Spring 2015

Isolation of Myogenic Nuclei from Whole Muscle Tissue

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Isolation of Myogenic Nuclei from Whole Muscle Tissue

By
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Molecular, Cellular, and Developmental Biology
University of Colorado, Boulder

Defense: April 1, 2015

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Abstract

Isolation of myogenic cell populations from whole skeletal muscle is necessary to elucidate the molecular repair mechanisms involved in skeletal muscle maintenance and regeneration that can be damaged when affected by diseases or aging, often severely impacting quality of life and lifespan. Skeletal muscle tissue is composed of hundreds of myofibers that are syncytia comprised of hundreds of nuclei named myonuclei. External to the sarcolemma (plasma membrane) of each myofiber is the basal lamina. Satellite cells, which reside between the basal lamina and the sarcolemma, are self-renewing muscle stem cells that maintain and repair muscle tissue. I have developed a method to purify myonuclei and satellite cell nuclei from non-myogenic nuclei in muscle to investigate molecular differences among nuclei present in muscle. I compared multiple methods of cellular lysis and homogenization to separate nuclei from the whole muscle tissue. Next, I tested two methods for nuclear isolation and found that a percoll gradient efficiently removed cellular debris from the nuclei. Finally, I stained for Pax7 and Myogenin, which are specific for satellite cells and myonuclei respectively. I found that percoll isolation effectively isolated 45% of myogenic nuclei from whole muscle tissue. A protocol for the isolation of myogenic nuclei will allow for nuclear sorting of myonuclei and satellite cell nuclei from non-myogenic nuclei, which has been previously difficult to achieve. Effective isolation and sorting of nuclei away from the intracellular environment without cell culture will allow for investigation of diseases that stem from errors in myogenic nuclei.
Acknowledgements

I would like to sincerely thank my thesis advisor and committee member Dr. Brad Olwin for mentoring me. Your help and guidance have been invaluable and allowed me to pursue my passion for research. I would also like to thank the other members of my committee, Dr. Jennifer Martin and Dr. Benjamin Teitelbaum, for providing me with support, advice, and guidance throughout my career at CU and during the process of my honors thesis. I genuinely appreciate your presence on my committee. I would like to extend special thanks to Dr. Monica Hall who has shown extraordinary effort to help me with this project from the beginning and for providing support throughout the process of writing this thesis. I am also very grateful to Kate Gadek who provided external guidance and answered all of my daily questions, helping me hone my lab bench skills. I would also like to thank the other members of the Olwin Lab for their daily help with my research tasks: Adam Cadwallader, Brad Pawlikowski, Tiffany Elston, Crystal Pulliam, Jason Doles, Thomas Vogler, Celeste Chitters, and Darian Williams. I would like to thank Brad Pawlikowski and Adam Cadawallader for providing me with images for which were used in my investigations and that appear below in Figure 3A and Figure 4A. All mice subjects were used in accordance with IACUC standards and with their approval.

Research support was provide by NIH 1R01AG040074 (Bradley B. Olwin), Howard Hughes Medical Institute (HHMI) Undergraduate Grant 2014-2015, and a Summer 2014 Biological Undergraduate Research Skills and Training (BURST) grant.
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### List of Abbreviations and Definitions

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>DAPI</td>
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<td>Sarcolemma</td>
<td>plasma membrane of myofiber</td>
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<td>TA</td>
<td>tibialis anterior muscle</td>
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Introduction

