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Review Article

Laser Capture Microdissection in the Genomic and Proteomic Era: Targeting the Genetic Basis of Cancer

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Abstract: The advent of new technologies has enabled deeper insight into processes at subcellular levels, which will ultimately improve diagnostic procedures and patient outcome. Thanks to cell enrichment methods, it is now possible to study cells in their native environment. This has greatly contributed to a rapid growth in several areas, such as gene expression analysis, proteomics, and metabolonomics. Laser capture microdissection (LCM) as a method of procuring subpopulations of cells under direct visual inspection is playing an important role in these areas. This review provides an overview of existing LCM technology and its downstream applications in genomics, proteomics, diagnostics and therapy.
Key Words: Laser capture microdissection, genomics, proteomics, carcinogenesis, clonality, biomarker discovery, prognosis

Introduction

The completion of the human genome sequencing initiative has revealed the inherent genetic diversity in individuals, with its associated functional genomic and proteomic diversity. Consequently, assays capable of monitoring genome, transcriptome and proteome in a highly parallel fashion have emerged [1, 2]. Molecular profiling is a collective term for such assays that provide information for the design of individualized therapies. For example, in clinical oncology, only a minority of cancer patients will respond to standard therapy, while others may suffer toxicity or drug resistance. Since experience has shown that many tumors with similar clinical stage have different clinical outcomes, the goal of molecular profiling is set up to provide a rational molecular basis for assessment of prognosis and therapy. Another common problem encountered in the genomic and proteomic analysis of tissue is its heterogeneous nature. Imagine a tissue section comprised of 80% tumor, 10% stroma and 10% infiltrating lymphocytes in which the lymphocytes may contribute more than 10% in overall signal. Because of direct microscopic visualization of the cells it is now possible to select normal, premalignant, malignant, or disease free cells as distinct populations from the heterogeneous background [3]. The need for obtaining pure samples of tumor tissue has resulted in several methods of cell enrichment including culturing of tumor cell lines, xenograft enrichment, cell sorting and microdissection, of which laser capture microdissection will be discussed in details in this review.

Historical Perspective

Culturing of tumor cell lines is a good tool for study of genetic changes in neoplasia and carcinogenesis [4-6]. The system is self-replicating and yields high-quality DNA, RNA and protein, but it is lengthy and expensive to establish, and is not always successful. Even though genetic alterations in xenografted tissues or cell lines usually reflect those present in the original tumor, additional

changes may develop during serial passage, or only an aggressive subset of tumor cells may be propagated [4]. The gene expression in cultured cells can be very different from the genes expressed in the tissue because they are no longer under the control of tissue elements that regulate gene expression, such as cell-cell communication, extracellular matrix molecules or various soluble factors [6]. For this reason, molecular events observed in cultured cells may not accurately represent those taking place in the actual tissue from which the cultured cells were derived [4]. Therefore, cellular heterogeneity represents a significant barrier to the molecular analysis of normal and diseased tissues as does the fact that normal cells, or preneoplastic lesions have rarely been successfully cultured [6, 8].

Xenograft enrichment is another method of cell enrichment. Immunodeficient rodents such as nu/nu or SCID mice are used to obtain human tumor cell populations whose non-malignant cells are of rodent origin. This is achieved by a serial passage of tissues through experimental animal [7-9]. The same limitations apply as in cell culturing, because the ability to propagate xenograft requires considerable expertise, a reasonable animal facility and the time for establishment. Another issue is that the tissue sample used for xenografting may not necessarily be representative of the primary lesion, since additional genetic changes may be introduced in the tumor cells during serial passage, or a subset of tumor cells with a selective growth advantage may propagate. The presence of large numbers of stromal cells of rodent origin may complicate subsequent molecular analysis. Xenografting is also limited to the study of tumor cells only. Preneoplastic lesions have rarely been xenografted.

Another approach to concentrate and purify cells of interest is to use cell sorting techniques, such as density gradients, fluorescence- activated cell sorting, antibody-labeled immunobeads, and affinity-labeled magnetic beads [5, 7, 8, 10]. For these methods there is a requirement to create suspensions of individual cells [6]. Samples from tumors amenable to formation of suspensions (hematolymphoid malignancies) can be easily prepared, whereas the technique is rarely applicable in solid tissue because intercellular adhesion prevents the disaggregation of cells, which is prerequisite for the formation of single cell suspension [8].

Selective isolation of tumor cells and their precursor lesion requires a technique where the cells can be isolated from the primary lesion itself, without an intervening step. This is even more true for preneoplastic lesions that may be spatially distinct from the tumor, and very often, sparse in quantity [6, 7]. Microdissection is a technique for isolation of specific cell subpopulations from a diverse background of cell types, cytological preparation, or live cell culture via direct visualization of the cells [6, 7, 10]. The need for microdissection was realized in the 1970s, when Lowry and Passonneau pioneered a procedure of quantitative biochemical analysis of specific cell types microdissected from lyophilized tissue sections [11, 12]. The procedure was done free hand under a dissecting microscope with splinters of a razor blade mounted on a flexible bristle. The dissected portions were then lifted and transferred with a short piece of human hair mounted to a pencil-shaped glass holder. With this approach, 15 - 20μm of sample size could be dissected.

