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Unicellular selection of living cells in liquid medium using laser microdissection and a pressure catapulting system

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To the editor of JOVE

Dear Editor,

Please find enclosed a manuscript entitled “Unicellular selection of living cells in liquid medium using laser microdissection and a pressure catapulting system” that we would like to submit for a video protocol in JOVE
We hope this manuscript will be suitable for publication in your journal and we are ready to make any modification suggested by the reviewers.

Sincerely Yours,

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TITLE:

Unicellular Selection of Living Cells in Liquid Medium Using Laser Microdissection and a Pressure Catapulting System

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KEYWORDS:

Laser microdissection, pressure catapulting, living cell selection, cancer stem cells, patient-derived tumor xenograft, tumor cell heterogeneity, cell transfection, CRISPR-Cas9

SUMMARY:

Here we describe a protocol for the use of laser microdissection and pressure catapulting to isolate living stem cells in an autophagic state, in a way that preserves their viability and tumorigenesis potential.

ABSTRACT:

The purpose of the method presented here is to isolate living cancer stem cells in an autophagic state using laser microdissection and pressure catapulting (LMPC). Isolating stem cells is challenging because of their small numbers, size heterogeneity, and fragility. Cell characteristics

are very specific under combined laser microdissection and immunohistochemistry staining, used here for the stem cell identification. LMPC is based on a contact-free dissection using an ultraviolet, pulsed N_2 (UV-A, λ = 337 nm) or, more recently, an Nd:YAG laser beam focused through the objective lens of a microscope to a diameter of 2 µm. With the laser beam, target excision and catapulting depend on plasma-mediated ablation (for the dissection process) and plasma-induced pressure (for catapulting). In this way, isolated cells are recovered by catapulting them into a capture fixture using the force of a more energetic defocused laser pulse.

LMPC is a contact-free and contamination-free method. Its precision depends on the laser characteristics (wavelength and beam quality) and the microscope (magnification and the numerical aperture of the objective). It enables the direct visualization of the studied cells without any cell-size limitation. On the other hand, it can be time-consuming and requires an experienced pathologist or adequate training in cellular morphology.

INTRODUCTION:

Laser-microdissection is a powerful tool that has been used for 20 years to precisely select specific cell populations from histological tissue sections in combination with immunohistology[1,2]. Its dissection and catapulting effects are plasma-mediated. Plasma-induced photochemical decomposition provides a large number of nuclei for the vaporization of heated tissue water through a photo-thermal effect[3]. Catapulting with defocused pulses, used here, is mediated by plasma-induced pressure formation if the irradiated spot size is ≤ 27 µm, whereas, for a spot size of > 27 µm, it is mediated by confined thermal ablation[4,5]. The early system used a nitrogen laser, whereas recent systems use frequency-tripled Nd:YAG lasers because of their better beam quality and lower pulse energy needed for dissection. The LMPC system includes a laser beam coupled to an inverted microscope with a computer-controlled stage. For live-cell catapulting, the system uses special dishes combined 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.

This system is of particular interest in the study of heterogeneity in cancer. However, it requires the expertise of a pathologist, or adequate training in cellular morphology, to identify the different cell populations within a malignant tumor. It also depends on molecular micro-methods for the genomic analyses of small numbers of cells[6-8] or even single cells[2].

Most LMPC applications have focused on mRNA and gene expression analyses on dead cells selected from formalin-fixed or frozen tissue sections[9]. It has also been used to isolate living cells[10,11,12], such as human embryonic stem cells, to preserve their karyotype and pluripotency[13,14]. However, LMPC still has a few applications for the selection of living cells and could, thus, be an interesting alternative when other selection methods are not applicable.

We recently demonstrated that the chemo-resistance of triple negative breast cancers is associated with the presence of autophagic cancer stem cells, in both patient biopsy samples and the corresponding patient-derived xenografts[15]. Particularly, we demonstrated that BECLIN1 expression was significantly correlated with an increased number of chemo-resistant breast

cancer stem cells. In addition, BECLIN1 is a well-known autophagy marker linked to hypoxia. We successfully established tumor spheres from tumor xenografts, in order to modulate autophagy gene expression *in vitro*. We used Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 technology rather than transfection to knock out the expression of the *BECN1* autophagy gene in tumor spheres, because of their short life. After transfection and efficient gene invalidation, we assessed the tumorigenicity of 200 selected living cancer stem cells. Then, we successfully demonstrated that autophagy pathway inhibition reduced the tumorigenicity of the cancer stem cells. To select living cancer stem cells one-by-one from a liquid culture medium, we cannot use fluorescence-activated cell sorting (FACS) for two reasons: the difficulty in separating cancer stem cells preventing their passage into the cytometer nozzle; and the enzymatic treatment necessary for cell separation and to make cell membrane permeable, which could result in false staining and/or cell death.

