Keep pace with the surge in fluorescent protein choices

Multicolor detection of fluorescent protein expression with the Attune™ NxT Acoustic Focusing Cytometer.

Since the discovery of Green Fluorescent Protein (GFP) in the 1960s, the use of fluorescent proteins (FPs) in biological studies has exploded. In its role as a reporter, GFP has added a new dimension to the analysis of protein function and localization, allowing real-time examination in live cells and processes that previously had been observed only through immunocytochemical "snapshots" in fixed specimens.

Fluorescent proteins are widely used to investigate gene expression, as well as protein localization, translocation, and trafficking within live cells. More advanced techniques include assessment of protein–protein interactions and spatial relation of proteins in live cells with fluorescence resonance energy transfer (FRET) techniques and fluorescence lifetime imaging microscopy (FLIM). In addition, far red–emitting FPs, which 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 photoswitchable FPs (e.g., Dendra2 and mEos2).

Versatile tools for flow cytometry

The use of FPs became popularized in the field of flow cytometry in the 1990s after mutation of wild-type GFP resulted in a variant optimally excited by the 488 nm argon-ion laser common to most flow cytometers [1]. Early studies using FPs in flow cytometry include 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 56 are commonly used in flow cytometry and exhibit excitation wavelengths between 355 nm and above 600 nm (Table 1). Flow cytometrists have taken advantage of this large set of choices—combined with the increased availability of alternate excitation sources such as the 405 nm, 532 nm, and 561 nm lasers—by developing multiparameter flow cytometry assays. These assays are designed to analyze cells expressing several different FPs [5,6], as well as FP-expressing cells that have also been labeled with fluorescent antibodies and functional probes [7,8]. →

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

Fluorescent protein	Excitation max (nm)	Emission max (nm)	Channel on the Attune™ NxT Acoustic Focusing 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)
T-Sapphire	399	511	VL2 (512/25)
LSS-mKate1, LSS-mKate2	463, 460	624, 605	VL3 (603/48)
TurboGFP, EGFP, TagGFP, Emerald GFP	482, 483, 484, 487	502, 506, 507, 509	BL1 (530/30) (510/10)*
TagYFP, TurboYFP, EYFP, Topaz, Venus, Citrine	508, 508, 514, 514, 515, 517	524, 524, 527, 527, 528, 529	BL1 (530/30) (510/10)* or BL2 (574/26) (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) (615/25)*
mPlum, HcRed, mRaspberry, mNeptune, E2Crimson	590, 592, 598, 599, 611	649, 645, 625, 649, 646	YL3 (695/40)

* Bandpass emission filter when using the fluorescent protein filter kit (optional, Cat. No. 100022775)

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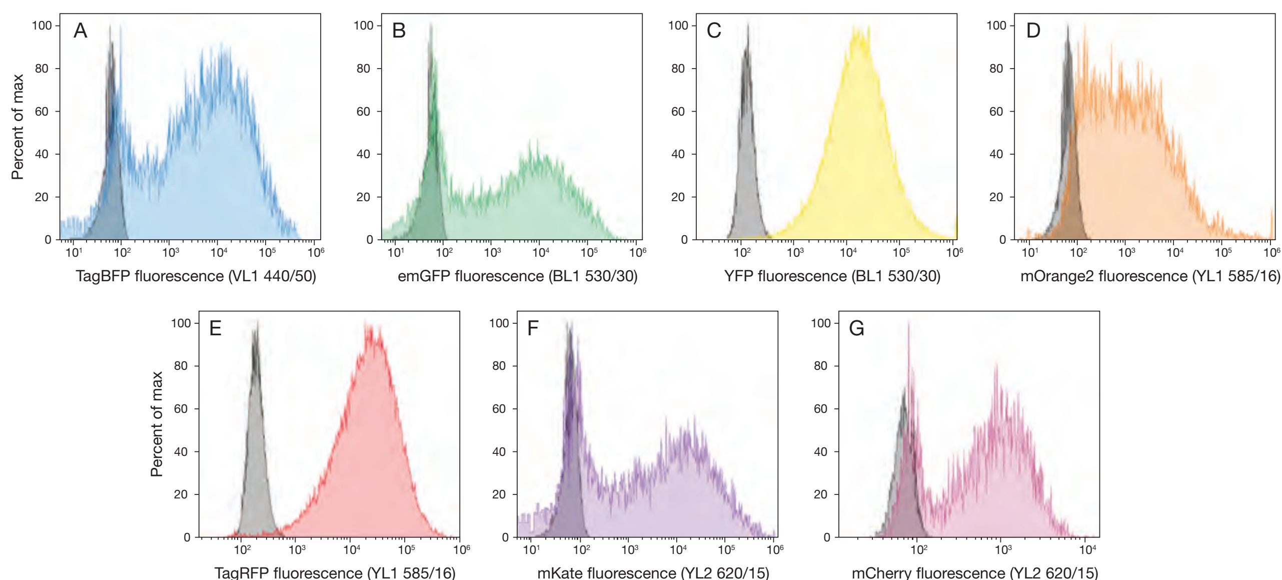

