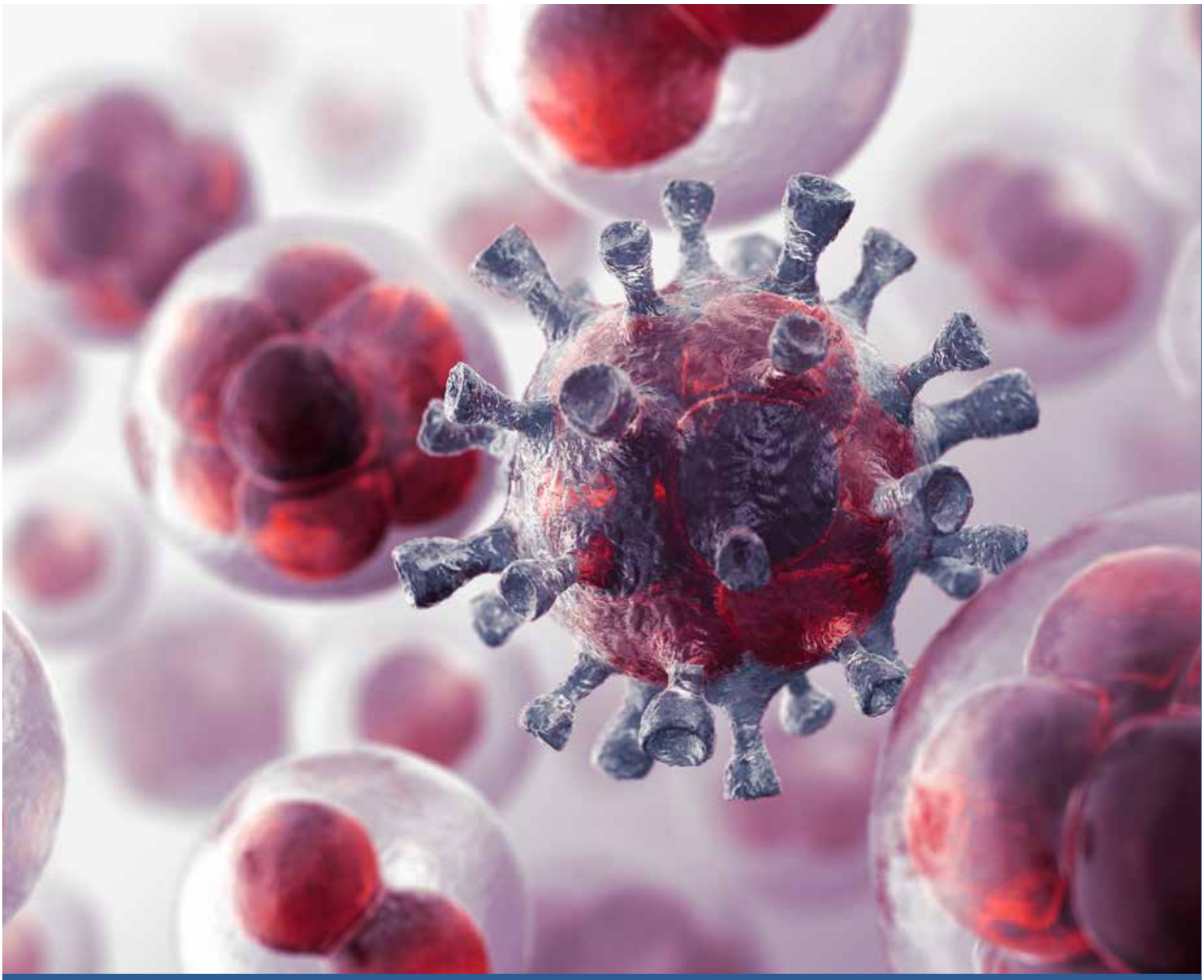


FUNCTIONAL ASSAYS BY FLOW CYTOMETRY

Brought to you by: FlowMetric™

Cells execute functions to divide, differentiate and communicate with the extracellular environment every moment of their existence. Immune system cells have evolved to execute numerous functions that defend the host against pathogens and other threats like tumors. Scientists have worked for decades to identify and measure cellular function and numerous experimental protocols have been developed to this end. In today's laboratory, flow cytometry has emerged as a precise, rapid, and customizable platform for many state-of-the-art functional assays.

