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Oleg V. Podgorny

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TECHNICAL NOTE

Live cell isolation by laser microdissection with gravity transfer

Oleg V. Podgorny[a,b]
[a]Koltzov Institute of Developmental Biology of Russian Academy of Sciences, Research scientist at Laboratory of Experimental Neurobiology, Vavilov Street, 26, Moscow 119334, Russia
[b]Faculty of Nano-, Bio-, Information and Cognitive Technologies, Institute of Physics and Technologies (State University), Research scientist at Brain Stem Cells Laboratory, Institutskii per. 9, Dolgoprudny, Moscow Region, 141700 Russia

Abstract. Laser microdissection by pulsing ultraviolet laser allows the isolation and recultivation of live cells based on morphological features or/and fluorescent labelling from adherent cell cultures. Previous investigations described only the use of the laser microdissection and pressure catapulting (LMPC) for live cell isolation. But LMPC requires complex manipulations and some skill. Furthermore, single-cell cloning using laser microdissection has not yet been demonstrated. The first evidence of successful application of laser microdissection with gravity transfer (LMDGT) for capturing and recultivation of live cells is presented. A new strategy for LMDGT is presented because of the failure to reproduce the manufacturer's protocol. Using the new strategy, successful capturing and recultivation of circle-shaped samples from confluent monolayer of HeLa cells was demonstrated. It was found that LMDGT is easier than LMPC because it doesn't require personal participation of investigator in transferring of isolated samples to final culture dishes. Moreover, for the first time, the generation of clonal colonies from single live cells isolated by laser microdissection was demonstrated. Data obtained in this study confirm that LMDGT is a reliable and high-yield method allowing isolation and expansion of both cell clusters and single cells from adherent cell cultures. [DOI: 10.1117/1.JBO.18.5.055002]

Keywords: laser microdissection; cell culture; recultivation; cell separation; isolation of live cells.

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1 Introduction

In biomedical research, selection and isolation of single live cells or subpopulations of live cells with identical characters from mixed populations is often necessary to obtain cell lines or homogeneous cell populations. Most strategies, including well known methods like fluorescence- and magnetic-activated cell sorting, cloning by limiting dilution, panning, affinity column chromatography, and new techniques based on microfluidic devices, dielectrophoresis and laser-induced forward transfer, work for selective isolation of non-adherent cells only.[1–4] A considerable disadvantage of these strategies is mechanical or enzymatic digestion of the cells required before isolation procedure, since it makes the separation based on morphological features of the cells impossible. Moreover, enzymatic digestion can trigger diverse signaling cascades, which affect the cell fate after plating. Currently, several techniques for selective isolation of live cells from adherent cell cultures exist: manual[5] and automated cell picking (commercial platforms: ClonePix from Molecular Devices, UK; and CellCelector from ALS Automated Lab Solutions GmBH, Germany), laser-mediated elimination (negative selection of the cells),[6] laser-based release of micropallets from arrays[7–10] and laser microdissection.[11]

Unlike other techniques, laser microdissection allows contact free capturing areas of various shapes and sizes containing live cells. Moreover, during the isolation process the cells remain attached to the special base (polymer foil). Automation of the procedure and high precision of cutting prevent contamination by unwanted cells. Initially, laser microdissection by pulsing UV-A (wavelength range 315 to 400 nm) lasers was developed for capturing defined cell clusters or individual cells from slices of fixed tissue for downstream molecular analysis. This technique allows isolation of purified cell populations from heterogeneous tissue slices. Genomic and mitochondrial DNA, RNA, proteins and metabolites can be extracted from the captured samples to perform various downstream molecular assays.[12–24] Laser microdissection allows the capture live cells from adherent cell cultures according to morphological criteria, growth behavior, defined surface markers or defined gene expression visualized by a reporter such as green fluorescent protein. Capturing of live cells according to a variety of different criteria allows accurate separation of mixed cell populations and subsequent analysis of different subpopulations.

Mayer and co-workers were the first to use laser microdissection and pressure catapulting (LMPC) for live cell capturing.[11] They demonstrated that clusters of the live cells EJ-28 (bladder carcinoma cell line) and TPC-1 (thyroid carcinoma cell line) survive after the LMPC procedure and continue to grow in culture.[11] The principle of LMPC for live cell capturing is as follows. The cells are grown onto a thin, UV-absorbing polymer foil that is mounted into a transparent culture dish (specimen). Under control of inverted microscope a region of interest with the live cells is separated from the rest of the specimen by ablation of the polymer foil using a sequence of UV-A laser pulses focused through objective lens. The dissected region

Address all correspondence to: Oleg V. Podgorny, Koltzov Institute of Developmental Biology of Russian Academy of Sciences, Research scientist at Laboratory of Experimental Neurobiology, Vavilov Street, 26, Moscow 119334, Russia. Tel: +7 499 135 30 55; Fax: +7 499 135 80 12; E-mail: olegpodgorny@inbox.ru

(sample) is catapulted into the cap of a microfuge tube filled with culture medium by application more energetic laser pulse. The microdissected and catapulted sample inside the collection cap is sealed with tube and centrifuged into tube. The investigator then gently transfers the sample by micropipette from the tube into a culture dish filled with medium.

