Video Article

Analysis of Skeletal Muscle Defects in Larval Zebrafish by Birefringence and Touch-evoke Escape Response Assays

Laura L. Smith[1], Alan H. Beggs[1], Vandana A. Gupta[1]

[1]Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Boston Children's Hospital, Harvard Medical School

Correspondence to: Vandana A. Gupta at vgupta@enders.tch.harvard.edu

URL: https://www.jove.com/video/50925
DOI: doi:10.3791/50925

Keywords: Physiology, Issue 82, birefringence, dystrophy, myopathy, touch-evoked escape, zebrafish, *Danio rerio*, microscopy

Date Published: 12/13/2013

Citation: Smith, L.L., Beggs, A.H., Gupta, V.A. Analysis of Skeletal Muscle Defects in Larval Zebrafish by Birefringence and Touch-evoke Escape Response Assays. *J. Vis. Exp.* (82), e50925, doi:10.3791/50925 (2013).

Abstract

Zebrafish (*Danio rerio*) have become a particularly effective tool for modeling human diseases affecting skeletal muscle, including muscular dystrophies[1-3], congenital myopathies[4,5], and disruptions in sarcomeric assembly[6,7], due to high genomic and structural conservation with mammals[8]. Muscular disorganization and locomotive impairment can be quickly assessed in the zebrafish over the first few days post-fertilization. Two assays to help characterize skeletal muscle defects in zebrafish are birefringence (structural) and touch-evoked escape response (behavioral).

Birefringence is a physical property in which light is rotated as it passes through ordered matter, such as the pseudo-crystalline array of muscle sarcomeres[9]. It is a simple, noninvasive approach to assess muscle integrity in translucent zebrafish larvae early in development. Wild-type zebrafish with highly organized skeletal muscle appear very bright amidst a dark background when visualized between two polarized light filters, whereas muscle mutants have birefringence patterns specific to the primary muscular disorder they model. Zebrafish modeling muscular dystrophies, diseases characterized by myofiber degeneration followed by repeated rounds of regeneration, exhibit degenerative dark patches in skeletal muscle under polarized light. Nondystrophic myopathies are not associated with necrosis or regenerative changes, but result in disorganized myofibers and skeletal muscle weakness. Myopathic zebrafish typically show an overall reduction in birefringence, reflecting the disorganization of sarcomeres.

The touch-evoked escape assay involves observing an embryo's swimming behavior in response to tactile stimulation[10-12]. In comparison to wild-type larvae, mutant larvae frequently display a weak escape contraction, followed by slow swimming or other type of impaired motion that fails to propel the larvae more than a short distance[12]. The advantage of these assays is that disease progression in the same fish type can be monitored *in vivo* for several days, and that large numbers of fish can be analyzed in a short time relative to higher vertebrates.

Video Link

The video component of this article can be found at https://www.jove.com/video/50925/

Protocol

1. *In vivo* Analysis of Skeletal Muscle Structure by Birefringence

1. Prepare mating cages separating the male(s) from the female(s) of the desired zebrafish line(s) late in the afternoon after feeding.
    1. Distinguish females by their bigger underbelly and slight blue/white coloration, and males by their slender body shape and pink/yellow hues.
    2. Success with pair-wise crosses (one male and one female) may only be about 50%. For a higher rate of success, mate one male with 2-3 females.
2. Remove cage dividers the next morning shortly after the onset of light and allow the fish to spawn undisturbed.
3. Collect the eggs using a strainer when sufficient numbers of fertilized eggs are laid at the bottom of the tank.
4. Transfer the embryos into a deep Petri dish by rinsing the strainer with fish water and return the parent fish to their tanks. Place dishes of embryos into a 28.5 °C incubator.
5. Clean out the inviable eggs and debris later that day or the following morning, and return the embryos to the incubator for further growth.
6. Embryos/larvae reach the appropriate age to observe birefringence at 3-7 days post-fertilization (dpf), as results prior to 3 dpf may potentially suffer from limited contrast. Wild-type embryos hatch from their chorions between 48-60 hr post-fertilization (hpf), followed by a straightening of the body axis. Late-hatching wild-type or mutant embryos may require manual dechorionation.
    1. Gently make a tear in the chorion with sharp forceps and turn it upside down so that the embryo falls out.
    2. Allow dechorionated embryos to straighten prior to beginning the assay.
    3. Anesthetize embryo(s) with tricaine (0.04% in fish water) to aid in their correct positioning.

