ELSEVIER

Genetic Analysis: Biomolecular Engineering
14 (1997) 1–8

GENETIC ANALYSIS
Biomolecular Engineering

Cut out or poke in—the key to the world of single genes: laser micromanipulation as a valuable tool on the look-out for the origin of disease

Karin Schütze [a,*], Ingrid Becker [b], Karl-Friedrich Becker [b], Stefan Thalhammer [c], Robert Stark [c], Wolfgang M. Heckl [c], Malte Böhm [d], Hans Pösl [a]

[a] *Städtisches Krankenhaus Harlaching, Applikatives Laserzentrum der I. Medizinischen Abteilung, Sanatoriumsplatz 2, D-81 545 München, Germany*
[b] *Technische Universität München, Klinikum Rechts der Isar, Institut für Pathologie, Ismaninger Straße 22, D-81 675 München, Germany*
[c] *Institut für Kristallographie der Ludwig Maximilians Universität, Theresienstraße 41, D-80 333 München, Germany*
[d] *Universitätsklinikum, Urologische Klinik und Poliklinik, Hufelandstraße 55, D-45 122 Essen, Germany*

Received 15 October 1996; accepted 19 November 1996

Abstract

The optical micromanipulation systems UV(ultraviolet)-Laser Microbeam and Optical Tweezers Trap, already proven to be powerful tools for 'non-contact' micro-manipulation of gametes, cells and organelles, have now made their way into the nanocosmos of genes and molecules. Force measurements of DNA transcription have been performed and selective DNA molecule micromanipulation gives insight into single molecule behaviour. Retrievement of selected single cells without contamination is an import prerequisite for further processing with modern methods of molecular biology. Laser micro-dissection allows to precisely eliminate any unwanted material or to isolate pieces of chromosomes or single cells of interest with high accuracy and efficiency. This enables the cell or chromosome specific molecular analysis of genes and genetic defects underlying disease, such as cancer or infection. This review article gives an overview of current topics of laser microbeam application in biological or medical research and advanced molecular diagnosis. © 1997 Elsevier Science B.V.

Keywords: Laser micro-dissection; Single cell preparation; Laser microbeam; Optical tweezers

1. Introduction

Lasers coupled into a microscope and focused to a spot size of less than one micrometer in diameter increasingly gain significance for 'non-contact' optical micro-manipulation in biological research and medical application. With the help of high photon densities and strong optical gradient forces microscopically small objects can be captured and positioned as well as micro-surgically processed with extremely high precision. The effective energy densities only arise at the small laser focal point. Since almost all biological specimens are transparent for the applied laser wavelength, it is possible to work within the depth of a cell without opening it. For a long time, the fascinating technique of laser micromanipulation has been the exclusive playground of physicists and laser experts. Huge setups with large lasers using high voltage power supplies, external water cooling and complicated coupling optics required extensive maintenance. Meanwhile, the development of smaller and less expensive lasers allows to manufacture compact, sturdy and easy to handle devices with no need for maintenance. Thus, the technique of optical micromanipulation now becomes amenable for non-physicists and an increasing number of different applications arise in the field of basic research and medical investigations.

* Corresponding author.

PII S1050-3862(96)00169-6

2. Optical trapping with visible laser light

A huge advantage of 'non-contact' laser microbeam application is the possibility, to manipulate specimen in a closed, entirely sterile environment without danger of evaporation, contamination or infection.

The mechanism of optical trapping utilizes the high photon density gradient of a strongly focused, continuous laser microbeam to catch, hold and move microscopically small particles. The force of the optical trap is depending on the shape and diffraction index of the trapped particle [1–4]. For example, motile sperm can be captured and dragged around [1,4]. Cell sorting is possible and even single bacteria can selectively be prepared out of a bulk of different species for subsequent cloning and DNA analysis [5].

Ashkin and Dziedzic [6] first used an Argon laser with 514 nm wavelength for optical trapping. When applied to living objects, they soon realized, that this wavelength was strongly absorbed and thus, detrimental to the specimen. Using an Nd:YAG laser with 1064 nm allowed trapping of bacteria, yeast cells and protozoa for a longer period without visible damage [7]. Meanwhile, some studies using laser traps working with different wavelengths revealed damaging effects also in the near infrared. Amongst those are impaired chromosome separation, cessation of sperm motility or strongly reduced cloning efficiency. These detrimental effects seem to be due to two photon absorption at 760 nm with resulting UVA like perturbations or water absorption with resulting heat formation at 1064 nm, respectively [8–10]. Recently, an optical tweezers trap was introduced using a visible, high power diode laser system working at 680 nm [11]. The Vis-Optical Trap works within the so called 'therapeutic window', which is located between 600–1200 nm, where only weak absorption of biomolecules occurs. Since water absorption starts from 800 nm and increases towards the infrared [12], trapping with the visible wavelength of 680 nm seems less harmful for living cells and organism as compared to optical tweezers working with wavelengths below 600 nm or in the near infrared. Interestingly, trapping with visible light is not protected by the Ashkin patent [13] of optical tweezers, which covers trapping systems in the infrared only, starting from 800 nm.

