

Improved FACS-Gal: Flow Cytometric Analysis and Sorting of Viable Eukaryotic Cells Expressing Reporter Gene Constructs¹

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The previously reported FACS-Gal assay (Nolan et al., Proc Natl Acad Sci USA 85:2603-2607, 1988) measures *E. coli lacZ*-encoded β -galactosidase activity in individual viable eukaryotic cells for a variety of molecular and cellular biological applications. Enzyme activity is measured by flow cytometry, using a fluorogenic substrate, which is hydrolyzed and retained intracellularly. In this system, *lacZ* serves both as a reporter gene to quantitate gene expression and as a selectable marker for the fluorescence-activated sorting of cells based on their *lacZ* expression level. This report details the following improvements of the original assay: 1) use of phenylethyl- β -D-thiogalactoside, a competitive inhibitor, to inhibit β -galactosidase activity; 2) reduction of false positives by two-color

measurements; and 3) inhibition of interfering mammalian β -galactosidases by the weak base chloroquine. We found an exponential relationship between fluorescence generated by β -galactosidase in this assay and the intracellular concentration of β -galactosidase molecules. Finally, we report conditions for optimal loading of the substrate (FDG) and retention of the product, fluorescein. Under these conditions, we found uniform loading of FDG in all cells of a clone in individual experiments. Together, these improvements make FACS-Gal an extremely powerful tool for investigation of gene expression in eukaryotic cells.

Key terms: Selectable marker, β -galactosidase, fluorescence-activated cell sorting

INTRODUCTION

We have previously reported the development of FACS-Gal (8), an enzyme assay of β -galactosidase (β -gal) activity encoded by the reporter gene *E. coli lacZ*. This system uses the fluorescence-activated cell sorter (FACS) to measure β -gal activity in large numbers of individual viable eukaryotic cells and can isolate viable cells based on their level of β -gal activity. FACS-Gal thus combines a reporter gene with a selectable marker, two independent tools commonly used in studies of eukaryotic transcriptional regulation.

Selectable markers are genes that allow the selection of infrequent cells expressing a particular phenotype encoded by that gene and are commonly used to isolate cells in which a different recombinant gene of interest (linked to the selectable marker) has integrated. Often the recombinant gene of interest is a reporter gene whose protein product has an enzyme activity not nor-

mally expressed by the cell and which is easily, sensitively, and quantitatively assayed. Reporter genes allow the precise functional analysis of specific regulatory DNA sequences by isolating these regulatory sequences from their normal genetic context and assaying their ability to control reporter gene transcription. The *lacZ* reporter gene can also be used to analyze the expression of endogenous genes *in situ* with gene fusions in which the reporter gene is introduced into an endogenous locus that controls its expression (6).

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Besides combining these independent tools, reporter genes and selectable markers, FACS-Gal has unique capabilities as a reporter gene assay. Since FACS-Gal measures β -gal activity in large numbers of individual cells, the *distribution* of activity within a population of cells is determined. This information can be used to analyze variation of gene expression within complex or clonal cell populations and to correlate such variation with physiological parameters such as cell cycle position (3) or expression of surface markers (6). Other common reporter gene systems, such as chloramphenicol acetyltransferase (4) or luciferase (2), which measure the activity of the reporter gene in lysates of cell populations, can provide information only on the mean expression level in the population. They provide no information on the distribution of expression within the population and it is impossible to correlate reporter gene expression with other parameters on a cell-by-cell basis. FACS-Gal is also unique among reporter gene systems in maintaining cell viability and therefore allowing further analysis and growth of the assayed cells. In addition, FACS-Gal is an extremely sensitive assay allowing convenient detection of very low levels of β -gal activity, with a wide dynamic range of <5 to $>50,000$ enzyme molecules per cell.

Selection by FACS-Gal also has advantages over standard drug-based selectable markers. Generally, selectable marker systems rely on a combination of toxic compounds and genes that encode resistance or sensitivity to the toxic compound. These systems work by killing or halting the growth of all cells that do not (or do) produce some threshold amount of the protein encoded by the selectable marker gene. Some of the disadvantages of such a survival-based selection system are that 1) the selection system is inherently toxic, often even to cells expressing the resistance gene; 2) the selection generally takes weeks; 3) the cells below (or above) the threshold amount of the selection protein are dead and unavailable for further study; 4) it is difficult or impossible to select for different levels of selectable marker expression; and 5) the cells must be able to reproduce in culture. FACS-Gal uses the sorting capabilities of the FACS to isolate cells based on virtually any level of expression of nontoxic β -gal. The FACS can analyze and sort from as many as 5,000 cells per second (9), so one cell in 10 million can be selected in an hour of sorting, making FACS-Gal as efficient as drug-based selectable markers, but considerably faster.

With a combination of technical abilities that are unique among both reporter genes and selectable markers, FACS-Gal creates new experimental possibilities such as those discussed above for the study of eukaryotic gene expression. In this report we examine various aspects of the FACS-Gal assay, such as parameters of substrate loading and the correlation with other β -gal assays, and introduce new techniques and information that improve and extend the capabilities of this assay.

