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Light sheet microscopy of fast cardiac dynamics in zebrafish embryos

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TITLE:

Light Sheet Microscopy of Fast Cardiac Dynamics in Zebrafish Embryos

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SUMMARY:

We describe optimized tools to study the zebrafish heart *in vivo* with light sheet fluorescence microscopy. Specifically, we suggest bright cardiac transgenic lines and present new gentle embedding and immobilization techniques that avoid developmental and heart defects. A possible data acquisition and analysis pipeline adapted to cardiac imaging is also provided.

ABSTRACT:

Embryonic cardiac research has greatly benefited from advances in fast *in vivo* light sheet fluorescence microscopy (LSFM). Combined with the rapid external development, tractable genetics, and translucency of the zebrafish, *Danio rerio*, LSFM has delivered insights into cardiac form and function at high spatial and temporal resolution without significant phototoxicity or photobleaching. Imaging of beating hearts challenges existing sample preparation and microscopy techniques. One needs to maintain a healthy sample in a constricted field of view and acquire ultrafast images to resolve the heartbeat. Here we describe optimized tools and solutions to study the zebrafish heart *in vivo*. We demonstrate the applications of bright transgenic lines for labeling the cardiac constituents and present novel gentle embedding and immobilization techniques that avoid developmental defects and changes in heart rate. We also propose a data acquisition and analysis pipeline adapted to cardiac imaging. The entire workflow presented here focuses on zebrafish embryonic heart imaging but can also be applied to various other samples and experiments.

INTRODUCTION:

To uncover the complex events and interactions in the early beating heart, *in vivo* imaging of the whole organ is required. With its minimal phototoxicity[1–3], low photobleaching[4], and high speed, light sheet microscopy has evolved as the primary imaging tool for embryonic and cardiac development[5,6]. It has delivered insights into cardiac form and function at a high spatial and temporal resolution[7–9] and has allowed researchers to image and track fluorescently labeled

parts of the heart at high speed, study hemodynamic forces, and follow the heart development directly inside the body of developing embryos[6,10–12].

To precisely and reproducibly constrain zebrafish in the field of view, a variety of embedding protocols for light sheet exist, for the short and long term, as well as single or multi-sample[13–19]. The most common protocol involves tricaine immobilization and agarose mounting inside a glass or plastic tube. However, as the heart rate can change due to the temperature, anesthetics, and embedding material used[20–22], zebrafish cardiac imaging requires specific, gentle protocols to ensure sample health[6,8,11,12,20–23]. For short-term imaging (up to an hour), one can anesthetize the fish in 130 mg/L tricaine and embed it in Fluorinated Ethylene Propylene (FEP) tubes with 0.1% agarose solution and a plug, as described in Weber et al. 2014[16]. However, as tricaine can lead to developmental defects[20,22], different protocols must be used for long-term imaging.

Here we describe a new strategy for long-term cardiac imaging. While many light sheet implementations exist[24], we recommend using a hanging sample in a T-SPIM microscope (one detection and two illumination lenses in a horizontal plane with the sample hanging vertically in the common focus). This gives the necessary freedom of movement and rotation for the precise sample positioning. The fish are immobilized by injecting 30 pg α-bungarotoxin mRNA at the one- or two-cell stage. α-bungarotoxin is a snake venom that paralyzes muscles without affecting cardiovascular development or physiology[22]. For precise, distortion-free imaging, we recommend mounting fish in tubes made of FEP, a polymer with a refractive index almost identical to water. We discuss how to best prepare the FEP tubes by straightening and cleaning them prior to imaging. The fish are then mounted in these tubes, head down, in media, and the bottom of the tube is sealed with a 2% agarose plug, on which fish heads rest. Cutting holes in the FEP tube facilitates gas exchange and ensures fish growth. The embedded fish can be kept in media until mounted onto a sample holder right before imaging. We also suggest a data acquisition and analysis pipeline for reproducible high-speed imaging. Further, we discuss the use of cytoplasmic versus membrane marker transgenic lines for robust heart cell labeling, as well as different options to stop the heart. These mounting techniques ensure sample health while allowing to constrain the heart precisely and reproducibly in the field of view.

PROTOCOL:

All zebrafish (*Danio rerio*) adults and embryos were handled in accordance with protocols approved by the UW-Madison Institutional Animal Care and Use Committee (IACUC).

1. Preparation of zebrafish

1.1. Handle zebrafish according to established protocols[25,26] and institutional guidelines. Breed adult fish of desired transgenic line (see Discussion). Collect the embryos and keep them at 28 °C in a Petri dish filled with fish medium, e.g., E3[27].

1.2. Choose a method of immobilization (see Discussion).

1.2.1. If using α-bungarotoxin mRNA to immobilize the fish, inject 30 pg mRNA[22] into the yolk of one- or two-cell stage embryos using a bore glass needle mounted onto a micromanipulator and connected to a picoinjector[28].

1.2.2. If using tricaine, make 0.4% stock solution buffered to pH 7.0-7.4 with 1 M Tris base and store at -20 °C until imaging.

1.3. Keep the eggs in an E3 filled Petri dish at 28 °C and transfer the eggs every 24 h to a new dish with fresh E3 until imaging.

1.4. To prevent pigment formation, if the zebrafish background is not albino, transfer fish at 24 h post-fertilization (hpf) to a new E3 dish with 0.2 mM tyrosinase inhibitor 1- phenyl 2-thiourea (see Discussion).

2. Preparation of FEP tubes

[Place **Figure 1** here]

2.1. Straighten the FEP tubes (**Figure 1a,b**) by placing them in a glass or steel autoclave-safe tubing (**Figure 1c**) with the correct inner diameter to fit FEP tubes, usually 1.6 or 2.4 mm, and autoclave to 180 °C for 2 h. Let the tubes cool down at room temperature for at least 5 h. Then, remove from the straightening tubes.

NOTE: Use gloves when manipulating the tubes and work with 50 cm tubing at a time.

2.2. Clean the FEP tubes.

NOTE: Syringes with blunt needle tip of the inner FEP tube size are recommended for safety, but a regular needle will work.

2.2.1. Flush the tubes with 1 M NaOH twice with a 50 mL syringe (**Figure 1d**).

2.2.2. Cut the FEP tubes to the size of a 50 mL centrifuge tube with a razor blade (**Figure 1e**), place cut tubes in 0.5 M NaOH filled centrifuge tubes, and ultrasonicate them for 10 min.

