



Generation of chromosome painting probes from single chromosomes by laser microdissection and linker-adaptor PCR

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Received 18 February 2004. Received in revised form and accepted for publication by Herbert Macgregor 12 March 2004

Key words: chromosome painting, laser microdissection, linker-adaptor PCR, molecular cytogenetics

Abstract

Fluorescence *in situ* hybridization (FISH) plays an essential role in research and clinical diagnostics. The versatility and resolution of FISH depends critically on the probe set used. Here, we describe an improved approach for the generation of specific DNA probes from single copies of chromosomes. Single chromosomes or single chromosomal regions were microdissected by laser pressure catapulting and amplified using linker-adaptor PCR. The probes were labeled and tested in various scenarios including multicolor-FISH experiments employing up to seven different fluorochromes. FISH confirmed the specific and even staining of the respective chromosomal regions. Furthermore, the capability of these probes to detect even small translocations (<3 Mb) suggests that the dissected regions are completely represented in the generated painting probes.

Introduction

For molecular cytogenetic applications there are various means for the generation of chromosome-specific painting probes (reviewed by Langer *et al.* 2004). Chromosome-specific DNA probes can be generated by flow sorting of whole chromosomes and subsequent universal amplification by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) (Wienberg *et al.* 1994, Carter *et al.* 1992). Flow sorting has been successfully applied to the generation of whole chromosome-specific libraries from various species, including painting probes for pig, dog, mouse, rat, and muntjacs. Traditionally, several hundreds

of chromosomes have been sorted to generate a painting probe by flow sorting (Telenius *et al.* 1992).

An alternative to flow sorting is microdissection. The conventional microdissection approach uses fine extended glass needles for the collection of chromosomes or subchromosomal regions of GTG (G-bands by trypsin using Giemsa)-banded chromosomes (Meltzer *et al.* 1992). Different authors reported various numbers of chromosomes needed for the generation of painting probes, ranging from more than 20 (Lengauer *et al.* 1991, Deng *et al.* 1992) to less than 10 (Müller-Navia *et al.* 1995, Weimer *et al.* 2001) or even single chromosomes (Guan *et al.* 1994). Newer developments include laser

micromanipulation and microdissection, which has considerably increased the ease and speed of isolating chromosomes (Thalhammer *et al.* 2003). The generation of painting probes from a single chromosome using laser pressure catapulting and subsequent amplification by DOP-PCR has been described (Schermelleh *et al.* 1999). However, with this procedure, the representation of the derived painting probe was incomplete.

The generation of chromosomal painting probes from a small number of chromosomes or even a single chromosome should be advantageous for many applications. Examples include the following scenarios: firstly, in clinical diagnostic cases of mosaicisms, in which the chromosome to be further analyzed is present only in a small subset of metaphase spreads; secondly, in tumor cytogenetics, if rearranged chromosomes in a heterogeneous cell population may be hard to identify; thirdly, for the generation of region-specific chromosome painting probes, in which the probe-specificity can be increased if microdissection is not done with numerous, differently condensed chromosomes.

New amplification protocols appear to make the generation of high-quality painting probes from single chromosomes feasible for routine applications. Using flow cytometry and a new commercially available amplification kit, Gribble *et al.* (2004) succeeded recently in the generation of such painting probes from single chromosomes. Here, we describe the generation of specific painting probes derived from single chromosomes or single chromosomal regions using laser microdissection. We amplified the single chromosomes by linker-adaptor PCR (Klein *et al.* 1999) and used them in various multi-color-FISH experiments with up to seven different fluorochromes. To test whether the generated painting probes are completely represented and yield an even hybridization pattern, probes were hybridized onto metaphase spreads of a male donor (46,XY) and a patient with known chromosomal translocation (46,XX, t(2;3)(q37;p25)). FISH revealed bright signals and specific painting of the respective chromosomal regions and allowed clear detection of translocated chromosomal parts.

Material and methods

Chromosome preparation

Heparinized human whole blood (3 ml) was cultivated at 37°C for 72 h in 10 ml RPMI 1640 Medium (Invitrogen, Germany) containing 20% FCS (Biochrom, Germany), 1% Penicillin/Streptomycin (5000 U/ml; 5000 µg/ml, ICN Biomedicals, USA), and 2% PHA (Murex, Germany). Cells were arrested with colchicine (10 µg/ml) for 30 min. Chromosome preparations were made by incubating the cell suspension in 0.075 mol/L KCl at 37°C for 10 min, followed by a fixation step in a freshly prepared mixture of 3:1 methanol:acetic acid at 4°C.

