11

Laser-Assisted Microdissection of Membrane-Mounted Tissue Sections

Lise Mette Gjerdrum and Stephen Hamilton-Dutoit

Summary

Biological tissues (in particular those affected by disease) are inherently complex mixtures of different cell types and matrices. This heterogeneity can complicate the interpretation of molecular biological studies performed on whole-tissue extracts if the precise cellular origin of the molecules being tested is not known. Laser-assisted microdissection (LAM) has emerged as a leading histological technique for obtaining samples enriched for specific target cell populations or tissue components for subsequent molecular (especially polymerase chain reaction-based) analysis. This method allows the identification and study of target-specific molecular alterations in heterogeneous specimens, and enables more accurate detection and quantification of target molecules. In this chapter, we focus on tissue microdissection performed with an ultraviolet laser system and describe protocols for the basic procedure and for handling of the samples.

Key Words: Laser-assisted microdissection; MOMeNT; frozen tissue; formalin-fixed; paraffin-embedded tissue.

1. Introduction

Advances in molecular biology have provided new tools for the analysis of the genetic processes that govern disease. A crucial factor for the reliability of the data obtained using tests based on tissue extracts is the cellular homogeneity of the study samples. This is particularly true for cancer tissues, which are inherently complex. In addition to the main tumor clone and subclones, they typically include a variable mixture of dead or dying cells, stroma, blood vessels, inflammatory cells, and other non-neoplastic tissue components. Many molecular biological assays (including detection of loss of heterozygosity, comparative genomic hybridization, high-throughput cDNA arrays, and proteomics) require relatively homogeneous test material to unmask tumor cell-specific genetic alterations ***(1–4)***. In cancers, the presence in the test material

From: *Methods in Molecular Biology, vol. 293: Laser Capture Microdissection: Methods and Protocols*
Edited by: G. I. Murray and S. Curran © Humana Press Inc., Totowa, NJ

of as a few as 20% nontumor cells may confound the correct result ***(5)***. Therefore, accurate and meaningful molecular analysis of tumors requires precise documentation of the cellular origin of the test DNA, RNA, or proteins in tissue extracts.

Laser-assisted microdissection (LAM) is a general term for methods that use a laser to selectively obtain homogeneous cell specimens, both from cytological preparations and from frozen or formalin-fixed, paraffin-embedded (FFPE) tissue sections.

These microdissected samples are suitable for a range of downstream molecular analyses of DNA, RNA, or proteins ***(1,6–9)*** (*see* **Note 1**). In laser microbeam microdissection (LMM), a pulsed ultraviolet (UV) laser is used to photoablate unwanted tissue and to cut out the area of interest (*see* **Note 2**). Mounting the section on a thin polyethylene membrane facilitates the microdissection and makes it possible to remove both large tissue fragments and individual target cells intact, while reducing the risk of contamination ***(1)***. The transfer of dissected material is accomplished in a variety of ways (*see* **Note 2**; **Fig. 1**). In this protocol, we use an UV laser system supplied by Molecular Machines & Industries (MMI, Glattbrugg, Switzerland) for microdissection. General protocols for microdissection of membrane-mounted native tissue (MOMeNT) using both FFPE and frozen tissues are described, together with instructions for improving tissue morphology during microdissection and notes on the storage of sections (*see* **Note 3**). Further, we provide protocols for sample collection with a needle and using the single-step collection device supplied by MMI (*see* **Fig. 1**). Protocols for RNA and DNA extraction from frozen and paraffin-embedded tissues are given, suitable for conventional polymerase chain rection (PCR)-based analysis, as well as for real-time quantitative PCR.

2. Materials

2.1. LMM Using Disposable Needles for Sample Collection

1. Polyethylene membranes (MMI).
2. Fixogum rubber cement (MMI).
3. 31 G × 5/16 8-mm Microfine Insulin pen needles (Becton Dickinson, Broendby, Denmark).
4. 0.2-mL MicroAmp tube (PE Biosystems, Foster City, CA) or 1.5-mL Eppendorf tube.
5. Cytotec (Schuco International, London, UK).

2.2. LMM Using Single-Step Collection

1. Membrane-mounted metal slides (MMI).
2. Tubes with adhesive lid, with or without diffuser (MMI).
3. Single-step collection robot-stage and cap-holder (MMI).

