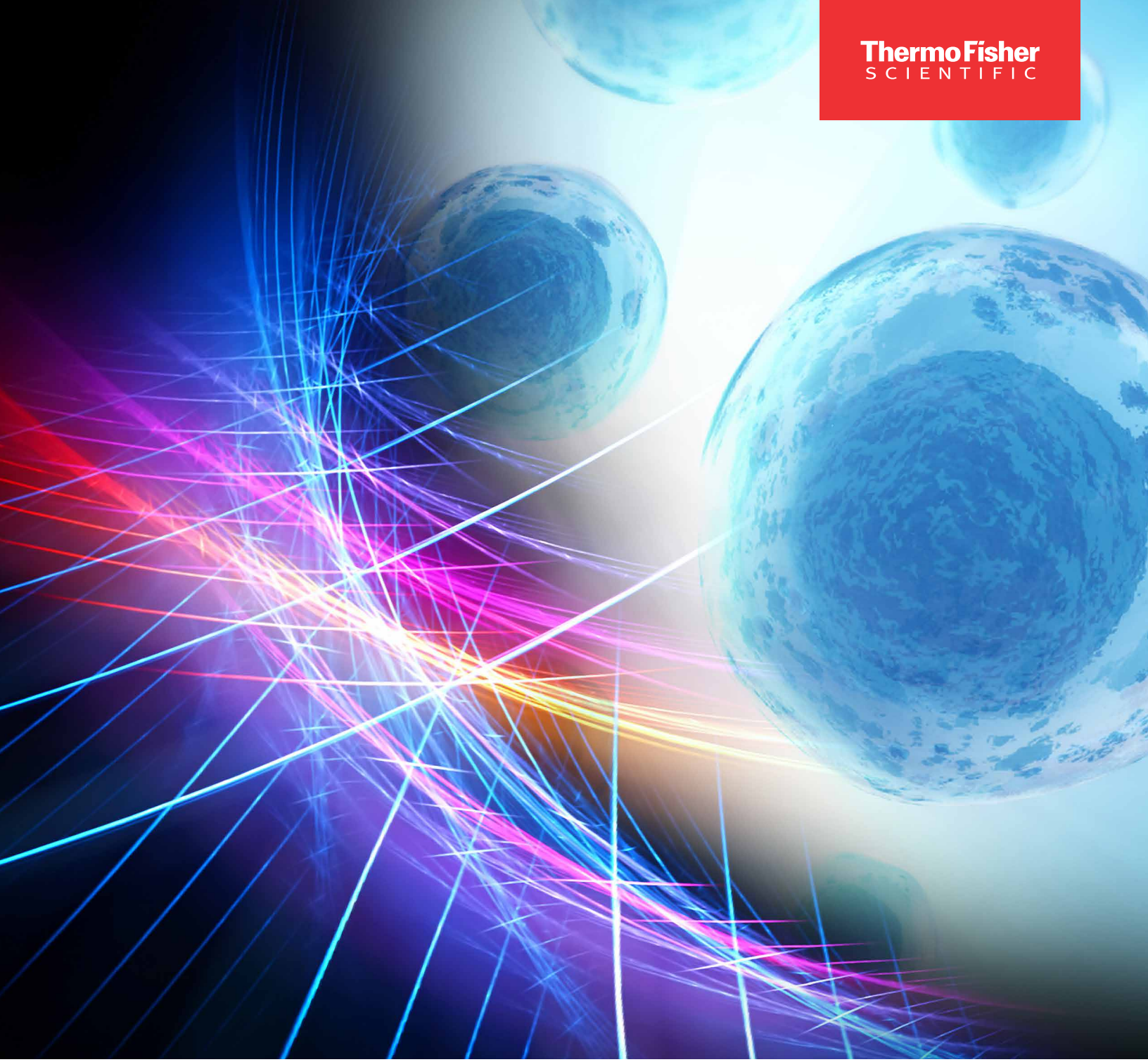


# Flow cytometry capabilities guide

Sample preparation | Fluorophore selection | Flow cytometry antibodies and assays | Attune flow cytometers | PrimeFlow RNA Assay | Bigfoot Spectral Cell Sorter

invitrogen

# Getting started

Flow cytometry enables the simultaneous analysis of multiple proteins, gene expression, and cell functions such as oxidation, viability, cell cycle, apoptosis, and proliferation from an individual cell. This technology makes it possible to obtain a statistically relevant amount of data by combining information from individual cells to gain insight into a heterogeneous sample. Whether you are identifying cell subpopulations or investigating cell functions, flow cytometry can make significant contributions to moving your research forward.

Building a flow cytometry experiment often requires combining products into a multicolor panel. Use this guide to understand the basics of Invitrogen™ eBioscience™ flow cytometry antibodies and Invitrogen™ flow cytometry assays and reagents. Then see how example panels are run on flow cytometers, including Invitrogen™ Attune™ flow cytometers, in the following areas:

- Immunology
- Inflammation
- Immuno-oncology
- Solid-tumor cancers
- Neuroinflammation
- Gene editing
- Microbiology

## Flow cytometry workflow—what you will need

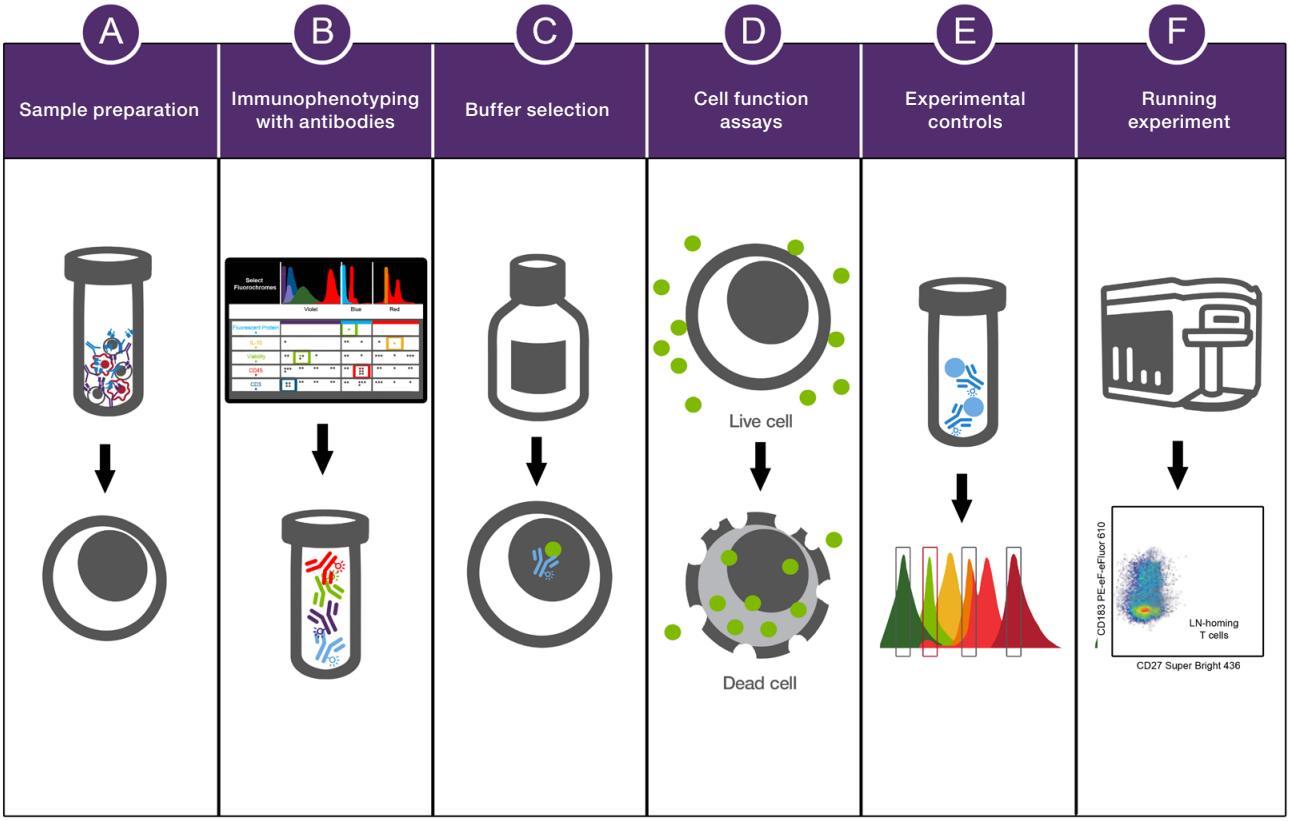



**Figure 1. Flow cytometry workflow.** Planning your workflow in advance as outlined will help generate a successful experiment.

Find out more about multipurpose flow cytometry experiments at [thermofisher.com/flowcytometry](thermofisher.com/flowcytometry)

# Sample preparation: reagents for immune cell activation

Stimulation or treatment of cells is usually required for activation of immune cells to proliferate and differentiate into mature cell types (Figure 2). Activated cells often express higher levels of transcription factors, cytokines, chemokines, and other mediators detected by flow cytometry. Choosing the appropriate activating reagent will depend on (1) cell type, (2) expression and kinetics of the protein of interest, and (3) experimental conditions.

