Quantitative visualisation of endocytic trafficking through photoactivation of fluorescent proteins

Manuela Ecker, Gregory Redpath, Philip Nicovich, and Jérémie Rossy

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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 #E20-10-0669
TITLE: Quantitative visualisation of endocytic trafficking through photoactivation of fluorescent proteins

Dear Dr. Rossy:

Thank you for submitting your work to MBoC. The reviewers of your paper both felt that further work was needed to clarify various aspects of your approach using photo activation to visualize endocytic trafficking. Please address these concerns in a revised paper. I will then consult the reviewers one more time before making a final decision regarding publication.

Sincerely,

Jennifer Lippincott-Schwartz
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Rossy,

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 haveopted out of publishing the review history.

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

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To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

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):

This submission by Ecker et al describes techniques for quantitating a variety of elements of endocytic trafficking using

photoactivatable fluorescent proteins. These assays include tracking the incorporation of surface receptors into various endosome populations, and the use of two-photon photoactivation to monitor recycling of intracellular receptors to the cell surface. These are applied to compare the intracellular trafficking of the T cell receptor and CD4. They conclude that TCR is efficiently and rapidly recycled to the cell surface via Rab11-positive recycling endosomes, while CD4 is not recycled via this pathway. They suggest instead that CD4 is degraded after endocytosis.
Overall, this paper does a good job of illustrating how photoactivation of GFPs can be used to dissect endocytic pathways. In particular, their successful use of two-photon photoactivation to selectively highlight receptors already inside the cell for recycling assays appears to be novel. The whole set of techniques they describe has the potential to be quite useful to investigators attempting to dissect the trafficking of proteins through endocytic pathways even when known ligands or antibodies don't exist. Their comparison of TCR to CD4 is interesting, and potentially of general interest to immunologists and cell biologists. While, in my opinion, there are issues with the study that need to be addressed, it is potentially of interest to the broad readership of MBOC.
There are some issues with the presentation and the data which are enumerated below. However, there is one major scientific issue that needs addressing. The demonstration of TCR recycling looks convincing. They argue that CD4 is degraded rather than recycled after endocytosis. However, what they mostly show is that CD4 isn't recycled over the timescale of the experiment, i.e, they demonstrate that it acts differently from TCR. However, they also fail to show progressive accumulation in Rab7-positive endosomes, and it could be worthwhile to repeat the experiment in the presence of protease inhibitors such as leupeptin. In my opinion, they need to show convincingly whether the photoactivated CD4 is destroyed, which could be done by monitoring whole-cell fluorescence of endocytosed CD4 after two-photon photoactivation, and comparing to a control such as TCR or TfR which avoids degradation after endocytosis. They should also discuss the lifetimes of these proteins in light of existing literature. E.g., Rhee and Marsh J. Virol. 68(8):5156-63 (1994) reports that CD4 lifetimes are normally about 24 hours, but reduced to a t1/2 of 6 hrs in Nef expressing cells. Such a lifetime is long relative to the experiments described here, so are the experiments here describing the fate of a small fraction of CD4 (slow endocytosis), or is there partial recycling via a Rab11-independent pathway, or are there differences in the experimental systems that lead to more rapid turnover?

1. The first paper describing a photoactivatable GFP (Patterson and Lippincott-Schwartz (2002) Science Vol. 297, Issue 5588, pp. 1873-1877) described photoactivation of lysosomally-located proteins. It seems this should be cited at least briefly in the introduction.

2. A variety of older techniques for following endocytosis are described in the introduction (e.g., antibody feeding). However, there is no mention of the use of fluorescent ligands (e.g., fluorescent Tf, LDL) despite the fact that this is one of the most important techniques employed, and there is a very extensive pre-existing literature. These techniques do get mentioned in the Results (lines 199-208), and it would be helpful to move the material covered in this paragraph into the introduction. Further, very few papers are referenced prior to 2000.

3. Related to the previous point, there were extensive attempts in a number of laboratories, most notably Fred Maxfield, to dissect endocytic pathways in detail by following fluorescent ligands. Much of this work is older (late 1980's, early 1990's). It should be acknowledged in the introduction, although the authors are free to point out that the methods described in the current paper can follow endocytosis and recycling of a protein even in the absence of any ligand.

4. Some of the graphs (Figure 2D, Figure 2G, Figure 5F) are quite noisy. The figure legend doesn't make it clear whether these graphs are representative data from single cells or averages from multiple cells. Further, for each protein, a thick line is visible bracketed on each side by thinner lines of the same color without explanation. The figure legends need some work to clarify these ambiguities. If these are representative traces from individual cells, it may be better to show a scatterplot (unconnected points).

