

## Selection of Chloroplasts by Laser Microbeam Microdissection for Single-Chloroplast PCR

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### ABSTRACT

*Laser microbeam microdissection and laser pressure catapulting offer the possibility of separating cell compartments, thus allowing for contamination-free analysis. Using these methods, we were able to select single chloroplasts of *Nicotiana tabacum*. Starting from homogenized leaf material, chloroplasts were purified by differential centrifugation and applied directly onto a poly-ethylene-naphthalate membrane that was mounted on a microscope slide. Single chloroplasts were dissected under microscopic control and catapulted into a PCR tube. Subsequent PCR of a spacer region between the *trnT* and *trnF* genes verified the successful amplification of DNA from a single chloroplast. The advantage of this method compared to the use of capillaries or optical tweezers is that one is able to prepare high numbers of samples in a short time.*

### INTRODUCTION

Laser-based systems are the state-of-the-art technologies for precise and noncontact micromanipulations with nanometer accuracy (1,2). The forces of light are utilized to catch and move, cut, or fuse microscopically small specimens. To accomplish this, lasers of high beam quality are interfaced into a research microscope and focused through the objective onto the sample. Most biological objects are transparent for the applied laser wavelength, and the effective light force acts only within the nanometer-sized laser focal spot (3). Thus, it is possible to handle biological materials without changing features or even to work inside living cells without disturbing their viability.

These systems became indispensable tools in modern medicine and biotechnology. Recent advances in molecular methods to analyze genes and their transcripts necessitated the development of technologies to extract homogeneous specimens of morphologically defined origin for the subsequent genetic or proteomic analysis without contamination. This was achieved by the capture of microdissected specimens using the technology of laser capture microdissection (LCM) (4). A recent improvement of this method is the combination of laser microbeam microdissection (LMM) with laser pressure catapulting (LPC) (5,6).

With the use of LMM, biological material can be isolated from neighboring tissue. The laser cuts around the target specimen, which yields a clear-cut gap between the selected and nonselected specimens. In addition, unwanted

material within a larger selected area can be selectively destroyed with a few laser shots. After this step, using LPC, the isolated material is ejected from the object plane with a single laser shot and catapulted directly into the cap of a standard reaction vessel. The basis of this technique is believed to be the laser pressure force that develops under the specimen and is caused by the extremely high photon density of the precisely focused laser microbeam (5). The isolated sample is driven with high speed along the wave front of the photonic stream and can be transported for several millimeters, even against gravity (6). After catapulting and capturing, the probes are suspended in appropriate buffers in the cap of the reaction vessel and subsequently centrifuged down into the tube for further processing.

The 337-nm nitrogen laser works within the UV-A range; nevertheless, damage of biological matter could occur. However, within the laser-catapulted specimen and the adjacent tissue, the recovery of DNA, RNA, or protein is not impaired (5), which makes this technique ideal for sample preparation before single-cell PCR (7).

Specimens of any shape and size from one to several micrometers in diameter can be captured in a quick and reliable manner. Catapulting can be performed on large areas of tissue or single cells from frozen or fixed histological tissue slices as well as from cytocentrifuged samples or cell smear. Even fragile structures such as chromosomes can be collected using the same principle (8).

The amplification of microdissected chromosomes or nuclei is already a

standard technique for the generation of specific DNA probes used in chromosome mapping and chromosome walking (9–11), for fluorescence in situ hybridization (12), or for the generation of genomic libraries of single or partial chromosomes via microcloning procedures (13,14). In these applications, several samples are pooled to increase the amount of DNA for subsequent use.

Applications of single-cell PCR are used when the material is limited, such as in prenatal diagnostics, where single embryonic cells are isolated, or when somatic cells are differentiated after mutation, as in cancer research (7). LMM and LPC are powerful tools for the isolation of single cells and organelles for subsequent molecular analysis and amplification of DNA via PCR (5,15,16). This technique is easier to use and results in a higher efficiency than systems that depend on microcapillary needles (7).

Although micromanipulation via laser microbeam and optical tweezers has been widely used on living plant cells (for an overview, see Reference 17) and on chloroplasts (18), the isolation of plant material via LPC for use in single-cell PCR has thus far only been reported for single pollen typing (19). In the context of experiments that test for

heteroplasmy in populations of chloroplasts, we were interested in the examination of high numbers of single chloroplasts. Here we describe the high-throughput and contamination-free isolation of single chloroplasts and their successful use in single-organelle PCR.

