Journal of Visualized Experiments

Visualizing ocular morphogenesis by Lightsheet microscopy using rx3:GFP transgenic zebrafish

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62296R2
Full Title:	Visualizing ocular morphogenesis by Lightsheet microscopy using rx3:GFP transgenic zebrafish
Corresponding Author:	Ann C Morris, Ph.D. University of Kentucky Lexington, KY UNITED STATES
Corresponding Author's Institution:	University of Kentucky
Corresponding Author E-Mail:	ann.morris@uky.edu
Order of Authors:	Rebecca Petersen
	Ann C Morris, Ph.D.
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please specify the section of the submitted manuscript.	Developmental Biology
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lexington, KY USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	Two video files are over the size limit; we uploaded lower resolution versions here but will need to provide the high resolution versions somehow
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Visualizing Ocular Morphogenesis by Lightsheet Microscopy using rx3:GFP Transgenic Zebrafish

AUTHORS AND AFFILIATIONS:

Rebecca A. Petersen, Ann C. Morris

Department of Biology, University of Kentucky, Lexington KY 40506

Email addresses of co-authors:

Rebecca A. Petersen (rebecca.petersen@uky.edu)

Ann C. Morris (ann.morris@uky.edu)

Corresponding author:

Ann C. Morris (ann.morris@uky.edu)

KEYWORDS:

time-lapse imaging, Lightsheet microscopy, live imaging, zebrafish, eye development, retina

ABSTRACT:

Vertebrate eye development is a complex process that begins near the end of embryo gastrulation and requires the precise coordination of cell migration, proliferation, and differentiation. Time-lapse imagining offers unique insight to the behavior of cells during eye development because it allows us to visualize oculogenesis in vivo. Zebrafish are an excellent model to visualize this process due to their highly conserved vertebrate eye and their ability to develop rapidly and externally while remaining optically transparent. Time-lapse imaging studies of zebrafish eye development are greatly facilitated by use of the transgenic zebrafish line Tg(rx3:GFP). In the developing forebrain, rx3:GFP expression marks the cells of the single eye field, and GFP continues to be expressed as the eye field evaginates to form an optic vesicle, which then invaginates to form an optic cup. High resolution time lapse imaging of rx3:GFP expression, therefore, allows us to track the eye primordium through time as it develops into the retina. Lightsheet microscopy is an ideal method to image ocular morphogenesis over time due to its ability to penetrate thicker samples for fluorescent imaging, minimize photobleaching and phototoxicity, and image at a high speed. Here, the study provides a protocol for time-lapse imagining of ocular morphogenesis using a commercially available lightsheet microscope and an image processing workstation to analyze the resulting data. This protocol details the procedures for embryo anesthesia, embedding in low melting temperature agarose, suspension in the imaging chamber, setting up the imaging parameters, and finally analyzing the imaging data using image analysis software. The resulting dataset can provide valuable insights into the process of ocular morphogenesis, as well as perturbations to this process as a result of genetic mutation, exposure to pharmacological agents, or other experimental manipulations.

INTRODUCTION:

Embryonic development is a complex process that requires the precise coordination of many different events. The formation of the vertebrate eye begins in the developing forebrain, where

a portion of the cells are specified as the eye field. These cells will evaginate toward the surface ectoderm, giving rise to two bilateral optic vesicles[1–10]. Contact with the surface ectoderm then induces an invagination of the optic vesicle into an optic cup. The surface ectoderm will give rise to the anterior structures of the eye, such as the lens and cornea, while the optic cup will give rise to the neural retina and retinal pigmented epithelium[1–3,5–12]. Disruptions in this process can lead to congenital defects such as microphthalmia, anophthalmia, and coloboma (MAC). At this time, there are no options to correct these defects[3,5–7,9–12]. Further studies of the mechanisms of ocular morphogenesis and the problems that can lead to MAC will provide a foundation of knowledge that will potentially lead to treatments. One powerful tool to investigate the dynamic behaviors of cells during eye development is time-lapse imagining, which allows this process to be visualized and characterized *in vivo* and in real time.

Zebrafish (*Danio rerio*) are an excellent model to visualize early ocular development using time-lapse imaging. They have a highly conserved vertebrate eye and possess the ability to develop rapidly and externally while remaining optically transparent[1]. Zebrafish provide a great resource for time-lapse imaging due to these characteristics that mammalian models lack. Time-lapse imaging studies of zebrafish eye development are greatly facilitated by use of the transgenic zebrafish line Tg(rx3:GFP). Rx3 (Retinal homeobox protein 3) is a transcription factor essential for eye development[13]. Rx3 is the first of the three rx genes in the zebrafish to be expressed, starting its expression mid-gastrulation, approximately 8 h post fertilization (hpf)[14,15]. The rx3:GFP transgene can be visualized in the developing forebrain starting at the 1 somite stage (ss), approximately 10 hpf[14,16–20]. In the developing forebrain, rx3:GFP expression marks the cells of the single eye field, and GFP (green fluorescent protein) continues to be expressed through the remainder of ocular morphogenesis. High resolution time lapse imaging of rx3:GFP expression, therefore, allows us to track the single eye field through time as it develops into the retina[16,17,19,20].

Time-lapse imaging studies of zebrafish development have primarily been performed using confocal or Lightsheet microscopy. Confocal microscopy is advantageous in that it allows for the precise imaging of samples along the z-axis and reduces fluorescent background signal. However, it is limited by the amount of time it takes to acquire an image, sample position rigidity, and its propensity toward photobleaching and phototoxicity of live samples. Lightsheet microscopy is an ideal method to image ocular morphogenesis over time due to its ability to penetrate thicker samples for fluorescent imaging, increased flexibility in sample orientation, minimized photobleaching and phototoxicity, and imaging at a high speed[20–30]. The spatial resolution achievable with current light sheet microcopy systems is approximately 250–500 nanometers (nm). Although this is not significantly different from what can be obtained with confocal microscopy, the sample can be freely rotated and imaged from multiple angles, improving both imaging depth and resolution, and offering much greater flexibility for in vivo time lapse imaging experiments than the confocal platform[26,31]. For these reasons, Lightsheet microscopy is quickly becoming the favored method for time-lapse imaging studies of zebrafish development. This protocol describes the steps of quantifying oculogenesis through the imaging of Rx3:GFP transgenic zebrafish using a commercially available Lightsheet microscope[32] and details a pipeline for image analysis using the arivis software platform.

PROTOCOL:

All experiments involving the use of zebrafish were carried out in accordance with protocols established by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

1. Sample preparation

1.1. Set up a mating pair of Tg(Rx3:GFP) zebrafish in a gated cross tank the night before imaging is planned. The following morning pull the gate as the lights come on in the fish facility[33,34].

NOTE: The mating pair of fish selected should be between 4 months to 2 years in age and are easily distinguished as male or female based on body shape and coloration[34].

1.2. Check the crosses every half hour for embryos and note what time embryos are first visualized in the bottom of the cross tank. Transfer the embryos to a Petri dish and maintain the embryos at 28.5 °C for approximately 10 h to develop to the 1–2 somite stage (ss). Begin to image at the 1–2 somite stage (ss).

1.3. To screen for the presence of somites, observe the embryos under a stereoscope at 10 hpf and count the number of somites[1].

NOTE: Any embryos that are beyond the single somite stage should not be used for this experiment.

1.4. Out of the embryos that are at the single somite stage, screen for GFP expression using a fluorescence adapter in combination with a stereomicroscope to confirm the presence of the Rx3:GFP transgene. Once 3–5 GFP positive individuals have been identified, use fine forceps to dechorionate the embryos and transfer them into a small Petri dish containing E3 embryo buffer with a glass pipette[34].

1.5. Anesthetize the embryos by transferring them into 0.5 mL E3 embryo buffer (pH 7) containing 0.168 mg/mL Tricaine (MS222) in a micro centrifuge tube[34,35]. Spontaneous muscle movements begin as early as 17 hpf[36]. Ensure that embryos are anesthetized to image beyond this timepoint.

1.6. Embed the embryos in 1% low melting temperature agarose in 0.168 mg/mL Tricaine and E3.

NOTE: This is the optimal concentration of low melting temperature agarose to allow for the embryo to be held in place but remain pliable enough to permit the growth of the embryo over the imaging time-course[29,30].

1.7. Prepare a 10 mL solution of 2% low melting temperature agarose in E3 buffer. Heat it in a microwave oven to dissolve agarose using 15 s intervals, to prevent the solution from boiling over. Allow the solution to cool enough to hold without discomfort but not so much that it solidifies, and not cause harm to the embryos. Once the agarose is sufficiently cool, add 0.5 mL to the embryos in the tube of Tricaine in E3 and gently pipet the solution and embryos to mix.

1.8. Using a 1 mm glass capillary and polytetrafluoroethylene (PTFE) plunger, pull up the embryos in the agarose solution into the capillary. Make sure to pull a total of 3–5 embryos (multiple embryos) into the capillary to increase the likelihood of having a well-positioned embryo.

NOTE: Due to the round nature of the embryos at this timepoint, it is challenging to guarantee a specific orientation in the capillary. Ideally, the body of the embryo will be positioned laterally in the capillary, allowing for the greatest ease of positioning within the microscope.

1.9. Let the agarose solidify over a period of 30–60 s at room temperature. Place the capillary in a beaker of 0.168 mg/mL Tricaine in E3 buffer until ready to image (**Figure 1A**).

NOTE: The excess 2% low melting temperature agarose solution can be allowed to solidify and subsequently reheated in future experiments.

1.10. Place the capillary into the sample holder (**Figure 1B–F**) as described in step 2.5.

2. Zeiss Lightsheet Z.1 setup

2.1. Switch on each component of the microscope and the computer in the following order: 1) System, 2) PC, then 3) Incubation (**Figure 2A**).

2.2. Place the 20x imaging objective and the 10x illumination objective into the microscope chamber. Match the objective settings in the Zen Software interface under the **Maintain** tab.

2.3. Slide the chamber (**Figure 2C**) into the housing on the track (**Figure 2B**) with the tubing facing out. The tubing connects to the appropriate ports on the right as shown in **Figure 2E**.

2.4. Attach the extension line to the syringe with the luer-lock mechanism (**Figure 2D**); fill it with Tricaine in E3 and place it in the holder attached to the right of the microscope[34]. Connect the extension attached to the syringe filled with Tricaine in E3 to the bottom right of the chamber with the luer-lock mechanism. Push the plunger to fill the chamber with the Tricaine/E3 buffer. Close the door to the chamber.

2.5. Place the capillary with the sample into the capillary sample holder. The capillary sample holder comprises two rubber sleeves, a metal sample holder disc, a metal stem, and a metal cap (**Figure 1B**). Click the metal sheath into the center of the metal disc (**Figure 1C**). Place the two rubber stoppers into the sheath, with the slits facing the ends of the sheath followed by the

capillary. Slide the capillary through the middle of the rubber stoppers. Fasten it in place with the metal cap once the marker is at the base of the metal sheath (**Figure 1D**).

2.6. Place the capillary sample holder onto the top of the microscope, with the white marks aligning (**Figure 1E**,**F**). Close the lid.

2.7. Click on the **Locate Capillary** button on the software interface (**Figure 3A**). Use the ErgoDrive control panel, a manual device that controls the capillary orientation (**Figure 3E**), to move the capillary and position it just above the objective (**Figure 3B**).

