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A W Krause, W W Carley and W W Webb
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Original Articles

Fluorescent Erythrosin B is Preferable to Trypan Blue as a Vital Exclusion Dye for Mammalian Cells in Monolayer Culture[1]

ANDREW W. KRAUSE,[2] WILLIAM W. CARLEY,[3] and WATT W. WEBB

School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853

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Erythrosin B and trypan blue are tested and compared for their effectiveness as vital exclusion stains for mammalian cells in monolayer culture. Both stains are supposed to mark cells that have lost membrane integrity. Fluorescein diacetate (FDA), an efficient vital inclusion stain, is used as a control, as it marks cells retaining membrane integrity. Erythrosin B and FDA are used as fluorescent dyes, whereas trypan blue colors via light absorption. The effectiveness of both vital exclusion stains is assayed by their ability to stain a high percentage of monolayer cells exposed to treatments lethal to an entire cell population. Two types of lethal treatment, severe heat and metabolic poison, are employed. Erythrosin B stains all monolayer cells immediately after complete lethal treatment. Trypan blue optimally stains only about 60% of monolayer cells. Cell staining by erythrosin B and by FDA are found to be mutually exclusive. This result demonstrates the coincidence of viability indications by erythrosin B and FDA and thus confirms the reliability of both viability stains as they probe membrane permeability via independent mechanisms. This study shows that erythrosin B is an effective, nontoxic, and convenient fluorescent vital exclusion dye for three mammalian cell lines in monolayer culture, but tends to disqualify trypan blue for this application.

KEY WORDS: Vital exclusion dye; Fluorescent erythrosin B; Monolayer cultures.

Introduction

The determination of cell viability after potentially harmful treatments (including radiation, heat, chemical, etc.) is often required in biological studies. The major criteria employed in viability assays include survival and growth in tissue culture, enzyme assays, transplantation potential, metabolite (usually nucleotide) incorporation, structural alteration, and membrane integrity. Typically, viability measurements derived from these different criteria correlate well with one another. However, the particular criteria for determining cell viability should be chosen to fit the needs of each particular study. For instance, a viability test based on membrane integrity judges cells with functionally undamaged membranes to be viable even if they have lost the ability to proliferate. Our objective here is to find suitable vital exclusion dyes for mammalian cells in monolayer culture.

By far the most convenient viability assays involve the determination of membrane integrity via dye exclusion from live cells. A variety of such dyes have been effectively employed, including eosin (6), Congo red (5), erythrosin B (2,9,10), and trypan blue (6,8,10). However, none of these dyes is recommended for use on monolayer cultures but rather they are intended for cells in suspension; thus monolayer cells must first be trypsinized (8–10). Nevertheless, trypan blue has been by far the most popular vital exclusion dye for mammalian cells in monolayer culture as well as in suspension even though limitations have been noted even in suspension (2,4,12,13). Therefore, one purpose of this study is to test trypan blue for vital exclusion staining of cells in monolayer culture.

Vital exclusion staining of cells in monolayer culture is frequently necessary or preferable because 1) it can distinguish the viability of specific cells in situ, and 2) an exclusion stain permits unperturbed continued observation of unstained live cells. Unfortunately the fluorochrome fluorescein diacetate (FDA), which has proven effective in reliably differentiating live from dead cells in monolayer culture, is retained by live cells (1,11). This dye is permeable through the membrane initially, and after being converted to impermeable fluorescein by cell esterases it promptly accumulates in live cells and is retained after washing. The live cells thus fluoresce a bright greenish-yellow when illuminated at 450–480 nm. Cells that have lost membrane integrity promptly lose the dye and fail

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[2]Present address: Department of Biophysics, Johns Hopkins University, Baltimore, Maryland 21218.
[3]Present address: Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510.

to fluoresce. Erythrosin B had previously been used as a fluorescent vital exclusion dye on yeast cells with excellent results (7). In this study, mutual exclusion of FDA and erythrosin B staining in mammalian monolayer cells is tested in order to determine the effectiveness of erythrosin B as well as to confirm the reported reliability of FDA.

