**Point-by-point responses**

**Editorial comments:**   
We thank the editors for considering our manuscript for publication and we have addressed the editors’ comments and made the following changes in the revised manuscript:

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

Our manuscript has been fully revised by an English translator, Mrs. Angela Swaine.

**2. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.**

We have now included a scale bar for the two images in Figure 1, and we have defined it in the corresponding figure legend.

**3. Figures 2 and 3: Please define the error bars in the figure legend.**

To follow the advice by the Editor, we have now defined the error bars, which indicate the standard deviation, in the figure legends.

**4. Table of Equipment and Materials: Please provide lot numbers and RRIDs of the antibody used, if available.**

To follow the advice by the Editor, we have now provided all lot numbers for the materials. However, the human CD133 microbeads kit (Miltenyi Biotec, ref. 130-050-801) is not available on the [Resource Identification Portal](https://scicrunch.org/resources) (RRIDs).

**5. Abstract: Please include a statement about the purpose of the method.**

To follow the advice by the Editor, we have included a statement about the purpose of our method in the abstract (line 46, page 1): “The purpose of our method is to isolate living cancer stem cells in autophagic state using LMPC.”

**6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.**

We have reviewed all the numbers and included a space between them and their corresponding unit.

**7. Please define all abbreviations before use (FITC, LS, etc.).**

We have now defined all the abbreviations before use.

**8. For culture media and buffer such as DMEM, PBS, etc., please spell out at first use and provide composition. If they are purchased, please cite the materials table.**

For culture media and buffers, we have now spelled out all names at the time of first use. We have also provided their detailed composition in the revised version of our manuscript:

* (line 114-133, page 2) : Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12): Glycine (18.75 mg/L), L-Alanine (4.45 mg/L), L-Arginine hydrochloride (147.5 mg/L), L-Asparagine-H2O (7.5 mg/L), L-Aspartic acid (6.65 mg/L), L-Cysteine hydrochloride-H2O (17.56 mg/L), L-Cystine 2HCl (31.29 mg/L), L-Glutamic Acid (7.35 mg/L), L-Histidine hydrochloride-H2O (31.48 mg/L), L-Isoleucine (54.47 mg/L), L-Leucine (59.05 mg/L), L-Lysine hydrochloride (91.25 mg/L), L-Methionine (17.24 mg/L), L-Phenylalanine (35.48 mg/L), L-Proline (17.25 mg/L), L-Serine (26.25 mg/L), L-Threonine (53.45 mg/L), L-Tryptophan (9.02 mg/L), L-Tyrosine disodium salt dehydrate (55.79 mg/L), L-Valine (25.85 mg/L), Calcium Chloride (CaCl2) (anhyd.) (116.6 mg/L), Cupric sulfate (CuSO4-5H2O) (0.0013 mg/L), Ferric Nitrate (Fe(NO3)3"9H2O) (0.05 mg/L), Ferric sulfate (FeSO4-7H2O) (0.417 mg/L), Magnesium Chloride (anhydrous) (28.64 mg/L), Magnesium Sulfate (MgSO4) (anhyd.) (48.84 mg/L), Potassium Chloride (KCl) (311.8 mg/L), Sodium Bicarbonate (NaHCO3) (1200.0 mg/L), Sodium Chloride (NaCl) (6995.5 mg/L), Sodium Phosphate dibasic (Na2HPO4) anhydrous (71.02 mg/L), Sodium Phosphate monobasic (NaH2PO4-H2O) (62.5 mg/L), Zinc sulfate (ZnSO4-7H2O) (0.432 mg/L), D-Glucose (Dextrose) (3151.0 mg/L), Hypoxanthine Na (2.39 mg/L), Linoleic Acid (0.042 mg/L), Lipoic Acid (0.105 mg/L), Phenol Red (8.1 mg/L), Putrescine 2HCl (0.081 mg/L), Sodium Pyruvate (55.0 mg/L), Thymidine (0.365 mg/L)
* (line 136-139, page 3) Phosphate Buffered Saline medium (PBS): The formulation for 1 liter is 8 g Sodium Chloride, 0.2 g Potassium Phosphate, monobasic, 1.15 g Sodium Phosphate, dibasic, and 0.2 g Potassium Chloride.
* (Line 177-182, page 4) B27-NeuroMix™ Supplement (50X), serum free : Biotin, DL Alpha Tocopherol Acetate, DL Alpha-Tocopherol, Vitamin A (acetate), BSA, fatty acid free Fraction V, Catalase, Human Recombinant Insulin, Human Transferrin, Superoxide Dismutase, Corticosterone, D-Galactose, Ethanolamine HCl, Glutathione (reduced), L-Carnitine HCl, Linoleic Acid, Linolenic Acid, Progesterone, Putrescine 2HCl, Sodium Selenite, T3 (triodo-I-thyronine). The concentration of the different components is confidential.

