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Examining Muscle Regeneration in Zebrafish Models of Muscle Disease

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Abstract

Skeletal muscle has a remarkable ability to regenerate following injury, which is driven by obligate tissue resident muscle stem cells. Following injury, the muscle stem cell is activated and undergoes cell proliferation to generate a pool of myoblasts, which subsequently differentiate to form new muscle fibers. In many muscle wasting conditions, including muscular dystrophy and ageing, this process is impaired resulting in the inability of muscle to regenerate. The process of muscle regeneration in zebrafish is highly conserved with mammalian systems providing an excellent system to study muscle stem cell function and regeneration, in muscle wasting conditions such as muscular dystrophy. Here, we present a method to examine muscle regeneration in zebrafish models of muscle disease. The first step involves the use of a genotyping platform that allows the determination of the genotype of the larvae prior to eliciting an injury. Having determined the genotype, the muscle is injured using a needle stab, following which polarizing light microscopy is used to determine the extent of muscle regeneration. We therefore provide a high throughput pipeline which allows the examination of muscle regeneration in zebrafish models of muscle disease.

Introduction

Skeletal muscle accounts for 30-50% of human body mass, and is not only indispensable for locomotion, but it also serves as a critical metabolic and storage organ[1]. Despite being postmitotic, skeletal muscle is highly dynamic and retains a tremendous regenerative capacity following injury. This is attributed to the presence of tissue resident stem cells (also called satellite cells), located under the basal lamina of myofibers and marked by the transcription factors *paired box protein 7* (*pax7*) and/or *paired box protein 3* (*pax3*), among others[2,3]. Following injury, the satellite cell is activated and undergoes cell proliferation to generate a pool of myoblasts, which subsequently differentiate to form new muscle fibers. The highly conserved cascade of pro-regenerative signals regulating satellite cell activation and robust muscle repair can be affected in various conditions such as myopathies and homeostatic ageing[4,5].

One such diverse group of myopathies is muscular dystrophy, characterized by progressive muscle wasting

and degeneration[6]. These diseases are the consequence of genetic mutations in key proteins, including dystrophin and laminin-α2 (LAMA2), responsible for the attachment of muscle fibers to the extracellular matrix[7,8]. Given that proteins implicated in muscular dystrophy play such a central role in maintaining muscle structure, for many years it was believed that a failure in this process was the mechanism responsible for disease pathogenesis[9]. However, recent studies have identified defects in the regulation of muscle stem cells and subsequent impairment in muscle regeneration as a second possible basis for the muscle pathology observed in muscular dystrophy[10,11]. As such, further studies are needed to investigate how an impairment in muscle stem cell function and associated niche elements contributes to muscular dystrophy.

Over the past decade, zebrafish (*Danio rerio*) has emerged as an important vertebrate model for disease modeling[12]. This is attributed to the rapid external development of the zebrafish embryo, coupled with its optical clarity, which allows the direct visualization of muscle formation, growth, and function. Additionally, not only is the development and structure of muscle highly conserved in zebrafish, they also display a highly conserved process of muscle regeneration[13]. Consequently, zebrafish represent an excellent system to study the pathobiology of muscle diseases, and explore how muscle regeneration is affected in it. To this end, we have developed a method that enables the timely study of skeletal muscle regeneration in zebrafish models of muscle disease. This high throughput pipeline involves a method to genotype live embryos[14], following which a needle-stab injury is performed and the extent of muscle regeneration is imaged using polarizing light microscopy. The utilization of this technique will therefore reveal the regenerative capacity of muscle in zebrafish models of muscle disease.

Protocol

Zebrafish maintenance was carried out as per the standard operating procedures approved by the Monash University Animal Ethics Committee under breeding colony license ERM14481.

