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Preparation of developing *Xenopus* muscle for sarcomeric protein localization by high-resolution imaging

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Abstract

Mutations in several sarcomeric proteins have been linked to various human myopathies. Therefore, having an *in vivo* developmental model available that develops quickly and efficiently is key for investigators to elucidate the critical steps, components and signaling pathways involved in building a myofibril; this is the pivotal foundation for deciphering disease mechanisms as well as the development of myopathy-related therapeutics. Although striated muscle cell culture studies have been extremely informative in providing clues to both the distribution and functions of sarcomeric proteins, myocytes *in vivo* develop in an irreproducible 3D environment. *Xenopus laevis* (frog) embryos are cost effective, compliant to protein level manipulations and develop relatively quickly (a week) in a petri dish, thus providing a powerful system for *de novo* myofibrillogenesis studies. Although fluorophore-conjugated phalloidin labeling is the gold standard approach for investigating actin-thin filament architecture, it is well documented that phalloidin-labeling can be challenging and inconsistent within *Xenopus* embryos. Therefore we highlight several techniques that can be utilized to preserve both antibody and fluorophore-conjugated phalloidin labeling within *Xenopus* embryos for high-resolution fluorescence microscopy.

Keywords

Immunofluorescence microscopy; *Xenopus laevis*; myofibrillogenesis; phalloidin; sarcomere

1. Introduction

The sarcomere is an ordered, semi-crystalline, macro-molecular complex consisting of thousands of contractile and regulatory proteins that interact with one another to create the smallest contractile unit of striated muscle. Sarcomeric and sarcomeric-associated proteins are involved in numerous cellular processes (e.g., calcium signaling, mechano-sensing and structural maintenance) to facilitate and maintain optimal acto-myosin interactions for

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muscle contraction. How the dynamic sarcomeric structure is assembled and maintained is currently an active area of investigation. There are a plethora of skeletal and cardiac cell culture studies that have demonstrated the temporal and spatial localization of contractile proteins. Although they have been extremely informative and have provided important insights to deciphering the events occurring during the stages of myofibrillogenesis, they fall short from providing an accurate *de novo* perspective of muscle development that a growing embryo can provide. Therefore, identifying models that allow for gene and/or protein manipulations within a developing embryo that are amenable to high resolution microscopy is crucial for obtaining direct insights into the functional significance of individual sarcomeric components during myofibril assembly.

For more than 40 years, the *Xenopus laevis* (frog) embryo has been an excellent model for studying and imaging the dynamic steps of embryonic development *in vivo* [e.g., 1–5]. Due to the *Xenopus* embryos' large size (~1mm), visualization of both gross morphology and subcellular structure during development is relatively straightforward. Since the embryo can develop into a tadpole quickly (within four days at room temperature) and externally (not internally like its mammalian counterparts), investigators can view any stage of embryonic development in less than a week. These features of *Xenopus* development allow investigators to observe potential effects of a protein knockdown or overexpression at every developmental time point from a single cell to a swimming tadpole. In fact, several groups have used *Xenopus* embryos or *Xenopus* myotomal cell cultures to study events occurring during myofibrillogenesis [6–9]. In all, *Xenopus laevis* is not only an emerging but also an excellent model for assessing protein localization and function during *de novo* myofibrillogenesis in a developing embryo.

Determining the timing and the localization of where a protein is expressed is an important first step to deciphering the physiological function of a protein. Whole mount immunofluorescence microscopy is an approach that most often uses combinations of unconjugated primary antibodies and fluorophore-conjugated secondary antibodies as molecule-specific probes to localize and image proteins of interest within different regions of the embryo and/or at the subcellular level. Fluorophore-conjugated phalloidin is also frequently used to localize filamentous actin (F-actin) in combination with antibodies generated against actin-binding proteins to provide important clues to how actin-binding proteins may functionally associate with F-actin. Staining for the Z-line component alpha-actinin is often used for identifying the specific stages of myofibrillogenesis [e.g., 10–12]. Informative immunofluorescence studies stem from acquiring clear and highly detailed images from transparent tissue samples. Unfortunately, tissues from *Xenopus* embryos are greatly pigmented and opaque due to the epidermis and yolk granules, respectively. In fact, younger embryos (stage 32 and younger) tend to have more yolk granules and thus are potentially more challenging with respect to unwanted autofluorescence signals. These features often pose challenges when acquiring images but can be overcome with several techniques that have been developed. Some of these approaches include the addition of a bleaching and/or a clearing step. Bleaching removes the embryo's pigmentation, while clearing makes the embryo (including the yolk granules) transparent. Transparency is achieved when the embryo's refractive index is matched by the mounting medium (i.e.,

Murray's clear) [13]. Although both approaches are effective in improving image quality, they compromise phalloidin labeling.

Here we discuss several different approaches for preserving both antibody and phalloidin staining in *Xenopus* embryo skeletal muscle. We highlight a variation of whole mount immunofluorescence staining which entails manually removing the epidermis of the embryo; this bypasses the bleaching step. Another approach is the preparation of cryosections. This method not only preserves the structural integrity of the myofibrils to be analyzed but also greatly increases the penetrance of both antibody and phalloidin into the samples. Finally, we describe a "clearing" technique that markedly improves microscopic analysis and preserves both antibody and phalloidin stains. Fortunately, as outlined above, there are many options that can enhance acquisition of high-resolution images for the study of sarcomeric protein localization in developing *Xenopus* embryos.

2. Fixation of Embryos

Fixation of embryos is a crucial step in capturing and preserving the fine structural detail of a tissue sample. In the process of holding molecules and structures in place, fixatives can also alter or mask the target antigen, making it poorly or completely unrecognizable by the antibody. Therefore, it is important to select a fixative according to the antibody being used.

After the *Xenopus* eggs are fertilized and de-jellied [i.e., removal of the gelatinous matrix coat of proteins and carbohydrates that surround the embryo via incubation in a L-cysteine solution in 0.2× Marc's modified buffer (MMR; pH 7.95) for 4–8 minutes], they are placed into a petri dish filled with 0.2× MMR for further development (see Section 2.1.1 for formula). Embryos develop between 15–25°C (the rate of development is temperature dependent). The development of the embryo is monitored via the use of a stereomicroscope. Once they reach the desired stage, they are fixed in the solution of choice.

There are two general types of fixatives used for immunofluorescence microscopy: alcohol- and aldehyde-based fixatives. In short, alcohol-based fixatives are "denaturing" fixatives due to their ability to reduce the solubility and frequently perturb the tertiary structure of proteins. Aldehyde fixatives function by creating covalent bonds between proteins, often preserving the tertiary structure. The chemistry behind each fixative type is well understood relative to their antigen-alteration and fixing mechanisms of action and has been described previously [e.g., 13–15].

2.1. Fixation Types and Components

There are several aldehyde- and alcohol-based fixative solutions that can be used to fix *Xenopus* embryos. Some of the aldehyde-based fixatives solutions are MEMPFA (MOPS/EGTA/magnesium sulfate/paraformaldehyde), 4% paraformaldehyde (PFA), 0.37% formaldehyde and Millonig's fix [16–18]. Alcohol-based fixative solutions include 100% methanol, 1 methanol: 1 acetone, and Dent's fix [19, 20].

Based on our experience and from others, immunofluorescence staining is often more effective on aldehyde-based fixed embryos than on alcohol-based fixed embryos. It is

important to note that the ability of an antibody to recognize its antigen can vary between fixatives. As an example, in our hands, anti- α -actinin antibodies and not anti-tropomodulin antibodies are effective at recognizing their respective proteins in Dent's-fixed embryos (data not shown). Thus, it is clear that in Dent's fix, some epitopes are preserved while others are not recognizable.

With respect to the fixatives we tested, MEMPFA was the most effective for antibody and phalloidin use. 0.37% formaldehyde fixed embryos exhibited similar results to MEMPFA; hence it is a viable alternative. Surprisingly, we observed that not all aldehyde-based fixatives that we tested were optimal for both antibody and phalloidin use. For example, *Xenopus* embryos fixed in Millonig's Fix, a fix specifically designed for preservation of actin filaments in sea urchins [18], exhibit strong phalloidin labeling but poor antibody staining. Phalloidin staining of alcohol-fixed embryos is well known to be poor since it loses its F-actin binding ability once exposed to methanol [17]. In certain instances, the variability in the contractile state of each sarcomere (i.e., the distance between Z lines) can prove to be challenging for accurate visualization and quantification of specific sarcomeric structures (for example, for high resolution of thin filament pointed ends) hence the use of a "relaxing" buffer (EGTA, K⁺ and ATP containing buffer) can be utilized before (~30 min incubation) and during the fixation step.

