

# A fluorescence microplate cytotoxicity assay with a 4-log dynamic range that identifies synergistic drug combinations

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## Abstract

**Purpose:** Cytotoxicity assays in 96-well tissue culture plates allow rapid sample handling for multicondition experiments but have a limited dynamic range. Using DIMSCAN, a fluorescence digital image system for quantifying relative cell numbers in tissue culture plates, we have developed a 96-well cytotoxicity assay with a >4-log dynamic range. **Methods:** To overcome background fluorescence that limits detection of viable cells with fluorescein diacetate, we used 2'4'5'6'-tetrabromofluorescein (eosin Y) to quench background fluorescence in the medium and in nonviable cells to enhance the reduction of background fluorescence achieved with digital image thresholding. The sensitivity and linearity of the new assay were tested with serial dilutions of neuroblastoma and leukemia cell lines. DIMSCAN was compared with other *in vitro* cytotoxicity assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, colony formation, and trypan blue dye exclusion. **Results:** Without background fluorescence reduction, scans produced a nearly flat curve across various cell concentrations from 100 to 10<sup>6</sup> cells per well. Either digital image thresholding or eosin Y dramatically reduced background fluorescence, and combining them achieved a linear correlation ( $r > 0.9$ )

of relative fluorescence to viable cell number over >4 logs of dynamic range, even in the presence of  $4 \times 10^4$  nonviable cells per well. Cytotoxicity of deferroxamine for neuroblastoma cell lines measured by the DIMSCAN assay achieved dose-response curves similar to data obtained by manual trypan blue counts or colony formation in soft agar but with a wider dynamic range. Long-term cultures documented the clonogenic ability of viable cells detected by DIMSCAN over the entire dynamic range. The cytotoxicity of two drug combinations (buthionine sulfoximine + melphalan or fenretinide + safingol) was tested using both DIMSCAN and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, and the wider dynamic range of DIMSCAN facilitated detection of synergistic interactions. **Conclusion:** DIMSCAN offers the ability to rapidly and efficiently conduct cytotoxicity assays in 96-well plates with a dynamic range of >4 logs. This assay enables rapid testing of anticancer drug combinations in microplates. [Mol Cancer Ther 2007; 6(3):886–97]

## Introduction

Testing antineoplastic agents on cell lines is a critical component of the drug development process, and achieving at least 2 logs of cell kill in preclinical studies increases the likelihood of a response in clinical trials (1). Thus, a meaningful assessment of tumor cell kill obtained with a drug, and especially a combination of drugs, requires an assay having a wide dynamic range (at least 3 logs). A number of different assays for measuring *in vitro* cytotoxicity have been developed, but all have disadvantages. Colony-forming assays in semisolid medium are employed by many investigators, but their use is limited by long incubation times, their labor intensive nature, the inability to exchange culture medium, and a limited dynamic range due to the poor clonogenicity of many cell lines in agarose (2, 3). Dye exclusion assays (4–6) and their adaptation, the differential staining cytotoxicity assay (7), have a limited dynamic range, are labor-intensive, and are subject to observer error (8).

Cytotoxicity assays involving cell lines conducted in 96-well tissue culture plates allow for rapid sample handling and the ability to change medium, permitting variable times of exposure to drug. As conditions in 96-well plates closely resemble standard cell culture conditions, the clonogenicity of most human tumor cell lines should be optimal in such a liquid culture setting. Cell numbers in 96-well tissue culture plates can be quantified using radioactive isotopes, such as <sup>51</sup>Cr release (9) or <sup>3</sup>H-uridine incorporation (10); colorimetric measurement of substrates

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altered by viable cells, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; refs. 8, 11–13); or protein-binding dyes, such as sulforhodamine (13, 14). However, many of these assays have a limited dynamic range (usually not greater than 2 logs), and due to required washing steps, they can be difficult to perform with suspension cultures or poorly attached adherent cell lines.

Alternatively, cytotoxicity can be measured with fluorochromes, such as fluorescein diacetate or 2',7'-bis(carboxymethyl)-5,6-carboxyfluorescein, which accumulate only in viable cells. Such fluorescent dyes are used either alone or in combination with DNA fluorescent dyes that do not penetrate viable cells (9, 15, 16). Fluorescence-based systems offer a number of advantages, such as the lack of radioactive waste, short incubation times, the ability to brightly stain individual cells, and the ability to rapidly measure cell numbers directly in microplates using a 96-well fluorescence reader (17, 18). However, commercially available 96-well fluorescence readers provide a limited dynamic range because they cannot discriminate between the fluorescence from viable cells and background fluorescence, a problem only partially overcome by use of expensive custom filtration plates (9).

Our fluorescence digital image microscopy system (DIMSCAN) allows for flexible and rapid analysis of viable or total cell numbers in tissue culture plates of any format and provides a 3-log dynamic range due to its ability to decrease background fluorescence using digital image processing (17). We have used DIMSCAN to develop a semiautomated 96-well cytotoxicity assay employing the fluorescent dye fluorescein diacetate to detect viable cells. Here, we report that the viable dye 2',4',5',6'-tetrabromofluorescein (eosin Y) quenched background fluorescence produced by fluorescein diacetate, and that using eosin Y and fluorescein diacetate with the DIMSCAN system provides an increase in the 96-well cytotoxicity assay to a >4-log dynamic range. We compared results produced by the DIMSCAN assay with other methods for measuring cytotoxicity *in vitro*, including the MTT assay, colony-forming assay, and trypan blue dye exclusion. Furthermore, we show the importance of a wide dynamic range for detecting synergistic drug interactions by comparing drug combination assays done using the MTT assay to those conducted with the new DIMSCAN assay described in this study.

## Materials and Methods

### Cell Lines

Neuroblastoma cell lines were established in our laboratory (SMS-KCNR, SMS-KAN, SMS-KANR, and CHLA-90; refs. 19, 20) or kindly provided by the originator of the lines: LA-N-1 and LA-N-5 (Dr. R. Seeger, Childrens Hospital Los Angeles, Los Angeles, CA; ref. 21), CHP-126 (Dr. A. Evans, Childrens Hospital Philadelphia, Philadelphia, CA; ref. 21), and SK-N-SH (Dr. J. Biedler, Memorial Sloan Kettering, New York, NY; ref. 22). Neuroblastoma cell lines were subcultured using Puck's Saline A + 1 mmol/L EDTA as previously described (19). The lymphoblastic

leukemia cell line MOLT-3 (23) was obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in RPMI 1640 + 10% (v/v) heat-inactivated (56°C for 60 min) fetal bovine serum (FBS) and 2 mmol/L L-glutamine (complete medium) in a 5% CO<sub>2</sub> humidified incubator. All cell lines were grown without antibiotics to allow ready detection of *Mycoplasma*.

### Reagents

Fluorescein diacetate (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO to produce a 1 mg/mL stock solution, filter sterilized through 0.8- $\mu$ m nylon, aliquoted into 1.5-mL microcentrifuge tubes, and stored frozen at -20°C in the dark. For individual assays, an intermediate stock was prepared by thawing and diluting an aliquot with RPMI 1640 + 10% FBS such that delivery of 50  $\mu$ L would produce the required final concentration of fluorescein diacetate in the well (8, 10, 12, or 16  $\mu$ g/mL). Because cleavage of fluorescein diacetate to free fluorescein by endogenous esterases in the FBS could increase the background fluorescence, intermediate dilutions were prepared immediately before loading each individual plate. The 1% (w/v) solution of eosin Y (Sigma Chemical) in 0.9% NaCl was stored at room temperature.

Deferoxamine mesylate (Desferal, CIBA Pharmaceutical Co., Summit, NJ) was dissolved in sterile water to produce a 10 mg/mL stock solution, sterile filtered through 0.22- $\mu$ m nylon, and stored at 4°C. For cytotoxicity experiments, the proper volumes of the deferoxamine stock were added to RPMI 1640 + 10% FBS to produce the desired drug concentrations.

Fenretinide [*N*-(4-hydroxyphenyl)retinamide (4-HPR)], safingol, melphalan [L-phenylalanine mustard (L-PAM)], hydroxyurea, and buthionine sulfoximine (BSO) were supplied by the National Cancer Institute.

