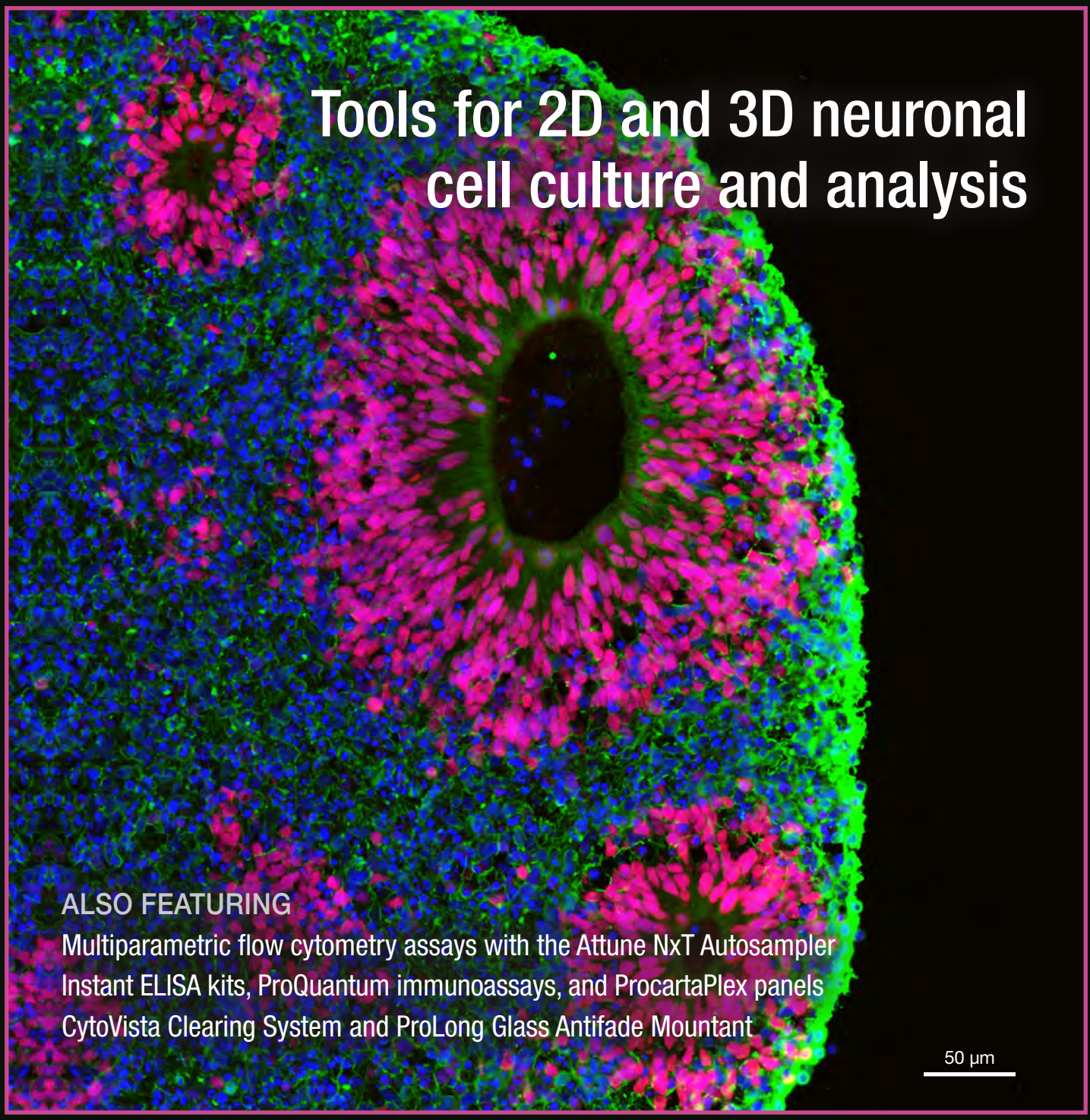
BIOPROBES 78

JOURNAL OF CELL BIOLOGY APPLICATIONS NOVEMBER 2018



Tools for 2D and 3D neuronal cell culture and analysis

ALSO FEATURING

Multiparametric flow cytometry assays with the Attune NxT Autosampler

Instant ELISA kits, ProQuantum immunoassays, and ProcartaPlex panels

CytoVista Clearing System and ProLong Glass Antifade Mountant

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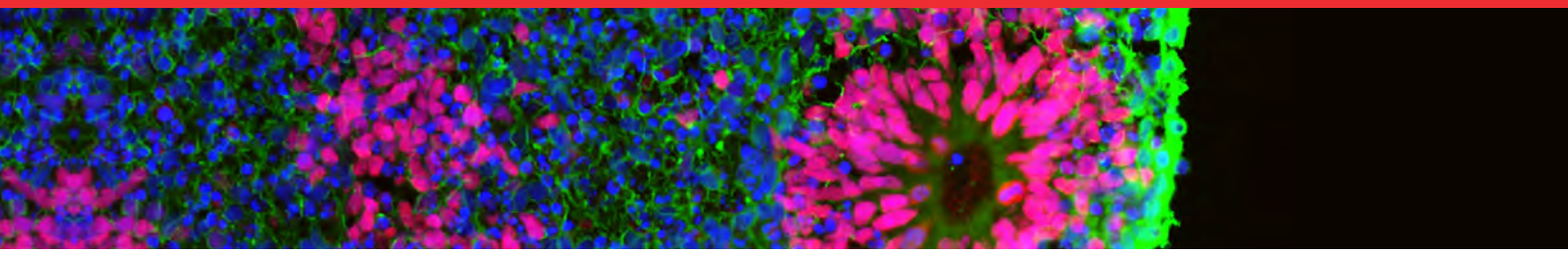

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BioProbes Journal, available in print and online at **thermofisher.com/bioprobes**, is dedicated to providing researchers with the very latest information about cell biology products and their applications. For a complete list of our products, along with extensive descriptions and literature references, please see our website.

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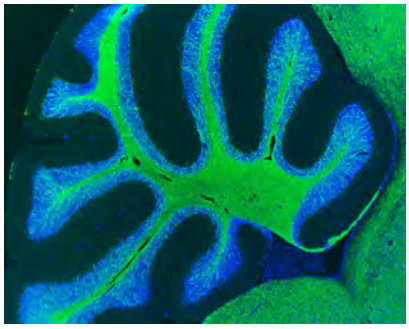
Watch. Apply. Publish: Five Steps for Publication-Quality Immunohistochemistry Imaging

In this on-demand webinar, Jason Kilgore, Technical Applications Scientist at Thermo Fisher Scientific, walks viewers through each of the steps in an immunohistochemistry (IHC) application workflow—from sample preparation, embedding, and blocking through antibody labeling, mounting, and imaging—and provides specific details and tips to obtain publication-quality images. Because IHC protocols use primary and secondary antibodies, it is critical that the concentrations and conditions be optimized to prevent artifacts, nonspecific background signals, and inadequate target detection. This presenter has firsthand experience developing these protocols and draws upon four decades of IHC product development by Thermo Fisher Scientific. Access the on-demand webinar now at **thermofisher.com/ihc5steps**.



Search and find your ELISA kits with ease: The updated ELISA web experience

Our ELISA search tool has been renovated to make it easier than ever to find the right ELISA kit for your experiments. New features include:

- Improved search—just type in the protein target (including synonyms), gene symbol, or analyte
- A search results display that compares kit features side by side, including sensitivity, assay range, sample volume, and time-to-result
- Revised product detail pages with easy-to-find product information

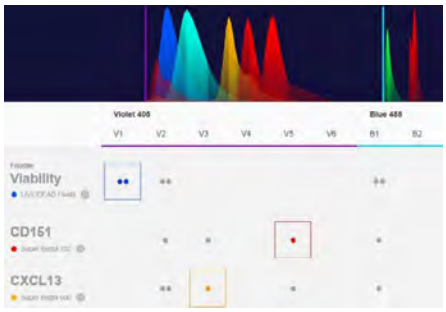
Use the ELISA search tool to choose your next round of ELISA kits. Try it for yourself at **thermofisher.com/elisa**.

Experience the iWestern workflow: An intelligent start-to-finish western blotting solution

Streamline your western blotting with the Invitrogen™ iWestern™ workflow, an inventive take on the decades-long problem of getting the desired results from the often elusive and time-consuming western blot. Each step of the iWestern workflow—from protein separation and transfer to detection and analysis—is addressed with the innovative Invitrogen™ Mini Gel Tank, Invitrogen™ iBlot™ 2 Dry Blotting System, Invitrogen™ iBind™ Western Systems, and Invitrogen™ iBright™ Imaging Systems. Each of these devices was meticulously developed to optimize processing efficiency, reproducibility, and robustness of results. Discover the iWestern workflow at **thermofisher.com/iwestern**.

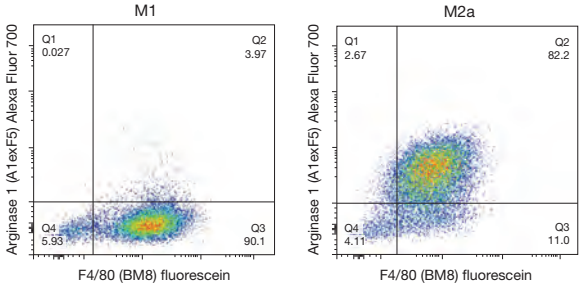
The Flow Cytometry Panel Builder—A tool for all flow cytometrists

Whether you are a novice or an expert, designing a panel for flow cytometry is a highly complex process. If you are a beginner, let the Invitrogen™ Flow Cytometry Panel Builder lessen your panel building anxiety by making the pairing of markers and fluorophores quick and simple using a highly visual format. Are you an expert? Then you will appreciate using the Flow Cytometry Panel Builder to easily review the spectral signals and filters per laser line and check fluorophore spillover values per channel. With access to over 13,000 antibodies for flow cytometry, this tool allows quick identification of antibodies for flow cytometry panels. Get started building your panel today at **thermofisher.com/flowpanel**.



Behind the Bench blog: A1exF5, a novel arginase 1 monoclonal antibody for flow cytometry

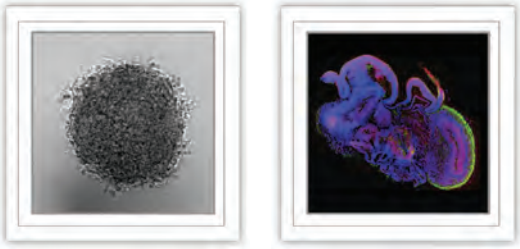
The Invitrogen™ Arginase 1 Monoclonal Antibody A1exF5 recognizes both the human and mouse forms of arginase 1, a 35 kDa cytosolic enzyme that catalyzes the conversion of arginine into ornithine and urea, the final step in the urea cycle. A1exF5 is the only clone commercially available that can detect human or mouse arginase 1, a specific marker for M2 macrophages, by flow cytometry. For more information about the A1exF5 clone, as well as tips on the use of antibody conjugates in multiplex flow cytometry applications, please read the interview with Nicolas Schrantz, Senior Manager at Thermo Fisher Scientific, who leads the team of R&D scientists responsible for the development of new antibody specificities, at **thermofisher.com/arginaseflowblog**.



Expression of arginase 1 in M2a macrophages from mouse bone marrow. Intracellular staining of mouse bone marrow–derived macrophages polarized to M1 (left) or M2a (right). As expected based on known relative expression patterns, the Arginase 1 Monoclonal Antibody A1exF5 stains most M2a macrophages and does not stain M1 macrophages.

Access the new Cancer Spheroid and Organoid eLearning course

The Cancer Spheroid and Organoid eLearning course is the latest addition to our protein and cell analysis eLearning modules. These free, self-paced, animated courses include knowledge checks and practical application exercises, as well as downloadable course materials and relevant supplementary resources. Our newest release is a two-part course presenting a succinct, contextual overview of spheroid and organoid culture techniques and applications for modeling organ physiology and disease. These interactive modules cover methods for analyzing 3D culture models using various imaging modalities including high-content analysis. To access the free, on-demand eLearning courses, visit **thermofisher.com/elearningcourses**.



Spheroid Organoid

Now available for on-demand viewing: The 3rd annual 7 Steps of Protein virtual event

The 7 Steps of Protein virtual event offers both broad and in-depth content that is designed to give you the information and insights you need to empower your protein research every step of the way. You will receive firsthand knowledge of the latest technologies, as well as tips and tricks for 7 protein research application areas—protein expression, protein isolation and purification, gel electrophoresis, western blotting, protein assays and analysis, mass spectrometry, and protein crosslinking. For on-demand viewing at your convenience, go to **thermofisher.com/7steps**.

Download the new Bioconjugation Technical Handbook

Now available for download, the Bioconjugation Technical Handbook provides helpful information and resources for the crosslinking, immobilization, modification, biotinylation, and fluorescent labeling of proteins and peptides. This 72-page handbook describes the chemical reactivity, molecular properties, and applications of bioconjugation reagents, while also providing detailed information on chemical structures, spacer arm length, and more. In addition, a glossary, reference list, and information on digital selection tools are included. Use this guide to find the right bioconjugation reagent for your application. Download your free copy today at **thermofisher.com/bioconjugationhandbook**.

The 2018 winners of the Thermo Fisher Scientific Antibody Scholarship Program

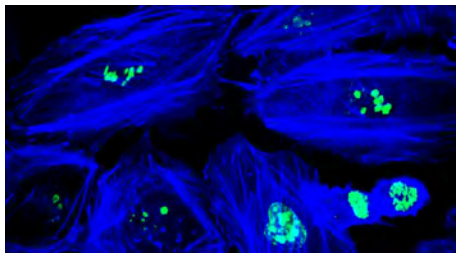
Over the past several years, the Thermo Fisher Scientific Antibody Scholarship Program has awarded $400,000 in scholarship funds to 60 deserving undergraduate and graduate students in the field of biological research. The goal of this program is to ensure that well-educated students are prepared for future scientific research, teaching, and technological development opportunities. We receive applications from students across the United States who show a passion for science and learning and have a wide variety of interests and achievements in science. Check out our website to read more about the scholarship, the application requirements, and the winners at **thermofisher.com/antibodyscholarship**. The Fall 2018 scholarship winners are:

- Rachael Adams, The Ohio State University
- Shannon Esswein, California Institute of Technology
- Veeraj Shah, University of Maryland
- Christopher Thang, University of Houston
- Jeffrey Zhou, Yale University

Introducing Zip Alexa Fluor Rapid Antibody Labeling Kits

The new Invitrogen™ Zip Alexa Fluor™ Rapid Antibody Labeling Kits provide reagents and a simple protocol to efficiently label your primary antibody with one of three bright fluorescent Alexa Fluor dyes in just 15 minutes. Not only is the antibody conjugate ready to use in this short time, but you will recover 100% of your antibody because there are no purification steps required. Using a directly labeled primary antibody often produces lower background fluorescence and less nonspecific binding than detection with a secondary antibody. In addition, multiple primary antibodies of the same isotype or derived from the same species can easily be used in the same experiment if they are directly labeled with compatible fluorophores.

The kit contains everything you need to perform three separate labeling reactions and produces fluorescent antibody conjugates that are ideal for a wide range of applications, including flow cytometry, fluorescent microscopy, immunohistochemistry, immunocytochemistry, ELISAs, and indirect FISH. Learn more about our diverse selection of antibody and protein labeling kits designed to fit your starting material and experimental setup at **thermofisher.com/antibodylabeling**.



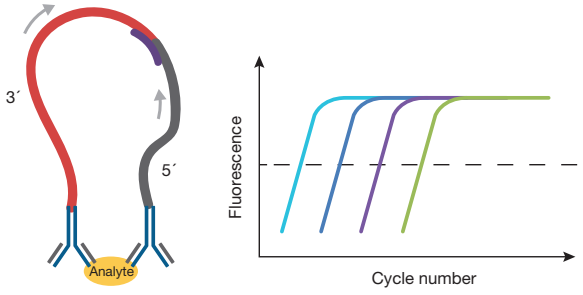
HeLa cells stained with directly labeled primary antibodies. HeLa cells were labeled with a mouse anti-Ki67 antibody directly conjugated to Alexa Fluor 488 dye using the Invitrogen™ Zip Alexa Fluor™ 488 Rapid Antibody Labeling Kit (Cat. No. Z11233) and with Invitrogen™ Alexa Fluor™ Plus 405 Phalloidin (Cat. No. A30104). Cells were imaged using a 40x objective on the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform (Cat. No. CX7A1110LZR) using spinning disk confocal microscopy.

Selected products	Quantity	Cat. No.
Zip Alexa Fluor™ 488 Rapid Antibody Labeling Kit	3 labelings	Z11233
Zip Alexa Fluor™ 555 Rapid Antibody Labeling Kit	3 labelings	Z11234
Zip Alexa Fluor™ 647 Rapid Antibody Labeling Kit	3 labelings	Z11235

Next-generation ProQuantum high-sensitivity immunoassays

Meet the newest easy-to-run immunoassay kits for measuring low levels of cytokine proteins using very small (2 µL) sample volumes. Leveraging a proximity-based amplification technology, the Invitrogen™ ProQuantum™ immunoassays combine the analyte specificity of high-affinity antibody–antigen binding with the high sensitivity and large dynamic range of real-time PCR amplification to achieve simple yet powerful protein quantitation. ProQuantum immunoassays are an open, affordable platform that can be run on any qPCR instrument. See page 16 for a more complete description of our selection of immunoassays, including the ProQuantum kits, and learn more at **thermofisher.com/proquantum**.

Selected products	Quantity	Cat. No.
EGF Human ProQuantum™ Immunoassay Kit	96 reactions	A35579
IFN gamma Human ProQuantum™ Immunoassay Kit	96 reactions	A35576
IL-1 beta Human ProQuantum™ Immunoassay Kit	96 reactions	A35574
IL-2 Human ProQuantum™ Immunoassay Kit	96 reactions	A35603
IL-5 Human ProQuantum™ Immunoassay Kit	96 reactions	A35588
IL-6 Human ProQuantum™ Immunoassay Kit	96 reactions	A35573
IL-8 Human ProQuantum™ Immunoassay Kit	96 reactions	A35575
IL-12 p40 Human ProQuantum™ Immunoassay Kit	96 reactions	A35577



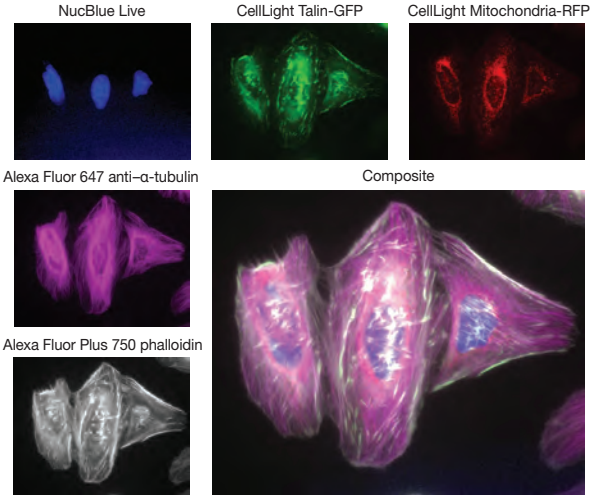
How ProQuantum immunoassays work. Paired antibody–oligonucleotide conjugates bind to the analyte of interest during a 1 hr incubation, followed by ligation of the two oligonucleotides in the presence of a splint oligonucleotide and 40 cycles of qPCR-based amplification. No wash steps are required, and the amount of analyte-specific antibody pair binding is directly proportional to the number of PCR amplicons generated.

