# Experimental toolbox for quantitative evaluation of clathrin mediated endocytosis in the plant model *Arabidopsis*

Alexander Johnson, Nataliia Gnyliukh, Walter A. Kaufmann, Madhumitha Narasimhan, Grégory Vert, Sebastian Y. Bednarek and Jiří Friml
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**Editor**: David Stephens

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Original submission: 28 April 2020
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**Original submission**

First decision letter

MS ID#: JOCES/2020/248062

MS TITLE: Experimental toolbox for quantitative evaluation of clathrin mediated endocytosis in the plant model Arabidopsis

AUTHORS: Alexander Johnson, Nataliia Gnyliukh, Walter A Kaufmann, Madhumitha Narasimhan, Greg Vert, Sebastian Y Bednarek, and Jiri Friml
ARTICLE TYPE: Tools and Resources

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

I consider these revisions relatively minor and nothing that should require any additional experiments. I think they are largely self-explanatory but should you require any clarification then please do just ask.

*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.*

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

Johnson et al recapitulate available quantitative imaging protocols and review pharmacological and genetic manipulation with the aim to provide standardized experimental approaches for studies of clathrin-mediated endocytosis (CME) in plants. This s a timely and much needed summary as many plant labs use different approaches to evaluate CME that might create discrepancies in results and their interpretation. Especially important is the stipulated benefits and drawbacks for each method.

*Comments for the author*

Overall the manuscript provides very useful information for the plant field, it is well written and illustrated. I only have some minor suggestions for improvement.

Clathrin mediated endocytosis should be written as “Clathrin-mediated endocytosis”

Line 71 ".. has been the use of biochemical methods such as pull-down assays coupled with mass spectrometry and in vitro binding studies"- mention the available protocol for CCVs isolation.

Line 74 "...Also, the identification of a major plant EAP complex, the TPLATE complex, was facilitated using similar approaches (Gadeyne et al., 2014)" - AP2 was identified through TAP and IP-MS (by Di Rubbo et al., 2013 and Yamaoka et al., 2013), please mention this.

Line 98 "While there many different" – replace with “While there are many different”

Line 51 "For example;" replace with “For example,..”

Line 120 “…composed to produce a 3D ultra-structural view of the cell. While TEM has been used routinely in plants, and CCVs are visible and detectable in plant samples (Bonnett and Newcomb, 1966,..” - Please also include Dejonghe et al., 2016

Line 253, it is very appropriate to consider expression levels and functionality of different fluorescently-tagged EAPs. Many plant labs use fluorescently-tagged EAPs without testing them for functionality. Please correct me if wrong but this includes the shown in Fig. 4 and Supplemental Figure 3 CLC2 line (Huge aggregation is observed). If a fluorescently-tagged EAPs is tested for functionality and respectively is shown to be functional it should be indicated in in Supplemental Table 2. This table should be referred to in the part
“Considerations for the use of fluorescent protein tagged reporters”. In addition, the Arabidopsis background should be indicated as for example early studies of CLC2 were done in ws (Konopka et al., 2008a) background.

The authors should comment on the use of transient tobacco expression system to study CME by overexpressing heterologous proteins, manly related to study of plant immunity and CME.
Figure 6 - can the authors provide some exemplary FM measurements to compare their method with what is routinely published so far? For example using known CME mutants or pharmacological treatments. In Supplemental Figure 4 (related to Figure 6), not clear what is plotted on the Y axis.

Line 335, in the section “Fluorescently labeled cargo uptake assays” the authors have to mention the use of fluorescently-labelled ligands, which was successfully utilized to study CME of BRI1, PEPR1 and FLS2 (Irani et al., 2012;
Ortiz-Morea et al., 2016, Mbengue et al., 2016). I disagree that transferrininternalization can be recommended as an universal method to evaluate CME in plants, which will require expression of

heterologous receptor and the use of protoplast. Until now only one lab (that of the authors) has employed this approach and for this I will not include it as separate part but fuse with the possible use of fluorescently-labelled ligands part.