### DIMSCAN System

The DIMSCAN system consisted of an inverted fluorescence microscope equipped with a stepper motor scanning stage. Using an intensified CCD camera, images of portions of a well in a 96-well plate were fed to a PC Vision frame grabber in a 80386 microcomputer. The microcomputer controlled stage movement and quantified fluorescence in the wells, examining 18 images of 512  $\times$  480 pixels per well and determining the relative number of total cells present due to fluorescence of fluorescein diacetate accumulated in the viable cells (17). Fluorescence was read from underneath the plate. Digital thresholding was employed to decrease background fluorescence, as previously described (17, 24). Calibration of the system was carried out by adjusting the iris controlling excitation light to the minimum needed to cause all cells in a representative field to exceed the digital threshold and generate an image on a monitor displaying the thresholded image in "real time." Such calibration was carried out using control wells containing viable cells present in each plate analyzed. Data from DIMSCAN were imported into SigmaPlot 2000 (SPSS Science, Chicago, IL) to produce graphic output. The Pearson linear correlation coefficient (*r*) was calculated using Microsoft Excel.

### Background Fluorescence Testing (Dynamic Range Testing)

**Serial Dilution of Viable Cells.** To determine the correlation of relative fluorescence and viable cell number, cells were loaded into Falcon 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) with an Electrapette multichannel pipettor (Matrix Technologies, Lowell, MA) set for serial 2-fold dilution and a 125- $\mu$ L mixing volume in three cycles. A range of cell concentrations from one million cells per well to two cells per well were plated in eight replicate wells per condition, in a final well volume of 125  $\mu$ L. Neuroblastoma cells were allowed to settle and attach for 2 to 8 h. Before scanning, the tissue culture plates were gently loaded by multichannel pipettor with 50  $\mu$ L per well of intermediate fluorescein diacetate stock and incubated at 37°C for 30 min. After incubation, plates were loaded with 30  $\mu$ L per well of 0.5% eosin Y solution (final eosin Y concentration, 0.083%); controls received medium without eosin Y. Relative fluorescence was determined with the DIMSCAN system, using a  $\times 4$  magnification objective lens (numerical aperture, 0.10) with an image intensifier and Omega Optical XF22 filters (17). To determine the sensitivity of the DIMSCAN assay, fluorescence data obtained from each cell concentration group were compared using a nonparametric Mann-Whitney *U* test (Statistica 6.0; StatSoft, Inc., Tulsa, OK), and the level of significance was expressed as *P*.

**Serial Dilution of Viable Cells with Additional Dead Cells.** To study the influence of large numbers of dead cells on the ability of DIMSCAN to detect small numbers of viable cells, identical plates with serial fourfold dilutions of cells were prepared as before. Before staining with fluorescein diacetate, half of the plates was loaded with  $4 \times 10^4$  nonviable cells per well by removing 50  $\mu$ L of medium and replacing it with  $4 \times 10^4$  dead cells resuspended in 50  $\mu$ L of RPMI 1640 + 10% FBS. Nonviable cells for these experiments were obtained from the identical cell line, resuspended in RPMI 1640 + 10% FBS, and frozen at -20°C for 4 h. Cell viability was determined after thawing by trypan blue dye exclusion counts to be <1%.

### Cytotoxicity Assay

**Dose Response to Deferoxamine.** To show the ability of DIMSCAN to measure the cytotoxic effect of a drug, increasing concentrations of the iron chelator deferoxamine (25) were tested on the SMS-KAN, SMS-KANR, SMS-KCNR, and LA-N-5 neuroblastoma cell lines. The 96-well plates were loaded with  $4 \times 10^4$  cells per well in 250  $\mu$ L of complete medium per well ( $1.2 \times 10^5$  per  $\text{cm}^2$ ). Each plate was divided into six conditions (16 wells for each), consisting of one control and five concentrations of deferoxamine (1, 5, 10, 20, and 50  $\mu$ g/mL). Duplicate plates for each cell line were incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator. Treated cells were exposed to deferoxamine during the entire incubation period. On days 4 and 7, plates were stained with fluorescein diacetate and analyzed with the DIMSCAN system, using  $\times 4$  magnification with the image intensifier. Digital thresholding was used in combination with eosin Y to reduce background fluorescence.

Controls for all DIMSCAN cytotoxicity experiments consisted of cells incubated in complete medium. A medium blank was used in a number of initial experiments and later abandoned, as the fluorescence readings in these controls were always zero, due to the combination of digital image thresholding and eosin Y quenching.

**Manual Counts.** Similar conditions were created in 25- $\text{cm}^2$  tissue culture flasks, using identical neuroblastoma cell lines and deferoxamine concentrations. Triplicate flasks with the same cell density as the 96-well tissue culture plates ( $3 \times 10^6$  per flask equivalent to  $1.2 \times 10^5$  per  $\text{cm}^2$ ) were incubated under identical conditions. Manual trypan blue dye exclusion counts using a hemocytometer were done after 4 and 7 days of treatment (4, 5).

**Colony-Forming Assays.** Identical cell lines and deferoxamine concentrations were used, plating  $0.5 \times 10^6$  per  $35 \times 10$  mm tissue culture dish in Iscove's DMEM/0.41% bacto agar (Difco, Detroit, MI), + 0.7  $\mu$ mol/L  $\alpha$ -thioglycerol, 1.3 mmol/L L-glutamine, 20% (v/v) heat inactivated FBS, and 85 units/mL of penicillin-streptomycin. Triplicate dishes were incubated under identical conditions, and colonies of  $\geq 50$  cells were counted after 14 days (2, 3). To compare the results of the DIMSCAN assay with the colony-forming assay, the cytotoxic effect of deferoxamine at the end of the treatment was expressed as the percentage of relative fluorescence intensity (DIMSCAN assay), or the percentage of colonies detected (colony-forming assay), relative to control (untreated cells).

**Determination of the Ability of the DIMSCAN System to Detect Small Numbers of Clonogenic Viable Cells.** Replicate 96-well tissue culture plates were loaded with  $2 \times 10^4$  SMS-KCNR neuroblastoma cells per well and treated for 7 days with increasing concentrations of deferoxamine (1, 3, 5, and 10  $\mu$ g/mL, respectively), 16 wells per concentration. After 7 days of exposure to deferoxamine, 150  $\mu$ L of the 250  $\mu$ L in each well was removed and replaced with complete medium without drug; every 7 days, one replicate plate was stained with fluorescein diacetate, and remaining plates had medium exchanged as described above. Plates stained with fluorescein diacetate were analyzed using DIMSCAN (with digital thresholding and eosin Y quenching) and observed by fluorescence microscopy for cell colonies, and representative fields of cells photographed.

### DIMSCAN Assay Reproducibility

To determine the intra-day variability of the DIMSCAN system, 96-well plates were loaded with CHP-126 or LA-N-1 cells at 40,000 per well in 250  $\mu$ L of complete medium and incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator. The combination deferoxamine + hydroxyurea was then added to the plates. Each plate was divided into six conditions (16 wells per condition), consisting of one control and five concentrations of deferoxamine + hydroxyurea (deferoxamine: 1, 5, 10, 20, and 50  $\mu$ g/mL; hydroxyurea: 50, 100, 200, 500, and 1,000  $\mu$ mol/L). All plates were analyzed on day 0 within 2 h from treatment initiation. The coefficient of variation for each group was calculated using Microsoft Excel 2000.

To determine the inter-day variability of DIMSCAN, we conducted two identical experiments on separate days, where 96-well plates were loaded with SMS-KCNR or SMS-KANR cell lines at 40,000 cells per well in 250  $\mu$ L of complete medium. Deferoxamine (1, 5, 10, and 20  $\mu$ g/mL) was added to the plates. Each condition was tested in replicates of 16 wells. Plates were incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator and analyzed on day 0 of each experiment. We compared fluorescence values (control plus four increasing deferoxamine concentrations) for each experiment and calculated the coefficient of variation using Microsoft Excel 2000.