Laser microdissection

For laser microdissection and isolation, metaphase spreads are transferred onto a microscope slide covered with a polyethylene-naphthalate (PEN) supporting membrane. In order to exclude DNA contamination and to improve metaphase spreading, the PEN membrane was treated with ultraviolet light for 30 min and incubated at -20°C for 30 min. To obtain metaphase chromosomes, the cell suspension was dropped onto the membrane that was fixed to the slides at the edges by nail polish. The chromosomes were stained using 5% Giemsa for 5 min. Slides were rinsed with water and air-dried. For microdissection we used the UV-Laser Microbeam System (PALM Micro-laser Technologies AG, Germany) equipped with an oil immersion objective (100×) of high numerical aperture to yield a spot size of less than 1 µm in diameter. The supporting membrane was cut with 0.5–0.6 µJ/pulse. We strongly recommend the use of PEN membranes; other tested materials (eg. polyester-membrane, POL) resulted in a low yield of successfully hybridized chromosomes. For laser extraction of the isolated chromosome-membrane islet, a single laser shot of 1–2 µJ/pulse was placed at the rim of the islet and the ejected islet was lifted into the cap of a 200 µl Eppendorf tube containing 4.5 µl digestion-mix. Collected chromosomal material was centrifuged into the tube at 13 000 rpm for 15 min (Mikro 22 R, Hettich, Germany).

Linker-adaptor PCR

Isolated chromosomal material was digested in 4.5 µl of proteinase K digestion buffer [0.5 µl of 10× Pharmacia One-Phor-All-Buffer-Plus, 0.29% Tween 20 (Sigma, Germany), 0.29% Igepal (Sigma), 0.29 mg/ml Proteinase K (Sigma)] for 15 h at 42°C in a MJ-Research PTC-200 thermocycler (Waltham, USA). Proteinase K was inactivated at 80°C for 10 min. *MseI* restriction endonuclease digest was performed in 5 µl by adding 0.25 µl of *MseI* (50 000 Units/µl, New England Biolabs, Germany) and 0.25 µl H₂O for 3 h at 37°C with subsequent inactivation at 65°C for 5 min (Klein *et al.* 1999). Pre-annealing of primers was achieved by adding 0.5 µl Lib1 primer (5'-AGT GGG ATT CCT GCT GTC AGT-3') and 0.5 µl ddMse11 primer (5'-TAA CTG ACA G-ddC-3') (100 µmol/L stock solution, Metabion, Germany), 0.5 µl of One-Phor-All-Buffer-Plus (Pharmacia, Germany) and 1.5 µl of H₂O. Pre-annealing was started at temperature of 65°C and was shifted down to 15°C with a ramp of 1°C/min. At 15°C, 1 µl of ATP (10 mmol/L) and 1 µl T4-DNA-Ligase (5 U/µl, Roche, Germany) were added and incubated overnight at 15°C.

For PCR amplification, 3 µl of 10 × PCR buffer (Expand Long Template, Buffer 1, Roche, Germany), 2 µl of dNTPs (10 mmol/L), 1 µl (3.5 U) of DNA polymerase mixture of Taq- and Pwo- polymerase (Expand Long Template, Roche) and 35 µl of H₂O were added to 10 µl reaction volume. The PCR program started with 68°C for 3 min and was subsequently programmed to 94°C (40 s), 57°C (30 s) and 68°C (1 min 30 s) (ramp + 1 s/cycle) for 14 cycles, 94°C (40 s), 57°C (30 s) (ramp + 1°C/cycle) and 68°C (1 min, 45 s) (ramp + 1 s/cycle) for 8 cycles; 94°C (40 s), 65°C (30 s) and 68°C (1 min 53 s) (ramp + 1 s/cycle) for 22 cycles followed by a final elongation step at 68°C for 3 min 40 s.

Reamplification and labeling

2 µl of chromosomal material were reamplified in a final volume of 50 µl using 5 µl BM Buffer 2 (Roche, Germany), 5.5 mmol/L MgCl₂ (Gibco, Germany), 0.2 mmol/L dNTP (Roche, Germany), 4 µmol/L Lib1 primer, 2.5 U/µl Taq-polymerase (Roche, Germany). After a denaturation

step of 95°C (10 min), 45 cycles were programmed to 95°C (30 s), 50°C (30 s), 72°C (2 min) and a final elongation step with 72°C (7 min) (Snijders *et al.* 2001). Reamplified chromosomes were labeled by standard nick-translation (Table 1) using Cy5-dUTP (1 nmol/L, Pharmacia, Germany), Biotin-16-dUTP (1 nmol/L, Roche, Germany), Fluorogreen-dCTP (Amersham, Germany), Digoxigenin-11-dUTP (2.5 nmol/L, Roche, Germany), DEAC-5-dUTP (1 nmol/L, NEN, Germany), Texas Red-12-dUTP (40 nmol/L, Molecular probes, Germany), and Cy3-dUTP (1 nmol/L, Pharmacia, Germany).