Thus, we designed a technology to select cells one-by-one using laser microdissection and pressure catapulting in a liquid medium.

PROTOCOL:

All tumors used in these experiments were patient-derived breast cancer xenografts[15-17]. After host euthanasia, a fresh fragment of the tumor was immediately processed to isolate cancer stem cells.

1. Mechanical Tissue Dissociation

Note: These steps are performed at room temperature, under the laminar flow.

1.1. Place a sample of 1 cm^3 of fresh patient-derived breast cancer xenograft in a culture dish of 10 cm in diameter. In 2 mL of serum-free, high-glucose culture medium, dissociate the fresh tumor by scraping, using a rubber policeman.

1.2. Use pipettes of successively smaller sizes (*i.e.*, 25, 10, and 5 mL) to dissociate the suspension obtained in the previous step by successive aspirations, to break residual fragments, and to obtain a homogeneous cell suspension. Change the size of the pipette when there is no longer any resistance to the aspiration.

1.3. Place a filter with pores of 70 µm on a 50-mL tube, transfer the cellular suspension obtained, and rinse it 1x with 40 mL of phosphate-buffered saline (PBS) to recover the largest possible number of cells.

1.4. Centrifuge for 2 min at 250 x *g* at room temperature and, then, remove the supernatant.

1.5. Rinse the cell pellet with 40 mL of 1x PBS to recover as many cells as possible.

1.6. Discard the supernatant and, then, resuspend the pellet with 5 mL of PBS.

1.7. Use 10 µL of the cell suspension for the cell count. Place the sample on a hemocytometer and count the living cells using trypan blue.

1.8. Centrifuge the cell suspension for 2 min at 250 x *g* and, then, discard the supernatant.

2. Cancer Stem Cell Selection

2.1. Resuspend the pellet with 5 mL of PBS, centrifuge it at 250 x *g* for 5 min, and remove the supernatant.

2.2. Resuspend the sample to obtain a concentration of 10^7 cells for 400 µL of cell-sorting buffer.

2.3. Add 100 µL of FcR blocking reagent (CD133 microbeads kit) and, then, 100 µL of CD133 antibody microbeads. Incubate for 30 min at 4 °C in the dark.

2.4. Before cell selection, place the column in a magnet. Add 3 mL of cell-sorting buffer in the column and resuspend the cells and the antibody microbeads in 5 mL of cell-sorting buffer.

2.4.1. Add 1 mL of the eluate to the suspension of cells. Then, wash it with 3 mL of cell-sorting buffer. Repeat the washing 2x. Remove the column and wash it with 5 mL of separation buffer.

2.5. Push the piston to recover 5 mL of eluate containing CD133-expressing cells in a 10-mL tube. Centrifuge at 250 x *g* for 5 min at room temperature to remove the supernatant and, then, resuspend the pellet in 1 mL of DMEM/F-12.

3. Cell Culture of Cancer Stem Cells Sorted from Patient-derived Xenografts

3.1. Prepare the culture medium. In 49 mL of DMEM/F-12, add 1 mL of 2% B27. 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%.

3.2. Place 5 mL of eluate containing CD133-expressing cells in a low-attachment 6-well plate. Ensure that the cell density is 200,000 cells/well. Add 4 mL of the culture medium prepared in step 3.1 in each well. Prepare two complete 6-well plates.

3.3. Place CD133-expressing cells for 48 h in a humidified chamber (37 °C, 5% CO_2) under normoxia (20% O_2). Check regularly after 36 h of incubation to see if spheres have formed. When more than five spheres for each well have formed, stop the incubation.

4. Transfection of Cancer Stem Cells to Invalidate Autophagy Gene Expression

Note: CRISPR-Cas9 technology was chosen in this case to invalidate the *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. The *BECN1* gene was chosen in this case since the BECLIN1 expression has been correlated with the number of breast cancer stem cells resistant to chemotherapy[15].

