Journal of Visualized Experiments

Force measurement during contraction to assess muscle function in zebrafish larvae

--Manuscript Draft--

Manuscript Number:	JoVE50539R1
Full Title:	Force measurement during contraction to assess muscle function in zebrafish larvae
Article Type:	Methods Article - JoVE Produced Video
Corresponding Author:	Susan V. Brooks University of Michigan Ann Arbor, MI UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Michigan
Corresponding Author's Secondary Institution:	
First Author:	Darcée D. Sloboda
First Author Secondary Information:	
Order of Authors Secondary Information:	
Abstract:	Zebrafish larvae provide models of muscle development, muscle disease and muscle-related chemical toxicity, but related studies often lack functional measures of muscle health. In this video article, we demonstrate a method to measure force generation during contraction of zebrafish larval trunk muscle. Force measurements are accomplished by placing an anesthetized larva into a chamber filled with a salt solution. The anterior end of the larva is tied to a force transducer and the posterior end of the larva is tied to a length controller. An isometric twitch contraction is elicited by electric field stimulation and the force response is recorded for analysis. Force generation during contraction provides a measure of overall muscle health and specifically provides a measure of muscle function. Although we describe this technique for use with wild-type larvae, this method can be used with genetically modified larvae or with larvae treated with drugs or toxicants, to characterize muscle disease models and evaluate treatments, or to study muscle development, injury, or chemical toxicity.
Corresponding Author E-Mail:	svbrooks@umich.edu
Order of Authors:	Darcée D. Sloboda
	Dennis R. Claflin
	James J. Dowling
	Susan V. Brooks
Author Comments:	Please note that this manuscript has co-corresponding authors, Dr. James Dowling and me (Dr. Susan Brooks).

Susan V. Brooks
109 Zina Pitcher Place, BSRB 2029
Ann Arbor, MI 48109
January 24, 2013

Alexa Meehan
Journal of Visualized Experiments (JoVE)
17 Sellers Street
Cambridge, MA 02139

Dear Ms. Alexa Meehan,

Please find our revised manuscript entitled: “Force measurement during contraction to assess muscle function in zebrafish larvae” by Darcée D. Sloboda, Dennis R. Claflin, James J. Dowling, and Susan V. Brooks. We feel that we have addressed all of the reviewers’ thoughtful and helpful comments. The specifics of our responses and revisions are described in a separate document.

Thank you again for your consideration of our work. Please do not hesitate to contact me if you have any additional questions or concerns.

Sincerely,

Susan V. Brooks
e: svbrooks@umich.edu
p: 734-936-2147

TITLE

Force measurement during contraction to assess muscle function in zebrafish larvae

AUTHORS AND AFFILIATIONS

Darcée D. Sloboda[1], Dennis R. Claflin[1,2], James J. Dowling[3], and Susan V. Brooks[1,4]

[1]Department of Biomedical Engineering
[2]Department of Surgery, Section of Plastic Surgery
[3]Departments of Pediatrics and Neurology
[4]Department of Molecular and Integrative Physiology

AUTHOR INSTITUTIONS

Darcée D. Sloboda
109 Zina Pitcher Place, BSRB 2318
Ann Arbor, MI 48109
University of Michigan
Ann Arbor, USA
ddnelson@umich.edu

Dennis R. Claflin
109 Zina Pitcher Place, BSRB 2023
Ann Arbor, MI 48109
University of Michigan
Ann Arbor, USA
claflin@umich.edu

James J. Dowling
109 Zina Pitcher Place, BSRB 5019
Ann Arbor, MI 48109
University of Michigan
Ann Arbor, USA
jamedowl@med.umich.edu

Susan V. Brooks
109 Zina Pitcher Place, BSRB 2029
Ann Arbor, MI 48109
University of Michigan
Ann Arbor, USA
svbrooks@umich.edu

CO-CORRESPONDING AUTHORS

James J. Dowling
Phone: 734-764-7022

Fax: 734-763-7275

Susan V. Brooks
Phone: 734-936-2147
Fax: 734-615-3292

KEYWORDS

Muscle, contraction, force, zebrafish, larvae

SHORT ABSTRACT

Force measurements can be used to demonstrate changes in muscle function due to development, injury, disease, treatment or chemical toxicity. In this video, we demonstrate a method to measure force during a maximal contraction of zebrafish larval trunk muscle.

LONG ABSTRACT

Zebrafish larvae provide models of muscle development, muscle disease and muscle-related chemical toxicity, but related studies often lack functional measures of muscle health. In this video article, we demonstrate a method to measure force generation during contraction of zebrafish larval trunk muscle. Force measurements are accomplished by placing an anesthetized larva into a chamber filled with a salt solution. The anterior end of the larva is tied to a force transducer and the posterior end of the larva is tied to a length controller. An isometric twitch contraction is elicited by electric field stimulation and the force response is recorded for analysis. Force generation during contraction provides a measure of overall muscle health and specifically provides a measure of muscle function. Although we describe this technique for use with wild-type larvae, this method can be used with genetically modified larvae or with larvae treated with drugs or toxicants, to characterize muscle disease models and evaluate treatments, or to study muscle development, injury, or chemical toxicity.

INTRODUCTION

Young zebrafish (*Danio rerio*) larvae, 3-7 days post-fertilization (dpf), are increasingly recognized as a useful organism for skeletal muscle research. Young larvae are used to model human muscle disease[1-9], evaluate drugs and therapeutic strategies[10-11], study muscle injury[12], understand muscle development[13-16], and investigate muscle-related chemical toxicity[17-19]. Typical studies in these areas examine the degree to which healthy muscle is rendered abnormal by genetic manipulation or exposure to toxicants, and some studies examine the degree to which abnormal muscle responds to treatment. Critical to the success of these studies is the ability to accurately assess muscle health.

While there are a variety of methods available to assess muscle health in zebrafish larvae, few provide direct information about muscle function. Muscle health is usually evaluated by appearance, as assessed by histological staining[6,8,11],

immunostaining[9,15,16,18], light microscopy[3,13], electron microscopy[3,4,14,16], or birefringence[7,9,11], but these techniques provide morphological information only. Trunk and tail displacements and swimming speed[4,17] evaluate motor function, but these are not direct measures of muscle function since they also reflect neural input, energy metabolism, and other processes.

In contrast, measuring force generation during contraction provides a direct assessment of muscle function and represents a measure of overall muscle health. Added benefits of this approach include straightforward data analysis and quantitative results. In this video article, we provide a detailed procedure for measuring force generation by larval muscles, in the hope that more researchers will use this method to complement existing measures of muscle health in their research.

The overall goal of this method is to measure force generation during contraction of zebrafish larval trunk muscle. To accomplish this goal, a zebrafish larva is anesthetized and placed into a chamber filled with a salt solution. The anterior end of the larva is tied to a force transducer and the posterior end of the larva is tied to a length controller. Muscle activation is accomplished by electric field stimulation, and the stimulation current and the length of the larva are adjusted to produce maximum twitch force. An isometric twitch contraction is elicited and the force response is recorded for analysis.

To be clear, this technique does not measure forces generated by larval muscles during swimming. Because both ends of the larva are tied to equipment and because the larva remains anesthetized, it cannot initiate movement during testing. Furthermore, field stimulation activates all the muscle fibers at the same time to induce a bilateral contraction, which is not what naturally occurs[,20]. Therefore, rather than measuring actual forces generated during swimming, this technique determines the force generating capability of the larval muscles.

We have used this technique to demonstrate muscle weakness in a zebrafish model of nemaline myopathy[21], as well as to evaluate the effect of antioxidant treatment on muscle function in a zebrafish model of multi-minicore disease[22]. Others have used a similar technique[23] to examine the effects of an environmental pollutant on muscle function[19].

PROCEDURE

Note: all procedures involving zebrafish should be performed in accordance with relevant guidelines, regulations, and regulatory agencies. All animal use procedures shown in this article were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

1) Make suture loops

1.1) Use forceps to separate non-sterile suture (USP 10/0 monofilament nylon, 3 ply) into three strands.

1.2) Begin to tie a double overhand knot in one of the strands. Stop before tightening the knot completely to make a small (~1 mm diameter) loop instead of a knot.

1.3) Use scissors to cut excess suture from the loop tails. An example of a finished loop is shown in Figure 1.

1.4) Place the loop on the sticky side of a Post-it Note for later use. The suture loops will be used to hold the larvae in place during force testing.

1.5) Repeat steps 1.1-1.4 as necessary. Make two suture loops for each larva that will be tested.