Materials and Methods

Cell culture. Three cell lines were used: 1) NRK (normal rat kidney) cells (3), 2) PRC-NRK [Rous sarcoma (Prague C strain) virus transformed NRK] cells (3), and 3) GM3348 cells (a human fibroblast strain) [The Human Genetic Mutant Repository, Camden, NJ]. All cells were grown on 22 × 22 mm glass cover slips. NRK and PRC-NRK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The GM3348 cells were grown in Ham's F-12 medium supplemented with 20% fetal calf serum. All cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. All media were obtained from the Grand Island Biological Co., Grand Island, New York (GIBCO).

Cell treatment. At 30–70% confluence, monolayer cells on cover slips were exposed to a heat or metabolic poison treatment either lethal to the entire cell population (complete lethal treatment) or lethal to a fraction of the entire cell population (incomplete lethal treatment). For complete lethal heat treatment, the cover slips of monolayer cells were dipped in phosphate-buffered saline (PBS) at 60°C for 90 sec followed by dipping in PBS at 0°C for 10 sec. For complete lethal medium treatment, the cells were washed 3 times with PBS and then incubated for 2.5 hr in a humidified 5% CO_2 atmosphere at 37°C in a medium containing 10^{-2} M NaF and 10^{-2} M NaN_3 in 90% PBS (by volume) and 10% distilled water (to roughly preserve the osmolarity).

For incomplete lethal (20–70% viability) heat treatment, the monolayer cells on cover slips were dipped in PBS at 60°C for 5–10 sec followed by dipping in PBS at 0°C for 2–4 sec. Incomplete lethal medium treatment required an exposure to the lethal medium previously described for 50–90 min.

Trypan blue staining. Trypan blue stain was prepared fresh daily as a 0.4% solution in PBS at pH 7.2 according to the method of Phillips (10) except that the methyl-*p*-hydroxybenzoate preservative was not added. For staining, cell monlayers subjected to complete lethal heat or medium treatment were washed three times in PBS. The cells were subsequently incubated in a humified 5% CO_2 atmosphere at 37°C for 0–3 hr. The cells were subsequently incubated with a 1:10 dilution of the 0.4% trypan blue solution in PBS for 5 min and were then washed three times in PBS and observed on a Nikon Optiphot microscope with a 40× power phase-contrast objective. Cells with blue nuclei were counted as stained.

Erythrosin B and FDA stain preparation. Two basic modes of fluorochrome staining were employed. One mode (normal mode) used the minimal concentration of erythrosin B and/or FDA necessary to quickly achieve adequate staining for usual purposes. Cell staining counts were done only on cell monolayers stained in this mode. The second mode (photographic mode) involved using much higher dye concentrations than normally necessary so that good photographs could be taken in a minimum amount of time. This was necessary because low concentrations of erythrosin B and, especially, FDA, although easily visible, are difficult to photograph due to photobleaching.

Erythrosin B and FDA were obtained from the Sigma Chemical Company (St. Louis, MO). For the normal mode of staining, FDA was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml. It was then diluted 1:200 in DMEM. Erythrosin B was used at 5 μg/ml in DMEM for the normal staining mode. Only 1 min was required for staining with either stain solution.

For the photographic staining mode, erythrosin B was used at a concentration of 30 μg/ml in DMEM, while FDA was used at the same concentration as for normal mode staining. Only 2 min were required for staining suitable for rapid photography. All stain solutions were made at pH 7.2.

Erythrosin B and FDA staining technique, observation, and general staining count procedure. After complete lethal treatment, cell monolayers were stained by erythrosin B. Three staining procedures were employed after incomplete lethal treatment. After either type of lethal treatment, the cell monolayers on cover slips were washed three times in PBS. The cell monolayers, in petri (35 mm diameter) dishes, were either 1) stained with a 50:50 (v:v) mixture of each stain (for 2 min in either the normal or photographic mode), 2) stained first with erythrosin B followed by FDA, or 3) stained first with FDA followed by erythrosin B. After the stain mixture, for procedure 1, or after each successive stain in procedures 2 and 3, was applied, the cell monolayers were washed three times with PBS. A stained cover slip was then left immersed in PBS in its petri dish (cells facing up).

Sequential staining was necessary in order to separate the staining by FDA from erythrosin B. This is due to the fact that erythrosin B apparently has a broad fluorescence emission wavelength range that completely overlaps the emission spectrum of FDA. Therefore, through proper choice of excitation and emission wavelengths, erythrosin B but not FDA fluorescence could be isolated in a field stained by FDA and erythrosin B.