2.1.1. Formulas for Fixatives—Unless mentioned otherwise, fixatives should be prepared fresh before use. All fixatives should be prepared in a chemical fume hood while wearing gloves, goggles and a mask. The institution's chemical waste disposal policies should be followed.

i. MEMPFA (MOPS/EGTA/magnesium sulfate/paraformaldehyde).

4% paraformaldehyde (PFA)

2mM EGTA

100mM MOPS, pH 7.4 (needs to be protected from light; once yellow, should be discarded).

1mM MgSO₄

ii. 4% Paraformaldehyde

4% PFA from an 8% stock (made from PFA prill: Electron Microscopy Sciences Cat#19202)

dH₂O at ~50°C

Stir and bring PFA prill into solution with 10N NaOH.

or

8% PFA from a 16% aqueous solution (from Electron Microscopy Sciences Cat#15710)

8% PFA can be stored in an airtight glass bottle at 4°C for two weeks.

iii. Dent's Fix [20]

4 methanol (100%):1 dimethyl sulfoxide (DMSO)

iv. 0.37% Formaldehyde Adapted from [6]

0.37% (w/v) formaldehyde (diluted from formalin containing 37% formaldehyde)

80mM NaCl

10mM EGTA

10mM MgCl₂

10mM HEPES, pH 7.4

v. Methanol

100% ice-cold methanol

vi. 1 Methanol: 1 acetone

1 methanol (100%): 1 acetone (100%)

vii. Millonig's Fix [18]

3% formaldehyde (diluted from formalin containing 37% formaldehyde)

136mM NaCl

200mM NaH₂PO₄, pH 7.4

2.1.2. Relaxing buffer formula (adapted from [16, 21])—100mM MOPS, pH 7.4

1mM MgSO₄

5mM EGTA

5mM ATP

2.1.3. Fixation Procedure—Materials

Petri dishes (60×15mm)

Glass storage vials (15×44mm)

2ml plastic transfer pipettes

Stereomicroscope

Protocol

1. Using a transfer pipet move embryos from 0.2× MMR in a petri dish into labeled glass vials and remove excess liquid.
2. With a transfer pipette, add fixative solution to each glass vial (substitute relaxing/fixative procedure if desired).
3. Cap and allow embryos to fix at room temperature for 2–4 hours.
4. Transfer vials to 4°C overnight.
5. Store fixed embryos the next day (see below). Avoid over-fixing the embryos (i.e., fixing embryos > 48 hours at room temperature).

2.1.4. Storage of Fixed Embryos—Traditionally, embryo storage entails transferring the fixed embryos to vials filled with 100% methanol at –20°C. This approach is effective at preserving embryos for approximately two months. Although this storage procedure is conducive for many antibody stains, the opportunity to stain with fluorescently-conjugated phalloidin is lost. In order to preserve phalloidin staining, fixed embryos can be stored in a lower percentage fixative (1% MEMPFA) for up to a month at 4°C.

3. Embryonic Tissue Preparation: Whole Mount and Cryosection Approaches

3.1. Tissue Whole Mount Preparation

Whole mount immunohistochemistry (IHC) typically involves staining the entire embryo and this approach takes at least 3 days. We discovered, however, that dissecting the somitic region of the embryo and removing the skin before staining not only increases antibody and phalloidin penetration into the skeletal muscle area (somites), but also reduces the protocol to 1.5 days without compromising the quality of the stain.

3.1.1. Somitic tissue dissecting procedure—Somites develop in the dorso-lateral region of the *Xenopus* embryo. Under a stereomicroscope, use two pairs of forceps (one in each hand) to dissect somitic tissue from fixed embryos. The No. 5 forceps provide a tip that is helpful in pinning down the tissue. The No. 55 forceps are much sharper with a curved point and are useful for grasping the somite tissue and epidermis during the dissection process.

Materials

Positively charged (poly-lysine coated) glass slides

Nail polish to make slide wells

Petri dishes (60×15mm)

Stereomicroscope

Dissection tools: Forceps No. 5 and No. 55

Plastic 1ml pipette tip box (i.e., humidifying chamber)

200 μ l pipette and tips

10 μ l pipette and tips

Reagents

i. Dissection Solution

1 \times Marc's Modified Ringers stock (MMR)

0.1M NaCl

2mM KCl

1mM MgSO₄

2mM CaCl₂

5mM HEPES, pH 7.8

0.1mM EDTA

(Note this is typically diluted to 0.2 \times for use).

ii. Permeabilization Solution

0.2% Triton-X100 in 1 \times PBS

Protocol

1. Fill a petri dish with 0.2 \times MMR and place a fixed embryo into the dish with a transfer pipette (Fig. 1a).
2. Using both forceps, remove the head and tail region from the trunk of the embryo (Fig. 1b).
3. Remove the yolky endodermal tissue by cutting just below the somites (Fig. 1c).
4. Separate the somitic regions on each side of the embryo. To do this, place the remaining trunk of the embryo dorsal side down against the petri dish, so that the somites are "up". With forceps, split the somites apart, cutting through the center of the dorsal region (Fig. 1d).
5. Pin the tissue down with the No. 5 forceps, and peel off the notochord (NC) and neural tube (NT) with the No. 55 forceps (Fig. 1e,f). Typically, one somitic side will have the notochord and neural tube attached to it.
6. Peel the epidermis away from somites with forceps. While pinning down the somites with the No. 5 forceps, the epidermis is peeled away with the No. 55 forceps (Fig. 1g); this should be done for both somite pieces. This process may take

several attempts. Complete removal of the epidermis is essential since its' high pigmentation obstructs acquisition of high-quality images (Fig. 4a & 4b).

7. Fill the circular “slide well” with 50 μ l of permeabilization solution and place the dissected pieces of somitic tissue into the well (Fig. 1h). Slide wells are created earlier on glass slides by layering several coats of nail polish (minimum of 3) to build the walls of the well (diameter approximately 1.5mm). Each layer is applied after the initial coat dries.
8. Section 4.1. describes the subsequent immunostaining procedure.

3.2. Tissue Cryosection Preparation

As an alternative to whole mount staining, we have found that utilizing frozen sections of developing *Xenopus* embryos preserves outstanding structural details of developing muscle. Although individual sections only provide a snapshot (single slice) within an embryo (in comparison to whole mounts), it does enhance antibody and phalloidin access. This approach also allows for the use of higher resolution (high numerical aperture (NA)) objectives since the working distance between the sample and the objective can be reduced (compared with whole mounts). Here, we describe a cryosection preparation optimized in our laboratory based on protocols described previously [22, 23].

Embryos are fixed as described (Section 2.1.2). Although embryos that have been fixed for days to weeks can be used for this procedure, the staining quality of stored embryos is often compromised (e.g., lower signal to noise ratio) in comparison with samples that are processed immediately after fixation. Gelatin-coated coverslips need to be prepared before sectioning [24]. Once samples are frozen, trained personnel on a cryostat can section them. For further details on cryosection preparation and sectioning, see reviews by [25, 26].

