Preface

Molecular morphologic methods—defined as molecular and cell biologic analyses of tissues in which the architectural integrity and spatial interrelationship of the cells being studied are preserved—have increased rapidly in number and versatility during the past few years. These changes have occurred both in diagnostic pathology and in basic scientific research. Several ongoing developments affecting the pathology and the scientific communities should make this book a valuable resource. First, it is usually difficult for pathologists and investigators interested in molecular morphology to learn rapidly from a single source about methods suitable to specific diagnostic and experimental questions. Second, the completion of the human genome project in the near future will provide the foundation to learn about the functions of myriad of genes with unique roles in specific cells and tissues, so a morphologic basis for the study of human genes and understanding human diseases will be in greater demand. There is no good single source available that discusses in detail the most significant aspects of recent cell biologic techniques by outstanding experts in their fields. Such a book is needed to keep up with scientific research in morphology and recent pathologic diagnostic techniques relevant in the twenty-first century.

Our objective was to produce a book addressing the major areas relevant to molecular morphology today. Many of the chapters include detailed protocols for setting up or performing techniques now in use. Potential pitfalls and anticipated problems are also discussed. Practicing pathologists interested in recent developments and researchers interested in molecular morphology for designing experiments, for teaching undergraduate, graduate, and professional students, or simply for keeping up with the literature detailing molecular morphologic approaches—all will find here the technical and scientific background to accomplish their objectives.

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2
Laser Capture Microdissection
Principles and Applications

Ricardo V. Lloyd, MD, PhD

INTRODUCTION

The acquisition of homogeneous or pure cell populations for cell biologic and molecular analyses has been a difficult challenge for many decades. A variety of approaches have been tried, including macroscopic dissection of tissues from frozen tissue blocks to increase the population of specific cell types *(1,2)*. Some investigators have used irradiation of manually ink-stained sections to destroy cells that were not of interest *(3,4)*. Microdissection with the aid of a microscope and needles has been used by many other investigators *(5,6)*. This latter approach can provide a great deal of precision in collecting homogenous cells, but it is slow and labor-intensive and requires a high degree of manual dexterity. Most of these approaches have not provided the speed, precision, and efficiency needed for research or routine clinical molecular diagnostic use.

The recent development of a laser capture microdissection (LCM) unit by the National Cancer Institute (NCI) group led by Liotta and his colleagues *(7,8)* and by other groups, mainly in Europe *(9,10)*, has provided a rapid and efficient method to capture pure cell populations for molecular and other studies.

Principles of LCM

Liotta's group at the NCI first reported on the development of a rapid and reliable method of obtaining homogenous population of cells from complex tissues *(7,8)*. The availability of commercial instruments resulted from a joint venture by the NCI and Arcturus Engineering (Mountain View, CA).

With LMC, a complex section of tissues or heterogeneous cell populations on a glass slide is placed on the stage of a specially designed microscope. After the areas or cells of interest are selected, an ethylene vinyl acetate (EVA) transparent film is apposed to the section, and an infrared laser beam is directed at the cell of interest. When the focused laser beam coaxial with the microscope optics is activated, the EVA film above the targeted area melts, surrounds, and holds the cells of interest, which are kept in the film after removal from the glass slide. The film can be placed directly

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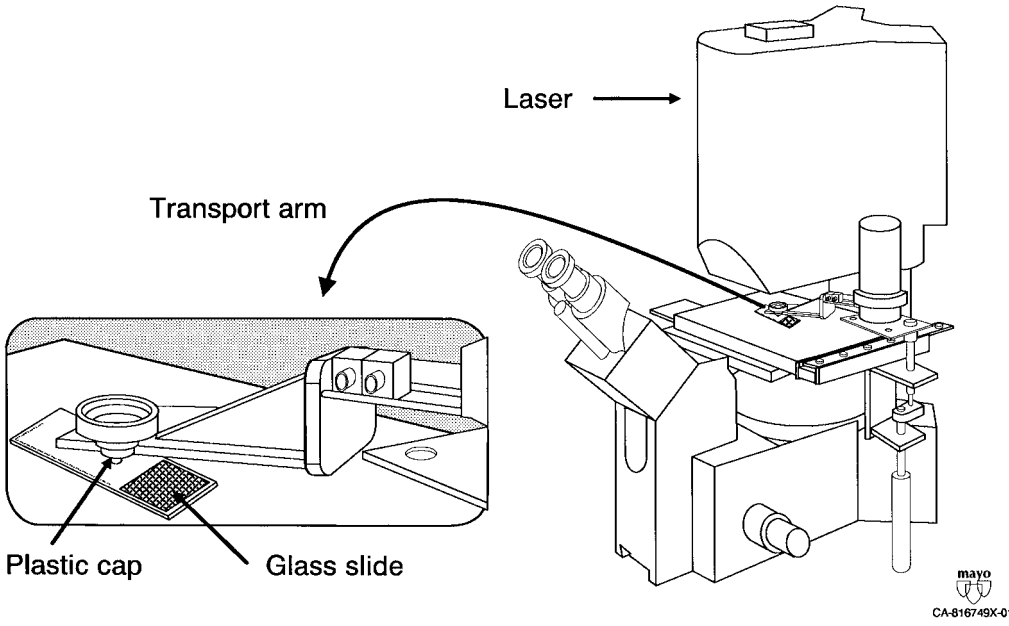