5. There are some references to the use of thresholding to eliminate background (e.g. lines 146-147; lines 221-222). Thresholding by itself will define the locations to be quantitated, but is not a background correction by itself. Is the threshold actually subtracted?

6. In line 303, PA-GFP is described as "inconsistently fluorescent". What does this mean?

7. As mentioned above, repeating some work e.g. in Figure 4 {plus minus}
a protease inhibitor such as leupeptin could establish more definitively whether CD4 is being degraded in lysosomes.

8. Scale bars are missing in 1C, 2C, 3C and 5C. While these are illustrating the quantitation procedures, they would not be difficult to add.

Reviewer #2 (Remarks to the Author):

Ecker et al. describe a method to quantify endocytic trafficking of receptors through various compartments using localized activation of photoactivatable fluorescent proteins. These rely on spatial control of the photoactivation either through the use

of TIRF illumination or 2-photon excitation. Localized control of photoactivaton is not necessarily novel, but the authors offer this experimental approach coupled with analysis tools as an improved approach to quantifying receptor trafficking through the endocytic pathway. Their reasoning for pitfalls of existing methods and arguments for how this method can overcome them seem solid and this work may be useful to a broader audience of cell biologists studying endocytic trafficking. I suggest the authors address the following points.

• It would be helpful and would adhere to the MBC checklist if the authors could state explicitly the samples sizes in each figure legend in addition to their inclusion of scatter plots to show each data point in the figures.

• In their quantifications for figures 1 and 2, the authors indicated the percent PA signal in a particular Rab compartment or the percent vesicles within 320nm at designated time point after photoactivation. It would be helpful for comparison to know the "steady-state" value by running the same analyses on cells in which the entire cell was activated. This could of course be performed with normal fluorescent protein tags but quantifying the PA versions would be a better control.

• How did the authors normalize the signal immediately after photoactivation? I realize that most of their analyses express the activated signal in one compartment or another as a percentage. However, one concern is that if the level of the beginning signal is too small, the signal may not be detectable in some compartments after trafficking. I suggest the authors can address this by determining the total signal in each cell immediately after the photoactivation events and document the average values for each receptor in each experiment in the manuscript.

• Testa et al. (Journal of Microscopy, 2008 230:48-60) also performed similar photoactivation experiments using TIRF and multi-photon to selectively activate populations of molecules including EGFR. Their data analyses were different than those performed in this study, but it would be helpful if the authors cited this work and highlighted their advances over these studies.

Quantitative visualisation of endocytic trafficking through photoactivation of fluorescent proteins

Point-by-point response to referees' comments

Line numbers refer to the revised version of the manuscript. Changes are underlined in the manuscript.

Reviewer #1

- There are some issues with the presentation and the data which are enumerated below. However, there is one major scientific issue that needs addressing. The demonstration of TCR recycling looks convincing. They argue that CD4 is degraded rather than recycled after endocytosis. However, what they mostly show is that CD4 isn't recycled over the timescale of the experiment, i.e, they demonstrate that it acts differently from TCR. However, they also fail to show progressive accumulation in Rab7-positive endosomes, and it could be worthwhile to repeat the experiment in the presence of protease inhibitors such as leupeptin. In my opinion, they need to show convincingly whether the photoactivated CD4 is destroyed, which could be done by monitoring whole-cell fluorescence of endocytosed CD4 after two-photon photoactivation, and comparing to a control such as TCR or TfR which avoids degradation after endocytosis.

The reviewer suggestions of additional experiments (including the use of a protease inhibitor in point 7 below) are excellent and would totally make sense if the submitted manuscript was focused on elucidating endocytic trafficking of CD4. However, it is not, and we are responsible for the confusion. Our intention was only to thoroughly describe a method that we have used in two previous publication (Compeer et al., 2018; Redpath et al., 2019). We used TCR and CD4 as examples to illustrate that this method allows the distinction between the endocytic trafficking of two surface receptors involved in the same cellular response. The fact is that we were so happy to see that CD4 trafficking was so distinct to TCR that we got a bit carried away and speculated with too much conviction that CD4 is degraded after endocytosis. In fact, we can only state that CD4 does not reach Rab11-positive compartments and is not recycled from Rab7-positive compartments, at least not at the same rates than TCR from these compartments.

