CHAPTER 5.2

Complementary Techniques:
Laser Capture Microdissection—Increasing Specificity of Gene Expression Profiling of Cancer Specimens

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Abstract

Recent developments in sensitive genome characterization and quantitative gene expression analyses that permit precise molecular genetic fingerprinting of tumoral tissue are having a huge impact on cancer diagnostics. However, the significance of the data obtained with these techniques strictly depends on the composition of the biological sample to be analyzed and is greatly enhanced by including a preprocessing step that allows the researcher to distinguish and isolate selected cell populations from surrounding undesired material. This may represent a remarkable problem: indeed, genomic and proteomic analysis in the context of cancer investigation is susceptible to contamination by nonneoplastic cells, which can mask some tumor-specific alterations. Moreover, the heterogeneity of the tissues of a histological section, in which the cell population of interest may constitute only a small fraction, can represent an insurmountable difficulty for the use of quantitative techniques that absolutely depend on genomic material strictly derived from the cells that require analysis. This is obviously not possible if DNA or RNA is extracted from entire biopsies.

In the past, this obstacle was partially overcome by manual dissection from slides with a needle or scalpel; however, this method is feasible only if there is a clear demarcation between the tissue under consideration and its surroundings and moreover, allows only an approximate separation of tissues. The recent development of microdissection systems based on laser technology has largely solved this important problem.

Laser microdissection is a powerful tool for the isolation of specific cell populations (or single cells) from stained sections of both formalin-fixed, paraffin-embedded and frozen tissues, from cell cultures and even of a single chromosome within a metaphase cell. Resulting material is suitable for a wide range of downstream assays such LOH (loss of heterozygosity) studies, gene expression analysis at the mRNA level and a variety of proteomic approaches such as 2D gel analysis, reverse phase protein array and SELDI protein profiling. This chapter describes the characteristics of the most widely utilized laser microdissection systems and their current applications.

Microdissection Technologies: The Past and the Present

The shift from the concept of cellular pathology, formulated by the German pathologist Rudolf Virchow in the second half of the 19th century, to the current concept of molecular pathology, made possible by remarkable developments in the knowledge of the molecular

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processes involved in human disease achieved in recent years through molecular biology techniques, represents the latest of several revolutions that pathological anatomy has faced during its long history. It appears now clear that, in the future, the skills of the pathologist and those of the molecular biologist will have to be more integrated. Laser microdissection is, without doubt, a key technique in this perspective.

The need to isolate specific cellular types from complex tissues with the aim of carrying out accurate molecular assays has been argued for decades. Beginning in the 1970s, several papers have described different techniques to accomplish this task. They were based on the manual dissection (under microscope control) using razor blades, needles or fine glass pipettes to isolate the cells of interest from the rest of the section.[1-4] However, manual dissection is too time consuming and moreover, it does not allow precise control of the material effectively selected. In the last decade, attempts have therefore been made to standardize more efficient techniques. A significant technological advance in microdissection procedures was proposed in 1993 by Shibata.[5] He published a study which described a technique that relied on a negative selection of material (SURF: Selective Ultraviolet Radiation Fractionation): this technique used an UV laser beam in order to destroy the DNA of all the undesired components of the tissue, while the cells to be studied were protected from the action of the laser by a dye. Obviously, this technique was applicable only for molecular analysis of DNA.

Subsequent improvements led to the development of more sophisticated techniques, all based on a laser beam and able to isolate even one single cell, with the possibility to obtain DNA, RNA or proteins for molecular studies.

In 1996, Emmert-Buck and colleagues of the National Institutes of Health (NIH) in Bethesda, MD, introduced the LCM (Laser Capture Microdissection) system,[6] which was later commercialized by Arcturus Engineering as the PixCell System. Other companies subsequently developed new systems for laser microdissection, with various characteristics regarding the method to collect cells, the laser, etc. Today, the systems produced by Arcturus Engineering, P.A.L.M. Microlaser Technologies and Leica Microsystems are among the most popular. The following section outlines the functional characteristics of these three systems, with the reminder that they are continuously being updated (Fig. 1).

Arcturus System (PixCell)

The LCM system by Arcturus utilizes a low-power infrared laser to melt a special thermoplastic film (ethylene vinyl acetate membrane - EVA) on top of the cells to be isolated. A glass slide with the sectioned tissue is placed on the stage of the microscope and an area of interest is selected by the user on a computer screen. A custom-designed PCR- tube cap, coated with thermoplastic film, is then placed on the tissue section by means of a transport arm. The laser is then directed through the cap to melt the film onto the target cells. Pulsing the laser through the cap causes the thermoplastic film to form a thin protrusion that bridges the gap between the cap and the tissue and adheres to the targeted cells: in this way they are embedded by the polymer. The laser diameter can be adjusted from 7.5 to 30 μm so that individual cells, or an entire cluster of cells, can be selected. When the cap is lifted off the tissue section, the selected cells are attached and captured, ready to be transferred into a microfuge tube containing the appropriate extraction buffer for further analyses. The rest of the section remains intact and ready for further dissections. The morphology of the transferred cells is preserved and can be visualized under the microscope. The entire process is easily documented by means of a database program able to record images of both the area of interest and the dissected cells. This system has the unquestionable advantage of being able to use normal glass slides and therefore, theoretically, material prepared routinely for diagnostic purposes is also utilizable, obviously after removing the coverslip. A problem in common with all microdissection methods is its suboptimal microscopic visualisation because of the absence of a mounting medium and a coverslip. However, this shortcoming does not pose a problem when the identification of the cell types for microdissection is performed by an experienced pathologist.

