Multiview tiling light sheet microscopy for 3D high-resolution live imaging

Mostafa Aakhte and Hans-Arno J Müller
DOI: 10.1242/dev.199725

Editor: Thomas Lecuit

Review timeline
Original submission: 18 April 2021
Editorial decision: 26 May 2021
First revision received: 31 July 2021
Accepted: 13 August 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/199725

MS TITLE: Multiview tiling light sheet microscopy for 3D high resolution live imaging

AUTHORS: Mostafa Aakhte and Hans-Arno J Mueller

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 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

Aakhte and Müller report on the development of a new form of light sheet microscopy (Multiview tiling light sheet microscopy, MT-SPIM). They examine the relative strengths of MT-SPIM versus non-tiling M-SPIM microscopy, and do a good job describing the light paths and construction parameters of the MT-SPIM (although a reviewer with an optical physics background would be better at critically evaluating this). They then apply MT-SPIM to image nuclei and Myosin II across the entire embryo, and compare these data with that of the non-tiling M-SPIM. Their approach permits the

simultaneous imaging of posterior morphogenetic movements (like the posterior midgut) and anterior movements (like cephalic furrow formation), which allows a better timing of the relative initiation of the events. The authors also see different Myosin II accumulation rates in different events, suggesting possible different initiating mechanisms. In total, MT-SPIM seems like a promising new approach to whole embryo imaging, and their work appears to be well-justified. A few more detailed comments are below:

Comments for the author

1) One critique – the authors only compare their approach to their own internal data using M-SPIM, so the reported advances in resolution are essentially against their own microscope (presumably minus the SLM module?). Is there a commercial light sheet scope available to them that they could compare their data to? I know it may not be an apples-to-apples comparison (e.g., in terms of field of view), and perhaps the improved MT-SPIM data is so obvious to the authors that they didn't feel it was warranted to make this kind of comparison, but it is notable that they are comparing their resolution standards to their own microscope. It also could be worth discussing this further in the manuscript.

2) page 11, "The lateral and axial resolution of the MT-SPIM is measured at 800 ± 0.04 μm and 1.4 ± 0.09 μm with deconvolution, respectively." I was confused by these numbers, this difference in resolution along the two axis cannot be correct, can it? Or please explain more thoroughly what is being measured here.

3) page 12, "MyoII-GFP is lacking at several anterior-posterior cell boundaries suggesting a partially planar polarized distribution at the onset of cellularization." Is there something in M-SPIM that introduces this bias? Seemed like a bit of an odd finding/statement that could be clarified.

4) Wasn't so sure about this statement, "The differences in the rate changes of MyoII-GFP intensities suggest different mechanisms or differences in the MyoII associated mechanics in these movements." (page 14), or maybe it could just be clarified a little. Or does it come down to semantics of what a "mechanism" is? But, to me, you could get different rates out of a similar biochemical mechanism (RhoGEF activation of Rho) depending on the previously reported regulation by GPCRs, etc. Maybe that differential GPCR regulation is itself the "mechanism", though that could be more explicitly referenced if that is the author's intent.

5) Fig. 3D – why does the FWHM improve with time? Is this due to a shift in Z (either in the scope or in the embryo?)?

Reviewer 2

Advance summary and potential significance to field

The work presented in this paper focuses on the development and testing of a novel light sheet microscope system, which combines Multiview SPIM and a tiled light sheet generated using a spatial light modulator. The usefulness of combining the two techniques is tested by systematically comparing the imaging of live Drosophila embryos with and without light sheet tiling. The authors show that light sheet tiling gives a significant improvement in the image resolution, in particular in the axial direction. The manuscript is well written and the images presented of high quality. Imaging examples include following cortical Myosin II dynamics at the scale of the whole Drosophila embryo during gastrulation. The authors also show that the quality of the imaging can improve subsequent analyses such as image segmentation, as shown by a rather impressive segmentation of nuclei at gastrulation. Although there are no biological results included in the paper, the high quality imaging of a whole embryo presented will be of broad interest to developmental biologists and microscopy scientists.

Comments for the author

Main suggestions:

1) To make their work more relevant to a developmental biologist audience, the authors need to discuss further the advantages of their system compared to other light sheet systems already in use, such as those developed by the Hufnagel and Huisken labs (Huisken and Stainier, 2007; Krzic et al, 2012). Both of these systems have been applied successfully in a number of developmental studies imaging whole Drosophila embryos (e.g Rauzi et al 2015; Streichan et al 2018; Lye et al 2015, Dicko et al, 2017). The authors need to discuss their imaging more directly in the context of these existing examples. Note that some of these studies have used sample rotation followed by image recombination successfully to generate usable whole embryo images, so when the authors highlight some potential drawbacks of rotation, it might be useful to also have a practical assessment of why no rotation is better (is it easier/cheaper to implement?)

