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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 X 10^2 to 2.6 X 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.*

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}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 fluoroscein probe, carboxyfluoroscein 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 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 cm^2 and 75 cm^2 polystyrene monolayer tissue culture flasks (Corning) containing bicarbonate-buffered Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat inactivated (56°C for 30 min) fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 110 µlg/ml sodium pyruvate and 2.5 µg/ml Fungizone (experimental growth medium (EGM)). Cells were incubated at 37°C in a humidified, 5% 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°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.

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Fluorometric linearity assay. Immediately following trypsinisation, cells were centrifuged, resuspended in EGM, counted and recentrifuged. The supernatant was removed and cells were resuspended in EGM at varying concentrations (n = 8 for each concentration) to yield cell numbers/well as described in the Results section. 125 µl of seeding medium with cells in suspension were placed into each well of 96-well tissue culture plates (Falcon). Seeding medium consisted of EGM without fetal calf serum. It was designed to be a holding medium (HM) in which an extended lag phase could be demonstrated. 125 µl of HM were also placed in another set of wells which served as blanks for the fluorometer. All cells were allowed to attach during incubation (4-6 hours) at 37°C in a humidified, 5% CO_2/95% air atmosphere.

Staining and fluorescence determination. Medium was removed by micropipettor and 80 µl of freshly diluted CFDA (25µM in PBS) were added as a stain to each well. After a 30 min incubation at room temperature the fluorescence of each well (average 3 readings/well) was determined in arbitrary units using an automatic microplate fluorometer (Cambridge Technology-Model 7620, Watertown, MA) equipped with 485 nm excitation and 530 nm emission bandpass filters.

Fluorometric inhibition assay. Plates were prepared as above, except that cells were seeded at a constant rate of 3,000 cells/well in EGM and incubated for 24 hr, after which EGM was removed and replaced with EGM supplemented with varying concentrations of menadione (n = 8 for each concentration, blank and control). After 3 or 6 days of incubation, supplemented medium was removed and cells were washed with 125 µl of PBS which was subsequently removed by aspiration. CFDA staining and fluorescence determination were performed as above.

Coulter counter inhibition assay. Duplicate plates were prepared as in the fluorometric inhibition assay. At the end of the growth period, supplemented medium was removed and cells were detached with 50 µl of a solution containing 0.2% trypsin (w/v) and 0.2 g/l EDTA (ethylene diamine tetraacetic acid). Cells were aspirated from wells and wells were washed twice with EGM containing FCS. Cell aspirate and EGM from seven wells were pooled and diluted with Isoton prior to counting with the Coulter counter. Three counts per sample were averaged. The eighth well for each concentration and control was used for trypan blue exclusion. In that well, medium was removed and cells were detached as above. 100 µl of trypan blue 0. 1% (w/v) were added to each well and allowed to stand for 5 min. Live cells were determined as a percentage of 200 cells excluding dye.

Supranormal fluorescence assay. Plates were prepared as in the fluorometric inhibition assay through the beginning of the growth period. U-87 MG cells were used. Two concentrations of menadione, 1 and 10 µg/ml, were used. Plates were incubated as in the fluorometric linearity assay. At variable intervals over a 7-day period, fluorescence was determined using the staining and fluorescence methods above (n = 8 for each menadione concentration, control, and blank).

Statistical methods. Regression coefficients, linear least square fit for standard curves, and Student t-test were calculated using analysis tools of Excel 4.0 for Windows software (Microsoft, Redmond, WA).

Fluorometric inhibition assay: Inhibition of cell proliferation (% inhibition) was evaluated as:
1 - [(Fluorescence of supplemented cells - mean fluorescence of blank) / (fluorescence of unsupplemented control cells - mean fluorescence of blank)] X 100%.

Counter inhibition assay: Inhibition of cell proliferation (% inhibition) was evaluated as: 1 - [(Cell count of supplemented cells X percent excluding trypan blue) / (cell count of unsupplemented controls X percent excluding trypan blue)] X 100%.