We offer an expansive list of high-quality cell stimulation products that include:

- Functional-grade antibodies and recombinant proteins to stimulate many types of immune cells
- Reagents in appropriate preservative-free buffers with low endotoxin levels to use in cell culture
- The Invitrogen™ eBioscience™ Cell Stimulation Cocktail at a ready-to-use concentration

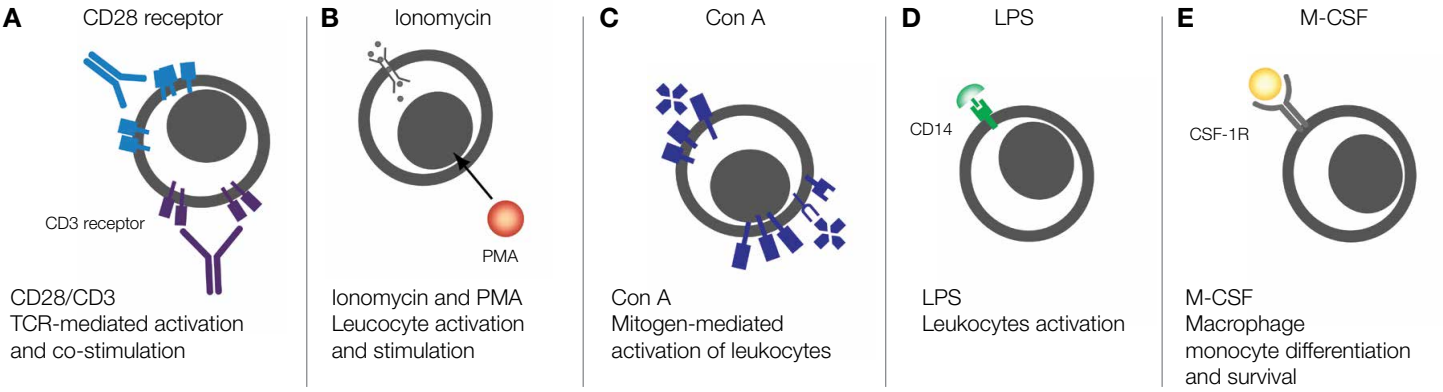



**Figure 2. Cell stimulation reagents. (A)** Functional-grade antibodies (e.g., anti-CD3 and anti-CD28) or Invitrogen™ Dynabeads™ magnetic beads for T cell activation and expansion. **(B)** eBioscience Cell Stimulation Cocktail comprising phorbol 12-myristate 13-acetate (PMA), a protein kinase activator, and ionomycin, a calcium ionophore, stimulate T cells to produce interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), and interleukin-4 (IL-4). **(C)** Concanavalin A (Con A) induces T cell activation and proliferation. **(D)** Monocytes can be activated by lipopolysaccharide (LPS) to secrete interleukin-6 (IL-6), interleukin-10 (IL-10), or TNF-α. **(E)** Macrophage colony-stimulating factor (M-CSF) is a growth factor that regulates the proliferation, differentiation, and functional activation of monocytes' differentiation into macrophages.

## Example: T cell activation

T cells require external signals for differentiation and expansion from a quiescent state (Figure 3). PMA and ionomycin or anti-CD3 and anti-CD28 antibodies are recommended to upregulate intracellular transcription factors for detection. Time-course profiling of cells with the cell-stimulating reagents is recommended, since cytokines have different kinetics and/or expression levels.

Identification of human Th17 cells within a CD4⁺ T cell population

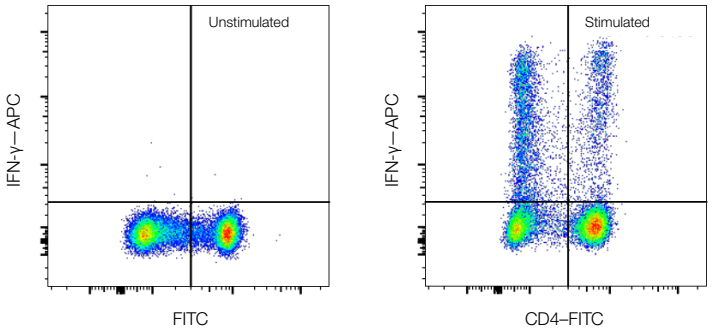



**Figure 3. Identification of human Th17 cells within a CD4⁺ T cell population.** Normal human peripheral blood cells were unstimulated (left) or stimulated with eBioscience Cell Stimulation Cocktail plus protein transport inhibitors (500X) (right). Cells were fixed and stained intracellularly with Invitrogen™ anti–human CD4 APC and anti–human IFN-γ conjugated to Invitrogen™ eBioscience™ PE-eFluor™ 610 dye, using the Invitrogen™ eBioscience™ Intracellular Fixation and Permeabilization Buffer Set and protocol. Cells in the lymphocyte gate were used for analysis.

Find out more at [thermofisher.com/flow-assays](thermofisher.com/flow-assays)

# Immunophenotyping with flow cytometry antibodies

A multicolor flow cytometry panel uses two or more primary conjugated antibodies to identify single cells by detecting multiple antigens. The goal of the panel is to get the maximum signal for effective visualization of cell populations. Use this section of the guide to aid in the selection of antibodies.

Flow cytometry antibodies cover:

- CD markers
- Transcription factors
- Cytokines, chemokines, and growth factors
- Signaling pathway markers, including phosphoproteins

## Marker selection

Select from one of the largest portfolios of primary conjugated antibodies specifically developed for flow cytometry applications. Each flow cytometry antibody search result contains data plots gathered from internal antibody validation* testing and published customer data accessible online. Use this online search tool to determine which antibody is applicable to find your cell population (Figure 4).

Our flow cytometry antibodies are conjugated to different fluorophores to allow for use on any instrument. These fluorophores simplify the optimization of panel design because of flexible dye selection for reduced spectral overlap.

**Choose dyes based on:**

- Laser and filter configuration of the flow cytometer
- Expression level or abundance of the target protein
- Fluorophore brightness
- Fluorescence excitation emission spectra

### Example: selecting the right fluorophore

Fluorophore selection is important for finding your cell of interest. Pick fluorophores with less spectral overlap to clearly identify two populations (Figure 5). Match brighter fluorophores with less abundant targets, and dimmer fluorophores with abundant targets for greater signal separation.

* The use or any variation of the word "validation" refers only to research-use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.

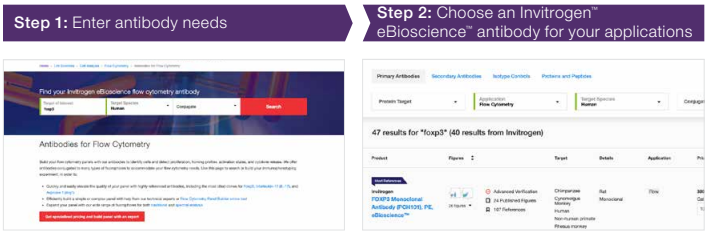



**Figure 4. Antibody search tool to find information and purchase antibodies.** Antibody application data from customer publications and internal testing data (left). A list of antibodies can be purchased, or saved and shared for later use (right).

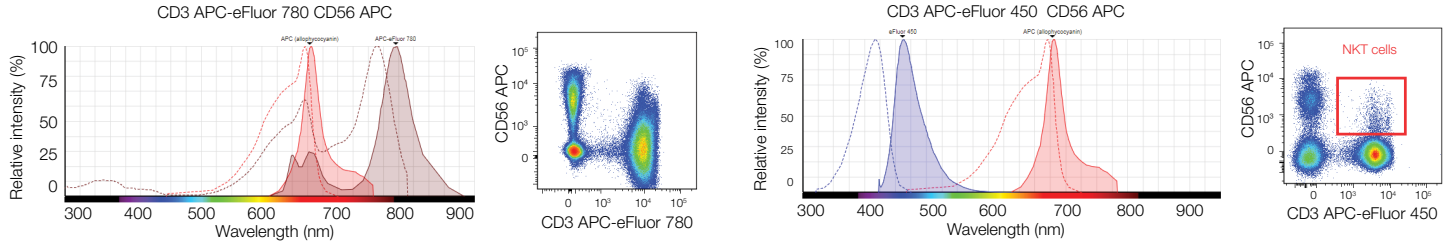



**Figure 5. Normal human peripheral blood cells were stained with anti–human CD3 antibody conjugated with Invitrogen™ eBioscience™ APC-eFluor™ 780 dye (left) or eFluor™ 450 dye (right), as well as anti–human CD56 antibody conjugated with APC dye.** Cells in the lymphocyte gate were used for analysis.

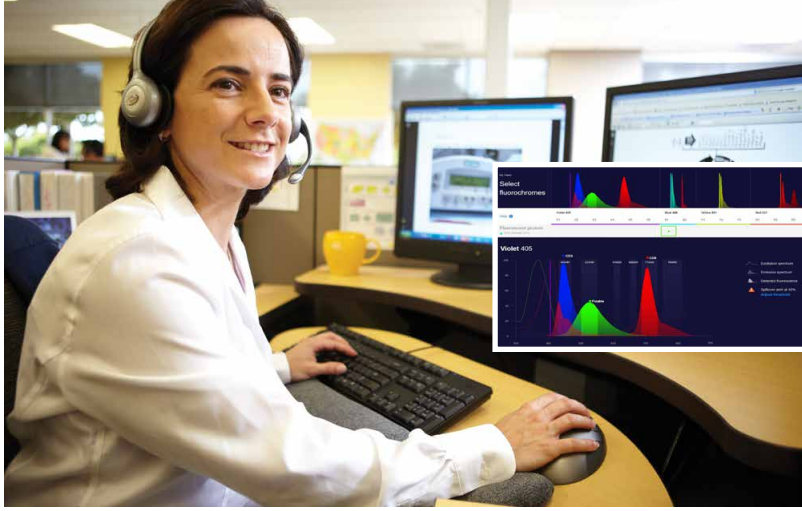
Find your flow cytometry antibodies at **thermofisher.com/flowantibodies**

