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Review Article

Flow cytometry of fluorescent proteins

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ABSTRACT

Fluorescent proteins are now a critical tool in all areas of biomedical research. In this article, we review the techniques required to use fluorescent proteins for flow cytometry, concentrating specifically on the excitation and emission requirements for each protein, and the specific equipment required for optimal use.

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1. Introduction

Fluorescent proteins (FPs) have revolutionized modern biomedical research [1,2]. The ability to transiently or stably incorporate the gene for an expressible fluorescent protein has become a critical technique for the study of gene expression, cellular and tissue development and a host of other biomedical phenomena. From the original purification, cloning and expression of wild type green fluorescent protein (GFP) in the 1990s, biomedical scientists now have access to a veritable palette of expressible markers ranging from the blue to the long red, with excitation and emission properties that can be selected or tailored to specific fluorescence detection technologies [1–3].

The original wild type green fluorescent protein derived from the coelenterate *Aequeora* possessed an excitation maxima in the long ultraviolet range, with emission in the green (approximately 510 nm) [4,5]. These excitation/emission properties were well-suited to imaging applications using microscopes equipped with mercury arc lamps. However, wild type GFP was less useful for the by then well-developed technology of flow cytometry. Commercial flow cytometry systems were usually equipped with argon-ion lasers emitting at the blue-green 488 nm wavelength, allowing the efficient excitation of fluorescein and other traditional fluorochromes. Wild type GFP could not be excited on these systems. Although some large-scale cytometers could be equipped with ultraviolet sources, these were expensive and were not common. The development of enhanced green fluorescent protein (EGFP) by the Tsien laboratory via site-directed mutagenesis produced a modified GFP that was optimally excited at 488 nm while retaining or enhancing the wild type emission, expression and photostability properties [5,6]. GFP was now a practical technique for flow cytometry, and became widely applied in a variety of systems. The development of enhanced yellow fluorescent protein (EYFP) and cyan fluorescent protein (ECFP) further enhanced the usefulness of these fluorescent protein technology for flow cytometry [6]. EYFP could also be excited at 488 nm, with a longer emission profile that could be detected simultaneously with EGFP. ECFP excited in the violet range (405–450 nm) and required a violet laser source. Although violet lasers had previously even less common than ultraviolet lasers, the development of small, inexpensive violet laser diodes made this laser wavelength practical for benchtop flow cytometers [7]. On a cytometer equipped with both 488 nm and violet lasers, ECFP could be combined with eGFP and EYFP for the analysis of up to three gene expression events simultaneously [8–10]. By the end of the 20th century, fluorescent protein analysis (particularly GFP) was a dominant technique in flow cytometry.

The subsequent isolation of the first red fluorescent protein DsRed by the Lukyanov laboratory in Russia also proved tremendously useful for flow cytometry. DsRed was the first fluorescent protein to be isolated from a non-photosynthetic organism, the coral genus *Discosoma* [11,12]. DsRed was optimally excited in the green range (500–560 nm), but had a minor excitation peak in the 480–500 nm range. Its emission extended into the red, a significantly longer emission than any previously isolated protein [11–13]. DsRed proved ideal for imaging; it could be optically

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excited using the green line from a mercury arc lamp, and emitted in a wavelength range with considerably lower autofluorescence than that of EGFP or EYFP [14,15]. While not optimal, DsRed could also be excited at 488 nm, allowing it to be used for flow cytometry. It could also be combined with eGFP and eCFP on flow cytometers equipped with violet lasers [9,10]. On more advanced cytometers with krypton-ion lasers that could produce green or yellow laser wavelengths, DsRed could be excited with even greater efficiency, and could be combined with EGFP, EYFP, and ECFP for up to four color FP cytometry [9,10]. As with imaging, DsRed emitted in a wavelength range with less autofluorescence than eGPF or eYFP, decreasing background fluorescence and improving efficiency. The tetrameric structure of DsRed resulted in slow maturation times and frequent misfolding errors, limiting its usefulness for many applications [12,13]. However, the development of tetramers with faster maturation times and more accurate folding have extended its usefulness, and the development of monomeric and dimeric forms have allowed its use in whole organism expression and fusion proteins [16].

The discovery of DsRed has led to a virtual explosion in fluorescent protein development. The "fruit" fluorescent proteins developed by site-directed mutagenesis and other molecular selection techniques from monomeric red fluorescent protein (mRFP) by the Tsien laboratory spanned a broad range of excitation and emission wavelengths from green to red [17–19]. Largely monomers and dimers, these proteins are relatively low in molecular weight, mature rapidly, and have good fluorescent characteristics. The "fruit" fluorescent proteins including dTomato and mCherry are now common tools in molecular biology [18]. The Anthrazoa coral fluorescent proteins, of which DsRed is one, have resulted in a variety of useful fluorescent proteins including ZsGreen, AmCyan, HcRed and others [20–22]. These too provide wide coverage of the visible spectrum, with excitation/emission properties ranging from the violet/blue range of eCFP to the red range of DsRed and beyond [23]. These proteins can be tailored to particular research needs. For example, the Turbo series of fluorescent proteins generated by Evrogen fold and mature rapidly, which the Tag series of proteins are have low molecular weights and small monomeric or dimeric properties, permitting their use in fusion proteins with minimal structural disruption [24]. Another aim of current fluorescent protein development is to push the emission of the probe into the long red and near-infrared range. For imaging, this emission range displays low cellular autofluorescence, and good tissue penetration, potentially allowing deep tissue imaging using two-photom microscopy technologies. For flow cytometry, it will allow the utilization of red laser lines, common fixtures on commercial flow cytometers. Recently developed long red fluorescent proteins like E2-Crimson, TagRFP657, mNeptune and eqFP670 all possess emission maxima greater than 640 nm, and have easily detectable fluorescence beyond 650 nm [24–31]. Newly developed fluorescent proteins should exceed even these values.

This review will provide a practical guide to the use of common fluorescent proteins in flow cytometry. Unlike imaging, flow cytometers have typically been limited to a small number of excitation wavelengths. Until recently, most commercial instruments were limited to a 488 nm blue-green laser, and in some cases a red laser source, either helium–neon (633 nm) or a red laser diode (~640 nm). Only large-scale cell sorters possessed the large argon- and krypton-ion lasers capable of emitting multiple wavelengths in the ultraviolet, violet, blue, green and yellow, and such instruments were very much in the minority [32]. Small violet laser diodes were introduced into less expensive benchtop instrument in the early 2000s; this permitted the easy excitation of eCFP and other violet-excited dyes [7,33]. However, many of the more recent red fluorescent proteins like mCherry, HcRed and mKate were still not accessible to most flow cytometers, and even shorter red fluorescent proteins like DsRed and dTomato could not be optimally excited. This has largely changed with the introduction of small green (532 nm) and yellow (561 nm) diode-pumped solid state (DPSS) lasers into flow cytometers [34,35]. Originally installed with the goal of providing better excitation of fluorescent probes traditionally used for immunolabeling (i.e. phycoerythrin), they have proven tremendously useful for exciting short and long red fluorescent proteins as well. Modern benchtop flow cytometers are now often equipped with four or more laser sources, and are very well-suited to exciting a wide variety of fluorescent proteins. The use of both traditional older instruments and high-end multi-wavelength instruments will be discussed in detail.

2. Materials and methods

2.1. Cells

Cells expressing a variety of fluorescent proteins were used to illustrate the best methods for detection. Two types of cells were used in these examples. For some experiments, SP2/0 cells expressing the indicated fluorescent protein (EGFP, EYFP, ECFP, DsRed or tdTomato). These cells were stably transduced with the appropriate pEGFP-1, pEYFP, pECFP, pDsRed1–1 or ptdTomato plasmid and retroviral vectors as previously described [9,10]. For the *Escherichia coli* samples, the genes encoding the indicated fluorescent protein were inserted into pBAD/His-B vector (Invitrogen Life technologies, Carlsbad, CA) using BglII and EcoRI restriction sites. The resulting plasmids were transformed into the electrocompetent *E. coli* bacterial strain LMG194 (Invitrogen). The protein expression was then induced with 0.02% (wt/vol) L-arabinose at 37.8 °C. For flow cytometry, bacterial cells expressing the proteins were washed with phosphate-based saline (PBS), fixed with 4% paraformaldehyde and resuspended in PBS with OD = 0.01 measured at 650 nm.

