

“Rainbow” Reporters for Multispectral Marking and Lineage Analysis of Hematopoietic Stem Cells

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ABSTRACT

Hematologic diseases potentially benefiting from gene-based therapies involving hematopoietic stem cells (HSCs) include hereditary hemoglobinopathies, immunodeficiency syndromes, and congenital bleeding disorders such as hemophilia A, as well as acquired diseases like AIDS. Successful treatment of these blood diseases with gene-modified HSCs requires high efficiency gene delivery to the target cell population and persistence of transgene expression following differentiation. We review flow cytometric procedures that permit simultaneous, noninvasive measurements of

transgene expression and phenotypic discrimination of hematopoietic cell subsets. Central to this approach has been the recent development of a spectrum of blue, cyan, and yellowish-green fluorescent reporters based on the jellyfish *Aequorea victoria* green fluorescent protein and the discovery of a red fluorescent protein in *Discosoma* coral. This methodology should facilitate the optimization of oncoretroviral and lentiviral vectorology and HSC transduction protocols for the ultimate purpose of HSC-directed gene therapy. *Stem Cells* 2001;19:118-124

INTRODUCTION

Hematopoietic stem cells (HSCs) are attractive targets for gene therapy because of their capacity for self-renewal and ability to replenish mature blood cell populations continuously throughout life [1, 2]. Sustained expression of transgenes at clinically relevant levels in the progeny of HSCs would provide novel and potentially curative treatments for a variety of inherited hematologic and immune disorders [3]. Indeed, multiple incremental improvements

in HSC-targeted oncoretroviral vector-mediated gene transduction protocols over the past few years culminated recently in the correction of severe combined immunodeficiency (SCID) disease in four infants [4]. However, continued advances in gene transfer technology are necessary if the inherent promise of HSC-directed gene therapy is to be fully realized. Ongoing efforts are focused on modifying oncoretroviral vector designs and pseudotyping with alternative envelope proteins [5]. In addition, because of their

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ability to transduce nondivided cells, safety-modified human immunodeficiency virus-based lentiviral vectors have emerged as promising tools for gene modification of HSCs, which reside primarily in the G_0/G_1 phase of the cell cycle [6, 7]. Irrespective of these advances, accumulated data indicate that stably integrated transgenes are frequently subject to position-effect variegation and extinction of expression [8]. Therefore, the extent to which genetic control elements such as chromatin domain insulators and scaffold/matrix attachment regions will result in enhanced probability and level of transgene expression is also under active investigation [9]. In order to accurately measure transduction of HSCs and conveniently evaluate stability of transgene expression in their differentiated progeny, reporter genes are routinely incorporated into the vectors under study.

DRUG RESISTANCE GENES, INTRACELLULAR ENZYMES, AND CELL SURFACE PROTEINS

Traditionally, genes encoding resistance to antibiotics such as neomycin, hygromycin, and puromycin have been used as selectable marker genes in oncoretroviral vectors [10]. Although antibiotic drug resistance is a useful way to estimate gene transfer and expression in colony-forming cells derived from transduced hematopoietic progenitors, the drug concentrations required to reduce the survival of the nontransduced cells to 1% of the initial number frequently inhibit progenitor cell proliferation and/or differentiation [11]. Moreover, it is difficult to measure the efficiency of functional gene delivery into more primitive hematopoietic precursors—i.e., long-term repopulating HSCs which are not clonogenic—using selection schemes based on antibiotic drug resistance. An alternative strategy has involved the use of genes encoding intracellular enzymes like β -galactosidase and in situ staining methods involving chromogenic or fluorogenic substrates [12, 13]. However, these markers have some intrinsic limitations. Histochemical-based detection of enzymatic activity generally requires tissue fixation, precluding subsequent biological analyses of the gene-modified cells, and there is false-positive background staining in some hematopoietic cell types due to expression of related cellular enzymes (e.g., lysosomal β -galactosidase activity in macrophages). Whereas vital staining procedures maintain cell function, there is a requirement to transport sufficient concentrations of fluorogenic substrates across cell membranes and retain the cleaved fluorescent products intracellularly. Genes encoding cell-surface proteins, including murine CD24 and the low-affinity human nerve growth factor receptor, have also been utilized to enumerate gene transfer efficiency and mark HSCs [14, 15]. But a potential disadvantage of this

approach includes the possibility of changing the biological properties of the transduced HSCs due to aberrant triggering of signal transduction pathways or alteration of their homing properties.

