Novel Reagents and Solutions for Flow Cytometry

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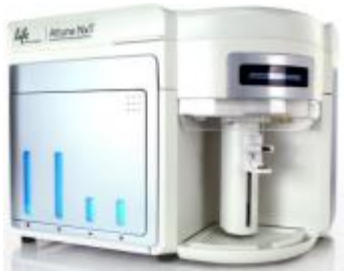
The Essence of Fluorescence

Cellular Analysis

Goal: enable scientists to visualize and analyze cells

- **Supplier of innovation**—reagents and relevant instrumentation to illuminate cellular processes/functions
- **Simplify the complicated**—reagent workflows and instrumentation interfaces
- **Customer connectivity**—partnership, education, and technical support

Assays, Reagents & Antibodies



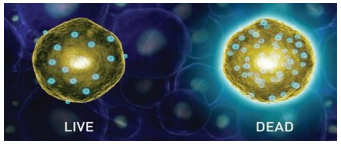
Flow Cytometry

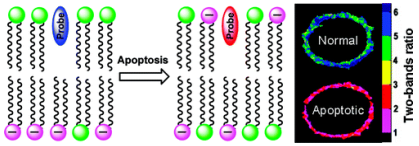
HCA/HCS

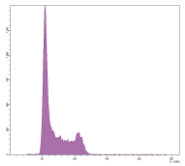
Imaging Systems

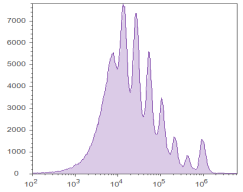
Today We Will Cover:

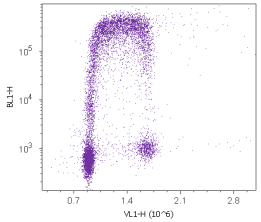
- Viability Reagents
- Apoptosis Assays
- Proliferation Assays
  - Dye Dilution
  - DNA Content Cell Cycle
  - Thymidine Analogues
- Attune® NxT Flow Cytometer

