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A Simple and Effective Method for Differentiating GFP and YFP by Flow Cytometry using the Violet Laser

To the Editor:

GFP and YFP are fluorescent proteins that are widely used in various cellular assays (1). The 488-nm blue laser, that is standard on virtually all flow cytometers, efficiently excites both proteins. GFP and YFP signals can be discriminated using a customized filter configuration involving a 550/30-nm band pass for YFP, a 510/20-nm band pass filter for GFP, with a 525-nm short-pass dichroic mirror between them (2). However, on most cytometers, GFP/YFP signals are detected using a single band pass filter (usually in the range 500–550 nm). The requirement for using a customized setup deters some investigators from using these fluorescent proteins in conjunction, and is a rate-limiting step for others. In addition, because this method relies on using 510/20-nm and 550/30-nm band pass filters instead of a 525/50-nm band pass filter, only a fraction of the photons emitted by GFP/YFP are detected, meaning sensitivity is suboptimal.

In recent years, 405-nm violet lasers have become standard on most flow cytometers, and these lasers are capable of exciting additional fluorochromes. The standard configuration of filters off the violet laser includes a band pass in the range of 500–550 nm, capable of detecting Amcyan, Quantum Dot 525, Brilliant Violet 510, and Aqua live/dead fixable stain. In this communication, we demonstrate that the 405-nm violet laser weakly excites GFP but not YFP, allowing discrimination between them using a 525/50-nm band pass filter.

NK cells from *Ncr1*/GFP and *Nkp46*^{iCRE}/*R26R*^{eYFP} mice express GFP and YFP, respectively (3,4). Discriminating between GFP and YFP would allow tracking distinct populations of NK cells that had been adoptively transferred to the same mouse, which could be very useful in multiple scenarios. Examining the excitation spectra of GFP, we noticed that the 405-nm violet laser caused the excitation of GFP, which could be detected using a 525/50-nm band pass filter off the 405-nm violet laser (the "prototypical" channel used for detecting the Amcyan/Aqua live/dead fixable stain). Indeed, a distinct signal could be detected in the "Amcyan" channel when *Ncr1*/GFP splenocytes were used, but a much lower or negligible signal was evident when *Nkp46-CRE/R26-YFP* splenocytes were used (Figs. 1a and 1b). Importantly, the GFP/YFP signals could still be discriminated when the cells were mixed together (Fig. 1c), providing evidence that GFP+ and YFP+ cells in a mixture can be separately analyzed or sorted using this method.

Concurrent use of GFP and YFP could be highly advantageous in many cellular assays, particularly when using cells of transgenic mice whose cells express fluorescent proteins, because generating mice with alternative fluorescent proteins can take a lot of time and effort. In this report, we demonstrated that the 405-nm violet laser excitation could be used to discriminate GFP and YFP signals. Using the violet laser to distinguish GFP and YFP has several advantages. First, the standard setup of the flow cytometer could be used, obviating the need for exchanging filters and dichroic mirrors. In the previous method the light coming off the blue 488-nm laser is partitioned into two channels corresponding to GFP and YFP. In our method the light is not partitioned, and a single channel (normally used for detecting FITC) is used to detect both proteins, allowing for detection of weaker signals. In addition, the previous method also required the use of an additional detector off the blue 488-nm laser, which must come at the expense another fluorochrome that is excited by this laser (such as PE, PE-TXRD, PE-Cy5, PE-Cy7, or Percp-Cy5.5). By contrast, our method exploits a channel (525/50-nm band pass filter off the 405-nm laser), for which far fewer

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Correspondence to: David H. Raulet; Department of Molecular Cell Biology, University of California at Berkeley, Berkeley, CA 94720-3200
E-mail: raulet@berkeley.edu

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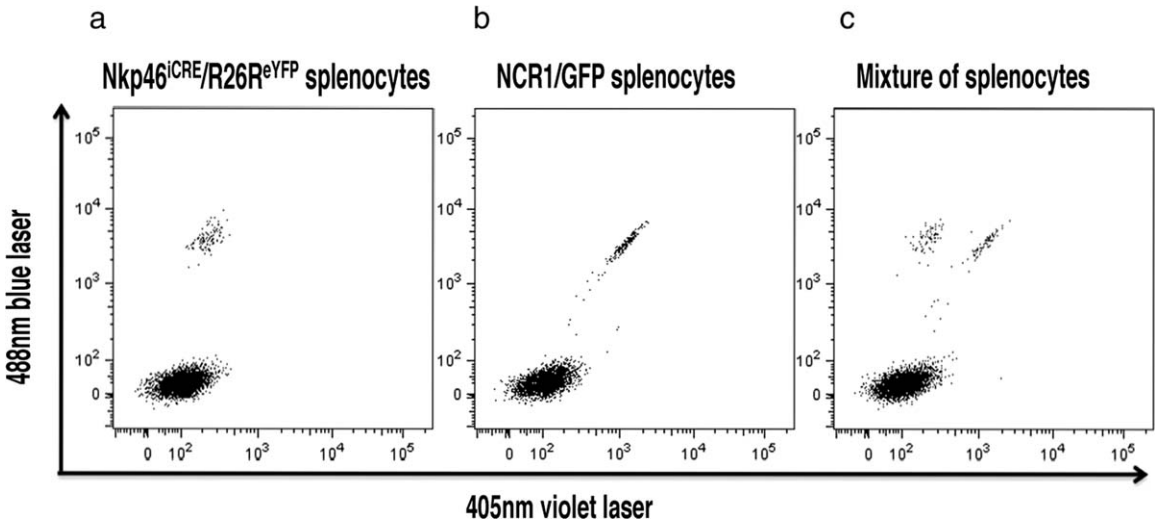

Figure 1. Splenocytes from (**a**) *Nkp46^{iCRE}/R26R^{eYFP},* (**b**) *Ncr1*/GFP, and (**c**) a mixture were analyzed on an LSR II Fortessa. The signals were detected with a 525/50BP off both the blue and violet lasers.

commercial antibody conjugates are available. Taken together, these results demonstrate a simple and effective method for discriminating GFP and YFP, which does not require a customized setup and allows for increased sensitivity. We predict that this method will be useful for discriminating GFP+ and YFP+ cells in many types of experiments.

Assaf Marcus and David H. Raulet*
Department of Molecular Cell Biology, University of California at Berkeley, Berkeley, California 94720-3200

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