2.8. Open the lid, and gently push on the plunger until the section of agarose containing the embryo is hanging below the capillary bottom and is in front of the objective (**Figure 3C**).

2.9. Turn off **Locate Capillary** and click on the **Locate Sample** button (**Figure 3A**). This switches the view from the sample chamber's webcam to the microscope objective (**Figure 3D**). Use this view to adjust the position of the sample more precisely. Turn off **Locate Sample** (**Figure 3A**).

2.10. Switch over to the **Acquisition** tab. Check the boxes for **Z-Stack** and **Time Series**.

2.11. In the **Acquisition Mode** parameters window, choose the **Dual Side Lightsheet** setting and check the boxes for **Online Dual Side Fusion** and **Pivot Scanning**.

2.12. In the **Channels** window, choose **488 channel**, set the laser power to 1, and the exposure time to 7.5 ms.

2.13. Next, click on the **Continuous** button to get a live view of the embryo. Use the ErgoDrive control panel to adjust the position of the embryo until the eye field is directly facing the camera. Continue adjusting the left and right lightsheets in the **Channels** parameters until the eye field is sufficiently in focus.

2.14. Set the **Z-Stack** parameters using the ErgoDrive control panel to move through the Z-plane. Set the first and the last Z-Positions around 500 µm beyond the last detectable fluorescent signal. This leaves room for the eye field to remain in frame as the embryo grows throughout the time-lapse imaging session. After setting the range of the Z-Stack, click on the **Optimal** button to set the step size to 0.477 µm, the optimal setting.

2.15. Set the **Incubation** parameters by checking the box for the Peltier Unit to keep the temperature at 28 °C. In the **Time Series** window, choose the frequency and time interval to acquire images. In this protocol, the parameters were set to image every 5 min, for a total of 166 intervals.

2.16. Click on the **Start Experiment** button. Choose the folder to save the image set. Set the image prefix and hit **Save** to start the imaging.

NOTE: The microscope will now run through each image set at the interval specified.

2.17. After the time-lapse imaging session has been completed, send the stage to the load position, and remove the capillary sample holder. Take apart the capillary sample holder in the reverse order that it was put together and use the plunger to remove the sample and excess agarose from the capillary. Open the chamber door. Use the syringe to remove the chamber liquid from the chamber, disconnect and remove the chamber, then rinse with water and air dry.

3. Image analysis

3.1. Open the arivis Vision4D software program.

3.2. Click on **File**, then choose **Import file**. Select all .czi files from the time-lapse imaging session and open. The next window opens with the options on how to import the files; select **Z-stacks as Frames** to order the z-stacks in the order obtained. Once the files have been imported, the software saves as a single .sis file. To specify the location for the file to be saved, select the folder prior to clicking on **Import**.

3.3. After the file is imported, arivis is now ready to render a video of the time-lapse images. Click on the **4D Viewer Cube** in the bottom-left corner and the **Scale Bar** icon (**Figure 4D–E**). Click on the video icon to bring the Storyboard taskbar to the bottom of the screen (**Figure 4C**).

3.4. In the **Storyboard** taskbar, choose **Add Keyframe Sequence** (**Figure 4F**). Specify the duration of the video in seconds, uncheck the **Create Rotation** box, and check the **Use Time Progression** to include the specific timepoints in the video. The software displays these parameters to the right of the Storyboard taskbar for any adjustments at any time. Save the **Storyboard** to apply the same parameters to multiple image sets (**Figure 4F**).

3.5. Click on **Export Movie** to save a video of the time-lapse imaging (**Figure 4F**). Specify the movie export settings, including the file name and location, video format (.mp4), video resolution (1,080 p), framerate (60 FPS), and data resolution (1,297 x 1,297 x 784). Add timestamps here, if desired. Once these parameters are set, choose **Record** (**Figure 4F**).

3.6. Steps 3.4 and 3.5 can be repeated with modification to Step 3.4 to create rotation videos at specific timepoints. When adding a keyframe sequence to the storyboard, check the **Create Rotation** box, and uncheck **Use Time Progression**. Then, follow the same instructions as in step 3.5.

3.7. To render a high-resolution image at any orientation at any individual time point, select the **Camera** icon in the 4D Viewer to obtain a high-resolution image (**Figure 4C**).

3.8. To build a pipeline for analysis, choose the **Flask** icon to access the **Analysis** panel (**Figure 4B**). In the **Analysis** panel, click on the **Analysis Operations** dropdown menu. As an example, the

protocol demonstrates below the sequence of operations for a volume analysis pipeline. Run or undo each step of the pipeline individually to fine tune each parameter (**Table 1**).

3.9. Click on the blue triangle at the top of the pipeline to allow the pipeline to run.

NOTE: This will take some time depending on the speed of the computer. Once the pipeline has been optimized, the pipeline can be exported and imported to arivis with ease and applied to multiple datasets in a batch analysis.

3.10. To access batch analysis, click on **Batch Analysis** under the **Analysis** tab.

3.11. After the pipeline runs, a window pulls up with all of the objects found. Access this manually through the **Table** icon (**Figure 4B**). At the top of this window, click on the box labeled **Feature Columns** to pull up a list of features that can provide information about the object of interest. For the eye field volume analysis, these features include Surface Area, Volume (Volume, VoxelCount), Intensities #1 (Mean), Attributes (Id, Type), and Time Point (First). Click on **Export** to export the data to a spreadsheet.

REPRESENTATIVE RESULTS:

The dataset displayed here was imaged using the protocol described above. A Tg(Rx3:GFP) embryo was imaged starting at the 1 somite stage (ss) through 24 hpf, a total time period of 14 h, with the images acquired at 5 min intervals. Time-lapse imaging allows for easy selection and comparison of any time-point that shows a phenotype of interest. **Figure 5** demonstrates a set of high-resolution images that were rendered from the dorsal vantage point at select developmental time points. The pipeline run in arivis Vision4D builds a mask that represents the developing eye as identified by fluorescent signal. In **Figure 5** and **Videos 1–6**, the mask can be visualized in comparison to the fluorescent rendering of the developing eye. Additionally, **Table 2** displays the volume data from the developing eye at every imaging point. This dataset includes the segment name, id, volume in both μm^3 and voxel count, the mean fluorescence intensity of the object, the time point the object was identified, and the object's surface area in μm^2. It is important to note that when the eye field separates into two optic vesicles (starting at Timepoint 64), there remains a third region that is Rx3:GFP positive in the forebrain, which will contribute to the hypothalamus[37–39] (**Figure 5D–M**). This shows up in the volume data represented in **Table 2** (highlighted in yellow starting at Timepoint 71) and can easily be separated out from the optic vesicles, since it is much smaller in volume than either optic vesicle.

FIGURE AND TABLE LEGENDS:

Figure 1: Sample preparation. (**A**) Positioning of embryos in a glass capillary. The arrow points to an embryo in the capillary. (**B**) Glass capillary and capillary holder parts. (**C**) Partially assembled capillary holder. (**D**) Fully assembled capillary holder. (**E**) Lightsheet mounting chamber. The arrow indicated the white line used to orient the capillary holder. (**F**) Capillary holder properly mounted in the Lightsheet. The arrow shows the matching white lines, indicating proper orientation of the capillary holder.

Figure 2: Lightsheet imaging set-up. (**A**) Switchboard to turn on the Lightsheet, computer, and incubation unit. The numbers indicate the order of operations. (**B**) Lightsheet objective chamber. (**C**) Imaging chamber. (**D**) Syringe and tubing that will be connected to the imaging chamber. (**E**) The imaging chamber properly positioned within the objective chamber with all of the tubes connected to the appropriate ports to the right.

Figure 3: Sample positioning. (**A**) **Locate Capillary** and **Locate Sample** buttons in Zen Software as indicated by the arrows. (**B**) The positioning on the glass capillary. The arrow indicates the edge of the glass capillary positioned just above the lens of the objective. (**C**) The embryo suspension beyond the glass capillary. The arrow indicated the embryo suspended in agarose beneath the glass capillary in front of the objective's lens. (**D**) View of the embryo through the objective. (**E**) The ErgoDrive control panel.

Figure 4: Important icons for navigating arivis Vison4D. Each panel has the icon function identified from left to right. (**A**) Open, Save, Close. (**B**) Analysis Panel, Show Objects Table, Open Track Editor. (**C**) Copy current viewer content as an image into clipboard, Toggle Bookmarks, Create a high-resolution image for the current view, Toggle Storyboard. (**D**) Show Measure Box, Show Orientation Cross, Show Legend, Show Scale Bar. (**E**) Show as 2D Viewer, Show as Gallery Viewer, Show as 4D Viewer, Show as Info Viewer, Show as Projection Viewer. **F**) Refresh all Keyframes, Add Keyframe, Add Keyframe sequence, Insert Keyframe, Remove all Keyframes, Export Movie, Load Storyboard, Save Storyboard, Adjust the target time of the entire movie, First Keyframe, Play, Pause, Stop, Last Keyframe.

Figure 5: High-resolution images and eye field masks. (**A**–**M**) A set of high-resolution images that were rendered from the dorsal vantage points; (**A'**–**M'**) the eye field masks for each corresponding timepoint. Each image set is notated by the time it was acquired from the start of imaging and the corresponding developmental stage in either somite stage (ss) or hours post fertilization (hpf).

Video 1: Time-lapse video of Tg(rx3:GFP) zebrafish embryo from 1 ss–24 hpf.

Video 2: Time-lapse video of Tg(rx3:GFP) zebrafish embryo from 1 ss–24 hpf with the eyefield as identified by the arivis Vision4D pipeline.

Video 3: 360° rotation of a Tg(rx3:GFP) zebrafish embryo at 1 ss.

Video 4: 360° rotation of a Tg(rx3:GFP) zebrafish embryo eye field mask at 1 ss.

Video 5: 360° rotation of a Tg(rx3:GFP) zebrafish embryo at 24 hpf.

Video 6: 360° rotation of a Tg(rx3:GFP) zebrafish embryo eye field mask at 24 hpf.

Table 1: arivis Vision4D pipeline for volume analysis of the developing eye field.

Table 2: Volume and surface area of the developing eye field acquired by arivis Vision4D. The rows pertaining to the presumptive hypothalamus are highlighted in yellow to distinguish them from the optic vesicles.

DISCUSSION:

In this protocol, the Lightsheet microscope was used to perform time-lapse imaging of eye development and the resulting data were analyzed. The resulting dataset can provide valuable insights into the process of ocular morphogenesis, as well as perturbations to this process as a result of genetic mutation, exposure to pharmacological agents, or other experimental parameters. Here the protocol demonstrated how this dataset can be obtained and provided an example of how to analyze the volume of the eye field through early development. This data was found to be reproducible and consistent (less than 10% variation in volume) across biological replicates, bearing in mind that slight differences in embryo staging prior to the start of the run can lead to some variation in final volume measurements.

Care should be taken in the initial positioning of the embryo in the capillary and in positioning the embedded embryo in front of the objective. Orientation plays an important role in preventing the embryo from growing and moving out of the view of the objective. The embryos have a round shape at 10 hpf, which makes it challenging to guarantee a specific orientation in the capillary. Ideally, the body of the embryo will be positioned laterally in the capillary. Loading multiple embryos in the capillary will increase the likelihood of having a well-positioned embryo.