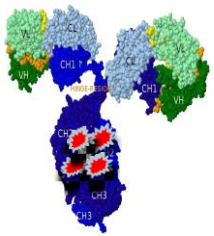




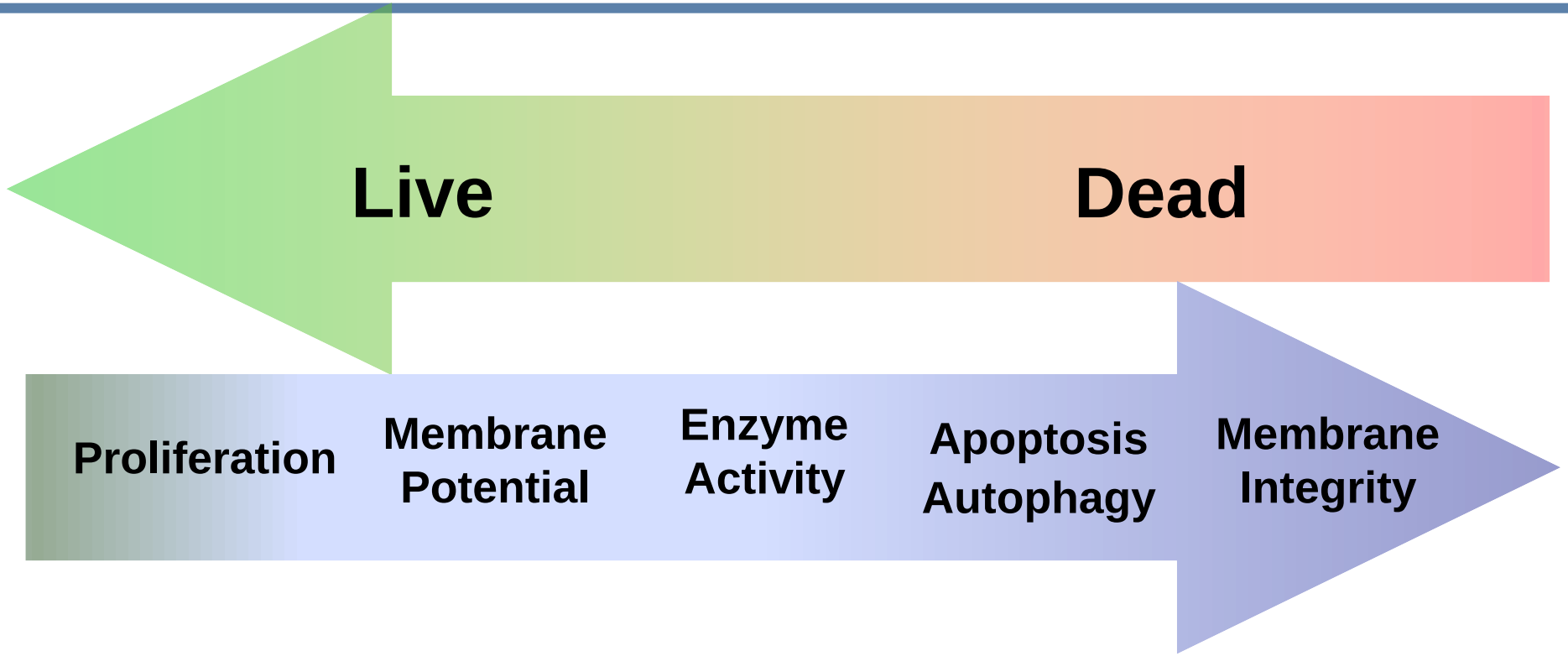






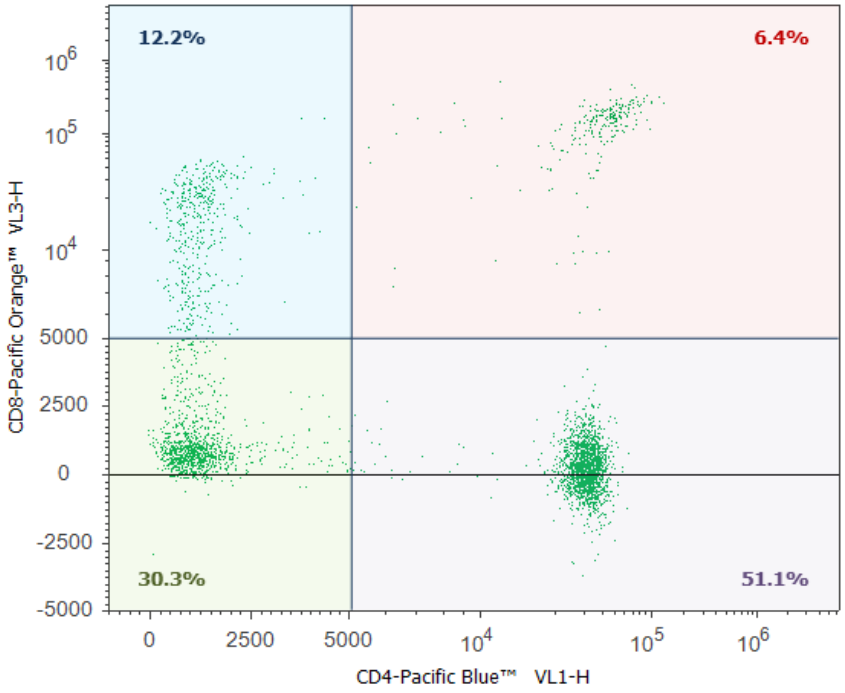


Cells exist on a continuum between healthy and dead

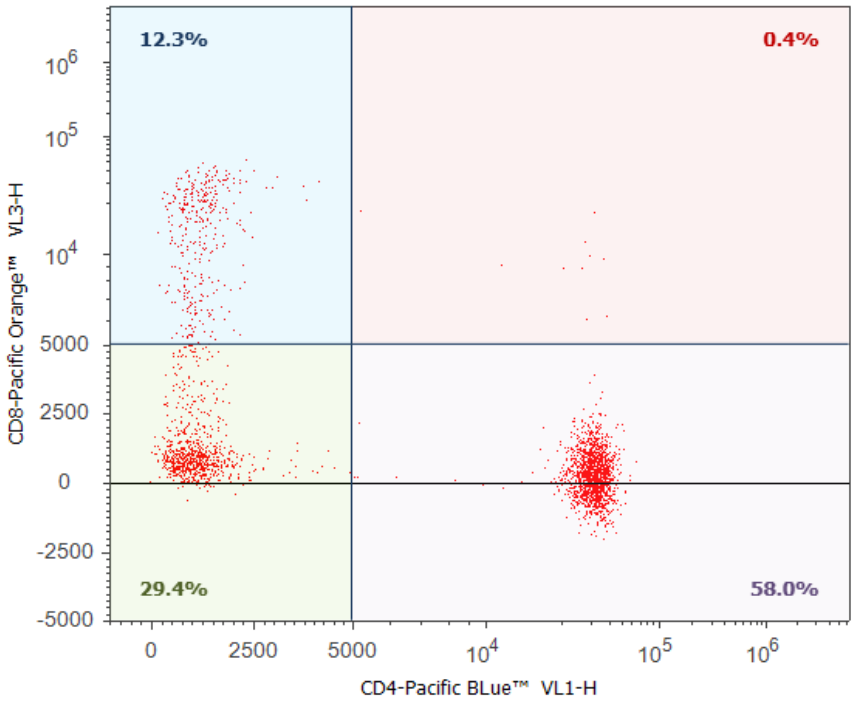


Why assess viability? Eliminate dead cells from analysis

A **Live + Dead Cells**

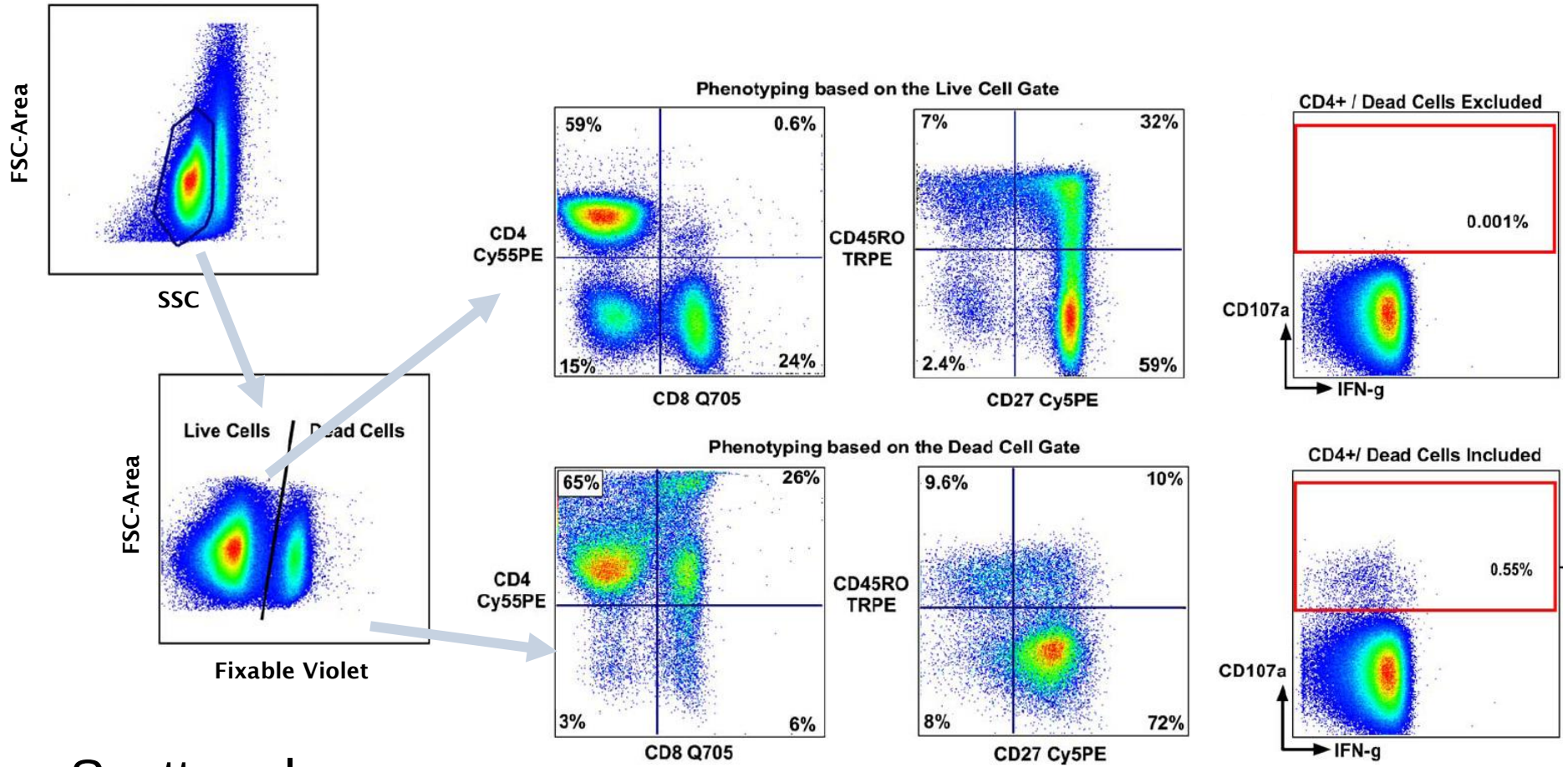


B **Live Cells**



Antibodies can bind nonspecifically to dead cells

Using a Viability Indicator to Improve Results Accuracy



Stimulated with SEB + Brefeldin A

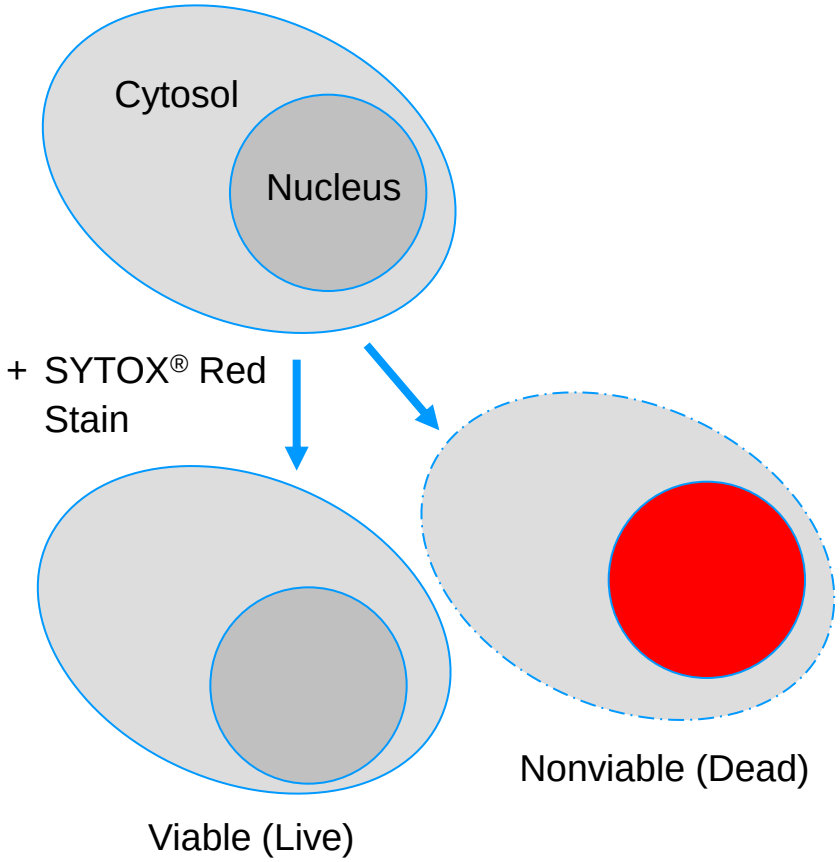
Scatter alone cannot accurately ID dead cells

Perfetto et al. (2006) J Immunol Methods *313*:199

Viability: Impermeant nucleic acid-binding dyes

Viability:

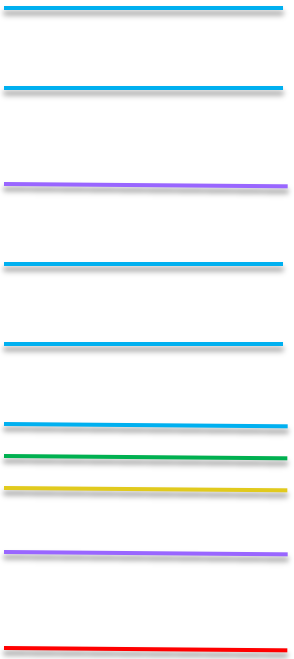
Integrity of plasma membrane



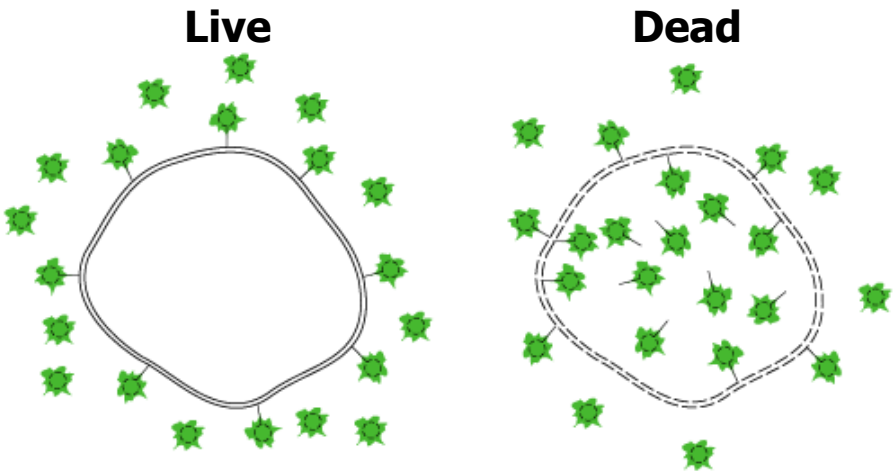
Impermeant Nucleic Acid Dyes for Flow Cytometry

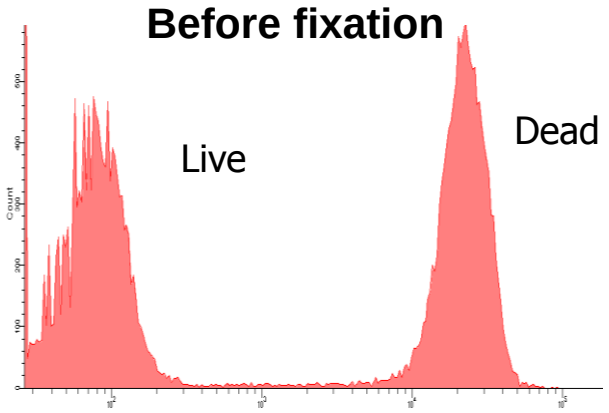
- Dyes which penetrate cells with a compromised cell membrane to stain nucleic acids, but do not cross the membranes of live cells
  - Can be used to identify dead cells in a population
  - Can be used to quantitate DNA content in fixed cells

- Propidium Iodide (488 nm ex)
- 7-AAD (488 nm ex)
- DAPI
- SYTOX® AADvanced™ dead cell stain (488 nm ex)
- SYTOX® Green dead cell stain (488 nm ex)
- SYTOX® Orange dead cell stain (488 /532/561 ex)
- SYTOX® Blue dead cell stain (405 nm ex)
- SYTOX® Red dead cell stain (633 nm ex)



Amine-reactive LIVE/DEAD® Fixable Dead Cell Stains

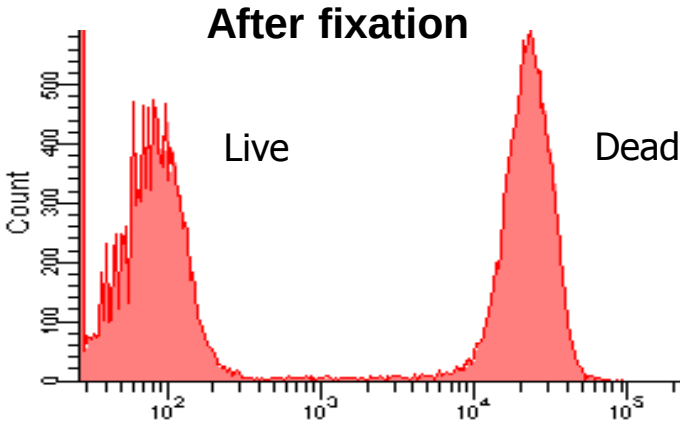




Live cells: react with the kit's fluorescent reactive dye only on their surface to yield weakly fluorescent cells.

Cells with compromised membranes: react with the dye throughout their volume, yielding brightly stained cells.

Viability = membrane integrity



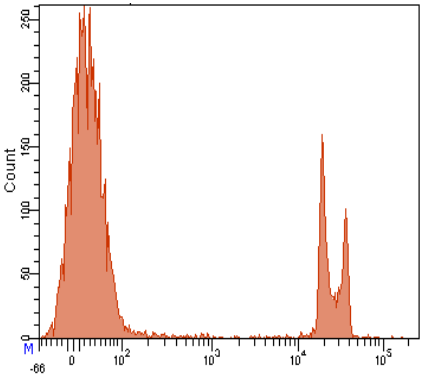
Fixable violet dead cell stain
405 nm Violet Excitation (440/40 BP)

(Staining done before fixation)

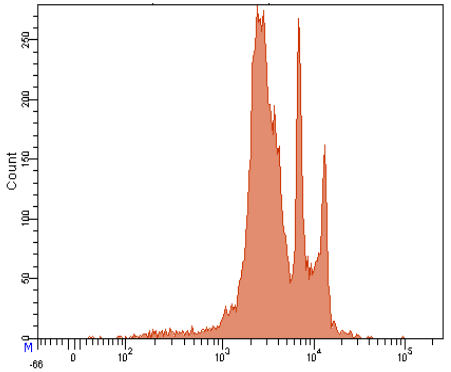
Effect of fixation on dead cell dyes

Propidium iodide
(not fixable)