3. Force measurements on single molecule level

Force measurements of motor molecule action or evaluation of elasticity and rigidity in conformational changes of biomolecules is one of the most exciting applications of optical tweezers. Beads attached to microtubules serve as handles and are held in the laser trap. If a motor molecule acts on the microtubule, the bead will be dragged out of the trap center. The translocation of the bead from the trap center together with the applied laser power gives a measure of the dragging force. Another approach are escape force measurements within living specimen. This way the motion forces of dynein, kinesin and myosin molecules have been determined to be in the range between 1 and 3 pN/binding site [14–19].

With optical tweezers Kurachi et al. (1995) measured the rigidity of microtubules, by trapping attached polystyrene beads [20]. Felgner et al. could bend microtubules, fixed to axonema, by just grabbing the free floating ends of the microtubules with tweezers [21].

Single DNA molecules, visualized with video fluorescence microscopy and equipped with 1-micrometer beads as handles were held in the tweezers trap and stretched to full extension in a flow. Their relaxation was measured, when the flow stopped [22,23]. A double beam trap with two counter propagating laser beams, coupled through opposing objectives (dual-beam laser trap), was used to study the elastic response of individual double-stranded and single-stranded DNA molecules after over-stretching [24]. A highly cooperative transition into a stable, about 70% longer DNA configuration was measured. It is supposed that the conformational changes following over-stretching may play a significant role in DNA recombination.

In a recently published article the force involved in the process of DNA transcription was determined [25]. One polymerase molecule, usually walking along the DNA to transcribe its information into RNA, was fixed to the coverslip. This way, the polymerase molecule tears on the DNA molecule during transcription. It was shown that an average force of 14 pN is required to reversibly stall the polymerase molecules. This is substantially larger as compared to the above mentioned force of motor molecule action.

4. UV-laser microbeam for assisted embryo hatching

The high photon density arising at the laser focus point of an extremely focused, pulsed UV-laser enables to physically ablate almost all material. Thus for example, cutting of molecules, cytoplasmic filaments, flagella or even sperm tail is possible with high precision. Cytoplasmic movement or motile organism can reversibly be stopped. Tiny holes can be poked into cell membranes or even into rigid cell walls without disturbing cell viability. Poking holes into the contacting membrane area of two adjacent cells can induce cell fusion [1–4].

We have studied optical micromanipulation of gametes and embryos with 'non-contact' laser microbeams (Fig. 1 (a) and (b)) as an alternate approach to the more common in vitro fertilization procedures

