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Journal

Microscopy and Microanalysis, 3(1)

ISSN

1431-9276

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[et al.](#)

Publication Date

1997

DOI

10.1017/s1431927697970033

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Laser Microdissection for Generation of a Human Chromosome Region-specific Library

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Abstract: A human chromosome pq34 region-specific, microdissected library was constructed by using laser microdissection techniques. This library contains over 10,000 clones with an average insert size of 450 bp. It has greater coverage of the dissected chromosome region as compared with the needle-dissected chromosome 9q34 library. The laser microdissection technique provides more accurate chromosome targeting and easier operation than existing needle microdissection techniques. To simplify the procedure for chromosome microdissection and chromosome fragment collection, a trapping-cutting system was developed. This technique involves the use of two trapping beams which hold a single chromosome in suspension, and a third cutting beam, which dissects the immobilized chromosome. A collection chamber allowing for the fast collection of dissected chromosome fragments needs to be developed. However, DNA can be cloned from trapped chromosome fragments with an insert size comparable to that of both needle-cut and laser-cut chromosomes.

Key words: laser microdissection, region-specific DNA library, microcloning, laser trapping, chromosome targeting

INTRODUCTION

High-resolution physical mapping and the efficient search for a disease-related gene in a particular chromosome region requires a large number of unique DNA sequence probes from the region of interest. Microdissection and the generation of chromosome region-specific libraries is one of the most straightforward approaches for this purpose. Since the introduction in 1981 by Scalenghe et al., of microdissection techniques to clone *Drosophila* polytene chromosomes, these techniques have been improved and successfully used for human chromosomes (Ludecke et al., 1989;

Jonson, 1990; Meltzer et al., 1992). Although the molecular cloning steps have been improved considerably, the microdissection step is still very tedious and difficult. It is important to simplify the procedure for dissection and collection of the chromosome fragments and at the same time improve the accuracy of the microdissection.

Based on the early results of Berns et al. (1969), who demonstrated that the laser can be used to dissect chromosomes, Greulich et al. perfected this technique for the generation of region-specific DNA libraries (Monajembashi et al., 1986). In order to compare laser microdissection with needle microdissection, we have constructed two microdissection libraries from the same chromosomal region by using both techniques. From our experience, the laser microdissection technique provides more accurate chromosome

Received July 7, 1996; accepted September 16, 1996.

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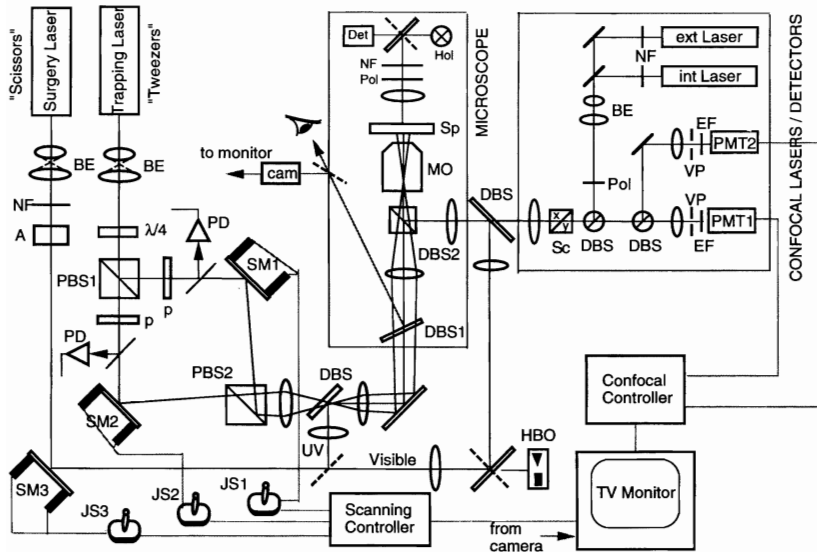


Figure 1. Confocal ablation trapping system (CATS). Two external lasers, tweezers, and scissors were brought into a Zeiss confocal microscope. The scanning mirrors, labeled SM1, SM2, and SM3, were used to control the movement of these three laser beams in the focal plane. The elements in the figure are as follows: BE, beam expander; NF, neutral density filter; A, attenuator; DBS, dichroic beam splitter; PBS, polarized beam splitter; SM, scanning mirror; JS, joystick; P, polarizer; PD, photo diode.

targeting because the beam of light is focused to a precise diffraction-limited spot, and the beam can be directed onto a specific chromosome region without physically having to manipulate and disturb the chromosome. The cloning results from both libraries show that the laser-microdissected library contains a larger average insert size and greater coverage of the dissected region. Additionally, we have demonstrated that an optical trapping laser system can potentially be used to replace the needle for collecting chromosomal fragments. This new combined “ablation” and “trapping” optical-based system could provide a major improvement in chromosome microdissection and collection in constructing chromosome region-specific DNA libraries.