4.1. Remove the culture medium delicately by slow aspiration of the supernatant using a pipette.

4.2. Meanwhile, prepare the following.

4.2.1. Mix 2 µg of the plasmid expressing *BECN1* sgRNA and Cas9 nuclease with 0.2 µg of plasmid expressing green fluorescent protein (GFP) and 7.5 µL of transfection reagent.

4.2.2. Add 500 µL of a reduced serum medium and leave the mixture for 5 min at room temperature; then, add it to the cell suspension.

4.3. Add 15 µL of lipid-based transfection reagent and leave it for 30 min at room temperature.

4.4. Put 500 µL of this mixture, together with 2 x 10^5 cells from spheres derived from tumor xenografts, in a 6-well plate on the agitator, and incubate it for 2 h at 37 °C.

4.5. Add 1 mL of culture medium, incubate it for 24 h, and then add 1% Kanamycin to select cells that have integrated the plasmid.

4.6. Control the transfection efficiency using a fluorescent microscope with a fluorescein isothiocyanate (FITC) filter to detect GFP in the cancer stem cells (50% - 60%).

5. Selection of Living Transfected Cells, One-by-one, Using Laser Microdissection and Pressure Catapulting

5.1. Pool two plates (*i.e.*, 12 wells) containing transfected cells from step 4.5 in a 50-mL tube.

5.2. Use a 1-mL pipette for pooling the cells in the wells to achieve sphere dissociation.

5.3. Centrifuge the tumor cells from the dissociated sphere in their culture medium at 250 x *g* for 10 min.

5.4. Suspend the cells to the concentration of 3,000 cells/µL of culture medium (see step 3.1).

5.5. Take 20 µL of this cell solution and place it on the membrane of the microdissection dish.

5.6. Smear about 0.5 cm^2 under the laminar flow hood and place the laser capture microdissection (LCM) caps in the microdissector cap holder filled with 20 µL of DMEM/F-12 in the already calibrated laser microdissector.

5.7. Draw the boundaries with a pencil around the smear and position the microscope directly over the target area.

5.8. Use the microdissector software with a GFP filter and delineate isolated GFP-positive cells using the freehand tool in the microdissector.

5.9. Set the laser to the cutting mode and activate it with a left click on the software button.

5.10. Cut delineated cells along with the membrane layer on the microdissection dish. Using commercial software (*e.g.*, PALM), perform the cutting with a speed of 15%, the laser microbeam energy at 95%, the focus at 69% with two cycles, and a z-focus delta of 1 µm.

5.10.1. Catapult the micro-dissected cells into the medium-filled cap of a microcentrifuge tube. Using the software, the laser pressure catapulting with a δ of 18 and a δ of -3 on the periphery of the dissected specimen

5.11. Unload the samples and caps containing the cells.

6. Assessment of Gene Expression in the Selected Cancer Stem Cells

6.1. Use a minimum of 10 living cells to assess the copy number of the *BECN1* gene using droplet digital PCR[15].

7. Tumorigenicity Assessment on Selected Living Cancer Stem Cells

7.1. For the tumorigenicity assessment, close the 200-µL tube containing the microdissected catapulted cells, and centrifuge it at 900 x *g* for 1 min.

7.2. Resuspend with 100 µL of NaCl 0.9% and recover the cells in a syringe for immediate grafting into nude mice by an intravenous injection.

REPRESENTATIVE RESULTS:

With laser microdissection and pressure catapulting, we selected breast cancer stem cells transfected with an invalidated *BECN1* autophagy marker.

[place **Figure 1** here]

Efficient invalidation of *BECN1* was verified using droplet digital PCR on a minimum of 10 cells. An invalidated *BECN1* autophagy marker and GFP genes were transfected in the same plasmid when the cells expressed GFP.

[place **Figure 2** here]

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.

[place **Figure 3** here].

After each microdissection, the catapulting success was systematically controlled with the dedicated device on the laser microdissector. Breast cancer stem cells selected by laser microdissection were successfully cultivated.