Before Fixation



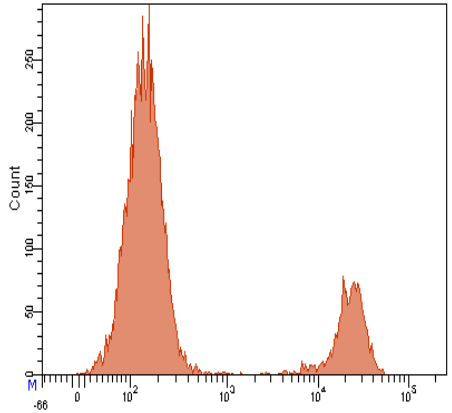
After Fixation



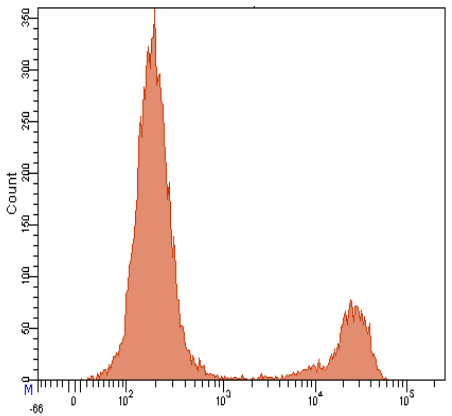
488 nm excitation, 610/20 filter

LIVE/DEAD®
Fixable Red stain
(fixable)

Before Fixation

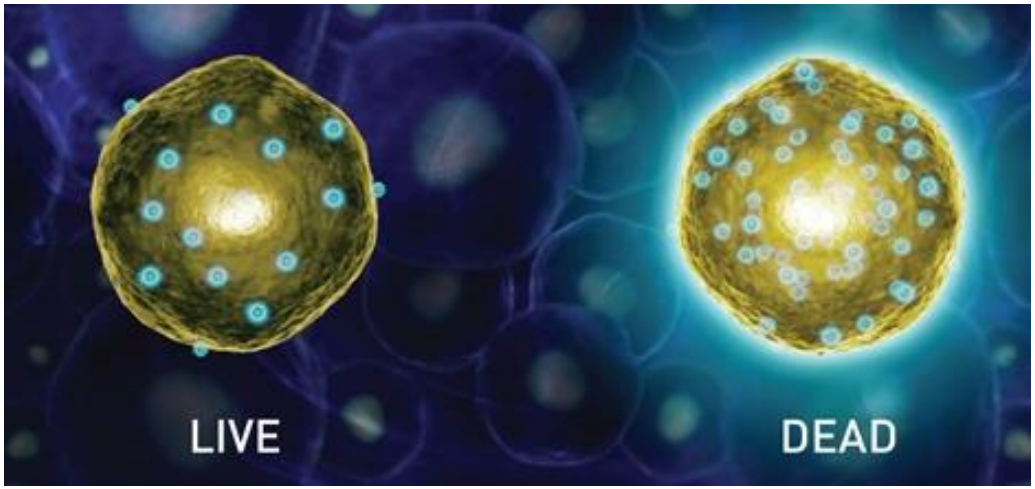


After Fixation



488 nm excitation, 610/20 filter

Dead cell identification in 8 color options



LIVE/DEAD® Fixable Dead Cell Stain Kits

Reactive dye	Excitation source	Ex*	Em*
blue fluorescent reactive dye (L23105)	UV	350	450
violet fluorescent reactive dye (L34955)	405 nm	416	451
aqua fluorescent reactive dye (L34957)	405 nm	367	526
yellow fluorescent reactive dye (L34959)	405 nm	400	575
green fluorescent reactive dye (L23101)	488 nm	495	520
red fluorescent reactive dye (L23102)	488 nm	595	615
far red fluorescent reactive dye (L10120)	633/635 nm	650	665
near-IR fluorescent reactive dye (L10119)	633/635 nm	750	775
*Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.			

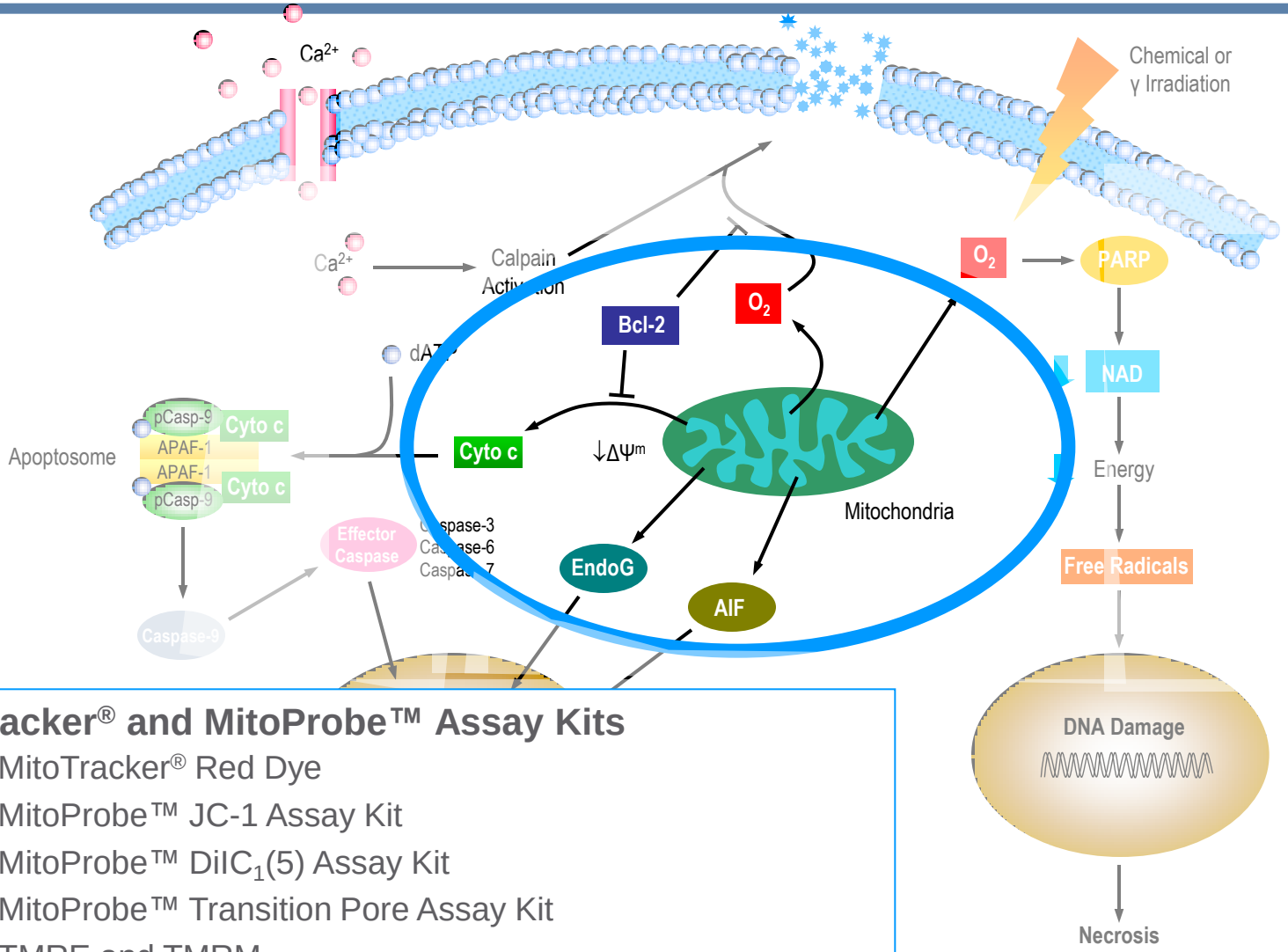
Apoptosis

Relative Timeframe – Apoptosis Molecular Details

Function	Reagent
Mitochondria	
Activity changes	MitoTracker® Red dye
Membrane potential	$DiOC_2(3)$, $DiIC_1(5)$, JC-1
Transition pore	MTP assay
PS translocation, Membrane Permeability	Annexin V, F2N12S YO-YRO™1, PO-PRO™-1
Caspase activity	Cell Event™ Capsase Green, Caspase substrates
Metabolic activity	C_{12} resazurin, Calcein AM
Nuclear condensation	Hoechst, DyeCycle™ Violet dye
Sub G_0 peak	DyeCycle™ Orange dye
Membrane integrity	PI, SYTOX® dead cell stains, LIVE/DEAD Fixable stains

(Jurkat cells induced with 10 µM camptothecin)

Detecting Mitochondrial Changes, Early in Apoptosis

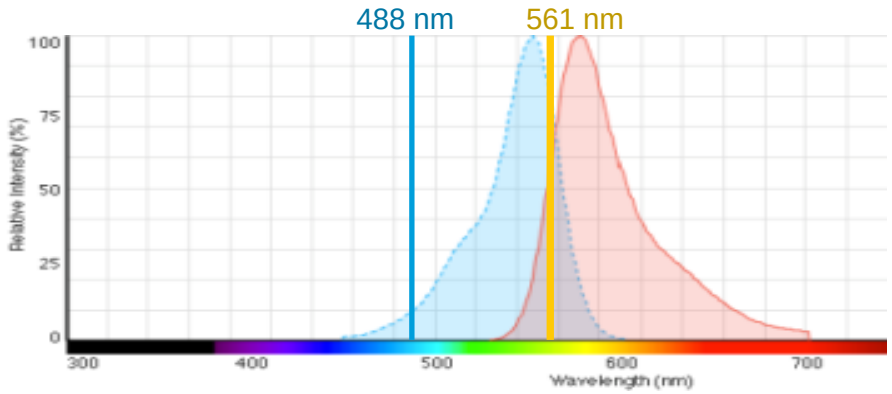


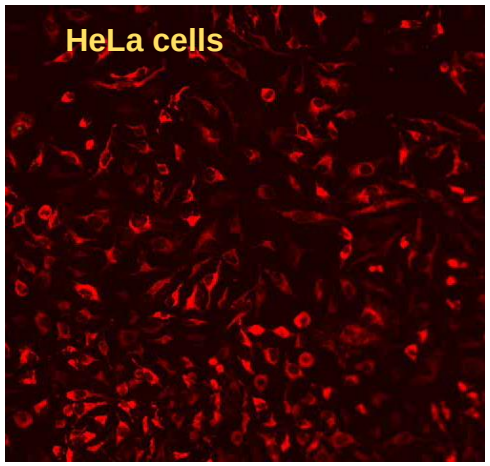
MitoTracker® and MitoProbe™ Assay Kits

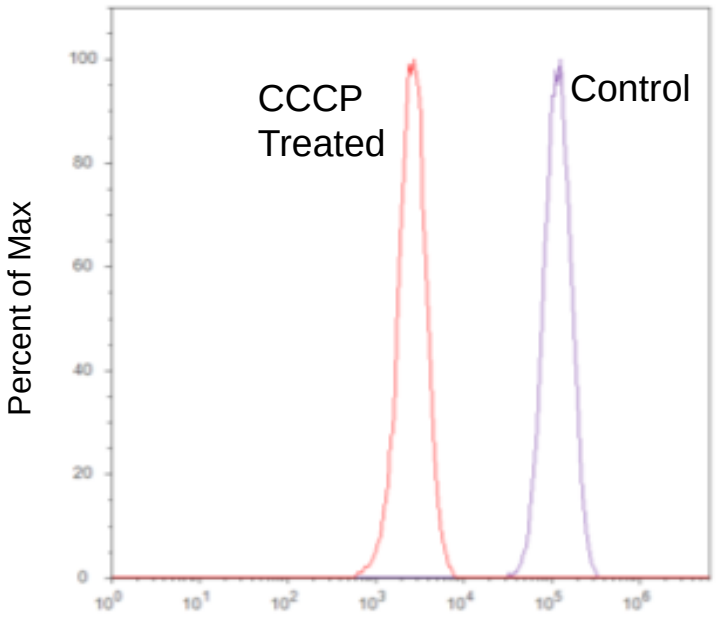
- MitoTracker® Red Dye
- MitoProbe™ JC-1 Assay Kit
- MitoProbe™ DiIC$_1$(5) Assay Kit
- MitoProbe™ Transition Pore Assay Kit
- TMRE and TMRM

