Multi-view confocal microscopy enables multiple organ and whole organism live-imaging

Olivier Leroy, Eric van Leen, Philippe Girard, Aurélien Villedieu, Christian Hubert, Floris Bosveld, Yohanns Bellaïche and Olivier Renaud
DOI: 10.1242/dev.199760

Editor: Thomas Lecuit

Review timeline
Original submission: 4 May 2021
Editorial decision: 29 July 2021
First revision received: 4 November 2021
Editorial decision: 1 December 2021
Second revision received: 17 December 2021
Accepted: 13 January 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/199760

MS TITLE: Multi-view confocal microscopy enables multiple organ and whole organism live-imaging

AUTHORS: Olivier Leroy, Eric Van Leen, Philippe Girard, Aurelien Villedieu, Christian Hubert, Floris Bosveld, Yohanns Bellaïche, and Olivier Renaud

I am terribly sorry and sincerely apologise for the very long delay before coming back to you. This is due to one of the reports not coming back to us and the fact that this wasn't somehow detected by us so the manuscript was stalled. I have now received two referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

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

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

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Leroy and colleagues describe a new imaging set up combining spinning disc imaging , dual objective at different magnification and full rotation of the sample without requirement for aqueous/gel mounting. This so called “multi-view confocal imaging” offers a nice alternative to light-sheet microscopy which combined the resolution and homogeneous excitation provided by spinning-disc scanning with the possibility to perform in toto imaging on rather large sample. This could be very useful to study the connection between body parts during morphogenesis specially for samples which cannot be mounted in liquid media, and should bridge the cellular scale with the full body scale. The manuscript is clear, provide very nice imaging data on various samples and a detail description of the set up. It could be of interest for rather a large number of people in the developmental biology community. I would only suggest a few clarifications in the text regarding the actual procedure of acquisition and maybe a more thorough discussion on the potential limitations of the system.

Comments for the author

1. The procedure of acquisition was not totally clear to me. I suspect the set up take a full stack and move to another angle. The method part was a bit confusing in this regard and it might help to describe each step of acquisition in temporal order. On the same line, the “rotation speed of 360°/sec” was confusing for me. I suspect this is the speed of rotation that is used to move to a finite number of angles, but it was not obvious on the first read.

2. While the method state that it is possible to use different XY position and stitching, it is not clear whether this was the case in all the data/movie presented in the paper. Could the author provide more details in the figure legend of each image (how many angles used, how many X-Y positions) ? Also while the combination of 10X and 40X offers a nice connection between cell resolution and in toto imaging, I suspect it should be possible to perform full reconstruction of the sample at the 40X magnification using multi XY positions and multiple angles. Is that a possibility and was it tested by the authors ?

3. While the discussion provides some explanations on future improvement of the system, I feel it would be fair to provide a list of weakness/limitations of the set up in the discussion (I am not necessarily requesting new data here, but more a clear discussion on potential limitation compared to other set up). For instance, how good is the set up to image rather deep structure compared to light-sheet (the comparison was only performed for surface signal such as E-cad)? I guess the muscle data shows that it is possible to image deep structure but it is not clear how good is the deep signal compared to what could be obtained using light-sheet microscopy. Also what about bleaching (in principle the light-sheet system should be more performant on this side) ?Is it possible to perform full 3D reconstruction and can it be processed with tools currently used for light-sheet microscopy (if I am correct right now the data in the manuscript just provide separate max projections of a limited number of angles but there is no proper 3D reconstruction) ?
Other minor point: The “lowest quality region” shown in the inset of the Figure 1B’ rather seems to be a problem of out of plane signal. If I am correct the intensity of the SOPs is not different from the neighbouring region where the epithelial cell contour is visible, so it seems unlikely to be just a problem of shadowing. I am not questioning the shadowing problem per se, but I am not sure the current inset is the best illustration of this.

