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Poji: a Fiji-based tool for analysis of podosomes and associated proteins

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First decision letter

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MS TITLE: Poji: a Fiji-based tool for analysis of podosomes and associated proteins

AUTHORS: Robert Herzog, Koen van den Dries, Pasquale Cervero, and Stefan Linder
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.organd click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers thought Poji is potentially a useful tool for the community. Nevertheless, the study received quite mixed reports. Reviewer 1 feels that it would be useful to have a couple examples or simple treatments to more fully demonstrate its usefulness and maybe learn something new about podosomes at the same time. Reviewer 1 also eludes to see to some of the issues raised by Reviewer 2, who feels the plugin has considerable limitations given its significant user intervention. Reviewer 2 does not recommend publication and believes that Poji could easily be improved to allow more automated analysis by the introduction of additional scripts that are already commonly used, for example to define cell outlines. Reviewer 3 is more positive but again feels that you need to go further with your analysis to demonstrate that Poji is actually superior. In light of their comments, I feelthat a revised version might prove acceptable, if you can address the reviewers concerns highlighted above with additional experiments. As suggested by reviewer 2 when revising the MS the script should be submitted as a plain text file or even better placed in an online repository such as GitHub (together with some test data). This would then enable a full assessment by the reviewers when we return the revised paper to them.

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

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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

The authors describe a Fiji based tool for quantifying localisation and distribution of proteins in podosomes relative to position in the cell. This tool builds on previous simpler podosome quantification software and adds features that allow new types of quantification. As described, it could potentially be useful for quantifying other similar sized objects, such as invadopodia or focal adhesions, but this is not shown. While a clear workflow outlining various uses of Poji is shown, it isn't clear how each component of the workflow is achieved and it would be easier to understand what Poji is useful for if an example of each step of the workflow was shown. Also, it feels that the authors haven't really demonstrated the usefulness of Poji. They show a couple of examples of how it can be used- but what have they learned from these example? Are there other simple treatments that they can do to show a bit of new biology with this tool? It would entice more people to give it a try if the outcomes shown led to a biological message.

Comments for the author

General Comments:
• Check spelling of Poji throughout manuscript.
• 'Potential smoothing steps and noise tolerance… defined empirically' Maybe explain this in more detail or make it clear that these settings need to be kept consistent between cells and experiments for a fair comparison.
• Applications: are there more applications of this plugin? Can it be used in different cell types. Can it be used to quantify invadopodia and correlate with areas of degradation? The tool publication would have more impact if another aaplication were demonstrated.
• Can inhibitors be used to disassemble podosomes/invadopodia to see bigger differences? E.g. MMP inhibitor.
• Generally well written. Honest account of pitfalls and troubleshooting is welcome.
• Can an example dataset and user guide be supplied with the plugin?
Figure 1
• 1B2: Can a macro or plugin be designed to define the cell outline?
• 1B7: This is very cool but the full power of this tool, is it used throughout the manuscript? It should be made clear which part of the workflow is used for each application and where possible please give clear examples of each step in the workflow.
Figure 2
• Can you explain the 65 groups? This whole section is confusing and should be clarified.
• The graph is confusing, can it be labelled where the nucleus is on the x-axis?
• There are no error bars, can this be done for multiple cells? Is this data only from one cell? One podosome?
• Perhaps a better first example would be with something that has a clearer difference in the number of podosomes, i.e. a more potent inhibitor. Then the authors can go on to show the Plugin can also pick out subtle differences that are more biologically relevant.
Figure 3
• Can this be used for multiple cells and podosomes? Again there are no error bars.
Figure 4
• I am surprised the cells do not change size on the different matrix stiffnesses. Perhaps this depends on the cell type.
Figure 5
• Can Figures 5J and 5K be further explained? Surely taking an average profile of all the podosomes in a cell is the best measure for comparison.

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Reviewer 2

Advance summary and potential significance to field

The authors present a tool, Poji, implemented as a FIJI script, for the analysis of protein enrichment at podosomes. A description of the workflow is presented, along with a "proof of concept" analysis of myosin IIA and vinculin recruitment to podosomes in primary human macrophages. While there would undoubtedly be interest among the broader cell biology community in a tool designed to automate the analysis of podosomes and other punctate structures, the tool suffers from some severe limitations, limiting its usefulness.

Comments for the author

First and foremost, the tool (Poji) relies on significant user intervention to manually define a cell and draw an appropriate region of interest. Assuming appropriate staining of cell bodies, automated cell segmentation is something that is relatively easily implemented in a script.

Secondly, related to the first point above, the analysis is limited to that one single cell defined by the user. The utility of the tool as a means of automating analysis, reducing workload and (more importantly) removing user bias, is therefore limited.

Finally, I have never seen code submitted with a manuscript in the form of a multi-column PDF! While I tried to copy and paste the contents into a text file to run the script in FIJI, not surprisingly, errors were encountered. I strongly recommend that, at the very least, the script is in future submitted as a plain text file, but would strongly encourage the authors to put their work in an online repository such as GitHub (together with some test data).

Specific comments:

Lines 114 - 115: I disagree that Poji is suitable for the analysis of super-resolution data derived from STORM or PALM experiments - the output from such experiments is typically a list of point localisations, not an image.

Lines 128 - 130: This seems a little time-consuming and unnecessary - again, splitting channels is something easily automated within a script.

Lines 150 - 153: I appreciate that this is somewhat related to the means used to detect the podosomes (local maxima detection), but the lack of sub-pixel registration at this stage, prior to averaging, likely results in a lower resolution intensity profile than could otherwise be achieved.

Lines 165 - 166: The authors seem to have quantified the number of "false positives" returned by Poji, but not the number of "false negatives"?

Lines 194 - 196: This is worded in a confusing manner. I believe the authors are referring to the binning of podosome intensities for the purposes of constructing a histogram?

Lines 198 - 201: This seems to be an assumption - was it actually quantified? It would not be difficult to relate intensities of detected podosomes to their position within the cell.

Lines 204 - 206: The description of myosin IIA distribution in blebbistatin-treated cells here ("loss of the pronounced myosin IIA network in the cell periphery") seems to contradict what was said earlier (Lines 186 - 187: "in the blebbistatin-treated cells, myosin II was absent from a large part of the cell, and was present mostly in the cell periphery").

Lines 262 - 265: If cell size and podosome-covered area are similar across all conditions, then how can the podosome density be significantly different?

Lines 339 - 347: The authors reveal a significant shortcoming of their analysis here - that is, the distribution of protein enrichment around a podosome may not be homogeneous or symmetrical and orientation may vary from one podosome to another. Averaging profiles together will therefore

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mask this inhomogeneity. While the authors are aware of this limitation, their solution seems to be to systematically enforce symmetry? Perhaps a better solution would be to assume inhomogeneous distributions and attempt to align profiles by rotation, prior to averaging?

Lines 348 - 411: I'm not sure the discussion adds a whole lot to the manuscript - it seems to be mostly repeating what has been stated previously.

Lines 372 - 376: Again, this seems cumbersome and unnecessary - it's relatively easy to check automatically what the bit-depth of an image is or what the maximum possible pixel value in a given image is. Asking a user to modify values within the script seems unwise and will likely cause problems.

Lines 410 - 411: I strongly disagree with the final line here - the "well-honed eyes of experienced researchers" are notoriously biased!

Line 434: Unless I am very much mistaken, the authors never specify which secondary antibodies (which fluorophores) they have used?

Reviewer 3

Advance summary and potential significance to field

Podosomes represent a subgroup of cell-matrix contacts, which is especially prominent in cells of the monocytic lineage such as monocytes, macrophages and dendritic cells. . The analysis of podosomes can be challenging because these structures can be densely displayed on the ventral surface. Podosome number, distribution and composition can be affected by experimental treatments - and thus a tool to investigate podosomes in an unbiased fashion is useful. The authors present a Fiji-based macro code termed "Poji" (podosome analysis by Fiji), and provide the plugin code which they suggest is superior to existing routines - in order to characterize a variety of cellular and podosomal parameters.
Fo example fluorescence intensity and relative enrichment in podosomal versus cytoplasm or cell membrane.

