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Real time and repeated measurement of skeletal muscle growth in individual live zebrafish subjected to altered electrical activity

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Real time and repeated measurement of skeletal muscle growth in individual live zebrafish subjected to altered electrical activity

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SUMMARY:

Optical clarity is a major advantage for cell biological and physiological work in zebrafish. Robust methods for measurement of cell growth in individual animals are described that permit novel insights into how growth of skeletal muscle and neighbouring tissues are integrated with whole body growth.

ABSTRACT:

A number of methods can be used to visualize individual cells throughout the body of live embryonic, larval or juvenile zebrafish. We show that live fish with fluorescently-marked plasma membranes can be scanned in a confocal laser scanning microscope in order to determine the volume of muscle tissue and the number of muscle fibres present. Efficient approaches for the measurement of cell number and size in live animals over time are described and validated against more arduous segmentation methods. Methods are described that permit the control of muscle electrical, and thus contractile, activity. Loss of skeletal muscle contractile activity greatly reduced muscle growth. In larvae, a protocol is described that allows reintroduction of patterned electrical-evoked contractile activity. The described methods minimize the effect of inter-individual variability and will permit analysis of the effect of electrical, genetic, drug, or environmental stimuli on a variety of cellular and physiological growth parameters in the context of the living organism. Long-term follow-up of the measured effects of a defined early-life intervention on individuals can subsequently be performed.

INTRODUCTION:

Regulated tissue growth, comprising increase in cell number (hyperplasia) and/or cell size (hypertrophy), is a crucial factor in development, regeneration and ecological and evolutionary adaptation. Despite huge advances in molecular genetic understanding of both cell and developmental biology over recent decades, mechanistic understanding of the regulation of tissue and organ size is still in its infancy. One reason for this lacuna in knowledge is the difficulty of quantifying tissue growth in living organisms with the necessary spatial and temporal accuracy.

Various aspects of growth of whole organisms can be measured repeatedly over time, revealing growth curves for each individual [1-5]. Increasingly sophisticated scanning methods, such as dual X-ray absorptiometry (DXA), computerized tomography (CT) and magnetic resonance imaging (MRI), permit the tracking of growth of whole organs and other body regions (for example, individual identified skeletal muscles) in single individuals, both human and in model organisms [6-10]. However, these methods do not yet have the resolution to reveal individual cells and thus the links between cellular behaviours and tissue level growth have been hard to discern. To make such links, traditional studies have often relied upon cohorts of similar individual animals, a few of which are sacrificed at successive timepoints and then analysed in cytological detail. Such approaches require averaging the observed changes across groups of (preferably similar, but nevertheless variable) individuals and thus suffer from a lack of temporal and spatial resolution, making it hard to find correlated events at the cellular level suggestive of cause and effect.

Studies on invertebrate model organisms, initially in *C. elegans* and *D. melanogaster*, have circumvented these problems by developing optical microscopy to achieve cellular resolution and accurately measure growth over time in single individuals. Such studies have revealed strikingly invariant cell lineage behaviours in the growth of these small model organisms [11-17]. However, many animals, including all vertebrates, have indeterminate cell lineages, and control tissue growth by mysterious feedback processes that serve to turn the genetically-encoded growth program into a functional three dimensional organism with all its constituent tissues and organs suitably matched in size. To understand these complex growth processes, it is desirable to image whole tissues or organs over time in single individuals that can be experimentally manipulated by genetic, pharmacological or other interventions at a time of choice and the effect subsequently analysed.

Each vertebrate skeletal muscle has a defined size, shape and function and well-characterised interactions with adjacent tissues, such as bone, tendon and nerves. Some muscles are small, lie just under the skin and are therefore good candidates for high-resolution imaging studies. Like most organs, each muscle grows throughout embryonic, postnatal and juvenile life, before reaching a stable adult size. Muscle, however, also has a unique ability to change size during adult life, dependent upon use and nutrition [18], and this property has a major impact on organismal fitness, sporting performance and independent living. Loss of muscle mass and function in old age, sarcopenia, is an issue of increasing concern for societies faced with an ageing population [19-21].

We and others have focused on the growth of defined blocks of skeletal muscle tissue in the segmentally-repeating body of zebrafish larvae, as an apparently closed system containing several hundred cells in which tissue growth, maintenance and repair can be observed and manipulated [22-26]. While some quantitative work has previously been reported [25-35], no detailed and validated method of measuring muscle growth in cellular detail in individual vertebrate organisms over time is available. Here an efficient protocol for how to perform such repeated

measurements is described, along with validation, and an example of its use to analyse changes in both hypertrophic and hyperplastic growth in response to altered electrical activity is provided.

PROTOCOL:

All research described was performed in compliance with institutional guidelines and under suitable licences from UK Home Office in accordance with the Animal (Scientific Procedures) Act 1986 and subsequent modifications. Embryos/larvae should be reared at 28.5ºC until completion of gastrulation, but may then be kept at 22-31ºC to control the rate of development. Fish may be scanned or stimulated at room temperature.

1. Anaesthetise zebrafish larvae

1.1. Cross suitable fluorescent reporter adult fish such as *Tg(Ola.Actb:Hsa.HRAS-EGFP)*^{vu119Tg} ref.[36] or *Tg(α-actin:mCherry-CAAX)*^{pc22Tg} ref.[37] and collect embryos as described [38].

1.2. At time of choice, such as 2 days post-fertilization (dpf), anaesthetise embryos briefly using Tricaine-containing fish medium (either fish water or E3 medium), and screen for EGFP or mCherry under a fluorescence microscope, such as a Leica MZ16F. If one has many embryos, select those with the brightest signal. Return embryos to normal fish medium immediately after screening.

2. Mounting fish for confocal scanning

2.1. Turn on the confocal laser scanning system and lasers, to let system stabilize for 30-60 min. A Zeiss LSM 5 Exciter microscope with an upright Materials stand (which enhances working distance) equipped with 20×/1.0W water-immersion objective may be used.

2.2. Freshly prepare 1% low melting agarose (LMA) and keep in a 37°C heat block for repeated use in a 1.5 mL 'Eppendorf' tube. To avoid heat-shock, it is best to let the LMA aliquot cool to just above setting before applying to the larva. Remove the tube containing the LMA from the heat block and allow it to cool, testing against one's skin to judge the appropriate temperature, as when assessing the temperature of baby formula milk.

2.3. Select fish to be mounted and transiently anaesthetise each fish in turn with Tricaine (0.6 mM in fish medium).

2.4. Take a 60 mm diameter Petri dish that has been coated with a layer of 1% agarose and place on stage of a dissecting microscope.

2.5. Transfer the larva with a 1 mL plastic Pasteur pipette onto the 60 mm coated Petri dish and remove as much transferred medium as possible. Then, still using the Pasteur pipette, place 5 to 10 drops of LMA onto the fish and rapidly position horizontally in lateral view with forceps (or a fire-polished fine glass needle) before the LMA sets.

2.6. Alternatively, using a 1 mL plastic Pasteur pipette, collect the larva with as little fish medium as possible, and transfer the larva into the aliquot of barely-liquid LMA. Allow the larva to sink for 5 s to become fully surrounded by LMA, then retrieve the larva and transfer it in a drop of LMA onto the agarose-coated Petri dish. Quickly orientate and position the larva as described above.

2.7. It is highly desirable to orient the larva with both its anteroposterior and dorsoventral axes within 10º of horizontal (see Note: On error and its correction at point 4.5 below). If larva is not correctly mounted horizontally near the surface of the agarose drop, it is better to

remove and re-embed. Larvae can be easily retrieved by gentle suction using a microfine 1 mL plastic Pasteur pipette, and LMA can be gently removed using Kimwipes. Practice really does make perfect in the mounting procedure; spend an afternoon embedding some unimportant larvae before trying this on a real experiment.

NOTE: On microscope design. Many labs use inverted confocal microscopes for imaging through a coverslip. We have found that the repeated embedded and removal of fish held in agarose under a coverslip for observation in an inverted microscope leads to greater loss of samples during repeated scanning than in the described procedure with an upright microscope. For this reason, the use of an upright system is recommended, if available. Nevertheless, a key to high quality data is the proper selection and use of objective and scan parameters, a subject too large for discussion here.

3. Confocal scanning

3.1. When LMA has set, flood the dish with around 10 mL of Tricaine-containing fish medium. If planning to capture confocal stacks, let the mounted fish rest for at least 10 min before proceeding to scanning, as some agarose swelling occurs.

3.2. Load sample dish to the stage of the confocal system, locate larva and focus on desired somite. Somite 17 may be chosen because of its ease of localization near the anal vent and ease of imaging. Check by counting somites from anterior. *NOTE*: the first somite is fused behind the ear and has no anterior border, but can be readily observed to have striated muscle fibres.

3.3. Set up as if to capture a *Z*-stack by defining top (i.e. just above the skin) and bottom (i.e. just below the notochord, so as to include the entire somite, even if the fish is mounted slightly skewed). Both left and right sides can be captured as desired. This will ensure that all the rapid *YZ* scans capture the desired region(s).

3.4. Capture an *XY* image. Orient the scan area with respect to the fish, as the confocal software permits. The fish is positioned with the anteroposterior axis parallel to the imaged *X* axis and dorsoventral axis parallel to the *Y* axis with somite 17 in the centre of the field as shown in **Supplementary File 1**. Focus on a mid-level plane in the uppermost myotome in which the whole epaxial and hypaxial somite halves together with the vertical and horizontal myosepta are visible and capture a high resolution *XY* image. Remember to name and save the image.

3.5. Capture one or more *YZ* images as follows. In the Representative Results (below) the accuracy of '2-slice' and '4-slice' methods is compared. In the 2-slice approach, single *XY* and *YZ* scans are employed. In the 4-slice approach, three *YZ* scans are averaged to give a more accurate estimate of myotome volume. If required by the confocal software, re-orient the scan field. Draw a precisely dorsal to ventral line across the chosen somite perpendicular to the fish anteroposterior axis at a selected anteroposterior position. Perform a *Z*-stack line scan. Repeat the *YZ* line scan three times at defined anteroposterior positions along the selected myotome to capture *YZa*, *YZm* and *YZp*. Representative results are shown in Figure 2A. Name and save these images together with the related *XY* image.

Note: On selection of *YZ* planes. The myotome is *V*-shaped and its form changes during growth. To obtain the most accurate assessment of myotome 17 volume with the 4-slice method, position *YZa* on the anterior tip of the myotome, *YXp* on the posterior tips of the myotome at dorsal and ventral extremes and *YXm* halfway between *YZa* and *YZp*. Assuming the somite tapers uniformly, the mean of measurements from each *YZ* section will represent the myotome as a whole. For the 2-slice method, the single *YZ* scan should be positioned at the posterior end of the horizontal myoseptum, which roughly corresponds to the anteroposterior centre of the myotome of interest (like *YZm*). Alternatively, a set of three *YZ*

sections may be taken at anterior, middle and posterior of horizontal myoseptum but, as shown below, such measurement will slightly over- or under-estimate myotome volume (for rostral and caudal somites, respectively, due to myotome tapering). Fundamentally, consistency in positioning of *YZ* slice plane(s) between fish and experiments is key to reproducibility.

4. Analysis

4.1. As the myotome changes size along the fish in a graded manner, it is essential to always work with the same somite in comparative studies.

4.2. To measure and calculate the myotome volume, use the confocal software (such as the '.lsm' files created using Zeiss ZEN microscope software) or open-source universal image analysis software, such as Fiji/ImageJ (National Institutes of Health, NIH). *NOTE*: if changing file formats, make sure the *Z*-step size is correctly transferred, as not all software can read proprietary confocal file formats correctly. For example, to import a ZEN line scan image into Fiji, first use the File/Export command to export as '.tif' in the 'Full resolution image window – single plane' format, then import into Fiji. Although '*YZ* scan.lsm' can be opened directly in Fiji, the resulting *YZ* images are generally compressed in the '*Z*' dimension due to incorrect evaluation of the *Z* step size.

4.3. Analysis using ZEN.

4.3.1. First, open the '*XY* scan.lsm' files in ZEN. Go to the 'Graphics' tab and select the 'Line' tool. Draw a line between the two vertical myosepta of somite 17 spanning the entire the myotome length (parallel to the anteroposterior axis of the fish). Check the 'M' box to reveal the values of the measurement (Length = 89.71 µm, see **Supplemental File 2**).

4.3.2. Open the '*YZ* scan.lsm' files. Under the 'Graphics' tab, select the 'Closed bezier' tool. Draw around the perimeter of the myotome. Once completed, check the 'M' box, this would reveal the value of the measurement (Area = 11980.01 μm^2, see **Supplemental File 3**).

4.3.3. Record the values of each measurement manually in a Microsoft Excel spreadsheet or similar. Average the CSA measurements as required. Volume of the myotome can be calculated as Volume = Myotome length × CSA. i.e. 89.71 µm × 11980.01 μm^2 = 1.075×10^6 μm^3.

