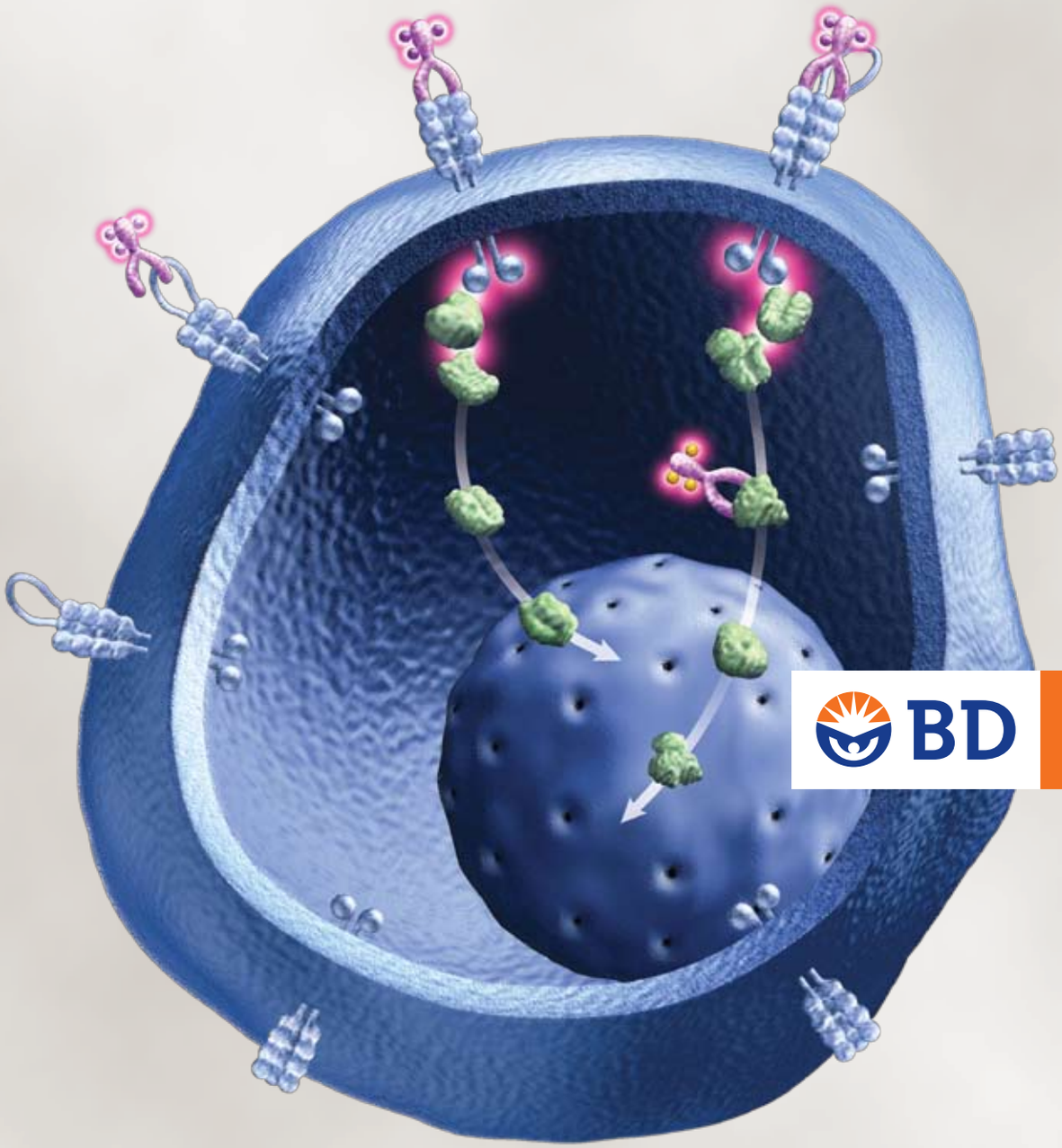
BD Phosflow

Tools for the study of phosphoprotein signaling



BD

Helping all people live healthy lives

BD Phosflow

An intracellular snapshot of protein phosphorylation

BD™ Phosflow technology is the first commercially available flow cytometry solution to reveal intracellular data on protein phosphorylation events. Using BD Phosflow, researchers can simultaneously analyze multiple intracellular phosphoprotein and cell surface markers for an information-rich view of cell signaling in discrete subpopulations of cells.

