CELL SAMPLING

Laser Capture Microdissection: Molecular Analysis of Tissue

Robert F. Bonner, Michael Emmert-Buck, Kristina Cole, Thomas Pohida, Rodrigo Chuaqui, Seth Goldstein, Lance A. Liotta

TECH VIEW

As the list of expressed human genes expands, a major scientific and medical challenge is to understand the molecular events that drive normal tissue morphogenesis and the progression of pathologic lesions in actual tissue. With the advent of polymerase chain reaction (PCR) and the development of high-throughput, automated microhybridization arrays and mutation screening methods, DNA or RNA can be extracted from tissue biopsies and analyzed with a parallel panel of hundreds or even thousands of genetic markers. Because cells in complex tissue are biochemically and physically affected by surrounding cells and by remote stimuli from greater distances, the task of analyzing critical gene expression patterns in development, normal function, and disease progression depends on the extraction of specific cells from their complex tissue milieu. The value of even the most sophisticated genetic testing methods will be limited if the input DNA, RNA, or protein is not derived from pure populations of cells or is contaminated by the wrong cells. Laser capture microdissection (LCM) (*1*) is a rapid, reliable method to procure pure populations of targeted cells from specific microscopic regions of tissue sections for subsequent analysis.

Several methods have been developed to microdissect stained tissue sections and amplify the procured material with PCR (*2*). The first category uses ablation to destroy or remove the unwanted regions, and the remaining desired tissue is then collected mechanically (*3*, *4*). These methods require a subsequent micromanipulation for collection of the cells of interest. A second category involves a tool under manual guidance used to mechanically separate cells of interest from the histologic section. The tool can be a pipette, pointed probe, fine needle, or blade (*3*) either hand-held or connected to a micromanipulator arm (*5*). Boehm *et al.* (*6*) placed the tissue section on an ultrathin film and then used an ultraviolet laser to cut out the tissue of interest.

In the third category, exemplified by LCM, a transfer surface is placed onto the tissue section and then focally bonded to the targeted tissue, allowing it to be selectively removed for molecular analysis (Fig. 1) (*1*). In the microscope, the operator views the tissue and selects microscopic clusters of cells for analysis, then activates a laser within the microscope optics. The pulsed laser beam is absorbed within a precise spot on the transfer film immediately above the targeted cells. At this precise location, the film melts and fuses with the underlying cells of choice. When the film is removed, the chosen cells remain bound to the film, while the rest of the tissue is left behind.

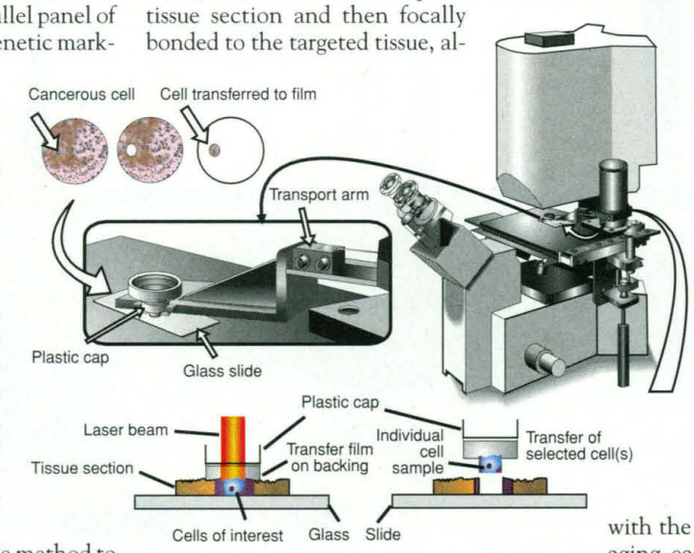

Fig. 1. The LCM apparatus.

LCM offers a number of advantages. In one step, the cells of interest are transferred to the polymer film. The separate fragmentation step used in conventional microdissection and the resulting contaminating debris are avoided. In addition, only the targeted cells are affected, with a precision of transfer that can approach 1 μm. Thus, the remaining tissue on the slide is fully accessible for further capture, allowing comparative molecular analysis of adjacent cells. The exact morphology of the procured cells is retained and held on the transfer film, so transfer images constitute a diagnostic record of the cells undergoing molecular analysis. In contrast, with manual microdissection, cells may be pulverized or lost (*5*). With LCM, the procurement of specific cells from a complex tissue section is reduced to a routine method amenable to widespread research and clinical diagnostic use.

