Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons

Attila Ignácz, Domonkos Nagy-Herczeg, Angelika Hausser, and Katalin Schlett

Corresponding author(s): Katalin Schlett, Eötvös Loránd University

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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 #E22-08-0331
TITLE: Comparative analysis of actin visualization by genetically encoded probes in cultured neurons

Dear Dr. Schlett:

Thank you for submitting your manuscript to MBoC. I am including the reviewer comments, which I hope you will find useful and constructive. As you can see, both reviewers express interest in the potential value of the study, but they also have several criticisms and suggestions. We would be interested in reconsidering the manuscript once revisions are undertaken to address the issues raised by both reviewers. In your revised manuscript, please respond to each of the reviewers' comments point-by-point. In particular, please address their concerns regarding expression levels of the various actin probes and the relationship of each probe to filamentous actin staining with phalloidin. Additionally, how these probes are useful for different actin based structures or are affected by different pharmacological perturbation are likely important considerations.

Sincerely,

Stephanie Gupton
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Schlett,

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.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

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

To submit the rebuttal letter, revised 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):

In "Comparative analysis of actin visualization by genetically encoded probes in cultured neurons" the authors compare expression of three different actin-labeling constructs in primary neurons with the goal of discerning how expression of these constructs may alter visual readouts of neuronal morphology and dynamics as well as how well these constructs faithfully label enrichment of actin within sub-compartments such as dendritic spines and changes in actin remodeling. The three constructs compared are: EGFP-actin (exogenous expression of fluorescently-tagged actin monomers), LifeAct-GFP, and AC-GFP (both of which are fluorescent probes that bind endogenous actin - LifeAct is protein-based while AC is nanobody-based).
The results of this study are important for guiding future studies of actin dynamics, particularly in the context of primary neurons. EGFP-actin and LifeAct-GFP are commonly used tools for live imaging of actin so it may be taken for granted that these constructs do not cause important biological alterations. However, direct binding to actin (LifeAct) and/or alteration of the actin monomer pool (EGFP-actin) may indeed alter the normal physiological properties of actin which could confound studies utilizing these constructs. The AC-GFP construct is relatively new and, therefore, further characterization of this probe is also warranted. The broad range of readouts studied here provide good context for the types of experiments where one construct may be preferable to another. Although the manuscript is well done overall, there are some issues that should be addressed.
Critiques:
Broad/general critiques:
The main issue I have is the manuscript lacks co-labeling with phalloidin to confirm the coverage of each of these probes. One could image live, then fix and stain with phalloidin, and then measure colocalization of the two probes in the shaft and spine to establish the accuracy of localization of the different GFP probes.
Also: it is generally understood that GFP-actin fusions prohibit formin-mediated actin assembly (see Chen, Nag, and Pollard 2012, J. Struct. Biol.). I find it difficult to accept simplistic conclusions as presented in Table 1 without a more extensive analysis of how each of these probes behave in the context of different actin-regulatory proteins and perturbations, such as SMIFH2, CK666, latrunculin, and cytochalasinD.
More specific critiques:
One important missing control is a comparison of the expression levels of each of the constructs. Overexpression artifacts can occur in many contexts including primary neurons. Typically, artifacts increase with increasing expression level. Currently, there are no data showing that the EGFP-actin, LifeAct-GFP, and AC-GFP are being expressed at similar levels. It is possible that the differences in the various physiological readouts when comparing constructs could be a result of differences in expression levels. These data could be collected by either measuring average GFP fluorescence via flow cytometry/imaging or by Western blotting with an anti-GFP antibody.
In Figure 1, the way the data are displayed could be improved. In panel A, the GFP signal is sometimes hard to see when overlayed with the mCherry signal. It would be ideal to show GFP-only versions of these panels either in this figure or an accompanying supplemental figure. This is also true of Supplemental Movie 1.
Also in Figure 1, it's odd that the graphs are displayed in a slightly different format in panel B compared to panel C. The format of panel C is preferable as it results in less data overlaid on top of each other which makes it easier to discern if there are any differences between thin and mushroom shaped protrusions.
In regard to Figure 2, the authors state: "We found no significant difference in center of mass displacement between actin probe expressing cells and EGFP expressing control filopodia (Figure 2B)". However, when inspecting Figure 2B, there do appear to be differences when comparing the Jaspl - conditions to each other. Specifically, the EGFP-actin values appear to be much higher than the EGFP control values. There are no statistics comparing the Jaspl - conditions to each other (which is also an issue as these comparisons are highly relevant), so it's possible that this difference is not statistically significant but it's odd that this obvious difference wasn't commented on. These results appear to be consistent as center of mass results are also shown in Supplementary Figure 1B and this difference between EGFP-actin and EGFP control is present there as well.
There is some confusion concerning the n-values in Figure 3. There are n-values listed in panel F. Are these also the n-values for panels C-E? If so, this should be clarified and if not, the n-values for panels C-E need to be provided. Also, the meaning of the n-values is described in the figure legend for all panels except panel F. Related to this, it would be preferable to show the individual values in the Figure 3 graphs as was done in previous figures. Figure 3B is an exception as the n-values are very high in this instance and showing all the individual data points would not be easy to interpret.
Also for Figure 3, various morphological readouts were measured (number of protrusions, spine length, head-to-neck ratio), however, no corresponding images are shown to support these results. Since these results are at odds with Wegner et al. 2017, it's important to convincingly demonstrate that morphological differences are visible in this context.
In the Image analysis methods section it says "Dendritic endpoints were counted, and image skeletons were analysed also by the corresponding Fiji functions". It would be nice to include the exact name of the Fiji function/plugin used.
Positive aspects:
This manuscript is very well written. The flow is logical and easy to follow. The introduction in particular is excellent - it provides both solid background and rationale for the experiments carried out. Also, aside from the minor issues mentioned regarding Figure 1, data in all figures are displayed clearly. The outline of the machine learning workflow used in Supplementary Figure 2 is much appreciated. It allows for a good understanding of the methodology and could also be used as a guideline for other studies involving similar measurements.

