

Video Article

Preparation of Primary Myogenic Precursor Cell/Myoblast Cultures from Basal Vertebrate Lineages

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Abstract

Due to the inherent difficulty and time involved with studying the myogenic program *in vivo*, primary culture systems derived from the resident adult stem cells of skeletal muscle, the myogenic precursor cells (MPCs), have proven indispensable to our understanding of mammalian skeletal muscle development and growth. Particularly among the basal taxa of *Vertebrata*, however, data are limited describing the molecular mechanisms controlling the self-renewal, proliferation, and differentiation of MPCs. Of particular interest are potential mechanisms that underlie the ability of basal vertebrates to undergo considerable postlarval skeletal myofiber hyperplasia (*i.e.* teleost fish) and full regeneration following appendage loss (*i.e.* urodele amphibians). Additionally, the use of cultured myoblasts could aid in the understanding of regeneration and the recapitulation of the myogenic program and the differences between them. To this end, we describe in detail a robust and efficient protocol (and variations therein) for isolating and maintaining MPCs and their progeny, myoblasts and immature myotubes, in cell culture as a platform for understanding the evolution of the myogenic program, beginning with the more basal vertebrates. Capitalizing on the model organism status of the zebrafish (*Danio rerio*), we report on the application of this protocol to small fishes of the cyprinid clade *Danioninae*. In tandem, this protocol can be utilized to realize a broader comparative approach by isolating MPCs from the Mexican axolotl (*Ambystomamexicanum*) and even laboratory rodents. This protocol is now widely used in studying myogenesis in several fish species, including rainbow trout, salmon, and sea bream¹⁻⁴.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51354/>

Introduction

Considerable understanding of mammalian myogenesis has been obtained through the recapitulation of this process in both primary mouse (*Mus musculus*) myoblast cultures and the well-described mouse-derived cell line, C2C12⁵. Beginning in the 1950s⁶, these cultures have led to much advancement in the understanding of the murinemyogenic program and, by extension, myogenesis in other vertebrates. Additionally, single cell myofiber explant techniques have increased our understanding of interactions between satellite cells and surrounding myofibers⁷⁻⁹. Cell cultures are particularly attractive for investigations of myogenesis due to the short time from precursor to differentiated cell¹⁰, relative ease of transfection for RNAi¹¹⁻¹⁴, transgenic^{15,16} and overexpression studies^{14,17,18}, *in vitro* expansion followed by *in vivo* transplantation¹⁸⁻²⁰, and even comparison of myogenic precursor cells and their regulating agents across taxa^{21,22}. While differences due to the artificial environment of the culture system have been described^{5,23}, these *in vitro* systems have proven to be indispensable to our dissection of the intricate program governing the formation of multinucleated, terminally differentiated myofibers from mononucleated proliferative progenitor cells known as myosatellite cells (MSCs) among the mammals.

Outside of the class *Mammalia*, however, the conservation and/or divergence of mechanisms controlling myogenesis are poorly understood, largely due to the difficulty in culturing myogenic precursor cells (MPCs) and myoblasts from various taxa. Indeed, primary myoblast cultures have only been described in three birds²⁴⁻²⁶, one reptile²⁷, a few amphibians²⁸⁻³⁰, and some fishes^{1,3,4,31-33}. Continuous myogenic cell lines from vertebrates other than rodents³⁴⁻³⁶ are even more rare, with the only non-mammalian myogenic cell line being derived from Japanese quail (*Cortunix japonica*), QM7³⁷. Despite many attempts at immortalization, a teleost myogenic cell line remains elusive and a protocol for efficient transfection of these cells was only published this year¹⁵. Thus, clear and well-optimized protocols for culturing primary MPCs and myoblasts from a variety of vertebrates are very much needed to not only further expand our knowledge of the evolution of the myogenic program, but to employ the power of comparative physiology to make breakthroughs in the treatment of human skeletal muscle diseases and disorders.

While the literature contains many reports of MPC/myoblast isolation³⁸⁻⁴⁹, it is common for authors to describe the protocols for such isolations in brief, often incomplete, formats. Further, the most instructive protocols reported have been developed for mice⁵⁰⁻⁵³, and some of these rely on antibody selection^{54,55} or fluorescence transgenes^{56,57}, making these protocols unusable or impractical in most non-rodent species utilized

by muscle biologists. With little known about piscine, amphibian, and reptile myogenesis, a detailed and thorough protocol, described with audiovisual guidance and with demonstrated efficiency in distantly related species, would be most helpful to the field.

