# The Many Roads to Cell Death
Gaining a Practical Understanding of Apoptosis, Necrosis, and Autophagy

## Instructions for Viewers
- Change the size of any window by dragging the lower right corner. Use controls in top right corner to close or maximize each window.
- What each widget does:

<table>
<thead>
<tr>
<th>Icon</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>🔊</td>
<td>shows the audio media player</td>
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<td>📤</td>
<td>opens the Ask a Question box</td>
</tr>
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<td>download slides and more info</td>
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<td>🌐</td>
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<td>tweeting bird</td>
<td>Twitter login (#ScienceWebinar)</td>
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<td>LinkedIn login</td>
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<tr>
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<td>if you need help</td>
</tr>
</tbody>
</table>
The Many Roads to Cell Death
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June 4, 2014

Participating Experts

John Abrams, Ph.D.
University of Texas
Southwestern Medical Center
Dallas, Texas

William Telford, Ph.D.
National Institutes of Health
Bethesda, MD
Cell Death - a holistic view

John Abrams, Ph.D.
Programmed Forms of Cell Death are Ubiquitous in Biological Systems

- Development: *sculpting produces morphologic patterns*
- Immunity: *negative selection removes auto reactive lymphocytes*
- Viral Infection: *molecular ‘arms race' between viral and host genes*
- Tissue Damage: *caused by environmental stressors, e.g. genotoxins*
Programmed Forms of Cell Death are Ubiquitous in Biological Systems

- Development  *sculpting produces morphologic patterns*
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Diseases can result when underlying pathways are deranged

- Cancer  *Failure in normal cell death programs*
- Degenerative Disorders  *Excessive cell death*
- AIDS  *Excessive cell death*
Classification Systems

Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012 (Cell Death and Differentiation (2012) 19, 107–120)

Appearance
Ultrastructural changes within the dying cell and nearby cells
Type I associated with heterophagy
Type II associated with autophagy
Type III no associated digestion

Programmed vs. incidental

*programmed* = “Naturally occurring cell death”
= predictable in development
= dedicated gene-directed pathways
= active, cellular process
= adaptive, not passive cytotoxicity
= not caused by damage, injury or insult
Active Forms of Cell Death

apoptosis, programmed forms of necrosis

specified by natural or non-natural inducers

suicide vs. murder

underlying genetics and biochemistry

dedicated death pathways vs. sabotage or signaling run ‘amok’
the ultimate irreversible reaction

Convicted

Condemned

Execution

Disposal

pardon

stay

reversible

no return

no return
Apoptosis- Greek for “falling off”. Historically referred to as pycnosis for shrunken, densely stained cells. Although originally derived as an ultrastructural term, its current meaning also implies a gene-directed cell suicide program.
Real Time Imaging in Culture
Live imaging with Stains, Vital Dyes
Acridine Orange
Live imaging with Stains, Vital Dyes

Acridine Orange
Fixed Tissue
Fixed Tissue
Apoptotic Cells are rapidly engulfed by phagocytes
Caspases are activated and functional during apoptosis

**Dormant Proenzyme**

- Active site cys, cleave asp
- Initiating 'platforms' launch amplifying cascade
- Substrates can be activated or activated
- Function in PCD, immunity
- Viral genomes encode inhibitors

**Processing and Maturation**

**Active Tetramer**
Peptide Substrates and Antibodies Detect Caspase Activity

In culture, bulk assays

![Untreated vs Smac-mimetic cells](image_url)
Peptide Substrates and Antibodies Detect Caspase Activity

In culture, bulk assays

In vivo, Immuno-histochemistry

Functional studies - Inhibitors, RNAi, mutants
<table>
<thead>
<tr>
<th>Feature</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size</td>
<td>Shrunken</td>
<td>Swollen</td>
</tr>
<tr>
<td>Cell fragmentation</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Specific DNA cleavage</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Engulfment</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Inflammatory*</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Caspases Required</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Altered</td>
<td>Swollen</td>
</tr>
<tr>
<td>Phosphatidylserine exposed</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
Methods of detection rely on characteristic features