7. Fit a dissecting microscope with a polarized lens that can be rotated to adjust the angle of polarized light (**Figure 1A**).
8. Place dechorionated, anesthetized embryo(s) directly on top of a second polarized lens along the lateral axis of the body. The second lens should be positioned below the top lens, on the microscope stage (**Figure 1B**). Do not use plastic Petri dishes at any point during the birefringence assay, as plastic is not an appropriate medium for the transmission of refracted light.
9. Rotate the top lens with the embryo of interest in view until the axes of polarization of the two lenses are oriented at 90° from one another and the background is completely dark.
10. The optimum output of the assay is dependent on the orientation of fish between two polarized lenses. Move the fish around to make sure that they are lying as flat as possible.
    1. If the fish is curved, only the segments with this flat orientation will pass the polarized light and exhibit birefringence. In such cases, measure the birefringence in the flat areas and record the birefringence of the corresponding area in the wild-type fish (*e.g.* somites 1-10 in both wild-type and mutant fish).

11. Observe the birefringent phenotype of the fish.
    1. Wild-type fish with highly organized skeletal muscle show bright birefringence, as the refractive index for the light parallel to the myofilaments is higher than the polarized light perpendicular to these structures.
    2. Fish with disorganized skeletal muscle suggestive of a nondegenerative myopathy or a developmental defect typically show an overall reduction in birefringence.
    3. Fish with disorganized skeletal muscle characteristic of a muscular dystrophy commonly exhibit a patch-like pattern of birefringence, with dark areas representing muscle degeneration among bright areas of normal muscle architecture.

12. Quantify birefringence by taking images of wild-type and mutant fish under polarized light at the same exposure settings and magnification. Save images as .tiff files.
    1. Open birefringence images in ImageJ software (http://rsbweb.nih.gov/ij/).
    2. For each image, select the area of zebrafish muscle by drawing a line around the body using the “Polygon Selections” option from the toolbar.
    3. Use “Set Measurements” under the “Analyze” drop-down menu to select the required statistics for the image.
        1. Minimum selection requires that boxes for “Area,” “Mean gray value,” and “Min & max gray value” are checked.
        2. A maximum gray value of >255 will indicate pixel saturation. Therefore, use images with maximum gray values less than 255.

    4. Normalize the mean intensity with the selected area and repeat this same quantification procedure for each birefringence image.

2. Touch-evoked Escape Behavior Assay

1. Set up mating pairs of the appropriate zebrafish line(s) and collect embryos as described above in steps 1.1-1.5.
2. At 2-7 days post-fertilization, place dechorionated embryos/larvae individually into a deep Petri dish or the chambers of multiwell plates.
3. Working with only one embryo at a time, center the embryo in the field of view of a Nikon SMZ1500 stereomicroscope with a SPOT RT3 digital camera system or similar, and begin sequential imaging.
    1. The same frequency of imaging should be used for both wild-type and mutant fish. Frame rates >30 Hz are recommended, as slower rates will typically not be sufficient to capture the fast swimming of wild-type fish.

4. Deliver mechanosensory stimuli to the embryo by touching the tail with an insect pin.
5. Stop imaging when the embryo has stopped swimming or has swam out of the field of view.
6. Convert sequential images into a video file, or analyze individual frames of time-lapse images off-line, using SPOT 5.1 Advanced software. Similar software packages include Open Lab, NIS Elements, or freely available ImageJ (http://rsbweb.nih.gov/ij/).
7. Quantify swimming behavior by performing the touch-evoked response in multiple embryos.
    1. This can be achieved by averaging the distance embryos are able to swim within a fixed time interval using software or a metric ruler to mark the start and end points of the swimming bout.
    2. Avoid embryos becoming habituated to the touch stimulus over time by performing the assay with a new embryo for each experimental repeat. Behaviors in wild-type and reliable mutant models tend to be highly reproducible.