First described by Powell and colleagues in 1989⁵⁸, the following protocol was initially developed to isolate MPCs and myoblasts from salmonid fishes (namely, rainbow trout, *Oncorhynchus mykiss*, and Atlantic salmon, *Salmo salar*) and some larger cyprinids (i.e. goldfish, *Carassius auratus auratus*). In 2000, Fauconneau and Paboeuf optimized a primary myoblast culture for rainbow trout⁵⁹, and minor optimizations made that protocol utilizable in several smaller minnows of the *Danioninae* clade (zebrafish, *Danio rerio*, and giant danio, *Devario aequipinnatus*)³² due to the many genetic tools available for zebrafish work and thus its close relatives. Teleost fish are attractive organisms for study due to their divergent growth strategy (at least in most species). Large salmonids, like most fishes, grow indeterminately, with growth potential unfettered by an asymptote at maturity, even in old age⁶⁰⁻⁶². Unlike zebrafish, large danionins such as the giant danio⁶³ and moustached danio display growth potentials typical of teleost fish, making their direct juxtaposition an ideal platform for understanding whether MPC cell fate choice plays a role in skeletal muscle hyperplasia versus hypertrophy.

Likewise, we have demonstrated that this protocol can be used with mice and axolotls, with relatively high cell yield and viability indices. Urodele salamanders, such as the Mexican axolotl (*Ambystoma mexicanum*), possess the remarkable ability to regenerate tissues, including entire limbs and tails⁶⁴⁻⁶⁶. This characteristic makes these amphibians interesting models of skeletal muscle wasting and aging. Using the protocol described below, a similar approach can be undertaken as has been done in many fish species, providing an even wider comparative context for such studies. As many truly comparative biologists appreciate, the most meaningful advances in basic biology and translational biomedicine can be made when data are analyzed within the widest spectrum (here, the entire vertebrate lineage).

Protocol

Ethics Statement: All experimentation involving vertebrate animals described herein was approved in advance by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and is consistent with guidelines established by the Office of Laboratory Animal Welfare, National Institutes of Health of the U.S. Department of Health and Human Services.

1. Preparation for Culture

1. Prepare the base medium as follows: 9 mM NaHCO₃ (1.51 g per 2 L), 20 mM HEPES (9.53 g per 2 L) in DMEM (13.48 g/L; 26.96 g per 2 L).
 1. Determine pH and adjust to 7.40 with HCl or NaOH.
 2. Determine osmolality and adjust to ~ 300 mOsm with NaCl (if needed).
 3. Filter sterilize using (2) 1 L vacuum sterilization systems and store at 4 °C protected from light for up to 2-3 months.

NOTE: See **Tables 1** and **2** for complete reagent, tools, consumables, and equipment information.

2. Prepare poly-L-lysine-treated plates by dissolving 5 mg of γ-irradiated poly-L-lysine (> 300,000 mw) in 50 ml of ultrapure sterile water. Mix gently by inversion for 10 min at room temperature, ensuring full dissolution of lysine polymers.
3. Pipette the necessary amount of poly-L-lysine solution into each well, according to the plate type (**Table 3**). Allow plates to stand in laminar flow hood for 5 minutes.
4. Next, remove poly-L-lysine solution and wash twice with sterile water. Allow plates to air dry in laminar flow hood for 20 minutes.
5. Obtain 1 mg of laminin (Engelbreth-Holm-Swarm origin) and dissolve in 50 ml of base medium to a final concentration of 20 µg/ml. Apply laminin solution to poly-L-lysine-treated cell culture plates at 2 µg/cm². (see **Table 3** for different plate sizes)
6. Gently swirl the solution to ensure full well coverage. Seal plates with laboratory tape and place in incubator for 24 hours. The incubator should be set to appropriate temperature for species being used (see **Table 7**), and no additional gases need to be added to incubator.

NOTE: Laminin must be allowed to adhere to plates for 24 hours before seeding cells. Failure to do so will result in poor to no cell adherence.

7. Prepare media as described in **Table 4**. Complete media is best prepared the day of use.
8. In preparation for tissue isolation, assemble the following items and autoclave: 300-500 ml beaker(s); glass Petri dishes; scalpel handles; forceps (fine and coarse); dissection scissors; and several sheets of water-repellant autoclave paper.

NOTE: For each person assisting with the dissection process, (2) scalpel handles, (1) forceps (type depending on personal preference), (1) dissection scissors, and (5-10) sheets of water-repellant autoclave paper are sufficient.

9. In addition to the autoclaved items above, assemble 70% ethanol, a weight balance, sterile 50 ml conical tubes (number depends on size of culture), and ice.

2. Tissue Dissection

1. Before beginning, ensure that a sufficient stock of fish is available.

NOTE: The age of the fish to be used is of critical importance. While fish of two different species may be of similar weights, a younger fish of one species will possess more MPCs than an older fish of another species. As a general rule, younger fish, especially when working with salmonids, are best. For danionins, fish as old as one year can be utilized optimally, while salmonids of age 4-6 months or younger (up to 15 g) are optimal.

2. For each 5 g of tissue to be dissected, aliquot 25 ml of isolation medium into a sterile 50 ml conical tube.
3. Weigh, record mass, and number the tube(s). Place tubes on ice.
4. Euthanize 2-3 fish at a time by immersion in > 300 mg/L sodium bicarbonate-buffered tricaine methanesulfonate. Allow opercular movement to cease for > 5 minutes and then submerge fish in 70% ethanol (contained in the sterile beakers) for 30 seconds.