2) Make testing solution

2.1) Make Tyrodes solution by adding 7.977 g sodium chloride, 0.373 g potassium chloride, 0.265 g calcium chloride dihydrate, 0.102 g magnesium chloride hexahydrate, 0.048 g sodium phosphate monobasic, 1.000 g sodium bicarbonate, and 0.037 g ethylenediaminetetraacetic acid disodium salt dihydrate to 1000 mL of purified water.

2.2) Stir the solution until the salts are completely dissolved. This solution can be stored for a month at 4 °C.

2.3) Add 2.1 mL of 4 mg/mL tricaine, prepared according to The Zebrafish Book[24], to 47.9 mL Tyrodes solution and mix. Protect this solution from light by storing it in a dark glass bottle or in a glass bottle covered with aluminum foil. This solution should be stored at room temperature and made fresh each day.

3) Tie anesthetized larva into experimental chamber

3.1) Place the testing apparatus (Figure 2) on the stage of a stereo microscope.

3.2) Connect the force transducer and length controller cables to the testing apparatus. Turn on the force transducer. Turn on the length controller so that it remains rigid. (Note: the length controller provides the ability to stretch or shorten a muscle preparation *during* a contraction. However, this feature of the length controller is not used in the method described herein. Therefore, the length controller can be thought of as a rigid attachment point mounted to an XYZ positioning system.)

3.3) With a disposable transfer pipette, fill the experimental chamber with testing solution.

3.4) Use forceps to pick up a suture loop by one of the tails and hang it on the force transducer tube. Hang a second suture loop on the tube attached to the length controller. (Note: gripping a suture loop on the curved part can kink the suture and cause it to break during subsequent steps.)

3.5) With a disposable transfer pipette, transfer a zebrafish larva to a small petri dish filled with testing solution. Wait for the anesthetic in the testing solution (tricaine) to take effect (~1 minute). With a forceps, gently nudge the tail and verify that the larva is anesthetized by a lack of touch-evoked swimming.

3.6) Use a glass pipette to transfer the larva to the experimental chamber.

3.7) By gently nudging the larva with a closed forceps, position the larva so its trunk is parallel to the force transducer tube. Guide the anterior portion of the larva through the suture loop on the tube. Grasp both suture loop tails with forceps and pull them simultaneously to tighten the suture loop posterior to the yolk sac or swimbladder (Figure 3A).

3.8) With a forceps, hold one suture loop tail and pull, causing the larva to swivel 90° around the tube until the lateral side of the larva faces up (Figure 3B). If the loop was tightened enough, there will be some resistance to the pull; the larva should not swivel easily. If the loop was tightened too much, the larva will not swivel around the tube.

3.9) Using the XYZ positioning device attached to the length controller, move the length controller tube along the X-axis (axis definitions in Figure 2A) and under the trunk and tail of the larva. Leave space between the ends of the length controller tube and the force transducer tube.

3.10) Guide the suture loop over the tail of the larva and tighten the suture loop as previously described (Figure 3C). You may need to swivel the posterior part of the larva so that the lateral side faces up. Trim the suture loop tails (Figure 3D).

4) Position larva in experimental chamber

4.1) Move the larva to an appropriate distance from the chamber bottom to ensure the larva will be within “working distance” of an inverted microscope objective during subsequent steps. To accomplish this, use the XYZ positioning devices to slowly lower the tubes (with attached larva) along the Z-axis until the tubes just touch the bottom of the chamber. Then, raise the tubes until the larva is an appropriate distance from the chamber bottom (~100 µm).

4.2) Using the XYZ positioning device attached to the length controller, adjust the length controller tube along the Y-axis to align the long axis of the larva with the long axis of the force transducer tube.

5) Record force during a maximal twitch contraction

5.1) Move the testing apparatus to the stage of an inverted microscope.

5.2) Adjust the chamber temperature to a desired value. To begin, connect the water bath circulator, thermometer, and temperature controller to the testing apparatus. Turn on the necessary components and adjust the setting on the temperature controller until the thermometer reports the desired value. Data included in this article were collected at 25 °C, but measurements may also be made at room temperature or at 28.5 °C.

5.3) Connect cables from the stimulator to the testing apparatus. Turn on the power to the stimulator but do not stimulate the larva until step 5.6.

5.4) Make sure the larva is parallel to the chamber bottom. Through a 40X objective, view the portion of the larva between the ends of the tubes. If parallel to the bottom, both ends of the larva will be in focus. If needed, adjust the force transducer tube along the Z-axis until both ends are in focus.

5.5) Verify that the larva length is shorter than optimal. Turn on the video sarcomere length system and rotate the video camera such that the striations are parallel to the sides of the video frame. This system monitors striation spacing by analyzing variations in pixel intensity along each horizontal row of pixels within a user-defined region of interest (ROI). The results for all rows within the ROI are averaged and reported with a frequency equivalent to the video frame-rate (≥80 s^{-1}). The striation spacing is used as an indicator of sarcomere length.

5.6) Adjust the microscope to focus on peripheral fibers and note the indicated sarcomere length. If necessary, use the XYZ positioning device attached to the length controller to adjust length of the larva (X-axis) until the sarcomere length is less than optimal (e.g. 1.90 µm).

5.7) Adjust the stimulation current to optimize twitch force. To begin, set the output current on the stimulator to a low magnitude (e.g. 100 mA). The stimulator can be triggered manually or by a computer running a custom LabVIEW program. Elicit a twitch of the larval muscleswith a current pulse of 0.2 ms in duration.

5.8) Use an oscilloscope to record the force output and measure the peak twitch force using the oscilloscope's cursors. Increase the current by 50 mA increments and measure the peak twitch force at each current level. Wait 30 s between twitches to prevent fatigue. As stimulation current increases, peak twitch force typically increases to a maximum and then gradually decreases. The current at which the larva generates the greatest force is the optimal stimulation current. Set the current amplitude to the optimal stimulation current.

5.9) Using the XYZ positioning device, adjust the length of the larva (and thus, sarcomere length) in order to elicit maximum twitch force. Wild-type zebrafish larvae (3-7 dpf) generate maximum twitch force at sarcomere lengths of 2.10 µm or 2.15 µm. However, the sarcomere length can be set to 2.08 µm to avoid excess strain on the larva.

5.10) Elicit a twitch of the larval muscles. Use the oscilloscope to record the force response and save the record for subsequent analysis.

6) Measure musculature dimensions with larva at optimal length

6.1) Move the testing apparatus back to the stereo microscope.

6.2) Using the eyepiece scale, measure the height of the musculature as viewed from the side. Then, taking care not to change the length of the larva, swivel the larva by 90° using the suture loop tails in order to view the larva from the bottom. Measure the width of the musculature as viewed from the bottom. Take the measurements at an anatomical landmark (e.g. urogenital opening) (Figure 4).

6.3) Cut the suture loops with a microblade to release the larva from the testing equipment.

REPRESENTATIVE RESULTS

In healthy wild-type zebrafish larvae, the muscle fibers should be parallel to one another without large gaps between them and have evident striations (Figure 5A). Wild-type zebrafish larvae that do not exhibit these features, or with evident damage such as detached fibers (Figure 5B), should be discarded.

A representative plot of peak twitch force versus stimulation current for a single zebrafish larva is shown in Figure 6. For wild-type zebrafish larvae between 3-7 dpf, the optimal stimulation current is typically between 400-600 mA, with 3 dpf larvae generally requiring greater stimulation current than 6-7 dpf larvae.

The raw force data (collected during step 5.8) has to be processed and analyzed with data analysis software. First, the baseline of the force record is set to zero. Second, the voltage output of the force transducer is converted to force (mN) (see manufacturer's instructions to generate a calibration curve for the force transducer). A representative force response collected during a maximal twitch contraction of a single larva is shown in Figure 7. Data analysis software can be used to measure peak force and other features of the force response.

A representative set of peak force data from maximal twitch contractions is shown in Figure 8A. Typical peak twitch force values for wild-type 3-7 dpf larvae range from 0.9 to 1.7 mN, with older larvae generating more force than younger larvae. Differences in

peak twitch force can be due to normal processes like growth and development (Figure 8) or abnormal processes such as gene mutation-related pathology[21,22].

