PROCEDURES FOR SUBCULTURING FISH CELLS AND PROPAGATING FISH CELL LINES

Submitted by

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I. INTRODUCTION

This method describes a rapid, simple and effective means of subculturing primary monolayers of fish cells and propagating lines of fish cells which have been established or are in the process of development. For standard terminology, see Fedoroff (1). The word *fish* as used here means vertebrates of the class Teleostomi, the bony fishes. The culture of cells and tissues of teleosts has been easier and has progressed further than the culture of materials from more primitive forms. By 1976 there were more than 40 cell lines from various fresh water or marine teleosts. The physiology and the blood plasma constituents of teleost fishes are very much like those of terrestrial vertebrates; therefore the methodology for culture of fish cells and tissues is also similar.

Procedures for the culture of fish cells and tissue were published by Sigel and Beasley (2) for marine teleosts and by Wolf (3) for fresh water teleosts. The most comprehensive review of all fish cell and tissue culture is that of Wolf and Quimby (4), who additionally set forth a system of precepts used in managing laboratory propagation of fish and other poikilothermic vertebrate cell lines (5). Compared with cultivation of avian and mammalian cell lines, fish cell culture differs somewhat in that the cultures generally grow through a greater range of temperature. Many fish cell cultures grow well at room temperature and therefore do not require special incubators. Also, fish cells seldom require a change of medium between subcultures and thus demand less time and effort than do avian or mammalian cells.

It is most important to note that conventional physiological solutions and culture media intended for mammalian or avian work are wholly appropriate without modification for use with fresh water teleost cells and tissues. The same solutions and media may be used for marine teleost cells and tissues, but most workers have found it advantageous to adjust osmolarity to isotonicity of marine teleosts.

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II. MATERIALS

Eagle's MEM (Earle's BSS) (GIBCO)[1] with 10% fetal bovine serum and 50 μg gentamicin (Schering)[2] and 25 IU nystatin (Squibb)[3] per ml (see NOTE at end of Materials and Table 1)
Penicillin
Streptomycin
Amphotericin B
Plastic culture flasks, sterile, 25-cm² (No. 3013) or 75-cm² (No. 3024) Falcon[4]; or comparable prescription bottles available from local pharmacies

[1] Grand Island Biological Co., Grand Island, NY.
[2] Schering Corp., Port Reading, NJ.
[3] E. R. Squibb and Sons, Princeton, NJ.
[4] Falcon Plastics, Oxnard, CA.
[5] Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, NJ.
[6] American Optical Corp., Buffalo, NY.

Trypsin (1:250) solution, 2.5%, sterile[1]
Ethanol or isopropanol, 70%
Versene solution, 1:5000, sterile. To 1000 ml of double-distilled or pyrogen-free water, add the following reagent grade chemicals: 8.0 g NaCl; 0.2 g KH_2PO_4; 0.2 g KCl; 1.15 g Na_2HPO_4; and 0.2 g disodium ethylenediamine tetraacetate S-311 Fisher[5]. Dissolve completely and decontaminate by membrane filtration or sterilize by autoclaving at 15 psi for 15 min. A similar product available commercially is No. 504.[1]
Wipes, laboratory, heavy-duty or gauze sponges
Dispersing solution: Aseptically mix 96 ml of versene solution with 4 ml of trypsin solution. Dispense in 10-ml portions and store at −20°C or lower. Thaw immediately before use.
Pipets, sterile, cotton-plugged, serological, 25-ml, 10-ml and 5-ml
Hemacytometer, Bright Line, No. 1490 A.O.[6]

TABLE 1

Osmolarity Adjustments of Eagle's Minimal Essential Medium[a]

Animal	Adjustment	Freezing point (Δ; °C)
Freshwater teleost	None	-0.6
Terrestrial reptile	None	-0.6
Marine reptile	Add 0.35 g% (0.06 M) NaCl[b]	-0.75
Amphibian	Dilute 4 parts of medium with 1 part water	-0.45
Marine teleost	Add 0.41 g% (0.07 M) NaCl[c]	-0.75

[a] In the absence of specific information to the contrary, a pH of 7.4 ± 0.2 is suggested for all cultures. Cultured cells and tissues often tolerate osmolarity somewhat above or below that of the intact animal, but a slightly hypotonic medium is usually better. Fresh-water teleost cells usually fare well in "frog strength" medium. Frog cells may not thrive in medium having a freezing point (Δ) of -0.6 °C, but they can be adapted to hypertonic medium.

[b] For this adjustment, use a 3.4 M (20% w/v) stock solution of sterile NaCl and add 17.6 ml per l of medium.

[c] For this adjustment, use a 3.4 M (20% w/v) stock solution of sterile NaCl and add 20.6 ml per l of medium.

Microscope, inverted, with 3X to 10X objective and 10X ocular lenses

Miscellaneous other equipment usually found in microbiological laboratories, such as:
Timer
Autoclave
Filtration apparatus
Bunsen burner
Magnetic stirrer
Container for ice-water bath
Sterile cotton-plugged pasteur pipets

NOTE: Although it may prove inadequate for some purposes, the following is an all-purpose medium for cell and tissue culture of teleost fishes, amphibians and reptiles.
Eagle's Minimum Essential Medium (Earle's balanced salt solution) 90%
Fetal bovine serum 10% (20% serum may be needed for leucocytes and some cell lines)
For routine purposes, the addition of 50 μg gentamicin (or 100 IU penicillin plus 100 μg streptomycin) and 5 μg amphotericin B (or 25 IU nystatin) per ml is suggested.