Reviewer 2

Advance summary and potential significance to field

The Authors of this manuscript provide an interesting new imaging technique whereby a spinning disc microscope is implemented with two independent optical paths and a rotational sample holder. This technique allows for high quality (cellular resolution), large-scale (whole organism) imaging without the use of dipping lenses, which are common in light-sheet microscopes. Thus, it accommodates organisms that require gas exchange and cannot be immersed in liquid, such as the

Drosophila pupae, a powerful model organism for tissue morphogenesis and organ growth. The authors show examples of how this technique enables very long-term live imaging (>90hr of development), as well as cellular resolution whole organism imaging (21hr of development every 30min, three regions, 5 views). They also demonstrate the versatility of the setup using different organisms. The microscopy setup is well explained and should allow for the reproduction of the system by the reader. I assume this article is meant for the Techniques section of Development.

In general, the technique is novel and could provide a powerful new way of looking at the organismal-wide coordination of organ growth and morphogenesis in certain model organisms, particularly insects. It is an important open question in developmental biology how organ morphogenesis and growth is systemically coordinated, and thus this technique should enable exciting new discoveries and would be of interest to the readers of Development. Suggestions for improvement are discussed below.

Comments for the author

Major:
1. The authors motivate the development of this technique in part by explaining that light-sheet microscopy, an established technique for rapid, gentle, large-scale imaging, is not suitable for certain organisms such as the Drosophila pupae. There are a couple of weaknesses in this argument:
a. If I understand correctly, the feasibility and quality of the commercial light sheet microscopes was only evaluated using dual-sided illumination with a single view (no sample orientation). A multi-view approach, where the sample is rotated and images acquired at multiple angles, would like increase image quality and result in a much more homogeneous illumination than that shown in Fig 1. The authors attribute the poor image quality to shadowing artifacts that are inherent to light-sheet microscopy. While shadowing undoubtedly has an effect, multi-view imaging, followed by fusion and deconvolution generally alleviates this problem to a certain extent. It is fair to argue that the later approach is heavy on data size and image processing. Nevertheless, for assessing image quality on the Z1 system, these optimization steps should not be neglected.
b. The authors state that the Drosophila pupae requires gas exchange and cannot be immersed in liquid, but no data are actually presented to show loss of viability or adverse effects on tissue development upon immersing the pupae for light-sheet imaging. Does the entire pupal development require gas exchange? Is it possible to immerse the pupae only periodically for imaging and remove it in between time points? Ideally, the authors would show data demonstrating that viability or tissue development are adversely affected by immersion. At the very least, however, the authors could comment on this point or cite references on the importance of gas exchange throughout pupal development.

2. No quantification is provided for any of the timelapse imaging presented. The authors only make descriptive observations of the timing of certain events (e.g., “Starting at ~17h APF…”) and annotate their images with arrows. There is no mention of whether the experiments were repeated, how many times, and what kind of variation there may be in the behavior from movie to movie, which would be helpful in assessing the reliability of the method. Some quantification at the cellular level would also be helpful – eg. Is the image quality of the wing/notum sufficient for cell segmentation and tracking? How does the cellular behavior/flows compare to the cited papers that used conventional spinning disc microscopy?

Minor:
1. LZ1 system/ PhaseView ALPHA3 system: for the images displayed in Figure1 and 2, the authors do not describe imaging settings, laser power, exposure, multi-view setup etc.

2. The figure and movie references are not always correct – as just one example, the legend for Figure 6 references movie 3, when it should be movie 4.

3. Figures are not always color blind friendly (Figure 3).

4. Movie 1 is very nice but could be improved with some text annotation.

5. The sample preparation process could be explained in more detail, ideally with a supplementary movie showing dissection and mounting.

6. There are quite a few grammar mistakes, for example (but also check for more):
a. (in Results): “…the lateral view allows to image the contraction the hinge starting…”
b. (in Discussion): “It’s conventional sample illumination…” (should be “its”)
c. (in Discussion): “…illumination without the need to post-acquisition image processing…”
d. (in Discussion): “multidirectional imaging enables to assess…”

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field...

