Video Article

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

Diaddin Hamdan[1], Guillaume Gapihan[1], Morad El Bouchtaoui[1], Wang Li[1,2], Anne Janin*[1,3], Guilhem Bousquet*[1,4,5], Christophe Leboeuf*[1]

[1]U1165, Université Paris-Diderot, INSERM

[2]Pôle de Recherches Sino-Français en Science du Vivant et Génomique, Shanghai Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine

[3]Laboratoire de Pathologie, AP-HP-Hôpital Saint-Louis

[4]Service d'Oncologie Médicale, AP-HP-Hôpital Avicenne

[5]Université Paris 13

*These authors contributed equally

Correspondence to: Diaddin Hamdan at diaddin_h@hotmail.com, Christophe Leboeuf at christophe.leboeuf@univ-paris-diderot.fr

URL: https://www.jove.com/video/58234
DOI: doi:10.3791/58234

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

Date Published: 8/6/2018

Citation: Hamdan, D., Gapihan, G., Bouchtaoui, M.E., Li, W., Janin, A., Bousquet, G., Leboeuf, C. Unicellular Selection of Living Cells in Liquid Medium Using Laser Microdissection and a Pressure Catapulting System. *J. Vis. Exp.* (), e58234, doi:10.3791/58234 (2018).

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[6,7,8] 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[15,16,17]. 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. 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.
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.
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.
4. Centrifuge for 2 min at 250 x *g* at room temperature and, then, remove the supernatant.
5. Rinse the cell pellet with 40 mL of 1x PBS to recover as many cells as possible.
6. Discard the supernatant and, then, resuspend the pellet with 5 mL of PBS.
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.
8. Centrifuge the cell suspension for 2 min at 250 x *g* and, then, discard the supernatant.

2. Cancer Stem Cell Selection

1. Resuspend the pellet with 5 mL of PBS, centrifuge it at 250 x *g* for 5 min, and remove the supernatant.
2. Resuspend the sample to obtain a concentration of 10^7 cells for 400 µL of cell-sorting buffer.
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.
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.
    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.
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

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%.
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. 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].

1. Remove the culture medium delicately by slow aspiration of the supernatant using a pipette.
2. Meanwhile, prepare the following.
    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.
    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.
3. Add 15 µL of lipid-based transfection reagent and leave it for 30 min at room temperature.
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.

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.
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

1. Pool two plates (*i.e.*, 12 wells) containing transfected cells from step 4.5 in a 50-mL tube.
2. Use a 1-mL pipette for pooling the cells in the wells to achieve sphere dissociation.
3. Centrifuge the tumor cells from the dissociated sphere in their culture medium at 250 x *g* for 10 min.
4. Suspend the cells to the concentration of 3,000 cells/µL of culture medium (see step 3.1).
5. Take 20 µL of this cell solution and place it on the membrane of the microdissection dish.
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.
7. Draw the boundaries with a pencil around the smear and position the microscope directly over the target area.
8. Use the microdissector software with a GFP filter and delineate isolated GFP-positive cells using the freehand tool in the microdissector.
9. Set the laser to the cutting mode and activate it with a left click on the software button.
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.
    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
11. Unload the samples and caps containing the cells.

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

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

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.
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.

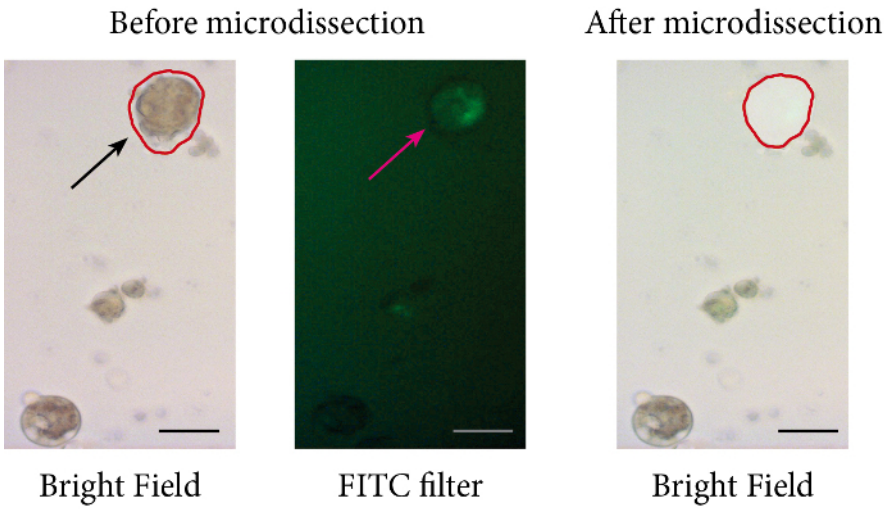

Figure 1: Laser microdissection of live GFP+ breast cancer stem cells. On the left panel, one GFP-positive breast cancer stem cell invalidated for the *BCEN1* gene is identified with the FITC filter of the laser microdissector (GFP gene and BECN1). In the photo on the left, the cell identified as positive 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.
Please click here to view a larger version of this figure.