Skeletal muscle is a self-repairing tissue that is necessary for voluntary movement. Aging and disease can affect the repair mechanisms that maintain the tissue leading to loss of skeletal muscle mass and function. Skeletal muscle contains many multinucleated cells called myofibers that are held together by connective tissue (Figure 1A). A myofiber is a syncytium formed by hundreds of myogenic cells that fuse together and share a cytoplasm. The nuclei within the syncytium are known as myonuclei. Satellite cells, found outside of the syncytium and under the basal lamina, also contain myogenic nuclei (Figure 1B). Satellite cells repair myofibers that have been damaged. If a myofiber is damaged, myonuclei activate mechanism for cellular repair. Satellite cells can differentiate and fuse to add additional nuclei to the myofiber in a process known as myogenesis. In muscle diseases, such as sarcopenia, and in the normal course of aging, satellite cells lose the ability to repair the myofibers. Isolation of myogenic cell populations from whole skeletal muscle would allow investigation of repair mechanisms that are affected by aging and disease.
Figure 1: Myonuclei within Skeletal Muscle

**A)** Skeletal muscle is formed from a collection of myofibers and contains large quantities of connective tissue.

**B)** Myofibers are multinucleated cells that share cytoplasm and have a plasma membrane (Sarcolemma). Satellite Cells are found between the basal lamina and the sarcolemma. Image source: (Cummings et al. 2001).

One key difficulty in studying satellite cells and myonuclei comes from attempting to separate the populations from whole muscle tissue. Although satellite cells and myonuclei have been isolated from myofibers, cell culture is the most common method of isolating these cell types.\(^9,15,16,17,25\) Analysis of differences between satellite cells and myonuclei requires isolation of myogenic nuclei from whole muscle tissue. Isolation of specific nuclear populations from whole tissue has been performed with neuronal tissue.\(^18,19\) However, the nuclei within muscle are more difficult to isolate, due to the presence of structural proteins and connective tissue that prevent the release of nuclei.\(^20,23\) Intracellular proteins, as well as extracellular matrix proteins, such as laminin in the basal lamina, provide structure and resist mechanical stress, making it difficult to extract the nuclei. In this paper, I will explore a method for the isolation of myogenic nuclei from other nuclei that are present within whole muscle tissue, without using cell culture.

Density gradients will be used to separate nuclei from other cellular components. When a liquid with suspended particles is spun at high speeds within a centrifuge, a density gradient is formed. I will use both a sucrose density gradient, and a percoll density gradient, to separate
nuclei from other cellular components. Centrifugation will separate dense cellular components, which will sediment to the bottom of the tube from lighter cellular components, which will float on top of the gradient. In addition to sucrose gradients, I employed Percoll, a colloidal silica mixture that forms gradients rapidly as heavier silica particles are forced to the bottom of the tube when the Percoll mixture is spun within a centrifuge. Use of a density gradient will allow me to separate the dense nuclei from the less dense cytoplasmic debris.

Myonuclei and satellite cells express transcription factors that distinguish myonuclei and satellite cell nuclei from each other, and from non-myogenic nuclei within muscle tissue.\textsuperscript{5,12} Transcription factors are proteins that bind to DNA and regulate the transcription of DNA into mRNA. During skeletal muscle differentiation, the transcription factors Pax7 and myogenin determine cell fate.\textsuperscript{2} Pax7 is a transcription factor that is expressed in quiescent satellite cells and is downregulated during myogenesis.\textsuperscript{6} Myogenin is a muscle specific transcription factor that is expressed in the late stages during myogenesis. Myogenin is not expressed within myogenic nuclei until Pax7 activation is downregulated.\textsuperscript{38,39,40,41} Myogenin is present in myonuclei after differentiation (Figure 2).\textsuperscript{21,39,40,41} Pax7 and Myogenin are transcription factors that will allow me to separate satellite cell nuclei from myonuclei and from other nuclei are present within muscle tissue.
Figure 2: Transcription Factors in Myogenic Cells
Satellite cells exhibit Pax7 which is lost after activation. During proliferation and differentiation, Pax7 expression decreases. As myogenic precursor cells finish differentiation and fuse, Myogenin(MyoG) becomes highly expressed within the nuclei.

