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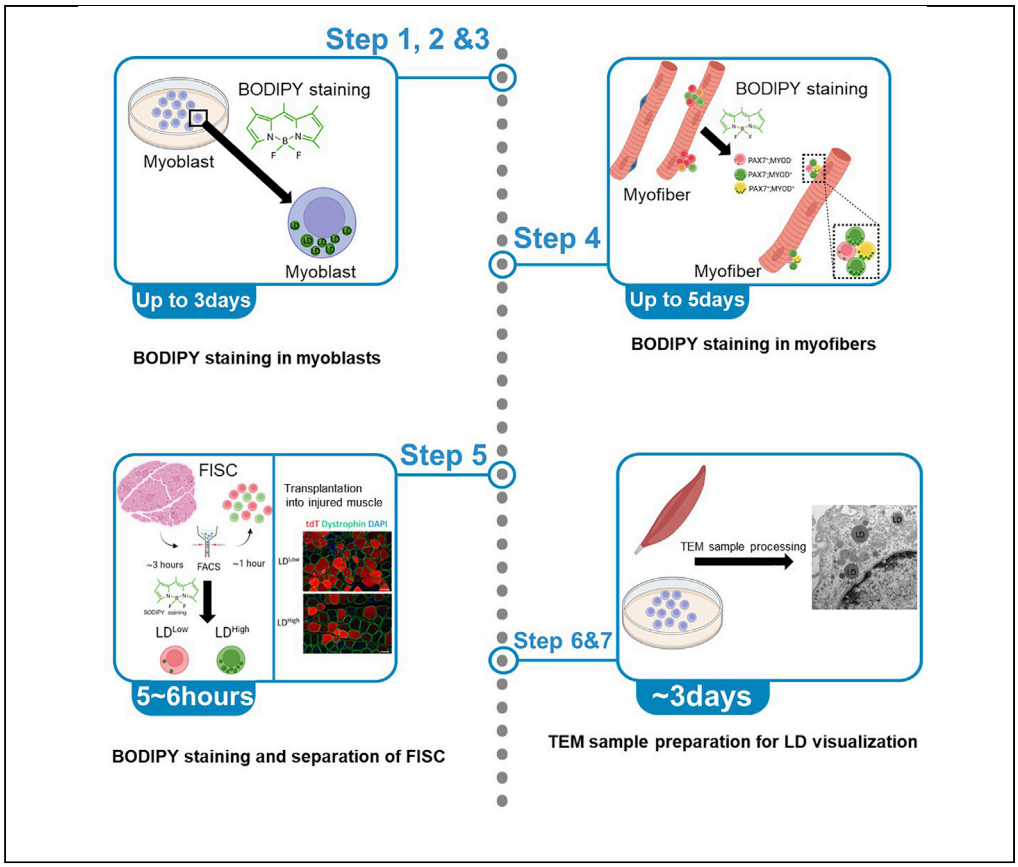
Labeling and analyzing lipid droplets in mouse muscle stem cells



Lipid droplets are emerging as an important and dynamic organelle whose metabolism controls stem cell behavior. Here we present a comprehensive protocol to visualize and quantify these organelles in mouse muscle satellite cells (MuSCs). This protocol includes steps for BODIPY/LipidSpot610 staining of freshly isolated MuSCs, *in vitro* cultured myoblasts, and single myofibers to label lipid droplets and subsequent analysis and quantification of fluorescence signals. This protocol can be modified to stain lipid droplets in other cell types of interest.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Jingjuan Chen, Feng Yue, Shihuan Kuang

chen2627@purdue.edu (J.C.)
skuang@purdue.edu (S.K.)

Highlights

Use BODIPY and LipidSpot610 to mark lipid droplets in mouse muscle satellite cells

Label lipid droplets in freshly isolated, cultured, or fixed cells

Quantitatively analyze lipid droplets with transmission electron microscopy

Chen et al., STAR Protocols *3*, 101849
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101849

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Labeling and analyzing lipid droplets in mouse muscle stem cells

Jingjuan Chen,[1,3,*] Feng Yue,[2] and Shihuan Kuang[1,4,*]

[1]Department of Animal Sciences, Purdue University, West Lafayette, IN 47907, USA

[2]Department of Animal Sciences, University of Florida, Gainesville, FL 32608, USA

[3]Technical contact

[4]Lead contact

*Correspondence: chen2627@purdue.edu (J.C.), skuang@purdue.edu (S.K.)
https://doi.org/10.1016/j.xpro.2022.101849

SUMMARY

Lipid droplets are emerging as an important and dynamic organelle whose metabolism controls stem cell behavior. Here we present a comprehensive protocol to visualize and quantify these organelles in mouse muscle satellite cells (MuSCs). This protocol includes steps for BODIPY/LipidSpot610 staining of freshly isolated MuSCs, *in vitro* cultured myoblasts, and single myofibers to label lipid droplets and subsequent analysis and quantification of fluorescence signals. This protocol can be modified to stain lipid droplets in other cell types of interest. For complete details on the use and execution of this protocol, please refer to Yue et al. (2022).[1]

BEFORE YOU BEGIN

MuSCs are muscle resident stem cells that are responsible for muscle growth and regeneration. This protocol focuses on lipid droplet staining on freshly isolated MuSCs, single myofibers and cultured myoblasts. BODIPY fluorophore (Invitrogen D-3922) is intrinsically lipophilic and stains neutral lipid. We routinely prepare BODIPY stock as 1 mg/mL in DMSO and store at −20°C. The working concentration we use in the lab is 1 μg/mL (1,000 ×). Similarly, LipidSpot610 (Biotium, T-0069) is used at 1:1,000 dilution from the stock purchased from Biotium. Before starting the protocol, ensure that the required reagents are purchased and ready for use.

For muscle stem cell isolation, please refer to Ling Liu et al. 2015.[2] The isolation procedure from one mouse takes approximately 3 h. The Fluorescence Activated Cell Sorting (FACS) process for one sample takes approximately 0.5–1 h.

For myoblast isolation, please refer to Kun Ho Kim et al. 2020.[3] The isolation procedure from one mouse takes approximately 2 h. The purification and establishment of primary myoblasts for experiment use might take up to two weeks.