In this manuscript, Leroy and colleagues describe a new imaging set up combining spinning disc imaging, dual objective at different magnification and full rotation of the sample without requirement for aqueous/gel mounting. This so called “multi-view confocal imaging” offers a nice alternative to light-sheet microscopy which combined the resolution and homogeneous excitation provided by spinning-disc scanning with the possibility to perform in toto imaging on rather large sample. This could be very useful to study the connection between body parts during morphogenesis specially for samples which cannot be mounted in liquid media, and should bridge the cellular scale with the full body scale. The manuscript is clear, provide very nice imaging data on various samples and a detail description of the set up. It could be of interest for rather a large number of people in the developmental biology community. I would only suggest a few clarifications in the text regarding the actual procedure of acquisition and maybe a more thorough discussion on the potential limitations of the system.

We thank the reviewer for his/her positive comments on our manuscript and experimental work as well as on the usefulness of the Multi-View confocal microScopy system (MuViScope). We have addressed his/her points below to improve manuscript clarity and further discuss the potential limitations of the system.

Reviewer 1 Comments for the author...

1. The procedure of acquisition was not totally clear to me. I suspect the set up take a full stack and move to another angle. The method part was a bit confusing in this regard and it might help to describe each step of acquisition in temporal order. On the same line, the “rotation speed of 360°/sec” was confusing for me. I suspect this is the speed of rotation that is used to move to a finite number of angles, but it was not obvious on the first read.

The reviewer is fully correct. The acquisition procedure is done in the following order: we acquire a full z-stack and then move to another angle. To clarify this point, we have now stated in the M&M: “The following parameters of acquisitions are available in the MuViScope control software (Metamorph) in a sequential order: xy coordinate of the image (xy), multi-channel imaging (ch), z-stack (z), multi-positions allowing tiling (P), multi-angles (θ) and two different magnifications (M). In brief, the order of acquisition is as follows: xy→ch→z→P→θ→M→t.” (page 14). Furthermore, we have provided a Supplementary table (Table S1) with all details regarding acquisition settings for each figure and movie reported in the manuscript.

Our statement regarding the rotation speed was unclear. The speed of rotation that we had indicated 360°/sec corresponds to the speed set in the control software of the rotation stage. In the revised version, we have now measured the actual rotation speed and found a speed of 212°/sec. To clarify and take into account this measurements, we indicate in M&M: “The rotation speed of the stage is 212°/sec in our experimental conditions. This represents the speed at which the stage can turn from one angle to the next and does not include the time necessary for image acquisition.” (page 15).

2. While the method state that it is possible to use different XY position and stitching, it is not clear whether this was the case in all the data/movie presented in the paper. Could the author

provide more details in the figure legend of each image (how many angles used, how many X-Y positions) ? Also, while the combination of 10X and 40X offers a nice connection between cell resolution and in toto imaging, I suspect it should be possible to perform full reconstruction of the sample at the 40X magnification using multi XY positions and multiple angles. Is that a possibility and was it tested by the authors?

We have clarified whether the acquisitions are performed by stitching multiple positions and/or angles in each figure. As aforementioned we have also added a Supplementary Table (Table S1) which summarizes all acquisition settings used in the manuscript.
To address the second point of the reviewer we have performed a full reconstruction of the head and thorax of the Drosophila pupa at 40x. We limited ourselves to the head and thorax because the pupa needs to be glued to the holder by the pupal case. To best display the result, we have provided a reconstruction as a Movie S3. This illustrates the capability of the microscope to achieve very large 3D reconstructions at cellular resolution.