Immunostaining for Pax7 and Myogenin will allow me to distinguish satellite cells and myonuclei from non-myogenic nuclei. I will use antibodies to fluorescently tag Pax7 and Myogenin within isolated nuclei. By combining different primary and secondary antibodies, I will be able to identify myogenic nuclei that express either Pax7 or Myogenin. I will use DAPI to mark the presence of any nuclei regardless of their cellular identity within my samples. Co-staining of nuclei for Pax7 and Myogenin will allow for separation of myogenic nuclei from whole muscle tissue.
**Methods:**

**Homogenization of muscle tissue**

Mice were euthanized with isoflurane and their spinal cords were severed. Next the tibialis anterior muscles were dissected and placed in a glass 35mm plate with 4 ml Buffer A (0.25M sucrose, 10mg/ml BSA, 5mM MgCl₂, Sigma protease inhibitor cocktail P8340). Muscles were mechanically chopped with a razor into 1mm pieces. The tissue and buffer was then placed into a 7 ml dounce homogenizer. The glass plate was rinsed with 1 ml of Buffer A which was added to the homogenizer. Tissue was ground for 50 strokes with the loose pestle (clearance of 0.114 mm). The mixture was then passed through a 100 micron filter to remove extracellular debris and placed back into a clean homogenizer. Triton x-100 was added to a concentration of 0.5%. The mixture was ground for an additional 50 strokes with the tight pestle (clearance of 0.076mm) and passed through a 40 micron filter. The resulting homogenates were analyzed for cellular lysis, the extent of nuclear lysis assessed and intact nuclei quantified by counting with a hemocytometer.

To test the efficiency of cellular lysis and nuclear isolation, a number of different methods were tested. A second protocol was used to homogenize tissue rather than the mincing, lysis and dounce homogenization presented above. Following a protocol for nuclear isolation from neural tissue, muscles were dissected, then frozen in liquid nitrogen. Muscles were then ground with a mortar and pestle while being rinsed with liquid nitrogen to keep the tissue frozen. The ground tissue was then suspended in PBS and passed through a 40 micron filter before analyzing samples for cellular lysis and quantifying intact nuclei with a hemocytometer.

A third protocol was also attempted in which, muscles were placed in phosphate buffered saline (PBS) and Triton x-100 was added to a concentration of 0.5%. Tissue was homogenized with an Ultra-Turax homogenizer at 20500 RPM for 2 cycles of thirty seconds. The slurry was then
passed through a 40 micron filter before checking samples for lysis and counting nuclei with a hemocytometer.

**Separation of Nuclei**

Muscle Tissue filtrate was centrifuged at 3000g and the pellet was resuspended in 10 ml of Buffer B (0.32M sucrose, 3mM CaCl, 2 mM magnesium Acetate, 0.1 mM EDTA, 1mM DTT, 10mg/ml BSA, 10 mM Tris HCl, Sigma Protease inhibitor cocktail P8340, pH 8.0). Percoll was added to create 27% solution and spun at 25000g for 15 minutes. The nuclear layer was then diluted into 10 volumes of Buffer B and centrifuged at 2000 rpm for 5 minutes. The pellet was resuspended in 5 ml of Buffer B. The intact nuclei were quantified by counting with a hemocytometer.

The second protocol was used in which, nuclei were resuspended in 3.3ml of Buffer C (10 mM NaCl, 3mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 1% NP-40). The nuclear suspension was layered over 6ml of Buffer D (1.8 M Sucrose, 3 mM Mg(Ac)₂, 10 mM Tris-HCl, Sigma Protease inhibitor cocktail P8340, pH8) in ultracentrifuge tubes. In all sucrose separation experiments, tubes were then placed in the ultracentrifuge for 3 hours at 4ºC and spun at 100,000g using slow acceleration and no brake to prevent disruption of the interfaces of the gradient. The nuclear pellet was resuspended in 10 volumes of Buffer B before pelleting and resuspending in 5 ml of Buffer B. The intact nuclei were quantified by counting with a hemocytometer.