Figure 1. Detection of a palette of fluorescent proteins using the Attune™ NxT Acoustic Focusing Cytometer. 293FT cells or U2OS cells were transfected or transduced with plasmid or viral constructs expressing different fluorescent proteins. Samples were acquired on the Attune™ NxT cytometer at a flow rate of 100 µL/min using 405 nm, 488 nm, or 561 nm excitation sources. **(A)** Blue Fluorescent Protein (TagBPF) fluorescence was collected in the VL1 channel using a 440/50 bandpass (BP) filter; **(B)** Emerald GFP (emGFP) fluorescence and **(C)** Yellow Fluorescent Protein (YFP, Venus variant) fluorescence (in cells transduced with the Premo™ Halide Sensor) were collected in the BL1 channel using a 530/30 BP filter; **(D)** Orange Fluorescent Protein (mOrange2) fluorescence and **(E)** Red Fluorescent Protein (TagRFP) fluorescence were collected in the YL1 channel using a 585/16 BP filter; **(F)** mKate fluorescence and **(G)** mCherry fluorescence were collected in the YL2 channel using a 620/15 BP filter. Control cells that do not express fluorescent protein are shown in each histogram overlay (gray peaks). TagBFP, mOrange2, TagRFP, YFP, and mCherry were expressed from the CMV promoter, and emGFP and mKate were expressed from the EF-1α promoter. YFP and RFP constructs were delivered to U2OS cells using the BacMam 2.0 transduction system, whereas TagBFP, emGFP, mKate, and mOrange2 constructs were transfected into 293FT cells using Lipofectamine™ 3000 reagent. The mCherry construct was transduced into U2OS cells using an adenovirus delivery system.

