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Laser Capture Microdissection: Methodical Aspects and Applications with Emphasis on Immuno-Laser Capture Microdissection

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Laser capture microdissection · Immunohistochemistry · RT-PCR · Gene expression · Polymerase chain reaction

Abstract

Laser capture microdissection (LCM) is an easy, extremely fast and versatile method for the isolation of morphologically defined cell populations from complex primary tissues for molecular analyses. However, the optical resolution is limited due to the use of dried sections without coverslip necessary for tissue capture, and routine stains such as hematoxylin and eosin are sometimes insufficient for precise microdissection, especially in tissues with diffuse intermingling of different cell types and lack of easily discernible architectural features. Therefore, several groups have adapted immunohistochemical staining techniques for LCM. In addition to providing high contrast targets for microdissection, immunostaining allows selection of cells not only according to morphological, but also phenotypical and functional criteria. In order to allow reliable tissue transfer on one hand and preserve the integrity of the target of analysis such as DNA, RNA and proteins on the other hand, immunostaining protocols have to be modified for the purposes of LCM. The following review gives an overview of immuno-LCM and describes some applications, e.g. in the field of hematopathology.

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Introduction

The molecular analysis of pathologically altered primary tissues has brought significant advances for our understanding of disease mechanisms and also resulted in the development of a whole array of new diagnostic tests with a significant impact on patient management and therapy. This is especially true of human neoplasms, and the advent of high-throughput analytical tools such as cDNA arrays will allow to establish individual molecular profiles of tumors, complementing and extending the diagnostic and prognostic information gained from conventional histopathological examination. However, surgically obtained specimens of tumors are a complex mixture of neoplastic cells and reactive cellular elements, and the reactive component frequently outnumbers the tumor cell population. Whereas some molecular tests, such as the detection of disease-specific chromosomal translocations by Southern blot or PCR, are moderately to highly sensitive and can detect a small minority of cells carrying the alteration in question, others such as the detection of loss of heterozygosity or identification of point mutations in oncogenes or tumor suppressor genes by direct sequencing of PCR products will yield false-negative results if contamination by reactive cells reaches a certain threshhold. On the RNA and protein level, an assignment of expressed genes and proteins to specific cell populations may be impossible if bulk tissue is used.

In the light of these problems, microdissection techniques were introduced as tools for obtaining homoge-

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neous cell populations from complex primary tissues [1–7]. Recently, the development of laser-based dissection technologies such as laser capture microdissection (LCM) or laser microbeam microdissection with laser pressure catapulting has resulted in a breakthrough in terms of speed, versatility and ease of use [8–13]. This transformed high-precision microdissection from a time-consuming technique restricted to highly skilled workers in devoted research labs into a simple, standard procedure easily applicable in any pathology laboratory.

However, one of the drawbacks of most laser-assisted dissection devices including LCM is the necessity for the use of dehydrated sections without coverslip, which leads to a significant decrease in optical resolution. Although the morphological details of routinely stained sections (e.g. hematoxylin-eosin, HE) are sufficient for many purposes, precise isolation of homogeneous cell populations from complex tissues lacking easily discernible architectural features such as lymphoma, inflammatory infiltrates or diffusely infiltrating carcinomas can be virtually impossible. Immunohistochemical and cytochemical stains potentially are an important path to circumvent this problem, since they can render easily discernible, high-contrast targets. Furthermore, they could allow the isolation of cell populations according to phenotypic and functional criteria, complementing and expanding morphology. However, the requirements of LCM on one hand and the desire for optimal preservation of the target of analysis (DNA, RNA or protein) on the other sometimes require significant adaptations of conventional immunohistochemical staining techniques.

DNA Analysis

DNA is currently still the most frequent substrate for molecular examinations in pathology. Although high molecular weight DNA can only be obtained from fresh or frozen tissue and nucleic acids are fragmented to a significant degree in conventionally formalin-fixed, paraffin-embedded tissue, these archival sources can still be used for a wide range of PCR-based tests. In our experience and that of others, conventional immunostaining protocols for paraffin-embedded tissues do not significantly influence DNA quality or tissue capture by LCM and can be used without major modifications [14]. Already before the introduction of laser-assisted microdissection, immunostains or nonradioactive in situ hybridization have been used successfully to identify and recover target cells from tissue sections for subsequent molecular analysis [15]. An example is the isolation of the neoplastic Reed-Sternberg cells of Hodgkin's disease by micromanipulation of immunostained single cells. The amplification of identical, clonal immunoglobulin gene rearrangements from multiple single cells dissected from frozen sections finally confirmed their derivation from germinal center B cells with the exception of rare cases of T cell origin [16–18].

