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Rapid purification of transfected porcine muscle cells

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Abstract. A detailed methodology is described for fluorescence-activated cell sorting (FACS) of porcine muscle cells that have been transfected to express green fluorescent protein (GFP). Cells are liberated from porcine skeletal muscle and primary cultures are transfected with DNA encoding GFP. Primary cultures are subjected to immunocytochemistry using a primary muscle-specific monoclonal antibody followed by incubation with a phycoerythrin-conjugated second antibody. Transfected myoblasts are sorted from fibroblasts using forward angle light scatter and ninety degree light scatter, phycoerythrin fluorescence, and GFP fluorescence. These procedures allow for isolation of genetically-engineered porcine muscle cells more rapidly than traditional clonal selection procedures. Consequently, FACS provides porcine myoblast populations that retain the majority of their replicative capacity and are not contaminated with non-myogenic cells.

Key words: Flow cytometry, Muscle cell, Porcine, Transfection

Abbreviations: GM = growth medium; MEM = minimal essential medium; FACS = fluorescence-activated cell sorting; FLS = forward angle light scatter; 90° LS = ninety degree light scatter; GFP = green fluorescent protein; PBS = phosphate-buffered saline; FBS = fetal bovine serum; PE = phycoerythrin

1. Introduction

A. *Myoblast-mediated gene transfer*

Ex vivo cell-mediated gene transfer has been defined as the isolation of primary cells *in vivo*, subsequent genetic manipulation and re-implantation [8]. Adult myoblasts (satellite cells) are mononucleated cells located between the basal lamina and sarcolemma of muscle fibers that donate their nuclei to existing muscle fibers to facilitate muscle hypertrophy and repair. These unique characteristics make myoblast-mediated gene transfer a potential mechanism for delivery of engineered myoblasts to animals for the study of gene expression in skeletal muscle. Purified myoblasts are capable of *in vitro* proliferation, however, traditional clonal isolation techniques may expend nearly half of their proliferative capacity in the selection process. Successful myoblast-mediated gene transfer is dependent on implantation of cells that retain much of their replicative capacity; thus, there is a need for development of techniques that rapidly isolate large numbers of genetically-modified myoblasts from mixed primary preparations prior to implantation.

B. *Myoblast identification*

Harvesting myoblasts from young animals results in the greatest number of isolated cells that retain their maximal proliferative capacity. Webster et al. [19] found that satellite cells isolated from young animals are capable of 35 to 40 cellular doublings before quiescence. However, as age of animals used for isolation increases, the number of replications each isolated myoblast is capable of decreases. Unfortunately, traditional muscle cell isolation procedures result in significant contamination with other cell types such as adipocytes, immune cells and fibroblasts. Therefore, there is a need for a rapid method of isolating muscle cells from mixed cell preparations. Isolation using clonal rings, pre-plating on treated tissue culture plates and muscle fiber teasing have all been used [9, 17], but these methods are time consuming and result in low yields of pure myoblasts. A possible solution to these methods is the utilization of a muscle-specific antibody and fluorescence activated cell sorting (FACS).

In 1980, Walsh [20] produced a myoblast-specific monoclonal antibody against a neural cell adhesion molecule (NCAM). This antibody (5.1H11) was utilized to study the involvement of NCAM in myoblast fusion. Three isoforms of the myoblast-specific neural cell adhesion molecule were identified and determined to be splice variants that belong

to the immonoglobulin super-family. All three isoforms are required for accurate myotube fusion [10]. Two of the isoforms are cell surface proteins, and the smallest isoform is secreted [6]. Similarly, monoclonal antibodies to a membrane glycoprotein (H36) were used to isolate rat myoblasts from mixed primary preparations by FACS [15]. This antigen was eventually identified as a skeletal muscle-specific integrin alpha-chain designated H36 $\alpha 7$ [18].

C. *Isolation of myoblasts through antibody detection*

Once a myoblast-specific antibody is created, isolation of cells via fluorescence-activated cell sorting (FACS) or magnetic bead separation is possible. Magnetic bead separation has been used to remove small proteins, nucleic acids and immune cells from liquid medium, but this technique has the potential for separating myoblasts from mixed primary muscle cell populations [1]. However, technology for the isolation of cells from mixed cell populations by flow cytometry has been extensively used in clinical applications and can be easily converted to myoblast separation [14]. In fact, 5.1H11 antibody has been used to separate myoblasts from a mixed populations via FACS [3, 4, 19].

