

Laser-Assisted Cell Microdissection Using the PALM System

Patrick Micke, Arne Östman, Joakim Lundeborg, and Fredrik Ponten

Summary

Laser-assisted microdissection has enabled the collection of morphologically defined cell populations from a tissue section. The PALM[®] Robot MicroBeam laser microdissection system provides a robust system for the retrieval of specified cells (including single cells). Due to the fragile nature of DNA, and in particular RNA, robust protocols are required to obtain reliable data from a limited number of cells (1–10,000 cells). This chapter describes the application of the PALM MicroBeam system to isolate RNA and DNA from cells in a complex tissue for subsequent molecular analysis. Protocols for successful analysis of RNA from 500 to 1000 cells, including steps to produce cDNA for subsequent polymerase chain reaction analysis, are given. The cDNA could also be used as a template for linear amplification in order to perform gene array analysis. Furthermore, a protocol for genomic analysis of p53 mutations from single cells is given. The described procedures emphasize preparation of tissue, laser microdissection including catapulting of cells, and extraction of RNA and DNA. Downstream experiments for validation are also shown.

Key Words: RNA isolation; real-time quantitative PCR; p53; PALM; microdissection.

1. Introduction

The PALM[®] Robot Microbeam laser microdissection system (P.A.L.M. GmbH, Bernried, Germany) provides a valuable tool for laser microdissection of selected cell populations and single cells from tissue sections. A need for large number of cells often limits the possibility of analyzing defined cell populations due to the mixture of cell types in complex tissues. However, recent technical developments to analyze nucleic acids and proteins from only limited amounts of cells has created a prerequisite for precise microdissection. Microdissection of cells defined under the microscope ensures a relevant

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selection of templates for subsequent molecular analysis. Microscopical evaluation remains the gold standard to determine morphological characteristics that define normal and abnormal cell populations in a tissue; thus, tools for noncontact microdissection are required to define genetic and transcriptional events underlying phenotypic differences between normal and abnormal cells.

The principle of the PALM system is based on a pulsed UVA laser that is focused through the microscope to allow laser ablation of cells and tissue on a tissue section. The physical mechanism for such laser beam function is photofragmentation, a photochemical process that “transforms” biological material into atoms that are blown away at supersonic velocities. This force is restricted to a minute laser focal spot ($<1 \mu\text{m}$), leaving adjacent material (cells, nucleic acids, proteins, etc.) fully intact (**1,2**). This chapter describes the application of the PALM MicroBeam laser microdissection system to isolate RNA and DNA for downstream molecular biological analysis.

The original quality and subsequent handling of tissue used as a template source is of fundamental importance. This is especially true for RNA, which is rapidly degraded by the widespread presence of ribonuclease. We have focused on using fresh, unfixed tissues to develop a robust methodology. Molecular analysis of material fixed in various fixatives is also possible. Formalin fixation degrades genomic DNA and may also create artifactual mutations (**3**). Although selected DNA gene fragments and to a lesser extent mRNA, can be amplified from only few or single fixed cells, more global approaches, e.g., transcription profiling using microarray technology, may require substantially more cells.

2. Materials

1. RNaseZap (Sigma, St. Louis, MO).
2. Superfrost plus charged glass slides (Menzel Gläser, Braunschweig, Germany).
3. PALM LiquidCoverglass (P.A.L.M. AG, Bernried, Germany).
4. Polyethylene membrane covered slides (P.A.L.M. AG.).
5. Zincfix buffer: 5 g ZnCl_2 , 6 g $\text{ZnAc}_2 \cdot 2 \text{H}_2\text{O}$, 0.1 g CaAc_2 , in 1 L 0.1 M Tris-HCl, pH 7.4, make fresh if required, stable at room temperature (RT) for approx 1 wk.
6. 70% Ethanol.
7. 95% Ethanol.
8. Hematoxylin solution.
9. PALM Laser-MicroBeam System (P.A.L.M. AG.).
10. RNase free water (Ambion, Austin, TX).
11. RNasin (Promega UK, Southampton, UK).
12. Zymogen micro RNA Isolation kit (Zymo Research, Orange, CA).
13. Agilent 2100 Bioanalyser (Agilent Biotechnologies, Palo Alto, CA).
14. RNA 6000 Pico Labchip kit (Agilent Biotechnologies).
15. RNA 6000 ladder (Ambion).

16. Oligo-dT primer.
17. Primer for real-time polymerase chain reaction (PCR) analysis of GAPDH.
18. Linear acrylamide (Ambion Ltd., Cambridgeshire, UK).
19. Ultrapure dNTPs (Clontech, Palo Alto, CA).
20. Reverse transcriptase 5X buffer (included in Superscript II kit, Invitrogen, Lidingö, Sweden).
21. DTT (included in Superscript II kit, Invitrogen).
22. Superscript II (included in Superscript II kit, Invitrogen).
23. Microcon 100 columns (Millipore AB, Sundbyberg, Sweden).
24. TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA)
25. PCR buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl.
26. Glass capillary (Femtotips, Eppendorf).

3. Methods

The methods described below outline (1) preparation of tissue samples, (2) staining of tissue sections for microdissection, (3) microchip gel electrophoresis, (4) laser microdissection and laser pressure catapulting, (5) RNA extraction, (6) cDNA synthesis, (7) quantitative real-time PCR, and (8) genetic analysis from single cells. Special care should be taken when handling RNA samples, especially when nanogram amounts are processed (*see Note 1*).