1. Determination of the genotype of live embryos using an embryo genotyping platform.

1. Anesthetize 3 days post fertilization (dpf) zebrafish embryos by adding tricaine methanesulfonate to a final concentration of 0.016% (v/v) in embryo medium (5 mM NaCl, 0.17mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in water). Wait for 10 minutes to ensure that the fish are completely anesthetized, evident when the fish stop swimming.
2. Prepare the 24 chamber DNA extraction chip by peeling the clear protective film from the top surface of the chip (**Figure 1A**).
3. Cut the tip of a 20 µL filter tip to widen the opening. Using this, pick a single embryo in a 13 µL volume of embryo medium and load into the first chamber of the chip. Repeat this process until embryos been dispensed into each chamber.
   NOTE: This step should be performed in an area with minimal air drafts, as high air flow may cause excessive evaporation of the fluid subsequently resulting in reduced genotyping sensitivity and survival of the embryos.
4. Once the desired number of embryos have been loaded onto the DNA extraction chip, load it onto the zebrafish

embryo genotyping platform. This is best achieved by placing in one side first, followed by the rest of it.

NOTE: Avoid adding too much pressure onto the platform as this may overstress and the underlying springs.

5. Affix the magnetic platform lid over the chip, which will prevent evaporation of embryo media during the DNA extraction protocol, and close the platform lid.

6. Set the base unit to 2.4 volts, 0.051 A, and 0.12 W and start the DNA extraction protocol by pressing the "ON/ OFF" button. The platform should start vibrating, which can be assessed by gently touching the lid. The protocol should be run for 8 minutes.

   NOTE: The vigorous vibration results in shedding of epidermal cells, from which genomic DNA is extracted.

7. While the program is running, prepare a 24 well plate by adding 1 mL of embryo medium to each well, which is necessary to separate individual embryos while downstream genotyping assays are being performed.

8. Additionally, appropriately label 8 well strip tubes, which will be used for collection of DNA material from each embryo.

9. At the completion of the 8 minute protocol, press the **ON/ OFF** button to stop the vibration of the platform.

10. Open the lid of the platform, and gently remove the magnetic lid ensuring minimal pressure is applied to the platform.

11. Carefully remove the DNA extraction chip from the platform and place it on a flat surface.

12. From the first chamber, remove 10 µL of embryo media surrounding the embryo and place it into the appropriate well of the 8-well strip tube. The media contains the genetic material from that embryo, which will be used for downstream assays.

    NOTE: Avoid touching the embryo with the pipette tip during media collection, as this can damage the embryo.

13. Using a plastic pipette, immediately add two drops of fresh embryo medium to the same chamber. Collect the embryo and move it to the first well of a 24 well plate.

14. Repeat steps 1.12 and 1.13 for all the remaining chambers. At the completion of this, the 24 well plate containing live embryos can be moved to a 28 °C incubator.

15. Perform appropriate downstream genotyping assays to determine the genotype of the embryos.

    NOTE: The DNA obtained from the embryo genotyping platform can be successfully amplified by PCR and can be used for subsequent analysis including sequencing, gel electrophoresis, or high-resolution melt-analysis[14]. To genotype the *lama2* strain described in the representative results, PCR, restriction digestion and gel electrophoresis were used. Given that the concentration of DNA obtained from each embryo is low, it is suggested to utilise most, if not all, of the genetic material obtained for the downstream assay.

16. Once the genotype of each larvae has been identified, transfer them to 90 mm Petri dishes, placing each genotype in a different dish. Fill the dish with 25 mL embryo medium and keep them in the 28 °C incubator.

2. Performing muscle injury using a needle stab

1. Once the larvae are 4 dpf, anesthetize them by adding tricaine methanesulfonate to a final concentration of 0.016% (v/v) in embryo medium. Wait for 10 minutes to

ensure that the fish are completely anesthetized, evident when the fish stop swimming.

NOTE: To avoid bias, the identity of the genotype should be blinded to the investigator performing the stabs and downstream analyses.