MATERIALS AND METHODS

Laser System Design

A schematic diagram of the two-beam optical tweezers and single-beam optical scissors combined with a laser scanning confocal microscope is shown in Figure 1. The system is designated as CATS for Confocal Ablation Trapping System. The Argon ion, laser-pumped CW Ti:Sapphire laser beam (Coherent, Model 899 Ring Laser), with an adjustable wavelength range from 700 nm to 1000 nm, is used as the trapping laser. The laser beam is first divided into two beams by a polarized beam splitter and then deflected individually into the microscope by two motorized scanning mirrors (SM1 and SM2). Each trapping beam can be independently controlled by the scanning mirror via electronic

and computer-controlled joysticks. The precise position of each trapping beam is controlled by a scanning controller (motion controller, cat. # PMC400-06; closed-loop actuator, cat. # 850B-05, Newport Inc., Irvine, CA). The resolution of beam movement was about 100 nm. To minimize the laser beam power loss and maximize the beam deflection angle, the scanning mirrors and the microscope objective lens were placed at corresponding conjugation planes of a converging lens. With this geometry, each beam can scan a field of $60 \times 60 \text{ mm}^2$ at the focal plane without loss of incident laser power. Each trapping laser beam can deliver up to 200 mW power in the focal spot, which produces enough force to hold individual chromosomes. For chromosome dissection, a frequency-doubled, Q-switched Nd:YAG laser (Surelite I-10, Continuum, Santa Clara, CA), which produces a 532-nm laser beam, was directed into the microscope through the epifluorescence microscope port. The focused spot of the cutting laser was $0.5 \mu\text{m}$ in diameter when a $100\times/1.3 \text{ N.A.}$ Zeiss (Thornwood, NY) Neofluar objective was used. The nJ- μJ focal spot energy was controlled by an optical attenuator (Karl Lambrecht Corp., cat. # QAGLS-10, Chicago, IL). The focal point of the cutting laser was also controlled by a joystick (JS3).

Laser Microdissection and Needle Collection

Chromosomes were prepared by using standard cytogenetic methods (Buckle and Kack, 1993). To identify the chromosome of interest, G-banding was performed on the day of the dissection. In each individual experiment the frequency-doubled YAG laser (532 nm) was first used to cut out the

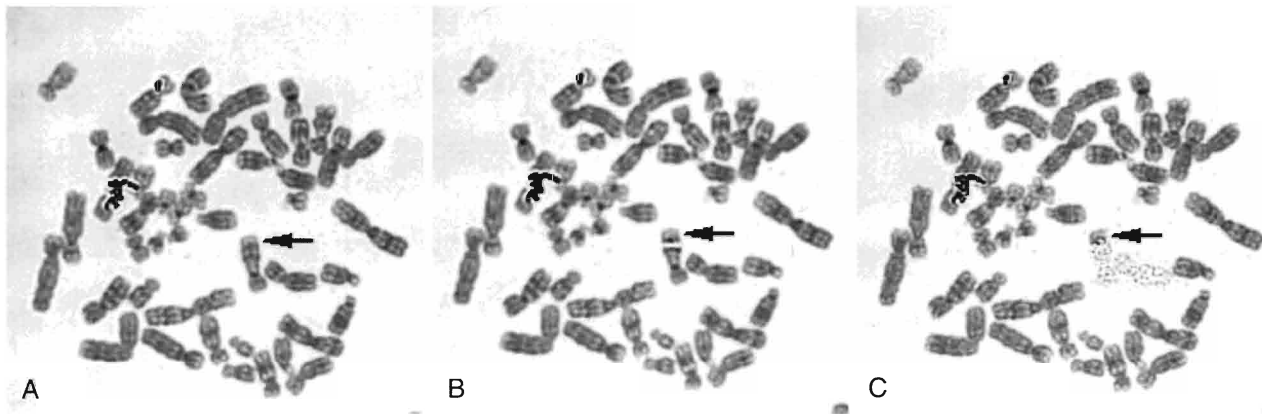


Figure 2. Laser chromosome microdissection. **A:** A G-banded metaphase spread. Chromosome 9 is indicated by the arrow. **B:** Chromosome 9q34 region was dissected out with a cutting laser. **C:** The rest of chromosome 9 and the surrounding chromosomes were damaged using a laser beam.