AEQUOREA GREEN FLUORESCENT PROTEIN

We and others have demonstrated that variants of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* are sensitive and convenient vital markers to monitor oncoretroviral and lentiviral vector-mediated transduction and maintenance of transgene expression in HSCs [7, 16-19]. Unlike other bioluminescent reporters, the chromophore in GFP is intrinsic to the primary structure of the protein, and GFP fluorescence does not require substrates or cofactors [20]. Wild-type GFP emits green light ($Em\lambda_{max} = 510$ nm) when excited by an ultraviolet source ($Ex\lambda_{max} = 395$ nm) or blue light ($Ex\lambda_{min} = 470$ nm). The minimal chromophore of GFP responsible for light absorption is located within a hexapeptide at amino acids 64 through 69 where cyclization of three of these residues, serine-65, tyrosine-66, and glycine-67 by an autocatalytic reaction, and oxidation of tyrosine to dehydrotyrosine, generates a *p*-hydroxybenzylideneimidazolidinone fluorophore. Wild-type GFP has several undesirable properties including low fluorescent intensity when excited by blue light at the minor 470 nm peak, a significant lag in the development of fluorescence after protein synthesis, complex photoisomerization, and poor expression in mammalian cells [21].

To improve upon these properties, mutagenic strategies yielded early generation GFP molecules such as GFP-S65T (containing a mutation that converts serine-65 to threonine) and RSGFP4 (containing mutations that convert phenylalanine-64 to methionine, serine-65 to glycine, and glutamine-69 to leucine) variants, which have excitation maxima that are red-shifted ($Ex\lambda_{max} = 490$ nm), fluorescein-like fluorescence spectra ($Em\lambda_{max} = 510$ nm), and brighter chromophores than wild-type GFP [22, 23]. Our group, in collaboration with *Linzhaio Cheng* and colleagues, was among the first to demonstrate that oncoretroviral vectors carrying these variant GFP genes produce sufficient fluorescent signals following transduction of mammalian cells to be detected by flow cytometric analysis [16].

Although substantial improvement in fluorescence was achieved with the GFP-S65T or RSGFP4 proteins (e.g., the GFP-S65T protein is up to 18-fold brighter than wild-type GFP when excited at 488 nm), the fluorescent signals from these GFP variants in stably transduced cells were not always well above background levels of cellular autofluorescence. Subsequently, an enhanced GFP (EGFP) gene was developed based on the GFPmut1 fluorescein-like mutant (which, in addition to the serine-65 to threonine

mutation, contains a second mutation that converts phenylalanine-64 to leucine) [24]. GFPmut1 exhibits \approx fourfold brighter fluorescence relative to GFP-S65T, and displays more efficient protein folding and improved solubility compared to the wild-type protein. The EGFP gene is a synthetic "humanized" derivative of the GFPmut1 gene containing a Kozak consensus translation initiation site and more than 190 silent base mutations which create an open reading frame containing codons preferentially found in highly expressed human proteins [25]. As a result of these combinatorial changes, EGFP exhibits over 300-fold brighter fluorescence relative to wild-type GFP when excited by blue light ($E_{\lambda\max} = 488 \text{ nm}$; $E_{m\lambda\max} = 507 \text{ nm}$). Using the MGIN oncoretroviral vector [17], which is based on the MSCV (murine stem cell virus) vector platform developed in our laboratory [10], EGFP transgene expression was readily detected in gene-modified human $CD34^+Lin^-$ hematopoietic progenitors by fluorescence microscopy (Fig. 1A). EGFP fluorescence also facilitates the accurate assessment of the percentage of vector-expressing progeny cells following establishment of human hematopoiesis in sublethally irradiated nonobese diabetic/SCID (NOD/SCID) mice. Figure 1B illustrates long-

term EGFP expression in human $CD45^+$ hematopoietic cells recovered from the bone marrow of a NOD/SCID mouse after transplantation with human $CD34^+$ cord blood progenitors transduced with a new self-inactivating lentiviral vector [7]. Flow cytometric analysis demonstrated that 18% of the human $CD45^+$ hematopoietic cell population continued to express the EGFP transgene at a high level.