## MATERIALS AND METHODS

### Plant Material

In vitro cultured material from *Nicotiana tabacum* was used for all of the experiments shown. Additional experiments were performed with material from *Taraxacum officinale*.

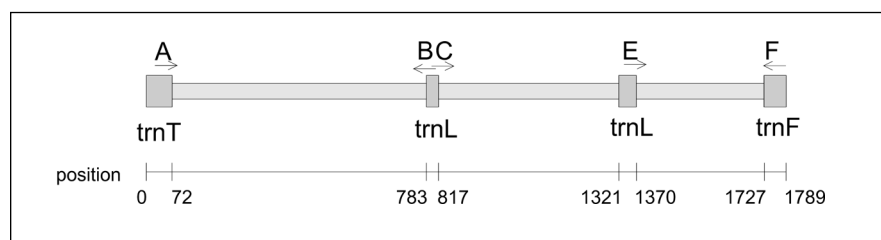
### Isolation of Chloroplasts

For the isolation of chloroplasts, a previously described buffer system (20) was used, and the following modifications were introduced to enable the micropreparation of plastids from low amounts of leaf material. Fresh leaf material (1–2 cm<sup>2</sup>) was cut into small pieces with a sharp razor blade on an ice-cold glass plate. The material was homogenized with a micropestle on ice in a 1.5-mL reaction vessel with 1 mL homogenization buffer (50 mM

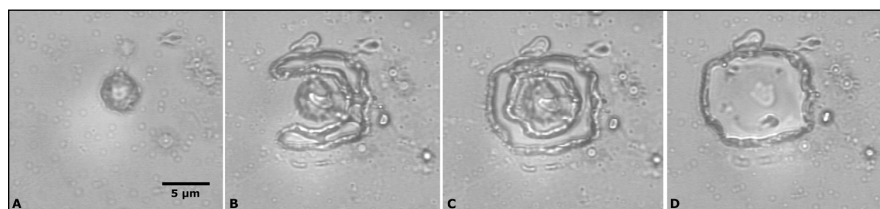
HEPES/KOH, pH 8.0, 400 mM sorbitol) and a small amount of inert sea sand (Merck, Munich, Germany). The suspension was cleared by centrifugation through a layer of nylon gaze (Ø 22 µm) at 3000× g for 1 min at 4°C. The pellet was resuspended in 100–200 µL homogenization buffer and layered onto an ice-cold Percoll™ gradient in a 1.5-mL reaction vessel (600 µL 40% Percoll on top of 600 µL 80% Percoll, both in homogenization buffer). After centrifugation in a swing-out rotor (HB4, Sorvall®; Kendro Laboratory Products, Newtown, CT, USA) at 4500× g for 10 min at 4°C, the chloroplast layer above the 80% Percoll layer was transferred to a new reaction vessel containing 1 mL homogenization buffer and pelleted by centrifugation at 3000× g for 1 min at 4°C. This was repeated twice to remove all of the remaining Percoll. The pellet was resuspended in 10 µL homogenization buffer and applied directly onto a poly-ethylene-naphthalate (PEN) membrane (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) fixed at its edge with nail varnish on a microscope slide. In subsequent experiments, the chloroplast suspension was further diluted with the homogenization buffer before application to the membrane (see Results and Discussion). The sample was carefully dispersed on the membrane with a pipet tip until it formed a thin layer and dried for several minutes at 37°C (see Figure 2A).

### LMM

A commercially available UV-laser microbeam system (ROBOT-Microbeam; P.A.L.M. Microlaser Technologies AG) was used as previously described in detail (21). This system consists of a 337-nm nitrogen laser that is coupled with the light path of an inverted microscope and focused through an oil immersion objective (100× magnification) with a high numerical aperture to yield a spot size of less than 1 µm in diameter (2). The positioning of the stage in the x- and y-directions is computer-controlled. The microscopic image is taken by a video camera, displayed on a video screen, and stored by the computer. The position of the laser beam during the isolation procedure can be controlled online on the video



**Figure 1. The *trnT-trnF* region of *N. tabacum*.** The dark gray boxes indicate conserved regions and the light gray boxes indicate variable regions. Oligonucleotide positions A, B, C, E, and F are as previously indicated (22) and also apply to the nested primers A2 E2, F2, and F3 designed for this study.