Current microdissection techniques can be divided into 3 broad categories: manual extraction of desired foci, selective ablation of unwanted regions and use of laser pulses to capture cells of interest, so-called laser capture microdissection (LCM). Manual extraction was described by Goelz *et al* in 1985 [13]. He removed areas of interest from the paraffin block under direct visual inspection, and used sample for DNA extraction. This was an important step forward because it showed that genomic material extracted from routinely fixed paraffin embedded tissue was amenable to PCR amplification and sequence analysis. In subsequent years, other manual methods of microdissection were described. For example, one of them used modified Pasteur pipettes or tungsten wire attached to mechanical micro-manipulators for extraction of DNA from lesions < 1 mm in size. However, methods of manual extraction are limiting because they are both tedious and operator dependent, they require manual dexterity to prevent contamination, and even minor air currents can cause the cells to be lost in the process of transferring from the pipette or needle tip into the microcentrifuge tube. Attempts to improve upon manual extractions have included the usage of adhesive sellotape in order to cover areas of interest, and thus prevent loss of microdissected material from tremor or air

Table 1 Principles and technical basis of LCM techniques

Method	Principle
IR LCM	IR laser pulse melts EVA polymer, cells adhere to the melted membrane. As the membrane cools it solidifies again and the cells are removed by peeling of the membrane from the cap
UV LCM	Unwanted tissue is photoablated with the very narrow UV laser beam

LCM, laser capture microdissection; IR, infrared; UV, ultraviolet

current. In contrast, selective ultraviolet radiation fractionation (SURF) use UV radiation to destroy unwanted regions, and the islands of tissue that are left behind are manually scraped off for molecular analysis. Shibata *et al* was the first to describe this kind of technology [14]. Becker *et al* described analogous technique [15]. They have used UV laser and manual micromanipulator to ablate surrounding non-neoplastic elements. It is worth noting that ablative technologies are applicable to formalin fixed samples.

Laser Capture Microdissection

The advent of laser-based microdissection techniques has marked a new era in microdissection. Isenberg *et al* were among the first to use primitive UV laser technology in the 1970s, but their approach required massive space occupying instruments to dissect subpopulations of cell types from a heterogeneous background [16]. LCM was devised at the NIH in the mid 1990 by Lance Liotta, Emmert-Buck and co-workers who recognized a need to develop a microscope-based microdissection system for accurately and efficiently dissection of cells from histological tissue sections of solid tumors to fully exploit emerging molecular analytical technologies [17]. The system rapidly moved into commercial production by Arcturus engineering (Mountainview, CA) and offers one of several laser-assisted dissection strategies that allow direct selection of cell types without the need for enzymatic processing or growth in culture [3, 7, 8, 10, 17, 18].

Principles and Technical Basis

It is important to emphasize that the term LCM is used generically. Although a variety of instruments exist, the term laser capture microdissection is the standard terminology used regardless of laser method type [2]. There are three general classes of laser capture microdissection systems: infrared (IR LCM), ultraviolet (UV LCM) and combined IR/UV system. LCM instruments exist in a form of manual and automated (robotic) platforms [3]. Regardless of the system used, the fundamental features of the laser microdissection process are visualization of the cells via microscopy, transfer of laser energy to the area of interest, and removal of the cells of interest from the heterogeneous tissue section. The transfer of laser energy may be to a thermolabile polymer thus forming a polymer cell composite as in IR systems, or photovolatilization of cells surrounding a selected area that is specific to UV systems. IR systems include both manual (Arcturus Pix cell, Bio-Rad Clonis) and automated (Arcturus AutoPix). Both the manual and automated versions of IR LCM work on the same principle. UV LCM includes automated systems [2, 3] such as PALM microbeam, LMD6000 and mmi CellCut. UV systems often employ cutting methods that combine UV laser microdissection and catapulting systems [7, 17]. An example of a combined IR/UV system is the automated Arcturus Veritas instrument [3].