2.2.3. Flush the FEP tubes with double-distilled H_2O, then repeat flushing with 70% ethanol (**Figure 1f**).

2.2.4. Transfer tubes to 70% ethanol and ultrasonicate for 10 min.

2.2.5. Flush the tubes with double-distilled H_2O and store them in centrifuge tubes in double-distilled H_2O (**Figure 1g**).

3. Preparation of 2% agarose dish

3.1. In a glass flask, dissolve low melting point agarose powder in E3. Heat the solution in a microwave and stir it every 20 s, until all powder is dissolved.

3.2. Pour agarose into a glass or plastic Petri dish to make a 1–2 mm coat. Wait until agarose is solidified.

3.3. To store, gently pour E3 on the top of the agar to prevent drying. Wrap in paraffin film and keep at 4 °C.

4. Preparation of embedding media

4.1. Prepare enough E3 to fill the sample chamber.

NOTE: Avoid using methyl blue if media is in contact with objective lenses.

4.2. If using tricaine, thaw stock solution and add 0.02% tricaine to E3.

5. Sample mounting

[Place **Figure 2** here]

5.1. With a disposable glass pipette, transfer fish to embedding media (**Figure 2a**). If using tricaine, transfer fish to Petri dish filled with tricaine-containing E3, 10 min before imaging. In both cases, view under a stereomicroscope to verify that the fish stopped moving and that the heart is beating at the similar speed when compared to the control.

5.2. Cut the FEP tube to the ideal length with a razor blade (**Figure 1h**).

NOTE: The length should be adjusted to the microscope's sample holder; the typical length is about 3 cm.

5.3. Prepare a syringe with a blunt end cannula. Fill the syringe with air, then mount the FEP tube onto the needle and gently flush out any remaining water by emptying the syringe (**Figure 2b**).

NOTE: Avoid making bubbles by slowly flushing out the air.

5.4. With the syringe mounted FEP tube, first, take up media to fill the FEP tube, then take up an embryo head down. Keep the fish head as close to the tube end as possible. Avoid making any bubbles; if a bubble is present, discard the sample.

5.5. With a razor blade, carefully cut the FEP tube at the edge of the blunt end cannula or needle (**Figure 2c**).

5.6. Discard any liquid on the top of the agar-coated dish. Plunge the FEP tube straight into the agar (**Figure 2d**). Rotate the tube and take it out to release the plug from the agarose bed.

5.7. Under a stereoscope, verify the presence of the agar plug at the end of the tube (**Figure 2e**).

5.8. For long-term imaging, cut 3–5 holes into the FEP tube at each cardinal direction, at least 5 mm above the end of the fish.

5.8.1. Under a stereoscope, use a razor blade perpendicular to the axis of the tube to make a 30° incision into the FEP tube until reaching the mounting media (**Figure 2f**).

5.8.2. Make a second cut at 180° to create a hole (**Figure 2g,h**).

5.9. Transfer mounted embryo head down into a 1.5 mL microcentrifuge tube with embedding media until ready to image (**Figure 2i**).

6. Sample positioning

[Place **Figure 3** here]

[Place **Figure 4** here]

6.1. At the microscope, mount the FEP tube in the sample holder (**Figure 3a**) and fill the imaging chamber with embedding media (**Figure 3b,c**). Next, place the sample holder on the stage with the sample dipping into the chamber (**Figure 3d**).

6.2. Check the sample's health. Visually assess heart rate to evaluate overall fish wellness, as specific heart rate is stage and temperature dependent, compared to non-mounted control fish. If the heartbeat is too slow, discard the fish.

NOTE: Ensure gentle handling of embryos, careful transfer to embedding media, imaging immediately after embedding, avoiding rapid temperature changes, avoiding tricaine, and lowering the exposure time to tricaine.

6.3. For reproducible imaging, always use the same sample position. Aligning the eyes and imaging at an angle is recommended.

6.4. Rotate the fish so that both eyes (**Figure 4a,c**) are in the focal plane (**Figure 4a',c'**)

6.5. From that position, further rotate the fish approximately 50 °–100 ° clockwise for 24 hpf imaging (**Figure 4b, b'**), and approximately 20°-30° counterclockwise for 48 hpf imaging (**Figure 4d**).

NOTE: The early heart, before 30 hpf, can be difficult to image due to its hidden position (**Figure 4b**).

7. Image acquisition

7.1. Choose the illumination side that gives the best image quality and adapt the laser power to every fish.

NOTE: Record the laser power used for subsequent image analysis.

7.2. At each z-plane, record 4–5 heartbeats at 300 frames per second (fps) or more.

NOTE: The field of view can be cropped to increase acquisition speed. For example, at 48 hpf the zebrafish heart beats two to three times per second, therefore, at 300 fps, between 300 and 600 frames are required to acquire four to six heartbeats.

7.3. To record the beating heart, move the sample stepwise through the light sheet. Use a z-spacing of 1–2 µm, covering the entire depth of the heart.

8. Image processing

8.1. Synchronize recorded movie to reconstruct a 4D (x,*y*,*z*, time) heart using a Fiji (Image J2[29,30]) plugin as previously described[6].

8.2. To explore data and generate movies of the rendered zebrafish heart, load the 4D file (*x*,*y*,*z*, time) into a 3D rendering software.

REPRESENTATIVE RESULTS:

[Place **Figure 5** here]

We have recorded the 48 hpf beating heart of Tg(kdrl:Hsa.HRAS-mCherry; myl7:lck-EGFP) zebrafish according to the protocol detailed above (**Figure 5**). A 488 nm and a 561 nm laser light sheet illuminated the sample simultaneously. The emitted fluorescence was detected perpendicularly using a 16x/0.8 W objective lens and a scientific metal oxide semiconductor (sCMOS) camera.

At 48 hpf, the heart has just undergone looping and has two chambers, the ventricle and the atrium but has yet to develop valves. In our movies, the different heart structures such as inflow tract, ventricle, atrioventricular canal (AVC), atrium, and outflow track are easily distinguishable (**Figure 5a,b**). These data show the precise beating and reveal complex interactions between the heart's two cell layers: the myocardium, a single-cell muscle layer contracting and generating

force (**Figure 5c**, red), and the endocardium, a single cell layer that connects the heart to the vasculature (**Figure 5c**, cyan).

The heartbeat reconstruction in x,y,z (3D) + time (4D) + color (5D) was performed according to Mickoleit et al.[6]. The reconstruction is based on two hypotheses: the motion of the heart is repetitive, and data should be acquired with a small z-step. The output is a reconstructed single heartbeat in 5D, measuring 30 GB to 80 GB per heartbeat. To render the data, we used the free, open-source tool FluoRender for in depth rendering[31] as it was designed to handle multidimensional datasets and easily renders 5D movies of both cell layers and individual layers (**Figure 5b**).

