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FACS-Gal: Flow Cytometric Analysis and Sorting of Cells Expressing Reporter Gene Constructs

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In this report, we present a detailed explanation of the FACS-Gal assay. This assay is used to measure the intracellular concentration of β-galactosidase, as expressed by an introduced *Escherichia coli lacZ* gene. *lacZ* is commonly used as a reporter gene to measure the activity of particular constructs, i.e., heterologous promoters and enhancers. With FACS-Gal, the activity of a construct can be measured in an individual viable cell. In addition to determining the gene activity distribution within a population, the FACS can be used to measure other cellular parameters and to sort cells on the basis of a specific range of β-galactosidase activity. This makes the reporter construct a nonlethal selectable marker. The biochemistry of the FACS-Gal assay is discussed, with an emphasis on sensitivity. Additionally, the utility of FACS-Gal compared to that of other reporter gene assays is discussed, especially its use as a selectable marker. Finally, we show two examples of how FACS-Gal has been used to answer questions that are unapproachable with standard reporter gene systems. In addition, we provide detailed protocols for the FACS-Gal assay and a sensitive assay for β-galactosidase measurements in bulk cellular lysates. © 1991 Academic Press, Inc.

Reporter genes have been used to analyze the expression of genetic control elements for several decades. One of the more widely utilized is the bacterial *lacZ* gene encoding β-galactosidase (β-gal), for a number of reasons: its enzymatic activity is easily and sensitively measured, it can be expressed and assayed in virtually any type of cell, and its activity is unaltered by making N-terminal fusion polypeptides.

The development of an assay to measure β-gal activity in individual, viable cells by FACS (1, 2) allows the determination of gene expression on a cell-by-cell basis. The advantages of this assay over simple bulk assay measurements are several (see Fig. 1): (i) determination of the distribution of enzyme activity (and thus gene activity) within a population of viable cells; (ii) resolution of heterogeneities in gene expression within a population; (iii) concomitant measurement of other cellular parameters, such as surface immunophenotype, DNA content, or intracellular glutathione content; and (iv) sorting of cells with defined levels of gene expression for subsequent studies, in essence making the expression of the reporter gene a selectable marker.

FACS-Gal has been used in a wide variety of systems to answer a number of questions and problems in modern biology, for instance: (i) control of gene expression by known promoters and enhancers (3–12); (ii) detection of novel developmentally or stress-regulated genes and enhancers (13–17); (iii) expression of a reporter gene in untransformed cells *in vivo* (14, 15, 18–20) and in embryonic stem cells (21); (iv) isolation of antigen-responsive T-cell hybridomas (11) and recombinase-active B cells (22); (v) development of cell lines responsive to viral particles (12, 23); (vi) the study of anti-viral agents (9, 10, 24); (vii) the study of gene expression during embryonic development of Drosophila (20); and (viii) quantitation of a novel DNA transfection method (19) and episomal DNA replication (25). FACS-Gal has been used successfully in a number of cell types other than mammalian cells, including chicken (19), Drosophila (20), yeast (26), and bacteria (26, 27).

In this article, we describe in detail the FACS-Gal assay as used in mammalian cells. The protocol that we used for the assay is given in Appendix A and is based on previous publications (1, 2). (In addition, we present a protocol in Appendix B for measurement of β-gal activity in bulk lysates using a fluorescence plate reader.) Here we show how a variety of parameters affect the FACS-Gal assay so that it can be tailored to any particular system. We then show two examples of how the measurement of β-gal activity in individual cells has led to surprising revelations that would have been undetected with bulk lysate measurements.

A. BIOCHEMISTRY OF FACS-Gal

I. Substrate Loading

The FACS-Gal assay depends on the introduction of a nonfluorescent fluorogenic substrate for β-gal, fluorescein di-β-D-galactopyranoside (FDG), into the cytoplasm of

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cells. β-gal hydrolyzes FDG, releasing highly fluorescent fluorescein. The resulting fluorescent product is trapped intracellularly in order to measure it in individual cells by FACS. The presumption of the assay is that the rate of hydrolysis (rate of accumulation of fluorescence) is proportional to the cellular concentration of β-gal, as long as the substrate (FDG) has not been exhausted.

To load the substrate, cells in suspension at 37°C are briefly exposed to a hypotonic medium containing FDG. Following this treatment, the cell suspension is diluted at least 10-fold with ice-cold isotonic medium to restore isotonicity and to chill the cells ("quenching"). These two effects serve to restrict further substrate entry and to prevent leakage of the hydrolyzed product. Fluorescein is membrane-permeable at 37°C (a temperature at which cells lose it with a half-time of about 3 min (1)) but not at 0°C (a temperature at which cells do not lose significant fluorescence even over a 17-h period (2)).

As Fig. 2 shows, the amount of FDG loaded into cells is roughly proportional to the length of time that cells are exposed to the hypotonic medium (after a short lag phase). We have found no significant loss in viability for most cell types with treatment times as long as 3 min. In general, however, we use only a 1-min treatment, for two reasons: it is adequately long to load enough FDG for the sensitivity required by most systems; and in cell lines with high activity, significant amounts of product can leak out of the cells during the longer loading time (since hydrolysis begins immediately upon introduction of substrate). This loading technique results in a very uniform loading of substrate, with the cell-to-cell variation in the amount of FDG being less than threefold (2): some of this variation may be cell volume dependent.

The other parameters affecting the amount of FDG loaded into cells are the degree of hypotonicity and the concentration of the substrate (Fig. 2). The greater the hypotonicity, the greater the amount of FDG loaded. However, viability can be affected below 50% of isotonicity. The amount of FDG loaded is also directly proportional to the concentration of FDG during the hypotonic treatment. We have estimated the intracellular concentration of FDG to be 5 μM when the standard loading conditions are used (i.e., 1 min, 50% hypotonic treatment, at 1 mM FDG) (2).

After the cells are loaded with FDG and quenched at 0°C (to restore isotonicity), the hydrolysis by β-gal proceeds linearly with time (data not shown). In fact, hydrolysis can continue for at least 17 h if the concentration of β-gal is low enough (2). During this time, cells can be stained for other types of analyses, i.e., surface staining with fluorescent monoclonal antibodies and staining with DNA-specific dyes. As long as the cell membrane is intact and the temperature of the cells is maintained below about 10°C, the fluorescein will not leak from the cytoplasm.

The two considerations to be addressed in optimizing a FACS-Gal experiment are the requisite sensitivity and the effect on viability. Sensitivity (i.e., the amount of fluorescence over background) can be increased by increasing the amount of FDG loaded: increasing the hypotonic treatment time, increasing the degree of hypotonicity, or increasing the FDG concentration (and thus DMSO) in the hypotonic treatment. Higher sensitivity can also be achieved by increasing the time of incubation after quenching. However, each of these could adversely affect viability; thus, if sensitivity is not an issue, then more gentle loading conditions can be utilized.

