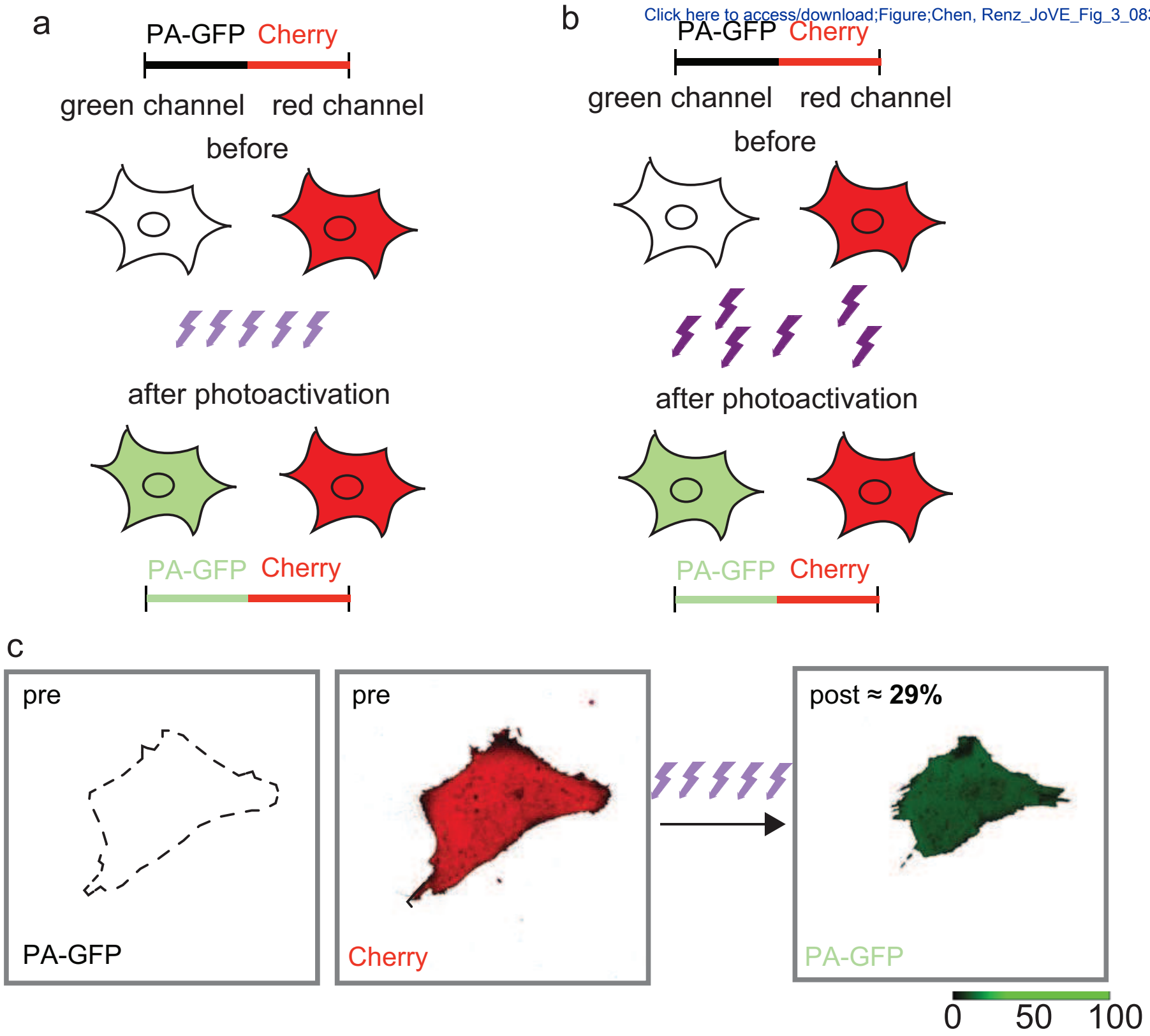


Table of Materials

Click here to access/download;Table of Materials;Chen, Renz_JoVE_Materials.xls.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
pEGFP-N1 mammalian cell expression vector	Clontech		
DMEM w/o phenol red	Thermo Fisher Scientific	11054020	
Trypsin w/o phenol red	Thermo Fisher Scientific	15400054	
L-Glutamine (200 mM)	Thermo Fisher Scientific	25030081	
HEPES	Thermo Fisher Scientific	15630080	
LabTek 8-well chambers #1.0	Thermo Fisher Scientific	12565470	
Fugene 6	Promega	E2691	

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: How to quantify the fraction of photoactivated fluorescent proteins in bulk and in live cells.