Line 468 the claim "..IKA may specifically inhibit CME mediated FM uptake, but not CIE.." is not supported by the data. The lack of complete inhibition of FM can be interpreted in many different ways. I would be very cautious proposing the use of IKA to the plant field without knowing the mode of action of this molecule to avoid another TyrA23 failure.

Supplemental Table 2 – Some data are incorrect and the table is incomplete.
pRPS5A:AP2A1-mTagRFP/Col-0 was made by Di Rubbo et al., 2013 pCLC1-CLC1-GFP/Col-0, pCLC2-CLC2-GFP/Col-0 and pCLC3-CLC3-GFP/Col-0 were made by Dejonghe et al., 2016 pRPS5A::CHC2-GFP/Col-0 was made by Ortiz-Morea et al., 2016 Not included are: pAP2M-AP2M-GFP/ap2m was made by Yamaoka et al., 2013; pAP2S-AP2S-GFP/ap2s was made by Fan et al., 2013; pTML-TML-GFP/tml-1 and TPLATE-TPLATE-GFP/tplate made by Gadeyne et al., 2014 (please double check the references for correctness). I would suggest to include the AGI code for each gene in Supplemental Table 2

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Supplemental Table 6 – CRISPR/Cas9-induced null mutation in the AP2M gene was reported by Yamamoto et al., 2018

Several labs are utilizing immunolocalization or biochemical approaches to study CME also quantitatively. As this manuscript id focused on plant CME resources,
perhaps is good to also list available "good" antibodies to study CME, which are very important tools.

Reviewer 2

*Advance summary and potential significance to field*

In this Tools & Techniques paper Johnson et al. present an overview of methods to study clathrin-mediated endocytosis in plants. Detailed methods are laid out for an EM method as well as two MATLAB-based imaging workflows for analysing CME. They also cover specificity of reagents used to inhibit CME.
Strictly speaking the manuscript does not present novel results. However, the gathered resources will be very useful to plant cell biology labs interested in membrane trafficking. I think this will be a very useful addition to this section of JCS.

*Comments for the author*

The manuscript was well written and very clear. I don't have suggestions for more work etc. I did review the code and associated resources carefully. My comments below are technical and should be addressed in the GitHub repo.

The MS states up front which platforms the code has been tested on (this is good). I use cmeAnalysis and this always breaks between versions of MATLAB. I tested the code on MATLAB 2019b on macOS Catalina. I could get everything to run but there are two issues:

1. Catalina uses a signing protocol which means that any users downloading the repo will not be able to run the software because the OS thinks the *.maxmaci64 files are malware. Users need to recursively approve all the files on the command line using

sudo xattr -dr com.apple.quarantine plantCmeMethods-master

I think a note about this should be added to the instructions because Catalina is not going to go away.

2. I ran the code with only plantCmeMethods and sub-directories in my MATLAB path. It doesn't run on MATLAB2019b under those conditions. It seems to be missing some files, I don't think this is related to 2019b (my guess is that it will run with cmeAnalysis also in the path). Anyhow, I pushed the latest code for cmeAnalysis from DanuserLab GH into the cmeAnalysisPackage. So the authors can fix this by getting their codebase level with the latest release. Alternatively, they could retest on a clean MATLAB install and try and fix at the level of the supplied files, but my feeling is that will be difficult to do. Bottom line is that this stuff should just run out of the box on latest OS, recent-ish MATLAB and only the authors code in the MATLAB path.
Above points apply to cellSurfaceAnalysis. fmUptakeAnalysis ran fine.

Minor issue:
The instructions say that the supplied data is 8 um pixel size??? Is this correct for an 100X objective this seems wrong. Please check.

Finally, the authors should consider doing a release of their code to zenodo to mint a doi to include in the paper. The data could also be uploaded to Figshare or equivalent. DOIs will help others to find and cite the work associated with the paper.

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**First revision**

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
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Reviewer 1 Comments for the Author:
Overall the manuscript provides very useful information for the plant field, it is well written and illustrated. I only have some minor suggestions for improvement.

