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Use of chromosome microdissection in fish molecular cytogenetics

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Abstract

Chromosome microdissection is a technique in which whole chromosomes or chromosomal segments are dissected under an inverted microscope yielding chromosome-specific sequences. Several protocol modifications introduced during the past 15 years reduced the number of chromosomes required for most applications. This is of particular interest to fish molecular cytogenetics, since most species present highly uniform karyotypes which make impossible the collection of multiple copies of the same chromosome. Probes developed in this manner can be used to investigate chromosome homologies in closely related species. Here we describe a protocol recently used in the gymnotiform species group *Eigenmannia* and review the major steps involved in the generation of these markers focusing on protocol modifications aiming to reduce the number of required chromosomes.

Key words: fluorescence *in situ* hybridization, chromosome painting, sex chromosomes, cross FISH.

Chromosome-specific sequences are highly desired in studies focusing on comparative genomics and genomic organization. The two most straightforward paths for obtaining chromosome-specific makers are *flow sorting*, commonly referred to as FACs (*fluorescence activated cell sorting*), and *chromosome microdissection*. In flow-sorting, chromosomes are sorted using a laser system that distinguishes chromosomes depending on their size and fluorochrome affinity (AT, GC base content) while in chromosome microdissection chromosomes or chromosomal segments are literally scraped and collected.

It is generally assumed that flow-sorting generates paints of greater complexity and coverage because this method allows the collection of massive amounts of chromosomes (around 300-500) in a highly automated procedure (Ferguson-Smith *et al.*, 1998). However, the costs of equipment and need of high quality cell cultures make this technique not suitable for most fish cytogenetics laboratories. Specifically concerning genetic studies in fish, a new difficulty arises, since most fish species present a highly uniform karyotype regarding chromosomal size and base content.

Chromosome microdissection was originally developed in studies involving *Drosophila* polytene chromosomes as a way to obtain DNA markers from specific chromosomal regions and was quickly applied to mammalian genomes during the 80s (Fan, 2002). The subsequent development of *in situ* hybridization procedures and establishment of DOP-PCR (Telenius *et al.*, 1992) allowed the application of chromosomal probes obtained by microdissection in FISH (fluorescence *in situ* hybridization) procedures (sometimes referred to as microFISH). This technique has been applied in several studies involving fish species, and has been particularly useful in studies focused on sex-chromosomes (Reed *et al.*, 1995; Harvey *et al.*, 2002; Liu *et al.*, 2002).

Microdissection is commonly regarded as a labor intensive method since most protocols require the collection of 20 chromosomal copies. However, protocol modifications have been made in order to reduce the number of chromosomes (one to five chromosomes are sufficient depending on the application), turning microdissection into a routine procedure whilst maintaining probe quality (Guan *et al.*, 1994; Engelen *et al.*, 1998; Christian *et al.*, 1999; Weimer *et al.*, 1999).

Amplification of the chromosomal libraries is done by degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius *et al.*, 1992). A pre-amplification step (eight cycles) has been found to increase probe coverage (Guan *et al.*, 1994). Pre-amplification involves the use of alternative DNA polymerases, particularly T7 DNA polymerase (commercially available as Sequenase) due to its higher pro-

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cessivity, particularly in the 25 to 35 °C range, which are the temperatures used in primer annealing and extension. Since Sequenase is not thermo-stable, small quantities (0.3 U) must be added at each of the eight cycles during annealing. The use of Thermosequenase has also been reported (Christian *et al.*, 1999). Since this enyme is thermostable, its use minimizes risk of contamination involved in adding additional enzyme each cycle. It should be noted however, that Thermosequenase is a modified *Taq* polymerase, and therefore presents highest processivity at elevated temperatures. Therefore, the use of temperature ramps between annealing and extension is recommended. The final 20 cycles (high-stringency cycles) can be performed using regular Taq polymerase, since incorporation of the 5' non-degenerate sequence of the primer insures specific amplification at high extension temperatures. (Guan *et al.*, 1994)

Here we describe a protocol, slightly modified from Weimer *et al.* (1999), recently used to microdissect and amplify autosome and X-linked sequences of *Eigenmannia virescens*. Samples used for microdissection and FISH present 2n = 38 XY and 31/32, as previously described by Almeida-Toledo *et al.* (2001). Direct kidney preparations was obtained from individuals collected at small tributaries of the Tietê and Parapanema river basins (Brazil) in 2006, and have been stored in fixative at -20 °C.

