

A method for the isolation and culture of embryonic cardiomyocytes from Mexican axolotl

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Previous experiments using cultures of Mexican axolotl cells have been reported for skeletal myoblasts (Duprat *et al.*, 1975), mesenchymal blastema cells (Boilly and Albert, 1988), and for various tissues including heart (Frost *et al.*, 1989). We have developed the following procedure for culturing cells from stage 38-41 embryonic hearts.

To enhance sterility, pre-hatching embryos were washed while still in their jelly coats, twice in filter-sterilized modified Steinberg's solution (Zackson and Steinberg, 1986), once in 95% ethanol, and then twice in filtered Steinberg's solution. The embryos were then dejellied and washed four more times in filtered Steinberg's solution. Post-hatching embryos were only washed four times in filtered Steinberg's solution.

Embryos were dissected in modified Steinberg's solution in a 60 mm Petri dish previously lined with three layers of parafilm and sterilized by ultraviolet light for 30 minutes. Hearts were removed with dry-heat sterilized watchmaker's forceps under a dissecting microscope and placed into calcium- and magnesium-free Steinberg's solution (CMF-Steinberg's= 59 mM NaCl, 0.67 mM KCl, 0.40 mM EDTA, 9.99 mM HEPES, pH to 7.4, with 1% antibiotic/antimycotic; Gibco stock composed of penicillin G sodium 10,000 units/ml, 10 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B). The CMF-Steinberg's solution was contained in a 1.5 ml microfuge tube. When all of the hearts were collected, they were centrifuged at 91g for 60 sec. The supernatant was replaced with a CMF-Steinberg's solution containing 774 units/ml collagenase (Sigma type II: *C. histolyticum*) and 1760 units/ml hyaluronidase (Sigma type I-S from bovine testis) for 20 min at room temperature on an orbital shaker. The heart tissue was centrifuged at 91g for 60 sec, after which the supernatant was

replaced by CMF-Steinberg's containing 0.0625% trypsin (Sigma, porcine pancreas, T-4424). Heart tissue was incubated for 20 minutes on the orbital shaker on slow speed at room temperature.

After trypsin digestion, the dissociated heart cells were centrifuged as above and the supernatant replaced by 50% L-15 Medium (Gibco) containing 10% fetal bovine serum (Gibco). This step was repeated with new medium, and then the clumps of cells were reduced by gentle trituration with a fire-polished glass pipette. The composition of the growth medium was 50% L-15 medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic (Gibco). The cells were plated onto Lab-Tek 8-well chambered coverglasses (Nunc, Naperville, Illinois) with 200 µl of cell suspension at a final concentration of 1×10^4 cells/ml. Before use, the chambered coverglasses were coated with the substrates laminin and fibronectin. Laminin (Gibco) at 18 µg/ml was added to the wells overnight at 4°C or for one hour at room temperature; fibronectin (Sigma, St. Louis) was added at a concentration of 32 µg/ml for one hour at room temperature. Substrates were removed and the wells rinsed with sterile water before placing the chambered coverglasses under ultraviolet light for 15 minutes. The optically clear vessel allowed us to monitor cell growth and analyze antibody distributions by confocal microscopy.

After 18-36 h, synchronous contractions appeared in the myocytes. By three days in culture, myocytes of hearts dissected at stage 41 had reformed striated myofibrils and formed projections. Muscle specific antibodies for tropomyosin (CH1, Lin *et al.*, 1985) and myosin (MF20, Bader *et al.*, 1982) were used to determine the presence of myofibrils in the cultured cells and to confirm the identity of the muscle cells. The staining procedure was the same as for whole hearts (LaFrance and Lemanski, 1994) but with shortened times.

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In conclusion, we have been able to use the described techniques to obtain isolated cardiomyocytes that retained their functional characteristics in short-term primary cultures.

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