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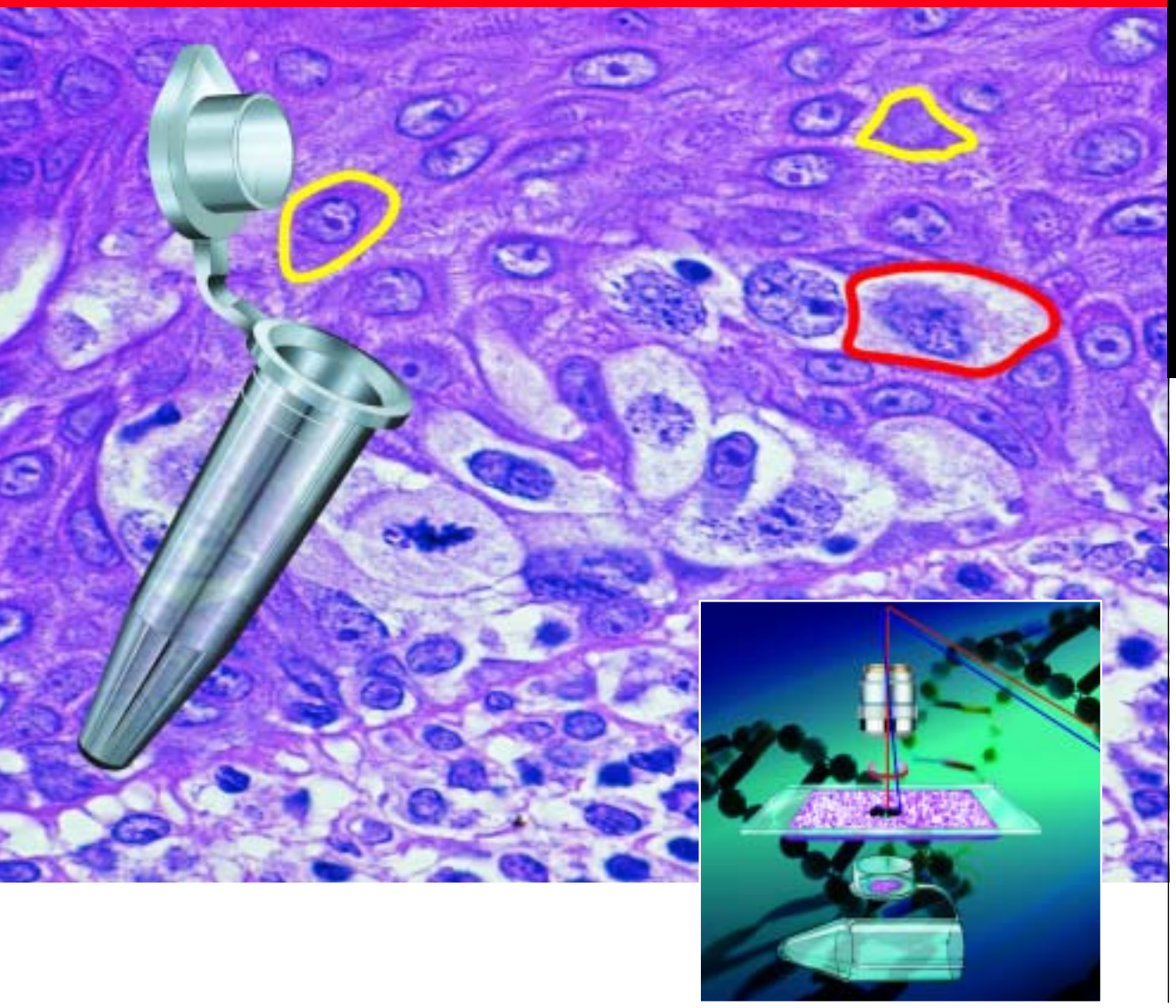
APPLICATION LETTER



Laser Microdissection

Leica
MICROSYSTEMS

Dear Reader

We are pleased to present the first edition of our new forum, the "Application Letter". Our plan is to publish this bulletin a couple of times every year.

Our aim is to provide you with basic information regarding different applications.

This edition gives more background on Leica Laser Microdissection AS LMD. "AS" stands for **A**pplication **S**olution and this is our concern – to offer the investigator a optimized tool for his application.
The first part describes possible downstream applications after cutting out cells of interest for DNA, RNA or protein analysis. It informs you about features and benefits of our system.

