Laser microdissection (LMD) is now a well established method for isolating individual cells or subcellular structures from a heterogeneous cell population. In recent years, cell, DNA, RNA, and protein based techniques has been successfully coupled to LMD and important information has been gathered through the analysis of the genome, transcriptome, and more recently the proteome of individual microdissected cells. The aims of this review are to summarize and compare the principles of different laser microdissection instruments and techniques, to discuss sample preparation procedures for microdissection, and to provide wide variety of examples of translational/clinical research applications of LMD. Novel techniques specifically developed for the improved isolation of stained cells, living cells, or rare cells are also discussed. LMD has become an indispensable tool in the preparation of homogenous samples for sophisticated cell or molecular assays. Despite major technological advances, the labor requirements of LMD are still relatively high. However, understanding the advantages and disadvantages of LMD technology and associated sample preparation procedures may aid in the earlier introduction of this method into the routine clinical diagnostics. © 2006 International Society for Analytical Cytology

Key terms: laser microdissection; gene expression; microarray; mutation analysis; proteomics

Both physiological and pathophysiological processes are governed by a complex array of cellular populations. However, modern cell and molecular biological methods require pure samples to allow reliable and statistically relevant analysis. The development of the LMD instrument made it possible to isolate defined cells from heterogeneous cell populations without contamination of unwanted cells and thereby ameliorated the obstacle of tissue complexity. If followed by high throughput genomic, transcriptomic, and proteomic analysis techniques, delineation of the expression profile of specific cell types or individual cells, which was so far hidden or masked in the background of surrounding cells, could be revealed. The advent of LMD procedure would help in the elucidation of biological processes and in the understanding and classification of diseases by identifying novel genes/proteins, pathways, and pathway networks not previously known to be associated with a particular disease.

The first report of a laser-assisted microdissection system that was capable to overcome the extremely time-consuming and highly operator dependent manual preparation of tissue sections was published in 1976 (1). A few hundred cells from heterogeneous tissue samples were successfully captured with this device and quantitative histochemical analysis was performed. The next big step in the development of LMD instruments was the employment of laser light for the negative selection of unwanted tissue areas by selective ultraviolet (UV) radiation fractionation by Shibata et al. (2). It was in the late 90s when almost concurrently two very different novel laser microdissection platforms were built. One was developed in the National Institutes of Health in Bethesda and became commercially available within 12 months of the first publication describing its use (3). This laser capture microdissection platform is based on the selective adherence of visually targeted cells to a thermoplastic membrane activated by a laser pulse. The other microdissection technique, the laser microbeam microdissection, was developed by Schütze and Lahr in Germany (4). The principle
underlying this technology is a highly focused laser beam that is used to cut out the cells of interest from the surrounding structures, and by increasing the power of the laser the desired cells are then ejected into a receptacle for subsequent analysis. Essentially, the methods that are employed in these two platforms constitute the base of current LMD systems.

In this review we intend to give an overview of the commercially available laser microdissection systems with a special emphasis on the consequence of operational differences on sample recovery. Furthermore, we discuss in detail various sample preparation procedures that were developed or optimized for the isolation of macromolecules from microdissected material. And last we provide examples of translational/clinical research studies that successfully used LMD technology in combination with cellular and molecular methods.

**PRINCIPLES OF LASER-MICRODISSECTION**

A typical LMD instrument consists of an inverted or an upright research microscope that is equipped with a CCD camera and a motorized computer-controlled stage. The microscope image of the specimen selected for microdissection is usually observable on a color computer monitor and overlaid with the user interface of the laser microdissection software. The positioning of the laser microbeam is achieved through the computer via joystick. Cells or tissue fragments desired to be harvested are marked out and cut manually or automatically by moving the laser light along a user-defined path or geometric pattern. The cutting process can be followed on the computer monitor in real-time. The spot size, the energy, the pulse duration, and in some instruments also, the precise focus of the laser can be selected for fine precision cutting. Most instruments allow for the selective destruction of unwanted cells by applying a few laser shots around the desired fragment and thus preparing a clear-cut gap between wanted and unwanted materials. Depending on the method of laser microdissection being used, the dissected specimen is transferred into a collection vial. To confirm the success of the microdissection and transfer processes, LMD instruments give a way of visually inspecting the collection vial with microdissected specimen and the empty patches at the tissue section corresponding to it. Once the correct identity and quality of the microdissected areas are verified, an archival workstation allows photographic documentation.

At this point of time there are five different widely available laser microdissector systems (Table 1). The different laser microdissectors can be categorized by the type of the laser used and the method how the dissected specimen is captured.

Except for Arcturus’ PixCell® microdissector, which uses an infrared (IR) laser, all these systems are fitted with a pulsed UV laser light source. The laser light is brought onto the specimens through the objectives of the microscope or from a separate top mounted source. For fine precision cutting the laser beam is either focused through the microscope optics or specially designed mechanical
parts. The shorter wavelength of the UV systems allow focusing of the laser light in the sub-micron range, while the focusing diameter of the IR laser beam in the Arcturus system can be adjusted from 7.5 (approximately the size of a cell) to 30 μm. Although, the narrower focusing width of the UV lasers enables more precise cutting and thus single cell or subcellular microdissection, there were concerns over the impact of the UV light on cellular macromolecules. More recently numerous studies have been published showing that the UV-A range (320–400 nm), where these systems operate, is not changing or harming nucleic acids and other cellular biomolecules, as their absorption peaks lies well outside of this region (10,11). While, in Arcturus’ PixCell device, because of the lower energy of IR lasers, the photochemical damaging effect is less of an issue, but what could potentially harm the cells is the heat generated by the absorption of the IR laser wavelength. However, according to some studies, the thermal effect that reaches the tissue is transient (last only for a few milliseconds) and mild, thus the DNA, RNA, or protein content of the cell is not altered in any measurable fashion (12,13).