TMRM and TMRE

- Dye accumulates in active mitochodria due to the membrane's negative charge
- Depolarized or inactive mitochondria have decreased membrane potential and are unable to sequester the positively charged dye



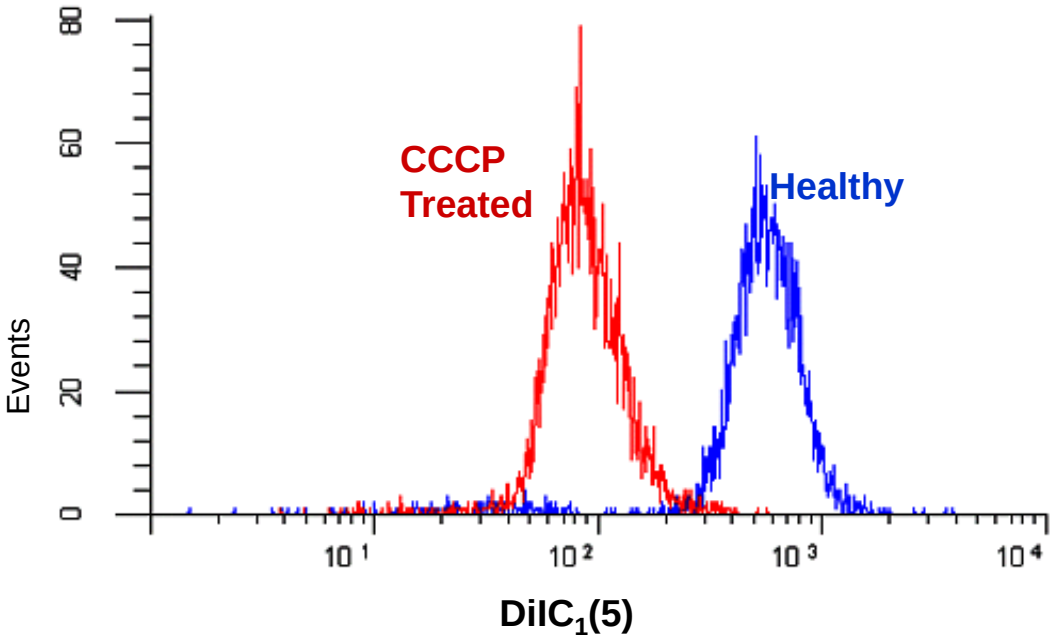


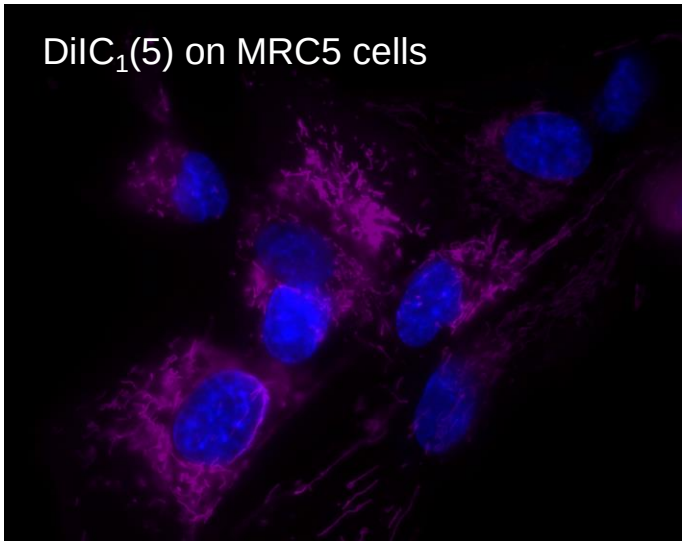


TMRE Fluorescence
561 nm Excitation

MitoProbe™ DiIC$_1$(5) dye

Use with the APC detection channel

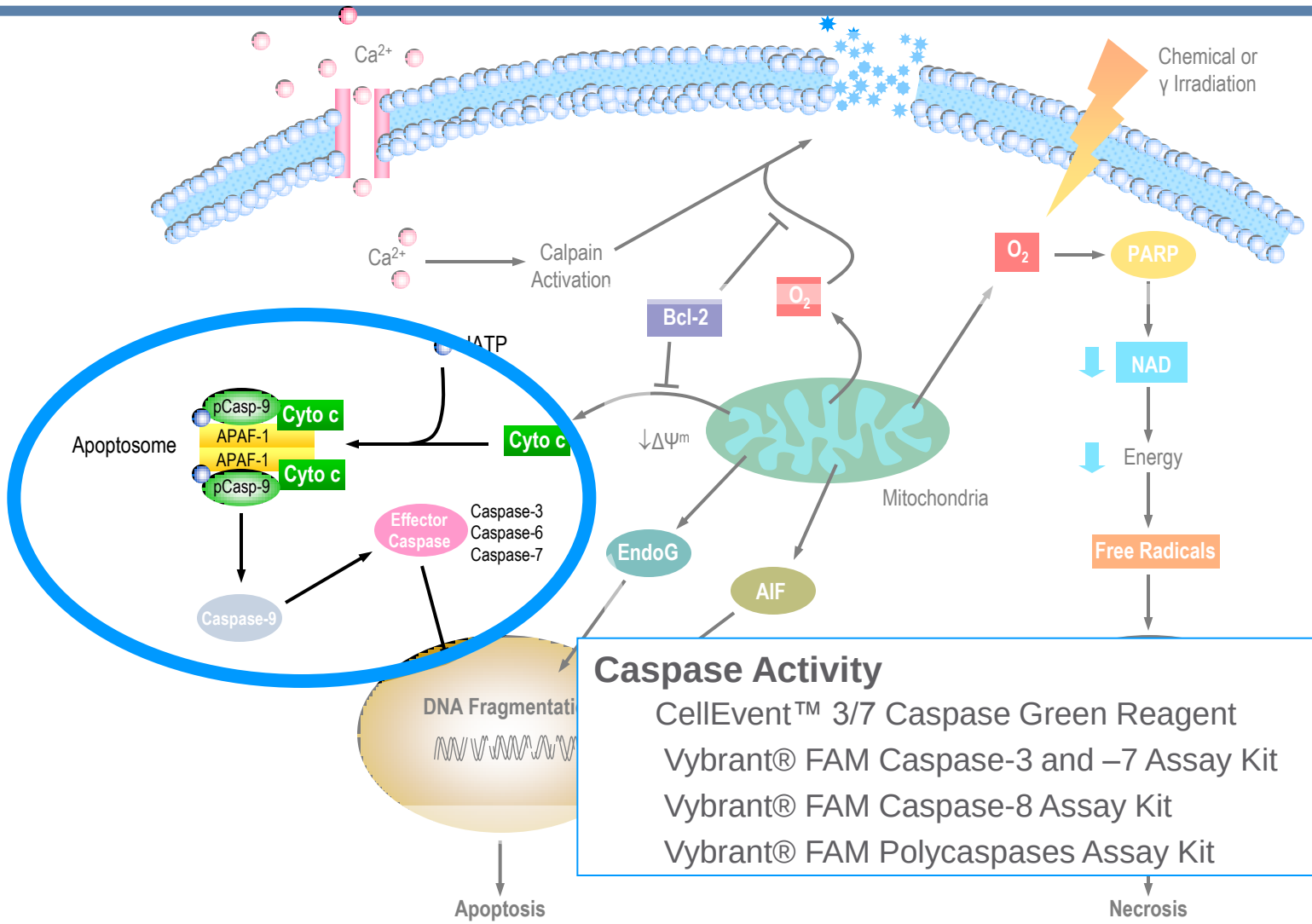




633 nm Excitation, 660/20 BP emission

50 nM DiIC$_1$(5)

Caspase Assays (mid-to-late stage apoptosis)



Caspase Activity

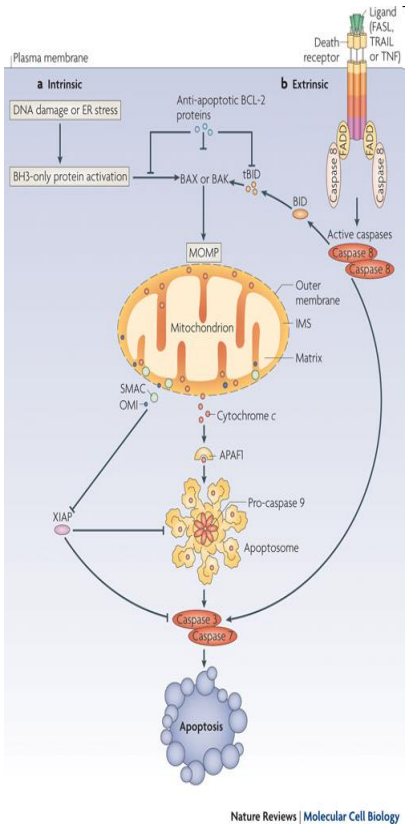
CellEvent™ 3/7 Caspase Green Reagent

Vybrant® FAM Caspase-3 and –7 Assay Kit

Vybrant® FAM Caspase-8 Assay Kit

Vybrant® FAM Polycaspases Assay Kit

CellEvent™ Caspase 3/7 Green Reagent

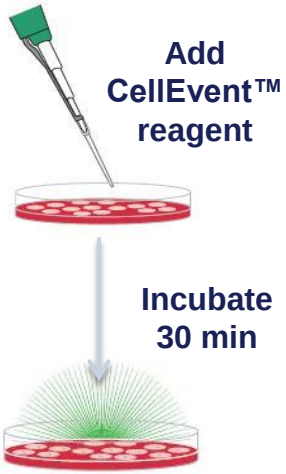


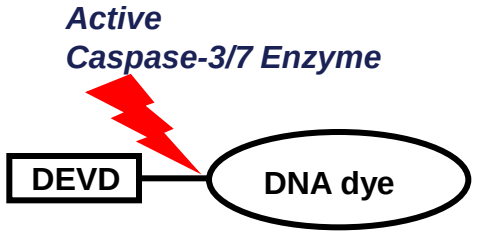
→Fluorogenic Caspase 3/7 Substrate

Active caspase 3/7 cleaves the DEVD peptide and the free nucleic acid dye binds to DNA.