It was found that application of LMPC does not affect the cell proliferation rate, and cells are still viable after two subsequent LMPC procedures.[25] Five cycles of cell isolation by LMPC and recultivation did not affect the karyotype of genetically stable colorectal cancer cell line HCT116.[26] It was also shown that LMPC had no influence on the karyotype of human embryonic stem cell lines H9.2 and I3.[27]

The comparison of the efficiency of LMPC and manual isolation of human embryonic stem cell colonies revealed no significant differences in cell viability ($80.6 \pm 8.7\%$ and $88.6 \pm 1.7\%$, respectively).[27] The study of cells replated by the LMPC procedure showed no significant differences from the controls in expression of pluripotency-associated markers. However, transfer efficiency and colony formation (replating efficiency) were lower after the LMPC procedure ($72.5 \pm 7.3\%$ versus $98.6 \pm 0.9\%$ and $36.4 \pm 9.2\%$ versus $53.3 \pm 11.5\%$, respectively).

Horneffer and co-workers[28] described the strategy of LMPC of live cells and discussed side effects of this technique. They suggested keeping of minimized level of the liquid layer above the cells allowing reduction in energy of catapulting laser pulse. They also found that catapulting by laser pulse focused at the periphery of the sample is preferable to catapulting by a defocused laser pulse. The recultivation rate of catapulted cells is much higher when a focused pulse is used. The focused laser pulse causes a fast rotational movement that minimizes the flow of culture medium parallel to the sample surface, thus decreasing the shear stress applied to live cells. They also revealed that side effects by heat and UV exposure applied to live cells play only a minor role in comparison to mechanical damage. This study allowed further optimization of LMPC process.

In spite of some successful applications of LMPC listed above, there is limited number of publications using LMPC for live cells capturing and recultivation. One probable explanation is that LMPC requires complex manipulations and some investigator's skill. To date publications describing alternative techniques of laser microdissection of live cells are also absent. One of these techniques, patented by Leica Microsystems (Germany), is laser microdissection with gravity transfer (LMDGT). The principle of LMDGT for capturing of live cells suggested by manufacturer is as follows. The cells are grown on a thin, UV-absorbing polymer foil that is mounted on the bottom of Petri dish. Before the laser microdissection, culture medium is removed as much as possible, leaving the liquid layer just covering the cells. Petri dish without a cover is mounted into upright microscope. A region of interest with the live cells is cut from the rest of specimen by a sequence of UV-A laser pulses focused through objective lens. Laser pulses not only ablate the polymer foil and the cells but also evaporate a small layer of liquid above the cells. As a result, the sample after cutting falls into a well of an 8-well strip or into another Petri dish filled with culture medium placed directly under specimen. The main advantage of LMDGT in comparison to LMPC is direct collection of samples into culture dish that makes this technique potentially more efficient and easier than LMPC. But the tries to reproduce manufacturer's protocol in our initial experiments failed. Because of this reason the goal of this study was to find a new strategy for LMDGT which allows efficient, easy and reproducible isolation of live cells for further recultivation. Additionally, we tested the possibility of single-cell cloning using laser microdissection, because this possibility has not yet been demonstrated.

2 Materials and Methods

2.1 *Laser Microdissection Instrumentation*

In this study, we used the Leica LMD7000 laser microdissection system (Leica Microsystems, Germany) installed on an upright microscope and equipped with a Nd:YLF laser ($\lambda = 349$ nm, pulse duration—4 ns, maximum pulse energy—120 μJ, tunable pulse frequency 10-5000 Hz). Two objectives were used: 10×/0.3 and 20×/0.4. The instrument was equipped with a climatic chamber providing temperature control.

2.2 *Cell Culture*

HeLa cells (human cervical carcinoma cell line) were used for the experiments. The cells were cultured in 25 cm^2 (50 ml) culture flasks in culture medium: DMEM with 4.5 g/l glucose (Invitrogen), 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) at 37°C with 5% CO_2. The cells were passaged and harvested using a standard trypsinization procedure. For microdissection 2 ml of cell suspension (4×10^5 cells/ml) were inoculated in special Petri dish (Leica Microsystems, diameter 50 mm). The polyethylene naphthalate (PEN) foil (2 μm thickness) is mounted on the bottom of this Petri dish. To improve cell adhesion, the inside surface of PEN foil was pre-coated with poly-L-lysine (0.01% poly-L-lysine solution, MW: 70000-150000, Sigma). The dish was placed into conventional plastic Petri dish (diameter 90 mm) to avoid contamination of downside surface of foil. Cells were grown until confluent monolayer formation (usually next day after plating), because, as was found in previous study, best results for quick regrowth are achieved if the source culture dish is almost confluent.[25] Before microdissection cells were washed two times in 2 ml of Hanks' balanced salt solution (HBSS). Then, 1 ml of fresh HBSS was added, and the dish without a cover was put into the specimen holder and mounted on the stage of the microscope. In this case the height of the liquid layer above the cells was 580 ± 18 μm, as determined by the difference of focal planes between the upside of liquid and the foil surface (measurements were performed at randomly selected 10 points on the working field of Petri dish using 40×/0.6 objective). The 8-well strips (Greiner Bio One) were used for collection of dissected samples. The strips were filled with 350 μl of the culture medium prior microdissecion. Then strips were mounted into the substage directly under the Petri dish. To capture single live cells, we inoculated low density cell suspension (5000 cells/ml) into the Petri dish with PEN foil and isolated individual cells by LMDGT 12 hours after plating.

