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Simple and efficient recovery of rare living lymphoid cells from a vast majority of dead cells

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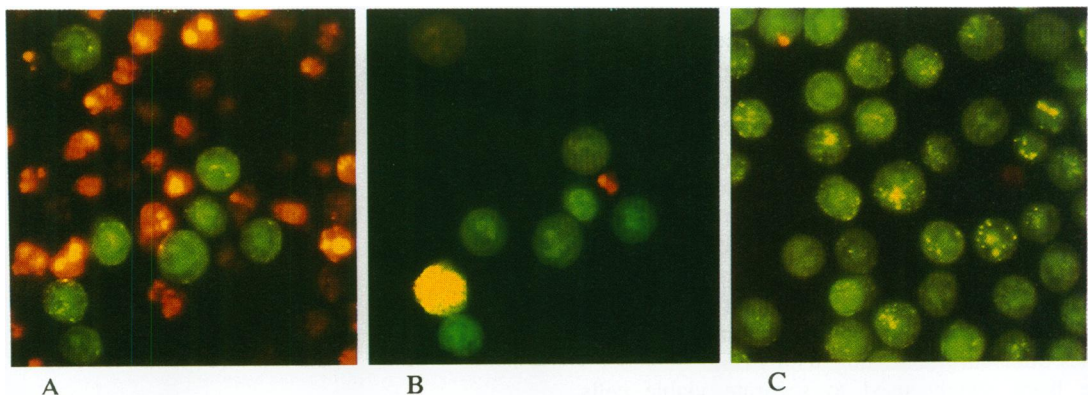
A critical problem associated with establishment of stable transformants of mammalian cells containing drug resistant markers is that many cell types are dependent on cell to cell interaction for growth. Thus, when the transfection efficiency is very low the successfully, but rare transfected drug resistant cells may be inhibited from growing due to low cell density. Moreover, cytotoxicity of the selection drug may reduce survival even further. When dealing with cells growing in monolayers it is sometimes possible to circumvent biological problems of this kind simply by replating the cells to restore the required minimal cell density. However, when experiments involve cells growing in suspension, it can be very difficult indeed to recover the extremely few live cells from the large number of dead ones. It does not help much to reduce the culture volume with the aim of increasing the density of the living cells, because massive amounts of dead or dying cells constitute a considerable source of deleterious lysozymal activity. Nor is it in practice possible to separate minute numbers of live cells from dead cells by centrifugation through a Ficoll gradient without losing the majority of the cells. Alternative methods have involved the use of more elaborate systems with co-transfection of surface markers and subsequent isolation by fluorescence activated cell sorting (6) or magnetic beads (2).

To overcome problems of this kind we report here a very simple and inexpensive panning method to separate live cells growing in suspension from an ocean of dead ones. The method is generally applicable and highly specific allowing practically 100% recovery of live cells irrespective of how minute a fraction they constitute of a given culture.

The method exploits the specificity of the plant lectin concanavalin A (con A) to α-glucosyl and α-mannosyl residues that are almost universally present on the outer surface of mammalian cell membranes. We observed that living cells, but not dead ones suspended in serum-containing media will adhere strongly to plastic or glass surfaces covalently coated with the lectin.

The protocol is as follows: The water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate, (WSC) is used to covalently couple con A to the cell culture surface (1). For a 35 mm-diameter petri dish 0.5 ml of WSC (75 mg/ml) (Sigma) and 0.5 ml of con A (15 mg/ml) (Sigma), both dissolved in phosphate buffered saline



Separation of live and dead mouse MPC11 plasmacytoma cells stained for viability using the nucleic acid specific dyes acridine orange and ethidium bromide (5). Acridine orange enters living cells that will appear bright green fluorescent, whereas ethidium bromide stains nuclei of dead cells orange. (**A**) Live and dead MPC11 cells plated in conA-WSC coated petri dish. (**B**) The same field photographed after gentle rinsing with PBS. All the live cells remain attached. Their fluorescence is more dim than that in panel A due to the rapid fading of acridine orange upon exposure to ultraviolet light. (**C**) MPC11 cells attached to conA-WSC coated petri dish for 3 hours. All cells are fully viable as demonstrated by their exclusion of ethidium bromide.

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(PBS: Na_2HPO_4, 6.5 mM; KH_2PO_4, 1.5 mM; NaCl, 136.9 mM; KCl, 2.7 mM; pH 7.3), are mixed and spread immediately over the entire surface. After 2 h of incubation at room temperature (RT), the liquid is aspirated and the dish washed several times with PBS. The dishes are subsequently sterilized by γ-irradiation (CsCl source, 490 Gy) or under ultraviolet light in a laminar hood for 15–30 min. The sterile dishes can be stored in PBS for at least 3 days at 4°C. Prior to use the dishes are rinsed with culture medium containing 10% foetal calf serum, and cells (10^6/ml) suspended in the same growth medium (1–4 ml) are then added. After sedimentation for 20 min at RT live cells have attached firmly to the bottom of the culture dish, whereas dead cells remain in suspension, a process that is easily monitored by microscopical inspection. Dead cells are removed by gentle rinsing twice with PBS.

Specificity of the interaction of con A with receptor sites on the cell surface is indicated by the fact that 0.1 M α-methyl-D-mannoside or incubation of cells in the presence of 0.1 M EDTA prevents the binding of living cells. Nor do cells attach when con A is substituted by bovine serum albumin (30 mg/ml) or when con A is omitted (data not shown). However, it should be emphasized that it is necessary to incubate the cell mixtures in serum-containing medium to avoid unspecific fixation of dead cells to the culture surface.

Recovery of live cells was assessed by plating cell mixtures in coated petri dishes containing platinum prints of microgrids. Randomly selected areas of the grids were photographed before and after removal of dead cells, and living cells were identified by their relative positions. To distinguish between live and dead cells we used phase contrast microscopy or a sensitive viability assay based on fluorescence microscopy (5). For the production of Figure 1 the viability assay was employed allowing live and dead cells to be discriminated by virtue of their green and red fluorescence, respectively. Figure 1a and 1b depict the same field photographed before and after removal of dead cells. Every single live cell (green fluorescence) in Figure 1a remains after rinsing the dish (Figure 1b). The recovery of live cells in Figure 1b is representative of recoveries in general being nearly 100% (97.2 ± 1.8%), irrespective of the cell line. Sensitivity of the method was established by retrieving all three live cells that had been placed in a 35-mm dish together with 4×10^6 dead cells (data not shown). Recovered cells are fully viable as demonstrated in Figures 1b and 1c, cells in the latter being photographed several hours after plating in a coated dish. The selection method has successfully been applied to the B cell lines Daudi (human), MPC11 (murine) and 70Z/3 (murine) and the erythroleukemia cell line K562 (human) (data not shown). Cells can be harvested shortly after attachment simply by flushing the tissue culture surface with a jet of growth medium produced by forced pipetting. However, it is not recommended to recover live cells by scraping or trypsinization because of considerable loss of cell viability. Alternatively, cells that have attached can be grown directly in the coated dishes after careful rinsing with PBS.

Lectins have been widely used to separate viable cells, particularly lymphocytes, bearing different surface carbohydrates (3,4). The basis for the difference between live and dead cells in binding of con A is not clear to us, but may be due to either masking of the sugar residues or to their absence on dead cells. We believe that our method is generally applicable whenever the need arises to completely recover a small fraction of live cells from a majority of dead ones, not only in transfection studies, but also in e.g. establishment of hybridoma cultures and EBV transformed lymphoid cultures. The method is time saving and obviates the need for expensive equipment and costly materials.

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