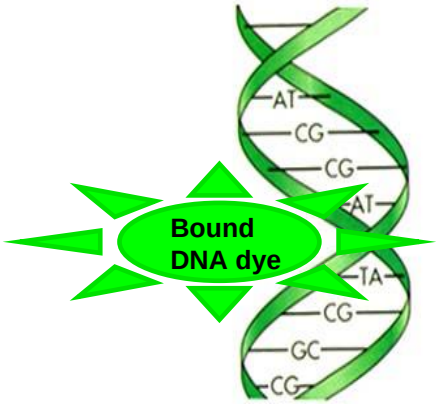
ADVANTAGES:

- **For live cell, no-wash protocols**
- **May be added to complete growth media**
- **Retained after fixation and permeabilization**
- **May be multiplexed with other live or fixed cell probes**

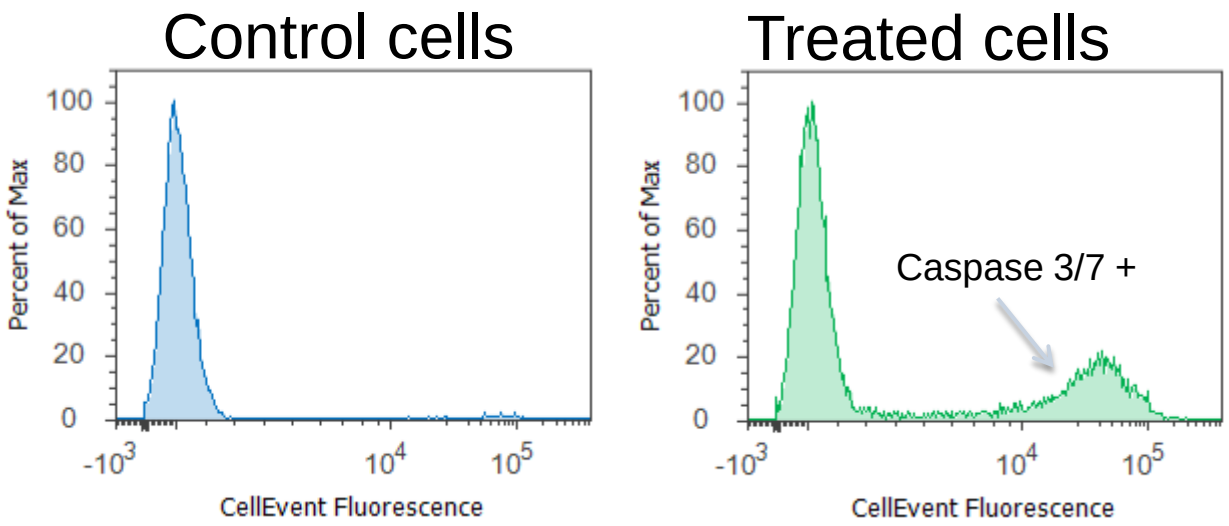


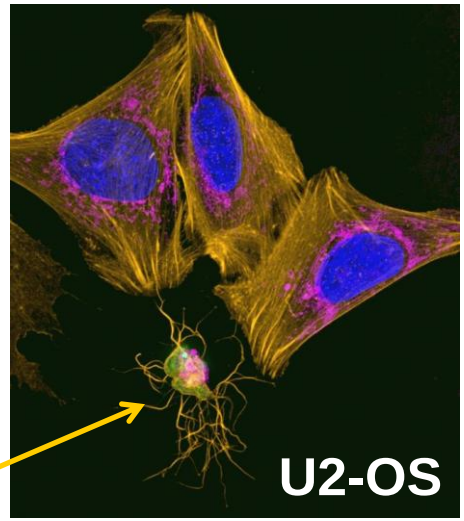


Non-fluorescent
No DNA binding



CellEvent® Caspase 3/7 Green Detection Reagent

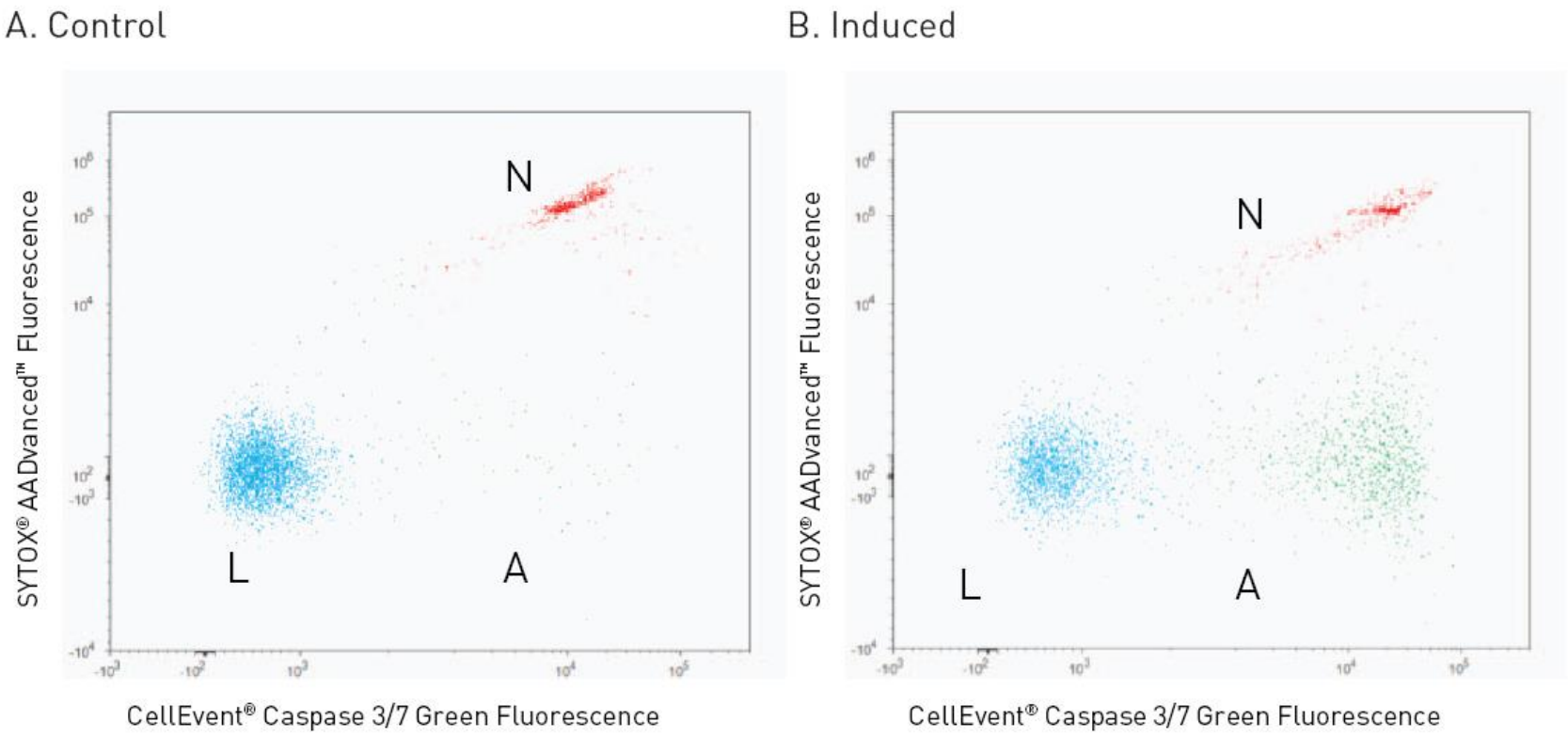




Analyze typically 30 minutes after addition of staining reagent

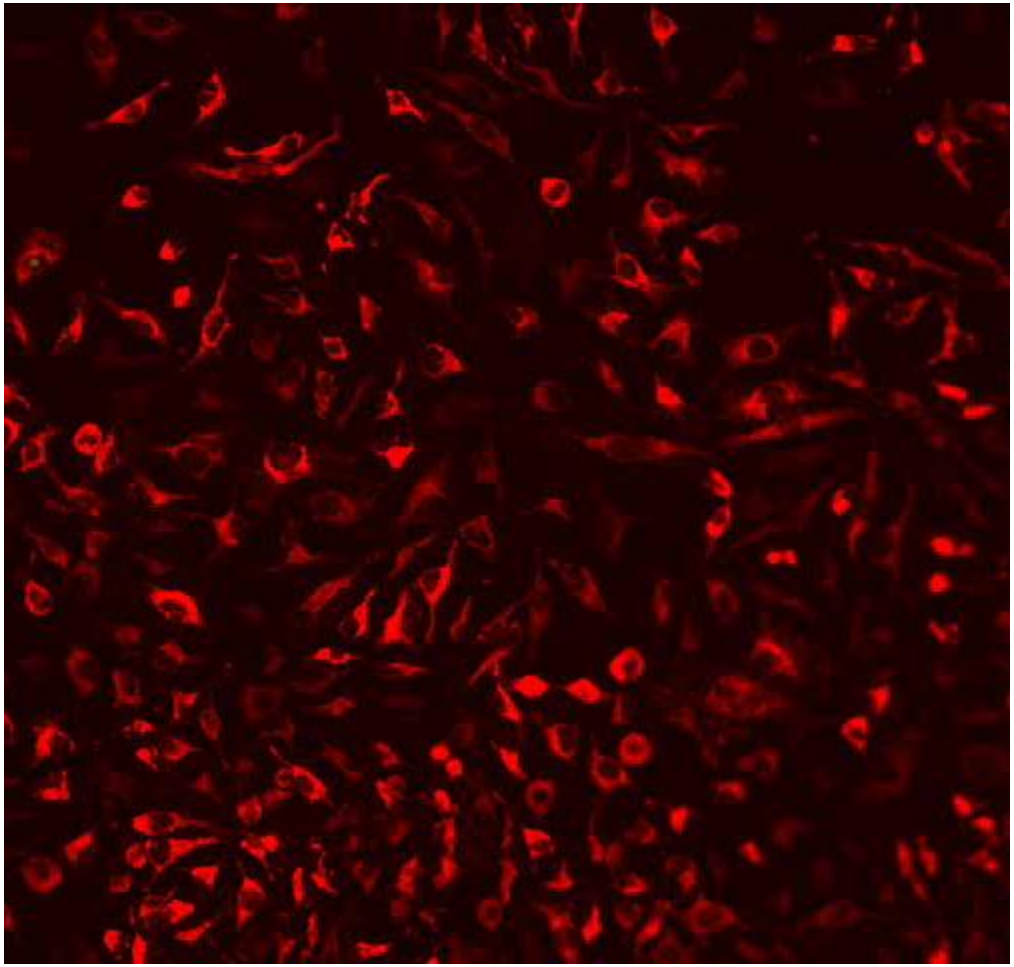
CellEvent™ Caspase-3/7 Green Detection Reagent

for the detection of activated* caspase 3/7



*Jurkat cells treated with 10 µM camptothecin for 3 hours before labeling with CellEvent® Caspase 3/7 Green Flow Cytometry kit. Stained samples analyzed on the Attune® Acoustic Focusing Cytometer equipped with a 488 nm laser.