FIGURE AND TABLE LEGENDS:

Figure 1: FEP tube cleaning and straightening. (**a**) FEP tubes on a cable drum. (**b**) FEP tubes before straightening. (**c**) FEP tubes in glass and steel autoclave-safe tubing. (**d**) Flushing of the FEP tubes after straightening and cool down. (**e**) FEP tube cut to the size of a centrifuge tube for sonication. (**f**) Flushing of FEP tubes after sonication. (**g**) Storage of the cleaned and straightened FEP tubes in a centrifuge tube. (**h**) Cutting the FEP tube prior to imaging.

Figure 2: Embryo mounting in FEP tube. (**a**) Anesthetized pigment-free fish in mounting media. (**b**) A syringe with blunt end needle and FEP tube attached. (**c**) Once media and fish are taken up in the FEP tube, cut the tube at the edge of the needle. (**d**) Dipping the cut tube into a dish coated with 2% agarose to plug its end. (**e**) A zebrafish in a plugged FEP tube. (**f**) Gently cut the FEP tube at 30° to create gas-exchange holes. (**g**) FEP tube with four holes above an embedded zebrafish. (**h**) Scheme of a zebrafish embedded in an FEP tube. Holes and agar plug are indicated. (**i**) Multiple embedded zebrafish ready for imaging.

Figure 3: Sample chamber. (**a**) FEP tube mounted on a sample holder. (**b**) The sample chamber with stages and objectives. (**c**) Top view of the media-filled sample chamber, with illumination and detection objective in a T-SPIM configuration. (**d**) Sample holder mounted on the microscope, with the sample in the chamber.

Figure 4: Embryo positioning for heart imaging. (**a**) 24 hpf Tg(kdrl:Hsa.HRAS-mCherry) zebrafish with eyes misaligned. (**a'**) Same fish, with eyes aligned. (**b**) Same fish rotated -100 ° and (**b'**) -50 ° for optimal heart imaging. (**c**) 48 hpf zebrafish with eyes misaligned. (**c'**) Same fish, with eyes aligned. (**d**) Same fish rotated by 30° for optimal heart imaging. Black arrows point to heart. Scale bar 100 µm.

Figure 5: The 48 hpf zebrafish heart. (**a**) Still of one *z*-frame, anterior-ventral view of 48 hpf Tg(kdrl:Hsa.HRAS-mCherry; myl7:lck-EGFP) zebrafish, imaged with LSFM, (**b**) 3D reconstruction of movie stacks, cut view through the atrium. (**c**) Montage of four frames over a full heartbeat at one z-plane. Pie charts indicate the time during heartbeat. Scale bar 50 µm.

Figure 6: Comparison of cytoplasmic- and membrane-marker zebrafish transgenic lines. Anterior-ventral view of 48 hpf zebrafish hearts imaged with LSFM. White arrows indicate structures visible only with a membrane-marker transgenic line. (**a**) Tg(kdrl:EGFP)[32] signal in cyan in the heart and (**a'**) in the ventricle. (**b**) Tg(kdrl:Hsa.HRAS-mCherry; myl7:dsRed)[33] signal in red in the heart and (**b'**) in the ventricle.(**c,c'**) merge of both Tg(kdrl:Hsa.HRAS-mCherry; myl7:dsRed) and Tg(kdrl:EGFP) signal. Scale bar 50 µm.

DISCUSSION:

Transgenic lines to image the heart

[Place **Figure 6** here]

Imaging the zebrafish heart requires precise heart-cell labeling. While the myocardial thickness is relatively constant throughout the cells, endocardial cells are thick around the nucleus but have thin membrane protrusions, in some regions thinner than 2 µm. Cytoplasmic transgenic lines such as Tg(kdrl:EGFP)[32] effectively label the regions around endocardial nuclei, but further away, the thin cytoplasm might not emit enough photons to be detected with such short exposure times, leading to artificial holes in the data (**Figure 6a**). In contrast, membrane marker transgenic lines such as Tg(kdrl:Hsa.HRAS-mCherry)[33] can effectively label the endocardium and reveal more details (**Figure 6b,c**). For each experiment, carefully choose the most appropriate transgenic line.

Zebrafish immobilization

The choice of immobilization technique depends on the length of the experiment and the age of the fish to image. Tricaine has commonly been used for zebrafish immobilization, mostly due to its ease of use. Indeed, simply adding 130 mg/L tricaine to the fish media results in their anesthetization in 10 min. As it can lead to developmental defects and affect heart physiology[20,22], we recommend using tricaine only for short experiments (less than 30 min). For longer imaging, α-bungarotoxin mRNA injections at the one- or two-cell stage paralyzes fish up to 3 days post fertilization (dpf) without affecting cardiovascular development or physiology[22].

Choosing the right FEP tubes

FEP tubes are available in various diameters and thicknesses. To image 0–5 dpf fish, 0.8 mm is a good inner diameter; choose either thick wall 0.8 x 1.6 mm tubes or thin wall 0.8 x 1.2 mm tubes. We recommend thin-walled tubes; however, thicker walls offer increased stability and rigidity, which can be important if the sample chamber has flowing media that could disrupt and move a thin tube. For larger samples, 1.6 x 2.4 mm and 2 x 3 mm can be used.

Temperature and gas exchanges

An essential aspect of the zebrafish embryo's well-being is temperature. Ideally, keep the fish at 28.5 °C while imaging, as the environment's temperature affects development and heart rate[34]. In our experience, oxygen exchange through the 2% agarose plug only maintains a stable heart rate until 3-4 dpf. Therefore, cutting holes in the tube ensures oxygen diffusion. It can also be necessary for drug delivery to the sample if desired.

Suspension of heartbeat.

The fast acquisition speeds of appropriately equipped light sheet microscopes allow recording of the beating heart *in vivo*. However, to acquire an undisturbed z-stack, one can slow down or stop the heart. However, stopping the heart leads to heart muscle relaxation and might result in the collapse of the heart[6]. Heartbeat suspension can be done by using morpholinos, low temperatures, an inhibitor of muscle contraction or optogenetics. These methods each have their drawbacks and must be carefully evaluated for every experiment.

The injection of 4 ng of *silent heart (sih)* morpholino at the one cell stage can stop the heartbeat by targeting the gene *tnnt2a* crucial for sarcomere formation[35]. *sih* zebrafish do not have a heartbeat and only survive until 7 dpf, when the embryos start to rely on circulating blood for oxygenation. As heart morphogenesis is driven by both genetic and biomechanical forces[36], these fish present heart malformations around 3 dpf.

