

Simultaneous fluorescence-activated cell sorter analysis of two distinct transcriptional elements within a single cell using engineered green fluorescent proteins

(genetics/transcription)

M. T. ANDERSON*, I. M. TJIOE*, M. C. LORINCZ*, D. R. PARKS*, L. A. HERZENBERG*, G. P. NOLAN†‡, AND L. A. HERZENBERG*§

Departments of *Genetics, †Molecular Pharmacology, and ‡Microbiology and Immunology, Stanford University School of Medicine, Stanford CA 94305

Contributed by Leonard A. Herzenberg, April 9, 1996

ABSTRACT Green fluorescent protein (GFP) is widely used as a reporter gene in both prokaryotes and eukaryotes. However, the fluorescence levels of wild-type GFP (wtGFP) are not bright enough for fluorescence-activated cell sorting or flow cytometry. Several GFP variants were generated that are brighter or have altered excitation spectra when expressed in prokaryotic cells. We engineered two GFP genes with different combinations of these mutations, GFP(S65T,V163A) termed GFP-Bex1, and GFP(S202F,T203I,V163A) termed GFP-Vex1. Both show enhanced brightness and improved signal-to-noise ratios when expressed in mammalian cells and appropriately excited, compared with wtGFP. Each mutant retains only one of the two excitation peaks of the wild-type protein. GFP-Bex1 excites at 488 nm (blue) and GFP-Vex1 excites at 406 nm (violet), both of which are available laser lines. Excitation at these wavelengths allows for the independent analyses of these mutants by fluorescence-activated cell sorting, permitting simultaneous, quantitative detection of expression from two different genes within single mammalian cells.

The GFP gene, isolated from the jellyfish *Aequorea victoria*, encodes a protein that fluoresces upon excitation with violet or blue-green light. This gene is useful in both prokaryotes and eukaryotes as a reporter or marker (reviewed in refs. 1–3) and is unique among reporters in that the green fluorescent protein (GFP) fluorophore spontaneously forms intracellularly without added cofactors. As a result, the gene product provides a direct readout of gene expression, avoiding the difficulty of introducing a substrate into live cells and the complexity of enzyme-substrate kinetics.

Fluorescence can be detected by flow cytometry in mammalian cells transiently transfected with a wild-type GFP (wtGFP) expression vector. However, detection of fluorescence from cells containing a single copy of GFP has not been described. We find that the high levels of autofluorescence inherent in mammalian cells, combined with excitation spectra that are suboptimal for flow cytometry, precludes the detection of wtGFP when a single copy of the gene is stably integrated. Furthermore, mammalian cells are typically grown at 37°C, well above the temperature that yields maximum wtGFP fluorescence (4). The generation of mutant GFPs with improved stability and therefore increased fluorescence at 37°C, in combination with spectral changes optimized for fluorescence-activated cell sorting (FACS) analysis, would allow use of this reporter where low levels of expression are expected.

wtGFP has a major excitation peak at 395 nm, a minor excitation peak at 475 nm and a single emission peak at 509 nm

(5). Several mutants were generated which retain one or the other of the excitation peaks when expressed in *Escherichia coli* (6–9). In addition, Bender *et al.* isolated a mutant (V163A) which retains the excitation and emission peaks of wtGFP but shows a 17-fold increase in fluorescence intensity (W. Bender, J. Kahana, A. Hudson, and P. A. Silver, personal communication) when it is expressed in *E. coli*. Presumably, this mutant forms a more stable GFP fluorophore in *E. coli* and other cells.

