Video Article

Laser-inflicted Injury of Zebrafish Embryonic Skeletal Muscle

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Abstract

Various experimental approaches have been used in mouse to induce muscle injury with the aim to study muscle regeneration, including myotoxin injections (bupivacaine, cardiotoxin or notexin), muscle transplantations (denervation-devascularization induced regeneration), intensive exercise, but also murine muscular dystrophy models such as the *mdx* mouse (for a review of these approaches see [1]). In zebrafish, genetic approaches include mutants that exhibit muscular dystrophy phenotypes (such as *runzel*[2] or *sapje*[3]) and antisense oligonucleotide morpholinos that block the expression of dystrophy-associated genes[4]. Besides, chemical approaches are also possible, *e.g.* with Galanthamine, a chemical compound inhibiting acetylcholinesterase, thereby resulting in hypercontraction, which eventually leads to muscular dystrophy[5]. However, genetic and pharmacological approaches generally affect all muscles within an individual, whereas the extent of physically inflicted injuries are more easily controlled spatially and temporally[1]. Localized physical injury allows the assessment of contralateral muscle as an internal control. Indeed, we recently used laser-mediated cell ablation to study skeletal muscle regeneration in the zebrafish embryo[6], while another group recently reported the use of a two-photon laser (822 nm) to damage very locally the plasma membrane of individual embryonic zebrafish muscle cells[7].

Here, we report a method for using the micropoint laser (Andor Technology) for skeletal muscle cell injury in the zebrafish embryo. The micropoint laser is a high energy laser which is suitable for targeted cell ablation at a wavelength of 435 nm. The laser is connected to a microscope (in our setup, an optical microscope from Zeiss) in such a way that the microscope can be used at the same time for focusing the laser light onto the sample and for visualizing the effects of the wounding (brightfield or fluorescence). The parameters for controlling laser pulses include wavelength, intensity, and number of pulses.

Due to its transparency and external embryonic development, the zebrafish embryo is highly amenable for both laser-induced injury and for studying the subsequent recovery. Between 1 and 2 days post-fertilization, somitic skeletal muscle cells progressively undergo maturation from anterior to posterior due to the progression of somitogenesis from the trunk to the tail[8, 9]. At these stages, embryos spontaneously twitch and initiate swimming. The zebrafish has recently been recognized as an important vertebrate model organism for the study of tissue regeneration, as many types of tissues (cardiac, neuronal, vascular etc.) can be regenerated after injury in the adult zebrafish[10, 11].

Video Link

The video component of this article can be found at https://www.jove.com/video/4351/

Protocol

1. Labeling Single Cells

Inject one-cell stage embryos with a plasmid encoding GFP or any GFP-fusion protein under the control of a β-Actin promoter. During development, GFP is then expressed in a mosaic fashion. Here, we used the transgenic construct Tg[*β-actin:α-actinin-GFP*], which places the α-Actinin-GFP fusion protein under the control of the *β-actin* promoter.

2. Embryo Embedding

a. Collect zebrafish embryos and maintain under normal conditions until 28 hpf (staging according to[12]).
b. Dechorionate embryos under the stereomicroscope with forceps (Dumont #5).
c. Prepare a microscope slide with a petroleum jelly ring.
d. Prepare a 1% low-melting point agarose /10% tricaine solution with E3 medium: boil 100 mg agarose in 10 ml E3 solution to obtain a liquid and homogeneous agarose solution; aliquot and store unused agarose solution at -20 °C for further use. For immediate use, let the boiled solution cool down and maintain at max. 39 °C. Add tricaine stock solution to obtain a final concentration of 10% tricaine. Both tricaine and agarose are necessary to prevent embryonic movements.

e. Embed a single embryo in 1% low-melting agarose / 10% tricaine / E3 medium within the petroleum jelly ring, with the embryo laying naturally on the side; gently cover with a coverslip just to maintain the embryo in place and to avoid a convex surface which would break the light. Ideally, the embryonic muscle tissue that will be targeted should touch the coverslip.

3. Embryo Laser-wounding

a. Prepare the micropoint laser and the microscope:
   i. Please refer to the micropoint laser manual for detailed instructions on how to set up and use the laser in conjunction with the microscope.
   ii. Select the correct dye for the generation of a 435 nm wavelength laser beam.
   iii. Adjust the laser power by using the attenuation slider. The laser power should be strong enough to break a glass surface with a single pulse (*e.g.* focus on the coverslip of your sample to test this). This is the laser power required for cell ablation.

b. Focus on the region or cell of interest using the reticle installed in the ocular of the microscope. Use a 20x objective (40x will allow more precise damage, but often the working distance is not sufficient for this lens).