MATERIALS AND METHODS

Cells and Cell Culture

BW5147 mouse T lymphocytes and Jurkat human T lymphocytes were maintained in RPMI. Mouse NIH3T3 fibroblasts or 293 human embryonic kidney cells were maintained in DMEM. All media were supplemented with 10% fetal calf serum, 108 U/ml penicillin, and 52 U/ml streptomycin, and all cells were maintained in an atmosphere of 7%CO₂/93%air. The cell lines with integrated *lacZ* used in these studies were mouse BW5147 T lymphocytes transduced with retroviral DNA constructs that express *lacZ* either from the SV-40 early region (8) or from the Moloney leukemia virus LTR (12) or Jurkat human T cells transfected with a construct that expresses *lacZ* from a modified interleukin-2 enhancer (3).

Chemicals

Fluorescein di- β -D-galactoside (FDG) was generously supplied by Molecular Probes (Eugene, OR) and stored frozen as a 2 mM stock in 98% H₂O, 1% dimethyl sulfoxide (DMSO), 1% ethanol. Phenylethyl- β -D-thiogalactoside (Sigma Chemical Co., St. Louis, MO) was stored frozen as a 50 mM stock in water. Chloroquine diphosphate salt (Sigma) was stored frozen as a 30 mM stock in water. 4-Methylumbelliferyl- β -D-galactoside (Sigma) was stored at -20°C as a 30 mM stock in dimethylformamide. All reagents were unaffected by multiple freeze-thaw cycles.

FACS-Gal Assay

The FACS-Gal assays were done basically as described elsewhere (8). Briefly, aliquots of cells to be assayed were suspended in 50 μl of growth medium or deficient RPMI and warmed to 37°C . FDG (2 mM in 98% distilled water) was warmed to 37°C , and 50 μl was added to each 50 μl aliquot of cells. The cells and FDG were rapidly mixed and immediately returned to a 37°C water bath. After incubation at 37°C for 1 min the cells were removed from the water bath and 1 ml or more of ice-cold growth medium or phosphate-buffered saline (PBS) was added to the cells and they were incubated on ice until FACS analysis. The presence of 0.1% sodium azide, which sometimes was added to the diluent, does not affect the assay or cell viability. Analysis was done on a FACSTAR Plus (Becton-Dickinson, San Jose, CA) configured for analysis of fluorescein as described elsewhere (11). The sensitivity of the assay was improved by autofluorescence compensation (1). Multiparameter data were analyzed by using FACS-Desk software (7). Dead cells were excluded from the analysis based on uptake of 1 μM propidium iodide added from a 100 \times stock after the cells were loaded with FDG.

Determination of Intracellular FDG Concentration

The intracellular FDG concentration was estimated as follows: BW 5147.l04 cells (8), which expresses β -gal,

were hypotonically treated as above, but with varying concentrations of FDG. The cells were incubated on ice until all intracellular substrate had been hydrolyzed, as evidenced by a lack of increase of fluorescence. The final median fluorescence was compared with a standard curve of fluorescence per fluorescein molecule derived from FACS measurements on microbeads with known numbers of fluorescein molecules (Flow Cytometry Standards Corporation, Research Triangle park, NC) to determine the number of intracellular fluorescein molecules (and thus the initial number of FDG molecules). This was corrected by a factor of 2, based on a previous report (17) indicating that the intracellular fluorescein fluorescence is 50% of the fluorescence of free fluorescein in solution. The original FDG concentration was then calculated based on an estimated volume of the cell of $520 \mu\text{m}^3$.

4-Methylumbelliferyl- β -D-Galactoside (MUG) In Vitro Assay of β -Gal Activity

The MUG assay is based on the conversion of the nonfluorescent β -gal substrate (MUG) into the fluorescent product 4-methylumbelliferone. The samples were prepared for the assay by suspension of 5×10^3 to 5×10^5 cells in $120 \mu\text{l}$ of Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , pH 7.0) containing 0.1% Triton X-100 (Sigma). The assay is started by the addition of $30 \mu\text{l}$ 3 mM MUG (diluted 1:10 in Z buffer from a 30 mM stock in dimethylformamide just prior to addition), which brings the reaction to 0.6 mM MUG. After between 5 min and 6 h of incubation at 37°C (depending on the β -gal activity of the cells), the assay was stopped by the addition of $75 \mu\text{l}$ of MUG stop buffer (15 mM EDTA, 300 mM glycine, pH 11.2). 4-Methylumbelliferone fluorescence was quantified in a 96-well tissue culture plate by a Titertek Fluoroskan II (Flow Labs, McLean, VA) 96-well plate fluorometer after blanking the machine on an assay done on an equal number of β -gal-nonexpressing cells. With this protocol, as long as the substrate is not depleted by the reaction, the 4-methylumbelliferone fluorescence is linearly related to both the β -gal enzyme concentration and duration of the assay and is insensitive to small initial MUG concentration differences (the K_m of β -gal for MUG is $120 \mu\text{M}$; data not shown).

RESULTS

Influence of Substrate Loading Parameters on the Assay

The basis of the FACS-Gal assay is the hydrolysis of the fluorogenic substrate FDG to the fluorescent product fluorescein by β -gal (15). The precision and accuracy of *in vivo* single cell measurements of β -gal activity by FACS-Gal is dependent on loading cells quickly and uniformly with FDG without impairing cell viability. These requirements are adequately fulfilled by exposing the cells briefly to a hypotonic solution of FDG (8). This is done by mixing cells in growth medium or PBS at 37°C with an equal volume of 2 mM FDG in

water at 37°C . This procedure mediates rapid and uniform uptake of FDG. Following the hypotonic treatment, the cells are brought back to isotonicity by the addition of at least $10 \times$ volume of ice-cold medium or PBS. Chilling the cells prevents the substrate, FDG, and the product, fluorescein, from leaking out of the cells. None of the cell types we have worked with showed any loss of viability after a brief (up to 3 min) hypotonic treatment as described here. To define better this critical aspect of the assay, we have examined the influence of the following hypotonic treatment variables on the loading of FDG and viability of the cells: duration, tonicity, and cell density.