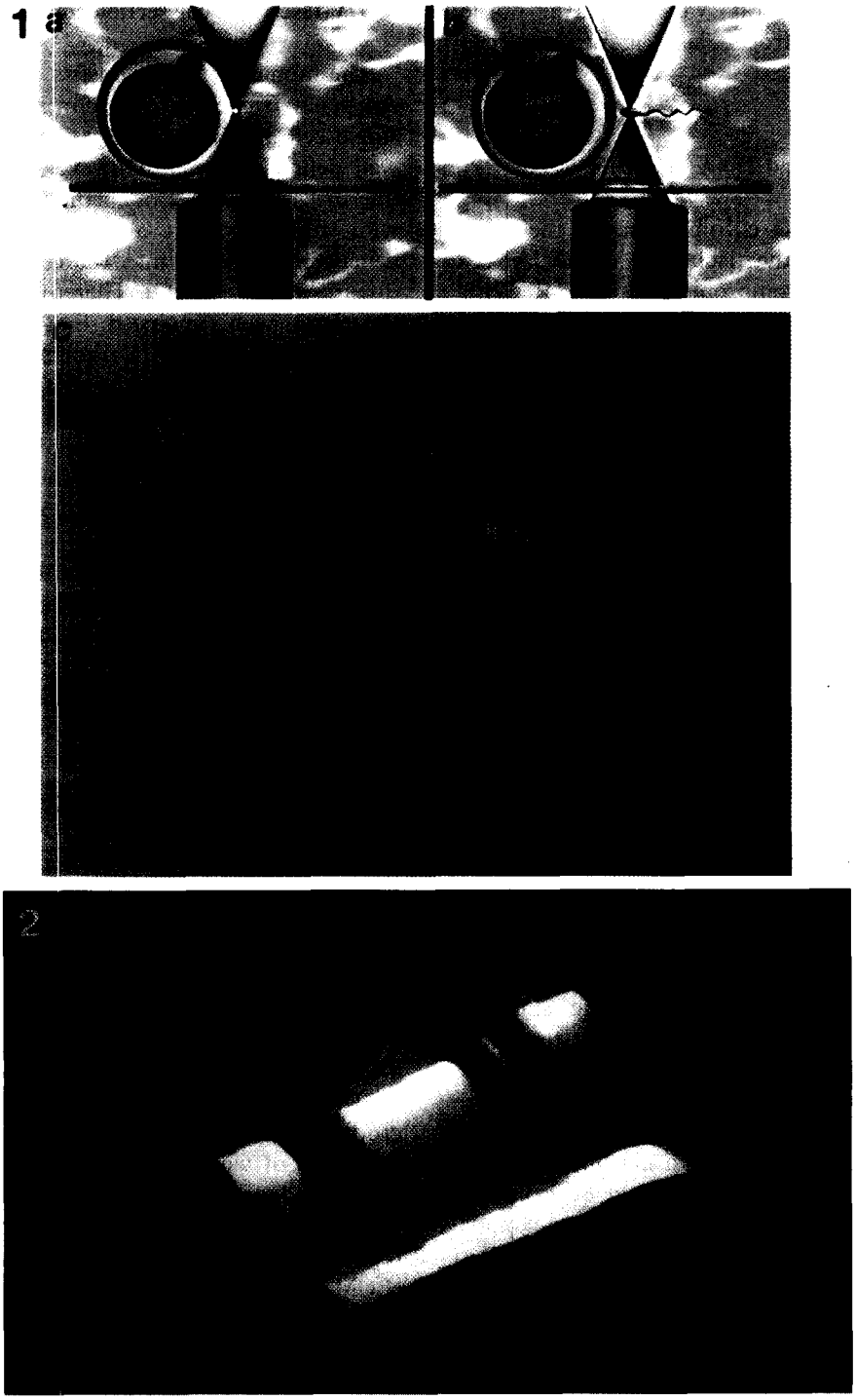

Fig. 1. (a) The UV-laser is focused through a high numeric objective. Due to the extremely high energy density at the focal point, the UV-laser microbeam can cut minute holes with high precision into the zona pellucida of an oocyte (video animation). (b) The optical forces arising at the laser focus of optical tweezers allow catching of highly motile sperm and enable sperm insertion into the perivitelline space of an oocyte without any mechanical perturbations (video animation). (c) Human embryo after non-contact laser zona drilling for assisted hatching. The embryo will hatch through the laser drilled hole (diameter of the hole is about 15 μm).

Fig. 2. Scanning force microscope image of a laser dissected Muntjac metaphase chromosome. Cutting size is about 700 nm, the thickness of the slices is around 1300 and 700 nm, respectively.

using mechanical microtools [26–30]. A laser drilled hole into the zona pellucida, for example, enables the embryo to hatch, and thus to implant [4] (Fig. 1 (c)). No detrimental effects but higher fertilization rates and increased hatching, have been found, when zona drilling was performed with a 337 nm nitrogen laser

[30–32]. Meanwhile, several women have borne healthy babies due to nitrogen laser assisted embryo hatching [33].

5. Cutting chromosomes and DNA filaments

Dissection of metaphase chromosomes is the most forward approach for the isolation of DNA sequences from specific chromosome regions [34]. With the high energy density of the focused laser beam, chromosomes on a metaphase or prometaphase spread or even within living cells can precisely be cut [35–37]. To evaluate the size of the laser induced cuts we generated a scanning force microscope (SFM) image of a laser microdissected Muntjac metaphase chromosome (Fig. 2). Several studies have demonstrated the excellent capability of SFM to monitor biological material like metaphase chromosomes [38–40], chromatin fibers and plasmids [41,42]. Two double cuts have been set with the UV-laser microbeam. No loose debris of biomaterial could be found in the SFM image. The laser cut pierces through the chromosome until it reaches the underlying glass substrate. Analysis of the cross section profile reveals a cut size of around 700 nm at a cutting angle of around 25 degrees with respect to the glass surface. The distances within the cuts of the two pairs measure 1300 and 700 nm, corresponding to about 13×10^6 and 7×10^6 bp, respectively [43]. The loss of beam quality due to lens imperfections together with the mechanism of photon absorption and/or heat transfer during ablation of the biomaterial may be responsible for the fact, that the theoretically diffraction limited spot size of around 200 nm cannot be reached.

