

Specific collection of adherent cells using laser release in a droplet-driven capillary cell

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Abstract. Laser pressure catapulting of adherent cells directly or cells grown on micropallets are two common methods of dislodgement. We describe a method where laser catapulting is performed as a flow is introduced orthogonally in a simple capillary chamber that is inexpensive and obviates flow-generating devices. The moving cells terminate near the contact line within the liquid medium, ensuring that they remain continuously hydrated and where the surface-tension forces hold them in place to permit a later collection process with a receptacle. By dislodging the cells close to the free edge of the liquid chamber, the amount of cell travel and, thus, contamination is minimized. The metrics of cell death and movement show that firing of the laser beam center a distance away from the cell to create a bubble that cavitates over time is more viable with the technique than directly on the cell.

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1 Introduction

Mammalian cells are extensively used in biomedical research to understand the function of the body and to improve the treatment of diseases. During the development of cell lines that are genetically engineered, single cells must be isolated and then cloned from a population. Typically, cells are cultivated on an adherent surface from which specific individual cells selected under the microscope can be studied for criteria such as the cell surface expression of specific proteins detected.¹ A postprocess of removing these cells from the surface allows for analysis of their deoxyribonucleic acid or ribonucleic acid.^{2,3} Mechanical and enzymatic techniques have been traditionally used for this. However, they are fraught by loss of viability and morphology, removal of surface markers, damage to the membrane, and alterations in physiology. In the last two decades, the technique based on laser-capture microdissection (LCM) followed by laser-pressure catapulting (LPC) has been touted to provide better adherent cell selection and collection.⁴⁻⁸ Nevertheless, in many instances, these processes still result in some degree of cellular injury. In LPC, for instance, initial specimen velocities of 50–60 m/s are typical⁷ and lead to strong shear forces developing in the cell. It has been found that damage from these shear stresses is much higher than that emanating from heat or UV exposure.⁷

A more recent alternative approach applies the use of SU-8 polymer micropallets mounted on a glass surface to separate the living cells.⁹ The top of these micropallets is coated with collagen or fibronectin to facilitate culture. The delivery of a pulsed laser beam then releases the micropallet from the glass surface into the liquid chamber. The advantages claimed for

this approach include the following: (i) side-step of photo-UV damage from LCM, (ii) a thicker substrate to withstand the mechanical stresses and heating damage during pallet release, (iii) immersion in liquid media in a chamber throughout, and (iv) an ability to address the coordinate of a specific pallet of interest for release. After the micropallet is removed, the cells are collected in a microplate well placed above the micropallets in a one-to-one corresponding fashion.¹⁰ The advantages claimed with this scheme include reduction in contamination via immediate collection, lowered cell damage from minimal mechanical contact, and the ability to match the micropallets to the coordinate of their release. Once collected inside the microplate well, the adherent cells are released from the micropallet via a second pulsed laser operation. Although this scheme, overall, promises to have degrees of cell damage lower than LPC, it is a more lengthy process and requires the fabrication of specialty structures.

In this work, we describe an approach that primarily offers reduction in contamination and can be conducted rather quickly in a single step without the fabrication of specialty structures. It is based on the concept of creating fluidics with microscope coverslips that we have developed.^{11,12}

2 Method

The method of specific collection of adherent cells in a capillary cell is described in Fig. 1. The system is constructed by trapping a droplet between two glass surfaces, the area of the chamber is defined by the area of the smaller glass piece and the height by the droplet volume.^{13,14} The lower glass surface is a coverslip that has been treated to permit good cell adhesion, the upper surface is created using a smaller shaped coverslip that is sealed at all edges except one and a small aperture for droplet dispensation [see top view in Fig. 1(a)]. The introduction of the droplet imposes a hydrostatic pressure on the solution in the chamber

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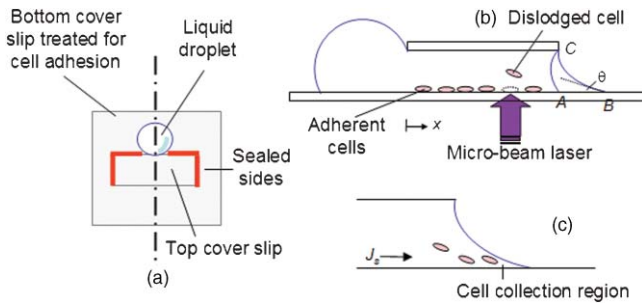