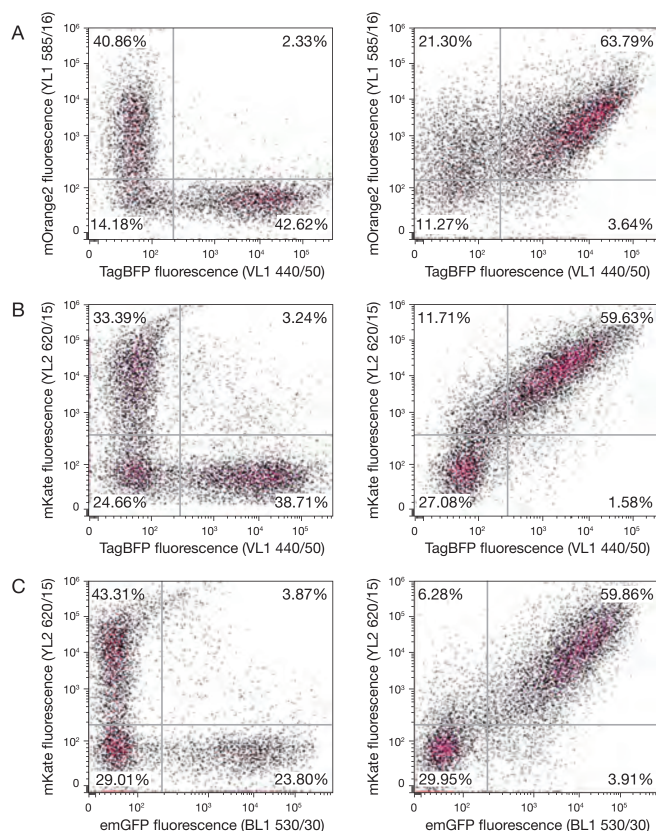

Figure 2. Detection of multiple FPs expressed in the same cell. Plasmid constructs were transfected alone (left panels) or simultaneously (right panels) in a 1:1 (w/w) mix into 293FT cells using Lipofectamine™ 3000 reagent. Transfected cells were grown for 48 hr prior to harvest and analysis by flow cytometry. Samples were acquired using an Attune™ NxT Acoustic Focusing Cytometer at a flow rate of 100 µL/min, and a minimum of 15,000 events were collected for each sample. **(A)** Plasmids encode the Blue Fluorescent Protein (TagBFP) or Orange Fluorescent Protein (mOrange2) under control of the CMV promoter. The 405 nm and 561 nm lasers were used for excitation of TagBFP and mOrange2, respectively. TagBFP fluorescence was detected in the VL1 channel using a 440/50 bandpass (BP) filter, and mOrange2 fluorescence was detected in the YL1 channel using a 585/16 BP filter. **(B)** Plasmids encode TagBFP under control of the CMV promoter or the mKate Fluorescent Protein (mKate) under control of the EF-1α promoter. The 405 nm and 561 nm lasers were used for excitation of TagBFP and mKate, respectively. TagBFP fluorescence was detected in the VL1 channel using a 440/50 BP filter, and mKate fluorescence was detected in the YL2 channel using a 620/15 BP filter. **(C)** Plasmids encode the Emerald Green Fluorescent Protein (emGFP) or mKate under control of the EF-1α promoter. The 488 nm and 561 nm lasers were used for excitation of emGFP and mKate, respectively. emGFP fluorescence was detected in the BL1 channel using a 530/30 BP filter, and mKate fluorescence was detected in the YL2 channel using a 620/15 BP filter. 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 fluorescent proteins are easily distinguished from non-expressing cells.

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Detecting multiple FPs using the Attune™ NxT Acoustic Focusing Cytometer

With the option to be configured with up to 4 lasers and 16 detection channels (14 colors and 2 scatter channels), the Attune™ NxT Acoustic Focusing Cytometer is ideally suited for the detection of multiple FPs. The Attune™ NxT instrument has a modular design with a 488 nm laser for excitation of the most commonly used FP (EGFP) and its variants (emGFP, TurboGFP), and can be upgraded to include optional laser lines including 405 nm, 561 nm, and 637 nm lasers. The 561 nm laser is particularly useful for exciting the orange- and red-fluorescent protein variants: mCherry, the popular monomeric red-fluorescent protein with superior brightness and photostability [9]; mKate, known for its fast maturation rate as well as its high pH stability and photostability [10]; and mOrange2, a bright monomeric orange-fluorescent protein. The 405 nm laser can be used to excite FPs such as TagBFP [11], the bright blue-fluorescent mutant created from site-specific and random mutagenesis of TagRFP, or others including Azurite and T-Sapphire. Figure 1 shows detection of TagBFP, emGFP, YFP, mOrange2, TagRFP, mKate, and mCherry using the Attune™ NxT Acoustic Focusing Cytometer.