Materials

2ml plastic transfer pipettes

Positively charged (poly-lysine coated) glass slides

Stereomicroscope

Embryo dissection tools: Forceps No. 5 and No. 55

Bench top liquid nitrogen container

Metal Ladle (~4” diameter)

Aluminum foil

Tissue-Tek Cryomolds (25 \times 20 \times 5mm) (Sakura® Finetek)

Delicate task wipers (e.g., most commonly used are Kimwipes from Kimberly-Clark)

Gelatin-coated (18×18mm) coverslips (See [24] for preparation, or commercially available from NeuVtro, Cat# GG-18-gelatin)

0.5 ml micro-centrifuge tubes

Reagents

Isopentane

Liquid nitrogen

30% sucrose in 1× PBS

Optimal cutting temperature (OCT) freezing medium (Sakura® Finetek)

Protocol

1. Rinse fixed embryos with 1× PBS. Embryos can be stored in 1× PBS at 4°C up to a week before moving on to step 2.
2. Incubate embryos overnight in 30% sucrose/PBS at 4°C. Sucrose acts as a cryoprotectant. Ensure embryos sink to the bottom or assist with a quick shake; this indicates that the embryos are saturated with sucrose. If the embryos remain afloat, more time is needed in the sucrose solution. Typically, embryos stage 37 or younger, require two days of saturation.
3. Set up the isopentane/liquid nitrogen ice bath. Fill container with liquid nitrogen. Place the ladle into the nitrogen-filled container and fill ladle with isopentane. Let the liquid nitrogen cool the isopentane. Ice sludge will form at the bottom of the ladle indicating that the isopentane has reached the optimal temperature for freezing samples (Fig. 2a). Alternatively, isopentane can be stored at –80°C until use.
4. Transfer 3–4 embryos onto a glass slide with a plastic transfer pipette. Absorb as much of the sucrose as possible with a kimwipe. Cover the embryos with OCT freezing medium (Fig. 2b).
5. Place a drop of OCT medium into a labeled cryomold. Do not fill mold up.
6. Transfer embryos from the glass slide into the cryomold with the OCT medium. Using forceps, gently lift the embryos one at a time and place them in the cryomold. Position the embryos on their side for sagittal sections. Align the embryos, ensuring that they are flat against the bottom of the mold. Fill mold with OCT (just enough to completely cover the embryos) (Fig. 2c). Re-align the embryos with forceps if necessary. Ensure that the cryomold is bubble free (i.e., bubbles can damage tissue during cryosections).
7. Freeze the samples. Immerse the cryomold with embryos into the cooled isopentane and hold until the OCT turns opaque (white). Hold for another thirty seconds to ensure the sample is completely frozen (Fig. 2d, e). Freeze-thaws will cause tissue damage.

8. Store samples in aluminum foil. Allow for a quick air dry of frozen samples before placing them onto a piece of foil. Wrap up samples tightly (Fig. 2e).
9. Store the aluminum foil-wrapped frozen samples. Place wrapped samples in a plastic bag. Remove as much air as possible from the storage bag. Store at -80°C (Fig. 2f).
10. Section samples in cryostat. Have a trained person generate $\sim 5\ \mu\text{m}$ thick sections and “melt” them onto gelatin-coated coverslips.
11. Store sections in $1\times$ PBS at 4°C , until ready for staining. For longer storage, keep sections well wrapped in foil in an airtight container at -80°C until use. For immunostaining of cryosections, see Section 4.2.

4. Immunostaining

Antibody stains not only provide important information about the localization of endogenous proteins but also can be used to monitor the localization of exogenous proteins produced in the embryo by mRNA injection. Most importantly, deliberate alterations in protein levels may result in perturbations of sarcomere structure, leading to important clues about a protein’s function that can be assessed by immunofluorescence staining. Fortunately, several groups engaged in deciphering the roles of sarcomeric proteins during striated muscle development have demonstrated that many antibodies raised against human, chicken or mouse sarcomeric proteins robustly recognize *Xenopus* orthologues due to the high conservation of contractile proteins across species [6–9, 27–36]. See Table 1 for a list of commercial anti-sarcomeric protein antibodies that recognize *Xenopus* skeletal muscle proteins. It is worth noting that antibodies raised against “cardiac-specific” isoforms (i.e., α -cardiac actin) may recognize a similar antigen in skeletal muscle. For example, anti- α -cardiac actin antibodies (clone Ac1-20.4.2 from Sigma-Adrich) effectively recognize the actin-thin filaments in *Xenopus* skeletal myotomal cells.

Immunofluorescence staining approaches allow investigators the ability to detect and gather information on multiple target antigens within a cell and/or tissues. Fluorescently conjugated-secondary antibodies are one of many ways to visualize a target protein. Described below is a straightforward “single” stain. However, most often double or triple localization of different molecules is required; these approaches take advantage of the wide range of fluorophores available as well as the different species that antibodies are generated in. The protocols provided below can be adapted seamlessly for multiple stain approaches. Specifically, multiple labeling can be done sequentially or in combination (e.g., mixing the primary antibodies).

Note that primary and secondary antibodies have to be carefully titrated since each lot can differ with respect to its concentration and fluorophore coupling efficiency. Additionally, “secondary alone” control samples (i.e., samples where the primary antibodies are not used) should be performed to determine optimal working concentrations; that is, dilutions that do not result in nonspecific “background” signals.

Fluorescently-conjugated phalloidin can also be added to any of the staining protocols listed below. Note, most vendors distribute fluorophore-conjugated phalloidin in a lyophilized form with instructions to re-suspend and store the lyophilized product in 100% methanol. Therefore to achieve optimal actin-filament staining, it is important to evaporate off the methanol before diluting the phalloidin. Dilute stock in wash buffer to a final concentration of 0.001 unit/ μ l for whole mounts and 0.0005 unit/ μ l for sections.

From our experience, the staining patterns demonstrated by fluorophore-conjugated phalloidins on *Xenopus* somitic tissue prepared in whole mount is often dependent upon how the sample was prepared. In dissected somitic tissue, phalloidin exhibit either a staining pattern that spans the entire actin-thin filament or a staining pattern that is devoid of detectable staining within the I band (on each side of the Z-disc) (Fig. 4). Interestingly with sections, fluorophore-conjugated phalloidins consistently stain the length of the actin-thin filaments, likely due to better accessibility (Fig. 5j-l).

4.1. Whole Mount Immunostaining Protocol

The approach described here is a cost effective way to do whole mount immunostaining on *Xenopus* embryo tissues. The entire immunostaining procedure is carried out in a slide well (Fig. 1h). To prevent the tissue from drying out during overnight incubations, slide wells are placed into a humidifying chamber constructed from a 1ml pipette tip box with a cover that is filled (half-way) with water. The slide wells are placed on top of the tip rack with tissue samples inside the wells (Fig. 3a).

As mentioned above, fluorophore-conjugated phalloidin can be added to this protocol, following any wash step. If the phalloidin stain does not appear to be optimal, transfer tissue samples into 0.5ml micro-centrifuge tubes filled with 100 μ l of the diluted phalloidin solution. Wrap tubes in aluminum foil to protect from the light and rotate on a neutator for two hours. Afterwards, remove tissue samples from each tube and place back into the slide wells for washes.

Materials

Charged slides with nail polish wells (described above)

1ml pipette tip box (humidifying chamber)

200 μ l pipette and tips

Forceps

Coverslips (18x18mm)

Stereoscope

Solutions

i. Permeabilization solution

0.2% Triton-X100 in 1× PBS

ii. Blocking solution

2% BSA plus 1% normal donkey serum in 1× PBS

iii. Wash solution

0.02% Triton-X 100 in 1× PBS

iv. Antibody solutions

Dilute primary antibodies (see Table 1) or secondary fluorescently-conjugated antibodies in blocking solution.

v. Mounting medium specialized for immunofluorescence microscopy (e.g., Aqua-Poly/Mount from Polysciences, Inc.)