2.2. Flow cytometry

All experiments were carried on a BD Biosciences LSR II flow cytometer (San Jose, CA). Mammalian cells or bacteria were excited using one or more of the following lasers: a DPSS 488 nm at 50 mW (Newport Spectra Physics, Irvine, CA), a violet laser diode 405 nm, a blue laser diode 440 nm (Coherent Laser, Santa Clara, CA), a green DPSS 532 nm (Laser-Export, Moscow, Russia), a green-yellow 550 nm fiber laser (Zecotek Photonics Ltd., Singapore), a yellow DPSS 561 nm at 50 mW (Oxxius, Lannion, France), yellow 580 nm, orange 592 nm and red 628 nm fiber lasers (MPB Communications, Pointe-Claire, QB, Canada) and a red HeNe 633 nm (JDS Uniphase, San Jose, CA).

2.3. Detection optics

For all examples appropriate laser wavelength from the list above will be indicated. The required bandpass filter will also be listed, using the center wavelength (i.e. 530 nm) followed by window size in nanometers, separated by a slash. So, a 530/30 nm filter will have a center transmission wavelength of 530 nm, with a 30 nm emission window. The emission of this particular filter will therefore range from approximately 515–545 nm. For some multicolor experiments, a dichroic mirror will be indicated for separating two fluorescence wavelength ranges. Shortpass dichroics (SP) transmit light below their indicated values and reflect longer wavelengths; longpass dichroics (LP) transmit light greater than their indicated values and reflect longer wavelengths. Therefore, a 560 SP dichroic mirror will transmit light less than 560 nm, and reflect light greater than 560 nm. Dichroics are uses at a particular angle of incidence. For the BD Biosciences LSR II flow

cytometer used here, the angle of incidence ranged from 10° to 18°. All filters and dichroic mirrors used in this study were manufactured by Omega Filter (Brattleboro, VT), Chroma (Bellows Falls, VT) or Semrock (Rochester, NY).

2.4. Data acquisition and analysis

Data was initially acquired using DiVa acquisition software version 5.0.3 (BD Biosciences) with data collected in FCS 3.0 file format. Data was analyzed using FlowJo for PC version 7.2.5. For multicolor experiments, fluorescence compensation or spillover was calculated automatically using single color controls and FlowJo. To determine the detection sensitivity of a particular fluorescent protein using an indicated laser and filter combination, a sensitivity index (SI) is used, calculated using the following formula:

$$SI = (\text{median fluorescent cells} - \text{median non-expressing cells})/(\text{robust SD})$$

where robust SD = (84 percentile non-expressing cells)/(median non-expressing cells).

These values are indicated on all histograms comparing excitation and emission conditions.

3. Results

A summary table of all discussed fluorescent proteins is shown in Table 1, including optimal lasers and filters. For the purposes of clarity, fluorescent protein descriptions will range from the lowest excitation and emission wavelengths to the highest, with excitation in the ultraviolet and emission in the blue range) will be reviewed first. As each wavelength range is discussed, multicolor analyses will be included where appropriate.

3.1. Blue fluorescent proteins

The original enhanced Blue Fluorescent Protein (EBFP) was developed at the same time as the original enhanced YFP and CFP variants, with the intention of creating an ultraviolet-excited fluorescent protein that would be spectrally compatible with EGFP for fluorescence microscopy. Unfortunately, EBFP was not particularly bright, and emitted in a region of strong autofluorescence. As a result, it saw little use. More recently developed blue fluorescence proteins include EBFP2, Sirius and Azurite, as well as the Evrogen proteins TagBFP and mTagBFP [36–38]. These proteins are much brighter than EBFP; however, the autofluorescence issue remains.

While not often used for flow cytometry, they are nevertheless compatible with any flow cytometry system equipped with an ultraviolet laser. The original large water-cooled gas lasers previously required to produce ultraviolet laser excitation have been largely superceded by smaller solid-state lasers, most based on a Nd:YAG crystal structure and frequency-tripled to an emission of 355 nm. These lasers are sometimes present on high-end benchtop instruments such as the BD Biosciences LSR II, Fortessa and FACSAria SORP systems, and the Beckman–Coulter Astrios cell sorter. Near-ultraviolet laser diodes that emit at 375 nm are also appropriate for the blue fluorescence proteins, although these are not common on flow cytometers. Bandpass filters appropriate for the DNA dye DAPI or the immunolabeling probes AMCA or Pacific Blue are appropriate for blue fluorescent proteins, with examples being 440/10, 450/50 and 460/50 nm.

3.2. Cyan fluorescent proteins

Enhanced cyan fluorescent protein (ECFP) has proven to be a valuable reagent, particularly for multicolor applications involving other fluorescent proteins or fluorescent markers. ECFP requires a violet or blue excitation source, and can take advantage of the ultraviolet and blue lines from a mercury arc lamp for fluorescence microscopy. Prior to 1999, the only way to generate a violet laser line on a flow cytometer was to use a water-cooled krypton-ion laser, which could produce strong 407 and 413 nm laser lines [9,10]. The 457 nm line generated from an argon-ion laser could also be used [8]. These lasers could only be accommodated on large cell sorters like the BD Biosciences FACStar series and FACSVantage, the Beckman–Coulter (formerly DakoCytomation) MoFlo, and the Beckman-Coulter Altra. As a result, ECFP analysis was largely limited to these instruments. After 2000, small violet laser diodes emitting from 395 to 415 nm were introduced into smaller benchtop instruments, making ECFP excitation far more accessible [7]. Violet laser diode excitation of ECFP is now the standard method for analyzing this protein [33]. Filters appropriate for the DNA dye DAPI (i.e. 450/50 nm) will work for ECFP, but slightly longer filters (i.e. 460/50 nm) are even closer to its emission maxima and will improve signal collection, as shown in the Fig. 1a top left histogram. Care should be taken however that the filter used does not allow the entry of 488 nm laser light, which will dramatically increase detector background and diminish sensitivity. For example, a 485/22 nm filter would normally allow 488 nm light and should be avoided. In the Fig. 1a top middle histogram, a 485 nm filter sandwiched with a 488 nm restriction bandpass (RB) or notch filter (which blocks 488 nm laser light) is superior to a 450/50 nm filter. However, notch filters are expensive. An even longer filter detecting in the green range (i.e. 510/20 or 530/30 nm) can also be used with minimal signal loss, although higher compensation values may result in multicolor experiments with EGFP (Fig. 1a top right histogram).

Prior to the introduction of small violet lasers, ECFP was also found to be excited with the 457 nm laser line that could be generated by a water-cooled argon-ion laser [8]. In fact, EGFP, EYFP and ECFP could be simultaneously excited with this laser source. However, again a large cell sorter was required to accommodate these lasers. The replacement of water-cooled 488 nm gas lasers with cheaper, more durable solid state units has resulted in the elimination of the 457 nm line from most systems. Solid state 457 nm lasers appropriate for flow cytometry do exist, but are rarely found on commercial systems. A few flow cytometers are equipped with 440 nm blue laser diodes, a concurrent development with the violet laser technology. In the bottom row of Fig. 1, a blue laser diode was found to provide superior excitation of ECFP when compared to a violet laser at a higher power level, using the same filters as for violet with the exception of the 450/50 nm. However, the violet laser diode with blue filter combination is much more commonly found on commercial instruments, and is adequate for most detection applications.

Several improved variants of ECFP have been developed, most notably Cerulean, Cerulean 2, Cerulean 3 and TagCFP [39,52]. CyPET is an engineered donor fluorescent protein for the FRET acceptor YPET [40]. All of these fluorescent proteins have improved brightness compared to ECFP. However, ECFP is still widely used. Several slightly longer wavelength cyan fluorescence proteins have also been isolated, including AmCyan (Clontech), Midori-Ishii Cyan and mTurquoise [53]. These proteins excite optimally at greater than 450 nm; although violet laser diodes will work for these proteins, a longer wavelength laser might be desirable.

3.3. Green fluorescent proteins

The *Aequeora*-derived enhanced GFP (EGFP) mutant remains the most commonly used fluorescent protein, and is still frequently used despite other GFP-like variants with better quantum efficiency and overall brightness [41,42]. With an excitation maxima

Table 1
Fluorescent proteins commonly used for flow cytometry.