**Table 1. Comprehensive list of available fluorophores based on their usage, benefits, and intended applications.**

| Family | Type | Benefit | Invitrogen™ fluorophore | |
|---|---|---|---|---|
| **Organic dyes—small, stable molecules** | Original | • Cost-efficient | FITC | |
| | Invitrogen™ Pacific dyes | • Some of the dimmest dyes | Pacific Blue | Pacific Orange |
| | Invitrogen™ Alexa Fluor™ dyes | • Photostable dyes that range across the visible spectrum<br>• Used in flow cytometry and imaging<br>• Named for their excitation wavelengths | Alexa Fluor 405<br>Alexa Fluor 488<br>Alexa Fluor 532<br>Alexa Fluor 561 | Alexa Fluor 647<br>Alexa Fluor 660<br>Alexa Fluor 700 |
| | Invitrogen™ eBioscience™ eFluor™ organic dyes | • Engineered for detection for flow cytometry<br>• Named for their emission wavelength | eFluor 450<br>eFluor 506 | eFluor 660 |
| **Large, protein-based molecules** | Original | • Cost-efficient<br>• Some of the brightest dyes available | APC (allophycocyanin)<br>PerCP (peridinin chlorophyll protein)<br>PE (phycoerythrin) | |
| | Tandem dyes | • Dyes occupy different channels from the donor molecule, and this can be used to build larger panels | APC-Cyanine5<br>APC-Cyanine7<br>PE-Cyanine5 (TRI-COLOR dye)<br>PE-Cyanine5.5<br>PE-Cyanine7<br>PE–Texas Red dye<br>PerCP-Cyanine5.5 | PE–Alexa Fluor 610<br>PE–Alexa Fluor 700<br>APC–Alexa Fluor 750<br>PE–eFluor 610<br>PerCP–eFluor 710<br>APC–eFluor 780 |
| **Polymer dyes** | Invitrogen™ eBioscience™ Super Bright dyes and their tandems | • Excited by the 405 nm violet laser<br>• Minimal spillover into other channels<br>• Add Invitrogen™ eBioscience™ Super Bright Complete Staining Buffer (Cat. No. SB-4401-42) when using two or more polymer dyes to lower background levels | Super Bright 436<br>Super Bright 600<br>Super Bright 645<br>Super Bright 702<br>Super Bright 780 | |
| **Polymer dyes—recent dye innovation** | Invitrogen™ eBioscience™ Brilliant Violet™ and Brilliant Ultra Violet™ dyes | • Excited by the violet and ultra violet lasers<br>• Add Invitrogen™ Brilliant Stain Buffer (Cat. No. 00-4409-75) when using two or more polymer dyes to lower background levels | Brilliant Ultra Violet 395<br>Brilliant Ultra Violet 496<br>Brilliant Ultra Violet 563<br>Brilliant Ultra Violet 615<br>Brilliant Ultra Violet 661<br>Brilliant Ultra Violet 737 | Brilliant Ultra Violet 805<br>Brilliant Violet 421<br>Brilliant Violet 480<br>Brilliant Violet 650<br>Brilliant Violet 711<br>Brilliant Violet 786 |
| **Nanocrystals** | Invitrogen™ Qdot™ dyes | • Narrow emission<br>• Large Stokes shift | Qdot 605<br>Qdot 655 | Qdot 705<br>Qdot 800 |
| **DNA-scaffold dyes—recent dye innovation** | Invitrogen™ eBioscience™ NovaFluor™ dyes | • Unique spectral signatures<br>• Variable brightness<br>• Low cross-laser excitation<br>• Named for the exciting laser and emission spectrum<br>• Use Invitrogen™ CellBlox™ Blocking Buffer (Cat. No. B001T03F01) for use with NovaFluor dyes to label cells to block nonspecific labeling and to reduce background | NovaFluor Blue 510<br>NovaFluor Blue 530<br>NovaFluor Blue 555<br>NovaFluor Blue 585<br>NovaFluor Blue 610-30S<br>NovaFluor Blue 610-70S<br>NovaFluor Blue 660-40S<br>NovaFluor Blue 660-120S<br>NovaFluor Blue 690<br>NovaFluor Yellow 570 | NovaFluor Yellow 590<br>NovaFluor Yellow 610<br>NovaFluor Yellow 660<br>NovaFluor Yellow 690<br>NovaFluor Yellow 700<br>NovaFluor Yellow 730<br>NovaFluor Yellow 755<br>NovaFluor Red 660<br>NovaFluor Red 685<br>NovaFluor Red 700<br>NovaFluor Red 710<br>NovaFluor Red 725<br>NovaFluor Red 755 |

For more information and sales, contact a technical sales specialist at **thermofisher.com/paneldesignhelp**

# Free Flow Cytometry Panel Design Service



## Panel Builder tool—for self-designing panels

The Invitrogen™ Flow Cytometry Panel Builder is a free online tool to help select antibody conjugates and reagents for a multicolor flow cytometry panel (Figure 6). This allows for improved panel design with greater separation and detection of individual cell populations of interest.

### With this tool, you can:

- Create a new immunophenotyping experiment or add antibodies and reagents to an existing panel
- Check fluorophore emission spectra with the built-in SpectraViewer
- Export an Excel™ document with your antibody choices, or order directly

### Key features of our free panel design service include:

- **Free and fast**—no purchase necessary; typical response time within one business day
- **Personalized**—one-on-one, customized assistance with a live specialist
- **Flexible**—accommodates antibodies that you already have, and those that you need

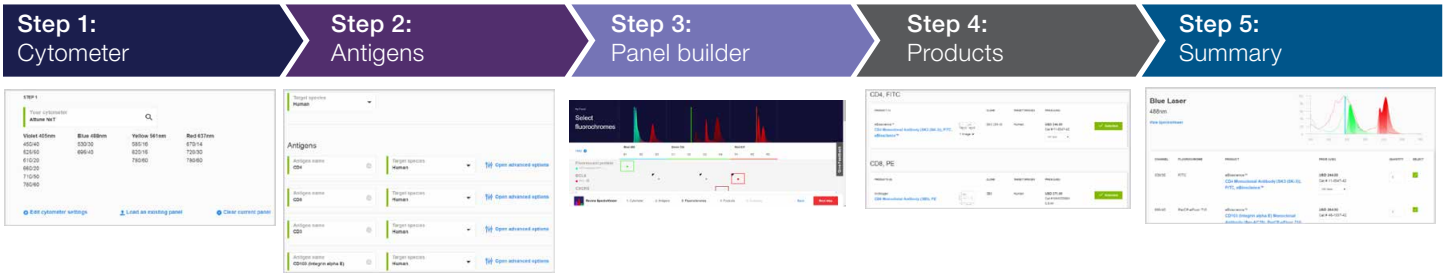



**Figure 6. The Flow Cytometry Panel Builder simplifies experimental design with a 5-step strategy.**

For panel design service and panel builder tool visit **thermofisher.com/flowpanel**

# NovaFluor dyes—add markers with minimal impact to compensation

NovaFluor dyes are designed for more resolution with narrow emission spectra and minimal cross-laser excitation. Lower spectral spillover or overlap lessens the need for compensation, decreases spreading error, and increases opportunities to add new markers. This aids in construction of flow cytometry panels with increased resolution while expanding the overall size of panels.

The CellBlox Monocyte and Macrophage Blocking Buffer is formulated to block nonspecific binding of NovaFluor dyes with cells. These nonspecific interactions can result in higher background labeling. The CellBlox buffer is a non-antibody, nonprotein-based blocking solution designed for use with NovaFluor dyes, cyanine-based dyes, or cyanine-based tandem dyes to block nonspecific interactions with monocytes, macrophages, and other cell types to minimize background labeling.

## Know why NovaFluor dyes should be in every panel:

- **Unique spectral signatures**—fluorophores with distinct brightnesses and signature patterns
- **Higher resolution**—decreased spillover spread from dyes with narrow emissions and minimal cross-laser excitation
- **Long-term storage**—stable dyes keep fluorescence better, compared to tandem dyes
- **Highly specific**—CellBlox blocking buffer is included with every NovaFluor conjugate to reduce nonspecific binding and background to monocytes and macrophages

**Table 2. Nonexclusive list of fluorophores with spread and separation index values.*,****

| Fluorescent label | Excitation max (nm) | Emission max (nm) | Primary detector (nm) | Laser line (nm) | Spread | Separation index |
|---|---|---|---|---|---|---|
| NovaFluor Blue 510 | 496 | 511 | B1 (498–518) | 488 | **727** | **55** |
| NovaFluor Blue 530 | 509 | 530 | B2 (516–533) | 488 | **1,128** | **12** |
| NovaFluor Blue 555 | 494 | 555 | B3 (533–550) | 488 | **691** | **21** |
| NovaFluor Blue 585 | 494 | 585 | B4 (571–590) | 488 | **1,527** | **9** |
| NovaFluor Blue 610-30S | 509 | 614 | B6 (605–625) | 488 | **2,343** | **30** |
| NovaFluor Blue 610-70S | 509 | 614 | B6 (605–625) | 488 | **3,384** | **71** |
| NovaFluor Blue 660-40S | 509 | 665 | B7 (652–669) | 488 | **3,418** | **37** |
| NovaFluor Blue 660-120S | 509 | 665 | B7 (652–669) | 488 | **5,971** | **119** |
| NovaFluor Blue 690 | 494 | 690 | B9 (688-707) | 488 | **5,226** | **136** |
| NovaFluor Yellow 570 | 552 | 568 | YG1 (567–587) | 561 | **1,878** | **52** |
| NovaFluor Yellow 590 | 552 | 590 | YG2 (588–608) | 561 | **810** | **254** |
| NovaFluor Yellow 610 | 552 | 612 | YG3 (605–625) | 561 | **4,257** | **117** |
| NovaFluor Yellow 660 | 552 | 663 | YG4 (652–669) | 561 | **6,824** | **96** |
| NovaFluor Yellow 690 | 552 | 690 | YG6 (687–706) | 561 | **3,123** | **190** |
| NovaFluor Yellow 700 | 552 | 700 | YG7 (706–735) | 561 | **3,299** | **214** |
| NovaFluor Yellow 730 | 552 | 731 | YG7 (706–735) | 561 | **5,775** | **120** |
| NovaFluor Yellow 755 | 552 | 755 | YG8 (735-765) | 561 | **6,012** | **182** |
| NovaFluor Red 660 | 637 | 659 | R2 (669–687) | 640 | **3,789** | **192** |
| NovaFluor Red 685 | 637 | 685 | R3 (688–707) | 640 | **3,734** | **270** |
| NovaFluor Red 700 | 639 | 700 | R3 (688–707) | 640 | **3,301** | **363** |
| NovaFluor Red 710 | 639 | 710 | R4 (707–727) | 640 | **4,256** | **108** |
| NovaFluor Red 725 | 636 | 725 | R5 (728-749) | 637 | **10,659** | **234** |
| NovaFluor Red 755 | 636 | 755 | R6 (749-772) | 637 | **7,209** | **236** |

* All spectral flow cytometry data shown were generated by Cytek Biosciences on a Cytek™ Aurora™ spectral flow cytometer five-laser system and analyzed using SpectroFlo™ software.

** Fluorescent labels for flow cytometry, including spectral spread (calculated as the sum of spectral spread added to all non-primary channels) and separation index (as a measure of brightness, measured using anti-human CD4-SK3 for all fluorescent labels).

Try today at **thermofisher.com/novafluor-dyes**

# Buffer selection: fixation and permeabilization reagents

Fixatives are necessary for saving samples to be used later or for looking at intracellular or intranuclear targets. Ready-to-use fixation kits are optimized for flow cytometry applications. Benefits of using these kits include the following:

- Methods used to stain cells take into consideration the location of the target proteins
- The fixation and permeabilization procedure keeps the morphological light-scattering characteristics of the cells intact
- The reagents in the kits help reduce background staining

**Table 3. Cell staining workflow.**

| | Cell-surface staining (CD markers) | Cytoplasmic staining (cytokines) | Nuclear and cytoplasmic staining (cytokines and transcription factors) |
|---|---|---|---|
| **Stain surface proteins** | ✓ | ✓ | ✓ |
| **Fix cells** | | ✓ | ✓ |
| **Permeabilize cells** | | ✓ | ✓ |
| **Stain cytoplasmic proteins** | | ✓ | ✓* |
| **Stain nuclear proteins** | | | ✓ |

* Cytoplasmic proteins may be stained with a nuclear staining kit, but it may not be optimal.