A 60 s hypotonic treatment adequately loads most cell types so that β -gal-expressing (β -gal⁺) cells that have converted all their intracellular FDG to fluorescein are well resolved from β -gal-nonexpressing (β -gal⁻) cells. However, we have noted that different cell lines vary in their rate of FDG uptake during the hypotonic treatment (data not shown). The relationship between duration of the hypotonic treatment and uptake of FDG was defined by using a cell line with uniform high β -gal activity (Fig. 1A). Following varying durations of the hypotonic treatment, isotonicity was restored and the cells were assayed after being incubated long enough on ice to convert all the intracellular FDG into fluorescein (30 min). Therefore, the final fluorescence is related only to the quantity of FDG introduced into the cell during the hypotonic treatment and allows quantitative analysis of parameters influencing the loading of FDG. Following a 15 s lag before loading commences, FDG enters the cell at a linear rate for up to 2 min, at which time the rate of loading begins to slow. Thus, by varying the duration of the hypotonic treatment, the amount of substrate taken up can be varied.

The tonicity of the medium during the substrate loading is another variable that affects the rate of FDG uptake into cells. The rate of uptake is inversely correlated to the percent of isotonicity during loading (Fig. 1B). Therefore, FDG can be loaded into the cells with a higher tonicity treatment (still below isotonicity) if a cell type is found to be harmed by the 50% isotonicity treatment used here. However, raising the tonicity of the loading reduces the rate at which FDG enters the cell and therefore, a longer hypotonic treatment is needed to introduce a particular concentration of FDG into the cells.

Increasing the duration of the hypotonic treatment can cause a problem when the cells have a high level of β -gal activity. During the hypotonic treatment, until the cells are returned to isotonicity and chilled, the cells are permeable to both the substrate, FDG, and the product, fluorescein. Fluorescein generated in β -gal⁺ cells during the hypotonic treatment leaks out of these cells and is taken up by other cells, reducing the separation between β -gal⁺ and β -gal⁻ cells. Problems caused by the leakage of fluorescein from β -gal⁺ cells during the loading are related not only to the duration

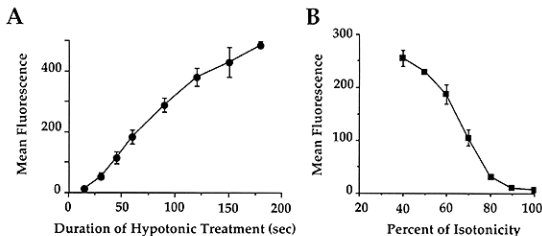


FIG. 1. Duration and tonicity of the hypotonic treatment influence the amount of FDG introduced into the cell. **A:** β -gal-expressing BW 5147.56 cells (8) were hypotonically treated for varying periods with 1 mM FDG in 37°C medium with a tonicity of 50% isotonic. After the hypotonic treatment, the samples were incubated on ice until all intracellular FDG had been hydrolyzed (30 min), and then the samples were analyzed by FACS. The geometric mean of the fluorescein flu-

orescence is plotted against the duration of the hypotonic treatment. **B:** BW5147.56 cells were hypotonically treated for 60 s with 1 mM FDG in media of varying tonicity as displayed. Cells were incubated as above until all intracellular FDG was hydrolyzed and then FACS analyzed. In both A and B, the values displayed are the average of three trials. Error bars indicate ± 1 S.D.

of the hypotonic treatment and the amount of β -gal activity in the cells but also to the concentration of the cells during the loading. We have found that, with respect to adequately introducing FDG into the cells, the basic hypotonic loading procedure works identically for virtually any concentration of cells (data not shown). However, when assaying cells that are high in β -gal activity (greater than about 2,000 molecules of enzyme per cell), the cell density must be kept low enough so that a significant concentration of fluorescein does not accumulate in the medium (and thus in β -gal⁻ cells) during the hypotonic treatment (less than about 10⁶ cells/ml during loading).

The amount of FDG introduced into the cells is proportional to the concentration of FDG in the hypotonic treatment (data not shown). We examined the rate of fluorescence increase as a function of the intracellular concentration of FDG in a clone of β -gal⁺ cells, and we find that these two parameters are linearly related (Fig. 2). The reaction rate is directly dependent on the concentration of substrate in these cells across a 16-fold range of FDG concentrations around the concentration loaded by the standard hypotonic treatment (5 μ M). For each FDG concentration, the increase of fluorescence was linear with time for all the data points taken in this experiment (data not shown). This extends previous results showing that, when cells were loaded with FDG by the standard loading technique, the increase of fluorescence proceeds linearly with time until the substrate concentration is markedly reduced by the reaction (8).