For single myofiber isolation, please refer to Caroline E. Brun et al. 2018.[4] The isolation procedure from one mouse takes approximately 2 h. The experiment time points to harvest the cultured MuSCs differ based on the experimental design, the most often selected time points to look at different stages of the MuSCs are: 24–36 h (activation), 48 h (proliferation) and 72 h (differentiation and self-renew).

Where applicable, room temperature (RT) in this protocol refers to around 22°C. Overnight (O/N) refers to around 10 h.

Institutional permissions

All animal procedures in this protocol were approved by the Purdue Animal Care and Use Committee. Before proceeding with the following protocol, please ensure that the procedures are approved by relevant institutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
BODIPY 493/503	Invitrogen	D3922
DAPI	Invitrogen	D1306
LipidSpot610	Biotium	T0069
Paraformaldehyde	Sigma-Aldrich	P6148
Glycine	Sigma-Aldrich	50046
BD Matrigel matrix (1:10)	BD Biosciences	356235
Collagen from rat tail	Sigma-Aldrich	G7021
Hoechst 33342	Invitrogen	H3570
Tween-20	Sigma	P1379
Bovine serum albumin	GeminiBio	700-105P
Phosphate buffered saline (PBS)	Gibco	21600-069
Goat serum	MP Biomedicals	08642921
Triton X-100	Sigma-Aldrich	X100
Sodium azide	Sigma-Aldrich	S2002
Glutaraldehyde	Sigma-Aldrich	354400
Sodium cacodylate	VWR	100504-840 11550043
F-10 Ham's nutrient mix	Gibco	MT35030CV
Horse serum	Corning	15070063
Penicillin/streptomycin (100×)	ThermoFisher	99990-086
Fluoromount	VWR	21600-069
Antibodies		
PE rat anti-mouse CD31 antibody (1:1000 dilution)	BD Biosciences	Cat#553373, RRID: AB_394819
PE anti-mouse CD45 antibody (1:1000 dilution)	eBioscience	Cat#12-0451-82, RRID: AB_465568
Pacific Blue anti-mouse Ly-6A/E (sca-1) antibody (1:1000)	BioLegend	Cat#122520, RRID: AB_2143237
APC anti-mouse CD106 antibody (1:500 dilution)	BioLegend	Cat#105718, RRID: AB_1877141
Alexa 568 goat anti-mouse IgG1 (1:1000 dilution)	Invitrogen	Cat# A-21124, RRID: AB_2535766
Alexa 647 goat anti-mouse IgG2b (1:1000 dilution)	Invitrogen	Cat# A-21242, RRID: AB_2535811
Mouse monoclonal anti-PAX7 (1:10 dilution)	DSHB	Cat#PAX7, RRID: AB_2299243
Mouse monoclonal anti-MyoD (1:500 dilution)	Santa Cruz Biotechnology	Cat# sc-377460, RRID: AB_2813894
Software and algorithms		
Prism 6.0	GraphPad Prism	RRID: SCR_002798
BD FACSDiva Software	BD Biosciences	RRID: SCR_001456
Experimental models: Organisms/strains		
Mouse: B6.Cg-Pax7tm1(cre/ERT2)Gaka/J	The Jackson Laboratory	JAX stock: #017763
Mouse: B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J	The Jackson Laboratory	JAX stock: #007909
Experimental models: Cell lines		
Muscle stem cell (myoblast)	N/A	N/A
Other		
Tek-Select Cover Glass	IMEB	#CG1-2450
Tek-Select Positive Charge microscope slides	IMEB	50-168-9503
Fisherbrand™ Disposable Cover Slips (22 × 22 mm)	Fisherbrand	S17525B
Hydrophobic pen	Newcomer Supply 6505	NC9827128
6 well culture plate	Fisherbrand	FB012927
24 well culture plate	Fisherbrand	FB012929
48 well culture plate	Fisherbrand	FB012930

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Glass Pasteur pipette	Research Products international	50-136-7739
Heraeus Heracell 240 CO_2 Incubator	Thermo Scientific	51021056a
Transmission electron microscope	Tecnai	T-12
Fluorescence microscope	Leica	DM6000B
Dissection microscope	Leica	M80
FACS system	BD Aria III FACS system	BD Biosciences

MATERIALS AND EQUIPMENT

Blocking Buffer

Reagent	Final concentration	Amount
Goat Serum	5%	2.5 mL
Bovine serum albumin	2%	1 g
Triton X-100	0.1%	0.05 mL
Sodium Azide	0.1%	0.05 g
1× PBS	N/A	46.5 mL
Total	100%	50 mL

Storage condition: 4°C for up to 1 month; −20°C for up to 6 months.

1 M glycine

Reagent	Final concentration	Amount
Glycine	1 M	75 g
1× PBS	N/A	Up to 1 L
Total	100%	1 L

Storage condition: 4°C for up to 6 months, working solution can be stored at RT.

4% PFA

Reagent	Final concentration	Amount
Paraformaldehyde	4%	4 g
10× PBS	1 ×	10 mL
H_2O	N/A	~85 mL
Total	100%	100 mL

⚠ CRITICAL: Weigh 4 g of paraformaldehyde (PFA) to 50 mL of H2O on 60°C heat block, and add drops of 1 M NaOH until all PFA is dissolved. Add 10 mL of 10× PBS and adjust pH to 7.4 with 1 M HCl. Adjust the final volume to 100 mL with H2O.

Storage condition: 4°C for up to 1 month; −20°C for up to 6 months.

1× PBST

Reagent	Final concentration	Amount
1× PBS	1×	50 mL
Tween-20	0.1%	50 µL
Total	100%	50 mL

Storage condition: RT for up to 6 months.

TEM fixative		
Reagent	Final concentration	Amount
Paraformaldehyde (10%)	1.5%	3 mL
Glutaraldehyde (25%)	2.5%	1.5 mL
Sodium cacodylate (0.2 M, pH 7.4)	0.1 M	7.5 mL
H_2O	N/A	3 mL
Total	100%	15 mL

Storage condition: 4°C, up to 1 week once paraformaldehyde is added.