Although the manuscript does not seem to contain new findings regarding podosome biogenesis or composition - based on use of the plugin - there is some merit in characterizing what the software can achieve and making the software available.

The authors should supply the image files for Fig. 1B, 2A and B, 3A and 5 A, B, G. These are needed to evaluate if the results obtainable with their software is indeed superior to pre-existing versions mentioned.

Comments for the author

Specific points.

The authors look at two conditions which have been investigated in some detail previously, namely changes in mysoin II contractility and different substrate rigidity.

1. Application 1. In the former case the authors are using a low concentration of blebbistatin (10 um) which really should be shown on figures or in the legend. It is hardly surprising that little change is noted under their experimental conditions.

The role of myosin IIA in podosome dynamics is complex question and it would be nice if authors can provide some new insights (using their software) on this issue. There also needs to be more balanced analysis of existing literature for example recent papers, show that myosin IIA and contractility plays inhibitory rather than stimulatory role in podosome formation. cf. Rafig, et al., 2019,; Dulyaninova, et al., 2018, Cervero, et al., 2018 (this paper by the author is mentioned) and van den Dries et al., 2013.

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2. Regarding the limitations of blebbistatin treatment, I would suggest alternate method(s) to interfer with the podosomes associated acto-mysoin complex such as inhibition of Rho/ROCK pathways (van Helden, 2008,). The authors are using what likely produces a slight inhibition of ATPase activity by blebbistatin - more convincing data might be forthcoming if they rather completely inhibited ROCK.

Perhaps the object of using low levels of myosin II inhibitor is to demonstrate the software is more "sensitive" versus the existing Poji-like macros. Nonetheless it is not clear what this redistribution of myosin IIA represents (ie. to what extent there is disassembly of myosin IIA filaments under these conditions?). If the authors have access to structured illumination super-resolution technology it would be simple to get much improved micrographs of myosin IIA (Fig. 2A'' and B") in order to properly discuss alteration in "ordered' (line 185) myosin IIA distribution after blebbistatin treatment.

3. In application 2. The authors look at the protein LSP1 which they have previously characterized in some detail. There does not seem to be any specific advantage in the analysis of LSP1 (in a different focal plane) using Poji , since a few much better resolution images would be of much more value in assessing the Z 'position' of various podosome components.

4. In Application 3 the authors investigate the effects of substrate rigidity on parameters extracted by Poji. Podosome formation can efficiently proceed in cells plated on fluid-supported lipid bilayer membrane, a substrate which does not permit development of traction forces exerted on integrin clusters. Thus, podosomes appear to self-assemble by default under conditions of deprivation of traction forces (Yu, et al., 2013). In view of these results, it is rather surprising to see (Fig. 4) that number of podosomes, podosomal area and subsequently podosome density significantly decreased on less rigid matrices (Fig. 4A-C). Additional literature (Yu et al., 2013, Changede, et al., 2015) should be taken into consideration and explanation provided. As it is I am not sure if this is an interesting new finding (in this cell type).

First revision

Author response to reviewers' comments

Dear Dr. Way, dear Michael,

We are submitting a revised version of our manuscript entitled "Poji: a Fiji-based tool for analysis of podosomes and associated proteins", and ask you to consider its suitability for *Journal of Cell Science*. We are very grateful for the reviewers´ constructive comments and also for your editorial guidance and have tried to address the raised points as closely as possible. Please find a point-by-point response below. The respective changes are marked in red in the manuscript.
As discussed at the recent ASCB meeting with the JCS editors, the macro code could be deposited on the currently developed microsopy website of the Company of Biologists or on a more traditional online source such as GitHub. We would be happy to follow the preferences of JCS here. Respective points in the manuscript that refer to the to-be-determined online depository are currently marked in yellow.

Reviewer 1
The authors describe a Fiji based tool for quantifying localisation and distribution of proteins in podosomes relative to position in the cell. This tool builds on previous simpler podosome quantification software and adds features that allow new types of quantification. As described, it could potentially be useful for quantifying other similar sized objects, such as invadopodia or focal adhesions, but this is not shown. While a clear workflow outlining various uses of Poji is shown, it isn't clear how each component of the workflow is achieved and it would be easier to understand what Poji is useful for if an example of each step of the workflow was shown. Also, it feels that the authors haven't really demonstrated the usefulness of Poji. They show a couple of examples of how it can be used- but what have they learned from these example? Are there other simple

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treatments that they can do to show a bit of new biology with this tool? It would entice more people to give it a try if the outcomes shown led to a biological message.

1) Check spelling of Poji throughout manuscript.
Thank you. The name is now spelled “Poji” throughout the manuscript

2) ‘Potential smoothing steps and noise tolerance… defined empirically’ Maybe explain this in more detail or make it clear that these settings need to be kept consistent between cells and experiments for a fair comparison.
Thank you for pointing this out. We have now added more detailed information about the two parameters that need to be determined by the user and also explained why an individual optimization per cell is preferable over a fixed set-up for all cells (p.6).

3) Applications: are there more applications of this plugin? Can it be used in different cell types. Can it be used to quantify invadopodia and correlate with areas of degradation? The tool publication would have more impact if another aaplication were demonstrated.
We haved now used Poji to analyse images of cortactin-positive invadopodia in MDA-MB-231 breast cancer cells and corresponding images of gelatin degradation, kindly supplied by Dr. Philippe Chavrier and Dr. Pedro Monteiro. Poji is indeed able to reliably identify also dot-like invadopodia and correlate them with areas of gelatin degradation. This additional application is now included as the new Fig. 5 and is also mentioned in the Results (p.12) and Discussion sections (p. 18).

4) Can inhibitors be used to disassemble podosomes/invadopodia to see bigger differences? E.g. MMP inhibitor.
Thank you for this good suggestion. We have now tried a variety of pharmacological substances and are now presenting new data on the loss of two prominent proteins from their specific substructures, namely loss of vinculin from the ring upon addition of cytochalasin D (new Figure 3) and loss of LSP1 from the cap upon addition of the Ca2+ ionophore ionomycin (new Fig. 4). Especially the latter application provides new information on the use of inhibitors to selectively influence podosome architecture and composition and could be helpful in futher analyses of the podosome cap.

5) Generally well written. Honest account of pitfalls and troubleshooting is welcome.
Thank you, we are grateful that this is appreciated.

6) Can an example dataset and user guide be supplied with the plugin?
This is a very good suggestion. We now added the image that was used for analysis of false positives and false negatives (new Fig. 6) as an example data set, together with a comprehensive userguide as a PDF, in the Supplementary material. We suggest that these files should be uploaded to the same online depository, where also the macro code and all associated tables will be available for download (see also comment 3 of reviewer 2).

7) Figure 1
1B2: Can a macro or plugin be designed to define the cell outline?
This is, in principle, possible. For example, we used this function in the earlier basic macro described in (Cervero et al., 2013). We ultimately decided against including this function as a fixed feature, as it limits the usefulness of the macro considerably, without providing a comparable boost in speed. This is mostly based on the exclusion of false positives by manually defining the area to be analysed, as shown in (Fig. 6A,B).
Still, we took this comment as an opportunity to add an option in the macro interface to not only define the cell area, but for optional additional definition of podosome clusters for each cell. This feature is now also shown in (Fig. 6A,B) and mentioned in the text (lines 373-881). We believe this is a valuable step that enables a more flexible analysis, which can be used to reduce false positive rates, but which also enables a better analysis of cells that show podosome cluster formation such as dendritic cells or osteoclasts. Moreover, it can be used to analyse differentially localized subpopulations such as the periphery-associated precursors and the more centrally located successor podosomes. See also points 1 and 12 of reviewer 2.

8) Figure 1
1B7: This is very cool but the full power of this tool, is it used throughout the manuscript?