What mostly determines how the dissected specimen is collected is the spatial arrangement of the stage, the specimen collector, and the laser light. In PALM’s inverted microscope system, the specimen collector lies above the stage and the laser light comes from the bottom through the microscope optics. Thus, after cutting, the isolated specimens need to be ejected out of the object plane, which is achieved with the help of a single defocused laser pulse. The gas pressure force developing under the dissected specimen pushes it out and transports it over centimeter-wide distances into a common collection vial lying above the stage. Lecia’s microdissector uses an upright microscope architecture with the laser coming from the top through the microscope objectives and the specimen collector is located right below the stage. The dissected material in this device simply falls in the receptacle by the force of gravity without the need of any additional physical forces. The design in the Arcturus’ inverted microscope system is such that the laser, mounted above the stage, has to be directed through specially developed caps that are coated with a thermoplastic film. The collection caps are placed directly on the top of the specimen, and so when the laser melted the film onto the cells of interest they remain attached and can be lifted with the cap for further analysis. Reportedly, the melting technique applied in the Arcturus system carries a higher risk of contamination with non-selected material sticking to the adhesive film (14). MMI has recently debuted with their new μCUT microdissector that is built around an inverted microscope system. In brief, the principle of their microdissection procedure is that specimen is placed on a special holder, which is covered by a membrane; the collection cap rests on the membrane and has no direct contact with the specimen. When the cut has been completed, the samples isolated can be removed with the aid of a special adhesive film on the lid of the test tube that ensures that only the cut out parts of the specimen remain adhered to the lid. For the collection of microdissected specimens, Cell Robotics offers a manual fragment retrieval system that consists of a specially coated microscope slide and a collector receptacle that is placed on top. Fragment separation from the tissue section is made possible by action of electrostatic forces between the slides and the collector cap.

It follows from the operational differences described earlier that some of these systems are more prone to contamination from environmental sources and from neighboring tissues than others, but most investigators agree that the purity of microdissected specimen in all of these systems allows for downstream analysis of cells and subcellular components at unprecedented accuracy.

General Aspects of Sample Preparation

Laser microdissection has been applied on several different sample types, such as snap frozen or formalin-fixed, paraffin-embedded tissue sections, cytospins, cell smears, and chromosome preparations. General pathways of retrieving biological information from microdissected material are shown in Figure 1.

Prior to microdissection, depending on the type of the specimen and the method of microdissection, samples are deposited onto routine glass object slides, super-frosted slides, charged-slides, or specially designed membrane mounted slides. Although, the latter is not compatible with all microdissection protocols, it facilitates the isolation of large tissue areas (up to several millimeters in diameter) and helps to preserve the original tissue morphology. Theoretically, from this point on, the sample could be subjected to microdissection without any further manipulation. However, precise sampling of microdissected material is often complicated by the poor visualization of the specimen. This is because standard microdissection protocols require direct physical access to specimens and thus they need to be uncovered and air-dried. To distinguish morphologically similar but phenotypically different cells or less prominent cell types on these preparations could be very challenging. Different strategies have been suggested to improve visualization of microdissected specimens.

Mické et al. recently described the use of a novel fluid cover medium that provided improved tissue morphology on both frozen and paraffin-embedded tissue sections without interfering with the subsequent microdissection procedure (15). Based on their results, the proposed method leaves DNA and RNA-dependent downstream applications unaffected.

Another strategy to improve the distinction of different cell types is to apply some sort of a staining procedure before LMD. However, the difficulties with standard staining protocols are several folds. First, for the successful performance of LMD, careful control of tissue adhesion to the slide is necessary to avoid detachment of sections during the staining procedure without compromising laser capture. Second, prolonged incubation times and harsh chemicals jeopardize the integrity of biomolecules, and thus the staining procedure may negatively affect the recovery
or the integrity of cellular proteins and nucleic acids and could be specially deleterious for the easily degradable RNAs.

Fend et al. developed a rapid and versatile immunostaining protocol that is sufficiently gentle to conserve minute quantities of RNA and can be applied on frozen sections prior to microdissection (16). This enables the targeted isolation and analysis of immunophenotypically different cell populations from complex lesions, with an intimate mixture of morphologically identical cell types. The feasibility of this immuno-LMD approach was demonstrated in their study with amplifying cell-specific transcripts with conventional reverse transcription (RT)-PCR from immunostained normal and neoplastic lymph node, breast, and prostate frozen sections. Since then, DNA, RNA, and proteins have been successfully recovered, with optimized protocols from minute quantities of immunohistochemically or immunofluorescently stained specimens (16).

To prevent contamination from neighboring tissue or cells in some cases, it is advisable to destroy undesired structures before collecting the microdissected specimens. This can be achieved by cutting around the target specimen, yielding a clear-cut gap between the selected and non-selected specimen or selectively burn unwanted objects with precise laser shots. Once the desired specimen has been selectively collected, its nucleic acid or protein content has to be extracted for downstream analysis.

Although LMD technology has proven itself as a valuable method for the rapid and efficient isolation of specific cells free of contaminants, a number of potential disadvantages exist. The LMD technology is examined from different perspectives, such as the ease of use, versatility, costs,
sample handling, and time efficiency, and the identified advantages and disadvantages are summarized in Table 2.

Because of its relatively high-cost, the LMD instrument is a part of a core facility in most institutions. Generally, microdissection services are provided to individual labs on a per sample or per hour basis. For neophytes in the field of LMD, a couple of hours of training is usually sufficient prior to independent operation of any type of LMD instrument. However, optimization of microdissection protocols for novel cell or tissue types can be a time-consuming process and experienced operators can help tremendously for beginners in the selection of the best-suited instrument and protocol for a particular application.

DNA Isolation and Amplification from Laser-Microdissected Sample

Because of the cumbersome procedure of phenol-chloroform-based DNA extraction, it is generally considered unsuitable for extraction of nucleic acids from microdissected material.

