

Combined Laser-Assisted Microdissection and Short Tandem Repeat Analysis for Detection of *In Situ* Microchimerism After Solid Organ Transplantation

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Summary

Following the transplantation of a solid organ leukocytes of donor origin migrate out of the organ, contributing to a chimeric blood cell population (“peripheral microchimerism”). At the same time, leukocytes and pluripotent precursor cells of the recipient migrate into the organ, creating an “*in situ* microchimerism.” A method is described for the identification of cells with the recipient’s genotype in the transplanted organ by combining laser-assisted microdissection and short tandem repeat analysis. The microdissection allows the contamination-free isolation of morphologically and immunohistochemically characterized cells or groups of cells from histological tissue sections. The subsequent analysis of highly polymorphic short tandem repeats enables unequivocal genotyping in nearly all donor-recipient instances. Employing this new methodological approach, we could identify in individual transplanted organs differentiated parenchymal cells of recipient’s origin, which most probably are derived from circulating precursor cells from the bone marrow.

Key Words: Organ transplantation; STR analysis; *in situ* microchimerism; microdissection.

1. Introduction

The transplantation of a solid organ inevitably creates a chimeric organism (“macrochimerism”), because donor and recipient have a different genotype (with the exception of identical twins). In addition to the transplanted organ, leukocytes are also transferred from the donor to the recipient. This creates a chimeric leukocyte population in the blood circulation of the recipient, a phenomenon called “peripheral microchimerism” (1,2). At the same time circulating cells from the recipient migrate into the new organ, thereby creating a so-called “*in situ* microchimerism.”

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It has been 40 yr since the hypothesis was proposed that cells from the recipient may stably populate the transplanted organ (3,4), thereby modulating the antigenicity of the foreign tissue in the recipient's body. However, to date experimental evidence has been lacking. In particular the question of whether parenchymal cells (e.g., hepatocytes or bronchial epithelium) of recipient origin can be found in the transplanted organ was denied until quite recently (e.g., ref. 5).

In animal models the discrimination between donor and recipient cells and the identification of parenchymal cells of recipient's origin can be achieved by selecting animals with differing genetic markers for transplantation experiments or by utilizing genetically engineered animals with easily detectable markers, such as the green fluorescent protein (GFP) (6-8). In humans this is obviously not possible. Earlier studies relied on the immunohistochemical analysis of blood group antigens; however, under many circumstances this methodology cannot discriminate unequivocally between donor and recipient, because the blood group antigens are usually very similar (9).

More recently, the detection of the Y-chromosome by fluorescence or chromogenic *in situ* hybridization (FISH or CISH) (10) in case of cross-gender transplantations has been successfully used by several groups (11-13). The limitation of this approach is the requirement for a sex mismatch. The preferred situation for Y-chromosome hybridization is the transplantation of a female organ into a male recipient because in this situation the cells of interest (the recipient's cells in the donor organ) are marked positively by the presence of a Y-chromosome signal. Because the majority of organ donors are younger males, less than one quarter of the archived biopsies can be analyzed by this method.

In order to develop a methodology that is completely independent of gender mismatch and that has the potential to analyze all available biopsies (especially formalin-fixed and paraffin-embedded biopsies), we combined the isolation of the cells of interest from histological sections using laser-assisted microdissection and the subsequent genotyping utilizing highly polymorphic genetic markers (so-called short tandem repeats or STRs [14]) for the identification of recipient's cells in the transplanted organ. Short tandem repeats are nucleotide repeats (di-, tri-, and tetranucleotide repeats), which are dispersed throughout the human genome. The lengths of these repeats show a quite high allelic variability and the combination of several STRs can be utilized for the unequivocal identification of any individual. In the situation of a transplanted organ only two individuals have to be differentiated by their genotype. Therefore, the analysis of only one highly polymorphic marker, such as the tetranucleotide repeat at the SE33 locus, is sufficient.