When the cantilever of the atomic force microscope was used to scrape through a chromosome, an even smaller chromosomal fraction could be isolated. We could demonstrate, that even such a small amount of chromosomal material was amenable for further PCR processing and DNA sequencing. Laser microdissection and subsequent micro-cloning of the dissected fragments seem to be a helpful tool for rapid and convenient isolation of specific chromosomal regions.

Recently, Endlich et al. [44] have demonstrated laser cutting of isolated DNA filaments under microscopical view. To visualize the molecules, they were stained with the fluorescence dye DAPI. If combined with optical tweezers, the so called laser microbeam trap might be suitable to selectively prepare a small piece out of a defined DNA region. Activation of 'caged' molecules with a few UV-laser pulses seems to be an interesting approach to study the kinetics and mechanism of DNA function [45,46].

6. UV-laser microdissection for single cell preparation

The human genome consists of about 100 000 different genes packed into 46 chromosomes. In a healthy, single cell around 10–20 000 genes work together in a well defined manner. However, if there is a defect in one or several genes or in the symphonic performance with molecules that are involved in translating the genetic information into the cellular products, serious diseases may result [2]. Therefore, scientists for a long time have been convinced, that the origin of disease is on the single cell level. Molecular biologists and pathologists are now looking for mutated genes that may be responsible for the disorders. They try to prepare and multiply the DNA sequences of interest to learn more about the reason for the genetic alteration. One of the most powerful molecular biological techniques developed during the last decade is the polymerase chain reaction (PCR), which allows the million fold amplification of specific DNA sequences. But, this highly sensitive technique requires absolutely, clean sample preparation.

Generally, a tumorous tissue consists of tumor cells intermingled with normal, non-tumorous cells. This tissue heterogeneity provides one of the major obstacles to the molecular diagnosis of disease. Therefore, numerous efforts were made to specifically prepare small tissue sections consisting solely of tumor cells. In 1993 Böhm et al. demonstrated the selective procurement of tumor cells by microdissection of membrane mounted native tissue (MOMeNT) [47,48] using a small scalpel. This method provides good correlation with standard histology but allows only straight cuts. Using fine glass pipettes sample areas with sizes of around 1 mm^2 were mechanically resected from freeze-dried tissue [49,50]. In 1995 Kube et al. demonstrated a laser based microdissection of an entire renal tube, which was an extension of work by Meier-Ruge et al., 1976 [51,52]. Recently, laser melting of interesting cell areas onto a thermoplastic film using an infrared laser beam was reported [53]. With the 'laser capture microdissection' (LCM)-technique the prepared regions had a diameter between 60 and 200 μm.

However, within these dissected huge tissue areas, there is a high risk of contamination due to 'non-tumorous' material. We used the ROBOT-MICROBEAM system to eliminate all 'unwanted' cells from a selected tissue area with high precision or to microdissect one single cell out of a histological tissue slice without contamination. The ROBOT-MICROBEAM (P.A.L.M. Wolfratshausen, Germany) consists of a small, sturdy nitrogen laser of high beam quality, which is coupled through the fluorescence illumination path of the microscope. A motorized, computer controlled microscope stage and micromanipulator is attached to this Microbeam (Fig.