2) The images comparing M-SPIM with MT-SPIM are impressive but the claim that axial resolution is increased by a factor 2 needs to be supported better by increasing n for the intensity profiles and using appropriate statistics, if applicable.

3) The discussion could be expanded to include other advances in the light sheet imaging field including OPEN-SPIM (Pitrone et al, 2013), use of Gaussian versus Bessel beams, lattice light sheet (eg Chen et al, 2014) and confocal multi-view SPIM (de Medeiro et al, 2015), in order to highlight better the specific contribution that MT-SPIM is making to the microscopy field.

4) The nuclear segmentation presented is impressive but more discussion of the challenges of performing segmentation on whole embryo imaging data would be useful.

Technical comments:

1) There is limited interpretation of the Myosin II whole embryo images in Figure 4, but for the few observations reported, are these based on imaging a single embryo?

2) A key advantage of light sheet imaging is to reduce phototoxicity, however this can still occur. Do the authors check that the embryos survive the imaging protocol?

3) Despite the known advantage that a klar mutant background reduces light scattering, it has not been widely employed for live imaging by the Drosophila imaging community. Would the nuclear segmentation be possible without the use of klar? Have the authors checked that klar embryos develop normally? The use of this genetic background should also be made clearer in the results and legends, in addition to being documented in methods.

Reviewer 3

Advance summary and potential significance to field

Past work on embryonic morphogenesis has used light sheet microscopy to track cell shape changes/movements and to look at myosin polarity throughout the embryo (Lye et al., PLOS Bio, 2015; Rauzi et al., Nat. Comm., 2015; Streichan et al., eLife, 2018). In this study, the authors present a method of light sheet microscopy that uses multiview tiling to enhance axial resolution. The authors illustrate the effectiveness of this imaging modality by imaging changes in myosin across the embryo during Drosophila cellularization and gastrulation and also by imaging nuclei during cellularization.

Comments for the author

My main criticism is that I do not see how the authors showed the improvement of their imaging system over previous studies. The authors claim to be able to image subcellular structure, but most of what they image are cell boundaries, which has been shown to be possible in numerous previous studies (Lye et al., PLOS Bio 2015; Rauzi et al., Nat. Comm., 2015; Streichan et al., eLife, 2018). Much of the manuscript focuses on cellularization, which occurs on time scales that are very long and are amenable to characterizing in fixed embryos (i.e. using furrow length as a proxy for time) (Figard et al., Dev. Cell, 2013, 2016). Also, the authors avoided regions of complex curvature, which would have benefited the most from enhanced resolution.

Can the authors observe the wave of myosin accumulation in the posterior midgut (Bailles et al., Nature 2019)? Imaging these temporal dynamics and the subcellular organization of myosin within the posterior midgut, which form rings (Chanet et al., Nat. Comm., 2017), would be a better test of the system's resolution.

Minor:
Page 13 : "MyoII-GFP intensities at the apical domains arise at about 10 min after the start of the cellularization" Do the authors mean that apical MyoII-GFP begins 10 min prior to start of gastrulation?
Apical myosin appears right before mesoderm invagination (Martin et al., 2009) and this is well after the start of cellularization, which takes ~ 50 min.

Page 14: "The differences in the rate changes of MyoII-GFP intensities suggest different mechanisms or differences in the MyoII associated mechanics in these movements." Couldn't it also reflect differences in signaling strength?

First revision

Author response to reviewers' comments

Reviewer 1

We thank the referee for commending our manuscript and our presentation of the MT-SPIM advantages over previous SPIM imaging techniques.

Comments for the author
1) One critique – the authors only compare their approach to their own internal data using M-SPIM, so the reported advances in resolution are essentially against their own microscope (presumably minus the SLM module?). Is there a commercial light sheet scope available to them that they could compare their data to? I know it may not be an apples-to-apples comparison (e.g., in terms of field of view), and perhaps the improved MT-SPIM data is so obvious to the authors that they didn't feel it was warranted to make this kind of comparison, but it is notable that they are comparing their resolution standards to their own microscope. It also could be worth discussing this further in the manuscript.