⚠ CRITICAL: paraformaldehyde should be freshly added before use.

Wash medium		
Reagent	Final concentration	Amount
F-10 Ham's nutrient mix	N/A	45 mL
Horse serum	10%	5 mL
Penicillin/Streptomycin (100×)	1×	500 µL
Total	100%	50 mL

Storage condition: 4°C up to 1 month.

STEP-BY-STEP METHOD DETAILS

Lipid droplet staining protocol in cultured myoblast

Timing: up to 3 days

Cultured myoblasts contain abundant lipid droplets (LD) in the cytosolic compartments. The morphology and size of these lipid droplets can be determined by the transmission electron microscopic images (Figure 1A). The LDs can be readily labelled with lipophilic dye or fluorophore such as BODIPY. In this scenario, LDs are visualized as fluorescent puncta in images captured with a 20× Plan Apochromat objective with a numerical aperture of 0.70 (Figure 1B). The LD numbers

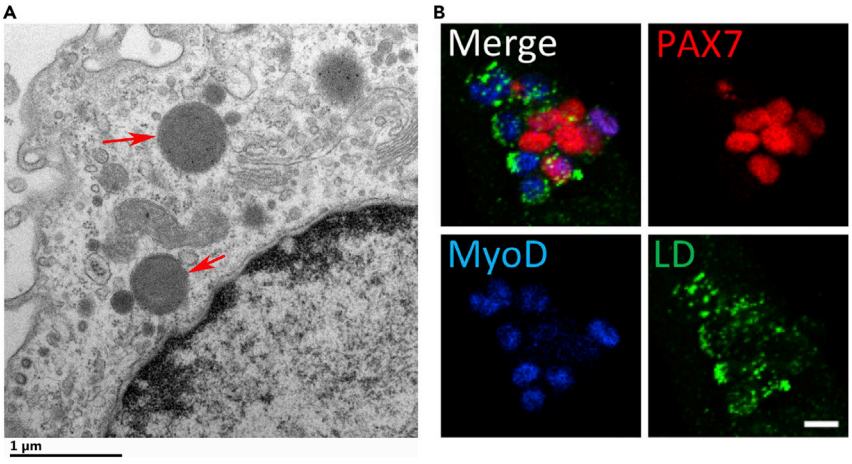

Figure 1. Presence of Lipid droplets in MuSCs under TEM and fluorescence images

(A) LDs in MuSCs at 5.5 days post injury in TA muscle (indicated by red arrow).

(B) BODIPY staining in satellite cells on myofiber; satellite cell fates are distinguished by PAX7 and MYOD1. LDs are marked by BODIPY (green).

Scale bar: 10 µm. Figures are adapted from Yue et al.[1] licensed under CC BY-NC-ND (https://creativecommons.org/licenses/by-nc-nd/4.0/).

on cultured myofibers are quantified on the same imaging plane and all fluorescently labeled LDs are quantified. From our staining results, LDs in cultured myofibers are well separated from one another, allowing unambiguous enumeration of the number of LDs. Quantitative analysis indicates that each cell contains an average of 5.1 LDs in LD^{High} satellite cells and 1.2 LDs in LD^{Low} satellite cells in myofiber culture; and the average size of the LDs are 0.6 μm as quantified by transmission electron microscopy (TEM) images. Here we describe the procedures and details to stain LD in cultured myoblasts.

Note: After myoblasts are successfully isolated from mice, we routinely use myoblasts within 10 passages.

Note: For other cell types with complex LD profiles and dynamics resulting in multiple intertwined LDs rendering the quantification difficult, accurate enumeration of the number of the LDs should be based on confocal images.

1. Plating myoblasts for imaging.
   a. Prior to staining, trypsinize myoblasts, and plate them onto culture vessels of choice. For reference, 6×10^4 myoblasts are seeded onto surface areas equal to one well in a 24-well plate for staining 24–48 h later.

   Note: Due to auto-fluorescence in the plastic culture plate, and/or conditions that allow confocal imaging, other vessels or materials such as chamber slides or coverglass should be considered to eliminate auto-fluorescence and improve signaling specificity.

   b. Coat positively charged glass coverslips with Matrigel to provide the extracellular matrix for myoblasts to attach and support proliferation.
      i. Sterilize the coverslips by covering in aluminum foil and autoclave before use. Carefully unwrap the slide and use hygiene practice in a cell culture hood to place the coverslip into a 6-well culture plate.
      ii. Dilute Matrigel in DMEM medium (without FBS) at 1:10 ratio and add about 1 mL to the coverslip to cover the entire coverslip, let the Matrigel sit for approximately 30-min at room temperature. Collect the excess Matrigel and leave the coverslip to dry for about 20-min in the cell culture hood.
      iii. After quantification of cell number after trypsinization, 40,000 myoblasts can be diluted in 200 μL of culture medium (F-10 Ham's nutrient mix, 20% Fetal Bovine Serum, 4 ng/mL basic Fibroblast growth factor, 1% penicillin-streptomycin) and added dropwise to the coated coverslip.
      iv. Carefully place the 6-well culture plate in the 5% CO_2 cell culture incubator (Heraeus Heracell 240 CO_2 Incubator) and check the cell status the next day. If the myoblasts have attached to the slide, add enough medium to submerge the entire coverslip in the well to prevent drying of the myoblasts. Harvest the myoblasts according to desired cell density, usually at around 60%–70% confluence.
   c. Chamber slides/coverglass (IMEB) are optimal for IF staining of cells.

   Note: They offer detachable chambers for staining as well as relatively small cell number for visualization. Check with the manufacturer if the slides are coated/treated for cell attachment, if not, the slides/coverglass can be coated with matrigel as shown in step 1.b.ii).

   d. Culture plates/dishes are sub-optimal for IF staining.

   Note: Due to the auto-fluorescence especially if the signals are weak and if microscope equipped with long distance lens is not available in the lab, these vessels should be avoided. However, if other choices are not available, culture plates can also be utilized. Myoblasts generally

attach very well in the plastic culture plates without coating but additional coating of the culture plate (collagen or Matrigel) can be up to user's discretion.