As the flow of Ca^{2+} is temperature sensitive, temperature influences heart rate in embryonic zebrafish[21]. Consequently, lowering the temperature in the imaging chamber slows down the heartbeat. Stopping the heartbeat requires temperatures below 15 °C. As zebrafish are usually kept at 28.5 °C, such low temperatures can only be maintained for brief periods (less than 10 min).

Drugs such as chemical inhibitors of muscle contractions, 2,3-Bu-tanedione 2-monoxime (BDM), can be added to the zebrafish media (50 nM[37,38]) to suspend the heartbeat temporarily. BDM is convenient to use as it stops heart contraction in under 15 minutes and can be washed away to restore cardiac function. However, as BDM alters the cardiac action potential, it must be used with a caution[37].

Finally, the heart of transgenic zebrafish expressing light-gated ion channels or pumps such as channelrhodopsin or halorhodopsin in their myocardium can be manipulated and stopped by illuminating the pacemaker at the inflow tract with light[39,7,40,41,9].

Outlook

The presented optimized tools and solutions to study the zebrafish heart *in vivo* allow long term, gentle imaging of ultrafast cardiac dynamics. The sample embedding can be adapted to suit different imaging modalities, such as confocal microscopy, two-photon microscopy, or optical projection tomography (OPT). Light sheet microscopy, however, is likely the preferred technique that offers optical sectioning at a speed sufficient to capture the dynamics of the heart. While this protocol focuses on zebrafish embryonic heart imaging, we believe that it could also be applied to various other samples and experiments. It will be interesting to see in the future if similar embedding and imaging techniques can also be used at later stages during development when the heart is more hidden and the larva less translucent.

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DISCLOSURES:

The authors have nothing to disclose.

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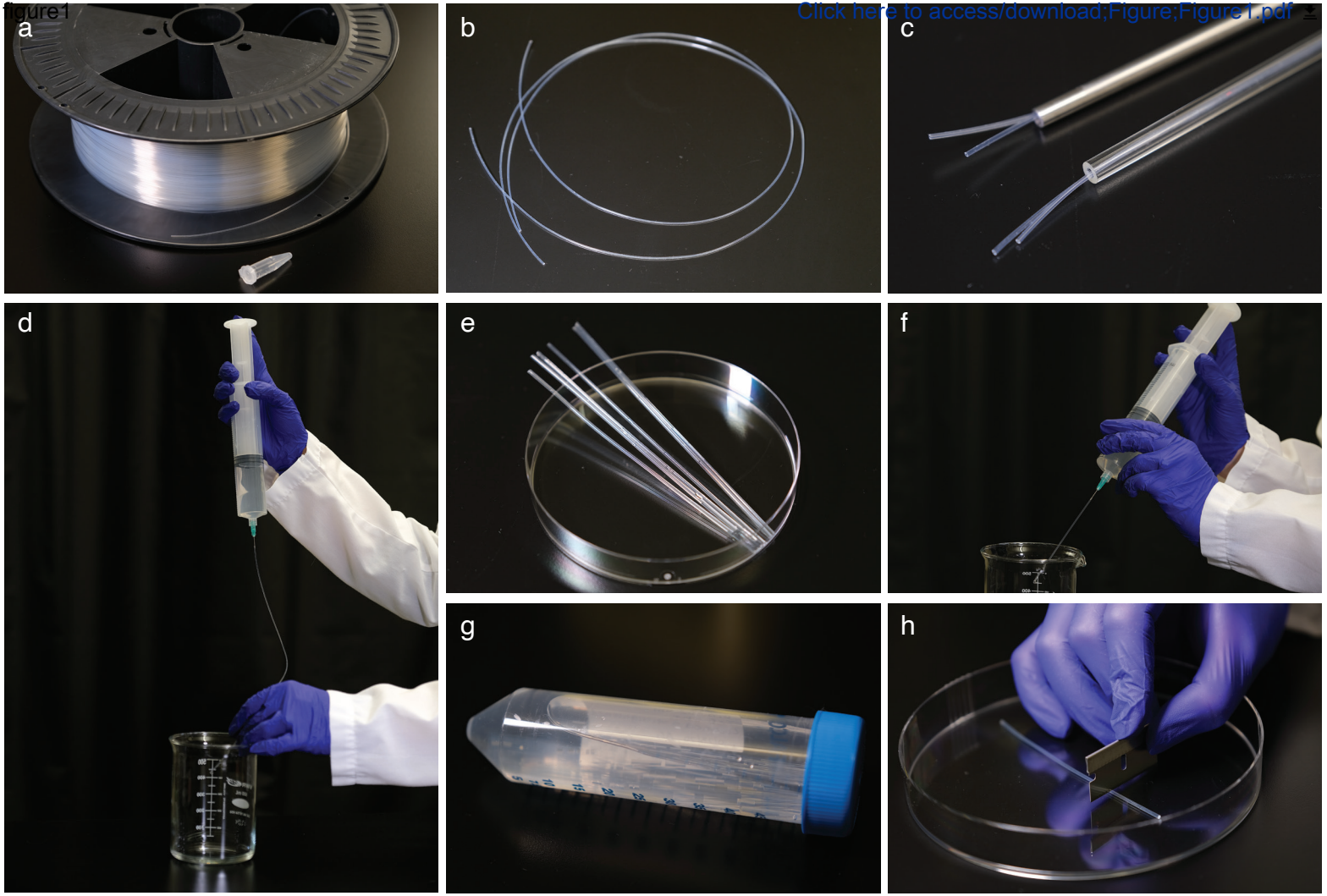


