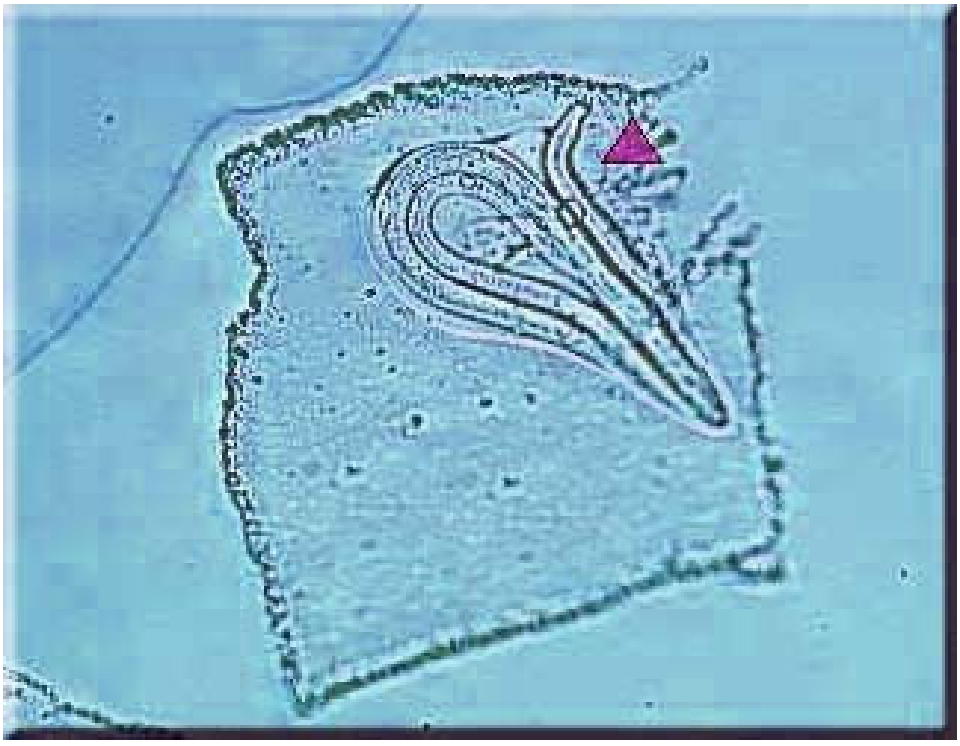
P.A.L.M.
Microlaser Technologies

Non-Contact Live Cell Laser Micromanipulation



with the

PALM® MicroLaser Systems

by Karin Schütze, Ph. D., Director Research&Development
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Title photo:
Living organism (Caenorhabditis elegans) first ever "beamed" only by the force of light without impairing its viability.

Not all applications are authorized to be carried out in all countries.

The following technologies have been patented by P.A.L.M. Microlaser Technologies AG:

Laser Pressure Catapulting LPC[pat.]
Patents: US 5,998,129, EP 879408 B1 and others.

3D laser beam positioning
Patents: US 5,689,109, EP 679325 B1 and others.

Additional patents pending.

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Non-Contact Live Cell Laser Micromanipulation

Non-Contact Live Cell Laser Micromanipulation

The PALM® MicroBeam is worldwide established in numerous research laboratories as state-of-the-art tool for non-contact laser microdissection and catapulting. More than 250 of published papers using the PALM® MicroLaser Systems demonstrate the successful and efficient capture of homogeneous tissue areas as well as pure single cells or subcellular parts from any kind of tissue sections, cyto-centrifuged specimens or cell smears for subsequent genetic or proteomic evaluation.

Recently, increasing interest is found to apply the unique cutting features of the PALM® MicroBeam laser to work with or even within living cells or tissue without harming the specimens viability and/or molecular biological information.

There are different possibilities for non-contact laser micromanipulation of living cells that could be summarized in the following four categories: microsurgery, cell fusion, microinjection and laser picking.

1 Laser Microsurgery

The laser can cut the cell membrane of mammalian cells, or drill holes into the solid cell wall of plant cells. Even within living cells entire organelles (e.g. chloroplasts), chromosomes or other cellular parts have selectively been opened, cut or destroyed without impairing cell viability.

Since the beginning of laser micromanipulation in 1962 several experiments using different lab-type laser systems have been published (Bessis 1962; Bereiter-Hahn 1971; McNeill and Berns 1981; Berns et al. 1981; Pool 1990; Greulich et al. 1991; Weber et al. 1992; Berns et al. 1992; Liang et al. 1993; Rajcan-Separovic 1995).

Single cells within an entire organism (e.g. Caenorhabditis elegans) have selectively been destroyed by applying distinct laser shots (Hutter and Schnabel 1994).

The zona pellucida of oocytes has precisely been laser drilled to facilitate sperm penetration or to assist embryo hatching (figure 1 a). Zona drilling is also helpful for preimplantation diagnosis, to examine polar bodies or blastomers for inherited disease (figure 1 b; see chapter 5.1.2; Clement-Sengewald et al. 2000 a, b and 2002). Cutting the sperm tail stops the sperm and facilitates in vitro fertilization using conventional glass needles and glass capillaries (figure 1 c).

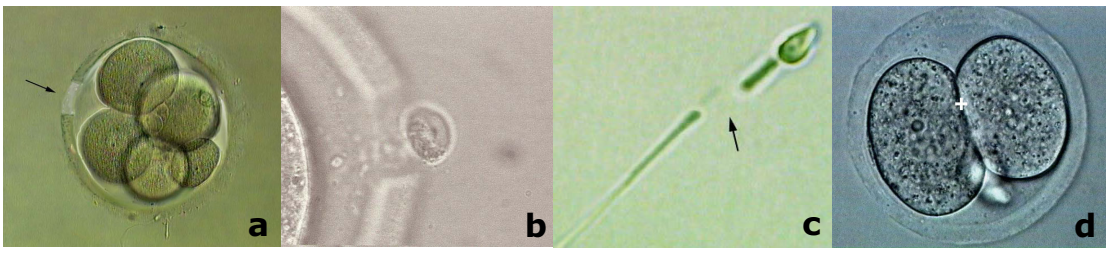


Figure 1: a) embryo drilling to assist hatching; b) polar body extraction; c) cutting sperm tail to facilitate in vitro fertilization; d) fusion of blastomers within 2-cell mouse embryo

Plant cells: Laser Microsurgery of cytoplasmic strands and cytoplasmic streaming within plant cells (e.g. onion cells) and protoplasts was one of the first experiments using cutting lasers with plant cells (Hahne and Hoffmann 1984). Removing the cell wall of pollen tubes for subsequent patch clamping is possible; poking holes into pollen grains or shaving off their tuff lignin structures has been reported (Kathy Heel, personal communication; Heel and Dawkins 2001).

Single laser shots could reversibly stop the cytoplasmic motion within lily pollen tubes. The mechanism behind seems to be membrane depolarization of internal calcium stores, and release of calcium into the cytoplasm, which causes actin depolymerization. As soon as the calcium is pumped back, cytoplasmic motion started again (Schütze et al. 1989).

Materials and Methods

Objectives: For laser microsurgery the laser spot size should be less than 1 µm in diameter. Therefore, highly magnifying objectives with numerical aperture NA >1 should be used, which usually have short working distances.

Disposables: We recommend culturing the cells in commercially available **Petriperm** dishes having a 20 µm thin bio-membrane bottom. This special, gas permeable Teflon-membrane enables laser transmission and allows the usage of objectives with short working distances.
For multiple experiments performed in parallel, special inserts are available to minimize the reaction volume (figure 2).