5. Remove fish from 70% ethanol and place on water-repellant autoclave paper. Directly behind the opercula, use a scalpel to make a superficial, shallow incision. Remove the scales and skin by grasping the skin at the aforementioned incision and pull toward tail of fish.
6. Following skin removal, excise the epaxial, fast-glycolytic 'white' muscle of the fish from both sides (avoiding the slow-oxidative 'red' muscle located near the lateral line) and place in isolation medium. Discard the remainder of the fish as required by institution.

NOTE: While it is entirely possible to culture MPCs originating from red muscle, nearly every publication using teleost MPCs has utilized cells isolated from the epaxial myotome.

7. Repeat steps 2.4-2.6 until a sufficient quantity of muscle is obtained. During dissection, ensure that pooled muscle tissue remains on ice to maintain cell viability.

3. Mechanical Dissociation

1. Pour one tube of 25 ml/5 g of muscle tissue into a glass Petri dish. Using two scalpel handles with attached #10 scalpel blades, mince tissue to a slurry or purée consistency. A "back-and-forth" motion of pulling the scalpel blades past each other works best.

NOTE: While tissue homogenizers may seem appropriate, the number of viable cells will be dramatically reduced, if not abolished.

2. Using a 25 ml serological pipette and serological pipettor, remove the slurried tissue and replace in the original conical tube.
3. Repeat steps 3.1-3.2 until all tissue in all tubes has been mechanically dissociated.
4. Centrifuge tissue for 5 minutes at 300 x g and 10 °C.
5. Following centrifugation, discard the 25 ml of media supernatant with a 25 ml serological pipette, vacuum aspirator, or by careful decanting.
6. Add 25 ml of wash medium to each tube, resuspend tissue, and recentrifuge as above.
7. Wash twice with wash medium, removing the supernatant each time.

4. Enzymatic Dissociation

1. During step 3.4, prepare collagenase solution by combining 0.22 g of Type IV collagenase and 44 ml of base medium (or sufficient quantity at 0.11 g/0.22 ml).
2. Stir in a beaker for 10-15 minutes at 4 °C. Filter sterilize with a 50 ml syringe and a 0.45 µm syringe filter.

NOTE: More than one syringe filter may be needed, depending on the collagenase preparation. Collagenase obtained from vendors other than Worthington Biochemical poses more difficulty during filtration. Collagenase is used to dissociate peptide bonds in collagen that constitute the endomysium. This digestion will remove that protective barrier of the myofibers.

3. For each g of muscle tissue, resuspend tissue in a 0.2% collagenase solution. For 5 g of muscle tissue, add 10 ml of collagenase solution and 15 ml of base medium.
4. Add 10 µl of PSF per ml of collagenase solution/base medium (e.g. 250 µl to 25 ml). Ensure that tissue is well suspended, as this will affect the efficiency of the enzymatic digestion.
5. Incubate muscle tissue in collagenase for 60 minutes at 18 °C with gentle rocking. Depending on the degree of mechanical dissociation and species, collagenase digestion may be extended to 90 minutes, although this may affect cell viability.
6. Following collagenase digestion, centrifuge conical tubes at 300 x g for 5 minutes at 12 °C. Discard the supernatant and resuspend tissue in 25 ml of wash medium.
7. Recentrifuge at 300 x g for 5 minutes at 12 °C. Repeat with washing medium a second time.
8. After washing tissue twice, resuspend muscle tissue in 25 ml of wash medium and triturate with a 10 ml serological pipette until the tissue/medium homogenate passes in and out of the pipette with relative ease. 5-10 triturations is usually sufficient.
 1. Repeat with a 5 ml serological pipette and then a 16 gauge metal cannula attached to a syringe.
9. Centrifuge conical tubes at 300 x g for 5 minutes at 12 °C. Decant supernatant.
10. During the above 5 minute centrifugation, prepare the trypsin digestion solution by combining 0.25 g of trypsin with 5 ml of base medium.
11. Stir at 4 °C for 10-15 minutes and filter sterilize with a 20 ml syringe and 0.45 µm syringe filter.
12. Retrieve conical tubes and add 100 µl of trypsin solution and 4.9 ml of dissociation medium for every 1 g of muscle tissue to every tube. For example, add 500 µl of trypsin solution and 24.5 ml of dissociation medium to 5 g of muscle tissue.

NOTE: Trypsin is a serine protease that will separate myogenic precursor cells from the basal lamina.

13. Resuspend tissue in the trypsin/dissociation medium well. Incubate for 20 minutes at 18 °C.
14. Following the first trypsin incubation, centrifuge conical tubes at 300 x g for 1 min at 12 °C.
15. Neutralize trypsin by combining the supernatants with *isolation medium* at a concentration of 1 volume of supernatant to 4 volumes of isolation medium. Store at 4 °C during the second trypsin digestion.
16. To the remaining pellet of tissue, repeat steps 4.12-4.15, combining the supernatant following step 4.15 with the supernatant/isolation medium from the first trypsin digestion.
17. **ALTERNATIVELY:** The collagenase and trypsin digestions may be combined to maximize cell yields.
 1. To do this, triturate the collagenase digest using a metal cannula (6 in length and 16 gauge works best) attached to a 20 ml syringe until the homogenate easily passes through the cannula.
 2. To the homogenate, add 500 µl of trypsin solution (prepared in step 4.10) and incubate at 18 °C for 20 minutes.
 3. Following the incubation, centrifuge conical tubes at 300 x g for 1 minute at 12 °C.
 4. Neutralize trypsin by combining the supernatants with *isolation medium* at a concentration of 1 volume of supernatant to 4 volumes of isolation medium.
 5. Store at 4 °C during the second trypsin digestion.
 6. Proceed with step 4.18 as with the standard protocol.
18. Dispense the final supernatant/isolation medium mixture into 50 ml conical tubes and centrifuge at 300 x g for 10 minutes at 12 °C.