Dual laser FACS instruments equipped with krypton ion and argon ion lasers are capable of simultaneous excitations at 406 nm and 488 nm. In this study, we combine a mutant that excites primarily at 488 nm [S65T (7)] or a mutant which excites primarily at 406 nm [S202F, T203I (6)], with the V163A mutation, to generate two GFP variants, termed GFP-Bex1 and GFP-Vex1, respectively. Both variants show brighter fluorescence than the wild-type protein when expressed in mammalian cells. Fluorescence levels of both mutants are sufficient to detect GFP expression by FACS in cells with a single proviral integration. Thus mammalian cells can be viably sorted based on the level of GFP fluorescence, making these GFP mutants valuable genetic markers for mammalian studies. Furthermore, these mutants are spectrally distinguishable using FACS, allowing for the simultaneous, quantitative analysis of expression from two different promoters within a single cell.

MATERIALS AND METHODS

Plasmids, Mutagenesis and Sequencing. MFG-wtGFP-pBR322 was a kind gift from R. Mulligan. Analysis of MFG-wtGFP-pBR322 revealed a sequence differing from the published sequence (10) by the substitution of a G for A at nucleotide 239. This MFG-wtGFP-pBR322 backbone was mutagenized by PCR to generate a double mutant (S65T and V163A), termed GFP blue-excited excitation mutant 1 (GFP-Bex1) and a triple mutant (S202F, T203I, V163A) termed GFP violet-excited excitation mutant 1 (GFP-Vex1). The PCR-based site-directed mutagenesis strategy was as described by Picard *et al.* (11), with the addition of a Qiaquick Spin PCR Purification (Qiagen, Chatsworth, CA) step following the generation of the megaprimer. Subsequently, the coding fragments of mutant GFP variants from MFG-GFP-Bex1 and MFG-GFP-Vex1 were cloned into *Xba*I-*Hind*III digested pGL3 (Promega) to create pGL3-(GFP-Bex1) and pGL3-(GFP-Vex1). The tetracycline (tet) inducible construct, pGL3-UHD10–3-(GFP-Bex1) was created by inserting a modified fragment from pUHD10–3 (12) containing heptamerized tet operators into the *Xho*I-*Hind*III site of pGL3-(GFP-Bex1).

Abbreviations: GFP, green fluorescent protein; wtGFP, wild-type GFP; FACS, fluorescence-activated cell sorter; tet, tetracycline; Bex1, blue-excited excitation mutant 1; Vex1, violet-excited excitation mutant 1; RSV, Rous sarcoma virus; IPTG, isopropyl β -D-thiogalactoside; moi, multiplicity of infection.

§To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The *Hind*III–*Sac*I fragment of pOPRSV1-CAT (Stratagene) containing the *lac* operator (*lac*O) and Rous sarcoma virus (RSV) promoter was inserted into pGL3-GFP(Vex1) upstream of the GFP coding region to create the *lac*-inducible construct pGL3-OPRSV1-GFP(Vex1). The *lac* repressor expression vector p3'SS and the tet repressor-*vp16* chimera expression vector, pUHD15-1 were purchased from Stratagene. GFP coding regions were sequenced by dyedeoxy terminator cycle sequencing (PRISM Ready Reaction Kit; Perkin-Elmer). DNA sequence analysis was performed with IntelliGenetics software.

Cells, Tissue Culture and Retroviral Infection. NIH 3T3 cells (ATCC CCL 163) and BOSC 23 (13) and Phoenix retroviral producer cells (G.P.N., unpublished data) were maintained as described (13). Transfection of BOSC 23 and Phoenix producer cells as well as retroviral infection of 3T3 were as described (13). For gene induction experiments, BOSC 23 cells were maintained in 1 μ g of tet per ml (Sigma) for 24 h and then transfected in the continued presence of tet. After 24 h, cells were harvested, split into four aliquots, and incubated for 48 h in the presence or absence of 1 μ g of tet per ml and/or 50 mM isopropyl β -D-thiogalactoside (IPTG; Life Technologies, Gaithersburg, MD).

