**Point-by-point responses**

**Reviewers' comments:**

**Reviewer #2:  
Manuscript Summary:  
UV-laser pressure catapulting is used to isolate live stem cells from patient derived breast tumor xenograft specimens. The rationale for the protocol is based on inhibiting autophagy in breast cancer stem cells to reduce tumorigenicity. The protocol consists of tumor tissue disruption, magnetic cell sorting with anti-CD133, cell culture, CRISPR-Cas9 gene knock-out, and LCM of the living cultured cells. The procedural steps are logical and easy to follow. The Introduction should be expanded to indicate the rationale for selecting only CD133 positive cells. The recommended revisions listed below will further enhance the continuity and procedures.**We thank Reviewer #2 for remarking that the procedural steps are logical and easy to follow.

**Major Concerns:   
1. Summary line 49: The Nitrogen laser in the Zeiss PAL Micro-beam is 337nm. Please correct this.**

To follow this advice, we have now corrected this (line 50, page 2).

**2. Summary lines 57-58: Microdissection can be performed by anyone with adequate training in histology/histomorphology. A pathologist can provide expert guidance but a pathologist is not required. Please change this sentence to "On the other hand, it can be time-consuming and requires an experienced pathologist, or adequate training in cellular morphology."**

In the abstract, lines 59-60 of page 2, we have modified the manuscript according to the advice by Reviewer 2: “On the other hand, it can be time-consuming and requires an experienced pathologist or adequate training in cellular morphology.”

**3. Introduction, lines 63-64: As mentioned above in point #2, please modify the sentence to show that anyone with adequate training can perform LCM. "However, it requires the expertise of a pathologist, or adequate training in cellular morphology."**

We have modified the manuscript in lines 76-78, page 2: “However, it requires the expertise of a pathologist, or adequate training in cellular morphology, to identify the different cell populations within a malignant tumor.”

**4. Introduction, lines 65: As the author's state, LCM has been commercially available for 20 years. During the past 20 years numerous micro methods of molecular analysis have been developed with extensive literature citations. Please delete the phrase "…on the development of… ".**

We agree with Reviewer 2 and have now deleted the phrase “on the development” in the modified manuscript (line 78 and 80, page 2): “It depends also on molecular micro-methods for genomic analyses of small numbers of cells 6-8, or even single cells 2. “

**5. Introduction or Discussion: Please add the rationale for using anti-CD133 to isolate stem cells rather than CD44 or another stem cell marker because CD133 negative cells may also be involved in growth and proliferation. See Li Experimental Hematology & Oncology 2013, 2:17 http://www.ehoonline.org/content/2/1/17.**

We agree with Reviewer 2 that CD133-negative cells may be involved in growth and proliferation. Cancer stem cells can be CD133-positive or negative (Shmelkov S.V. et al. J Clin Invest. 2008, Ricci-Vitiani L. et al. Nature. 2007, Singh S.K. et al. Cancer Res. 2003, Collins A.T. et al. Cancer Res. 2005, Zhou J. et al. Mol Cancer. 2011, O'Brien C.A. et al. Nature. 2007), but CD133-positive cells have a higher proliferation index and chemo/radioresistance properties (Beier D. et al. Cancer Res. 2007, Wang L. et al. Int J Biol Sci. 2013, Reyes E.E. et al. J Transl Sci. 2015). CD133 also has a prognostic and predictive value for disease-free survival, overall survival and progression-free survival (Grosse-Gehling P. et al. J Pathol. 2013). In the revised manuscript, we have now added the rationale for using an anti-CD133 antibody to isolate stem cells (lines 300-305, page 7): “CD133-positive cells have a higher proliferation index and chemo/radioresistance properties 18,19. CD133 also has a prognostic and predictive value for disease-free survival, overall survival and progression-free survival 20. We focus here on the proof of concept, and homogeneous cell populations can be isolated, on the basis of a specific molecular marker. In addition, they remain viable, enabling their in culture or xenograft.”

**6. Protocol line 103: Please specify if the recovered cells are being washed or if the filter is being washed. "…then wash with PBS to recover a maximum number of cells".**

We wanted here to recover stem cells in tissue debris. We first filtered the cell suspension with a filter, and then we used PBS to wash. Thus we recovered the remaining cells, maximizing the number of cells obtained. We have modified the text (line 148, page 4): "Rinse cells with 40 mL of PBS to recover the largest possible number of cells.”

