mBeRFP: a versatile fluorescent tool to enhance multichannel live imaging and its applications

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AUTHORS: Emmanuel Martin and Magali Suzanne

I apologize for the delay before getting back to you. I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Martin and Suzanne describes the implementation in Drosophila of two new long Stokes shift red fluorescent proteins: mBeRFP and LSSmKate2; which offer new opportunities for multicolor imaging. They demonstrated that mBeRFP is not toxic and can be used together with other fluorescent proteins such as RFP or GFP without any cross reactivity. Interestingly, this

protein displays low photobleaching making it relevant for long term imaging; but also for quantitative applications using FRAP or laser ablation. Most importantly, when combined with other fluorescent proteins it offers the possibility of using a single source of light excitation and scanning at two different wavelength which can be beneficial for photo toxicity and substantially speeds up image acquisition. We think mBeRFP is a valuable tool that will benefit the field. Overall this is a well written manuscript, with clear and well laid out out figures, the data is convincing, and the progress it reports is valuable.

Comments for the author

I have a few remaining concerns which are outlined below:

Major points:
- The authors did a good job demonstrating the relevance of using mBeRFP as a fluorescent protein for live imaging in Drosophila, to do so they are mostly utilise a fusion of mBeRFP and αcatenin. They then simultaneously image this construct with GFP fused to myosin. First, the choice of these two markers appears arbitrary so the authors should explain why they chose them. Second, While the paper nicely covers the technical aspects of this tool such as spectra, crosstalk toxicity; it could do a better job demonstrating the biological usefulness of this tool. Specifically, using a specific biological context would be helpful to illustrate the advantage multi marker imaging discussed in the study. For example, something along the lines of showing how catenin (marking adhesions) and myosin (imaging the cell division apparatus) can highlight cell divisions in neuroblasts, or cultured wing discs.
- In fig. 1 B,C and FigSup1A, the authors argue that the expression of mBeRFP and LSSmKate2 do not cause developmental defects due to cytotoxicity. However, without comparison to a control this remains an unsubstantiated claim. We would suggest to the authors to show images of appropriate controls that did not express mBeRFP but another fluorescent protein such as RFP known to not be toxic or affecting development.
- The authors argue that the main advantage of mBeRFP is when used in combination with other fluorescent protein sharing a similar excitation spectrum they can use only one laser for excitation thus limiting toxicity and bleaching. They nicely showed mBeRFP can be used with GFP or RFP without crosstalk which is indeed an interesting advantage but there is no experiment in the paper directly showing how using only one laser for emission would be advantageous. We think that performing an experiment demonstrating this phenomenon would greatly benefit the story. To do so the authors could perform a long-term imaging experiment with a quite high frequency of acquisition between two samples: one having expressing GFP and mBeRFP, and the other one expressing GFP and RFP. Ideally the sample expressing GFP and RFP would start to bleach before the other one. Or, the authors could perform a FRAP experiment comparing one sample expressing MyoII-GFP and α-cateninmBeRFP with another sample expressing MyoII-GFP and α-cateninTagRFP. In addition can they give an example of how faster could be an acquisition would be with only one light source.
- In FigSub1B, the authors show a lower fluorescent intensity for LSSmKate2 when under the expression of ap-Gal4, and use this result to discard LSSmKate2 from the story. Is this phenomenon specific to this Gal4 or generalizable to all Gal4s? We would recommend repeating the same experiment using another Gal4, such as DII-Gal4. Also, was the laser intensity used for mBeRFP and LSSmKate2 similar? If yes is it possible to mention it in the text?

Minor points:
- "Results and Discussion". It sounds confusing to name this part "Results and Discussion" while there is a "Discussion" part later on.
- The composition of the figure 1 is confusing: The first 2 vectors in A are actually cited in the text at the very end of the figure description. Maybe put these vectors at the end of the figure or in supplementary would be helpful.
- Fig. 1D,E, we think it would offer more clarity to show the vector of α-catenin-mBeRFP above the corresponding staining.
- Fig. 2B, the promoter used is not mentioned in the figure or the legend.
- Test related to Fig. 2B, "We imaged at the same time alpha-catenin-mBeRFP expressed under the control of an apterous-Gal4", it is just apterous-Gal4, not "an".
- FigSup. 2A,B, The illumination microscopy is very interesting to address the spatial distribution, we think it would benefit the story if the authors could justify why they need this particular

technique. They can mention/discuss the difference of scale between the previous staining and the new ones. Also, in this particular experiment there is no cross talk calculations, while the text mentions it.
- FigSup. 2C, the spectra of mBeRFP and tagRFP would be very useful in the main figure and would offer a nice comparison with the spectrum of GFP: mBeRFP and GFP have similar excitation properties while mBeRFP and tagRFP have similar emission properties.
- FigSup. 2D, the image of the α-catenin-mBeRFP on the "confocal mode" is missing while present if Fig. 2B. The square boxes to calculate the crosstalk are in the TRITC panel while for Fig. 2B,C they were in the mBeRFP panels; Can you explain why is this different? Also is it "Confocal mode" or "Classic mode" (like in Fig. 2B)?
- Fig. 2E, This is indeed a beautiful staining. But it would be really interesting if the protocol/settings would be explained. Could you explain the spectra and laser used in supplementary and/or Material and methods?
- Legend Fig. 2B,C, the number of samples is not mentioned.
- At the end of the first paragraph there is a reference to Fig.3A while it should be Fig.3A'.
- For the Fig.3B the protocol is a bit unclear: in the text it is mentioned that there are 1800 frames per second while the legends mentioned 1s per frame for a total of 1800. Please clarify.
- Fig.3B, what is the genotype? How many samples have been used?
-FigSup.3, The genotype is not mentioned. Are the Ecad-GFP and the NLS-mBeRFP expressed in the same flies? How many samples? Also, the mBeRFP is localized in the nucleus (NLS), would the result be the same with a cytoplasmic localization or at the membrane (fused with α-catenin)? We suggest here to repeat the same experiment but with MyoII-GFP and α-catenin-mBeRFP.
- What does FRAP stand for? What does this experiment show? We think it would increase the impact of these experiment if the authors would explain in more details the principle and goal of it.
- Fig.3C legend, how many FRAP experiments were conducted?
- Fig.3 legend: in the figures some panel are labeled with ' (for example C', D' or D"), but in the legends they are referred as "Top", "middle" or "bottom", please homogenize.
- Fig.3D legend, how many experiments were conducted?
- In the Material and Methods, RIM stands for random illumination microscopy? put the abbreviation in the text.
- In the Material and Methods, the protocol for photobleaching is not described (Fig.2B).
- In the Material and Methods, please give the parameters used for the Fig.2E.