Infrared LCM

The basic principle of LCM is the capture of groups or individual cells onto a thermoplastic membrane from histological sections of stained tissue (frozen or formalin-fixed paraffin-embedded) (**Table 1**) [17, 18]. The system consists of an inverted microscope that is connected to a personal computer for additional laser control and image archiving, a solid state near infrared laser diode, a laser control unit, a joy stick controlled microscope stage with a vacuum chuck for slide immobilization, a CCD camera, and a color monitor [19-21] (**Figure 1** for an example). A 100μm thick ethylene vinyl acetate (EVA) film, impregnated with a dye that absorbs light in the near-IR spectrum, is attached to a rigid 6mm optically clear cap with a diameter of 6 mm. The cap fits on the standard 0.5 mL microcentrifuge tubes for further tissue

Figure 1 Components of the Artcurus laser capture microdissection system (PixCell II).

processing [5, 7, 10, 17-20, 22]. A dye absorbs laser energy, preventing damage to the cellular constituents. It also aids visualizing areas of melted polymer and it aids visualizing areas of melted polymer [2, 3]. The cap, that is suspended on a mechanical transport arm and placed on the desired area of the dehydrated tissue section, acts as an optic. It focuses the laser and brings it to the same plane as the tissue section, and then is lowered in exact apposition to the area of interest [3, 17, 19, 20]. Laser activation raises temperature to 90°C [7] which leads to focal transient melting of the ethylene vinyl acetate (EVA) membrane [20, 21]. The polymer melts only in the vicinity of the laser pulse, expands into the section and fills the extremely small hollow spaces present in the tissue. Cells then selectively adhere to the thermoplastic membrane activated by a low energy infrared laser pulse [20]. Under standard working conditions, the area of polymer melting corresponds quite exactly to the laser spot size [19-22]. As the temperature decreases, it solidifies again within milliseconds and forms a polymer-cell composite that embeds the cells into the plastic membrane [2, 19, 20]. The selected tissue fragments are harvested by simple removal of the polymer from the tissue surface that serves to shear the embedded cells of interest away from the tissue section [2, 3, 19, 20]. The cap with the dissected cells is then placed in a 0.5mL Eppendorf tube containing lysis buffer [7, 8, 19, 20]. The long chain polymers within the EVA film then dissolve in the lysis buffer and the cells are released into the solution [7]. Laser impulses, usually between 0.5 and 5 ms in duration, can be repeated multiple times across the whole cap surface, thus allowing the rapid isolation of large numbers of cells [19, 20]. Up to 3000-5000 cells can be isolated onto a single cap in this fashion [7, 17] (**Figure 2**). In the Arcturus Pix Cell II instrument, a current model of the laser micro capture system, the laser beam has 3 settings of diameter [5, 12] (**Figure 1**). Varying laser spot size within a narrow range ensures the specificity of dissection [4, 7, 10, 17-21], and usage of a laser spot at its narrowest diameter (7.5 μm) permits microdissection of a single cell [5, 12, 23]. Important, because most of the energy is absorbed by the membrane, the maximum temperatures reached by the tissue upon laser activation are in the range of 90°C for several milliseconds, thus leaving biological macromolecules of interest intact [1, 4, 7, 18-20]. The low energy of the infrared laser also avoids potentially damaging photochemical effects [19, 20]. The joystick is used for movement of the laser cap around the tissue in order to select multiple areas on the same cap [6, 8]. A caveat of the membrane slides is that they are not cover slipped which makes visualization fuzzy [8, 17]. However, newer versions of LCM systems have a built in optical system that makes it possible to confirm the histology of the area to be microdissected [8, 17]. When using glass slides, they need to be non-charged and non-coated since either feature can interfere with the transfer of tissue from the slide onto the cap.