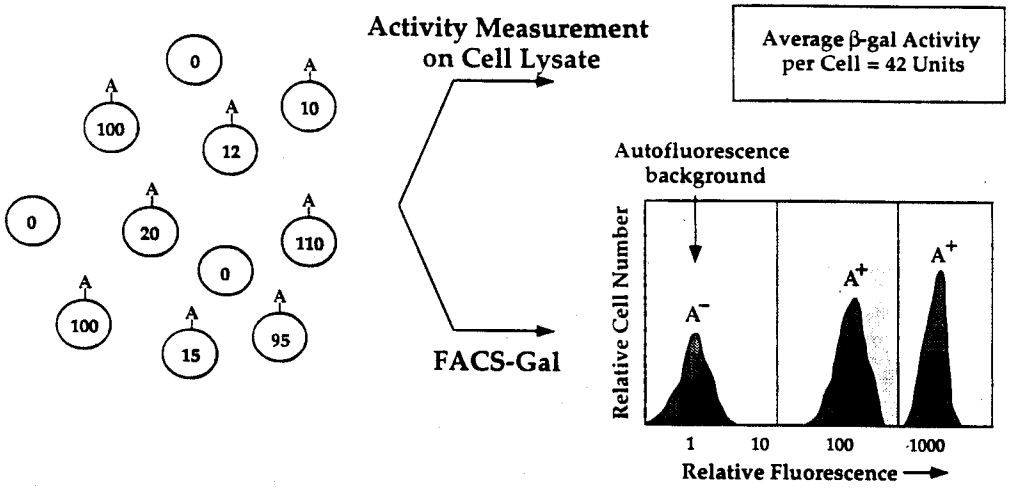

FIG. 1. Advantage of FACS-Gal over other reporter gene systems. In this hypothetical system, cells of two phenotypes (expressing surface antigen "A" or not) express various levels of β-gal activity (numbers within the cells). A bulk lysis measurement would show only an average activity per cell (in fact, an activity not present in any cells). FACS-Gal resolves three populations by activity (quantitative analysis would determine the number of β-Gal molecules per cell in each subpopulation), and could further demonstrate the difference in phenotype of these populations. If desired, the medium-level-expressing cells (in shaded region) could be sorted for further study or cloning, since FACS-Gal is a viable cell assay.

II. Background Considerations

The FACS-Gal assay has three common sources of background that detract from the sensitivity of the assay. The first is from autofluorescence of the cells. Sensitivity can be significantly improved by performing "autofluorescence compensation"; see Alberti *et al.* (28) for more information. The second is the presence of endogenous β-gal activity, contributed by the mammalian enzyme which localizes to endosomes and lysosomes. The third background activity is the generation of "rare brights," rare cells that falsely appear to have reasonably high *lacZ* activity. In this section, we discuss the latter two backgrounds and what can be done to reduce their effect on the assay.

Most mammalian cells possess endogenous β-gal activity in the lysosomes. This enzyme has a pH optimum that is acidic (lysosomal pH being at or below 5). Since it can hydrolyze FDG, this enzyme contributes to background activity (non-*lacZ*-dependent hydrolysis of the substrate). The amount of endogenous activity varies considerably from cell type to cell type. In general, lymphocytes and lymphocytic cell lines have very low activity; macrophage and adherent cell lines tend to have higher activity; primary fibroblasts can have very high activity. Culture conditions can also affect the endogenous activity. Since confluent cells accumulate lysosomes (29), cells that have been maintained at high density or allowed to reach confluency have high activity (Fig. 3). Cells that have been serum-starved or otherwise "mistreated" also tend to accumulate lysosomal activity and thereby have higher endogenous activity.

To reduce the background activity of the lysosomal β-gal, we rely on its relatively acidic pH optimum. Treatment of cells with a lysosomotropic weak base such as chloroquine will raise the pH of acidic compartments (chloroquine freely crosses cell membranes, unless protonated; thus, it accumulates in acidic vesicles and buffers the pH toward neutrality). As Fig. 3 shows, treatment of cells with chloroquine significantly reduces the hydrolysis of FDG by the endogenous β-gal. Since chloroquine does not inhibit hydrolysis of FDG by the *lacZ*-encoded β-gal (data not shown), its use increases the signal-to-background activity in cell types in which endogenous activity

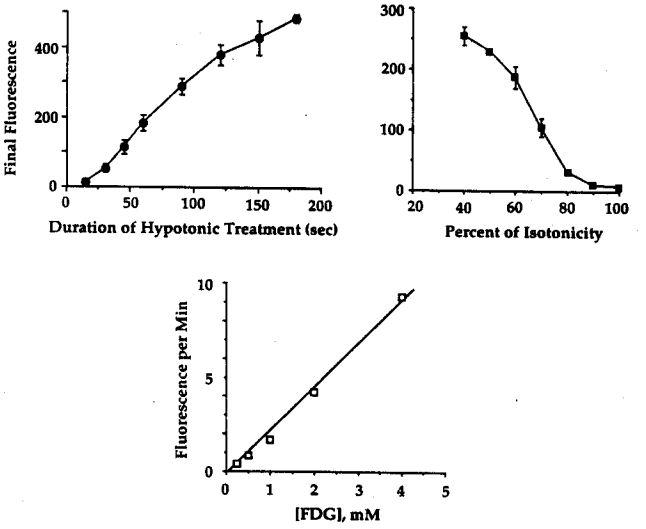

FIG. 2. Effect of substrate loading parameters on the FACS-Gal assay. A *lacZ*-positive cell line was loaded with FDG under different conditions. Incubation was continued until all FDG had been hydrolyzed to fluorescein; thus, the final fluorescence is proportional to the amount of FDG loaded. Top left: The amount of FDG loaded increases linearly with time of hypotonic treatment, after a 15-s lag. Top right: The amount of FDG loaded increases with increasing degrees of hypotonicity. At 50% of isotonic for 1 min, cell viability for all lines we have tested is unaffected. Bottom: The rate of FDG hydrolysis is proportional to the amount of substrate present during the hypotonic shock. The amount of FDG loaded is also proportional to the concentration in the medium (not shown). Since the FDG stock solution was 200 mM in DMSO, the highest concentration point had 2% (v/v) DMSO during the 1-min hypotonic treatment.

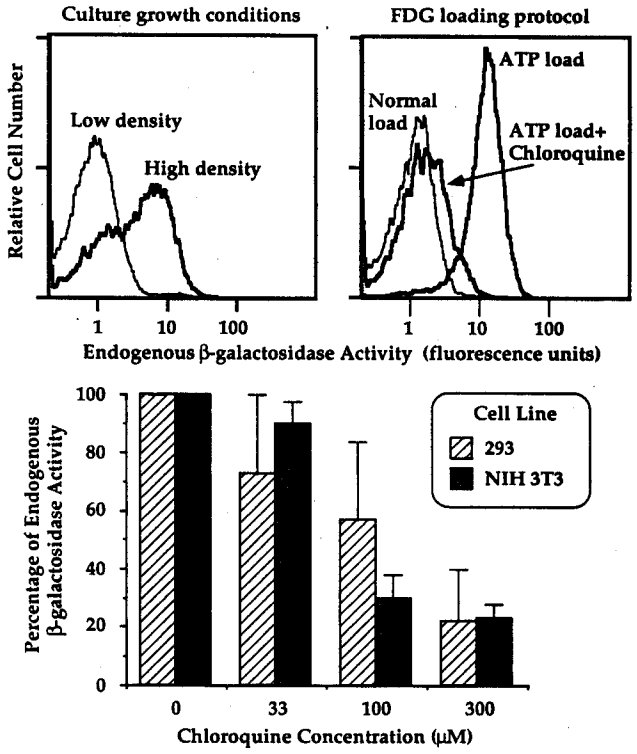

FIG. 3. Mammalian cells have endogenous β-galactosidase activity. For these experiments, cell lines not expressing the bacterial *lacZ* were used. Top left: Culturing 3T3 cells at high density for 2 weeks increases the endogenous (background) activity significantly over that of cells maintained in exponential growth. Top right: Incubation of 3T3 cells with 10 mM ATP and 1 mM FDG at 37°C introduces substrate into the cells. However, much of it enters lysosomes, as evidenced by the high endogenous activity compared to that of the "Normal" loading procedure. Note that chloroquine significantly inhibited this activity. Bottom: Chloroquine reduces the lysosomal endogenous activity in a dose-dependent fashion. At 300 μM, chloroquine does not inhibit the *lacZ*-encoded β-gal activity (data not shown).