In the second part we have collected protocols referring to the microdissection process. You can also find these protocols on the AS LMD – Demo CD ROM.
We hope you enjoy this first and the following editions of our new Application Letters.

Market Segment Team

Application Letter – Laser Microdissection

Since the Human Genome Project offers researchers a lot of basic information about the sequences of the complete genome, they now want to examine the functions, the expression and the regulation of gene activity. The pure knowledge about the sequence of a gene is the theory. It has to be applied to processes within the cell (cell cycle, development, growth, diseases etc.). In order to do this, DNA sequences, the messenger RNA and proteins are the targets of the investigation. Further investigations of DNA and RNA are often carried out by the Polymerase chain reaction or RT-PCR, Southern- and Northern blotting. Protein analysis is done e.g. by SDS-PAGE, two-dimensional gel electrophoresis and Western blot analysis.

Laser microdissection offers researchers a tool for precise non-contact and contamination-free preparation of cell groups or single cells cut out of histological tissue sections.

The Leica Laser Microdissection Microscope – AS LMD

Leica uses the DM LA upright microscope including motorized nosepiece, motorized stage, the xyz-control element and all other advantages of the new DM LA microscope. The laser used is a UV laser of 337 nm wavelength. The movement during cutting is done by the optics, while the stage remains stationary. The region of interest can be marked on the monitor and is cut out by PC control. The sample falls down into PCR tubes without extra forces. The result of the cutting can be easily checked by an automated inspection mode.

Specimen stage with removable slide holder

Ergonomic controls for stage movements (xy) focus (z), objective change and light intensity

Lower plane of the AS LMD stage.
Motor controlled positioning of 4 PCR tubes.

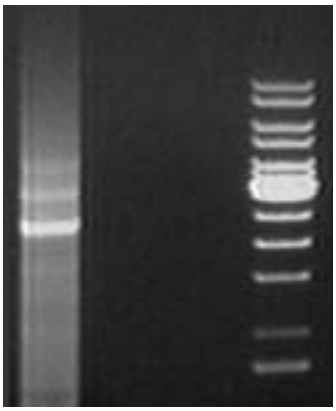
Sample preparation uses foiled slides, i.e. a rectangular UV-sliceable piece of foil fixed at the margins to a normal glass slide. An alternative are the frame slides covered with foil. These foiled frames are handled like glass slides. Paraffin sections, frozen sections, blood smears, stained and immunolabelled specimens can be cut. Also, metaphase chromosomes can be cut out. The advantages of the Leica AS LMD are:
It is an easy to use automated upright microscope, easy to handle, easy to operate, easy to adjust and to calibrate by the user. The cut area falls into the tube without further (destroying) energy. Simultaneous cutting and observation in fluorescence with the standard RF4 fluorescence module is possible. The system is manufactured by **one** company with a high level of experience in microscopy.
(www.leica-microsystems.com)

The Leica AS LMD was developed together with pathologists to suit their specific applications. This includes the use of paraffin or frozen sections of human tissue. Pathologists want to cut cell groups or single cells out of a tissue area identified as tumor tissue. Together with this sample they cut out one or two reference tissue areas. Further investigations include the use of PCR (Polymerase Chain Reaction) to amplify specific (e.g. oncogen) sequences.

The applicative possibilities given by Leica laser microdissection are not limited to the application of PCR. The AS LMD is also useful for investigations related to DNA, RNA, protein analysis, chromosomes and many more (e.g. living cells).

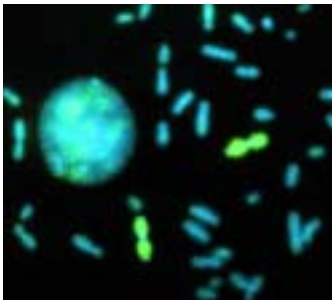
DNA:
After cutting a cell or cell groups out of a specimen the DNA has to be extracted. This can be done by an enzyme called proteinase K which digests the proteins of the cells.
Alternatively, an instant DNA isolation kit is usable (e.g. Qiagen).
After extraction the DNA can be used as a template in PCR.