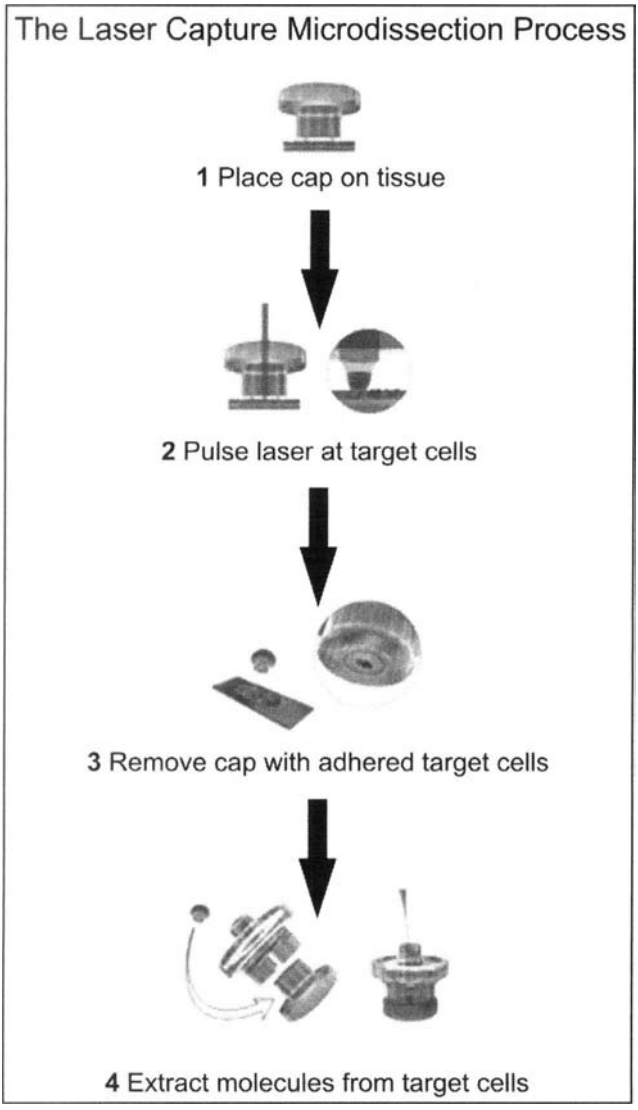

Figure 1A. Outline of the laser microdissection systems' characteristics: 1A) Arcturus Pixcell.

Arcturus has recently commercialized a new system (Veritas^{TM} microdissection) that combines the LCM system, based on infrared laser, with UV laser cutting.

(For more details, go to the following web site: www.arctur.com.)

P.A.L.M. System (MicroBeam)

The system of P.A.L.M. Microlaser Technologies is based on the Laser Microdissection and Pressure Catapulting (LMPC) technology. Mounted on an inverted microscope, the system selects the areas of interest in tissue sections mounted on a microscope slide coated by a Polyethylennaphtalate (PEN) membrane and catapults them into a collection tube by means of a pulsed ultra-violet (UV-A, 337 nm wavelength) laser. This laser is coupled with the

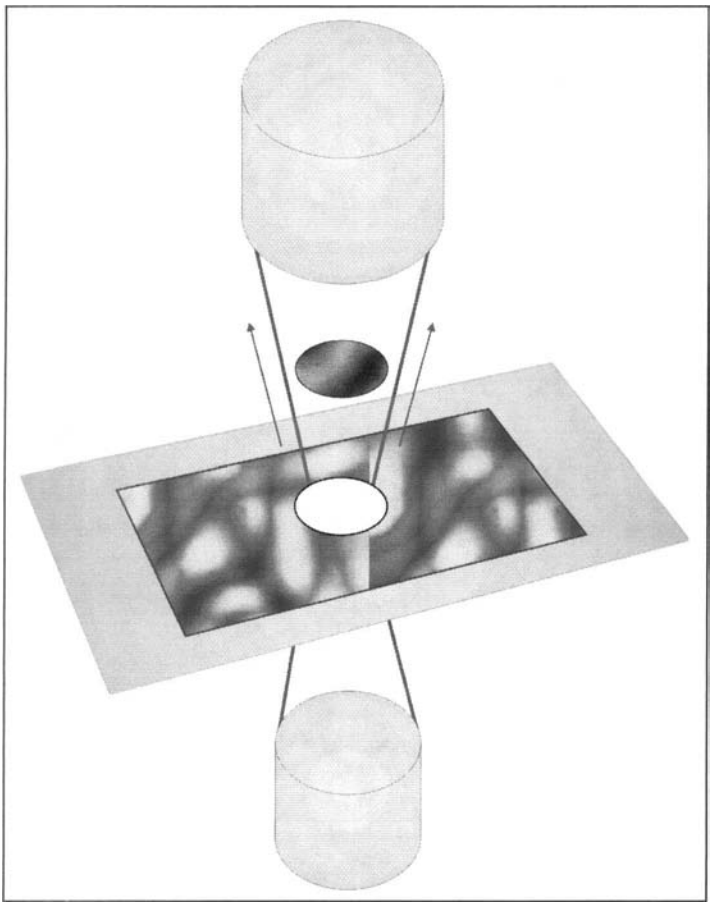

Figure 1B.Outline of the laser microdissection systems' characteristics: P.A.L.M. Laser microdissection and pressure catapulting (LMPC).