Fig. 1 Schematic of method of specific collection of adherent cells using low laser release in a capillary cell. (a) Top view depicts release of droplet at one end of the liquid chamber that causes adherent cells at the bottom coverslip selectively dislodged by the microbeam laser to flow to the right in (b) the sideview. The cells collect at the proximity of the triple phase contact line in a hydrated state.

that produces a movement of the contact line at the free edge of the chamber [depicted as being from A to B in Fig. 1(b)] The contact angle is denoted by θ . The ability of the meniscus to deform in such a manner is caused by a pinning effect at the top sharp edge of the coverslip C, which allows the contact angle there to have a large range. The ensuing effect is a flowrate J_s through the liquid chamber.¹² This flow rate arises from evaporation that occurs at the free edge of the coverslip; hence, the flow pattern is unusual in that it leads to a single line location rather than a more typical swirling pattern.^{15–17}

A microbeam laser delivered from underneath the bottom coverslip serves to selectively dislodge the adherent cells to enter into the flow. The travels of these cells terminate near the contact line within the liquid medium, ensuring that they remain continuously hydrated. If the cells to be dislodged are located close to the free edge of the liquid chamber, then the amount of cell travel and, thus, contamination is minimized. Essentially, this method enables us to capitalize on the ability to detach an extended choice of cells from their positions in proximity to the edge of the liquid chamber for maximal harvest while keeping contamination to a minimum. Such a scheme has not been reported before to the best of our knowledge. More importantly, it can be accomplished rather easily in the laboratory, wherein the flow is conveniently created by a droplet onto the liquid chamber made using a coverslip and glass slide rather than with a pump and expensive fluidic channels. The surface tension forces at the free edge keep the cells in place somewhat for a later collection process with a receptacle. It is conceivable that the flow generated orthogonal to cell dislodgement may introduce a shear peeling action on the cells and thus reduce the amount of force needed to dislodge them from the surface.

3 Materials and Methods

3.1 Cell Culture Preparation

The human glioblastoma cell line U-251 cultures were grown using Dulbecco's Modified Eagle Medium (Gibco BRL, USA) (supplemented with 10% (v/v) fetal bovine serum (CSL, Australia), 100 units/ml penicillin (CSL, Australia), and 0.1% (w/v) streptomycin (CSL, USA), pH 7.2) in a biosafety class II hood to ensure sterility. A small concentration of the cells were then transferred and placed in a 5% CO₂ humidified incubator at

37°C to grow. The cells were prepared on 15 × 15 mm coverslips using malignant gliomas (U-251) cells, which had an average size of 15 μm. These coverslips were precleaned using 100% ethanol and placed in well dishes where 2 ml of [phosphate saline buffer (PBS), comprised of 8 mM Na₂HPO₄, 17.5 mM K₂HPO₄, 2.7 mM KCl, 137 mM NaCl, in distilled water, pH 7.4] was poured into the wells. Fibronectin [constituting 1 μg/ml fibronectin (Calbiochem) in 1X PBS] of 20 μl was added in, giving a concentration of 0.01. Following this, the cells were placed in the incubator for 3 h for the cells to grow before they were transferred into separate Petri dishes with PBS to be used in the experiment.

3.2 Trypan Blue Exclusion Test of Cell Viability

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm, whereas a non-viable cell will have a blue cytoplasm. One part of 0.4% trypan blue (Sigma-Aldrich, USA) and one part of the cell suspension were mixed. This mixture was allowed to incubate for 3 min at room temperature. The cells were evaluated within 3–5 min of mixing with trypan blue. Longer incubation periods may lead to viable cells taking up the dye as well, leading to an erroneous deduction of cell death.