Immunohistochemical labeling prior to laser microdissection enables the clear distinction of circulating leukocytes from the recipient in the transplanted organ (CD45 or CD68 positive) from parenchymal cells of recipient's origin (e.g., cytokeratin positive).

Using this new methodology we sought to find out when and to what extent liver cell chimerism after transplantation occurs, how it is correlated with rejection, and whether it influences the long-term fate of the graft (15).

2. Materials

Manufacturers or distributors are specified only if reagents or laboratory equipment might be important for the outcome, or if a source might be difficult to identify. All chemicals were purchased in analytical grade quality from Merck, Roth, or Sigma and kept strictly separate from the postamplification area in our institute.

1. Glass slides.
2. Cyanacrylate glue (UHU, Buehl, Germany).
3. Soft brush, for smoothing the foil on the glass slides before fixing it with glue.
4. Poly-L-lysine (0.1% aqueous solution, Sigma, Taufkirchen, Germany), stored at 4°C.
5. Poly-propylene foil, 1.2 µm (PALM, Bernried, Germany).
6. "Xylol-Ersatz," a xylene substitute, which is less toxic and smells less unpleasant (Vogel, Karlsruhe, Germany).
7. Ethanol (100%, 96%, 70%).
8. Glass cuvetts.
9. ABC Vectastain-Kit (Vector Laboratories, Burlingame, CA).
10. Methylene blue (Loeffler's Methylene blue, Merck, Darmstadt, Germany).
11. Methyl green (Merck). Staining solutions are stored at room temperature in the dark.
12. Antibodies: LCA (1:100, Dako, Hamburg, Germany), CD68 (1:100, Dako), Cytokeratin 8/18 (1:100, Dako).
13. Liquid wax (MJ Research, Boston, MA).
14. Proteinase K-buffer: 50 mM Tris-HCl, pH 8.1, 1 mM EDTA, 0.5% Tween-20.
15. Proteinase K, stock solution: 20 mg/mL in water, aliquots stored at -20°C (Merck).
16. TE buffer: 10 mM Tris-HCl, pH 8.1, 1 mM EDTA
17. 0.5-mL Tubes with transparent lid and lowered inner lid, for collecting dissected and catapulted cells (PALM).
18. Taq-Polymerase: Hot Start Taq (Qiagen, Hilden, Germany).
19. Polymerase chain reaction (PCR)-buffer: supplied with the enzyme.
20. Nucleotides for PCR: 10 mM dNTP-Mix (MBI, Fermentas, St. Leon-Roth, Germany).
21. PCR tubes: 0.2-mL PCR-tubes, colorless (no. 710900, Biozym, Hessisch Oldendorf, Germany).

22. PCR primer: 5'-6FAM-AGAGAGAGAAAGGAAGGAAGG 5'-CTACC GCTATAGTAACTTGC One primer is labelled at the 5' end with 6-FAM (in our case the forward primer, but the reverse primer can also be labeled) (*see Note 1*).
23. Formamide: deionized, minimum 99.5% (GC), for molecular biology (F-9037, Sigma).
24. Septa for 0.5-mL sample tubes (no. 401956, Applied Biosystems, Darmstadt, Germany).
25. GeneScan350 ROX Size Standard (no. 401735, Applied Biosystems).
26. Matrix for capillary: Performance Optimized Polymer (POP-4, Part no. 402838, Applied Biosystems).
27. Tips with aerosol protection, DNase-, RNase-free (Sarstedt, Nümbrecht, Germany).
28. HPLC-water (no. 4218, JT Baker, Deventer, Holland).
29. 3 M Sodium acetate, pH 7.0, containing 100 µg/mL dextran T500 (Sigma).
30. Hypochlorite solution, for use diluted 1:4 with water (Roth).
31. PCR bench with UV lamp, for decontamination of racks and irradiation of polypropylene foil.
32. Laser microdissection system (PALM).
33. Sequencer ABI310 (Applied Biosystems).
34. Refrigerated table-top centrifuge for 0.2- to 2.0-mL tubes (max. 14,000g).
35. Vortex.
36. 40°C Incubator.
37. Thermoshaker with heated lid (CLF, Emersacker, Germany).

