
Technical Notes

Simultaneous Analysis of the Cyan, Yellow and Green Fluorescent Proteins by Flow Cytometry Using Single-Laser Excitation at 458 nm

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Background: Development of spectrally distinct green fluorescent protein (GFP) variants has allowed for simultaneous flow cytometric detection of two different colored mutants expressed in a single cell. However, the dual-laser methods employed in such experiments are not widely applicable since they require a specific, expensive laser, and single-laser analysis at 488 nm exhibits considerable spectral overlap. The purpose of this work was to evaluate detection of enhanced cyan fluorescent protein (ECFP) in combination with the enhanced green (EGFP) and enhanced yellow (EYFP) fluorescent proteins by flow cytometry.

Methods: Cells transfected with expression constructs for EGFP, EYFP, or ECFP were analyzed by flow cytometry using excitation wavelengths at 458, 488, or 514 nm. Fluorescence signals were separated with a custom optical filter configuration: 525 nm shortpass and 500 nm long-

pass dichroics; 480/30 (ECFP), 510/20 (EGFP) and 550/30 (EYFP) bandpasses; 458 nm laser blocking filters.

Results: All three fluorescent proteins when expressed individually or in combination in living cells were excited by the 458 nm laser line and their corresponding signals could be electronically compensated in real time.

Conclusions: This method demonstrates the detection of three fluorescent proteins expressed simultaneously in living cells using single laser excitation and is applicable for use on flow cytometers equipped with a tunable argon ion laser. Cytometry 37:68–73, 1999. © 1999 Wiley-Liss, Inc.

Key terms: green yellow cyan fluorescent proteins; EGFP; EYFP; ECFP; single laser excitation; flow cytometry; 458 nm excitation

The green fluorescent protein (GFP) (reviewed in 1) has become a powerful tool in both mammalian and microbiological systems for a multitude of applications, including assessment of gene expression, protein trafficking, or localization and general cell labeling (2–7). The wild type GFP (wtGFP) has a complex excitation spectrum with major and minor absorption peaks at 395 and 475 nm, respectively, but exhibits a single emission peak at 510 nm (8). The fluorescence of wtGFP can be observed by spectroscopy, fluorimetry, and fluorescence microscopy. However, its utility in flow cytometry is limited because most flow cytometers are not equipped with an excitation wavelength at 395 nm, and the intensity of the fluorescence emission for wtGFP is low following excitation at 488 or 351–364 nm (9–11). However, site directed mutations of the GFP fluorophore have yielded additional GFP molecules with enhanced spectral properties (12–14). Some of these GFP mutants exhibit altered excitation spectra with unimodal absorption in either the blue or

violet region of the spectrum and a corresponding emission at 510 nm (10,15,16). One of the most useful mutants is the “red-shifted” GFP variant resulting from a serine to threonine substitution at amino acid 65 which displays increases in extinction coefficient and quantum yield compared to wtGFP when excited at 488 nm (17,18). This enhanced-GFP (EGFP) is now widely used in flow cytometry for many different applications including cell sorting, gene therapy, and the identification of transfected cells during cell cycle analysis (19–23). Recently, additional EGFP variants have been constructed that, as well as retaining high quantum yields, have excitation and emission maxima shifted to different regions of the visible

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spectrum. Two such mutants are the enhanced cyan fluorescent protein (ECFP) and the enhanced yellow fluorescent protein (EYFP). ECFP and EYFP have absorption peaks at 453 and 513 nm, respectively, which closely match the commonly available excitation wavelengths of the argon ion laser utilized on most standard flow cytometers.

Imaging any one of these three fluorescent proteins (EGFP, EYFP, ECFP) by microscopy is straightforward provided that the correct filter sets are employed. However, dual and triple labeling experiments, especially with EGFP and EYFP, are difficult due to the spectral overlap of these signals (24). Flow cytometers allow for electronic compensation of the spectral overlap arising from different fluorochromes and this should provide for the ability to detect and resolve these fluorescent proteins when expressed in cells either alone or in combination. Here, a method is presented for the simultaneous detection of ECFP, EGFP and EYFP in living cells using single-laser excitation at 458 nm. This protocol is applicable for use on flow cytometers equipped with a tunable multiline argon ion laser and should increase the utility of these fluorescent proteins for all types of biological applications.

