Published in final edited form as:

Methods Mol Biol. 2016 ; 1411: 291–312. doi:10.1007/978-1-4939-3530-7_19.

Imaging Approaches to Investigate Myonuclear Positioning in *Drosophila*

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Abstract

In the skeletal muscle, nuclei are positioned at the periphery of each myofiber and are evenly distributed along its length. Improper positioning of myonuclei has been correlated with muscle disease and decreased muscle function. Several mechanisms required for regulating nuclear position have been identified using the fruit fly, *Drosophila melanogaster*. The conservation of the myofiber between the fly and vertebrates, the availability of advanced genetic tools, and the ability to visualize dynamic processes using fluorescent proteins in vivo makes the fly an excellent system to study myonuclear positioning. This chapter describes time-lapse and fixed imaging methodologies using both the *Drosophila* embryo and the larva to investigate mechanisms of myonuclear positioning.

Keywords

Drosophila; Muscle; Nuclear movement; Embryo; Larvae

1 Introduction

The skeletal muscle provides an important system in which to study mechanisms of myonuclear positioning. Myofibers, the cellular units of the skeletal muscle, are multinucleated and position their myonuclei to reside above the sarcomeres at the cell periphery and along the length of the fiber to maximize internuclear distance. Moreover, mispositioned myonuclei correlate with muscle disease [1], and recent data suggest that mispositioned myonuclei may cause muscle weakness [2–4], illustrating the functional importance of proper positioning.

To identify the cellular mechanisms of myonuclear movement in vivo, we have utilized the musculature of *Drosophila* embryos and larvae [2–6]. *Drosophila* muscles share the conserved myofiber structure found in vertebrates: *Drosophila* myofibers are multinucleated, maintain particular sizes and shapes, attach to particular tendon cells, become innervated by specific motoneurons, build sarcomeres, and are essential for locomotion (reviewed in [7, 8]). The *Drosophila* system also offers unparalleled genetic approaches to manipulate genes of interest within the whole organism or specifically within muscle tissue. In addition to their powerful genetics, *Drosophila* embryos are also amenable to drug treatments commonly used to manipulate cell biological processes in culture, providing additional

opportunities to probe the cellular machinery responsible for myonuclear movement [6]. Lastly *Drosophila* embryos are amenable to time-lapse microscopy without perturbing development such that cell biological processes, including myonuclear dynamics, can be assessed [5].

There are 30 individual muscles per abdominal hemisegment of the *Drosophila* embryo (Fig. 1). Formation of these individual body wall myofibers depends on the specification and fusion of two myoblast cell types: founder cells (FCs) and fusion-competent myoblasts (FCMs). Each FC contains the information necessary to direct the formation of a specific muscle. FCs can be identified by the expression of identity genes, such as the transcriptional regulators *even-skipped* and *apterous* [9–11]. The combination of identity genes and chromatin regulators expressed by a particular FC regulates the final morphology of the specific muscle. In contrast, FCMs are more naïve cells. Upon fusion to an FC, FCMs become reprogrammed to the specific developmental program of the FC, as evidenced by the observation that each newly incorporated FCM nucleus begins to express the same combination of identity genes as the FC [7]. Myoblast fusion is an iterative process; depending on the particular muscle, body wall muscles in *Drosophila* embryos contain between 2 and 25 myonuclei. As myoblast fusion concludes, the polarized syncytial myotube extends processes toward specific tendon cells and forms stable myotendinous junctions. Specific motoneurons innervate each muscle, leading to coordinated contraction at the final stage of embryogenesis [8, 12].

Although mispositioned nuclei are observed in all muscles when certain genes are disrupted, myonuclear positioning has been best characterized in the lateral transverse (LT) muscles of the developing embryo. We have shown that LT myonuclei undergo not one but a series of movements to maximize internuclear distance in vivo (Fig. 2; [2–5]). Post-fusion (stage 14), the myonuclei are located in a single group within the myotube. Over the course of development, these nuclei segregate into smaller groups that follow characteristic migration patterns (stages 15, 16) before evenly distributing throughout the myofiber at the end of embryonic stage 17 [2, 8]. During larval development, in which the muscles grow up to 40-fold [13], the myonuclei in the individual muscles maintain regular spacing along the muscle fiber. These myonuclei are positioned above the sarcomeres along the periphery of the muscle fiber (Fig. 3; [2]).

To date, there are several mechanisms required for the different movements of myonuclei in *Drosophila*. These mechanisms require microtubules; microtubule-associated proteins, such as ensconsin; the microtubule motor protein s, kinesin and dynein; and motor protein adaptors, such as kinesin light chain, dynein light chain, p150/Glued, cytoplasmic linker protein-190 (CLIP190), sunday driver (Syd or JNK-interacting protein 3 (JIP3)), and JNK signaling [2–5]. We refer the reader to Schulman et al. [8] in which we discuss our current thinking about the different mechanisms at work during each stage of myonuclear movement. To identify these mechanisms, we have developed various techniques to define and quantify myonuclear position in *Drosophila* [2–5]. This chapter describes these methodologies in both fixed and live *Drosophila* embryonic and larval preparations.