The cell monolayers were observed by both phase-contrast and fluorescence microscopy with the 40× or 100× power phase-contrast objective immersed in the PBS of the petri dish. Only the 40× power phase-contrast objective was used in making staining counts. After any type of staining procedure (including trypan blue staining), all cells of a particular cell field were first counted (usually 20–30 cells) under phase-contrast microscopy before making staining counts for that field.

After concurrent staining with FDA and erythrosin B, a FDA staining count was performed first by counting all cells appearing greenish-yellow through a 515 nm barrier cutoff filter with excitation at 450–480 nm from an Osram HBO/50 mercury lamp (MacBeth Sales, Newburgh, NY). Because erythrosin B fluoresces yellowish-orange under those conditions, it was observed whether or not all cells were stained by either dye by direct comparison with the same cell field viewed under phase-contrast microscopy. An erythrosin B staining count was then made using fluorescent excitation at 535–550 nm and a 580 nm barrier cutoff filter. Under such conditions, erythrosin B staining glows red while FDA fails to fluoresce. These conditions were also used in making erythrosin B staining counts on cell monolayers exposed to complete lethal treatment.

After successive staining, fluorescent staining was observed through the 515 nm barrier cutoff filter with excitation at 450–480 nm. Particular cell fields were observed for each set of successive stainings. It was sufficient to count fluorescent cells as stained by the last stain applied because the fluorescence of the previous stain was bleached out under the same excitation (in about 10–30 sec using normal mode stains and 80–120 sec using stains in the photographic mode).

In all staining counts, no cells were counted as stained if the dye appeared sequestered in small vesicles. After trypan blue or erythrosin B staining of completely lethally treated cell monolayers, or after FDA–erythrosin B concurrent staining of incompletely lethally treated cell monolayers, staining counts were made on 10 cell fields on each of at least 10 cover slips (2000–3000 cells) for each particular combination of cell type and lethal treatment. Our procedure resulted in

Table 1. *Quantitative cell staining results for each staining procedure*[a]

Lethal exposure[b]	Stained with	Number of cells observed				% Cells (± SD) stained by			
		NRK	PRC-NRK	GM 3348	total	TB	EB	FDA	total
C[c]	TB	4224	4982	4292	13,498	58 ± 13	—	—	—
C[c]	EB	4404	4873	4366	13,643	—	100 ± 0	—	—
I[c]	EB + FDA mixture	4414	4801	4171	13,386	—	54 ± 15	48 ± 14	102 ± 1
IH	EB then FDA sequentially	253	287	—	540	—	52 ± 11	54 ± 10	106 ± 4
IH	FDA then EB sequentially	289	350	—	639	—	53 ± 17	55 ± 17	108 ± 4

[a]The excess of total staining over 100% tests the results for mixed and sequential staining by indicating the degree of (contradictory) FDA/erythrosin B staining overlap.
[b]Abbreviations: C, complete lethal treatment; I, incomplete lethal treatment; IH, incomplete lethal heat treatment; TB, trypan blue; EB, erythrosin B.
[c]Data for lethal medium and heat treatment as well as for each cell type are grouped together because they gave no significant difference (less than 1%) in total % staining.

12,000–14,000 cells being counted for each cell type or staining procedure (see Table 1). However, following the tedious successive staining procedure (either staining order), staining counts were made for only one cell field on each of 10 cover slips (about 200–400 cells) for NRK and PRC-NRK cells exposed to incomplete lethal heat treatment. The percentage of all cells in a particular field stained by a certain stain was determined. Each staining percentage for a particular stain and cell field was averaged with those of every other cell field of every cover slip in the same category with respect to cell type, staining procedure, and lethal treatment (heat or metabolic poison). The standard deviation in each average staining percentage was then determined among all the cell fields examined in a particular category.

The procedure for taking photographs was analogous to the staining count procedure in that photographs were taken at points in the procedures above that originally suggested for staining counts. Images were photographed directly onto Kodak Ektachrome 400 ASA color slide film. The film was pushed to 3200 ASA in the developing process. Black and white negatives were made after photographing the color slides with Kodak Tri-X film so that black and white prints could be made for publication.