Normalization by muscle cross-sectional area (CSA) can be used to determine the degree to which differences in peak twitch force are simply due to differences in size of the musculature[21,22]. Muscle CSA can be estimated using the formula: CSA = $\pi(A/2)(B/2)$, where A is the height of the musculature as viewed from the side, B is the width of the musculature as viewed from the bottom, and an elliptical cross-section is assumed. Typical CSA values for wild-type 3-7 dpf larvae range from 0.027 to 0.034 mm^2, with 3-4 dpf larvae generally showing smaller CSA values than 5-7 dpf larvae. A representative set of normalized peak force data from maximal twitch contractions is shown in Figure 8B. Typical normalized peak twitch force values for wild-type 3-7 dpf larvae range from 34 to 51 mN/mm^2, with 4-7 dpf larvae generally showing greater values than 3 dpf larvae.

FIGURE LEGENDS

Figure 1 – Suture loop. Arrows point to the suture loop tails.

Figure 2 – (A) Testing apparatus with labeled components. (B) Close-up views of the experimental chamber. (a) Experimental chamber with transparent bottom. (b) Force transducer. (c) Length controller. (d) XYZ positioning devices. The X, Y, and Z axes are defined in the upper right corner. (e) Temperature control system utilizing thermoelectric modules. Tubing accommodates water flow for cooling of thermoelectric modules. (f) Stainless steel tube attached to force transducer. (g) Stainless steel tube attached to length controller. (h) Thermometer microprobe. (i) Platinum parallel plate electrodes, spanning the length of the chamber. Platinum plates are 2.5 mm high and 0.255 mm thick.

Figure 3 – Tying larva into experimental chamber. (A) Larva tied on at anterior end but not yet swiveled 90°. (B) Larva after swiveled 90°. (C) Larva tied on at posterior end but not yet swiveled. (D) Larva after swiveled and suture loop tails are trimmed.

Figure 4 – Measurements for cross-sectional area estimation. Musculature as viewed from the (A) side and (B) bottom. Placement of the red bars indicate the location of the urogenital opening. The length of the red bars indicate the height and width of the musculature as viewed from the side and bottom respectively..

Figure 5 – Lateral view of zebrafish larvae trunk musculature. (A) Healthy tissue. (B) Tissue with evident damage. Contractures resulting from fiber detachments are marked with asterisks.

Figure 6 – Representative plot of peak twitch force versus stimulation current. The optimal stimulation current is 500 mA.

Figure 7 – Representative force record for a single twitch contraction. This contraction was elicited with a stimulus pulse at 0 ms. The peak force is 1.56 mN.

Figure 8 – Representative force data from 3-7 dpf larvae. (A) Peak force data from maximal twitch contractions (B) Peak force data from maximal twitch contractions normalized to CSA. Older larvae (6-7 dpf) were fed Hatchfry Encapsulon Grade 0 with Spirulina (Argent Laboratories) starting on 5 dpf. Means + standard deviations are reported with N=5 in each group. Groups significantly different from 3 dpf larvae (∗) and 4 dpf larvae (#) are indicated (ANOVA, $P<0.05$). The significant increase in normalized force between 3 and 4 dpf (B) indicates an increase in the intrinsic force generating capability during this time period, whereas the increase in force between 4 and 6-7 dpf (A) is attributed to growth based on no change from 4 to 7 dpf in normalized force.

DISCUSSION

This method measures force generation during a twitch to assess muscle function in trunk muscles of zebrafish larvae. Although tetanic contractions can be elicted in zebrafish larvae (e.g. by 200 stimulation pulses per second for a duration of 0.2 seconds), the maximum tetanic force is only 10-15% greater than the maximum twitch force. Therefore, the force generated during a twitch is a reasonable measure of force generating capability. Twitches are preferred over tetanic contractions because twitches are less likely to cause ripping or slipping at the suture ties.

In order to generate meaningful data with this technique, maximum twitch force should be achieved for each larva and the variability between experimental groups should be minimized. With these goals in mind, we offer the following suggestions. First, take care when tying the larva to the force transducer and the length controller tubes. If the suture loops are tightened too much, the suture will cut through the muscle tissue. If the suture loops are not tightened enough, the force generated by the larva will not be fully transmitted to the force transducer. Both situations, but especially the latter, underestimate maximum twitch force. Second, since testing multiple experimental groups can take several hours (20-30 minutes per larva), alternate between groups because larvae will continue to develop during the testing period.

While some of the mentioned equipment is essential for measurement of maximum twitch force (e.g. force transducer, current stimulator), other items are not absolutely necessary. The video sarcomere length system is desirable but not required. As an alternative, a series of twitches can be used to find optimal length, during which the length of the larva is adjusted until maximum twitch force is achieved. A temperature control system is also not absolutely necessary. Temperature control is critical when measuring twitch kinetics, which are highly sensitive to temperature, whereas maximum twitch force is not particularly sensitive to small changes in temperature and could be measured at room temperature. Note that regardless of the temperature in the chamber during force testing, the larvae should be maintained at the optimal growth temperature of 28.5 °C[24] prior to force testing for accurate staging.

The larvae are tested in a Tyrodes solution containing tricaine. We use 0.02% (w/v) tricaine, the concentration recommended for anesthesia[24], to eliminate spontaneous contractions evoked by the nervous system and thus prevent fatigue during force testing. Tricaine also facilitates the tie-on step and reduces overall testing time. However, we observe that including tricaine in the testing solution consistently reduces the maximum twitch force by approximately 30%. A similar effect has also been observed in tadpole tail muscle, where tricaine reduced force generation after neuromuscular transmission was blocked, suggesting that tricaine has a direct effect on muscle[25]. Tricaine may reduce muscle cell excitability by reducing sodium conductance across the cell membrane, as it does in nerve cells[26]. Other options for blocking activation by motoneurons are *d*-tubocurarine and α-bungarotoxin but, unlike tricaine, these compounds are not skin-permeable and must be injected directly into the head, spinal cord, or heart[27]. Individual investigators will need to assess whether or not tricaine is desirable for their specific application. If tricaine is included in the testing solution, the concentration should be consistent between experiments and researchers should verify that the effect of the tricaine does not vary between experimental groups.

We describe this method for larvae as young as 3 dpf and as old as 7 dpf. Although muscle fibers appear to be functional as early as 17 hours post-fertilization, when spontaneous tail movements begin[27], the short length of the tail before 3 dpf hinders tying the larva to the testing equipment. We typically do not test larva after 7 dpf since many disease models do not survive much longer than this time. If testing larvae beyond 5 dpf, the larvae should be fed. We have observed that unfed larvae have smaller muscles and generate less maximum twitch force than fed larvae, likely due to the diminishing yolk sac. Thus it may be desirable to test larvae between 3-5 dpf, to avoid the additional variable of external feeding.

In summary, we describe a quantitative and reliable method for measuring force generation during a maximal twitch contraction of zebrafish larval trunk muscle. This method can be used to assess the overall health of zebrafish larval muscle and specifically provides information about muscle function. In addition to providing information about the magnitude of force generation, this technique can be used to study the kinetics of force generation or be adapted to study muscle fatigue[22]. Although we describe this technique for use with wild-type larvae, this method can be used for genetically modified larvae or for larvae treated with drugs or toxicants, to characterize muscle disease models and evaluate treatments, or to study muscle development, muscle injury, or muscle-related chemical toxicity.

ACKNOWLEDGMENTS

The authors thank Angela Busta for assistance with zebrafish husbandry. This work was supported by the National Institutes of Health (AG-020591 to S.V.B. and 1K08AR054835 to J.J.D.).

DISCLOSURES

The authors declare that they have no competing financial interests.

REAGENTS

Name	Company	Product Number	Comments
Tricaine powder	Sigma-Aldrich	A5040	
Sodium chloride	Sigma-Aldrich	S7653	
Potassium chloride	Sigma-Aldrich	P9541	
Calcium chloride dihydrate	Sigma-Aldrich	223506	
Magnesium chloride hexahydrate	Sigma-Aldrich	M2670	
Sodium phosphate monobasic	Sigma-Aldrich	S0751	
Sodium bicarbonate	Sigma-Aldrich	S6297	
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma-Aldrich	E5134	

EQUIPMENT

Name	Company	Product Number	Comments
Nonsterile-suture	Ashaway Line & Twine	S30002	USP 10/0 monofilament nylon (3 ply)
Forceps	Fine Science Tools	11251-20	Dumont #5
Spring scissors	Fine Science Tools	15000-08	Vannas

Stereo microscope	Leica Microsystems	MZ8	Illuminated with Fostec EKE ACE I light source
Force transducer	Aurora Scientific	400A	
Length controller	Aurora Scientific	318B	
XYZ positioning devices	Parker Hannifin	3936M	
Thermometer	Physitemp	BAT-12	
Disposable transfer pipette	Fisher Scientific	13-711-9AM	Cut end to widen opening and facilitate larva transfer
Petri dish	Fisher Scientific	08-757-11YZ	
Glass pipette	Fisher Scientific	13-678-8B	Cut end (and fire-polish) to widen opening and facilitate larva transfer
Inverted microscope	Carl Zeiss Microscopy	Axiovert 100	
Water bath circulator	Neslab Instruments	RTE-111	
Temperature controller	Alpha Omega Instruments	Series 800	
Stimulator	Aurora Scientific	701C	High-power, follow stimulator
Video sarcomere length system	Aurora Scientific	900B-5A	
LabVIEW software	National Instruments		
Oscilloscope	Nicolet Technologies	ACCURA 100	

Microblade	Fine Science Tools	10050-00	
Microblade holder	Fine Science Tools	10053-13	
Data analysis software (Signo)	Alameda Applied Sciences		

REFERENCES

1. Bassett, D.I., Bryson-Richardson, R.J., Daggett, D.F., Gautier, P., Keenan, D.G., Currie, P.D. Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. *Development* **130** (23), 5851-5860, doi: 10.1242/dev.00799 (2003).

