StarBright Dyes: Fluorophores with Superior Performance for Multiplexing in Flow Cytometry and Western Blotting

S. Sanderson, O. Rosenwasser, and M. Blundell
Bio-Rad Laboratories Inc., Endeavour House, Kidlington, OX5 1GE, UK

Abstract # 84

New StarBright™ Dyes from Bio-Rad are bright, fluorescent fluorophores ideal for many antibody-based applications, for example, western blotting and flow cytometry. Traditional fluorophores can be dim, have high cross laser excitation, or are incompatible with all protocols, leading to poor data. StarBright Dyes have been designed to address these pain points.

For flow cytometry, we have a full range of 32 StarBright Dyes excited by the most common laser lines: 355 nm, 405 nm, 488 nm, 561 nm, or 640 nm (Figure 1). They have narrow excitation and emission spectral profiles, which reduce compensation and spread. This, combined with their exceptional brightness (Figure 2), enables high resolution of cell populations (Figure 3). They work in all buffers and fixatives (Figure 4A and B), therefore including them in existing panels does not require a change in protocol. They can also be premixed and stored at 4ºC as a master mix for over six months with no loss in performance (Figure 4C). Premixes are ideal when using the same panel over an extended period, such as in clinical and longitudinal studies, to ensure reproducible results, also saving time and money.

Western blotting secondary antibodies are available conjugated to StarBright Blue 700 and StarBright Blue 520 Dyes. Their brightness, short exposure time, and low background make them ideal for detection of a single target (Figure 5), or multiplex detection of several proteins in combination with other common fluorophores (Figure 6).

StarBright UltraViolet, Violet, Blue, Yellow, and Red Dye Range for Flow Cytometry

The full range consists of 32 superior dyes excited by the most common laser lines; 355 nm, 405 nm, 488 nm, 561 nm, or 640 nm (Figure 1).

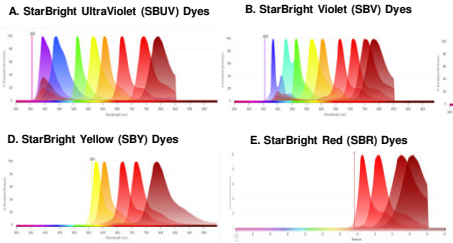

Fig. 1. Emission spectra for StarBright Dyes. StarBright Dyes excitable by **A,** the 355 nm laser, **B**. the 405 nm laser, **C**. the 488 nm laser **D,** the 561 nm laser, **E,** the 640 nm laser.

Materials and Methods

Flow cytometry staining conditions. Red blood cell lysed human peripheral blood was blocked with 10% human serum. Cells were incubated with an antibody panel or a single antibody, for compensation control tubes. Cells were stained in a 96-well plate for 1 hr at room temperature (RT), washed 3X in PBS + 1% BSA (PBS/BSA) and resuspended in PBS/BSA. A live/dead dye, DAPI (#1351303), was added 5 min prior to acquisition. To compare different buffers, cells were stained with CD4 (#MCA1267SBV515) in PBS/BSA, Bio-Rad Stain Buffer (#BUF073), or Brilliant Stain buffer (#00-4409, Thermo Fisher). To compare signal after fixation, cells were acquired fresh or after fixation with Bio-Rad Fixation Buffer (#BUF071), 2% paraformaldehyde, or 70% EtOH.

Flow cytometry multiplex panels. Antibodies used in the StarBright Dye and PE/PE tandem panel are shown in Table 1. Antibodies used in the premix panel are shown in Table 2. All antibodies in the panels were titrated to determine the optimal staining concentration prior to use.

Flow cytometry data collection and analysis. Cells were acquired on a 5-laser, 30-parameter ZE5 Cell Analyzer with option A, 355 nm laser upgrade (Bio-Rad). 300,000 cells were acquired for the multiplex panels and 60,000 cells for the single stained sample. Analysis was performed using FCS Express 7 Software. (De Novo Software by Dotmatics).

Western blotting. Cell lysate was mixed with Laemmli Buffer (#1610737) and heated at 95ºC for 5 min. The reduced cell lysates and protein ladder (#1610373) were separated on a Stain Free Criterion Gel (#5678125) at 300V until the dye reached the bottom of the gel. The gel was transferred onto nitrocellulose membrane (#1704159). Membranes were blocked with Caesin Blocking Buffer (#1610782) for 1 hr at RT. Blots were probed with a primary antibody for 1 hr at RT and washed 5 x 5 min in Tris buffered saline (#170-6435) with 0.1% Tween 20 (#161-0781) (TBST). Membranes were probed with a secondary antibody for 1 hr at RT and washed 5 x 5 min in TBST. The membrane was imaged directly when using a fluorescently conjugated secondary antibody or following incubation with Clarity Max Western ECL Substrate (#1705061) when using an HRP-conjugated secondary antibody. Images were acquired using a ChemiDoc MP Imaging System (Bio-Rad).

