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Selection of Rare Event Cells Expressing β-Galactosidase

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The ACAS Interactive Laser Cytometer was used to screen and select mammalian cells in culture infected with a retrovirus construct containing the *Escherichia coli lacZ* and neomycin resistance genes. The product of expression of the *lacZ* gene, β-galactosidase, can be quantitated using the fluorescent substrate fluorescein di-β-D-galactopyranoside (FDG). NIH 3T3 cells infected with the retrovirus construct were selected for neomycin resistance and individual colonies were cloned. These cells served as a positive control, and uninfected parent NIH 3T3 cells were used as the negative control. Autofluorescence and background fluorescence using FDG were examined on uninfected NIH 3T3 cells and found to be negligible. The fluorescence assay was performed on individual adherent cells in culture. Our results indicate that cells expressing the recombinant retrovirus product, β-galactosidase, can be viably stained in culture, analyzed, sorted, and cloned without the cells being trypsinized or removed from their natural growth surface. The assay is sensitive enough to detect *lacZ*$^+$ and *lacZ*$^-$ cells and also capable of discriminating between high and low expressers of β-galactosidase. This technology could be a powerful tool in gene therapy and gene regulation studies. By using the β-galactosidase gene as the reporter molecule in the constructs containing the gene of interest, selection of infected/transfected cells, infection/transfection efficiency, promoter strength, and specificity can be measured with minimal manipulation soon after the infection/transfection process. This technique can be further extended for sorting mutant cells, hybridomas, and rare event occurrences.

The development of eukaryotic gene transfer systems has greatly impacted many areas of biology. Genes that encode reporter functions such as luciferase, chloramphenicol acetyltransferase, human growth hormone, and β-galactosidase have been used to study the function of DNA elements that control transcription. Reporter functions that can be detected at a cellular level have been extremely useful in dissecting complex lineage relationships in developmental biology.

A variety of viral and nonviral techniques for transducing genes into cultured cells are available. The most commonly used nonviral methods involve the use of calcium phosphate, DEAE-dextran, cationic liposomes, anionic liposomes, electroporation, spheroblast fusion, and direct microinjection. Although these techniques are extremely useful for studying the transient expression of a recombinant gene, the efficiency of stable transfection is usually low.

An alternative approach to gene transfer utilizes recombinant viruses. These include adenoviruses, adeno-associated viruses, retroviruses, herpesviruses, and vaccinia viruses. The vaccinia virus system is capable of high-level gene transfer and transient expression; however, it is limited by the fact that it is toxic to the infected cells. The adeno-associated viruses, adenoviruses, and herpesviruses are less well characterized.

Gene transfer using the retrovirus system is less efficient, but the efficiency of stable transduction is much higher. This system has several advantages. First, the integrated viral DNA form, the provirus, becomes indistinguishable from other cellular genes and hence is a template for expression of the recombinant genes. Second, the expression of these recombinant genes has no effect on the viability or proliferative capacity of infected cells. Retroviruses are therefore the vector of choice for shuttling genes of interest into mammalian cells both *in vivo* and *in vitro*. By replacing viral structural genes with the genes of interest linked to a reporter molecule, one can take advantage of the stable introduction of the recombinant genes due to the efficient viral infection process.

The reporter molecule of choice should be one that can be easily identified. In the past, CAT (chloramphenicol acetyltransferase) was used as a reporter molecule to study gene activity because the radioactive CAT assay had been developed for biochemical measurements (1). However, this technique required the use of bulk populations of cells to study gene activity and did not provide any information on expression at the single-cell level.

Later, a chromogenic β-galactoside analogue, *O*-nitrophenyl β-D-galactopyranoside (ONPG), was used to assay gene activity (2, 3). The molecule in this assay was β-galactosidase, the product of the *Escherichia coli lacZ* gene.

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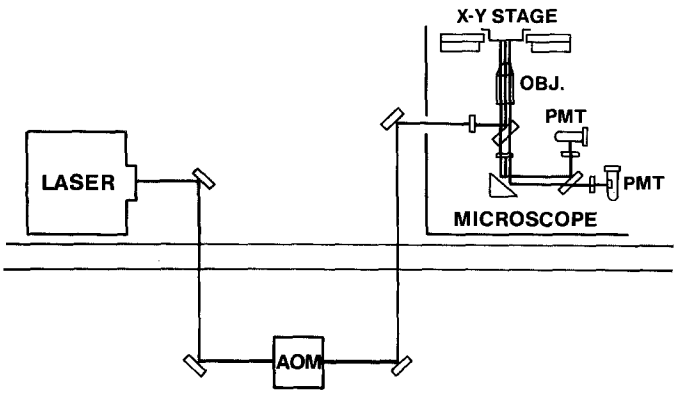


FIG. 1. From signal to image—the basics. Cells are positioned above the microscope objective on the XY scanning stage. When the stage moves, the excitation laser beam passes through a set of mirrors via an AOM (which pulses the laser beam at μs intervals) to the sample positioned on the stage. The fluorescence emission is captured by photomultiplier tubes (PMT) and the signal is digitized by the computer.

β-Galactosidase expression was detected *in situ* using an enzymatic histochemical stain, X-gal, which stains the cell cytoplasm blue (4). Again, the heterogeneity of expression within a population at a single-cell level could not be quantitated.

More recently, the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG), available from Molecular Probes, has been used by various groups to select positive populations of β-galactosidase-expressing cells by using the flow cytometer (5–7). This requires trypsinization of cells, and again information on gene activity and gene regulation is determined on bulk populations.