3. While the discussion provides some explanations on future improvement of the system, I feel it would be fair to provide a list of weakness/limitations of the set up in the discussion (I am not necessarily requesting new data here, but more a clear discussion on potential limitation compared to other set up). For instance, how good is the set up to image rather deep structure compared to light-sheet (the comparison was only performed for surface signal such as E-cad) ? I guess the muscle data shows that it is possible to image deep structure but it is not clear how good is the deep signal compared to what could be obtained using light-sheet microscopy. Also what about bleaching (in principle the light-sheet system should be more performant on this side) ? Is it possible to perform full 3D reconstruction and can it be processed with tools currently used for light-sheet microscopy (if I am correct right now the data in the manuscript just provide separate max projections of a limited number of angles but there is no proper 3D reconstruction) ?

We thank the reviewer for raising these points. We have extended our discussion by providing a description of the pros and cons of the MuViScope relative to light-sheet microscopy (please see second discussion paragraph). The discussion is accompanied by a table summarizing the characteristics of the two imaging modalities for non-immersed samples (Table S2). Based on optical principles, we detail why the MuViScope gives a clear advantage over light-sheet microscopy for non-immersed samples and why the advantage increases with imaging depth. In a nutshell, the high mismatch of refraction index between air and the sample (around 40%) induces a much larger z-offset for light-sheet microscopy that furthermore increases with depth of the imaging. While this z-offset results in strong reduction of resolution in the case of light-sheet imaging, the impact is far less when excitation and acquisition are both done with the same objective in the case of the MuViScope. To best illustrate this point, we have added a figure (Fig. S6) to document the impact of light refraction for MuViScope and light-sheet microscopy imaging and we describe the underlying optical principles in its legend.
Last, we have provided a 3D reconstruction movie where we highlight how the 8 acquired angles are used to perform a 360° sample reconstruction (Movie S2).
Other minor point: The “lowest quality region” shown in the inset of the Figure 1B’ rather seems to be a problem of out of plane signal. If I am correct the intensity of the SOPs is not different from the neighbouring region where the epithelial cell contour is visible, so it seems unlikely to be just a problem of shadowing. I am not questioning the shadowing problem per se, but I am not sure the current inset is the best illustration of this.

We have carefully checked the original image and the lowest quality in this region is not due to a problem of out plane signal. To best illustrate the quality obtained by LSFM, we have added an inset of another region where the signal to noise ratio is better (Figure 1B’).

Reviewer 2 Advance summary and potential significance to field...
The Authors of this manuscript provide an interesting new imaging technique whereby a spinning disc microscope is implemented with two independent optical paths and a rotational sample holder. This technique allows for high quality (cellular resolution), large-scale (whole organism) imaging without the use of dipping lenses, which are common in light-sheet microscopes. Thus, it accommodates organisms that require gas exchange and cannot be immersed in liquid, such as the Drosophila pupae, a powerful model organism for tissue morphogenesis and organ growth. The

authors show examples of how this technique enables very long-term live imaging (> 90hr of development), as well as cellular resolution whole organism imaging (21hr of development every 30min, three regions, 5 views). They also demonstrate the versatility of the setup using different organisms. The microscopy setup is well explained and should allow for the reproduction of the system by the reader. I assume this article is meant for the Techniques section of Development.

In general, the technique is novel and could provide a powerful new way of looking at the organismal-wide coordination of organ growth and morphogenesis in certain model organisms, particularly insects. It is an important open question in developmental biology how organ morphogenesis and growth is systemically coordinated, and thus this technique should enable exciting new discoveries and would be of interest to the readers of Development. Suggestions for improvement are discussed below.

We are grateful to this reviewer for her/his positive comments on our imaging technique. We have now addressed his/her points to improve our manuscript, which is intended for the Techniques and Resources section of Development.

Reviewer 2 Comments for the author...

