**DATA NOTE**

**Long-term in toto cell tracking using lightsheet microscopy of the zebrafish tailbud [version 2; peer review: 1 approved, 1 approved with reservations]**

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**Abstract**

In toto light-sheet imaging allows the tracking of entire growing tissues with high spatial and temporal resolution for many hours. However, this technology requires a sample to be immobilised to ensure that the tissue of interest remains within the field of view throughout the image acquisition period. We have developed a method of mounting and image capture for long-term light-sheet imaging of a growing zebrafish tailbud from the 18 somite stage through to the end of somitogenesis. By tracking the global movement of the tailbud during image acquisition and feeding this back to the microscope stage, we are able to ensure that the growing tissue remains within the field of view throughout image acquisition. Here, we present three representative datasets of embryos in which all nuclei are labelled and tracked until the completion of somitogenesis.

**Keywords**

Zebrafish, Tailbud, Axial elongation, Lightsheet, Tracking, Online Registration

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**Open Peer Review**

**Reviewer Status**

Invited Reviewers

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**version 2**

(revision)

15 Jul 2019

**version 1**

23 Dec 2018

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Any reports and responses or comments on the article can be found at the end of the article.
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Author roles: Fulton T: Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation; Lenz MO: Investigation, Methodology, Software; Muresan L: Methodology, Software; Andrews T: Investigation, Methodology; Lancaster C: Investigation; Horton E: Writing – Original Draft Preparation; Steventon B: Conceptualization, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Writing – Review & Editing

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Introduction

Early embryonic development is characterised by large-scale cell movements that together generate tissues of the correct shape and size. Furthermore, these highly dynamic processes must be coordinated between neighbouring tissues in order to establish the connections required to build functioning organs. Recent advances in light-sheet imaging have vastly increased the speed at which whole embryos can be imaged during development, allowing for all cells to be tracked for long time periods, a processed termed “in toto imaging” (Megason, 2009). In toto light-sheet imaging has allowed for single-cell tracking across multiple tissues with high time resolutions during axis formation in both mouse and zebrafish embryos (Keller et al., 2008; McDole et al., 2018). Recently, this has allowed the tracking of cells in the gastrulating zebrafish from 30% epiboly to 12 somites (Attardi et al., 2018a; Shah et al., 2017). In this period of development, the embryo remains stationary with most growth being derived from cellular rearrangements. In later developmental stages, however, in toto light-sheet imaging has previously been impossible due to the large amounts of growth and global movements of the tailbud which results in the object leaving the field of view rapidly (Hirsinger & Steventon, 2017; Steventon et al., 2016).

To follow the cell movements contributing to multi-tissue morphogenesis during posterior body elongation, we have therefore developed a mounting technique for an upright, single view scanning light-sheet microscope and online tracking tool to follow the growing tailbud of zebrafish from 18 somites through to the end of somitogenesis. We have implemented an online tracking tool similar to that of (McDole et al., 2018) to retain the sample within the field of view. This uses an image-based registration to calculate the XYZ shift of the tailbud between the N0 and N4+4 frame and then centres this object back into the field of view. With imaging every 2 minutes, registration therefore occurs every 10 minutes, which is demonstrably sufficient to permit tracking of the tailbud over an extended period of time to allow tracking of cells with single cell accuracy.

Methods

Mounting and imaging

Embryos were obtained from an incross of a heterozygous constitutive Histone 2B-fused GFP line (H2B::GFP) and screened for strong green fluorescence at 50% epiboly. Embryos were then grown to the 16 somite stage prior to mounting which is described here, and graphically, in Figure 1.

To mount the embryos, 10-cm petri dishes were filled with a 5-mm layer of 1% agarose made in E3 media. Next, two glass rings were placed in the centre of these dishes, 5 mm apart, and the agarose was allowed to set. These glass rings were then removed alongside the contained agarose, leaving a bottom layer of agarose with two holes inside, separated from one another by an agarose bridge.

On this bridge, a small embryo-sized hole was cut using size 5 forceps to allow for correct orientation of the embryo relative to the light-sheet excitation objective and detection objectives. The hole was then lined, by filling and removing 1% low-melting-point agarose made in E3.

