Journal of Visualized Experiments

Isolation of primary murine skeletal muscle microvascular endothelial cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58901R2
Full Title:	Isolation of primary murine skeletal muscle microvascular endothelial cells
Keywords:	Cell isolation; murine microvascular endothelial cells; skeletal muscle cells; blood muscle barrier; myovascular unit; myopathy.
Corresponding Author:	Tobias Ruck Universitatsklinikum Munster Klinik fur Allgemeine Neurologie Munster, Northrhine-Westphalia GERMANY
Corresponding Author's Institution:	Universitatsklinikum Munster Klinik fur Allgemeine Neurologie
Corresponding Author E-Mail:	Tobias.Ruck@ukmuenster.de
Order of Authors:	Thomas Müntefering
	Alexander P.E. Michels
	Steffen Pfeuffer
	Sven G. Meuth
	Tobias Ruck
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Munster, Northrhine-Westphalia, Germany

University Hospital Muenster . Department of Neurology . 48129 Münster

JoVE
Ronald Myers
Science Editor

Department of Neurology

Univ.-Prof. Prof. h. c. Dr. med. Heinz Wiendl
Director

with

Institute of Translational Neurology
Univ.-Prof. Dr. med. Dr. rer. nat. Sven Meuth

Dr. med. Tobias Ruck

Albert-Schweitzer-Campus 1, Building A1
48149 Münster

T +49 (0)2 51 - 83 – 41125

F +49 (0)2 51 - 83 - 4 68 12

Münster, 02 August 2018

Dear Dr. Myers,

we would like to thank you again for your invitation to submit our article **„Isolation of primary murine skeletal muscle microvascular endothelial cells"** for consideration to JoVE.
Microvascular endothelial cells shape the inner wall of muscle capillaries and regulate fundamental processes in the skeletal muscle such as the exchange of fluids and nutrients or immune cell migration.
Therefore, the isolation of primary murine skeletal muscle microvascular endothelial cells is an essential method in muscle research, and thus would be of general interest to a wide audience.

As potential reviewers, we would like to suggest:

1) Christoph Kleinschnitz, Universitatsklinikum Essen, Germany, christoph.kleinschnitz@uk-essen.de
2) Ralf Gold, University of Bochum, Germany, ralf.gold@ruhr-uni-bochum.de
3) Werner Stenzel, Charite Universitatsmedizin Berlin, Germany, werner.stenzel@charite.de
4) Tim Magnus, Universitatsklinikum Hamburg-Eppendorf, Germany, t.magnus@uke.de

Please do not hesitate to contact me in case of any additional questions.

Sincerely,

Tobias Ruck, MD

TITLE:

Isolation of Primary Murine Skeletal Muscle Microvascular Endothelial Cells

AUTHORS AND AFFILIATIONS:

Thomas Müntefering[1] *, Alexander P.E. Michels[1] *, Steffen Pfeuffer[1], Sven G. Meuth[1] *, Tobias Ruck[1] *

[1]Institute for Translational Neurology and Neurology Clinic, University of Münster, Münster, Germany

*These authors contributed equally.

Corresponding Author:

Tobias Ruck (tobias.ruck@ukmuenster.de)
Phone +49 251 83 41125
Fax: +49 251 83 46812

E-mail Addresses of the Co-authors:

Thomas Müntefering (thomas.muentefering@ukmuenster.de)
Alexander P.E. Michels (a_mich20@uni-muenster.de)
Steffen Pfeuffer (steffen.pfeuffer@ukmuenster.de)
Sven G. Meuth (sven.meuth@ukmuenster.de)

KEYWORDS:

Cell isolation, murine microvascular endothelial cells, skeletal muscle cells, blood muscle barrier, myovascular unit, myopathy

SUMMARY:

Microvascular endothelial cells of skeletal muscles (MMECs) shape the inner wall of muscle capillaries and regulate both the exchange of fluids/molecules and the migration of (immune) cells between muscle tissue and blood. The isolation of primary murine MMEC, as described here, enables comprehensive *in vitro* investigations of the "myovascular unit".

ABSTRACT:

The endothelial cells of skeletal muscle capillaries (muscle microvascular endothelial cells [MMECs]) build up the barrier between the bloodstream and skeletal muscles regulating the exchange of fluids and nutrients, as well as the immune response against infectious agents by controlling immune cell migration. For these functions, MMECs form a functional "myovascular unit" (MVU) with further cell types, such as fibroblasts, pericytes, and skeletal muscle cells. Consequently, dysfunctions of MMECs and, therefore, the MVU contribute to a vast variety of myopathies. However, the regulatory mechanisms of MMECs in health and disease remain insufficiently understood and their elucidation precedes more specific treatments for myopathies. The isolation and in-depth investigation of primary MMEC functions in the context of the MVU might facilitate a better understanding of these processes. This article provides a

protocol to isolate primary murine MMECs of the skeletal muscle by mechanical and enzymatic dissociation, including purification and culture maintenance steps.

INTRODUCTION:

Via the bloodstream, cells and organs are supplied with oxygen, substrates, and other necessary molecules. This interchange takes place in capillaries, the smallest vessels. Capillaries are formed by an inner endothelial cell (EC) layer whose integrity remains a prerequisite to the successful regulation of muscle homeostasis between the intravascular and interstitial space. To ensure a selective transition of soluble factors and cells, ECs constitute a monolayer interconnected by tight and adherens junctions[1]. Besides their role as a barrier for nutrients or metabolic products, ECs regulate the recruitment of leukocytes in inflammatory processes. Inflammation or tissue damage leads to an upregulation of adhesion molecules on the EC surface and the production of chemokines facilitating leukocyte attachment and transmigration into the target tissue[2]. Consequently, ECs are critically involved in the regulation of inflammatory processes, such as the defense against pathogens or tissue repair.

Dysfunctions of ECs are directly associated with vascular diseases, chronic kidney failure, and venous thrombosis severe pathogen infections. Furthermore, ECs are virtually always involved in organ-specific autoimmunity, such as diabetes mellitus or multiple sclerosis[3]. The barrier function between the bloodstream and organs is, therefore, controlled by a concerted interplay of different cell types. In the skeletal MMECs, together with muscle cells, fibroblasts and pericytes form a functional unit, the MVU. Therefore, dysfunctions of the MVU might play a critical role in the pathophysiology of myopathies. However, a deeper understanding of these regulatory mechanisms is still missing and currently precludes the identification of new, urgently needed, therapeutic targets in myopathies.

To investigate the complex physiological and pathophysiological mechanisms, animal models are commonly used. However, *in vitro* models offer the advantage of focusing on the subject of interest by excluding a variety of confounding factors. To investigate processes *in vitro*, it is necessary to isolate pure and viable primary cells. In contrast to cell lines, primary cells isolated from transgenic animals enable researchers to investigate the consequences of genetic modifications *in vitro*.

Here, a method to isolate primary murine MMECs is described by using mechanical and enzymatic dissociation followed by magnetic-activated cell sorting (MCS) techniques for purification. For this purpose, magnetic beads against specific surface markers are used. Platelet endothelial cell adhesion molecule-1 (PECAM1, CD31) is mainly expressed on ECs and can be used to enrich this cell type. To warrant a high cell purity, cells of hematopoietic origin are excluded by a negative selection for protein tyrosine phosphatase receptor type C (PTPRC, CD45). Further, quality controls, the cultivation of primary murine MMECs, potential applications, and limitations, as well as special considerations, are presented.

