Ivana Viktorinová

Max Planck Institute of Molecular Cell Biology and Genetics

Pfotenhauerstr. 108

01307 Dresden

Germany

Alisha DSouza  
Senior Review Editor  
JoVE

Cambridge, MA

USA

18th December 2018

Dear Alisha,

Please find attached our revised manuscript Nr. 58587 titled *‘*Analysis of actomyosin dynamics at local cellular and tissue scales in cultured *Drosophila* egg chambers*’* that we resubmit to Journal of Visualized Experiments (JoVE) after the first round of revision.

We would like to thank you, your colleagues and all three reviewers for your and their thorough comments and suggestions. Upon revising our manuscript according to the reviewers’ wishes, we resulted with a protocol that far exceeded the allowed length. This prompted us to restructure the protocol section and place several instructions (dissection and imaging of egg chambers) into the supplement. Therefore, the main part of this revised protocol focuses on actomyosin analysis at the local and tissue scale.

We provide only a few links to publicly accessible git-lab repository (<https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics>) and other external sources. However, this can be re-arranged according to your policies. We hope that through this restructuring, our protocol is now easy to follow and user-friendly to a broad spectrum of readers, even novices.

We also provide high resolution figures. We apologize for the previous issues, which must have happened when converting our files into the formats required by JoVE. Please, let us know in case this remains an issue.

Please, also find attached our detailed responses to the reviewers below.

We thank you again for considering our manuscript for publication and are looking forward to hearing from you.

Yours sincerely,

Ivana Viktorinová and co-authors

CC: [rhaase@mpi-cbg.de](mailto:rhaase@mpi-cbg.de), [pietzsch@mpi-cbg.de](mailto:pietzsch@mpi-cbg.de), [henry@mpi-cbg.de](mailto:henry@mpi-cbg.de), [tomancak@mpi-cbg.de](mailto:tomancak@mpi-cbg.de)  
  
Dear Dr. Viktorinova,  
  
Your manuscript, JoVE58587 Analysis of actomyosin dynamics at cellular and tissue scales in cultured Drosophila egg chambers, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.  
  
After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.  
  
Your revision is due by **Jul 31, 2018**.  
  
To submit a revision, go to the [JoVE submission site](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
Best,  
  
Alisha DSouza, Ph.D.  
Senior Review Editor  
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The manuscript has been modified to include line numbers and minor formatting changes. The updated manuscript **58587\_R0.docx** is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file.**Please download the .docx file and use this updated version for future revisions.** The file is also attached.  
  
You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.  
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.   
  
**Editorial Comments:**  
  
• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.  
  
• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) your protocol steps.**Furthermore, there should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples of missing details:

1) 1.1: Is the fly food placed in a vial? How much per vial? We now provide this info.  
2) 2.2: Remove particles by pouring through the filter? We now state to remove particles by using the filter.  
3) 2.8: Use the word “anesthetized” in place of “sleeping” We now use the word ‘anesthetize’, thank you.  
4) 3.2.1.2: Split channels how? We now describe how to do it.   
5) 3.2.1.2: Merge how? We now also added this information.  
6) 3.2.2.2: Run how? We now provide this info.  
7) Line 234: how? We now describe this.  
8) 3.5: Is this done in FIJI? Yes, all steps are Fiji-based as we state now so.  
9) 3.5.3: how? We now describe this in the note.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.  
ok  
• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
  
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.  
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.  
5) Notes cannot be filmed and should be excluded from highlighting.  
6) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.  
  
• **Discussion:** Please avoid using a number list in this section.  
ok  
• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Millex®, MatTek, CellMask, MatLab, all items listed in the supplementary materials list following the protocol.  
1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.  
  
• **Table of Materials:**Please remove the Supplementary material section from the manuscript and list all materials in the table of materials. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file.   
  
• Please define all abbreviations at first use.  
  
• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.  
  
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**Comments from Peer-Reviewers:**   
  
**Reviewer #1:**  
  
Manuscript Summary:  
An important step early in Drosophila oogenesis is rotation of follicles around their A/P axis. Breaking of Myosin II symmetry leads to directional actomysin activity that generates the forces necessary to drive rotation. Understanding the action of these forces can provide insight into cellular movement in general. Ideally, in vivo analysis at the level of individual cells and/or at a tissue level is required to understand the role actomyosin. The manuscript provides alternate procedures to prepare samples for live imaging in a way that circumvents distortion of cell shape that occur with previously published live imaging methods. Beginning with dissection of ovaries, this procedure describes the isolation of early stage eggs for analysis and employs a coverslip-free method to culture egg chambers during imaging to eliminate potential sources of cellular distortion while minimizing sample movement, thereby providing a more accurate depiction of actin dynamics. It then provides a step-by-step guide in the use of image analysis techniques for the analysis of actomyosin dynamics in time lapse images for both single plane and thick tissue samples. This manuscript is highly suitable for publication in JoVE as both the dissection and image analysis steps will benefit from the video documentation features. However, the manuscript suffers from a lack of clarity about both the rationale and the experimental protocol. Thus, extensive changes are required. Listed below are specific suggestions to assist the authors.  
  
Lack of clarity:  
1) The issue of tissue curvature and the need to correct for this to analyze the image data is not clearly explained. This aspect need to be discussed in the introduction and discussion.

Thank you for this point. We now explain why is that important in the text.  
2) It would be extremely helpful to provide an example data files for the reader to analyze using the methods (one for 3 and one for method 4).

We agree and now provide example data sets to individual steps.  
3) Larger and more detailed figures are needed.

We are sorry that you obtained low resolution data. Our psd files are 600 dpi and generally 3000x5000 pixels, the low resolution must have happen by a conversion of those file. We hope that now we provide high resolution figures that can be easily read.  
4) The output data and their purpose are not clear (i.e. 3.3.1 and 3.3.2). An example of how the data is used would help (i.e. comparison of wild-type and mutant).

We hope that in the revised manuscript it is clear.

5) The image adjustment and analyses the settings used are not explained, nor are alternatives discussed.

We now explain.  
6) What is actually being done in the methods steps are unclear. A short sentence stating something like: Set up an image export path by…..would dramatically improve the clarity of the methods. This type of change is needed throughout.  
We now provide snapshots/screenshots which should help the clarity and detail action guidance.