In this procedure, the embryo is embedded in agarose in order to suspend it in front of the imaging and illumination objectives. Choosing the correct concentration of the low melting temperature agarose is critical. Too high of a concentration will constrict the embryo and prevent it from properly developing; too low of a concentration will result in the agarose falling apart and not holding the embryo. The concentration optimal for this protocol is a final concentration of 1% low melting temperature agarose[29,30].

Another element that should be taken into consideration is the level of saturation. As the eyefield grows and differentiates, the strength of the Rx3:GFP signal intensifies. Therefore, when setting the initial imaging parameters, the exposure and laser power should be reduced to undersaturate the image. This will prevent the image from becoming oversaturated as the Rx3:GFP gets brighter over time. Modifications can be made to correct for undersaturation in image processing but oversaturation cannot be corrected after the images have been acquired.

There are a few additional modifications that can be made to this protocol that may be advantageous to some projects that are not described in this paper. For example, it is possible to set up Multiview imaging in the image acquisition set up. This parameter would allow multiple embryos at different positions along the y-axis to be sequentially imaged at each time interval. While adding complexity to the data set, it would increase the rate of data collection. Additionally, in terms of image processing, it is possible to quantify the eye field by other parameters. Here, we described how to quantify the data in terms of the eye field volume.

Alternatively, a pipeline could be made to quantify and track individual cells or determine the rate of optic vesicle evagination.

As previously mentioned, both confocal and Lightsheet microscopy have been used to perform time-lapse imaging studies of zebrafish. Lightsheet was specifically chosen for this project due to its superior ability to image through a thick (>1 mm) sample, because it is equipped with an incubation unit to maintain an ideal temperature environment for the zebrafish embryo, and because its ability to image at a faster rate than confocal microscopy allows for image acquisition at the numerous time intervals required for this protocol no accompanying damage or photobleaching of the embryo[20–30]. It is also important to note that the Lightsheet microscope is equipped to image the signal from multiple fluorophores. The Lightsheet microscope used in this study has solid state laser excitation lines at 405, 445, 488, 515, 561, and 638 nm, which could be useful for imaging transgenic embryos expressing more than one fluorescent reporter transgene.

While this protocol details instructions for image acquisition analysis specifically using the Lightsheet Z.1 Dual Illumination Microscope System and arivis Vision4D analysis software, there are other commercially available Lightsheet microscopes made by Leica, Olympus, and Luxendo, as well as image analysis software by Imaris, that could be used to achieve similar results. The selection of equipment and software for this protocol was determined by the availability at our institution.

In summary, it is anticipated this protocol will provide a solid starting point for conducting time-lapse imaging using Lightsheet microscopy, and for image quantification of early eye development in zebrafish.

DISCLOSURES:

The authors declare no competing financial interests.

ACKNOWLEDGMENTS:

Research reported in this publication was supported by the Office of The Director of the National Institutes of Health (NIH) under Award Number S10OD020067 and by NIH award R01EY021769 (to A.C.M.). We are grateful for the assistance of Doug Harrison and Jim Begley in the Arts & Sciences Imaging Center at the University of Kentucky, and to Lucas Vieira Francisco and Evelyn M. Turnbaugh for expert zebrafish care.

REFERENCES:

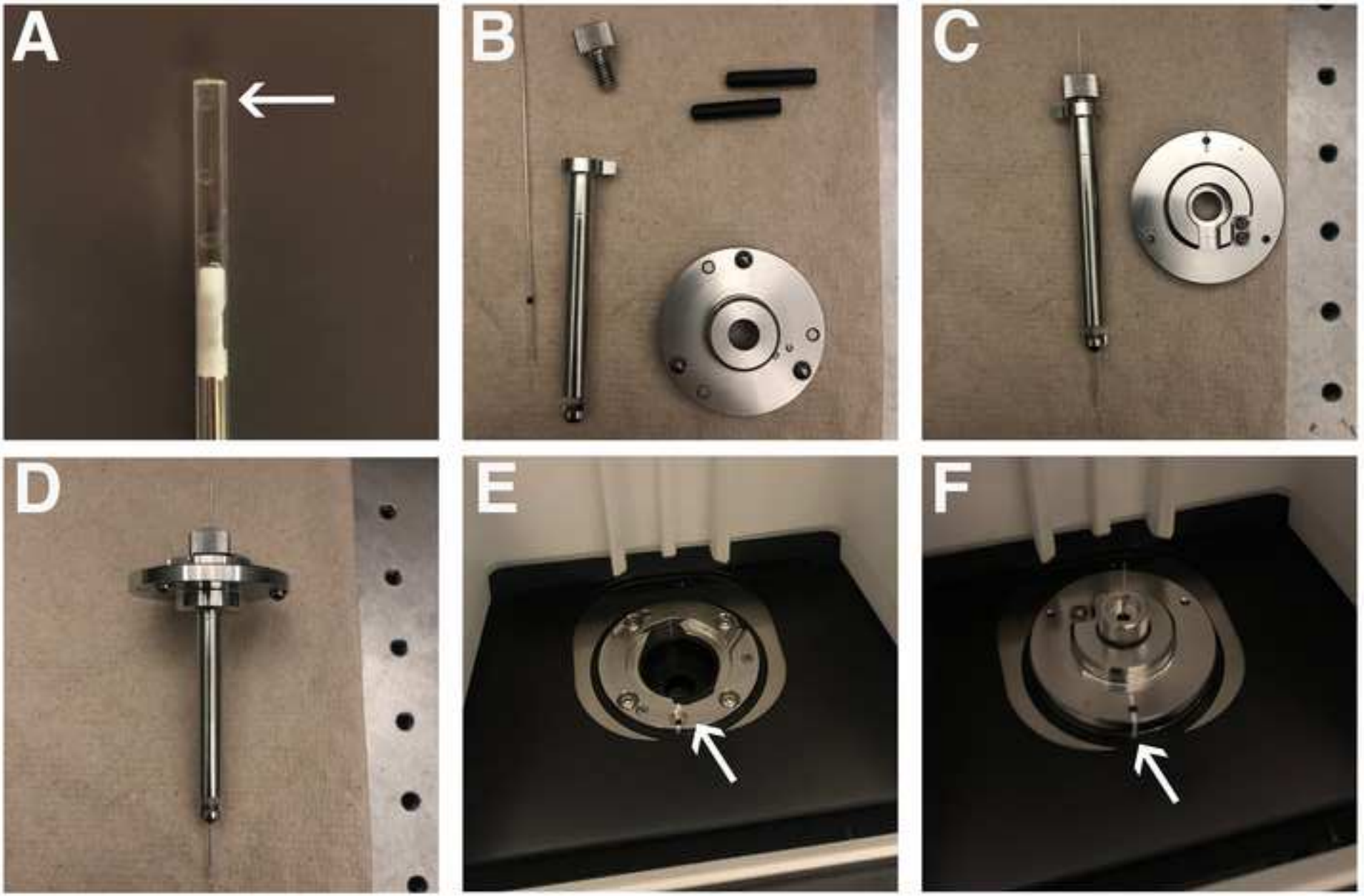
1. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. Stages of embryonic development of the zebrafish. *Developmental Dynamics*. **203** (3), 253–310 (1995).
2. Li, Z., Joseph, N. M., Easter, S. S. The morphogenesis of the zebrafish eye, including a fate map of the optic vesicle. *Developmental Dynamics*. **218** (1), 175–188 (2000).
3. Chow, R. L., Lang, R. A. Early eye development in vertebrates. *Annual Review of Cell and Developmental Biology*. **17**, 255–296 (2001).
4. Wilson, S. W., Houart, C. Early steps in the development of the forebrain. *Developmental Cell*. **6** (2), 167–181 (2004).

438 5. Picker, A. et al. Dynamic coupling of pattern formation and morphogenesis in the
439 developing vertebrate retina. *PLoS Biology*. **7** (10), e1000214 (2009).
440 6. Fuhrmann, S. Eye morphogenesis and patterning of the optic vesicle. *Current Topics in*
441 *Developmental Biology*. **93**, 61–84 (2010).
442 7. Kwan, K. M. et al. Bin A complex choreography of cell movements shapes the vertebrate
443 eye. *Development*. **139** (2), 359–372 (2012).
444 8. Heermann, S., Schütz, L., Lemke, S., Krieglstein, K., Wittbrodt, J. Eye morphogenesis driven
445 by epithelial flow into the optic cup facilitated by modulation of bone morphogenetic protein.
446 *eLife*. **4**, e05216 (2015).
447 9. Eckert, P., Knickmeyer, M. D., Schütz, L., Wittbrodt, J., Heermann, S. Morphogenesis and
448 axis specification occur in parallel during optic cup and optic fissure formation, differentially
449 modulated by BMP and Wnt. *Open Biology*. **9** (2) (2019).
450 10. Yoon, K. H., Fox, S. C., Dicipulo, R., Lehmann, O. J., Waskiewicz, A. J. Ocular coloboma:
451 Genetic variants reveal a dynamic model of eye development. *American Journal of Medical*
452 *Genetics Part C: Seminars in Medical Genetics*. **184** (3), 590–610 (2020).
453 11. Sidhaye, J., Norden, C. Concerted action of neuroepithelial basal shrinkage and active
454 epithelial migration ensures efficient optic cup morphogenesis. *eLife*. **6**, e22689 (2017).
455 12. Pillai-Kastoori, L., Wen, W., Morris, A. C. Keeping an eye on SOXC proteins. *Developmental*
456 *Dynamics*. **244**, 367–376 (2015).
457 13. Loosli, F. et al. Loss of eyes in zebrafish caused by mutation of chokh/rx 3. *EMBO Reports*.
458 **4** (9), 894–899 (2003).
459 14. Chuang, J. C., Mathers, P. H., Raymond, P. A. Expression of three Rx homeobox genes in
460 embryonic and adult zebrafish. *Mechanisms of Development*. **84** (1–2), 195–198 (1999).
461 15. Cavodeassi, F. et al. Early stages of zebrafish eye formation require the coordinated
462 activity of Wnt11, Fz5, and the Wnt/β-catenin pathway. *Neuron*. **47** (1), 43–56 (2005).
463 16. Ivanovitch, K., Cavodeassi, F., Wilson, S. W. Precocious acquisition of neuroepithelial
464 character in the eye field underlies the onset of eye morphogenesis. *Developmental Cell*. **27** (3),
465 293–305 (2013).
466 17. Ebert, A. M., Childs, S. J., Hehr, C. L., Cechmanek, P. B., McFarlane, S. Sema6a and Plxna2
467 mediate spatially regulated repulsion within the developing eye to promote eye vesicle cohesion.
468 *Development (Cambridge)*. **141** (12), 2473–2482 (2014).
469 18. Emerson, S. E. et al. Identification of target genes downstream of semaphorin6A/plexinA2
470 signaling in zebrafish. *Developmental Dynamics*. **246** (7), 539–549 (2017).
471 19. Hehr, C. L., Halabi, R., McFarlane, S. Polarity and morphogenesis of the eye epithelium
472 requires the adhesion junction associated adaptor protein Traf4. *Cell Adhesion and Migration*. **12**
473 (5), 489–502 (2018).
474 20. Jemielita, M., Taormina, M. J., Delaurier, A., Kimmel, C. B., Parthasarathy, R. Comparing
475 phototoxicity during the development of a zebrafish craniofacial bone using confocal and light
476 sheet fluorescence microscopy techniques. *Journal of Biophotonics*. **6** (11–12), 920–928 (2013).
477 21. Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J., Stelzer, E. H. K. Optical sectioning deep
478 inside live embryos by selective plane illumination microscopy. *Science*. **305** (5686), 1007–1009
479 (2004).
480 22. Reynaud, E. G., Kržič, U., Greger, K., Stelzer, E. H. K. Light sheet-based fluorescence
481 microscopy: More dimensions, more photons, and less photodamage. *HFSP Journal*. **2** (5), 266–