A third protocol was attempted and discarded in which, nuclei were resuspended in 3ml of Buffer E (Buffer B with 1.85M sucrose). Four milliliters of Buffer F(Buffer B with 2.15M sucrose) was layered on four milliliters of Buffer G (Buffer B with 2.8M sucrose) to create a sucrose gradient. The nuclei in Buffer E, were layered on top. Samples were placed in the ultracentrifuge for 3 hours at 4ºC and spun at 100,000g using slow acceleration and no brake. The nuclear pellet
was resuspended in 10 volumes of Buffer B before pelleting and resuspending in 5 ml of Buffer B. The intact nuclei were quantified by counting with a hemocytometer.

**Plating and immunostaining the nuclei**

Nuclei were diluted and 1,000,000 nuclei were then placed onto collagen-coated coverslips in 6 well cell plates. The collagen allowed the nuclei to attach to the coverslips without lysing. Tissue culture plates containing nuclei were then spun at 2000g for 30 minutes with no brake. The nuclei were then fixed with 4% paraformaldehyde and rinsed 3 times with PBS.

Nuclei were then stained with two antibody/secondary antibody pairs. First, nuclei were incubated for 1 hour at room temperature with mouse pax7 monoclonal antibodies (2.5 mg/ml, stored at -20º C, 1:1000 in PBS). The nuclei were rinsed three times with phosphate buffered saline (PBS) and then they were incubated for 1 hour at room temperature with Myogenin M-225 polyclonal antibodies made in rabbits (1:1000 in PBS). The nuclei were next rinsed 3 times with PBS and incubated for 1 hour at room temperature with Goat-anti-mouse 555 Alexa antibodies (1:500 in PBS). Finally, nuclei were rinsed 3 times with PBS and incubated for 1 hour at room temperature with Donkey-anti-rabbit 647 Alexa antibodies (1:500 in PBS). After rinsing 3x with PBS, nuclei were incubated with DAPI (1:1000 in PBS) to stain nuclei and placed in 0.02% azide in PBS at 4ºC. Coverslips with nuclei were mounted on slides and imaged with a fluorescent microscope.

In experiment 11, nuclei were incubated with both primary antibodies for 24 hours each at 4ºC. Next, nuclei were incubated for 1 hour with both secondary antibodies before incubation with DAPI, mounting and imaging.
**Immunofluorescence Analysis**

Fluorescence microscopy was performed using a widefield, Olympus IX81 Inverted Microscope. Fluorescent proteins were excited at the following wavelengths: Pax7(555nm), Myogenin (647nm) and DAPI(350nm). Images were captured using Slidebook (Intelligent Imaging Innovations). Fluorescent images were taken using Slidebook and summed using Image J software (v1.47). All images were globally processed for size, brightness and contrast using Image J. Figures were prepared using Microsoft Powerpoint(15.8) and Adobe Illustrator (14.0)
**Results:**

**Estimation of Theoretical Yield from a Single Mouse Tibialis Anterior Muscle**

I estimated the number of nuclei and ratio of myogenic nuclei to total nuclei within a single mouse Tibialis Anterior muscle to determine the theoretical yield of nuclei I could obtain after nuclear isolation. Five images of muscle sections from wild type (WT) mice, which had been stained for both Laminin and DAPI, were scored to determine the number of myogenic nuclei and total nuclei within each section. Nuclei that were found within the basal lamina (as visualized by laminin staining), were counted as myogenic while nuclei outside of the basal lamina were counted as non-myogenic (Figure 3A). On average, each image contained 200±20 myogenic nuclei and 80±10 non-myogenic nuclei as well as 160±10 myofibers (Figure 4B). The average number of myofibers per TA muscle is 2500 and the average length of a TA muscle is 17.39mm for a 5 month old mouse.31,32 Using this information, I estimated that the average TA muscle contains 9.6±0.9 x 10^6 total nuclei, of which 7.1±0.4 x 10^6 are myogenic (Figure 3C). I also estimated that 74±4% of nuclei in muscle are myogenic (Table 1).
Figure 3: Myogenic Nuclei Identification

