Detection of fluorescent proteins using the Attune NxT Flow Cytometer

Introduction

Since the discovery of Green Fluorescent Protein (GFP) in the 1960s, fluorescent proteins (FPs) have gained great prominence as tools for biological studies. Their role as reporters has added new dimensions to the analysis of protein function and localization. The use of FPs allows for real-time examination, in live cells, of proteins that previously had only been observed through immunocytochemical "snapshots" in fixed specimens. The utility of fluorescent proteins has expanded, and they are widely used in the investigation of gene expression as well as protein localization, translocation, and trafficking within live cells. More advanced techniques include assessment of protein–protein interactions and spatial relationships of proteins in live cells using fluorescence resonance energy transfer (FRET) techniques and fluorescence lifetime imaging microscopy (FLIM). In addition, far red–emitting FPs that exhibit emissions beyond typical autofluorescence wavelengths have proven useful for imaging of tissues. Even more recently, FPs have been used to assess intracellular topology in live cells using super-resolution localization imaging.

The quest for new FPs with unique excitation and emission profiles has been the focus of many distinguished laboratories, and mutation and evolution of such proteins has led to the creation of variants including the "fruit" FPs (e.g., tdTomato, mStrawberry, mCherry, mPlum, and

mRaspberry), specialized FPs such as the fluorescent timer protein Kusabira Green Orange, long–Stokes shift (LSS) fluorescent proteins such as LSS-mKate2, and photo-switchable FPs such as Dendra2 and mEos2. The large variety of fluorescent proteins opens up significant opportunities to increase the depth and intensity of cell analysis, but this also requires the proper tools and knowledge to do so. The Invitrogen™ Attune™ NxT Flow Cytometer was developed with fluorescent protein analysis in mind; it enables easy and accurate analysis of multiple fluorescent proteins simultaneously in the same cell. This application note discusses a variety of available fluorescent proteins, their advantages, and how the Attune NxT Flow Cytometer enables their detection.

Table 1. Spectral characteristics of fluorescent proteins commonly used in flow cytometry.

Fluorescent protein	Excitation max (nm)	Emission max (nm)	Channel on Attune NxT Flow Cytometer
Azurite, TagBFP, mTagBFP, mTagBFP2, Cerulean, ECFP, TagCFP, AmCyan	383, 400, 400, 400, 433, 439, 458, 458	450, 456, 456, 456, 475, 476, 480, 489	VL1 (440/50), VL2 (512/25)
T-Sapphire	399	511	VL3 (603/48)
LSS-mKate1, LSS-mKate2	463, 460	624, 605	BL1 (530/30 or 510/10*)
TurboGFP, EGFP, TagGFP, emerald GFP (emGFP)	482, 483, 484, 487	502, 506, 507, 509	BL1 (530/30 or 510/10*)
TagYFP, TurboYFP, EYFP, Topaz, Venus, Citrine	508, 508, 514, 514, 515, 517	524, 524, 527, 527, 528, 529	BL2 (574/26 or 590/40)† or (540/30)*
mKOm, mOrange, mOrange2, Kusabira Green Orange, E2 Orange	548, 548, 549, 548, 540	559, 562, 565, 561, 561	YL1 (585/16)
DsRed, DsRed2, DsRed-Express, tdTomato, TagRFP, mStrawberry, mCherry, mKate, mKate2, TurboFP635 (Katushka)	553, 553, 553, 554, 555, 574, 587, 588, 588, 588	583, 583, 584, 581, 584, 596, 610, 635, 633, 635	YL2 (620/15 or 615/25*)
mPlum, HcRed, mRaspberry, mNeptune, E2Crimson	590, 592, 598, 599, 611	649, 645, 625, 649, 646	YL3 (695/40)

*Bandpass emission filter used with the Invitrogen™ Attune™ NxT Fluorescent Protein Filter Kit (optional, Cat. No. 100022775). †The 574/26 filter set is standard on all Attune NxT instruments that are not configured with a yellow laser. The 590/40 filter set is included on Attune NxT instruments configured with the yellow laser.

