DATA NOTE

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

Timothy Fulton ¹, Martin O. Lenz ², Leila Muresan ², Toby Andrews ³, Courtney Lancaster ¹, Elizabeth Horton¹, Benjamin Steventon ¹

¹Department of Genetics, University of Cambridge, Cambridge, CB2 3EH, UK

²Cambridge Advanced Imaging Center, University of Cambridge, Cambridge, CB2 3EH, UK

³Department of Zoology, University of Cambridge, Cambridge, CB2 3EJ, UK

v2 First published: 23 Dec 2018, 3:163
<https://doi.org/10.12688/wellcomeopenres.14907.1>
 Latest published: 15 Jul 2019, 3:163
<https://doi.org/10.12688/wellcomeopenres.14907.2>

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

Open Peer Review

Approval Status

	1	2
version 2 (revision) 15 Jul 2019		 view
version 1 23 Dec 2018	 view	

1. **Reto Fiolka**, University of Texas Southwestern Medical Center, Dallas, USA
2. **Johannes Stegmaier** , RWTH Aachen University, Aachen, Germany

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Benjamin Steventon (bjs57@cam.ac.uk)

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

Competing interests: No competing interests were disclosed.

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 BSDB/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.

Copyright: © 2019 Fulton T *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Fulton T, Lenz MO, Muresan L *et al.* **Long-term *in toto* cell tracking using lightsheet microscopy of the zebrafish tailbud [version 2; peer review: 1 approved, 1 approved with reservations]** Wellcome Open Research 2019, 3:163 <https://doi.org/10.12688/wellcomeopenres.14907.2>

First published: 23 Dec 2018, 3:163 <https://doi.org/10.12688/wellcomeopenres.14907.1>

REVISED 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

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 process 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 & Stevenon, 2017](#); [Stevenon 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 N^{th} and $N+4^{\text{th}}$ 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 orientated 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 downsampled, so that a voxel represents $1 \mu\text{m}^3$, 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 neuromesodermal 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

Figure 1. A schematic of mounting and downstream processing for 4D lightsheet imaging of the zebrafish tailbud. (A) Set up of the 1% agarose base plate with two circular holes punched inside using glass rings generating a bridge onto which the embryo is mounted. (B) The embryo is mounted inside a hole cut from the agarose and lined with 1% low-melting-point agarose. The embryo is fully covered with low-melting-point agarose, which is allowed to set. (C) The growing tail and tailbud are then cut free from the low melting point agarose and (D) placed under an upright scanning lightsheet microscope under E3 plus tricane. (E) A lateral view of the mounting demonstrating the hole cut into the agarose baseplate and orientation of the embryo to permit optimal illumination and imaging by the objectives which are mounted at 45 degrees to the baseplate. (F, G) The online mid-imaging registration software allows tracking of the centre of mass of the tailbud in both X, Y and Z dimensions. (H) A medial 1-μm slice of the downsampled and registered data from the first timepoint of the acquisition which is (I) segmented using the TGMM software to permit tracking of single cells.

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, downsampled .tiff, downsampled .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).

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 BSDB/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.

References

- Amat F, Lemon W, Mossing DP, *et al.*: **Fast, accurate reconstruction of cell lineages from large-scale fluorescence microscopy data.** *Nat Methods*. 2014; 11(9): 951–958.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Attardi A, Fulton T, Florescu M, *et al.*: **Neuromesodermal progenitors are a conserved source of spinal cord with divergent growth dynamics.** *Development*. 2018a; 145(21): pii: dev166728.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Attardi A, Fulton T, Florescu M, *et al.*: **Dataset for Neuromesodermal Progenitors are a Conserved Source of Spinal Cord with Divergent Growth Dynamics.** *Image Data Resource*. 2018b.
<http://www.doi.org/10.17867/10000117>
- Hirsinger E, Steventon B: **A Versatile Mounting Method for Long Term Imaging of Zebrafish Development.** *J Vis Exp*. 2017; (119): e55210.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Keller PJ, Schmidt AD, Wittbrodt J, *et al.*: **Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy.** *Science*. 2008; 322(5904): 1065–1069.
[PubMed Abstract](#) | [Publisher Full Text](#)
- McDole K, Guignard L, Amat F, *et al.*: **In Toto Imaging and Reconstruction of Post-Implantation Mouse Development at the Single-Cell Level.** *Cell*. 2018; 175(3): 859–876.e33.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Megason SG: **In toto imaging of embryogenesis with confocal time-lapse microscopy.** *Methods Mol Biol*. 2009; 546: 317–332.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Shah G, Thierbach K, Schmid B, *et al.*: **Pan-embryo cell dynamics of germlayer formation in zebrafish.** *BioRxiv*. 2017; 173583.
[Publisher Full Text](#)
- Steventon B, Duarte F, Lagadec R, *et al.*: **Species-specific contribution of volumetric growth and tissue convergence to posterior body elongation in vertebrates.** *Development*. 2016; 143(10): 1732–1741.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Wolff C, Tinevez JY, Pietzsch T, *et al.*: **Multi-view light-sheet imaging and tracking with the MaMuT software reveals the cell lineage of a direct developing arthropod limb.** *eLife*. 2018; 7: pii: e34410.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Open Peer Review