3. Methods

The protocols described below for STR analysis after laser-assisted microdissection concentrate on the following steps:

1. Organization of the laboratory.
2. Preparation of foil-coated glass slides for microdissection.
3. Cutting and staining histological tissue sections.
4. Laser microdissection and specimen recovery.
5. Isolation of DNA from microdissected cells.
6. STR-PCR.
7. Analyzing fluorescent-labeled PCR products.

3.1. Organization of the Laboratory

In order to prevent any cross-contamination of samples that could lead to false-positive results (indicating chimerism of cell populations that are actually not chimeric), strict guidelines for the laser-assisted microdissection and the setup of the PCR mixtures, as well as for the physical separation of the analysis of reaction products (postamplification) from all stages of sample preparation (preamplification), have to be implemented.

For these reasons, strictly enforced protocols concerning the cleaning of instruments and the handling of samples before and after amplification must be followed by all personnel involved. We perform all preamplification steps, including the laser-assisted microdissection, in a separate laboratory consisting of two rooms: one for setting up the PCR master mix (a “template-free” room) and the other for preparation of tissue sections, microdissection, nucleic acid extraction, and adding DNA to the PCR mixes. Plastic labware and the benches are cleaned regularly using a 3% hypochlorite solution. The PCR products are analyzed in a separate laboratory. Under no circumstances should amplified samples or equipment from this working area be brought back to the pre-PCR area.

3.2. Preparation of Foil-Coated Glass Slides

1. The tissue section is mounted on a glass slide coated with a very thin foil. This foil serves as a carrier for the tissue and is glued to the glass slide only at the four corners. Therefore, after microdissection the dissected piece of tissue can be easily removed together with the supporting foil, which is completely inert and does not interfere with any subsequent analysis.
2. Because manufactured slides coated with polypropylene foil are very expensive, we buy the foil from PALM and prepare the coated slides ourselves.
3. For coating with poly-L-lysine a drop of the solution (0.1% in sterile water) is spread over the membrane with a sterile pipet tip, carefully avoiding any damage of the very thin foil. (Any leakage underneath the membrane might result in problems with the laser pressure catapulting technology, because the poly-L-lysine will glue the foil onto the glass slide.)
4. The foil, pretreated in this way, is cut into appropriately sized pieces and mounted onto the glass slides.
5. It is very important to use a very sharp scalpel blade to avoid damaging the foil.
6. Any fold in the foil is removed with a clean soft brush and it is fixed at the four corners with cyanacrylate glue from UHU (Buehl, Germany) (*see Note 2*).
7. After curing overnight, the foil-coated glass slides are irradiated for 45 min with short-wavelength UV light to destroy all traces of DNA or RNA (*see Note 3*).

3.3. Specimen Preparation and Staining

1. Formalin-fixed paraffin-embedded biopsies are cut using a conventional microtome and sections are mounted on foil-covered slides.
2. After cutting a biopsy the cryotome blade and the sample holder are cleaned meticulously before the next biopsy is cut to avoid any cross-contamination.
3. To improve adhesion, the slides with sections are incubated for 15 min at 55°C.
4. Afterward, the sections are dewaxed and rehydrated using “Xylol-Ersatz” and ethanol (2X xylene substitute for 10 min, 2X 100% ethanol for 5 min, 2X 96% ethanol for 5 min, 1X 70% ethanol for 5 min, sterile water for 5 min).

3.3.1. Conventional Staining

For staining with methylene blue, the hydrated sections are covered with staining solution for 30 s, rinsed twice with sterile water, dehydrated with a drop of absolute ethanol, and allowed to dry at room temperature.