Finally, the embryo was mounted into this hole by aspirating the dechorionated embryo in low melting point agarose and placing it into the lined hole on the petri dish. The embryo was oriented to that the embryo is ventral-side-up and laying at a 45 degree angle to the agarose layer. The agarose was then allowed to set fully before filling the dish with E3 media plus tricane methanesulfonate. Using a fine glass capillary needle, the agarose was cut away from the tail whilst leaving the anterior of the embryo fixed in place. The embryo was then imaged on the light-sheet microscope as described by Attardi et al. (2018a).

This mounting technique permits the embryo to be fixed in position, from the anterior agarose, which prevents the sample moving as the objectives scan the sample whilst also allowing unrestrained growth of the tissue posteriorly. This technique also ensures that the embryo tail is correctly aligned for optimal illumination from the illumination objective and viewing from the collection objective which both sit at 45 degrees to the stage.

Post-image-acquisition processing

Following image acquisition, the data was downscaled, so that a voxel represents 1 µm³, and registered to remove the visible stage movements caused by the tracking program. Finally the data was tracked using the Tracking with Gaussian Mixture Models (TGMM) software vOct-17 (Amat et al., 2014) as described in Attardi et al. (2018a) followed by manual validation of tracks using Mamut v0.27.0 for Fiji v1.52d (Wolff et al., 2018). Figure 1 shows a representative binary output from automatic segmentation of the three-dimensional image from the starting timeframe from which lineage inferences were made.

While this dataset was of ample spatial and temporal resolution to follow cell lineages within the neumesdermal progenitor region (Attardi, 2018a), there was a significant amount of blurring within more anterior portions of the tail. It is likely that this could be further improved with the use of additional

Amendments from Version 1

Since publishing our article online, the responses that we have received (including the reviewer), have indicated that an unexpected impact of the work relates to describing how we mount zebrafish embryos for imaging on the light-sheet microscope. For this reason, we have decided to include Toby Andrews as an author on the version 2, as he made a significant contribution to developing the mounting technique.

We have now updated Figure 1 to indicate the different viewpoints for the diagrams of embryo mounting. In addition, we have cited (McDole et al., 2018) within the introduction to indicate the similarity with their AutoPilot method to maintain sample position within the field of view during imaging. We have also returned to this paper in the discussion to mention how their adaptive optics approach has the potential to further improve image quality in our system.

See referee reports
adaptive optics that would dynamically account for local differences in optical conditions, such as that recently applied during the imaging of early mouse development (McDole et al., 2018).

The complete dataset generated is available on the Image Data Resource (Attardi, 2018b).

**Ethics policies**

This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

**Dataset validation**

Of the automatic tracks generated, a subset was validated. A detailed description of this validation is available from Attardi et al. (2018a). Approximately 75% of automatic tracks correctly followed a single cell to the termination of the track, with the remaining 25% requiring some level of human intervention to either correct or discard the track.
Data availability
The imaging data as registered, downscaled .tiff, downscaled .klb files and associated tracking data, as .xml, are available from the Image Data Resource. DOI: https://doi.org/10.17867/10000117 (Attardi et al., 2018b)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

References

Grant information
B.S. and T.F. were supported by a Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (109408). L.M. is supported by the EPSRC EP/R025398/1 grant. C.L is supported by a BDSE/Company of Biologists/Gurdon Summer Studentship. T.A. is supported by a Wellcome Trust studentship.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Open Peer Review

Current Peer Review Status: ✔️  ❓

Version 2

Reviewer Report 08 August 2019

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Johannes Stegmaier
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Fulton et al. present a method for embedding zebrafish embryos that were raised to the 16-somite stage such that tailbud development can be imaged with an upright light-sheet microscope. The pipeline involves automatic estimation of global shifts of the specimen in 3D using image registration techniques that are applied in fixed time intervals and are used to readjust the field of view of the microscope. The final registered data set is used for single cell tracking in 3D with an established software tool (TGMM by Amat et al., 2014). The data note is generally well understandable and I agree with Reviewer 1 (Fiolka, 2019) that it’s indeed nice to see that even such large-scale data sets are becoming publicly available. However, the note lacks a bit of technical detail for the involved software aspects that would make it difficult to reproduce the presented results. Moreover, I have some doubts that the segmentation/tracking quality is indeed as good as the authors describe it.