Reviewer #2 (Remarks to the Author):

Probes for visualizing the actin cytoskeleton are used extensively yet often without knowing their full capabilities and limitations. Therefore, studies like the one presented here by Ignácz et al. have the potential to provide real value to the cytoskeletal community by doing a deep dive into how these probes effect their experimental system. However, in its current state, this manuscript is more of a collection of supplementary data justifying the use of GFP-actin to visualize the actin cytoskeleton in the dendrites of cultured mouse hippocampal neurons over other actin probes than it is a generally useful body of knowledge for the neuronal cytoskeletal community that warrants a standalone publication. So, while I appreciate the rigor in which the experiments here have been performed, I cannot recommend this manuscript for publication in the way that it is presented, and I am not sure if the work required to warrant a standalone paper will be worth it to the authors. The specific reasons for this assessment are:

1. It is not clear how the expression levels of each probe are chosen, assessed, and compared between experiments. Additionally, it is not as simple as making sure that all cells have generally the same level of green fluorescence. Every biological probe has a different range in which visualization and perturbation of the experimental system need to be balanced. For example, one of the conclusions of this paper is that expression of GFP-actin does not perturb dendritic arborization (Figure 4). But what if you compared cells expressing different amounts of GFP-actin? It is almost certain that at some point you would see an effect. There needs to be some justification that this study is making "apples to apples" comparisons. Plotting fluorescence levels vs. phenotype quantifications would help to reveal these correlations, though this would require many measurements.