Selfmade: It is possible to use regular plastic petridishes, if a cover glass insert is manufactured within the bottom (glass thickness about 170 µm)!
Sew a hole into the plastic bottom and cover it with a glass slide that should be fixed and sealed using tissue culture glue.

Procedure: Non-contact laser microsurgery can be performed within the closed dish, so that there is no danger of contamination and further cultivation of the cells after laser treatment is possible.

Sometimes only a small 10-50 µl droplet may be applied into the petridish or just on a thin glass slide, wherein the experiment is performed. To avoid evaporation, these droplets should be covered by a thin film of light weight mineral oil (e.g. PCR oil). Simply focus onto the area of interest and apply the laser shots with appropriate energy. It is advisable to first adjust laser focus and laser energy within a test object.

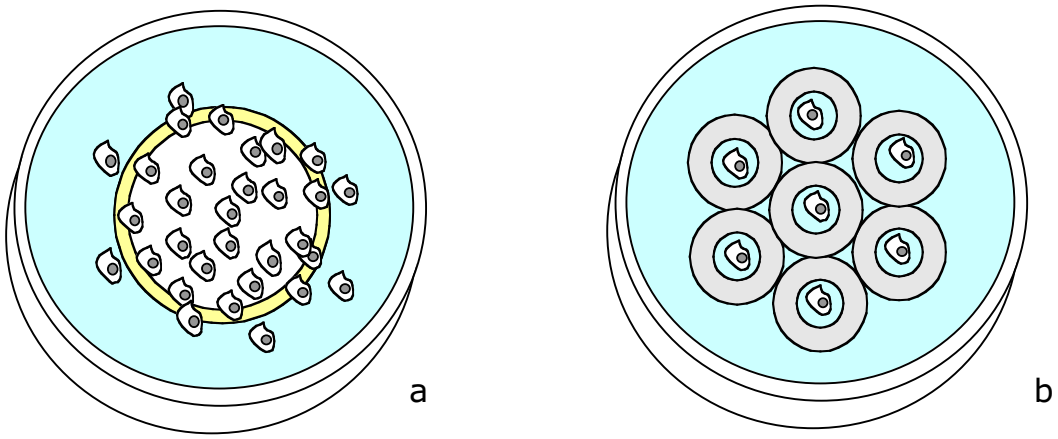


Figure 2: a) routine plastic Petridish with glass insert;
b) Petriperm dish with silicon-inserts to divide the volume into small reaction compartments

Figure 3: single 10-50 µl droplets within a) a Petriperm dish or
b) on a glass slide, covered with mineral oil to prevent evaporation

Note: Some experiments (e.g. polar body extraction or plant cell experiments) are performed within small droplets of the medium, which are pipetted onto thin glass slides (170 µm), a Petriperm dish or onto PALM® MembraneSlides (all available through P.A.L.M.) (figure 3).

Note: If the manipulated cells should be picked after the experiments, the specimen have to be seeded onto the LPC-membrane (see chapter 4).

There are some precautions to perform reliable cell culturing on LPC-membranes. The membranes should be treated to improve cell attachment and cell growth and should also be sterilized to avoid cell culture contamination (autoclaved at 121 °C for 20 minutes or irradiated with 254 nm for 30 minutes).

For cell culturing the glass slides may be emerged in culture medium within sufficient large petridishes or within special 4-well plates (figure 4). Make sure to prevent them from floating-up!

It is also possible to apply culture chambers on top of the membrane slides (figure 5). P.A.L.M.`s especially developed DuplexDishes (bio-membrane bottom plus LPC-membrane) might also be very useful.

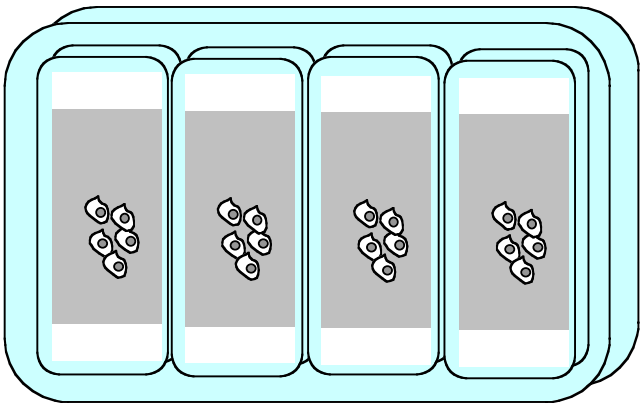


Figure 4: 4-well plate for culturing cells on PALM® MembraneSlides

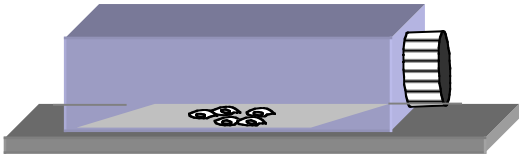


Figure 5: Cell culture chamber on PALM® MembraneSlides (PEN or POL)

2 Cell Fusion

Placed at the membrane contact site of two adjacent cells, laser shots can induce cell fusion (figure 6). Mammalian cells should be held in tight contact using biochemical bondings like avidin-biotin bridging, which prevents the cells from separating during laser application (Wiegand et al. 1987).

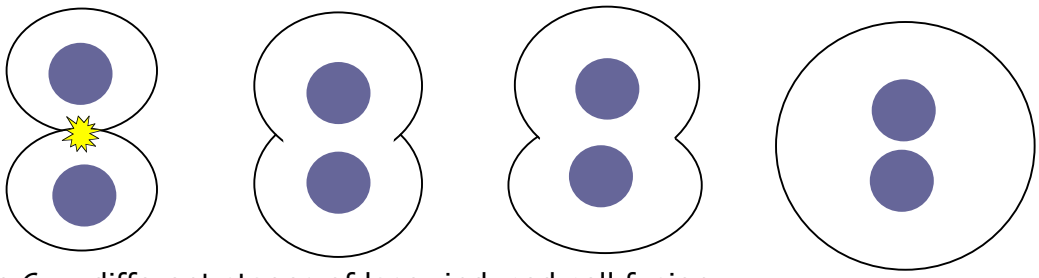


Figure 6: different stages of laser induced cell fusion

The fusion of individual blastomers within embryos (figure 1 d) or within nematodes as well as the fusion of artificial cytoplasts with the oocyte do not require biochemical bridging, as the target cells are hold in tight contact due to the zona pellucida (Clement-Sengewald 1993 and 2000 b). Interestingly, the laser can even work within the depth of the huge oocyte, as laser application usually is performed at the equatorial plane of the object (i.e. 50 to 60 µm in depth).

The fusion of plant protoplasts is even easier. If sufficiently concentrated and thus slightly pressed together, the protoplasts are fusing easily after one or a

few individual laser shots focused on the membrane contact site (Wiegand et al. 1987; Weber 1992).

Materials and Methods

Objectives: For laser induced cell fusion the laser spot size could vary in diameter depending on the target specimen. Highly magnifying objectives with numerical aperture NA >1 are recommended for tiny or fragile cells, but 40x or 63x oil immersion or long distance objectives might be used as well for larger or less critical specimen.

Disposables: PALM® MembraneSlides or Petriperm dishes (see chapter 1).

Selfmade: Regular plastic petridishes with glass insert (see chapter 1).