Note 1: The laser will not only injure cells within the focus plane, but also cells above and underneath that are within the laser beam. Make sure before the experiment that the laser is optimally set up and adjusted for a maximal focus within the z-axis. It is best to determine the depth of the laser injury before performing the experiment in order to know which cells exactly will be injured.

c. Set up the light needed for visualizing the experiment: brightfield for normal use, or fluorescence if targeting a fluorescently-labeled cell.
d. Send a pulse of laser light to the targeted region, or several pulses, depending on the desired degree of injury.

4. Analysis of Wounding and Recovery

a. If desired, the procedure of laser injury can be recorded live (*e.g.* using the stream acquisition option of the Metamorph software).[6]
b. Immediately after wounding, the embryo should be removed gently from the agarose with forceps and placed back into egg water for recovery.

Note 2: Muscular activity (swimming and twitching) is especially important for skeletal muscle recovery. Since the embryo cannot move because of tricaine exposure and the stiff agarose embedding, it is not recommended to maintain the embryos between slide and coverslip for too long.

c. After recovery, the embryo can be fixed and analyzed as desired by light-, fluorescence- or confocal microscopy as indicated.

Representative Results

Laser-mediated injury was performed on immobilized 1 day-old embryos. As shown in **Figure 1**, a few laser pulses can generate a small wound, easily recognizable by the damaged, coiled, Actin-rich myofibrils that are normally stretched between the somite boundaries. A higher number of laser pulses will however result in a massively damaged somite block, where most myofibrils are destroyed. Nevertheless, we can literally observe that "time heals all wounds", with small injuries healing faster than larger ones. After recovery, Actin stainings show that myofibrils again span the somite normally; however, additional information can be gained by staining for Xirp1. The Xirp1 protein is a good marker for detecting where injury and recovery have occurred, as it is ectopically localized along recovering myofibrils within formerly injured muscle cells[6].

For some purposes, it is important to determine which cells exactly are injured with the laser; therefore, it might be useful to genetically label some cells (*e.g.* by mosaically expressing a GFP-tagged protein) in order to be able to visualize precisely which cell is targeted and then to assess whether its neighboring cells have been affected as well by the laser pulse (**Figure 2**). After injury recovery and antibody staining, it becomes possible to find and analyze the targeted cell again thanks to the pattern of mosaically labeled cells surrounding it.

One interesting approach is to monitor regularly over time how the laser-mediated injury affects the somitic muscle. For this, the laser injury of the immobilized embryo can be recorded live to see the immediate effects, *e.g.* within the first minute (**Figure 3**). Furthermore, timelapse recordings can be performed with any desired time interval (*e.g.* 5 min interval), in order to follow closely how the cells rearrange around the wound.

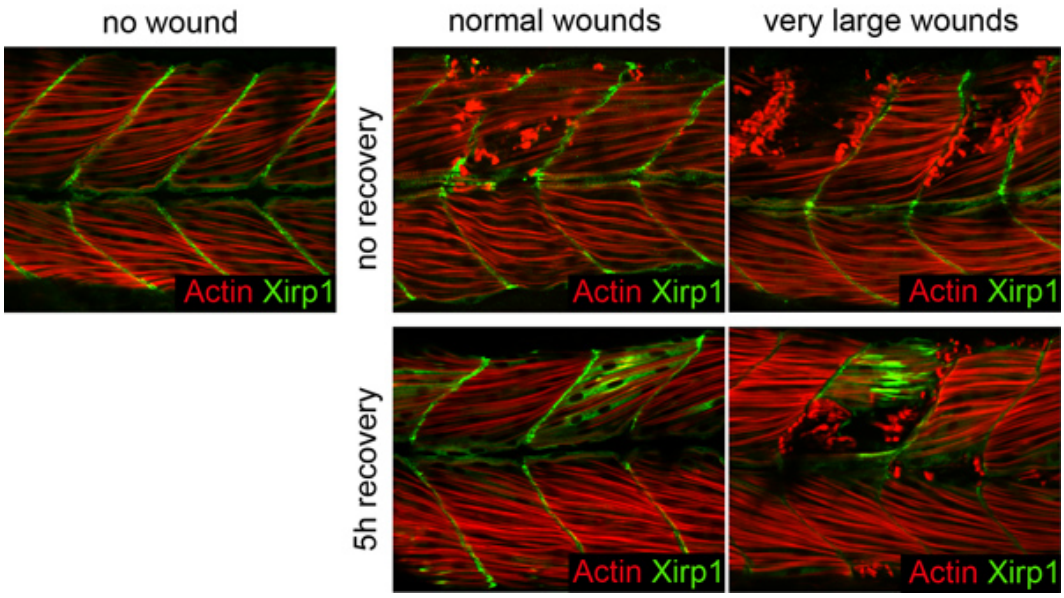

Figure 1. The severity of the injury inflicted can vary greatly, depending on the number of laser pulses applied. Accordingly, recovery from injury will proceed at difference paces, but eventually, most wounds are closed. We have recently shown that Xirp1 (green) is a good marker for muscle regeneration, since it is upregulated upon injury and localizes to non-sarcomeric Actin (red) of damaged myofibrils at 32 hpf[6].