3.3.2. Immunohistochemical Staining

1. For labeling cells with antibodies before microdissection we use the Vectastain kit from Vector Laboratories (*see Note 4*). As outlined above, all reagents brought into contact with samples before amplification (pre-PCR) have to be strictly separated from all other reagents used in the laboratory (post-PCR). The great advantage of ready-to-use kits is that the components are free of any potentially contaminating PCR products and completely separated from all reagents normally used in the laboratory. This justifies the higher costs.
2. Antibodies directed against CK8/18 and LCA and CD68 are used in a 1:100 dilution.
3. Nonspecific binding to the sections is prevented by preincubation for 20 min with horse serum provided with the kit.
4. For incubation with antibodies and the detection reaction, the manufacturer's instructions are followed.

3.4. Microdissection Using PALM Laser Microdissection Microscope

1. The width of the laser cut can be altered by adjusting the laser energy and/or the focus of the laser.
2. In our laboratory, the optimal focus for using the 40× long-distance objective from Zeiss (40x/0.60 Korr /0–2) is around 980 (arbitrary units); for using the 10× objective from Zeiss (10x/0.50 /0.17) the optimal value is 680.
3. Depending on the thickness of the section and the tissue type, the energy for cutting is between 920 and 1000 (arbitrary units), for catapulting greater than 1020.
4. The actual numbers for the energy and focus setting may vary slightly for different instruments (*see Note 5*).
5. For recovery of dissected cells using the laser pressure catapulting technology of the PALM system, the 0.5-mL tubes distributed by PALM itself are most suitable because of the lowered inner lid, which shortens the distance the catapulted cells have to travel. Also the specimens are readily visible in the transparent lid (*see Fig. 1*).
6. For catapulting a dissected cell or a group of cells, the laser is focused slightly below the section and the laser energy is increased.
7. A single short laser pulse is sufficient for catapulting the specimen into the lid of a reaction tube placed directly above the section.
8. The lid of the reaction tube is conveniently positioned in the holder of the micro-manipulator (*see Note 6*).
9. Despite having the very sophisticated (and also often necessary) laser pressure catapulting technology we recover dissected pieces of tissue with a sterile needle

