New applications for Vybrant® DyeCycle™ stains

IDENTIFYING STEM CELL SIDE POPULATIONS AND CELL CYCLE–BASED SORTING.

Live-cell studies of cellular DNA content and cell cycle distribution are useful to detect variations of growth patterns due to a variety of physical, chemical, or biological means, to monitor apoptosis, and to study tumor behavior and suppressor gene mechanisms. The Vybrant® DyeCycle™ stains, available as Vybrant® DyeCycle™ Violet stain, Vybrant® DyeCycle™ Green stain, and Vybrant® DyeCycle™ Orange stain, were designed to report DNA content in living cells. All Vybrant® DyeCycle™ stains are DNA selective, cell membrane permeant, and nonfluorescent until bound to double-stranded DNA. These dyes take advantage of the commonly available 488 nm and violet excitation sources, placing cell cycle studies within reach of all flow cytometrists.

Recent studies have shown the utility of these dyes in two new application areas:

- identification of stem cell side populations (SP) [1] (Figure 1)
- sorting potential based on cell cycle phase (Figure 2)

Identification of Hoechst side population using DyeCycle™ Violet stain

Hoechst 33342 is widely used as a reagent to identify stem cells and early progenitors in mammalian hematopoietic tissues. After loading a cell population with Hoechst 33342, a cell-permeant nucleic acid stain, the side population (SP) containing stem cells and early progenitors is identified in the cytometer as a low-fluorescence "tail" (arising as the dye is pumped out of the cell via an ABCG2 membrane pump–dependent mechanism). This technique requires a flow cytometer equipped with a UV laser, an uncommon and relatively costly option. As violet diode lasers become more prevalent secondary excitation sources on flow cytometers, the opportunity arises for performing this technique using a violet laser–excited cell-permeant nucleic acid stain. DyeCycle™ Violet stain exhibits the same pump specificity as Hoechst 33342 while being excited by the violet laser.

With DyeCycle™ Violet stain in mouse hematopoietic cells, side populations similar to those observed with Hoechst 33342 can be resolved using either violet or UV excitation (Figure 1). Fumitremorgin C, an ABCG2-specific inhibitor, blocked the appearance of this cell population. Further characterization of the cells by immunophenotyping using mouse bone marrow stem cell markers confirmed that the identified DyeCycle™ Violet stain SP is restricted to the stem cell LSK population (Lineage^{negative} Sca-1^{positive} c-kit^{positive}), similar to the Hoechst 33342 SP. These results strongly suggest that DyeCycle™ Violet stain efflux identified the same stem cell population as Hoechst 33342 efflux.* SP analysis on flow cytometers equipped with violet lasers should therefore be possible by substituting DyeCycle™ Violet stain for Hoechst 33342.

* For details of this experiment, visit **www.invitrogen.com/flowcytometry** and follow the link under the poster entitled "Cell Cycle Analysis in Live Cells Using Novel Vybrant® DyeCycle™ Stains."

Cell sorting using Vybrant® DyeCycle™ stains

Flow cytometry testing is useful for quantifying the distribution of cell populations across the different nuclear phases of the cell cycle. Analysis of the cell cycle is widely used in cell growth and cell cycle regulation studies, oncology research, and DNA ploidy determinations. These applications require dyes that bind to DNA in a stoichiometric manner. With the exceptions of UV-excited dyes such as Hoechst 33342, cells have generally required fixation and permeabilization as well as treatment with RNase to obtain DNA-specific cell cycle information. The Vybrant® DyeCycle™ stains are DNA-selective, cell membrane–permeant dyes that show greatly enhanced fluorescence when bound to DNA and are available in versions that can be excited by 405 nm, 488 nm, or 532 nm lasers. Cell cycle analysis using the Vybrant® DyeCycle™ stains has been performed on a wide variety of cells, including Jurkat, CHO, 3T3, and HL60 cells, and peripheral blood lymphocytes, monocytes, and neutrophils.

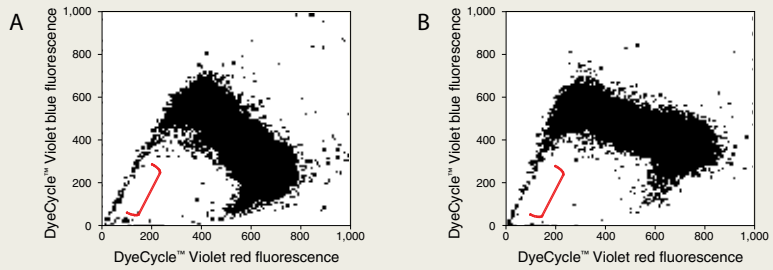

Figure 1—DyeCycle™ Violet stain side population analysis in human cord blood. Cells were incubated with 10 µM DyeCycle™ Violet stain for 90 minutes at 37°C, then washed and stored on ice until analysis. Results were the same using UV excitation (A) or violet diode laser excitation (B). Data courtesy of William Telford, NIH.