2.3 *Laser Microdissection Procedure*

Isolation of circle-shaped samples containing either confluent cell monolayer or single cells and capturing them into wells of 8-well strips was performed according to the new strategy for LMDGT (see Sec. 3).

2.4 *Recultivation Efficiency Assay*

The recultivation efficiency was determined as relation of amount of samples which gave rise to new cell colonies to overall amount of microdissected samples. The recultivation efficiency was measured for samples with diameters of 50, 100, 200, 300 and 600 μm. The recultivation efficiency was determined in five unrelated cutting sessions. Twenty samples were cut into five wells of the strip in each unrelated session. Colony counting was performed at day five after isolation. The criterion of growing colony was the expansion of cells outside the foil piece.

2.5 *Cell Viability Assessment after Laser Microdissection*

To assess cell viability after laser microdissection, we used double staining with propidium iodide (PI) and Sybr Green (SG).[29] Before microdissection, HeLa cell monolayers were stained for 3 min with 1 μg/ml of PI (Sigma, USA) in HBSS followed by triple washing in fresh HBSS. Then, circle-shaped areas with different diameters (50, 100, 200, 300 and 600 μm) contained only viable cells (all cells in the area were PI-negative) were selected and microdissected. Immediately after microdissection, captured samples (40 samples of each size) were incubated in HBSS containing two dyes: PI (1 μg/ml) and Sybr Green (1:2000, 10000× stock solution, DNK-Sintez, http://www.oligos.ru) during 3 min followed by triple washing with fresh HBSS. Stained samples were visualized and photographed with an Olympus IX51 epifluorescent microscope. Live and dead cells were manually counted on images.

3 Results

3.1 *New Strategy for Laser Microdissection with Gravity Transfer*

The principle LMDGT for live cell isolation suggested by manufacturer has an essential disadvantage as we found in our initial tries to capture clusters of live cells. A very thin layer of liquid left in the Petri dish is evaporated quickly without cover at 37°C. Because of this reason, the liquid layer should be gently controlled during the procedure to avoid drying of cells. We found that control of liquid layer is very difficult during the LMDGT procedure. It makes LMDGT of live cells inefficient. To overcome this disadvantage we used an amount of liquid which excluded fast evaporation providing the prolonged cutting session. We found that 1 ml of HBSS (the height of the liquid layer above the cells is 580 ± 18 μm) is enough for about 40 minutes of cutting session without interruption. If a longer time was necessary, we completely removed the liquid and added 1 ml of fresh HBSS every 40 min.

If 1 ml of liquid is used, the sample does not fall into the collector (8-well strip) because the force of surface tension of the liquid holds the sample at the plane of foil. Because of this reason we performed LMDGT in two steps: (1) we cut the region of interest from the rest of specimen (cutting), and then (2) shot down the dissected sample into the collector by applying a laser pulse to the rim of the sample (extracting). In automatic mode "Draw and Cut" of the microdissection software, we circumscribe the circle regions of interest on the live image from CCD camera installed on microscope and then press "Start Cut" bottom. The laser beam focused through the objective [Fig. 1(a)] is guided over the cutting line and the circle

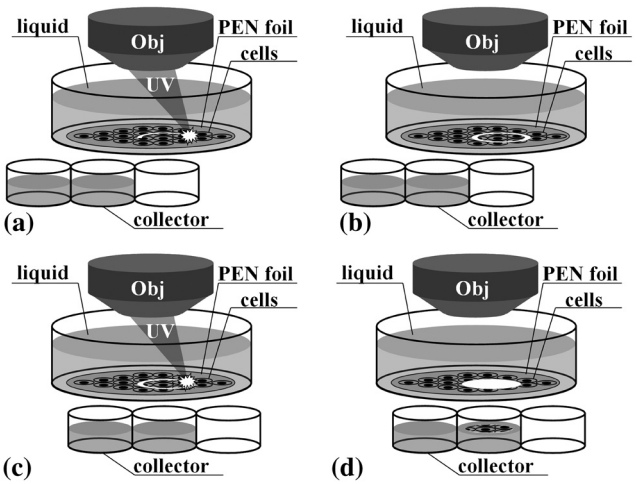


Fig. 1 New strategy of the laser microdissection with gravity transfer.

region is dissected from the rest of specimen. After dissection the sample, which is held by the force of the surface tension of the liquid [Fig. 1(b)], is shot down into a well of the 8-well strip by application of the laser pulse focused at the rim of sample [Fig. 1(c)]. To make it we use manual mode "Move and Cut" of the microdissection software, adjust the mouse cursor at the rim of sample on live image and click left button of the mouse to apply shot laser pulse. This procedure results in the falling of the sample onto the surface of liquid in the well placed directly under the Petri dish [Fig. 1(d)].