3.3 Fluidic Chamber Using Coverslips on Slides with Flow

A fluid cell was first prepared by depositing a volume of phosphate buffer solution on a glass slide and lowering a cut coverslip onto this fluid such that it fully wets the coverslip's area the cell height was 8 μm. After it had settled, standard varnish was placed gently on the three sides and left to dry up. A small opening was left at the center of the sealed edge opposite the free side. To ensure the cells did not dry up during this process, a drop of PBS was introduced at the entry point. This also created a flow in the chamber for cell harvesting at the open side.¹²

3.4 Selective Laser Dislodgement of Adherent Cells and Collection

The laser microbeam laser system used was equipped with an N₂ laser (wavelength 337 nm) emitting pulses of 90 μJ of energy in 4-ns duration (PALM Microlaser Technologies). The laser power can be attenuated as percentage of the energy of each pulse delivered. In this system, the laser beam is coupled through the beam path for epi-illumination into an inverted microscope (Axiovert 200, Carl Zeiss Micro Imaging). The microscope objective used in this study was 40X with 0.6 NA. A moveable lens for focus control could be used for purposeful defocusing by making the laser beam before the microscope objective slightly divergent or convergent. The z shifts of the focus corresponding to given settings at the control unit were measured by producing laser effects in microscope glass slides,

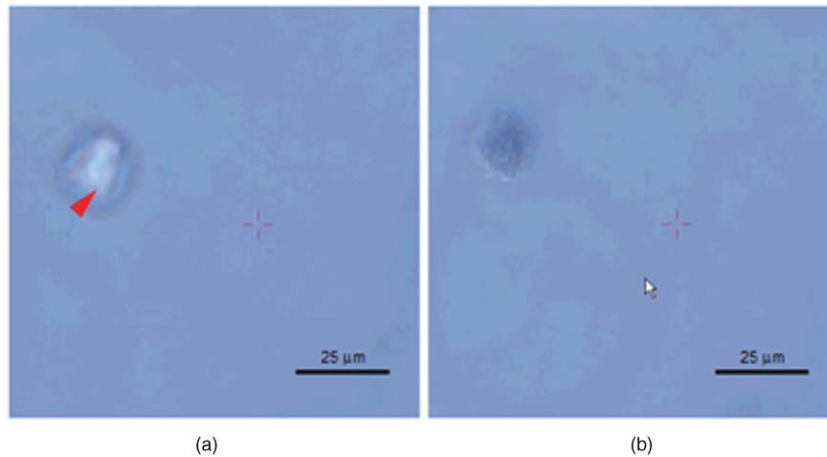


Fig. 2 Images sequences (a) before and (b) after 14-s delivery of the laser at 60% power directly hitting the cell (spot indicated by arrow). The cell is seen to experience death as evidenced by its color change to blue.

using the lower surface of the slide as reference. The distance between surface and laser effects was determined from the distance of the positions of the microscope stage for which either plane is in focus. For removal of the adherent cells, the microscope objective was focused in two ways: (i) directly onto the cell, and (ii) in proximity with the cell wherein a cavitating bubble is created such that the force generated dislodged the cell. The manner of microbubbles induced via pulsed laser delivery on water has been identified¹⁸ and studied.¹⁹

3.5 Data Analysis

The selectively removed cells were evaluated on viability based on the trypan blue exclusion test. Cell death probability was determined by evaluating a sample of six at specific power levels and mode of delivery of the laser (i.e., directly impinging the cell or generating a cavitating bubble at the proximity of the cell). The cells were also evaluated on their ability to be displaced via these two modes by averaging the distance in which the six cells moved. This was made possible by the micrometer scale provided on the software screen by the PALM Robosoftware (PALM Microlaser Technologies). This displacement measurement was taken from the cell periphery to the position that it moved to after delivery of the laser beam.

4 Results and Discussion

Figure 2 provides image sequences of a typical adherent cell before and 14 s after the laser beam was delivered to remove it via a direct delivery on the cell. The cell was found to be dislodged from the surface (in an out-of-focus sense), but cell death occurred in this case as evidenced by its color change to blue. Observation of the video footage enabled us to determine that the cell was brought to the edge of liquid chamber with a droplet induced flow. Figure 3 gives image sequences associated with the laser beam delivered 30 μm from the nearest cell at 63% maximum power, wherein no visible effect on the liquid was seen as well as the cell failed to dislodge. Having the laser beam delivered 35 μm from the nearest cell at 61% maximum power produced a bubble growth that cavitated in time to produce an indirect force needed that dislodged the cell. This is evident in the image sequences of Figs. 4(a)–4(c). In this case, the cell remained viable.

