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3/6/14, Thursday

1. Engraftment of stem cells: 1:15 – 2:15 PM

Intramuscular injection in TA muscle on mice with PBS. (CB)

2. Luciferase live imaging (meet at Clark auditorium, CB or TN will escort)
   Tim Doyle will give a ~30' presentation about the IVIS (2:30 – 3:15 PM) followed by demonstration of satellite cell engrafted mouse (CB, TN).

3/11/14, Tuesday

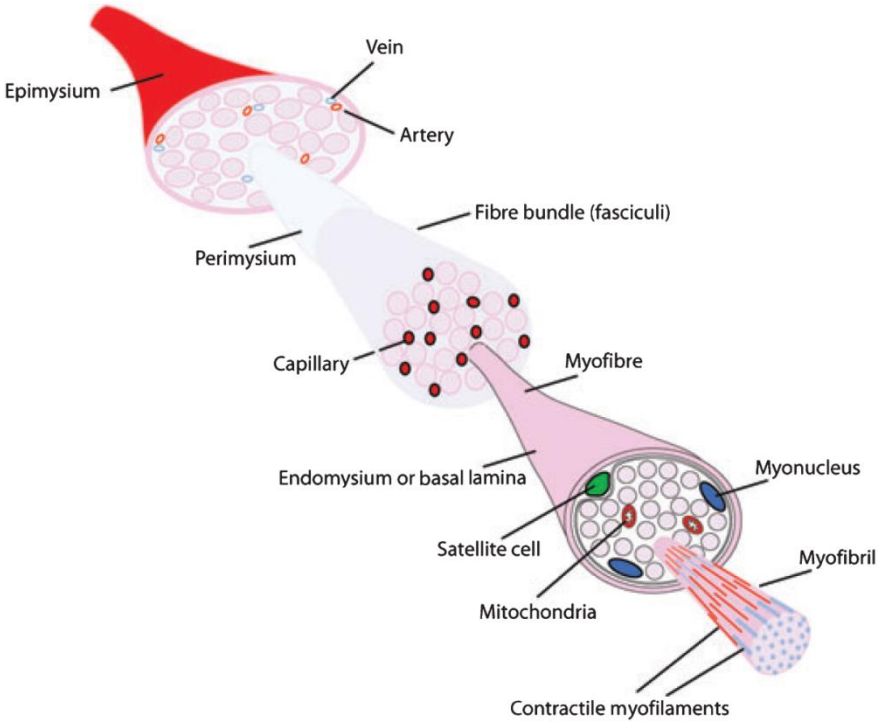
1. Nucleofection of DNA into mouse embryonic fibroblast (1:15 – 2:15 PM)
   Students will nucleofect GFP plasmid, then detect transfected cells with Scanford on 3/13/14. (CZ and JG)

3/13/14, Thursday

Checking the nucleofected cells from 3/11/14 to analyze transfection efficiency with FACS (1:15-3:15 PM)

1. View on the GFP scope that is in the facility (1:15-1:30)
2. Isolate and prep cells for FACS (1:30 – 2:15)
3. FACS analysis to quantify GFP expressing cells, (2:15 – 3:15)