Color	Fluorescent protein	EX max (nm)	EM max (nm)	Recommended laser (nm)	Recommended bandpass filters (nm)
Blue	Azurite	383	447	355, 375	450/50, 460/50
	eBFP2	383	448		
	TagBFP	400	456		
	mTagBFP, mTagBFP2	400	456		
	T-Sapphire	399	511		510/20
Cyan	Cerulean, Cerulean 2, Cerulean 3	433	475	405, 440, 457	450/50, 460/50, 485/22 with 488 nm block
	ECFP	439	476		
	TagCFP	458	480		
	TagCFP	458	480		
	AmCyan	458	489		
	CyPet	435	477		
	Midori-Ishii Cyan	470	496		
	LSS-mKate1	463	624		630/22
	LSS-Kate2	460	605		610/20
Green	Wild type GFP	396	510		
	EGFP	484	507	488	510/21, 530/30
	TagGFP	483	506		
	TagGFP2	483	506		
	TurboGFP	482	502		
	Emerald GFP	487	509		
	Monster Green	505	518		
	Azami Green	492	505		
	ZsGreen, ZsGreen1	493	505		
	hrGFP, hrGFP2	502	505		
	Renilla GFP	472	540		
	Verdi GFP (monomer, dimer)	491	503		
Yellow	EYFP	514	527	488, 505, 514, 517, 532	530/30, 550/30, 556/21, 575/26, 585/42
	Topaz	514	527		
	Venus	515	528		
	Citrine	516	529		
	YPet	517	530		
	TagYFP	508	524		
	TurboYFP	508	524		
Orange	mKOm	548	559	488, 532, 561	575/26, 585/42
	mKOk	551	563		
	mOrange, mOrange2	548	562		
	E2 Orange	540	561		
Short red	DsRed	553	583	532, 550, 561	585/42, 590/20, 610/20
	DsRed2	553	583		
	DsRed-Express	553	584		
	DsRed monomer	556	586		
	dTomato	554	581		
	tdTomato	554	581		
	TagRFP	555	584		
	mStrawberry	574	596		
	mCherry	587	610		
	TagFP635 (mKate)				
	mKate2	588	633		
	TurboFP635 (Katushka)	588	635		
Long red	HcRed	592	645	550, 561, 592	660/20
	mPlum	590	649		
	eqFP650	592	650		
	mRasberry	598	625		
	mNeptune	599	649		
	eqFP670	605	670		
	E2 Crimson	611	646		
	TagRFP657	611	657		

at 488 nm, it is ideally suited for flow cytometry using the traditional 488 nm laser. The emission of maxima of 509 nm suggests a can be accommodated by a 510/20 nm or similar bandpass filter; however, the typical fluorescein bandpass filter found on many flow cytometers, usually at 530/30 nm, will work well for most applications with minimal loss of signal, as shown in Fig. 1b. An exception to this is when combining EGFP with a yellow fluorescent protein variant, where a 530/30 nm filter will admit too much EYFP signal (see upcoming section on combining EGFP and EYFP). Keep in mind that wild-type GFP is still in use, as are several other GFP variants (such as GFP2) that still require an ultraviolet or violet excitation source, despite their green emission.

A variety of other green fluorescent proteins are available, although most of them have spectral characteristics almost identical to EGFP. Emerald GFP (Invitrogen Life technologies, Carlsbad, CA) is an Aequora mutant. TagGFP, TagGFP2, ZsGreen, ZsGreen1 (Clontech), Azami Green (MBL Laboratories), Monster Green (Promega) and monomeric and dimeric Verde Green are all coral-derived. Renilla GFP, hrGFP and hrGFP1 are derived from the sea pansy [43]. These alternatives will often be chosen for increased

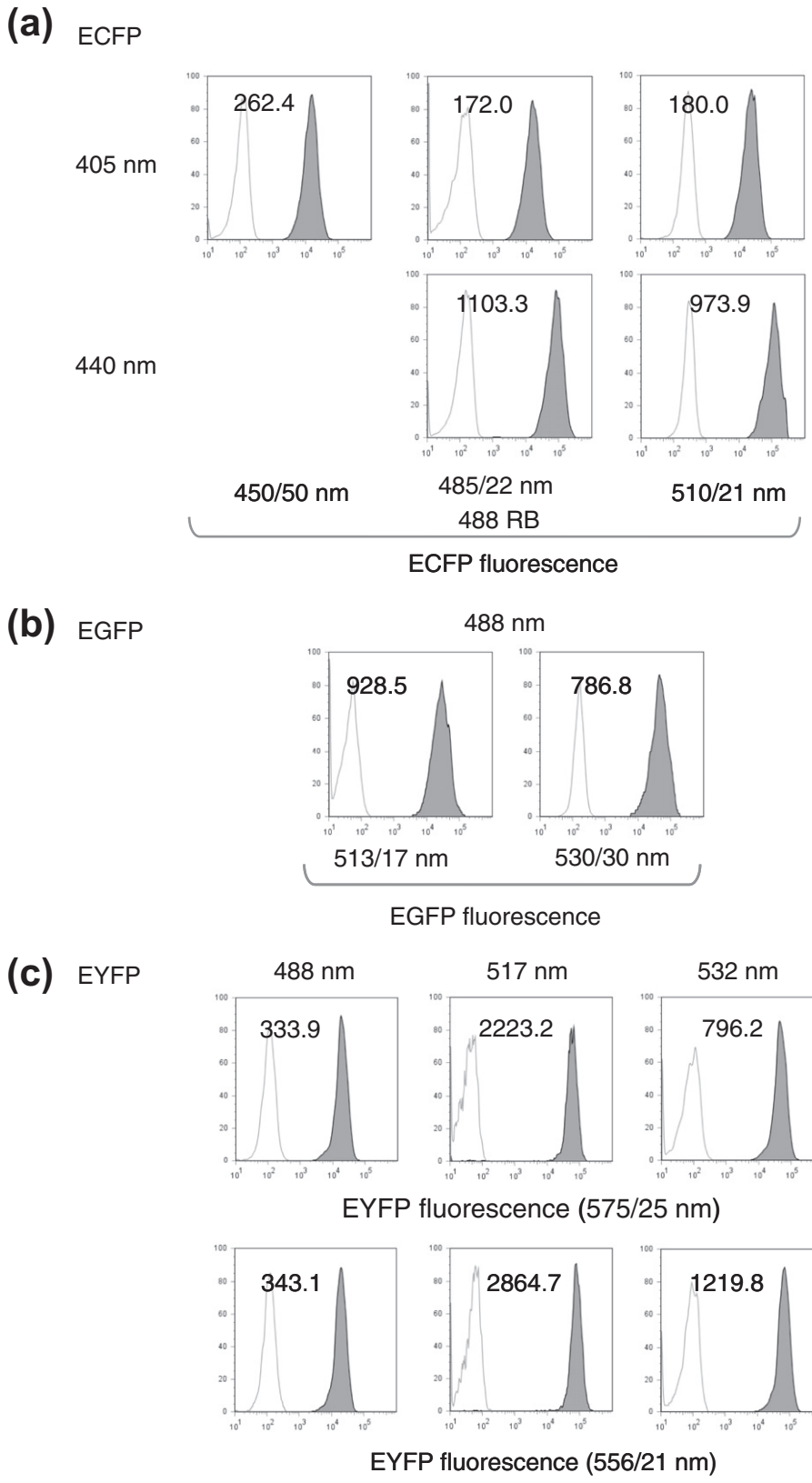


Fig. 1. Single color analysis of ECFP, EGFP and EYFP. (a) SP2/0 cells wild-type (open peak) or expressing ECFP (filled peaks) were analyzed on a BD LSR II using the indicated lasers (top row with a violet laser diode, 405 nm at 25 mW, bottom row with a blue laser diode 440 nm at 16 mW) and bandpass filters. Sensitivity index for each sample is indicated, and was calculated as described in the Section 2. (b) SP2/0 cells wild-type (open peak) or expressing EGFP (filled peaks) were analyzed on a BD LSR II using a 488 nm laser and the indicated filters. (c) SP2/0 cells wild-type (open peak) or expressing EYFP (filled peaks) were analyzed on a BD LSR II using the indicated lasers (left, default 488 nm at 50 mW; middle, green laser diode 517 nm at 20 mW; right, DPSS green 532 nm at 50 mW) and bandpass filters (top row, 575/26 nm; bottom row, 556/21 nm).