New and improved tools for labeling the actin cytoskeleton

Fluorescent phallotoxin derivatives, which stain F-actin selectively at nanomolar concentrations, are powerful labels for identifying and quantifying actin in fixed cells, tissue sections, or cell-free preparations. Thermo Fisher Scientific offers over 26 different fluorescent phalloidins conjugated either to Invitrogen™ Alexa Fluor™ dyes or to classic fluorophores. With recent protocol improvements for making stock solutions, our phalloidin products now produce up to 60% more signal than previous formulations, with an improved workflow.

In addition, we have just added two very bright fluorescent phalloidin conjugates to the portfolio, increasing your labeling choices and facilitating 4- to 6-plex imaging without interference from spectral overlap. The near-infrared–excitable Alexa Fluor Plus 750 Phalloidin can be detected with the 790 nm laser line of the Thermo Scientific™ CellInsight™ CX7 LZE High-Content Analysis Platform or other confocal microscope, the Invitrogen™ EVOS™ Cy®7 Light Cube, or a Cy7 filter set. The violet light–excitable Alexa Fluor Plus 405 Phalloidin can be detected with the 405 nm laser line, the EVOS DAPI Light Cube, or a DAPI filter set. To find out more about our complete set of actin-specific probes, visit **thermofisher.com/phalloidin**.

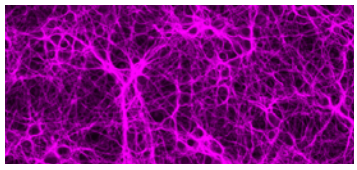
Selected products	Quantity	Cat. No.
Alexa Fluor™ Plus 405 Phalloidin	300 units	A30104
Alexa Fluor™ Plus 750 Phalloidin	300 units	A30105



HeLa cells labeled with Alexa Fluor Plus 750 Phalloidin. HeLa cells were labeled overnight with Invitrogen™ CellLight™ Talin-GFP (BacMam 2.0, Cat. No. C10611), Invitrogen™ CellLight™ Mitochondria-RFP (BacMam 2.0, Cat. No. C10601), and Invitrogen™ NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605), and then fixed, permeabilized, and labeled with Invitrogen™ anti–α-tubulin mouse monoclonal antibody (clone DM1A, Cat. No. 62204) in conjunction with Invitrogen™ Alexa Fluor™ 647 goat anti–mouse IgG secondary antibody (Cat. No. A21235) and Invitrogen™ Alexa Fluor™ Plus 750 Phalloidin (Cat. No. A30105). Cells were mounted in Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36984). Images were acquired using an Invitrogen™ EVOS™ FL Auto 2 Imaging System (Cat. No. AMAFD2000) with an Olympus 60x Aprochromat Oil Objective (Cat. No. AMEP4694) and Invitrogen™ EVOS™ DAPI (Cat. No. AMEP4650), GFP (Cat. No. AMEP4651), RFP (Cat. No. AMEP4652), and Cy®7 (Cat. No. AMEP4667) light cubes.

New Tubulin Tracker Deep Red for labeling polymerized tubulin

The Invitrogen™ Tubulin Tracker™ reagents are cell-permeant fluorescent dyes that specifically label polymerized tubulin in live cells. The new Tubulin Tracker Deep Red—a conjugate of docetaxel and a very bright and photostable far-red fluorophore—easily enters live cells and stains tubulin with deep red fluorescence. Tubulin Tracker Deep Red (excitation/emission = 652/669 nm) can be visualized with standard Cy5 filter settings using almost any fluorescence imaging instrument. This far-red–fluorescent tubulin probe can be multiplexed with blue, green, orange, red, and near-IR fluorophores. Both Tubulin Tracker Deep Red and the original Tubulin Tracker Green are fluorescent derivatives of cytoskeletal drugs (docetaxel and paclitaxel, respectively) that promote and preserve tubulin polymerization. To learn more, visit **thermofisher.com/tubulintracker**.

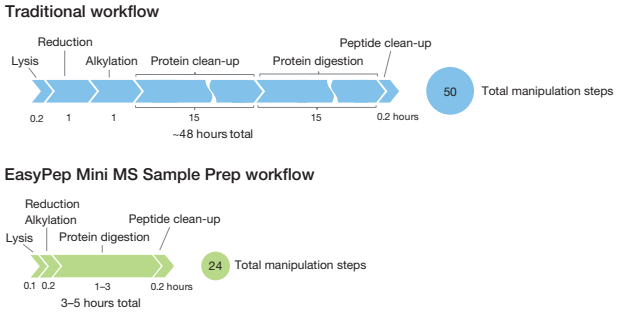


Rat cortical neurons stained with Tubulin Tracker Deep Red. Gibco™ Primary Rat Cortex Neurons (Cat. No. A1084001) were stained with Invitrogen™ Tubulin Tracker™ Deep Red (Cat. No. T34077) and imaged in Invitrogen™ Live Cell Imaging Solution (Cat. No. A14291DJ) on an Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective and an Invitrogen™ EVOS™ Cy®5 Light Cube.

Product	Quantity	Cat. No.
Tubulin Tracker™ Deep Red	60 slides 300 slides	T34077 T34076
Tubulin Tracker™ Green (Oregon Green™ 488 Taxol, bis-acetate)	60 slides 300 slides	T34078 T34075
Tubulin Tracker™ Variety Pack	2 x 60 slides	T34079

Rapid sample prep for mass spectrometry analysis

Traditional protein sample preparation for mass spectrometry (MS) analysis requires numerous and variable steps and can take up to 2 days to complete. The new Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit provides preformulated reagents, an MS-grade enzyme mix, peptide clean-up columns, and a robust method for the preparation of high-quality samples for MS analysis in just 3 to 5 hours. This kit enables efficient and reproducible processing of cultured mammalian cells, plasma, and tissue (10–100 µg), providing more protein identifications and higher peptide yields with fewer steps than traditional methods. Learn more at **thermofisher.com/easypep**.



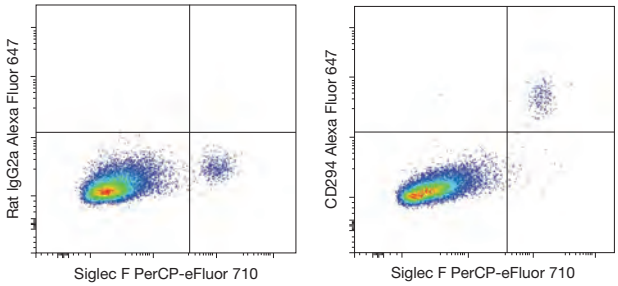
Comparison of the traditional workflow for mass spectrometry sample preparation and the new EasyPep Mini MS Sample Prep workflow.

Flow cytometry antibodies: More markers, more clones, more formats

As a committed supplier of flow cytometry antibodies, Thermo Fisher Scientific has already released more than 1,300 flow cytometry antibody conjugates in 2018, providing you with more options when designing flow cytometry panels. Highlights of recently released markers include:

- Anti–mouse CD294 (CRTH2) antibody—a unique monoclonal for studying innate immunity, for use with eosinophils and mast cells
- Anti–human and anti–mouse VISTA (B7-H5, PD-1H) antibodies—for studying hematopoietic cells in immuno-oncology applications
- Anti–arginase 1 antibody (clone A1exF5)—the only clone available that can detect human or mouse arginase 1 by flow cytometry

Additionally, we've continued to support and extend our portfolio of violet light–excitable Invitrogen™ eBioscience™ Super Bright antibody conjugates, and we now offer Invitrogen™ eBioscience™ Super Bright Staining Buffer in two convenient sizes. Search the entire portfolio of 13,000 flow cytometry antibodies quickly and easily at **thermofisher.com/flowantibodies**.



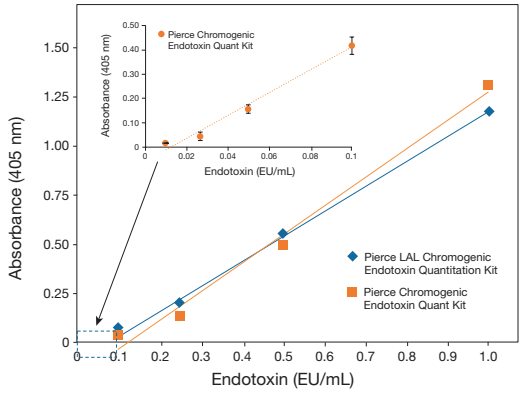
Expression of CD294 (CRTH2) in lysed whole blood cells from mice. Swiss Webster mouse lysed whole blood cells were stained with Invitrogen™ CD170 (Siglec F) Monoclonal Antibody (clone 1RNM44N), PerCP-eFluor™ 710, eBioscience™ (Cat. No. 46-1702-82) and either 0.125 µg of Invitrogen™ Rat IgG2a kappa Isotype Control, Alexa Fluor™ 647, eBioscience™ (left, Cat. No. 51-4321-81) or 0.125 µg of Invitrogen™ CD294 (CRTH2) Monoclonal Antibody (clone No3m1scz), Alexa Fluor™ 647, eBioscience™ (right, Cat. No. 51-2941-82). Total viable cells were used for this flow cytometry analysis.

Selected products*	Quantity	Cat. No.
Arginase 1 Monoclonal Antibody (A1exF5), Alexa Fluor™ 488, eBioscience™	100 µg	53-3697-82
Arginase 1 Monoclonal Antibody (A1exF5), Alexa Fluor™ 700, eBioscience™	100 µg	56-3697-82
Arginase 1 Monoclonal Antibody (A1exF5), APC, eBioscience™	100 µg	17-3697-82
Arginase 1 Monoclonal Antibody (A1exF5), eFluor™ 450, eBioscience™	100 µg	48-3697-82
Arginase 1 Monoclonal Antibody (A1exF5), PE, eBioscience™	100 µg	12-3697-82
Arginase 1 Monoclonal Antibody (A1exF5), PE-Cyanine7, eBioscience™	100 µg	25-3697-82
Arginase 1 Monoclonal Antibody (A1exF5), PerCP-eFluor™ 710, eBioscience™	100 µg	46-3697-82
Super Bright Staining Buffer, eBioscience™	100 tests 1,000 tests	SB-4400-42 SB-4400-75

*To see the complete set of flow cytometry antibodies for CD294 (CRTH2) and VISTA (B7-H5, PD-1H), please visit **thermofisher.com/flowantibodies**.

New Pierce Chromogenic Endotoxin Quant Kit with improved sensitivity and broad-range detection

Recognizing the need for a more sensitive endotoxin detection method, Thermo Fisher Scientific has extended its endotoxin detection line to include the Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit, which accurately detects endotoxins at levels as low as 0.01 EU/mL in samples. This kit is an endpoint amebocyte lysate assay that quantifies endotoxins (lipopolysaccharides) in a variety of sample types, including protein, vaccine, plasmid, DNA, and RNA samples, with no interference from β-glucans. The kit offers high sensitivity with two linear dynamic ranges of 0.01–0.1 and 0.1–1.0 EU/mL, and reproducibility from test to test and operator to operator with a coefficient of variation (CV) of 3%. Find out more at **thermofisher.com/endotoxin**.

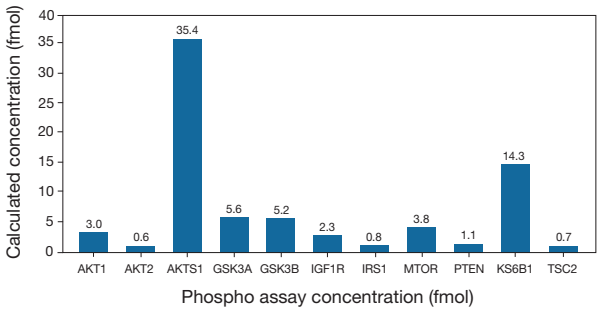


Comparison of Pierce endotoxin quantitation kits. Compared with the Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, the new Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit provides a nearly identical linear response curve but with an increased dynamic range of 0.01–0.1 EU/mL. Assays were performed according to the manufacturer's protocols. The standard curves for both kits produced $R^2 \geq 0.98$, and a CV < 5%. The Pierce Chromogenic Endotoxin Quant Kit's standard curve at the lower range (n = 17) showed exceptional linearity, with $R^2 \geq 0.99$ and CV < 5%.

Product	Quantity	Cat. No.
Pierce™ Chromogenic Endotoxin Quant Kit	30 reactions	A39552S
	60 reactions	A39552
	240 reactions	A39553

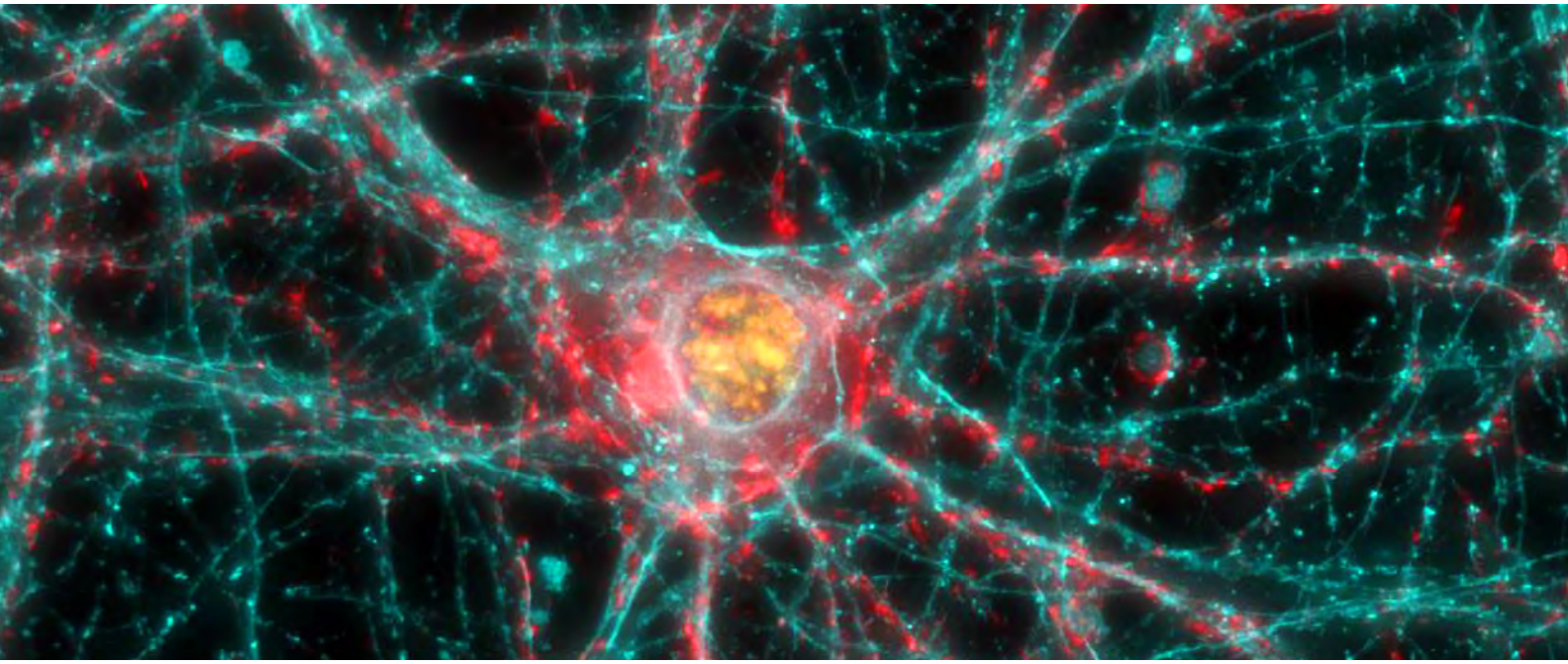
Quantitative mass spectrometry–based targeted assays for cancer signaling pathways

Thermo Scientific™ SureQuant™ Panels enable multiplex immunoprecipitation to mass spectrometry (mIP-MS) analysis for simultaneous enrichment and quantitation of multiple total and phosphorylated proteins in the AKT/mTOR signaling pathway. Each multiplex panel for absolute or relative quantitation contains two modules: 1) the IP and MS Sample Prep Module, which includes all reagents necessary to immunoenrich AKT/mTOR pathway proteins and perform in-solution MS sample preparation; and 2) the Absolute or Relative Quantitation Module, which includes a system suitability standard and AQUA Ultimate Heavy and Light Peptide (or, in the Relative Quantitation module, only Heavy Peptide) mixtures. The immunoenriched and digested samples spiked with internal standards can be processed using the discovery MS method (nanoLC-MS/MS) or targeted MS method (nanoLC-PRM/MS). See our full portfolio of mass spectrometry kits for cancer signaling pathways at **thermofisher.com/ms-targeted-assays**.



Absolute quantitation of AKT (phospho) signaling pathway proteins in mammalian cells using the SureQuant AKT Pathway (Phospho) Multiplex Panel (Absolute Quantitation). Absolute quantitation of AKT/mTOR phospho pathway proteins was performed using the mammalian cell line MCF7 treated with IGF1. AKT/mTOR pathway proteins were enriched through multiplex immunoprecipitation, followed by nanoLC-PRM/MS analyses on a Thermo Scientific™ Q Exactive™ HF-Orbitrap™ Mass Spectrometer. All targets were quantified by standard curves generated for each target peptide included in the Quantitation Modules.

Product	Quantity	Cat. No.
SureQuant™ AKT Pathway Multiplex Panel (Absolute Quantitation)	10 reactions	A40011
SureQuant™ AKT Pathway Multiplex Panel (Relative Quantitation)	10 reactions	A40080
SureQuant™ AKT Pathway (Phospho) Multiplex Panel (Absolute Quantitation)	10 reactions	A40084
SureQuant™ AKT Pathway (Phospho) Multiplex Panel (Relative Quantitation)	10 reactions	A40085



Tools for 2D and 3D neuronal cell culture

From the B-27 Plus Neuronal Culture System to fluorescent probes for cell analysis.

Traditional culture and analytical methods in the neuroscience field have long relied on rodent cell model systems to emulate the behaviors of higher mammalian neural network activity, with the aim of discovering, characterizing, and testing pharmaceuticals for safety and toxicology. These and most excitable, postmitotic cell types are typically dissociated from freshly prepared tissues or cryopreserved for later study using a variety of media and extracellular matrix formulations intended to recapitulate native or *in vivo* conditions. To this end, the commercial availability of large-scale batches of quality-controlled, cryopreserved primary cell types has helped advance the study of brain and nerve function. Also available are improved media formulations specifically developed to increase neuronal survival and enhance maturation of neuronal phenotypes in extended cultures of primary and induced pluripotent stem cell (iPSC)-derived neurons, as well as functional probes designed for use with 2D and 3D neuronal cultures.

Figure 1 (above). Visualizing a mouse cortical neuron culture. Cryopreserved Gibco™ Primary Mouse Cortical Neurons (Cat. No. A15585) were grown in culture for 3 weeks using the Gibco™ B-27™ Plus Neuronal Culture System (Cat. No. A3653401). Cells were fixed and labeled with Invitrogen™ NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606), anti–β-tubulin mouse monoclonal antibody (Cat. No. 32-2600) in conjunction with Invitrogen™ Alexa Fluor™ 488 goat anti–mouse IgG antibody (Cat. No. A11029), and Invitrogen™ ActinRed™ 555 ReadyProbes™ Reagent (Cat. No. R37112). Cells were mounted in Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980) and imaged on an Invitrogen™ EVOS™ FL Auto 2 Imaging System using a 60x oil immersion objective.

