[26] Flow Cytometric Analysis of Transgene Expression in Higher Plants: Green Fluorescent Protein

By DAVID W. GALBRAITH, LEONARD A. HERZENBERG, AND MICHAEL T. ANDERSON

Introduction

The green fluorescent protein (GFP) of *Aequorea victoria* provides a unique means to study living cells, because transgenic expression of the coding sequence is sufficient to produce fluorescence within the cells of many different pro- and eukaryotic species.[1] The availability of GFP as a transgenic marker has spurred interest in methodologies and instruments that can accurately measure cellular fluorescence emission, of which flow cytometry is one. Flow cytometric instrumentation[2,3] is designed to examine the optical properties of suspensions of single cells or cellular homogenates as they are constrained to pass rapidly in a fluid stream through focused illumination. The individual cells or subcellular particles scatter light and, if they contain fluorochromes, also absorb light that is subsequently reemitted in the form of fluorescence. These signals are detected using sensitive photomultiplier tubes (PMTs) and photodiodes screened by appropriate wavelength-selective filters. After conversion to voltage-versus-time pulse waveforms, the intensity values are digitized and stored for analysis or further processing; the values are stored either on a cell-by-cell basis ("list-mode"), or in the form of frequency distributions. Flow cytometric data are then presented in the form of multidimensional population distributions, which are graphically represented as histograms. This method of data accumulation and display, because it preserves differences between cells, is different from that commonly encountered in biochemical determinations, which provide only population means. Flow cytometry therefore allows identification of subpopulations of cells having different optical characteristics; these typically are observed as individual clusters within two-dimensional representations. Flow cytometry is also inherently quantitative, in that the intensities of the individual light scatter and fluorescent signals are accurately recorded. Either linear or logarithmic scales can be employed, the latter being preferred when signals of large dynamic ranges are encountered.

[1] P. M. Conn (ed.), *Methods Enzymol.* **302**, 99 (1999).
[2] M. R. Melamed, T. Lindstrom, and M. L. Mendelsohn, "Flow Cytometry and Cell Sorting," John Wiley & Sons, New York, 1990.
[3] H. M. Shapiro, "Practical Flow Cytometry," 3rd ed. John Wiley & Sons, New York, 1995.

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ome of the optical characteristics of cells, from which flow cytometric surements derive, reflect the physical properties of the cells, for exam- he intensities of light scattered at angles close to the axis of illumination vard-angle light scatter, FALS) or orthogonally to the axes of illumina- and fluid flow (90° light scatter). Fluorescent signals produced from reflect either the presence of endogenous autofluorescence or result addition of fluorochromes (synthetic chemicals or natural products). e of these have intrinsic specificity, such as the various fluorochromes tightly bind to DNA or intercalate nucleic acids. Others have no isic specificity, but through being covalently linked to other molecules, as antibodies, can be provided with particular binding specificities. he basis of the commercial availability of many different types of escent molecules, flow cytometric methods and reagents have been ed to provide a sensitive measure of a large variety of cellular param-

low cytometry finds particular power when it is combined with cell ig. Sorting is done following laser interrogation and analysis of the stream, which technically occurs either in air or within an enclosed te. In either case, the stream subsequently emerges through a precisely eered flow orifice, having a typical diameter in the range of 40–400 The technique of cell sorting relies on the principle that cylindrical streams in air are unstable and, as a consequence of minimizing surface nergy, decay into droplets. Imposition of a periodic oscillation on the stream at the point of emergence, using a piezoelectric or electrome- cal drive, constrains the stream to break into droplets at a precisely d point below the flow tip, which therefore corresponds to a precise in time after a given object has been analyzed. An electrical voltage lied to the stream at the precise time that the desired object is entering last-attached" droplet. This leaves a residual charge on the newly d droplet surface, and the droplet can then be collected following ostatic deflection by passage through a fixed high-voltage field. Flow g is implemented through computerized sort decisions, based on re- selected on the population distributions that were previously col- . Switching of the sort charge can be done rapidly, and this means igh rates of sorting can be achieved, and large numbers of purified btained for further characterization.[2,3]

nsiderable effort has been devoted to the development of flow cytom- nd cell sorting for the analysis of the fluorescence derived from enic expression of GFP.[4,5] This chapter describes transient and

lsh and S. A. Kay, *Curr. Opin. Biotechnol.* 8, 617 (1997).
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transgenic expression of GFP in plants, targeting of GFP to the nucleus, and the use of flow cytometry to analyze this expression in protoplasts and in isolated nuclei.

Materials. Enzymes for modification of DNA molecules are available from a number of sources. Macerase and cellulysin are obtained from Calbiochem (La Jolla, CA), and MS (Murashige and Skoog) medium from GIBCO (Grand Island, NY). Other chemicals are available from the Sigma Chemical Company (St. Louis, MO). Sterile plastic centrifuge tubes (Falcon 2098; Becton Dickinson Labware, Lincoln Park, NJ) and culture dishes (Falcon 3047) are used in the preparation and manipulation of protoplasts. Disposable microcentrifuge tubes, pipette tips, and 12 × 75 mm test tubes are available from general scientific supply companies.

Methods

Recombinant DNA Molecules

Routine manipulation of DNA follows the general methods of Sambrook *et al.*[6] The design and construction of the plasmids employed in this work have already been described: 35SCPPDK-GFP comprises[7] a hybrid promoter formed between the cauliflower mosaic virus (CAMV) 35S enhancer and the basal promoter and 5′ nontranslated region of the maize C4PPDK gene. This hybrid promoter was used to drive expression of the wild-type GFP coding sequence.[8] pRJG2 comprises[9] the wild-type GFP-coding sequence under the transcriptional regulation of a doubled CAMV 35S promoter and the polyadenylation signal of the same gene.[10] In pRJG8, the CAMV 35S promoter is placed upstream from a modified version (mGFP[11]) of the GFP coding sequence, in which the codon usage is altered to prevent improper mRNA processing. The GFP-coding sequence is followed by the transcriptional terminator from nopaline synthetase. In pRJG23, the doubled CAMV 35S promoter and the polyadenylation sequence regulate expression of a chimeric protein,[12] containing the minimal