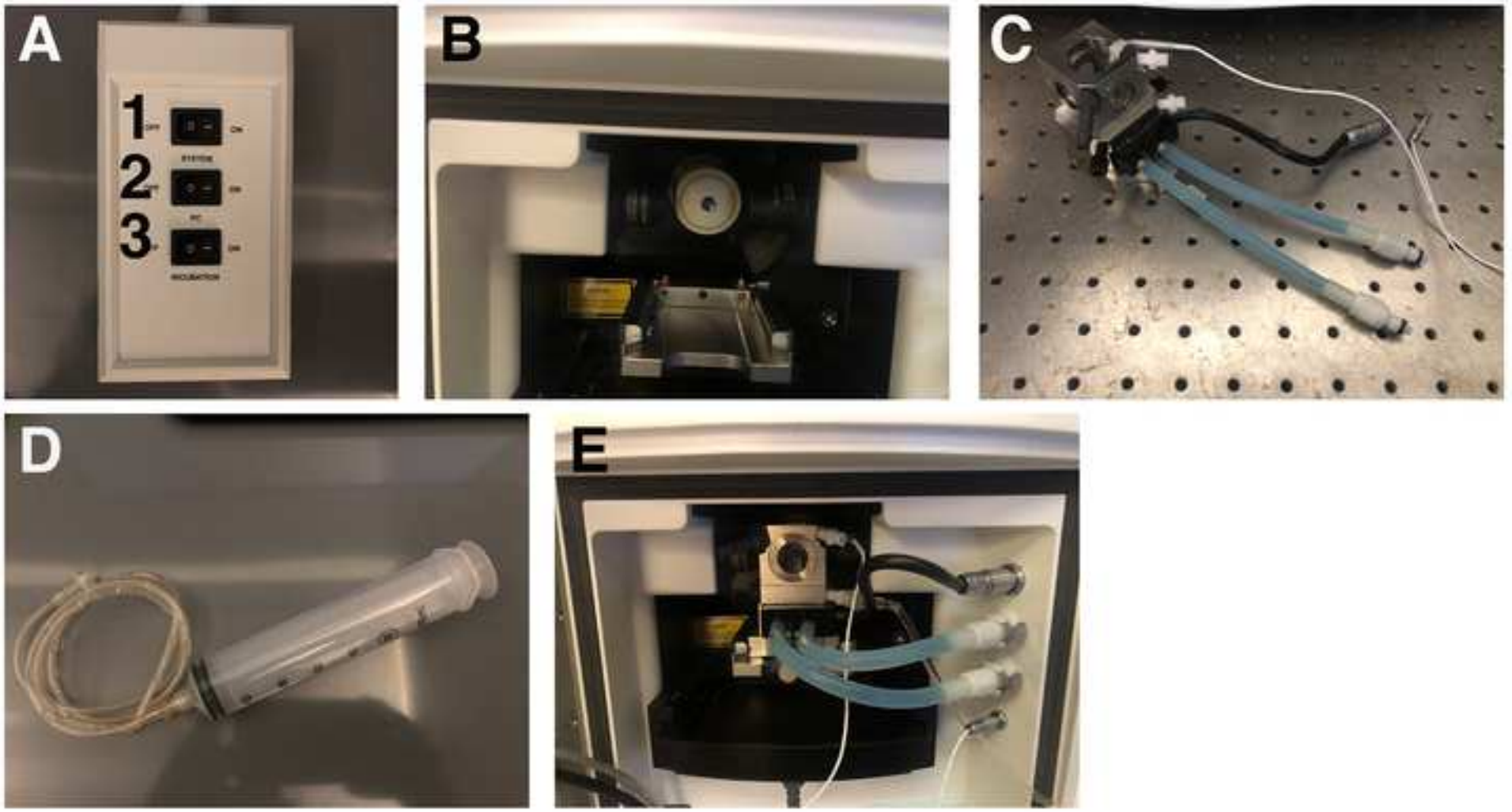
275 (2008).
23. Icha, J. et al. Using light sheet fluorescence microscopy to image zebrafish eye development. *Journal of Visualized Experiments:JoVE*. **110**, e53966 (2016).
24. Keller, P. J., Dodt, H. U. Light sheet microscopy of living or cleared specimens. *Current Opinion in Neurobiology*. **22** (1), 138–143 (2012).
25. Pampaloni, F., Chang, B. J., Stelzer, E. H. K. Light sheet-based fluorescence microscopy (LSFM) for the quantitative imaging of cells and tissues. *Cell and Tissue Research*. **360** (1), 129–141 (2015).
26. Pantazis, P., Supatto, W. Advances in whole-embryo imaging: A quantitative transition is underway. *Nature Reviews Molecular Cell Biology*. **15** (5), 327–339 (2014).
27. Park, O. K. et al. 3D light-sheet fluorescence microscopy of cranial neurons and vasculature during zebrafish embryogenesis. *Molecules and Cells*. **38** (11), 975–981 (2015).
28. Royer, L. A. et al. Adaptive light-sheet microscopy for long-term, high-resolution imaging in living organisms. *Nature Biotechnology*. **34** (12), 1267–1278 (2016).
29. Keller, P. J., Schmidt, A. D., Wittbrodt, J., Stelzer, E. H. K. Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science*. **322** (5904), 1065–1069 (2008).
30. Keller, P. J. et al. Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy. *Nature Methods*. **7** (8), 637–642 (2010).
31. Santi, P. A. Light sheet fluorescence microscopy: A review. *Journal of Histochemistry and Cytochemistry*. **59** (2), 129–138 (2011).
32. Reynaud, E. G., Peychl, J., Huisken, J., Tomancak, P. Guide to light-sheet microscopy for adventurous biologists. *Nature Methods*. **12** (1), 30–34 (2014).
33. Avdesh, A. et al. Regular care and maintenance of a Zebrafish (*Danio rerio*) laboratory: An introduction. *Journal of Visualized Experiments: JoVE*. **69**, e4196 (2012).
34. Westerfield, M. *The Zebrafish Book. A Guide for the laboratory use of zebrafish (Danio rerio)*, 5th Edition. University of Oregon Press, Eugene (2007).
35. Hirsinger, E., Steventon, B. A versatile mounting method for long term imaging of zebrafish development. *Journal of Visualized Experiments: JoVE*. **119**, e55210 (2017).
36. Saint-Amant, L., Drapeau, P. Time course of the development of motor behaviors in the zebrafish embryo. *Journal of Neurobiology*. **37** (4), 622–632 (1998).
37. Cavodeassi, F., Ivanovitch, K., Wilson, S. W. Eph/Ephrin signalling maintains eye field segregation from adjacent neural plate territories during forebrain morphogenesis. *Development (Cambridge)*. **140** (20), 4193–4202 (2013).
38. Muthu, V., Eachus, H., Ellis, P., Brown, S., Placzek, M. Rx3 and Shh direct anisotropic growth and specification in the zebrafish tuberal/anterior hypothalamus. *Development (Cambridge)*. **143** (14), 2651–2663 (2016).
39. Rojas-Muñoz, A., Dahm, R., Nüsslein-Volhard, C. chokh/rx3 specifies the retinal pigment epithelium fate independently of eye morphogenesis. *Developmental Biology*. **288** (2), 348–362 (2005).