Fig. 1. Laser capture microdissection (LCM) with the Pix Cell instrument. The laser beam is focused on the cut section of cells on the glass slide. The transport arm carries the plastic cap over to align it with the specimen and laser beam. The focused laser beam captures the cells of interest, which adhere to the ethylene vinyl acetate (EVA) film that binds to the captured target cells. After collecting the cells of interest, the transport arm carries the cells back to the side.

into DNA or RNA extraction buffers for nucleic acid extraction and analysis or into other buffers for protein analysis. While in the buffer, the cellular material becomes detached from the film and can be used for cellular or molecular analyses after extraction (see Protocols).

Based on numerous reported studies in the literature and our own observations, the LCM procedure does not lead to significant alterations in the morphologic or apparent molecular features of the cells of interest.

Technical Aspects

The prototypic instrument used for LCM is the Pix Cell™ developed by Arcturus Engineering and the NCI group **(Figs. 1 and 2)**. The instrument consists of a laser optics deck and illumination tower with a halogen bulb housing, a microscope, and a slide stage with a vacuum chuck. A joystick with XY control and video camera attachment are attached to the microscope. The XY control is for fine positioning of the sample. The laser can be controlled by amplitude and pulse width adjustments using digital controls on the front panel of the electronic box. When the laser fires, an emission indicator is lit, and the power supply emits a beeping sound. Typical operating parameters for the laser for a 30-µm spot is an amplitude of 30 mW and a pulse width of 5 ms; for a 60-µm spot, an amplitude of 50 mW and a pulse width of 5 ms is used.

When the operator is ready to capture the cells of interest, a plastic cap is transported with the film carrier and placed on the desired position of the tissue or cells. The laser

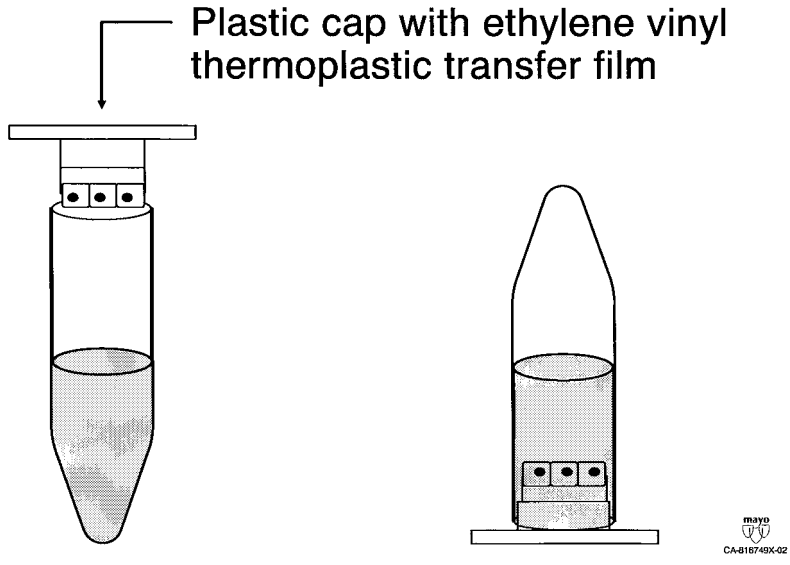

Fig. 2. The cells on the EVA film are placed in the microfuge tube and immersed in the appropriate buffer for DNA, RNA, or protein extraction and analysis.

is then activated, and the EVA film binds to the captured target on the tissue. The laser can be activated as often as is needed to capture the desired numbers of cells. Multiple clusters of homogenous cells can be accumulated into the same polymer EVA film, and individual single laser shots can be used to procure specific cell clusters or individual cells **(Fig. 3)**. In addition, multiple shots can be combined to procure complex but homogenous tissue structures. Up to 3000 shots can be captured on one transfer film cap, which may include up to 6000 cells. Since each shot takes less than 1 s to perform, a large number of cells can be captured in a relatively short period.

Technical Variables

Technical variables with LCM include the types of analyses that will be done with the tissues relative to fixation and processing, use of frozen versus paraffin tissue sections, and combination of LCM with other techniques such as immunohistochemistry (Immuno-LCM).