Using the ACAS 570 Interactive Laser Cytometer, it is possible to study gene activity at a single-cell level without trypsinizing the cells and with very little manipulation. By combining the three technologies (recombinant retrovirus-mediated gene transfer, a fluorogenic dye, and the ACAS 570) it has been possible to isolate, clone, and quantitate individual cells expressing the gene of interest without perturbing the cells under their natural growth conditions.

INSTRUMENTATION

The ACAS 570 Interactive Laser Cytometer, an instrument for the fluorescence analysis, selection, and sorting of attached cells, is composed of:

1. An inverted microscope for ocular examination of living cells and a focusing element for monitoring fluorescence emissions generated from labeled cells.
2. A 5-W argon ion laser, which provides a coherent, high-intensity illumination of a wide range of fluorescent dyes and stains.
3. Photomultiplier tubes, which translate emitted fluorescence to electronic signals.
4. An acousto-optical modulator and a series of neutral density filters used to control light intensity.
5. A two-dimensional moving stage to locate, analyze, and clone individual attached cells in tissue or tissue culture.
6. A computer that coordinates laser and instrument functions, as well as provides data collection, analysis, and organization.

A schematic view of the ACAS 570 Interactive Laser Cytometer is presented in Fig. 1. The system components have been described previously in detail (8, 9).

Cell samples for analysis were positioned on the microscope stage. During scanning, the microscope stage moves in an XY direction. The stationary laser beam, about 1.5 μm in diameter, excites the fluorescence in the cells at varying step intervals. The emissions are captured by photomultiplier tubes (PMT) and digitized by a 32-bit microcomputer to produce pseudocolor images of fluorescence distribution.

The confocal option of the ACAS 570, which was used for some of the studies described here, has a variable pinhole which enables the collection of either optical slices or transmitted image scans that can be stored. The fluorescent images and transmitted light images of the same cells can then be superimposed to identify and correlate signals in subcellular organelles. A direct co-localization of morphological structure and emitted fluorescence can be examined for correlation with intracellular structures. Confocal analysis also permits the reconstruction of optical slices for three-dimensional fluorescence analysis.

FLUORESCENT PROBE

FDG, available from Molecular Probes, is a fluorogenic substrate for β-galactosidase. FDG is taken up by cells and upon hydrolysis by the enzyme, fluorescein is released. The chemical structure and site of hydrolysis are shown

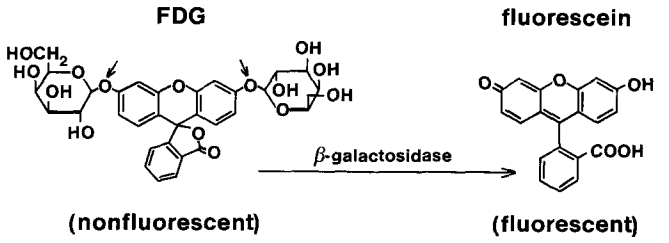


FIG. 2. Chemical reaction of FDG and β-galactosidase. The chemical structure of fluorescein di-β-D-galactopyranoside (FDG), the nonfluorescent molecule, is shown on the left. Arrows indicate sites of hydrolysis by β-galactosidase. The chemical structure of the fluorescent product, fluorescein, is shown on the right.

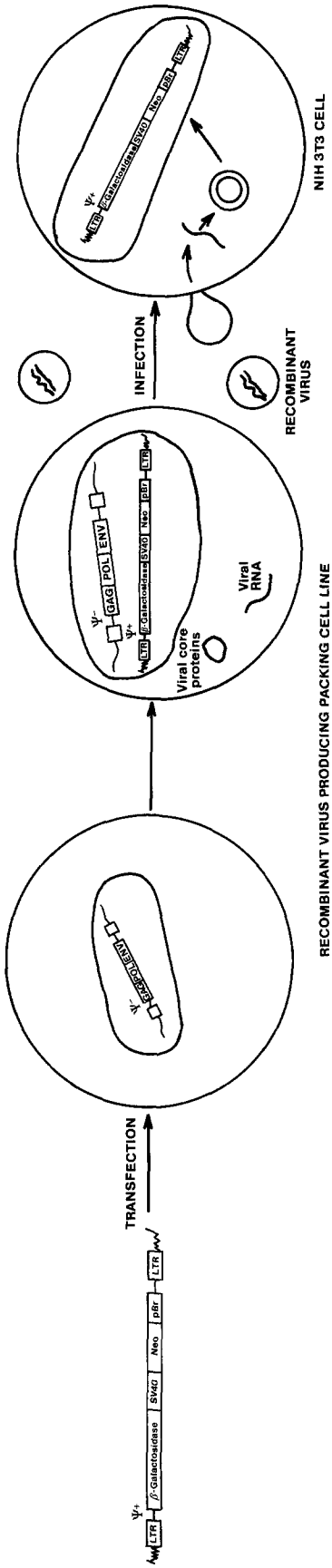


FIG. 3. Construction of recombinant cells expressing β-galactosidase. The packaging cell line containing retroviral *trans* functions is transfected with the recombinant retrovirus construct containing LTR and *cis* function, including the genes of interest and β-galactosidase (left). The resulting recombinant virus (infection defective) is used to infect the NIH 3T3 cells and the genes of interest are stable when integrated into the NIH 3T3 genomic DNA.

in Fig. 2. Imagene (another substrate for β-galactoside, from Molecular Probes) was tested in our experiments without success.