4.4. Analysis using Fiji/ImageJ.

4.4.1. Open the '*XY* scan.lsm' files in Fiji/ImageJ. Check that *XY* images directly opened in Fiji are correctly calibrated in scale, as they should be.

4.4.2. Select the 'Straight Line' tool from the icons. Draw a line along the length of somite 17 as described in 4.3.1. Set measurement parameters by going to 'Analyze', then select 'Set Measurements…', and check the following boxes 'Area' and 'Display label'. To measure, simply press the hot key 'M', or go to 'Analyze' menu and select 'Measure'. A resulting pop-up window lists all measurement values (i.e. Length = 90.023 µm; see **Supplemental File 4**). The results can be saved in form of '.csv' and opened in Microsoft Excel or similar for subsequent analysis.

4.4.3. To measure CSA on the *YZ* images, open *YZ* images in '.tif' format as described in 4.2.

4.4.4. Calibrate the *YZ* '.tif' images as they are uncalibrated when exported. Parameters for the calibration can be obtained in ZEN by going to the 'Info' of the selected images: record the 'Scaling X' (0.489 µm) and 'Scaling Z' values (0.890 µm; see **Supplemental File 5**). Next, while the images are open in Fiji, go to 'Image' and select 'Properties…'. Input '0.489 µm' for the 'Pixel width' and 'Pixel height' and '0.890 µm' for the 'Voxel depth'. Check the 'Global' box to apply the calibration

universally if repeated measurement of *YZ* images is anticipated (see **Supplemental File 6**). *NOTE:* Make sure all *YZ* images are captured using the same scanning parameters; restart Fiji/ImageJ or modify the calibration values if a new set of calibration is required.

4.4.5. To measure the CSA of the calibrated *YZ* images, select the 'Polygon selections' tool from the icons. Draw around the perimeter of the somite, and press 'M' to reveal the values of the measurement (Area = 11980.395 μm^2; see **Supplemental File 7**). Volume of the myotome can be calculated as Volume = Myotome length × CSA. i.e. 90.023 µm × 11980.395 μm^2 = 1.079×10^6 μm^3.

4.5 Repeat the measurements on the other *XY* and *YZ* images. It is recommended to use the same software for all measurements within an experimental series for consistency. The volume estimate from each software is similar but not identical due to the distinct drawing tools i.e. ZEN = 1.074×10^6 μm^3 and Fiji/ImageJ = 1.079×10^6 μm^3. Growth of the myotome between two time points (i.e. 3 to 4 dpf) can be calculated as: $(\text{Volume}_{4\,dpf} - \text{Volume}_{3\,dpf}) / \text{Volume}_{3\,dpf} \times 100\%$.

NOTE: On error and its correction. During mounting, the fish should be orientated with its sagittal plane (i.e. the anteroposterior and dorsoventral axes) as close as possible to horizontal, to avoid yaw and roll, respectively. This is because both the myotome length L measured from the *XY* scan and the CSA measured from a *YZ* scan will be over-estimated if the fish shows yaw (rotation around the dorsoventral axis) due to oblique anteroposterior mounting. Neither pitch nor roll during mounting should affect measurements after scanning as described in section 3. Nevertheless, dorsoventral rotation (roll) degrades image quality. Simple trigonometry shows that up to 10° of yaw will give 3% error in volume measurement, as measured L and CSA each increase in proportion to $(\cos\theta)^{-1}$, where θ is the angle away from anteroposterior horizontal (yaw). 15° and 20° off will give 7% and 13% over-estimates of volume, respectively.

As the notochord is cylindrical, inclusion of the whole notochord in the *YZ* scan can be used to calculate the angle and extent of obliquity from the orientation and magnitude of the major and minor axes and thereby correct the measured L and CSA to maximize accuracy. Corrected CSA = Measured CSA x Notochord minor axis/Notochord major axis. Corrected L = Measured L x Notochord minor axis/Notochord axis in microscope *Z* direction.

A further consideration permits additional correction of L. As the myotome grows it skews in the coronal plane (normal to the dorsoventral axis) such that the medial myotome is slightly anterior to the lateral myotome. Viewed from dorsal, the vertical myosepta on left and right sides form a broad chevron pointing anterior. If yaw is low, this morphology does not affect measurement of L. But if yaw is significant trigonometrical correction becomes challenging and a better approach is to measure True L directly by estimating the *XYZ* coordinates of the two points where the anterior and posterior vertical myosepta meet the notochord at the horizontal myoseptum. Simple trigonometry permits calculation of True L from these coordinates as $L = \text{SQRT}[(X_2 - X_1)^2 + (Y_2 - Y_1)^2 + (Z_2 - Z_1)^2]$. Weaknesses of this last approach are that a) selection of the points can vary with operator and b) no visual record of the points chosen is retained. This consideration does not affect CSA correction.

5. Optional method: Remove and re-introduce muscle electrical activity

5.1. At 3 dpf, split fish into three conditions: fish medium Control, Inactive and Inactive+Stim.

1.3.1 For Inactive and Inactive+Stim groups, anaesthetise larvae at 9 am with Tricaine (0.6 mM). *NOTE*: Following [38], frozen aliquots of tricaine stock are thawed and diluted (40 µl/ml fish medium, to a final concentration of 0.6 mM) before adding to

fish. Do not add tricaine straight into the water containing fish, as some fish could receive high doses. Tricaine stock should be used within a month and never be re-frozen.

1.3.2 For the fish medium Control fish, leave them un-anaesthetised.

5.2. At selected time(s) after the onset of tricaine exposure (i.e. at 80 hpf), prepare the Inactive+Stim group for stimulation.

5.2.1. Create a stimulation chamber. Take a 6 × 35 mm-well plate, create two small openings (<5 mm in diameter) on each side of each well (see Figure 1) using a narrow soldering iron. *NOTE*: handle the hot soldering iron with care and work in a fume hood if desired to avoid inhaling vapour.

Thread a pair of silver or platinum wires (~20 cm-long) through the openings of each well (see Figure 1). Reusable adhesive material e.g. BluTack can be applied near the openings to keep the wires in place, and ensure a >1-cm separation between the wires (see Figure 1).

5.2.2. Prepare 60 mL of 2% agarose (1.2 g agarose powder in 60 mL fish medium), and melt fully using microwave, cool, add tricaine and pour 4 mL into each well of the stimulation chamber (Figure 1).

5.2.3. Immediately add custom-made 4-well combs in between the electrodes, (created by cutting out plastics e.g. polypropylene of desired dimensions and sticking together using Superglue; see Figure 1). Allow 10 min for gel to set. Remove combs carefully to create four rectangular wells.

5.2.4. Fill each well with tricaine water and place a single anaesthetised 'Inactive+Stim' larva in each well using a micropipette, with their anteroposterior axis perpendicular to the electrodes (see Figure 1).

5.2.5. Check under the dissecting fluorescent microscope that each fish is fully anaesthetised within each well of the chamber.

5.3. Connect an adjustable electrophysiological pattern-generating stimulator to the chamber via a Polarity Controller, using crocodile clips connected to each of the electrodes on one side of the chamber (see Figure 1). *NOTE*: The polarity controller is used to reverse the polarity every 5 seconds, so as to prevent electrolysis and corrosion of the electrodes.

5.4. Stimulate fish. For example, 1 s with a train of 200, 20 V pulses of alternating polarity, with 0.5 ms pulse duration and 4.5 ms pulse separation, once every 5 seconds gives an effective repeated-tetanic contraction 'resistance' regime.

5.5. Regularly check under the microscope that the fish are being stimulated; the example electrical stimulus should induce a visible bilateral contraction and slight movement, once every 5 seconds.

5.6. For a 'resistance/high force' regime, stimulate the fish for a bout of 5 mins, three times, with each bout separated by 5 mins of rest. *NOTE*: While fish on one side of the chamber are resting, the crocodile clips can be connected to the electrode pair on the other side of the chamber, and those additional fish stimulated.

5.7. After stimulation, carefully remove fish from each well by gently flushing them out with a plastic pipette and return to incubator in fresh tricaine-containing fish medium.

5.8. Pour away the tricaine water from within the chamber and use Forceps to cut around and remove the agarose from each well. Rinse the wells with tap water and allow to dry.

NOTE: If using silver wire electrodes, occasionally silver oxide may accumulate on the surface of the wire after a stimulation experiment. As silver oxide is less conductive than silver, to maintain reproducibility, careful rub silver oxide off the wire using Kimwipes before re-using the set-up.

REPRESENTATIVE RESULTS:

A rapid and precise measure of somite volume

A method of sample preparation, data acquisition and volumetric analysis that allows the rapid measurement of muscle growth in zebrafish larvae is described. Muscle size can be measured in live animals using fish labelled on their plasma membranes with a membrane-targeted GFP (*β-actin:HRAS-EGFP)* or mCherry *(α-actin:mCherry-CAAX).* Larvae were transiently anaesthetised using tricaine, mounted in low-melting-point agarose and imaged using confocal fluorescence microscopy. Somite 17 was chosen for analysis of muscle size given its accessibility at the trunk-tail interface [31]. In practice, as in theory, myotome volume can be calculated as the product of myotome length (L) and the average of three cross-sectional area measures (CSA) (Figure 2A, which is referred to as the 4-slice method).

To validate this method, whole myotome confocal Z-stacks of live zebrafish larvae (Figure 2B) were obtained and somite volume was calculated by multiplying the sum of somite 17 profile areas in each slice (80-100 slices) by the inter-slice distance (1-1.2 µm). A strong correlation was observed between the 4-slice and full stack methods of calculation (Figure 2C). However, the 4-slice method generally gave a slightly larger volume estimate (~2% larger on average) (Figure 2D). This difference could be due either to a) obliquity of the samples giving erroneously large volume measurement or b) the observation that zebrafish myotomes taper along the anteroposterior axis, being smaller towards the tail of the animal. As the *YZa*, *YZm* and *YZp* CSA measurements are towards the anterior portion of the somite 17 myotome chevron (Figure 2A), the latter interpretation was tested by volumetric analysis of the myotomes of somites 16 and 18. Each somite was about 7% larger than the one behind (Figure 2E). Further analysis revealed that a '2-slice' method requiring only a single *YZ* measurement, located in the middle of the somite where the epaxial and hypaxial halves meet at the horizontal myoseptum (*YZm*), and the *XY* slice, gives a reasonably accurate estimate of myotome volume (Figure 2F,G). The 2-slice approach enables more rapid data acquisition when time constraints limit the number of fish that can be scanned. In summary, these data show that myotome volume can be measured rapidly and accurately in live zebrafish larvae. Single larvae were successfully repeatedly measured over a six day period with this method.

For comparative study of growth of an identified tissue unit, the described method provides reliable and precise volume estimates. To obtain accurate absolute volumes, however, a number of modifications can be applied. First, correction can be made for errors caused by oblique mounting either by tilting dish or microscope stage to obtain better transverse *YZ* and parasagittal *XY* slices during imaging or through using the notochord profile in *YZ* slices; the cross-sectional minor axis reveals the true diameter of the cylindrical notochord, whereas its major axis reveals the angle and magnitude of obliquity (see Note in point 4.5 above). Second, the location of *XY* and *YZ* sectioning during scanning must be selected to reflect accurately the desired myotome(s). (See Note in point 4.5 above). Note, however, that the form of the myotome chevrons changes depending on developmental stage and this must be taken into account when selecting *YZ* scans. Lastly, to determine if changes in myotome 17 reflect muscle growth throughout the axis, myotomes further into the trunk or tail regions may be measured by the described method.

Repeated measurements reveal somite growth

An advantage of the described method is the ease of repeated analysis on single fish. Individual embryos and larvae can be repeatedly embedded, measured and released without suffering

obvious long-term effects (Figure 2H). The myotome grows detectably both in L and CSA between 2 and 5 dpf, leading to steady increase in volume (Figure 2H). Growth was imaged in this way between 1 and 8 dpf and larvae were also released after imaging and grown to adulthood. Analyses further into the late larval period are expected to be possible, although effects of repeated imaging in feeding behaviour would need to be monitored carefully in comparison with siblings that are not imaged. Importantly, development of pigmentation can obscure imaging. Whereas pigmentation is not a problem in properly orientated fish, as the melanophore stripes do not prevent the required measurements, pigment is more problematic in obliquely mounted samples. The use of pigmentation mutant lines, such as *roy;mitfa* [39], is anticipated, which would extend the time window of growth measurements until the limits of practicable confocal scan depth are reached.

A further advantage of the described procedure is the ease of detailed analysis of growth of single fibres in comparison with their whole myotome over short periods. By mosaic labelling of fibres through DNA injection, a method was developed to detect nuclear acquisition and growth in individual identified fibres over four hours and then permit re-analysing at later times (Figure 2I). Moreover, growth of the whole myotome can be measured over 12 h or less [26] (and data not shown). By repeatedly measuring the same fish, inter-individual variability is eliminated and small numbers of animals can yield statistically robust results [26].