Tissues fixed in buffered formalin and embedded in paraffin can be used for routine analysis of DNA and in some cases mRNA as well. Tissues are routinely stained with hematoxylin and eosin for optimum visualization of cellular details during LCM. These stains do not affect the integrity of the DNA or RNA. For optimum RNA analysis, frozen tissue sections are preferred. Surprisingly, the nucleic acids are not adversely affected by exposing the tissues to xylene for a short period. RNAse-free conditions are important for obtaining high-quality RNA samples.

The section for frozen tissue should be cut at 10 μm or less for optimum transfer, because with thicker sections it is more difficult to visualize single cells. Sections have to be completely dry and not cover-slipped for effective LCM transfer so the final xylene rinse facilitates efficient LCM transfer. After the frozen sections are cut, the slides can be used immediately or stained at −80°C until use. We have observed that

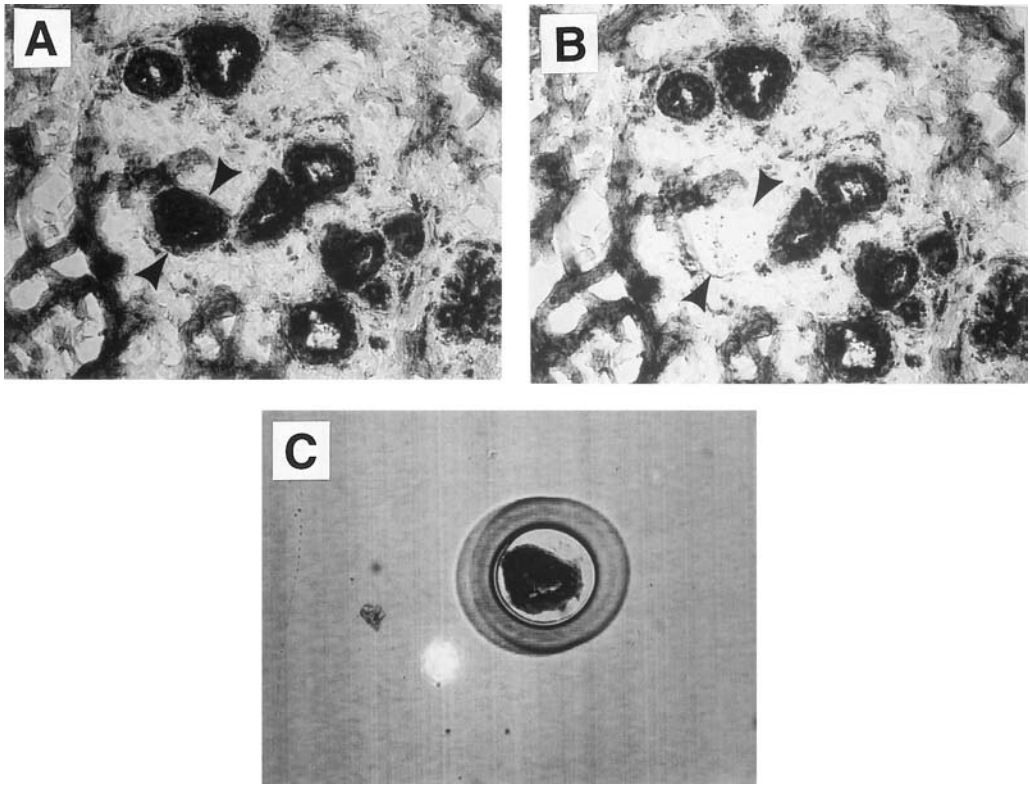

Fig. 3. LCM capture of cells from normal breast tissue. (**A**) Groups of normal epithelial cells were selected for LCM (arrowheads). (**B**) Arrowheads indicate the space from which the cells were captured. (**C**) Captured cells on the cap with the transfer EVA film. (Reproduced with permission from ref. *13.*)

starting with fresh alcohols and xylene for each set of experiments avoids many of the technical problems associated with difficult transfers during LCM.

The procedure of combining LCM with immunophenotyping for RNA analysis (immuno-LCM) was first reported from the NCI *(11)* and subsequently by our groups and others for analyzing single cells *(12)* and tissues *(13–15)* **(Fig. 4)**. The critical requirements include using RNAse-free condition and using RNAse inhibitors during the immunostaining procedures. A rapid immunostaining procedure minimizes the time of tissue exposure to RNAse.