PROTOCOL:

All animal experiments were approved by the local authorities and conducted according to the German animal welfare act (84-02.05.20.13.097).

1. General Remarks on Animal Experiments

1.1. Perform all mouse experiments in accordance with the guidelines of the respective institutional animal care and use committee.

1.2. Keep the mice under standardized conditions and according to international guidelines, such as the Federation for Laboratory Animal Science Associations (FELASA).

NOTE: In general, this isolation technique can be used for mice independent of age, gender, or genetic background. To obtain a sufficient cell number, 4- to 10-week-old males are preferred, because biological properties can vary with age and gender.

2. Preparation of the Solutions, Media, and Coating

2.1. Prepare digestion solution (DS) by mixing 2.2 mL of Dulbecco's modified Eagle's medium (DMEM) with 200 µL of collagenase-dispase and 45 µL of deoxyribonuclease (DNase).

2.2. Prepare 500 mL of endothelial cell medium (ECM) by mixing 450 mL of DMEM with 50 mL of fetal calf serum (FCS; approximately 10%), 0.25 mL of basic fibroblast growth factor (bFGF; 20 µg/mL; approximately 0.05%), and 5 mL of penicillin-streptomycin (approximately 1%). Perform a sterile filtration in a glass tube (with filter pores of 0.2 µm in diameter). Afterward, store the solution at 4 °C.

2.3. Coat cell culture plates as described below.

2.3.1. For the cultivation of freshly isolated primary murine MECs, cover the whole surface of a 6-well cell culture plate with 1 mL per well of a gelatin-based speed coating solution (see **Table of Materials**) according to the manufacturer's instructions, for 3 - 5 min at room temperature (RT).

2.3.2. Aspirate and discard the coating solution. Rinse each well with 2 mL of sterile phosphate-buffered saline (PBS), pH 7.1 - 7.5, in two consecutive washing steps.

2.3.3. Aspirate and discard the PBS. Fill up the wells with a 1 mL volume of ECM.

3. Isolation of Primary Murine MMECs

3.1. Euthanize one adult, 4- to 12-week-old male mouse by cervical dislocation without any anesthesia. The aim is to quickly separate the spinal cord from the brain so as to provide the animal with a fast and painless death.

3.2. Sever the extremities.

3.2.1. Use surgical sharp/blunt (with a cutting edge of 42 mm, straight) and sharp/sharp (with a cutting edge of 23 mm, straight) scissors and straight (serrated, 2 mm x 1.25 mm x 12 cm) and curved (serrated, 1.3 mm x 1 mm x 13 cm) forceps.

3.2.2. Disinfect all surgical instruments with 70% ethanol.

3.2.3. Position the animal on its back and moisten the legs with 70% ethanol. Sever the whole leg by cutting at the hip joint with the sharp/blunt surgical scissors. Place the extremities in a closed cell culture dish (35 mm x 10 mm).

NOTE: From now on, perform every step under a sterile laminar flow hood and work with sterilized instruments.

3.3. Isolate the muscle tissue (preferentially, use the musculus quadriceps femoris or musculus triceps surae from both legs).

3.3.1. Cut the skin open from the hip to the tip of the toe by using sharp/sharp scissors and curved forceps. Hold the toe or footpad with the straight forceps. Peel off the skin with the curved forceps from the toe to the hip.

3.3.2. Isolate the musculus quadriceps femoris by cutting the tendon from the knee and sever the muscle along the femur to the hip. Isolate the musculus triceps surae by severing the Achilles tendon. Hereafter, cut along the tibia to the popliteal fossa and remove the muscle.

NOTE: If large vessels (*Arteria femoralis*, *Arteria tibialis anterior*, *Arteria tibialis posterior*, *Arteria fibularis*) are visible in the isolated muscles, remove the vessels to avoid contamination by macrovascular endothelium.

3.3.3. To remove large vessels, hold the part of muscle not containing large vessels with curved forceps and cut it off next to the vessel. For this protocol, 1 g or less muscle tissue equal to both quadriceps femoris and triceps surae is recommended.

3.3.4. Add 2445 µL of DS (from step 2.1) to a cell culture dish (35 mm x 10 mm) and determine the weight.

3.3.5. Transfer all muscle pieces to this cell culture dish containing the 2445 µL of DS and determine the weight. The difference of both measured values provides the dry weight of the muscle tissue, which must not exceed 1 g.

3.3.6. Cut the whole muscle tissue into small pieces (≤2 mm cubes, about 100 pieces) by using the sharp/sharp scissors.

3.4. Dissociate the muscle tissue.

NOTE: This step takes about 120 min.

3.4.1. Store the muscle/DS suspension at 37 °C (in an incubator with 5% CO_2) for 1.5 h. Mix the suspension carefully every 20 min for about 5 min, using a 1 mL insulin syringe.

3.4.2. Transfer the suspension to a 70 µm nylon cell strainer placed on a 50 mL tube and collect the flow through. Wash the cell strainer with 8 mL of DMEM and collect the flow through.

3.4.3. Discard the cell strainer and centrifuge the suspension for 10 min at 300 x *g* at 20 °C. Carefully remove the supernatant.

CAUTION: The pellet is easy to lose.

3.4.4. Resuspend the cell pellet in 1 mL of an ammonium-chloride-potassium (ACK) lysing buffer (see **Table of Materials**) for the lysis of red blood cells and incubate for 30 s at RT. Add 9 mL of DMEM + 10% FCS to stop the reaction and transfer the cell suspension to a 15 mL tube.

3.5. Deplete $CD45^+$ cells, following the instructions of the CD45 microbeads' manufacturer. For all following steps of $CD45^+$ depletion, use MCS buffer (see **Table of Materials**).

NOTE: This step takes about 60 min.

3.5.1. Determine the cell numbers using a Neubauer cell counting chamber (expect about 5 x 10^6 cells per gram of muscle tissue).

3.5.2. Centrifuge the suspension at 300 x *g* for 10 min at 4 °C. Take off the supernatant completely and resuspend the cell pellet in 90 µL of MCS buffer (per 10^7 cells or less). Add 10 µL of CD45 microbeads (per 10^7 cells or less).

3.5.3. Mix the cell suspension and incubate it for 15 min in the fridge at 4 - 8 °C.

3.5.4. Add 1 mL of MCS buffer (per 10^7 cells or less). Centrifuge at 300 x *g* for 10 min at 4 °C. Remove the supernatant completely and resuspend the cells in 500 µL of MCS buffer.

3.5.5. For magnetic cell separation, use a large magnetic column (LMC) with a reservoir volume of 8 mL and a capacity of up to 2 x 10^9 total cells. Position the LMC in the magnetic field of the separator.

3.5.6. Rinse the reservoir of the LMC with 3 mL of MCS buffer. After rinsing, place a 15 mL conical tube below the column to collect the flow through. Apply the whole cell suspension (500 µL) onto the column and let it flow through completely.

3.5.7. Wash the column 3x by adding 3 mL of MCS buffer into the reservoir, and wait until the column's reservoir is empty before performing the next washing step.

3.5.8. Collect the unlabeled cells passing the column to use them for further separation steps (representing the $CD45^-$ fraction). Labeled cells (representing the $CD45^+$ fraction) that have accumulated in the column can be discarded.

3.6. Accumulate $CD31^+$ cells, following the instructions of the CD31 microbeads' manufacturer. For all following steps of $CD31^+$ accumulation, use MCS buffer.

NOTE: This step takes about 60 min.