3.1. Preparation of Tissue Samples

After surgical resection, samples are dissected and snap-frozen in liquid nitrogen. Frozen tissue is stored at -70°C until further processing (*see Note 2*). Frozen tissue is cut into 10- μm -thick cryosections and mounted either on glass slides or slides covered with a thin polyethylene membrane (*see Note 3*). The slides are immediately stored at -70°C until further use.

3.2. Staining of Tissue Sections for Laser Microdissection

1. Add to the tissue section mounted on the slide 50 μL hematoxylin mixed with 1 μL RNasin (5000 U/L) on (*see Note 4*).
2. Incubate slide in a cuvet filled with 70 mL Zincofix for 30 s.
3. Incubate in 70% and 95% ethanol for 30 s consecutively.
4. Place the section directly onto the PALM device; after 3 min of air-drying the tissue is ready for laser microbeam microdissection (**Fig. 1**).
5. To improve the poor morphology in air-dried tissue sections, 50 μL of a newly developed PALM LiquidCoverglass diluted in isopropanol (1:3) can be applied directly on the tissue section (**Fig. 1B**). Laser microdissection can be performed after 5 min drying of the section (**4**; *see Note 5*).

3.3. Laser Microdissection and Laser Pressure Catapulting

Sections are laser microdissected following the manufacturer's protocol for the PALM Laser-MicroBeam System. The UV laser microbeam is coupled to

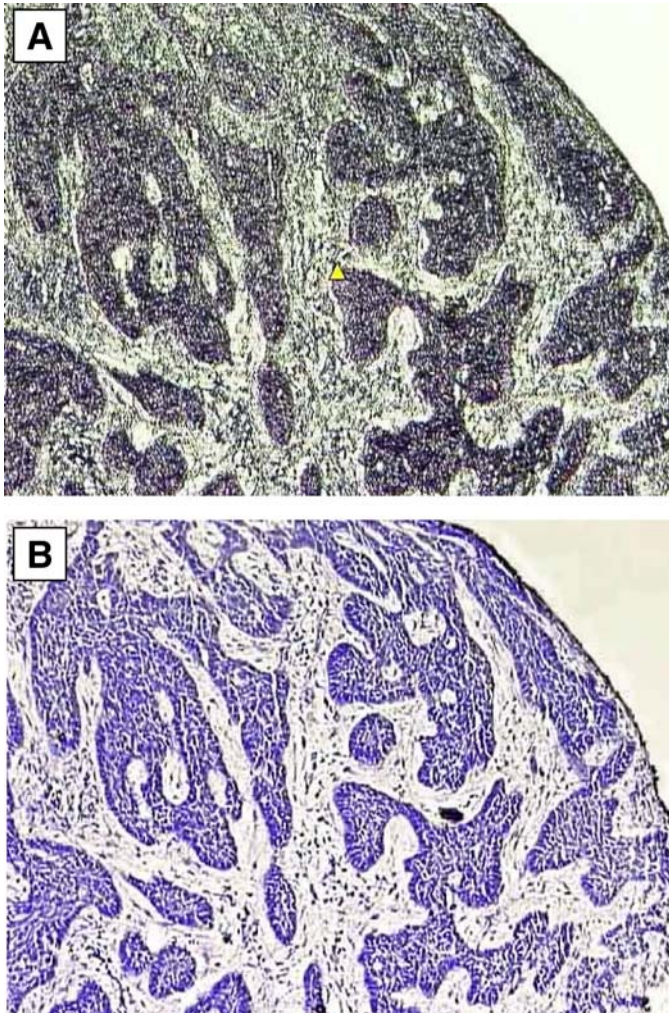


Fig. 1. Morphology of sections for microdissection. Sections of snap-frozen basal cell cancer were stained with hematoxylin. Pictures were taken without cover (A) and with PALM LiquidCoverglass (B).

the epifluorescence illumination port of the microscope. A motorized controlled microscope stage is attached to the microscope and a frame grabber enables the observation of the microscopic image on a computer screen (**Fig. 3A**). The image is overlaid with a graphical user interface enabling the user to perform laser manipulation of tissue directly on the screen (**Fig. 3B**). A microfuge cap moistened with 1 μ L mineral oil and 1 μ L RNAsin (*see Note 7*) is mounted

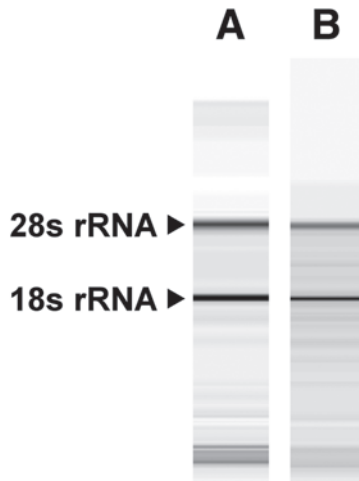


Fig. 2. RNA analysis of unstained and stained tissue. The RNA was extracted from tissue sections and separated with a Pico Labchip in an Agilent 2100 Bioanalyzer. The size range of RNA transcripts is estimated by the ladder marker. The electropherograms were converted to a gel-like image and showed total RNA of the original tissue (A) and from tissue after staining and storage at -70°C (B).

upside down just above the tissue section. To select and isolate areas of interest, microdissection is performed by cutting with a fine focused laser beam producing a gap of 0.5 to 1.2 μm (Fig. 4A). Single unwanted cells can selectively be eliminated with single “shots” of pulsed laser. Following isolation of cells, a high-energy pulse of the focused laser beam just below the focal plane of the tissue specimen is used to create a pressure wave separating the targeted tissue and catapulting it into the microfuge cap. When membrane-mounted slides are used, one single shot is sufficient to catapult the target tissue into the microfuge cap (Fig. 4). When normal glass slides are used multiple pulses are necessary, each catapulting only a fragment of the isolated tissue cells (Fig. 4). In each session approx 500–5000 cells are collected within 30–60 min. Extraction buffer is added into the microfuge tube, vortexed briefly, and centrifuged for 5 min to spin down cells from the lid. The samples are stored on ice or frozen at -70°C .