Table 1. Antibodies used in the multicolor StarBright Dye and PE/PE tandem panels

Laser	Filter	Marker	StarBright Panel	PE and PE Tandem Panel
488	525/35	CD20	MCA1710 A488	MCA1710 A488
488	692/52	CD19	MCA1940 SBB580	MCA1940 SBB580
488	692/80	CD45RA	MCA88 SBB675	MCA88 SBB675
561	582/30	CD4	MCA1267 SBY575	PE
561	616/24	CD3	MCA463 SBY605	PE-Dazzle 594
561	670/30	CD45RO	MCA461 SBY665	MCA461C PE-Cy5
561	728/30	CD45	MCA87 SBY720	MCA87 PE-Cy5.5
561	780 P	CD14	MCA1568 SBY820	MCA1568 PE-Cy7
640	670/30	CD8	MCA1226 A647	MCA1226 A647

AXXX, Alexa Fluor; PE, phycoerythrin; PE-Cy5, phycoerythrin-cyanine5; PE-Cy5.5, phycoerythrin-cyanine5.5; PE750, phycoerythrin Alexa Fluor 750; SBB, StarBright Blue; SBY, StarBright Yellow.

Table 2. Antibodies used in the multicolor premix panel

Marker	Fluorescent Dye	Bio-Rad Catalog number
CD14	SBUV400	MCA1568SBUV400
CD45RA	SBUV665	MCA88SBUV665
CD19	SBV440	MCA1940SBV440
CD45RO	SBV610	MCA461SBV610
CD4	SBB580	MCA1267SBB580
CD33	SBB675	MCA1271SBB675
CD8	SBB810	MCA1226SBB810
CD20	SBG525	MCA1710SBG525
CD3	SBG745	MCA463SBG745

SBB, StarBright Blue; SBY, StarBright Yellow. SBUV, StarBright UltraViolet; SBV, StarBright Violet..

Multiplexing with StarBright Dyes in Flow Cytometry

Challenges in Multiplexing in Flow Cytometry	Solution	StarBright Dyes
Cells hard to detect as not all dyes are bright	Bright dyes	✓
High levels of compensation and spreading	Narrow excitation and emission	✓
Amending workflow to incorporate special buffers	Dyes that work in any buffer	✓
Errors introduced when assembling panels	Premixing large panels for later use	✓
Inconsistent performance with beads or when fixed	Consistent dyes that generate reproducible data	✓

StarBright Dyes have been designed to be bright (Figure 2) with narrow excitation and emission profiles. This results in multiplexing data with clear, bright populations with low spread and high cell resolution (Figure 3).

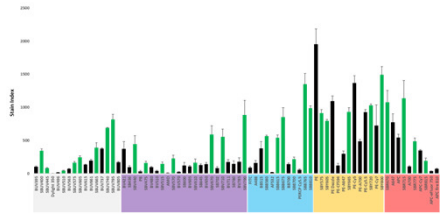

Fig. 2. Stain Index values of mouse anti-human CD4 conjugated to StarBright Dyes (green) and other fluorescent dyes (black). Red blood cell lysed human peripheral blood was stained with fluorescently labeled mouse anti-human CD4 and acquired on the ZE5 Cell Analyzer. Cells were gated on live, single cell lymphocytes and the stain index calculated. Data shown as 3 donors +/- SD.

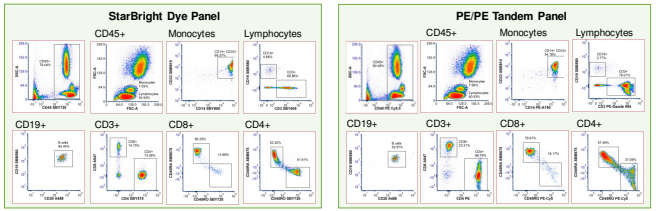

Fig. 3. Panels including StarBright Yellow Dyes have less spreading compared to a panel using alternative PE and PE-tandem dyes with similar excitation and emission max. Red blood cell lysed human peripheral blood was stained with the antibody panels (Table 1) and a live/dead dye and acquired on a ZE5 Cell Analyzer. Cells were gated on live, single cells.