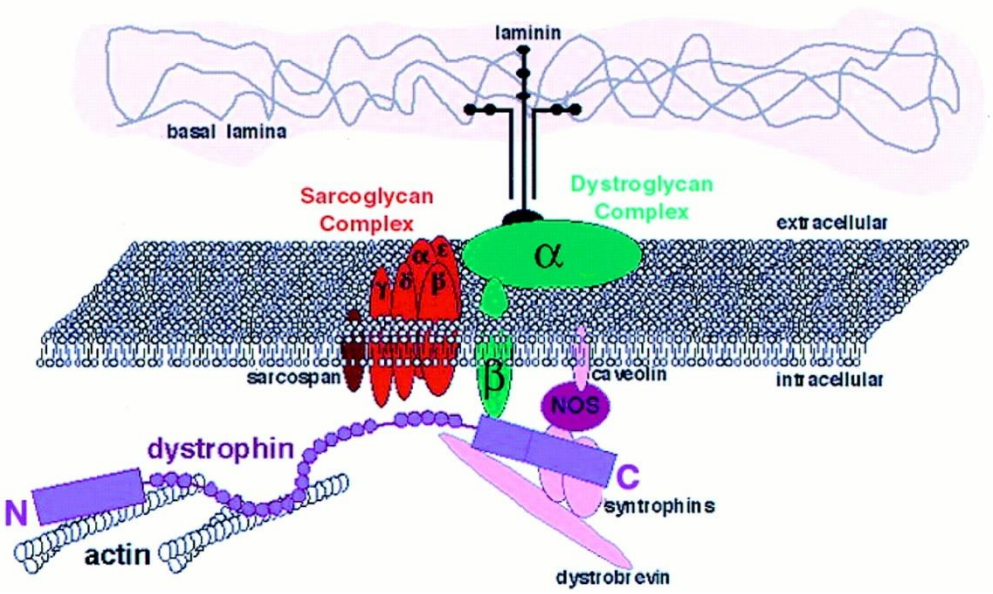
Muscle Architecture



Otto *et al.*, 2010

Skeletal muscle is organized as bundles within bundles. The smallest unit is referred to as a myofiber. The myofiber is a syncitium surrounded by two membranes; the outer basal lamina, and the inner sarcolemma. The integrity of muscle is maintained by a stem cell population termed the satellite cells, located between the basal lamina and sarcolemma. Satellite cells are quiescent in resting muscle. In response to injury or disease, satellite cells enter the cell cycle and produce myoblasts that are used to repair damaged tissue. The regenerative potential, relative ease of engraftment, and accessibility are characteristics that make skeletal muscle a valuable tissue for pursuit of cell-based therapeutics.

The dystrophin signaling complex



Sweeny, 2000

Dystrophin is a large protein (~417 kDa) that connects the cytoplasm of muscle cells with the extracellular space. Dystrophin interacts with a large number of cytoplasmic proteins that collectively form the dystroglycan complex (DGC). Mutation of any component of the DAG causes muscular degeneration, or dystrophy. Specifically, mutations in the dystrophin gene lead to Duchenne Muscular Dystrophy (DMD), which is the most common, serious, and intensively studied of the muscular dystrophies. DMD patients gradually lose the ability to walk and are wheelchair bound by their teens. DMD patients typically die by their mid to late 20s. Therapies exist to extend lifespan, but there is currently no cure for DMD.

By taking advantage of the highly regenerative characteristic of muscle, cell replacement is a promising approach for the treatment of DMD.

Specific Protocols:

Irradiation

In the adult organism, stem cells from all tissues reside in specialized compartments called niches. Elimination of endogenous stem cells will leave a niche for engrafted stem cells to occupy. In skeletal muscle, experimental approaches can be taken to open the niche – either through the use of a myotoxin, such as barium chloride or cardiotoxin, or through localized γ-irradiation. A myotoxin is used 24 hours before engraftment, while mice are irradiated 72 hours prior to cell engraftment.

1. Mice to be irradiated are first anesthetized using a solution of ketamine/xylazine (1:9, use 10 μL/g body weight, injected IP).

2. Place anesthetized mice in a custom-made irradiation jig that limits exposure to only the hind limb muscles. Use Scotch tape to secure the legs and tail (Figure 2).

Figure 2

3. Administer a total dose of 14 Gy (~25 min). 6 mice can be irradiated at a time.

4. Mice are allowed to recover overnight, and their health is assessed the next morning.

Engraftment

Stem cells are engrafted into the Tibialis Anterior muscle (TA). The TA is easily accessible and, like all skeletal muscle, is highly regenerative. TA muscles can accept up to 50 μL of injected volume; however, a volume of 30 μL or less is preferred. Cells can be suspended in many different types of media for engraftment purposes, though PBS is the most commonly used.