MATERIALS AND METHODS

Plasmids, Cells and Transfections

The pEGFPN-1, pEYFPN-1 and pECFPN-1 are from ClonTech (Palo Alto, CA). U-2 OS cells and primary human foreskin fibroblasts (PHFF) were maintained in DMEM (Sigma, St. Louis, MO) containing 10% FBS (Hyclone Laboratories Inc., Logan, UT). U-2 OS or PHFF cells were transfected with 5 μ g of the indicated plasmid(s) by electroporation as described previously (19). For flow cytometric analysis, cells were collected by trypsinization, resuspended in PBS and analyzed immediately.

Flow Cytometry

Cells were analyzed for fluorescence using a FACS Vantage (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with Coherent Innova 300, Spectrum and dye lasers (Coherent Inc, Santa Clara, CA). The OmniCompensation option permitting interlaser compensation was used for dual laser analysis. For single laser analysis, the primary laser (Innova 300) was tuned to 458 or 488 nm. For dual laser analysis, the second laser (Spectrum) was tuned to 488 or 514 nm. Laser output powers were set at 50 mW and verified using a power meter (Coherent, model 210).

The ECFP fluorescence signal was separated from the EGFP and EYFP fluorescence with a 500 nm longpass dichroic (LPDi) and collected with a 480/30 nm bandpass (BP) filter. The EGFP and EYFP signals were separated with a 525 nm shortpass dichroic (SPDi) and collected with a 510/20 nm BP and 550/30 nm BP, respectively, as described previously (25). A 458 nm longpass laser blocking (LPLB) filter was placed in front of each of the bandpass filters. All optical filters were purchased from Omega Optical (Burlington, VT).

Table 1
*Excitation and Emission Maxima for ECFP, EGFP and EYFP**

Protein	Color	Excitation maximum (nm)	Emission maximum (nm)
ECFP	Cyan	433, 453	475
EGFP	Green	490	509
EYFP	Yellow-green	513	527

*Data from ref. (26).

RESULTS AND DISCUSSION

Initially, U-2 OS cells transfected separately with each expression construct for the three fluorescent proteins were analyzed by flow cytometry to determine the optimal excitation wavelength and optical configuration for the system. The excitation maximum for each of the fluorescent proteins has been previously determined (26) and is summarized in Table 1. Using single laser analysis, ECFP was excited well at 458 nm producing bright fluorescence collected at 480 nm (Fig. 1A). The EGFP fluorescence, collected at 510 nm, was detected following excitation at 458 or 488 nm. In each case, bright fluorescence was observed with resolution of the different levels of EGFP expression (Fig. 1B,C). The percentage of EGFP(+) cells detected at either excitation wavelength was comparable, but the mean fluorescence intensity (MFI) was higher when excited at 488 nm (Table 2). As expected, the EYFP fluorescence was optimal following excitation at 514 nm with some loss of fluorescence observed following excitation at 488 nm (Fig. 1E, F). Interestingly, when cells expressing EYFP were analyzed with excitation at 458 nm, a bright emission signal detected at 550 nm was still observed (Fig. 1D). The percentage of EYFP-expressing cells detected at each of these three excitation wavelengths was comparable, but the MFI of these positive cells decreased 2.5- and 9-fold when excited at 488 and 458 nm, respectively, when compared to excitation at 514 nm (Table 2). Excitation of EYFP was achieved even though the protein shows less than 35% of maximum absorbance at 488 nm and 12% at 458 nm (25). The ability to detect the EYFP fluorescence following excitation at 458 nm may also be due to the lower levels of autofluorescence at wavelengths below 488 nm (27-29). Indeed, this was observed as being a major factor in the ability to achieve increased signal resolution from blue-emitting fluorochromes when using the 407 nm line from the violet-enhanced krypton laser (9,15).