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It should be made clear which part of the workflow is used for each application and where possible please give clear examples of each step in the workflow.
Thank you for this comment. We are now mentioning specific steps of the Poji macro in the respective results sections of the presented applications, thus clarifying which steps of the workflow are necessary to gain specific data sets.

9) Figure 2
Can you explain the 65 groups? This whole section is confusing and should be clarified.
The 65 groups were used to refer to the process of binning. However, we agree that this section was written in a confusing manner. We have thus rephrased this section for clarification and to explain the binning process of the data better, by mentioning that podosomes were "…binned in groups with a fluorescence intensity interval of 500 a.u. per group" (p.9). See also point 8 of reviewer 2.

10) Figure 2
The graph is confusing, can it be labelled where the nucleus is on the x-axis?
The graph depicts the intensity distribution of the respective podosome-localized proteins. It does not give information on the localization of individual podosomes. For example, podosomes in the area around the nucleus do tend to be associated with lower levels of myosin IIA. However, individual podosomes in the cell periphery can exhibit similar levels of myosin II A intensity and their fluorescence intensity levels thus localize to a position on the graph that is similar to the ones close to the nucleus. Fluorescence intensity of podosome-associated proteins is thus not directly linked to subcellular localization, and indicating the general intensity of podosomes around the nucleus on the x-axis of the graph would be misleading.
However, we have now added an analysis of myosin IIA fluorescence intensities for podosomes under the nucleus as well as for all podosomes in the new Supplementary Figure 1. The respective graph shows clearly that the low intensity peak of myosin IIA-based fluorescence is mostly due to the podosomes located under the nucleus. See also reviewer 2, point 9.

11) Figure 2
There are no error bars, can this be done for multiple cells? Is this data only from one cell? One podosome?
Figure 2 shows one cell and data from all of its podosomes. The manuscript is now structured in a way that we first show the ability of the macro to report fluorescence intensities and distribution of proteins in a single cell (Fig. 2), and then in multiple cells (new Fig, 3) as well as in different z planes (new Fig. 4), and finally also for invadopodia in cancer cells (new Fig. 5). In these applications, we show controls and pharmacological interventiony that influence the distribution of the respective proteins shown, including myosin IIA and blebbistatin (Fig. 2), vinculin and cytochalasin D (Fig. 3), as well as LSP1 and ionomycin (Fig. 4). Moreover, we also show that Poji is useful for the detection of invadosome-associated matrix degradation (new Fig. 5). See also reviewer 1, point 14 and reviewer 2, point 11.

12) Figure 2
Perhaps a better first example would be with something that has a clearer difference in the number of podosomes, i.e. a more potent inhibitor. Then the authors can go on to show the Plugin can also pick out subtle differences that are more biologically relevant.
Thank you for this suggestion. We indeed considered the suggested flow of the manuscript, but ultimately decided against it. First, we took a similar approach in presenting a previous, basic macro in (Cervero et al., 2013). Second, with the addition of new material, the manuscript is now structured to proceed from a more global effect in a single cell (myosin IIA redistribution upon use of blebbistatin in Fig. 2) to specific dislocalization of podosome components such as vinculin upon use of cytochalasin D (new Fig. 3) and LSP1 upon use of ionomycin (new Fig. 4) from their respective substructures, to gelatin degradation by invadopodia in cancer cells (Fig. 5). At the same time, using these different examples, we show that Poji allows the analysis of subtle differences and changes in globular parameters in both single, as well as in multiple cells, and also in multiple confocal planes, and finally also in different cell types. We hope that the reviewer agrees with us on this structure of the manuscript.

13) Figure 3
Can this be used for multiple cells and podosomes? Again there are no error bars.
The macro can indeed be used for multiple cells. Please see our replies to points 11 and 12.

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14) Figure 4
I am surprised the cells do not change size on the different matrix stiffnesses. Perhaps this depends on the cell type.
We also think this likely depends on the cell type, and that this point should be investigated in more depth. However, this figure was mainly intended to show that macro can be used for the analysis of multiple cells. As this is now shown in the new Figure 3, the data from cells seeded on matrices of different stiffness have now been removed from the manuscript.

15) Figure 5
Can Figures 5J and 5K be further explained? Surely taking an average profile of all the podosomes in a cell is the best measure for comparison.
Figures 5J and 5K (now Fig. 7D,E) are given as examples of profiles from individual podosomes. The point we want to make with these examples is that podosome size and fluorescence intensity distribution of podosome components may vary greatly, especially in regard to the mentioned podosome subpopulations or to fusion and fission processes. These differences may be overlooked when values are just averaged. To clarify the difference between the profiles of single podosomes and the average of all podosomes, we have now added to the respective paragraph on p.14: “For examples of these individual differences in podosome size and fluorescence intensity distribution, see (Fig. 7C-E) and compare them to the average of all podosomes from this cell in (Fig. 7B).”.

Reviewer 2
The authors present a tool, Poji, implemented as a FIJI script, for the analysis of protein enrichment at podosomes. A description of the workflow is presented, along with a "proof of concept" analysis of myosin IIA and vinculin recruitment to podosomes in primary human macrophages. While there would undoubtedly be interest among the broader cell biology community in a tool designed to automate the analysis of podosomes and other punctate structures, the tool suffers from some severe limitations, limiting its usefulness.

1) First and foremost, the tool (Poji) relies on significant user intervention to manually define a cell and draw an appropriate region of interest. Assuming appropriate staining of cell bodies, automated cell segmentation is something that is relatively easily implemented in a script.
We generally agree with the reviewer on this point and refer to discussion of new features of the macro in point 7 of reviewer 1.

2) Secondly, related to the first point above, the analysis is limited to that one single cell defined by the user. The utility of the tool as a means of automating analysis, reducing workload and (more importantly) removing user bias, is therefore limited.
The reviewer addresses an important point here. We originally designed Poji to analyse single, highly resolved cells per individual image to ensure sufficient resolution of podosomes and associated structures. However, by appropriate adjustment of the resolution during microscopy, it is possible to also acquire sufficiently resolved multi-cell images. We thus took this comment as inspiration to edit the macro to process multiple cells per image and introduced this option by adding a section about this in the chapter “overview of the macro”. We also used the new option of multi-cell selection by analysing at least 15 cells per condition, with several cells per image, for the new Figure 3 and added a section in the manuscript to highlight this option. This new feature reduces workload even more, while at the same time enabling the analysis of a high number of cells in a reasonable time frame. Independently of this feature, random selection of a sufficiently high number of representative cells during microscopy is the most important way to reduce user bias and to ensure comparability of results.

3) Finally, I have never seen code submitted with a manuscript in the form of a multi-column PDF! While I tried to copy and paste the contents into a text file to run the script in FIJI, not surprisingly, errors were encountered. I strongly recommend that, at the very least, the script is in future submitted as a plain text file, but would strongly encourage the authors to put their work in an online repository such as GitHub (together with some test data).
Thank you for this good suggestion. We are now submitting the code as a plain text and as a .ijm-macro file. We would also like to deposit the code, together with test data and a comprehensive user guide, online. One possibility, as discussed at this year´s ASCB meeting with the editor, would be the new microscopy-focused-website of JCS, which is currently under construction at the Company of

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Biologists. Another possibility would be an online depository such as GitHub. For a final decision, we would be grateful if the JCS editorial office would indicate a preferred site of deposition.

4) Lines 114 - 115: I disagree that Poji is suitable for the analysis of super-resolution data derived from STORM or PALM experiments - the output from such experiments is typically a list of point localisations, not an image.
We agree with the reviewer and have removed this part.