Author(s): Vanessa Chen and Malte Renz

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [x] Standard Access [ ] Open Access

Item 2 (check one box):

[x] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

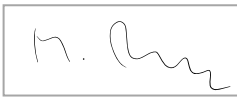
11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Malte Renz
Department:	Gynecologic Oncology Division
Institution:	Stanford University School of Medicine
Article Title:	How to quantify the fraction of photoactivated fluorescent proteins in bulk and in live cells.
Signature:	 Date: 06/07/2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

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 μm^2, 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)

1. Plasmid construction

1.1. Generate two-color fusion probes. Use a Clontech N1 mammalian cell expression vector in which mCherry1 [12] and PA-mCherry1 [13] have been inserted with the restriction sites *AgeI* and *BsrGI.*

1.2. Order custom oligo-nucleotides to amplify the monomeric variants of eGFP and PA-eGFP containing the A206K mutation, i.e. mEGFP and PA-mEGFP [14] without a stop codon as a *SalI-BamHI* fragment. Use the N-terminal primer 5'-AAT TAA CAG TCG ACG ATG GTG AGC AAG GGC GAG G 3' and the C-terminal primer 5'-AAT ATA TGG ATC CCG CTT GTA CAG CTC GTC CAT GC 3' and insert this *SalI-BamHI* fragment into the multiple cloning site of the Clontech N1 vector. This will create the five amino acid linker RNPPV between the green and red fluorescent protein.

2. Cell culture and transfection

2.3. Grow cells of a standard cell line in 8-well LabTek chambers with #1.0 cover glass for fluorescence live-cell microscopy.

2.4. Transfect cells 24 h after plating using Fugene 6 (Roche) per distributer's protocol with the GFP—Cherry, PA-GFP—Cherry, and GFP—PA-Cherry chimeras.

2.5. Image cells after a total of 20 h post transfection to allow for protein expression, folding and maturation.

3. Imaging and photoactivation

3.1. Image cells in a humidified and heated environmental chamber at 37 degrees Celsius. To buffer the cell media at physiological pH and render it CO2-independent, add 20 mM HEPES; or use CO2 gas set to 5% flow.

3.2. First, image cells expressing the GFP—Cherry construct. Set parameters that define time-integrated laser intensity per pixel in a confocal image, i.e. pixel dwell time in microseconds, acousto-optical tunable filter (AOTF) transmission in percent, and digital zoom. Using a 60x objective and a digital zoom of 3x allows imaging of a cell in its entirety while providing sufficient magnification. Set pixel dwell time to 2-4 μs and AOTF transmission for the 488-nm and 561-nm laser such that images show a good signal-to-noise ratio without any bleaching and no pixels indicating fluorescence intensity saturation.

3.3. Image with set laser power, AOTF transmission, pixel dwell time and digital zoom 15-20 cells expressing GFP—Cherry.

3.4. Then, image with the same set laser power, pixel dwell time, AOTF transmission and digital zoom cells expressing GFP—PA-Cherry and PA-GFP—Cherry. Search for expressing cells in the green channel or red channel, respectively. Avoid long exposure of the cells during the search for expressing cells in order to not bleach the fluorescent proteins.

3.5. Set up a mini-time series with one pre-activation image and three post-activation images. The post-activation images will help identify potential transient dark states due to the exposure to UV-light.

3.6. To determine photoactivation efficiency, i.e. the fraction of PA-FPs that is switched on to be fluorescent, in the specific photoactivation experiments that you have already established, apply the

photoactivation settings you have been using to 15-20 cells that are expressing the internal rulers. If you are beginning to set up photoactivation experiments, find here a few different settings based on our experimental experience with PA-GFP and PA-Cherry. Modify as needed for your own experiments.

3.6.1. For instantaneous photoactivation of PA-GFP and PA-Cherry using a confocal laser scanning microscope, apply 90 μW of 405-nm laser light in 3 or 5 iterations, respectively. With our microscopic set-up, using a 38% AOTM transmission and a 2 μs pixel dwell time, we measured this 405-nm laser power in line-scan mode at the objective lens. With these settings, about 8% and 16% of the PA-GFP and PA-Cherry expressed can be photoactivated to be fluorescent[17].

3.6.2. If a higher fraction of photoactivated PA-GFP and PA-Cherry fluorophores is advantageous e.g. to achieve a higher signal-to-noise ratio, and photoactivation does not have to be immediate, apply 40 μW of 405-nm laser light with a 2 μs pixel dwell time and 6% AOTF transmission for 450 iterations. Then, photoactivation will take up to 4 min as opposed to only 1-2 seconds, but photoactivation efficiency for PA-GFP will be 29% instead of 8%, allowing for a higher signal-to-noise ratio.

3.7. If a Mosaic Digital Illumination System that contains micro mirror arrays in a spatial light modulator is available, 405-nm laser light can be used for widefield-photoactivation. This allows for efficient photoactivation within milliseconds. With 1.6 mW laser power as measured at the objective lens and an exposure time of 250 ms, 29% of PA-GFP can be photoactivated to be fluorescent.