Representative Results

Birefringence can be used as an efficient, noninvasive assay to shed light on the state of myofibrillar organization in living zebrafish embryos. Examples of wild-type zebrafish as well as zebrafish with decreased expression of genes critical to skeletal muscle development and function are presented. Wild-type zebrafish at 5 dpf display highly birefringent skeletal muscles under polarized light due to the ordered array of myofilaments (**Figures 2A-B**). In contrast, an age-matched embryo homozygous for a pathogenic mutation in the dystroglycan gene (*dag1*) displays patchy birefringence, indicative of areas of muscle degeneration (**Figures 2C-D**)[2]. This patch-like pattern and rapid progression of muscle degeneration throughout early development is consistent with other dystrophic fish models[1,3]. Quantifying the birefringence of 5 dpf dystrophic *dag1* mutants identifies a significant reduction in brightness to 27.9%±5.3 of the maximal birefringence seen in their wild-type siblings. This reduction is more severe than the reduction to 52.4%±5.5 observed in 3 dpf myopathic *mtm1* morphants (wild-type/*dag1*/*mtm1*: n = 3; Student's t-test, $P < 0.001$) (**Figure 4**). Myopathic fish models (**Figures 3C-D**) instead tend to show an overall reduction in birefringence compared to wild-type (**Figures 3A-B**), as there is often myofibrillar disorganization in the major axial skeletal muscles but no evidence of degenerative changes.

Touch-evoked escape behaviors can be used to demonstrate that zebrafish models of skeletal muscle disease display locomotive defects and swimming difficulties. The live swimming phenotypes of wild-type and *dag1* fish were examined by video microscopy with a touch-evoked escape behavior assay (**Videos 1** and **2**)[2]. Videos were then analyzed frame by frame to confirm that *dag1* mutants exhibit impaired movement compared to wild-type controls in response to tactile stimuli (**Figure 5**). The distances both types of embryos were able to move within a

fixed time interval were averaged (wild-type: 6.2±0.4 cm/0.1 sec, n = 10; mutant: 0.75±0.08 cm/0.1 sec, n = 10; Student's t-test, $P < 0.001$) and indicate diminished motor function in *dag1* mutants. However, reduced touch-evoke responses in neuromuscular mutants could also be attributed to defects in the central nervous system and/or the neuromuscular junction. Therefore, electrophysiological measurement of the voltage response in muscles evoked by tactile stimulation may help to distinguish whether touch-evoke impairments are due to a sensory deficit or to a primary defect in skeletal muscle[13].

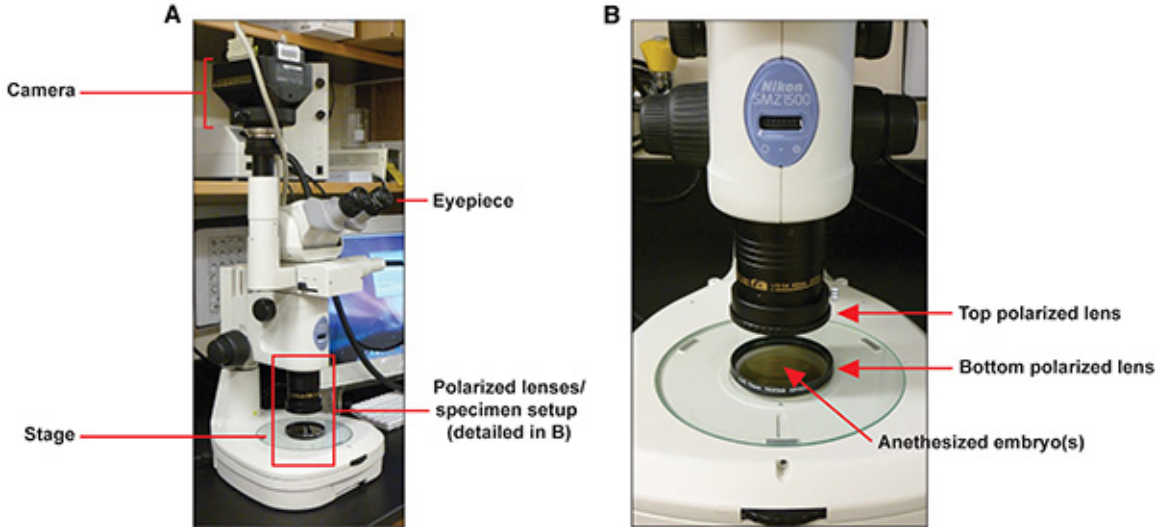

Figure 1. Diagram of a standard dissecting microscope fitted with a camera and polarized lenses (A). Sample set-up with polarized lenses and anesthetized zebrafish embryos is presented larger in (**B**). Click here to view larger image.