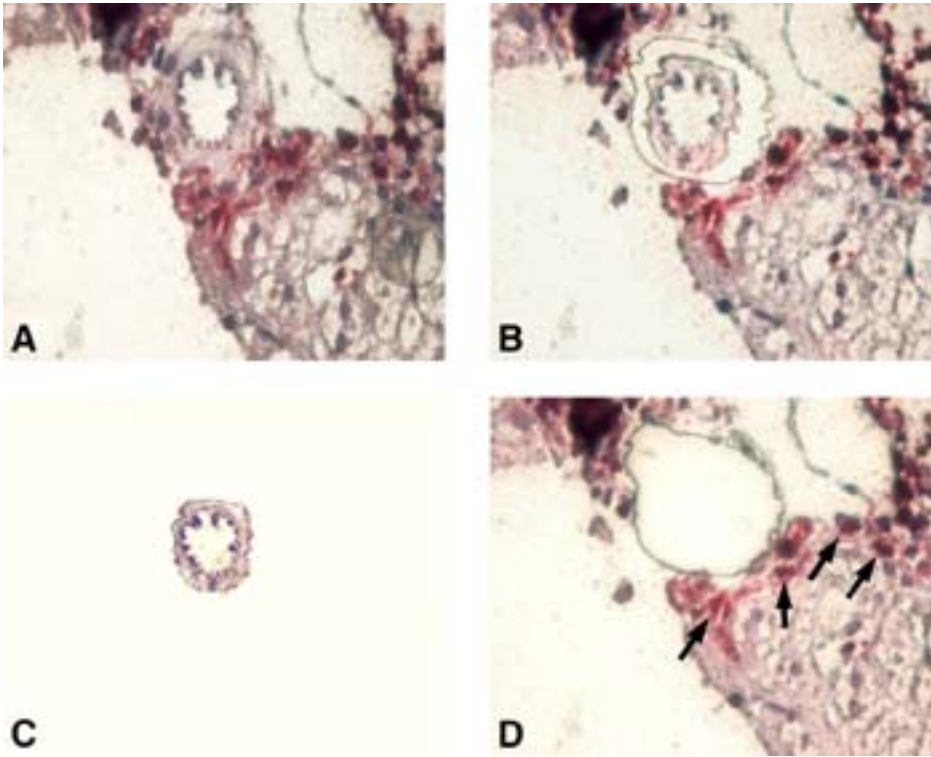


Fig. 1. Laser-assisted microdissection and isolation of endothelial cells from a liver vessel. Inflammatory cells are labelled immunohistochemically (CD68 and LCA, *see* arrows). (A) Section before microdissection. (B) Endothelial lining dissected. (C) Isolated endothelial cells in the lid of a reaction tube. (D) Section after removal of endothelial cells (fast red and hematoxylin counterstain; original magnification: $\times 100$). The reduced optical quality is because the tissue section is dried and not cover-slipped.

if they are large enough. This can be done easily by hand without any technical support. After some practice this turns out to be straightforward for each person working with the laser microscope.

3.5. Isolation of DNA

1. Small numbers of cells are lysed in the lid of the reaction tube by adding 10–30 μL TE-buffer containing 40 μg proteinase K.
2. The closed tubes are incubated in a small incubator in an inverted position at 45°C overnight.
3. The next day samples are centrifuged and heated for 8 min at 95°C for inactivation of proteinase K and efficient denaturation of the DNA.
4. This lysate is used directly for subsequent PCR analysis.

5. If thousands of cells are isolated the samples are lysed in a larger volume of proteinase K buffer (100–300 μL) containing 500 $\mu\text{g}/\text{mL}$ proteinase K.
6. The samples are incubated in a vigorously shaking thermoshaker at 56°C overnight.
7. The next day the lysate is transferred to a new tube (*see Note 7*) and the DNA is precipitated by adding sodium acetate (pH 7.0) containing dextran T500 as a carrier (100 $\mu\text{g}/\text{mL}$) and ethanol.
8. This precipitation step almost completely removes contaminating dyes and cell debris.
9. After centrifugation and washing of the pellet with 70% ethanol the DNA is air-dried and dissolved in 30–50 μL of sterile water.

3.6. Short Tandem Repeat-PCR

1. The amplification reaction is performed in a final volume of 25 μL containing 200 nM of each primer, 0.5 U Hot Start Taq Polymerase, 1.5 mM MgCl_2 , 250 nM of dNTP, and up to 10 μL DNA lysate.
2. The forward primer is labeled with 6-FAM at the 5' end.
3. The reaction mixture is preheated at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with a final elongation step at 72°C for 10 min (*see Note 8*).
4. As described above (**Subheading 3.1.**), the PCR is set up in a completely separate room under strict guidelines to prevent cross-contamination.

3.7. Analysis of Fluorescence-Labeled PCR Products

1. For the analysis of PCR products, 1 μL of the reaction mix is mixed with 0.3 μL size standard (GeneScan350) and 12 μL formamide.
2. This mixture is heated for 2 min at 90°C and chilled immediately on ice (*see Note 9*).
3. The samples are placed in the sample holder and electrophoresis is started and analyzed as described in detail by the manufacturer (*see Fig. 2*).
4. We use routinely an injection time of 5 s.

4. Notes

1. This new primer pair for the SE33 locus was designed to reduce the length of the PCR products by 85 bp compared to the original SE33 primers (**16,17**) to obtain fragments ranging from 140 to 236 bp in length.
2. We tested several glues and tapes, following tips and hints from colleagues, and PALM. Cyanacrylate glue (“two-component glue”) turned out to be the best, almost completely resisting xylene and 100% ethanol. All other glues or tapes tested dissolved in one of these solvents. But even this glue starts dissolving after prolonged incubations of longer than 45 min.
3. It is very important to wait until the glue is completely cured, before UV irradiation starts, because otherwise the glue will never set and will dissolve rapidly in xylene or the xylene substitute.