Specific comments:  
1.Introduction  
Line 82: change media to medium

Thank you. We have corrected as suggested.  
Line 100: In relation to the discussion of pressure in other live imaging mounting techniques, it seems important to consider that the egg chambers are normally under pressure from the muscle sheath and adjacent chambers. Thus, no pressure may be artificial.

We believe that classical glass cover slips provide a different type of pressure than surrounding soft muscle sheet and/or adjacent chambers. In fact, the question is whether egg chambers are really pressed in ovaries. One has also to take into a consideration that glass material is not present in ovaries and may have a certain antistatic properties. Nevertheless, we agree with the reviewer’s point that certain pressure might be ok and actually cause no harm to egg chambers. However, it would be necessary to empirically test to prove/disprove that no pressure is artificial in this case.

Line 102: change life to live  
Thank you for finding this typo. Now, it is corrected.

2.Protocol  
  
For this section, it might be useful to have a figure that shows what ovaries look like and a schematic of the steps in dissection, particularly ovarioles with and without muscle sheath.  
Thank you. We describe it hopefully better in detail.  
Line 135: How are you taking the female?

Now specified.  
Line 140: Wording that the ovaries and other organs will "fall off" perhaps isn't the best choice of phrasing. It might be better to state that the ovaries, along with other organs will be pulled out of the abdomen.

Arranged as suggested.  
Line 142-143: Phrasing. I would not describe ovarioles as fibers, rather they are chains or strings of developing egg chambers. We use now the word string. Additionally, how are the ovaries being moved and how are the ovarioles being dissected?

We now describe, how to pull out one ovariole out of ovaries.  
Line 142-156: You need to explain the rationale for why it is necessary to dissect the ovaries in this manner- is it necessary to reduce the risk of damage, etc.? Now we include that by dissection ovarioles can be damaged.

Why not separate the ovarioles in the original drop, then transfer the chosen ovarioles to the MatTek dish? This would reduce/eliminate debris in the MatTek dish and reduce the risk of ovarioles drying out when replacing dirty SMI. We agree that this approach would avoid debrits in the dish, however, to transfer a single ovariole is a risk. It can stick on the forcep and by trying to release it means another danger to damage egg chambers in ovarioles. Therefore, we prefer to let ovarioles to only gently pull out from ovaries and limit their touching.

Line 152: What is meant by remnants released from the female abdomen? Now we removed remnants and specified what we mean. Also, since ovaries are the topic in this paper, it is not necessary to specify female abdomen.  
Line 158: Can the dye be added to the SMI prior to adding the ovaries? This would reduce the risk of damaging the egg chambers when mixing the dye. We agree that it is also the way to do it. However, the SMI often needs to be replaced/cleaned from debris and old egg chambers. Our way, therefore, requires to place the dye only once to the SMI. Mixing with a forcep is a gentle step. To make sure that the reader should be careful, we add: ‘Avoid contact of ovarioles with the forcep…’

Line 161: Phrasing. Change "You might need to figure out how long and what dilution works the best in your case." To "Optimization may be required for different applications."

Thank you for this suggestion. We have replaced our text with your suggested sentence.  
  
3.Analysis of actomyosin at the cellular scale  
  
Line 175-181: It seems that six to seven ovarioles would be a lot to have in 100ul of SMI, and it thus be difficult to prevent them from contacting each other. We understand your concern, but based on our experience this number of ovarioles is the perfect number as it is still possible to nicely spread ovarioles in the dish and at the same time it provides enough material for imaging session of 2 h without a need to dissect new ovarioles. The dish has relatively wide well for a good spreading of ovarioles and the bleaching of unimaged ovarioles is close to zero. Also, the cactus tool is not in the reagent list. We apologize for this. We have add the cactus tool to our reagent list.  
Line 184-185: Is a Z-stack being collected? If so, specify. If this method does not require a z-stack, then be sure to specify as such. It is unclear if a single plane or a z-stack are being acquired. If it is a single slice, is the use of a confocal necessary? Additionally, given that all the image analyses assume 63x, it seems like you should indicate what objective to use and why. Thank you for this point. We now specify why to use 63x and provide detailed instructions. To acquire actomyosin signal behaviour, we obtained the best results by using the confocal microscope in terms of signal resolution.

Line 187. What "selected cells" are us unclear? Since the protocol is about follicle cell imaging, a more detailed description is needed here. What cells should be focused on and in what plane? We now explain in detail to focus on the most central part of an egg chamber and the basal (outside) side of follicle cells.

Line 191-192: Change "avoid a negative impact of floor resonation…." to "reduce vibration that could affect imaging" Thank you, we have replaced our text with your suggested sentence.  
Line 199-200: These formats are specific to specific microscopes. However, many of those who use this protocol may have instruments from other manufacturers. It would be better to state to make sure the TLMs are saved in the confocal manufacture software format and why this is important. We now state that any formats that can be opened in Fiji are possible to use.   
  
3.2 Data processing  
  
Overall, more detail/explanation is needed. A flowchart of the process would be useful in providing an overview of the analyses and the purpose of each step or data. I think this section should begin with ensuring that the user has first installed Fiji and the required plugins, if they have not already done so. It would also be helpful to specify at the beginning what plugins are necessary to complete this procedure so the user of this protocol can easily follow along with an example. Furthermore, a sample image and script would also be beneficial and allow the user to work through the protocol. Finally, figures for each of these steps would be extremely helpful, particularly easy to read images/screenshots highlighting options required for successful image processing.  
We now reworked our manuscript in the way that the dissection and imaging part is in the Supplement. We therefore focues on the data processing what was the main aim of this publication. We provide improved instruction accompanied with test files and screenshots in figures.

Line 204: why is bleaching correction required? Setting choice is unclear.

Acquired TLMs suffer from bleaching due to the high-speed imaging and therefore, bleaching correction is highly recommendable to be able to properly see the actomyosin signal later in movie frames during the manual analysis. Setting choice of 0.0 is the standard one. No difference was observed when the value was changed to e.g.10.  
Line 206-207: Simple ratio works best if the intensity does not change dramatically. Would histogram matching be a better option for time lapse? You are right. Thank you for this detail. We now provide this info as a note.  
Line 211: it is unclear what "TissueCellSegmentMovie-3.ijm" is. Is it a file? Is it a script?