Results

Trypan Blue Staining of Monolayer Cells

Monolayer NRK, PRC-NRK, and GM3348 cells exposed to completely lethal heat or medium treatment stained poorly with trypan blue. It was often difficult to distinguish the blue-stained nuclei from unstained nuclei. Furthermore, it required from 50 min to 1.5 hr after lethal treatment for the best staining results (60–70%) to occur. The best results are given in Table 1 where the delay between lethal treatment and stain application was 1.5 hr. Nevertheless, on the average, only about 60% of the dead cells were stained. Results varied greatly from trial to trial and appeared to be independent of the cell type and treatment. The photographs in Figure 1 for NRK cells killed by heat or lethal treatment are examples of the best trypan blue staining that was observed (72% of the heat-killed cells and 70% of the lethal medium-killed cells were stained). No relationship between any trypan blue fluorescence and viability was observed (results not shown).

Erythrosin B and FDA Staining after Complete Lethal Treatment

Erythrosin B stained 100% of the cells within 1 min immediately after either complete lethal treatment (see Table 1). The result was independent of cell type or staining mode. Such staining is depicted in Figure 2 where NRK monolayers were completely heat killed (Figure 2a,b) and PRC-NRK monolayers were completely killed by 2.5 hr of lethal medium exposure

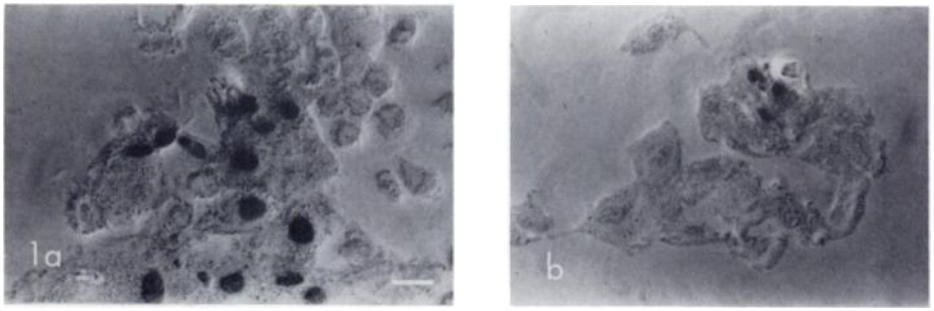

Figure 1. NRK monolayers exposed to complete lethal treatments were stained with trypan blue. Phase-contrast photomicrographs show NRK cells that were all killed by heat treatment (a) or metabolic poisons (b) and stained with trypan blue. Approximately 72% of the heat-killed cells and 70% of the metabolic poison-killed cells were stained. Cells were cultured, lethally treated, stained, and photographed as described under Materials and Methods. Bar = 25 μm.