Suspensions were dropped on coverslips and subjected to regular Giemsa staining. The use of coverslips is necessary since the objectives and material are positioned underneath and on top of the coverslip, respectively, in an inverted microscope. Slide thickness impairs such cross focusing with 40x and 100x objectives. Cell suspensions should be diluted to ensure good distribution of metaphases, and special attention should be given to spreading and pre-treatment. Usually, fresh suspensions can be used without any treatment. (Henegariu *et al.*, 2001).

The needles were prepared from borosilicate rods (Harvard Apparatus – Massachusetts, USA) using a two-step pipette puller (Narishige, Tokyo, Japan). Glass capillaries can also be used, although they are less stable and more susceptible to breakage. Needles were loaded to a micromanipulator attached to the microscope. Siliconized pipettes were also prepared with the pipette puller or by manual pulling after heat exposure, and the tip was gently broken in order to create a small opening where the chromosomes and collection solution should are stored during microdissection. The pipette was fixed to the microscope table using commercially available modeling clays. All equipment was exposed to UV light prior to use.

Centralizing the needle and pipette before loading the coverslip has been found to considerably facilitate procedures. While manipulating the coverslip and microscope table, it is recommended to maintain the needle at the high position to avoid breakage. Detachment of the chromosomes from the coverslip was achieved by gently scraping the chromosome along its vertical axis, causing the chromosome to fold around itself and finally bind to the needle.

Chromosomes were transferred to the pipette containing a collection solution (10 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5-8.0, 0.1% SDS, 0.1% Triton X100, 500 µM proteinase K, and 30% glycerol). The collection solution was added by capillarity to the pipette prior to use. The use of topoisomerase instead of proteinase has also been reported (Guan, 2002).

The chromosomes were transferred by inserting the tip of the needle into the pipette containing the collection solution. The same needle was used to pick up further chromosomes. The pipette containing the chromosomes was incubated in a humid chamber at 60 °C for one hour. Incubation in a humid chamber and the use of glycerol are both effective in controlling evaporation. The chromosomes were then transferred to the pre-amplification mixture described bellow by breaking the tip of the pipette inside the microtube containing the PCR solution.

The pre-amplification PCR mix used consisted of 24 mM Tris- pH 7.5, 12 mM $MgCl_2$, 30 mmol/L NaCl (0.6x Reaction Buffer), 0.2 mM dNTP's, 5 µM 6-MW primer (5' CCG ACT CGA GNN NNN NAT GTG G 3'), in a 5 µL final volume. The reaction profile used is 90 °C for 1 min, 25 °C for 2 min, and 4 °C for 2 min. As mentioned before, 0.3 U of Sequenase should be added at each cycle during annealing. A first step of denaturation (5 min at 90 °C) is necessary to inactivate proteinase activity in the collection solution.

For the standard DOP amplification after the pre-amplification step (30 cycles), 50 µL of the following mix is added to the previous PCR product: 10 µL of 10x NH_4 Reaction Buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20), 0.2 mM dNTP's, 5 µM 6-MW primer and 0.1 U Taq polymerase. The high-temperature reaction profile is 92 °C for 1 min, 56 °C for 2 min, and 70 °C for 2 min. A final 5 min extension step at 72 °C should be added.

The probe was PCR labeled with Biotin-16-dUTP (Roche) in 28 DOP-PCR cycles using as template DNA-deletar 1 µL of the primary DOP-PCR product as template DNA. Control of non-specific signals was achieved using salmon sperm DNA (15 mg) and a pre-hybridization step (15-30 min at 37 °C).