U-2 OS cells that expressed two fluorescent proteins were also analyzed. Either cells expressing a single type of fluorescent protein were co-cultured after transfection (mixed) or cells were cotransfected with equal amounts of each of the indicated expression plasmids (cotransfected). For the mixed samples separately expressing pairs of fluorescent proteins ECFP/EGFP, EGFP/EYFP, or ECFP/EYFP, all fluorescence signals were well resolved when excited at 458 nm, but the latter pair exhibited less spectral overlap as seen from analysis of the uncompensated data (Fig. 2A1-A3). This suggests that for dual color analysis, ECFP and EYFP may provide for better resolution

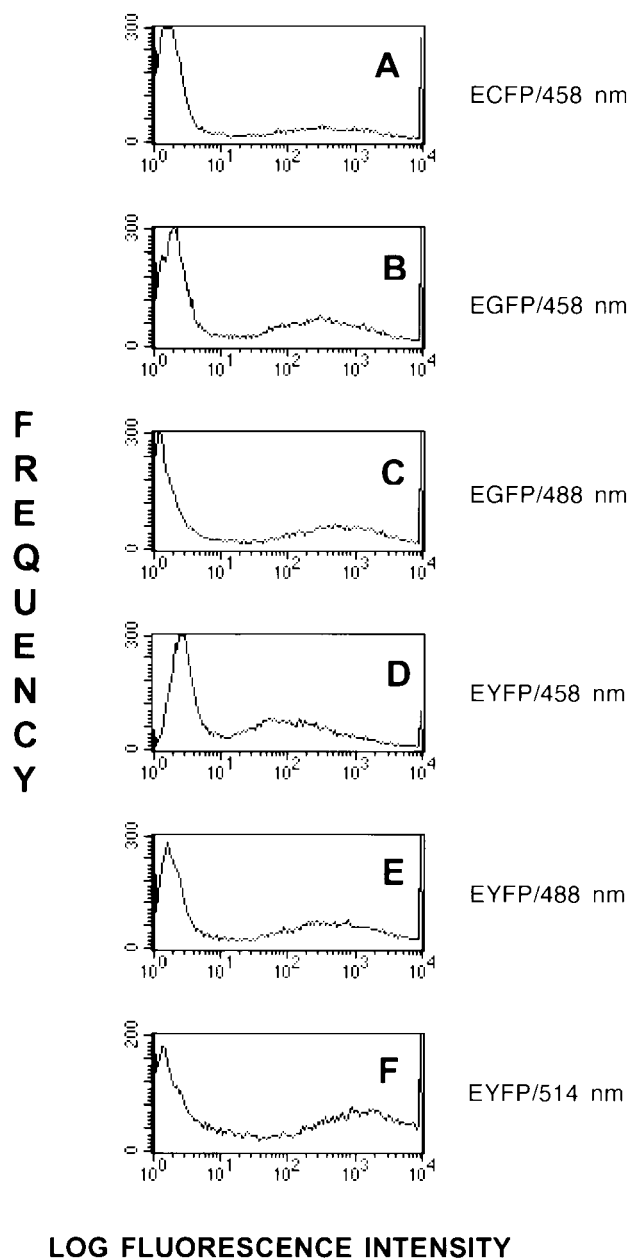


FIG. 1. Flow cytometric analysis of U-2 OS cells expressing EGFP, EYFP, or ECFP. Cells were transfected with pECFPN-1 (A), pEGFPN-1 (B,C) or pEYFPN-1 (D-F), harvested 48 h later and analyzed for fluorescence on a FACS Vantage using an excitation wavelength at 458 nm (A,B,D), 488 nm (C,E) or 514 nm (F) at 50 mW. The ECFP fluorescence emission was separated with a 500 LPDi and collected as the FL3 parameter with a 480/30 BP filter. The EGFP (FL1) and EYFP (FL2) signals were separated with a 525 SPD_i and collected with a 510/20 BP and a 550/30 BP, respectively. For each analysis, the respective laser blocking filter was placed in front of the BP filter. The data are representative of three experiments.

than ECFP and EGFP or EGFP and EYFP. However, the signals from all three pairs of fluorescent proteins could be compensated and the single color subpopulations resolved (Fig.2B1-B3). Analysis of cotransfected samples expressing two fluorescent proteins indicated that the double-

Table 2
Quantitation of the Expression of EGFP, EYFP or ECFP in U-2 OS Cells by Flow Cytometric Analysis Using Three Separate Excitation Wavelengths*

Fluorescent protein	Parameter	Excitation wavelength (nm)	Percent positives	MFI ^a (positives)
ECFP	FL3	458	29.4	207.4
EGFP	FL1	458	47.9	195.0
		488	48.1	317.3
EYFP	FL2	458	51.9	92.9
		488	52.3	322.0
		514	52.9	878.6

*Data are representative of three experiments. Cells were analyzed for fluorescence at each excitation wavelength with detector settings constant. The percentage of cells positive for fluorescence was determined by comparison to cells transfected with control vector pSP72.