Protocol

1. **Permeabilization.** This is a continuation from step 8 of 3.1.1 (somitic tissue dissecting procedure). Incubate in permeabilization solution for at least one hour at room temperature.
2. **Blocking.** Remove permeabilization solution and add blocking solution (50µl) to each well. Block for at least 2 hours at room temperature. If nonspecific background is a problem, the samples can be blocked initially at 37°C for 30min, prior to the 2 hour room temperature incubation. This step is used to prevent non-specific binding of antibodies by binding to any “sticky” parts of the cells/tissue that are being stained.
3. **Primary Antibody.** Remove as much blocking solution from the wells as possible to prevent dilution of the subsequent primary antibodies. Add 50µl of the diluted primary antibodies to each well. Close humidifying chamber to prevent samples from drying out and incubate overnight at 4°C. If time is limited, immunofluorescence staining can be successful with a one-hour incubation in primary antibody; this is a viable option for antibodies with high affinity and/or when the antigen is abundant.
4. **First Wash.** Rinse tissue of excess primary antibodies with five 50µl washes, each for 5 minutes. Be careful when adding and removing liquid from the wells. If not, tissue will be lost.
5. **Secondary Antibody.** Remove as much washing solution as possible and add 50µl of the secondary antibody solution to each well. Close humidifier and cover samples to prevent exposure to light. Incubate tissue for at least 45 minutes at room temperature.
6. **Second wash.** Wash as described for First Wash.
7. **Continue protocol or go to 4.1.1 for Clearing Procedure.**

8. Preparation for Mounting of Stained Tissue. Place a glass slide on the platform of the stereomicroscope. Adjust the light source to best view the tissue. Using a 200 μ l pipette, transfer the tissue to the center of the slide. At this stage, remove any remaining epidermis with forceps. Gently shear apart the somitic tissue with the forceps. This flattens the tissue to be viewed and thus, enhances imaging of myofibrils. Remove as much wash buffer as possible as to not dilute the mounting medium (i.e., this will cause premature fading of the fluorescent signal). Rinse tissue with dH₂O to clear of excessive salts from wash buffer (i.e., this will reduce background when imaging).
9. Final Mounting Steps. Place ~4 drops of mounting media in close proximity, surrounding the stained tissue. Gently place the coverslip on top of the tissue. Once the coverslip has been laid down, with forceps tap the four corners of the coverslip. This approach removes air bubbles and keeps the tissue centered under the coverslip. Dry the mounted slides away from light. For long-term storage, place slides at 4°C in the dark.

4.1.1. Clearing Procedure—As mentioned above, it is difficult to acquire clear images from *Xenopus* embryos due to the abundance of yolk granules that auto-fluoresce. Implementing a clearing step before imaging can alleviate this problem. The clearing protocol includes dehydrating the tissue after staining, with a series of alcohol washes. Methanol is often the method of choice (over other alcohols) since there is little or no shrinkage of the tissue; however, the ability to stain with phalloidin is compromised. Isopropanol can replace methanol, and if done quickly, phalloidin labeling is preserved [17]. The unfortunate tradeoff of using isopropanol is that there is significant tissue shrinkage.

Materials

2ml Plastic transfer pipette

Positive charged (poly-lysine coated) glass slides

Stereomicroscope

Coverslips (18×18mm)

10 μ l and 200 μ l pipettes and tips

1.5ml microcentrifuge tube rack

Aluminum foil

Self-sealing film (e.g., Parafilm[®] M)

Kimwipes

Permanent Marker

Solutions

Alcohol Dilution Series (these can be stored and reused)

100% 95% 85% 70%, 50%, and 25% methanol (or isopropanol)

Clearing solution

1 benzyl alcohol: 2 benzyl benzoate (BABB): protect from light and make fresh each time. BABB is highly corrosive and toxic.

Protocol

1. Create wells for alcohol dehydration. Place a piece of parafilm on top of a 1.5 ml plastic tube rack and tape it in place (Fig. 3c,d). Use fingers to press the parafilm into the holes of the tube rack (do not break the parafilm). This will create “wells”. If necessary, leave empty wells between the alcohol solutions to prevent inadvertent mixing.
2. Dehydration. Each incubation is 15 seconds long. Transfer between wells using a 200 μ l pipette. The series is as follows: 25%, 50%, 70%, 85%, 95%, 100% and 100%. Ensure all water is extracted from the tissue. If there is residual water, it will mix with the subsequent clearing solution (see below) and create a precipitate that renders the tissue opaque.
3. Transfer dehydrated tissue. With a 200 μ l pipette, transfer tissue to a marked area on a prepared charged slide under a stereomicroscope. Remove excess 100% isopropanol from the tissue. To prepare the slide, prepare draw a circle to designate the location for the tissue to be placed.
4. Clear the tissue. Add 2–5 μ l of BABB to the tissue and incubate for 5 min. Repeat three times with fresh BABB solution. Watch the tissue “disappear”. The circle helps with identifying the location of the tissue once it becomes transparent. Finally, add 5–10 μ l of BABB to the tissue for mounting.
5. Place a coverslip onto the tissue. Remove excess BABB surrounding coverslip with a kimwipe. Seal the coverslip with nail polish. Let mounted slides air dry away from light.

4.2. Cryosection Immunostaining Protocol

The immunostaining approach for cryosections follows a similar protocol as for whole mount staining: the solutions are the same, while some different materials are used. Unlike the whole mount approach, cryosections have increased tissue and antigen accessibility; therefore antibodies can be used more dilute and for shorter incubation times. For example, the anti-sarcomeric α -actinin antibody (Sigma-Aldrich) is used at a dilution factor of 1:3000 with an overnight incubation period when conducting whole mount immunofluorescence staining. Conversely with staining sections, the anti- α -actinin antibody works well at 1:6000 with an incubation time of one hour.

As with whole mount staining, fluorescently-conjugated phalloidin can be added or substituted into the protocol. Specifically, add 50 μ l of diluted phalloidin solution onto the

coverslip and incubate for two hours at room temperature. Cover the humidifying chamber to protect coverslips from light.

Materials

Standard 6-well cell culture plate, non-treated, flat-bottom with lid

200µl pipette and 1ml pipette with tips

Forceps

Humidifying chamber (container with lid)

Rubber stoppers (flanged rubber stoppers for vaccine bottles) (Plasticoid Corp: Cat#20190)

beaker

Filter paper (0.34mm)

Parafilm

Kimwipes

Slides

Mounting Medium for Fluorescence Microscopy

Light Box

Solutions

Same as described above for Whole Mount staining without Clearing

- 1. Permeabilization.** Place one coverslip (section facing up) into each well of a 6 well culture dish with forceps. Gently fill wells with 2 ml of permeabilization solution. Incubate for 15 minutes at room temperature on a light box (i.e., this step photo-bleaches the tissue, often reducing autofluorescence from the tissue, particularly the yolk granules). Check and make sure coverslips do not float to the top (sections will dry out and compromise image quality if not submerged). If they float up, poke down with forceps.
- 2. Blocking.** Remove permeabilization buffer and add 2ml of blocking solution to each well. Incubate for 45 minutes at room temperature on a light box.
- 3. Primary antibody.** Set up the humid chamber (Fig. 3b). Place filter paper into a plastic container (humid chamber) and wet it completely with water (pour off excess water). Place the rubber stoppers on top of the wet filter paper. The wet filter paper holds the rubber stoppers in place. With a pair of forceps, remove the coverslip from the well, remove excess blocking buffer by touching a corner of the coverslip on a kimwipe and place the coverslip face-up on top of a rubber stopper. Note, care must be taken to assure that sections remain on coverslips during the

staining procedure. Add 50 μ l of primary antibody solution onto the coverslip. Incubate for one hour at room temperature (or overnight at 4°C). For overnight incubations, parafilm the humidifier to prevent sections from drying out.

4. First wash. Remove primary antibody by touching edge of coverslip to a Kimwipe. Place the coverslips back into 6-well cell culture dish for washing steps. Wash with 2ml of wash buffer, 3 times for 5 minutes each.
5. Secondary Antibody. Transfer the coverslip from the six well plate to the rubber stoppers in the humid chamber. Remove excess wash buffer and place the coverslip on top of the rubber stopper. Add 50 μ l of the secondary antibody solution onto each coverslip. Cover humidifying chamber to protect coverslips from light. Incubate 45 minutes at room temperature.
6. Second wash. Remove secondary antibody by touching edge of coverslip to a Kimwipe. Wash as described for First Wash.
7. Rinse coverslip several times in beaker filled with dH₂O. Remove excess dH₂O from the coverslip with a Kimwipe.
8. Place a glass slide onto the platform of the stereomicroscope. Add a single drop of mounting medium onto the slide. Work quickly to prevent mounting medium from drying. Drying reduces the medium's spreading ability and will prevent complete immersion of the tissue.
9. Place coverslip, section down, onto mounting medium drop. Gently press the coverslip against the glass slide with the back-end of the forceps to ensure equal spreading of the mounting medium around the sample and to remove trapped air. Let mounted slides air dry away from light.