Manipulations which change somite volume can readily be detected

The current protocol allows one to interrogate changes in muscle growth under various biological and physiological conditions, such as altered physical inactivity. Anaesthetic tricaine, which blocks nerve action potentials by inhibiting voltage-gated Na^+ channels [40], was used to induce muscle inactivity in *β-actin:HRAS-EGFP* larvae. As shown previously [26], inactivity for 24 h between the third and fourth dpf greatly reduced myotome volume, indicating that larval muscle growth is activity-dependent (Figure 3A). The effect of inactivity can also be investigated using other detection methods which reveal the structure of the somite, such as mCherry-CAAX (either in a transgenic line or by mRNA injection) or by overnight immersion of larvae in BODIPY dye (Figure 3A). The latter approach, while removing the need to cross fish onto transgenic backgrounds or inject embryos cannot be used for repeated measurements due to toxicity of BODIPY. Thus, the current method allows one reproducibly to measure changes in the volume of muscle tissue.

As described above, genetic marking methods can be used to make repeated measurements of myotome volume, permitting tracking of change in muscle size over successive days in individual fish. As individual fish and entire lays at the same development stage differ in absolute myotome volume (Figure 3A; perhaps due the size or health of eggs), the ability to measure growth of each individual reduces the effects of individual variation by permitting paired sample statistical analyses. Repeatedly measuring the same fish reduces the number of fish needed to detect effects robustly. To illustrate this effect, results from analysing the growth of each individual from 3 to 4 dpf in populations of active and inactive larvae were compared with analysis of the same two populations using only the single measure of myotome size made at 4 dpf. From lay to lay, larger variation in apparent reduction of myotome size was observed when measuring myotome volume at 4 dpf only compared to measuring 4/3 dpf volume for each individual (Figure 3B). Note the greater range of reduction (68-91%) in the 4 dpf only measurements, compared to the 4/3 dpf method (78-89%) and the weak correlation between the two measures. Although, as expected, the mean reduction across all 13 biological replicates was similar in each assessment (Figure 3C) being 82.62 ± 1.01% (mean ± SEM, n=13) for the 4/3 dpf method and 82.31 ± 1.92% for the 4 dpf only method, the estimated error with the 4 dpf only method was almost double that with the

4/3 dpf method. Thus, quantifying individual growth through repeated measurement is the more accurate method, by eliminating size variability between fish within the same lay, as demonstrated previously [26]. Nonetheless, as no significant difference in the reduction in myotome volume caused by inactivity was observed when measuring myotome volume at 4 dpf only (Figure 3C), the data suggest that ~6-8 fish are sufficient to average out inter-individual size difference within a lay. Clearly, when size changes are small the 4/3 dpf method is preferred.

Analysis of the cellular basis of growth

Myotome CSA is determined by muscle fibre number and fibre size. Fibre number can be estimated by counting the number of fast and slow fibres on three *YZ* sections (*YZ*a, *YZ*m, *YZ*p). Although regions of two adjacent myotomes are contained in such *YZ* sections, as shown by the presence of vertical myosepta (VM) in most sections (Figure 2A), these counts accurately reflect fibre number. Over-counting occurs at VMs due to the tapering of pairs of fibres from each adjacent segment at the VM (Figure 4A). Such overcounting can be accounted for using the following equation: Fibre number = Total fibre count – (fibres in contact with VM)/2 ref [33]. Furthermore, average fibre volume can be determined by dividing myotome volume by the number of fibres. We have used these analyses to reveal that activity controls both cellular aspects of growth (Figure 4B,C).

It is evident from Figure 2A that fibres vary in size across the myotome, a reality that is not reflected in calculated average fibre volume measurements. By drawing around each fibre of somite 17 from two fish, measured fibre cross-sectional area was shown to range from 28 μm^2 to 217 μm^2 (Figure 4D). In reality, however, many fibres are angled obliquely within the myotome, so such CSA measures do not reflect the true CSA of a fibre perpendicular to its long axis. Conversely, due to the varying angles of fibres within the myotome, all fibres running at orientations that are not aligned anteroposteriorly have lengths that differ from the myotome length. Despite these caveats, which can only be circumvented either by complete segmentation of the myotome into single fibre volumes or by calculation after measuring the angle of obliquity of each fibre, the measured CSAs provide an estimate of fibre size diversity in each fish. For example, individual fibres decreased in CSA with inactivity, resulting in a shift to the left in the cumulative frequency curve with respect to active (un-anaesthetised) control larvae (Figure 4E). As inactive fish have ~10 fewer fibres than active fish (Figure 4B), either the ten smallest fibres from active un-anaesthetised fish (on the assumption that they are the new ones) or the ten largest (as the alternative extreme) were omitted from the comparison, which showed that most loss of the myotome volume is due to lack of fibre growth, rather than failure of new fibre formation (Figure 4F). Taken together, these data show that the described method allows detailed investigation the role of physical activity on the formation and growth of muscle tissue.

Reimposition of activity by electrical stimulation

Physical activity is required for muscle growth (Figure 3A). A method to re-impose muscle contractions by electrical stimulation in otherwise inactive larvae, evoking a strong contractile response is described (Figure 5A, Supplemental File 8). Although precise stimulation parameters are described here (Figure 5B) to maximally activate the musculature, the protocol can be altered (by changing current amplitude, frequency, pulse duration etc) to control muscle activation and 'exercise' dosage. Thus, the current method provides a controlled activity stimulus with standardised behaviour between activity bouts, overcoming an important limitation of current animal models of exercise [18].

The described methods demonstrate the potential of using zebrafish larvae to study various aspects of muscle growth e.g. hyperplasia and hypertrophy. In particular, myogenesis in zebrafish larvae is shown to be amenable to analysis through pharmacologically-induced inactivity and electrically-induced contractility. The approach allows the study of the molecular mechanisms by which physical activity leads to muscle growth *in vivo*.

FIGURE AND TABLE LEGENDS:

Figure 1. Design of a stimulation chamber for re-introduction of muscle electrical activity to zebrafish larvae. A six well cell culture plate fitted with electrodes permits maintenance of larvae in wells made within 2% agarose gel. Custom four-well combs are made to create rectangle wells (dimensions as indicated) in agarose to maintain position and orientation of individual fish larvae, and to prevent them from contacting the silver wires during vigorous twitching upon electrical stimulation.

Figure 2. Measuring muscle volume in live zebrafish *α-actin:mCherry-CAAX* (A,B,E) or *β-actin:HRAS-EGFP* transgenic larvae. Larvae imaged from lateral view and shown dorsal to top, anterior to left in upper panels (A,B,E). **A.** In the 4-slice method, myotome volume was calculated by multiplying the length of somite 17 (L) measured between vertical myosepta (VM) on an *XY* plane (blue arrow) by the average of three cross-sectional areas (CSA) measured on *YZ* planes (*YZm*, *YZa* and *YZp;* dashed yellow lines). **B.** In the Full Stack method, the area of somite 17 myotome was measured (examples outlined in yellow) in each slice of a whole *Z*-stack of a live zebrafish larva and the sum of myotomal areas was multiplied by the inter-slice distance. **C.** High concordance between the 4-slice and Full Stack methods. Colours denote individual β-actin:GFP (squares) or α-actin:mCherry-CAAX (circles) fish. **D.** Myotome volume is slightly, but significantly, larger when calculated using the 4-slice method. **E.** In the tapering zebrafish larvae, more anterior somites are larger than posterior somites, as measured by the 4-slice method. **F,G.** Strong correlation between volume measurements made using the average of three CSA sections (4-slice method) or a single *YZm* CSA (2-slice method)**.** *p*-values show results of two tailed t-tests with equal variance (D,G) or one way ANOVA with Bonferroni *post hoc* tests (E). ns, not significant. **H.** Measurement of myotome 16 volume, length (L) and cross-sectional area (CSA) from two to five dpf in three individual fish (colours) expressing β-actin:GFP and myog:H2B-mRFP. *YZm* images of the green individual at each timepoint are shown above. **I.** Single fibre growth measured by automated constant-threshold segmentation. A *β-actin:HRAS-EGFP;myog:H2B-mRFP* fish mosaically labelled by injection of a CMV:Cerulean plasmid at the 1-2 cell stage. A single Cerulean marked fibre in somite 10 was scanned with high resolution full *XYZ* stacks repeatedly on a Zeiss LSM880. Images shown are representative single slices (left) and the projection of the three dimensional segmented volume (right). Each datapoint on the graph represents a single scan of the same fibre, at 3 dpf (0 h), after 4 hours, and at 5 dpf (54 h). Triplicate scans were made and segmented per time-point to show reproducibility. White arrowheads point to fibre nuclei; note that two nuclei are added between 3 and 5 dpf.

Figure 3. Activity-dependent muscle growth in somite 17. **A.** Inhibiting activity for 24 h by application of tricaine (pink) between 3 and 4 dpf reduces myotome volume both in transgenic lines and in non-transgenic fish stained with BODIPY compared to vehicle controls on siblings (blue). Volume quantified by 4-slice method. Symbol shape indicates replicate experiments from distinct lays (biological replicates). Large symbols denote mean ± SEM values. Small faint symbols show the volume of individual replicate larvae. **B.** Comparison of myotome volume

reduction in inactive fish compared to control siblings determined by single measurement of myotome volume at 4 dpf (4 dpf only, upper schematic) or change in myotome volume between 3 and 4 dpf (4/3 dpf, lower schematic). Each symbol represents the mean volume of myotome 17 in ~5 inactive fish from a single lay divided by the mean myotome 17 volume of ~5 active control siblings. **C**. No difference was observed in the mean reduction in muscle growth in inactive fish, when measuring by the 4 dpf only or 4/3 dpf methods. Numbers within bars represent total number of fish analysed. *p*-values show results of two way ANOVA with Bonferroni *post hoc* tests (A) or two tailed t-test with unequal variance (C).

Figure 4. Cellular level changes in muscle growth caused by inactivity. **A.** Schematic showing how overcounting occurs at VMs due to double counting of tapering fibers where the blue and red myotomes meet. Note that the average fibre count is 6 but the true mean value is 5.5. The corrected count gives a better approximation. **B,C** Fibre number (B) and average fibre volume (C) are reduced in inactive(pink) compared to active (bue) larvae. Symbol shape indicates replicate experiments from distinct lays (biological replicates). Large symbols denote mean ± SEM values. Smaller faint symbols show the value of individual replicate larvae. Numbers within bars represent total number of fish analysed. **D**. In single larvae, each fibre profile in myotome 17 was outlined and CSA determined. Boxes show mean ± SEM. *p*-values show results of two-way ANOVA with Bonferroni *post hoc* tests (B,C) or two tailed t-test with equal variance (D). **E,F.** Cumulative frequency curves showing fibre size distribution in active control (blue) and inactive (pink) larvae. Comparison of all fibres (E), or after omission of presumed-nascent small or, at the alternative extreme, large fibres (F) shows that fibre size increase, not increase in fibre number, primarily accounts for activity-driven growth of the myotome.

Figure 5. Brief electrical stimulation evokes tetanic muscle contractions in anesthetised zebrafish larvae. **A**. Sequential images (captured from video in Supplemental File 8) showing how electrical stimulation triggers maximal contractions of an anaesthetised larva at 3 dpf. Time scale in seconds. Red boxes indicate movements at start of three successive 1 s stimulation trains. Each image is a 40 ms exposure. **B**. Schematic showing the electrical stimulation regime, in which a 1 s train of 200 high frequency, 20 V electrical impulses is given every 5 seconds.

Supplemental File 1. Schematics of captured images of perfectly (top left) and imperfectly mounted larvae with respect to the microscope *XYZ* reference frame (black axes). Myotome (green), notochord (yellow), neural tube (tan), measured myotomal parameters (red), measured notochord parameters (black arrows) and possible or actual fish rotations (blue).

Supplemental File 2. Screenshot showing somite length measurement from *XY* images in ZEN.

Supplemental File 3. Screenshot showing CSA measurement from *YZ* images in ZEN.

Supplemental File 4. Screenshot showing somite length measurement from *XY* images in Fiji/ImageJ.

Supplemental File 5. Screenshot showing extraction of calibration parameters for *YZ* images from ZEN.

Supplemental File 6. Screenshot showing calibration of *YZ* images in Fiji/ImageJ.

Supplemental File 7. Screenshot showing CSA measurement from *YZ* images in Fiji/ImageJ.

Supplemental File 8. Representative video showing muscle contraction evoked by direct

electrical stimulation (length 1 s) of a tricaine-anaesthetised 3 dpf larva.

TABLE OF MATERIALS:

See attached.