For genomic analysis of single cells a small glass capillary (Femtotips) are used to transfer the isolated cell to the microfuge tube. The tip of the glass capillary with the attached cell is broken off against the bottom of the microfuge tube containing 10 μL of 1X PCR buffer and the samples are covered with 50 μL of mineral oil.

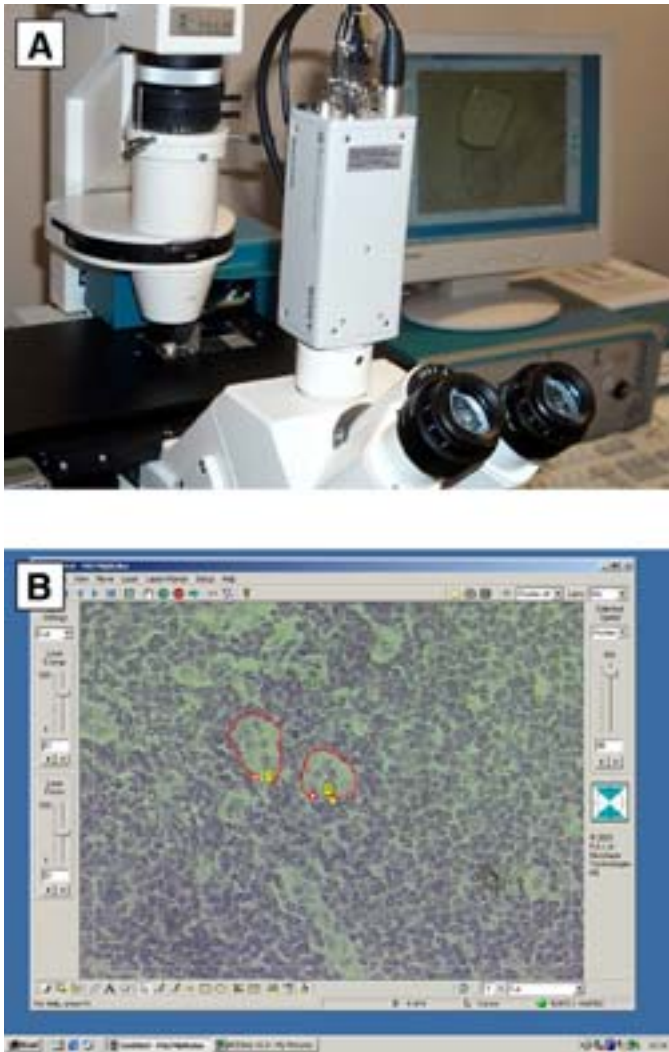
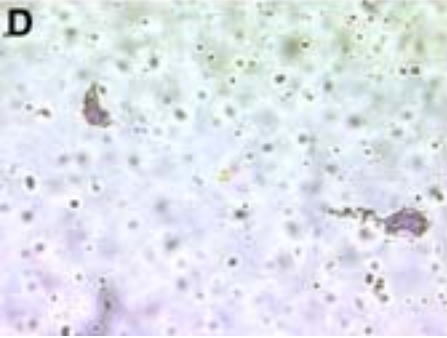
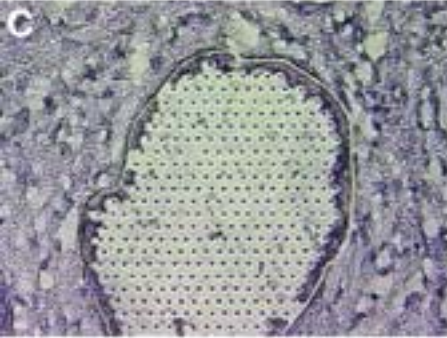
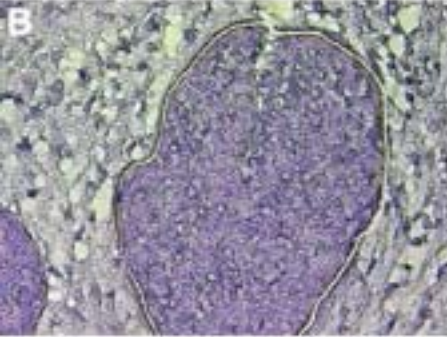
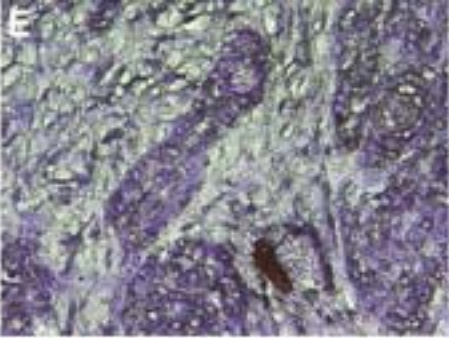
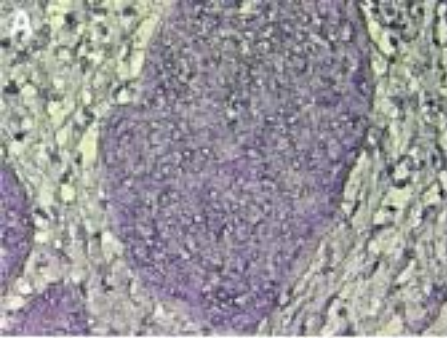


Fig. 3. Image of the PALM microbeam system. The laser system is coupled to a Zeiss light microscope (A). The slides are positioned on a stage, that can be computer controlled (B).