This was a mistake as the submitted work is intended only as a description of a methodology and not as an investigation of CD4 intracellular trafficking. To avoid further confusion, we have removed from the text the speculations about CD4 trafficking or made very clear that these are only speculations, which require further investigation (line 473-481 and 521-530).

We envisage building up on the CD4 results (and the reviewer's suggestions!) to draw a more comprehensive picture of CD4 recycling and degradation in resting and activated T cells. Furthermore, as rightfully mentioned by the reviewer in their comment just below, we will likely need to adjust the imaging parameters to capture endocytic steps that happen much later after internalisation. We actually touch on this problem when we mention the fast-bleaching time of PAmCherry and the necessity to adjust time intervals between imaging frames to visualise endocytic processes with different timeframes (lines 314-317 and 523-525).

- They should also discuss the lifetimes of these proteins in light of existing literature. E.g., Rhee and Marsh J. Virol. 68(8):5156-63 (1994) reports that CD4 lifetimes are normally about 24 hours, but reduced to a t1/2 of 6 hrs in Nef expressing cells. Such a lifetime is long relative to the experiments

described here, so are the experiments here describing the fate of a small fraction of CD4 (slow endocytosis), or is there partial recycling via a Rab11-independent pathway, or are there differences in the experimental systems that lead to more rapid turnover?

As mentioned just above, because of the bleaching of PAmCherry, we cannot exclude that there is a fraction of CD4, internalised by slow endocytosis, that we did not observe with the imaging parameters we used in this study (the fraction we observe was internalised by a fast – we could detect and count endocytic vesicles almost immediately after photoactivation – and clathrin-independent mechanism).

Furthermore, it is indeed possible that we did not observe incorporation of CD4 into Rab11-positive compartments because CD4 needs much longer than TCR to reach these compartments. This is however unlikely in light of the quantification of the amount of total photoactivated signal suggested by reviewer 2 (Fig. S1), which shows that there was no photoconversion of CD4-PSCFP2 in Rab11-positive compartments, suggesting very low levels of CD4 in these endosomes. But the data in figure S1D further show that there is CD4 in Rab7 endosomes, which leaves the possibility of a slow recycling of CD4 from these compartments.

Nevertheless, a slow Rab7-mediated recycling of CD4 should not depend on the lifetime of CD4: no matter how long CD4 needs to get to the compartment it recycles from or how slowly it is degraded, we should be able to visualise the return of CD4 from this compartment to the cell surface if it does happen, unless CD4 requires a much longer time to recycle from Rab7 than TCR.

We have to further mention here that CD4 could potentially recycle from Rab4-positive compartments. To sum up, there are many questions about CD4 trafficking that would require further investigation: is there a slow clathrin-dependent endocytosis of CD4, is CD4 recycled from Rab4-positive endosomes, is there a slow Rab7-mediated CD4 recycling or is there no recycling but instead invariable degradation after endocytosis (what would be exciting would be that the slowly internalised fraction of CD4 is recycled and the fast one degraded!). This is why we feel that CD4 endocytic trafficking deserves a bit more investigation than what we can do in a methodological study.

Nevertheless, we have added a reference to the work of Rhee and Marsh in a comment on the long half-life of CD4 and on the importance of the time it might take for CD4 to recycle when considering the meaning of our data (lines 473-481). We have also amended the conclusion to include these considerations (lines 521-530)

1. The first paper describing a photoactivatable GFP (Patterson and Lippincott-Schwartz (2002) Science Vol. 297, Issue 5588, pp. 1873-1877) described photoactivation of lysosomally-located proteins. It seems this should be cited at least briefly in the introduction.

The reviewer is correct; we should have cited this paper. We do it in the revised version in line 109.

2. A variety of older techniques for following endocytosis are described in the introduction (e.g., antibody feeding). However, there is no mention of the use of fluorescent ligands (e.g., fluorescent Tf, LDL) despite the fact that this is one of the most important techniques employed, and there is a very extensive pre-existing literature. These techniques do get mentioned in the Results (lines 199-208),

and it would be helpful to move the material covered in this paragraph into the introduction. Further, very few papers are referenced prior to 2000.

3. Related to the previous point, there were extensive attempts in a number of laboratories, most notably Fred Maxfield, to dissect endocytic pathways in detail by following fluorescent ligands. Much of this work is older (late 1980's, early 1990's). It should be acknowledged in the introduction, although the authors are free to point out that the methods described in the current paper can follow endocytosis and recycling of a protein even in the absence of any ligand.