**Figure 2. Separation of one single chloroplast.** (A) A single chloroplast from a chloroplast solution fixed on a microscope slide-mounted PEN membrane before the microdissection process; (B) LMM of the supporting PEN membrane around the single chloroplast; (C) microdissected PEN membrane islet with the single chloroplast; and (D) region of the supporting membrane after LPC of the selected specimen into a PCR tube cap. The size bar in panel A applies to all pictures.

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screen. An energy of 0.2–0.3  $\mu\text{J}$  per pulse was used for ablation, and an energy of 0.5–0.6  $\mu\text{J}$  per pulse was used for membrane dissection. The area around one chloroplast visible on the video screen was cut with the laser beam until it was completely separated from the neighboring membrane. Chloroplast-free islets were isolated in the same manner and used as a control.

## LPC

For catapulting, the laser was focused slightly below the membrane. The isolated sample-membrane islets were ejected and hovered on a photonic cloud from the object slide with a single laser shot of 1–2  $\mu\text{J}$  per pulse, as shown (see Figure 2). The collecting device consists of the flat cap of a 0.2-mL reaction vessel mounted onto the LPC collector. The LPC collector was positioned at a distance of about 1 mm above the slide with the help of the computer-controlled Robot-Manipulator. The microfuge caps were covered with 1  $\mu\text{L}$  mineral oil to improve the adhesion of the captured specimen and to facilitate their release into the reaction tube.

## Proteinase K Incubation

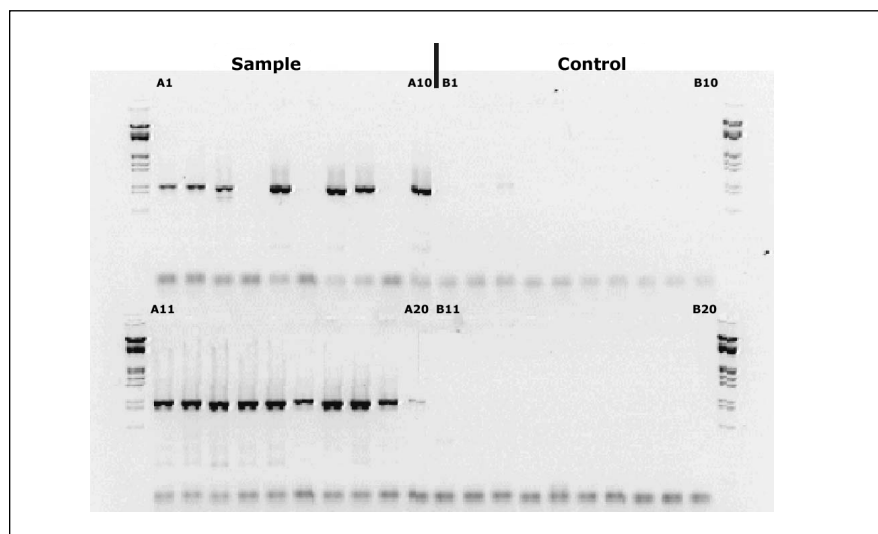
The cap was removed immediately from the LPC collector, and 25  $\mu\text{L}$  1 $\times$  PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ ) with 0.23  $\mu\text{g}/\mu\text{L}$  proteinase K (PCR-grade; Roche Applied Science) were added to the middle of the cap. The cap was covered with the corresponding reaction vessel and incubated until all of the samples had been dissected. After centrifugation at 14,000 $\times g$  for 10 min at room temperature, the samples were incubated for 60 min at 65 $^\circ\text{C}$  and subsequently for 10 min at 94 $^\circ\text{C}$  to inactivate the proteinase K.

## PCR Amplification

The DNA amplified was the plastome region between the *trnT* and *trnF* genes, consisting of the two conserved coding regions of *trnL*, the variable *trnL* intron, and two variable intergenic regions. Primers were placed in the conserved regions (Figure 1). Because of the high sequence variability, we were