Fig. 4. (*opposite page*) Laser microdissection of basal cell cancer. When glass slides without membrane were used (A), areas of interest were circumscribed and separated from unwanted cells (B). Multiple laser shots are necessary to catapult the tissue into a lid of a PCR tube (C). Cell fragments are visible in the cap (D). If membrane mounted slides are processed (E) and separated (F), the target cells can be catapulted often with a single shot (G). The membrane avoids fragmentation and preserves morphology after catapulting (H).



3.4. RNA Extraction

RNA isolation is performed using a column-based RNA extraction method according to the manufacturer's instructions (Mini RNA isolation Kit, *see* **Notes 8** and **9**). In brief:

1. Dissolve collected cells in 200 μ L RNA extraction buffer.
2. Incubate on ice for 20 min; vortex briefly every 10 min.
3. Add 200 μ L 100% ethanol, mix briefly.
4. Incubate on ice 10 for min.
5. Transfer mixture to spin column and spin at maximum speed for 1 min.
6. Wash twice with 200 μ L wash buffer and repeat 1 min centrifugation.
7. Add 8 μ L RNase-free water directly onto the dry filter and wait for 2 min.
8. Elute in a new collection tube by centrifugation at maximum speed for 1 min.
9. Add 1 μ L RNasin and store at -70°C .

The purified RNA is used for quantitative real-time PCR analysis or, after linear amplification, for global expression analysis on gene arrays. Since these methods are detailed in other chapters, they are only presented brief in this chapter (*see also* **ref. 4**).

3.5. Microchip Gel Electrophoresis

The Agilent 2100 Bioanalyser and a RNA 6000 Pico Labchip kit are utilized to evaluate RNA quality of cells from tissue sections (*see* **Note 6**). One μ L corresponding to 1% (v/v) of each RNA probe is transferred to the Pico Labchip, together with 1 μ L of RNA 6000 ladder. The analysis is performed according to the manufacturer's instructions and an example of results are shown as a gel-like image (**Fig. 2**).

3.6. cDNA Synthesis From Microdissected Cells

cDNA synthesis is performed according to a method that is part of a T7-based linear RNA amplification protocol of Scheidl et al. (**5**). An oligo-dT primer extended with the T7 promoter sequence is used (*see* **Note 10**).

1. Add 1 μ L (1 $\mu\text{g}/\mu\text{L}$) oligo-dT primers (5'-AAA-CGACGGCCAGTGAATTGT AATACGACTCACTATAGGCGCTTTTTTTTTTTTTTTT-3') (**5**) and 0.5 μ L (5 $\mu\text{g}/\mu\text{L}$) of linear acrylamide to the eluted 9 μ L of total RNA.
2. Heat sample to 70°C for 5 min and rapidly cool the sample on ice.
3. Add 4 μ L reverse transcriptase buffer, 2 μ L DTT, 2 μ L ultrapure dNTPs, 1 μ L RNasin, and 2 μ L Superscript II reverse transcriptase.
4. Perform reverse transcription at 42°C for 1 h.
5. When small amounts (<1 ng) of cDNA are expected, purification of cDNA should be considered. This can be performed by phenol extraction followed by three times washing with water on Microcon 100 columns. The volume of the purified cDNA is adjusted to 20 μ L (*see* **Note 11**).

