Review

Microdissection Techniques for Cancer Analysis

(microdissection / cancer / microarray)

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Abstract. One difficulty in studying molecular changes of tumours has been the inability to isolate DNA and RNA from a homogeneous cell population. The combination of several new technologies should help overcome these hurdles. Microdissection is a technique for rapid and easy procurement of a pure cellular subpopulation away from its complex tissue milieu. Laser-assisted microdissection has recently been identified as a quick, simple and effective method by which microdissection of complex tissue specimens can be routinely performed for molecular analysis. With the advent of laser microdissection, cDNA libraries can be developed from pure cells obtained directly from stained neoplastic tissue, and microarrays of thousands of genes can now be used to examine gene expression in microdissected tumour tissue samples. This review will concentrate on the application of different microdissection techniques in the area of cancer research.

There have been dramatic advances in the last two decades in our understanding of the molecular processes involved in cancer. This understanding has revealed numbers of exciting new targets for the development of effective therapies, some of which have already entered clinical practice. There is a pressing need to integrate this knowledge with structural and architectural data derived from conventional morphological approaches (Surivatanauksorn et al., 1999). Molecular analysis of cells in their native tissue environment provides the most accurate picture of the *in vivo* disease state. However, accomplishing this goal is more difficult than just grinding up a piece of tissue and applying the extracted molecules to a panel of assay.

Tissues are complicated three-dimensional structures, composed of a large number of different types of interacting cell populations. The cell subpopulations of interest might constitute a tiny fraction of the total tissue volume. For example, a biopsy of breast tissue harbouring a malignant tumour usually contains the following types of cell populations: 1) fat cells in the adipose tissue surrounding the ducts; 2) normal epithelium and myoepithelial cells; 3) fibroblasts and endothelial cells in the stroma and blood vessels; 4) premalignant carcinoma cells in the "*in situ*" lesions and 5) clusters of invasive carcinoma. Analysis of a tumour or preneoplastic genomic alteration can, therefore, be compromised by the presence of surrounding normal cells. If the goal is to analyse the genetic changes of premalignant cells, this subpopulation usually occupies less than 5% of the tissue volume (Simone et al., 1998). The accurate analysis of molecular changes associated with tumours and their precursor lesions requires precise isolation of the specific cell types from a heterogeneous background of non-neoplastic elements such as normal epithelium, desmoplastic stroma, inflammatory cells and blood vessels (Dean-Clower et al., 1997).

Culturing the cell population is one approach to reducing contamination. However, cultured cells might not accurately represent the molecular events taking place in the actual tissue they were derived from. The cell culture condition can never duplicate the environment of the cells in the actual tissue from which they were derived. Cell sorting techniques have also been used as a means of cell selection. They can be easily applied to tumours amenable to the formation of a suspension but cell sorting techniques are rarely applicable in solid tissue, in which intercellular adhesion prevents the disaggregation of cells. The problem of cellular heterogeneity has thus been a significant barrier to the molecular analysis of normal and diseased tissue. It is obvious that the selective isolation of tumour cells and their precursor lesions requires a technique by which the cells of interest can be isolated from the primary lesion itself, without any intervening step. This problem can now be overcome by new developments in the field of tissue microdissection.

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Abbreviations: LCM – laser capture microdissection, LMM – laser microbeam microdissection, LPC – laser pressure catapulting.

Microdissection Techniques

Tissue microdissection techniques enable the isolation of specific subpopulations from a diverse background of cell types, usually obtained under direct visual inspection. Microdissection techniques can be divided into three categories: 1) selective ablation of unwanted regions; 2) manual extraction of desired foci and 3) use of laser pulses to capture cells of interest.

The first category requires the use of an ablative technology, such as ultraviolet radiation, to destroy the unwanted regions, and the islands of tissue that are left behind are manually scraped off for molecular analysis. While ablative technologies are readily applicable to formalin-fixed archival material and can be used to isolate microscopic lesions with reasonable precision, the surrounding tissue is by definition unsuitable for PCR and cannot be reused.

The early microdissection techniques, involving manual or micromanipulator guidance of the needle to scrap off the cells of interest under a microscope, have been applied for some years to isolate a few hundred cells from heterogeneous tissue samples. One of the earliest examples of this technique was described by Goelz et al. (1985), who removed areas of interest from the paraffin block itself for molecular analysis, using before and after histological section to assess the purity of the DNA extraction. Manual methods of microdissection allow the extraction of DNA from lesion less than 1 mm in size under direct inspection (Zhuang et al., 1995). Since the number of premalignant cells is usually small, this method is not suitable for premalignant lesion analysis. Precision, avoidance of contamination and efficiency of the procedure are the most important parameters in tissue microdissection. Manual methods are rather tedious, operator-dependent and require considerable manual dexterity to prevent contamination with unwanted surrounding tissue elements.