Response
In order to obtain an accurate comparison of the operational parameters between the MT-SPIM and the M-SPIM, we chose to directly compare the performance of the two microscopic modes of operation on the same system and with precisely the same specimen. To our understanding this is the best possible way to directly compare the performance of microscopic techniques. In particular, different specimens of the same kind of living samples, their orientation and/or their transparency are difficult to control and thereby will render a direct comparison from data of two different systems rather unreliable. Also, the direct comparison to a commercially available set up is complicated by the different specifications of individual components in the two systems including the magnification and numerical aperture of the objective lenses, or the application and the type of spatial light modulator (SLM), which in fact is unique to our MT-SPIM system. Because of these variables that complicate the comparison of the MT-SPIM to commercial M-SPIM systems, we decided to use the advantage of the fast SLM to directly compare optical parameters in imaging living Drosophila embryos in a near-simultaneous fashion. Importantly, the application of the SLM enables us to simulate the M-SPIM situation as well as other beam engineering techniques in the context of either SPIM mode by using SSPIM (Aakhte et al., 2019, Sci. Rep.). We added a comment to the manuscript to emphasize the advantage of comparing the two microscopy techniques in the same system.

2) page 11, "The lateral and axial resolution of the MT-SPIM is measured at 800 ± 0.04 μm and 1.4 ± 0.09 μm with deconvolution, respectively." I was confused by these numbers, this difference in

resolution along the two axis cannot be correct, can it? Or please explain more thoroughly what is being measured here.

Response
We are very grateful to the referee for pointing this out and are sorry for making a mistake here. The sentence must read as: “The lateral and axial resolution of the MT-SPIM is measured at 0.74 ± 0.05 µm and 1.55 ± 0.14 µm with deconvolution, respectively.” We have corrected this sentence in the revised manuscript.

3) page 12, “MyoII-GFP is lacking at several anterior-posterior cell boundaries suggesting a partially planar polarized distribution at the onset of cellularization.” Is there something in M-SPIM that introduces this bias? Seemed like a bit of an odd finding/statement that could be clarified.

Response
We thank the referee for pointing us to our incomplete interpretation of the data. There is indeed a difference in MyoII-GFP-distribution in the primary phase of cellularization, that even may occasionally appear as if MyoII-GFP exhibits a planar polarized distribution early in cellularization. This uneven distribution of MyoII-GFP around the early furrows may arise from the reported difference in MyoII intensities based on the formation of new cell boundaries (He et al. 2016). These new cell boundaries have been described to emerge gradually in the early priming phase (Acharya et al., 2014, He et al. 2016). In the M-SPIM mode, the uneven MyoII-GFP distribution is indeed visualized partially as a planar polarized MyoII-GFP distribution. However, when comparing the MyoII-GFP localization with the MT-SPIM, we do not see this effect, but instead we can resolve the uneven distribution of MyoII and the new cell boundaries being formed at the entry of cellularization. The comparison of the two modes at this stage of development does in fact illustrate another example for how a poor resolution in SPIM can mislead the interpretation of a dynamic subcellular distribution of proteins. We have slightly expanded this discussion in the manuscript to emphasize this problem and how to improve the imaging with the different techniques.

4) Wasn’t so sure about this statement, “The differences in the rate changes of MyoII-GFP intensities suggest different mechanisms or differences in the MyoII associated mechanics in these movements.” (page 14), or maybe it could just be clarified a little. Or does it come down to semantics of what a “mechanism” is? But, to me, you could get different rates out of a similar biochemical mechanism (RhoGEF activation of Rho) depending on the previously reported regulation by GPCRs, etc. Maybe that differential GPCR regulation is itself the “mechanism”, though that could be more explicitly referenced if that is the
author’s intent.

Response
We agree with the referee that our discussion on the different rate changes in different morphogenetic events was a bit obscure with respect to potential differences in the underlying biochemical mechanisms. We slightly expanded the discussion of this aspect and added references pointing out differential biochemical regulation of MyoII by cell surface receptors.

5) Fig. 3D – why does the FWHM improve with time? Is this due to a shift in Z (either in the scope or in the embryo?)?

