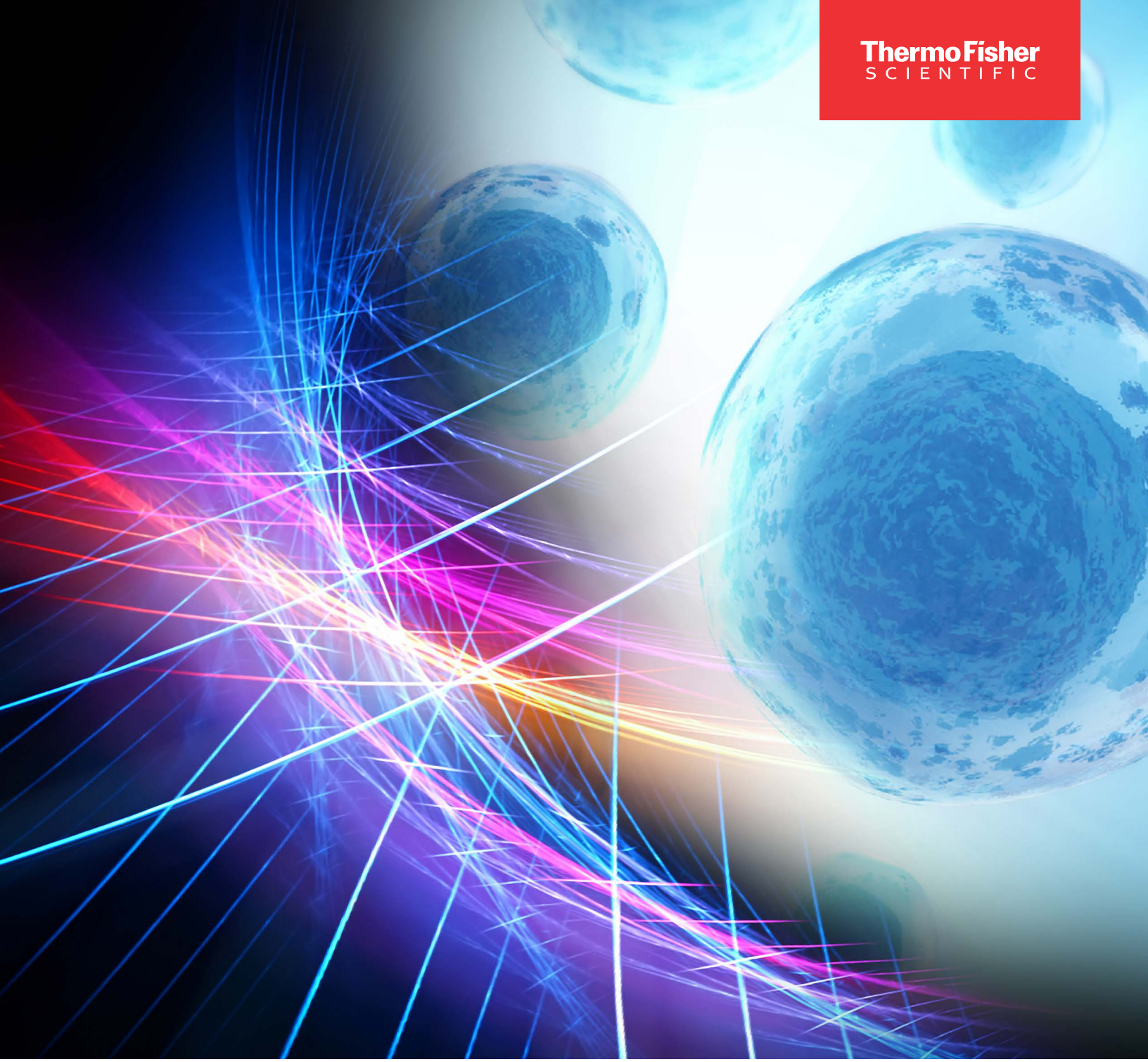


ThermoFisher SCIENTIFIC

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

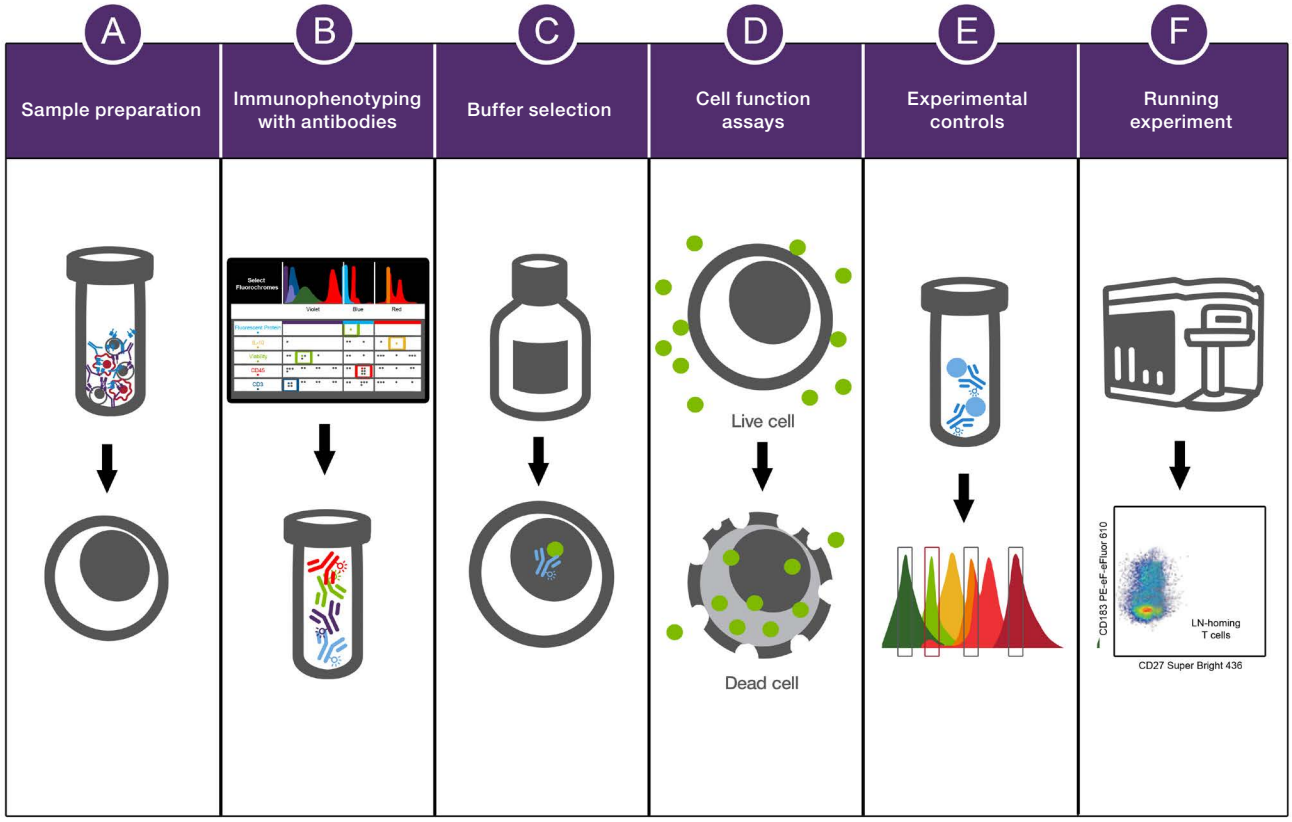


Figure 1. Flow cytometry workflow. Planning your workflow in advance as outlined will help generate a successful experiment.

Find out more about multipurpose flow cytometry experiments at thermofisher.com/flowcytometry

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

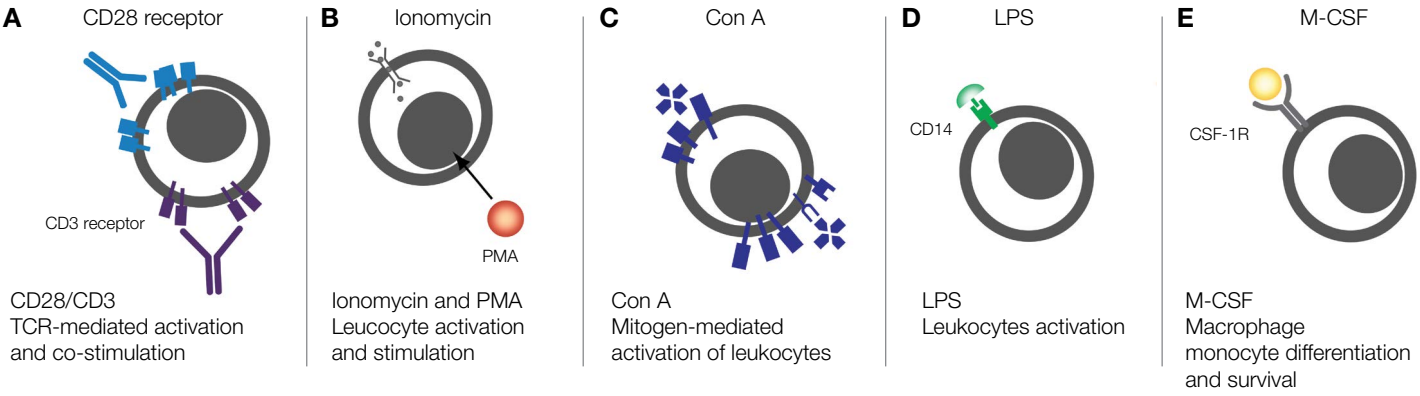


Figure 2. Cell stimulation reagents. (A) Functional-grade antibodies (e.g., anti-CD3 and anti-CD28) or Invitrogen™ Dynabeads™ magnetic beads for T cell activation and expansion. **(B)** eBioscience Cell Stimulation Cocktail comprising phorbol 12-myristate 13-acetate (PMA), a protein kinase activator, 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