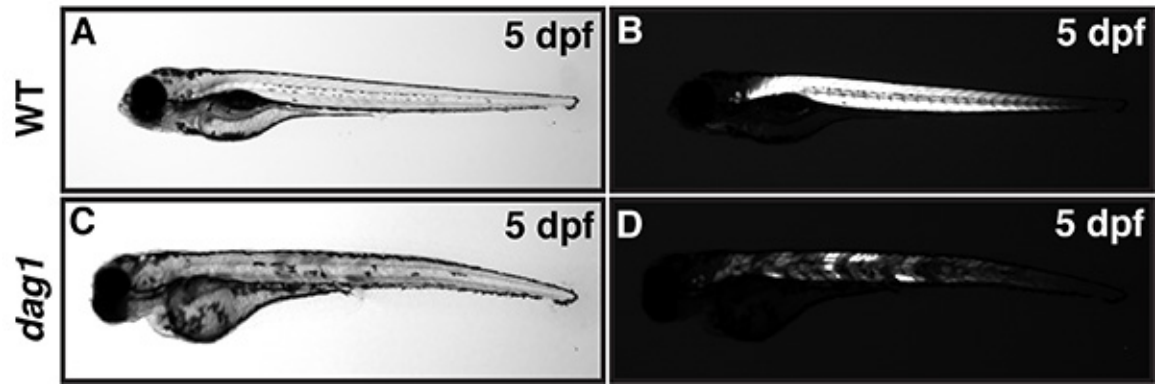

Figure 2. Wild-type fish show bright birefringence indicative of highly organized muscles at 5 dpf (B). Age-matched *dag1* mutant fish appear grossly normal in normal light whereas extensive muscle degeneration in most somites is demonstrated by the loss of birefringence under polarized light and is consistent with a dystrophic phenotype (**D**). Brightfield images of the wild-type fish (**A**) and the *dag1* mutant (**C**) are shown for reference. Click here to view larger image.

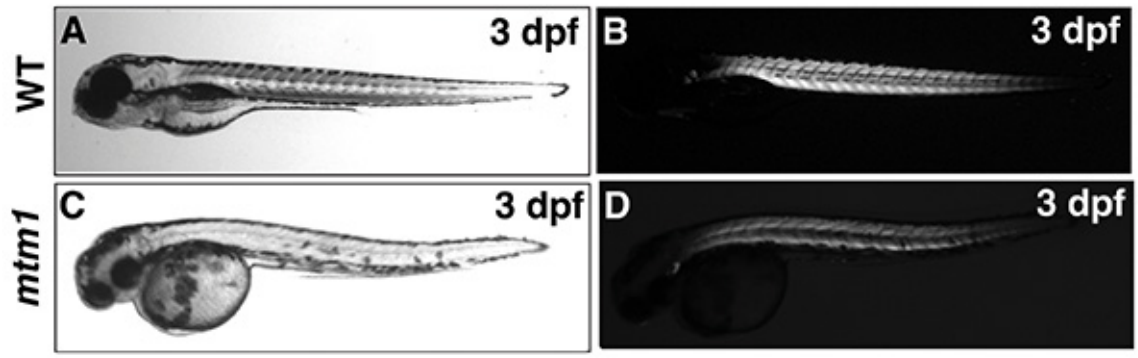

Figure 3. Morpholino-based knockdown of myotubularin (*mtm1*) in zebrafish results in defects in skeletal muscle at 3 dpf. An overall reduction in birefringence is observed in *mtm1* morphant fish (**D**) in comparison to wild-type fish (**B**), consistent with a myopathic phenotype. Brightfield images of the wild-type fish (**A**) and the *mtm1* morphant (**C**) are shown for reference. Click here to view larger image.