is a significant fraction of total β-gal activity. We have found variation in the amount of inhibition of endogenous activity by chloroquine from experiment to experiment (but not within an experiment) (2).

One of the major advantages of the hypotonic treatment for loading FDG is that only a small amount of substrate penetrates into lysosomes. We have investigated a different method for loading FDG, in which the substrate is introduced into cells by a 3- to 5-min incubation at 37°C in isotonic medium containing 10 mM adenosine triphosphate and 1 mM FDG (2). This technique loads an adequate amount of FDG for use in the assay, although there is more cell-to-cell variation in the amount of substrate loaded (data not shown). This technique results in a substantial amount of substrate loaded into lysosomes (Fig. 3).

Endogenous activity contributes to the background present in all cells in a population. This reduces the signal-to-background ratio, thus reducing the sensitivity to *lacZ* activity. Another type of background activity, the presence of "rare brights," reduces the ability to detect and sort infrequent *lacZ*-positive cells. When the FACS-Gal assay is performed on cells without *lacZ*, there can be a small fraction of rare cells that are higher in fluorescence than the rest of the population (Fig. 4). We generally find these cells at a frequency of between 0.01 and 1%. We do not know why or how these rare brights arise; however, they arise only when cells are loaded with FDG.

The presence of rare brights decreases the purity with which *lacZ*-expressing cells can be sorted. However, successive sorts (after cells have been grown and reassayed) will enrich for the true, *lacZ*-expressing cells. This is because the progeny of FACS-sorted rare brights (which are viable) are not themselves enriched for rare brights; i.e., this property is not heritable (14). FACS-Gal has been used to select for *lacZ*-expressing cells occurring at a frequency of less than 0.1%, even in the presence of rare brights (15, 16, 22).

Two fluorescence characteristics of rare brights help differentiate them from *lacZ*-expressing cells (Fig. 4): they are generally less fluorescent than cells with enough activity to have completely hydrolyzed loaded FDG, and they tend be brighter than negative cells for emission in both the yellow wavelength band (562–588 nm) and the green (fluorescein) band (515–545 nm). (The green emission from *lacZ*-positive cells does not correlate with the yellow emission if compensation is appropriately adjusted for fluorescein.) Thus, the highest ratio of *lacZ*-positive cells to rare brights is obtained by sorting cells with high 530-nm emission and low 575-nm emission (see Fig. 4).

The presence of rare brights is cell-type dependent and has a weak correlation with endogenous activity. Adherent cells, which typically have higher endogenous activity, include more rare brights. The percentage of rare brights is increased in high-density or confluent cultures. For FACS-Gal assays, we recommend maintaining cultures in exponential growth, since we find that these growth conditions minimize both endogenous activity and the frequency of rare brights.

III. Quantitation

In order for FACS-Gal to be a quantitative measure of β-gal activity per cell, several criteria must be met: (i) the substrate must be uniformly and rapidly loaded; (ii) the product must not leak from the cells; (iii) the hydrolysis of the substrate must proceed at a constant rate such that the final fluorescence can be related to absolute β-gal activity; (iv) the measurement of fluorescence must be made at a known time after addition of the substrate; (v) the measurement of fluorescence must be made prior to the exhaustion of substrate by any cells in the population; and (vi) appropriate controls for background activity must be performed. Points (i) and (ii) have been covered; point (iii) is covered in the section on nonlinear kinetics, below; and points (iv–vi) are addressed in this section.

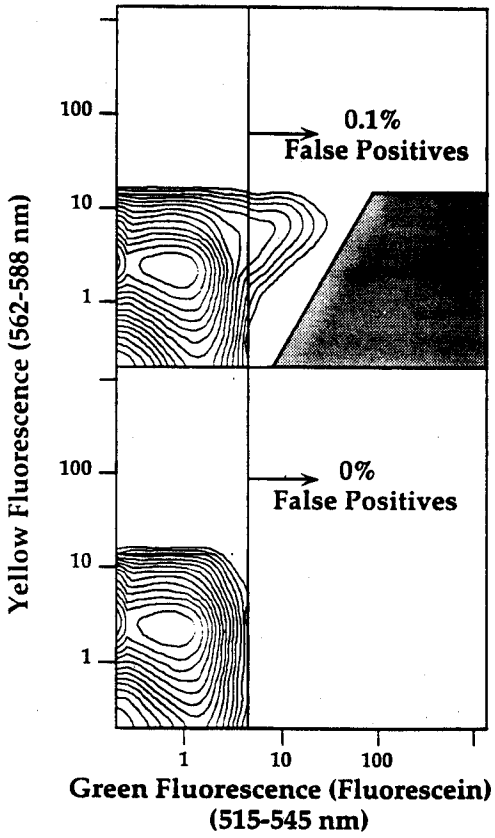

FIG. 4. "Rare brights" have a fluorescence characteristic different from that of *lacZ*-positive cells. *lacZ*-negative cells were treated with FDG either hypotonically (top) or isotonically (bottom). Under isotonic conditions, FDG does not enter cells. This demonstrates that the appearance of rare brights depends on the hypotonic loading. Rare brights tend to be bright in the yellow emission band compared to *lacZ*-positive cells, which have uncorrelated green and yellow fluorescences. Thus, the highest enrichment of *lacZ*-positive cells over rare brights can be obtained by sorting from the shaded region.

Because FACS-Gal used as a quantitative assay is a kinetic assay, precise timing of incubations is required. When a large number of samples are being used, it is too difficult to analyze each sample at known times with respect to substrate addition. Therefore, we have developed the use of a competitive inhibitor to stop the hydrolysis of FDG by β-gal. Phenylethyl-β-D-thiogalactoside (PETG) is ideal for this purpose for several reasons (2): it is sufficiently hydrophobic to enter cells rapidly even at 0°C, but is sufficiently hydrophilic to dissolve stably in aqueous solution; it is a competitive inhibitor of *lacZ*-encoded β-gal, with a low K_i (2.5 μM); it has a sulfur atom in place of an oxygen atom at the hydrolytic site, making it nonhydrolyzable by glycosidases; it is a reversible inhibitor and can be washed out of the cells at room temperature or above, allowing β-gal activity to proceed (surprisingly, even though it readily enters cells at 0°C, it cannot be washed out of the cells at 0°C for unknown reasons (2)); it is nontoxic, allowing viable cell sorting; and it is inexpensive and readily available. PETG only slightly inhibits the lysosomal mammalian β-gal activity.

