A Non-enzymatic Method for Subculturing Monolayer Cell Cultures[1] (35612)

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Cell cultures grown as monolayers conventionally may be removed from the surface of the culture vessel by physical or by chemical methods. Physical methods, such as scraping the cells from their growth substrate, generally yield heterogenous suspensions ranging from single cells to cell aggregates of varying size to monolayer membranes. Furthermore, many cell types, such as human fibroblasts, do not yield readily to removal by physical means. Most chemical methods for the removal of cell monolayers employ proteolytic enzymes. Although chemical techniques tend to produce more uniform single cell suspensions, the possibility exists that repeated exposure of cell cultures to proteolytic enzymes may have deleterious effects upon cell membranes. However, it is also possible that cell membranes are effectively "rejuvenated" with each division; in which case one could consider that individual cells are exposed to proteolytic enzymes only once and that membrane damage therefore might not be cumulative.

Here we introduce a new physical method for subculturing monolayer cell cultures, one in which the cells are not removed from occupied space, but the occupied space is removed and replaced with available and accessible space.

Materials and Methods. Cell cultures of human fibroblasts were initiated by trypsinization of neonatal foreskins obtained at the time of circumcision. They were maintained at 37° and 5% CO_2. Cells were fed twice a week using Eagle's minimum essential medium fortified with calf serum, penicillin, streptomycin, and Mycostatin. Cultures were doubled when the cell monolayers became confluent.

The standard enzymatic method for subculturing monolayers is as follows: spent culture medium is aspirated from plastic tissue culture dishes (Falcon No. 3002) as completely as possible and the cultures are washed briefly with 2 ml of phosphate buffered saline. The saline is aspirated and 1 ml of 0.25% trypsin in calcium- and magnesium-free Hanks' balanced salt solution is added to the dish. After about 1 min most, but not all, of the trypsin is decanted. Cultures are then incubated at 37° for 10 to 15 min until the cells become detached. The resulting suspensions are adjusted to the desired density in growth medium, and culture dishes are reinoculated.

The nonenzymatic method for subculturing is executed by growing cells on the surface of 500-μ glass beads. When cell monolayers approach confluency, the growth medium is aspirated and half the beads are removed; an appropriate volume of sterile virgin beads and fresh culture medium are added. At this point the cultures are considered to have been doubled or subcultured at a 1:2 split ratio, *i.e.*, half the number of cells per unit area. In order to discourage separate but unequal cell populations from growing on the surface of the dishes, plastic petri dishes, not designed specifically for tissue culture use (Falcon No. 1007), are employed. Human fibroblasts tend not to grow well on the surface of these dishes, but to be confined to the surfaces of the glass beads. Furthermore, new dishes are introduced after about every fourth transfer.

The number of beads must be adjusted to form a contiguous layer on the bottom of the dish, thereby providing optimal opportunity

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TABLE I. Comparison of Cell Cultures Doubled by Trypsinization and by the "Beads" Method.

	Cell strain	No. of times subcultured	Av interval between subculturing (days)[a]	Days required for (3 doublings): First	Last
Control group (trypsinized)	129C	18	4.7	13	13
	130C	14	6.0	11	13
	148C	16	5.2	10	13
Exptl group (beads)	129B	16	5.2	13	10
	130B	16	5.2	13	10
	148B	16	5.2	13	10

[a] These numbers are larger than one-third the number of the last two columns because of a temporary slow down in growth rate from about the fifth to the twelfth subculture.

for cell growth onto adjacent beads. For a 60 × 15-mm culture dish this requires a volume of about 0.9 ml of beads, each 500 μ in diameter. Since the beads, by virtue of their spherical configuration, greatly increase the effective surface area for cell growth, a greater volume of medium must be added to maintain the same cell (no.):medium (vol) ratio as in control dishes. It is advisable to gently agitate these cultures daily and to subculture them just before they reach full confluency. This procedure prevents the beads from becoming stuck together by rather strong cellular bridges.

Glass beads 500 μ in diameter (LaPine Scientific Co., Chicago, Ill.) are prepared for cell culture by soaking overnight in 7X washing compound (Linbro Chemical Co., New Haven, Conn.) followed by an 8-hr rinse in running water, and about 10–12 rinses in double-distilled water. Beads are oven dried, aliquoted in amounts suitable for use, and autoclaved.

Results. Three diploid strains of human fibroblasts were used in this study. They were each divided into two groups (Table I). The control group was grown as conventional monolayers on the surface of the dish and subcultured by trypsinization. The experimental group was grown as cell monolayers on the surface of glass beads and subcultured without the use of proteolytic enzymes. Cultures were maintained for 3 months and subcultured each time they approached confluency (14–18 times). For the control group the doubling interval averaged 5.3 days, with a range of ±0.65 days between the individual strains vs 5.2 days, and a range of 0 days, for the experimental group (Table I). In both groups the cell growth rate was not significantly different at the end of the study than at the beginning.

We were able to harvest cells from the beads, by a single trypsinization, for use in routine tissue culture procedures such as determination of mitotic index, contact inhibition, chromosome cytology, general histology, and freezing of the cells in a viable state.

Discussion. The chief advantage of the bead method over the trypsinization method lies in its applications to culture systems in which it may be undesirable to repeatedly expose cells to proteolytic enzymes, and yet still maintain them in an exponential growth phase. Long-term experiments are required to establish to what extent cells in bead culture are inclined to undergo transformation *in vitro* (1–3), and to establish whether such cells "age" *in vitro* (4). In studies which focus on cell surface factors, the applications of the bead method are obvious. Use of the bead method may shed further light on the declining growth rates and doubling efficiencies of cell cultures noted in relation to age *in vitro* (4–6).

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