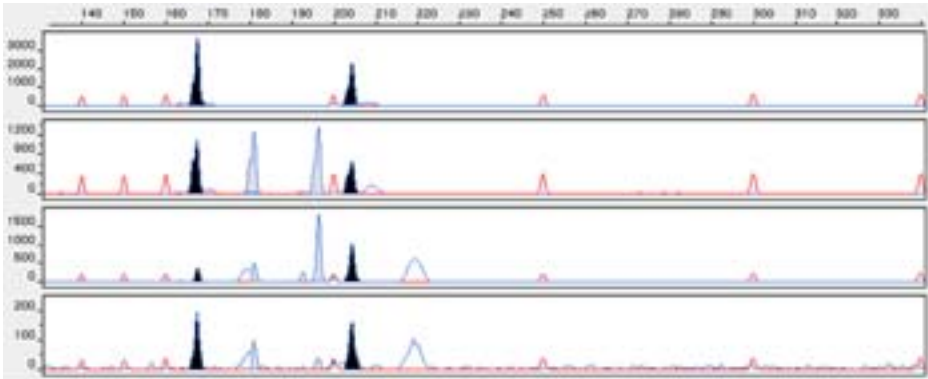


Fig. 2. Examples of PCR product analysis employing the ABI310 capillary sequencer from Applied Biosystems. **(A)** Recipient's genotype (explanted organ). **(B)** Genotyping of a whole section. Chimerism is clearly discernible: All four alleles from the donor and the recipient are visible. **(C)** Laser microdissected cholangiocytes displaying *in situ* microchimerism. **(D)** Laser microdissected leukocyte infiltrate displaying primarily the genotype of the recipient.

4. Only if one routinely performs immunohistochemical staining for laser microdissection on a large scale is the "in-house" set-up of detection reagents under PCR contamination-free conditions cost-effective. We didn't perform a comprehensive comparison of commercially available staining kits; therefore other kits might work as well.
5. The width of the laser cut depends very much on the thickness of the section and the type of tissue structure that has to be cut (e.g., adipose tissue is very easily cut; connective tissue is often quite resistant). Therefore, the energy sufficient for a fine cut through fat tissue will not be sufficient for cutting connective tissue structures and the energy adjusted to the latter tissue type will create a quite broad, irregular cut through fat tissue. However, adjustment of the appropriate laser focus and energy must be learned by trial and error; the values given in the text are only a guide.
6. Dissected cells can also be catapulted into the lid of a reaction tube without changing the focus of the laser, but this will create a "bullet hole" in the specimen. This is no problem if larger structures are dissected and the laser "bullet hole" can be placed in an irrelevant part of the specimen (e.g., the lumen of a vessel dissecting the vessel lining endothelial cells). When dissecting single cells or very small cell clusters it is essential to change the focus of the laser. This adjustment of the laser focus for catapulting has to be learned by trial and error; the correct adjustment is a delicate balance between the size of the specimen and the laser focus and the laser energy. A tiny drop of liquid wax from MJ Research is distributed in the lid of the reaction tube. This wax film ensures that the catapulted specimens will adhere firmly to the lid.

7. The transfer of the lysate of larger groups of cells to a new tube before precipitation is necessary in order to separate the pieces of supporting membranes from the cell lysate. These pieces are isolated together with the dissected cells. They are not lysed and interfere physically with precipitation of nucleic acids by preventing the formation of a compact pellet at the bottom of the tube during centrifugation.
8. Following this PCR protocol we can achieve detection sensitivity of 2–4%, i.e., one to two cells of recipient's origin are reproducibly detected in a background of 50 donor cells.
9. For the analysis of the PCR products utilizing the capillary sequencer from Applied Biosystems we use ordinary colorless 0.5-mL tubes from which we cut off the lid. But we recommend the use of specialized (and quite expensive) septa from Applied Biosystems (Part no. 401956) to close these tubes before placing them in the sample holder.

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