A) Both myogenic nuclei and non-myogenic nuclei are present within whole muscle. Mouse TAs were dissected, frozen and the 8 micron sections were stained with Laminin and DAPI. Nuclei within the basal lamina (as seen with laminin staining) were counted as myogenic nuclei while nuclei found between laminin borders were considered non-myogenic. (Image Credit: Brad Pawlikowski) B) After counting 5 representative images of muscle sections, 74±4% of nuclei were found to be of myogenic origin. C) An estimated 9.6±0.9 x 10^6 nuclei are present within the whole TA. 7.1±0.4 x 10^6 myogenic nuclei are estimated to be in the average mouse TA.

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Table 1: Estimation of the Number of Myogenic Nuclei in a Mouse TA Muscle

5 images were scored to determine the number of myogenic nuclei and the total number of nuclei per myofiber in each 8 micron section. This number of nuclei per myofiber was then multiplied by the average number of myofibers per TA muscle and by the average length of the TA muscle based on the ages of the mice. I estimated the total theoretical number of nuclei and the proportion of myogenic nuclei within a single mouse TA muscle.
**Figure 4: Nuclear Isolation from Mouse Tibialis Anterior Muscle**

**A)** TA muscles were dissected from WT mice and muscles were minced and placed in a Dounce homogenizer with a lysis buffer. Next, the slurry was filtered and resuspended in a secondary buffer and separated with either a percoll or sucrose gradient. Nuclei were centrifuged at 25000g and the layers were isolated and resuspended. Finally, nuclei were plated onto collagen-coated coverslips and stained with antibodies to Pax7 and Myogenin. **B)** Cultured satellite cells that were isolated through a sucrose gradient show incomplete membrane digestion. Satellite cell nuclei were stained with DAPI and imaged with fluorescence and phase microscopy. **C)** Myogenic nuclei isolated from whole muscle tissue are not contained within myofibers. The nuclei were stained with DAPI and Myogenin (not shown) and imaged with fluorescence and phase microscopy. **D)** Percoll was effective at separating myofibrils from nuclei.
Homogenization and Isolation of Nuclei from Whole Muscle

To isolate nuclei from whole muscle tissue, TA muscles were dissected from mice and homogenized. Nuclei were filtered to remove large debris and connective tissue. Sucrose and percoll gradients were used to remove cellular debris. After separation of nuclei from cellular debris and extracellular components through either a sucrose gradient or a Percoll gradient, nuclei were resuspended and plated onto coverslips before staining (Figure 4A).

Three methods of cellular lysis and homogenization were tested and two were subsequently discarded. Although freezing and grinding of tissue was found to release nuclei in neurons, I found the muscle tissue required multiple cycles of freezing and grinding to achieve a homogenous mixture and that few nuclei were released (Data not shown). I used a blade homogenizer but found that this method of tissue disruption led to aggregation of the myofibers and myofibrils as well as inadequate release of the nuclei.

I successfully used a lysis buffer and dounce homogenization to lyse myofibers and extract nuclei from their niche. To test the lysis efficiency of Buffer C, I first used Chicken fibroblast cells (DF1), which were cultured and then exposed to the lysis buffer. Buffer C contained NP-40, a detergent, which was able to break down the cell membranes to release the nuclei (Data not shown). I then tested the lysis solution on cultured satellite cells and found Buffer C was sufficient to lyse cells and release the nuclei although some cytoplasmic components remained (Figure 4B). When I attempted homogenization with Buffer C and dounce homogenization with whole muscle however, the lysis buffer was not sufficient to dissolve the extracellular components to release the nuclei. Instead, dounce homogenization was used in conjunction with Buffer B, containing Triton, to break up the extracellular matrix and plasma membrane (Figure 4A). Dounce homogenization with Buffer B was highly effective at extracting the nuclei from the myofibers and the introduction
of BSA prevented nuclear clumping and aggregation of cytoplasmic components. (Figure 4C). The process of cellular lysis and homogenization with Buffer B that I developed is critical for optimal extraction of nuclei from muscle tissues because the intracellular and extracellular proteins present in muscle tissue are resistant to published methods for homogenization of other tissues.