SPECTRAL VARIANTS OF GFP AND CORAL RED FLUORESCENT PROTEIN

A number of spectral variants of GFP have been created that can be used in combination with EGFP for analysis of hematopoietic cells by multiparameter flow cytometric methodology [26-31]. Among these are several commercially available versions which incorporate the human codon-optimized open reading frame of EGFP: a blue fluorescent protein, EBFP (containing mutations that convert phenylalanine-64 to leucine, serine-65 to threonine, tyrosine-66 to histidine, and tyrosine-145 to phenylalanine); a blue-shifted emission variant termed enhanced cyan fluorescent protein ([ECFP] containing mutations that convert phenylalanine-64 to leucine, serine-65 to threonine, tyrosine-66 to tryptophan, asparagine-146 to isoleucine,

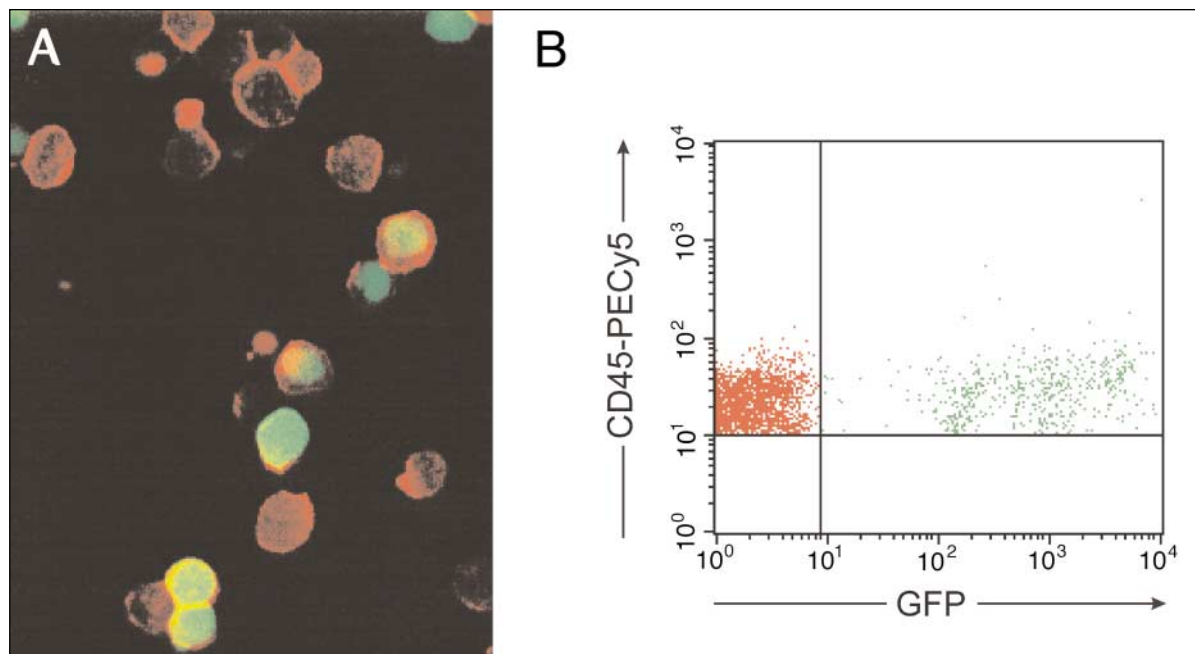


Figure 1. Fluorescence microscopy and flow cytometric analysis of EGFP expression in transduced human hematopoietic cells. (A) Highly purified $CD34^+Lin^-$ human hematopoietic progenitors were stained with an anti- $CD34$ antibody conjugated to Texas Red four days after transduction with the MGIN oncoretroviral vector (Fig. 2A), and analyzed for $CD34$ (red) and EGFP (green) expression by confocal fluorescence microscopy. Reproduced in part by permission of Nature Publishing Group from Cheng et al. [17]. (B) High-level EGFP expression in human hematopoietic cells present in the bone marrow of a NOD/SCID mouse. Six weeks after receiving human $CD34^+$ cord blood progenitors transduced with a lentiviral vector in which EGFP expression is driven by an internal MSCV long terminal repeat, murine bone marrow was recovered, stained with an antihuman $CD45$ antibody conjugated to PE-Cy5, and analyzed for $CD45$ and EGFP expression by flow cytometry. Reproduced in part by permission of Academic Press from Ramezani et al. [7].