2. Preparing for IF staining with different culture vessels.

Note: BODIPY is compatible with either fixed samples or live staining. Depending on the assay, different strategies can be used.

a. Live staining of BODIPY.
   i. Dilute BODIPY (1:1,000) directly in the culture medium, rock the plate gently so that BODIPY spread evenly in the medium.
   ii. Incubate the plate for 30 min at 37°C in the cell culture incubator. At this step, if no other staining is required for imaging, nuclei can be counterstained with DNA stains such as DAPI at 1 μg/mL or Hoechst-33342 at 10 μg/mL final concentration.
   iii. If further staining is desired, please refer to the below instructions as to how to prepare the different vessels for future staining.

⚠ CRITICAL: Care should be taken to avoid the exposure of the culture plate to light as this may bleach the BODIPY signal. Culture plate can be wrapped in aluminum foil to avoid light exposure during the transportation from culture room to dark room designated for staining.

b. BODIPY staining after fixation.

If post-fixation staining is desired, directly take out the culture vessels at the time of choice, aspirate the medium off, wash the cells 3 times with 1 × PBS and fix with 4% PFA for 10 min at RT. In this method, BODIPY will be stained together with secondary antibodies against chosen primary antibody. Here we describe the different methods to prepare the culture vessels for staining.

   i. Matrigel coated coverslips.

   At the desired time point, take out the cell culture plate and transfer the coverslip out from the culture dish with the help of a tweezer. Carefully blot away the excessive medium underneath and on the periphery of the desired staining area with Kimwipes. Use a hydrophobic pen to draw a boundary so that the liquids will remain on the slide during the staining procedure. The slide can now be fixed for staining.

Note: Drawing with hydrophobic pen will require a dry surface. Kimwipes can be carefully wrapped around a 1,000 μL pipette tip (or simply substitute using a Q-tip), and used to blot dry any wet areas. This step may wipe away cells in the area.

   ii. Chamber slides/coverglass.

   The myoblasts can be stained with the chambers intact to better help distinguish different wells/treatments if such experimental design is desired. Aspirate the medium off and wash the cells with 1× PBS for 3 times. Fix the myoblasts on the chamber slides with 4% PFA for 10 min and proceed with next steps.

   iii. Culture plates/dishes.

   Aspirate the medium from the plates/dishes and wash 3 times with PBS. The myoblasts can now be fixed with 4% PFA for 10 min and proceed with next steps.

3. Staining primary antibodies of choice and BODIPY in cultured myoblasts.

Note: Depending on the staining strategies, BODIPY can already be stained at this step. If that is the case, take care not to bleach the signals by exposing the vessels to any light source before vessels are ready for imaging.

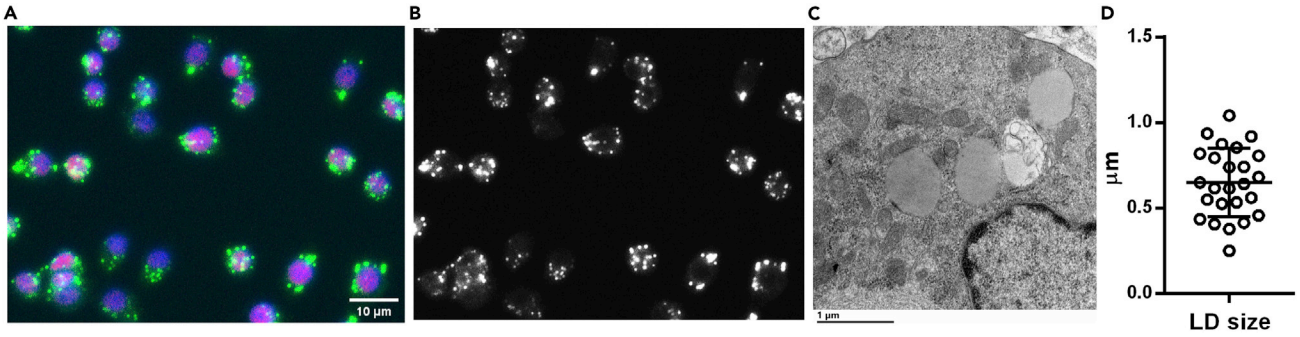

Figure 2. Lipid droplet staining and quantification by BODIPY staining and TEM

(A) LD staining in cultured myoblasts; myoblasts are marked by PAX7 (purple), nuclei are counter stained by DAPI (blue), LD are marked by BODIPY (green).

(B) BODIPY staining in myoblasts. Scale bar = 10μm.

(C) Visualization of LD in cultured myoblasts by TEM.

(D) Quantification of the size of LD under TEM.

Figures are adapted from Yue et al.[1] licensed under CC BY-NC-ND (https://creativecommons.org/licenses/by-nc-nd/4.0/).

a. Fix the myoblasts with 4% PFA for 10-min at RT, wash with 1× PBS 3 times and quench any residual PFA with 100 mM Glycine for 10 min. Then wash the myoblasts another 3 times with 1× PBS before blocking with blocking buffer for at least 1 h at RT.

Pause point: cells can now be kept in blocking buffer for up to 1 week.

b. Dilute PAX7 antibody (marker for myoblasts) in blocking buffer at ratio determined empirically and incubate in the cold room (4°C) overnight.

Note: Other primary antibodies can be used depending on the cell types of choice at ratios determined by the lab accordingly.

c. Wash the myoblasts with 1× PBST with 0.1% Tween-20 for 3 times, 5 min each time.
d. Prepare Alexa 568 goat anti-mouse IgG1 against PAX7, BODIPY and DAPI mixture in 1× PBST and stain the myoblasts for 1 h at RT in the dark room.

Note: Pay attention to the choice of fluorophore of the secondary antibody against the primary antibody so that the fluorophore does not overlap with that of BODIPY.

Note: The choice of secondary antibody should correspond to the species and isotypes of the primary antibody used in step b).

e. Then wash the myoblasts with 1× PBST for 3 times, 5 min each, before mounting with mounting media.
f. Seal the slides with nail polish, let dry and use for imaging.

