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rent pattern. Using a porous agarose layer to support the sample, we frequently measure proton currents traveling towards the dish floor, suggesting that this provides a more physiological environment in which ions are free to move from all surfaces of the ovariole unimpeded (Figure 3).

The recording chamber provides a physiological environment and specimen manipulation and orientation possibilities that are well suited to extracellular electrophysiological investigation of a variety of tissues and can be adapted to other studies requiring precise sample orientation.

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Storage of Various Cell Lines at -70°C or -80°C in Multi-Well Plates While Attached to the Substratum

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Cryopreservation of higher eukaryotic cell lines is generally accomplished by addition of a cryopreservative (generally glycerol or DMSO) to the cell suspension. This combined with controlled cooling and thawing rates is thought to prevent dehydration and lethal injury during the freezing and thawing stages (1-3,10,11,13,14). When screening panels of clones derived from mammalian cells, it is useful to store the panel in a frozen state during screening to prevent contamination, to eliminate constant maintenance, or to facilitate absences from the laboratory. This report describes a simple method of freezing cells while still attached to the substratum of a multi-well plate. There have been previous reports describing the frozen storage of hybridoma cell lines in 96-well plates (8,21), one of them while cells were still attached to the substratum. Our experiments differ from the previous reports in five major ways. (i) We have simplified the previously reported methods by eliminating time-consuming media addition steps. (ii) We used a standard cryopreservative consisting of 10% DMSO, 20% FBS, and 70% RPMI 1640 (Invitrogen, Carlsbad, CA, USA) for storage at -70°C. (iii) We avoided use of feeder cells. (iv) We carried out experiments to assess recovery at four different DMSO:FBS ratios. (v) Importantly, our observations with six commonly used mammalian cell lines, including human breast cancer, keratinocytes, and rhabdomyosarcoma, show that the ability to store cells in a frozen state while attached to a substratum is not unique to hybridomas, making this technique widely applicable in modern molecular biology laboratories.

We demonstrated effective storage of six mammalian cell lines for up to one month in a DMSO-containing storage solution at -70°C or three months at

-80°C, while still attached to the substratum of a multi-well plate (Table 1). The technique bypasses the detachment and centrifugation steps required by the standard storage protocols and offers a significant savings of time when cryopreserving panels of single-cell clones.

Cells were seeded into multi-well dishes (Falcon® low-evaporation, flat-bottom tissue culture plates; Fisher Scientific, Pittsburgh, PA, USA) and grown in RPMI 1640 + 10% vol/vol NUSERUM™ IV (Becton Dickinson Labware, Franklin Lakes, NJ, USA) or 9% FBS (Sigma, St. Louis, MO, USA), at indicated confluencies for 24-30 h at 37°C in a 5% CO₂ incubator. The medium was then aspirated, and cells were overlaid with a warm cell freezing solution [10% vol/vol DMSO (Sigma), 20% FBS (Invitrogen), and 70% RPMI 1640]. Either 1 mL/well or 100 µL/well of freezing solution was applied to 12-well or 96-well plates, respectively. Each plate was then either sealed in a hybridization bag (Invitrogen) or double-bagged with Zip-Loc® freezer bags to prevent water condensation and immediately placed into a Styrofoam box (18 × 18 × 9 cm, with wall thickness of 5 cm) packed with paper towels. A lid of 2.5 cm thickness was then taped on, and this "freezing box" was placed in the ultralow freezer (-70°C or -80°C); after 18-24 h, the plate was removed from the box and stored in ultralow freezer until thawing.

One month or three months after freezing, plates were removed from hybridization bags and placed in a 37°C + 5% CO₂ incubator for 20 min. The freezing solution was then aspirated, and the well was rinsed once with PBS before the addition of 1 mL (12-well plate) or 300 µL (96-well plate) RPMI 1640 + 10% NUSERUM IV or RPMI 1640 + 9% FBS. Plates were then returned to the incubator, and growth was monitored throughout the following week.

To assess survival after freezing in multi-well plates, an SV-40-transformed human NB cell line, NB324K (6,16,17), was seeded into 12- and 96-well plates at either 40% or 90% confluency. Twenty-four hours later, the culture medium was removed, and the attached cells were overlaid with freezing solution (70% RPMI 1640, 20%

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Table 1. Survival and Extent of Detachment of Five Cell Lines Stored for One Month at -70°C in 10% DMSO/20% FBS/70% RPMI 1640

Cell Line	Survival in Well	Detachment into Freezing Solution
NB324K (human kidney)	Yes	<1%
HaCaT (human keratinocyte)	Yes	<1%
RH18 (human muscle)	Yes	90%
CRFK (feline kidney)	Yes	<1%
A72 (canine fibroma)	Yes	90%

With all lines, those cells remaining attached after thawing quickly resumed growth. The percent detachment was estimated by a visual determination of the confluency of cells remaining in the originally seeded well versus the confluency at which they were originally seeded.

Table 2. Comparison of Recovery with Varying Ratios of FBS/DMSO

Cell Line	Recovery: 0% DMSO	Recovery: 10% DMSO	Recovery: 20% DMSO	Recovery: 100% DMSO
HaCaT	none	good	good	none
MCF7	none	good	good	none
CrFK	none	good	good	none
A72	none ^a	good	good	none
NB324K	none ^a	good	good	none

Each cell line was seeded into a 96-well plate at several confluencies ranging from 10% to 90%. The next morning, the culture medium was aspirated and replaced with indicated cryopreservative. Three months later, cells were thawed and observed.