figure2

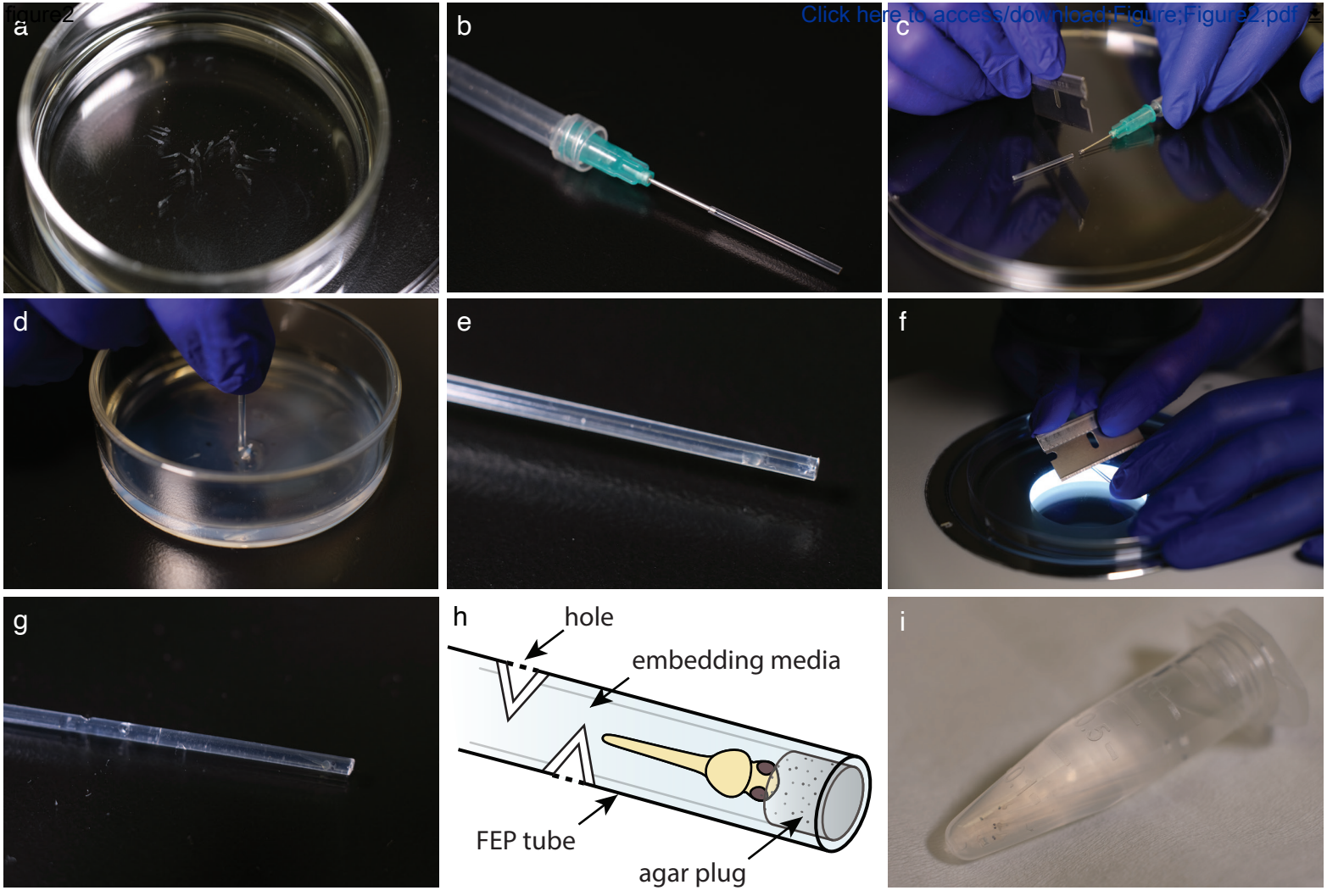
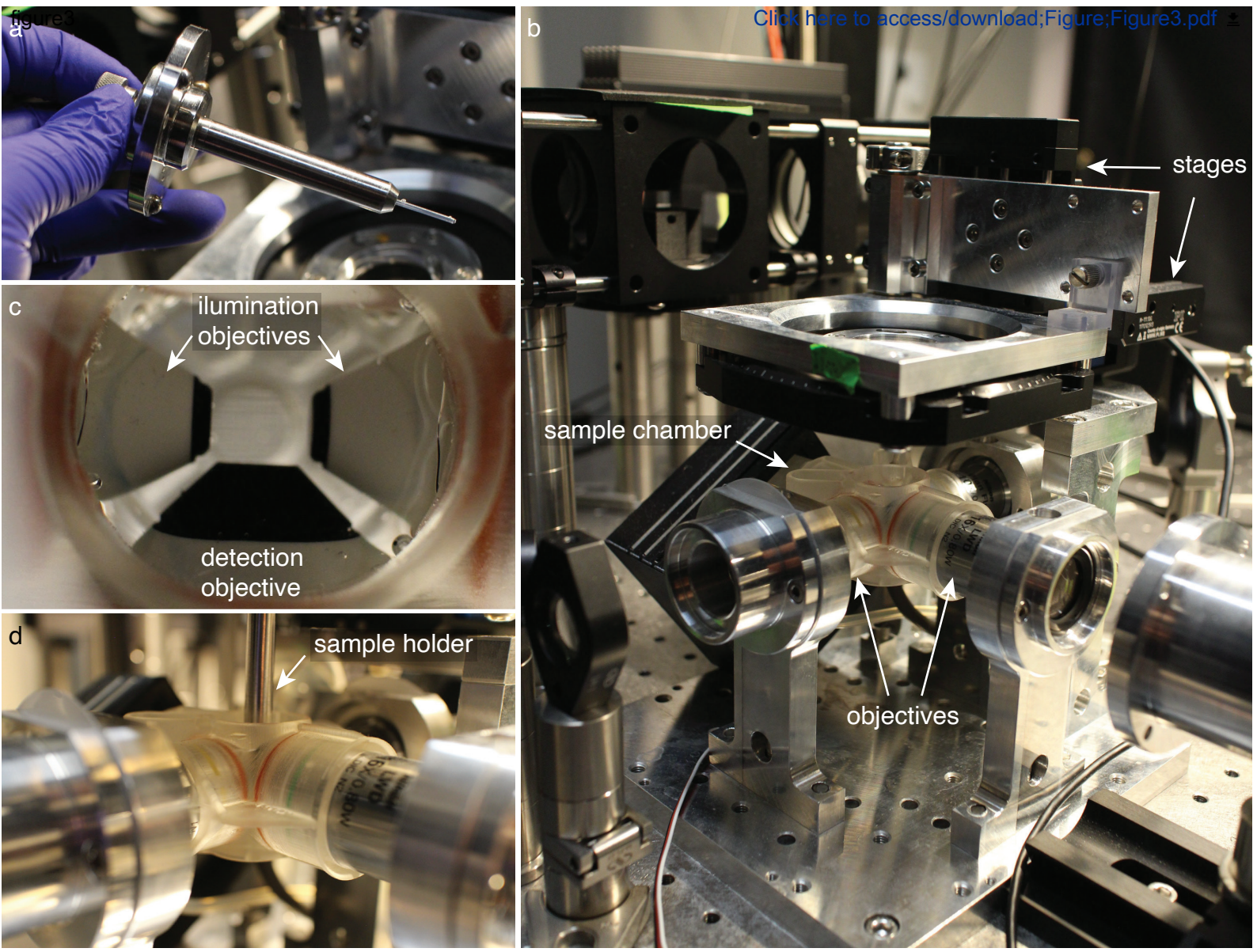
