CHAPTER 19

Flow Cytometric Analysis and FACS Sorting of Cells Based on GFP Accumulation

David W. Galbraith*, Michael T. Anderson†, and Leonard A. Herzenberg†

* Department of Plant Sciences
University of Arizona
Tucson, Arizona 85721

† Department of Genetics
Stanford University Medical School
Beckman Center, B007
Stanford, California 94305-5125

I. General Introduction
  A. Principles of Flow Cytometry and Cell Sorting
  B. Green Fluorescent Protein (GFP)
  C. Fluorescence Characteristics of GFP
II. Methods and Specific Applications
  A. Preparation of GFP-Expressing Cells of Higher Eukaryotes
  B. Preparation of GFP-Expressing Bacteria
  C. Flow Cytometry and Sorting
III. Typical Results
  A. Mammalian Cell Systems
  B. Plant Cell Systems
  C. Bacteria
IV. Discussion and Conclusions
  References

I. General Introduction

A. Principles of Flow Cytometry and Cell Sorting

The technique of flow cytometry involves the optical analysis of biological particles and cells, constrained to flow within a fluid stream through the focus

METHODS IN CELL BIOLOGY, VOL. 58

0091-679X/99 $25.00

of an intense source of light, thereby absorbing and scattering light. The resultant scatter and fluorescence signals are detected as intensity-versus-time waveforms using photomultipliers (PMTs) and photodiodes. These pulse waveforms are digitized, and the values are stored either on a cell-by-cell basis or in the form of population frequency distributions. The data produced by flow cytometry preserves optical differences between cells, and subpopulations of cells having different optical characteristics can be recognized and their numbers determined.

The optical characteristics of cells are determined in part by physical parameters, which affect the intensities of light scattered both at angles close to the axis of illumination (forward-angle light scatter, FALS) and orthogonally to the axes of illumination and fluid flow (90° light scatter). Fluorescence emission by cells reflects either endogenous autofluorescence or application of exogenous fluorochromes. A great variety of fluorochromes are available either as synthetic chemicals or as purified natural products. These either have intrinsic specificity, for example, the various dyes that bind to DNA, or are covalently linked to carriers with particular binding characteristics (the prototypical example being the linking of fluorescein to antibodies). Based on the specificities of these fluorescent molecules, flow cytometry can therefore provide a sensitive measure of a large variety of cellular parameters.

From a practical viewpoint, the type of illumination that can be applied to the cells depends on the resources available to the flow cytometric facility. Commercial instruments, under increasing cost constraints, are minimally equipped with an air-cooled argon laser emitting light of 488 nm. With increasing fiscal resources, flow cytometric facilities can be equipped with instruments having water-cooled argon and krypton lasers. These provide tuned illumination either in the UV or at various visible wavelengths from about 400 to 800 nm. Fully equipped, flow cytometric facilities have instruments with multiple independent sources of laser illumination, each tuned to a specific wavelength.

From a technical viewpoint, the point of illumination and analysis of the fluid stream either is done in air or within an enclosed cuvette. In either case, emergence of the fluid stream into air involves passage through an orifice of precise diameter, typically 40–400 μm). The technique of cell sorting derives from the principle that fluid streams in air are unstable and necessarily decay into spherical droplets as a consequence of minimizing free energy. Imposition of a harmonic oscillation on the fluid stream precisely constrains the point of droplet formation, which therefore corresponds to a precise point in time after a given cell has been analyzed. Application of a voltage to the fluid stream at the time this cell occupies the last-attached droplet imposes a corresponding charge on the surface of the newly forming droplet. The detached droplet then can be deflected by passage through a fixed HV electrostatic field. Because the process of fluid charging can be rapidly and reversibly switched, cell sorting at high rates is readily achieved.

B. Green Fluorescent Protein (GFP)

GFP, the endogenous fluorochromatic protein found in the marine invertebrate *Aequorea victoria,* is a single polypeptide of 238 amino acids having a molecular

mass of 26 kDa. The cloning of the full-length cDNA (Prasher *et al.,* 1992) led to the discovery that GFP expression, resulting in cytoplasmic fluorescence, can be readily achieved for a variety of prokaryotic and eukaryotic species (Chalfie *et al.,* 1994). Fluorochrome formation involves a series of posttranslational intramolecular reactions, involving cyclization and autoxidation of amino acids 65–67 (Ser-Tyr-Gly) to generate a *p*-hydroxybenzylidine–imidazolidinone fluorescent chromophore (Heim *et al.,* 1994). This reaction requires neither cellular cofactors nor prosthetic groups. At the moment, GFP occupies a unique position as an *in vivo* marker of gene expression and has been extensively employed in studies of transcription, translation, and protein targeting.

C. Fluorescence Characteristics of GFP

The fluorophore of wild-type GFP has an excitation spectra that is complex with absorption peaks at 395 nm (major), and 475 nm (minor). In contrast, its emission spectrum is unimodal, peaked at approximately 509 nm. Biochemical analysis indicates the bimodal excitation spectrum is a consequence of two distinct states of the fluorophore (Cody *et al.,* 1993). When electrically neutral, the fluorophore exhibits maximal absorbance at 395 nm, whereas when the fluorophore is negatively charged, this peak of absorbance shifts to 475 nm. In its native context, the ionization state of the fluorophore is determined by a hydrogen bond network formed between the fluorophore and the side chains of the surrounding amino acids (Brejc *et al.,* 1997). The extent to which this network forms determines the ratio of the excitation maxima at these two wavelengths. Amino acid substitutions either in the fluorophore or in the surrounding sequences can influence the extent to which this network forms and thereby alters the excitation peak ratio.

Variants that are altered in their 395/475-nm excitation ratios or those that possess new wavelengths of excitation and/or emission form the spectral class of GFP variants. The mutations found in this class have significantly contributed to the construction of several distinct GFP reporters suitable for use with the flow cytometer. The first subclass of spectral variants is characterized by a unimodal absorption spectrum peaked at 395 nm. Members of this subclass have been designated Vex, reflecting their violet excitation. The two Vex variants thus far described share an threonine to isoleucine substitution at amino acid 203 (Heim *et al.,* 1994; Ehrig *et al.,* 1995). This substitution disrupts the hydrogen bond network formed with the fluorophore (Ormo *et al.,* 1996; Brejc *et al.,* 1997). As a consequence, tyrosine 66 is fully protonated, leading the fluorophore to be electrically neutral. For flow cytometric applications, the recently introduced Innova 300 krypton laser, which produces a powerful 407-nm line, is particularly suited for excitation of GFP variants of the Vex subclass (Anderson *et al.,* 1996).

A second subclass of GFP spectral variants is characterized by unimodal excitation spectra peaked at wavelengths near the 475-nm excitation peak of wild-type GFP. Variants of this subclass are designated as Bex due to their excitation in

the blue. To date, nearly a dozen different Bex variants have been isolated (Delagrave *et al.*, 1995; Heim *et al.*, 1995; Cormack *et al.*, 1996). Among these is the popular "red-shift" variant, possessing a serine to threonine substitution at amino acid 65 (Heim *et al.*, 1995). The amino acid substitutions in the Bex variants stabilize the hydrogen-bonding network formed with the fluorophore (Brejc *et al.*, 1997). This strengthened network promotes dissociation of a proton from the phenolic hydroxyl on tyrosine 66, leaving this amino acid negatively charged. The excitation peaks of the Bex variants tend to be at slightly longer wavelengths than the 475-nm peak of wild-type GFP. This shift partly accounts for the increase in brightness found with Bex variants (up to sevenfold higher), as compared to wild-type GFP, when they are excited by the 488-nm argon line (Heim *et al.*, 1995). This increase also results from a greater proportion of the Bex fluorophores being in an ionization state that is excitable in the blue. The extinction coefficients and quantum yields of both Bex and Vex are at least as great as those of wild-type GFP and comparable to those of fluorescein.

Members of the third subclass of GFP spectral variants have excitation and/or emission spectra that differ in their peak wavelengths from those found for wild-type GFP. One member of this subclass has an excitation peak in the UV at 382 nm; its emission peak is at 488 nm. It possesses a tyrosine to histidine substitution at amino acid 66 (Heim *et al.*, 1994). Application of a consistent nomenclature to this variant designates it as Uex. Although readily distinguishable from wild-type GFP and from the Vex and Bex variants, the Uex variant has a lower extinction coefficient and quantum yield (Heim and Tsien, 1996). Its relative dullness in terms of flow cytometric analysis is compounded by the absence of an available strong laser line coincident with the Uex excitation peak (this lies midway between the 350-nm argon and the 407-nm krypton laser lines) (Ropp *et al.*, 1995, 1996). Recently another member of this subclass has been described; it has an excitation peak at 513 nm and an emission peak at 527 nm (Ormo *et al.*, 1996). It was constructed by substituting glycine for serine at amino acid 65 and histidine for serine at amino acid 203. The histidine substitution is thought to increase the excitation and emission wavelengths by extending the electron conjugation system of the fluorophore. Applying a consistent nomenclature to this variant designates it as Gex due to its excitation peak in the green.

In addition to the class of spectral variants, two other classes of GFP variants have provided important contributions in for the construction of reporters suitable for fluorescence-activated cell sorting (FACS). One of these classes is characterized by greater than wild-type thermostability in terms of establishment of the native three-dimensional structure of the protein molecule. Whereas a significant proportion of wild-type GFP molecules fail to properly fold and cyclize when synthesized at 37°C, variants of this class fold correctly and therefore are significantly brighter. This effect, not seen at 22°C, is conferred by several different amino acid substitutions, and in some instances these mutations can function additively (Anderson *et al.*, 1996; Crameri *et al.*, 1996; Heim and Tsien, 1996; Siemering *et al.*, 1996; Kimata *et al.*, 1997). One member of this class of variants

has a valine to alanine substitution at amino acid 163, which increases its fluorescence by about fivefold when expressed in mammalian cells (Anderson *et al.*, 1996). Combinatorial incorporation of multiple mutations of this class of mutations can lead to an overall tenfold increase in fluorescence (Anderson *et al.*, 1998b).