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size</td>
<td>imaging</td>
</tr>
<tr>
<td>Cell fragmentation</td>
<td>imaging</td>
</tr>
<tr>
<td>DNA cleavage</td>
<td>TUNEL</td>
</tr>
<tr>
<td>Engulfment</td>
<td>markers, sensors</td>
</tr>
<tr>
<td>Inflammatory*</td>
<td>markers, sensors</td>
</tr>
<tr>
<td>Caspases</td>
<td>antibodies, substrates</td>
</tr>
<tr>
<td>Phospatidylserine</td>
<td>annexin V</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>imaging, markers, biosensors</td>
</tr>
</tbody>
</table>
INTERNUCLEOSOMAL CLEAVAGE OF DNA IS ASSOCIATED WITH APOPTOTIC CELL DEATH

30 nm nucleoprotein fiber

Nucleosomes

Histone H1

DNA helix

Spacer region
TUNEL - terminal deoxytransferase dUTP nick end labeling

Enzyme adds labelled nucleotides to ‘free ends’ that occur at sites of cleaved DNA
TUNEL - terminal deoxytransferase dUTP nick end labeling

*Enzyme adds labelled nucleotides to ‘free ends‘ that occur at sites of cleaved DNA*
Defining the mode of cell death

Multiple criterion typically needed

Descriptive criteria vs. Functional criteria

Functional studies define events necessary for killing
Defining the mode of cell death

Multiple criterion typically needed

Descriptive criteria vs. Functional criteria

Functional studies define events necessary for killing
Cell death genes prevent death when eliminated

Cell death defective macrophages
normal engulfment normal
Cell death genes prevent death when eliminated.

Cell death defective macrophages normal engulfment normal

Produce extra cells
Cell death defective phenotypes observed in real time through *in vivo* imaging

Transgenic labels: Green fluorescent protein (GFP); Red fluorescent protein RFP

Genetic mosaic tissue cell death defective genes
Pivotal apoptosis regulators impact the extrinsic and/or intrinsic pathway. e.g. Bcl2 family members, caspases, death receptors.

Processed Caspases are physically inhibited by IAPs.

IAP antagonists are also pivotal.
Genome scale screens for cell death genes

‘Hits’ reverse killing by smac mimetic
Genome scale screens for cell death genes

Seed cells in triplicate (13,071 dsRNAs x 3) → 3 days → Induce apoptosis → 2 days → Assay cell viability

‘Hits’ reverse killing by smac mimetic
The Lexicon of Cell Death includes:

- **Anoikis** - apoptotic response seen when adherent cells lose matrix interactions. believed to be major tumor suppressive mechanism.
- **Pyroptosis** - associated with inflammation, involves Casapse 1 (Accounts for loss of T cells associated with AIDS).
- **Necroptosis** - TNFR1 signaling through RIP1 that occurs when Caspase 8 is inhibited.
- **Secondary necrosis** - necrosis occurring after full apoptotic program. Seen in culture and/or when engulfment is compromised.
- **Programmed forms of necrosis** -
  - Parthanatos - excessive poly(ADP-ribose) polymerase activity depletes ATP and NAD+
  - Ferroptosis - dependent upon intracellular iron.
- **Autophagic cell death** - induced cytoplasmic vacuolization
  - Autophagy is typically a survival adaptation.
  - Death by autophagy vs. death with autophagy? Is autophagy the lethal event?
The Autophagy Regulatory Network Engages Cell Death Regulators

Douglas Green & Beth Levine
Cell, Volume 157, Issue 1, 2014, 65 - 75
Many thanks to

Nichole Link
Anwesha Gosh
Su Kit Chew
Alex Rodriguez
Mark Carlson
Beth Levine
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Detecting Apoptosis by Flow Cytometry

Where are we after 25 years?

Bill Telford, Ph.D.

