

Multiparameter Flow Cytometry of Fluorescent Protein Reporters

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Summary

Reporters based on the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and GFP-like proteins from other marine organisms provide valuable tools to monitor gene transfer and expression noninvasively in living cells. Stable cell lines were generated from the Sp2/0-Ag14 hybridoma that express up to three spectral enhanced versions of GFP, the enhanced cyan fluorescent protein (ECFP), the enhanced green fluorescent protein (EGFP), and the enhanced yellow fluorescent protein (EYFP), and/or a variant of the *Discosoma* coral red fluorescent protein (DsRed). The panel of lines was used to demonstrate a flow cytometric procedure for simultaneous analysis of all four fluorescent proteins that utilizes dual-laser excitation at 488 nm and 407 nm. Additional schemes for simultaneous detection of two, three or four of these fluorescent proteins are also presented.

Key Words

Discosoma coral red fluorescent protein (DsRed), enhanced cyan fluorescent protein (ECFP), enhanced green fluorescent protein (EGFP), enhanced yellow fluorescent protein (EYFP), gene transfer, multiparameter flow cytometry.

1. Introduction

Derivatives of the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* have been demonstrated to be convenient and sensitive vital markers of transgene expression in mammalian cells, including hematopoietic stem cells (1–9). A number of spectral variants of GFP have been developed in mutagenesis studies that are suitable for simultaneous use in multiparameter flow cytometric applications (1–3,8–16). The ability to detect multiple fluorescent proteins simultaneously by flow cytometry provides the

opportunity to differentiate noninvasively among various cell populations, or to assess gene function and monitor protein–protein interactions in individual cells (8,9,17–19) (see also Chapter 10 by Pruitt et al., Chapter 14 by Galbraith et al., and Chapter 15 by Chan and Holmes, *this volume*). In this regard, we and others have found the combination of enhanced GFP (EGFP) (3), a blue-shifted emission variant of GFP termed enhanced cyan fluorescent protein (ECFP) (10), and a red-shifted emission variant referred to as enhanced yellow fluorescent protein (EYFP) (14) to be useful for this purpose (9,15,16).

GFP orthologs have been isolated from other bioluminescent organisms, such as the Atlantic sea pansy *Renilla reniformis* (20), the Gulf of Mexico sea pansy *Renilla mulleri* (21), and the sea pen *Ptilosarcus gurneyi* (21). However, in no instance has it been possible to produce stable far-red fluorescent GFP derivatives. It was noteworthy, therefore, when a red fluorescent protein, drFP583 (commonly referred to as *Discosoma* coral red fluorescent protein [DsRed]), was identified among a group of GFP-like proteins discovered in nonbioluminescent Anthozoa reef corals (22). DsRed has an emission maximum that is shifted by more than 50 nm toward the red end of the spectrum in comparison with the most red-shifted GFP mutant (22). Approximately 30 GFP-like proteins have now been cloned and spectroscopically characterized (23,24). While additional red fluorescent proteins have been identified or generated by mutagenesis of non-fluorescent purple-blue chromoproteins (23,25–27), new versions of DsRed that exhibit improved folding and spectral properties make them well suited for simultaneous multicolor flow cytometric analyses with the ECFP, EGFP, and EYFP variants (28).

This chapter will describe the derivation of cell lines stably expressing ECFP, EGFP, EYFP, and/or DsRed. These cell lines are used to illustrate one particular flow cytometric detection strategy for simultaneous analysis of all four of these fluorescent proteins. Additional detection strategies for different combinations of these fluorescent proteins are discussed as well.

2. Materials

1. Sp2/0-Ag14 cells (American Type Culture Collection, Manassas, VA, cat. no. CRL-1581).
2. GP+E-86 supernatant containing retroviral vectors carrying the ECFP, EGFP, EYFP, or DsRed fluorescent protein gene plus the bacterial neomycin resistance (*neo*) gene (see **Note 1**).
3. 0.45- μ m Sterile filters.
4. Hexadimethrine bromide (polybrene): Make up a stock solution of 1 mg/mL in distilled water. Filter sterilize. Store at 4°C.
5. Geneticin (G418): Make up a stock solution of 40 mg/mL in distilled water. Filter sterilize. Aliquot and store at –20°C.

6. Growth medium for Sp2/0-Ag14: Dulbecco's modified Eagle medium (DMEM) with high glucose, and 5% (v/v) fetal bovine serum.
7. Growth medium for GP+E-86: DMEM with high glucose, and 10% (v/v) calf serum.
8. Analysis buffer: Phosphate-buffered saline (PBS) and 2% (v/v) fetal bovine serum.
9. Flow cytometer equipped with:
 - a. Excitation wavelengths: 488 nm as the primary beam and 407 nm (*see Note 2*) as the secondary or tertiary beam.
 - b. Detection optics: 488/10-nm bandpass (BP) filters, 470/20-nm BP filter, 510/20-nm BP filter, 550/30-nm BP filter, 610/20-nm BP filter, 525-nm short-pass (SP) dichroic mirror, and 610-nm SP dichroic mirror (BD Biosciences; Omega Optical Inc., Brattleboro, VT; *see Note 3*).
 - c. Intra- and inter-laser compensation capability (*see Note 4*).
10. Calibration-grade alignment beads (*see Note 5*).

3. Methods

The methods described below outline: (1) the derivation of cells stably expressing ECFP, EGFP, EYFP, and/or DsRed; (2) simultaneous detection of all four of these fluorescent proteins; and (3) additional schemes for simultaneous detection of two, three, or four of these fluorescent proteins.

3.1. Derivation of Cells Stably Expressing Individual or Multiple Fluorescent Proteins

Configuring the flow cytometer for simultaneous detection of multiple fluorescent proteins requires cells expressing the individual fluorescent protein genes (*see Note 6*). In addition, cells expressing multiple fluorescent protein genes can be used to confirm correct delineation of different populations.

3.1.1. Cells

Many cell types can be used to express stably the fluorescent protein genes. Sp2/0-Ag14 cells were chosen because of their ease of culture.

3.1.2. Introduction of the Fluorescent Protein Genes Into Cells

Retroviral vectors were used to introduce the fluorescent protein genes into Sp2/0-Ag14 cells. Construction of the retroviral vectors and generation of the producer cell lines have been previously described (**16**). Because of space limitations, they are not described here in detail. In brief, each retroviral vector was engineered by standard recombinant DNA methodology to coexpress the fluorescent protein gene and the bacterial *neo* gene. The latter confers resistance to the neomycin analog G418. The retroviral vectors were transfected into GP+E-86 ecotropic packaging cells (**32**). Supernatant from transfected GP+E-86 cells was

harvested and passed through 0.45- μ m sterile filters. Filtration removed any contaminating cells from the retroviral vector particles.

