0014-4754/93/090810-04$1.50 + 0.20 © Birkhäuser Verlag Basel, 1993

A method for chromosome preparations from large fish specimens using in vitro short-term treatment with colchicine[1]

F. Foresti[a], C. Oliveira[a] and L. Foresti de Almeida-Toledo[b 2]

[a]Departamento de Morfologia, Instituto de Biociências, UNESP, Campus de Botucatu, 18618-000, Botucatu, SP (Brazil), and [b]Departamento de Biologia, Instituto de Biociências, USP, São Paulo, SP (Brazil)
Received 22 March 1993; accepted 28 June 1993

Abstract. This paper describes a new technique for preparing mitotic fish chromosomes using short-term in vitro treatment with colchicine. The results show that a large number of good quality metaphases (many suitable for chromosome banding) can be obtained by this technique, which requires an average of 1 h and 30 min for all steps. The procedure considerably reduces the time normally required for chromosome preparations in fish.
Key words. Fish cytogenetics; mitotic chromosomes; technique.

Fish chromosome data have great importance in studies on evolution, systematics and mutagenesis, and in aquaculture. Although many techniques employing the cell culture technique to obtain chromosome preparations have been described[3,4], they are usually expensive and time consuming, a fact which limits their use in many laboratories. Direct preparations are less expensive and quicker to prepare but require injection of mitotic inhibitors into and killing of the animals[5–7].

The air-drying technique, the most common procedure used in chromosome preparations, was initially developed for mammalian chromosome studies[8] and was first used in fishes by Ojima and Hitotsumachi[9]. Although the basic steps remain the same, many small modifications have been added to this technique in recent years[10].

The present paper describes a modified method for obtaining fish chromosome preparations using the air-drying technique preceded by short-term colchicine treatment of the cells in vitro. The method results in a reasonable quantity and quality of chromosome spreads and is particularly useful for large fish specimens.

Material and methods

Mitotic chromosome preparations were obtained from cephalic kidney or gills or regenerated fin epithelium according to the following procedures:

The animals were anaesthetized by immersion in a solution of 0.01% benzocaine. As soon as respiratory activity stopped, tissue was obtained either from the gills, by opening the operculum and pulling a forceps over the filaments of the branchial arches, or by cutting a piece of the fin epithelium, or by dissecting out pieces of cephalic kidney. Tissues were washed once in a Petri dish containing Hanks' saline solution (HSS) to remove any adhering fat or blood, and minced in 5–8 ml of clean HSS at room temperature. After discarding residual tissue lumps, the clean cell suspension was placed into a 20-ml conical centrifuge tube and 1 drop of 0.03% colchicine was added. The suspension was stirred lightly and the tube placed in an incubator at 37 °C for 15 min. After colchicine treatment, the cell suspension was centrifuged at 800–1000 rpm for 7 min. The supernatant was removed by suction and the pellet resuspended by vigorous tapping of the tube, before adding 7 ml of a 0.075 M KCl hypotonic solution. The tube was then stirred lightly and placed in an incubator at 37 °C for 30–40 min. After hypotonization, 5 drops of a freshly prepared ice-cold fixative (3 methanol:1 acetic acid) were added to the tube and the cell suspension stirred gently at room temperature. After 5 min, 7 ml of ice-cold fresh fixative was added and the cell suspension was gently stirred with a Pasteur pipette and centrifuged at 800–1000 rpm for 10 min. After centrifugation, the supernatant (KCl solution + fixative) was removed by suction and the cell pellet resuspended by gently tapping the tube before the addition of 7 ml of ice-cold fixative for a new centrifugation period of 7 min. This procedure was repeated once or twice. After the final centrifugation, the pellet was resuspended in about 1–2 ml fixative according to the density of cells. 1–2 drops of the concentrated cell suspension were dropped onto clean slides, supported inside a water-bath at 60 °C, and allowed to dry.

For standard Giemsa staining, the slides were dried and stored for one day and then immersed in 1 N HCl solution at 60 °C for 3 min (this step is optional), washed in distilled water and covered with 3% Giemsa in sodium phosphate buffer, pH 6.8, for 7 min. The slides were then washed in distilled water and allowed to dry. To locate the nucleolus organizer regions by silver staining, slides should be kept at room temperature overnight and then stained, following the technique described by Howell and Black[11]. For C-banding, slides

must remain at room temperature for 5–8 days and then stained according to the technique described by Sumner[12].