Reviewer 2

Advance summary and potential significance to field

This technical report by Martin and Suzanne is reporting a new tool to better visualise multiple proteins live in living Drosophila tissue.
Live imaging of proteins is an invaluable way to report the in vivo dynamics of proteins. However, the current limitations in separating the different fluorescent excitation and emission spectra of the different available genetically encoded proteins makes it difficult to image the dynamics of several proteins at the same time in Drosophila.

Here the authors have generated transgenic lines using the large Stokes shift red fluorescent protein mBeRFP in Drosophila and explored its value for live imaging in combination with an existing GFP fusion protein. The long stokes shift allowed excitation of mBeRFP and GFP with one laser line (458nm, a common laser line present in the standard argon laser) and easy separation of the emission spectra. Also, valuable combinations of mBeRFP with either GFP or RFP were demonstrated as well as for mBeRFP FRAP experiments that can assess mBeRFP tagged protein dynamics at same time as GFP tagged protein dynamics. This is really cool!

In combination with the generated UAS and non-UAS cloning vectors containing mBeRFP, these tools will be valuable for the Drosophila and the imaging community in general.

Comments for the author

1.Fluorescent proteins are particularly useful for live imaging as the authors of course appreciate. Hence, the authors should make clearer that Figures 1 and 2 analyse fixed samples (which would not have needed live dyes). I would have preferred to see these quality assessments of mBeRFP in live samples, as it would be more meaningful. Also, fixation may differentially affect the intensity of different fluorescent proteins; from the figure legends it is unclear but from the methods I suppose that FigSup1 was using fixed samples too which might not be optimal to distinguish the 2 proteins in living samples.
2. Figure 2D/Suppl Figure 2D: to better appreciate the intention of the experiment I would appreciate if also the images acquired with 458nm excitation were shown. This makes clearer that the combination of mBeRFP with TagRFP or TRITC needs sequential excitation while collecting at the same emission window. Again, live imaging would be better.
3. The authors convincingly showed that mBeRFP can be imaged successfully together with GFP or RFP protein versions (in fixed samples). However, it is stated in the text that the authors combine mBeRFP with GFP AND RFP, which implies imaging of the three at the same time. I guess this should be possible, however was only shown with the synthetic TRITC dye in fixed images. Please amend the text.
4. Figure 3 shows nicely that mBeRFP can be actually used for live imaging in cultured leg discs, either as single colour fused to alpha-Cat or together with NMII-GFP. It would be nice to annotate the overview Movie 1 with arrows similar as in the 3A' to illustrate the cell extrusions in the movie directly.
The authors state: "we are able to follow apoptotic cell extrusions from the apical surface of the epithelial leg at the expected rate" – I am missing how this rate was measured.
5. To fully assess the data of the FRAP and laser cuts, please provide the missing example movies for Figures 3C and D. Was general bleaching of the sample during the 'postbleach' acquisition taken into account when calculating the fluo recovery rate? The alphaCat-mBeRFP looks brighter at 0 sec compared to 60 sec. It might be better to show ratios comparing the recovering junction to neighbouring unbleached junctions.

Reviewer 3

Advance summary and potential significance to field

In this technical report, Martin and Suzanne introduce a series of mBeRFP-fused fluorescent constructs to use in Drosophila. mBeRFP is a red fluorescent protein with a long Stokes shift, and thus can be excited simultaneously with CFG/GFP variants, and its emission can be independently detected. The authors show that cytoplasmic mBeRFP is brighter than LSSmKate2, another long Stokes shift RFP. mBeRFP can be imaged (and simultaneously excited) with eGFP with no crosstalk and it displays low crosstalk with TagRFP ro TRITC. mBeRFP construct expression does not affect cellular dynamics, and only displays moderate photobleaching, and can be used for FRAP and laser ablation assays.

This is a nice, short technical report that presents fluorescent constructs and plasmids that are likely to be useful for the wider Drosophila community. I have a few suggestions of some additional controls to validate the level of crosstalk of mBeRFP and eGFP in regular confocal mode, and a few additional comparisons that I think would be important to validate the constructs made and their dynamics.

Comments for the author

1. To determine how widely applicable these constructs are to "classic" confocal mode, with no spectral detector or linear unmixing, the authors should quantify how GFP fluorescence (e.g. alpha-catenin:eGFP) changes in the presence of mBeRFP (e.g. alpha-catenin:mBeRFP) and vice versa (e.g. comparing the fluorescence in each of the channels in embryos expressing one or two of the fluorescent proteins). This is important, as not every reader will have acccess to spectral imaging.
2. Figs. 3B and S3: to evaluate the significance of the photobleaching of mBeRFP a much more interesting and practical comparison would be similar to that shown in Fig. S3 for MyoII-GFP and

NLS-mBeRFP, but comparing fusions to the same protein (e.g. alpha-catenin-GFP vs. alpha-catenin-mBeRFP).
3. Fig. 3C-C': the FRAP experiments should be conducted comparing (quantitatively) alpha-catenin-GFP vs. alpha-catenin-TagRFP vs. alpha-catenin-mBeRFP. Are the mobile fractions and half times of fluorescence recovery similar for all three markers. This is really important to verify that mBeRFP tagging does not affect protein turnover or dynamics in a way different from GFP or TagRFP.
4. Figure 1: similar to the comparison to LSSmKate2 (Fig. S1), I would appreciate a comparison between mBeRFP and TagRFP. For example, for the alpha-catenin construct shown in Figure 1D-E, the TagRFP fusion seems brighter. Presenting quantitative data (maybe normalized to the MyoII-GFP signal) would be useful for people trying to decide between a TagRFP or an mBeRFP fusion (when Stokes shift is not a consideration).
5. Fig. S3: the labels indicate that the images beelong to E-cadGFP and NLS-mBeRFP, and the quants to MyoII-GFP and NLS-mBeRFP. Unless the labels are wrong (and in that case they should be corrected), the images should correspond to the markers shown in the quants.

TYPOS Page 9: "2-photons" should be "2-photon".

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Martin and Suzanne describes the implementation in Drosophila of two new long Stokes shift red fluorescent proteins: mBeRFP and LSSmKate2; which offer new opportunities for multicolor imaging. They demonstrated that mBeRFP is not toxic and can be used together with other fluorescent proteins such as RFP or GFP without any cross reactivity. Interestingly, this protein displays low photobleaching making it relevant for long term imaging; but also for quantitative applications using FRAP or laser ablation. Most importantly, when combined with other fluorescent proteins it offers the possibility of using a single source of light excitation and scanning at two different wavelength which can be beneficial for photo toxicity and substantially speeds up image acquisition. We think mBeRFP is a valuable tool that will benefit the field.
Overall this is a well written manuscript, with clear and well laid out out figures, the data is convincing, and the progress it reports is valuable.

