24. Preparation of Cell-cultures for Chromosome Studies of Fishes

By Yoshio OJIMA

Department of Biology, Kwansei Gakuin University, Nishinomiya

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Cell cultures of fishes which provide important materials for studies in various fields of cell biology have been carried out being promoted under the influence of mammalian techniques since the initial work by Wolf (1960), and the establishment of the *in vitro* cell-line by Clem *et al.* (1961).

The development of cytogenetic techniques for fish chromosomes was made within a few years since 1961 by introducing the methods of tissue culture into this field. The cell-culture techniques become available with the use of leucocytes and cells derived from a variety of sources such as blood, fin, scale, ovary, kidney and eyed embryos, and were designed advantageously to apply to cytogenetic investigations of fishes.

Especially, recent advances of new differential staining techniques have made possible the demonstration of the characteristic banding patterns of fish chromosomes from the cultured cells. Now, new technical approaches have proved effectiveness in understanding of the characterization of individual chromosomes, as well as for the standardization of karyotypes.

In response to the request for laboratory investigations of the fish chromosomes, up-to-date technical procedures for preparing slides from the cultured cells, are presented here in a form of summary; they were mostly devised in our laboratory to contribute toward the development of fish chromosome studies.

1) *Procedures of the blood culture.* The blood-culture method is processed as follows: a) Whole blood culture: Whole blood about 0.2 ml in volume was derived from ventriculus cordis of a young fish and suspended in a medium consisting of 2 ml calf serum and 0.1 ml PHA-M in TC-199 (Difco) with penicillin and streptomycin. b) Separation of leucocytes: 1 ml of blood was obtained from ventriculus cordis and allowed to stand in an ice bath for 1–2 hours. Then the sedimentation of erythrocytes was accomplished following the centrifugation at 300–350 rpm for 10 minutes at 5°C. The supernatant plasma containing leucocytes, 0.2–0.3 ml in volume, was suspended in a culture bottle containing a mixture of 2 ml of calf serum, 8 ml of

TC-199 (Difco), and 0.1 ml of PHA-M, together with penicillin and streptomycin. The initial concentration of cells was 1 to 2×10^6 /ml of the culture medium. The pH was adjusted as 7.0–7.2 initially. c) Harvesting: Examination of growing cells *in vitro* revealed that an optimal mitotic activity was obtained on the 3rd day of culture at 25–30°C. For chromosome slides, the culture was terminated in

Figs. 1-6. 1: C-banded metaphase from kidney culture of *Carassius auratus langsdorfii*. For technique see 3). 2: A single cell layer from Oviparous culture of *Gambusia affinis affinis*, hematoxylin-stain. 3: Fin-culture from *Carassius auratus cuvieri*, hematoxylin-stain. 4: Cells growing in scale-culture, from *Carassius auratus cuvieri*. 5: A single cell layer in scale-culture from male *Carassius auratus* subsp., hematoxylin-stain. 6: Metaphase of *Carassius auratus* subsp., from the single cell layer as shown in Fig. 5. Giemsa-stain.

this condition. To accumulate a large number of cells at metaphase, colchicine was added at a 1×10^{-8} M concentration (approximately one drop of 100 $\mu\text{g}/\text{ml}$ colchicine for a culture bottle) at an interval of 5–6 hours prior to termination.

2) *Procedures of the scale culture.* The scale culture method is scheduled as follows: Several scales were scraped from a fish and the scale tissues were immersed in PBS (phosphate-buffered saline after Dulbecco and Vogt 1954). The scales were then washed three times with PBS containing penicillin (100 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and kanamycin (60 $\mu\text{g}/\text{ml}$) for ten minutes in each. The scales were placed in a Petri dish (6 cm in diameter) as to be set at surface level of the dish with a perforated cellophane. They were cultivated at 28–30°C in a humidified incubator about 24 hrs. The medium for culture consists of a mixture of 85 ml of NaHCO_3 -free TC-199 containing penicillin (100 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), kanamycin (60 $\mu\text{g}/\text{ml}$), mycostatin (50 IU/ml), and HEPES (15 mM), together with 15 ml of fetal calf serum. The initial pH of the culture medium was adjusted to about 7.4 by NaOH. After 24 hours of cultivation and growth, the scale epithelium is spread on the glass wall of the Petri dish. Two or three hours before harvesting, colchicine solution was added to the Petri dish at a final concentration of 1 $\mu\text{g}/\text{ml}$. At the termination of the culture, the cellophane paper and scales were removed from the Petri dish and the sheets of epithelial cells were washed by Ca^{++} - and Mg^{++} -free PBS followed by 0.1% trypsin for 10 minutes at 37°C.