Eagle's medium may be purchased in liquid form (both working strength and as concentrates) or as a lyophilized or powdered preparation. Powdered forms must be dissolved in high purity water; either double-glass-distilled deionized water or USP "Water for Injection" (obtainable at pharmacies or hospital supply houses) is recommended.

III. PROCEDURE

A. Selection and examination of culture

1. Work with only one cell line or kind of primary culture at a time.
2. Make certain that culture medium over cells is optically clear and free of evidence of microbial contamination.
3. Examine cell sheet microscopically and determine that cell morphology agrees with that of the description (if known) and that culture has no foci or areas of necrosis or other factors that indicate culture is suspect. Evaluate general vigor and quality of the starting culture; ideally, culture should be confluent, but mitoses should be evident.

B. Preparations

1. Thaw dispersing solution.
2. Verify clarity of fresh culture medium.
3. Wipe down entire work area with 70% alcohol.
4. Wash hands and wrists thoroughly and further decontaminate with 70% alcohol.

C. Dispersing of cell sheet

1. Read and understand the following instructions *before* proceeding further. *Total treatment time should not exceed 10 to 12 min.*
2. Decant old medium.
3. At a rate of about 5 ml per 75-cm² flask or about 2 ml per 25-cm² flask, add a rinsing volume of dispersing solution and quickly wash all internal surfaces of the culture vessel. Allow solution to contact cell sheet proper for 2 or 3 min; then decant or aspirate all. The purpose of this preliminary rinse and treatment is to initiate separation of cell sheet and to dilute residual antitryptic activity of serum in the culture medium. Cell sheet often shows slight whitening or opacity at the end of the 2- or 3-min treatment.
4. At a rate of about 2 to 3 ml per 75-cm² flask or 1 to 2 ml per 25 cm² flask, add fresh dispersing solution and make certain that it contacts the entire cell sheet. Occasional tilting of culture vessel during

TABLE 2

Guide for Subculturing Representative Established Lines of Fish Cells

Cell line		Nominal Split Ratio	Suggested Seeding Rate (per ml)	Incubation Temp. (°C)	
ATCC[a] Designation	Common Name			Suggested	Range
CCL 42	FHM	1:4-6	500,000	25-30	14-34
CCL 55	RTG-2	1:3-4	200,000	15-20	4-26
CCL 59	BB	1:3-4	300,000	25-30	20-30
CCL 91	BF-2	1:2-3	200,000	25-30	20-30

[a] American Type Culture Collection, Rockville, MD.

the next 5 to 7 min insures contact between dispersing solution and cell sheet and expedites cell separation.

5. Observe cell sheet at about 1-min intervals; when cells begin to shed—usually in 4 to 7 min—strike a narrow side of the vessel against the heel of your hand to help dislodge cells.
6. Pipet cells in dispersing solution several times.
7. Add 5 ml or less fresh medium to 25-cm² flasks or 10 ml or less to 75-cm² flasks, and pipet several times to disperse uniformly and minimize foaming.
8. Add enough additional growth medium to make at least two daughter cultures of a size similar to that of the original. A 1:2 split ratio is suggested. After the operator gains experience, greater ratios can be routinely used (Table 2).

NOTE: Alternatively, count cells in a hemacytometer and seed at recommended levels (Table 2).

9. If cells are to be enumerated, chill the suspension in an ice bath to inhibit reattachment or keep in motion on a magnetic stirrer, or do both.
10. Adjust pH of cultures to about 7.3 to 7.5 with alveolar air or sterile 5% CO_2.
11. Incubate at appropriate temperature (Table 2).

IV. DISCUSSION

The method we describe works well with the many kinds of teleost cells that we have cultured, providing that they are not allowed to become overly dense. Dispersion of dense cultures may be incomplete, and the result is a mixture of monodisperse cells and monolayer fragments. The fragments produce outgrowth, however, and uniformity can be achieved in subsequent subculturing. Dense or old cell sheets that resist removal from the growth surface of culture vessels may be freed by scraping the surface with a rubber scraper or similar tool.

The explanation of the combined versene/trypsin approach is that divalent cations essential to cell adhesion and cohesion are chelated at the same time that the proteolytic enzyme attacks the cells' cementing substance. The advantage of the method is that it is rapid and simple and does not require centrifugation. One need not be apprehensive about using a scraper to remove tenacious cell sheets; in fact, if glass culture vessels are used, it is possible to subculture with scrapers exclusively. Damage to cells is then rather great, however, and the daughter cultures are not uniform or amenable to careful quantification by enumeration.

Commercial trypsin solutions may contain bacteriophage or homeotherm viruses. We know of no problems that such agents pose to fish cell culture, but if you wish to employ a truly sterile cell dispersant, we suggest autoclaved versene. Cultures can be treated with sufficient versene solution to permit dislodging of cells by vigorous pipetting or scraping. Too lengthy (over 10 to 12 min) exposure to versene is apt to damage cells, but the chelating action is simply arrested by adding an equal volume of culture medium and thus providing an excess of divalent cations to "neutralize" the versene. Such dispersion has a place in maintaining stock cultures, but cells must be sedimented by centrifugation (200 to 500 × g for 10 min at 4°C is suggested.) The resulting pellet of cells should be resuspended in an appropriate volume of fresh growth medium.

V. REFERENCES

1. Federoff, S. 1975. Proposed usage of animal tissue culture terms. Procedure 81169. TCA Manual 1: 53-57.

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Approved by Senior Author
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