Reviewer 1 Comments for the Author:
I have a few remaining concerns which are outlined below:

Major points:

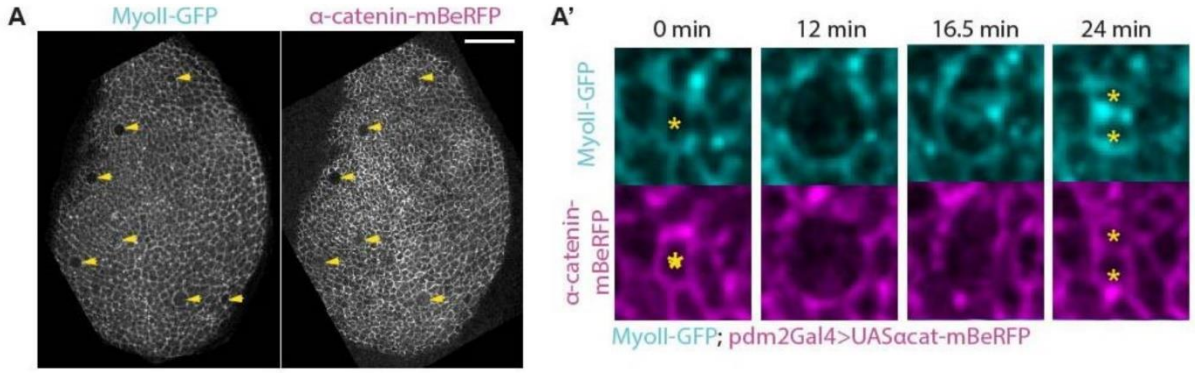
- The authors did a good job demonstrating the relevance of using mBeRFP as a fluorescent protein for live imaging in Drosophila, to do so they are mostly utilise a fusion of mBeRFP and αcatenin. They then simultaneously image this construct with GFP fused to myosin.
First, the choice of these two markers appears arbitrary so the authors should explain why they chose them. Second, While the paper nicely covers the technical aspects of this tool such as spectra, crosstalk, toxicity; it could do a better job demonstrating the biological usefulness of this tool. Specifically, using a specific biological context would be helpful to illustrate the advantage multi marker imaging discussed in the study. For example, something along the lines of showing how catenin (marking adhesions) and myosin (imaging the cell division apparatus) can highlight cell divisions in neuroblasts, or cultured wing discs.

Re: We agree with the referee that we should have justified our choice. To do so, we added the following sentences at the beginning of the 'Live imaging and micromanipulation experiments using α-catenin-mBeRFP and MyoII-GFP' section:

"In the following experiments, we chose to focus on the acto-myosin cytoskeleton (MyoII-GFP) together with the adherens junctions (aCat-mBeRFP), two very dynamic markers, as an example to evaluate simultaneously tissue mechanics and cell shape changes, a central interest in the field of

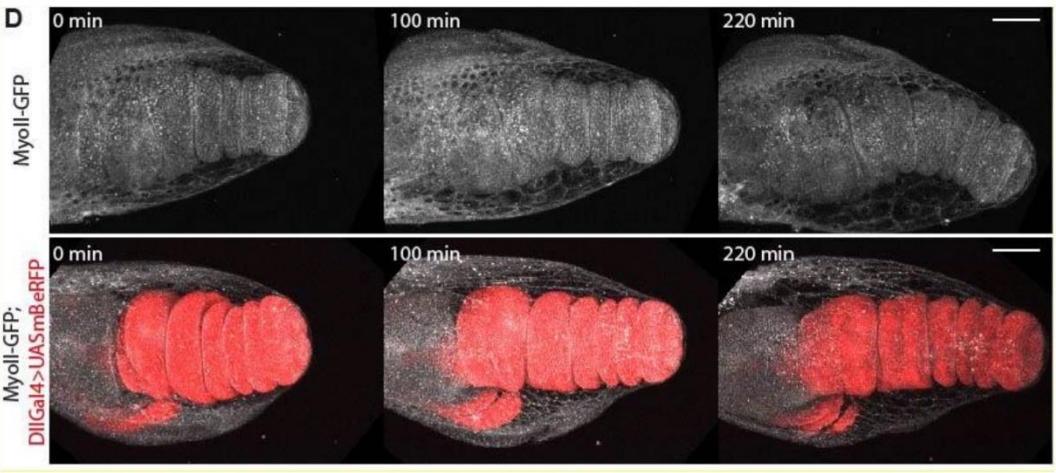
mechanobiology. Our aim was to show that this new tool could be of interest for long term live imaging experiments and could be combined easily with other markers."

We agree with the referee that we could have illustrate the advantage of multimarker imaging with a single laser source and chose to do so following cell division in a wing disc as suggested (see new fig 4A).



- In fig. 1 B,C and FigSup1A, the authors argue that the expression of mBeRFP and LSSmKate2 do not cause developmental defects due to cytotoxicity. However, without comparison to a control this remains an unsubstantiated claim. We would suggest to the authors to show images of appropriate controls that did not express mBeRFP but another fluorescent protein such as RFP known to not be toxic or affecting development.

Re: We agree that this information was missing. We now show a leg expressing MyoII-GFP as a control in Fig1D and a movie to compare the development of leg expressing MyoII-GFP or MyoII-GFP; DllGal4; UAS α-catenin-mBeRFP (see Movie1), demonstrating that the development is not affected by the expression of mBeRFP.



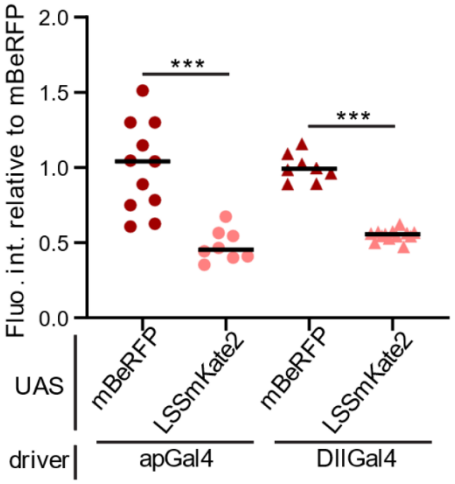
- The authors argue that the main advantage of mBeRFP is when used in combination with other fluorescent protein sharing a similar excitation spectrum they can use only one laser for excitation thus limiting toxicity and bleaching. They nicely showed mBeRFP can be used with GFP or RFP without crosstalk which is indeed an interesting advantage but there is no experiment in the paper directly showing how using only one laser for emission would be advantageous. We think that performing an experiment demonstrating this phenomenon would greatly benefit the story. To do so the authors could perform a long-term imaging experiment with a quite high frequency of acquisition between two samples: one having expressing GFP and mBeRFP, and the other one expressing GFP and RFP. Ideally the sample expressing GFP and RFP would start to bleach before the other one. Or, the authors could perform a FRAP experiment comparing one sample expressing MyoII-GFP and α- cateninmBeRFP with another sample expressing MyoII-GFP and α-cateninTagRFP. In addition can they give an example of how faster could be an acquisition would be with only one light source.