2. The focus of the work is much narrower than the title suggests. First, most actin structures within the neuron are being ignored, with only dendritic protrusions being assessed. So those wanting to compare how different probes could alter the properties of growth cones, dynamic axonal actin structures (reviewed by Roy, JCB, 2016), the periodic cytoskeleton, presynaptic actin structures (ex. Bingham et al, bioRxiv 2022), somatic actin (ex. Meka et al, EMBO Reports 2019), etc. are not going to find answers in this publication. Likewise, the study has been restricted to one type of neuron from a single species. Is even the most robust result, the inhibition of dendritic arborization by AG-GFP (Fig. 4), generally applicable to different types of neurons? At least even from the same species?

3. While Table 1 addresses the point of "general actin labelling", there are no experiments in this manuscript which do. I would have liked to see a quantitative co-localization of these probes with F-actin labeled with phalloidin. Do they label all of the same structures equally or are there differences? There is strong evidence to believe that they would (references provided by authors in the Introduction). This would also be an important point in choosing the correct probe.

4. Other popular actin binding probes like the ones based off of Ftractin and Utrophin (both mentioned in the Introduction) are not included here. Adding them to the study would have significantly increased the potential value of the work.

Dear Dr Gupton,

Thank you very much for sending us the reviewers' reports on our E22-08-0331R manuscript, entitled "Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons". We greatly appreciate the reviewer's comments and helpful suggestions. As requested, we performed additional analyses to compare relative expression levels of the probes tested and to correlate the relative fluorescence intensity values with morphological features and filopodial motility. We also performed F-actin labelling with phalloidin and analysed colocalization with the genetically encoded actin probes. Accordingly, 3 figures were modified, a new supplementary figure was added, and the criticised table was removed. In addition, the title of the manuscript was changed in accordance with the suggestions of Reviewer #2 to make it clear that the focus of our studies was on the analysis of the dendritic consequences of the expression of actin-labelling probes. We hope that our work will provide the scientific community with valuable guidance and image analysis methods for selecting the appropriate F-actin labelling tool in neurons, especially in studies focusing on structural plasticity in dendritic spines.

Below we list our detailed point-by-point responses to the reviewers' questions. We are convinced that the changes made in the text and figures have improved the quality of our work and hope that our manuscript will be accepted for publication.

With best regards

Katalin Schlett

Corresponding author

Responses to Reviewer #1:

We would like to thank the Reviewer for his/her positive comments and constructive and helpful suggestions. We have done our best to address the points raised accordingly and list our detailed responses below.

The main issue I have is the manuscript lacks co-labeling with phalloidin to confirm the coverage of each of these probes. One could image live, then fix and stain with phalloidin, and then measure colocalization of the two probes in the shaft and spine to establish the accuracy of localization of the different GFP probes.

We thank the Reviewer for this suggestion and agree that phalloidin staining provides an essential control to determine potential differences between the F-actin binding of the tested actin labelling constructs. Therefore, we performed the suggested additional experiments. As expected, phalloidin signal was enriched in dendritic spines (Figure 3B). In addition, all genetically encoded actin probes tested colocalized with phalloidin to a similar extent, which was significantly higher than in control, EGFP-expressing cells (Figure 3C, D). Thus, we can conclude that all tested actin labelling constructs similarly mirror the intra-dendritic distribution of F-actin.

Also: it is generally understood that GFP-actin fusions prohibit formin-mediated actin assembly (see Chen, Nag, and Pollard 2012, J. Struct. Biol.). I find it difficult to accept simplistic conclusions as presented in Table 1 without a more extensive analysis of how each of these probes behave in the context of different actin-regulatory proteins and perturbations, such as SMIFH2, CK666, latrunculin, and cytochalasinD.

We accept the criticism and has removed Table 1. Instead, we provide a summary in the text regarding the advantages and disadvantages of using the tested actin-probes. Unfortunately, it was beyond the scope of our manuscript to test the influence of various drugs on different actin-labelling proteins.

One important missing control is a comparison of the expression levels of each of the constructs. Overexpression artifacts can occur in many contexts including primary neurons. Typically, artifacts increase with increasing expression level. Currently, there are no data showing that the EGFP-actin, LifeAct-GFP, and AC-GFP are being expressed at similar levels. It is possible that the differences in the various physiological readouts when comparing constructs could be a result of differences in expression levels. These data could be collected by either measuring average GFP fluorescence via flow cytometry/imaging or by Western blotting with an anti-GFP antibody.