Advancing neuronal culture

For over 25 years, researchers have relied on Gibco™ B-27™ Supplement and Gibco™ Neurobasal™ Medium for their neuronal culture applications [1]. The original serum-free B-27 Supplement was developed for the long-term culture of rat hippocampal and cortical neurons. However, the publication history of B-27 supplements demonstrates that researchers are expanding their use far beyond these applications. Progress in the development of iPSC-derived neurons has led to the need to maintain a higher density of neurons over longer periods of time than B-27 Supplement and Neurobasal Medium were designed to support. We have applied our decades of experience with culture media to develop the Gibco™ B-27™ Plus Neuronal Culture System—a serum-free culture system that provides increased neuronal survival, accelerated neurite outgrowth, and improved electrophysiological activity and maturation of neurons.

A closer look at neuronal subtypes

The B-27 Plus Neuronal Culture System improves upon the classic culture environment through both raw material and manufacturing upgrades and minor formulation modifications. Here we look at how improved neuronal culture conditions affect subpopulations of neurons in primary neuronal cultures and the functional implications of enhanced subpopulation survival and maturity. Preparations of neurons from rodent brains contain different types of cells, including excitatory (glutamatergic) and inhibitory (GABAergic) neurons, and a subset of GABAergic interneurons that are positive for parvalbumin (PV). *In vivo*, the numbers of excitatory and inhibitory neurons are tightly regulated, and their interactions are the foundation for neural network regulation. Further, PV interneurons play a critical role in controlling synchronous activity across networks. *In vitro*, neuronal survival and robust maturation are vital to neuronal subpopulation maintenance and the formation of networked and synchronous activity in primary cortical and hippocampal cultures.

The impact of culture conditions on subpopulations of excitatory and inhibitory rat cortical neurons is shown in Figure 2, using comparisons between 28-day cultures grown in the classic B-27 Supplement and Neurobasal Medium and in the B-27 Plus system. The total numbers of neurons in each culture were determined by counting HuC/D-positive cells. Using neuronal subpopulation–specific antibodies, we further characterized these cells as glutamatergic neurons (VGLUT2-positive), GABAergic neurons (GABA-positive), and PV-positive interneurons.

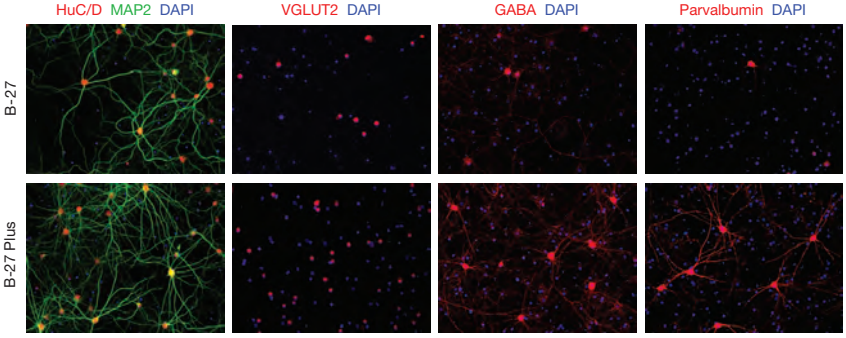


Figure 2. Defining neuronal subtypes in cultures of primary rat neurons. Rat cortical neurons were cultured for 28 days in either the classic Gibco™ B-27™ Supplement and Gibco™ Neurobasal Medium (top row) or the Gibco™ B-27™ Plus Neuronal Culture System (bottom row), and then immunostained for MAP2 (green, Cat. No. PA5-17646) and HuC/D (red, Cat. No. A21271), VGLUT2 (red, Cat. No. 42-7800), GABA (red, Cat. No. PA5-32241), or parvalbumin (red, Cat. No. PA1-933) and the corresponding Invitrogen™ Alexa Fluor™ 488 donkey anti–rabbit IgG (Cat. No. A21206) and Alexa Fluor™ 594 donkey anti–mouse IgG and anti–rabbit IgG (Cat. No. A21203, A21207) secondary antibodies. Nuclei were labeled with DAPI (blue).

Quantitative image analysis performed on the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform showed approximately 50% more HuC/D-positive cells in the B-27 Plus system than in the classic B-27 system. In addition, 90% of all neurons in the classic B-27 system stained positive for VGLUT2, compared with 81% in the B-27 Plus system, and 12% of the neurons in the B-27 cultures were GABA-positive neurons versus 14% in the B-27 Plus system. Strikingly, the percentage of PV-positive interneurons was less than 1% in the classic B-27 system, compared with 7% in the B-27 Plus system. These results are in line with estimates of 80% glutamatergic and 20% GABAergic for cortical neurons *in vivo*. Qualitative analysis of the MAP2, GABA, and PV staining indicates significantly more staining of developed neurites in cells cultured in the B-27 Plus system, suggesting enhanced maturation of the neurons and their subpopulations. Taken together, these data indicate significant improvements in the maintenance and maturation of neuronal subpopulations in the B-27 Plus system.

Challenges to neuronal maturation and analysis of 2D and 3D cultures

One of the principal barriers to generating useful data with neural culture systems is the length of time required for stem cell–derived and primary cultures to reach maturation for downstream functional analysis. Typically, culture medium must be refreshed 2–3 times

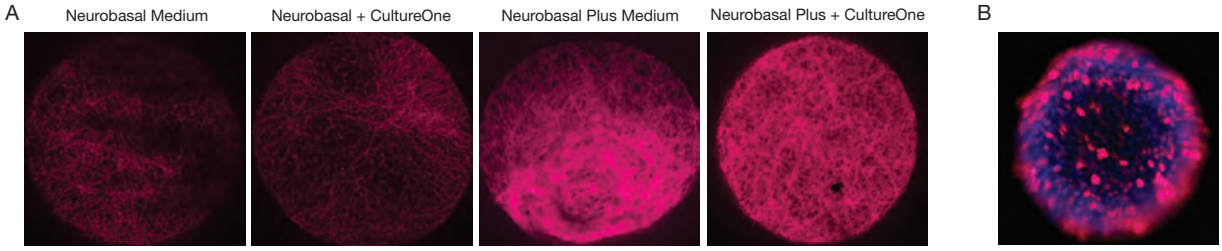


Figure 3. 3D neuronal cultures stained with Tubulin Tracker Deep Red. (A) Neuronal spheroids were cultured using Gibco™ Neurobasal™ (Cat. No. 21103049) or Gibco™ Neurobasal™ Plus (Cat. No. A3582901) Medium, with and without Gibco™ CultureOne™ Supplement (Cat. No. A3320201), in Thermo Scientific™ Nunclon™ Sphera™ 96U-Well Microplates, and then stained with Invitrogen™ Tubulin Tracker™ Deep Red reagent (Cat. No. T34077). Images were generated using a 10x objective on the Thermo Scientific™ CellInsight™ CX7 High-Content Analysis (HCA) Platform. **(B)** Neuronal spheroids were labeled with Tubulin Tracker Deep Red and Invitrogen™ NucBlue™ Live ReadyProbes™ (Cat. No. R37605) reagents. Cells were imaged in Gibco™ HBSS containing calcium and magnesium (Cat. No. 14025134), supplemented with probenecid (Cat. No. P36400). Images were generated using an Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective and an Invitrogen™ EVOS™ Cy®5 Light Cube.

per week for a minimum of 2 weeks for synapses to form and stable networks to emerge in the cultures. This challenge becomes exceptionally difficult in the context of 3D cultures, where medium must be carefully removed and replaced without disturbing or accidentally aspirating the spherical organoids formed by immature progenitors. Neural stem cell (NSC)-derived cultures can take 4 weeks or more to become functionally responsive to depolarizing stimuli, and longer still to form synapses.

To address these challenges, the Neurobasal Plus culture system was specifically developed to accelerate the maturation rate of primary cultures and increase the yield of neurons that survive the long road to differentiation. Additionally, the Gibco™ CultureOne™ Supplement was designed to halt proliferation of progenitor cells and redirect them toward a neural lineage in roughly half the time of unsupplemented cultures. Culturing NSCs with the Neurobasal Plus system and CultureOne supplement resulted in improved functional responses from terminally differentiated cells that are almost entirely lacking progenitor phenotypes and greatly enriched for markers of neuronal character.

The advantages of using CultureOne Supplement are especially important in 3D NSC cultures. To form 3D neuronal spheroids, NCSs were differentiated in Neurobasal or Neurobasal Plus Medium, with and without CultureOne Supplement, for 2 weeks and then stained with Invitrogen™ Tubulin Tracker™ Deep Red reagent to label neuronal processes. This cell-permeant reagent provides a far-red–fluorescent, tubulin-selective dye that requires only a single stain-and-wash step. Because media changes and staining protocols alike are pain points for 3D culture protocols, Tubulin Tracker Deep Red reagent is an ideal cell analysis tool, eliminating the need to fix and permeabilize spheroids before analysis (in contrast to immunostaining protocols). As demonstrated by TubulinTracker Deep Red staining and imaging on the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform, neuronal spheroids show enhanced neurite growth when grown in Neurobasal Plus Medium with CultureOne Supplement, compared with those grown in the original Neurobasal Medium without supplement (Figure 3).

Along with improvements in neuronal media, recent developments in 3D culture systems and supports have enhanced the ability to grow neurons in a matrix that more closely resembles the state of nervous system tissue in intact organisms, without requiring the use of expensive and time-consuming animal models. The neuronal spheroids shown in Figure 3 were grown on Thermo Scientific™ Nunclon™ Sphera™ Microplates, which have a surface with very low cell-attachment properties. Nunclon Sphera plates consistently promote 3D spheroid and organoid culture.

Explore 2D and 3D culture and analysis

Thermo Fisher Scientific offers a suite of culture media, cultureware, cell analysis reagents, and fluorescence instrumentation for 2D and 3D neuronal cell cultures. Learn more at **thermofisher.com/neurobasal**. ■

Reference

1. Brewer GJ, Torricelli JR, Evege EK et al. (1993) *J Neurosci Res* 35:567–576.

Product	Quantity	Cat. No.
B-27™ Plus Neuronal Culture System	1 system	A3653401
B-27™ Plus Supplement (50X)	10 mL	A3582801
CultureOne™ Supplement (100X)	5 mL	A3320201
Neurobasal™ Plus Medium	500 mL	A3582901
Nunclon™ Sphera™ 96U-Well Microplate	1 case of 8	174925
Tubulin Tracker™ Deep Red	60 slides 300 slides	T34077 T34076
GABA Polyclonal Antibody	100 µL	PA5-32241
HuC/HuD Monoclonal Antibody (clone 16A11)	100 µg	A21271
MAP2 Polyclonal Antibody	100 µL	PA5-17646
β-Tubulin Monoclonal Antibody (clone 2 28 33)	100 µg	32-2600
Parvalbumin Polyclonal Antibody	100 µg	PA1-933
VGLUT2 Polyclonal Antibody	100 µg	42-7800

Improve image quality in 2D and 3D biological samples

CytoVista Clearing System and ProLong Glass Antifade Mountant.

The development of reliable 2D and 3D cell and tissue culture techniques and reagents has produced a plethora of options for studying the brain and other neural tissues. However, as the tissues and cultures become more complex, the experimental samples become more challenging to investigate. New approaches are needed for imaging spheroids, organoids, and thicker tissue sections.

One key challenge with imaging neural tissue is uneven light scattering caused by cell organelles and components such as nuclei, mitochondria, and membranes [1], as well as by lipids, which are heterogeneously distributed. It is this heterogeneity of the biological sample that contributes to the uneven scattering of light, making the sample appear milky white or opaque under the microscope [2]. Opaqueness is the result of an inherent mismatch between the refractive indices of the objective, medium, and cells or tissue. In fluorescence microscopy, opaqueness limits axial (z-dimension) resolution and focal depth during imaging. Therefore, it becomes imperative to match the refractive index of each component in order to capture high-quality images.

Optical clearing for sharp images

Several clearing techniques have been developed to match the refractive index of the sample to that of coverslips, immersion oil, and objectives. An effective clearing treatment for cells, spheroids, organoids, and tissue must meet specific criteria. First, it cannot change the overall morphology of the sample. Second, it must be compatible with immunofluorescence (IF), immunocytochemistry (ICC), and immunohistochemistry (IHC) techniques, including incubations with fixatives, permeabilization reagents, and antibodies. Third, the resulting refractive index from the clearing treatment needs to be closely matched to common microscope objectives, and the instrumentation needed to process and image the sample must be easily available in research laboratories. With the increased use of fluorescent proteins in neuronal studies, it is also important that clearing treatments do not diminish the fluorescence of these proteins [1].

Table 1. Selection guide for clearing reagents.

	ProLong Glass Antifade Mountant	CytoVista 3D Cell Culture Clearing/ Staining Kit	CytoVista Tissue Clearing Reagent and Enhancer
Form	Hard-setting, ready to use	Soft-setting, ready to use	Soft-setting, ready to use
Media type	Aqueous	Solvent	Solvent
Refractive index	~1.52 after curing	1.48	Reagent alone: 1.50 With enhancer: 1.53
Sample archiving time frame	Months to years	Weeks to months	Weeks to months
Imaging depth	Up to 150 µm	Up to 1 mm	Up to 10 mm
3D cell culture	Yes	Yes	No
Tissue sections up to 150 µm	Yes	No	Yes
Tissue sections up to 10 mm	No	No	Yes
Signal-to-noise ratio	Best	Good	Good
Photobleaching protection	Best	None	None
Mounted microscope slides	Yes	Yes	Yes
Microplate imaging	No	Yes	No
Sample preparation time	Overnight to 4 days	30 min to a few hours	1–3 days
Reversibility/tissue recovery	Yes	Yes	Yes
Cat. No.	P36980	V11325	V11324

CytoVista Clearing Reagents

Thermo Fisher Scientific recently released the Invitrogen™ CytoVista™ Clearing System (Table 1), a family of products developed to minimize the impact of refractive index

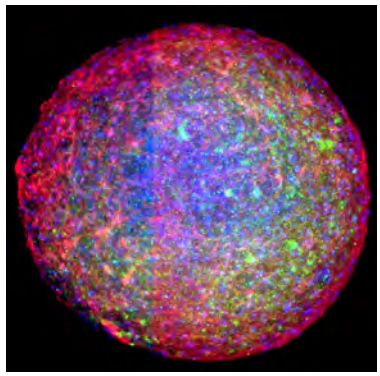


Figure 1. Clearing and fluorescent staining of brain spheroids. StemoniX™ 3D microBrain™ cortical neuronal spheroids (~500 µm diameter) were generated from induced pluripotent stem cell (iPSC)-derived neural progenitor cells. Neuronal bodies were stained with Invitrogen™ NeuroTrace™ Green Fluorescent Nissl Stain (green, Cat. No. N21480). Glial fibrillary acidic protein (GFAP) was detected with an anti-GFAP primary antibody and labeled with Invitrogen™ Alexa Fluor™ Plus 647 goat anti–rabbit IgG secondary antibody (red, Cat. No. A32733). Nuclei were stained with DAPI (blue, Cat. No. D21490). The tissue was cleared and mounted with the Invitrogen™ CytoVista™ 3D Cell Culture Clearing/Staining Kit (Cat. No. V11325). This image is a composite of z-stack imaging using the Thermo Scientific™ CellInsight™ CX7 LZR HCA Platform and HCS Studio™ Software. Videos of the z-stack images can be found at **thermofisher.com/cytovista**.

mismatch when imaging cells, spheroids, organoids, or tissue. The CytoVista clearing workflow is compatible with most fluorophores, including fluorescent proteins, that are detected with common fluorescence imaging instruments such as widefield, confocal, and light sheet microscopes, and high-content analyzers. Features of the CytoVista family of tissue and 3D cell culture clearing reagents include rapid clearing of fluorescently labeled cells, spheroids, organoids, and tissue for 2D and 3D imaging, as well as minimal shrinkage, expansion, or other morphological changes to cells and tissue. In addition, cell and tissue clearing with CytoVista reagents does not require any special equipment and is compatible with IF, ICC, and IHC protocols. After the fluorescence analysis is complete, if needed, the cells or tissue clearing can be reversed and samples can be further analyzed for other histological studies.

The CytoVista 3D Cell Culture Clearing/Staining Kit clears fluorescently labeled 3D cultured cells such as organoids and spheroids, enabling the acquisition of sharp, bright images on samples up to a depth of 1,000 µm using fluorescence instrumentation (Figure 1). This kit includes CytoVista 3D Cell Culture Clearing Reagent, penetration buffer, wash buffer, blocking buffer, and antibody dilution buffer, and can be used to clear and image samples on microscope slides or in microplates or chambers. With this system, most samples can be cleared in as little as 30 minutes, depending on the thickness of the sample.

For thicker samples, the CytoVista Tissue Clearing/Staining Kit can be used to clear fluorescently labeled tissue up to 10 mm thick prior to fluorescence imaging. This kit contains both CytoVista Tissue Clearing Reagent and CytoVista Tissue Clearing Enhancer, which together can be used to clear most fluorescently labeled tissue types, as well as the buffers needed for IHC protocols. The clearing process is relatively fast, again depending on the thickness of the sample. For example, a whole mouse brain, which is approximately 8 mm thick, can be cleared in 24 hours, while a 1 mm section can be cleared in 2 hours.

ProLong Glass Antifade Mountant

Invitrogen™ ProLong™ Glass Antifade Mountant (Table 1) is a glycerol-based, hard-setting, ready-to-use mountant that can be applied directly to fluorescently labeled cells or tissue samples on microscope slides or coverslips. Prolong Glass mountant has a refractive index of 1.52 after curing, similar to that of glass coverslips, compatible immersion oil, and oil immersion objectives. Due to the close match in the refractive indices of the objective, medium, and cells or tissue, ProLong Glass mountant enables superior axial and lateral resolution in fluorescence imaging applications. ProLong Glass mountant also provides exceptional photobleaching protection across the visible to near-infrared spectra, and it is compatible with most organic dyes and fluorescent proteins. ProLong Glass mountant is ideal for producing bright, high-resolution z-stack, 3D, and 2D images of any cell or tissue sample up to 150 µm in thickness without adding extra steps to the fluorescence imaging workflow. Figure 2 shows the use of ProLong Glass mountant for imaging along the axial dimension of a rat brain section (100 µm thick) without loss of target resolution throughout the entire sample.

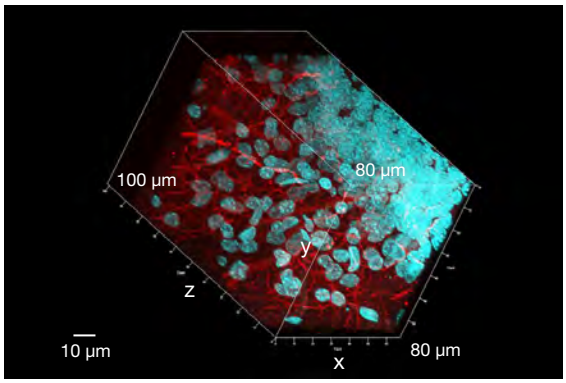


Figure 2. 3D imaging using ProLong Glass Antifade Mountant. Formalin-fixed, paraffin-embedded (FFPE) 100 µm rat brain sections were stained with Invitrogen™ β-3 tubulin primary antibody (Cat. No. MA1-118) and Invitrogen™ Alexa Fluor™ 594 goat anti–mouse IgG secondary antibody (red, Cat. No. A11032), with overnight incubations for each antibody. Nuclei were stained with DAPI (blue, Cat. No. D1306). The stained tissue sections were placed on a coverslip, and Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980) was added. The samples were allowed to air-dry uncovered overnight on a flat, dry, dark surface. The following day, ~30 µL of 100% glycerol was applied on the surface of the hardened mountant, microscope slides were placed on top, and the samples were left to cure for 1 hr. Sections were imaged on a Zeiss™ LSM 710 confocal microscope using a Plan-Apochromat 63x/1.4 NA oil immersion objective. Images were processed using Ziess™ ZEN software.

