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Following endocardial tissue movements via cell photoconversion in the zebrafish embryo

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Dear Dr Myers,

Thanks again for your invitation to revise our protocol on new techniques for studying embryogenesis. We have now completed our revisions and are happy to send it to you. As you will see, we have now addressed your comments and the reviewers' ones. Given the increasing appreciation of the impact of live imaging in understanding cell behavior and the mechanisms of morphogenesis, we expect our results to find his readers in the field of development and cell biology. We therefore hope that you will consider our protocol for publication in Jove.

Thank you for your consideration and we look forward to hearing from you.

Yours Sincerely,

Julien Vermot, PhD

TITLE:

Following Endocardial Tissue Movements via Cell Photoconversion in the Zebrafish Embryo

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KEYWORDS:

Developmental Biology, Heart development, Cell tracking, in vivo imaging, photoconvertible fluorescent proteins, *Danio rerio*, zebrafish, embryo

SHORT ABSTRACT:

This protocol describes a method for the photoconversion of Kaede fluorescent protein in endocardial cells of the living zebrafish embryo that enables the tracking of endocardial cells during atrioventricular canal and atrioventricular heart valve development.

LONG ABSTRACT:

During embryogenesis, cells undergo dynamic changes in cell behavior, and deciphering the cellular logic behind these changes is a fundamental goal in the field of developmental biology. The discovery and development of photoconvertible proteins have greatly aided our understanding of these dynamic changes by providing a method to optically highlight cells and tissues. However, while photoconversion, time-lapse microscopy, and subsequent image analysis have proven to be very successful in uncovering cellular dynamics in organs such as the brain or the eye, this approach is generally not used in the developing heart due to challenges posed by the rapid movement of the heart during the cardiac cycle. This protocol consists of two parts. The first part describes a method for photoconverting and subsequently tracking endocardial cells (EdCs) during zebrafish atrioventricular canal (AVC) and atrioventricular heart valve development. The method involves temporally stopping the heart with a drug in order for accurate photoconversion to take place. Hearts are allowed to resume

beating upon removal of the drug and embryonic development continues normally until the heart is stopped again for high-resolution imaging of photoconverted EdCs at a later developmental time point. The second part of the protocol describes an image analysis method to quantify the length of a photoconverted or non-photoconverted region in the AVC in young embryos by mapping the fluorescent signal from the three-dimensional structure onto a two-dimensional map. Together, the two parts of the protocol allows one to examine the origin and behavior of cells that make up the zebrafish AVC and atrioventricular heart valve, and can potentially be applied for studying mutants, morphants, or embryos that have been treated with reagents that disrupt AVC and/or valve development.

INTRODUCTION:

The zebrafish is currently one of the most important vertebrate models to study cellular and developmental processes *in vivo*. This is largely due to the zebrafish's optical transparency and amenability to genetics, which makes it a powerful model for applying optical techniques involving genetically encoded photoresponsive protein technologies[1]. Specific to the study of heart development, zebrafish receive sufficient oxygen via diffusion such that even mutants without heartbeat can survive through the first week of development, permitting analyses on the effects of developmental genes and perturbed blood flow on heart morphogenesis not possible in most vertebrates[2].

The zebrafish heart tube is formed by 24 hours post fertilization (hpf). Shortly after its formation, the heart tube starts actively beating. By 36 hpf, a clear constriction separates the atrium from the ventricle. This region of constriction is called the atrioventricular canal (AVC), and cells in this region change from a squamous morphology to a cuboidal morphology[3]. Zebrafish atrioventricular valve morphogenesis starts around 48 h post fertilization. By 5 days post fertilization, two valve leaflets extend into the AVC and prevent the back flow of blood from the ventricle to the atrium during the cardiac cycle[4]. Tracking cells during AVC and valve formation is challenging as the rapid beating of the heart makes it difficult to follow cells via traditional time-lapse microscopy[5,6]. This protocol, adapted from Steed *et al.*, 2016[7], describes a method that uses the *Tg(fli1a:gal4FF^{ubs}, UAS:kaede)*[8] zebrafish transgenic line, in which the photoconvertible protein Kaede is expressed in endothelial cells, including the endocardium. The drug 2,3-butanedione-2-monoxime (BDM) is used to temporarily stop the heart beating, allowing accurate photoconversion of EdCs between 36 and 55 hpf, and high-resolution imaging of photoconverted EdCs at specific developmental time points. It has previously been shown that EdCs photoconverted using this method can remain distinguishable from their unconverted neighbors for five days or more after the time of photoconversion[7]. This protocol also details a method used for image analysis of photoconverted EdCs in embryos younger than 48 hpf, which has successfully been used to follow tissue movements during AVC development (Boselli *et al.*, in press)[9]. We hope that readers would find this protocol useful for studying AVC and valve development in normal embryos, and in mutants, morphants, or drug-treated embryos. For a more general protocol relating to cell tracking using photoconvertible proteins during zebrafish development, please view the article by Lombardo *et al.*, 2012[10].

PROTOCOL:

1. Preparing molds and mounting agarose

1.1. Create a mold with the dimensions shown in **Figure 1** using either a 3D printer or traditional mechanical workshop tools.

1.2. Pipette about 1.5 mL of melted 1 % agarose into a 35-mm plastic mounting dish. Place the plastic mold in the liquid agarose, taking care to avoid trapping air between the mold and the agarose. Place the dish at 4 °C, wait till the agarose hardens (this takes about 5 min), then remove the plastic mold.

1.3. To store the mounting dish for later use, cover the dish with its lid, then wrap both the dish and the lid tightly with parafilm. The mounting dish can then be stored lid side facing down for up to 5 days at 4 °C.

1.4. Prepare in advance 1 mL aliquots of 0.7 % low melting point agarose in 1.5 mL Eppendorf tubes to use on the day of mounting.

Note: The agarose wells of the mounting dish will deform over time as water evaporates if parafilm is not wrapped around the dishes tightly.

2. Obtaining Embryos for the Photoconversion

2.1. Incross the *Tg(fli1a:gal4FF^{ubs}, UAS:kaede)* line and grow approximately 200 embryos in embryo medium in the dark at 28.5 °C. For more details on how to cross zebrafish lines, please refer to the JoVE Science Education Database[11].

2.2. After 5 h but before 24 h, treat embryos with 1-phenyl-2-thiourea (PTU) to prevent pigment formation (0.003 % PTU in embryo medium).

2.3. About 1 h before the start of photoconversion, screen embryos under a fluorescent stereoscope and select 5 healthy embryos that appear to express the brightest green fluorescence. The use of low-intensity light when visualizing embryos is recommended to avoid exposure of the embryos to ambient light in order to avoid photoconverting Kaede in non-specific cells.