2. Nixon, S. J., Wegner, J. *et al.* Zebrafish as a model for caveolin-associated muscle disease; caveolin-3 is required for myofibril organization and muscle cell patterning. *Hum Mol Genet* **14** (13), 1727–1743, doi: 10.1093/hmg/ddi179 (2005).

3. Hall, T. E., Bryson-Richardson, R. J. *et al.* The zebrafish *candyfloss* mutant implicates extracellular matrix adhesion failure in laminin α2-deficient congenital muscular dystrophy. *PNAS* **104** (17), 7092-7097, doi: 10.1073/pnas.0700942104 (2007).

4. Hirata, H., Watanabe, T. *et al.* Zebrafish *relatively relaxed* mutants have a ryanodine receptor defect, show slow swimming and provide a model of multi-minicore disease. *Development* **134**, 2771–2781, doi: 10.1242/dev.004531 (2007).

5. Dowling, J. J., Vreede, A. P. *et al.* Loss of myotubularin function results in t-tubule disorganization in zebrafish and human myotubular myopathy. *PLoS Genet* **5** (2), e1000372, doi: 10.1371/journal.pgen.1000372 (2009).

6. Berger, J., Berger, S., Hall, T. E., Lieschke, G. J. & Currie, P. D. Dystrophin-deficient zebrafish feature aspects of the Duchenne muscular dystrophy pathology. *Neuromuscul Disord* **20** (12), 826–832, doi: 10.1016/j.nmd.2010.08.004 (2010).

7. Kawahara, G., Guyon, J. R., Nakamura, Y. & Kunkel, L. M. Zebrafish models for human FKRP muscular dystrophies. *Hum Mol Genet* **19** (4), 623–633, doi: 10.1093/hmg/ddp528 (2010).

8. Wallace, L. M., Garwick, S. E. *et al. DUX4*, a candidate gene for facioscapulohumeral muscular dystrophy, causes p53-dependent myopathy in vivo. *Ann Neurol* **69**, 540-552, doi: 10.1002/ana.22275 (2011).

9. Sztal, T. E., Sonntag, C., Hall, T. E. & Currie, P. D. Epistatic dissection of laminin-receptor interactions in dystrophic zebrafish muscle. *Hum Mol Genet* doi: 10.1093/hmg/dds312 (2012).

10. Kawahara, G., Karpf, J. A., Myers, J. A., Alexander, M. S., Guyon, J. R. & Kunkel, L. M. Drug screening in a zebrafish model of Duchenne muscular dystrophy. *PNAS* **108** (13), 5331–5336, doi: 10.1073/pnas.1102116108 (2011).

11. Berger, J., Berger, S., Jacoby, A. S., Wilton, S. D. & Currie, P. D. Evaluation of exon-skipping strategies for Duchenne muscular dystrophy utilizing dystrophin-deficient zebrafish. *J Cell Mol Med* **15** (12), 2643–2651, doi: 10.1111/j.1582-4934.2011.01260.x (2011).

12. Seger, C., Hargrave, M., Wang, X., Chai, R. J., Elworthy, S. & Ingham, P. W. Analysis of Pax7 expressing myogenic cells in zebrafish muscle development, injury, and models of disease. *Dev Dyn* **240**, 2440–2451, doi: 10.1002/dvdy.22745 (2011).

13. Postel, R., Vakeel, P., Topczewski, J., Knöll, R. & Bakkers, J. Zebrafish integrin-linked kinase is required in skeletal muscles for strengthening the integrin-ECM adhesion complex. *Dev Biol* **318** (1), 92–101, doi: 10.1016/j.ydbio.2008.03.024 (2008).

14. Zoeller, J. J., McQuillan, A., Whitelock, J., Ho, S. Y. & Iozzo, R. V. A central function for perlecan in skeletal muscle and cardiovascular development. *J Cell Biol* **181** (2), 381–394, doi: 10.1083/jcb.200708022 (2008).

15. Kim, H. R. & Ingham, P. W. The extracellular matrix protein TGFBI promotes myofibril bundling and muscle fibre growth in the zebrafish embryo. *Dev Dyn* **238**, 56–65, doi: 10.1002/dvdy.21812 (2009).

16. Beqqali, A., Monshouwer-Kloots, J. *et al.* CHAP is a newly identified Z-disc protein essential for heart and skeletal muscle function. *J Cell Sci* **123** (7), 1141–1150, doi: 10.1242/jcs.063859 (2010).

17. Huang, H., Huang, C. *et al.* Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). *Aquat Toxicol* **98** (2), 139–147, doi: 10.1016/j.aquatox.2010.02.003 (2010).

18. Sylvain, N. J., Brewster, D. L. & Ali, D. W. Zebrafish embryos exposed to alcohol undergo abnormal development of motor neurons and muscle fibers. *Neurotoxicol Teratol* **32** (4), 472–480, doi: 10.1016/j.ntt.2010.03.001 (2010).

19. Chandrasekar G., Arner, A., Kitambi, S. S., Dahlman-Wright, K. & Andersson-Lendahl, M. Developmental toxicity of the environmental pollutant 4-nonylphenol in zebrafish. *Neurotoxicol Teratol* **33** (6), 752–764, doi: 10.1016/j.ntt.2011.09.009 (2011).

20. Buss, R. R. & Drapeau, P. Activation of embryonic red and white muscle fibers during fictive swimming in the developing zebrafish. *J Neurophysiol* **87** (3), 1244-1251, 10.1152/jn.00659.2001 (2002).

21. Telfer, W. R., Nelson, D. D., Waugh, T., Brooks, S. V. & Dowling, J. J. *neb*: a zebrafish model of nemaline myopathy due to *nebulin* mutation. *Dis Model Mech* **5**, 389–396, doi: 10.1242/dmm.008631 (2012).

22. Dowling, J. J., Arbogast, S. *et al.* Oxidative stress and successful antioxidant treatment in models of RYR1-related myopathy. *Brain* **135** (4), 1115–1127, doi: 10.1093/brain/aws036 (2012).

23. Dou, Y., Andersson-Lendahl, M. & Arner, A. Structure and function of skeletal muscle in zebrafish early larvae. *J Gen Physiol* **131**, 445-453, doi: 10.1085/jgp.200809982 (2008).

24. Westerfield, M. The zebrafish book: a guide for the laboratory use of zebrafish (Danio rerio). 4th ed., Univ. of Oregon Press, Eugene, Oregon (2000).

25. Herr, V. D., Sonnenburg, D. C., Courogen, P. M., Fiamengo, S. A. & Downes, H. Muscle weakness during tricaine anesthesia. *Comp Biochem Physiol Part C* **110** (3), 289–296, doi: 10.1016/0742-8413(94)00102-G (1995).

26. Frazier, D. T. & Narahashi, T. Tricaine (MS-222): effects of ionic conductances of squid axon membranes. *Eur J Pharmacol* **33** (2), 313–317, doi: 10.1016/0014-2999(75)90175-2 (1975).

27. Saint-Amant, L. & Drapeau, P. Time course of the development of motor behaviors in the zebrafish embryo. *J Neurobiol* **37** (4), 622-632, doi: 10.1002/(SICI)1097-4695(199812)37:4<622::AID-NEU10>3.0.CO;2-S (1998).