Sp2/0-Ag14 cells were incubated with supernatant containing ECFP, EGFP, EYFP, or DsRed retroviral vector particles for 4 h at 37°C, in the presence of 2 μ g/mL polybrene. The procedure was repeated with fresh vector supernatant. Two days later, cells expressing individual fluorescent protein genes were selected by the addition of 750 μ g/mL of G418. In addition, cells expressing four fluorescent protein genes were generated by incubation with mixtures of the retroviral vector supernatants using the same transduction protocol (*see Note 7*).

3.2. Simultaneous Detection of Four Fluorescent Proteins

Because of overlapping excitation spectra, multiple fluorescent proteins can be excited by a single excitation wavelength (**15**). As few as two laser lines are sufficient to excite ECFP, EGFP, EYFP, and DsRed, and we have previously described a protocol for simultaneous detection of these four fluorescent proteins on a flow cytometer equipped with tunable lasers (**16**). However, the two lasers were tuned to 458 nm and 568 nm, excitation wavelengths that are not widely available on current commercial flow cytometers. Many flow cytometers are equipped with lasers providing fixed excitation wavelengths, most commonly 488 nm and 633 nm. With the recent introduction of violet laser diodes, new flow cytometers can also provide 405/407/408-nm excitation (exact wavelength depends on the manufacturer of the laser). These small violet laser diodes can also be retrofitted onto existing flow cytometers. The protocol described here for the simultaneous detection of ECFP, EGFP, EYFP, and DsRed utilizes dual-laser excitation at 488 nm and 407 nm. It is applicable to many flow cytometers (*see also* Chapter 13 by Pruitt et al., *this volume*).

3.2.1. Configuring the Flow Cytometer (*see Note 8 and Fig. 1*)

1. Install the appropriate filters in front of detectors:
 - a. 488/10-nm BP filters for forward scatter (FSC) and side scatter (SSC).
 - b. 470/20-nm BP filter for the detection of ECFP.
 - c. 510/20-nm BP filter for the detection of EGFP.
 - d. 550/30-nm BP filter for the detection of EYFP.
 - e. 610/20-nm BP filter for the detection of DsRed.
 - f. 525-nm SP dichroic mirror between the 510/20-nm and 550/30-nm BP filters.
 - g. 610-nm SP dichroic mirror between the 510/20-nm and 610/20-nm BP filters.
2. Using calibration-grade microspheres, align both lasers to obtain maximum fluorescence intensity and minimum coefficient of variation (*see Note 9*).

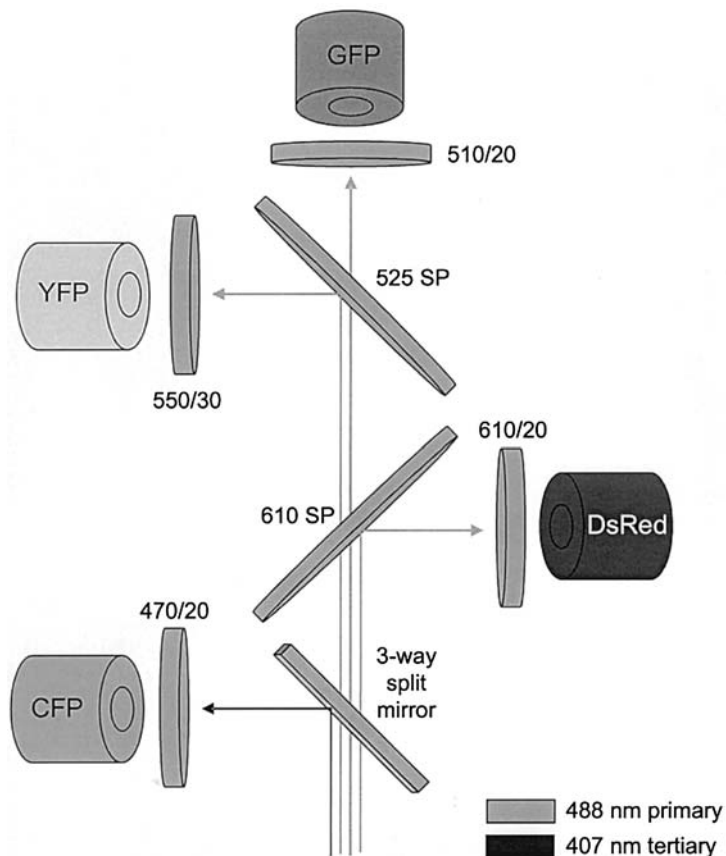


Fig. 1. Optical configuration for simultaneous detection of ECFP, EGFP, EYFP, and DsRed using dual-laser excitation at 488 nm and 407 nm on a FACSVantage SE/FACS-DiVa equipped with Innova 70C-Spectrum argon-ion and Innova 302C krypton-ion lasers. The two beams were spatially separated. The three-way split mirror directed signals from the primary beam (488 nm) to the GFP, YFP, and DsRed detectors, and signals from the tertiary beam (407 nm) to the CFP detector. 488/10 BP filters were placed in front of the FSC and SSC detectors to enable triggering from the primary beam (not shown). 470/20, 510/20, 550/30, 610/20 BP filters were used to capture ECFP, EGFP, EYFP, and DsRed fluorescence, respectively. The 525 SP dichroic mirror was used to separate the GFP and YFP signals. The 610 SP dichroic mirror was used to separate the GFP/YFP and DsRed signals.