If a well of the strip filled with culture medium is placed directly under Petri dish, the evaporating liquid condenses at the downside of the foil. This affects searching of the region of interest and the laser microdissection procedure. Because of this reason at least one well should always be kept empty in each strip. For the cutting step, the empty well of the 8-well strip is placed directly under the Petri dish [Fig. 1(a) to 1(b)]. This empty well excludes liquid drops scattering during laser microdissection and also prevents falling of foil and cell fragments onto the condenser. To capture a sample, the well with the culture medium is placed right under the Petri dish [Fig. 1(c)], and the dissected sample is shot down by laser pulse into the well [Fig. 1(d)].Using this strategy, we can fill seven wells of an 8-well strip with individual samples. After laser microdissection, the strip is removed from the substage of the system, and samples floated on surface of liquid are sent to the bottom by adding the extra droplet of culture medium into each well of strip. Then the strip is inserted into the strip-plate (Grainer Bio One), covered by lid and placed into incubator for further recultivation.

3.2 *Laser Settings for Cutting and Extracting*

Correct laser settings are crucial to cut and shoot down samples. The main strategies for empirical searching of optimal laser settings for the cutting step were as follows. The first one was minimization of laser power. The value of setting "Power" should be sufficient to cut and any decreasing of this value will result in a failure of cutting. The second one was the exclusion of cavitation bubbles formation. Cavitation bubbles are generated in a consequence of laser-induced plasma formation in liquid during microdissection.[27,28] They have a strong negative effect on the cutting process by laser microdissection system installed on an

Table 1 Optimal values of laser settings for live cell laser microdissection from HeLa cell monolayer determined for objectives with magnifications 10× and 20×. RU—in relative units.

Setting	10×	20×
Power (RU):	40	40
Aperture (RU):	1	1
Speed (RU):	6	6
Head current (%):	100	100
Pulse frequency (Hz):	120	120
Offset (RU):	30	120

upright microscope, because they are formed into the laser beam pathway and, as a consequence, scatter the light. To overcome this trouble, the laser settings "Power," "Speed" (speed of laser movement), and "Pulse Frequency" should have such values which allow ablation of the foil across its thickness by every laser pulse. In this case, the generated plasma will not be confined between the foil and the liquid that prevents formation of the cavitation bubbles.

The laser setting "Aperture" sets the angle of the cone of the laser beam that exits the lens. A low value of "Aperture" is preferable because in this case, the laser focal spot has high extension along Z axis. This excludes strong dependence of Z axis position of laser focal spot from the gradually declining height of the liquid above the cells in consequence of its evaporation during the laser microdissection procedure.

Empirically found optimal laser settings for 10× and 20× objectives are presented in Table 1. Almost identical values of settings for 10× and 20× objectives (differences only in parameter "Offset") can be explained by close values of numerical apertures of objectives (0.3 and 0.4, respectively). These settings provided reproducible dissection with widths of cut 11 ± 1 and 7 ± 1 μm, respectively as measured in ten unrelated cuts for both objectives.

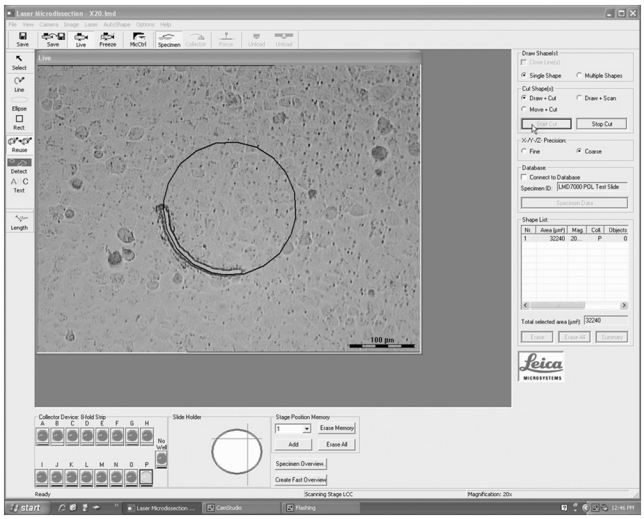
We performed an empirical searching of laser settings to shoot down the circle samples with different diameters in manual mode "Move and Cut" of microdissection software. The main strategy for searching was minimization of laser power to shoot down sample, i.e., laser power should be sufficient only to eject the sample from plane of foil of the Petri dish. Then the ejected sample freely falls down into well by the force of gravity. To achieve this condition, it was accepted to set "Pulse Frequency" always to the minimal value of 10 Hz. To find the optimal settings for "Power" and "Aperture," a sequential decreasing of their values to shoot down samples with different diameters was performed. The optimal settings for "Power" and "Aperture" were accepted when these settings had achieved such values that any decreasing resulted in failure of extracting of the sample. These values are presented in Table 2. It was found that circle-shaped samples with a diameter of 800 μm could not be shot down with any laser settings. The samples with diameter of 700 μm were shot down with an efficiency lower than 20% (measured from 100 attempts).