*Figure 1
Click here to download high resolution image

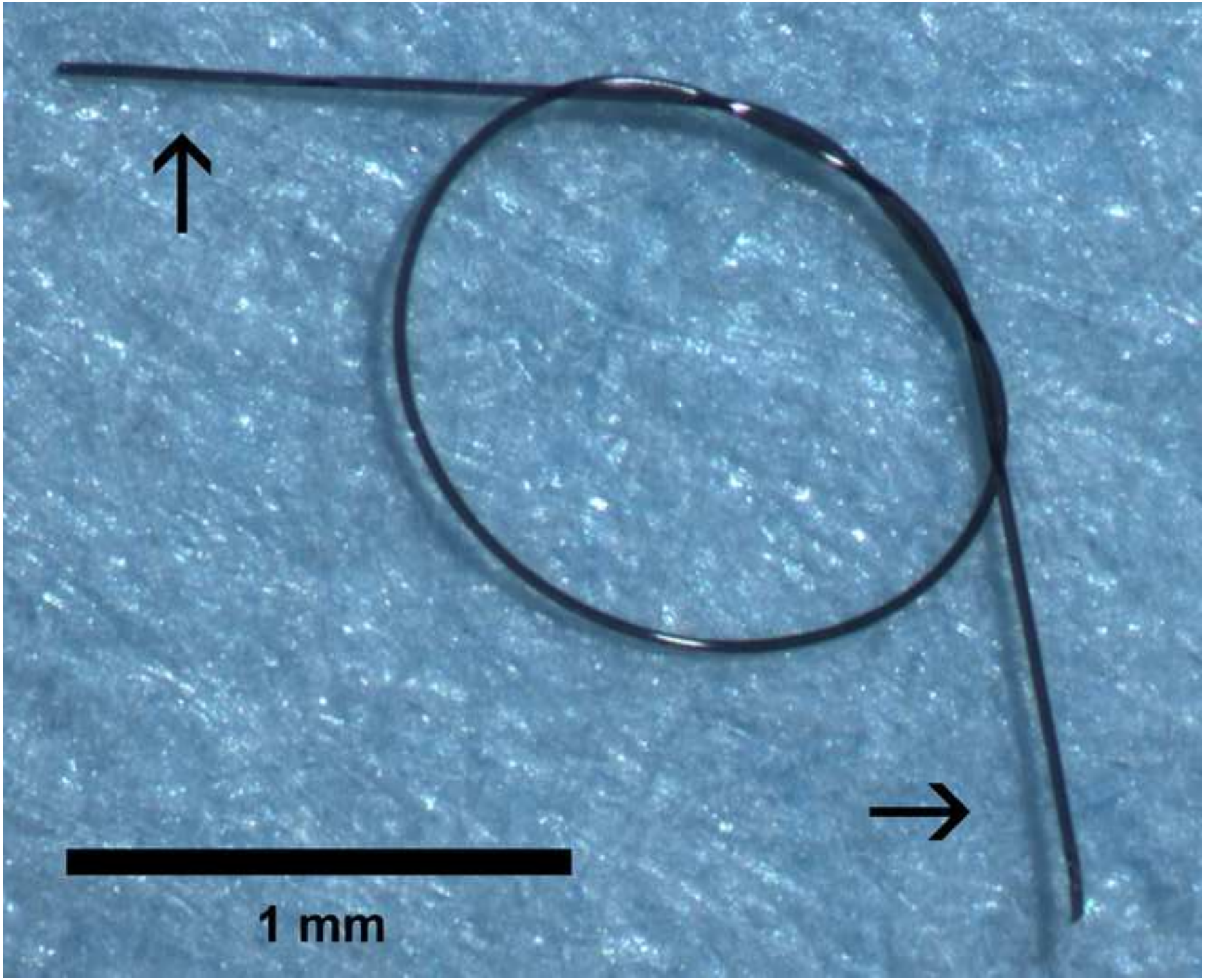

*Figure 2
Click here to download high resolution image

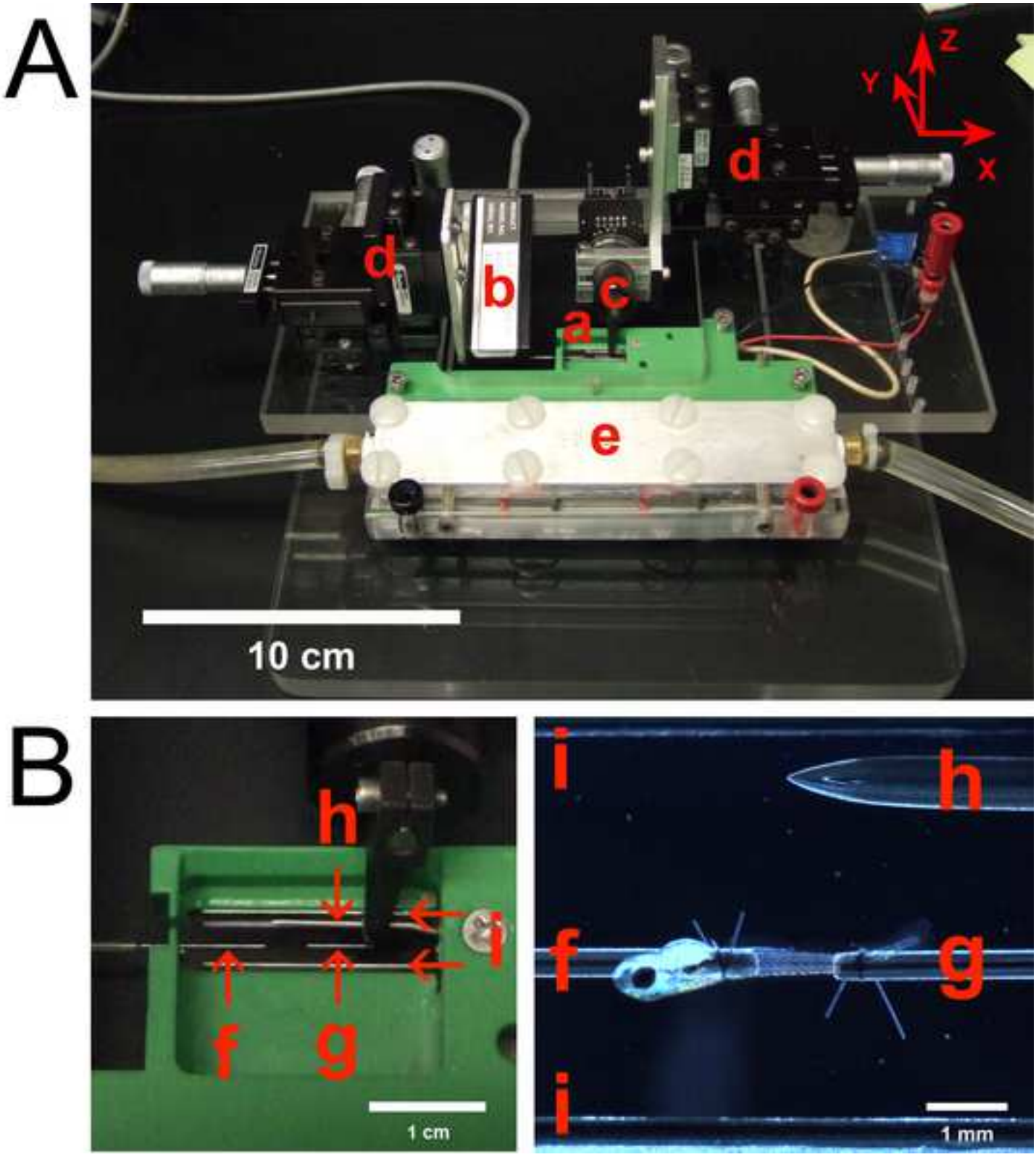

*Figure 3
Click here to download high resolution image

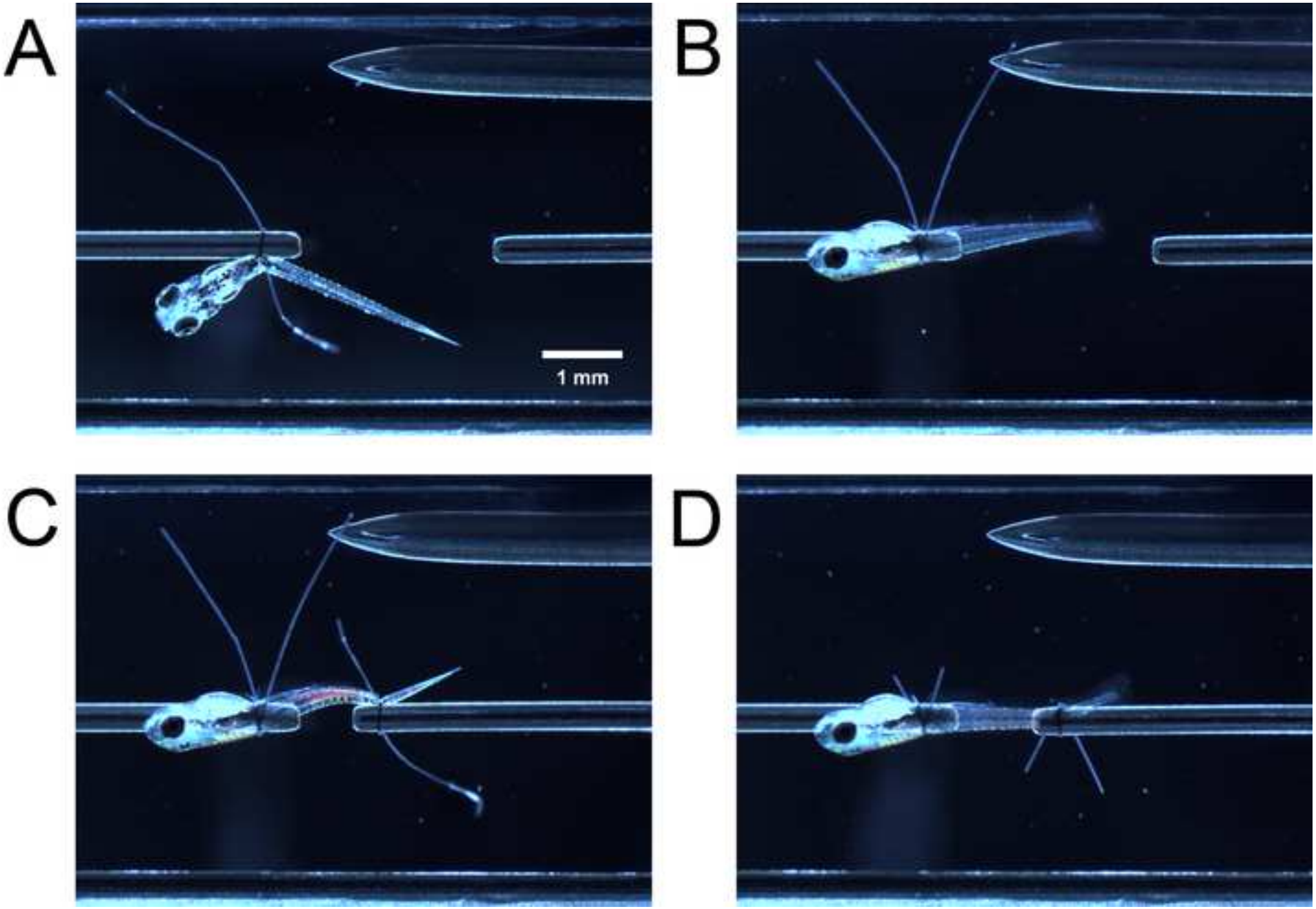

*Figure 4
Click here to download high resolution image

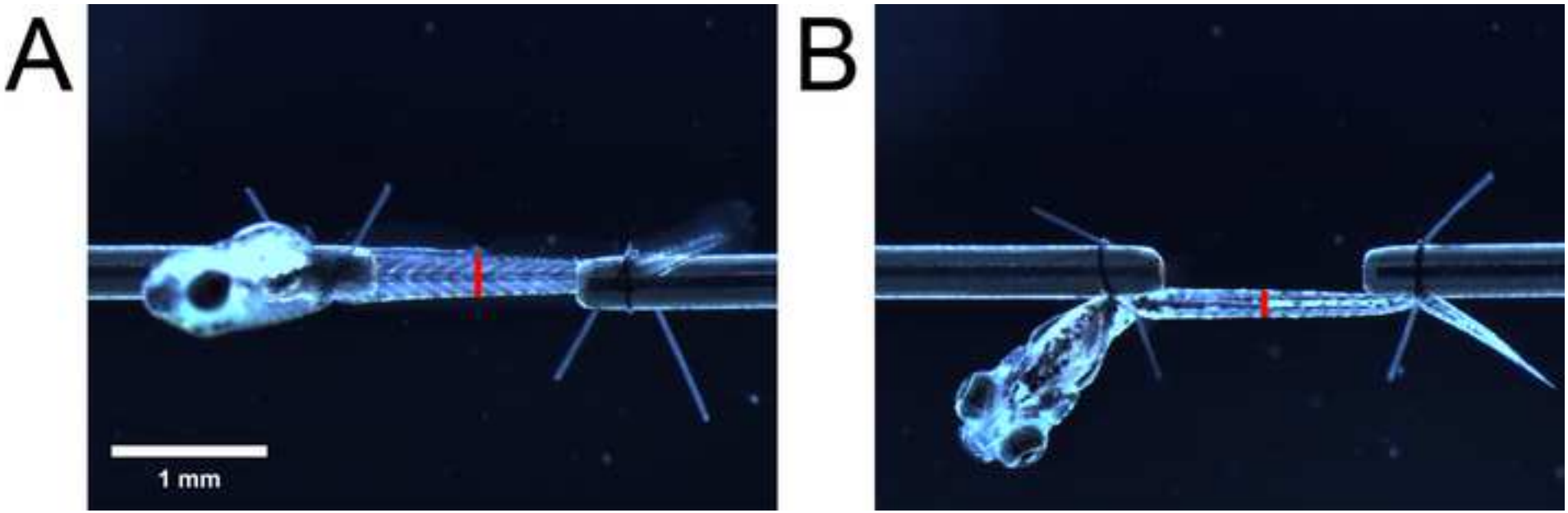

*Figure 5
Click here to download high resolution image

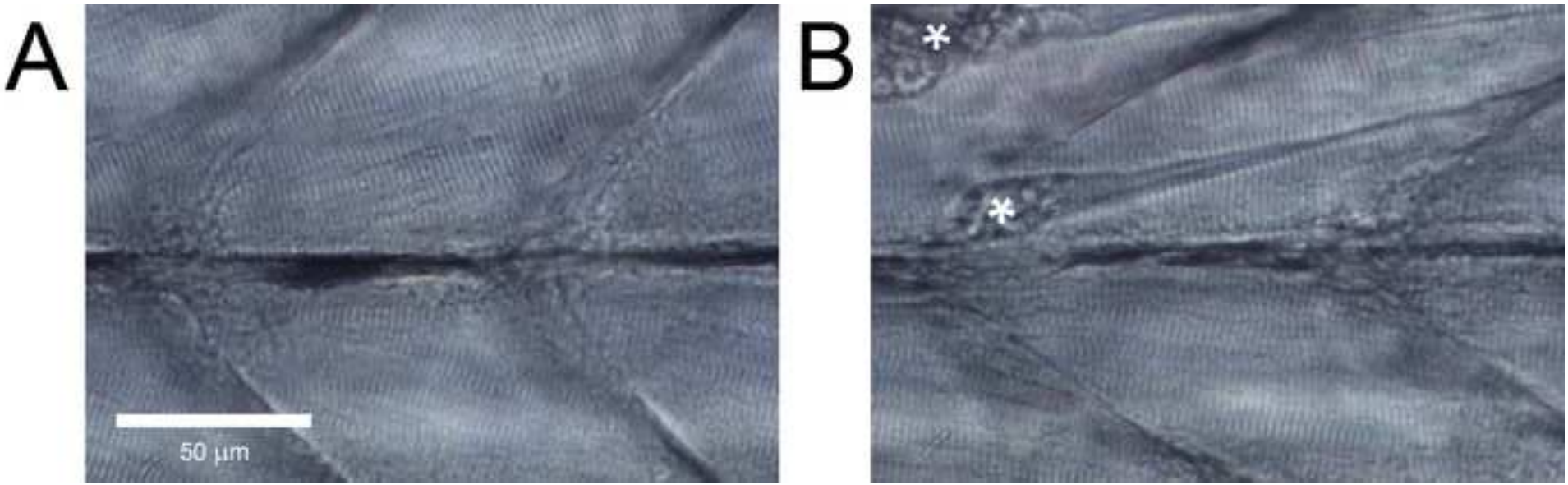

*Figure 6
Click here to download high resolution image

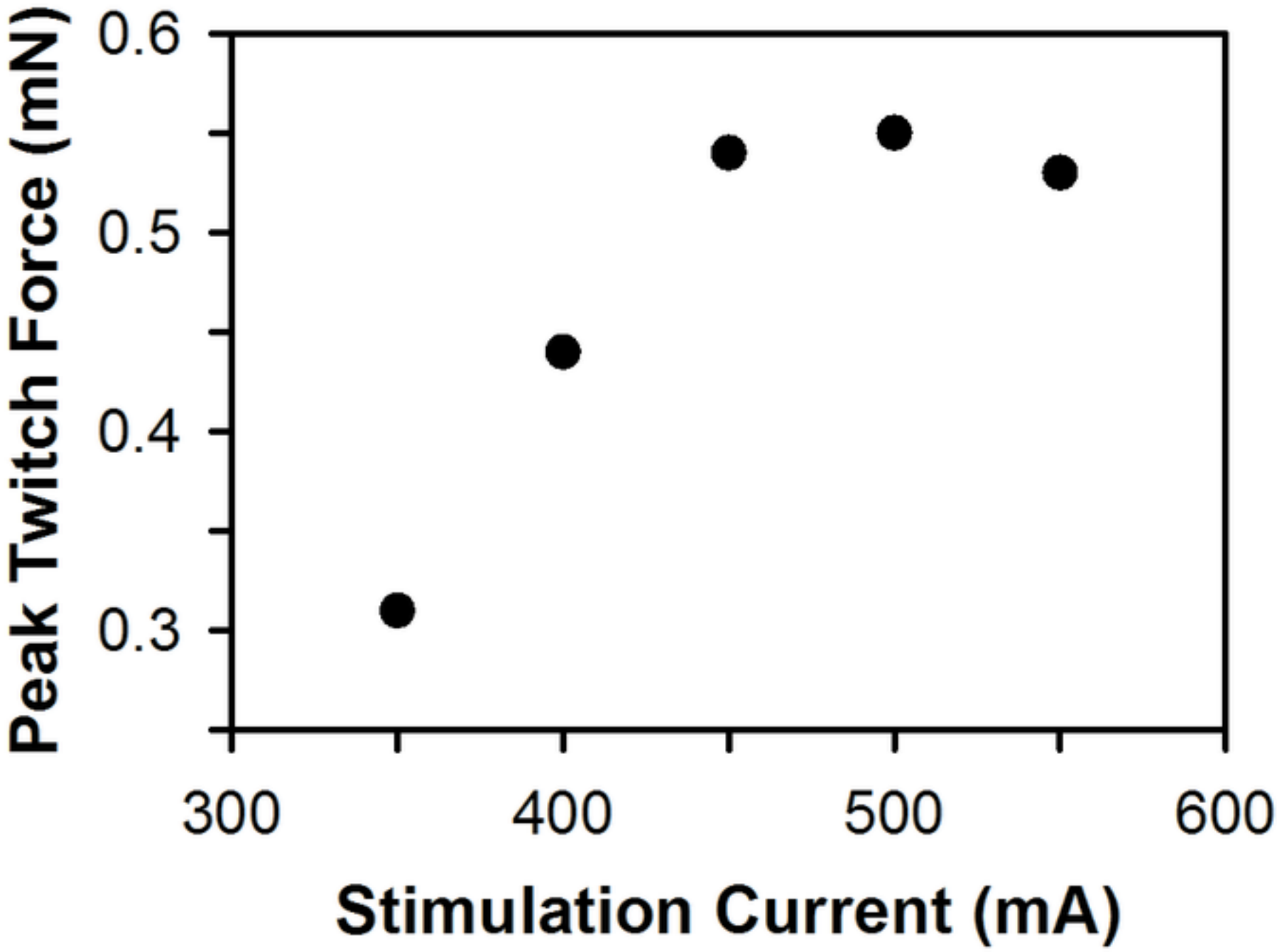

*Figure 7
Click here to download high resolution image

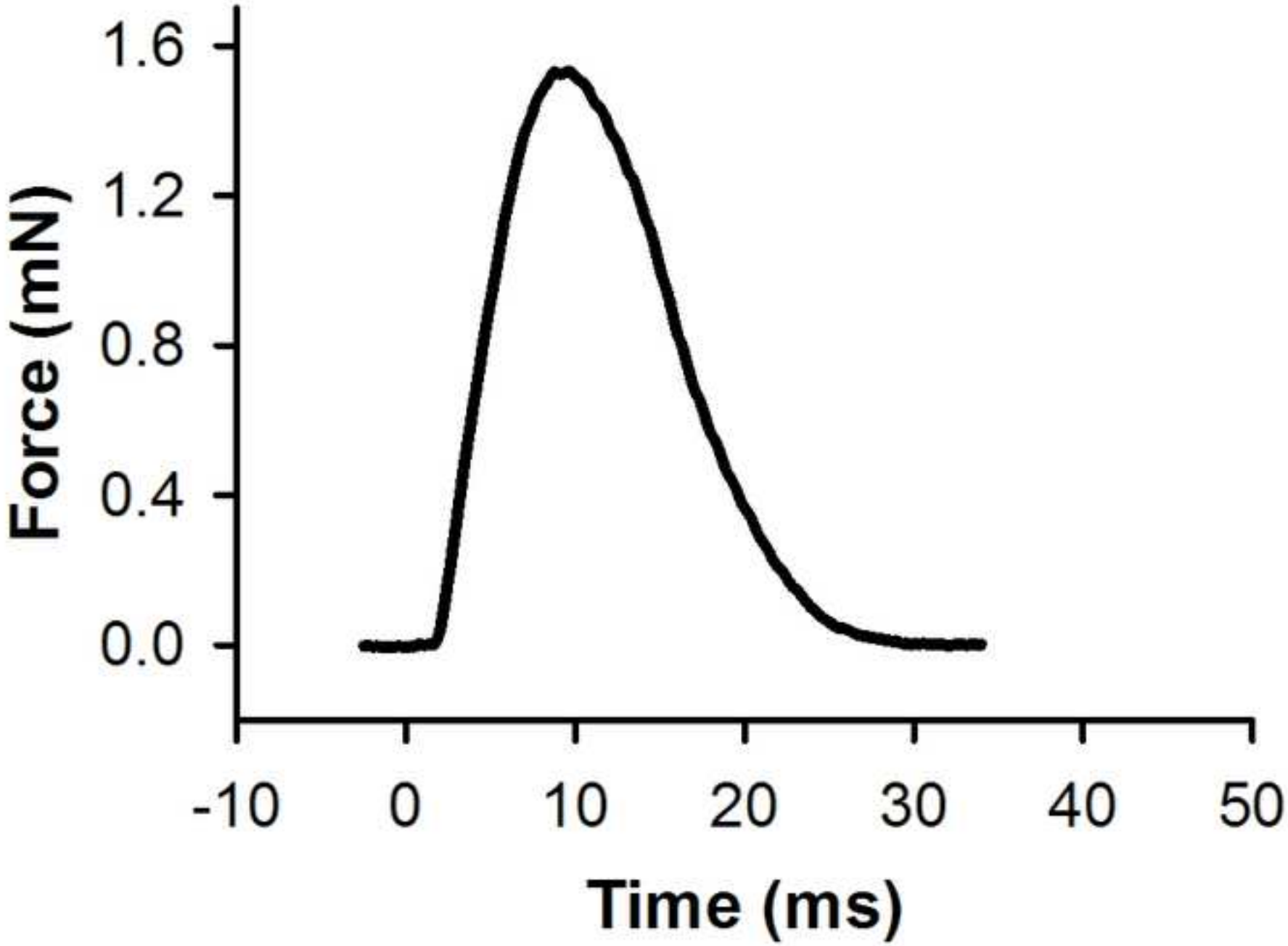

*Figure 8
Click here to download high resolution image

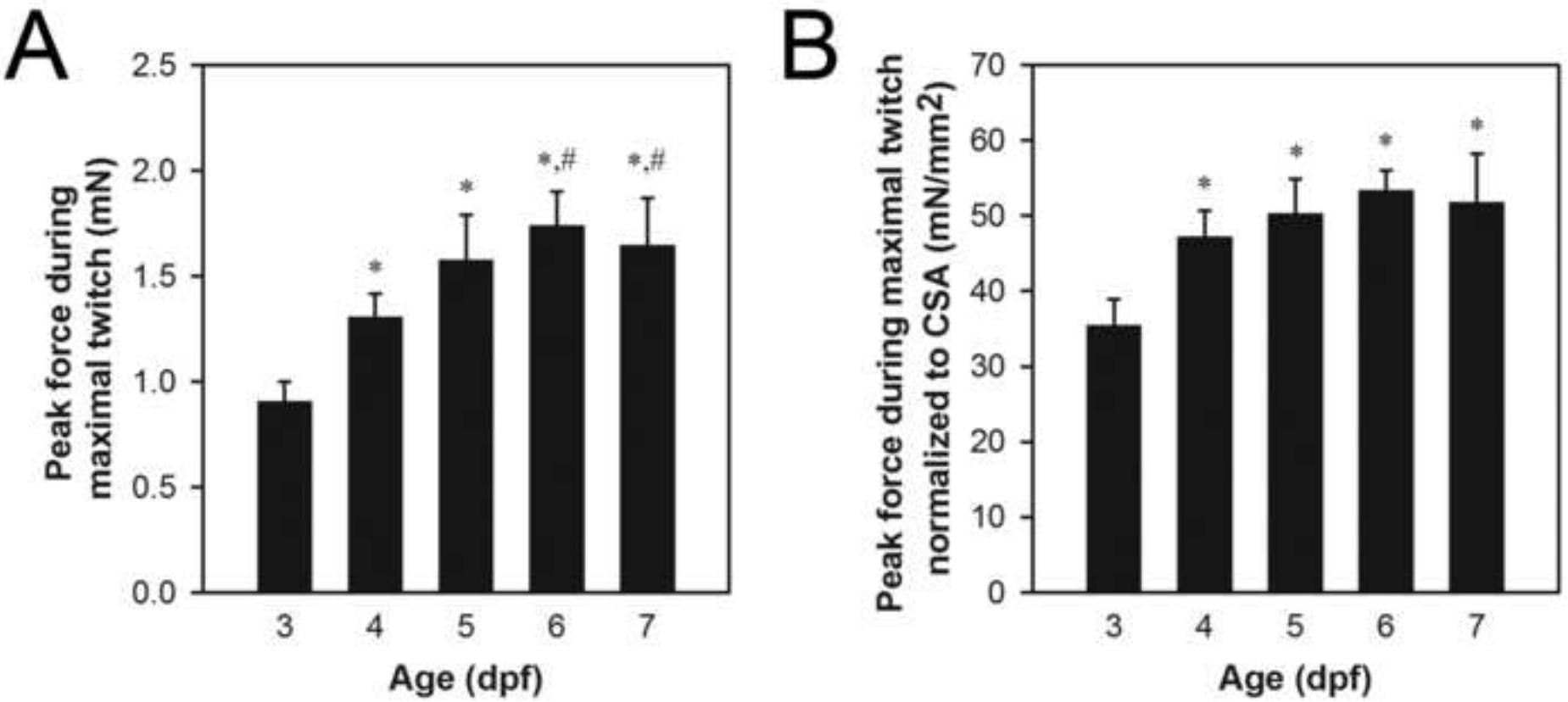

*Table of Reagents/ Materials Used
Click here to download Table of Reagents/ Materials Used: REAGENTS AND EQUIPMENT.xlsx

REAGENTS

Name	Company	Product Number
Tricaine powder	Sigma-Aldrich	A5040
Sodium chloride	Sigma-Aldrich	S7653
Potassium chloride	Sigma-Aldrich	P9541
Calcium chloride dihydrate	Sigma-Aldrich	223506
Magnesium chloride hexahydrate	Sigma-Aldrich	M2670
Sodium phosphate monobasic	Sigma-Aldrich	S0751
Sodium bicarbonate	Sigma-Aldrich	S6297
Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma-Aldrich	E5134

EQUIPMENT

Name	Company	Product Number
Nonsterile suture	Ashaway Line & Twine	S30002
Forceps	Fine Science Tools	11251-20
Spring scissors	Fine Science Tools	15000-08
Stereo microscope	Leica Microsystems	MZ8
Force transducer	Aurora Scientific	400A
Length controller	Aurora Scientific	318B
XYZ positioning devices	Parker Hannifin	3936M
Thermometer	Physitemp	BAT-12
Disposable transfer pipette	Fisher Scientific	13-711-9AM
Petri dish	Fisher Scientific	08-757-11YZ

Glass pipette	Fisher Scientific	13-678-8B