**Table 4. Flow cytometry buffer and reagent selection guide.**

| Staining buffer | Description | Location |
|---|---|---|
| **eBioscience Flow Cytometry Staining Buffer** | Cell-surface markers are often used to identify cell types. Permeabilization techniques can damage or denature cell-surface antigens and prevent antibodies from binding to surface epitopes. It is advisable to stain for cell-surface antibodies separately. Cell-surface markers can also be stained first, and then protocols for cytoplasmic or nuclear staining should be followed. | Cell surface |
| **Invitrogen™ FIX & PERM™ Cell Permeabilization Kit** or **Intracellular Fixation and Permeabilization Buffer Set** | Cytoplasmic proteins can include cytokines, organelles, and cytoplasmic transcription factors. These proteins are easily accessible with gentle fixation and light permeabilization. Fixation of cytoplasmic proteins often requires a crosslinking agent to have the protein trapped within the cell. | Cytoplasm |
| **eBioscience Foxp3/ Transcription Buffer Set** | Transcription factors, DNA-binding proteins, and modified proteins make up the bulk of nuclear proteins. A quick fixation combined with a stringent permeabilization allows antibodies to penetrate into the nucleus. Fixation reagents can include either crosslinking agents or organic solvents. This type of protocol is also appropriate when examining proteins found both in the cytoplasm* and nucleus. | Nucleus |

* Cytoplasmic proteins may be stained with a nuclear staining kit, but it may not be optimal.

Find out more about buffers at **thermofisher.com/flow-sample**

# Cell function assays: dyes and reagents

Flow cytometry is more than just panels with antibodies. Fluorophore reagents can be used to label cell functionalities such as viability and mitochondrial oxidation.

These reagents and assays can be incorporated into a flow cytometry panel just like a flow cytometry antibody. Use the chart below to determine which assays can be incorporated into a panel (Figure 7).

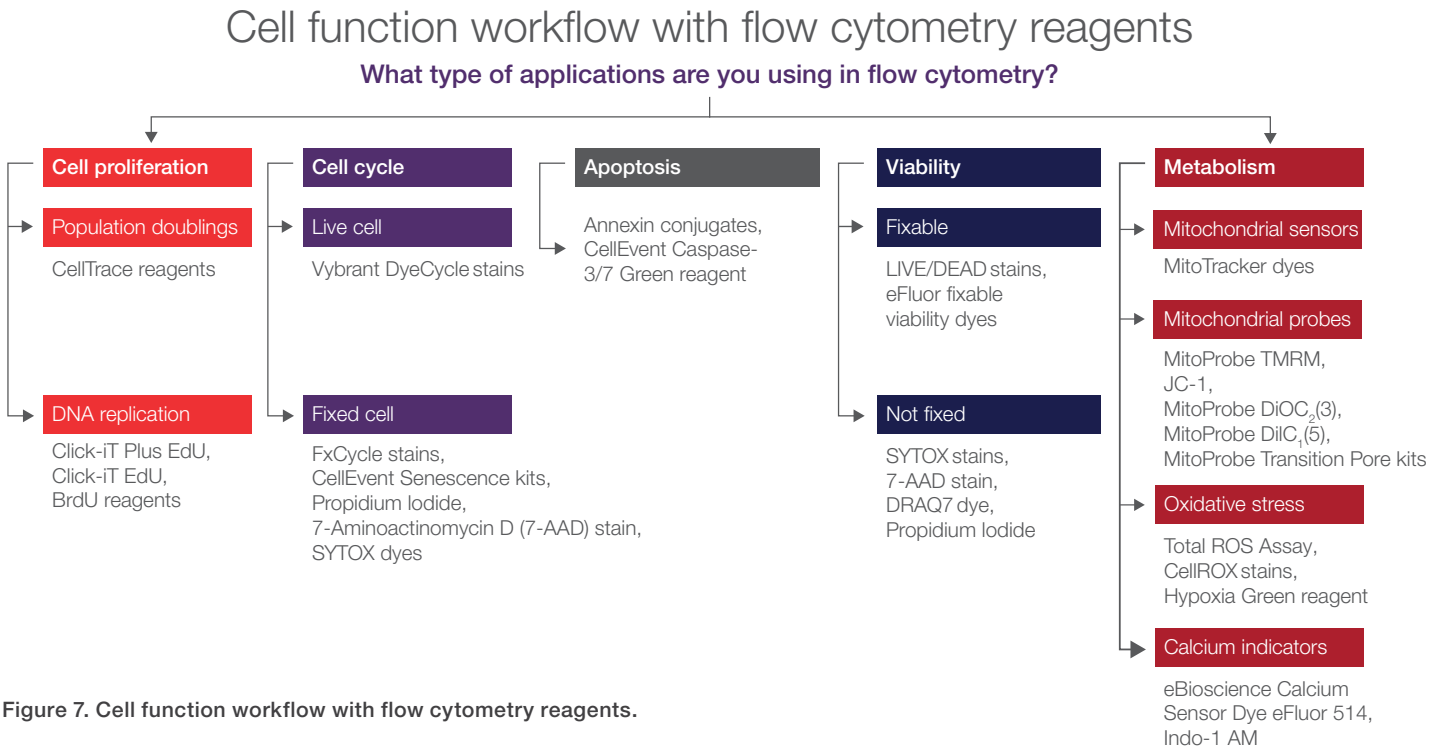



**Figure 7. Cell function workflow with flow cytometry reagents.**

## Cell viability

Cell viability assays can be used to simply distinguish between live and dead cell populations, to correlate with other cell functions or treatments, or to exclude dead cell populations from analyses. Our assays are all one- or two-step processes and can be used in cell sorting or analysis applications.

### Membrane dyes to characterize extracellular vesicles (EVs)

Uniformly label a population of EVs from cell culture. These reagents stain lipids, which is useful for EV detection.

- Lipophilic styryl dye: Invitrogen™ FM™ dye
- Long-chain lipophilic carbocyanine dyes: Invitrogen™ DiI, Vybrant™ CM-DiI (fixable), DiO, and DiD dyes, or Vybrant™ Multicolor Cell Labeling Kit
- Invitrogen™ Di-8-ANNEPS dyes

**Table 5. Cell viability dyes selection guide.**

| Laser | Nonfixable stains | Fixable stains |
|---|---|---|
| UV | DAPI (470) | LIVE/DEAD Fixable Blue (450) |
| 405 nm | SYTOX Blue (480) | LIVE/DEAD Fixable Violet (451) |
| | | LIVE/DEAD Fixable Lime (506) |
| | | LIVE/DEAD Fixable Aqua (526) |
| | | LIVE/DEAD Fixable Yellow (575) |
| 488 nm | SYTOX Green (523) | LIVE/DEAD Fixable Green (520) |
| | Propidium Iodide (617) | LIVE/DEAD Fixable Olive (557) |
| | SYTOX AADvanced (647) | LIVE/DEAD Fixable Red (615) |
| 561 nm | SYTOX Orange (570) | LIVE/DEAD Fixable Orange (602) |
| | SYTOX AADvanced (647) | |
| 633/5 nm | SYTOX Red (660/20*) | LIVE/DEAD Fixable Far Red (665) |
| | | LIVE/DEAD Fixable Scarlet (723) |
| | | LIVE/DEAD Fixable Near IR (775) |
| | | LIVE/DEAD Fixable Near IR (780) |
| 808 nm | | LIVE/DEAD Fixable Near IR (876) |

* Emission maximum (nm).

Find out more at thermofisher.com/flow-assays

### Example: avoiding inaccurate analysis with a LIVE/DEAD assay

When choosing a viability dye to stain cells after fixation, it is important to select one that is retained in the cell post-fixation to preserve the staining pattern. Excluding dead cells from the data allows cleaner separation and identification of cell populations. Brightfield images collected using the Invitrogen™ Attune™ CytPix™ Flow Cytometer confirm your gating to give you more confidence in your data (Figure 8). Invitrogen™ LIVE/DEAD™ fixable dead cell stains are fixable viability dyes that help you accurately assess the viability of cells in samples after fixation and/or permeabilization (Figure 9).

### Application spotlight—bacterial cell viability workflow

Flow cytometry methods can shorten bacterial phenotyping and counting time.

- To obtain a single bacterial cell suspension, beverages and solid foods should be weighed and homogenized
- Serial dilution is not necessary—just take a stained sample, dilute, and analyze
- Invitrogen™ LIVE/DEAD™ *Bac*Light™ kits can be used to quickly determine bacterial cell viability

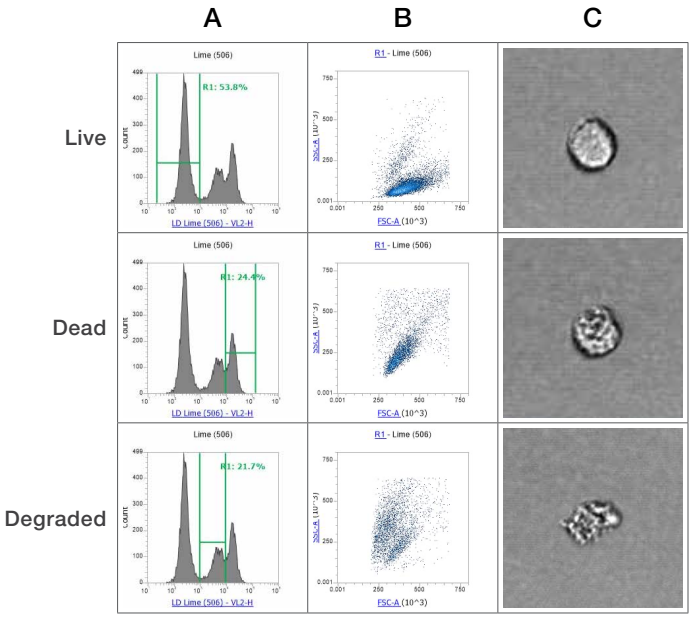



**Figure 8. Gating strategy using LIVE/DEAD stains. (A)** Histograms showing fluorescence from Jurkat cells stained with LIVE/DEAD Lime 506 stain. **(B)** Gating each peak reveals live, dead, and degraded populations. **(C)** Representative brightfield images showing morphological features that are consistent with the data in B. The images and flow cytometry data were collected simultaneously on the Attune CytPix Flow Cytometer.