Procedure: Carefully focus the laser beam to the contacting membrane slide and apply one shot of sufficient energy (the minimum amount has to be found by trial and error at test objects). In some cases a few shots might be required to initiate cell fusion. With one or two shots only, the cells can immediately close and repair the hole(s) within a fraction of a second, i.e. before fusion could start.

Note: If the manipulated cells should be picked after the experiments, the specimen have to be seeded onto the LPC-membrane (see chapter 4).

3 Laser Microinjection

The laser pokes minute holes into cells and nuclear envelopes (figure 7), which enables injection of drugs or genetic material without viral vectors (Schütze et al. 1995).

Some previous publications using self-constructed lab-type laser systems already reported significantly higher efficiency of laser-mediated microinjection as compared with needle microinjection (Kurata 1986; Tao 1987; Tsukakoshi 1984).

The new PALM® MicroBeam generation in combination with the PALM® RoboSoftware allows to predefine the place of laser injection with computer graphic tools, which speeds up the microinjection procedure, and the superior precision further increases the efficiency of laser-based microinjection.

Materials and Methods

Objectives: For laser microinjection a 100x high numerical aperture objective is required.

Disposables: Petriperm dishes, PALM® DuplexDishes or PALM® MembraneSlides (see chapter 1).

Note: If subsequent sampling of the injected cells is planned, the cells have to be cultured on the LPC-membrane (see chapter 4).

Selfmade: regular plastic petridishes with glass insert (see chapter 1).

Procedure*:* A single cell layer is cultured within a "Petriperm" dish (see also chapter 1, "microsurgery"). Laser focus and laser energy have to be accurately set to yield the smallest available laser spot (less than 1 micron in diameter!).

Provide drugs, genetic material or other biologically active substances into the culture medium. A single laser shot aimed at the nuclear area causes a tiny opening of the cell membrane and the underlying nuclear envelope. The cell will swallow whatever is in its close surrounding, before repair mechanisms start to close the opening.
Successful microinjection can be demonstrated either by certain staining procedures or using selection medium – i.e. only the injected cells can survive.

With the **LPC-dot** function of the PALM® RoboSoftware, the desired cells can be preselected, so that microinjection can be performed automatically, i.e. the stage will bring the marked spot into the line of laser fire and microinjection is done immediately.

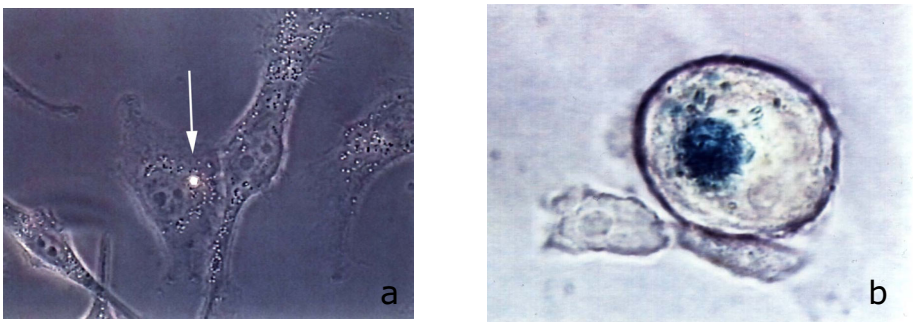


Figure 7: a) laser microinjection of cultured fibroblasts;
b) microinjection of a plasmid encoding for β-galactosidase results in blue-stained cytoplasm three days after microinjection

4 Laser Based Isolation and Picking of Cultured Cells

4.1 Collection After Fixation

Cultured cells or parts of them may selectively be isolated by laser microdissection and catapulting. Prior to the laser procedure, the cells may be fixed and eventually be stained to perform genetic or proteomic analysis (figure 8), but the cells could also be collected alive for subsequent culturing or cloning using the non-contact LPC technology (see chapter 4.3).

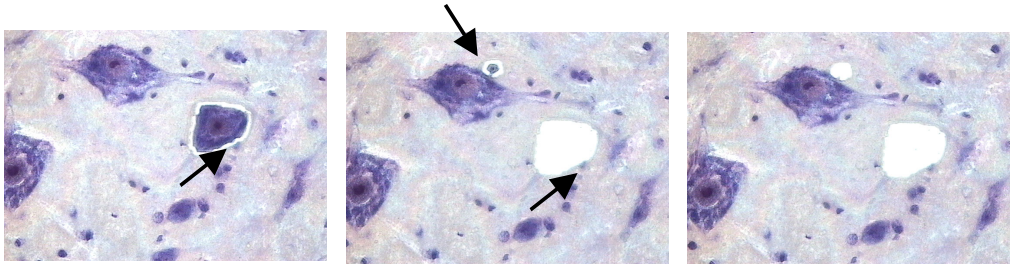


Figure 8: Motoneurons are grown on the LPC-membrane, fixed and stained. The large motoneurons are microdissected and catapulted. The tiny, closely attached glial cells (∅ 5 µm) are circumcised and collected separately (pure and uncontaminated sample collection as demonstrated by Bernd Meurers, Johnson Pharmaceutical Research Inst., San Diego, CA).

Materials and Methods

Objectives: For laser microdissection and catapulting of tiny single cells or subcellular compartments, a 100x high numerical aperture objective is required. Larger cells might be cut and catapulted using the long distance 40x as well.

Disposables: PALM® DuplexDishes, PALM® MembraneSlides (figure 4).

Selfmade: Chamber slides: Nunc-Chambers mounted on thin (170 µm) LPC-slides.

Procedure: Cells are grown on one of P.A.L.M.´s LPC-membranes (PEN or POL), which may be mounted on glass slides. The usage of thin glass slides (cover glass thickness of about 170 µm) enables working with high magnifying objectives like 100x oil immersion, that usually provide only short working distances. The PEN-membrane is used, if the specimen are directly cut and catapulted. If, however, unwanted material has to be laser-ablated prior to catapulting, the POL-membrane is preferable.

4.2 The "Donut"-Preparation

The "donut"-isolation is a special method of this kind of laser application (figure 9). After cell culturing and experimental set-up, cells are fixed and stained. First the nuclei of selected cells are circumcised and separately captured. Then the surrounding cytoplasm is isolated and catapulted in an extra collection device.
The "donut"-method enables discrimination of cell nuclei from the rest of the cytoplasm. This way kinetics of drug delivery or viral infections might be studied. The idea of partly cell capture might be of use in additional biological experiments (see Protocol 5.3).

Materials and Methods

Objectives: 100x high numerical aperture objective.

Self-made: Chamber slides: Nunc-Chambers mounted on thin (170 µm) LPC-slides.

Procedure: Cells are grown on PEN-membrane in Nunc-Chambers, fixed and stained. Nuclei and cytoplasm are circumcised and captured separately.