[6] J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
[7] J. Sheen, S. Hwang, Y. Niwa, H. Kobayashi, and D. W. Galbraith, *Plant J.* **8,** 777 (1995).
[8] M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher, *Science* **263,** 802 (1994).
[9] D. W. Galbraith, J. Sheen, G. M. Lambert, and R. J. Grebenok, *Methods Cell Biol.* **50,** 3 (1995).
[10] F. Guerineau, S. Woodston, L. Brooks, and P. Mullineaux, *Nucleic Acids Res.* **16,** 11380 (1988).
[11] J. Haseloff, K. R. Siemering, D. C. Prasher, and S. Hodge, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 2122 (1997).
[12] R. J. Grebenok, E. A. Pierson, G. M. Lambert, F.-C. Gong, C. L. Afonso, R. Haldeman-Cahill, J. C. Carrington, and D. W. Galbraith, *Plant J.* **11,** 573 (1997).

uclear targeting sequence from the C2 protein[13] of *Nicotiana tabacum*, GFP,[14] and β-glucuronidase (GUS[15]). pRJG37 was produced[16] by transfering the expression cassette of pRJG23 into *Kpn*I-cut pBin19.[17]

Plant Growth and Maintenance

Maize hybrid lines FR9^{cms} × FR37 or FR992 × FR637 are from Illinois Foundation Seed (Champaign, IL). Seeds are germinated (25 seeds per pot, 6 inches in diameter) and plants grown either in sterile potting soil (Hyponex, Marysville, IL) or a 2:1 vermiculite:peatlite mix (Grace Sierra, Milpitas, CA) within an environmental chamber (Revco or Conviron) in darkness at 25°. Etiolated leaves are harvested 11–12 days after germination. Plantlets are watered, but no additions of fertilizer are made during growth.

Axenic *Nicotiana tabacum* cv. Xanthi plants are individually grown from surface-sterilized seed in Magenta boxes on 50 ml agar-solidified MS medium containing 3% sucrose. Plants are maintained within standard growth chambers (Revco) at 22°, under constant illumination. Plantlets are subcultured by excising the apical meristem with one or two lateral nodes and subtending leaves, which is then transferred onto fresh medium.

Preparation of Transgenic Plants and of Nuclei

Transgenic tobacco plants are produced by *Agrobacterium*-mediated transformation, using routine methods.[18] For preparation of homogenates, tobacco leaves (200–500 mg) are excised and transferred into a plastic petri dish (60-mm diameter), placed on a prechilled (4°) ceramic tile. Ice-cold chopping buffer (3 ml) is added, and the tissues chopped for 1.5 min using a single-edged razor blade. The chopping buffer (CB) comprises 45 m*M* $MgCl_2$, 30 m*M* sodium citrate, 20 m*M* morpholinepropanesulfonic acid (MOPS), and 0.1% (w/v) Triton X-100, pH 7.0. Prior to analysis, the homogenates are filtered through 15-μm pore size nylon mesh (Tetko, Briarcliff Manor, New York).

[13] C. L. Afonso and D. W. Galbraith, *In Vitro Cell Dev. Biol. (Plants)* **30**, 44 (1994).
[14] W. L. Chiu, Y. Niwa, W. Zeng, T. Hirano, H. Kobayashi, and J. Sheen, *Curr. Biol.* **6**, 325 (1996).
[15] R. A. Jefferson, T. A. Kavanagh, and M. W. Bevan, *EMBO J.* **6**, 3901 (1987).
[16] R. J. Grebenok, G. M. Lambert, and D. W. Galbraith, *Plant J.* **12**, 685 (1997).
[17] M. Bevan, *Nucleic Acids Res.* **12**, 8711 (1984).
[18] G. Rogers, R. B. Horsch, and R. T. Fraley, *Methods Enzymol.* **118**, 627 (1986).

Maize protoplasts are prepared using the methods described by Sheen *et al.*[7] Second leaves from 11- to 12-day-old maize plants are excised, and stacked (20–25 high) in a sterile plastic 9-cm-diameter petri dish. The leaves are trimmed to remove 4 cm from the top. The remainder is cut into 0.5-mm strips using single-edged razor blades sterilized with 95% ethanol. The strips are placed in a 250-ml sterile side-arm filtration flask containing 20 ml of filter-sterilized enzyme solution: 1% cellulase RS, 0.25% macerozyme R10, 0.6 *M* mannitol, 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), 1 m*M* $CaCl_2$, 5 m*M* 2-mercaptoethanol, and 0.1% bovine serum albumin (BSA). Vacuum is then applied for 15 min with constant swirling. The flask is placed on a rotary shaker at 40 rpm for 2 hr, and then at 100 rpm for 10 min. The suspension is filtered through a funnel covered with 60-μm pore size nylon mesh into a 50-ml sterile plastic centrifuge tube (Falcon 2098), and centrifuged at 100 *g* for 5 min. The pelleted protoplasts are resuspended in electroporation buffer comprising 0.6 *M* mannitol, 20 m*M* KCl, 4 m*M* MES, and 5 m*M* EGTA, pH 5.7. The centrifugation step is repeated and the protoplasts resuspended to a concentration of 1–2 $\times$ 10^6/ml.

Tobacco protoplasts are prepared from wild-type and transgenic plants according to the following procedure; all steps are done under standard sterile conditions. Fully expanded leaves are excised (~500 mg) and transferred into a sterile plastic petri dish containing 20 ml of filter-sterilized [Millipore (Bedford, MA) GSWP 047] digestion medium (0.1% driselase, 0.1% macerase, and 0.1% cellulysin, dissolved in 0.5 *M* mannitol, 10 m*M* $CaCl_2$, and 3 m*M* MES, pH 5.7). Incubation is continued overnight (18–24 hr) at 22° in darkness. The protoplast suspension is filtered through two layers of sterile cheesecloth into a 50-ml disposable plastic centrifuge tube, and is centrifuged at 50 *g* for 10 min. The pelleted protoplasts are gently resuspended in 20 ml of 25% (w/v) sucrose dissolved in modified T0 medium.[19] The suspension is overlaid with 5 ml of W5 medium,[20] and centrifuged at 50 *g* for 10 min. Viable protoplasts accumulate as a band at the interface, and are removed using a pasteur pipette. The protoplasts are diluted by addition of 2 vol of W5 medium, collected by centrifugation at 50 *g* for 10 min, and resuspended to a final concentration of 10^6/ml in W5 medium; protoplast numbers are determined by hemocytometry. Samples are filtered through 50-μm pore size nylon mesh immediately prior to flow analysis.

[19] K. R. Harkins, R. A. Jefferson, T. A. Kavanagh, M. W. Bevan, and D. W. Galbraith, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 816 (1990).