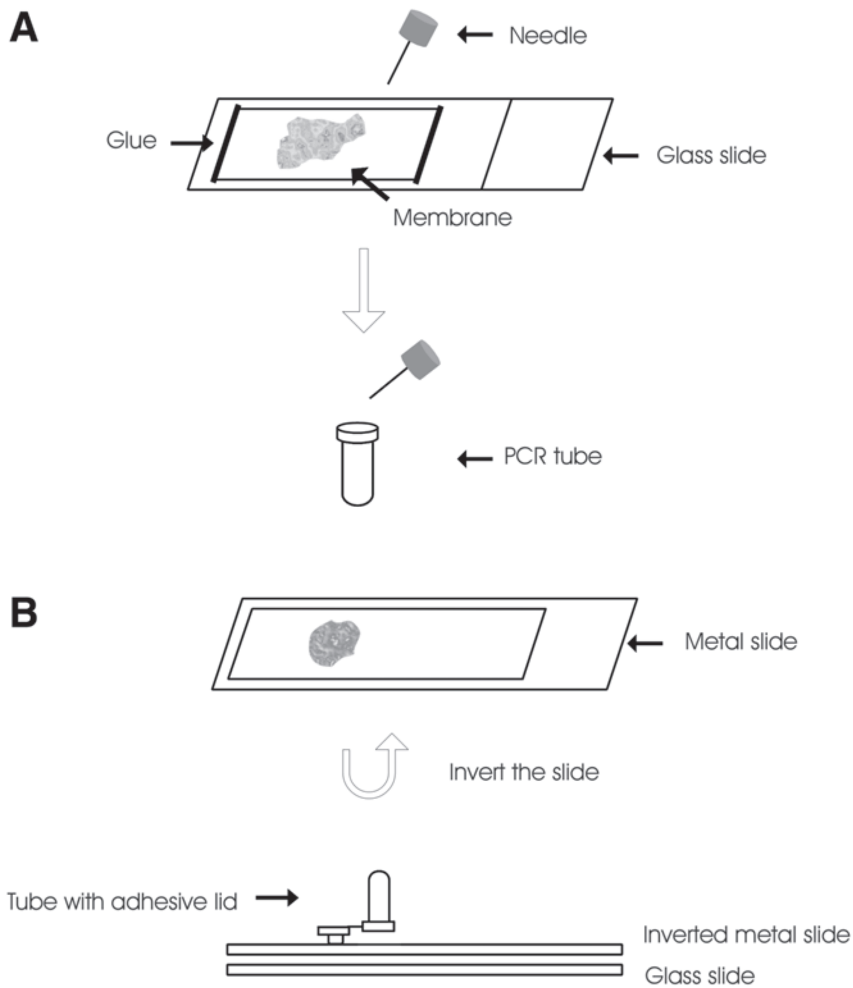

Fig. 1. **(A)** Laser microbeam microdissection (LMM) with needle collection of target. **(B)** LMM with single-step target collection.

2.3. Disposables Shared by Both Methods

1. Laser microdissection system: SLµCUT (MMI) (*see* **Note 2** for suitable alternative systems).
2. Noncharged Superfrost glass slides (Menzel-Glaser, Braunschweig, Germany).
3. Xylene; store at room temperature (RT).
4. Graded alcohols (99%, 96%, 70%); store at RT.
5. Absolute acetone store at 4°C.
6. Histological stain (Mayer's hematoxylin, methyl green, or toluidine blue).

2.4. DNA Extraction

1. DNA extraction buffer containing 50 m*M* Tris-HCl, pH. 8.1, 1 m*M* EDTA, pH.8.0, and 0.5% Tween-20; store at RT.
2. Proteinase K, 5 mg/mL, stock solution at –20°C.
3. Glycogen 20 mg/mL (Invitrogen A/S, Taastrup, Denmark) at –20°C for single-cell collection.