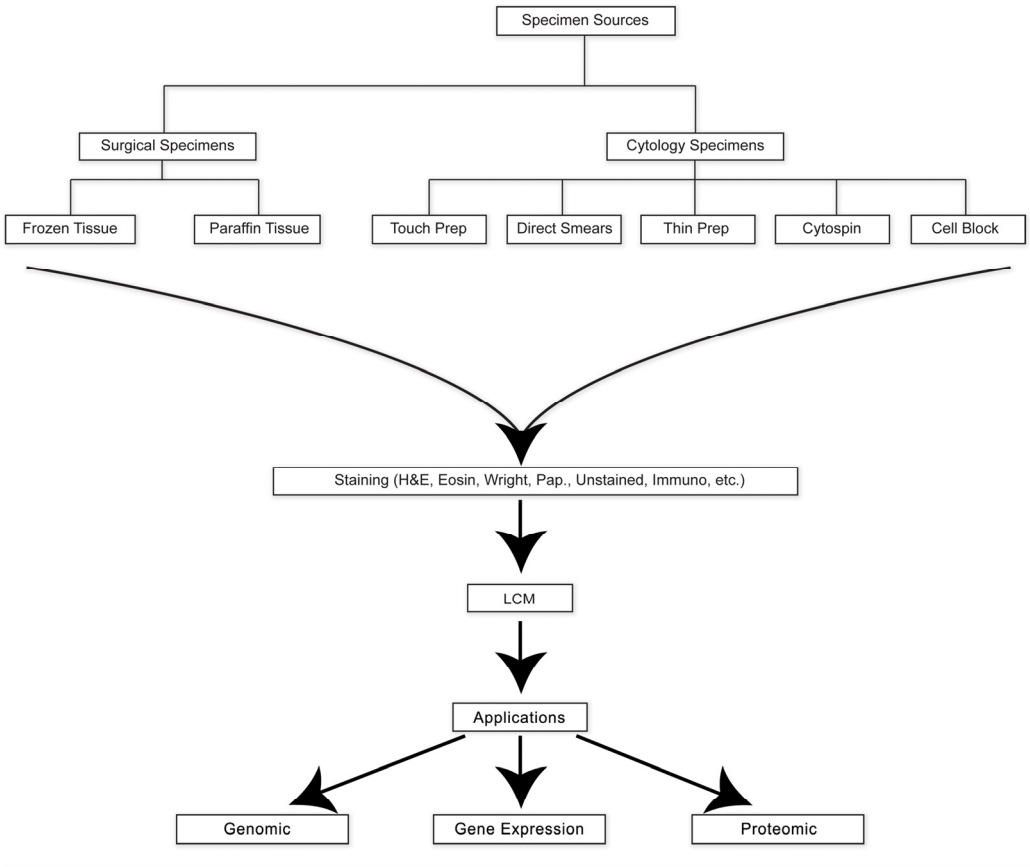

Figure 2 Tissue sources and applications of LCM

The cell samples that are obtained can be used for any molecular analytical methods. DNA and RNA can easily be extracted and used for PCR, gene expression analysis, and proteomics [8]. About 50-100 cells are adequate for PCR analysis from microdissected material [7] and can yield nanogram quantities of nucleic acids [8, 24]. Of note, histological staining that is required for visualization during microdissection will not affect the quality of the biomolecules In the sample, nor does the process of acquiring cells onto the thermoplastic membrane alter or damage the integrity of DNA, RNA and protein.

Protocols used for molecular analysis from LCM - captured cells are standardized and are available for use by the public at large on the NIH web site (http://dir.nihcd.nih.gov/lcm.htm) [7, 8, 17]. Newer version for single cell extraction is called cylinder LCM.

Ultraviolet LCM – Laser Microbeam Microdissection with Laser Pressured Catapulting (LMM-LPC)

UV-based methods for LCM operate on a quite different principle than IR LCM (**Table 1**). Current LMM-LPC platforms uses tissue that has been mounted on a 6 µm membrane and placed on a glass slide, onto which the operator directs an UV laser beam under direct visualization [7, 17, 18]. The narrow-beam UV laser is used to draw around the cell or cells of interest leaving the desired cell population intact while simultaneously ablating away unwanted tissue [7, 17]. The cells of interest are then isolated by catapulting them under pressure onto an overhanging cap. There are two major advantages of this method. First, it avoids any intricate operator dependent step, and second, by ablating the adjacent rim of unwanted tissue, non-specific adherence of tissue to the cap is avoided. Another important feature of LMM-LPC is that the UV laser has high photon density (cold laser). Thus, the heat generated during microdissection is minimal, which reduces the risk of damaging extracted

reagents [7].

3 different manufacturers have produced laser microbeam microdissection systems, all of which vary in their precise details of operation regarding the method of collection and transfer of the microdissected tissue for subsequent molecular analysis. The PALM system offers the advantage that there is no physical contact between the cells and plastic because the laser is used to catapult the microdissected cells into a collecting tube under the influence of gravity [19]. This process avoids the potential risk of modification of molecules of interest due to heating and cooling of the thermoplastic membrane. Of course, the original quality and subsequent handling of the tissue is of fundamental importance [25]. Laser microbeam microdissection systems use a much finer laser beam diameter (0.5 μm) contrary to the IR LCM where the smallest laser beam size is 7.5 μm. Thus, UV LCM systems are ideally suited for the precise microdissection of single cells although this is potentially more time-consuming, especially when a large number of cells are required to be microdissected. This is unlikely to be a significant issue for nucleic acid based molecular analysis, as it can often be performed on very few cells, but the time taken to acquire the large number of cells for proteomic studies may become a significant factor in experimental planning and design. In this instance, UV cutting systems such as Veritas, PALM or Leica MMI are particularly useful for microdissection of tissue sections up to 200μm of thickness. However, damage induced by UV lasers to the final cell population poses a potential limitation of the UV laser systems, because these cells may contribute significantly to the final molecular signal if their number in the perimeter of the cut area is high (>10%) compared to the overall microdissected area [20]. Newer generation instrument such as the mmi CellCut system employs a "touchless" microdissection platform in which the UV laser is used to cut tissue from a polyethylene tretraphtalate (PET) membrane slide that is protected from contact with the environment during microdissection. In this method, an adhesive lid of a microcentrifuge tube is placed onto the cut area and the selected cells are removed from the tissue.