Major:

1. The authors motivate the development of this technique in part by explaining that light-sheet microscopy, an established technique for rapid, gentle, large-scale imaging, is not suitable for certain organisms such as the Drosophila pupae. There are a couple of weaknesses in this argument:

a. If I understand correctly, the feasibility and quality of the commercial light-sheet microscopes was only evaluated using dual-sided illumination with a single view (no sample orientation). A multi-view approach, where the sample is rotated and images acquired at multiple angles, would like increase image quality and result in a much more homogeneous illumination than that shown in Fig 1. The authors attribute the poor image quality to shadowing artifacts that are inherent to light-sheet microscopy. While shadowing undoubtedly has an effect, multi-view imaging, followed by fusion and deconvolution generally alleviates this problem to a certain extent. It is fair to argue that the later approach is heavy on data size and image processing. Nevertheless, for assessing image quality on the Z1 system, these optimization steps should not be neglected.

We fully agree that multi-view imaging should increase the light-sheet resolution and overcome some of the shortcoming associated with shadowing effect. We had stated this point in the introduction and now further indicated it in the discussion when comparing the light-sheet and MuViScope capabilities (please see second discussion paragraph). To best illustrate this point and avoid undermining the capability of light-sheet microscopy, we have added a fusion and deconvolution image of the immersed pupa imaged by the Z1 light-sheet (Zeiss) with dual illumination and multiple angles. As expected by the reviewer, this increases resolution in the medial part of the tissue (Fig. S1B). More importantly, the pupa does not develop upon immersion as shown in Movie S1; thus, precluding the use of light-sheet imaging to perform time-lapse microscopy of the Drosophila pupa using the Z1 light-sheet (Zeiss).

b. The authors state that the Drosophila pupae requires gas exchange and cannot be immersed in liquid, but no data are actually presented to show loss of viability or adverse effects on tissue development upon immersing the pupae for light-sheet imaging. Does the entire pupal development require gas exchange? Is it possible to immerse the pupae only periodically for imaging and remove it in between time points? Ideally, the authors would show data demonstrating that viability or tissue development are adversely affected by immersion. At the very least, however, the authors could comment on this point or cite references on the importance of gas exchange throughout pupal development.

Previous publications reported the importance of gas exchange for the proper development of the pupa (Fourche, 1967; Merkey et al., 2011). We therefore based our reasoning on these publications. However, the reviewer is fully right that we did not include an analysis of the effect of immersion on pupal development. To address this point, we have performed the following experiment using

spinning disk microscopy: we let the pupa develop for 50 minutes to ensure good pupal viability and stable imaging. Then PBS was added to one side of the pupa using a pipette, which resulted in an acute stop of cell cycle progression (after 5 min of immersion). This confirms that immersion is incompatible with live imaging of Drosophila pupae. We have provided this additional data as Movie S1 and have incorporated the results in the main text.

2. No quantification is provided for any of the timelapse imaging presented. The authors only make descriptive observations of the timing of certain events (e.g., "Starting at ~17h APF…") and annotate their images with arrows. There is no mention of whether the experiments were repeated, how many times, and what kind of variation there may be in the behavior from movie to movie, which would be helpful in assessing the reliability of the method. Some quantification at the cellular level would also be helpful – eg. Is the image quality of the wing/notum sufficient for cell segmentation and tracking? How does the cellular behavior/flows compare to the cited papers that used conventional spinning disc microscopy?

We thank the referee for pointing out this lack of information. We have improved our manuscript by stating the number of times each experiment was performed in each figure. In particular, the analyses of tissue dynamics by MuViScope microscopy were performed at least 3 times to ensure reproducibility of the presented results. We agree that our initial indications of developmental timings were too vague. We have now provided more precise indications regarding developmental timings in the main text (mean values rounded to the half hour) and in the figure legends (mean values and standard errors).

To determine the reproducibility of tissue flows by the MuViScope and to compare it to spinning disk microscopy, we have compared the tissue flows in the notum and apical cell area variation in the wing during pupal morphogenesis. These quantitative analyses show that MuViScope imaging gives results similar to the ones obtained by spinning disk microscopy both for mean and standard error values (Figure S3 and S4). Last, we have shown that MuViScope time-lapses can be used for cell tracking (Figure S5). These additional quantitative analyses confirm the quality and relevance of MuViScope imaging for developmental studies.