inverted microscope and focused through the objective lenses to a micron-sized spot diameter. The narrow laser focal spot allows the ablation of the material while the surrounding tissue remains fully intact. At the focal point, unwanted material is photo fragmented into molecules and atoms, a phenomenon called "cold ablation".[7] Photo ablation was first described by Srinivasan, who used the ablative forces of an excimer laser to ablate polymers.[8] He later employed the ablative photodecomposition device (APD) for the ablation of biological matter.[9] The focused laser leaves nothing behind that could be analyzed as a bio molecule. All the matter onto which the laser is focused is in the state of fragments of molecules, ions or other debris, cut into remnants of low molecular weight or even atoms. Since this cutting is a fast, photochemical process without heat transfer, the adjacent biological matter or bio molecules such as DNA, RNA and proteins are not affected. Moreover, the 337 nm nitrogen laser works within an UV-A range, where no damage of biological matter occurs. Therefore, these molecules can be isolated from the specimen for downstream analyses and applications. The noncontact capture of homogeneous tissue samples or individual cells is achieved by means of catapulting using P.A.L.M.'s patented Laser Pressure Catapulting technology. With the same laser, the separated cells, or the selected tissue area, can be directly catapulted into the cap of a common microfuge tube in an entirely noncontact procedure with the help of a single

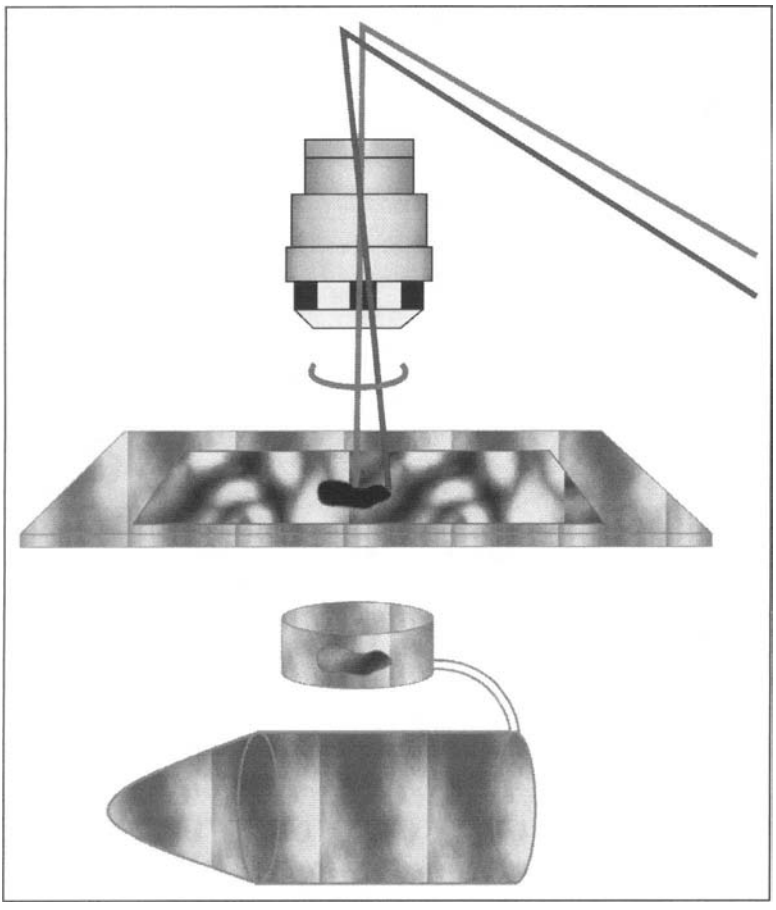

Figure 1C.Outline of the laser microdissection systems' characteristics: Leica AS LMD (modified from respective web sites).

defocused laser pulse. The sample is driven at high speed along the wave front of the powerful photonic stream and can be "beamed" several millimetres away, even against gravity.

(For more details, go to the following web site: www.palm-microlaser.com).

Leica System (AS LMD)

The Leica AS LMD Laser Microdissection System is based on an automated laboratory microscope integrated with an UV laser. Through this system, the tissue sections that are to be microdissected are mounted on polyethylennaphtalate (PEN)-foiled slides, which are microscope glass slides that support a thin plastic (PEN) film (cell culture samples are grown in special Petri dishes with a PEN surface). After selecting the area of interest on a computer screen, a pulsed UV-A laser (337.1 nm wavelength) cuts the plastic film by "cold ablation" along the drawn line and the excised section falls by gravity into a PCR-tube cap located directly beneath the slide. This technique avoids direct UV irradiation of the dissected cells (even if no interaction with DNA or RNA and UV radiation used would take place) or mechanical contact that could cause contamination. The result of the cutting can be easily checked by an automated inspection mode. To perform the cut, the laser beam moves over the specimen, with its direction along the cutting line controlled by two rotating prisms. In this