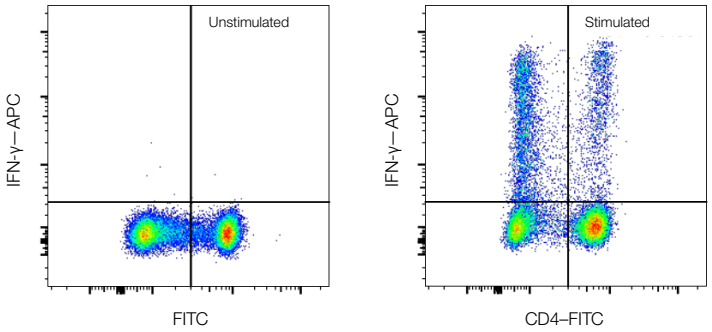


Figure 3. Identification of human Th17 cells within a CD4⁺ T cell population. Normal human peripheral blood cells were unstimulated (left) or stimulated with eBioscience Cell Stimulation Cocktail plus protein transport inhibitors (500X) (right). Cells were fixed and stained intracellularly with Invitrogen™ 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

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.

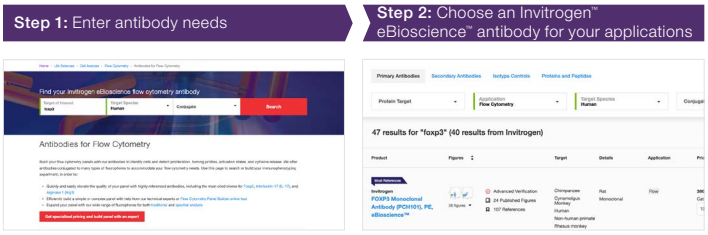


Figure 4. Antibody search tool to find information and purchase antibodies. (Left) Antibody application data from customer publications and internal testing data. (Right) A list of antibodies can be purchased, or saved and shared for later use.

