

Improved resolution by mounting of tissue sections for laser microdissection

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Isolated nucleic acids and proteins are the basic materials used for most current molecular techniques. To obtain reliable results, it is important that this starting material is derived from a pure cell population. Two laser assisted microdissection methods are used to separate the specific cells of interest from infiltrating and surrounding other cells. In laser capture microdissection, a thermoplastic membrane is placed above the tissue and is attached to the cells of interest after melting the foil by a low energy near infrared laser pulse. The cells remain embedded in the film and are collected by removal of the foil from the section.¹ The second method is known as laser microbeam microdissection (LMM) and uses a pulsed ultraviolet (UV) laser as an optical knife to cut out target cells. Separated cells are transferred to a reaction tube by needle, by laser pressure catapulting, or by gravity.² The advantage of LMM is that unwanted adjacent cells can be easily photoablated by the UV laser beam. The availability of laser assisted microdissection devices has greatly facilitated the isolation of pure cell populations and even made it possible to identify mutations in single cells.^{3,4} However, all microdissection methods use tissue sections without a coverslip. Most of the light passing through the tissue is scattered, resulting in obscured cellular detail and a poor light microscopic image. This limited detail hampers the recognition of specific cells at the histological level.^{5,6} To overcome this limitation, several approaches have been tried so far. The use of microdissection of membrane mounted native tissue has been suggested as a solution by Bohm *et al.*⁷ Using this technique, tissue is mounted on to a membrane and subsequently attached upside down on to a glass slide. This approach does result in improved morphology, although it is quite labourious, and the morphological quality decreases during microdissection. Alternatively, immunohistochemical detection or in situ hybridisation of specific cells can be of help.^{8,9} In many cases, however, antibodies or in situ probes that discriminate between cells of interest and other cells may not be available. For example, the capability to distinguish between premalignant cells and

Background: Laser microbeam microdissection has greatly facilitated the procurement of specific cell populations from tissue sections. However, the fact that a coverslip is not used means that the morphology of the tissue sections is often poor.

Aims: To develop a mounting method that greatly improves the morphological quality of tissue sections for laser microbeam microdissection purposes so that the identification of target cells can be facilitated.

Methods: Fresh frozen tissue and formalin fixed, paraffin wax embedded tissue specimens were used to test the morphological quality of mounted and unmounted tissue. The mounting solution consisted of an adhesive gum and blue ink diluted in water. Interference of the mounting solution with DNA quality was analysed by the polymerase chain reaction using 10–2000 cells isolated by microdissection from mounted and unmounted tissue.

Results: The mounting solution greatly improved the morphology of tissue sections for laser microdissection purposes and had no detrimental effects on the isolation and efficiency of amplification of DNA. One disadvantage was that the mounting solution reduced the cutting efficiency of the ultraviolet laser. To minimise this effect, the mounting solution should be diluted as much as possible. Furthermore, the addition of blue ink to the mounting medium restores the cutting efficiency of the laser.

Conclusions: The mounting solution is easy to prepare and apply and can be combined with various staining methods without compromising the quality of the DNA extracted.

malignant cells relies on morphological characteristics, thus warranting optimal resolution.

“The availability of laser assisted microdissection devices has greatly facilitated the isolation of pure cell populations and even made it possible to identify mutations in single cells”

Therefore, we have developed a mounting method that solves the refraction problem and results in excellent morphology of tissue sections for LMM purposes.

METHODS AND MATERIALS

Fresh frozen tissue and paraffin wax embedded tissue specimens from the files of the department of pathology of the University Medical Centre Nijmegen, the Netherlands, were used in our study. From these tissue specimens, 4–20 µm thick tissue sections were cut and mounted directly on to glass slides for isolation by laser microdissection of areas larger than 1 mm², or mounted on crosslinked polyethylene (PEN) foil attached to a glass slide or to a carrying frame (Leica Microsystems GmbH, Wetzlar, Germany; <http://www.leica-microsystems.com/website/lms.nsf>) for laser microdissection of small groups of cells or single cells. The paraffin wax embedded tissue sections were dewaxed and both fresh frozen tissue and paraffin wax embedded sections were washed twice with distilled water and stained with haematoxylin or by immunohistochemistry.