Immuno-LCM

It is very often hard to visualize cells for microdissection because non-coverslipped slides are used. This is particularly difficult when isolating cells that are morphologically very similar (B and T lymphocytes), or present within a heterogeneous background (such as Reed Sternberg cells in Hodgkin lymphoma) [3, 7]. Immuno-LCM can help to overcome this issue. Immunohistochemistry (HICK) is performed to mark the cells expressing a type-specific antigen (such as CD3 for T-cells or prolactin for pituicytes), and the cells are captured under direct visualization. The common detection reagents used in HICK such as DAB do not adversely affect PCR retrieval [7, 26]. It is also possible to simultaneously detect multiple different messages in pathologically altered tissue with the immunohistochemical tagging. One variation of immuno LCM is prelabeling of cells *in vivo* in an animal model, by injection of a fluorogold label. It has been used to label cells to avoid RNA degradation due to immuohistochemical staining [7].

Types of Samples

The nature of investigation is determining the choice of fixative. Formaldehyde is an efficient fixative for DNA, but acetone or ethanol fixation yields better quality of RNA. RNAse free reagents should be used at all times for RNA based investigations, because protection of sample from degradation is important. It is also important to note that the specimens need to be dehydrated as well, since the presence of water interferes with the bonding of polymer to the captured cells. Types of samples typically used for LCM include tissue sections (cut from paraffin and frozen specimens), cytology preparations (touch preps, direct smears, cytospins and cell blocks), and HICK or hybridization labeled cells (**Figures 2** and **3**). LCM is compatible with most cell/tissue staining techniques such as HE, HICK, toluene blue, fluorescent dyes, and in situ hybridization [27].

Applications

At present, 3 distinct classes of biomolecules can be analyzed in LCM specimens: DNA, RNA and proteins [28]. Less material is required for DNA and RNA analyses than for protein analyses. Therefore, it is possible to perform genomic analyses on samples derived from one single cell, whereas for protein this may not be possible with the current generation of proteomic tests.

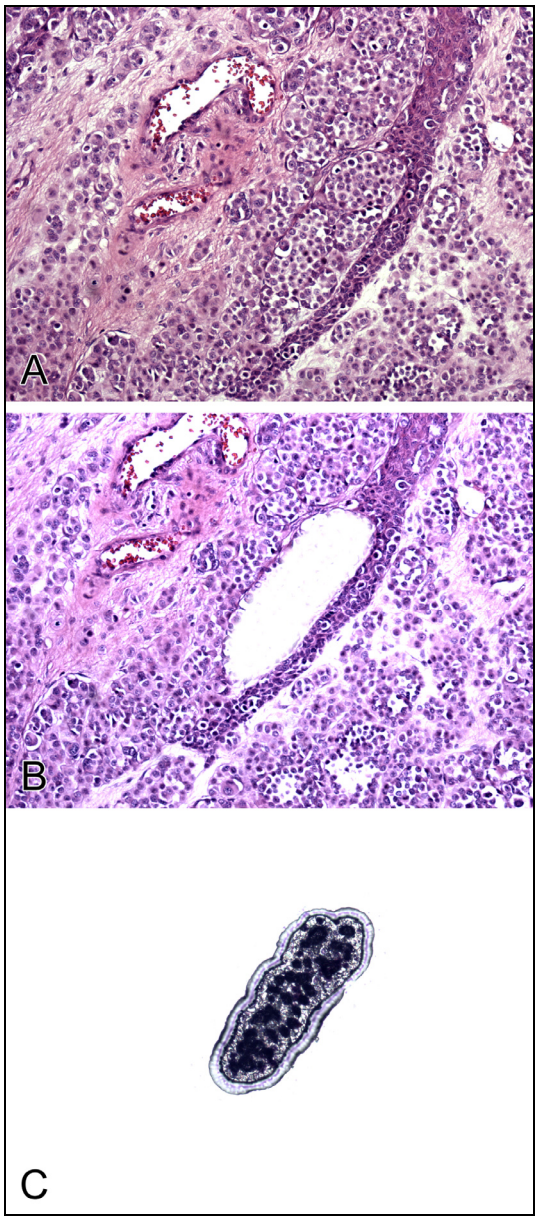

Figure 3 Example of microdissected cancer cells with LCM. Melanoma cells before (**A**) and after (**B, C**) microdissection.

Cancer Research - Cancer Genome Anatomy Project

The Cancer Genome Anatomy Project (CGAP) at the National Cancer Institute (www.ncbi.nlm.nih.gov/ncigap) relies on micro-dissection in the search for the molecular progression of cancer, with the fundamental objective to identify genetic differences between normal, preneoplastic and cancer tissues by comparing and contrasting expression profiles from microdissected regions in the same patient [7, 17].