Supranormal fluorescence assay: Fluorescence relative to control was evaluated as: [(Fluorescent of supplemented cells - fluorescence of blank) / (fluorescence of unsupplemented control cells - fluorescence of blank) X 100%.

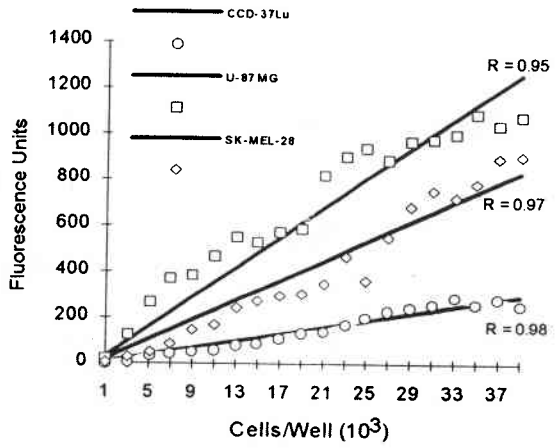

Figure 1. *Least squares lines for 3 cell lines. Fluorescence is linear over the assay range. Each point is the mean of 8 samples.*

Results

Fluorometric linearity assay. The linearity of the microplate fluorometer method for the estimation of viable cell numbers was determined first on one normal (CCD-37Lu) and two tumor cell lines (U-87 MG and SK-MEL-28) using HM for seeding (4-6 hr incubation). Figure 1 shows the linearity of the cell count *vs.* fluorescence units between 1 X 10^3 and 3.9 X 10^4 cells/well. The correlation coefficients for all three standard curves are R $\geq$0.95 when the least squares lines are forced through zero. Serial dilutions of U-87 MG cells (relatively small cells) showed linearity between 500 and 256,000 cells per well (Figure 2).

Critical to the assay is the ability to remove all FCS-containing medium due to the inherent esterase activity of the FCS. Multiple seeding media and washing conditions were tested to determine which yielded the best results. Initial attachment with EGM as a seed medium (4 hr) and subsequent changing medium to HM (4 hr) yielded results slightly better than HM alone, but the small increase in accuracy did not warrant the extra handling (data not shown). Direct CFDA staining of a suspension of detached cells in PBS yielded a correlation coefficient of R >.99. However, the fluorescence determined by the microplate reader was over 2 fold greater than attached cells (data not shown). This effect was probably due to the increased surface area of the suspended cells and rendered this method ineffective for development of a standard curve.

Comparison of fluorometric and Coulter counter inhibition assays-3 day incubation. Initial comparisons (duplicate plates) between the fluorometric and cell counter inhibition assays of U-87 MG cells are given in Figure 3. It shows dose response inhibition (percent) of cells by the cytotoxic agent, menadione, compared to controls. Menadione (vitamin K_3), a quinone, exhibits cytotoxic activity by inducing intracellular lipid peroxidation (16). Others have reported that a quinone (adriamycin) and other, chemically unrelated cytotoxic agents, at

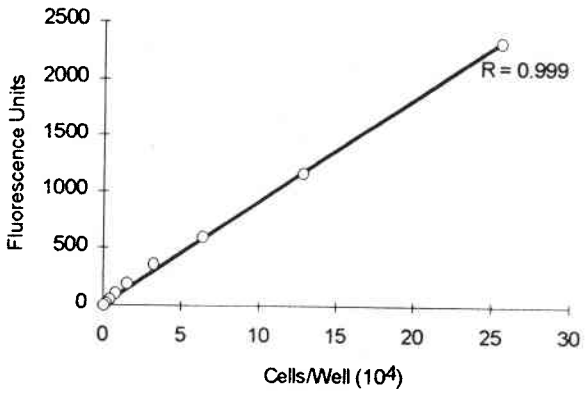

Figure 2. *Least squares line for serialty diluted U-87 MG c.ells. Fluorescence is linear over the assay range. Each point is the mean of 8 samples.*