The result of the PCR is proven by **agarose-gel electrophoresis** for the positive amplification process. A small amount of the PCR sample is loaded to the gel and the DNA molecules inside the sample are separated on the gel according to its molecular weight with the help of voltage. The negatively charged DNA molecules move to the positive pole of the gel chamber. With e.g. ethidium bromide, which stains DNA molecules,

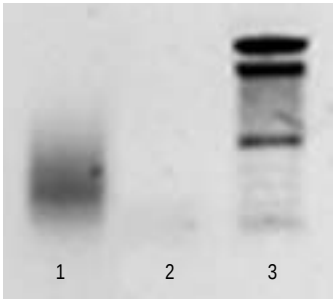


PCR-Product Marker

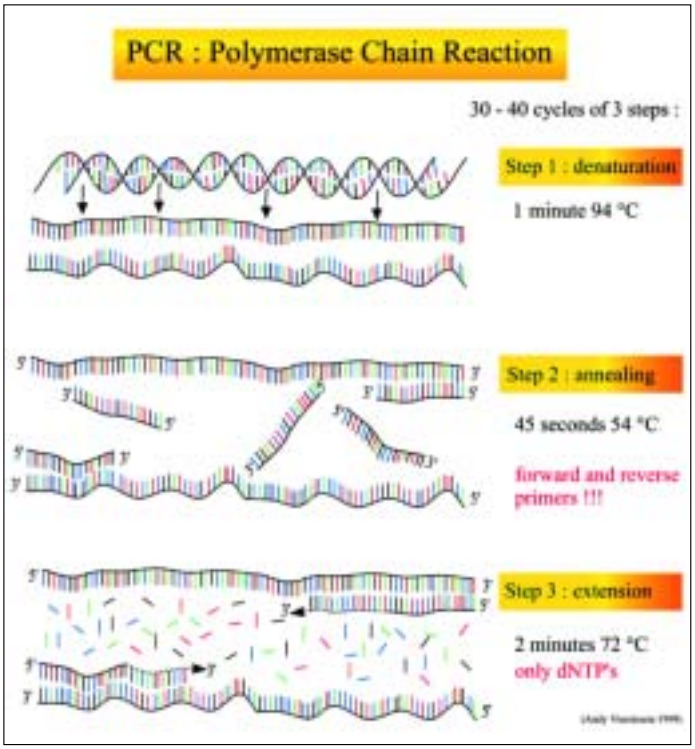
Agrose-gel electrophoresis
of DNA amplified by PCR



Metaphase chromosome specimen with fluorescence-labelled chromosomes.
A chromosome fragment was selected for laser microdissection and subjected to PCR (see right-hand illustration).



DOP-PCR products
1 Microdissection product after 2 rounds of PCR
2 Negative control
3 100bp ladder