#### Drug Combination Studies

For a direct head-to-head comparison of DIMSCAN with the MTT assay, two different drug combinations were evaluated after 4 days of *in vitro* treatment. Duplicate Falcon 96-well tissue culture plates (Becton Dickinson) were seeded on day -1 with CHLA-90 neuroblastoma cell line at 6,000 per 150  $\mu$ L of complete medium per well and allowed to attach overnight at 37°C in a 5% CO<sub>2</sub> humidified incubator. On day 0, 4-HPR, safingol, and BSO in various concentrations were added in 50  $\mu$ L of complete medium per well to assigned wells (12 wells per condition, one dose-response curve of a drug or a drug combination per plate). L-PAM was added under similar conditions on day 1, 24 h after BSO. Duplicate plates for each cell line were incubated for a period of 4 days. Treated cells were exposed to drugs during the entire incubation period. BSO, L-PAM, safingol, and 4-HPR were tested at the concentrations achievable in human plasma, with the highest tested concentration of L-PAM achievable clinically in myeloablative settings (26–28).

**DIMSCAN Analysis.** Plates were stained on day 4 with fluorescein diacetate (final concentration, 10  $\mu$ g/mL), incubated at room temperature for 20 min, and analyzed using  $\times 4$  magnification. Digital thresholding was employed in combination with eosin Y to reduce background fluorescence (see Background Fluorescence Testing).

**MTT Assay.** Cell viability was assessed by the uptake of MTT (thiazolyl blue tetrazolium bromide; Sigma Chemical; ref. 29). On day 4, the culture medium in each well was substituted with 200  $\mu$ L of fresh medium containing MTT (final concentration, 250  $\mu$ g/mL). Plates were then incubated for an additional 2-h period at 37°C. Subsequently, the medium was carefully removed in such a way that no loosely adherent cells were removed. Cells containing the trapped MTT crystals were then solubilized in 200  $\mu$ L DMSO at 37°C for 15 min. Absorbance was determined in a microtiter plate reader (Molecular Devices, Menlo Park, CA) at 550 nm and subtracted from absorbance at 650 nm.

The results from both DIMSCAN and MTT assays were analyzed and expressed as survival fractions by a comparison of the quantified fluorescence (DIMSCAN) or the absorbance (MTT) of surviving cells to that of untreated control cells in each plate, as described previously (27). The limits of detection for DIMSCAN in this study were determined according to the estimated number of cells

present in control wells at the time of treatment initiation ( $\sim 1 \times 10^4$  per well on day 0), for a detection limit for cytotoxicity of one live cell at the end of the assay per 10,000 starting cells (27). Results for the DIMSCAN survival fraction are graphed in SigmaPlot 2000 (SPSS Science) with a detection limit of  $10^{-4}$ .

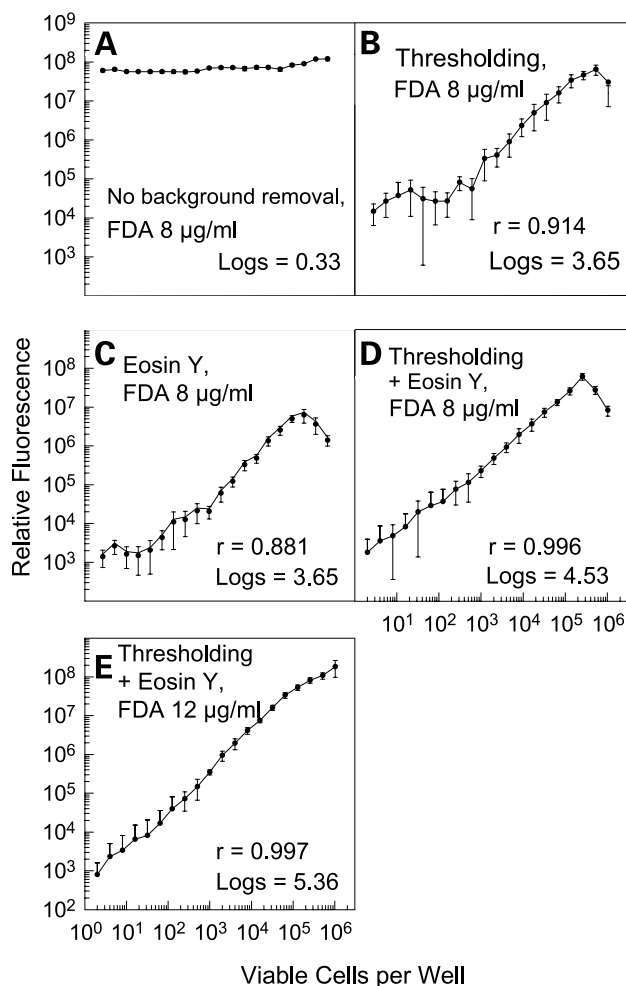
Statistical analyses were done using Microsoft Excel. Differences of means were analyzed by two-sided Student's *t* test with significance defined as  $P < 0.05$ .

**Combination Index Method.** The combination index method for quantifying drug synergism, developed by Chou and Talalay, was used to analyze the drug combinations tested by both DIMSCAN and MTT assay (27, 30). Employing the "Dose-Effect Analysis with Microcomputers" software (Biosoft, Great Shelford, Cambridge, United Kingdom), the drug combination data were evaluated for possible additive or synergistic effects based on the following combination index values, calculated for each concentration level: <1.0, synergistic effect; 1.0, additive effect; >1.0, antagonistic effect. Based on the combination index values for each concentration level, the synergistic effect was further refined as marginal synergism (between 0.7 and 1.0), synergism (between 0.3 and 0.7), strong synergism (between 0.1 and 0.3), and very strong synergism (lower than 0.1; refs. 27, 30).

## Results

Using DIMSCAN without employment of a method for reducing the background fluorescence across, scans of 96-well plates containing various concentrations of SMS-KCNR neuroblastoma cells stained with fluorescein diacetate produced a nearly flat curve due to the viable cell fluorescence being obscured by background fluorescence in the medium (Fig. 1A). Next, we analyzed replicate plates using digital image thresholding (Fig. 1B), which decreased the background fluorescence and allowed us to detect varying numbers of viable cells over 3.6 logs of dynamic range. In an attempt to further decrease background fluorescence, we tested the ability of dyes that do not penetrate viable cells (trypan blue, eosin Y) to quench the fluorescence in the medium and in dead cells. We found that trypan blue slightly decreased background fluorescence, without reducing fluorescence in the viable cells, but eosin Y markedly decreased background fluorescence without obscuring fluorescence from viable cells if used at an optimal final concentration of 0.083%. Figure 1C shows the reduction in background fluorescence obtained when eosin Y was added to a replicate of the plate in Fig. 1A.

The combination of both thresholding and eosin Y presented a further and marked decrease in background fluorescence, wherein the scan detected a linear increase in relative fluorescence intensity corresponding to increasing numbers of viable cells over nearly 5 logs (Fig. 1D). With a fluorescein diacetate concentration of 8  $\mu$ g/mL, fluorescence tapered off at cell densities above  $1 \times 10^5$  cells per well, due to cell overgrowth and exhaustion of the



**Figure 1.** Correlation of relative fluorescence measured by DIMSCAN with viable neuroblastoma cell numbers in 96-well plates. Serial 2-fold dilutions of SMS-KCNR neuroblastoma cells (87% viable by trypan blue exclusion) were prepared in 96-well tissue culture plates, stained with fluorescein diacetate (FDA), incubated at 37°C for 30 min, and analyzed with the DIMSCAN system at  $\times 4$  magnification with image intensifier (one plate per condition). Points, average of eight wells; bars, SD. The Pearson linear correlation coefficient ( $r$ ) values presented here were calculated for the fraction of the curve representing serial dilution of viable cells from 1,000 to 2 cells per well. When  $r$  values for the complete viable cell serial dilution curve were calculated, only the combination of higher fluorescein diacetate concentration (12  $\mu\text{g}/\text{mL}$ ) with digital thresholding and eosin Y to quench the background fluorescence (E) achieved an  $r > 0.9$ . A, final fluorescein diacetate concentration of 8  $\mu\text{g}/\text{mL}$ ; no methods to decrease the background fluorescence were used (logs of dynamic range, 0.33). B, fluorescein diacetate concentration of 8  $\mu\text{g}/\text{mL}$ ; threshold set to a gray-scale pixel value of 120 (all pixels below 120 forced to zero) to decrease background fluorescence. The Pearson linear correlation coefficient was  $r = 0.914$  ( $r = 0.681$  for the entire curve; logs of dynamic range, 3.65). C, fluorescein diacetate concentration of 8  $\mu\text{g}/\text{mL}$ ; 30  $\mu\text{L}$  of 0.5% eosin Y per well was added before analysis to quench background fluorescence;  $r = 0.881$  ( $r = 0.406$  for the entire curve; logs of dynamic range, 3.65). D, fluorescein diacetate concentration of 8  $\mu\text{g}/\text{mL}$ ; digital threshold set to a gray-scale pixel value of 50, and 30  $\mu\text{L}$  of 0.5% eosin Y per well added before analysis to decrease background fluorescence;  $r = 0.996$  ( $r = 0.378$  for the entire curve; logs of dynamic range, 4.53). E, fluorescein diacetate concentration of 12  $\mu\text{g}/\text{mL}$ ; digital threshold set to a gray-scale pixel value of 90, and 30  $\mu\text{L}$  of 0.5% eosin Y per well added to remove the background;  $r = 0.997$  ( $r = 0.973$  when calculated for the entire 20-point serial dilution curve; logs of dynamic range, 5.36).

