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| University Hospital Muenster . Department of Neurology . 48129 Münster  **JoVE**  **Alisha DSouza**  **Senior 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, 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 > 2mm3 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.*