We thank the Reviewer for the useful suggestions. Due to the technical limitations of the low transfection rate in our neuronal cultures, Western Blotting would not be sensitive enough to compare expression levels and could not provide data from individual cells. Flow cytometry requires dissociation of established neuronal networks, resulting in loss of all neurites, which affects cell survival and prevents direct comparison between morphological features and expression levels. Therefore, we approximated relative expression levels by measuring GFP fluorescence intensities within the soma under the same imaging settings in all cells analyzed. Because TagGFP2 and EGFP, the fluorescent proteins fused to the tested actin-tagging proteins in a 1:1 ratio, have similar fluorescent properties, the differences between GFP intensities in the soma from cell-to-cell are likely due to differences in the amount of GFP expressed.

Our results show a wide expression range in EGFP and LifeAct-GFP expressing cells, which is more restricted in case of EGFP-actin and AC-GFP expression. This by itself suggests that high level expression of the latter two constructs might impair cell survival, implicating a dose-dependent artifact as the Reviewer suggested. On the other hand, when we compared morphological parameters with the relative expression levels in the cells that exhibited healthy morphology (i.e., cells that possessed dendritic spines and continuous non-fragmented neurites), we found no correlation between expression level and the extent of dendritic tree (Figure 4F, G) or parameters describing the morphology of the dendritic spines (Supplementary Figure 1C, D). Nevertheless, correlations have been found between relative expression levels and filopodial motility and protrusion density in case of LifeAct-GFP and EGFP-actin, respectively. Thus, we conclude that expression levels should be indeed carefully monitored in experiments using these protein constructs, as noted by the Reviewer.

In Figure 1, the way the data are displayed could be improved. In panel A, the GFP signal is sometimes hard to see when overlayed with the mCherry signal. It would be ideal to show GFP-only versions of these panels either in this figure or an accompanying supplemental figure. This is also true of Supplemental Movie 1.

Also in Figure 1, it's odd that the graphs are displayed in a slightly different format in panel B compared to panel C. The format of panel C is preferable as it results in less data overlaid on top of each other which makes it easier to discern if there are any differences between thin and mushroom shaped protrusions.

Figure 1 has been updated as requested. In the current version, Figure 1B contains all FRAP recovery curves in the same format. To preserve the original fluorescence images, Supplementary Video 1 was not modified.

In regard to Figure 2, the authors state: "We found no significant difference in center of mass displacement between actin probe expressing cells and EGFP expressing control filopodia (Figure 2B)". However, when inspecting Figure 2B, there do appear to be differences when comparing the Jaspl - conditions to each other. Specifically, the EGFP-actin values appear to be much higher than the EGFP control values. There are no statistics comparing the Jaspl - conditions to each other (which is also an issue as these comparisons are highly relevant), so it's possible that this difference is not statistically significant but it's odd that this obvious difference wasn't commented on. These results appear to be consistent as center of mass results are also shown in Supplementary Figure 1B and this difference between EGFP-actin and EGFP control is present there as well.

Live cell imaging experiments have been repeated, and new data have been added to the already existing Figure 2. In addition, to circumvent the signal-to-noise sensitivity of the used FIJI plugin (Dendritic Filopodial Motility Analyzer), automatically segmented time-lapse recordings were used to calculate the center of mass displacement. In the updated Figure 2 datasets, no significant differences were found between the control filopodial motility values. The lack of significant difference between the control groups is not shown in the figure for clarity.

There is some confusion concerning the n-values in Figure 3. There are n-values listed in panel F. Are these also the n-values for panels C-E? If so, this should be clarified and if not, the n-values for panels C-E need to be provided. Also, the meaning of the n-values is described in the figure legend for all panels except panel F. Related to this, it would be preferable to show the individual values in the Figure 3 graphs as was done in previous figures. Figure 3B is an exception as the n-values are very high in this instance and showing all the individual data points would not be easy to interpret.