Visualization and quantification of the lipid droplets in MuSCs attached on myofibers

Timing: up to 5 days, depending on the collection time points

Myofibers are isolated based on the published protocol.[4] Upon isolation, single myofibers are resuspended in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 20% Fetal Bovine Serum, 1% penicillin-streptomycin (Sigma-Aldrich) and 4 ng/mL basic fibroblast growth factor (FGF, Promega) and split into three parts. Transfer two parts of myofibers to two 6-cm horse serum coated culture dishes. These two plates will be used for 48 h and 72 h collection respectively. Rock

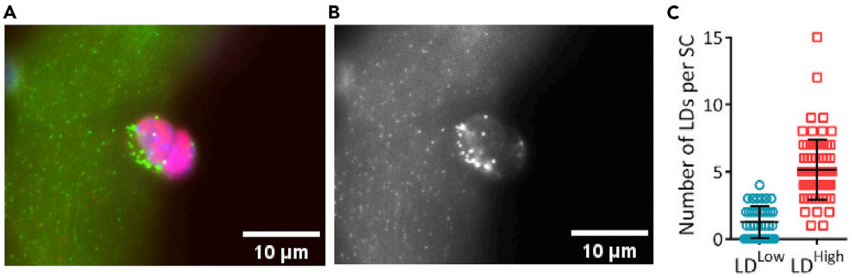

Figure 3. Lipid droplet staining in cultured myofiber harvested at 72 h

(A) BODIPY staining in satellite cells on myofiber; satellite cells are marked by PAX7 (purple), nuclei are counter stained by DAPI (blue), LDs are marked by BODIPY (green).

(B) BODIPY staining in satellite cells on myofiber. Scale bar = 10μm.

(C) Quantification of LD number in cultured myofiber.

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the plate a couple of times so that the myofibers are spread evenly in the dishes to ensure that the myofibers are not intertwined after 48 h or 72 h in culture. Representative images of BODIPY staining on satellite cells associated with myofiber culture are shown in Figure 3.

⚠ CRITICAL: Keep in mind that the myofiber staining procedure require using a dissection microscope and lighting to better visualize the myofibers and prevent losing the myofibers in between washes. Yet too much exposure of the BODIPY (if BODIPY has been stained already at this step) to the lights would diminish the signals and cause the quenching of fluorescence before imaging. Therefore, BODIPY staining after PFA fixation is preferred when staining LD in myofibers.

4. Staining of myofibers collected from different time points.
   a. The remaining one part of the myofiber (at 0 h) can be collected and fixed immediately in 4% PFA by transferring the myofibers with a glass Pasteur pipette to a well containing approximately 500 μL of 4% PFA in a 48-well plate. Collect the remaining myofibers at 48 and 72 h after culture. Similarly, collect the single myofibers from the culture with a large-cut Pasteur pipette to empty wells containing 500 μL 4% PFA in the 48-well plate used for collecting the 0 h myofibers. Pay attention to replace with fresh 4% PFA if excessive medium is transferred. Fix the myofibers and wash with 1× PBS for 3 times.

Note: From this point on, the same procedure can be conducted on the myofibers collected from the 3 time points.

Note: If too much medium is transferred to the well, remove the medium/PFA mixture after the myofibers settled to the bottom of the well under the dissecting microscope with a fine cut Pasteur pipette and then add 500 μL fresh 4% PFA and fix for 10 min at RT. Then wash the myofiber with 1× PBS for 3 times.

Pause point: the myofiber can be stored at 4°C until all the myofibers are collected.

   b. Quench residual PFA with 500 μL of 100 mM Glycine for 10 min at RT in the myofibers from different time points, then wash the myofibers with 1× PBS for 3 times.
   c. Block the myofibers with blocking buffer for 1 h at RT.
   d. Then dilute the PAX7 and MyoD antibodies and stain the myofibers overnight at 4°C. PAX7 and MyoD are used to distinguish the different cellular status ($PAX7^+MyoD^-$: self-renewing; $PAX7^+MYOD^+$: proliferating; $PAX7^-MyoD^+$: differentiating).

e. On the next day, wash the myofibers three times with 1× PBST for 5 min and then incubate with corresponding secondary antibody (anti-mouse IgG1-568 for PAX7, anti-mouse IgG2b-647 for MyoD1 (SCBT-G1), DAPI for nuclei), plus BODIPY (493/503) for 1 h at RT. Wash the myofibers with 1× PBST for 3 times before mounting the myofibers onto a positively charged glass slides (IMEB).
f. Briefly, pre-wet the central region of glass slide with 1× PBST, then transfer the myofibers to the slide using the large-bore Pasteur pipette, pay attention to not transfer too much 1× PBST on the slide. Then use a fine-cut Pasteur pipette to remove the 1× PBST from the slide with one end slightly tilted. Then add mounting medium onto the slide and use two 30-G syringe needle to separate the intertwined myofibers into individual myofibers. Add mounting medium if necessary, then carefully cover the slide with a coverglass. Place the coverglass at a 30 degree angle relative to the slide with one edge touching the slide, gradually lower the higher edge of the coverglass until it lies flat onto the slide. Blot with Kimwipes to remove the excessive mounting medium and seal the slide with nail polish.

Note: Pay attention to slowly and carefully cover the coverglass to prevent formation of bubbles and movements of myofibers.

g. Myofibers can now be visualized under the microscope.

Visualization and analysis of lipid droplets in freshly isolated MuSCs

Timing: Up to 1 day

Muscle-derived single cell suspension preparation should be performed before sorting. Different labs utilize different surface markers for sorting MuSCs. In our lab, we use CD45, CD31 and SCA-1 as negative selection markers for hematopoietic, endothelial and mesenchymal cells respectively, and VCAM-1 (CD106) as a positive selection marker for MuSCs according to the method described by Liu et al.[2] Representative FACS gates for isolation of MuSCs from WT mice are shown in Figure 4.

⚠ CRITICAL: One important consideration is the fluorophore of the antigen used to stain and sort MuSCs should not overlap with the fluorophore of lipid stain such as BODIPY or LipidSpot in your lab.