2.5. RNA Extraction

1. RNA digestion buffer containing 20 m*M* Tris-HCl, pH 7.5, 20 m*M* EDTA, pH.8.0, and 1% SDS at RT.
2. Proteinase K, 20 mg/mL, stock solution at –20°C.
3. TRIzol (Life Technologies, Gaithersburg, MD) at 4°C.
4. Chloroform at RT.
5. Isopropanol at RT.
6. 3 *M* Sodium acetate, pH 5.2, at –20°C.
7. Glycogen 20 mg/mL (Invitrogen) at –20°C.
8. Absolute and 70% ethanol at RT.
9. Sterile H_2O.

3. Methods

The methods described include: (1) LMM performed with disposable needles, including preparation of the membrane-mounted glass slides and instructions for improving the quality of morphology; (2) single-step collection; (3) preparation of sections from FFPE tissues and preparation of frozen sections; (4) laser microdissection using both sample collection methods; (5) DNA extraction; and (6) RNA extraction.

3.1. LMM Performed Using Disposable Needles

3.1.1. Preparation of Membrane-Mounted Glass Slides

1. The polyethylene membrane (1.5 µm, MMI) is layered between two sheets of A4 paper and cut to size (~2 × 3 cm) using a paper guillotine. The noncharged Superfrost glass slides (Menzel-Glaser) are dipped in 70% ethanol to make wrinkle-free application of the membranes easier.
2. After removing one of the papers, the precut membrane is applied to the wet glass with the help of the remaining paper support ***(10)***. This is then removed, and the membranes are smoothed out using sterile cotton swabs.
3. Subsequently, the membrane is attached to the glass slide using Fixogum rubber cement along the two short opposing sides (*see* **Notes 3** and **4**). Gloves should be worn throughout the whole procedure.
4. The membrane-mounted slides are then put under UV light overnight (*see* **Note 5**).

3.1.2. Instructions for Improving Quality of Morphology

Improvement of morphology can be achieved using several different volatile fluids, applied using a simple plastic spray bottle. We use Cytotec, which is a commercially available fixative for cytology specimens (*see* **Note 6** for alternatives).

3.2. Single-Step Collection

Commercially available metal slides are placed under UV light for 30 min (*see* **Note 5**). Each metal slide is used together with a single Menzel glass slide.

3.3. Preparations Common to Both Methods

3.3.1. Preparation of Sections From FFPE Tissues

1. Gloves should be worn and changed regularly.
2. The microtome is cleaned with ethanol before each new case.
3. Serial 5-μm sections are cut with a fresh knife, floated out in a hot water bath for 30 s, and finally mounted on the prepared slides.
4. Subsequently, they are incubated at 60°C for 15 min to adhere the tissue to the membrane (*see* **Note 7**).
5. Removal of paraffin is carried out for 2 × 2 min in xylene, then 2 min each in 99%, 96%, and 70% ethanol.
6. The sections are stained for either 3 min in Mayer's hematoxylin, or for 10 s in methyl green or toluidine blue (*see* **Note 8**), rinsed in distilled water for 1 min, and finally dehydrated in 99% ethanol for 1 min.
7. When the membrane is glued on to the glass slide, the sections are dried at RT for approx 30 min (*see* **Note 9**).
8. The metal slides for single-step collection should be dried for approx 5 min at RT.

3.3.2. Preparation of Frozen Sections

1. The procedure for cleaning and cutting is essentially the same as for FFPE sections.
2. Frozen sections are fixed in acetone for 3 min immediately after being cut, air-dried for 15 s, stained with either Mayer's hematoxylin for 3 min, or methyl green or toluidine blue for 10 s (*see* **Note 8**).
3. Solutions are washed in distilled water for 1 min, and dehydrated consecutively in 96% and 99% ethanol for 1 min each.
4. Finally, the sections are dried at RT for 30 min or 5 min, depending on the type of slide used (*see* **Note 9**).

3.4. Laser-Assisted Microdissection

3.4.1. LMM Using Disposable Needles

1. We use the SLμCUT UV laser system for microdissection.
2. Before each new sample, the robot-stage is cleaned with ethanol.