Combining isolation and analysis into a single step, BD Phosflow enables research involving complex cell mixtures. Scientists can monitor the effect of cell stimuli in their near-native conditions, reducing the time spent on dead ends and discrepancies that are common artifacts of in vitro analysis. Even rare cell subtypes, which may reveal an alternative signaling mechanism or off-target drug effect, can be identified and analyzed with a single streamlined experiment.

Using BD Phosflow, complex signal pathway analysis can be completed in less than a day, providing a richer data set in a shorter period of time than with traditional methods such as Western blotting and immunoprecipitation. The BD Phosflow portfolio also offers a 96-well protocol for the additional speed required for high throughput cell-based screening.

Backed by a world-class service and support organization with unmatched flow cytometry experience, BD Phosflow solutions provide an integrated approach with robust protocols for different cell types, as well as deep libraries of high-quality phosphoprotein and empirically tested cell-surface antibody markers.

From clinical research, to drug efficacy screening, to leading-edge research on cell signaling networks, BD Phosflow analysis accelerates breakthroughs that depend on complex systems analysis.

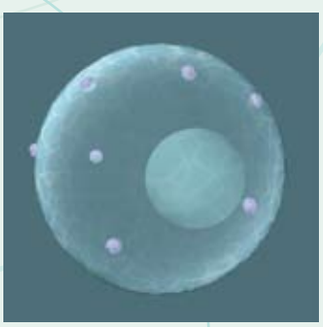
A revolutionary advance in techniques for cell-signaling research

BD Phosflow allows scientists to narrow down analysis to small cellular subsets in complex samples, such as whole blood or peripheral blood mononuclear cells (PBMCs). Unlike traditional methods such as Western blotting, researchers can use BD Phosflow to distinguish and analyze phosphoprotein signaling in single cells through the use of multiple cell surface markers. Even rare cell subtypes can be uncovered and isolated without additional upfront methods.

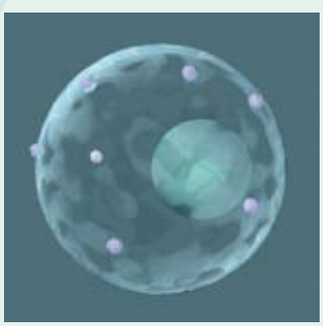
Only BD Phosflow provides phospho-signaling data on cell subpopulations in clinical research samples or primary cell types. The ability to study complex events under near-native conditions provides greater accuracy for understanding the effect of a disease or stimulus. For drug discovery, BD Phosflow is also a unique secondary cell-based screening tool for cell types such as mouse splenic cells.

By enabling investigators to analyze phosphorylation states in whole blood or other complex cell mixtures, BD Phosflow helps streamline discovery. It eliminates the need for lengthy and costly experiments designed to reconcile unexpected effects stemming from rare cell populations or discrepancies between in vivo and in vitro results.

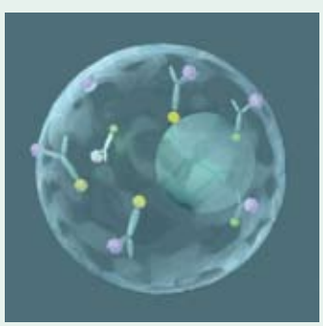
Rapid, high complexity analysis
Providing static or kinetic state information for multiple intracellular phosphoproteins at one time enables BD Phosflow to yield an intracellular snapshot of high complexity systems much faster than Western blotting, immunoprecipitation, or immunofluorescence microscopy. Additionally, intracellular phosphorylation state analysis can be performed sequentially using 96-well plates to rapidly screen multiple phosphoproteins.



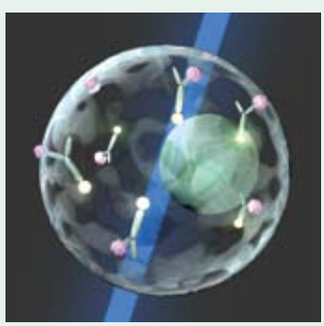
Step 1: Fix cells with one of the BD Phosflow Fixation Buffers for 10 minutes.