Multiplex Time Lapse Imaging of Apoptosis and Mitochondrial Health: CellEvent™ Caspase 3/7 Green and TMRM



HeLa cells, 0-7 hrs treatment with 50 nM staurosporine

Red: TMRM mitochondrial membrane potential indicator

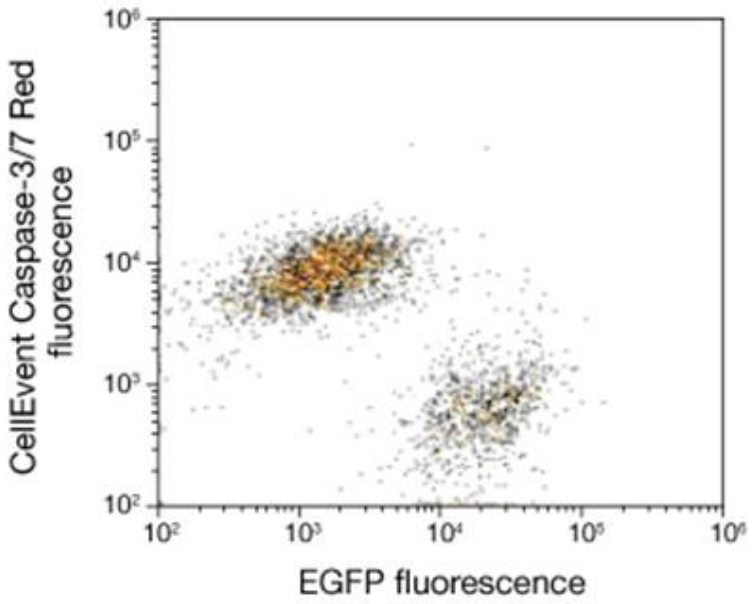
- **Fades with apoptosis**

Green: CellEvent™ Caspase 3/7 (5 μM)

- **Fluorogenic with apoptosis**

CellEvent™ Caspase-3/7 Red Detection Reagent

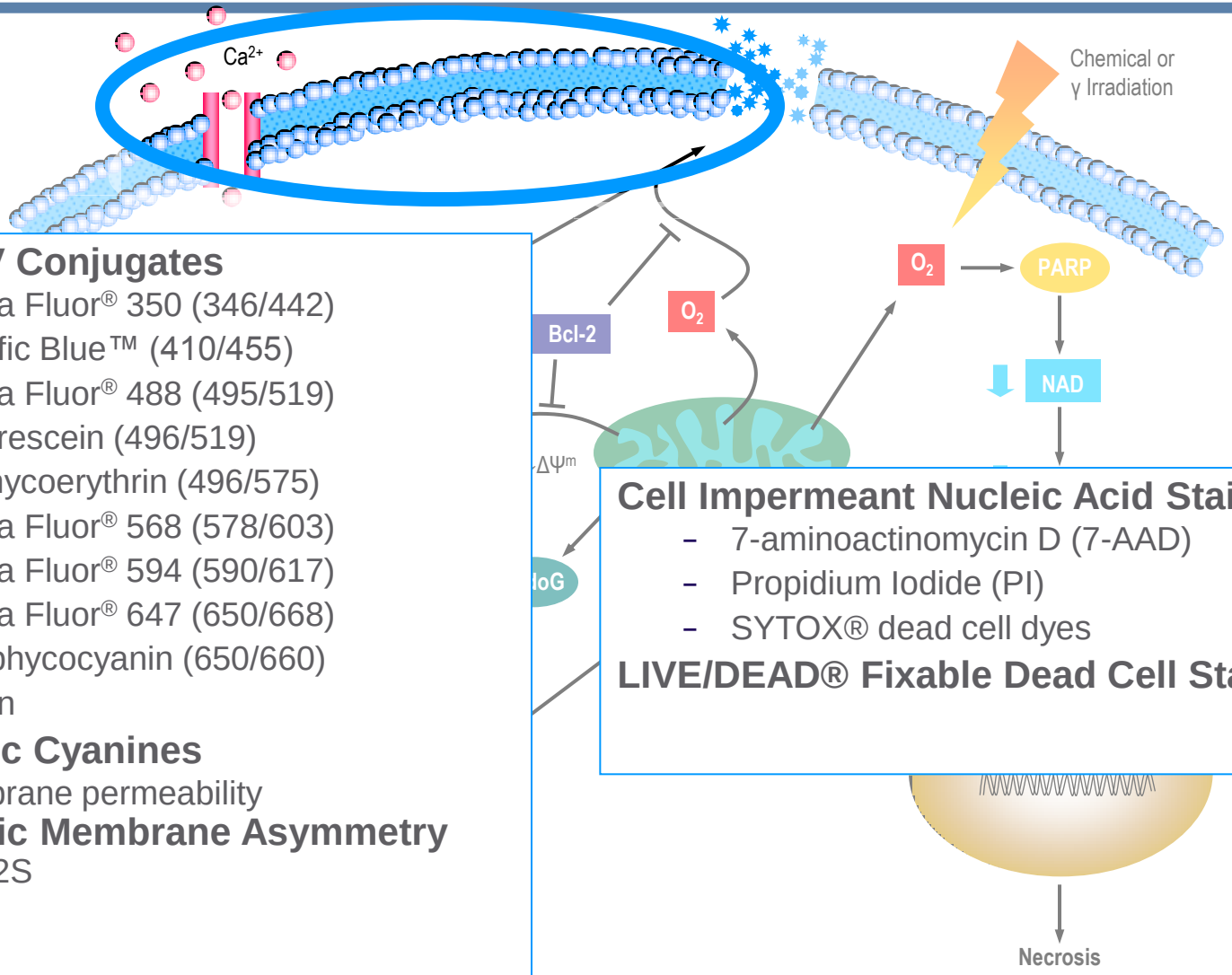
for the detection of activated caspase 3/7



Catalog #	Name	Size
C10427	CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit	100 assays
C10740	CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit	20 assays
C10747	CellEvent™ Caspase-3/7 Red Flow Cytometry Assay Reagent	20 assays
C10748	CellEvent™ Caspase-3/7 Red Flow Cytometry Assay Kit	100 assays

BJAP cells expressing EGFP, treated with 100 ug/mL gentamicin for 24 hr to induce apoptosis, followed by treatment with CellEvent™ Caspase 3/7 Red. EGFP detected using 488 nm laser, CellEvent™ Red using 637 nm laser (670/14 nm emission filter).

Loss of Membrane Asymmetry and Integrity



Annexin V Conjugates

- Alexa Fluor® 350 (346/442)
- Pacific Blue™ (410/455)
- Alexa Fluor® 488 (495/519)
- Fluorescein (496/519)
- R-phycoerythrin (496/575)
- Alexa Fluor® 568 (578/603)
- Alexa Fluor® 594 (590/617)
- Alexa Fluor® 647 (650/668)
- Allophycocyanin (650/660)
- Biotin

Monomeric Cyanines

- Membrane permeability

Ratiometric Membrane Asymmetry

- F2N12S

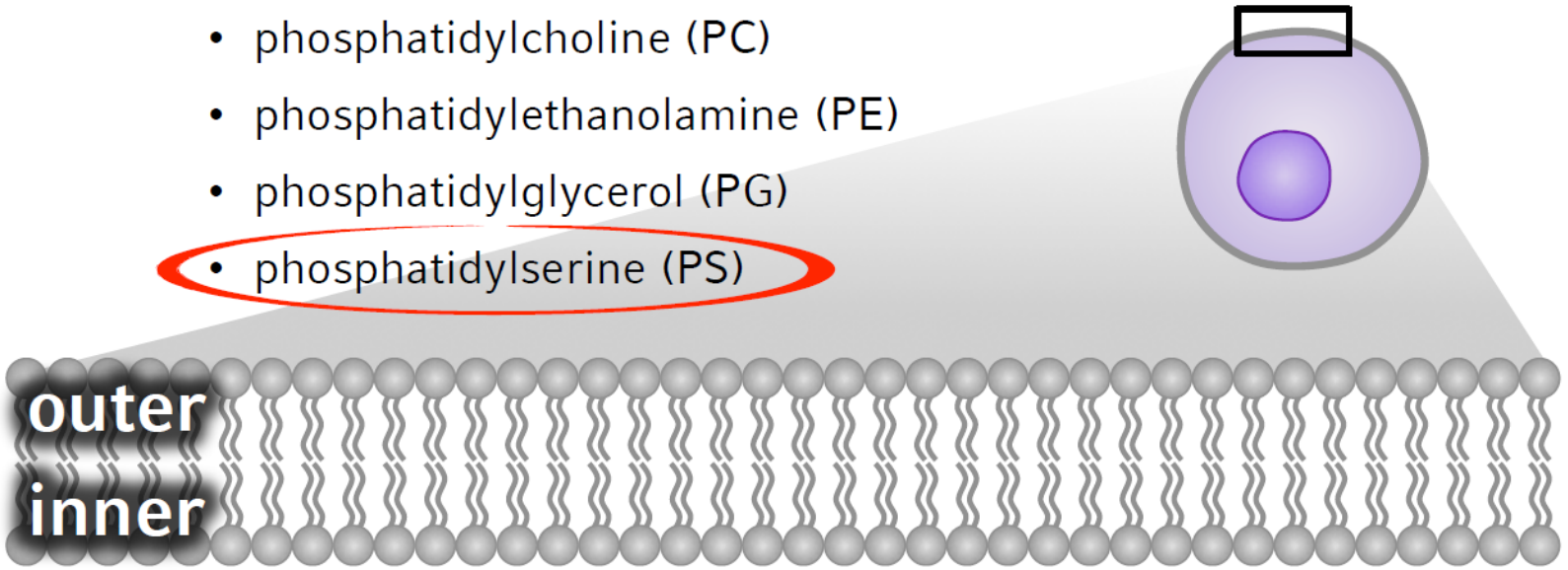
Cell Impermeant Nucleic Acid Stains

- 7-aminoactinomycin D (7-AAD)
- Propidium Iodide (PI)
- SYTOX® dead cell dyes

LIVE/DEAD® Fixable Dead Cell Stains

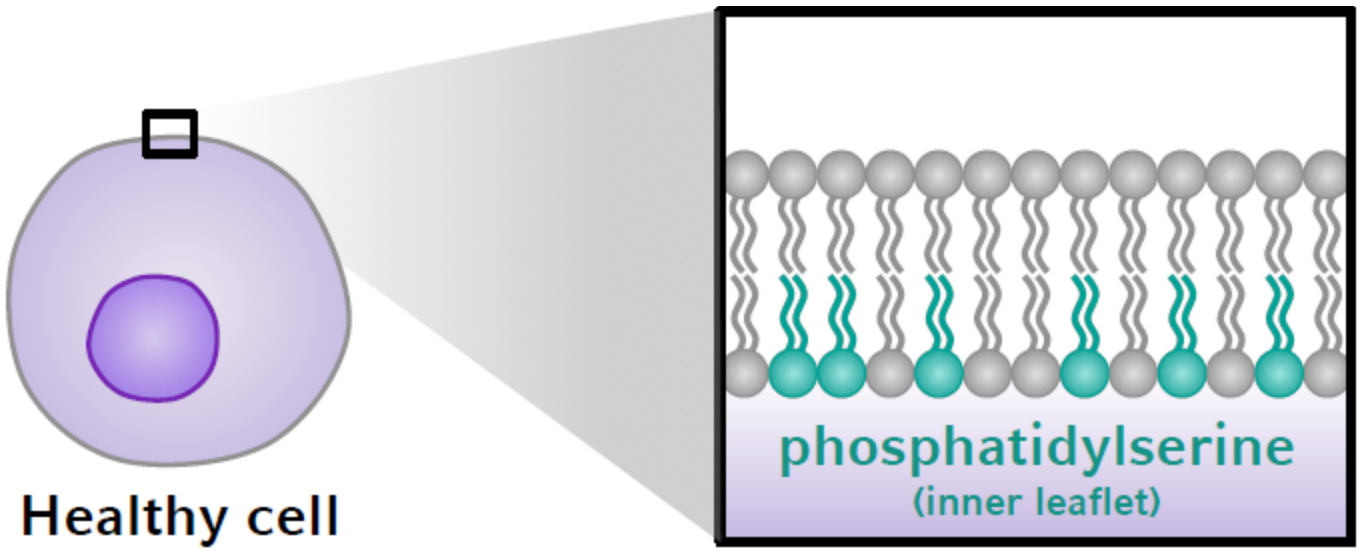
Phospholipid asymmetry

- The plasma membrane
  - lipid bilayer (inner/outer leaflets) composed of different phospholipids
    - phosphatidylcholine (PC)
    - phosphatidylethanolamine (PE)
    - phosphatidylglycerol (PG)
    - phosphatidylserine (PS)