The final class comprises GFP variants that provide enhanced levels of intracellular fluorescence over that of wild-type GFP, but without changes in the amino acid sequence. Variants of this class produce mRNA species that are more efficiently translated than mRNA produced from the wild-type coding sequence. This appears largely a consequence of codon bias; some codons found in the GFP cDNA from the jellyfish *Aequorea victoria* are infrequently utilized in yeast, plants, insects, and mammals. Replacement of these codons with ones preferred within the organism of interest increases GFP production (Chiu *et al.*, 1996; Ha *et al.*, 1996; Pang *et al.*, 1996; Yang *et al.*, 1996; Zhang *et al.*, 1996; Zolotukhin *et al.*, 1996; Cormack *et al.*, 1997; Kimata *et al.*, 1997; Muldoon *et al.*, 1997; Rouwendal *et al.*, 1997). For mammalian cells, the increase is approximately four- to fivefold.

Because independent mechanisms underly the increases in fluorescence seen in these three classes of variants, combining mutations from these different classes has resulted in the production of several spectrally distinct GFP reporters with greatly enhanced levels of fluorescence (up to 100-fold higher than that of wild-type GFP). This is important in terms of sensitivity because at most only a single fluorophore can be formed for each GFP molecule that is synthesized. In flow cytometry, a rule of thumb is that at least 500 fluorescein molecules are required for a particle or cell to produce a fluorescent signal that is above the background derived from cellular autofluorescence and instrument noise. It is considered that optimized S65T GFP and fluorescein produce roughly equivalent amounts of fluorescence, which renders GFP approximately 100-fold less sensitive than the FACS–GAL enzymatic assay. However, the versatility of GFP (at least three spectrally distinct variants are available for simultaneous flow cytometric analysis) provides obvious theoretical and practical advantages.

Considerable interest is emerging in the use of flow cytometry and sorting for the analysis of GFP fluorescence in prokaryotic and eukaryotic systems. This chapter does not claim to comprehensively cover all species, tissues, and cell types, and the reader is recommended to consult the following references for alternative methods (Ropp *et al.*, 1995, 1996; Lybarger *et al.*, 1996; Subramanian and Srienc, 1996; Bierhuizen *et al.*, 1997; Gervaix *et al.*, 1997; Kain and Kitts, 1997; Mosser *et al.*, 1997; Muldoon *et al.*, 1997).

II. Methods and Specific Applications

We have grouped the methods according to organism type, separately describing specific applications for higher eukaryotes (animal and plants) and for prokaryotes.

A. Preparation of GFP-Expressing Cells of Higher Eukaryotes

1. Mammalian Cells

Several methods are available to introduce GFP expression constructs into mammalian cells. The most widely used method is to transiently transfect the cells using lipofectin, calcium phosphate, or electroporation. In the majority of the transfected cells, the DNA construct is maintained as an extrachromosomal episome. In the rare cell, the construct stably integrates into the chromosome. Those cells with stable integrants can be selected by cotransfection of a drug resistance marker. However, retroviral infection is a more efficient method of producing cells with constructs stably integrated into their chromosomes.

In this chapter, we detail a calcium phosphate–mediated transfection protocol. It is a generally useful protocol for transiently introducing GFP expression constructs into adherent cells. A more specific application of this protocol is to introduce defective retroviral constructs into either the BOSC 23 or the Phoenix producer lines (Pear *et al.*, 1993; Kinsella and Nolan, 1996). These lines are derivatives of the highly transfectable 293 human cell line. Each of these producer lines has two constructs integrated within its chromosomes. These constructs function to complement the genes absent from defective Moloney retroviruses. One of these integrants constitutively expresses the viral *gag* and *polymerase* genes; the other constitutively expresses the viral *envelope* gene. When either producer line is transiently transfected with defective retroviral constructs, it can rapidly produce high-titer retrovirus, which can be used for infection of susceptible cells (Pear *et al.*, 1993; Kinsella and Nolan, 1996).

The marrying of GFP reporters with this method of retroviral production is likely to gain considerable popularity as biology moves toward efficient assay of the function of genes through complementation analysis. Together, these methodologies permit the selection of cells expressing the gene of interest within 4 days of preparation of the retroviral construct.

a. Biological Materials

We have used the Moloney retrovirus derivative MFG (Anderson *et al.*, 1996; Krall *et al.*, 1996) as a retroviral vector. Within many cell lines, the MFG LTR directs high levels of expression from genes that are inserted within this vector. Other useful retroviral vectors include the pBABE series of SIN retroviruses, which lack functional LTRs and express inserted genes from internally placed *cis*-acting sequences (Hofmann *et al.*, 1996; Naviaux *et al.*, 1996). The transcripts from either series of retroviruses can be made dicistronic by inclusion of an internal ribosomal entry site (IRES) within the transcript (Hofmann *et al.*, 1996; Mosser *et al.*, 1997). This allows two distinct proteins to be expressed from the same transcript, one of which can be a FACS-selectable GFP reporter.

b. Transfection Procedure

This procedure is detailed for the Phoenix or the BOSC 23 cell lines, but is applicable to many different mammalian cell lines.

a. Producer lines are cultured in supplemented Dulbecco's Minimal Essential Medium (sDMEM), supplemented to 10% (v/v) with a 1:1 mixture of fetal calf to horse serum, 100 U/ml each of penicillin and streptomycin, 2 m*M* glutamine, and 50 μM β-mercaptoethanol. The day before transfection, 2.5×10^6 cells of the producer line are plated onto each 60-mm tissue Falcon culture dish (Becton Dickinson, Franklin Lakes, NJ). The plate of cells is then placed overnight in a 37°C incubator at 5% CO_2. Immediately prior to transfection, chloroquine is added to the plates at a final concentration of 25 μM.

b. To prepare the transfection mixture, 10 μg of the DNA construct is pipetted into a FALCON 12- × 75-mm tube. Sufficient H_2O is added to bring the final volume to 438 μl. Then, 62 μl of 2*M* $CaCl_2$ is added followed by 500 μl of 2× HBS (50 m*M* HEPES, pH 7.05, 10 m*M* KCl, 12 m*M* dextrose, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4).

c. This solution is mixed by bubbling air from a 2-ml pipette through it for 10–20 s, and then the solution is added dropwise to the plate of producer cells. After this addition, the cells are returned to the incubator.

d. Between 8 and 12 h after transfection, the fluid in the plate is replaced with 3 ml of fresh sDMEM. This process is repeated at 24 h after transfection.

e. At 48 h after transfection, the retrovirus containing supernatant is decanted from the plate of cells and then centrifuged at 500 × *g* at 4°C for 5 min. The supernatant is then ready for use in infecting susceptible cells.

This protocol routinely results in transfection rates of 60–100%; such high rates are necessary to produce high-titer retrovirus. To achieve these rates, several aspects of the transfection process are critical. One of these aspects is the pH of the culture medium. This pH is established by the CO_2 concentration of the incubator. Therefore, the cell culture plates should spend only minimal amounts of time outside of the incubator. In practice, only the particular dish being transfected is transferred from the incubator to the tissue culture transfer hood. A second critical aspect is the pH of the HBS solution; although we define a pH of 7.05 as optimal for the 2x HBS solution, it is necessary to routinely prepare several different batches of this medium differing stepwise from pH 7.00 to 7.10. Each batch should then be evaluated as to which confers the highest transfection efficiency. A final critical aspect of this protocol is the length of time that transfection mixture is bubbled with air. Each individual bubbles this mixture with a slightly different level of vigor. Consequently, the length of bubbling time that produces the highest transfection efficiencies varies between individuals.

c. Viral Infection of Cells

a. For the retroviral infection of adherent murine cell lines such as NIH 3T3 with retrovirus, approximately 5×10^5 cells are seeded onto a 60-mm Falcon plate on the day before they are to be infected. To infect the cells, an aliquot of viral supernatant (titer $0.5–2.0 \times 10^6$ pfu/ml is added to the plate of cells and then the liquid on the plate is adjusted to 4 μg/ml in polybrene.

b. To infect nonadherent murine cells such as lymphocyte cell lines grown in suspension, we have incorporated a modification of the preceding protocol described by Bunnell *et al.* (1995). For the infection of cells, the virus and the cells are pelleted together by centrifugation at 1000 x *g* at 22°C.

In general we have been able to detect by flow cytometry GFP expression in as little as 18 h following retroviral infection.