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.

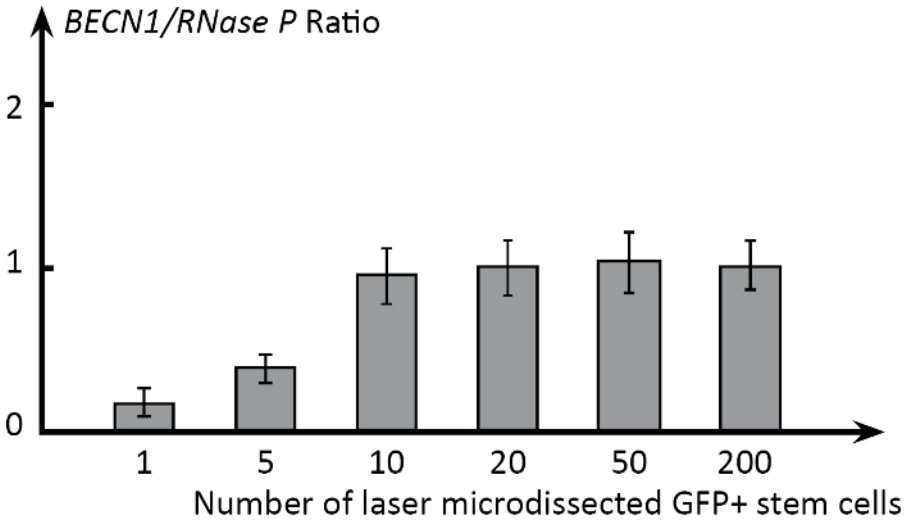

Figure 2: Copy number variation (CNV) of the *BECN1*/*RNase P* ratio using digital droplet PCR.
This panel shows the ratio of the autophagy gene *BECN1* to the standard CNV reference *RNase P* using droplet digital PCR on a minimum of 10 GFP-positive breast cancer stem cells. The error-bars represent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

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.

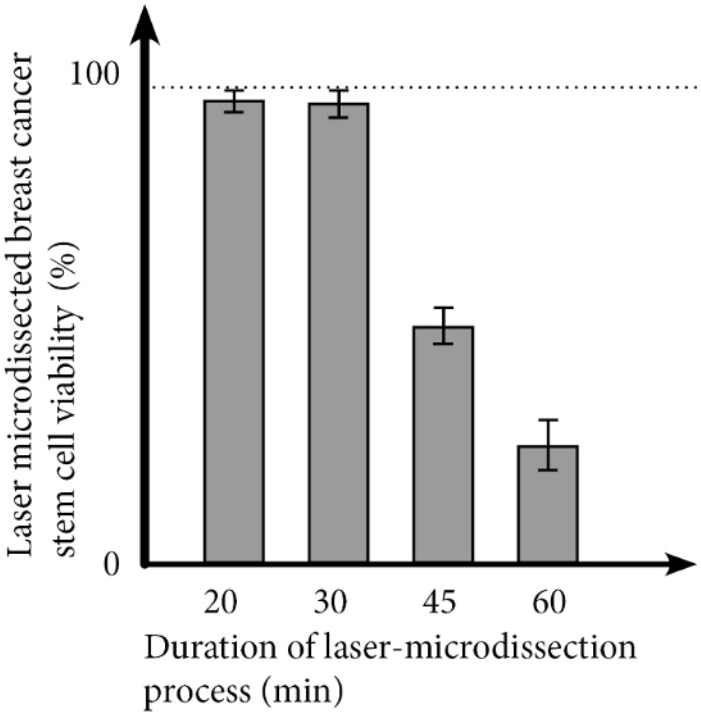

Figure 3: Assessment of the breast cancer stem cell viability. To calculate live-cell percentages after different durations of laser microdissection and after a 24-h incubation period in culture medium, breast cancer stem cell viability was assessed on 200 cells on a special slide, using trypan blue. The error-bars represent the standard deviation. Please click here to view a larger version of this figure.

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.

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 1: Composition of DMEM medium.

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

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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors would like to thank S. Leyshon and A. Swaine for revising the English text written here. The work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), Paris Diderot University.