Our finding that the reaction rate is dependent on substrate concentration raises the possibility that variation of intracellular FDG concentrations affects the apparent variation of β -gal activity within a population of cells. Although different cell types do vary in the

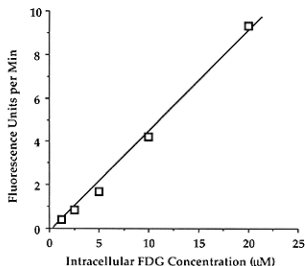


FIG. 2. Rate of fluorescence increase is linearly related to the intracellular concentration of FDG. BW5147.104 cells (8) with a low level of β -gal activity were loaded with FDG by hypotonic treatment of varying durations and FDG concentrations to produce a range of intracellular FDG concentrations. Hydrolysis of FDG was stopped with PETG in aliquots of cells at 2, 6, 19, 54, and 90 min after substrate loading and then all samples were FACS analyzed. The increase of median fluorescence per unit time was linear for all samples in this time range. The graph displays the increase in fluorescence units of the median per min plotted against the estimated intracellular concentration of FDG (see Materials and Methods). We estimate that BW5147 cells loaded by the standard hypotonic loading (60 s, 50% of isotonicity, 1 mM FDG) have an intracellular FDG concentration of 5 μ M. Irrespective of the accuracy of the concentration measurement, the linear dependence of the reaction rate on FDG concentration over this concentration range demonstrates that the intracellular concentration of FDG is still below its apparent K_m for β -gal.

concentration of FDG introduced by the hypotonic treatment (and therefore comparison of FACS-Gal measurements of different cell types should take this into account), the experiments shown in Figure 3 indi-

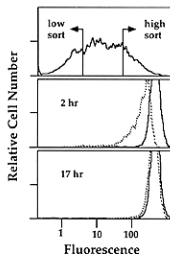


FIG. 3. The hypotonic treatment introduces uniform concentrations of FDG into a population of cells. BW5147.28 *lacZ*⁺ cells (8) were loaded with FDG, and, immediately after loading, 100,000 of the 25% most fluorescent and 25% least fluorescent cells were sorted into ice-cold media. The cells were kept on ice and reanalyzed periodically. The top panel shows the fluorescence distribution of the sorted populations 2 h after substrate loading. The middle panel shows the fluorescence distribution of the sorted populations 2 h after loading (low sort is the broken line and high sort is the solid line). With enough time to hydrolyze the substrate, the two histograms are almost identical, demonstrating that the intracellular FDG concentration is uniform and does not contribute to the observed differences in measured β -gal activity.

cate that cells of a given type that are loaded in the same hypotonic treatment are virtually identical in intracellular FDG concentration. For this experiment, cells of a *lacZ*⁺ clone were loaded with FDG and then sorted according to fluorescence levels (brightest 25% and dimmest 25%). Following the sort, these populations were kept on ice, and at various times their fluorescence distributions were reanalyzed. If the intracellular FDG concentration differed significantly among the sorted cells we would have seen significant differences in their levels of fluorescence when all the intracellular FDG was hydrolyzed, indicating that variation in intracellular substrate concentration was systematically influencing the rate of FDG cleavage. The reanalysis revealed that initially the median of the two populations differed approximately two fold in fluorescence. However, this was due to the fact that the cells with less β -gal activity were hydrolyzing the FDG more slowly, since, when assayed after 17 h, all sorted populations had almost identical fluorescence distributions (means within 25% of each other, Fig. 3). Further evidence that the hypotonic loading introduces uniform concentrations of FDG into a population of cells comes from noting that the distribution of fluorescence following complete hydrolysis of intracellular FDG was very narrow, with 90% of the cells found within a three-fold range of fluorescence. This experiment also demonstrates that the leakage of either fluorescein or FDG from cells incubated on ice is negligible and that cells

with low levels of β -gal activity slowly increase in fluorescence over many hours.

Use of Phenylethyl- β -D-Thiogalactoside (PETG), a Competitive Inhibitor of β -Gal Enzyme Activity

Standard biochemical assay of enzyme activity requires that the reaction be stopped before the substrate approaches exhaustion and that all samples in an experiment are allowed to react for known lengths of time. To satisfy these requirements conveniently, we sought a competitive inhibitor of β -gal to slow or stop the reaction. We utilized a previously characterized compound (13), PETG. PETG has the following properties that make it an ideal β -gal inhibitor for use in this assay: It is a competitive inhibitor of *lacZ*-encoded β -gal; it is sufficiently hydrophobic to enter cells rapidly even after the cells have been chilled to 0°C but is sufficiently hydrophilic to be soluble and stable in aqueous solution; it has a thiol linkage, making it non-hydrolyzable by β -gal and thus remain at constant concentration; it is a reversible inhibitor and comes out of the cells at room temperature or above to allow β -gal activity to proceed (surprisingly, it will not wash out of cells kept at 0°C, data not shown); and it is nontoxic, readily available, and inexpensive. The use of PETG improves the convenience and accuracy of the assay and increases the maximum number of β -gal molecules per cell that can be quantitated.

The ability of PETG to enter cells kept on ice and to inhibit completely β -gal activity after the cells are loaded with FDG is demonstrated in Figure 4A. In this experiment a cell line with moderate β -gal activity was loaded with FDG and assayed by FACS at various times after loading. At the indicated time points during the assay, aliquots of the cells being assayed were removed and PETG was added to 1 mM. At the final time point, 2 h after loading the cells, all the PETG-treated samples were assayed and showed a fluorescence distribution that, compared with the distribution of the samples assayed earlier, indicates that no fluorescence was generated after the PETG was added. Therefore, when 1 mM PETG is applied externally to cells kept on ice, the concentration that rapidly enters the cells is sufficient to stop immediately hydrolysis of FDG by β -gal.