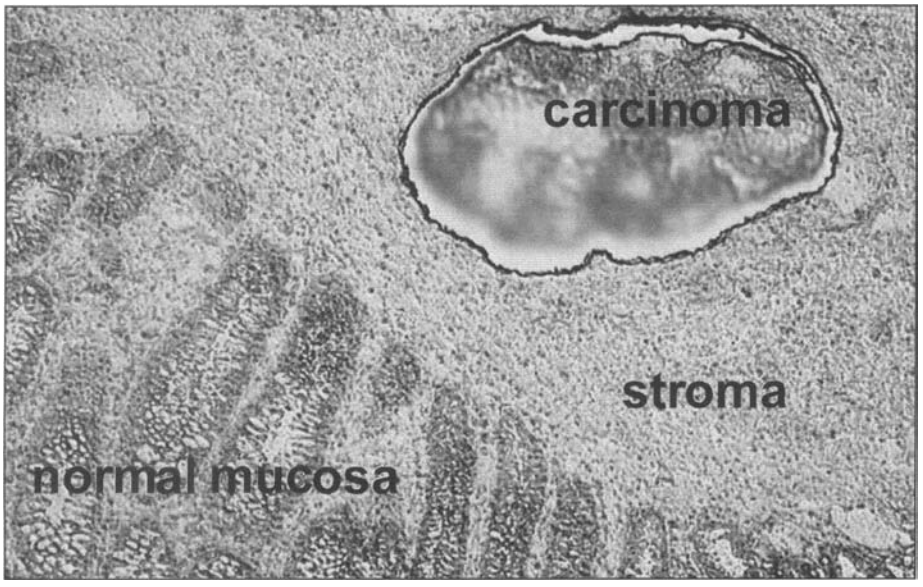

Figure 2A. Selective microdissection of neoplastic cells from a frozen section of primary colon cancer stained with haematoxylin (Leica AS LMD - original magnification 10X): the laser cuts along the line drawn by the operator, isolating the neoplastic cells.

way, the specimen remains stable so that it can be clearly observed during the cutting process. All steps are documented by means of an image archiving software. (For more details, go to the following web site: www.leica-microsystems.com).

All three systems are equally able to isolate living cells, can be used in fluorescence and allow creation of a database of archived images.

Why Microdissection?

The aim of tissue microdissection is to select and isolate single cells or groups of cells from a heterogeneous tissue sample in order to perform molecular analyses. The development of tissue microdissection techniques and the increasing interest towards them are a consequence of the refinement of PCR techniques which permit molecular analyses from very limited amounts of biological material, but require very pure preparations to avoid any risk of contamination. Microdissection techniques are useful in the analysis of heterogeneous tissues containing numerous cell types. For instance, a tumor sample is obviously constituted of tumor cells, but also of stromal cells (fibroblasts and endothelial cells), inflammatory cells and red blood cells; some tumors, e.g., pancreatic adenocarcinoma, in which a prominent desmoplastic reaction and often an evident lymphocytic infiltrate are observable, the number of tumor cells may actually be much lower than that of the noncancerous cells. Conventional techniques for molecular analyses based on whole tissue dissociation therefore introduce an initial contamination problem that reduces the specificity and sensitivity of the downstream molecular techniques, thus making the interpretation of the results more difficult. On the contrary, laser microdissection represents an ideal method for the extraction of cells from samples in which the exact morphology of both isolated cells and surrounding tissues is observable and preserved (Fig. 2). In this way, laser microdissection represents a very interesting technique in molecular pathology and creates a link between histology and molecular analysis.

Due to limits connected to sample preservation methods described below, tissue microdissection is currently more widely employed to analyze DNA than RNA or proteins, which are

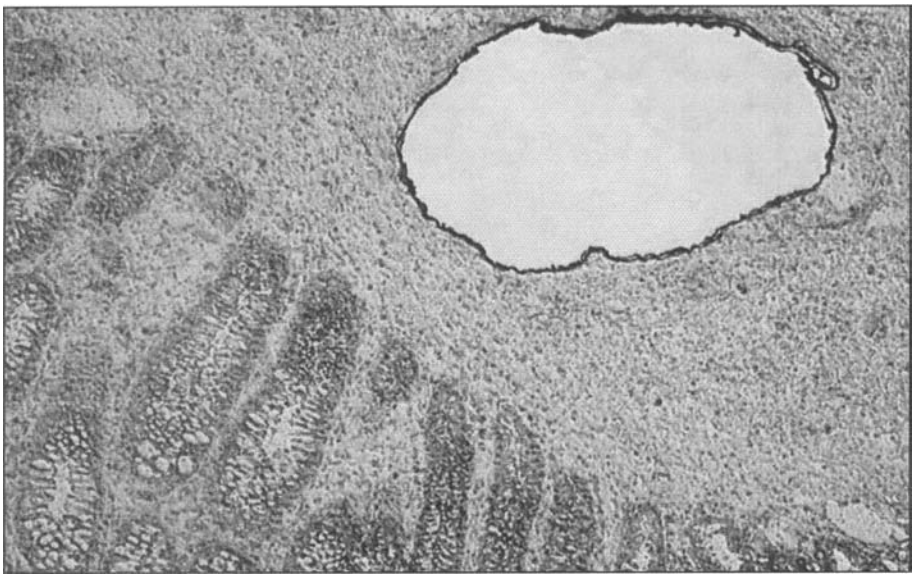

Figure 2B. Selective microdissection of neoplastic cells from a frozen section of primary colon cancer stained with haematoxylin (Leica AS LMD - original magnification 10X): the neoplastic cells fall into a PCR tube cap.