3. When working with single cells, we irradiate the same working area with a portable UV lamp.
4. The power, focus, and speed of the laser should be adjusted for each new specimen.
5. After visual identification of the target, the computer mouse and software are used to select and cut out the chosen area.
6. This procedure and the transfer of the cut fragment will differ between the various available systems (*see* **Note 2** for alternatives).
7. We use a 31G needle (Becton Dickinson) clamped in a holder on the microscope to collect the specimen.
8. The computer mouse and software position the microdissected fragment under the needle point.
9. The needle is then lowered to pick up the cut fragment, which is transferred to a PCR tube (*see* **Note 10**; **Fig. 1A** in this chapter; **Fig. 1A–D** in Chapter 12).

3.4.2. Single-Step Collection

1. The same UV laser system and software are used, and the preparation of slides and tissues is essentially the same.
2. The metal slide containing the membrane and cut tissue is inverted and put on top of a Menzel glass slide.
3. The tissue now lies between the membrane and glass slide (*see* **Note 11**, **Fig. 1B** in this chapter and **Fig. 2A–D** in Chapter 12).
4. This sandwich is placed on the robot stage with the metal slide on top, and clamped in a holder.
5. The adhesive cap is placed in the cap-holder, and centered over the light beam.
6. The lid is then lowered onto the membrane surface.
7. The procedure of adjusting the laser and cutting the target is carried out as described above.
8. After cutting one or more samples, the cap (with adherent microdissected fragments) is lifted away from the stage, and the tube closed for transportation.
9. Digestion buffer is added to the cap, and incubation takes place in a hot-air oven with the tubes inverted (*see* **Note 11**).

3.5. DNA Extraction

1. The extraction method is essentially the same, regardless of which sampling method is used.
2. Using a disposable needle, the dissected tissue is transferred into a 0.2-mL MicroAmp tube containing 28.6 μL of DNA extraction buffer and 1.4 μL of 5 mg/mL proteinase K solution (*see* **Note 12**).
3. This is incubated overnight at 56°C.
4. Following single-step collection, 10 μL of digestion buffer is added to the cap.
5. The digestion buffer for single-cell microdissection contains, in addition, 1 μL of 20 mg/mL glycogen (*see* **Note 11**).
6. After inversion, the tubes are placed in a hot air oven at 48°C overnight.

7. In both protocols, the enzyme is inactivated at 95°C for 5 min before subsequent PCR analysis is performed.
8. The samples can be stored at –20°C for months.
9. For larger dissected samples, an ethanol precipitation may be advisable (*see* **Note 13**).

3.6. RNA Extraction

1. The same RNA extraction protocol is used with both methods.
2. Microdissected tissue samples are either placed in a reagent tube containing 200 μL of digestion buffer and 5 μL of 20 mg/mL proteinase K, and are subsequently allowed to incubate at 60°C overnight ***(11)*** (*see* **Note 12**), or the inverted tube containing the tissue and same amount of digestion buffer are incubated overnight in an hot air oven.
3. The enzyme is inactivated at 95°C for 5 min.
4. The RNA is purified using phase separation by 500 μL TRIzol and 100 μL chloroform (*see* **Note 14**).
5. The samples are left for 15 min on ice, after which they are centrifuged at 12,200*g* for 15 min.
6. The aqueous phase is removed to a new tube, and RNA is precipitated by an equal volume of isopropanol, 0.1 vol 3 *M* sodium acetate and 1 μL of glycogen.
7. The samples are now kept overnight at –20°C and can be stored for several weeks.
8. When required, the samples are centrifuged at 12,200*g* for 30 min, the supernatant is removed, and the pellet is washed once with 500 μL 70% ethanol.
9. After centrifugation at 7500*g* for 5 min, the supernatant is removed, and the pellet is left to air-dry with the tube open on a thermomixer at 65°C for 5–15 min.
10. The pellet is resuspended in 10 μL of sterile water, and can be stored at –80°C for several weeks.