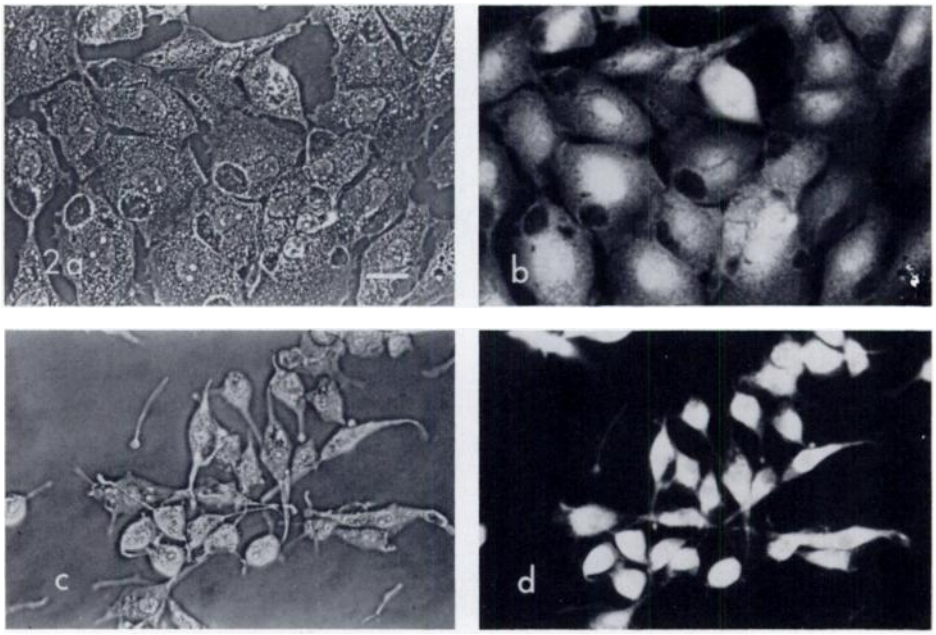

Figure 2. NRK and PRC-NRK monolayers exposed to complete lethal treatments were stained with erythrosin B. NRK cells were exposed to lethal heat treatment (a and b) and PRC-NRK cells were metabolically poisoned (c and d). In both cases, erythrosin B fluorescently stained 100% of the dead cells (b and d), as evidenced by comparison with the respective phase-contrast photomicrographs (a and c). Cells were cultured, lethally treated, stained, and photographed as described under Materials and Methods. Bar = 25 μm.

(Figure 2c,d). By direct comparison of the phase-contrast photomicrographs in Figure 2a and c with their fluorescently stained counterparts in Figure 2b and d, all cells appear stained. Staining the same monolayers with FDA, after photobleaching erythrosin B fluorescence, yielded no fluorescence and no photographic exposures.

Erythrosin B and FDA Staining after Incomplete Lethal Treatment

When any of the three live cell types after incomplete lethal treatment were stained with the 50:50 mixture of erythrosin B and FDA stain (at concentrations for either the normal or photographic mode), all cells appeared stained on illumination at 450–480 nm and observation through the 515 nm barrier cutoff filter. Furthermore, under these conditions, the yellowish-orange fluorescence of erythrosin B-stained cells could easily be distinguished from the bright greenish-yellow fluorescence of FDA-stained live cells. FDA-stained cells could also be distinguished because they photobleached much faster than the erythrosin B-stained cells. The red erythrosin B fluorescence observed through a 580 nm barrier cutoff filter with illumination at 535–550 nm coincided only with the cells (dead) previously seen above to fluoresce yellowish-orange. Typical observations are displayed in Figure 3. Figure 3a–c shows a particular field of a NRK monolayer exposed to incomplete lethal medium treatment, whereas Figure 3d–f shows a particular field of a PRC-NRK monolayer exposed to incomplete lethal heat treatment. When the phase-contrast photomicrographs in Figure 3a and d are superimposed on their stained counterparts (FDA and erythrosin B) in Figure 3b and e (fluorescence induced by 450–480 nm illumination), respectively, all cells appear stained. However, when they are superimposed on their counterparts where only erythrosin B fluorescence can be excited in Figure 3c and f (illuminated at 535–550 nm), respectively, only the red fluorescence of erythrosin B can be observed in a fraction of the cells. These selective observations of erythrosin B fluorescence suggest that erythrosin B does not significantly enter FDA-stained cells.

All cells in every field observed immediately after staining (and washing) in the normal and photographic mode were stained by at least one of the two dyes. However, if more than about 3 or 4 min elapsed between staining and observation, increasing numbers of cells failed to fluoresce. If the same monolayer was restained with erythrosin B and observed immediately, all cells again appeared stained. These results suggest that cells die and lose their FDA in the interim between staining and observation. Therefore, speed was required in the procedures in order to minimize the effects caused by continuous cell death in monolayers exposed to incomplete lethal treatment. The cell staining count results (see Table 1) demonstrated that, even though the percentage of cells stained by FDA or erythrosin B varied considerably from field to field (large standard deviation), the percentage of FDA-stained cells plus the percentage of erythrosin B-stained cells for each cell field (total staining) remained relatively constant (low standard deviation) at almost 100%. As shown in the last column of Table 1, the total staining exceeded 100% by less than 1–3%, indicating that only about 2–6% of FDA-stained cells were also significantly stained with erythrosin B. However, to make sure that fluorescein hydrolyzed from FDA does not significantly accumulate in cells stained by erythrosin B, sequential staining was used to determine the amount of FDA/erythrosin B staining overlap.