^aMFI, mean fluorescence intensity (arbitrary units).

positive and single-positive subpopulations could also be resolved for each pair of fluorescent proteins expressed in combination (Fig.2C1-C3). Identical results were observed with the PHFF cells (not shown).

Additionally, the ability to analyze expression of three fluorescent proteins simultaneously was then determined using single or dual laser excitation. Once again, both mixed and cotransfected samples of either U-2 OS or PHFF cells were utilized. Single laser excitation of the three fluorescent proteins was performed using a 458 nm laser line. Typical instrument settings used for this analysis of the PHFF cells are given in Table 3. Note that it is important to be able to independently compensate the spectral overlap between FL1 (EGFP) and FL3 (ECFP). All three fluorescence signals could be resolved and compensated in real time, which allows for optimal identification of the possible multicolor combinations for the PHFF cells (Fig. 3). A dual laser analysis with configurations for the first and second lasers tuned to either 458 and 488 nm or 458 and 514 nm was also employed. The initial configuration was used to collect ECFP excited at 458 nm and EGFP and EYFP excited at 488 nm, whereas the latter configuration was used to excite ECFP and EGFP at 458 nm and EYFP at 514 nm. Due to the excitation of one or more fluorescent proteins in both lasers, crossbeam compensation was required to eliminate this spectral overlap (not shown). However, it was not possible to compensate the fluorescence emission of the EYFP (a second laser parameter) that resulted from actual excitation of EYFP by the first laser, producing spectral overlap with the EGFP signal (a first laser parameter). Although it may be performed in software after acquisition, this would preclude the ability to accurately sort any subpopulations of cells expressing the EYFP. This suggests that the ability to simultaneously quantitate the expression of EGFP, EYFP, and ECFP using single laser excitation is preferable. Identical results were obtained with U-2 OS cells (not shown).

The development of fluorescent proteins with diversity in their spectral properties has provided for the ability to independently analyze expression of two reporter con-