chromosome band of interest (laser power 10–40 μJ in the focal spot). The laser power was then increased to $\sim 100 \mu\text{J}$ in order to destroy the rest of the chromosome arm as well as surrounding chromosomes (Fig. 2). After this step, a clean, UV-sterilized needle attached to a micro-manipulator (model 5170, Eppendorf, Hamburg, Germany) was used to pick up the chromosome fragment of interest and transfer it to a PCR tube containing 10 μl $1 \times$ PCR buffer. A new needle was used for each chromosome fragment to reduce the contamination. A total of 20 fragments from chromosome 9q34 were collected into the PCR tube with this method.

Needle Microdissection

The same cytogenetic method as that previously described in laser microdissection was used to prepare chromosomes for needle microdissection. The microdissection needles were produced using a pipette puller (model 700C, David Kopf Instrument, Tujunga, CA). Average tip size of the needle was about 1 μm . Microdissection was performed by directly scratching the chromosome fragments from the slide and placing them into a sterile siliconized microtube containing $1 \times$ PCR buffer. Technically, it was very important to position the needle perpendicular to the chromosome of interest for precise microdissection. A total of 7 chromosome fragments was collected for PCR amplification.

Microlibrary Construction

The collected chromosomal fragments were PCR amplified by using degenerate oligonucleotide primer

(--CCGACTCGAGNNNNNNATGTGG--). The PCR reaction started with 8 cycles of low annealing temperature (30°C) followed by 28–35 cycles of standard annealing temperature (56°C) (Telenius et al., 1992; Meltzer et al., 1992). After PCR, the products were directly cloned into a blunt end plasmid vector using a PCR cloning kit from Pharmacia Biotech (SureClone™ ligation kit, Uppsala, Sweden). Recombinant clones were transferred to 96-well plates.

Preparation of Chromosome Sample Suspension

The human-rodent hybrid cell line GM10611, which contains only human chromosome 9, was used. Mitotic cells were shaken off from T75 flasks and collected in a 1.7 ml microtube. After two washes with $1 \times$ PBS, the pellet was resuspended in 800 μl of KCl (40 mM) and incubated at room temperature for 15 min. Four hundred μl of Triton-X (1%) and 20 μl of propidium iodide (1 mg/ml) were subsequently added. After incubation for another 3 min at room temperature, the cell suspension was pushed through a 23G needle to release chromosomes. Fluorescence dye, 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) was used (10 $\mu\text{g}/\text{ml}$ final concentration) to generate a unique staining pattern which permitted identification of chromosome 9.

Two-beam Trapping and Cutting of Chromosomes in Suspension

The chromosome suspension was pipetted into a specially designed chamber and placed on the microscope stage (Fig. 3). Each individual trapping laser beam was applied to opposite ends of the chromosome. In this way the trapped

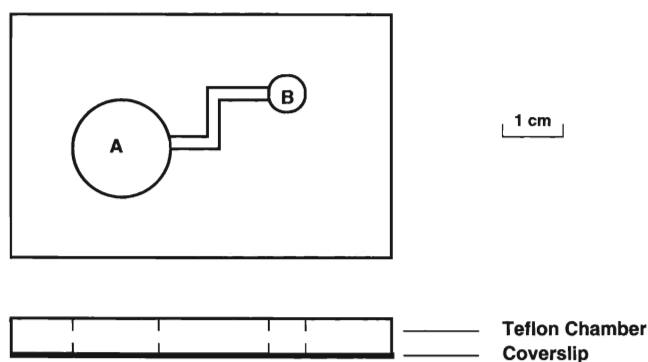


Figure 3. Collection chamber design. The collection chamber has two components: a Teflon plate with two wells and a connecting channel (cut through the whole piece); and a coverslip that adheres to the bottom of the Teflon plate with vacuum grease. The chromosomes are dissected in the dissection well (A). The desired fragments are moved from the dissection well to the collection well (B) by using the trapping laser.

chromosome was manipulated into a horizontal position so that the cutting laser could be easily moved across the desired chromosome region. The three different laser beams (two trapping and one cutting) were manipulated by three separate joystick controllers while the entire process was projected on the video monitor.