However, most of the currently available commercial kits that use magnetic glass particles, silica membrane, gel beads, or glass filter to bind DNA are appropriate to isolate DNA from minute amount of starting material. It is still important though to minimize the possibility of DNA degradation during the isolation procedure and include a nuclease inhibitor (17). The DNA yield from microdissected clinical samples is frequently limited and thus for genetic studies a whole genome amplification method (WGA) has to be used to substitute the genomic DNA with WGA genomic DNA. However, complete and faithful amplification of the entire genome is difficult, and the requirements and limits of WGA with respect to cell number and cell processing are poorly established (18). The number of cells and the recovered amount of DNA is especially critical when characterizing the allele status at chromosomal loci demonstrating microsatellite instability (MSI) or loss of heterozygosity (LOH) as insufficient quantities of cells or genomic DNA may result in unequal allelic amplification (19). Furthermore, artificial losses of chromosomes or chromosome parts could occur during microdissection, leading to artificial allelic losses (pseudo-LOH) (20).

RNA Isolation and Amplification from Laser-Microdissected Sample

Typically, laser microdissection samples are so small that the effective isolation of the easily degradable RNA fraction becomes challenging. Purifying RNA from these samples with conventional methods is often inefficient and results in the loss of precious RNA. To limit the loss of RNA during the isolation procedure, many manufacturers assembled specially designed kits for microdissected samples. These kits usually contain DNAses and RNase inhibitors and allow the elution of purified total RNA in small volumes (10–30 μl). It is suggested to add the lysis solution directly to the microcentrifuge cap, and thus the captured cells are immediately stabilized and purification can proceed as normal.

As endogenous RNases also pose a risk to the integrity of the RNA, appropriate handling of the tissue is necessary to prevent endogenous nucleases to be activated. Thus processing time and storage conditions may also be important factors in influencing RNA recovery. Although there are existing protocols designed to extract acceptable quality of RNA from formalin-fixed laser microdissected tissue material, ideally RNA recovery is best performed on freshly frozen tissue (21,22). Low, medium, and high-abundance transcripts are consistently recovered from fresh frozen tissue in relatively high quantities. Some investigators achieved acceptable RNA recovery by using precipitative fixatives, such as ethanol and acetone. As for example, in the study of Su et al. the RNA yield from ethanol-fixed brain tissues was 70% of the yield from fresh frozen specimens (23). According to almost all studies, precipitative fixatives constantly produce more RNA than cross-linking fixatives such as formalin (20,21,24).

The average amount of total RNA per cell from a human tissue section is estimated to be about 10 pg, while most expression profiling platforms require microgram quantities (25,26). Thus, regardless of the extraction method, the recovered quantity of RNA is usually insufficient for reliable microarray analysis. This underlies the necessity to employ RNA amplification methods that does not introduce major distortion of expression profiles obtained after amplification. More recently a number of techniques have been presented for the amplification of scant amounts of RNA, including linear amplification and PCR based exponential amplification methods. Most currently used linear amplification protocols are based on the patented Eberwine’s antisense RNA (aRNA) amplification protocol (27).
The detailed description of these approaches is beyond the scope of this review article, but in brief the principle steps of Eberwine's procedure is a reverse transcription using an oligo(dT) primer containing a T7 RNA polymerase promoter sequence, next the small RNA fragments serve as primers during the second-strand synthesis reaction, producing a double-stranded cDNA template for transcription. cDNA template is then used in a large scale in vitro transcription (IVT) reaction to produce linearly amplified aRNA. When using microdissected sample material, the amount of aRNA at the end of the first amplification cycle is usually insufficient for gene expression analyses. Then, the aRNA can be used as template for cDNA synthesis followed by a second round of amplification. Because these second round primers are random primers, transcript sizes will generally decrease. Using more than two amplification rounds is not recommended as the risk of distortion of the original gene expression profile becomes too high (28). Because of their high fidelity, linear amplification protocols became an integrated part of several standard labeling protocols for microarray hybridization.

An interesting novel method for analyzing mRNA transcripts from microdissected cryostat sections of frozen tissues without the need for RNA isolation has been published by To et al. (29). In brief, the principle of the technique is to subject microdissected cells to freeze-thaw cycles and collect the cell lysate into a solution designed to minimize RNA degradation. Subsequent RT-PCR reactions can be performed from the lysate without the need of any additional processing. With this technique the authors demonstrated that a small microdissected region, containing only a couple of hundred cells, can provide adequate RNA template for 80–100 RT-PCR reactions. In their study they successfully amplified different size cDNA fragments of the β2 microglobulin, β2I*variant, and BRCA1 genes from microdissected regions of frozen breast carcinoma sections.

With the use of internal controls of defined copy numbers, Luzzi et al. demonstrated that mRNA from 15,000 LMD isolated breast epithelial cells can be used for high-density oligonucleotide array based expression profiling with similar analytical sensitivity and precision than routine methodologies, using 200 and 1,000 times greater input RNA (30).

In a recent study combining microdissection with high-density oligonucleotide array on breast tissue, King et al. showed unequivocally that even when the starting RNA material is in the nanogram range the biological variability in gene expression between independent specimens, and between histologically distinct samples within a specimen, is greater than the technical variability associated with the procedures (31).

Sanna et al. analyzed the gene expression profiles of anatomically-defined brain regions by comparing LMD-derived samples subjected to 2 rounds of IVT and manually-dissected brain regions subjected to 1 or 2 rounds of IVT (32). As expected, target RNAs prepared with the double IVT protocol were shorter (∼200–1,000 bases) than those prepared with a single cycle of IVT and thus resulted in lower number of “present calls”. However, regardless of the number of IVT cycles, gene expression ratios between manually-dissected and LMD samples were highly concordant.

Following from the aforementioned we can conclude that the feasibility of using cDNA/oligonucleotide microarray on microdissected samples is well established. The number of cells required for analysis is dependent on many factors, such as tissue preparation, type of laser used for microdissection, RNA isolation, and amplification strategies, etc. While, RT-PCR reactions have been successfully performed from RNA from as few as 10–100 microdissected cells, microarray analysis still requires a couple of thousand cells. If the goal is to achieve a less noisy global gene expression profile from microdissected material, protocols must call for the procurement of more than 10,000 cells, harvesting of which requires a substantial time commitment.