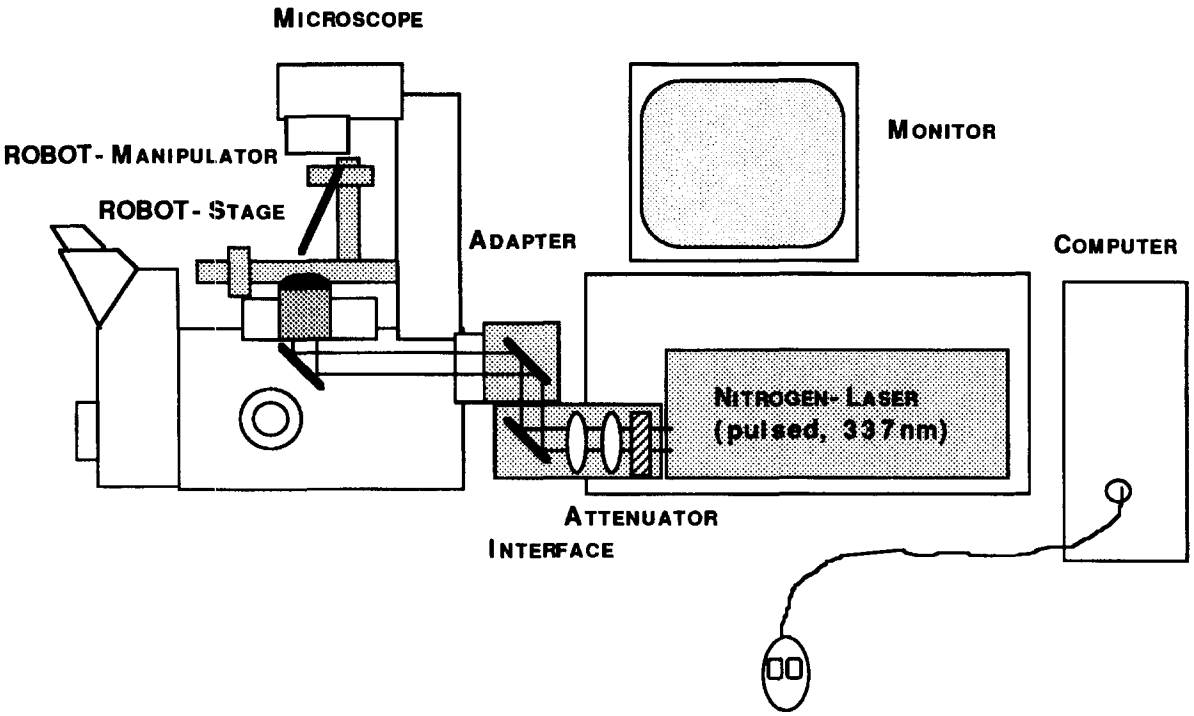

Fig. 3. PALM® ROBOT–MICROBEAM, a schematic drawing.

3). Laser micromanipulation with nanometer precision requires minute object positioning with extremely high resolution. The axis of ROBOTStage and ROBOTManipulator can travel with a speed of a few hundred nanometers up to several millimeters per second. Their hybrid stepping motors allow to travel distances up to 10 cm with a minimum step size of 20 nm. An integrated frame grabber enables simultaneously to observe the microscope image on the screen overlaid with a graphical user's interface to show the control functions of stage and manipulator. Also mandatory for precise laser micromanipulation is the coincidence of the laser focus with the optical focus of the microscope. Therefore, an important feature of the ROBOT-MICROBEAM is the possibility to individually move the laser focus position to correct for chromatic aberrations due to light absorbing material within the laser path.

The cells or cellular material that would interfere with PCR-amplification, are brought into the line of laser fire by conveniently moving the microscope stage via the computer mouse. One or a few UV-laser pulses are sufficient to destroy any biological material. The high photon density within the laser focus causes the material to be photolysed. This is a so called 'cold ablation' without any heating effect.

If combined with the above described MOMeNT-technique, retrievement of a large, previously laser cleaned, homogeneous tissue area, can be performed with high accuracy. The track of the UV-laser microbeam is preselected freely on the video screen. Thus, the laser dissects the membrane with the attached tissue along the edge of the interesting area, allowing to exactly respond to the irregular features of the histological section (Fig. 4 (a),(b) and (c)). The laser dissected membrane–tissue stack may be retrieved using a conventional discardable, sterile syringe needle. Another possibility is to use the laser pressure force to catapult the probe into a collecting tube or onto an adhesive foil without any physical contact (Fig. 4 (d)). This procedure could even be partly automated by several computer controlled steps.

The combined MICROBEAM-MOMeNT technique utilizes the force of high photon density, i.e. the ablative effect is due to photolysis and not to a heating effect [54]. This is different to the above mentioned LCM method, that melts the cells at about 90°C onto the thermoplastic film. Since the diameter of the melting laser beam is about 30 μm and has a round, circular shape, it seems to be difficult to selectively prepare irregular shaped tumor areas or to isolate one single cell only.

The ROBOT-MICROBEAM reveals a laser focus of less than 1 μm in diameter. This allows not only the preparation of one single cell or one cell nucleus out of a heterogeneous tissue slice, but even enables the isolation of a nucleus freed cytoplasm.