⚠ CRITICAL: FACS-based separation of BODIPY-labeled fresh-isolated satellite cells (FISCs) often bleaches out the green BODIPY signal during the prolonged washing and sorting steps. Therefore, we used a more stable far-red fluorophore LipidSpot for FACS-based applications. Readers are encouraged to test if BODIPY signals are stable throughout the sorting procedure in their system.

5. Staining and separating MuSCs based on lipid contents.
   a. Sort MuSCs by BD-FACS Aria III FACS system (BD Biosciences) into FACS wash medium (HAM'S F-10 medium supplemented with 10% Horse Serum, 1% P/S).
   b. Perform BODIPY/LipidSpot610 non-stained control by aliquoting a small volume of the sorted MuSCs.
      i. Analyze the non-stained control cells first to establish the threshold of the BODIPY non-stained sample at 503 nm or LipidSpot610 non-stained sample at 638 nm.
      ii. Stain the remaining cells with BODIPY/LipidSpot610 as described below.
   c. For BODIPY/LipidSpot610 staining, centrifuge the MuSCs at 4°C at 2,500 rpm (~600 g) for 5 min. Resuspend the MuSCs with 1× PBS and then stain with BODIPY/LipidSpot610 at RT for 30 min, wash with 1× PBS again before analyzing the MuSCs with the FACS sorter again.

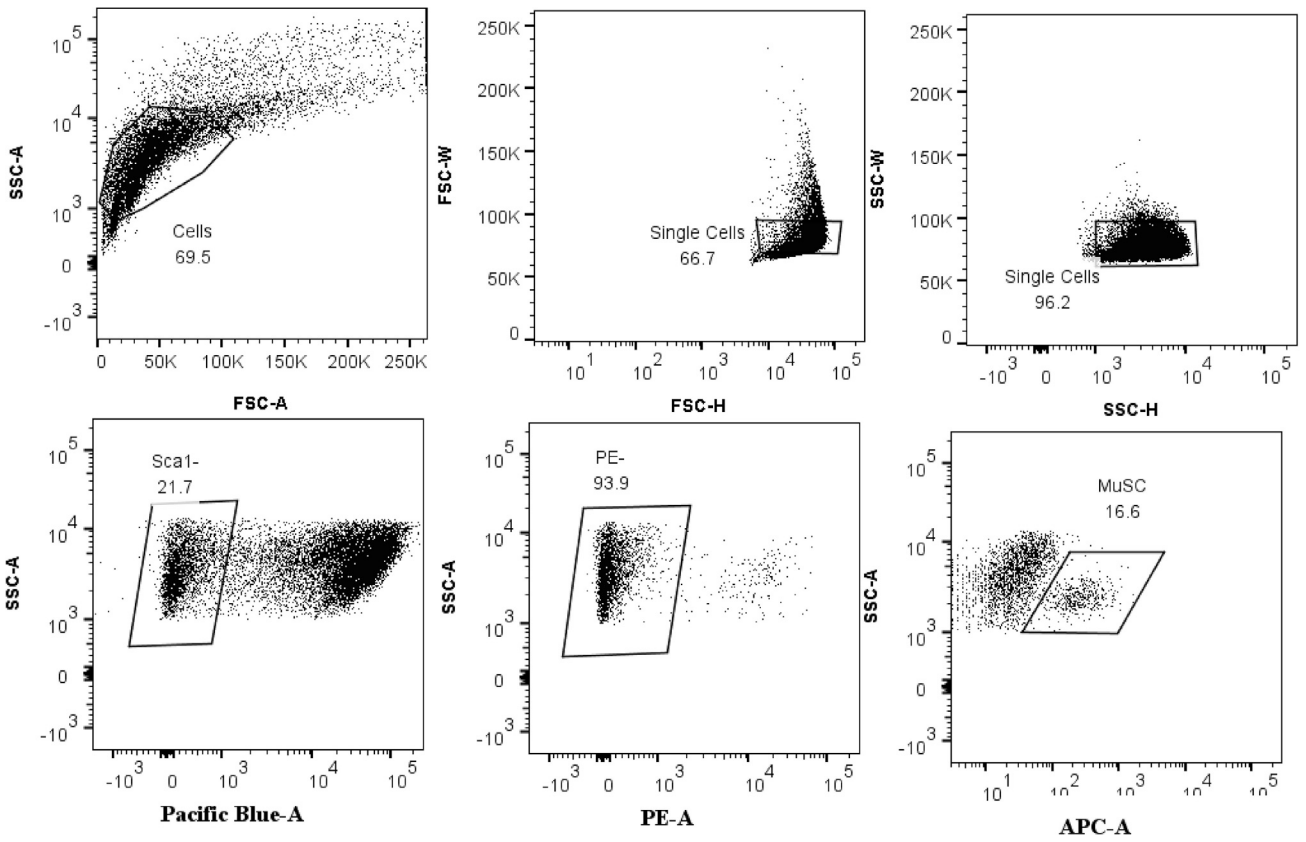

Figure 4. FACS strategies and gates to sort out MuSCs from hind limb muscle single cell suspension

Cells are first plotted based on FSC-A and SSC-A.

The bulk of cells are selected (cells). Then singles cells are gated based on FSC-H against FSC-W as well as SSC-H against SSC-W. This is to get rid of doublets and debris. Then based on the sorting strategy, we use SCA1 to sort out the mesenchymal stem cells and CD31&CD45 to get rid of the epithelial cells and hematopoietic cells. Lastly, MuSCs are sorted based on VCAM1 conjugated to APC.

d. Different intensities of the BODIPY/LipidSpot610 signals can be gated into low, medium and high, respectively, and the corresponding cells can be sorted based on the BODIPY/LipidSpot610 signals.

Note: In our study, the LD^{Low} MuSCs display higher self-renew capacity while the LD^{High} MuSCs display higher differentiation potentials. Therefore, we separated the MuSCs into LD^{Low} and LD^{High} and sorted them out for transplantation experiments.

e. Plate the resulting cells onto Matrigel coated plates to examine their proliferative and differentiation potentials or inject them into muscle-injured immune-deficient mice to assess their regenerative capacities.