FISH

Labeled chromosomes were coprecipitated with 80 µl human Cot-1 DNA (1 mg/ml, Roche, Germany) and 5 µl salmon sperm DNA (9.7 mg/ml, Sigma, Germany). The pellet was washed in 70% ethanol, resuspended in 6 µl 100% formamide and incubated at 42°C for 45 min. Subsequently 6 µl 40% dextran sulfate were added and the solution was incubated at 42°C for 30 min. The labeled probes were denatured at 78°C for 7 min, with 25 min pre-annealing at 42°C. After RNase- and pepsin-digestion, target metaphase spreads were denatured in a hybridization solution containing 2× SSC and 70% formamide, pH 7.0, at 72°C for 1 min 45 s. The probe was dropped onto the metaphase spread, covered, sealed with rubber cement and hybridized in a moist chamber at 37°C for 2 nights.

The slides were then washed three times in 4× SSC/0.2% Tween at 42°C for 5 min and three times in 1× SSC at 60°C for 5 min. To avoid non-specific binding of the antibody, the slides were briefly equilibrated in 4× SSC/0.2% Tween at 42°C and incubated with 3% albumin dissolved in 4× SSC/0.2% Tween for 30 min at 42°C. Biotin-labelled probes were detected with Avidin-Cy5.5 Fab Fragments (200 µg/ml, Roche, Germany) and Digoxigenin-labeled probes with anti-Dig-Cy7 (200 µg/ml, Roche, Germany). 200 µl of Avidin-Cy5.5 or anti-Dig-Cy7 diluted 1:50 in 4× SSC/0.2% Tween/1% albumin solution were dropped on the slides and incubated for 45 min at 37°C. After three wash steps in 4× SSC/0.2% Tween at 42°C (5 min each), the slides were counterstained

with 4',6-diamidino-2-phenylindole, (DAPI) for 2 min and mounted in 50 μ l antifade.

Image recording was performed using a Leica DMXA-RF8 microscope (Leica acquisition program QFISH, Germany) equipped with a Sensys CCD camera (Photometrics, USA). Processing and karyotyping was done with Leica software package Q-CGH.

Results and discussion

Primary amplification of single isolated chromosomes was performed with linker-adaptor PCR, as previously described (Klein *et al.* 1999). Probes were directly and indirectly labeled by nick-translation and used for FISH. Reverse painting on respective metaphase spreads resulted in a specific and homogenous staining of chromosomes regardless of size, here exemplarily shown for chromosome 3 (fluorochrome Cy5.5) and chromosome 4 (FITC) resp. chromosome 14 (SC8), chromosome 15 (Cy 3.5), chromosome 20 (Cy3) and chromosome Y (Cy7) (Table 1, Figure 1). Also partly dissected material (chromosome 2q, Cy5) and combined isolation of numerous chromosomes (here chromosome 4 and Y, Cy7) resulted in an intensive and cross-hybridization-free detection (Figure 1).

Chromosome	2	3	4	14	15	20	Y
Color	Red	Blue	Yellow	Pink	Green	Orange	Purple
DEAC				×			
FITC			×				
Cy3						×	
Cy3.5					×		
Cy5	×						
Cy5.5		×					
Cy7			×				×

Table 1. Labeling scheme with the color combination for each fluorochrome. The first row shows the chromosome numbers 2, 3, 4, 14, 15, 20 and sex chromosome Y. The second row indicates the classification colors which were assigned to the respective chromosomes as shown in Figures 1c–d. Rows 3–9 show the color combinations used in the experiments and the composition of individual fluorochrome pools. For example, chromosome 2 is labeled only with Cy5, chromosome 3 with Cy5.5 and so on.