Inverted microscope	Carl Zeiss Microscopy	Axiovert 100
Water bath circulator	Neslab Instruments	RTE-111
Temperature controller	Alpha Omega Instruments	Series 800
Stimulator	Aurora Scientific	701C
Video sarcomere length system	Aurora Scientific	900B-5A
LabVIEW software	National Instruments	
Oscilloscope	Nicolet Technologies	ACCURA 100
Microblade	Fine Science Tools	10050-00
Microblade holder	Fine Science Tools	10053-13
Data analysis software (Signo)	Alameda Applied Sciences	

Comments

Comments
USP 10/0 monofilament nylon (3 ply)
Dumont #5
Vannas
Illuminated with Fostec EKE ACE I light source
Cut end to widen opening and facilitate larva transfer

Cut end (and fire-polish) to widen opening and facilitate larva transfer
High-power, follow stimulator

jove JOURNAL OF VISUALIZED EXPERIMENTS
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Force measurement during contraction to assess muscle function in zebrafish larvae

Author(s): Darcee D. Sloboda, Dennis R. Claflin, James J. Dowling and Susan V. Brooks

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: Susan V. Brooks

Department: Molecular and Integrative Physiology and Biomedical Engineering

Institution: University of Michigan

Article Title: Force measurement during contraction to assess muscle function in zebrafish larvae

Signature: Susan V Brooks Date: 10/29/12

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

*Rebuttal Comments
Click here to download Rebuttal Comments: Response to reviewers DDS DRC JJD SVB Jan 2013.docx

“Force measurement during contraction to assess muscle function in zebrafish larvae” by Darcée D. Sloboda, Dennis R. Claflin, James J. Dowling, and Susan V. Brooks

Editorial comments:

* Please remove all trademark symbols and use of the word "we" from the protocol text. We removed the trademark symbol and the use of the word “we” from the protocol text.

