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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