DISCUSSION:

Here we report a method for accurate and efficient estimation of the muscle volume of live zebrafish larvae at stages or in genetic variants in which pigmentation is not a big hinderance to imaging and when transient anaesthesia and/or immobilisation is well tolerated. Whereas we have employed laser scanning confocal microscopy, the approaches described are applicable to spinning disk confocal or light sheet microscopy and to any other method that creates stacks of images at distinct focal planes. A series of increasingly sophisticated approaches to tissue size and cell content estimation is described. Each method has advantages and limitations, which we show can be quantified. A major limitation in studying tissue growth is the difficulty of analysing growth changes in real time as growth rates alter in response to a series of molecular or sub-cellular events catalysed by acutely administered stimuli. Moreover, individual variation can create problems when separate individuals are compared. The current approach allows measurement of tissue growth over periods of less than a day in single live individuals. Application of the approach can be envisaged on the minute timescale.

The described methods permit analyses that were hitherto impracticable. Using the 4-slice method, the imaging portion of the muscle growth assay can be completed on a sample size of around 20 fish within an hour by a trained operator. This is in stark contrast to the conventional Full Stack method, which takes at least 3 hours for the same number of fish (i.e. three times longer). If high resolution images are required for subsequent sub-cellular analyses the Full Stack method can easily require 30 min per fish, making growth assays of cohorts of animals at a similar developmental stage impossible. In contrast, the 2- or 4-slice methods permit rapid high quality image capture. Moving to consideration of image analysis, the advantages of the 2- or 4-slice method in saving of operator time (in the absence of automated image segmentation) over the Full Stack method are enormous. Each fish requires about 20 min for Full Stack analysis, but only 3-4 min for 4-slice analysis. Operator time can be further conserved by using the almost-as-accurate 2-slice method. Thus, the described method is efficient and thereby increases flexibility in experimental design.

The major limitation of the 4- or 2-slice methods are their being estimates, due to the overlapping chevron shape of somites, of the volume of regions of two neighbouring somites e.g. in the examples shown, myotome 16 and 17. It is shown that this can over-estimate the actual myotome 17 volume by around 2%, depending on precisely where *YZ* slices were selected. Moreover, manual tracing of the somite borders during measurements might contribute to variations in estimates, although little inter-experimenter difference was found (data not shown). Measurement errors might be addressed using thresholding, filtering, and segmentation algorithms to acquire surface area in a more objective and reproducible manner. However, customisations will still be required to account for variations in the background fluorescence (such as due to embedding and/or thickness of the LMA) and the expression level of fluorescence proteins of individual larva over time. Note that such automated measurements will be even more challenging if a non-muscle-specific reporter is used, such as the *β-actin:HRAS-EGFP* line. Nonetheless, under many

circumstances, for example when comparing effects of manipulations between fish subjected to different treatments that are expected to affect all muscle tissue, the inaccuracy may be immaterial. However, if maximal accuracy is required for comparison to fibre or nuclear numbers counted solely from myotome 17, for example, the 'slice' methods can be improved. This can be achieved either by using the arduous Full Stack method, by mathematical correction by multiplying the measured 4-slice volumes by 0.98, or by moving the location of the *YZ* CSA scans posteriorly to reflect more accurately the true CSA of myotome 17.

A second limitation of the method is its sensitivity to the mounting orientation of the fish. In practice, skilled operators can orient fish within reasonable limits most of the time, even when working quickly to embed many samples. Modifications to equipment on the microscope stage can be envisaged that would allow correction of yaw and roll prior to scanning. Without such apparatus, a method to quantify misorientation that can then be used to correct the measured volumes is described. Moreover, even if misorientation increases variability in measured volume, and thus reduces the chance of observing small effect sizes, in many situations such variation will affect control and experimental samples similarly. So false positive results are unlikely, if operators are aware of the issue.

The described methods have initially been applied to analyse the role of electrical activity in muscle growth, a subject with a long history of analysis in a wide range of species (reviewed in [18]). To this end, simple methods to block endogenously-triggered activity are described in detail and replaced with controlled patterned electrical stimulation in the zebrafish larva. Advantages of this approach are the removal of neural feedback controls [40], the elimination of the effects of altered nutrition and the ability to analyse circadian effects on growth itself, rather than on growth proxies, such as protein turnover [26]. As different patterns of electrical activity, trigger distinct muscle responses, regulating fibre type, size and metabolism [41-48], the current methods open the zebrafish to such analyses.

The methods described offer a suite of techniques with which many aspects of muscle physiology, cell biology and pathology can be analysed in unprecedented temporal and spatial resolution by taking advantage of the relatively unexplored zebrafish. The current approaches could clearly be applied to other species, regions of the body, and developmental stages. The rapid early growth of zebrafish larvae makes detection of acute effects of manipulations on tissue growth and morphogenesis particularly attractive areas of study. Moreover, zebrafish muscle is shown to share various growth mechanisms and controls with mammals. While zebrafish are vertebrates that conserve many aspects of muscle molecular genetics, cell and developmental biology with humans, there are also significant differences in the control of muscle growth. For example, slow and fast fibre types are more clearly spatially segregated in fish and the innervation of muscle shows differences. Furthermore, it must be borne in mind that, so far, we have only been able to analyse the early stages of development. Similar analyses at later stages are envisaged.

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DISCLOSURES:

The authors declare no competing interests.