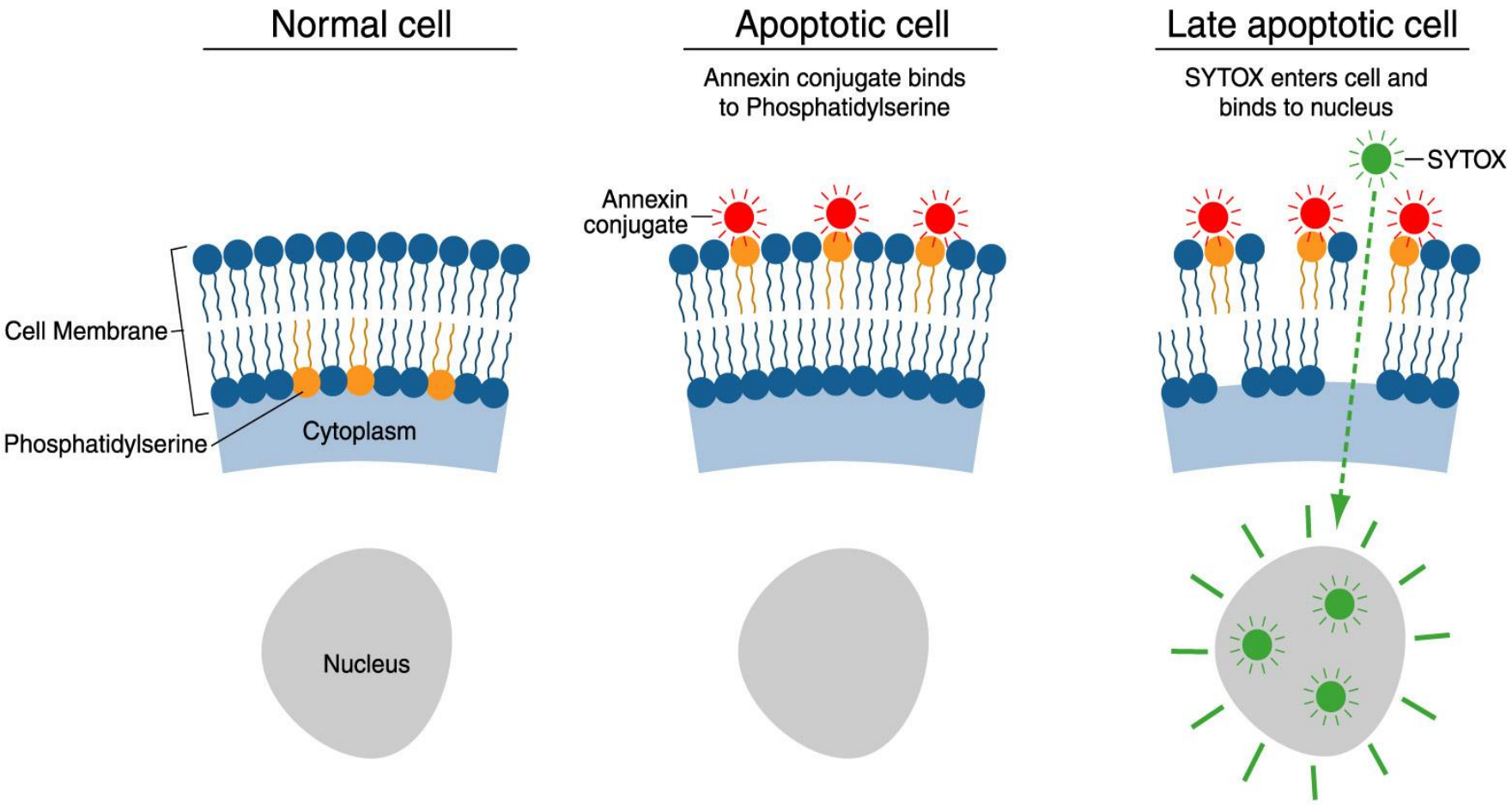


Phospholipid asymmetry

Phosphatidylserine (PS) is found predominately on the inner membrane leaflet



Loss of Membrane Asymmetry: Annexin V



Combine to confirm/distinguish late apoptotic cells from necrotic or dead cells

Pacific Blue™ AnnexinV + SYTOX AADvanced Dead Cell Stain

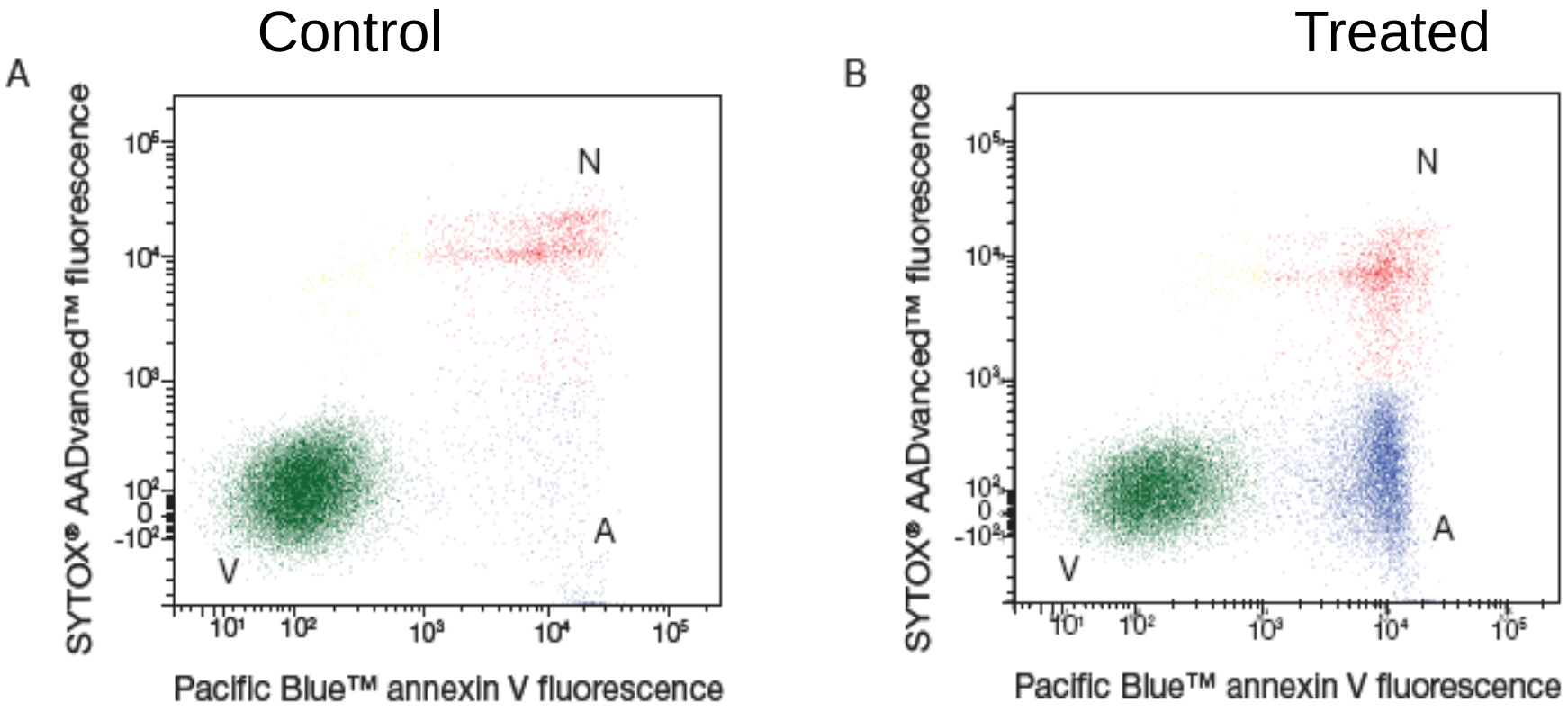
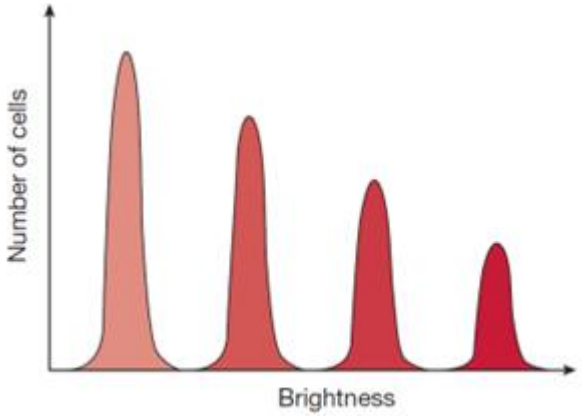


Figure 1. Jurkat cells (T-cell leukemia, human) treated with 10 µM camptothecin for four hours (panel B) or untreated control (panel A). Cells were treated with the reagents in Apoptosis Kit – Pacific Blue™ annexin V/SYTOX® AADvanced™ and analyzed by flow cytometry using 405 nm and 488 nm excitation. Note that the camptothecin-treated cells have a higher percentage of apoptotic cells (panel B) than the basal level of apoptosis seen in the control cells (panel A). A = apoptotic cells, V = viable cells, N = necrotic cells.

Selecting an Apoptosis assay

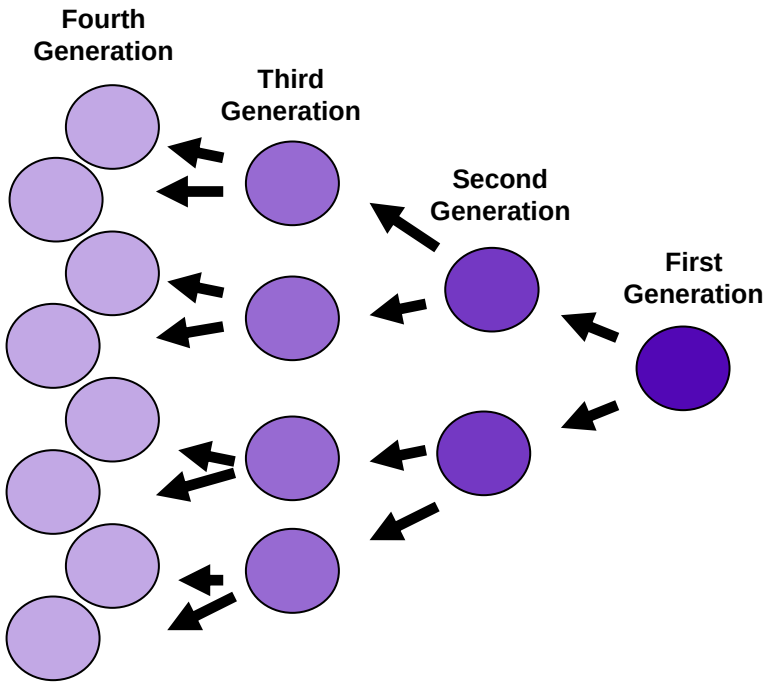
- Apoptosis is a variable process. It can differ greatly between cell types and even within the same cell type with different modes of induction
- Select assays that are suitable for your model system, which may mean trying a few
- Combine multiple assays of apoptosis together to help elucidate the apoptotic process. The multiparametric nature of flow cytometry is ideal for this!
- Perform time courses to track the progression of cells through apoptosis