3. Embedding

3.1. Prepare 10 mL of mounting media: 0.02 % tricaine and 50 mM BDM in embryo medium.

3.2. Add 2 mL of mounting medium to the mounting dish and allow 15-30 min for the solution to diffuse into the agarose.

3.3. In the meantime, melt a 1 mL aliquot of 0.7 % low melting point (LMP) agarose by placing the tube in a 70 °C heat block for about 5 min.

3.3.1. Wait for the LMP agarose to cool slightly, then add 25 µL of 8 mg/mL tricaine solution and 40 µL of 1M BDM solution to the tube of low melting point agarose. Mix by

pipetting up and down, then transfer the tube to a 38 °C heat block to keep the LMP agarose in its liquid state.

3.4. In a Petri dish with embryo medium, carefully dechorionate the pre-selected embryos under the stereomicroscope using forceps, taking care not to damage the embryos.

3.5. Transfer the embryos to a separate dish containing 2 mL of mounting media.

3.5.1. When the hearts of the embryos are stopped (takes about 5-10 min), transfer the embryos to the mounting dish and arrange embryos in the wells using forceps so that they lie at a 25 degree angle, tails pointing towards the deeper part of the well, yolk facing up.

3.5.2. When embryos are roughly in place, remove the media, embed the embryos in ~200 µL of 0.7 % LMP agarose containing tricaine and BDM, and readjust the embryos' positions, tilting the embryos to the left or right as necessary.

3.6. Wait until the LMP agarose sets (takes about 5 min), then add mounting medium to the dish. The embryos are now ready to be photoconverted.

Note: The tricaine and the BDM are used to anesthetize the embryos and to stop the embryos' heart, respectively. Mounting media can be prepared the day before imaging, but make sure to protect the media from light.

Note: The correct positioning of the embryos is important to allow a clear laser path to the cells one wishes to photoconvert. Position the head of the embryo close to the start of the well so that laser light does not have to travel far to reach the embryo, and so that the embryos can be easily unmounted after photoconversion.

4. Photoconversion

4.1. On an upright confocal microscope (like Leica SP8) equipped with a 405 nm, 488 nm and 561 nm laser source, locate the embryo's heart using transmitted white light (brightfield) and an objective lens (like Leica HCX IRAPO L, 25 ×, N.A. 0.95 objective). Check for red Kaede fluorescence using the 561 nm laser (*i.e.*, DPSS 561 laser, at ~5 % intensity, detector set at 589-728 nm). Then, visualize the endocardium by using the 488 nm laser (*i.e.*, 30 mW multiline Argon laser at ~5 % intensity, detector set at 502-556 nm).

4.2. Enter "FRAP" mode on the microscope acquisition software. Select ~1 % laser power for both 488 nm and 561 nm lasers.

4.3. Focus on the plane of interest, select a region of interest (ROI), then photoconvert cells using the 405 nm diode laser at 25 % laser power, scanning over the ROI three times. Should insufficient Kaede be photoconverted in the ROI, scan the 405 nm laser over the area three times again. Repeat this step for all ROIs.

4.4. Return to the “TCS SP8” mode on the software and acquire a z-stack, using the 561 nm and 488 nm lasers sequentially. Make sure to select the ‘bidirectional’ option to increase imaging speed.

Note: How long it takes to photoconvert and image each embryo varies. Heart beat is known to be important for normal heart valve development, so avoid keeping the heart stopped for more than 30 min, even if it means you do not have time to photoconvert all 5 embryos.

Note: During the photoconversion process, use PMT detectors, while after photoconversion, it is recommended to use hybrid detectors set to ‘counting’ mode. This is because the hybrid detectors are more sensitive and are capable of producing better quality images, but are easily damaged by overexposure.

5. Unmounting photoconverted embryos

5.1. After photoconversion, use a glass pipette to press down slightly just above the head of the embryo to break the LMP agarose, then gently suck up the embryo.

5.1.1. Eject the embryo from the glass pipette into a 35-mm Petri dish containing embryo medium with PTU (to wash away BDM containing medium).

5.1.2. Transfer the embryo to a well in a 6-well plate containing embryo medium with PTU. During this step, make sure to keep note of which embryo goes into which well, as this is essential to correlate the position of photoconverted cells at later developmental stages.

5.2. Now that the embryos are removed from the BDM containing medium, their hearts should start beating again in about 5 min. Return embryos to the dark at 28.5 °C to allow embryos to continue to develop normally.

6. Imaging photoconverted cells at later embryonic stages

6.1. At the desired stage, stop the heart and re-embed the embryos like under Step 3 of this protocol, with the important difference that embryos must be treated with BDM in separate, marked dishes to keep track of embryos.

6.2. For embryos up to 62 hpf, acquire z-stacks of the endocardium using 561 nm and 488 nm for red and green fluorescent signals, respectively. For embryos at stages older than 62 hpf, use a multiphoton laser set to 940 nm to image green Kaede instead of the 488 nm laser.

7. Image analysis for embryos under 48 hpf

Note: Tissue movements of the AVC can be difficult to analyze due to its complex three-dimensional structure. To quantify the length of a photoconverted region in the AVC between two non-photoconverted regions or the length of a non-photoconverted region in the AVC between two photoconverted regions, it can be desirable to map the three-dimensional

structure onto a two-dimensional map. Images of photoconverted embryos obtained prior to 48 hpf can be analyzed using the following method.

7.1. Import the acquired images in Matlab or any similar software.

7.2. Delimitate manually the ROI, *i.e.* the endocardium, and apply a mask to remove the signal outside this region.

7.3. Apply an intensity threshold on the image to identify the points on the endocardium and plot them in three-dimensions. Isolate the points corresponding to the narrowest and most straight part of the AVC, by erasing manually the points of no interest on the atrium and the ventricle using the erase command in the software.

7.4. Use a cylinder to fit the points representing the AVC so obtained.

7.5. Define a reference system to compare several samples. For example, adopt the center of mass of the AVC points as the origin of the reference system and the axis of the fitted cylinder as the z-axis.

7.5.1. Make sure that positive z-positions correspond always to the same side of the heart (ventricular or atrial).

7.5.2. Project the AVC points on the x-y plane and use an ellipse to fit them.

7.5.3. Rotate the endocardial points around the z-axis so that the major axis of the ellipse and the y-axis of the reference system are parallel and positive y-values correspond to the internal side of the AVC with respect to the embryo.

7.6. To segment the AVC, cut the three-dimensional dataset with some planes obtained by rotating the half-plane {y=0, x>0} in the z-direction.