Addition of PETG at 1 mM final concentration to cells hydrolyzing FDG completely stops further hydrolysis (2), thereby "freezing" the reaction at that time point. Thus, multiple samples can all be stopped at the same time relative to the addition of substrate, and analyzed even several hours later. Furthermore, cell sorts requiring a large amount of time can be performed without the problem of constantly increasing fluorescence during the sort (requiring continual adjustment of sort gates). Finally, cells with high β-gal activity can be analyzed easily, as the reaction can be stopped immediately after loading (i.e., PETG is included in the quenching solution).

Because a limited amount of substrate is loaded into cells, the assay has a limited range. Thus, given enough time, cells will hydrolyze all available FDG into fluorescein and will appear uniformly bright. Although useful for distinguishing *lacZ*-positive from *lacZ*-negative cells, saturation completely obviates quantitation of activity. The reaction must be stopped (or at least measured) prior to any cells reaching saturation. Saturation can be determined by letting a *lacZ*-expressing cell line incubate until fluorescence no longer increases (this point will vary for different cell lines and depends on the amount of FDG loaded).

When cells with very high levels of β-gal activity are assayed, accurate measurements of activity are impossible because the cells will have hydrolyzed all available FDG prior to the end of the 1-min loading time (i.e., before PETG can be added). This problem can be surmounted by adding concentrations of PETG to the loading mixture that slow the reaction. Since the K_i of PETG is 2.5 μM (data not shown), the presence of 25 μM PETG in the loading and quenching solutions will slow the reaction approximately 10-fold; 1 mM PETG can be added later to (essentially) stop the reaction.

Finally, accurate quantitation requires the determination of the background fluorescence, which will be subtracted from sample fluorescences. Background fluorescence has three contributions: (i) autofluorescence, (ii) free fluorescein contamination in the FDG stock, and (iii) hydrolysis of FDG by endogenous β-gal. Each background can be relatively easily determined by performing appropriate controls.

Autofluorescence should be determined on cells not loaded with FDG. Autofluorescence compensation (28) should be performed to yield maximum sensitivity for the FACS-Gal assay. This is especially important with cells that have significant autofluorescence, such as fibroblasts or macrophages.

Free fluorescein in the FDG is generally not a problem with the availability of very pure FDG from Molecular Probes (Eugene, OR). However, it is possible that old stocks of FDG can accumulate some hydrolyzed product. To test this, simply load cells in the presence of high concentrations of PETG. Use cells with little or no endogenous β-gal activity, since it is not well-inhibited by PETG. By monitoring the cells over time, e.g., for a couple of hours, the initial contribution to the fluorescence from free fluorescein can be assessed (endogenous activity, if present, will result in increasing fluorescence as a function of time; the contaminating fluorescence is that found by extrapolating to zero time and comparing to samples mock-loaded with FDG).

Endogenous activity can be determined on a parental cell line that is *lacZ*-negative. As discussed above, chloroquine can be used to lessen the contribution of endogenous activity and increase the sensitivity of FACS-Gal.

In general, the most accurate quantitation of β-gal activity is obtained by measuring the rate of fluorescence increase after quenching. By removing aliquots of the quenched sample at various time points (e.g., 2, 5, 10, 30 min) and adding to tubes containing PETG, this rate can be easily determined. For FACS analysis, it is probably best to use the median fluorescence for each time point, primarily because the median is less sensitive to some of the cells having exhausted the FDG substrate. The rate of fluorescence accumulation can be calibrated to the number of enzyme molecules per cell (see section C.I, below).

B. UTILITY OF FACS-Gal

I. Comparison to Other β-gal Assays

There are three other commonly used biochemical assays for β-gal activity (Table 1). Each has advantages and disadvantages. For assays on bulk lysates of cells, the MUG assay is the most convenient and sensitive. A protocol for this assay is given in Appendix B. It is especially useful for screening large numbers of cell aliquots or clones

growing in 96-well trays because it is suitable for use with a fluorescence plate reader. Since it is a nonviable assay, plates must be replicated prior to assay if cell recovery is desired (for more details, see (16), in this issue).

The two assays performed on intact cells are the X-gal assay and the FACS-Gal assay. X-gal is considerably less sensitive, with cells requiring about 500 molecules of enzyme to appear blue (data not shown). It is also a nonviable stain, requiring cells to be fixed. The advantage of X-gal is that subcellular localization of the *lacZ* can be ascertained; this is useful if localization sequences have been introduced on the *lacZ* coding sequence.

We have determined that the FACS-Gal assay can detect *lacZ*-expressing cells that contain as few as 5 molecules of β-gal; thus, it is possible that the assay could detect a cell with a single *lacZ* mRNA molecule. The upper limit of quantitation by FACS-Gal without the use of PETG is approximately 10,000 molecules of β-gal per cell (because cells will hydrolyze all FDG before even the loading step is finished); with PETG to slow the reaction, we have analyzed cells expressing in excess of 10^6 molecules of enzyme.

II. Use as Selectable Marker

Using *lacZ* as a reporter gene with FACS-Gal has two major advantages over other reporter gene systems: one is the determination of the distribution (and possible heterogeneity) of expression of the reporter gene within a population; the other is the use of the FACS to sort (select) desired cells from within a subpopulation without killing either sorted or nonsorted cells, thereby making *lacZ* a nontoxic selectable marker.

Selectable markers are genes that allow the selection of infrequent cells expressing a particular phenotype encoded by that gene. Selection by FACS-Gal has several 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: (i) the selection system is inherently toxic, often even to cells expressing the resistance gene; (ii) the selection generally takes weeks; (iii) the cells below (or above) the threshold amount of the selection protein are dead and unavailable for further study; (iv) it is difficult or impossible to select for different expression levels of the marker; and (v) the cells must be able to reproduce in culture. FACS-Gal uses the sorting capabilities of the FACS to isolate cells on the basis of virtually any level of expression of nontoxic β-gal. The FACS can analyze and sort from as many as 5000 cells per second (30); thus, 1 cell in 10 million could be selected in an hour of sorting, making FACS-Gal potentially as efficient as drug-based selectable markers.

The ability to select cells on the basis of a particular level of expression is a big advantage over standard selection methods, for several reasons. First, cells expressing very low levels of the enzyme (e.g., 10 molecules) can be detected and sorted; such low levels might not be sufficient to confer drug resistance. Second, cells expressing both an upper and a lower limit of enzyme level can be selected, for instance (as depicted in Fig. 1), sorting cells with only an intermediate level of expression. Third, cells that rapidly change expression of the reporter gene in response to a stimulus can be selectively sorted. These capabilities allow the selection of cells that would not be possible using standard drug selection methods.

Finally, the ability to sort cells based on gene expression levels allows the concomitant determination of other parameters that can only be determined by (e.g.) biochemical assays on lysates. For instance, by sorting cells with different expression levels and then doing mRNA quantitation on the sorted cells, a comparison of message levels within defined subsets of a population can be made (for example, see (6)).