2. Plant Cells

For flow cytometric analysis and sorting of plant cells according to GFP accumulation, we require production of single-cell suspensions that are expressing GFP. Two strategies are available: the first is the production of protoplasts, which are subsequently transfected, then examined for GFP expression; the second is the production of transgenic plants expressing GFP, which are then reduced to protoplasts prior to flow analysis and sorting. The first strategy is detailed in this chapter. This is followed by an analysis of transgenic accumulation of GFP, specifically employing nuclear targeting as a means for generating fluorescent nuclei that are suitable for FACS analysis.

a. GFP Expression in Transfected Plant Protoplasts

Preparation of protoplasts for different plant species follows defined protocols optimized for the different species and tissue types that are under study. The reader is referred to comprehensive references (see, for example, Gamborg and Philips, 1995).

i. Protoplast Preparation. Protoplasts are most commonly prepared from leaf tissues, which provide adequate supplies of starting materials that can be obtained easily in axenic form. The following protocol works well for two model plant systems: *Arabidopsis thaliana* and *Nicotiana tabacum. Arabidopsis* is accepted as a model for plant molecular genetic analysis, and tobacco is particularly amenable to tissue culture and transformation. The given protocol illustrates the important general concepts of protoplast preparation: use of an osmoticum, selective solubilization of the cell wall using polysaccharidases, recovery and purification of the protoplasts, and viability determinations.

a. Macerase, Cellulysin, and propidium iodide were obtained from Calbiochem, Inc. (La Jolla, CA), and fluorescent microspheres from the Coulter Corporation (Miami, FL). MS (Murashige and Skoog) medium was from Gibco (Grand Island, NY). All remaining chemicals were obtained from the Sigma Chemical Co. (Saint Louis, MO).

b. Plants are grown within standard growth chambers (Conviron) under sterile conditions in Magenta boxes on MS medium containing 3% sucrose. Tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants are maintained at 22°C under continuous light (Harkins *et al.*, 1990). *Arabidopsis* plants (*Arabidopsis thaliana* L. ecotype Columbia) are maintained at 22°C under continuous illumination.

c. All procedures are done using standard sterile technique. Fully expanded leaves are excised from axenic tobacco or *Arabidopsis* plants (approximately 600 mg wet weight), and are transferred into 20 ml of digestion medium, contained in a sterile plastic Petri dish. (*Note:* Repeated harvesting of leaves from individual plants is not recommended. Molecular changes to the cell wall and plasma membrane structure occur, probably as a consequence of wound-induced signal transmission through the body of the plant. Thus, second and subsequent harvests of leaves do not produce protoplasts at yields and viabilities comparable to those from the leaves of the first harvest.) The digestion medium comprises 0.1% driselase, 0.1% macerase, and 0.1% cellulysin, dissolved in a buffer containing 0.5 *M* mannitol, 10 m*M* $CaCl_2$, and 3 m*M* MES, pH 5.7. This medium is sterilized by Millipore filtration (GSWP 047). Incubation is continued at 22°C overnight (18–20 h) in darkness without agitation.

d. The protoplast suspension is filtered through two layers of sterile cheesecloth into a 50-ml sterile centrifuge tube, and centrifuged at 50 × *g* for 8 min.

e. Protoplasts are purified by centrifugal flotation using a step sucrose gradient. The pelleted protoplasts are gently resuspended in 20 ml of 25% (w/v) sucrose dissolved in modified TO medium (Harkins *et al.,* 1990). This is overlaid with 5 ml of W5 medium (Negrutiu *et al.,* 1987) and centrifuged at 50 × *g* for 10 min. Viable protoplasts, found at the interface, are carefully removed using a wide-bore Pasteur pipet and are diluted with two volumes of W5 medium. Protoplasts are counted using a hemocytometer and are recovered by centrifugation at 50 × *g* for 5 min.

ii. Preparation of Recombinant GFP Constructions. For transient expression, recombinant GFP constructs are prepared in high-copy-number vectors. Features of the constructs include promoter sequences for regulated or constitutive expression [the Cauliflower Mosaic Virus (CaMV) 35S promoter is frequently used for constitutive expression], the GFP coding region, and a transcriptional terminator. For expression in *Arabidopsis,* elimination of a cryptic splice site is critical (Haseloff *et al.,* 1997). Use of targeted gene fusions (translation fusions that direct GFP to specific cellular compartments) can also enhance fluorescence emission (Grebenok *et al.,* 1997a,b; Haseloff *et al.,* 1997). Preparation of these recombinant constructions is done according to routine procedures of molecular biology (Sambrook *et al.,* 1989).

iii. Protoplast Transfection. Protoplasts can be transfected by addition of polyethylene glycol (PEG).

a. For PEG-mediated transfection, protoplasts are resuspended in 600 μl W5 to a concentration of ~1.6×10^6 protoplasts/ml. Plasmid DNA and carrier (calf thymus) DNA are then added to a concentration of 20 and 50 μg/ml, respectively.

b. Tap the side of the tube to ensure that the protoplasts are well resuspended, then immediately add 1.5 volumes of PEG solution, comprising 40% w/v PEG 4000 (Baker Chemical Co.), 0.4 *M* mannitol, and 0.1 *M* $Ca(NO_3)_2 \cdot 4H_2O$, pH 7.0. Gently mix by resuspension using a 1-ml disposable Pipetman pipet tip.

c. Incubate at room temperature for 25 min.

d. Add eight volumes of culture medium [medium NTTO (Harkins *et al.*, 1990)], modified to contain 0.18 *M* glucose and 0.15 *M* mannitol, and supplemented with 75 mg/ml ampicillin).

e. Incubate for 18 to 24 h in darkness at room temperature.

Protoplasts can also be transfected by electroporation (for details of maize protoplast preparation, see Galbraith *et al.*, 1995).

a. For electroporation, the protoplast pellet is resuspended in an electroporation buffer comprising 0.6 *M* mannitol, 20 m*M* KCl, 4 m*M* MES, and 5 m*M* EGTA, pH 5.7.

b. The protoplasts are pelleted once by centrifugation at 100 x *g* for 10 min. Protoplasts (1.5×10^5) are resuspended in 0.3 ml of electroporation buffer and transferred into a plastic electroporation cuvette (gap width 4 mm; BTX, San Diego, CA).

c. Plasmid DNA is added (25–50 μg in 30–60 μl TE). Electroporation conditions involve a 10-ms pulse length, a field strength of 400–500 V/cm, a 200-mF capacitance setting, and a total of three pulses.

d. The protoplasts are transferred into plastic 6-well culture plates (Falcon 3046), kept on ice for 10 min after electroporation, and diluted by addition of electroporation solution (0.7 ml/well).

e. The tissue culture plates are covered with foil and incubated at room temperature for 15 to 18 h.

b. GFP Expression in Transgenic Plants

Transgenic tobacco plants expressing GFP are prepared via agrobacterium-mediated transformation. The methods are well established and routine, and need not be reiterated here (see, for example, Rogers *et al.*, 1986, and for the GFP constructions described in this chapter, Grebenok *et al.*, 1997a,b).

B. Preparation of GFP-Expressing Bacteria

When GFP is expressed in bacteria from appropriate transcriptional/translational controls, it generally produces a FACS-detectable fluorescent signal. This results from GFP folding properly within the reducing environment of the bacterial cytosol. To express GFP in bacteria, we and others have used the tight transcriptional/translational controls of the T7 bacteriophage (Heim *et al.*, 1994). These controls are present within the pET vector, which is available from Novagen Inc. (Madison WI). To express GFP from this vector, the GFP coding sequence is inserted between the BamHI site downstream of the T7 promoter/lac operator and the Xho I site upstream of the T7 terminator; this typically requires PCR-based manipulation to introduce flanking restriction sites. The resulting GFP expression vectors are then transfected into the BL21(DE3) strain

of bacteria by standard methodologies such as electroporation (Dubendorff and Studier, 1991). This bacterial genotype contains a lambda DE lysogen, which expresses the T7 polymerase under the control of the lacUV5 promoter. Growth of transfectants under noninductive conditions (high glucose, no IPTG) leads to little or no GFP expression. In contrast, significant levels of fluorescence reflecting increases in GFP expression are obtained under inducing conditions (removal of glucose and addition of IPTG).

C. Flow Cytometry and Sorting

a. Analysis and Sorting of Mammalian Cell Lines Expressing GFP

The following methods are platform independent but are detailed for a custom flow cytometer and FACS constructed in the Herzenberg laboratory. It is capable of excitation with three laser lines and possesses sufficient detectors to assay the fluorescent signal from Bex, Vex, and six immunofluorescence parameters. It consists of a FACStar Plus optical bench (Becton-Dickinson, San Jose, CA), MoFlo electronics (Cytomation, Fort Collins, CO), which collect and digitize the signals from the optical bench, and custom-designed electronics designed and built in the Herzenberg laboratory that control the PMT voltages and that handle data transfer to the computers.

a. The 488-nm line and the 595-nm dye laser line are both powered by a 5-watt Innova-90 argon-ion laser (Coherent, Sunnyvale, CA), run in "all lines" mode. The light from this laser is split by a dichroic mirror into its shorter and longer wavelength components. The shorter wavelengths are bandpass filtered to provide only 488-nm illumination. The longer wavelength lines are directed to a dye head tuned to 595 nm. The third laser line comes from an Innova-300 krypton laser tuned to produce light at 407 nm.

b. The signals collected include forward-angle light scatter (FALS), 90° light scatter (SSC), 488-nm excited GFP–Bex fluorescence (495–535 nm) and 407-nm excited GFP–Vex fluorescence (495–535 nm). A more detailed description of the configuration of this optical bench including the optical filters used to collect the immunofluorescence signals can be found in Roederer *et al.* (1998) and in Anderson *et al.* (1997a). The components of the optical bench are aligned with 3.2-μm multidye polystyrene test particles obtained from Spherotech Inc. (Libertyville, IL). PMT voltages are selected that result in the cells with and without GFP expression both being on scale.

c. Cells are analyzed at a flow rate of between 200 and 1000 events/s in a stream formed from a nozzle with a 70- to 80-μm orifice. Generally PBS is used as the sheath fluid; however, when cells are to be sorted for high viability, RPMI-deficient medium (Hyclone Laboratories, Logan, UT) lacking phenol red and biotin, is substituted.

d. To sort cells, a nozzle pressure of 12 psi is established. The electronics are set for a drop drive frequency of 30 kHz, the drop delay is set between 14 and 16, and the sorting setting is set at the two drops mode.

b. Analysis and Sorting of Plant Protoplasts Expressing GFP

The following two methods are also platform independent but are in this case described for the instrument currently available in the Galbraith laboratory (Elite, Coulter Electronics, Miami Lakes, FL). This is equipped with a 20-mW 488-nm argon-ion laser and a 100-μm-diameter sense-in-quartz flow tip.

a. Signals are collected for forward-angle light scatter (FALS), 90° light scatter (PMT1), and fluorescence [PMTs 2 (green; 505–545 nm) and 4 (red; 670–680 nm)]. Typical high-voltage and amplification settings are FALS, 260/10; PMT1, 350/7.5; PMT2, 850/10; and PMT4, 950/5. The FALS discriminator is set to 50, and all other discriminators are turned off.