**7. Protocol line 116: Please indicate what the abbreviation "LS" indicates.**

In line 169 in the revised manuscript, page 4, LS is part of the commercial name according to the manufacturing company. We have modified the text and removed it.

**8. Protocol lines 128-135: The order of the steps should be revised. It appears that DMEM-F12 is supplemented with B27-NeuroMix, BSA, insulin, EGF, FGFb, hydrocortisone and penicillin/streptomycin. Then the complete media is added to the cells in a 6 well plate. The steps currently listed as 3.2 and 3.2 should be 3.1 and 3.2, respectively. The current step 3.1 should become step 3.2.**

To follow the advice by Reviewer 2, we have changed the order of the steps (lines 183-198): “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: 20 µL 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%.

* 1. Place CD133-expressing cells in a low-attachment six-well plate at a density of 200000 per well, and add 4 mL of the previously prepared culture medium per well.

**9. Protocol line 129: 49mL of DMEM-F12 appears to be a typographical error as this is too much volume for a 6 well plate.**

We have revised the protocol. The corresponding step in the revised manuscript is now step 3.2., lines 200-201, page 5: “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.”

**10. Protocol line 145: Please list the sequence for the forward and reverse oligos or list them in the Material/Equipment table.**

For the transfection step and the use of CRISPR-Cas9, we have now modified the manuscript to indicate that this step can be done with any plasmid expression technique (lines 206-207, page 5). However, as advised by Reviewer 2, we have now listed the sequence of the forward and reverse oligos used in our CRISPR-Cas9/transfection steps in the revised Table of Materials.

**11. Protocol line 177: In step 5.The cells are to be resuspended at 3000cells/µL. However, in step 4.6, the input number of total cells is less than 3000cells/µL. This is prior to a 24 hour incubation and prior to selection of cells that integrated the plasmid. In step 4.6, 200,000cells are placed in 500 µL, which is only 400cells/µL. Even if the cell population doubles in 24 hours and transfection is 100% effective, the total number of available cells is less than 3000cells/µL. I guess multiple wells in the 6 well plate would need to be seeded with 200,000cells to have an adequate number of cells. Please either correct the initial number of cells/volume used, the final cell count, or state the number of wells in the 6 well plate that need to be seeded.**

We thank Reviewer 2 for this relevant comment. In the revised version of the manuscript, we have added the number of 6-well plates to be seeded. And we have modified step 3.2. to indicate that we need to prepare two 6-well plates (lines 196-198, page 5). We have added an additional step 5.1. (line 235, page 5) for the pooling of the 2 plates (12 wells).

**12. Protocol line 195: Please describe how the cap of a microcentrifuge tube, that is positioned with the open end facing down, can hold medium for catapulting the cells.**

The small diameter 4.6 mm of the cap of the microcentrifuge tube enables a drop of DMEM/F-12 medium to stay in the cap, open and facing down, for 30 minutes for the laser microdissection procedure.

**13. Protocol line 209: How quickly do you have to inject the cells into a mouse xenograft after LCM? Figure 3 shows the effect of the duration of the LCM process on cell viability. Taking this reduction in viability over time into consideration, how long can you wait prior to injecting the cells into a mouse?**

The laser microdissected cells are immediately injected into the mouse. We have now modified the corresponding step in the revised manuscript to explain this (line 272, page 6).

**14. Results line 218-219: This sentence appears to contradict statements in the Introduction. Based on statements in the Introduction, cells that survived after BECN1 knock out had reduced tumorigenicity. The following sentence implies that knock out of BECN1 does not affect cell growth: "Breast cancer stem cells selected by laser microdissection were successfully cultivated or transplanted into nude mice.". Please clarify this statement.**

The transplantation here was considered as a sign of cell viability, not as a sign of tumorigenicity. To prevent any misunderstanding, we have removed “transplanted into mice” in the revised version of the manuscript (line 284, page 8): “Breast cancer stem cells selected by laser microdissection were successfully cultivated”.

**15. In Figures 2 and 3, please indicate what the error bars represent. SD, SEM, or CI?**

In Figures 2 and 3, error bars indicate the Standard Deviation. The manuscript has been modified to indicate this.