TABLE 1

A Comparison of β-Galactosidase Assays

Substrate	Instrument	Sensitivity[a]	Quantitative?
Cell extracts			
MUG	Fluorometer	10^5	Yes
ONPG	Photometer	10^8	Yes
FDG	Fluorometer	10^6	Yes
Intact cells			
X-gal	Microscope	500	No
FDG	FACS	≤5	Yes

[a] Sensitivity is defined as the minimum number of β-gal molecules detectable. For intact cells, this is the minimum number of molecules per cell.

III. Use in Transgenic Mouse Tissues

A basic technique of gene expression studies is to isolate regulatory elements that control expression of a given gene and attach these elements to a reporter gene. Mammalian gene expression studies in the past decade have frequently utilized transgenic mice to study the expression in an *in vivo* context. Studies of the control of gene expression in the functioning immune system have been hampered by a lack of appropriate reporter gene systems (i.e., those that do not interfere with function and are sufficiently sensitive). Since FACS-Gal combines the measurement of gene expression with cell identification (e.g., cell surface immunophenotyping; see (16)), it would seem to be the ideal assay for studying gene expression *in vivo*.

Unfortunately, we have made the completely unexpected finding that *lacZ* is not expressed in the immune system of transgenic mice (31). This is completely unexpected because *lacZ* has been expressed in a wide variety of (nonhematopoietic) tissues of transgenic mouse embryos from many different constructs (32, 33). *lacZ* has been expressed in some adult tissues, but often only under control of very strong promoters.

We and others have tested for *lacZ* expression in a variety of transgenic mouse lines. This information is summarized in Tables 2 and 3. None of these lines showed any expression in the lymphoid system, even though most of these promoters should be very active. Twenty-eight separate integration sites from 12 independent constructs are represented. The enhancer/promoter of one construct, NFAT/IL-2, did not express in 2 of 2 lines when attached to *lacZ*, but did express in 4 of 4 lines when attached to SV40 T-antigen (34). This result is not due to transformation by T-antigen since expression was obtained only by activation of T cells. Ravid *et al.* have recently demonstrated the expression of β-gal in megakaryocytes of transgenic mice (35), indicating that the block is not present in all hematopoietic lineages. Expression in myeloid cells has also been detected (16).

Analysis of RNA from 4 separate constructs in 9 independent lines revealed no expression of *lacZ* mRNA; only 1 construct yielded demonstrable transcription (Table 2). Since the methods used to quantitate RNA assayed steady-state levels, it is not possible to state whether there is a block to transcription or whether there is a post-transcriptional problem. Interestingly, Perlmutter demonstrated the presence of *lacZ* transcripts in thymocytes from transgenic animals with *lck* promoter fusions; however, no β-gal activity was detected (R. Perlmutter, personal communication). The block to expression may involve mRNA instability sequences shown to cause instability of c-*myc* and c-*fos* (36). The consensus sequence conferring this instability is "ATTTA" repeated in a generally AT-rich region. The sequence of *lacZ* from pCH110 has a single ATTTA embedded in a generally AT-rich region, with other sequences similar but not identical to ATTTA. Another possibility is that the prokaryotic *lacZ* RNA message is not efficiently processed and exported from the nuclei in these cells.

Beddington (from the CGR, Edinburgh, Scotland) has supplied us with data demonstrating that the expression of *lacZ* in transgenic animals is blocked only in some tissues (R. Beddington, personal communication). Chimeric mice were made by injection of embryonic stem (ES) cells expressing *lacZ* under control of the β-actin promoter into a developing blastula. The ES-derived (donor) cells could be distinguished from the recipient-derived (host) cells on the basis of a variant glucose phosphate isomerase (GPI) isozyme. Data in Table 3 show that β-gal is not expressed (as assayed by X-gal) in the spleen, gut, or blood even though there is significant contribution from the donor ES cells to these tissues. In the remaining tissues, β-gal expression is well correlated with the fraction of donor ES cells. This experiment also demonstrates that the block of β-gal expression is not a by-product of transmission through the germline. Although *lacZ* remains a useful reporter gene for many tissues in the mouse, it is at this time unusable for stable expression in lymphoid (and possibly some other) tissues.

TABLE 2

Transgenic Mice Do Not Express β-gal in the Immune System

Promoter/enhancer	*lacZ* source	No. of lines	RNA[a]	Constructing lab
		Analyzed in the Herzenberg lab		
c-*fes*	pCH110	2		Greer
HIV LTR	pCH110	6	0/6; RNase	Skowronski
MHC class I K[b]	pCH110	2	0/2; Northern	Tokuhisa
MHC class I	pCH110	1		Breitman
IgH enhancer/class I		1		Loh
β-Actin	pSDK	1		Rossant
NFAT/IL-2	pCH110	2	0/2; RNase	Herzenberg
Ig fusion	pCH110	1		Kohler
		Personal communication		
lck	pLacF	3	2/3[b]; S1 nuclease	Perlmutter
IL-2	pSDK	4		Weissman
IL-4	pSDK	4		Weissman
Granzyme A	pRS414	1	0/1; Northern	Weissman

[a] An entry in this column indicates that the presence of *lacZ* RNA was assayed by the method noted; the numbers refer to the number of positive lines/number of lines tested.

[b] Expression of *lacZ* transcripts assayed in thymocytes. No β-gal protein was detected.

C. EXAMPLES

Following are two examples of results that we obtained using FACS-Gal. The conclusions that we can draw on the basis of the data would have been virtually impossible to arrive at without the ability to measure reporter gene activity on a cell-by-cell basis. These examples highlight the power and utility of FACS-Gal.

I. Nonlinear Enzyme Kinetics in the Cytoplasm of Mammalian Cells

To correlate the FACS-measured fluorescence with the absolute enzyme activity within cells, we adopted the experimental scheme depicted in Fig. 5. In this scheme, a particular cell line expressing *lacZ* was assayed by FACS-Gal. The reaction was stopped with PETG at a time before any cells had reached saturation. Normally, cells with exogenously introduced *lacZ* vectors show a broad distribution of fluorescence. Cells from very narrow ranges of fluorescence were sorted from the population into individual wells of a 96-well plate. Each sort was done from a different range of fluorescences; normally, about 20 partially overlapping "slices" were sorted from a particular clone.

The sorted cells were then assayed by the MUG assay (with reference to dilutions of purified β-gal) to determine the exact number of enzyme molecules per sort, and thus per cell. This number was correlated with the original FACS-measured, viable cellular fluorescein fluorescence generated during the incubation with FDG. In this manner, the fluorescence measured on the FACS was correlated with the cytoplasmic enzyme content.

This correlation is shown in Fig. 6. Quite unexpectedly, the amount of fluorescence measured by FACS-Gal correlates nonlinearly with the concentration of enzyme in the cell. In fact, the data are best fit by the equation

$$F = C \cdot E^{1.8}, \qquad [1]$$

where F is the fluorescence measured by FACS, C is a constant of proportionality, and E is the number of β-gal molecules per cell. Figure 6 also demonstrates that the best linear fits does not adequately describe the product fluorescence to enzyme level relationship.

The nonlinear relationship of enzyme activity (fluorescence generated in the assay) to enzyme content was true within 21 independently derived cell lines (including B and T cell lines, NIH 3T3 fibroblasts, and 293 embryonic kidney epithelial cells). These included 6 different constructs, one of which localizes β-gal to the nuclear membrane. The use of PETG in the FACS-Gal assay did not contribute to the observed nonlinear relationship, as experiments conducted without it gave identical results. Finally, we have found that this nonlinearity holds true for cells with as few as 5 molecules of β-Gal per cell, as well as those with greater than 5000 per cell.