5. Results

Traditionally, bleaching and clearing approaches have been useful in optimizing immunofluorescence imaging of *Xenopus* embryos. The methods we describe here illustrate different approaches investigators can utilize for optimal imaging of proteins and filamentous actin structures within *Xenopus* somitic tissue. If removing the epidermis is done correctly (Fig. 1h), the whole mount dissection yields intact tissue and clear visualization of sarcomeric components [e.g., α -actinin and phalloidin (Fig. 5d,e)]. If the epidermis is not removed properly, it will obstruct viewing of the myofibrils within the somitic tissue (Fig. 5a,b). The presence of abundant yolk granules can also be detrimental for imaging of the myofibrils. This is especially true for stage 32 and younger embryos, where large numbers of yolk granules can block the visualization of subcellular structures and add to the autofluorescent background (Fig. 5e). Clearing tissue with an isopropanol dehydration/BABB protocol after immunofluorescence staining effectively masks yolk granules, and preserves phalloidin labeling (Fig. 5g,h). Unfortunately, somitic tissue undergoes shrinkage after the isopropanol/BABB process therefore compromising quantitative analysis of any subcellular structure. Of the plethora of protocols tested by our laboratory, we found that generating *Xenopus* embryo cryosections was the best approach for optimal imaging of developing muscle tissue. Because the cryosection technique yields a

~5 μ m section of the embryo, the amount of autofluorescence from other structures is greatly reduced. This advantage along with the enhancement of tissue penetrance results in robust and clear imaging of sarcomeric components within the somitic tissue (e.g., Fig. 5j,k).

Although this article focuses primarily on studying myofibril assembly in developing skeletal muscle tissue, these techniques can affectively be applied to studying myofibril assembly during heart development. However, while many cardiac studies in *Xenopus* have primarily focused on elucidating the molecular network leading to cardiac induction or capturing the morphological transformation of the myocardial tube into a threechambered heart [e.g., 9, 37–38], there is great potential for assessing the temporal and spatial localization of sarcomeric proteins at the subcellular level [9, 39].

6. Concluding Remarks

One of the most fascinating examples of cytoskeletal assembly is the sarcomere; perhaps the most highly ordered macromolecular structure in eukaryotic cells. When striated muscle cells differentiate, thousands of structural and regulatory molecules assemble to the precision of single molecules. In the last several decades, significant progress has been made in understanding the complex process involved in discerning the steps and specific molecules required for myofibrillogenesis. Initial investigations stemmed from remarkable, in-depth electron microscopy studies that allowed investigators to visually identify the stages involved in myofibrillogenesis, without the identification of most molecules involved [e.g., 40–42]. These findings were followed by a plethora of immunofluorescence and biochemical studies in isolated myocytes (from rat, mouse, chick and frog) and various model organisms including *Drosophila melanogaster*, zebra fish, *C. elegans* as well as the gold standard of many laboratories, genetically altered mice. With these studies providing an important framework for understanding the molecular players involved in myofibrillogenesis and the *Xenopus laevis* embryo in place as an *in vivo* developmental model of *de novo* myofibril assembly, the field is now poised to investigate the mechanisms by which perturbations in specific sarcomeric components lead to the development of human myopathies. The optimized protocols for preparing embryonic *Xenopus* somitic tissue, phalloidin labeling and immunofluorescence staining for high resolution imaging presented in this publication lay a solid foundation for these mechanistic, disease-related investigations in the *Xenopus* embryo.

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References

1. Muntz L. Myogenesis in the trunk and leg during development of the tadpole of *Xenopus laevis* (Daudin 1802). *J. Embryol. Exp. Morph.* 1975; 33:757–774. [PubMed: 1176869]

2. Boucaut J, Thierry D. Fibronectin in early amphibian embryos. *Cell Tissue Res.* 1983; 234:135–145. [PubMed: 6640612]
3. Fagotto F, Gumbiner BM. Beta-catenin localization during *Xenopus* embryogenesis: accumulation at tissue and somite boundaries. *Development.* 1994; 120:3667–3679. [PubMed: 7821229]
4. Schohl A, Fagotto F. Beta-catenin, MAPK and Smad signaling during early *Xenopus* development. *Development.* 2000; 129:37–52. [PubMed: 11782399]
5. Kieserman EK, Lee C, Gray RS, Park TJ, Wallingford JB. High-magnification in vivo imaging of *Xenopus* embryos for cell and developmental biology. *Cold Spring Harbor Protocols.* 2010; 5 pdb-prot5426.
6. Li H, Cook JD, Terry M, Spitzer NC, Ferrari MB. Calcium transients regulate patterned actin assembly during myofibrillogenesis. *Dev. Dyn.* 2004; 229:231–242. [PubMed: 14745949]
7. Campbell NR, Podugu SP, Ferrari MB. Spatiotemporal characterization of short versus long duration calcium transients in embryonic muscle and their role in myofibrillogenesis. *Dev. Biol.* 2006; 292:253–264. [PubMed: 16460724]
8. Geach TJ, Zimmerman LB. Paralysis and delayed Z-disc formation in the *Xenopus tropicalis* unc45b mutant dicky ticker. *BMC Dev. Biol.* 2010; 10:75. [PubMed: 20637071]
9. Kolker SJ, Tajchman U, Weeks DL. Confocal imaging of early heart development in *Xenopus laevis*. *Dev. Biol.* 2000; 218:64–73. [PubMed: 10644411]
10. Wang SM, Greaser ML, Schultz E, Bulinski JC, Lin JJ, Lessard JL. Studies on cardiac myofibrillogenesis with antibodies to titin, actin, tropomyosin, and myosin. *J. Cell Biol.* 1988; 107:1075–1083. [PubMed: 3047149]
11. Dukhee R, Sanger JM, Sanger JW. The premyofibril: evidence for its role in myofibrillogenesis. *Cell Motil. Cytoskel.* 2005; 28:1–24.
12. Schultheiss T, Lin ZX, Lu MH, Murray J, Fischman DA, Weber K, Masaki T, Imamura M, Holtzer H. Differential distribution of subsets of myofibrillar proteins in cardiac nonstriated and striated myofibrils. *J. Cell Biol.* 1990; 110:1159–1172. [PubMed: 2108970]
13. Klymkowsky, MW.; Hanken, J. Whole mount staining in *Xenopus* and other vertebrates. In: Kay, BK.; Peng, HB., editors. *Methods in Cell Biology.* Vol. 36. San Diego: Academic Press Inc.; 1991. p. 419-441.
14. French, D.; Edsall, JT. The reactions of formaldehyde with amino acids and proteins. In: Edsall, ML.; Anson, JT., editors. *Advances in Protein Chemistry.* Vol. 2. New York: Academic Press Inc.; 1945. p. 277-335.
15. Pearse AGE. Histochemistry: theoretical and applied. *Am. J. Med. Sci.* 1961; 241:136.
16. Sive, HL.; Grainger, RM.; Harland, RM. Early development of *Xenopus laevis*: a laboratory manual. Cold Spring Harbor Laboratory Press; 2000.
17. Strickland, L.; Dassow, G.; Ellenberg, J.; Foe, V.; Lenart, P.; Burgess, D.; Charles, A.; Etensohn, GAW.; Gary, MW. Light Microscopy of Echinoderm Embryos. In: Etensohn, CA.; Wray, GA.; Wessel, GM., editors. *Methods in Cell Biology.* Vol. 74. San Diego: Elsevier Academic Press Inc.; 2004. p. 371-409.
18. Millonig G. A study on the formation and structure of the sea urchin spicule. *J. Submicrosc. Cytol.* 1970; 2:157–165.
19. Klymkowsky MW, Maynell LA, Polson AG. Polar asymmetry in the organization of the cortical cytokeratin system of *Xenopus laevis* oocytes and embryos. *Development.* 1987; 100:543–557. [PubMed: 2443336]
20. Dent JA, Polson AG, Klymkowsky MW. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. *Development.* 1989; 105:61–74. [PubMed: 2806118]
21. Gokhin D, Kim N, Lewis S, Hoenecke HR, D’Lima DD, Fowler VM. Thin-filament length correlates with fiber type in human skeletal muscle. *Am. J. Physiol. Cell.* 2012; 302:555–565.
22. Brown D, Martz SN, Binder O, Goetz SC, Price BMJ, Smith JC, Conlon FL. Tbx5 and Tbx20 act synergistically to control vertebrate heart morphogenesis. *Development.* 2005; 132:553–563. [PubMed: 15634698]
23. Fischer, AH.; Jacobson, KA.; Rose, J.; Zeller, R. Cryosectioning Tissues. Vol. 8. Cold Spring Harbor Protocols; 2008. pdb-prot4991.