Versatile tools for flow cytometry

The use of FPs became popular in the field of flow cytometry in the 1990s after a mutation of wild-type GFP resulted in a variant that is optimally excited by the 488 nm argon-ion laser common to most flow cytometers [1]. Early studies using FPs in flow cytometry included the quantitative detection and monitoring of gene expression in yeast [2], bacteria [3], and eukaryotic cells [4]. Today there are over 70 FPs available, of which approximately 56 are commonly used in flow cytometry and exhibit excitation wavelengths between 355 nm and 600 nm (Table 1). Cell biologists have advanced their research by taking advantage of this large set of choices of FPs, combined with the increased availability of alternate excitation sources such as the 405 nm, 532 nm, and 561 nm lasers, and developing multiparameter flow cytometry assays. These assays are designed to analyze cells with multiple labels, including FPs expressed in the cells as well as fluorescent antibody conjugates and functional probe reagents [5–8].

Detecting multiple fluorescent proteins using the Attune NxT Flow Cytometer

The simultaneous detection of multiple FPs in the same cell has traditionally been more difficult than the detection of multiple fluorophore-labeled antibodies. This is in part because FPs have a different, broader emission spectrum than the traditional cell dyes and fluorophores used in the labeling of antibodies. Recent advances in flow cytometry instrumentation, such as the increased number of lasers and corresponding filter sets in the Attune NxT Flow Cytometer, have alleviated most of these problems. This has resulted in the greater enablement of multiparametric single-cell analysis. The Attune NxT Flow Cytometer is ideally suited for the detection of multiple FPs and fluorescently labeled antibodies (separately or in combination), with configurations allowing up to 4 lasers and 16 detection channels (14 colors and 2 scatter channels). This markedly advances the field of single-cell analysis, as described in this application note.

Detecting additional FPs using various laser options

The modular design of the Attune NxT Flow Cytometer enables a choice of lasers, including the 488 nm laser for excitation of the most commonly used FP (EGFP) and its variants (emGFP, TurboGFP), and can be upgraded to include optional 405 nm, 561 nm, and 637 nm lasers. These additional lasers enable more choices for the use of combinations of fluorescent proteins and labeled antibodies (Figure 1). The 561 nm laser is particularly useful for exciting the orange- and red-fluorescent protein variants such as mCherry, mKate, and mOrange2. mCherry is a popular monomeric red-fluorescent protein that exhibits superior brightness and photostability [9]. mKate is known for its fast maturation rate as well as its high pH stability and photostability [10]. Finally, mOrange2 is a bright monomeric orange-fluorescent protein that expands the viable options. The 405 nm laser provides many options as well, such as excitement of TagBFP [11], a bright blue-fluorescent protein created by site-specific and random mutagenesis of TagRFP. The 405 nm laser can also be used to excite other FPs such as Azurite and T-Sapphire. Figure 1 shows detection of a variety of FPs using the Attune NxT Flow Cytometer.

Method for detecting multiple FPs on the Attune NxT Flow Cytometer

Many experiments today involve the simultaneous analysis of multiple FPs in the same cell, but as mentioned earlier, this can be problematic due to their broad emission spectrum and resulting spectral overlap. It is therefore important to understand the appropriate laser and filter set configurations