For clarity, “N” and “n” values are now displayed either within the subfigures or in table format.

Also for Figure 3, various morphological readouts were measured (number of protrusions, spine length, head-to-neck ratio), however, no corresponding images are shown to support

these results. Since these results are at odds with Wegner et al. 2017, it's important to convincingly demonstrate that morphological differences are visible in this context.

Figure 3A has been updated and the protrusions that fall into the different morphological categories have been labelled in the panel. Wegner et al (2017) showed qualitative data indicating that high expression levels of Actin-Chromobody and LifeAct, conjugated to different fluorescent proteins, leads to a marked decrease in dendritic spine density. The authors however, did not provide quantitative analysis on spine density or different morphological parameters in relation to expression levels. In our work, we present machine learning based automated spine morphology quantification that circumvents human bias inherent in manual or qualitative analysis. Our results partially overlap with Wegner and colleagues' as we also concluded that only lower AC-GFP expression levels were tolerated by transfected neurons. However, LifeAct-GFP expressing cells reached a higher expression level while retaining healthy morphology. These differences, potentially originating from the different expression systems (viral transduction vs. lipofection) or the different expression times (2-4 weeks vs. 24 hours post-transfection time) should also be taken into consideration.

In the Image analysis methods section it says "Dendritic endpoints were counted, and image skeletons were analysed also by the corresponding Fiji functions". It would be nice to include the exact name of the Fiji function/plugin used.

The used plugins have been named in the text and the used FIJI macros have been uploaded to GitHub.

Responses to Reviewer #2:

We appreciate the Reviewer's positive comments and are grateful for raising our attention to better specify the scientific aims of our manuscript. Our focus was to examine how the tested actin probes interfere with dendritic morphological features especially relevant during structural plasticity of the neurons, rather than to give an overview on the general usefulness of the actin labelling probes in different cell types. Structural plasticity in dendritic spines is often accompanied by only subtle changes in dendritic or spine morphology, therefore choosing an appropriate actin probe is important to avoid experimental artifacts that hinder the correct interpretation of the results. Therefore, we modified the title of the manuscript to "Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons". We are convinced that the unbiased methodology in combination with the measurement of expression levels will provide useful information for colleagues planning to conduct studies on actin-dependent functions in dendritic spines or dendrites.

Our specific answers addressing the Reviewer's questions are listed below.

1. It is not clear how the expression levels of each probe are chosen, assessed, and compared between experiments. Additionally, it is not as simple as making sure that all cells have generally the same level of green fluorescence. Every biological probe has a different range in which visualization and perturbation of the experimental system need to be balanced. For example, one of the conclusions of this paper is that expression of GFP-actin does not perturb dendritic arborization (Figure 4). But what if you compared cells expressing different amounts of GFP-actin? It is almost certain that at some point you would see an effect. There needs to be some justification that this study is making "apples to apples" comparisons. Plotting fluorescence levels vs. phenotype quantifications would help to reveal these correlations, though this would require many measurements.

The dependence of the morphological effects on expression levels was also questioned by Reviewer #1. We thank both Reviewers for raising this important point. As we explained in detail in response to Reviewer #1, we decided to measure and compare the fluorescence intensities within the soma of transfected neurons, as a proxy to quantify relative expression levels. As suggested by Reviewer #2 , we plotted the extent of the dendritic tree (Figure 4F, G) and morphometric measurements of spines (Supplementary Figure 1) against fluorescence levels. Our analysis revealed that transfected neurons retained healthy morphological properties only when expressing EGFP-actin or AC-GFP within a limited range. Furthermore, EGFP-actin expression showed a dose-dependent effect on dendritic protrusion density, as shown in Supplementary Figure 1B. Although directly labelled actin monomers are advantageous in visualizing actin dynamics and colocalize well with phalloidin, expression levels should be carefully monitored when this probe is chosen to label F-actin in dendritic spines. In contrast, cells tolerated LifeAct-GFP expression in a higher range, with only slight morphological alterations, which were not dose-dependent. Dendritic filopodial motility in LifeAct-expressing cells, on the other hand, correlated negatively with the higher expression level. Consistent with the Reviewer's suggestion, our results highlight the importance of

testing the dose-dependent effects of actin-labelling constructs in a particular experimental setting.