Before the cells were plated, the "Cookie Cutter" culture dishes were sterilized under a uv lamp for 20 min and the dishes were rinsed with sterile phosphate-buffered saline (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl, pH 7.2).

Cultures were grown in D medium containing 10% calf serum and 0.5% gentamycin. D medium is modified Eagle's medium containing Earle's balanced salt solution with a 50% increase in vitamins and essential amino acids, 1 mM sodium pyruvate, 5.5 mM glucose, 17.3 mM NaCl, and 11.9 mM $NaHCO_3$. Overnight cultures were washed with staining medium (D + 4% calf serum + 10 mM Hepes, pH 7.2) to remove excess serum. Cells were loaded by hypotonic shock (1:1 (v/v) staining medium and water) with prewarmed FDG for 5 min in a 5% CO_2 incubator at 37°C, at a final concentration of 0.5 mM. Loading was terminated by washing the cells with ice-cold staining medium and incubating on ice for 1 h before scanning and selection (6). Dye retention was found to be better at 4°C than at 37°C or RT. Prior to selection by Cookie Cutter, 20 mM cysteamine (final concentration) was added to the dishes to scavenge free radicals.

MODEL SYSTEM

Replication-defective retrovirus with amphotrophic host range was used to introduce a reporter gene, *E. coli lacZ,* into the genomic DNA of NIH 3T3 cells as shown in Fig. 3. The BAG virus produced (10) was used to infect NIH 3T3 cells. The structure of the retrovirus construct used for transfecting a replication-defective packaging cell line has been previously described (11).

Briefly, in the retrovirus construct, most of the internal sequence of the retrovirus was replaced with *E. coli lacZ,* SV40, and neomycin genes. The recombinant retroviral DNA (vector) contains the two ends of the viral genome called long terminal repeats (LTR) and the most important and necessary portion of the viral genome for replication, the *cis* function. The hybrid DNA was introduced into a packaging cell line, especially engineered *in vitro* to be defective in *cis* function but containing all the *trans* functions, which are the proteins necessary for the formation of infectious virions. These packaging cells express viral proteins (GAG, POL, ENV) but cannot encapsulate viral RNA into a virion due to the lack of the *cis* functions.

By transfecting the packaging cells with the recombinant vector DNA by the calcium phosphate technique, the packaging cells can complement the *trans* functions that are missing in the incoming vector DNA. The vector DNA is transcribed into the RNA and encapsulated into virions. This results in the production of a recombinant

infectious defective retrovirus which is secreted into the medium.

The gene transfer occurs when the target cells are infected with this recombinant infectious defective virus. These infectious defective virus particles were used to infect NIH 3T3 cells, and the *E. coli lacZ* gene was stably integrated into the NIH 3T3 cell genomic DNA. Cells were then selected for β-galactosidase expression using G418 (selection process for neomycin resistance in eukaryotic cells).

EXPERIMENTAL DESIGN

In the mixing experiment, various ratios of NIH 3T3 cells containing the recombinant β-galactosidase gene and uninfected parent NIH 3T3 cells were plated at a density of 1×10^5, as shown in Table 1. Approximately 30% of the positive cells plated were detected. However, when checked, the cloning efficiency of both infected and uninfected NIH 3T3 cells was found to be between 30 and 35% (data not shown).

When cells were plated at a ratio of 1 in 10^5, single colonies were identified in 2 out of 10 dishes. Given the plating efficiency, it was concluded that positive cells could easily be selected when the mixture was 10:100,000. The limitation to the selection process was that cells had to be plated at subconfluent densities. NIH 3T3 cells at confluency express endogenous β-galactosidase and this can blur the discrimination between positive and negative cells. Also, the QUIKLOOK program scans a maximum area of 2×2 cm; therefore, the ideal subconfluent plating density was 1×10^5 cells per 35-mm dish.

Toxicity due to the Selection Process

The cells were plated on a film-lined tissue culture dish treated with the heat-absorptive material. For cookie cutting and selecting β-galactosidase expressers, cells were stained with FDG by hypotonic shock as described above. Cells were rinsed and exposed to staining medium supplemented with cysteamine or varying concentrations of FDG. Cells in other dishes were subjected to hypotonic shock without FDG and/or left on ice in staining medium for several hours. The dishes were rinsed with staining medium, and growth medium (D + 10% FCS + 0.5% gentamycin) was added back to the cells. The cells were allowed to proliferate in a 5% CO_2 incubator at 37°C and were monitored over a period of 5–7 days for toxicity to these agents. No effect on cell viability and growth was observed when cells were exposed to the conditions listed in Table 2.

Identification of lacZ$^+$ Cells

Sorting of anchored cells under conditions of attachment and cell integration is of vital importance to cell biologists. Previously, various methods such as those involving the use of specific media, cloning rings or toothpicks and trypsinization of large quantities of cells for use in a flow cytometer have been employed to separate one cell type from a mixture of cells. Anchorage-dependent cells have physical properties that mandate that quantitative analytical measurements be conducted under these conditions. The ACAS Cookie Cutter method offers the versatility and flexibility to perform multiple experiments on the same cell with long-term monitoring (12). In employing this strategy, it is critical that cells be analyzed under appropriate conditions of spreading, differentiation, and growth and that cell sorting be conducted under the same *in vitro* growth conditions.

