MEDICAL UNIVERSITY OF VIENNA

Dr. Beate Lichtenberger

Principal Investigator
Skin & Endothelium Research Division (SERD)
Department of Dermatology
Medical University of Vienna

Anna Spiegel Center of Translational Research
Lazarettgasse 14, 1090 Vienna, Austria
Phone: +43 (0)1 40400-73809
Mobile: +43 69918205693
Email: beate.lichtenberger@meduniwien.ac.at
www.meduniwien.ac.at
www.lichtenbergerlab.org

Dr. Jaydev Upponi
Science Editor, Immunology and Infection
Editorial Department
Journal of Visualized Experiments (JoVE)
1 Alewife Center, Suite 200
Cambridge, MA 02140, USA
Email: jaydev.upponi@jove.com

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Dear Drs. Upponi and Dsouza,

Many thanks again for inviting us to submit our manuscript entitled "Isolation of papillary and reticular fibroblasts from human skin by FACS sorting" (JoVE59372) to JoVE, and for giving us the opportunity to revise the manuscript.
We have changed the text and figures according to the editor's and reviewers' suggestions. Please find the point-by-point response to these comments attached. Our response is labelled in blue.

We hope that the manuscript is now acceptable for publication at JoVE and look forward to hearing from you soon.

Yours sincerely,

Beate Lichtenberger

POINT-BY-POINT RESPONSE

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the text thoroughly and corrected typos etc..
2. Please shorten the Summary to no more than 50 words.
The summary is now shorter than 50 words.
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: GlutaMAX, falcon, AmnioMAX, etc.
Commercial terms in the text have been replaced.
4. Please place the ethics statement (lines 434-437) before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
The position of the ethics statement has been changed accordingly.
5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Personal pronouns have been removed.
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.
We have changed the protocol according to the editor's suggestions and moved any discussion sentences from the protocol to the discussion.
7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
We have added more details wherever necessary.
8. Line 116: Please specify the source of skin piece epidermis.
The source has been added.
9. 4.3: Please specify the incubation temperature.
This has been specified.
10. 5.4: Please provide the composition of ACK Lysis buffer.
This has now been provided.
11. 8.1: Please specify the cell numbers.
Cell numbers have been added.
12. 8.3: At what temperature are the cells fixed?
At ambient temperature.
13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
The text for the filming has been highlighted.
14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
16. Figure 2: Please label different panels to facilitation their explanation in the figure legend.
This has been changed.
17. Please revise the Discussion to explicitly cover the critical steps within and protocol and any limitations of the technique.
The discussion has been revised.
18. References: Please do not abbreviate journal titles.
We have used the endnote output style provided by JoVE. Although in the style options the

term ‘use full journal name” is ticked, some Journal titles are abbreviated.
19. Table of Materials: Please provide lot numbers and RRIDs of antibodies, if available, and sort the items in alphabetical order according to the name of material/equipment.
The materials list has been changed accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a clear and useful protocol to isolate human skin fibroblasts from reticular and papillary dermis by enzymatic digestion followed by FACS (avoiding performing explant culture). It is a improvement from what is described so far in the field. Authors are experts who have troubleshooted a great new technique.
We are glad that this reviewer thinks our protocol is an improvement compared to other available methods, and are grateful for his/her minor comments, which we addressed to improve the manuscript.

Major Concerns:
No major concern.

Minor Concerns:

In the introduction regarding the structure of the skin, hypodermis should be mentioned.
This has been added.
Overnight storage of other layer samples could be clarified : floating on PBS or immersed in PBS?
This has been clarified in the revised manuscript.
Similarly, lanes 352/353, section 3.1 "place the epidermis/papillary dermis from 2.1. with the epidermis facing upward..." from 2.1 or from 1, correct?
This has been corrected.
Finally, in section 4.3 providing a ratio of weight to volume of digestion mix could be helpful
This has been added.