3.2.2. Instrument Settings on the FACSVantage SE/FACSDiVa (see **Note 10**)

1. Laser power: 70 mW of 488 nm (primary beam) and 50 mW of 407 nm (tertiary beam).
2. Voltages: CFP, 430 log; GFP, 450 log; YFP, 540 log; DsRed, 580 log.
3. Spectral overlap (%):

<u>Colors/Channel</u>	<u>CFP</u>	<u>GFP</u>	<u>YFP</u>	<u>DsRed</u>
CFP	100.0	2.72	1.81	0.90
GFP	0.52	100.00	43.96	11.52
YFP	0.04	58.75	100.00	33.65
DsRed	0.04	0.86	17.45	100.00

3.2.3. Data Acquisition and Analysis

1. Format a bivariate histogram correlating forward scatter (FSC) and side scatter (SSC) using linear scales. Draw a region to exclude debris (typically low FSC and SSC) and unhealthy cells (typically low FSC and intermediate-to-high SSC).
2. Format bivariate histograms (gated on cells of interest) encompassing all combinations of fluorescence parameters using logarithmic scales.
3. Suspend cells in analysis buffer. While running the “negative” cells (cells not expressing any fluorescent protein), adjust voltage for each fluorescence detector so that the cells are confined to the first decade of the log scale on each histogram. Acquire at least 5000 events.
4. Run the following “positive” cells sequentially: cells expressing ECFP, EGFP, EYFP, or DsRed. Acquire at least 5000 events of each sample.
5. Perform compensation among all parameters (see **Note 11**). A properly compensated sample should have equivalent median values of fluorescence intensities for negative and positive cells with respect to each parameter (**33–35**) (see **Note 12**).
6. Draw nonrectilinear markers to delineate different populations (see **Note 13** and **Fig. 2**).
7. Mix in equal proportions: Negative cells, ECFP-positive cells, EGFP-positive cells, EYFP-positive cells and DsRed-positive cells. Run the sample. Acquire 50,000 events (**Fig. 3A**). Apply compensation (**Fig. 3B**).
8. (Optional) Mix in equal proportions: Negative cells, cells expressing individual fluorescent proteins, and cells expressing all four fluorescent proteins. Acquire 50,000 events (**Fig. 3C**). Apply compensation (**Fig. 3D**).

3.2.4. Data Display

In cytometry, all measurements of signals are subject to at least two fundamental sources of variability: photon counting (counting error) and analog-to-digital conversion (digital error), both of which are intensity dependent and symmetric (see **Note 14**). Subtle but important problems result when data are compensated and presented in a log display. Because signal compensation is a

subtractive process, some of the events could have negative computed channel values after compensation. If the data could be visualized adequately in linear space, the measurement variability would manifest as an intensity-dependent symmetric increase in the breadth of a control population. When these compensated data are transformed back to a log display, negative and zero channel values are normally set to 1, as the log function is undefined at less than or equal to 0. This truncation of data has at least two undesirable consequences. First, the truncated data are plotted on the axis and are not easily visualized in the display. Second, the variability is no longer symmetric and, as a result, the data appear to be undercompensated (**Figs. 3D** and **4A**). The first problem can be circumvented by adding a small random number to the data (**Fig. 4B**, WinList v5.0 “log bias” feature). Although this feature allows the visualization of events that are on the axis, it does not address the more important problem of the loss of symmetry and subsequent misinterpretation of the data. The second problem has been recently solved by two new types of transforms, biexponential and hyper-log, that have all the benefits of the log transform but are defined over the entire real domain, which includes 0 and negative numbers (*see Note 15* and cover illustration). The advantage of these transforms is that the symmetry of the measurement error is preserved, making it far easier to visualize properly compensated data.

3.3. Additional Schemes for Simultaneous Detection of Two, Three, or Four Fluorescent Proteins

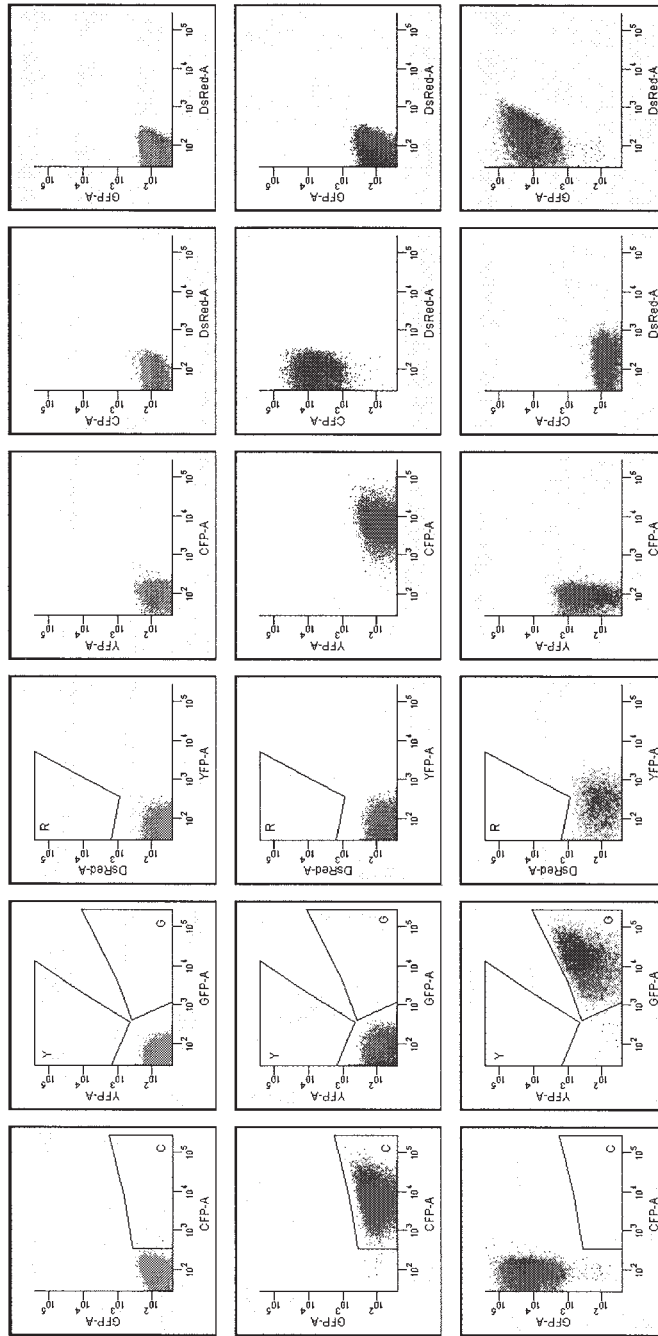
Because of their broad excitation and emission spectra, detection of various combinations of ECFP, EGFP, EYFP, and DsRed can be achieved with different schemes. Some of the possibilities are described below.

3.3.1. Excitation and Emission Spectra of the Four Fluorescent Proteins (**39**)

	<u>Excitation maximum (nm)</u>	<u>Emission maximum (nm)</u>
ECFP	434	477
EGFP	489	508
EYFP	514	527
DsRed	558	583

3.3.2. Feasible Excitation Wavelengths (nm) (*see Note 16*)

1. ECFP: 407, 413, or 458 (*see Note 17*).
2. EGFP: 458 or 488.
3. EYFP: 458, 488, or 514.
4. DsRed: 488, 514, 531, or 568.