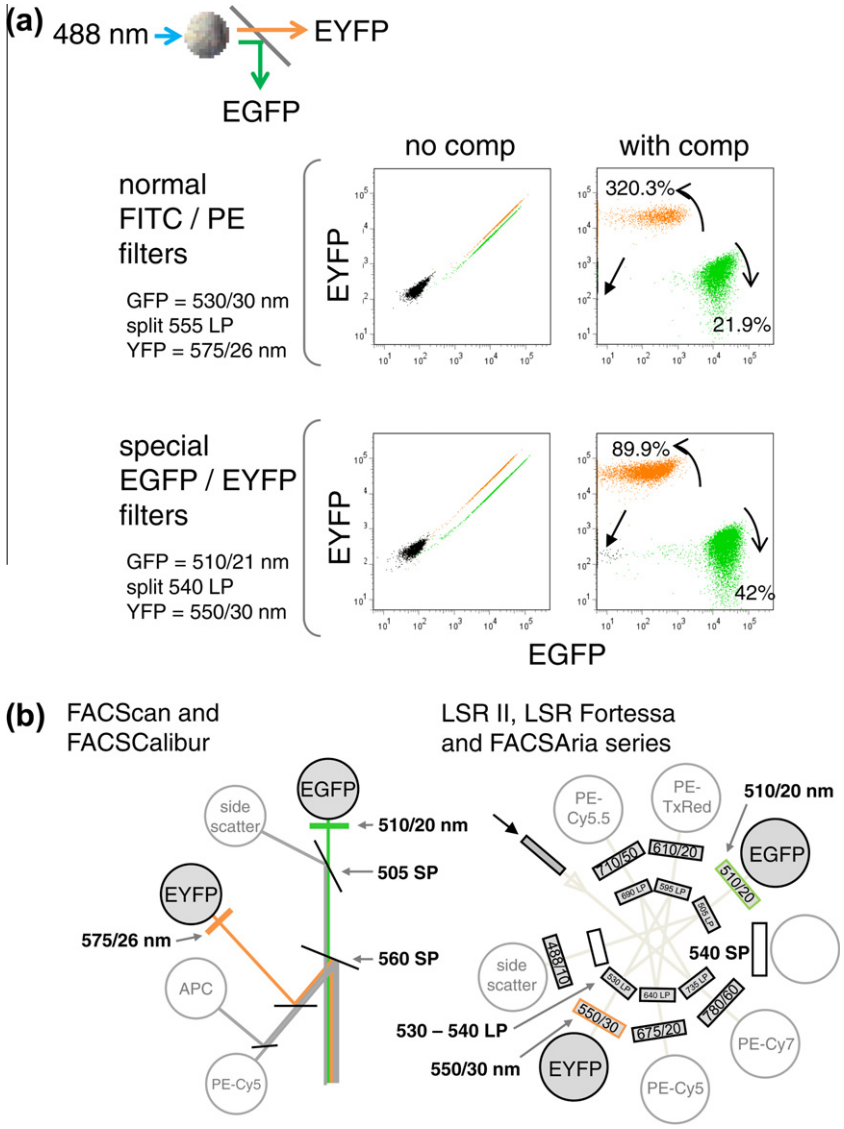


Fig. 2. Simultaneous analysis of EGFP and EYFP. (a) Mixture of wild type, EGFP and EYFP expressing SP2/0 cells analyzed on a BD LSR II. Excitation with a single 488 nm laser. Top row, detection with EGFP = 530/30 nm, EYFP = 575/26 nm, 560 SP dichroic), bottom row, detection with EGFP = 510/21 nm, EYFP = 550/30 nm, 540 LP dichroic. Both uncompensated and compensated analyses are shown (left and right, respectively). The required compensation values are shown on each scatterplot. (b) Recommended filter and dichroic combinations for a BD Biosciences FACScan or FACSCalibur (left) and LSR II, Fortessa or Aria series (right).

brightness, improved photostability, low molecular weight or improved expression characteristics. They can all be analyzed as above.

3.4. Yellow fluorescent proteins

The *Aequeora* mutant EYFP was until recently the most common yellow fluorescent protein; it has now been largely supplanted by more recent variants with enhanced photostability and brightness, although it is still often seen in some expression systems, particularly as a FRET acceptor to ECFP [5]. With a broad excitation maxima of 514 nm, it can be well-excited with a 488 nm laser, and is optimally detected through a fluorescein or slightly longer filter (530/30 and 550/30 nm commonly used). However, using a laser wavelength more closely matched to the excitation peak will result in significant improved sensitivity. Earlier water-cooled argon lasers produced a 514.5 nm line, and more recent solid state lasers emitting at 505 and 515 nm are available. These are rarely incorporated into flow cytometers due to a lack of applications; however, they are available from at least one instrument manufacturer. A recently developed 517 nm green diode laser (Pavilion Integration Corporation, San Jose, CA) gave significantly better EYFP sensitivity when compared to 488 nm (Fig. 1). Interestingly, a 532 nm green laser found on many modern instruments also gave somewhat better of excitation of YFP than a 488 nm laser. When analyzing EYFP fluorescence alone, a green 532 nm paired with a 556/21 or longer filter can be used for EYFP detection (Fig. 1).

Since it has historically been paired with EGFP for two-color analysis, EYFP filters have tended to be longer than absolutely optimal, with a 550/30 nm or similar usually used. However, it can be detected reasonably well through the fixed filters for phycoerythrin found on many commercial cytometers, usually a 575/26 or 585/42 nm or similar.

Recently developed yellow fluorescent proteins include Citrine (Clontech), Topaz, Venus, TagYFP and TurboYFP (Evrogen) [44,45]. All of these proteins have increased quantum efficiency and brightness in comparison to EYFP, and are more photostable. They can be analyzed as for EYFP.

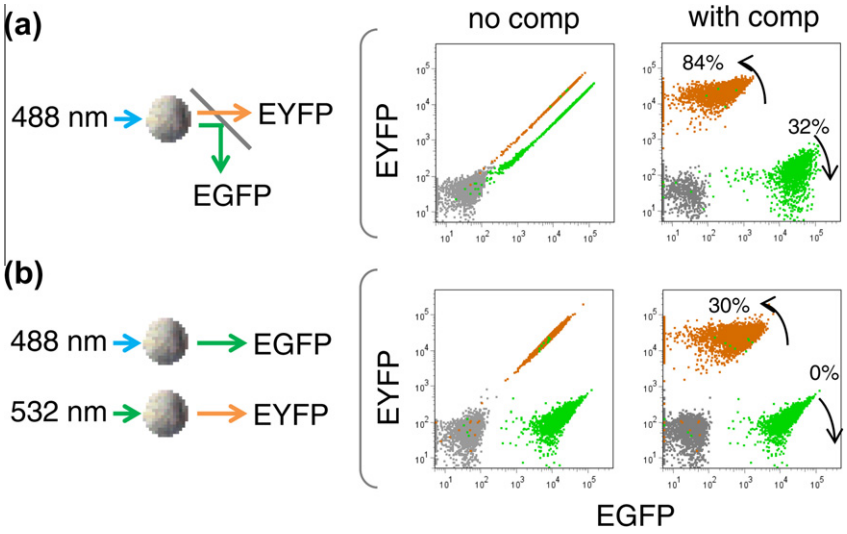


Fig. 3. EYFP analysis with 532 nm excitation. Mixture of wild type, EGFP and EYFP expressing SP2/0 cells analyzed on a BD LSR II. Top row, excitation of both fluorescent proteins with a single 488 nm laser, EGFP = 510/21 nm, EYFP = 550/30 nm, 540 LP dichroic; bottom row, EGFP excitation with a 488 nm laser, EYFP excited with a spatially separated 532 nm laser, detection of EGFP with a 510/21 nm filter, detection of with EYFP = 550/30 nm on separate signals paths. Both uncompensated and compensated analyses are shown (left and right, respectively). The required compensation values are shown on each scatterplot.