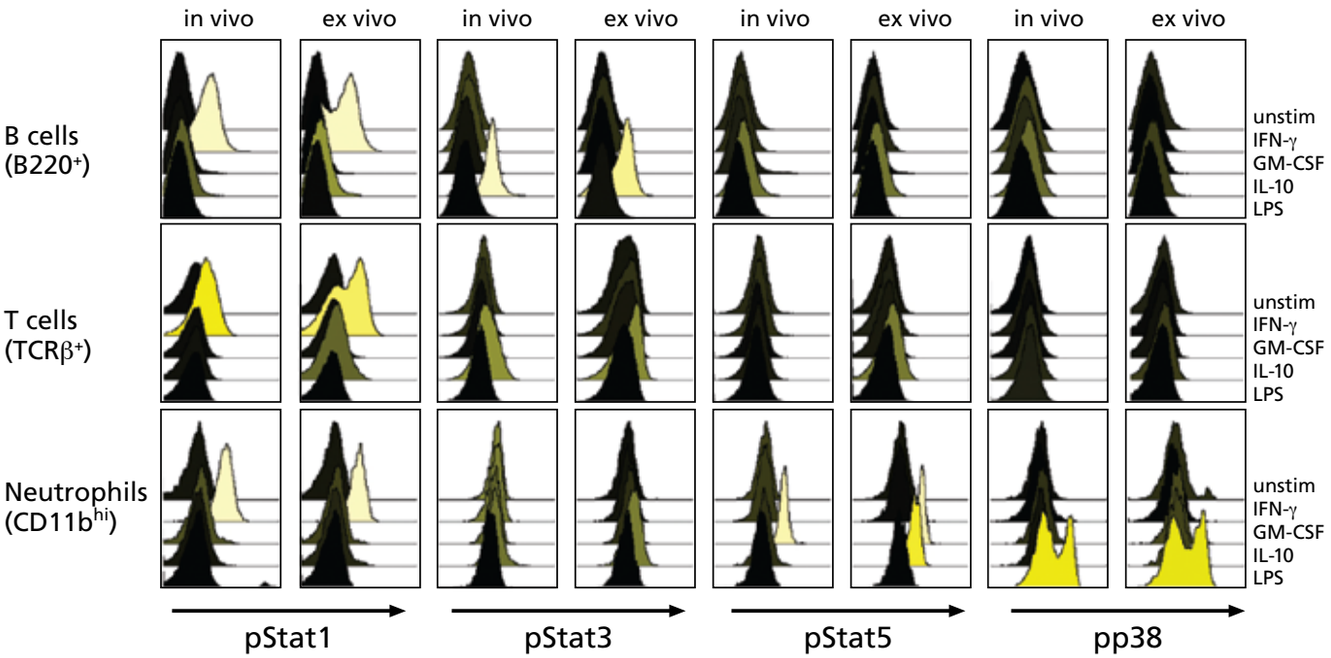
Step 2: Permeabilize cells with one of the BD Phosflow Permeabilization Buffers for 30 minutes.



Step 3: Stain cells with directly conjugated BD Phosflow antibodies in BD Pharmingen Stain Buffer.



Step 4: Analyze cells on a BD FACS™ Instrument.



Multiple phosphoproteins in different cell types studied by BD Phosflow

This figure illustrates how BD Phosflow makes it possible to conduct the simultaneous analysis of multiple phosphoproteins in complex cell mixtures. In this experiment, BD Phosflow was used to analyze the effect of four different stimuli on cell signaling pathways in mouse splenic cell subsets (B cells, T cells, and CD11b^{high} cells). Differences in signaling pathway responses were uncovered between mouse splenocyte cultures stimulated in vitro and splenic cells stimulated in vivo, underscoring the importance of conducting studies in close to native conditions. Data were analyzed in Cytobank software (www.cytobank.org). Histograms are colored according to fold change in phosphorylated protein relative to unstimulated samples.

Data courtesy of Dr. Peter Krutzik and Dr. Matt Hale, Stanford University.