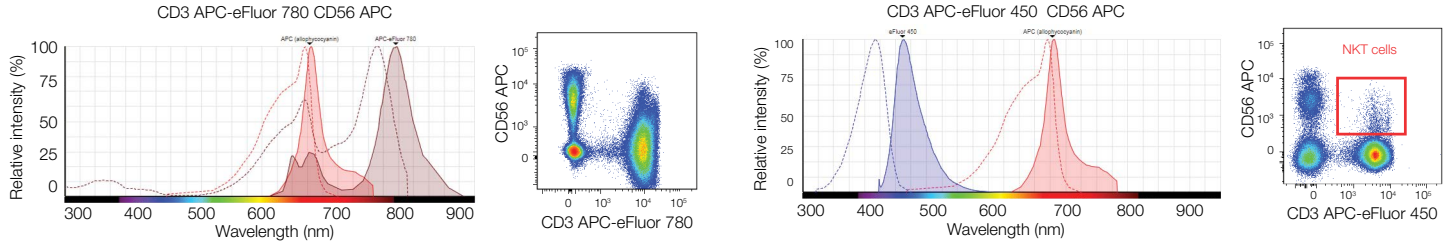


Figure 5. Normal human peripheral blood cells were stained with anti–human CD3 antibody conjugated with Invitrogen™ eBioscience™ APC-eFluor™ 780 dye (left) or eFluor™ 450 dye (right), as well as anti–human CD56 antibody conjugated with APC dye. Cells in the lymphocyte gate were used for analysis.

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 • Used in flow cytometry and imaging • Named for their excitation wavelengths	Alexa Fluor 405 Alexa Fluor 488 Alexa Fluor 532 Alexa Fluor 561	Alexa Fluor 647 Alexa Fluor 660 Alexa Fluor 700
	Invitrogen™ eBioscience™ eFluor™ organic dyes	• Engineered for detection for flow cytometry • Named for their emission wavelength	eFluor 450 eFluor 506	eFluor 660
Large, protein-based molecules	Original	• Cost-efficient • Some of the brightest dyes available	APC (allophycocyanin) PerCP (peridinin chlorophyll protein) PE (phycoerythrin)	
	Tandem dyes	• Dyes occupy different channels from the donor molecule, and this can be used to build larger panels	APC-Cyanine5 APC-Cyanine7 PE-Cyanine5 (TRI-COLOR dye) PE-Cyanine5.5 PE-Cyanine7 PE–Texas Red dye PerCP-Cyanine5.5	PE–Alexa Fluor 610 PE–Alexa Fluor 700 APC–Alexa Fluor 750 PE–eFluor 610 PerCP–eFluor 710 APC–eFluor 780
Polymer dyes	Invitrogen™ eBioscience™ Super Bright dyes and their tandems	• Excited by the 405 nm violet laser • Minimal spillover into other channels • 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 Super Bright 600 Super Bright 645 Super Bright 702 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 • 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 Brilliant Ultra Violet 496 Brilliant Ultra Violet 563 Brilliant Ultra Violet 615 Brilliant Ultra Violet 661 Brilliant Ultra Violet 737	Brilliant Ultra Violet 805 Brilliant Violet 421 Brilliant Violet 480 Brilliant Violet 650 Brilliant Violet 711 Brilliant Violet 786
Nanocrystals	Invitrogen™ Qdot™ dyes	• Narrow emission • Large Stokes shift	Qdot 605 Qdot 655	Qdot 705 Qdot 800
DNA-scaffold dyes—recent dye innovation	Invitrogen™ eBioscience™ NovaFluor™ dyes	• Unique spectral signatures • Variable brightness • Low cross-laser excitation • Named for the exciting laser and emission spectrum • Use Invitrogen™ CellBlox™ Blocking Buffer (Cat. No. B001T03F01) for use with NovaFluor dyes to label cells to block non-specific labeling and to reduce background	NovaFluor Blue 510 NovaFluor Blue 530 NovaFluor Blue 555 NovaFluor Blue 585 NovaFluor Blue 610-30S NovaFluor Blue 610-70S NovaFluor Blue 660-40S NovaFluor Blue 660-120S NovaFluor Yellow 570	NovaFluor Yellow 590 NovaFluor Yellow 610 NovaFluor Yellow 660 NovaFluor Yellow 690 NovaFluor Yellow 700 NovaFluor Yellow 730 NovaFluor Red 660 NovaFluor Red 685 NovaFluor Red 700 NovaFluor Red 710

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

Creating a flow cytometry panel

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

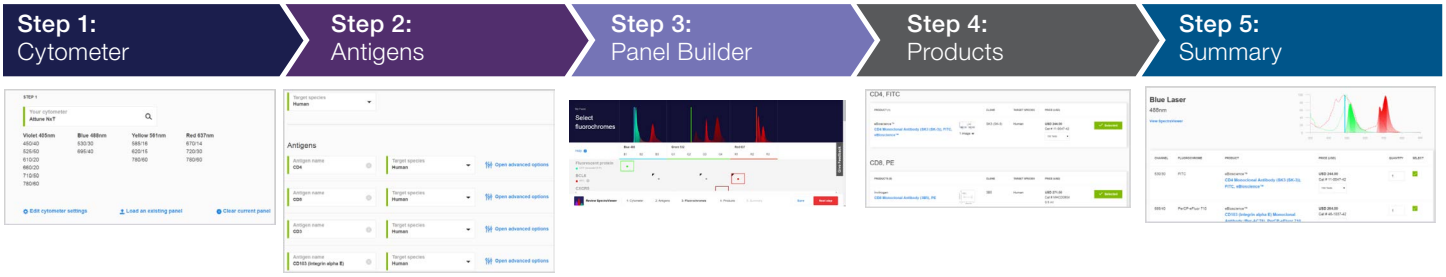


Figure 6. The Flow Cytometry Panel Builder simplifies experimental design with a 5-step strategy.