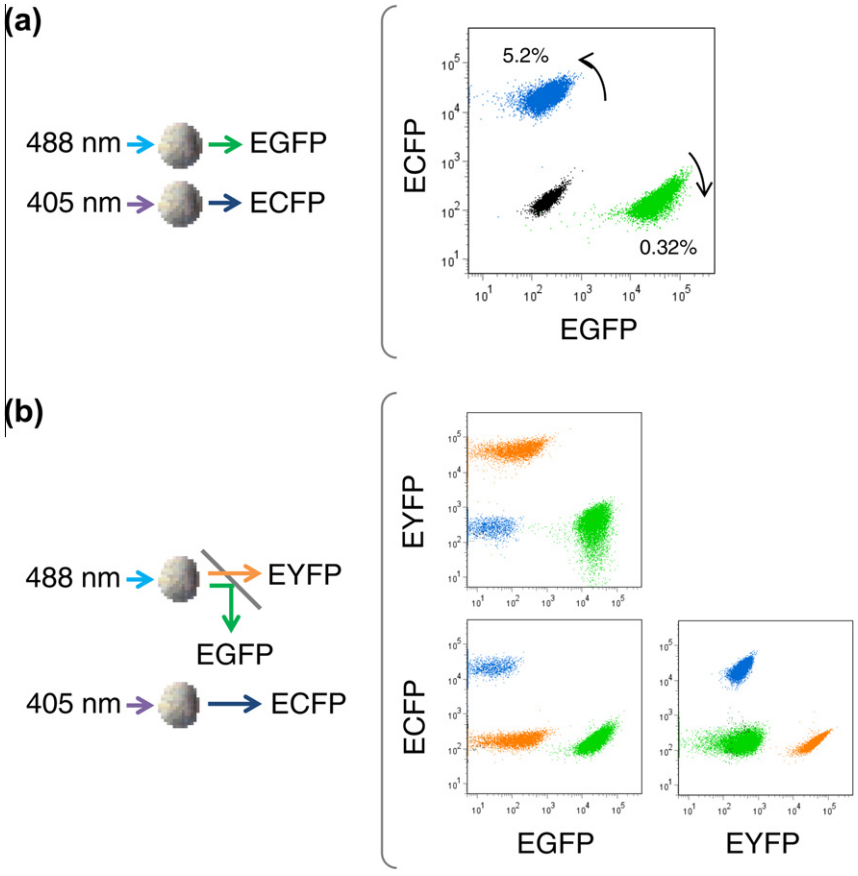


Fig. 4. Simultaneous analysis of EGFP, EYFP and ECFP. (a) Mixture of wild type, EGFP and ECFP expressing SP2/0 cells analyzed on a BD LSR II with 488 nm excitation of EGFP and spatially separated 405 nm excitation of ECFP. Compensation values are shown. (b) Mixture of wild type, EGFP, EYFP and ECFP expressing SP2/0 cells analyzed on a BD LSR II with 488 nm excitation of EGFP and EYFP and spatially separated 405 nm excitation of ECFP. Compensation values are shown. Filters for both analysis were EGFP = 510/21 nm and EYFP = 556/21 nm, separated with a 540 LP dichroic, ECFP = 450/50 nm. The EYFP filter was modified from Fig. 2 to exclude 532 nm laser light from the EYFP detector.

3.5. Combining green and yellow fluorescent proteins

In the early days of fluorescent protein development, the choices for combining more than one protein for analyzing multiple gene expression events were limited. In addition, most flow cytometers had at most two lasers, one of which was a red HeNe or laser diode, which at that time could not excite any known fluorescent proteins. That left the blue-green 488 nm laser, which

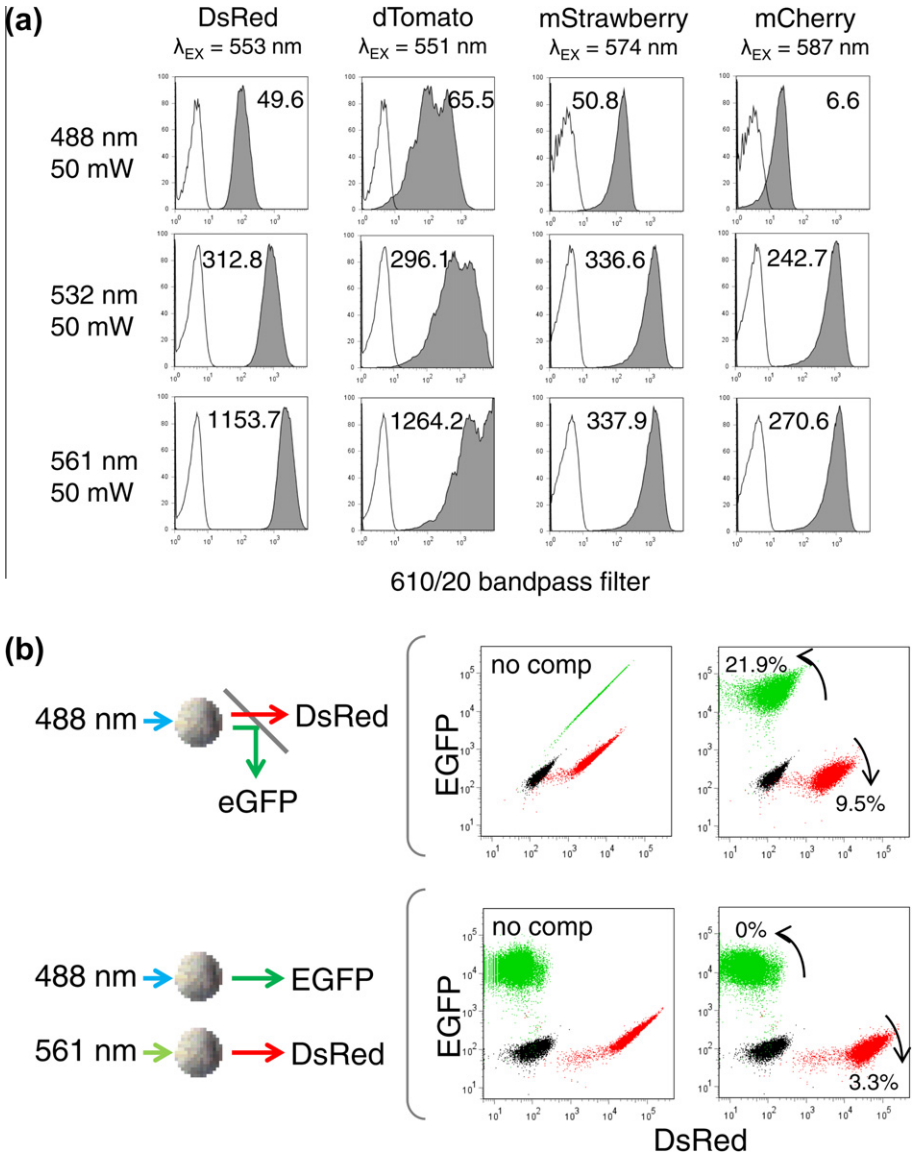


Fig. 5. Red fluorescent proteins. (a) Analysis of *E. coli* expressing DsRed, dTomato, mStrawberry and mCherry using laser excitation at 488, 532 and 561 nm, all at 50 mW. All fluorescent proteins detected through a 610/20 nm filter. Unfilled peaks, wild type *E. coli*, filled peaks, fluorescent protein expressing *E. coli*. Sensitivity index values are indicated on each histogram. (b) Mixture of wild type, EGFP and DsRed expressing *E. coli* analyzed on a BD LSR II with 488 nm excitation of both EGFP and DsRed (top scatterplots) or 488 nm excitation of EGFP and spatially separated 561 nm excitation of DsRed. Compensation values are shown. Filters were EGFP = 510/21 nm, DsRed = 610/20 nm.

in theory allowed the combination of EGFP and EYFP. In reality, the emission spectra of EGFP and EYFP overlapped considerably, making it difficult to design an optical system that could distinguish the two proteins. Most commercial flow cytometers are designed to detect fluorescein (i.e. FITC) and the orange-emitting phycobiliprotein phycoethrin (PE). While not completely dissimilar, the emission bandwidth of PE peaks at a longer wavelength than EYFP, and EGFP is slightly shorter than fluorescein. Fluorescein and PE were typically detected with a 530/30 and 575/26 or 585/42 nm filter, with the signals separated with a 550–560 shortpass or longpass dichroic mirror. This was not ideal for EGFP and EYFP detection; EYFP sensitivity would be suboptimal, and too much EYFP signal would overlap into the EGFP detector. The result is shown in Fig. 2a upper scatterplots. While the signals can be distinguished, an extremely high level of fluorescent compensation (over 300%) to subtract EYFP signal from the EGFP detector can be required, and the fluorescence patterns are extremely distorted. Unfortunately, many dual FP experiments were historically analyzed under these non-optimal conditions due to apparently fixed optics in these systems.

A more optimal system consists of a shorter EGFP filter (typically 510/20 nm or similar) and a shorter EYFP filter (typically 550/30 nm or similar), with a shorter bandpass (530 nm longpass or shortpass, depending on the system). The critical element here is the dichroic mirror, which excludes more EYFP signal from the EGFP detector. The EYFP filter can remain in the 575 or 585 nm range if necessary, if a loss of sensitivity can be tolerated. Typical results are shown in Fig. 2a lower scatterplots, with less than 90% compensation required to subtract EYFP signal from the EGFP detector. This is still high, but much less than with the standard fluorescein/PE filters. Most modern flow cytometers (including instruments manufactured by BD Biosciences, Beckman–Coulter, Partec, Sony-iCyt, Stratedigm, etc.) are equipped with interchangeable optics, allowing the removal of default filters and replacement with more optimal alternatives. Optical layouts for the FACScan/FACSCalibur and LSR II cytometers are shown in Fig. 2b. All major