This is a script. As it is not our script, we did not wanted to change its name. We now specify that this file is a script in the text. We provide the link to the original webpage as well as direct link to download it.  
Line 212: What is this script, where did it come from, is it specific to a particular image, and does the user have to write this script? If the user must write a script, you will want to address that. How does the user run the script?

As mentioned above, this file is a script developed in other institution (Barcelona by Sebastien Tosi) and you can open this script in Fiji. We now state how to run the script in the text more in detail. Please follow our new instructions.  
Line 214: How were these settings chosen and what do they mean?

These settings are the best observed for 63x magnification time-lapse movies and work the best for cell segmentation in stage 6-8 egg chambers.

Line 215: Is this applicable only to a particular brand of 63X objective, or is it universal? Also, if a user wishes to use a 20X or 40X how would they determine the necessary noise tolerance?

It is right that these settings are applicable for 63x objective, we state now that different parameters are required for other objectives. We also state that the cleaner the background, the lower noise tolerance is required.

Line 216-217: Maybe change "clear outlines" to "well-defined outlines". We use now well-defined. How are the cells selected? We state now: well-defined and complete cell outlines.

When was the mask created? Generally, a better description of creating and editing the mask is required (i.e. where is the merge tool).We now provided detail description how to create and edit the mask in the text.  
Line 218: Is ParticleStack the filename generated by the segmentation process? If so, it is recommended that you state what files are generated by the program. We included it into the text.  
Line 223: Note that the user must download and install this plugin. It seems like a Fiji set up method is needed.

We now start the section of cell segmentation with telling the user to open Fiji and download the script.   
Line 234: Describe how to remove unwanted cells. We now describe it.  
Line 235: A more detailed description of how to correct the cell outlines and where the "brush" tool is needed.

We now added a whole new section how to do it.  
Line 238: You assume the reader knows a lot - Where are the tools you are referring to? How is a cell mask created? Where is the "+add"? Added.  
Line 246. the phrasing is unclear "the subject to an analysis" We removed this part of the sentence.  
Line 252: Method 3.3 needs a short introduction to explain the purpose of the analyses

We have now included brief explanation.  
Line 254: It is unclear which file should be opened. Also, why are these cell parameters needed? What can they be used for? We now explain in the introduction and provide instructions in detail.  
Line 255: Again, what is the data used for? What is surface manager (where is it in Fiji)? Is it Surface Manager 3D? What is different about the data in 3.3.1 vs 3.3.2? When performing the 'Statistics' function, is it possible to select particular values of interest for analysis? If so, explain how this is done. what is different about the data in 3.3.1 vs 3.3.2? We apologize if our instructions were confusing. We now specify and explain more in detail. We have changed the statistic parameters to relevant ones that are related to our previous paper Viktorinova et al. (2017) PLoS Genetics.   
Line 265: Method inconsistencies - you don't always state what program to open the file in.

We now guide the reader hopefully better.  
Line 271. Are all the plugins in Fiji generally loaded? If not, you need to add that as a step in the method. Also explaining what each change is in non-Fiji language would be helpful. We now include at the beginning of Data processing section/instruction tutorials how to download and update Fiji to have required plugins.

In general, why is this method important for the later image analysis?

To correct for drift/movement prior to the manual analysis could be probably skipped, but generally, it is easier to detect signal movement relatively to the rigid cell membrane than without the drift correction. Even the short signal movement is then prominent. We included a short explanation by this point in the manuscript.

Line 274: Explain why you use the default values for Gaussian Blur here and if it would be necessary to change this. We tested the MultiStagReg also without Gaussian Blur and it works too. We now put the Guassian Blur and conversion to mask as an optional step.   
Line 278-279: What is the purpose of these steps? These steps serve to create the mask based on which the registration/drift correction will be done.  
Line 287: it is unclear what step this is an alternative for. We now specify in the text.  
Line 291: Method 3.5 An introduction of what the purpose of the analysis is and this data is useful for would be helpful. An example of using the data could increase clarity. We now included a brief explanation of this method.   
Line 298: How is the movie divided into submovies? We now explain how to create submovies.  
Line 302: What do you mean by signal line? How is a signal line identified? We now specify that a signal line is the trajectory of one actomyosin signal movement over time…  
Line 311: Are the same signal lines being analyzed in each submovie? No, we state in the manual that this must be avoided. How many lines are analyzed per cell? How many cells per image? We now state that all visible actomyosin signals should be analysed and it should be aimed for 20-30 signals per one analysed cell. All cells with well-defined outlines should be analysed as we newly state.

Line 312: unclear. We corrected it.

Line 315-317: Is this done in Fiji or exported and analyzed elsewhere? Please explain further. We provide detail instructions.

4. Analysis of actomyosin at the tissue scale  
  
As with the section above, this protocol would benefit by the addition of more explanation of what is required for, and what is accomplished by each step. Again, more figures of dialog boxes, etc. and another example file for the reader to work with would be beneficial.  
  
Line 322: delete e.g. Ok.  
Line 331: How big of a Z stack is needed and why. What magnification is needed? Should it be optimal Z-stacks or over/under sampled? What cells are the focus? We now specify to use 40x to acquire tissue scale (half of an egg chamber) that individual planes should sufficiently overlap. As we think that the settings may differ for different spinning discs, we try to avoid too much of a detail.

Line 335-337: This is confusing. May need to clarify point 3.1 to indicate that only a single plane is being collected. We now specify.

Here in the Note, "set up additionally a z-stack" implies that both a single plane and a z-stack are being acquired. We now removed additionally. Is the 60 second acquisition time for your particular settings? It would seem scan speed, step size, number of channels, etc. could all influence this. Yes, we agree. We have corrected it and state that it all depends on the used microscopic settings.  
Line 343: change designed to designated We rather deleted this word.  
Line 346: Fiji can automatically update upon starting. Is this step absolutely necessary prior to running this protocol? You are right, in this case, it is not required, but we state to install the required plugin.  
Line 347: What is the file? What is the purpose of this step? You jump in here with no real explanation. Is there a file open? We corrected this. Thank you for pointing this out.  
Line 355: In the note - grammar issues make it confusing We rewritten this part.  
Line 360-361: It is unclear what the bounding box should be selecting. No point has told us what to focus on. We now state that the focus in on the egg chamber in the bounding box. Additionally, it would be helpful to reference a zoomed in figure of the dialog boxes with entries highlighted for the next steps. We now provide instruction tutorial with screenshots.