24. Fischer, AH.; Jacobson, KA.; Rose, J.; Zeller, R. Preparation of slides and coverslips for microscopy. Vol. 5. Cold Spring Harbor Protocols; 2008. pdbprot4988.
25. Webster, P. The production of cryosections through fixed and cryoprotected biological material and their use in immunocytochemistry. In: Hajibagheri, N., editor. Methods in Molecular Biology. Vol. 117. Totowa, NJ: Humana Press Inc.; 1999. p. 49-76.
26. Tokuyasu KT. A technique for ultracryotomy of cell suspensions and tissues. J. Cell Biol. 1973; 57:551–565. [PubMed: 4121290]
27. Vernon AE, Philpott A. A single cdk inhibitor, p27Xic1, functions beyond cell cycle regulation to promote muscle differentiation in *Xenopus*. Development. 2003; 130:71–83. [PubMed: 12441292]
28. Schwartz LM, Kay BK. Differential expression of the Ca²⁺-binding protein parvalbumin during myogenesis in *Xenopus laevis*. Dev. Biol. 1988; 128:441–452. [PubMed: 3294065]
29. Lee Y, Saint-Jeannet JP. Cardiac neural crest is dispensable for outflow tract septation in *Xenopus*. Development. 2011; 138:2025–2034. [PubMed: 21490068]
30. Sugita S, Adachi T, Ueki Y, Sato M. A novel method for measuring tension generated in stress fibers by applying external forces. Biophys. J. 2011; 101:53–60. [PubMed: 21723814]
31. Hocking JC, Hehr CL, Bertolesi G, Funakoshi H, Nakamura T, McFarlane S. LIMK1 acts downstream of BMP signaling in developing retinal ganglion cell axons but not dendrites. Dev. Biol. 2009; 330:273–285. [PubMed: 19361494]
32. Cary RB, Klymkowsky MW. Desmin organization during the differentiation of the dorsal myotome in *Xenopus laevis*. Differentiation. 1994; 56:31–38. [PubMed: 8026644]
33. Sadikot T, Hammond C, Ferrari MB. Distinct roles for telethonin N- versus C- terminus in sarcomere assembly and maintenance. Dev. Dyn. 2010; 239:1124–1135. [PubMed: 20235223]
34. Mudry RE, Perry CN, Richards M, Fowler VM, Gregorio CC. The interaction of tropomodulin with tropomyosin stabilizes thin filaments in cardiac myocytes. J. Cell. Biol. 2003; 162:1057–1068. [PubMed: 12975349]
35. Pappas CT, Bhattacharya N, Cooper JA, Gregorio CC. Nebulin interacts with Cap Z and regulates thin filament architecture within the Z-disc. Mol. Biol. Cell. 2008; 19:1837–1847. [PubMed: 18272787]
36. Nishikawa A, Hayashi H. Spatial, temporal and hormonal regulation of programmed muscle cell death during metamorphosis of the frog *Xenopus laevis*. Differentiation. 1995; 59:207–214. [PubMed: 8575642]
37. Mohun TJ, Leong LM, Weninger WJ, Sparrow DB. The Morphology of Heart Development in *Xenopus laevis*. Dev. Biol. 2000; 218:74–88. [PubMed: 10644412]
38. Kaltenbrun E, Tandon P, Amin NM, Waldron L, Showell C, Conlon FL. *Xenopus*: an emerging model for studying congenital heart disease. Birth Defects Res. A Clin. Mol. Teratol. 2011; 91:495–510. [PubMed: 21538812]
39. Smith SJ, Ataliotis P, Kotecha S, Towers N, Sparrow DB, Mohun TJ. The MLC1v gene provides a transgenic marker of myocardium formation within developing chambers of the *Xenopus* heart. Dev. Dyn. 2005; 232:1003–1012. [PubMed: 15736168]
40. Raworth AE, Pepe FA. Ultrastructure of developing muscle cells in the chick embryo. Am. J. Anat. 1965; 116:115–147. [PubMed: 14283278]
41. Peng BH, Wolosewick JJ, Cheng P. The development of myofibrils in cultured muscle cells: a whole-mount and thin-section electron microscopic study. Dev. Biol. 1981; 88:121–136. [PubMed: 7197240]
42. Fischman DA. An electron microscope study of myofibril formation in embryonic chick skeletal muscle. J. Cell Biol. 1967; 32:557–575. [PubMed: 6034479]

Highlights

- Staining protocols were optimized to study myofibrillogenesis in *Xenopus* embryos.
- A dissection approach was developed for ideal staining of *Xenopus* somitic regions.
- Optimized phalloidin labeling and immunostaining for *Xenopus* embryo cryosections.
- Demonstrated methods to preserve phalloidin labeling after Murray's clear.

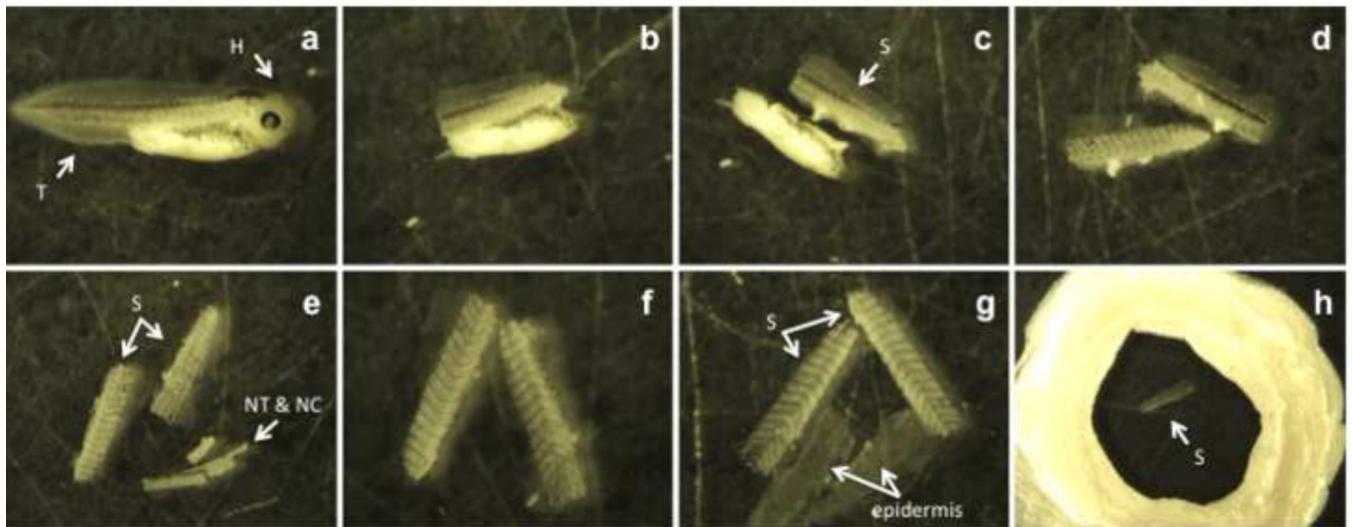
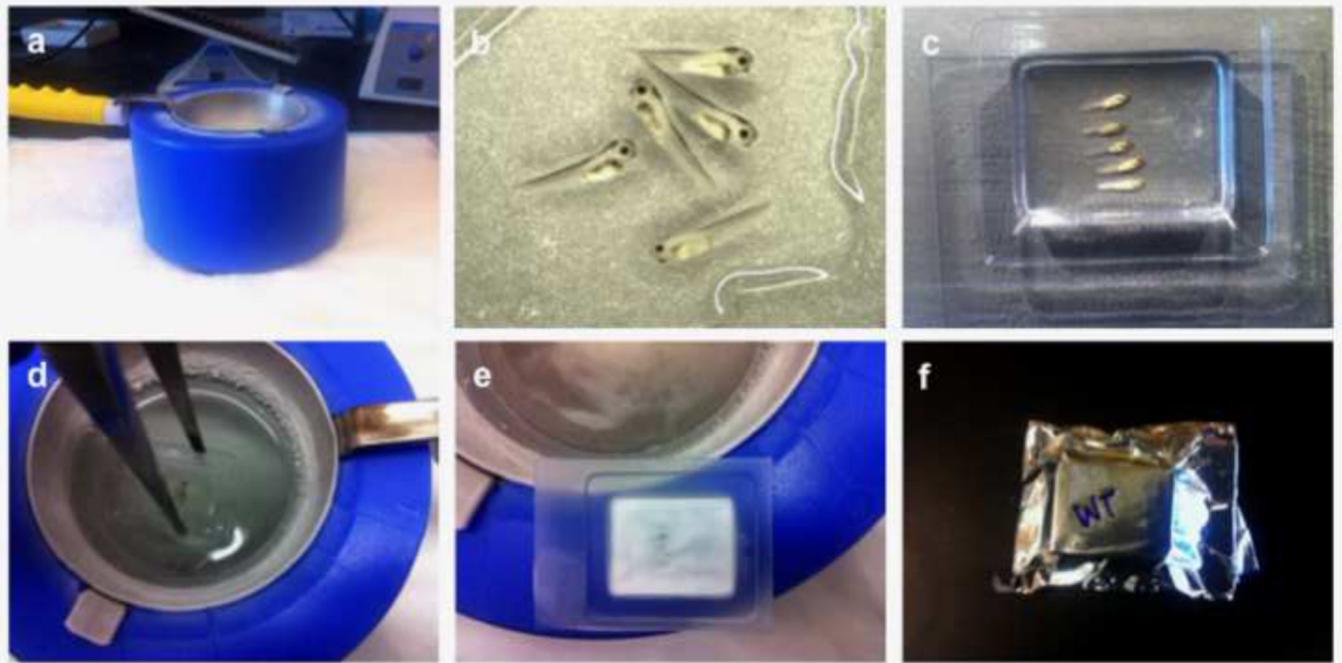