A

B

C

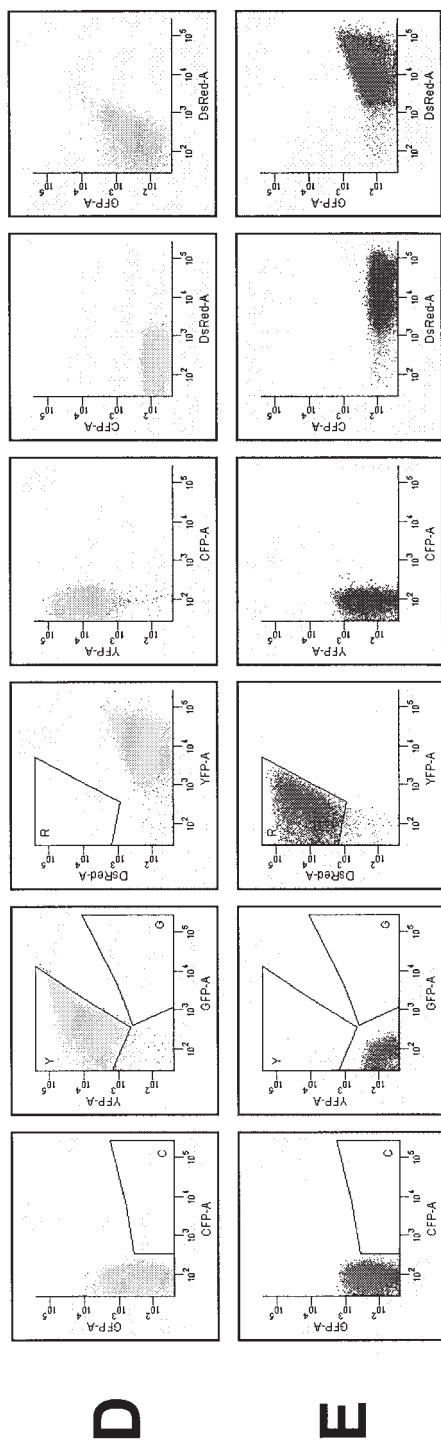
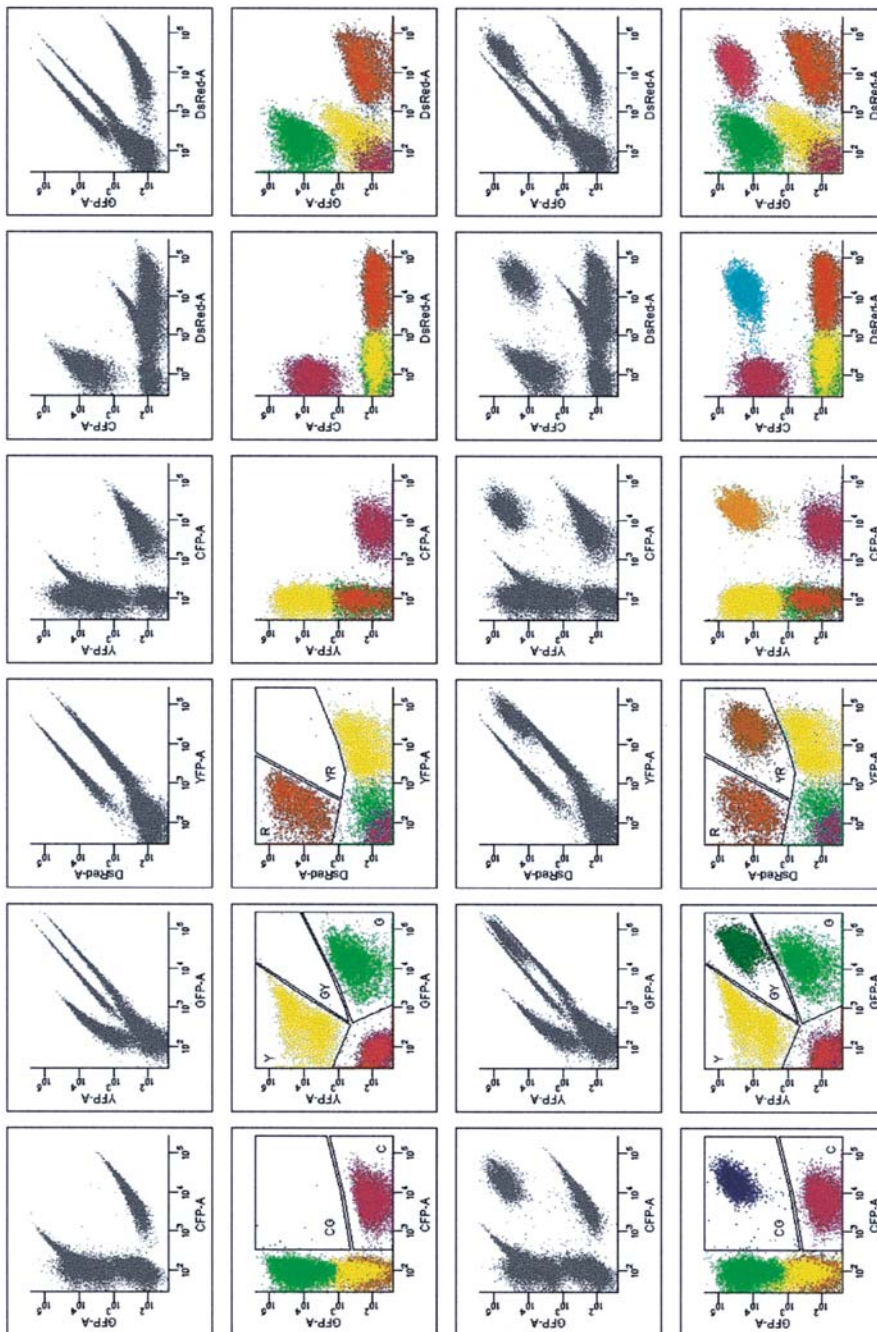


Fig. 2. Bivariate histograms depicting detection of parental Sp2/0-Ag14 cells (A), Sp2/0-Ag14 cells expressing ECFP (B), EGFP (C), EYFP (D), or DsRed (E) using 488-nm and 407-nm excitation as described in Fig. 1. The area of the pulse was used to measure fluorescence intensity. Data was acquired and analyzed using FACSDiVa software. The software calculated spectral overlap among all parameters and applied compensation post-acquisition. Nonrectilinear markers were used to delineate different populations in the first three plots (from left to right). “C,” “Y,” and “G,” and “R” represent ECFP-positive, EGFP-positive, EYFP-positive, and DsRed-positive, respectively.