2 Materials

2.1 Imaging of Fixed Embryonic Muscles

2.1.1 Embryo Collection and Fixation (Fig. 4)

1. Embryo laying pot: 100 ml plastic beaker punched with holes to allow air exchange and prevent condensation. To make holes use a hot syringe needle with gauge (Sub-Q 26G5/8).
2. Embryo collection plates (apple juice plates): Microwave 1500 ml of ddH₂O, 50 g of granulated sugar, and 45 g of agar until a homogeneous solution is formed. Add 500 ml of cold apple juice, cool solution to 65 °C, and add 40 ml of 10 % Tegosept in 100 % ethanol. Pour solution into petri dishes (60 × 15 mm); make approximately 200 plates. Secure these plates to the laying pots with rubber bands.
3. Yeast paste: Distill water plus dry baker's yeast and stir to make a paste. Store at 4 °C.
4. Paintbrush (size 0).
5. Collection baskets: Cut the top off of a 15 ml Falcon tube at approximately the 12 ml line and cut out the center of the tube cap.
6. Nylon membrane (pore size, 140 µm) screwed onto the collection basket with the cap.
7. 3 % sodium hypochlorite (50 % bleach).
8. Small funnel.
9. Eppendorf tubes (1.5 ml).
10. Heptane.
11. Methanol.
12. PFA: 4 % EM grade paraformaldehyde diluted in PBS (phosphate-buffered saline).
13. Platform shaker.
14. Vortex with 1.5 ml Eppendorf tube adaptor.
15. PFA/heptane waste receptacle.
16. Methanol/heptane waste receptacle.
17. Glass Pasteur pipets.

2.1.2 Embryo Staining

1. 3 % Triton X-100: For 200 ml, add 6 ml of Triton X-100 and 194 ml ddH₂O.

2. 10× PBS (phosphate-buffered saline; 0.01 M KH_2PO_4 , 0.1 M Na_2HPO_4 , 1.37 M NaCl, 0.027 M KCl, pH 7.0).
3. PBT (phosphate-buffered saline containing 0.3 % Triton): For 100 ml, add 10 ml 10× PBS, 10 ml 3 % Triton X-100, and 80 ml ddH_2O .
4. PBT-BSA (phosphate-buffered saline containing 0.3 % Triton and 0.1 % BSA): For 100 ml, add 10 ml 10× PBS, 10 ml 3 % Triton X-100, 1 ml 10 % BSA, and 79 ml ddH_2O .
5. Primary and secondary antibodies of interest. Refer to Table 1 for a suggested list of antibodies and probes routinely used to label *Drosophila* muscle and nuclei.
6. Three-dimensional rotating mixer such as a Nutator.
7. Eppendorf tubes (0.5 ml).
8. Glass Pasteur pipets.

2.1.3 Embryo Mounting

1. Microscope slides.
2. Scotch tape.
3. Cover slips: size 22×40 mm with thickness 0.16–0.19 mm.
4. Mounting medium, such as ProLong Gold antifade reagent or Vectashield.
5. Razor blades.
6. Metal washers (any hardware store).

2.1.4 Embryo Imaging—All images in the lab are acquired on a Leica SP5 laser-scanning confocal microscope equipped with a $20\times$ 0.7NA HC PL Apochromat oil objective, $63\times$ 1.4NA HCX PL Apochromat oil objective, and LAS AF 2.2 software. Equivalent microscopes and software may be used.

2.2 Time-Lapse Imaging of Embryonic Muscles

2.2.1 Embryo Collection and Mounting (Fig. 5)—Materials described in Subheading 2.1.1, plus:

1. Plain-tipped applicator (wooden stick).
2. Halocarbon oil 700.
3. Air-permeable Teflon membrane mounted on a circular Perspex frame (designed by E. Wieschaus; [14, 15]).
4. Cover slips: size 22×40 mm with thickness 0.16–0.19 mm.

2.2.2 Embryo Imaging—Mounted embryos are imaged on a Leica SP5 laser-scanning confocal microscope equipped with a $63\times$ 1.4NA HCX PL Apochromat oil objective and

LAS AF 2.2 software (for imaging nuclear clusters or individual nuclei [5]) or on a Zeiss AxioPlan2 epifluorescence imaging system with light microscopy with a 40× Plan-NeoFluar 0.75NA objective and AxioVision Rel 4.8 software (for myonuclear movement in the whole embryo; [2]). Equivalent microscopes and software may be used.

2.3 Imaging of Formalin-Fixed Larval Muscles

2.3.1 Larvae Collection

1. Laying pot as described in Embryo Collection and Fixation from Imaging of Fixed Embryonic Muscles section (*see* Fig. 4).
2. Lightly yeasted apple juice plates (*see* Subheading 2.1.1, **step 2**).
3. 15 % sucrose solution: sucrose diluted in water.
4. Embryo hook or straight forceps (tip dimensions, 0.1 × 0.06 mm).
5. Dissection microscope equipped with fluorescence.
6. Paintbrush.