Current Peer Review Status:

Version 2

Reviewer Report 08 August 2019

<https://doi.org/10.21956/wellcomeopenres.16774.r36157>

© 2019 Stegmaier J. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Johannes Stegmaier

Institute of Imaging and Computer Vision, RWTH Aachen University, Aachen, Germany

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, 2019¹) 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

1. Fiolka R: Referee Report For: Long-term in toto cell tracking using lightsheet microscopy of the zebrafish tailbud [version 1]. *Wellcome Open Research*. 2019; **3** (163). [Reference Source](#)

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.

Author Response 12 May 2020

Benjamin Steventon, University of Cambridge, Cambridge, UK

We thank both reviewers again for their time in reviewing our Data Note and are pleased they agree with us the importance of publishing short descriptive articles of large datasets and the importance of making them freely available to the community.

The reviewer is indeed correct in saying that parts of the image are under segmented. These regions tend to be the anterior regions of the tail and therefore outside of the region of interest for our published work in Attardi & Fulton et al. (2018a). The parameter search used to set up the initial image segmentation was not exhaustive however, parameters were identified that gave sufficiently high image segmentation to the dorsal posterior wall of the tailbud, where we wanted to focus our studies. The automatic segmentation was not the end of the pipeline in our image analysis as we then subset tracks from this dorsal posterior wall region and manually validated whether the track followed a single cell until the termination of the track. As no conclusions in Attardi & Fulton et al (2018a) were drawn from automatic tracks which had not been manually validated, we felt it was not required to undertake an exhaustive parameter search. The reviewer is correct in stating that the track may not terminate at the end of the experiment, and an accurate track would be any one which did not switch cell along the track duration. We have better described the methods in the main text.

We thank the reviewer for seeking further clarity regarding the registration process. The microscope is controlled using an in-house developed Labview software. Initial image handling within the acquisition software was integrated into the bespoke software utilising Matlab scripts. The images stacks were downsampled immediately after acquisition to a voxel size of $1\mu\text{m}^3$. The downsampled stacks were then used to estimate the relative translative offset between the Nth and (N+4)th stack using image phase correlation for 3D registration. The computation time for determining the shift between two image stacks is about 4-5 sec on the microscope workstation. In combination with the fact that the calculation and successive position correction is performed only every 10 min, this does not slow down the acquisition. The above mentioned phase correlation based Matlab scripts were also used for the image registration post acquisition and is applied to every timeframe. These scripts are available from the authors upon request.

The full dataset is available from the Image Data Repository. Detailed instructions on how to download the raw files from the IDR are available on their website, linked here <https://idr.openmicroscopy.org/about/download.html>. We also thank the reviewer for noting the mislabelling of panel 1I, we have corrected this in the text.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 29 April 2019

<https://doi.org/10.21956/wellcomeopenres.16250.r34929>

© 2019 Fiolka R. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Reto Fiolka

Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA

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.* (2018¹). 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

1. McDole K, Guignard L, Amat F, Berger A, et al.: In Toto Imaging and Reconstruction of Post-Implantation Mouse Development at the Single-Cell Level. *Cell*. 2018; **175** (3): 859-876.e33 [PubMed Abstract](#) | [Publisher Full Text](#)

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

Yes

Are the protocols appropriate and is the work technically sound?

Yes

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?

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.