Laser-Assisted Microdissection of Membrane-Mounted Paraffin Sections for Polymerase Chain Reaction Analysis

Identification of Cell Populations Using Immunohistochemistry and in Situ Hybridization

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Laser microbeam microdissection (LMM) is an increasingly important method for obtaining pure cell samples for genetic and proteomic analysis. Immunohistochemistry (IHC) and in situ hybridization (ISH) are useful techniques for targeting specific cell populations for microdissection but are difficult to apply with the tissue support membranes often used during LMM. Using detection of cytokeratins and Epstein-Barr virus gene products in head and neck carcinoma as a model, we describe optimized protocols for membrane and section preparation and for low temperature antigen retrieval that allow IHC and ISH to be used reliably on membrane mounted paraffin tissue sections. Visualization of cellular targets was markedly improved by staining and this could be further improved using a variety of optical media before microdissection. Tissue fragments thus stained were suitable for subsequent polymerase chain reaction analysis of extracted DNA using standard techniques. These IHC and ISH procedures are generally applicable and will be useful for detecting a wide range of antigens and nucleic acids in paraffin sections in conjunction with LMM. (*J Mol Diag* 2001, 3:105-110)

The molecular revolution has provided new tools for the analysis of the genetic processes that govern disease. However, the quality of data obtained using tests based on tissue extracts will often be dependent on the cellular homogeneity of the study samples. For example, the detection of loss of heterozygosity, and the use of comparative genomic hybridization and high-throughput DNA microarray and proteomic techniques in tumors are sen-

sitive to "contamination" by non-neoplastic cells, which may mask tumor specific alterations.¹⁻³ Pathologically altered tissues, and in particular, neoplastic tissues, are inherently complex and there is a need for techniques that make it possible to analyze separately subpopulations of cells from heterogeneous specimens. Laser-assisted microdissection (LAM) has emerged recently as a key methodology for this purpose. It enables precise and rapid procurement of homogeneous cell samples from both frozen and formalin-fixed, paraffin-embedded tissue sections and from cytological smears, suitable for cell-specific analysis of DNA, RNA and proteins.^{1,4-7} Two advanced systems are available for LAM. In laser capture microdissection (LCM), visually targeted tissue fragments are bonded to a thermoplastic membrane activated by a low energy near-infrared laser pulse.⁴ In contrast, laser microbeam microdissection (LMM) uses a pulsed ultraviolet (UV) narrow beam focus laser to cut out target cells, and to photoablate unwanted adjacent tissue.^{1,5} When using LMM, tissue sections are often mounted on a thin supporting polyethylene membrane that is cut together with the target tissue, so called microbeam microdissection of membrane-mounted native tissue (MOMeNT).¹ This facilitates dissection and transfer of large intact tissue fragments and reduces the risk of contamination of the target sample. The quality of data obtained using microdissection is heavily dependent on the precision with which target cells can be identified. Since no coverslip or mounting medium is used during LAM, cellular detail is poor and this may make it difficult to distinguish different cell types reliably by ordinary morphology in routinely stained sections. In these circum-

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stances, cell specific labeling using immunohistochemistry (IHC) or *in situ* hybridization (ISH) would greatly increase the precision with which particular cell populations could be sampled. IHC has been used in conjunction with LAM.^{7,8} However, there are considerable technical difficulties to be overcome when applying these methods and detailed protocols for IHC and ISH on membrane mounted sections have not been published. This combination of techniques gives rise to specific problems. Both membranes and tissue sections are easily damaged and may be completely lost during the staining process. Heat induced epitope retrieval (HIER) exacerbates these problems making it unsuitable for use, thus limiting the sensitivity of IHC possible in paraffin sections.

The aim of this study was to develop reliable protocols for performing IHC and ISH on membrane-mounted paraffin sections before LMM. As a model, we stained head and neck carcinomas, including cases of nasopharyngeal carcinoma (NPC) using labels specific for epithelial markers and for Epstein-Barr virus (EBV) to identify tumor islands. We removed these cells using microdissection, and we tested their suitability for performing subsequent polymerase chain reaction (PCR) analysis.