Figure

Click here to access/download;Figure;Figure 1 revised.tif

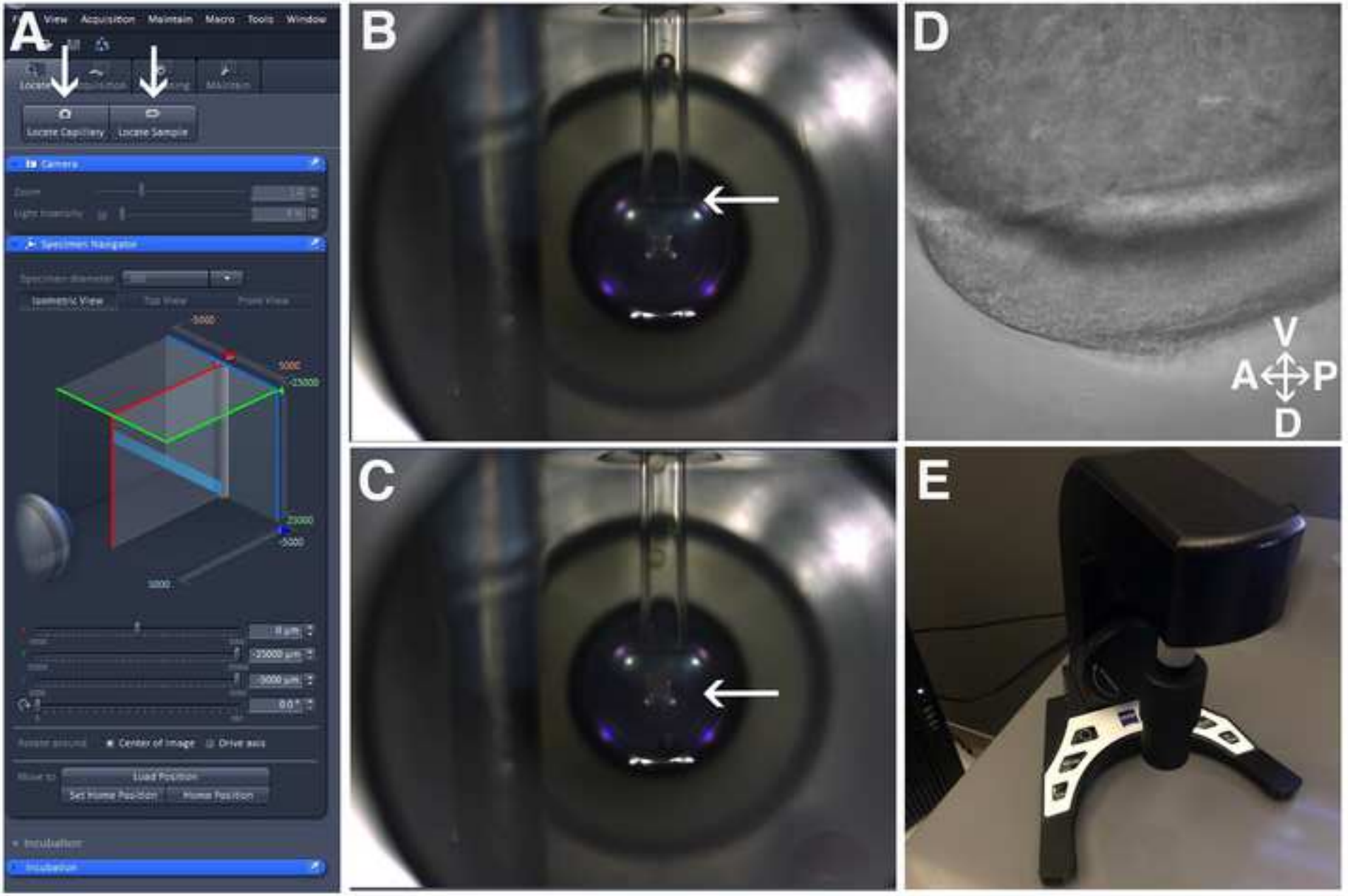


Figure

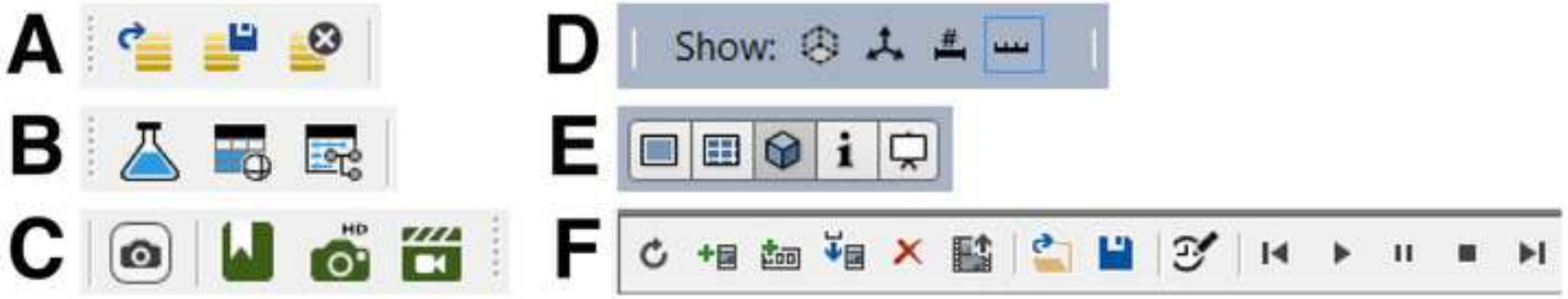


Figure

Click here to access/download;Figure;FIgure 3 revised.tif

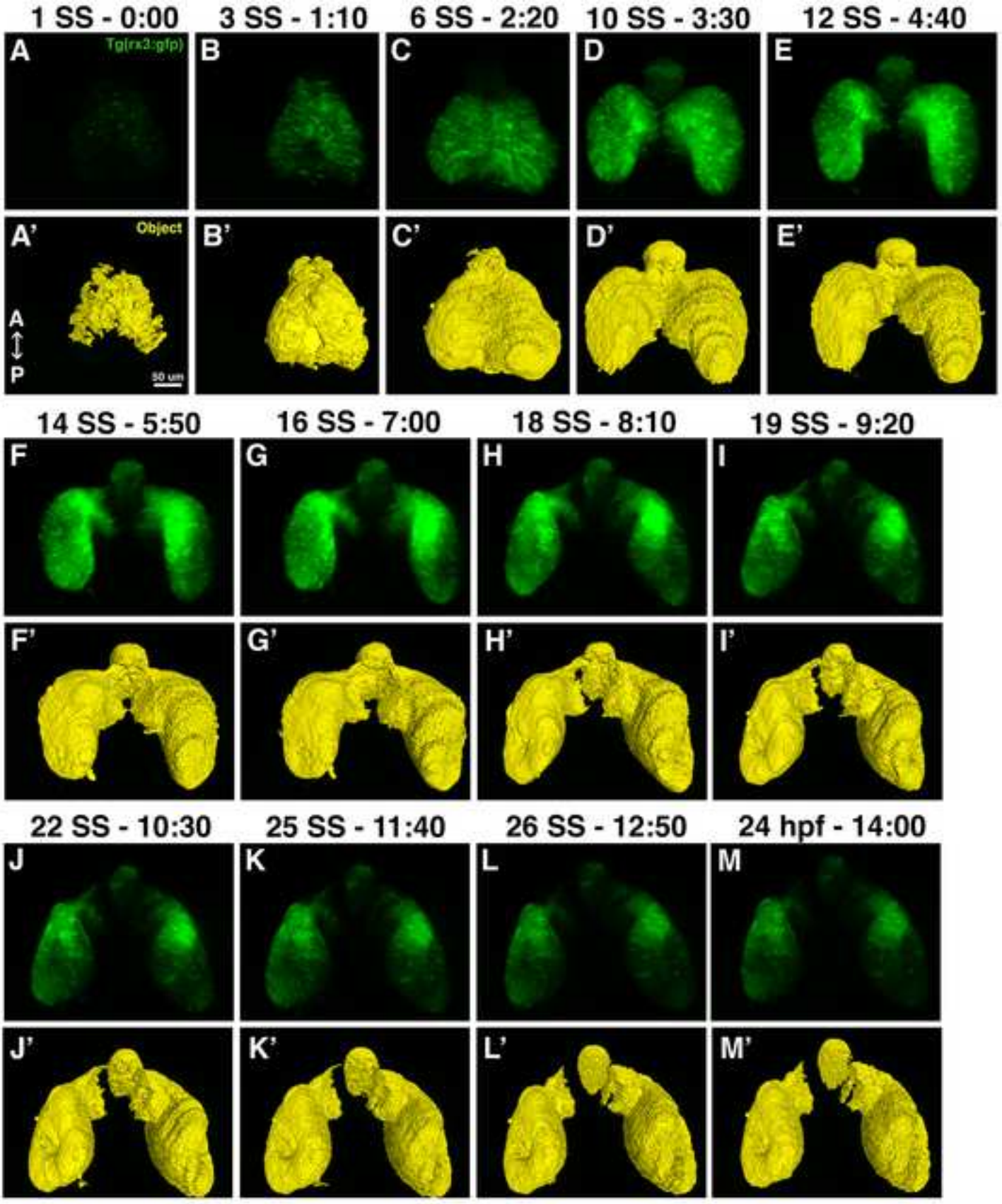


Figure



Figure

Click here to access/download;Figure;Figure 5 revised.tif



Click here to access/download

Video or Animated Figure

Video1_timelapse_rx3-GFP_1ss-24hpf MED RES.mov

Click here to access/download

Video or Animated Figure

Video3_360-rotation_rx3-GFP_1ss(1).mp4

Click here to access/download

Video or Animated Figure

Video4_360-rotation_rx3-GFP_eyefieldmask_1ss.mp4

Click here to access/download

Video or Animated Figure

Video5_360-rotation_rx3-GFP_24hpf.mp4

Click here to access/download

Video or Animated Figure

Video6_360-rotation_rx3-GFP_eyefieldmask_24hpf.mp4

Input ROI	Sets boundaries of the data set that will be included in the analysis.
Denoising Filter	Removes some autofluorescence or “noise” from data set.
Intensity Filter	Sets the min/max of what fluorescent signal is “real”
Segment Filter	Excludes objects that are not part of the eyefield based on object volume.
Export Objects Feature	Where to export the “object” information and in what format.
Store Objects	Which data to include in and save in “object” export.

Table

Replicate	Name	Volume, Volume (µm³)	VoxelCount, Volume	Id
1	Segment #1260701 (GFP)	1028939.669	41295736.000	0
1	Segment #7829 (GFP)	1152001.002	46234712.000	421
1	Segment #17826 (GFP)	1272342.664	51064536.000	422
1	Segment #29524 (GFP)	1499282.320	60172592.000	423
1	Segment #41164 (GFP)	1890770.055	75884664.000	424
1	Segment #52607 (GFP)	2108515.971	84623736.000	425
1	Segment #62254 (GFP)	2330058.742	93515192.000	426
1	Segment #70697 (GFP)	2542123.726	102026264.000	427
1	Segment #76771 (GFP)	2725779.070	109397136.000	428
1	Segment #81112 (GFP)	2908866.919	116745232.000	429
1	Segment #84868 (GFP)	3031648.989	121673000.000	430
1	Segment #88643 (GFP)	3127641.382	125525584.000	431
1	Segment #92783 (GFP)	3221042.074	129274152.000	432
1	Segment #97196 (GFP)	3332524.274	133748408.000	433
1	Segment #101709 (GFP)	3389340.365	136028680.000	434
1	Segment #106434 (GFP)	3431260.855	137711128.000	435
1	Segment #111221 (GFP)	3495480.297	140288528.000	436
1	Segment #115913 (GFP)	3544208.934	142244216.000	437
1	Segment #120606 (GFP)	3599083.343	144446560.000	438
1	Segment #125475 (GFP)	3628484.057	145626536.000	439
1	Segment #130255 (GFP)	3694449.239	148274000.000	440
1	Segment #134993 (GFP)	3738757.115	150052264.000	441
1	Segment #139697 (GFP)	3799753.176	152500296.000	442
1	Segment #144452 (GFP)	3837325.063	154008216.000	443
1	Segment #149425 (GFP)	3880379.746	155736184.000	444
1	Segment #154551 (GFP)	3910809.605	156957464.000	445
1	Segment #159692 (GFP)	3960766.520	158962448.000	446
1	Segment #165187 (GFP)	3997880.544	160451992.000	447
1	Segment #170678 (GFP)	4018720.193	161288376.000	448
1	Segment #176198 (GFP)	4055257.154	162754760.000	449
1	Segment #182011 (GFP)	4069912.960	163342960.000	450
1	Segment #187894 (GFP)	4117391.394	165248472.000	451
1	Segment #194320 (GFP)	4157979.556	166877448.000	452
1	Segment #200803 (GFP)	4210256.883	168975560.000	453
1	Segment #207535 (GFP)	4234519.244	169949312.000	454
1	Segment #214624 (GFP)	4288882.170	172131128.000	455
1	Segment #221818 (GFP)	4330089.852	173784968.000	456
1	Segment #229112 (GFP)	4353463.995	174723072.000	457
1	Segment #236562 (GFP)	4385886.366	176024320.000	458
1	Segment #244042 (GFP)	4407662.074	176898272.000	459
1	Segment #251710 (GFP)	4438206.947	178124168.000	460
1	Segment #259873 (GFP)	4453849.840	178751984.000	461

1	Segment #268260 (GFP)	4468190.704	179327544.000	462
1	Segment #276833 (GFP)	4460321.916	179011736.000	463
1	Segment #286033 (GFP)	4465836.406	179233056.000	464
1	Segment #295020 (GFP)	4457720.448	178907328.000	465
1	Segment #304184 (GFP)	4461704.675	179067232.000	466
1	Segment #313611 (GFP)	4469957.773	179398464.000	467
1	Segment #322889 (GFP)	4463272.612	179130160.000	468
1	Segment #332305 (GFP)	4447875.095	178512192.000	469
1	Segment #341821 (GFP)	4462623.590	179104112.000	470
1	Segment #351284 (GFP)	4460291.618	179010520.000	471
1	Segment #360718 (GFP)	4455696.841	178826112.000	472
1	Segment #370163 (GFP)	4433207.728	177923528.000	473
1	Segment #379775 (GFP)	4419629.105	177378560.000	474
1	Segment #389115 (GFP)	4406482.633	176850936.000	475
1	Segment #398871 (GFP)	4412017.654	177073080.000	476
1	Segment #408552 (GFP)	4400267.494	176601496.000	477
1	Segment #418320 (GFP)	4376477.747	175646712.000	478
1	Segment #427613 (GFP)	4366965.077	175264928.000	479
1	Segment #437082 (GFP)	2273684.766	91252664.000	480
1	Segment #437087 (GFP)	2087461.841	83778744.000	481
1	Segment #446355 (GFP)	4357100.788	174869032.000	482
1	Segment #455460 (GFP)	4349977.298	174583136.000	483
1	Segment #464716 (GFP)	4331594.003	173845336.000	484
1	Segment #473857 (GFP)	2235506.515	89720408.000	485
1	Segment #473860 (GFP)	2086628.438	83745296.000	486
1	Segment #482880 (GFP)	2220197.103	89105976.000	487
1	Segment #482885 (GFP)	2087651.803	83786368.000	488
1	Segment #491424 (GFP)	4302046.183	172659456.000	489
1	Segment #494415 (GFP)	90882.345	3647496.000	490
1	Segment #499876 (GFP)	4180852.581	167795440.000	491
1	Segment #502858 (GFP)	91767.175	3683008.000	492
1	Segment #508160 (GFP)	4172356.698	167454464.000	493
1	Segment #511267 (GFP)	90211.995	3620592.000	494
1	Segment #516550 (GFP)	4167497.409	167259440.000	495
1	Segment #519550 (GFP)	91118.353	3656968.000	496
1	Segment #524651 (GFP)	4160943.209	166996392.000	497
1	Segment #527581 (GFP)	91107.190	3656520.000	498
1	Segment #532660 (GFP)	2087162.845	83766744.000	499
1	Segment #532662 (GFP)	2060942.854	82714424.000	500
1	Segment #532682 (GFP)	2087563.898	83782840.000	501
1	Segment #535640 (GFP)	92779.975	3723656.000	502
1	Segment #540635 (GFP)	2048093.385	82198720.000	503
1	Segment #540661 (GFP)	2079263.161	83449696.000	504