Figure 5(a) presents results depicting cell death probability of six cells against the laser power (i.e., six cells per data point) used by direct delivery of laser on the cell as well as inducing a bubble 60 μm away from the cell. Six cells were used per data point in order to keep the total experimentation time from being too extended. The latter approach is clearly able to sustain lower cell death probabilities in relation to an equal amount of laser

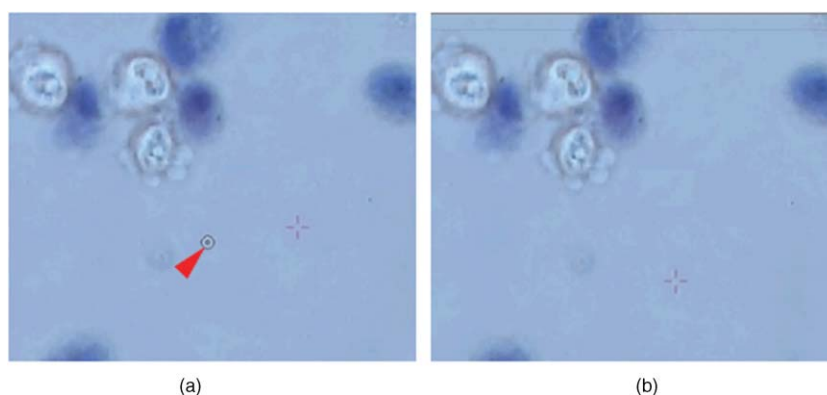


Fig. 3 Images sequences (a) before and (b) after delivery of the laser at 63% power delivered 30 μm from the nearest cell (spot indicated by arrow).

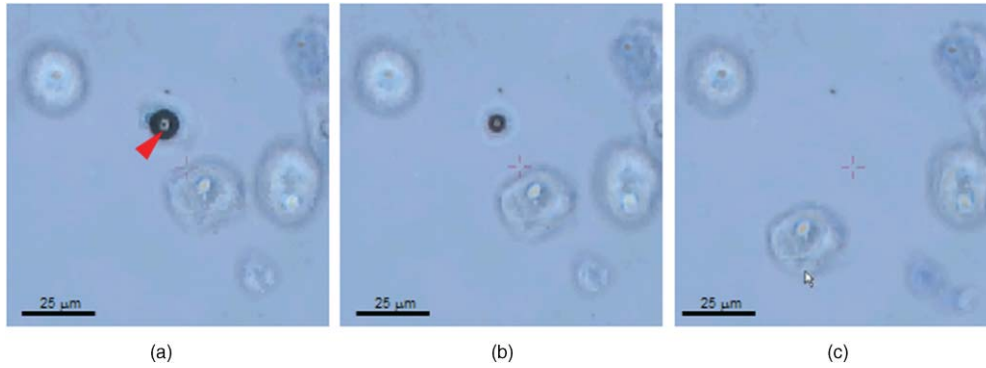


Fig. 4 Images sequences (a) at the instant, (b) 3 s, and (c) 11 s after laser at 61% power delivered 35 μm from the nearest cell. A bubble is seen to instantaneously form that diminished in size over time. In this case, the cell was dislodged, but no reduction in cell viability was observed.

power used. However, the ability of the cell to survive better is of little consequence if the cell cannot be dislodged sufficiently for the fluid flow to carry it to the collection region of the liquid chamber. Figure 5(b) gives the average movement of six cells against the laser power used by direct delivery of laser on the cell and inducing a bubble 60 μm away from the cell. In this case, the former approach is able to deliver greater average cell movement in relation to the laser power used. This result is largely expected

due to the larger shear forces delivered to the cell⁷ by this means. Again, the ability for cell movement is inconsequential if the cell does not survive the dislodgement process. A comparison of Figs. 5(a) and 5(b) together is thus necessary in order to arrive at an apt conclusion. By considering the direct laser delivery approach alone, it would appear that there is no laser power range wherein both cell survival and cell movement are possible. The laser power ranges between 50 and 60% of the maximum