2.3.2 Larvae Dissection and Fixation

1. Dissection pins (diameter, 0.1 mm).
2. Two pairs of straight forceps (tip dimensions, 0.1 × 0.06 mm).
3. Dissection plate: petri dish (150 × 15 mm) coated with a thick layer of Sylgard.
4. Vannas spring scissors (sharp tips; non-serrated; cutting edge, 3 mm; tip diameter, 0.05 mm).
5. Cold HL3.1 buffer: 70 mM NaCl, 5 mM KCl, 0.2 mM CaCl₂, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES; pH 7.3 [16].
6. 1× PBS.
7. Formalin solution, 10 %.
8. Glass Pasteur pipets.

2.3.3 Larvae Staining and Mounting—As in Subheadings 2.1.2 and 2.1.3, plus:

1. Straight forceps (tip dimensions, 0.1 × 0.06 mm).
2. Kimwipes tissues.
3. Dissection scope.

2.3.4 Heat-Fixed Larvae

1. Water (distilled) at 65 °C.
2. Halocarbon oil or 50 % glycerol.

3. Microscope slides.
4. Cover slips: size 22 × 40 mm with thickness 0.16–0.19 mm.

2.3.5 Larval Imaging—Images are acquired using the 40× 1.25–0.75NA HXC PL Apochromat oil objective of a Leica SP5 laser-scanning confocal microscope and the LAS AF 2.2 software. Equivalent microscopes and software may be used.

2.4 Fly Stocks

The *Drosophila* community has developed a rich collection of genetic reagents to probe various cellular processes, including myonuclear positioning. In addition to classic mutations, there is a wide range of available fly stocks that allow tissue-specific manipulation of a gene of interest. Spatial and temporal manipulation is accomplished using the bipartite GAL4/UAS system [17]. Reagents useful for the study of myonuclear positioning are listed in Table 2. New reagents are constantly being developed, particularly with the development of CRISPR genome-editing approaches in *Drosophila*. We refer the reader to FlyBase (<http://flybase.org/>), a community resource that provides a comprehensive, searchable compendium of genes, mutations, expression patterns, available stocks, and references.

3 Methods

3.1 Imaging of Fixed Embryonic Muscles

3.1.1 Embryo Collection

1. Set up a laying pot with 60 females/female virgins and 30 males at 22 °C (room temperature), 25 °C, or 29 °C, depending on the experiment (see Note 3).
2. Label and change the apple juice plates on laying pots, keeping the old ones for embryo collection.
3. Pour enough 50 % bleach to cover embryos on each plate and remove dead flies with a paintbrush. Dechorionate embryos for 4 min at room temperature. During this period, label 1.5 ml Eppendorf tubes and add 500 µl heptane to each.
4. Gently disturb the embryos on the plate with a paintbrush and transfer them to a collection basket using a small funnel. Rinse the plate with water to collect any remaining embryos and transfer them to the collection basket. Rinse the embryos in the collection basket liberally with additional distilled water to remove any traces of bleach.
5. Move the embryos to the Eppendorf tube containing the heptane with the paintbrush.

³Although the ideal number of flies in a laying pot is 60 females/female virgins and 30 males, laying pots can be set up with as few as 25 females/female virgins and 15 males. Using a large number of flies in a laying pot is important when collecting large amounts of embryos.

3.1.2 Embryo Fixation

1. Thaw an aliquot of 4 % PFA.
2. Add 500 µl of PFA to the Eppendorf tube containing the heptane and embryos.
3. Using a platform shaker set at 250 rpm, incubate the embryos for 20 min at room temperature.
4. Remove both the PFA and heptane phases, leaving the embryos in the Eppendorf tube.
5. Add 500 µl of fresh heptane and 500 µl of 100 % methanol (for an alternative fixation method to label fixed embryos with phalloidin, *see* Note 4).
6. Vortex for 1 min at room temperature. Embryos that have been devitellinized will fall to the bottom of the tube, whereas those that remain at the interface or float are not devitellinized and should be discarded.
7. Remove all liquid.
8. Rinse embryos in methanol three times. To rinse: add solution, invert tube, let settle, remove, and repeat.
9. Add fresh 100 % methanol for storage at –20 °C. Embryos can be stored and used reliably for up to 6 months.

3.1.3 Embryo Staining

1. Transfer embryos to a 0.5 ml Eppendorf tube and remove excess methanol.
2. Rinse three times with PBT-BSA.
3. Add fresh PBT-BSA and incubate for 30 min on Nutator at room temperature. BSA serves to reduce nonspecific binding of antibodies.
4. Add primary antibodies diluted in PBT-BSA (Table 1). As an example, use antibodies directed to tropomyosin or myosin heavy chain to visualize muscles and DsRed to label the nuclei of the lateral transverse (LT) muscles expressing the *apterousME::NLSdsRed* (Figs. 1 and 2). Incubate primary antibodies with the embryos on the Nutator for 1 h at room temperature or overnight at 4 °C.
5. Remove primary antibodies and store at 4 °C (most can be reused up to three times).
6. Rinse embryos with PBT-BSA three times.