2. The focus of the work is much narrower than the title suggests. First, most actin structures within the neuron are being ignored, with only dendritic protrusions being assessed. So those wanting to compare how different probes could alter the properties of growth cones, dynamic axonal actin structures (reviewed by Roy, JCB, 2016), the periodic cytoskeleton, presynaptic actin structures (ex. Bingham et al, bioRxiv 2022), somatic actin (ex. Meka et al, EMBO Reports 2019), etc. are not going to find answers in this publication. Likewise, the study has been restricted to one type of neuron from a single species. Is even the most robust result, the inhibition of dendritic arborization by AG-GFP (Fig. 4), generally applicable to different types of neurons? At least even from the same species?

As mentioned earlier, the title has been modified because we are focusing on dendritic effects. Our focus was to examine how the tested actin probes interfere with dendritic morphological features especially relevant during structural plasticity of the neurons, rather than to give an overview on the general usefulness of the actin labelling probes in different cell types or model organisms. Thus, in this brief report manuscript, it is beyond our possibilities to extend our investigations towards different neuronal types or model organisms.

3. While Table 1 addresses the point of "general actin labelling", there are no experiments in this manuscript which do. I would have liked to see a quantitative co-localization of these probes with F-actin labeled with phalloidin. Do they label all of the same structures equally or are there differences? There is strong evidence to believe that they would (references provided by authors in the Introduction). This would also be an important point in choosing the correct probe.

As requested by both Reviewers, we performed phalloidin staining and colocalization studies with GFP-labelled fusion proteins. As shown in Figure 3C and D, all three tested constructs showed similar Manders' colocalization coefficients with phalloidin in the dendrites and spines. In contrast, relative enrichment of LifeAct-GFP was significantly lower compared to EGFP-actin and AC-GFP or phalloidin itself (Figure 3B), pointing to the limitations of LifeAct-GFP when visualizing F-actin dependent effects specifically within dendritic protrusions.

In agreement with changing the title and focusing on the dendritic effects of the tested actin probes, Table 1 has been removed.

4. Other popular actin binding probes like the ones based of Ftractin and Utrophin (both mentioned in the Introduction) are not included here. Adding them to the study would have significantly increased the potential value of the work.

The use of Utrophin was already strongly discouraged by Patel and colleagues in 2017, therefore, we did not consider it relevant to investigate this actin probe further. Moreover, F-Tractin is extensively studied in that paper and elsewhere, in experiments like ours, therefore the addition of these probes would not have provided new scientific data. On the other hand,

fewer quantitative investigations have been carried out with LifeAct and Actin-Chromobody, therefore we focused on these two actin labelling tools.

RE: Manuscript #E22-08-0331R
TITLE: "Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons"

Dear Dr. Schlett:

We have received comments from both reviewers. As you can see, both reviewers agree that manuscript has improved after revision, but also mention that there were several concerns that had not been addressed even textually in the revision. We request that you address each of these concerns in the text of your manuscript, detailing caveats mentioned by both reviewers. Please provide a point-by-point response of how and where in the text you have addressed all these concerns in a revised version.

Sincerely,
Stephanie Gupton
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Schlett,

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

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

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

Authors are allowed 15 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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

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

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

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

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The manuscript is much improved now. It is frustrating that the discussion did not include some of the important points addressed in the review process. Namely, that there are big differences in the transfection conditions between Wegner 2017 and the current manuscript (i.e. lipofectamine 24h vs viral transduction 2-4 weeks). This should be acknowledged in the manuscript so readers can properly evaluate this work in context.