Materials and Methods

Tissues

For validation of the technique, routine formalin-fixed, paraffin-embedded blocks from seven carcinomas were selected from the archives of the Institute of Pathology, Aarhus. Four cases were undifferentiated NPCs; the other cases were non-keratinizing carcinomas from the tongue ($n = 1$) and tonsil ($n = 2$). A clear cell sarcoma of the kidney arising in a 4-year-old child was used as a negative tissue control for viral analyses. This tumor was chosen after screening by PCR, ISH, and IHC confirmed it to be negative for EBV genomes and gene products, and because adequate tissue was available for multiple experiments. Since the prevalence of EBV infection increases with age, a childhood tumor was selected to expedite identification of the EBV-negative control.

Sectioning, Immunohistochemistry, and *In Situ* Hybridization

Polyethylene membranes (1.5 μm ; SL-Microtest, Jena, Germany) were cut to size and mounted on non-charged Superfrost Color glass slides (Menzel-Glaser, Germany). To ensure wrinkle-free application, the glass slide was first dipped in 70% alcohol after which the pre-cut membrane was applied to the wet glass with help of a paper support. The paper was then removed and the membrane attached using Fixo gum rubber cement (SL-Microtest) along two opposing edges. Slides were sterilized in UV light overnight. Slides for IHC and ISH staining were treated with 8% and 2% 3-aminopropyltriethoxysilane (APES) solution in acetone, respectively. Serial 5- μm paraffin sections were cut with a fresh knife, mounted on prepared slides, and incubated at 60° C for 2 hours to

achieve better tissue adhesion to the membrane. Deparaffinization was carried out by prolonged incubation in xylene (4 $\times$ 7 minutes), followed by prolonged washing and rehydration in ethanol (99.9% ethanol 4 $\times$ 5 minutes, 96% ethanol 2 $\times$ 5 minutes, 70% ethanol 5 minutes). For IHC, antigen retrieval was achieved with HIER at low temperature (60° C) in TEG buffer (Tris-EGTA buffer, pH 9.0). Retrieval times of 12, 16, 24, and 48 hours were tested and optimized according to the antigen of interest. Primary mouse monoclonal antibodies to pancytokeratins (AE1/AE3, Roche A/S, Hvidovre, Denmark and KL1, Serotec, Oxford, UK), high molecular weight cytokeratin (CK-H; 34 β BE12, DAKO A/S, Glostrup, Denmark) and Epstein-Barr virus latent membrane protein LMP-1 (CS-1, DAKO A/S) were used for staining. Sections were stained with the avidin-biotin complex method (Vectastain ABC universal kit, Vector Laboratories, Inc., Burlingame, CA), according to the manufacturer. Briefly, slides were blocked with horse serum (5 minutes), incubated in a humid chamber for 60 minutes with primary antibody (AE1-3 1:250, CK-H 1:100, KL1 1:100, CS 1-4 1:2000), and for 30 minutes each with biotinylated secondary antibody and avidin-biotinylated horseradish peroxidase complex (ABC). Endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide (H_2O_2) in methanol for 10 minutes. Gentle intervening washing was carried out using TBS buffer (pH 7.6, 3 $\times$ 3 minutes) without shaking, and excess fluid was removed with a sterile gauze, care being taken not to touch the tissue sections. Color development was obtained with 3,3-diaminobenzidine-tetrahydrochloride (DAB, Kem-En-Tec A/S, Copenhagen, Denmark) for 10 minutes. Sections were counterstained in hematoxylin for 3 minutes, washed for 4 minutes in tap water, and dried in ethanol.

For CS 1-4 staining, avidin-biotin blocking was performed according to the manufacturer (DAKO Biotin Blocking System, DAKO A/S) to minimize background staining. Immunohistological signals were amplified by catalyzed reporter molecule deposition (CARD) with biotinylated tyramine.⁹ Biotinylated tyramine was produced as described by Adams.⁹ In brief, 31.2 mg tyramine hydrochloride (Sigma, St. Louis, MO) was dissolved in 40 ml of 50 mmol/L borate buffer (pH 8.0) containing 100 mg sulfo-NHS-LC-Biotin (Pierce, Rockford, IL), agitated overnight at room temperature, filtered through a 0.45- μm filter and stored as frozen aliquots. CARD amplification was performed by incubating the ABC-stained sections in biotinylated tyramine diluted 1:500 in 50 mmol/L Tris (pH 7.6) containing 0.03% H_2O_2 for 10 minutes. Following washing, sections were incubated in ABC for 30 minutes, washed, and developed in DAB.