Re: We agree that the advantage of using only one laser source (instead of two) to image two different fluorophores should have been explained more clearly. Although the experiment suggested by the referee could be interesting, we reasoned that it would have been complex to demonstrate the difference in phototoxicity between the two conditions proposed. To further support this point, we discuss it:

"Moreover, using the mBeRFP/GFP pair with a single excitation source rather than GFP/RFP pair using two different lasers sequentially could (1) greatly reduce phototoxicity since sample are illuminated at a single wavelength (instead of two different wavelengths) and (2) reduce twice the acquisition time, a critical parameter to study the protein dynamics."

- In FigSub1B, the authors show a lower fluorescent intensity for LSSmKate2 when under the expression of ap-Gal4, and use this result to discard LSSmKate2 from the story. Is this phenomenon specific to this Gal4 or generalizable to all Gal4s? We would recommend repeating the same experiment using another Gal4, such as DII-Gal4. Also, was the laser intensity used for mBeRFP and LSSmKate2 similar? If yes is it possible to mention it in the text?

Re: We repeated the experiment using Dll-Gal4 and quantified the fluorescence using the 458nm laser with the same intensity. We obtained similar results that those obtained with ap- Gal4. We added the quantification in FigSup1D



Moreover, we now specify in the text that the laser intensity was the same in these experiments: "and showed that, at the same laser power, the fluorescence intensity of the LSSmKate2 was not as high as the one of mBeRFP (FigSup1C-D), so we focused on mBeRFP."

Minor points:

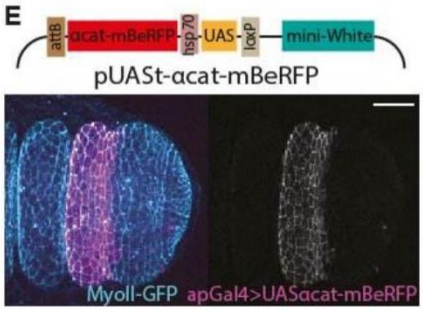
- "Results and Discussion". It sounds confusing to name this part "Results and Discussion" while there is a "Discussion" part later on.

Re: We agree and rename the "Discussion" part "To go further with mBeRFP"

- The composition of the figure 1 is confusing: The first 2 vectors in A are actually cited in the text at the very end of the figure description. Maybe put these vectors at the end of the figure or in supplementary would be helpful.

Re: We reorganized figure 1 and put the promoter-free vectors in supplementary figure 1E.

- Fig. 1D,E, we think it would offer more clarity to show the vector of α-catenin-mBeRFP above the corresponding staining.
Re: We agree and modified Fig1E accordingly.



- Fig. 2B, the promoter used is not mentioned in the figure or the legend.

Re: We added the promotor used (ap-Gal4) in the new Fig3B. (Figure 2 is now Figure 3)

- Test related to Fig. 2B, "We imaged at the same time alpha-catenin-mBeRFP expressed under the control of an apterous-Gal4", it is just apterous-Gal4, not "an".

Re: We deleted “an”.

- FigSup. 2A,B, The illumination microscopy is very interesting to address the spatial distribution, we think it would benefit the story if the authors could justify why they need this particular technique. They can mention/discuss the difference of scale between the previous staining and the new ones. Also, in this particular experiment there is no crosstalk calculations, while the text mentions it.

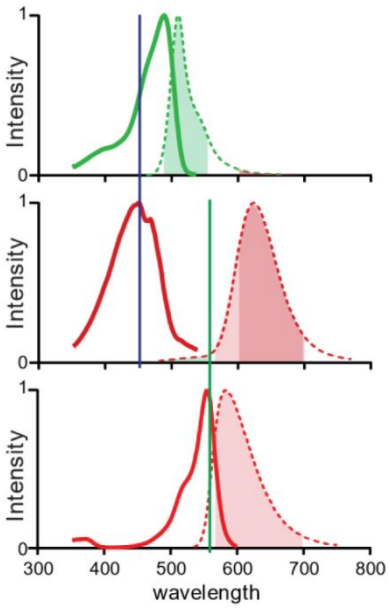
Re: We added a sentence to justify the RIM:

“[…] we used random illumination microscopy. This newly developed super-resolution technique offers the possibility to reach unprecedented spatial resolution (around 100 nm) in living tissues (Mangeat et al., 2021).”

The mention to crosstalk calculation was a mistake and was removed from the text.

- FigSup. 2C, the spectra of mBeRFP and tagRFP would be very useful in the main figure and would offer a nice comparison with the spectrum of GFP: mBeRFP and GFP have similar excitation properties while mBeRFP and tagRFP have similar emission properties.

Re: We reorganized this figure (Fig 2 is now Fig 3) and added the spectra of TagRFP in the main figure 3A to offer a better comparison of GFP, mBeRFP and TagRFP spectra.



- FigSup. 2D, the image of the α-catenin-mBeRFP on the "confocal mode" is missing while present if Fig. 2B. The square boxes to calculate the crosstalk are in the TRITC panel while for Fig. 2B,C they were in the mBeRFP panels; Can you explain why is this different? Also is it "Confocal mode" or "Classic mode" (like in Fig.2B)?

Re: We agree with you that these panels could be confusing. The objective here was to analyze the potential crosstalk using different modes of imaging. To clarify this point, we change the title of the new figure 3:

“mBeRFP can be combined with green- and red-emitting fluorescent proteins without any crosstalk”

In new Fig3B, we imaged at the same time MyoII-GFP and α-catenin-mBeRFP (with a 458nm laser) using either the classical confocal mode with two different emission windows as shown in Fig3A (GFP and mBeRFP emission windows), or the spectral mode based on GFP and mBeRFP spectra. This explains why we have images of MyoII-GFP and α-catenin-mBeRFP in each mode.

Regarding the quantification in Fig 3B,C, we wanted to know if GFP was detected in mBeRFP emission window so we compared the fluorescence of the GFP in mBeRFP in classic mode, which could allow crosstalk, and spectral mode, which accurately separate FP.

In FigSup 3D, we excited the sample (apGal4>UAS α-catenin-mBeRFP + phalloidin-TRITC staining) with a 561nm laser. In the classic mode of detection, we used a single emission window for mBeRFP and TRITC because their emission spectra overlap, as shown in Fig3A. The result is a single image of all the detected fluorescent proteins excited with the 561 nm laser and detected in this emission window, so, in the one hand, only TRITC in the other hand, a mix of TRITC and mBeRFP. In the spectral mode, we detected fluorescent proteins based on their spectra, here TRITC and mBeRFP spectra. This explains why we can obtain two images in this condition. Finally, the quantitative comparison (formula detailed in Fig 3C) of the spectral and classic mode showed that mBeRFP is not detected in the classic mode suggested that there is no crosstalk between TRITC and mBeRFP using these parameters.