For comparative purposes, four specimens of *Orechromis niloticus* (two males and two females) were tested using four different conditions each. Thus, 24 ml of kidney cell suspension in HSS was initially prepared from each fish. The first aliquot (6 ml) did not receive the addition of colchicine and the second, third and fourth (6 ml each) were treated with colchicine for 15, 30 and 60 min, respectively. All metaphases in each drop of cell suspension dropped on the slides were counted and classified as good when chromosomes were isolated and well distended, or poor when it was impossible to determine the diploid number of the cell or when the chromosomes were excessively condensed. To standardize cell concentration on each slide, all the cells in ten microscope fields were counted and the average number of cells on each slide was estimated. The final number of metaphases was then corrected for all slides from each animal. The data obtained are shown in the table. To develop the technique further, several other fish species were used and some results are shown in figure 2.

Results and discussion

The results of the experimental test of viability and applicability of the method described using *Oreochromis niloticus* cells revealed several interesting peculiarities of the technique proposed. Analysis of the table shows that treatment of the cell suspension with colchicine for 15 or 30 min increased the number of good metaphases, but more prolonged treatment (as long as 60 min) resulted in a low number of cells suitable for cytogenetic analysis.

In cell suspensions prepared without colchicine treatment a consistent number of cells in metaphase was also found (25.7%). However, the degree of chromosome distension in such preparations was so uneven that it was only possible to determine the diploid number in 18.9% of cells (figs. 1a and 1b; table).

The greatest number of cells in metaphase for the four treatments was observed after 15 min of colchicine treatment (32.9%). The number of good metaphases in this treatment was about five times the number in the suspensions without colchicine (table). The number of metaphases suitable for the establishment of karyotypic formulae and for chromosome banding studies was as high as after the 30-min colchicine treatment (table). Nevertheless, in the 15-min treatment some cells with highly condensed chromosomes were found (fig. 1c), suggesting that this treatment may be long enough to block mitosis. The ability of colchicine to inhibit metaphase and condense chromosome is the fundamental basis for its use in chromosome preparations. However, short-term treatment of cells with colchicine, even if it does not increase the number of cells in metaphase, can facilitate chromosome condensation so that most metaphases can reach a level of chromosome condensation suitable for karyotype studies even after such short treatment (figs. 1c and 1d; table).

The 30-min treatments provided a large number of good cells in metaphase but in most cases chromosomes were overcondensed, which limits their use in cytogenetic analysis (figs 1e and 1f). 60-min treatments made the chromosomes very condensed (figs 1g and 1h) and only a small number of dividing cells were found. The rapid action of colchicine could be explained by its direct action on the cells in suspension. The reduction in number of dividing cells in longer treatments is probably due to the fact that even if cells can be maintained alive in HSS, some cell activities could be blocked, including the cell cycle.

The HSS method has been employed with good results in different fish species to which banding chromosome techniques have been successfully applied, as shown in figure 2.

The total time needed to perform the HSS technique was about 1 h and 30 min (without considering handling time). This time is much less than that required by the widely employed technique described by Kligerman

Number of metaphase cells of *Oreochromis niloticus* obtained in different treatments

Fish no. and sex	Treatments							
	Without colchicine		With colchicine					
			15 min		30 min		60 min	
	G*	P**	G*	P**	G*	P**	G*	P**
1727 ♀	39	126	143	77	22	7	1	5
1728 ♂	17	213	249	184	20	3	13	3
1729 ♀	57	342	232	133	516	158	295	74
1730 ♂	32	86	80	70	161	38	117	35
Total	145	767	704	464	719	206	426	117

*G = number of metaphases for which it was possible to determine the diploid number;
**P = number of metaphases for which it was not possible to determine the diploid number.