b. The cytometer is aligned using fluorescent calibration particles (DNA Check, Coulter Electronics). Biparametric histograms are accumulated [log 90° light scatter versus log green fluorescence, or log red (chlorophyll) fluorescence versus log green fluorescence] to a total count of 100,000. Uniparametric histograms can also be acquired.

c. A sample flow rate of 100–200/s is employed for analysis of the protoplasts. The sheath fluid comprises 0.47 *M* mannitol, 50 m*M* KCl, 10 m*M* $CaCl_2$, and 4 m*M* MES, pH 5.7, filtered through a 0.22-μm filter (Millipore GSWP047) prior to use.

d. For sorting, the Elite is operated at a sheath pressure of 8.0 psi, a sample pressure of 7.3 psi, a drive frequency of 15 kHz, and a drive amplitude of 11.5%. This typically gives a sort stream having six free drops above the deflection plate assembly. Delay settings are systematically optimized by sorting batches of 25 particles onto a microscope slide for various delay settings. The proportion of recovered particles is then determined under the microscope. DNA Check calibration particles (diameter 10 μm) and Lycopodium spores (diameter 28 μm, Polysciences, Warrington, PA) are used for this procedure. Delay settings of 17 to 19 gave stable sort streams, using a 70% deflection amplitude. Samples were sorted at a rate of 25 to 50/s into 12- × 75-mm plastic tubes.

c. Analysis of Plant Nuclei Accumulating GFP

a. Nuclei are isolated from transgenic plants by chopping (Galbraith *et al.*, 1983). Leaf material (250 mg) is transferred into 2 ml of ice-cold homogenization medium and homogenized by chopping for approximately 1 min using a single-edged razor blade. The homogenization medium comprises 0.225 *M* mannitol, 50 m*M* potassium acetate, 20 m*M* HEPES–KOH, pH 7.3, 5 m*M* sodium acetate, 2 m*M* magnesium acetate, 2 m*M* PMSF, 0.125 m*M* spermine, 0.125 m*M* spermidine, and 5 mg/ml each of aminocaproic acid, aprotinin, leupeptin, and pepstatin A (Hicks *et al.*, 1996).

b. The homogenate is filtered through nylon mesh (pore size 60 μm, Tetko, NY).

c. Flow cytometry is done based on detection of FALS, 90° light scatter (PMT1), 505- to 545-nm fluorescence (green; PMT2), 555- to 595-nm fluorescence (orange; PMT3), and 670- to 680-nm fluorescence (red; PMT4), at a sample rate of 100/s. Typical high-voltage and amplification settings are FALS, 260/10; PMT1, 350/7.5; PMT2, 900/7.5; PMT3, 900/7.5; PMT4, 750/7.5. The PMT3 integral signal discriminator is set to 50, and all other discriminators are turned off.

d. Triggering is done on PMT3 fluorescence, enabling detection of signals both from nuclei containing GFP and from chloroplasts. Triggering should not be done on based on FALS, due to the excessive proportion of nonfluorescent, light-scattering particles in the homogenate. Uniparametric and biparametric histograms are collected to a total count of 5–10 $\times$ 10^5 particles, or for defined periods of time.

e. For sorting of nuclei using the 100-μm flow tip, the sheath and sample pressures are set to 12 and 11.5 psi. Typical settings are an analysis rate of 100/s, a drive frequency of 16.9 kHz and amplitude of 70%, and a delay setting of 16.4. Sorted nuclei are allowed to settle prior to observation under the microscope.

d. Analysis and Sorting of GFP-Expressing Bacteria

To analyze bacteria with the flow cytometer, the bacteria must be prepared as a single-cell suspension. To prepare a suspension from clonal colonies present on 100- $\times$ 150-mm Petri plates, 10 ml of LB are pipetted onto the plate, which is then incubated at 22°C, with gentle rocking for 10–20 min. An important consideration in this process is that none of the agar from the plate contaminate the suspension, because it can clog the fluidic lines of the flow cytometer. Immediately prior to FACS analysis, the suspension is diluted with phosphate buffered saline (PBS), to an extent that leads to a data acquisition rate on the flow cytometer of about 200 to 1000 events/s.

The analysis of bacteria on the flow cytometer is complicated by their small size. However, in contrast to debris, bacteria have a characteristically high side scatter, allowing this parameter to be employed as the discriminator. Bacteria can then be further distinguished with a forward-scatter gate set with a log gain. In general, the analysis of bacteria is done under a similar instrumentation set up to that used for mammalian cells. In the studies shown here, a standard dual-laser FACStar$^+$ was used. Excitation was from a Innova 70 argon laser producing 200 mW at 488 nm. In all other respects, except for the use of side scatter as the discriminator, the optical filters, analysis conditions, and sorting conditions were identical to those used with mammalian cells.

The analysis of bacteria and other tissue culture contaminants on the flow cytometer often raises concerns about the ability to sterilize the instrument for subsequent use with mammalian cells. After the analysis of bacteria on the flow

cytometer, we sterilize it by passing through the fluidic lines diluted dish soap followed by a 70% solution of alcohol.

III. Typical Results

A. Mammalian Cell Systems

The original GFP gene (WT-GFP) of *Aequorea victoria* produces too little fluorescent signal for it to be useful in FACS either as a selectable marker or as a transcriptional reporter. This is illustrated in Fig. 1, which employs FACS to compare the fluorescence emissions of uninfected 3T3 cells with those of 3T3 cells infected at a high multiplicity of infection (MOI) with MFG–WT-GFP; at this MOI, nearly every cell is productively infected. Comparison of their fluorescence intensity distributions shows that these distributions overlap, their median values differing by only a factor of two. This is in part due to the majority of the WT-GFP being improperly folded and in part due to only a minority of the productively folded GFP having fluorophores in a state excitable by the 488-nm line. It is also in part due to a high background from autofluorescence. For lymphocytes, this background signal is similar in intensity to that of at least 500 fluorescein molecules (Alberti *et al.*, 1988). GFP that is properly folded and that has a fluorophore in a state optimally excitable by the 488-nm line produces a fluorescent signal comparable to that of fluorescein (Cubitt *et al.*, 1995). Consequently, many more than 500 molecules of WT-GFP need to be expressed in order to exceed the background due to autofluorescence.

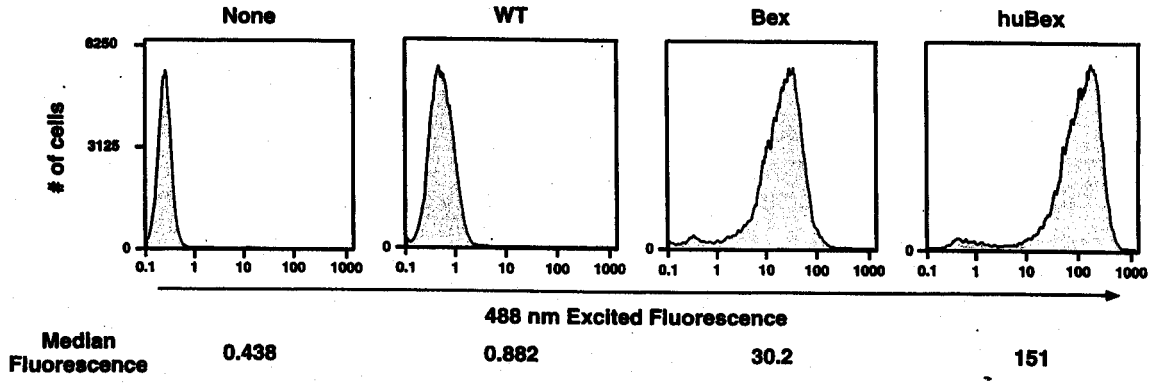

Fig. 1 FACS histograms of NIH/3T3 cells infected with either WT-GFP or improved GFP variants. NIH/3T3 cells were infected with (A) WT-GFP, (B) Bex–GFP, or (C) HuBex–GFP retrovirus and then analyzed by flow cytometery 48 h after infection. The MOI of each virus was similar and sufficient to infect nearly every cell. Emission data were collected with 488-nm excitation and emissions were collected between 495 nm and 535 nm. Representative histograms are shown.

This difficulty has been largely overcome with brighter versions of the GFP reporter obtained through either genetic screens or directed mutagenesis. These brighter versions of GFP produce adequate levels of signal to be used as selectable markers with flow cytometry and sorting. For example, 3T3 cells infected with MFG–GFP–Bex are completely distinguished from controls as a consequence of their GFP expression (Fig. 1). In these experiments, the increased brightness of the Bex variant derives from improved fluorochrome excitation at 488 nm because of the S65T mutation and an increase in the proportion of correctly folded GFP molecules because of the V163A mutation. When the Bex variant coding sequence is further modified to incorporate only those codons that are frequently used in mammalian cells (huGFP-Bex), even greater levels of fluorescence are produced by the infected cells (Fig. 1).

Questions do remain as to whether these bright GFP variants will turn out to be generally applicable as transcriptional reporters. We have shown their utility as transcriptional reporters in transient transfection analysis using flow cytometry (Anderson *et al.*, 1996). They have also proven useful as reporters of inducible constructs stably integrated into the chromosome (Gervaix *et al.*, 1997). However, even the brightest GFP reporters are likely to be less sensitive than other reporters used in flow cytometry, such as β-galactosidase and β-glucuronidase; these GFPs all lack the property of signal amplification inherent to substrate to product conversion by enzymatic reporters (Nolan *et al.*, 1988; Lorincz *et al.*, 1996). The overall sensitivity of GFP reporters is also a function of the stability of the GFP protein and its mRNA. Both the GFP mRNA and GFP protein appear relatively stable, because the GFP signal progressively increases until 36–48 h after infection (Fig. 2).