Response
The difference in FWHM is based on the underlying MyoII signal measured in the embryo. The time parameter indicates simply the progression through cellularization and the imaging of the dynamic MyoII structures that are imaged. During the process of cellularization, the MyoII at cell boundaries are getting more confined over time and eventually form individually defined rings. The confinement of MyoII at boundaries can only be completely resolved by applying the MT-SPIM mode which has a higher resolution compared to the non-tiling method and the reconstructed data is also expected to give considerably more information about the MyoII distribution. Since the axial resolution of the MT-SPIM is approximately 1.5 µm which is more than 80 percent better than the M-SPIM in an identical same situation with 2.7 µm, the imaging component’s size is going to be considerably more important when its size lies in between these two resolutions. If we measure the distance between two opposing cell borders in a single cell during cellularization, the findings of

the cell boundaries also should differ by approximately 1 μm between the two methods of measurement. This measurement is becoming important when the radius of the MyoII ring shape is getting much smaller than the M-SPIM resolution resulting the better data achievement with the MT-SPIM with higher resolution. These is exactly the reason why the FWHM measured from MT-SPIM data is seemed to be enhanced over the same measurement from M-SPIM which is also independent of the undesired shift either in embryo or scope.

Reviewer 2

We greatly appreciate the overall positive assessment by the reviewer on the potential and significance of our findings for the scientific community and in particular developmental biologist and microscopy scientists.

Reviewer 2 Comments:
1) To make their work more relevant to a developmental biologist audience, the authors need to discuss further the advantages of their system compared to other light sheet systems already in use, such as those developed by the Hufnagel and Huisken labs (Huisken and Stainier, 2007; Krzic et al, 2012). Both of these systems have been applied successfully in a number of developmental studies imaging whole Drosophila embryos (e.g Rauzi et al 2015; Streichan et al 2018; Lye et al 2015, Dicko et al, 2017). The authors need to discuss their imaging more directly in the context of these existing examples. Note that some of these studies have used sample rotation followed by image recombination successfully to generate usable whole embryo images, so when the authors highlight some potential drawbacks of rotation, it might be useful to also have a practical assessment of why no rotation is better (is it easier/cheaper to implement?)

Response
We thank the referee for these remarks. As explained in the response to referee #1, we decided to directly compare the performance of the two microscopic modes of operation on the same system and with precisely the same specimen. We think that this is the best way to directly compare the performance of two microscopic techniques, because of comparable system and specimen parameters. One of the major differences of our set up to the previously reported Multiview systems is the application and type of spatial light modulator (SLM), which in fact is unique to our MT-SPIM system. The application of the SLM enables us to simulate the M-SPIM situation as well as other beam engineering techniques in the context of either SPIM mode by using SSPIM (Aakhte et al., 2019).
For example, the application of the SLM facilitates the increase of the FOV in our MT-SPIM configuration by adding more tiles, which is is not easy when using the previously introduced MuVi-SPIM or SIM-light sheet microscopes. Our overall goal was to obtain a high-resolution image of a three-dimensional sample over time in such a way that the sample is imaged as smoothly as possible to avoid loss of information during dynamic processes. To accomplish this, the standard MuVi-SPIM introduces a technique of imaging through rotation, which is reasonable but has three significant drawbacks. The first limitation is that the sample may move unintended due to the rotation, resulting in a blurred image. Second, the mechanical sample stage should move slowly enough that the agarose and the sample do not drift out of the imaging and light sheet FOV. Third, because the sample is rotated multiple times at a specific time point for Multiview imaging, which can take approximately 10-12 seconds (Mvi-SPIM paper), the time delay between each rotated volume can result in a time misalignment between the imaged volume and a motile subcellular structure throughout the live sample, resulting in an incorrect image fusion, which also has been shown earlier by Roju Tomer (Tomer et al., 2012). All of these constraints have been addressed in our tiling approach in conjunction with a rotation-free Multiview imaging light sheet microscope. Because the sample is illuminated using the tiling method with several thin light sheets, the exposure time of each light sheet is confined to 20 ms. The initial imaging plane is illuminated with about six tiles and requires a total of 120 milliseconds. Following that, the adjacent plane is imaged with six tiles directly following the first plane, and this process is repeated 200 times to cover the embryo. Thus, the time delay between neighboring planes is only approximately 120 ms, significantly less than the 10 seconds time delay between the rotation method, which significantly improves the homogeneous timing in single volume imaging. Additionally, because the sample is fixed in place, minimal image post-processing and registration are necessary to match the various views of the recorded images, making our technique simple to use for biologists.

2) The images comparing M-SPIM with MT-SPIM are impressive but the claim that axial resolution is increased by a factor 2 needs to be supported better by increasing n for the intensity profiles and using appropriate statistics, if applicable.