The most recent advance in the field of microdissection has been the advent of laser-based microdissection techniques. Laser-assisted microdissection has been developed to procure precisely the cells of interest in a tissue specimen, in a rapid and practical manner. The current prototypes of these techniques include laser microbeam microdissection (LMM) coupled with laser pressure catapulting (LPC) and laser capture microdissection (LCM) (Fig. 1).

The LMM with LPC technique uses tissue that has been mounted on a 6 μm polyethylene membrane and placed on a glass slide, onto which the operator directs an ultraviolet laser beam under direct visualization, by a light microscope. The membrane supports the section and is of good optical quality, non-stretchable and it sinks in extraction buffers (Bohm et al., 1997). The laser beam burns the rim of the membrane and ablates the underlying unwanted tissue around the area of interest, leaving the desired cell population intact. The latter is then isolated by catapulting under pressure onto an overhanging cap (Figs. 2A, B, C). A great advantage is the well-preserved morphology of the transferred cells, which can be readily visualized under the microscope (Figs. 2D, E). Laser isolation and cell pick-up procedures are quick and easy to perform.

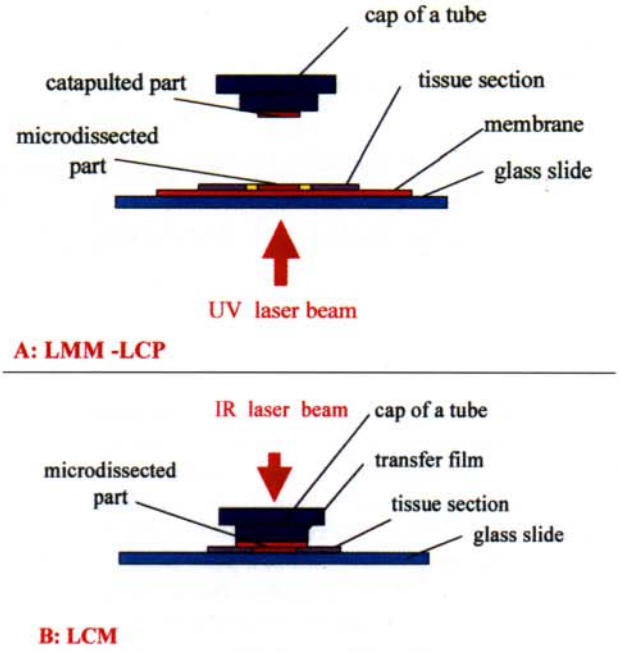

Fig. 1. Principle of A: LMM coupled with LPC and B: LCM, IR – infrared, UV – ultraviolet.

The LCM technique uses transparent ethylene vinyl acetate thermoplastic transfer film containing a near-infrared absorbing dye, attached to a 6 mm-diameter rigid, flat cap that is placed in contact with the tissue. An infrared laser, focused to the size of the desired target, melts the film directly above the target cells. The selected cells become adherent to the melted film on the cap, which absorbs the energy of the laser pulse. Removal of the cap from the tissue section effectively procures the targeted cells. The cap is placed directly into a fitted tube containing the buffer for molecular processing (Pappalardo et al., 1998). Up to 3000–5000 cells can be isolated onto a single cap in this fashion. The greatest advantage of this method is that it avoids any intricate operator-dependent step and the procurement of the material is in a non-contact manner, which minimizes the risk of contamination.

To positively identify the specific cells desired for microdissection, histochemical fixation must be used to preserve the tissue morphology. In general, existing methods of tissue processing focus mainly on morphological preservation for pathological diagnosis, not molecular analysis. Procedures that involve manipulation of tissue, including fixation, dehydration, clearing, embedding, staining and sectioning are all factors that could adversely affect the quality of the desired