1. Prepare stem cells for engraftment and suspend in a final volume of 30 μL. Cells are kept on ice until engrafted.

2. Recipient mice are anesthetized in an induction chamber using an isoflurane/O_2 mixture. Once anesthetized, mice are placed on a sterile surgical pad on their back, and a nose cone is used to give constant isoflurane/O_2, though at a lower dose than that given for induction.

3. The region of engraftment is shaved and gently swabbed with iodine or alcohol.

4. Cells to be engrafted are briefly warmed to room temperature – the low volume permits rapid warming. Gently flick the tube to resuspend the cells.

5. Coat the needle of an insulin syringe (31 gauge, 5/8" length) with horse serum by pulling the plunger up and down a few times while the needle is immersed in the serum. Coating the syringe with serum lubricates the walls of the syringe and needle and prevents cell loss.

6. Pull up the entire volume of solution containing the cells into the syringe.

7. Inject the TA muscle by injecting directly into the muscle, piercing the skin. An incision is not required. A larger proportion of the TA is engrafted by injecting 4 or 5 different areas with a volume of 5-10 μL per injection. Immediately following the injection of cells, the position of the needle is kept constant to prevent the solution from leaking through the needle track.

8. Once injected, remove the mouse from the nose cone and place it in a fresh cage that contains new bedding. Repeat the engraftment with the remaining mice.

Tissue harvest

1. Euthanize engrafted mice.

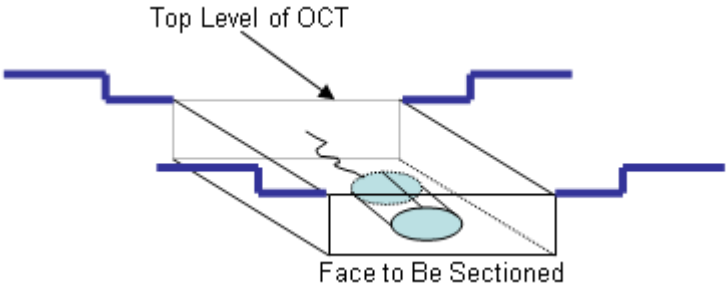
2. Harvest TAs from the engrafted side, as well as the contralateral side. The contralateral tissue will be your control.

3. Fix tissue at room temperature for 4 to 5 hours in 0.5% electron microscopy-grade paraformaldehyde. Tissue should be gently agitated during fixation by using a shaker or rocker. Tissue will become rigid and whitened as fixation proceeds.

4. Remove PFA and wash once in PBS. Remove PBS and replace with 20% sucrose to dehydrate. Leave tissue overnight at 4ºC. Tissue will sink to the bottom overnight.

5. The next day, aspirate sucrose and place dehydrated tissue on a paper towel and pat tissue dry.

6. Place dried tissue in cryomold and fill with optimal cutting solution (OCT). Fill just to the top so the OCT is level with the molding.



7. Fill a 600 mL beaker with 400 mL 2-methylbutane. Fill a styrofoam container with enough liquid nitrogen so the level is about the same as the level of the 2-methylbutane in the beaker.

8. Place the 2-methylbutane containing beaker in the liquid nitrogen. Once vapor is no longer being produced, the 2-methylbutane temperature has cooled enough to freeze the TA in OCT.

9. Freeze the OCT/TA by touching the cryomold to the top of the 2-methylbutane. The OCT in contact with the 2-methylbutane (throught the cryomold) will turn white.

10. Once the OCT across the entire bottom of the mold has turned white, immerse the entire mold in 2-methylbutane for 30 seconds. Submerge the cryomold just below the surface of 2-methylbutane.

11. Shake off remaining 2-methylbutane and store the cryomold block on dry ice for short term storage. Store at -80ºC for long term storage. Muscle blocks can be sectioned using a cryostat and processed for either H&E staining or immunocytochemistry.