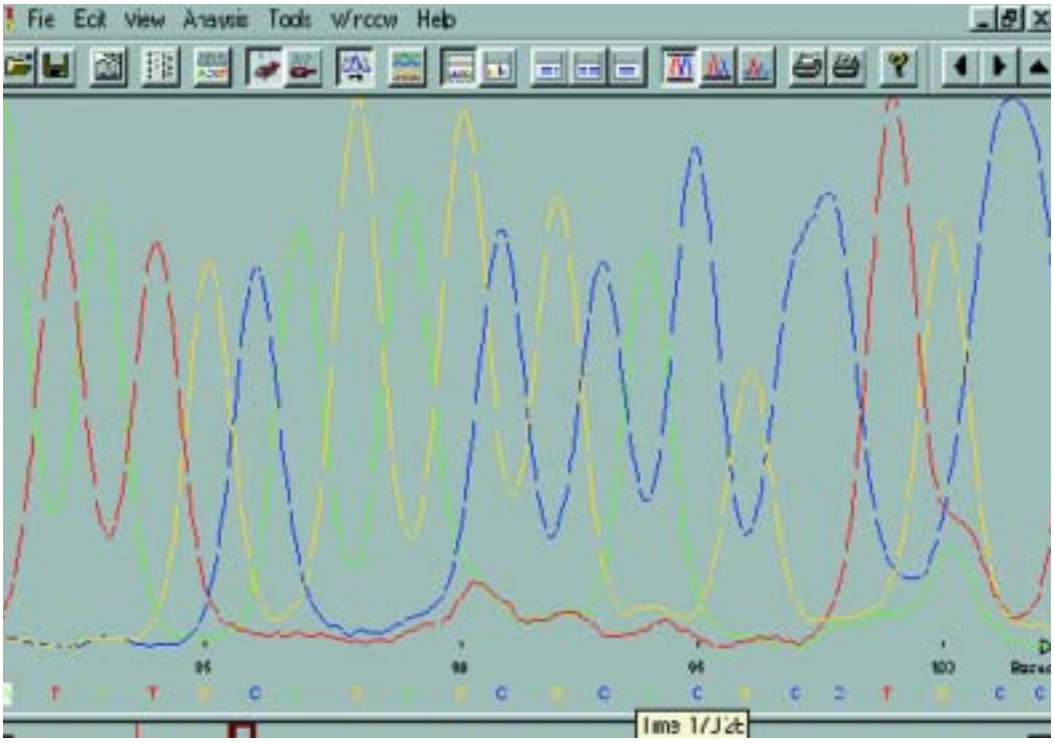


the separated DNA fragments become visible in UV light.
Faster results are possible by using **quantitative PCR** (real time PCR [e.g. light cycler, Roche]) where a fluorescence DNA stain is included in the PCR reaction which binds to the amplified product. The more DNA is produced, the more fluorescence is measured. In this way the result of the reaction can be checked.

At the last stage the DNA can be sequenced or it can be used for Southern blotting.

Sequencing of DNA today is not as difficult as it was at the beginning in the mid seventies (Sanger and Barrel, Maxam and Gilbert). At that time radioactivity was used, today it is done with the help of fluorochromes. An easy way to sequence DNA is to do it by PCR. The origin DNA molecule is amplified in many fragments of different length which carry a fluorescence molecule at their end. A PCR sequencing needs 4 reactions according to the 4 bases. Every reaction is directed to one base of the DNA. This mixture of DNA fragments is separated via gel electrophoresis. A sequencing gel has 4 lanes equivalent to the 4 bases of the DNA molecule (A,C,G,T). During this gel run the fluorochromes at the end of the DNA fragments are activated by a laser beam.
The speed of the DNA fragments is dependent on their length. Short fragments run faster than longer fragments. They are detected by their moment of arrival at the laser locus. The sequence of the 4 bases is shown on a monitor by a software process.

Southern blotting means to transfer DNA to a filter (membrane) (e.g. nitrocellulose, nylon). The DNA is separated before by gel electrophoresis. That means that the same pattern of bands is transferred to the filter. Using a probe one can detect specific sequences of the separated DNA fragments. A probe is a single stranded DNA molecule. It has the opposite sequence to this DNA sequence which it is supposed to hybridize with. The hybridization of the probe to its specific loci is done during a hybridization procedure. A probe also carries a molecule which is detectable at an X-ray film (e.g. ^{32}P radioactivity, digitoxin, biotin – peroxidase, alkaline phosphatase detection). As the result of this procedure a band is visible on the X-ray film. This band is analog to the DNA band of the gel. This is the evidence that a specific sequence is present.



DNA base sequencing

RNA:
In principle, the lab work with RNA is a little bit more complicated than the handling of DNA. The reason is the instability of the RNA molecule. In comparison with DNA, RNA is a single stranded molecule and not as stable as the double stranded DNA (DNA is a very stable molecule existing for thousands of years e.g. extractable out of old Egyptian mummies).

In the way a protein has its own specific charge (pI – isoelectrical point) it disposes itself in an electric field. This is done in a capillary or on special stripes. The gel inside the capillary is shaped like a thin sausage and transferred to the second dimension after the gel run. The polyacrylamid gel will be stained by Coomassie blue or silver to visualize the proteins. Unlike the protein bands of a one-dimensional gel, a 2D-gel shows protein spots.

Proteins can be identified by a mass spectrometer, e.g. **MALDI** (Matrix-Assisted Laser Desorption/Ionization)-**TOF** (Time Of Flight). Special prepared samples of proteins are ionized and the time of their "flight" from the ionization source to a detector is measured. This time is an efficient measure of the mass of a protein. A sequencing of the protein is possible.

Features and Benefits:

The feature and benefits list demonstrates the advantages of the Leica AS LMD.