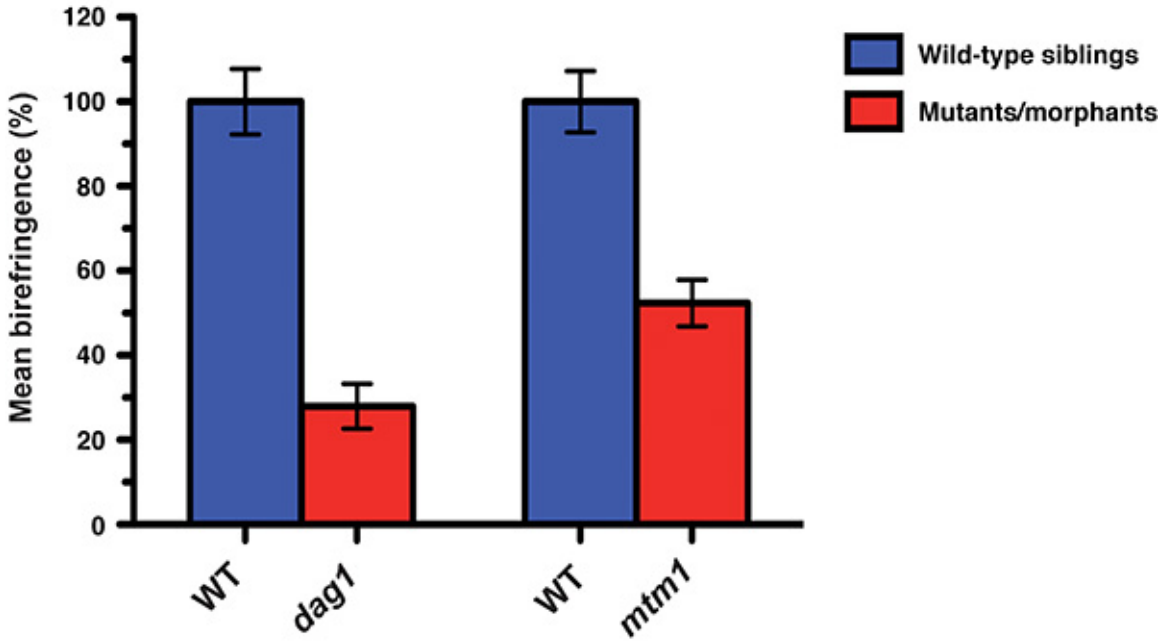

Figure 4. Zebrafish embryos with skeletal muscle defects show a highly significant reduction in birefringence compared to wild-type controls. Quantification data are calculated by dividing the mean intensity by the selected area of the fish (ImageJ), and normalizing the percentage values to wild-type. Data are presented as means±SEM and ** indicates $P < 0.001$ by Student's t-test. Click here to view larger image.