Choosing the right clearing solutions

The CytoVista Clearing System and ProLong Glass Antifade Mountant offer unique solutions for improving 2D and 3D imaging quality and resolution. Table 1 provides a selection guide to help you choose the right product for your experiment. For more information on clearing reagents and mountants, go to **thermofisher.com/cytovista**. ■

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Hitting the mark with histone antibodies

Specificity analysis of antibodies that recognize histone posttranslational modifications.

The nucleosome is the fundamental repeating unit of the eukaryotic chromosome. It functions to package DNA into units of ~150 base pairs wrapped around two copies each of histones H2A, H2B, H3, and H4, while also significantly contributing to the regulation of gene expression. Histones have a remarkable assortment of posttranslational modifications (PTMs)—including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, and ribosylation [1]. Due to the large number of modified histone residues, and the additional complexity resulting from the different methylation states of lysine or arginine residues, studying the epigenome requires a set of highly specific and validated tools [1].

Antibodies specific for histone PTMs are essential reagents in a variety of experimental techniques, including chromatin immunoprecipitation (ChIP), western blotting, immunofluorescence, and immunohistochemistry. ChIP is extensively used to assess protein–DNA interactions and to analyze the occupancy of chromatin modifications on a genome-wide scale. Because certain histone modifications may display similar DNA-binding patterns, the accuracy of a ChIP experiment depends upon the specificity of the antibody and its ability to distinguish between subtly different PTMs, such as dimethylation versus trimethylation. Recent studies aimed at testing the quality of commercially available histone PTM antibodies have raised concerns regarding their specificity, which is of paramount importance when analyzing the association of histone modifications and disease [2-5]. Thus, rigorous specificity analysis and functional validation of histone PTM antibodies are needed.

Here we describe our analysis of Invitrogen™ histone PTM antibodies. We are conducting extensive specificity testing of our antibodies using peptide arrays and functional ChIP validation assays, and comparing their performance to that of other widely cited, commercially available histone PTM antibodies. Table 1 lists a set of recently tested Invitrogen histone PTM antibodies that performed as well as or better than corresponding histone PTM antibodies from other manufacturers in histone peptide arrays; a detailed explanation of how this comparison was performed is discussed in Figure 1.

Table 1. Invitrogen histone PTM antibodies validated on peptide arrays.

Antibody target	Abbreviated target name	Host and class	Cat. No.*
Methylation antibodies			
Methyl-Histone H3 (Lys4)	H3K4me1	Rabbit monoclonal	701763
		Rabbit oligoclonal	710795
Methyl-Histone H3 (Lys9)	H3K9me1	Rabbit oligoclonal	710814
Methyl-Histone H3 (Lys27)	H3K27me1	Rabbit polyclonal	491012
Di-Methyl-Histone H3 (Lys4)	H3K4me2	Rabbit monoclonal	701764
		Rabbit oligoclonal	710796
Di-Methyl-Histone H3 (Lys9)	H3K9me2	Rabbit polyclonal	491007
Di-Methyl-Histone H3 (Lys36)	H3K36me2	Rabbit monoclonal	701767
Tri-Methyl-Histone H3 (Lys9)	H3K9me3	Rabbit polyclonal	491008
Tri-Methyl-Histone H3 (Lys27)	H3K27me3	Rabbit monoclonal	MA511198
Acetylation antibodies			
Acetyl-Histone H3 (Lys9)	H3K9ac	Rabbit monoclonal	701269
		Rabbit oligoclonal	710293
Acetyl-Histone H3 (Lys14)	H3K14ac	Rabbit polyclonal	720094
Acetyl-Histone H3 (Lys18)	H3K18ac	Rabbit polyclonal	720095
Acetyl-Histone H4 (Lys8)	H4K8ac	Rabbit polyclonal	720105
		Rabbit oligoclonal	710828
Phosphorylation antibodies			
Phospho-Histone H3 (Ser10)	H3pS10	Rabbit monoclonal	701258
Phospho-Histone H4 (Ser1)	H4pS1	Rabbit polyclonal	720100

*All antibodies listed here perform as well as or better than corresponding antibodies from other suppliers.

Methods for comparing histone PTM antibodies

To compare the specificity of antibodies for a particular histone PTM, we tested antibodies from various manufacturers using histone peptide arrays, which contain 384 peptides from the N-terminal tails of histones featuring 59 posttranslational modifications. This peptide array assay was followed by an analysis of the functional performance of the histone PTM antibody in ChIP. Commercially available antibodies were chosen based on citations and their applicability in peptide arrays, peptide dot blots, ChIP-qPCR, and ChIP-Seq, as well as in additional applications such as western blotting and immunocytochemistry.

Specificity analysis using peptide arrays

Figure 1A shows representative peptide arrays that have been incubated with either the Invitrogen™ Di-Methyl-Histone H3 (Lys4) Antibody (anti-H3K4me2 rabbit oligoclonal antibody) or a commercially available antibody from another supplier purported to have the same specificity.

The Invitrogen anti-H3K4me2 antibody only binds to peptides that contain the H3K4me2 modification, whereas the other supplier's antibody binds to peptides that contain the specified modification as well as peptides containing other modifications (Figure 1A). The data can also be represented as a graph of the "specificity factor" for each modification, which is the ratio of the average intensity of all spots containing a particular PTM to the average intensity of all spots lacking that PTM on the peptide array (Figure 1B). "Specific" antibodies are defined as those showing greater than a two-fold difference in the specificity factors for binding at the target site versus at the best nontarget site. Invitrogen antibodies that performed as well as or better than competitors showed equal or higher fold differences, respectively, between the specificity factors for binding at the target and at the best nontarget site (listed in Table 1).

Functional analysis in ChIP assays

Chromatin pull-down in a ChIP assay is evidence of the presence or absence of a specific histone PTM at a particular genomic locus. Use of a specific histone PTM antibody helps ensures that chromatin is not pulled down by nonspecific interactions. As this assay requires recognition of the modification in the context of nucleosomes, functional validation of a histone PTM antibody for use in ChIP is required to establish both that target epitopes are accessible and that the antibody is binding to expected loci.

Figure 1C shows the performance of the two anti-H3K4me2 antibodies in a ChIP assay. The Invitrogen anti-H3K4me2 antibody shows the expected enrichment of H3K4me2 on the active but not the silent loci, whereas the other supplier's anti-H3K4me2 antibody shows much lower fold enrichment of the active loci as compared with the silent loci. These data clearly emphasize that antibodies for ChIP should be chosen not only for their ability to pull down chromatin but also for their ability to show enrichment of target histone PTMs at the expected genomic regions.

Find the right antibody for your experiments

When choosing a histone PTM antibody, two major considerations are the stringent verification of antibody specificity and the functional validation in ChIP. To address reports of the lack of reproducibility in commercially available histone PTM antibodies [2-5], histone PTM antibodies in the Invitrogen portfolio are undergoing extensive specificity verification and functional ChIP validation.* This comprehensive study underscores the breadth of histone PTM antibodies offered by Thermo Fisher Scientific and should serve as a useful guide when

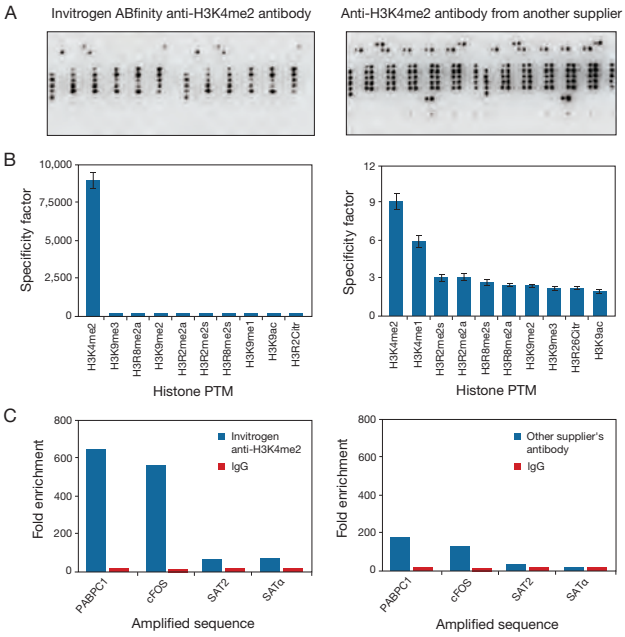

Figure 1. Specificity analysis and functional validation of two anti-H3K4me2 antibodies. (A) The specificity of the Invitrogen™ ABfinity™ Di-Methyl-Histone H3 (Lys4) Antibody (anti-H3K4me2 rabbit oligoclonal antibody, Cat. No. 710796) and a corresponding antibody (right) from another supplier was determined using MODified™ Histone Peptide Arrays (Active Motif Inc.) and manufacturers' protocols. **(B)** The results of the peptide array analysis are displayed as graphs of specificity factor (ratio of the average intensity of all spots containing H3K4me2 to those lacking H3K4me2) vs. modification. **(C)** ChIP analysis was performed on sheared chromatin from 2 x 10^6 HeLa cells using the Applied Biosystems™ MAGnify™ Chromatin Immunoprecipitation System (Cat. No. 492024); nonspecific rabbit IgG was used as a negative control. The purified DNA was analyzed using the Applied Biosystems™ 7500 Fast Real-Time PCR System (Cat. No. 4351106) with optimized PCR primer pairs for the promoters of the active PABPC1 and cFOS genes (positive controls) and for the regions of the inactive SAT2 and SATα satellite repeats (negative controls). Data are presented as fold enrichment of the antibody signal vs. signal from the negative control IgG.

choosing the right antibody for a particular experiment. To learn more about individual histone PTM antibodies or search our entire portfolio of over 80,000 antibodies, visit **thermofisher.com/antibodies**. ■

*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. The product(s) was not validated for clinical or diagnostic use.

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Time-saving immunoassay tools

Instant ELISA kits, ProQuantum high-sensitivity immunoassays, and ProcartaPlex multiplex panels.

For over 40 years, the well-established enzyme-linked immunosorbent assay (ELISA) has been considered the gold standard for target-specific protein measurement in classic immunology studies. With reagents conveniently packaged in ready-to-use or coat-it-yourself kits, microplate-based ELISAs offer a larger selection of targets than any other immunoassay platform available, as well as a long list of published references that validate their use. Despite the useful kit formats, however, it can still take weeks or months to optimize a working protocol and complete the experimental assay runs needed for a research study. With an increased urgency to understand and develop treatments for a multitude of devastating human diseases, today's life science researcher needs assay platforms that can quantify proteins with the same sensitivity and specificity as conventional ELISAs but in a faster and easier way.

The Invitrogen™ family of immunoassays offers a number of tools with which to explore all facets of the immune system and to monitor changes in immune responses for pathophysiological conditions. In addition to conventional ELISAs, we have developed Instant ELISA™ kits, ProQuantum™ high-sensitivity immunoassays, and ProcartaPlex™ multiplex panels, which provide advanced features to support time savings and increased productivity in the lab (Figure 1, Table 1).

Instant ELISA kits provide the same specificity and sensitivity as ELISAs but with less hands-on time

In a typical sandwich ELISA, the antigen of interest is immobilized on a solid surface via capture by an antigen-specific antibody that has been precoated on the wells of a microplate. The bound antigen is then quantified after binding with a second antigen-specific antibody that is linked to an enzyme (direct ELISA) or that is detected with either an enzyme-linked secondary antibody or a biotinylated secondary antibody in conjunction with enzyme-linked streptavidin (indirect ELISA). The amount of antigen is reported by determining the conjugated enzyme's activity after incubation with a substrate that produces a measurable product.

In contrast to conventional ELISA kits, Instant ELISA kits come with plates that contain more than just the precoated capture antibody. The 96-well plates in the Instant ELISA kits contain lyophilized detection antibody, streptavidin-HRP, and sample diluent, greatly reducing pipetting time during the assay. These kits also provide four additional

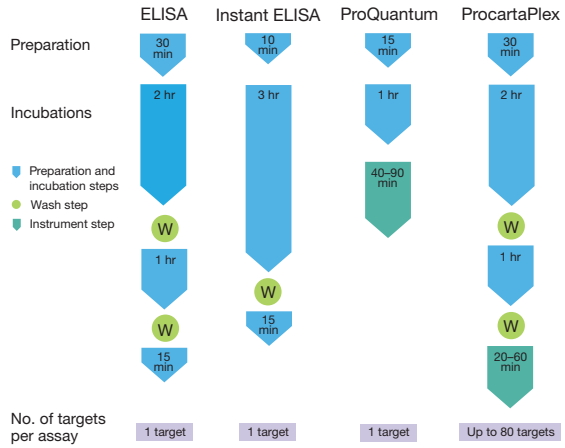

Figure 1. Comparison of the workflows for the Instant ELISA kit, ProQuantum immunoassay, and ProcartaPlex multiplex panel with that of a conventional ELISA. The Invitrogen™ Instant ELISA™ protocol saves hands-on time with simplified preparation steps and a single wash step, compared with that of a conventional ELISA. The Invitrogen™ ProQuantum™ immunoassays feature a streamlined workflow and consume as little as 2 µL sample per result. The Invitrogen™ ProcartaPlex™ multiplex panels can provide the same information as up to 80 individual ELISAs.

Table 1. Comparison of the protocol and assay requirements of a conventional ELISA with those of three alternative ELISAs.

	ELISA	Instant ELISA	ProQuantum	ProcartaPlex
Time-to-result	4 hr	4 hr	2 hr	4.5 hr
Hands-on time	80 min	40 min	40 min	80 min
Wash steps	Yes, multiple	One wash	No wash	Yes, multiple
Multiplexing	No	No	No	Yes, up to 80 targets
Sample volume	50–100 µL	50–100 µL	2–5 µL	25–50 µL
Readout	HRP-TMB	HRP-TMB	FAM (fluorescence)	RPE (fluorescence)
Instrument	Microplate reader	Microplate reader	qPCR instrument	Luminex® instrument
Instrument read time	2 min	2 min	40–90 min	20–60 min

8-well strips of lyophilized, serially diluted protein standards that are ready to use. Therefore, each Instant ELISA kit can run more samples (96 tests, plus 4 strips or 32 wells of standards) than a conventional ELISA kit, and no plate setup or serial dilutions are required. Simply reconstitute the reagents in the wells with water, and add your unknown samples. After a 3-hour incubation and one wash step, add the enzyme substrate and then end the reaction with stop solution. With the Instant

ELISA workflow, both preparation time and hands-on time are much shorter than with a conventional ELISA. Moreover, instead of attending to your plate every hour to do washes, you have a 3-hour window to focus on something else before wrapping up the assay run. Learn more about our ELISA kit formats, including the Instant ELISA kit, at thermofisher.com/instantelisa.

ProQuantum immunoassays use qPCR to provide a next-generation alternative

Our newest immunoassay platform innovation—the ProQuantum high-sensitivity immunoassay—features a streamlined workflow with no wash steps and consumes as little as 2 µL of sample per result. The small sample-volume requirement means less work, not just during the assay run itself but also during sample collection procedures. In addition, the readout of the ProQuantum immunoassay is on a qPCR instrument, leveraging the high sensitivity and large dynamic range of qPCR technology. The ProQuantum immunoassays can quantify analytes over a concentration range of up to 5 orders of magnitude or more, minimizing the need for sample dilutions.

ProQuantum immunoassays are similar to ELISAs in that they both use matched antibody pairs to detect the target analyte (Figure 2). The assay is based on an optimized pair of antibodies that bind to specific epitopes in close proximity on the analyte. These antibodies are pre-conjugated at the 3′ end of a 60-base oligonucleotide or the 5′ end of a 40-base oligonucleotide (Figure 2A). When added to a sample suspension containing the specific analyte to be quantified, the two antibodies bind to their respective epitopes, which results in the two oligonucleotide strands being brought into proximity of one another (Figure 2A). This antibody binding provides structural stability such that in the presence of DNA ligase and a third splint oligonucleotide (complementary to the ends of the other two DNA oligonucleotides), the two antibody-conjugated oligonucleotides are ligated together to create a 100-base strand that can serve as a DNA amplification template (Figure 2B). Following a temperature increase to 95°C to inactivate the ligase and denature the analyte proteins and antibodies, the template is amplified through 40 cycles of Applied Biosystems™ TaqMan® fluorescence-based qPCR. The amount of amplification after each cycle (fluorescence increase, see Figure 2C) is directly proportional to the number of ligated templates created by the antibody–analyte binding (Figure 2D).