Also, the authors seemingly ignored our comment about EGFP-actin not being polymerized by formin proteins. There is no mention of this limitation anywhere in the manuscript, and furthermore the final two paragraphs of the discussion go so far as to say that EGFP-actin should be the "gold standard".

This sentence does not make sense and should be clarified/corrected (i'd offer a suggestion but am actually unclear what is meant here): "While neurons can tolerate high expression levels of LifeAct-GFP over 24h, they may be more sensitive to ectopic expression of EGFP-actin and AC-GFP because of the lack of high somatic GFP fluorescence levels in surviving transfected cells."

I believe these issues can probably be vetted in a revised version by the editor and thus do not think a revise-and-resubmit is required, but I do believe these issues need to be addressed before publication.

Reviewer #2 (Remarks to the Author):

The authors have sufficiently addressed the concerns brought up by myself and the other reviewer about the integrity of the data and the conclusions that were drawn from it. However, the authors did not do anything to address the concern that the results they achieved using different actin binding probes in mouse hippocampal neurons were applicable to different types and/or species of neurons. That, combined with the fact that there is no real novel biology being presented, nor are there novel/ generally applicable tools being developed, I fear that this manuscript is too narrow in focus to be a standalone paper in MBoC and will not appeal to that journal's broad readership. However, that is just my opinion and whether or not this paper is suitable for MBoC is ultimately up to the editorial staff. The science is sound and should be published somewhere.

Dear Dr Gupton,

Thank you very much for the feedback on our #E22-08-0331R manuscript, entitled "Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons". We were pleased to read that both Reviewers acknowledged the improvement in our manuscript. We apologise that due to space limitations, we did not include all reflections on their comments into the manuscript text.

We have modified the manuscript text as requested - the rewritten sentences are now highlighted in the revised manuscript text (uploaded as a supplemental material) to facilitate evaluation. We also added three additional references to important previously published findings on the limitations of actin labelling methods and related our findings in more detail to a previously published in vivo comparison of LifeAct and Actin-chromobody. We are convinced that unbiased quantitative evaluation of the effects of the tested actin probes in dendrites and dendritic spines also provides a toolbox for readers interested in automated morphology analyses. Thus, we consider our work a useful comparison for those wishing to carry out detailed actin-related analyses within the dendrites and dendritic spines of cultured neurons.

We hope that the additional changes are satisfactory and that our manuscript will be accepted for publication.

Please see our point-by-point reflections below.

With best regards

Katalin Schlett

Corresponding author

Responses to Reviewer #1:

We would like to thank the Reviewer for his/her positive comments on our manuscript and constructive and helpful suggestions during the revision.

The manuscript is much improved now. It is frustrating that the discussion did not include some of the important points addressed in the review process. Namely, that there are big differences in the transfection conditions between Wegner 2017 and the current manuscript (i.e. lipofectamine 24h vs viral transduction 2-4 weeks). This should be acknowledged in the manuscript so readers can properly evaluate this work in context.

As requested, we have extended the comparison of our work and that of Wegner et al as follows to address how our work supplement their previous findings:

p3, lines 8-10: “LifeAct and Actin-Chromobody were shown to cause spine loss upon strong overexpression in vivo, but they have not been yet quantitatively compared and analysed

regarding their effects on dendritic branching, spine morphology and motility under more physiological expression levels (Wegner et al., 2017)".

p5, lines 15-19: "Our results are in line with previous work using AAV-mediated and long-term, hSyn promoter driven expression of LifeAct and AC in vivo as well as in primary cell cultures (Wegner et al., 2017). Regardless that we used 24h-long survival time of liposome-based transfection and a general CMV promoter, our more detailed quantitative analysis indicates that moderate level expression of EGFP-actin, LifeAct-GFP or AC-GFP does not result in drastic changes of dendritic morphology."