methionine-153 to threonine, and valine-163 to alanine); and a red-shifted emission variant referred to as enhanced yellow fluorescent protein ([EYFP] containing mutations that convert serine-65 to glycine, valine-68 to leucine, serine-72 to alanine, and threonine-203 to tyrosine) [28-31]. Of the blue-shifted emission variants, ECFP, which emits light at 475 nm (with a smaller emission peak at 501 nm), is superior to EBFP for most applications owing to a higher fluorescence quantum yield (0.40 versus 0.18); it is also more resistant to photobleaching than the latter because it is excited at a longer, less destructive wavelength ($Ex\lambda_{max} = 434$ nm versus $Ex\lambda_{max} = 380$ nm). EYFP emits a bright yellowish-green light ($Em\lambda_{max} = 527$ nm) upon excitation at 514 nm, which reflects a high fluorescence quantum yield (0.61) similar to that of EGFP (0.60). Due to overlapping excitation spectra, ECFP, EGFP, and EYFP can be analyzed simultaneously on a flow cytometer equipped with an argon ion laser tuned to 458 nm [31].

Recently, a red fluorescent protein gene with homology to GFP (drFP583) was cloned from *Discosoma* coral [32], and a humanized version of drFP583, called DsRed, is now available commercially. Although sharing only $\approx 30\%$ amino acid similarity with GFP, two of the crucial residues that contribute to the chromophore of GFP (tyrosine-66 and glycine-67) are conserved in DsRed (the corresponding amino residues are numbered tyrosine-67 and glycine-68). Notably, DsRed has an emission maximum ($Em\lambda_{max} = 583$ nm) that is shifted by more than 50 nm toward the red end of the spectrum in comparison with EYFP. When excited at its 558-nm absorbance maximum, fully matured DsRed has a fluorescence quantum yield of 0.7 and emits a brilliant red light that is similar to rhodamine dyes in terms of wavelength and brightness [33, 34].

The ability to introduce up to four fluorescent protein genes into HSCs provides the opportunity to simultaneously evaluate multiple variables—tissue sources, enrichment strategies, ex vivo culture conditions, transduction protocols, and vector platforms and configurations—in molecular and cellular biological investigations of HSC function and gene therapy modeling. Toward this goal, we have developed three- and four-color flow cytometry procedures to detect DsRed in conjunction with EYFP, EGFP, and ECFP [35]. Together with another recent report [36], our studies have demonstrated that: A) The DsRed signal is bright enough to permit detection from stably introduced transgenes in viable mammalian cells by fluorescence microscopic and flow cytometric methodology; B) sustained expression of DsRed is not toxic to the cells under the conditions employed, and C) the spectral properties of this red fluorescent protein are suitable for simultaneous multicolor flow cytometric analyses with the above GFP variants.

Visualization by fluorescence microscopy of the four fluorescent proteins in oncoretroviral vector producer cell lines is shown in Figure 2. Note that although the DsRed signal