Immunocytochemistry

Day 1

1. Cut appropriate section and control slides on Cryostat.

Note: *Thinner sections yield better staining and, since the cells are on the order of 5 µm thick, provide better resolution. Normally, sections between 6 – 8 µm are sufficient. The best way to achieve these cuts is to follow the fixing protocol outlined. Cryostat angle of the stage between 7.5 – 10º, set the temperature to around -26 – -28ºC and -very important- adjust the glass cutting guide so that the clearance between the stage and the glass guide is just enough to allow the section to slide through, but not too much so that the specimen hits the glass guide. Most importantly, these are only tips, and each person will end up developing a personal cutting technique as comfort level increases – cryosectioning is an art!*

2. After cutting all the sections on a slide, use a PAP pen to outline the sections on the slide.

Note: *Make sure to reduce the time that sections are exposed to air. Drying out of the sections by exposure to air throughout the staining significantly increases α-mouse antibody backgrounds. Also, the more slides that are prepared, the more tedious staining becomes and the higher the risk of the slides drying out in between washes. It is best not to cut and stain more than 12 slides at a time, depending on the level of proficiency.*

3. After completing each slide, either post-fix immediately with 2% PFA (EM grade not necessary) or add 0.3% PBS-Triton to keep sections hydrated.

4. If not already performed, post-fix all sections on all slides with 2% PFA for 5-10 minutes.
5. Wash 2-3X for 1-2 mins each (the more washes, the better; do not aspirate all the liquid, as this will dry out the sections and result in higher background)

6. Block for 20 mins in 5% Donkey Serum (in 0.3% PBS-Tween).
7. Prepare primary antibodies in 5% Donkey serum.

rat α-Laminin (Sigma cat L9393)	1:1000
rb α-GFP (Invitrogen cat A11122)	1:250
rb α-Dystrophin (Abcam cat 15277)	1:200

8. Incubate primary antibodies on overnight @ 4 ºC in a humid chamber.

9. Wash 2-3X for 1-2 mins each.

10. Prepare secondary antibodies in 5% donkey serum:

Alexa dyes α-rabbit	1:500
Alexa dyes α-rat	1:500
DAPI (5mg/ml in DMF)	1:2500-3000

11. Incubate secondary @ RT for 1 hr in the dark.

12. Wash 2-3X with PBS-Tw for 1-2 min each.

13. Wash 1XPBS for 1 min.

14. Aspirate and mount using fluorogel (or some other mounting media that will reduce fading of fluorophore).

15. Seal with clear nail polish and store at 4ºC.

Hematoxylin & Eosin staining

H&E staining is a fast method of determining tissue histology using bright field microscopy. Hematoxylin (purple) stains nuclei and Eosin (pink) stains tissue containing protein. H&E staining typically works better on unfixed/non-dehydrogenated tissue on thicker (~10-12 mm) cryosections. Incubation times are estimates and depend on staining desired and freshness of solutions used.

Hematoxylin	5 – 10 minutes
Water	1 minute
Water (under running dH_2O)	3 minutes
Scotts Water ($1:1000\ NH_3OH$)	≥ 1 minute
Eosin	1 - 5 minutes
95% EtOH	1 minute
95% EtOH	1 minute
100% EtOH	1 minute
100% EtOH	1 minute
Xylene	1 minute
Xylene	1 minute