D. *Isolation of myoblasts using structural characteristics*

Unfortunately, antibodies do not react with the same epitope across different species. To eliminate the need for antibody production, Baroffio et al. [2] found that myoblasts could be separated from a mixed human primary muscle cell preparation based on size (forward angle light scatter) and cytoplasm/nuclear ratio (90° light scatter). Baroffio et al. [2] and Blanton [3, 4] found that illuminating cells with a 488 nm argon laser could distinguish two populations of cells based on size. A population with a smaller cell diameter expressed myoblast markers, and fused to form multinucleated myotubes, indicating a myoblast lineage, whereas, a population with a larger cell diameter contained cells (fibroblast) that did not express these characteristics.

E. *Detection of genetically-modified myoblasts*

Several researchers have utilized the β-galactosidase gene products to identify implanted muscles post-fusion [11, 12, 16]. This requires fixation, permeabilization or the incorporation of a hypotonic solution in order to internalize β-galactosidase products. The fixation and permeabilization process prevents isolation and separation of transformed cells from non-transformed cells and utilization of hypotonic solutions can be technically difficult. Jellyfish, *Aqueous victoria*, possesses a green fluorescent protein (GFP) that fluoresces without the addition of substrates when illuminated with ultraviolet light. This makes GFP an ideal candidate for the isolation and selection of viable cells [21]. Transfected cells expressing GFP can be rapidly identified and isolated by FACS. Functional mutagenesis of GFP has produced a red shifted GFP (rsGFP), whose excitation maximum is 490 nm [7] and a substitution of Ser 65 to Thr (GFPS65T) which results in a six-fold greater fluorescence [13]. A variant containing two substitutions produced a protein that is 35-times brighter than wild type GFP, with a 488 nm excitation peak that is identical to the emission wavelength of the argon laser most commonly used in FACS and confocal microscopy [5]. The FACS optimized variant is available commercially as pEGFP[13]. This variant is synthesized efficiently in mammalian cells and has a wider stokes shift than the original GFP. This shift in emission and excitation allows detection and isolation of transfected cells by flow cytometry with minimum background fluorescence.

2. Materials

A. Equipment
1. Laminar safety cabinet (Bellco-Glass, Inc.).[1]
2. Centrifuge (IEC).[2]
3. Inverted microscope (Nikon).[3]
4. Light and ultraviolet inverted microscope (Nikon).[3]
5. 37 °C, 5% CO_2, humidified incubator (Nuaire).[4]
6. –80 °C freezer (Forma Scientific).[5]
7. 4 °C refrigerator (Sear, Roebuck, Inc.).[6]
8. 37 °C water bath (GCA/Precision Scientific).[7]
9. Liquid nitrogen storage freezer (Taylor-Wharton).[8]
10. Coulter Epics Elite flow cytometer (Coulter).[9]

B. Glass and plastic supplies
1. 10-cm tissue culture plates (Falcon; 08-772-4F).[10]
2. 15 ml and 50 ml conical tubes (Corning; 430790, 430828).[11]
3. 0.22 μm bottle top filter (Corning; 431117).[11]
4. 500 ml and 1000 ml bottles (Corning; 1395 500, 1395 1L).[11]
5. 500 ml and 1000 ml Erlenmeyer flasks (Corning; 5100 500, 5100 1L).[11]
6. 1.8 ml freezing vial (Corning-Costar; 430488).[11]

C. Biological Reagents
1. LipofectAMINE (Life Technologies; 10552-016).[12]
2. pEGFP (Clontech; 6084-1).[13]
3. Phosphate buffered saline (PBS; Life Technologies; 21300-025).[12]
4. Pronase (Calbiochem; 53702).[14]