Two types of density gradients were used to isolate muscle nuclei and a percoll gradient was selected because the percoll was efficient at separating myofibrils from the nuclei. Sucrose gradients were effective in isolating nuclei from cellular debris but samples contained high numbers of myofibrils (Figure 4B). In subsequent experiments, myofibrils were removed with a percoll gradient (Figure 4D).

**Immunostaining to Confirm Myogenic Origin**

To illustrate representative immunostaining, cultured satellite cells were incubated with a mouse Pax7 monoclonal primary antibody and a goat anti-mouse Alexa 555 secondary antibody. Cultured satellite cells exhibit nuclear localization of the Pax7. Cultured myogenic precursor cells were exposed to the M-225 Rabbit Myogenin antibody and a donkey anti-rabbit 647 secondary antibody. Cultured myogenic precursor cells also exhibit nuclear localization of Myogenin (Figure 5A).
Figure 5: Myogenin and Pax7 Immunostaining

A) Example of Pax7 (green) and Myogenin (red) immunostaining for intact cells showing nuclear steady-state localization of transcription factors with DAPI (blue) B) Magnification of Pax7 Positive cell from B showing colocalization of DAPI and Pax7. C) Magnification of Myogenin Positive cell from C showing colocalization of DAPI and Myogenin. D) Pax7 staining of nuclei shows nonspecific staining of non-nuclear cellular debris. Nuclei were isolated with Percoll gradient and stained with mPax7 hybridoma antibody. E) Myogenin immunostaining of nuclei exhibits nonspecific background staining. Nuclei were isolated with Percoll gradient and stained with M-225 Myogenin antibody and DAPI

After the isolation of our nuclei with a percoll gradient, nuclei were stained with a Pax7 antibody. The pax7 antibody was present in 1% of nuclei and localized to the nucleus (Figure 5D). Upon scoring, 73% of nuclei were found to contain nuclear myogenin (Figure 5E). Nuclear localization of either Pax7 or Myogenin in myogenic nuclei, was evident and appeared similar to antibody staining examples (Figure 5B,C)
Figure 6: Calculating Myogenic Yields

A) Ten random photos of isolated stained nuclei were chosen and the number of myogenic nuclei that stained positive for either Pax7 or Myogenin were counted and compared to the total number of nuclei. B) Percoll was successful in isolating 45±9% of estimated number of myogenic nuclei and 50±10% of the estimated total number of nuclei.

Calculation of Final Myogenic Yields from a Single Mouse Tibialis Anterior Muscle

I calculated the number of myogenic nuclei and total nuclei isolated after percoll isolation. Pax7 positive nuclei and DAPI positive nuclei as well as nuclei that were Myogenin positive and DAPI positive were considered myogenic while nuclei that were only DAPI positive were considered non-myogenic (Figure 6A). The average number of myogenic nuclei, obtained by percoll isolation was 3.2±0.7 x 10^6 and the average total number of nuclei was 5±1 x 10^6 (Table 2). The ratio of myogenic to non-myogenic nuclei was found to 60±20% and percoll isolation allowed for the collection of 45±9% of myogenic nuclei (Figure 6B).
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Table 2: Calculating Final Myogenic Yield

Ten random samples from two percoll isolation experiments were imaged and the percentage of myogenic nuclei (Pax7 positive and Myogenin positive nuclei) in each sample was scored. Concentrations of nuclei per ml were counted with a hemocytometer and the total nuclei obtained per experiment. Percoll isolation was found to extract $5\pm1 \times 10^6$ nuclei from a single TA muscle and extract $3.2\pm0.7 \times 10^6$ myogenic nuclei.
Discussion