19. Remove the supernatants taking care not to disturb the cell pellets. Pipette 2 ml of *complete medium* into each tube, dissolving each cell pellet.
20. Combine cells suspended in complete medium in one 50 ml conical tube.
21. Rinse each tube with 1 ml of complete medium, combining with the pool of cells resuspended previously.
22. Triturate with a metal cannula and syringe 5-10 times.
23. Filter cell suspension through a 100 μ m cell strainer, rinsing with complete medium.
24. Filter the cell suspension through a 40 μ m cell strainer.
25. Add sufficient complete medium to 50 ml and centrifuge at 300 x g for 10 minutes at 15 °C.

5. Counting, Dilution, and Seeding of Cells

1. Following the final centrifugation in step 4.25, remove supernatant by careful decanting or with a serological pipette. Resuspend the cell pellet in 5 ml of complete medium.
2. With cells fully resuspended, remove 20 μ l of the cell suspension and place in a 1.5 ml microcentrifuge tube.
3. Add 5 μ l of Trypan blue dye and wait 5 minutes. Using a hemocytometer, determine the number of viable cells.
4. Upon determination, dilute cells to the needed concentration. Teleost MPCs are best seeded at 150,000-200,000 cells per cm². For a six-well plating strategy, diluting cells to 1.5×10^6 - 2.0×10^6 per ml supports sufficient proliferation and differentiation.
5. Retrieve poly-L-lysine-treated, laminin-coated plates and seed cells. (See **Table 5** for plate-specific dilutions and plating volumes.) NOTE: **Table 6** depicts average number of cells isolated per gram of muscle tissue isolated from various teleost species. Rainbow trout (*O. mykiss*) exhibit fewer MPCs per gram of muscle tissue than do zebrafish (*D. rerio*) and will thereby require more fish to isolate from for proper seeding.
6. Seal plates with laboratory tape and place in chilling incubator at the appropriate temperature. MPCs from all species listed here (save for mice) may be incubated under normal atmospheric conditions without carbon dioxide (CO₂) supplementation. (See **Table 7**.)
7. **ALTERNATIVELY:** Some laboratories have adopted a protocol that calls for allowing MPCs to adhere to the culture substrata for 40 minutes.
 1. Then rinse plates with washing media to remove any loosely attached and non-adherent cells. Warning: This step is very important to avoid "non-specific" adhesion of other cells (e.g. fibroblasts) to the culture substrata.
 2. Add complete media and place cells in the incubator and care for as detailed below.

Representative Results

Twenty-four hours post-seeding, myogenic precursor cells (MPCs) should be visible attached to the laminin substratum (see **Figures 1a** and **1d**). Following seeding, cells (MPCs) adopt a spindle-like shape, indicative of this cell type (**Figure 1**) and are MyoD1+ (**Figure 2**). In *Danio* species, MPCs appear to be more compact with smaller bipolar processes than do MPCs from *Oncorhynchus* and *Salmo* species. However, over four days of culture, MPCs from all piscine species examined adopt similar morphologies and look distinctly like myoblasts (**Figures 1b** and **1e**). Complete media should be removed, and MPCs should be washed twice with wash medium. Myofibroblasts can contaminate myogenic cultures and are readily distinguishable from MPCs/myoblasts by their star-like morphology (versus the spindle shape of the myoblast). Washing of the plates greatly improves the removal of such fibroblasts.

In the species described here (see **Table 7**), daily media changes produce the best results. MPCs and progeny (myoblasts and nascent myotubes) should be washed once or twice prior to the addition of fresh complete media. Alternatively, media changes can be reduced to every other day after the cells have reached the definitive myoblast stage (approximately day 4 of culture). Myotubes should form within 2-3 days (**Figures 1c** and **1f**) and be myogenin+ (**Figure 2**), especially when cultured in differentiation medium (see below). While periods of weeks to months have been reported in several mammalian species, piscine MPCs and myoblasts do not appear to tolerate such periods of time. Cells proliferate for the first six days of culture (**Figure 3**), with subsequent differentiation between days 7 and 9-11 of culture. Afterward, cells senesce or apoptose.