Various studies have shown that precipitating fixatives such as ethanol and acetone produced better quality reverse transcriptase-polymerase chain reaction (RT-PCR) product amplification compared with crosslinking fixatives such as formaldehyde and glutaraldehyde *(11,12,14).* However, precipitating fixatives are less effective in some immunostaining procedures compared with crosslinking fixatives *(12).*

Other Laser-Based Microdissection Systems

Although LCM is the principal system used, other systems have been developed, mainly in Europe, that are somewhat similar to LCM *(16–21).* Schutze and Lahr *(16)*

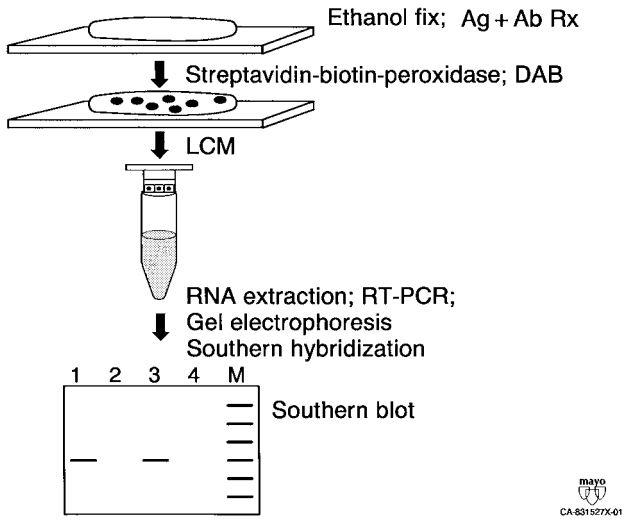

Fig. 4. Immuno-laser capture microdissection (LCM). Combined immunohistochemistry and LCM to capture immunophenotyped cells. After ethanol fixing, the tissues are exposed to the specific antibody for about 15 min followed by a streptavidin-biotin-peroxidase reaction and diaminobenzidine (DAB). The immunostained cells (black circles) can be used for LCM and reverse transcriptase-polymerase chain reaction (RT-PCR) and the products analyzed by gel electrophoresis and Southern hybridization.

used a low-power laser to create a gap between the cells of interest and adjacent cells; with an increase in the laser power, they were able to "catapult" the microdissected cells into the cap of a microfuge tube for further analysis without requiring any direct contact with the cells of interest. Using this approach, they could amplify simple cells from archival tissues in the analysis of Ki-ras mutations *(16).*

Fink and colleagues *(17)* used an ultraviolet light laser to ablate cells that were not of interest and subsequently collected the cells of interest with a stent needle under the control of a micromanipulator *(17),* which is a modification of the original method of Shibata et al. *(3,4).* Other investigators have utilized this approach to study microsatellite instability in breast cancer at the single cell level *(18).* A combination of ultraviolet microbeam microdissection with laser pressure catapulting for the isolation of single chromosomes for development of chromosome-specific paint probes has been reported *(19).* Becker et al. *(20)* used a combination of ultraviolet laser microbeams with microdissection and molecular characterization of individual cells to detect a novel mutation in the E cadherin gene in single tumor cells *(20).* A recent study used the PALM Laser-Microbeam System, which allows the contact-free isolation of single cells or groups of cells using the laser pressure catapulting technique and real-time PCR to study the Her-2/Neu gene and topoisomerase IIα gene in breast cancer specimens *(21).*

Disadvantages of LCM and Related Techniques

Although LCM is faster and easier to perform than manual microdissection, there are a few disadvantages. The principal one is that the tissue is not cover-slipped during LCM, so the refractive indices of the dry sections have a refractile quality that obscures

cellular details at higher magnifications. This can be partially overcome with a diffusion filter on the instrument or by using a drop of xylene on the tissues, which provides wetting and refractive-index matching; the xylene usually evaporates rapidly before microdissection *(22)*. Another disadvantage for investigators with a tight budget is the costs of the instrument. Finally, although LCM is faster than manual methods, a great deal of time is still required to collect cells for an experiment compared with biochemical analysis, especially when thousands of cells are collected *(23)*.

APPLICATIONS OF LCM AND RELATED MICRODISSECTION METHODS

General Applications

The first reports of the development of LCM illustrated the wide applicability of the technique *(7,8)*. The NCI investigators used this technique to analyze loss of heterozygosity (LOH) of the *BRCA1* gene in familial breast cancer, chromosome 8p in prostate cancer, the *p16* gene in invasive esophageal cancer, and the *MEN1* tumor suppressor gene on chromosome 11q13 in gastrinomas *(7)*. Other reported applications using the original carbon dioxide laser included detection of a single base mutation in exon 2 of the von Hippel-Lindau (VHL) gene in hemangioblastoma RT-PCR amplification of actin, prostate-specific antigen, and matrix metalloproteinase-2 in frozen prostate cancer samples and analysis by gelatin zymography, showing the applicability of the LCM technique for enzyme assays *(7)*.

Suarea-Quian et al. *(24)* used an LCM technique with 1-ms laser pulses focus of 6 μm to demonstrated rapid capture of single cells from different types of tissues including immunostained cells *(24)*.

Glasow and colleagues *(25)* utilized LCM to study the leptin receptor in the adrenal by RT-PCR and were able to identify the cell types producing the leptin receptor. In a subsequent study of prolactin receptor (PRL-R) in the adrenal, LCM was used to demonstrate that PRL-R was produced by cortical, but not medullary, cells in the adrenal gland *(26)*.