5. Minimal essential medium (MEM; Life Technologies; 11900-24).[12]
6. Fetal bovine serum (FBS; Harlan Bio-products).[15]
7. Antibiotic/antimycotic (Sigma; A7292).[16]
8. Gentamycin (Life Technologies; 15750-060).[16]
9. Type A porcine gelatin (Sigma; G6144).[16]
10. Dimethylsulphoxide (Sigma; D2650).[16]
11. Bovine serum albumin (BSA; Sigma; A9418).[16]
12. Muscle-specific monoclonal antibody (5.1H11; Developmental Studies Hybridoma Bank).[17]
13. Phycoerythrin conjugated second antibody (PE; Jackson ImmunoResearch; 715-106-150).[18]
14. Ethylenediamine tetraacetic acid (EDTA; Sigma; E9884).[16]
15. Trypsin (Sigma; T4424).[16]

3. Procedure

A. Isolation, freezing, and thawing of porcine muscle cells
   1. Isolation of muscle cells for primary preparations [9]:
      a) Euthanize 4 to 8 week old pigs and aseptically remove the hind limb and transport to cell culture laboratory. Remove skin from medial surface of hind limb.
      b) Remove gracilis muscle and discard.
      c) Blunt dissect semimembranosus, remove adipose and connective tissue, and place in ice-cold phosphate-buffered saline (PBS).
      d) Thoroughly grind muscle in aseptically-prepared food processor.
      e) Place 10 g ground tissue into a 50-ml conical tube and add 10 ml pronase solution (0.8 mg/ml PBS).
      f) Incubate in a water bath at 37 °C for 40 min. Samples should be vigorously vortexed every 10 min during the incubation period.
      g) Centrifuge sample for 15 min at 1,200 ×*g*. Remove and discard supernatant.
      h) Resuspend pellet in 10 ml PBS and centrifuge for 15 min at 1,200 ×*g*. Remove and discard supernatant. This step ensures the complete removal of the pronase solution which if not thoroughly removed will decrease overall number of viable muscle cells isolated.
      i) Resuspend pellet in 10 ml cold minimal essential medium (MEM, pH 7.2) and gently vortex. Centrifuge 5 min at 300 ×*g*, and collect supernatant in a 50-ml conical tube. Repeat this step two times in order to maximize total number of cells collected.
      j) Centrifuge pooled supernatants at 1,200 ×*g* for 15 min.
      k) Discard supernatant using caution not to disrupt the pellet. Resuspend pellet in 10 ml Growth Medium (GM; MEM containing 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic and 0.1% gentamycin).
      l) Plate cell suspensions on culture dishes (10 g tissue equivalent per 10-cm dish) coated with 0.1% type A porcine gelatin and incubate in culture incubator (humidified environment; 5% CO_2, 95% air).
   2. Freezing isolated cells:
      a) Suspend 10^6 cells in 1 ml MEM containing 20% FBS, 1% antibiotic/antimycotic and 0.1% gentamycin in 1.8 ml cryovials.
      b) Gradually add 111 µl dimethyl sulphoxide (for final concentration of 10% v/v) while gently agitating cell suspension. It is important to add dimethyl sulphoxide very slowly for equilibration.
      c) Place vials at –80 °C for 24 h. For long term storage, transfer vials to nitrogen freezer.
   3. Thawing stored primary muscle cells:
      a) Remove vials from liquid nitrogen and immediately place in 37 °C water bath.
      b) Once thawed, transfer cell suspension to a 15-ml conical tube containing 10 ml MEM and centrifuge at 500 ×*g* for 5 min. Remove and discard supernatant.
      c) Resuspend cells in appropriate volume of GM and plate cell suspension on gelatin-coated tissue culture dishes, and incubate in culture incubator.

B. Transfection and immunocytochemistry
   1. Transient LipofectAMINE transfection of porcine muscle cells with pEGFP:
      a) Grow cells until 60 to 80% confluent in a 10-cm gelatin-coated tissue culture dishes.
      b) Incubate 83 µg pEGFP in 10 ml MEM at room temperature (RT) for 30 min.
      c) Add 53 µg pEGFP to lipofectAMINE solution for 15 min at RT.
      d) Wash cells with PBS and transfer lipofectAMINE:pEGFP solution to cells.
      e) Incubate for 5 h in culture incubator. Incubations exceeding 5 h result in extensive cell death due to the lack of FBS.
      f) Remove lipofectAMINE:pEGFP solution and wash cells with PBS. Add the appropriate volume of GM.
      g) A minimum of 24 h is required for post-

translational modification of pEGFP prior to functional assay.