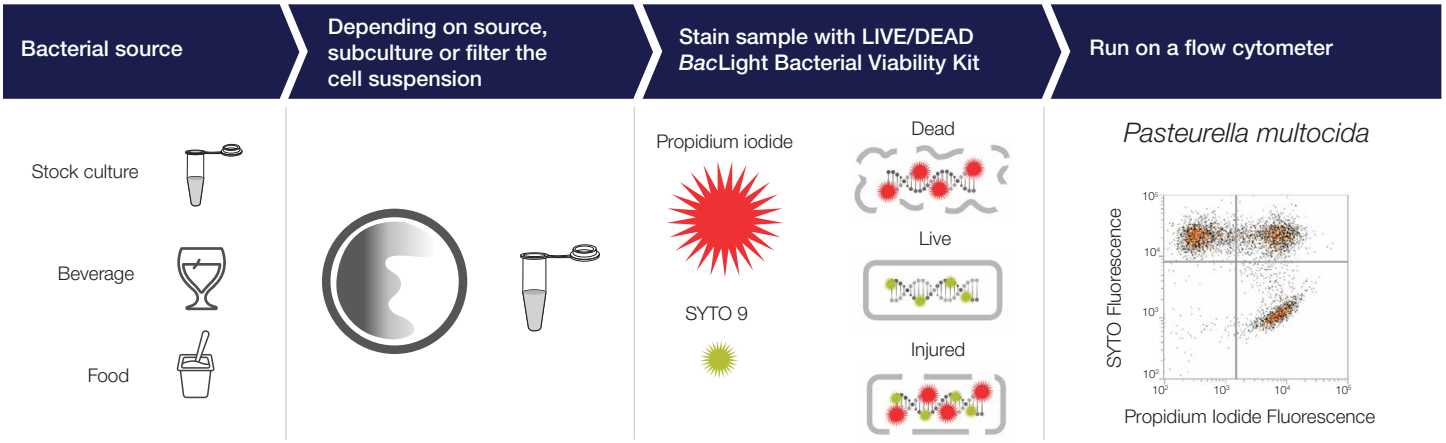



**Figure 9. Pasteurella multocida bacteria labeled with LIVE/DEAD *Bac*Light kit stains for 15 min.** Sample was analyzed on an Attune flow cytometer.

Find out more about cell viability dyes at **thermofisher.com/flow-cellviability**

## Cell proliferation

Cell proliferation analysis is important for drug development and cell tracing applications. Proliferation measurements are typically made based on average DNA content or on cellular metabolism parameters. Assays can report either total live-cell numbers or measure DNA synthesis in single cells. We offer dyes, kits, and antibodies to track proliferation. Use our guide to find suitable reagents for flow cytometry assays or multicolor panels.

### Example: generational tracing with CellTrace reagent

Invitrogen™ CellTrace™ reagents track cell division by analyzing cell subsets for dye dilution in successive generations (Figure 10). When cells proliferate, the fluorescence of each proliferating generation is half as bright compared with the previous generation. The CellTrace reagents help to monitor and visualize distinct generations of proliferating cells. With these reagents, you can observe one uniformly labeled cell population for each generation.

**Table 6. Flow cytometry reagent selection guide for cell proliferation assays.**

| Product | Target | Fixable | Live-cell analysis | Application |
|---|---|---|---|---|
| **Click-iT Plus EdU Flow Cytometry Assay Kits** | Incorporation into newly synthesized DNA | Yes | Yes | Cell proliferation |
| **BrdU** | Incorporation into newly synthesized DNA | Yes | Yes | Cell proliferation |
| **CellTrace Cell Proliferation Kits** | Lysine-containing proteins | Yes | Yes | Generational analysis |
| **Ki-67 antibody** | Nuclear protein expressed in proliferating cells | Yes | Yes | Cell proliferation and cell cycle |
| **Minichromosome maintenance (MCM2) antibody** | Nuclear protein expressed in proliferating cells | Yes | No | Cell proliferation and cell cycle |
| **Proliferating cell nuclear antigen (PCNA) antibody** | Nuclear protein expressed in proliferating cells | Yes | No | Cell proliferation and cell cycle |

"CellTrace Violet is the best reagent for tracking proliferation in any amenable cell type by fluorescent dye dilution and flow cytometry. Compared to CFSE, which is cytotoxic to cells when used at higher concentrations, CellTrace Violet labels cells brightly, with low toxicity and is faithfully distributed to daughter cells, ensuring the best possible peak resolution."

– Andrew Filby, Flow Cytometry Core Facility Manager and ISAC SRL Emerging Leader, Newcastle University

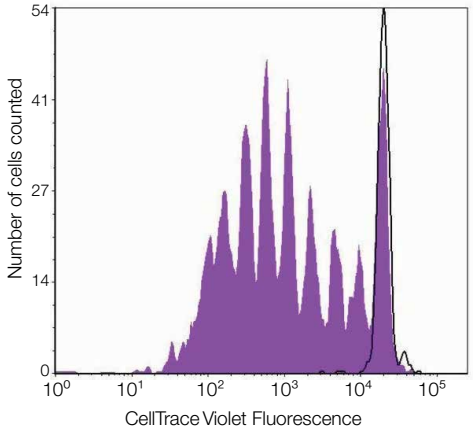



**Figure 10. Tracing cell divisions with CellTrace reagent.** Human peripheral blood lymphocytes were harvested and stained using the Invitrogen™ CellTrace™ Violet Cell Proliferation Kit. The violet peaks represent successive generations of cells stimulated with Invitrogen™ mouse anti–human CD3 and interleukin-2, and grown in culture for 7 days. The peak outlined in black represents cells that were grown in culture for 7 days with no stimulus.

Find out more about cell proliferation reagents at **thermofisher.com/flow-cellproliferation**

# RNA detection by flow cytometry

With the novel Invitrogen™ PrimeFlow™ RNA Assay, scientists can now reveal the dynamics of RNA and protein expression simultaneously within millions of single cells (Figure 11). This assay employs a proprietary fluorescence *in situ* hybridization (FISH) and branched DNA (bDNA) amplification (Figure 12) technique for simultaneous detection of up to four RNA transcripts labeled with Invitrogen™ Alexa Fluor™ 488, Alexa Fluor™ 568, Alexa Fluor™ 647, and Alexa Fluor™ 750 dyes, in a single cell using a standard flow cytometer. RNA detection may be combined with intracellular and cell-surface antibody staining to elevate the understanding of single-cell dynamics to a new dimension.

### Novel product applications:

- Unmask gene expression heterogeneity at the single-cell level
- Correlate RNA and protein levels in the same cell
- Detect noncoding RNA, microRNA (miRNA), and long noncoding RNA (lncRNA)
- Evaluate viral RNA in infected cells
- Analyze mRNA expression when antibody selection is limited
- Analyze up to four RNA transcripts simultaneously
- Detect telomere DNA

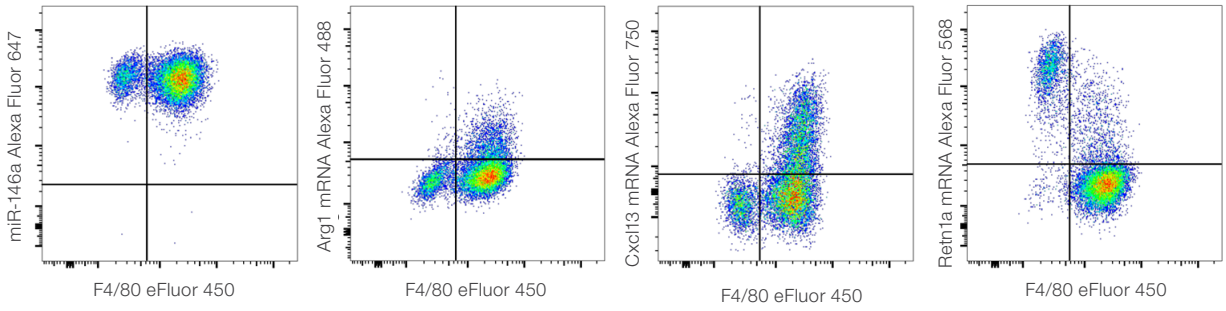



**Figure 11. PrimeFlow RNA Assay detection of miR-146a, Arg1 mRNA, Cxcl13 mRNA, and Retn1a mRNA in mouse peritoneal cells.** C57Bl/6 mouse resident peritoneal exudate cells were analyzed using the PrimeFlow RNA Assay. Cells were stained with Invitrogen™ eBioscience™ Anti–Mouse F4/80 eFluor 450 and Anti–Mouse CD11b PE-Cyanine7 antibodies, then fixed and permeabilized using PrimeFlow RNA Assay buffers and protocols. Cells were then hybridized to label RNA with Invitrogen™ Type 1 Human/Mouse miR146a Alexa Fluor 647, Type 4 Mouse Arg1 Alexa Fluor 488, Type 6 Mouse Cxcl13 Alexa Fluor 750, and Type 10 Mouse Retn1a Alexa Fluor 568 target probes. Viable $CD11b^+$ cells were used for analysis. Data show that both small peritoneal macrophages (SPM, $F4/80^-$) and large peritoneal macrophages (LPM, $F4/80^+$) were positive for miR-146a. SPM expressed high levels of Retn1a (Relm-alpha) mRNA, whereas LPM were positive for Cxcl13 mRNA and expressed low levels of Arg1 mRNA.