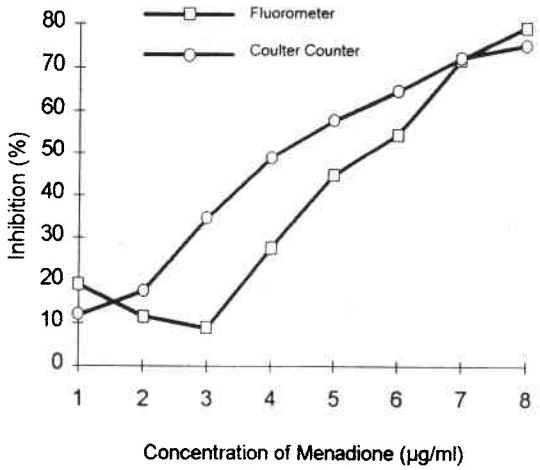

Figure 3. *Comparison of results from fluorometer method with Coulter method. Duplicate plates of U-87 MG cells exposed to varying conconcentration of menadione for 3 days. Each point for fluorometer method is mean of 8 samples. Counter points are the average of a pool of 7 samples.*

sub-lethal dosages, could induce supranormal CF fluorescence in CFDA stained cells for a short time after exposure (10). Although Massaro used a flow cytometer, the possibility of supranormal fluorescence would question the reliability of any fluorescent assay of cytotoxic or cytostatic agents using CFDA. Three days after exposure to menadione, we found supranormal cellular fluorescence of U-87 MG cells exposed to less than 11 µg/ml of menadione as evidenced by the spuriously low inhibition (Figure 3).

Supranormal fluorescence assay. In an effort to increase the reliability of this fluorometric assay, we studied the time dependence of supranormal CF fluorescence. Figure 4 shows the time variation of relative CF fluorescence of U-87 MG cells exposed to 1 and 10 µg/ml of menadione compared to duplicate controls.

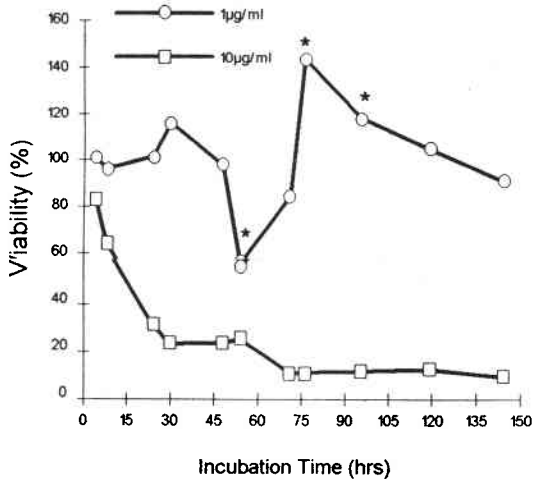

Figure 4. *CF fluorescence of U-87 MG cells exposed to 1 and 10 µg/ml of menadione over time relative to control wells. Each point is the mean of 8 samples relative to 8 control samples. *P<0.05 between the mean of 1 µg/ml and control groups.*

At the lethal dose of 10 µg/ml, relative fluorescence was suppressed within 4 hr and continued downward for 71 hr when it remained stable. The sub-lethal dose (1 µg/ml) exhibited fluorescence similar to control values for 48 hr. Fluorescence decreased at 54 hr, followed by supranormal fluorescence at 76 and 96 hr. A stabilization of fluorescence values was seen from 119 to 168 hours.

Comparison of fluorometric and Coulter counter inhibition assays, 6-day incubation. Given this information, we repeated the comparison of cell counter and fluorometer viability determinations for U-87 MG cells, using an end point (144 hr-6 days) when the CF fluorescence was relatively stable. In contrast to the disparity between the two methods shown in Figure 3, Figure 5 shows good agreement between the dose response curves. Figure 6 shows similar agreement for a different cell line (Caov-3) after a 144 hr exposure to menadione.