5) Lines 128 - 130: This seems a little time-consuming and unnecessary - again, splitting channels is something easily automated within a script.
Thank you for this suggestion. We also realized that manual splitting of channels is more time-consuming. Therefore, we initially inserted a piece of code to automatically split and save the fluorescent channels. Ultimately, however, we decided against adding this function as a fixed feature of the macro, as it limits the amount of different analyses that can be conducted by Poji. As the macro uses the podosome core channel to define podosome localization, the performance of the analysis depends, of course, on the quality of the corresponding images. If this channel is always used unaltered, due to an automatic splitting in the main Poji macro, easily avoidable problems can occur. First, since certain experiments are designed to compare fluorescence intensity levels, it might be necessary to acquire images at the same settings throughout several biological conditions. This can ultimately lead to a decrease in imaging quality of the core channel and thus reduce the podosome detection performance. By manually splitting the channels, it is possible to duplicate the data and thus measure the core channel twice in the same analysis, once as reference channel after preprocessing to ensure optimal podosome detection, and once as original images that are used for comparing fluorescence intensities. Furthermore, manual splitting allows for a better analysis of stacks. As z stacks are analysed plane by plane, the number and intensity of podosomes can change throughout the stack, leading to potentially wrong results. Splitting prior to analysis again enables the creation of a reference channel (where the core channel from one plane is duplicated several times to match the number of z planes in the stack. Poji then refers to always the same image as reference for identical podosome detection throughout the stack, while it simultaneously analyses the core channel in its individual planes). Both experimental designs are frequently used in podosomes analysis. It would thus be very difficult, if not impossible, to conduct respective analyses with a Poji version that had the splitting of channels and planes included by default.
Still, we think the reviewer has a raised a valid point, and we now also include the small code to split and save channels, as an optional feature of the Poji macro. We also added information about the necessity to spilt channels, as well as about the possibility to automate this with the additional code to the overview section on p.6.

6) Lines 150 - 153: I appreciate that this is somewhat related to the means used to detect the podosomes (local maxima detection), but the lack of sub-pixel registration at this stage, prior to averaging, likely results in a lower resolution intensity profile than could otherwise be achieved.
To address the potential impact of sub-pixel registration, we have enlarged images by 3x3 prior to podosome detection to include a sub-pixel registration to podosomes, with averaging and calculated respective intensity profiles afterwards, to avoid loss of profile resolution. However, image enlargement results in artefacts, as F-actin profiles show artificial enhancement of central maxima. To illustrate this point, we include Figure 1 for referees, which shows fluorescence intensity profiles of F-actin, myosin IIA and vinculin, one according to the original Poji profile, and one according to the profile from an enlarged image. Sub-pixel registration, therefore, does not seem to give a significant advantage over regular detection of podosomes. We thus decided not to implement a respective feature in the Poji macro, which also results in a more streamlined analysis. We hope the reviewer agrees with us on this point.

7) Lines 165 - 166: The authors seem to have quantified the number of "false positives" returned by Poji, but not the number of "false negatives"?
Thank you for this good point. We have now included both false positive and false negative analysis, with both absolute numbers and percentages, in the new Figure 6.

8) Lines 194 - 196: This is worded in a confusing manner. I believe the authors are referring to the binning of podosome intensities for the purposes of constructing a histogram?
We agree with the reviewer that this section lacked clarity. We have now rephrased this section in both manuscript and figure legends. See also Reviewer 1, comment 9.

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9) Lines 198 - 201: This seems to be an assumption - was it actually quantified? It would not be difficult to relate intensities of detected podosomes to their position within the cell.
This is a very good point. Therefore, we now added an analysis of myosin IIA fluorescence intensities for podosomes under the nucleus, as well as for all podosomes, in the new Supplementary Figure 1. The respective graph shows clearly that the low intensity peak of myosin IIA-based fluorescence is mostly due to the podosomes located under the nucleus
See also reviewer 1, point 10

10) Lines 204 - 206: The description of myosin IIA distribution in blebbistatin-treated cells here ("loss of the pronounced myosin IIA network in the cell periphery") seems to contradict what was said earlier (Lines 186 - 187: "in the blebbistatin-treated cells, myosin II was absent from a large part of the cell, and was present mostly in the cell periphery").
In human macrophages, myosin IIA is mostly present as a striated network in the cell periphery (Fig. 2A). This network is disrupted upon addition of blebbistatin. Instead, myosin IIA becomes more pronounced around single podosomes (Fig. 2B). Please note that the peripheral striated myosin IIA network is not directly associated with podosomes, as mentioned. We have now rearranged this part for clarity.

11) Lines 262 - 265: If cell size and podosome-covered area are similar across all conditions, then how can thepodosome density be significantly different?
There is a trend in cells seeded on matrices of 7 and 04.kPa for lower podosome numbers that are significantly different from those of cells on higher stiffness (previous Fig. 4A). In the calculation of podosome density, this resulted in significant differences of podosome density (previous Fig. 4B). However, the previous Fig. 4, originally intended to show that the macro can be used for the analysis of multiple cells, has now been removed, as this ability is now demonstrated by the new Figure 3 and the analysis of multiple planes in Figure 4 (see reviewer 1, points 11 and 14).

12) Lines 339 - 347: The authors reveal a significant shortcoming of their analysis here - that is, the distribution of protein enrichment around a podosome may not be homogeneous or symmetrical and orientation may vary from one podosome to another. Averaging profiles together will therefore mask this inhomogeneity. While the authors are aware of this limitation, their solution seems to be to systematically enforce symmetry? Perhaps a better solution would be to assume inhomogeneous distributions and attempt to align profiles by rotation, prior to averaging?
The reviewer raises an important point. Podosome biology is complex and is associated with a certain degree of variability in morphology. First, podosomes in macrophages come in two flavors, larger and more dynamic precursors in the cell periphery and smaller and less dynamic successor podosomes in the inner parts of the cell (Evans et al., JCB, 2003; Kopp et al., JCS, 2006). This is also addressed in the paragraph relating to Figure 7C-E, where we point out the potential importance of analysing this heterogeneity. Importantly, we have now added a feature to analyse not only the whole cell area, but also to define ROIs within the cell, and this feature can also be used to differentially analyse these heterogeneous subpopulations (see also point 7 of reviewer 1). Further, it is possible to also analyse single podosomes without averaging, as shown in the new Figure 4.
Second, podosomes are not static but undergo oscillation and also fusion and fission events, which leads to differences in size and/or asymmetrical or bi-lobed appearance over time. Following these dynamics in live cell imaging can give important insights into podosome regulation. However, these are transient events that occur to the same degree within a specific subpopulation at a given time point, and the general composition of a subpopulation remains unchanged over time. The usefulness of the Poji macro lies in its ability to simultaneously analyse a high number of podosomes, thus revealing the general composition/architecture of podosomes, while live cell imaging of a limited number of podosomes is suitable to detect inhomogeneities over time. For this reason, generation of a symmetrical profile by the Poji macro as a description of average podosome morphology seems appropriate. Indeed, symmetrical profiles of podosomes have, therefore, been used in all recent attempts of automated podosomes analysis or podosome modelling (Bouissou et al., ACS Nano, 2017; Joosten et al., Front Immunol, 2018).

13) Lines 348 - 411: I'm not sure the discussion adds a whole lot to the manuscript - it seems to be mostly repeating what has been stated previously.
We agree with the reviewer and have now extensively reworked the discussion section. We included

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new discussion points on problem solving using the macro (pitfalls and solutions), on the implications of the altered distribution of vinculin and LSP1 following treatment with cytochalasin D or ionomycin, respectively, as well as the newly included analysis of invadopodia and associated matrix degradation.

14) Lines 372 - 376: Again, this seems cumbersome and unnecessary - it's relatively easy to check automatically what the bit-depth of an image is or what the maximum possible pixel value in a given image is. Asking a user to modify values within the script seems unwise and will likely cause problems.
We agree with the reviewer and added now added a function that automatically 1) detects the bit-depth of supported images (8- and 16-bit) and 2) determines the pixel range for normalisation. If an unsupported image (e.g. 32-bit or RGB) is analysed, the macro stops and returns an error message

15) Lines 410 - 411: I strongly disagree with the final line here - the "well-honed eyes of experienced researchers" are notoriously biased!
We do understand the reviewer´s concern. Researchers are indeed biased, despite their best efforts. Still, as the analysis of false positives and negatives (Fig. 6) shows, even a complex macro is not able to take into account all intricacies that enable the unequivocal identification of podosomes or other structures that are easily identified by an experienced researcher. It needs both machine code for high throughput and re-checking by a researcher to achieve optimal results. We have now rephrased the last paragraph as "Still, it should be kept in mind that the individual variety between podosomes is higher than average values or profiles can depict. It is thus possible that individual differences, for example indicating podosome subgroups, get masked by use of the macro. Despite having a human bias, the well-honed eye of a researcher thus remains a vital resource that can not be fully replaced by even the best tool." We hope the reviewer agrees with us on this point.