We must thank the reviewer for pointing out the large body of work that has been done before 1995 using fluorescently labelled ligands and lipids and that is indeed highly relevant for the experiments we report. And we must shamefully confess that the great care we took to align our work with the latest advances in the field made us overlook the key findings that were made at the time. I guess this is what peer-reviewing is for!

We have added a new paragraph in the introduction to mention this work (lines 85-95). In term of references, we cite two reviews from Fred Maxfield that we feel represent an excellent and thorough overview of the work done in late 80s and early 90s.

4. Some of the graphs (Figure 2D, Figure 2G, Figure 5F) are quite noisy. The figure legend doesn't make it clear whether these graphs are representative data from single cells or averages from multiple cells. Further, for each protein, a thick line is visible bracketed on each side by thinner lines of the same color without explanation. The figure legends need some work to clarify these ambiguities. If these are representative traces from individual cells, it may be better to show a scatterplot (unconnected points).

We thank the reviewer for spotting this mistake and have specified the number of cells per experiment in the revised version of the manuscript. Furthermore, we now clearly mention the figure legends that the error bars in all the charts represent SEM (Figure 2D, G and F).

5. There are some references to the use of thresholding to eliminate background (e.g. lines 146-147; lines 221-222). Thresholding by itself will define the locations to be quantitated, but is not a background correction by itself. Is the threshold actually subtracted?

The reviewer is correct- the threshold is not background correction in itself and is used to define locations to be quantified. We have changed the wording in lines 146-47 (now lines 160-161) and lines 221-222 (now lines 240-242). We have further modified how we refer to the threshold in all figure legends from “thresholding” to “identification by threshold” to make it clear the threshold is used to identify the endosomal regions of interest.

6. In line 303, PA-GFP is described as "inconsistently fluorescent". What does this mean?
The phrasing is indeed not accurate, and we thank the reviewer for spotting it. For unknown reasons, PA-GPF proved to be difficult to photoactivate when fused to TCRζ or CD4, even more when using two-photon illumination. In writing so, we merely wanted to warn other researchers potentially willing to use the same approach that PA-GFP did not work for us in combination with TCR and CD4. However, as determining why so is far beyond the scope of this study, we have removed from the text any reference to the unpredictable photoconversion of PA-GFP in our hands (lines 342-343).

7. As mentioned above, repeating some work e.g. in Figure 4 {plus minus} a protease inhibitor such as leupeptin could establish more definitively whether CD4 is being degraded in lysosomes.

Please see our response to the first comment above.

8. Scale bars are missing in 1C, 2C, 3C and 5C. While these are illustrating the quantitation procedures, they would not be difficult to add.

We thank the reviewers for pointing this out. We added all missing scale bars to Fig. 1C, 2C, 3C and 5C.

Reviewer #2:

• It would be helpful and would adhere to the MBC checklist if the authors could state explicitly the samples sizes in each figure legend in addition to their inclusion of scatter plots to show each data point in the figures.

We fully agree with the reviewer and apologize for this omission. We have now added the sample size and number of experiments in each figure legend.

• In their quantifications for figures 1 and 2, the authors indicated the percent PA signal in a particular Rab compartment or the percent vesicles within 320nm at designated time point after photoactivation. It would be helpful for comparison to know the "steady-state" value by running the same analyses on cells in which the entire cell was activated. This could of course be performed with normal fluorescent protein tags but quantifying the PA versions would be a better control.

We agree with the reviewer that our quantification would be strengthened by a comparison to a control scenario where we know:

a) that all vesicles that contain the protein labelled with PA-mCherry also contain the protein labelled with EGFP in the case of the endocytosis quantification (vesicles within 320nm) in Fig. 1.

or

b) that 100% of the cargo labelled with PA-mCherry is directed to Rab5 (or Rab11) in the case of the sorting experiments shown in Fig. 2.

We have performed the control mentioned in **a)** when we first used this approach in (Compeer et al., 2018). We used the membrane protein flotillin-1 and flotillin-2, which form heterodimers in leukocytes to measure the percentage of vesicles within 320 nm in a scenario where all PA-mCherry-positive vesicles should also be positive for EGFP (Supplemental Figure 3 of Compeer et al.). The percentage of nearest-neighbour below 320 nm was 54.6% in this instance. We have modified the text to now refer to this control (line 175-178).

As for the control mentioned in **b)**, we do not think it is possible to find a molecule that would entirely be incorporated in Rab5, Rab11 or Rab7 endosomes after endocytosis. Performing photoactivation of the whole cell or using classic fluorescent proteins would only generate more vesicles with no certainty that these vesicles are coming or going or targeted to a given compartment. Activating only a delimited region should not influence

the percentage of the signal generated by photoactivation that can be found in a given Rab compartment, as this percentage depends on the sorting mechanism and not on the sheer number of proteins whose internalisation has been revealed by photoactivation.