Discussion

We have described a rapid, sensitive, and reliable method for *in situ* estimation of viable cell number using CFDA and a microplate fluorometer. One advantage of this assay is its ability to estimate cell densities as low as 10^3 cells/well in 2 cell lines and 500 cells/well in another. Secondly, the assay allows for *in situ* estimation of viable cells, reducing the amount of steps in which the cells must be manipulated. In itself, this *in situ* approach allows fewer chances for error and increases reliability and reproducibility. This method is much safer than using radioactive tracers. By being rapid and efficient it can also expand the ability of a laboratory to screen cytotoxic agents many fold.

We have also presented a technique to ensure reliable results when using this and other fluorescence assays to assess cell proliferation after exposure to cytotoxic agents. Cytotoxic

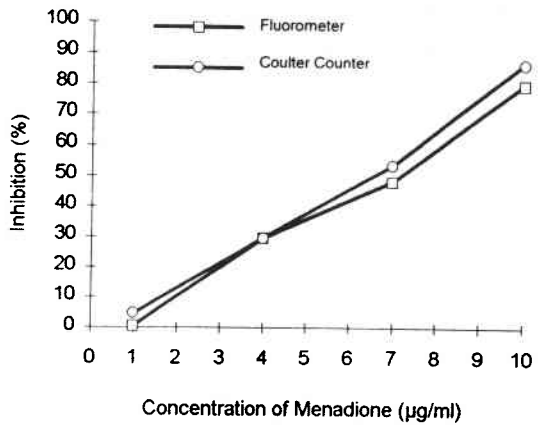

Figure 5. *Comparison of results from fluorometer method with Coulter counter method. Duplicate plates of U-87 MG cells exposed to varying concentration of menadione for 6 days. Each point for fluorometer method is the mean of 8 samples. Counter points are the average of a pool of 7 samples.*

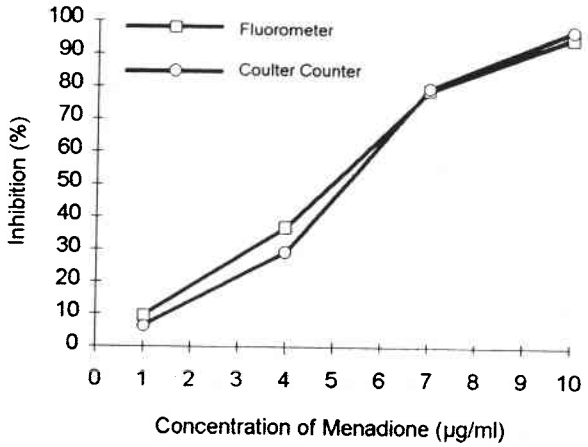

Figure 6. *Comparison of results from fluorometer method with Coulter counter method. Duplicate plates of Caov-3 cells exposed to valying concentration of menadione for 3 days. Each point for fluorometer method is the mean of 8 samples. Counter points are the average of a pool of 7 samples.*

agents have been shown in the past to induce sub- and supranormal fluorescence which could lead to spurious results in a fluorescent assay. Supranormal CF fluorescence is attributed to a retardation of CF efflux (10). The cause of subnormal fluorescence in CFDA stained cells is not known, but decreased esterase activity is probable. By assessing, and then avoiding, the sub- and supranormal fluorescence for our particular combination of cell line (U-87 MG) and toxic agent (menadione), we have demonstrated good agreement between this method and standard cell counting methods. We expect that this approach will work as well for other cell lines and other toxic agents.

One disadvantage of the assay is the inability to use it for suspension cultures. Without the addition of specialized centrifugation equipment and microtiter centrifuge plates, washing of cells in suspension presents a problem. Well contents would have to be removed from microplate wells, centrifuged and resuspended prior to being placed back in a microplate for evaluation.