able to eliminate the possibility of contaminations from other plant species during the first experiments. Each of the amplifications was performed with the same number of samples, containing chloroplasts and chloroplast-free samples as control, generally 20 samples of each. In addition, a negative PCR control was performed. Modified oligonucleotides A2 (5'-CATTACAAATGCGATGC-3') and F2 (5'-ATTTGAACTGGTGACAC-3') were used as described previously (22) to amplify the entire region in the first PCR. The reaction mixture, consisting of 25 pmol each primer, 2.5  $\mu\text{L}$  dNTPs (2.5 mM each nucleotide), 2.5  $\mu\text{L}$  10 $\times$  PCR buffer, and 2 U *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany) in a total volume of 25  $\mu\text{L}$ , was added directly to the sample. All PCRs were run on a Primus 96 thermal cycler (MWG, Ebersberg, Germany). After 2 min of denaturation at 94 $^\circ\text{C}$ , the following "touch-down" profile was used: two cycles of 55 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 2.5 min, and 94 $^\circ\text{C}$  for 1 min; two cycles of 50 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 2.5 min, and 94 $^\circ\text{C}$  for 1 min; 10 cycles of 48 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 2.5 min, and 94 $^\circ\text{C}$  for 1 min; 20 cycles of 48 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 2.5 min, with an elongation step of 2 s for each cycle, and 94 $^\circ\text{C}$  for 1 min. Finally, elongation at 72 $^\circ\text{C}$  for 10 min was performed. The reaction products were used as template in a second amplifica-

tion using either oligonucleotides A (5'-CATTACAAATGCGATGCTCT-3') and B (5'-TCTACCGATTCGCCATATC-3') (21) or C (5'-CGAAATCGGTAGACGCTACG-3') (21) and F3 (5'-CAGTCCTCTGCTCTACCAAC-3'). The expected product sizes were approximately 800 and 1,000 bp, respectively. The reactions were performed in a total volume of 25  $\mu\text{L}$  with 12.5 pmol each primer, 1.25  $\mu\text{L}$  dNTPs (2.5 mM each nucleotide), 2.5  $\mu\text{L}$  10 $\times$  PCR buffer, 1 U *Taq* DNA polymerase, and 1  $\mu\text{L}$  reaction product from the first PCR. We used the following temperature profiles. For amplification with A and B, the initial denaturation was at 94 $^\circ\text{C}$  for 2 min; 35 cycles of 59 $^\circ\text{C}$  for 30 s, 72 $^\circ\text{C}$  for 1.5 min, and 94 $^\circ\text{C}$  for 30 s; and final elongation at 72 $^\circ\text{C}$  for 5 min. For amplification with C and F3, initial denaturation was at 94 $^\circ\text{C}$  for 2 min; 35 cycles of 53.2 $^\circ\text{C}$  for 30 s, 72 $^\circ\text{C}$  for 1.5 min, and 94 $^\circ\text{C}$  for 30 s; and final elongation at 72 $^\circ\text{C}$  for 5 min. Additional experiments were performed using the primers E2 (5'-GGTTCAAGTCCCTCTAT-3') and F2 in the first PCR, with the following temperature profile: initial denaturation at 94 $^\circ\text{C}$  for 2 min; 2 cycles of 60 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 1 min, and 94 $^\circ\text{C}$  for 1 min; 2 cycles of 55 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 1 min, and 94 $^\circ\text{C}$  for 1 min; 2 cycles of 50 $^\circ\text{C}$  for 1 min, 72 $^\circ\text{C}$  for 1.25 min, and 94 $^\circ\text{C}$  for 1 min; and final elongation at 72 $^\circ\text{C}$  for 5 min.



**Figure 3. Amplification products after secondary PCR.** Twenty samples each from the regions containing chloroplasts (lanes A1 to A20, samples) and from chloroplast-free regions (lanes B1 to B20, controls) were used. PCR was performed using oligonucleotides C and F3.  $\lambda$ -phage DNA cut with *EcoRI* and *HindIII* serves as size standard.

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Reaction products were separated on 1.5% standard agarose gels. Gel and running buffer were 1× TBE (90 mM Tris-borate, pH 8.3, 2.5 mM EDTA) containing 5 µg/mL ethidium bromide. To verify the specificity of the PCR, samples of positive PCR of one experiment were pooled and sequenced using the same oligonucleotides as for the PCR. Sequencing was performed with biotin terminator-labeled ddNTPs in cycle sequencing reactions with Thermo Sequenase™ (Amersham Biosciences Europe GmbH, Freiburg, Germany) using a GATC-direct blotter (GATC, Konstanz, Germany).

## RESULTS AND DISCUSSION

### Chloroplast Isolation

The aim of this study was to establish a method for the rapid isolation of

single chloroplasts for single-organelle PCR analysis, sufficient for the preparation of numerous samples, which would allow one to work on a level of statistical significance. In the context of a phylogenetic study, we had co-amplified two paralogous sequences from the *trnK* intron of various *Nepenthes* species. Because this locus is normally chloroplast encoded, both copies could be located in chloroplasts. Alternatively, one copy could be of nuclear origin or mitochondrially encoded. It was important to distinguish between these possibilities and to determine which copy is chloroplast encoded. To establish the methodology, we chose to use the leaf material of *N. tabacum*.