I developed a method of isolating nuclei from whole skeletal muscle utilizing a percoll gradient and confirmed my results with immunostaining. First, I tested three methods of cellular lysis and homogenization and I found that a lysis buffer to extract nuclei from myofibers before using a dounce homogenizer ensured removal of connective tissue. Next, I tested the efficiency of a sucrose gradient as opposed to a percoll gradient and found the percoll gradient to be much more effective at removing cellular debris and myofibrils from the nuclear fractions. Finally, I used Pax7 and Myogenin to separate the myonuclei and the satellite cells from the other non-myogenic nuclei. Percoll isolation of nuclei from whole muscle was found to be highly efficient at isolating and concentrating myogenic nuclei.

Cell lysis and tissue homogenization

Muscle tissue contains high levels of connective tissue and structural proteins that prevent cellular lysis and homogenization. Extracellular proteins such as laminin and collagen provide structural support to muscle during movement and exercise.\textsuperscript{42} The extracellular proteins within muscle are highly resistant to mechanical stress. Inside myofibers, many structural proteins including actin and dystrophin also resist mechanical stress and provide cellular structure.\textsuperscript{29,30} I found that both freezing and grinding of muscle tissue and blade homogenization was ineffective in releasing intact nuclei. Freezing and grinding, and blade homogenization were either unable to break apart the extracellular matrix and intracellular structural proteins or they were too violent to allow preservation of intact nuclei. Dounce homogenization of muscle tissue with a lysis buffer containing Triton effectively released intact nuclei from the extracellular and intracellular structural proteins present within muscle tissue.
Sucrose gradient vs Percoll gradient

Sucrose gradients were unable to remove debris from nuclei and caused clumping of nuclei. Although much of the cellular debris was less dense than the sucrose and floated, high quantities of non-nuclear debris was seen within samples isolated via a sucrose gradient. The presence of non-nuclear debris led to extensive background staining making it impossible to differentiate myogenic from non-myogenic nuclei. Sucrose isolates also contained myofibrils that would pellet with the nuclei. Pelleting of nuclei also led to clumping which was prevented with the use of BSA. Sucrose gradients were found to allow excessive cytoplasmic and extracellular debris to pellet and left high quantities of myofibrils within samples.

To obtain a cleaner nuclear pellet, I used a percoll gradient to isolate nuclei. Upon isolation of nuclei with percoll, I found that the cellular debris was decreased. Most importantly, I saw a decrease in myofibrils in the nuclear fraction, which had contaminated our nuclei isolated through a sucrose gradient. During percoll isolation, nuclei do not pellet but rather form a layer that floats thus allowing a separation of the nuclei from a greater proportion of the cellular debris.

A third method using multiple densities of sucrose was also attempted but was discarded when no viable nuclei could be obtained. I used three densities of sucrose including one that had a density of 1.86 g/ml. The scarcity of viable nuclei could be due to nuclear lysis during extraction. Another issue faced during this method was collection of nuclei after centrifugation because the nuclei did not pellet and were instead suspended above the high density buffer. Multiple density sucrose gradients were discarded because no intact nuclei could be obtained.
**Pax7 and Myogenin Immunostaining**

I showed that both Myogenin and Pax7 are present in nuclei after lysis, homogenization and percoll isolation. Myogenin and Pax7 are rarely present in the same nuclei but they are both transcription factors that are located within the nucleus of myogenic cells. I compared the staining of our isolated nuclei with whole cells that had been stained with Pax7 and Myogenin. I found that Pax7 and Myogenin were found in many of the nuclei and the nuclei appeared to have similar staining to the cells. Scoring of nuclear localization of Pax7 or Myogenin along with DAPI allowed estimation of the efficiency of percoll isolation.