Note: Additional sorting strategies can be used to sort out MuSCs utilizing reporter mouse lines. Both sexes of mice at 2–3 months were used for this experiment. Here we briefly describe the sorting strategy using *Pax7*$^{CreERT2(Gaka)}$;*Rosa26*$^{LSL\text{-}tdTomato}$ mouse model. In this model, after TMX (Tamoxifen) injection, all the MuSCs are labeled by tdTomato. Single cell preparation from hind limb muscles from this mouse should be directed to the ref. 2, and the staining protocol of LipidSpot610 can be modified from step 3. Please note the emission wavelength of LipidSpot610 is at 638 nm. LD^{High} population is defined as the top 27.5% and LD^{Low} population is defined as the lower 27.5% based on the fluorescence intensity. Sorted MuSCs are visualized under the microscope to visualize the success of the gating (Figure 5).

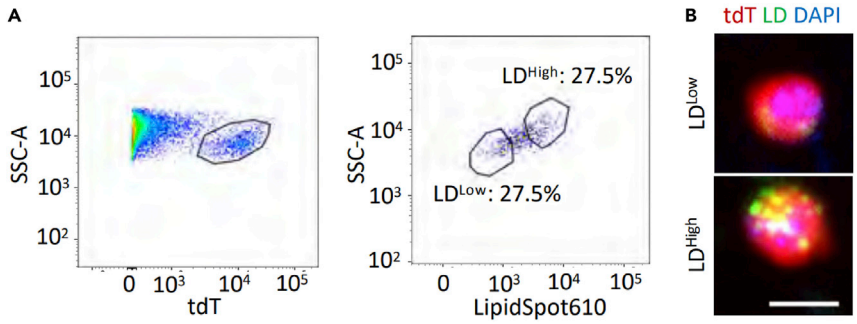

Figure 5. Representative image of LD^{Low} and LD^{High} MuSCs after FACS

(A) Gating of the tdT^{+} MuSCs from *Pax7*$^{CreERT2(Gaka)}$;*Rosa26*$^{LSL\text{-}tdTomato}$ mouse model and the subsequent separation of LD^{High} and LD^{Low} MuSCs after LipidSpot610 staining.

(B) Example image of the LD^{High} and LD^{Low} cells after sorting. Scale bar: 10 μm.

Figures are adapted from Yue et al.[1] licensed under CC BY-NC-ND (https://creativecommons.org/licenses/by-nc-nd/4.0/).

Identification of lipid droplets with transmission electron microscopy (TEM)

Timing: Up to 1 week

Due to the hydrophobic nature of LDs, their presence can be easily identified by Osmium Tetroxide treatment which binds to lipids and generates great contrast when visualized under electron microscope. Here we have described two procedures to prepare myoblasts and muscle samples for the visualization of lipid droplets under electron microscopy. Detailed TEM sample processing and troubleshooting can be found in a comprehensive protocol by Graham et al.[5]

Note: For reference, the concentrations and duration of different reagents for TEM sample preparation can be found in Yue et al.[1]

⚠ CRITICAL: For this procedure, highly toxic and inhalable substances such PFA and glutaraldehyde may cause allergies in the respiratory tract or skin if exposed. Therefore, researchers should wear respirators and perform the designated steps in a fume hood or a well-ventilated area. Dispose of the hazardous waste according to the institute's instructions.

⚠ CRITICAL: During muscle injury induced by cardiotoxin (CTX), resident muscle satellite cells quickly activate, followed by proliferation and differentiation to fuse with myofibers to repair the injury. During this process, MuSCs may exit their niche underneath the basal lamina. This challenged the identification of MuSCs among other infiltrating cells from the circulation. Researchers are encouraged to use this method with caution for their cell types of interest.

6. Preparing myoblast samples for TEM.

Note: TEM sample processing will require pelleting and processing the myoblasts in agarose. It is recommended to have approximately 1 million myoblasts for easier handling during sample preparation and enough myoblasts to quantify under the electron microscope. To this end, myoblasts can be grown in a 10-cm culture dish. Myoblasts can be harvested after desired treatments or can directly be subjected to TEM sample preparation.

a. After myoblasts have established in the culture dish, take out the culture dish from the incubator and immediately wash with ice-cold 1× PBS once.

b. Aspirate the excess 1× PBS quickly and immediately add 2 mL of TEM fixation buffer (0.1 M sodium cacodylate, 2.5% glutaraldehyde, 1.5% paraformaldehyde). Since there was residue PBS from the previous wash, aspirate the TEM buffer off and replace with 1.5 mL of fresh TEM buffer.
c. Incubate the myoblasts (on the plate) for 5 min for initial fixation. Then use a cell scraper to carefully scrape the myoblasts off from the culture dish. Collect the myoblasts in a 1.5 mL Eppendorf tube.

⚠ CRITICAL: This step should be conducted on ice for better preservation of the ultrastructure such as mitochondria in the myoblast.

d. The myoblasts can now be stored in 4°C until TEM sample processing.

7. Preparing TA muscle for TEM.

Note: We have previously demonstrated that lipid droplets in the muscle stem cells are dynamically remodeling during muscle injury to facilitate the regeneration.[1] Studying MuSCs in their native environment *in vivo* provide invaluable information about their physiology. MuSCs under homeostasis are unique as they are wedged between the basal lamina and the sarcolemma.[6] Besides, quiescent MuSCs also manifest heterochromatin structure compared to myonuclei.[7] These characteristics will aid in the quick identification of homeostatic MuSCs under electron microscopy.

a. Perform cervical dislocation to quickly sacrifice the mouse, pinch the toes to ensure the death of the mouse before procedures.
b. In a timely fashion, remove the skin with surgical scissors and expose the TA muscle. Quickly add two drops of fixative to the muscle before dissecting the TA muscle out from the mouse. This will greatly help retain the ultrastructure such as lipid droplets and mitochondria.
c. Carefully dissect the TA muscle by cutting the distal tendon first, take care not to stretch the muscle too much as this may damage the sarcomeres.
d. Transfer the TA muscle to a 1.5 mL Eppendorf tube with 1 mL of fixative. To ensure effective penetration of osmium tetroxide in the TA muscle, the TA muscle should be trimmed along the long axis to reduce the width to about 5–10 mm. The muscle can now be stored at 4°C until ready for TEM sample processing.