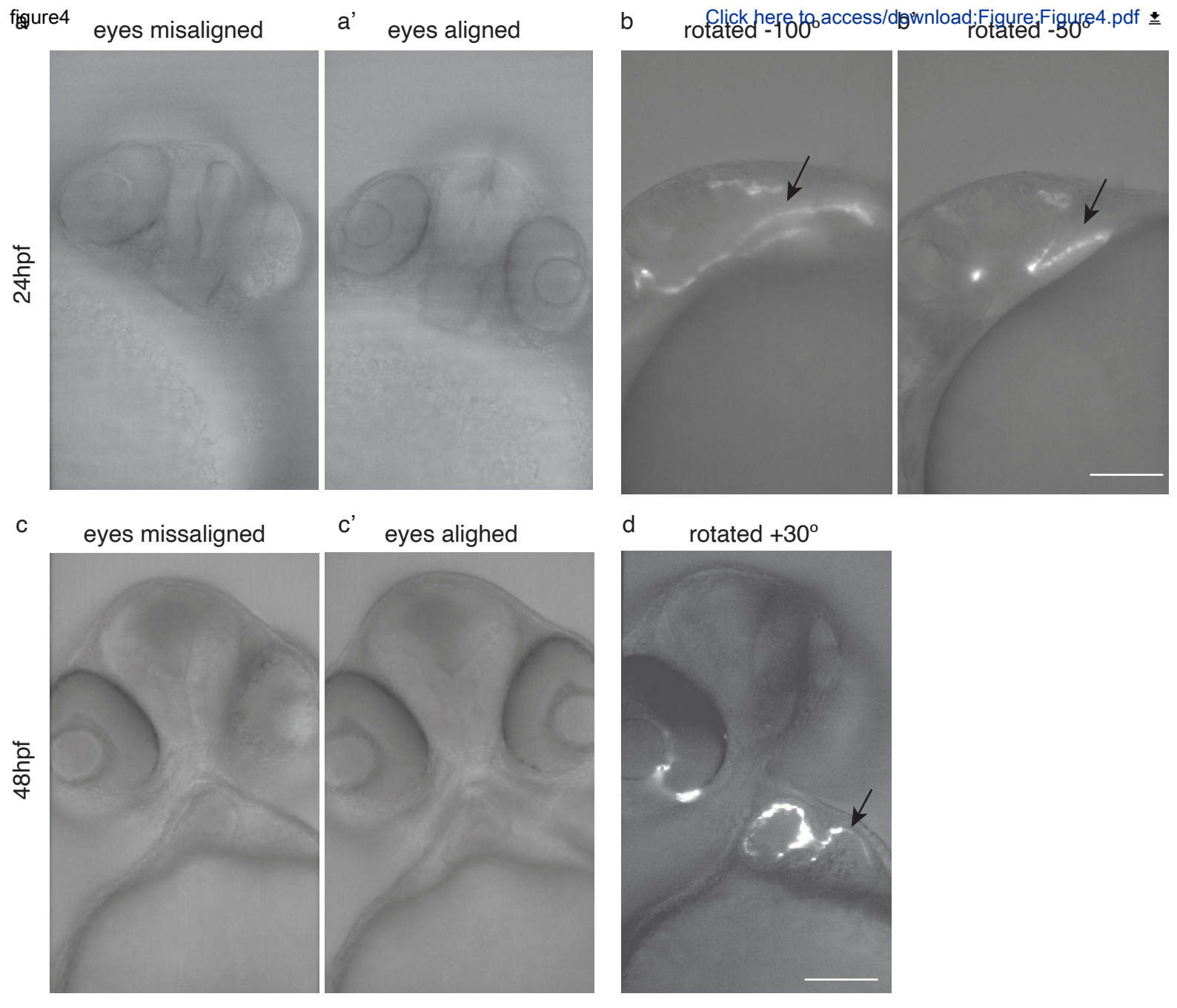