⁴For labeling fixed embryos with phalloidin, an alternative fixation method in which methanol is substituted with ethanol should be used. After fixing in heptane and PFA, add 100 % ethanol, vortex for 45 s, discard the ethanol, add 100 % ethanol, vortex for 45 s, collect embryos to a new tube, and store in fresh 90 % ethanol at –20 °C.

7. Wash embryos with PBT-BSA three times for 10 min at room temperature on the Nutator.
8. Remove PBT-BSA and add secondary antibodies (Table 1), diluted in PBT-BSA, to the embryos and incubate on the Nutator for 1 h at room temperature. As fluorescent antibodies are light sensitive, cover them with aluminum foil. From this point forward, keep them covered as much as possible until the staining protocol is finished.
9. Remove and discard secondary antibodies.
10. Rinse embryos with PBT (no BSA) three times.
11. Wash embryos with PBT three times for 10 min on the Nutator at room temperature.

3.1.4 Embryo Mounting

1. While washing the embryos (**step 12** above), prepare slides for mounting. To mount embryos, first make a “tape bridge,” comprised of two superimposed pieces of Scotch tape, on two opposite sides of the cover slip (Fig. 5a).
2. Label the slide with date, genotype or cross, and the antibodies used.
3. Place a metal washer on the slide and rest the cover slip on the washer with the tape bridge facing up.
4. Cut 1 cm off of the end of a p200 pipet tip for each sample so that embryos can be pipetted without damage.
5. Remove PBT from the last wash (**step 12** above).
6. Add 80 µl of ProLong Gold antifade reagent to embryos using the truncated p200 tip (ProLong Gold is extremely viscous) and slowly mix them together.
7. Transfer embryos to the cover slip. Remove any visible bubbles.
8. Place a new cover slip over the tape bridge and line up both cover slips to form a sandwich.
9. Store overnight in the dark until the ProLong Gold sets.
10. Remove the washer and tape the cover slip sandwiches to the slide. These slides are ready to be imaged.
11. Slides can be stored at -20°C for up to 6 months.

3.1.5 Confocal Microscopy—While the data indicate that similar movements and mechanisms are active in other *Drosophila* muscle subsets, our lab has focused on studying myonuclear movements in a subset of muscles, the lateral transverse muscles (LT). Hence, the methodology for confocal imaging described below is focused on this subset.

1. On the confocal microscope, identify an embryo at the correct stage (stages 14–16) positioned with its lateral side facing up using the low-magnification objective. Staging is done based on overall embryo shape, the intensity of DsRed and tropomyosin signal when used, gut morphology, and the morphology of the trachea [2, 3, 18]. At stage 16, a control LT muscle shows two clusters of nuclei (3–4 nuclei in each cluster) near each longitudinal pole of the muscle.
2. After finding a correctly staged embryo for imaging, move to a higher magnification objective, such as the 63× objective, and increase the zoom to 1.5×. On the computer, position the embryo in a horizontal position with four hemisegments visible in the imaging field. Choose hemisegments 3–7 for imaging (*see* ^{Note 5}).
3. To set the Z-stack, begin by selecting the first slice in which the LT muscles are visible, and after moving through the muscle, stop and select the last slice in which these muscles are no longer visible. The recommended step size is 0.5 μm. The total size of the stack will vary between embryos, but the step size should be kept constant. The gain for each channel should be adjusted according to the intensity of the corresponding signal.

3.1.6 Image Analysis—Multiple software packages, including Volocity Visualization Software 6.1.1 (Improvision), Imaris (Bitplane), and Adobe Photoshop CS4, are used for image processing. ImageJ (NIH) software is used for measurements. With the appropriate plug-in (*bioformats_package*), however, files from Leica or Zeiss confocal microscopes can be opened directly in ImageJ, and all rendering, processing, and analysis can be done within ImageJ. Therefore, the instructions below will refer to ImageJ software:

1. Open your files by dragging them into ImageJ toolbar.
2. For the nuclear positioning analysis, select the extended focus view to visualize whole muscles. Select: Image > Stacks > Z Project and select Standard Deviation as the projection type (*see* ^{Note 6}). Adjust the number of slices to be included in the final projection.
3. Merge channels by selecting Image > Color and Merge Channels.
4. Brightness and contrast, as well as other settings, can be adjusted by selecting Adjust under the Image menu (Image > Adjust > Brightness/Contrast).
5. For analysis, position the image with the anterior side of the muscle set to the left and dorsal to the top of the viewing field. The image can be rotated

⁵When imaging embryos, it is recommended to focus on hemisegments 3–7 because these hemisegments are developmentally more similar in age and are less prone to variations in muscle structure.

⁶When rendering a Z-stack in ImageJ, a number of projection types are available (average intensity, maximum intensity, minimum intensity, sum of slices, standard deviation, and median). The type selected depends on what is being measured. Maximum intensity is suggested if the user is measuring that particular signal. We use standard deviation as it renders an excellent image of the sarcomeric structure in the muscle; however, we are measuring distances between the nuclei.

by selecting Transform under the Image menu (Image > Transform > Rotate).