Four Steps of BD Phosflow

BD Phosflow technology relies on four steps to investigate phospho-signaling in cellular subsets. First, cell suspensions or adherent cells are fixed with one of the BD Phosflow fixation buffers. Second, cells are permeabilized. In step three, cells are stained with fluorescently conjugated phospho-specific and extracellular cell-type-specific antibodies. Finally, the sample in solution is introduced into a flow cytometer where the stream of single cells is interrogated.

The flow cytometer analysis software counts the cells in the sample, estimates a size distribution, and quantitates the fluorescence signal emitted by the fluorescently conjugated antibodies. Specific subsets of cells can be analyzed for the presence or absence of multiple cell-surface markers or intracellular phosphoprotein signals, enabling many different potential combinations. Typically, BD Phosflow procedures and analysis take less than a day.

Advantages of BD Phosflow

Western Blot	Flow Cytometry	Flow Cytometry Advantages
Population analysis Obtain average value of multiple cells	**Single cell analysis** Collect data for each individual cell	• Enables direct analysis of cell subsets in whole-blood, PBMCs • Enables direct analysis of other complex primary samples • Enables direct analysis of rare cell subtypes
One parameter Obtain data sets individually	**Multiparameter** Correlate multiple intracellular phosphoprotein markers or cell surface markers simultaneously	• Speeds analysis • Provides correlations between markers
Low throughput 8 to 12 samples processed/analyzed in a day	**96-well plate capable** Hundreds of samples processed/analyzed in a day	• Amenable to screening

Accelerate analysis with optimized reagents and protocols

BD Biosciences provides a comprehensive portfolio of tested antibody reagents, optimized buffers, and robust protocols for conducting phosphoprotein signaling analysis in different sample types.

A wide choice of high quality antibodies to phosphoprotein and cell-surface markers empowers researchers to explore a diverse range of signaling pathways and cell types, with consistent results. Robust protocols and study guidelines help reduce the guesswork of experimental setup for researchers.

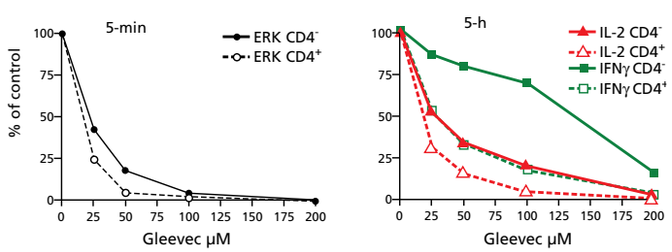
Flexible analysis with a library of tested antibodies to phosphoprotein markers
High quality monoclonal anti-phosphoprotein antibodies from more than a dozen signaling pathways provide flexibility for studying complex signaling networks. Additionally, antibodies that recognize either phosphorylated or non-phosphorylated protein states offer multiple analysis permutations.

Strict validation criteria for monoclonal antibodies, including verification by Western blot, optimal signal to noise ratios, and signal blocking by specific phosphopeptides, ensure dependable results and consistency with legacy Western blotting data.

Tested antibodies to surface markers reduce guesswork
BD Biosciences has tested antibodies against cell-surface antigens for resistance to fixation and permeabilization procedures of BD Phosflow. This continuously expanding library of monoclonal antibodies includes markers for human and mouse leucocyte differentiation and offers access to a wide range of cell types.

Human whole blood analysis with BD Phosflow

In this experiment, human whole blood was activated using anti-CD3, anti-CD28, and anti-CD49d in the presence of increasing concentrations of the tyrosine kinase inhibitor imatinib mesylate (Gleevec®). Phospho-ERK expression was measured in $CD4^+$, and $CD4^-$ T cells after 5 minutes of stimulation and compared with IL-2 and IFN-γ intracellular cytokine expression after a 5-hour incubation with the activating antibody cocktail. The percentage of cells that expressed phospho-ERK or IL-2 was decreased in both $CD4^+$ and $CD4^-$ T cell populations in the presence of increasing concentrations of Gleevec. However, the number of IFN-γ expressing cells was less affected by Gleevec in $CD4^-$ T cells.