In a detailed analysis of B-cell lymphomas, Fend et al. *(27)* used LCM to isolate cell populations with different antigen expression patterns. They combined this with PCR and sequencing of clonal immunoglobulin heavy chain rearrangements and clonal rearrangement to show that low-grade B-cell lymphomas with two distinct morphologic and immunophenotypic patterns in the same anatomic site were frequently biclonal *(27)*. This study demonstrates the power of LCM to detect molecular microheterogeneity in complex neoplasms.

Our laboratory has used LCM from dissociated cells to study gene expression in individual pituitary cells *(12)*. We have also analyzed normal and tumorous breast tissue by LCM and RT-PCR to show that PRL-R was expressed not only in the epithelial component of normal and tumorous breast tissues but also by stromal cells *(13)*, suggesting modulation of mammary stromal cells by PRL **(Figs. 5 and 6)**. In more recent studies, our laboratory has for the first time obtained pure populations of pituitary folliculo-stellate cells and used these for molecular and cell biologic studies that have provided new insights into the role of these cells in pituitary function *(28)*.

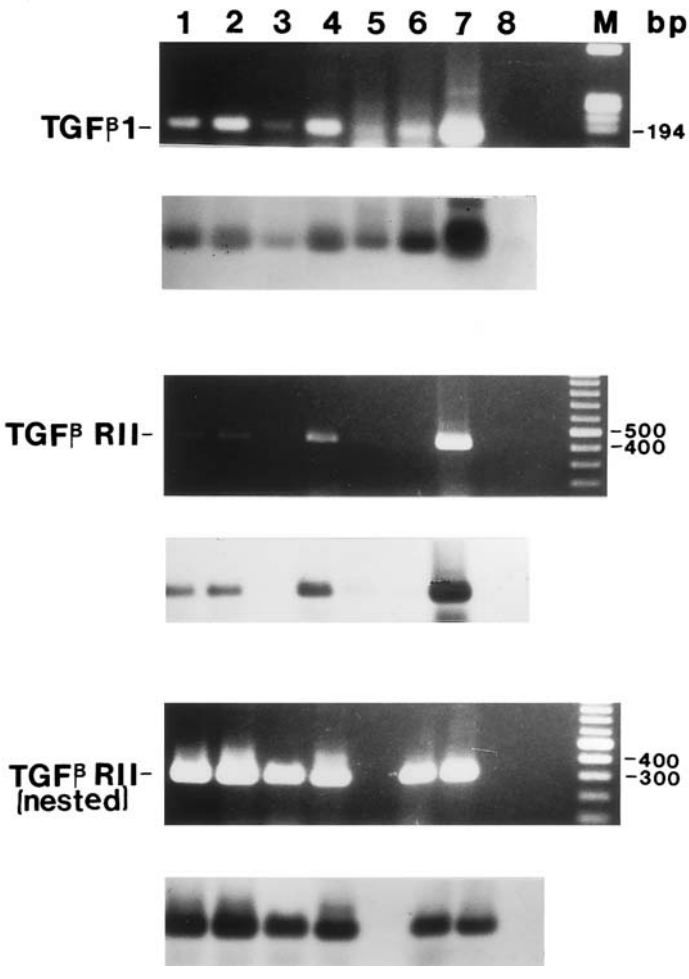

Fig. 5. RT-PCR and Southern hybridization examination of transforming growth factor-β1 (TGF-β1) and TGF-β-RII with RNA obtained by LCM for normal and neoplastic breast tissues showing representative examples of the analyses. Lanes 1, 3, and 5 are normal breast tissues. Lanes 2, 4, and 6 are invasive carcinoma. Lane 7 represents a positive control breast tissue, and lane 8 is a negative control without reverse transcriptase. Lanes 1–3 and 4–6 represent matching normal and tumor tissues, respectively. Lane 7 is a positive breast tissue control, and lane 8 is a negative control without reverse transcriptase. M, molecular size marker. The top part of each panel represents the RT-PCR results on the gel after ethidium bromide staining. The bottom part is the Southern hybridization with the internal probes. (Reproduced with permission from ref. *13*.)

In a recent study of synovial sarcoma, Kasai et al. *(29)* used LCM and RT-PCR with paraffin wax-embedded tissues to show that the SYT-SSX fusion transcript was present in both the spindle cell and epithelial areas of biphasic synovial sarcomas. This study also reinforced the idea that RT-PCR can be performed on RNA obtained from formalin-fixed, paraffin-embedded tissue sections. In a related study, Shibutani et al.