3.8. Image with any set photoactivation parameters 15-20 cells expressing PA-GFP—Cherry and GFP—PA-Cherry, respectively.

4. Image analysis and algorithm for ratiometric intensity-based quantification of photoactivation efficiency

4.1. Image analysis can be done with the open source image processing platforms ImageJ or Fiji. Determine the background fluorescence intensity in non-transfected cells in the green (B_1) and red (B_2) channel. Avoid perinuclear or any areas showing increased auto-fluorescence.

4.2. To determine the fluorescence intensity in a transfected cell, outline the cell body with the freehand selections tool. Again, avoid perinuclear or any other areas showing auto-fluorescence.

4.3. Subtract the background from the measured fluorescence intensity in each channel.

$I_G = I_{Green\_measured} - B_1$

$I_R = I_{Red\_measured} - B_2$

4.4. Use the GFP—Cherry construct to calculate the red-to-green ratio (RtoGr) and correct for donor-quenching due to fluorescence resonance energy transfer (FRET). The FRET efficiency E was determined to be 0.3 in previous experiments for the GFP—Cherry construct using the same amino acid linker between the two fluorophores [18].

$RtoGr = (I_{Red\_measured} - B_2) / (I_{Green\_measured} - B_1)$

$RtoGr_{corr} = RtoGr * (1 - E)$

4.4.1 Caveat: In this intensity- based approach, donor quenching for mEGFP and PA-mGFP may be different since the rate of FRET (kET) and the Foerster distance (R0) depend upon the quantum yield of

the donor which have not been characterized for mEGFP and PA-mGFP.

4.5. Use the GFP—PA-Cherry construct to assess the fraction of photoactivated PA-Cherry. Determine the expected fluorescence intensity of PA-Cherry by multiplying the measured unquenched green fluorescence intensity of the GFP—PA-Cherry construct *prior* to photoactivation with the corrected red-to-green-ratio ($RtoGr_{corr}$).

$I_{Red\_expected} = (I_{Green\_measured} - B_1) * RtoGr_{corr}$

4.5.1. Caveat: As indicated above, the molecular brightness of the always-on FP and the photoactivatable FP may be different. The molecular brightness (MB) is the product of quantum yield (QY), extinction coefficient (EC) and percent absorbance at the given excitation wavelength relative to the absorbance peak. For Cherry [12] and PA-Cherry [13], respective values of QY and EC have been published. The percent absorbance at the given excitation wavelength of 543 nm relative to absorbance peak is 0.5 and 0.7, respectively.

4.5.1.1. $MB_{Cherry} = 0.22 * 72{,}000 * 0.5 = 7{,}920$

$MB_{PA\text{-}Cherry} = 0.46 * 18{,}000 * 0.7 = 5{,}796$

Thus, the lower molecular brightness of PA-Cherry compared to Cherry can be taken into account by dividing $I_{Red\_expected}$ by 1.37.

4.5.1.2. However, it is unknown under which photoactivation conditions the published molecular brightness of PA-Cherry has been determined. This is important, since we show here that the mode of photoactivation changes the measured fraction of photoactivated PA-FPs. Furthermore, for the monomeric versions comprising the A206K mutation. i.e. mEGFP and PA-mEGFP, no molecular brightness has been published.

4.5.1.3. In this ratiometric intensity-based approach, the molecular brightness of the PA-FPs and the always-on FP counterparts in a first approximation have been considered identical. We decided on this approach, since (i) for some FPs no molecular brightness has been reported, and (ii) 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. Furthermore, (iii) for a comparative analysis the knowledge of the molecular brightness is not necessary, it is only needed for the intensity-based determination of the absolute fraction of photoactivated PA-FPs which can be calculated as shown above.

4.6. Calculate the PA-Cherry photoactivation efficiency as a fraction of the measured red fluorescence intensity *after* photoactivation and the expected fluorescence intensity in the red channel.

$(F_{PA\text{-}Cherry}) = (I_{Red\_measured} - B_2) / I_{Red\_expected}$

4.7. Use the PA-GFP—Cherry construct to assess the fraction of photoactivated PA-GFP. Determine the expected fluorescence intensity of PA-GFP by dividing the measured red fluorescence intensity of the PA-GFP—Cherry construct *prior* to photoactivation by the red-to-green-ratio (RtoGr). Here, the RtoGr does not need to be corrected for donor quenching, because GFP and PA-GFP are subject to donor quenching to the same amount.

$I_{Green\_expected} = (I_{Red\_measured} - B_2) / RtoGr$

4.8. Calculate the PA-GFP photoactivation efficiency as a fraction of the measured green fluorescence intensity *after* photoactivation and the expected fluorescence intensity in the green channel.

$(F_{PA\text{-}GFP}) = (I_{Green\_measured} - B_1) / I_{Green\_expected}$