Recent studies of the identification of prostate specific genes by the analysis of prostate expression sequence tags (ESTs) have shown the power of LCM in creating tissue specific expression libraries. For example, it was initially believed that highly expressed T cell receptor found in prostate libraries generated from microdissected tissue stems from the contaminating T cells in the prostatic interstitium, but the subsequent analyses showed that the transcript did in fact originate from prostate epithelial cells. In the future similar revealing results can be expected for other tissues. Of course, to produce useful information, it is most important to have primary tissues of superior quality [1].

Genomic Analyses from Microdissected Materials

In most cases, solid tumors progress in a multistep pathway. A continuous accumulation of genetic aberrations within the indigenous cells result in neoplastic transformation [17]. In carcinomas, this continuum of changes may involve amplification or gain of function mutations in dominant oncogenes, or loss of function by deletion, mutation or methylation in recessive tumor suppressor genes. In Knudson's two hit hypothesis of tumor suppressor gene function, one parental allele is lost by deletion, while the second is inactivated by mutation or by some other mechanism such as aberrant methylation. This means that in order to examine the integrity of parental alleles at a given polymorphic locus of tumor suppressor genes in non-cancer tissue, two different (or heterozygous) alleles would be present: a normal allele, and an abnormal allele (i.e. one which generates a dysfunctional protein product). In cancer tissue, the normal allele is lost, *i.e.,* there is a loss of heterozygosity (LOH) [17, 29-41]. LOH has been used for a long time in cancer research both for mapping of tumor suppressor genes, as well as for studying the frequency of involvement of known or putative tumor suppressor genes in various cancers using flanking or intragenic polymorphic markers. For LOH analysis pure populations of tumor cells or preneoplastic foci are required because the contamination by even few unwanted cells would mean the second allele "lost" in the cell population of interest will be amplified in the PCR reaction. The use of microdissection in the study of cancer has made a significant difference in the application of LOH analysis

[31, 37-40, 42-57]. LOH analysis has been invaluable for mapping of tumor suppressor genes (Tags), localization of putative chromosomal "hot spots" and the study of sequential genetic changes in preneoplastic lesions [7, 33, 35, 37, 48, 50-59]. Before the widespread availability of microdissection techniques, many valuable samples had to be discarded because the desired purity could not be achieved. The study of genetic losses in preneoplastic lesions was virtually impossible to perform. Microdissection had revolutionized the approach to LOH research in many ways. First it made this study feasible. Moreover, it also has shown that frequencies of LOH in non-microdissected material often under-estimates the true incidence of genetic alterations, and that there may be heterogeneity present within a single tumor where some genetic changes often begin very early, including histologically "normal" or mildly abnormal tissue.

Besides LOH analysis, other genome analyses can be performed from microdissected samples, such as analysis of patterns of X-chromosome inactivation to assess clonality, restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) analysis for assessment of mutation in critical genes such as *Ki-ras* and *P53*, and most recently, the analysis of promoter hypermethylation. Hypermethylation of promoter sequences in tumor suppressor genes is a frequent and early event in carcinogenesis. It is a potentially reversible, and thus can be used as a surrogate biomarker in chemoprevention trials [7]. With the help of combination of microdissection with a primer extension preamplification (PEEP) and whole genome amplification (WGA), it became possible to use smaller and smaller samples of cells, thereby refining the study of preneoplastic lesions, which has opened a whole new frontier in cancer research. For example, the comparative genomic hybridization (CGH), until recently, was possible only with large amounts of DNA extracted from tumor tissues. Now, thanks to the new technology, CGH can be performed on 20-100 microdissected cells from FFPE tissue and even fewer in material obtained from precipitating fixatives such as methanol [7]. Microarrays, popularly known as a "gene chips" have generated the most excitement of all current expression technologies, especially in combination with LCM. Formalin fixed archival tissue can be used in all of these methods. The sample size is very small, no more than 50 - 100 dissected cells per PCR and even fewer cells if material is obtained from cryostat sections or methanol- fixed specimens [7].

Gene Expression Analysis from Microdissected Material

A useful parameter to determine how tumors differ from the normal tissues they are derived from is differential gene expression [7]. It is quite possible that the identification of gene expression patterns related to neoplastic transformation, inflammation or tissue repair will have far reaching consequences in the prognostic and diagnostic field, preventive medicine and for novel treatments tailored for specific genetic alterations [19, 20]. Gene expression can be studied by a variety of available methods such as differential display, representation difference analysis (RDA), SAGE, ESTs, and differential gene chips [4, 7]. Similar issue as in DNA-based studies in the analysis of gene expression is the problem of contamination with inflammatory and stromal cells [7, 20, 60]. Therefore, there has been an increasing need to apply microdissection methods in the expression studies as well, because confirmation by HICK or in situ hybridization is laborious, time-consuming and might not always be possible. mRNA in situ hybridization lacks sensitivity for the detection of low abundance transcripts [7, 60], thus there is an increased need for single cell LCM as well [7].