1	Segment #543489 (GFP)	91944.978	3690144.000	505
1	Segment #548621 (GFP)	2050314.131	82287848.000	506
1	Segment #551455 (GFP)	92322.312	3705288.000	507
1	Segment #556555 (GFP)	2091276.038	83931824.000	508
1	Segment #556558 (GFP)	2059392.658	82652208.000	509
1	Segment #559601 (GFP)	91675.084	3679312.000	510
1	Segment #564430 (GFP)	2081649.550	83545472.000	511
1	Segment #564434 (GFP)	2045085.282	82077992.000	512
1	Segment #567493 (GFP)	91386.054	3667712.000	513
1	Segment #572284 (GFP)	2084208.760	83648184.000	514
1	Segment #572290 (GFP)	2049323.257	82248080.000	515
1	Segment #575158 (GFP)	88731.764	3561184.000	516
1	Segment #579949 (GFP)	2074223.875	83247448.000	517
1	Segment #579956 (GFP)	2040707.775	81902304.000	518
1	Segment #582653 (GFP)	89406.897	3588280.000	519
1	Segment #587334 (GFP)	2070318.385	83090704.000	520
1	Segment #587336 (GFP)	2036658.168	81739776.000	521
1	Segment #590057 (GFP)	89700.512	3600064.000	522
1	Segment #594781 (GFP)	2059724.743	82665536.000	523
1	Segment #594784 (GFP)	2030468.544	81491360.000	524
1	Segment #597491 (GFP)	89659.250	3598408.000	525
1	Segment #602326 (GFP)	2064190.354	82844760.000	526
1	Segment #602336 (GFP)	2029431.425	81449736.000	527
1	Segment #605002 (GFP)	91134.897	3657632.000	528
1	Segment #609757 (GFP)	2057902.061	82592384.000	529
1	Segment #609762 (GFP)	2033082.371	81596264.000	530
1	Segment #609776 (GFP)	2035896.525	81709208.000	531
1	Segment #612400 (GFP)	90598.697	3636112.000	532
1	Segment #617201 (GFP)	2027744.089	81382016.000	533
1	Segment #619716 (GFP)	92427.758	3709520.000	534
1	Segment #624585 (GFP)	2052076.216	82358568.000	535
1	Segment #624589 (GFP)	2027173.006	81359096.000	536
1	Segment #627074 (GFP)	91812.224	3684816.000	537
1	Segment #632017 (GFP)	2047870.732	82189784.000	538
1	Segment #632019 (GFP)	2018106.638	80995224.000	539
1	Segment #634509 (GFP)	93927.723	3769720.000	540
1	Segment #639442 (GFP)	2050128.952	82280416.000	541
1	Segment #639446 (GFP)	2026285.385	81323472.000	542
1	Segment #641909 (GFP)	95027.232	3813848.000	543
1	Segment #646886 (GFP)	2052583.314	82378920.000	544
1	Segment #646888 (GFP)	2024199.786	81239768.000	545
1	Segment #649361 (GFP)	93526.071	3753600.000	546
1	Segment #654488 (GFP)	2049095.820	82238952.000	547

1	Segment #654493 (GFP)	2026395.017	81327872.000	548
1	Segment #656842 (GFP)	95730.870	3842088.000	549
1	Segment #661932 (GFP)	2047701.500	82182992.000	550
1	Segment #661934 (GFP)	2023197.949	81199560.000	551
1	Segment #664339 (GFP)	94014.233	3773192.000	552
1	Segment #669458 (GFP)	2041357.793	81928392.000	553
1	Segment #669464 (GFP)	2021506.426	81131672.000	554
1	Segment #671874 (GFP)	94137.220	3778128.000	555
1	Segment #677043 (GFP)	2041616.924	81938792.000	556
1	Segment #677045 (GFP)	2020314.228	81083824.000	557
1	Segment #679541 (GFP)	95530.543	3834048.000	558
1	Segment #684634 (GFP)	2040928.833	81911176.000	559
1	Segment #684642 (GFP)	2014541.804	80852152.000	560
1	Segment #687057 (GFP)	99701.742	4001456.000	561
1	Segment #692206 (GFP)	2031952.563	81550920.000	562
1	Segment #692210 (GFP)	2010180.642	80677120.000	563
1	Segment #692211 (GFP)	2014480.410	80849688.000	564
1	Segment #694567 (GFP)	98241.643	3942856.000	565
1	Segment #699588 (GFP)	2006193.227	80517088.000	566
1	Segment #699592 (GFP)	2006302.460	80521472.000	567
1	Segment #702041 (GFP)	99760.345	4003808.000	568
1	Segment #707186 (GFP)	1994454.230	80045952.000	569
1	Segment #707187 (GFP)	2006232.893	80518680.000	570
1	Segment #709429 (GFP)	103942.308	4171648.000	571
1	Segment #714601 (GFP)	1998929.209	80225552.000	572
1	Segment #714604 (GFP)	1999095.850	80232240.000	573
1	Segment #716811 (GFP)	99239.892	3982920.000	574
1	Segment #721920 (GFP)	1982452.116	79564256.000	575
1	Segment #721923 (GFP)	2001860.171	80343184.000	576
1	Segment #724133 (GFP)	101090.879	4057208.000	577
1	Segment #729183 (GFP)	1987588.076	79770384.000	578
1	Segment #729186 (GFP)	2004802.495	80461272.000	579
1	Segment #731501 (GFP)	100666.902	4040192.000	580
1	Segment #736712 (GFP)	1984824.553	79659472.000	581
1	Segment #736713 (GFP)	2003797.069	80420920.000	582
1	Segment #739061 (GFP)	101490.936	4073264.000	583
1	Segment #744435 (GFP)	1978081.187	79388832.000	584
1	Segment #744443 (GFP)	1998326.632	80201368.000	585
1	Segment #746592 (GFP)	101355.591	4067832.000	586
1	Segment #751927 (GFP)	1964272.139	78834616.000	587
1	Segment #751932 (GFP)	1988046.936	79788800.000	588
1	Segment #754096 (GFP)	99574.369	3996344.000	589
1	Segment #756173 (GFP)	1961926.213	78740464.000	590

1	Segment #759487 (GFP)	1994246.327	80037608.000	591
1	Segment #761622 (GFP)	102755.691	4124024.000	592
1	Segment #767223 (GFP)	1964302.238	78835824.000	593
1	Segment #767226 (GFP)	1994131.114	80032984.000	594
1	Segment #769361 (GFP)	104010.479	4174384.000	595
1	Segment #774780 (GFP)	1958888.011	78618528.000	596
1	Segment #774787 (GFP)	1989871.612	79862032.000	597
1	Segment #776986 (GFP)	104787.072	4205552.000	598
1	Segment #782282 (GFP)	1952585.366	78365576.000	599
1	Segment #784295 (GFP)	107292.862	4306120.000	600
1	Segment #789707 (GFP)	1984900.298	79662512.000	601
1	Segment #789710 (GFP)	1944746.678	78050976.000	602
1	Segment #791736 (GFP)	106996.058	4294208.000	603
1	Segment #797193 (GFP)	1990338.843	79880784.000	604
1	Segment #797198 (GFP)	1943062.531	77983384.000	605
1	Segment #799190 (GFP)	108358.485	4348888.000	606
1	Segment #801439 (GFP)	1943949.952	78019000.000	607
1	Segment #804715 (GFP)	1990669.932	79894072.000	608
1	Segment #806860 (GFP)	110852.713	4448992.000	609
1	Segment #809025 (GFP)	1935622.304	77684776.000	610
1	Segment #812407 (GFP)	1990204.295	79875384.000	611
1	Segment #814400 (GFP)	112033.350	4496376.000	612
1	Segment #819881 (GFP)	1990097.653	79871104.000	613
1	Segment #819886 (GFP)	1929405.571	77435272.000	614
1	Segment #821918 (GFP)	109891.140	4410400.000	615
1	Segment #827249 (GFP)	1984175.531	79633424.000	616
1	Segment #827254 (GFP)	1923347.705	77192144.000	617
1	Segment #829393 (GFP)	111064.801	4457504.000	618
1	Segment #831494 (GFP)	1921922.888	77134960.000	619
1	Segment #834835 (GFP)	1989349.763	79841088.000	620
1	Segment #837035 (GFP)	111619.339	4479760.000	621
1	Segment #839232 (GFP)	1913945.465	76814792.000	622
1	Segment #842335 (GFP)	1983345.318	79600104.000	623
1	Segment #844446 (GFP)	106686.497	4281784.000	624
1	Segment #846611 (GFP)	1903108.640	76379864.000	625
1	Segment #849673 (GFP)	1978834.060	79419048.000	626
1	Segment #849678 (GFP)	1968556.956	79006584.000	627
1	Segment #851799 (GFP)	107191.402	4302048.000	628
1	Segment #853836 (GFP)	1898431.539	76192152.000	629
1	Segment #857004 (GFP)	1970650.928	79090624.000	630
1	Segment #859128 (GFP)	109504.638	4394888.000	631
1	Segment #861235 (GFP)	1885538.615	75674704.000	632
1	Segment #864425 (GFP)	1963928.691	78820832.000	633