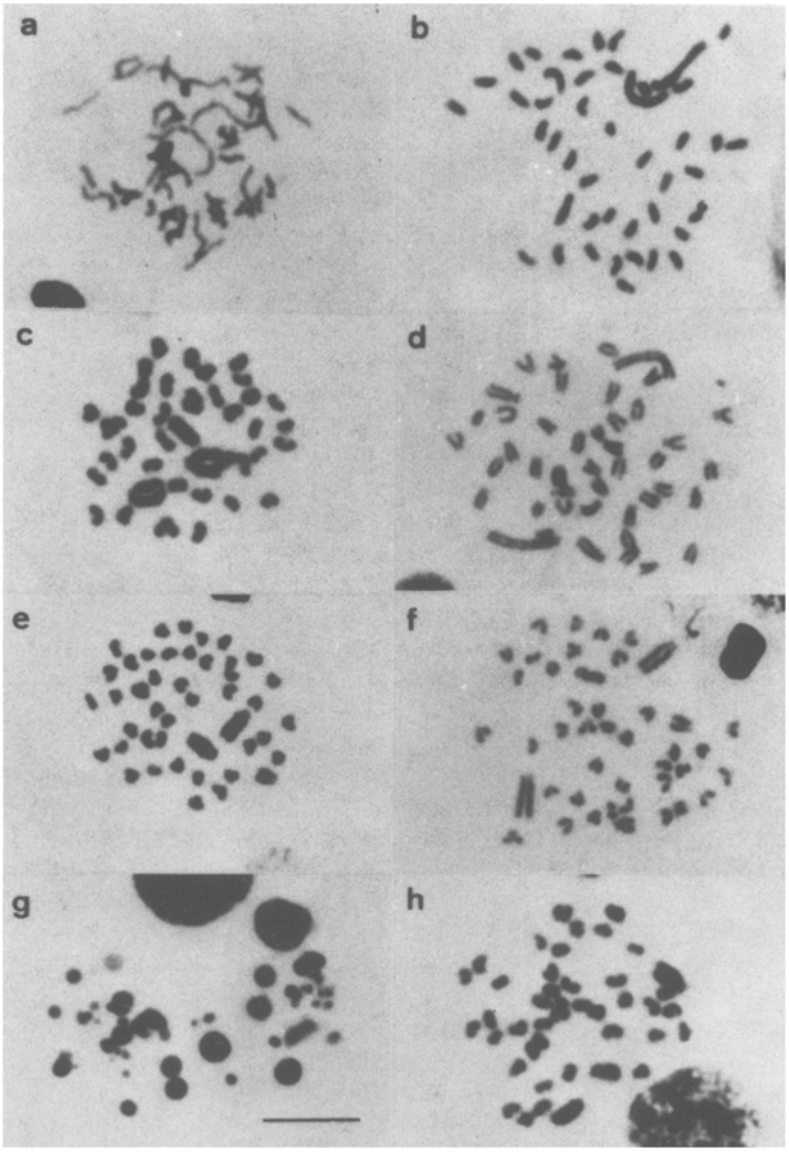

Figure 1. Somatic metaphases of *Oreochromis niloticus* obtained in four treatments: *a* and *b* – without the use of colchicine; *c* and *d* – with 15 min of colchicine treatment; *e* and *f* – with 30 min of colchicine treatment; and *g* and *h* – with 60 min of colchicine treatment. All metaphases were photographed in the same magnification. Bar = 10 μm.

and Bloom[13] which lasts about 7 h and 30 min, a time similar to that reported by Lozano et al.[6]. The technique described by Gold et al.[3] is rapid but still requires about 2 h and 30 min without considering pretreatment with phytohaemagglutinin (18–24 h) and handling of the cell suspension.

The HSS method can also be very useful in cytogenetic studies of large fish specimens since it does not require injections of large colchicine volumes into the animal's body, and permits the karyotypic analysis of large and heavy individuals at reduced cost. Furthermore, for large specimens, the use of cells obtained from branchial arches or regenerated fin epithelium has the advantage of permitting karyotypic studies without killing the animals; this could be especially useful for chromosome analysis of broodstock. The method has also provided good results in small specimens, mainly those which are difficult to handle and frequently die during intraperitoneal colchicine injection.

In other vertebrate species, i.e. mammals *(Mesocricetus auratus, Cavia porcelus, Felis catus* and *Canis familiaris)*, birds *(Gallus domesticus)*, reptiles *(Hemidactylus mabouia)*, and amphibians *(Bufo paracnemis, Bufo crucifer* and *Hyla faber)*, this in vitro colchicine treatment technique has also yielded a large number of good metaphases for cytogenetic studies.