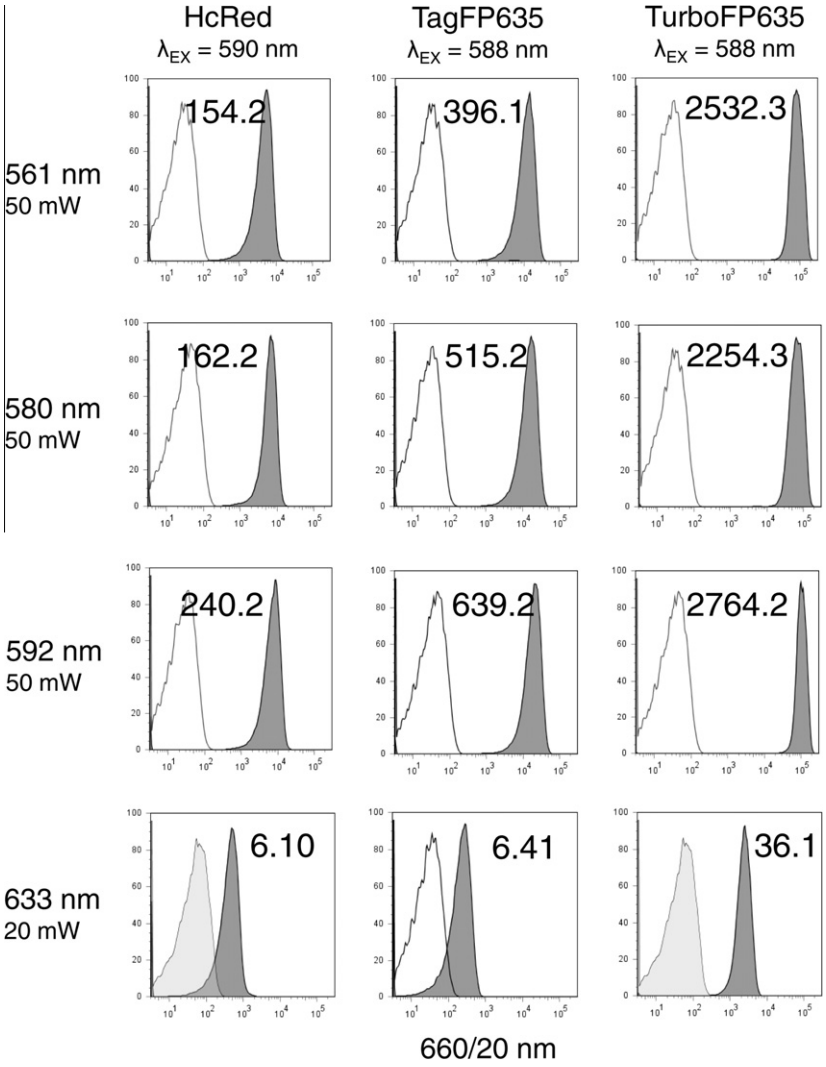


Fig. 6. Red fluorescent proteins. Analysis of *E. coli* expressing HcRed, TagFP635 (mKate) and TurboFP635 (Katushka) using laser excitation at 561, 580, 592 or 633 nm, all at 50 mW except 633 nm at 25 mW. All fluorescent proteins detected through a 660/20 nm filter. Unfilled peaks, wild type *E. coli*, filled peaks, fluorescent protein expressing *E. coli*. Sensitivity index values are indicated on each histogram.

manufacturers of optics for flow cytometers produce filters and dichroics optimized for the major fluorescent proteins. Most of these systems use standard 25 mm bandpass filters, with dichroic mirrors ranging from 25 mm diameters with 45° of angle of incidence (most instruments) to 18 mm diameters with 10°–18° angle of incidence (BD Bioscience LSR II, Fortessa and FACSAria series). Even earlier benchtop cytometers with supposedly fixed optical systems can be modified. The BD Biosciences FACScan and FACSCalibur have removable bandpass filters (10 mm square), and replacements can be obtained from the filter manufacturers.

A green solid state laser emitting at 532 nm can reduce the need for EYFP compensation even further. In Fig. 3b, this laser was used to excite EYFP with a 556/21 nm bandpass filter, while a spatially separated 488 nm was used to excite EGFP with a 510/20 nm filter. Only 30% compensation required to subtract EYFP signal from the EGFP detector compared to almost 90% with a single 488 nm laser (shown in Fig. 3a) with EYFP sensitivity similar to 488 nm excitation. This technique requires a solid state green laser; however, many high-end cytometers are now equipped with these laser modules.

With the advent of cyan and red fluorescent proteins, there is less cause to combine EGFP and EYFP, and other combinations with less spectral overlap are often preferable.

3.6. Combining green, cyan and yellow fluorescent proteins

Shortly after the development of ECFP, it was possible to measure three fluorescent proteins simultaneously if one had access to a large cell sorter with a water-cooled krypton-ion, which could produce strong 407 and 413 nm violet laser lines [9,10]. When smaller and cheaper violet laser diodes became available at the turn of the century, violet laser light became readily on flow cytometers, and constituted a third major laser line in addition to blue-green and red [7,33]. EGFP and ECFP could therefore be readily combined on such instruments. Unlike EGFP and EYFP, there is virtually no spectral overlap between EGFP and ECFP. Violet lasers produce almost no excitation in EGFP, and the 488 nm line does not excite ECFP; this absence of "crossbeam excitation" results in almost complete spectral isolation between the two fluorochromes. This is shown in Fig. 4a, where the required compensation between the two fluorochromes is extremely low. By using the same detector configuration described for EGFP and EYFP, EYFP can be added for three color analysis, albeit with the high levels of compensation required above (Fig. 4b). However, like EGFP, EYFP similarly does not overlap spectrally with ECFP. ECFP and EYFP are often used as a FRET pair, and the lasers and detectors used in Fig. 4 can be applied.

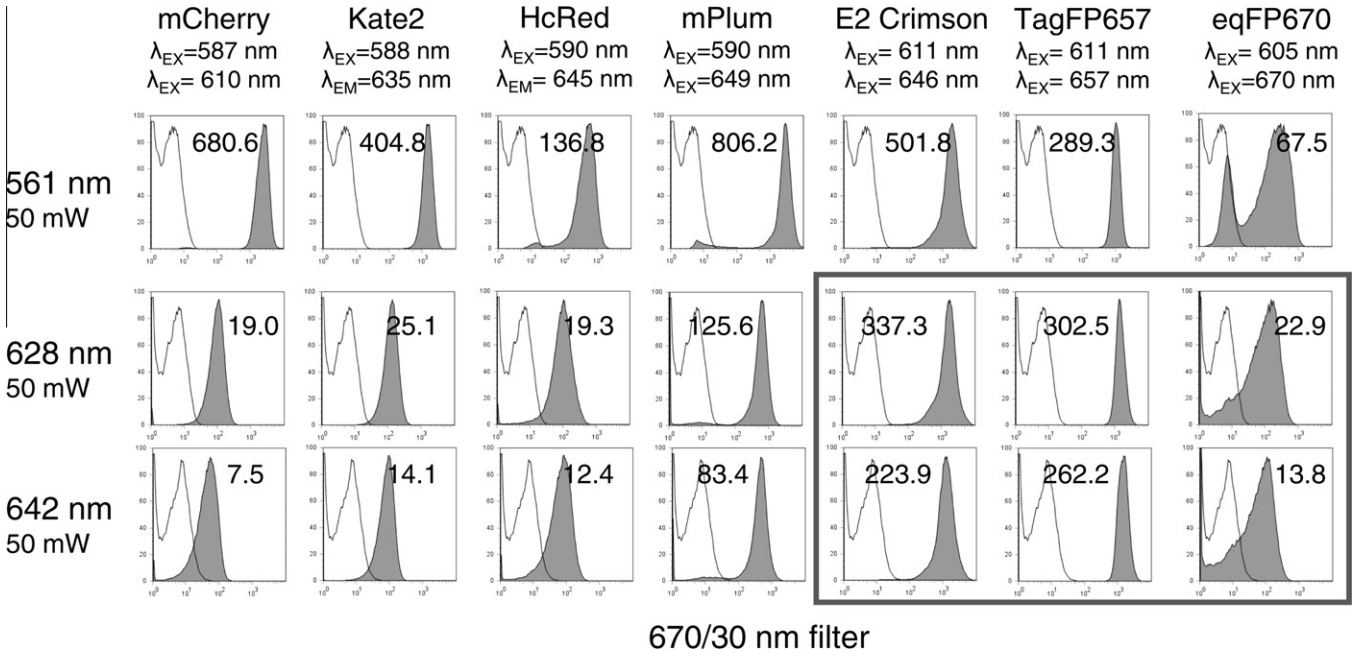


Fig. 7. Red fluorescent proteins. (a) Analysis of *E. coli* expressing mCherry, mKate2, HcRed, mPlum, E2 Crimson, TagRFP657 and eqFP670 using laser excitation at 561, 628 and 642 nm, all at 50 mW. All fluorescent proteins detected through a 660/20 nm filter. Unfilled peaks, wild type *E. coli*, filled peaks, fluorescent protein expressing *E. coli*. Sensitivity index values are indicated on each histogram.