A 2×2-cm area was scanned on the ACAS using the laser beam diffuser and the QUIKLOOK program to generate a list of areas of fluorescence. The 1.5-μm-diameter laser beam was expanded to about 120 μm. The fluorescent locations were registered in the computer as XY coordinates with respect to the zero limit of the stage and a cell list was constructed automatically. Subsequently, cells in the cell list were sorted using the Cookie Cutter process (see below).

Using the ACAS 570, there are two methods for cell selection, laser ablation and Cookie Cutter. Both methods require growing cells on coverslips (ablation) or film-lined tissue culture dishes (Cookie Cutter) that have been treated with a special heat-absorptive material.

Cloning lacZ$^+$ Cells

For selection of rare events the Cookie Cutter procedure uses a high-intensity laser beam to isolate single cells. The cells of interest, growing on film-lined tissue culture dishes, are encircled with the laser such that the plastic film is fused to the surface of the tissue culture dish (12). The large unwanted piece of film is removed under sterile conditions, leaving "cookies" containing the desired cells attached to the tissue culture dish. This has advantages

TABLE 1

Mixing Experiment to Identify β-Gal-Positive Cells Using the ACAS 570

No. of NIH 3T3 cells	No. of NIH 3T3 BAG-infected Cells	Ratio +:− cells	No. of + cells identified
1×10^5	1000	$1:10^2$	>100
1×10^5	100	$1:10^3$	30
1×10^5	10	$1:10^4$	2
1×10^5	1	$1:10^5$	0.2[a]

Note. Numbers of β-galactopyronoside expressing cells identified in a background of NIH 3T3 parent cells are shown. The number of positive cells identified was approx 30% of the number of cells plated. All experiments were performed in triplicate.

[a] Ten dishes were scanned and single clones identified in two dishes.

TABLE 2

Treatments Tested for Cell Viability

Parameters tested	Time	Concentration (mM)
Ice	2 h	
	4 h	
	6 h	
Staining media	1 h	
	2 h	
	6 h	
Hypotonic shock at RT and 37°C	1 min	
	2 min	
	5 min	
	10 min	
FDG staining		0.5
		1.0
		2.0
Cysteamine		20.0

Note. Cookie dishes plated with mixed population of cells in triplicate were tested for different parameters. The dishes were rinsed, supplemented with growth medium, and allowed to continue to proliferate in a 5% CO_2 incubator at 37°C. The cells grew to confluency.

over other current methods, which require removal of cells from their natural growth surface and may result in changes in membranes, alteration of receptors, and loss of cell–cell interactions. Using the Cookie Cutter technique, cells that are difficult to dissociate into viable single-cell suspensions, cells that bind lectins differentially, cells with different cytoskeleton components, different receptors, or other membrane markers, and/or cells that can be visually separated by morphological differences can be easily selected in their attached state from within large populations. The advantages of this method include speed, low amounts of cells and substrate requirement, and the ability to select cells in their natural state.

Cells at various ratios of positive and negative expression of β-galactosidase were cultured on a heat absorptive film-lined tissue culture dish at a standard density of 1×10^5 cells for the experiments described here.

In this study, cells expressing β-galactosidase were fluorescently labeled with FDG. Each fluorescent location was registered in the computer with regard to an XY coordinate, as described in QUIKLOOK (Fig. 4). Following detection by QUIKLOOK, the laser beam was switched to higher intensity and cookies were cut around the desired positive cells. The outermost octagon welded the film to the culture dish; optional "killing" octagons and manual laser pulsing were used to eliminate unwanted cells. Within a cookie, laser power for the inner octagons was kept at a lower intensity. The purpose of the inner rings was to minimize the heat and at the same time kill unwanted cells further away from the cell of interest. This resulted in a small cookie remaining on the dish to which the cells remained anchored. Unwanted cells outside the cookies were removed under sterile conditions using forceps to pull the film off the tissue culture dish, leaving behind cookies with the attached cells undisturbed in the dish. The dish with the cookies was rinsed, supplemented with growth medium, and placed in a 5% CO_2 incubator at 37°C to continue proliferation. The sequence of events followed by a demonstration of viability after cell isolation is shown in Fig. 5. The proliferation of these cells was followed over several days and it was found that when the laser intensity required for welding was kept at a suf-