The movie demonstrating LMDGT procedure (cutting and extracting) of the 200 μm circle-shaped sample from HeLa cell monolayer is presented in Video 1 (MPEG, 9.8 MB).

3.3 *Recultivation Efficiency and Cell Viability after Laser Microdissection*

The new strategy of LMDGT allows capture of live cell clusters from confluent cell monolayer. The estimation of recultivation efficiency from the circle-shaped samples of different diameters is presented in Table 3. We found that no colonies formed from samples with a diameter of 50 μm. About 50% of the samples of 100 μm gave rise to growing colonies, and 100% of samples with larger diameter gave rise to growing colonies. Generation of new cell colonies from samples with different diameters: 100 (a), 200 (b), 300 (c) and 600 (d) μm is presented in Fig. 2.

We found that it is possible to cut and capture more than 20 samples during 40 min of cutting session. To prolong a cutting



Video 1 LMDGT procedure (cutting and extracting) of the 200 μm circle-shaped sample from HeLa cell monolayer (MPEG, 9.8 MB) [URL: http://dx.doi.org/10.1117/10.1117/1.JBO.18.5.055002.1.

Table 2 Optimal values of 'Power' and 'Aperture' settings for extracting of circle-shaped samples with different diameters using 10× and 20× objectives. RU—in relative units.

Sample diameter (μm)	10× objective					20× objective					
Adjustment	**700**	**600**	**500**	**400**	**300**	**300**	**250**	**200**	**150**	**100**	**50**
Power (RU)	60	50	45	40	40	40	40	40	50	45	40
Aperture (RU)	55	55	55	55	30	40	27	20	1	1	1

Table 3 Recultivation efficiency after laser microdissection of circle-shaped samples with different diameters.

Sample diameter, μm	**50**	**100**	**200**	**300**	**600**
Recultivation efficiency, %	0	55 ± 6	100	100	100

session, Hanks' solution was renewed as described above. It was found that renewal can be repeated at a minimum of two times (total duration of cutting session in this case is 3×40 min) without a decrease in recultivation efficiency (analyzed for samples with different diameters). Additional renewals were not tested in our study.

To investigate damaging effect during cutting and extracting, we stained a HeLa cell monolayer with PI before LMDGT procedure to mark dead cells and then collected samples with different diameters from areas which didn't contain dead cells (Fig. 3). Double staining with PI and SG immediately after LMDGT procedure revealed that dead cells (stained with PI) were predominantly distributed along the periphery of the samples and around the hole which appeared as a consequence of the application of a laser pulse for extracting at the rim of sample (Fig. 4). Dead cells appeared as a one-cell wide layer along the periphery of sample. The damage zone (dead cells) around the hole was extended according to sample size [Fig. 4(b) to 4(d), arrows], because more powerful laser pulses were applied to shoot down larger samples. The analysis of live cell distribution through the samples revealed that cells remain viable when they are positioned as close as 10 μm to the rim of the sample [Fig. 4(b), arrowhead].

The diameters of the holes appeared in consequence of the application of laser pulse for extracting were 10 ± 2 μm for the 100 μm samples, 15 ± 2 μm for the 200 μm samples,