Line 360: Are the coordinates obtained by mousing over the image? We now specify how to do it in the text.  
Line 366-373: Again, referencing a zoomed in figure of the appropriate dialog box would be helpful. We specify it now.  
Line 366-373: This line is not a method. What are blobs? Why is this step crucial? Perhaps more explanation is required here. We agree and changed it in the text with providing more explanation.

Line 368-369 could be left as a note, but the rest of this information seems it is important enough to be separated out of the Note section (insert Line 369-373 above line 368). Thank you, we corrected it as suggested.  
Line 375: What is being done and what is its purpose? Is the ellipsoid drawn by the program? How are samples chosen? We explain now.  
Line 377:Do you mean designated or desired rather than designed? To avoid confusion, we use the word ‘desired’ ellipsoid.  
Line 381: See above comment for Line 377 Corrected to desired ellipsoid.  
Line 392: See above comment for Line 377 Corrected to desired ellipsoid.  
Line 400-401: What would the log file be used for? One can easily see the parameters in case of repetition. We explain why is that important.

Line 403: when is this necessary. Now we state when it is required.  
Line 413-421: Again, it is unclear what this data is used for….what is the script for? What is the output? Again, a figure showing the dialog boxes would be helpful. We now explain.  
Line 428-429: Either describe how this is done in PIV Lab, or merely state that manual analysis is not suitable for tissue scale images. We excluded this from our revised manuscript.

Line 485: Spelling-change cytoplasmatic to cytoplasmic, change "its" to "their" Ok, corrected.  
Line 485-495: It think this figure would benefit from editing. Screenshots of dialog boxes are difficult to read. I may be better to create separate figures and reference these in the appropriate steps in the text. OK  
Line 499: Is figure 2 being referenced in figure 3, or do you mean figure 3A? As in Figure 2, it may be better to create separate figures and enlarge screenshots of dialog boxes so they can be read. OK  
Line 511-519: You reference AP axis, but it is not indicated in the movies or the legend. Thank you, we have now added that anterior is on the left in the movie.  
  
5. Discussion  
  
The discussion could benefit by expanding on the benefits of this method over other published methods for visualizing actin. At some point in the manuscript you need to discuss what the data is useful for. The discussion would be a great place to do this, with an example of wild-type vs mutant data.

We hope that we provide this now.

Line 579: Delete "and body's remains" as it is redundant with debris. Thank you, we have now deleted it.  
Line 609-611: The wording is repetitive, there are grammar issues, and it is unclear. Re-word. Thank you, this is our mistake and now is corrected.  
Line 617: change "your" to "the" OK  
Line 617-619: Why can't the file be closed? This is a little confusing. Does ParticleStack.tif have to be open when correcting cell outlines in the original movie? We agree, this is our mistake after changes applied. We now deleted it.  
Line 623- 627: True false positives need to be defined by statistics. We specify better.

Line 632-635: Perhaps this should be pointed out when discussing how to make the mask of cell outlines. We do now.  
Line 656-667: It might be helpful to provide a schematic or picture illustrating an ideal ellipsoid and an ellipsoid that is not good to aid in explaining how this step affects blob identification. OK, we now provide this.  
Lines 676-682: This info on the images is needed much earlier in the paper. We hope it is now.  
Line 686-690: Expansion of the alternative uses to include specific examples - other organisms and tissue development would be appropriate and informative.

We now mention a few exmaples.   
  
**Reviewer #2:**  
  
Manuscript Summary:  
Viktorinova et al describe methods for live-imaging the epithelial follicle cells of the Drosophila ovary such that apical or basal protein dynamics can be observed. This type of imaging has provided insight to actinomyosin dynamics and could be useful for further analysis in addition to characterizations of many other dynamic proteins. The title is appropriate; the abstract could be shortened but is also accurate. Overall the protocol is relatively easy to follow, even for someone not used to the Fiji software, which is a plus. All materials and equipment are listed appropriately, and the procedure should be expected to lead the predicted outcomes. The sample images and movies provided in the protocol are impressive and useful.  
We are very grateful for your valuable review and comments.

I have no major concerns, but I do have a number of comments and questions that would help make the manuscript maximally clear and useful, as follows:  
  
Minor Concerns:  
In the protocol description:  
The authors should clarify that the starting flies need to be transgenics with fluorescently labeled protein(s) of interest (in this case, for myosin, MRLC-GFP). Since MRLC-GFP is used throughout, the protocol should include information on what this strain is (in text and figure legend) and a reference.

Thank you. We now provide information on MRLC::GFP in the text and legends as well as reference to the stocks used in the protocol.

It would also be helpful to comment on the larger blob of myosin in each cell- what is this thought to be?

We now state that the protocol focuses on the smaller Myosin II signals and users should avoid analysing the big ones. We would love to know what these big Myo-II signals are, however, we haven’t found it yet. We think that it could be a sort of an adhesion type that links to the follicle epithelium to the surrounding extracellular matrix.  
  
Are the flies fed fresh food each day (or just left with day one food) in step 1.2?

We now specify that flies should be kept in the same vials as prepared..  
  
In my experience, the dissection strategy described in step 2.9 would lead to removing the abdomen completely. Is that the intention? We now changed this and specify that the abdomen should be pulled on the dorsal tip of the female abdomen in this point. However, it can sometimes happen that the whole abdomen gets separated from the thorax. Therefore, we added a note to this point and state what to do in such a case.

Are the ovaries falling out at the anterior of the abdomen? If not, the authors may want to explain how this is avoided or suggest holding at the top of the abdomen and not the thorax.

We added to hold one forcep on the thorax with the adjunct part of the abdomen. We believe that this will be clear from the video.  
  
What is a cactus tool, and where can it be obtained?  
We specify this in the Table of Materials.

TissueCellSegmentMovie-3.ijm script was a bit hard to find as it is embedded in text - more details on the website link would be helpful (eg, it is different than "tissue cell geometry stats"), as well as direction to the Supplementary Information given in Suppl.2.

We now provide detailed information.

Line 464 in the protocol itself. We included this information where to download the script into the protocol as you suggested.  
  
I don't have the "surface manager" as an option under the "segmentation" plug-ins in Fiji, so I was unable to test the steps that required this. Where should I get this? Is it a Pc/mac problem? Perhaps the authors can advise in the protocol.