A

B

C

D

3.3.3. Feasible Detection Optics (nm) (see **Note 18**)

1. ECFP: 470/20- or 485/22-BP filter.
2. EGFP: 510/20- or 530/30-BP filter.
3. EYFP: 530/30-, 546/20-, or 550/30-BP.
4. DsRed: 585/42- or 610/20-BP filter.
5. Dichroic mirrors are used to separate the appropriate wavelengths: 500-longpass (LP), 525-SP, 560-SP, or 610-SP filters.
6. Filters for scatter parameters: BP filters capturing the (primary) excitation wavelength.
7. Laser line restriction band (RB) filters or notch filters, if necessary (see **Note 19**).

3.3.4. Feasible Detection Schemes (see **Note 20**)

<u>Combination</u>	<u>Excitation (nm)</u>	
EGFP/EYFP	488	(Fig. 5A)
EGFP/DsRed	488	(Fig. 5B)
EYFP/DsRed	488	(Fig. 5B)
ECFP/EGFP/EYFP	458	(Fig. 5C)
EGFP/EYFP/DsRed	488	(Fig. 5D)
ECFP/EYFP/DsRed	514 (primary) and 413 (tertiary)	(Fig. 5E)
ECFP/EGFP/EYFP/DsRed	458 (primary) and 568 (tertiary)	(Fig. 5F)

4. Notes

1. The pECFP, pEGFP-1, pEYFP-N1, and pDsRed-1 plasmids encoding ECFP, EGFP, EYFP, and DsRed, respectively, were obtained from BD Biosciences Clontech (Palo Alto, CA). DsRed.T1 and DsRed.T4 (developed by Benjamin Glick, University of Chicago, Chicago, IL) are improved versions of DsRed (**28**). DsRed.T1, known commercially as DsRed-Express, is available from BD Biosciences Clontech. BD Biosciences Clontech also sells other Anthozoa reef coral GFP-like proteins (**26,29**). Other commercially available GFP-like proteins that may serve as useful alternatives to the fluorescent proteins employed in this study include the

Fig. 3. (see opposite page) Bivariate histograms depicting simultaneous detection of ECFP, EGFP, EYFP, and DsRed using 488-nm and 407-nm excitation as described in **Fig. 1**. Data were acquired and analyzed using FACSDiVa software. A mixture of Sp2/0-Ag14 cells and cells expressing the individual fluorescent proteins is shown before (**A**) and after (**B**) spectral overlap compensation. A mixture of Sp2/0-Ag14 cells, cells expressing the individual fluorescent proteins and cells expressing all four fluorescent proteins is shown before (**C**) and after spectral overlap compensation (**D**). In addition to the markers shown in **Fig. 2**, nonrectilinear markers were drawn to delineate double-positive populations in the first three plots (from **left to right**). “CG,” “GY,” and “YR” represent ECFP/EGFP-positive, EGFP/EYFP-positive, and EYFP/DsRed-positive, respectively. Cells expressing all four fluorescent proteins appeared as a double-positive cluster in every histogram.

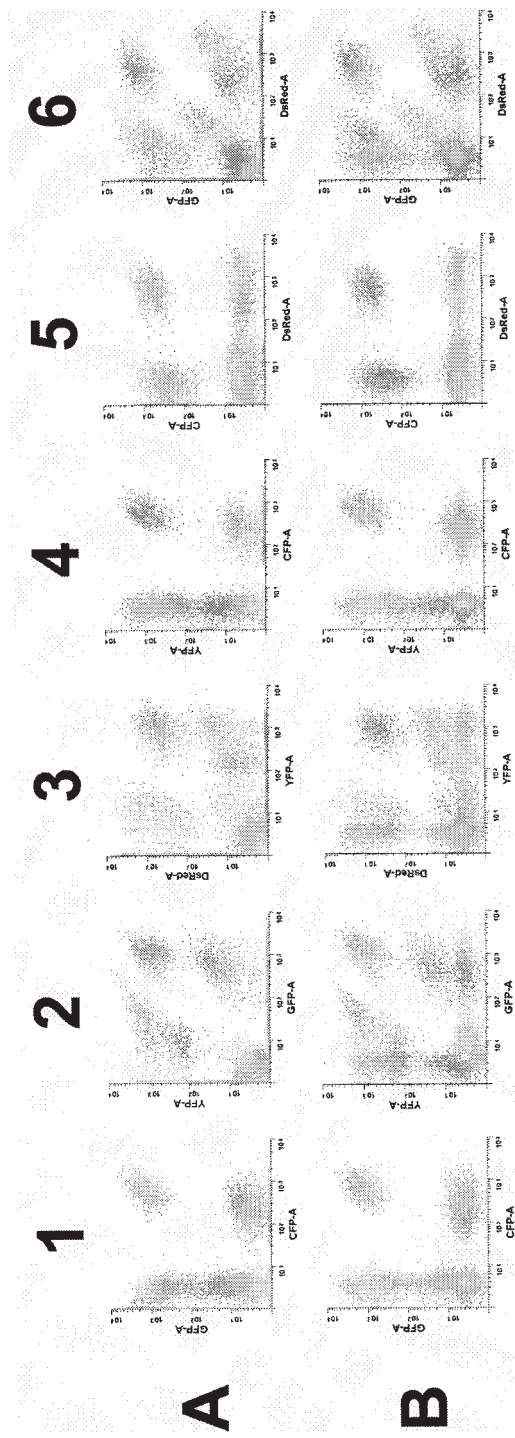
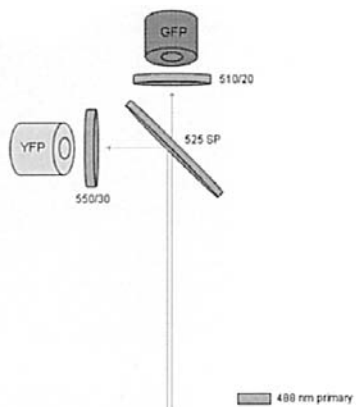


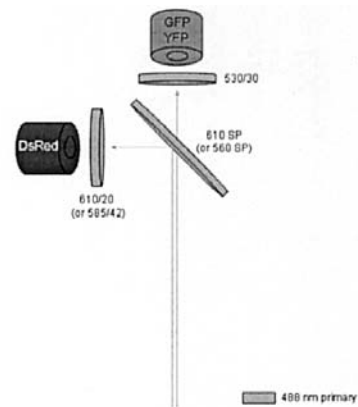
Fig. 4. Visual effect of log bias. These bivariate histograms were generated from high-resolution (262,144 channel) files (the same data files as described in **Fig. 3C**) using WinList's option to display in four decades. They were color compensated using WinList's n-color compensation system, displayed with log bias on (**A**) or log bias off (**B**). With log bias enabled, compensated values that have 0 intensity are assigned a random value between 0 and the log bias setting, 3 by default. This feature allows the visualization of events that are on the axes. This visual effect can be appreciated particularly well in (**B**), histograms 2, 3, and 6.