Contamination free single cell microdissection can be performed out of freeze dried or paraffin embedded tissue sections mounted directly on the glass slide. All cellular material adjacent to the cell of interest is en-

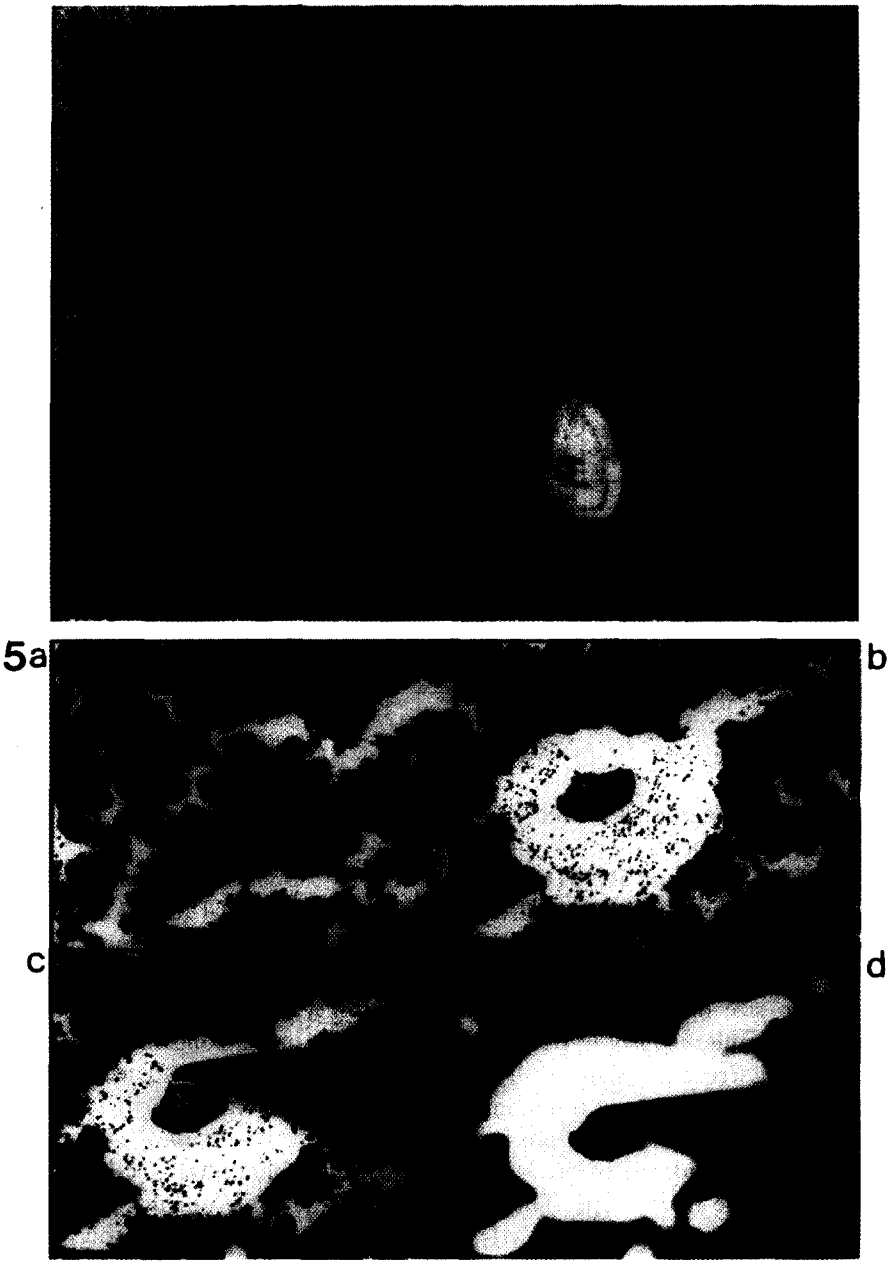

Fig. 4. (a) MICROBEAM–MOMeNT technique: the tissue section is mounted on a 6 μm thin supporting membrane. (b–c) The laser cuts along a preselected path, exactly corresponding to the various features of the tissue section. (d) For further DNA evaluation the laser dissected membrane-tissue stack is catapulted into a collecting vial or onto an adhesive foil using the laser pressure force (size of the dissectates = 30 × 50 μm).