Note: LD appears as a circular/round electron opaque structure lined with a single membrane under our TEM sample processing procedure. LDs can be in close proximity with mitochondria or nucleus. For reference, the average size of LDs in cultured myoblasts was around 0.6 μm (Figure 3D).

EXPECTED OUTCOMES

Images for expected outcomes at various steps of the protocol are described below.

The presence of LD can be detected by either fluorescence staining using lipophilic stains such as BODIPY (Figure 1B) and LipidSpot or transmission electron microscope (Figure 1A). LDs appear as spherical structures both under TEM and fluorescence images in cultured myoblasts (Figure 2). The average size of LDs under TEM images is around 0.6 μm (Figure 2D). During myofiber culture, satellite cells associated with the myofibers accumulate LDs and the number of the LDs is associated with different cell fate in the satellite cell clusters (Figure 3). Representative gating of the FACS plots are shown in Figure 4. After the MuSCs are sorted out, they are stained with LipidSpot and can be separated based on the intensity of the LipidSpot signals (Figure 5A). The representative images of the LD^{High} and LD^{Low} MuSCs after sorting are shown in Figure 5B.

QUANTIFICATION AND STATISTICAL ANALYSIS

Experiments involving mice were performed with a minimum of three biological repeats. Statistical analyses and graphing were performed using Graphpad Prism 6.0 (Graphpad Software). All experimental data are represented as mean ± standard deviation. Statistical analysis was done using Student *t* test under two-tailed and *p* value less than 0.05 was considered significant.

LIMITATIONS

Due to the word limit, detailed protocol for isolation of myoblasts, myofibers and muscle derived single cells were not provided in this protocol. Readers should refer to the references cited in this protocol before starting BODIPY staining.

The concentration of FBS affects the dynamics of LD, therefore to ensure repeatability of results, FBS (vendor and concentration) supplementation needs to be consistent across different batches of experiment.

In our hands, BODIPY-488 staining signals were not very stable and bleached rapidly during FACS applications. We encourage the fellow researchers to test whether BODIPY staining would provide consistent result in their own experimental settings. We used LipidSpot610 for a better separation of signals in FACS applications.

Single myofiber isolation and culture are delicate procedures. The dissociation of myofibers using enzyme (collagenase) digestion followed by mechanical trituration requires optimization since over digestion and too harsh trituration will also dissociate the satellite cells from the myofiber, yet insufficient digestion or not adequate digestion time will result in difficulty in dissociating myofibers. The proliferation of satellite cells on myofiber also require optimal culture condition, including but not limited to the FBS supplementation, bFGF addition, and sodium pyruvate presence. These conditions also affect the dynamic of lipid droplets which may affect experimental results.

In this protocol, we only discussed BODIPY and LipidSpot610 labeling of neutral lipids (lipid droplets). There are other staining protocols for polar lipids, readers are encouraged to explore different staining procedures that suit their experimental use.

TROUBLESHOOTING

Described below are some potential problems and recommendations for troubleshooting.

Problem 1

No BODIPY signal in myoblasts, related to step 3.

Potential solution

BODIPY staining usually gives consistent results.

- In the case where BODIPY signals are not present, first ensure that the BODIPY reagent is not expired or gone bad during storage.
- Then consider the following steps during which BODIPY signal might be quenched. During transport of myoblasts live-stained from cell culture incubator, wrap the plates with aluminum foil to avoid exposing to room lights.
- When imaging the myoblasts under fluorescence microscope, ensure that BODIPY signal is imaged before imaging other fluorophores and exposed to UV light as briefly as possible as BODIPY signals appear to be bleached by UV light rather quickly during image acquisition.

Problem 2
No BODIPY signal in myofiber, related to step 4.

Potential solution

- Since myofiber culture requires supplementation of FBS, basic FGF and sodium pyruvate, if the satellite cells are not supplemented with enough nutrients, LDs will be scarce in the satellite cells resulting in the reduction of the BODIPY signals.
- On the other hand, ensure that the signals are not bleached during staining of myofibers as illumination with fiberoptic lights under a dissection microscope will quench the signals. Dim the light as much as possible after the staining of BODIPY.
- Ensure that BODIPY is imaged/viewed first and quickly during image acquisition.

Problem 3
No BODIPY signal in FISC, related to step 5.

Potential solution

- Depending on the amount of LDs in the cell type, the staining time and concentration of the BODIPY or LipidSpot should be optimized.
- Increase the detection voltage on the flow cytometer detector.
- Ensure all the proper flow cytometry staining controls are included before setting up the FACS gates.

Problem 4
Not enough FISC for BODIPY/LipidSpot staining, related to step 5.

Potential solution

- Ensure adequate digestion of the muscle tissue during single cell suspension preparation. Enzyme concentration might need to be optimized for better results.
- Reduce the aggregation of myoblasts after the single cell preparation. Buffer may be supplemented with 1 mM EDTA to reduce cell aggregation.
- Perform compensation controls and negative staining controls before sorting.

Problem 5
Loss of FISC during BODIPY/LipidSpot staining, related to step 5.

Potential solution

- Low retention tubes and pipette tips must be used to minimize the FISC sticking to the wall of the tube/tip.
- The speed of the centrifuge must not be set too high to avoid crashing the cells during washes.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shihuan Kuang (skuang@purdue.edu).

Materials availability
All the materials used in this protocol are commercially available and no mouse lines were generated for this protocol.

Data and code availability

No new data or codes were generated for this protocol.

ACKNOWLEDGMENTS

This work was supported by NIH R01AR079235 and R01AR078695 to S. K. We would like to acknowledge Jill Hutchcroft at Purdue University's flow cytometry and cell separation facility for assistance with fluorescence-activated cell sorting, Laurie Mueller and Robert Seiler for TEM sample processing, and Jun Wu for technical support.

AUTHOR CONTRIBUTIONS

J.C. wrote the protocol, F.Y. assisted with the protocol development, and S.K. conceived the protocol and revised the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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