Each ProQuantum immunoassay kit provides enough reagents to scale an assay up or down depending on the user's comfort level with

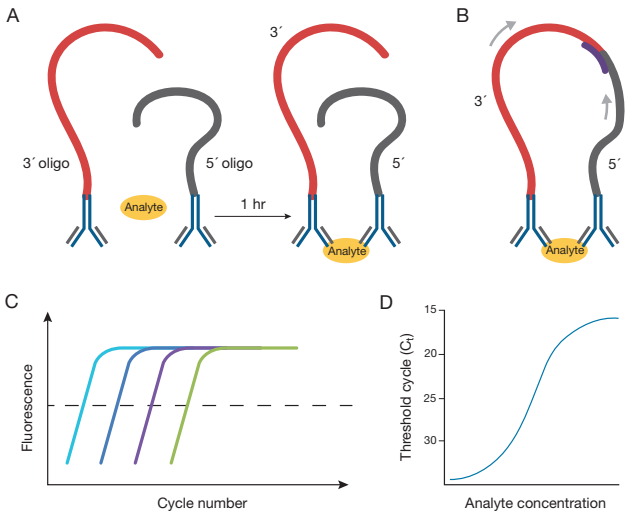

Figure 2. How ProQuantum immunoassays work. ProQuantum immunoassays utilize a matched pair of analyte-specific antibodies, each conjugated to a DNA oligonucleotide. During antibody–analyte binding, the two DNA oligonucleotides are brought into close proximity **(A)**, which then allows for ligation of the two strands by DNA ligase in the presence of a third splint oligonucleotide to create a 100-base template for amplification **(B)**. **(C)** Once the ligase is inactivated, the sample is amplified through 40 qPCR cycles, and the amount of DNA produced, measured via fluorescence, is directly proportional to the number of amplicons generated. This graph shows the fluorescence vs. cycle number curves for four different starting analyte concentrations; the dashed line represents the fluorescence threshold. **(D)** The cycle number required to reach the fluorescence threshold (threshold cycle, or C_t) is plotted vs. analyte concentration to create a standard curve.

small-volume pipetting. Run one 96-well plate if pipetting 5 µL/well, two 96-well plates if pipetting 2 µL/well, or even one 384-well plate for liquid automation handling of 1 µL/well. PCR plates are not included because they should be compatible with the qPCR instrument that will be used. In the ProQuantum protocol, we recommend using a "setup plate" to prepare and organize solutions, samples, and standards. Once the solutions and samples are prepared, use a multichannel pipette to transfer the prepared solutions from the setup plate to the actual PCR plate; this step helps to avoid evaporation when working with small volumes. Incubate the PCR plate at room temperature for 1 hour to allow for antibody–analyte binding, then add the ProQuantum master mix and ligase solutions directly to each well and place the plate into the qPCR instrument for the ligation and qPCR amplification steps. The new free ProQuantum companion software enables easy standard curve generation and data analysis and is accessible from Thermo Fisher™ Cloud. To learn more about ProQuantum immunoassays and software, visit thermofisher.com/proquantum. →

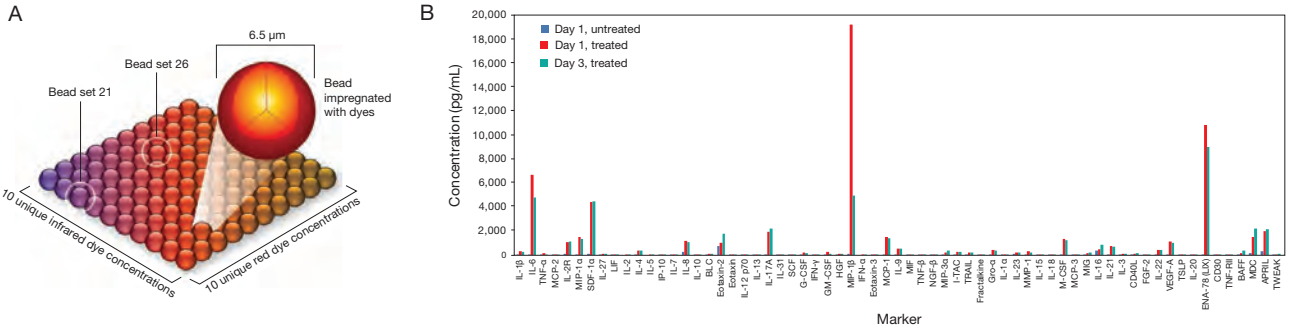

Figure 3. ProcartaPlex multiplex immunoassay system. (A) xMAP magnetic beads are color-coded to enable differentiation of proteins in a single well. **(B)** Using the Invitrogen™ Immune Monitoring 65-Plex Human ProcartaPlex™ Panel (Cat. No. EPX650-10065-901), we quantitatively analyzed 65 soluble protein biomarkers in human peripheral blood mononuclear cells (hPBMCs, collected from a single individual) before treatment, and 1 day and 3 days after treatment with lipopolysaccharide (LPS).

ProcartaPlex panels enable high-level multiplexing to screen proteins

ProcartaPlex multiplex immunoassays are antibody-based magnetic-bead reagent kits and panels for multiplex protein quantitation using the Luminex® instrument platform and associated xMAP® magnetic bead technology. The Luminex® MagPlex® superparamagnetic microsphere beads in the ProcartaPlex assays are internally dyed with precise proportions of red and infrared fluorophores to create 100 spectrally unique signatures (Figure 3A), which can be identified by the Luminex xMAP detection systems, including the Luminex® 200™, FLEXMAP 3D®, and MAGPIX® systems. Similar to a sandwich ELISA, the ProcartaPlex assay uses matched antibody pairs to identify the protein of interest. In a ProcartaPlex multiplex assay, each spectrally unique bead is labeled with antibodies specific for a single target protein, and bound proteins are identified with biotinylated antibodies and streptavidin–R-phycoerythrin (RPE). The conjugation of protein-specific antibodies to a distinct bead allows for analysis of multiple analytes in a single well (Figure 3B). The biggest difference between the two types of immunoassays is that the capture antibody in the ProcartaPlex assay is conjugated to a magnetic bead and not adsorbed to the microplate well as in a conventional ELISA, and therefore the ProcartaPlex assay reagents are free-floating in the solution. For detection, the Luminex 200 instrument, for example, contains two lasers, one to distinguish the spectral signature of each bead and the second to quantify the amount of RPE fluorescence, which is proportional to the amount of protein present in the sample.

ProcartaPlex assays are designed for quantitation of more than 200 cytokine, chemokine, growth factor, and other protein targets from a variety of species (human, mouse, rat, nonhuman primate, canine, and porcine). ProcartaPlex assays allow simultaneous measurement of up to 80 proteins in a single well, which would be equivalent to running 80 individual ELISAs, and a ProcartaPlex multiplex panel can be run in the same amount of time as a single ELISA, with a very similar workflow. In addition, with a ProcartaPlex panel, only 25 µL of sample is required to screen for up to 80 proteins, providing significant sample volume savings. This approach is amenable to high-throughput screening and ideal for looking at many proteins before narrowing down to a handful of specific proteins to interrogate. Select from over 60 preconfigured multiplex panels (2- to 65-plex) or blend singleplex kits (90% are combinable) to create custom multiplex assays that can screen for up to 80 different target proteins. Learn more at **thermofisher.com/procartaplex**.

Learn more

To find out more about the Instant ELISA kits, ProQuantum high-sensitivity immunoassays, and ProcartaPlex multiplex panels and the protein targets currently available for each assay, visit **thermofisher.com/immunoassays** (where you can also download a free biomarker quantitation assay guide). ■

Selected products	Quantity	Cat. No.
Instant ELISA kits		
TNF alpha Human Instant ELISA™ Kit	128 tests	BMS223INST
IL-6 Human Instant ELISA™ Kit	128 tests	BMS213INST
ProQuantum high-sensitivity immunoassays		
IFN gamma Human ProQuantum™ Immunoassay Kit	96 tests	A35576
IL-8 Human ProQuantum™ Immunoassay Kit	96 tests	A35575
ProcartaPlex multiplex panels		
Immune Monitoring 65-Plex Human ProcartaPlex™ Panel	96 tests	EPX650-10065-901
Cytokine & Chemokine 36-Plex Mouse ProcartaPlex™ Panel 1A	96 tests	EPX360-26092-901

Taking photometry to the cyber-physical space

Multiskan Sky Microplate Spectrophotometer with Thermo Fisher Cloud connection.

Scientists are embracing the phenomenon known as the Internet of Things (IoT), which refers to technology driven by machine-to-machine communication. In the life sciences context, the IoT translates into research and medical laboratory instruments that are connected to the internet [1]. Integral to the IoT is the cyber-physical space—the interconnected system of physical devices and computer-based algorithms that control and regulate the instrumentation. By 2020, the IoT is projected to comprise approximately 20 billion discrete devices, surpassing the number of human internet users [2].

In the midst of this digital revolution, Thermo Fisher™ Cloud offers an innovative ecosystem tailored to the needs of life science researchers. It harbors well-designed, user-friendly tools for secure remote instrument access, scientific analysis, and data storage. As the first microplate reader connected to Thermo Fisher Cloud, the Thermo Scientific™ Multiskan™ Sky Microplate Spectrophotometer enables researchers to perform photometric measurements while also taking advantage of the cyber-physical space provided by the IoT.

Multiskan Sky microplate reader: Redefining photometry

The Multiskan Sky Microplate Spectrophotometer (Figure 1) is ideal for multiuser environments and is compatible with a variety of endpoint, kinetic, and spectral assays. This UV/Vis microplate reader is exceptionally convenient for virtually any photometric application, especially nucleic acid and protein analyses. Here we highlight two Multiskan Sky spectrophotometer models (without and with a cuvette port) that have a touchscreen user interface (Figure 1) as well as access to Thermo Fisher Cloud–based tools. These models offer:

- Monochromator-based detection, with a freely adjustable selection of wavelengths from 200 nm to 1,000 nm (in 1 nm steps)
- Compatibility with 96- and 384-well microplates (with and without lids), cuvettes, and the Thermo Scientific™ µDrop™ Plate, a 16-well reusable plate for microvolume DNA, RNA, and protein quantitation
- Onboard shaking and incubation (up to 45°C) for temperature-sensitive assays
- Touchscreen access to built-in protocols for nucleic acid and protein quantitation
- Access to more complex protocols (e.g., cell viability assays) in the Online Protocols Library of Thermo Scientific™ SkanIt™ Software v5.0

Figure 1. Multiskan Sky Microplate Spectrophotometer with touchscreen.

To access cloud-based tools for secure, powerful remote data management, users can link the Multiskan Sky Microplate Spectrophotometer to their Thermo Fisher Cloud accounts via the instrument's touchscreen, from a laptop using the Thermo Fisher Cloud site, or from a cell phone using the Instrument Connect app. Once linked, users can log in to their Multiskan Sky instrument with a simple access code, perform photometric measurements, and upload their data directly to the cloud, where it can be shared within their team or with colleagues around the world. When several Multiskan Sky instruments are used together in an application, users can manage them from the cloud dashboard. In a multiuser setting, a Multiskan Sky instrument can be booked via the "Schedule Instrument" tool of Thermo Fisher Cloud.

Propelling photometry into the IoT space

The cloud-connected Multiskan Sky Microplate Spectrophotometer allows you to access research results remotely, facilitating data analysis, management, and storage. To learn more and stay up to date as resources expand, go to **thermofisher.com/multiskansky**. ■

References

1. Perkel JM (2017) *Nature* 542:125–126.
2. Joyce J (2018) *Laboratory Manager*, 07 May 2018. labmanager.com/laboratory-technology/2018/05/how-the-internet-of-things-is-affecting-laboratory-equipment

Product	Cat. No.
Multiskan™ Sky Microplate Spectrophotometer, with touchscreen	51119600 51119600DP*
Multiskan™ Sky Microplate Spectrophotometer, with touchscreen and cuvette-reading capability	51119700 51119700DP*
µDrop™ Plate	N12391

*Also includes a µDrop™ Plate.

An absorbance-based assay for cell health and proliferation

CyQUANT XTT Cell Viability Assay.

Measuring changes in cell viability is fundamental when assessing cell health, determining gene toxicity, and evaluating anticancer drugs. Many assays are available for determining different parameters of cell health such as metabolism, membrane integrity, and enzyme and channel activity. These assays can be used to provide a snapshot of cell viability or, if used on sequential days, to monitor cell proliferation. Choosing the viability assay that best addresses your research question requires a closer look at the differences between these assays, including the mechanistic readout of cell health status, the detection methods, and the assay sensitivity.

The fastest and easiest way to determine cell health is with an add-and-read (continuous) assay designed for detection with a microplate reader. Ideal for high-throughput applications, the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader paired with Thermo Scientific™ SkanIt™ Software allows for fast readout of absorbance and fluorescence signals, and instantaneous access to data processing steps such as curve fitting, cell viability calculations, and cytotoxicity potencies.

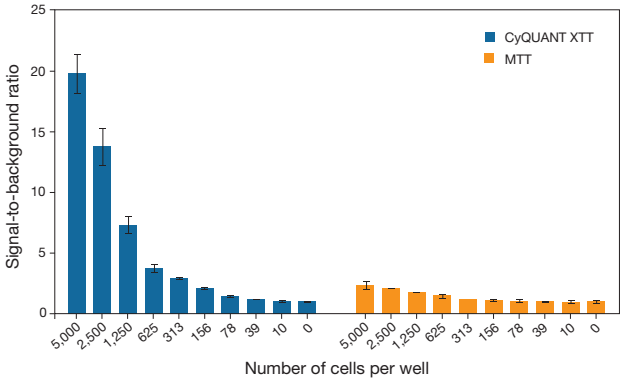

Figure 1. Comparison of the signal-to-background ratios provided by XTT- and MTT-based viability assays. A549 cells were assayed using either the Invitrogen™ CyQUANT™ XTT Cell Viability Assay (Cat. No. X12223) or a commercially available MTT assay according to their respective manufacturers' protocols. The signal-to-background ratios provided by the CyQUANT XTT assay were much greater than those produced with the MTT assay. See Figure 2 for a definition of signal-to-background ratio for the XTT assay product. The signal-to-background ratio for the MTT assay product is calculated as the absorbance of the sample at 540 nm divided by the absorbance of the blank at 540 nm. All measurements were made using a Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

Assay cell viability with tetrazolium salts

Metabolically active cells can oxidize or reduce a variety of chromogenic or fluorogenic probes, providing a measure of cell viability and overall cell health. Tetrazolium salts are widely used for detecting the redox potential of cells for absorbance-based viability, cytotoxicity, and proliferation assays. Following reduction, these water-soluble, colorless compounds are converted to uncharged, brightly colored (nonfluorescent) formazans. Several of the formazans precipitate out of solution and are useful for histochemical localization of the site of reduction or, after solubilization in organic solvent, for quantitation by standard spectrophotometric techniques.

The reduction of MTT remains the most common assay for tetrazolium salt–based viability testing. However, the typical MTT assay requires more than 8 hours to perform, including two separate 4-hour incubation steps. Additionally, because the purple-colored formazan product formed from the reduction of MTT is insoluble, treatment with an acid or DMSO is required to solubilize the formazan before acquiring the data on a microplate reader. Unlike MTT's formazan product, the extremely water-soluble formazan product of XTT does not require solubilization prior to quantitation, thereby reducing the assay time in most viability assay protocols. Moreover, the sensitivity of the XTT reduction assay is reported to be similar to or better than that of the MTT reduction assay [1].

The Invitrogen™ CyQUANT™ XTT Cell Viability Assay provides the XTT reagent for assessing cell viability as a function of cellular redox potential. In the presence of actively respiring cells, XTT is converted to a water-soluble, orange-colored formazan product. In addition, because the CyQUANT XTT assay does not require cell lysis and uses a noninvasive probe, stained cells can be further analyzed with other cell function probes. Compared with an MTT-based assay, the CyQUANT XTT assay displays up to an 8-fold increase in signal-to-background ratios, offering significant advantages when high sensitivity is required for detection of small cell populations or poorly metabolizing cells (Figure 1). This assay can be completed in as little as 2 hours, though 4 hours is recommended for optimal results.

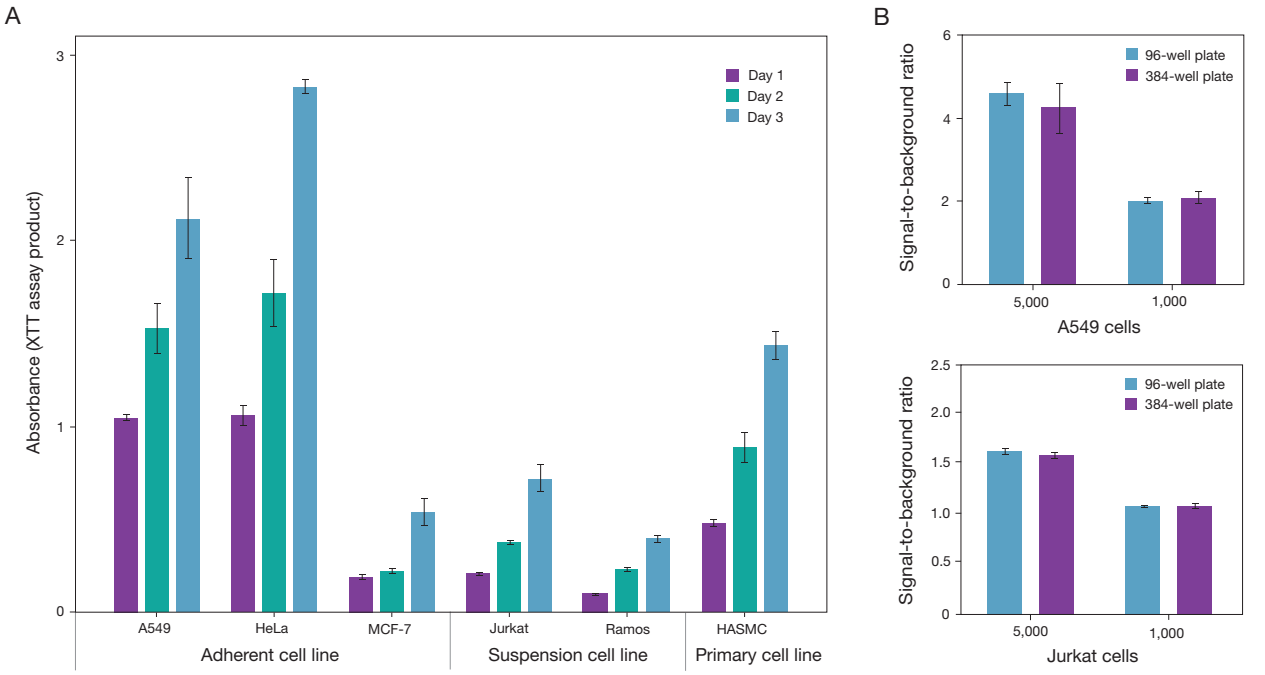

Figure 2. Versatility of the CyQUANT XTT assay. (A) The Invitrogen™ CyQUANT™ XTT Cell Viability Assay (Cat. No. X12223) was used with a variety of cell types, including adherent cells (A549, HeLa, and MCF-7 cells), suspension cells (Jurkat and Ramos cells), and primary cells (human aortic smooth muscle cells, HASMCs), to evaluate cell viability and proliferation. Each cell line was seeded onto three independent plates on day 1, and baseline viability was measured using the CyQUANT XTT assay. The assay was repeated on days 2 and 3, and the absorbance increase over the 3-day period is indicative of cell proliferation. **(B)** The CyQUANT XTT Cell Viability Assay produced nearly identical results when used to evaluate viability of A549 and Jurkat cells in 96- and 384-well assay plates and at two different cell densities. Absorbance of the XTT assay product is reported as the absorbance of the sample at 450 nm minus the absorbance of the sample at 660 nm (this subtracted value is the signal) minus the absorbance of the blank at 450 nm. The signal-to-background ratio is calculated as the signal divided by the absorbance of the blank at 450 nm. All measurements were made using a Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

Simplify viability measurements with the CyQUANT XTT assay

The CyQUANT XTT Cell Viability Assay is a complete and easy-to-use kit for the detection of mammalian cell viability. This kit includes the XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) and an Electron Coupling Reagent, each provided in single-use vials; sufficient reagents are provided for ten 96-well plates, and the kit is stored at –20ºC. The reduction of XTT is facilitated by incorporation of the Electron Coupling Reagent, an electron mediator that scavenges readily available electrons on the plasma membrane, leading to the formation of a reactive intermediate that then reduces XTT to its intensely colored formazan product.

The CyQUANT XTT viability assay protocol is simple: once cells are seeded and treated with the metabolite or drug of interest, the XTT reagent and the Electron Coupling Reagent are thawed, mixed together, and added to cells. After the cells are incubated at 37ºC, their absorbance is measured at 450 nm (the absorption maximum of the orange XTT reduction product) and at 660 nm (to measure background signals due to cell debris and excess coupling reagent) using an absorbance-based plate reader such as the Varioskan LUX Multimode Microplate Reader.