The previous version of the figure may have been confusing. We modified Fig2 (new Fig3) and FigSup2 (new FigSup3) to make them clearer for the reader.

Regarding the quantifications in FigSup3B, to test if mBeRFP was detected when excited with a 561nm laser, we compare the detection in classical mode (possible crosstalk) and the spectral mode (no crosstalk).

- Fig. 2E, This is indeed a beautiful staining. But it would be really interesting if the protocol/settings would be explained. Could you explain the spectra and laser used in supplementary and/or Material and methods?

Re: We explained the protocol in the Material and Methods section:

"Fig.3G was acquired using three different tracks. MyoII-GFP and α-catenin-mBeRFP were imaged simultaneously (track 1) using 458 nm laser with 2 emission windows (490-510 nm and 642-695 nm respectively for GFP and mBeRFP), the nuclei (DAPI) and the actin (Phalloidin-TRITC) were imaged in a second track using 405 nm and 561 nm laser, and E- cadherin (Alexa 647) with 633 nm laser in a third track."

- Legend Fig. 2B,C, the number of samples is not mentioned.

Re: We added the number of samples in the legend (n=9)

- At the end of the first paragraph there is a reference to Fig.3A while it should be Fig.3A'.

Re: Thank you to point that out. This has been corrected.

- For the Fig.3B the protocol is a bit unclear: in the text it is mentioned that there are 1800 frames per second while the legends mentioned 1s per frame for a total of 1800. Please clarify.

Re: There was indeed a mistake. It was 1 frame per second for a total of 1800 frames, but we chose to delete this panel in the current version of the manuscript because we think that it is redundant with the live experiment that we added in Fig 2F-G. However, if the referee think that we have to keep this panel, we will add it.

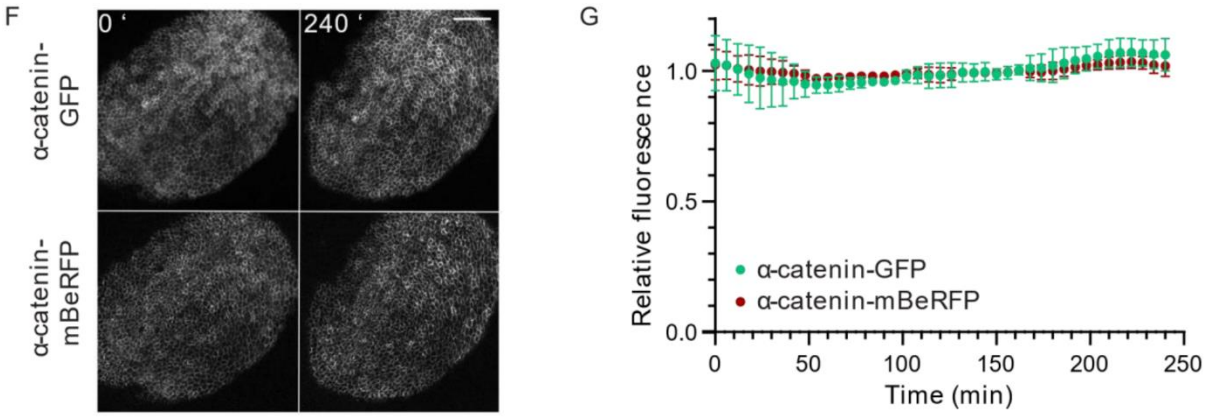
- Fig.3B, what is the genotype? How many samples have been used?

Re: We deleted this panel in the new version of the Figure and the manuscript.

-FigSup.3, The genotype is not mentioned. Are the Ecad-GFP and the NLS-mBeRFP expressed in the same flies? How many samples? Also, the mBeRFP is localized in the nucleus (NLS), would the result be the same with a cytoplasmic localization, or at the membrane (fused with α-catenin)? We suggest here to repeat the same experiment but with MyoII-GFP and α-catenin-mBeRFP.

Re: We added the genotype (Ecad-GFP; pdm2Gal4; UAS NLS-mBeRFP) and the number of samples in the legend (n=3).

According to your suggestion, we repeated this experiment but using UAS α-catenin-GFP and UAS α-catenin-mBeRFP expressed under the control of pdm2Gal4 promoter. Here, we compared exactly the same protein (α-catenin) fused with two different FP and showed that the fluorescence of both GFP and mBeRFP is quite stable all along the live. We added this experiment in Fig 2F, G.



- What does FRAP stand for? What does this experiment show? We think it would increase the impact of these experiment if the authors would explain in more details the principle and goal of it.

Re: Frap stands for “Fluorescence Recovery After Photobleaching”. This technique allows to evaluate the mobility of a protein of interest. The velocity of the recovery of fluorescence gives an idea of the turnover of the protein at a particular location of the cell while from the percentage of recovery, the protein stability can be deduced. We realize that this experiment should be explained in more detail and add the following in the text:

“Then, we assessed the impact of the mBeRFP fusion on the protein dynamics by performing FRAP experiments (Fluorescence Recovery After Photobleaching) on α-catenin fused to mBeRFP, TagRFP or GFP (Fig.3B-E). This technique allows to evaluate the kinetics of diffusion of a protein of interest, through the analysis of the fluorescence recovery after photobleaching. FRAP on α-catenin-mBeRFP junctions indicates that the mobile fraction and the half time recovery were not significantly different from those of α-catenin-TagRFP or α- catenin-GFP suggesting that the protein dynamics is mainly unaffected by the fusion of mBeRFP. Of note, the mobile fraction of α-catenin-TagRFP is significantly lower than the α- catenin-GFP one.”

- Fig.3C legend, how many FRAP experiments were conducted?

Re: We added the number of samples in the legend (n=5). Of note, Fig3C is now Fig.4A.

- Fig.3 legend: in the figures some panel are labeled with ' (for example C', D' or D"), but in the legends they are referred as "Top", "middle" or "bottom", please homogenize.

Re: Thanks for pointing that out. We modified the legends accordingly.

- Fig.3D legend, how many experiments were conducted?

Re: We added the number of samples in the legend (n=7). Fig3D is now Fig.4B.

- In the Material and Methods, RIM stands for random illumination microscopy? Put the abbreviation in the text.

Re: This has been done.

- In the Material and Methods, the protocol for photobleaching is not described (Fig.2B).

Re: This experiment has been removed from the current manuscript (see above) but we added a photobleaching experiment section in the Material and Methods to describe new experiments: “Photobleaching was performed on the LSM880 confocal microscope. GFP and/or mBeRFP were excited simultaneously using the 458 nm laser source and photons were collected by GaAsP detectors. Images were acquired from imaginal wing discs every 6 minutes during 240 minutes (41 time-frames).”

- In the Material and Methods, please give the parameters used for the Fig.2E.