We thank the reviewer for their valuable comments to help improve the manuscript and address all their suggestions below.

Clathrin mediated endocytosis should be written as “Clathrin-mediated endocytosis”

We have made sure that this is correct throughout the whole manuscript.

Line 71 ".. has been the use of biochemical methods such as pull-down assays coupled with mass spectrometry and in vitro binding studies"- mention the available protocol for CCVs isolation.

We do not feel referencing them at this point in the manuscript is appropriate, as much of the biochemical studies to date have been conducted on whole plant lysates and not CCV preparations. But we agree that these are important and very useful references and have already referred to them later in the text:

“..protocols for the enrichment of CCVs from plant tissues (for detailed methods see (Mosesso et al., 2018, Reynolds et al., 2014)).” – Line 130

Line 74 "...Also, the identification of a major plant EAP complex, the TPLATE complex, was facilitated using similar approaches (Gadeyne et al., 2014)" - AP2 was identified through TAP and IP-MS (by Di Rubbo et al., 2013 and Yamaoka et al., 2013), please mention this.

We have updated the manuscript to include the suggested references – Line 71.

Line 98 "While there many different" – replace with “While there are many different”

Corrected as suggested – Line 94.

Line 51 "For example;" replace with “For example,..”

Corrected as suggested – Line 51.

Line 120 “…composed to produce a 3D ultra-structural view of the cell. While TEM has been used routinely in plants, and CCVs are visible and detectable in plant samples (Bonnett and Newcomb, 1966,..” - Please also include Dejonghe et al., 2016

We have added the suggested reference – Line 142.

Line 253, it is very appropriate to consider expression levels and functionality of different fluorescently-tagged EAPs. Many plant labs use fluorescently-tagged EAPs without testing them for functionality. Please correct me if wrong but this includes the shown in Fig. 4 and Supplemental Figure 3 CLC2 line (Huge aggregation is observed). If a fluorescently-tagged EAPs is tested for functionality and respectively is shown to be functional it should be indicated in in Supplemental Table 2. This table should be referred to in the part “Considerations for the use of fluorescent protein tagged reporters”. In addition, the Arabidopsis background should be indicated as for example early studies of CLC2 were done in ws (Konopka et al., 2008a) background.

We agree with the reviewer that this is a critically important point. We believe we highlight the importance of testing functionality already in the text:

“It is therefore good practice to use fluorescent fusion proteins that have been demonstrated to be functional (e.g. through their ability to rescue the phenotype of corresponding loss-of- function mutant lines)” – Line 272.

Regarding the CLC2 data shown in Figure 4 and the Supplemental figures, we chose to use a line which has been accepted and utilized in many publications and as thus become a standard in the field. While there are large aggregations seen in the example figures, these represent early endosomes. Recent work has shown that the CCV uncoating reaction is slower than those seen in other systems and thus CLC reaches the early endosome, creating these large aggregations – which are visible in SEM images of unroofed Col-0 protoplasts (Narasimhan et al., 2020). Additionally, these aggregations are found in every CLC2 line published to date and are also highlighted by immuno-staining using antibodies for differing clathrin isoforms. For example, Dhonukshe et al., 2007, Gadeyne et al., 2014, Ito et al., 2012, Konopka et al., 2008 and Wang et al., 2017.

We have updated the supplemental table to include the suggestions made and referred to it in the suggested section – Line 288.

The authors should comment on the use of transient tobacco expression system to study CME by overexpressing heterologous proteins, manly related to study of plant immunity and CME.

We have added a short statement highlighting that tobacco systems have been used to transiently express CME related proteins – Line 364.

Figure 6 - can the authors provide some exemplary FM measurements to compare their method with what is routinely published so far? For example using known CME mutants or pharmacological treatments. In Supplemental Figure 4 (related to Figure 6), not clear what is plotted on the Y axis.

We have specified further what the axis represents in all FM uptake figures and expanded it in the figure legends (Figure 6 and now supplemental figure 3).