Reviewer #2:

Manuscript Summary:
The protocol presented by Korosec et al describes the step-wise isolation of human papillary and reticular fibroblast using FACS sorting. This is the first protocol to isolate distinct fibroblast populations with very high purity from human skin. Given the importance of fibroblasts in tissue homeostasis and disease, and analyzing human tissue, this protocol is highly valuable. The protocol itself is clearly explained and good to follow (the very few improvement suggestions are given below). Taken together, I recommend publication of this protocol in JoVE.
We are grateful to this reviewer for recommending publication of our manuscript and his/her comments and suggestions of how to improve the manuscript.

Major Concerns:
None.

Minor Concerns:

Suggestions for minor textual changes to further improve clarity:

Lines 117-120:
Authors wrote "Then slice the tissue into 5 mm wide strips in order to facilitate the penetration of the Dispase II solution (cf. 3. Separation of epidermis and dermis) into the tissue before putting the skin pieces into a petri dish if epidermis needs to be separated from the whole dermis. Please skip

2. Sectioning of human skin papillary and reticular layers with a dermatome and directly refer to 3. Separation of epidermis and dermis."

Suggested change:
Then slice the tissue into 5 mm wide strips before putting into a petri dish. This facilitates the penetration of the Dispase II (cf. 3. Separation of epidermis and dermis), and is used if the epidermis needs to be separated from the entire dermis. Please skip 2. Sectioning of human skin papillary and reticular layers with a dermatome and directly refer to 3. Separation of epidermis and dermis.

We have introduced these changes as suggested.

Line 124: Perhaps some readers would be concerned if cleaning the tissue with ethanol would cause lots of cell death in the tissue. Could you please make a comment here (if true) that the caused death of cells is minor (or something similar).

A comment concerning cell death has been added.

Line 152: In 3.1 it would add to clarity to say: 3.1. Prepare a 3 U/mL Dispase II solution in sterile 1xPBS and place the 5 mm skin stripes (from 1.), or the epidermis/papillary dermis (from 2.1.), with the epidermis facing upward in a 10 cm petri dish with 10 mL Dispase II solution....

This has been changed according to the reviewer's suggestion.

Line 168: It may be helpful to introduce "Dermal layer 1, 2 and 3 (or similar)" in the points 2.2, 2.3 and 3.2 (see below), so that here you could refer to: "Mince all dermal layers (Dermal layers 1, 2, and 3) separately with scissors/scalpel as thoroughly.....". This would have helped me not to search back in the text which exact steps/layers/tissue parts were meant.

For example:
2.2. Repeat 2.1. by adjusting the dermatome to a cutting thickness of 700 μm and slice the remaining dermis. Place upper slice which is defined as the upper reticular dermis ("Dermal layer 2") into a separate petri dish.

2.3. Scrape away the subcutaneous fat layer with a scalpel from the residual lower reticular dermal layer (> 1000 μm) and discard it. Collect the lower reticular dermis ("Dermal layer 3") in another petri dish.

3.2. After incubation, transfer the epidermis/papillary dermis to the dry lid of the petri dish and separate epidermis from upper dermis ("Dermal layer 1") with two forceps each holding the edge of either epidermis or dermis.

Alternatively (if this is an option for JoVE) you could show a simple cartoon marking the 3 layers.

We have added a cartoon showing the three dermal layers (Figure 2) and changed the text according to this reviewers suggestions.

Line 212: Since you give an exact time for the lysis, this statement may lead to confusions. Either it would need one more sentence like: you can adjust the time for erythrocyte lysis if it would appear incomplete; however, don't keep cells too long in buffer .... Also, I could not easily find the self-made ACK Lysis buffer (i.e. contents); could you please refer to it?

The contents of the ACK lysis buffer has been added and we have changed the text according to the suggestions.

Since it is apparent that the protocol is new/first time of its nature, it feels not necessary to use the word "novel" repeated times; a suggestion is to use it only in the abstract and remove elsewhere (lines 90, 103, 416), but this is of course free for the authors to decide.

We have removed repeated claims of novelty.