The burgeoning area of immune-based therapies has brought the importance of functional assays to the forefront of preclinical and clinical research. Candidate molecular and cell-based therapies must be fully characterized, including determining the specificity of the immune response being targeted as well as the scope of nonspecific or off-target responses that may cause devastating side effects. Functional assays are becoming an indispensable complement to other assays that profile the immune system.

This white paper provides a summary of flow cytometry functional assays currently being used in basic and clinical research settings. Many of these assays can be adapted for use in preclinical development or clinical trials so that they meet the appropriate regulatory criteria. Consider incorporating a flow cytometry functional assay into your current research project.

Proliferation

Cell proliferation is a fundamental process directed by the cell cycle and is required for the survival of all multicellular organisms[1]. Mutations that arise in cells can dysregulate the cell cycle, and if left unchecked, tumors can form and proliferate uncontrollably. In fact, cell cycle inhibitors, particularly those that target cyclin-dependent kinase (CDK), are being tested in clinical trials as novel cancer therapies. The cell cycle is a tightly regulated process in which a cell duplicates its genome and synthesizes a variety of proteins needed for cell division, and then the cell divides into two cells in a process called mitosis. This process assures that cells divide equally and each cell contains the genetic material and essential proteins to perform all necessary cellular functions, after which cells may undergo additional steps of differentiation.

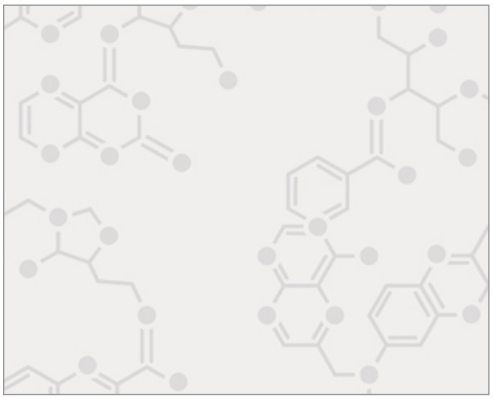
Normal cell proliferation is essential to the success of cell-based immunotherapies in which specific immune cells are isolated from a patient, induced to proliferate and differentiate under very precise and controlled conditions in the lab and then are reinfused into the patient.

Cell proliferation must be monitored when these cells are being handled in the lab and after they are reinfused into patients in order to determine if cells are dividing normally and reaching a level that is associated with a therapeutic effect.

[1] *Matson JP and Cook JG. Cell cycle proliferation decisions: the impact of single cell analyses. FEBS J. 2016. doi:10.1111/febs.13898*

Flow cytometry has been used to measure cell proliferation for over two decades[2,3]. 5-bromo-2'-deoxyuridine (BrdU) is a synthetic thymidine analog that can be added to cells in vitro and incorporated into genomic DNA during the S-phase of the cell cycle. Fluorescently labeled anti-BrdU antibodies are used to stain BrdU-treated cells and can be combined with other fluorescently labeled antibodies in a flow cytometry staining panel. Flow cytometric analysis shows discrete populations of cells with decreasing levels of BrdU staining that correlate with successive rounds of cell division.

CFSE (5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester) is a non-fluorescent molecule that is converted into a fluorescent form inside a cell. CFSE can be taken up by live cells and is activated into a fluorescent molecule in the intracellular space upon cleavage by intracellular esterases. This reaction forms dye-protein conjugates that cannot exit the cell and can be diluted upon subsequent rounds of cell division. Similar to BrdU, CFSE staining can be used alongside other fluorescent antibodies in a flow cytometry staining panel. CFSE's fluorescence is generated by carboxyfluorescein, which can be easily detected and measured on all standard flow cytometers.