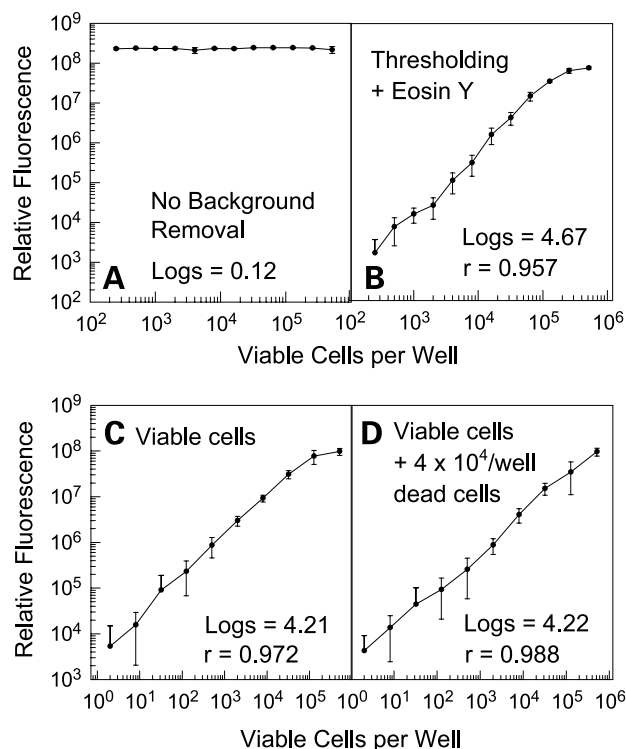
fluorescein diacetate (Fig. 1B–D). When we increased the fluorescein diacetate concentration to 12  $\mu\text{g}/\text{mL}$  (Fig. 1E), scans showed a further improvement in linearity ( $r = 0.973$ ), covering  $\sim 5$  logs of fluorescence intensity and 4 logs of cell density ( $1 \times 10^6$  to  $1 \times 10^2$  cells per well). Although the graphs seem linear across a full 5 logs of cell density, the SDs for wells containing  $<100$  cells per well were in excess of the mean; thus, the assay may not be truly linear at very low cell concentrations. The optimal concentration of fluorescein diacetate was dependent on the cell line used and the highest cell concentration in an assay; fluorescein diacetate concentrations  $> 12 \mu\text{g}/\text{mL}$  increased background fluorescence; concentrations from 8 to 12  $\mu\text{g}/\text{mL}$  were found to be optimal.

To investigate the sensitivity of the DIMSCAN assay at low-end fluorescence values, we conducted a statistical analysis of the data from Fig. 1E using a nonparametric Mann-Whitney  $U$  test. We determined that the difference between the fluorescence values at the extreme low-end concentration (4 cells per well) and the values obtained with the concentrations of 8 to 32 cells per well was not significant ( $P > 0.24$ ); yet, there was a statistically significant difference between the values obtained with the 4 and 64 cells per well groups ( $P < 0.04$ ). Similarly, the difference between the fluorescence values obtained with 64 and 32 or 125 cells per well was not significant ( $P > 0.09$ ); yet, there was a statistically significant difference between values obtained from 64 and 250, 16, 8, 4, 2 and cells per well, respectively ( $P < 0.03$ ). At concentrations above 250 cells per well, differences among all groups were statistically significant.

We also tested the ability of DIMSCAN to quantify viable cells using nonattached (suspension) cell cultures. Representative results are shown in Fig. 2A and B using the MOLT-3 leukemia cell line. Analysis conducted without using techniques that reduce background fluorescence resulted in a nearly flat line across various cell concentrations (Fig. 2A). With a combination of digital image thresholding and eosin Y, the assay produced a linear increase in relative fluorescence intensity corresponding to increasing cell number ( $r = 0.957$ ) over 4.5 logs of fluorescence and 3.5 logs of cell density, from  $0.512 \times 10^6$  to 250 cells per well (Fig. 2B).

Background fluorescence resulted from addition of large numbers of nonviable cells that release esterases (which convert fluorescein diacetate to fluorescein) and possibly by esterases normally present in fetal bovine serum. Figure 2C and D shows the effectiveness of the combination background reduction method (digital image thresholding plus eosin Y addition) when a large excess of nonviable SK-N-SH neuroblastoma cells were added to the culture plates containing serial dilutions of viable SK-N-SH cells ( $10^6$  down to 2 cells per well) before staining with fluorescein diacetate. Both control and test plates were stained and analyzed with DIMSCAN under identical conditions. The control plates produced a linear increase in fluorescence intensity with increasing viable cell number ( $r = 0.972$ ; Fig. 2C). The scans of a replicate plate with  $4 \times 10^3$

additional nonviable cells per well produced a comparably linear relationship ( $r = 0.988$ ), and  $<100$  viable cells per well were easily detected even in the presence of a 10,000-fold excess of nonviable cells (Fig. 2D).



**Figure 2.** **A** and **B**, correlation of relative fluorescence measured by DIMSCAN with viable leukemia cell numbers in 96-well plates. Serial 2-fold dilutions of viable MOLT-3 leukemia cells (98% viable by trypan blue exclusion) were prepared in 96-well tissue culture plates, stained with fluorescein diacetate, incubated at 37°C for 30 min, and analyzed with the DIMSCAN system at  $\times 4$  magnification with image intensifier. *Points*, average of eight wells; *bars*, SD. *Y-axis*, relative fluorescence measured by DIMSCAN; *X-axis*, number of viable cells (according to trypan blue exclusion at time of plating) seeded per well. **A**, final fluorescein diacetate concentration of 8  $\mu\text{g}/\text{mL}$ ; no methods to remove the fluorescence background were used (logs of dynamic range, 0.12). **B**, fluorescein diacetate concentration of 8  $\mu\text{g}/\text{mL}$ ; digital threshold set to a gray-scale pixel value of 90, and 30  $\mu\text{L}$  of 0.5% eosin Y per well was added before analysis of decrease background fluorescence. The Pearson linear correlation coefficient was  $r = 0.957$  (logs of dynamic range, 4.67). **C** and **D**, detection of small numbers of viable cells with DIMSCAN in the presence of excess numbers of dead cells. Duplicate 96-well tissue culture plates with serial 4-fold dilution of viable SK-N-SH neuroblastoma cells were prepared. Nonviable SK-N-SH cells ( $<1\%$  viable by trypan blue dye exclusion) were added to each well ( $4 \times 10^4$  dead cells per well) of a duplicate plate to determine the effect of the dead cells on background fluorescence. Both plates were stained with fluorescein diacetate, incubated at 37°C for 30 min, and analyzed with the DIMSCAN system at  $\times 4$  magnification with image intensifier. *Points*, average of eight wells; *bars*, SD. *Y-axis*, relative fluorescence measured by DIMSCAN; *X-axis*, number of viable cells (according to trypan blue exclusion at time of plating) seeded per well. **C**, fluorescein diacetate concentration of 12  $\mu\text{g}/\text{mL}$ ; threshold set to 90, and 30  $\mu\text{L}$  of 0.5% eosin Y per well was added before the reading to remove the fluorescence background. The Pearson linear correlation coefficient was  $r = 0.972$  (logs of dynamic range, 4.21). **D**,  $4 \times 10^4$  dead SK-N-SH cells per well were added before staining. Fluorescein diacetate concentration of 12  $\mu\text{g}/\text{mL}$ ; threshold set to 90, and 30  $\mu\text{L}$  of 0.5% eosin Y per well was added before the reading to remove the fluorescence background ( $r = 0.988$ ; logs of dynamic range, 4.22).

