Original articles

A simple and efficient method for microdissection and microFISH

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Abstract

A simple and efficient method for the dissection of (marker) chromosomes, (micro)nuclei, and chromosome regions is presented. Before microdissection, metaphases are overlaid with milli-Q water to rehydrate the chromosomes, which makes them soft and sticky. The dissected chromosome fragments are dissolved without proteinase-K or topoisomerase treatment and directly amplified using a degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR). The advantages of this microFISH method over previously reported methods are: (1) microdissection in this way is very fast; (2) a chromosome, marker, (micro)nucleus, or chromosome region is collected as a whole using only one microneedle; (3) the dissected material sticks tightly to the needle without the risk of getting lost; (4) no Sequenase is used in the DOP-PCR reaction which reduces the risk of contamination.

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Microdissection was introduced in human cytogenetics by Bates *et al.*¹ who constructed a chromosome 2 library using many (>100) chromosome fragments that were dissected from unbanded and unstained metaphases. Ludecke *et al.*² described an improved procedure for microdissection and microcloning and introduced the PCR technique to amplify the minute amounts of dissected chromosome fragments. This method was improved further by Senger *et al.*³ who successfully dissected single bands from GTG banded chromosomes. MicroFISH, a technique developed by Meltzer *et al.*⁴ at present represents the most straightforward method for the synthesis of chromosome region specific and chromosome band specific paints and thus for the characterisation of chromosome aberrations. In microFISH, four important steps can be distinguished: (1) the physical dissection of (part of) a chromosome or a marker chromosome, (2) the incubation of the dissected material with proteinase-K or topoisomerase to relax the supercoiled DNA of the metaphase chromo-

some parts, (3) a degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) to amplify the dissected material, and (4) the labelling of the PCR product with biotin or digoxigenin followed by fluorescence in situ hybridisation (FISH) with the probe obtained.

In this study we describe an improved and simplified method for microdissection, dissolving of the dissected material, and DOP-PCR with less risk of contamination.

Material and methods**PREPARATION OF METAPHASE CHROMOSOMES**

Chromosomes were prepared from peripheral blood lymphocytes using the synchronisation method of Dutrillaux and Viegas-Pequignot⁵ with only minor modifications. Chromosome spreads for microdissection were made on 45 × 65 mm coverslips according to routine procedures. The coverslips were rinsed in distilled water and stored in 98% ethanol at -20°C (for three days to two weeks). GTG banding with trypsin-Giemsa was performed before microdissection.

MICRODISSECTION OF BANDED CHROMOSOMES

Microdissection was performed with glass microneedles (tip diameter <0.5 µm) controlled by a Narishige micromanipulator (MO-202). To eliminate the risk of amplification of contaminating extraneous DNA, microneedles, microcentrifuge tubes (including the collection drop), and pipette tips were treated with ultraviolet light (254 nm) for 30 minutes. Before microdissection of a (marker) chromosome, a metaphase with well spread chromosomes was selected and covered with 5 µl milli-Q water (fig 1A, B). Because of the rehydration, the chromosomes became sticky and could be collected as a whole using only one microneedle. For the dissection of a chromosome region or a chromosome band, a slightly different procedure had to be followed. First, the chromosome region or band of interest was isolated from the rest of the chromosome by dissecting the chromosome parts proximal/distal to the region or band of interest (fig 1E, G). This manipulation was performed when the chromosome was still dry and hard. Next, a new microneedle was installed, positioned slightly above the metaphase, which was then covered with 5 µl milli-Q water. Then the chro-

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Figure 1 Microdissection procedure. (A) Localisation of a well spread metaphase and installation of the microneedle. (B) Covering of the metaphase with milli-Q water. (C, D) Dissection of chromosome 1. (E) Removal of the centromere of chromosome 2. (F) Dissection of the long arm of chromosome 2. (G) Removal of the chromosome bands 7q11.2 and 7q22. (H) Dissection of chromosome region 7q21 after covering the metaphase with milli-Q water. (I, K) Dissection of a micronucleus of a patient with ICF syndrome.

mosome region or band was dissected using only one needle (fig 1F, H). Finally, the needle tip was moved out of the water drop and the dissected band or region dried instantly and stuck firmly to the needle tip. Routinely, five chromosome bands or regions were collected in this way and transferred to a 20 μ l collection drop (containing 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 0.1 mg/ml gelatine, 0.1% (v/v) Brij-35 (Sigma)) in a 0.5 ml microcentrifuge tube. As the needle tip holding the microdissected chromosome part was broken off, a fresh microneedle was used for each dissection.