To assess the outcome, we used electron microscopy, assessing the numbers of autophagosome vesicles within the cytoplasms of the tumor cells. We compared tumor cells transfected with the empty plasmid and tumor cells transfected with sb1BECN1. We found a striking difference with large numbers of autophagosomes in the cytoplasm of sg1BECN1-transfected tumor cells. For each experimental condition, we counted 25 cancer cells, and we found that the mean number of autophagosomes per 1,000 μm^2 of cytoplasmic area was 35.8 *versus* 6.5 (in cells transfected with empty plasmid or with sg1BECN1, respectively; $p < 0.05$)[15].

Then, we assessed the tumorigenicity of the cancer cells cotransfected with GFP and sg1BECN1. After the dissociation of spheres, we laser-selected 200 fluorescent cancer cells expressing GFP and injected them subcutaneously in eight mice. We did not observe any successful engraftment[15]. Conversely, when we laser-selected 200 GFP-negative cancer cells, they were tumorigenic in 6/8 and 7/8 grafted mice.

FIGURE AND TABLE LEGENDS:

Figure 1: Laser microdissection of live GFP+ breast cancer stem cells. On the left panel, one GFP-positive breast cancer stem cell invalidated for the *BCEN1* gene is identified with the FITC filter of the laser microdissector (GFP gene and BECN1). In the photo on the left, the cell identified as positive is surrounded manually in the microdissector software with a red line; this line represents the path of the laser around the cell. On the right panel, a picture of the same area after microdissection is shown: the cell was sent into the cap. The scale bar = 10 µm.

Figure 2: Copy number variation (CNV) of the *BECN1*/*RNase P* ratio using digital droplet PCR. This panel shows the ratio of the autophagy gene *BECN1* to the standard CNV reference *RNase P* using droplet digital PCR on a minimum of 10 GFP-positive breast cancer stem cells. The error-bars represent the standard deviation.

Figure 3: Assessment of the breast cancer stem cell viability. 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. The error-bars represent the standard deviation.

Table 1: Composition of DMEM medium.

Table 2: Composition of 1x PBS.

DISCUSSION:

Combining the laser microdissection of cancer cells and molecular micromethods adds considerable value to the routine translational research. We developed the technique so as to provide the quickest procedure possible to preserve cell viability. It should be noted that qualified persons are required to resolve machine-related technical problems. We used an anti-CD133 antibody to isolate cancer stem cells. CD133-positive cells have a higher proliferation index and chemo/radio-resistance properties than any other markers[18,19]. CD133 also has a prognostic and predictive value for disease-free survival, overall survival, and progression-free survival[20]. We focus on the proof of concept that homogeneous cell populations can be isolated on the basis of a specific molecular marker. In addition, they remain viable, enabling their culture or xenograft.

The protocol presented here is a convincing alternative to other cell-sorting technologies for three reasons. First, FACS is limited by cell size and can alter cell characteristics because of the enzymatic dissociation in a separate environment before analysis[21]. LMPC, on the other hand, can handle all cell sizes and uses a small number of cells. The cell mortality rate is low. Second, the total chemical analysis system (TAS) utilizes microfluidics and is limited by cell size[21]. Third, cell or cytosol harvesting by glass capillary is limited by the contamination of the surrounding extracellular solution[21]. Taken together, LMPC offers the possibility of identifying cells both morphologically and fluorescently.

There are three critical steps and limitations of this methodology. First, dissection is less precise in a liquid medium than on a tissue section. Microdissection in a liquid medium requires more energy because of cavitation bubbles around the laser focus[3,5,22,23]. Cavitation bubbles can induce shear stress[24], which is compensated for by delivering the pulses on the cell periphery. This minimizes the flow of culture medium parallel to the cell surface[5]. Second, thermal and UV light damage depends on the cell fraction exposed, and on the exposure duration[4]. The laser thermal effect is minor since cells can survive at temperatures as high as 180 °C for a heat exposure time of 300 µs[4,22]. In focused pulses, the fraction exposed is small. Defocused catapulting exposes cells to laser light. However, polyethylene naphthalate (PEN) polymer foil protects them[4,22]. It has been shown that the carrier foil of the cells in conjunction with the rapid cooling upon plasma expansion protects the cells from thermal damage[4]. In addition, a nitrogen laser wavelength of 337 nm is far from the UV-C wavelength of 200 - 290 nm necessary to induce DNA damage and cell death[14,25,26]. Third, mechanical stress is the most likely source of cell damage[5]. The RNA content may be affected, but quantitative data on absolute mRNA loss compared to fixation and staining techniques is still lacking[4]. In addition, the assistance of a qualified pathologist is essential for cell identification, and the procedure must be performed in a short time to avoid cell stress[27].