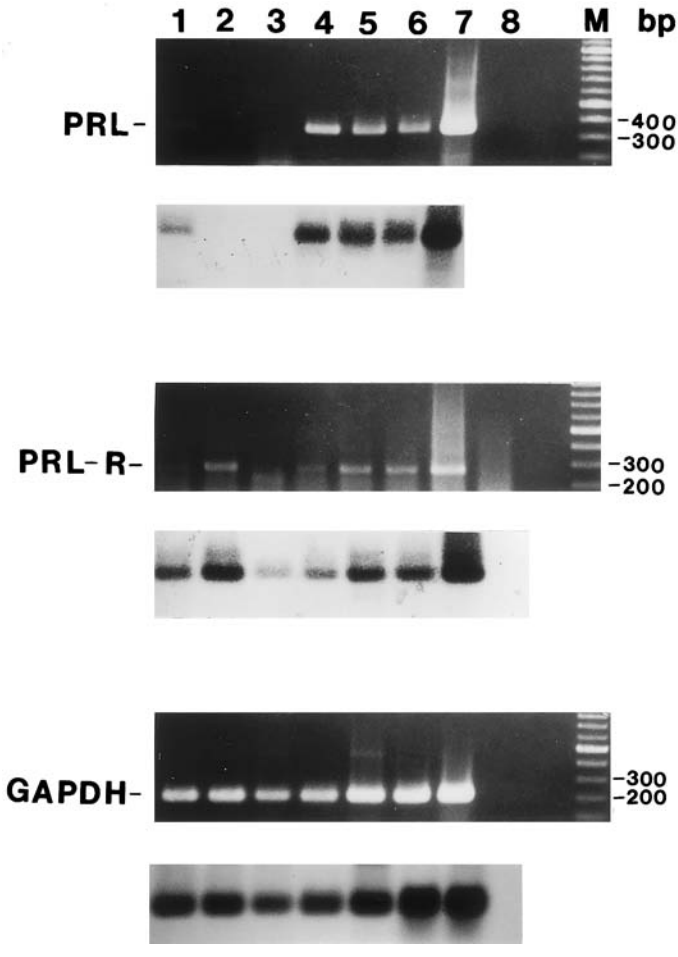

Fig. 6. RT-PCR and Southern hybridization examination of prolactin (PRL), prolactin receptor (PRL-R), and GAPDH RNA obtained by LCM showing representative examples of the analyses. Lanes 1, 3, and 5 are normal breast tissues. Lanes 2, 4, and 6 are invasive ductal carcinoma. Lane 7 represents a positive pituitary tissue control, and lane 8 is a negative control without reverse transcriptase. Lanes 1–3 and 4–6 represent matching normal and tumor tissues, respectively. M, molecular size marker. The top part of each panel represents the RT-PCR results on the gel after ethidium bromide staining. The bottom part of each panel is the Southern hybridization with the internal probes. (Reproduced with permission from ref. *13*.)

(30) demonstrated that methacarn-fixed, paraffin-embedded tissue could be used for RNA analysis by RT-PCR and for protein analysis by Western blotting after LCM using rat liver as an experimental model.

In a study of renal glomerular cells, Kohda et al. *(31)* used LCM with RT-PCR to detect podoplanin in renal glomerular cells harvested by LCM. They were also able to use LCM to capture pure cell populations from different portions of the tubules, but they could not do this from the collecting ducts.

In a study of papillary and follicular thyroid carcinoma Gillespie et al. *(32)* used LCM and loss of heterozygosity (LOH) analysis of highly polymorphic chromosomes to show that LOH was more common in follicular than papillary carcinoma. In a related study on practical clinical applications of LCM, a group of investigators analyzed a primary duodenal carcinoid as well as tumors in the scalp and cervical lymph nodes from the same patient by LCM with 22 markers. The 3p12 marker showed loss in all three tumors, and the 3pl4.2 marker showed an identical shift in the three tumors indicative of a common microsatellite alteration and supporting the notion that the shared molecular abnormalities indicated a common clonal origin with the duodenal carcinoid as the primary tumor *(33).*

Combining LCM with Other Techniques

A combination of other techniques with LCM has been reported by various investigators. Jones et al. *(34)* used LCM and comparative genomic hybridization to study myoepithelial cell carcinomas of the breast and found common alterations such as loss at 16q (3/10 cases) and 17 p (3/10 cases), but they also reported fewer genetic alterations compared with ductal carcinomas of the breast. Shen et al. *(35)* used LCM and genome-wide searching in a study of breast cancers to show that LOH was seen only in ductal carcinoma *in situ* but not in the invasive component of ductal carcinoma *in situ.* LCM was advantageous in reducing the heterogeneity within tumors *(35).* A combination of LCM with fluorescence *in situ* hybridization (FISH) and flow cytometry was used in a study of normal breast and breast carcinoma from formalin-fixed, paraffin-embedded tissues in which the nuclei were microdissected by LCM *(36).* Using probes for cyclin-D1 and RB1, this group showed amplification of cyclin-D1 of the two cases studied when the results were compared with touch preparations of nuclei from the same fresh tumor specimens. Unfortunately, the numbers of samples analyzed were too small to evaluate, but the preliminary results are promising.

