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Fiber FISH reveals different patterns of high-resolution physical mapping for repetitive DNA in fish

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ABSTRACT

Fiber fluorescence *in situ* hybridization (fiber-FISH) has improved physical gene mapping very much since it allows ascertaining the relative position of two or more genes or repetitive DNA sequences. But this technique has never been employed in fish. Here we show that the standard fiber-FISH protocol can be employed on normally fixed cells stocked in many laboratories. We analyze here possible outcomes of this technique in three species of fish from two different orders. In every case, we performed double fiber-FISH for two different repetitive DNAs and observed three different patterns depending on whether the two probes were at independent (separately in interphase nuclei and on two different pairs of chromosomes), adjacent or interspersed locations. This high-resolution method can thus be employed on the same cell suspensions routinely used for chromosome analysis, and this can be an excellent complementary resource for compositional, structural and functional analysis of DNA sequences in the genomes of fish and other aquatic organisms.

1. Introduction

Fluorescence *in situ* hybridization (FISH) has become a standard procedure for physical mapping of DNA sequences on eukaryotic chromosomes. Most studies usually employ probes for coding and non-coding repetitive DNA, especially multigene families of ribosomal DNA (rDNA). These probes have provided efficient markers for comparative analysis, chromosome mapping and chromosome rearrangement characterization, as well as ascertaining homologies in different groups (Cabrero et al., 2003; de Jong et al., 1999; Ferguson-Smith et al., 1998; Vicari et al., 2010). However, resolution is limited to 1–3 megabases (mb) due to the multifolded structure of the DNA strand when the chromosome is at metaphase (Raap et al., 1996). The advent of FISH on extended chromatin fibers (fiber-FISH) increased the resolution of physical mapping to 1–400 Kb (Raap et al., 1996; Schwarzacher and Heslop-Harrison, 2000). This method has thus attracted considerable interest in genomic studies in different eukaryotes, such as *Arabidopsis thaliana* (Fransz et al., 1996; Jackson et al., 1998), tomato (Fransz et al., 1996), cattle (Brunner et al., 1998), maize (Adawy et al., 2002), rice (Dong et al., 1998; Nagaki et al., 2004), humans (Heiskanen et al., 1995; Iafrate et al., 2004; Teitz et al., 2000; Vaandrager et al., 1996), plants (Lavania et al., 2005), bivalves (García, 2010) and grasshoppers (Muñoz-Pajares et al., 2011). The method using nuclear halo preparations was developed by Wiegant et al. (1992), who modified the version described by Vogelstein et al. (1980) with the aim of relaxing the chromatin fibers of the nuclear scaffold. A number of changes in the methodology of this technique were subsequently developed (Fidlerova et al., 1994; Heiskanen et al., 1994, 1996; Heng et al., 1992; Mann et al., 1997; Parra and Windle, 1993), with applications in the analysis of sequence overlapping, detection of chromosome rearrangements and can be useful to establish the relative position of a gene to another in chromatin fibers.. The application of fiber-FISH may also be useful to understanding processes of gene degeneration and structural differentiation involved in the evolution of sex and supernumerary chromosomes, due to the accumulation of several classes of repetitive DNA in these chromosomes (Acosta et al., 2011; Singh et al., 1980; Vicari et al., 2010). Hence, considering their highly variable evolutionary dynamics, repetitive DNA sequences can provide useful information regarding both evolution and genome organization. Moreover, with advances in the understanding of the chromatin composition of sex and supernumerary chromosomes in fish, double fiber-FISH may be useful in the analysis of the arrangement of fibers in the scaffold. However, fiber-FISH is not routinely used on fish and other aquatic organism. We provide here an adaptation of the techniques described in Fidlerova et al. (1994), Pinkel et al. (1986) and Muñoz-Pajares et al. (2011), which provides analyzable extended chromatin fibers and probe detection by FISH, from stocks of normally fixed cells that had been stored for long

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time periods. As an example, we show here the results obtained in three fish species, illustrating three different outcomes between the two probes, namely separate, adjacent or interspersed locations.