We believe that the robustness of the FM analysis method presented here is well demonstrated. For example, we show that it robustly confirms FM4-63 and FM1-43 produce similar amounts of uptake and we show that it is capable of detecting a visible difference in the IKA FM experiments (Figure 7).

Line 335, in the section "Fluorescently labeled cargo uptake assays" the authors have to mention the use of fluorescently-labelled ligands, which was successfully utilized to study CME of BRI1, PEPR1 and FLS2 (Irani et al., 2012; Ortiz-Morea et al., 2016, Mbengue et al., 2016). I disagree that transferrininternalization can be recommended as an universal method to evaluate CME in plants, which will require expression of heterologous receptor and the use of protoplast. Until now only one lab (that of the authors) has employed this approach and for this I will not include it as separate part but fuse with the possible use of ligands part.

We have reduced the Transferrin section in to the 'fluorescently labelled cargo' section suggested and put less emphasis on using it to study plant CME – Line 358.

Line 468 the claim "..IKA may specifically inhibit CME mediated FM uptake, but not CIE.." is not supported by the data. The lack of complete inhibition of FM can be interpreted in many different ways. I would be very cautious proposing the use of IKA to the plant field without knowing the mode of action of this molecule to avoid another TyrA23 failure.

We agree and have further added some clarification and further statements to address that its action is not known, and thus caution is required if using it. Lines – 463 and 468

Supplemental Table 2 – Some data are incorrect and the table is incomplete. pRPS5A:AP2A1-mTagRFP/Col-0 was made by Di Rubbo et al., 2013 pCLC1-CLC1-GFP/Col-0, pCLC2- CLC2-GFP/Col-0 and pCLC3-CLC3-GFP/Col-0 were made by Dejonghe et al., 2016 pRPS5A::CHC2-GFP/Col-0 was made by Ortiz-Morea et al., 2016 Not included are: pAP2M- AP2M-GFP/ap2m was made by Yamaoka et al., 2013; pAP2S-AP2S-GFP/ap2s was made by Fan et al., 2013; pTML-TML-GFP/tml-1 and TPLATE-TPLATE-GFP/tplate made by Gadeyne et al., 2014 (please double check the references for correctness). I would suggest to include the AGI code for each gene in Supplemental Table 2

We thank the reviewer for their corrections and suggestions. As such, we have updated the table as suggested and entitled it 'Examples of...' to highlight that it is not completely exhaustive.

Supplemental Table 3 - Pitstop2 was also tested in Arabidopsis (see Dejonghe et al., 2019), is good to mention this in the table for completion. Because Pitstop2 decreased the FM4-64 fluorescence with increasing its concentration, is perhaps inaccurate to say it was inactive (line 437).

We have added Pitstop 2 to Supplemental Table 3 and further explained the results as reported in Dejonghe et al., 2019 – Line 438.

Supplemental Table 4 - XVE»AUXILIN-LIKE1/2 was first tested by Ortiz-Morea et al., 2016, please include this reference

We have updated the table to include this reference.

Supplemental Table 6 – CRISPR/Cas9-induced null mutation in the AP2M gene was reported by Yamamoto et al., 2018

We have updated the table to include this information.

Several labs are utilizing immunolocalization or biochemical approaches to study CME also quantitatively. As this manuscript id focused on plant CME resources, perhaps is good to also list available "good" antibodies to study CME, which are very important tools.

We agree that this would be a great addition, however we feel at present it is not possible to present an exhaustive list of 'good' antibodies. One major reason for this is that many labs use different immuno-staining protocols, and each affects the efficiency of staining, thus we have avoided this.

However, we have added a statement into the discussion stating how important antibodies have been, and still are, in investigating plant CME. Further, we suggest that researchers could add their favorite antibodies to online open resources, like Antibodypedia (on online database of submitted available antibodies and reviews from users – although mammalian orientated, it could become a very useful resource for plant researchers).