To minimize adverse photochemical effects, it was sufficient to stain quickly, photobleach, and observe each cell field using fluorescence excitation illumination as low in intensity

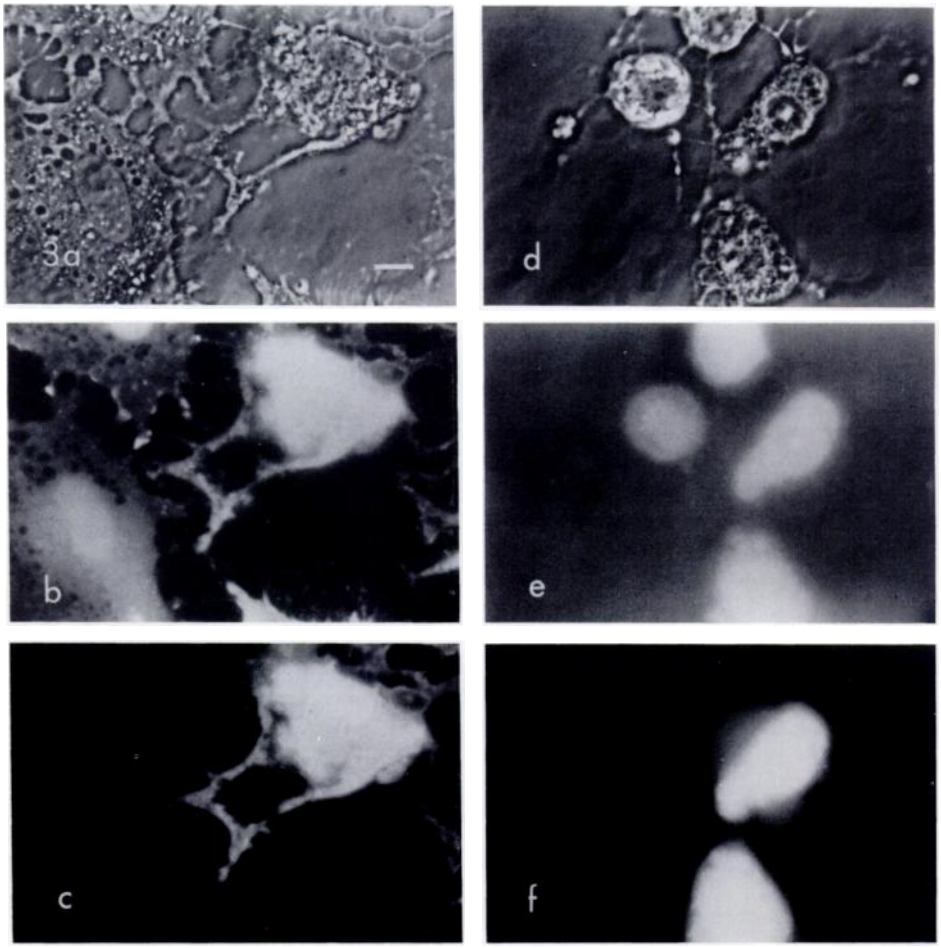

Figure 3. NRK and PRC-NRK monolayers exposed to incomplete lethal treatments were stained with a 50:50 (v:v) mixture of erythrosin B and FDA stains. A particular field of a NRK monolayer (a–c) and a PRC-NRK monolayer (d–f) incompletely killed by metabolic poisons and heat treatment, respectively, are shown. With fluorescence excitation at 450–480 nm observed through a 515 nm barrier filter, all cells appear stained by the FDA and erythrosin B mixture (b and e) when compared to the corresponding phase-contrast photomicrographs (a and d). Fluorescence excitation at 535–550 nm using a 580 nm barrier filter reveals only erythrosin B staining (c and f) in a fraction of all the cells seen in the respective phase-contrast photomicrographs (a and d). Cells were cultured, lethally treated, stained, and photographed as described under Materials and Methods. Bar = 10 μm.

as possible. Because the results of the concurrent staining experiments conclusively demonstrated that all monolayer cells, whether dead or alive, become stained either by FDA and/or erythrosin B, photochemical effects in sequential staining counts were controlled by observing that this same total staining result be retained.

After following all precautions, the results obtained (see Table 1) demonstrated that erythrosin B- and FDA-stained cells were mutually exclusive to a very high degree of reliability (about 93%). The staining overlap (defined as total staining over 100% when all cells were stained by FDA and/or erythrosin B) was only about 7%. The standard deviation in total staining (4%) was higher than that observed for concurrent staining, and probably reflects the limitations of the sequential staining procedure.