Thus, the HSS method may be very useful in fish cytogenetics because it is extremely simple, faster and less expensive than similar techniques, and because it has proved to be effective for obtaining a sufficient

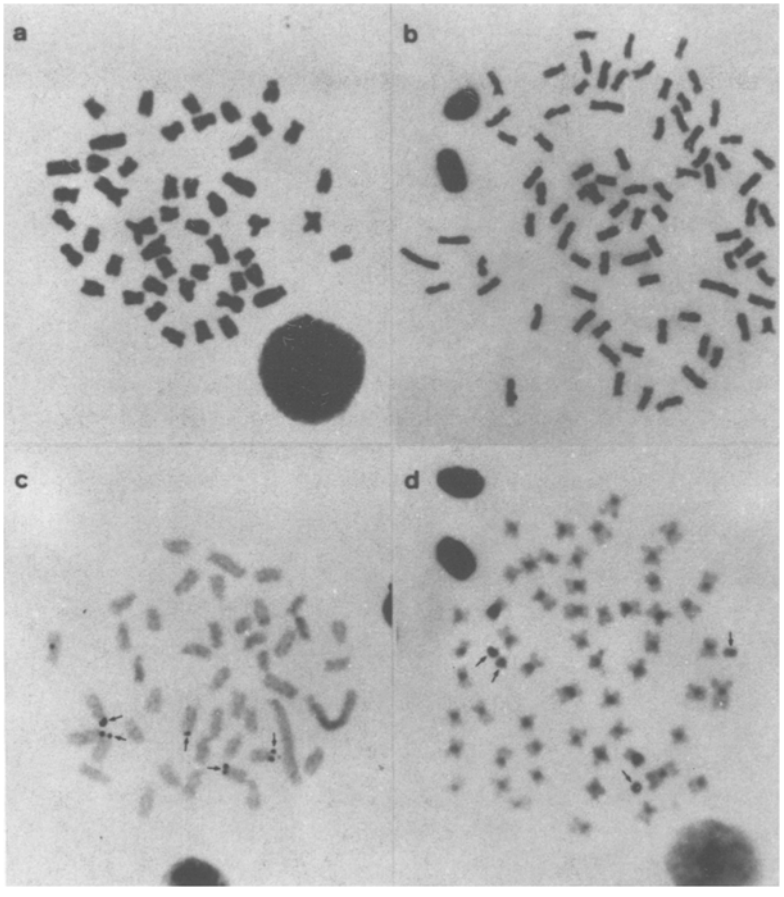

Figure 2. Kidney metaphases from: *a* a bighead carp, *Aristichthys nobilis*, with 2n = 50, and *b* a triploid specimen of *Piaractus mesopotamicus* with 3n = 81 stained by the Giemsa technique; *c* a female of *Oreochromis niloticus* with 2n = 44 stained by the silver staining technique (the arrows point to the chromosomes with NORs); and *d* a female of *Prochilodus lineatus* with 2n = 54 plus 4 supernumerary microchromosomes (arrows) stained by the C-banding technique. Note the heterochromatic nature of the supernumerary chromosomes.

number of good metaphase figures in all specimens analysed.

1 This work was supported by grants FUNCUNESP, FAPESP and CNPq.
2 The authors are grateful to Mr Renato Devidé for technical assistance.

3 Gold, J. R., Li, Y. C., Shipley, N. S., and Powers, P. K., J. Fish Biol. *37* (1990) 563.
4 Alvares, M. C., Otis, J., Amores, A., and Guise, K., J. Fish Biol. *39* (1991) 817.
5 Rivlin, K., Rachlin, J. W., and Dale, G., J. Fish Biol. *26* (1985) 267.
6 Lozano, R., Ruiz-Rejon, C., and Ruiz-Rejon, M., Stain Technol. *63* (1988) 335.
7 Cucchi, C., and Barufaldi, A., J. Fish Biol. *37* (1990) 71.
8 Hsu, T. C., Human and Mammalian Cytogenetics. An Historical Perspective. Springer-Verlag, New York 1979.
9 Ojima, Y., and Hitotsumachi, S., Jap. J. Genet. *42* (1967) 163.
10 Klinkhardt, M. B., Cytobios *67* (1991) 193.
11 Howell, W. M., and Black, D. A., Experientia *36* (1980) 1014.
12 Sumner, A. T., Exp. Cell Res. *75* (1972) 304.
13 Kligerman, A. D., and Bloom, S. E., J. Fish. Res. Board Canada *34* (1977) 266.