16) Line 434: Unless I am very much mistaken, the authors never specify which secondary antibodies (which fluorophores) they have used?
We have now added also secondary antibodies to both the Materials and Methods section as well as to the figure legends.

Reviewer 3
Podosomes represent a subgroup of cell-matrix contacts, which is especially prominent in cells of the monocytic lineage such as monocytes, macrophages and dendritic cells. . The analysis of podosomes can be challenging because these structures can be densely displayed on the ventral surface. Podosome number, distribution and composition can be affected by experimental treatments - and thus a tool to investigate podosomes in an unbiased fashion is useful. The authors present a Fiji-based macro code termed "Poji" (podosome analysis by Fiji), and provide the plugin code which they suggest is superior to existing routines - in order to characterize a variety of cellular and podosomal parameters. For example fluorescence intensity and relative enrichment in podosomal versus cytoplasm or cell membrane.
Although the manuscript does not seem to contain new findings regarding podosome biogenesis or composition - based on use of the plugin - there is some merit in characterizing what the software can achieve and making the software available.

Thank you for the positive comments on the macro. In addition to the description of the macro, we are now also presenting additional information on podosome composition in response to treatment with cytochalasin D (new Fig. 3) and ionomycin (new Fig. 4). Using Poji, we show that LSP1 is lost from podosomes in all analysed confocal planes upon treatment with ionomycin. This is the first indication that a podosome cap protein can be selectively removed by pharmacological means. It is also the first report that podosome architecture can be influenced by ionomycin. Poji-based analysis of several confocal planes thus appears to be particularly suitable for further investigation of podosome cap proteins.

The authors should supply the image files for Fig. 1B, 2A and B, 3A and 5 A, B, G. These are needed to evaluate if the results obtainable with their software is indeed superior to pre-existing versions mentioned.
The image files have now been added to the Supplementary material.

The authors look at two conditions which have been investigated in some detail previously, namely

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changes in mysoin II contractility and different substrate rigidity.

1. Application 1. In the former case the authors are using a low concentration of blebbistatin (10 um) which really should be shown on figures or in the legend. It is hardly surprising that little change is noted under their experimental conditions. The role of myosin IIA in podosome dynamics is complex question and it would be nice if authors can provide some new insights (using their software) on this issue. There also needs to be more balanced analysis of existing literature for example recent papers, show that myosin IIA and contractility plays inhibitory rather than stimulatory role in podosome formation. cf. Rafig, et al., 2019,; Dulyaninova, et al., 2018, Cervero, et al., 2018 (this paper by the author is mentioned) and van den Dries et al., 2013.

We fully agree with the reviewer that the role of myosin II is likely more inhibitory. In fact, we also cited our paper (Bhuwania et al., JCS, 2013), where this is already part of the title: "…myosin-dependent contractility…enables podosome turnover". We have now added a paragraph on the role of myosin II to the discussion (p.16) and added the respective citations to the reference list.

2. Regarding the limitations of blebbistatin treatment, I would suggest alternate method(s) to interfer with the podosomes associated acto-mysoin complex such as inhibition of Rho/ROCK pathways (van Helden, 2008,). The authors are using what likely produces a slight inhibition of ATPase activity by blebbistatin - more convincing data might be forthcoming if they rather completely inhibited ROCK. Perhaps the object of using low levels of myosin II inhibitor is to demonstrate the software is more "sensitive" versus the existing Poji-like macros. Nonetheless it is not clear what this redistribution of myosin IIA represents (ie. to what extent there is disassembly of myosin IIA filaments under these conditions?). If the authors have access to structured illumination super-resolution technology it would be simple to get much improved micrographs of myosin IIA (Fig. 2A and B) in order to properly discuss alteration in "ordered' (line 185) myosin IIA distribution after blebbistatin treatment.

Redistribution of myosin IIA upon blebbistatin treatment was mostly included to show that also more global cellular alterations in protein distribution and fluorescence intensity can be detected by the macro. As the reviewer points out, podosome-associated myosin IIA can only be fully visualized by the use of superresolution microscopy such as SIM. However, some of the potential users most probably lack access to SIM. As a more accessible example for Poji analysis of protein re-distribution, we are now including new data on vinculin loss from the podosome ring upon treatment with cytochalasin D (new Fig. 3) and of loss of LSP1 from the cap upon ionomycin treatment (new Fig. 4). Especially the loss of LSP1 from its localization at the cap due to ionomycin treatment, and thus the selective influence of Ca2+ on podosome composition, provides novel information on podosome regulation and composition (see also reviewer 1, point 11). This is now also discussed in more detail (p.17).

3. In application 2. The authors look at the protein LSP1 which they have previously characterized in some detail. There does not seem to be any specific advantage in the analysis of LSP1 (in a different focal plane) using Poji since a few much better resolution images would be of much more value in assessing the Z 'position' of various podosome components.

This example was included to indicate that analysis by Poji can be performed in multiple planes of imaging, which is necessary to detect changes in the relative composition of numerous podosomes in the z-axis. We think it is worthwhile to point this out, as it might not immediately occur to everyone who wants to image podosomes in a high-throughput mode.

4. In Application 3 the authors investigate the effects of substrate rigidity on parameters extracted by Poji. Podosome formation can efficiently proceed in cells plated on fluid- supported lipid bilayer membrane, a substrate which does not permit development of traction forces exerted on integrin clusters. Thus, podosomes appear to self-assemble by default under conditions of deprivation of traction forces (Yu, et al., 2013). In view of these results, it is rather surprising to see (Fig. 4) that number of podosomes, podosomal area and subsequently podosome density significantly decreased on less rigid matrices (Fig. 4A-C). Additional literature (Yu et al., 2013, Changede, et al., 2015) should be taken into consideration and explanation provided. As it is I am not sure if this is an interesting new finding (in this cell type).

We agree with the reviewer that this finding would need more in-depth investigating. As critical points were also raised by the other reviewers (reviewer 1, point 14; reviewer 2, point 11), we have now removed the part on Application 3 on matrices of different stiffness, including the previous Figure 4 from the manuscript.

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We are grateful for the time and energy that the referees have invested in the review of our manuscript. We hope that the current version is now suitable for publication in *Journal of Cell Science*.

Kind regards,
Stefan Linder, on behalf of all coauthors

Second decision letter

MS ID#: JOCES/2019/238964

MS TITLE: Poji: a Fiji-based tool for analysis of podosomes and associated proteins

AUTHORS: Robert Herzog, Koen van den Dries, Pasquale Cervero, and Stefan Linder
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.organd 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 more favourable reports but also had mixed responses with reviewer 2 indicating that the study is not appropriate for publication. I think reviewer 2 and 3 still raise some very valid points that will need to be addressed including clarifications to the text. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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

The authors have put in a serious effort to revise this according to the referees comments and I think that they have made all of the major improvements/ revisions suggested. I have no further requests for revision. Poji will be an interesting tool for the community and should find multiple uses for analysis of podosomes and invadopodia.

Comments for the author

n/a

Reviewer 2

Advance summary and potential significance to field

The authors present a tool, Poji, implemented as a FIJI script, for the analysis of protein enrichment at podosomes. A description of the workflow is presented, along with several "proof of

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concept" analyses. I appreciate that the authors have gone to great lengths to address the points raised by the reviewers. However, several of my previously-stated concerns remain, particular those related to the necessity for user intervention when using Poji - this, in my opinion, places severe limitations on its utility.

Comments for the author

Specific points:

L 111 - 112: Poji cannot reasonably be described as automatic, as significant user-intervention is required during analysis.