Some more specific remarks and suggestions:

- The authors claim that an “image-based registration is used to calculate XYZ shift”. Could you comment a bit more on what registration technique was used? How is it incorporated to the imaging workflow? How much time does the registration need to align two sequential 3D images? Are there any special hardware requirements to do it in the constrained time interval?

- The segmentation result presented in Figure 1I does not look very convincing and it seems that there are many undersegmentation errors. There are a few reasons why this could happen: (1) Ideally, it’s just a visualization issue due to a limited number of label colors, (2) it could be caused by insufficient spatial resolution considering the relatively high density of nuclei at this later developmental stage or (3) the parameterization of the TGMM algorithm was not performed properly. Of course, it could also be a combination of all three issues. Can you comment on this and explain how/if the parameters of the method were adjusted for that particular data set?

- Mini note: The type of image visualized in Figure 1I is usually termed “label image” or “label map” rather than “binary output” (which would only distinguish background vs. foreground).
To provide an easier assessment of the segmentation quality presented in Figure 1I, you could superimpose label boundaries on top of the raw image shown in Figure 1H. This would also allow to see if the apparent undersegmentations are actually only a visualization issue or indeed an error of the segmentation. You could simply compute the intensity gradient of the label image, binarize it by setting everything above zero to 1 and then superimpose the raw image with the cell region boundaries.

For the tracking validation you mention “75% of automatic tracks correctly followed a single cell to the termination of the track”. Does this mean a track spans the entire duration of the experiment or would a track that only connects two time points successfully also be considered as a correct automatic track? In Attardi et al. (2018a), the tracking validation seems to be described only for the 30%-75% epiboly stage – how was selection of the subset of cells used for validation of the tailbud segmentation/tracking performed?

Availability of the registration software is unclear: is this a module that can be easily added to any upright microscope setup/software? If yes, where can it be obtained from?

Availability of the data seems to be provided. However, I only managed to view the data online but was not allowed to download anything else than PNG images of the current view. The tracking files are listed in the tables overview, but it was not obvious how those files can be downloaded from the repository. Maybe comment on how to actually obtain the data in case there are any special things to consider.

References

Is the rationale for creating the dataset(s) clearly described?
Yes

Are the protocols appropriate and is the work technically sound?
Partly

Are sufficient details of methods and materials provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 3D Image Analysis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
In this note, Fulton et al. detail their workflow to image and track nuclei in the tailbud of a growing Zebrafish. The methods detail a novel way of mounting the Zebrafish in 1% Agarose such that the tail is not embedded and can be imaged in an inverted light-sheet geometry. To me this is the biggest innovation in this note; the other steps are using tools described previously by the authors and other groups.

I appreciated that there is an online data repository where raw data can be viewed. I think this is very valuable for the community, as too often raw data is not available and only finished and processed data is shown. As such, I think this can be very useful to other groups to compare their image quality.

Some questions and comments:

- The authors claim that tracking of the movement of the sample and growth have not previously been accounted in acquisition software. However, such capabilities are described in the Autopilot method by the Keller lab. A description of the algorithm for centering the embryos and adjusting the imaging volume (in conjunction with adjusting the optical model of the AutoPilot itself) is available on pages 24-26 in McDole et al. (2018\(^1\)). This publication should be cited and it would be great if the authors could comment if they think Autopilot could be used here too.

- Upon looking at the online data, it looks that in some cases, not the whole image is sharp – some nuclei are blurry. Is this a problem for the tracking algorithm and would a multiview implementation (i.e. diSPIM) help?

- For Figure 1 A-D, coordinate axes would be helpful. I was not fully sure if E shows the same plane as A-C.

References

Is the rationale for creating the dataset(s) clearly described?
Yes

Are the protocols appropriate and is the work technically sound?
Yes
Yes

Are sufficient details of methods and materials provided to allow replication by others?
Partly

Are the datasets clearly presented in a usable and accessible format?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Optical microscopy

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.