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826

Figure 1

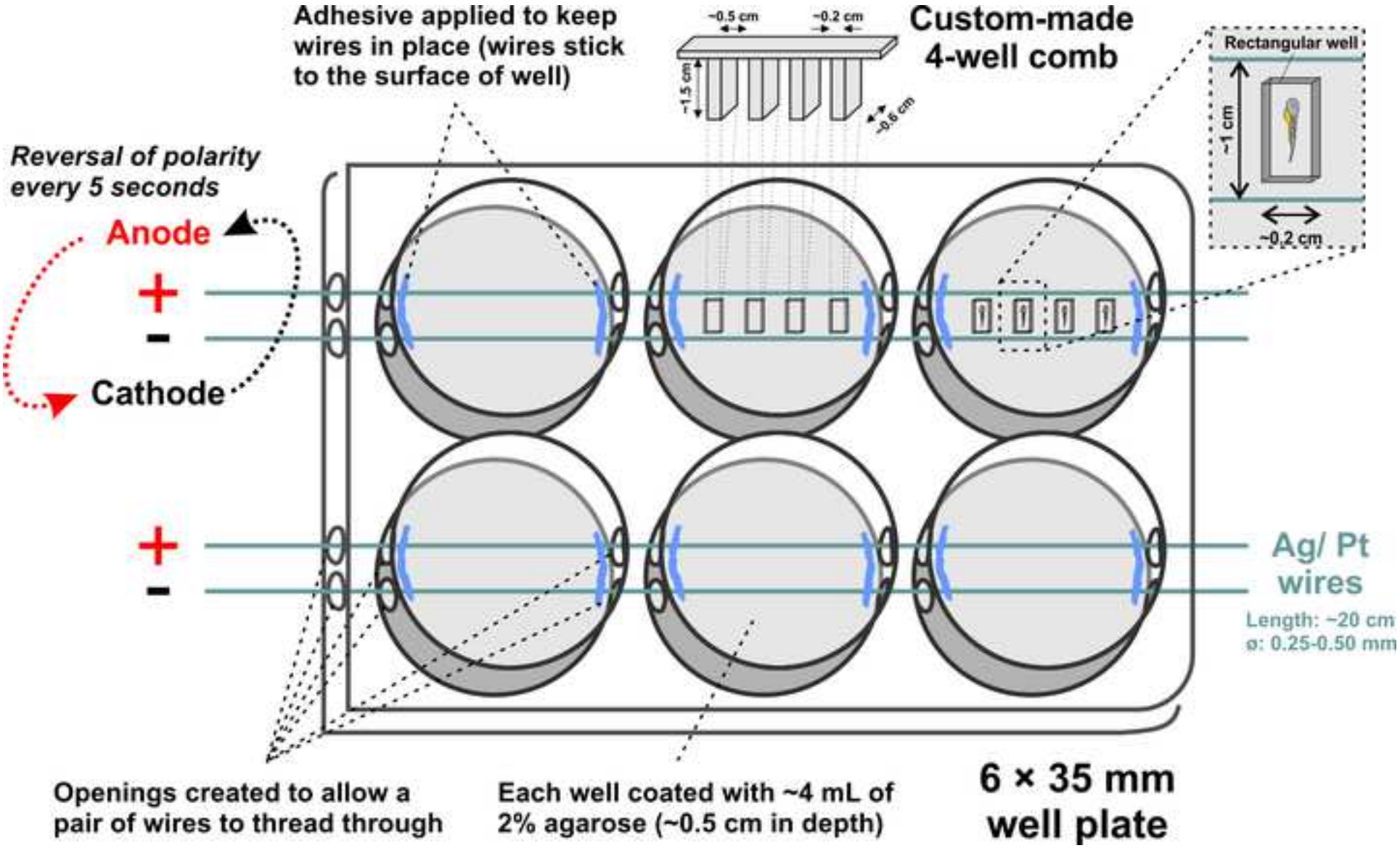


Figure 2

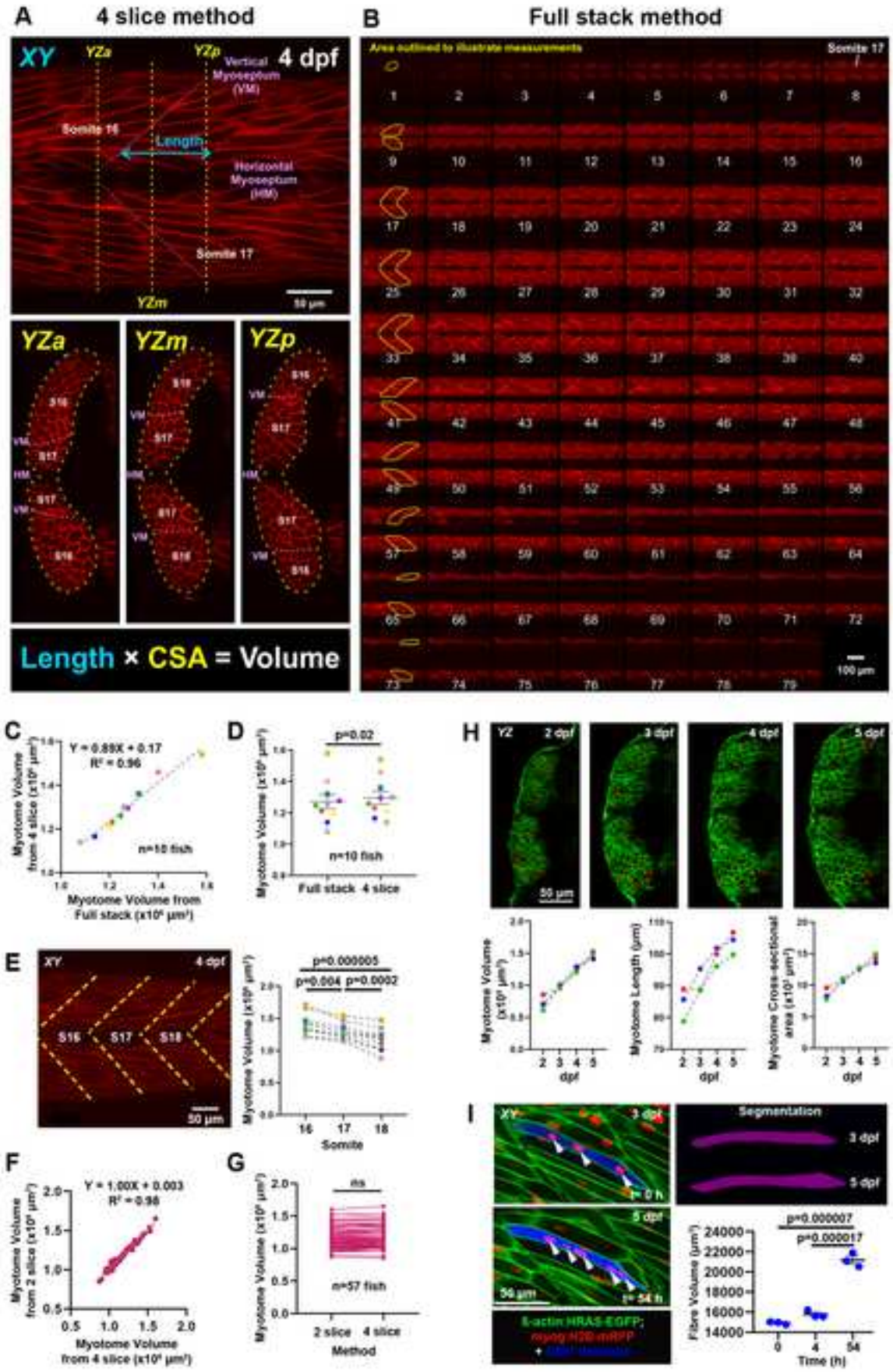


Figure 3

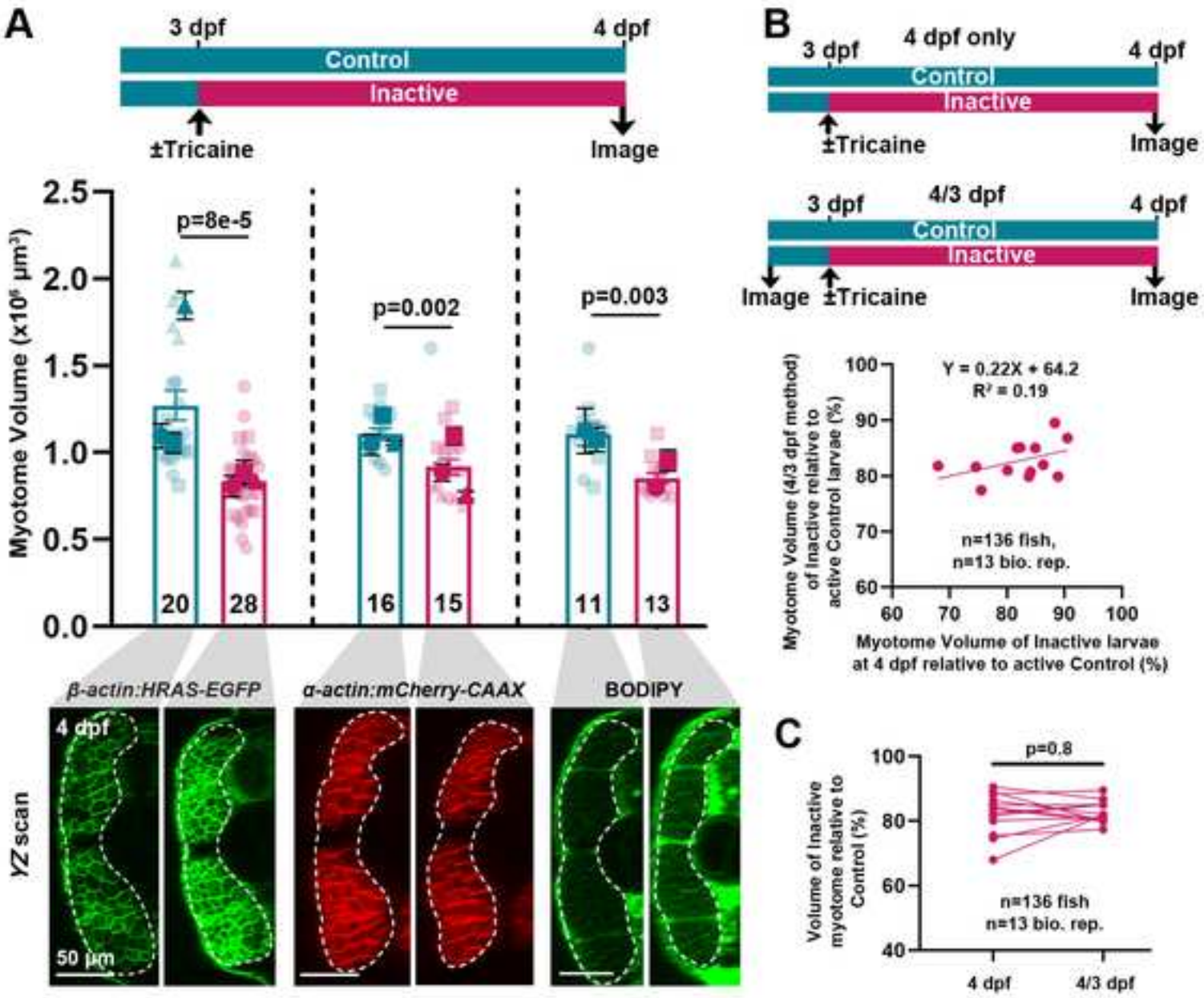


Figure 4

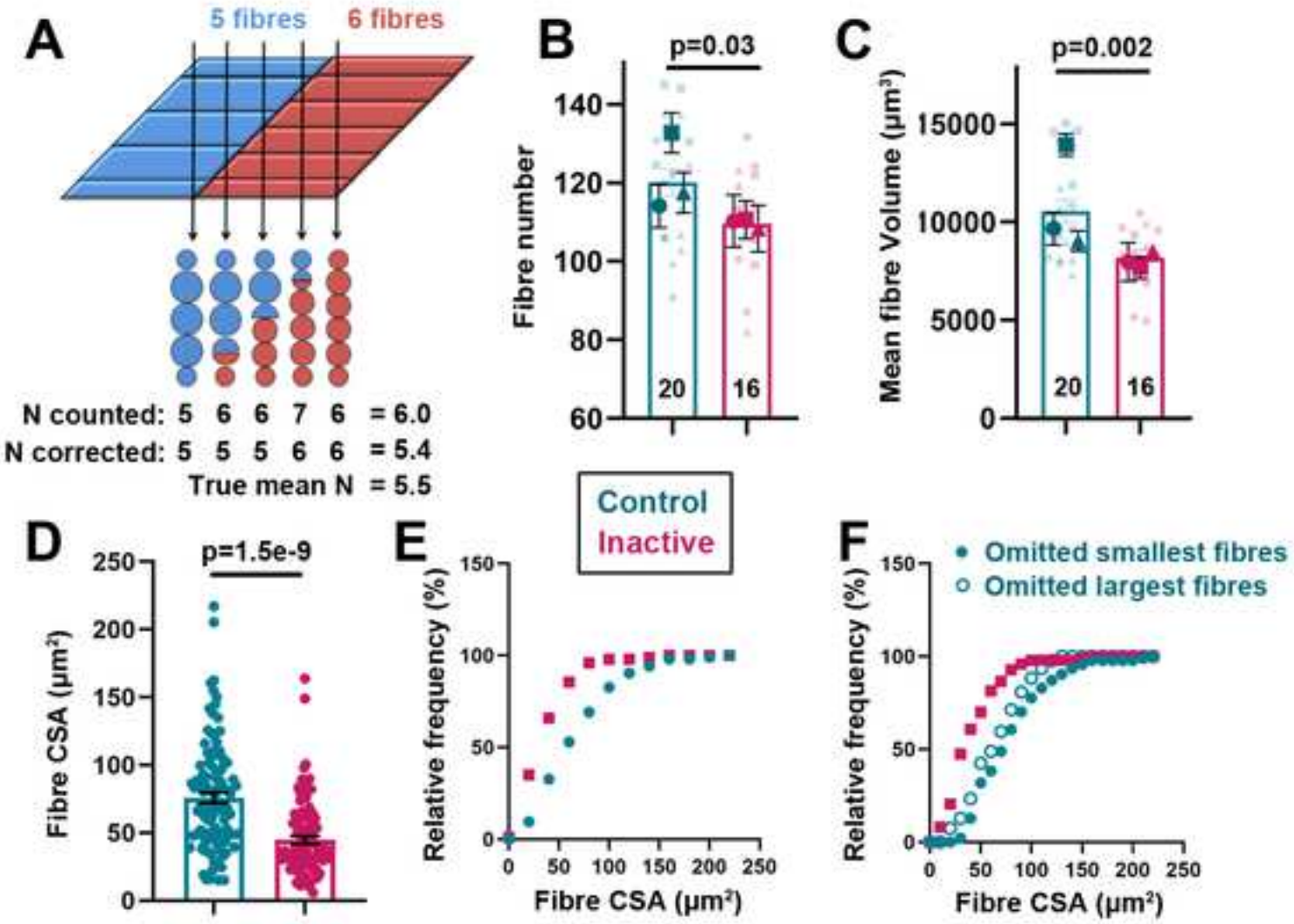
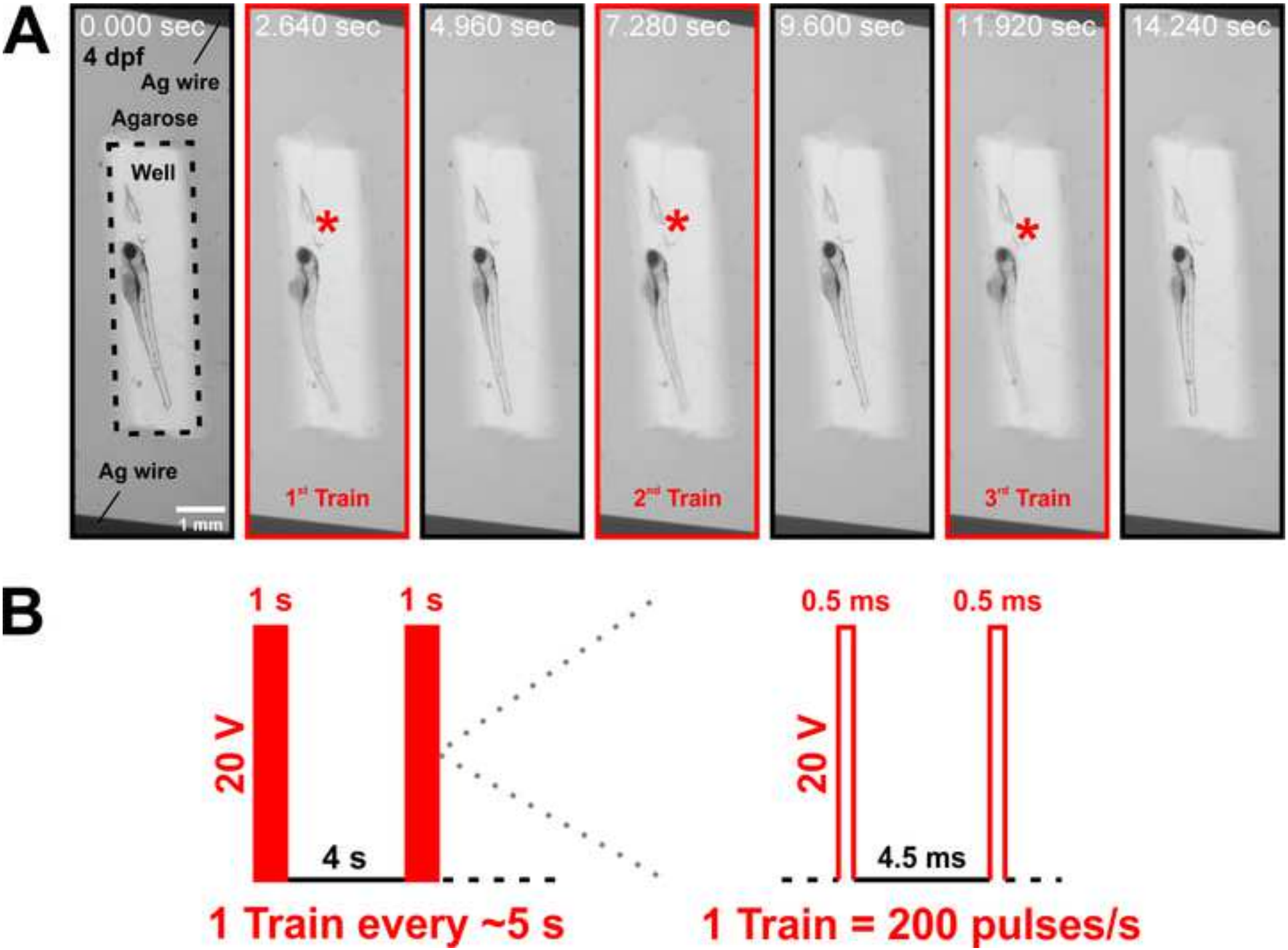


Figure 5



Dear JoVE

Thanks for the extensive Reviewer comments which have helped to improve significantly our presentation. We have removed some data (Figs 5 and 6), as suggested by Reviewer 3, but have retained our methodology for imposing both loss and gain of function of electrical activity on zebrafish larvae as we believe this is a powerful method for understanding neuromuscular growth control.

Thanks for your consideration of our revised manuscript.

Best wishes, Simon

Reviewer #1:
Manuscript Summary:
The authors describe and compare several approaches to zebrafish larval muscle measurement using confocal imaging. Technical details of image acquisition and analysis are given, along with instructions how to avoid potential caveats. The authors further expand on the subject by including an experiment with inactive and stimulated larvae, as a demonstration of the protocol efficacy. The manuscript could be of potential use to researchers coming into the field of zebrafish muscle biology. The manuscript is well written, although some parts could be revised to improve readability (particullarly the several longer sentences in the introduction). However, certain issues need clarification before publishing.

Major Concerns:
The title is not entirely appropriate. Altough the authors did perform live imaging, the experiments did not actually require "real time" readout, as imaging on several timepoints was sufficient. Furthermore, in order to observe muscle growth in real time, which was not the case here, more challendges would have to be addressed (heating bed/chamber, effect of imobilisation/anestesia on muscle development etc.).