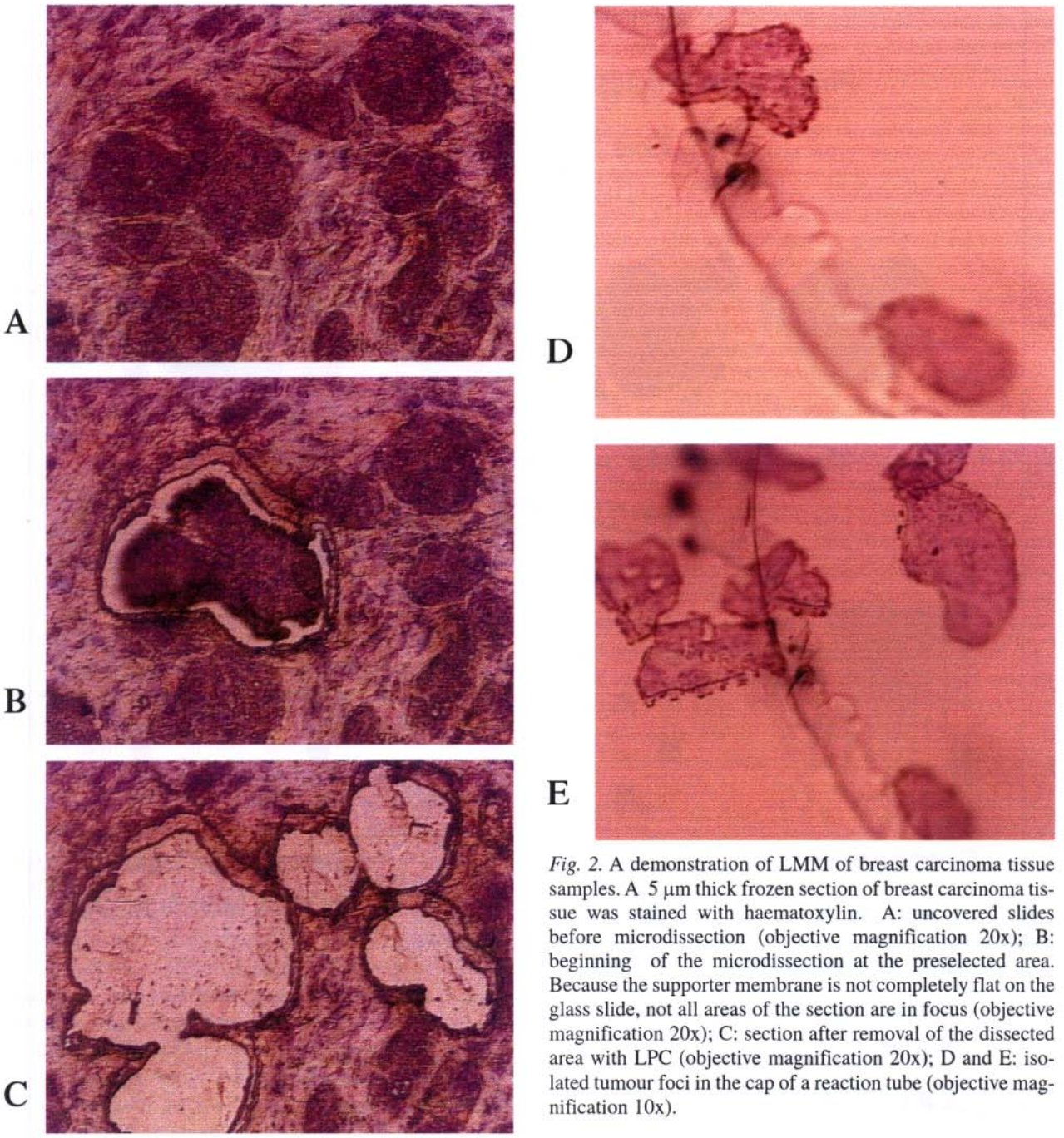

Fig. 2. A demonstration of LMM of breast carcinoma tissue samples. A 5 μm thick frozen section of breast carcinoma tissue was stained with haematoxylin. A: uncovered slides before microdissection (objective magnification 20x); B: beginning of the microdissection at the preselected area. Because the supporter membrane is not completely flat on the glass slide, not all areas of the section are in focus (objective magnification 20x); C: section after removal of the dissected area with LPC (objective magnification 20x); D and E: isolated tumour foci in the cap of a reaction tube (objective magnification 10x).

macromolecules (Goldsworthy et al., 1999). Although the morphology of frozen sections is not optimal, snap-frozen tissue, cryostat-sectioned and mounted onto glass slides, is suitable for laser-microdissection techniques (Serth et al., 2000). Unfortunately, the surgical pathology archives of most institutions contain paraffin-embedded tissue that has been fixed in neutral buffered formalin. Formalin fixation causes widespread cross-linkage between nucleic acids and protein, which often interferes with polymerase chain reaction (PCR) amplification. The DNA extracted from such tissue is extensively fragmented, varying in size from 100–200 bp, and usually limits the size of the PCR-derived amplification. For DNA analysis 50–100 sectioned cells are required per PCR reaction.