Feature	Benefit
Untouched stress-free preparation. Transport by gravity (upright microscope). No mechanical or physical forces (laser light) are needed	no contamination no damage of DNA by laser shot
UV laser cutting	precise cutting
Laser movement by optics not by mechanics	allows high precision cutting by a wide range of objectives (increasing precision with increasing objective magnification)
Stable and compact design	no realignment by service technicians needed
Built in calibration features	easy to handle, user friendly fast alignment of laser beam
Unlimited definition of shapes with draw function	selection of every structure in tissue/ what you see is what you get
Compatible with a wide range of objectives (10x – 100x)	allows a wide selection of different areas
Motorized UV offset correction	fast change of objectives without recalibration
Fully motorized and automated laser dissection module	ease of use/ reproducible experiments/ high throughput
User defined profiles for laser cutting	fast switch between different experiments/ short set-up time
Automated multi-well positioning	ease of use/ high throughput/ effective selection and documentation/ defined choice of selection
Drawing on screen	what you see is what you get
Easy and affordable apparatus for fluorescence applications	no high investments for upgrade/ no need for realignment and service/ can be done by user
Simultaneous UV cutting and fluorescence observation	allows observation and selection of cells and tissues in immuno fluorescence
Fully automated microscope functions with ergonomic control	convenient for microscope and screen observation
Compact system solution	saves valuable lab space/ small footprint
Versatile and easy to use windows software	no need to learn new environments/ short training times/ multi user friendly
Optimized optics and 3 CCD RGB camera	superb image quality
Automated inspection mode	control of cutting result in PCR tube
100 % microscope integrated design	optimal performance/ responsibility by one company
Worldwide service and support organization	fast service and support
Active application support	support on preparation issues

Laser microdissection could be a valuable tool in:

– Molecular Medicine
– Pathology
– Cancer Research/Diagnostics
– Cell Biology
– Plant Research
– Neurobiology
– Forensic Medicine
– Single Cell Preparation
– PCR, RNA-, DNA-, Protein Analysis

Leica Protocols

Preparation protocols for Leica laser microdissection

Slide-holder and tube holder are autoclavable!

1. Preparation of membrane-coated glass slides

Membrane-coated slides (1 mm for 20 – 40x; 0.17 mm for 100x magnification [oil]) can be prepared as follows:

Gloves must be worn at all times.

Conventional microscopic slides are cleaned meticulously with acetone/ethanol and left to dry in a dust-free environment.

Slides are dipped in distilled water and mounted with appropriately sized membranes (approx. 21 mm x 42 mm). Watch out for wrinkles.

Make sure to leave at least 2 mm uncovered glass surface around the edges.

After complete evaporation of water from the uncovered slide areas the membranes are sealed around the edges with clear nail varnish and left to dry for at least 1 hour at room temperature.

Optional: Siliconizing of glass slides by dipping into dimethyldichlorosilane and drying for 10 min. at 40 °C. Siliconizing of glass slides may improve efficiency of cutting/falling for larger samples.

Optional: apply nail varnish only to two sides of the foil and put the slide in an oven at 40 °C overnight. Then fix the other two sides of the foil with the nail polish. This will improve the drying and make sure that there is no water between foil and slide.

Leica offers prepared PEN (polyethylennaphtalate) foil slides ready for step 2.

Immunohistochemical detection requires use of Leica membrane-coated slides.

2. Applying specimen to slide

For reduction of electrostatical charge of the foil and possible destruction of RNases the slide is incubated for 30 min. in a UV chamber (max. power 254 nm). UV will improve the fixation of the foil.

Optional (frozen sections): For better attachment of tissue/cells to foil, tissue adhesive can be applied as follows. Apply shortly before use one drop of diluted tissue adhesive (Novacastra, Novobond-slite adhesive NCL-BOND; www.novocastra.co.uk) to foil. Spread gently over foil with pipette tip and remove excess TA. Shake vigorously until only a thin film of TA remains on the foil. Allow the TA to dry for 15 min. at 37 °C.

Collect tissue section (± 5 µm) on the foil. Allow tissue section to dry for 30 min. or 1 h at room temperature.

The foiled slides can be treated with Poly-L-Lysin to improve the attachment of the sections to the foil.

3. Staining of sections on membrane-coated slides

3.1 Paraffin-embedded tissue

Dewax in xylol for 45 seconds (max. 3 minutes), as otherwise the adhesive fixing the foil to the specimen slide will dissolve. As an alternative, intermedium substitutes such as Paraclear can be used instead of xylol.

For rehydration, the sections are immersed for 30 seconds each in absolute ethanol (3x), 96 % ethanol (1x), 70 % ethanol (1x) and finally in distilled water.

Nucleus staining with haemalum (Mayer technique) for 5 min.

Rinse in tap water.