DNA Libraries and Arrays

Various studies have used LCM to obtain cells as the starting material to clone cDNAs and to analyze genes by cDNA arrays. Several laboratories have used LCM in the initial phase of generating cDNA libraries. The principal leader in this area is the NCI Cancer Genome Anatomy Project (CGAP) *(37,38),* under which cDNA libraries from human cancers of prostate, ovary, breast, lung, and the gastrointestinal tract will be produced and sequenced.

cDNA array technology has been combined with LCM in various studies *(39–41).* Segroi et al. *(40)* used LCM and high-density cDNA arrays to study gene expression in purified normal, invasive, and metastatic breast cell populations from a single patient and combined this approach with real-time quantitative PCR and immunohistochemistry to study tumor progression in this tumor model. Leethanakul et al. *(41)* used LCM and cDNA arrays in studies of squamous cell carcinomas of the head and neck using 5000 cells from normal and tumor tissues. They observed a consistent decrease in cytokeratins and an increase in expression of signal-transducing, cell cycle regulatory proteins, and angiogenic factors, as well as tissue-degrading proteases. Unexpected findings included

overexpression of the wnt and notch growth and differentiation regulatory systems by these squamous cell carcinomas *(41)*.

Proteomic Analysis

The use of LCM for protein analysis had been reported in the original descriptions of LCM *(7)* in which gelatin zymography was used to study gelatinase A (MMP-2) in frozen sections of prostate cancer. In a more recent study, Banks et al. *(42)* used LCM and two-dimensional electrophoresis to examine protein profiles of selected tissue areas. In the cervix they were able to show enrichment of some proteins compared with the whole tissue using these combined techniques. Emmert-Buck et al. *(43)* used normal and neoplastic esophageal squamous mucosa as a model to study proteins captured by LCM followed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). They were able to use 50,000 cells to resolve 675 distinct proteins or isoforms with molecular weights between 10 and 200 kDa and isoelectric points between pH 3 and 10. Although 98% of the proteins in normal and tumor samples were identical, 17 of the tumors showed tumor-specific alterations. Seven of these proteins were present only in normal tissues, and 10 of the proteins were present only in the tumors. Cytokeratin I and annexin I were identified as two of the altered proteins.

In a study of LCM-captured prostate tissue analyzed with a quantitative chemiluminescent assay, Simone et al. *(44)* studied prostate-specific antigen (PSA) distribution in normal prostatic intraepithelial neoplasia and carcinoma and found a range of 2×10^4 to 6.3×10^6 PSA molecules per microdissected tissue cells. These observations were concordant with the immunohistochemical staining intensity of tissue sections from these different tissue areas *(44)*. Ornstein et al. *(45)* used LCM and PAGE (1D and 2D) to analyze PSA α_1-antichymotrypsin (ACT) by Western blotting studies and showed a 30-kDa band that was the expected molecular weight of unbound PSA. Binding studies revealed that PSA recovered from LCM-procured cells had the full ability to bind ACT as in the normal prostate. Electrophoretic studies also showed that the PSA/ACT complex was stable, indicating that the complex was similar in normal and tumorous tissues.

FUTURE DIRECTIONS

Future advances in LCM will include technologic advances in instrumentation and application of LCM to solve difficult and/or challenging problems in cell biology and biomedical sciences. The further development of laser fields small enough to capture single cells or specific parts of cells such as the nucleus are currently under way. Automation of the LCM system for automatic performance of cell capture is also being developed. A major challenge for the technology is to develop methods to capture live cells that can be used for cell culture and other in vitro studies with living cells.

Major advances can be anticipated in combining LCM with other techniques such as flow cytometry, FISH and comparative genomic hybridization. Significant advances can be anticipated in gene expression studies with microarrays to profile specific human diseases starting from clearly defined histopathologic cell populations. The technique of “molecular fingerprinting” of entire organs such as the prostate has been initiated by one group *(38)*. Cole et al. *(38)* have proposed a model for integrating in three

dimensions the data obtained by LCM and microarrays for the in vivo molecular anatomy analysis of normal and neoplastic cells. These types of approaches will rely heavily on LCM for further advances in understanding the molecular aspects of diseases as they relate to tissue structure and function.

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PROTOCOLS

H&E Staining

For paraffin sections, start with **step 1.** For frozen sections that have been previously fixed, start with **step 5.**

1. Xylenes to deparaffinize the slides 2 × 5 min each.
2. 100% ethanol 30 s.
3. 100% ethanol 30 s.
4. 95% ethanol 30 s.
5. Purified water rinse.
6. Mayer's hematoxylin 30 s.
7. Purified water rinse.
8. Eosin Y 30 s.
9. 95% ethanol 30 s.
10. 95% ethanol 30 s.
11. 100% ethanol 30 s.
12. Xylene 2 × 3 min.
13. Shake off excess, wipe the slide carefully, and air-dry for at least 2 min.