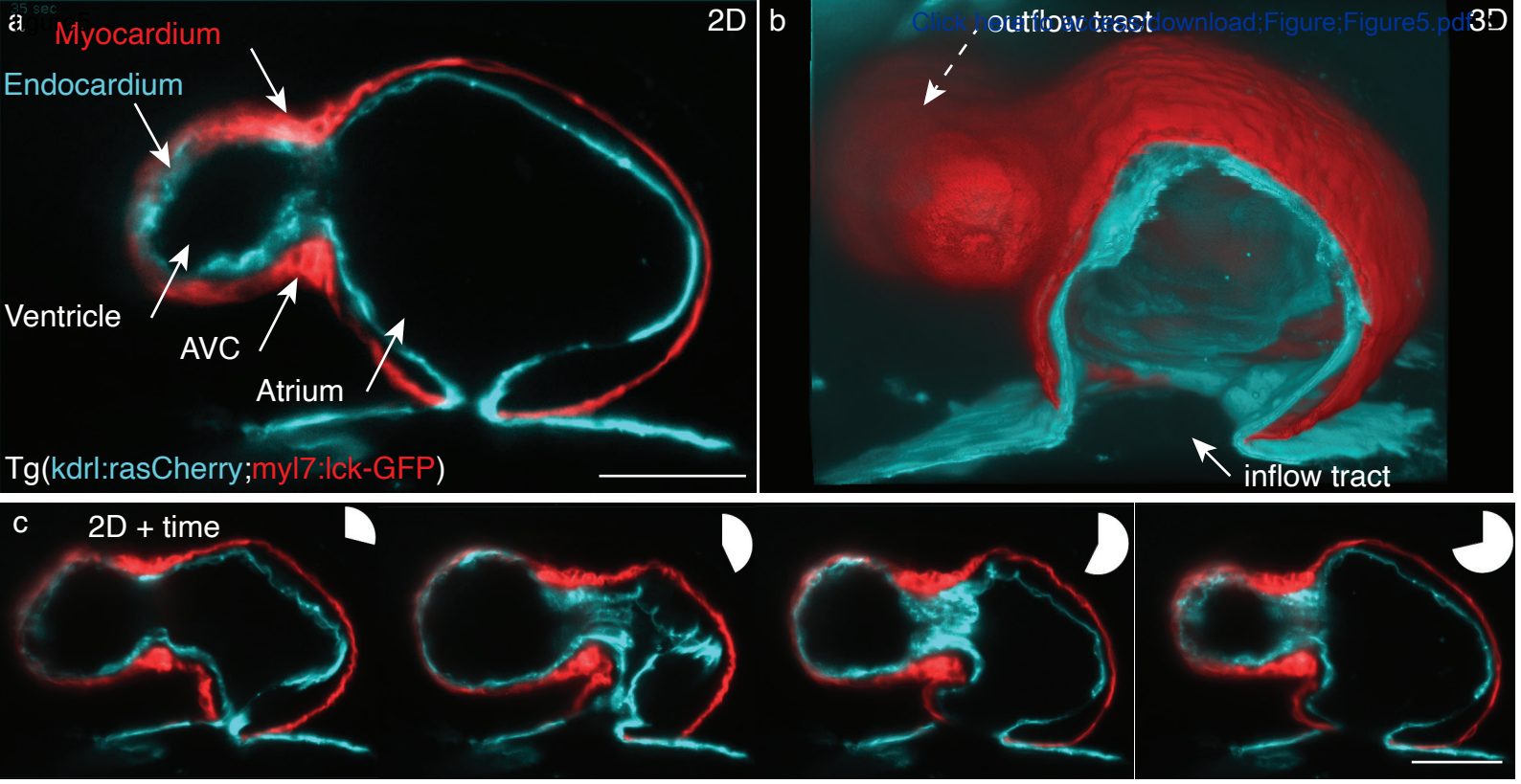
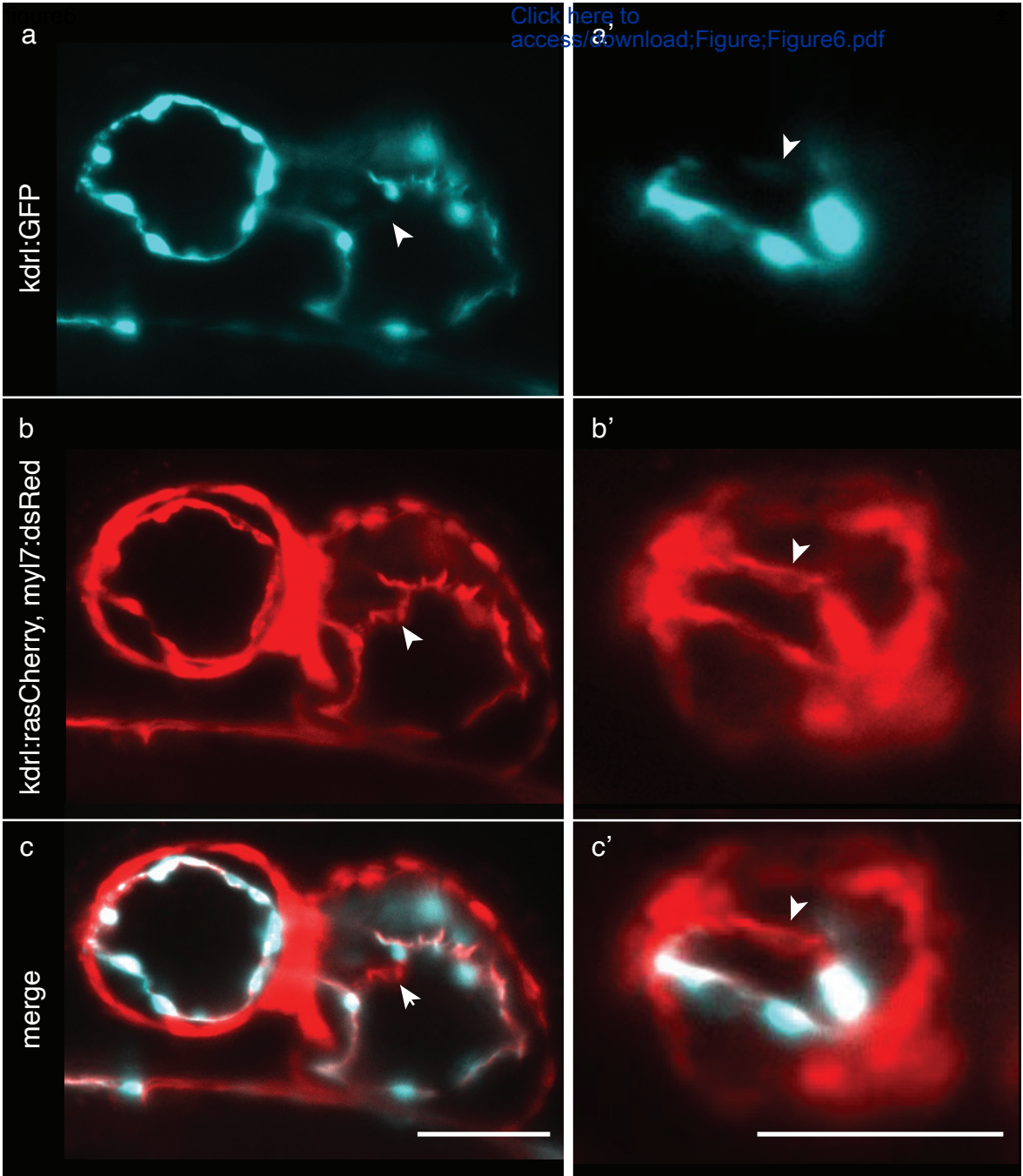