When cells with very high levels of β -gal activity are assayed, accurate β -gal measurements are impossible because the cells have hydrolyzed all the available substrate during the 1 min loading time. This problem can be surmounted by using concentrations of PETG that slow but do not completely inhibit β -gal activity. For the experiment shown in Figure 4B, cells were loaded with FDG in the presence of varying concentrations of PETG, and after loading, the cells were diluted with cold isotonic medium that contained the same varying PETG concentrations. The cells were kept on ice and assayed 30 min after loading. These cells had sufficient

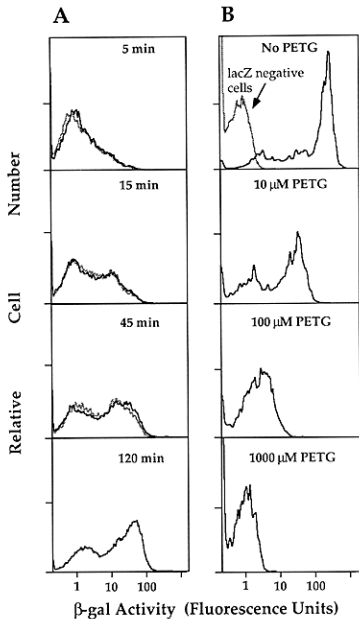


FIG. 4. PETG can be used to stop or slow the hydrolysis of FDG by β -gal. A: Jurkat NFATZ human T lymphocytes with an inducible *lacZ* construct were stimulated to produce β -gal by treatment with ionomycin and PMA as described elsewhere (3). A single sample of cells was loaded with FDG and incubated on ice. At the indicated time-points, the cells' fluorescence was assayed on the FACS; simultaneously, an aliquot was removed and PETG was added to 1 mM. At the final time point (120 min), the aliquots previously treated with PETG were assayed. The histograms displayed demonstrate that the PETG-treated aliquots (broken lines) assayed at 120 min after loading, compared with the assays done at the time of PETG addition (solid lines), have not increased in fluorescence since the addition of PETG. B: BW5147.42 *lacZ*⁻ cells (8) were loaded with FDG in the presence of the indicated concentrations of PETG. The hypotonic treatment was terminated by the addition of $10 \times$ ice-cold buffer containing PETG at the same concentrations as were used during the loading. After 30 min of incubation on ice the cells were analyzed by FACS. Displayed are histograms of that analysis. The broken line in the top panel shows the fluorescence of *lacZ*⁻ BW5147 cells loaded with FDG without any PETG.

activity so that during the 30 min incubation without PETG treatment most of the population had accumulated in a narrow fluorescence peak above 100 fluores-

cence units, which is indicative of complete substrate hydrolysis. Thus information about relative β -gal activities of cells in this peak is lost. Using a range of PETG concentrations between 10 μ M (partial inhibition) and 1 mM (complete inhibition) slows the reaction and allows determination of the relative β -gal activities among all the cells in this population. Estimates of relative reaction rates can be made based on a value of 2.5 μ M for the K_i of PETG (data not shown).

Experiments that entail lengthy sorts based on β -gal activity are difficult and potentially inaccurate without PETG, since, unless the cells have exhausted their substrate, they increase in fluorescence during the sort and therefore cells with a given fluorescence level sorted at different times after loading have different levels of β -gal activity. The use of PETG alleviates this problem. When working with uncharacterized cells, to reveal most effectively maximal information about the distribution of β -gal activity, it is best to PETG stop aliquots of the cells at different times after loading (for example, 2 min, 15 min and 120 min) so that the range of activities can be clearly discerned.

Correlation of FACS-Gal With In Vitro Assays of β -gal

Previous experiments, in which FACS-Gal measurement of β -gal activity of different clones of β -gal expressing cells was compared with in vitro measurement of the β -gal activity of the clones by the colorimetric orthonitrophenylgalactoside (ONPG) β -gal assay, indicated that the β -gal measurements of the two assays were roughly proportional (8). We have repeated these experiments in a more precise manner by using PETG to stop accumulation of fluorescence, sorting cells based on their fluorescein fluorescence, and subsequently assaying the β -gal activity of the sorted cells with the MUG assay. The MUG in vitro β -gal assay is at least 100-fold more sensitive than the previously used ONPG assay (data not shown) and allows measurement of β -gal activity from 5,000 cells or less. These experiments, an example of which is shown (Fig. 5), reveal that the relationship between FACS-Gal in vivo measurement of β -gal activity and in vitro measurement of β -gal activity in extracts of the same cells is not linearly proportional. The exponential relationship is described by the following equation:

$$F = K \times B^{1.8},$$

where F is the fluorescence measured by the FACS-Gal assay, K is a constant of proportionality, and B is the number of β -gal molecules per cell (measured by MUG hydrolysis in extracts). This relationship is not dependent on the cell line used for the study nor on the subcellular localization of β -gal (data not shown).

We used purified β -gal enzyme to construct a standard curve for the MUG assay to enable us to determine the number of β -gal molecules per cell. By carrying out experiments as shown in Figure 5 and correlating fluorescence generated by FACS-Gal with

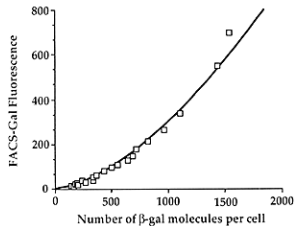


FIG. 5. FACS-Gal fluorescence is nonlinearly related to the number of β -gal molecules per cell. BW5147 clone BV1.24 transduced with a recombinant Moloney retrovirus expressing *lacZ* under the control of the retroviral LTR (12) was used in this experiment. Cells were loaded with FDG by the standard technique, and the enzyme activity was inhibited with PETG after a 5 min incubation on ice. For each data point, 50,000 cells with fluorescence values within a narrow range were sorted into a well of a 96-well tray. The cells were kept on ice during the sort. Following the sort, each sorted population was assayed by MUG for total β -gal activity in the 50,000 cells. In other wells on the same tray, dilutions of purified β -gal enzyme were assayed in parallel to quantitate absolute enzyme levels in the sorted cells. The number of β -gal molecules per cell is plotted against the median fluorescein fluorescence for each sorted population. The line shows a best fit to the data with $[\text{FACS-Gal fluorescence}]$ proportional to $[\beta\text{-gal molecules per cell}]^2$.