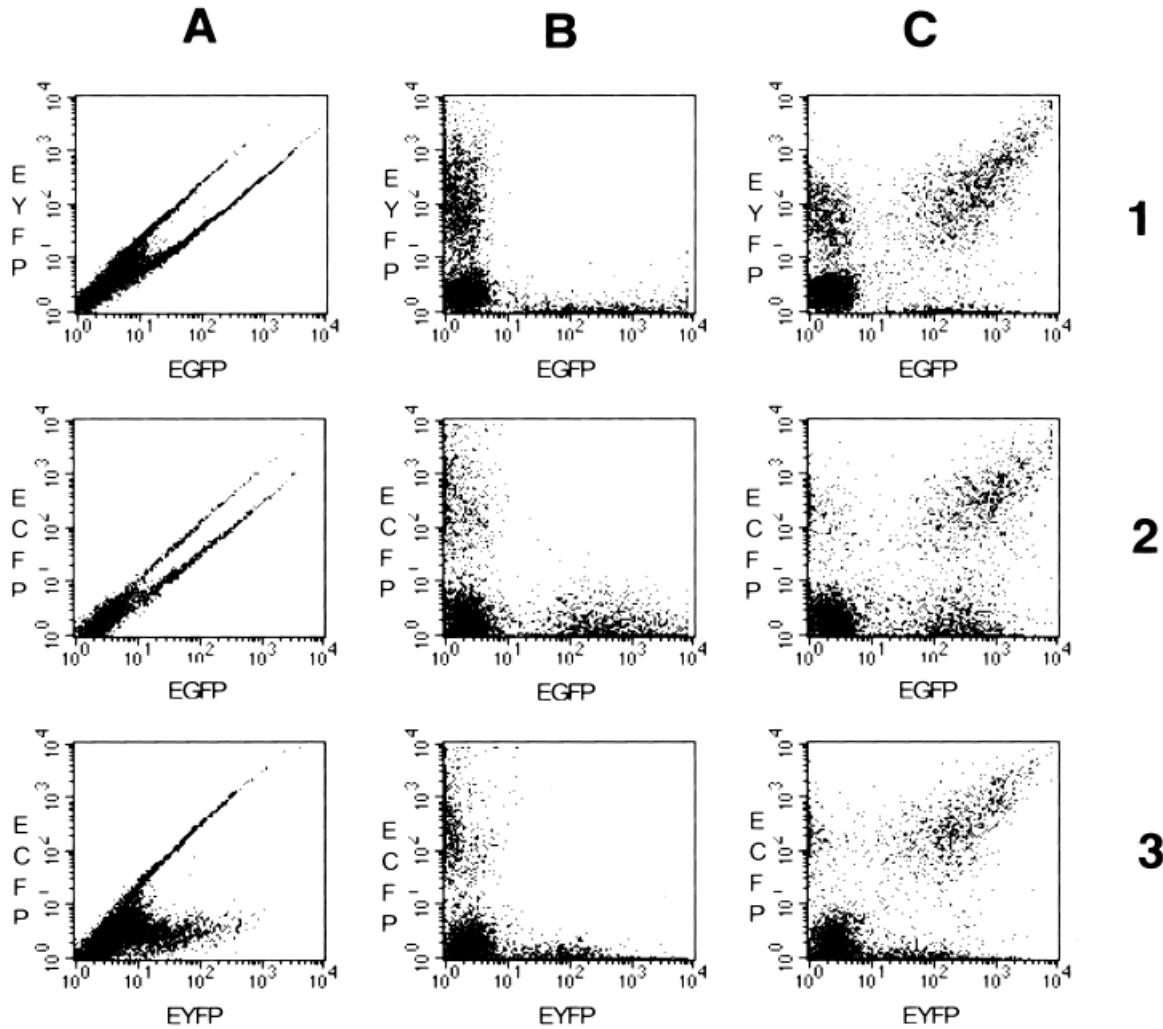


FIG. 2. Flow cytometric analysis of two fluorescent proteins expressed in U-2 OS cells using excitation at 458 nm. Co-cultured (mixed) cells expressing a single fluorescent protein or cells cotransfected with equal amounts of each of two of the expression plasmids for EGFP, EYFP, and ECFP were analyzed for fluorescence as described. For the mixed samples, two-color dotplots illustrating the spectral overlap of EGFP, EYFP and ECFP fluorescence are shown (uncompensated data: A1–A3). When compensation is applied (B1–B3), the fluorescence emissions of these fluorescent proteins can be completely resolved. Two-color analysis (compensated) of cells cotransfected with expression plasmids for pairs of the fluorescent proteins (C1–C3) indicates that cells expressing two of the fluorescent proteins simultaneously can be resolved from the single-positive cells for each pair of fluorescent proteins expressed in combination. Data are representative of three experiments. Due to the bright fluorescence of cells which appear in the upper channel or just off scale, compensation required to eliminate spectral overlap resulted in an apparent over-compensation of cells expressing intermediate levels of the fluorescent proteins. However, this does not limit resolution of the EGFP, EYFP, or ECFP fluorescence signals.

structs within a single cell. This has been demonstrated using a dual-laser, flow cytometric technique (10,15,30) but requires that each fluorescent protein be excited by a separate wavelength. These techniques have required the use of either a 407 nm line from the violet-enhanced krypton laser or ultraviolet (351–364 nm) excitation. More recently, dual-color flow cytometric analysis of cells expressing EGFP and EYFP was demonstrated using a single 488 nm laser line and a custom set of optics for optimal resolution of the fluorescence signals (25). Here, we describe a method to assess the expression of EGFP, EYFP, and ECFP in live cells using one excitation wavelength at 458 nm. Although the excitation spectrum of EYFP suggests that it has minimal absorbance at 458 nm, practical

Table 3
*Instrument Settings for Simultaneous Analysis of Three Fluorescent Proteins Expressed by PHFF Cells Using Single-Laser (458 nm) Flow Cytometry**

Parameter	Fluorescent Protein	PMT Voltage	Compensation		
			(-%FL1)	(-%FL2)	(-%FL3)
FL1	EGFP	400	—	55.6	58.4
FL2	EYFP	400	51.6	—	28.2
FL3	ECFP	575	45.6	3.4	—

*Parameter settings were adjusted for cells expressing each of the fluorescent proteins to position fluorescence signals on scale. Compensation was set using CellQuest software and OmniCompensation. This allows for the spectral overlap of the FL1 and FL3 signals to be adjusted independently of the FL2 parameter.