NCI Flow Cytometry Core Laboratory
National Cancer Institute
National Institutes of Health
Apoptosis was first identified as a distinct morphological phenomenon in the 1960s (and probably earlier), and was well-accepted as an important regulatory process by the 1970s...

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From Kerr, J.F.R., J Pathology 105, 13-20, 1971 (!)
Institute of Theoretical And Experimental Biophysics

Pushchino, Serphkov region, Russia

Institute of Cell Biophysics
ОПРЕДЕЛЕНИЕ РАДИОЧУСТОВИТЕЛЬНОСТИ ИНТЕРФАЗНО ГИБНУЩИХ КЛЕТОК ТИМУСА, СЕЛЕЗЕНКИ И КОСТНОГО МОЗГА КРЫС МЕТОДОМ ПРОТОЧНОЙ ЦИТОМЕТРИИ

УМАНСКИЙ С. Р., АФАНАСЬЕВ В. Н., КОРОЛЬ Б. А., ПЕЧАТНИКОВ В. А.

Рис. 1. ДНК-гистограммы клеток тимуса (A), селезенки (B) и костного мозга (B) крыс

1 — контроль, 2 — 3 ч; 3 — 6 ч после облучения в дозе 10 Гр. По оси абсцисс — относительное содержание ДНК на клетку; по оси ординат — количество клеток

Hoechst 33258
FLOW CYTOMETRY USED TO STUDY INTERPHASE DEATH OF THYMUS, SPLEEN AND BONE MARROW CELLS OF IRRADIATED RATS

UMANSKY S. R., AFANASIEV V. N., KOROL' B. A., PECHATNIKOV V. A.

Institute of Biological Physics, USSR Academy of Sciences, Pushchino

Fig. 1. Block-scheme of flow cytometer LAKS-1

Hoechst 33258
Flow cytometry assays for apoptosis are now almost 25 years old...

The earliest flow cytometry assays for apoptosis analyzed changes in forward and side scatter, and DNA fragmentation / loss following ethanol treatment. Unlike earlier assays, flow cytometry analyzed apoptosis in individual cells.

From Telford et al., Applied Fluorescence Technology 4, 12-17 (1992)
Signal transduction of apoptosis

**Signals**
- Death ligands (TNF, TRAIL, Fas ligand)
- Stress, DNA damage

**Caspase activator**
- TNFR1, Fas, DR4/5
- FADD

**Initiator caspase**
- caspase 8, 10

**Effector caspase**
- caspase 3, 6, 7

**Morphology**
- Autophagy
- Cytoskeletal breakdown
- Membrane asymmetry
- Chromatin condensation
- DNA fragmentation

**Extrinsic pathway**
- cytochrome C release

**Intrinsic pathway**
- Stress, DNA damage
- Bax, Bam, Bid
- Bcl-2, Bcl-xl

**From Z. Darzynkiewicz**
Types of assays...

Flow cytometry assays now target almost every stage of apoptosis, from the earliest mitochondrial changes to caspase activation, membrane changes and DNA damage.