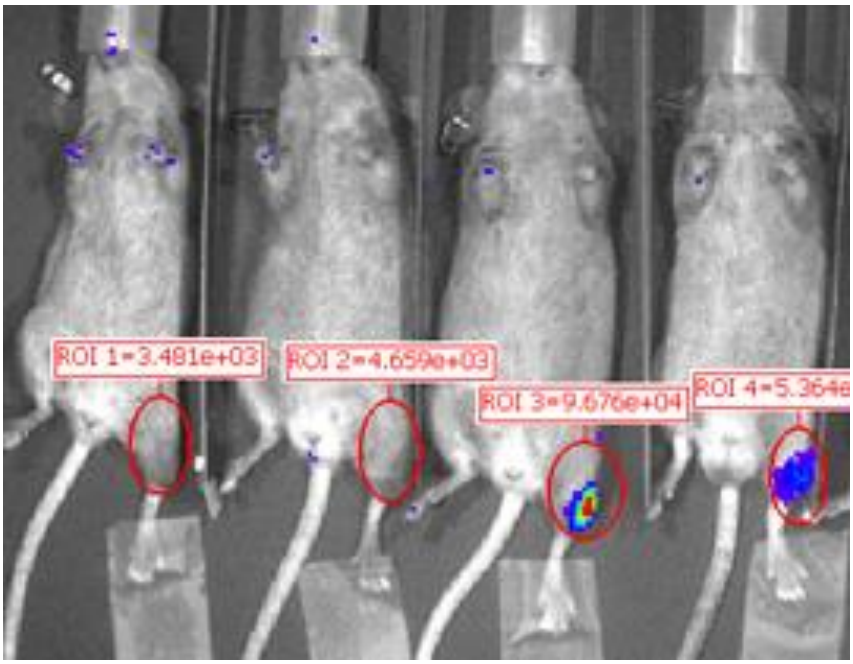
Dry slides for one hour in a fume hood. Mount using Permount.

Luciferase imaging

Using the in vivo imaging system (IVIS) it is possible to non-invasively monitor engrafted cells labeled with firefly luciferase. Engrafted mice are injected with the luciferase substrate, luciferin, and anesthetized using isoflurane. Anesthetized mice are placed in a light-sealed detection chamber. The enzymatic product of luciferase acting on luciferin emits infrared luminescence. Photons emitted are detected using a camera, and pixels produced are quantifed. Because of the non-toxic nature of anesthesia and luciferin, engrafted mice can be monitored indefinitely.

1. Inject engrafted mice with luciferin intraperitoneally (10 μL per gram of body weight, stock luciferin = 30 mg/mL in PBS).
2. 10 minutes after injection, place mice in induction chamber and anesthetize using a mixture of isoflurane/O_2.
3. Once unconscious, place mice on a piece of black paper in the IVIS chamber, and use nose-cones to maintain anesthesia. Appendages can be immobilized using tape to expose engrafted regions. Removal of fur allows better photon detection.
4. Image mice starting approximately 15 to 20 minutes following luciferin injection.
5. Begin imaging with just a photograph of the mice in the chamber to optimize placement.
6. Image mice by activating the luciferase detection, along with photographic detection. For reference see Figure 1.

Figure 1

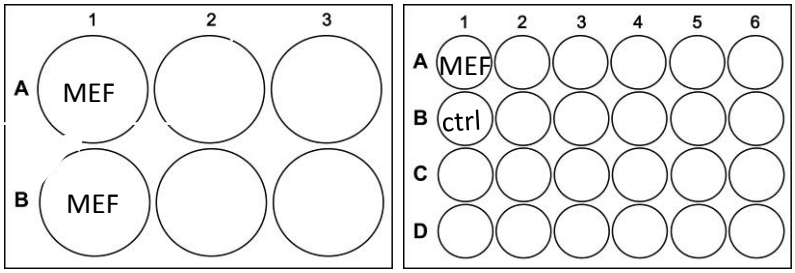


7. Acquire image. Make adjustments to the length of exposure, binning, etc to optimize photon detection.

8. Once imaging is complete, place unconscious mice back in their cage. Be careful not to bury the snout in bedding, as it may lead to suffocation when the mice are still unconscious.

Mouse embronic fibroblast Nucleofection with GFP Plasmid and FACS Analysis

Before Nucleofection After Nucleofection



Note: Control group means non-nucleofected cells, just plain cells.