The microdissection procedure for RNA isolation is more intensive compared to that for DNA since a large number of cells are required. Most of the recent work on RNA analysis from microdissected tissue has been performed on frozen sections. RT-PCR for the detection of single analytes has been successfully performed on single cells extracted by laser-assisted microdissection (Schutze and Lahr, 1998). However, for *in vivo* analysis of gene expression using microarrays, the limited quantity and quality of RNA isolates using laser-assisted microdissection continues to be a technical obstacle.

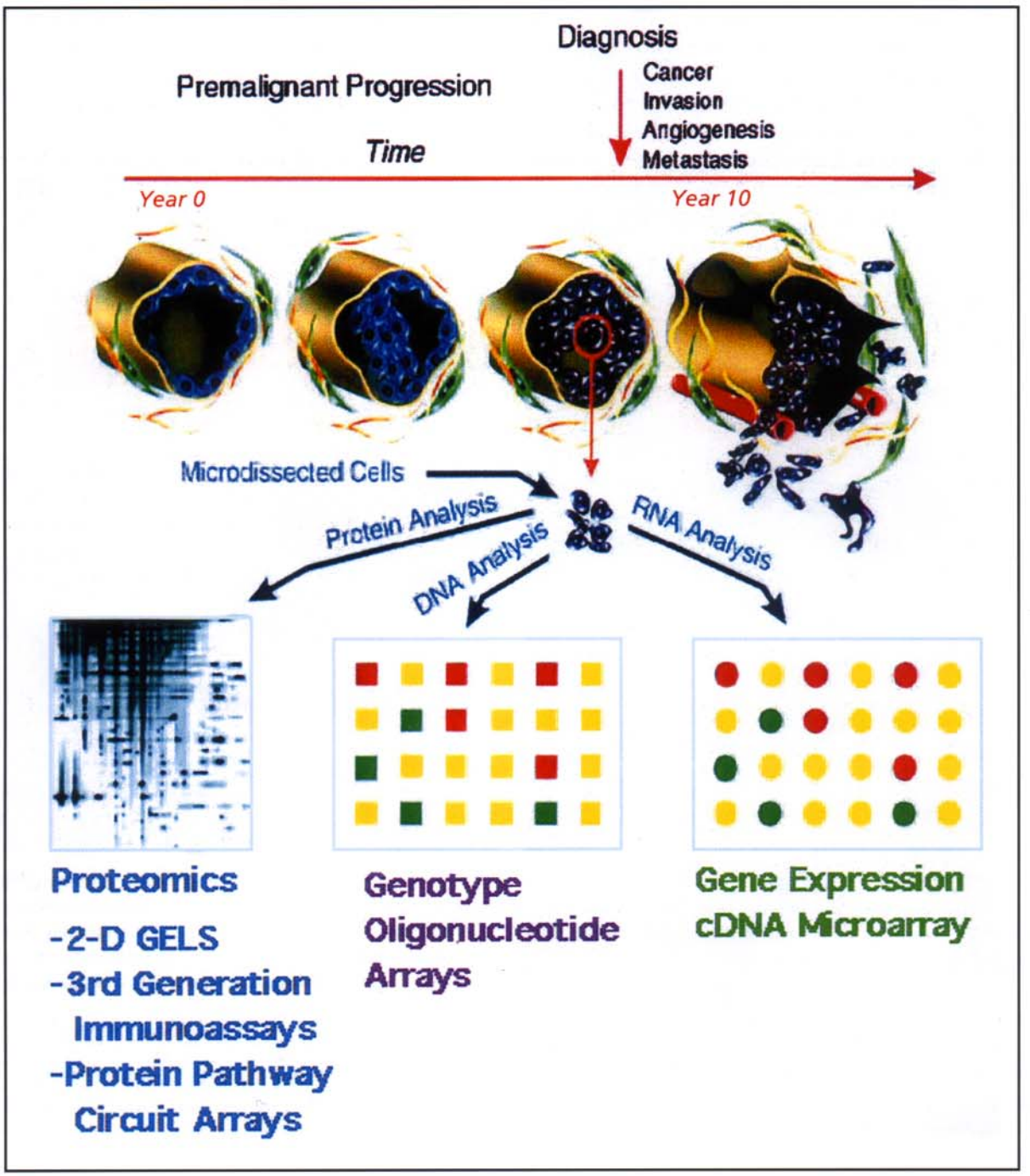

Fig. 3. Application of laser microdissection for studying the molecular events associated with cancer progression. The expression pattern of proteins, the DNA genotype or gene-expression pattern by RNA can be compared with the same tissue of a single patient. In the same tissue section, normal epithelium, stroma, premalignant lesions, invasive cancer foci and host response cells can all be sampled and compared (reprinted with permission of Dr. Michael R. Emmert-Buck from the National Cancer Institute Bethesda).