The myogenic nature of MPCs and their progeny isolated using this protocol has been determined^{3,32,33} based on gene expression. Unlike mammalian culture systems, proliferation of MPCs and myoblast progeny is relatively low during the initial days of culture (when compared to systems utilizing primary murine myoblasts or C2C12), with rapid increases detected as the cells form immature myotubes during days 6-9 of culture in *Danio* species³². Reports from rainbow trout have utilized a differentiation medium (common in mammalian myoblast cultures, but not required in piscine systems) and indicate that proliferation indices decrease as expected with myotube formation³³. In our hands, the resultant myotubes can remain in culture for up to 9-11 days (*Danio* species) and 11-13 days (*Oncorhynchus* species) before cellular senescence or apoptosis occurs.

Reagent	Company (Preferred v. Alternate)	Catalog Number (Preferred v. Alternate)	Quantity per Culture
γ -irradiated poly-L-lysine	Sigma-Aldrich (MP Biomedicals)	P5899 (ICN19454405)	5 mg
DMEM (high glucose)	Sigma-Aldrich (cellgro)	MT-50-003-PB (D7777)	2 L
Laminin	BD Biosciences (Sigma-Aldrich)	CB-40232 (L2020)	1 mg
Sodium Bicarbonate (NaHCO_3)	Fisher Scientific (Sigma-Aldrich)	BP328-500 (S5761)	1.51 g
HEPES ($\text{C}_6\text{H}_{18}\text{N}_2\text{O}_4\text{S}$)	Fisher Scientific (Sigma-Aldrich)	BP310-1 (H6147)	9.53 g
Antibiotic/Antimycotic	Thermo Scientific (Sigma-Aldrich)	SV3007901 (A5955)	17-20 ml
Gentamicin Sulfate	Lonza (Sigma-Aldrich)	BW17-519Z (G1397)	2-3 ml
Donor Equine Sera	Thermo Scientific (Sigma-Aldrich)	SH3007403 (H1270)	75 ml
Fetal Bovine Sera	Thermo Scientific (Sigma-Aldrich)	SH3007103 (F2442)	25 ml
Collagenase (Type IV)	Worthington (Sigma-Aldrich)	LS004189 (C9891)	0.44 g
Trypsin (from Pancreas)	MP Biomedicals (Sigma-Aldrich)	ICN15357125 (T5266)	1 g

Table 1. Detailed reagent list with preferred manufacturer and catalog number.

Consumable	Tools	Equipment
Cell Culture Plates	Forceps (Coarse)	Serological Pipettor
Sterile 50 ml Conical Tubes	Forceps (Fine)	pH Meter
Laboratory Tape	Scalpel Handles	Chilling Incubator (Ecotherm)
0.2 μm Vacuum Sterilization Systems	Scalpel Blades (#10, #11)	Laminar Flow Hood
Water-repellant Autoclave Paper	Surgical Scissors	Vacuum Manifold
Serological Pipettes	Glass Petri Dishes	Microosmolality Meter
	12-16 G Cannulas with Luer Locks	

Table 2. Consumables, tools, and equipment needed for cell culture.

Plate Size	cm^2 per Well	Poly-L-lysine*	Laminin**
6 well	9.5	1.6 ml	1
24 well	1.9	0.32	0.2
48 well	0.95	0.16	0.1
96 well	0.32	0.06	0.03
*0.1 mg/ml concentration	** 0.020 mg/ml concentration		

Table 3. Optimized volumes for coating cell culture plates with poly-L-lysine and laminin.

Reagent	Isolation	Wash	Dissociation	Complete
Base Medium	419.25 ml	395.40 ml	297.00 ml	178.00 ml
PSF*	5.00 ml	4.00 ml	3.00 ml	2.00 ml
Gentamicin Sulfate**	0.75 ml	0.60 ml	-	-
Donor Equine Serum	75.00 ml	-	-	-
Fetal Bovine Serum***	-	-	-	20.00 ml
* PSF: penicillin/streptomycin/fungizone cocktail (100x); ** 50 mg/ml concentration; *** Characterized				

Table 4. Different media preparations for isolation and culture of myogenic precursor cells (MPCs).

Plate Size	cm ² per Well	Dilution	Plating Volume
6 well	9.5	1.5 - 2.0 x 10 ⁶ cells/ml	1 ml
24 well	1.9	1.5 - 2.0 x 10 ⁶ cells/ml	250 µl
48 well	0.95	1.5 - 2.0 x 10 ⁶ cells/ml	150 µl
96 well	0.32	1.5 - 2.0 x 10 ⁶ cells/ml	50-100 µl

Table 5. Recommended dilutions and plating volumes by cell culture plate well size.

Species	Average # cells/g tissue
<i>Danio rerio</i>	6,400,000
<i>Danio dangila</i>	1,783,000
<i>Devario aequipinnatus</i>	1,797,000
<i>Oncorhynchus mykiss</i>	66,800

Table 6. Average number of myogenic precursor cells isolated from 1 gram of muscle tissue from various teleost species: *Danio rerio* (zebrafish), *Daniodangila* (moustacheddanio), *Devario aequipinnatus* (giant danio), and *Oncorhynchus mykiss* (rainbow trout).