[20] I. Negrutiu, R. Shillito, I. Potrykus, G. Biasini, and F. Sala, *Plant Mol. Biol.* **15,** 363 (1987).

Protoplast Transfection

Protoplast transfection is done either by using polyethylene glycol (PEG), or by electroporation. For PEG-mediated transfection, tobacco leaf protoplasts are resuspended in a sterile 15-ml disposable centrifuge tube in 600 μl of W5 medium at a concentration of ~1.6×10^6 protoplasts/ml. Plasmid DNA and carrier (calf thymus) DNA are added to final concentrations of 20 and 50 mg/ml, respectively. The side of the tube is tapped to ensure the protoplasts are thoroughly resuspended, then 1.5 vol of PEG solution is added with gentle stirring using a 1-ml disposable pipette tip. The PEG solution comprises 40% (w/v) PEG 4000, 0.4 *M* mannitol, and 0.1 *M* $Ca(NO_3)_2 \cdot 4H_2O$, pH 7.0. Incubation is done at room temperature for 25 min, then 8 vol of culture medium [medium NTTO modified[19] to contain 0.18 *M* glucose and 0.15 *M* mannitol, and supplemented with ampicillin (75 mg/ml)] is added. Incubation is then continued overnight (18–24 hr) in darkness at room temperature.

For transfection of maize protoplasts by electroporation,[9] the protoplast pellet is resuspended in electroporation buffer comprising 0.6 *M* mannitol, 20 m*M* KCl, 4 m*M* MES, and 5 m*M* EGTA, pH 5.7. The protoplasts are collected by centrifugation at 100 *g* for 10 min, are resuspended (1.5×10^5) in 0.3 ml of electroporation buffer, and are transferred into a plastic electroporation cuvette (gap width, 4 mm; BTX, San Diego, CA). Plasmid DNA is added [25–50 μg in 30–60 μl of Tris–EDTA (TE)]. Electroporation is done using three pulses, each of 10-msec duration, a field strength of 400–500 V/cm, and a 200-mF capacitance setting. The protoplasts are transferred into plastic six-well culture plates (Falcon 3046), kept on ice for 10 min after electroporation, and diluted by addition of electroporation solution (0.7 ml/well). The culture plates are covered with foil, and incubated in darkness at room temperature overnight.

Setting up Flow Cytometers

Coulter Elite. The Coulter (Hialeah, FL) Elite flow cytometer is operated using a single 20-mW air-cooled argon laser (488-nm emission), a forward-angle light scatter (FALS) detector, and four photomultiplier tubes (PMTs). The standard optical path assigns 90° light scatter to PMT1, 505 to 545-nm fluorescence (green) to PMT2, 555- to 595-nm fluorescence (orange) to PMT3, and 670- to 680-nm (red) fluorescence to PMT4. The laser beam intercepts the flow stream within a 250-μm square quartz cuvette, and emerges through a 100-μm diameter orifice.

The sheath pressure is adjusted to 8.0 lb/in^2 and biparametric histograms of log 90° light scatter versus log green fluorescence, and of log green fluorescence versus log red fluorescence (chlorophyll) are collected at a

sample flow rate of 70 particles/sec, with the FALS discriminator being set to 50, and all other discriminators being switched off. PMT voltages and amplification settings should be empirically adjusted, but typical values are as follows: FALS, 260/10; PMT1, 350/7.5; PMT2, 850/10; PMT4, 950/5.

Cytomation MoFlo. The Cytomation (Fort Collins, CO) MoFlo flow cytometer is equipped with a single water-cooled argon laser providing ultraviolet (UV) and 488-nm excitation, an FALS detector, and 5 PMTs. The optical path is organized such that 90° light scatter is assigned to PMT1, 505- to 545-nm fluorescence (green) to PMT2, 555- to 595-nm fluorescence (orange) to PMT3, and 670- to 680-nm (red) fluorescence to PMT4. The laser beam intercepts the flow stream in air after it emerges from a 70- or 100-μm-diameter orifice.

The laser is adjusted to produce 488-nm light at a power output of 180 mW. The sheath pressure is adjusted to 15 lb/in^2, and biparametric histograms collected of log 90° light scatter versus log green fluorescence, and log green versus log red fluorescence, to a total event count of 10^5, at a sample flow rate of 100–200/sec. Typical photomultiplier voltages and amplification settings are as follows: PMT1, 400/1.0; PMT2, 520/1.0; PMT3, 430/1.0; PMT4, 600/1.0. The system is triggered on 90° light scatter, with the threshold being adjusted to eliminate detection of debris.

For protoplasts, the sheath fluid comprises 154 m*M* NaCl, 125 m*M* $CaCl_2$, 5 m*M* KCl, and 5 m*M* glucose, pH 5.7; it is filtered through a 0.22-μm pore size filter prior to use. For nuclei, the sheath fluid comprises CB lacking Triton. The cytometers are aligned using calibration fluorospheres (DNA Check; Coulter Electronics) diluted by addition of 10 vol of deionized water. Adjustment of the optics is continued until population coefficients of variation (CVs) for the various fluorescence and light scatter signals are minimized; these are typically <2% for fluorescence, and slightly higher (2–3%) for light scatter.

Analyzing Protoplasts and Subcellular Organelles

For analysis of protoplasts, biparametric histograms of log 90° light scatter versus log green fluorescence, and log red fluorescence (chlorophyll) versus log green fluorescence, are collected to a total count of 0.1–1 × 10^5. The sample flow rate was 100–200/sec. For analysis of subcellular homogenates,[16] biparametric histograms are similarly collected, with the exception that it is recommended that triggering be done on fluorescence signals rather than light scattering. Homogenates contain a large majority of nonfluorescent, light-scattering particles, and triggering on light scatter

can obscure the contributions made by the minor populations of fluorescent organelles.

Sorting

Data from experiments involving sorting are not presented, because they are beyond the scope of this chapter. However, for completeness, the experimental setup required for sorting is provided below.