2. During this time, prepare 24 well plates by filling each well with 1 mL of fresh embryo medium, and set up the stereomicroscope with a black background and high-power light, to facilitate the visualization of the somite borders.

3. Using a plastic pipette, transfer one anesthetized larva into a new Petri dish.

4. Carefully remove excess embryo medium with a pipette, and under a dissecting microscope, orient the fish such that the head is on the left, tail on the right, dorsal region up and ventral region down (**Figure 1B**).

5. Working under a dissecting microscope, use a 30-gauge needle to perform a quick but precise stab in the epaxial muscle located above the horizontal myoseptum. To ensure consistency in anterior-posterior position, aim for 1-2 somites located above the anal pore (**Figure 1B**).

NOTE: Avoid stabbing the neural tube and notochord, and work quickly to avoid the drying of the fish during the process.

6. Using a plastic pipette, pour a drop of embryo medium on the stabbed zebrafish and carefully transfer it into a well of the 24 well plate.

7. Repeat steps 2.3 to 2.6. until all the fish have been stabbed.

NOTE: Several larvae can be stabbed together depending on the processing speed and proficiency of the investigator, as long as the fish do not dry out during the process.

8. Once all the larvae have been stabbed, place the 24 well plate in the 28 °C incubator until subsequent imaging is performed.

3. Imaging of muscle injury and recovery

1. At 1 day post injury (1 dpi), anaesthetize the stabbed larvae by adding tricaine methanesulfonate to a final concentration of 0.016% (v/v) in embryo medium. Wait for 10 minutes to ensure that the fish are completely anesthetized, evident when the fish stop swimming.

NOTE: At 0 dpi, the wound site contains a large amount of cellular debris, which makes it difficult to quantify the extent of muscle injury (**Supplementary Figure 1A-B**). It takes approximately 18-20 hours for this debris to be cleared from the wound site, and as such it is more reliable to image larvae at 1 dpi, rather than 0 dpi, to determine the extent of injury elicited.

2. Place a clean and empty glass bottom based dish on the stage of the polarizing microscope and set background using the integrated software.

NOTE: Do not use plastic Petri dishes to image birefringence, as they do not appropriately transmit refracted light. Depending on the polarized microscope used, additional settings may be required as per the manufacturer's guidelines.

3. Having set the background, remove the glass bottom based dish from the microscope stage, and using a glass pipette, transfer the anesthetized, stabbed fish onto the glass bottom based dish.

4. Carefully remove the excess of embryo medium using a pipette, and orient the fish as per 2.5 - the head is on the left, tail on the right, dorsal region up and ventral region down.

NOTE: Ensure the larvae is mounted as flat as possible, as uneven mounting results in different birefringence intensities across different somites.

5. Place the glass bottom based dish with the anesthetized larvae on the microscope stage and image the muscle using polarized light (**Figures 1C & 1D**).
   NOTE: Depending on the type of microscope/polarized lens used, the fish orientation on its anterior-posterior axis can affect the overall birefringence[15]. Ensure to include at least 5 somites on either side of the injury site when imaging.
6. Using a plastic pipette, pour a drop of embryo medium to rehydrate the fish and place the fish in a well of a 24 well plate filled up with embryo medium.
   NOTE: It is important to perform the imaging as quickly as possible to prevent the fish from drying.
7. Repeat steps 3.3. to 3.6. until all the fish have been imaged.
8. When all images have been acquired, save them in .tiff format for subsequent analyses.
9. Put the fish back into the 28 °C incubator until performing subsequent imaging at 3 dpi (7 dpf).
10. When the fish are 3 dpi, image the fish as per 3.3 to 3.8.
11. Once all fish have been imaged, euthanize them by adding tricaine methanesulfonate to a final concentration of 0.2% (v/v) in embryo medium. Wait for at least 10 minutes to ensure that the fish are euthanized, evident by the loss of swimming ability, pumping of the gill covers, and lack of a flight response following touch.