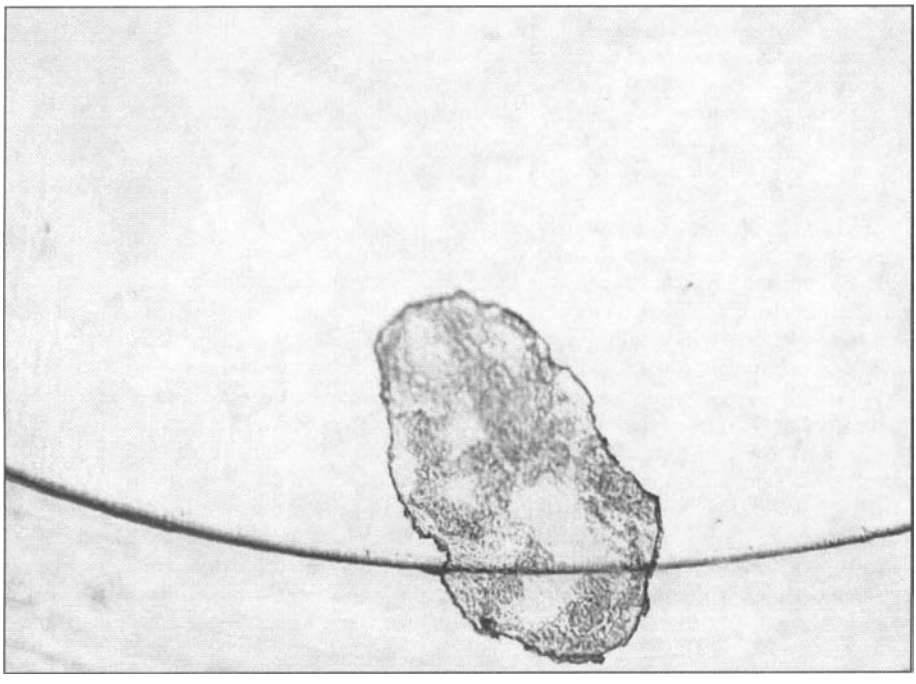

Figure 2C. Selective microdissection of neoplastic cells from a frozen section of primary colon cancer stained with haematoxylin (Leica AS LMD - original magnification 10X): inspection of the tube cap showing the isolated cells.

much more sensitive to degradation and fixation. However, tissue microdissected from carefully preserved frozen samples is suitable for protein analysis, and can also be employed for refined investigations of RNA expression using sensitive methods such as quantitative RT-PCR and microarray analysis.[10-18]

Microdissection is currently most commonly applied to analyze molecular alterations in tumors, with the majority of studies focused at the DNA level to detect loss of heterozygosity, microsatellite instability and the presence of mutations in tumor suppressor genes and oncogenes. In addition to enriching for tumor cells by eliminating surrounding stroma, microdissection permits comparison of distinct zones of tumor cells within a given lesion, the tumor cell population with neighboring normal cells and different stages of tumor progression coexisting in the same primary tumor sample (dysplasia, in situ carcinoma, invasive carcinoma) as well as metastases.

Laser microdissection can be applied to routinely formalin fixed - paraffin embedded tissues, frozen tissues, cytological preparations as well as cultured cells. Obviously, each one of these biological materials presents its own peculiarities and specific problems when dealing with the optimization of protocols upstream and downstream of microdissection.

In the case of histological preparations, it is certainly better to utilize samples that have been snap-frozen and stored in liquid nitrogen (or alternatively at -80°C). Formalin (4% buffered formaldehyde), the standard fixative routinely used in pathology laboratories, is an "additive" fixative that creates cross-links between itself and proteins and between nucleic acids and proteins. This can interfere with the recovery of nucleic acids and proteins, as well as with the amplification of DNA and RNA by PCR. As a consequence of these cross-links, the nucleic acids isolated from these specimens are highly fragmented, the extent of fragmentation mainly depending on the fixation conditions. This problem often occurs when using years-old archived material, especially since pathology laboratories did not pay much attention to fixation times in the past. In fact, the longer the fixation time, the stronger the cross-linking will become. The optimal fixation time in buffered formaldehyde solution is 24 hours. While fixation for up to 48 hours is still compatible with subsequent molecular analysis, soaking in formalin for more than 1 week destroys nucleic acids.

It is advisable to choose alcoholic rather than additive fixatives, as alcohols fix the tissues by dehydrating them but without creating chemical links; however, in the majority of laboratories, this is feasible only if microdissection is considered from the start as one of the possible options for processing the sample. Frozen sections obtained by cryostat cutting have the advantage of not undergoing cross-links due to fixatives but, on the other hand, show poor histological definition; not to mention that frozen material is not always easily obtainable.

For these reasons, it would be very important to find a standardized procedure that allows adequate extraction and eventual amplification of nucleic acids from routinely processed material. Some publications propose methods for this purpose. All these papers mainly deal with the problem of DNA fragmentation due to formalin fixation and the necessity for increased purity of the isolated nucleic acids. For instance, in a study of urinary bladder cancers and gliomas, Zhi-Ping Ren and coauthors[19] suggest that the key to successful DNA recovery is to completely digest all the proteins in the tissue sample. In their opinion, any leftover proteins associated with chromosomes would seriously affect the quality as well as the quantity of the DNA template. They underline the importance of strictly controlling the Proteinase K concentration and incubation time. The other side of this problem is the optimization of the PCR conditions. When dealing with formalin-fixed microdissected material, it is sometimes difficult to amplify the gene of interest using primer pairs that work very well for cell lines or fresh frozen materials. This is probably due to the fact that the DNA double helix has been broken into smaller fragments. This can be circumvented by employing a new set of primer pairs that amplifies a shorter fragment e.g., about 120 base pairs.