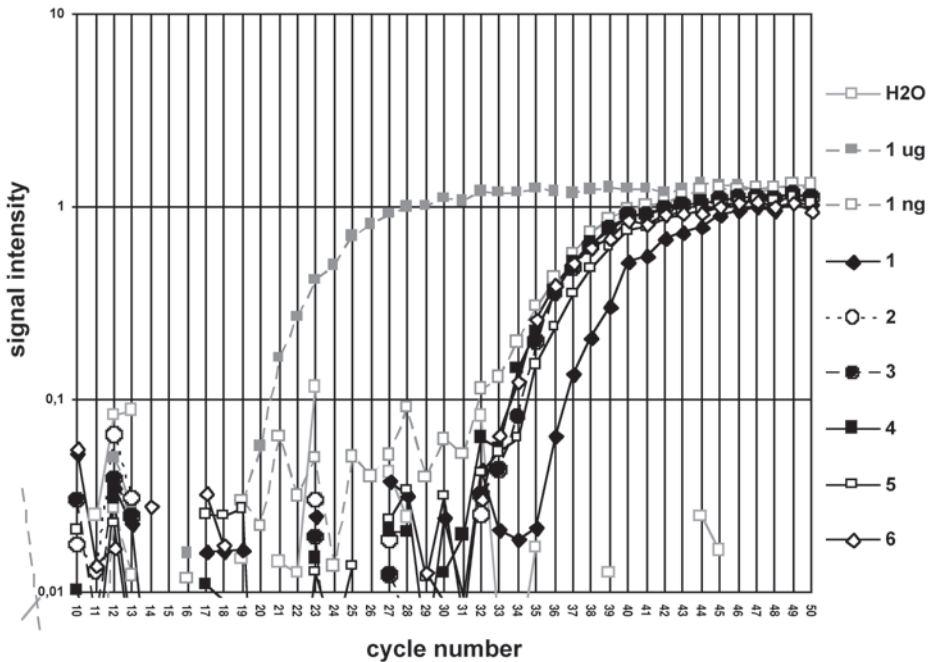


Fig. 5. Real-time PCR of cDNA derived from microdissected tissue. Extracted RNA from microdissected tissue samples (Sample 1: 800 cells; 2: 500 cells; 3: 300 cells; 4: 300 cells; 5: 300 cells; 6: 400 cells) was used for cDNA synthesis. 10% of cDNA was used for TaqMan real-time PCR (A). Amplification curves represent the mean from duplicates. For positive controls 1 μ g or 1 ng of total RNA extracted from a cell line was used. As negative control (H₂O) a sample of water was processed exactly in the same way as the tissue (i.e., RNA extraction, cDNA synthesis, real-time PCR). Please note that sample 2 failed to give an adequate amplification curve.

3.7. Quantitative RT-PCR

In order to validate the RNA extraction procedure and to test its suitability for RT-PCR analysis, primers for the housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are used in TaqMan real-time PCR (Fig. 5). Two microliter (10%) is added to a 50-cycle TaqMan PCR assay using the TaqMan Universal PCR Master Mix with following primers for GAPDH mRNA sequence: Forward primer, 5'-CCCATGTTCTGTCATGGGTGT (200 nM); Reverse primer, 5'-TGGT CATGAGT CCTTCCACGATA (200 nM); Probe, 5'-FAM-CTGCACCAC CAACT GCTTAGCACCC-TAMRA (150 nM). The reaction is performed with the ABI PRISM 7000HT real-time PCR cycler (Applied Biosystems, Foster City, CA) under conditions recommended by the manufacturer (Fig. 5).

3.8. Genetic Analysis of Single Cells

Genomic analysis of the *p53* gene (exons 4–11) can be performed from microdissected single cells by amplification in a multiplex/nested configuration (**Fig. 6, 6,7**). Primers for the mitochondrial sequence are incorporated in the multiplex PCR to generate a specific sequence for each individual (*see Note 13*). The outer multiplex amplification is performed in one tube with 18 primers for 30 cycles. After a 100-fold dilution, inner region specific amplifications for 30 cycles are performed. All primers used are listed in **Table 1**.

1. For the outer multiplex amplification prepare the reagent mix on ice. The volume of each sample is 10 μL consisting of:
 - (a) 2 μL 10X Pfu buffer; (b) 2 μL (2 mM), dNTP; (c) 1 μL outer primer mix (consisting of 5-pmol/ μL of each of the 18 outer primers in water); (d) 0.7 μL Pfu Turbo Polymerase 2.5 U/ μL and (e) 4.3 μL Millipore water. Always prepare sufficient mix for a couple of additional samples, since a small amount is lost during multiple pipetting. Also include some negative controls without DNA—at least 3 for every 10 samples is appropriate, in each run.
2. Add 10 μL of the mix to each single cell sample, just letting the tip touch the liquid surface beneath the oil. Keep samples on ice.
3. Initiate the PCR by denaturation at 98°C for 2 min. Then amplify the samples in two steps. First run 4 cycles of denaturation at 98°C for 15 s; annealing at 55°C for 4 min, and extension at 72°C for 30 min. This is followed by 26 cycles of denaturation at 98°C for 15 s; annealing at 55°C for 30 s, and extension at 72°C for 1 min. End the program with an extension step at 72°C for 10 min and a hold step at 4°C.

Each outer PCR product is used as a template in nine inner amplifications, one for each exon and one from the mitochondrial sequence.

4. For nested amplification a 50- μL reaction is used. Prepare reagent mixes (on ice), one for each fragment. For *p53* exons 4, 6–11 and Mito, the mix per sample consists of:
 - (a) 5 μL 10X PCR buffer II; (b) 5 μL dNTPs (2 mM); (c) 4 μL MgCl_2 (25 mM); (d) 1 μL inner primer (forward)(10 pmol/ μL); (e) 1 μL inner primer (reverse) (10 pmol/ μL); (f) 0.2 μL AmpliTaq DNA polymerase 5 U/ μL ; and (g) 33.3 μL Millipore water. For *p53* exon 5 the mix consists of: (a) 5 μL 10X PCR buffer II; (b) 5 μL dNTPs (2 mM); (c) 5 μL MgCl_2 (25 mM); (d) 1 μL inner primer (forward)(10 pmol/ μL); (e) 1 μL inner primer (reverse)(10 pmol/ μL); (f) 0.7 μL Pfu Turbo polymerase (2.5 U/ μL); and (g) 31.8 μL Millipore water.
5. For each sample dispense 49.5 μL of the reagent mix in an Eppendorf tube (0.5 mL) and cover with 30–50 μL mineral oil.
6. Add 0.5 μL of the outer reaction product as template. Make sure that the oil layer is penetrated when dispensing.