The CyQUANT XTT Cell Viability Assay has been developed to work across a wide range of cell lines, including adherent, suspension, and primary cell types (Figure 2A), and with both 96- and 384-well plates (Figure 2B). The sensitivity and dynamic range of the CyQUANT XTT assay is significantly increased by the inclusion of the Electron Coupling Reagent. In the protocol, we recommend that the XTT/Electron Coupling Reagent stock solution be used soon after thawing and mixing; assay performance is compromised if this stock solution is kept at room temperature for extended periods of time or subjected to freeze/thaw cycles. To help ensure stability, these reagents are provided in single-use vials and stored at –20ºC until thawed for use. →

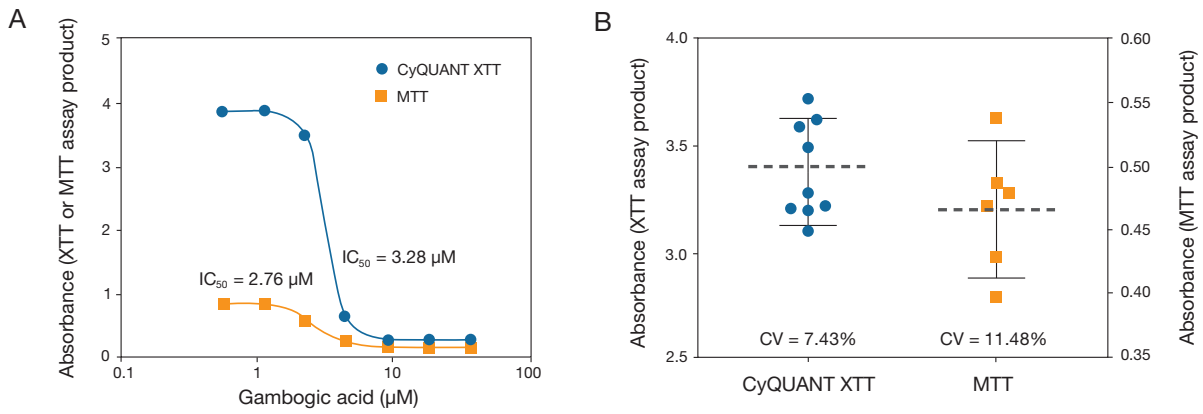

Figure 3. Comparison of CyQUANT XTT and MTT assay sensitivity and variability. (A) The Invitrogen™ CyQUANT™ XTT Cell Viability Assay (Cat. No. X12223) and a commercially available MTT assay were used according to their respective manufacturers' protocols to generate drug dose response curves for A549 cells exposed to increasing concentrations of gambogic acid for ~18 hr at 37°C and 5% CO_2. The IC_{50} value for gambogic acid generated with the CyQUANT XTT assay (3.28 µm) was similar to that obtained with the MTT assay (2.76 µm). **(B)** Variability of the assays was compared by measuring the reduction of either CyQUANT XTT or MTT in multiple wells of untreated cells. The data shown represent an example in which the mean of the assay absorbance (dashed lines) is plotted along with the absorbance of the individual wells. The CV of the CyQUANT XTT assay was smaller than that of the MTT assay. Absorbance of the XTT assay product is reported as the absorbance of the sample at 450 nm minus the absorbance of the sample at 660 nm minus the absorbance of the blank at 450 nm. Absorbance of the MTT assay product is reported as the absorbance of the sample at 540 nm minus the absorbance of the blank at 540 nm. All measurements were made using a Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader, and data were analyzed using GraphPad Prism software.

Generate drug dose response curves with the CyQUANT XTT assay

We used the CyQUANT XTT Cell Viability Assay to generate drug dose response curves for A549 cells exposed to gambogic acid, a xanthanoid compound extracted from the bark of an evergreen tree native to Southeast Asia. Gambogic acid has been shown to inhibit cell growth through the induction of apoptosis and cell death.

Figure 3A shows a comparison of the drug dose response curves obtained with the CyQUANT XTT assay and the MTT assay. The CyQUANT XTT Cell Viability Assay has an IC_{50} for gambogic acid of approximately 3 µM, similar to that of the MTT assay. Furthermore, this study shows that the CyQUANT XTT assay measures cell viability with greater sensitivity (Figure 3A) and less variability (Figure 3B) than does the MTT assay.

Add the XTT assay to your lab's repertoire

The CyQUANT XTT Cell Viability Assay is a complete, optimized, easy-to-use kit for sensitive colorimetric detection of viable mammalian cells using a microplate reader. Unlike other commercially available viability assays, the CyQUANT XTT assay displays a large dynamic range and low well-to-well variability and can be multiplexed with other cell function probes. For more information on absorbance- or fluorescence-based microplate assays for viability, visit **thermofisher.com/microplate-viability**. ■

Reference

1. Meshulam T, Levitz SM, Christin L et al. (1995) *J Infect Dis* 172:1153–1156.

Product	Quantity	Cat. No.
CyQUANT™ XTT Cell Viability Assay	1 kit	X12223
Varioskan™ LUX Multimode Microplate Reader, absorbance and fluorescence intensity measurements, top-reading	1 each	VL0000D0 VL0000D1
Varioskan™ LUX Multimode Microplate Reader, absorbance and fluorescence intensity measurements, top- and bottom-reading	1 each	VLB000D0
Varioskan™ LUX Multimode Microplate Reader, absorbance, fluorescence intensity, and luminescence measurements, top-reading	1 each	VL0L00D0
Varioskan™ LUX Multimode Microplate Reader, absorbance, fluorescence intensity, and luminescence measurements, top- and bottom-reading	1 each	VLBL00D0
SkanIt™ Software for Microplate Readers, Research Edition	1 each	5187139

Put the Attune NxT Autosampler to work for high-throughput screening

Multiparametric flow cytometry assays for determining pharmacological effects.

High-throughput screening (HTS) is an extremely effective method for identifying potential candidates for therapeutics. Initial compound screening is often performed on a microplate reader because multiwell plate assays tend to be easy to perform, cost-effective, and robust, and they enable the screening of a large number of compounds in a single run. Another useful platform for HTS is flow cytometry, which offers the additional benefit of cell-by-cell analysis. A flow cytometer paired with an autosampler—such as the Invitrogen™ Attune™ NxT Flow Cytometer equipped with the Invitrogen™ Attune™ NxT Autosampler—provides an extremely effective platform for performing HTS assays using multiwell plates (Figure 1). The Attune NxT Flow Cytometer is up to 10 times faster than traditional flow cytometers and has 16 detection channels available, allowing analysis of up to 35,000 events per second. These features greatly benefit studies of protein–protein interactions, metabolic activity, and DNA content in a single or multiparametric assay format.

Figure 1. Setup of Attune NxT Flow Cytometer with Autosampler for high-throughput screening experiments. (A) Invitrogen™ Attune™ NxT Flow Cytometer with Invitrogen™ Attune™ NxT Autosampler. **(B)** Plate heat-mapping is available on Invitrogen™ Attune™ NxT Software to monitor results as they are acquired.

HTS on the Attune NxT Flow Cytometer

Multiparametric flow cytometry provides a means of analyzing large numbers of individual cells for several cell health parameters in a short amount of time. The ability to perform multiple secondary and tertiary assays at the same time as the initial compound screening assay helps to minimize sample collection and preparation steps while also allowing for the robust analysis of cell health in a population. Multiparametric analyses provide a more complete picture of the complex effects on cell function that result from a particular compound treatment.

The toxicity of a pharmaceutical compound can be assessed in less than 45 minutes per plate (acquisition time, with real-time visualization as results are collected) on the Attune NxT Flow Cytometer with Autosampler. When running multiple samples, Invitrogen™ Attune™ NxT Software allows easy transitions between tube and plate format using a one-click command; no disassembly is required. The many advantages to running the Attune NxT Flow Cytometer with Autosampler in high-throughput mode include:

- Compatibility with a variety of plate types, including 96- and 384-well, deep-well, and round- and V-bottom plates
- Optimized mixing to enable maximal sample throughput with minimal effects on cell viability
- Live heat maps for rapid screening and the ability to monitor results while the instrument acquires data
- Real-time numeric overlay text for assay feedback in live heat maps
- Consistent data with minimal variation, regardless of sampling method and collection rate
- Automated compensation calculations

Initial screening for compounds that affect cell viability

In a typical initial screening of potential pharmaceutical drugs, "hit" compounds—defined as molecules that have the desired activity based on the mechanistic readout of the functional assay used—are identified and then further analyzed using a variety of cell health assays and compound concentrations to establish potency. In the HTS flow cytometry experiment shown in Figure 2, we used a cell viability assay to initially identify hit compounds that affected the health of Ramos cells (B cells) and Jurkat cells (T cells, data not shown) cultured at different oxygen levels. In this example, the viability was determined based on membrane integrity using the Invitrogen™ LIVE/DEAD™ Fixable →

Aqua Dead Cell Stain, a membrane-impermeant amine-reactive dye that only enters cells with compromised membranes. Thus, in dead cells with compromised membranes, the dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining; in live cells, the dye's reactivity is limited to cell-surface amines, resulting in less-intense fluorescence.

Cells were cultured at both normoxic (≥19% O_2) and hypoxic (1% O_2) conditions to mimic the variable oxygen levels found in tumors. Figure 2 shows an example of the results of this initial screening, with a few compounds marked as hits. In this experiment, a hit is defined as a compound that produces different effects on the viability of cells exposed to the two different oxygen levels. These hit compounds were then tested further to assess their potency, including determining the drug dose response and their apparent EC_{50}.

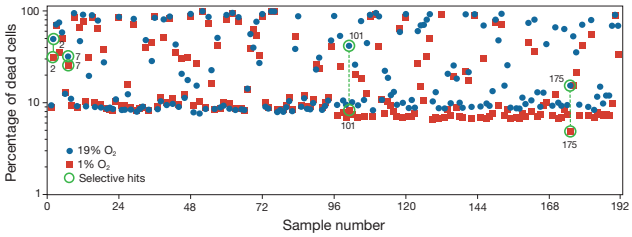

Figure 2. High-throughput screening of Ramos cells stained with LIVE/DEAD Fixable Aqua Dead Cell Stain using the Attune NxT Flow Cytometer with Autosampler. Identical plates containing Ramos cells (40,000 cells per well) were treated with 10 µM of the different drugs in the Killer Collection (MicroSource Discovery Systems Inc.) and cultured under standard conditions with either hypoxic (1% O_2) or normoxic (19% O_2) levels using a Thermo Scientific™ Heracell™ VIOS 160i CO_2 Incubator. Cells were stained with Invitrogen™ LIVE/DEAD™ Fixable Aqua Dead Cell Stain (for 405 nm excitation, Cat. No. L34957) and analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer with Autosampler. Cell viability is expressed as percentage of dead cells (which are >50-fold more fluorescent than live cells); ~Z′ = 0.8.

Secondary screening to define mechanism of action

Once initial hit compounds were identified, secondary assays were performed to help define the mechanism of action of each compound on the cell model. To better characterize these compounds, we determined the drug dose dependency using assays for cell cycle phase (DNA content), membrane integrity, mitochondrial function, and apoptosis. For example, here we show the secondary screening of the hit compound amsacrine, a potent chemotherapeutic drug known to inhibit DNA replication, in both Ramos cells (data not shown) and Jurkat cells (Figures 3 and 4) at both normoxic and hypoxic oxygen conditions. To perform this battery of cell health assays, we incubated cells with a dye cocktail containing four different fluorescent cell function probes, using a single-step, multiplex high-throughput workflow.

The drug dose response can be visualized on a heat map that is generated by Attune NxT Software and referenced to any statistic such as cell count, cell percentage, or cell concentration in each well of the microplate. To obtain statistically relevant data sets, each sample (and well) should contain the same number of cells, and the same number of events across all samples should be collected to avoid sample-to-sample variation that can result from differences in cell number and fluorescence signal.

To monitor the effect of amsacrine on cell health, the dye cocktail included Hoechst™ 33342 dye, a cell-permeant nucleic acid stain that can be used to measure DNA content and to distinguish the cell cycle phase of each cell by flow cytometry (Figure 3A). Because data are acquired at the single-cell level, histogram plots generated during this

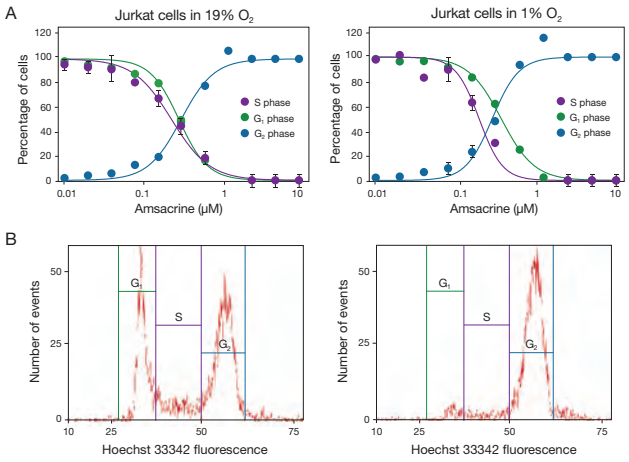

Figure 3. Determination of the effective dose response to pharmaceutical drugs in each phase of the cell cycle by staining with Hoechst 33342 dye. (A) Using Jurkat cells stained with Invitrogen™ Hoechst™ 33342 Ready Flow™ Reagent (Cat. No. R37165), EC_{50} values can be calculated for each phase of the cell cycle. There are no significant differences in the cell cycle for Jurkat cells treated with amsacrine at either 19% or 1% O_2. **(B)** The G_1, S, and G_2/M phases are observed in healthy replicating cells (left). Cells treated with 2.5 µM amsacrine become apoptotic and accumulate in the G_2 phase (right).

flow cytometry analysis also allow visualization of the distribution of cells in each cell cycle stage at a given amsacrine concentration (Figure 3B).

To investigate the effect of amsacrine on membrane integrity and mitochondria, Invitrogen™ SYTOX™ Red Dead Cell Stain and tetramethylrhodamine methyl ester (TMRM) were also included in the dye cocktail. SYTOX Red stain is a cell-impermeant nucleic acid stain that easily

penetrates cells with compromised membranes, where it becomes highly fluorescent upon binding DNA, but that will not cross the membranes of live cells. TMRM is a cell-permeant, red-orange–fluorescent dye that accumulates in functioning mitochondria with intact membrane potentials; as mitochondrial membranes depolarize, fluorescent signals decrease. Jurkat cells exposed to either 19% or 1% oxygen and treated with increasing concentrations of amsacrine showed a similar response, with no significant differences in EC_{50} values between the two oxygen levels (Figure 4A) for cell viability and mitochondrial health.

Similarly, oxygen levels had no effect on apoptosis, which was detected using Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent (Figure 4B), also included in the dye cocktail. The cell-permeant CellEvent reagent comprises the four–amino acid peptide DEVD—which contains the recognition site for caspases 3 and 7—conjugated to a nucleic acid–binding dye. Because the DEVD peptide inhibits the ability of the dye to bind to DNA, CellEvent Caspase-3/7 Green reagent is intrinsically nonfluorescent. In the presence of activated caspases 3 and 7, key markers in the early stages of apoptosis, the dye is cleaved from the DEVD peptide and free to bind DNA, producing a bright green-fluorescent signal indicative of apoptosis.

Benefits of multiparametric flow cytometry screening

Using the Attune NxT Flow Cytometer with Autosampler in combination with a dye cocktail to assess cell health, we assayed several cell health parameters simultaneously in multiple samples in a multiwell plate. The Attune NxT Autosampler provides the functionality to run a drug dose response assay across a 96-well plate and obtain six independent EC_{50} values at two different oxygen levels, all in under 45 minutes (Table 1, Figure 5). See our comprehensive suite of products for flow cytometry, from instruments and standards to antibodies and cell function reagents, at **thermofisher.com/flowcytometry**. ■

Product	Quantity	Cat. No.
Attune™ NxT Autosampler	1 each	4473928
Attune™ NxT Flow Cytometer, blue/red/violet6/yellow	1 each	A29004
CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit	100 assays	C10427
Hoechst™ 33342 Ready Flow™ Reagent	120 assays	R37165
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	80 assays 200 assays 400 assays	L34965 L34957 L34966
SYTOX™ Red Dead Cell Stain, for 633 or 635 nm excitation	1 mL	S34859
MitoProbe™ TMRM Assay Kit for Flow Cytometry	100 assays	M20036

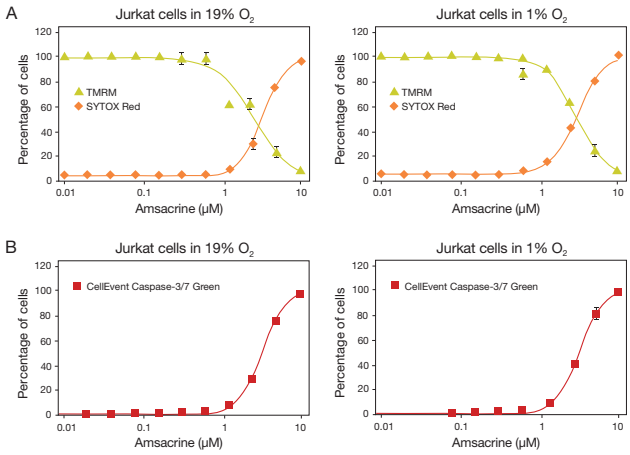

Figure 4. Secondary assays to examine cellular responses to amsacrine treatment. (A) Jurkat cells were exposed to a range of amsacrine concentrations overnight and then stained with Invitrogen™ SYTOX™ Red Dead Cell Stain (for 633 or 635 nm excitation, Cat. No. S34859) and tetramethylrhodamine methyl ester (Invitrogen™ MitoProbe™ TMRM Assay Kit for Flow Cytometry, Cat. No. M20036). As the amsacrine concentration increases, the SYTOX Red signal intensifies, indicating an increase in cell death as the cell membrane integrity is lost, while the TMRM signal decreases, indicating a loss in mitochondrial membrane potential. **(B)** In Jurkat cells stained with the Invitrogen™ CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Cat. No. C10427), an increase in CellEvent Caspase-3/7 Green signal, indicative of apoptosis, is seen in response to increasing concentrations of amsacrine.

Table 1. Summary of EC_{50} values in cell function assays using Jurkat cells.

Fluorescent probe	Cell function detected	EC_{50} (µM) in 1% O_2	EC_{50} (µM) in 19% O_2
Hoechst 33342	S phase	0.20	0.24
	G_1 phase	0.37	0.30
	G_2 phase	0.28	0.31
CellEvent Caspase-3/7 Green	Apoptosis	2.84	3.30
TMRM	Mitochondrial health	2.89	2.60
SYTOX Red	Membrane integrity	2.93	3.48

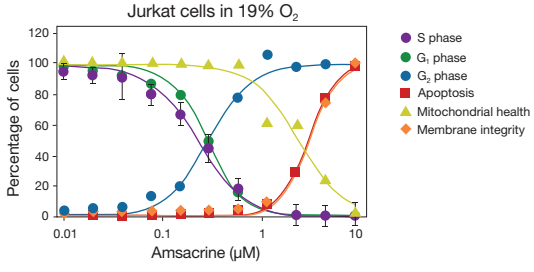

Figure 5. Summary of multiplex staining of Jurkat cells in a range of amsacrine concentrations under normoxic (19% O_2) conditions. Jurkat cells were stained with a cocktail containing four different cell health indicator dyes, and six EC_{50} values were generated. At >0.3 µM (EC_{50}) amsacrine, Jurkat cells are arrested in the G_2 phase and no longer replicate. Measurements of mitochondrial health and caspase activity based on amsacrine treatment correlate with one another, supporting the role of mitochondria in the apoptosis pathway.

Flow cytometry compensation tools for a host of GFP variants

GFP BrightComp eBeads Compensation Bead Kit.