The Reviewer makes a good point. We have now added two kinds of data (in part in response to Reviewer 3) showing a) repeated measures over many days in the same fish (Fig. 2H) and growth of a single fibre over 4 hours in a continuous timelapse session (Fig. 2I). The Reviewer is correct that use of anaesthetic during embedding will slow growth (as we showed in our Kelu et al 2020 PNAS paper) but in the current analysis, after brief anaesthesia during embedding, one can then remove the tricaine and keep the fish embedded. 'Real time' for growth does not need to be on the second timescale, but more like the hour. This is achievable, as we show.

Line 223 - 224: Differences in estimates mentioned here can also be attributed to manual drawing of muscle borders. A more reproducible results can be obtained by combining filters (such as median) and segmentation algorithms to acquire surface area. This would work well with muscle cell-specific reporter lines, but could be problematic if the reporter is more widely expressed. Authors should reflect on this issue.

Good point. We have now included discussion of this issue at ln 642-650.

Line 229-231: In case of yaw (rotation around D-V axis), myotome lenght can only be under-estimated, as its projection on the xy plane (in which you measure) would be shorter.

This was also our initial thinking, but it is not correct. When one takes an *XY* plane photo of a fish embedded with yaw, one is taking a photo of a real plane at an angle through the myotome. One is not observing a projection of the myosepta from the 'desired' perfectly aligned plane (a parasagittal section) onto the *XY* plane of the microscope. Thus, the distance between the two myosepta can only be elongated (in exactly the same way as, in the *YZ* plane, the notochord becomes elongated in the direction of the major axis but retains its true diameter in the orthogonal minor axis, a phenomenon many people find easier to visualize). No line between two myosepta can ever be shorter than a line normal to the plane of those myosepta, and thus

a correction to correct L for its artificial elongation is required, as we describe.

Lines 238-244: Would it be more appropriate to call it "Corrected/Adjusted" CSA rather than "True"? This part would be much clearer if accompanied by an additional illustration.

Good idea. We now include such an illustration (new Supplemental File 1) and further explanation, as the rationale is not always obvious, as illustrated by the confusion on the previous point.

Minor Concerns:
Line 121: Authors should emphasise they are using an upright microscope. Working with an inverted setup would require a slightly different approach.

This is true. We now mention it at ln 149-155.

Line 128: Authors should describe this step in more details. Which tools to use? Micropipette, Pasteur pipette? Should care be taken not to dilute the agarose further in the process...This is an important and delicate step. Inexperienced experimenters might encounter difficulties here.

We have done as suggested.

Lines 386-392: Authors discuss estimating fibre number by using YZ sections parallel to dorsoventral axis. What about using sections in YZ plane parallel to the VM? This way only fibres from myotome 17 would be counted. If authors have a Z-stack of myotome 17, they could use the "reslice" option in ImageJ/Fiji (use line tool to define the plane angle) to obtain these kind of "optical sections" and count the cells. If a membrane marker is used, one should be able to count individual cells despite the fact that the cross-sections appear slightly skewed. This would have to be done separately for dorsal and ventral muscle mass, sacrificing speed. But it would be interesting to compare fibre number obtained in this way to the authors estimate.

These things are all true. It seems we failed to make clear that a big advantage of our method is that many individual fish can be analysed in a single experiment. It is precisely to avoid having to capture entire stacks (which slows both imaging and analysis markedly unless one has access to some unusually fancy kit) that we developed our methods, which can be employed on even a basic LSM confocal.

Figures 5 and 6: Yellow colour on white background (and vice versa) is hard to read due to lack of contrast. I suggest using different colour here.

We have now deleted these Figures.

Reviewer #2:
Manuscript Summary:
The title and abstract are appropriate and should attract interest of researchers using zebrafish to model muscular dystrophies or mechanisms of skeletal muscle development. It could possibly be of used to also investigate interactions of muscle with nervous system where motor neuron innervation might be visualised and assessed using this method by scientists modelling motor neuron disease, using additional transgenic lines.
The materials and equipment needed listed is complete and the protocol steps are clearly and fully explained. More detail on controls is not required as the application maybe used in conjunction with different approaches (genetic/ gene targeting/drug treatment) each would need different consideration of appropriate controls. It is good that the authors have highlighted the critical steps; this is very important when using a new protocol.
The results shown are really beautiful and the extraction of such useful data from beautiful images is great. Being able to measure and calculate differences in muscle growth will be useful to reseachers in the field of developmental biology and medical research. The range of references included is good.

Major Concerns:

None

Minor Concerns:
None

Reviewer #3:
Manuscript Summary:
The article "Real time measurement of skeletal muscle growth in live zebrafish" by Attwaters et al, describes an imaging based technique to examine myotome volume in zebrafish, and potentially examine growth stretches such as hypertrophy and hyperplasia. This topic is of great importance, and I clearly see merit in publishing this protocol in Jove. Although the technique is very simple, I think the authors have identified the various challenges faced in examining muscle growth in zebrafish, and importantly have performed detailed validation/optimisation which has addressed potential flaws of the technique.

Major Concerns:
However, I strongly feel that in its current form, the manuscript is more suited for a research article and not written appropriately for JOVE, which is focused on sharing cutting-edge experiments enabling efficient learning and replication of new research methods and technologies. The manuscript has too much scientific data which although is interesting, it is complex, difficult to read and follow, and importantly, the methodology in question seems to be diluted. For example, the authors need to consider if the electric activity protocol is really required in this study? To me, it seems like a cool protocol to stimulate the muscle activity but it does not provide a way to examine growth - which is the goal of the protocol. In line with this, I strongly feel that Figures 5 and 6 (and all related text) can be omitted as they do not necessarily demonstrate validity of the protocol. This will make the protocol and all the optimisation experiments (Figures 1-4) a lot clearer to understand and follow.

While we are gratified that the Reviewer finds our method has merit, we think that a simple demonstration of the ability to combine the growth assay with manipulations that alter growth is in the spirit of the section entitled 'Representative Results'. The data included in no way constitute a meaningful and informative study for the primary research literature. Nevertheless, we take the Reviewer's point, so we have now made three significant changes to the data presented:

1. **We have added further characterisation of the timecourse of growth in Fig. 2H and I, as requested by Reviewer 1.**
2. **We have removed the RNAseq data (Fig. 5)**
3. **We have removed the stimulated growth data (Fig. 6). However, we think retention of the detailed description of the stimulation apparatus and method is desirable in a JoVE video context. Such a full description would not be possible, or easily discoverable, in more focussed research paper/s. So we have replaced Figs 5 and 6 with a new Fig 5 showing how the stimulation of the fish triggers movement.**

Abstract needs to be rewritten to clearly describe the focus of the protocol. As it is written, it is very difficult to follow what the article is about.

We have now refocused the Abstract as suggested.

Up until the discussion section, it is not clear what the goal/aim of step 5 "Optional method: Remove and re-introduce muscle electrical activity" is. This needs to be clarified early on, perhaps when step 5 is listed. Given that it is not really a protocol for examining growth, consider removing this part from the protocol.

We prefer to retain the protocol, but we have now re-written the Abstract and also included altered electrical activity in the title to highlight that this is also described.

It is unclear why a Z stack is set up (step 3.3) but after this only snap images of says that a Z-Stack is set up, steps 3.4 and 3.5 suggest that only 2 dimensional images are captured. Is this correct, and if yes, please justify why this is the case (apart from saving time?).

We now explain more clearly that it is indeed to save time, as measuring many larvae is the key to robust statistical analysis. On a Zeiss scope (with ZEN software) it is necessary to define a stack in order to obtain a *YZ* slice image of the desired dimensions.

How exactly are the YZ slices determined to ensure consistency between each fish? With the way the protocol is currently written, it appears that the YZ slices are selected without any real measure and are quite subjective. This of course can be different for each fish, and as such, it would be better to have a more definitive way of defining each YZ slice.

Thanks for pointing out this serious omission. We are indeed careful about reproducibility in this respect, and have now explained precisely the issues and how they should be resolved in the text at ln 188-200.

As shown by the investigators, different somites can have significantly different volumes and potentially growth dynamics. As such, while I appreciate that it is not possible to examine the entire trunk of the fish, perhaps, it is better to examine at least 3 neighbouring myotomes rather than just relying a single one?

Good point. As illustrated, we have done some of this, and we now make the issue clear at ln 370-373 and in Fig. 2E.

Line 333: "We have successfully measured single larvae daily for over 5 days with this method". Was the same fish followed over a 5 day period? If yes, please include this data and include images to show comparison of growth of the same myotome at each day? This will significantly strengthen the paper and demonstrate the power of the approach.

We have now included daily somite volume data from three fish between 2 and 5 dpf in Fig. 2H and discussed what further extension may be possible in ln 397-409. In addition, we have added new relevant data in Fig. 2I.

Figure 3A: While the graph clearly demonstrates that inactivity reduces myotome volume, representative images of control and tricaine fish are needed to show what the myotome looks like in each case - rather than just of the control fish. The authors may choose to do this for each of the 3 strategies they have used - HRA-eGFP, mcherry-CAAX, and Bopidy.

We now include such images in Fig. 3A.

Figure 3B: It is not exactly clear what the graph represents and how these calculations have been made. Have the authors examined the same fish at 3 days, before addition of tricaine, and then again at 4 days (after approximately 24 hours of tricaine). If this is the case, please provide more explanation of what exactly was done, and what the graphs represent. The low R2 value suggests that there is weak correlation between the 4 dpf and 4/3 method but this does not support the usability of one approach over the other. The more detailed explanation should help clear this up.

Thanks for pointing the lack of clarity out. We have now expanded the description to explain why (and how) the 4/3 dpf method is demonstrated to be superior (if more time-consuming) to the 4 dpf only method in ln 434-455.

Line 409: "As inactive fish have ~10 fewer fibres than active fish (Figure 4B), we also omitted from the comparison the ten smallest fibres from active un-anaesthetized fish (on the assumption that they are the new ones)" which showed that most loss of the myotome volume is due to fibre shrinkage, rather than failure of new fibre formation." This seems like a circular argument and without more data whereby the identity and lineage of a cell is followed before and after tricaine, the conclusion that "most loss of the myotome volume is due to fibre shrinkage" is unwarranted. Given that the aim of this manuscript is to provide a detailed protocol on how to examine growth, rather than identify mechanisms of growth, I strongly feel that this statement needs to be removed. Only data pertaining to the validity of the protocol needs to be included, and this data (4E and 4F) doesn't do that. It in fact, leaves the reader with more questions than answers.

We disagree with the Reviewer; it is in no way a circular argument. We think it important to alert readers to the potential pitfalls in growth analyses, and this is one of them. By including this analysis (it is not extra data *per se*) we show how to deal logically with the issue of distinguishing growth due to new fibre formation from that due to fibre expansion. We omitted the smallest fibres in our calculation because this is likely to reflect reality: new fibres are small. However, if we instead omit the ten largest fibres (a very unlikely scenario: that the largest fibres are the most recently-formed ones and are missing in inactive fish), we now show in the revised Fig. 4F that the reduction in fibre CSA of the residual fibres still makes a large contribution to the reduction in muscle mass.

Figure 5: It is unclear how the protocol described permits the study of molecular mechanisms. The protocol described allows the examination of myotome volume and does not reveal any molecular changes. Please delete this data and if you choose to include it, please amend the protocol appropriately.

Now deleted.

Figure 6: Again, this data seems unnecessary and seems more appropriate for a research article rather than for JOVE, which is geared to sharing protocols rather than scientific data.

Now deleted as requested, and instead illustrative data of how the stimulation protocol triggers movement is included.

Minor Concerns:

Line 26: "A further protocol is described that controls muscle contractile activity". This does not make sense

The Abstract has now been significantly altered and this sentence clarified'.

4.4.1 - Please elaborate what the authors mean by "correctly calibrated". Ie scale?

Now explained.

Line 320: "observed between both methods of calculation". Given that the 4-slice method is discussed in the next statement, it is not clear what "both" is referred to here.

Clarified.

Line 50. Change advance to advances

Done.

Please add discussion on hypertrophic and hyperplastic growth in the introduction, as the technique described clearly has the potential of addressing this.

We have now added this to the Introduction.

What is the maximum age of fish that this technique can be used on - given the obvious challenges faced in imaging larger fish? Please add this as a limitation.

Good question. Now addressed in ln 402-409. We have done this on 8 dpf larvae and anticipate it could be used much later in fish carrying pigmentation mutations, such as *roy;mtifa.*

Reviewer #4:
Manuscript Summary:
In this study the investigators developed an effective approach to analyze live fish embryonic and

larval muscle growth using confocal laser scanning microscopy to visualize muscle fibers with fluorescently marked plasma membranes. By using the approach, they demonstrated that muscle inactivity dramatically reduced muscle growth. The reduced muscle growth was largely rescued by a brief electrical stimulation. Overall, this was a well-designed and executed studies. The data are of high quality and support the conclusions. The described methods provide a powerful tool to study the effect of genetic, drug or environmental factors on a variety of cellular and physiological muscle growth parameters in living organism. A few minor comments may help clarify potential questions from future users.

