**Response to editorial and reviewers comments Icha et al.:**

We would like to start by thanking the editor and reviewers for their constructive comments that in our opinion helped to improve this manuscript and made it more reader friendly.

Our response to all comments follows below:

**Editorial comments:**

•Grammar: 7.1.2 – Please correct the grammar in the first sentence, and make sure it is in imperative tense.

We corrected the sentence.

•Formatting:

-6.1 should not be a note. The note should be 6.1.1.

This note was deleted. There is an action to be performed-the data transfer.

-Remove “Note” from 6.4.6, as these are actions to be performed.

This has been removed.

-References – Please abbreviate all journal titles.

This has been applied.

•Additional detail is required:

-What is the composition of E3 medium?

E3 composition was added to the ‘Checklist of materials needed before starting’ document.

-1.2 – What stage embryos are used?

We added a sentence: This protocol is optimized for 16-72 hours old embryos.

We also state the different stages used in the figure legends.

-5.3.1 – How large is the Z step used here?

This information was added: 1 µm.

-6.2.4 – What should these values be?

We rephrased the sentence to clarify that the number of the angles, channels, illuminations and the voxel size are read from the metadata.

-6.4.7 – When is the amount of detections appropriate?

We added a range of numbers of detections that is appropriate (600-several thousand per view).

-6.5.7 – What is “adequate” registration?

Adequate registration has now been explained as: when no shift between different views as observed on fine structures within the sample, e.g. cell membranes is observable.

•There is unnecessary branding in the protocol which should be removed:

-Keywords, Long abstract, Introduction, Note under Protocol header, 5.2, 6.2.3 - Lightsheet Z.1; Please also remove the link to the manual in the introduction. This can be included in the materials table.

-2.3.5, Discussion – ZEN

-6.2.3 – Zeiss

We removed the terms: ZEN, Zeiss, Lightsheet Z.1 everywhere except at the step 6.2.3. This is due to the fact, that here it describes a name of a menu option in the software that the readers are supposed to click onto.

•Discussion: The discussion section should be written entirely in paragraph format. The settings and hardware specifications can be included as tables and cited in the text appropriately.

Imaging settings and Hardware specifications are now included as Table 3 and 4 respectively.

Please also remove the links, which should be included in the materials table.

This has been done.

Significance with respect to other methods should be discussed in more detail with appropriate citations for comparison with other techniques.

We discuss the significance already in the introduction, where we compare LSFM to spinning disc and point scanning confocal microscopy, which are the relevant and previously used alternatives for presented applications.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

This has been done.

•Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

This has now been done.

\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

We now included DOIs for all the references.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

The authors describe the use of a Zeiss Lightsheet Z1 for the imaging of the development of zebrafish eye. The main points of the manuscript are 1. a description of how to carry out the data acquisition; 2. a description of the numerical processing of the raw data and the tools they use to do this; and 3. a demonstration of the results that they obtain in their imaging of zebrafish eye development.

The manuscript is well written, and, with the exception of the concerns raised below, provides a tool that I believe will be valuable to scientists who are interested in getting started with light sheet microscopy as users rather than instrument developers. I see no reason that the manuscript should not be accepted for publication if the issues below can be addressed.

*Major Concerns:*

(Note: these concerns are not so much "major" as more general ones that don't refer to specific lines in the manuscript.)

The authors describe the use of the commercially available Zeiss Z1 instrument for data acquisition, but then use open source FIJI software for data processing. It is not clear why this choice was made, other than because FIJI software is freely available. Surely anyone who has access to a Z1 will also have access to the Zeiss data processing software. I do not expect a quantitative comparison of the different software choices, but the authors should at least explain in general terms the pros & cons of each, and give the reader an idea of which should be chosen under particular circumstances or for particular applications.

We now comment on this in the Discussion section. We there present two alternatives to our image processing pipeline and describe benefits of Fiji over the Zeiss software.