3.6.1. Determine the cell number, using a Neubauer cell counting chamber (expect about 4×10^6 cells per gram of muscle tissue).

3.6.2. Centrifuge the obtained unlabeled cell suspension from step 3.5 for 10 min at 300 x *g* at 4 °C.

3.6.3. Remove the supernatant completely. Resuspend the pellet in 90 µL of MCS buffer and add 10 µL of CD31 microbeads (per 10^7 total cells or less). Mix the whole suspension and incubate for 15 min in the fridge at 4 - 8 °C.

3.6.4. Add 1 mL of MCS buffer and centrifuge at 300 x *g* for 10 min at 4 °C. Take off the supernatant and resuspend the cells in 500 µL of MCS buffer.

3.6.5. For the magnetic cell separation, use a medium magnetic column (MMC) with a reservoir volume of 3.5 mL and a capacity of up to 2×10^8 total cells.

3.6.6. Position the MMC in the magnetic field of the separator. Rinse the MMC with 500 µL of MCS buffer. After rinsing, place a 15 mL conical tube below the column to collect the flow through.

3.6.7. Apply the whole cell suspension (500 µL) onto the column and let it completely flow through.

3.6.8. Wash the column 3x by adding 500 µL of MCS buffer, and wait until the column's reservoir is empty before performing the next washing step. Unlabeled cells passing the column represent the $CD45^-$ $CD31^-$ fraction. Store them for further quality controls.

3.6.9. Remove the column from the separator and place it on a suitable collection tube (15 mL tube). Pipette 2 mL of MCS buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. This $CD45^-$ $CD31^+$ fraction represents the enriched primary murine ECs.

3.6.10. Centrifuge the cell suspension for 5 min at 350 x *g* at 20 °C. Remove the supernatant completely and resuspend the cells (expect around 0.6 x 10^6 cells per gram of muscle tissue) in 1 mL of ECM (see step 2.2). Transfer the cells (0.5 x 10^6 to 0.7 x 10^6 cells) to a single well of a coated 6-well culture plate (from step 2.3) containing 1 mL of ECM.

3.7. For cultivation, incubate the cells at 37 °C and 5% CO_2 in a sterile incubator. Refresh the ECM every 2 - 3 days.

4. Primary Murine MMEC Purification

4.1. Perform a second cycle of $CD31^+$ accumulation, following the manufacturer's instructions (CD31 microbeads protocol; see step 3.6).

4.1.1. Detach the cells with trypsin/EDTA solution when they are at 80% - 90% confluence (usually after 7 days). For this, rinse each well with 2 mL of sterile PBS, in two consecutive washing steps. Use 800 µL of trypsin/EDTA solution per well of a 6-well plate and incubate at 37 °C and 5% CO_2 in a sterile incubator for 3 - 5 min. Stop enzymatic activity by using 1,200 µL of DMEM containing a minimum of 10% FCS.

4.1.2. Centrifuge the cell suspension for 5 min at 350 x *g* at 20 °C.

4.1.3. Perform the second CD31-MCS step to increase the purity (according to the instructions of the CD31 microbeads' manufacturer; see step 3.6).

4.1.4. Observe cell confluence *via* bright-field or standard phase-contrast microscopy using a 20X magnification and 0.35 lens numerical aperture. See **Figure 1**.

NOTE: The cells can be used for respective experiments or kept in culture by passaging. Use the cells for experiments promptly, in lower passages. It is not recommended to use the cells after passages 8 - 10.

5. Quality Control

5.1. Perform a flow cytometry of primary murine MMECs after the second CD31-MCS step.

5.1.1. Prepare a fluorescence-activated cell sorting (FACS) tube by adding 1 mL of flow cytometry (FC) buffer (see **Table of Materials**).

5.1.2. Detach the cells with trypsin/EDTA solution when they are at 100% confluence (usually after 14 days). For this, rinse each well with 2 mL of sterile PBS, in two consecutive washing steps. Use 800 µL of trypsin/EDTA solution per well of a 6-well plate and incubate at 37 °C and 5% CO_2 in a sterile incubator for 3 - 5 min. Stop the enzymatic activity by using 1,200 µL of DMEM containing 10% FCS.

5.1.3. Determine the cell number, using a Neubauer cell counting chamber, and add a minimum of 1 x 10^5 cells to the prepared FACS tube.

5.1.4. Centrifuge the cell suspension for 10 min at 300 x *g* at 4 °C. Perform two washing steps by removing the supernatant, resuspending the cells in 1 mL of FC buffer, and centrifuging for 10 min at 300 x *g* at 4 °C.

5.1.5. Prepare a staining mix by adding anti-mouse CD31-FITC antibody (1:100) and anti-mouse CD45-PE (1:100) with FC buffer.

5.1.6. Resuspend the cells in 100 µL of staining mix in the FACS tubes. Incubate for 30 min at 4 °C. Add 1 mL of FC buffer. Centrifuge the cell suspension for 5 min at 300 x *g* at 4 °C. Carefully remove the supernatant and resuspend the cells in 200 µL of FC buffer.

5.1.7. Measure the cells in a flow cytometer, following the gating strategy as shown in **Figure 2A**.

5.2. Alternatively, perform immunofluorescence staining of primary murine MMECs after the second CD31-MCS step.

5.2.1. Coat the coverslips.

5.2.1.1. Transfer a sterile 13 mm-diameter round glass coverslip into a well of a 24-well plate. Coat the coverslip by adding 300 µL of speed-coating solution per well. Incubate for 3 - 5 min at RT.

5.2.1.2. Aspirate and discard the coating solution. Rinse each well with 2 mL of sterile PBS in two consecutive washing steps. Aspirate and discard the PBS.

5.2.1.3. Seed 1 x 10^5 cells in 1 mL of ECM on the coated coverslip and incubate at 37 °C and 5% CO_2 in a sterile incubator until the cells are 99% confluent.

5.2.2. Perform fixation and immunofluorescence staining.

5.2.2.1. Remove the ECM medium of the cultivated cells from step 5.2.1.3.

5.2.2.2. Fix the cultured cells by adding 300 µL of 4% paraformaldehyde (PFA) with a pH of 7.4 per well, for 10 min at 4 °C.

CAUTION: PFA is toxic and must be handled with care.

5.2.2.3. Perform three washing steps by adding 1 mL of PBS per well, and remove the supernatant after 5 min at RT.

5.2.2.4. Prepare a blocking solution containing 5% bovine serum albumin (BSA), 0.2% Triton-X, and 1% goat serum in PBS.

5.2.2.5. Add 300 µL of blocking solution to each well and incubate for 1 h at RT.

5.2.2.6. Remove the supernatant and add 200 µL per well of an anti-PECAM-1 (CD31) antibody (1:200) in 5% BSA and 1% goat serum in PBS, and incubate at 4 °C overnight.

5.2.2.7. Perform three washing steps by adding 1 mL of PBS per well and removing the supernatant after 5 min at RT in each step.

5.2.2.8. Prepare a secondary antibody solution containing Cy3-conjugated anti-rat IgG antibody (1:500) in 1% BSA and PBS. Add 300 µL per well of the secondary antibody solution and incubate for 1 h at RT in the dark.

5.2.2.9. Perform three washing steps by adding 1 mL of PBS per well and removing the supernatant after 5 min at RT in each step.

5.2.3. Carefully take out the round glass coverslips with forceps and transfer them to a microscopy slide. Add an appropriate amount of mounting medium containing 4',6-diamidino-2-phenylindol (DAPI) on the cell layer and carefully put a cover glass on it (see **Table of Materials**).