We tested whether the representation of our microdissected painting probes is sufficient to identify small interchromosomal rearrangements. To this end we used metaphase spreads of a female patient with a known, small chromosomal translocation involving chromosomes 2 and 3 [karyotype: 46,XX,t(2;3)(q37;p25)]. We had estimated earlier that the size of the translocated material is in the range of 3–5 Mb (Azofeifa *et al.* 2000). Our microdissected probes readily identified this translocation (Figure 2) suggesting that these probes maintain a high probe complexity during the generation procedure.

Our results demonstrate that the laser energy used for membrane dissection, a few μ m around the target chromosome, and ejection does not alter the chromosomal material for adaptor-linked PCR. This is due to the fact that the applied laser wavelength of 337 nm is far from the absorption maximum of DNA of 260 nm and the effective laser energy is limited within the focal area (Greulich & Leitz 1994).

Our approach paves the way to acquire DNA with ease from a single isolated chromosome or parts of a chromosome for the generation of specific probes for FISH applications. It does not require sophisticated instrumentation, which is needed for isolation of chromosomes by flow-cytometry. Laser microdissection working units have become increasingly popular in recent years. As a consequence more investigators will have access to microdissection equipment than to a flow-cytometer. Another improvement, as compared to previous protocols (Schermelleh *et al.* 1999, Kubickova *et al.* 2002), is the amplification by linker-adaptor PCR, which yields a better representation of the probe than DOP-PCR.

The homogenous amplification of small amounts of DNA has evolved into a rapidly moving field. This is reflected in the launch of several commercial kits for DNA amplification (e.g. GenomePlex from Rubicon Genomics, GenomiPhi from Amersham and Repli-G from Molecular Staging). The usefulness of the GenomePlex-kit for the generation of painting probes from single chromosomes was recently demonstrated (Gribble *et al.* 2004). We have not yet explored in detail the capabilities of the GenomiPhi and the Repli-G kits. Both kits are identical but distributed by different manufacturers. To date, we have had the