With these adjustments, the authors maintain that they were able to recover amplifiable DNA from virtually all investigated formalin-fixed, paraffin-embedded microdissected samples (99%).

The scenario regarding RNA extraction is quite different, as protocols that allow the use of RNA isolated from microdissected formalin-fixed paraffin-embedded cells require further improvements and validation.

The literature includes a few articles that describe RNA extraction from whole, fixed biopsies. Gloghini et al[20] published an interesting study that investigated whether RNA can be efficiently isolated from Bouin-fixed (a fixative that incorporates picric acid) or formalin-fixed lymphoid tissue specimens. Using a combination of Proteinase K digestion and column purification, they were able to obtain RNA that yielded accurate real time quantitative RT-PCR results.

Finally, it is important to remember that several companies have produced kits specifically devoted to the extraction of RNA from small amounts of material obtained by microdissection.

A product named RNAlater (Ambion - web site: www.ambion.com) is currently in wide use to improve RNA preservation in biological samples. RNAlater is an aqueous, nontoxic, tissue storage reagent that quickly permeates the tissues in order to stabilize and protect RNA in fresh specimens. RNAlater eliminates the need to immediately process or freeze the samples; the specimen can simply be submerged in RNAlater and stored for extended periods (up to 1 week at room temperature, 1 month at +4°C, indefinitely at -20°C) and thus allows the investigator to analyze the sample at a later time. While there is no doubt about the effectiveness of RNAlater in preserving nucleic acids from degradation, there are contrasting data about the product's effects on morphological preservation and subsequent microscopic observation.[21,22] In our experience, it is very difficult, if not impossible, to obtain satisfactory cryostat sections from some tissues, thus making RNAlater unsuitable for the preservation of samples destined to microdissection. Therefore, one needs to carefully choose the storage modality of the samples in connection with the type of analysis to be performed. As we have seen above, histological samples are routinely formalin fixed in clinical practice for diagnostic purposes. This procedure does not preserve DNA or RNA from degradation and cannot be used for proteomic analyses, since formalin extensively crosslinks proteins, thus preventing subsequent molecular studies.[23] Today pathologists understand the need to provide fresh tissue samples for research purposes, but the preservation method becomes of utmost importance in order to guarantee the feasibility of future molecular studies. The optimization of specific preservation methods compatible with the widest possible spectrum of assays on a given sample would be accelerated by the combined input of surgeons, pathologists and molecular biologists.

Slide Preparation for Microdissection

It is not the purpose of this chapter to provide a detailed description of technical procedures; the following are only indications about specific issues in slide preparation for laser microdissection.

The brochures provided by Arcturus, P.A.L.M. and Leica emphasise the possibility of utilizing routine standardized staining procedures, but recommend protocols characterised by very brief treatment times. In fact, in agreement with the rather obvious general rule that we have drawn—i.e., the least manipulation in the shortest time yields the best results—some adjustments are required in order to shorten the staining procedure, both for frozen and for fixed and embedded tissues.

If the material to be microdissected is destined for RNA extraction, care must be taken in order to create a ribonuclease (RNase)-free environment to avoid RNA degradation; RNases are ubiquitous, very stable and difficult to inactivate. Hand contact, laboratory glassware and dust particles are the most common sources of RNase contamination. To prevent contamination from these sources, it is necessary to wear powder-free gloves at all times when handling reagents and RNA samples and to sterilize glassware by heat. When dealing with frozen tissue, one must keep in mind that endogenous RNases may still be active even after short-time fixation phases. Therefore, it is advisable to keep all histochemistry incubation steps as short as possible. RNase-free water, solutions and ethanol series should be used. RNase-free solutions can be obtained by treatment with DEPC (diethylpyrocarbonate), which destroys the activity of RNases.

Another open issue regards the choice of the histological staining protocol. Ideally, staining should provide an acceptable morphology to allow the selection of target cells and without interfering with the macromolecules of interest or with the subsequent molecular techniques. A series of nuclear dyes have been examined but, up to now, they have not yielded univocal results. Ehrig et al[24] examined the effect of four dyes (methyl green, haematoxylin, toluidine blue O, azure B) on DNA extraction from fixed and frozen tissues. They concluded that DNA recovery from a microdissected tissue is not connected to the histological stain chosen. Burgemeister et al[25] compared haematoxylin/eosin, methylene blue, methyl green and nuclear fast red on frozen sections for RNA isolation. In their experience, the best results were achieved using methyl green and nuclear fast red stains; haematoxylin/eosin results were similar to nuclear fast red and methylene blue staining yielded partially degraded RNA. Okuducu et al[26] stained frozen sections from prostatic tissue with haematoxylin, methyl green, toluidine blue O and May-Grunwald in order to identify a reliable stain for RNA analysis. Results of real-time quantitative RT-PCR performed after laser microdissection showed that methyl green yielded more RT-PCR product than the other dyes. On the other hand, the main protocol provided by Leica suggests the use of haematoxylin but in an alternative protocol reports that there are indications of better PCR results when using methyl green or toluidine blue. Arcturus proposes its own kit, but does not specify the dye used. P.A.L.M. hosts customers' protocols on its website; for histological staining of frozen sections, a rapid haematoxylin stain is recommended.