StarBright Dyes are compatible with most protocols. They work in all buffers (Figure 4A), alcohol and PFA based fixatives (Figure 4B). For experiments requiring repeated measurements over several days, preparing and storing a mastermix for an extended period has clear advantages in terms of reproducibility, time and money. Figure 4C shows data from a panel containing nine StarBright Dye conjugated antibodies, that were stored for six months (premix), are comparable to a freshly prepared panel.

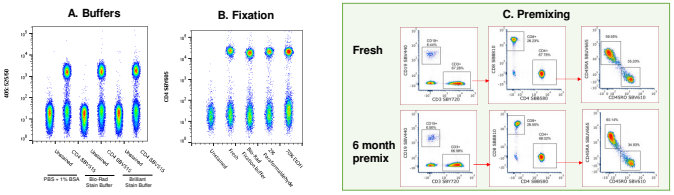

Fig. 4. Reproducibility of StarBright Dyes in different buffers, fixatives and premixes. Red blood cell lysed human peripheral blood was stained with antibodies conjugated to StarBright Dyes and acquired on a ZE5 Cell Analyzer. In **A,** different buffers **B,** different fixatives or, **C,** with a fresh or premixed antibody panel (Table 2) and a live/dead dye. Example plots shown, full data can be found at **bio-rad-antibodies.com/starbright-premix**

Multiplexing with StarBright Dyes in Western Blot

Challenges in Multiplexing in Western Blotting	Solution	StarBright Dyes
Sensitivity	Highly sensitive dyes; 2-4-fold lower limit compared to traditional fluorophores	✓
Long exposure time	Bright dyes requiring short exposure times	✓
High background	Low background	✓
Limited dye choice	More multiplexing options	✓
Compatibility with other fluorophores	Compatible with Bio-Rad's Stain-Free Gels, hFAB Rhodamine and antibodies labelled with traditional fluorophores	✓

StarBright Blue 520 and 700 conjugated secondary antibodies are available conjugated to goat anti-mouse IgG and goat anti-rabbit IgG for the western blotting application. They can detect low levels of protein after a shorter time than can be seen with chemiluminescence with minimal background (Figure 5). They are suitable for multiplexing and increase the choice of fluorophores (Figure 6).

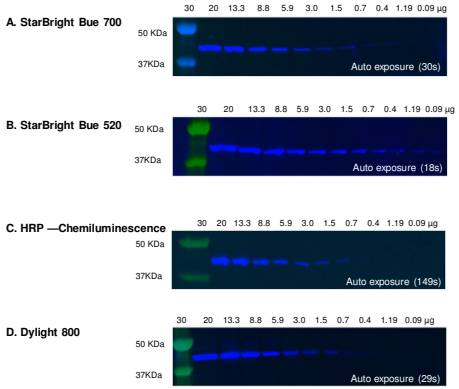

Fig 5. Western blots of anti-actin beta using different secondaries for detection. Western blot analysis of Hela lysates at decreasing dilutions (1.5 x serial dilutions) were probed with Anti-Actin Beta (#VMA00048) and detected with **A,** Goat Anti-Mouse IgG SBB700 (#12004158) **B,** Goat Anti-Mouse IgG SBB520 (#12005866) **C,** Goat Anti-Mouse IgG HRP (#STAR117P) or **D,** Goat Anti-Mouse IgG Dylight 800 (#STAR117D800GA).

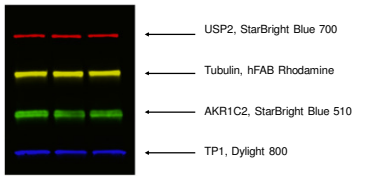

Fig 6. Western blot composite image of four proteins. In red USP2 (#VPA00475) detected with Goat Anti-Rabbit IgG SBB700 (#12004161) , in yellow Tubulin hFAB-Rhodamine (#12004165), in green AKR1C2 (#VMA00346) detected with Goat Anti-Mouse IgG SBB520 (#12005866), in blue TP1 (biotinylated) detected with DyLight 800 Streptavidin (#STAR152D800GA).

Conclusions

StarBright Dyes are ideal for multiplexing assays in both western blotting and flow cytometry and provide clear advantages over other common dyes currently being used.

BIO-RAD and STARBRIGHT are trademarks of Bio-Rad Laboratories, Inc. in certain jurisdictions.
All trademarks used herein are the property of their respective owner. © 2024 Bio-Rad Laboratories, Inc.

Bulletin Number 3660

Bio-Rad Laboratories, Inc. | Life Science Group | 2000 Alfred Nobel Drive | Hercules, CA 94547 USA