Figure 1. Somitic tissue dissecting procedure

The fixed stage 40 embryo is placed into a petri dish (a). With forceps, both the head (H) and tail (T) are removed (b). The yolk-filled belly region is separated from the somitic tissue (S) (c). The somites are split into two pieces (d). One somitic side will have the neural tube (NT) and notochord (NC) still attached and both structures are removed with forceps (e, f). The epidermis is peeled away from the somitic regions (g). The somitic tissue (S) is placed into a well formed from nail polish for immunostaining (h).

**Figure 2. Cryosection preparation**

A liquid nitrogen bath setup with an isopentane filled ladle (a). 4–5 embryos are cleared of all residual sucrose solution and covered with OCT freezing medium (b). The embryos are transferred to, and aligned in a cryomold (c) and then immersed into the isopentane bath (d) until they turn opaque (e). Samples are quickly allowed to dry, wrapped in aluminum foil and stored (e, f).

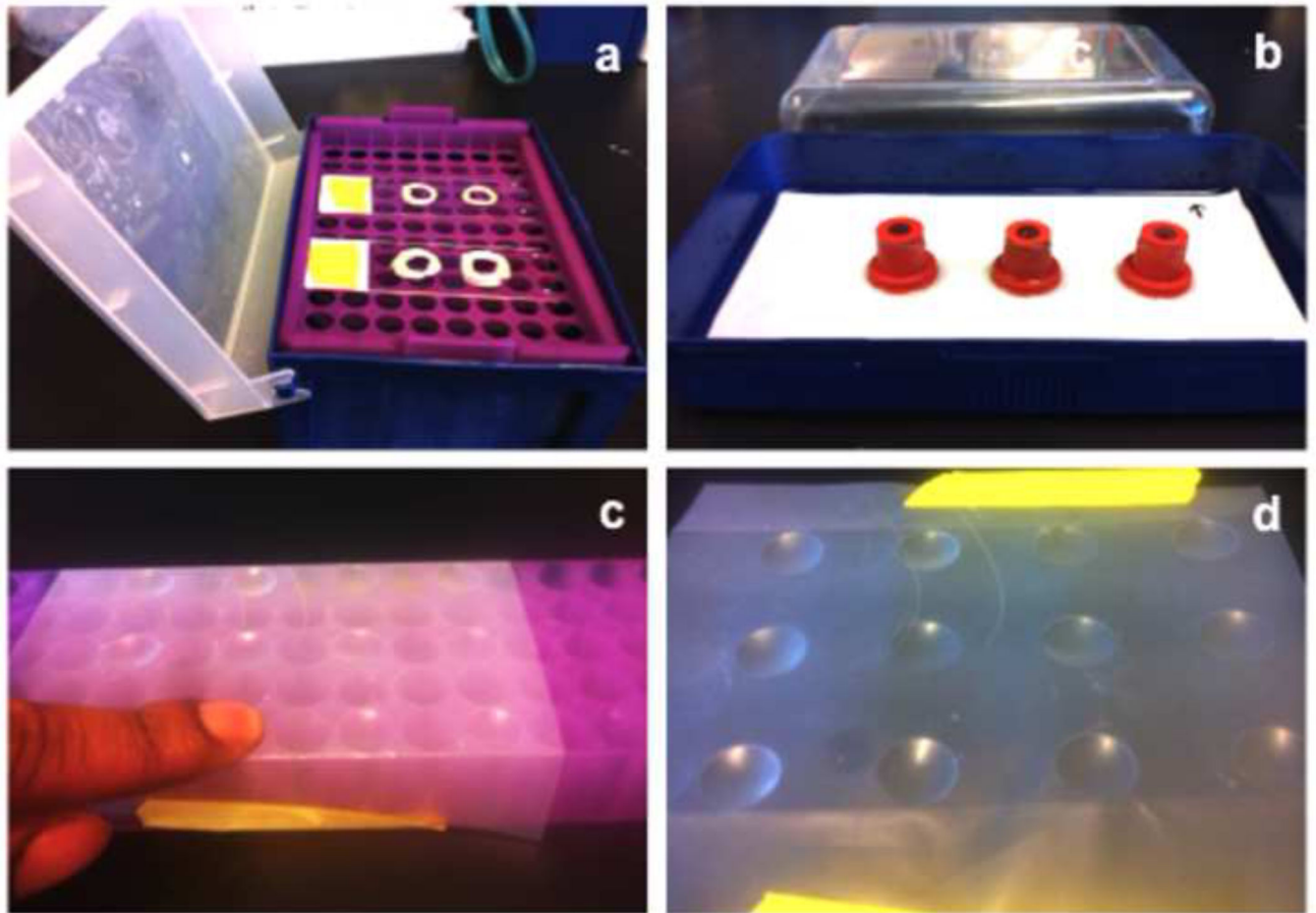


Figure 3. Setup for whole-mount immunohistochemistry (IHC), cryosection IHC and BABB preparation

For whole-mount IHC, the entire procedure is executed in a nail polish slide well (a). For cryosection IHC, sections on coverslips are incubated on rubber stoppers in a moist covered chamber (b). For BABB preparation of the somitic tissue, the isopropanol washes are carried out in parafilm wells (c, d).