Coulter Elite. Using the 100-μm flow tip, sheath and sample pressures are set to 12 and 11.5 lb/in^2, respectively, and the piezoelectric drive to about 17 kHz. For large-particle sorting (>10 μm diameter), the sheath and sample pressures are lowered to 8.0 and 7.5 lb/in^2, respectively. Conditions that produce a stable sorted stream can then be found within the range of 12–17 kHz at a 70% drive amplitude. Lower drive frequencies in general improve the efficiency of recovery of larger particles.[21,22] Sort optimizing involves the following: the transducer is allowed to warm up for 60 min at 70% amplitude. The droplet deflection assembly is moved upward to a point as close as possible to the flow cell tip without blocking the laser beam. The video field is adjusted so that both the ground plate of the deflection assembly (to the left edge of the screen) and the laser beam intercept (to the right) can be observed using only the "pan" function. The drive frequency and amplitude are adjusted to provide the shortest possible droplet breakoff point. In sort test mode, the delay setting is adjusted in 0.1-drop increments ("phase" adjustment) to give a sorted stream that is stable. The deflection plate assembly is then lowered so that two or three free droplets are visible above the ground plane. The last-attached droplet should have a well-rounded profile on the left-hand (non-attached) side, and should be obviously connected to the flow stream on the right-hand side. If smaller satellite droplets are observed, it should be established whether these are "fast" or "slow," i.e., whether they merge with the major droplet ahead of or behind the satellite. The amplitudes and frequencies of the piezoelectric transducer are then adjusted until only "fast" satellites are produced. The cursor is next moved to superimpose the second undulation to the right of the last-attached droplet. A point is marked between the first two free drops above the ground plane. The number of droplets separating the two cursors is counted and entered into the delay-calculation program. Histograms are acquired, using the particles of interest, and appropriate sort windows are defined. The delay setting is now empirically optimized through doing a "sort matrix analysis," which

[1] K. R. Harkins and D. W. Galbraith, *Physiol. Plantarum* **60**, 43 (1984).
[2] D. W. Galbraith and G. M. Lambert, *in* "Flow Cytometric Applications in Cell Culture" (M. Al-Rubeai and A. N. Emery, eds.), p. 311. Marcel Dekker, New York, 1995.

aims to achieve a maximal (near 100%) sort efficiency. This involves batch sorting of groups of 25 particles onto standard glass microscope slides, counting the numbers actually recovered under the microscope as a function of the sort delay setting. Between each batch, the sort delay setting is adjusted in one-step increments to span a range of ± five around the calculated delay setting. For one-drop sorts, the delay is adjusted in 0.1-drop increments. In the case of large, fragile particles, such as protoplasts, sorting efficiencies must be optimized using indestructible particles of similar sizes, such as pollen.[22] The deflection plate high-voltage and phase settings are adjusted to obtain side streams lacking droplet "fanning." Histograms are accumulated for the particles of interest, the appropriate sort windows are defined, and sorting is enabled. For larger tips, droplet formation usually occurs below the field of view of the video camera, and the cursor-based computation cannot be done. In this case, the sort delay has to be determined through a sort matrix.

If protoplasts are to be cultured after sorting, sterile sorting procedures are required. Sheath and rinse tanks are cleaned with detergent, then filled with 70% ethanol. Ethanol is back-flushed through the sample uptake opening, then a sample tube containing 70% ethanol is run through the system for 15 min. These steps are repeated with sterile water, before filling the system with sterile sheath fluid. Samples are sorted into sterile 15-ml centrifuge or microcentrifuge tubes. In standard, three-droplet sort mode, we routinely obtain 100% sort efficiencies using a transducer drive frequency of 16.7 kHz and a sort delay of 15. Three-droplet sorting provides the greatest margin of error for recovery of the desired particles, but it also increases the probability that unwanted particles will be included within the sorted droplets. The unwanted particles can be eliminated by enabling the anticoincidence circuit, as long as they trigger data acquisition, although this may result in unacceptably low recoveries. If the unwanted particles produce signals lower in value than the threshold set on the active discriminator, they will not trigger data acquisition. Under these circumstances the only available means to eliminate contamination is to perform one-droplet sorts. Because the error margin for sorting the desired particle is reduced in one-droplet mode, optimizing sort recoveries requires careful adjustment of the phase setting.

Cytomation MoFlo. For sorting of protoplasts or nuclei using the 100-μm tip, we employ sheath and sample pressures of 15 and 14.3 lb/in^2, a drive frequency of 21.5 kHz, and a drive amplitude of 60 V. These settings produce symmetrical droplets having six to eight fast satellites visible below the last attached droplet. In this system, the deflection assembly is fixed in position so no adjustment is required. Using the test pattern function, the charge phase and deflection amplitude values are adjusted to produce stable

reams with minimal fanning. An estimate of the delay is first deter-
from the stroboscopic camera image of the flow stream, by counting
mber of undulations observed between the last attached droplet and
w cell tip. This is then used as a starting point for performing a sort
, using the methods described in the previous section. It is particularly
l, when employing the one-drop sorting mode, to find via the sort
: the nearest one-sixteenth drop setting that gives 100% recovery.
r sterile sorting, the sheath tank is filled with 70% ethanol and the
nent is allowed to run for 15 min, thereafter being rinsed with sterile
before filling the sheath tank with sterile sheath fluid. Samples are
into sterile 12 × 75 mm tubes.

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Cytometric Detection of Green Fluorescent Protein in nsfected Protoplasts

gure 1 illustrates the flow cytometric detection of accumulation of
asmic GFP following transfection of maize leaf protoplasts with
?PDK-GFP. Biparametric analysis of red versus green fluorescence
ion intensity indicates that control (nontransfected) protoplasts char-
stically occupy a discrete cluster having a low level of green autofluor-
:e and a somewhat higher level of red autofluorescence (Fig. 1A).
s a consequence of the presence of endogenous pigments within these
ted cells. In contrast, protoplasts examined under the microscope
hr after transfection with the GFP constructs exhibit green fluores-
.[12] and flow cytometric analysis of these protoplasts reveals a second
r, having a greatly increased level of green fluorescence but unaltered
iorescence (Fig. 1B). This is consistent with expression of GFP within
protoplasts. The proportion of protoplasts expressing GFP is variable
:en experiments. In the illustrated case, approximately 14% express
and 86% do not. However, the lower cluster includes nonviable proto-
. and this means that the overall transfection percentages are almost
nly higher than 14%.
ow cytometric analysis of surface granularity (90° light scatter) can
)e combined with measurement of green fluorescence emission for
tion of GFP expression in protoplasts. Again, nontransfected proto-
occupy a single cluster (Fig. 1C), whereas protoplasts analyzed 18 hr
:ansfection are distributed between two clusters (Fig. 1D), the upper
ich (in this experiment, 34% of the protoplasts) represents the GFP-
ssing cells. Statistical analysis can be automatically done on the clusters
)vide mean, median, and mode values. The mean fluorescence emission