3) *Procedures of the kidney cultures.* The process of the kidney culture is as follows: Any kinds of fishes were sterilized by rinsing out the body with 70% ethanol. The kidney was aseptically removed and minced with scissors. The tissue fragments thus obtained were transferred into a test tube containing Eagle's MEM. The cells were liberated from the renal intertubular tissue by gentle pipetting. Then, the test tube was allowed to stand for several minutes at room temperature to obtain cell suspension. The upper part of the cell suspension, free of coarse tissue fragments, was collected in another test tube. The cell density (without erythrocytes) was determined with a hemacytometer. Initial concentration of cells was $2 \times 10^6/\text{ml}$ of the culture medium. The medium for culture consists of 85% of HEPES-buffered Eagle's MEM (HEPES: 15 mM, antibiotics; penicillin: 100 IU/ml, streptomycin: 100 $\mu\text{g}/\text{ml}$ and kanamycin: 60 $\mu\text{g}/\text{ml}$) and 15% of fetal calf serum. Then PHA-M (Difco) was added to the medium at a concentration of 10 $\mu\text{l}/\text{ml}$. The culture was kept in a humidified incubator for 3 days at $29 \pm 1^\circ\text{C}$. Four hours prior to harvest, colchicine was added to the culture at a final concentration of 1 $\mu\text{g}/\text{ml}$.

4) *Procedures for the scale-fin culture.* The schedule of the scale and fin culture is as follows: The body surface of the fish was sterilized by immersing it in sodium hypochloride (1%) for a few seconds followed by the flush sterilization in 70% ethanol. Scales and fins were aseptically removed and put into a test tube. They were washed three times with balanced salt solution, and finally transferred to a flask containing 5 ml of trypsin solution (0.15% trypsin, 0.3 mM EDTA · 2Na) with a small magnetic bar. Cells were liberated by low speed stirring at 4°C for 4 hours. Liberated cells were pelleted by centrifugation at 1,000 rpm for 3–5 minutes. The cells were suspended in growth medium and transferred to a stoppered-growth-tube. The growth medium consists of balanced salt solution containing fourfold concentration of Eagle's amino acids and vitamins, 20 mM of HEPES, 60 µg/ml of kanamycin, and 100 IU/ml of penicillin being supplemented with 20% of fetal calf serum. Cultures were continued at 32°C. To obtain primary mono-sheet of cells, about one half of the volume of growth medium was freshened every 3 to 4 days. Trypsin-EDTA solution was used at 4°C in the first transplantation to detach cells from the glass surface. After the second transplantation, when the cell sheets were confluent, cells were suspended mechanically with a fine Pasteur pipette in the old medium, and then transferred into two Petri dishes supplemented with the same volume of the fresh medium.

5) *Procedures for the embryo-culture.* The embryo-culture from ovo- and viviparous fishes was carried out as follows: a) For ovo-viviparous fishes: The body surface of a female with big belly was wiped with 70% ethanol. Several viviparous embryos were aseptically removed and washed by a digestion mixture containing 0.15% trypsin and 0.3 mM EDTA · 2Na, adjusted to be pH 7.2. Embryos were transferred into a Petri dish and minced with scissors. The cells were soaked in the 2 ml of trypsin solution for 5–10 minutes at room temperature. The tissue fragments were transferred into a test tube. Then 2 ml of culture medium with serum were added in order to stop the trypsin action. The cells were liberated by gentle pipetting. The cells were washed once with a growth medium and seeded in a plastic Petri dish (Wako Pure Chemical Co.). The culture was kept at 29±1°C. The composition of the growth medium is as below:

NaCl	7.5 g	Eagle A. & V.*	3.44 g
KCl	0.2 g	Kanamycine	60 mg
NaHCO ₃	0.2 g	Phenol Red	6 mg
CaCl ₂	0.2 g	GDW	up to 1 liter
Glucose	1.0 g		

* Eagle MEM amino acid vitamin (Nissui Seiyaku Co. Ltd.).

The medium is filtered through a 0.22 or 0.45 micron membrane filter, and supplemented with fetal calf serum at a final concentration of 15%. b) For oviparous fishes: The egg surface was sterilized by immersion in sodium hypochloride (1%) for a second followed by the dipping in 70% ethanol. For the culture procedure, the same method as that for the ovoviviparous fishes was applied. c) Establishment of the cell-line: To obtain primary monosheet of cells, about one half of the volume of growth medium was freshened every 3 to 4 days. Trypsin-EDTA solution was used at 4°C in the first transplantation to detach cells from the glass surface. For chromosome investigations, colchicine was added 2 to 3 hours prior to harvest to the culture at a final concentration of 0.2 µg/ml.

6) *Procedures for the fin culture.* The culture of the fin is processed as follows: The fins were sterilized by immersing them in sodium hypochloride (1%) and immediately followed by dipping in 70% ethanol. After sterilization, they were washed three times with fish-BSS. The fins were cut into the small pieces, removed into a dish plate and covered with pieces of glass. After the culture medium was added, they were cultured at $29\pm 1^{\circ}\text{C}$. About one half of the volume of growth medium was freshened every 3 to 4 days. In this condition the cells grew up and were ruled around the fins within 3 weeks. The glass pieces on the tissues were removed. On the next day, the trypsin-EDTA solution was used at 4°C for the first transplantation to detach cells from the glass surface. Two to three hours prior to harvest, colchicine was added to the cultures at a concentration of 0.2 µg/ml, and then the chromosome slides were prepared with the cultures thus made.

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