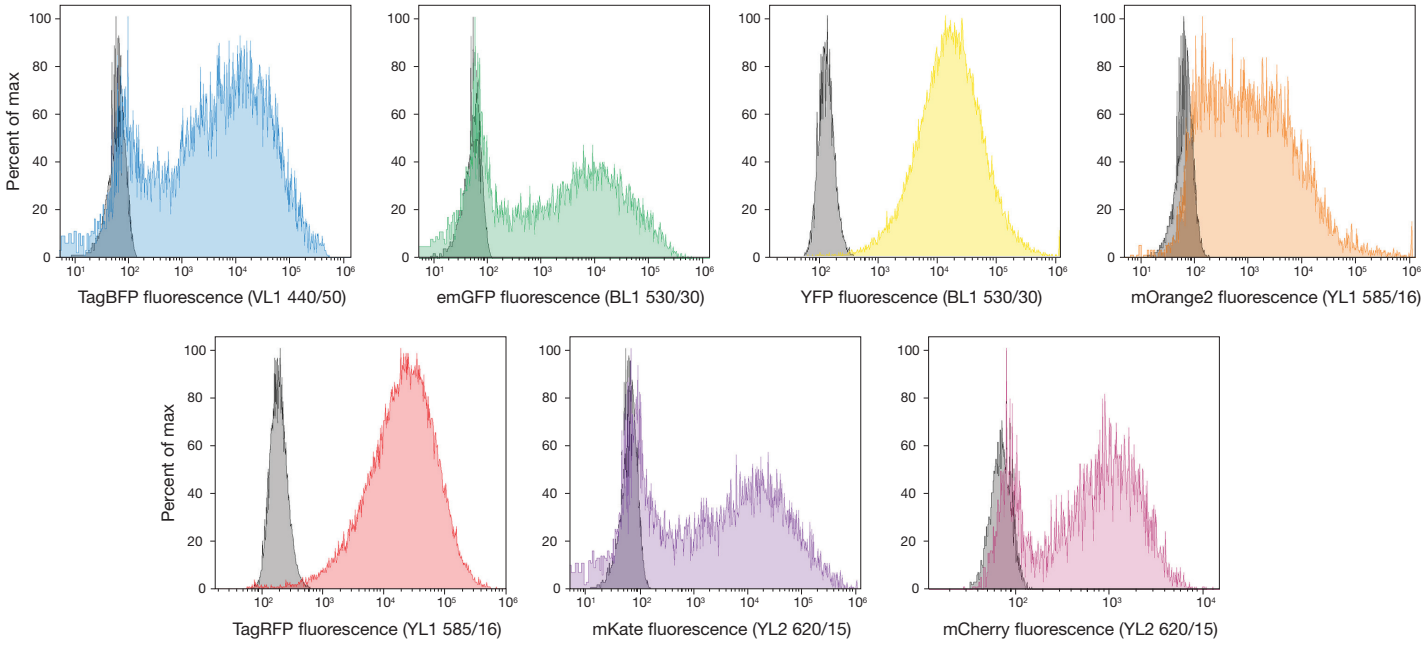

Figure 1. Detection of a palette of fluorescent proteins using the Attune NxT Flow Cytometer. Cells were transfected or transduced with vectors expressing different fluorescent proteins. Samples were acquired on the Attune NxT Flow Cytometer at a flow rate of 100 µL/min using 405 nm, 488 nm, or 561 nm excitation sources. The gray peaks represent control cells that do not express fluorescent proteins.

to use with each combination of FPs. Figure 2 demonstrates how experiments involving multiple FPs can be performed to achieve the clear separation of cells expressing one of the FPs, both FPs, or neither FP.

As an example of spectral overlap, GFP and YFP are two FPs that can both be excited and detected by the same set of laser and bandpass filters. This is important because GFP and YFP are both widely used. In this case, the 488 nm laser efficiently excites both of these FPs and both can also be detected with the 530/30 nm bandpass filter typical on most flow cytometers [12]. The simultaneous use of both of these FPs requires appropriate filter sets to accurately discriminate the signals coming from each FP. The GFP and YFP signals can be appropriately discriminated using the Invitrogen™ Attune™ NxT Fluorescent Protein Filter Kit. This is demonstrated in Figure 3. The full protocol for this experiment, using U2OS cells transfected with both Invitrogen™ CellLight™ Histone 2B-GFP and Premo™ Halide Sensor, is described below in the "Materials and methods" section.

In addition to target-specific FP experiments, FPs are also valuable tools for labeling organelles in the cell. CellLight™ and Premo™ reagents offer simple and efficient baculovirus-mediated (BacMam) delivery of genes for FP constructs