The mounting solution was prepared by diluting PinPoint solution (Pinpoint slide DNA isolation system; Zymo Research, Orange, USA; www.zymor.com), an adhesive gum, in

Abbreviations: FSH, follicle stimulating hormone; LMM, laser microbeam microdissection; PCR, polymerase chain reaction; PEN, polyethylene; UV, ultraviolet

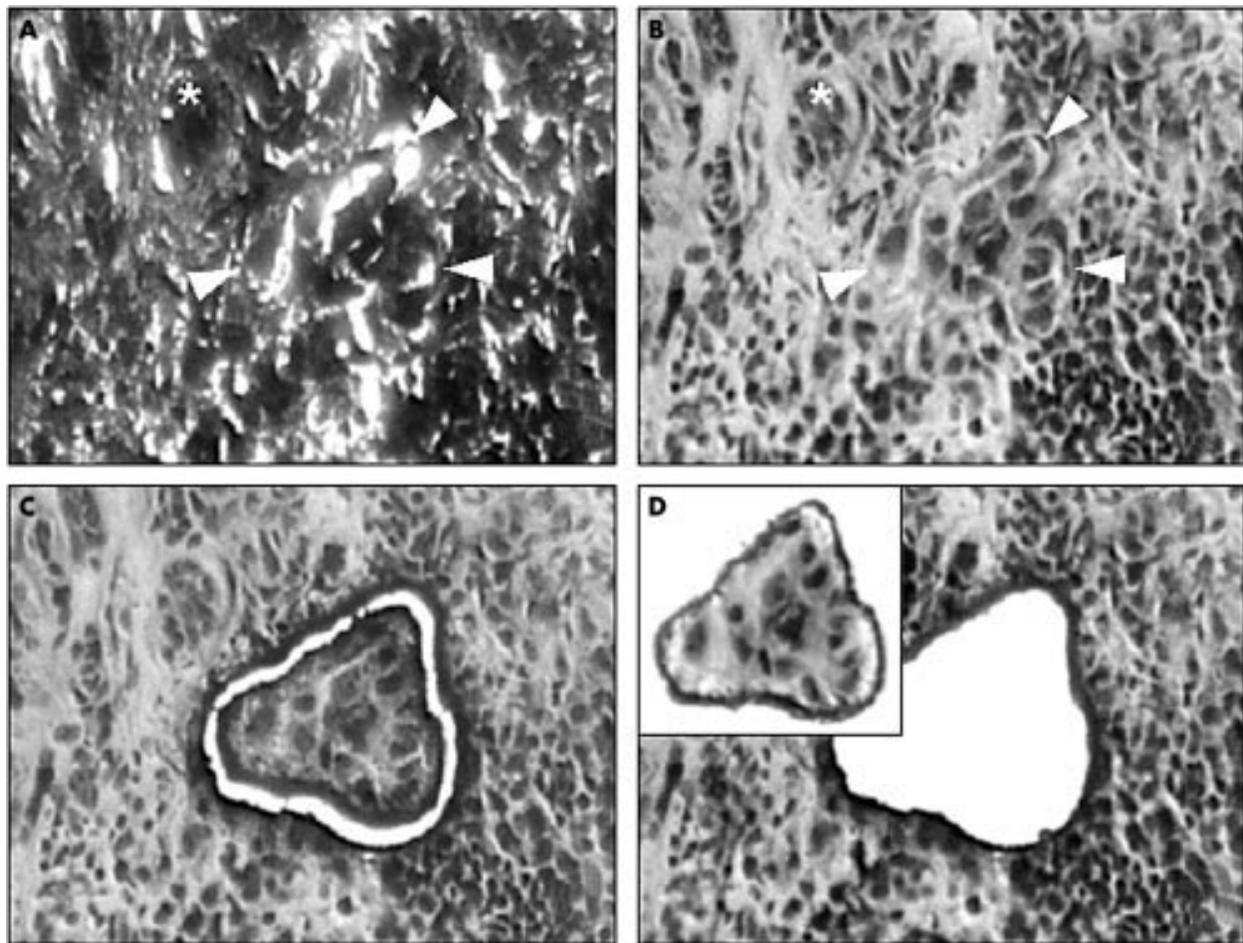


Figure 1 (A) An unmounted 10 μm paraffin wax embedded tissue section showing melanoma cells juxtaposed to benign naevocellular naevus cells. (B) The same area as (A) after the addition of the mounting solution. Note the same cell nest (*) in (A) and (B). The arrowheads indicate the melanoma cells selected for microdissection. (C) The same area as in (A) and (B) after laser microdissection of the melanoma cells. (D) The targeted cells have been removed (inset) and the residual tissue remains intact. Haematoxylin staining; original magnification, $\times 250$.

distilled water and the addition of dark blue ink (Quink solv-X, Parker). The optimal ratio of the different components of the mounting solution is dependent on the structure of the target tissue and was tested at ratios of 16/4/1 to 20/2/1 for water, pinpoint, and blue ink. For tissue with a relatively high cell density and little intercellular spaces, such as kidney tissue, a high dilution factor of the mounting solution of 1/20 should be used. In contrast, tissue with a relatively low cell density with large intercellular spaces and loose connective tissue, such as dermis, requires a higher concentration (1/5) of the mounting solution. Dark blue ink was added to the mounting solution at a ratio of 1/20. Finally, the mounting solution was added to and distributed over the tissue section with a clean disposable pipette tip and air dried for 30 minutes.

A Leica AS LMD microdissection system with a UV laser (Leica Microsystems GmbH) was used to separate a group of target cells from the surrounding tissue. Samples microdissected from tissue mounted on glass slides were procured by needle and transferred to a reaction tube (frosted flat top tube 0.2 ml; Biozyme, Landgraaf, The Netherlands) and samples microdissected from tissue mounted on PEN foil were procured by the force of gravity into a reaction tube.