We now provide detailed instruction how to get this plugin and how to install it into Fiji.

I don't have "MultistackReg" from step 3.4.5 in my plugins? Can I used "correct 3D drift"?  
Thank you for this information. Yes, you can use Correct 3D drift as well. We have added this information to the protocol as an alternative option. To obtain MultiStackReg, it is required to update Fiji as we describe in the protocol now.

The authors may want to explain best way to make submovies in step 3.5 without overlapping any information.

Thank you, we have now added the requested information.  
  
More details needed for how to set up z-stack in step 4.1. Does "ca 60s" mean you should have 60 between acquisition of z-stacks? It is the time for one z-stack. We now state more in detail in this step.

How many microns is a typical required distance for the z-stack? We now specify the depth for a z-stack.  
  
Steps 4.2.1 are a bit harder to follow. I think the authors acquire focal planes below the nurse cells but only use lateral information on the follicle cells, is that correct? We now improved this part. We first acquire the information for follicle cells and nurse cells in the z-stack. Then, we extract defined part of the surface based on the ellipsoid fitting and then from obtained surface projection select the layer of the interest.

See comments and questions for figure 3. Also, does the data to be inputted for 4.2.1.7 slice distance and projection information come from the confocal settings for z-stack? We recommend to use slice distance 1.  
  
The majority of critical steps are highlighted, but authors may want to include making fresh media+insulin as a critical step. Also, some processing may not need to be listed as critical steps (for example the "Notes"), although they are probably needed for successful digital analysis.

Thank you for your kind comments. We provide additional information on this critical step in the dissection part.  
  
Figure/Movie comments:  
It would be helpful to have all egg chambers oriented the same way in figures, including schematics and movies, especially since in some there is no way to know the orientation as they are small sections.  
Thank you for this point. We have rearranged our figures accordingly.

Figure 2 and legend- Overall this figure is extremely helpful, especially panels in F. It is not clear how helpful the screenshots are since they are so tiny and almost illegible. These may be better to include elsewhere and larger so they are more readable.

Thank you. We now provide larger figures.

Also, consider using "cytoplasmic" instead of "cytoplasmatic" in the figure. Thank you, we have changed it.

The legend should reference the corresponding movie so that the readers can see how the arrows in the analysis correspond to the dynamics of the movie.

Than you, we provide this now.  
  
Figure 3 and legend- more detail here would be helpful. In B, where are the blobs and fit ellipsoid in these examples? We have now improved this part and replace by instruction tutorials.

Could the authors indicate this on the figure?

It seems like focal planes cuts through nurse cells for part of this - are the areas of nurse cells dropped out to capture lateral epithelial cells at this deeper focal plane, or are only very shallow optical sections used? These screenshots show lower planes of captured egg chamber in order to extract lateral follicle cells. We now state this in figure legends.

In fig3b, it looks like there is an intensity change in the very center - is that from nurse cells? Would that area be avoided in analysis? This is exactly why we have developed the surface extraction method. Myo-II expression is stronger at the apical side of follicle cells (i.e. closer to the nurse cells) than at their basal (i.e. outer) side. Therefore, this apical Myo-II information mixes with the basal Myo-II upon classical z-stack projection (see Fig. 1). Using the surface extraction, the apical Myo-II information is omitted.

For all movies, timestamps or total elapsed time needs to be specified on the movies or in the legends.

Thank you for this missing information. We have added it to all movies.  
  
Some myosins seem to move across 2 cells. Why? Is this significant? Would they not be counted?

We agree that it is sometimes the case. We do not know, however, as to why is that/what is their origin and how come this at all. We are sorry that it is not clear from our protocol instruction (cytoplasmic, subcellular) and now we specifically point that these signals should be left out from the analysis.

Which way is movie 2 oriented?  
Thank you for this missing information. We state now that anterior is on the left for both movies, 1 and 2.

In discussion section 2, it is suggested to have about 6 ovarioles or egg chambers per set up- are multiple egg chambers imaged at once? Or just one? Can a different egg chamber be visualized after one is used, or would it also be damaged from light exposure? This issue could be clarified.

We apologize for confusion if unclear in the text. We now state that only one egg chamber is imaged and discuss that there is no damage to other egg chambers in the same ovariole or other ovarioles in the SMI during imaging.

Other:  
More explanation about why the processing steps are needed, especially the point of each digital manipulation, would be helpful for a novice.

For example, why is the surface manager needed?

Is this just to choose the subset of cells to analyze further?

If so, how many are needed? Since a number of manipulations are performed it is worth pointing out to a novice that original, unprocessed data should be kept separately.  
We hope that through our new instruction it became clear now. The aim of this protocol is to convey how analyse actomyosin at the local and tissue scale. The dissection and imaging is leading towards it and may be even skipped as we now provide example data and test files.

How are the statistics obtained (as in fig 2D, steps 3.3.1-2) to be used?

Are these values needed later in the protocol? If it is just for quantification purposes, an example of that processed data (maybe in a graph) might make the utility clearer.  
Since fat2 mutants are used to demonstrate proof of principal and differences observed in the mutant case, more background about this in the text would be helpful. The authors should reference their own paper as well as others on this, eg, Barlan (2017) Chen (2016). References should list all authors unless the journal style is different.  
We now provide a bit background in the figure legends what to observe in control and fat2 mutant egg chambers. However, we are limited here onto methodological part.

Discussion of future applications and limitations is quite good, however, it could be broadened to mention other protein analysis in egg chambers (in addition to actinomyosin applications in other tissues).  
We now state that this protocol is suitable for actomyosin and similarly sized subcellular dynamic particles and mention other tissues that can be subject to this analysis.  
  