Also, the authors seemingly ignored our comment about EGFP-actin not being polymerized by formin proteins. There is no mention of this limitation anywhere in the manuscript, and furthermore the final two paragraphs of the discussion go so far as to say that EGFP-actin should be the "gold standard".

We agree with the Reviewer that it is important to note previously reported artefacts caused by the expression of actin labels, therefore we added additional references (Chen et al., 2012; Courtemanche et al., 2016; Kumari et al., 2020). We also agree that due to the limitations on using EGFP-actin, the used "gold standard" phrase was not appropriate and we removed it from the text. Rewritten parts read now as:

p2, lines 36-38: "Furthermore, fluorescent label can interfere with formin-mediated actin incorporation into the contractile rings during cytokinesis (Chen et al., 2012)."

p5, lines 11-14: "Comparing somatic fluorescence intensity to morphometric data revealed that only EGFP-actin intensity was positively correlated with total protrusion density, while none of the other morphometric parameters depended on relative expression levels (Supplementary Figure 1B-D)."

This sentence does not make sense and should be clarified/corrected (i'd offer a suggestion but am actually unclear what is meant here): "While neurons can tolerate high expression levels of LifeAct-GFP over 24h, they may be more sensitive to ectopic expression of EGFP-actin and AC-GFP because of the lack of high somatic GFP fluorescence levels in surviving transfected cells."

We apologise for this sentence which remained in the text only by an editing mistake – it was removed from the revised text.

I believe these issues can probably be vetted in a revised version by the editor and thus do not think a revise-and-resubmit is required, but I do believe these issues need to be addressed before publication.

Thank you again for the clarifying and helpful remarks which helped to polish our manuscript.

Responses to Reviewer #2:

The authors have sufficiently addressed the concerns brought up by myself and the other reviewer about the integrity of the data and the conclusions that were drawn from it. However, the authors did not do anything to address the concern that the results they achieved using different actin binding probes in mouse hippocampal neurons were applicable to different types and/or species of neurons.

We appreciate the Reviewer's positive comments on the value of the added evaluations. Nevertheless, extending our analysis to additional species or additional cell types was clearly beyond our possibilities, regarding both temporal as well as manuscript length limitations as a Brief Report. We believe that cultured mouse hippocampal neurons provide a well-established model system to detect fine morphological or motility changes in dendrites potentially evoked by the tested actin labelling probes, which can be relevant to those interested in visualizing dendritic actin reorganization in neurons. Although we cannot exclude that some of these processes are cell-type specific, many regulatory steps are executed in a cell-autonomous manner and therefore, can provide important comparisons of the actin labelling probes relevant to other neuronal subtypes, as well.

That, combined with the fact that there is no real novel biology being presented, nor are there novel/ generally applicable tools being developed, I fear that this manuscript is too narrow in focus to be a standalone paper in MBoC and will not appeal to that journal's broad readership. However, that is just my opinion and whether or not this paper is suitable for MBoC is ultimately up to the editorial staff. The science is sound and should be published somewhere.

Thank you again for reassuring us that our analysis was careful enough and has scientific merit. We believe that unbiased comparison on the dendritic consequences of expressing the tested actin labelling probes leads to novel biological data, even if relatively subtle changes are detected. In addition, we hope that our AI-based analysing toolset, made available to the scientific community, can additionally attract attention and might be useful for the broad audience. Therefore, we hope that our manuscript will be accepted by MBoC.

RE: Manuscript #E22-08-0331RR
TITLE: "Dendritic effects of genetically encoded actin labelling probes in cultured hippocampal neurons"

Dear Dr. Schlett:

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

Thank you for addressing these last reviewer concerns. Are you interested in including this in the recently announce special issue of MBoC, Cell Biology of the Nervous System? It won't change publication date, but will allow for additional highlight of the article in this collection

Sincerely,
Stephanie Gupton
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Schlett:

Congratulations on the acceptance of your manuscript.

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

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

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