AMPLIFICATION OF CHROMOSOMAL DNA

The samples, containing five copies of the chromosomal material of interest, were cycled for 15 cycles at 30°C for one minute and at 50°C for one minute to dissolve the dissected chromosome parts. Subsequently, 30 μ l of PCR

buffer containing 100 μ mol/l of each dNTP, 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 5 ng acetylated BSA, 4 μ mol/l universal primer, and 0.5 U super *Taq* DNA polymerase (HT Biotechnology) were added to each sample. The universal primer 5' CCGACTCGAGNNNNNNATGTGG 3' was used, as suggested by Telenius *et al.*⁶ For initial denaturation the mixture was heated to 93°C for three minutes, followed by eight cycles of one minute at 94°C, one minute at 30°C, one minute at 45°C, and three minutes at 72°C, and by 28 cycles of one minute at 94°C, one minute at 56°C, three minutes at 72°C, with a final extension step at 72°C for 10 minutes. The DOP-PCR experiments always contained a negative control consisting of all PCR components except microdissected DNA and a positive control with 25 pg total human DNA. PCR reactions were performed in a Biometra personal cyler with a heated lid. PCR products were stained with ethidium bromide and analysed for yield and probe size (200–900 base pairs) on an agarose gel. Experiments were continued only if DNA synthesis was not apparent in the negative control. The PCR product was precipitated and purified with NH_4Ac (4 mol/l) and isopropanol, washed with ethanol (70%), and dissolved in 60 μ l T0.1E buffer (10 mmol/l Tris-HCl/0, 1 mmol/l Na_2EDTA , pH 8.0).

FLUORESCENCE IN SITU HYBRIDISATION (FISH)

PCR products were labelled by nick translation with biotin-14-dATP (BioNick™ labelling system (Gibco BRL)). Metaphase spreads for FISH were prepared from PHA stimulated lymphocytes using standard methanol:acetic acid (3:1) fixation. In situ hybridisation was performed following the protocol of Guan *et al.* with only minor modifications. Briefly, for hybridisation, 200 ng of probe was used in 10 μ l of hybridisation mixture containing 50% formamide, 2 $\times$ SSC, 10% dextran sulphate, 50 mmol/l phosphate, and 20 μ g human Cot 1 DNA (BRL). Probe and Cot 1 DNA were denatured at 70°C for five minutes and reannealed at 37°C for 90 minutes. Slides were denatured in 70% formamide, 2 $\times$ SSC (pH 7.0) at 70°C for three minutes, dehydrated, and hybridised with probe at 37°C overnight in a moist chamber. Slides were then washed four times in 2 $\times$ SSC (pH 7.0) at 45°C for five minutes and once in PN buffer (0.1 mol/l sodium phosphate, 0.1% (v/v) Nonidet P-40, pH 8.0) at 45°C for 10 minutes. The hybridisation signal was detected by three layers of fluorescein-avidin DCS (Vector) amplified by two layers of biotinylated antiavidin D (Vector), followed by two washes with 4 $\times$ SSC, 0.5% Tween, and one wash with PBS. Slides were dehydrated and counterstained with DAPI/PI in antifade solution. Analysis was carried out using a Zeiss Axiophot microscope and photographs taken with Scotchchrome 640 ASA colour slide film.