figure3






a
2D
Myocardium
Endocardium
Ventricle
AVC
Atrium
Tg(kdrl:rasCherry;myl7:lck-GFP)
b
3D
outflow tract
inflow tract
c
2D + time



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5mL Eppendorf	Eppendorf	22364111	To carry embedded samples
15mL Falcon tubes	Falcon	352095	To carry embedded samples
50mL centrifuge tubes	Falcon	352070	50ml tubes for sonication step, and storing cleaned, straightened FEP tubes
50mL syringe	BD	309654	50ml syringe for FEP cleaning
Agarose, low gelling temperatu	Sigma Aldrich	39346-81-1	To make plug
Blunt Tip Needles, 21 gauge	VWR	89500-304	Blunt end needle for 0.8 inner diameter FEP tube
Borosilicate glass tube	McMaster-Carr	8729K33	Tubing for FEP tube straightening 9.5mm outer diameter, 5.6 inner diameter, 3
Borosilicate glass tube	McMaster-Carr	8729K31	Tubing for FEP tube straightening 6.35mm outer diameter, 4mm inner diamete
Conventional needles, 21 Gauge	BD	305165	Conventional needle for 0.8 inner diameter FEP tube
Disposable glass pipette	Grainger	52NK56	To transfer fish, use with pipette pump
E3 medium for zebrafish embryos	Sigma Aldrich	64-17-5	Ethanol for FEP cleaning
Ethanol	ProLiquid	2001048	FEP tube with thick wall, other sizes available
FEP tube, 0.8 / 1.2 mm	Bola	S1815-04	FEP tube with thin wall, other sizes available
FEP tube, 0.8 / 1.6 mm	BGB	211581	Large FEP tube with thick wall, other sizes available
FEP tube, 2/3mm	Cole-Parmer	691000	To carry hot equipment after autoclaving
Hot Hand Rubber Mitt	B Braun	9161406V	1ml syringe for embedding
Omnifix 1mL Syringes	Dot Scientific	PD-94050	To make agarose plug
Petri dish, small	Argos Technologies	04395-05	To transfer fish, use with disposable glass pipette
Pipette pumps	Sigma Aldrich	103-85-5	Also known as: N-Phenylthiourea, 1-Phenyl-2-thiourea, Phenylthiocarbamide
PTU			

Razor blades	Azpack Dot	11904325	To cut FEP tubes
Sodium Hydroxide	Scientific	DSS24000	NaOH for FEP cleaning
Tricaine	Sigma Aldrich	E10521	Also known as: MS-222, Ethyl 3-aminobenzoate methanesulfonate, Tricaine

30cm long, other sizes available

er, 30cm long, other sizes available

Editorial comments

Thank you very much for these helpful comments and thorough review. We have addressed all the comments and made the necessary adjustments to the manuscript. Below is a detailed description of the changes we have made.

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

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. Done

3. Please define all abbreviations during the first -time use. Done

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Done

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Sigma Aldrich, falcon, fluorender, etc. Done

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. Done

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes. Done

8. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). Done

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Done

10. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Thank you, we have added additional text for clarity.

11. 1.2: What kind of RNA is injected and how? This step now reads:
"If using *α*-bungarotoxin mRNA to immobilize the fish, inject 30 pg mRNA[22] into the yolk of one or two cell stage embryo using a bore glass needle mounted onto a micromanipulator and connected to a picoinjector[28]."

12. Please include a single line space between each step of the protocol. Done

13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Done

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
All the figures are original materials.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol, b) Any modifications and troubleshooting of the technique, c) Any limitations of the technique, d) The significance with respect to existing methods, e) Any future applications of the technique. We have added a paragraph in the discussion, to add information on how to choose the best immobilization method. It reads:
"Zebrafish immobilization
The choice of immobilization technique depends on the length of the experiment and the age of the fish to image. Tricaine has commonly been used for zebrafish immobilization, mostly due to its ease of use. Indeed, simply adding 130mg/L tricaine to the fish media results in their anesthetization in 10 minutes. As it can lead to developmental defects and affect heart physiology[20,22], we recommend using tricaine only for short experiments (less than 30 minute). For longer imaging, α-bungarotoxin mRNA injections at the one- or two-cell stage paralyses fish up to 3 days post fertilization (dpf) without affecting cardiovascular development or physiology[22]. "

16. Please sort the materials table in alphabetical order. Done

Reviewer #1:

Manuscript Summary:
Schlaeppi and colleagues present a detailed protocol for imaging the larval zebrafish heart and its movements using light-sheet microscopy. The important considerations that readers would have to make before attempting these experiments (mounting, immobilization, transgenic lines, microscope configuration, pausing heartbeats) are covered in a comprehensive and circumspect manner. The quality of the writing and figures is high. I believe that this will be a valuable resource for readers interested in this method, and feel that JoVE is a highly suitable venue for this work.
Ethan Scott, The University of Queensland

Major Concerns:
No Major Concerns.

Minor Concerns:
- Line 303: "Channelrhodopsin" should be one word, and this sentence should be reworded to be clearer.

- I do not like the practice of using "ca." in place of "approximately" or some other qualifier. I would reword sentences using "ca." to so that the sentences flow better.

We would like to thank the reviewer for their thoughtful review and suggestions, we have implemented all the changes suggested.

Reviewer #2:

Manuscript Summary:

The article "Light sheet microscopy of fast cardiac dynamics in zebrafish embryo", by Schlaeppi and colleagues, is an accurate protocol describing how to optimize the in vivo documentation of the zebrafish heart using light sheet fluorescent microscopy.

We would like to thank the reviewer for their thorough review and comments, which have certainly helped make this a stronger manuscript.

Major Concerns:

I have no major concerns toward this work.

Minor Concerns:

- line 87: as a matter of taste, and to ensure a more generalized use of the protocol, I would suggest using the "albino" term, instead of focusing only on the "casper" strain, as several albino lines are available for Danio rerio, with sufficient optical clarity to perform cardiac imaging in vivo. This has been changed to the suggested nomenclature throughout the manuscript.

- line 163: the paragraph is not mentioning in the text the a' and c' panels of Figure 4. Thank you for spotting this oversight.

- lines 208-209: for some reasons, the 5d and 5e panels of Figure 5 (mentioned in the lines 208-209) are not present in the set of figures received by this reviewer. Thank you, we updated the text.

Also, it seems that "Figure 6" is not mentioned in the section "Representative Results". Figure 6 is mentioned in the discussion.

- lines 236-239 on Figure 4: in the version received by this reviewer, a strange, lighter rectangular area is present inside the 4a' panel of Figure 4. We are not sure what the reviewer is referring to. In our version we cannot see a lighter rectangular region. If they are referring to the subtle horizontal stripes in the middle of panel 4a', they are due to our use of a pulse-width modulated LED for positioning of the sample. The pulses are not synchronized to our ultra-fast camera's rolling shutter and appear as intensity fluctuating stripes. These images are for illustration purposes only.

Also, the black arrows in the panels are not described. Thank you for spotting this oversight, we added a sentence to the description.

- lines 247-251: in the legend of Figure 6, "c)" should be in lowercase. The a', b', c' panels are not described (e.g.: they are enlargements of … region… ?) Thank you for spotting this oversight, we modified the description accordingly.

- line 216: what does "6Sa" mean? Was Figure 6 a supplementary figure in previous versions of the manuscript? Thank you for spotting this typo, it was meant to be figure 6a.

- Table of material: 15ml falcon tubes: "Falcon" should have the "F" in uppercase. We agree. This has been changed throughout the manuscript.

Also, throughout the manuscript, I would suggest using "L" in uppercase for Liter (Litre). We agree and have updated the manuscript accordingly.