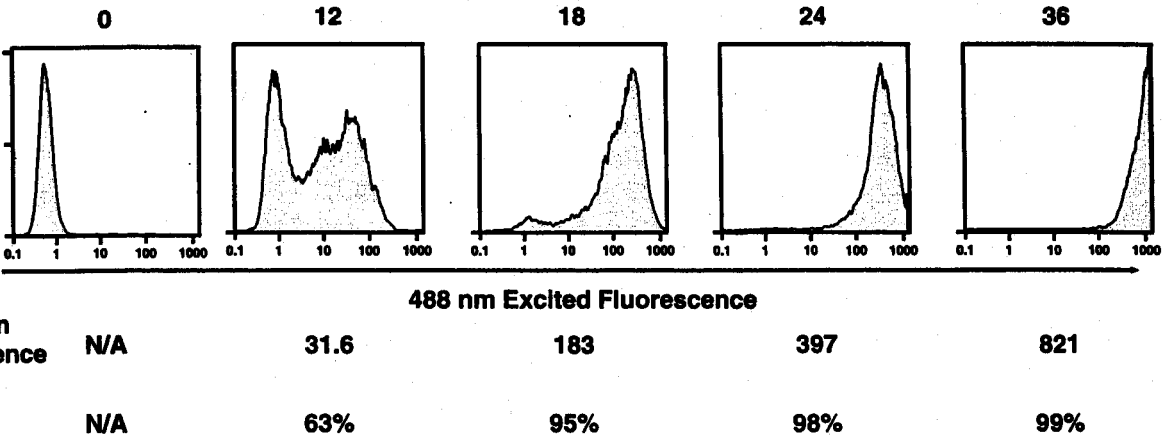

Fig. 2 FACS histograms depicting the increased huGFP–Bex fluorescent signal with increases in time after infection. Aliquots of NIH/3T3 cells were infected identically with huGFP–Bex retrovirus.

Despite these questions, the GFP variants provide unique capabilities, particularly the ability to simultaneously detect the activities of two different reporters within a single living cell. This makes it possible to acquire data simultaneously about two processes or pathways—for example, different transcriptional responses to given stimuli within single cells—and is a result of the availability of the Bex and Vex series of GFP variants. These variants differ from each other in excitation spectra but not in their emission spectra. Thus, Bex is optimally excited by the 488-nm argon line but only negligibly by the 407-nm krypton line. In contrast, Vex is well excited by the 407-nm krypton line but not by the 488-nm argon line. It is not intuitively obvious that flow cytometric instrumentation can be configured to analyze fluorochromes differing only in excitation spectra. Nonetheless, this is the case, and the fluorescent signal from fluorophores differing only in excitation can be as distinctly and quantitatively measured as the signal from fluorophores differing only in emission (Fig. 3). In this case, biparametric population frequency distributions are presented for 3T3 cells infected singly or simultaneously with the MFG–GFP–Bex and MFG–GFP–Vex retroviruses. Only 3% crosstalk between the fluorescent signals of these variants is observed for the uncompensated data. This is small enough to allow ready and unambiguous recognition of the various populations of cells expressing either one or both of these variants (Fig. 4).

As a demonstration of the power and unique information obtainable with the dual-GFP-reporter system, we tested the relative susceptibility to infection of members of a 3T3 cell population and whether infection with one retrovirus precluded infection with a second. The methodology necessary to answer these questions required discrimination of multiply infected cells. This is best done with multiple distinguishable reporters. To test this, we infected NIH/3T3 cells with equal amounts of MFG–GFP–Vex1 and MFG–GFP–Bex1 at 3 different MOIs (Fig. 4). FACS analysis of the 3T3 cells infected with the lowest MOI, 0.064 of each variant, reveals three populations of cells: one with low levels of fluorescence characteristic of uninfected cells and two with higher levels of fluorescence. The latter clearly distinguishable populations were excited exclusively with 488- or 407-nm light, representing cells infected by a single retrovirus of either the GFP–Bex1 or GFP–Vex1 type, respectively. Infection with a twofold greater amount of retroviral supernatant yields the predicted MOI of 0.13 for each virus and generates, in addition to the two singly infected population, a new "doubly positive" population showing bright fluorescence with both 488-nm and 406-nm excitation, consistent with cells expressing both GFP variants due to coinfection. With a fivefold further increase in the amount of retrovirus used for the infection of the 3T3 cells, the number of double positives increases. These results are consistent with those predicted by the Poisson distribution with an MOI of 0.65. Therefore, with the dual-reporter system we were able to show that each of the cells is equally infectable with retrovirus and that infection with one retrovirus does not alters the cell's infectability by a second retrovirus.

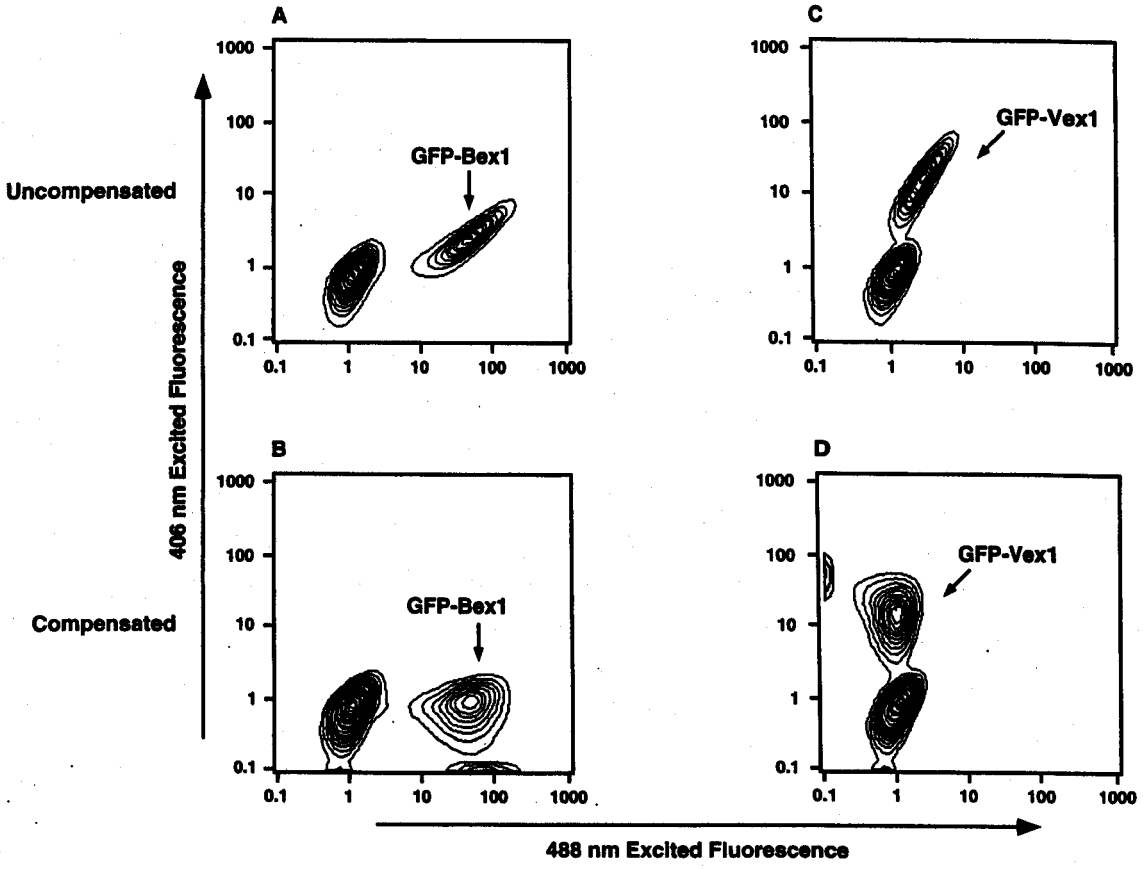

Fig. 3 Raw and compensated data of GFP–Bex1 and GFP–Vex1 fluorescence in NIH/3T3 cells. NIH/3T3 cells were infected with MFG–GFP–Bex1 (A and B) or MFG–GFP–Vex1–GFP (C and D) retrovirus and analyzed by flow cytometry 48 h after infection. Emission data collected with 407-nm and 488-nm excitation are displayed on the abscissa and ordinate, respectively, in uncompensated (A and C) and compensated (B and D) form. Representative plots from three independent experiments are shown. (Reproduced from Anderson *et al.*, 1996, with permission.)

B. Plant Cell Systems

1. Analysis and Sorting of WT-GFP-Expressing Protoplasts

Plant protoplasts in general have larger diameters than mammalian cells and consequently have much larger cytoplasmic volumes. For this reason, perhaps, it is relatively easy to accumulate levels of cytoplasmic GFP to give fluorescent signals that are detectable through flow cytometry. Expression of levels of GFP sufficient for flow analysis is illustrated for transfected maize protoplasts in Fig. 5. In this biparametric analysis, nontransfected protoplasts occupy a single cluster