References

1. Datta, S. *et al.* Laser capture microdissection: Big data from small samples. *Histology and Histopathology.* **30** (11), 1255-1269 (2015).
2. Schutze, K., Lahr, G. Identification of expressed genes by laser-mediated manipulation of single cells. *Nature Biotechnology.* **16** (8), 737-742 (1998).
3. Vogel, A., Venugopalan, V. Mechanisms of pulsed laser ablation of biological tissues. *Chemical Reviews.* 1**03** (2), 577-644 (2003).
4. Vogel, A. *et al.* Mechanisms of laser-induced dissection and transport of histologic specimens. *Biophysical Journal.* **93** (12), 4481-4500 (2007).
5. Horneffer, V., Linz, N., Vogel, A. Principles of laser-induced separation and transport of living cells. *Journal of Biomedical Optics.* **12** (5), 054016 (2007).
6. Verneuil, L. *et al.* Donor-derived stem-cells and epithelial mesenchymal transition in squamous cell carcinoma in transplant recipients. *Oncotarget.* **6** (39), 41497-41507 (2015).
7. Furet, E. *et al.* Increased risk of brain metastases in women with breast cancer and p16 expression in metastatic lymph-nodes. *Oncotarget.* **8** (23), 37332-37341 (2017).
8. Bousquet, G. *et al.* Tracking sub-clonal TP53 mutated tumor cells in human metastatic renal cell carcinoma. *Oncotarget.* **6** (22), 19279-19289 (2015).

9. Chung, C. Y. *et al.* Cell type-specific gene expression of midbrain dopaminergic neurons reveals molecules involved in their vulnerability and protection. *Human Molecular Genetics.* **14** (13), 1709-1725 (2005).
10. Stich, M. *et al.* Live cell catapulting and recultivation. *Pathology Research and Practice.* **199** (6), 405-409 (2003).
11. Mayer, A., Stich, M., Brocksch, D., Schutze, K., Lahr, G. Going *in vivo* with laser microdissection. *Methods in Enzymology.* **356**, 25-33 (2002).
12. Chaudhary, K. W. *et al.* Embryonic stem cells in predictive cardiotoxicity: laser capture microscopy enables assay development. *Toxicological Sciences.* **90** (1), 149-158 (2006).
13. Terstegge, S. *et al.* Laser-assisted selection and passaging of human pluripotent stem cell colonies. *Journal of Biotechnology.* **143** (3), 224-230 (2009).
14. Langer, S., Geigl, J. B., Ehnle, S., Gangnus, R., Speicher, M. R. Live cell catapulting and recultivation does not change the karyotype of HCT116 tumor cells. *Cancer Genetics and Cytogenetics.* **161** (2), 174-177 (2005).
15. Bousquet, G. *et al.* Targeting autophagic cancer stem-cells to reverse chemoresistance in human triple negative breast cancer. *Oncotarget.* **8** (21), 35205-35221 (2017).
16. Bousquet, G. *et al.* Individual xenograft as a personalized therapeutic resort for women with metastatic triple-negative breast carcinoma. *Breast Cancer Research.* **16** (1), 401 (2014).
17. Paquet, C. *et al.* Effect of anti-cancer drugs on microglia in patient-derived breast cancer xenografted mouse models. *Neuropathology.* **37** (1), 91-93 (2017).
18. Beier, D. *et al.* CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Research.* **67** (9), 4010-4015 (2007).
19. Reyes, E. E. *et al.* Molecular analysis of CD133-positive circulating tumor cells from patients with metastatic castration-resistant prostate cancer. *Journal of Translational Science.* **1** (1), (2015).
20. Grosse-Gehling, P. *et al.* CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. *The Journal of Pathology.* **229** (3), 355-378 (2013).
21. Hodne, K., Weltzien, F. A. Single-Cell Isolation and Gene Analysis: Pitfalls and Possibilities. *International Journal of Molecular Sciences.* **16** (11), 26832-26849 (2015).
22. Vogel, A. *et al.* Principles of laser microdissection and catapulting of histologic specimens and live cells. *Methods in Cell Biology.* **82**, 153-205 (2007).
23. Venugopalan, V., Guerra 3rd, A., Nahen, K., Vogel, A. Role of laser-induced plasma formation in pulsed cellular microsurgery and micromanipulation. *Physical Review Letters.* **88** (7), 078103 (2002).
24. Rau, K. R., Quinto-Su, P. A., Hellman, A. N., Venugopalan, V. Pulsed laser microbeam-induced cell lysis: time-resolved imaging and analysis of hydrodynamic effects. *Biophysical Journal.* **91** (1), 317-329 (2006).
25. de With, A., Greulich, K. O. Wavelength dependence of laser-induced DNA damage in lymphocytes observed by single-cell gel electrophoresis. *Journal of Photochemistry and Photobiology B.* **30** (1), 71-76 (1995).
26. Bertling, C. J., Lin, F., Girotti, A. W. Role of hydrogen peroxide in the cytotoxic effects of UVA/B radiation on mammalian cells. *Photochemistry and Photobiology.* **64** (1), 137-142 (1996).
27. Frost, A. R., Eltoum, I. E., Siegal, G. P., Emmert-Buck, M. R., Tangrea, M. A. Laser Microdissection. *Current Protocols in Molecular Biology.* **112** (1), 25A.1.1-25A.1.30 (2015).