…Currently, there exist several possibilities for processing multiview data from a light sheet microscope that are well documented and relatively easy to adopt. We use the multiview reconstruction application, which is an open source software implemented in Fiji32 (Stephan Preibisch unpublished, **Link 1** in the Table of Materials). This plugin is a major redesign of the previous SPIM registration plugin20, reviewed by Schmied et al.42, integrating the BigDataViewer and its XML and HDF5 format33 with the SPIM registration workflow (**Figure 1B**, **Link 2**, **Link 3**). This application can be also adapted for high performance computing cluster, which significantly speeds up the processing43. This multiview registration application is actively developed further and keeps improving. In case of problems or features requests for the described software, please file issues on the respective github pages ([Link 4](https://github.com/bigdataviewer/SPIM_Registration/issues) for Multiview Reconstruction and [Link 5](https://github.com/bigdataviewer/bigdataviewer_fiji/issues) for BigDataViewer).

The second option is to use the commercial software available together with the microscope. This solution works well and employs the same principle of using fluorescent beads to register the different views. However, it lacks the option to visualize the whole dataset fast like with the BigDataViewer. Also the software cannot be adapted to the cluster and furthermore the processing blocks the microscope for other users, unless additional license for the software is purchased.

The third option, which is also an open source software, was recently published by the Keller lab44 and provides a comprehensive framework for processing and downstream analysis of the light sheet data. This software is using information from within the sample to perform multiview fusion, therefore it does not require the presence of fluorescent beads around the sample. But at the same time it assumes orthogonal orientation of the imaging views (objectives), so it cannot be used for data acquired from arbitrary angles44….

One of the main selling points of LSFM is that it can collect high-resolution data within large 3D structures at a reasonable speed. But of course, one can also zoom in on smaller regions of interest. Where appropriate, it should therefore be made clear for each figure/movie whether the data has been digitally cropped out of a full data set covering the entire eye, or whether an optical zoom-in on a smaller region of interest was made.

We now specify in the figure legends that the data has been digitally cropped out of a dataset covering large part or the whole eye. Which is true for all of our figures.

*Minor Concerns:*

line 85. To the best of my knowledge the "original" LSFM technique published was Orthogonal-Plane Fluorescence Optical Sectioning (Voie, Burns & Spelman, J.Microscopy 170, 1993) and not SPIM (Huisken et al, 2004).

We have now rephrased this section and added the Voie et al. citation. Now it reads: To fulfill all these requirements, a modern implementation(Huisken et al, 2004) of the orthogonal plane illumination microscope(Voie, Burns & Spelman, J.Microscopy 170, 1993) was developed.

line 498. The term "RANSAC" is first used here, but it isn't defined until line 1040.

We now defined the term the first time it is used.

line 559. The sentence "Observe the bounding box... will be displayed" is unclear.

We deleted the sentence and the section should be now clear. We immediately start with an action to be performed in that step: Move the slider for min and max in every axis to determine the region of interest and then press OK.

line 610. The data "... has been converted to 16-bit". Is the full 32-bit data saved in case the user wants to go back to it?

Currently, unfortunately only the 16-bit data are available as stated in the protocol.

line 611. There is a section 7.3.3) and a 7.3.5), but no 7.3.4).

We now corrected the numbering.

line 654. The figure shows "selected time points of one optical slice from one view out of an unfused multiview dataset". Is there some reason for not showing the fusion? The authors go to considerable effort to show that in general multi-view fusions are superior to single views, so why isn't this used here, especially if the data was available?

line 658. In movie4, it would be useful to show, beside the multi-view deconvolution, an unprocessed single-view of the same region, so that a direct comparison can be made.

We reworked this section and now we are showing the comparison of single view, weighted-average multiview fusion and multiview deconvolution next to each other in the new Figure 5 for a single time point, and throughout the whole time lapse recording in the Movie 3. The new Movie 3 replaced Movie 3 and 4, which were showing similar aspects of imaging and biological processes.

line 687. "... of the same view after weighted-average fusion". Fusion with what? How many views? What angles?

We now specified this in the figure legend to figure 2. We refer to a fusion of 4 views, 20 degrees apart.

line 737. Is the fluorescence part of movie 1 single-view data or a multi-view fusion?

It is single view data. This was clarified in the respective figure legend.

line 951. "sufficient overlap" needs to be defined/explained.

We now explain, in the section “Multiview” in the discussion, that the sufficiency of overlap is best tested empirically. After acquisition of the first time point, it should be immediately confirmed that the multiple views can be successfully registered.

line 1206. The authors should be "Huisken, J. et al."

We now corrected this reference.

line 1235. The authors should be "Pinto-Teixeira, F. et al."

We now corrected this reference.

Figure2. The images are probably single optical slices, but this should be clarified.