Purchased materials are cited in the table of Materials.

**9. 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.  
For example: KOVA, Miltenyi, B27-NeuroMix, Lipofectamine, etc.**

To follow the advice by the Editor, we have removed all commercial names in the revised version of the manuscript and replaced them by generic terms. We have also modified the Table of Materials and Reagents accordingly.

**10. Please add more details to your protocol steps. 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.**

In the revised version of the manuscript, we have added more detail to the protocol steps. In part 1, there are now 8 steps, with 3 new steps 1.3, 1.6 and 1.8. In part 2, there are now 7 steps, 2.3 and 2.2 being the new ones. In part 5, step 5.1 is a new step.

**11. 1.1: It is unclear how the dissociation is done. Please revise.**

After sampling from tumor xenograft, tissue dissociation is obtained after two successive steps: a first step through scraping using a policeman, and the second step through repeated aspirations using three sizes of pipettes successively, 25 mL, 10 mL and then 5 mL.

In the revised version of the manuscript, we have now detailed this procedure (lines 113-138, pages 2 and 3):

“1.1. Place tumor sample in a culture dish and dissociate the tissue by scraping using a policeman in serum-free, high glucose culture medium:…

1.2. Use pipettes of successively smaller sizes, 25, 10 then 5 mL to dissociate the suspension obtained in the previous step by aspiration to break residual fragments and to obtain a homogeneous cellular suspension. Change the size of the pipette when there is no longer any resistance to the aspiration.”

**12. 1.3: Please specify the volume of PBS used to wash.**

We have now specified the volume of PBS in step 1.5 (line 148, page 3): “Rinse cells with 40 mL of PBS to recover the largest possible number of cells.”

**13. 2.2: Please provide the composition of cell-sorting cell buffer.**

Unfortunately, it is impossible to provide the composition of the cell-sorting cell buffer in this step (line 161, page 4) since this is a commercial buffer of unknown composition.

**14. 2.3: Is a vortex used for mixing?**

In part 2 of the protocol, we have revised the steps to be clearer (lines 157-177, page 4). No vortex is used for mixing.

**15. 2.4: Please specify the buffers used in this step.**

We have modified this step (step 2.5. in the revised manuscript). There is only one buffer used here for different purposes, to hydrate, elute and rinse. This buffer is part of the commercial kit by Miltenyi. It is now defined in the Table of materials.

**16. 3.2: Do you mean add 1 ml of 2 % B27-NeuroMix and 0.4 % BSA as a mixture, or 1 mL of each sequentially?**

We agree with the Editor that this step needed clarification, and we have now modified it in the revised version of the manuscript (lines 181-198, page 4 and 5): “3.1. Prepare culture medium

3.1.1 In 49 mL of DMEM/F-12, add 1 mL of 2 % B27-NeuroMix which contains: Biotin, DL Alpha Tocopherol Acetate, DL Alpha-Tocopherol, Vitamin A (acetate), BSA, fatty acid free Fraction V, Catalase, Human Recombinant Insulin, Human Transferrin, Superoxide Dismutase, Corticosterone, D-Galactose, Ethanolamine HCl, Glutathione (reduced), L-Carnitine HCl, Linoleic Acid, Linolenic Acid, Progesterone, Putrescine 2HCl, Sodium Selenite, T3 (triodo-I-thyronine). The concentration of the different components is confidential.