Plan your experiment at **thermofisher.com/flowpanel**

NovaFluor dyes for immunophenotyping

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

Benefits of NovaFluor dyes include:

- **Unique spectral signatures**—there are four fluorophores with individual 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 non-specific 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 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 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

* 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 (RUO and clinical research–grade) or **Intracellular Fixation and Permeabilization Buffer Set (RUO)**	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).

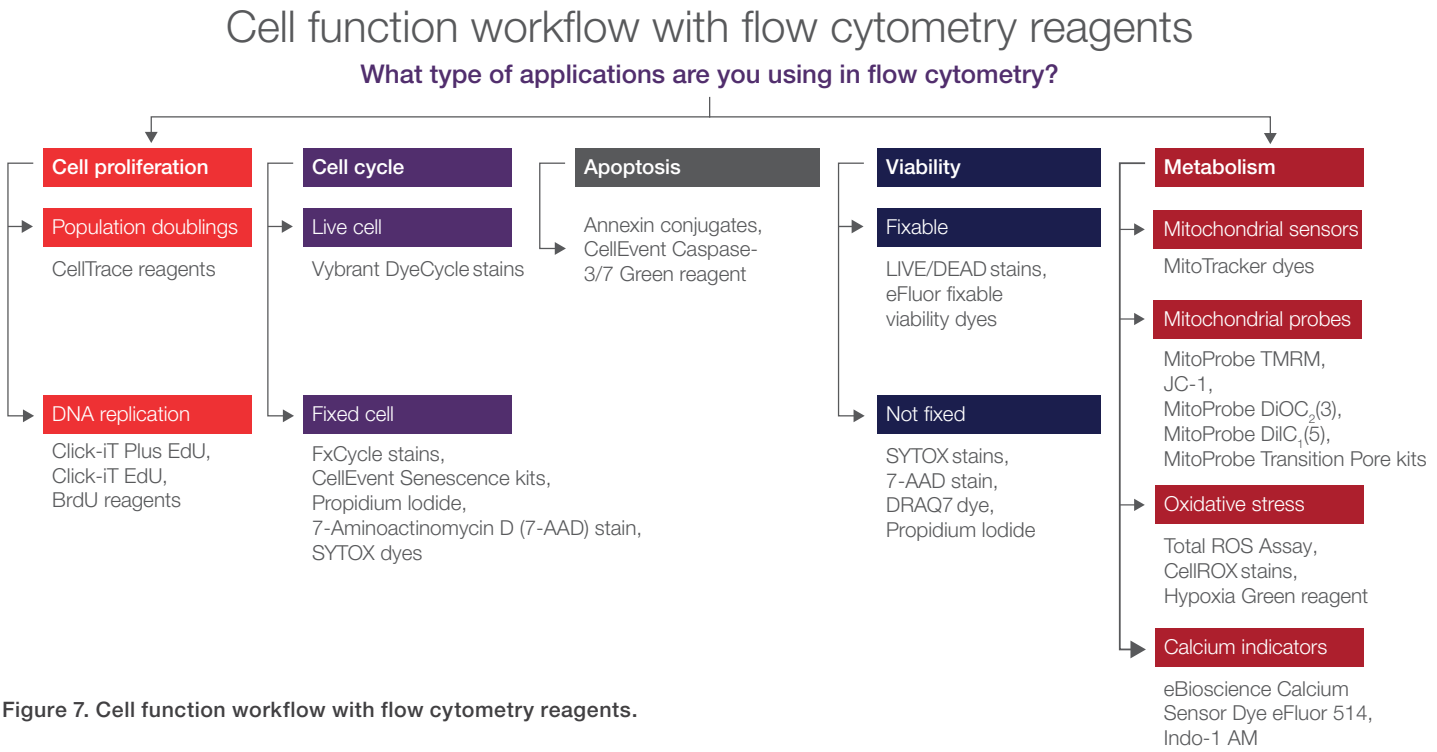


Figure 7. Cell function workflow with flow cytometry reagents.

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

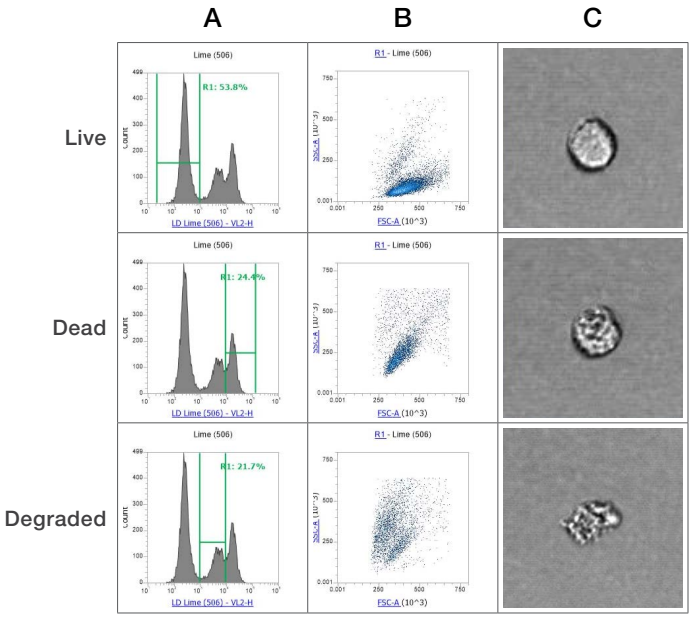


Figure 8. Gating strategy using LIVE/DEAD stains. (A) Histograms showing fluorescence from Jurkat cells stained with LIVE/DEAD Lime 506 stain. **(B)** Gating each peak reveals live, dead, and degraded populations. **(C)** Representative brightfield images showing morphological features that are consistent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