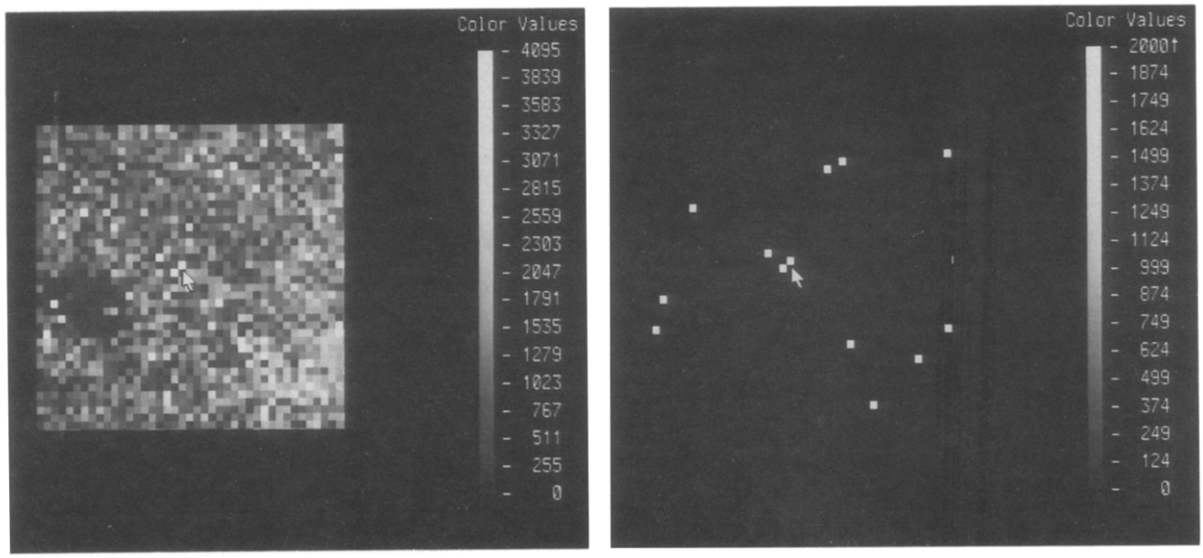


FIG. 4. QUIKLOOK—raster scan to identify locations of fluorescence. Left: An area of 2×2 cm was scanned using the laser beam diffuser. Each pixel in this scan has an area of 120×120 μm. Right: After thresholding to remove background fluorescence, only the brightest fluorescent locations remain to be included in the cell list for either cloning or ablation sorting.

ficient distance from the desired cells, the cells remaining on the cookie could proliferate, overgrowing the welded points onto the clear tissue culture dish surface. The diameter of the cookie may differ from cell type to cell type because some cells are more fragile than others. The laser intensity used for welding is very high, which may result in the generation of heat and possibly free radicals from the plastics. For optimal results, cold medium was used to neutralize the heating effect and the medium was supplemented with 20 mM cysteamine to scavenge free radicals. In our mixing experiment using QUIKLOOK and FDG staining, we were able to identify 1 positive cell in 10^4 negative cells.

Differences in clonability, growth rates, and heat sensitivity were diminished by altering parameters, including laser intensity, size of the cookie, etc. In our mixing experiments with NIH 3T3 cells, we found that when we left a single cell in the cookie, we needed to add conditioned medium to the dish for the cell to proliferate and grow. However, if we had 3–5 cells, they grew well in conditioned or nonconditioned medium. We also observed that when we subjected the same cells twice to hypotonic shock within 24 h, the cells no longer proliferated. These cells were checked for viability with carboxyfluorescein diacetate and were found to be viable (data not shown).

Enriching Cells for High Expressors of β-Galactosidase in a Heterogeneous Population

To enrich cells from a heterogeneous population, ablation sorting is the method of choice. The dark surface on the coverslip converts high-intensity laser beam illumination into heat. Using the ablation software, one can opt to kill either fluorescently labeled or unlabeled cells by the choice of threshold parameters. Cells anchored to the growth surface, which are to be excluded, are killed by high-intensity laser illumination. Cells that are to be selected are spared from killing intensity illumination and can then proliferate. NIH 3T3 cells that contained the β-galactosidase were initially selected with G418 and colonies expanded; however, β-galactosidase expression in a cloned population was found to be very heterogeneous. Figure 6 shows a typical ablation sort to enrich for high expressers of β-galactosidase. In this experiment the cells were imaged using a photomultiplier detector coupled with a 12-bit ADC (analog digital converter), resulting in a fluorescence data range of 0–4095. Highly fluorescent cells were saved by setting parameters to kill at a threshold value of fluorescence below 2500. The border distance around the saved cells (set at 25 μm) and the blast distance (frequency of kill lines, 10 μm), can be set independently and are dependent on the cell type being used. For NIH 3T3 cells, a 25-μm border distance was set to keep the killing intensity laser beam from coming any closer than 25 μm to the high expressers. Each pixel that is below 2500 and outside a border will be killed by the laser beam, thereby eliminating the low expressers (Fig. 6).

Image Analysis

Fluorescence image analysis was performed on NIH 3T3 cells infected with β-galactosidase and on uninfected controls. Two-dimensional pseudocolor images were gathered. Quantitative fluorescence analysis of live cells