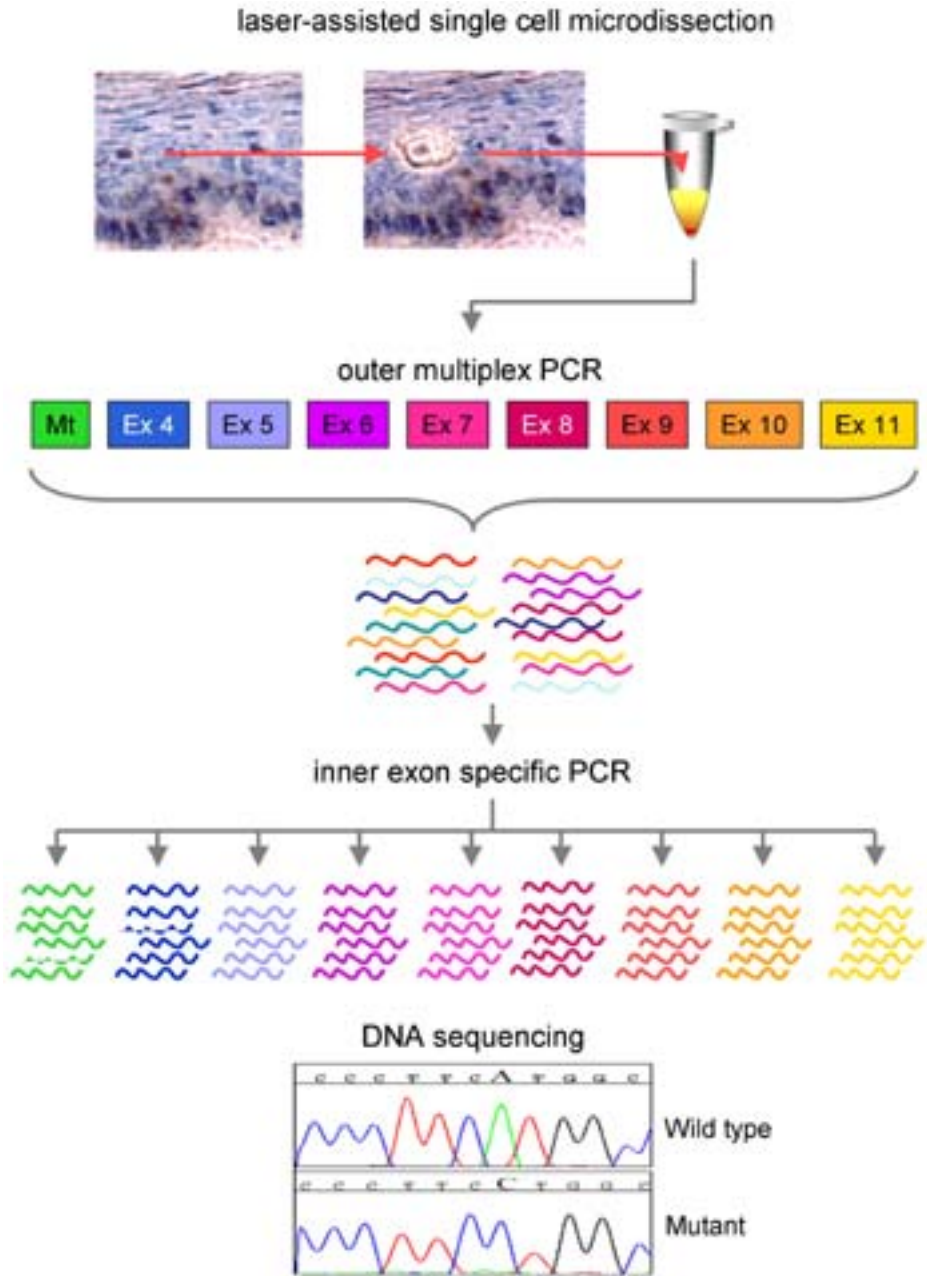


Fig. 6. Schematic illustration of the different steps involved in genetic analysis of the p53 gene from single cells. The strategy of multiplex/nested PCR followed by direct DNA sequencing is depicted.

Table 1
Primers Used for Multiplex PCR of Genomic DNA From Single Cells

Target	Amplification	Primer sequence	
		Forward	
Exon 4	Outer	5'-CTGGGACCTGGAGGGCTGGG	5'-AGAGGAATCCCAAGTTCCA
	Inner	5'-CTGAGGACCTGGTCCTCTGAC	5'-ATACGGCCAGGCATTTGAAAGT
Exon 5	Outer	5'-TGCTGCCGTGTTCCAGTTGC	5'-CAATCAGTGAGGAATCAGAGG
	Inner	5'-TTCACTTGTGCCCTGACTT	5'-ACCAGCCCTGTCGTCTCTCC
Exon 6	Outer	5'-GGCTGGAGAGACGACAGGGC	5'-CGGAGGGCCACTGACAAACCA
	Inner	5'-TTGCCAAGGTCCCCAGGCC	5'-CTTAACCCCTCCTCCCAGAG
Exon 7	Outer	5'-CCTCCCCTGCTTGCCACAGG	5'-GGAAGAAATCGGTAAGAGGTGG
	Inner	5'-CGCACTGGCCCTCATCTTGGG	5'-CAGCAGGCCAGTGTGCAGGG
Exon 8	Outer	5'-ACAGGTAGGACCTGATTTCC	5'-TGAATCTGAGGCATAACTGC
	Inner	5'-GCCTCTTGCTTCTCTTTTCC	5'-CCCTTGGTCTCCTCCACCGC
Exon 9	Outer	5'-AGCAAGCAGGACAAGAAGCG	5'-GTTAGCTACAACCAAGGACC
	Inner	5'-GCCTCAGATTCACTTTATCACC	5'-CTGGAAACTTTCCACTTGTAT
Exon 10	Outer	5'-GATCCGTCATAAAGTCAAAC	5'-TTGACCATGAAGGCAGGATG
	Inner	5'-CTTGAAACCATCTTTTAACCTCAGG	5'-AATCCTATGGCTTCCAACTTAGG
Exon 11	Outer	5'-CTTCAAAGCATTTGGTCAGGG	5'-GGGTTCAAAGACCCAAAACC
	Inner	5'-CACAGACCCCTCACTCATG	5'-GCAGGGGAGGAGAGATGGG
Mito.	Outer	5'-CCTGAAGTAGGAACCAAGATG	5'-ACACCAAGTCTTGTAAACCGG
	Inner	5'-CTCCACCAATTAGCACCCAAAG	5'-TGATTTACGGGAGGATGGTGG