7.6.1. Project the intensity of the neighboring pixels on these planes and define the endothelium in a consistent manner. For example, draw manually a line from the atrial to the ventricular side of the AVC at half thickness of the endothelium.

7.6.2. Interpolate the points of the lines representing the endothelium with a three-dimensional spline using the spline toolbox to represent the AVC with a surface.

Note: The number of the cutting planes determines both the accuracy of the segmentation and the time consumption of this step. Usually, 8 planes are enough to achieve good results.

7.7. Define the azimuthal position of each point on the AVC surface using cylindrical coordinates.

7.7.1. At each azimuthal position define a parametric curve along the surface of the AVC by calculating the arc length on the surface between consecutive points.

7.7.2. For each parametric curve, define the point on the curve closest to the center of the AVC to zero. The points of the AVC are thus fully described by their azimuthal position and their position on the parametric curve.

7.8. Unfold the AVC into a two-dimensional image by using the parametric position of the points.

7.9. Find the edges of the region to quantify as the edges of the binary image thresholding the ratio between the intensities of the photoconverted and the non-photoconverted channels. Manually correct the result if necessary.

7.10. Calculate the arc length between the edges to compute the length of the tissue as a function of the azimuthal position.

REPRESENTATIVE RESULTS:

An example of an embryo photoconverted at 48 hpf and imaged again at 80 hpf is shown in **Figure 2**, **Movies 1** and **2**. Exposing Kaede to 405 nm light irreversibly converts from the protein from its fluorescent green form to fluorescent red form, enabling the behavior of cells labeled with either the green or their red form to be followed with respect to their differentially colored neighbors during valve formation. It can be seen that the few photoconverted cells of the 48 hpf AVC later proliferated and later resided predominantly on one side of the valve leaflet.

An example of the results that can be achieved by applying the image analysis method described on a 36 hpf embryo after photoconverting EdCs in the atrium and in the ventricle is shown in **Figure 3**. The segmentation of the AVC allows the parametrization of the three-dimensional structure that is then projected in two-dimensions for easier visualization and analysis. The method allows one to quantify the distance between the two photoconverted regions. By repeating this process at different developmental times it is possible to study the migration of cells toward the AVC (Boselli *et al.*, in press)[9].

FIGURE AND TABLE LEGENDS:

Figure 1: Mold used to create agarose wells. A) Front view. **B)** Back view. **C-D)** Side views. **E-F)** Oblique views. Dimensions are shown in millimeters. The .STL file for the mold template is provided in the Supplementary Materials.

Figure 2: Representative results of a *Tg(fli1a:gal4FF^{ubs}, UAS:kaede)* photoconverted embryonic heart. A) The embryonic heart just after photoconversion at 48 hpf. Cytoplasmic Kaede within several cells of the superior side of the AVC has been converted from its green form to its red form. **B)** The same embryo at 80 hpf. It can be seen that the previously photoconverted cells have proliferated and have come to reside in the inner side of the superior atrioventricular valve. Scale bars: 20 µm.

Figure 3: Representative results of the image analysis process on a 36 hpf embryo. A) The result of the three-dimensional segmentation of the AVC in a reference system that is common among the embryos; the signal from the photoconverted (magenta) and non-photoconverted (green) Kaede is projected onto its surface and marks the photoconverted and non-

photoconverted regions. **B)** The AVC is unfolded according to the parametric position of its point and the signal is projected in two-dimensions for easier visualization. The edges of the non-photoconverted region are labeled in white. **C)** The length of the non-photoconverted region is plotted as a function of the angular position.

Movie 1: The embryonic heart just after photoconversion at 48 hpf. Related to Figure 2A. The movie goes through the acquired z stack of the 48 hpf embryo shown in Figure 2A z slice by z slice. Only one z plane was chosen during the photoconversion process, but it can be seen here that cells at various depths have been photoconverted. Z slices are spaced 2 μm apart.

Movie 2: The photoconverted embryonic heart at 80 hpf. Related to Figure 2B. The movie goes through the acquired z stack of the 80 hpf embryo shown in Figure 2B z slice by z slice. Z slices are spaced 2 μm apart.

Supplementary file: Related to Figure 1. The .STL file for the mold template.

DISCUSSION:

Timing of photoconversion: Although Kaede remains brightly expressed in EdCs even at 96 hpf, it should be noted that as the embryo grows, laser light diffuses more before it reaches the AVC, making confined photoconversion of Kaede more difficult. At embryonic stages later than 55 hpf, the ballooning of the ventricle and the atrium also means that the violet laser beam used for photoconversion cannot reach AVC cells without first passing through either the atrium or ventricle. This means that beyond 55 hpf, in order to photoconvert EdCs of the AVC, one must also photoconvert additional EdCs in the atrium and ventricle.

BDM and tricaine: Accurate photoconversion of EdCs requires temporarily stopping the heart from beating. BDM (2,3-butanedione monoxime) is an electro-contractile uncoupler that is well known to suppress cardiac muscle contraction[12]. We do not observe differences between the morphology of embryos that have been subject to this protocol, and embryos that have been growing normally and have not had their hearts stopped for imaging. However, the risk remains that the drug treatment is altering normal development[13]. Concentrated tricaine can also be used to temporarily stop the heart[14]. We find that we can effectively stop the heart by placing embryos in embryo medium with 0.32 % tricaine for 1 min, and maintain the heart in its stopped state by transferring the embryos to embryo medium with 0.16 % tricaine. Similar to the BDM treatment, the heart can be stopped this way for 30 min and heart beat can be seen to return within 5 min after returning embryos into normal embryo medium. Higher concentrations of tricaine should be avoided; low concentrations of tricaine for prolonged periods (h/days) has been shown to affect normal embryonic development[15,16]. Tricaine solution used for stopping the heart is best prepared fresh. If frozen aliquots are used, make sure to keep the solution well protected from light and to defrost the tricaine without heating the solution above 30 °C. Finally, blebbistatin has been reported to stop the heart without severe side effects[13]. We have not tested this last method in our lab.

Mounting and unmounting: Correct positioning of the embryos is critical during photoconversion. The mold for making agarose wells facilitates the mounting of embryos such that the anterior-posterior axis of the embryo consistently lies at 25° relative to the surface of the dish. We have found the mold to be applicable for photoconversion at all developmental

stages between 36 and 55 hpf. In mounting, the tilt of the embryo in the left-right direction needs to be adjusted according to the embryo's developmental stage and the area one wishes to photoconvert. For example, to photoconvert the AVC at 36 hpf, the embryo should face roughly 15° to the left, while at 55 hpf, the embryo should face roughly 10° to the left. Under the stereomicroscope, you should be able to see the AVC unobscured by the ventricle or the atrium. If, for example, the atrium lies over the AVC, it would not be possible to photoconvert just the AVC or just the atrium. For imaging purposes, the same mold is used to position embryos between 36 and 96 hpf. The mold could be made using a variety of materials using a 3D printer or using traditional mechanical workshop tools. Stainless steel or chemical and heat resistant plastic are both suitable materials. Should the reader wish to create the mold using a 3D printer, please see supplementary information for the .STL file of the mold template.