2. Immunocytochemical staining of porcine myoblasts using a muscle-specific monoclonal antibody:
   a) Grow primary muscle cell preparations until 70% confluent on 10-cm gelatin-coated tissue culture dishes.
   b) Remove medium and wash with 10 ml PBS. Block plates with 5% bovine serum albumin (BSA) in PBS for 30 min at 37 °C in culture incubator. Following incubation gently remove blocking buffer. Do not wash plates.
   c) Dilute the primary muscle-specific monoclonal antibody (5.1H11) 1:2,500 in PBS containing 10% FBS and add to primary muscle cell preparation. Incubate at 37 °C in culture incubator for 30 min.
   d) Wash cells three times with PBS and incubate with phycoerythrin-conjugated (PE) second antibody diluted 1:1,000 in PBS containing 10% FBS solution for 30 min at 37 °C in culture incubator.
   e) To remove cells from tissue culture plate without disrupting antibody binding, incubate for 15 min in 10 ml calcium phosphate-free PBS solution containing 0.53mmol/l EDTA. Aggressive agitation is often required to completely remove cells from tissue culture plate.
   f) Cells are then centrifuged at 500 ×*g* for 5 min and resuspended in MEM prior to flow cytometric analysis.

C. Flow cytometric isolation and analysis of porcine fibroblasts and muscle cells
   1. Isolation based on size (forward light scatter; FLS) and nuclear:cytoplasmic ratio (90° light scatter; 90°LS; Figure 1).
      a) Grow primary muscle cell preparation until 60 to 80% confluent on 10-cm gelatin-coated tissue culture plates.
      b) Wash cells three times with PBS followed by incubation with 5 ml trypsin solution (1 mg trypsin/ml PBS) for 5 min at 37 °C in culture incubator. Periodic gentle agitation may be required to remove cells from tissue culture plate.
      c) Add 10 ml GM to inactivate trypsin. Collect solution in a 15-ml conical tube and centrifuge at 500 ×*g* for 5 min. Discard supernatant and resuspend cell pellet in 200 µl MEM.
      d) Immediately process cells on a Coulter Epics Elite flow cytometer employing a 488 nm argon laser to detect FLS and 90°LS. Fluorescent beads of 10, 20 and 40 µm diameters should be used to calibrate FLS for size measurements. Electronic compensation is used to correct for fluorescence overlap where necessary.
      e) Following sorting, cells are plated on 10-cm gelatin-coated tissue culture plates for