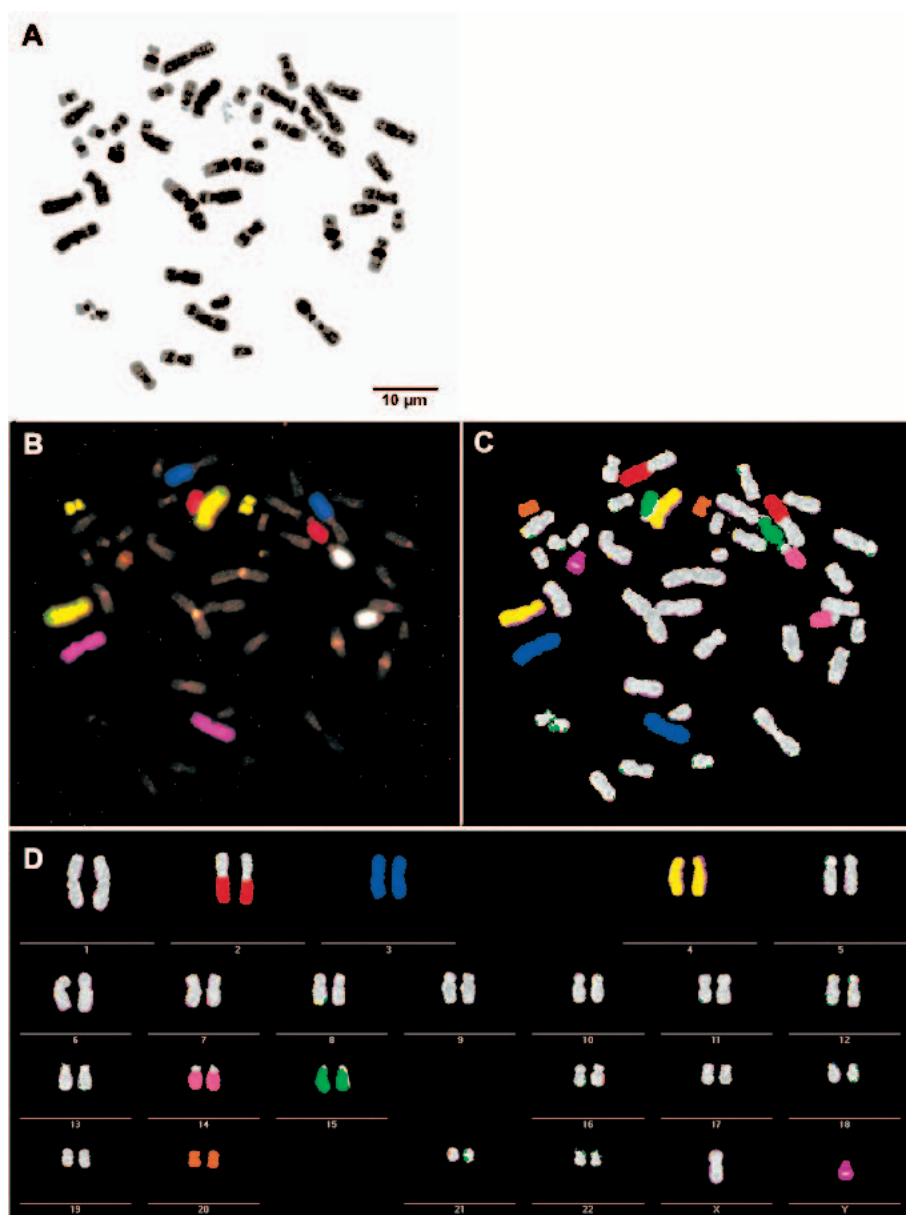


Figure 1. Metaphase spread and karyogram after simultaneous hybridization of 7 probes, each generated from a single microdissected chromosome or chromosomal region and each labeled with a different fluorochrome combination (chromosome 3 (Cy5.5), chromosome 4 (FITC and Cy7), chromosome 14 (DEAC), chromosome 15 (Cy 3.5), chromosome 20 (Cy3), chromosome Y (Cy7), and chromosome arm 2q (Cy5). (A) inverted DAPI counterstain; (B) “true colors” (generated by overlying the pseudo-colored source images), (C) classification colors and (D) karyogram. Scale bar in (A) 10 µm.

opportunity to use the GenomiPhi/Repli-G kits for the DNA amplification of single cells. We have not yet achieved a homogenous painting pattern with the single cell amplification product. However, these are very preliminary results which have to be confirmed in further experiments. The use of

kits may have the advantage that the amplification procedure is less laborious. However, more detailed comparisons are needed to establish whether the reproducibility and robustness of these kits is comparable to the linker-adaptor approach used in this study.

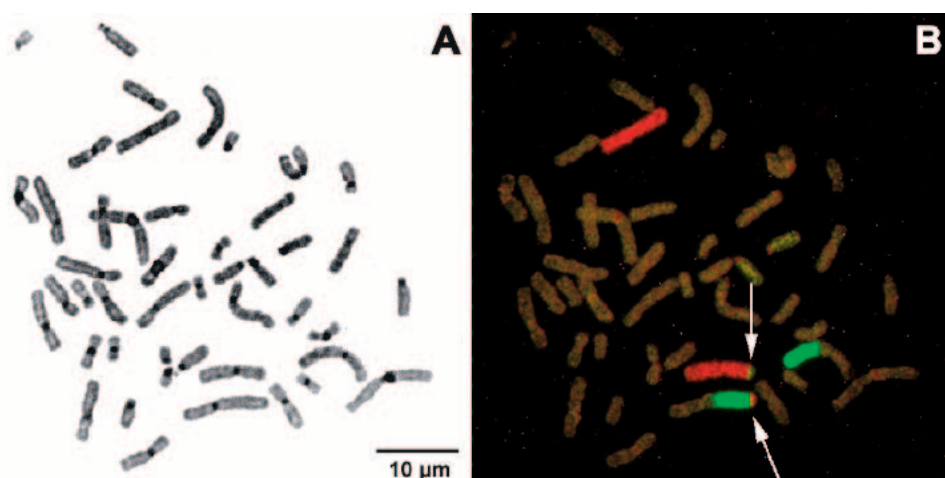


Figure 2. FISH analysis with microdissected probes applied to metaphase spreads of a patient, who is carrier of a balanced translocation between chromosomes 2 and 3 [karyotype: 46,XX,t(2;3)(q37;p25)]. (A) inverted DAPI counterstain; (B) hybridization patterns of the chromosome-arm 2q (green) and chromosome 3 probes, arrows indicate translocation site. Scale bar in (A) 10 µm.

In summary, we describe an approach which should be attractive for a number of applications, including the examination of rare translocations in tumor patients, classification of marker chromosomes, and haplotype studies.

Acknowledgements

We thank Dr Heide Seidel, Institute for Human Genetics, LMU Munich, for clinical cooperation, and Gaby Lederer and Doris Sollacher for excellent technical assistance. This work was supported by a Deutsche Forschungsgemeinschaft (DFG) SFB 486 grant, by DFG grant (Sp 460/5-1), and the Bundesministerium für Bildung und Forschung (NGFN KB P06T5, P06T6).

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