NOTE: Chain reagents (xylenes and alcohols) each day that LCM staining is done and filter hematoxylin before use.

Operating the PIX Cell System

1. If entire instrument is shut off, turn on power strip located behind the computer monitor. Also, turn on power for the TV monitor.
2. On computer screen, click mouse on "shortcut to Arc 100."
3. When instrument serial # is given, click "continue."
4. Select name or enter new name and click "acquire data."
5. Highlight study or enter new study and click "select."
6. Enter slide #, spot size, cap lot, and thickness (usually 10 µm, but this may vary depending on slides cut), and click "continue."
7. Enter laser power of approx. 45 and pulse of approx. 55 (to start) length needed (see procedures for RNA and DNA extractions for this information).
8. Place slide on microscope, and, using TV monitor, find an appropriate starting area that will be easy to find after finishing the microdissection.
9. Click on "before" to obtain a picture of the area selected.
10. Insert a row of arcturus caps with transfer film into slot on the right side of the microscope.
11. If the laser power chosen is below 60, place the optic beam adjust piece without the filter in the indentation on the end of the placement "arm." If laser power chosen is above 60, place the beam adjust piece with the filter on the arm.
12. Using the placement arm, pick up a cap, move the arm all the way over to the left (this will put it directly above the slide), and gently release it so that the cap slowly drops onto the slide.
13. Pick up the white cord with the red button on the end and press the button to get a laser pulse. A dark circle will appear around the area if it "melted." (However, just because an area melts does not necessarily mean that it transferred to the cap.)
14. Use the joystick located to the bottom left of the microscope to move the slide around and get pulses in different areas.
15. After finishing the microdissection, use the arm to pick up the cap very gently (so as not to pick up any other tissue), move it over to the right, and place it on a sterile 0.5-mL microcentrifuge tube.
16. Find the original starting area and clock on "after" to get an image of the completed microdissection. Then look at the slide under a microscope to make sure that most of the areas transferred.
17. Click "done" and then "exit" or "continue."

DNA Extraction From Collected Cells

1. Strain slides with hematoxylin and eosin according to the written procedure for staining.
2. Aliquot 100 µl 0.05 *M* Trizma buffer, pH 8.3, and 4 µL proteinase K (10 mg/mL) into sterile 0.5-mL Eppendorf microfuge tubes (see **Digestion Buffer** below).
3. Use LCM to capture the desired number of cells from the slide.
4. Place the film cap containing the captured cells on the top of the microfuge tube and use the black cap tool to snap the cap in. (It is *critical* to place the bottom of the film cap just

inside the lip of the microfuge tube, leaving about a 1-cm gap. Using the black cap tool ensures the correct spacing needed to prevent leakage during incubation.)
5. Invert the tubes and incubate in a 55°C water bath for 48 h.
6. Remove tubes from water bath and spin down in microcentrifuge. Discard the film cap and transfer the solution to a new sterile 0.5-mL tube.
7. Boil samples for 8 min at 95°C in a hot block and place on ice. Store DNA at 4°C until ready to use.

Digestion Buffer

1. 3.03 g Trizma base.
2. 500 mL water.
3. Adjust pH to 8.3.

RNA Extraction From Collected Cells

1. Obtain samples in cap with transfer film using LCM.
2. Aliquot 200 µL TRIzol reagent into a *sterile* 0.5-mL microcentrifuge tube, then place cap with film on top, and invert tube.
3. Leave samples with TRIzol inverted at room temperature for more than 1 h. (At this point, samples may be stored at –70°C for up to 1 month).
4. Take off cap, add 1 µL glycogen and 40 µL chloroform to each tube, and shake vigorously for 15 s.
5. Incubate samples for 3 min at room temperature.
6. Using Eppendorf centrifuge in cold room, spin for 15 min at 10,000 rpm.
7. Transfer aqueous phase to a new 0.5-mL tube, add 100 µL isopropanol, and vortex.
8. Incubate at room temperature for 10 min.
9. Place in a –70°C freezer for 1 h or longer. Take out and allow to thaw.
10. Centrifuge for 10 min at 10,000 rpm in Eppendorf centrifuge.
11. Very carefully discard the supernatant, as you will probably not see a pellet.
12. Add 200 µL 75% ethanol and vortex.
13. Centrifuge at 8,000 rpm for 5 min in Eppendorf centrifuge.
14. Carefully pour off the supernatant, invert the tube, and air-dry for approximately 5 min.
15. Resuspend the pellet in 10 µL diethyl pyrocarbonate (DEPC) water and use this directly for the RT reaction.