Determination of the total signal after photoactivation in each cell as suggested by the reviewer in the next comment appear to confirm this interpretation. While there are differences between proteins in the amount of signal revealed by photoactivation (Fig. S1), these differences do not correlate with how much of the protein reaches Rab-positive compartments. For example, TCRζ-PAmCherry was photoactivated far more than CD4-PAmCherry in the Rab5 sorting experiments (Fig. S1), yet identical proportions of each sorted into Rab5 endosomes after photoactivation (Fig. 2). This indicates that the amount of photoactivated signal does not correlate with the percentage of protein observed in a given Rab compartment- if it did, we would expect proportionately more TCRζ present in Rab5, which we do not. We have added additional results and discussion covering these points for both methods of photoactivation in lines 297-307 and 452-459, in addition to Figure S1.

• *How did the authors normalize the signal immediately after photoactivation? I realize that most of their analyses express the activated signal in one compartment or another as a percentage. However, one concern is that if the level of the beginning signal is too small, the signal may not be detectable in some compartments after trafficking. I suggest the authors can address this by determining the total signal in each cell immediately after the photoactivation events and document the average values for each receptor in each experiment in the manuscript.*

We thank the reviewer for this comment, as we indeed did not sufficiently explain the strategy used to normalise the signal after photoactivation in the original submission. For sorting experiments (Fig .2), we calculate the percentages by first determining the intensity of photoactivated signal of the protein of interest that is in endosomes in each frame of the image. Then we quantify the intensity of this endosomal signal that is present in the Rab mask, and finally we divide the intensity of photoactivated signal in the Rab mask by the total endosomal photoactivated signal. Therefore, we do not "normalise" strictly immediately following photoactivation, but you could say that we "normalise" in every frame of the image by performing this calculation. For recycling experiments (Fig. 4), we normalise to the background signal present within the cell mask at time zero and then express the change in photoactivated PSCFP2 membrane signal as the percentage change over this initial background.

We have amended our wording in lines 240-242, 247-249, 367-368, 376-377, 432-435 and 442-444 to make every step of the analysis clearer and explain how these percentages are determined.

Furthermore, we understand the reviewers concern regarding the possibility that if the signal is too weak right after photoactivation, it may not be detectable further on in the experiment. We have therefore performed the suggested analysis, quantifying the total photoactivated signal in each cell (Fig. S1). We have discussed the implications of these results in the answer to the previous comment, but in the context of this comment we would like to add that this additional analysis shows that sufficient photoactivated signal was present in sorting experiments to detect proteins of interest trafficking to a given Rab compartment. We wish to thank the reviewer for this suggestion as the analysis proved to be highly relevant and helped strengthen our conclusions. Of note, it also yielded information that we used to answer a comment of reviewer 1: if there is no signal after two-

photon photoactivation in a given compartment, it indicates that there is no or very little of the protein fused to PSCFP2 in this compartment. This is typically the case for CD4 in Rab11-positive compartments.

• *Testa et al. (Journal of Microscopy, 2008 230:48-60) also performed similar photoactivation experiments using TIRF and multi-photon to selectively activate populations of molecules including EGFR. Their data analyses were different than those performed in this study, but it would be helpful if the authors cited this work and highlighted their advances over these studies.*

Here we must respectfully disagree with the reviewer. In the original submission, we already cited two publications that are anterior to the work of Testa et al and that represent the conceptual framework for the method we describe. Luo et al., in 2006 (line 326) performed two-photo-activation of fluorescent protein to visualise cargo sorting the Golgi and Caswell et al., in 2007 (line 111), used quantification of photoactivation to investigate integrin trafficking in migrating cells. These papers have been published earlier and, more importantly, they report a usage of photoactivation of fluorescent protein to investigate protein dynamics that is more relevant to the work we describe than Testa et al., which mostly focuses on how photoactivation can be localised in cells using various illumination approaches.

Nevertheless, we have added one key publication in the field of photoactivation that was missing in the first version of this manuscript, Patterson and Lippincott-Schwartz 2002 (line 108-109).

RE: Manuscript #E20-10-0669R
TITLE: "Quantitative visualisation of endocytic trafficking through photoactivation of fluorescent proteins"

Dear Dr. Rossy:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Jennifer Lippincott-Schwartz
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Rossy:

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.

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