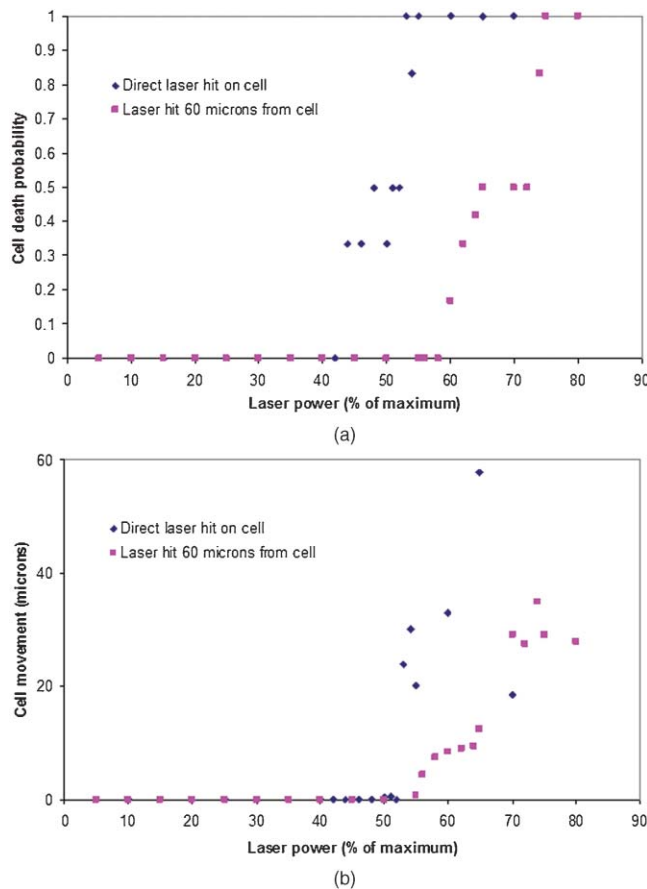


Fig. 5 Plots of (a) comparative cell death probability of six cells against the laser power used by direct delivery of laser on the cell and inducing a bubble 60 μm away from the cell. The latter approach is able to sustain lower cell death probability in relation to the laser power used. Alternatively, (b) comprises plots of comparative average cell movement of six cells against the laser power used by direct delivery of laser on the cell and inducing a bubble 60 μm away from the cell. The former approach is able to deliver greater average cell movement on the basis of dislodgement in relation to the laser power used.

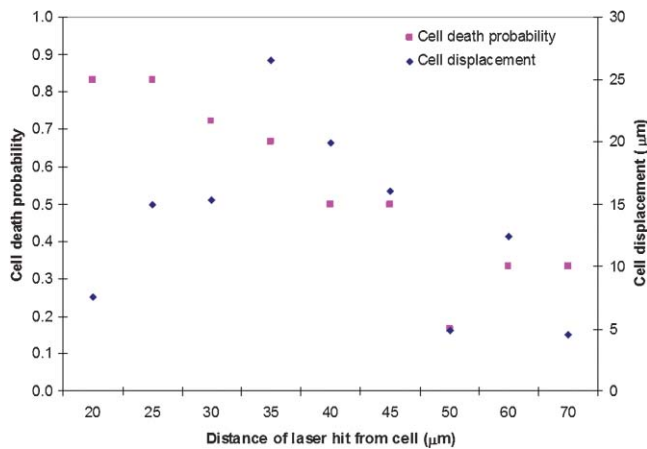


Fig. 6 Cell death probability and average cell movement (taken from six cells) determined by inducing a bubble at various distances away from the cell using a constant laser power of 60% of the maximum. The cell death probability distribution follows a distinct reducing trend with distance but not the average cell movement distribution.

with the bubble created 60 μm away, alternatively, offers such a possibility. We have further investigated the effect of creating the bubble at different distances away from the cell by keeping the laser power at 60% of its maximum.