Fig. 5. (a) ROBOT–MICROBEAM technique: formalin fixed and paraffin embedded, heterogeneous tissue sample, consisting of tumor cells intermingled with non-tumorous cells. (b) Single cell isolation: using the extremely high photon density within the focal spot of the UV-laser microbeam all cells adjacent to the cell of interest have entirely been eliminated. (c) A conventional injection needle, mounted to a micromanipulator, gently scrapes over the target cell. (d) The cell tightly sticks to the needle and can easily be transferred to a microfuge for further PCR based DNA amplification and subsequent DNA analysis.

tirely destroyed with laser ablation to yield a material free gap around the target (Fig. 5 (a) and (b)). To retrieve the cell from the object slide, a conventional sterile syringe needle is mounted to the ROBOT-Manipulator. When the needle gently scrapes over the laser isolated cell, it will stick and adhere tightly to the needle (Fig. 5 (c) and (d)) and can then be transferred into a microfuge tube for further DNA evaluation. Laser pressure catapulting for non-contact and quick single cell preparation as described above is possible as well. Laser irradiation does not affect the genetic information of the laser prepared cells, as proven by PCR

based amplification and subsequent DNA sequencing of the gene coding for the cell adhesion molecule e-cadherin in a diffusely growing gastric carcinoma [55].

The laser microbeam based, contamination-free preparation of one single or a few homogeneous cells out of a heterogeneous cell population is an important step towards highly precise molecular analysis of genetic defects, underlying cancer, infection or disease. Furthermore, the possibility to also use formalin-fixed and paraffin-embedded samples for single cell based, genetic analysis opens the way to also examine archival material of resected tissue samples collected over decades for routine histopathological diagnosis. A large number of patients with usual and unusual disease of known clinical outcome could retrospectively be examined to gain more information about the molecular defects.

7. Future perspectives

An interesting application of the UV-laser microbeam is material injection into a living cell without destroying cell viability. The precisely focused UV-laser microbeam can poke minute holes into the membrane of an adherent growing or free floating cell. The hole will stay open for a fraction of a second only, before it is repaired by the cell. This is sufficiently long to allow genetic material dissolved in the surrounding medium to be soaked. If the hole is poked directly into the nuclear region, the success rate of DNA transfection as well as the efficiency of cell modification was increased by orders of magnitude [56,57]. The selectivity and increased efficiency of laser based material injection without chemicals or viral vectors might serve as a first step towards 'optical' gene therapy.

One of the major advantages of 'non-contact' laser micromanipulation for single cell or single molecule based gene diagnosis is its high precision and selectivity, as well as the purity of sample preparation. Combined with a laser scanning microscope micromanipulation of nuclear domains as well as chromosome territories might be possible in an environment close to physiological conditions [58]. Further details of DNA alterations could be studied in combination with scanning force or near field microscopy [59,60]. With the FOLANT technique [61] (focusing of laser radiation in the near field of a tip), which is a modified version of scanning tunneling microscopy, 'non-contact' laser microdissection of biomolecules with only a few nanometers of cutting size is possible.

In conclusion, we believe that 'non-contact' laser micromanipulation will become a mandatory tool in modern gene and biotechnology.

Acknowledgements

We are grateful to Art Ashkin and Karl–Otto Greulich, who introduced the fascinating technique of laser micromanipulation to us and to Raimund Schütze (P.A.L.M. GmbH, Wolfratshausen, Germany) for instrumental support and his untiring technical assistance. We also thank Dirk J. Ruiter and Henry Dijkman, Nijmegen, Netherlands as well as Heinz Höfler, München for transferring the laser microdissection technique to molecular pathological questions and Christoph Köppen for stimulating discussions and helpful comments.