Photographs demonstrating mutual exclusion of the two dyes were difficult to obtain because the conditions necessary to obtain them aggravated the limitations of the procedure, i.e., long photographic exposure and photobleaching times and higher fluorescence illumination intensity. Nevertheless, good representative photographs were obtained. Figure 4 shows such observations for both staining orders. Figure 4a–c shows a particular field of a NRK monolayer exposed to incomplete lethal heat treatment, whereas Figure 4d–f shows a particular field of a PRC-NRK monolayer exposed to incomplete lethal medium exposure. Figure 4a and d are simply the phase-contrast photomicrographs for each respective cell field. The NRK cells were stained first with FDA (Figure 4b), photobleached, and then stained by erythrosin B (Figure 4c). Alternatively, PRC-NRK cells were first stained with erythrosin B (Figure 4e), photobleached, and then stained by FDA (Figure 4f). By comparing the appropriate corresponding photomicrographs, it is apprent that FDA and erythrosin B staining are mutually exclusive.

Discussion

The efficacy of erythrosin B fluorescent staining for detecting mammalian monolayer cells (NRK, PRC-NRK, and GM3348) that have lost membrane integrity has been demonstrated. First, erythrosin B stains all the cells of monolayers exposed to a complete lethal treatment. Secondly, in cell monolayers exposed to incomplete lethal treatment, erythrosin B stains cells that have lost membrane integrity, as indicated by the lack of fluorescein accumulation in live cells via FDA hydrolysis. Both mixed staining using the ability to discern erythrosin