Results and discussion

In the procedure that is nowadays used to prepare metaphase chromosomes for microdissec-

Figure 2 Partial metaphase after reverse painting to a normal metaphase with DOP-PCR product, (A) derived from the long arm of chromosome 2, (C) derived from chromosome region 7q21, and (E) derived from the micronucleus of an ICF patient. (B, D, F) DA/DAPI counterstaining of fig 2A, C, E.

tion, special attention is paid to prevent degradation of the DNA.⁸ When preparing slides for microdissection, they are washed with distilled water to remove acetic acid and stored immediately in 98% ethanol at -20°C . Because of the fixation procedure and the storage in ethanol, the chromosomes are dehydrated and become hard.⁹ This hampers the microdissection, especially of whole chromosomes and chromosome regions. An additional problem that frequently occurs is the repulsion of the dissected fragments from the needle. Bussey¹⁰ described it as the "Mexican jumping bean" phenomenon. The procedure reported here overcomes these problems. Before microdissection of a whole chromosome, a marker chromosome, or a (micro)nucleus, the micro-needle is brought into the field within a few μm of a well spread metaphase (fig 1A). This metaphase is covered with 5 μl milli-Q water (fig 1B), the microneedle is then moved down onto the slide, and the (marker) chromosome of interest or (micro)nucleus is detached entirely from the slide using just one needle (fig 1C, D). Besides the fact that this procedure is very fast

and dissection of a chromosome is completed within a minute, a second advantage is that the microdissected chromosome part sticks tightly to the needle tip (particularly after the tip is removed from the water drop) avoiding the risk of losing the dissected fragment when transferring it to the microcentrifuge tube containing the collection drop.

The second step in the microFISH procedure is treatment of the dissected material to dissolve chromosomal DNA. Proteinase-K treatment^{4 11 12} is frequently used to digest the proteins that are associated with the DNA of mitotic chromosomes and also pretreatment with topoisomerase I is recommended¹³ to enhance the accessibility of the dissected DNA for primers and polymerase during the subsequent PCR. In our experiments the samples were cycled for 15 cycles at 30°C for one minute and at 50°C for one minute, without proteinase-K or topoisomerase treatment, which led to a yield of DNA after DOP-PCR comparable with the proteinase-K method we used previously.¹⁴⁻¹⁶ The most crucial step in microFISH is, without any doubt, the amplification of the dissected material using DOP-PCR.

Although various protocols for DOP-PCR exist, most authors at present prefer the method developed by Guan *et al.*¹³ During the initial eight cycles of this method, when the annealing temperature is low, T7 DNA polymerase (Sequenase) is used. However, Sequenase is heat inactivated at the denaturation temperature of 94°C and consequently active Sequenase has to be added eight times. For this reason, these steps are a prime target for contamination. In the method described here, the use of Sequenase is omitted, which reduces the risk of extraneous contamination considerably.

For standard applications a primer concentration between 0.1 and 1 $\mu\text{mol/l}$ is recommended¹⁷ and the primers are rarely completely used up during the reaction. However, the primers have to compete with the accumulating product in finding the target sequences, which could become a limiting factor for the reaction, certainly in the late cycles. We decided to use a high primer concentration (4 $\mu\text{mol/l}$) in our experiments, as Czerny¹⁸ showed that primer limitation can make a critical contribution to the attenuation of amplification rates observed for late cycles of PCR.

As an example, fig 2A shows the results of microFISH of the long arm of chromosome 2 dissected in fig 1E and F, and fig 2C shows the chromosome 7q21 band specific paint dissected in fig 1G and H. In fig 1I and K, a micronucleus is dissected from a patient with ICF syndrome.¹⁹ Patients with this syndrome can be recognised cytogenetically by the presence of triradial and multiradial chromosomes and many micronuclei in routine cultures, owing to instability of the pericentromeric heterochromatin of, in particular, chromosomes 1, 9, and 16. Fig 2E shows that a micronucleus from a patient with ICF syn-

drome contains only DNA derived from the long arm of chromosome 1.

In conclusion, the modified microdissection method described here enables fast and reliable dissection of (marker) chromosomes, (micro) nuclei, and regions or bands of chromosomes. Furthermore, relaxation of the dissected material was achieved without extra additives, an efficient DOP-PCR reaction mix was composed, and reduction of the risk of extraneous contamination was attained by omitting the addition of Sequenase. In clinical cytogenetics, microFISH is an elegant method for characterising chromosome aberrations and constructing region and band specific libraries. Unique clones from these libraries can be used to isolate YAC clones and to isolate candidate genes from cDNA clones. In genetic toxicology and radiation genetics, microFISH is a valuable tool for determining the chromosomal content of micronuclei and sensitive sites for chromosome breakage can be detected in this way.

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