Depending on the recipe of the haemalum used, differentiation must be made in 0.5 – 1 % HCl alcohol under microscope control (Mayer's haemalum, prepared with the following recipe: dissolve 1 g haematoxylin in 1000 ml distilled water, then add, one after the other: 200 mg sodium iodate, 50 g potash alum, 50 g chloral hydrate and 1 g citric acid).

Bleach nuclei blue in tap water for 5 to 10 min. (microscope control).
The staining of the cytoplasm

can be done with eosine or erythrosine for 1 min. A 1:1 mixture of the two solutions is recommended.

Rinse the sections well in distilled water then air-dry. If necessary, it is also possible to differentiate in 70 – 96 % ethanol and dry after returning to distilled water.

3.2 Fresh frozen tissue

Follow protocol for paraffin sections after rehydration step.

Alternative protocol:
Stain tissue section for 1 min. with haematoxyline or 10 seconds with methyl green or toluidin blue and rinse in sterile water. There are indications that methyl green or toluidin blue give better PCR results than haematoxylin. Carefully remove excess water by placing a filter on the sample and striking it gently with your finger. Allow to dry further for 30 min. at 37 °C.

4. Immunostaining

4.1 Paraffin-embedded tissue

4.1.1 Optional heat-induced epitope retrieval (HIER)

Transfer the rehydrated 3 µm paraffin sections into 1000 ml HIER citrate pH 6.0 buffer.

10x stock	7.65 g Citric acid
	48.2 g Sodium citrate
	water ad 2000 ml

Bring to the boil in a pressure cooker for 1 – 3 minutes, let equilibrate to room temperature and transfer into TBS pH 7.4 (50 mM Tris, 150 mM NaCl).

4.1.2 Alkaline Phosphatase Immunolocalization

Incubate sections in DAKO Protein Block (No. X0909) to reduce unspecific binding. Incubate the section in 100 µl TBS/0.5 % BSA with the first antibody for 1 h at room temperature or 37 °C (alternatively overnight at 4 °C).

Wash section once with TBS-Tween 0.01 %, once with TBS.

Incubate the section in 100 µl TBS/0.5 % BSA with the second biotinylated antibody for 30 min. at room temperature or 37 °C.

Wash section once with TBS-Tween 0.01 %, once with TBS.

Incubate the section in 100 µl Streptavidin-Alkaline Phosphatase conjugate (ABC-Kit Vector No. AK5000) for 30 min. at 37 °C.

Wash section once with TBS-Tween 0.01 %, once with TBS.

Develop with Sigma Fast Red chromogen under microscopic control.

Wash section once with distilled water.

Counter stain with Mayer's Haemalum.

Bleach nuclei blue in tap water for 5 to 10 min. (microscope control).

Coverslip the section in Aquatex.

4.2 Fresh frozen tissue

Frozen tissue is cut at preferably 3 – 5 µm thickness in a cryostat.

Sections are fixed in acetone 0.1 % NP40 for 5 min.

Incubate in DAKO Protein Block (No. X0909) to reduce unspecific binding as above under 4.1.

5. Preparation of microdissectate for DNA analysis

Microdissectates (frozen or paraffin-embedded) are collected individually or pooled in 20 – 100 µl of lysis buffer (10 mM Tris-HCl pH 8.0, 1 % Tween-20).

After capping the solution is spun for 15 s and 1 – 5 µl of Proteinase K stock solution (e.g. 100 mg/ml) is added.

The microdissectate solution is kept at 55 °C for a minimum of 60 min.
Proteinase K is heat inactivated at 99 °C for 10 min.

Use 1 – 10 µl aliquots in subsequent PCR analyses.

Alternatively spin column extraction (QIAGEN DNA blood/tissue kits) can be used.

6. Preparation of microdissectate for RNA analysis/cDNA synthesis

It is recommended to wear gloves, to work, if not otherwise stated, on ice and to use RNase-free tubes and DEPC-water.

Microdissectates (frozen or paraffin-embedded) are collected individually or pooled in 20 – 100 µl of QIAGEN RNAeasy Mini Kit (Cat. No. 74106) LGT/ß-ME buffer dispensed centrally in the lid of an 0.5 ml Eppendorf cup.

After capping, the solution is spun for 15 s and transferred into a 1.5 ml Eppendorf cup.

Follow QIAGEN RNAeasy protocol for further procedures.

Elute into 30 µl of distilled water and keep at – 80 °C.