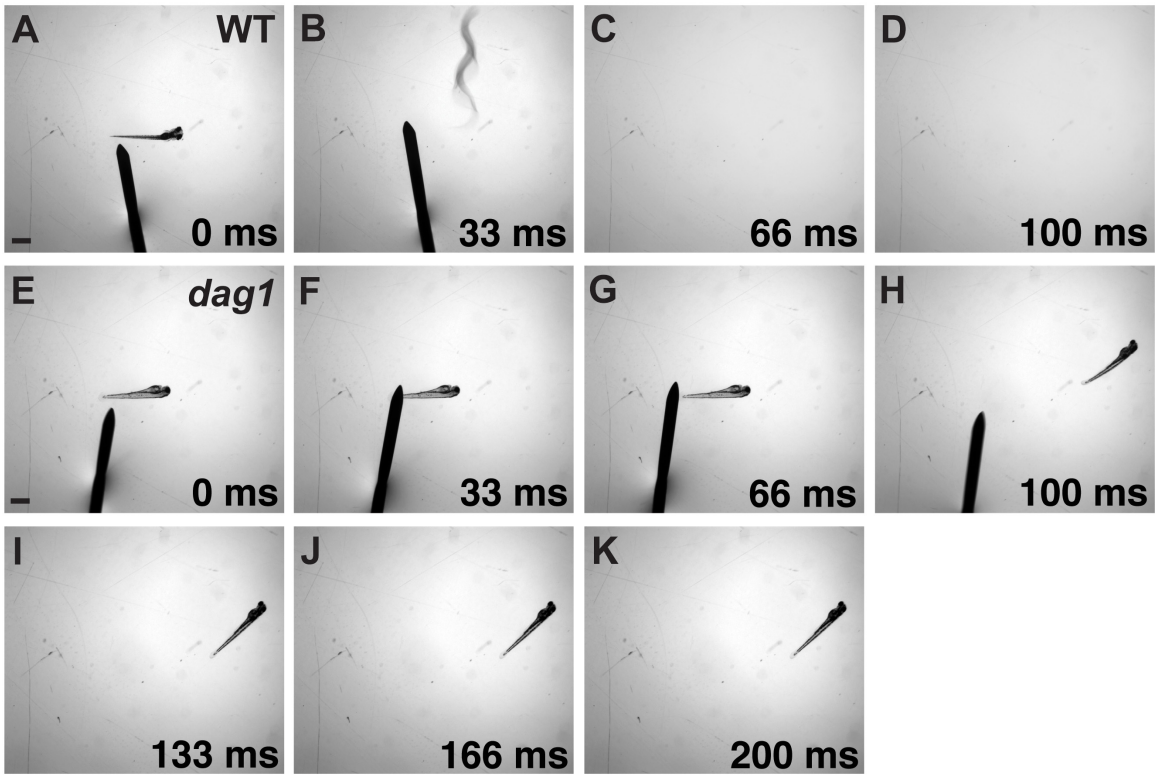

Figure 5. *Dag1* mutant embryos exhibit reduced touch-evoked response at 5 dpf (E-K) while mechanosensory stimulation causes 5 dpf wild-type embryos to swim away rapidly and fully exit the field of view (A-D). Scale bar = 1 mm. Click here to view larger image.

Video 1. Touch-evoked escape behavior assay on wild-type zebrafish. Normal fry (5 dpf) swim away very rapidly in response to touch. Click here to watch video.

Video 2. Touch-evoked escape behavior assay on *dag1* mutant zebrafish. *Dag1* fry (5 dpf) show impaired response to touch and fail to exit the field of view. Click here to watch video

Discussion

Primary neuromuscular disorders are traditionally classified as dystrophic or nondystrophic processes. Muscular dystrophies are characterized by myofiber degeneration followed by repeated rounds of regeneration, which ultimately leads to an end stage process typified by fibrosis and replacement by adipose tissue[14]. Nondystrophic myopathies, in contrast, are not associated with necrosis or regenerative changes, but do result in disorganized myofibers and overall skeletal muscle weakness.

Zebrafish models have emerged in recent years as a tremendously valuable resource for better understanding the molecular pathways and pathogenesis of different neuromuscular diseases. In addition to the general advantages of this model system, including rapid *ex utero* development, translucent embryos, high reproductive capacity, and a genome closely related to that of human, the zebrafish disease model often closely mimics the human disease. Although many zebrafish with skeletal muscle defects have morphological abnormalities, some mutant phenotypes are grossly normal and require finer characterizations. For these and all cases, the extent of muscle damage and weakness can quickly and easily be evaluated in zebrafish using noninvasive *in vivo* assays for birefringence and touch-evoked escape behavior. Birefringence is a sensitive indicator of muscle integrity in translucent zebrafish embryos that has been used to noninvasively identify skeletal muscle mutants since early in the establishment of the zebrafish model system[15]. The assay itself involves placing a zebrafish embryo between two polarizing filters and visualizing the fish in an otherwise dark background[16]. Wild-type fish with highly organized sarcomeric arrays appear bright, whereas muscle mutants usually present with dark patches or an overall reduction in birefringence, suggesting myofibrillar disorganization within the major axial skeletal muscles. As described above, birefringent images may be readily quantified using publically available ImageJ software.

Touch-evoked escape behaviors in zebrafish involve many steps, beginning with the sensing of tactile stimuli and culminating in the contraction of muscles. Zebrafish embryos swim in response to touch by 26 hpf, but the frequency of muscle contractions during swimming does not increase to a value comparable to that of adult zebrafish until 36 hpf[12,18]. The touch-evoked response is optimally captured between 2-7 dpf. The time at which the touch stimulus is applied to the tail is set as the experimental “start” point. Whereas both wild-type and mutant larvae begin stationary, video microscopy is able to capture that wild-type larvae respond to mechanosensory stimuli with rapid and vigorous swimming in a straight line. Muscular mutants instead tend to weakly flex, swim in circles, or otherwise move for only a short distance. It should be noted that while there is an inherent degree of variability in the timing and intensity of manual touch stimuli, quantitative measurements of touch-evoke behaviors and swimming episodes tend to be highly reproducible within wild-type and reliable fish models of skeletal muscle disease.

Both birefringence and touch-evoked escape assays provide a fast and noninvasive way to analyze defects in skeletal muscles, and are therefore appropriate for analyzing a large number of embryos when performing high-throughput mutagenesis or chemical screens.

Disclosures

No conflicts of interest declared.