To test the ability of the system to quantify cytotoxicity, the DIMSCAN assay was used to measure the cytotoxic effect of the iron chelator deferoxamine (DFO) on neuroblastoma cell lines *in vitro*. Four different neuroblastoma cell lines were treated with increasing concentrations of deferoxamine, the plates were stained with fluorescein diacetate, and relative numbers of viable cells were measured with DIMSCAN, using digital thresholding and eosin Y quenching. Figure 3A to D shows the relative fluorescence (directly proportional to viable cell numbers) evaluated on days 4 and 7 after initial exposure to deferoxamine. The cytotoxic effect of deferoxamine was dose dependent, with increasing cytotoxicity from 1 to 20  $\mu\text{g}/\text{mL}$  of deferoxamine for all cell lines tested. Deferoxamine was effective in a time-dependent manner, as a 7-day exposure to deferoxamine markedly increased the cytotoxic effect compared with a 4-day exposure (Fig. 3).

We compared DIMSCAN assay results for deferoxamine dose-response curves to similar dose-response experiments done in 25-cm<sup>2</sup> tissue culture flasks (Fig. 3E–H). Cells were treated with deferoxamine for 4 and 7 days, and cell viability was determined by the trypan blue dye exclusion method using a hemocytometer. Results from trypan blue counting showed dose-response curves to deferoxamine similar to those produced by the DIMSCAN assay, but the trypan blue assay had a more narrow dynamic range (Fig. 3).

We also compared the DIMSCAN cytotoxicity assay to a colony formation assay in semisolid medium using identical neuroblastoma cell lines and deferoxamine concentrations (Fig. 4). The dose-response curves to deferoxamine using the colony formation assay were similar to those produced by DIMSCAN, although with a narrower dynamic range in two of four lines tested (Fig. 4).

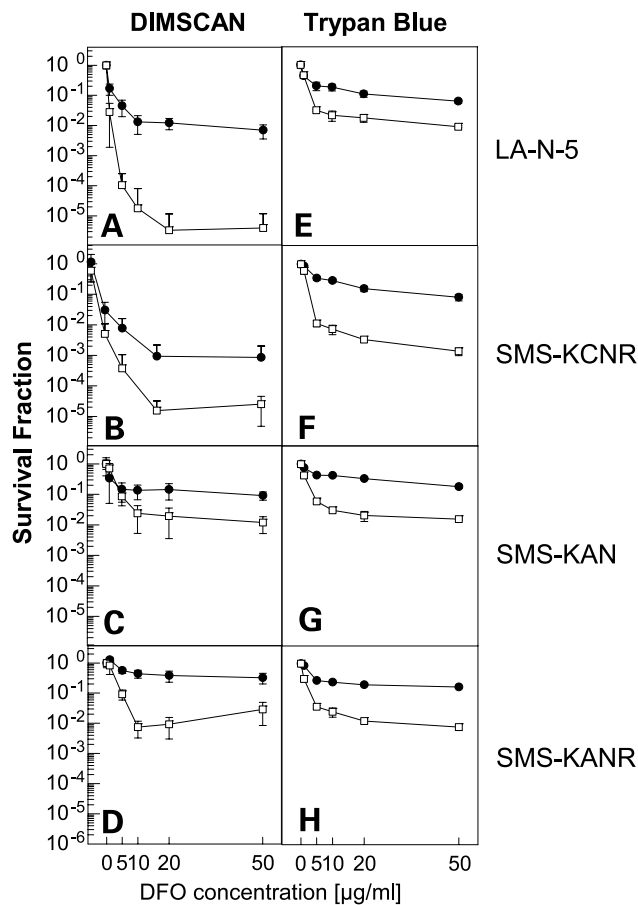
To exclude the possible fluorescence-quenching properties of deferoxamine and hydroxyurea that could confound the DIMSCAN testing, set of plates were read on day 0, immediately after adding drugs. Nearly identical fluorescence averages obtained across all groups (untreated controls plus five increasing drug concentrations) on day 0 indicated that deferoxamine and hydroxyurea had no fluorescence-quenching properties (data not shown).

To test the intra-day reproducibility of the DIMSCAN system (with eosin Y quenching and digital image thresholding), plates containing CHP-126 or LA-N-1 cells were treated with deferoxamine + hydroxyurea and analyzed on the same day. When all six groups from a plate (five treatment groups and controls) were compared, the coefficients of variation ranged from 1.9% to 9.8%. When all 24 groups within one experiment were compared (tested on a total of four plates), the coefficients of variation were 9.3% for CHP-126 and 8.1% for LA-N-1.

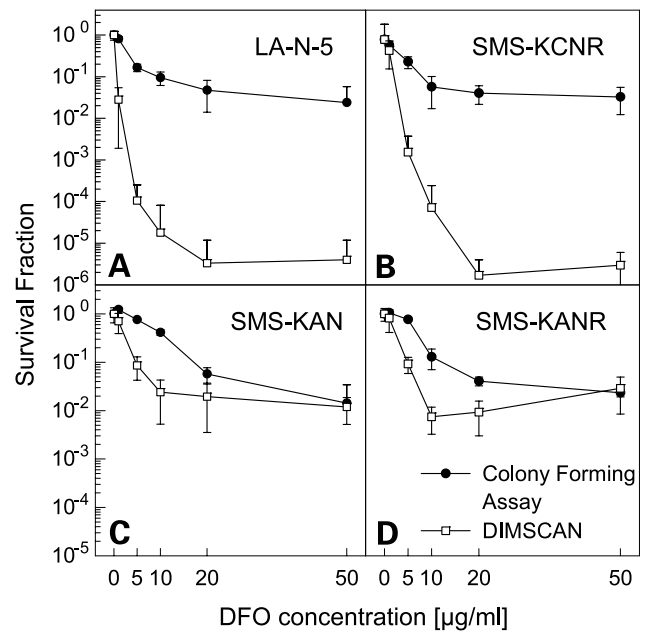
We have also evaluated the DIMSCAN system with the new dye combination (fluorescein diacetate + eosin Y) for inter-day reproducibility using deferoxamine-treated plates containing either SMS-KCNR or SMS-KANR cell lines. We compared five average fluorescence values (four deferoxamine-treated groups and controls) in identical

experiments conducted on separate days and observed a maximum coefficient of variation of 9.1%. The data from the highest concentration group (50  $\mu\text{g}/\text{mL}$ ) were omitted from analysis due to the immediate cytotoxic effect that resulted in a decrease in fluorescence in both cell lines.

A major advantage for a high dynamic range in a cytotoxicity assay is the ability to assess the increase in cell kill when combining two or more drugs (i.e., accurate assessment of drug synergism). We therefore compared the ability of DIMSCAN to measure drug synergism with the



**Figure 3.** Comparison of DIMSCAN to the trypan blue dye exclusion method in determining the dose response of neuroblastoma cell lines to deferoxamine mesylate (DFO). **A** to **D**, DIMSCAN assay. Two 96-well tissue culture plates per cell line were loaded with  $4 \times 10^4$  cells per well, divided in six conditions per plate, and treated with increasing concentrations of deferoxamine (0, 1, 5, 10, 20, and 50  $\mu\text{g}/\text{mL}$ ). On days 4 and 7, respectively, one of the duplicate plates for each cell line was stained with fluorescein diacetate (final concentration, 8  $\mu\text{g}/\text{mL}$ ), incubated at 37°C for 30 min, and analyzed with the DIMSCAN system at  $\times 4$  magnification with image intensifier. Digital image threshold (set to a gray-scale pixel value of 90) and addition of 30  $\mu\text{L}$  per well of 0.5% eosin Y were used to quench the fluorescence background. Points, average of 16 wells; bars, SD. ●, day 4; □, day 7. **E** to **H**, trypan blue exclusion. Neuroblastoma cell lines identical to those tested with the DIMSCAN system were plated in 25-cm<sup>2</sup> tissue culture flasks and treated over a period of 4 d with increasing concentrations of deferoxamine (0–50  $\mu\text{g}/\text{mL}$ ). The cells were then harvested, and viable cell numbers for each condition were determined by counting >200 cells using a hemocytometer and 0.04% trypan blue. Points, average of three flasks; bars, SD. ●, day 4; □, day 7.