For each stain, two sets of sections mounted directly on glass slides were processed in parallel as controls of the procedure. HIER was performed using a domestic microwave (3 $\times$ 5 minutes at maximum power) or a hot air oven (60° C), respectively.

For ISH, EBV-encoded small RNAs (EBER 1 and 2) were detected using an in house, digoxigenin-labeled, PCR generated single-stranded DNA probe (Zhou et al, submitted). Following deparaffinization, the tissue sections were transferred to TBS-DEPC (Tris-buffer, 0.1%

diethylpyrocarbonate, pH 7.6) and incubated at room temperature for 12 hours to wash out excess APES. Sections were treated with proteinase K (DAKO S3004 40 μ l/ml) and refixed in 0.4% paraformaldehyde in 0.1% DEPC. Twenty μ l of probe was applied to each section, distributed evenly using a cover glass and slides were incubated in a humid chamber for 90 minutes at 55°C. The probe was visualized by immunostaining using anti-digoxigenin 1:20000 (D1-22, Sigma-Aldrich Chemie) as the primary antibody and ABC as described.

Microdissection and Polymerase Chain Reaction

Microdissection of the membrane-mounted sections was performed with a high-resolution nitrogen UV-laser (SL-Microtest) at 337 nm. The dissected cells were captured using a microscope-mounted sterile 31gauge needle (Becton Dickinson, Heidelberg, Germany). Completeness of cutting and target removal were controlled visually. Approximately 1000 cells were cut after being identified by AE1-3, CK-H, CS 1-4, or EBER1-2, and for each stain the procedure was repeated three times in each case. The tip of the needle and the attached tissue fragment and membrane were broken off into a 0.2 ml Micro-Amp tube (PE Biosystems, Foster City, CA) containing 25 μ l of 0.28 mg/ml proteinase K solution, incubated for 3 hours at 55°C, and inactivated at 95°C for 10 minutes. PCR was performed on a PTC 200 Thermocycler (MJ Research, Waltham, MA) with a reaction mixture containing 5 μ l of template, 0.2 mM of each dNTP, 500 pmol of each primer, 1 U Biolase Diamond (Bioline, London, UK), 2 μ l of 10X PCR buffer (Bioline), 1 mmol/L MgCl₂, 1 mM cresol red (60% sucrose) loading buffer and distilled water up to 20 μ l. Analysis of the housekeeping gene nucleophosmine (NPM, 194 bp,¹⁰) was used to verify the quality of DNA with primers 5'-tcccttgggggcttgaataacacc and 3'-gctaccacctccaggggcaga. The PCR program consisted of 3 cycles of 95°C for 90 seconds, 55°C for 60 seconds, and 74°C for 120 seconds followed by additional 37 cycles of 95°C for 90 seconds, 50°C for 60 seconds, and 74°C for 120 seconds. A final extension step was carried out at 72°C for 10 minutes. EBV genome detection and subtyping was done by PCR with primers specific for EBV nuclear antigen-3C (EBNA-3C,¹¹); (5'-agaaggggagcgtgtgtgtgt and 3'-cggctgcagttttgctcgg. EBV type 1 gave a PCR 153-bp PCR product and EBV type 2 resulted in a 246-bp product. The PCR program consisted of 37 cycles of 94°C for 60 seconds, 56°C for 120 seconds, 72°C for 120 seconds, and extension at 72°C for 10 minutes. DNA from whole, unstained sections from NPC were included in each PCR run as positive control. A nude area of the membrane adjacent to the stained tissue, a cut sterile membrane, and whole tissue sections of the EBV negative clear cell sarcoma were used as negative controls in each experiment. Amplified products were separated electrophoretically on a 2% agarose gel stained with ethidium bromide.

The quality of morphology during microdissection was systematically examined, using a series of approaches

Table 1. Quality of Morphology, Ease of Cutting, and Ease of Transfer using Various Optical Media

Stain and optical medium	Morphology*	Ease of cutting†	Ease of transfer‡
H	1	2	2
H + 99% ethanol	2-3	2	2
H + 70% ethanol	2-3	2	0-2
H + Cytotec	2-3	2	2
H + xylene	3	2	2
H + H ₂ O	1-2	0-2	0-2
IHC	2	2	2
IHC + 99% ethanol	2-3	2	2
IHC + 70% ethanol	2-3	2	2
IHC + Cytotec	2-3	2	2
IHC + xylene	3	1	2
IHC + H ₂ O	2	1	2
ISH	2-3	2	2
ISH + 99% ethanol	3	2	2
ISH + 70% ethanol	3	1-2	1-2
ISH + Cytotec	3	2	2
ISH + xylene	3	1	2
ISH + H ₂ O	2-3	1	2

*Morphological identification of tumor: 0, identification not possible; 1, identification of approximate borders of tumor complexes; 2, clear identification of borders of tumor complexes but not individual cells; 3, identification of individual cell boundaries.