MUG-derived β -gal activity per sorted cell, we have found that FACS-Gal can identify cells with fewer than five molecules of β -gal per cell as having β -gal enzymatic activity (data not shown). This is not surprising in light of previous experiments in which single molecules of β -gal in droplets were revealed with FDG (15).

Background Due to Endogenous Mammalian β -Gal Activity

Mammalian cells have endogenous β -gal activity that can hydrolyze FDG. This activity is localized in the lysosomes, and these enzymes have acidic pH optima of approximately lysosomal pH (pH 4–5) (19). In most cell lines that we have studied, the fluorescence due to endogenous β -gal activity is not significant. This is because either the substrate, FDG, is not effectively loaded into the lysosomes by the hypotonic treatment or the total lysosomal β -gal activity is low. Nevertheless, we found that this endogenous β -gal activity can impair measurement of low levels of *lacZ*-encoded β -gal in some cell lines, particularly adherent cell lines. In many cell lines the amount of endogenous β -gal activity is related to the density at which the cells are cultured (Fig. 6). NIH3T3 fibroblasts passaged for 2 weeks at sufficiently high cell density to limit their growth rate have much higher endogenous β -gal activity than the same cell line after being maintained in exponential growth at low cell density. This is undoubtedly due to the fact that confluent NIH3T3 cell cultures have an

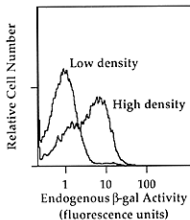


FIG. 6. Cells maintained in exponential growth conditions have less endogenous β -galactosidase activity than cells whose growth is limited by cell density. NIH3T3 mouse fibroblast cells (*lacZ*⁻) were either maintained at low density (5–60% confluent) and in exponential growth phase or at high density (50–100% confluent) in which their growth was generally limited by contact inhibition. After 2 weeks of culturing in these conditions, the cells were harvested, and a FACS-Gal assay was performed. Following a 2 h incubation on ice, the cells were analyzed; the growth-inhibited cultures display far greater β -gal activity.

increased accumulation of hydrolytic compartments compared with nonconfluent cultures (14).

One approach we have explored for inhibiting the endogenous β -gal activity without inhibiting *lacZ*-encoded β -gal is treating the cells with a weak base that concentrates in the lysosomes and raises the lysosomal pH above the optimum for the endogenous β -gal. We have examined a variety of such weak bases and found chloroquine to be the most useful inhibitor of endogenous lysosomal β -gal activity. The effect of chloroquine on fluorescence generated by endogenous β -gal is presented in Figure 7. *lacZ*⁻ cells were incubated for 30 min at 37°C in media containing the noted chloroquine concentrations and then loaded with FDG by the standard hypotonic treatment. The loading was terminated by the addition of ice-cold, isotonic media containing the same chloroquine concentrations as used for the incubations. The increase in mean fluorescein fluorescence per unit time is used as a measure of endogenous β -gal activity. The extent of chloroquine inhibition of endogenous β -gal activity increased with the duration of preincubation in chloroquine up to 20 min of preincubation. Preincubation for more than 20 min did not further increase the inhibition of endogenous β -gal (data not shown). After a 30 min preincubation, chloroquine at a concentration of 300 μ M inhibited endogenous β -galactosidase activity by an average of 75% in both cell lines. Inhibition of endogenous β -galactosidase activity with chloroquine shows variation between experiments, with 300 μ M chloroquine in some experiments giving 95% inhibition and in other experiments giving only 50% inhibition; we have not determined the cause of this variation. Treatment of the cells with 300 μ M chloroquine as described here had no

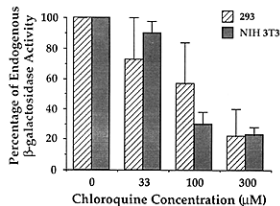


Fig. 7. Chloroquine can inhibit endogenous lysosomal β -gal activity. Cells without *lacZ* (NIH3T3 mouse fibroblasts or 293 human embryonic kidney cells) maintained in standard growth conditions (split 1/10 when 90% confluent) were incubated in growth medium with the indicated concentrations of chloroquine at 37°C for 30 min and then loaded with FDG by hypotonic treatment. The hypotonic treatment was terminated by the addition of 10 \times volume of ice-cold media containing the same chloroquine concentrations as were used for the incubations. The cells were kept on ice, and their fluorescence was assayed by FACS at various time points. Increase in the geometric mean fluorescence per hour was used as a measure of endogenous β -gal activity. Full (100%) endogenous activity was defined by activity of cells with no chloroquine treatment for each experiment, since the fluorescence generated by endogenous β -gal activity in the cells varied between experiments. Results from four independent experiments are displayed with error bars denoting \pm 1 S.D.

discernible toxic effect on the cells, but treatment with 1000 μ M chloroquine was toxic and caused some cell death (data not shown). Importantly, treatment with 300 μ M chloroquine did not inhibit *lacZ*-encoded β -gal activity (data not shown). Thus chloroquine can be used to increase the signal-to-background ratio of the assay when endogenous lysosomal β -gal activity is high.