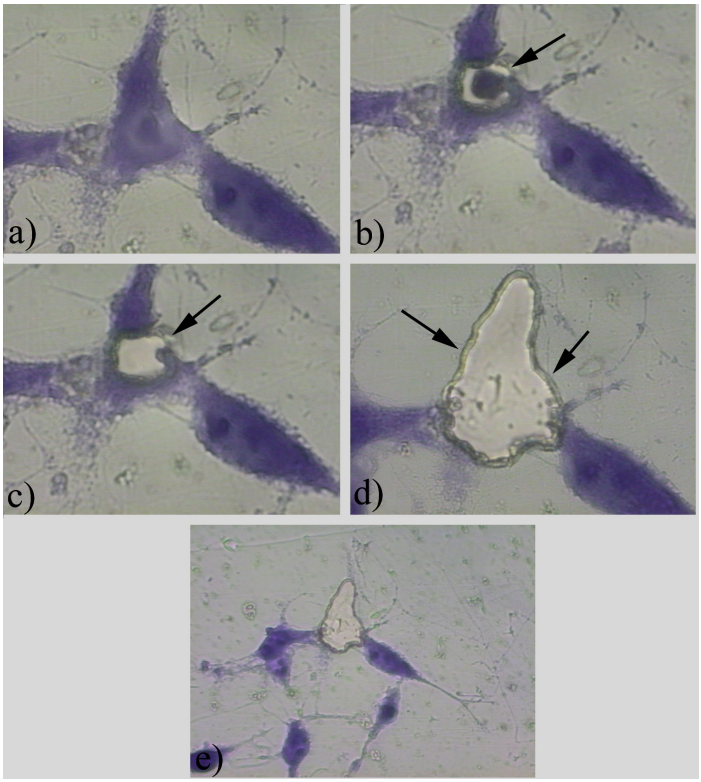


Figure 9: Donut – isolation:
a, b: circumcision of the nucleus
c: after catapulting
d: isolation of the rest of the cytoplasm
e: overview with 40x magnification

4.3 Laser Catapulting of Cultured Living Cells for Subsequent Growth or Cloning

An old dream of cell biologists is to isolate living cells from tissue culture or unfixed and unfrozen sections of living tissues. But until now, laser-based microdissection of living cells resulted in the destruction of the isolated cells (Schindler et al. 1985).

Now, a modification of the LMPC laser technique and a newly developed cell culture protocol allows microdissection and "ejection" of living single cells or cell clusters with ongoing cultivation for potential treatment and analysis (Mayer et al. 2002). The protocol was applied to culture cells (TPC-1, a papillary thyroid carcinoma cell line; EJ28 and RT112, bladder carcinoma cell lines), and selected cells were microdissected, ejected and recultivated. The captured and catapulted cells are not affected in their viability. They enter the cell cycle and proliferate.

To perform LMPC of living cells, these cells were cultivated in a PALM® DuplexDish. Cell clusters or single cells were circumcised using a 20x (figure 10 and 11) objective lens. In cases where the selected specimen area contained undesired cells, these can be eliminated by a direct laser shot. LPC was done with one single laser shot positioned at the border of the circumcised membrane-cell stack. Even large cell-membrane stacks can be catapulted (figure 10). Microdissection and catapulting of cell clusters took less than 2 minutes.

The manipulation of single living cells was done within seconds. The isolated and catapulted cells were finally suspended in an appropriate volume of supplemented medium.
The following day the aggregated cells could be seeded in a droplet of supplemented medium on the bottom of a standard culture dish. Due to the spherical shape of the droplet, the aggregated cells were forced to move to the tension-free center of the droplet and after one day in culture, the cells showed the typical flat shape of epithelial cells (figure 10 c). Cells entered the cell cycle and started to proliferate. Depending upon the density of the seeded cells this took 3 days up to 3 weeks.

This new approach can be used to establish a homogeneous cell population out of a heterogeneous cell population (e.g. after transfection experiments). Now it's possible to obtain 100 % homologous cell populations for expression studies by recultivation of transfected cells or destruction of unwanted cells by precise laser shots. In addition this protocol serves as a basis to develop the procedure to isolate homologous living cells from biopsies for real in vivo studies.

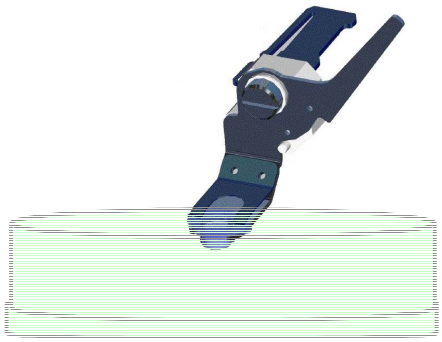
Culturing or cloning individual single cells has always been a problem in cell culture biology. Especially now, as catapulting of individual cells has become possible, special cell culturing techniques have to be developed, if less than 10 cells are available in one vial. Some ideas have been tested successfully.

Materials and Methods

Objectives: 20x or 40x objective.

Disposables: PALM® DuplexDishes with double membrane layer; special PALM® DuplexDishHolder for the PALM® CapMover.

Procedure: Cells are grown and cultured on the LPC-membrane. Shortly before picking, the culture medium is soaked away leaving a thin film of liquid. Cutting and catapulting is performed as usual directly into a medium-filled collection cap.



PALM® DuplexDish with duplex-membrane
and PALM® DuplexDishHolder

For further details please directly contact P.A.L.M. Microlaser Technologies AG.

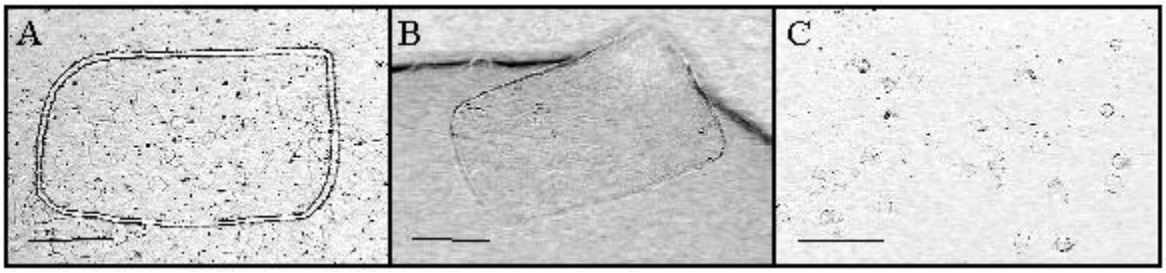


Figure 10: Microscopic illustration using LPC to capture EJ28 cells. The images show the cells after microdissection (A), catapulted membrane with the cells (B) and cells after one day in culture (C). Bar equals 100 µm; objective lens: 20x

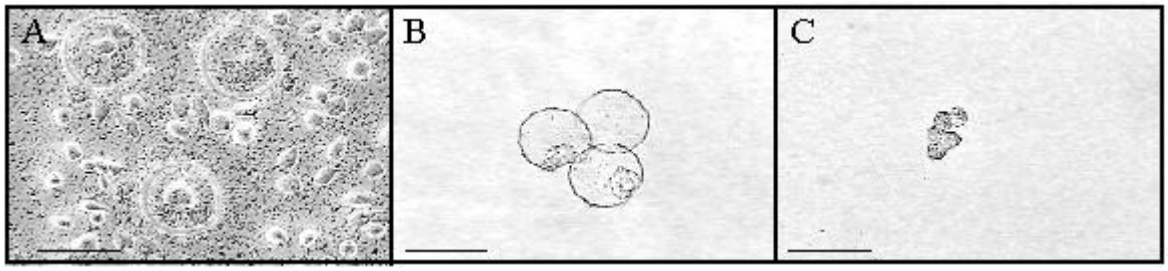


Figure 11: Images of pooled single EJ28 cells. The sequence shows the cells after laser microdissection (A). Three catapulted membranes with single cells (B, C). Bar equals 100 µm in A and B, 50 µm in C. Objective lens in A and B: 20x, C: 40x

4.4 Laser Catapulting of Living Specimen Using the "Sandwich" Method

Recent experiments demonstrated the possibility to capture living endosymbionts from the cytoplasm of Amoeba proteus (figure 12 a), to isolate and collect phytoplankton (figure 12 b, c) or to even catapult entire organisms, like e.g. Caenorhabditis elegans alive (figure 12 d, e).