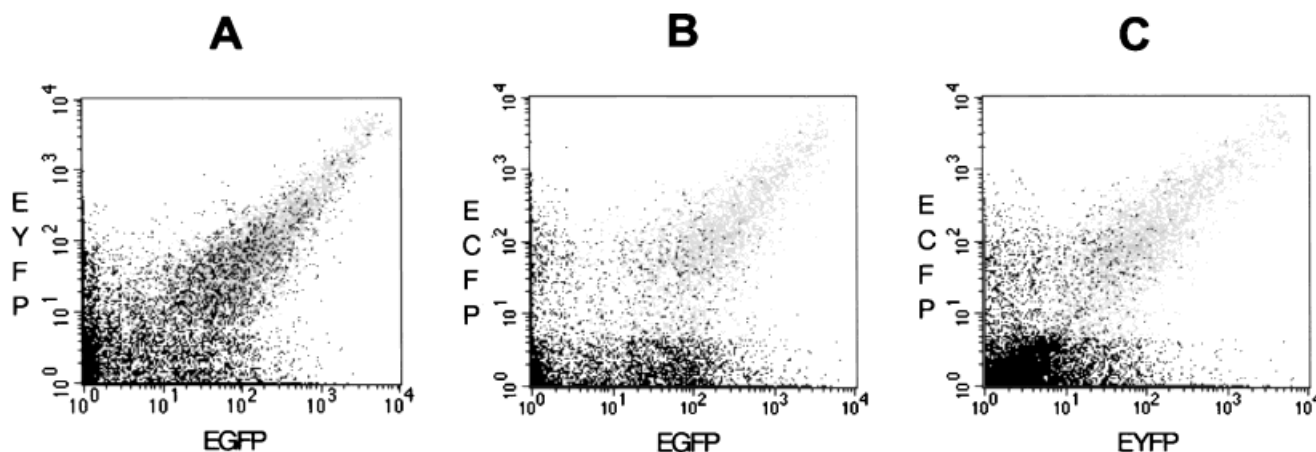


FIG. 3. Simultaneous analysis of three fluorescent proteins expressed in PHFF cells using single-laser excitation at 458 nm. PHFF cells cotransfected with pEGFPN-1, pEYFPN-1, and pECFPN-1 were cultured for 48 h and subsequently analyzed for fluorescence on a FACS Vantage using an argon ion laser tuned to 458 nm at 50 mW. Compensation for spectral overlap was set using cells expressing each fluorescent protein individually with CellQuest software and OmniCompensation. The dual-parameter dotplots for EGFP versus EYFP (A), EGFP versus ECFP (B), and EYFP versus ECFP (C) are shown. Cells expressing all three fluorescent proteins [EGFP(+)/ECFP(+)/EYFP(+)] simultaneously are shown in gray. The possible subpopulations were enumerated by Boolean gate logic: EGFP(+)/ECFP(-)/EYFP(-) = 4.8%; EGFP(-)/ECFP(+)/EYFP(-) = 1.4%; EGFP(-)/ECFP(-)/EYFP(+) = 5.2%; EGFP(+)/ECFP(+)/EYFP(-) = 1.6%; EGFP(+)/ECFP(-)/EYFP(+) = 8.0%; EGFP(-)/ECFP(+)/EYFP(+) = 1.6%; EGFP(+)/ECFP(+)/EYFP(+) = 13.2%.

examination indicates that EYFP can be excited well in the violet-blue region of the spectrum producing bright emission at 550 nm (Fig. 1D). However, the intensity of fluorescence is reduced compared to excitation at 514 nm (Fig. 1F), so this may place limitations on the use of the EYFP when excited at 458 nm. It may be preferable to use EYFP in cases when expression levels are expected to be high analogous to the manner in which bright and dim fluorochromes are matched for the detection of low or high density antigens, respectively (31).

This method provides clear advantages for the analysis of EGFP and its variants since the 458 nm laser line is available on flow cytometers equipped with a tunable argon ion laser. Furthermore, since the EGFP, EYFP and ECFP fluorescence signals are collected over a narrow range of wavelengths from 480 to 550 nm, a wide band of the spectrum is still available for multicolor analysis (32) in combination with other fluorochromes that could be excited in a second and/or third laser configuration. The ability to simultaneously quantitate expression of fluorescent proteins other than EGFP may be particularly useful in analyses where EGFP is already employed to label or identify specific cell types. Thus, EYFP and ECFP could then be used to study gene expression or protein localization in such EGFP-positive cells using single laser excitation.

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