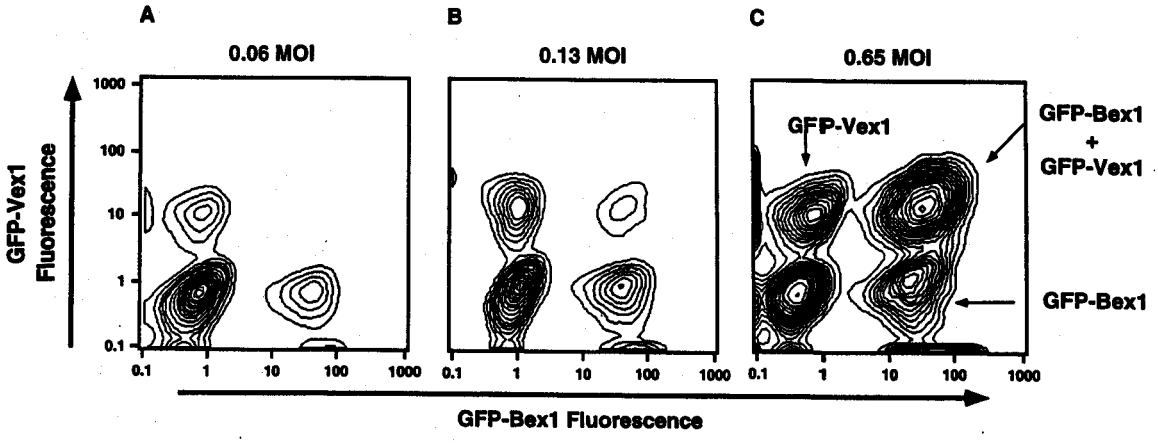

Fig. 4 Simultaneous detection of GFP–Bex1 and GFP–Vex1 fluorescence. Increasing amounts of a 1:1 mixture of the GFP–Bex1 and GFP–Vex1 viruses were used to infect NIH/3T3 cells. Cells were infected with an MOI for each of the two viruses of (A) 0.06 MOI, (B) 0.13 MOI, or (C) 0.65 MOI, as determined by multiplying the MOI measured in (A) by the relative amount of supernatant used in (B) and (C). FACS analysis was conducted as described for Fig. 2B and D. Representative plots from three independent experiments are shown. (Reproduced from Anderson *et al.*, 1996, with permission.)

having a very low level of green autofluorescence and a slightly higher level of red autofluorescence. This autofluorescence derives from the cellular pigments other than GFP (Fig. 5A). Flow analysis of protoplasts 10–18 h after transfection with GFP reveals a second cluster, characterized by greatly enhanced green

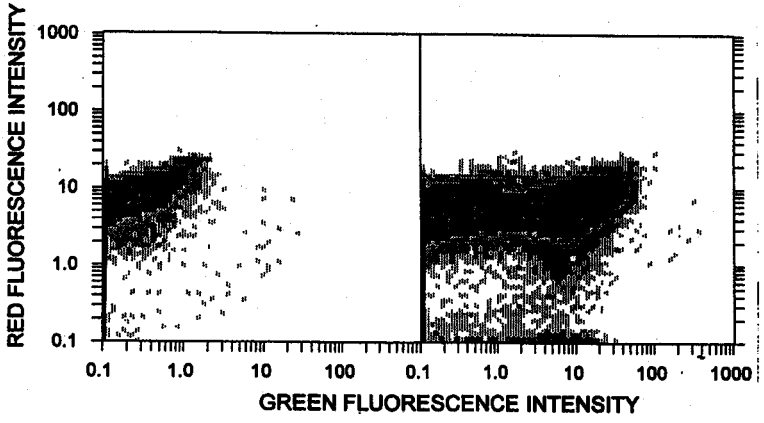

Fig. 5 Biparametric flow analysis of GFP expression in maize protoplasts, based on green and red fluorescence emission. (A) Control (nontransfected) protoplasts, (B) Protoplasts 24 h after transfection. (Reproduced from Galbraith *et al.*, 1995, with permission.)

fluorescence but unaltered red fluorescence (Fig. 5B). This corresponds to those protoplasts expressing GFP, which, in this experiment, approximates 14% of the population. The variability in transfection efficiencies between experiments was quite high, with the proportion of protoplasts expressing GFP ranging from 4 to 34% of the total. The fluorescence emission intensity from GFP-expressing protoplasts averaged about 80-fold more than that from nontransfected cells.

The maize protoplasts are about 25 μm in diameter and, being prepared from etiolated tissues, lack mature chloroplasts. Similar results were obtained following expression of the S65T humanized variant of GFP in protoplasts prepared from tobacco leaves (diameters about 40 μm), although the proportion of GFP-expressing protoplasts was lower, ranging from 2 to 10%. It is not possible to directly compare these results to extract information about the GFP constructions most appropriate for all situations because the experiments differ in protoplast types and sizes, in their relative biosynthetic capacities, and particularly in transfection efficiencies. Different plant species also appear to differ in their abilities to excise the cryptic intron found in the WT-GFP coding sequence. A systematic comparison of those that are most suitable for flow cytometric detection for specific situations should, however, be relatively straightforward.

We have previously described conditions for the sorting of intact maize protoplasts according to GFP expression (Galbraith *et al.,* 1995). Sort conditions were optimized using *Lycopodium* spores, which are of approximately the same size as the protoplasts. After placing a bit-mapped sort window around the cluster corresponding to the GFP-expressing protoplasts and enabling the sorting circuitry, protoplasts were sorted that remained intact and exhibited green fluorescence.

2. Analysis and Sorting of GFP-Accumulating Nuclei

Levels of GFP sufficient for detection using flow cytometry can be accumulated within subcellular organelles (Fig. 6). In this example, nuclear accumulation of GFP [the S65T "humanized" version (Chiu *et al.,* 1996)] requires an effective nuclear localization signal. It also requires enlargement of the molecular size of GFP, which in the native state is too small to be prevented from passive diffusion into and out of the nucleus (Grebenok *et al.,* 1997). Identification of nuclei accumulating GFP requires biparametric flow analysis in which green fluorescence emission is combined with 90° light scatter or with orange fluorescence emission. In the latter case, the population of GFP-accumulating nuclei falls on a distinct diagonal, due to spillover of the GFP-derived fluorescence into PMT3.

C. Bacteria

GFP was first heterologously expressed in *Escherichia coli.* Subsequently, because of the superior genetic tractability of this organism, it has been used as the expression vehicle in the selections for brighter GFPs. Many of the brighter

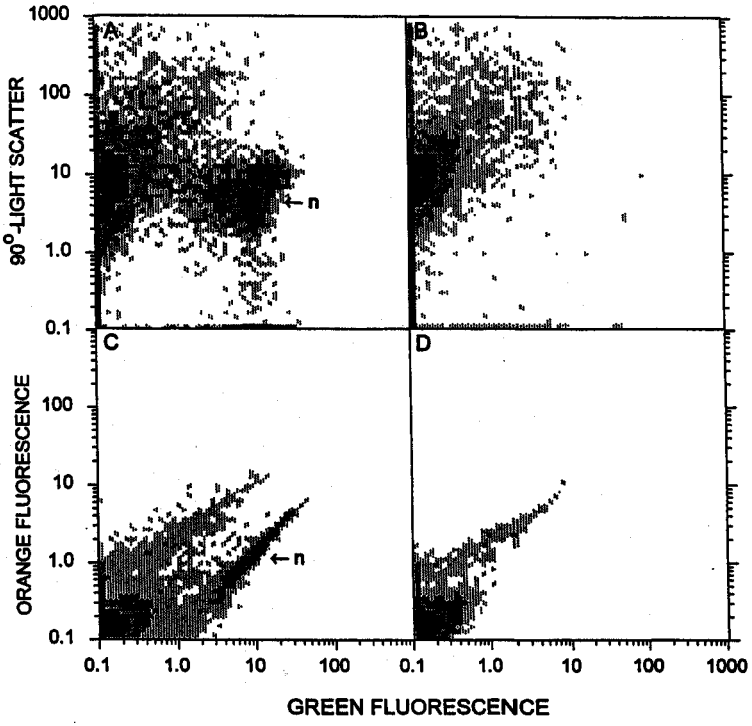

Fig. 6 Flow cytometric analysis *in vitro* of GFP targeting to nuclei. Homogenates from leaves of transgenic plants (A and C) or nontransgenic controls (B and D) were subjected to biparametric analysis of 90° light scatter versus green fluorescence (A and B) or of orange versus green fluorescence (C and D). The regions corresponding to nuclei containing GFP (key: n, nuclei) are absent from the controls. (Reproduced from Grebenok *et al.*, 1997b, with permission.)

GFP variants were selected by visual examination, but some were selected by FACS, allowing greater quantitation of their signals and greater efficiency in their selection. When huWT-GFP is expressed in *E. coli*, it is, as expected, dull. As shown in Fig. 7, the induced expression of huWT-GFP leads to only a 3.5-fold increase in the fluorescent signal compared to the signal produced with uninduced bacteria. This relative dullness is partly due to the time (approx. 3 h) required for this protein to fold and cyclize (Heim *et al.*, 1995). It is also partly due to the majority of GFP folding improperly at 37°C as well when properly folded, most of its fluorophores being in a state not well excited by the 488-nm line.

However, the brighter GFP variants produce signal levels markedly higher than those obtained with WT-GFP. For example, the induced expression of huBex–GFP leads to 80-fold higher fluorescence levels than are obtained in the absence of induction. In addition to humanized codons, this variant has the S65T spectral mutation and the V163A thermal stability mutation. The S65T mutation

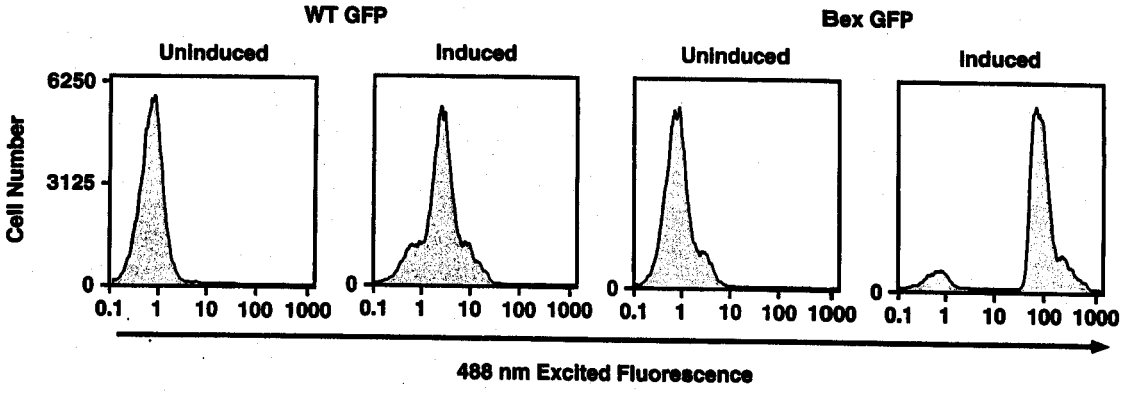

Fig. 7 Inducible expression of huWT-GFP and huBex–GFP *E. coli.* The BL21(DE3) strain of *E. coli* was transformed with pET expression vectors containing either huWT-GFP (A and B) or huBex–GFP (C and D). Suspensions of these transfectants were grown for 3 h at 37°C under induced conditions (2 m*M* IPTG, no glucose) or uninduced conditions (no IPTG, 2% glucose). Suspension cultures were diluted with PBS and analyzed on the flow cytometer. Emission data were collected with 488-nm excitation, and emissions were collected between 495 nm and 535 nm. Representative histograms are shown.

converts the fluorophore to a state better excited by the 488-nm laser line (Fig. 6) and causes GFP to more rapidly fold correctly (average time less than 30 min) (Heim *et al.,* 1995). The length of time required for folding influences the GFP signal levels because, for example with WT-GFP, this time significantly exceeds the cell doubling time. The influence of the other spectral mutations and thermal stability mutations on the rate of folding still remains to be explored. A particular advantage in the use of flow cytometer in the selection of new GFP variants is that this instrument can quantitatively detect not only the magnitude of the GFP fluorescent signal but also spectral changes in its excitation and emission (Cormack *et al.,* 1996; Crameri *et al.,* 1996).