2. Materials and methods

2.1. Cell preparation and fibers extension

Cell suspensions fixed in methanol and acetic acid (3:1) were obtained following the protocol described by Bertollo et al. (1978) for fish, and then were stored in the cell suspension bank of the Cytogenetics and Evolution Laboratory of the Universidade Estadual de Ponta Grossa (Brazil). We analysed three species from different fish families: *Steindachneridion melanodermatum* (Pimelodidae, Siluriformes), *Isbrueckerichthys duseni* (Loricariidae, Siluriformes) and *Astyanax janeiroensis* (Characidae, Characiformes). The material in cell suspension was dropped on glass slides moistened in water at 65 °C and air dried. Usually 10 ± 5 nuclei per visual field at the light microscope (100× magnification) was a favorable condition to obtain good fiber-FISH stretched. The slides were washed with phosphate buffered saline (PBS) for two minutes at room temperature (25 °C). In the upper part of the slides (previously dried and in the horizontal position), 200 µl of sodium hydroxide (NaOH) 0.15 M diluted in 30% alcohol was added. The slide with then inclined between 30 and 40° and a continuous smear was made on the preparation from the top downward with the aid of a clean slide in such a way as to cover the entire surface of the slide, spreading out the NaOH solution. Next, 500 µl of 100% ethanol was immediately applied to the slide, which was maintained inclined until complete evaporation. This fixation procedure increases the number of attachment points between the DNA fibers and surface of the slide.

2.2. Probe preparation

The 18S rDNA probe was obtained from nuclear DNA of *Astyanax janeiroensis* through polymerase chain reaction (PCR) using the primers for the initial sequence (18S IF 5′-TCCGCAGGTTCACCTACGGA-3′ and 18S IR 5′-ACGCGAGATGGAGCAATAAC-3′) and final portion (18S FF 5′-TGGTCACCATGGTAGTCCTCT-3′ and 18S FR 5′-GTAGTCATATGCTTGTCTCA-3′) of the gene. Both sequences were submitted to PCR in 35 amplification cycles in a BJI® thermocycler (1 min at 95 °C, 45 s at 56 °C, 1 min at 72 °C and 5 min at 72 °C). The 5S rDNA probe was obtained from *S. melanodermatum* through PCR with primers described for the rainbow trout (Komiya and Takemura, 1979; Martins and Galetti, 1999), 5′-TACGCCCGATCTCGTCCGATC-3′ and 3′-CAGGCTGGTATGGCCGTAAGC-5′, in 35 amplification cycles in a BJI® thermocycler (1 min at 95 °C, 30 s at 55 °C, 45 s at 72 °C and 5 min at 72 °C). The As51 satellite DNA contains 59% A + T and monomeric units of 51 bp (Mestriner et al., 2000). It was obtained from nuclear DNA from *Astyanax scabripinnis* by PCR using the primers As51F 5′GGTCAAAAAGTCGAAAAA3′ and As51R 5′GTACCAATGGTAGACCAA3′ in 35 amplification cycles in a BJI® thermocycler (1 min at 95 °C, 45 s at 56 °C, 1 min at 72 °C and 5 min at 72 °C). The probes were labeled through PCR, using the haptens biotin-16-dUTP (Roche®) for 18S rDNA or digoxygenin-11-dUTP (Roche®) for the 5S and *As*51 rDNA probes. The labeling PCR with specific primers was performed for the rDNA using 20 ng of template DNA, 1X *Taq* Reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 40 µM dATP, dGTP and dCTP, 28 µM of dTTP, 12 µM biotin-16-dUTP or digoxygenin-11-dUTP, 1 µM of the primers, 2 mM $MgCl_2$ and 0.05 U/µL of *Taq* DNA Polymerase (Invitrogen®).