**Reviewer #3:**  
  
Manuscript Summary:  
Viktorinova et al present a protocol for ex vivo culturing of Drosophila egg chambers and a pipeline for biological image analysis of actomyosin dynamics in the follicular epithelium. JoVE videos are particularly useful for manipulative techniques such as tissue micro-dissections, so this topic is appropriate, particularly because the preparation and ex vivo imaging of Drosophila egg chambers is used by a growing number of labs, but videos of this method are not readily available. An additional strength of the manuscript is that the protocol for tissue preparation and ex vivo culture is coupled with a detailed image analysis pipeline, an essential linkage for researchers that study the morphogenesis of the follicular epithelium, particularly cytoskeletal dynamics in the epithelium of the early egg chamber. However, in its current incarnation, the protocol is minimally detailed, often vague and unclear, and unlikely to lead to the described outcome, particularly in the hands of novices. Essential revisions are needed to render this protocol 1) discoverable: abstract does not adequately articulate the focus and usefulness of the protocol; 2) Informative: as written, readers have to read through the entirety of the protocol to know what data they will acquire; and 3) complete: essential details are missing for successful completion of the protocol. In the opinion of this reviewer, the key innovation to the tissue preparation portion of the protocol is the minimalist method to mount tissues for imaging. The authors of this manuscript could expand this section, as well as the time-lapse imaging section and the subsequent image analysis pipeline section, by reducing or eliminating details of the dissection and culture conditions portions. These portions of the tissue preparation protocol are elaborated more thoroughly in the cited 2007 Nature Protocols paper by Prasad et al., and an uncited 2014 Methods in Molecular Biology paper (Cetera, Llewellyn, and Horne-Badovinac, "Cultivation and Live Imaging of Drosophila Ovaries", Chapter 12 of "Drosophila: Methods and Protocols" in Methods in Mol. Biol. Vol. 148, Dahmann ed., pp. 215-226. The authors cite a less detailed 2014 paper by Cetera et al, in Nature Protocols.)

Thank you for your suggestions. We have now moved dissection and imaging section into a supplement and focus on the data analysis. We also cite the other protocol, although not suitable for this analysis, in the relevant place.

One major oversight in the protocol presentation is the absence of a description of the statistics that are calculated. "Press Statistics" is stated in several steps of the protocol. Without an explicit discussion of the statistical analysis performed, it is difficult to generalize this protocol to other applications, or to have confidence in the data generated for this application. This information is not visible in the screenshots in data processing steps shown the figures.  
Thank you for this point. We improved this part and now added corresponding screenshots and explain what parameters ‘Statistics’ button provides in the instruction material.

Major Concerns:  
  
----Are the title and abstract appropriate for this methods article?  
  
The title is appropriate, but the abstract is not. The authors give a scientific description of the subject for their investigation, but do not provide specific information about the tissue preparation protocol and image analysis pipeline that are the actual subject of the methods described. They do not state the specific applications of their protocol beyond stating that it is useful for "in vitro live imaging" and analysis of "dynamic actomyosin machinery…at the cellular and tissue level."

We now provide a changed abstract that hopefully better reflects our protocol.  
  
Rather than simply saying "cytoskeletal dynamics", the authors should include: 1) how actin and myosin are visualized in live fluorescence microscopy; and 2) The specific types of cellular and behaviors and cytoskeletal dynamics that can be measured with their protocol and analysis pipeline. It is not clear until late in the protocol that the image analysis pipeline  
We now provide this information.

----Are there any other potential applications for the method/protocol the authors could discuss?  
  
As written, the protocol is specific for imaging a very specific set of cell biological events during epithelium circumferential migration. The authors state that it is designed for detailed analysis of actomyosin machinery, but the image analysis protocol seems to be specific to periodic myosin localization into "blobs."

Thank you for this point. We now specify what myosin signals should be analysed.

The tissue preparation and imaging protocol appears to be optimized for imaging of pre-vitellogenic egg chambers (stages 1-8). Furthermore, the specific protocol for time-lapse imaging is adapted to view fast events over a very short time period (See Lines 187-188). This creates a limitation that should be presented early in the introduction, because many morphogenetic events in the egg chambers take periods greater than 1 hour. At some point in the protocol or discussion, the authors should discuss the pros and cons of changing the time-lapse specifications to encompass slower or longer events.  
We completely agree that this was not clear in our first version. We now specify or the local cellular scale and for the tissue scale what time and resolution can be gained and what are limitations of these approaches.

There are many, widely accessible, transgenic reagents used to visualize actin and myosin; the authors should specify which reagents have worked in their hands, and particularly the genetic tools they visualized for the movies they show in the supplementary data, and which haven't if these have been encountered.

Thank you for this point. We have now specified what transgenic lines we have used.

At a minimum, authors should cite Spraklen et al., 2014, Dev Biol 393, 209, which critically assesses the utility of common expression drivers and actin labeling tools for in vivo studies of oogenesis.

Thank you. We added this citation to our text.

In contrast to potential limitations of the live imaging protocol, the image analysis pipeline, including cell segmentation, and the measurements of "statistics" such as cell area, cell shape, etc. could be useful to researchers studying later stages of egg chamber development. It would be important to state which parts of the ex vivo culture methods are restricted to use with inverted microscopes, and what parts of the image analysis pipeline can be used for time-lapse image data acquired using other culture and imaging conditions.  
Thank you for this suggestion. We now specify what microscopes are needed for which part of the protocol. We have also corrected the statistics parameters and avoided unnecessary ones.

To summarize the comments in this section, it is not clear how adaptable this protocol will be. Assessing the adaptability for either the imaging or the image analysis pipeline will require additional notes and comments on the range for variation in parameters that are detailed under items 3 and 4, below.  
We now state in the introduction and in the discussion how can this protocol be adapted and for what signals it can be used.

----Do you think the steps listed in the procedure would lead to the described outcome?  
  
It seems unlikely, more on this is listed under the minor comments. One major issue is that the authors do not describe controls that could be used to check on proper performance of the technical steps in their protocol.  
We now provide new instructions with screenshots and test files, which should hopefully make it easier for users to follow our protocol. Beside this, we are more specific about preparation and culturing of egg chambers.

The authors do not state the percentage of imaged egg chambers that yields a useable time-lapse data set, which will be needed to estimate the time needed for repeated rounds of the experiment.

Thank you for this point. We now included that the rate of successfully imaged undamaged egg chambers in the discussion part in troubleshooting for this section.

The authors don't explain how they avoid damaging egg chambers during imaging, nor do they summarize a list of specific precautions that are needed to avoid movement of the egg chambers in the culture medium.

We state in the dissection troubleshooting part what precautions and how avoid damaging of egg chambers.

The authors state that other published protocols introduce additional steps to limit egg chamber motion, but that the interventions compromise the egg chambers. However, this protocol does not clearly state how their methods avoid small movements that would impact the imaging session. It seems likely that the authors avoid slight movements of free egg chambers by imaging only one egg chamber during one time-lapse imaging session, due to inevitable slight motions caused by stage movements. If the authors do not encounter these limitations to their imaging sessions, they should explicitly say so, and then describe the important features of their protocol, stage, and stage control mechanisms, as well as the limits to distances travelled by the stage. If only one egg chamber is imaged per session, they should say so. Such a condition would necessitate access to the confocal or spinning disk microscope "on demand", rather that through advanced sign-up (a common condition for use of shared microscope systems).