Figure 6 outlines the results obtained. The cell death probability distribution follows a distinct reducing trend with distance. The average cell movement distribution, however, shows that there is an optimal distance away (30–40 μm), where laser delivery produces the best effect for this. We do not fully understand the reason for this but postulate that it may be due to the cell being flexible rather than rigid and the force induced by the bubble having a vertical as well as a horizontal component. When the cell is close to the bubble, the resultant force may be large in magnitude but more vertically directed; resulting in the cell deforming but not being able to move much horizontally. If the cell is far from the bubble, then the resultant force is lower in magnitude despite being more horizontally oriented, again resulting in a smaller cell displacement. The lack of correlation between the two graphs appears to show that mechanical forces may not be the contributing cause for cell death from dislodgement via the bubble approach. This is in contrast to the findings with the direct laser delivery on cell approach.⁷ In fact, a plausible cause for the cell death probability distribution following a reducing trend with laser delivery distance may, in fact, be due to phototoxicity. It should be noted that the distances mentioned refer to the center of the laser beam and must not be taken to mean that the laser beam is confined to an infinitesimally small spot. The specified diameter of the laser beam for sharpest focus at its waist is 1 μm . However, this is used only for dissection purposes. We estimate that for catapulting purposes, the plane containing the cell corresponds to a beam diameter of 10 μm . If we assume a Gaussian profile, the percentage of energy delivered at the cell center is then around 10% of the laser spot center at this particular plane.

Figure 7 presents an image of cells collected at the collection region of the fluidic chamber. It can be seen that the cells remain hydrated throughout. Closer inspection of these harvested cells using trypan blue indicates the presence of both viable and

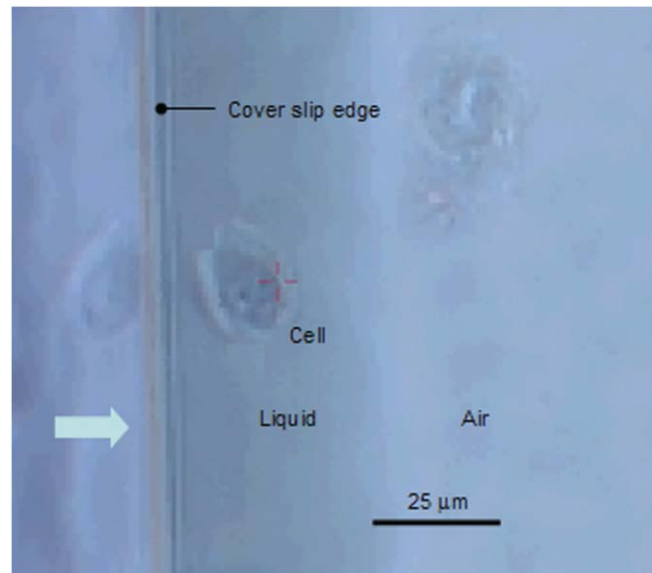


Fig. 7 Image of cells collected at the collection region of the fluidic chamber. It can be seen that the cells remain hydrated throughout and that they can be kept viable. The arrow shows the direction of liquid flow in the chamber.

nonviable numbers. We believe that the nonviable cells are a consequence of the cell dislodgement process using the micro-laser and not due to the fluid flow and collection process. To check for this, we have conducted tests of collecting nonadherent cells using the liquid chamber. With this, we observed no nonviable cells at the collection region. It is also important to note that the cells were located beyond the coverslip edge, which would enable physical removal if required.

5 Conclusion

We demonstrate that the approach of a microbeam laser delivered from underneath the bottom coverslip serves to selectively dislodge the adherent cells to enter into a flow. The transport of these cells terminate near the contact line within the liquid medium, ensuring that they remain continuously hydrated. By dislodging the cells close to the free edge of the liquid chamber, the amount of travel and thus contamination is minimized. The investigation of the modes of dislodgement via the metrics of cell death and movement show that the firing of the laser beam center a distance away from the cell to create a bubble that cavitates with time is more viable. The results obtained also show that phototoxicity appears to play a greater role than mechanical shear stresses in determining cell viability. The flow process was found to cause no death effects on the cells as they traveled to the coverslip edge for collection. More importantly, this technique can be readily performed in the laboratory without expensive fluidic channels and external flow creating sources (e.g., pumps).

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