Proteomic Analyses from Microdissected Material

Proteins perform all the necessary functions of the cell. The existence of a DNA sequence does not guarantee the synthesis of a corresponding protein, nor is it sufficient to describe its function and cellular locations. DNA sequence also does not give information about context dependent posttranslational processes such as glycosylation, phosphorylation or sulphatation, or how proteins link together into networks and functional machines in the cell [4]. Proteomics is a complementary approach to study gene expression and provide additional information regarding the effects of post-translational modification. It aims at determining the overall set of proteins (“proteome”) that are important in normal cellular physiology or altered by disease

process such as cancer. A variety of techniques such as western blotting, high resolution two dimensional polyacrylamide gel electrophoresis (2-D PAGE), mass spectrometry and peptide sequencing can be used for the analysis [7, 61, 62]. Mass spectrometry, such as surface enhanced laser desorption ionization (SELDI) mass spectrometry, has facilitated the study of gene expression at the protein level leading to the recent expansion of proteomics-based research [4, 5, 7, 61]. In the context of protein analysis of LCM procured samples, a number of factors must be considered, such as tissue type, molecule(s) being studied or the method of the downstream analysis. There are limitations resulting from the amount of samples available for analysis, because there is no amplification step for protein analysis [5]. Electrophoresis of cell samples in 2-D PAGE first separates individual proteins by charge, and then by size. Proteins have a distinct advantage compared to RNA; they are much more stable and this disparity has important consequences for the measurement of these molecules in biological materials, especially in clinical samples.

Cellular heterogeneity can affect and limit proteomic analysis in the same way as it does in DNA and RNA analysis. All of the current techniques require tissue homogenization and hence do not account for the cell of origin contributing the measured protein content. HICK has been used for a long time to identify cell-specific protein expression. However, there still remains to be resolved issue of artifacts secondary to fixation technique, antigen-antibody affinity, extent of antigen retrieval and absence of a reliable quantification protocol.

Recently, LCM has been applied to the study of protein alterations in tumors and their preneoplastic lesions, which is an important step toward formulating treatment and intervention strategies [4, 7]. Prostate specific antigen (PSA) levels in microdissected benign and malignant tissues can be measured with a rapid, sensitive and quantitative chemo-luminescent assay. This quantitative technique can potentially be extended to analyze a large variety of proteins in pure populations of normal and tumor cells. More than 600 proteins or their isoforms from a sample of about 50,000 microdissected cells can be resolved in 2-D PAGE and the dysregulated products in cancer cells identified. Novel tumor specific alterations can be identified by sequencing of the altered peptide products in cancer cells [7]. Based on the chemical characteristics of proteins (i.e. hydrophobic, hydrophilic, cationic, anionic) or biochemical ligands such as receptors, antibodies or oligonucleotides, it is possible to apply proteins of interest directly to the surface where they can be analyzed by SELDI time-of-flight (TOF) mass spectrometry. Using SELDI biochip, Paweletz *et al* have successfully classified protein population into molecular weight classes and shown distinct protein expression patterns of normal, premalignant and malignant cancer cells procured by LCM from human tissues [4].

Application in Diagnostics

Sporadic gene mutations in tumor often correlate with prognosis and/or therapeutic response. Efficient detection of gene mutations is becoming increasingly important in the pathological diagnosis, classification and treatment of tumors. The current detection method, however, is labor intensive, a major barrier of tumor mutational analysis for routine clinical use. LCM plays a major role in this area because captured tumor tissue is enriched in tumor-associated genetic alterations prior to molecular analysis, which eliminates the time consuming intermediate steps in mutational analysis, and thus allows more rapid and efficient tumor genotyping [63]. For example, the prostate gland is composed of epithelial and stromal cells. The normal epithelial component often represents only about 10% of the entire prostate. Prostatic carcinomas (PCA) often grow in an infiltrative pattern, with individual tumor acini infiltrating through stroma and directly adjacent to benign prostatic epithelium. LCM was initially used to evaluate the genetic alterations in PCA. Lutchman *et al* analyzed dermatin, a cytoskeleton protein encoded by a gene on chromosome 8p21 [64]. Rubin *et al* studied loss of heterozygosity at 10q23, a region that has been associated with many tumors including glioblastoma multiforme, melanoma, endometrial carcinoma, and PCA [65, 68]. 10q23 is also the site of PTEN and MMAC which have been found to be mutated in prostate cancer cell lines, xenografts, and hormone refractory PCA tissue specimens.