Adding sufficient LMP agarose to cover the entire embryo is advised to ensure that the embryos do not become dislodged between mounting and photoconversion/imaging, especially if the microscope is not located directly adjacent to the mounting station. In general, provided that the embryos are properly mounted, LMP agarose does not stick to the embryos after unmounting. However, should some LMP agarose remain stuck on the embryo, the LMP agarose can be removed by gently teasing the embryo from the agarose using forceps.

Embryos, especially embryos less than 48 hpf, can be easily damaged during mounting and unmounting. For embryos less than 48 hpf, we recommend fire polishing the end of the glass pipette to prevent damaging embryos during pipetting.

Microscope: This protocol was performed using a Leica SP8 microscope. However, photoconversion is a well-established process and this protocol should be easily convertible to other upright microscopes equipped with lasers capable of producing 405 nm, 488 nm and 561 nm light. For embryos up to 62 hpf, images were acquired using an argon excitation laser and a DPSS 561 laser for green and red Kaede, respectively. For embryos at stages older than 62 hpf, green Kaede can be excited using a multiphoton laser set to 940 nm, which has a greater penetration depth than 488 nm light. In theory, red Kaede should have a two-photon excitation spectra similar to that of DsRed, and could be imaged at 950 nm[17].

An objective lens with a high numerical aperture is necessary to efficiently photoconvert EdCs and to acquire high-resolution images. Since the embryos are kept in media, it is important to choose a microscope equipped with a water objective lens.

Photoconverting the region of interest: It is advisable to be conservative when selecting the ROI to photoconvert – the 405 nm laser diffuses as it travels through tissue and can photoconvert cells in an area greater than that selected. If, for example, you wish to label a cell red but would like the cell directly adjacent to remain green, it may be prudent to select only the part of the cell lying further away from the adjacent cell as your ROI. Since Kaede is expressed in the cytoplasm of EdCs, photoconverted protein will diffuse to parts of the cell not selected by the ROI.

Photobleaching and phototoxicity: The Kaede protein, both its green and red forms, is extremely bright and photobleaching is generally not a limiting factor in cell tracking experiments. However, laser light can impact embryonic development and should be

minimized, especially during early stages of development. Signs of phototoxicity include developmental delay and, in more serious cases, improper heart looping and malformed valves.

The power of the violet laser (25 %) and the number of iterations the violet laser scans over the ROI (3-6 times) used in this protocol were determined empirically. At these settings, we saw almost complete conversion of Kaede from its green to red form without noticeable developmental delay. The amount of laser power required in other microscopy setups would depend on the laser's intensity. It should be noted that the red form of Kaede can be bleached by intense 405 nm illumination, so longer photoconversion does not necessarily yield more red fluorescence.

Image analysis: The method proposed for the image analysis is a newly developed process to describe the three-dimensional geometry of the AVC and compare it among embryos. It is intended to be applied to monolayer tubular structures, such as the AVC before 48 hpf and potentially the heart tube, the dorsal aorta and others. It is mainly automatic, even though the segmentation on the cutting planes is performed manually by the user and other steps could potentially require manual correction. The manual segmentation represents the most critical part of the process, since knowledge of the structure geometry and training are needed to achieve good results. After practicing, the time-consumption of this step is considerably reduced.

The protocol described here provides an effective way to track endocardial tissue movements during AVC and atrioventricular heart valve development using the *Tg(fli1a:gal4FF^{ubs}, UAS:kaede)* zebrafish transgenic line.

Currently, one of the key limitations of this method is that the violet laser beam is not confined in the axial dimension. This makes targeting of single cells for photoconversion, and hence clonal analyses and single cell tracking, difficult. Two years ago, it was discovered that the photoconvertible protein Dendra2 can be primed converted – the protein can be converted from its green form to its red form by first irradiating the protein with blue light, and then with near-infrared light[18]. Microscope setups that exploit this property can photoconvert Dendra2 expressing cells in a spatially confined manner in complex 3D tissue structures while avoiding many of the phototoxicity problems associated with intense near-UV irradiation[18-20]. With the recent discovery of the molecular mechanism behind this phenomenon, variants of many green-to-red photoconvertible fluorescent proteins, including Kaede, have been engineered so that are also capable of entering a primed intermediate state[21]. To our knowledge, zebrafish transgenic lines where a promoter such as *fli1a* or *kdrl* drives the expression of protein Dendra2 or other primed convertible fluorescent proteins in endothelial cells are not yet available. However, lines that express Dendra2 ubiquitously are available and may prove useful in certain applications [22,23].

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DISCLOSURES:

No conflict of interest declared.