This relationship also held when 24 independent T-cell clones were assayed by FACS-Gal and MUG. The average fluorescence for each clonal population by FACS-Gal correlated with the average number of β-gal molecules per cell for that clone (Fig. 6). Thus, cells from clone to clone as well as cells within a clone share the same property: those cells with higher enzyme levels hydrolyze a disproportionate amount of FDG per unit time. It is interesting to note that Jongkind *et al.* noted a similar nonlinear dependence when they measured FDG hydrolysis by the endogenous galactosidase (37).

The nonlinear dependence of hydrolysis rate on enzyme concentration is not predicted by Michaelis–Menten kinetics nor by any measurements of β-galactosidase activity *in vitro*. One might predict that cooperativity is responsible for the nonlinear dependence (as the hydrolysis of FDG is a two-step process). However, four lines of evidence counter this hypothesis: (i) The second hydrolysis (fluorescein monogalactoside (FMG) to fluorescein) is considerably faster than the first (FDG to FMG); thus, the reaction is first order (38). (ii) Cooperativity is not seen *in vitro*, in cell extracts or with purified enzyme,

TABLE 3

Expression of *lacZ* in ES Cell Chimeric Mice Is Limited to Certain Tissues

	Muscle	Liver	Spleen	Kidney	Lung	Gut	Brain	Blood
Female, 3 months								
ES %[a]	25	2	nd[b]	30	5	20	23	nd
lacZ %[c]	50	1	nd	40	5	0	25	0
Male, 12 months								
ES %	40	24	13	37	6	nd	27	25
lacZ %	80	20	0	30	2	0	30	0

Note. Underlined values are those which show lack of *lacZ* expression in tissue.

[a] Percentage of donor-derived cells in tissue.

[b] nd, not done.

[c] Percentage of cells expressing *lacZ* (can come only from donor).

even when the reaction is measured using FDG as the fluorogenic substrate at enzyme concentrations in excess of 3×10^{13} molecules/ml (a concentration roughly equivalent to 3000 molecules/cell; data not shown). (iii) The nonlinearity is identical over at least 3 orders of magnitude of enzyme concentration; i.e., it is true even for cells that have less than 10 molecules of enzyme in the entire cytoplasm. (iv) The reaction order is consistently 1.8, rather than an integral number, 2, predicted by cooperativity.

These results provide an interesting window into the cytoplasmic structure. It is not surprising that the enzyme kinetics in the cytoplasmic of cells cannot be modeled by dilute solution criteria or follow the predictions of Michaelis–Menten kinetics. However, the observation that the reaction rate increases faster than an increase in enzyme concentration is surprising; it might be modeled by fractal reaction kinetics with the assumption that reaction is diffusion-limited (39). In terms of the FACS-Gal assay, it means that cells with 10 times the generated fluorescein fluorescence, for example, only have 3 times as much enzyme.

II. Heterogeneous T-Cell Activation

The FACS-Gal assay was used to assess T-cell activation on a cell-by-cell basis (6). A Jurkat T-cell clone that had a stably transfected inducible *lacZ* construct was selected. This construct consisted of a trimer of NF-AT (nuclear factor of activated T cells; a DNA-binding enhancer protein) binding sites fused to the *lacZ* coding region. In this cell line, β-gal is not expressed unless the T cells are activated (6).

Figure 7 shows the kinetics of activation of this construct by phorbol ester and the calcium ionophore ionomycin. There is a "biomodal" response at early time points in that some cells have substantial β-gal activity, whereas most of the rest have none. Determination of the absolute β-gal activity in these cells reveals that the cells that have induced the construct have similar (within 5-fold) levels of the enzyme; thus, there appear to be two states during activation: "off," in which no enzyme has been synthesized, and "on," in which a specific amount of transcription and translation has occurred fairly rapidly. Few cells fall into the intermediate range at any time point.

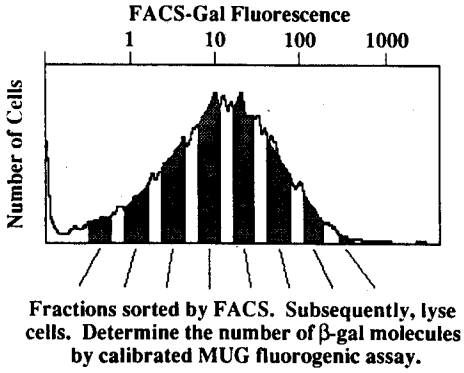

FIG. 5. Scheme for calibration of FACS-Gal fluorescence to absolute β-gal activity. Narrow "slices" of the fluorescence distribution of cells assayed by FACS-Gal are sorted into individual wells of a 96-well plate and assayed by the biochemical MUG assay. The absolute number of molecules of β-gal determined by MUG is then correlated against the FACS-measured fluorescence. The experiment was most often done by collecting the fluorescein fluorescence with linear amplification; however, it was also done with logarithmic amplification as depicted here (both kinds of experiment yielded identical results).

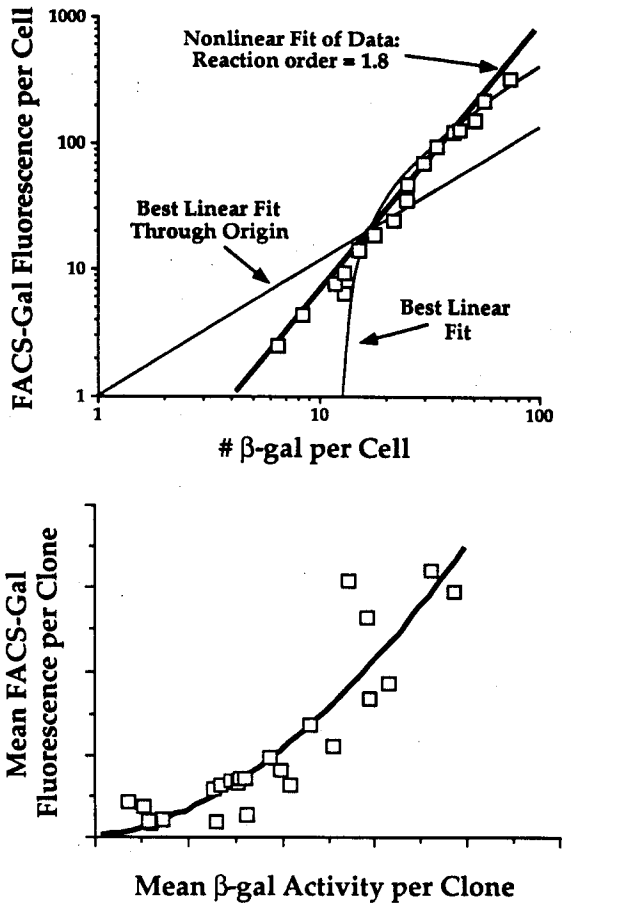

FIG. 6. Hydrolysis of FDG by β-gal in mammalian cells follows nonlinear kinetics. Top: Using the experimental scheme depicted in Fig. 5, we obtained a reaction order of 1.8 in β-gal concentration for the hydrolysis of fluorescein (the reaction order is 1 in FDG concentration; see Fig. 2). Thus, the cells with greater amounts of β-gal hydrolyze a disproportionate amount of FDG in the same amount of time. Since background activities were subtracted from both assays, any fit is expected to intersect the origin (as the nonlinear fit does; see bottom plot). Bottom: This reaction order was not an artifact of nonuniform substrate loading in the cells: when 24 independent clones with varying activities were compared for the average FACS-Gal fluorescence and average β-gal molecules per cell in the whole of each population, an identical relationship was found. Eight of the cell lines used in this comparison were tested individually as in the top panel. The reaction order for these 8 lines (tested a total 17 times) was 1.82 ± 0.08 (SD). In both panels, the dark line is the best fit of Eq. [1] (see text) to the data (the reaction order was a parameter to the fits).