1	Segment #866497 (GFP)	107449.536	4312408.000	634
1	Segment #868544 (GFP)	1884405.618	75629232.000	635
1	Segment #871592 (GFP)	1960303.858	78675352.000	636
1	Segment #873680 (GFP)	109190.492	4382280.000	637
1	Segment #875777 (GFP)	1879012.122	75412768.000	638
1	Segment #878991 (GFP)	1958415.597	78599568.000	639
1	Segment #881083 (GFP)	110935.036	4452296.000	640
1	Segment #883309 (GFP)	1879392.046	75428016.000	641
1	Segment #886527 (GFP)	1964969.597	78862608.000	642
1	Segment #888510 (GFP)	109175.941	4381696.000	643
1	Segment #890597 (GFP)	1869830.142	75044256.000	644
1	Segment #893677 (GFP)	1963530.229	78804840.000	645
1	Segment #895927 (GFP)	107722.220	4323352.000	646
1	Segment #897908 (GFP)	1871807.903	75123632.000	647
1	Segment #901060 (GFP)	1961484.097	78722720.000	648
1	Segment #903320 (GFP)	105835.553	4247632.000	649
1	Segment #905331 (GFP)	1868342.336	74984544.000	650
1	Segment #908560 (GFP)	1958360.581	78597360.000	651
1	Segment #910781 (GFP)	104326.618	4187072.000	652
1	Segment #912635 (GFP)	1865524.195	74871440.000	653
1	Segment #915784 (GFP)	1948825.387	78214672.000	654
1	Segment #917771 (GFP)	106064.784	4256832.000	655
1	Segment #919790 (GFP)	1857593.017	74553128.000	656
1	Segment #923075 (GFP)	1948486.325	78201064.000	657
1	Segment #925139 (GFP)	104384.225	4189384.000	658
1	Segment #927031 (GFP)	1852678.513	74355888.000	659
1	Segment #930253 (GFP)	1944024.701	78022000.000	660
1	Segment #932255 (GFP)	103891.479	4169608.000	661
1	Segment #934209 (GFP)	1852592.601	74352440.000	662
1	Segment #937536 (GFP)	1936502.550	77720104.000	663
1	Segment #939674 (GFP)	102732.170	4123080.000	664
1	Segment #941600 (GFP)	1840871.743	73882032.000	665
1	Segment #944864 (GFP)	1939226.606	77829432.000	666
1	Segment #947117 (GFP)	101221.242	4062440.000	667
1	Segment #948856 (GFP)	1838366.353	73781480.000	668
1	Segment #952093 (GFP)	1937401.732	77756192.000	669
1	Segment #954405 (GFP)	102354.239	4107912.000	670
1	Segment #956201 (GFP)	1831359.472	73500264.000	671
1	Segment #959424 (GFP)	1927029.746	77339920.000	672
1	Segment #961509 (GFP)	100146.449	4019304.000	673
1	Segment #963346 (GFP)	1828031.842	73366712.000	674
1	Segment #966654 (GFP)	1928564.594	77401520.000	675
1	Segment #968775 (GFP)	99025.412	3974312.000	676

1	Segment #970480 (GFP)	1819609.113	73028672.000	677
1	Segment #973806 (GFP)	1928195.832	77386720.000	678
1	Segment #975821 (GFP)	96744.668	3882776.000	679
1	Segment #977586 (GFP)	1818510.401	72984576.000	680
1	Segment #980870 (GFP)	1926169.434	77305392.000	681
1	Segment #982868 (GFP)	97646.640	3918976.000	682
1	Segment #984632 (GFP)	1813442.413	72781176.000	683
1	Segment #987958 (GFP)	1915817.979	76889944.000	684
1	Segment #990060 (GFP)	96684.071	3880344.000	685
1	Segment #991703 (GFP)	1802702.861	72350152.000	686
1	Segment #995149 (GFP)	1924117.721	77223048.000	687
1	Segment #997303 (GFP)	97022.934	3893944.000	688
1	Segment #999017 (GFP)	1805252.503	72452480.000	689
1	Segment #1002337 (GFP)	1913981.145	76816224.000	690
1	Segment #1004411 (GFP)	95241.314	3822440.000	691
1	Segment #1006176 (GFP)	1807742.744	72552424.000	692
1	Segment #1009475 (GFP)	1906248.301	76505872.000	693
1	Segment #1011662 (GFP)	94731.425	3801976.000	694
1	Segment #1013177 (GFP)	1797108.838	72125640.000	695
1	Segment #1016640 (GFP)	1907460.233	76554512.000	696
1	Segment #1018824 (GFP)	94570.964	3795536.000	697
1	Segment #1020489 (GFP)	1790368.861	71855136.000	698
1	Segment #1023853 (GFP)	1901580.967	76318552.000	699
1	Segment #1025973 (GFP)	93209.334	3740888.000	700
1	Segment #1027681 (GFP)	1787979.282	71759232.000	701
1	Segment #1031253 (GFP)	1900854.007	76289376.000	702
1	Segment #1033398 (GFP)	93105.084	3736704.000	703
1	Segment #1035090 (GFP)	1785827.106	71672856.000	704
1	Segment #1038613 (GFP)	1902572.041	76358328.000	705
1	Segment #1040851 (GFP)	94705.512	3800936.000	706
1	Segment #1042510 (GFP)	1780819.514	71471880.000	707
1	Segment #1046112 (GFP)	1904008.619	76415984.000	708
1	Segment #1048324 (GFP)	92400.848	3708440.000	709
1	Segment #1049757 (GFP)	1783528.621	71580608.000	710
1	Segment #1053092 (GFP)	1889903.364	75849880.000	711
1	Segment #1055395 (GFP)	90309.867	3624520.000	712
1	Segment #1056830 (GFP)	1770130.194	71042872.000	713
1	Segment #1060431 (GFP)	1893404.014	75990376.000	714
1	Segment #1062645 (GFP)	91551.100	3674336.000	715
1	Segment #1064123 (GFP)	1775102.703	71242440.000	716
1	Segment #1067653 (GFP)	1892732.867	75963440.000	717
1	Segment #1069972 (GFP)	89234.078	3581344.000	718
1	Segment #1071392 (GFP)	1758716.904	70584808.000	719

1	Segment #1075051 (GFP)	1890803.343	75886000.000	720
1	Segment #1077140 (GFP)	89173.680	3578920.000	721
1	Segment #1078671 (GFP)	1757239.064	70525496.000	722
1	Segment #1082300 (GFP)	1893143.090	75979904.000	723
1	Segment #1084642 (GFP)	89630.945	3597272.000	724
1	Segment #1086026 (GFP)	1765365.188	70851632.000	725
1	Segment #1089561 (GFP)	1891544.456	75915744.000	726
1	Segment #1091885 (GFP)	88870.697	3566760.000	727
1	Segment #1093430 (GFP)	1774457.070	71216528.000	728
1	Segment #1097136 (GFP)	1887930.387	75770696.000	729
1	Segment #1099406 (GFP)	89177.866	3579088.000	730
1	Segment #1100892 (GFP)	1766202.777	70885248.000	731
1	Segment #1104549 (GFP)	1883103.389	75576968.000	732
1	Segment #1106724 (GFP)	88489.975	3551480.000	733
1	Segment #1108182 (GFP)	1756914.354	70512464.000	734
1	Segment #1111829 (GFP)	1876879.281	75327168.000	735
1	Segment #1114145 (GFP)	87474.783	3510736.000	736
1	Segment #1115479 (GFP)	1742148.916	69919864.000	737
1	Segment #1119261 (GFP)	1880348.237	75466392.000	738
1	Segment #1121503 (GFP)	87657.968	3518088.000	739
1	Segment #1122927 (GFP)	1738874.108	69788432.000	740
1	Segment #1126688 (GFP)	1877713.680	75360656.000	741
1	Segment #1128992 (GFP)	88488.979	3551440.000	742
1	Segment #1130329 (GFP)	1736921.662	69710072.000	743
1	Segment #1133963 (GFP)	1875022.912	75252664.000	744
1	Segment #1136390 (GFP)	89303.644	3584136.000	745
1	Segment #1137653 (GFP)	1733132.381	69557992.000	746
1	Segment #1141267 (GFP)	1865073.508	74853352.000	747
1	Segment #1143608 (GFP)	87328.275	3504856.000	748
1	Segment #1144936 (GFP)	1719357.816	69005160.000	749
1	Segment #1148735 (GFP)	1856574.835	74512264.000	750
1	Segment #1151089 (GFP)	84806.938	3403664.000	751
1	Segment #1152326 (GFP)	1708795.071	68581232.000	752
1	Segment #1155992 (GFP)	1857875.469	74564464.000	753
1	Segment #1158354 (GFP)	86507.828	3471928.000	754
1	Segment #1159638 (GFP)	1723493.534	69171144.000	755
1	Segment #1163264 (GFP)	1856745.462	74519112.000	756
1	Segment #1165596 (GFP)	86652.343	3477728.000	757
1	Segment #1166885 (GFP)	1720670.211	69057832.000	758
1	Segment #1170535 (GFP)	1857850.752	74563472.000	759
1	Segment #1172864 (GFP)	86283.581	3462928.000	760
1	Segment #1174175 (GFP)	1709701.030	68617592.000	761
1	Segment #1177932 (GFP)	1850082.626	74251704.000	762

1	Segment #1180268 (GFP)	85640.340	3437112.000	763
1	Segment #1181628 (GFP)	1704773.171	68419816.000	764
1	Segment #1185269 (GFP)	1842010.521	73927736.000	765
1	Segment #1187527 (GFP)	84508.141	3391672.000	766
1	Segment #1188960 (GFP)	1690649.578	67852976.000	767
1	Segment #1192790 (GFP)	1847023.096	74128912.000	768
1	Segment #1195008 (GFP)	86698.389	3479576.000	769
1	Segment #1196361 (GFP)	1701929.117	68305672.000	770
1	Segment #1200096 (GFP)	1836594.700	73710376.000	771
1	Segment #1202537 (GFP)	84512.127	3391832.000	772
1	Segment #1203865 (GFP)	1682646.043	67531760.000	773
1	Segment #1207688 (GFP)	1841745.610	73917104.000	774
1	Segment #1210001 (GFP)	84988.528	3410952.000	775
1	Segment #1211312 (GFP)	1688520.724	67767536.000	776
1	Segment #1215113 (GFP)	1831631.360	73511176.000	777
1	Segment #1217407 (GFP)	83319.730	3343976.000	778
1	Segment #1218584 (GFP)	1674423.642	67201760.000	779
1	Segment #1222279 (GFP)	1829991.863	73445376.000	780
1	Segment #1224726 (GFP)	81612.859	3275472.000	781
1	Segment #1225832 (GFP)	1675216.580	67233584.000	782
1	Segment #1229772 (GFP)	1839373.772	73821912.000	783
1	Segment #1232269 (GFP)	80558.199	3233144.000	784
1	Segment #1233511 (GFP)	1680313.671	67438152.000	785
1	Segment #1237332 (GFP)	1835029.553	73647560.000	786
1	Segment #1239797 (GFP)	81923.018	3287920.000	787
1	Segment #1241009 (GFP)	1674368.029	67199528.000	788

First, Time Point	Mean, Intensities #1	Surface Area (µm²)
0	337.342	352334.188
1	344.431	347758.943
2	350.783	373863.702
3	352.487	431076.283
4	358.361	447171.626
5	363.787	446697.618
6	370.018	439180.109
7	376.289	412783.237
8	382.728	359062.449
9	394.884	334083.006
10	407.203	322143.230
11	421.543	319384.366
12	434.522	323998.480
13	448.853	345465.414
14	456.689	352656.930
15	461.741	360158.481
16	467.791	367597.436
17	471.172	372238.433
18	474.210	377434.958
19	479.310	378330.677
20	483.551	385825.510
21	488.864	393533.880
22	494.248	400184.340
23	496.832	401578.022
24	500.234	408091.033
25	500.695	422248.974
26	503.217	431400.761
27	505.968	436518.811
28	505.629	442238.325
29	508.706	452699.062
30	508.034	463560.862
31	509.239	471456.827
32	507.414	492148.924
33	506.406	505299.447
34	507.169	514660.859
35	506.293	532341.265
36	507.405	544524.631
37	506.648	551530.377
38	506.998	556617.596
39	506.186	565824.369
40	507.055	578489.645
41	508.186	591255.974