To test the efficiency of DNA isolation, 10–20 cells from fresh frozen tissue and 50–2000 cells from paraffin wax embedded tissue were microdissected. DNA isolated from paraffin wax embedded tissue sections was pretreated by incubation in 100 μl P buffer (50mM Tris/HCl, pH 8.2, 100mM NaCl,

1mM EDTA, 0.5% Tween-20, 0.5% NP40, and 20mM dithiothreitol) at 90°C for 15 minutes to partially reverse the effect of the formalin fixation. DNA was isolated from fresh frozen tissue and paraffin wax embedded tissue after protein digestion with 0.5 mg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for three days and fresh proteinase K (0.5 mg/ml) was added every 24 hours. Subsequently, genomic DNA from paraffin wax embedded tissue and fresh frozen tissue was isolated using the DNeasy tissue kit (Qiagen, GmbH, Hilden, Germany). DNA was eluted with 50 μl TE buffer (10mM Tris, pH 8.2, 1mM EDTA, pH 8.0). Primers specific for the follicle stimulating hormone (FSH) receptor gene (forward primer sequence, CTA CCC TGC ACA AAG ACA GTG; reverse primer sequence, GTG TAC GTC ATG TCA AAT CCT CTG C) were used, resulting in a 210 bp polymerase chain reaction (PCR) product. PCR reactions were performed in a total volume of 15 μl , and contained 2.5 μl diluted template DNA, buffer IV (Integro, Dieren, the Netherlands), 200 μM of each deoxynucleotide triphosphates, 1.5mM MgCl_2 , 1 pmol of each primer, and 30 μg bovine serum albumin (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). DNA amplification was performed in a PTC 200 (MJ Research, Waltham, Massachusetts, USA). An initial denaturation at 92°C for three minutes was followed by 35 cycles of denaturation at 92°C for 30 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 45 seconds.

(Part of this protocol will also be published in the addendum of *Molecular cloning*, Cold Spring Harbor, "Laser Pressure Catapulting", Chapter 5, Part 2 (in press).)