FACS Analysis. FACS analyses were carried out on a two-laser FACStar Plus platform (Becton Dickinson) modified by the Stanford FACS Development Group. An Innova 302 krypton ion laser (Coherent Radiation, Santa Clara, CA) tuned to 406 nm and an argon ion laser (Coherent Radiation) tuned to 488 nm were used in the dual laser experiments. To optimize detection of GFP fluorescence by FACS, the standard fluorescein filter was replaced with a 515/40-nm interference filter. Furthermore, to allow subtraction of the autofluorescence background signal, a detector with a 630/30 bandpass filter was used for autofluorescence compensation (14). On the FACS, the same emission fluorescence can be collected from two different laser excitations because the laser-stream intercepts are separated and the light emitted from each laser excitation follows a separate light path. Multiparameter data were collected and analyzed by using FACS-DESK configured as described (15). Where indicated, software compensation was applied to the collected data by using software from A. Treister (Stanford University, personal communication).

RESULTS

While wtGFP fluorescence can be detected by FACS in mammalian cells transiently transfected with the MFG-GFP plasmid [data not shown and Ropp *et al.* (16)], quantitative interpretation of these results is impossible due to the variable distribution of the reporter plasmid within a population of transfected cells. To determine whether the GFP expressed from a single GFP gene per cell fluoresces at levels sufficient for detection by FACS, retroviral gene transfer (13) was used to stably incorporate the MFG-wtGFP construct in NIH 3T3 cells. Using 488-nm excitation, FACS analysis of MFG-wtGFP transduced cells revealed a single peak on a fluorescence histogram (Fig. 1B). While the median fluorescence value of the MFG-wtGFP-infected population was 2-fold greater than that of uninfected cells (Fig. 1A), the difference in fluorescence was not sufficient to resolve infected from uninfected cells.

We next transduced a new GFP variant, including the S65T and V163A mutations (termed GFP-Bex1) under conditions yielding infection efficiencies comparable to those achieved with the MFG-wtGFP (data not shown). In contrast to the results found with wtGFP, FACS analysis of NIH 3T3 cells infected with MFG-GFP-Bex1 revealed two populations of cells (Fig. 1C), one with fluorescence levels indistinguishable from uninfected cells, and the second with a median fluorescence \approx 50-fold greater than uninfected cells.

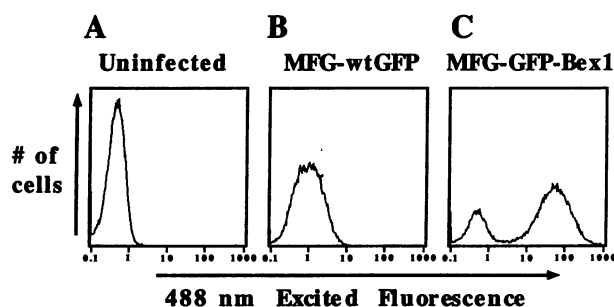


FIG. 1. Comparison of the wtGFP and GFP-Bex1 fluorescence levels in mammalian cells. NIH 3T3 cells infected with wtGFP or GFP-Bex1 retrovirus were analyzed 48 h after infection. Shown is fluorescence from 488-nm excitation of (A) uninfected NIH 3T3 cells, (B) MFG-wtGFP, or (C) MFG-GFP-Bex1-infected cells. This fluorescence was collected at 495–535 nm and is displayed as a histogram. Representative plots from three independent experiments are shown.

The latter population of cells exhibited 25-fold greater fluorescence than cells infected with wtGFP (compare Fig. 1B to C), an increase sufficient to allow for the complete resolution of infected from uninfected cells. This population represents 60% of the total cells. Poisson statistics predicts that the probability of an infected cell carrying only a single viral integrant is \approx 0.36. As the fluorescence levels of all of the GFP positive cells are distinguishable from nontransduced cells, this mutant can be used to accurately measure infection efficiencies.

The fluorescence levels achieved with GFP-Bex1, and the narrow excitation profile (with a peak at 488 nm) of the S65T mutation when expressed in *E. coli* (7), prompted us to explore the possibility of establishing a second GFP mutant with a distinct excitation spectrum for use on the FACS. To show that GFP-Bex1 is excited primarily with 488-nm light when expressed in mammalian cells, we configured a dual laser flow cytometer to excite cells at 488 nm and 406 nm, successively, and measure the emission at 495–535 nm in both cases (described in the *Materials and Methods*).