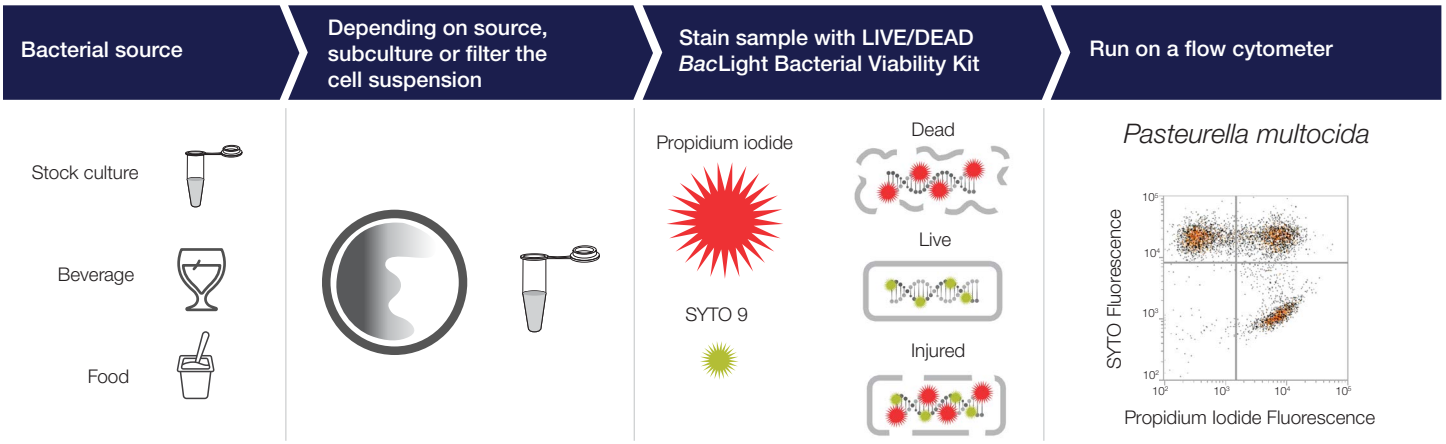


Figure 9. Pasteurella multocida bacteria labeled with LIVE/DEAD *Bac*Light kit stains for 15 min. Sample was analyzed on an Attune flow cytometer.

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

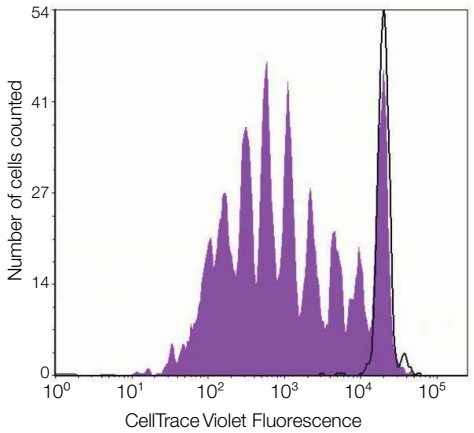


Figure 10. Tracing cell divisions with CellTrace reagent. Human peripheral blood lymphocytes were harvested and stained using the Invitrogen™ CellTrace™ Violet Cell Proliferation Kit. The violet peaks represent successive generations of cells stimulated with Invitrogen™ mouse anti–human CD3 and interleukin-2, 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

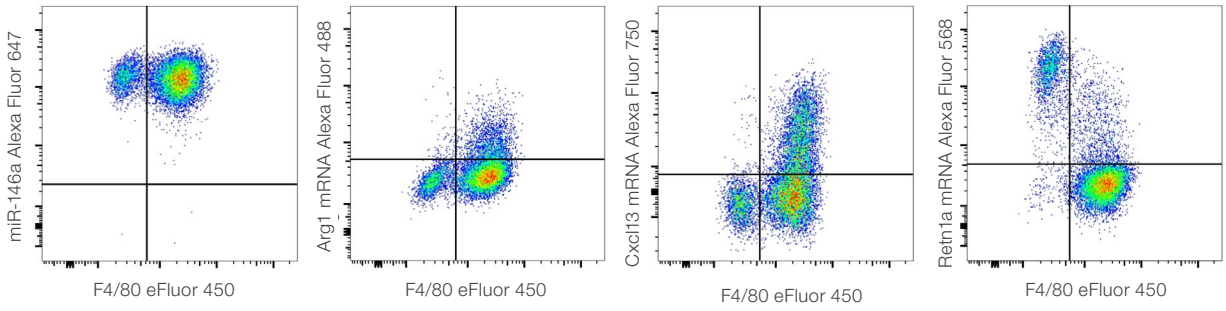


Figure 11. PrimeFlow RNA Assay detection of miR-146a, Arg1 mRNA, Cxcl13 mRNA, and Retn1a mRNA in mouse peritoneal cells. C57Bl/6 mouse resident peritoneal exudate cells were analyzed using the PrimeFlow RNA Assay. Cells were stained with Invitrogen™ eBioscience™ Anti–Mouse F4/80 eFluor 450 and Anti–Mouse 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.