In a future application, the method presented here could be used to isolate a specific cell type, cultivate it, and reutilize it in tissue or organ replacement and transplantation.

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DISCLOSURES:

The authors have nothing to disclose.

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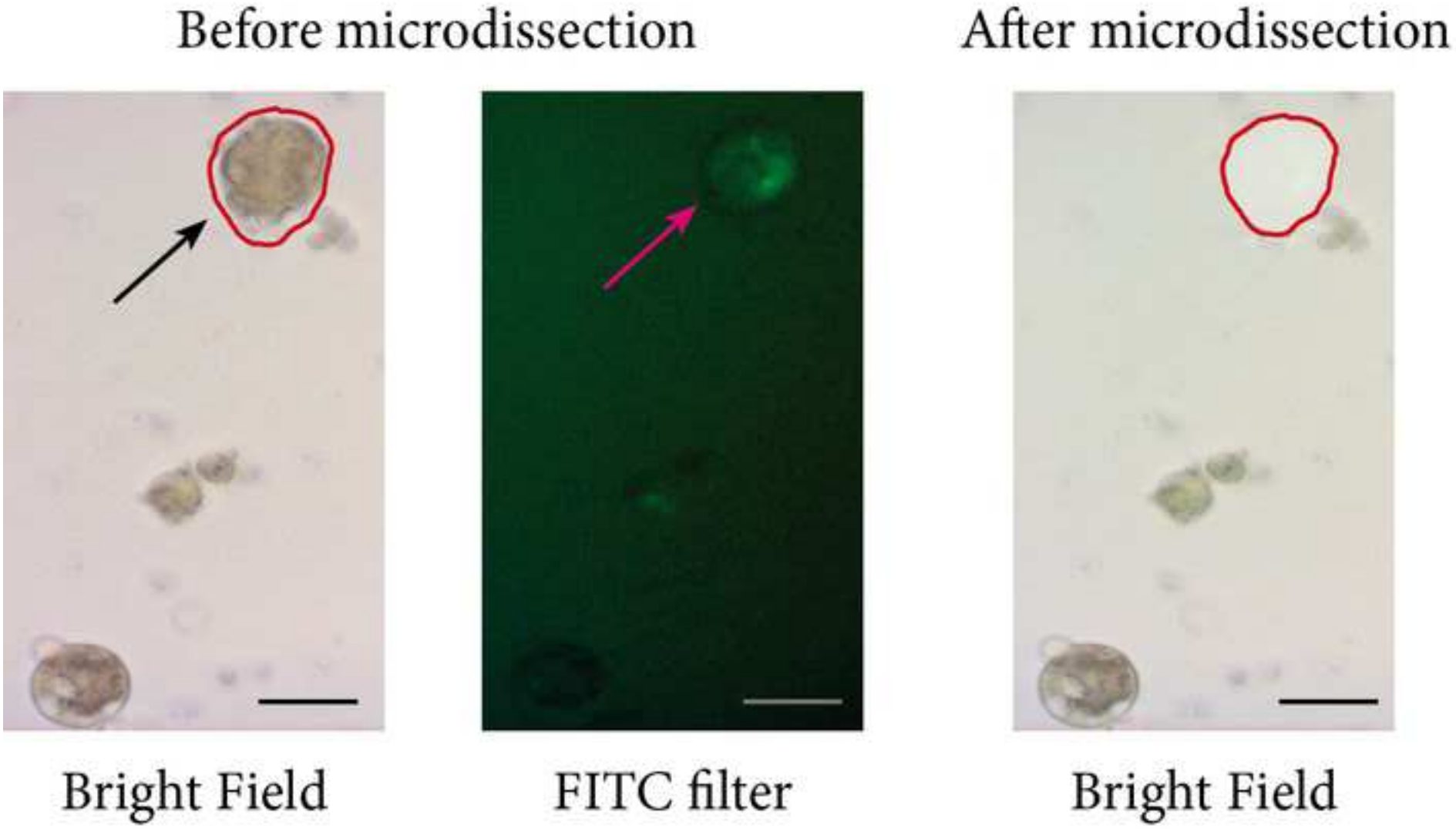