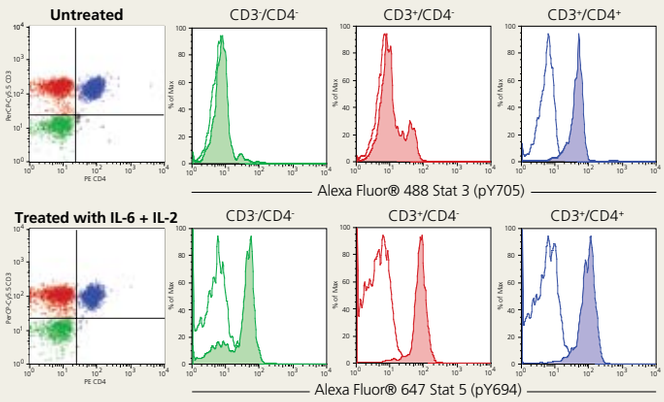


Dependable analysis for different sample types
Optimized buffers and protocols for a variety of sample types, including human whole blood, human PBMCs, mouse thymocytes, and mouse splenocytes reduce the need for time consuming optimization. Available data on different stimulations tested for whole blood and PBMCs, as well as 4-color combinations for multiplexed analysis of T cells and B cells in whole blood and PBMCs, reduces upfront troubleshooting.

Tested protocols for high throughput screening
BD Phosflow technology is readily scalable to 96-well plate processing and analysis. With the protocols provided, hundreds of samples can be analyzed per day, providing the most information rich cell based phosphoprotein screen available for drug discovery and development.

Discrete cell types in human PBMCs are distinguished and analyzed using tested protocols, buffers, and reagents from BD Biosciences.

In this experiment, human PBMCs were either untreated (open histogram) or treated with IL-6 + IL-2 (shaded histogram), 100 ng/mL each for 15 min at 37°C. The cells were then fixed using BD Cytofix™ buffer for 10 min at 37°C, and followed by BD Phosflow Perm Buffer III for 30 min on ice. PBMCs were stained with PerCP-Cy™5.5 anti-human CD3, PE anti-human CD4, Alexa Fluor® 488 anti-Stat 3 (pY705), and Alexa Fluor® 647 anti-Stat 5 (pY694).



Examples of tested protocols for two cell types

It is critical to select the optimal BD Phosflow protocol for the cell type studied in a particular experiment. Please refer to **bdbiosciences.com/phosflow** for more information on choosing the right products for your experiment.

Whole blood	Stimulate cells	Fix cells with BD Phosflow Lyse/Fix Buffer	Incubate cells with appropriate BD Phosflow Perm Buffer	Stain cells with phosphoprotein and surface antibody	Wash and resuspend cells in BD Pharmingen™ stain buffer	Flow cytometry analysis	
Adherent cell line	Stimulate cells	Detach cells	Fix cells with BD Phosflow Fix Buffer	Incubate cells with appropriate BD Phosflow Perm Buffer	Stain cells with phosphoprotein and surface antibodies	Wash and resuspend cells in BD Pharmingen stain buffer	Flow cytometry analysis

Opening new avenues of exploration

BD Phosflow excels in a wide variety of applications including clinical research, drug discovery, and basic cell signaling research. In clinical research, scientists are focusing on identifying blood based biomarkers for disease. In drug discovery, BD Phosflow is being used as a secondary screening assay as well as for kinase target discovery. In basic signaling applications, researchers have discovered new mechanisms for disease and uncovered discrepancies between in vitro and in vivo systems.

Novel biomarker discovery with BD Phosflow
Using BD Phosflow, University of Antwerp scientists are researching novel biomarkers for allergic disease that might be more reliable than the current diagnostic methods of history taking, skin testing, and specific IgE quantitation in certain types of allergies.

In a proof of concept study, the researchers examined markers for basophil activation—CD63 and phospho-p38 MAPK—in whole blood samples induced with recombinant Bet v1 (*Betula verrucosa*) in a group of birch pollen (BP) allergic, healthy controls, and grass pollen (GP) allergic individuals. The team found that these markers showed excellent sensitivity and specificity in discriminating BP allergic individuals from GP allergic individuals and healthy controls. The researchers are currently investigating the use of this biomarker for assessing drug allergies and for follow-up of venom immunotherapy (right).