References

[1] Schütze K, Clement–Sengewald A. Nature 1994; 368: 667–670.
[2] Greulich KO, Leitz G. Exp Tech Phys 1994; 40: 1–14.
[3] Greulich KO et al. Video C-1897 Inst. Sci. Film, Göttingen, Germany 1995.
[4] Schütze et al. Video C-1921 Inst. Sci. Film, Göttingen, Germany 1996.
[5] Huber et al. Nature 1995; 376: 57–58.
[6] Ashkin A, Dziedzic JM. Science 1987; 235: 1517–1520.
[7] Ashkin A, Dziedzic JM, Yamane T. Nature 1987; 330: 6150: 769–771.
[8] Vorobjev IA et al. Biophys J 1993; 64: 533–538.
[9] König K et al. Nature 1996; 377: 20–21.
[10] Liang H et al. Biophys J 1996; 70: 1529–1533.
[11] Product review. Nature 1996; 381: 813.
[12] Boulnois JL. Laser Med Sci 1986; 1: 47–66.
[13] Ashkin A, Dziedzic JM. Patent no. 4893886, 1990.
[14] Ashkin A et al. Nature 1990; 348: 346–348.
[15] Svoboda K et al. Nature 1993; 365: 721–727.
[16] Kuo SC, Sheetz MP. Science 1993; 260: 232–234.
[17] Finer JT, Simmons RM, Spudich JA. Nature 1994; 368: 113–119.
[18] Warshaw DM. News Physiol Sci 1996; 11: 1–7.
[19] VanBuren P et al. Biophys J 1995; 68: 256s–259s.
[20] Kurachi M, Hoshi M, Tashiro H. Cell Motil Cytoskeleton 1995; 30: 221–228.
[21] Felgner H, Frank R, Schliwa MJ. Cell Sci 1996; 109: 509–516.
[22] Perkins TT et al. Science 1994; 264: 822–826.
[23] Perkins TT et al. Science 1995; 268: 83–87.
[24] Smith SB, Cui Y, Bustamente C. Science 1996; 271: 795–799.
[25] Yin H et al. Science 1995; 270: 1653–1657.
[26] Schütze K, Clement–Sengewald A, Ashkin A. Fertil Steril 1994; 61: 783–786.
[27] Tadir Y, Neev J, Berns MW. Semin Reprod Endocrinol 1994; 12: 169–176.
[28] Schütze KA, Clement–Sengewald A, Berg FD. Hum Reprod Update 1995; 1: 1.
[29] Clement–Sengewald A et al. J Assisted Reprod Genet 1996; 13: 259–265.
[30] Neev et al. Hum Reprod 1993; 8: 939–944.
[31] Enginsu E et al. Hum Reprod 1995; 10: 1761–1764.
[32] Liow SL, Bongso A, Ng SC. Hum Reprod, 1996; 11(6): 1273–1280
[33] Antinori S, Selman HA, Caffa B, Panci C, Dani GL, and Versaci C. Zona opening of human embryos using a non-contact UV laser for assisted hatching in patients with poor prognosis of pregnancy. Hum Reprod 1996; 11(11): 2488–2492.

[34] Senger G et al. Hum Genet 1990; 84: 507–511.
[35] Monajembashi S et al. Exp Cell Res 1986; 167: 262–265.
[36] Liang et al. Exp Cell Res 1993; 204: 110–120.
[37] Ponelis N et al. J Biotechnol 1994; 35: 109–120.
[38] Hansma HG et al. Science 1992; 256: 1180–1184.
[39] de Grooth BG, Putman CA. J Microsc 1992; 168: 239–247.
[40] Rasch P et al. Proc Natl Acad Sci USA 1993; 90: 2509–2511.
[41] Schaper A, Pietransanta LI, Jovin TM. Nucleic Acids Res 1993; 25: 6004–6009.
[42] Allen MJ et al. Chromosoma 1993; 102: 623–630.
[43] Thalhammer S et al. Biomed Optics, 1997; 2: 3–5.
[44] Endlich N, Harim A, Greulich KO. Exp Tech Phys 1994; 40: 87–93.
[45] Endlich N et al. Exp Tech Phys 1995; 41(2): 298–308.
[46] Meldrum RA, Meaking WS, Wharton CW. Nucleic Acids Res 1994; 22: 1234–41.
[47] Böhm M, Wieland I, Totzeck B. Cancer Genet Cytogenet 1993; 65: 83–87.
[48] Böhm M and Wieland I. Int J Oncol 1997; 10: 131–139.
[49] Zhuang Z et al. Am J Pathol 1995; 146: 620–625.
[50] Speicher MR et al. Am J Pathol 1995; 146: 1332–1340.
[51] Meier–Ruge W et al. Histochem J 1976; 8: 387–401.
[52] Kubo Y et al., Cancer Res 1995; 55: 989–990.
[53] Emmert–Buck et al. Science 1996; 274: 998–1001.
[54] Srinivasan R. Science 1986; 234: 559–565.
[55] Becker I et al. Lab Invest 1996; 75(6): 801–807.
[56] Tsukakoshi M et al. Appl Phys 1984; B35: 135–140.
[57] Tao W et al. Proc Natl Acad Sci USA 1987; 84: 4180–4184.
[58] Cremer T et al. In: Brandham PE, Bennett MB (eds) Kew Chromosome Conference. Kew: Royal Botanic Gardens, 1995; IV: 63–81.
[59] vanHulst N, Moers M, Bölger B. J Microsc 1993; 171: 95–105.
[60] Heckl WM. In: Fischer EP, Klose (eds) The Diagnostic Challenge, The Human Genome. München: Piper Verlag, 1995: 99–145.
[61] Jersch J, Dickmann K. Appl Phys Lett 1996; 68: 868–870.