4. Quantification of muscle regeneration

1. Open a 1 dpi image on an imaging analysis software such as the freely available Image J software.
2. Using the polygon tool, draw around the wound site, and measure the area and mean birefringence intensity of this region (**Figures 1C & 1D**). Copy these values to cells D3 and E3 in the template provided (**Supplementary Table 1**).
3. Draw two additional regions, each spanning 1-2 uninjured somites, and measure the area and mean birefringence intensities of each of these regions (**Figures 1C & 1D**). Copy these values to cells D4-D5 and E4-E5 in the template provided (**Supplementary Table 1**).
   NOTE: While it is preferable to select the same uninjured somites in the 1 dpi and 3 dpi images, the sporadic detachment of muscle fibres and subsequent reduction in birefringence in mutants may make this impossible. Therefore, in the event the same somites cannot be selected at 1 dpi and 3 dpi due to the reduction in muscle integrity in mutants, select two different but unaffected areas at each of the timepoints.
4. Calculate the normalized birefringence for each region by dividing the mean birefringence intensity of that region by the area - displayed in column F in the template provided (**Supplementary Table 1**).
5. Repeat steps 4.1-4.4 for all 1 dpi and 3 dpi images.
   NOTE: When using the template provided (**Supplementary Table 1**), the 1 dpi area and mean birefringence intensity values should be inserted in columns D and E respectively, and the 3 dpi area and

mean birefringence intensity values should be inserted in columns J and K respectively.

6. For each time point, calculate the average normalized birefringence of the two uninjured regions. This value provides a reference point of uninjured muscle.

   NOTE: In the template provided (**Supplementary Table 1**), this value is computed in columns G and M, for the 1 dpi and 3 dpi images respectively.

7. Next, determine the extent of muscle injury at 1 dpi, by dividing the normalized birefringence of injury region by the average normalized birefringence of the uninjured regions (calculated in 4.6).

   NOTE: Using the above detailed needle stab procedure, wildtype larvae typically show a normalized birefringence of 48.5 ± 14.3% at the wound site at 1 dpi, indicating that the birefringence has reduced by approximately 50% when compared to uninjured somites. When using the template (**Supplementary Table 1**), this value is computed as a percentage in column H.

8. To determine the extent of muscle regeneration, divide the normalized birefringence of the injury region in the 3 dpi image, by the average normalized intensity of the uninjured regions at this stage.

   NOTE: At 3 dpi, wildtype larvae typically show a normalized birefringence of 60 ± 15.3% at the wound site. Given that the normalized birefringence within the wound site at 1 dpi was 48.5 ± 14.3% (step 4.7), the increase in birefringence at 3 dpi to 60 ± 15.3% indicates a recovery of approximately 11.5%. When using the template (Supplementary Table 1), this value is computed as a percentage in column N.

9. Keeping fish within each genotype separate, perform a paired t-test comparing the normalized birefringence of the wound site at 3 dpi (step 4.8) with that of 1 dpi (step 4.7). This will reveal the trajectory of muscle regeneration displayed by each fish in each genotype, and highlight if the extent of muscle regeneration displayed by each genotype has significantly altered.

10. Finally, calculate the regenerative index by dividing the value obtained in step 4.8, which is the extent of muscle regeneration at 3 dpi, by the value obtained in step 4.7, which is the extent of muscle injury at 1 dpi. A regenerative index of 1 indicates that the injury at 1 dpi is comparable to 3 dpi and that muscle regeneration has not occurred; a value above 1 indicates that at 3 dpi new muscle has formed in the wound site highlighting that the muscle has regenerated; and a regenerative index of less than 1 highlights that the wound at 3 dpi is worse than 1 dpi, and that muscle regeneration in impaired. A t-test or a one-way ANOVA can be performed to statistically compare the extent of muscle regeneration between the different genotypes. When using the template provided (**Supplementary Table 1**), this value is calculated in column O.