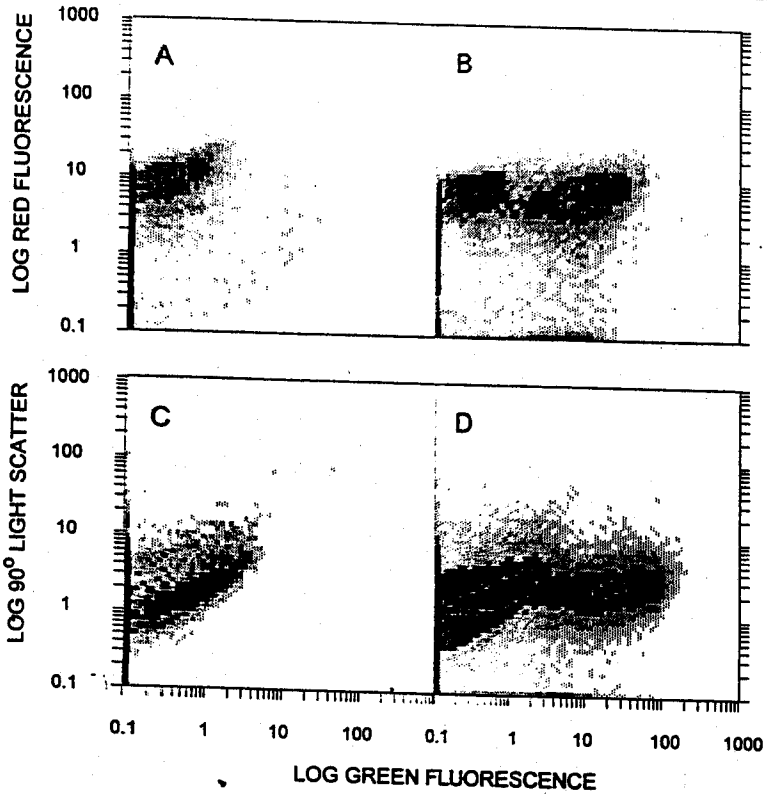

FIG. 1. Transient expression of GFP in maize leaf protoplasts. (A) Biparametric flow analysis of control protoplasts, based on green and red fluorescence emission. (B) Biparametric flow analysis, based on green and red fluorescence emission of transfected maize protoplasts. (C) Biparametric flow analysis of control protoplasts, based on green fluorescence and 90° light scatter. (D) Biparametric flow analysis of transfected protoplasts, based on green fluorescence and 90° light scatter (from Ref. 9, with permission).

intensity from individual GFP-expressing protoplasts in these cases is about 80-fold that of the nontransfected controls.

Similar results are obtained using pRJG2, pRJG8, and 35SCPPDK-GFP, and using tobacco leaf protoplasts, although the proportion of tobacco protoplasts that express GFP is generally lower than for maize. Particularly important to successful transfection is the use of plants growing under optimal conditions. Such plants produce high yields of protoplasts, which are intact and viable, and can tolerate transfection without excessive cell rupture. As a general rule, if initial protoplast yields are poor, transfection efficiencies will be drastically reduced.

Flow Cytometric Detection of Green Fluorescent Protein in Protoplasts Prepared from Transgenic Plants

Protoplasts from the leaves of dark-grown maize seedlings contain etioplasts, rather than chloroplasts, and therefore appear as translucent spheroids approximately 25 μm in diameter, within which is the nucleus, a large vacuole, and cytoplasmic strands containing numerous granules.[7] In contrast, protoplasts from tobacco leaves are about 35 μm in diameter, and contain approximately 70 mature chloroplasts.[21] Biparametric analysis of tobacco protoplasts based on 90° side scatter versus log red fluorescence identifies two clusters (Fig. 2A and C). The cluster of higher fluorescence comprises intact protoplasts. The cluster of lower red fluorescence comprises free chloroplasts that are derived from broken protoplasts that, owing to the large numbers of chloroplasts within individual cells, inevitably contaminate protoplast populations, even those purified by sucrose gradient flotation.[21]

Tobacco plants transformed with pRJG37 accumulate green fluorescence within the nuclei.[16] No differences are observed in biparametric analyses of 90° side scatter versus red fluorescence between the control and transgenic populations (Fig. 2A and C)..Biparametric analysis of 90° light scatter versus log green fluorescence of control tobacco protoplasts also reveals two clusters (Fig. 2D). However, for the transgenic population (Fig. 2B), an additional cluster is observed, having an approximately fourfold increase in green fluorescence. It can be assumed that all tobacco protoplasts produce low levels of endogenous green fluorescence due to contributions from chloroplast and vacuolar pigments having emission spectra overlapping that of GFP. This modest overall increase in green fluorescence may also reflect in part the small size of the nuclear compartment within which the GFP molecules are accumulated. Interestingly, there is evidence that the transgenic population contains some protoplasts that express little or no GFP (in Fig. 2B, some protoplasts remain in a cluster occupying the same position as that of the nontransgenic protoplasts in Fig. 2D).

Flow Cytometric Detection of Green Fluorescent Protein in Nuclei Isolated from Transgenic Plants

The problem of high endogenous fluorescent backgrounds provided by protoplasts can be alleviated by analysis of subcellular homogenates. When leaves from transgenic tobacco plants transformed with pRJG37 are homogenized by chopping, intact nuclei are released. Biparametric flow analysis of 90° side scatter versus log green fluorescence identifies a discrete cluster of green-fluorescent nuclei that are not present in homogenates prepared