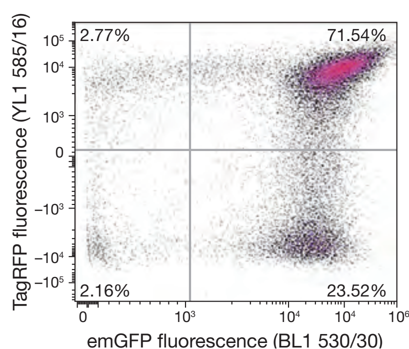

Figure 3. Live-cell detection with CellLight™ reagents. Baculovirus constructs encoding Emerald GFP (emGFP) or Red Fluorescent Protein (TagRFP) under control of the CMV promoter were co-transduced into U2OS cells using the BacMam 2.0 delivery system. Samples were acquired using a four-laser Attune™ NxT Acoustic Focusing Cytometer equipped with 405 nm, 488 nm, 561 nm, and 637 nm lasers. emGFP fluorescence was collected in the BL1 channel using a 530/30 bandpass (BP) filter, and TagRFP fluorescence was detected in the YL1 channel using a 585/16 BP filter. 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%).

Simultaneous detection of paired FPs expressed in the same cell is shown in Figure 2, demonstrating clear separation of cells expressing one of the FPs, both FPs, or neither FP. For no-hassle labeling of specific organelles in live cells, CellLight™ ready-to-use constructs express fluorescent fusion proteins targeted to specific intracellular structures. With these reagents, the FP is introduced using a simple transduction step that doesn't require molecular biology techniques to carry out—they work like cell stains and can be detected easily using flow cytometry (Figure 3).

More resources for your fluorescent protein 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

1. Ropp JD, Donahue CJ, Wolfgang-Kimball D et al. (1995) *Cytometry* 21:309–317.
2. Atkins D, Izant JG (1995) *Curr Genet* 28:585–588.
3. Kremer L, Baulard A, Estaquier J et al. (1995) *Mol Microbiol* 17:913–922.
4. Anderson MT, Tjioe IM, Lorincz MC et al. (1996) *Proc Natl Acad Sci U S A* 93:8508–8511.
5. Hawley TS, Telford WG, Ramezani A (2001) *Biotechniques* 30:1028–1034.
6. Hawley TS, Telford WG, Hawley RG (2001) *Stem Cells* 19:118–124.
7. Gundry RL, Riordon DR, Tarasova Y (2012) *Mol Cell Proteomics* 11:303–316.
8. Yang N, Huang B, Tsinkalovsky O et al. (2014) *Cancer Cell Int* 14:541–549.
9. Shaner NC, Steinbach PA, Tsien RY (2005) *Nat Methods* 2:905–909.
10. Shcherbo D, Merzlyak EM, Chepurnykh TV et al. (2007) *Nat Methods* 4:741–746.
11. Subach OM, Gundorov IS, Yoshimura M et al. (2008) *Chem Biol* 15:1116–1124.

Product	Quantity	Cat. No.
Attune™ NxT Acoustic Focusing Cytometer—Blue/Red/Violet/Yellow Lasers	1 each	A24858
Attune™ NxT Fluorescent Protein Filter Kit	1 kit	100022775
Attune™ Performance Tracking Beads	3 mL	4449754
Attune™ Focusing Fluid (1X)	10 L	A24904
CellLight™ Plasma Membrane-RFP, BacMam 2.0	1 mL	C10608
CellLight™ Nucleus-RFP, BacMam 2.0	1 mL	C10603
CellLight™ Nucleus-GFP, BacMam 2.0	1 mL	C10602
GeneArt™ CRISPR Nuclease Vector with OFP Reporter Kit (with competent cells)	10 reactions	A21178
Lipofectamine™ 3000 Transfection Reagent	0.1 mL	L3000-001
Premo™ Halide Sensor	1 kit	P10229
293FT Cell Line	3 x 10^6 cells	R700-07

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