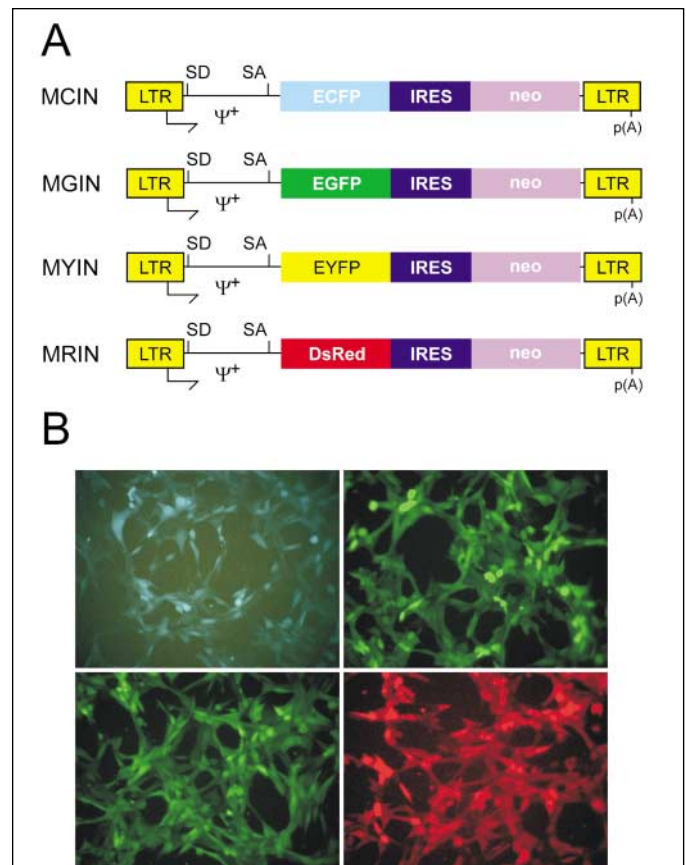


Figure 2. Oncoretroviral vectors for expression of ECFP, EGFP, EYFP, and DsRed transgenes in hematopoietic cells. (A) The MCIN, MGIN, MYIN, and MRIN oncoretroviral vectors contain the ECFP, EGFP, EYFP, and DsRed genes (Clontech Laboratories, Inc.; Palo Alto, CA; <http://www.clontech.com>), respectively, under the control of the MSCV long terminal repeat (LTR) on a bicistronic transcript that also contains a downstream neomycin resistance (*neo*) gene linked via an encephalomyocarditis virus internal ribosome entry site [17, 35]. Other abbreviations: SD = splice donor; ψ^+ = extended packaging signal; SA = splice acceptor; p(A) = polyadenylation site. (B) Stable producer cell lines expressing the MCIN, MGIN, MYIN, and MRIN oncoretroviral vectors were generated by transduction of GP+E-86 packaging cells followed by G418 selection (400 μ g/ml Geneticin; Life Technologies, Inc.; Gaithersburg, MD; <http://www.lifetech.com>) and/or cell sorting. GP+E-86 cells individually expressing ECFP (top left panel), EGFP (top right panel), EYFP (bottom left panel), and DsRed (bottom right panel) were analyzed by fluorescence microscopy using standard filter sets that closely matched the excitation and emission maxima of the respective fluorescent proteins. Images were collected on a Nikon Eclipse TE300 inverted fluorescence microscope (Nikon Inc.; Melville, NY) equipped with a 100 W mercury arc lamp and a Sony DKC-5000 Digital Photo Camera System (Sony Electronics Inc.; San Jose, CA). ECFP was detected with a BV-1A filter set (EX 435/10, DM 455, BA 470), EGFP and EYFP with a B-2E/C filter set (EX 465-495, DM 505, BA 515-555), and DsRed with a G-2A filter set (EX 510-560, DM 575, BA 590). Note that EGFP and EYFP fluorescence could not be distinguished with the B-2E/C filter set, which is normally used for fluorescein isothiocyanate-like emission. Adapted and reproduced in part by permission of Eaton Publishing from Hawley et al. [35].

can be discriminated reasonably well from the other fluorescent protein signals using standard filter sets, ECFP and EYFP emissions cannot be separated from EGFP fluorescence due to extensive spectral overlap. In Figure 3, we demonstrate that all four fluorescent protein signals can be successfully resolved in real time by multiparameter flow cytometric methodology.

CONCLUDING REMARKS

We have also described a protocol to distinguish DsRed fluorescence from that of R-phycoerythrin (PE)-Cy5 or PE-Cy7 antibody conjugates using single-laser excitation at 488 nm—a laser line that is widely available on flow cytometers—allowing multicolor analyses involving the EGFP/EYFP/DsRed combination to be performed with either of these fluorochromes [35]. It is important to point out, however, that DsRed in its present form has some shortcomings that limit its utility as an all-purpose reporter for gene transfer applications involving hematopoietic progenitor cells. Chief among these is a