2.3. Fluorescence in situ hybridization (FISH)

Hybridization was accomplished by using a modification of the protocol described by Pinkel et al. (1986) under high stringency conditions (2.5 ng/mL probes, 50% deionized formamide, 10% dextran sulfate, 2 × SSC at 37 °C overnight). After hybridization, the slides were washed in 15% formamide/0.2 × SSC at 42 °C for 20 min, 0.1 × SSC at 60 °C for 15 min and 4 × SSC/0.05% Tween at room temperature for 10 min; the latter consisting of two 5-min washes. The signal detection was performed using streptavidin-alexa fluor 488 (Molecular Probes®) for the 18S rDNA and anti-digoxigenin-rhodamine (Roche®) for the 5S rDNA and *As*51 probes. The chromosomes were analyzed under an epifluorescence microscope (Olympus® BX41) coupled to an image capturing system (Olympus® DP71). The extended DNA fiber images were captured in TIFF format with a resolution of 432 dpi and 1000× magnification.

3. Results and discussion

The fiber-FISH technique yielded good quality hybridizations, thereby allowing the determination of several specific signals on a single slide prepared for each species. The result was reproduced in additional assays a number of times. Moreover, the probes proved to be easily distinguishable from the background, even under conditions of high stringency. The fiber-FISH analyses performed here revealed different patterns for the relative location of the two probes for repetitive DNA employed: i) independent location, ii) adjacent syntenic location, and iii) interspersed syntenic location. *S. melanodermatum* shows 18S and 5S rDNA located separately in interphase nuclei and on two different pairs of chromosomes (Matoso et al., 2011) (Fig. 1a,d).

Double fiber-FISH with these two probes demonstrated the independent location of these two kinds of ribosomal genes in different segments of chromatin fiber, corresponding to different chromosomes (Fig. 1b,c,e,f). This independent location of 18S and 5S rDNA seems to be the most common situation in fish and other vertebrates, when analyzed by FISH on mitotic chromosomes (Lucchini et al., 1993; Martins and Galetti, 1999; 2001; Suzuki et al., 1996). FISH analysis on mitotic chromosomes of *I. duseni* has shown that this species carries 18S and 5S rDNA in synteny on a same chromosome in an apparently adjacent location (Ziemniczak, 2011) (Fig. 1g). Double fiber-FISH has shown that these two kinds of rDNA are actually located close together in the same chromosome, although they are not contiguous (Fig. 1h,i). Similar situations have been described, by conventional FISH, in fish species such as *Salmo salar* and *Oncorhynchus mykiss* (Móran et al., 1996; Pendás et al., 1994). In these species, double fiber-FISH could throw much light on the precise arrangement of these genes.

In *A. janeiroensis*, conventional FISH has shown that the 18S rDNA and the As51 satellite DNA seem to occupy the same chromosome domain appearing to be interspersed (Fig. 1j). Double fiber-FISH showed the syntenic interspersed co-localization of these two repetitive DNAs in this species (Fig. 1 k,l). Vicari et al. (2008) claimed that, under these conditions, ribosomal genes would be silenced by the As51 insertion. Whereas the low resolution of FISH on metaphase chromosomes did not allow differentiating both fluorescent probes (Fig. 1j), the double fiber-FISH technique provided a clear pattern of interspersion for both kinds of repetitive DNA, which may be informative in respect to the possible silencing effect of As51 on 18S rDNA.

Therefore, high resolution mapping with fiber-FISH may be very informative to understand the organization and function of the genome, since it may reach a 1000-fold magnification with respect to conventional FISH. In the bivalve mollusk *Mytillus galloprovincialis*, FISH with the H1 histone gene and the 5S rDNA probes on mitotic chromosomes showed independent or overlapping patterns for these two DNA sequences at different physical places, but fiber-FISH indicated that they are actually interspersed in the latter (García, 2010). Many genes or repetitive DNAs that appear to be overlapping, when analysed on mitotic chromosomes, demand analysis at higher resolution, for which purpose fiber-FISH is a valuable tool. Double or multiple fiber-FISH essays may be highly informative to analyze the physical basis for position effects, epigenetic regulation and chromosome rearrangements. The ability of performing the technique in the same cell suspensions used for conventional analysis to mitotic chromosomes makes it a goldmine for performing additional