**Point-by-point responses**

**Reviewers' comments:**   
  
**Reviewer #1:**   
**Manuscript Summary:   
The authors report the use of laser microdissection and pressure catapulting (LMPC) to isolate living stem cells in autophagic state, in a way that preserves their viability and tumorigenesis. Thus, they are using a well-established technique for a novel and important biomedical application. For this purpose, they use a commercial LMPC device in combination with up to date protocols for stem cell sorting and cultivation, transfection of the cells to invalidate autophagy gene expression, selection of the transfected cells, and assessment of the gene expression and tumorigenicity. This way, a homogeneous cell population was produced that could be successfully cultivated and transplanted into nude mice for further studies.**  
**Major Concerns:   
The importance of the application and the overall scientific quality of the approach presented in this manuscript makes it suitable for publication in the Journal of Visualized Experiments. However, shortcomings in the description of the mechanisms of LMPC, some missing details in the description and evaluation of the protocols and the need for a few additional references require revision before the paper can be accepted for publication, as detailed below.**

We would like to thank Reviewer #1 for remarking on the overall scientific quality of this approach.

**1. LMPC mechanisms are described only in the abstract but not in the body of the paper, and the description given in the abstract is partially wrong. In line 51 the authors state that "the target is excised in a process called cold ablation", which is wrong. Firstly, "cold tissue ablation" with UV laser pulses does not exist at all as has been shown in detail in sections V. H and I, and sections VIII. B. and D of Ref. [18] quoted by the authors. Secondly, LMPC with UV-A pulses (337 nm and 355 nm) has been shown by Vogel et al. to rely on plasma-mediated ablation (for the dissection process) and plasma-induced pressure (for catapulting) as described in Ref. [16] quoted by the authors.**

We thank Reviewer 1 for this comment and as advised we have modified the manuscript to describe the LMPC mechanisms accurately [18] (Lines 52-54, Page 2): “With the laser beam, target excision and ejection depend on plasma-mediated ablation (for the dissection process) and plasma-induced pressure (for catapulting).”

**2. A (correct) description of LMPC mechanisms should be included also in the body of the paper, best after Reference 1 in Line 63. Here it should also be mentioned that early system included a nitrogen laser, whereas at present frequency-tripled Nd:YAG lasers are used because of their better beam quality and lower pulse energy needed for dissection [for a comparison see Vogel et al. Biophys. J. 93:4481-4500 (2007)].**

We agree with the comment by Reviewer 1 that today Nd:YAG lasers are now used. Vogel et al. compared Nd:YAG and nitrogen lasers, and the former offers better beam quality and lower pulse energy requirements for dissection. As suggested by Reviewer 1, we have now modified our manuscript to include this information as well as a description of the LMPC mechanisms in the introduction (lines 65-72, page 2): "Its dissection and catapulting effects are plasma-mediated. Plasma-induced photochemical decomposition provides a large number of nuclei for vaporization of heated tissue water through a photo-thermal effect3. Catapulting with defocused pulses, used here, is mediated by plasma-induced pressure formation if the irradiated spot size is ≤ 27 µm, whereas for spot size > 27 µm it is mediated by confined thermal ablation4,5. The early system used nitrogen laser, whereas recent systems use frequency-tripled Nd:YAG lasers because of their better beam quality and lower pulse energy needed for dissection.” However, we acquired the microdissector in 2005 and we have used it very frequently with very good results in different research programs (Bousquet G. et al. Oncotarget 2017; Bousquet G. et al. Breast Cancer Res 2014; Verneuil L. et al. Oncotarget 2015, Brugière C. et al. J Invest Dermatol 2018). It is for this reason we mentioned only the nitrogen-based laser microdissector.

**3. Reference [1] refers to a recent review of laser capture microdissection (LCM), which is not used by the authors. It should be complemented be a quotation of the seminal paper by Schütze and Lahr (Nature Biotechnology 16:737-742) on Gene identification by means of LMPC, the technique used by the authors.**

We have added the reference by Schütze and Lahr in the revised version of our manuscript, line 65, page 2.

**4. For the description of the LMPC mechanisms, the authors refer to a book chapter authored by Vogel et al. (Ref. [16]). They should also quote the respective Journal articles, which are easier accessible: Vogel et al., Mechanisms of laser-induced dissection and transport of histologic specimens, Biophys. J. 93:4481-4500 (2007), and Horneffer et al., Principles of laser-induced separation and transport of living cells, J. Biomed. Optics 12:054016, 1-13 (2007).**

To follow this advice by Reviewer 1, we have now added these two references (line 70, page 1): “Catapulting with defocused pulses, used here, is mediated by plasma-induced pressure formation if the irradiated spot size is ≤ 27 µm, whereas for spot size > 27 µm it is mediated by confined thermal ablation4,5 “

**5. A paragraph describing the principle design of an LMPC system (laser beam coupled into an inverted microscope with a computer-controlled stage) as well as the design of the Duplex dishes used for live cell catapulting should be included in the introduction.**

To follow the advice by Reviewer 1, we have now fully described the LMPC system and the dishes used for catapulting living cells.

In the introduction of the revised version of our manuscript, we have now specified (lines 72-75, page 2): “the LMPC system includes a laser beam coupled with an inverted microscope and a computer-controlled stage. For living cell catapulting, the system uses special dishes with a membrane ring; it has a special foil bottom adapted to the growth of many cell types; the plastic ring is used for the isolation of living cells.”