**Protein Isolation from Laser-Microdissected Sample**

Several reasons necessitate the direct examination of proteins rather than the genome or transcriptome. First, post-translational modifications significantly affect the function and activity of proteins and disease-specific post-translational modifications may have particular importance in clinical applications. Second, mRNA expression levels are not necessarily correlating with protein quantities and by means of alternative splicing different splice variants may encode for diverse proteins. Third, deciphering the changes in the proteome could potentially aid in the identification of novel diagnostic and therapeutic targets.

In recent years, several investigators established conditions for obtaining proteomic information from laser capture microdissected specimens. Proteins in these studies were analyzed by two-dimensional (2D) gel electrophoresis (33,34) immunoassay (35,36), and more recently by mass spectrometry based technologies (37,38). Nevertheless, the amount and quality of proteins retrieved after microdissection primarily impacts the successes of downstream analysis. Usually, a few hundred to a few thousand cells are required for mass spectrometry, 20,000–40,000 cells per immunoassay, and a couple of hundred thousand cells per gel electrophoresis experiment. To accommodate the low number of cells available for analysis after LMD, interests have shifted towards methods requiring less sample material. Moreover, the amount of retrieved proteins is particularly important as in contrast to nucleic acid based methods they are not amplifiable. According to most studies, the LMD procedure itself does not interfere with the subsequent proteomic analysis and global protein profile could be obtained from microdissected material (39). However, the preparation of tissue for microdissection, such as the choice of fixative and the staining procedure, may significantly influence both the quantity and quality of the retrieved material. As formalin extensively
crosslinks proteins, formalin fixed tissues generally cannot be used. There is no consensus on the matter of fixative and staining agent, but most researchers advocate the use of fresh frozen tissue with or without ethanol fixation and a low concentration of toluidine blue, methylene green, or hematoxylin staining (40,41). To be on the safe side and avoid any possible detrimental effect of staining procedure, some people suggest performing LMD on unstained fresh-frozen tissue while a mirrored-stained section is used for identification of distinct architectural features. This navigated LMD procedure proved to be a practical and powerful method for performing proteomic studies in specifically defined brain regions (42).

### Live and Rare Cell Isolation from Laser-Microdissected Sample

The recently opened possibility to specifically isolate individual living cells from mixed cultures opens new frontiers in cell and molecular biology (43). It has been demonstrated that LMD have no influence on the viability, metabolism, and proliferation rate of isolated living cells (44). Even recultivated cell colonies, trypsinized and seeded out again, are still viable after multiple rounds of LMD-procedure. Novel cell culturing equipment for live cell isolation and recultivation is available from several manufacturers. Even an entire living organism, such as the nematode Caenorhabditis elegans, is successfully transported without impairing the biological information or the viability (45).

Another rapidly developing application is the combined automated identification of rarely occurring cells, followed by immediate computer-controlled LMD. The combination of automated scanning software with an automated LMD system allows for fast access and pooling of the desired rare cells for subsequent analysis. Tested applications include but not limited prenatal diagnosis and minimal residual cancer detection (46,47). Current methods for the prenatal diagnosis of aneuploidy and monogenic disorders require invasive testing, which carries a significant procedure related risk of miscarriage, and the potential of this technology could be enormous in prenatal diagnosis. With this approach rarely occurring, fetal cells could be safely recovered from maternal blood or from transcervical swabs and may be an attractive addition to the non-invasive prenatal diagnosis repertoire (48,49).

Detection of circulating cancer cells from the peripheral blood or from other extra-tumorous compartments holds promise for the early diagnosis of cancer and monitoring the effectiveness of anti-cancer therapy. Vona et al. recovered circulating tumor cells on filtration membranes after isolation by size of epithelial tumor cells from peripheral blood of cancer patients (50). Using this method, epithelial tumor cells can be isolated individually by filtration because of their larger size as compared to peripheral blood leukocytes. The circulating tumor cells are retained on a routine blood filter and are circumscribed by the laser and catapulted into a collection tube for further analysis. In another study, dysplastic cells were collected from fixed Pap-stained smears of various grades by laser microdissection and analyzed for genetic lesions typical for cervical carcinoma (51). The LOH pattern as published for cervical carcinoma tissue was successfully detected from such samples.

### TRANSLATIONAL/CLINICAL RESEARCH

**APPLICATIONS OF LASER-MICRODISSECTION DNA Based Assays in Translational/Clinical Research**

Until recently, LMD was most commonly used to study cancerous tissue and the DNA isolated from microdissected cancer and normal cells was used successfully for mutation analysis and expression profiling. Ling et al. sequenced 172 laser pressure catapulted single keratinocyte samples from human skin for p53 mutations and found that p53 mutations are common in normal skin and that a clone of keratinocytes with a mutated p53 gene prevailed despite two months of total protection from UV light (52).

Pontén et al. isolated single tumor cells from immuno-histochemically stained cytossections of basal cell carcinoma with laser-assisted capture microscopy to record p53 gene mutations (53). To improve the accuracy of their assay, before picking up the individual microdissected tumor cells with a tip of a glass capillary, surrounding cells were destroyed with the laser beam. After performing a multiplex/nested PCR amplification, the mutation status of Exons 4-9 of the p53 gene was determined by direct sequencing. In their assay system only about 50% of the dissected cells produced an amplification product, probably because of the loss of cell material during handling. However, they determined by this approach that immunoreactivity in basal cell carcinoma cells is not correlated to p53 genotype and the same missense mutations can be found in widely spread areas of the tumor, suggesting clonal proliferation originating from one cell.

Zitzelsberg et al. combined LMD with degenerated oligonucleotide-primed (DOP)-PCR and CGH for the demonstration of intratumor genetic heterogeneity at various primary tumor areas and metastases of prostatic adenocarcinoma (54). Moreover, cytogenetic changes in prostatic intraepithelial neoplasia (PIN), which is often present adjacent to invasive tumors, were also recognized. As DOP-PCR has been considered to produce relatively low molecular weight DNA that may not be representative of the entire genome,

In the study of Cardoso et al. DNA was amplified from 1,000 cells with multiple strand displacement amplification (MDA) that generates thousands of high-molecular weight copies of genomic DNA in a robust simple protocol (55,56). In this study colorectal adenomatous polyps were studied with array-CGH and the sensitivity of their approach was demonstrated by the detection of a small 5q deletion subsequently validated by PCR-based LOH analysis.