We have also investigated an alternative loading system in which FDG is introduced into the cells by a 3–5 min incubation at 37°C in isotonic media containing 10 mM adenosine triphosphate (ATP) and 1 mM FDG. This method does load adequate amounts of FDG into cells (although there is a much greater cell-to-cell variation in the amount loaded; data not shown), but it also introduces large amounts of FDG into the lysosomes. Figure 8 shows the distribution of fluorescence for NIH3T3 cells (*lacZ*⁻) that have been loaded with FDG by the ATP technique or by the normal hypotonic loading technique. Figure 8 also shows that the high level of fluorescence caused by ATP-mediated loading is greatly reduced by preincubation in 300 μ M chloroquine. Taken together, these data show that the background of FDG hydrolysis by endogenous β -gal is minimized by the hypotonic loading, which apparently does not introduce a significant amount of FDG into the lysosomes where endogenous β -gal is found.

Rare False-Positive Cells

When the FACS-Gal assay is done on cells without *lacZ*, there is a "background" of rare cells that are

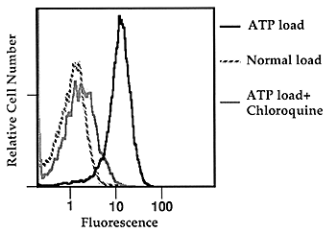


Fig. 8. The hypotonic loading system minimizes the amount of FDG that enters lysosomes compared with ATP-mediated loading. NIH3T3 (*lacZ*⁻) cells were either loaded with FDG by the normal hypotonic treatment (50% isotonic, 1 mM FDG, 1 min), by incubation for 4 min in isotonic media containing 10 mM ATP and 1 mM FDG, or by 4 min incubation in isotonic media with 10 mM ATP and 1 mM FDG after 30 min preincubation in 300 μ M chloroquine as described for Figure 7. After the loading reactions, all samples were chilled by the addition of 10 \times volume of ice-cold isotonic media. The cells were analyzed 15 min after loading, and the fluorescence histograms of those analyses are overlaid.

higher in fluorescence than the rest of the population and appear to be weakly positive for β -gal activity. We refer to these cells as "rare false positives" and generally find them at a frequency of between 0.01% and 1% (Fig. 9, upper panel). The ability of FACS-Gal to select β -gal-expressing cells occurring at a frequency of less than 0.1%, even in the presence of rare false positives, has been demonstrated previously (6,20). However, these rare false-positive cells can reduce the effectiveness of the FACS-Gal assay as a selection system since they reduce the purity when selecting for infrequent positive cells.

We have examined these rare false-positive cells and the conditions under which they arise. The appearance of these cells is dependent on loading the cells with FDG (Fig. 9, compare upper and lower panels). There is no heritable component responsible for the difference between rare false-positive cells and the rest of the population. FACS-sorted "false-positive" cells (from a *lacZ*⁻ population) are viable, and their progeny are not enriched for cells that have this characteristic (data not shown). Culture density during growth often has an influence on the frequency of these cells. As with endogenous activity, maintaining a cell line in exponential growth reduces the frequency of rare false-positive cells in some cell lines but not in all (data not shown). Since we have seen no indication in any cell line that exponential growth can increase either the endogenous β -gal activity or the frequency of rare false-positive cells, it is generally a good practice to maintain cells in exponential growth for FACS-Gal studies.

Two fluorescence characteristics of the rare false positives help to differentiate them from β -gal⁺ cells (Fig. 9): They generally are not as fluorescent as cells

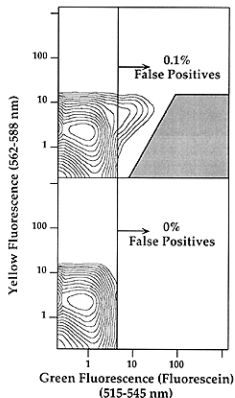


Fig. 9. Rare false-positive cells appear only in cells loaded with FDG, are moderate in fluorescein fluorescence, and are above average in yellow fluorescence. The top panel displays a logarithmic contour plot of cells without *lacZ* (NIH3T3) after a standard hypotonic treatment (60 s, 50% of isotonicity). The bottom panel displays the same type of plot on NIH3T3 cells that were treated identically except that an isotonic FDG solution was used instead of the normal hypotonic FDG solution. The contour plots are 50% logarithmic, a representation that emphasizes areas of the plot with very low cell density, since each line delineates a cell frequency 50% of that marked by the line immediately within it (10). The shaded area depicts the fluorescence levels from which the highest ratio of rare β -gal expressing cells over false positives can be selected.

with enough β -gal activity to have hydrolyzed all of their FDG substrate, and they tend to be bright for emission in the yellow wavelengths (562–588 nm) as well as the fluorescein (green, 515–545 nm) emission wavelengths, whereas the green emission from β -gal⁺ cells is not correlated with emission in the yellow wavelength if the fluorescence compensation is appropriately adjusted for fluorescein. Avoiding cells with low green fluorescence and above-average yellow fluorescence is the best approach we have found to select true β -gal-expressing cells when they are very infrequent in the population.