IV. Discussion and Conclusions

The use of GFP as a reporter in flow cytometry and cell sorting is in its infancy. A major benefit provided by GFP is that it can be measured noninvasively. Alterations to the primary structure of the molecule have provided variants that are readily detectable in both prokaryotic and eukaryotic cells and within subcellular organelles. Further spectral variants that can be combined for multiparametric analyses will find considerable future utility in flow cytometry.

In animal cell systems, GFP is particularly suited as an indicator of viral infection. When expressed in viruses, either as a gene fusion or when substituted

for a nonessential viral gene, GFP fluorescence provides a sensitive and accurate measure of the proportions of infected cells and the progress of viral infection (Anderson *et al.*, 1996; Balague *et al.*, 1997; Bierhuizen *et al.*, 1997; Chen *et al.*, 1997; Lee *et al.*, 1997). Coupling this approach for viral detection with concerted mutagenesis of virus provides a screen for the roles of the various viral components within the infection process.

Flow cytometric detection of GFP also finds practical application in gene therapy. By marking the viral vectors containing therapeutic gene products with GFP, the members expressing these products within a population of cells can be identified, selected, and used exclusively for the reconstitution of organisms. One recent innovation has allowed this approach to employed with retroviral vectors. It involves the insertion of an IRES element within the retroviral transcript allowing both the therapeutic gene product and GFP to be expressed from the transcript (Mosser *et al.*, 1997). Cells infected with such dicistronic viruses can be not only stochastically selected for GFP expression but also dynamically selected for specific levels of GFP expression. These levels are anticipated to correlate with the levels of therapeutic product within the cells. Although this strategy is not unique for the GFP reporter, the noninvasive measure of its level and the small size of its gene greatly aid its implementation.

It can be anticipated that the methodologies used in the gene therapeutic approaches can also provide a generalized approach for the basic study of cell function. In this approach, exogenous genes introduced by virus are tested not only for their effects on cell viability and cell proliferation but for their effects upon less dramatic end points of cellular processes, the transcription of gene products. This can be measured as the activity of *cis*-acting elements linked to a reporter such as Bex–GFP or β-galactosidase integrated into the genome (Nolan *et al.*, 1988; Anderson *et al.*, 1996). This activity should be measurable either with GFP–Bex or with the FACS–GAL assay of β-galactosidase activity. GFP–Vex, which is spectrally distinct from the fluorescence derived from these two reporters, would then mark those cells within the population that have been infected with the retrovirus genetically complemented by virus and that are therefore expressing the protein being tested (Mosser *et al.*, 1997). This approach and similar ones made possible by pairs of FACS-detectable markers should greatly expand the genetic analysis of cell function, which is particularly relevant in an age of gene discovery driven by large-scale genome sequencing projects.

Recently, it has been demonstrated that pairs of GFP variants can be coupled through energy transfer and, when appropriately engineered, provide real-time readouts of changes in cellular physiology (Romoser *et al.*, 1997). This is specifically seen when Uex and Bex variants are brought into close proximity. The light absorbed by the Uex molecule transfers nonradiatively to the Bex molecule, which subsequently emits its characteristic fluorescence. Changes in the proximity of the two GFP molecules are detected as alterations in the proportions of fluorescence produced at the Uex and Bex emission maxima. Such changes are measurable continuously within single cells through ratiometric fluorescence

microscopy, but they can also be surveyed in entire cell populations using flow cytometry, which is well suited for the simultaneous detection of fluorescence at two different wavelengths. Thus far, GFP energy transfer couples have been successfully employed as assays for proteolytic cleavage of a peptide linker linking the two GFP domains in a single fusion protein and for calcium-dependent three-dimensional rearrangements in a calmodulin domain arranged to link the two GFP domains. Further developments of this novel technology, particularly in assays designed to monitor protein–protein interactions (such as the two-hybrid system) are expected to emerge.

In higher plants, transient expression of GFP is readily detected in transfected protoplasts and these protoplasts can be recovered in purified form through FACS sorting. One of our particular interests is the development of methods for the analysis of global patterns of gene expression in plants. One possible way to achieve this involves the use of GFP as a means to specifically highlight cell types having developmentally interesting patterns of gene expression, thence to employ FACS as a means to purify protoplasts prepared from these cells or tissues. Although we and others have previously established that protoplast-derived reports can be representative of the tissues from which the protoplasts are prepared (Harkins *et al.,* 1990; Sheen *et al.,* 1995), it is not a simple task to prepare and sort protoplasts. Further, although reports of high-level expression of GFP within transgenic plants have emerged (Chiu *et al.,* 1996; Pang *et al.,* 1996; Haseloff *et al.,* 1997), a systematic study employing flow cytometry will be needed to establish the dynamic range over which GFP expression within transgenic protoplasts is suitable for the study of cell-specific gene expression.

We have been pursuing an alternative strategy to protoplast sorting. This is based on our observation that, through construction of translational fusions, GFP chimeric proteins can be used to monitor the process of targeting to the nucleus (Grebenok *et al.,* 1997a,b). Our work indicates it is possible to employ FACS for the isolation of nuclei that have accumulated GFP. This leads to the idea of examining the RNA transcripts within sorted nuclei as a way to monitor gene expression. Coupling nuclear accumulation of GFP to cell-, tissue-, or developmentally specific promoters would allow characterization of patterns of gene expression within these subpopulations of cells. The strength of the approach relies on the observation that nuclei are intrinsically nonautofluorescent, hence give a low background, and are highly homogeneous in terms of physical dimensions. This renders them easy to analyze and sort using FACS, as has been well established for analyses of genome sizes (Galbraith *et al.,* 1983). In principle, this approach is applicable to any eukaryotic system and may prove particularly important in the study of the interactions of different organisms, such as in infections, or in disease states characterized by abnormal patterns of gene expression and/or abnormal genome sizes.

GFP as a transcriptional reporter in bacteria should prove particularly important in the development of therapeutics against bacterial pathogens. In the past, it has been difficult to clone virulence factors from invasive bacterial pathogens.

However, because GFP is an intrinsically fluorescent reporter, it allows the identification of bacterial transcription units up-regulated in response to engulfment. This has allowed Valdivia and Falkow (1997) to screen a library of *Salmonella typhimurium* with GFP gene fusions to identify genes upregulated in this organism as a response to engulfment by host macrophages (Valdivia and Falkow, 1997). From this genetic screen they were able to clone four of the virulence factors of *S. typhimurium.* This strategy should find wide applicability to a range of host/pathogen interactions.

Acknowledgments

D.G. would like to thank Georgina Lambert for valuable technical assistance. This work was supported by grants to D.G. from the U.S.D.A.-N.R.I. Competitive Grants Program (Plant Genome), the Instrumentation and Instrument Development Program of N.S.F., and the N.S.F./U.S.D.A./D.O.E. Triagency Program in Plant Biology.

L.A.H. and M.T.A. would like to thank the Stanford Shared FACS Facility (David Parks, Director) for the custom instrumentation and custom software used in many of these analysis. In addition, we greatly appreciate the assistance of Iwan Tijoe in the construction of the GFP variants. This work was supported by a grant CA-42509 to L.A.H.

References

Alberti, S., Parks, D. R., and Herzenberg, L. A. (1988). A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry* **8,** 114–119.

Anderson, M. T., Tjioe, I. T., Lorincz, M. C., Parks, D. R., Herzenberg, L. A., Nolan, G. P., and Herzenberg, L. A. (1996). Simultaneous fluorescence-activated cell sorter analysis of two distinct transcriptional elements within a single cell using engineered green fluorescent proteins. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8508–8511.

Anderson, M. T., Gerstein, R., Haugland, R., Tjioe, I., Herzenberg, L. A., and Herzenberg, L. A. (1998a). Three violet-excited fluorophores for immunofluorescence analysis on the flow cytometer (in preparation).

Anderson, M. T., Baumgarth, N., Haugland, R. P., Gerstein, R. M., Tjioe, I. T., Herzenberg, L. A., and Herzenberg, L. A. (1998b). Submitted.

Balague, C., Kalla, M., and Zhang, W. W. (1997). Adeno-associated virus Rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *J. Virol.* **71,** 3299–3306.

Bierhuizen, M. F., Westerman, Y., Visser, T. P., Wognum, A. W., and Wagemaker, G. (1997). Green fluorescent protein variants as markers of retroviral-mediated gene transfer in primary hematopoietic cells and cell lines. *Biochem. Biophys. Res. Commun.* **234,** 371–375.