Re: Re: We explained the protocol in the Material and Methods section:

“Fig.3G was acquired using three different tracks. MyoII-GFP and α-catenin-mBeRFP were imaged simultaneously (track 1) using 458 nm laser with 2 emission windows (490-510 nm and 642-695 nm respectively for GFP and mBeRFP), the nuclei (DAPI) and the actin (Phalloidin-TRITC) were imaged in a second track using 405 nm and 561 nm laser, and E- cadherin (Alexa 647) with 633 nm laser in a third track.”

Reviewer 2 Advance Summary and Potential Significance to Field:
This technical report by Martin and Suzanne is reporting a new tool to better visualize multiple proteins live in living Drosophila tissue. Live imaging of proteins is an invaluable way to report the

in vivo dynamics of proteins. However, the current limitations in separating the different fluorescent excitation and emission spectra of the different available genetically encoded proteins makes it difficult to image the dynamics of several proteins at the same time in Drosophila.

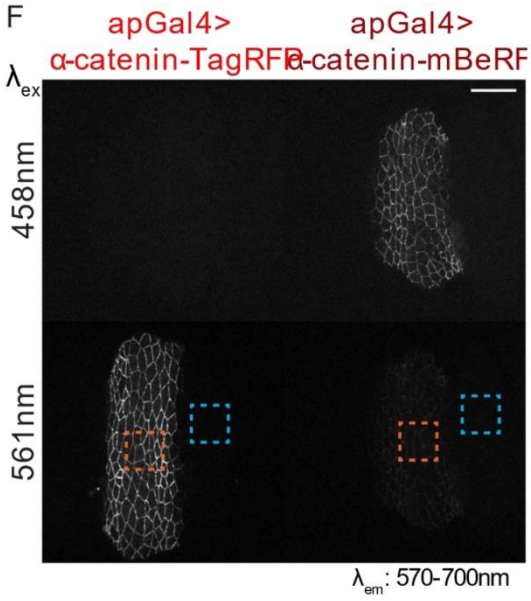
Here the authors have generated transgenic lines using the large Stokes shift red fluorescent protein mBeRFP in Drosophila and explored its value for live imaging in combination with an existing GFP fusion protein. The long stokes shift allowed excitation of mBeRFP and GFP with one laser line (458nm, a common laser line present in the standard argon laser) and easy separation of the emission spectra. Also, valuable combinations of mBeRFP with either GFP or RFP were demonstrated as well as for mBeRFP FRAP experiments that can assess mBeRFP tagged protein dynamics at same time as GFP tagged protein dynamics. This is really cool!
In combination with the generated UAS and non-UAS cloning vectors containing mBeRFP, these tools will be valuable for the Drosophila and the imaging community in general.

Reviewer 2 Comments for the Author:
1. Fluorescent proteins are particularly useful for live imaging as the authors of course appreciate. Hence, the authors should make clearer that Figures 1 and 2 analyse fixed samples (which would not have needed live dyes). I would have preferred to see these quality assessments of mBeRFP in live samples, as it would be more meaningful. Also, fixation may differentially affect the intensity of different fluorescent proteins; from the figure legends it is unclear but from the methods I suppose that FigSup1 was using fixed samples too, which might not be optimal to distinguish the 2 proteins in living samples.

Re: The first figures were effectively done in fixed samples to describe the different constructs and test the possible crosstalk with other fluorescent proteins. We reasoned that since the fluorescence intensity is higher in fixed sample, it would be ideal to reveal possible crosstalks. Indeed, if there is no crosstalk on fixed sample, we can reasonably assume that this will also be the case in living sample. This has been clarified in the text.
However, we agree with the referee that this tool is particularly useful for live experiments and that the interest of mBeRFP in living sample should be highlighted earlier in the manuscript. A time lapse showing that leg development is not affected by the presence and illumination of mBeRFP is now presented in Figure 1 and Movie1 (combining MyoII-GFP with DllG4>UAS- mBeRFP). In addition, a time lapse showing the division of an epithelial cell in the wing imaginal disc, combining MyoII-GFP and α-catenin-mBeRFP illuminated by a single laser at 458nm is now presented in Figure 4A and Movie 4, illustrating that mBeRFP can be easily followed in living sample.

2. Figure 2D/Suppl Figure 2D: to better appreciate the intention of the experiment I would appreciate if also the images acquired with 458nm excitation were shown. This makes clearer that the combination of mBeRFP with TagRFP or TRITC needs sequential excitation while collecting at the same emission window. Again, live imaging would be better.

Re: We added images acquired with 458 nm laser in the figure (Fig2 is now Fig3). As mentioned above, we reasoned that since the fluorescence intensity is higher in fixed sample than in living samples, it would be ideal to reveal possible crosstalk. This has been clarified in the text.



3. The authors convincingly showed that mBeRFP can be imaged successfully together with GFP or RFP protein versions (in fixed samples). However, it is stated in the text that the authors combine mBeRFP with GFP AND RFP, which implies imaging of the three at the same time. I guess this should be possible, however was only shown with the synthetic TRITC dye in fixed images. Please amend the text.

Re: We totally agree and modified the text accordingly.

4. Figure 3 shows nicely that mBeRFP can be actually used for live imaging in cultured leg discs, either as single colour fused to alpha-Cat or together with NMII-GFP. It would be nice to annotate the overview Movie 1 with arrows similar as in the 3A' to illustrate the cell extrusions in the movie directly. The authors state: "we are able to follow apoptotic cell extrusions from the apical surface of the epithelial leg at the expected rate" – I am missing how this rate was measured.

Re: We annotated the corresponding movie (now Movie 2) to highlight cell extrusions.
We mentioned the "rate of extrusion", while we were referring to the duration of the process (lasting around 1h) which is similar to what has been observed in our previous work (Schott et al, 2017). Thanks for pointing this out. We modified the text as follow and corrected the reference:

"At a cellular scale, we are able to follow the extrusion of an apoptotic cell from the apical surface of the leg disc (Fig.2A'), with a duration similar to our previous observations (Schott et al, 2017). "
Of note, Fig3 is now Fig2.

5. To fully assess the data of the FRAP and laser cuts, please provide the missing example movies for Figures 3C and D. Was general bleaching of the sample during the 'postbleach' acquisition taken into account when calculating the fluo recovery rate? The alphaCat-mBeRFP looks brighter at 0 sec compared to 60 sec. It might be better to show ratios comparing the recovering junction to neighbouring unbleached junctions.

Re: We furnished the movie of FRAP and laser ablation (respectively Movie 5 and 6).
There is indeed some photobleaching. However, for the quantification of the recovery, the general photobleaching was take into account to avoid misleading conclusion as done in Sidor et al, 2020 Dev Cell. We added a brief description in the Mat & Meth section:
"The analysis of the FRAP experiments was done using a homemade macro in ImageJ based on the publication of (Sidor et al., 2020). Briefly, fluorescence intensity (I) was measured in a ROI at the site of bleach and, to account the general photobleaching, measures were normalized by a photobleaching correction factor ($C_{photobleaching}$) such as:

$$F_t = I_t \times C_{photobleaching\ t}$$

with $C_{photobleaching\ t} = \frac{background\ intensity\ at\ t_0}{backgroung\ intensity\ at\ (t)}$

and t_0 corresponding to the time frame of the photobleaching.