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Figure1

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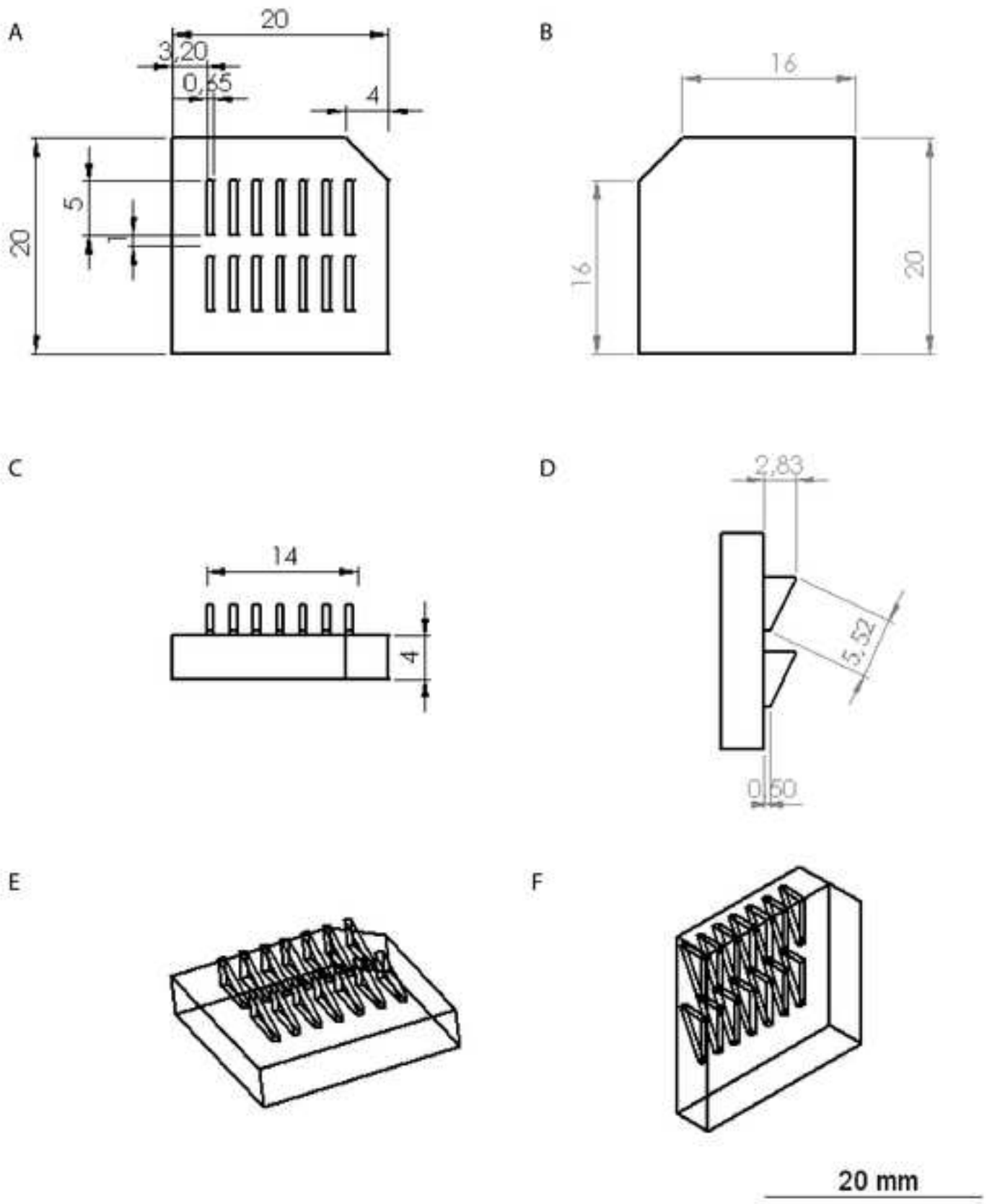