Minor Concerns:
1. In Fig. 2A, it appears that slow fibers in the horizonal myoseptum were not clearly labeled by a-actin:mCherry-CAAX. Is this true? Any explanation?

This is due to the presence of pigment cells in the horizontal myoseptum in the fish shown.

2. In Fig. 6 the investigators showed that a brief electrical stimulation could rescue the growth reduction caused by 24 h of inactivity. Was this due to increased CSA or numbers of myofibers or both?

At the request of Reviewer 3, we have now removed this data from the manuscript.

3. All studies were performed on 3-4 dpf fish larvae. Could this method be used analyze myofibers in older fish larvae?

Good point. We now show a time course study in Fig 2H and discuss this issue at ln 397-409.

Reviewer #5:
Manuscript Summary:
The authors describe a live imaging protocol of skeletal muscle cells in zebrafish larvae. This detailed protocol includes procedures for sample preparation, data acquisition, and volumetric analysis. The muscle cell size can be measured very elegantly using a transgenic fluorescent report that target plasma membranes. The author thoughtfully provided methods and discussions to correct the over-counting of fiber numbers due to the presence of vertical myosepta and obliquity within the myotome. One potential problem of live-imaging muscle growth is that inactivity will affect the growth. They demonstrated that an electrical stimulation can maintain the growth of skeletal muscle. The manuscript is very well-written and the data are of high quality. Due to some of the caveats, the authors have to make a few assumptions in their measurement. Nonetheless, the manuscript has demonstrated a feasible protocol of live imaging of zebrafish skeletal muscle cells in larvae.

Major Concerns:
NA

Minor Concerns:
1. Please clarify the concentration of the agarose. It is 1% in the text and 2% in Figure 1.

Thanks for noticing this. We have now clarified, in Fig. 1 the 2% agarose is used to make water-filled chambers to maintain the orientation of the larvae in the stimulation electric field, not for embedding. In the text, by contrast, we describe embedding in 1% low melt agarose for confocal scanning.

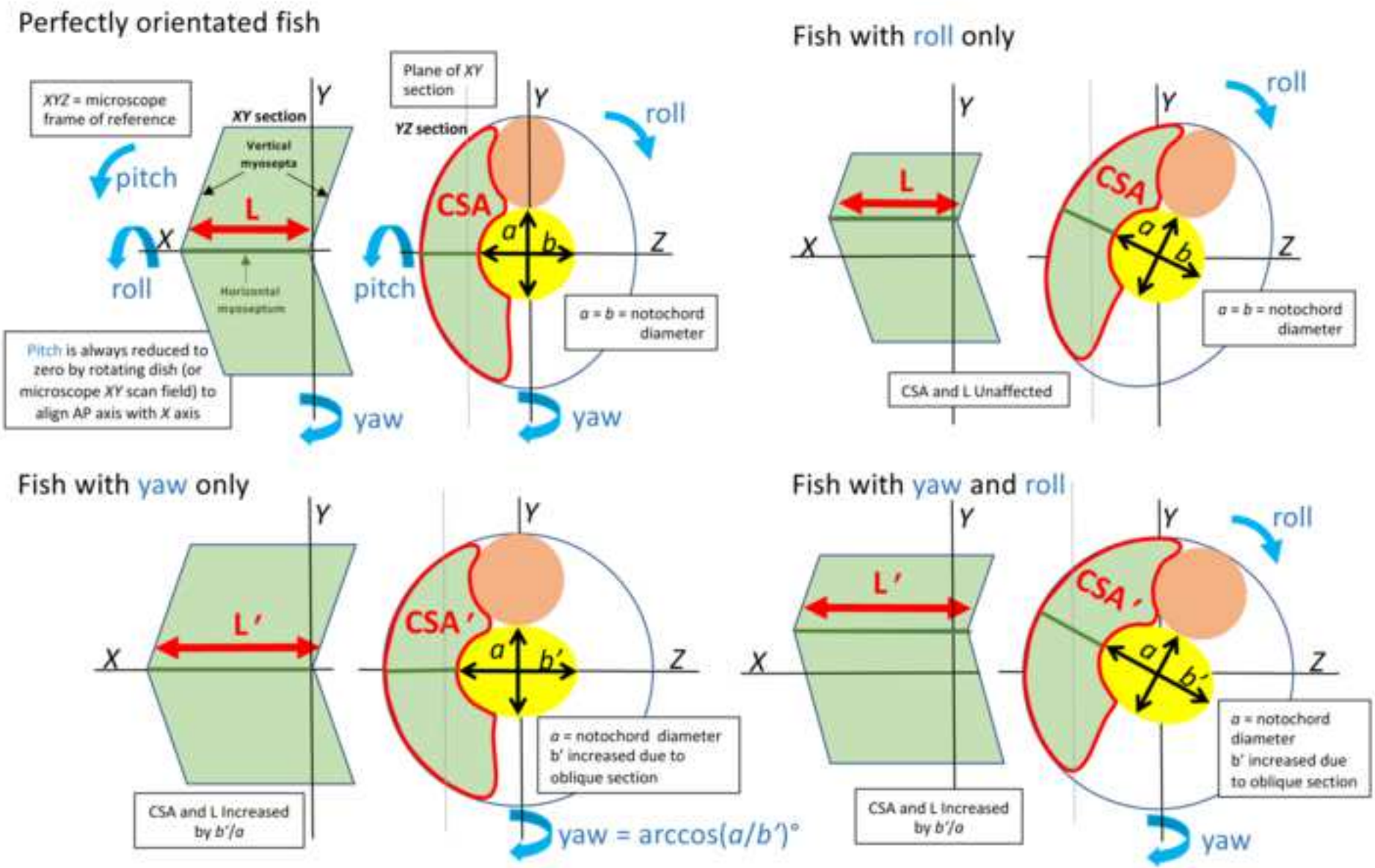
2. The authors mention that this protocol is well-tolerated for zebrafish larvae. Can the fish larvae be recovered after imaging and continue to grow? The authors might comment on this since it will be very helpful if the larvae can be recovered and perhaps analyzed at later stage.

Good point. Yes. We now show a time course study in Fig 2H and discuss this issue at ln 397-409.

3. General writing suggestions- The authors should consider to keep the description of each step (for

instance 3.4, 3.5, 4.2, 4.4.) succinct. This will be easier for the readers to follow the protocol. Some of the descriptions might be incorporated into representative results.

We have tried to abbreviate where possible.



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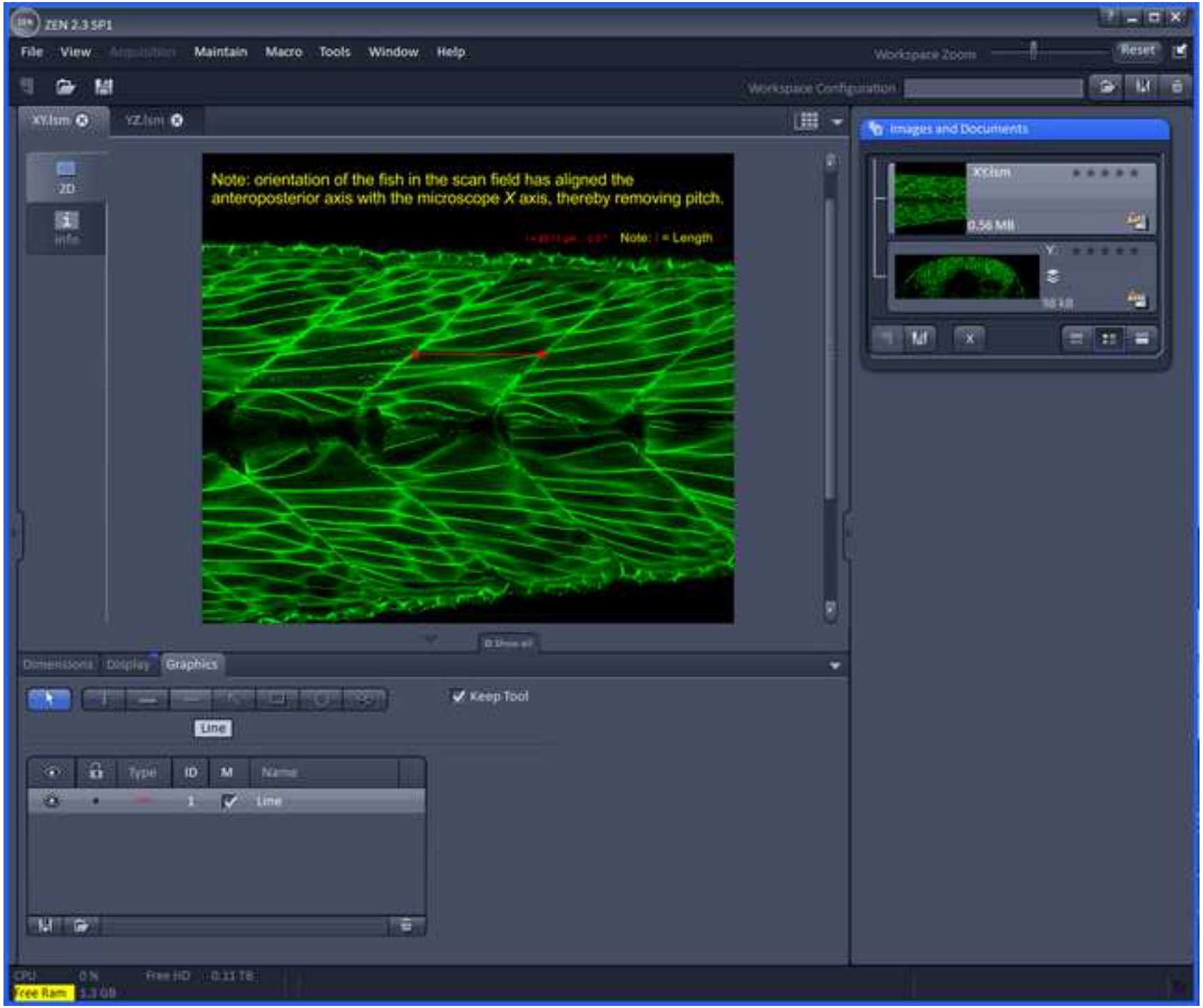

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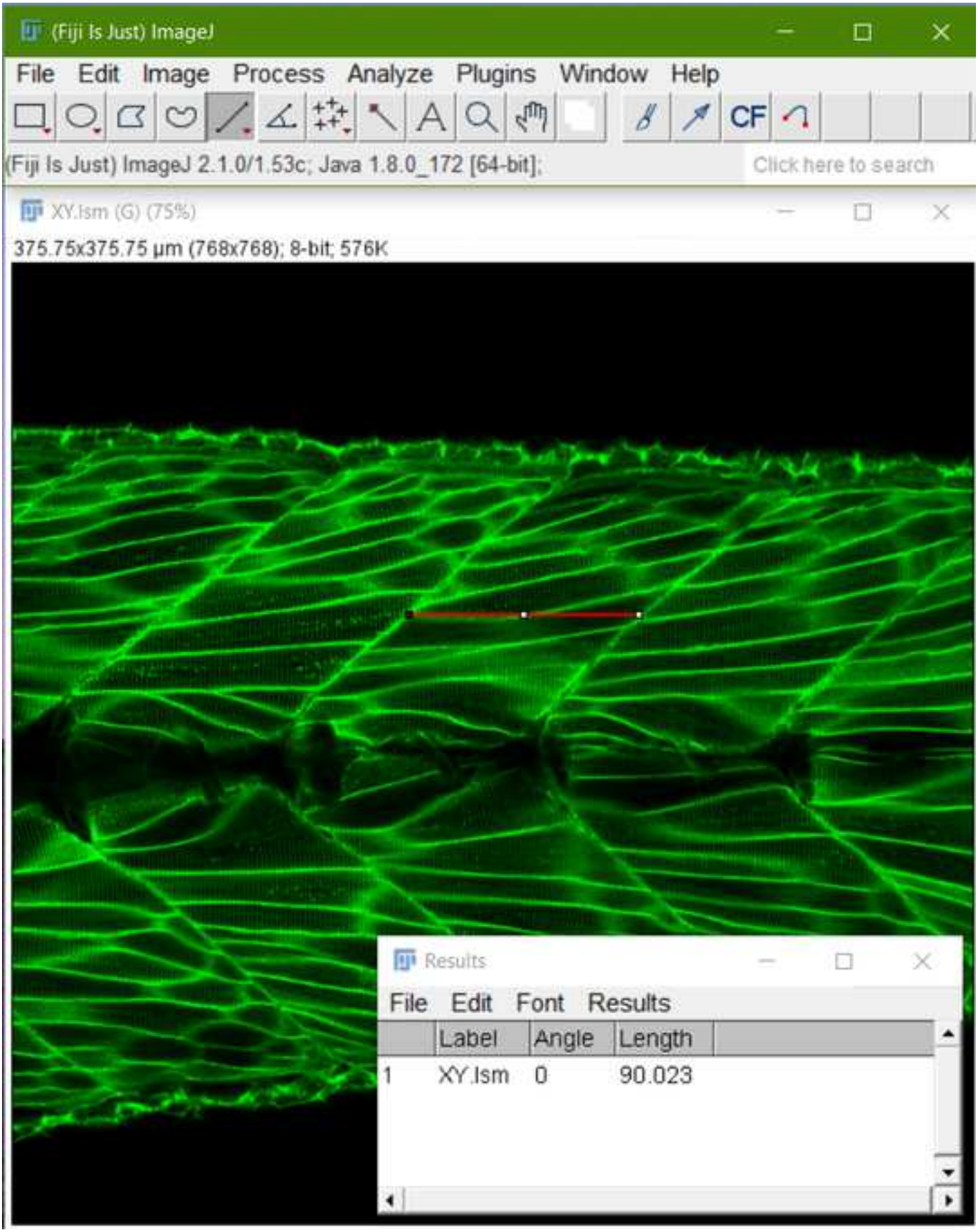