Because DyeCycle™ dyes can be used for cell cycle analysis on living cells, experiments were performed to determine whether these dyes could be used to sort cell populations based on their position within the cell cycle. HEK and NIH3T3 cells were stained with DyeCycle™ Violet and DyeCycle™ Orange stains, respectively, then sorted using a BD FACSVantage™ flow cytometer. Figure 2 shows NIH3T3 cells stained with DyeCycle™ Orange stain. While the Vybrant® DyeCycle™ stains caused some retardation of cell division, they did not produce the toxicity reported with DRAQ5™ stain (Biostatus Ltd.) and have been used to sort viable cells from G_0/G_1 and G_2/M populations. Resolution of cell cycle information in viable cells allows evaluation against the dynamic background of live-cell activity, as well as the possibility of cell sorting based on position in the cell cycle. ■

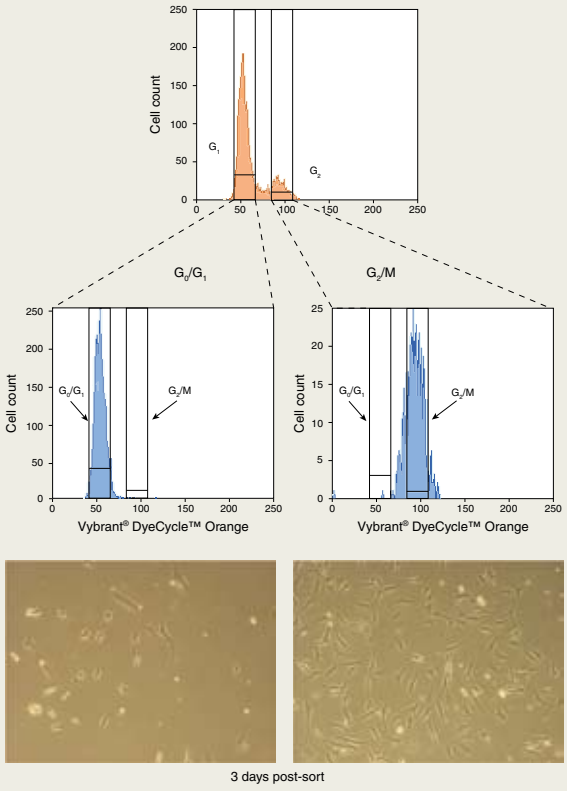

Figure 2—Sorting of live-cell populations. NIH3T3 cells were stained with 10 µM Vybrant® DyeCycle™ Orange stain. Cells were sorted based on G_0/G_1 and G_2/M gates using a FACSVantage™ flow cytometer (BD Biosciences) with 488 nm excitation and a 585/42 nm bandpass filter. Cells were cultured after sorting.

Reference

1. Telford, William G. et al. (2007) *Stem Cells* (in press).

Product	Excitation	Quantity	Cat. no.
Vybrant® DyeCycle™ Violet stain *5 mM in water* *200 assays*	UV, 405 nm	200 µl	V35003
Vybrant® DyeCycle™ Green stain *5 mM solution in DMSO* *200 assays*	488 nm	400 µl	V35004
Vybrant® DyeCycle™ Orange stain *5 mM solution in DMSO* *200 assays*	488 nm, 532 nm	400 µl	V35005

JOURNAL HIGHLIGHT

A new approach to cell-based multiplexing expands the drug screening capabilities of flow cytometry

Krutzik, P.O. and Nolan, G.P. (2006) Fluorescent Cell Barcoding in Flow Cytometry Allows High-Throughput Drug Screening and Signaling Profiling. *Nature Methods* 5:361–368.

Can high-throughput, high-content flow cytometry be economically applied to large-scale drug screening? Flow cytometry is a widely utilized and powerful method for the analysis of multiple antigens in cell populations. However, the use of flow cytometry in drug screening applications, which can involve hundreds or thousands of samples, can quickly become cost and time prohibitive, due to the amounts of antibodies required and the throughput limitations of cytometers. The authors present a cell-based multiplexing approach—fluorescent cell barcoding (FCB)—that uses varied staining intensities to allow the analysis of complex samples in a single flow cytometry run.

In this technique (based on standard phospho flow protocols), cell samples that have undergone an initial treatment (e.g., unstimulated, stimulated, and stimulated in the presence of an inhibitor drug candidate) are "barcoded" by general staining with different levels of a reactive fluorophore (Pacific Blue™, Alexa Fluor® 488, Alexa Fluor® 700, and Alexa Fluor® 750 fluorophores were all shown to be effective barcoding dyes). Following barcoding, the samples are then recombined, stained with fluorescently labeled antibodies to detect the effects of the treatment, and analyzed as a single sample. Deconvolution of the results clearly resolves the differentially treated cells into discrete, quantifiable populations. The authors successfully demonstrate the utility of the method for real-world drug screening applications. In an inhibitor-titration experiment using the Pacific Blue™ fluorophore as the barcoding dye, U937 monocyte cells were pretreated with four small-molecule inhibitors of JAK kinases, then stimulated to induce pStat1, pStat3, and pStat5 production. The effect of these inhibitors on the degree of phosphorylation of the three Stat transcription factors was clearly revealed in a single flow cytometry run.