FACS analysis with 488-nm excitation reveals that 3T3 cells infected with MFG-GFP-Bex1 at a 5-fold lower multiplicity of infection (moi) than used in Fig. 1 show 40-fold greater fluorescence than uninfected cells, whereas excitation with 406-nm light yields fluorescence levels only 3-fold greater than uninfected cells (Fig. 2A). The low level of spectral overlap can be corrected by using software compensation, which corrects for the 406-nm excited fluorescence component of GFP-Bex1 from the 488-nm excitation value (compare Fig. 2A and B).

The low level of intrinsic 406-nm excitation exhibited by GFP-Bex1 prompted us to establish a second composite GFP variant that has high excitation at 406 nm but minimal excitation at 488 nm, for use in combination with GFP-Bex1 on the FACS. The double-mutant GFP (S202F, T203I) was previously shown to be maximally excited at 395 nm, with only minimal excitation at 488 nm (6). To increase the fluorescence intensity of this mutant, we added the V163A mutation to generate the triple mutant MFG-GFP (S202F, T203I, V163A), termed GFP-Vex1.

We produced MFG-GFP-Vex1 retrovirus and infected 3T3 cells as before. FACS analysis revealed that excitation of cells with 406-nm light results in 16-fold greater fluorescence levels than those found in uninfected cells. In contrast, 488-nm excitation of GFP-Vex1 results in only 3-fold greater fluorescence levels (Fig. 2C). This low level of 488-nm excited fluorescence can be corrected for by software compensation, as shown in Fig. 2D.

The distinct spectral properties of GFP-Vex1 and GFP-Bex1 indicates that these fluorophores can be used in combination for dual reporter analyses. To confirm that these variants can be detected simultaneously within a single cell, we infected

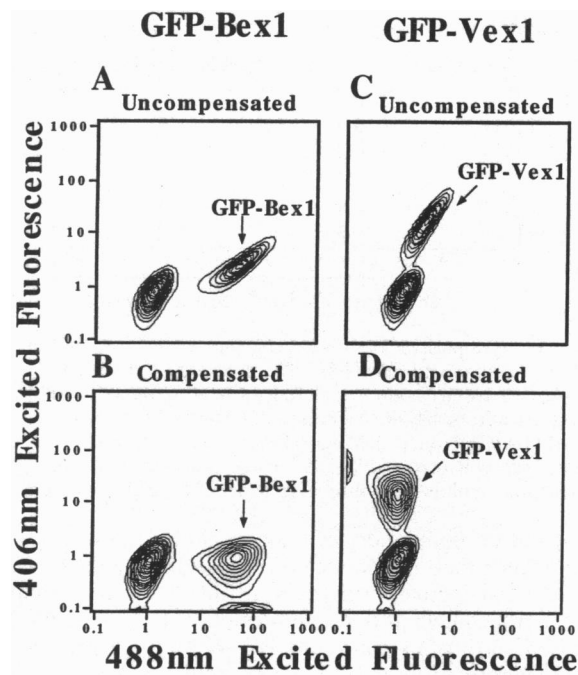


FIG. 2. Raw and compensated data of GFP-Bex1 and GFP-Vex1 fluorescence in NIH 3T3 cells. NIH 3T3 cells were infected with MFG-GFP-Bex1 (A and B) or MFG-GFP-Vex1-GFP (C and D) retrovirus and analyzed by flow cytometry 48 h postinfection. The moi was $\approx 20\%$ of that used in Fig. 1. Emission data collected with 406-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.