Wang et al. has developed a high-throughput strategy, with combining LMD and WGA, to determine LOH profile.
in small quantities of archival tumor tissue samples of early-stage epithelial ovarian carcinomas (57). In their study, the DNA content of about 5,000 tumor and non-tumor stromal cells was amplified with Primer-Extension-Preamplification (PEP)-PCR and a detailed LOH analysis was performed with using 20 microsatellite markers. Findings from this study suggest that tumor suppressor genes are located on five distinct regions on chromosomes 5 and 6 and may be involved in the early development of ovarian carcinomas.

Using laser-microdissection followed by DNA amplification and direct sequencing, Becker et al. found a novel mutation in the gene coding for the cell adhesion mole-cule E-cadherin in single tumor cells that was absent in the adjacent single epithelial cells of a patient with early gastric cancer of the diffuse type (58). Successful DNA amplification of a 246-bp fragment from the E-cadherin gene was seen in 15 to 35% of the selected single cells.

With combining LMD and WGA Dietmaier et al. systematically investigated on both frozen and formalin-fixed paraffin-embedded tissue sections the minimum cell number requirements for reliable MSI, LOH and sequencing studies (18). In their assay system the critical cell number to perform reliable microsatellite and mutation analysis was 10 and 30 cells from frozen and formalin-fixed paraffin-embedded tissue sections, respectively.

The potential power of LMD in forensic applications, when high specificity of a particular analyzed cell population or tissue is required, has lately been recognized. In the study of Di Martino et al. the LMD instrument was used to collect the bulb of a single hair separately from the rest of the keratin, which is a strong inhibitor of the following PCR reactions (59). With this method they were able to accurately extract adequate amount of DNA for Short Tandem Repeat (STR) profiling and thus were able to determine an almost complete identifier profile from these few cells. In another study from the same author STR profiles were obtained from single sperm cells by LMD in order to test the applicability of this methodology for forensic applications (60). They concluded that if an STR profile was obtained from single sperm cells, like sperm cells may be possible.

Budimlija et al. report on the use of LMD as a precise tool for the isolation of placental chorionic villi from aborted material and subsequent genetic analysis for paternal determination in cases of sexual assault (61). The authors also present the evaluation of microdissection to separate maternal (decidua) and fetal (chorionic villus) components on archival formalin-fixed human abortus material and demonstrate the reliability and utility of LMD in forensic DNA-based paternity testing when a specific cell population is required.

**RNA Based Assays in Translational/Clinical Research**

Using laser-microdissection and immunomagnetic separation techniques followed by specific RT-PCRs of metastasis-associated genes, Ito et al. found that primary oral squamous cell carcinoma cells (SCC) highly express matrix-metalloproteinases (MMP 1, 2, and 7) compared to circulating squamous oral carcinoma cells, while circulating tumor cells show overexpression in CD44 and its variant forms (CD44s, v6, v9) compared to the primary tumor cells (62).

In the study of Jakob et al., the correlation between gene expression levels of 5-FU metabolizing enzymes, such as thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase, and histological tumor regression was determined on microdissected rectal cancer specimens after 5-FU-based neo-adjuvant chemoradiotherapy (63). They found that without microdissection there is no correlation between histopathological tumor response and mRNA expression level of the three genes. However, with the use of microdissection the quantity of two genes, thymidylate synthase and thymidine phosphorylase, showed marked correlation with histolopathological response. The lack of any correlation between gene expression levels and tumor regression in non-microdissected samples supports the thesis that gene expression levels are strongly influenced by the degree of tissue heterogeneity, such as fibrosis, inflammation, or necrosis, which are marked features after chemoradiotherapy.

With the selective isolation of megakaryocytes from the bone marrow, Bock et al. examined whether the expression of TGF-β1 in these cells is involved in the pathogenesis of idiopathic myelofibrosis (64). Bone marrow was stained with methylene blue, which allowed satisfactory identification and consecutive isolation of megakaryocytes by LMD. By analyzing 100-150 laser-microdissected megakaryocytes by real-time RT-PCR the authors concluded that TGF-β1 gene and protein expression does not correlate with the risk of bone marrow fibrosis in chronic myeloproliferative disorders.

Luo et al. examined the differential gene expression between adjacent Nissl stained large- and small-sized neurons in the dorsal root ganglia with cDNA microarrays and successfully identified 40 mRNAs preferentially expressed in either large or small neurons (65).

Lechner et al. applied a novel gene expression analysis strategy, combining differential display approach of RNA arbitrarily primed-PCR fingerprinting and cDNA expression array, on LMD prepared crypts of colonic adenomas for the identification and validation of distinct gene expression profiles within the areas of low grade dysplasia (66). They found the upregulation of proliferation associated genes, such as p21-waf1 and MAPK p38α, as well as downregulation of apoptosis related genes, such as FAST kinase and p53, most likely reflects specific alterations in adenomas with low grade dysplasia. Using a similar approach Judex et al. mapped the area-dependent differential regulation of gene expression in histologically defined areas of rheumatoid arthritis synovium (67).

Microdissected sections of rheumatoid arthritis synovial tissue containing ~600 cells yielded enough RNA to produce a reproducible RNA fingerprint pattern and several
genes could be identified as being expressed differentially between the synovial lining and the sublining. This study was the first to demonstrate the potential of this analytical strategy in the investigation of nonmalignant, multifactorial inflammatory disease.

Kitahara et al. pooled cells with LMD from cancerous and corresponding non-cancerous colonic epithelia and compared their gene expression profile with a cDNA microarray consisting of more than 9,000 gene transcripts (68). With this approach they found 235 colon cancer related genes, among which 44 were up- and 191 were downregulated, whose expression was changed during development and progression of colorectal cancer.

In the study of Kim et al., the gene expression profile of laser microdissected gastric cancers was analyzed with 14K cDNA microarray chips specially designed for gastric cancer analysis (69). The difficulty with molecular grouping of gastric cancers stems from their complexity and variety but also the precise microdissection of single-infiltrating cancer cells of the diffuse type cancers poses a significant challenge. Kim’s data showed that gastric cancers could be effectively clustered into relevant subgroups by using stomach-specific microarrays and pairwise analysis of tumor/non-tumor mucosa samples.