5.2.4. Observe the cells with an appropriate fluorescence microscope equipped with a fluorescence light source (120 W) and two filter sets: an excitation/emission filter set with 550/25 nm and 605/70 nm wavelengths to detect Cy3 540/562 nm.

5.2.5. Use a second filter set with an excitation/emission wavelength of 365/50 nm and 445/50 nm to detect DAPI 350/440 nm. Use a magnification of 40X and 80% intensity of 14.5 mW/cm^2 for DAPI with an acquisition duration of 30 ms. For the detection of Cy3, use 80% of 67.5 mW/cm^2 with an acquisition duration of 60 ms.

5.3. Perform quantitative PCR.

5.3.1. Isolate mRNA.

5.3.1.1. Follow standard procedures, using an acid guanidinium thiocyanate-phenol (AGTP) reagent to isolate mRNA. Use a minimum of 0.5 x 10^6 cells from the CD45$^-$ CD31$^-$ (step 3.6.8) or CD45$^-$ CD31$^+$ (step 3.6.9) fractions and cultivated primary murine MMECs (step 4.1.3).

5.3.1.2. Centrifuge the cell suspension for 10 min at 300 x *g* at 20 °C. Take off the supernatant completely. Resuspend the cell pellet in 500 µL of AGTP reagent and transfer the cell suspension to a 2 mL reaction tube. Incubate for 5 min at RT.

5.3.1.3. Add 100 µL of chloroform and shake vigorously for 15 s. Incubate for 3 min at RT.

5.3.1.4. Centrifuge the suspension for 15 min at 12,000 x *g* at 4 °C.

5.3.1.5. Carefully take off the upper aqueous phase (containing RNA) and transfer it to a 1.5 mL reaction tube. Add 250 µL of isopropanol and incubate for 10 min at RT.

5.3.1.6. Perform a centrifugation step at 12,000 x *g* for 10 min at 4 °C.

5.3.1.7. Take off the supernatant and add 1 mL of 75% ethanol carefully. Centrifuge at 7,500 x *g* for 5 min at 4 °C.

NOTE: The pellet is easy to lose.

5.3.1.8. Remove the supernatant completely. Let the pellet dry on air for 5 - 10 min.

NOTE: Ethanol needs to be removed, but do not dry the pellet too much.

5.3.1.9. Dissolve the pellet in 30 µL of diethylpyrocarbonate-treated water and heat it for 10 min at 55 °C.

NOTE: The RNA concentration and purity can be measured by a spectral photometer.

5.3.2. Perform cDNA synthesis (reverse transcriptase PCR).

5.3.2.1. Prepare a master mix containing 10 µL of PCR buffer (5x), 2.5 µL of deoxyribonucleoside-triphosphate (dNTP [10 mM]), 0.5 µL of a random mixture of single-stranded primer (0.2 µg/µL), 1 µL of RNase inhibitor (40 U/µL), 0.4 reverse transcriptase (200 U/µL), and 5.6 µL of diethylpyrocarbonate-treated water (see **Table of Materials)**.

5.3.2.2. Dilute 500 ng of RNA in 30 µL of diethylpyrocarbonate-treated water in 0.2 mL PCR tubes and add the whole 20 µL of the master mix.

5.3.2.3. Use a PCR cycler to perform the following steps: 10 min at 25 °C, 30 min at 50 °C, 5 min at 85 °C, and hold at 4 °C.

5.4. Perform quantitative PCR (qPCR).

NOTE: Perform the qPCR of the samples in duplicates or triplicates.

5.5. Use a primer with two different fluorescence dyes. Use a primer with an absorption of 495 nm and an emission of 517 nm for the gene of interest.

5.6. For the detection of the satellite cell marker gene expression, use primers for paired box protein 7 (*Pax7*) and M-cadherin (*Cdh15*) (see **Table of Materials**).

5.7. For the detection of the endothelial cell marker gene expression, use primers for claudin-5 (*Cld5*), occludin (*Ocln*), and zonula occludens-1 (*Tjp1* or *ZO1*) (see **Table of Materials)**.

5.8. As a reference gene for 18S rRNA, use a primer with an absorption of 538 nm and an emission of 554 nm in wavelength.

5.9. Prepare a qPCR master mix for each gene of interest with the respective primer. The master mix contains 10 µL of qPCR buffer (2x), 1 µL of the primer for the gene of interest (10 µM), 1 µL of the primer for 18S rRNA, and 4 µL of nuclease-free water.

5.10. Add 16 µL of the master mix to a single well of a 96-well reaction plate. Add 4 µL of cDNA (obtained from step 5.3.2) per well containing the master mix.

5.11. Use a real-time PCR cycler, performing the following steps: the holding stage with 50 °C for 2 min and 95 °C for 10 min and the cycling stage with 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

REPRESENTATIVE RESULTS:

One day after isolation, primary murine MMECs and residual other cells form conglomerates and adhere to the bottom of culture dishes (**Figure 1A**, day 1). From day 7 onward, flat and elongated cells can be observed. However, contamination of other, mostly spheroid cells, is still visible (**Figure 1A**, day 7). Thus, another cycle of CD31 positive selection *via* MCS is required. Hereafter, primary murine MMECs proliferate to a density of approximately 80% - 90%. Upon confluence, they typically form a nonoverlapping monolayer of longitudinally aligned cells (**Figure 1A**, day 14). Proliferation stops upon confluence due to contact inhibition. After 14 days, about 5 x 10^5 to 10 x 10^5 cells can be used for further investigations.

Quality control *via* flow cytometry, using fixable viability dye FVD780 (to stain for dead cells), PECAM1 (to stain for CD31), and PTPRC (to stain for CD45, as a marker for cells of hematopoietic origin), showed values for both viability and purity ranging around 70% each for cells immediately after the isolation (**Figure 2A**). Cells cultivated after another CD31 positive selection *via* MCS showed satisfying values for purity, as well as for viability, ranging up to 95% each (**Figure 2B**).

To evaluate the accuracy of the selection steps, obtained cells were further investigated for the gene expression of the muscle satellite cell marker genes paired box protein 7 (*Pax7*) and M-cadherin (*Cdh15*) on mRNA level by quantitative PCR (qPCR). Primary murine MMECs (pmMMEC) and differentiated primary murine muscle cells (pmMC) were used as negative and positive control, respectively. Murine muscle cells were commercially purchased. As expected, only the $CD45^-$ $CD31^-$ fraction, as well as the pmMC, expressed *Pax7* and *Cdh15*, whereas $CD45^-$ $CD31^+$ and the primary murine MMECs were negative for these markers (**Figure 2C**).

ECs derived from capillaries in the endomysium of skeletal muscle express tight junction proteins[4,5]. By using qPCR, the expression of claudin-5 (*Cld5*), occludin (*Ocln*), and zonula

occludens-1 (*Tjp1* or *ZO1*) of confluent primary murine MMECs after the second CD31-MCS step was evaluated. PmMC were used as controls (**Figure 2D**). Primary murine MMECs expressed high levels of *Cld5*, *Ocln*, and *Tjp1*, whereas pmMC only show a low expression of *Tjp1*. Furthermore, immunofluorescence staining as quality control confirmed the surface expression of the endothelium-specific marker PECAM1 in primary murine MMECs (**Figure 2E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Morphology of primary murine MMECs. (**A**) Representative image of cultured primary murine MMECs by phase-contrast microscopy from days 1 to 14. d1 = day 1, d7= day 7, d14 = day 14.