Line 539 - "Another important area for the standardization of plant CME investigations is the highlighting of robust EAP antibodies. To date, many antibodies have led to key insights in to the interactions and localization of EAPs in both pull-down assays and immunohistochemical staining of plant tissues (Dejonghe et al., 2019, Dhonukshe et al., 2007, Di Rubbo et al., 2013, Gadeyne et al., 2014, Gao et al., 2019). Therefore, to further broaden the standardization of plant CME tools to cover antibodies, researchers should be encouraged to submit their routinely used antibodies to online databases, such as Antibodypedia (Bjorling and Uhlen, 2008)".

Reviewer 2 Advance Summary and Potential Significance to Field:
In this Tools & Techniques paper Johnson et al. present an overview of methods to study clathrin-mediated endocytosis in plants. Detailed methods are laid out for an EM method as well as two MATLAB-based imaging workflows for analysing CME. They also cover specificity of reagents used to inhibit CME. Strictly speaking the manuscript does not present novel results. However, the gathered resources will be very useful to plant cell biology labs interested in membrane trafficking. I think this will be a very useful addition to this section of JCS.

Reviewer 2 Comments for the Author:
The manuscript was well written and very clear. I don't have suggestions for more work etc. I did review the code and associated resources carefully. My comments below are technical and should be addressed in the GitHub repo.

We thank the review for their efforts in checking the code, helping us improve it and providing us with some very helpful technical comments and suggestions.

The MS states up front which platforms the code has been tested on (this is good). I use cmeAnalysis and this always breaks between versions of MATLAB. I tested the code on MATLAB 2019b on macOS Catalina. I could get everything to run but there are two issues:

1. Catalina uses a signing protocol which means that any users downloading the repo will not be able to run the software because the OS thinks the *.maxmaci64 files are malware. Users need to recursively approve all the files on the command line using

sudo xattr -dr com.apple.quarantine plantCmeMethods-master

I think a note about this should be added to the instructions because Catalina is not going to go away.

Thank you very much for this help. We had failed to test Catalina and did not realize that so much had changed compared to Mojave – where the analysis was tested. We have added a note about this to the manuals instructing people how to install and get it running on their system.

2. I ran the code with only plantCmeMethods and sub-directories in my MATLAB path. It doesn't run on MATLAB2019b under those conditions. It seems to be missing some files, I don't think this is related to 2019b (my guess is that it will run with cmeAnalysis also in the path). Anyhow, I pushed the latest code for cmeAnalysis from DanuserLab GH into the cmeAnalysisPackage. So the authors can fix this by getting their codebase level with the latest release. Alternatively, they could retest on a clean MATLAB install and try and fix at the level of the supplied files, but my feeling is that will be difficult to do. Bottom line is that this stuff should just run out of the box on latest OS, recent-ish MATLAB and only the authors code in the MATLAB path.
Above points apply to cellSurfaceAnalysis. fmUptakeAnalysis ran fine.

We thank the reviewer for the suggestion and their effort in testing the code with the latest cmeAnalysisPackage. We have updated our code to include this version and made a few changes to ensure that it now works on Windows, Catalina and Ubuntu for all the cell surface analyses.

Minor issue:
The instructions say that the supplied data is 8 um pixel size??? Is this correct for an 100X objective this seems wrong. Please check.

We have corrected this.

Finally, the authors should consider doing a release of their code to zenodo to mint a doi to include in the paper. The data could also be uploaded to Figshare or equivalent. DOIs will help others to find and cite the work associated with the paper.

We have reserved DOIs for the code, example data and for the raw data on Zenodo and provided their links in the manuscript - Lines 555 and 910.

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Second decision letter

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AUTHORS: Alexander Johnson, Nataliia Gnyliukh, Walter A Kaufmann, Madhumitha Narasimhan, Greg Vert, Sebastian Y Bednarek, and Jiri Friml
ARTICLE TYPE: Tools and Resources

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Thank you for your careful revisions. I did not consider it necessary to return this to the reviewers so there are no further comments from them.