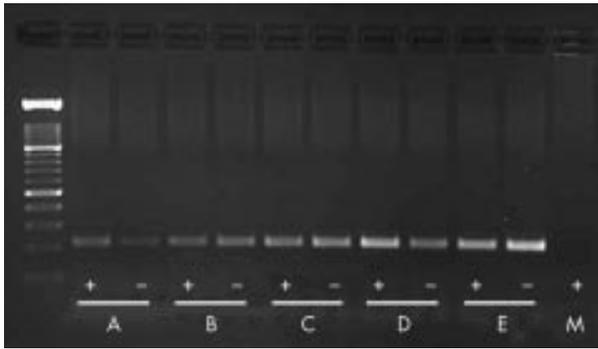


Figure 2 Aliquots (10 μ l) of the polymerase chain reaction (PCR) products were run on an agarose gel and stained with ethidium bromide. The left hand lane contains a 100 bp DNA marker (Invitrogen BV, Breda, the Netherlands). (A–E) PCR analysis of DNA isolated from five different paraffin wax embedded tissue sections, M, PCR with mounting solution but without tissue; +, DNA isolated from tissue sections covered with mounting solution; –, DNA isolated from tissue sections not covered with mounting solution. The mounting solution did not affect the PCR results.

RESULTS

Standard mounting of tissue on a glass slide or a PEN foil covered glass slide or frame, as used for laser microdissection purposes, results in poor tissue morphology. To overcome this problem we used a mounting solution that results in excellent morphology of tissue specimens for laser microdissection. Figure 1 demonstrates the benefit of using this mounting solution. It shows a primary cutaneous melanoma lesion with benign naevocellular naevus cells and stromal cells juxtaposed to melanoma cells before and after the use of the mounting solution and before and after the microdissection of a group of melanoma cells. In the tissue section without mounting solution, the melanoma cells could hardly be distinguished from

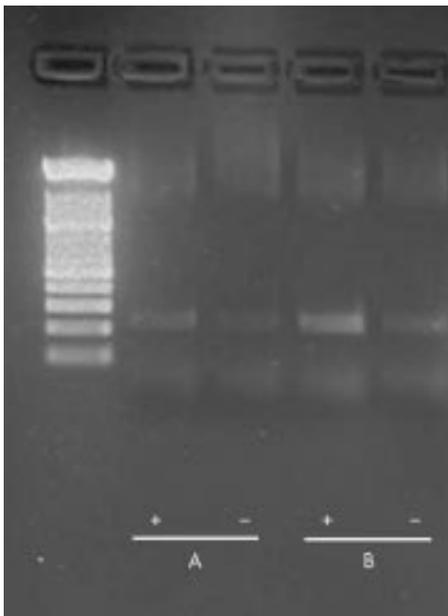


Figure 3 Aliquots (10 μ l) of the polymerase chain reaction (PCR) products were run on an agarose gel and stained with ethidium bromide. The left hand lane contains a 100 bp DNA marker (Invitrogen BV); (A, B) PCR analysis of DNA isolated from two different fresh frozen tissue sections containing 10 and 20 cells for each PCR, respectively. +, DNA isolated from tissue sections covered with mounting solution; –, DNA isolated from tissue sections not covered with mounting solution. The mounting solution did not affect the PCR results, even at critical DNA concentrations.

the naevus cells or stromal cells, whereas this was not a problem when the tissue section was covered with mounting solution.

To test the efficiency of DNA isolation, we microdissected 10 and 20 cells from fresh frozen tissue sections and 50, 100, and 2000 cells from paraffin wax embedded tissue sections that were covered with our mounting solution. Genomic DNA was isolated as described in the materials and methods section. PCR amplification of the FSH receptor gene was used to compare the quality and quantity of DNA from mounted and unmounted tissue. As shown in fig 2, the mounting solution did not interfere with the isolation of DNA or the efficiency of PCR in paraffin wax embedded tissue sections. The quantity of product amplified was comparable between the mounted and unmounted tissues. We also performed similar analyses on small numbers of cells from fresh frozen tissue sections. No negative influence of the mounting solution was detected for fresh frozen tissue or for paraffin wax embedded tissue sections at DNA concentrations where PCR is critical. Results are shown in fig 3.