Acknowledgements

We thank Behzad Moghadaszadeh for his wonderful help with quantification of birefringence images. This work was funded by the Muscular Dystrophy Association USA (MDA201302) and the National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01 AR044345), as well as generous support from A Foundation Building Strength, Cure CMD, and the AUism Charitable Foundation. VAG is supported by K01 AR062601 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, and LLS is supported by F31 NS081928 from the National Institute of Neurological Disorders and Stroke.

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**, 5851-5860, 10.1242/dev.00799 (2003).
2. Gupta, V., *et al*. The zebrafish dag1 mutant: a novel genetic model for dystroglycanopathies. *Hum. Mol. Genet*. **20** (9), 1712-1725, 10.1093/hmg/ddr047 (2011).
3. Hall, T.E., *et al*. The zebrafish candyfloss mutant implicates extracellular matrix adhesion failure in laminin α2-deficient congenital muscular dystrophy. *Proc. Natl. Acad. Sci. U.S.A*. **104** (17), 7092-7097, 10.1073/pnas.0700942104 (2007).
4. Dowling, J.J., *et al*. Loss of myotubularin function results in T-tubule disorganization in zebrafish and human myotubular myopathy. *PLoS Genet*. **5** (2), e1000372, 10.1371/journal.pgen.1000372 (2009).
5. 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** (3), 389-396, 10.1242/dmm.008631 (2012).
6. Ferrante, M.I., Kiff, R.M., Goulding, D.A., Stemple, D.L. Troponin T is essential for sarcomere assembly in zebrafish skeletal muscle. *J. Cell Sci*. **124** (4), 565-577, 10.1242/jcs.071274 (2011).
7. Hawkins, T.A., *et al*. The ATPase-dependent chaperoning activity of Hsp90a regulates thick filament formation and integration during skeletal muscle myofibrillogenesis. *Development*. **135** (6), 1147-1156, 10.1242/dev.018150 (2008).
8. Barbazuk, W.B., *et al*. The syntenic relationship of the zebrafish and human genomes. *Genome Res*. **10** (9), 1351-1358, 10.1101/gr.144700 (2000).
9. Schapira, G., Dreyfus, J.C., Joly, M. Changes in the flow birefringence of myosin as a result of muscular atrophy. *Nature*. **170** (4325), 494-495, 10.1038/170494b0 (1952).

10. Kimmel, C.B., Patterson, J., Kimmel, R.O. The development and behavioral characteristics of the startle response in zebrafish. *Dev. Psychobiol*. **7**, 47-60 (1974).
11. Eaton, R.C., Bombardieri, R.A., Meyer, D.L. The Mauthner-initiated startle response in teleost fish. *J. Exp. Biol*. **66**, 65-81 (1977).
12. Saint-Amant, L., Drapeau, P. Time course of the development of motor behaviors in the zebrafish embryo. *J. Neurobiol*. **37** (4), 622-632, 10.1002/(SICI)1097-4695(199812)37:4<622::AID-NEU10>3.0.CO;2-S (1998).
13. Hirata, H., *et al*. Zebrafish relatively relaxed mutants have a ryanodine receptor defect, show slow swimming and provide a model of multi-minicore disease. *Development*. **134** (15), 2771-2781, 10.1242/dev.004531 (2007).
14. Wallace, G.Q., McNally, E.M. Mechanisms of muscle degeneration, regeneration, and repair in the muscular dystrophies. *Annu. Rev. Physiol*. **71**, 37-57, 10.1146/annurev.physiol.010908.163216 (2009).
15. Granato, M., *et al*. Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development*. **123**, 399-413 (1996).
16. Berger, J., Sztal, T., Currie, P.D. Quantification of birefringence readily measures the level of muscle damage in zebrafish. *Biochem. Biophys. Res. Commun*. **423** (4), 785-788, 10.1016/j.bbrc.2012.06.040 (2012).
17. Low, S.E., *et al*. TRPM7 is required within zebrafish sensory neurons for the activation of touch-evoked escape behaviors. *J. Neurosci*. **31** (32): 11633-11644, http://dx.doi.org/10.1523/JNEUROSCI.4950-10.2011 (2011).
18. Liu, D.W., Westerfield, M. Function of identified motoneurones and co-ordination of primary and secondary motor systems during zebrafish swimming. *J. Physiol*. **403**, 73-89 (1988).