Microarray usually requires an input of 50–100 μg of total RNA, which can be difficult to obtain from microdissected tissue. A number of techniques have recently been presented for the amplification of small quantities of total RNA from laser-assisted microdissection material (Luo et al., 1999). However, a major challenge in this line of investigation still remains to be the ability to generate a sufficient amount and quality of the desired RNA from biopsied material (Ohyama et al., 2000; Baugh et al., 2001).

The slides used for microdissection are not coverslipped, which makes visualization fuzzy. Routinely stained frozen sections without coverslip as necessary for microdissection show greatly reduced cellular detail, which diminishes the ability to distinguish and isolate specific cell populations from complex lesions

with an intimate mixture of morphologically similar cell types. Immunohistochemical staining of tissue sections could help to identify and isolate specific cell populations, even of identical morphology, according to their antigen expression. Fend et al. (1999) developed a rapid immunostaining procedure for frozen sections followed by laser microdissection and RNA extraction. They reported that the RNA recovered from immunostained tissue was of high quality.

Molecular Analysis of Cancer Progression

Tumour development and progression are dynamic processes accompanied by accumulation of molecular genetic alterations. Genetic changes may involve amplification or gain of functional mutations in dominant oncogenes, or may involve loss of function by deletion, mutation or methylation in recessive tumour suppressor genes (Walch et al., 2000). To complicate the picture, accumulations of mutant genes in neoplasms tend to be accompanied by other genetic and epigenetic changes, including loss of heterozygosity or loss of imprinting genes and/or gene amplifications, all of which can alter gene expression profiles. Genome-wide monitoring of gene expression is therefore of great importance to disclose the numerous and diverse events associated with carcinogenesis (Kitahara et al., 2001). Before microdissection techniques, tumours were screened for purity of cancer cells, and valuable samples had to be discarded because the proportion of tumour cells was overwhelmed by lymphocytes or stromal cells. It is obviously an advantage to use microdissected cell samples in molecular analysis, because the confounding effect of contaminating cells is eliminated. In cancer, laser-assisted microdissection provides the capacity for isolating specific cells, including normal, precancerous, malignant and metastatic cells. Applied to cancer research, microdissection in conjunction with a variety of highly sensitive molecular techniques has the potential to assess genetic changes associated with each of the various morphological stages of tumour progression (Fig. 3). Sgroi et al. (1999) described that using carefully controlled conditions, *in vivo* subpopulations of malignant cells from multiple stages of cancer progression can be simultaneously screened for thousands of genes.

Traditionally, tumours have been categorized on the basis of histology. However, the staining pattern of cancer cells viewed under the microscope is insufficient to reflect the complicated underlying molecular events that drive the neoplastic process. Biomedical researchers are demanding more sophisticated platforms for studying the activity of many genes or proteins in parallel - an approach known as molecular profiling (Liotta and Petricion, 2000). Currently, some sophisticated and powerful technologies for molecular analysis include the use of microarrays. Within the last year, several publications have described the use of microarrays to profile differences in expression patterns between cancers and normal cells (Alon et al., 1999; Alizadeh et al., 2000; Luzzi et al., 2001). A distinctive feature discovered in all of these studies was that the same types of cancer from different individuals differed extensively in their gene expression patterns. In the case of B-cell lymphoma, the results were particularly dramatic in that the disease previously recognizable as a single entity by conventional pathology could be divided into two separate categories with different survival by molecular pathology (Alizadeh et al., 2000).

Using RNA and DNA arrays with appropriate experimental models, the ultimate goal is to move beyond correlation and classification to achieve new insights into tumour mechanisms and treatment targets. Gene profiling, in addition to contributing to the basic understanding of cancer cell biology, may also play a role in strategies for drug development. In the future it would appear that knowledge of the molecular profile of a tumour will be required prior to therapeutic intervention, so that more aggressive therapies can be tailored to the more aggressive tumours (Bertram, 2000). However, one of the conditions for this is the analysis of a homogeneous tumour cell population. Laser-assisted microdissection is therefore potentially one of the most useful techniques in molecular cancer research.

Conclusion

The new technologies becoming available for studying DNA, RNA and proteins will have an enormous impact in cancer research, and the ability to isolate homogeneous populations of cells with laser-assisted microdissection will add real value to these approaches.

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