Then, normalized measurements (F_t) were used to determine and plot the percentage of fluorescence recovery as follows:

$$F(t) = \frac{F_t - F_0}{F_{prebleach} - F_0}$$

with $F_{prebleach} = avg\ F_{t-10\ to\ t-1}$

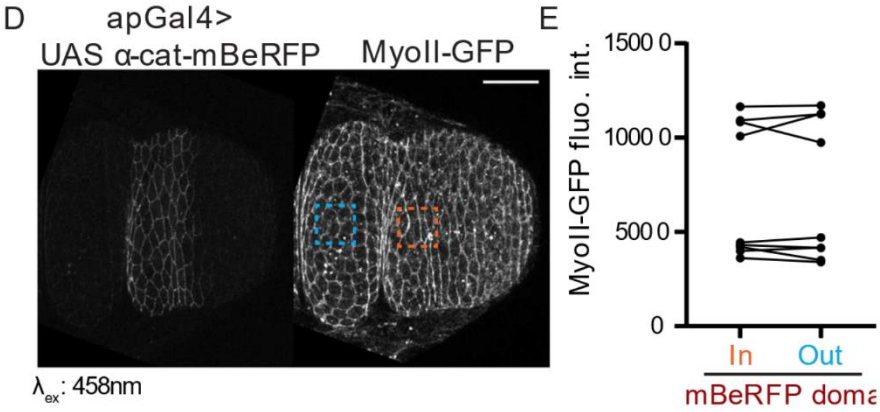
Finally, using Prism 8 (Graph Pad) we modeled the recovery using the one phase association equation ($a(1 - e^{-kt})$) and extract the mobile fraction (a, corresponding to the plateau) and the half time."

Reviewer 3 Advance Summary and Potential Significance to Field:
In this technical report, Martin and Suzanne introduce a series of mBeRFP-fused fluorescent constructs to use in Drosophila. mBeRFP is a red fluorescent protein with a long Stokes shift, and thus can be excited simultaneously with CFG/GFP variants, and its emission can be independently detected. The authors show that cytoplasmic mBeRFP is brighter than LSSmKate2, another long Stokes shift RFP. mBeRFP can be imaged (and simultaneously excited) with eGFP with no crosstalk, and it displays low crosstalk with TagRFP ro TRITC. mBeRFP construct expression does not affect cellular dynamics, and only displays moderate photobleaching, and can be used for FRAP and laser ablation assays.

This is a nice, short technical report that presents fluorescent constructs and plasmids that are likely to be useful for the wider Drosophila community. I have a few suggestions of some additional controls to validate the level of crosstalk of mBeRFP and eGFP in regular confocal mode, and a few additional comparisons that I think would be important to validate the constructs made and their dynamics.

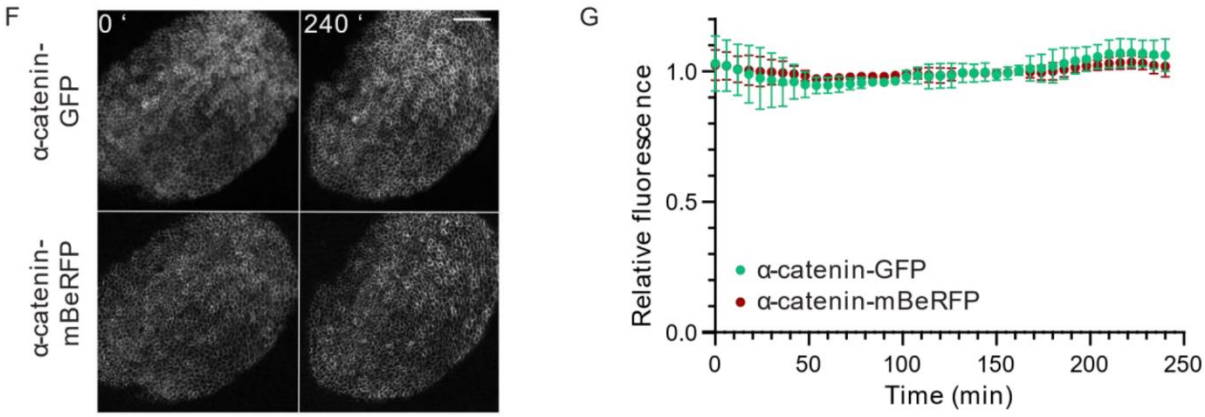
Reviewer 3 Comments for the Author:
1. To determine how widely applicable these constructs are to "classic" confocal mode, with no spectral detector or linear unmixing, the authors should quantify how GFP fluorescence (e.g. alpha-catenin:eGFP) changes in the presence of mBeRFP (e.g. alpha-catenin:mBeRFP) and vice versa (e.g. comparing the fluorescence in each of the channels in embryos expressing one or two of the fluorescent proteins). This is important, as not every reader will have acccess to spectral imaging.

Re: To address this concern and quantify how GFP fluorescence is affected by the presence of mBeRFP, we measured the fluorescence of the MyoII-GFP inside or outside the expression domain of alpha-catenin:mBeRFP. This quantification revealed no difference between these domains suggesting that the presence of mBeRFP does not affect the GFP fluorescence. We add panel D and E in Figure 3. Of note, Fig2 is now Fig3.



2. Figs. 3B and S3: to evaluate the significance of the photobleaching of mBeRFP, a much more interesting and practical comparison would be similar to that shown in Fig. S3 for MyoII-GFP and NLS-mBeRFP, but comparing fusions to the same protein (e.g. alpha- catenin-GFP vs. alpha-catenin-mBeRFP).