**Figure 4.** Comparison of DIMSCAN to colony formation in soft agar for determining the dose response of neuroblastoma cell lines to deferoxamine mesylate. Two 96-well tissue culture plates per cell line were loaded with  $4 \times 10^4$  cells per well, divided in six conditions per plate, and treated with increasing concentrations of deferoxamine (1–50  $\mu\text{g}/\text{mL}$ ). On day 7, one of the replicate plates for each cell line was stained with 8  $\mu\text{g}/\text{mL}$  of fluorescein diacetate and analyzed with the DIMSCAN system. Digital image threshold (set to a gray-scale pixel value of 90) and addition of 30  $\mu\text{L}$  per well of 0.5% eosin Y were used to quench the fluorescence background. Points, average of 16 wells; bars, SD. Identical neuroblastoma cell lines and deferoxamine concentrations were used for colony-forming assays, in which  $0.5 \times 10^6$  cells were plated into 35  $\times$  10 mm tissue culture dishes in soft agar at the appropriate concentration of drug. Colonies of  $\geq 50$  cells were counted after 14 d of incubation. Points, average of three tissue culture dishes; bars, SD. ●, colony-forming assay data (day 14); □, DIMSCAN system data (day 7).

commonly used MTT assay. Duplicate plates treated with increasing concentrations of BSO, L-PAM, and their combination were analyzed after 4 days of exposure to drugs using both the DIMSCAN and the MTT assays. The dose-response curves produced by MTT were similar to those obtained with DIMSCAN, but DIMSCAN provided a markedly wider dynamic range (Fig. 5A and B). As shown in Fig. 5A and B, the MTT assay showed that the addition of BSO increased the cytotoxicity of L-PAM by <0.2 log ( $P = 0.017$ ), whereas DIMSCAN detected an increase in cytotoxicity of >2.5 logs ( $P = 0.0001$ ) at the same concentration level (400  $\mu\text{mol}/\text{L}$  BSO + 40  $\mu\text{mol}/\text{L}$  L-PAM). When analyzing the DIMSCAN data with the combination index method for quantifying drug synergism, the cytotoxic effect of BSO + L-PAM was determined as synergism (combination index, 0.40 for 300  $\mu\text{mol}/\text{L}$  BSO + 30  $\mu\text{mol}/\text{L}$  L-PAM; synergism defined as combination index, 0.3–0.7). At higher concentrations, DIMSCAN detected very strong synergism (combination index, 0.07 for 400  $\mu\text{mol}/\text{L}$  BSO + 40  $\mu\text{mol}/\text{L}$  L-PAM; very strong synergism defined as combination index, <0.1). However, identical analysis of

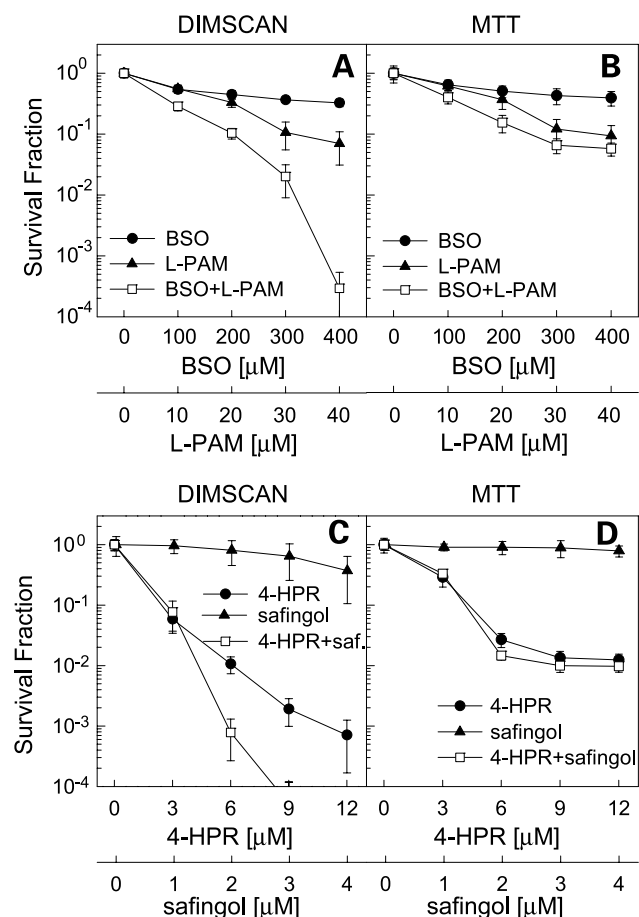
MTT data suggested that the effect of the tested drug combination was only marginally synergistic (combination index, 0.73 for 300  $\mu\text{mol/L}$  BSO + 30  $\mu\text{mol/L}$  L-PAM; combination index, 0.90 for 400  $\mu\text{mol/L}$  BSO + 40  $\mu\text{mol/L}$  L-PAM; marginal synergism defined as combination index, 0.7–1.0).

Similarly, dose-response curves of another drug combination (4-HPR + safingol) were comparable following analysis with both the DIMSCAN and the MTT assays, with DIMSCAN providing a wider dynamic range (Fig. 5C and D). As shown in Fig. 5C and D, the MTT assay showed that the addition of safingol increased the cytotoxicity of 4-HPR by  $<0.1$  log ( $P = 0.015$ ), whereas DIMSCAN detected an increase in cytotoxicity over 2 logs ( $P = 0.001$ ) at the same concentrations (12  $\mu\text{mol/L}$  4-HPR + 4  $\mu\text{mol/L}$  safingol). The drug combination of 4-HPR + safingol showed a synergistic effect when DIMSCAN data were used to determine the combination index (combination index, 0.41 for 9  $\mu\text{mol/L}$  4-HPR + 3  $\mu\text{mol/L}$  safingol; combination index, 0.55 for 12  $\mu\text{mol/L}$  4-HPR + 4  $\mu\text{mol/L}$  safingol; synergism

defined as combination index, 0.3–0.7). The same analysis of data obtained with the MTT assay determined the cytotoxic effect of both drugs in combination as being marginally synergistic at the lower drug concentration level (combination index, 0.81 for 9  $\mu\text{mol/L}$  4-HPR + 3  $\mu\text{mol/L}$  safingol; marginal synergism defined as combination index, 0.7–1.0). However, MTT suggested that 4-HPR + safingol were marginally antagonistic at higher concentrations (combination index, 1.07 for 12  $\mu\text{mol/L}$  4-HPR + 4  $\mu\text{mol/L}$  safingol; antagonism defined as combination index,  $>1.0$ ).

To show that small numbers of cells detected by fluorescein diacetate and the DIMSCAN system were clonogenic, and that those cells considered nonviable by DIMSCAN were not clonogenic, we treated replicate plates with various concentrations of deferoxamine, resulting in a range of cell kill over 4 logs at day 7 (Figs. 6 and 7). We then analyzed replicate plates from this experiment at days 7, 14, 21, and 28. Medium was renewed every 7 days during the course of the experiment. As shown in Figs. 6 and 7, the small numbers of viable cells detected after a  $>4$ -log cell kill were able to proliferate into colonies after drug removal. Moreover, the regrowth of cells paralleled the amount of cell kill, indicating that the assay accurately distinguished viable from nonviable cells after 7 days of exposure to a cytotoxic agent.