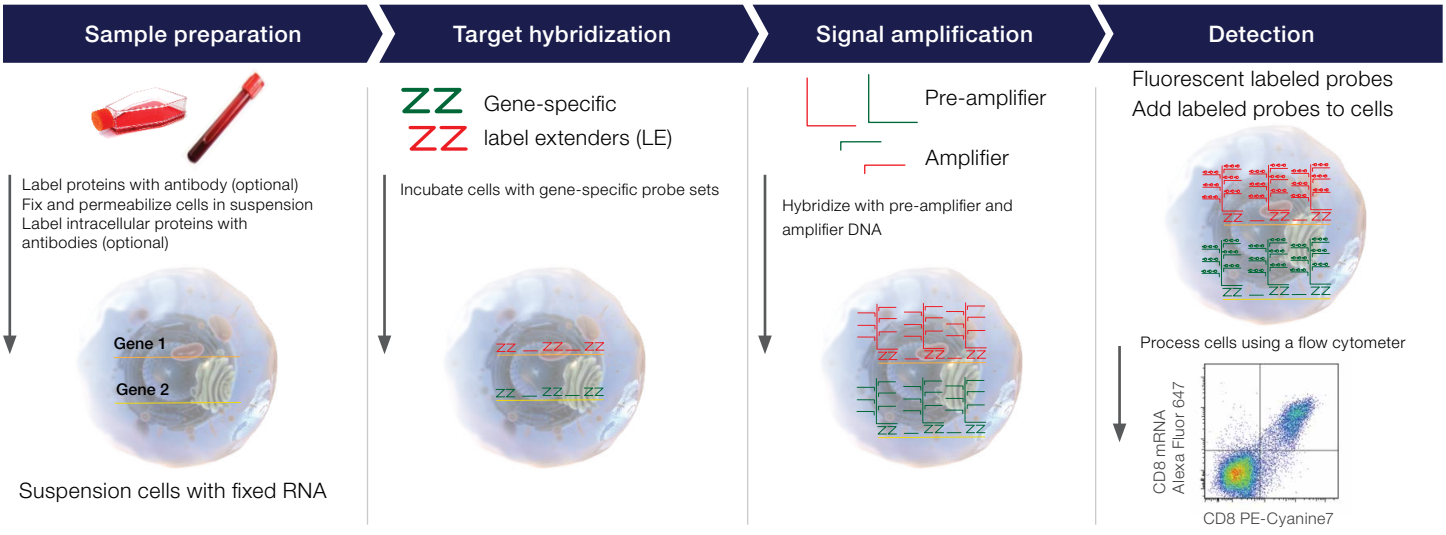



**Figure 12. The PrimeFlow RNA Assay workflow.** The assay workflow contains several steps: antibody staining; fixation and permeabilization, including intracellular staining, if desired; and target hybridization with a target-specific probe set containing 20–40 oligonucleotide pairs.

Find out more at **thermofisher.com/primeflow**

# Compensation and instrument beads

## Compensation beads for flow cytometry

Emission profiles of fluorophores are broad, which can result in overlapping profiles that require compensation for signal correction. Compensation can be set using beads, particularly when cell samples are limited or when a positive population is needed.

## The latest generation of compensation beads

Build flow cytometry panels with more accurate compensation using new Invitrogen™ UltraComp eBeads™ Plus Compensation Beads. When a fluorophore-conjugated antibody is added to the beads, both positive and negative populations result. UltraComp eBeads Plus Compensation Beads now offer:

- Increased species reactivity including rabbit- and human-origin antibodies (Figure 13)
- Compatibility with fluorophores excited by ultraviolet (355 nm), violet (405 nm), blue (488 nm), green (532 nm), yellow–green (561 nm), and red (633–640 nm) lasers
- Better compensation resolution for antibodies conjugated with Invitrogen™ eBioscience™ Super Bright 780, Brilliant Violet 711, or Brilliant Violet 786 dyes

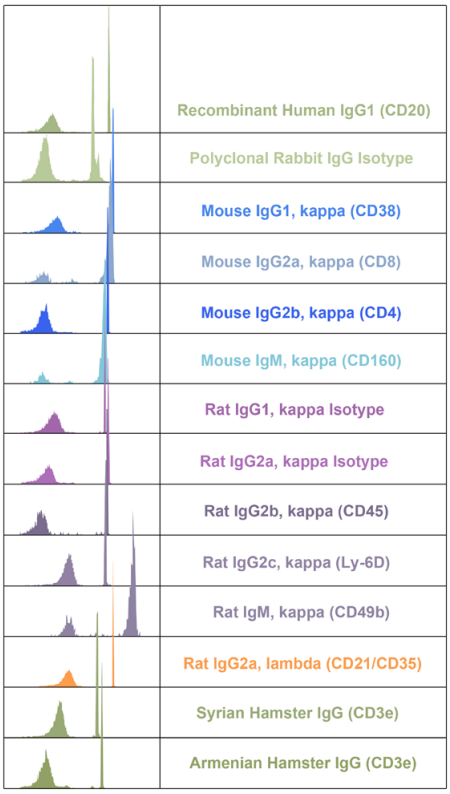



**Figure 13. Staining of UltraComp eBeads Plus Compensation Beads with 14 different antibody species.** Beads were stained with 0.25 µg of each antibody and analyzed by flow cytometry.

### Table 7. Invitrogen™ antibody compensation beads.

| | UltraComp eBeads™ Plus beads | UltraComp eBeads™ beads | OneComp eBeads™ beads | AbC™ Total Antibody Compensation Bead Kit* | ArC™ Amine Reactive Compensation Bead Kit | BrightComp eBeads™ beads (for FP) |
|---|---|---|---|---|---|---|
| **Application** | Immunophenotyping | | | | Cell viability assay | GFP, RFP, mCherry, YFP, and CFP expression; beads are present at 3 levels of the respective FP-like intensity |
| **Reactivity** | Human, rabbit, hamster, mouse, and rat antibodies | Hamster, mouse, and rat antibodies with recognition of the kappa and lambda chains | | Hamster, mouse, rabbit, and rat antibodies | LIVE/DEAD™ fixable dead cell stains* | GFP, RFP, mCherry, YFP, and CFP isoforms |
| **Format** | One vial: dispense as a single drop | | | 1 vial positive beads, 1 vial negative beads | | One vial: dispense as a single drop |
| **Laser compatibility** | Compatible with most standard lasers, UV to 633 nm; improved for polymer dye use from the violet laser | Compatible with most standard lasers, UV to 633 nm | Compatible with most standard lasers, but not with UV or violet lasers | Compatible with most standard lasers, UV to 633 nm | | 405 nm, 488 nm, and 561 nm |
| **Quantity** | 25 tests or 100 tests | | | | | 25 tests |
| **Cat. No.** | 01-3333-41<br>01-3333-42 | 01-2222-41<br>01-2222-42 | 01-1111-41<br>01-1111-42 | A10513<br>A10497 | A10628<br>A10346 | A10514, A54740, A54741, A54742, and A54743 |

* Also applicable to similar amine-reactive dyes.

## Counting beads

Absolute cell counts is a method for quantifying cell concentration or absolute count of cells in a sample. Benefits of our absolute counting beads include:

- Wide range of fluorophores to fit a broad spectrum (Figure 14)
- Accommodates most cell sizes with increased percentage of singlets

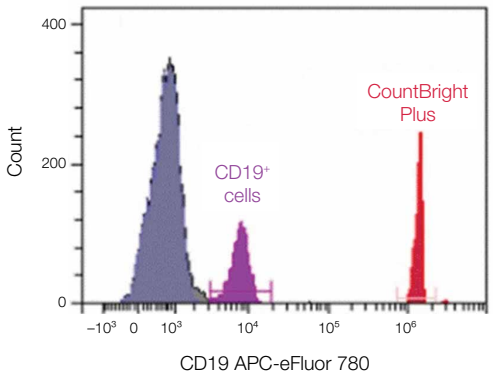



**Figure 14. CountBright Plus beads can be used with a broader range of fluorophores.** CountBright Plus beads (red) can be detected simultaneously with cells stained with Invitrogen™ CD19 APC-eFluor™ 780 antibody (pink) in lysed whole blood when excited with an IR laser (808 nm) with an 840/20 nm emission filter.

### Table 8. Invitrogen™ absolute counting beads.

| | CountBright™ Plus beads* | AccuCheck™ beads | | LIVE/DEAD™ *Bac*Light™ Bacterial Viability and Counting Kit** |
|---|---|---|---|---|
| **Parameters measured** | Cell concentration in sample | • Cell concentration in sample<br>• Pipetting accuracy | | • Viability<br>• Bacterial concentration in sample |
| **Sample type** | Any type | Whole blood | | Bacteria |
| **Bead size** | 4 µm | Bead A<br>6.40 µm | Bead B<br>6.36 µm | 6 µm |
| **Range** | Ex: UV–800 nm<br>Em: 385–860 nm | Bead A<br>Ex: 488 nm<br>Em: 575–585 nm | Bead B<br>Ex: 635 nm<br>Em: 660–680 nm | Ex: 488 nm<br>Em: 617 nm, 498 nm |
| **Cat. No.** | C36995 | PCB100 | | L34856 |

* The original Invitrogen™ CountBright™ Absolute Counting Beads are still available, but not compatible with IR-excitable fluorophores.
** Stains all cells, so a pure bacterial sample is required for accurate results.

## Calibration and size beads

Instrument calibration is critical to collecting and analyzing accurate experimental data. Our beads are designed to help ensure robust flow cytometer performance.

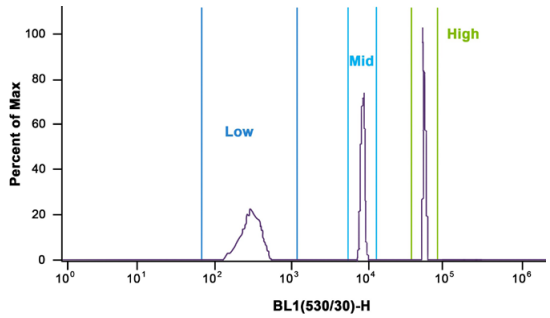



**Figure 15. ERF particles provide three fluorescence intensities.**

### Table 9. Invitrogen™ calibration beads.

| | Size calibration | | Instrument control | Alignment control | Fluorescence standardization |
|---|---|---|---|---|---|
| **Product** | Flow Cytometry Size Calibration Kit | Flow Cytometry Sub-micron Particle Size Reference Kit | Rainbow Calibration Particles | Alignflow™ Flow Cytometry Alignment Beads | AccuCheck ERF and Virocheck ERF Reference Particles |
| **Use** | Size reference | Size reference | Routine calibration of flow cytometers | Calibrate laser alignment | Standardization and calibration for inter- and intra-instrument data comparisons |
| **Emission** | No fluorescence | Green fluorescence | 400–680 nm | 3 types: 400–470 nm (for UV lasers), 515–660 nm (for blue lasers), or 645–680 nm (for red lasers) | AccuCheck ERF Beads, 415–910 nm<br>ViroCheck ERF Beads, 390–910 nm |
| **Bead size** | 6 sizes:<br>1.0–15 µm range | 6 sizes:<br>0.02–2.0 µm | 3.0–3.4 µm | 2 sizes: 2.5 or 6.0 µm diameter | AccuCheck ERF Beads, 3.2 µm<br>ViroCheck ERF Beads, 100 nm, 200 nm, 500 nm |
| **Cat. No.** | F13838 | F13839 | A34305 | 2.5 µm: A16502, A16500, A16501<br>6.0 µm: A16505, A16503, A16504 | AccuCheck ERF Beads, A55950<br>ViroCheck ERF Beads, V10425 |

Find out more about flow cytometry beads and controls at thermofisher.com/flow-controls

# Sample analysis: Attune flow cytometers, CytKick autosamplers, and automation

Run samples faster and achieve greater resolution—with minimal concern about sample loss due to clogging. Pairing an Attune flow cytometer with an Invitrogen™ CytKick™ Autosampler or an Invitrogen™ CytKick™ Max Autosampler combines precision and performance. Our benchtop flow cytometers are configurable with up to 4 lasers and 16 detection parameters. The Attune CytPix Flow Cytometer includes a high-speed brightfield camera and image analysis software capability that help you confirm that your gates contain cells of interest and discover new insights into your sample biology.