Schedule:

March 10: 1) change medium before nucleofection

2) Coat 24 well plates with gelatin

March 11: Nucleofection

Required materials:

Lonza nucleofector device	GFP plasmid
MEF Nucleofector kit 1	Certified cuvettes (10)
15 ml Falcon tube (10)	Pipettes(nucleofection) (10)
TrypLE express (GIBCO cat#12605-010)	24 well culture plates coated with Gelatin (7)
PBS	Complete mouse MEF culture medium (10)
Timer	Cell counter (2)
Hemocytometer (10)	foam box (FACS)
Trypan blue	blue cap FACS tube (10)

FACS-tube compatible metal storage block aluminum foil

Protocol:

Material preparation

- put nucleofection kit and DNA plasmid at room temperature
- Test plasmid DNA concentration
- Warm up medium, trypLE and PBS in water bath
- Calculate DNA amount and aliquot in Eppendorf tube for each reaction
- Make nucleofection solution 100ul/reaction (82ul of nucleofector solution + 18 ul of supplement)

1. Harvest of mouse embryonic fibrobalst

Wash cells with PBS and add 1 ml of TrypLE, incubate for 5 minutes at 37°C. Dissociate the cells into a single cell suspension by pipetting the suspension carefully up and down 4 – 6 times. Add medium to stop TrypLE. Centrifuge the required number of cells (2 x 10^6 cells per sample) at 115 xg for 5 minutes at room temperature

2. Nucleofection

One Nucleofection sample contains

- 2 x 10^6 cells
- 3 – 10 µg highly purified plasmid DNA (in H_2O or TE, in max. 5ul)
- 100 µl MEF Nucleofector Solution 1

[Note]

- Perform each sample separately to avoid storing the cells longer than 20min in MEF nucleofection solution1
  - Please make sure that the entire supplement is added to the Nucleofector Solution

2.1 Prepare cell culture plates by filling appropriate number of wells with desired volume of recommended culture media, coated with gelatin and pre-incubate/equilibrate plates in a humidified37°C/5%CO_2.

2.2 Combine 100 µl of cell suspension with 3-10 µg DNA

2.3 Transfer cell/DNA suspension into certified cuvette; sample must cover the bottom of the cuvette without air bubbles. Close the cuvette with the cap

2.4 Select the appropriate Nucleofector Program T-20

2.5 Insert the cuvette with cell/DNA suspension into the Nucleofector Cuvette Holder and apply the selected program

2.6 Take the cuvette out of the holder once the program is finished

2.7 Add ~500 µl of the pre-equilibrated culture media to the cuvette and gently transfer the sample immediately into the 24-well plate coated with gelatin with MEF culture medium

[Note] Remove sample from the cuvette immediately after program has finished.

2.8 Plate non-nucleofected cells in separate well as control

3. Post Nucleofection

3.1 Incubate the cells in a humidified 37°C/5% CO_2 incubator for 24 hours.

3.2 Run FACS analysis the following day.

March 13 FACS

4. FACS

4.1 prior to running FACS analysis, check GFP expression under fluorescence microscope

4.2 Harvest cells

Detach the nucleofected MEF from the gelatin coated plates by incubation with TrypLE Express for 5 minutes at 37°C. Dissociate the cells into a single cell suspension by pipetting the suspension carefully up and down 4 – 6 times. Transfer to a 15mL conical tube. Add medium to dilute out TrypLE Express.

4.3 Centrifuge cells at 115xg for 5 minutes at room temperature

4.4 Resuspend the cell pellet carefully in 200-300 ul PBS. The goal is have the cell suspension be just slightly cloudy. As a general rule, the cloudier the suspension, the more likely it will be to cause a clog in the FACS machine.

4.5 Transfer cells to blue filter-cap FACS tube on ice or chilled metal block and reduce light with aluminum foil.

4.6 for control cells, repeat steps 4.2-4.5.

4.7 go to FACS facility to run FACS. The operator should be trained and certified before experiment.

4.8 analyze data with Flowjo software.

Supplement:

Nucleofection kit (Lonza) :

MEF Nucleofector kit 1

(cat# VPD-1004)