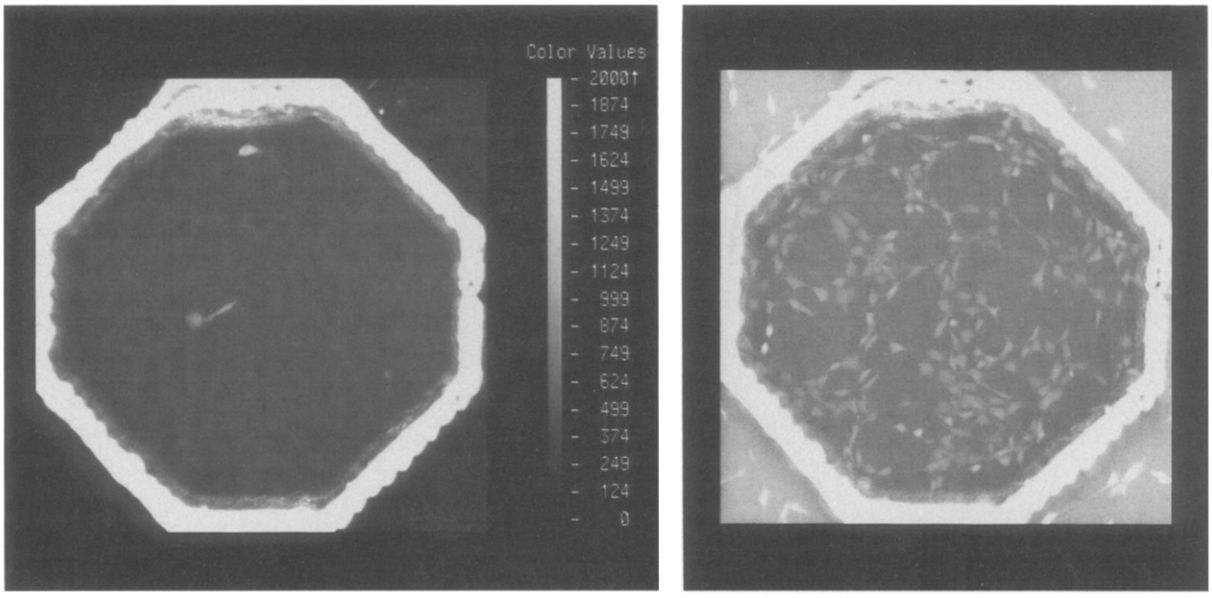


FIG. 5. Cookie Cutter—cloning cells. NIH 3T3 cells were grown on Cookie Cutter film-lined dishes and stained for FDG. An octagonal cookie was cut around cells expressing β-galactosidase and the remainder of the film containing unwanted cells was peeled away. Left: Fluorescence scan of a cookie showing a few cells. Right: Same cookie 8 days later. Note that all the cells are expressing β-galactosidase.

provided information on the expression and subcellular localization of the β-galactosidase over a large population.

Several fields were scanned and the image data gathered were consolidated into a histogram representing the number of cells analyzed versus the average fluorescence within a population. The distribution data of β-galactosidase activity indicate that the probe was uniformly distributed within a cell, and that heterogeneity exists in levels of expression even within a cloned population. Figure 7A shows one such image scan of NIH 3T3 cells infected with β-galactosidase demonstrating heterogeneity. Figure 7B is a consolidated histogram of several scans showing several distinct peaks in this case. The software provides statistical information on each peak, which is also shown in the figure.

Fluorescence and transmitted image scans were also done using the ACAS 570 confocal accessory. A positive population of NIH 3T3 cells expressing β-galactosidase, negative parent NIH 3T3 cells not expressing β-galactosidase, and a mixed culture of a few positive cells in a background of negative cells were used.

Fluorescence image scans were gathered first to avoid photobleaching and loss of fluorescence signal. This was followed by a transmitted scan of the same field. In order to acquire a transmitted scan, the laser path was closed, the microscope light was used as the source of illumination, and the variable pinhole on the confocal option was set at 50 μm. All other parameters were kept the same for both fluorescence and transmitted images to obtain perfect registration when the two scans were superimposed as shown in Fig. 8.

FUTURE APPLICATIONS

Interest in developing recombinant retroviruses has stemmed from the stable introduction of genes in a wide variety of recipients both *in vivo* and *in vitro*. The beneficial feature of the system is the capability to shuttle recombinant vectors between animal and bacterial cells (13).

The practical applications described in this paper are broad. The clonal selection of recombinant cells without trypsinization and soon after the infection process enables one to bypass the neomycin selection process. This in itself is advantageous because neomycin (G418) selection, as already reported, alters the cellular morphology (14). We have used this application to select caterpillar cells expressing β-galactosidase 24 h after infection with a recombinant baculovirus (data not shown).

The rapid isolation and clonal expansion with this method would be useful in purifying DNA from genomic DNA and/or mRNA in large quantities for further analysis and characterization.

Other possible applications include the study of gene regulation. By using different promoter sequences to drive the recombinant vector DNA, it is possible to access the functional efficiency of the promoter. We have applied this technique in our vector DNA construct used to infect primary rabbit hepatocytes. Our results indicate significant and consistent patterns of differences in levels of expression of β-galactosidase (unpublished data). On the other hand, infecting a variety of cell types using the same promoter may help delineate information on specificity