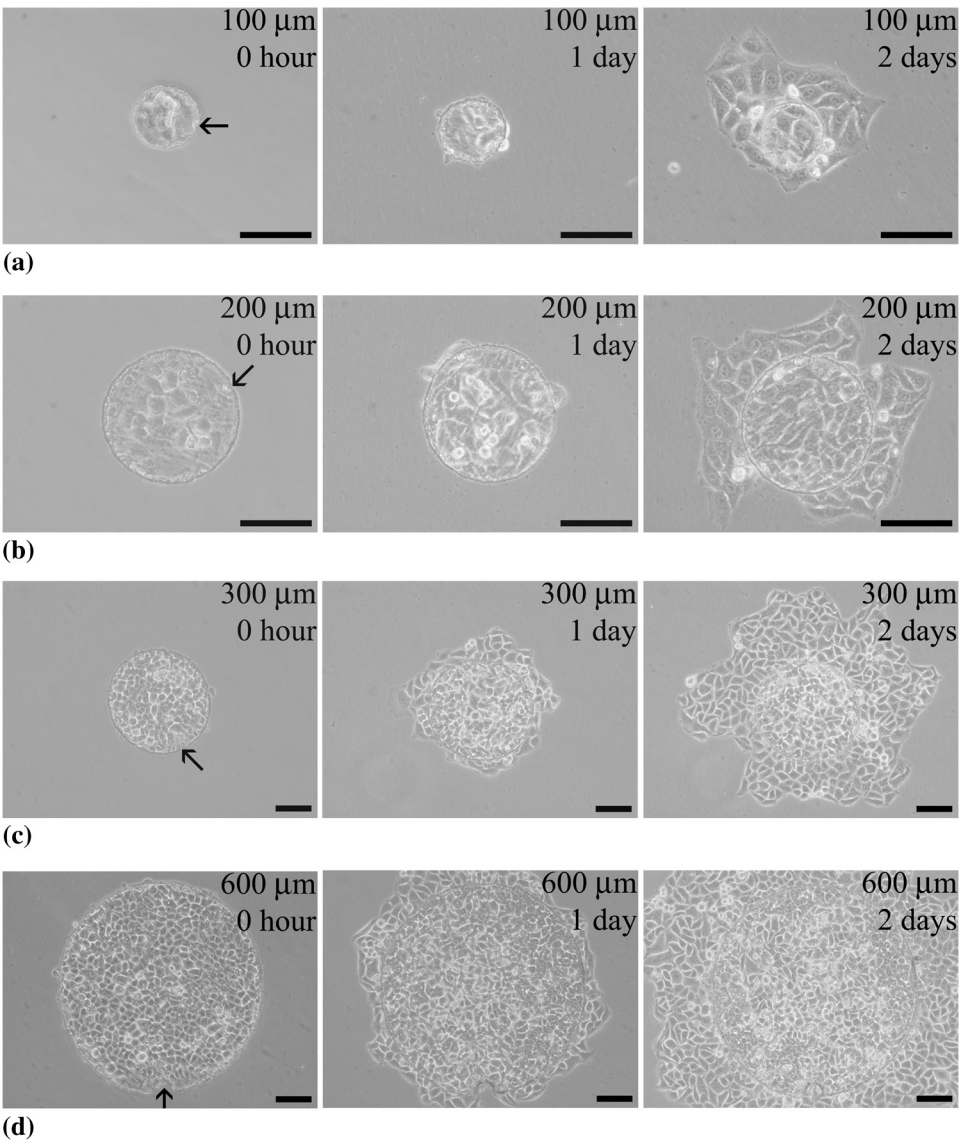


Fig. 2 Formation of new cell colonies from samples with different diameters: (a) 100, (b) 200, (c) 300 and (d) 600 μm. Microphotographs for samples of each diameter were obtained at 0 h, 1 day and 2 days after LMDGT. Arrows show holes appeared after application of laser pulse for extracting of the sample. Scale bars: 100 μm.

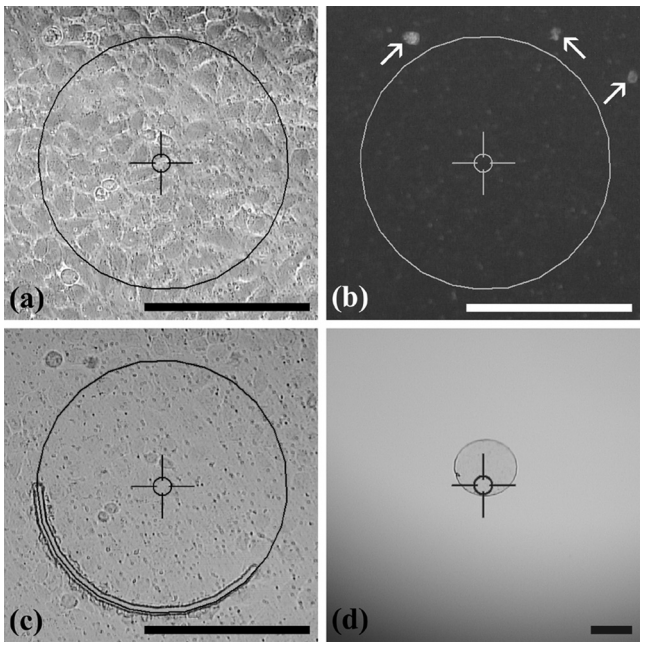


Fig. 3 (a) Circle area of confluent HeLa cell monolayer selected for cutting (phase contrast). (b) Selected area does not contain dead cells [prestaining with PI (arrows)], fluorescent mode of laser microdissection instrument. (c) Cutting process of selected area (bright field). (d) Sample into collector immediately after cutting and extracting. Scale bars: 200 μm.

20 ± 4 μm for the 300 μm samples and 50 ± 8 μm for the 600 μm samples.

Double staining (PI and SG) of samples with different diameters (40 samples for each diameter) revealed that about 50% (19 from 40) of 100 μm samples contained no viable cells. Samples with diameters of 200, 300 and 600 μm always contained viable cells. The cell viability was determined for samples contained viable cells (Table 4). The cell viability increases according to the size of sample from $15 \pm 7\%$ for the 100 μm samples to $83 \pm 5\%$ for the 600 μm samples.

3.4 *Single-Cell Cloning*

We found that the new strategy of LMDGT not only allows capturing of live cell clusters from a confluent monolayer, but can also be used for isolation of single live cells to generate cell clones. To illustrate this possibility we captured 30 circle-shaped samples with diameters of 200 μm containing a single cell each. All manipulations were performed in the same manner as capturing of live cell clusters from a confluent monolayer. The movie demonstrating the LMDGT procedure of isolation of a sample containing a single cell is presented in Video 2 (MPEG, 10.2 MB). After isolation, the cells grew into clonal colonies expanding on surrounding surface (Fig. 5). In 7 days, individual cells from 21 samples (70% of the total amount of captured samples) gave rise to clonal colonies.