Figure2

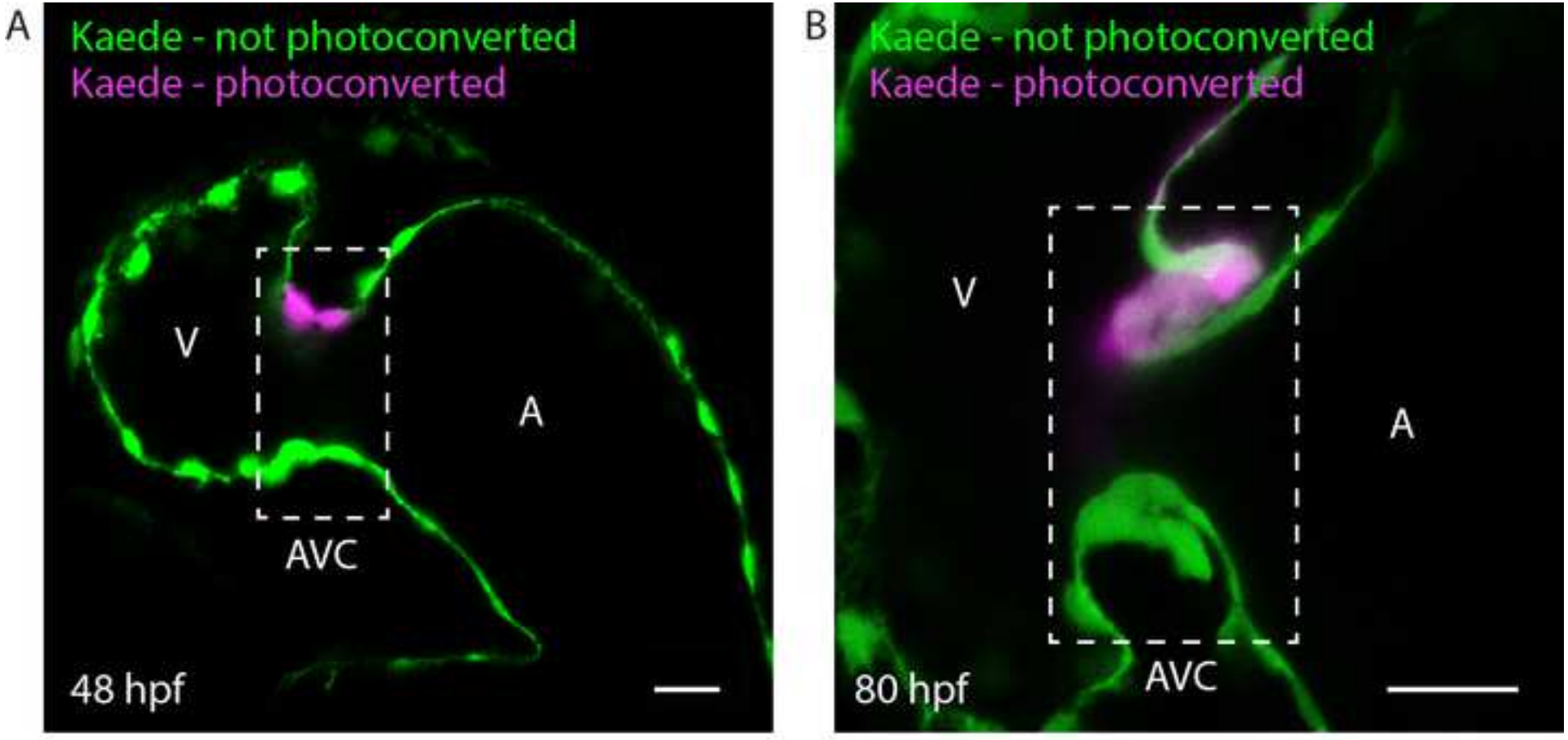

Figure3

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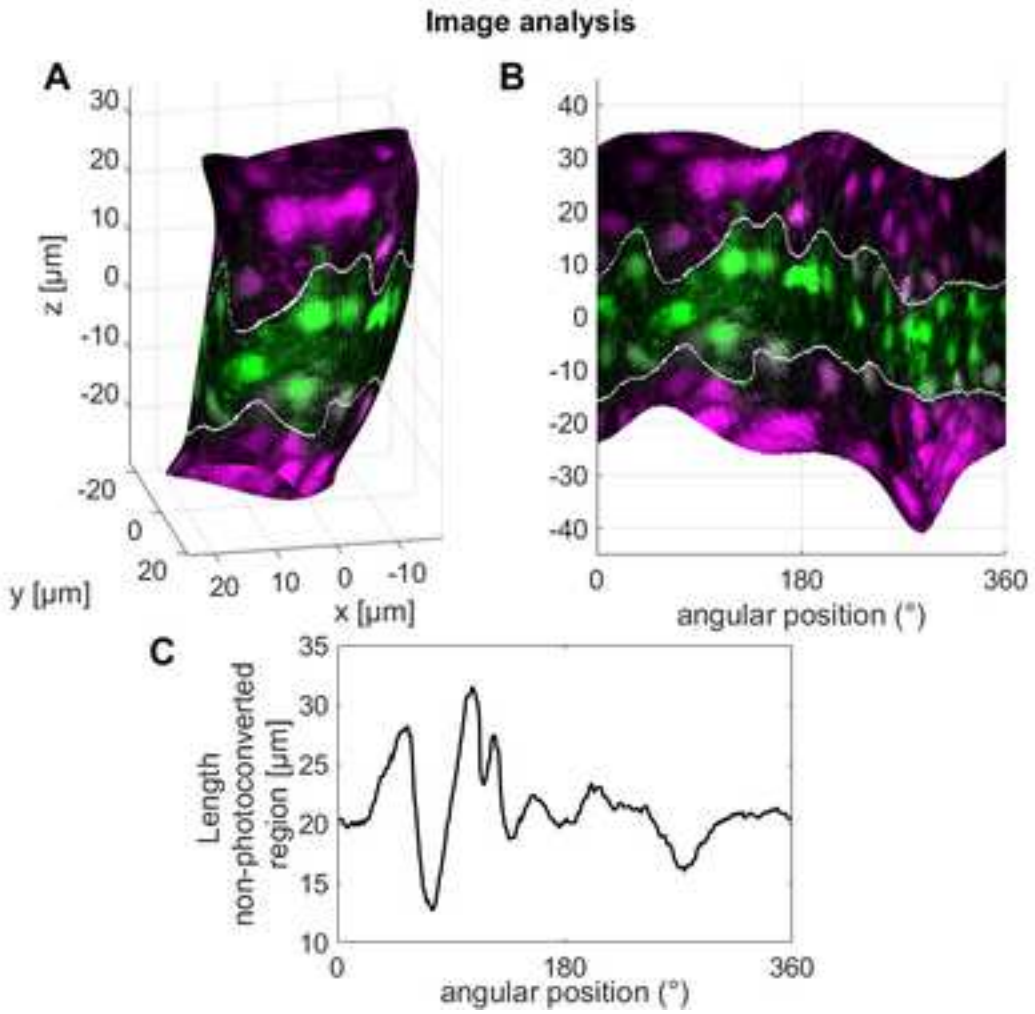


Materials Table

Click here to download Materials Table Materials and Reagents.xlsx

Name	Company
Materials	
Necessary equipment for raising fish and collecting eggs (see the Zebrafish Book22 for details).	
Stereomicroscope	
Upright confocal microscope (Equipped with lasers operating at 488 nm, 561 nm, and 405 nm, a tunable multiphoton laser, and a Leica HCX IRAPO L, 25 ×, N.A. 0.95 objective)	Leica
Heat block	ThermoScientific
35 mm x 10 mm tissue culture dish	Falcon
6 well plate	Falcon
Petri dish	
Forceps	
Glass pipette	
Mold	
Reagents	
8 mg/mL Tricaine stock solution	
1 M BDM stock solution	
UltraPure low melting point agarose	Invitrogen
Agarose	Lonza
PTU (1-phenyl-2-thiourea)	Sigma Aldrich
Embryo medium: 30x stock solution	
Software	
Matlab equipped with the Image Analysis, Curve Fitting, Bio-Formats Toolboxes	MathWorks

8 mg/mL Tricaine stock solution	
200 mg of tricaine powder (Ethyl 3-aminobenzoate methanesulfonic acid)	Sigma-Aldrich
25 mL Danieau	
Adjust to pH 7, aliquot and store at -20 °C	

1 M BDM stock solution	
1 g BDM	Sigma-Aldrich
10 mL ddH_2O	
Aliquot and store at -20 °C	

Embryo medium: 30x stock solution	
50.7 g NaCl	Sigma-Aldrich
0.78 g KCl	Sigma-Aldrich
1.47 g Magnesium sulfate	Sigma-Aldrich
2.1 g Calcium nitrite tetrahydrate	Sigma-Aldrich
19.52 g HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich
Adjust to pH 7.2 and store at room temperature	

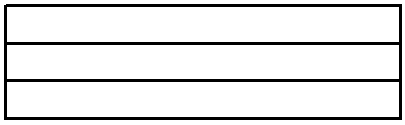
Mold	
PlasCLEAR resin	Asiga
Plus 39 Freeform Pico 3D printer	Asiga

Catalog Number
Leica SP8
88870001
353001
353046
16520-050
50004
P7629

E10521

112135

7647-14-5
7447-40-7
7487-88-9
13477-34-4
7365-45-9



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Author(s): Renee Wei-Yan Chow, Paola Lamperti, Emily Steed, Francesco Boselli and Julien Vermot

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Department:	
Institution:	IGBMC
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Responses in purple

Editorial comments:

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

2. Please be consistent with access type selected: You have chosen standard access in Editorial Manager but checked off open access in the ALA.

3. Please provide an email address for each author (missing email address of Paola Lamperti).
We will add her email address.

4. Please refrain from using personal pronouns (we, our, etc.) throughout the manuscript.
We have removed personal pronouns from the protocol section, but kept some in the discussion. We looked at a number of other published JoVE articles and they all contained personal pronouns, so we hope this is acceptable. We found that their use in the discussion helps to distinguish which parts of the protocol are designed the way they are due to theory, and which parts are designed the way they are because of first-hand experience.

5. Please use SI units, e.g. use “µL” instead of “µl”.
Done

6. 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.
The specific 3D printer and resin we used to make the mold been removed from the main text and moved to the materials and reagents table. The other two specific references to a company were the name of the microscope we used (Leica SP8), and the software we used (Matlab). We have noted that other JoVE articles also refer to specific microscopes and software. Removing the reference to Leica SP8 would mean that we cannot give step-by-step instructions, which are specific to the Leica graphical user interface. We also didn't delete the reference to Matlab, since our script is written in Matlab and would not work in other software.

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Some of the Notes have been moved to the discussion section or incorporated into the protocol steps.

8. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 35-38, 48-50, 157-159, 217-220 and etc. Please also see the attached iThenticate report and revise accordingly.
This has been changed.