6.1 First strand cDNA synthesis of RNA purified from microdissected tissue

Customary precautions for RNA handling should be observed.
Per reaction the Reverse Tran-

scription Master Mix consists of

4 µl dNTP-Mix; 10 mM each (Applied Biosystems N8080260)
4 µl $MgCl_2$; 25 mM
2 µl GeneAmp 10x Buffer II (Applied Biosystems N8080010)
1 µl Random hexamer primers (Applied Biosystems N8080127)
1 µl MuLV reverse transcriptase (Applied Biosystems N8080018)
1 µl RNAse Inhibitor (optional) (Applied Biosystems N8080119)

Distilled water ad 13 µl, mix at RT and use up to 8 µl of the RNA solution (see 5.) in a 20 µl total reaction volume.

Leave 10 min. at RT, then move to 42 °C for 60 min.

Use 2 – 10 µl aliquots in subsequent PCR analyses.

7. PCR (Polymerase Chain Reaction)

(Mullis et al. 1987, Meth. Enzymol 155, 335 – 350; Sambrook et al. 1989, Molecular cloning: A laboratory manual, Cold Spring Harbour, New York; Cold Spring Harbour Laboratory Pres.)

The PCR is used to amplify defined DNA sequences using special oligonucleotid primer. The cyclic reaction is carried out in three steps: The denaturation of the DNA by heat, the sequence specific annealing of the primer and the polymerization by the heat stable (Taq)-polymerase.

For example:

Cycle	Denaturing	Annealing	Polymerization
1	95 °C, 2 min.		
30	94 °C, 30 sec.	50 °C, 45 sec.	72 °C, 1 min.
1			72 °C, 5 min.

After these cycles a small amount of the amplified DNA is ready for agarose gel analysis.

PCR reaction:
1x Taq-buffer complete with 1.5 mM $MgCl_2$
dNTP-mix (200 µM)
(Taq)-polymerase (1 unit)
Primer 1 (2 µM)
Primer 2 (2 µM)
Template DNA
A.dest

8. Protein Analysis

The cells of the section also allow protein analysis,
e.g.
SDS-PAGE
Western blotting
2D-gel electrophoresis.

For example, the cells can be directly cut into SDS-sample buffer (10 % [v/v] glycerol, 5 % [v/v] 2-mercaptoethanol, 2 % [w/v] SDS, 0.05 % [w/v] Bromphenolblue, 2 mM Tris-base), heated and loaded to a normal polyacrylamid gel (Laemmli, U.K. 1970, Nature 227, 680 – 685).

For two-dimensional gelelectrophoresis IEF-buffer (e.g. 9.5 M urea, 2 % NP40, 5 % 2-mercaptoethanol) (O'Farrell 1975, J Biol Chem, 250, 4007 – 4021; O'Farrel, Goodman 1977, Cell, 12, 1133 – 1142; Shoeman, Schweiger 1982, J Cell Sci, 58, 23 – 33) can be used.
Western blot analysis can be carried out following standard procedure (e.g. Towbin, Staehelin, Gordon, 1979, Proc Natl Acad Sci USA, 76, 4350 – 4354).

9. In situ hybridization

Standard protocols for in situ hybridisation. The foil is heat stable for more than 120 °C.

10. Cytospins

Spin cells with a centrifuge to the slide using standard protocol. It is also possible to spin cells to Menzel Superfrost slides without foil.
Human white blood cells:
Cytospin methanol fixed human white blood cells on microdissection slide. A drop of fixed cells is pipetted into 1 ml of 50 % acetic acid (to remove cytoplasmic proteins) contained in a specially designed centrifuge bucket. After centrifugation, the slide is air dried and Giemsa stained.

11. Blood smear

One drop of freshly collected full blood is smeared on a foiled microdissection slide, air dried overnight and Giemsa stained.