^aOne colony was observed to grow in one of the high-density wells.

FBS, 10% DMSO). Cells were then placed in the ultralow freezer and thawed one month later. Approximately 40% of the cells, as estimated by pre- and post-freezing confluencies, survived and grew to confluency within two days. While the lower seed densities required more time to reach confluency, no differences in recovery were observed at the two confluencies. Similar results were obtained after three months of cold storage. To extend this observation to clonal populations, six neo-transfected NB324K clones were selected with G418, expanded in 12-well plates, and transferred into a fresh 12-well plate. These clones were then frozen as described above. After one month, good recovery was observed with every clone at both densities.

Crandell feline kidney (CRFK) cells, a renal line derived from domes-

tic cat (7,15,18,19), were seeded into a 12-well plate at densities of 50%, 80%, or 100% and frozen as described above. Excellent recovery was observed, with 50%–80% of original cells surviving the process. Efficient recovery of CRFK seeded into a 96-well plate at densities of 1%, 10%, 20%, and 50% was also observed, although post-thawing attachment was somewhat lower in the 96-well plate (approximately 10% of original confluency). Cells grew to confluency at all densities in both plates within one week. CRFK cells were also seeded into either the middle or along the plate's edge and showed no discernible difference in survival. Frozen storage of CRFK clones was also investigated. All of the 15 clones frozen at confluencies varying from 40% to 100% were efficiently recovered, again with no observable differences due to

the original seeding density.

The A72 cells, derived from a canine fibroma (4,15,18,19), were seeded into a 12-well plate at a density of 80% and frozen as described above. Upon thawing one month later, most of the cells (approximately 90%) detached into the freezing solution but were recovered by centrifugation at 250× g for 5 min at room temperature, followed by resuspension in 1 mL fresh RPMI 1640 + 10% NU-SERUM IV and seeding into a fresh well. After thawing, 5%–10% of these cells remained attached and grew to confluency within seven days. To extend this observation to clonal populations, 15 clones were grown to confluencies varying from 20% to 90% and frozen as described above. Upon thawing one month later, efficient recovery was observed with all 15 lines. No effects of cell seeding density were observed.

Two other cell lines, the human alveolar rhabdomyosarcoma RH18 (9,12) and a human keratinocyte line, HaCaT (5), also showed good survival in 12- and 96-well plates with original seeding densities of either 30%–40% or 90%. RH18 exhibited a high degree of detachment into the cell freezing solution upon thawing (Table 1), but the remaining cells quickly grew to confluency. As with other lines, no effects due to density at the time of freezing were observed.

A72, NB324K, HaCaT, CrFK, and a human breast cancer line, MCF7, were seeded at high and low densities into 96-well plates and the following morning overlaid with one of the following freezing solutions: 100% FBS, 90% FBS/10% DMSO, 80% FBS/20% DMSO, or 100% DMSO. They were then frozen as described in the Materials and Methods section and stored for three months in an ultralow freezer.

Table 2 shows the results of a representative experiment. Repeated experiments at various seeding densities yielded similar results regardless of the initial density of cells. These results indicate no major differences in recovery of all lines tested after storage for three months in 10% or 20% DMSO. A72, NB324K, CrFK, and HaCaT showed comparable recovery in either 90% FBS/10% or 80% FBS/20% DMSO. With both treatments, the densely seeded wells were all nearly confluent after

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thawing and quickly became confluent. The lower-density seeds (10%–50%) grew to confluency within 10 days, with the exception of MCF7, which had only reached 20%–30% confluency in that time, likely because of its low seeding density (Table 2).

Storage in 100% DMSO resulted in nearly complete cell killing, although with all lines the nonviable cells remained attached after thawing. All cells also failed to survive 100% FBS, although in one experiment a colony of A72 cells was observed in one well after a week. HaCat cells stored in 100% FBS remained attached upon thawing but did not grow; the attached cells eventually deteriorated.

We have demonstrated that six different cell lines survive frozen storage for up to three months while attached to the substratum of a multi-well plate. While this is relatively short in term, in our experience, -70°C and -80°C temperatures allow storage of cultured cells for up to three years. However, there have been studies showing that efficient long-term storage of embryos or epithelial grafts requires colder temperatures (20,22). It is likely that the same will apply to at least some cultured cell lines stored while attached to the substratum.

Efficient recovery of all cell lines was observed using 20% or 10% DMSO in FBS but not in either pure DMSO or pure FBS. All cells except A72 behaved identically at -70°C and -80°C; for unknown reasons, A72 cells displayed greater frequency of detachment at -70°C. This difference could have been caused by lot variation either among the plates or among the various reagents.

Recent studies suggest that recovery of at least some cell lines after frozen storage is influenced by cell-cell contact (1–3). Our experiments with different densities at both storage temperatures suggests that within the confines of our scoring system, differences in seeding densities had no influence on recovery. However, it is possible that subtle differences in efficiency of recovery might be observed upon employment of more quantitative scoring methods.

In situations where large panels of cell clones require storing, this methodology offers a savings of time and effort by avoiding the need for expansion in culture dishes before frozen storage.

The method is simple and can likely be extended to many cell types.

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