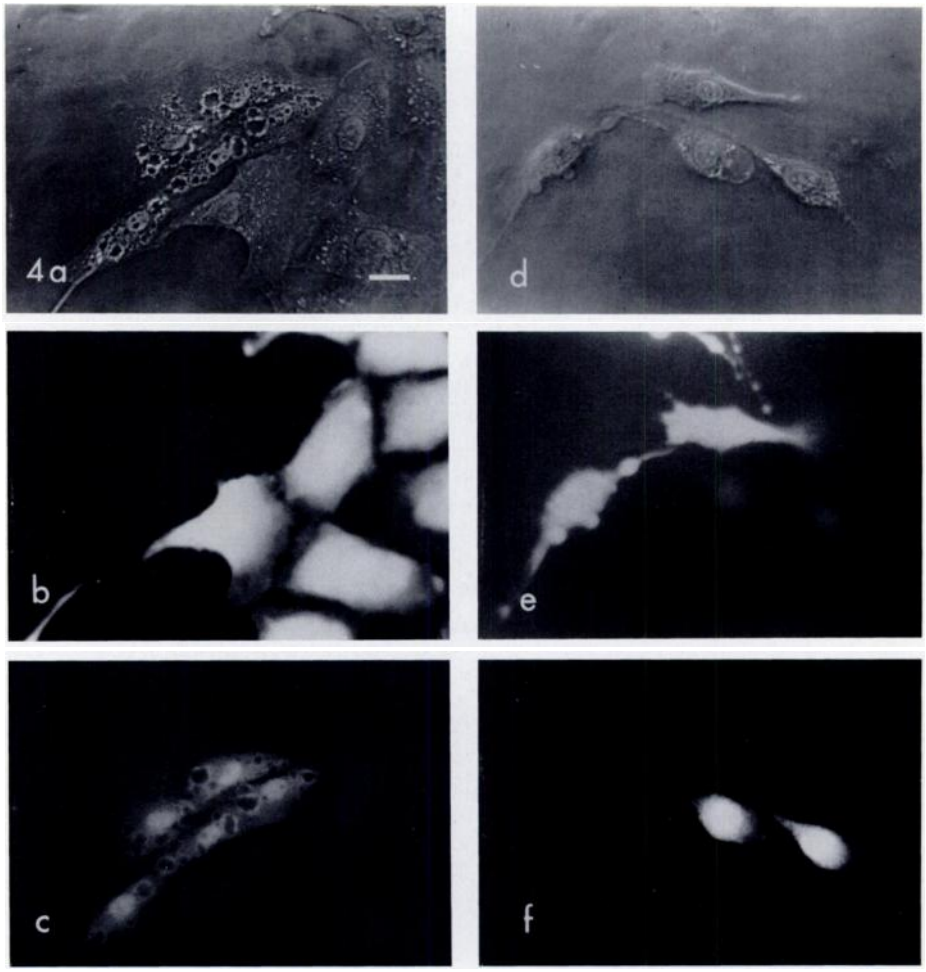

Figure 4. NRK and PRC-NRK monolayers exposed to incomplete lethal treatments were sequentially stained with erythrosin B and FDA. A particular field of a NRK monolayer (a–c) and a PRC-NRK monolayer (d–f) incompletely killed by heat treatment and metabolic poison, respectively, are shown. NRK cells stained first with FDA (b) followed by erythrosin B (c) and PRC-NRK cells stained first with erythrosin B (e) followed by FDA (f) reveal that FDA and erythrosin B staining is mutually exclusive. By comparing the photomicrographs of stained cells (b and c or e and f) with the corresponding phase-contrast photomicrographs (a or d), it is also apparent that all the cells in a particular field become stained by either erythrosin B or FDA. Cells were cultured, lethally treated, stained, and photographed as described under Materials and Methods. Bar = 25 μm.

B from FDA staining by changing the fluorescence excitation wavelength and sequential staining with intervening photobleaching clearly show that FDA-stained monolayer cells are mutually exclusive with those stained by erythrosin B. In fact, from staining count results (see Table 1), the two dyes are, on average, 92–96% mutually exclusive with a small standard deviation of no more than 4%. This result was independent of lethal treatment and cell type. Because erythrosin B and FDA staining is accomplished via different independent mechanisms, these results also give further credence to FDA as a vital inclusion dye.

Trypan blue staining (for visible-light microscopy) is found to be a poor measure of membrane integrity for mammalian cells in monolayer culture where only about 60% of monolayer cells exposed to complete lethal treatment could be stained. Furthermore, optimal staining requires a period of at least 50 min between lethal treatment and stain application. Other studies had found that trypan blue is an unreliable vital exclusion stain for mammalian cells in suspension (2,4,12,13).

We also tried erythrosin B as a red vital exclusion stain for visible-light microscopy on monolayer mammalian cells exposed to complete and incomplete lethal heat or metabolic poison treatment. Monolayer cells were stained for 5 min using the same concentration (0.4 mg/ml) as recommended by Phillips (10) for cells in suspension. However, the color contrast between stained and unstained cells was usually too weak to be reliable. Increasing erythrosin B concentration or staining times did not significantly improve the results.

The question of whether staining effectiveness depends on the type of lethal treatment is also of concern. Two types of lethal treatments, heat and metabolic poison, were used in this study. The heat treatment directly damages the plasma membrane, often causing loss of membrane integrity resulting in cell death. Alternatively, metabolic poison treatment kills the cell from within, ultimately resulting in the loss of membrane integrity. The effectiveness of erythrosin B vital exclusion staining was confirmed with both lethal treatments, suggesting that it is not dependent on the basic cause of cell death. We have also found that fluorescent erythrosin B is an effective vital exclusion stain for cell monolayers exposed to intense electric field or osmotic gradients [unpublished results].

Although erythrosin B does appear to be an effective vital exclusion stain, it may be prudent in some circumstances to employ a spectrum of viability assays, perhaps including ra-

dioactive nucleotide incorporation (4) and measures of continuation of growth in cell culture. The use of erythrosin B as a fluorescent vital exclusion stain to test membrane integrity has significant advantages over other viability assays. It is convenient to prepare and use, is water soluble, and does not stain serum proteins (8) as does trypan blue. We found erythrosin B to be nontoxic to the extent that cells incubated with erythrosin B for over 2 hr will continue to grow further in culture and will stain with FDA. Apparently, erythrosin B does not readily cross the cell plasma membrane because its pK_a is approximately 4 (14), which means it retains a negative charge at pH 7.2. As a fluorophore it can be used effectively at a relatively low concentration (5 μg/ml) compared to trypan blue (400 μg/ml) and other visible-light vital exclusion stains (5,6). Most importantly, erythrosin B staining is completed very quickly, within 1 min after a potentially lethal exposure. For many purposes, the essential advantage of erythrosin B over FDA is that it is an exclusion stain. This property permits the observation of live cells unobscured by a fluorescent substance.

Acknowledgments

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