12. Chromosome preparation

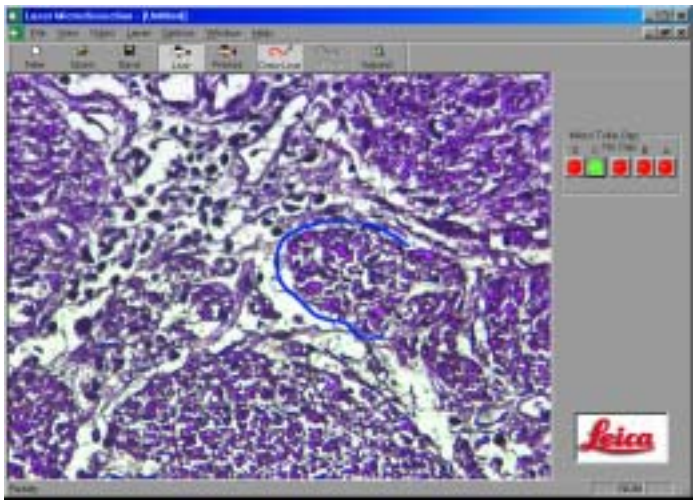
Chromosomes from white blood cells from human peripheral blood. Cells were cultured for 72 hours, then treated with colcemid (to arrest cells in metaphase), then treated with a hypotonic solution (to let them swell), and finally fixed in methanol/acetic acid (3 + 1, v/v). Fixed cells were dropped on the foiled microdissection slides and were air dried overnight. Then they were stained with Giemsa staining solution (1 : 20 diluted in Gurr buffer, which is a standard buffer used for Giemsa staining made by dissolving commercially available tablets) for 3 minutes. After washing in water they were air dried.

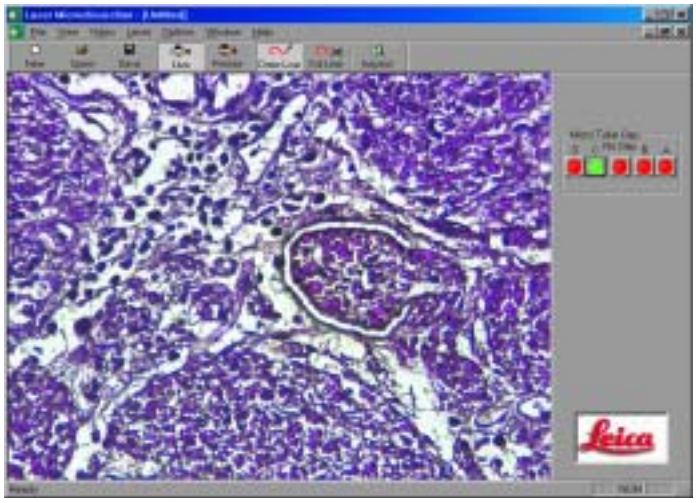
13. Living cells

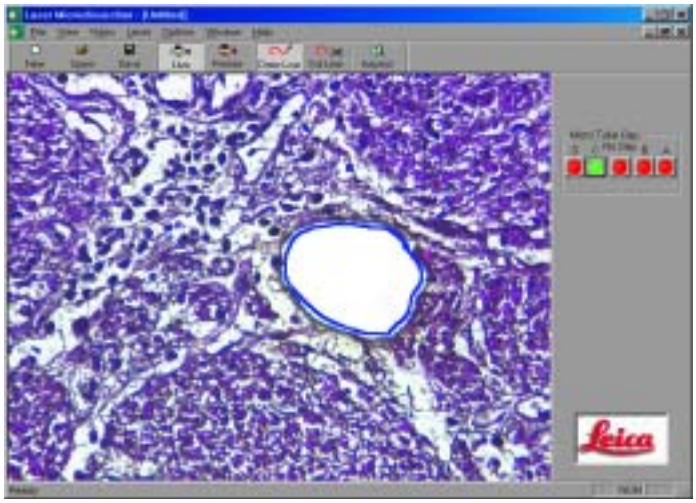
It is recommended to UV treat the PEN foil for sterilization. Then cells can be grown in culture medium on the foil. It is important to choose the right volume of culture medium. Single cells can be selected by laser cutting.

14. Embedding of tissue in paraffin using an automatic embedder

	Over night	Weekend
Formalin	1 h 30 min.	16 h 00 min.
70 % Ethanol	1 h 00 min.	1 h 00 min.
80 % Ethanol	1 h 00 min.	1 h 00 min.
90 % Ethanol	1 h 00 min.	1 h 00 min.
96 % Ethanol	1 h 00 min.	1 h 00 min.
Absolute Ethanol	1 h 00 min.	1 h 00 min.
Absolute Ethanol	1 h 00 min.	1 h 00 min.
Xylol	1 h 30 min.	1 h 30 min.
Xylol	1 h 30 min.	1 h 30 min.
Paraffin	0 h 50 min.	0 h 50 min.
Paraffin	1 h 05 min.	1 h 05 min.







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