    NOTE: It is recommended to perform the entire experiment in triplicates, with fish from each experiment obtained from different biological parents, and each experiment performed on different days, to avoid any bias.

Representative Results

The ability to quantify birefringence of skeletal muscle provides a non-invasive but highly reproducible method to examine and compare levels of muscle damage, and examine muscle regeneration *in vivo*. Birefringence results from the diffraction of polarised light through the pseudo-crystalline array of the muscle sarcomeres[15], and following injury or damage to the muscle, a reduction in birefringence is evident. Likewise, the activation and differentiation of stem cells results in the formation of new muscle fibres within the injury site, subsequently increasing birefringence intensity within this region. Using this system, we have examined muscle regeneration in a zebrafish model of congenital muscular dystrophy type 1A (MDC1A), caused by a deficiency in Lama2[16]. A clutch of embryos from a cross between two *lama2*$^{+/-}$ zebrafish was collected, and at 3 dpf, the embryos were transferred to a DNA extraction chip (**Figure 1A**) and subsequently genotyped using a embryo genotyping technology. Having identified the genotype, *lama2*$^{-/-}$ larvae, which model MDC1A[16], and *lama2*$^{+/+}$ siblings were injured using a needle stab as per **Figure 1B**, and imaged on a polarizing microscope at 1 dpi, and 3 dpi, and the birefringence intensities were quantified. While muscle injury results in a reduction in birefringence intensity at 1 dpi (**Figure 1C^{i} and D^{i}**), the successful regeneration of muscle results in increased birefringence in the same region (**Figure 1C^{ii} and D^{ii}**). It is also noteworthy that while *lama2*$^{+/+}$ larvae display uniform birefringence intensity (**Figure 1C**), due to normal muscle patterning, the birefringence intensity in the muscle of *lama2*$^{-/-}$ was uneven and highly sporadic (**Figure 1D**), attributed to reduced muscle integrity.

Using this approach, we reveal that both wildtype larvae (*lama2*$^{+/+}$; **Figure 2A**), and larvae deficient in *lama2* (*lama2*$^{-/-}$; **Figure 2B**), show significantly increased birefringence intensity in the wound site at 3 dpi compared to 1 dpi (**Figure 2C**), indicating that the muscle has regenerated. To compare the regenerative potential of larvae in each genotype, the regenerative index was determined, and we reveal that *lama2*$^{-/-}$ larvae displayed a striking increase in muscle regeneration compared to *lama2*$^{+/+}$ larvae (**Figure 2D**; mean in *lama2*$^{-/-}$ = 1.30 ±- 0.251; mean in *lama2*$^{-/-}$ = 1.83 ± 0.439). To further validate the improved regeneration in *lama2*$^{-/-}$ larvae, we stained the muscle with an antibody against F-Actin (Supplementary **Figure 1B-D**). While these results confirm that *lama2*$^{-/-}$ do indeed regenerate, evident by the presence of differentiated muscle fibres within the wound site, the inability to examine the same fish at 1 dpi and 3 dpi limits the ability to quantify and compare the regenerative response between *lama2*$^{+/+}$ and *lama2*$^{-/-}$ fish. Although the mechanistic basis for this improved regeneration capacity in *lama2*$^{-/-}$ larvae remains elusive, we believe that the loss of *lama2* increases the number of activated stem cells, which subsequently results in improved muscle regeneration. However, further studies are needed to determine this. Collectively, these results highlight that ability of the described technique to identify changes in muscle regeneration in zebrafish models of muscle disease.