RESULTS

Comparison of Laser and Needle Microdissection

The chromosome 9q34 region, which contains the *Tuberous sclerosis 1* gene, was chosen as the region of interest. Two libraries of this region were generated by using either laser microdissection or needle microdissection. The laser microdissected library contained over 10,000 clones with an average insert size of 450 bp (Fig. 4). The needle-microdissected library had an average insert size of ~200 bp. Further Southern blot analysis of randomly selected clones from the laser-microdissected library indicated that about 50% of the clones detected single-copy sequences from genomic DNA. Several probes mapped to the *Tuberous sclerosis 1* gene region on chromosome 9q34 were isolated from the laser-microdissected library (data not presented here).

Laser Trapping and Laser Microdissection

Figure 5 presents four live phase contrast images at different stages of the laser trapping and cutting procedure. The

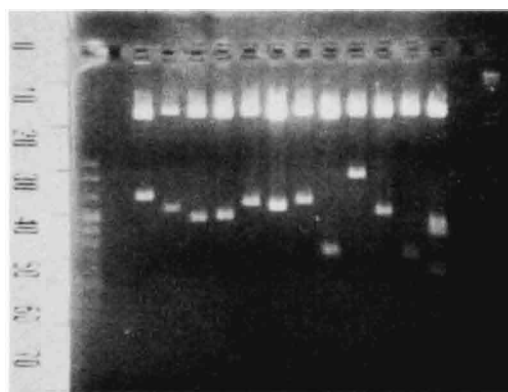


Figure 4. Analysis of insert size in 12 clones. Twelve clones randomly selected from the laser-microdissected chromosome 9q34 library were digested with *Hind*III and *Eco*RI to recover the inserts. The size of these inserts ranges from 300 bp to 1,000 bp. The top band represents the vector.

chromosome was held horizontally by the two independent trapping beams (arrows). The chromosome was trapped and dissected in the dissection well (Fig. 3) and then moved from the dissecting well to the collection well through the channel by using one of the trapping beams. To prevent the random flow from dissection well to collection well, it was necessary to design a narrow zigzag channel to connect these two wells. Due to the distance between the two wells, the time required to move one chromosome fragment from the dissection well to the collection well was about 15 min under current trapping laser powers. An improved chamber design in addition to a larger trapping force is necessary to improve the collection speed.

To demonstrate that the trapping beam did not cause enough damage to interfere with the DNA cloning process, we selected a total of 40 laser-microdissected chromosome fragments on one G-banded slide and applied the trapping laser to 20 of those fragments (9200 mW at focal plane for 10 min on each fragment). Needles were used to collect 20 trapped and 20 untrapped fragments into two separate PCR tubes. The result of the universal PCR reaction (as described in Methods and Materials) demonstrated base-pair insert sizes in the range of 300–1,000 for both groups.

DISCUSSION

Tedious needle-based chromosome microdissection and microcloning procedures have been developed for the generation of region-specific DNA libraries.