In some cases a special trick has to be applied to be able to perform laser-micromanipulation on the vigorously moving specimen. They have to be "trapped" inbetween two LPC-membranes, thus forming a duplex-membrane sandwich-slide. The laser cuts both of the membranes and using LPC, the membrane-sandwich with the enclosed specimen is transferred into the buffer filled cap (figure 12).

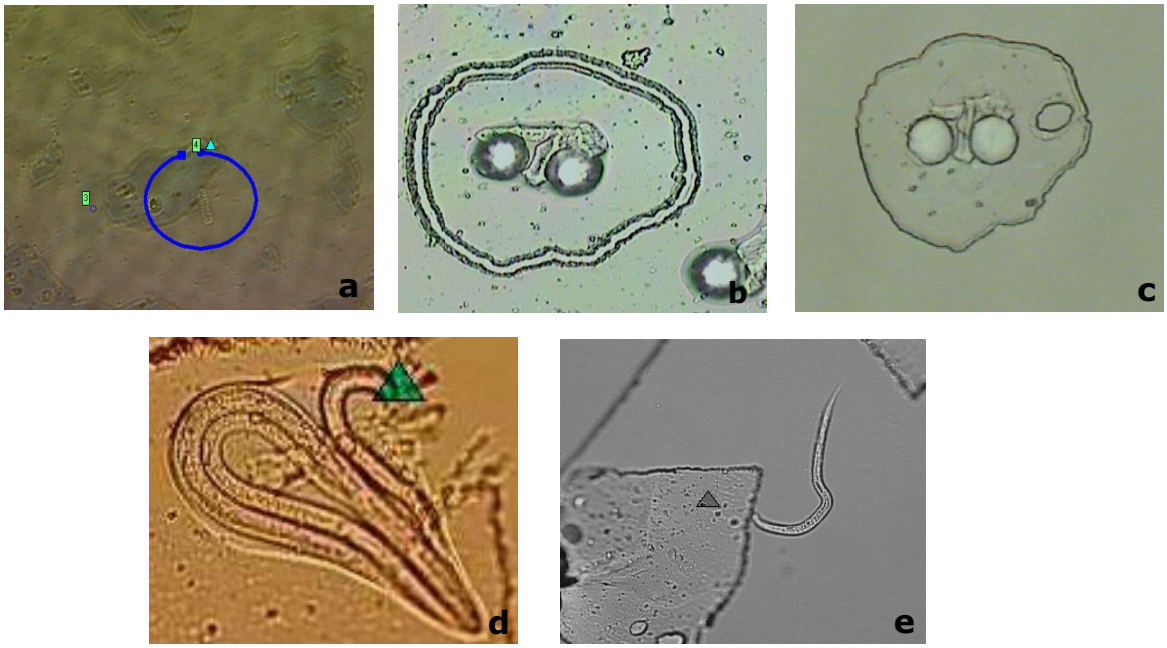


Figure 12: Catapulting of living specimen trapped in between two LPC-membranes. Endosymbionts from the cytoplasm of Amoeba proteus (a); phytoplankton (b, c); one Caenorhabditis elegans after catapulting moving on the membrane (d) and another one moving away from the membrane piece (e)

5 Protocols

5.1 Laser Micromanipulation of Gametes

Clement-Sengewald, Schütze, Buchholz, Berg U, Berg F-D: Non-contact, laser-mediated extraction of polar bodies for prefertilization genetic diagnosis; Journal of Assisted Reproduction and Genetics, 2002, in press

5.1.1 Fusion of Blastomeres and Cytoplasts

Introduction

Fusion of embryonic cells is a helpful technique for basic research, gene technology and biotechnology in order to gain more knowledge about polyploidy phenomenons, oocyte maturation mechanisms, and about the regulation of embryo development. Blastomeres may be fused within one embryo to produce diploid-tetraploid mosaics. Cytoplasts (cytoplasm surrounded by a cytoplasmic membrane) can be used as a vehicle to introduce substances into cells, and the fusion of a blastomere with an enucleated oocyte is the standard procedure for cloning experiments.

In order to induce fusion of blastomeres and cytoplasts, various experimental techniques have been described. The most important prerequisite for fusion of cells is, that their membranes have first to be brought in close contact, and secondly that fusion subsequently has to be induced by disruption of the membrane bilayers. The disruption is followed by reparation processes, which result in rearrangement of the membrane bilayers. This allows confluence of the cell contents, which yields in one round fused cell. Disruption of the membrane bilayers may be induced chemically or by a virus, but the most convenient techniques are induced physically like electrofusion or laser-induced fusion. Electrofusion acts with an electrical field on the whole blastomere, whereas fusion by laser pulses acts only in the laser focus in an area between one to two micrometers.

Materials

- Suction pump (Labotect, Göttingen, Germany)
- Object slide (thickness 170 µm)
- Culture medium + phytohaemagglutinine (300-400 µg/ml) + HEPES
- Ham's F 10 medium + 10 % fetal calf serum + Gentamycin (ICN Biomedicals, Aurora, OH, USA)
- Parker's medium (MPM) + 10 % fetal calf serum + 0.01 U/ml follicle-stimulating hormone (FSH) + 0.01 U/ml luteinizing hormone (LH) (SIOUX, Iowa, USA)
- HEPES buffer
- Trypsin/EDTA (1 % in 0.8 % in PBS buffer)
- Bovine oocytes from fresh ovaries: slaughterhouse material

Methods

In order to obtain experiences with laser-induced fusion, two-cell mouse or *in vitro* produced bovine embryos are a convenient model, as the two big blastomeres show a large contact area, and furthermore stick tightly to each other.
Any other cells to fuse have to be brought into close contact prior to fusion induction, either by placement of the two cells under the same zona pellucida by micromanipulation or chemically by sticking the cells together in medium containing phytohaemagglutinine (300-400 µg/ml) and simultaneous suction into a narrow pipette to bring them into close contact.

Note: As the laser pulses won't influence the surrounding manipulation media, any kind of medium may be used for placement of the embryos or cytoplasts under the laser microscope, as it would usually be used for micromanipulation purposes. HEPES-buffered media don't request special gas atmosphere and may therefore be used in air, which facilitates handling. If other media are used, a special gas atmosphere might be requested, which surrounds the manipulation droplet on the slide.

For the experiments use an oil immersion objective of high numerical aperture (e.g. 100x).

1. Collect bovine oocytes from fresh ovaries (slaughterhouse material). Puncture follicles with a diameter between 2-8 µm by a needle connected to a suction pump, with a maximum negative pressure of 80 mm Hg. Select cumulus/oocyte-complexes, wash and collect the complexes in Ham's F 10 medium + 10 % fetal calf serum + Gentamycin.
2. Perform *in vitro* maturation of the selected oocytes in 400 µl-drops (30 oocytes per drop) of modified Parker's medium (MPM) + 10 % fetal calf serum + follicle-stimulating hormone (FSH) + 0.01 U/ml luteinizing hormone (LH) for 20-22 hours.
3. To select matured oocytes, remove cumulus masses by incubation in Trypsin/EDTA solution (1 % in 0.8 % in PBS buffer) and pipette gentle. Oocytes showing an extruded polar body are classified as matured and can be selected for the laser experiments.
4. Place the cells to be fused into a droplet of medium (about 200 µl) on an object slide. Use the transfer pipette to rotate the cells on the slide, so that the contact area between the cells is in laser focus.
5. Apply several single laser pulses (max. 10) on several different sites in the contact area with laser energy of about 5 µJ and a pulse length of 3 ns.
6. Immediately after laser treatment, transfer the pulsed cells back into culture medium and record their fusion process every 10 minutes.