Since pure tumor samples are very difficult to isolate, prostate tumor cell lines were initially used for gene expression assay. Vaarala *et al*

examined cDNA expression between androgen-dependant and androgen-independent LNCaP prostate tumor cell lines [67]. Bubendorf *et al* were one of the first researchers who investigated amplification of androgen receptor gene with FISH on tissue microarrays of PCA. They found that both *Myc* and androgen receptor genes were over expressed in hormone refractory PCA with respect to clinically localized hormone-sensitive PCA. They also found that syndecan-1 was prognostically significant in PCA [69].

LCM can also aid in the diagnosis of many dermatological diseases. Routine diagnosis of cutaneous B- or T-cell lymphomas is challenging. Yazdi *et al* have introduced a LCM-based clonality assay to overcome these diagnostic dilemmas [66]. Using this technique, the authors were able to determine whether clonal T-cell receptor (TCR) gene rearrangement obtained by PCR stems from lymphoma or some inflammatory skin diseases [66].

Cancer Chemoprevention

The so-called intermediate endpoint biomarker (IEB) can serve as a reliable surrogate at the target site to monitor success of chemoprevention of cancer. Selection of an IEB requires that there is a consistent association between the biomarker and cancer and that biomarker modulation should be observed after chemotherapeutic interventions. Examples of IEBs for cancer are PIN for prostate carcinoma, CIN for cervical carcinoma and adenomas for colorectal cancer. These biomarkers can be used to select high risk patients for cancer development, and for evaluation of the efficacy of novel chemopreventive agents, which can greatly decrease the cost of clinical trials. It has been shown that phenotypic biomarkers alone, such as dysplasia, are insufficient to evaluate efficacy of chemoprevention; genotypic changes in "normal" epithelia adjacent to dysplasia also need to be evaluated. For example, in the chemoprevention study of oral leukoplakia by Hong *et al,* 54% of cases showed regression of dysplastic changes following treatment with 13-cis-retinoic acid. However, as many as 50% of cases relapsed after cessation of drug therapy, showing persistence of the malignant clone. In a follow up chemoprevention trial, biopsy was taken again from oral premalignant lesions and the testing has shown that as 89% of patients with histological or clinical remission had persistence of LOH at the 9p21 (p16 locus) [70]. In a similar study, Mao *et al* have observed that years after active smoking cessation genetic changes similar to those in lung cancers can persist in histologically normal or mildly abnormal bronchial epithelium [71]. These data suggest that there exists a definite time lag between phenotypic and genotypic response in epithelial tissues. In the absence of molecular data to reinforce histological remission, it is likely that chemopreventive intervention may be prematurely terminated in many "at risk" patients.

Biomarker Discovery

LCM can be applied for biomarker discovery in multiple human tissue types and organ systems. LCM in combination with DNA transcriptome profiling has been successfully used to identify differentially expressed genes between the supragranular and infragraunlar cellular layers of human neocortex [72]. 6 of the 69 differentially expressed genes were found to show specificity to neuronal distribution [72], a finding important for the understanding of brain structure and function in the cortical region. Demuth *et al* used LCM in brain biomarker research. In a so-called 3D spheroid *in vitro* invasion assay, they have evaluated the transcriptome of invasive glioma cells and their stationary equivalents [73]. LCM and whole genome expression microarrays, coupled with quantitative RT-PCR revealed that the activity of MAPK3, a key activator of p38 in glioma, and p38 were strongly correlated in their study set. The activity of MAPK3 and p38 could be decreased by inhibition of either MAPK3 or p38, suggesting potential treatment targets for glioma. With the help of LCM, Buckanovich *et al* isolated RNA from frozen or formalin-fixed paraffin-embedded breast tissue sections and identified a set of 12 novel ovarian cancer biomarkers termed as tumor vascular markers, which were distinct in comparison with vascular markers from either normal ovary or other tumor types [2, 74].

Other areas where LCM has also been used include evaluation of tumor microenvironment, forensic analysis of fixed cell samples and hair follicles, studies in developmental biology and embryology, animal model xenografting, infectious disease biology, plant cell biology,

spermatogenesis [1, 2].

Conclusions

The profound impact of molecular profiling on biomedical research and disease management is already taking place. Biomedical research community at no previous time has been more poised for rapid discovery and application of discoveries toward improved patient care. Consequently, it is of the utmost importance that efforts in molecular profiling be maximized for accuracy and relevance.

For decades many questions in biomedical research have been waiting to be answered. The use of microdissection methods, optimal tissue fixation and database integration for molecular profiling will yield many answers to those questions. Several challenges in these areas must be addressed. Protocols that maximize biomolecule preservation without sacrificing histopathology, and rescue biomolecules from cross–linking must be developed and improved. The benefits of rapid and precise microdissection, such as those provided by LCM, must be actualized not only through research, but also in a diagnostic capacity. Besides microdissection, methods must be developed that provide high-throughout analysis of tissue without sacrificing information regarding spatial relationships. The next few years hold great promise for the use of molecular information in disease management, including design of optimal lower risk, patient tailored treatment.

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