We now specified in the figure legend that single optical slices are shown.

Figure 5. It would be helpful for the non-fish-eye-expert if the structures described in the caption (RNE, REP, etc.) were labelled in the actual figure.

We now added labels of the most prominent structures of the zebrafish embryo head directly into the Figure 5.

*Additional Comments to Authors:*

N/A

**Reviewer #2:**

*Manuscript Summary:*

The submission by Icha et al describes how to use light sheet microscopy and appropriate software to monitor developmental processes in live embryos over a range of spatial and temporal scales. Unlike many previous publications and online resources, this manuscript is targeted at experimental biologists who may not be microscope builders but rather are interested in using light sheet imaging to tackle biological questions. The methodology is described in detail using clear language and the rationale behind most of the experimental choices is clearly explained. The Discussion section is especially valuable and provides numerous helpful tips and advice to avoid pitfalls. The example data is beautiful and convincingly demonstrates the utility of the method. Overall I consider this is an excellent manuscript that will be of considerable interest and value to the community.

I have a small number of specific comments:

\* In general, a number of statements are made in the Introduction that need to be supported with references. Some examples:

- The statement at line 96/97 in the Intro requires reference.

- Similarly 98/99.

- Similarly 101/102.

We now added appropriate references to support our statements.

\* Intro: The paragraph starting at Ln106 suggests lightsheet mounting strategies are preferable to those used for conventional imaging methods. I do not buy this. There is no reason one cannot mount a sample in an agarose column and then perform conventional confocal imaging. Either the authors should present a more compelling case or adjust this section to simply explain that lightsheet mounting can keep the specimen in near physiological conditions.

We now adjusted the wording in this paragraph. It now reads: LSFM comes with alternative sample mounting strategies well suited for live embryos…

\* Section 3.2.1: The description of the 20µl capillaries and "black mark" is a bit unclear. Is this explained further in the microscope manual? If not, please provide more standard specs for the capillaries (ID/OD, glass type, length etc) and explain the mysterious black mark in detail.

Additional information about the capillaries is provided in the table of materials.

\* 5.2.2: Consider showing some images of "good" versus "bad" PSFs in the Figures. I think this would be useful for other users to know what to aim for.

Images of PSFs in case of well aligned versus misaligned light sheet position with respect to the detection objective were added to Fig. 1, showing that users should aim for the “hour glass shape” in xz or yz.

\* Figure legends: please move the reference to figure panels (eg (A)) to the beginning of the relevant section of the legend (rather than the end).

We now moved the references to figure panels to the beginning of the sections.

Minor comments:

\* Maybe cut Danio Rerio from the title to make it a little shorter?

We now removed Danio rerio from the title.

\* Consider cutting "mother" from line 205

"mother" has been removed.

\* Ln224: "ether-ethanol-1:4" Please correct terminology

This has been corrected.

\* 7.1.2: Sentence does not make sense - please double-check.

We deleted the sentence and the section should be now clear. We immediately start with an action to be performed in that step: Move the slider for min and max in every axis to determine the region of interest and then press OK.

*Major Concerns:*

N/A

*Minor Concerns:*

N/A

*Additional Comments to Authors:*

N/A

**Reviewer #3:**

*Manuscript Summary:*

This manuscript describes in detail a method to image the zebrafish eye using light-sheet microscopy. Despite the existence or many such published protocols, this one is especially welcome as it focuses on the commercial Lightsheet Z.1 microscope, whereas other protocols focus on home-made microscopes. The manuscript is well written and easy to follow. The images are outstanding.

*Major Concerns:*

None

*Minor Concerns:*

Line 126. This statement needs a reference "LSFM further enables imaging of weakly fluorescent samples…"

We now added the reference to Sarov et al., in which they imaged transcription factors at endogenous expression levels using LSFM.

Line 1136. "In a typical LSFM experiment only one sample per experiment is imaged." This is not strictly true.

Even though people are working on improving this, currently usually one sample is typically imaged. This is true for zebrafish, Drosophila, Tribolium, Parhyale or Platynereis embryos. We add references to home built high throughput LSFM systems:

High throughput imaging of multiple embryos has been recently achieved in home built LSFM setups 46-48, although typically at the expense of freedom of sample positioning and rotation.

Line 1144. "zebrafish embryos are almost transparent…" The zebrafish embryo is translucent, not transparent.

This has now been corrected.