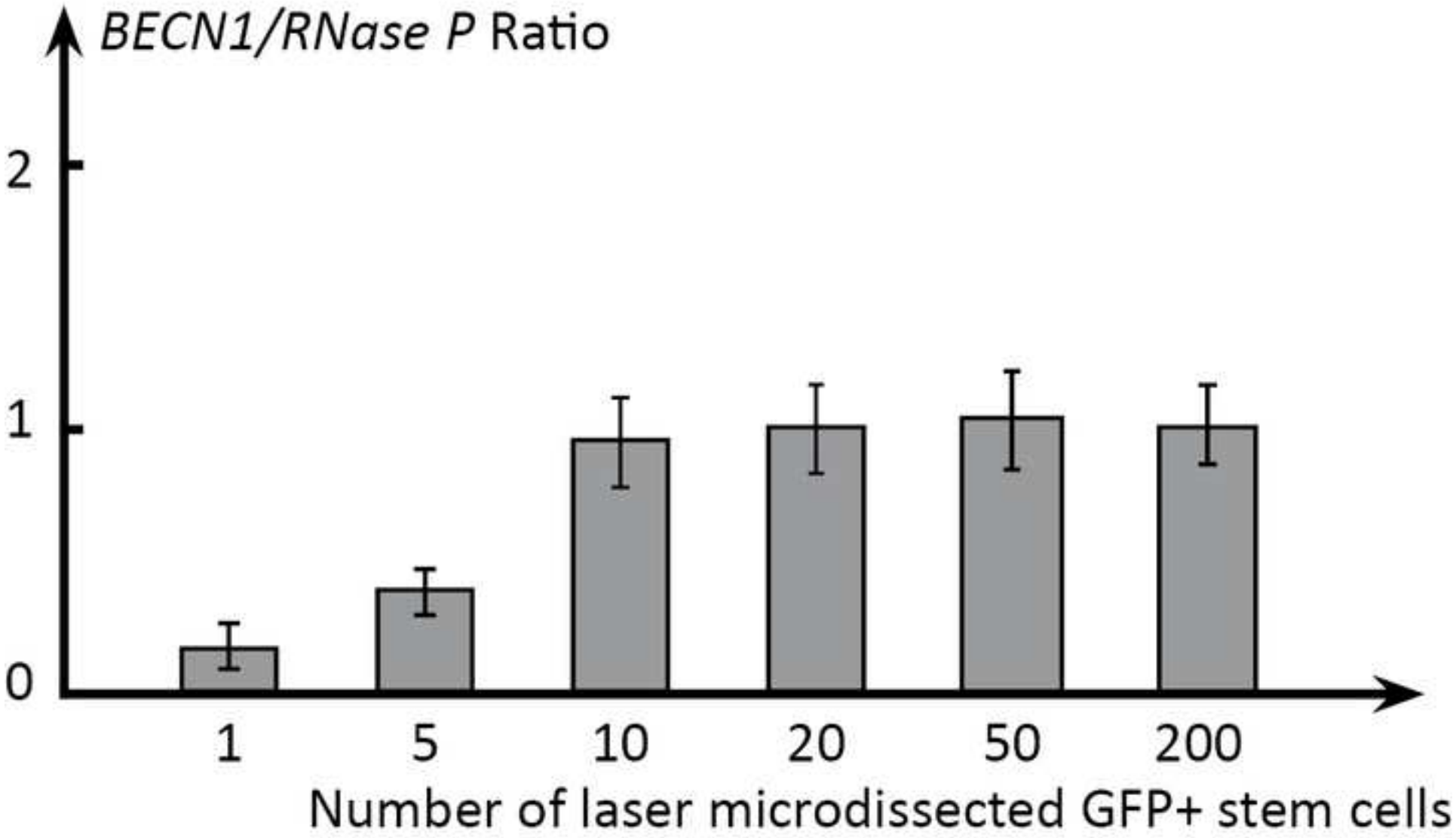

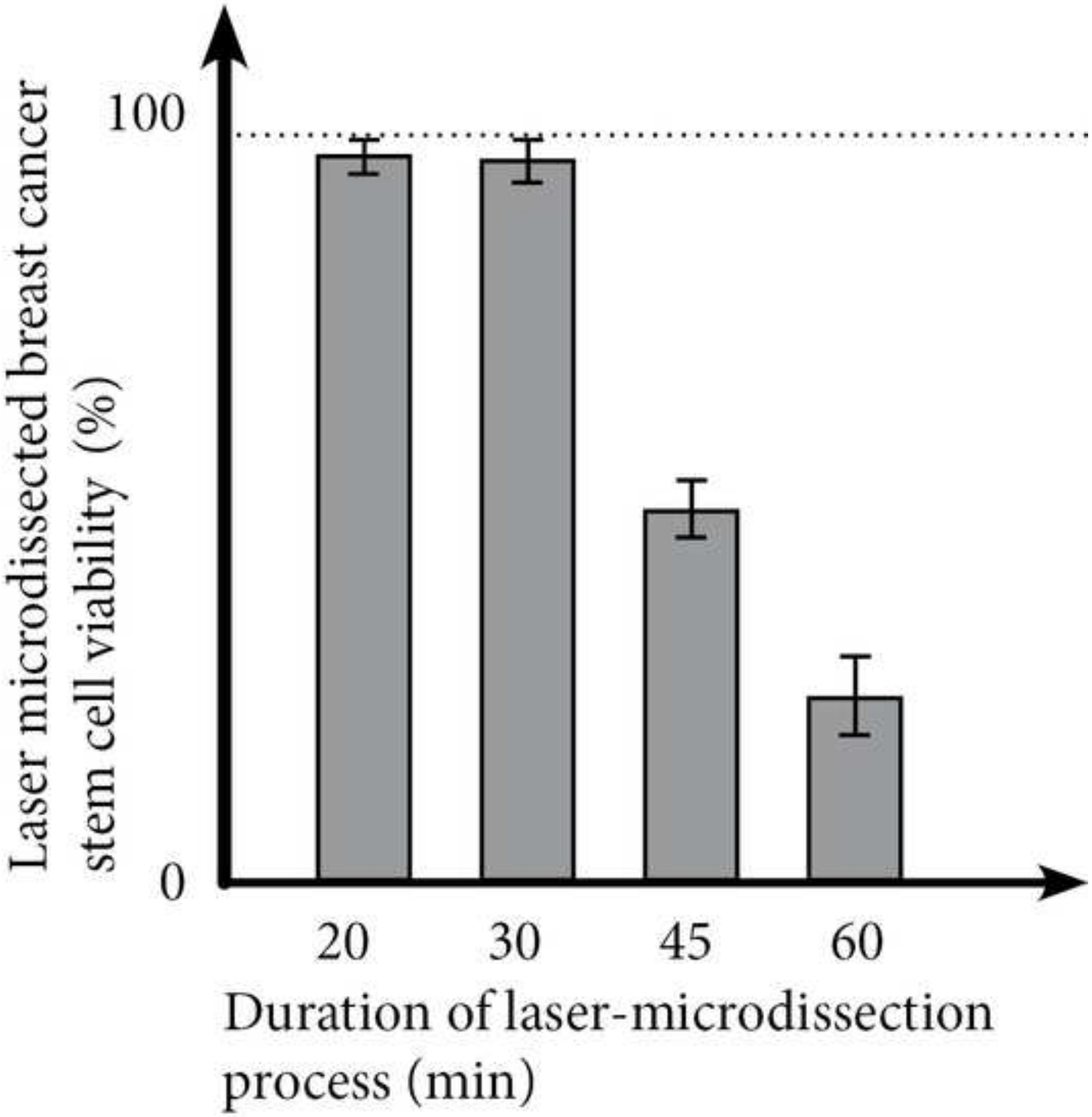

Table 1 Click here to download Table table 1.xlsx

Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture (DMEM

component	concentration (mg/L)
Glycine	18.75
L-Alanine	4.45
L-Arginine hydrochloride	147.5
L-Asparagine-H2O	7.5
L-Aspartic acid	6.65
L-Cysteine hydrochloride-H2O	17.56
L-Cystine 2HCl	31.29
L-Glutamic Acid	7.35
L-Histidine hydrochloride-H2O	31.48
L-Isoleucine	54.47
L-Leucine	59.05
L-Lysine hydrochloride	91.25
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	17.25
L-Serine	26.25
L-Threonine	53.45
L-Tryptophan	9.02
L-Tyrosine disodium salt dehydrate	55.79
L-Valine	25.85
Calcium Chloride (CaCl2) (anhyd,)	116.6
Cupric sulfate (CuSO4-5H2O)	0.0013
Ferric Nitrate (Fe(NO3)3"9H2O)	0.05
Ferric sulfate (FeSO4-7H2O)	0.417
Magnesium Chloride (anhydrous)	28.64
Magnesium Sulfate (MgSO4) (anhyd,)	48.84
Potassium Chloride (KCl)	311.8
Sodium Bicarbonate (NaHCO3)	1200
Sodium Chloride (NaCl)	6995.5
Sodium Phosphate dibasic (Na2HPO4) anhydrous	71.02
Sodium Phosphate monobasic (NaH2PO4-H2O)	62.5
Zinc sulfate (ZnSO4-7H2O)	0.432
D-Glucose (Dextrose)	3151
Hypoxanthine Na	2.39
Linoleic Acid	0.042
Lipoic Acid	0.105
Phenol Red	8.1
Putrescine 2HCl	0.081
Sodium Pyruvate	55
Thymidine	0.365

Table 2

phosphate-buffered saline (PBS) 1x

component	ation (mg/L)
Sodium Chloride	1.15
Potassium Phosphate, monobasic	1.15