Response
To determine the resolution of the M-SPIM and MT-SPIM, we conducted a set of experiments to measure the point spread function of each system by imaging 400 μm diameter fluorescent microspheres at random locations. We combined 1% microspheres with 1.5% low melting agarose gel to have microspheres in fixed positions, allowing us to scan microspheres using two modes of the SPIM at the same situation. The microspheres are scanned in the same manner as the Drosophila embryo is imaged, by collecting 200 images with a 1 μm z-step. Following imaging, the data from the stack are resliced from the top view to provide an axial image of the microspheres. As predicted, the intensity distribution of each individual microsphere along the axial axis is significantly longer than along the lateral axis. As a standard parameter in resolution measurement, we used the full width at half maximum of the microsphere's center of intensity in both axial and lateral directions. The results reveal that the FWHM of the measured PSF of the MT-SPIM is about 2 and 1.6 times smaller than the PSF of the M-SPIM before and after deconvolution along the 200 μm imaging depth, respectively. These results demonstrate the increased axial resolution by the MT-SPIM method. We have added these results as a new figure in the supplementary material (Supplementary Figure S6).

3) The discussion could be expanded to include other advances in the light sheet imaging field including OPEN-SPIM (Pitrone et al, 2013), use of Gaussian versus Bessel beams, lattice light sheet (eg Chen et al, 2014) and confocal multi-view SPIM (de Medeiro et al, 2015), in order to highlight better the specific contribution that MT-SPIM is making to the microscopy field.

Response
An earlier manuscript draft actually contained these aspects, which were left out in the submission upon the advice by our colleagues. The OPEN-SPIM platform (Pitrone et al., 2013) was actually already mentioned in the introduction. We now also commented on the potential of improving the SPIM performance by beam engineering or confocal applications and added the respective references to the introduction section.

4) The nuclear segmentation presented is impressive but more discussion of the challenges of performing segmentation on whole embryo imaging data would be useful.

Response
An important limiting parameter in commonly used algorithms in segmentation is based on fluorescence intensity thresholds and the imaging resolution. In the case of segmenting dynamic whole volume embryo imaging has additional limitations along the axial axis due to SPIM’s poor axial resolution. We show that segmentation using data obtained by the M-SPIM mode is also particularly limited because of its lower resolution, such that nucleus which are in the axial view of the embryo cannot be solved reliably. But, in the same situation all individual nuclei imaged with MT-SPIM are resolved and segmented successfully. We also encountered a similar problem in the case of MyoII GFP imaging in cellularization, where the dynamics of new furrow formation was mistaken as a planar polarized distribution of MyoII GFP when applying the M-SPIM mode. We added a more detailed discussion of these problems to the discussion.

Technical comments:
1) There is limited interpretation of the Myosin II whole embryo images in Figure 4, but for the few observations reported, are these based on imaging a single embryo?

Response
The MyoII data are based on three independent imaging experiments and we added a note of this fact to the figure legend. The primary focus of this part of the study was on imaging of known MyoII distributions and localizations to assess improvements in the dynamics of subcellular and supracellular MyoII structures. We appreciate the fact that in depth analyses of any specific morphogenetic event in the context of the entire embryo would mean to quantify the results of

multiple independent samples. However, we believe that this was not the main purpose of the manuscript and would go well beyond its scope.

2) A key advantage of light sheet imaging is to reduce phototoxicity, however this can still occur. Do the authors check that the embryos survive the imaging protocol?

Response
All embryos that are imaged in our imaging system were further cultured after imaging and hatched to larval stages. We added a note to the methods section.

3) Despite the known advantage that a klar mutant background reduces light scattering, it has not been widely employed for live imaging by the Drosophila imaging community. Would the nuclear segmentation be possible without the use of klar? Have the authors checked that klar embryos develop normally? The use of this genetic background should also be made clearer in the results and legends, in addition to being documented in methods.