NIH 3T3 cells with equal amounts of GFP-Vex1 and GFP-Bex1 virus at three different mois (Fig. 3). FACS analysis of cells infected at an moi of 0.065 for each variant reveals three populations of cells: one with low levels of fluorescence characteristic of uninfected cells, and two with higher levels of fluorescence (Fig. 3A). The latter, clearly distinguishable populations were excited exclusively with 488- or 406-nm light (Fig. 3A), consistent with infection by a single retrovirus of either the GFP-Bex1 or GFP-Vex1 type, respectively.

Infection with 2-fold more retroviral supernatant yields the predicted moi of 0.13 for each virus, and generates, in addition to the two single positive populations shown in Fig. 3A, a new "double positive" population showing bright fluorescence with both 488-nm and 406-nm excitation (Fig. 3B), which is consistent with cells expressing both GFP variants due to coinfection. With a 5-fold further increase in the amount of retroviral supernatant used for infection, the number of double positives increases. These results are consistent with those predicted by the Poisson distribution with an moi of 0.65.

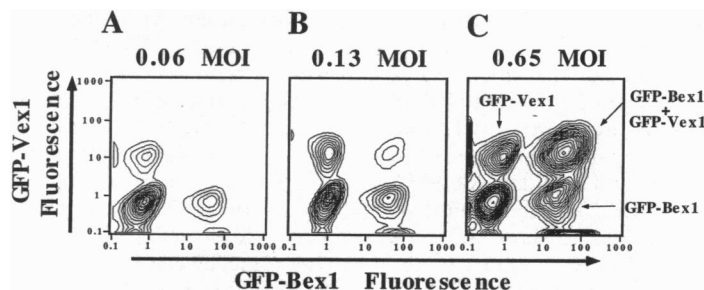


FIG. 3. Simultaneous detection of GFP-Bex1 and GFP-Vex1 fluorescence. Increasing amounts of a 1:1 mixture of the GFP-Bex1 and GFP-Vex1 viruses were used to infect NIH 3T3 cells. Cells were infected with an moi for each of the two viruses of 0.06 moi (A), 0.13 moi (B), or 0.65 moi (C), as determined by 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. 2 B and D. Representative plots from three independent experiments are shown.

We next tested whether the two GFP variants can be used simultaneously as reporters of two distinct regulatory elements. We developed a tet transactivator controlled GFP-Bex1 expression system and a *lac* repressor controlled GFP-Vex1 expression system for this purpose. Cotransfection of a vector with GFP-Bex1 under the control of the tet operator (12) with a second plasmid, pUHD15-1, encoding the tet transactivator (a chimera of the tet repressor and the VP16 activation domain) leads to the induction of GFP-Bex1 expression. However, in the presence of tet, the tet activator dissociates from the tet operator, repressing transcription of GFP-Bex1 (data not shown).

Transfection of a plasmid encoding GFP-Vex1 under the control of the *lac* operator and RSV promoter (17) with p3'SS, a plasmid constitutively expressing a modified *lac* repressor, leads to the repression of GFP-Vex1 expression. However, in the presence of IPTG, the *lac* repressor-DNA complex is disrupted, thereby inducing transcription of GFP-Vex1 (data not shown).

We cotransfected BOSC 23 cells with all four plasmids simultaneously, divided the cells into four populations, and tested the effects of inducing one, the other, both, or neither forms of GFP by the addition or removal of tet and/or IPTG. Examination of the two-dimensional contour and histogram plots reveals that the fluorescence generated with 488-nm excitation increases only for the populations cultured under inducing conditions for GFP-Bex1 (i.e., -tet) (Fig. 4 B and D versus A and C). This increase is not influenced by inducing conditions for GFP-Vex1 expression (i.e., +IPTG) (Fig. 4D). In contrast, the fluorescence generated with 406-nm excitation increases only for the populations cultured under inducing conditions for GFP-Vex1 (i.e., +IPTG) (Fig. 4 C and D versus A and B). This increase is not influenced by inducing conditions for GFP-Bex1 expression (i.e., -tet) (Fig. 4D). Induction of GFP-Bex1 and GFP-Vex1 simultaneously (i.e., -tet, +IPTG) increases the fluorescences at both 406-nm and 488-nm excitation (Fig. 4D).