The application of no-touch laser-assisted microdissection techniques for single-cell procurement should result in a significant reduction of dissection time without compromising dissection precision. Although it was not primarily designed for single cell capture, LCM can be used to pool larger numbers of single cells on one dissection cap for subsequent analysis, in contrast to the cell-by-cell technique used with micromanipulation [19–21]. We have recently used this approach for the analysis of the clonal relationship between the neoplastic cells of Hodgkin's disease and a cutaneous T cell lymphoma arising in the same patient. Cloning and sequencing of PCR products obtained from different groups of RS cells identified by CD30 immunostaining revealed identical Ig heavy chain gene rearrangements in all PCR reactions, confirming them to be clonal B cells unrelated to the neoplastic T cell clone [22]. We used the same strategy to analyze the clonality of EBV-infected, B cell marker-positive RS cells arising in the background of a peripheral T cell lymphoma. The presence of multiple bands of different sizes obtained from groups of immunostained RS cells indicated that this phenomenon represents an expansion of EBV-infected reactive B cells rather than a true composite lymphoma [21].

The analysis of groups of isolated cells can save time and cost by significantly reducing the amount of necessary PCR reactions. Although it carries a higher risk of contamination by unwanted cells, we are confident that the pooling of single cells by LCM is of sufficient precision for most analyses. In addition, it may have further advantages by reducing artifacts due to sectioned nuclei or excessive numbers of amplification cycles.

Furthermore, recent technical developments for LCM such as cap surfaces not in contact with the tissue section, special extraction chambers for small fluid volumes or the membrane-covered, rotating cone replacing the conventional LCM cap developed by Suarez-Quian et al. [23] will further improve the precision of single cell microdissection.

Apart from Hodgkin's disease and related disorders, both normal and neoplastic lymphoid tissues in general are good examples for the necessity of phenotype-based microdissection, since they usually contain an intricate

mixture of different subsets of lymphocytes lacking discriminating morphological features. We used LCM of immunostained paraffin sections to examine clonality in rare cases of composite B cell lymphomas showing two morphologically and phenotypically distinct cell populations [14]. PCR amplification of rearranged immunoglobulin genes was performed on DNA obtained both from gross tissue as well as on the two distinct cell populations microdissected from immunostained tissue sections. Whereas gross tissue-derived DNA showed a single band in all cases, PCR of the microdissected specimens amplified two distinct bands derived from two unrelated B cell clones as confirmed by sequencing. Since both populations were present in the gross tissue in roughly equivalent amounts, one would expect to be able to amplify both rearrangements simultaneously with the consensus primers used in this study. However, preferential amplification of one clone occurred in gross tissue extracts, possibly due to different priming efficiencies, wrongly suggesting monoclonality. These experiences indicate that microdissection to obtain purer cell populations may also be beneficial for analyses in which the percentage of the target population may seem high enough for conventional examination of bulk tissue.

In addition to the analysis of single or few genes by PCR, DNA obtained from microdissected paraffin-embedded tissue sections can be used for genome-wide screening techniques such as comparative genomic hybridization or genome-wide loss of heterozygosity screening, after a step of whole genome amplification if necessary [24, 25]. Further studies are needed to confirm the representativity of the amplified material in comparison to native DNA when a preanalysis random amplification step is used, and to establish the optimal conditions and the minimal amount of cells needed for reproducible results.

Analysis of Gene Expression

The establishment of gene expression signatures for different normal and pathologically altered tissues will enhance our abilities to understand and classify human disease and might provide us with tools for better prognostication and more refined, individually tailored treatments. However, assignment of expressed genes to the various cell populations present in heterogeneous primary tissues can be difficult or impossible, and quantification of RNA expression frequently is strongly influenced by the variable prevalence of the target population (e.g. tumor cells). Confirmation by in situ techniques such as immunohistochemistry or in situ hybridization may not always be possible, especially for low abundance transcripts, and is cumbersome and time-consuming when a large number of transcripts have to be examined. Therefore, many groups have tried to develop microdissection protocols that yield RNA of sufficient quality for downstream applications such as RT-PCR, expression library construction and cDNA array hybridization [9, 11, 13, 19, 26–37]. However, mRNA extraction poses more stringent requirements to tissue preservation and handling due to its higher sensitivity to fixation and degradation by ubiquitously present RNases unless stringent RNase-free conditions are observed. Nevertheless, several groups have demonstrated that microdissected frozen tissue can render good quality RNA from microdissected frozen tissue. Laser-based techniques have the advantage of performing the microdissection on completely dehydrated tissue sections or cell preparations, thus probably blocking endogenous RNases, and being significantly faster than manual or micromanipulation-based approaches. Cells isolated by LCM or laser microbeam microdissection are suitable mRNA sources for quantitative real-time PCR, both fluorescent or radioactively labelled probes for cDNA array hybridization, or expression library construction [19, 27, 30, 31, 34, 35, 38]. Linear amplification with T7 RNA polymerase has been shown to generate sufficient template for cDNA array hybridization [19]. As many as 5,000 cells are sufficient for generating reproducible results with radioactively labelled probes hybridized to commercial nylon filter arrays [32]. Although fresh frozen tissue remains the source of choice for RNA extraction, paraffin-embedded, formalin-fixed tissue can be used for certain applications such as RT-PCR, if the size of the chosen amplicons is small. Using a sensitive nested RT-PCR approach, Schütze and Lahr [11] have amplified expressed gene fragments from single cells microdissected from paraffin tissue sections. However, using such small amounts of starting material, the potential for technical artifacts caused by sectioning, contamination through fragments of attached cells and the high numbers of amplification cycles has to be kept in mind. Specht et al. [39] recently demonstrated the feasibility of quantitative real-time RT-PCR from microdissected formalin-fixed, paraffin-embedded tissue sections. Highly reproducible results were obtained with small amplicon sizes down to a level of approximately 2,000 cells, with an increasing variation below that number.