The thermoplastic polymer film contains special infrared (IR) absorbing dyes to allow use with near-IR gallium arsenide laser diodes, which are easily and economically integrated into normal microscopes. The polymer and its substrate do not absorb the visible wavelengths used to examine and target cells within the stained tissue section in the microscope. The thermoplastic polymer is chosen to have a steep decrease in viscosity with rising temperature, so that mild focal heating is sufficient to cause it to flow into the interstices of the targeted tissue and rapidly form a bond that is stronger than the bond between the tissue section and the glass slide. The mild, brief thermal transients experienced by the tissue in the capture process do not damage DNA, messenger RNA, or proteins. Thermoplastic bonding avoids chemical reactions that can cross-link biological molecules in the tissue and alter subsequent molecular analysis. Focusing the laser diode beam with the microscope objectives used for imaging easily allows focal activation of the polymer in spots as small as 3 μm (*7*). The transferred tissue is firmly embedded in the film, but fully accessible to aqueous solutions necessary for the extraction of DNA, RNA, and proteins from it.

LCM was conceived and first developed as a research tool at the National Institutes of Health (NIH) and in the last year, through a Collaborative Research and Development Agreement (CRADA) partnership with Arcturus Engineering (Mountain View, CA), was made into a commercial instrument. The handling of transfers has been simplified by bonding the transfer film to a flat or conical vial cap. This allows multiple homogeneous samples within the tissue section or cytological preparation to be targeted and pooled for extraction of molecules and analysis. A mechanical arm precisely posi-

R. F. Bonner is in the Laboratory of Integrative and Medical Biophysics, National Institute of Child Health and Human Development, Bethesda, MD. M. Emmert-Buck, K. Cole, R. Chuaqui, and L. Liotta are in the Laboratory of Pathology, National Cancer Institute, Frederick, MD. T. Pohida is in the Computational Bioscience and Engineering Laboratory and S. Goldstein is in the Biomedical Engineering Program, NIH, Bethesda, MD 20852. E-mail: bonner@helix.nih.gov

tions the transfer surface onto the tissue. The microscope focuses the laser beam to discrete sizes, delivering precise pulsed doses to the targeted film. Targeted cells are transferred to the cap surface, and the cap is placed directly onto a vial for molecular processing. Single cells can be individually targeted and transferred (Fig. 2). Laser transfer spots can be overlapped to capture complicated microstructures such as neoplastic clusters and the end buds of developing breast ducts embedded in adipose and connective tissue.

NIH has developed computer software to control the microdissection process and to store data, including digital images of the microdissected cells before and after transfer. This record can be integrated with subsequent molecular analysis results, which is critical in research and clinical applications. We are currently developing computer-based automation of the entire LCM process, including targeting specific cells, capture, and molecular analysis.

LCM is being used in the Cancer Genome Anatomy Program effort to catalog the genes that are expressed in human tissue as normal cells undergo premalignant changes and further develop into invasive and metastatic cancer (8). Microarray hybridization panels (9) containing these index sets are being used to obtain a molecular fingerprint of gene expression in microdissected human tissue biopsies. The fluctuation of expressed genes or alterations in the cellular DNA that correlate with a particular disease stage can be compared within or between individual patients. Such a fingerprint of gene expression patterns may provide crucial clues for etiology and may contribute to diagnostic decisions and therapies tailored to the individual patient. Molecules found to be associated with a defined pathological lesion may serve as future imaging or therapeutic targets.

LCM-based molecular analysis of histopathological lesions can be applied to any disease process that is accessible through tissue sampling. Examples include mapping the field of genetic changes associated with the progression of microscopic premalignant cancer lesions; analysis of gene expression patterns in multiple sclerosis, atherosclerosis, and Alzheimer's disease plaques; infectious microorganism diagnosis; typing of cells within disease foci; and analysis of genetic abnormalities in utero from selected rare fetal cells in maternal fluids.

Many informative uses involve studies of normal, healing, developing, or differentiating cells. Virtually nothing is known about the tissue-specific gene expression patterns for resting normal epithelium or endothelium. Assuming linear reverse transcriptase–dependent (RT)–PCR amplification of microdissected RNA, it is quite practical to prepare complementary DNA libraries and microarray probes from LCM samples of normal ovarian surface epithelium, regenerating muscle or nerve, embryologic organs, healing wounds, pancreas islets, glomeruli, parathyroid cells, and subcellular elements.

LCM allows routine analysis of DNA, RNA, or protein within selected pure cells from complex mixtures such as tissue sections or cytological smears. This places additional demands, however, on histopathology methods and on molecular analysis techniques. The condition of the macromolecules to be analyzed within the tissue sections must be preserved. Flash freezing, cryosectioning, and optimal processing of the transferred tissue are required for the most stringent and quantitative molecular analysis. More conventional fixation and paraffin-embedding of tissue lead to greater variability in the quantity and quality of macromolecules recovered for analysis, but this can usually be controlled for by comparing different cells from the same slide (for instance, normal epithelia and tumor cells). LCM can capture the selected cells without damaging the enzymatic functions of proteins. Consequently, an important area for future development will be the adaptation of specialized immunoassay, enzymatic, and two-dimensional gel panels to LCM samples.