†Ease of cutting: 0, not possible; 1, repeated laser cutting with bridges >10% of the circumference; 2, successful cutting with minimal bridges.

‡Ease of transfer: 0, not possible; 1, repeated attempts necessary; 2, successful at first attempt.

Abbreviation: H, hematoxylin.

with, either hematoxylin, IHC or ISH stain with or without an optical medium (xylene, 99% and 70% ethanol, Cytotec (Schuco International, London, UK) or distilled water) applied with a sprayer. The following parameters were semiquantitatively evaluated as defined in Table 1: morphology (0-3), ease of cutting (0-2), ease of transfer (0-2).

Results

Preparation of Membranes and Sections

Glass slides suitable for performing MOMeNT could be easily and economically produced using polyethylene membranes cut to size and mounted with rubber cement. In initial experiments using standard high temperature microwave HIER, membranes were either lost or were unusable because of severe heat damage. This problem was solved by modifying the protocol for epitope retrieval. Similarly, detachment of sections from the membranes during IHC and ISH was unacceptable unless APES was used to promote adherence. On the other hand, high concentrations of APES interfered with the ease of tissue fragment transfer following microdissection. By testing various dilutions of APES in acetone, optimal concentrations of APES for IHC and ISH of 8% and 2%, respectively, were found. Staining protocols were performed manually. It was important to avoid excessive handling of the slides during the procedures and

Figure 1. **A:** AE1/3 stain of nasopharyngeal carcinoma. **B:** EBER *in situ* hybridization of the same case, showing EBER transcripts in the carcinoma nuclei. **C and D:** EBV LMP-1 stain of undifferentiated NPC before and after laser-assisted microdissection. **E and F:** EBV LMP-1 staining of NPC without (**E**) and with (**F**) xylene. Use of xylene as an optical medium improves the quality of morphology. All photographs were taken without using a cover slip; magnification, $\times 20$.

in particular to perform gentle washing only to avoid damage to membranes and sections.

Low Temperature HIER for Immunohistochemistry

Prolonged incubation of sections in a high pH buffer (TEG pH 9) at 60°C in a hot air oven gave strong, sensitive immunostaining with low background, comparable to or even better than that in control sections stained with standard microwave high temperature HIER. With this modification, membrane loss and section detachment were not a problem. Optimal retrieval times for detecting different antigens varied. Incubation for 24 hours gave strong staining of all carcinomas for cytokeratins (with AE1/AE3, CK-H, KL 1)(Figure 1). Antigen retrieval for 16 hours or less gave unacceptably weak staining, which could not be improved by increasing the concentration of primary antibody or prolonging incubation (up to 2 hours). Optimal epitope retrieval for LMP-1 was 48 hours (Figure 1), after which strong EBV positivity was detected in the four viral positive NPC cases. Staining of membrane-mounted sections was successfully performed using CARD with biotinylated tyramine to amplify signals

Figure 2. Selected examples of the PCR analysis. **Lane 1:** Φ X 174 RF DNA/*Hae*III fragments (GIBCO BRL, Life Technologies A/S, Taastrup, Denmark) molecular weight marker; **lane 2:** EBV positive control; **lanes 3–5:** amplified products from triplicate microdissections of individual cases; **lanes 6–7:** negative membrane controls; **lane 8:** clear cell sarcoma of the kidney, EBV negative control; **lane 9:** water. **A:** PCR analysis for EBNA-3C in microdissected NPC. EBV-positivity is seen in three separate tissue fragments. **B:** The same analysis in a microdissected non-keratinizing carcinoma showing EBV-negativity. **C:** Housekeeping gene NPM.

without increasing background and without causing problems of section detachment. While standard CS 1–4 staining was weak, CARD amplification gave intense, specific reactions.