Figure 2: Quality control of primary murine MMECs. Gating strategy for flow cytometric analysis of primary murine MMECs (**A**) immediately after isolation and (**B**) on day 15, using (I) FSC/SSC, (II) FVD780, and (IIIa) CD45 and (IIIb) CD31 (dashed lines = isotype control). (**C**) Expression level of *Pax7* and M-cadherin (*Cdh15*) in $CD45^-$ $CD31^-$ and $CD45^-$ $CD31^+$ fractions after MCS isolation, as well as in primary murine MMECs (pmMMEC) and primary murine muscle cells (pmMC). Expression levels are shown as ΔC_t values (sample–18S rRNA); n.d. = not determined. (**D**) Expression levels of tight junction proteins claudin-5 (*Cldn5*), occludin (*Ocln*), or zonula occludens-1 (*Tjp1* or *ZO-1*) of pmMMEC and pmMC, shown as ΔC_t values. (**E**) Immunofluorescence staining for PECAM1 (red) in cultivated primary murine MMECs, 24 h after the second CD31 positive selection. Nuclear staining with DAPI-containing (blue) mounting medium; left: anti-PECAM1/DAPI, right: negative control/DAPI.

DISCUSSION:

Microvascular ECs provide barrier functions in all tissues, and their dysfunction results in the disease of the associated organs[3]. Moreover, organ-specific studies of microvascular ECs could pave the way for new therapeutic strategies. Therefore, a deeper understanding of microvascular EC function under physiological and pathophysiological conditions is of great scientific interest. The modulation of leukocyte/endothelium interaction is successfully used to treat multiple sclerosis patients with natalizumab, an antibody which prevents lymphocyte adhesion and, thereby, transmigration[6]. However, various myopathies, including the inflammatory subtypes, still remain only poorly treatable to date. Therefore, the elucidation of EC properties, specifically in skeletal muscle endothelium, could help to expand available treatments to myopathy or result in novel therapeutic approaches.

Immortalized endothelial cell lines (IECLs) have been established and used for several *in vitro* models. These cell lines offer some advantages compared to primary microvascular ECs due to immortalization and fast proliferation. Furthermore, primary cultured cells can undergo senescence and lose or change their morphology or physiological function after some time or passages. However, IECLs only maintain some endothelial cell properties and cannot resemble the variety of different organ-borne primary microvascular ECs. Of note, they are rarely derived from skeletal muscles. To date, only a single publication describes a human skeletal microvascular endothelial cell line, named TSM15[5]. In contrast, murine microvascular ECs have not been

described so far. Finally, primary cells from transgenic animals provide the opportunity to study genetic modifications *in vitro*. However, primary cell cultures also hold certain pitfalls. First, contamination with cells of no interest can interfere with the validity of experimental findings. Therefore, the accuracy of several selection steps and a high purity of the isolated cells must be guaranteed. Cell suspension after dissociation of muscle tissue contains erythrocytes and different mononuclear cell types, such as immune cells, satellite cells, pericytes, fibroblasts, and ECs[7]. Therefore, cell fractions after the CD31-MCS step were tested for markers exclusively expressed by muscle satellite cells. As expected, $CD45^-$ $CD31^-$ fraction and primary muscle cells showed *Pax-7* and *Cdh15* expression, whereas $CD45^-$ $CD31^+$ and cultivated primary murine MMECs were negative for these markers after a second CD31-MCS step. Furthermore, a quality control of isolated or cultured cells is inevitable, to guarantee reliable and reproducible experiments. Several methods for quality control of primary murine MMECs are available: besides the microscopy of morphological properties, flow cytometry, PCR, and immunofluorescence staining for characteristic EC markers (*e.g.*, CD31, Sca-1) can be used. The purity assessment of freshly isolated primary murine MMECs was only about 60% - 70%, whereas a viability of about 70% could be detected. The microscopy of these cells partially shows cell conglomerates, which might consist of contaminating cells such as fibroblasts or pericytes attached to ECs. After 7 days, mainly flat and elongated cells and spheroid cells can be observed. However, two CD31-MCS passages resulted in a high viability and purity. Alternative approaches to increase purity, such as puromycin-containing media, failed in primary murine MMECs while being effective in murine brain microvascular ECs[8].

Since microvascular ECs derived from capillaries express tight junction proteins, the gene expression of *Cld5*, *Ocln*, and *Tjp1* in primary murine MMECs compared to differentiated muscle cells was examined. As expected, all tight junction molecules were detected in primary murine MMECs. In comparison, muscle cells demonstrated only a low expression of *Tjp1*, whereas no expression of *Cld5* and *Ocln* could be determined. Similar expression patterns were previously demonstrated in whole murine skeletal muscle. Here, an expression on gene and protein level could be detected for *Tjp1* but not for occludin[9]. Further functional features, such as transendothelial resistance, could be measured.

The herein described protocol features only the isolation of microvascular ECs from murine muscle tissue. However, it might be transferred to various other species as, for example, a similar protocol was published for rat microvascular ECs derived from epididymal fat pads. Here, a CD31 positive selection *via* MCS was preceded by density gradient centrifugation[10]. Similar approaches were successful in macrovascular EC isolation from rat femoral arteries[11]. A recently published method to isolate microvascular ECs of heart and lung tissue uses CD31 and endoglin (CD105) as antigens for MCS[12]. However, the purity of ECs could not be increased further by using additional CD105 magnetic bead separation. Another possibility to isolate and purify primary murine MMECs is multicolor FACS. A distinct population of Sca-1^+, $CD31^+$, $CD34^{dim}$, and $CD45^-$ cells from murine muscles could be isolated and was characterized as primary murine MMECs[13]. An advantage of this method is a pure EC population which can be directly used or cultured for further experiments (note that FACS leads to increased cell death due to higher cell stress). Further, dead cells or conglomerates of different cell types can significantly reduce the isolated

cell numbers by FACS. Additionally, it is reported that primary murine MMECs isolated by FACS were unsuccessfully cultured at commonly used culture conditions of 21% O_2 and 5% CO_2. Here, the oxygen level had to be adjusted to 5% for a sufficient cultivation[13]. Moreover, the FACS technique is more complex and time-consuming, and the infrastructural prerequisites are expensive.

The musculus quadriceps femoris and the musculus triceps surae of male mice in the age of 4 - 12 weeks are most suitable for the isolation of primary murine MMECs as they are easily accessible and large enough for the isolation of sufficient cell numbers (5×10^5 to 10×10^5 per gram of muscle). Therefore, it must be ensured that conditions are comparable in independent experiments. As described above, primary cells can undergo senescence. Hence, use cells for respective experiments promptly, in lower passages. After passages 8 - 10, primary murine MMECs change their morphology, demonstrate slow proliferation rates, and lose their contact inhibition as signs of dedifferentiation and senescence.

Further, there are some notes about troubleshooting for this protocol. Sterile working is essential to avoid contaminations. Quality controls are necessary to monitor the purity and viability of isolated cells. Used materials in this protocol must be stored and applied according to the manufacturer's instructions. Additionally, the age and sex of the used animals, as well as different muscle groups, might influence the quality and comparability of the isolated cells in experiments.

The described protocol should be considered as a platform method to isolate the primary microvascular ECs of skeletal muscles. Isolated cells can be used for several applications to gain further insights into blood-muscle-barrier function. The cells are suitable for both protein and RNA expression studies, as well as functional assays (including transmigration and adhesion studies). However, this protocol was optimized for studies focusing on inflammatory processes, and hence, slight modifications might be necessary with respect to different research areas.