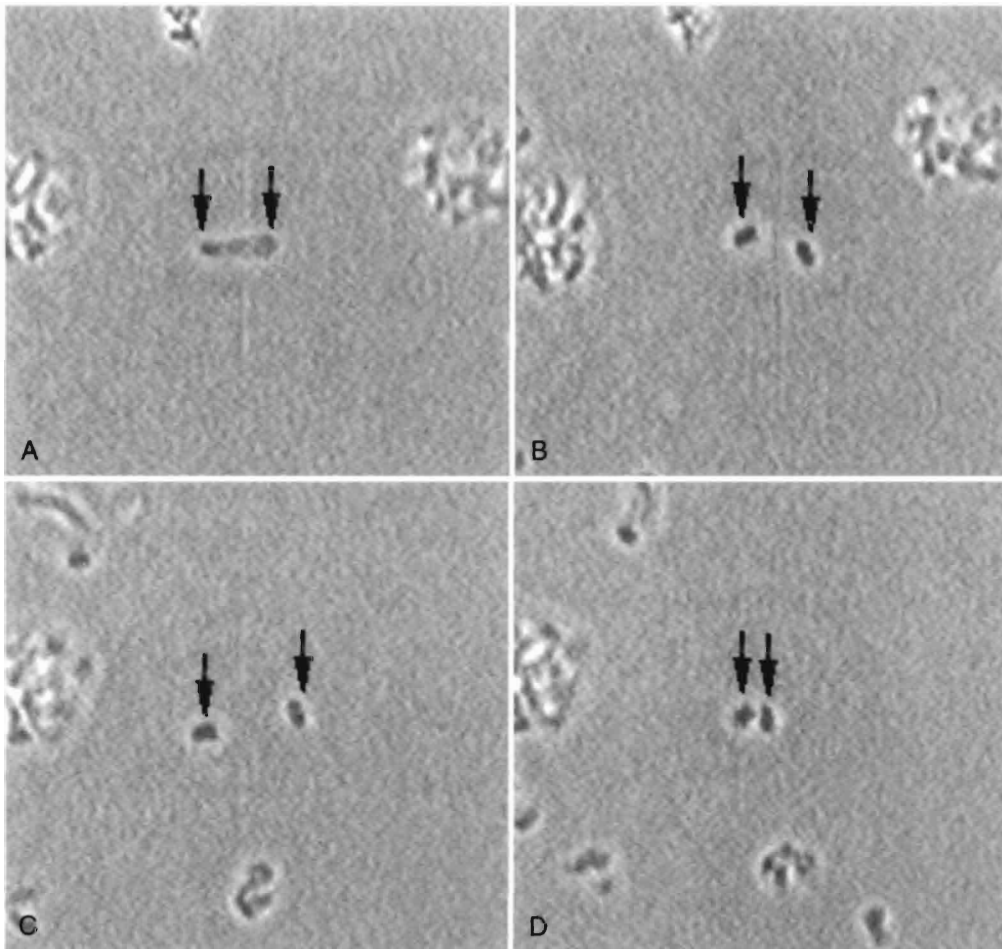


Figure 5. Laser trapping and cutting chromosomes in suspension. The following four images were taken during this process. **A:** A single chromosome was held in position by using two trapping beams. These held the ends of the chromosome, as indicated by arrows. **B:** A cutting laser was used to dissect this chromosome into two pieces which were individually held by each trapping beam. **C:** The trapping beams were used individually to move the chromosome fragments away from each other. **D:** The trapping beams were used individually to move the chromosome fragments towards each other.

The use of a laser microbeam to cut small regions from chromosomes in living cells was demonstrated over 27 years ago by Berns et al. (1969). In our study, we examined the question of whether laser microsurgery in combination with needle pick-up, or in combination with optical trapping, can be applied to DNA cloning in such a way as to improve the technology above present capabilities.

It is clear from the data that laser microsurgery can be applied to specific regions of isolated G-banded or DAPI-stained chromosomes; the chromosome fragment can be readily picked up by using microneedles or moved to a collection well by using an optical trap; the DNA from the laser-cut fragments can be subjected to successful PCR; DNA libraries with average insert sizes of 450 base pairs can be generated from the laser cut fragments as compared with 200 base pairs from the needle cut fragments; laser-trapped chromosome fragments can undergo PCR and generate insert sizes in the 300–1,000 base-pair range.

The results of these experiments demonstrate that the laser technique not only provides more accurate chromo-

some region targeting and easier operation than does needle microdissection, but it also generates larger average insert sizes. A human chromosome 9q34 region-specific, microdissected library was constructed using laser microdissection techniques. This library contains over 10,000 clones with an average insert size of 450 bp. It has greater coverage of the dissected chromosome region compared to the needle dissected chromosome pq34 library.

Further improvement of the laser-trapping collection system will involve increasing the laser-trapping power (so that the chromosome fragments can be moved at a faster rate) and shortening the connecting arm between the microsurgery well and the collection well. These improvements should result in the ability to cut, trap, and pool enough fragments in a short enough period of time so as to eliminate the need for needle pick-up. Ultimately, it should be possible to introduce the chromosome suspension into a sterile, closed chamber where the entire chromosome microdissection and collection process can be performed.

ACKNOWLEDGMENTS

We thank Kathy Handa and Tatiana Kracieva for their technical assistance. This work was supported by NIH grant 5P41-RR01192, which supports the NIH LAMMP Resource.

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