6. For measuring nuclear positioning, turn on the segmented line option. To do this, go to the ImageJ toolbar, right-click on the line option (represented as a straight diagonal line), and select Segmented Line.
7. Three different measurements are made for each muscle (Fig. 2a): Dorsal (D)—Distance from the dorsal end of each muscle to the nearest edge of the closest nucleus; Ventral (V)—Distance from the ventral end of each muscle to the nearest edge of the closest nucleus; and Length (L)—Length of each muscle following the curvature of the muscle. The edges of the nuclei and ends of the myofibers are defined by a clear change from signal to background within the relevant viewing channel. Each LT muscle is considered individually in all cases. To register each measurement, hit the M key (a new window will pop up when this is assessed for the first time).
8. For the analysis, a total of three measurements (dorsal, ventral, and length) per muscle, from four muscles per hemisegment in four hemisegments per embryo, are measured in each of ten embryos per genotype.
9. Copy all measurements to an Excel sheet. To calculate the dorsal and ventral distances, represent each value as a percent of the total muscle length to account for variation in muscle size. This will provide a quantification of the proximity of each cluster to each end of the muscle, which can be compared to control values. To calculate the nuclear spread, use the measurement values in the following formula:

$$\frac{\text{Length} - \text{Dorsal} - \text{Ventral}}{\text{Length}}$$
 This quantifies the distance that both clusters have traveled relative to each other and to the muscle length.
10. For statistical analysis, use either Excel or an advanced statistical software, such as SPSS (Statistical Package for the Social Sciences, IBM) or GraphPad Prism. The standard statistical test used is the Student's *t*-test.

3.2 Time-Lapse Imaging of Embryonic Muscles

3.2.1 Embryo Collection and Mounting

1. Follow **steps 1–4** from Subheading 3.1.1.
2. Place the collection basket on a small petri dish and fill it up with water so that the embryos are floating.
3. Dip a wooden stick into halocarbon oil and use the halocarbon oil on the end of the stick to collect the floating embryos from the collection basket. Then transfer them to an air-permeable Teflon membrane mounted on a circular Perspex frame (Fig. 5b). Place two pieces of tape on each side of the embryos to create a bridge that will prevent embryo damage. Place a cover slip on top of both pieces of tape.

3.2.2 Confocal Microscopy

1. For time-lapse imaging of whole embryos, Z-stacks are taken with optical sections set at 3 μm on the Zeiss AxioPlan2. To set the stack, the first slice is set when the LT nuclei are in view in multiple hemisegments. The final slice is set below the nuclei. A frame rate of 20 s is used. Only a single Z-section with the greatest number of nuclei in focus is selected for each time point. Images are processed using ImageJ and compiled into a video using QuickTime [2] (*see* ^{Note 7}).
2. For time-lapse imaging of myonuclear clusters and individual myonuclei, Z-stacks of two hemisegments are imaged simultaneously, using a high-magnification objective, such as the 63 $\times$ 1.4 NA HCX PL Apochromat oil objective (*see* Subheading 2.2.2) and increasing the zoom to 2–2.5 $\times$. Set the stack starting slightly above the nuclei and finishing slightly below the nuclei. Set the step size to 0.5 μm and the time interval to be between 20 s and 3 min [5] (*see* ^{Note 7}).
3. Depending on the types of nuclear movement analyzed, collect data from 10 h (whole embryo) to 60 min (individual nuclear movements). Record a minimum of five time-lapse series per genotype.

3.2.3 Image Analysis

1. Quantification of myonuclear movements and myonuclear morphology changes are completed manually using measurement functions in ImageJ (NIH) as described in Subheading 3.1.6.
2. *Nuclear translocation speed*: The velocity at which myonuclear clusters in the LT muscles separate during stage 15 is assessed by measuring the distance between the dorsal and ventral clusters of nuclei at $t = 0$ h and at $t = 1$ h and determining the increased distance between the two clusters. By tracking individual nuclei as they moved relative to a fixed position in the embryo over the course of 10 min, the speed of individual myonuclei is determined. One hundred individual myonuclei from five different embryos are assayed for each genotype.
3. *Nuclear rotations*: The number of LT myonuclei that rotate is determined by examining the time-lapse videos. A myonucleus that rotates, stops, and then rotates again is counted as one rotating myonucleus. Two hundred and fifty myonuclei are normally examined.
4. *Nuclear translocation directionality*: The leading edge of a myonucleus is defined as the edge of the myonucleus furthest in the direction of translocation. A myonucleus is judged to have changed direction if it persistently moves a distance of at least one nuclear radius in the opposite

⁷While acquiring time-lapse images, check every 10 min to adjust focus as embryos will move and the nuclei will shift out of focus. However, note that adjusting this focus means restarting the time-lapse sequence. This cannot be done within a time-lapse. After a few adjustments, the focal plane will essentially stabilize and it can be left for hours to get the desired time-lapse sequence.

direction from its previous direction of translocation. Two hundred and fifty myonuclei are examined.