Finally, haematoxylin and methyl green seem to have no effect on protein migration and therefore should be suitable for staining tissues to be microdissected for protein analyses.[27]

Sections prepared for microdissection are dehydrated and kept without a coverslip, which results in reduced cellular detail. This makes it hard to distinguish and isolate specific cell populations from lesions where morphologically similar cell types are strictly intermingled, such as lymphomas or carcinomas with a diffused growth pattern. Immunohistochemical staining of sections could help in identifying and isolating specific cell populations, even of identical morphology, according to their antigen expression, thus allowing a more precise microdissection. However, standard immunohistochemical staining protocols need several hours, which can lead to significant degradation of the macromolecules of interest, especially RNA by RNases activated in aqueous environments. In 1999, Fend et al[28,29] proposed a rapid immuno-staining procedure (total processing time from 12 to 25 minutes) for frozen sections followed by laser capture microdissection (LCM) and RNA extraction, which allows a targeted mRNA analysis of immunophenotypically defined cell populations. In 2000, Fink et al proposed the use of immunofluorescence applied to microdissection;[30,31] along this line of thought, a paper published by Burbach et al in 2004[32] described a rapid immunofluorescence staining approach combined with laser microdissection on frozen sections of mouse brain that does not interfere with RNA recovery and integrity for quantitative RT-PCR.

Another important issue concerns the number of cells that must be dissected. In the literature, this number ranges broadly, depending on the macromolecules to be analyzed, the methodology of their extraction (using "home-brewed" protocols or one of numerous commercial kits dedicated to extraction from small quantities of cells), the downstream bio molecular techniques adopted, fixed or frozen samples and last but not least, the operator's technical skill. When dealing with methods of linear RNA amplification, it is possible to perform gene expression profiling analyses even from a very limited number of cells, as the most critical parameters for the success of such an experiment seem to be the integrity and purity of the RNA.

In Our Laboratory

Our laboratory has direct experience with the Leica microdissection system, which is available in our Department. We have carried out a series of trials aimed at identifying the best conditions both for the conservation of the samples and to obtain an acceptable amount and quality of the extracted genomic material (DNA or RNA), which led us to introduce some modifications into the manufacturer's protocols. The following points concern the protocols that we now utilize in our laboratory.

Microdissection for DNA Extraction

DNA can be extracted from both frozen and formalin-fixed paraffin-embedded tissues. In the latter case, 4 μm microtome sections are obtained and mounted on polyethylennaphtalate (PEN)-foiled slides (Leica Microsystems). Immediately after slicing, the sections are placed at 60°C for 30 min, then de-paraffinazed in 3 histoclear baths (3 × 1 min), rehydrated in decreasing alcohols (100%, 95%, 70%, 50%, each for 30 seconds) and washed for 30 seconds in distilled water. They are then lightly stained with Mayer's haematoxylin (30 seconds), rapidly washed in tap water, stained with eosin for 30 seconds, rapidly washed in distilled water, dehydrated in increasing alcohols (70%, 95%, 100%, each for 30 seconds) and finally air dried for 10 minutes and microdissected at once.

Microdissection for RNA Extraction

In a cryostat set, snap-frozen specimens are anchored on cryostat supports using diethylpyrocarbonate (DEPC)-water (without OCT embedding) and sliced into 7-μm sections using a disposable blade. Immediately after slicing, the sections are fixed for 1 min in 70% alcohol, lightly stained with Mayer's haematoxylin (30 sec), washed in 2 DEPC-water baths (5 min each), dehydrated in increasing alcohols (80%, 95%, 100%), placed at 37°C for 30 min and then promptly microdissected. To suppress RNase activity, DEPC-water is also used for alcohol dilutions.

Another technical aspect concerns the possibility of storing slides at -20°C, or better at -80°C after the cryostat cut. This would allow the operator to perform the time-consuming microdissection at a later time or in more than one sitting, which would be especially helpful when a large number of small groups of cells need to be microdissected. To test this possibility, we prepared multiple sections from the same specimen and then stained and microdissected them either on the same day or after one day's storage at -80°C. Unfortunately, we found that the stored samples yielded much lower quantities of RNA compared to the freshly processed samples. Therefore, in our opinion, it is currently advisable to carry out all the phases of the microdissection process in the same day.

Conclusions

Laser microdissection is an extremely valuable tool for isolating and analyzing specific cell populations or subcellular material from sections of frozen tissues, paraffin embedded material, cytological preparations, living cells and even chromosome spreads. Coupled with state-of-the-art molecular analyses, the technique has already made a major contribution to studies aimed at understanding normal cell functions and at revealing the molecular changes underlying neoplastic progression. With anticipated improvements in preservation and staining protocols, laser microdissection should become even more valuable in the future.