To model the complex spatial and functional cellular interactions within the MVU, primary murine MMECs can be cultivated in more complex three-dimensional cell culture systems together with other cell types. Prospectively, it should also be possible to generate MVU organoids as it already has been described for other tissues[14]. However, the successful isolation of primary murine MMECs is vital when aiming for this next step.

ACKNOWLEDGMENTS:
This work was supported by the "Else Kröner-Fresenius-Stiftung" (2018_A03 to T.R.), "Innovative Medizinische Forschung (IMF) Münster" (I-RU211811 to T.R.), and the German Research Foundation (DFG, INST 2105/27-1, ME 3283/5-1, and ME 3283/6-1 to S.G.M.).

DISCLOSURES:
The authors have nothing to disclose.

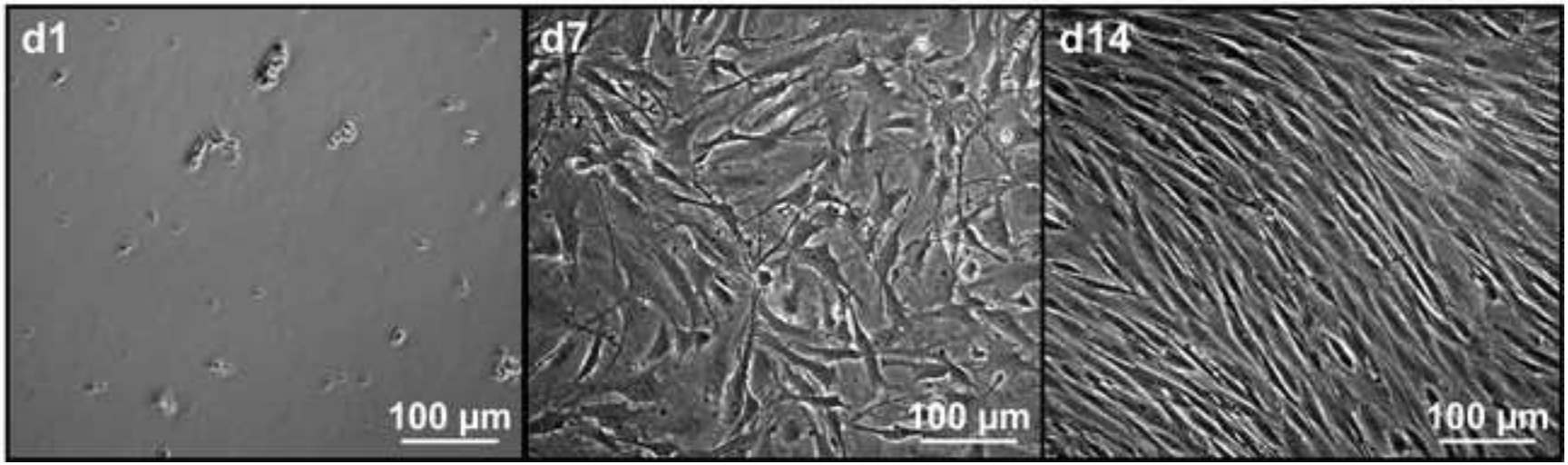
REFERENCES:

1. Rodrigues, S.F., Granger, D.N. Blood cells and endothelial barrier function. *Tissue Barriers*. **3** (1-2), e978720 (2015).
2. Michiels, C. Endothelial cell functions. *Journal of Cellular Physiology*. **196** (3), 430-443 (2003).
3. Pierce, R.W., Giuliano Jr., J.S., Pober, J.S. Endothelial cell function and dysfunction in critically ill children. *Pediatrics*. **140** (1), 20170355 (2017).
4. Nasdala, I. *et al.* A transmembrane tight junction protein selectively expressed on endothelial cells and platelets. *Journal of Biological Chemistry*. **277** (18), 16294-16303 (2002).
5. Sano. H. *et al.* Establishment of a new conditionally immortalized human skeletal muscle microvascular endothelial cell line. *Journal of Cellular Physiology*. **232** (12), 3286-3295 (2017).
6. Kappos, L. *et al.* Natalizumab treatment for multiple sclerosis: Updated recommendations for patient selection and monitoring. *Lancet Neurology*. **10** (8), 745-758 (2011).
7. Birbrair, A. *et al.* Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Research*. **10** (1), 67-84 (2013).
8. Ruck, T., Bittner, S., Epping, L., Herrmann, A.M., Meuth, S.G. Isolation of primary murine brain microvascular endothelial cells. *Journal of Visualized Experiments*. (93), e52204 (2014).
9. Hwang, I. *et al*. Tissue-specific expression of occludin, zona occludens-1, and junction adhesion molecule A in the duodenum, ileum, colon, kidney, liver, lung, brain, and skeletal muscle of C57BL mice. *Journal of Physiology and Pharmacology*. **64** (1), 11-18 (2013).
10. Frye, C.A., Patrick Jr., C.W. Isolation and culture of rat microvascular endothelial cells. *In Vitro Cellular & Developmental Biology - Animal*. **38** (4), 208-212 (2002).
11. Le, G.Y., Essackjee, H.C., Ballard, H.J. Intracellular adenosine formation and release by freshly-isolated vascular endothelial cells from rat skeletal muscle: Effects of hypoxia and/or acidosis. *Biochemical and Biophysical Research Communications*. **450** (1), 93-98 (2014).
12. Cheung, K.C., Marelli-Berg, F.M. Isolation of microvascular endothelial cells. *Bio-protocol*. **8** (12) (2018).
13. Ieronimakis, N., Balasundaram, G., Reyes, M. Direct isolation, culture and transplant of mouse skeletal muscle derived endothelial cells with angiogenic potential. *PLoS One*. **3** (3), e0001753 (2008).
14. Pham, M.T. *et al.* Generation of human vascularized brain organoids. *Neuroreport*. **29** (7), 588-593 (2018).