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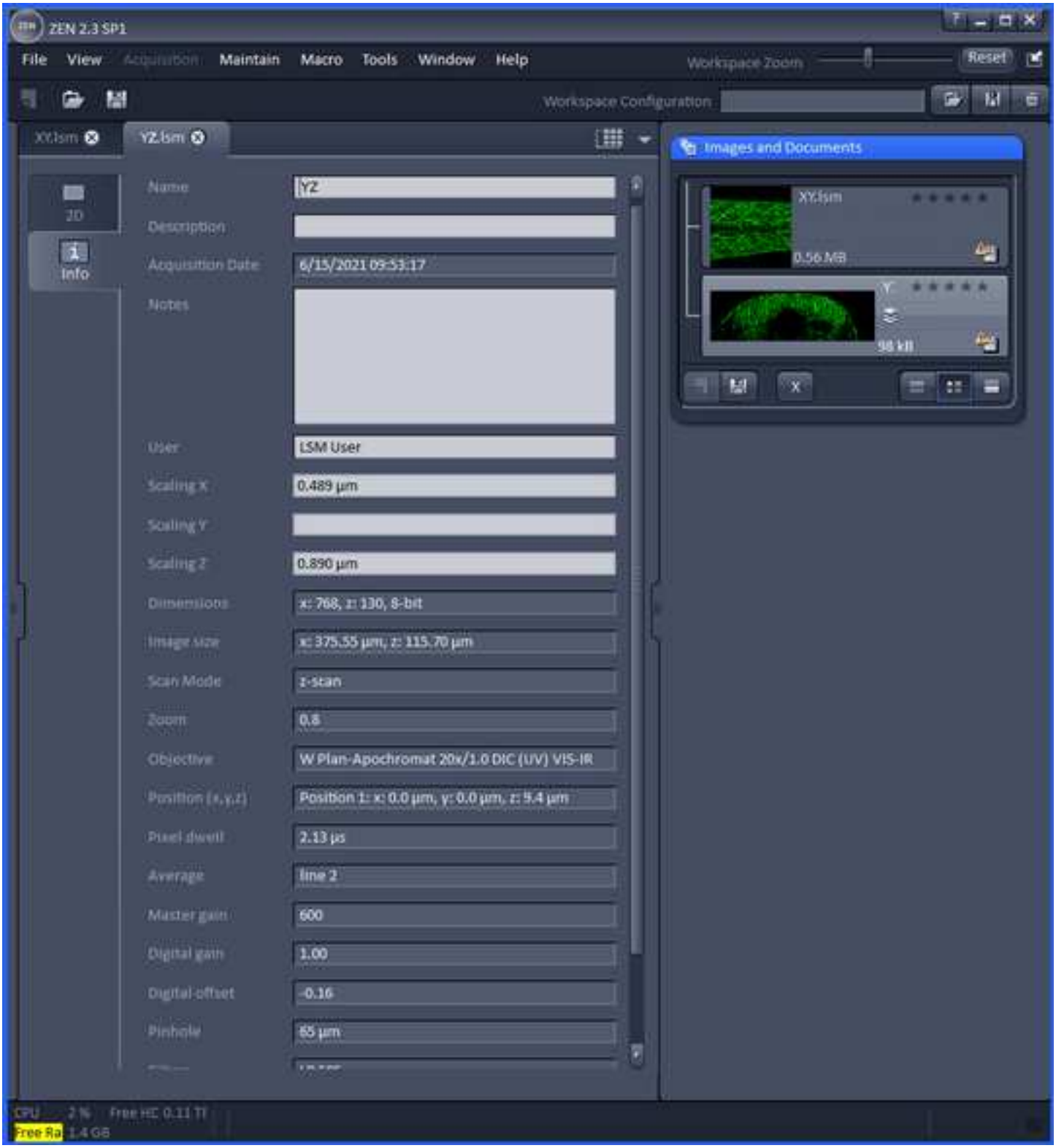

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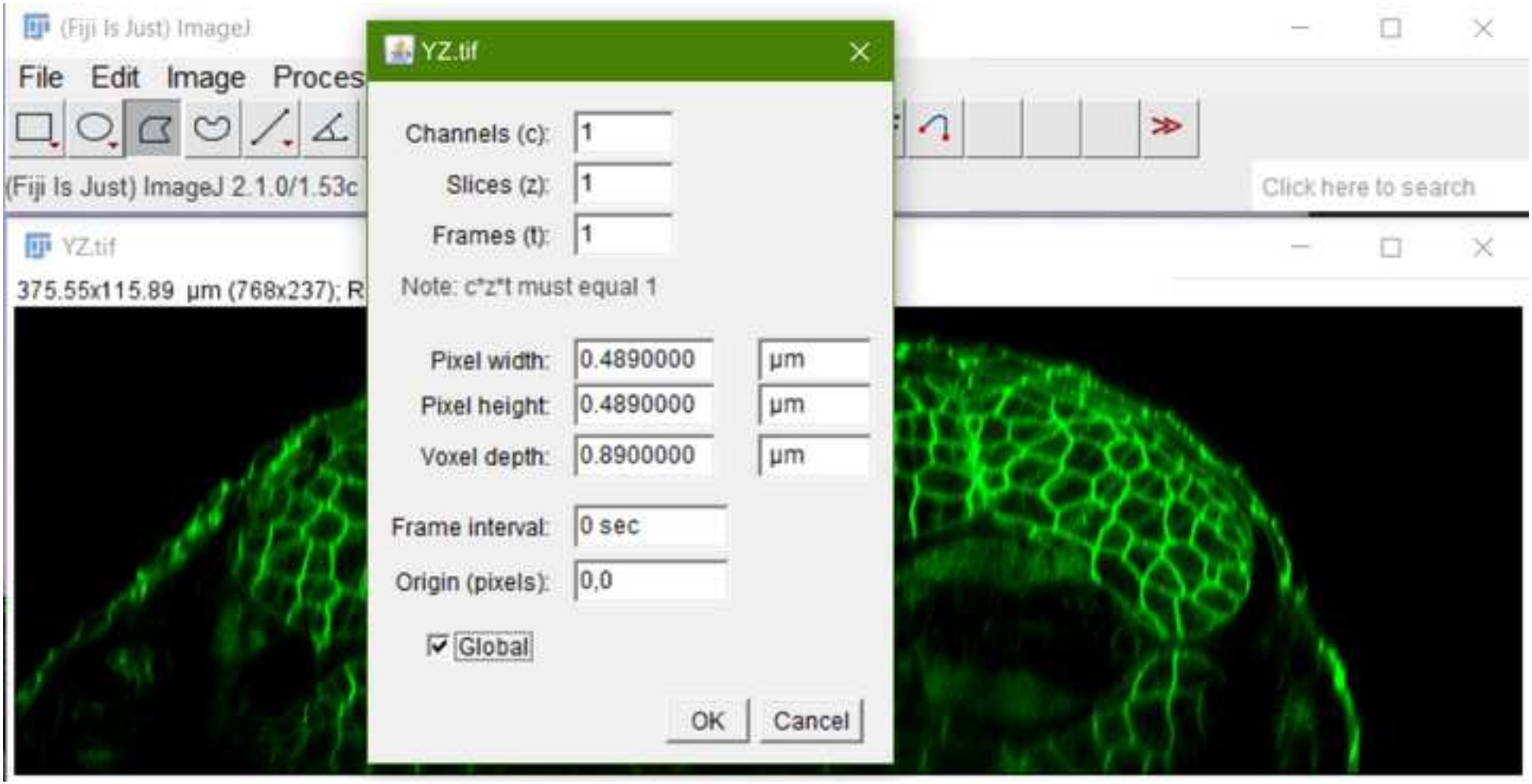

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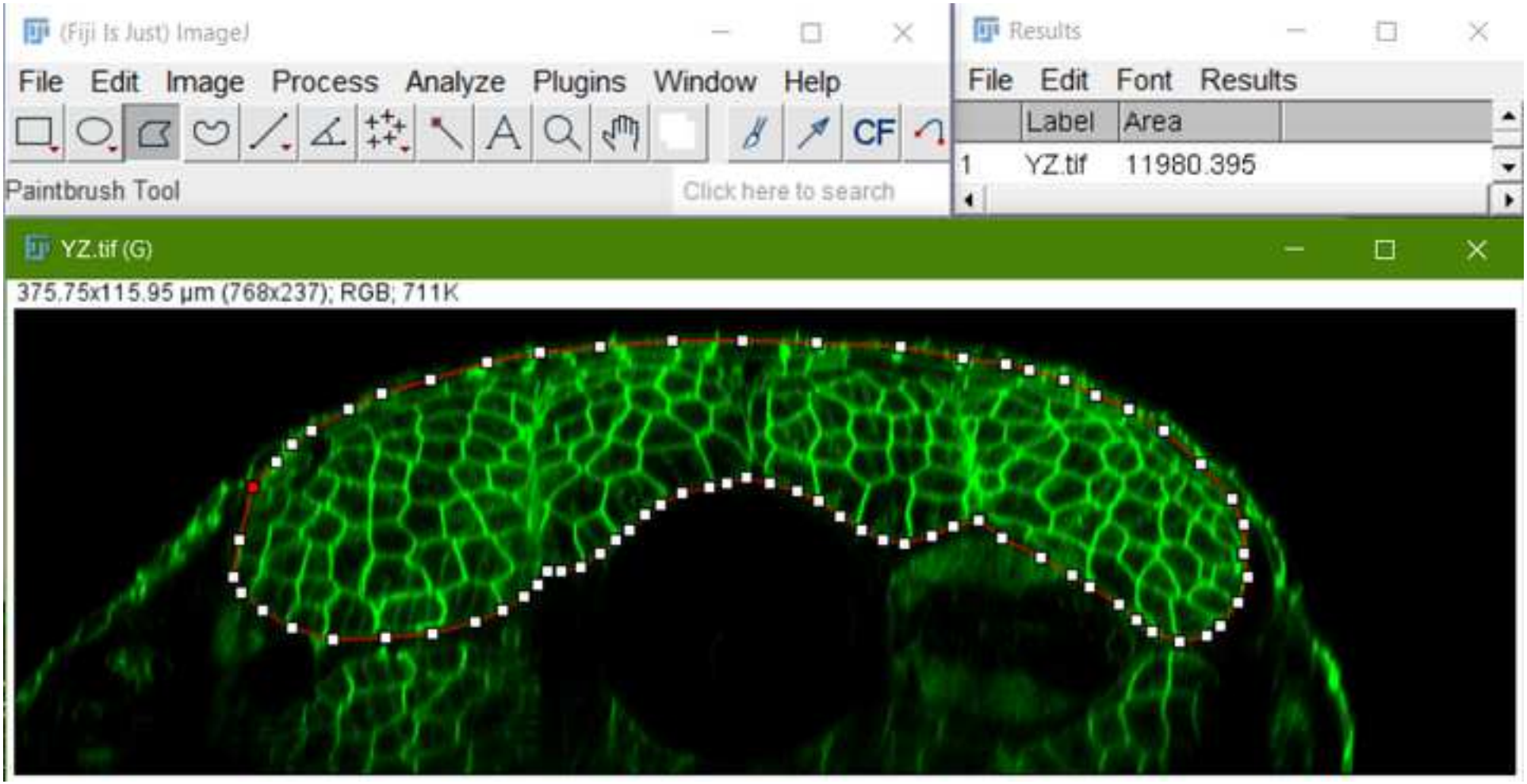

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