<table>
<thead>
<tr>
<th>characteristic</th>
<th>flow cytometry assay</th>
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</thead>
<tbody>
<tr>
<td>“early” apoptotic events</td>
<td>“late” apoptotic events</td>
</tr>
<tr>
<td>Cell volume fluctuations</td>
<td>Organelle changes</td>
</tr>
<tr>
<td>Cytochrome C release</td>
<td>PS membrane “flipping”</td>
</tr>
<tr>
<td>Changes in cell membrane potential</td>
<td>Transglutaminase crosslinking</td>
</tr>
<tr>
<td>Mitochondrial potential changes</td>
<td>Changes in chromatin organization</td>
</tr>
<tr>
<td>Signaling events (bcl-2, Bax, etc.)</td>
<td>DNA strand breaks</td>
</tr>
<tr>
<td>Initiator (proximal) caspase activation (1,9,10,8)</td>
<td>Membrane “blebbing”</td>
</tr>
<tr>
<td>Effector (distal) caspase activation (3,6,7)</td>
<td>Global chromatin damage</td>
</tr>
<tr>
<td>Minor changes in scatter</td>
<td>Loss of membrane permeability</td>
</tr>
<tr>
<td>Cytochrome C release assay</td>
<td>Immunolabeling of active caspases</td>
</tr>
<tr>
<td>Membrane potential probes</td>
<td>Organelle-specific probes</td>
</tr>
<tr>
<td>Mitochondrial potential probes</td>
<td>Annexin V, structure-specific plasma membrane probes</td>
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<tr>
<td>Bax translocation</td>
<td>Immunolabeling of histones and histone associated proteins</td>
</tr>
<tr>
<td>Fluorigenic caspase substrates</td>
<td>TUNEL assays</td>
</tr>
<tr>
<td>PhiPhiLux</td>
<td>Major changes in scatter</td>
</tr>
<tr>
<td>FLICA</td>
<td>Loss of DNA dye binding</td>
</tr>
<tr>
<td>CellEvent Green</td>
<td></td>
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</tbody>
</table>
Take-home lessons...

**Apoptosis is a highly variable process.** There is a lot of variation in the apoptotic process and phenotype between cell types, and even the same cell type at different levels of activation or differentiation.

You therefore need to find the best method for measuring apoptosis for your particular cell system. Don’t just choose a method at random!

Never use only one assay for apoptosis. And combine multiple assays wherever possible! Always measure cell death using several different methods, *preferably in the same sample*. Multiparametric flow cytometry is ideal for this. Combine biochemical and morphological assays when possible. The *process* of apoptosis can be observed.

Let your assay not only *measure* cell death, but *characterize* it as well. You can learn interesting things about your cells and your system.

**Take pictures!** Visualizing the cells is important and very educational! Many new options in image cytometry make this possible.
Combining multiple apoptosis assays

Annexin V and a DNA binding dye is an excellent example of combining two assays into a multiparametric method.

**EL4 cells**

*actinomycin D*

4 hours

At least two stages of apoptotic death are being measured here.

- **FITC annexin V**
  - 16%
  - 7%
  - 75.1%

- **propidium iodide**
  - **annexin V+ PI+** (late apoptotic/necrotic)
  - **annexin V+ PI-** “early apoptotic”
  - “viable” cells

PS “flipping” occurs prior to 7-AAD permeability

At least two stages of apoptotic death are being measured here.
Combining multiple apoptosis assays

With scatter, four parameters are being measured here. Combining assays allows the progression of apoptosis to be studied, and provides a much richer picture than any one assay can give.

Annexin V binding **precedes** both scatter changes and 7-AAD incorporation.

We are looking at a relatively *early* apoptotic event compared to other assays.
Combining multiple apoptosis assays

FLICA caspase detection, annexin V and a cell-impermeant DNA binding dye (as well as scatter) are combined into a powerful, multistage assay for apoptosis.

EL4 cells

no treatment

topotecan
2 μM 16 h
Apoptosis and image cytometry

Visualizing apoptotic cells is an excellent idea. Why?

→ Apoptosis is highly variable and pleiotropic. Imaging can give verification that apoptosis is occurring, and characterize it.

→ Imaging gives additional analysis options (like pixel-by-pixel analysis) that are useful for apoptotic analysis.

→ Imaging allows analysis of adherent cells without removal of the cells from their substrate.

Many options now exist for performing image cytometry, where cytometric data and correlated cell images can be collected simultaneously. Many laboratories prefer this technology for the analysis of apoptotic cells (particularly for adherent cells).
Apoptosis and image cytometry

Compucyte iCys analysis of apoptotic EL4 cells

Fluorescein PPL caspase 3

7-AAD

Alexa Fluor 647 annexin V

Compucyte iCys field scans

no treatment

camptothecin 6 h

7-AAD

viable
eviable

early apoptotic

advanced apoptotic

fluorescein PPL caspase 3

fluorescein PPL caspase 3
Apoptotic cell analysis with laser scanning cytometry

Direct correlation between the cytometric data and the imagery (relocation analysis).