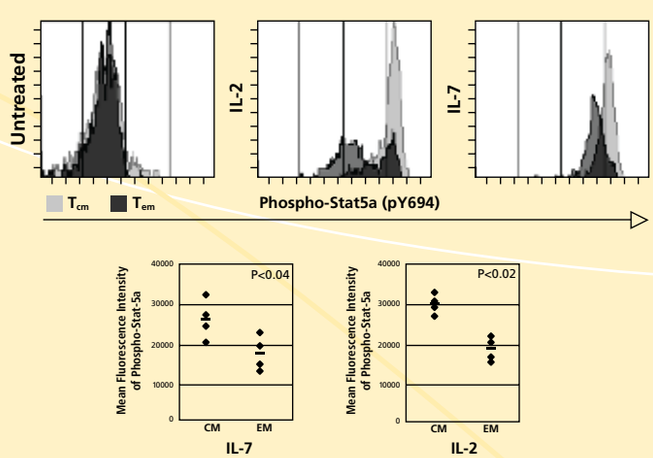
Identifying new signaling mechanisms with BD Phosflow
University of Montreal researchers have used BD Phosflow to uncover intracellular signaling mechanisms that enable certain memory T cells to resist apoptosis and persist long term to provide protective immunity against reinfection. Their results might help researchers develop more effective treatments against viral infection, in which memory T cells have ceased to function correctly.

The researchers profiled the phosphorylation events in highly purified CD4 central memory cells (T_{CM}) from human PBMCs. They found that T_{CM} cells depend on the activation and phosphorylation of STAT5a and FOXO3a signaling pathways for long term survival.

Phosphoprotein signaling studied with BD Phosflow

Using BD Phosflow, researchers found that levels of phosphorylated STAT5a (pY694) (Cat. No. 612599) in human PBMCs were significantly higher in central memory (T_{CM}) cells than effector memory (T_{EM}) cells, revealing that the STAT signaling pathway is critical for long term protective immunity in humans.

Data courtesy Dr. Rafick-Pierre Sekaly, University of Montreal.

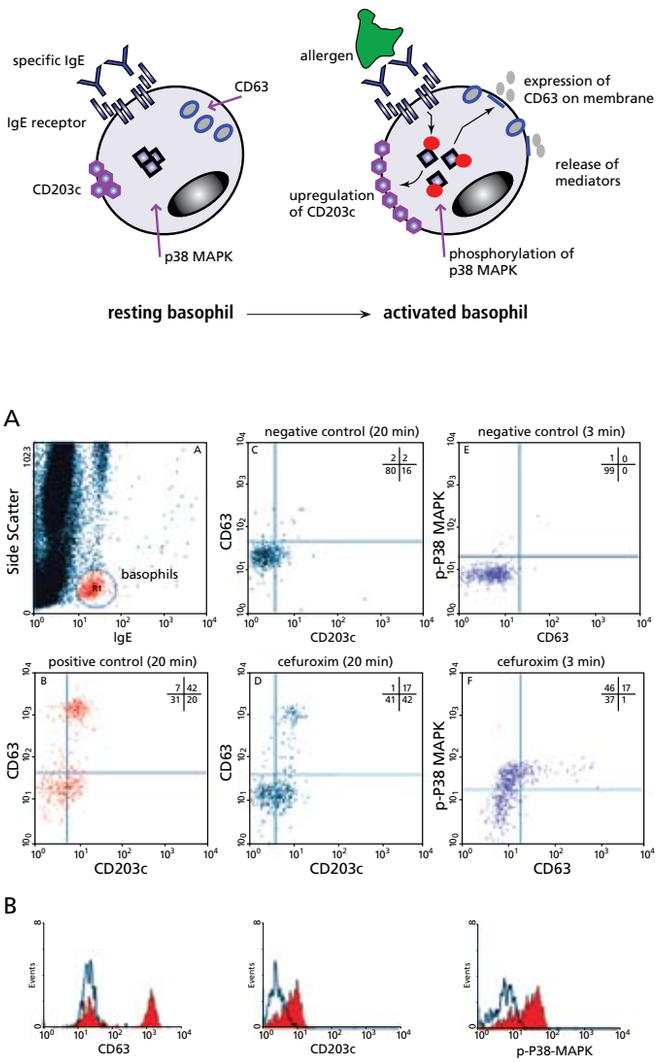


Intracellular biomarkers for allergic disease

The top right illustration shows how basophils not only secrete particular mediators, but will also upregulate specific activation markers such as CD63 and CD203c upon encountering specific allergens that cross-link membrane bound IgE. The exact mechanism(s) that govern basophil degranulation remain elusive, but it has been demonstrated that phosphorylation of p38 MAPK exerts a pivotal role in cell activation.