   Note: the fusion process may be recognized by disappearance of the membrane structures in the contact area and subsequent rounding up of the fusion product within one hour. If there are no signs of fusion after half an hour, pulsing may be repeated in another trial.

5.1.2 Drilling of the Zona Pellucida

Introduction

A few years ago, the opening of the zona pellucida of oocytes has been performed by micromanipulation methods using a sharp micropipette to microdissect the zona or, chemically by use of Tyrode's acid in order to destroy the structure of the zona.

The purpose of first experiments in oocytes to physically microdissect the zona pellucida by laser light (laser zona drilling) was the facilitation of sperm penetration to obtain better *in vitro* fertilization results. In the last years, laser zona drilling has been reported to facilitate the hatching process in expanded blastocysts, and to enable extraction of polar bodies by means of a blunt-ended pipette.

Materials

- Object slide (thickness 170 µm)
- Culture medium + phytohaemagglutinine (300-400 µg/ml) + HEPES
- Ham's F 10 medium + 10 % fetal calf serum + Gentamycin (ICN Biomedicals, Aurora, OH, USA)
- HEPES buffer
- Bovine oocytes from fresh ovaries: slaughterhouse material

Methods

For fusion experiments, any medium may be used for laser zona drilling experiments if the demanded external gas atmosphere is considered. For the experiments use an oil immersion objective of high numerical aperture (e.g. 100x).

1. Set the oocyte or embryo to be zona drilled into a droplet of medium (200 µl) onto an object slide.
2. Move the transfer pipette that the oocyte or embryo is set into the requested position (i.e. polar body visible, or where the perivitelline space is big enough).
3. Move the oocyte or embryo forwards and backwards into the focus of the laser beam and trigger series of pulses (laser frequency about 30 pulses per second, laser energy about 3-5 µJ). As the focus of the beam can be brought to less than 1 µm, small paths in the zona are visible and allow precise control of the manipulation procedure.
4. Transfer the laser-drilled oocyte or embryo back into culture medium or perform further procurement.

5.1.3 Polar Body Extraction for Prefertilization Diagnosis

Introduction

As polar bodies result from meiosis and are extruded into the perivitelline space, they contain the complementary genetic material to the oocyte, and therefore may be used for prefertilization genetic diagnosis. Polar body analysis helps to identify oocytes bearing chromosomal disorders or mutations. These altered oocytes result in failure of fertilization, abnormal embryo development, failure of implantation, and early or late fetal loss. Extracted polar bodies can subsequently be analyzed by fluorescence in situ-hybridization (FISH) for chromosomes or polymerase chain reaction (PCR) and related techniques to identify single genes and their disorders.
For polar body removal by a blunt-ended pipette, the zona pellucida of the oocyte has first to be microdissected with an UV-A laser beam (see chapter 5.1.2: Drilling of the Zona Pellucida). Then, a blunt-ended pipette can be introduced through the laser microdissected hole and pushed forward to the polar body. Then, the polar body is carefully sucked into the pipette and subsequently released into a separate drop of manipulation medium.
Polar bodies can be extracted solely by laser light, combining laser zona drilling and extraction of the polar bodies by means of a trapping laser instead of a pipette. This non-contact method, combined with laser pressure catapulting of the extracted polar bodies, is helpful especially if PCR is performed afterwards, as the risk of contamination is decreased by lack of contact to pipettes. Thus, a non-contact proceeding can diminish the danger of resulting misdiagnosis.

Materials

- Transfer pipette
- Object slide (thickness 170 µm)
- Culture medium + phytohaemagglutinine (300-400 µg/ml) + HEPES
- Ham's F 10 medium + 10 % fetal calf serum + Gentamycin (ICN Biomedicals, Aurora, OH, USA)
- HEPES buffer
- Bovine oocytes from fresh ovaries: slaughterhouse material

Methods

For the experiments use an oil immersion objective of high numerical aperture (e.g. 100x).

1. Set one oocyte into a 50 µl-drop of medium (see chapter 5.1.1: Fusion of Blastomeres and Cytoplasts) onto an object slide. Orientate the oocyte that way that the polar body is in the optical focus by using the transfer pipette.
2. The zona pellucida is laser-drilled close to the first (and second) polar body. Make sure that the opening results slightly bigger than the polar body to avoid its sticking during the extraction process.
3. Adjust the laser trap focus to the polar body and activate the trap. Move the trapped polar body through the laser-drilled hole.
   Note: If the polar body sticks to the membrane of the oocyte or to the zona pellucida, release it by carefully located UV-laser shots at the contact area.
4. Switch off the trap after the polar body is completely extracted out of the zona pellucida.
   Note: As a result, the polar body sinks to the bottom of the dish. In case of a fertilized oocyte, the second polar body is subsequently trapped and extracted through the same laser drilled hole.
5. Immediately, after manipulation, transfer the oocyte back into the culture medium.
6. The extracted polar body is either sucked into a transfer pipette and used for further analysis, or, in case of laser pressure catapulting, left on the slide until it is dried by evaporation (see chapter 5.1.4: Laser pressure catapulting of extracted polar bodies).

5.1.4 Laser Pressure Catapulting of Extracted Polar Bodies

Introduction

Proceeding with a non-contact manipulation method, laser pressure catapulting of extracted polar bodies enables their sterile transfer into PCR tubes. After extracted bovine polar bodies are dried on a LPC-membrane, the membrane in close vicinity around the polar body is microdissected by the UV-laser and the membrane fragment is catapulted into the top of a PCR tube.

Materials

- Polyethylene naphthalene (PEN) membrane mounted object slide (thickness 170 µm)
- ddH_20
- Mineral oil
- Requested buffers for subsequent biochemical processing

Methods

For the experiments use an oil immersion objective of high numerical aperture (e.g. 100x).

1. Transfer one extracted polar body in a small amount (0.5 µl) of highly purified water onto the polyethylene naphthalene (PEN) membrane, which is mounted on an object slide.
2. Watch the drying process through the microscope to make sure that the polar body can be identified again after drying. After complete drying, the polar body is prepared for laser pressure catapulting.
3. For the collection of the polar body place 1 µl of mineral oil into the cap of a PCR-tube. By using laser microdissection isolate the polar body without irritation of the adjacent areas. For targeted *LPC*, a small membrane cap connection is left between the polar body containing membrane island and its surroundings.
4. Catapult the polar body into the cap.
5. Add the requested buffer for further biochemical processing to the tube and spin down with 13000 rpm for 5 min.

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Clement-Sengewald et al.: Journal of Assisted Reproduction and Genetics. 2002

5.2 DNA Transfection

Schütze, Handschuh, Becker I, Minkus, Röhr, Becker K-F, Hitzler: Optical Tweezers and UV-laser MicroBeam for single cell preparation and nucleid acid transfection; European biomedical optics, 1995, 2628-19

The UV-laser PALM® MicroBeam was used to generate self-healing holes in the membrane of cells, through which substances like DNA can move into the cytoplasm. The cell line used for transfection was a dedifferentiated human breast cancer cell line, MDA-MB-435, with spindle-shaped morphology. Cells were cultured in hyperosmotic medium (DMEM, 10 % FCS; isoosmotic 330 mOs; increased to 630 mOs with NaCl) either overnight or for two hours. Before laser microbeam treatment, 2 µg of a plasmid containing the coding sequence for β-galactosidase was added to a 60 mm^2 cell culture dish. Cells were incubated for 24 hours, fixed and the activity of β-galactosidase was evaluated. The expressed enzyme converts the colorless cytoplasm to bright blue. Laser microbeam treatment resulted in significantly more blue stained cells than found in untreated areas.