- Vitality[®] humanized *Renilla reniformis* GFP ($Ex_{\lambda_{max}} = 500$ nm; $Em_{\lambda_{max}} = 506$ nm) distributed by Stratagene (La Jolla, CA) (**20**), and the humanized CopGFP[™] ($Ex_{\lambda_{max}} = 482$ nm; $Em_{\lambda_{max}} = 502$ nm), *Aequorea coerulescens* AceGFP[™] ($Ex_{\lambda_{max}} = 480$ nm; $Em_{\lambda_{max}} = 505$ nm) (**24**), and PhiYFP[™] ($Ex_{\lambda_{max}} = 525$ nm; $Em_{\lambda_{max}} = 537$ nm) fluorescent proteins marketed by Evrogen (Moscow, Russia).
2. The tunable water-cooled krypton-ion laser can provide 407 nm. Violet laser diodes can provide 405, 407, or 408 nm (depending on the manufacturer), and can be retrofitted onto existing flow cytometers (**30**). At present, the following flow cytometers are equipped with (or can be upgraded as an option) air-cooled lasers that provide excitation wavelengths including 488 nm and 405/407 nm: LSR, LSR II, FACSAria (all from BD Biosciences, San Jose, CA), CyAn (Dako-Cytomation, Fort Collins, CO), and CyFlow ML (Partec GmbH, Münster, Germany). The model of flow cytometer and the type of laser are not critical (**31**).
 3. Chroma Technology Corp. (Rockingham, VT) is another supplier of optical filters.
 4. Compensation for spectral overlap among all fluorescence parameters can be performed by utilizing hardware user-manipulated pairwise corrections during acquisition or software computer-aided calculations post-acquisition. For flow cytometers not equipped with inter-laser compensation electronic circuitry, software post-acquisition compensation is the only option. Available commercial software includes WinList (Verity Software House, Topsham, ME), FlowJo (Tree Star Inc., San Carlos, CA) and FCS Express (De Novo Software, Thornhill, Ontario, Canada). Some free software can perform compensation as well. A catalog of free cytometry software is available on the website managed by the Purdue University Cytometry Laboratories at <http://www.cyto.purdue.edu/flowcyt/software/catalog.htm>.
 5. Calibration-grade alignment beads are available from many suppliers including Molecular Probes (Eugene, OR), Polysciences Inc. (Warrington, PA), Spherotech Inc. (Libertyville, IL), Beckman Coulter (Miami, FL), BD Biosciences, and Bangs Laboratories Inc. (Fishers, IN).
 6. The authors welcome requests for Sp2/0-Ag14 cells expressing the various fluorescent protein genes.
 7. (Optional) Pure populations of cells expressing all four fluorescent proteins were isolated by fluorescence-activated cell sorting. Cell sorting was performed on a FACSVantage SE/FACSDiVa (BD Biosciences) equipped with two tunable lasers, an Innova 70C-Spectrum argon-ion laser and an Innova 302C krypton-ion laser (both from Coherent Inc., Santa Clara, CA), providing excitation wavelengths of 488 nm and 407 nm, respectively. A three-way split mirror allowed the 488-nm beam to be directed along the first laser pathway (primary position) and the 407-nm beam along the third laser pathway (tertiary position), maximizing their spatial separation and minimizing laser noise as well as crosstalk between the signals. Data were acquired and analyzed using FACSDiVa software.
 8. Cells were analyzed on the same flow cytometer as described in **Note 7**.
 9. AlignFlow Plus 6- μ m flow cytometry alignment beads (Molecular Probes) were used for 488-nm excitation. Fluoresbrite Calibration Grade 6- μ m YG microspheres (Polysciences Inc.) were used for 407-nm excitation.

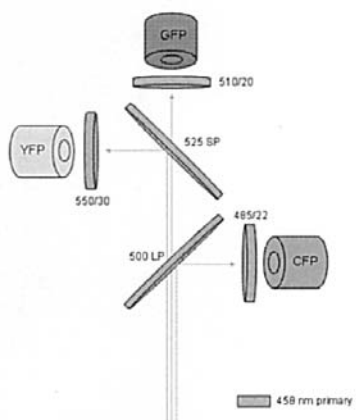
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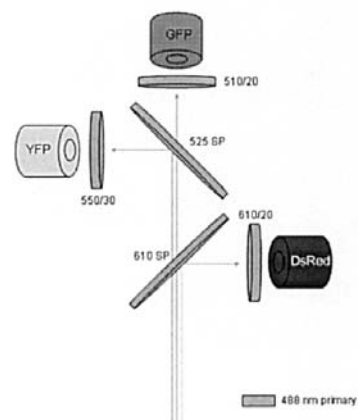
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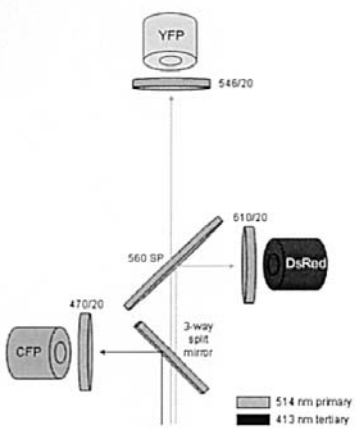
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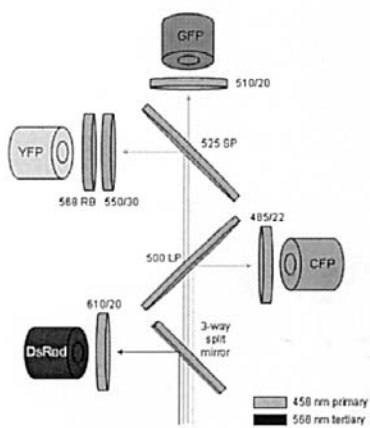
D