Figure

Click here to access/download;Figure;JoVE figure 1.tif

A



Figure

Click here to access/download;Figure;JoVE figure 2.tif

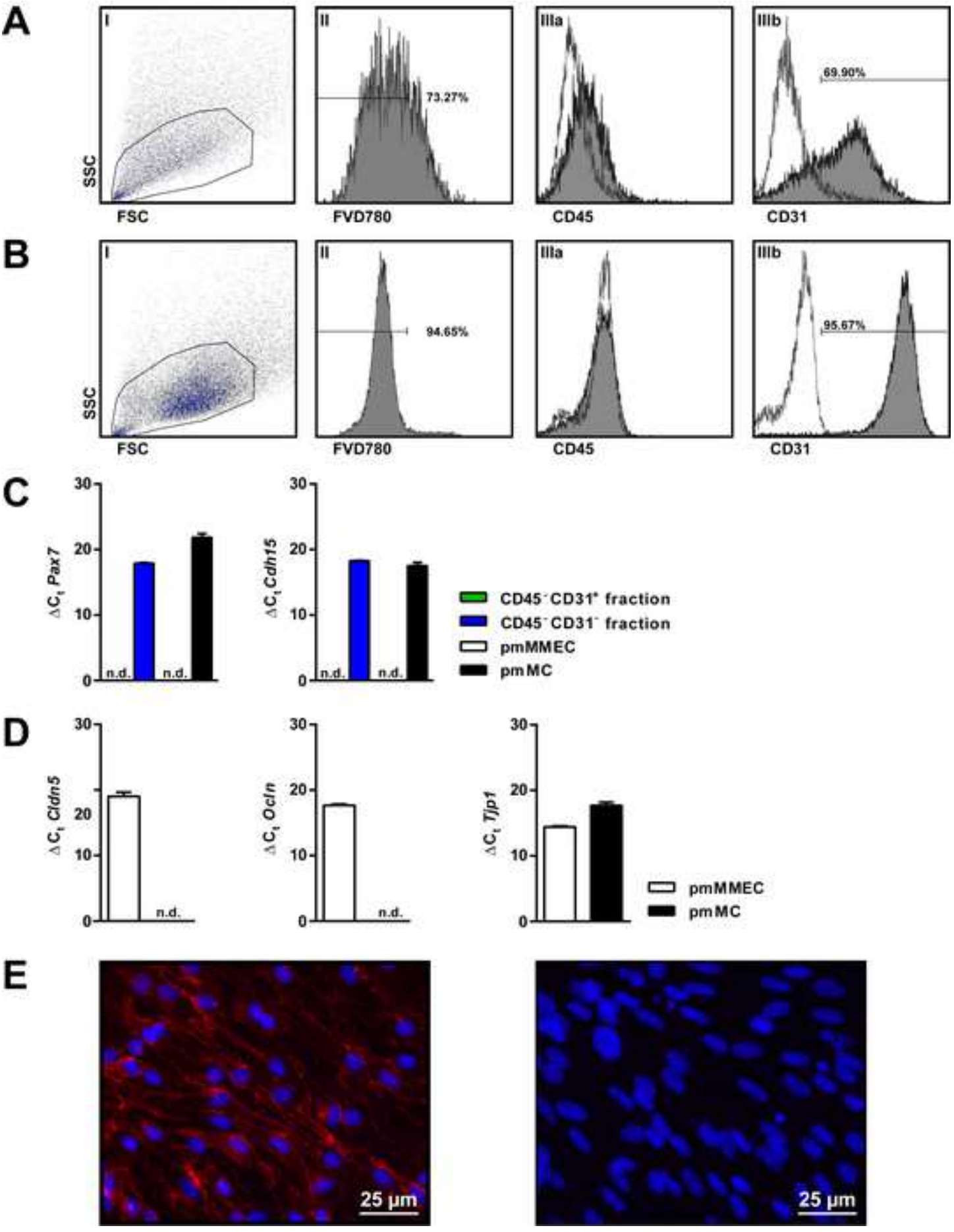


Table of Materials

Click here to access/download;Table of Materials;JoVE_primary_murine_MMEC_Table_of_materials_revision2.xlsx

Name of Material/ Equipment	Company	Catalog Number
0,25% Trypsin-EDTA	Thermo Fisher	25200-056
ACK buffer		
Anti-mouse CD31-FITC (clone MEC13.3)	Biolegend	102506
Anti-mouse CD45-PE (clone 30-F11)	Biolegend	103106
bFGF	Peprotech	100-18B
BSA	Sigma Aldrich	A4503
CD31 MicroBeads mouse	Miltenyi Biotec	130-097-418
CD45 MicroBeads mouse	Miltenyi Biotec	130-052-301
Collagenase-Dispase	Roche	10269638001
Corning Costar TC-Treated Multiple 6-Well Plates	Corning	3516
Cy3-conjugated anti-rat IgG antibody	dianova	712-166-153
DAPI (ProLong Gold antifade reagent with DAPI)	Thermo Fisher	P36935
Desoxyribonuclease	Sigma Aldrich	D4513
Diethylpyrocarbonat treated water	Thermo Fisher	AM9916
DMEM, containing Glutamin Supplement and pyruvate	Thermo Fisher	31966-021
dNTP Mix (10 mM)	Thermo Fisher	R0192
EDTA	Sigma Aldrich	E5134
FACS tubes	Sarstedt	551,579
Falcon 70 µm Cell Strainer	Corning	352350
FC buffer		
Fetal calf serum	Sigma Aldrich	F6178
Fixable Viability Dye eFluor780	Thermo Fisher	65-0865-14
Forceps (serrated, straight, 12 cm)	Fine Science Tools	11002-12
Forceps (serrated, straight, 12 cm)	Fine Science Tools	11009-13
Insulin syringe 100 Solo 1ml (Omnifix)	Braun	9161708V
large magnetiv columns (LS columns)	Miltenyi Biotec	130-042-401
MCS buffer		
Medium magnetic column (MS column)	Miltenyi Biotec	130-042-201
Nuclease free water	Thermo Fisher	R0581
PBS	Sigma Aldrich	
PCR buffer (5x)	Thermo Fisher	EP0742

Pecam1 rat α-mouse	SantaCruz	Sc-52713
Penicillin-Streptomycin	Sigma Aldrich	P4333
primary murine muscle cells	celprogen	66066-01
Primer Cdh15 (M-Cadherin)	Thermo Fisher	Mm00483191_m1
Primer Cldn5 (claudin-5)	Thermo Fisher	Mm00727012_s1
Primer Ocln (occludin)	Thermo Fisher	Mm00500912_m1
Primer Pax-7	Thermo Fisher	Mm01354484_m1
Primer Tjp-1 (Zonula occludens 1)	Thermo Fisher	Mm00493699_m1
Primer 18s rRNA (Eukaryotic endogenous control)	Thermo Fisher	4310893E
qPCR buffer (Maxima Probe/ROX qPCR Master Mix (2X)	Thermo Fisher	K0231
Random mixture of single-stranded primer	Thermo Fisher	SO142
Reverse Transcriptase (200 U/µL) + PCR buffer (5x)	Thermo Fisher	EP0742
Rnase Inhibitor (40 U/µL)	Thermo Fisher	EO0381
Scissor (cutting edge 23 mm, sharp/sharp))	Fine Science Tools	14088-10
Scissor (cutting edge 42 mm, sharp/blunt)	Fine Science Tools	14001-13
Speed Coating solution	PeloBiotech	PB-LU-000-0002-00

	Comments/Description
	ready to use
	150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in water at a pH of 7.3
	Isotype control: FITC Rat IgG2a, κ Isotype Ctrl
	Isotype control: PE Rat IgG2b, κ Isotype Ctrl
	Basic fibroblast growth factor
	Collagenase from *V. alginolyticus,* Dispase from *B. polymyxa*
	Deoxyribonuclease I from bovine pancreas
	warm up to 37 °C before use
	1 mL
	0.1% BSA, 0.2% NaN_3, 2 mM EDTA
	Fetal calf serum
	for CD45-MACS-step
	0.5% BSA, 2 mM EDTA in PBS at a pH of 7.2
	for CD31-MACS-step
	Phosphate buffered saline, ready to use
	in a kit with the reverse transcriptase

100 µg/mL

FAM labeled
FAM labeled
FAM labeled
FAM labeled
FAM labeled
VIC labeled
2 x 1,25 mL; for 200 reactions each
Random Hexamer Primer

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Isolation of primary murine skeletal muscle microvascular and otheliad cells

Author(s): Thomas Müntefering, Alexander P.E. Michels, Steffen Pfuetzer, Steven G. Meuth, Tobias Ruck