L 141 - 148: All of this could be made a whole lot easier by including a preview button on the dialog, allowing users to test their specified parameters within Poji

L 149 - 152: If two cells within the same experiment have been analysed using completely different parameters, then how can the results obtained from those two cells be considered in any way comparable?

L 259 - 260: I don't know what the statement in brackets means?

L 326 - 329: I'm not sure about this - looks to me like actin and vinculin recruitment is reduced in Fig 4D

L 222 - 225: If, as the authors claim, most of the myosin IIA measured as being associated with podosomes is not actually functionally associated with podosomes, doesn't that suggest that inhibition of myosin IIA is a poor choice of experiment for the purposes of demonstrating Poji? Doesn't it place a big question mark over the usefulness of the data in Figure 2, from the point of view of assessing the utility of Poji?

L 536 - 537: A tool that requires optimisation on a cell-by-cell basis really cannot be considered suitable for "fast analysis of high numbers of cells"

Responses to points in rebuttal:

1. I think the authors have misunderstood - they have implemented functionality to allow the user to manually define their cell. I suggested implementing an automated approach to cell detection? While it's possible for the user to generate ROIs defining cell boundaries, I'm struggling to understand why the authors are unwilling to incorporate this functionality? It would surely increase throughput while reducing bias?

2. With regard to the online hosting of the code, there's no reason why it should only be hosted in one place? I would suggest GitHub as the default option, but a copy can also be uploaded to the JCS website. That way, development of the GitHub-hosted version can continue, safe in the knowledge that JCS has a copy of the version used for this manuscript. Another option is Zenodo (https://zenodo.org), which generates a DOI for datasets and software (and also integrates with GitHub, I believe).

5. I don't really understand what the authors are saying here, but appreciate that the option to automate the splitting of channels is now included.

6. Again, I don't really understand what the authors have done here? Registering profiles prior to averaging does not necessitate enlarging the images?

10. See point above about lines 222 - 225.

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Reviewer 3

Advance summary and potential significance to field

Podosomes represent a subgroup of cell-matrix contacts, which is especially prominent in cells of the monocytic lineage such as monocytes, macrophages and dendritic cells. . The analysis of podosomes can be challenging because these structures can be densely displayed on the ventral surface. Podosome number, distribution and composition can be affected by experimental treatments - and thus a tool to investigate podosomes in an unbiased fashion is useful. The authors present a Fiji-based macro code termed "Poji" (podosome analysis by Fiji), and provide the plugin code which they suggest is superior to existing routines - in order to characterize a variety of cellular and podosomal parameters. Fo example fluorescence intensity and relative enrichment in podosomal versus cytoplasm or cell membrane.

In this new version of the manuscript the authors have removed some controversial data regarding the role of myosin II. The software has now been tested, and found suitable for analysis of podosome biogenesis or composition. It has been noted that the plugin needs to be made Mac compatible. In summary there us merit in characterizing what this software can achieve, and making the software available.

Suggestions for authors are in file Poji R2.pdf

Comments for the author

In this new version of the MS the authors have made extensive changes, largely in line with various reviewers' comments. Their rebuttal (blue) to our points (italic) are provided below, & I have added some notes thereafter (red). The additional points 6-14 will need to be addressed in revision.

(1) The authors should supply the image files for Fig. 1B, 2A and B, 3A and 5 A, B, G. These are needed to evaluate if the results obtainable with their software is indeed superior to pre- existing versions mentioned.

The image files have now been added to the Supplementary material.

1a. Having downloaded these files, neither Poji and 'Split and Save Channels' Macros would run. We have resolved this Mac incompatability - because the file separator “\\”, is Windows specific. In order to enable macros using Windows and Mac platforms “\\” should be changed to “/”. This is a relatively trivial but important change to the code. We have now been able to confirm the utility of the macros.

1b. After implementing changes both macros performed as expected. In the revision the 'Split and Save Channels' **macro** added as additional automated step. Please the corresponding work flow box as black since now it is automated step (instead of blue, which defined as manual step in legend) (Fig.1A).

(2) We fully agree with the reviewer that the role of myosin II is likely more inhibitory. In fact, we also cited our paper (Bhuwania et al., JCS, 2013), where this is already part of the title: “...myosin-dependent contractility...enables podosome turnover”. We have now added a paragraph on the role of myosin II to the discussion (p.16) and added the respective citations to the reference list. Good.

(3) In application 2. The authors look at the protein LSP1 which they have previously characterized in some detail. There does not seem to be any specific advantage in the analysis of LSP1 (in a different focal plane) using Poji since a few much better resolution images would be of much more value in assessing the Z 'position' of various podosome components.

This example was included to indicate that analysis by Poji can be performed in multiple planes of imaging, which is necessary to detect changes in the relative composition of numerous podosomes in the z-axis. We think it is worthwhile to point this out, as it might not immediately occur to everyone who wants to image podosomes in a high-throughput mode.

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Good. In data used to illustrate applications it is not clear whether various z-projection were used, or if analysis was performed for only one z-plane. I suggest authors should clearly specify this in each figure legends for easy reference.

(4) We agree with the reviewer that this finding would need more in-depth investigating. As critical points were also raised by the other reviewers (reviewer 1, point 14; reviewer 2, point 11), we have now removed the part on Application 3 on matrices of different stiffness, including the previous Figure 4 from the manuscript.

Good. Removing data on substrate rigidity (in response to our comments) as these were divergent with literature data.

5. The authors wrote: “The manuscript is now structured in a way that we first show the ability of the macro to report fluorescence intensities and distribution of proteins in a single cell (Fig 2), and then in multiple cells (new Fig 3) as well as in different z planes (new Fig. 4), and finally also for invadopodia in cancer cells (new Fig 5).”

It will be useful to mention the structure of manuscript in introduction, so reader would also follow these reasonings.

Other points relating to missing information or clarity.

6. **Line 217**. I suggest not use “absent” on Fig. 2B, since myosin IIA still present at cell edge and at the lower part of the cell.

7. **Fig. 2.** Frankly myosin IIA here looks like just background, I would suggest to remove myosin IIA images and corresponding analysis from this figure.

8. **Fig. 3 - 7**. The information on actin marker fluorophores and secondary antibodies are missing from figure legends. Material and methods section does not provide clear reference on which exactly fluorophore conjugated phalloidin or secondary antibody was used in each case.

9. **Lines 266, 271**. Application 2. The fact that cytochalasin D does not disrupt F-actin is rather surprising. Cytochalasin D is a potent inhibitor of actin polymerization, so unless the F-actin is not dynamic, cytochalasin D should affect this unless used at a lower level than normal (Fig. 3B). The authors can provide referencing for this if already published.

10. **Line 285.** Full name shall be used for “LSP1” with abbreviation in brackets, namely “Lymphocyte specific protein 1 (LSP1)”, the first time mentioned on line 58.

11. **Fig. 4.** Check bars on A and B, they have different lengths.

12. **Fig. 4.** The LSP1 ring-like structures are somewhat visible on representative images of 0.5 and 0 µm planes planes in A, which described in text (Lines 290 – 292). Poji analysis does not pick up ring-like structures for 0.5 µm plane at all and shows very slight dent in 0 µm plane graph. This discrepancy shall be addressed.

13. **Lines 306-307**. “with a local minimum in the center of the podosomes, corresponding to a more ring-like distribution (Fig. 4C)”. To make this dent a little bit more visible consider to change graph y axis dimensions.

14. **Fig. 6**. Legend. No scale bar information.

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Second revision

Author response to reviewers' comments

Dear Dr. Way, dear Michael,

We are submitting a revised version of our manuscript entitled "Poji: a Fiji-based tool for analysis of podosomes and associated proteins", and ask you to consider its suitability for *Journal of Cell Science*. We are very grateful for the reviewers´ constructive comments and also for your editorial guidance and have tried to address the raised points as closely as possible. Please find a point-by-point response below. The respective changes are marked in red in the manuscript.