slow and incomplete maturation process on the order of days which makes it less than ideal, for example, as an indicator of transduction efficiency in short-term gene expression assays [33, 34, 37]. Protein folding and chromophore formation occur more rapidly in EGFP (GFPmut1) than in wild-type GFP [22, 24]. Thus it is anticipated that the newly solved atomic resolution structure of DsRed will guide systematic mutagenesis efforts to isolate faster maturing variants of this red fluorescent protein [38]. In the interim, despite considerable spectral overlap, the most convenient fluorescent protein labels currently available are EGFP and EYFP. When used together with antibodies conjugated to fluorochromes such as PE-Cy5 and allophycocyanin, doubly transduced immunophenotypically defined hematopoietic progenitor cell subsets can be readily analyzed by four-color flow cytometry on most cell sorters and certain benchtop machines after only slight modifications to standard configurations [39]. On the other hand, for instruments capable of collecting more than four fluorescent signals and which are equipped with

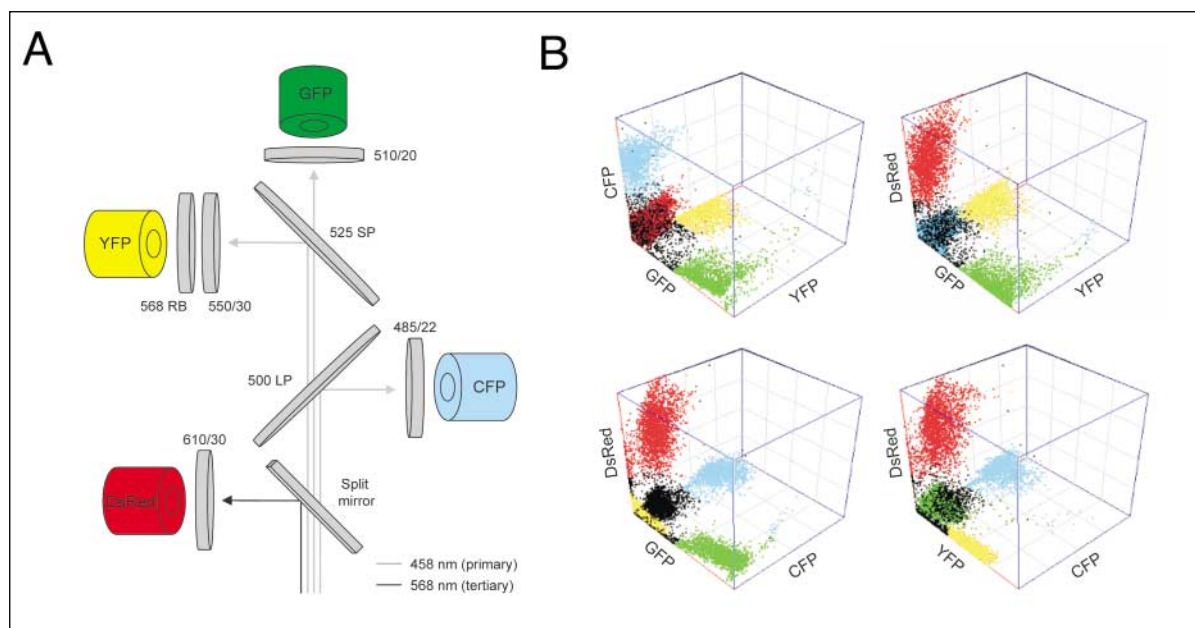


Figure 3. Real-time flow cytometric analysis of four fluorescent proteins. (A) Optical configuration for detection of ECFP, EGFP, EYFP, and DsRed using dual-laser excitation at 458 and 568 nm. Cells were analyzed on a FACSVantage SE (BD Biosciences; San Jose, CA; <http://www.bd.com>) equipped with Coherent I-90 argon-ion and Coherent I-302C krypton-ion lasers (Coherent Inc.; Santa Clara, CA). The primary argon-ion laser was tuned to 458 nm (100 mW) and the krypton-ion laser was tuned to 568 nm (35 mW). The three-beam separation option allowed the krypton-ion laser beam to be directed along the third laser pathway (tertiary position), maximizing spatial separation of the two beams, and minimizing both laser noise and crosstalk between the signals. Data were acquired using CELLQuest. The OmniCompensation option allowed real-time intra- and inter-laser compensations. The ECFP signal was split off from the EYFP/EGFP signals using a 500 nm long-pass (LP) dichroic filter and collected with a 485/22 nm bandpass filter. The EYFP and EGFP signals were separated with a 525 nm shortpass (SP) dichroic filter and collected with a 550/30 nm bandpass filter and a 510/20 nm bandpass filter, respectively. A 568 nm restriction band filter was placed in front of the 550/30 nm bandpass filter to block off stray laser light. The DsRed signal was collected with a 610/30 nm bandpass filter with a sharp cutoff at 595 nm. [35]. (B) Three-dimensional plots showing real-time detection of ECFP, EGFP, EYFP, and DsRed signals in a mixture of GP+E-86 cells individually expressing the MCIN, MGIN, MYIN, and MRIN oncoretroviral vectors (together with non-transduced parental GP+E-86 cells). Plots were generated using WinMDI v2.7 (J. Trotter, The Scripps Research Institute; La Jolla, CA).