Minor:

1. LZ1 system/ PhaseView ALPHA3 system: for the images displayed in Figure1 and 2, the authors do not describe imaging settings, laser power, exposure, multi-view setup etc.

We have now included all the acquisition information and experimental conditions in the Table S1.

2. The figure and movie references are not always correct – as just one example, the legend for Figure 6 references movie 3, when it should be movie 4.

This mistake has been corrected.

3. Figures are not always color blind friendly (Figure 3).

We have modified all necessary figures so that all figures are color blind friendly.

4. Movie 1 is very nice but could be improved with some text annotation.

We have added text annotation to Movie 1.

5. The sample preparation process could be explained in more detail, ideally with a supplementary movie showing dissection and mounting.

A step-by-step protocol for sample preparation for the MuViScope and is available in the supplementary document (Supplementary Material, Method S1).

6. There are quite a few grammar mistakes, for example (but also check for more):
a. (in Results): "…the lateral view allows to image the contraction the hinge starting…"
b. (in Discussion): "It's conventional sample illumination…" (should be "its")

c. (in Discussion): “…illumination without the need to post-acquisition image processing…”
d. (in Discussion): “multidirectional imaging enables to assess…”

These mistakes have been corrected and we have extensively proofread our manuscript.

Bibliography:

Fourche, J. (1967). La respiration chez Drosophila melanogaster au cours de la métamorphose. Influence de la pupaison, de la mue nymphale et de l’émergence. Journal of Insect Physiology 13, 1269–1277.
Merkey, A. B., Wong, C. K., Hoshizaki, D. K. and Gibbs, A. G. (2011). Energetics of metamorphosis in Drosophila melanogaster. Journal of Insect Physiology 57, 1437–1445.

Second decision letter

MS ID#: DEVELOP/2021/199760

MS TITLE: Multi-view confocal microscopy enables multiple organ and whole organism live-imaging

AUTHORS: Olivier Leroy, Eric Van Leen, Philippe Girard, Aurelien Villedieu, Christian Hubert, Floris Bosveld, Yohanns Bellaïche, and Olivier Renaud

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

The overall evaluation is very positive and we would like to publish your manuscript in Development. Reviewer 1 makes some useful suggestions for your consideration. It would be good and rather quick to address them before proceeding with formal acceptance.

Reviewer 1

Advance summary and potential significance to field

The authors have adressed all my concerns and significantly improved the clarity of the manuscript and the imaging procedure description. They also provide much more data documenting the resolution, reproducibility and capacitiy to process the output (segmentation and tracking). I therefore fully support the publication of the manuscript.

Comments for the author

See above

Reviewer 2

Advance summary and potential significance to field

This is a revised version of a previously submitted manuscript describing the development of a multi-angle spinning disc confocal microscope (named “MuViScopy”). The authors describe its benefits over light-sheet microscopy for examining the development of animals that require air exchange. They demonstrate its effectiveness using mostly Drosophila pupal development, but also two other arthropods. The authors have improved the manuscript by implementing many changes proposed by the reviewers in the first round. The technique will be valuable for the community,

enabling many types of experiments aimed at bridging the cell to organismal length scales. The manuscript now looks ready for publication.