The bottom figure illustrates the simultaneous analysis of the surface markers CD63 and CD203c and the novel biomarker, phosphorylated p38 MAPK, in an individual with Cefurim® drug allergy using BD Phosflow. Alexa Fluor® 488-conjugated anti-IgE positive basophils were gated out in a circular region (panel A, upper left). Prewarmed basophils were stimulated for 3 or 20 minutes at 37°C with anti-IgE as a positive control (panel A, lower left), washing solution to measure spontaneous CD63, CD203c, and phosphorylated p38 MAPK expression (negative control, panel A, upper center and upper right), and cefuroxim (Cefurim®, TEVA Pharma, Wilrijk, Belgium) (panel A, lower center and lower right). Note the clear bimodal upregulation of CD63 and the more homogenous upregulation of CD203c and phosphorylation of p38 MAPK in a larger proportion of the cells. This is also clear from the histograms in panel B (open histogram spontaneous expression by resting cells, closed histogram expression after stimulation of cells with cefuroxim).

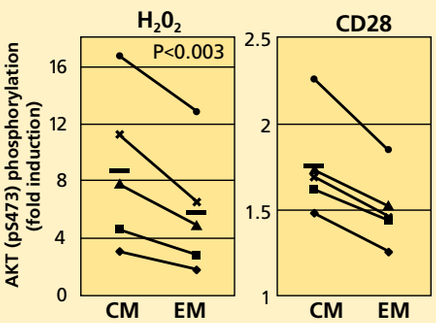
Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ.
The basophil activation test in immediate drug allergy. *Acta Clinica Belgica.* In press.



BD Phosflow for complex cell-signaling analysis

BD Phosflow analysis reveals that phosphorylation levels of AKT (pS473), a protein known to phosphorylate FOXO3a, are higher in human T_{CM} cells than T_{EM} cells in response to H_2O_2 and anti-CD28 treatment. CD28 triggered an additional increase in AKT phosphorylation in T_{CM} compared to T_{EM}. Intracellular staining was performed using CD4 (Cat. No. 557871), CD27 (Cat. No. 555441), CD45RA, and AKT (pS473) (Cat. No. 560404) -specific antibodies. The results are represented as the mean fold increase ± SD of five independent experiments, calculated as follows: (MFI of stimulated cells/MFI of unstimulated cells). P values (determined by the two-tailed t test) are shown.

Data courtesy Dr. Rafick-Pierre Sekaly, University of Montreal.



Committed to customer success

BD Biosciences is fully committed to the success and satisfaction of its customers. To help scientists take full advantage of our offerings, BD Biosciences products are backed by a world-class service and support organization with unmatched experience in flow cytometry, cell biology, and antibody reagent development.

Technical application support
BD Biosciences technical application support specialists are available to provide field- or phone-based assistance and advice. Expert in a diverse array of topics, BD technical application support specialists are well equipped to address customers' needs in both instrument and applications support.

Custom services
Mobilizing technology for research applications requires close collaboration. The Custom Technology Team (CTT) at BD Biosciences works with customers to provide solutions through custom reagents, panels, or assay protocols.

Staffed by leading scientists with both breadth and depth of scientific and technical expertise, the CTT team will coordinate with researchers to study the problem at hand, make recommendations, and help implement the solutions. In this way, BD Biosciences technical know-how is translated into practical solutions that allow customers to focus on research.

A Selection of BD Phosflow Cited Publications

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3. Krutzik PO, Nolan GP. Fluorescent cell bar coding in flow cytometry allows high throughput drug screening and signaling profiling. *Nature Methods.* 2006;3:361-368.
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14. Krutzik PO, Clutter MR, Nolan GP. Coordinate Analysis of Murine Immune Cell Surface Markers and Intracellular Phosphoproteins by Flow Cytometry. *J Immunol.* 2005;175:2357-2365.

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