

## Improved Microplate Fluorometer Counting of Viable Tumor and Normal Cells

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**Abstract.** An improved method has been developed to count cells *in situ* based on the measurement of esterase activity with carboxyfluorescein diacetate. This sensitive, semiautomated microplate fluorometer assay was able to estimate viable cell numbers over a range of  $5 \times 10^2$  to  $2.6 \times 10^5$  cells/well in a tumor cell line. Sensitivity to  $10^3$  was demonstrated in two other cell lines. Sub- and supranormal fluorescence events which can be responsible for unreliable readings when using a fluorescence assay for cell counting were quantified in a menadione (cytotoxic agent)/U-87 MG (cell line) model. There was a close correlation between the fluorometer method and Coulter counter method for two different tumor cell lines when this method was performed on cells after sub- and supranormal fluorescence events had ceased.

also present possible solutions to sub- and supranormal fluorescence events using the cytotoxic agent menadione with human glioblastoma cells as a model. Viable cell number is estimated by measuring the esterase activity of viable cells.

Fluorescein-derived probes such as fluorescein diacetate (FDA) and CFDA are nonpolar, non-fluorescing molecules. They readily penetrate the cell membrane, where they are intracellularly deacetylated by cellular esterases to yield the polar fluorescent molecules fluorescein (F) and carboxyfluorescein (CF), respectively (11,12). Intact cellular membranes are relatively impervious to F and CF so they accumulate in the viable cell (8). Although both FDA and CFDA are useful fluorogenic markers of cell viability (8,12-15), CFDA is preferred because it exhibits less extracellular leaching (9).

Under appropriate conditions, CFDA is loaded into the cells, *in situ*, where it is converted to CF. After a stabilization period, the fluorescence of the fluorogenic intracellular and leached, extracellular CF is measured on a microplate fluorometer.

### Materials and Methods

**Cell lines.** Caov-3 (human ovarian adenocarcinoma), CCD-37Lu (human, normal lung fibroblast), SK-MEL-28 (human malignant melanoma) and U-87 MG (human glioblastomaastrocytoma) were acquired from American Type Culture Collection (Rockville, MD). Stock cells were grown in  $75 \text{ cm}^2$  and  $75 \text{ cm}^2$  polystyrene monolayer tissue culture flasks (Corning) containing bicarbonate-buffered Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat inactivated ( $56^\circ\text{C}$  for 30 min) fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 110  $\mu\text{g}/\text{ml}$  sodium pyruvate and 2.5  $\mu\text{g}/\text{ml}$  Fungizone (experimental growth medium (EGM)). Cells were incubated at  $37^\circ\text{C}$  in a humidified, 5%  $\text{CO}_2/95\%$  air atmosphere. Cell lines were routinely tested for mycoplasma contamination using Mycotrim-TC (Irvine Scientific, Santa Ana CA). Cell counting was performed with a model ZM Coulter counter (Coulter Electronics, Hialeah, FL) or by Neubauer hemocytometer. The gain and threshold settings on the Coulter counter were selected for each cell line by matching the results to those of the hemocytometer.

**Stock solutions.** 5'-(and 6')-carboxyfluorescein diacetate (CFDA) (Molecular Probes, Eugene, OR) for the fluorometric assay was prepared as a 5.0 mM stock solution in dimethylsulphoxide (DMSO) and stored desiccated at  $-20^\circ\text{C}$ . Sterile phosphate-buffered saline (PBS) was prepared at pH 7.1. Menadione (Sigma, St. Louis, MO) working solution (1 mg/ml [w/v] in EGM) was prepared on the day of supplementation.

A commonly used end-point for measuring the cytotoxic and cytostatic effects of antineoplastic and toxic agents is the number of viable cells remaining after treatment (1). Standard methods for determining absolute numbers of viable cells include hemocytometer, Coulter counter,  $^3\text{H}$ -thymidine incorporation (2) and the MTT assay (3). Although these methods can accurately count viable cells, they are all time consuming (particularly when large numbers of samples are processed) and lack sensitivity (4). Newer methods employing microplate fluorometer-based assays which use fluorescein derived (5-9) and other (4,5) fluorescent probes for viable cells have also been described. Although these microplate fluorometer methods are less time consuming, none has been described with sensitivity greater than  $10^4$  cells/well. In addition, the reliability of fluorescein and other enzyme-dependent fluorescent indicators of cell viability is questionable due to sub- and supranormal fluorescence caused by sub-lethal doses of cytotoxic agents (10). We present here, a rapid, sensitive, *in situ*, microplate fluorometer method for measuring the proliferation of normal and tumor human cell lines using a fluorescein probe, carboxyfluorescein diacetate (CFDA). Results obtained with this method show that it is accurate at low cell densities ( $10^3$  cells/well) and compares well with Coulter counting. We

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