Morphological analysis using light scatter or absorption.
Discrimination of adherent apoptotic cells by image cytometry

Image cytometry can analyze site-specific fluorescence from images, improving sensitivity. Trypsin or accutase detachment, which can “muddy” apoptotic labels, is not necessary.

Apoptotic cells “round up” and can be lost from the growth substrate.
Analyzing apoptosis on the Amnis ImageStream

A stream-based scanning cytometry system like the Amnis ImageStream or FlowSight similarly is an another excellent way to combine cytometric and morphological analysis.

Again, direct correlation between cytometry and imagery.

Daudi cells induced with camptothecin

Data from Brian Hall and Tad George, Amnis, EMD Millipore
Analyzing apoptosis on the Amnis ImageStream

untreated

Images of live cells

Images of PhiPhiLux positives

Data from Brian Hall and Tad George, Amnis, EMD Millipore
Analyzing apoptosis on the Amnis ImageStream

Data from Brian Hall and Tad George, Amnis, EMD Millipore
Autophagy

U2OS cells expressing GFP-LC3
EMD Millipore
Autophagy detected by GFP-LC3 translocation

During induction, a lysozyme inhibitor is added to block destruction of autophagosomes by lysosomes.

If autophagy occurs, GFP-LC3 will accumulate in autophagosomes when the inhibitor is present.

The cells are then permeabilized.

In the absence of autophagy, the GFP-LC3 will be in the cytoplasm and will be released into the media.

With autophagy, the GFP-LC3 will be trapped in the autophagosomes and will not be released into the media.

Autophagosome associated GFP-LC3 can be detected in the intact cells by flow cytometry.

Assay courtesy EMD Millipore
Autophagy detected by GFP-LC3 translocation

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**Take pictures!** Visualizing the cells is important and very educational! Many new options in image cytometry make this possible.
Acknowledgements

NCI ETIB Flow Lab

Veena Kapoor
Nga Tu Voong
Elena I. Kovalenko

EMD Millipore

Karen Tamul
Brian Hall
Thad George

Molecular Probes, Thermo Fisher

Jolene Bradford
Gayle Buller
Suzanne Buck
Jeff Croissant
Mike Olszowy
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To submit your questions, type them into the text box and click

Sponsored by:
The Many Roads to Cell Death:
Simplified Solutions for Cell Death and Cell Health Analysis with the Muse® Cell Analyzer

Kamala Tyagarajan, Ph.D., EMD Millipore
June 4th, 2014
Study of Cell Death Pathways is Enabled by a Variety of Technologies

- Dissecting and understanding different cell death pathways requires information from multiple steps in the pathway.
- A range of technologies can provide information on different cell death pathways:
  - Flow cytometry
  - Imaging based cytometry
  - Technologies for adherent cell imaging
- Time courses and dose responses can provide deeper insights into mechanisms at play and provide a more comprehensive picture.
There are Common, Inherent Drawbacks with Current Methods for Cell Health Analysis

- Home-brew reagents and commercial kits
- Microscopy
- Western Blot, ELISA, Antibodies
- Flow Cytometry
- Dedicated Instrumentation for Cell Health Applications

- Limited Access to Instrumentation
- Greater Expertise and Planning Needed
- Expense
- Lack of Reproducibility
- Accuracy of Results
Muse™ Cell Analyzer: Simple, Affordable, Miniature Flow Cytometry

- Closed Platform (instrument and 23 assays) allowing instant assessments of Cell Health, Apoptosis, Cell Signaling, Immunology, Cell Cycle
- Instrument utilizes novel miniaturized flow cytometric technology
- Novel, intuitive software and touchscreen interface = novel, simplified user experience
- Simple, effortless operation and sample preparation = accessibility to flow novices
- Innovative “personal” cell analysis
- Accessible, simplistic approach to complex questions
Muse® Provides Multiple Solutions for Evaluation of Cell Death Mechanisms