Using cDNA expression microarrays containing 1,176 cancer-related genes, Sugiyama et al. detected 32 up- and 58 downregulated candidate genes in endometrium carcinoma cells compared to normal cells and found 45 upregulated type-I specific and 24 upregulated type-II specific genes between type-I (hyperplastic) and type-II (non-hyperplastic) endometrial tumors (70).

Nakamura et al. prepared purified populations of pancreatic cancer cells and normal pancreatic ductal cells by means of LMD and analyzed genome-wide expression profiles of 18 pancreatic tumors using cDNA microarray representing 23,040 genes (71). Through the expression profiles a set of genes whose expression was significantly associated with clinical parameters such as lymph node and liver metastasis of pancreatic cancer was identified. In addition, expression levels of 30 genes were related to the recurrence of the disease.

Irič et al. used laser microdissection and cDNA microarray to study the gene expression profile in oral SCC (72). They identified genes that were selectively up- or downregulated in metastasis positive and metastasis negative cases with the intention to identify genes important for acquisition of invasion, proliferation capability, and metastatic potential. These genome-wide expression profiles should provide useful information for finding candidate genes whose products might serve as specific tumor markers or as molecular targets for treatment of patients with cancer or other disease.

Mueller et al. isolated pure populations of the three major mouse gastric epithelial cell lineages by LMD and examined their gene expression patterns by cDNA array (73). Their study revealed that mucus-producing cells, but not parietal or chief cells, are responding with specific transcriptional changes in response to Helicobacter pylori infection in vivo. This data illustrates that transcriptional profiling of defined cell populations by laser microdissection is a powerful tool that is sensitive enough to pick up subtle changes that occur during the earliest stages of bacterial infection in vivo.

Watahiki et al. used laser microdissection on hard tissues for the first time by conducting time-dependent analysis of the mandibular condylar cartilage of young postnatal mice (74). By using real-time PCR and cDNA microarray technique they monitored in vivo changes in gene expression levels in normal growing mandibular condylar cartilage before, during, and after the initiation of mastication, revealing that the expression levels of several genes change markedly with initiation of mastication.

The first study that identified global gene expression profiles during the process of multistep carcinogenesis by integrating the technologies of LMD and cDNA microarray was published by Nishida et al. (75). In their study authors used their previously established rat esophageal SCC model, in which papilloma, dysplasia, and invasive carcinoma were successfully developed and sampled by means of LMD. In their study they identified 50 genes whose expression level had either significantly increased or decreased in a step-by-step manner from the normal epithelium to dysplasia and then to invasive carcinoma, suggesting that these sets of genes play an important role in rat carcinogenesis.

Huddleston et al. describe the use of a microarray containing 2,400 known human cDNAs and LMD to identify creatine kinase B (CKB) as an upregulated gene in ovarian cancer and further validate this by measuring CKB RNA in tumor specimens and cultured cells (76). The authors also demonstrate the usefulness of CKB as a possible serum marker by showing upregulated levels of CKB in the serum of patients with ovarian cancer compared to normal controls and patients with benign pelvic masses. As CKB enzyme activity is significantly elevated in sera from ovarian cancer patients, including those with stage I disease, their findings suggest a potential role for CKB as a marker for early diagnosis of ovarian cancer.

The combination of LMD and cDNA microarray analysis has been applied in the study of Takata et al. to predict the response of invasive bladder cancer patients to a certain neoadjuvant chemotherapy regimen (77). Using genome wide information obtained on 27 cases on a cDNA microarray consisting of 27,648 transcribed elements, they established a system to predict response to neoadjuvant chemotherapy based on the differential expression of 14 genes between responders and nonresponders. This system accurately predicted the drug responses of 8 of 9 test cases.

Large scale gene expression profiling was carried out on high-density oligonucleotide arrays in the study of Alevizos et al. (78). The mRNAs were harvested from 120,000–200,000 paired tumorous and non-tumorous oral epithelial cells and were subjected to three rounds of linear amplification, and then hybridized to microarrays according to the Affymetrix GeneChip® technology. The percent transcript detected ranged from 26 to 40%, indicating satisfactory quality and representation of the har-
vested RNA. Their analysis revealed 600 oral cancer associated genes (oncogenes, tumor suppressors, transcription factors, xenobiotic enzymes, metastatic proteins, and differentiation markers) that have not been implicated in oral cancer before.

Datson et al. used the combination of LMD with linear RNA amplification and oligonucleotide microarray containing 8,000 transcripts to generate a comprehensive inventory of genes with differential expression between hippocampal subregions (79). Analysis of data revealed that a large number of genes are differentially expressed supporting the idea that profiling in hippocampal subregions with this approach should improve detection of genes with subregion-specific expression or regulation.

A similar approach was employed by Fukushima et al. to study the global gene expression profile of an uncommon pancreatic tumor, the mucinous cystic neoplasms (80). Because this type of cystic tumors contains a small number of neoplastic cells, very little is known about their molecular background. To better understand the biology and to identify candidate diagnostic markers of these tumors they used LMD to precisely collect cells from frozen tissue sections. Cells were obtained from 10–20 sections to have sufficient RNA for subsequent analysis. If after the second round of IVT procedure the RNA yield was below 15 μg, additional cells were laser microdissected from the same neoplasms. The authors identified several that were overexpressed in the neoplastic epithelium compared to normal pancreatic ductal epithelium, including genes member of the Notch signaling pathways.

Wild et al. investigated the gene expression of papillary superficial urothelial tumors in patients with known clinical course using a combination of oligonucleotide and tissue microarray technologies (81). RNA from 2,000 laser microdissected cells were hybridized to custom-designed Affymetrix® microarrays and class prediction algorithm provided genomic basis for diagnosis of noninvasive papillary low-grade tumors with subsequent progressive behavior. Novel genes with clinical utility to select patients more likely to develop aggressive disease were identified.