Species	Temperature
<i>Danio/ Devario</i> spp.	26 - 28 °C
<i>Oncorhynchus/Salmo</i> spp.	10* - 18 °C
<i>Ambystoma mexicanum</i>	18 °C

* Lower temperatures support lower proliferation rates.

Table 7. Recommended incubation temperatures for piscine and amphibian myogenic precursor cells (MPCs).

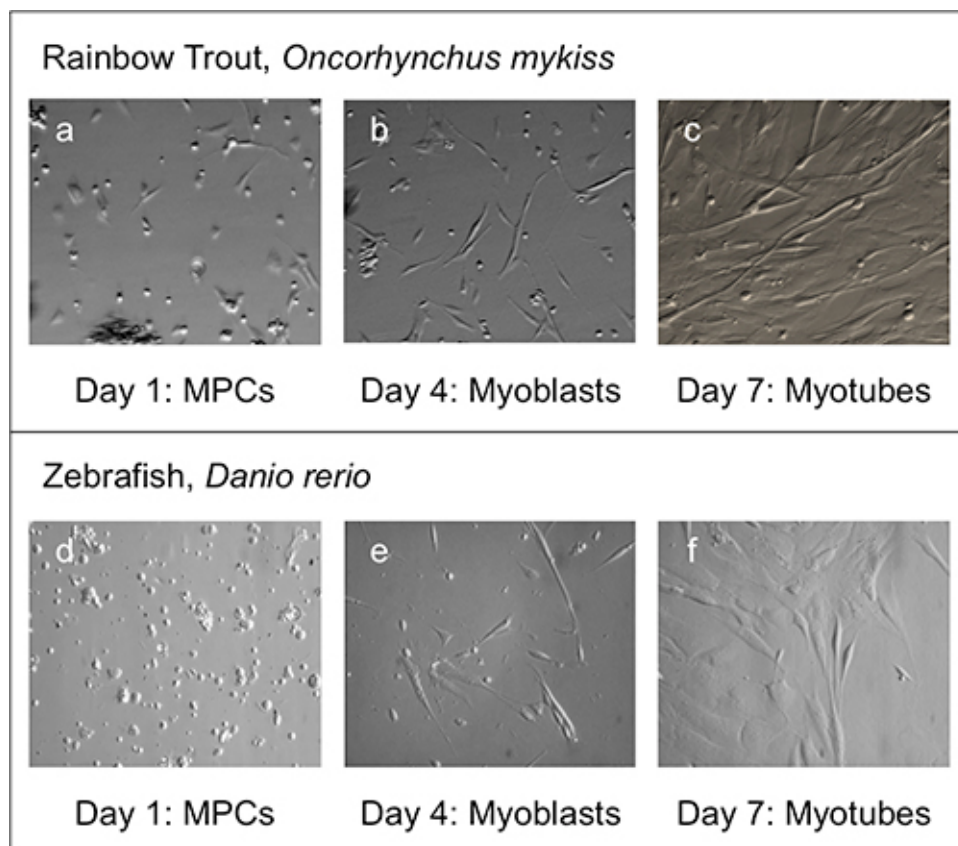


Figure 1. Representative bright field images of MPCs (leftmost), myoblasts (middle), and early myotubes (rightmost) from two species, rainbow trout (*Oncorhynchus mykiss*, top) and zebrafish (*Danio rerio*, bottom).