5. *Nuclear shape changes:* The aspect ratios of moving myonuclei are determined by dividing the length of the dorsal-ventral axis of a myonucleus by the length of the anterior-posterior axis of a myonucleus using ImageJ software (NIH). Aspect ratios are considered to change if a difference of 0.7 was observed in these measurements. Two hundred and fifty myonuclei are examined with at least ~80 % exhibiting the changes in noted behavior.

3.3 Imaging of Formalin-Fixed Larval Muscles

3.3.1 Larval Collection (Fig. 4)

1. Set up a laying pot with 60 females/female virgins and 30 males at 22 °C (*see* Note 3).
2. Label and change the apple juice plates on laying pots, keeping the old ones for embryo collection.
3. Day 0: Under the dissection scope, select stage 16/17 embryos and transfer them to a half-yeasted plate with the help of an embryo hook. Arrange the embryos in a line next to a very thin layer of yeast. This will make it easier to find the 1st instar larvae on the next day.
4. Day 1: Within 24 h, transfer the hatched larvae to a vial with food and store the vial at 25 °C for an additional 3 days or 22 °C for 4 days.
5. On day 4 or day 5 (depending on the temperature), larvae will be 3rd instar larvae. Pour room temperature 15 % sucrose solution in the vial. Gently disturb the food at the bottom of the vial with a spatula. The larvae will float and can be collected using a paintbrush. In the event that some larvae are stuck in the food, release by gentle agitation. Transfer the larvae to an apple juice plate.
6. If larvae expressing a fluorescent protein that was not previously visible in the embryo are desired, select the appropriate larvae under the fluorescence scope before starting dissecting.
7. Prepare separate Eppendorf tubes with PBS for each genotype/cross and keep them in ice.

3.3.2 Larval Dissection and Fixation with Formalin

1. Before starting the dissections, gather at least six dissection pins on the dissection plate.
2. Transfer one larva to the dissection plate and add a drop of cold HL3.1 buffer using a glass pipet. This “relaxing buffer” will slow larval movements.

3. Orient the larva with its dorsal side up (identified by visualization of the main trunks of the trachea) and pin both the anterior and posterior ends of the larva to the plate. Specifically, after securing one end, use the other pin to stretch the larva to its normal length, being careful to not overstretch it, before securing the other end.
4. Using the scissors, make small incisions perpendicular to the length of the larva close to each pin (cutting both trunks of the trachea). Then, cut along the larval body, ideally between the two main trunks of the trachea, to expose the inner organs.
5. With forceps, gently remove the gut, trachea, and neurons, being careful to not damage muscles.
6. Pin each corner of the larval body to the plate, making it flat. Once more, stretch out the tissue, but avoid overstretching. The flatter the larva during preparation, the easier the image acquisition later.
7. Remove the HL3.1 buffer, add a few drops of 10 % formalin, and incubate for 20 min at room temperature.
8. Rinse with cold PBS three times.
9. Add fresh PBS and remove pins.
10. Transfer to an Eppendorf tube with cold PBS.
11. Store at 4 °C for a maximum of 1 week (for optimal staining results). Do not store dissected larvae at −20 °C as this will damage the structure of the muscle.

3.3.3 Larval Staining and Mounting

1. Staining larvae is similar to staining embryos (*see* Subheading 3.1.3), except when using fluorescence-conjugated probes such as phalloidin or Hoechst. If these types of probes are used, there is only one incubation of 1 h at room temperature followed by PBT washes. If antibodies are also used, incubate with primary antibodies first, as in the embryo staining protocol, followed by incubation with appropriate secondary antibodies and the aforementioned probes.
2. After completing the staining, transfer a maximum of six larvae to a microscope slide.
3. Under a dissection scope, position larvae with the head at the top and flatten them by exposing the muscles facing up. Spread out the larvae evenly on the slide. While doing this, do not let larvae dry out. Add more PBT if necessary.
4. Remove any excess PBT by absorbing it with a Kimwipes.
5. Place a drop of ProLong Gold antifade reagent on top of each larva and remove any visible bubbles.

6. Cover with a cover slip and let it dry at room temperature in the dark. Note that there is no bridging of the cover slips for the mounting of larvae.
7. Slides can be stored at -20°C for up to 6 months.

3.3.4 Confocal Microscopy

1. For formalin-fixed larval muscles, we image the ventral lateral muscles 3 and 4 (VL3 and VL4) because they are easily observed and are more resistant to damage during dissection. For simplicity, it is recommended to focus on VL4, with its single row of myonuclei, rather than VL3, which contains two rows of nuclei.
2. A VL4 muscle should fit in the imaging field on the microscope computer. Set the Z-stack, starting a little above the muscle and finishing just prior to the slice when other muscles first become apparent. Set the Z-step size to $0.5\text{ }\mu\text{m}$. Image six muscles from each of five larvae per genotype.