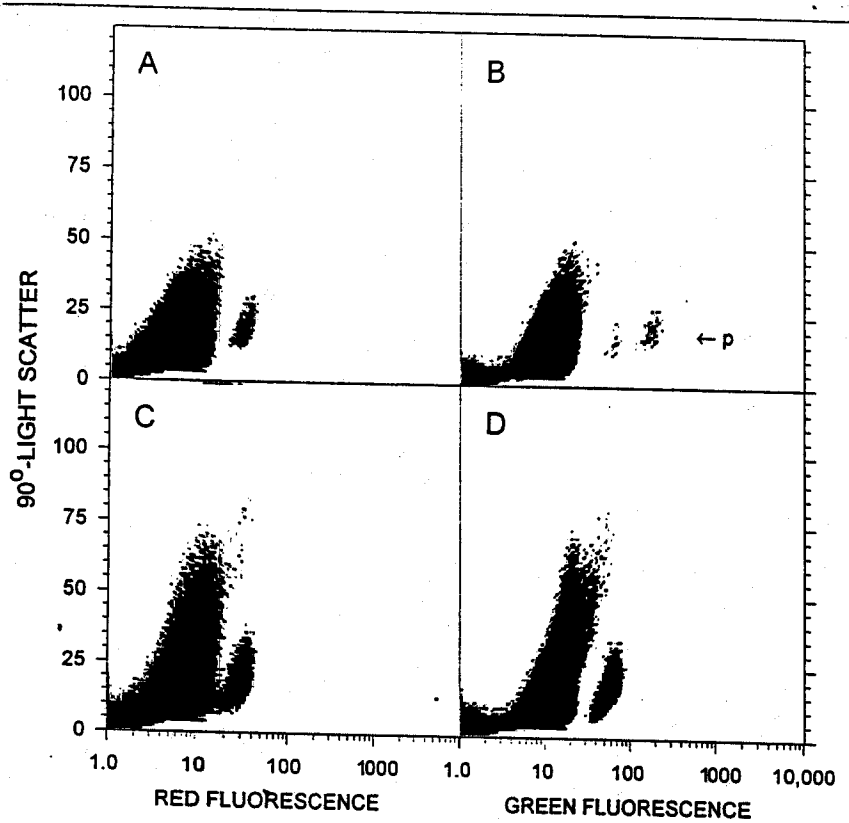

FIG. 2. Flow cytometric analysis *in vivo* of GFP targeting to nuclei. Protoplasts from leaves of transgenic plants (A and B), or nontransgenic controls (C and D), were subjected to biparametric analysis of 90° light scatter either versus red fluorescence (A and C), or versus green fluorescence (B and D). No differences are observed between the transgenic and nontransgenic populations in terms of red fluorescence. In the transgenic population an additional cluster of protoplasts (p) is visible; this cluster has an enhanced level of green fluorescence that is absent from the nontransgenic control.

from the nontransgenic controls (Fig. 3A and B). A similar analysis can be done employing log green fluorescence versus log orange fluorescence (Fig. 3C and D). In this case, the nuclei appear as a cluster oriented diagonally. This is due to spillover of the long-wavelength portion of the GFP fluorescence into the orange fluorescence detector (555–595 nm). In comparison with the situation observed in analysis of intact protoplasts, the cluster of nuclei is completely separated from the other cluster corresponding to subcellular organelles and miscellaneous debris, indicating the endog-

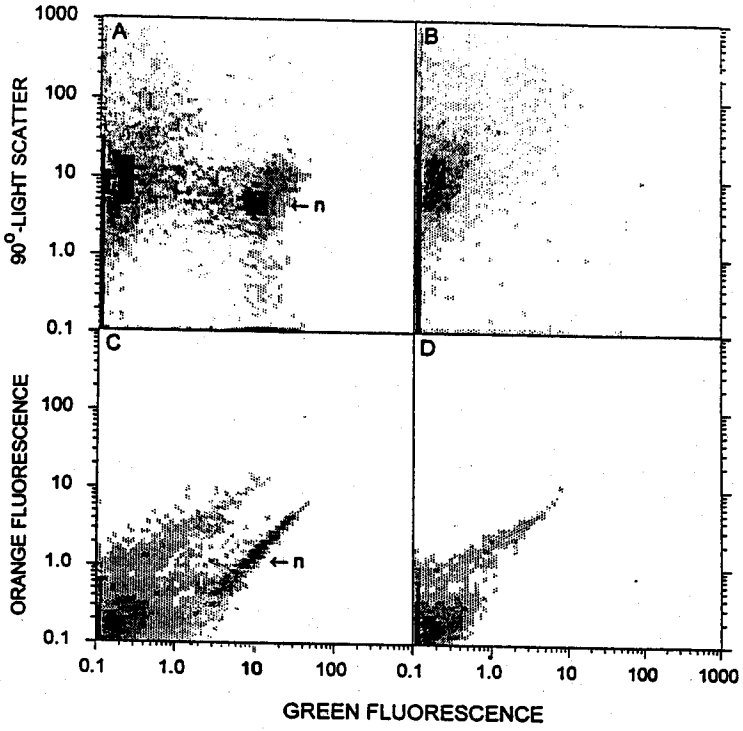

FIG. 3. Flow cytometric analysis *in vitro* of GFP targeting to nuclei. Homogenates from ıves of transgenic plants (A and C), or nontransgenic controls (B and D), were subjected biparametric analysis of 90° light scatter versus green fluorescence (A and B), or of orange rsus green fluorescence (C and D). The regions corresponding to nuclei (n) containing GFP : absent from the controls (from Ref. 16, with permission).

ıous levels of non-GFP green fluorescence within cellular homogenates 'e low.

Examination of different transgenic plants from independent transfor-ations reveals variation in the level of GFP fluorescence produced by the ıclei, which can be quantitatively analyzed through reference to fluores-:nt beads as internal standards.[16] The degree of nuclear GFP accumulation significantly correlated ($r^2 = 0.81$) with GUS activity measured in total :llular homogenates. GUS activity is also topologically restricted to the ıclei as indicated by histochemical staining.[16] The expression cassette ɔntained in pRJG23 and pRJG37 therefore represents an example of a uly bifunctional reporter molecule.

Discussion

Flow cytometry provides a powerful means for the accurate analysis of the amounts of fluorescence contained within populations of cells and within subcellular organelles.[2,3] For flow cytometry to be employed for the analysis of transgenic expression of marker proteins, it is necessary to link detection of a fluorescent signal to the level of expression of the transgene. Traditionally, this has been done through use of cell surface markers, which are subsequently detected via immunofluorescence labeling.[2,3] The emergence of GFP as a marker transgene has considerably expanded the range of experiments that can be done using flow cytometry. The unique attributes of GFP, which provide particular advantages over other markers, are primarily that the production of fluorescence requires only the translation of the primary sequence of the protein, and again that observations can be made *in vivo*, and do not require fixation or other cellular perturbations. A further, practical feature of GFP is that it can be efficiently excited using the argon lasers with which most flow cytometers are routinely equipped.[5] Finally, in the specific case of higher plants, the characteristic fluorescence emission of GFP is, for many tissues and plant species, spectrally distinct from other autofluorescent pigments found within the cells, most notably the pigments of the photosynthetic apparatus.