The discovery in the early 1960s [1] and subsequent development of Green Fluorescent Protein (GFP) as a reporter gene has greatly advanced the study of gene expression, protein localization, and cell and tissue development in a multitude of disciplines. GFP, an intrinsically fluorescent protein originally isolated from the jellyfish *Aequoria victoria*, enables real-time examination in live cells of processes that have conventionally been observed through immunocytochemical snapshots in fixed specimens.

Although enhanced Green Fluorescent Protein (EGFP, Ex/Em = 488/510 nm) has emerged as the most widely used GFP derivative, a number of other GFP variants have been isolated or engineered, each with minor variations in extinction coefficient, quantum yield, and excitation and emission wavelengths [2]. Concerns that these variations have the potential to impact compensation values in flow cytometry experiments have led many researchers to require that the emission spectrum of the compensation control and the sample fluorophore be identical. This requirement necessitates the use of sample to collect compensation data, an arduous and sometimes costly addition to the experimental protocol. Here we show that the Invitrogen™ GFP BrightComp eBeads™ Compensation Bead Kit—designed to be used to collect compensation data for EGFP—can also be used with a number of popular variants of GFP.

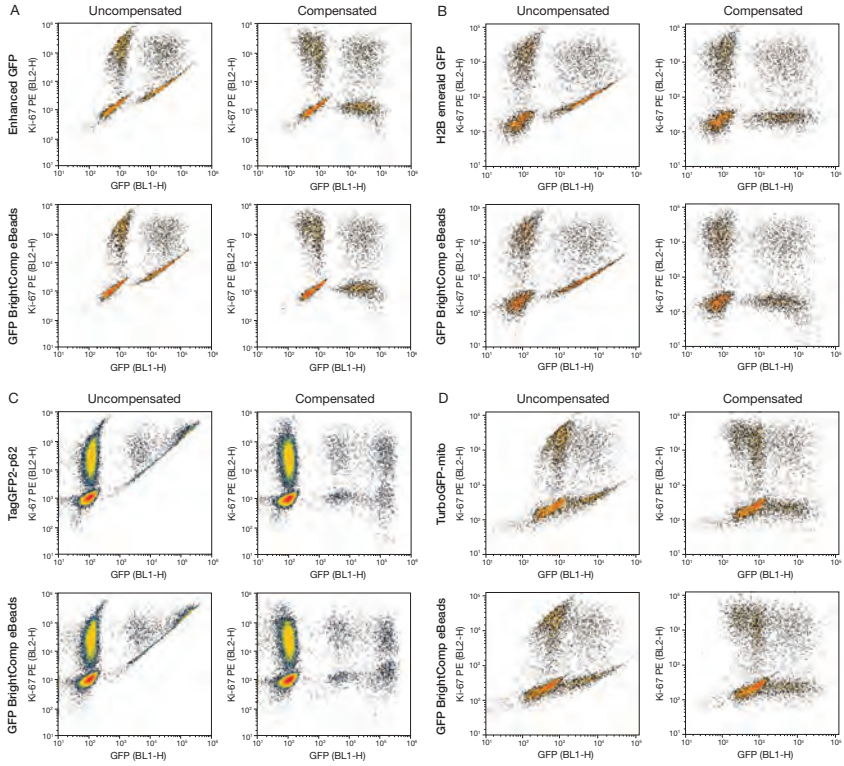

Figure 1. Flow cytometric analysis of GFP-expressing U2OS cells using either sample or GFP BrightComp eBeads Compensation Beads for compensation. U2OS cells expressing a variant of GFP were harvested, stained with Invitrogen™ LIVE/DEAD™ Fixable Far Red Dead Cell Stain (Cat. No. L10120), fixed, permeabilized, and then stained with Invitrogen™ Ki-67 Monoclonal Antibody (clone 20Raj1), PE conjugate (Cat. No. 12-5699-41). Data for each of the samples were acquired twice at the same voltages: first using the respective GFP-expressing U2OS cells for compensation (top row of each panel), and second using the Invitrogen™ GFP BrightComp eBeads™ Compensation Bead Kit (bottom row of each panel). Dual-parameter density plots (expressed GFP variant vs. PE-Ki-67 antibody staining) for each sample acquisition are presented without any compensation (uncompensated, left-hand columns of each panel) or with compensation with either cells or beads (compensated, right-hand columns of each panel). **(A)** For enhanced GFP expression, U2OS cells were transduced with an adenovirus containing enhanced GFP under the control of a CMV promoter (Vector Biolabs). **(B)** For emerald GFP expression, U2OS cells were transduced with Invitrogen™ CellLight™ Histone 2B-GFP (BacMam 2.0, Cat. No. C10594). **(C)** For TagGFP2 expression, U2OS cells were transduced with Invitrogen™ Premo™ Autophagy Sensor GFP-p62 (BacMam 2.0, Cat. No. P36240). **(D)** For TurboGFP expression, U2OS cells were transfected with the pTurboGFP-mito vector (Evrogen) using the Invitrogen™ Neon™ Transfection System (Cat. No. MPK5000). Compensation values for the LIVE/DEAD Fixable Far Red Dead Cell Stain were determined using 1:1 live and heat-killed U2OS cells labeled with the cell stain; compensation values for PE were determined using Invitrogen™ AbC™ Total Antibody Compensation Beads (Cat. No. A10513) labeled with PE anti–Ki-67 antibody. Samples were acquired on the Invitrogen™ Attune™ NxT Flow Cytometer at a flow rate of 200 µL/min; data were analyzed using the Attune NxT Software v2.6.

GFP compensation beads are compatible with GFP variants

Although the GFP BrightComp eBeads Compensation Beads were developed to compensate for EGFP, they are compatible with several variants of GFP, including emerald GFP, TagGFP2, and TurboGFP (Figure 1), as well as AcGFP, a monomeric GFP isolated from *Aequorea coerulescens*. To demonstrate their compatibility, we analyzed U2OS cells expressing four different GFP derivatives and used either GFP-expressing cells from the actual sample or GFP BrightComp eBeads Compensation Beads to obtain compensation values.

Figure 1 shows the complete set of uncompensated and compensated dual-parameter density plots of four multiplexed samples, each compensated with either the particular GFP variant–expressing U2OS cells or GFP BrightComp eBeads Compensation Beads. In all cases, the multiplexed samples were acquired twice at the same voltages using the Invitrogen™ Attune™ NxT Flow Cytometer at a flow rate of 200 µL/min.

For each GFP variant tested, we found that the GFP BrightComp eBeads Compensation Beads can be used as a replacement for traditional compensation methods that require the use of sample. The emission spectra of these GFP variants are not significantly different from that of EGFP, and use of the GFP BrightComp eBeads Compensation Beads has been shown to not impact compensation results in multiplexed flow cytometry experiments. In addition to their use to compensate multiple GFP variants, these compensation beads are effective with both transduction and transfection methods and for a variety of expression targets, including GFP fusion proteins.

GFP BrightComp eBeads Compensation Beads are easy to use

The GFP BrightComp eBeads Compensation Beads provide a simple method for the compensation of GFP and its variants in flow cytometry experiments. Provided in a convenient dropper vial, these compensation beads are embedded-dye microspheres with a diameter of approximately 5 µm (actual size for each lot is listed on the vial label) that can be used to determine compensation values in samples with different levels of GFP expression. Each drop of beads contains negative control (unstained) beads, as well as beads stained with a dye that is excited with a blue (488 nm) laser and exhibits three intensity levels to match a variety of GFP expression levels, with an emission spectrum that is a nearly identical match to that of EGFP (Figure 2).

Simplify GFP compensation in your lab

The GFP BrightComp eBeads Compensation Beads provide a reliable, accurate, and simple-to-use technique for setting flow cytometry compensation when analyzing GFP-expressing samples. Here we show that these compensation beads can be used as a replacement for traditional methods that use sample to obtain compensation values. In addition, GFP BrightComp eBeads Compensation Beads are effective with multiple GFP variants, expression targets, and transduction and transfection methods.

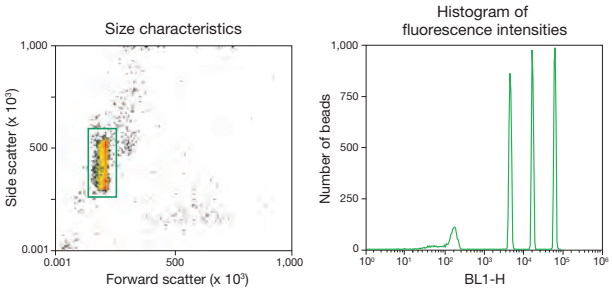

Figure 2. Size and fluorescence characteristics of the GFP BrightComp eBeads Compensation Beads. Fluorescent proteins are expressed in cells at varying levels, producing a range of fluorescence intensities. The Invitrogen™ GFP BrightComp eBeads™ Compensation Bead Kit (Cat. No. A10514) provides a suspension of beads that includes negative control (unstained) beads and beads stained at three levels of intensity with a dye that is a near-identical spectral match to GFP. Data were acquired on an Invitrogen™ Attune™ NxT Flow Cytometer using a 488 nm laser; emission was collected using a 530/30 nm bandpass filter for GFP.

Learn more about the diverse selection of flow cytometry compensation beads

Thermo Fisher Scientific offers a number of other flow cytometry compensation beads for use with a range of fluorophores. The Invitrogen™ UltraComp and Invitrogen™ OneComp eBeads™ Compensation Beads each contain a mixture of antibody-coated positive compensation beads and uncoated negative compensation beads in a single vial for quick and easy fluorescence compensation. The Invitrogen™ AbC Total Antibody and Invitrogen™ ArC Amine-Reactive Compensation Beads provide positive beads—which either bind all isotypes of the specific immunoglobulin or bind any of the amine-reactive dead cell stains in the Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stain Kits—and negative beads with no binding capacity or reactivity, for use in setting fluorescence compensation. These two components are provided in separate vials such that negative beads can be added after the positive beads are labeled, in order to avoid any transfer of fluorescence over time. Find out more about our wide selection of flow cytometry compensation tools at **thermofisher.com/brightcomp**. ■

References

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2. Day RN, Davidson MW (2009) *Chem Soc Rev* 38:2887–2921.

Product	Quantity	Cat. No.
GFP BrightComp eBeads™ Compensation Bead Kit	25 tests	A10514

Optimize detectors for high-quality flow cytometry data

Evaluation of different techniques for PMT optimization using the Attune NxT Flow Cytometer.

In a flow cytometry experiment, high-quality data can only be obtained when the instrument and its individual components are optimized. The detector is one of the critical components of a flow cytometer. A detector captures the photons that are emitted by the excited fluorophores and scattered laser light and converts them into photocurrent, which is then passed to the electronics system.

The most commonly used detectors for flow cytometry are the photomultiplier tubes (PMT) that reside in each channel of the instrument (Figure 1). In addition to converting the photons to photocurrent, the PMT amplifies the signal, a process that requires the application of a steady-state voltage to the detector. PMT sensitivity is controlled by the material used to construct it and the wavelength of light entering it, as well as the amount of voltage applied to it. Therefore, optimization of that applied voltage (or gain) is required for each PMT detector in order to obtain the best-quality data in each channel of the flow cytometer.

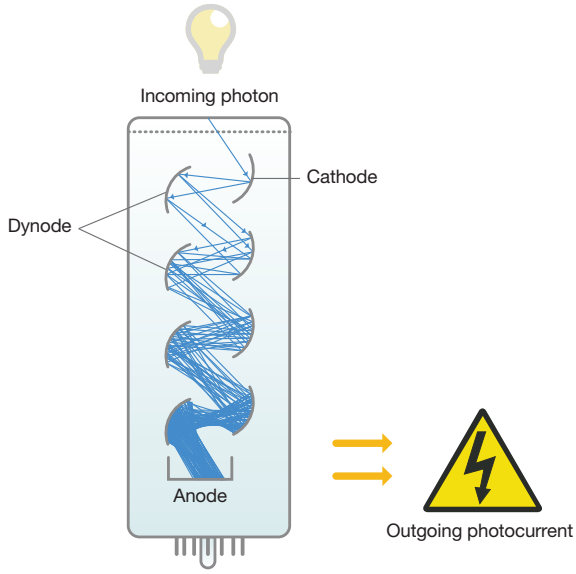

Figure 1. Schematic of a photomultiplier tube detector for a flow cytometer. An electron is emitted when a photon enters the photomultiplier tube (PMT) at the cathode. It then travels through the PMT, being amplified at the dynodes (electrodes) throughout and ending up at the anode, which is a collecting electrode.

Optimizing the voltage

As the voltage applied to the PMT is increased, the fluorescent signal is increasingly separated from background noise, providing greater resolution of the positive signal. At a certain voltage, however, the increasing separation of fluorescent signal from background will plateau, and the separation of fluorescent signal from background will remain constant. This voltage level is called the minimum voltage requirement (MVR); an ideal MVR setting will amplify dim signals above background but will not be so high that the fluorescent signals exceed the upper range of PMT detection linearity. Additionally, the MVR should allow the fluorescence signal of unstained and brightly stained cells or beads to be seen in plots on the same numeric scale. If the voltage is set too far above the MVR, the dim signal will still be separated from background but the positive signal may go off scale. Conversely, setting the voltage too far below the MVR will most likely result in compromised detection of dim signals.

Flow Cytometry Basics Module—Molecular Probes School of Fluorescence

What are the main components of a flow cytometer? How does a flow cytometer focus the sample stream and interrogate individual cells? How is the photocurrent from the detector digitized and processed? Find answers to these questions and more in the Invitrogen™ Molecular Probes™ School of Fluorescence—Flow Cytometry Basics module, available online. The Flow Cytometry Basics module is designed to help you understand the fundamentals of a flow cytometry experiment. This online content covers the three main components of a flow cytometer—fluidics, optics, and electronics—and was written by the bench scientists who created the *Molecular Probes Handbook*. Learn more at **thermofisher.com/mpsf-flow**.

Voltration

A common method for setting the MVR is the "Peak 2" method [1]. With this method, dimly fluorescent beads are run using a series of different voltage settings (also called voltration or voltage walk), and the spread of the signal (or the coefficient of variation, CV) is plotted against the voltage series. An example of a voltration experiment is shown in Figure 2. While this method does result in setting the MVR to get clear resolution of the dim fluorescent signal from the background noise of the detector, it does not address the need to ensure that the brighter fluorescent signals do not exceed the upper limit of the PMT detection range. This drawback has led to the development of a variety of methods in which unstained and brightly stained cells or beads are both used to determine the MVR [2-4].

Comparison of methods to determine MVR

Recently our scientists presented a study in which they compared different sample types as well as various calculated parameters to determine the MVR on the Invitrogen™ Attune™ NxT Flow Cytometer and any significant differences between the methods [5]. The samples tested included: different types of internally dyed microspheres (hard-dyed beads) that are detected in all detectors but do not include fluorophores used for typical experimentation; antibody-capture beads labeled on the bead surface with fluorescent antibody conjugates that are detected in a specific detector; lymphocytes that were either unstained or stained with fluorescent antibody conjugates; and a combination of the different samples.

The parameters derived from the data included calculation of the standard deviation of the electronic noise (SDEN), as well as of the staining index (SI), alternative staining index (Alt SI), and voltration index (VI) (Figure 3). In this article we will focus on a subset of these data, mainly the comparison of SI, Alt SI, and VI using antibody-capture beads (Invitrogen™ AbC Total Antibody Compensation Beads), lymphocytes (unstained and stained Beckman Coulter CYTO-TROL™ Control Cells), and a combination of the two (Table 1). You can view the scientific poster of the entire study at thermofisher.com/pmtposterbp78. →

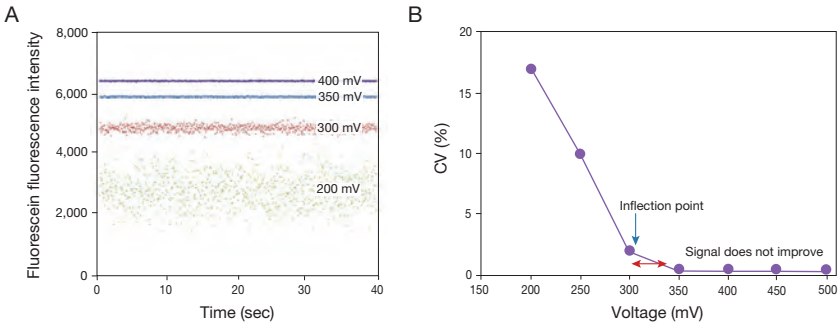

Figure 2. Principle of voltration using the Peak 2 method. Fluorescein-labeled beads were run at increasing PMT voltage settings. **(A)** The fluorescence events in the green channel were detected at different PMT voltages (indicated in the plot for each data set) and plotted against time. **(B)** The coefficient of variation (CV) for each data set in **A** was plotted against the PMT voltage setting. The point on the curve where the CV begins to level out is the inflection point, which marks where there is decreasing variation of the data at higher voltages. The red arrow indicates the optimal PMT voltage range for this fluorescence channel.

$$\text{Staining index (SI):} \quad \frac{\textit{(Median positive – Median negative)}}{\textit{(2 x SD negative)}}$$

$$\text{Alternative staining index (Alt SI):} \quad \frac{\textit{Median positive}}{\textit{SD negative}}$$

$$\text{Voltration index (VI):} \quad \frac{\textit{Alt SI}}{\sqrt{\textit{Voltage}}}$$

Figure 3. Equations used for MVR calculations. Three different equations were used to determine MVR. Median positive and median negative refer to the median fluorescence signal from the stained and unstained, respectively, beads or cells. SD negative is the standard deviation of the fluorescence measurements from the unstained beads or cells.

Table 1. Minimum voltage requirement (MVR) for the BL1 channel, determined with different parameters and different sample types.

Sample composition	Parameter used to calculate MVR for BL1*		
	Staining index	Alternative staining index	Voltration index
AbC Total Antibody Compensation Beads (unstained and stained beads)	400 mV	400 mV	400 mV
CYTO-TROL lymphocytes (unstained and stained cells)	425 mV	450 mV	450 mV
AbC Total Antibody Compensation Beads (stained) and CYTO-TROL lymphocytes (unstained)	450 mV	450 mV	450 mV

*The MVR determinations for the BL1 detector (fluorescein channel) using fluorescein-stained cells and beads are shown. The MVR for each detector must be determined separately.

The resulting MVR values

The results of the study are shown in Figure 4, and the MVR obtained for the BL1 detector (the fluorescein channel) using each method on three different sample types is summarized in Table 1. On the Attune NxT Flow Cytometer, the MVR for the AbC Total Antibody Compensation Beads alone (Figure 4A and 4B) using SI, Alt SI, or VI values was found to be ~400 mV. The MVR for the CYTO-TROL lymphocytes alone (Figure 4C and 4D) and for labeled AbC Total Antibody Compensation Beads with unstained CYTO-TROL cells (Figure 4E and 4F) was determined to be ~450 mV. All three methods (SI, Alt SI, VI) provide a working range of voltages that can be used to achieve high-quality flow cytometry data.