3.1.2 Then add: 20µL of 0.4 % bovine serum albumin (BSA), 250 µL of insulin at 5 µg/mL, 6.25 µL of epidermal-growth-factor at 25 ng/mL, 12.5 µL of basic fibroblast growth factor at 25 ng/mL, 10 µL of hydrocortisone at 0.5 µg/mL and 500 µL of penicillin/streptomycin at 1%.

3.2. Place CD133-expressing cells in a low-attachment six-well plate at a density of 200,000 cells per well, and add 4 mL of the previously prepared culture medium per well.”

**17. 4.2: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol to complete this protocol.**

We agree with the Editor that the protocol here cannot stand alone. We in fact chose CRISPR-Cas9 as a method of transfection, but the protocol can be performed with any transfection method using a plasmid expression technique. Consequently we have now modified the manuscript to explain this (lines 205-207, page 5): “We chose CRISPR-Cas9 technology to invalidate BECN1 gene expression because the lifespan of the spheres is less than 14 days. This can be done with any type of plasmid expression technique.”

**18. 4.3: Please describe how the medium is delicately removed.**

We have modified the manuscript to explain how the medium is delicately removed (line 209, page 5): “Remove culture medium delicately by slow aspiration of the supernatant with a pipette.”

**19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:**

We agree with the Editor that the following points are important for a methods journal and we have revised the discussion to back up what was already included in our manuscript.

**a) Critical steps within the protocol**

We have included a critical-steps paragraph in the protocol (lines 332-340, page 8). This includes:

* The special characteristics of microdissection in a liquid medium that require an adjustment of energy to avoid cell damage.
* Choosing the appropriate pulse energy and duration to avoid thermal damage to cells.
* Experience in pathology is necessary to recognize the specific type of cells.

**b) Any modifications and troubleshooting of the technique**

As advised by the Editor, we have now included the following in our revised manuscript (lines 304-306, page 7):

* “We developed the technique so as to provide the quickest procedure possible to preserve cell viability.
* Qualified persons are required to resolve machine-related technical problems.”

**c) Any limitations of the technique**

The limitations of the technique are included in the manuscript as a complement to the critical steps (lines 326-340, pages 7-8). They include:

* We need to increase energy for microdissection in a liquid medium to obtain reliable results, so the microdissector has to offer the possibility of increasing laser pulse energy.
* The microdissector has to offer the possibility of controlling laser pulse energy and duration to avoid cell damage.
* The personnel needs to be experienced in pathology to recognize specific cell types.
* The procedure has to be performed fairly rapidly to avoid cell stress.
* RNA content may be affected but quantitative data on absolute mRNA loss compared to fixation and staining techniques is still lacking.
* It can be very expensive to acquire a laser microdissector.

**d) The significance with respect to existing methods**

We have included three points of comparison between our method and other existing methods to show the advantages of this method (lines 314-323, page 7).

* This method does not need a large number of cells to perform the molecular analysis
* A high percentage of cell viability
* It is adapted to all cell sizes
* It combines results from fluorescent staining and morphologic characteristics

**e) Any future applications of the technique**

Finally, as advised by the Editor, we have now added a statement regarding possible future applications of the technique (lines 349 and 350, page 8): “In a future application, our method could be used to isolate a specific cell type, cultivate it and reutilize it in tissue or organ replacement and transplantation.”

**20. References: Please do not abbreviate journal titles.**  
We have utilized the type file of EndNote specific to JoVE for the references. We now include full journal titles.