Thank you for this point. We image early egg chambers that are mainly cross-linked in the ovariole, only occasionally there are egg chambers of stage 6-8 that are individually placed in the culturing medium. We image up to 10 egg chamber in one imaging (2h) session without changing media in a dish with egg chambers. We now describe this in the protocol and in the discussion troubleshooting part.

----Are any important steps missing from the procedure?  
  
\* Line 124, step 3, item 4: Other published protocols state that insulin must be dissolved in acidified water prior to addition to the Schneiders medium. They list both reagents in their list of materials (lines 444 and 445), but they do not state that the acidified water is specifically for the insulin solution. Within the protocol, they perform the pH measurement on the medium, prior to addition of the insulin. Do the authors intend for the insulin powder to be added directly to the medium. This is an example of details from other published protocols that are ambiguous in this protocol.

As we state in our Table of Materials, we use human insulin solution, no powder, i.e. there is no need to add acidified water into insulin. We add this insulin solution as described in the protocol, i.e. firstly after the Schneider medium mix and its pH adjustment is done. The volume of added insulin is ca. 0.1% of the total SMI volume and therefore does not change the final pH of the final used culturing medium.

\* Lines 142-145: Step 2, items 11-12: The authors list a cactus tool in the Materials table, but do not say what it is used for. It would most likely be used for these steps, but no details are provided. Why use such a rare tool, available only to those with access to cactus spines? Why not use tungsten needles or other materials that can be ordered from microscopy suppliers?

Thank you. We now describe how to create cactus tool in the Table of Materials. You are right that one can use other similar tools if no cactus spines are available.

\* Sections 3.2 (begin on line 202) and 4.2 (begin on line 339) on data processing: For each step in the analysis, it would be helpful to begin with an item for "Ensure fiji and the following plugins are downloaded", "Ensure that code is installed and working properly for \_\_\_\_\_\_\_\_", or "Use a computer with Matlab and the following toolboxes installed."

Thank you, we now guide the readers in detail and instruct them to download and open the corresponding script/code in indicated sections.  
  
----Is there any additional information that would be useful to include?  
  
\* There are many points in the protocol that suggest that this pipeline is optimized for image data acquired with a 63X immersion objective, with at least 1.3 NA. This implies that other investigators who use a different objective might need to optimize settings differently. Is optimizing settings straightforward for each step, or will other investigators struggle to apply this protocol in a different setting?

Thank you for this point. We now state in the manuscript that it is not recommended to use other objectives with lower resolution and lower NA. We believe that this restriction should be no limitation for majority of scientist as such objectives can be easily purchased or are a state-of-the-art equipment of confocal microscopic settings.

\* The authors state that images should be saved in .lsm or .czi formats, but it is not clear what these file formats are. Is .lsm proprietary to Zeiss microscopes? Do the authors mean that image data should be stained in the native file format for the microscope image acquisition software? If so, that would include other formats, such as Nikon's .nd2 format.

Thank you. Yes, you are right .lsm and .czi are Zeiss output formats. However, you can use any formats that can be opened in Fiji as now stated in our revised protocol.

\* The intended purpose for some of the data analysis steps is not explained. "Bleach correction", introduced in line 204, is one example. A table or diagram that outlines the purpose of each step in the analysis pipeline would be very helpful, for example: Bleach correction does this; cell segmentation does this; surface extraction does this. Gaussian blur does this, a blob represents that. Such a table should include notes on critical parameter adjustment, if appropriate.  
Thank you. We agree that this will be helpful and therefore, we now added this information to individual steps in the protocol.

Details on Vague or Problematic Steps of the Protocol:  
  
Either in the abstract or in the introduction, the authors should inform readers that the protocol is only compatible with inverted microscopes. Similarly, the time limitation for the time-lapse imaging should be stated up front, so that investigators can assess whether the protocol can be used with their major equipment.  
We agree and we hope it is clear in our revised manuscript.

------Table of materials needed lacks key information:  
\* The authors do not indicate that live imaging of cytoskeletal dynamics requires the use of fluorescently-labeled proteins. To use this protocol, investigators must have appropriate fly strains available, and these live materials are not mentioned at all.

Thank you for this point. We now added this information in the protocol and the Table of Materials.

\* The authors do not list the MATLAB programming environment, nor do they list the MATLAB Statistics and Image Processing toolboxes.

We now decided to retract MatLab information as this is a preliminary outcome and may be misleading for readers.

The use of the Statistic analysis is described in step 3.3.2 lines 254 and 269, step 4.3 in line 416, "Critical Steps and Troubleshooting" lines 624 and 639, and Figure Legend 2 in line 491. The Image Processing toolbox needed to access the Particle Image Velocity" (PIV) analysis in step 4.4, line 429. On lines 698-699 they state that this is available in "the up-to-date" MATLAB version, but they don't give a version number. They do list a 2014 paper on MATLAB and the Statistics Toolbox in the reference list, but I could not find where this is cited in the text. It is not clear whether this reference also describes the algorithms for the PIV analysis.

We apologize for this confusion. As stated in the point above, we now retracted MatLab information. The reason is that our whole protocol in the manuscript is NOT based on the MatLab software, but on exclusively Fiji-based plugins. Hopefully, the removal of the MatLab program will dismiss this confusion.

\* Line 452: Holder for cactus spine should be specified in more detail.  
We provide this information in the Table of Materials.

-----Many steps in the described feature are vaguely described:  
  
Many section headings in the protocol section are uninformative (i.e. "data processing"; "later analysis" "cellular scale" "tissue scale" (for the later two, the reader must do a lot of work to know precisely, how the authors are applying these terms. This needs to be spelled out in the introduction)  
Thank you for this suggestion, we now changed and improved titles so that they are hopefully clear what the part will be about.

For reagent preparation, the authors should indicate final solution concentrations.

We now specify the concentration of our dye solutions. The other concentrations should be easy to follow.