Reviewer 1

The authors have put in a serious effort to revise this according to the referees comments and I think that they have made all of the major improvements/ revisions suggested. I have no further requests for revision. Poji will be an interesting tool for the community and should find multiple uses for analysis of podosomes and invadopodia.

Thank you for your positive comments and the approval of the revised version.

Reviewer 2

1) L 111 - 112: Poji cannot reasonably be described as automatic, as significant user- intervention is required during analysis.

We now have amended "automatic" to "semi-automatic" when referring to Poji).

2) L 141 - 148: All of this could be made a whole lot easier by including a preview button on the dialog, allowing users to test their specified parameters within Poji

Thank you for this good suggestion. We have now included a preview button for a more convenient optimization of detection parameters. This additional feature is now also mentioned in the text (lines 151-152) and described in the user guide.

3) L 149 - 152: If two cells within the same experiment have been analysed using completely different parameters, then how can the results obtained from those two cells be considered in any way comparable?

The reviewer raises a very important point here, as comparability of data is the backbone of every tool. As we stated in the revised manuscript, we prioritized minimal false-positive and false-negative rates over consistent detection parameters. Podosomes of different cells, but within the same image, can vary in intensity and size, which has a serious impact on the local maximum recognition that is used by Poji to detect podosomes. Also, using just one static noise threshold for the maxima detection may be sufficient to detect the podosomes in one cell, but can result in a dramatically decreased recognition performance for the other cells. To illustrate this, we included a table for referees where we listed the results of the Poji analysis of four different cells within the same image with six different noise threshold values and compared it to the manual confirmation of one expert to determine false-positive and false-negative rates. The results show that none of the six randomly chosen noise values enable optimal detection conditions for all four cells simultaneously. As shown in the table, when different conditions were used for individual cells, the rates of false-positive and false-negative signals were more comparable between all cells, leading to a more consistent result compared to keeping analysis conditions identical. Yet, other parameters like the size for profile calculations, should indeed be kept constant to ensure comparability. We have now clearly pointed this out in the manuscript (lines 156-158). Moreover, should the user still wish to keep all analysis parameters constant, Poji does offer this option in the macro interface.

3) L 259 - 260: I don't know what the statement in brackets means?

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We have now removed the statement in brackets, which pointed to the respective steps of the macro depicted in Fig.1, as it is not necessary for understanding of the text.

4) L 326 - 329: I'm not sure about this - looks to me like actin and vinculin recruitment is reduced in Fig 4D

We are now stating that both F-actin and vinculin show their typical distribution, although at slightly reduced values (line 322).

5) L 222 - 225: If, as the authors claim, most of the myosin IIA measured as being associated with podosomes is not actually functionally associated with podosomes, doesn't that suggest that inhibition of myosin IIA is a poor choice of experiment for the purposes of demonstrating Poji? Doesn't it place a big question mark over the usefulness of the data in Figure 2, from the point of view of assessing the utility of Poji?

Figure 2 was included to illustrate the point that Poji-based analysis can yield detailed information on fluorescence intensity distribution of proteins, both at podosomes, but also in the context of the whole cell. We think this is a valuable feature of the macro that is worth demonstrating.

6) L 536 - 537: A tool that requires optimisation on a cell-by-cell basis really cannot be considered suitable for "fast analysis of high numbers of cells"

We have now rephrased this sentence, stating that Poji „…enables the analysis of statistically relevant numbers of cells and invadosomes.“. Still (see point 3), if a user decides that detection conditions can be kept constant, analysis speed is further increased.

Responses to points in rebuttal:
1. I think the authors have misunderstood - they have implemented functionality to allow the user to manually define their cell. I suggested implementing an automated approach to cell detection? While it's possible for the user to generate ROIs defining cell boundaries, I'm struggling to understand why the authors are unwilling to incorporate this functionality? It would surely increase throughput while reducing bias?

We do follow the reviewer´s reasoning and have indeed considered including this function, which is also present in a previous macro used for podosome detection (Cervero et al., 2013). However, we found that automatic detection of macrophages does not necessarily reduce bias, as overlapping cells were counted as a single cell, and vice versa, single cells with several podosome clusters were counted as several cells. Also, apoptotic/contracted cells were included in the analysis, leading to skewed values. Thus, for automatic cell detection to perform optimally, several parameters including thresholding, binary masks, and watershedding have to be adjusted, previewed and confirmed by the user and potentially corrected for individual cells before proceeding. We tried to keep this step as simple as possible, as some users may have limited experience with Fiji. Moreover, definition of podosome clusters, which we included into the new version of Poji, still has to be done manually. We therefore decided to not automate cell detection.
While we do recognise the existence of other automated cell segmentation tools that require less user interaction, we preferred keeping Poji independent of any other tool, to enable easier utilization. Still, manual detection of cell areas is technically not mandatory, as it is possible to load pre-defined ROIs into the Poji analysis. Cell detection can thus be automated by using pre- existing tools of personal preference with the output being used in Poji. This possibility is already mentioned in the user guide

2) With regard to the online hosting of the code, there's no reason why it should only be hosted in one place? I would suggest GitHub as the default option, but a copy can also be uploaded to the JCS website. That way, development of the GitHub-hosted version can continue, safe in the knowledge that JCS has a copy of the version used for this manuscript. Another option is Zenodo (https://zenodo.org), which generates a DOI for datasets and software (and also integrates with GitHub, I believe).

The reviewer makes a good point and we thank them for their suggestions. Following acceptance, we will upload the Poji code and user guide on GitHub, and also on the JCS microscopy website, at

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the journal´s discretion.

5. I don't really understand what the authors are saying here, but appreciate that the option to automate the splitting of channels is now included.

Thank you for your good suggestion for adding the script to split channels. We refer to the fact that there is an option for automatic splitting, although this has not been included in the Poji code, as splitting has to be done prior to starting the analysis. We rephrased the section slightly, to point this out more clearly (lines 125-126).

6. Again, I don't really understand what the authors have done here? Registering profiles prior to averaging does not necessitate enlarging the images?
We are sorry for not having explained this clearly before. We also took this opportunity to adjust the Poji code slightly, in order to ensure correct subpixel resolution of profile analysis. First, we have now included the command in Poji to activate the Fiji plot option to interpolate the profile line in sub-pixel resolution, so that each rotation of the profile line returns more precise values. In previous analyses, we manually activated this plot option in Fiji.
Second, we also altered the profile analysis method slightly, to enable correct translation of sub-pixel values from the profile line to the results table (Please note that the results obtained by the older version of Poji are still viable. The results of both versions were compared, and results of the newer version differed only marginally from the older version). Thus, subpixel resolution of all profiles is automatically enabled and correctly measured in the latest version of Poji.
Third, we already included the option to create profiles of individual podosomes (see Fig. 4 and 5) in the revised version of both code and manuscript, to circumvent the limitation of only obtaining values after averaging. In the current version, we have now also added the function to save the results of single rotations of the profile line in both individual podosome analysis and profile analysis after average intensity z projection. While this option is not automatically activated (as it would increase size of the results for a small and little-used feature), it can be used to obtain detailed information of the analysis. This not only makes the analysis more transparent, as additional results are always given out as single values, together with mean and standard deviation, but it also enables the user to choose the preferred level of analysis precision, as not only the mean of all rotations in one average z projection, but also results of individual podosomes and even single rotations of the profile analysis can be obtained and used. We hope that these changes are seen as useful additions to Poji.

10. See point above about lines 222 – 225

Please see point 5 above.

Reviewer 3
In this new version of the MS the authors have made extensive changes, largely in line with various reviewers' comments. Their rebuttal (blue) to our points (italic) are provided below, & I have added some notes thereafter (red). The additional points 6-14 will need to be addressed in revision.

(1) The authors should supply the image files for Fig. 1B, 2A and B, 3A and 5 A, B, G. These are needed to evaluate if the results obtainable with their software is indeed superior to preexisting versions mentioned.
The image files have now been added to the Supplementary material.
1a. Having downloaded these files, neither Poji and 'Split and Save Channels' Macros would run. We have resolved this Mac incompatability - because the file separator "\\", is Windows specific. In order to enable macros using Windows and Mac platforms "\\" should be changed to "/". This is a relatively trivial but important change to the code. We have now been able to confirm the utility of the macros.