4 Discussion

In this study we developed a new strategy for LMDGT that has several important differences compared to the strategy suggested by the manufacturer. First, our strategy suggests the using of a sufficient amount of the liquid covering the cells that excludes fast evaporation and provides non-interrupting work during 40 min. To prolong cutting it is only necessary to renew the liquid. The total amount of renewals and, as a consequence, the duration of cutting session depend on survival of the cells without 5% CO_2 administration and must be determined empirically for each type of cells. Second, the procedure of LMDGT is performed in two steps: cutting of the area of interest and extracting of dissected sample.

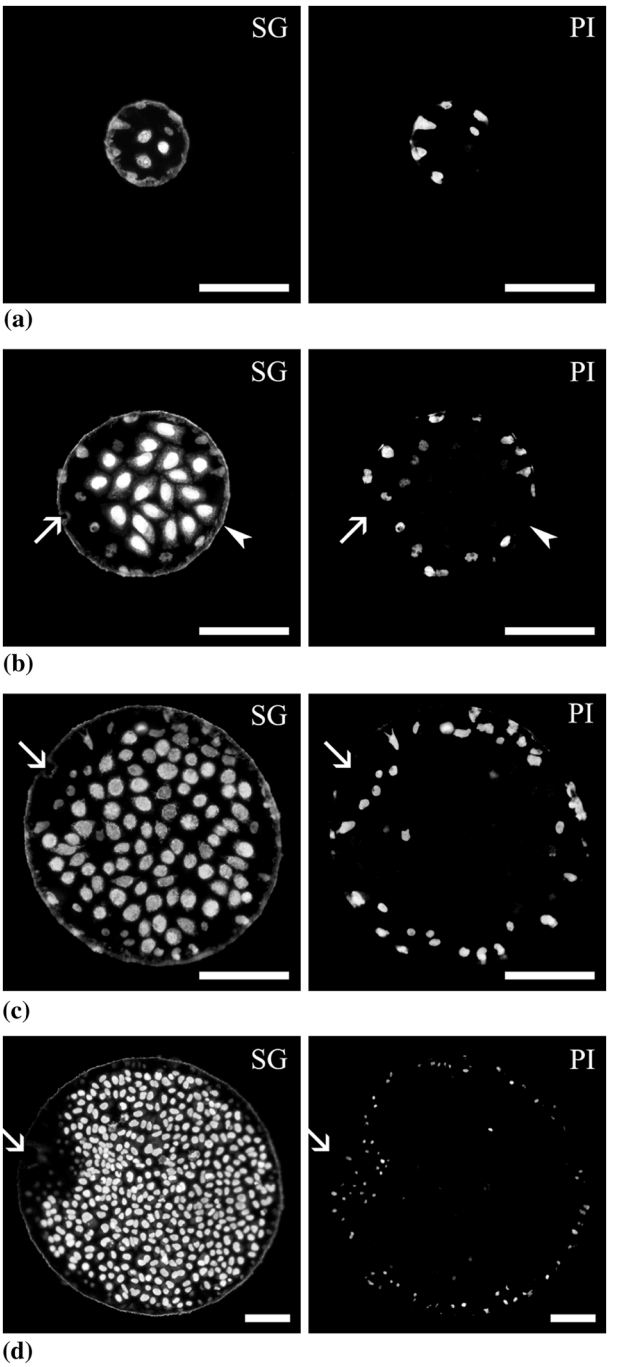
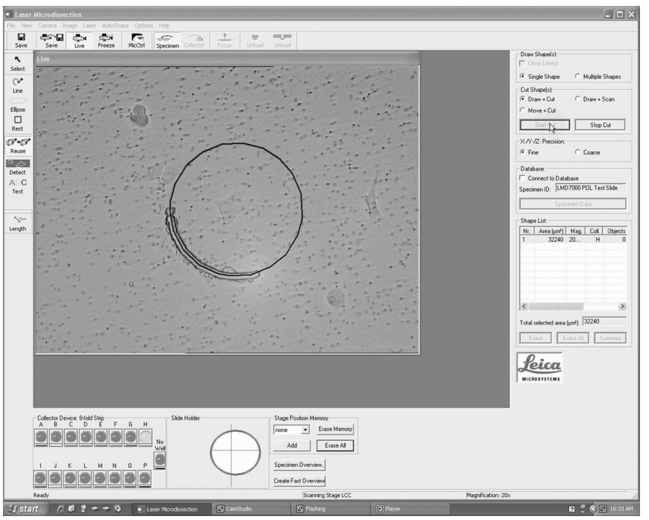


Fig. 4 Distribution of viable and dead cells on samples with different diameters after laser microdissection. Samples were double stained with SYBR Green (SG) and propidium iodide (PI). Sample diameters: (a) 100 μm, (b) 200 μm, (c) 300 μm, (d) 600 μm. Arrows (b) to (d) shoes the hole appeared after application of laser pulse for extracting of the sample. Arrowhead (b) shows the viable cell positioned as close as 10 μm to the rim of sample. Scale bars: 100 μm.

Table 4 The cell viability estimation after laser microdissection for samples of different diameters.