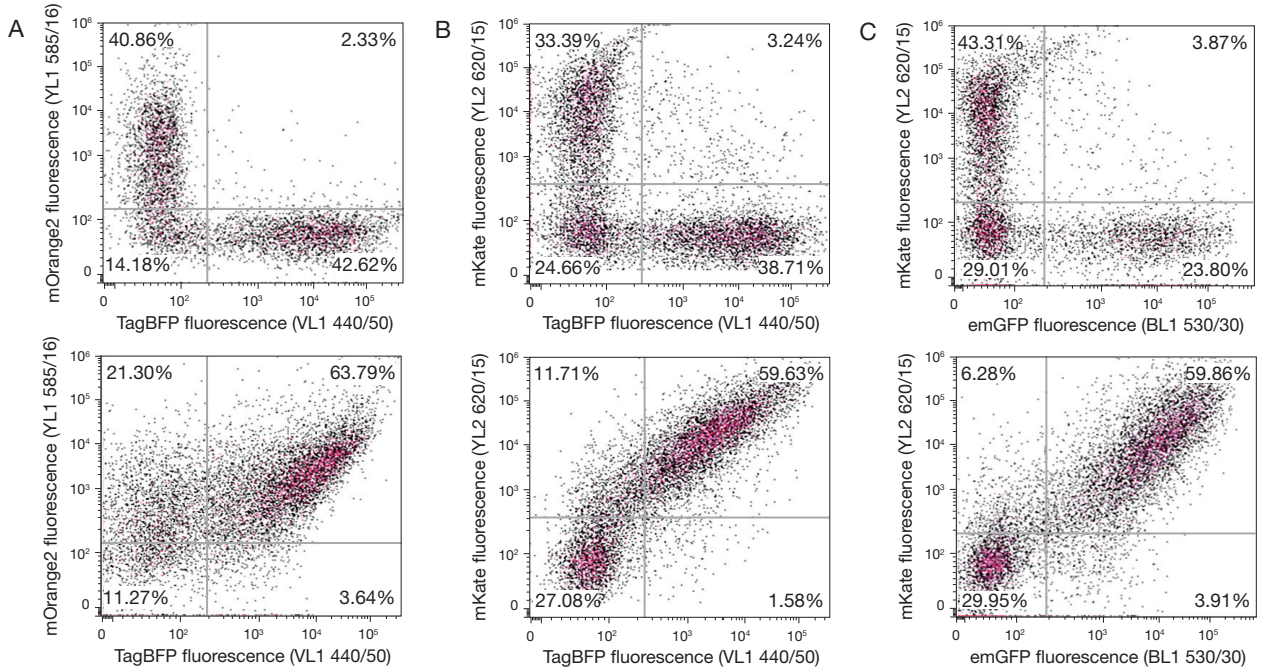

Figure 2. Detection of multiple FPs expressed in the same cell. 293FT cells were transfected with two plasmids, either by sequential delivery of each plasmid separately (top panels), or in 1:1 (w/w) mixes (bottom panels), using Invitrogen™ Lipofectamine™ 3000 reagent. Transfected cells were grown for 48 hr prior to harvest and analysis by flow cytometry. Samples were acquired using the Attune NxT Flow Cytometer at a flow rate of 100 µL/min, and a minimum of 15,000 events were collected for each sample. All major cell populations are detected: cells expressing one of the FPs, both FPs, and neither FP (percentages are indicated on the plots). Cells expressing the FPs are easily distinguished from non–FP-expressing cells. **(A)** The 405 nm and 561 nm lasers were used for excitation of TagBFP and mOrange2, respectively. **(B)** The 405 nm and 561 nm lasers were used for excitation of TagBFP and mKate, respectively. **(C)** The 488 nm and 561 nm lasers were used for excitation of emGFP and mKate, respectively.

for a variety of applications amenable to fluorescence imaging and flow cytometry. These reagents enable simple and specific labeling of organelles in live cells. With these reagents, the FP is introduced using a simple transfection step that does not require molecular biology techniques. They can be used like standard cell stains and can be detected easily by flow cytometry (Figure 4).

Materials and methods

A procedure for the simultaneous detection of GFP and YFP using the Attune NxT Fluorescent Protein Filter Kit is described here.

Materials

- Attune NxT Flow Cytometer—Blue/Red/Violet/Yellow Lasers (Cat. No. A24858)
- Attune NxT Fluorescent Protein Filter Kit (Cat. No. 100022775)
- Attune™ Performance Tracking Beads (Cat. No. 4449754)
- Gibco™ PBS, pH 7.4 (Cat. No. 10010023)
- U2OS cell line (ATCC, Cat. No. HTB-96)
- CellLight Histone 2B-GFP, BacMam 2.0 (Cat. No. C10594)
- Premo Halide Sensor (Cat. No. P10229)
- Gibco™ McCoy's 5A Medium (Cat. No. 16600108)
- Gibco™ TrypLE™ Express Enzyme (Cat. No. 12605093)
- 6-well plates
- 12 x 75 mm test tubes

Compensation guidelines

- Single-color compensation controls should be used with all multicolor flow cytometry experiments.
- Compensation controls need to be at least as bright as the relevant samples.
- Background fluorescence should be the same for the positive and negative control populations for any given parameter.
- The compensation color must be matched to the experimental color.