3.7. Orange fluorescent proteins

Only a few orange fluorescent proteins (classified as longer than yellow but shorter than red) exist, but they can be relatively bright and are used for some imaging and FRET donor applications. The "fruit" fluorescent proteins mOrange and mOrange2 are bright and are well excited by both blue-green 488 and green solid state lasers [18]. A recent DsRed-Express2 variant E2-Orange has also been developed [46].

3.8. Short red fluorescent proteins

The isolation of DsRed has proven to be as valuable to flow cytometry as it was to the imaging field. While not optimal from an excitation point of view, these proteins could be combined with EGFP, EYFP and ECFP for multicolor protocols [9,10]. The addition of green 532 nm and yellow 561 nm lasers however greatly expanded the usefulness of these proteins. Exciting DsRed at 532 or 561 nm rather than 488 nm could enhance its signal-to-background ratio from five to 20 times, greatly increasing sensitivity as measured by sensitivity index between expressing and non-expressing cells [35] (Fig. 5a). Green and yellow laser sources also excite less autofluorescence in cells, further enhancing this increase in sensitivity. The derivation of the "fruit" fluorescent proteins by the Tsien laboratory resulted in a large number of red fluorescent proteins with increasing excitation and emission maxima [18]. As with DsRed, the "short" red fluorescent proteins (here classified arbitrarily as those with excitation maxima less than 590 nm) were far better excited at longer green and yellow laser wavelengths (Fig. 5). This was particularly true of proteins like mCherry with longer excitation maxima than DsRed [47].

As with EYFP and ECFP, DsRed can be readily combined with EGFP for two color analysis. And as with EYFP, the best way to do this is with independent laser sources for each protein. In Fig. 5b, mixtures of cells were analyzed either using a single 488 nm to excite both proteins, or 488 and 561 nm lasers to excite EGPF and DsRed, respectively. Spatially separating the excitation of EGFP and DsRed greatly reduced the required fluorescence compensation, since EGFP was not excited by the 561 nm laser. Green 532 nm can be substituted for the yellow 561 nm used here if it is part of an existing system. EYFP and ECFP can also be combined into this labeling system as previously demonstrated [9,10].

The "short" wavelength red fluorescent proteins with the longest excitation maxima (including mCherry, mPlum, HcRed, TagFP635, etc.) can benefit from even longer wavelength laser excitation. As with all fluorochromes, the best results are achieved by matching the laser wavelength with the excitation maxima and range of the probe. In Fig. 6, fiber lasers emitting at 580 and 592 nm improve on the sensitivity observed with the shorter wavelength 561 nm laser when exciting "short" red fluorescent proteins with longer excitation maxima [48,49]. Of course, a 561 nm laser (much more common on flow cytometers than 580 or 592 nm sources) should be adequate for these proteins. However, orange solid state lasers in the 592–594 nm range are becoming increasingly common fixtures on flow cytometers and are optimal for these proteins if they are available. It should be noted that a red 633 nm HeNe laser was too long for these proteins; excitation was very poor, as shown in the bottom row of histograms in Fig. 6. This group of proteins therefore cannot be used with red lasers found on most modern flow cytometers.

The optimal excitation filters for the "short" red fluorescent proteins are shown in Table 1. For DsRed, dTomato and tdTomato, a PE filter (575/26 or 585/42 nm) can be used IF a 532 nm laser source is used. For 561 nm, however, laser light will impinge on these filter bandwidths, causing an intolerable increase in background. A slightly longer filter than does not admit 561 nm light should be used, 590/20 or 610/20 nm being examples. For mStrawberry, mCherry and other slightly longer emitting proteins, a 630/22 nm filter or similar can be used. The longest "short" reds, including mPlum, HcRed, TagFP635 (mKate in the literature) and TurboFP635 (Katushka in the literature), a bandpass filter appropriate for the phycobiliprotein allophycocyanin (APC), such as a 660/20 nm can be used [25]. It should also be noted that some of the dimmer red fluorescent proteins such mPlum and HcRed have been largely superceded by brighter proteins.