References

1. Abeln EC, Smit VT, Wessels JW et al. Molecular genetic evidence for the conversion hypothesis of the origin of malignant mixed mullerian tumours. J Pathol 1997; 183:424-31.
2. Perren A, Roth J, Muletta-Feurer S et al. Clonal analysis of sporadic pancreatic endocrine tumours. J Pathol 1998; 186:363-71.
3. Saxena A, Alport EC, Custead S et al. Molecular analysis of clonality of sporadic angiomyolipoma. J Pathol 1999; 189:79-84.
4. Looijenga LH, Rosemberg C, van Gurp RH et al. Comparative genomic hybridization of microdissected samples from different stages in the development of a seminoma and a non-seminoma. J Pathol 2000; 191:187-92.
5. Shibata D. Selective ultraviolet radiation fractionation and polymerase chain reaction analysis of genetic alterations. Am J Pathol 1993; 143:1523-6.
6. Emmert-Buck MR, Bonner RF, Smith PD et al. Laser capture microdissection. Science 1996; 274:998-1001.
7. Vogel A, Venugopalan V. Mechanisms of pulsed laser ablation of biological tissues. Chem Rev 2003; 103:577-644.

8. Srinivasan R, Leigh WJ. Ablative photodecomposition: Action of far ultraviolet (193 nm) laser radiation on poly (ethyleneterephthalate) films. J Amer Chem Soc 1982; 104:6784-5.
9. Trokel SL, Srinivasan R, Braren B. Excimer laser surgery of the cornea. Am J Ophthalmol 1983; 96:710-5.
10. Di Cristofano C, Mrad K, Zavaglia K et al. Papillary lesions of the breast: A molecular progression? Breast Cancer Res Treat 2005; 90:71-6.
11. D'Arrigo A, Belluco C, Ambrosi A et al. Metastatic transcriptional pattern revealed by gene expression profiling in primary colorectal carcinoma. Int J Cancer 2005; 115:256-62.
12. Coco S, Defferrari R, Scaruffi P et al. Genome analysis and gene expression profiling of neuroblastoma and ganglioneuroblastoma reveal differences between neuroblastic and Schwannian stromal cells. J Pathol 2005; 207:346-357.
13. Burgemeister R. New aspects of laser microdissection in research and routine. J Histochem Cytochem 2005; 53:409-12.
14. Schneider-Stock R, Boltze C, Jaeger V et al. Significance of loss of heterozygosity of the RB1 gene during tumour progression in well-differentiated liposarcomas. J Pathol 2002; 197:654-60.
15. Sethi N, Palefsky J. Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection. FASEB J 2004; 18:1243-5.
16. Cowherd SM, Espina VA, Petricoin IIIrd EF et al. Proteomic analysis of human breast cancer tissue with laser-capture microdissection and reverse-phase protein microarrays. Clin Breast Cancer 2004; 5:385-92.
17. Sugiyama Y, Farrow B, Murillo C et al. Analysis of differential gene expression patterns in colon cancer and cancer stroma using microdissected tissues. Gastroenterology 2005; 128:480-6.
18. Ma XJ, Salunga R, Tuggle JT et al. Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci USA 2003; 100:5974-9.
19. Ren ZP, Sallstrom J, Sundstrom C et al. Recovering DNA and optimizing PCR conditions from microdissected formalin-fixed and paraffin-embedded materials. Pathobiology 2000; 68:215-7.
20. Gloghini A, Canal B, Klein U et al. RT-PCR analysis of RNA extracted from Bouin-fixed and paraffin-embedded lymphoid tissues. J Mol Diagn 2004; 6:290-6.
21. Florell SR, Coffin CM, Holden JA et al. Preservation of RNA for functional genomic studies: A multidisciplinary tumor bank protocol. Mod Pathol 2001; 14:116-28.
22. Roos-van Groningen MC, Eikmans M, Baelde HJ et al. Improvement of extraction and processing of RNA from renal biopsies. Kidney Int 2004; 65:97-105.
23. Kunz Jr GM, Chan DW. The use of laser capture microscopy in proteomics research—a review. Dis Markers 2004; 20:155-60.
24. Ehrig T, Abdulkadir SA, Dintzis SM et al. Quantitative amplification of genomic DNA from histological tissue sections after staining with nuclear dyes and laser capture microdissection. J Mol Diagn 2001; 3:22-5.
25. Burgemeister R, Gangnus R, Haar B et al. High quality RNA retrieved from samples obtained by using LMPC (laser microdissection and pressure catapulting) technology. Pathol Res Pract 2003; 199:431-6.
26. Okuducu AF, Janzen V, Hahne JC et al. Influence of histochemical stains on quantitative gene expression analysis after laser-assisted microdissection. Int J Mol Med 2003; 11:449-53.
27. Craven RA, Totty N, Harnden P et al. Laser capture microdissection and two-dimensional polyacrylamide gel electrophoresis: Evaluation of tissue preparation and sample limitations. Am J Pathol 2002; 160:815-22.
28. Fend F, Emmert-Buck MR, Chuaqui R et al. Immuno-LCM: Laser capture microdissection of immunostained frozen sections for mRNA analysis. Am J Pathol 1999; 154:61-6.
29. Fend F, Kremer M, Quintanilla-Martinez L. Laser capture microdissection: Methodical aspects and applications with emphasis on immuno-laser capture microdissection. Pathobiology 2000; 68:209-14.
30. Fink L, Kinfe T, Seeger W et al. Immunostaining for cell picking and real-time mRNA quantitation. Am J Pathol 2000; 157:1459-66.
31. Fink L, Kinfe T, Stein MM et al. Immunostaining and laser-assisted cell picking for mRNA analysis. Lab Invest 2000; 80:327-33.
32. Burbach GJ, Dehn D, Nagel B et al. Laser microdissection of immunolabeled astrocytes allows quantification of astrocytic gene expression. J Neurosci Methods 2004; 138:141-8.