MVR methods and more

Instrument settings play a critical role when designing a flow cytometry experiment and must be refined for each application depending on the target specificity and abundance, specific antibodies, cell function probes, and fluorophores in the study. Using MVR settings that have been optimized for each PMT detector in each flow cytometer channel helps to minimize day-to-day variation in instrument performance and ensure that the resolution sensitivity for each parameter will be consistent from experiment to experiment. For a more complete description of all MVR methods evaluated, visit thermofisher.com/pmtposterbp78. ■

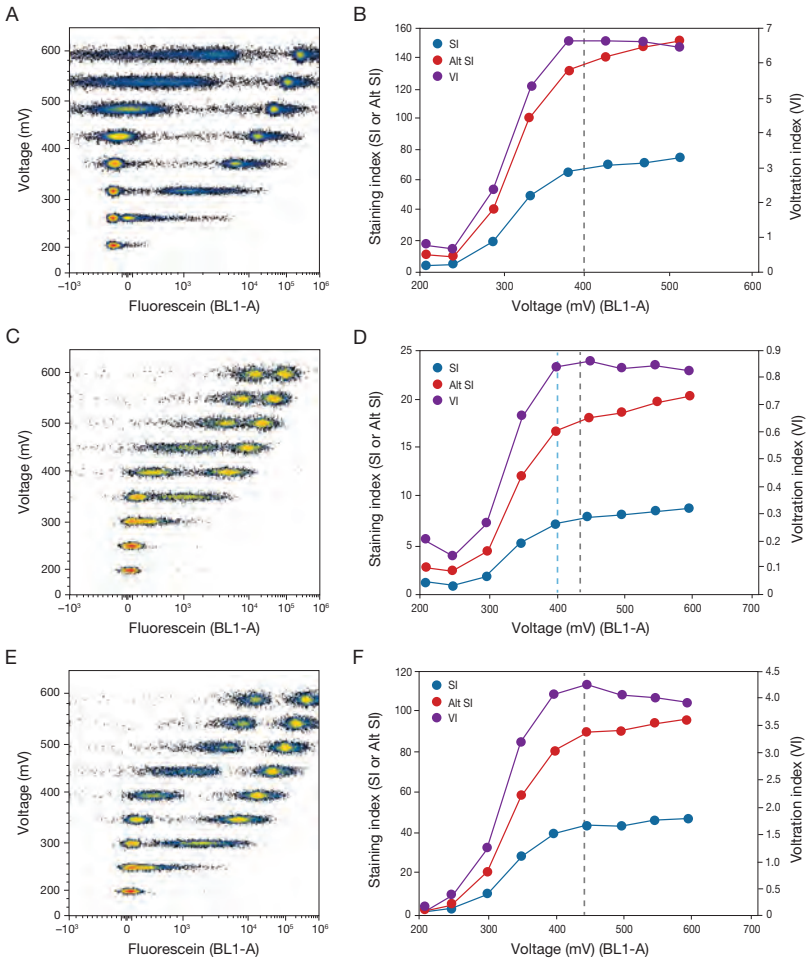

Figure 4. Comparison of different methods for MVR determination. The data for each sample type were acquired on the Invitrogen™ Attune™ NxT Flow Cytometer (BRVY and BRV6Y configurations) at a flow rate of 200 µL/min using a FSC threshold. A gate was placed around the main cell population, and a stop criterion of 10,000 gated events was used. Area, height, and width parameters were collected for all data points. Samples were recorded over a range of voltage settings, at 1 mV and from 50 to 650 mV, recorded at 50 mV increments for each detector. Data files were concatenated and plotted in the same graph **(A, C, E)**. The SI, Alt SI, and VI values were then calculated for each sample type and were plotted as a function of PMT voltage setting **(B, D, F)**. **(A, B)** Data were collected using Invitrogen™ AbC Total Antibody Compensation Beads labeled with a fluorescent antibody conjugate, and the unstained negative AbC beads. The black dashed line in **B** indicates the MVR for all three methods of calculation. **(C, D)** Data were collected using the autofluorescent signal of unlabeled CYTO-TROL™ lymphocyte cells (Beckman Coulter) and cells labeled with a fluorescent antibody conjugate. The blue dashed line in **D** represents the MVR for the SI calculations, and the black dashed line represents the MVR for both the Alt SI and the VI. **(E, F)** Data were collected using a combination of labeled AbC Total Antibody Compensation Beads and unlabeled CYTO-TROL lymphocytes. The black dashed line in **F** represents the MVR for all three methods of calculation (SI, Alt SI, and VI).

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Product	Quantity	Cat. No.
Attune™ NxT Flow Cytometer, blue/red/violet6/yellow (BRV6Y configuration)	1 each	A29004
Attune™ NxT Flow Cytometer, blue/red/violet/yellow (BRVY configuration)	1 each	A24858
AbC™ Total Antibody Compensation Bead Kit	25 tests 100 tests	A10513 A10497

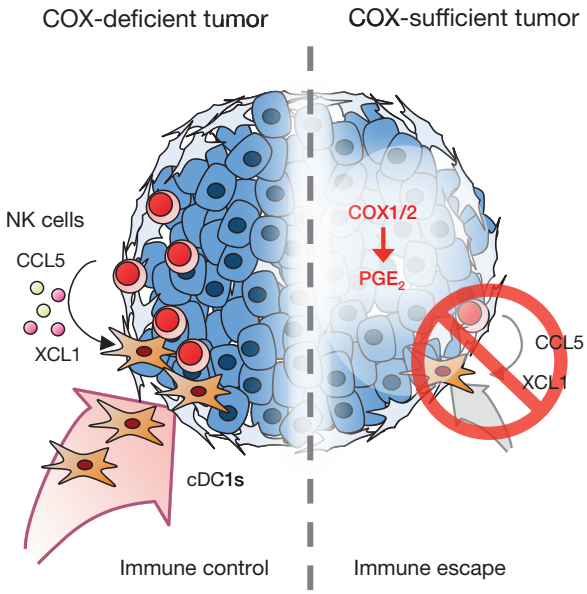
Tracking intratumoral NK cell function by flow cytometry

Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, Rogers NC, Sahai E, Zelenay S, Reis e Sousa C (2018) NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control. *Cell* 172:1022–1037.

Dramatic advances in immuno-oncology (I-O) research have led to the development of cancer immunotherapies that target the host immune system to attack tumor cells. These immunotherapies allow the successful treatment of significant fractions of cancer patients who do not respond to gold-standard "designer" cytotoxic therapies, which are tailored to target cancer based on specific molecular and pathological criteria [1]. I-O therapeutic approaches target cancer-associated immune responses to neutralize tumor-promoting chronic inflammation, block immunosuppressive mediators in tumors, or stimulate the antitumor activity of innate and adaptive immune cells such as antigen-specific cytotoxic T cells (CTLs) [2,3]. While powerful, the effectiveness of these strategies is limited by the nature of cancer cells, which can evade antitumor immunity and become invisible to the immune system through a variety of mechanisms [4].

Effective antitumor CTL responses are a key component of immune system–mediated control of cancer. Like all adaptive immune responses, antitumor CTL responses are dependent on conventional dendritic cells (cDCs), which are specialized in the presentation of tumor antigens and secrete cytokines that regulate CTL survival and effector function within the tumor. Conventional type 1 dendritic cells (cDC1s), in particular, excel at taking up dead tumor cells and transporting tumor antigens to tumor-draining lymph nodes where they cross-prime antitumor CTL responses [5,6]. They also play a role within the tumor itself, where they attract T cells and can restimulate tumor-infiltrating T cells [7,8]. Many tumors build resistance to CTL responses by secreting the prostanoid prostaglandin E2 (PGE_2), which inhibits the recruitment of intratumoral cDC1s and the development of effective antitumor CTL responses, leading to tumor growth. The ablation of the PGE_2-producing cyclo-oxygenase genes *Ptgs1* and *Ptgs2*, in turn, restores cDC1-dependent CTL-mediated tumor control [9].

Böttcher and colleagues recently reported their use of the Invitrogen™ PrimeFlow™ RNA Assay (**thermofisher.com/primeflow**) to identify a key role of intratumoral NK cells in the development of antitumor $CD8^+$ CTL responses by secreting XCL1 and CCL5 and recruiting CTL-priming cDC1s into the tumor microenvironment in mouse tumors [10] (Figure 1). They demonstrate that PGE_2-mediated tumor suppression of anticancer immunity works by diminishing NK cell survival and chemokine production and downregulating cDC1 responsiveness to XCL1 and CCL5 (Figure 1).



Highlights

- NK cells recruit cDC1s into the tumor microenvironment
- cDC1 recruitment depends on NK cell–derived chemokines CCL5 and XCL1
- The NK cell/chemokine/cDC1 axis is associated with cancer patient survival
- Tumor-derived PGE_2 impairs NK cells and cDC1s, resulting in cancer immune evasion

Figure 1. Graphical abstract from "NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control." Reprinted with permission, from Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, Rogers NC, Sahai E, Zelenay S, Reis e Sousa C (2018) *Cell* 172:1022–1037, and under the Creative Commons Attribution 4.0 International License (**creativecommons.org/licenses/by/4.0**).

Characterization of XCL1 mRNA–expressing intratumoral NK cells

In their publication, Böttcher et al. characterize XCL1-producing intratumoral NK cells using PrimeFlow Type 1 (Alexa Fluor™ 647) oligonucleotide probe sets specific for XCL1 mRNA. This analysis of intracellular XCL1 mRNA transcripts was done in combination with extensive protein immunophenotyping by flow cytometry.

They also examined tumor-infiltrating leukocyte (TIL) extracts from PGE_2-sufficient and -deficient (*Ptgs1/Ptgs2*$^{-/-}$) tumors in mice and found that the presence of PGE_2 led to decreased intratumoral NK cell presence and inhibited their ability to produce cDC1 chemoattractants XCL1 and CCL5. This, in turn, reduced CTL numbers in the tumor, resulting in increased tumor mass. These results were confirmed by inmmunofluorescence microscopy staining of tumor tissue sections and also by multiplex ELISA assays.

The authors further explored tumor gene expression data from The Cancer Genome Atlas (TCGA) and found strong evidence that a similar NK cell/chemokine axis exists in multiple types of human cancers and correlates positively with patient survival, suggesting this mechanism bears direct clinical relevance.

Future directions for I-O therapies

The development of increasingly specific and effective I-O therapies requires progressively deeper understanding of the cellular and biochemical mechanisms of immunological control of cancer, and of how cancers manage to evade surveillance by immune cells such as NK cells and cDC1s. Achieving this deeper understanding will entail meticulous mapping of immune cell function within the tumor microenvironment (TME), in addition to tracking how immune cell networks behave and interact with tumor cells during the cycle of tumor progression, remission during treatment, development of treatment resistance, and resurgence. ■

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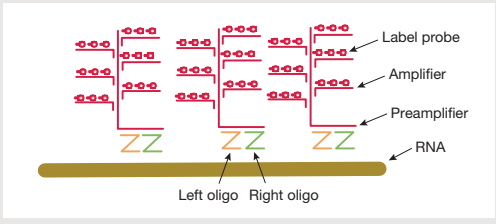
More about the PrimeFlow RNA assay

With the Invitrogen™ PrimeFlow™ RNA Assay Kit, researchers can reveal the dynamics of RNA transcription together with protein expression patterns at the single-cell level by multicolor flow cytometry. The PrimeFlow RNA assay employs fluorescence *in situ* hybridization (FISH) with branched DNA (bDNA) signal amplification for the simultaneous detection of up to 4 RNA targets, and it can be used in combination with immunolabeling of both cell-surface and intracellular proteins using fluorophore-conjugated antibodies.

In the PrimeFlow RNA assay workflow, cells are first labeled with cell-surface antibodies, fixed and permeabilized, and then labeled with intracellular antibodies. Next, the cells are hybridized with oligonucleotide probes specific for the RNA targets. Hybridized targets are detected after bDNA amplification, which is achieved through sequential hybridization steps with preamplifiers, amplifiers, and fluorophore-conjugated label probes. A fully assembled amplification "tree" has 400 label probe–binding sites, and can produce >8,000-fold signal amplification.

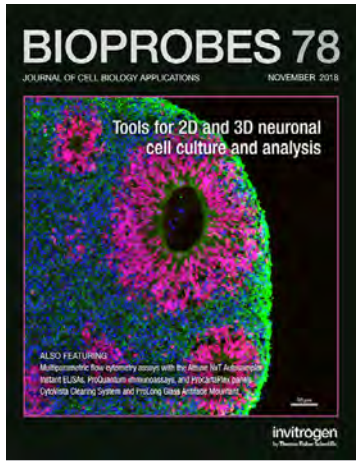
With target-specific probe sets, the PrimeFlow RNA assay can be used to detect miRNA, lncRNA, and mRNA, as well as viral RNA and telomere DNA. The PrimeFlow RNA Assay Kit provides reagents for detecting up to 4 RNA transcripts in mammalian cells optionally labeled with antibodies that recognize cell-surface or intracellular proteins. For more information, including catalog probe sets and ordering guidelines, visit **thermofisher.com/primeflow**.



Branched DNA (bDNA) amplification scheme used in the PrimeFlow RNA assay.

Product	Quantity	Cat. No.
PrimeFlow™ RNA Assay Kit	40 tests	88-18005-204
	100 tests	88-18005-210

Cover image

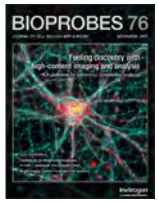


Immunofluorescence analysis of β-3 tubulin (green) and PAX6 (magenta) in human iPSC–derived forebrain organoids. At day 40, the human iPSC–derived organoids were fixed with 4% formaldehyde for 1 hr at room temperature and incubated in a 30% sucrose solution overnight at 4°C. The organoids were then embedded in OCT (optimal cutting temperature compound) and cryosectioned at 5 µm, permeabilized with 0.2% Triton™ X-100 detergent for 20 min, and blocked with 10% donkey serum in PBS for 30 min at room temperature. Organoid slices were incubated with a 1:500 dilution of Invitrogen™ anti–β-3 tubulin mouse monoclonal antibody (clone 2G10, Cat. No. MA1-118) and Invitrogen™ anti-PAX6 rabbit polyclonal antibody (Cat. No. 42-6600) in blocking buffer overnight at 4°C, then stained with a 1:1,000 dilution of Invitrogen™ Alexa Fluor™ 488 donkey anti–mouse IgG ReadyProbes™ secondary antibody (green, Cat. No. R37114) and Invitrogen™ Alexa Fluor™ 568 donkey anti–rabbit IgG secondary antibody (magenta, Cat. No. A10042), as well as DAPI (blue), in blocking solution at room temperature for 1 hr. Images were taken on a Nikon™ Inverted Eclipse™ Ti-E Microscope at 20x magnification. Scale bar: 50 µm. Reprinted with permission from Zhexing Wen, Assistant Professor, Emory University School of Medicine, Atlanta, Georgia, USA.

Previous issues

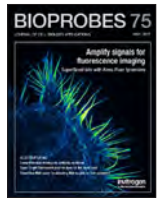
BIOPROBES 77

This issue focuses on advances in immuno-oncology such as immune checkpoint inhibitors and T cell immunotherapy, and the use of stem cell differentiation models to verify antibody specificity. Also described are tools for flow cytometry and high-content imaging and analysis, as well as Invitrogen™ iWestern™ workflow instrumentation and the updated Invitrogen™ Qubit™ 4 Fluorometer and RNA IQ assay.



BIOPROBES 76

This issue highlights recent publications citing the use of Thermo Scientific™ high-content analysis (HCA) platforms. Also discussed are fluorescent probes for autophagy, antibody internalization, and low-density lipoproteins, as well as a 14-color T cell immunophenotyping flow cytometry panel, ProQuantum™ immunoassays, and iBright™ Imaging Systems for western blot analysis.



BIOPROBES 75

In this issue, we describe SuperBoost™ Kits with Alexa Fluor™ tyramides for signal amplification, as well as immune checkpoint antibodies, Super Bright antibody conjugates for the violet laser, and the PrimeFlow™ RNA assay for detecting RNA targets by flow cytometry. A center insert includes a fluorophore and reagent selection guide for flow cytometry.

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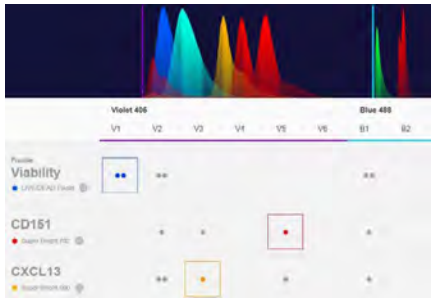

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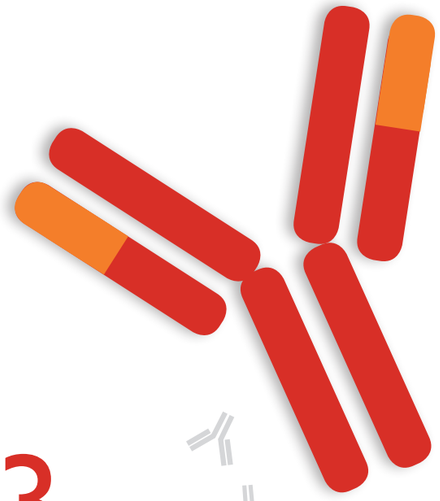
Making the most of your **antibodies**

Antibodies are an essential part of many modern biology labs. Used correctly, they are a powerful tool ... but they need to be treated with care and respect.

Here are some tips for getting the most out of your precious antibody reagents.



1

SETUP

- confirm that your antibody has been **validated** for the intended application
- **titrate** your antibody to determine the optimal working conditions and concentrations
- **carefully store** your reagents according to manufacturers' recommendations
- limit **excessive handling** by aliquoting antibody reagents.

2

DESIGNING THE EXPERIMENT

- do all **dilution calculations** in advance (and double-check them)
- carefully select **controls** (e.g., positive, negative, and nonspecific binding)
- prepare and review your **protocol** sheet
- **set up and label** all tubes and plates in advance.

3

DOING THE EXPERIMENT

- **follow your protocol** carefully, checking off each step as it's done
- **handle antibody reagents with care**—don't overmix or leave at room temperature
- **pipet reagents** carefully and accurately.

4

TROUBLESHOOTING

If things didn't turn out as expected:

- carefully **check** all calculations and dilutions
- **check your protocol** against manufacturers' recommendations
- do your **controls** help you identify the source of the problem?
- confirm **compatibility** of your primary antibody with secondary antibody and other reagents
- are all of your reagents **fresh** (especially blocking agents)?
- were the proper **incubation times** used?

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Verification of **antibody performance**

Researchers need antibodies that bind to the right target and work in their applications every time. To help ensure superior antibody results, Thermo Fisher Scientific has expanded their specificity and validation* testing methodologies using a 2-part approach for advanced verification.

The challenge

Antibodies are some of the most critical research reagents used in the lab. Poor specificity or application performance can lead to inconsistent results, a lack of reproducibility, and a waste of time and money.

Invitrogen™ antibodies are currently undergoing a rigorous 2-part testing approach

TARGET SPECIFICITY VERIFICATION

Helps ensure the antibody will bind to the correct target. Invitrogen antibodies are being tested using at least one of the following methods:

- Immunoprecipitation/mass spectrometry
- Knockout
- Knockdown
- Independent antibody verification
- Cell treatment
- Relative expression
- Neutralization
- Peptide array
- Orthogonal

2

FUNCTIONAL APPLICATION VALIDATION

These tests help ensure the antibody works in particular applications of interest, which may include (but are not limited to):

- Western blotting
- Immunofluorescence imaging
- Flow cytometry
- Chromatin immunoprecipitation
- Immunohistochemistry

The solution

Thermo Fisher Scientific is working to redefine antibody performance with a comprehensive approach to how antibodies are evaluated and validated. By combining specificity testing with extensive application validation data, Thermo Fisher helps ensure that Invitrogen antibodies will help enable superior performance for researchers.

*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.

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