Our results demonstrate that flow cytometry can be readily employed to monitor the expression of GFP within higher plants, both within intact protoplasts and, following targeting, within subcellular organelles.[7,9,12,16] In the flow cytometric analysis of plants, the major problems first concern reducing native organs and tissues into suspensions of cells or subcellular organelles that can pass through the restricted diameter of the flow cell tip. This problem is avoided either through enzymatic production of protoplasts, which are spherical, wall-less cells, or through mechanical tissue disruption to produce homogenates containing suspensions of intact organelles. The second problem is the production of a GFP-specific fluorescent signal that can be readily detected over the background of cellular fluorescence. Endogenous autofluorescence is largely associated with three cellular structures, the cell wall, the vacuole, and the plastids; other objects within the cell, for example, nuclei, mitochondria, and miscellaneous vesicles, are largely translucent and nonfluorescent. The fluorescent contribution of cell walls is irrelevant in the analysis of both protoplasts and subcellular homogenates. For homogenates, tissue disruption converts the large vacuoles typical of plant cells into a myriad of tiny vesicles, the individual fluorescent contributions of which turn out to be negligible. For subcellular homogenates, the problem then reduces to providing a GFP molecule that is efficiently targeted to a location that is structurally distinctive, and that can

e preserved in an intact state following homogenization. Targeting of GFP) the nucleus satisfies these criteria, and it then becomes a question of istinguishing green fluorescent nuclei from other organelles having endog- nous autofluorescence that overlaps the emission spectrum of GFP. For rotoplasts, the problem is more complex, because a single protoplast ontains a fluorescent background comprising the autofluorescent contribu- ion of its vacuole plus the summed contributions of all its plastids. It should e noted that, for some tissues (tobacco roots), the presence of green ıtracellular autofluorescence significantly impedes examination of nontar- eted (i.e., cytoplasmic) GFP expression in transgenic plants.[16] In this re- ard, targeting of GFP to the nucleus provides an unambiguous means to letect transgene expression over the background autofluorescence, because his background is diffusely dispersed throughout the cell, and never occu- ies punctate regions as seen for accumulation of GFP within the nucleus.

The means that we have employed to achieve GFP expression involves ne of two possible experimental procedures: transfection of protoplasts esulting in transient expression, or production of transgenic plants. We ıave shown that both approaches result in GFP expression that can readily e observed using epifluorescence and confocal microscopy, and, through ncluding appropriate modifications to the GFP primary sequence, allows ınambiguous observation of targeting to the nucleus. Two modifications ıre needed. The first is to include an effective nuclear localization signal (NLS) translationally fused to the GFP coding sequence. We have employed ı short NLS taken from an orphan tobacco transcriptional factor (the C2 protein[13]), which appears functional in a wide variety of different tissues, and in at least two plant species. The second modification involves increas- ing the size of the GFP molecule. Native GFP approximates 26 kDa in size, which is smaller than the exclusion limit of nuclear pores. For this reason, GFP appears free to diffuse into and out of the nucleus,[12] and is not retained within the nucleoplasm even by an effective NLS. We increased the size of GFP through fusing it to the complete coding region of GUS; the chimeric protein has a molecular size in excess of 100 kDa. Correspondingly, attachment of the C2 NLS efficiently targets this chimeric molecule into the nucleus. We have also reported the construction of GFP fusions with NLS-containing polypeptide sequences taken from the tobacco etch virus.[12] These effectively target GFP to the nucleus, and a common feature is an increase in molecular mass, respectively, to 48 and 76 kDa. Reports relating to the interaction of the molecular size of GFP with the process of nuclear targeting include analysis of the process of signal-mediated nuclear export.[23] In this case, a fusion protein (~68 kDa) was synthesized comprising a GFP

[23] K. Stade, C. S. Ford, C. Guthrie, and K. Weis, *Cell* **90**, 1041 (1997).

dimer coupled to the simian virus 40 (SV40) T antigen NLS and to the leucine-rich nuclear export signal (NES) of the inhibitor of the cAMP-dependent protein kinase. The increased size of this reporter prevented passive diffusion of the molecule across the nuclear pore, and thereby permitted genetic analysis of the mechanisms of NLS- and NES-mediated nucleocytoplasmic shuttling.

The presence of GUS and GFP within a contiguous polypeptide provides a bifunctional reporter. As far as we are aware, this is the first study of bifunctional reporter molecules in higher plants. Because the two different functions can be separately assayed, this means that an examination is possible of the interactions of these domains. Similar values were observed for the specific activities of GUS, c2-NLS-GUS, and c2-NLS-GFP-GUS in transgenic tobacco plants, and in transfected protoplasts.[12,15] The different transgenic plants produced in these experiments, derived from separate transformation events, displayed different levels of GFP accumulation. We observed a strongly positive correlation between the fluorescence intensity of the leaf nuclei and the total GUS activity measured in leaf extracts, implying that all of the bifunctional reporter is located in the nuclei. This also suggests that gene expression, rather than protein targeting, limits the levels of fluorescence that were observed. The residual variance in the regression analysis might represent interference between the two protein domains during folding, because GUS forms homo-tetramers,[15] whereas GFP has the ability to dimerize under various conditions,[24–26] which results in fluorescence quenching.

For the protoplasts isolated from transgenic plants, in contrast to the situation observed for transfected protoplasts, the total levels of cellular green fluorescence measured through flow cytometry were only modestly increased over those found in the nontransgenic controls. This is in part due to the presence of endogenous autofluorescence, which overlaps the emission spectrum of GFP. In part, the low contribution may also reflect the possibility that the nucleus provides a limited storage capacity for GFP. It is also possible that transgenic expression of GFP might be, at some level, toxic to the cell. A persistent level of comment on the Fluorescent Proteins Newsgroup on Bionet (*www.bio.net*), concerns the potential toxic effects of cytoplasmic accumulation of GFP. This contrasts with published reports from various groups of high levels of cytoplasmic GFP expression in

[24] A. B. Cubitt, R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross, and R. Y. Tsien, *Trends Biol. Sci.* **20**, 448 (1995).
[25] M. Ormö, A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien, and S. J. Remington, *Science* **273**, 1392 (1996).
[26] F. Yang, L. G. Moss, and G. N. Phillips, Jr., *Nature Biotechnol.* **14**, 1246 (1996).

transgenic plants.[27,28] In common with most studies in transgenic organisms, qualitative rather than quantitative methods are typically employed for estimating the amounts of GFP fluorescence. This can be fluorescence microscopy, although there is considerable interest in the possibility of employing hand-held illumination sources for large-scale screening of transformants in the greenhouse or in the field. From the results that have appeared, it is impossible to determine whether specific limits exist as to the levels of GFP that can accumulate within individual plant cells without encountering toxicity. It is self-evident that the expression of any protein beyond specific limits will, by definition, prove toxic to any cell. The situation for GFP is further complicated by photochemical effects, which are almost certainly a function of the intensity of the incident illumination.