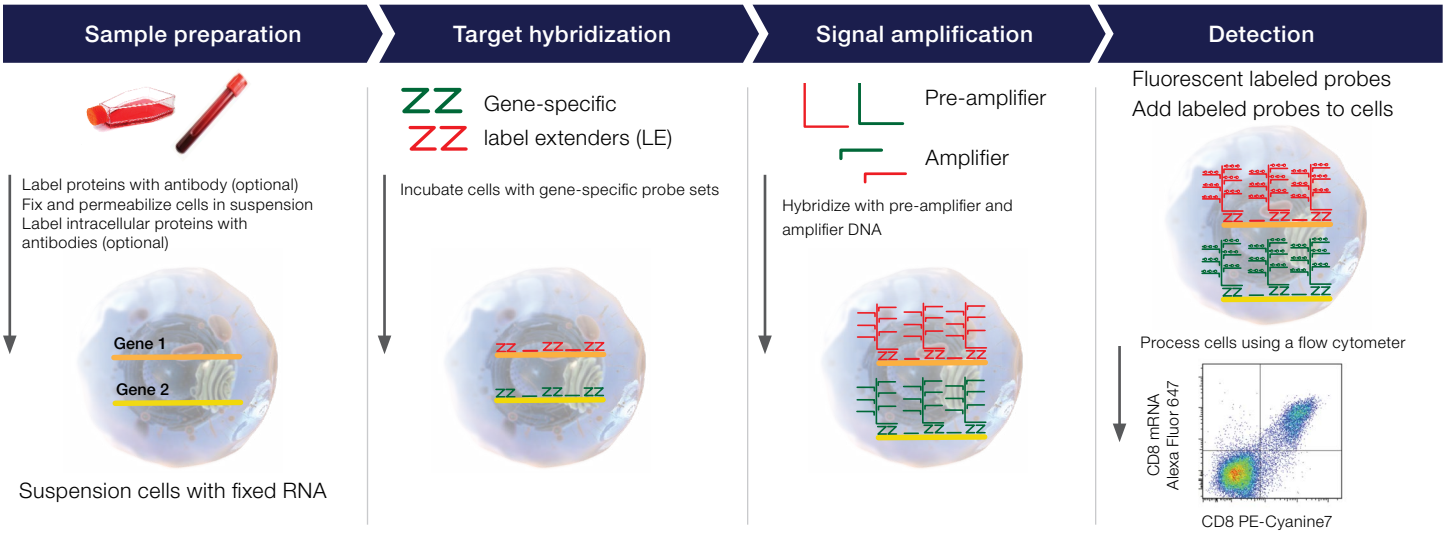


Figure 12. The PrimeFlow RNA Assay workflow. The assay workflow contains several steps: antibody staining; fixation and permeabilization, including intracellular staining, if desired; and target hybridization with a target-specific probe set containing 20–40 oligonucleotide pairs.

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

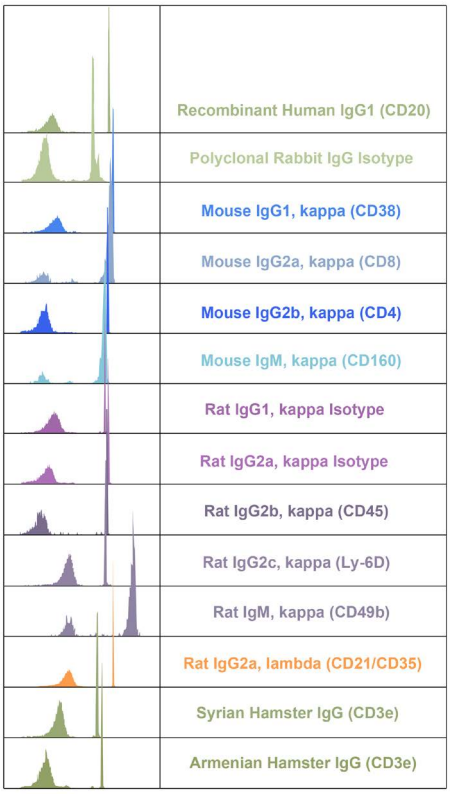


Figure 13. Staining of UltraComp eBeads Plus Compensation Beads with 14 different antibody species. Beads were stained with 0.25 µg of each antibody and analyzed by flow cytometry.

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	GFP BrightComp eBeads™ beads
Application	Immunophenotyping				Cell viability assay	GFP expression; beads are present at 3 levels of GFP-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 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		488 nm
Quantity	25 tests or 100 tests					25 tests
Cat. No.	01-3333-41 01-3333-42	01-2222-41 01-2222-42	01-1111-41 01-1111-42	A10513 A10497	A10628 A10346	A10514

* 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

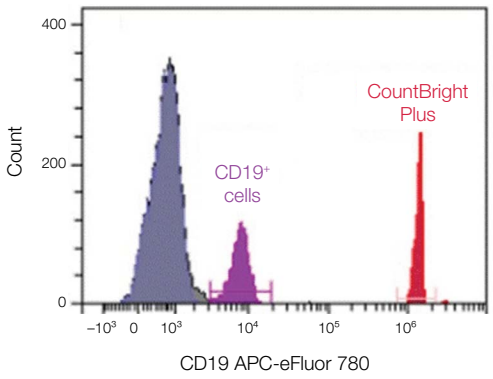


Figure 14. CountBright Plus beads can be used with a broader range of fluorophores. CountBright Plus beads (red) can be detected simultaneously with cells stained with Invitrogen™ CD19 APC-eFluor™ 780 antibody (pink) in lysed whole blood when excited with an IR laser (808 nm) with an 840/20 nm emission 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 • Pipetting accuracy		• Viability • Bacterial concentration in sample
Sample type	Any type	Whole blood		Bacteria
Bead size	4 µm	Bead A 6.40 µm	Bead B 6.36 µm	6 µm
Range	Ex: UV–800 nm Em: 385–860 nm	Bead A Ex: 488 nm Em: 575–585 nm	Bead B Ex: 635 nm Em: 660–680 nm	Ex: 488 nm 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.