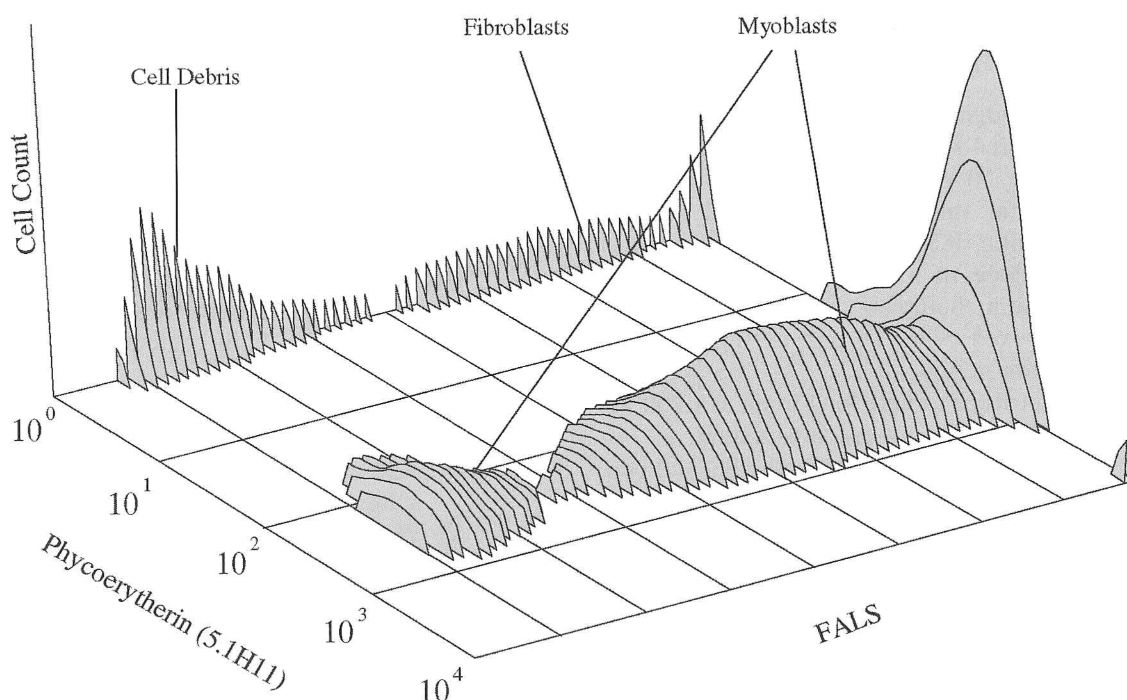

Figure 1. Three-dimensional contour plot of mixed primary porcine muscle cell preparation using 90°LS, 5.1H11 and cell count analysis for isolation of porcine myoblasts.

24 h. Cell populations are verified as myogenic by myotube fusion assays.

f) Cells larger than 10 µm are porcine non-myoblasts (fibroblasts) whereas cells smaller than 10 µm are porcine myoblasts [4].

2. Isolation of Porcine myoblasts using size (FLS) nuclear:cytoplasmic ratio (90°LS), a muscle specific monoclonal antibody (5.1H11), and pEGFP fluorescence (Figure 2).
   a) Using the previously described antibody staining procedure, primary porcine muscle cells transfected with pEGFP were incubated with the 5.1H11 antibody.
   b) Incubate cells for 15 min in 0.53 mM EDTA:PBS at 37 °C in culture incubator. Vigorous agitation may be required to remove cells from plates. This technique prevents antibodies from being stripped.
   c) Collect cells in a 15-ml conical tube and centrifuge at 300 ×*g* for 5 min. Supernatant is discarded and cells are re-suspended in 0.5M EDTA:PBS to prevent clumping.
   d) Cells are immediately analyzed on a Coulter Epics Elite flow cytometer employing a 488 nm argon laser to detect FLS, 90°LS, PE and GFP fluorescence. Fluorescent beads of 10, 20 and 40 µm diameters are used to calibrate FALS for size measurements. Electronic compensation should be used to correct for fluorescence overlap as necessary.
   e) Cells that are positive for GFP and PE fluorescence are sorted and placed in 10-cm gelatin-coated cell culture plates. Green fluorescent protein fluorescence is verified 24 h post-isolation followed by myotube fusion assays.

4. Results and discussion

We have discussed two methods of purifying porcine myoblasts from primary muscle preparations. Of these methods, isolation based on FLS is the most convenient, but least efficient. Purification by size revealed that small cell populations of 2-day-old primary muscle cell preparations are 92% myogenic. However, as days in culture increases number of myoblasts isolated from the small cell decreases to 78% by day 28 [4]. Caution should be used with this method to control non-myogenic cell contamination. Under conditions used in our laboratory we have found that fibroblasts divide every 18 to 20 h, whereas the myoblasts replicate every 24 to 28 h. Unfortunately, purification by population size results in some non-myogenic cell contamination, which will eventually outgrow isolated myoblasts. Additionally, the two populations are only detectable prior to first passage [4].

We have found that the purification of porcine myoblasts using a muscle-specific monoclonal antibody (5.1H11) [20] and fluorescence-activated cell sorting (FACS) is efficient and independent of passage (Figure 1). Using these techniques, we have repeatedly demonstrated that >80% of cells in our primary muscle cell preparations are myogenic, as confirmed by myotube fusion assay and desmin