For visualization of target cell populations sufficient for many types of tissues, conventional stains such as HE are fast to perform, and do not lead to a major loss or

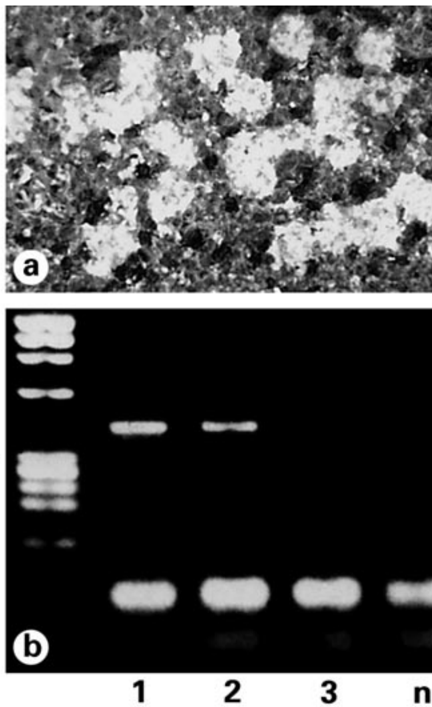

Fig. 1. a LCM of a frozen section of a reactive lymph node immunostained for CD3. The holes created by the LCM procedure are clearly visible. The immunostained T cells are left behind. **b** RT-PCR amplification of a 424-bp fragment of CD19 mRNA from a larger area of microdissected B cells after immuno-LCM. Lanes 1–3 show products with undiluted cDNA, a 1:5 dilution and a 1:25 dilution. N = Negative control.

RNA. If for reasons outlined above a higher level of optical resolution or identification of phenotypically diverse but morphologically similar cell types are desirable, immunolabelling can be applied.

In contrast to immunohistochemistry on paraffin sections, application of immunological staining techniques to frozen sections or cell preparations suitable for subsequent LCM requires a significant deviation from standard staining protocols and always results in a reduction of the RNA recovery compared to conventional stains. Reduction of staining times to less than 15 min in aqueous media and RNase-free conditions preserves sufficient high-quality RNA, allowing the amplification of cell-specific mRNA of more than 400 bp from captured cells with conventional single-step RT-PCR (fig. 1) [40]. Jin et al. [20] amplified specific mRNAs encoding for pituitary hormones from single immunostained cells isolated by LCM from cytospins with a sensitive nested RT-PCR. In addition to RNA recovery, a second crucial point for successful LCM from immunostained frozen sections is transfer efficiency, since frozen sections tend to adhere strongly to the glass slides, especially after drying steps. Good tissue transfer usually can be ensured by avoiding prolonged drying of sections, careful complete dehydration or pretreatment with glycerol [20, 40].

In order to further reduce the time of exposure to aqueous media and thus improve RNA recovery, Murakami et al. [41] have developed an ultrafast immunofluorescence staining technique which relies on the detection of weak fluorescent signals not visible by conventional means with the help of a very sensitive CCD camera. This makes possible staining times of 1 min, bringing them into the time span of conventional staining procedures.

If the problem of RNA recovery from immunostained sections can be resolved satisfactorily, gene expression can be correlated with phenotypic and functional properties of the examined cell population, such as proliferation, maturation stage and oncoprotein expression. Approaches such as RT-PCR or quantitative RT-PCR are probably more suited for partially degraded samples such as cells obtained by immuno-LCM rather than screening techniques like cDNA array hybridization, which are more likely to be influenced by partial degradation and bias introduced through template amplification.

Outlook

The spread of laser-assisted microdissection techniques will speed up identification of molecular alterations associated with human disease. The ability to separately analyze heterogeneous populations in complex primary tissues allows us to retrace the genetic evolution of neoplasms. The accumulation of genetic changes from metaplasia, to preinvasive changes and finally to invasive carcinoma can elegantly be demonstrated on microdissected tissue areas from a single case [7, 42].

The application of proteomics to microdissected tissues has opened a new bridge to 'molecular morphology'. The feasibility of two-dimensional gel electrophoresis, immunoblotting and immunoassays performed on cells obtained by LCM has been demonstrated by several groups [43–48]. The development of protocols to optimize recovery of nucleic acids and proteins from various microdissected sources, fast immunostaining methods for phenotype-directed microdissection and expression analysis and technical developments in terms of increased precision and automatization of laser-assisted microdissection will have a significant impact on tissue-based research and diagnostics.

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