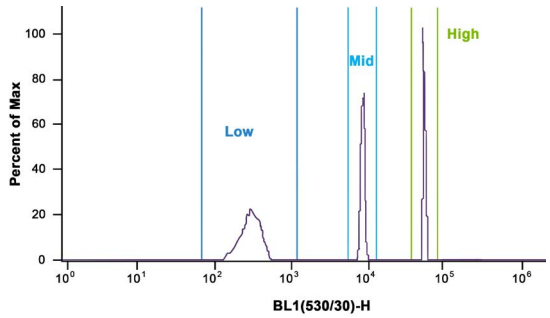


Figure 15. ERF particles provide three fluorescence intensities.

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 ViroCheck ERF Beads, 390–910 nm
Bead size	6 sizes: 1.0–15 µm range	6 sizes: 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 ViroCheck ERF Beads, 100 nm, 200 nm, 500 nm
Cat. No.	F13838	F13839	A34305	2.5 µm: A16502, A16500, A16501 6.0 µm: A16505, A16503, A16504	AccuCheck ERF Beads, A55950 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 little fear of 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 that helps you visually confirm that your gates contain cells of interest and discover relevant morphology.

- **Transform your research**—a flow cytometry analyzer with brightfield imaging capabilities; morphological information from images adds 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

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

Find out more about instruments and robotics at thermofisher.com/attune

Cell sorting and analysis: Bigfoot Spectral 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

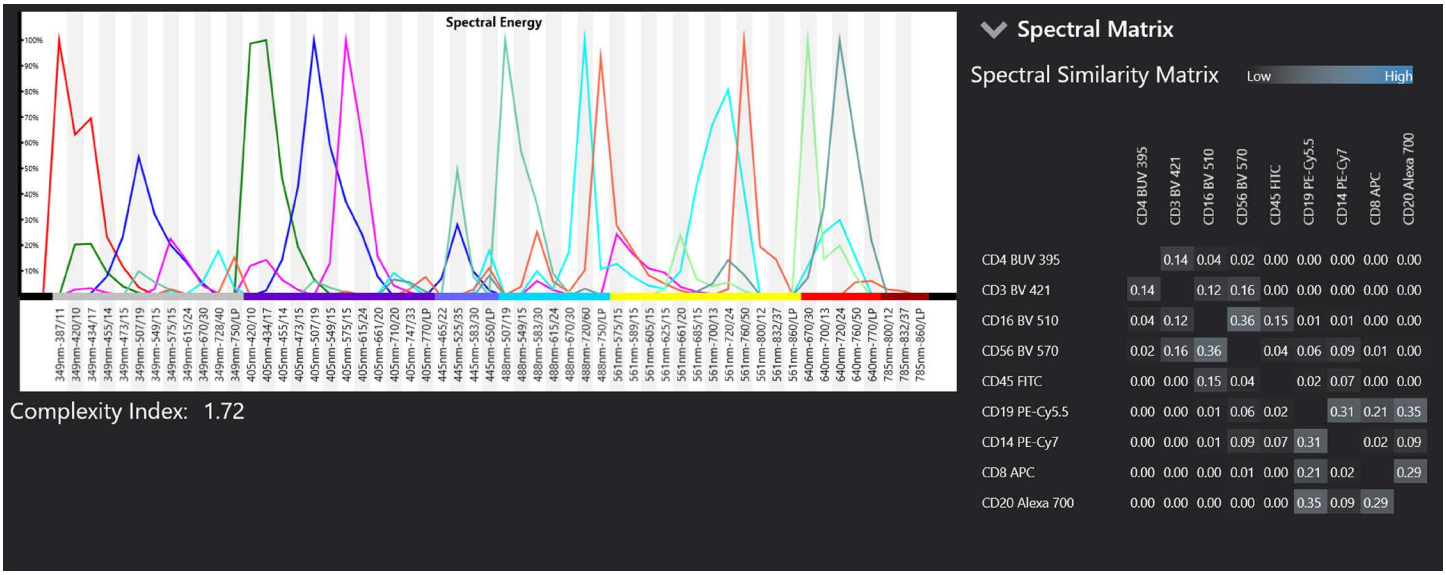


Figure 16. Spectral signature graph. In Sasquatch Software, you can see a visual representation of the selected panel of fluorophores. The graph provides a clear picture of where additional fluorophores can be added without overlapping with the current selections. A specific emission curve can be shaded 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 Invitrogen™ 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.