In a separate experiment—a 96-well plate–based drug candidate screening application—the authors employed a three-dye FCB barcoding scheme to label 96 samples. They used this scheme to screen a library of 70 small-molecule inhibitors for their effect on T cell receptor–mediated ERK phosphorylation/Stat1 phosphorylation in response to interferon-γ (IFN-γ) treatment. This screening experiment was completed in a single 5 minute flow cytometry run, and identified two compounds that selectively inhibited one or the other pathway and three compounds that nonselectively affected both pathways.

Overall, the authors report up to 100-fold reduction in antibody consumption, with significantly less acquisition time required for complex sample analyses. Owing to its improved throughput and greatly reduced consumption of antibodies, the FCB methodology may prove useful for drug candidate screening as well as for clinical monitoring of patient samples during late-stage drug trials.

Product	Quantity	Cat. no.
Alexa Fluor® 488 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg	A20000
Alexa Fluor® 488 carboxylic acid, succinimidyl ester *mixed isomers*	5 mg	A20100
Alexa Fluor® 700 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg	A20010
Alexa Fluor® 700 carboxylic acid, succinimidyl ester *mixed isomers*	5 mg	A20110
Alexa Fluor® 750 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg	A20011
Alexa Fluor® 750 carboxylic acid, succinimidyl ester *mixed isomers*	5 mg	A20111
Pacific Blue™ succinimidyl ester	5 mg	P10163

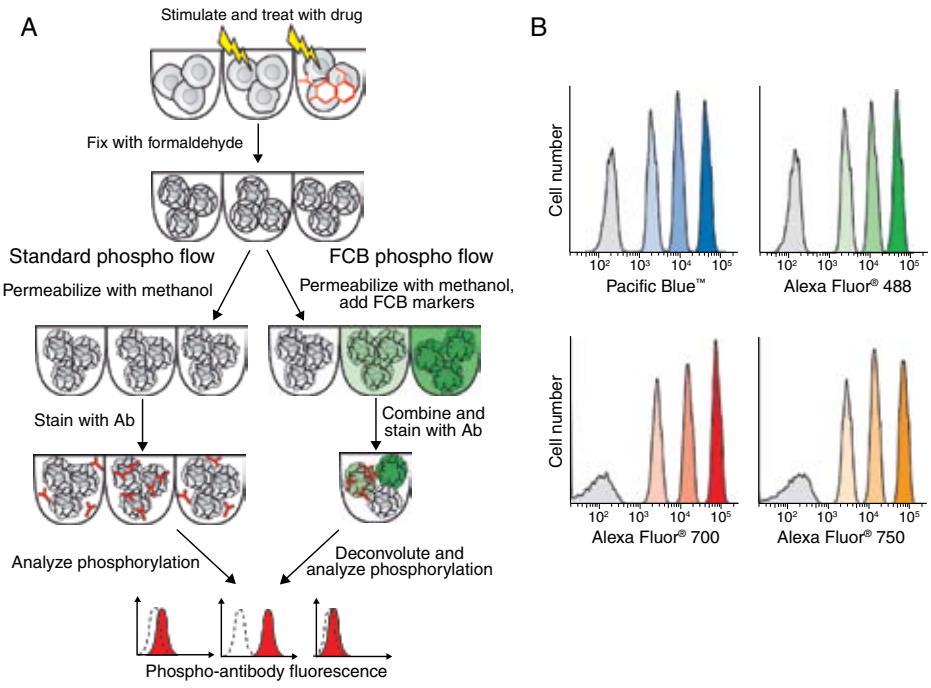

Figure 1—The fluorescent cell barcoding (FCB) technique. (A) Sample one was unstimulated, sample two was stimulated, and sample three was treated with a small-molecule inhibitor before stimulation. After fixation, cells in standard phospho flow (left) were permeabilized with cold methanol, washed, and stained with phospho-specific antibodies. In the FCS technique (right side), each sample was permeabilized with 20–25°C methanol containing a different concentration of amine-reactive fluorescent dyes (FCB markers), yielding a unique fluorescence signature for each sample. Samples were then washed, combined into one tube, and stained with antibodies. During software analysis of the acquired data, the samples were deconvoluted back to the original samples based on their FCB signature. In both standard and FCB phospho flow techniques, fluorescence of the phospho-specific antibody in each sample was measured. In the plots, dotted lines indicate autofluorescence and red histograms represent sample fluorescence. (B) Efficient labeling of four samples per marker with the FCB technique. U937 cells were fixed, then permeabilized in methanol containing 0, 0.04, 0.2, or 1 µg/ml Pacific Blue™–NHS, Alexa Fluor® 488–NHS, Alexa Fluor® 700–NHS, or 0, 0.4, 2, or 10 µg/ml Alexa Fluor® 750–NHS for 15 minutes at 20–25°C. After washing twice, samples stained with each FCB marker were combined and analyzed. Shown are histograms identifying the four original samples barcoded with each FCB marker. Gray peaks represent unlabeled samples (zero FCB marker). Colored peaks represent samples receiving low, medium, and high amounts of the FCB marker, with color intensity correlating to FCB marker staining level. Reprinted by permission from Macmillan Publishers, Ltd.: *Nature Methods* 3:361–368 (2005).