The mounting solution described in our paper can be used in combination with various histochemical staining methods and with immunohistochemistry. However, it is not compatible with fluorescent methods because of the autofluorescence of the pinpoint solution.

DISCUSSION

Laser microdissection has greatly facilitated the procurement of specific cell populations from complex tissues. However, because tissue sections are used without a coverslip, the resulting morphology is often poor, with obscured cellular detail. Here, we describe a mounting solution, consisting of adhesive gum and blue ink diluted in water, which greatly improves the morphology of tissue sections for laser microdissection purposes. The mounting solution coats the tissue section in a varnish-like manner and therefore obviates the need to use a coverslip for optimal morphology. The refractory index of the dried adhesive gum is similar to that of the tissue. This results in optimal morphological detail so that target cells can be selected more easily. In tissue with a low cell density, intercellular material, and interstitial fluid is lost during the fixation process, resulting in gaps in the tissue. Larger gaps require less dilution of the pinpoint solution, resulting in a higher viscosity of the mounting solution.

One disadvantage, however, was that the mounting solution reduced the cutting efficiency of the UV laser, as a result of poorer absorption of the laser energy. Consequently, microdissection of tissue sections covered with mounting solution required a higher setting of the laser energy, with a resulting larger laser diameter and a lower cutting specificity. To minimise this effect, the mounting solution should be diluted as much as possible. However, the benefit of an increased morphology using diluted mounting solution is dependent on the tissue structure of the tissue specimen used. Searching for ways to counteract the reduced laser cutting efficiency caused by the mounting solution, we found that the addition of dark blue ink (Quink solv-X; Parker) to the diluted mounting solution, at a ratio of 1/20, facilitated absorption of the UV laser light and resulted in restoration of the laser cutting efficiency. The blue ink stained the tissue slightly blue, but it did not interfere with the recognition of different cell types.

“We found that the addition of dark blue ink to the diluted mounting solution, at a ratio of 1/20, facilitated absorption of the ultraviolet laser light and resulted in restoration of the laser cutting efficiency”

The optimal composition of the mounting solution depends on the structure of the tissue. For the tissue types that we have

tested, either a 16/4/1 or a 20/2/1 ratio of water, adhesive gum, and blue ink, respectively, was required. It is possible that in specific cases a slightly different ratio of the components may yield better results. In general, as high as possible a dilution of the adhesive gum that gives an optimal compromise between morphology and laser cutting efficiency should be used. Occasionally, some loss of laser energy and thus fine specificity may occur; however, for most purposes a sufficient laser cutting efficiency is still obtained. When a higher cutting specificity is required than is obtained with a given composition of the mounting solution, the addition of more blue ink will increase the laser cutting efficiency. We tested the effect of our mounting solution on the isolation and amplification efficiency of DNA and found that the mounting solution had no detrimental effects. The adhesive gum used in our mounting solution was originally marketed for use in a micropipetting dissection method for the isolation of DNA and RNA from areas of tissue sections and therefore should not interfere with DNA isolation and amplification efficiency.

Because PCR artefacts are dependent on the number of target molecules in the first round of DNA amplification, it is important to start with as many target cells as possible.¹⁰ Because of the improved morphology, similar cell groups can easily be recognised in serial sections and can be pooled for DNA isolation. We have now successfully used this approach for the isolation of DNA from normal and tumour tissue using serial sections from melanocytic tumours for allelic imbalance analysis and to dissect morphologically different tumour parts to investigate tumour heterogeneity.¹¹

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Take home messages

- The mounting solution greatly improved the morphology of tissue sections for laser microdissection purposes and had no detrimental effects on the isolation and efficiency of amplification of DNA
- One disadvantage was that the mounting solution reduced the cutting efficiency of the ultraviolet laser, resulting in a lower cutting specificity, but the addition of dark blue ink to the diluted mounting solution, at a ratio of 1/20, facilitated absorption of the ultraviolet laser light and resulted in restoration of the laser cutting efficiency
- In addition, to minimise this effect, the mounting solution should be diluted as much as possible

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