A simple method for *in situ* freezing of anchorage-dependent cells including rat liver parenchymal cells

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**Abstract**

We developed a simple method for freezing anchorage-dependent cells, including primary cultured rat liver parenchymal cells, without detaching the cells from the culture dish. The method consists of preculture of the cells to confluence, changing the growth medium to a conventional freezing medium, packaging in a container, and storage at −80°C. After thawing and changing the freezing medium to regular growth medium, cell growth was nearly identical to that of cells freshly seeded into a new dish.

**Introduction**

The conventional method for freezing anchorage-dependent cells consists of several tedious steps, i.e. trypsinization, centrifugation, mixing with freezing medium, transfer to ampoules, sealing, and programmed slow-freezing. Thawing of the cells requires careful handling of the ampoule such that rapid thawing in a water-bath occurs, and opening then transferring the cell suspension to a centrifuge tube or culture dish.

A method of freezing the cells as an *in situ* monolayer, and simplification of the steps of freezing and thawing, would extend the use of frozen cells in biological studies. This prompted us to re-examine the freezing conditions of anchorage-dependent cells.

**Materials and methods**

Cells were cultured by a standard method with growth media as follows; L929, DM-160 (Katsu- ta and Takaoka, 1976) + 10% calf serum (CS); BALB/c 3T3 and NIH-3T3, MEM + 10% fetal bovine serum (FBS); IMR-90, hormone-free RITC 80-7 (Yamane, 1981) + 10% FBS; HUC-F, hormone-free RITC 80-7 + 10% FBS; Vero, MEM + 10% FBS.

Rat liver parenchymal cells were prepared and cultured as previously described (Aoki and Suzuki, 1985). Basal medium used was William’s medium E without CuSO₄, Fe(NO₃)₃, MnCl₂, and ZnSO₄. At cell seeding, 5% FBS was added together with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml fungizone, 10⁻⁶ M dexamethazone, 10 mM sodium pyruvate, and 30 ng/ml epidermal growth factor. Growth of these cells was monitored by measuring OD₂₈₀ after the
cells were dissolved with 0.1 N NaOH-0.1% sodium dodecylsulfate and neutralized with an equal volume of 0.1 N HCl – 0.05 M Tris-HCl.

For a simplified conventional method of freezing, BALB/c 3T3 cells were trypsinized and suspended in MEM containing 20% FBS and 10% dimethyl sulfoxide (DMSO) at a cell density of $4.0 \times 10^6$/ml. A portion of the cell suspension (0.8 ml) was transferred into a plastic ampoule, put into a styloform cylindrical box (95-mm outer diameter, 80-mm height, 11-mm wall thickness; a protective box for isotope shipping), and then set directly in −80°C freezer. Programmed slow-freezing of BALB/c 3T3 cells was carried out according to the manuals attached to each machine, i.e. TNP 82 (Nippon Freezer Co. Ltd., Tokyo) and BF2090 (Keltorr Co. Ltd.). Surviving fraction of cells were determined by standard trypan-blue dye exclusion method or by counting attached cells after the thawed cells were washed twice with MEM containing 10% FBS, seeded into a 35-mm dish and incubated for 8 hrs.

For in situ freezing, the medium in a dish was changed to freezing medium consisting of each growth medium containing 10% DMSO or, in the case of rat liver cells, growth medium containing additionally 0.1% methylcellulose (Ohno, 1988). The volume of freezing medium added is proportional to 0.1 ml/cm². Dishes were tightly sealed with Lab Label Protection Tape (Bel-Art Products, Pequannock, NJ 07440, USA) and placed in a freezing container as shown in Fig. 1, and then stored in a −80°C freezer. To rapidly thaw the frozen cells, the dishes were put on an aluminium block pre-warmed at 37°C. The cells were very fragile and weakly attached to the culture surface immediately after thawing. They were washed twice with regular growth medium with extreme care to avoid cell detachment from the culture surface, then cultured as usual. Attached cells were counted 8–16 hrs after thawing. Long-term cryopreservation was improved by the use of an inner floating lid (the inner lid of a conventional reagent bottle) made with polypropylene-coated styloform (Horiuchi Glass Ltd., Tokyo). Thickness and diameter were 1 mm and 34 mm, respectively.

Results

We have been noted by experiments that, for freezing by conventional method of trypsinized cells, use of the expensive programmable freezer was not necessarily advantageous for cell survival than a simplified method that sealed ampoules were put into a stylofoam box with approximately 1 cm wall thickness, then stored directly in −80°C deep freezer for overnight. When BALB/c 3T3 cells suspended in MEM containing 20% FBS and 10% DMSO were frozen by programmed slow-freezing at a rate of −1°C/min from 5°C to −50°C followed by a rate of −10°C/min from −50°C to −80°C, no essential difference in cell survival was observed to a simplified conventional method described in ma-

| Table 1. Comparison of programmed slow-freezing and a simplified conventional method of freezing of BALB/c 3T3 cells |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Freezing machine | Trypan blue dye exclusion | Attached cells after thawing |
| Programmed slow-freezing | TNP-82 | 76.9 | 58.3 |
| | BP2090 | 83.0 | 54.1 |
| Simplified conventional method | – | 81.5 | 53.0 |

All of the cells were frozen and stored at −80°C for overnight. Each figure represents the mean of triplicated ampoules. See materials and methods.