4. Notes

1. There are two main systems for performing laser-assisted microdissection. In laser capture microdissection (LCM), an infrared laser is used to melt a special transfer film, which binds to cells identified microscopically. The transfer film with the attached cells is then lifted off and can be used for subsequent analysis. This technology is described extensively in other chapters. In contrast, laser microbeam microdissection (LMM) uses a UV laser to cut selected cells out of a cytological or histological preparation. These two systems each have advantages and disadvantages and the choice of technology will be decided by the purpose of the study and, of course, by the availability of equipment. For example, LCM is particularly suited to projects in which many samples are to be rapidly collected from large tissue areas. In contrast, the superior resolution of the laser cutting in LMM means that this technique is better suited for precise microdissection, particularly of single cells. By mounting tissue sections on support membranes, large tissue fragments can also be dissected intact using LMM.
2. At least three UV laser-based systems for histological microdissection (LMM) are currently commercially available: (1) PALM MicroBeam system (PALM

Microlaser Technologies, Bernried, Germany); (2) SLμCUT (Molecular Machines & Industries, Glattbrugg, Switzerland [formerly SL Microtest]); (3) Leica Laser Microdissection—AS LMD (Leica, Wetzlar, Germany).

These are essentially similar in principle and use similar types of UV lasers to microdissect target cells and ablate adjacent unwanted tissue. They differ in the specifics of the technology used, particularly with regard to the mode of collection and transfer of the microdissected specimen for downstream analysis. Thus, in the PALM system, the laser itself can be used to catapult the microdissected cells into the lid of a microfuge tube in a "no touch" technique—laser pressure catapulting (LPC) ***(7)***. In the SLμCUT MMI system, transfer is by needle or glass pipet, or by single-step collection (either using a version of LPC or by picking the cells up directly using an adhesive film in the lid of a reaction tube). In the Leica AS LMD system, the section is inverted so that after cutting, the microdissected cells fall into the reaction tube under the influence of gravity. The latest versions of these systems have improved computer software and a number of new features that together allow more sophisticated microdissection techniques to be applied. These include (though not in all systems) automatic dissection of preselected fields with sorting into multiple reagent tubes, automatic multiple sampling from within a larger preselected area, and variable cut techniques. Each system appears to be adequate for most basic microdissection tasks, although each has its proponents and opponents.

3. Ready-made MOMeNT slides can be purchased from MMI, PALM, or Leica. We also make membrane-mounted slides ourselves; it is not particularly laborious and it allows us to prepare the exact number required each time. This avoids a possible source of contamination and minimizes the risk of introducing additional RNase/DNase. It is possible to keep the mounted slides in a dust-free, light-tight glass-slide storage box at RT, and tissue sections on membrane-mounted slides can be stored in a similar way. We have been able to extract and PCR-amplify DNA from sections stored in this way (so far for at least 2 yr). Similarly, we can retrieve RNA for PCR-based analysis from slides stored for up to at least 3 mo.
4. We have used different kinds of glue, including nail polish, but Fixogum rubber cement works best in our hands, especially when MOMeNT is used in combination with immunohistochemistry or *in situ* hybridization (*see* Chapter 12).
5. There are different protocols for overcoming the hydrophobic nature of the membranes. Both PALM and Leica recommend irradiating the membranes with UV light for 30 min. Coating the membranes with 0.1% poly-L-lysine may also be used. We treat the membrane-mounted slides under UV light overnight to eliminate any contaminating nucleic acids. To increase adherence of the tissue section to the membranes it is also possible to combine UV light with poly-L-lysine, or when extra adhesion is required, with 2% or 8% 3-aminopropyltriethoxysilane (APES) ***(10)*** (*see* Chapter 12). Extra precautions must be taken when applying these fluids, as they may interfere with LPC and transfer of the cut segment in cases where the fluids have leaked under the membrane. The commercially avail-

able metal slides for single-step collection, are purchased individually wrapped to ensure a DNase- and RNase-free environment, as well as to prevent any contamination with foreign nucleic acids. In this situation, we find treatment with UV light for 30 min to be sufficient.

6. A disadvantage of LAM is inferior tissue morphology during microdissection caused by not being able to use cover slips. This can make it difficult to identify targets by morphology alone (*see* Chapter 12). We have tried several different volatile fluids as "optical media" to compensate for this. Xylene is best for giving optimal morphological details, but is more difficult to distribute than Cytotec, and its use should be restricted on safety grounds. Cytotec is an alcohol-based fixative used in the preparation of cytological smears. We use Cytotec in routine LMM (*see* **Fig. 1E–F** in Chapter 12) and xylene when optimal morphology is important, e.g., when dissecting single cells.