Figure 1 shows hybridization of a probe developed from a single microdissected autosome of *Eigenmannia* sp. 2. Note that the probe distinctively hybridizes to the entire chromosome pair. Figure 2 shows hybridization of X-heterochromatin and Y-specific probes obtained by microdissection (three and four copies of each chromosome, respectively) from *E. virescens* and *Eigenmannia* sp. 2, respectively. The probe derived from the heterochromatic segment of *E. virescens* X chromosome hybridizes successfully to the X specific heterochromatin as shown in Figure 2A.

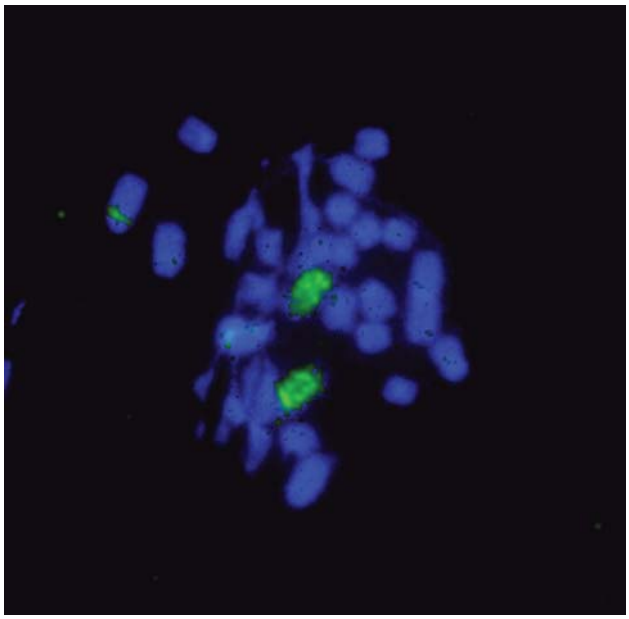

Figure 1 Hybridization of a probe developed from a single micro dissected autosome of *Eigenmannia* sp. 2.

Furthermore, DAPI (4'-6-Diamidino-2-phenylindole) counterstain on *E. virescens* (Figure 2B) revealed a distinct striped pattern of X heterochromatin, where AT-rich sequences seem to be alternated with GC-rich sequences. The Y-derived probe hybridizes to the Y, X_1, and X_2 chromosomes (Figure 2C). Hybridizations to X chromosomes, as seen in Figure 2C, were not total, adding further evidence to the presumable a loss of repetitive sequences during the fusion event that originated the Y chromosome (Almeida-Toledo *et al.*, 2000).

Recent modifications from the original protocols have made the microdissection procedure applicable for molecular-cytogenetics and comparative genomic research. Probes developed using microdissection have been reported to show properties similar to those obtained by flow-sorting (Griffin *et al.*, 1999), although this may not hold in cross-FISH experiments with distant species microdissection is a suitable procedure for comparison of closely related species groups. Probes obtained using this protocol have successfully revealed chromosome homologies in