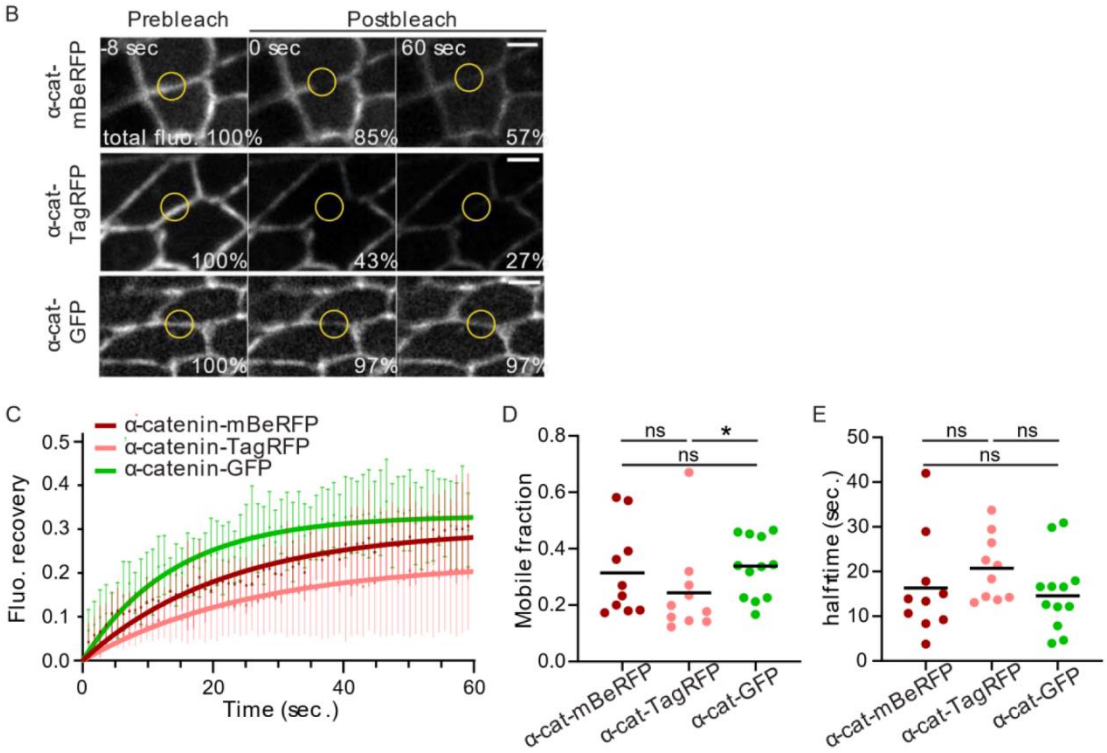
Re: According to your suggestion, we repeated this experiment using UAS α-catenin-GFP and UAS α-catenin-mBeRFP expressed under the control of pdm2Gal4 promoter in the wing pouch. Here, we compared exactly the same protein (α-catenin) fused with two different FP and showed that the stability of both GFP and mBeRFP fluorescence is quite similar in time. We added this experiment in Fig 2F, G. (Fig3 is now Fig2).



3. Fig. 3C-C': the FRAP experiments should be conducted comparing (quantitatively) alpha-catenin-GFP vs. alpha-catenin-TagRFP vs. alpha-catenin-mBeRFP. Are the mobile fractions and half times of fluorescence recovery similar for all three markers. This is really important to verify that mBeRFP tagging does not affect protein turnover or dynamics in a way different from GFP or TagRFP.

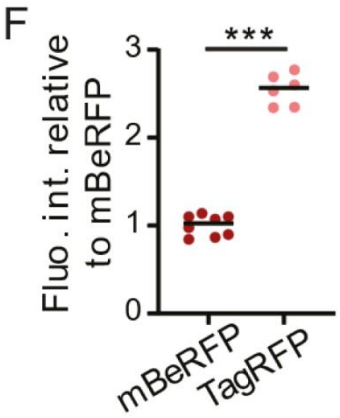
Re: To agree that the impact of the fluorophore on protein dynamics should have been done using the same molecule fused to different fluorophores. We added a comparative analysis of the dynamics of UAS alpha-catenin-GFP vs. UAS alpha-catenin-TagRFP vs. UAS alpha- catenin-mBeRFP in Fig.2B-E. This analysis showed that although alpha-catenin-TagRFP and alpha-catenin-GFP show a slightly different motile fraction, the motile fraction of alpha-catenin- mBeRFP is not significatively different from the one of alpha-catenin-TagRFP on the one hand or alpha-catenin-GFP on the other hand. In addition, no significant differences are observed between the halftimes of the three proteins. This leads us to conclude that:

"This technique allows to evaluate the kinetics of diffusion of a protein of interest, through the analysis of the fluorescence recovery after photobleaching. FRAP on α-catenin-mBeRFP junctions indicates that the mobile fraction and the half time recovery were not significantly different from those of α-catenin-TagRFP or α-catenin-GFP suggesting that the protein dynamics is mainly unaffected by the fusion of mBeRFP. Of note, the mobile fraction of α- catenin-TagRFP is significantly lower than the α-catenin-GFP one."



4. Figure 1: similar to the comparison to LSSmKate2 (Fig. S1), I would appreciate a comparison between mBeRFP and TagRFP. For example, for the alpha-catenin construct shown in Figure 1D-E, the TagRFP fusion seems brighter. Presenting quantitative data (maybe normalized to the MyoII-GFP signal) would be useful for people trying to decide between a TagRFP or an mBeRFP fusion (when Stokes shift is not a consideration).

Re: We agree that this comparison could be interesting to have an idea of the relative intensities of the different fluorophores. To address this point, we imaged either apGal4>UAS alpha-catenin-mBeRFP excited with 458 nm or apGal4>UAS alpha-catenin-TagRFP excited with 561nm. We ensured that the laser power of 458nm or 561nm was the same by measuring it using a power meter. We then quantified the fluorescence of mBeRFP and TagRFP and showed that mBeRFP is less bright than TagRFP (Fig.1F). Of note however, the fluorescence intensities of two different proteins with two different lasers, even adjusted at the same power, is difficult to compare.



5. Fig. S3: the labels indicate that the images belong to E-cadGFP and NLS-mBeRFP, and the quants to MyoII-GFP and NLS-mBeRFP. Unless the labels are wrong (and in that case they should be corrected), the images should correspond to the markers shown in the quants.

Re: We apologize for the mistake. The labels were wrong and have been corrected.

TYPOS
Page 9: "2-photons" should be "2-photon".
Re: This has been corrected.

Second decision letter

MS ID#: DEVELOP/2022/200495

MS TITLE: mBeRFP, a versatile fluorescent tool to enhance multichannel live imaging and its applications

AUTHORS: Emmanuel Martin and Magali Suzanne
ARTICLE TYPE: Techniques and Resources Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors did a great job addressing reviewer comments.

Comments for the author

All my comments were thoroughly addressed.

Reviewer 2

Advance summary and potential significance to field

This revised manuscript by Martin and Suzanne is now convincingly showing that the large Stokes shift red fluorescent protein mBeRFP is a very valuable tool for live imaging in Drosophila as it can be combined with standard GFP fusion proteins using a single laser line.

Comments for the author

I congratulate the authors for their effort to address all the reviewers' comments. I am looking forward to test mBeRFP in our group.

Reviewer 3

Advance summary and potential significance to field

This is an excellent revision, congratulations to the authors. The tools presented here will be a great addition to the Drosophila imaging community toolbox.

Comments for the author

I just have a minor suggestion that the authors may want to add a "discussion" line about why do they think the mobile fraction of alpha-catenin-TagRFP is lower than for the GFP-tagged construct.