   The fluids are applied using a plastic spray bottle. After identifying and marking the target cells, the fluid is left to evaporate before laser microdissection is performed. The specially designed tubes for single-step collection may contain a light diffuser in the cap, improving the morphology. Similarly, the glass slide below the metal slides also helps to improve morphology. Since this described method is one in which the membrane is stuck on the adhesive cap, a volatile medium cannot be used.
7. In most cases, we find it adequate to incubate the slides at 60°C for 15 min to secure adhesion of tissue to the membrane-mounted slides, but in some cases prolonged incubation (up to 2 h) is required in combination with coating of the membranes (*see* **Note 3**).
8. Mayer's hematoxylin binds to DNA and several papers have reported that this dye may interfere with PCR-based amplification of DNA ***(12–14)***. In contrast, other groups have not found this to be a problem ***(15)*** and single-cell microdissection of hematoxylin-stained sections with subsequent PCR-based analysis of DNA is possible ***(16)***. It appears that RNA is not affected in the same way; several papers have reported successful RT-PCR on single cells microdissected from hematoxylin-stained FFPE ***(7,8)***. We find that Mayer's hematoxylin does not affect RNA quantification, and it is our preferred histological stain because it gives improved morphological detail. However, when working with small numbers of (<50) microdissected cells or single cells, it might be advisable to use a different stain, such as methyl green or toluidine blue, when amplifying DNA or when performing quantitative analysis of DNA. Eosin is frequently used together with hematoxylin, but we find that this quite often results in a very dark stain with inferior morphology, especially when staining lymph nodes.
9. A major obstacle to successful LMM is wet sections, or fluid leaking under the membranes, as this interferes with the cutting efficiency of the laser. After the slides have been prepared with FFPE or frozen tissue sections, they should be allowed to dry at RT for approx 30 min. We then normally perform LMM immediately. In special cases, the slides can be dried at 37°C for 30 min. Some authors ***(17)*** and PALM recommend that sections be dried at 37°C overnight, but we

believe that the hands-on time should be as short as possible to diminish problems with external and internal RNase/DNase. The metal slides for single-step collection take less time to dry, and only 5–10 min is required.

10. The needle with the microdissected fragment can be transferred in different ways. We previously cut off the entire needle together with the microdissected cells, and placed everything in the reagent tube. Now we prefer to use the needle to place the cut fragment into a drop of fluid on the wall of the PCR tube. If the sample is placed directly onto the wall, static electrical forces can make it very difficult to get the microdissected sample into the digestion buffer.
11. In single-step collection, the tissue lies protected between the membrane and the opposing clean Menzel glass slide. The adhesive film in the cap is not in contact with the tissue, only with the membrane, to which the tissue is adherent. Single-step collection allows for sampling of several segments or single cells in one cap without removing the cap in between. We prefer to use single step collection for microdissection of single cells. Sampling of 10 cut cells from paraffin-embedded tissue, with subsequent I-PEP-PCR, is sufficient for PCR of a housekeeping gene (*see* **Fig. 2E** in Chapter 12). Digestion of sampled single cells or small tissue fragments (<100 cells), can preferentially be accomplished by adding 10 μL of buffer directly onto the cells. Since single cells are not visible to the eye, the cells should be sampled centrally in the cap. Similarly, the digestion buffer should also be placed centrally. Digestion of single cells for DNA analysis is accomplished using our DNA digestion buffer with glycogen, and incubation is performed overnight. Glycogen is used as a carrier to prevent nucleic acid binding to the plastic wall of the reaction tube. When dissecting larger fragments for subsequent RNA extraction, we prefer to use our standard total RNA extraction protocol.
12. We usually perform proteolytic digestion overnight. Shorter incubation times result in products suitable for PCR. We have obtained amplifiable DNA from pooled single cells after 2 h incubation; however, further incubation increases the DNA yield and the chances for successful amplification. Using the single-step collection system, it is possible to check that all cells present have been totally digested. To do this, the cap is replaced on the cap-holder, lowered onto a glass slide, and the digestion status is controlled visually. Proteinase K digestion can be performed using different concentrations of the enzyme. For DNA and RNA extraction from larger cut areas, we use 0.3 mg/mL. When digesting single cells, we use 4 mg/mL ***(18)***.
13. When microdissecting 1500 cells or less for DNA analysis, we use the template in the digestion buffer, without any precipitation steps. For larger samples, an ethanol precipitation should be considered.
14. We have tested different commercially available kits for total RNA extraction, and we prefer the TRIzol reagent for this procedure. However, new kits are being produced on a regular basis and readers should choose the method with which they are most familiar.