E



F



10. The degree of spectral overlap correction depends on the voltages applied to the detectors. Depending on laser power and the orientation of dichroic mirrors (especially the 525 SP between the GFP and YFP detectors), comparable results can be achieved with different settings on different instruments.
11. The FACS Vantage SE/FACSDiVa has digital electronics. Data are stored uncompensated in the linear domain. Compensation is performed by FACSDiVa software with matrix algebra. For flow cytometers equipped with analog circuitry and logarithmic amplifiers, compensation is performed by pulse subtraction. The percentages of compensation are adjusted by the user while viewing the data in a log display during acquisition. In this case, some negative cells should be added to each sample of positive cells so that the median values of fluorescence intensity for both populations can be compared. Alternatively, compensation can be applied using third-party software post-acquisition (*see Note 4*).
12. The photon counting statistics error is one of the errors intrinsic to the measurement process. It contributes to the spread of compensated data on a log scale. High degrees of spillover (as in the case of fluorescent proteins) and increased number of parameters exacerbate the error, especially for high fluorescence intensity populations. Consequently, visual adjustment of spectral overlap is prone to overcompensation. Computer-aided adjustment is highly recommended (**36**).
13. Owing to the spread of compensated data on a log scale, the use of quadrant markers will erroneously classify some single positives as double positives. Nonrectilinear markers should be used instead.
14. It is beyond the scope of this chapter to describe all of the potential errors associated with compensation. A discussion on software compensation can be found in the classic paper by Bagwell and Adams (**37**).
15. The biexponential transform (**38**) is a derivative of the hyperbolic sine and its inverse has many of the properties of the log distribution. The hyper-log transform developed by Verity Software House (Bruce Bagwell, personal communication) is a log-type transformation that smoothly moves from a log domain to a linear

Fig. 5. (*see opposite page*) Alternative optical configurations for simultaneous detection of different combinations of fluorescent proteins. Some of the optical filters shown here represent filters that are commonly used in other applications and are supplied with flow cytometers as standard filters. In some cases, resolution of the fluorescent proteins can be improved by custom-designed filters. **(A)** Detection of EGFP and EYFP using 488-nm excitation. **(B)** Detection of EGFP and DsRed, or EYFP and DsRed using 488-nm excitation. **(C)** Detection of ECFP, EGFP and EYFP using 458-nm excitation as described in **ref. 15**. **(D)** Detection of EGFP, EYFP, and DsRed using 488-nm excitation. **(E)** Detection of ECFP, EYFP, and DsRed using 514-nm and 413-nm excitation. **(F)** Detection of ECFP, EGFP, EYFP, and DsRed using 458-nm and 568-nm excitation as described in **ref. 16**.

domain around the first decade. In addition, the FCOM feature in WinList can be used to define properly all combinations of fluorescence (2^x , where x is the number of fluorescence parameters). This approach is readily scalable to as many parameters as required.

16. The required laser power for each wavelength depends on the detection sensitivity of the flow cytometer. In general, 15–35 mW is adequate for cuvet flow cell detection while 50–70 mW may be necessary for stream-in-air detection. Other factors also contribute to the efficiency of light collection. For example, lowering the stream velocity will increase the dwell time of the particle in the laser beam and enhance signal detection.
17. With the Innova 302C krypton-ion laser, the power output at 407 nm is half of that at 413 nm. However, at 50 mW of laser power, both wavelengths of excitation generate comparable data (data not shown).
18. The filter of choice for each fluorescence parameter is the one that allows the lowest spillover signals from other fluorescent proteins while capturing adequate signal from the primary fluorescent protein. It is not necessarily the filter that captures the maximal primary signal. Some of the optical filters suggested here represent filters that are commonly used in other applications (such as immunophenotyping) and are supplied with flow cytometers as standard filters. In some cases, resolution of the fluorescent proteins can be improved by using custom-designed filters.
19. It is critical to ensure that the detection optics do not capture the excitation wavelengths; under certain circumstances, notch filters that block out the excitation wavelengths should be included. For example, in the absence of a 514-nm notch filter, using 514 nm as the excitation wavelength precludes the detection of EGFP with a 510/20-BP filter. For simultaneous analysis of ECFP/EGFP/DsRed utilizing excitation wavelengths of 488 nm and 407 nm, a 488-nm notch filter is required for the detection of ECFP if a 485/22 BP filter is used (31). When the excitation wavelength is in close proximity to the wavelengths spanned by the detection filter, addition of a laser line restriction band filter to remove stray laser light may be required. As shown in Fig. 5F, the 568-nm RB filter ensures that detection of EYFP with a 550/30-BP filter is free of laser noise (16). When Beavis and Kalejta developed the detection scheme depicted in Fig. 5C, they installed 457-nm RB filters in front of all three fluorescence detectors (15).
20. Fluorescence resonance energy transfer studies utilizing CFP and YFP can be performed with excitation wavelengths of 413 nm and 514 nm (19) (see also Chapter 15 by Chan and Holmes, *this volume*). In addition, the detection schemes described here can be combined (depending on the availability of detectors and excitation wavelength) with red-shifted fluorochromes in other studies. Commonly used red-shifted fluorochromes excited by 488 nm include PerCP-Cy5.5 and tandem conjugates of phycoerythrin (PE); those excited by 633 nm include allophycocyanin (APC) and tandem conjugates of APC. Alexa Fluor dyes and their tandem conjugates (Molecular Probes) of the appropriate excitation and emission wavelengths can be used as well.

Acknowledgments

This work was supported in part by National Institutes of Health grants R01 HL65519 and R01 HL66305 (to R. G. H.). We thank Ali Ramezani for constructing the ECFP, EGFP, EYFP, and DsRed retroviral vectors, Bill Telford for fruitful collaborations, Dave Houck and Steven Merlin for their suggestions, and John Swanson for his support. Most of all, we are grateful to Bruce Bagwell and other members of the Verity Software House team (Ben Hunsberger, Christopher Bray, and Mark Munson), and to Joe Trotter at BD Biosciences for analyzing the data with the hyper-log transform and biexponential transform, respectively, prior to release.

References

1. Tien, R. Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544.
2. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33–38.
3. 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.
4. Cheng, L., Fu, J., Tsukamoto, A., and Hawley, R. G. (1996) Use of green fluorescent protein (GFP) variants to monitor gene transfer and expression in mammalian cells. *Nat. Biotech.* **14**, 606–609.
5. Cheng, L., Du, C., Murray, D., et al. (1997) A GFP reporter system to assess gene transfer and expression in viable human hematopoietic progenitors. *Gene Ther.* **4**, 1013–1022.
6. Persons, D. A., Allay, J. A., Riberdy, J. M., et al. (1998) Use of the green fluorescent protein as a marker to identify and track genetically modified hematopoietic cells. *Nat. Med.* **4**, 1201–1205.
7. Dorrell, C., Gan, O. I., Pereira, D. S., Hawley, R. G., and Dick, J. E. (2000) Expansion of human cord blood CD34+CD38- cells in *ex vivo* culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood* **95**, 102–110.
8. Horn, P. A., Topp, M. S., Morris, J. C., Riddell, S. R., and Kiem, H. P. (2002) Highly efficient gene transfer into baboon marrow repopulating cells using GALV-pseudotype oncoretroviral vectors produced by human packaging cells. *Blood* **100**, 3960–3967.
9. Hawley, T. S., Telford, W. G., and Hawley, R. G. (2001) “Rainbow” reporters for multispectral marking and lineage analysis of hematopoietic stem cells. *Stem Cells* **19**, 118–124.
10. 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.