3.3.5 Image Analysis—As for embryo image analysis (*see* Subheading 3.1.6), different software packages can be used. All the instructions herein will refer to ImageJ software (NIH):

1. Follow **steps 1–4** from Confocal Microscopy in the Imaging of Fixed Embryonic Muscles (*see* Subheading 3.1.6).
2. For assessing myonuclear position in larvae, different types of measurements are made: muscle length, internuclear distance (Fig. 6a, b), nearest neighbor (Fig. 6c), largest gap (Fig. 6d), and myonuclear number.
3. *Muscle length*: Use the straight line option on the ImageJ toolbar and hit the M key to open a new window with the values.
4. *Internuclear distance*: Measure the distance between every nucleus. In control muscles or muscles without nuclear clumping, the resulting standard deviation will be very small compared to the measurements from muscles with nuclear clumping.
5. *Nearest neighbor*: Measure the distance to the nearest edge of the closest nucleus from each nucleus. Assume distance equals 0 when two nuclei are touching. The closer the nuclei, the lower the final average will be.
6. *Largest gap*: Measure the longest distance between two nuclei within a given muscle. For more severe defects in myonuclear positioning, this distance is bigger, whereas in muscles with control-like nuclear distribution, this value is smaller.
7. *Number of nuclei per muscle*: A control VL4 muscle contains 7–9 nuclei.
8. Copy all measurements to an Excel sheet. To calculate each of the parameters mentioned above, always consider the length of the muscle and express values as a percentage of muscle length.

9. For statistical analysis, either use Excel or a more advanced statistical software, such as SPSS (Statistical Package for the Social Sciences, IBM) or GraphPad Prism. The standard statistical test used is the Student's *t*-test.

3.4 Heat-Fixed Larvae

An alternative method of fixing larvae for microscopy is heat fixation (adapted from [19]). For this approach, use larvae at any developmental stage. Since there is no staining with this protocol, larvae expressing fluorescent markers in both the muscles and the nuclei are ideal (see Table 2):

1. Pick a larva using forceps. Be careful not to damage muscles.
2. Dip the larva in water at 65 °C for 1–2 s.
3. Mount 5–6 larvae in halocarbon oil or 50 % glycerol in a microscope slide.
4. Cover with a cover slip.

Acknowledgments

We thank the members of the Baylies Lab for advice, particularly, Krista Dobi, Jonathan Rosen, and Stefanie Windner for providing images for Fig. 3A (Dobi) and 2A Stages 14/15 and Stage 17 (Rosen and Windner, respectively). We also acknowledge our funding agencies, SFRH/BD/52041/2012 (Azevedo), and MDA and NIH NIAMS RO1-068128 (Baylies).

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Fig. 1.

The *Drosophila* embryonic musculature. The *Drosophila* embryo is divided in hemisegments, each containing 30 muscles. (A) Stage 16 embryo showing the musculature in green (Tropomyosin) and the nuclei of the lateral transverse (LT) muscles in red (DsRed). (B) Image of a single hemisegment. (C) Schematic representation of all the muscles present in one hemisegment; the LT muscles are highlighted in red. For additional detail see Dobi et al. [7]

Fig. 2.

Myonuclear positioning in the LT muscles during embryonic development. **(A)** Myonuclear position from stage 14 to stage 17. In the images of stages 14–16, the LT muscles are stained with Tropomyosin (*green*) and DsRed (*red*) to show the muscle structure and the nuclei, respectively. In the stage 17 image, the LT muscles are labeled with *Tropomyosin-GFP* (*green*) and the nuclei are labeled with *apterousME::NLSdsRed* (*red*) (see Table 2). At stage 16, image measurements are taken: D represents the dorsal measurement, V represents the ventral measurement, and L represents the total length of the muscle. Scale bar, 10 μ m. **(B)** Schematic representation of myonuclear movements from stage 14 to stage 17 as shown in panel **(A)**. LT muscles 1–3 have 6–8 nuclei each, whereas LT4 has 4–6 nuclei

Fig. 3.

Larval musculature and myonuclear positioning in a 3rd instar larva. **(A)** Image of a live *Drosophila* 3rd instar larva expressing *Tropomyosin-GFP*, a GFP gene trap that labels all muscles. Note the repeated muscle pattern in each hemisegment. Scale bar, 100 μ m. **(A')** Higher magnification of one hemisegment. Scale bar, 25 μ m. **(B, B')** Dissected flat prep of a larva carrying *Tropomyosin-GFP* with all the muscles exposed. Scale bar, 50 μ m. **(B)** Bright field image. **(B')** Wide field fluorescence image. **(C)** Orthogonal view of a ventral longitudinal 4 muscle (VL4). *Red*, actin; *white*, nuclei. Scale bar, 25 μ m

Fig. 4. Materials used for embryo collection. (A) Embryo laying pot perforated on the *bottom*. (B) Small funnel. (C) Collection baskets: *top* and *side* views. (D) Yeasted apple juice plate. (E) Paintbrush. (F) Embryo hook

Fig. 5.