For absolute counts of cells, standard curves can be established using this method as long as strict attention is paid to the following conditions: time of incubation, volume of CFDA/PBS stain solution, and concentration of CFDA. Even small variations in any of these conditions may greatly vary the fluorescence reported.

For measurement of cytotoxic events, this method can be coupled with an exclusionary dye (trypan blue or propidium iodide) method. As long as diligence is used in reproduction of the method and assessment of supranormal and subnormal fluorescence are addressed in a given cell and toxic agent model, the results of the assay are in good agreement with standard cell counting methods.

This method for counting viable cells is faster and safer than other methods. It allows for the rapid quantification of inhibitory effects of chemical agents on normal and tumor cells and should be valuable for screening potential antineoplastic agents.

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References

1 Patterson Jr MK: Measurement of growth and viability of cells in culture. *In:* Jakoby WB and Patan IH (eds), Cell Culture. Methods in Ezymology. San Diego, Academic Press, 1979, pp 141-152.

2 Freshney R: Quantitation and experimental design: *In:* Freshney R (ed.), Culture of animal cells: A manual of basic technique. 2nd (Ed. New York, Alan R. Liss, Inc. 1987, pp 227-237.

3 Carmichael J, Degraff WG, Gazdar AF *et al:* Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res *47:* 936-943, 1987.

4 Huschtscha LI, Lucibello FC and Bodmer WF: A rapid micro method for counting cells *in situ* using a fluorogenic alkaline phosphatase enzyme assay. In Vitro Cell Dev Biol *25:* 105-108, 1989.

5 Essig-Marcello JS and Van Buskirk RG: A double-label *in situ* cytotoxicity assay using the fluorescent probes neutral red and BCECG-AM. In Vitro Toxicol *3:* 219-227, 1990.

6 Hansson Y, Jacobson E, Ortlund J, Paulie S and Perlmann P: A rapid method for detection of cellular proliferation using carboxyfluorescein. J Immunol Methods *100:* 261-267, 1987.

7 Provinciali M, Di Stefano G and Fabris N: Optimization of cytotoxic assay by target cell retention of the fluorescent dye carboxyfluorescein diacetate (CFDA) and comparison with conventional ^{51}CR release assay. J Immun Methods *155:* 19-24, 1992.

8 Smith MD, Barbenel JC, Courtney JM and Grant MH: Novel quantitative methods for the determination of biomaterial cytotoxicity. Int J Artif Org *15:* 191-194, 1992.

9 Smith MD, Shearer MG, Srivastava S, Scott R and Courtney JM: Quantitative evaluation of the growth of established cell lines on the

surface of collagen, collagen composite and reconstituted basement membrane. Urol Res *20:* 285-288, 1992.

10 Massaro EJ, Elstein KH, Zucker RM and Bair KW: Limitations of the fluorescent probe viability assay. Mol Toxicol *2:* 271-284, 1989.

11 Rotman B and Papermaster BW: Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Natl Acad Sci USA *55:* 134-141, 1966.

12 Sontag W: A comparative kinetic study on the conversion of fluoresceindiacetate to fluorescein in living cells and *in vitro*. Rad Environm Biophys *14:* 1-12, 1977.

13 Ross DD, Joneckis CC, Ordonez JV, Sisk AM, Wu RK, Hamburger AW and Nora RE: Estimation of cell survival by flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number. Cancer Res *49:* 3776-3782, 1989.

14 Gray DWR and Morris PJ: The use of fluorescein diacetate and ethidium bromide as a viability stain for isolated islets of langerhans. Stain Technology *62:* 373-381, 1987.

15 Schmalz G and Netuschil L: A modification ofthe cell culture agar diffusion test using fluoresceindiacetate staining. J Biomed Mater Res *19:* 6S3-661, 1985.

16 Noto V *et al:* Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K3) treatment on human tumor cell growth *in vitro*. Cancer *63:* 901-906, 1989.

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