Our results indicate that transgenic nuclear accumulation of GFP can be achieved at levels sufficient to provide an easily measured signal that can be recorded using epifluorescence or confocal microscopy, or flow cytometry. Under these conditions, the plants appear normal, and progress through their life cycles in a normal manner. It should be noted that the use of different promoters, coding sequences, targeting signals, and terminators prevents determination of the reasons for the different levels of expression exhibited between the transfected protoplasts and transgenic plants. This is further complicated by the known occurrence of position effects in transgenic plants, as well as between-experiment differences in transfection efficiencies. In our experiments, the highest levels of expression were typically found in transfected protoplasts. Variation was associated with transfection efficiencies, but not with the type of plasmid that was employed. From this, we conclude that, at least in tobacco, the levels of expression of cytoplasmic GFP that were achieved are not toxic over the short term, and are not seriously compromised by improper mRNA processing. The systematic use of flow cytometry to compare overall levels of GFP expression between different constructions, target locations, and tissue types, should be instructive. It should also allow an unambiguous determination as to whether transgenic GFP expression beyond a certain concentration limit becomes toxic to the cell, and whether targeting to various subcellular compartments ameliorates or augments this toxicity.[11]

Future developments in the flow cytometric analysis of GFP expression in plants will take advantage of an increasing number of spectral variants of this molecule. The wild-type GFP fluorophore has a bimodal excitation

[27] C. Reichel, J. Mathur, P. Eckes, K. Langenkemper, C. Koncz, J. Schell, B. Reiss, and C. Maas, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5888 (1996).

[28] S. Z. Pang, D. L. DeBoer, Y. Wan, G. Ye, J. G. Layton, M. K. Neher, C. L. Armstrong, J. E. Fry, M. A. W. Hinchee, and M. E. Fromm, *Plant Physiol.* **112**, 893 (1996).

spectrum, having peaks of absorption at 395 and 475 nm. Its emission spectrum is unimodal, with a maximum at about 509 nm. Amino acid substitutions either in the fluorophore or in the surrounding sequences are found to alter the emission and excitation spectra, the photostability, and the quantum yield of GFP. Spectral variants include those characterized by a unimodal absorption spectrum in which one of the two wild-type absorption peaks is eliminated, but without an alteration in the emission spectrum. One class of these variants has an absorption maximum at 395 nm. These variants[29,30] share a threonine-to-isoleucine substitution at amino acid 203, and for flow cytometric applications can be conveniently excited using the Innova 300 krypton laser.[31] Members of a second, large class of spectral variants exhibit unimodal excitation spectra having a maximum near 475 nm.[32–34] The excitation maxima of these variants are typically at a slightly longer wavelength than the 475-nm peak of wild-type GFP. This means that they are more efficiently excited by the 488-nm argon laser line, and therefore have an increased brightness over wild type. The extinction coefficients and quantum yields of both of these classes of variants are at least as great as wild-type GFP and comparable to that of fluorescein. As a general rule of thumb, in flow cytometry it has been found that at least 500 fluorescein molecules must be associated with a specific particle or cell to produce a fluorescent signal that is above the background derived from cellular autofluorescence and instrument noise. In this respect, optimized[14] S65T GFP (a member of the second class of variants) produces roughly equivalent amounts of fluorescence as fluorescein. GFP variants also have been described that absorb in the UV and emit in the blue regions of the spectrum,[29] and that absorb in the green and emit in the yellow.[25] In these cases, modifications to codon usage to increase overall levels of expression are also incorporated; such variants are typically termed "enhanced." Although the codon usage is human, we have found that such enhanced GFPs provide excellent signals in higher plant cells. Biparametric analysis of simultaneous expression of two GFP variants has been described[31,35] in

[29] R. Heim, D. C. Prasher, and R. Y. Tsien, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12501 (1994).
[30] T. Ehrig, D. J. O'Kane, and F. G. Prendergast, *FEBS Lett.* **367**, 163 (1995).
[31] M. Anderson, I. M. Tjioe, M. C. Lorincz, D. R. Parks, L. A. Herzenberg, G. P. Nolan, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8508 (1996).
[32] S. Delagrave, R. E. Hawtin, C. M. Silva, M. M. Yang, and D. C. Youvan, *Bio/Technology* **13**, 151 (1995).
[33] B. P. Cormack, R. H. Valdivia, and S. Falkow, *Gene* **173**, 33 (1996).
[34] R. Heim, A. B. Cubitt, and R. Y. Tsien, *Nature (London)* **373**, 663 (1995).
[35] J. Ropp, C. Donahue, D. Wolfgang-Kimball, J. Hooley, J. Chin, R. Hoffmann, R. Cuthbertson, and K. Bauer, *Cytometry* **24**, 284 (1996).

nimal cell systems, and it will be of interest to determine whether these :chniques can be applied to higher plants.

In terms of instrumentation, the illumination that can be applied to the ells is only limited by the financial resources available to the flow cytometric acility. Base model flow cytometers are equipped solely with an air-cooled rgon laser, emitting light of 488 nm. This evidently restricts analysis of FP molecules to those absorbing at that wavelength. Upgrades to these nstruments provide additional lasers with illumination either in the UV r at various visible wavelengths from 456 to 799 nm. Various numbers of asers can be arranged to independently intersect the flow stream. The nofficial record of numbers of independent flow cytometric parameters imultaneously made on a single population of cells is nine, and required hree-laser excitation.[36] The availability of multiple different excitation vavelengths considerably expands the numbers of different GFP variants hat might simultaneously be examined using flow cytometry, although he present costs of such equipment will inevitably be restrictive. Further levelopments in laser technologies, particularly for diode lasers, are cer- ainly likely to reduce future costs, and increase the different numbers of ines available for use in flow cytometry. An alternative strategy is to search or novel, spectrally distinct variants that can be simultaneously excited using 488-nm light. An example of this is a report describing dual-color flow cytometric analysis of expression in murine cells of enhanced GFP (i.e., E_{max} = 490 nm; codon optimized) and enhanced yellow fluorescent protein (E_{max} = 513 nm), using argon laser excitation at 488 nm.[37] Finally, it is most likely that additional novel fluorochromatic polypeptides will be recovered from oceanic species other than *Aequorea,* and these may well prove useful in flow cytometric analyses.

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