Sodium Phosphate, dibasic	0.2
Potassium Chloride (lyophilized powder)	0.2

Table of Materials

Click here to download Table of Materials Materials table.xlsx

Name of Material / Equipment	Name in the article	Company	Catalog Number	ents/Description
DMEM/F-	lture medi	Gibco life technologies, France	61331-020	lot: 134998C
Kova-slide	Special counting slide	Dutscher, France	50126	lot: K301533
MACS smartStrainers 70 µm		MACS Mil	120-018-898	lot: 5141106083
Miltenyi cell sorting buffer	cell sorting buffer			
CD133 Microbeads k		MACS Mil	130-050-801	lot: 5100531034
LS column	column	MACS Mil	130-042-4	lot: 5161007015
MACS Buffer		MACS Mil	130-091-221	lot: 5160411268
Quadromacs Separator		MACS Mil	130-090-976	lot: 13277
B27-Neur	B27	PAA	F01-002	lot: F00212-2873
BSA		Sigma, Fra	A2058	lot: 41F9730K
PBS 1x pH 7.4		Gibco, life	14200-075	lot: 1848915
Insulin		Sigma, Fra	I9278	lot: 5LBC3574
Epidermal growth fa		R&D syste	236-EG-01	lot: HLM4210101
Hydrocortisone		Sigma, Fra	H08888-5	lot: 028K1215
Penicilline/streptom		Gibco life	15140-22	lot: 1014003
Fibroblast growth fa		PeproTech	100-18B 5	lot: 060908-1
CRISPR-CAS9		System Bi	CAS740G-1	lot: N/A
BLOCK-IT fluorescent		Invitrogen	2013-44-2	lot: 1477937
Opti-MEM	d serum m	Gibco, life	11058-02	lot: 867419

Lipofectamine	Lipofectamin	Invitrogen	15338-100	lot: 178168
PLUS Reagent	fection rea	Invitrogen		
G418 sulfate		PAA	P02-012	lot: P01211-2715
Lumox dish	dissection	Carl Zeiss	94.6077.3	lot: 13070655

Palm software	Carl Zeiss microscopy GmBH	PALMRobot 4.6

BECN1 primers Forward (CCG-AAG-ACT-GAA-GGC-AAG-TC)	Eurogentec	custom

BECN1 primers Revers (GAC-GTT-GAG-CTG-AGT-GTC-CA)	Eurogentec	custom

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Title of Article: Unicellular selection of living cells in liquid medium using laser microdissection and pressure catapulting system

Author(s): Diaddin Hamdan, Guillaume Gapihan, Morad El Bouchtaoui, Wang Li, Anne Janin, Guilhem Bousquet, Christophe Leboeuf

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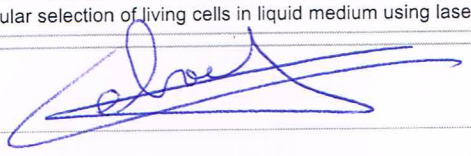
Signature:	 Date: 16/03/2018

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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 (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.

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 effect[3]. 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 ablation[4,5]. 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 ablation[4,5] "

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, CO_2 in the culture chamber, temperature at 37 °C) (Cree AI. Methods Mol Biol. 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, CO_2, 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].”

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%.

3.2. 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.