**Cell Death/Stress Indicators**

- **↑** ROS/NOS Stress
- **↓** Mitochondrial Membrane Potential
- **↑** Mitochondrial Protein Release
- Phosphatidylserine Translocation to Outer Membrane
- **↓** Metabolic Activity/Cell Vitality
- **↑** Caspase Activity
- **↑** DNA Condensation
- **↑** DNA Fragmentation
- **↑** Plasma Membrane Disintegration

**Early**

- Oxidative Stress
- Nitric Oxide Stress
- MitoPotential
- Annexin V & Dead Cell Dye
- Multi-Caspase
- Caspase 3,7
- Count & Viability Assay (2)

**Late**

- LC3 (Autophagy)
- Cell Cycle
- Cell Proliferation

**Other Indicators**

- Cell Death/Stress Indicators
- Muse Assays
Example: *Muse® MitoPotential Assay*

- A simple, no-wash assay that provides percentage and concentration of cells demonstrating mitochondrial membrane depolarization and cell death.
- Assay is based on detection of mitochondrial potential changes using a cationic lipophilic dye.
- Depolarized cells are detected by a decrease in fluorescence.
- 7AAD enters cells that are compromised in dead or late apoptotic cells.

![Muse® MitoPotential Assay Procedure](Image)

Add Reagent → Add Cells → Add 7-AAD → Incubate for 20 minutes at 37°C → Incubate for 5 minutes at Room Temperature → Run on Muse™ Cell Analyzer

![Muse® MitoPotential Results](Image)

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Sample Info</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Gated</td>
<td>Cell Conc. (cells/mL)</td>
</tr>
<tr>
<td>Total Depolarized</td>
<td>66.80 %</td>
</tr>
<tr>
<td>Total Cell Concentration</td>
<td>3.28E+05</td>
</tr>
</tbody>
</table>

![Muse® MitoPotential Graph](Image)

Results

Finish (Save & close data set)  Next Run (Run next sample)
Example of Time Course Studies in Cell Death Analysis

Time course of Jurkat cells treated with Staurosporine (top) and Gambogic Acid (bottom) and analyzed with the Muse® MitoPotential and Muse® Annexin V & Dead Cell Assay.
### Muse® Assay Offerings Span Multiple Areas

#### Cell Health
- Muse® Count & Viability Kit
- Muse® Cell Cycle Kit
- Muse® Annexin V & Dead Cell Kit
- Muse® Caspase 3,7 Kit
- Muse® MultiCaspase Kit
- Muse® MitoPotential Kit
- Muse® Nitric Oxide Kit
- Muse® Oxidative Stress Kit
- Muse® Ki67 Proliferation Kit
- Muse® LC3 Autophagy (Ab Based)
- Muse® LC3 Autophagy (Reporter Cell Line)

#### Cell Signaling
- Muse® **H2A.X** Activation Dual Detection Kit
- Muse® **MAPK** Activation Dual Detection Kit
- Muse® **EGFR-RTK** Activation Dual Detection Kit
- Muse® **PI3** Activation Dual Detection Kit
- Muse® **Bcl-2** Activation Dual Detection Kit
- Muse® Multi-Color DNA Damage Kit
- Muse® PI3K/MAPK Activation Dual Detection Kit

#### Immunology
- Muse® Human CD4 T Cell Kit
- Muse® Human CD8 T Cell Kit
- Muse® Human B Cell Kit
- Muse® Human CD25 Lymphocyte Kit
- Muse® Human CD69 Lymphocyte Kit
The Muse Cell Analyzer: Simplified Platform for Rapid Cell Health Analysis

- Small footprint platform based on miniaturized, microcapillary flow cytometry
- Optimized kits for convenient analysis of multiple cell death mechanisms
- Easy-to-follow guided software on touchscreen
- Extremely Affordable ($14,750)

Fast & Easy Sample Prep  Load and Run on Muse® Instrument  Quickly Analyze Results!
www.millipore.com/muse
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