DISCUSSION

We have examined a variety of characteristics of the previously reported FACS-Gal assay (8). The accuracy and precision of the FACS-Gal assay is dependent on the rapid introduction of uniform concentrations of FDG into all the cells of the sample. The hypotonic treatment accomplishes this, and the parameters that can be adjusted, i.e., tonicity, duration, and cell den-

sity, permit accurate measurements of β -gal activity in any cell line. We find that the rate of fluorescence increase is proportional to the substrate concentration. Thus fluorescence generated by the system is not dependent solely on enzyme concentration. This is not a limitation since the hypotonic loading technique is able to introduce uniform concentrations of substrate into all cells of a given cell type loaded in the same experiment. Precise comparison of the amount of fluorescence generated in cells in the FACS-Gal assay and the number of β -gal molecules per cell reveals that the relationship is nonlinear but well defined. Furthermore, the assay has a broad dynamic range from fewer than five molecules up to almost any number of β -gal molecules per cell.

We introduce the use of inhibitors of either *lacZ*-encoded β -gal or endogenous lysosomal β -gal. The use of PETG to inhibit *lacZ*-encoded β -gal activity increases the measurable range of enzyme activity and the convenience and utility of the assay. PETG provides the convenience of stopping the reaction in a number of samples after a precise incubation time and subsequently analyzing all the samples in one FACS session. In addition, the use of PETG allows lengthy sorts based on β -gal expression without changes in fluorescence during the sort. This ability opens new approaches to investigating gene expression such as correlating reporter gene activity with the concentrations and activities of particular transcription factors in sorted cells (3).

We report the presence of rare false-positive cells and the influence of growth conditions on their frequency. Although the cause for appearance of these cells is unclear and we have found no technique that completely inhibits their appearance, they emit a spectrum of fluorescence different from that of β -gal⁺ cells and can be screened out using two detection channels and appropriate optical filters.

Since the assay does have a background associated with endogenous lysosomal activity, it is important to distinguish weak *lacZ*-encoded β -gal activity from endogenous β -gal activity or from the fluorescence of rare false positives. This can be done by utilizing 1 mM PETG. Endogenous lysosomal β -galactosidase activity is inhibited only approximately 50% by treatment with 1 mM PETG and rare false positives are not inhibited at all (data not shown). Thus, in cells loaded with FDG in the presence of PETG, any fluorescence detected above control samples is due to endogenous β -galactosidase or rare false positives.

We previously reported (8) that the FACS-Gal assay is more sensitive than the commonly used X-gal single-cell chromogenic assay (16) for β -gal activity. We have determined that, to be recognized as expressing β -gal by the X-gal assay, a cell must have at least 500 molecules of β -gal per cell (data not shown). As noted above, the limit of FACS-Gal sensitivity is fewer than five molecules of β -gal per cell. Many of the β -gal-expressing cell lines we routinely study with FACS-Gal are below the sensitivity of the X-gal assay system.

Therefore, depending on X-gal staining to determine whether a cell expresses *lacZ* can produce false-negative results.

As shown above, the *in vivo* FACS-Gal assay is nonlinearly related to *in vitro* measurement of β -gal activity. Similar results were obtained by another group using FDG and the FACS to assay the endogenous lysosomal β -gal activity in mammalian cells *in vivo* they reported that the activity value provided by their FACS based assay was approximately the square of the activity measured by an *in vitro* assay (5). The cytoplasm in which *in vivo* enzyme reactions take place is vastly different from the dilute aqueous solutions of *in vitro* assays, and it is not too surprising that these *in vivo* assays show consistent kinetic differences in comparison to *in vitro* assays for the same enzymes.

To use effectively the unique capabilities of FACS-Gal, the *lacZ* construct must be stably, rather than transiently, introduced into the nucleus of the cells of interest. Generally, reporter gene studies use transient transfection systems in which the reporter gene is only transiently present in the cell, since it is neither integrated into the genome nor carried on a replication-competent episome. The use of stably integrated or episomal reporter gene constructs allows the exploitation of the single cell assay and selection capabilities of FACS-Gal, capabilities that are less useful in transient assays in which cell-to-cell variation of expression is due to stochastic variation in uptake of DNA and is not a stable genetic property of the cells. Furthermore, although studies using transient assays have historically been very productive, the cells are severely stressed by the transfection procedure, and the DNA being studied is in an unnatural configuration that does not reveal all aspects of gene expression (18). There are also limitations associated with using stably integrated reporter genes to study gene expression, namely, the time and effort required to select and characterize multiple integrants and the need to control for the influence of the integration site on gene expression. Although FACS-Gal is able to assay expression of transiently introduced constructs, we find that the system is most useful for experiments with stably integrated constructs in which obtaining the distribution of expression or using its selection capabilities is advantageous.

FACS-Gal makes flow cytometry a powerful tool for a variety of new applications by analyzing and sorting viable cells expressing a transduced reporter gene. Such a reporter gene may be used to study an isolated regulatory segment of DNA (3) or as a gene fusion to investigate an endogenous locus (6). As a reporter gene assay system, FACS-Gal has the advantage of providing information on the distribution of expression in the population. As a selectable marker, FACS-Gal allows immediate selection (sorting) of cells with virtually any level of β -gal activity without the delay, loss of potentially valuable cells, and physiological stress associated with drug selection procedures. This system

has unique abilities as either a reporter gene or a selectable marker, and, alone or combined, these abilities facilitate new experimental approaches to studies of gene regulation and cellular differentiation.

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