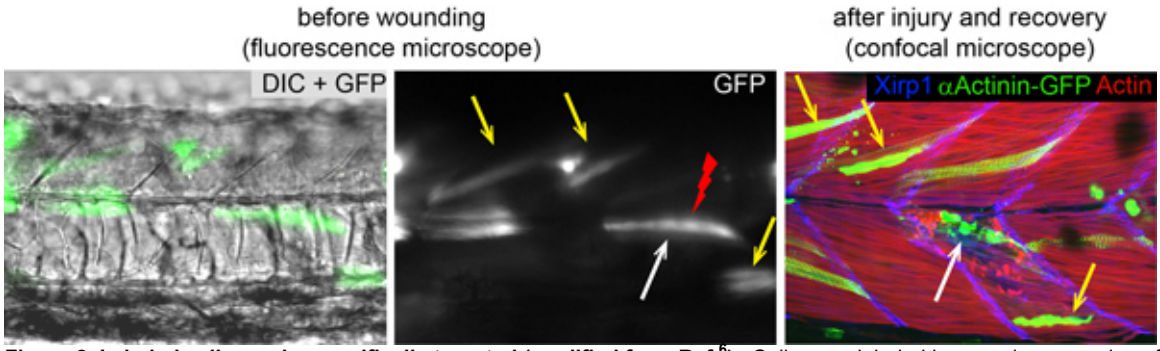

Figure 2. Labeled cells can be specifically targeted (modified from Ref [6]). Cells were labeled by mosaic expression of Tg[*β-actin:α-actinin-GFP*] (green or monochrome). One such cell was targeted here at 32 hpf and severely damaged by several laser pulses, as indicated by the red bolt. The white arrow indicates the injured cell; yellow arrows indicate other GFP-labeled cells. The picture on the right was taken after recovery and antibody staining of the same wound.

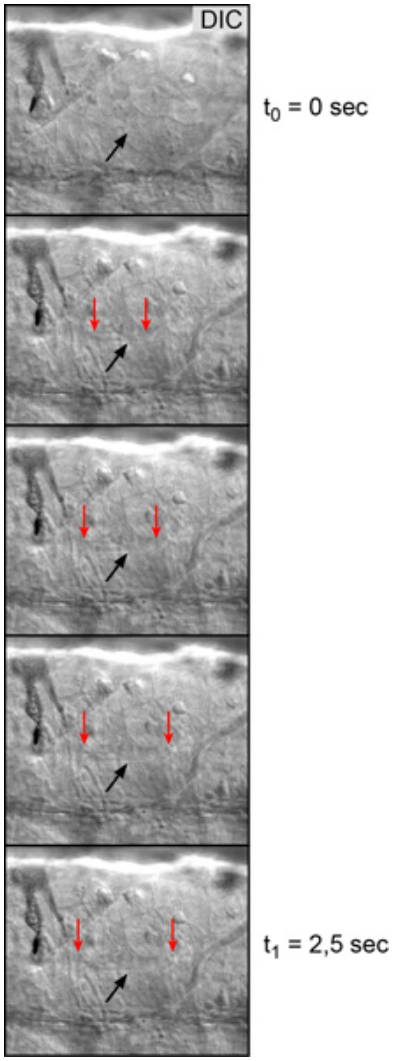

Figure 3. Live stream acquisition of laser injury reveals how the injury affects the entire somite block. Montage of single pictures recorded at the Axioplan in the live stream mode (*i.e.* about 16 frames / sec) with DIC. The black arrow indicates where the laser hits the somitic muscle at 28 hpf. Red arrows indicate the retracting ends of a damaged myofibril. These dynamic changes within one somite block occurred within 2.5 sec.

Discussion

Laser-mediated injury is a powerful method for inflicting wounds of a desired size by ablating cells in order to study regeneration under controlled conditions in the zebrafish embryo. Notably, cells can be targeted precisely (**Figure 2**) and both the injury area as well as timing can be controlled. Subsequently, the injury site and regeneration processes are easily monitored, recorded (**Figure 3**) and analyzed (**Figure 1**). However, although the laser is well focused in the x- and y-axis, it is not completely focused in the z-axis. This may cause damage to cells located underneath or above the cells of interest. Nevertheless, this approach opens up a vast field of *in vivo* investigations of muscle repair.

Disclosures

No conflicts of interest declared.

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