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

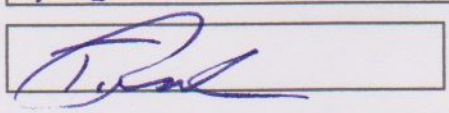
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Tobias Ruck
Department:	Department of Neurology
Institution:	Institute for Translational Neurology
Title:	MD
Signature:	
Date:	02. 08. 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

University Hospital Muenster . Department of Neurology . 48129 Münster

Department of Neurology

Univ.-Prof. Prof. h. c. Dr. med. Heinz Wiendl
Director

with

Institute of Translational Neurology
Univ.-Prof. Dr. med. Dr. rer. nat. Sven Meuth

Dr. med. Tobias Ruck

Albert-Schweitzer-Campus 1, Building A1
48149 Münster

T +49 (0)2 51 - 83 – 41125

F +49 (0)2 51 - 83 - 4 68 12

JoVE
Alisha DSouza
Senior Editor

Münster, 17 October 2018

Dear Dr. DSouza,

we would like to thank you for considering our manuscript for publication and your positive and constructive feedback. We are now happy to submit a 2nd revised version of our manuscript „**Isolation of primary murine skeletal muscle microvascular endothelial cells**" by Müntefering et al. to the *Journal of Visualized Experiments.* In this revision following key points were addressed:

1. We modified the structure of the manuscript and removed all trademarked names.
2. Protocols for mRNA Isolation, cDNA synthesis and qPCR were added.
3. Further details for the different steps are now provided increasing comprehensibility of the protocol.
4. Text indicated in blue fonts shows changes in the manuscript.
5. We highlighted steps (3 and 4) in yellow for filming.

We think that we were able to sufficiently address all your editorial comments (for details see point-to-point reply below) and are looking forward to hearing from you in due course.

Please do not hesitate to contact me in case of any additional questions.

Sincerely,
Tobias Ruck, MD

Point-to-point-reply

Editorial comments (EC):

EC1: MACS is a trademarked name and has been replaced with MCS throughout.

➔ *We totally agree.*

EC2: The structure and language has been modified slightly to meet JoVEs style requirements. We have highlighted portions for filming.

➔ *We agree.*

EC3: After anesthetization? Mention anesthesia technique and any drug dosage

➔ *Mice are euthanized without any anesthesia. The aim is to quickly separate the spinal cord from the brain so as to provide the animal with a fast and painless death.*

EC4 How is sterility maintained? Are tools sterilized? Is the surgical area prepped? Mention animal position.

➔ *Sterile working is maintained by working under a sterile laminar flow hood directly after severing of the extremities. Surgical instruments are disinfected by 70% ethanol. Animal position is now described.*

EC5: How? Mention surgical tools used?

➔ *We now specify how to sever the leg and which tools are required.*

EC6: How? Unclear exactly what is done. Please mention tools used.

➔ *Large vessels can only be removed by isolating parts of the muscle which do not contain large vessels. We clarified this point in the protocol.*

EC7: Please tools used for isolation.

➔ *We added all used surgical tools and describe how to use them for the isolation of the muscle groups.*

EC8: Cubes?

➔ *Ideally the muscle is cut into cubes with an edge length of >2 mm.*

EC9: How many pieces per well?

➔ *The muscle can be cut into more than 100 pieces. Important is the size of > 2mm^3 of the pieces.*

EC10: Dry weight? Or weight in PBS?

➔ *The protocol is designed for up to 1 g of dry weight muscle. Therefore, we added necessary steps to determine the dry weight of the muscle.*

EC11: How much per gram of tissue?

➔ *The volume of dissociation solution was added.*

EC12/EC18/EC27: How? Mention counting technique used.

➔ *To determine the cell number we used a Neubauer cell counting chamber. This is now indicated.*

EC13/EC17/EC19: Text indicated in red font shows significant overlap with previous publications or user manuals. Please use novel text.

➔ *We apologize for this mistake. We now use a novel text to describe the steps.*

EC14/EC20: What kind of column? Mention specifications and also add it to the table of materials.

➔ *To avoid trademarked names, we now describe the column specifications as reservoir volume and capacity for total cells to isolate.*

EC15: Define? Lymphocyte separation?

➔ *LS column has been replaced by large magnetic column (LMC) for a better understanding.*

EC16: How much?

➔ *Cell suspension volume is now described.*

EC21: Define.

➔ *The term MS columns is replaced by medium magnetic column (MMC) and defined by reservoir volume and capacity of total cells to isolate.*

EC22: How much? Are the unlabeled cells collected/discarded after this? It looks like you use them much later. Is there a step missing? Is this the cell suspension from 3.4.9?

➔ *We apologize for this misunderstanding. We amended the description to clarify the correct sequence of steps.*

EC23: How long after cell suspension application?

➔ *We now describe the washing steps after application of the cell suspension in more details.*

EC24: The appropriate amount or 2 mL? conflicting

➔ *We revised the conflicting description.*

EC25: Refresh?

➔ *„Change" was replaced by "refresh".*

EC26: Mention magnification and lens N.A.

➔ *Magnification and lens numerical aperture were added.*

EC28: Do you centrifuge again? Please mention the settings.

➔ *We describe the washing steps now in more details.*

EC29: Define this and also add it to the table of materials.

➔ *The FC buffer was described at the beginning of the steps5.1.1. and is defined in the table of materials.*

EC30: If this step is to be filmed, the gating strategy needs to be described in detail here. Please mention all software selections and button clicks.

➔ *This step should not be filmed. Only the isolation steps of the primary endothelial cells.*

EC31: WE require figures to be listed in the order that they are referenced. Please update the order of listing. Currently Figure 2 is called out before figure 1.

➔ *To avoid misunderstanding this information was deleted at this point.*

EC32: This sound come before 5.2.1, right?

➔ *We apologize for this misunderstanding. We describe these steps now in more details.*

EC33: Here you described how the plates are coated not cover slips. Please clarify.

➔ *The coating step is for cover slips. The paragraph was amended accordingly.*

EC34: Until what confluency%?

➔ *We added the percentage of confluence (=99%).*

EC35: After reaching what time point?

➔ *Cells are used after reaching 99% confluence. We clarified this.*

EC36: How much PFA per well?

➔ *The volume of PFA per well is now mentioned.*

EC37/EC38/ EC39/EC43: How much? / To each well?/ What is the volume added per well?

➔ *The volume for each well was added to the protocol.*

EC40/EC44: Please update

➔ *The washing steps were updated accordingly.*

EC41/EC42: Secondary?

➔ *Second antibody was replaced by secondary antibody.*

EC45: Mention microscope magnification, fluorescence excitation settings (wavelength and intensity), emission filter settings, acquisition duration etc.

➔ *We thank the editor for this suggestion and added the missing details.*

EC46: Please describe how this is done. If you use a kit, please say so and add the kit to the table of materials.

➔ *We inserted the missing steps for mRNA isolation.*

EC47: Collection of CD31- cells was not described.

➔ *We added the mentioned collection of the CD45$^-$ CD31$^-$ cell fraction to the respective step 3.6.14.*

EC48/EC50: Please describe the steps. Mention PCR primers and reaction conditions.

➔ *We added the missing steps and primer to perform the PCR.*

EC49: Please remove or replace the commercial name.

➔ *TaqMan probes were removed and replaced with by non-commercial names.*

EC51: What were the sources of these cells?

➔ *The cells were commercially purchased, the corresponding information was added to the table of materials.*

EC52: Define the color coding. What do blue and red stains represent?

➔ *Color coding was defined and added to the figure legend as well.*

EC53: References?

➔ *An appropriate reference was added.*