Specific examples of vague statements in the protocol:  
\* Lines 126-127 should specify a period of hours during which the SMI is "good" instead of saying "it is possible to use the same SMI on the day of preparation" but not the next day. Does this mean the solution must be used within 8 hours? 16 hours?

Thank you for this point. We now added this information.

\* Line 147: this description of an ovariole that lacks a muscular sheath is not sufficient. This is a key step of sample preparation and the authors give minimal instruction in terms of how to handle the ovarioles (they save this problem for the discussion) This should be clearer within the protocol. The statement also brings up the issue of similarity between this protocol and the protocol described in Cetera, Llewellyn, and Horne-Badovinac, "Cultivation and Live Imaging of Drosophila Ovaries", Chapter 12 of "Drosophila: Methods and Protocols" in Methods in Mol. Biol. V 148, Dahmann ed., pp. 215-226. The authors do not cite this paper, nor do they acknowledge any of the authors.

Thank you. We have changed it accordingly in the protocol. Due to the space limitation given by JoVE policies and also our initial intended focus of the protocol, we have now moved the dissection and imaging part of egg chambers into the supplement and focus only on the actomyosin analysis for both, local and tissue scales. We now also cite the mentioned paper in the relevant place. Their protocol, however, is not suitable for this analysis due to the final mounting of egg chambers and thus differs from our protocol.

\* Line 151, Step 13. The authors do not state how much medium can be removed, they just state "avoid that the ovarioles dry out." Is there a point where you add more medium? If so, does additional dye need to be added as well?

Thank you. We state this now. There is no need to add dye as it firstly comes in the following step.

\* Line 158, Step 14: The authors simply say to add dye, but they do not explain what CellMask or FM4-64 dyes stain, nor do they explain why these dyes must be used for the protocol. As one proceeds through the protocol, it becomes clear that all of the image analysis begins with segmentation, but the authors never mention in the abstract or the introduction that a cell membrane marker is essential if one wants to quantify cell level actin dynamics.

Thank you, you are right. We now corrected it and added this information.

Furthermore, they don't comment on whether commonly used membrane marking genetic tools such as shg::GFP or Indy::GFP could be used instead.

We agree that this could be better placed. We provide new version with this information.

\* Lines 292-293, Step 3.5: These lines introduce step 3.5 as the most demanding part of the protocol. However, this section of the protocol encompasses only about 25 lines, with no explanation of some the terms, such as "time project" in line 298 and "signal line" in line 302.

Thank you, we provide now detailed protocol, which explain this.

\* Line 266 and lines 378-373: "Find blobs": The authors do not explain what a blob is, either visually in the image nor in terms of the stained material it represents. Specific instructions are given, but the actual processes are not clear and leave a lot up to the interpretation of the reader.

Thank you, we now explain what is a blob and provide new detailed instruction for this part.

\* Lines 335-337, Step 4.1: The authors state the time limit for acquisition of a z-stack, but they specify neither the number of optical sections nor the step size. They mention that you can image halfway through the egg, but do not state at which stage they do so, nor do they mention whether they routinely image this deeply for their tissue scale analysis?

We have improved this part and provide more information. However, how many optical sections or step size to choose is dependent on the microscopic setting of the user and may vary. We therefore avoid this information.

Yes, it is required to image that deeply for the tissue scale due to the circumferential curvature of egg chambers. We explain this in the revised manuscript.

\* Line 429, section 4.4: It is not clear what "(1.4)" refers to in this line. There is no step 1.4 in the protocol. Is this a typo?  
We apologize for this confusion. This number refers to the PIV and we now removed this from the revised manuscript.

\* Troubleshooting steps #2 and #3 do not have much substance as to how to solve the problems listed. Examples:  
o Lines 593: "gently correct an unstable egg chamber" is a non-specific statement. Is the egg chamber manipulated, or is the microscope manipulated, and how?

Thank you. We agree that this is ambiguous and corrected it in the discussion part that it is done by moving the stage of the microscope.

o Line 602 - This sentence makes no sense in this context: "Count that high speed imaging results in fast bleaching of used fluorescent signals"

Thank you. We corrected it.

o Lines 610-611: "Reduce or increase the parameters based on the generated mask." How much should they be changed, and how is the mask assessed to know how to change them?  
"it is crucial to create a clean mask" - what exactly does this mean? How does one ensure the mask is clean? What does it look like?  
Thank you, you are right, we are really vague in our explanation. We apologize for this and provide a new version with more insights.

Minor Concerns:  
  
Presumably the figures and movies are intended to indicate the anticipated results and desired outcomes, but these are not clearly explained. Figures are low resolution and cannot be read. Legends for Figures 1, 3, and 4 are insufficient to understand. The captions for the Movies discuss the overall biological interpretation, but do not relate them to the protocol, or provide sufficient information for a novice investigator to understand what they are seeing.

We are sorry to hear that you also had issues with figures. It must have happened by conversion of .psd files (600dpi, ca. 3000x5000pixels) into the wished ones by the journal. We now provide, hopefully, figures with decent resolution.

\* Line 77 - Egg chambers are described as "cross-connected", which seems to imply a matrix of connected egg chambers, rather than a linear arrangement. This is stated again on line 143.

We removed the word “cross-connected’ and specify now that egg chambers are connected by stalk cells.  
  
-----Are any important references missing and are the included references useful?  
  
The included references are useful, but the cited references do not always appear to be the most relevant paper by the first author. For example, references #9, by Cetera et al 2016 is cited for biological observations of the same follicle cell migration, but the highly related methods paper by Cetera et al, 2016, published in Methods in Molecular Biology, is not cited (see above under #5, Line 147.

We don’t see a reason to cite this paper. This protocol is not suitable for live actomyosin network analysis presented in our manuscript.

The Spradling review from 1993 is cited for the presence of the muscle sheath, instead of either the primary literature or more recent research publications that used modern imaging methods imaging to study this structure.

We are sorry for this. We now added hopefully the right literature. Thank you for this improvement.

At least one other lab has performed related computational image analyses: Chen et al, 2016 (Cell Rep 15, 1125) used a different computational approach to derive a surface extraction from the entire egg chamber to evaluate events occuring during the circumferential rotation. The authors do not discuss the advantages and disadvantages of their methods relative to this, or any other approaches that have been used to evaluate dynamic events during circumferential migration.

Thank you. We understand your concerns and we now provide this citation and discuss what advantages our approach has over this one published recently by Chen et al.