**6. Detailed information about the devices, and consumables used in the protocol must be provided in order to enable readers to reproduce results, or to utilize the protocol for other goals where it may also be useful. Wherever specific devices or agents are used that are not common knowledge, name and manufacturer must be mentioned. For example, the authors should precisely specify, which device they used for LMPC and mention that Zeiss is now the manufacturer producing and distributing the PALM Microbeam. Details should also be given for the microdissection DuplexDish - how is anyone otherwise supposed to know where to buy them?**

We agree with Reviewer 1 that detailed information about the devices and consumables used in the protocol should be provided. However, the editors require all commercial names to be omitted and they are listed in a dedicated table of materials used where name, manufacturer, and necessary technical information are given in order to enable readers to reproduce the results by following the protocol. In this table, we have indicated that Zeiss is the manufacturer producing and distributing the PALM Microbeam and have given details on the microdissection Lumox® dish.

**7. In point 5.8 of the protocol it should be mentioned how the laser beam was focused on the dissected specimen (because Horneffer et al., JBO 12:054016, 1-13 (2007) showed that the transfer rate larger when the focus is located on the periphery of the sample rather than under its center).**

We agree with Reviewer 1 that describing the way the laser beam was focused on the dissected specimen is important, since focusing on the periphery causes a fast rotational movement minimizing the flow of culture medium and the resulting shear stress compared to delivering pluses under the center, as shown by Vogel, A. et al., Methods Cell Biol. 82:153-205, (2007) and Horneffer et al., JBO 12:054016, 1-13 (2007). So, according to the advice of Reviewer 1, we have modified the manuscript to specify that: “The laser beam was focused on the periphery of the dissected specimen.” (line 257 and 258 of the revised manuscript, page 6).

**8. In the results section, the success rates of both catapulting and recultivation should be given. When the laser beam is focused on the periphery of the specimen, it may not be captured which would compromise the catapulting success rate.**

To follow this advice, we have added the success rates of catapulting and re-cultivation (lines 282-283, page 6): “After each microdissection, the catapulting success was systematically controlled on the cap with the dedicated device on the laser microdissector.” We have also demonstrated that re-cultivation success was linked to laser-microdissected breast cancer stem cell viability. Figure 3 shows that the number of viable cells sharply declines when the laser microdissection procedure lasts more than 30 minutes.

**9. Why did cell viability drop after 30 min. (Fig. 3)?**

We repeatedly experienced a drop in cell viability after 30 minutes. We think that this time lapse corresponds to the longest time the cells can live outside optimal culture conditions (culture medium, CO2 in the culture chamber, temperature at 37 °C) (Cree AI. [Methods Mol Biol.](https://www-ncbi-nlm-nih-gov.gate2.inist.fr/pubmed/21516394) 2011).

**10. How did the drop of viability after > 30 min affect the success of stem cell cultivation or the events after transplantation into nude mice?**

The drop in viability when microdissection lasted more than 30 min was significantly related to the success rate of stem cell cultivation. The engraftment rate after transplantation into nude mice was also impacted.

**11. Lines 216/17 and Caption of Fig. 3: The sequence of events is unclear to me. Was cell selection by LMPC performed after the 24 h incubation period, and viability then assessed 30 min after LMPC?**

Our aim was to determine the optimal time of LMPC to obtain 100 % stem cell viability. LMPC was performed over periods of 20, 30, 45 and 60 minutes on different clusters of stem cells. The stem cells were then cultured in the optimal conditions (temperature, CO2, DMEM/F-12 culture medium) for 24 h. After that, we tested the viability of the stem cells by using the trypan blue test. We obtained the highest percentage of viability at 30 minutes of LMPC duration. We have modified the manuscript to explain this better (line 279-282, page 6): “Breast cancer stem cells were laser-microdissected over different durations and incubated in a culture medium for 24 h. Cell viability was assessed using trypan blue. Breast stem cell viability dropped significantly when the microdissection duration exceeded 30 min”. We have also modified the caption of Figure 3 (line 296-299, page 7): “To calculate live cell percentages after different durations of laser microdissection, and after a 24-h incubation period in culture medium, breast cancer stem cell viability was assessed on 200 cells on a special slide, using trypan blue”.

**12. Line 246, formation of a cavitation bubble. Quote also Horneffer et al. JBO 12:054016, 1-13 (2007).**

To follow this advice, we have also quoted Horneffer et al. JBO 12:054016, 1-13 (2007) (line 327 in the revised manuscript, page 7).

**13. Line 247. The role of focus location (middle or periphery of specimen) for shear-induced membrane denuding should also be mentioned.**

As advised by Reviewer 1, we have modified the manuscript to mention the role of focus location (line 328-331, page 7-8): “Shear stress, induced by defocused pulses, is compensated by delivering the pulses on the periphery of the specimen. This minimizes the flow of culture medium parallel to the sample surface, and the corresponding shear stress (Horneffer et al., JBO 12:054016, 1-13 (2007))”.

**14. Lines 250 and 254. Typo. It should be 300 µs, not 300 ms (see page 193 in Ref. [16]).**

We thank Reviewer 1 for his kindly correction of this typographic error. We have now modified the manuscript accordingly (line 333 and 334 in the revised manuscript, page 8): “The laser thermal effect is small since cells can survive at temperatures as high as 180° C for a heat exposure time of 300 µs.”

**15. It should also be mentioned that the carrier foil of the cells in conjunction with the rapid cooling upon plasma expansion protects the cells from thermal damage, as shown by thermal modeling in Vogel et al. Biophys. J. 93:4481-4500 (2007).**To follow this advice, we have modified the manuscript to add this explanation (line 336-338, page 8): “It has been shown that the cell carrier foil of the cells in conjunction with the rapid cooling upon plasma expansion protects the cells from thermal damage 4.”