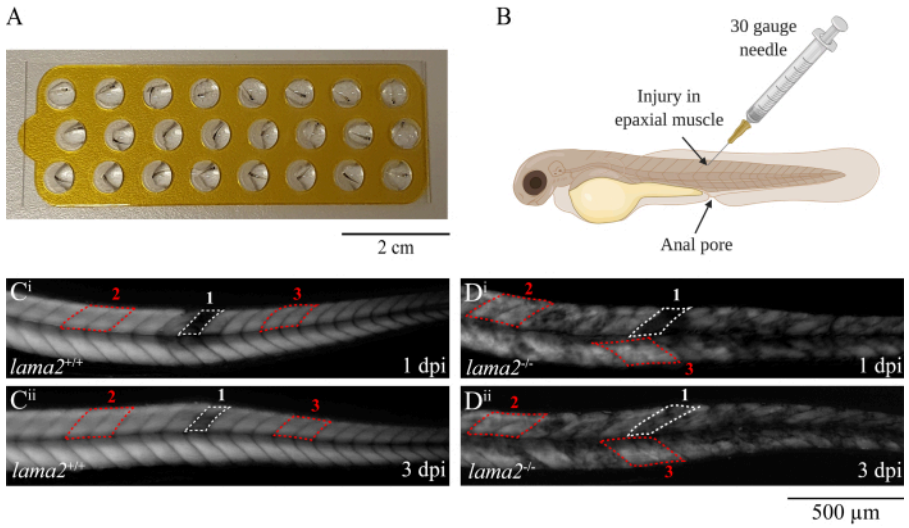

Figure 1: Overview of the genotyping and muscle regeneration protocol. (A) Image of a DNA extraction chip containing 24, 3 dpf zebrafish larvae. (B) Schematic of the orientation in which the 4 dpf larvae should be placed to perform the needle stab, with the head on the head, tail on the right, dorsal region up and ventral region down. The needle stab should be performed using a 30-gauge needle, targeting 1-2 somites of epaxial muscle. Created with BioRender.com. (C-D) Images of birefringence in a *lama2*$^{+/+}$ and *lama2*$^{-/-}$ larvae at 1 dpi and 3 dpi. Regions shown in white and red reflect the areas used to quantify the birefringence in the wound site and uninjured somites respectively. Please click here to view a larger version of this figure.