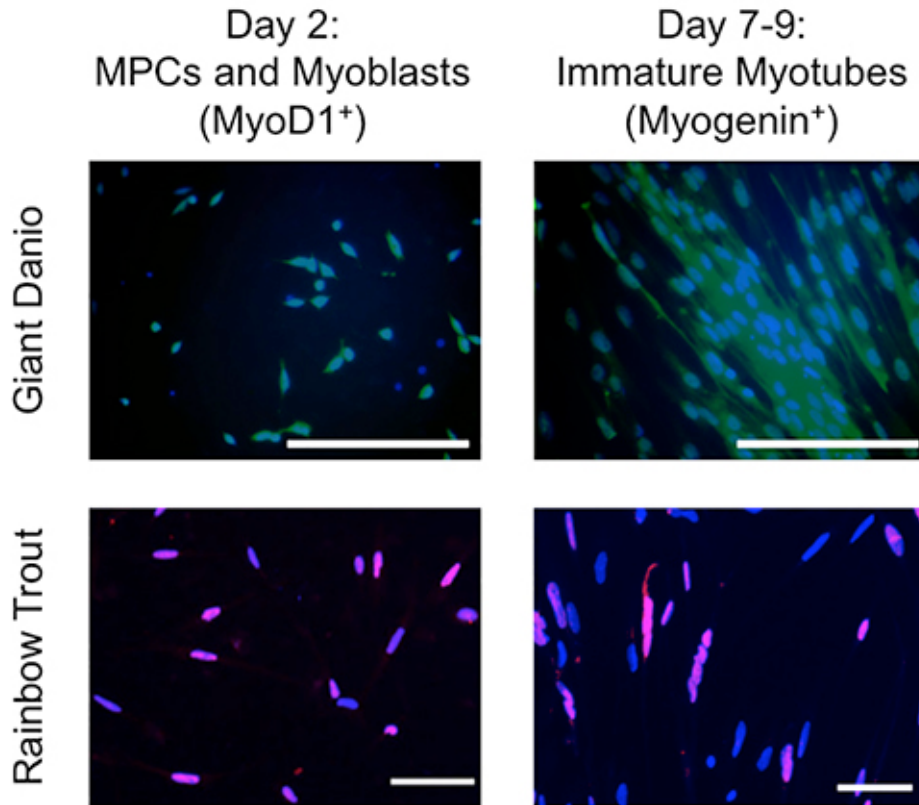


Figure 2. Representative immunocytochemical staining of MPCs (a, c) and myotubes (b, d) isolated and cultured from giant danio (*Devario aequipinnatus*, top) and rainbow trout (*Oncorhynchus mykiss*, bottom). In culture, myoblasts are MyoD1⁺ positive (a, c) as visualized using commercially available MyoD1⁺ antibodies (danio: C-20, Santa Cruz Biotechnology; trout: NB100-80899, Novus Biologicals). Additionally, as myoblasts differentiate, they express myogenin (b, d) as visualized using commercially available myogenin antibodies (danio: M-225; trout: SC-567; both from Santa Cruz Biotechnology).

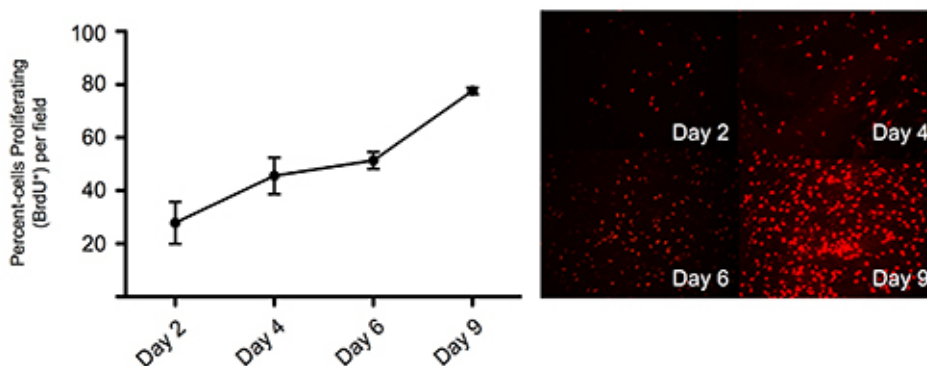


Figure 3. Representative cell proliferation data using BrdU to measure cell proliferation rates over time in culture. Data obtained from MPCs isolated and cultured from giant danio⁶⁷.

Discussion

The myogenic program, in whichever species examined, can be most easily studied through an *in vitro* system. Indeed, upon isolation, myogenic precursor cells (MPCs) in fish or myosatellite cells (MSCs) in mammals readily enter this highly regulated process involving the proliferation, cell cycle withdrawal, and terminal differentiation of myoblasts and the fusion of those myoblasts into nascent myotubes⁶⁷. The general lack of transgenic gene reporter strains of piscine species (with the possible exception of the zebrafish⁶⁷ and rainbow trout⁶⁹) constrains *in vivo* work of MPC/MSC activation, proliferation, and differentiation, and thus the *in vitro* system presented here is an attractive platform for studies in fish species.

Successful culture of MPC/MSCs, no matter the species, is highly dependent on a) rigorous attention to sterility; b) thorough mechanical dissociation of skeletal muscle tissue; and c) optimization of enzymatic digestion. During the initial steps of muscle tissue isolation, great care must be taken to ensure that the necessary quantity of tissue is excised without contamination. It is of utmost importance that fish (or any animals being utilized, for that matter) are properly disinfected in 70% ethanol and for sufficient time. In our hands, 30 seconds works best; shorter periods of time can result in contamination and longer, loss of tissue integrity and cell viability. Ethanol can be used as a fixative, so care must be taken not to dehydrate the fish before dissection. Dissection can be done outside of a laminar flow cell culture hood; however, we recommend that this process be done within a hood to minimize any potential bacterial or fungal contamination.

While the mechanical dissociation described above may seem crude and/or tedious at first, it is critical to the isolation procedure. Two large scalpel blades, pulled past each other in a sliding motion (as shown in the video protocol), produces the best results. Once finished, the consistency of the homogenate should be that of a slurry or purée, and easily collected with a wide-bore serological pipette (*i.e.* 25 ml). Simply, the better the mechanical dissociation, the higher the MPC/MSC yield and the better the resulting culture. Poor dissociation will hinder enzymatic digestion and decrease cell yield. Although it may be tempting to consider, the use of electric tissue homogenizers dramatically lowers cell viability despite its obvious convenience, at least with piscine MPCs.