Brejc, K., Sixma, T. K., Kitts, P. A., Kain, S. R., Tsien, R. Y., Ormo, M., and Remington, S. J. (1997). Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2306–2311.

Bunnell, B. A., Muul, L. M., Donahue, R. E., Blaese, R. M., and Morgan, R. A. (1995). High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7739–7743.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.

Chen, B. K., Feinberg, M. B., and Baltimore, D. (1997). The kappaB sites in the human immunodeficiency virus type 1 long terminal repeat enhance virus replication yet are not absolutely required for viral growth. *J. Virol.* **71,** 5495–5504.

Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996). Engineered GFP as a vital reporter in plants. *Curr. Biol.* **6,** 325–330.

Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W. (1993). Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. *Biochemistry* **32,** 1212–1218.

Cormack, B. P., Bertram, G., Egerton, M., Gow, N. A., Falkow, S., and Brown, A. J. (1997). Yeast-enhanced green fluorescent protein (yEGFP) a reporter of gene expression in *Candida albicans*. *Microbiology* **143,** 303–311.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33–38.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* **14,** 315–319.

Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995). Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* **20,** 448–455.

Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M., and Youvan, D. C. (1995). Red-shifted excitation mutants of the green fluorescent protein. *Bio/Technology* **13,** 151–154.

Dubendorff, J. W., and Studier, F. W. (1991). Creation of a T7 autogene. Cloning and expression of the gene for bacteriophage T7 RNA polymerase under control of its cognate promoter. *J. Mol. Biol.* **219,** 61–68.

Ehrig, T., O'Kane, D. J., and Prendergast, F. G. (1995). Green-fluorescent protein mutants with altered fluorescence excitation spectra. *FEBS Lett.* **367,** 163–166.

Galbraith, D. W., Harkins, K. R., Maddox, J. R., Ayres, N. M., Sharma, D. P., and Firoozabady, E. (1983). Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* **220,** 1049–1051.

Galbraith, D. W., Lambert, G. M., Grebenok, R. J., and Sheen, J. (1995). Flow cytometric analysis of transgene expression in higher plants: green-fluorescent protein. *Methods Cell Biol.* **50,** 3–14.

Gamborg, O. L., and Philips, G. C. (1995). "Plant Cell, Tissue, and Organ Culture: Fundamental Methods." Springer-Verlag, New York.

Gervaix, A., West, D., Leoni, L. M., Richman, D. D., Wong-Staal, F., and Corbeil, J. (1997). A new reporter cell line to monitor HIV infection and drug susceptibility *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4653–4658.

Grebenok, R. J., Pierson, E., Lambert, G. M., Gong, F. C., Afonso, C. L., Haldeman-Cahill, R., Carrington, J. C., and Galbraith, D. W. (1997a). Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *Plant J.* **11,** 573–586.

Grebenok, R. J., Lambert, G. M., and Galbraith, D. W. (1997b). Characterization of the targeted nuclear accumulation of GFP within the cells of transgenic plants. *Plant J.* **12,** 685–696.

Ha, D. S., Schwarz, J. K., Turco, S. J., and Beverley, S. M. (1996). Use of the green fluorescent protein as a marker in transfected *Leishmania*. *Mol. Biochem. Parasitol.* **77,** 57–64.

Harkins, K. R., Jefferson, R. A., Kavanagh, T. A., Bevan, M. W., and Galbraith, D. W. (1990). Expression of photosynthesis-related gene fusions is restricted by cell type in transgenic plants and in transfected protoplasts. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 816–820.

Haseloff, J., Siemering, K. R., Prasher, D. C., and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2122–2127.

Heim, R., Prasher, D. C., and Tsien, R. Y. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12501–12504.

Heim, R., Cubitt, A. B., and Tsien, R. Y. (1995). Improved green fluorescence [letter]. *Nature* **373,** 663–664.

Heim, R., and Tsien, R. Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* **6,** 178–182.

Hicks, K. A., Millar, A. J., Carre, I. A., Somers, D. E., Straume, M., Meeks-Wagner, D. R., and Kay, S. A. (1996). Conditional circadian dysfunction of the *Arabidopsis* early-flowering 3 mutant. *Science* **274,** 790–792.

Hofmann, A., Nolan, G. P., and Blau, H. M. (1996). Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette [see comments]. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 5185–5190.

Kain, S. R., and Kitts, P. (1997). Expression and detection of green fluorescent protein (GFP). *Methods Mol. Biol.* **63,** 305–324.

Kimata, Y., Iwaki, M., Lim, C. R., and Kohno, K. (1997). A novel mutation which enhances the fluorescence of green fluorescent protein at high temperatures. *Biochem. Biophys. Res. Commun.* **232,** 69–73.

Kinsella, T. M., and Nolan, G. P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* **7,** 1405–1413.

Krall, W. J., Skelton, D. C., Yu, X. J., Riviere, I., Lehn, P., Mulligan, R. C., and Kohn, D. B. (1996). Increased levels of spliced RNA account for augmented expression from the MFG retroviral vector in hematopoietic cells. *Gene Ther.* **3,** 37–48.

Lee, A. H., Han, J. M., and Sung, Y. C. (1997). Generation of the replication-competent human immunodeficiency virus type 1 which expresses a jellyfish green fluorescent protein. *Biochem. Biophys. Res. Commun.* **233,** 288–292.

Lorincz, M., Roederer, M., Diwu, Z., Herzenberg, L. A., and Nolan, G. P. (1996). Enzyme-generated intracellular fluorescence for single-cell reporter gene analysis utilizing *Escherichia coli* beta-glucuronidase. *Cytometry* **24,** 321–329.

Lybarger, L., Dempsey, D., Franek, K. J., and Chervenak, R. (1996). Rapid generation and flow cytometric analysis of stable GFP-expressing cells. *Cytometry* **25,** 211–220.

Mosser, D. D., Caron, A. W., Bourget, L., Jolicoeur, P., and Massie, B. (1997). Use of a dicistronic expression cassette encoding the green fluorescent protein for the screening and selection of cells expressing inducible gene products. *Biotechniques* **22,** 150–154.

Muldoon, R. R., Levy, J. P., Kain, S. R., Kitts, P. A., and Link, C. J. Jr. (1997). Tracking and quantitation of retroviral-mediated transfer using a completely humanized, red-shifted green fluorescent protein gene. *Biotechniques* **22,** 162–167.

Naviaux, R. K., Costanzi, E., Haas, M., and Verma, I. M. (1996). The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* **70,** 5701–5705.

Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G., and Sala, F. (1987). Hybrid genes in the analysis of transformation conditions. *Plant Mol. Biol.* **15,** 363–373.

Nolan, G. P., Fiering, S., Nicolas, J. F., and Herzenberg, L. A. (1988). Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-D-galactosidase activity after transduction of *Escherichia coli* lacZ. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2603–2607.

Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996). Crystal structure of the *Aequorea victoria* green fluorescent protein [see comments]. *Science* **273,** 1392–1395.

Pang, S. Z., DeBoer, D. L., Wan, Y., Ye, G., Layton, J. G., Neher, M. K., Armstrong, C. L., Fry, J. E., Hinchee, M. A., and Fromm, M. E. (1996). An improved green fluorescent protein gene as a vital marker in plants. *Plant Physiol.* **112,** 893–900.

Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8392–8396.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111,** 229–233.

Roederer, M., DeRosa, S., Gerstein, R., Anderson, M., Bigos, M., Stovel, R., Nozaki, T., Parks, D., Herzenberg, L., and Herzenberg, L. (1997). 8 color, 10-parameter flow cytometry to elucidate complex leukocyte heterogeneity. *Cytometry* **29,** 328–339.

Rogers, S. G., Bisaro, D. M., Horsch, R. B., Fraley, R. T., Hoffmann, N. L., Brand, L., Elmer, J. S., and Lloyd, A. M. (1986). Tomato golden mosaic virus A component DNA replicates autonomously in transgenic plants. *Cell* **45,** 593–600.

Romoser, V. A., Hinkle, P. M., and Persechini, A. (1997). Detection in living cells of Ca2+-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators. *J. Biol. Chem.* **272,** 13270–132704.

Ropp, J. D., Donahue, C. J., Wolfgang-Kimball, D., Hooley, J. J., Chin, J. Y., Hoffman, R. A., Cuthbertson, R. A., and Bauer, K. D. (1995). *Aequorea* green fluorescent protein analysis by flow cytometry. *Cytometry* **21,** 309–317.

Ropp, J. D., Donahue, C. J., Wolfgang-Kimball, D., Hooley, J. J., Chin, J. Y., Cuthbertson, R. A., and Bauer, K. D. (1996). *Aequorea* green fluorescent protein: simultaneous analysis of wild-type and blue-fluorescing mutant by flow cytometry. *Cytometry* **24,** 284–288.

Rouwendal, G. J., Mendes, O., Wolbert, E. J., and Douwe de Boer, A. (1997). Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. *Plant Mol. Biol.* **33,** 989–999.

Sambrook, J., Fritsch E. F., and Maniatis, T. (1989). "Molecular Cloning, a Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D. W. (1995). Green-fluorescent protein as a new vital marker in plant cells. *Plant J.* **8,** 777–784.

Siemering, K. R., Golbik, R., Sever, R., and Haseloff, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* **6,** 1653–1663.

Subramanian, S., and Srienc, F. (1996). Quantitative analysis of transient gene expression in mammalian cells using the green fluorescent protein. *J. Biotechnol.* **49,** 137–151.

Valdivia, R. H., and Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* **277,** 2007–2011.

Yang, T. T., Cheng, L., and Kain, S. R. (1996). Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res.* **24,** 4592–4593.

Zhang, G., Gurtu, V., and Kain, S. R. (1996). An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem. Biophys. Res. Commun.* **227,** 707–711.

Zolotukhin, S., Potter, M., Hauswirth, W. W., Guy, J., and Muzyczka, N. (1996). A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J. Virol.* **70,** 4646–4654.