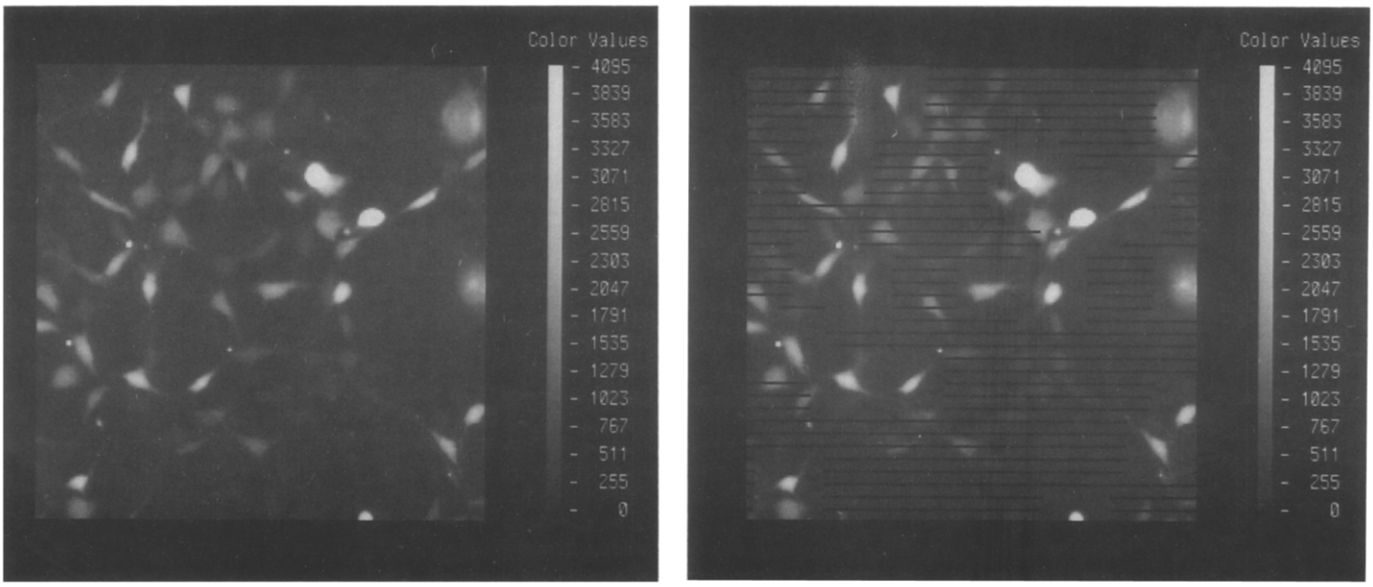
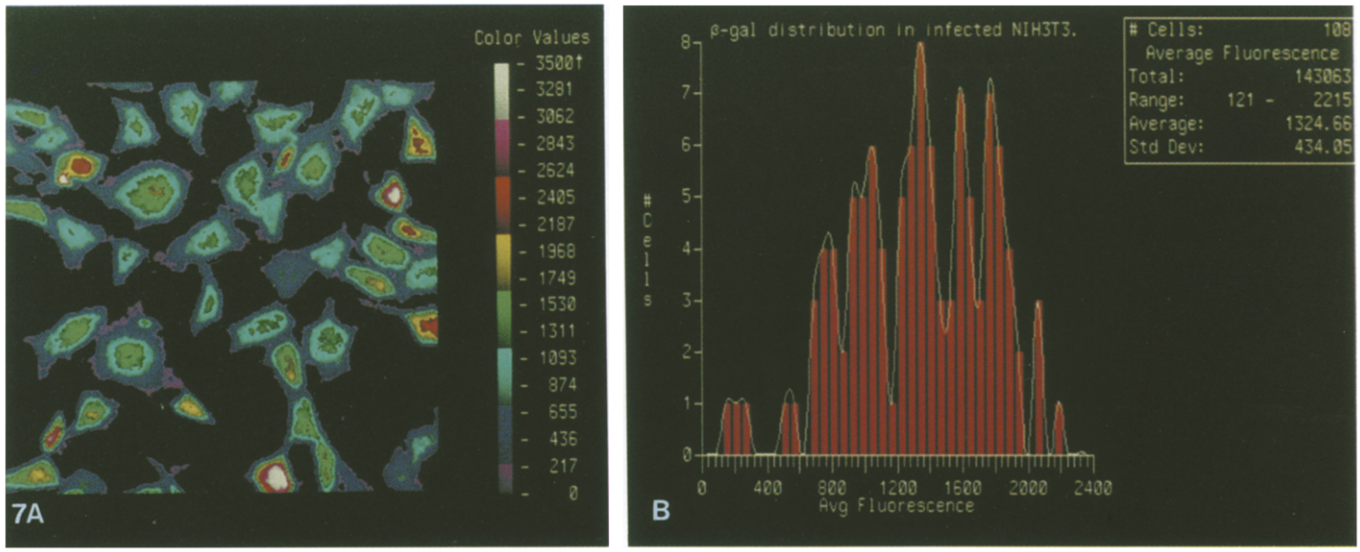


FIG. 6. Ablation sorting. Left: A 360 × 360-μm area is scanned at a step size of 1 μm and image data are gathered. Right: The same image is shown after the killing, with the high expressers saved and the low expressers eliminated. A 25-μm border was left around the cells of interest.