Meanwhile we learnt that hyperosmotic medium is not necessary for material ingestion. Microinjection works perfectly within normal medium, which is anyhow better for the cells.

With the same principle fluorescence labeled substances (e.g. latex beads) could be introduced into attached growing tumor cells and the fluorescence distribution after laser perforation can be observed with confocal laser microscopy.

5.3 "Donut-Isolation" – Separation of Nuclei and Cytoplasm

Stefan Thalhammer, Wolfgang Heckl

Thalhammer, Kölzer, Frösner, Heckl: Laser-based isolation of cells and cell clusters for virus specific PCR analysis; European Biophysics Journal, 2000, Vol 29, Number 4-5, 12D-5

For some purposes it is of big advantage to isolate cell specific regions, e.g. nucleus from the remaining cytoplasm. This so-called "donut"-isolation can be used in various fields of application like virology, pharmacy and especially in pharmacokinetics and pharmacodynamics. The principle idea is to first isolate the desired region by laser circumcision with a cutting width of about 1 µm and catapult it into one collection vial. Thereafter, the remaining cytoplasm is isolated and collected within a second vial. This way the cell specific regions are separated from each other and can be processed specifically for certain experimental follow-ups.

In this protocol, cells, e.g. HEP-G2, DMSZ Braunschweig, were grown on the supporting polyethylene-naphthalate membrane, fixed with methanol and stained with Giemsa solution to increase contrast. The nuclei were separately collected from the remaining cytoplasm to study the mode of viral penetration.

Materials

- Poly-L-Lysin
- Slide Flask or Flasket (NUNC: 170920 or 177453)
- Cover slips (25x75 mm, thickness 1)

Methods

All steps should be performed under sterile conditions.

If you like, you can prepare membrane chamber-slides yourself:

Homemade cell culture chambers

1. Fix the polyethylene-naphthalate membrane (PEN) on the thin object slide with nail polish. After drying, seal the nail polish with fixogum.
2. Incubate the membrane 30 min with Poly-L-Lysine solution, rinse with sterile water and let the membrane air-dry.
3. Cover the membrane with the top of the chamber and fix it with fixogum.

The membrane-chamber system is ready for **cell-culture**.

1. Perform cell-culture as described for the selected species.
2. After cells are grown to a confluent layer, remove the media and the top of the chamber, wash cells with PBS buffer and fix the cells with methanol for 5 min and let the cells air-dry.
3. To achieve a higher contrast between membrane and the fixed cells, perform a Giemsa staining in a 0.1 % Giemsa solution for 10 min. Rinse the slides thoroughly with sterile filtered water and let the slide air-dry.

Laser microdissection and catapulting

1. The collecting cap is filled with 0.1-0.3 µl of pure glycerol and positioned in a distance of about 1 mm above the slide.
2. For laser microisolation use oil immersion objectives (63x or 100x magnification) with high numerical aperture to yield a spot size of less than 1 µm in diameter. The nucleus is circumcised with the underlying membrane cut simultaneously. The laser energy should be adjusted to yield less than 1 µm thin cutting line (about 0.4 µJ per pulse). It might be preferable to use less energy and repeat cutting a few times yielding an even smaller cut. Catapulting can be performed separately with slightly increased energy (LPC-function) or automated using the RoboLPC function.
3. For the isolation and capture of the remaining cytoplasm use a separate collection cap.

5.4 Isolation of Living Cells: A New Approach to Produce Homogeneous Cell Populations

Introduction

Non contact **L**aser **M**icrodissection and **P**ressure **C**atapulting (LMPC) allows microdissection of cell clusters or single cells and their transfer directly into the cap of a microfuge tube (Schütze and Lahr 1998). Up to now, microdissection methods have been limited to cells from fixed or frozen tissues (Meier-Ruge et al. 1976, Emmert-Buck et al. 1996, Schütze et al. 1998, Lahr 2000). These isolated cells can then be analyzed further for RNA, DNA, proteins etc. (Schindler 1998, Lahr et al. 2000). An old dream of cell biologists is to isolate living cells from tissue culture or unfixed and unfrozen sections of living tissues. But until now, laser-based microdissection of living cells resulted in the destruction of the

isolated cells. Now, a modification of the LMPC technique and a newly developed cell culture protocol allows microdissection and "ejection" of living single cells or cell clusters with ongoing cultivation for potential treatment and analysis (Mayer et al. 2002). The protocol was applied to cell culture cells, and selected cells were microdissected, ejected and recultivated. The captured and catapulted cells are not affected in their viability. They enter the cell cycle and proliferate.
To perform LMPC of living cells, HEP-G2 liver cells were cultivated in a PALM® DuplexDish. Cell clusters or single cells were circumcised using a 40x (Fig. 1a-b) objective lens. In cases where the selected specimen area contained undesired cells, these can be eliminated by a direct laser shot. LPC was done with one single laser shot positioned at the border of the circumcised film-cell stack (Fig. 1c). Microdissection and catapulting of cell clusters took less than 2 minutes. The manipulation of single living cells was done within seconds. The isolated and catapulted cells were finally suspended in an appropriate volume of supplemented medium. If only up to 10 cells were isolated, best results were achieved by bringing these few cells in close contact within a so-called "hanging droplet" (Mayer et al. 2002). The cells - still in a spherical shape - aggregated at the bottom of the droplet due to the gravity and started to form cell-cell contacts (Fig. 2). The following day the aggregated cells could be plated in a droplet of supplemented medium on the bottom of a standard culture dish. Using more than 10 captured cells, this was directly done without forming a "hanging droplet". Due to the spherical shape of the droplet, the aggregated cells were forced to move to the tension-free center of the droplet and after one day in culture, the cells showed the typical flat shape of epithelial cells. Cells entered the cell cycle and started to proliferate. Depending upon the density of the plated cells this took 3 days (30 cells) or up to 3 weeks (10 cells).

This new approach can be used to establish a homogeneous cell population out of a heterogeneous cell population (i.e. after transfection experiments). Now it's possible to obtain 100 % homologous cell populations for expression studies by recultivation of transfected cells or destruction of unwanted cells by precise laser shots. In addition, this protocol serves as a basis to develop the procedure to isolate homologous living cells from biopsies for real in vivo studies.

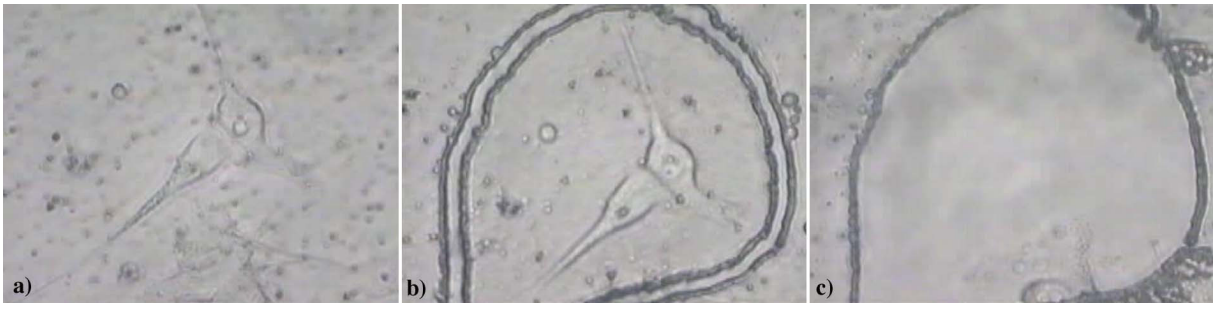

Figure 1: Laser Microdissection and Pressure Catapulting (LMPC) of living cells grown on the PEN membrane: **a)** HEP G2 cells grown on the supporting PEN membrane; **b)** isolation of selected cells; **c)** laser pressure catapulting of the isolated cells with the remaining hole in the supporting membrane (40x magnification) images courtesy of S. Thalhammer and W.M. Heckl, AG NanoScience, Munich.