**Figure 5.** Cytotoxic effect of drug combinations based on DIMSCAN versus MTT assay analysis. **A** and **B**, BSO + melphalan (L-PAM). **A**, 96-well tissue culture plates seeded with the CHLA-90 neuroblastoma cell line at 6,000 cells per well and treated with various concentrations of BSO (100, 200, 300, and 400  $\mu\text{mol/L}$ ), L-PAM (10, 20, 30, and 40  $\mu\text{mol/L}$ ), and the combination of both BSO plus L-PAM (BSO/L-PAM, 10:1, fixed concentration ratio). Treated cells were exposed to drugs for an incubation period of 4 d (BSO) or 3 d (L-PAM), added 24 h after BSO at 37°C in a 5%  $\text{CO}_2$  humidified incubator. Following treatment, plates were analyzed using the DIMSCAN assay. Each dose-response curve is from one tissue culture plate. Points, average of 12 wells; bars, SD. ●, BSO only; ▲, L-PAM only; □, BSO + L-PAM (10:1 concentration ratio). Using the combination index method for quantifying drug synergism, the cytotoxic effect of BSO + L-PAM was determined by DIMSCAN as synergism (combination index, 0.40 for 300  $\mu\text{mol/L}$  BSO + 30  $\mu\text{mol/L}$  L-PAM; synergism defined as combination index, 0.3–0.7) and very strong synergism at the higher concentration level (combination index, 0.07 for 400  $\mu\text{mol/L}$  BSO + 40  $\mu\text{mol/L}$  L-PAM; very strong synergism defined as combination index,  $<0.1$ ). **B**, duplicates of the plates analyzed in **(A)** assayed by the MTT assay. The cytotoxic effect of BSO + L-PAM determined by MTT was marginally synergistic (combination index, 0.73 for 300  $\mu\text{mol/L}$  BSO + 30  $\mu\text{mol/L}$  L-PAM; combination index, 0.90 for 400  $\mu\text{mol/L}$  BSO + 40  $\mu\text{mol/L}$  L-PAM; marginal synergism defined as combination index, 0.7–1.0). **C** and **D**, fenretinide (4-HPR) + safingol. **C**, 96-well tissue culture plates seeded with the CHLA-90 neuroblastoma cell line at 6,000 cells per well and treated with various concentrations of 4-HPR (3, 6, 9, and 12  $\mu\text{mol/L}$ ), safingol (1, 2, 3, and 4  $\mu\text{mol/L}$ ), or the combination of 4-HPR + safingol (4-HPR/safingol; 3:1, fixed concentration ratio). Treated cells were exposed to drugs for an incubation period of 4 d at 37°C in a 5%  $\text{CO}_2$  humidified incubator. Following treatment, duplicate plates were analyzed using the DIMSCAN assay. Each dose-response curve is from one tissue culture plate. Points, average of 12 wells; bars, SD. ●, 4-HPR only; ▲, safingol only; □, 4-HPR + safingol (3:1 concentration ratio). Using the combination index method for quantifying drug synergism, the cytotoxic effect of 4-HPR + safingol determined by DIMSCAN was synergistic (combination index, 0.41 for 9  $\mu\text{mol/L}$  4-HPR + 3  $\mu\text{mol/L}$  safingol; combination index, 0.55 for 12  $\mu\text{mol/L}$  4-HPR + 4  $\mu\text{mol/L}$  L safingol; synergism defined as combination index, 0.3–0.7). **D**, duplicates of the plates analyzed in **(C)** assayed by the MTT assay. The cytotoxic effect of 4-HPR + safingol was marginally synergistic at the lower concentration level (combination index, 0.81 for 9  $\mu\text{mol/L}$  4-HPR + 3  $\mu\text{mol/L}$  safingol; marginal synergism defined as combination index, 0.7–1.0) and marginally antagonistic at the high concentration level (combination index, 1.07 for 12  $\mu\text{mol/L}$  4-HPR + 4  $\mu\text{mol/L}$  safingol; antagonism defined as combination index,  $>1.0$ ).



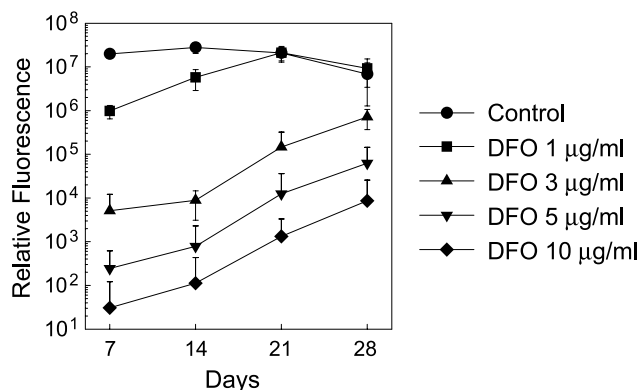


## Discussion

The use of fluorescent labels to measure cell-mediated cytotoxicity has been employed for a number of years (9, 31). A fluorochrome assay using the carboxyfluorescein derivative 2',7'-bis(carboxymethyl)-5,6-carboxyfluorescein was reported to be similar to the frequently used  $^{51}\text{Cr}$  release assay (31). Another fluorescent dye, fluorescein diacetate, is converted in viable cells by intracellular esterases to fluorescein, which becomes trapped within cells having intact membrane integrity (15), causing the viable cells to exhibit bright green fluorescence. Fluorescein diacetate has been used mostly in combination with dyes that penetrate nonviable cells and cause red fluorescence (fluorescein diacetate/ethidium bromide, fluorescein diacetate/propidium iodide) to identify viable cells on microscope slides (32) or with flow cytometry (15, 16).

We have previously described the development of the DIMSCAN digital image system for quantifying relative cell numbers by fluorescence *in situ* in a variety of tissue culture dishes, particularly in a 96-well format (17). The system allows flexible yet simple analysis of viable or total cell numbers. However, the linearity and dynamic range in detecting viable cells using fluorescein diacetate was limited in the original DIMSCAN system. Therefore, we have used DIMSCAN in combination with the dye eosin Y to selectively reduce background fluorescence, while preserving the detection of viable cells, producing a rapid and robust cytotoxicity assay with an excellent dynamic range.

To determine the sensitivity and dynamic range of DIMSCAN for detecting changes in viable cell numbers,

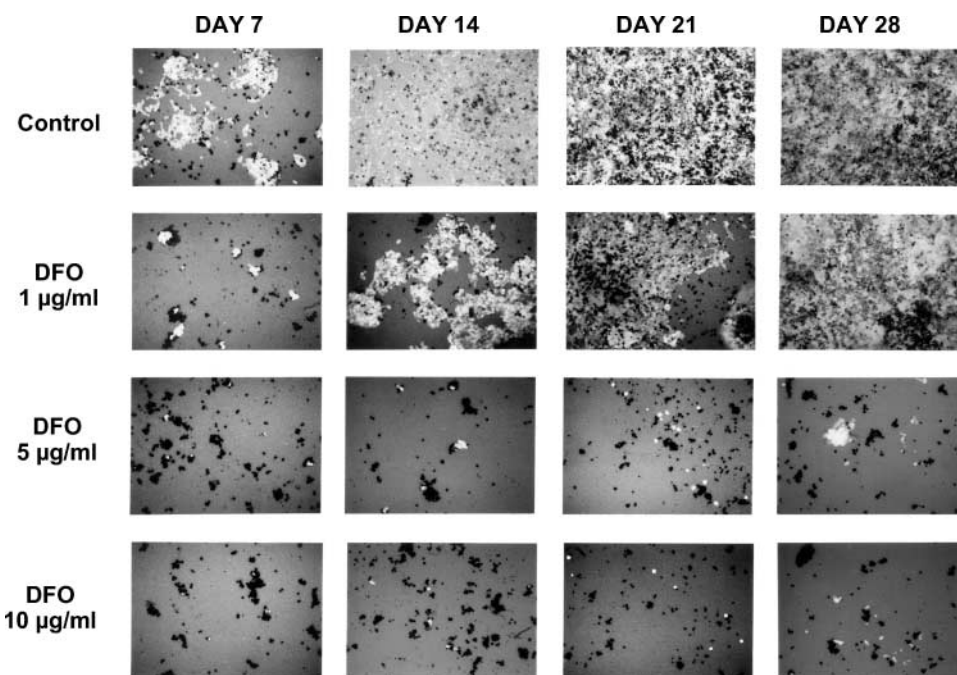


**Figure 6.** Small numbers of viable cells capable of cell proliferation are detectable by the DIMSCAN assay. Replicate 96-well tissue culture plates were loaded with  $2 \times 10^4$  SMS-KCNR neuroblastoma cells per well and treated with increasing concentrations of deferoxamine (DFO), 16 wells per concentration. After a 7-d exposure to deferoxamine, medium was exchanged, and this was repeated every 7 d. One plate was analyzed for fluorescence by DIMSCAN each week. Deferoxamine concentrations: ●, 0  $\mu\text{g}/\text{mL}$  (control); ■, 1  $\mu\text{g}/\text{mL}$ ; ▲, 3  $\mu\text{g}/\text{mL}$ ; ▼, 5  $\mu\text{g}/\text{mL}$ ; ◆, 10  $\mu\text{g}/\text{mL}$ . Growth in control wells saturated in relative fluorescence during the first 7 d, and due to overgrowth, viable cell numbers declined beginning at day 21. Cells in the deferoxamine-treated wells showed increasing amounts of fluorescence after drug removal, with the magnitude of cell regrowth paralleling the degree of cell kill and the amount of deferoxamine used per well. Photomicrographs of wells from this experiment are in Fig. 7.