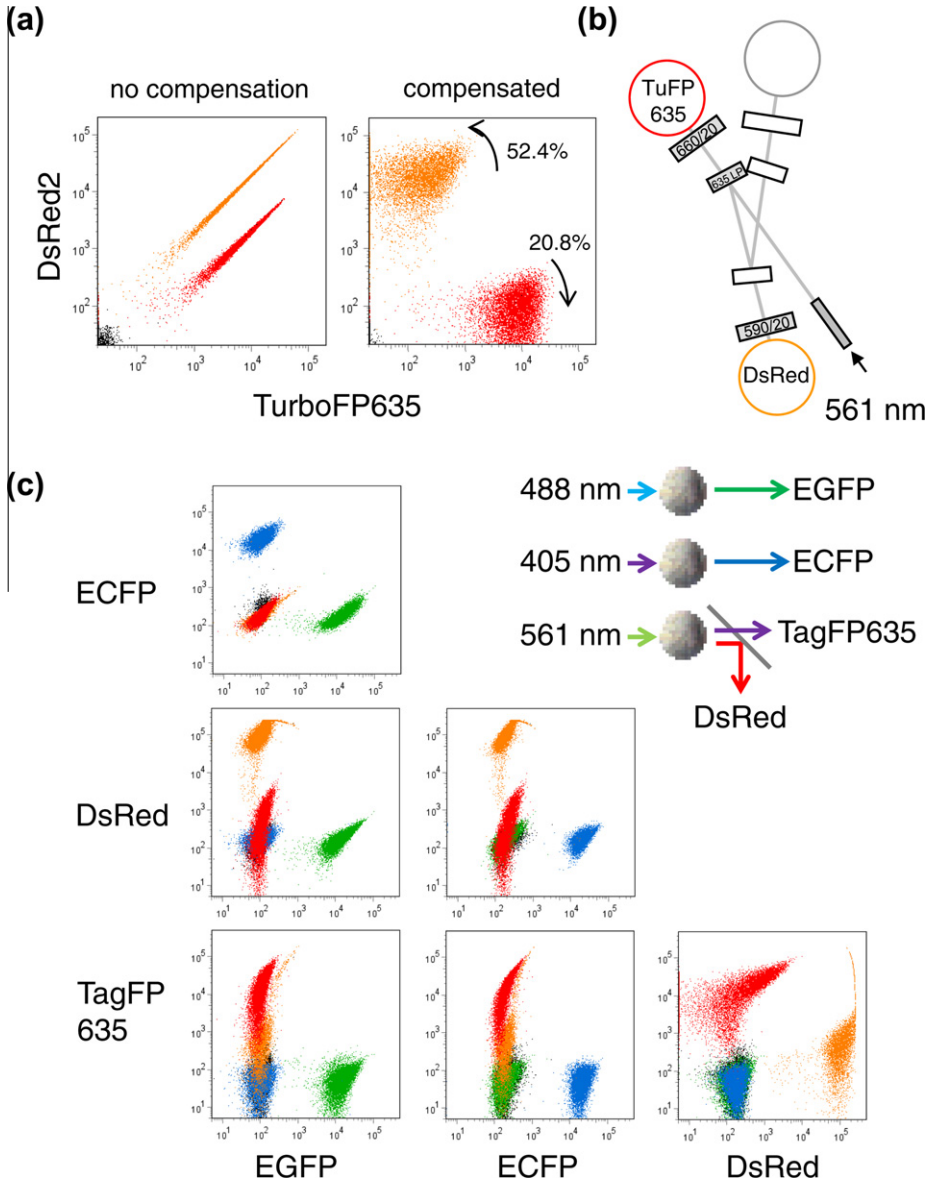


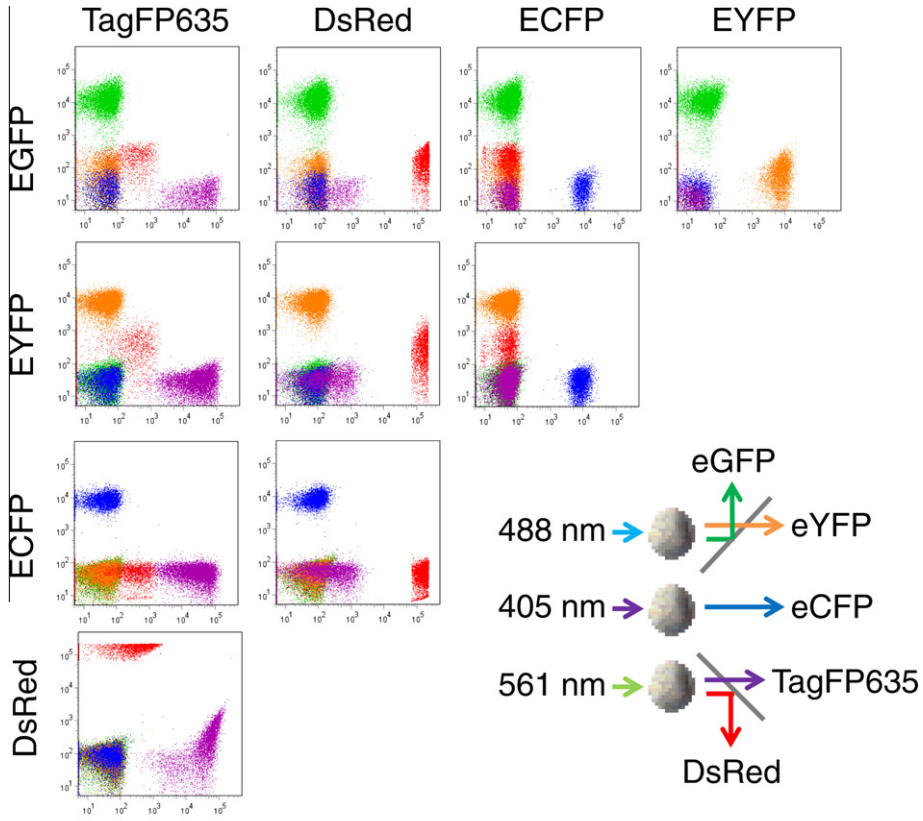
Fig. 8. Simultaneous analysis of EGFP, ECFP and red fluorescent proteins. (a) Mixture of wild type, DsRed2 and TurboFP635 (Katushka) expressing *E. coli* analyzed on a BD LSR II using 561 nm excitation for both proteins. DsRed2 was detected with a 610/20 nm, filter, TurboFP635 with a 660/20 nm filter, split with a 635 LP dichroic. Compensation values are shown. (b) Filter configuration for DsRed2 and TurboFP635 detection on a BD LSR II. (c) Mixture of wild type, EGFP, ECFP, DsRed and mKate expressing SP2/0 cells analyzed on a BD LSR II with 488 nm excitation of EGFP, and spatially separated 405 nm excitation of ECFP and 561 nm excitation of DsRed and mKate. Filters were EGFP = 510/21 nm, ECFP = 450/50 nm, DsRed = 610/20 nm and mKate = 660/20 nm, with a 635 LP dichroic to separate the red fluorescent proteins.

3.9. Long red fluorescent proteins

Recent advances in fluorescent protein isolation and technology have given us a series of "long" red fluorescent proteins that are approaching the characteristic of being efficiently excited by a true red laser. TagFP635 (mKate), TurboFP635 (Katushka), mKate2, mPlum and HcRed approach this characteristic, but are all poorly excited at 633 nm. More recently isolated FPs including mNeptune (λEX = 600 nm, λEX = 650 nm), E2 Crimson (λEX = 600 nm, λEX = 649 nm), TagRFP657 (λEX = 611 nm, λEX = 657 nm) and eqFP670 (λEX = 615 nm, λEX = 670 nm) all fall into this category [26–31]. Like the "short" long red proteins, these FPs have great potential for imaging applications, with their emission in a region of low autofluorescence, and their ability to penetrate thicker tissues. For flow cytometry, they are most optimally excited by an orange laser as described above. However, they also show some excitation in the red range. In Fig. 7, cells expressing the indicated fluorescent protein were analyzed with a 561 nm, a novel 628 nm fiber laser (with slightly shorter emission than a traditional red HeNe), and a 642 nm red laser diode. Although not as good as observed with 561 nm excitation, the red lasers did excite E2 Crimson, TagRFP657 and eqFP670 at roughly 40% at the 561 nm level. These very long red proteins can therefore be excited with a red laser if instrument limitations dictate. They also demonstrate progress toward the development of red FPs with even longer red and near-infrared excitation and emission properties. For all of these proteins, the same APC filter used for mKate above (660/20 nm) is appropriate.

3.10. Combining short and long red fluorescent proteins with EGFP, EYFP and ECFP

Short and long red fluorescent proteins can be paired for two color analysis with the appropriate laser source, usually a



	DsRed	TagFP635	GFP	YFP	CFP
DsRed	1.00000	0.23612	0.00031	0.02514	-0.00002
TagFP635	0.33112	1.00000	0.00030	0.00389	0.00028
GFP	0.00003	-0.00048	1.00000	0.11999	0.00272
YFP	0.03351	0.00237	1.03973	1.00000	0.00111
CFP	-0.00021	-0.00015	0.06462	0.01674	1.00000

Fig. 9. Simultaneous analysis of EGFP, EYFP, ECFP and red fluorescent proteins. Mixture of wild type, EGFP, EYFP, ECFP, DsRed and mKate expressing SP2/0 cells analyzed on a BD LSR II with 488 nm excitation of EGFP and EYFP, and spatially separated 405 nm excitation of ECFP and 561 nm excitation of DsRed and mKate. Filters were EGFP = 510/21 nm and EYFP = 550/30 nm with 540 LP to separate EGPF and EYFP signals, ECFP = 450/50 nm, and DsRed = 610/20 nm and mKate = 660/20 nm, with a 635 LP dichroic to separate the red fluorescent proteins. Compensation values for all five fluorescent proteins are shown, with high values boxed.

561 nm yellow laser. In Fig. 8a, the filter configuration for simultaneous analysis of DsRed and TurboFP635 (Katushka) is shown, namely a 590/20 nm filter for Dsred and a 660/20 nm filter for TurboFP635, with a 635 nm longpass dichroic mirror separating the signals. DsRed and TurboFP635 signals can be readily detected and separated with reasonable levels of compensation (Fig. 8b). EGFP and ECFP can be readily added to the analysis with 488 and violet excitation, respectively (Fig. 8c). EYFP can then be added, either using 488 nm as the excitation source, or with a green 532 nm laser if the analyzing instrument is so equipped. A typical five color analysis using three lasers is shown in Fig. 9. The compensation matrix is shown at the bottom of the figure. The primary compensation issues in this five color analysis were between TagFP635 (mKate) and DsRed, and between EGFP and EYFP; however, levels were adequate for good signal separation. Addition of additional longer red fluorescent proteins is not practical due to the close proximity of emission wavelengths between them and mKate; however, longer red and near infrared fluorescent proteins may be able to achieve larger numbers of colors.

4. Discussion

Choosing the correct lasers and filter configurations for fluorescent protein analysis is like designing the detection optics for any fluorescent probe or probes. The excitation wavelength should be as close to the excitation maxima as possible, although the broad excitation curves for fluorescent proteins allows some flexibility in laser choice. The recent trend of adding multiple lasers to benchtop flow cytometers and sorters has proven to be immensely useful in fluorescent protein analysis; fewer compromises in excitation conditions are required, and the proteins(s) in question can be excited under nearly optimal conditions. Green 532 nm and yellow 561 nm lasers are particularly common additions to modern flow cytometers, and are almost ideal excitation sources for all but

the longest red fluorescent proteins [34,35]. Orange 592–594 nm lasers are becoming increasingly common on multi-laser instruments and optimally accommodate the longest reds, although a 561 nm laser will usually work too [48,49].

Detection optics should also be matched as carefully as possible to the emission spectra of the proteins. The precise specifications of the filters will be dependent on the number of fluorescent parameters to be analyzed. For example, if single color EYFP detection is needed, using a bandpass filter with a very broad window (i.e. a 560/50 nm) will maximize sensitivity. If EGFP and Dsred are to be simultaneously measured, then a filter with a narrow window (a 550/30 nm, for example) will probably necessary to prevent signal spillover.

Although less common in flow cytometry, photoactivatable fluorescent proteins (i.e. PA-GFP, PA-mCherry, etc.) are often used in imaging work and occasionally need to be verified by cytometry. These proteins generally need to be photoactivated using an ultraviolet light source, and can emit at one or more wavelengths depending on their activation state [50]. They can usually be detected by flow cytometry, but usually cannot be activated by the brief exposure to light that flow cytometers afford. Fluorescence resonance energy transfer (FRET) is another technique involving at least two donor and acceptor fluorescent proteins where imaging is the dominant mode of data collection, but where flow cytometry is periodically required. Physiological sensors based on fluorescent proteins are also common in imaging, and may make appearances in flow cytometry as well [51]. For proteins used in imaging, the optics required for microscopy are usually a good guide for what is required in flow cytometry. The development of new fluorescent proteins continues as a dramatic pace, and improvements in both the utilization of current reagents and the development of new ones is constant [54–56]. For example, near-infrared fluorescent proteins have been reported for imaging and may soon see use in cytometry as well.

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