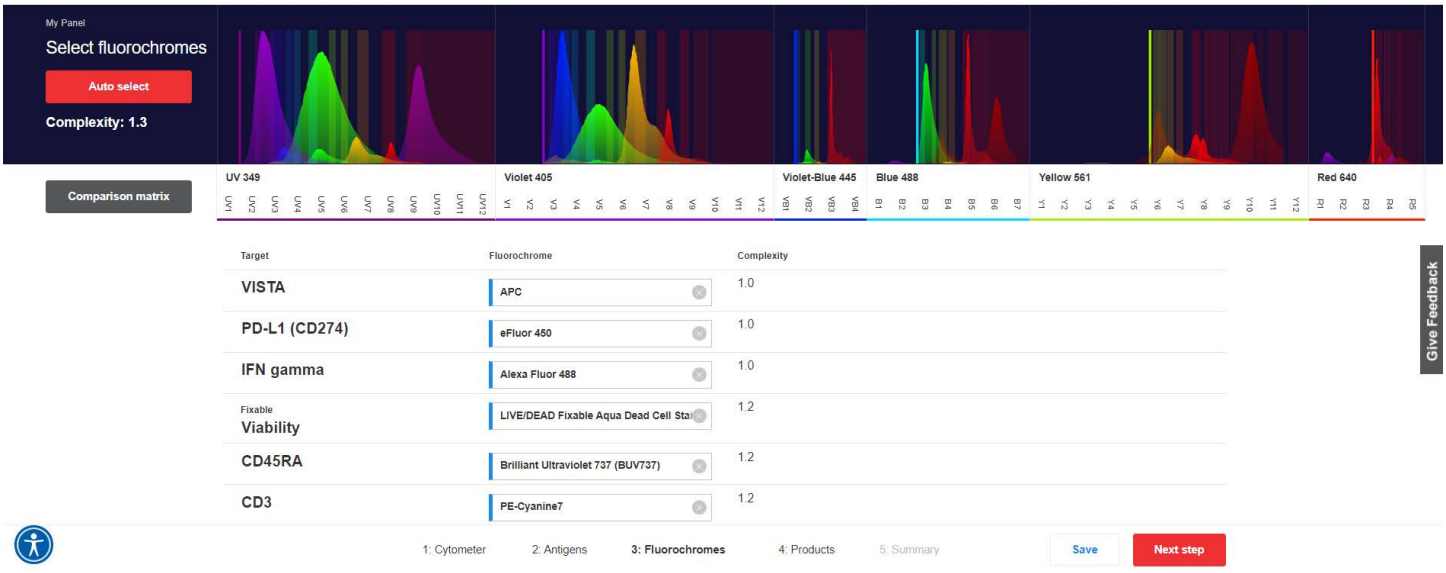
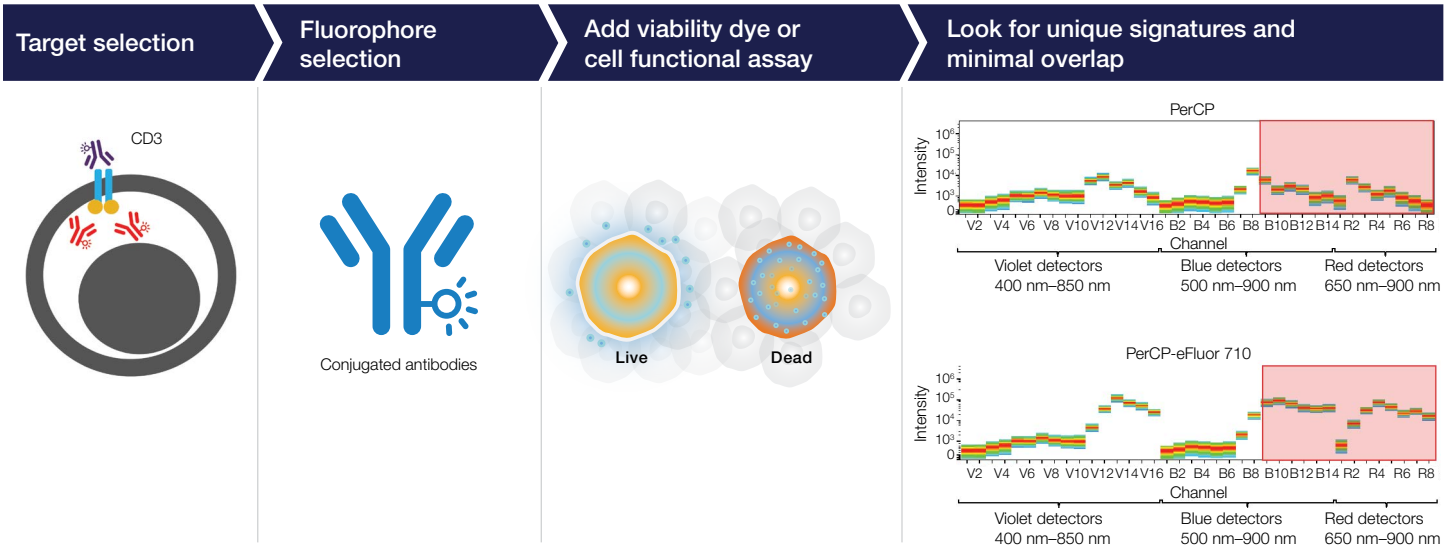


Figure 17. Flow Cytometry Panel Builder. Step 3 of the panel builder uses similarity and complexity index measurements to pick dyes with maximum individuality of signatures.

Use the tool at 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 Blue (450) Viability Kit, for UV excitation	L34961
LIVE/DEAD Fixable Violet (451) Viability Kit, for 405 nm excitation	L34963
LIVE/DEAD Fixable Lime (506) Viability Kit, for 405 nm excitation	L34989
LIVE/DEAD Fixable Aqua (526) Viability Kit, for 405 nm excitation	L34965
LIVE/DEAD Fixable Yellow (575) Viability Kit, for 405 nm excitation	L34967
LIVE/DEAD Fixable Green (520) Viability Kit, for 488 nm excitation	L34969
LIVE/DEAD Fixable Olive (557) Viability Kit, for 488 nm excitation	L34977
LIVE/DEAD Fixable Orange (602) Viability Kit, for 561 nm excitation	L34983
LIVE/DEAD Fixable Red (615) Viability Kit, for 488 or 561 nm excitation	L34971
LIVE/DEAD Fixable Far Red (665) Viability Kit, for 633 nm excitation	L34973
LIVE/DEAD Fixable Scarlet (723) Viability Kit, for 633 nm excitation	L34986
LIVE/DEAD Fixable Near IR (775) Viability Kit, for 633 nm excitation	L34975
LIVE/DEAD Fixable Near IR (780) Viability Kit, for 633 nm excitation	L34992
LIVE/DEAD Fixable Near IR (876) Viability Kit, for 808 nm excitation	L34980
Bead controls	
UltraComp eBeads Compensation Beads	01-2222-41
UltraComp eBeads Plus Compensation Beads	01-3333-42
AbC Total Antibody Compensation Bead Kit	A10497
ArC Amine Reactive Compensation Bead Kit	A10346
GFP BrightComp eBeads Compensation Beads	A10514
CountBright Plus Absolute Counting Beads	C36995
AccuCheck Counting Beads	PCB100
AccuCheck ERF Reference Particles	A55950
LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit	L34856
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**