Comments for the author

The authors have well addressed all of my previously mentioned comments. I think it is ready for publication. I have only the following minor comments for consideration:
1. There are still overlapping labels at the bottom of Fig 2A.
2. Fig S3: the overall flow patterns seem similar, but the different grid sizes used to analyze the two movies makes it difficult to compare exactly. If it is possible to have them on the same grid size, it would be easier for the reader.
3. Are the authors aware that there is a commercially available light-sheet microscope from Brucker (MuViSpim) with a very similar name to the one being proposed here (MuViScope)(https://www.bruker.com/en/products-and-solutions/fluorescence-microscopy/light-sheet-microscopes/muvi-spim-family/muvi-spim-ls.html)? The authors may want to consider revising the name for their technique to avoid confusion? Just a suggestion.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
The authors have adressed all my concerns and significantly improved the clarity of the manuscript and the imaging procedure description. They also provide much more data documenting the resolution, reproducibility and capacitiy to process the output (segmentation and tracking). I therefore fully support the publication of the manuscript.

We thank the reviewer for her/his positive review and for supporting the publication of our work.

Reviewer 2 Advance summary and potential significance to field

This is a revised version of a previously submitted manuscript describing the development of a multi-angle spinning disc confocal microscope (named “MuViScopy”). The authors describe its benefits over light-sheet microscopy for examining the development of animals that require air exchange. They demonstrate its effectiveness using mostly Drosophila pupal development, but also two other arthropods. The authors have improved the manuscript by implementing many changes proposed by the reviewers in the first round. The technique will be valuable for the community, enabling many types of experiments aimed at bridging the cell to organismal length scales. The manuscript now looks ready for publication.

Reviewer 2 Comments for the author

The authors have well addressed all of my previously mentioned comments. I think it is ready for publication. I have only the following minor comments for consideration:

We thank the reviewer for her/his positive comments, and we have now corrected all minor errors.

1. There are still overlapping labels at the bottom of Fig 2A.

The overlapping label has been corrected.

2. Fig S3: the overall flow patterns seem similar, but the different grid sizes used to analyze the two movies makes it difficult to compare exactly. If it is possible to have them on the same grid size, it would be easier for the reader.

We are grateful to the reviewer for pointing out this difference. To average and compare animals, we use the macrochaetae as spatial landmarks to register each tissue in space and time. We then use this information to determine each tissue origin and scaling factors to be applied in the analysis. Accordingly, the grid compartments in which we calculated the PIV cover the same regions in each pupa. One of the pupae displayed in Fig. S3 is smaller than the other, leading to smaller grid compartments on this animal; thus, upon rescaling of the images for the figure, the PIV box appears bigger in the smaller animal. Based on this reviewer comment, we have decided to show two pupae of more similar sizes to facilitate the comparison and remove any ambiguities (see new S3A-B).

Furthermore, we realized that we had made a very minor mistake in the averaging of PIV flows: for two animals among the six ones used, we had not applied the appropriate scaling factor needed to correct for differences in animal sizes. The correction of this scaling factor being small (changing the compartment size of about 10% in those two animals), the change did not affect the overall interpretation of the data and all conclusions are correct and remain valid.

To improve the figure and the manuscript, we have modified Fig S3A-B. We also added the averaged flows for three spinning disk pupa movies versus three MuViScopy pupa movies for one time-point (Fig. S3D,E) and also displayed them as Movie S4. Last, we have better detailed the registration and averaging procedures in M&M.

3. Are the authors aware that there is a commercially available light-sheet microscope from Brucker (MuViSpim) with a very similar name to the one being proposed here (MuViScope)(https://www.bruker.com/en/products-and-solutions/fluorescence-microscopy/light-sheet-microscopes/muvi-spim-family/muvi-spim-ls.html)? The authors may want to consider revising the name for their technique to avoid confusion? Just a suggestion.

We thank the reviewer for indicating this point. We were aware of the existence of MuViSpim. We believe that the name MuViScope is different and therefore we rather keep it.

Third decision letter

MS ID#: DEVELOP/2021/199760

MS TITLE: Multi-view confocal microscopy enables multiple organ and whole organism live-imaging

AUTHORS: Olivier Leroy, Eric Van Leen, Philippe Girard, Aurelien Villedieu, Christian Hubert, Floris Bosveld, Yohanns Bellaïche, and Olivier Renaud
ARTICLE TYPE: Research Article

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