* If any material is added to the protocol text, please check to make sure the highlighted portion is under 3 pages. We removed highlighting over a small portion of the protocol (muscle measurements) to keep the highlighted portion under 3 pages. The muscle measurements portion of the protocol is sufficiently described through the text and the corresponding figure.

Reviewers' comments:

Reviewer #1:

Minor Concerns:
* Did not see how the zebrafish larva were anesthetized. Tricaine, included in the testing solution, is the anesthetic. We altered the protocol text (step 3.5) to clarify this point for the readers.
* Are the electrodes described? Are they just the Aurora platinum electrodes that are used for EDLs? Are they modified in any way (e.g., size)? Our testing system actually pre-dates the Aurora system. The electrodes are described as “platinum parallel plate electrodes” in the Figure 2 legend. We added dimensions to the description.
* Step 6.2 suggests taking measurements at an anatomical landmark (e.g., urogential opening). The legend for Figure 4 states "Red bars indicate measurement locations". Is this the urogential opening that was used in this example (Fig 4)? Yes. We modified the protocol text in step 6.2 and the Figure 4 legend to clarify this point.
* Authors do a nice job reporting optimal current and representative forces. I was going to ask if force-frequency curves ever been generated for the zebrafish larva, but I presume one can only examine twitch? What happens if a tetanic contraction is attempted (e.g. a 200 ms train)? Tetanic contractions (e.g. 200 ms trains) can be elicited and a force-frequency curve can be generated. However, the maximum force generated during a twitch or at lower frequencies is not much different than the