Under GFP-Bex1 inducing conditions, the median fluorescence of the transfected population excited at 488 nm increased ≈ 10 -fold, while fluorescence excited at 406 nm (GFP-Vex1) showed no increase. In contrast, under inducing conditions for GFP-Vex1, fluorescence excited at 406 nm increased 2-fold, with no increase in fluorescence excited at 488 nm (GFP-Bex1). Based on these quantitative and qualitative comparisons, we conclude that GFP-Bex1 and GFP-Vex1 can be analyzed independently on the FACS, allowing for the simultaneous analysis of two distinct regulatory elements controlling these reporter genes.

DISCUSSION

Our studies show that GFP-Bex1 and GFP-Vex1 have sufficient fluorescence signal to be readily detected by FACS, and that both variants can be quantitatively detected indepen-

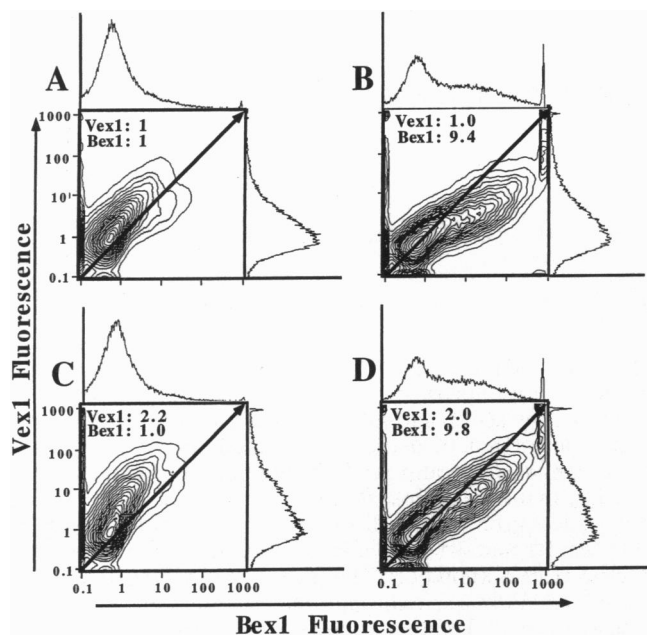


FIG. 4. Simultaneous analysis of two independently inducible transcription elements. BOSC cells were transiently transfected with the *lac*-inducible construct pGL3-OPRSV1-(GFP-Vex1), the tet-inducible construct; pGL3-UHD10-3-(GFP-Bex1), the tet transactivator expression plasmid pUHD15-1; and the *lac* repressor expression plasmid p3'SS in the presence or absence of tet and/or IPTG, as described. After 48 h of culture under inducing and/or noninducing conditions, transfected BOSC cells were analyzed on the FACS as described in Fig. 2B and D. Contour and histogram plots of uninduced GFP-Bex1 (A and C) and induced GFP-Bex1 (B and D) transduced cells under each culture condition are shown. The populations represented in C and D were also cultured under GFP-Vex1-inducing (+IPTG) conditions. The diagonal line through each contour plot is included as a visual reference. We calculated that $\approx 70\%$ of the transfected cells were transduced by comparing the fluorescence histograms of mock transfected and transfected cells under inducing conditions. The 65th percentile fluorescence value of each population thus represents the median fluorescence of successfully transduced cells. To calculate the fold increase in fluorescence, these values were normalized to those obtained for the population cultured in the absence of inducing conditions depicted in A. These values are displayed in the upper left corner of each contour plot. Data representative of three independent experiments is shown.

dently, permitting their simultaneous detection at the single cell level. As GFP(S65T) and GFP(V163A) are only 5- to 6-fold brighter than wtGFP when expressed in 3T3 cells (data not shown), the 25-fold increase in brightness achieved with the composite GFP(S65T,V163A) fluorophore, GFP-Bex1, reflects a synergy of the S65T and V163A mutations. We use a FACS with a krypton ion laser for excitation at 406 nm for exciting the GFP(S202F,T203I) fluorophore. Unfortunately, this GFP is not significantly brighter than wtGFP when excited at 406 nm (6). However, the addition of the V163A substitution generates a GFP (GFP-Vex1) that is bright enough when excited with the krypton ion laser at 406 nm to yield a signal-to-noise ratio sufficient for quantitative analysis.