As with all protocols, optimization of the procedure detailed above is often necessary. This has proven true in our work with several danionin species. Results from our laboratory indicate that smaller danionins (*e.g.* the zebrafish) yield more MPCs per gram of skeletal muscle than do larger species (*e.g.* giant or moustached danio). However, this is only realized if a higher concentration of collagenase (0.3%) is used with tissue from smaller species. In our hands, a concentration of 0.2% is appropriate for salmonid species (*e.g.* rainbow trout; cutthroat trout [*Oncorhynchus clarki*], Chinook salmon [*Oncorhynchus tshawytscha*]), the larger danionins, axolotl (*Ambystoma mexicanum*) limb and tail, and even mouse skeletal muscle. Likewise, care must be taken to select the appropriate collagenase. In our experience, type IV collagenase preserves cell viability far better than collagenases recommended for fibrous tissues such as bone and muscle (*i.e.* collagenase type II)⁷⁰. While digestion may be more complete in a shorter time period, cell membrane receptor integrity is likely compromised by increased tryptic activity in these preparations. With respect to trypsin, our laboratory has always utilized the cruder trypsin preparation purified from porcine pancreas, rather than the 'purer' preparations available. In our culture activities with various species, we have noticed little beneficial effect of varying trypsin concentrations.

Trituration is critical to this protocol. It both dissociates the fibrous matrix of skeletal muscle and increases surface area for tryptic digestion all the while manually disrupting the structural integrity of the myofibers. When performing the triturations, successively using smaller bore pipettes and cannulas is much easier than using the cannula and syringe straightaway. Proceeding directly to triturations with a cannula and syringe can result in plugged cannulas and/or excessive pressure being applied to cells. Applying tryptic digestion without adequate trituration will dramatically reduce cell yields.

Once isolated, the culture process is quite straightforward. Most publications to date have utilized a protocol with one media for both proliferation and differentiation^{33,71-75}, including our own; however, more recent articles written by several French investigators have described a two media protocol, with separate based media and serum content for proliferation and differentiation³³. This 'newer' protocol more closely mimics those used in the culture of murine MSCs and myoblasts and appears to enhance proliferation of early stage myoblasts³³ and may be more appropriate to control proliferation and/or differentiation of cells. However, without extensive characterization of gene expression, it is difficult to conclude whether such a 'proliferation' media also conserves a more 'myoblast-like' state than does the traditional one media methodology. Previous publications describing gene expression, whether at the transcript or protein level, reported using the traditional one media protocol³. Alternatively, a single media (DMEM described herein) can be supplemented with differing concentrations of fetal bovine serum (FBS): 10% for proliferation and 2% for differentiation.

While investigators employing mammalian cell culture systems may be acclimated to using carbon dioxide atmospheres for the cultivation of these cells, the intrinsic differences between terrestrial and aquatic gas exchange call for a different approach when culturing piscine or water-confined urodele cells, including MPCs. Media detailed here are buffered with a piperazine-derived, zwitterionic organic compound [HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid] and sodium bicarbonate (NaHCO₃). Thus, an incubator with gas injection is not needed or required for culture of these cells. More importantly, such incubators should possess the ability to cool rather than heat, as the temperatures needed to culture piscine and urodele MPCs range between 18-26 °C. Further, salmonid MPCs can be cultured at lower temperatures if needed, further highlighting the need for a cooling incubator. Additionally, we have found (as have other investigators, as communicated previously) that sealing of culture plates is necessary to preserve cellular viability. Simply wrapping the interface between the culture lid and plate with standard laboratory tape is sufficient to realize this goal.

While passaging of both primary MPCs/MSCs/myoblasts is common in mammalian cell culture, it does not appear to be possible with piscine cells, at least before the late Mb stage (around day 6 of culture). Therefore, the appropriate number of cells sufficient to support proliferation and to reach the goals of the experiment must be seeded at the initiation of the culture. Further, if less than expected cell yields are obtained, it is not possible to propagate cells and then replate the progeny later. We have attributed this characteristic to the increased reliance on the extracellular matrix (ECM) of piscine MPC/myoblasts. Alternatively, it is possible that this is an artifact of the laminin substrate and thus further empirical determination of ECM components necessary for piscine MPC/Mb proliferation and differentiation is warranted. We do note, however, that several of our collaborators have successfully removed late-stage myoblasts (~ day 6 of culture) from the culture substratum and replated these cells (J. M. Froehlich and P. R. Biga, personal communication).

Using this protocol or one similar to it, experimentation involving RT-PCR^{3,71-74,76-78}, Western blot^{32,79-81}, immunocytochemistry^{32,33}, proliferation assays^{32,33,59}, gene transfection¹⁵, morphometric analysis⁷⁸, toxicology screening⁸², and chromatin immunoprecipitation (ChIP; J. M. Froehlich and P. R. Biga, unpublished results) has been done. However, further descriptions of additional *in vitro* protocols, namely those involving passaging and clonal propagation, are very much needed. Indeed, the Rodgers laboratory¹⁵ made significant advancements in the culture of piscine MPCs/myoblasts by optimizing a protocol for inducing transfection of rainbow trout myoblasts. Based on this methodology, further investigations involving RNAi or overexpression of skeletal muscle-specific targets, especially those postulated to be involved with the lifelong growth trajectories of teleost fishes, are not only possible, but may lead to significant advancements in the aquaculture and biomedical fields of science.

Disclosures

There is nothing to disclose.

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