11. Heikal, A. A., Hess, S. T., Baird, G. S., Tsien, R. Y., and Webb, W. W. (2000) Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: coral red (dsRed) and yellow (Citrine). *Proc. Natl. Acad. Sci. USA* **97**, 11,996–12,001.
12. Nagai, T., Ibata, K., Park, E. S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**, 87–90.
13. Anderson, M. T., Tjioe, I. M., Lorincz, M. C., Parks, D. R., Herzenberg, L. A., and Nolan, G. P. (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. USA* **93**, 8508–8511.
14. Lybarger, L., Dempsey, D., Patterson, G. H., Piston, D. W., Kain, S. R., and Chervenak, R. (1998) Dual-color flow cytometric detection of fluorescent proteins using single-laser (488-nm) excitation. *Cytometry* **31**, 147–152.
15. Beavis, A. J. and Kalejta, R. F. (1999) Simultaneous analysis of the cyan, yellow and green fluorescent proteins by flow cytometry using single-laser excitation at 458 nm. *Cytometry* **37**, 68–73.
16. Hawley, T. S., Telford, W. G., Ramezani, A., and Hawley, R. G. (2001) Four-color flow cytometric detection of retrovirally expressed red, yellow, green and cyan fluorescent proteins. *BioTechniques* **30**, 1028–1034.
17. De Sepulveda, P., Okkenhaug, K., Rose, J. L., Hawley, R. G., Dubreuil, P., and Rotapel, R. (1999) Socs 1 binds to multiple signalling proteins and suppresses Steel factor-dependent proliferation. *EMBO J.* **18**, 904–915.
18. Leung, B. L., Haughn, L., Veillette, A., Hawley, R. G., Rottapel, R., and Julius, M. (1999) TcR $\alpha\beta$ independent CD28 signaling and costimulation require non-CD4-associated Lck. *J. Immunol.* **163**, 1334–1341.
19. Chan, F. K., Siegel, R. M., Zacharias, D., et al. (2001) Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein. *Cytometry* **44**, 361–368.
20. Felts, K., Rogers, B., Chen, K., Ji, H., Sorge, J., and Vaillancourt, P. (2000) Recombinant *Renilla reniformis* GFP displays low toxicity. *STRATEGIES Newsletter* **13**, 85–87. http://www.stratagene.com/vol13_3/p85-87.htm.
21. Peelle, B., Gururaja, T. L., Payan, D. G., and Anderson, D. C. (2001) Characterization and use of green fluorescent proteins from *Renilla mulleri* and *Ptilosarcus guernyi* for the human cell display of functional peptides. *J. Protein Chem.* **20**, 507–519.
22. Matz, M. V., Fradkov, A. F., Labas, Y. A., et al. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* **17**, 969–973.
23. Labas, Y. A., Gurskaya, N. G., Yanushevich, Y. G., et al. (2002) Diversity and evolution of the green fluorescent protein family. *Proc. Natl. Acad. Sci. USA* **99**, 4256–4261.
24. Gurskaya, N. G., Fradkov, A. F., Pounkova, N. I., et al. (2003) A colourless green fluorescent protein homologue from the non-fluorescent hydromedusa *Aequorea coerulea* and its fluorescent mutants. *Biochem. J.* **373**, 403–408.

25. Lukyanov, K. A., Fradkov, A. F., Gurskaya, N. G., et al. (2000) Natural animal coloration can be determined by a nonfluorescent green fluorescent protein homolog. *J. Biol. Chem.* **275**, 25,879–25,882.
26. Gurskaya, N. G., Fradkov, A. F., Terskikh, A., et al. (2001) GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Lett.* **507**, 16–20.
27. Wiedenmann, J., Schenk, A., Rocker, C., Girod, A., Spindler, K. D., and Nienhaus, G. U. (2002) A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from *Entacmaea quadricolor* (Anthozoa, Actinaria). *Proc. Natl. Acad. Sci. USA* **99**, 11,646–11,651.
28. Bevis, B. J. and Glick, B. S. (2002) Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). *Nat. Biotechnol.* **20**, 83–87.
29. Richards, B., Zharkikh, L., Hsu, F., Dunn, C., Kamb, A., and Teng, D. H. (2002) Stable expression of Anthozoa fluorescent proteins in mammalian cells. *Cytometry* **48**, 106–112.
30. Telford, W. G. (2003) How to mount a violet laser diode on the FACSVantage/FACSDiVa. <http://home.ncifcrf.gov/ccr/flowcore/index.htm>.
31. Telford, W. G., Hawley, T. S., and Hawley, R. G. (2003) Analysis of violet-excited fluorochromes by flow cytometry using a violet laser diode. *Cytometry* **54A**, 48–55.
32. Markowitz, D., Goff, S., and Bank, A. (1988) A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* **62**, 1120–1124.
33. Roederer, M. (May 24, 2000) Compensation (an informal perspective). <http://www.drmr.com/compensation/index.html>.
34. Roederer, M. (2001) Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry* **45**, 194–205.
35. Baumgarth, N. and Roederer, M. (2000) A practical approach to multicolor flow cytometry for immunophenotyping. *J. Immunol. Methods* **243**, 77–97.
36. Stewart, C. C. and Stewart, S. J. (2003) A software method for color compensation, in *Current Protocols in Cytometry* (Robinson, J. P., Darzynkiewicz, Z., Dean, P., et al., eds.), John Wiley & Sons, New York, pp. 10.15.1–10.15.12.
37. Bagwell, C. B. and Adams, E. G. (1993) Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *Ann. NY Acad. Sci.* **677**, 167–184.
38. Shapiro, H. M. (2003) Afterword, in *Practical Flow Cytometry*, 4th edit., Wiley-Liss, New York, pp. 561–566.
39. BD Fluorescence Spectrum Viewer. <http://www.bdbiosciences.com/spectra>.