By combining laser capture microdissection and microarray analysis of gene expression (microSAGE), Cho-Vega et al. demonstrated that a total of 20 genes were differentially expressed between normal and cancerous prostate cells obtained from fresh-frozen prostatectomy specimens (82). Large-scale expression profiles of known and unknown transcripts was generated successfully with the LMD-microSAGE approach from heterogeneous mixtures of cell types.

**Protein Based Assays in Translational/Clinical Research**

A group of researchers at the National Cancer Institute were among the first publishing proteomic profiles of histopathologically defined cell populations recovered with LMD from microscopically heterogeneous cell populations of human tissue specimens (33). In one of their pivotal trials, fresh frozen hematoxylin/eosin stained matched normal and malignant prostate epithelial cells were analyzed with 2D-polyacrylamide gel electrophoresis (PAGE). Microdissection of 40–50,000 cells permitted the reliable detection of ~750 distinct proteins as visualized by silver staining. Their data demonstrated that LMD-derived cells can reliably detect alterations in protein expression associated with prostate cancer. Furthermore, in the same study they showed that immortalized in vitro grown prostate cancer cell lines derived from the same patients were strikingly different from their in vivo counterpart in their proteomic profiles (33).

The experimental conditions using cells obtained by LMD for proteome analysis has been further refined by many investigators. Lawrie et al. performed 2D-PAGE, followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) mass spectrometry (MS) analysis on microdissected fresh frozen toluidine blue-stained colon cells (83). In this study the effect of histological processing and LMD on protein recovery were carefully evaluated with appropriate controls. It was found that the expression of the most abundant proteins is not altered in any measurable fashion by the pre-analytical steps.

The first real global quantitation of differential protein expression analysis between laser microdissected cancer cells and normal cells from the same human tumor–tissue sample was performed by Zhou et al. (84). The authors used a novel modified PAGE technology, called differential in-gel electrophoresis (DIGE), to simultaneously analyze in the same gel two pools of fluorescent dye labeled patient-matched esophageal cancer and normal cell protein extracts. The analysis identified 58 protein spots that were overexpressed and 107 proteins that were downregulated greater than threefold in cancer cells. Proteins with greater than threefold change in cancer versus normal were identified by capillary high performance liquid chromatography (HPLC)-MS and further confirmed by Western blotting analysis to validate the differential expression. The authors of this study also concluded that more reproducible protein expression profiles can be obtained with this novel technique and that it allows for more accurate quantitation between two populations of proteins than the traditional separate gel method.

In recent years even more detailed biological questions were successfully answered with the combination of LMD and proteomic analysis. Wulfkuhle et al. studied the proteomic profile of the earliest detectable form of breast cancer, ductal carcinoma in situ (DCIS), with 2D-PAGE and MS sequencing (85). The proteomic analysis of matched normal ductal/lobular units and DCIS revealed numerous and heterogeneous alterations in actin-binding protein expression, lipid, vesicular, and membrane trafficking and in the expression of chaperone proteins. Furthermore, this study also provided the first indication that proteomic trends from 2D gels can be validated using techniques common in clinical practice such as immunohistochemistry. Interestingly, when this proteomic data is compared to nucleic acid-based microarray analysis of Luzzi et al., very minimal overlapping identification can be found.
Thus suggesting caution in the assumption that mRNA levels reflect those of functional proteins.

In the study of Zhang et al., tumor cells were procured from both HER-2/neu-positive and -negative tumors in order to identify proteins that are associated with the aggressive phenotype of HER-2/neu-positive breast cancer (86). Tissue sections were fixed in ethanol and staining with hematoxylin was done in the presence of a protease inhibitor cocktail. Proteins were solubilized from microdissected cells and the solubilized proteins were separated by 2D gel electrophoresis: protein spots were characterized by peptide mass mapping using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). From each sample 10,000 shots, corresponding to 50,000–70,000 cells, were collected for analysis. With this approach they successfully identified 21 different protein spots with at least a fivefold change in volume in the HER-2/neu-positive tumors. These proteins play significant role in various metabolic, stress-responsive, antioxidative, and detoxification processes within the tumor microenvironment. The validity of their results was confirmed and further verified by immunohistochemistry on tissue microarrays.

In the study of Melle et al., surface-enhanced laser desorption/ionization (SELDI)-MS based ProteinChip technology was used to compare protein patterns of 2D gels from normal pharyngeal epithelium and squamous tumor tissue (87). From native air-dried cryostat tissue sections areas corresponding to 3,000–5,000 cells/tissue, probe were excised and three proteins, calgranulin A, calgranulin B, and calgizzarin, were identified as potential biomarkers of pharyngeal cancer. These proteins, as members of group S100 proteins, are involved in the Ca2+ signaling network and regulate intracellular activities such as cell growth and motility, cell cycle progression, transcription, and cell differentiation. Subsequently, the localization and