Sample diameter, μm	100 (19 samples)	200 (40 samples)	300 (40 samples)	600 (40 samples)
Total number of cells on sample	12 ± 3	39 ± 8	107 ± 20	444 ± 39
Number of live cells on sample	2 ± 1	20 ± 4	71 ± 15	370 ± 41
Number of dead cells on sample	10 ± 3	20 ± 5	36 ± 7	74 ± 22
Cell viability, %	15 ± 7	50 ± 6	66 ± 4	83 ± 5

We also developed strategies for searching optimal laser settings for cutting and extracting. In contrast to the previous investigation where the authors recommended the application of low energy laser pulses and a high repetition rate ($\geq$1 kHz) for obtaining best results during the cutting step in the LMPC technique,[28] in our case, for a system installed on the upright microscope we found that only application of high energy laser pulses with a low frequency of repetition led to the reproducible cutting without cavitation bubbles formation.



Video 2 LMDGT procedure of the 200 μm circle-shaped sample containing a single HeLa cell (MPEG, 10.2 MB) [URL: http://dx.doi.org/10.1117/1.JBO.18.5.055002.2.

Application of the extracting pulse results in the hole at the periphery of the sample, increasing in diameter in relation to the size of sample, because to shoot down greater samples it was necessary to apply laser pulse with higher energy. In our study holes appeared in 100 and 300 μm samples were sufficiently smaller than holes appeared after catapulting laser pulse application for capturing samples having similar diameters 100 and 350 μm in LMPC technique.[27,28] This evidence suggests that for extracting of the sample in our case it is necessary to apply laser pulses with lower energy than for catapulting in LMPC technique. Importantly, the energy of the catapulting laser pulse should be sufficient to lift out the sample from liquid layer and to overcome both the force of gravity and air resistance having opposite direction to sample movement. In contrast to LMPC, the energy of laser pulse for extracting of the sample in our case should be sufficient only to overcome the force of surface tension holding the sample within the plane of foil, because after extraction the sample freely falls down into the collector. Application of a laser pulse for extracting with lower energy will result in less damage of live cells on the sample.

Our findings indicate that it is impossible to cut and collect the intact colony with a diameter more than 700 μm entirely, i.e., without dissecting to smaller parts. A similar result was obtained in a previous study using the LMPC technique.[30]

Estimation of the recultivation efficiency revealed that all of samples with a diameter of more than 200 μm always gave rise to growing cell colonies. Samples having 50 μm diameter never gave rise to new cell colonies. The recultivation efficiency of 100 μm samples was about 50% (55 ± 6%). That is in strong correlation with cell viability assessment showing that about 50% samples contained no one viable cell after cutting.

For the first time, in this study we demonstrated the successful isolation of single live cells by laser microdissection and generation of clonal colonies from the captured cells. Our data show that the new strategy for LMDGT provides great flexibility for live cell isolation and allows to capture both single cells for generation of cell clones from mixed samples as well as cell clusters for subsampling of heterogeneous cell populations. Utilizing the combination of transmission light and fluorescence microscopy, the technique of LMDGT provides reliable identification, capturing, and expansion of individual cells or cell clusters with

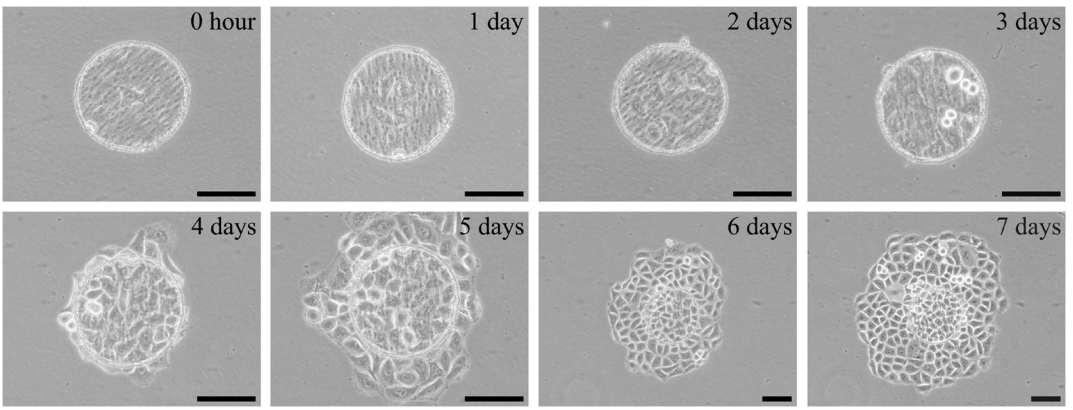


Fig. 5 Formation of clonal colony from a single HeLa cell captured by LMDGT. Sample diameter is 200 μm. Scale bars: 100 μm.

specific characteristics. This will be of use for a variety of biomedical purposes.

Thus, our study presents the first evidence of successful application of LMDGT for live cell isolation. We describe the strategy of laser adjusting for dissection and extracting of circle-shaped samples containing live cells, and show that this strategy results in successful and efficient laser microdissection. Direct automatic collection of the samples into final culture dishes without interference of the investigator is a great advantage of LMDGT and makes it much easier than LMPC. For the first time, we demonstrate that our new strategy for LMDGT allows isolation and capturing of single live cells followed by generation of clonal colonies. Our data suggest that LMDGT is a flexible, reliable, and high-yield method for live cell isolation and capturing.

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