7. Initiate PCR by denaturation at 94°C for 5 min (Taq Polymerase)/98°C for 2 min (Pfu polymerase). Amplify the samples for 30 cycles by denaturation at 94°C for 30 s/ 98°C for 15 s, annealing at 63°C for 30 s and extension at 72°C for 1 min. The program is ended with an extension step at 72°C for 10 min and a hold step at 4°C.
8. The resulting PCR products are analyzed on a 1% agarose gel.

4. Notes

Since we started to work with the PALM system many modifications and variations have been made to facilitate techniques and improve protocols.

1. The work with RNA requires lab work under RNase-free conditions. The working place and pipets should be decontaminated with RNaseZap. Gloves should be worn at all times and be changed frequently. Only RNase-free filter pipette tips should be used. If possible, all reagents used should be RNase-free.
2. The quality of the original material is most important for all kinds of RNA analyses. Best results were obtained with tissue specimens, which were immediately frozen after resection. Other possibilities that were suggested involved storage in Zincfix (8) or commercially available buffers (e.g., RNAlater from Ambion). Formalin fixation and paraffin embedding leads to rapid RNA degradation, making downstream applications very difficult (8,9). Although there are several descriptions of successful RNA amplification from formalin-fixed and paraffin-embedded tissue using PCR methods (10–12), this option should be considered only if no other materials are available. If OCT or other freezing media are used it is important to remove them before laser microdissection (e.g., incubation in water). If the tissue specimens are embedded the OCT is usually removed by incubation in staining and dehydrating solutions.
3. In order to shorten and facilitate the staining procedure the tissue specimens can also be stained directly after cutting in the cryostat and then stored, after dehydration, at –70°C.
4. In general the use of membrane-coated slides is preferable for several reasons: (1) The membrane serves as a backbone underlying the tissue and facilitates the laser catapulting. (2) It protects the sample from contaminating tissue fragments. (3) After catapulting the complete tissue fragment can be found with excellent preserved morphology in the lid. (4) Our experience indicates higher amounts of extracted RNA and more reproducible results when membrane-coated slides were used. However, some users state that no differences were observed when both slide types were compared (membrane coated vs non-membrane-coated).
5. Instead of hematoxylin, other staining reagents could be considered. A recent study suggested Nuclear Fast Red to be best in terms of preserving RNA integrity (13). In general incubation times should be kept as short as possible in any staining procedure.
6. Analysis of quantity and integrity of RNA is an important and necessary step prior to later applications. This avoids laborious and expensive processing of RNA probes of low quality or RNA subjected to degradation during handling.

Furthermore it circumvents misinterpretation of results. A very useful tool in this respect is the Bioanalyzer (Agilent). The combination of capillary electrophoresis, microfluidics, and nucleic acid binding dyes allows for evaluation of both concentration and quality of RNA. However, since the RNA has to be diluted in pure water, salt and proteins remaining in the RNA sample could lead to false negative results. Moreover the detection limit is around 1–0.1 ng/ μ L. Therefore the application for microdissected cells is most often limited to tissues with very high RNA content (e.g., liver) or high cell numbers (>5000 cells).

7. The morphology of air-dried uncovered sections under the light microscope is poor, which limits the repertoire of cell types that can be isolated without contamination. Single cells are hardly distinguished, and counter staining or other forms of labeling are less effective. A method to overcome some of these problems and to improve morphology is to apply PALM LiquidCoverglass, a fluid that was developed by Tone Bjørnsen and has been shown not to affect RNA integrity (4).
8. When normal glass slides (without membrane covering) are used for laser microdissection, it is recommended to apply at least 30 μ L of fluid in the lid for laser catapulting. Preferentially the buffer for the later RNA extraction should be applied (e.g., Zymogen RNA extraction buffer, TRIzol, etc.).
9. The method of RNA extraction can be crucial. We tested different microcolumn-based RNA isolation kits and saw significant differences in RNA quantity and quality. Inexpensive and effective alternatives are phenol-based extraction methods (e.g., TRIzol), followed by an alcohol precipitation with a coprecipitant (e.g., linear acryl amide or Pellet Paint). However, the recovery rate of RNA samples of known concentration should be tested before including a new method or kit in the RNA extraction process.
10. The amount of isolated RNA depends not only on the amount of microdissected cells but also on the type of cells that were isolated. For instance, metabolic active liver cells contain much more RNA than inactive fibroblasts or osteoblasts (Ambion, technical notes 8/2003).
11. The cDNA synthesis was performed according to a method, that is part of a T7-based linear RNA amplification protocol of Scheidl et al. (5). It includes an oligo-dT primer with the T7 promoter sequence. This enabled us not only to perform quantitative real-time PCR, but also to amplify good quality preparations to μ g amounts of Cy-labeled RNA for gene array analysis. If this application is not appropriate, a common poly-dT primer can be used instead. The sensitivity might improve by using random hexamers. However, this could also result in shorter transcripts and increase the risk of artifacts caused by DNA contaminations.
12. If very small amounts of RNA (<10 ng) are reverse transcribed and analyzed by real-time PCR, purification of cDNA before amplification could be advantageous. The unfavorable ratio between cDNA on one hand and enzymes, poly dT-primers, and nucleotides on the other hand can negatively affect the polymerase chain reaction.
13. Mitochondrial DNA can be used as a specific signature for each sample and thus serves as a control for contamination between samples. Since there are approx