By sorting the cells that are on from those that are off, Fiering *et al.* (6) determined the amount of the enhancer-binding protein NF-AT that induced the transcriptionally active state. Interestingly, only a 5-fold difference in the amount of NF-AT in the nucleus was capable of inducing a greater than 100-fold difference in the amount of β-gal per cell. Two possible explanations for this are that there is a threshold amount of the nuclear factor necessary for transcriptional activation and that activation of the enhancer requires simultaneous binding to all three sites, making activation third order in NF-AT concentration (i.e., a 5-fold increase in NF-AT concentration could result in a 125-fold increase in the amount of fully occupied enhancer). Binding of NF-AT to the trivalent binding site was not cooperative (6).

Finally, the relationship of activation to cell cycle was determined (Fig. 7). Cells were simultaneously stained with Hoechst 33342 (which quantitatively binds to DNA) and FDG. Quantitative analysis demonstrated that the first cells to respond to a brief (90 min) stimulation are enriched for cells in S and G_2 phases of the cell cycle (6). Further studies can determine whether it is the dividing cells that are more easily stimulated or whether the stimulation induces some activated cells to enter S phase.

D. SUMMARY: ADVANTAGES OF FACS-Gal

FACS-Gal has two major advantages over all other reporter gene systems based on different principles: the determination of activity on a cell-by-cell basis, and the use of an expression level as a selectable phenotype. The measurement of activity on individual cells within a population resolves the rich heterogeneity in gene expression. Two examples were presented in this article: the demonstration of nonlinear enzyme kinetics within the cytoplasm of mammalian cells, and the heterogeneous activation of T cells from a single clone. These types of results are impossible to obtain from bulk measurements of gene activity. The ability to use the FACS to viably sort cells based on β-gal expression allows the use of reporter gene expression as a nontoxic selectable marker. Because of the high sensitivity of FACS-Gal (5 molecules of enzyme per cell) and the almost unlimited dynamic range, virtually any level of expression can be selected (with upper and/or lower limits of expression). Furthermore, by sorting cells on the basis of expression level, followed by biochemical assays on the sorted populations, expression levels can be correlated with almost any desired parameter (e.g., RNA quantitation, presence of DNA constructs by polymerase chain reaction, metabolic enzyme activity).

The other main advantage of using FACS to measure gene activity is the ability to simultaneously determine cell phenotype, based on size, surface antigens, DNA content, or other FACS-measurable parameters. This allows the determination of gene activity in very heterogeneous cell populations, such as those in transgenic mice. Although *lacZ* is not expressed in the lymphoid tissues of transgenic mice, we hope that the development of new enzyme/substrate reporter genes that can be measured by FACS will overcome this problem.

We are currently developing an excellulase gene for use as a second FACS-based reporter gene (Brice, Roederer, Ela, and Herzenberg, in preparation). This system will have several advantages over the *lacZ*-based system: there is no background exocellulytic activity in mammalian or Drosophila cells, allowing extremely high sensitivity of detection; it is probable that the excellulase will be expressed in lymphocytes, making it useful for studies in transgenic mice; and the potential use of a different fluorophore as a substrate (other than fluorescein) will allow the simultaneous measurement of two different gene activities (together with *lacZ* and FDG) within individual, viable cells. Such a system will yield a wealth of information about the regulation and kinetics of gene expression in wide variety of cell systems.

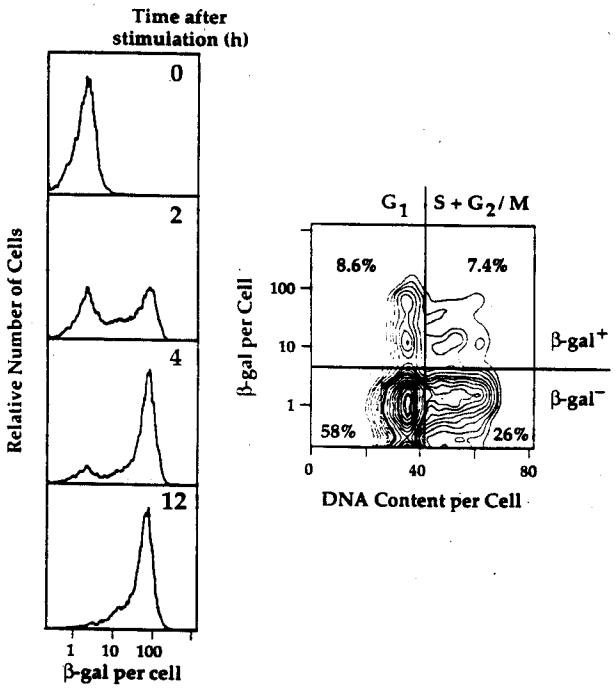

FIG. 7. The kinetics of T-cell activation within a clone is heterogeneous. The β-gal activity of a T-cell clone with a construct expressing *lacZ* under control of a tri-NF-AT enhancer was monitored as a function of time after stimulation with phorbol ester (10 ng/ml) and ionomycin (2.25 μM). At intermediate times, the expression is bimodal, demonstrating that not all cells have activated the construct. The panel to the side is a bivariate histogram correlating β-gal expression with DNA content; the percentage of cells in each quadrant is shown. For this sample, cells were stimulated for 90 min.

APPENDIX A: PROTOCOL FOR FACS-Gal ASSAY

Materials and Stock Solutions

FDG can be obtained from Molecular Probes (catalog number F1179; Eugene OR). PETG and chloroquine can be obtained from Sigma (catalog numbers P4902 and C6628, respectively; St. Louis, MO).

FDG. FDG is dissolved at 200 mM in DMSO and diluted in H_2O to a final concentration of 2 mM. Store at −20°C; stable for several months.

PETG. PETG is dissolved at 50 mM in H_2O and kept at −20°C; stable indefinitely.

CHL. Chloroquine is dissolved at 10 mM in H_2O and kept at −20°C; stable for several months.

PI. Propidium iodide (for exclusion of dead cells) is dissolved at 100 μg/ml in H_2O and kept at −20°C; stable indefinitely.

SM. The staining medium we have used is deficient RPMI (deficient in biotin, phenol red, and riboflavin, for minimal contribution to autofluorescence), supplemented with 4% serum and 10 mM Hepes (pH 7.4). PBS or non-deficient RPMI can be used instead.

Before the start of each experiment, make *Quench:* add 10 μl/ml PI to SM. If chloroquine is to be used, add 30 μl/ml CHL. One milliliter of Quench is needed for each sample. Keep on ice.