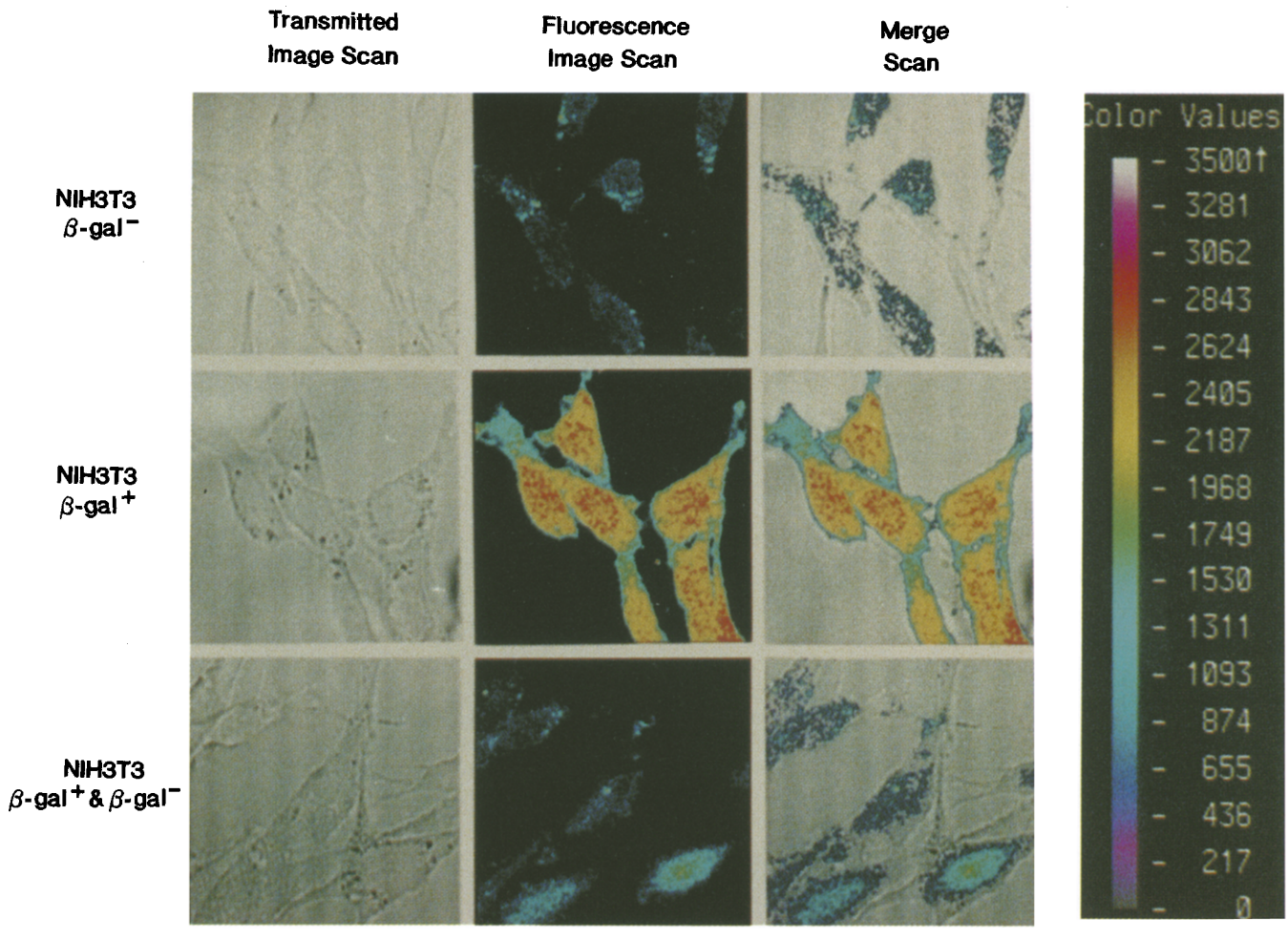


FIG. 7. Image analysis and histogram data presentation. (A) A two-dimensional, pseudocolor image scan of FDG-labeled NIH 3T3 cells expressing β-galactosidase. (B) Several image scan data are consolidated into a histogram to show average fluorescence distribution of β-galactosidase in a cloned population with statistics for this population in upper right corner.

FIG. 8. Transmitted, fluorescence, and merge images using confocal ACAS. Transmitted (phase contrast) images were gathered using the ACAS confocal accessory. Left: The pinhole was closed to 50 μm and the overhead microscope light was used as the source of illumination. Center: Fluorescence images were gathered at the same settings using the laser as the source of illumination. Right: Using the Merge Scan option in the software, the two images are superimposed, resulting in precise registration of the two scans.

and differential integration sites, to better understand mechanisms of gene regulation. This could lead to the development of a clinical assay using the ACAS 570 to quantitate the distribution and levels of the gene product on a per cell basis.

This technology will be a powerful tool in conducting gene therapy, gene replacement, and studies for drug delivery systems due to the ease, minimal manipulation, and time required to obtain a virtually pure and potentially homogeneous population of the recombinant cells.

Currently, methods for cell lineage analysis include the use of recombinant cells with *E. coli lacZ* (reporter molecule), FDG or X-gal (staining), and the flow cytometer to select for positive population and to monitor cellular differentiation (10). The advantages of using the ACAS assay system for cellular differentiation and developmental studies are severalfold. First, the cells are not put through any physiological stress and/or biochemical changes due to trypsinization. Second, cells can be viably stained and the progress of the same cell can be monitored over a period of time. Finally, quantitative data on individual cells can be obtained during the course of development.

Specific applications of this technology include, but are not limited to, the secretion of hormones, drugs, and/or proteins to specific organ sites to circumvent genetic disease processes. Such studies are in progress in many laboratories (14–16). Recombinant endothelial cells are being used for implantation of grafts (17), delivery systems for biologics, hormones (rat growth hormone), and proteins (adenosine deaminase) which prevent the metabolite, adenosine, from reaching toxic levels in the body (14). These are a few examples of the described technology possible in the research of human genetic disorders.

SUMMARY

Using the ACAS 570 Interactive Laser Cytometer and the fluorogenic β-galactosidase substrate FDG, expression of β-galactosidase in adherent individual mammalian cells was analyzed, permitting discrimination between $lacZ^+$ and $lacZ^-$ cells. One positive cell in 10,000 negative cells was identified and selected using the QUIKLOOK program.

Quantitative fluorescence measurements on individual viable cells were performed on the basis of levels of fluorescence to identify low and high expressers of β-galactosidase. Cells expressing specified levels of β-galactosidase activity were cloned by the ACAS Cookie Cutter program without the cells being removed from their environment. For NIH 3T3 cells cloned by this technique, conditioned medium was necessary for growth if there was only a single cell on the cookie, whereas groups of three to five cells grew well in either conditioned or non-conditioned medium. The FDG hypotonic shock, cysteamine, and cold temperatures were shown to be nontoxic to cells. Dye retention in cells was better at 4°C than at room temperature or 37°C.

Selection and sorting of cells expressing the reporter molecule β-galactosidase will be important in studying differentiation, gene regulation, selection of hybridomas, gene arrangements and rearrangements, and transcription controlling factors.

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