9. Short abstract should not contain more than 50 words, current version has 56! Please shorten the short abstract to maximum 50 words.
The short abstract is now 35 words.

10. The current Long Abstract is the Introduction. Please include a Long Abstract that clearly states the goal of the protocol in 150-300 words. In addition, Abstracts cannot contain any citation.
We have added a long abstract. It is 278 words.

11. Please highlight 2.75 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Done

12. 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.
Done

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Done

14. Protocol: 1.2: What is the approximate temperature of the fridge
The text has been changed to “4'C”

15. Protocol: 2.1: How is the incross done? How many embryos are grown?
The section now reads:
1. Incross the *Tg(fli1a:Gal4FF^{ubs}, UAS:Kaede)* line and grow approximately 200 embryos in egg water in the dark at 28.5 °C. For more details on how to cross zebrafish lines, please refer to this article in the JoVE Science Education Database[11].

16. Protocol: 3.2: How much time is approximately needed?
The section now reads:
1. Add 2 mL of mounting medium to the mounting dish and allow 15-30 minutes for the solution to diffuse into the agarose.

17. Protocol: 3.3: How much time is needed for melting and cooling processes?
The section now reads:
In the meantime, melt a 1 mL aliquot of 0.7 % low melting point (LMP) agarose by placing the tube in a 70 °C heat block for about 5 minutes.

18. Protocol 5: How is the heart stopped? How are the embryos re-embedded? There are two section 3's of the protocol.
The numbering problem has been corrected.

19. Protocol: 6: Is any special toolbox required? Please provide more information about the software and explicitly explain the steps of using it. We need explicit step wise details in a graphical user interface. I would recommend un-highlighting step 6 of the protocol.
The toolboxes required is now listed in the Materials and Reagents spreadsheet. The Matlab script does not come with a graphical user interface. Considering that the script is not user friendly, we prefer not to make the Matlab script freely available at this point, and so explicit step-wise details of how to use the script are not possible.

20. Figure 1: Please include the units for scale. Please label the panels and the parts.
This has been done.

21. Figure 3: Please provide the unit for all the axes.
They have been added.

22. Please provide a more descriptive title for the two movie files.
This has been done.

23. Please revise the table of 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.
This has been done.

Reviewers' comments:
Reviewer #1:
Summary:
The article is a nice protocol that illustrates the use of photoconvertible proteins to understand developmental processes in vivo. One can follow the steps in the protocol easily and it gives sufficient information to reproduce the procedure. The results are representative and sufficient to illustrate the power of the method.
I only have some minor comments, which could help to make the manuscript even more

useful for the readers.

General comments:
- Can the authors also make a statement on how convertible this protocol is in case other microscopes than the Leica SP8 is used?
This is now discussed in the *Microscope* section.

Introduction
- It should already be mentioned in the intro that the experiment will lead to a temporary stop of the heart.
This is now mentioned in the introduction and in the abstract.

- AVC is used as abbreviation before it is defined.
This has been fixed.

- The protein Kaede should be spelled with capital K throughout the manuscript.
This has been done. We also changed *Tg(fli1a:Gal4FF^{ubs}, UAS:Kaede)* to *Tg(fli1a:gal4FF^{ubs}, UAS:kaede).*

Protocol
- Step 1.4 The authors probably mean 0.7% agarose and not 0.07% agarose
Yes, thank you. This has been fixed.

- Step 2.1 I don't think this step is necessary to show in the video. It is a very standard procedure. It would be better to dedicate more time to the photoconversion itself and how to troubleshoot it.
We agree. We've removed this step from the video.

- Step 2.2 The authors probably mean fluorescent stereoscope, not fluorescent microscope
Yes, thank you. This has been changed.

- Step 3 There are two steps 3
This has been fixed.

- Step 3.4 I don't think this step is necessary to show in the video as again it is a very standard procedure.
We agree. We've removed this step from the video.

- Step 3.5 It is not clear how the embryo should be positioned, say explicitly that it lies on its side or on its belly. Did the authors experiment with mounting only the tails of the fish in the agarose and keeping the heads and trunks completely outside of the agarose? This would be something that could be tried in order to improve the imaging conditions. If the authors tried and it did not work, it would be still an interesting piece of information.
We have now specified that the yolk side faces up.
Regarding mounting only the tails of the embryos - we tried and it didn't quite work. Embryos tend to become dislodged when we had to walk from our lab to the room containing the microscope located in a separate building. This could potentially be avoided if

we had a mounting station directly next to the microscope. We are developing new molds that would allow us to either embed only the tails or to not use LMP agarose at all, but we haven't managed to do so yet.
The following sentence has been added to the discussion:
Adding sufficient LMP agarose to cover the entire embryo is advised to ensure that the embryos do not become dislodged between mounting and photoconversion/imaging, especially if the microscope is not located directly adjacent to the mounting station.

- Step 3.1 The objective magnification should be given with the × sign instead of letter X
This has been fixed.

- Step 3.1 'We have experience with mild conversion of Dendra2 also with 488 nm light.' Is this the case also for Kaede? In that case it would be beneficial to add a note that the red channel should be imaged before the green channel for taking the "before" picture.
We have not noticed mild conversion of red Kaede with 488 nm light, but imaging the red channel first is better practice and we have made changes in the text.

- Step 3.3 The details of the photoconversion settings could be discussed in more detail, either here as a note or in the discussion. How did the authors conclude 25% laser power and scanning 3 times? Why is this better than e.g. 35% laser and scanning 2 times etc.
- Is there a danger of killing the cells with this laser power? How is phototoxicity in the converted cells identified? In our experience the newly converted red version of the protein can be easily bleached with the intense 405 nm illumination, so longer photoconversion does not yield more red fluorescence. The authors could comment on that as well.
Discussion of these two points have now been added to the *Photobleaching and phototoxicity* section. Re: killing of cells - we've never been able to kill cells directly using this method.

Discussion
- Mounting section: It is not currently clear what the authors mean by "embryo facing a little to the left".
This part has been made more specific:
In mounting, the tilt of the embryo in the left-right direction needs to be adjusted according to the embryo's developmental stage and the area one wishes to photoconvert. For example, to photoconvert the AVC at 36 hpf, the embryo should face roughly 15° to the left, while at 55 hpf, the embryo should face roughly 10° to the left.

- Future directions: The information that one can detect the converted cells for up to five days is not really a future direction. However, this information should definitely be included in the manuscript either as a note in the protocol part or early in the discussion.
This information has been moved to the introduction.

Language:
- The last sentence in the abstract is very long and hard to understand, should be rephrased.
- Line 47, is 'morph' a real word?