In Situ Hybridization

Initially, enzyme incubation led to overdigestion and weak or negative signals, irrespective of the concentration of proteinase K. This problem was overcome by reducing the concentration of APES used to coat sections to 2%, and by prolonged washing in TBS-DEPC for 12 hours to remove excess APES. With this protocol, strong nuclear staining for EBERs 1 and 2 was seen in carcinoma cells from the four NPC cases (Figure 1). Stromal cells were negative, with the exception of rare EBV-positive non-neoplastic “passenger”

lymphocytes, which are commonly seen in tissues of EBV seropositive individuals.¹² The three non-keratinizing carcinomas, and the control clear cell sarcoma were consistently negative for EBER.

Optimization of Morphology

A variety of liquid media were tested on routine, IHC and ISH stained sections to optimize visualization of NPC targets before microdissection. The influence of these media on laser cutting and subsequent transfer of target fragments was also assessed. The results are shown in Table 1. Both IHC and ISH staining gave sufficient contrast to allow clear discrimination between tumor complexes and the surrounding tissue. This could be further improved using various optical media, although this was not essential for adequate microdissection. With standard hematoxylin staining, contrast was poor and use of an additional liquid medium was necessary. For this purpose, the alcohol-based media were adequate but xylene gave the best results (Figure 1). For single cell microdissection, cell borders could be precisely identified by combining either IHC or ISH with an optical medium. Xylene gave a slightly reduced efficiency of cutting compared with both 99% alcohol and Cytotec, the latter being easier to apply (Tone Bjoernsen, unpublished data). In some experiments, use of 70% ethanol interfered with the removal of the microdissected fragments. Excess fluids made cutting difficult; this problem was solved by applying the media as a light spray. This had an additional advantage that laser cutting could be performed immediately after applying the medium, without having to wait for the fluid to evaporate.

Polymerase Chain Reaction

Microdissected tissue fragments could be consistently used in standard PCR reactions. Strong bands were seen for viral gene EBNA-3C and weaker bands for house-keeping gene NPM, after 37 and 40 cycles, respectively (Figure 2). The difference in signal intensity can be explained by the relatively high number of EBV genomes in each tumor cell compared with the reported low copy number of NPM in each cell. Although various routine and IHC tissue section stains are known to interfere with the efficiency of subsequent PCR,¹³ this did not adversely affect our PCR analysis. Similarly, the various optical media used did not interfere with the PCR reactions.

Discussion

Thin polyethylene membranes are used to support tissue sections in a variety of UV laser microdissection protocols, facilitating target transfer and reducing problems of target contamination. A major disadvantage of such membranes has been that they are difficult to use in conjunction with traditional IHC and ISH techniques, particularly those using high-temperature HIER which is an often essential step in performing highly-sensitive IHC on

paraffin sections. We now describe optimized protocols for membrane and section preparation and for antigen retrieval that allow IHC and ISH to be applied reliably with membrane mounted tissue sections. Tissue fragments stained by IHC and ISH were suitable for subsequent PCR analysis of extracted DNA using standard techniques (Figure 2). These IHC and ISH procedures are generally applicable and will be suitable for detecting a wide range of antigens and nucleic acids (primarily DNA) in paraffin sections in conjunction with LMM.

Staining markedly improved visualization of cellular targets and compensated for the inferior morphology that results from not using either coverslips or mounting medium during LMM. This improvement was sufficient for precise microdissection of cell groups and larger tissue fragments (Figure 1). Visualization could be further improved by applying various liquid media to sections before target selection to reduce scattering of light passing through tissue sections (Table 1). Volatile media were convenient for this purpose applied as a spray. Although xylene gave better morphology overall (Figure 1), alcohol-based optical media were almost as good. The latter had the advantage that they gave somewhat better cutting efficiency. In our experiments, the alcohol-based fluid Cytotec was the best all-round optical medium. Use of such a medium was essential for accurate target selection using standard hematoxylin sections without IHC or ISH staining. It was also important in combination with IHC or ISH to give optimal definition of individual cell borders, a necessity when performing single-cell microdissection. Xylene was the best medium for this purpose.

The methods we describe here significantly improve identification of targets for microdissection, thus increasing the efficiency and reliability of cell sampling. Since they are for use on formalin-fixed, paraffin-embedded tissue sections they can be widely applied to study archival material.

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