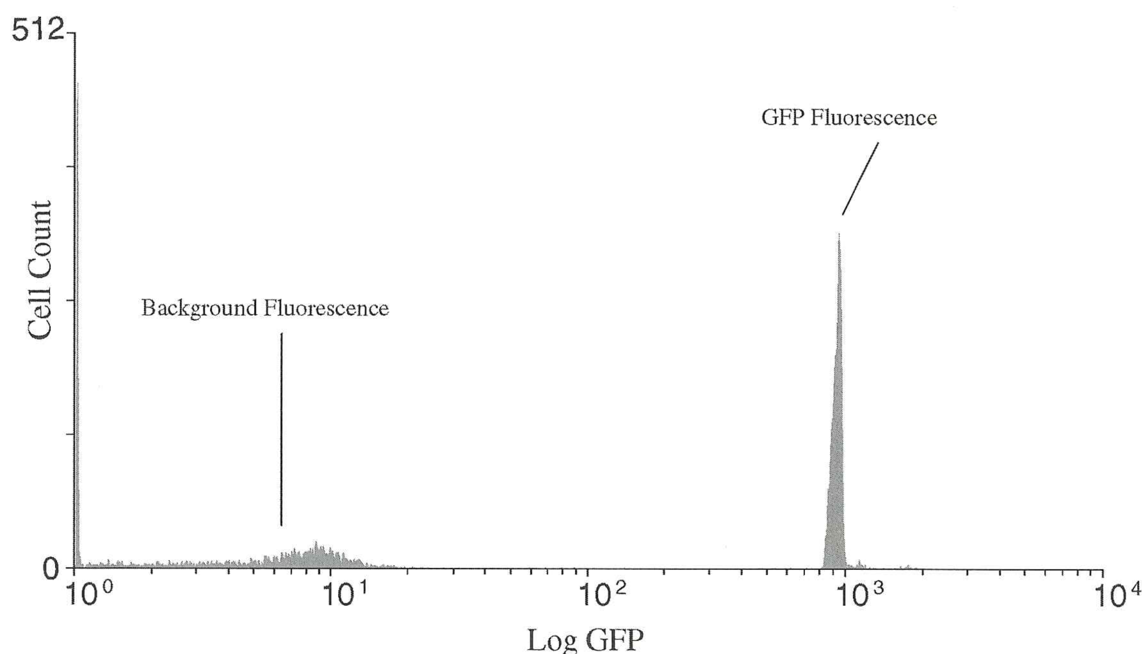

Figure 2. Flow cytometric analysis of GFP fluorescence of isolated porcine myoblasts transfected with pEGFP.

antibody staining. The yield of myoblasts isolated decreases in later passages, but FACS can enrich populations resulting in purities of >93%.

Successful myoblast-mediated gene transfer requires the isolation of transfected myoblasts that are positive for the gene of interest. To accomplish this we transfected primary muscle cell preparation with GFP constructs that can be detected by FACS (Figure 2). Combining fluorescence from 5.1H11 and pEGFP, we were able to purify GFP-positive porcine myoblasts from mixed primary preparations. It is likely that these FACS procedures are suitable for cells genetically-engineered using various transfection/transduction methods. For example, we [3] recently demonstrated that retrovirally-transduced muscle cells can also be detected and purified by FACS.

Overall, the techniques described above allow for the rapid isolation of genetically modified porcine myoblasts from mixed primary muscle cell preparations while maintaining the majority of their replicative capacity. These techniques of myoblast purification enable ex vivo cell mediated gene transfer and satellite cell biology experiments without non-myogenic cell contamination.

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Notes on suppliers

1. Bellco-Glass Inc., Vineland, NJ
2. International Equipment Company, Needham Heights, MA
3. Nikon Corporation, Torrance, CA
4. Nuaire, Plymouth, MN
5. Forma Scientific, Marietta, OH
6. Sears, Roebuck, Inc., Hoffman Estates, IL
7. GCA/Precision Scientific, Chicago, IL
8. Taylor-Wharton, Quincy, IL
9. Coulter, Hileah, FL
10. Falcon, Becton-Dickinson Laboratories, Franklin Lakes, NJ
11. Corning Science (Costar), Acton, MA
12. Life Technologies Inc., Rockville, MD
13. Clontech, Palo Alto, CA
14. Calbiochem, San Diego, CA
15. Harlan Bioproducts for Science, Indianapolis, IN
16. Sigma Chemical Corp., St. Louis, MO
17. Developemntal Studies Hybridoma Bank, University of Iowa, IA
18. Jackson ImmunoResearch Laboratories, Inc., West Grove, PA

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