Numerous proteins are critical to cell cycle regulation and function as checkpoints for detecting DNA damage, such as p53, or have enzymatic functions, like cyclin dependent kinases (CDKs) which phosphorylate cell

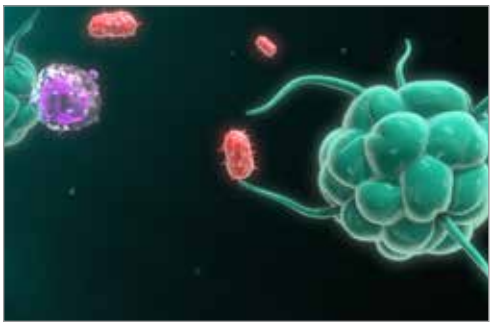
[2] Rabinovitch PS, Kubbies M, Chen YC, Schindler D, Hoehn H. BrdU-Hoechst flow cytometry: a unique tool for quantitative cell cycle analysis. Exp Cell Res. 1988. Feb;174(2):309-18.

[3] Lyons AB and Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods. 1994. May 2;171(1):131-7.
those from a mouse. Nature. 1986. 321: 522-525.

cycle-stage specific cyclins and allow for cell cycle progression. These different cell cycle proteins can be detected by flow cytometry and also provide insight into the cell cycle for a particular cell type.

Endocytosis and Exocytosis

Large macromolecules cannot readily cross the plasma membrane, so cells use vesicle-mediated active transport to move such particles across the plasma membrane and within the intracellular space.[4] Cell biologists and biochemists first characterized these membrane-bound vesicle structures and intracellular trafficking events using microscopy and Western blot analysis, but now flow cytometry has emerged as a tool for analyzing and quantifying these events. Two primary forms of cellular trafficking can be measured by flow cytometry: endocytosis and exocytosis.

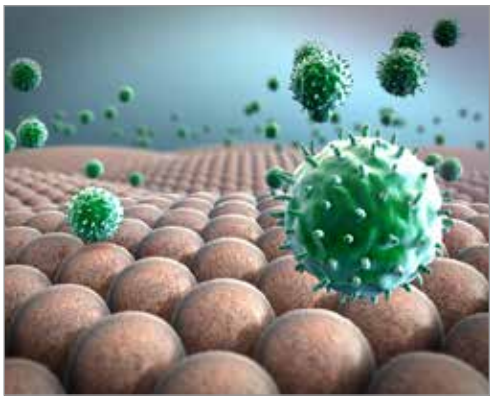


Endocytosis is the overall process by which cells take up macromolecules at the cell surface into vesicles that are trafficked into the cytoplasm. Pinocytosis or "cell drinking" (a form of endocytosis) is a process by which dissolved molecules are taken up into channels on the surface of the plasma membrane, which then form vesicles that are taken into cell. Fluorescently labeled molecules like FITC-dextran can be used to measure pinocytosis by flow cytometry because only dextran internalized into vesicles will be detected. Phagocytosis or "cell eating"

[4] *http://www.ck12.org/biology/Exocytosis-and-Endocytosis/lesson/Exocytosis-and-Endocytosis-BIO/*

is a form of endocytosis by which the plasma membrane engulfs larger structures, such as extracellular pathogens, and trafficks them into cells for targeted destruction. Similar to pinocytosis detection, fluorescently labeled bacteria or macromolecules can be used to measure phagocytosis by flow cytometry. Some reagents for measuring vesicle trafficking are commercially available, although unique reagents can be labeled with fluorescent molecules in order to measure trafficking of a specific particle or pathogen.

Exocytosis is the trafficking of vesicles from inside the cell to the plasma membrane, resulting in the fusion of membranes and the release of the vesicle contents to the extracellular domain. Degranulation is a specialized form of exocytosis by which immune cells like T cells or mast cells release vesicular contents extracellularly as a form of immune defense. Degranulation results in specific markers that are present within the granule to be left on the cell surface, where they can be detected with specific antibodies and measured by flow cytometry[5].