Thank you for taking the time to test the macros. Compatibility with Mac platforms is an important point and will make the macros useful to a much wider community. The file separator has how been changed in the codes, and we have tested the new Poji version on both Windows and Mac. Another slight incompatibility was detected (the header of the dialog windows to define location of the analysed images can disappear) and is now mentioned in the userguide.

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1b. After implementing changes both macros performed as expected. In the revision the 'Split and Save Channels' macro added as additional automated step. Please the corresponding work flow box as black since now it is automated step (instead of blue, which defined as manual step in legend) (Fig.1A).

Thank you for pointing this out. We took your comment as opportunity to slightly adjust the colours and legend of Figure 1. The colour of the mentioned box was not changed, but the blue colour now represents “user-dependent” steps, in contrast to the analysis that is automatically conducted (now represented by black, red and green boxes).

(2) We fully agree with the reviewer that the role of myosin II is likely more inhibitory. In fact, we also cited our paper (Bhuwania et al., JCS, 2013), where this is already part of the title: “...myosin-dependent contractility...enables podosome turnover”. We have now added a paragraph on the role of myosin II to the discussion (p.16) and added the respective citations to the reference list.
Good.

Thank you

(3) In application 2. The authors look at the protein LSP1 which they have previously characterized in some detail. There does not seem to be any specific advantage in the analysis of LSP1 (in a different focal plane) using Poji since a few much better resolution images would be of much more value in assessing the Z 'position' of various podosome components.
This example was included to indicate that analysis by Poji can be performed in multiple planes of imaging, which is necessary to detect changes in the relative composition of numerous podosomes in the z-axis. We think it is worthwhile to point this out, as it might not immediately occur to everyone who wants to image podosomes in a high-throughput mode.
Good. In data used to illustrate applications it is not clear whether various z-projection were used, or if analysis was performed for only one z-plane. I suggest authors should clearly specify this in each figure legends for easy reference.

We are now stating in the legend of Fig. 4C,D: “Profiles were calculated for all podosomes in ROIs of both cells individually, with mean ± SEM of projections of all podosome profiles shown (at z planes of 1 µm, 0.5 µm, and 0 µm distance to the most ventral F-actin signal of podosomes…)”

(4) We agree with the reviewer that this finding would need more in-depth investigating. As critical points were also raised by the other reviewers (reviewer 1, point 14; reviewer 2, point 11), we have now removed the part on Application 3 on matrices of different stiffness, including the previous Figure 4 from the manuscript.
Good. Removing data on substrate rigidity (in response to our comments) as these were divergent with literature data.

We agree with the reviewer.

5. The authors wrote: “The manuscript is now structured in a way that we first show the ability of the macro to report fluorescence intensities and distribution of proteins in a single cell (Fig 2), and then in multiple cells (new Fig 3) as well as in different z planes (new Fig.4), and finally also for invadopodia in cancer cells (new Fig 5).”
It will be useful to mention the structure of manuscript in introduction, so reader would also follow these reasonings.

Thank you for this good suggestion. We are now mentioning the structure of the manuscript in the overview (lines 192-196).

6. Line 217. I suggest not use “absent” on Fig. 2B, since myosin IIA still present at cell edge and at the lower part of the cell.

We have now rephrased this as „..the striated pattern of myosin II was no longer visible, and myosin II was mostly present at the cell periphery and the trailing edge of the cell (lower part of cell in Fig. 2B).“ (lines 209-210)

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7. Fig. 2. Frankly myosin IIA here looks like just background, I would suggest to remove myosin IIA images and corresponding analysis from this figure.

Figure 2 was included to illustrate the point that Poji-based analysis can yield detailed information on fluorescence intensity distribution of proteins, both at podosomes, but also in the context of the whole cell. We think this is a valuable feature of the macro that is worth demonstrating. We would thus suggest to keep Fig. 2 as part of the manuscript. Please also see point 5 of reviewer 2.

8. Fig. 3 - 7. The information on actin marker fluorophores and secondary antibodies are missing from figure legends. Material and methods section does not provide clear reference on which exactly fluorophore conjugated phalloidin or secondary antibody was used in each case.

We apologize for this. Information on fluorophores and antibodies was removed by us during the quality control step after resubmission to the JCS office, as the manuscript exceeded the word limit. This information has now been re-added, and we tried to shorten the text of the introduction to not exceed the word limit again.

9. Lines 266, 271. Application 2. The fact that cytochalasin D does not disrupt F-actin is rather surprising. Cytochalasin D is a potent inhibitor of actin polymerization, so unless the F- actin is not dynamic, cytochalasin D should affect this unless used at a lower level than normal (Fig. 3B). The authors can provide referencing for this if already published.

The results shown are in line with a previous report (van den Dries et al. 2013a), as mentioned in the Results (p.10) and Dsiscussion section (p.16). Addition of 2 microM cytochalasin D does not disrupt podosomes immediately, but first stops podosome growth, thus inhitibing the contractile oscillations of the podosome core that exert forces on the lateral cables of podosomes, thus driving mechanosensitive recruitment of vinculin to the ring, as referred to in the discussion. At the dose and time point chosen, this treatment thus primarily affects vinculin recruitment to podosomes.

10. Line 285. Full name shall be used for “LSP1” with abbreviation in brackets, namely “Lymphocyte specific protein 1 (LSP1)”, the first time mentioned on line 58.

This information has now been added (line 56).

11. Fig. 4. Check bars on A and B, they have different lengths.

Thank you for pointing this out. The ROIs shown were of different size, resulting in different lengths of the space bars. We have now adjusted the ROI size. Space bars are now of equal length.

12. Fig. 4. The LSP1 ring-like structures are somewhat visible on representative images of 0.5 and 0 µm planes planes in A, which described in text (Lines 290 – 292). Poji analysis does not pick up ring-like structures for 0.5 µm plane at all and shows very slight dent in 0 µm plane graph. This discrepancy shall be addressed.

We thank the reviewer for this good observation of discrepancy. This apparent discrepancy originated from averaging the results of the two podosome subpopulations, larger precursor and smaller successor podosomes, which were both present in the same cells, thus masking these faint localisation differences. We have now changed the order of the respective results and also introduced new results to address this point. In the last version, we showed a detailed ROI of the cell as a micrograph in Figure 4, together with the data of the whole cell, with the micrograph of the whole cell being shown in Suppl. Figure 2. For the current version, we created profile results of the podosomes shown in ROIs in Figure 4 and included them in this Figure. The results of the whole cells are now shown in Suppl. Figure 2, together with their corresponding micrographs. We also used this new structure to again point to the possibility of local differences being masked by averaging all results (as already mentioned in the chapter “limitations”) (lines 308-311).

13. Lines 306-307. “with a local minimum in the center of the podosomes, corresponding to a more ring-like distribution (Fig. 4C)”. To make this dent a little bit more visible consider to change graph y axis dimensions.

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According to our reply to point 12, we now present data in Figure 4 that show the described LSP1 localisation better than before. We ultimately decided for setting all y axes to the same range of absolute values, as we would like to also show the differences in overall fluorescence intensity, including the slightly reduced localisation of F-actin and vinculin (see also reviewer 2, comment 4).

14. Fig. 6. Legend. No scale bar information.

Scale bar information (10 μm) has now been added.

We are grateful for the time and energy that the referees have invested in the review of our manuscript. We hope that the current version is now suitable for publication in Journal of Cell Science.

Kind regards,

Stefan Linder, on behalf of all coauthors

Third decision letter

MS ID#: JOCES/2019/238964

MS TITLE: Poji: a Fiji-based tool for analysis of podosomes and associated proteins

AUTHORS: Robert Herzog, Koen van den Dries, Pasquale Cervero, and Stefan Linder
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.

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