Response:
The mutation klar has indeed been rarely applied to fluorescent live imaging in Drosophila, which may be explained by the fact that the advantages described below apply to early morphogenetic stages and the imaging of deep cells in embryos, but not in other tissues.
In the early embryo, lipid droplets are present in the periphery of the blastoderm stage, move towards the center early during interphase of mitotic cycle 14 and back to the periphery at the end of cellularization (Welte et al., 1998; Arora et al., 2016). Because of their high diffractive index and light scattering properties, lipid droplets reduce the excitation and the emission of fluorescence in the sample. The klarsicht (klar) mutation affects microtubule-dependent transport of lipid droplets and in particular impairs their outward movement late in cycle 14; as a result, the majority of lipid droplets is trapped in the central yolk cell (Welte et al., 1998). This severe reduction of lipid droplet numbers in the newly formed cells of the cellular blastoderm improves fluorescent imaging particularly of cells beneath the surface layer of cells in the embryo, such as the developing mesoderm and endoderm tissues. In the case of nuclear imaging in syncytial division cycles, the mutant klar background may not make a large difference, as the klar phenotype only occurs after the onset of gastrulation. However, at gastrulation stages, the klar mutant background is advantageous for imaging using His2AV.GFP, as lipid droplets also accumulate histones and peripheral His2Av-GFP may affect imaging, image processing and quantification or segmentation of nuclei deeper in the cytoplasm.
Females homozygous for the loss-of-function allele klar1 are viable and fertile and except for the lipid droplet clouding defect, no other developmental defects have been reported in embryos obtained from klar1 homozygotes (Welte et al., 1998). However, a fraction of embryos from mothers homozygous for another klar allele arrest before the blastoderm stage, likely due to altered oskar mRNA localization during oogenesis (Gaspar et al., 2014). Even if klar1 displays similar defects, it is unlikely to affect the studies described here because we only follow embryos from blastoderm onwards. In later stages, lack of Klar affects some additional processes, such as positioning of nuclei in photoreceptors and muscles in larvae and adults and the growth of the apical membrane in the development of embryonic salivary glands.

Reviewer 3
Comments for the author
My main criticism is that I do not see how the authors showed the improvement of their imaging system over previous studies. The authors claim to be able to image subcellular structure, but most of what they image are cell boundaries, which has been shown to be possible in numerous previous studies (Lye et al., PLOS Bio, 2015; Rauzi et al., Nat. Comm., 2015; Streichan et al., eLife, 2018). Much of the manuscript focuses on cellularization, which occurs on time scales that are very long and are amenable to characterizing in fixed embryos (i.e. using furrow length as a proxy for time) (Figard et al., Dev. Cell, 2013, 2016). Also, the authors avoided regions of complex curvature, which would have benefited the most from enhanced resolution.

Can the authors observe the wave of myosin accumulation in the posterior midgut (Bailles et al., Nature, 2019)? Imaging these temporal dynamics and the subcellular organization of myosin within

the posterior midgut, which form rings (Chanet et al., Nat. Comm., 2017), would be a better test of the system's resolution.

Response:
We thank the referee for this criticism and agree that we might not yet take full advantage of the whole volume embryo imaging over time in all aspects. The imaging of rapid processes and events happening in areas of complicated geometries like the anterior and posterior poles of the embryo are indeed particularly challenging for confocal fluorescent imaging, while those areas can be revealed much better using SPIM-based techniques. To address the referee's comment, we have slightly extended the analyses on the posterior midgut invagination as suggested. As the referee suggested, we now demonstrate that the previously published subcellular details in the initiation of the posterior midgut invagination can be dynamically followed providing information on the position of the subcellular MyoII-GFP accumulations over time. We demonstrate the initial accumulation of MyoII-GFP at apical junctions and their aggregation into subcellular MyoII-GFP rings in the posterior midgut primordium. The resolution of these rings (Chanet et al. 2017) is at the limit of lateral resolution that our system is currently able to provide. In addition, we demonstrate the more amorphic MyoII-GFP accumulations during the propagation phase as demonstrated in Bailles et al., 2019. We added these data as Figure S10 to the Supplemental Material section and a movie (movie 7) as additional examples for how the MT-SPIM provides an advantage to previous studies in imaging subcellular events at areas of complex geometries.

Minor:
Page 13 : "MyoII-GFP intensities at the apical domains arise at about 10 min after the start of the cellularization" Do the authors mean that apical MyoII-GFP begins 10 min prior to start of gastrulation? Apical myosin appears right before mesoderm invagination (Martin et al., 2009) and this is well after the start of cellularization, which takes ~ 50 min.

Response:
We thank the referee for pointing out this error and we corrected this statement in the revised manuscript.

Page 14: "The differences in the rate changes of MyoII-GFP intensities suggest different mechanisms or differences in the MyoII associated mechanics in these movements." Couldn't it also reflect differences in signaling strength?

Response:
We appreciate the concern, which was also raised by by referee 1 and we have expanded the discussion of this point to include alternative explanations.

Second decision letter

MS ID#: DEVELOP/2021/199725

MS TITLE: Multiview tiling light sheet microscopy for 3D high resolution live imaging

AUTHORS: Mostafa Aakhte and Hans-Arno J Mueller
ARTICLE TYPE: Research Article

I have looked carefully at the answers to the referees' comments and in light of this I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.