Yes, it is a word. But we agree that it is better to use simpler words unless necessary. We changed the word to 'change'.

- Line 62 lack a 'by'.
This has been added.

- line 136 lack a 'the' in front of photoconversion.
This has been added.

- Line 68, I would rephrase to 'taking care of avoiding bubbles'
We are worried that 'taking care of avoiding bubbles' would imply that bubbles are already there, and that you should avoid touching them. What we mean is that one should avoid trapping air when they place the mold into the melted agarose. We changed the wording to "Place the plastic mold in the liquid agarose, taking care to avoid trapping air between the mold and the agarose. " Hope that makes the section clearer.

- Line 159 lack a 'the' embryos.
This has been added:
1. At the desired stage, stop the heart and re-embed **the** embryos like under Step 3 of this protocol, with the important difference that embryos must be treated with BDM in separate, marked dishes to keep track of embryos.

- Line 258- 260, this is a very weird sentence.
We have changed it to:
At embryonic stages later than 55 hpf, the ballooning of the ventricle and the atrium also means that the violet laser beam used for photoconversion cannot reach AVC cells without first passing through either the atrium or ventricle. This means that beyond 55 hpf, in order to photoconvert EdCs of the AVC, one must also photoconvert additional EdCs in the atrium and ventricle.

- Is there a more scientific term for 'egg water'?
We changed 'egg water' to 'embryo medium' throughout the text.

Reviewer #2:
Manuscript Summary:
The manuscript addresses a major challenge in cardiac development, namely tracing at cellular resolution single atrioventricular valve cells. This will be particularly interesting for a number of researchers trying to analyze and interpret valvular phenotypes. The manuscript is well - written and detailed enough.

Minor Concerns:
A minor concern, although touched upon very briefly in the text (Discussion, 249) is the area of labeling and the challenges when trying to photoconvert at later stages. It will be very helpful if the authors give a little bit more details on photoconverting beyond the 48hours post-fertilization and some guidelines on how to achieve this in the most efficient way.

Unfortunately, we have no special tips for photoconverting embryonic hearts older than 55 hpf beyond what we have already written. We have tried specifically photoconverting atrioventricular valve cells at 72 hpf, but found that the light diffuses too much for the images to be useful. Thus, we have found it simplest to 'guess' where cells visualised at 48-55 hpf would end up, and image later to see if we are right. Beyond 55 hpf, it may be possible to label AVC cells via exclusion (ie. photoconvert all cells around the AVC cells). One can potentially access ventricular/atrial EdCs without photoconverting AVC EdCs by mounting the embryo at different angles. However, we have never tried this ourselves. We think that photoconversion between 28 and 36 hpf is possible, though we haven't tried it ourselves. At 28 hpf, we mount the fish embryo on its side for imaging.

We realised we may have misled the reader into thinking that we typically photoconvert embryos all the way up to 96 hpf. We have reworded the section on mounting embryos such that we clarify that the mold is used for imaging embryonic hearts up to 96 hpf, but only up to 55 hpf for photoconversion. We have also clarified the stages we have tried in the Introduction.

Reviewer #3:

Manuscript Summary:

In this manuscript Chow et al., the authors describe a method to photoconvert Kaede for lineage tracing of atrioventricular canal (AVC) cells in the developing zebrafish heart using a transgenic line that restricts expression of Kaede to the endocardium. The authors describe a workflow that includes the mounting, photoconversion, image and image analysis of the linear heart tube. The step-by-step protocol is informative and has the potential for phenotyping in various mutant background, however, there are some points that should be address before publication.

Major Concerns:

1. A potential use of this approach is for phenotyping mutants or embryos that have been treated with agents to disrupt AVC development. It would be good for the authors to point out this potential usage of their methods.

This is a very important point and we now discuss it in the abstract and introduction.

2. In the experimental pipeline, it will be important to keep track of each embryo that is photoconverted to correlate the position of the photoconverted cells at each development stage examined. This information is not noted in the text.

We have made changes to the text to make this point:

"After photoconversion, use a glass pipette to press down slightly just above the head of the embryo to break the LMP agarose, then gently suck up the embryo. Eject the embryo from the glass pipette into a 35 mm petri dish containing egg-water with PTU (to wash away BDM containing medium), then transfer the embryo to a well in a 6 well plate containing embryo medium with PTU. During this step, make sure to keep note of which embryo goes into which well, as this is essential to correlate the position of photoconverted cells at later developmental stages."

3. For the image analysis, the authors describe the use of Matlab to segment and project the 3D data into 2D space. The Matlab script should be provided.
Unfortunately, at this stage the Matlab script is not user-friendly enough to be included in the manuscript. We are happy to provide the Matlab script to anyone who requests it, and to go through it with them over Skype.

4. This may have been a technical problem with the upload but the two movies were not available for viewing
The movies are in .avi format.... We are not sure why they were not available for viewing.

Minor Concerns:
1. Authors discuss the use of 2P for imaging of later stage embryos put only provide parameters for exciting green Kaede. What wavelength should one use for exciting red Kaede?
In theory, red Kaede should have a two photon excitation spectra similar to that of DsRed, and could be imaged at 950 nm. This is now mentioned in the discussion section. We are not sure why, but we have not been able to perform 2P imaging of red fluorescent proteins on our microscope set up.

2. Line 74 describes preparing "0.07% low melting point agarose". Is this a typo as the rest of the text "0.7%"?
Yes, it is a typo. It has now been fixed.

Reviewer #4:
Manuscript Summary:
This is a timely protocol that will help the community performing in vivo cell tracking in the specific case of endocardial cells.

Major Concerns:
None

Minor Concerns:
-Line 62: The authors should mention also the following detailed protocol for performing near UV photoconversion of single cells using live zebrafish: Dempsey, Qin, and Pantazis, (2014) Methods Mol Biol 1148:217-28.
Reference has been added in Future Directions.

-The authors rightfully mention that photoconversion using nearUV light illumination is not confined, yet miss to mention its potentially cytotoxic effect, as photoconversion typically requires intense and extended nearUV illumination. Please include a note.
This has been added.

-Line 321: Please cite also the original publication of primed conversion: Dempsey et al., (2015) Nature Methods 12:645-648.
This reference has now been added.

-Recently, Kaede has been engineered to be primed convertible - please mention: Mohr et al., (2017) Angewandte Chemie International Edition 56:11628.
This is very interesting and has been added.

Supplemental File 1 - Movie 1.avi

This file could not be included in the PDF because the file type is not supported.

Supplemental File 2 - Movie 2.avi

This file could not be included in the PDF because the file type is not supported.

Supplemental File 3 - SuppInfoMold.STL

This file could not be included in the PDF because the file type is not supported.