The two GFPs used in our studies differ in sequence by only 3 amino acids. Because of this structural similarity, these variants should be very similar in their transcription, translation, and protein stability characteristics. Therefore, the levels of fluorescence of GFP-Bex1 and GFP-Vex1 can be used to accurately determine the relative activity of two independent transcriptional elements by FACS.

The cotransfection experiments described here establish that GFP reporter genes can be used to quantitatively study gene regulation from two different gene regulatory elements. Recently, Rizzuto *et al.* (18) demonstrated that two different GFPs could be qualitatively distinguished within single cells by fluorescence microscopy, adding to the potential uses of the GFPs described here. Thus, GFP-Bex1 and GFP-Vex1 should be useful tools for dissecting signal transduction pathways, determining hierarchies of gene expression and in marking cells for genetic complementation.

We thank Richard Mulligan for the MFG-GFP retrovirus and Wellcome Bender for sharing unpublished information on the V163A GFP variant. We also thank Rachel Gerstein, Daniel Kalman, and Mario Roederer for critical reading of the manuscript and helpful comments in its preparation. This work was supported by National Institutes of Health Grant CA 42609 to L.A.H. G.P.N. is a Scholar of the Leukemia Society of America and a Burrough's Wellcome New Investigator in Pharmacology.

- Peters, K. G., Rao, P. S., Bell, B. S. & Kindman, L. A. (1995) *Dev. Biol.* **171**, 252-257.
- Rizzuto, R., Brini, M., Pizzo, P., Murgia, M. & Pozzan, T. (1995) *Curr. Biol.* **5**, 635-642.
- Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A. & Tsien, R. Y. (1995) *Trends Biochem. Sci.* **20**, 448-455.
- Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K. & Umesono, K. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 11899-11903.
- Ward, W. W., Cody, C. W., Hart, R. C. & Cormier, M. J. (1980) *Photochem. Photobiol.* **31**, 611-615.
- Heim, R., Prasher, D. C. & Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12501-12504.
- Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995) *Nature (London)* **373**, 663-664.
- Heim, R. & Tsien, R. Y. (1996) *Curr. Biol.* **6**, 178-182.
- Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M. & Youvan, D. C. (1995) *Biotechnology* **13**, 151-154.
- Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. (1992) *Gene* **111**, 229-233.
- Picard, V., Ersdal-Badju, E., Lu, A. & Bock, S. C. (1994) *Nucleic Acids Res.* **22**, 2587-2591.
- Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5547-5551.
- Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8392-8396.
- Alberti, S., Parks, D. R. & Herzenberg, L. A. (1988) *Cytometry* **8**, 114-119.
- Moore, W. & Kautz, R. (1986) in *The Handbook of Experimental Immunology*, eds. Weir, D. M., Herzenberg, L. A., Blackwell, C. C. & Herzenberg, L. A. (Blackwell, Edinburgh), pp. 30.1-30.11.
- Ropp, J. D., Donahue, C. J., Wolfgang-Kimball, D., Hooley, J. J., Chin, J. Y., Hoffman, R. A., Cuthbertson, R. A. & Bauer, K. D. (1995) *Cytometry* **21**, 309-317.
- Fieck, A., Wyborski, D. L. & Short, J. M. (1992) *Nucleic Acids Res.* **20**, 1785-1791.
- Rizzuto, R., Brini, M., DeGiorgi, F., Rossi, R., Heim, R., Tsien, R. Y. & Pozzan, T. (1996) *Curr. Biol.* **6**, 183-188.