References

1 Bohm, M., Wieland, I., Schutze, K., and Rubben, H. (1997) Microbeam MOMeNT: non-contact laser microdissection of membrane-mounted native tissue. *Am. J. Pathol.* **151,** 63–67.

2 Aubele, M., Zitzelsberger, H., Schenck, U., Walch, A., Hofler, H., and Werner, M. (1998) Distinct cytogenetic alterations in squamous intraepithelial lesions of the cervix revealed by laser-assisted microdissection and comparative genomic hybridization. *Cancer* **84,** 375–379.

3 Luo, L., Salunga, R. C., Guo, H., et al. (1999) Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med.* **5,** 117–122.

4 Weber, R. G., Scheer, M., Born, I. A., et al. (1998) Recurrent chromosomal imbalances detected in biopsy material from oral premalignant and malignant lesions by combined tissue microdissection, universal DNA amplification, and comparative genomic hybridization. *Am. J. Pathol.* **153,** 295–303.

5. Bohm, M. and Wieland, I. (1997) Analysis of tumour-specific alterations in native specimens by PCR: How to procure the tumour cells! (review). *Int. J. Oncol.* **10,** 131–139.

6 Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., et al. (1996) Laser capture microdissection. *Science* **274,** 998–1001.

7 Schutze, K. and Lahr, G. (1998) Identification of expressed genes by laser-mediated manipulation of single cells. *Nat. Biotechnol.* **16,** 737–742.

8 Lahr, G. (2000) RT-PCR from archival single cells is a suitable method to analyze specific gene expression. *Lab. Invest.* **80,** 1477–1479.

9 Banks, R. E., Dunn, M. J., Forbes, M. A., et al. (1999) The potential use of laser capture microdissection to selectively obtain distinct populations of cells for proteomic analysis—preliminary findings. *Electrophoresis* **20,** 689–700.

10 Gjerdrum, L. M., Lielpetere, I., Rasmussen, L. M., Bendix, K., and Hamilton-Dutoit, S. (2001) Laser-assisted microdissection of membrane-mounted paraffin sections for polymerase chain reaction analysis: identification of cell populations using immunohistochemistry and in situ hybridization. *J. Mol. Diagn.* **3,** 105–110.

11 Specht, K., Richter, T., Muller, U., Walch, A., Werner, M., and Hofler, H. (2001) Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am. J. Pathol.* **158,** 419–429.

12 Burton, M. P., Schneider, B. G., Brown, R., Escamilla-Ponce, N., and Gulley, M. L. (1998) Comparison of histologic stains for use in PCR analysis of microdissected, paraffin-embedded tissues. *Biotechniques* **24,** 86–92.

13 Murase, T., Inagaki, H., and Eimoto, T. (2000) Influence of histochemical and immunohistochemical stains on polymerase chain reaction. *Mod. Pathol.* **13,** 147–151.

14 Serth, J., Kuczyk, M. A., Paeslack, U., Lichtinghagen, R., and Jonas, U. (2000) Quantitation of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. *Am. J. Pathol.* **156,** 1189–1196.

15 Ehrig, T., Abdulkadir, S. A., Dintzis, S. M., Milbrandt, J., and Watson, M. A. (2001) Quantitative amplification of genomic DNA from histological tissue sections after staining with nuclear dyes and laser capture microdissection. *J. Mol. Diagn.* **3,** 22–25.

16 Kuppers, R., Zhao, M., Hansmann, M. L., and Rajewsky, K. (1993) Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *EMBO J.* **12,** 4955–4967.

17. Lehmann, U. and Kreipe, H. (2001) Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. *Methods* **25,** 409–418.

18. Dietmaier, W., Hartmann, A., Wallinger, S., et al. (1999) Multiple mutation analyses in single tumor cells with improved whole genome amplification. *Am. J. Pathol.* **154,** 83–95.