maximum force generated during a tetanic contraction (e.g. 85-95% of maximum force with stimuli delivered at 50 Hz vs. 100% of maximum force with stimuli delivered at 200 Hz). We use twitches as a measure of muscle strength since there is less risk of the larvae ripping or slipping at the suture ties during a twitch compared with a tetanic contraction. We added a paragraph at the beginning of the discussion section to address this point.

Reviewer #2:

Minor Concerns:

i) I would like to see more information on the "video sarcomere length system" - it is unclear how this works. Text was added to the protocol (step 5.5) to provide more detail, describing how the system works. Use of the system will be shown on the video and this system is commercially available (indicated in the equipment list) so readers/viewers can also refer to the manufacturer for more information.

ii) There needs to be a good reason for performing the analysis at a non-standard temperature (ie 25 rather than 28.5 degrees). A good reason is not given. We recognize that 28.5 °C is the standard housing temperature for zebrafish. However, the slightly colder temperature does not alter force and allows the method to be used more broadly, by researchers who may not have temperature control on their force testing system. We did alter the protocol text (step 5.2) and added a sentence in the discussion section to let the readers know that measurements can be collected at 28.5 °C and that zebrafish should be maintained at 28.5 °C prior to testing for accurate staging.

iii) The classic model of zebrafish muscle disease was the development of the dmd mutant (Bassett et al 2003, Development 130(23):5851-60.). This should be referenced on p2 in the introduction. This reference was added and the numbering of other references was changed.