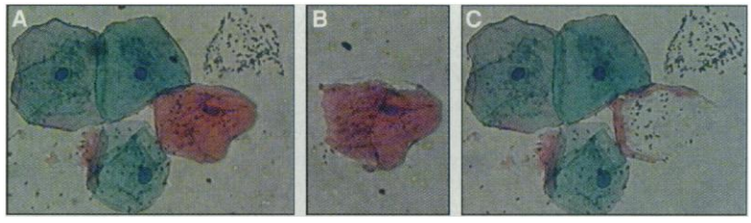

Fig. 2. LCM transfer of a single cervical squamous cell from a Pap smear. **(A)** Cluster of four cells on the slide. **(B)** Targeted cell transferred to polymer film. **(C)** Remaining cells on the slide after transfer.

Quantitative procurement of targeted tissue requires careful process control to ensure that the bond between the targeted tissue and the activated polymer exceeds the adherence of the cells to the slide. As the target size is reduced to that of single cells, the accuracy of targeting decisions becomes more difficult and may require immunohistochemical or molecule-specific fluorescent labels (such as fluorescent in situ hybridization) and improved sample imaging to identify the cell population of interest. Although, in principle, it would seem highly desirable to procure a single tissue cell for analysis, this will require that the cell is whole and representative of the pure subpopulation of interest. Cell cycle and other differences can be averaged out by the capture of 20 or more single cells with the same morphologic type (for example, Hodgkin's cells) on the same LCM transfer film for molecular analysis. A new NIH prototype (7) is able to target and concentrate such single cells onto a conical surface.

In order to apply LCM to routine clinical diagnosis, integration of LCM procurement with higher throughput, ultrasensitve molecular analysis will be required. We can envision efficient and rapid analysis of the procured cells directly on the polymer film. This could be accomplished by extraction and molecular analysis in microvolumes using microfluidics-based PCR, RT-PCR, and capillary-gel electrophoresis. As appropriate microanalytic techniques are improved, it may be possible to provide high spatial resolution of the extraction directly in the molecular analysis, thereby obviating the need for a separate LCM transfer step. For example, microelectrophoresis of molecules from a tissue section into an overlying gel matrix can extract and separate molecules at specific sites by using a "patch clamp" type approach. In a further refinement of this approach, parallel microchannel plate electrophoresis might permit rapid mapping of the distribution of multiple molecules within the tissue.

Quality control of multiplexed molecular microanalysis poses extreme challenges for clinical laboratories, medical diagnostic manufacturers, and regulatory agencies. Therefore, diagnostic tests on cells procured with LCM may rely on quantitative analysis of a small set of molecules specific for certain disease processes or cellular pathways.

References and Notes

1. M. R. Emmert-Buck *et al.*, *Science* **274**, 998 (1996).
2. E. R. Fearon, S. R. Hamilton, B. Vogelstein, *ibid.* **238**, 193 (1987); D. Radford *et al.*, *Cancer Res.* **53**, 2947 (1993); M. Emmert-Buck *et al.*, *Am. J. Pathol.* **145**, 1285 (1994).
3. D. Shibata *et al.*, *Am. J. Pathol.* **141**, 539 (1992).
4. I. Becker *et al.*, *Lab. Invest.* **75**, 801 (1996).
5. L. H. Whetsell *et al.*, *Oncogene* **7**, 2355 (1992).
6. M. Boehm *et al.*, *Am. J. Pathol.* **151**, 63 (1997).
7. R. F. Bonner, S. R. Goldstein, P. D. Smith, T. Pohida, in preparation.
8. The Cancer Genome Anatomy Web site is www.ncbi.nlm.nih.gov/ncicgap/
9. S. P. A. Fodor, *Science* **277**, 393 (1997); J. DeRisi *et al.*, *Nature Genet.* **14**, 457 (1996).
10. LCM development at NIH also included S. Bova, P. D. Smith, J. I. Peterson, J. Lee, C. Seshradi, M. Huckabee, and N. Simone. Arcturus staff (T. M. Baer, D. Head, M. Enright, and C. Todd) working with NIH have developed a commercial LCM instrument. Additional information is available on the Web at http://dir.nichd.nih.gov/lcm/lcm.htm

TechWire Forum:
www.sciencemag.org/dmail.cgi?53421