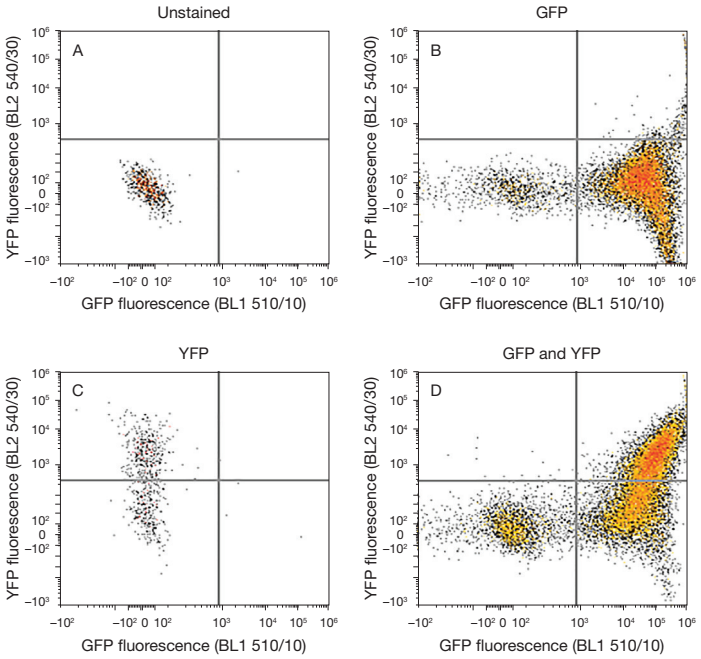

Figure 3. Flow cytometric detection of dual expression of GFP and YFP. U2OS cells were transfected with vectors encoding GFP or YFP, either individually **(B, C)** or in combination **(D)**. Untransfected cells are shown in **(A)**. Samples were acquired and analyzed using the Attune NxT Flow Cytometer at a flow rate of 200 µL/min. A total of 400,000 cells were collected for the sample coexpressing both FPs, and a minimum of 5,000 events were collected for each control sample. The 488 nm laser was used for excitation of both FPs. Coexpression of GFP and YFP is shown in the upper-right quadrant of **(D)**, and the lower-right quadrant shows cells expressing only GFP.

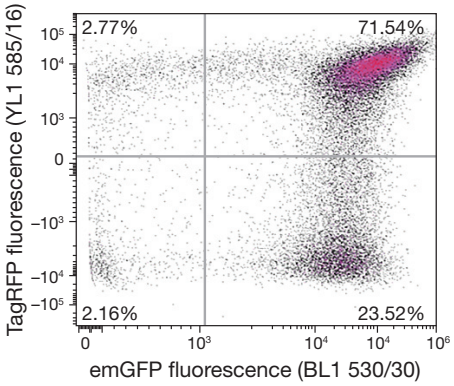

Figure 4. Live-cell detection with CellLight reagents. Baculovirus constructs encoding emGFP or TagRFP were cotransduced into U2OS cells. Samples were acquired using a four-laser Attune NxT Flow Cytometer equipped with 405 nm, 488 nm, 561 nm, and 637 nm lasers. As expected, the majority of cells in the sample are co-positive for both FPs ($emGFP^+/TagRFP^+$, 71.54%). There are two minor populations of cells that only express one FP: $emGFP^+/TagRFP^-$ cells are 23.52% of the population and $emGFP^-/TagRFP^+$, cells are 2.77% of the population. Only a small number of cells do not express either protein (2.16%).

Cell labeling

1. Plate U2OS cells at the desired density and allow them sufficient time to adhere.

2. Determine the appropriate volume of CellLight or Premo reagent for the number of cells, using this formula or Table 2.

$$\text{Volume of CellLight or Premo reagent (mL)} = \frac{\text{Number of cells} \times \text{desired particles/cell}}{1 \times 10^8 \text{ CellLight or Premo particles/mL}}$$

Table 2. Amount of reagent to add to each sample.

Sample	Reagent	Amount per 1 mL sample
Unstained control	NA	NA
GFP control	CellLight reagent	10 µL
YFP control	Premo reagent	20 µL
GFP and YFP	CellLight and Premo reagents	10 µL, 20 µL

3. Mix the CellLight or Premo reagent several times by inversion to ensure a homogeneous solution. Do not vortex.

4. Add the volume of CellLight or Premo reagent directly to the cells in McCoy's 5A Medium, and mix gently.

5. Return the cells to the culture incubator for ≥16 hours.

6. Remove the medium and rinse the cells with PBS. Add 1 mL of TrypLE enzyme and allow the cells to detach from the plate.

7. Add 4 mL of fresh medium to the plate and mix well. Remove the cell suspension and place it in a centrifuge tube.

8. Wash each sample twice with PBS.

9. Resuspend the cells of each tube in 2 mL PBS and place into appropriately labeled tubes for flow cytometry analysis, and place on wet ice.

Instrument preparation

1. Turn on the instrument; run startup and performance test.

2. Using the Attune NxT Fluorescent Protein Filter Kit, replace filters according to Table 3 and Figure 5.

3. Create a new experiment and set up your workspace as desired.

4. Compensation controls are needed to calculate the amount of compensation required for the experiment. We recommend that you optimize the instrument settings for compensation controls in the Compensation Workspace [13].