1000 copies of mitochondrial DNA in each cell compared to two copies of the *p53* gene, amplification of mitochondrial DNA can also be used to distinguish between loss of the cell (no amplified fragments) and degradation of the template (amplification only of mitochondrial DNA) in cases of amplification failure.

14. Two potential problems with single-cell PCR include risk of contamination and random amplification failure of one allele, i.e., allele dropout (ADO) (6,7). Preparation of PCR reagent mixture, adding PCR reagent mixture to the single cell sample and adding outer PCR product to the reagent mix for the inner PCR should be performed in separate rooms to avoid contamination. Coats and gloves should be used at all times. All pipetting should be performed with filtertips and the bench area where the template is added should be decontaminated using UV light. Negative controls without DNA should be included in abundance.
15. The random frequency of ADO, using the outlined procedure with frozen sections, has been approx 50%. ADO is important to consider when interpreting data. The ADO rate in cultured cells was lower compared to cells retrieved from tissue sections. The reported ADO rates, usually based on analysis of cultured cells, are between 10 and 80% and are known to be affected by fragment length and number of fragments amplified in parallel.

Useful Links

16. www.ambion.com/techlib/index.html: Very useful technical notes about the work with RNA.
17. <http://www.palm-mikrolaser.com>: Technical notes and protocols for the use of the PALM laser systems.

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References

- 1 Schutze, K., Burgemeister, R., Clemment-Sengewald, A., Ehnle, S., Friedemann, G., Lahr, G., et al. (2003) Non-contact live cell laser micromanipulation using PALM microlaser systems. *P.A.L.M Scientific Edition*, 11.
2. Schutze, K., Posl, H., and Lahr, G. (1998) Laser micromanipulation systems as universal tools in cellular and molecular biology and in medicine. *Cell. Mol. Biol. (Noisy-le-grand)* **44**, 735–746.
- 3 Williams, C., Ponten, F., Moberg, C., Soderkvist, P., Uhlen, M., Ponten, J, et al. (1999) A high frequency of sequence alterations is due to formalin fixation of archival specimens. *Am. J. Pathol.* **155**, 1467–1471.
- 4 Micke, P., Bjørnsen, T., Scheidl, S., Strömberg, S., Demoulin, J. B., Ponten, F., et al. (2004) A fluid cover medium provides superior morphology and preserves

- RNA integrity in tissue sections for laser microdissection and pressure catapulting. *J. Pathol.* **202**, 130–138.
- 5 Scheidl, S. J., Nilsson, S., Kalen, M., Hellstrom, M., Takemoto, M., Hakansson, J., and Lindahl, P. (2002) mRNA expression profiling of laser microbeam microdissected cells from slender embryonic structures. *Am. J. Pathol.* **160**, 801–813.
 - 6 Persson, Å., Ling, G., Williams, C., Bäckvall, H., Ponten, J., Ponten, F., and Lundeberg, J. (2000) Efficient analysis of single cells obtained from histological tissue sections. *Anal. Biochem.* **287**, 25–31.
 - 7 Ling, G., Persson, Å., Berne, B., Uhlen, M., Lundeberg, J., and Ponten, F. (2001) Persistent p53 mutations in single cells from normal human skin. *Am. J. Pathol.* **159**, 1247–1253.
 - 8 Wester, K., Asplund, A., Backvall, H., Micke, P., Derveniece, A., Hartmane, I., et al. (2003) Zinc-based fixative improves preservation of genomic DNA and proteins in histoprocessing of human tissues. *Lab. Invest.* **83**, 889–899.
 - 9 Vincek, V., Nassiri, M., Knowles, J., Nadjji, M., and Morales, A. R. (2003) Preservation of tissue RNA in normal saline. *Lab. Invest.* **83**, 137–138.
 - 10 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.
 - 11 Becker, I., Becker, K. F., Rohrl, M. H., and Hofler, H. (1997) Laser-assisted preparation of single cells from stained histological slides for gene analysis. *Histochem. Cell Biol.* **108**, 447–451.
 12. 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.
 13. Burgemeister, R., Gangnus, R., Haar, B., Schutze, K., and Sauer, U. (2003) High quality RNA retrieved from samples obtained by using LMPC (laser microdissection and pressure catapulting) technology. *Pathol. Res. Pract.* **199**, 431–436.