42	506.485	597233.136
43	506.596	602081.255
44	506.841	613740.813
45	507.333	619123.280
46	509.089	622005.985
47	507.786	624386.132
48	507.175	627519.586
49	505.872	634730.432
50	506.618	634892.303
51	504.456	633375.557
52	504.532	631949.469
53	502.888	629687.517
54	502.620	637668.529
55	502.020	627483.648
56	501.827	631748.555
57	501.711	627156.282
58	500.618	625108.827
59	499.683	622271.853
60	484.028	340190.607
60	515.436	276608.118
61	498.660	616703.091
62	498.154	611468.820
63	497.280	608141.765
64	479.455	336662.089
64	514.313	269681.840
65	479.191	326636.346
65	514.101	270326.192
66	495.853	593538.894
67	361.725	32317.582
67	498.320	551075.811
68	362.557	32777.309
68	496.832	545034.681
69	363.614	30340.022
69	496.641	545497.212
70	360.907	32016.127
70	495.806	543237.574
71	361.102	32109.416
71	481.040	279705.875
71	510.483	257793.078
72	478.676	280167.569
72	361.024	32076.350
72	509.958	255286.122
73	477.610	276221.555

73	360.670	32995.868
73	507.961	255471.959
74	360.959	34043.190
74	477.940	277186.489
74	508.507	256365.709
75	359.904	32655.899
75	477.601	274494.932
75	507.179	254317.107
76	361.352	32964.775
76	477.764	271752.318
76	507.371	255631.876
77	361.879	32058.919
77	475.369	273961.658
77	504.586	253709.513
78	360.869	32338.081
78	474.863	274472.131
78	504.441	254495.758
79	363.277	32053.957
79	474.586	270556.759
79	504.885	253360.370
80	363.689	30742.800
80	474.054	270101.512
80	504.872	253520.896
81	363.237	31749.307
81	472.942	270684.335
81	503.824	252152.873
82	474.002	256663.649
82	363.635	30807.698
82	502.218	253071.292
83	363.621	32436.416
83	472.415	267762.567
83	501.564	250574.903
84	361.715	33552.083
84	471.013	268103.397
84	499.273	250944.027
85	364.306	33172.963
85	470.239	268070.266
85	499.038	250813.894
86	364.138	33025.354
86	470.015	269080.344
86	498.048	250804.938
87	363.874	33457.746
87	468.556	268577.024

87	495.450	252142.820
88	362.146	34994.230
88	468.705	269706.719
88	495.476	251903.007
89	362.726	33997.718
89	469.221	266207.274
89	495.715	249804.776
90	361.250	34306.397
90	468.766	266440.843
90	494.831	249101.545
91	363.212	34543.485
91	468.578	266539.376
91	493.551	249835.058
92	363.227	37320.436
92	466.382	266627.211
92	490.343	249675.856
93	467.222	255141.373
93	362.619	36644.533
93	488.462	247504.720
94	466.460	254198.628
94	363.433	35328.172
94	487.494	248819.166
95	466.330	254924.098
95	363.869	37815.606
95	486.301	249140.361
96	465.576	252440.228
96	365.468	36020.803
96	484.893	247286.886
97	465.993	252869.962
97	365.700	36949.247
97	484.095	247606.481
98	465.312	252964.945
98	365.789	36066.145
98	482.012	247913.538
99	464.599	252491.488
99	366.849	36635.512
99	479.767	248381.915
100	465.133	252212.304
100	365.890	37032.096
100	478.518	246577.831
101	464.196	248554.678
101	365.843	36940.428
101	477.251	245628.404

102	463.896	250010.949
102	364.902	40596.458
102	476.042	245866.360
103	462.639	250618.508
103	365.458	39566.500
103	473.866	247360.213
104	461.678	248897.457
104	364.454	39073.210
104	472.937	245832.886
105	364.550	41348.275
105	460.886	249375.041
105	470.395	245738.926
106	365.128	41075.339
106	460.399	250961.235
106	469.357	244134.570
107	365.079	41339.824
107	467.528	243603.977
107	459.816	251257.871
108	363.520	42951.371
108	465.667	243175.932
108	458.599	250590.534
109	362.159	43584.685
109	458.531	250141.227
109	464.119	242832.340
110	363.680	42305.841
110	459.144	250202.534
110	463.012	242777.351
111	366.263	42357.543
111	461.979	241827.534
111	457.741	250978.278
112	366.497	42185.242
112	460.500	240750.384
112	456.421	250533.481
113	365.534	40683.402
113	459.096	239819.375
113	456.308	249152.980
114	456.510	246915.063
114	366.179	39736.471
114	457.741	240486.289
115	455.130	248212.879
115	365.301	42056.628
115	454.437	237107.293
116	452.882	247846.415

116	365.723	41830.443
116	452.355	238185.126
117	450.918	246546.136
117	364.783	39913.532
117	449.013	237888.050
118	449.397	246280.637
118	363.916	41894.372
118	447.033	237751.497
119	447.409	247130.049
119	362.823	40823.524
119	445.108	236722.300
120	446.590	245627.058
120	363.027	39484.663
120	443.790	239234.815
121	444.162	247783.934
121	361.416	40399.700
121	441.345	238557.143
122	443.779	246048.406
122	361.602	39480.093
122	440.267	240345.490
123	442.515	244167.605
123	359.976	41293.166
123	438.648	238984.499
124	442.071	245766.198
124	360.538	39405.471
124	437.721	238024.745
125	440.528	245272.743
125	360.540	39285.249
125	436.871	240593.702
126	439.224	244936.022
126	360.256	38868.390
126	435.305	236470.649
127	439.635	246487.666
127	360.904	37555.634
127	434.378	238048.432
128	437.925	245830.694
128	360.288	37166.943
128	433.079	237176.084
129	435.755	245040.230
129	357.956	37420.681
129	431.730	237113.686
130	436.079	245340.078
130	357.052	37794.513

130	430.961	238769.524
131	435.271	245214.255
131	355.561	37193.471
131	430.043	238350.796
132	434.561	245228.669
132	355.146	36858.753
132	428.931	237906.482
133	433.028	244397.583
133	355.317	36286.108
133	426.901	238405.606
134	433.783	246751.003
134	356.190	36568.693
134	426.435	238574.460
135	432.494	243968.983
135	356.722	35762.432
135	426.272	239477.668
136	431.304	246208.920
136	356.839	36833.859
136	424.434	239974.036
137	430.916	246052.459
137	354.254	36876.754
137	423.250	240525.896
138	429.079	247644.829
138	352.783	36408.326
138	421.957	239145.457
139	428.415	247310.315
139	353.624	36205.527
139	421.274	239190.494
140	428.352	246211.125
140	354.438	36484.872
140	421.668	237489.012
141	427.149	244989.107
141	354.565	34215.070
141	421.904	238064.401
142	425.066	245749.236
142	351.951	35839.875
142	419.050	238881.438
143	425.184	245795.575
143	351.625	35367.845
143	418.359	238832.690
144	424.176	245714.185
144	352.140	34520.935
144	416.709	236716.145

145	424.156	246270.589
145	350.588	36055.183
145	416.696	238387.679
146	422.837	249381.520
146	352.659	34316.511
146	417.788	237979.043
147	423.146	246304.749
147	351.473	35673.937
147	417.511	239157.799
148	422.305	247092.428
148	351.823	35848.684
148	416.958	239886.620
149	421.942	246489.162
149	352.581	34370.591
149	415.914	237717.028
150	421.556	247775.000
150	351.990	33931.790
150	413.392	238023.996
151	422.206	246969.729
151	351.026	34085.562
151	414.689	236721.148
152	421.891	245967.212
152	351.247	34932.460
152	415.657	236632.019
153	420.288	247607.543
153	351.184	34221.269
153	414.621	236974.439
154	419.673	245502.298
154	349.848	34009.640
154	412.742	235996.554
155	417.640	248294.933
155	349.396	33186.119
155	411.381	235919.335
156	416.039	248139.721
156	349.067	33481.632
156	410.873	236472.491
157	416.736	247616.071
157	348.858	33789.310
157	411.326	236611.924
158	416.869	249061.962
158	348.202	34098.574
158	410.767	235946.985
159	416.388	249070.523

159	347.848	34077.888
159	410.020	235562.596
160	414.694	247493.232
160	346.251	34326.465
160	407.550	235659.874
161	414.518	248611.643
161	348.187	34372.877
161	408.666	233527.686
162	411.818	247825.600
162	346.483	33544.239
162	404.678	236489.127
163	411.556	248206.304
163	346.833	34114.275
163	403.625	238686.071
164	408.128	245732.654
164	345.782	33536.398
164	400.774	238689.557
165	407.371	248315.486
165	344.072	33297.952
165	399.272	241816.431
166	407.657	249531.465
166	345.024	33219.210
166	399.302	240696.236
167	406.234	249489.053
167	346.051	33378.595
167	398.491	242800.276

Name	Reference Number
60mL Syringe Luer-Lok Tip	309653
Agarose, Low Melting Temperature	V2111
arivis Vision4D Software	https://www.arivis.com/en/imaging-science/arivis-vision4d
Dumoxel N3C Forceps	0203-N3C-PO
E3 fish buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$)	
Glass Capillary – Size 2 (~1mm)	Black/701904
Heidelberger Extension Line 100cm	4097262
Light & Filter Set – Royal Blue	SFA-LFS-RB
Petri Dish (100 x 15 mm)	25384-302
Stereomicroscope Fluorescence Adapter	
Teflon Tipped Plunger – Size 2	#701997
Tg(rx3:GFP) zebrafish	
Tricaine (MS222)	A-5040
Z.1 Lightsheet	
Zen Software	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html

Company
BD
Promega
arivis
Dumont
Zeiss
B. Braun
Night Sea
VWR
NightSea
Zeiss
Sigma
Zeiss
Zeiss

Response to editorial and peer review comments

We appreciate the thoughtful and constructive comments provided by the editors and reviewers. Our responses are detailed below.

Reviewer #5:

Minor Concerns:
I appreciate the highlighting in Table 2 to better explain the data, but I think there are errors in the lines that have been highlighted. It looks like the highlighting should start earlier and in some places is incorrect. If I am wrong, then further explanation is needed. It would also help if the authors could point out to the reader the transition from measuring a single eye field to measuring separate eye fields.
If highlighting is fixed, then the manuscript is acceptable for publication.

We thank the reviewer for catching the errors in highlighting – these have now been corrected, and we have clarified in the text at which time point the highlighting begins.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

This has been done

2. Line 97-101: Please include the details of how the mating pair is selected. Is there a specific age of the fish which determines its ability to mate? Are the embryos transferred to a separate tank?

These details have now been added, along with a relevant citation.

3. Line 111: Please include more details on dechorionation? A citation would suffice.

This has been done.

4. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

This has been done.

5. Please ensure that the Table of Materials covers all the essential supplies, reagents,

and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

The Table has now been arranged in alphabetical order. We have included catalog numbers for all items that had one (the Nighsea base and Lighsheet microscope did not have associated reference numbers, so we left those fields blank).