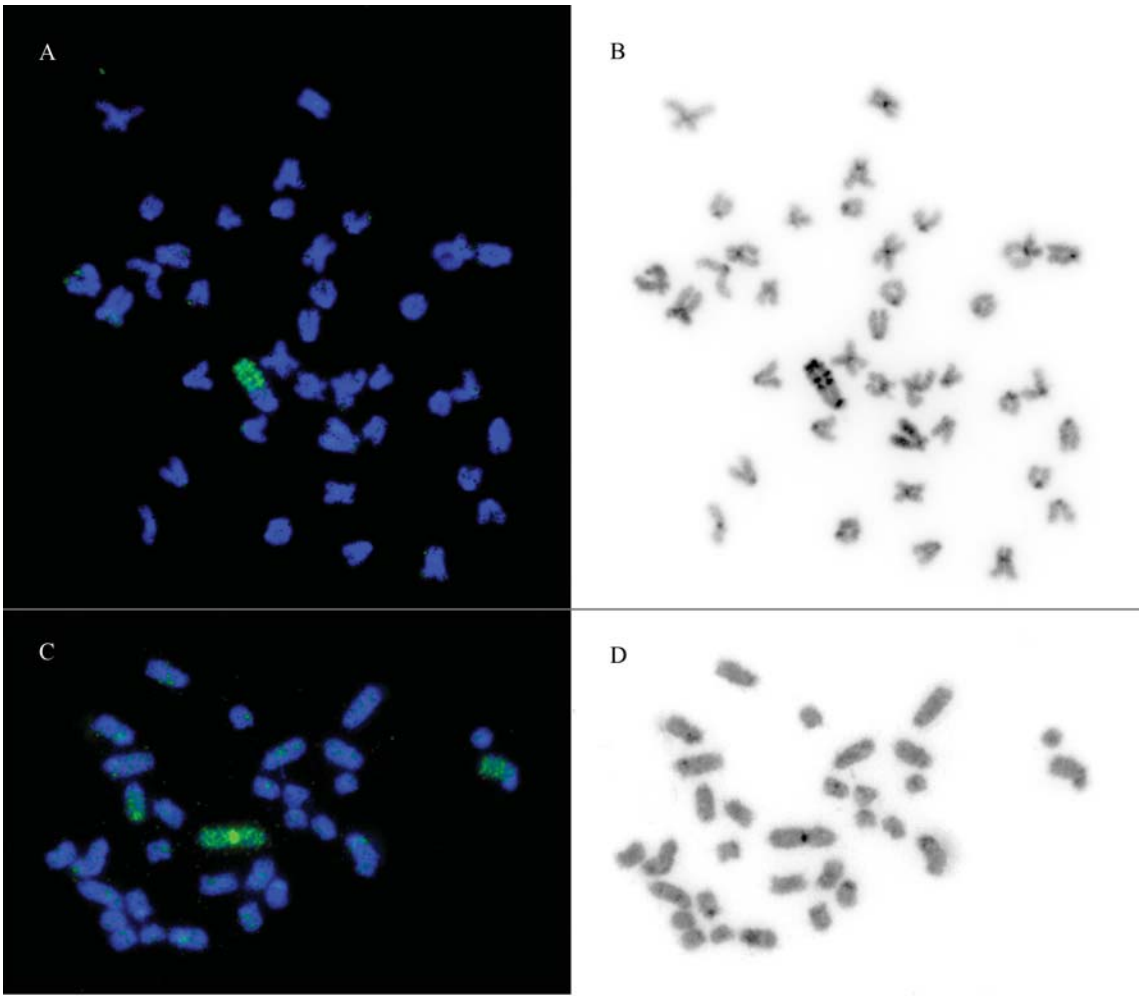

Figure 2 Hybridization patterns using A) a probe developed from three copies of X chromosome heterochromatin of *Eigenmannia virescens*; B) DAPI counterstain of A; C) a probe derived from *Eigenmannia* sp. 2 Y chromosome; D) DAPI counterstain of C.

Eigenmannia species (Henning F, Trifonov V, Ferguson-Smith MA and Almeida-Toledo, unpublished data).

An interesting application of microdissection is the construction of plasmid library from the DOP-amplified fragments. DOP-PCR fragments are, however, considerably small (200-500 bp) and fragmented (the 5' sequence of the 6-MW primer is expected to occur at each 4 kb). These libraries can however, be useful for isolation and sequencing of repetitive DNA using a combination of FISH and blotting techniques (Southern, dot or slot blotting) for screening (Shibata *et al.*, 1999; Madalena and Gorab, 2005) or as probes for retrieving region specific sequences from a BAC library. This procedure is highly desired in studies focused on B-chromosome investigations, since sequence data can help assessing the evolutionary origins of supernumerary chromosomes (Bugrov *et al.*, 2004, Rubstov *et al.*, 2004) and is of potential use in fish breeding (Yi *et al.*, 2003).

Despite being a numerous and highly diverse group, chromosome painting has been applied to only a handful of species (Phillips *et al.*, 2001; Harvey *et al.*, 2002; Liu *et al.*, 2002). The possibility of developing paints from few, or even single microdissected chromosomes is promising, considering common characteristics of fish chromosomes such as uniformity in size and morphology.

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