5. Optimize the instrument settings for compensation control samples, open the Compensation Setup dialog box, and select the BL1 and BL2 parameters.

6. In this experiment the Unstained Control Method for compensation was used (Figure 6) with the compensation controls:

- Unstained (untransfected) cells
- BL1 GFP single expression
- BL2 YFP single expression

Table 3. Filter configurations.

Channel	Original filter configuration (nm)	FP filter configuration (nm)
BL1	530/30	510/10
BL1	555DLP	525DLP
BL2	590/40	540/30
YL2*	620/15	615/25

* Not used for this experiment.

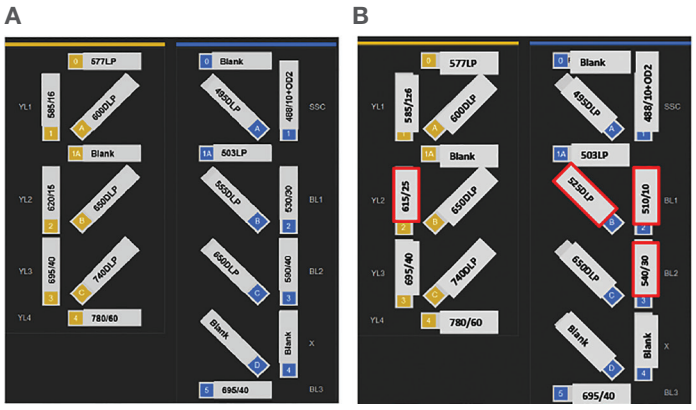

Figure 5. Use of the Attune NxT Fluorescent Protein Filter Kit. The standard configuration for the 561 nm yellow and 488 nm blue laser optical filter blocks is shown in **(A)**, and the same optical filter blocks using the Attune NxT Fluorescent Protein Filter Kit are shown in **(B)**, with changes outlined in red. To use the filter kit, remove the 530/30 nm BP in BL1 (slot 2) and replace it with the 510/10 nm BP filter; remove the 590/40 nm BP filter from BL2 (slot 3) and replace it with the 540/30 nm BP filter; remove the 555DLP filter (slot B) and replace it with the 525DLP filter. This allows detection of GFP with the 510/10 nm BP filter in the BL1 detector and YFP with the 540/30 nm BP filter in the BL2 detector.

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7. Once the instrument settings have been optimized for each single-expressing FP, the compensation controls can be acquired and recorded according to the step-by-step instructions provided in the Status Notification Bar above the Main Workspace.

8. After the recording of all three compensation samples, the compensation is applied and the GFP and YFP dual-expressing cells may be acquired and recorded.

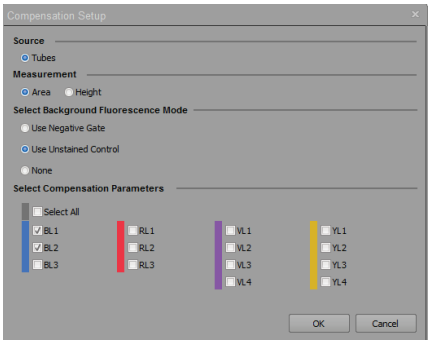

Figure 6. Compensation setup.

9. Collect at least 50,000 total events.

10. The use of HyperLog scale is recommended when displaying compensated multicolor data. The HyperLog scale uses log-linear hybrid transformations to display flow cytometry data that frequently contain negative values due to compensation. Logarithmic transformations cannot properly handle negative values, and they poorly display normally distributed cell types [14].The width of the linear area of the HyperLog plot can be adjusted by entering a value in the Transition Value in the Customize menu.

Conclusion

Fluorescent proteins are a valuable tool for researchers. A world of possibilities has been opened through their discovery and incorporation into biological research. The ability to monitor both live and dead manipulated cells allows researchers to better understand the mechanisms that regulate cellular processes. However, in order to utilize this, it is necessary to be able to analyze these processes on an individual cellular basis. Fortunately, with the increasing abilities of flow cytometers like the Attune NxT Flow Cytometer with up to 16 parameters of detection and clog-resistant design, researchers can now explore what was overly difficult or simply impossible only a few years ago.

More resources for your FP studies

Get the most out of your FP experiments. Visit our Flow Cytometry Resource Center at **thermofisher.com/flowresourcesbp71** for useful tutorials, webinars, application notes, fluorophore guides, and more information on reagents and instrumentation for flow cytometry.

References

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