Materials used for mounting embryos. **(A)** Cover slip with a Scotch tape bridge used to mount fixed embryos for imaging. The image shows a cover slip with two pieces of tape on both ends, before the excess is removed (*left*) and after (*right*). **(B)** Frame used to mount embryos for time-lapse imaging. The image shows an air-permeable Teflon membrane (*red dashed line box*) mounted on a circular Perspex frame. Two pieces of tape provide support for a cover slip bridge where the embryos are placed (*center of the frame*). The orange solid line box represents the cover slip; it is placed on top of both sides of the tape as shown in the image

Fig. 6.

Myonuclear positioning measurements in larval muscles. In a control VL4 muscle (**A**) and in a VL4 muscle with disrupted myonuclear positioning (**B–D**), white lines represent different measurements made to determine myonuclear positioning defects. (**A**) Internuclear distance. Nuclei are approximately equidistant. (**B**) Internuclear distance. The distance between nuclei is variable. (**C**) Nearest neighbor. Two different scenarios: the nearest neighbor of nucleus 5 is nucleus 6 and vice-versa; the nearest neighbor of nucleus 4 is nucleus 3 but the opposite is not the case. Nucleus 2 is the nearest neighbor to nucleus 3. (**D**) Largest gap. In this muscle, the largest gap between two nuclei is the distance between nuclei 6 and 7. Scale bar, 25 μ m

Table 1Antibodies and probes used to probe *Drosophila* myonuclear positioning^a

Primary antibodies ^b	Expression detected	Company, reference #
<i>Muscle markers</i>		
Tropomyosin	Actin cytoskeleton	Abcam, ab50567
Myosin heavy chain (MHC)	Myosin, actin motor protein	DSHB, EB165-s
<i>Nuclear markers</i>		
Lamin Dm0	Nuclear envelope	DSHB, ADL67.10
Lamin C	Nuclear envelope	DSHB, LC28.26
<i>Specific muscle nuclear markers</i>		
Even-skipped	DA1	[20]
Vestigial	DA1, DA2, DA3, LL1, VLs1-4	[21]
<i>Others</i>		
DsRed	Red fluorescent protein	Clontech, 632496
GFP	Green fluorescent protein; useful to identify the present of fluorescent balancers (e.g., D ^G y, CTG)	Clontech, 632381 Invitrogen, A-11120
α-Tubulin	Microtubules	Sigma, T9026
Secondary antibodies	Expression detected	Company, reference#
Alexa Fluor 488 conjugated		Life Technologies
Alexa Fluor 555 conjugated		Life Technologies
Alexa Fluor 647 conjugated		Life Technologies
Probes antibodies	Expression detected	Company, reference#
Alexa Fluor 546 conjugated phalloidin	F-actin	Life Technologies, A22283
Hoechst	DNA	Invitrogen, 33342

^aWe refer the reader to several papers for reagents that probe specific proteins involved in myonuclear positioning [2–6, 22]^bSee Notes 1 and 2

Table 2*Drosophila* stocks useful for the study of myonuclear positioning

Fly stock	Use	Reference
<i>GAL4 lines</i>	Available from stock centers/ <i>Drosophila</i> labs	
Twist-GAL4	Somatic mesoderm expression from gastrulation to stage 14	[23]
Mef2-GAL4	Somatic mesoderm expression from stage 7 and throughout larval and adult development	[24]
24B-GAL4	Somatic mesoderm expression from stage 7	[17]
MHC-GAL4	Somatic muscle expression—stage 13 throughout larval and adult development	[25]
G7-GAL4	Muscle expression in larvae	[26]
Alpha-GAL4	Muscle expression in larvae	K. Broadie lab, Vanderbilt University
ApterousME-GAL4	LT muscle-specific expression in embryo	[27]
5053-GAL4	VL1 muscle-specific expression in embryo	[28]
Rp298-GAL4	Founder cell-/myotube-specific expression in embryo/ pupa	[29]
sns-GAL4	Fusion competent myoblast in embryo/pupa	[30]
<i>UAS lines</i>	Available for gene of interest at <i>Drosophila</i> stock centers (i.e., Bloomington or Vienna stock centers) or from individual <i>Drosophila</i> labs	
UAS-RNAi	Examples: UAS-DhcRNAi, UAS-SydRNAi	[3, 4]
UAS-fluorescent reporter lines targeted to nuclei or other organelle	Examples: UAS-cytoplasmic GFP, UAS-nuclear localized GFP; UAS-lamin GFP labels nuclear envelope; UAS-EB1-GFP labels microtubule plus ends	[3, 5]
<i>Fluorescent reporter lines</i>		
<i>apterousME::NLSdsRed</i>	Labels all myonuclei in the LT muscles	[2, 27]
Tropomyosin-GFP	GFP gene trap in tropomyosin. Labels all muscles	<i>Drosophila</i> FLYTRAP collection
Zasp66-GFP	GFP gene trap in Zasp66. Labels all muscles	<i>Drosophila</i> FLYTRAP collection
<i>Gene mutants</i>	Available for gene of interest at stock centers or from <i>Drosophila</i> labs: (http://flybase.org/)	