we conducted serial dilutions of neuroblastoma or leukemia cells in 96-well plates from 2 cells to  $1 \times 10^6$  cells per well and then measured the correlation of relative fluorescence to viable cell number. Fluorescein produced from fluorescein diacetate by reaction with the esterases in fetal bovine serum in the medium, and diffusion of fluorescein out of dead cells produced significant background fluorescence, which masked the fluorescence of viable cells. We have previously reported that digital image thresholding decreased the background fluorescence of fluorescein diacetate, providing a dynamic range of  $\sim 3$  logs (17). Here, we show that the addition of eosin Y just before scanning of plates with DIMSCAN quenches the background fluorescence in the medium and dead cells. As eosin Y penetrates and stains cells that lack membrane integrity, but not viable cells (33), it enhanced the detection of viable cells, which remain the sole source of fluorescence. Combining both eosin Y quenching and digital thresholding provided the greatest sensitivity and linearity range. The mechanism by which eosin Y quenches the background fluorescence is currently unknown. However, as eosin Y is a nonfluorescent derivative of fluorescein (34, 35), quenching likely occurs by absorption of the same spectrum of light (peak, 488 nm) that excites fluorescein. Exclusion of eosin Y from the viable cells prevents the quenching effect of eosin Y from occurring in the viable cells, enabling bright fluorescein diacetate-mediated fluorescence (which exceeds the digital threshold), thereby allowing DIMSCAN to selectively quantify small numbers of viable cells, even in the presence of an excess of dead cells.

When used with appropriate concentrations of fluorescein diacetate, and with eosin Y to quench background fluorescence, the DIMSCAN system provided a semi-automated assay for quantifying viable cells in 96-well plates over 4 logs of cell concentration ( $1 \times 10^6$  to  $1 \times 10^2$  cells per well), using either adherent (neuroblastoma) or suspension (leukemia) cell lines. High SDs were seen within the lower cell numbers ( $<500$  cells per well or approximately a value of relative fluorescence of  $10^3$ ). These increased SDs are the result of "spotty" cell distributions in the wells containing lower cell numbers and the increased effect of fluorescent artifacts (dust particles, etc.) in wells with low cell numbers. More attentive analysis of the data after scanning, using Chauvenet's criterion or other analytic methods to reject the outlier wells, can decrease the variance (36, 37). Employing 16 wells per condition nevertheless provides a meaningful dose-response curve in a single 96-well plate and allows rejection of one or two wells without affecting the final data. In spite of the increased variance when measuring wells with very small numbers of surviving viable cells, the dynamic range of the system is higher than reported for most cytotoxicity assays (Table 1). Moreover, the combination of digital image thresholding and eosin Y quenching to remove background fluorescence protects the linearity of quantifying viable cells throughout the entire 4-log dynamic range, even in the presence of excessive numbers of dead cells.

**Figure 7.** Photomicrographs of experiment in Fig. 6. Using fluorescent microscopy as described in Materials and Methods, representative wells from each plate were photographed each time a plate was analyzed by DIMSCAN (magnification,  $\times 100$ ). Photomicrographs show that the growth of surviving viable cells measured by DIMSCAN was due to growth of tumor colonies that correlated in size to the measured amount of fluorescein diacetate fluorescence.



As described in our previous studies (9, 17, 19, 21, 38, 39), the detection limits for DIMSCAN were defined according to the estimated number of cells present in control wells at the time of treatment initiation (minimum of  $10 \times 10^3$  cells per well on day 0) and by the ability of the system to detect one live cell at the end of the assay. Based on these definitions, the detection limit of DIMSCAN is in excess of 4 logs. Because low-end fluorescence readings (at cell concentrations of  $<100$  cells per well) yield coefficients of variation that are close to 100%, we analyzed whether there was a statistical difference between the fluorescence values obtained at these low cell concentrations. Although these results show that this system has limitations in sensitivity at the extreme low-end readings, the differences between values obtained with increasing logs of cell concentrations (as documented by the differences among 4, 64, and 250 cells per well) were statistically significant.

Although changing the incubation time for fluorescein diacetate staining from 30 to 60 min did not significantly influence DIMSCAN assay results (data not shown), a 30-min incubation seems sufficient, avoids excessive background, and lowers the risk of cytotoxic effects of the fluorescein diacetate and eosin Y dyes. The incubation should exceed a minimum 15 min to allow for dye absorption by the cells. The use of large cell numbers per well can require increased fluorescein diacetate concentrations, but for the vast majority of experiments, a final fluorescein diacetate concentration of  $8 \mu\text{g}/\text{mL}$  produced excellent results.

To exclude the potential for drugs being tested causing fluorescence quenching of fluorescein diacetate, duplicate plates can be set up to allow the acquisition of data from

one plate on day 0, as we have shown with deferoxamine. One can then measure the fluorescence of cells stained with fluorescein diacetate together with simultaneous exposure to the drug, and the plate can be read immediately, not allowing time for the drug to cause cytotoxicity.

**Table 1. Dynamic range of cytotoxicity assays**

Type of assay	Dynamic range	Ref.
Clonogenic assay in semisolid medium	3 logs	(2, 3, 47)
Dye exclusion assay (trypan blue)	2 logs	(4–6)
MTT colorimetric assay	2 logs	(11–13, 48)
Sulforhodamine-B colorimetric assay	2 logs*	(14)
WST-1 + neutral red + crystal violet assay	2 logs*	(49)
ATP cell viability assay	2 logs*	(50–52)
ChemoSelect test (cytosensor microphysiometer)	2 logs*	(53)
Differentiated staining cytotoxicity assay	2 logs*	(54, 55)
Collagen gel-embedded culture and image analysis	2 logs*	(56, 57)
[ $^3\text{H}$ ]uridine assay	2-3 logs	(10)
Fluorometric microculture cytotoxicity assay	2-3 logs	(58–60)
Flow cytometric quantification of fluorescein diacetate/propidium iodide viable cell number	4 logs	(15)
DIMSCAN assay	4-5 logs	

\*When a linear scale was used for data presentation rather than a common log scale, the dynamic range was assessed as no greater than 2 logs.

The DIMSCAN cytotoxicity assay combines the advantages of the previously described DIMSCAN system (17), with the use of eosin Y to further decrease background fluorescence, providing an effective method for quantifying relative viable cell numbers *in situ* by fluorescence over a linear >4-log dynamic range. The ability to quantify cytotoxicity accurately and rapidly over such a wide dynamic range should present a clear advantage for an assay used in preclinical antitumor drug development (1). We showed that the DIMSCAN system could quantify cytotoxicity for both adherent and suspension cell cultures, and due to the use of 96-well culture plates, it allows processing of large numbers of samples rapidly and conveniently. This assay has already proven useful in showing drug resistance patterns in neuroblastoma (20, 40, 41) and in identifying single agents or synergistic combinations of agents active against cell lines from neuroblastoma (26, 27, 42–45), a variety of solid tumors (27), and leukemias (46).

In the single drug *in vitro* cytotoxicity studies presented here, the DIMSCAN system produced data consistently comparable with results obtained by other methods of *in vitro* cytotoxicity evaluation, such as the MTT assay, colony-forming assay, and trypan blue dye exclusion counting, but with a wider dynamic range. However, it is in the drug combination studies where the wider dynamic range of the DIMSCAN assay provides a distinct advantage, as it enables more readily distinguishing drug synergism from an additive effect. Due to its sensitivity for small numbers of residual viable cells, the DIMSCAN assay may magnify predictions of synergy at the higher ends of dose-response curves. The actual value of such predicted interactions will ultimately need to be established by independent means, such as *in vivo* animal models or ultimately in clinical trials.

Based on preclinical *in vitro* tests done with the DIMSCAN system, both drug combinations presented here (BSO combined with L-PAM and 4-HPR combined with safingol) have been further developed (26, 27). Those drug combinations have moved into clinical trials that would have unlikely been undertaken if relying only on results obtained with the MTT assay. In the future, as data from such clinical trials become available, it will be possible to assess the ability of high-dynamic range cytotoxicity assays, such as DIMSCAN, to predict clinical activity from drug combinations.

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