5.4.1 Materials

IMPORTANT: To reduce the chance of contamination wear gloves during the whole cell culture procedures. Don't keep the cell cultures outside the incubator longer than necessary.

1. Culture dish plates for cell culture
2. Gassed incubator for cell culture
3. PALM® DuplexDish
4. PALM® DuplexDishHolder
5. Microfuge tubes, (P.A.L.M. Microlaser Technologies AG, Bernried, Germany)
6. Buffers and Solutions: Hanks solution (Sigma-Aldrich GmbH, Deisenhofen, Germany)
   Trypsin-EDTA solution (Sigma-Aldrich GmbH, Deisenhofen, Germany)
   PBS buffer (Sigma-Aldrich GmbH, Deisenhofen, Germany)
7. Cell Culture Media: Appropriate cell culture medium including 1x antibiotic-antimycotic solution (Sigma-Aldrich GmbH, Deisenhofen, Germany) and conditioned medium

5.4.2 Methods

Cell culture

(1) Seed the cell culture cells at the desired density onto the PALM® DuplexDish bottom. After 1-2 days in culture at 37 °C in a gassed incubator the cells are ready for microdissection.

Laser Microdissection and Pressure Catapulting (LMPC)

(2) Remove the medium completely from the PALM® DuplexDish before laser microdissection.

(3) Microdissect the desired cell-membrane samples. The parameters concerning laser energy and laser focus during microdissection are depending on the used Laser-Microscope system and have to be optimized before.

(4) Pipet a 5 µl droplet of Hanks solution into the center of the cap of a microfuge tube and place the cap directly above the selected cells into the PALM® DuplexDish by using the PALM® DuplexDishHolder (see **figure 2**).

(5) Catapult the cell-membrane stack with one single laser shot that is positioned at the border of the circumscribed film-cell stack. Energy settings should be sufficiently high to catapult the microdissected cells with the membrane into a cap. Even large cell-film stacks (for example 380 x 250 µm) can be catapulted.

(6) After LMPC remove the PALM® DuplexDish from the microscope stage and inspect the catapulted cells in the cap (see **note 1**).

Recultivation of isolated cells

(7) Cover the catapulted cells in the cap with 25 µl of Hanks solution.

(8) Close the cap with the remaining tube and store like this up to 30 min at room temperature to detach the cells from the membrane.

(9) Centrifuge the tube for 1 min at 8000 g and discard the supernatant.

(10) Resuspend the cells in 20 µl Trypsin-EDTA solution and incubate for 10 min at room temperature.

(11) Centrifuge for 1 min at 8000 g.

(12) After centrifugation the trypsinized cells are resuspended in 15 µl (< 10 cells) or 20 µl of supplemented medium (4 parts medium: 1 part conditioned medium) and the droplets are placed onto the bottom of a culture dish (see **note 2**).

(13) 24 h later mark the droplets with a permanent pen. This facilitates tracing of the cells.

(14) Remove the medium and fill the culture plate with 4-10 ml of fresh medium, depending on the diameter of the culture dish.

(15) The growing of the cells can be observed on next days and weeks. A regular change of cell culture medium is recommended.

(16) With less than 10 cells in the suspension form a so called "hanging droplet": Place the 15 µl drops inside the lid of a culture dish. Turn the lid and mount it on the remaining culture dish. (This results in "hanging droplets" where single cells come in close contact and start aggregation, see **figure 2**).

(17) Incubate the cells within the "hanging droplet" overnight at 37°C in a gassed incubator.

(18) The next day flip around the lid again and add 5 µl of supplemented medium to the droplet (total: 20 µl).

(19) Place the 20 µl droplets with the cell suspension with a sterile micro-pipet onto the bottom of a culture dish, either alone or with sufficient space to other droplets. Incubate overnight at 37°C.

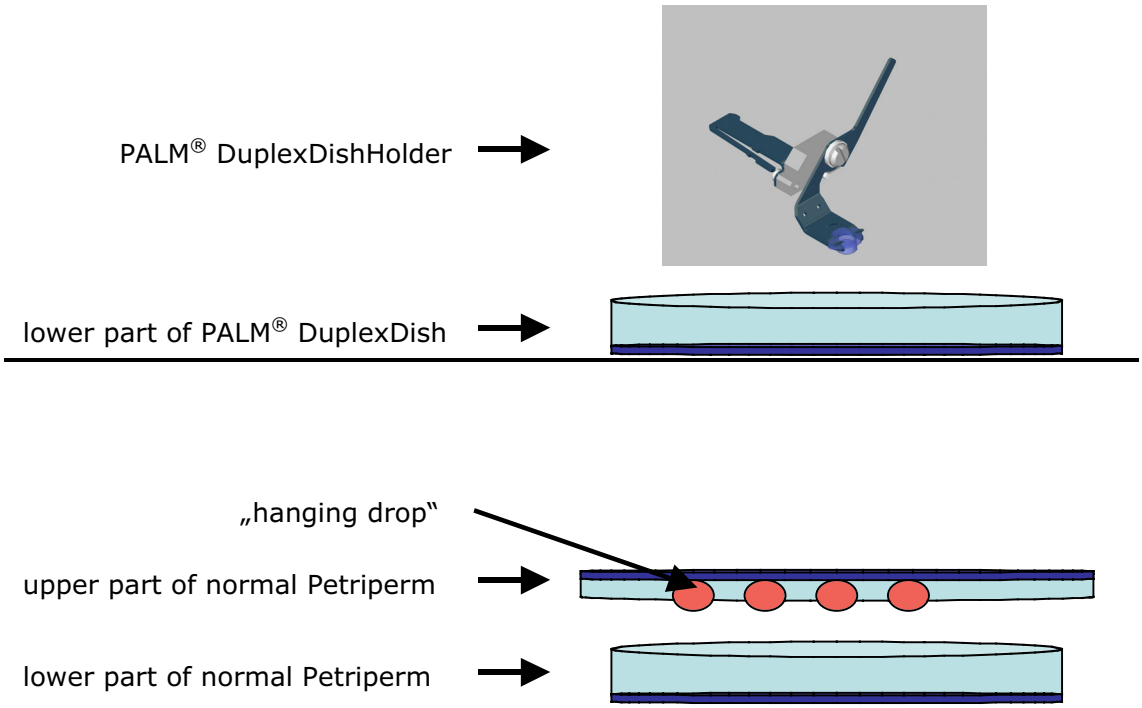

Figure 2: scheme of the isolation and „hanging droplet" recultivation

5.4.3 Notes

(1) In case of several experiments allow cells in the PALM® DuplexDish to recover from dryness by adding medium back to the cells. For microdissection go to **step 2**.

(2) If entirely sterile conditions are requested or the MicroBeam should be equipped with a small incubator on top of the stage, it should be placed within a hood (sterile working bench).

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