- **Flow and imaging data together**—a flow cytometry analyzer with brightfield imaging capabilities; morphological observations and image parameter measurements add to the richness of flow cytometry data more than multiplexed staining alone
- **Six fluorescence channels off the violet laser**—expand your capabilities in multicolor flow cytometry

- **Simplified sample prep**—no-wash, no-lyse sample prep options streamline your workflow
- **Flexibility**—switch between tubes and plates with a simple click of the mouse
- **Option for automation**—designed for walkaway performance with clog-resistant fluidics and robust data analysis software
- **Compatible**—mammalian cells, algae, bacteria, yeast, parasites, and plant cells can be successfully analyzed
- **Optional 21 CFR Part 11–compliant software**—adds security and auditing features to enable regulatory compliance in electronic record keeping

**Table 10. Specifications of Attune flow cytometers.**

| Attribute | Specification | Attune CytPix Flow Cytometer | Attune NxT Flow Cytometer |
|---|---|---|---|
| **Optics: fluorescence** | Laser excitation (nm) | Violet 405, blue 488, yellow 561, red 637 | Violet 405, blue 488, green 532, yellow 561, red 637 |
| | Emission filters | Up to 14 color channels with wavelength-tuned photomultiplier tubes (PMTs); user-changeable keyed filters | |
| **Optics: imaging** | Laser excitation | 405 nm | N/A |
| | Pulsed laser power | No warm-up delay; fiber not affected by “on/off” | N/A |
| | Pulse width | <50 ns | N/A |
| **Fluidics** | Flow cell | Quartz cuvette gel coupled to 1.2 numerical aperture (NA) collection lens, 200 x 200 µm | |
| | Sample analysis volume | 20 µL to 4 mL | |
| | Custom sample flow rates | 12.5–1,000 µL/min | |
| | Sample delivery | Positive displacement syringe pump for volumetric analysis | |
| **Performance: fluorescence detection** | Fluorescence sensitivity | ≤80 molecules of equivalent soluble fluorochrome (MESF) for FITC, ≤30 MESF for PE, ≤70 MESF for APC | |
| | Fluorescence resolution | CV below 3% for the singlet peak of propidium iodide–stained chicken erythrocyte nuclei (CEN) | |
| | Data acquisition rate | Up to 35,000 events/sec; 34 parameters; based on a 10% coincidence rate per Poisson statistics | |
| | Maximum electronic speed | 65,000 events/sec with all parameters | |
| | Carryover | Single-tube format: <1% | |
| | Forward and side scatter sensitivity | Able to discriminate platelets from noise | |
| | Minimum particle size | 0.2 µm on side scatter using the submicron bead calibration kit from Bangs Laboratories. 0.1 µm on side scatter under the following conditions: use of an Attune NxT Flow Cytometer with standard 0.5 mm blocking configuration, an Invitrogen Attune NxT 488/10 Filter (Cat. No. 100083194), and Attune Focusing Fluid (Cat. No. 4488621, 4449791, or A24904) that has been passed through a 0.025 µm filter. | |
| **Performance: imaging** | Pixel resolution | 0.3 µm per pixel | N/A |
| | Objective magnification | 20x | N/A |
| | Objective numerical aperture (NA) | 0.45 | N/A |
| | Theoretical resolution | 0.6 µm | N/A |
| | Detection limit | Visually detect 800 nm particles | N/A |
| | Image capture rate | 3,000–6,000 images per second (image size–dependent) | N/A |
| | Image size | 96 x 96 pixels to 248 x 248 pixels | N/A |
| | Field of view | 29 x 29 µm to 74 x 74 µm | N/A |
| **Image analysis software feature** | Trained models | leukocytes and beads | N/A |
| | Parameters/features | More than 25 image-derived parameters for event features categories: shape, intensity, object, pixel, and system | N/A |
| | Sample size range of models | 5–20 µm | N/A |
| | Image processing speed | Up to 1,000 images/second depending on image size and complexity | N/A |

Find out more about instruments and robotics at [thermofisher.com/attune](thermofisher.com/attune)

# Cell sorting and analysis: Bigfoot Cell Sorter

The Invitrogen™ Bigfoot™ Spectral Cell Sorter with Sasquatch Software (SQS) enables high-speed cell sorting. The Bigfoot Spectral Cell Sorter can be configured with up to nine lasers and 60 detectors for both standard fluorescence detection and spectral unmixing (Table 11). This cell sorter is:

- **Fast**—sort rates >70,000 events per second (EPS) and analysis rates of >100,000 EPS
- **Flexible**—capable of six-way sorting into tubes, four-way sorting into 96-well plates, eight-way sorting into 384-well plates, or straight-down sorting into 1,536-well plates, and multiple input options with temperature control, giving flexibility for all sorting applications
- **Precise**—custom-designed, programmable-logic hardware with algorithms developed specifically for the challenges presented by sorting; the resulting architecture allows operators to use either compensation or spectral unmixing in real time at a sort rate of >70,000 EPS
- **Safe**—integrated biocontainment system and aerosol management system (AMS) are designed to be fully integrated parts of the cell sorter


- **Easy to use**—automated software provides quick start-up, automated calibration, and accurate quality control (QC) combined with an experiment designer, intuitive interface, and efficient shutdown; remote access capability allows you to start up your instrument before reaching your lab, and system health information and email notifications save time and streamline your workflow

**Table 11. Specifications for a Bigfoot Spectral Cell Sorter.**

| | |
|---|---|
| **Excitation lasers (nm)** | 349, 405, 445, 488, 532, 561, 594, 640, and 785 |
| **Optical power** | Free space delivery of 349 nm (100 mW), 405 nm (100 mW), 445 nm (200 mW), 488 nm (125 mW), 532 nm (100 mW), 561 nm (100 mW), 594 nm (100 mW), 640 nm (100 mW), and 785 nm (100 mW) |
| **Beam alignment** | Fixed, 7 spatially separated pinholes |
| **Detection parameters** | 55 fluorescence + 5 scatter |
| **Scatter parameters** | Standard FSC and SSC, 488 nm; small particle FSC, 405 nm; depolarized FSC and SSC, 488 nm |
| **Scatter resolution** | <0.2 µm scatter resolution from background with small particle detector |
| **Pulse measurement** | Simultaneously measures peak, area, and width for sample input and output of every channel |
| **Fluorescence sensitivity** | <100 MESF for FITC, PE, and APC |

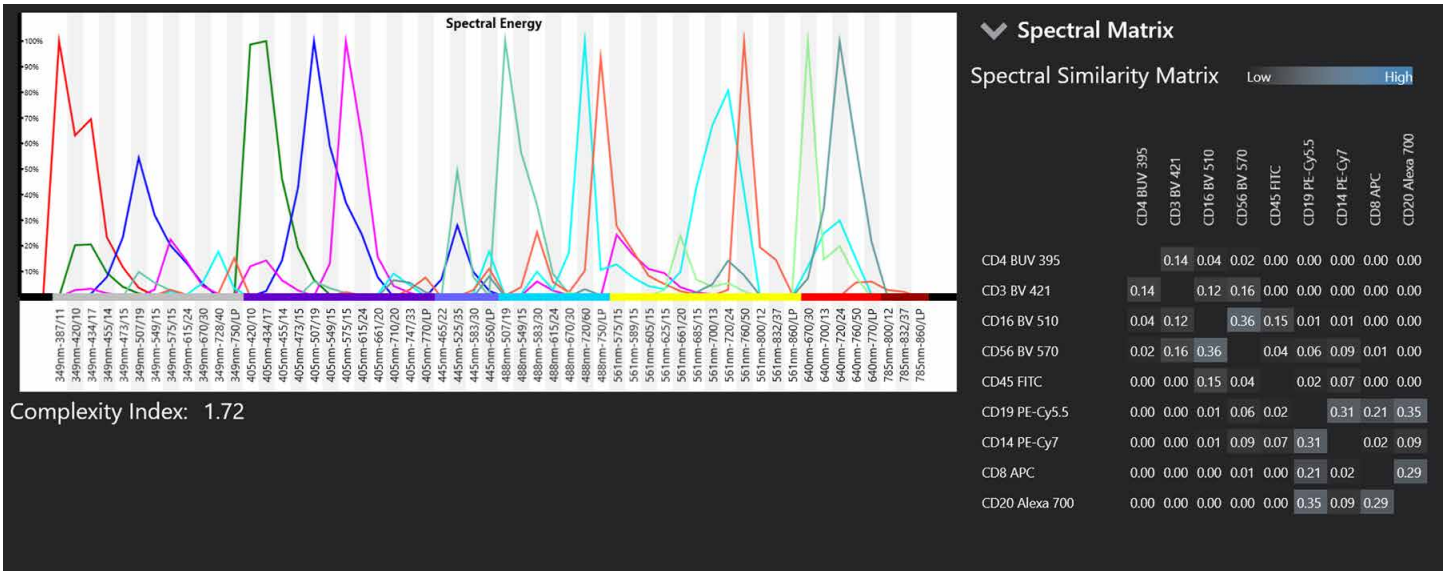



**Figure 16. Spectral signature graph.** In Sasquatch Software, you can see a visual representation of the selected panel of fluorophores. The graph provides a clear picture of where additional fluorophores can be added without overlapping with the current selections. A specific emission curve can be shaded by highlighting it in the Selected Fluorophores list.

"We welcomed the Bigfoot Spectral Cell Sorter to Babraham Institute in late August 2020, and it soon became apparent that the instrument was going to be a key piece of technology for our core facility. The designers of this cell sorter engaged with expert core managers when designing the machine, and this certainly shows now that it's in the lab. The machine is designed flexibly for a core facility. In fact, I call it the Swiss army knife of cytometers.