Assay

If cells have relatively high endogenous activity, then use chloroquine to quench it. The contribution of endogenous activity should first be assessed without chloroquine. If chloroquine is to be used, then add it to the SM (at 30 μl/ml) that will be used to resuspend the cells.

1. Harvest cells by appropriate method, e.g., trypsinize and centrifuge. An appropriate number of cells per sample is 10^6. There is virtually no reasonable upper limit on the number of cells that can be used per sample.
2. Resuspend the cells in 50 μl of SM (with chloroquine, if needed); move to 37°C water bath. If chloroquine is included, then incubate for 20 min in the water bath; otherwise, only 5 min is needed to equilibrate the temperature. Warm the FDG to 37°C and keep in the water bath.
3. Add 50 μl FDG to each sample.
4. After 60 s at 37°C, add 1 ml ice-cold Quench and then keep the tube on ice. The timing of this incubation is critical; the quenching must be done as close to 1 min later as possible. With a large number of samples, it is most convenient to have two persons perform the assay: one adds FDG every 10–15 s and the other adds Quench to each sample 1 min later.
5. Incubate for an additional period on ice (the length of time depends on the activity of the cells), during which FDG continues to be hydrolyzed. To stop the reaction, add 20 μl PETG. If cells have very high activity, PETG can be included with the Quench. Typical reaction times are 10–120 min (e.g., for cells with 100 molecules of enzyme per cell, use a 120-min reaction time).
6. After addition of PETG, cells can be stained with monoclonal antibodies by standard methods. The cells must be kept at 0–4°C to prevent leaking of fluorescein from the cells. Although PETG is difficult to wash out of cells (2), is probably best to include PETG during antibody stains and washes. If long incubations are desired (step 5), then antibody staining can be done during this incubation: repeated washing of the cells has no effect on the assay and FDG will continue to be hydrolyzed (for example, see (16)).

FACS Analysis

Dead cells can be excluded on the basis of propidium iodide fluorescence (excitation by 488-nm laser; emission at 562–588 nm). Fluorescence compensation between the fluorescein and PI channels can be used to reduce the contribution of autofluorescence and increase sensitivity of the assay (28). If cells are to be viably sorted, then all solutions need to be sterile-filtered prior to the assay.

When the FACS is used to quantitate absolute enzyme levels, it must be remembered that there is a nonlinear relationship between enzyme content and generated fluorescence; i.e., the fluorescence per cell is proportional to roughly the square of the number of β-gal molecules per cell (see section C.I).

APPENDIX B: PROTOCOL FOR MUG ASSAY

This assay is performed on bulk lysates of cells and relies on the conversion of the nonfluorescent substrate 4-methylumbelliferyl-β-D-galactoside (MUG) to the highly fluorescent product 4-methylumbelliferone. The resulting solution can be measured on any fluorometer capable of uv excitation. The assay as given below has been tailored for measurement by a fluorescence plate reader (96-well plates). The major advantage of using a plate reader is that several thousand assays can be easily performed in a few hours. This allows for a high degree of replication of samples. Another advantage comes from the fact that we routinely grow, stimulate or treat, and lyse our cells in the 96-well plates; thus, no cell transfers need to be done prior to the biochemical assay. For batch screening of clones, in which precise quantitation (e.g., activity per cell) is less important, the assay can be modified to be used in culture medium directly (16).

Materials and Stock Solutions

MUG can be obtained from Sigma (catalog number M1633); all other chemicals are obtained from standard supply companies.

MUG stock (50×). 30 mM MUG in DMF (10 mg/ml) is made in large batches, aliquoted, and kept at −20°C. This preparation is stable for several months.

Z-buffer (1×). 60 mM $Na_2HPO_4 \cdot 7H_2O$ (16.1 g/liter; or, alternatively, 21.5 g/liter of $\cdot 12\ H_2O$); 40 mM $NaH_2PO_4 \cdot H_2O$ (5.5 g/liter); 10 mM KCl (0.75 g/liter); 1 mM $MgSO_4 \cdot 7H_2O$ (0.246 g/liter); pH to 7.0, with NaOH or HCl. Stable at room temperature indefinitely.

TX-100 (10×). 1% (v/v) Triton X-100 in Z-buffer. Stable at room temperature indefinitely.

Stop buffer (3×). 15 mM EDTA (4.4 g/liter; 300 mM glycine (22.5 g/liter); pH to 11.2, with NaOH. Stable at room temperature indefinitely.

PETG (10×). PETG is dissolved at 50 mM in H_2O and kept at −20°C; stable indefinitely.

At the start of the experiment, prepare 5× MUG by diluting the MUG stock 1:10 with Z-buffer. 5× MUG will only remain in solution for a limited time (approximately 30–60 min at room temperature).

Assay

The general protocol for the assay is as follows: Resuspend the cells in 0.125% TX-100/Z-buffer. Add 0.25 vol of 5× MUG and incubate for the desired time. Stop the reaction by addition of 0.5 vol of stop buffer. The stop buffer completely halts further hydrolysis of substrate and increases the pH to greater than 10, required for deprotonation to obtain maximal 4-methylumbelliferone fluorescence. The specific volumes noted below are what we use with 96-well plates and a Titertek Fluoroskan II fluorescence plate reader (Flow Labs, McLean, VA).

1. Prepare cells. Cells can be grown (and stimulated or treated) in the 96-well plates used for the assay. For adherent cells, simply aspirate the medium before addition of the resuspension mixture. For nonadherent cells, centrifuge 10 min at 1000*g* in a plate centrifuge. Aspirate medium and resuspend.

Alternatively, cells grown in other carriers can be harvested and resuspended prior to deposition in the wells. Finally, cells can also be sorted directly from a cell sorter into individual wells and assayed directly. These should be centrifuged and resuspended or the volume of the sorted cells needs to be measured for appropriate additions of solutions.

The number of cells required per well depends on the activity per cell. The Fluoroskan can easily detect 10^6 molecules of β-gal per well with this assay. To determine the number of enzyme molecules per cell, standard dilutions of purified enzyme (Sigma, catalog number G6512) can be included on the same plate (dilute the enzyme into the same volume as the cells).

2. Prepare a 105:15 mixture of Z-buffer:TX-100. Resuspend the cells in 120 μl of this mixture. To one of the wells on each plate, add 15 μl of PETG. This will be the control (blanking) well.

3. Using a multichannel pipet add 30 μl of 5× MUG to each well. Because the increase in fluorescence is directly proportional to time, note the time of addition of the MUG and of the stop buffer carefully.

4. The plate may be read on the plate reader during the reaction to assess the progress. The reaction must be stopped before the substrate has been exhausted and before measurement of fluorescence by the plate reader saturates. On the Fluoroskan, this is at approximately 5000 fluorescence units after the reaction has been stopped. The addition of stop buffer increases the fluorescence at least sixfold; thus, the reactions should be stopped before the wells reach 1000 fluorescence units, unstopped.

The reaction proceeds linearly with time for at least 96 h (data not shown). To increase the reaction rate approximately fourfold, incubate at 37°C.

5. Using a multichannel pipet, add 75 μl of stop buffer to all wells. The resulting fluorescence is stable and can be read several hours later, if needed.

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