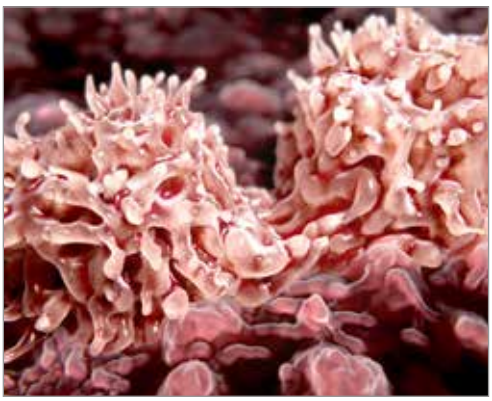
Calcium Signaling

Calcium ion concentrations are tightly controlled within the cell cytoplasm by calcium ion pumps in the plasma membrane or endoplasmic reticulum to maintain the baseline calcium concentrations, but calcium can be rapidly released from its intracellular stores in the endoplasmic reticulum[6]. Numerous signaling events can trigger an

[5] *Betts MR1, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003 Oct 1;281(1-2):65-78.*

[6] *Shin DM, Son A, Park S, Kim MS, Ahuja M, Muallem S.The TRPCs, Orais and STIMs in ER/PM Junctions. Adv Exp Med Biol. 2016;898:47-66.*

increase in cytoplasmic calcium levels, and this calcium flux induces diverse cellular functions including cellular differentiation, gene transcription and activation. Immune cells, particularly lymphocytes, are activated by changes in calcium ion concentrations, which is a critical step for triggering various effector functions[7]. An array of dyes can detect changes in intracellular calcium, and these calcium sensor dyes can be used to measure calcium flux using flow cytometry[8]. These dyes, which include Indo-1 AM, Fluo-3, and Fura Red AM, can bind to calcium ions and their excitation and emission spectra may differ depending on binding of free or bound calcium ions. Selection of an appropriate calcium sensor dye for flow cytometry is dependent on the type of flow cytometer in use, and the concentration of free calcium ions trying to be detected in cells.



Oxidative Metabolism

Oxidative metabolism occurs during normal physiological processes and can produce reactive oxygen species such as peroxides, superoxide, and hydroxyl radical[9]. Inflammatory processes such as those associated with cancer and autoimmune disorders are associated with a dramatic increase in oxidative metabolism, and high levels of these oxidative byproducts can cause oxidative stress and cause damage to cells.

Measuring oxidative byproducts is critical to understanding inflammation and is also critical to determining how experimental treatments may cause undesirable or toxic side effects.

[7] *Feske S. Calcium signalling in lymphocyte activation and disease. Nat Rev Immunol. 2007. Sep;7(9):690-702.*

[8] *Posey AD Jr, Kawalekar OU, June CH. Measurement of intracellular ions by flow cytometry. Curr Protoc Cytom. 2015. Apr 1;72:9.8.1-21.*

[9] *FHayyan M, Hashim MA, AlNashef IM. Superoxide Ion: Generation and Chemical Implications, Chem. Rev., 2016. 116 (5), pp 3029–3085*

Flow cytometry can be used to measure many different molecules and cellular components associated with oxidative stress. These oxidative stress detection techniques can be included with most flow cytometry staining panels depending on the flow cytometer being used, and include reagents that measure free radicals, glutathione, and lipid peroxidation. Intracellular free radicals can be measured with specific reagents that detect total reactive oxygen species or specific molecules like superoxide, hydrogen peroxide or peroxynitrate. Glutathione is an intracellular tripeptide (glutamyl-cysteinyl-glycine) with a free sulphydryl group that is an important target of attack for free radicals. Flow cytometry can be used to measure the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and serves as an indicator of oxidative stress. Lipid peroxidation is the process by which lipids are degraded and can be initiated by reactive oxygen species. The byproducts of lipid peroxidation can damage cell membranes and shifts in peroxidation status can be measured by flow cytometry.

Conclusion

Flow cytometry allows for unrivaled analysis of a cell's phenotype and functions, and many of these measurements can be made during a single experiment. The functional assays described here have be developed years ago and have become widely used by the research community. Many other novel functional assays are being developed and published each year, which highlight the flexibility of flow cytometry as an analysis tool. Functional assays can be and have been validated for use in preclinical and clinical trials and provide critical information needed to determine the success of an experimental drug or biologic-based therapy. Flow cytometry-based functional assays are certain to remain critical to biomedical research for years to come.

Quantifying Biological Response
Through Cytometry