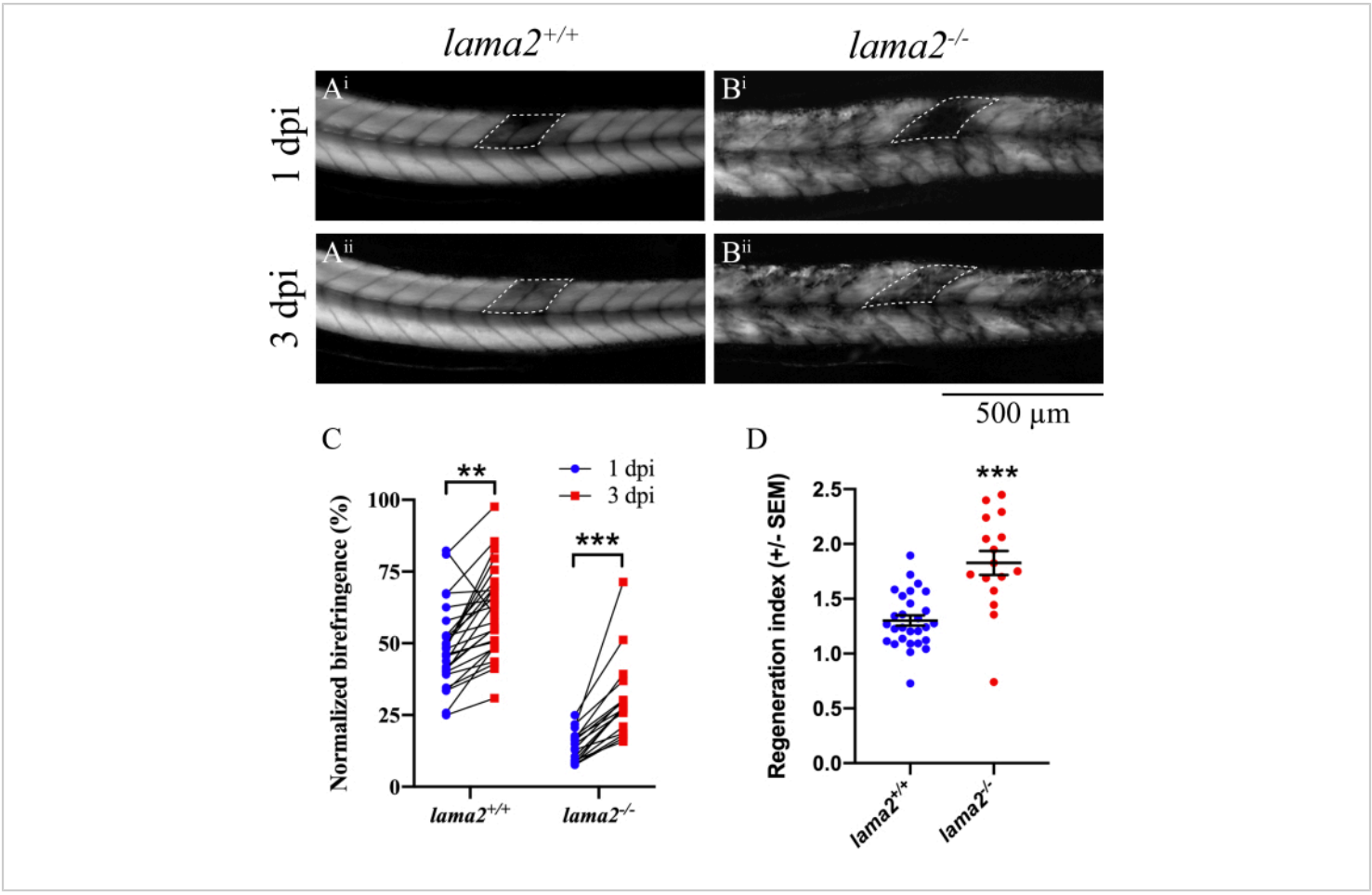

Figure 2: Quantification of muscle regeneration in *lama2* deficient zebrafish larvae. Images of birefringence in *lama2*$^{+/+}$ (A), and *lama2*$^{-/-}$ (B) larvae at 1 dpi and 3 dpi. The wound at 3 dpi in both, *lama2*$^{+/+}$ *and lama2*$^{-/-}$ larvae is filled with new muscle. (C) Graph of the normalized birefringence of each larvae at 1 dpi and 3 dpi. The normalized birefringence in the wound site in *lama2*$^{+/+}$ *and lama2*$^{-/-}$ larvae is significantly increased at 3dpi, as determined using a paired t-test. (D) Regenerative index in *lama2*$^{+/+}$*, and lama2*$^{-/-}$ with the latter showing increased muscle regeneration, as determined using a t-test. Error bars represent SEM with larvae from three independent experiments *(lama2*$^{+/+}$n=28, and *lama2*$^{-/-}$ n=16). Please click here to view a larger version of this figure.

Supplementary Figure 1: Examination of muscle regeneration in *lama2* deficient larvae. Images of birefringence in *lama2*$^{+/+}$ (Ai), and *lama2*$^{-/-}$ (Aii) larvae at 0 dpi demonstrating the presence of cellular debris within the wound site. Maximum projection confocal images of the larval myotome stained for F-actin at 0 dpi (B), 1 dpi (C) and 3 dpi (D). At 3 dpi, the wound site of *lama2*$^{+/+}$ and *lama2*$^{-/-}$ larvae is characterized by the appearance of F-actin labelled muscle fibers. Please click here to download this file.

Supplementary Table 1: Template for the quantification of muscle regeneration. Please click here to download this file.