Table 1. Sample fluorescent protein-surface fluorochrome combinations

| Fluorescent Proteins | Surface Fluorochromes | Excitation Lines (lasers) | Filters | Refs. |
|---------------------------|--|------------------------------------|--|-------------------|
| EBFP | N.D. ^a | 360 nm (Argon) | 405/20 nm BP | [30] |
| EGFP, EYFP | | 488 nm (Argon) | 525 nm SPD; 510/20 nm BP, 550/30 nm BP ^b | |
| EGFP, EYFP | PE-Cy5 APC | 488 nm (Argon) 635 nm (Diode) | 560 nm SPD; 510/20 nm BP, 550/30 nm BP, 650 nm LP 661/16 nm BP | [39] |
| ECFP, EGFP, EYFP | APC, APC-Cy7 ^c | 458 nm (Argon) 633 nm (HeNe) | 500 nm LPDi, 525 nm SPD; 480/30 nm BP, 510/20 nm BP, 550/30 nm BP 690 nm LPDi; 660/40 nm BP, 745 nm EFLP | [31] |
| EGFP, EYFP, DsRed | PE-Cy5 (or PE-Cy7) APC-Cy7 (or APC) ^d | 488 nm (Argon) 633 nm (HeNe) | 525 nm SPD; 560 nm SPD; 610 nm SPD; 510/20 nm BP, 550/30 nm BP 585/42 nm BP, 675/20 nm BP (or 745 nm EFLP) 745 nm EFLP (or 660/40 nm BP) | [35] |
| ECFP, EGFP, EYFP DsRed | N.D. | 458 nm (Argon) 568 nm (Krypton) | 500 nm LPDi, 525 nm SPD; 485/22 nm BP, 510/20 nm BP, 550/30 nm BP 610/30 nm BP | [35] ^e |

Abbreviations: APC = allophycocyanin; BP = bandpass; Cy = cyanine; Di = dichroic; EFLP = longpass edge filter; HeNe = helium neon; LP = longpass; N.D. = not done; PE = R-phycoerythrin; SP = shortpass.

^aFluorochromes that can be excited by a 360 nm laser line include Alexa Fluor 350 and Marina Blue.

^bGFP/YFP Flow Set (XF500; Omega Optical Inc.; Brattleboro, VT).

^cPossible additions to referenced method. Other fluorochromes that can be excited with a 633 nm HeNe laser include Alexa Fluor 633, APC-Cy5.5, CryptoFluor-1, CryptoFluor-2, Cy5, and PBXL-3.

^dPossible additions to referenced method. Note that the availability of a 407 nm violet-enhanced krypton laser would allow the use of fluorochromes such as Cascade Blue, Cascade Yellow, Lucifer Yellow, and Alexa Fluor 430. Detailed information on potential surface fluorochrome combinations that can be used for multicolor flow cytometric analyses is provided by *Baumgarth et al.* [40].

^eFigure 3.

more versatile lasers, the introduction of novel fluorochromes plus improvements in spectral compensation hardware make it feasible to significantly extend this scenario [40]. Examples

of some potentially useful fluorescent protein-surface fluorochrome combinations for HSC marking and fate mapping are provided in Table 1.

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