### Table 3

**Various Applications of Laser Microdissection**

<table>
<thead>
<tr>
<th>Macromolecules</th>
<th>Downstream analysis</th>
<th>Tissue type</th>
<th>Number of LMD cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>RFLP, SSCP, LOH, CGH</td>
<td>Gastric cancer</td>
<td>1</td>
<td>Becker et al. (1996) (58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>50–100</td>
<td>Zitzelsberg et al. (1998) (54)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian cancer</td>
<td>5,000</td>
<td>Wang et al. (2001) (57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colorectal polyps</td>
<td>1,000</td>
<td>Cardoso et al. (2004) (56)</td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>Skin</td>
<td>172</td>
<td>Ling et al. (2001) (52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>Pontén et al. (1997) (53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast, colon,</td>
<td>10–100</td>
<td>Dietmaier et al. (1999) (18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gall bladder,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pancreas cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>RT-PCR</td>
<td>Breast cancer</td>
<td>200</td>
<td>To et al. (1998) (29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>200</td>
<td>Fend et al. (1999) (16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral cancer</td>
<td>na</td>
<td>Ito et al. (2003) (62)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rectal cancer</td>
<td>na</td>
<td>Jakob et al. (2004) (63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bone marrow</td>
<td>100–150</td>
<td>Bock et al. (2005) (64)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nervous system</td>
<td>1,000</td>
<td>Luo et al. (1999) (65)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colon cancer</td>
<td>na</td>
<td>Lechner et al. (2003) (66)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rheumatoid synovium</td>
<td>10,000</td>
<td>Kitahara et al. (2001) (68)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric cancer</td>
<td>600</td>
<td>Judek et al. (2003) (67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endometrial cancer</td>
<td>na</td>
<td>Kim et al. (2003) (69)</td>
</tr>
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<td></td>
<td></td>
<td>Pancreatic cancer</td>
<td>na</td>
<td>Sugiyama et al. (2003) (70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast cancer</td>
<td>200</td>
<td>Fukushima et al. (2004) (80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bladder cancer</td>
<td>2,000</td>
<td>Wild et al. (2005) (81)</td>
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<tr>
<td></td>
<td></td>
<td>Breast cancer</td>
<td>15,000</td>
<td>Luzzi et al. (2001) (30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain tissue</td>
<td>na</td>
<td>Datson et al. (2004) (79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>30,000</td>
<td>Cho-Vega et al. (2005) (82)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colon cancer</td>
<td>na</td>
<td>Lawrie et al. (2001) (85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Esophageal cancer</td>
<td>250,000</td>
<td>Zhou et al. (2002) (84)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>40,000–50,000</td>
<td>Ornstein et al. (2000) (33)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast cancer</td>
<td>50,000–100,000</td>
<td>Wulffkuhle et al. (2002) (85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50,000–70,000</td>
<td>Zhang et al. (2005) (86)</td>
</tr>
<tr>
<td></td>
<td>SELDI-MS</td>
<td>Head and Neck SCC</td>
<td>3,000–5,000</td>
<td>Melle et al. (2004) (87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>3,000–6,000</td>
<td>Cazares et al. (2002) (88)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colorectal Cancer</td>
<td>500–1,000</td>
<td>Krieg et al. (2004) (89)</td>
</tr>
</tbody>
</table>

na, data are not available.
tissue sections of the SCCs of the head and neck by IHC. With the intention to find potential markers for early detection or risk factors for development of prostate cancer (PA), Cazares et al. procured pure epithelial cell populations from organ-matched normal luminal epithelia, benign prostatic hyperplasia (BPH), and PA (88). A total of 1,000 laser shots totaling 3,000–6,000 cells were collected for each cell type and proteomic profiles were compared by SELDI-MS. Profiles suggested that most of the markers found in the PA profiles were also present in the prostate intraepithelial neoplasia profiles, and thus, the ability to discriminate between these two cell types was difficult. However, better discrimination was achieved between the benign cell types (normal, BPH) and the diseased cell types (PIN or PCA combined). A combination of markers was effective in distinguishing normal/BPH from PIN/PCA with a sensitivity and specificity of 93%.

Proteomic profiles were prepared from as few as 500–1,000 cells in the study of Krieg et al. (89). In conjunction with LMD and the SELDI technique, the authors were seeking to detect potential new diagnostic markers for the existence and invasive properties of colorectal carcinoma. By using this approach several differences were identified in protein mass spectra in benign vs. malignant epithelium and benign vs. malignant stroma. The technique applied allowed the sensitive detection of proteins from less than a thousand cells originating from different compartments of malignant tumors and associated normal counterparts.

In the study of Li et al. LMD was combined with isotope-coded affinity tag (ICAT) technology and 2D liquid chromatography to investigate the qualitative and quantitative proteomes of hepatocellular carcinoma (90). Selected references demonstrating a variety of applications for laser microdissection are shown in Table 3.

**DISCUSSION**

The technology of LMD has undergone a tremendous development since laser-assisted microdissection of tissue sections was first published in 1976 (1). It is currently being used in various interdisciplinary programs, as for example the National Cancer Institute’s Cancer Genome Anatomy Project to catalog the protein profiles of normal, precancerous, and cancer cells.

It may be argued that examining the expression profile of the complete cellular microenvironment of disease states is more representative to the ongoing process than the separation of individual cells but such gross sampling has not provided much diagnostic utility so far. Studies that gradually break down the complex interactions that exist in vivo between neighboring cell types would greatly facilitate our understanding of normal and disease states. However, at this point of time it is commonly believed that homogeneous isolation of defined, constant cell number is a prerequisite for precise qualitative and quantitative molecular analysis. On the other hand, the user-dependent selection of cells and objects for microdissection introduces the problem of sampling bias. To a certain extent this is unavoidable, but to reduce this type of bias some microdissection softwares offer automation for complex experiments that allows the automated identification of target objects as well as storing and retrieving predefined target areas for microdissection.

Another consideration concerns that for which area of the specimen is procured for analysis. As for example in case of malignant tumors, the tumor itself is heterogeneous and this inherent heterogeneity is a hallmark of most disease processes. To circumvent these problem different regions of the sample may need to be analyzed separately in order to be able to extract biologically relevant information.

LMD allowed us to prepare samples with an unprecedented homogeneity, a prerequisite for functional genomics and proteomic studies. Despite of the exciting results that came out from this research, it is important to note that LMD technology has only been used as “translational/clinical research” tool so far, as none of these applications has yet made the transition to routine clinical diagnosis or clinical practice. Probably, one of the major obstacles of this transition is that it takes a long time to pick up a sufficient amount of cells for a sample intensive assay, as for example an immunoassay or a 2D gel. Therefore, interest has shifted towards downstream analysis methods (e.g. microarray, SELDI-TOF-MS) that require less starting material but can still provide high-throughput analysis.

Nevertheless, the development and refinement of the LMD technology is still ongoing. For example, an interesting recent innovation is the combination of LMD with atomic force microscopy (91). In a one-step procedure, human metaphase chromosomes were dissected optically by UV-laser ablation and mechanically by manipulation with the atomic force microscope. With both methods, sub-400-nm cuts could be achieved routinely. Thus, nanomanipulation and nanoextraction on a scale close to and beyond the resolution limit of light microscopy was achieved.

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