After isolation, chloroplasts could be applied directly onto a dry PEN membrane, with no need to prebind adhesive buffers. Plastids kept their spherical shape and could be dissected easily (Figure 2). Good amplification

results were achieved only with fresh preparations, indicating that DNase activities persist even in dried material. The addition of EDTA to the isolation medium for the inhibition of DNases was not possible since the hygroscopic nature of EDTA prevents the complete drying of the membrane and disturbs the dissection procedure. The same effect was caused by excess amounts of sorbitol from the homogenization buffer when the samples were not distributed evenly over the membrane.

### PCR Amplification

Amplification with one isolated chloroplast as template with oligonucleotides A2 and F2 in the first PCR never resulted in visible amounts of the expected 1,800 bp product. Since amplification with oligonucleotides E2 and F2 with an expected product size of about 300 bp gave the same result, we

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concluded that a second round of PCR was necessary.

The second round of PCR led to products in 90% of the samples, with sizes of about 800 and 1000 bp for the oligonucleotides A and B, and C and F3, respectively. Sequencing of these products was performed to confirm the specificity of the PCR. A comparison with the *N. tabacum* chloroplast genome DNA sequence (accession no. gi2924257) revealed 100% identity.

To determine whether DNA of the isolated visible chloroplasts or DNA dispersed in the preparation served as template in the PCR amplifications, chloroplast-free membrane regions were dissected as controls. Amplifications with these control samples resulted in PCR products in 60% of the cases. This contamination demonstrated that either plastid DNA of ruptured chloroplasts is not quantitatively removed by separation via the Percoll gradient or that

chloroplasts not visible under the microscope are present in membrane regions used as control. To circumvent this problem, we diluted the pelleted chloroplasts in homogenization buffer before fixation onto the PEN membrane.

Dilution of the pellet from 1- to 10-fold resulted in PCR products in only 30% of the control reactions, while all of the chloroplast-containing samples gave a positive result. After a further 2-fold dilution, 85% of the reactions with samples that contained visible chloroplasts still led to products, while all controls remained negative (Figure 3). With this, we could confirm specific amplification from a single, morphologically intact chloroplast. Additional experiments with *T. officinale* led to analogous results (data not shown).

## Application of the Technique

The advantage of LPC compared to

other techniques, such as limited dilution or needle-based micromanipulation, for preparation of single chloroplasts lies in the speed and effectiveness of the procedure. It is possible to verify directly the isolation of a single chloroplast and reduce co-isolated contaminations. To our knowledge, the data presented here are the first reported amplification of DNA using the plastomes of one organelle as template. It is also feasible that thin-layer preparations of leaf sections may be sufficient for the LPC of single chloroplasts, which is a subject currently under investigation. Our data indicate that the quality of the DNA should be sufficient for the amplification of longer fragments. With this isolation procedure, whole plastome analysis using primer-extension-pre-amplification strategies (23) from one chloroplast could also be successful. This has been demonstrated by Matsunaga et al. (19) in the case of single

LPC-isolated pollen grains, in which the target sequences are present in three copies only. Due to the high copy number of plastomes in a single chloroplast, amplification should be easily achieved.

Therefore, it might also be possible to sample single mitochondria via LPC. Kuroiwa et al. (24) isolated mitochondria using optical tweezers from a suspension and performed PCR from DNA of at least nine mitochondria dried on a glass slide. LPC allows for the collection of single organelles directly in the reaction vessel. Thus, the separation and sampling of single organelles is very efficient with this method. The advantages of LPC as shown for separating single pollen grains (19) will also apply to fragile cell compartments.

The procedure we present here allows for the direct comparison of plastomes from single organelles to detect the phenomenon of heteroplasmy, such as those described for chloroplasts of *Senecio vulgaris* (25) or for plastid transformants. The same applies for mitochondria that seem to coexist rather than to segregate in species with biparental inheritance, after fusion experiments and due to mutations of the chondrom (26).

Apart from its use to differentiate organelle populations, we assume this method will be of great benefit for the direct verification of successful transformation of chloroplasts and mitochondria, such as for addressing questions of mitochondrial exclusion or inheritance.

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