Discussion

Skeletal muscle regeneration is driven by obligate tissue resident muscle stem cells, whose function is altered in many muscle diseases such as muscular dystrophy, subsequently impeding the process of muscle regeneration. Here, we describe a high throughput protocol to examine muscle regeneration in live zebrafish models of muscle disease. The first step of the pipeline utilizes a embryo genotyping platform[14], which is a user-friendly and accurate method to determine the genotype of live larvae, before performing the downstream regeneration assay. A direct benefit of this is that it allows the selection of genotypes that are of interest only, and/or comparable number of fish within each genotype group, therefore significantly reducing the number of fish that need to be processed through the rest of the protocol. However, it is noteworthy that the quantity of genomic DNA obtained from the embryo genotyping device is relatively low, which may compromise downstream genotyping assays. It is therefore important that all genotyping assays are tested and optimized before using it for the main experiment. Additionally, the source of the DNA is primarily epidermal, and as such, the embryo genotyping system cannot be successfully used to genotype larvae displaying tissue specific mutations.

The second part of the protocol demonstrates the use of a needle-stab injury, which is a cost-effective and high throughput technique that not only results in a highly reproducible injury size, but is also sufficient to trigger the activation of muscle stem cells resulting in muscle regeneration. An alternative approach to inducing skeletal muscle injury in zebrafish is to use laser mediated cell ablation[13,17,18] . While this provides the ability to focus on specific x, y and z planes and subsequently target single muscle cells, the extremely small wound size induced limits accurate quantification of muscle regeneration, especially in muscle disease models whereby the integrity of muscle is already compromised. We therefore favor the use of needle stab injuries when examining muscle regeneration in zebrafish muscle disease models.

To accurately quantify the extent of muscle regeneration, we take advantage of the birefringent nature of skeletal muscle, which can easily be imaged using a standard polarizing microscope. There are two critical points that need to be considered while using this approach. Firstly, depending on the type of microscope and/or polarized lens used, the orientation of the fish in its anterior-posterior axis may affect overall birefringence intensitites[15], and this needs to be considered while imaging. Finally, although muscle regeneration is not fully complete by 3 dpi, the extent of recovery is sufficient to enable the distinction of regeneration capacities in different strains[13] (**Figure 1**). Our previous work has demonstrated that using the protocol we have outlined, wildtype larvae fully regenerate after 14 dpi[19], and therefore, to determine if a strain cannot regenerate or if muscle regeneration is delayed, it may be necessary to examine the birefringence intensities beyond 3 dpi.

It must be noted that in teleost fishes, muscle growth occurs throughout the life of the animal[20], and as such, it is very important to normalize the birefringence intensities of the wound site, with that of uninjured somites within the same fish. This step provides an internal control and removes bias in the analyses due to differences in the amount of muscle at the different timepoints, or differences in imaging parameters during different sessions. This normalization step is also imperative to accurately quantify muscle regeneration in models of muscle disease, which may inherently have reduced myofibrils and/or display

compromised muscle integrity, subsequently reducing overall birefringence intensities. Additionally, while this protocol can effectively reveal changes in the regenerative capacity of muscle, it is possible that they are as a result of indirect effects on stem cell function. Muscle regeneration is a complex process involving signals from multiple cell types including muscle cells, macrophages, fibro-adepogenic progenitors, and interstitial cells. It is possible that the altered capacity of muscle to regenerate maybe explained by changes in the biology of any of these other cell types which subsequently influence muscle stem cell function and the regenerative process. Therefore, while this protocol can identify alterations in muscle regeneration in models of muscle disease, downstream cellular and molecular analyses need to be performed to identify the mechanism(s) responsible for the changes observed.

In conclusion, the regenerative capacity of muscle in various muscle diseases is not fully understood, and the emergence of new techniques to examine muscle regeneration *in vivo* provides a platform to tackle such questions. The method can be used as a basis for the exploration of cellular and molecular cues that regulate muscle regeneration in zebrafish models of muscle disease.

Disclosures

The authors have nothing to disclose.

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