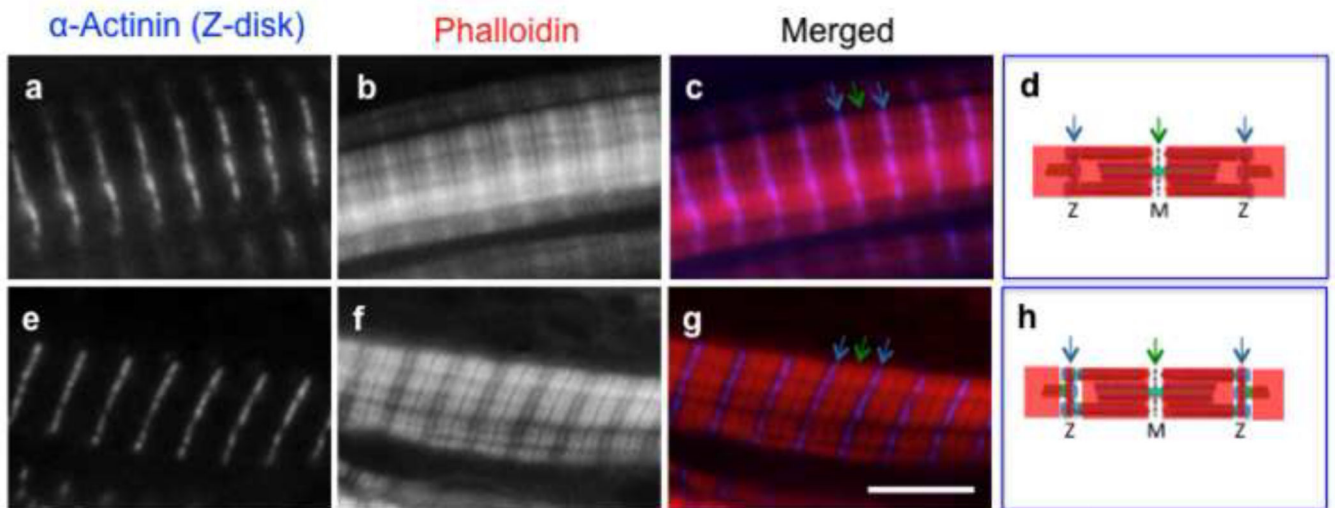


Figure 4. Two different phalloidin staining patterns are observed in somitic tissue
 Fluorescently-labeled phalloidin can exhibit two distinct staining patterns in the *Xenopus* muscle cells. Green arrows point to the Z-disk (Z) and the blue arrows point to the M-line (M) (c, d, g, h). For whole-mounts, phalloidin exhibits a staining pattern that spans the length of the actin-thin filament (b, d). Phalloidin will also exhibit a staining pattern that spans the length of the actin-thin filament except for a region within the I-band in close proximity to the Z-disk (f, h). With cryosections, fluorophore-conjugated phalloidins consistently stain the entire actin-thin filaments (Fig. 5, j–l). Scale bar is 5 μ m.

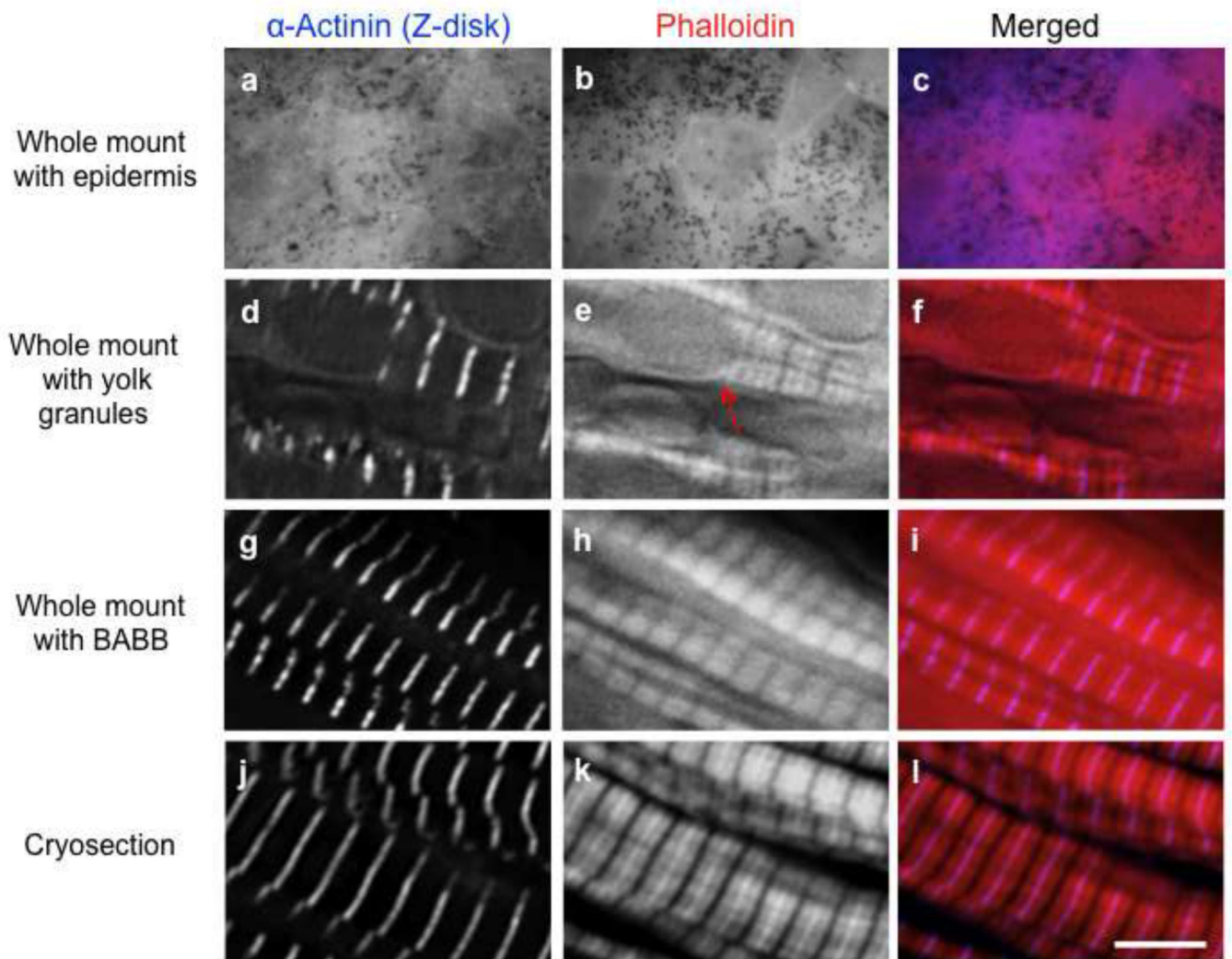


Figure 5. Comparison of stains from whole-mount, cryosection and BABB approaches
 When the epidermis is left on the whole-mount tissue, it obstructs the observation of myofibrils/sarcomeric proteins (a–c). Whole-mounts free of the epidermis exhibit staining for sarcomeric proteins (d–f) but yolk granules remain visible and obstruct subcellular structures (e, arrow). After staining, clearing somitic tissue with BABB masks yolk platelets; phalloidin labeling is preserved (g–i). Cryosections are superior for staining of actin filaments and sarcomeric proteins (j–l). Scale bar is 5 μ m.

Table 1
Some primary antibodies against sarcomeric proteins that cross-react with *Xenopus* orthologues

In most cases the concentrations of the antibodies are not given, thus only the working dilutions for whole mount IHC are provided.

Antibody	Species	Supplier & Catalog No#	Final Dilution	Reference
Anti- α -actinin EA-53	Mouse monoclonal	Sigma A7811	1:200	[33]
Anti- α -cardiac actin Acl-20.4.2	Mouse monoclonal	American Research Products 03-61075	1:400	[34]
Anti-pan α -actin	Rabbit polyclonal	Sigma A 2103	1:200	[35]
Anti-actin 5C5	Mouse monoclonal	Sigma A2172	1:500	[27]
Anti- α -skeletal actin JLA20	Mouse monoclonal	Developmental Studies Hybridoma Bank	unknown	[28]
Anti- α -tropomyosin TM311	Mouse monoclonal	Sigma T 2780	1:200	[36]
Anti-tropomyosin CH1	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:50	[9]
Anti-leiomodin	Rabbit polyclonal	Proteintech 14948-1-AP	1:400	
Anti-titin 9D10	Mouse monoclonal	Developmental Studies Hybridoma Bank	unknown	[6]
Anti-myosin F59	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:50	[6]
Anti-myosin MF20	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:1	[29]
Anti-myosin IIa 56-396-5	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:300	[30]
Anti-cofilin2	Rabbit polyclonal	Millipore 07-300	1:300	[31]
Anti-phospho cofilin1	Rabbit polyclonal	Santa Cruz SC-12912-R	1:3000	[31]
Anti-phospho cofilin2	Rabbit polyclonal	Abcam AB14134	1:300	[31]
Anti-desmin DB-E-5	Mouse monoclonal	Millipore MAB3430	1:200	[32]
Anti-cardiac troponin T CT3	Mouse monoclonal	Developmental Studies Hybridoma Bank	1:10	[9]
Anti-capZ 1E5.25	Rabbit polyclonal	Developmental Studies Hybridoma Bank	unknown	[6]