"Automation is key for us. The automated start-up, alignment, and QC on the Bigfoot instrument is amazing—17 minutes from start to finish. Automated start-up saves us at least an hour a day. That is an hour's more sorting that I can charge for in my core facility. That equates to 20 hours a month more capacity and more income into my facility. The Bigfoot Spectral Cell Sorter can run for hours with little intervention and it's quiet and bijou. The machine is so incredibly quiet that sometimes we have to question whether it's on and the hood is working—but we know it is. This makes working on the machine a dream, especially when you compare this to the long sorts my team has to do with ear defenders on whilst using other sorters which have separate hoods. What has impressed us most at Babraham is the size of the machine and its integrated hood. In fact, the Bigfoot instrument has a small footprint."

**Rachael Walker, Head of Flow Cytometry**
**Babraham Institute, Babraham**
**Cambridgeshire, England**

Find out more about the Bigfoot Spectral Cell Sorter at **thermofisher.com/bigfoot**

# Large multicolor experiments: instruments and reagents

Instruments with spectral capabilities enable larger multicolor experiments. Using a spectral flow cytometer and reagents maximizes detail and information about cell types and functions within a model system. Benefits include:

- Save limited sample by using many antibodies and functional dyes within one experiment
- Cohesive in-depth characterization of the immune system
- Easily design 20+ immunophenotyping panels on instruments with advanced capabilities

## Webtool for designing large experiments

The Flow Cytometry Panel Builder can be used to design panels for spectral flow cytometers. Experiments with more than 15 colors often require intimate knowledge of signatures and emission spectra. This webtool allows you to build complex experiments in a simplified program.

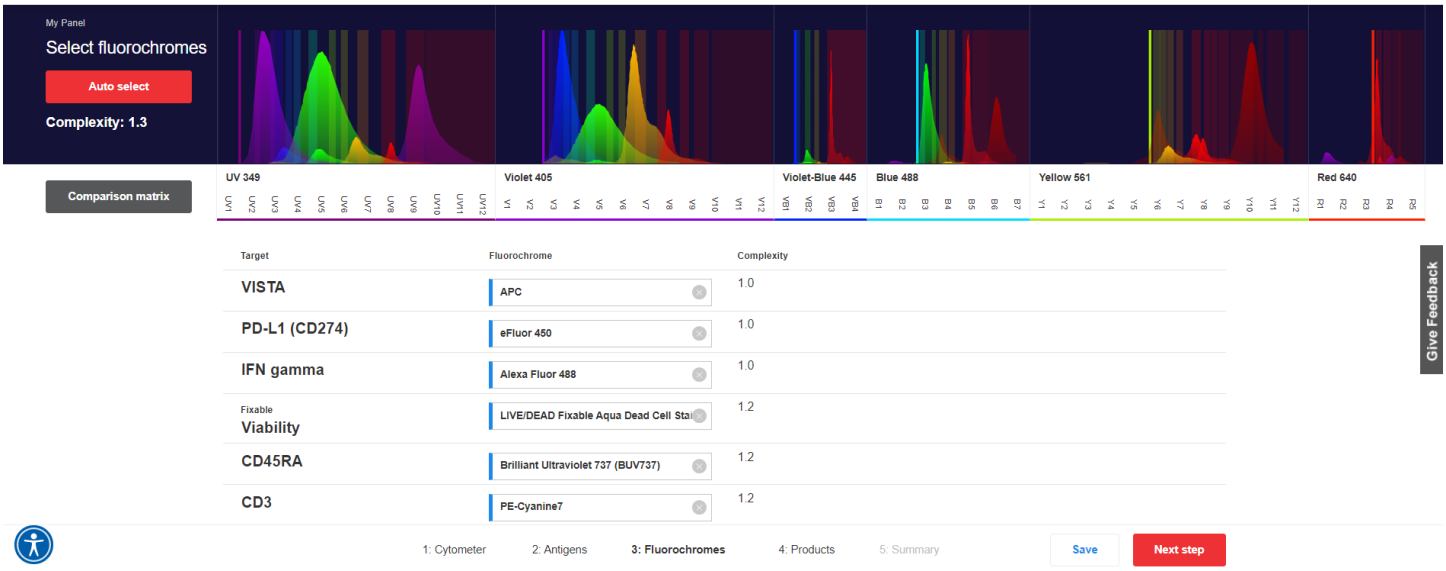


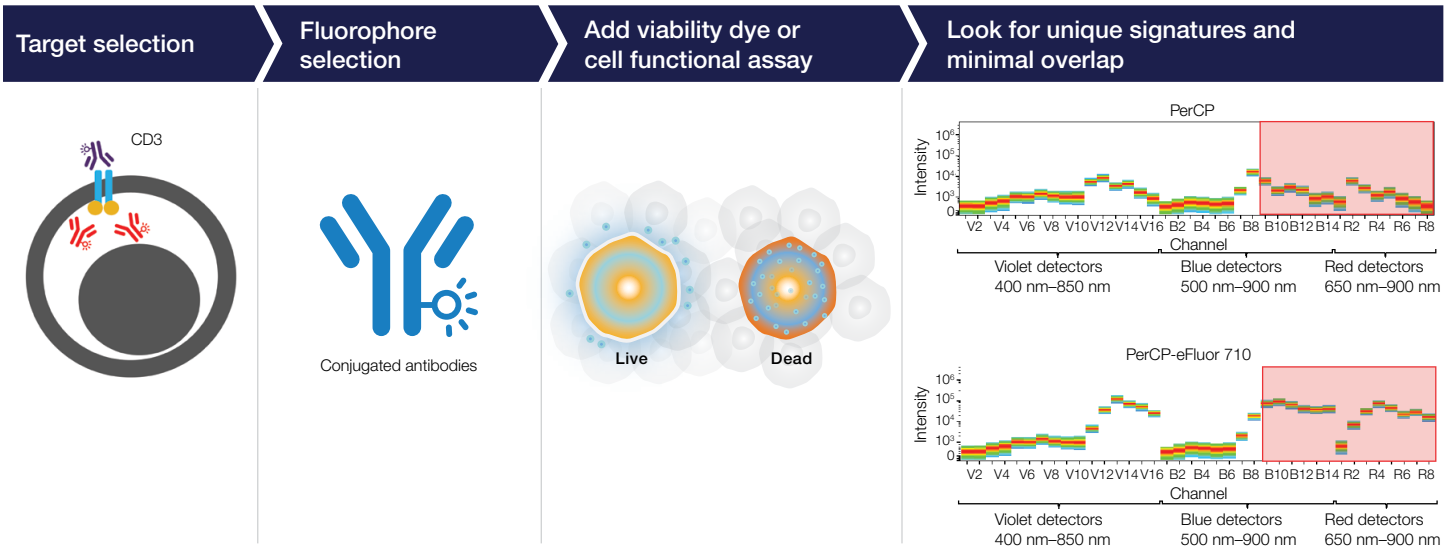

**Figure 17. Flow Cytometry Panel Builder.** Step 3 of the panel builder uses similarity and complexity index measurements to pick dyes with maximum individuality of signatures.

Our free, professional Flow Cytometry Panel Design Service includes building high-parameter panels for spectral flow cytometry.

For panel design service and the panel builder tool, visit [thermofisher.com/panelbuilder](thermofisher.com/panelbuilder)

## Application spotlight—immunophenotyping on a spectral flow cytometer

- Standard panel design rules apply
- Invitrogen™ fluorescent probes and reagents are suitable for all cytometry instrumentation, including spectral flow cytometers; use fluorophores designed for use with spectral instruments including NovaFluor dyes, Alexa Fluor 561 dye, and Alexa Fluor 660 dye
- Many previously incompatible labeling dyes and functional reagents, including PerCP and PerCP eFluor 710 dyes, can now be used together in your expanded multicolor application
- Expand your panel with Alexa Fluor 532, Pacific Orange, eFluor 450, and Super Bright 436 labels
- Dump channels are not recommended, as variations within spectral signatures can be detected by flow cytometry instruments with spectral capabilities




# Services and support

### Instrument service plans and warranties

Extended-coverage service plans are available at the time of instrument purchase. These service plans can help you can maximize system uptime, reduce overall repair costs, get rapid repair by a manufacturer-trained and certified field service engineer (FSE), extend instrument life, and help keep it running at peak performance. Choose from a variety of service options that balance budget, productivity, uptime, and regulatory requirements. Plans start with the most basic repair models and scale to premium offerings, including advanced support and compliance services.

### Technical support for flow cytometry experiments

Technical support and specialists assist with panel design and help you choose the correct antibodies for your needs, including new experiments and quality control. Each specialist helps you troubleshoot experiments and product performance issues. Our specialists also assist customers in designing and implementing complex flow cytometry panels (>30 colors), all remotely via phone or email. Services are available globally.

Build a personalized service quote at **thermofisher.com/serviceselector**

> "Our team includes a variety of experienced professionals with an average of 14 years of research experience. While we are technically oriented, our focus is the achievement and satisfaction of our customers and that is how we measure our own success."
>
> – Ricky Williams, Commercial Global Service and Support

## Ordering information

| Product | Cat. No. |
|---|---|
| **Cell stimulation reagents** | |
| Cell Stimulation Cocktail | 00-4970-93 |
| Concanavalin A (Con A) Solution (500X) | 00-4978 |
| Lipopolysaccharide (LPS) Solution (500X) | 00-4976 |
| Anti-Human CD3, Functional-Grade Purified (clone OKT3) | 16-0037 |
| Anti-Human CD28, Functional-Grade Purified (clone CD28.2) | 16-0289 |
| Macrophage Colony-Stimulating Factor (M-CSF) | PHC9504 |
| **Flow cytometry antibodies** | |
| eBioscience flow cytometry antibodies | thermofisher.com/flowantibodies |
| **Fixatives** | |
| eBioscience Flow Cytometry Staining Buffer | 00-4222-57 |
| FIX & PERM Cell Permeabilization Kit | GAS003 |
| eBioscience Intracellular Fixation and Permeabilization Buffer Set | 88-8824-00 |
| eBioscience Foxp3/Transcription Buffer Set | 00-5523-00 |
| **Viability dyes** | |
| LIVE/DEAD fixable dyes viability kits | thermofisher.com/livedead |
| **Bead controls** | |
| Compensation beads | thermofisher.com/compbeads |
| Counting beads | thermofisher.com/countingbeads |
| Calibration beads | thermofisher.com/calibrationbeads |
| **Instruments** | |
| Attune CytPix Flow Cytometer | thermofisher.com/cytpix |
| Attune NxT Flow Cytometer | thermofisher.com/attune |
| Bigfoot Spectral Cell Sorter | thermofisher.com/bigfoot |
| CytKick Autosampler | A42901 |
| CytKick Max Autosampler | A42973 |

Learn more at **thermofisher.com/flow**