The levels of debris within our samples led to high levels of background staining within our nuclear samples, especially those isolated with sucrose gradients. Antibodies can bind nonspecifically to other proteins leading to high background levels of fluorescence. Nonspecific binding of secondary antibodies could be eliminated by reducing the amount of secondary antibody and by reducing the amount of non-target debris within our samples. In nuclei isolated with a sucrose gradient, the levels of background staining were so high that scoring of these images was impossible. A western blot could be used to test if Pax7 and Myogenin are present in within the nuclei after isolation. A western blot would allow me to determine if isolation reduces the prevalence of Pax7 and Myogenin. Although there was evidence of background staining, Pax7 and Myogenin staining were sufficient for evaluation of percoll isolation efficiency.

**Myogenic Isolation Efficiency**

I estimated $9.6 \times 10^6$ as the theoretical number of nuclei and 74% as the myogenic proportion of nuclei in a single 5 month old mouse TA muscle. My estimate for the number of nuclei agrees with published findings and is also consistent with the average density of cells.
within muscle tissue.\textsuperscript{31,32,33,34,35,43} My estimate for the proportion of cells that are myogenic could be improved to obtain more precise values. During scoring of sections, nuclei that were found contained within a muscle fiber, as marked by laminin staining were considered myogenic. However, the cells that I counted, contains some cells that were counted as myogenic but that would not be positive for either Pax7 or Myogenin in my later experiments. In order to obtain a more accurate estimate, sections could be stained for Pax7 and Myogenin and only cells that stained positive for these transcription factors would be counted. Estimates for the number of myofibers within a muscle were obtained by counting the number of myofibers within a single cross-sectional plane of the TA muscle.\textsuperscript{31} Multiple cross-sectional planes and comparisons between the length, weight and age of the TA muscles could have allowed more accurate estimates. Finally, my estimate assumed that the density and proportion of myogenic nuclei remained constant throughout the TA muscle, which is unlikely as tendons, blood vessels and nerves take up more proportional area at the end of the muscle. Although my estimates for the number of nuclei in a TA muscle and the proportion of myogenic nuclei could be made more precise, my estimates agreed with other published values.

I calculated that percoll isolation was able to isolate 45\% of myogenic nuclei and 50\% of nuclei found within a single TA muscle. These values were not found to be significantly different from each other, indicating that half of nuclei are lost during lysis, homogenization, and percoll isolation. Optimization of a protocol for percoll isolation may allow for higher yields of nuclei from whole tissue. A disproportionally large number of nuclei are lost during homogenization. Further mincing of tissue could allow for the breakup of connective tissue within muscle. Filtering of homogenates was necessary but also reduced the number of nuclei found within our samples many nuclei became trapped by extracellular proteins, myofibrils or within contracted myofibers.
**Future Directions**

Successful isolation of myogenic nuclei is required for future investigations into satellite cells and myonuclei. I plan on continuing with this project in order to complete isolation and separation of satellite cells and myonuclei through fluorescence-activated nuclei sorting (FANS). FANS machines sort nuclei according to their fluorescent signature. The FANS machine will sort the nuclei into collection tubes based on their color. FANS requires isolated nuclei, which are fluorescently labeled. This isolation and sorting of myogenic nuclei from whole muscle tissue provides an important tool for the investigation of myogenesis and muscular disease because it will allow for better *in vivo* studies of muscle homeostasis and regeneration.

Sorting of myogenic nuclei will also allow for investigations into the heterogeneity of the satellite cell population which has been suggested in numerous studies.\(^2,6,10,24,26\) Understanding this heterogeneity will be attempted through lineage tracing of satellite cells during injury and muscle repair. The isolation of myogenic nuclei and separation of satellite cells from myonuclei will also allow for investigations into protein expression, epigenetic changes and mRNA splicing that contribute to myogenesis during muscle repair. In the development of a better understanding of muscle repair and homeostasis, especially during aging and disease, we will be able to develop treatments which may be able to help patients who experience negative side effects of these processes.
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