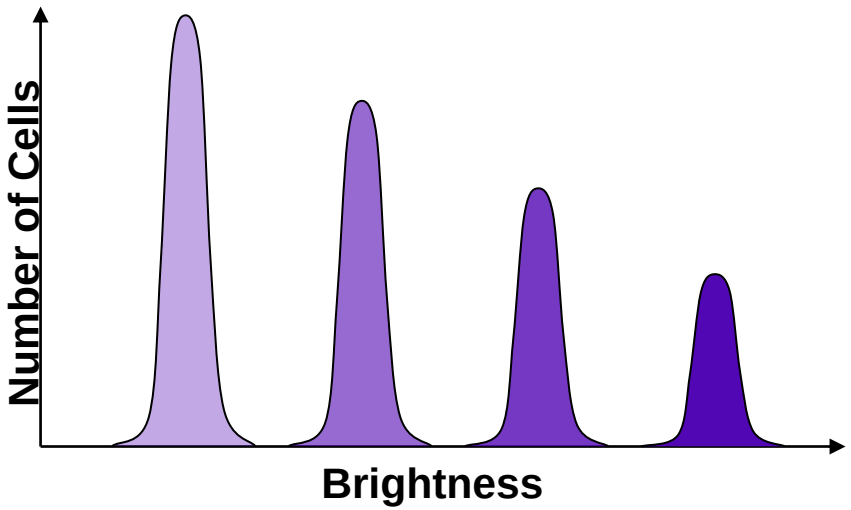
Proliferation: Dye Dilution



Cell Proliferation Analysis by Dye Dilution

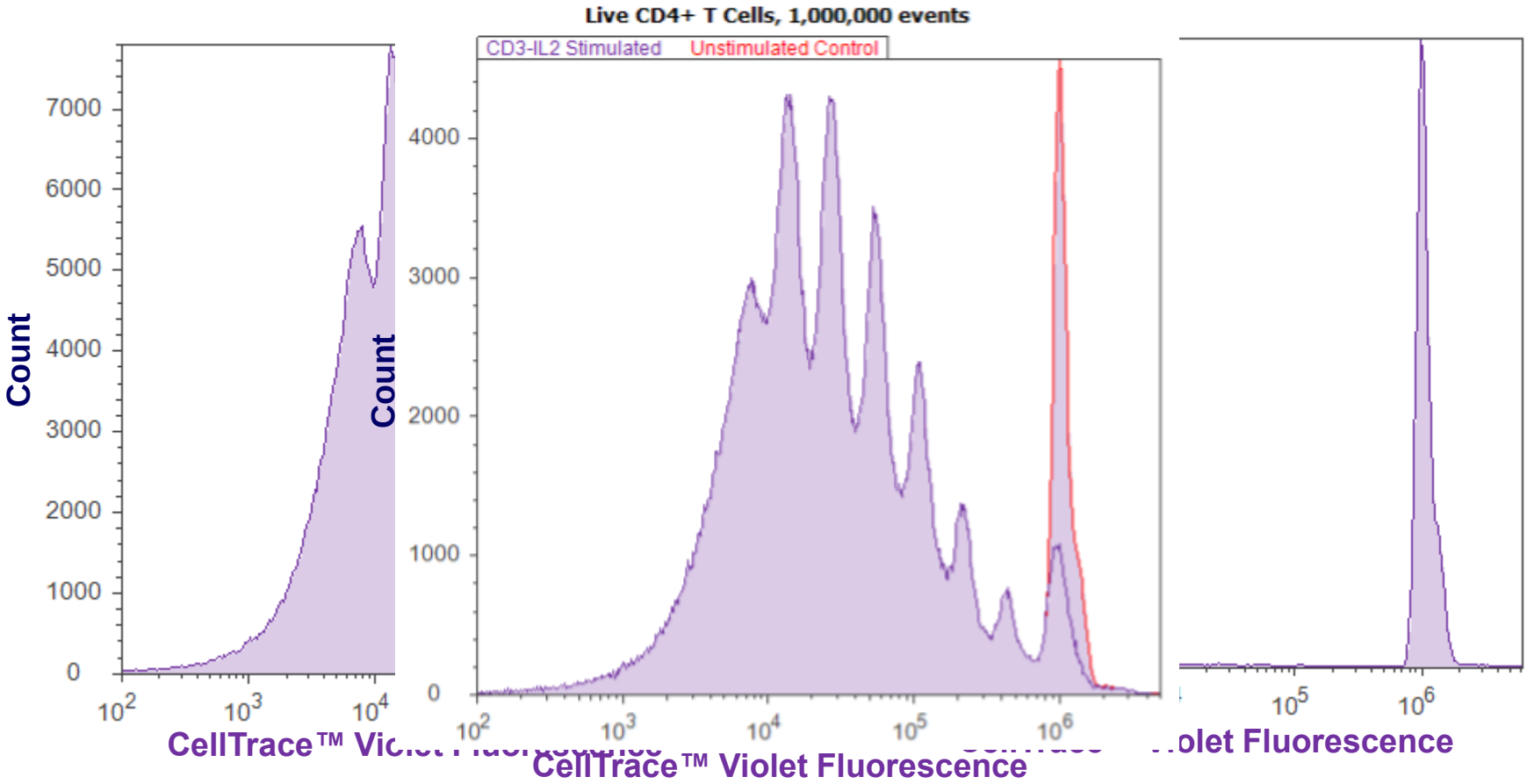
- Cell permeant, fluorogenic dyes give bright homogenous fluorescence that is well retained compound
- Cell division results in equal partitioning of dye between daughter cells
- Fluorescence of daughter cells is half that of parent cell



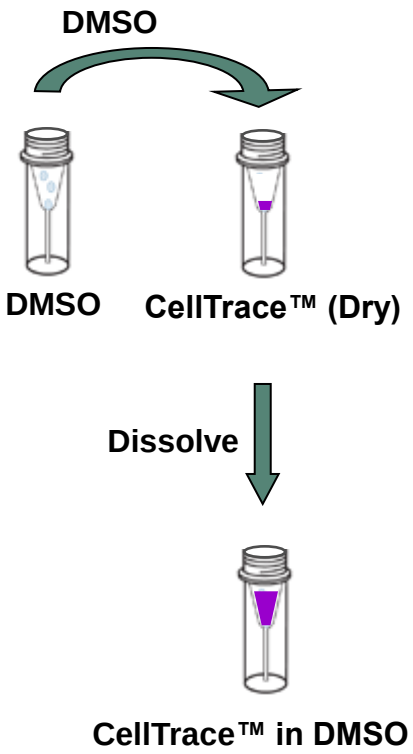


Cytometric Analysis

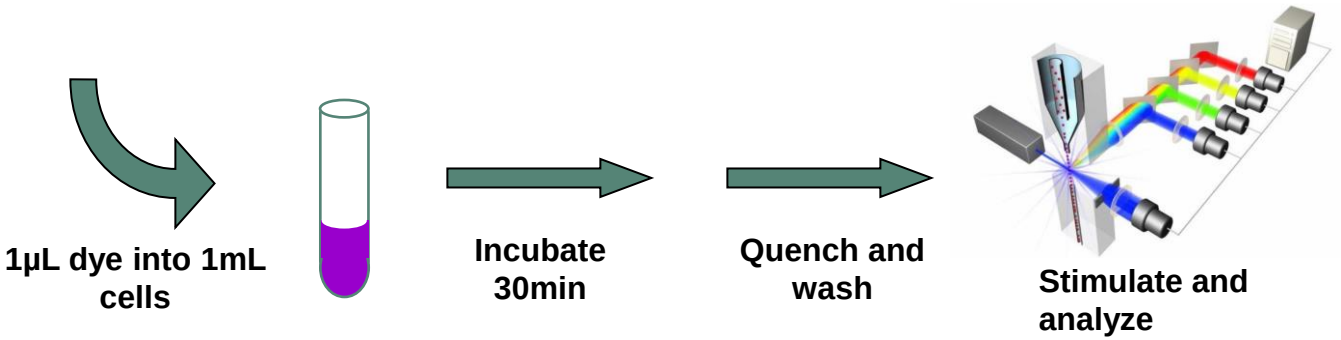
Including an unstimulated control helps determine the fluorescence of undivided cells



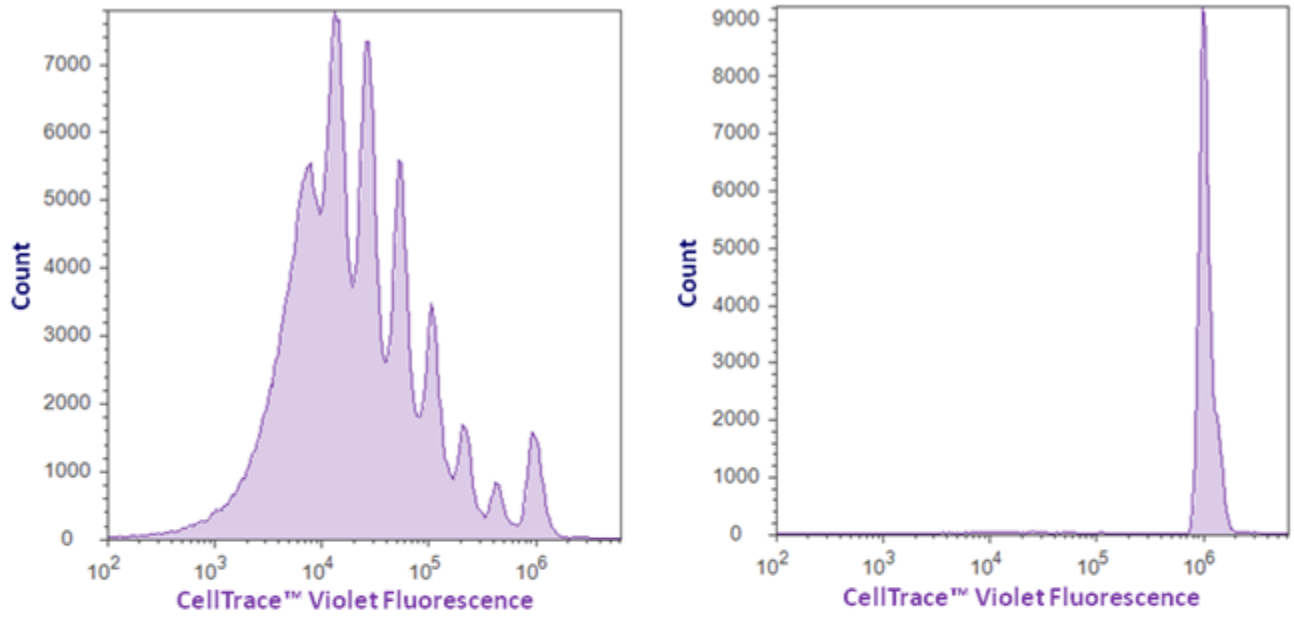
CellTrace™ Experimental Protocol



1. Bring a vial of CellTrace™ dye to room temperature.
2. Add anhydrous DMSO to prepare a stock solution.
3. Add 1 µL of stock solution to 1 mL cells for final concentration
4. Incubate 30 minutes.
5. Quench and wash.
6. Proceed with stimulation and analysis.

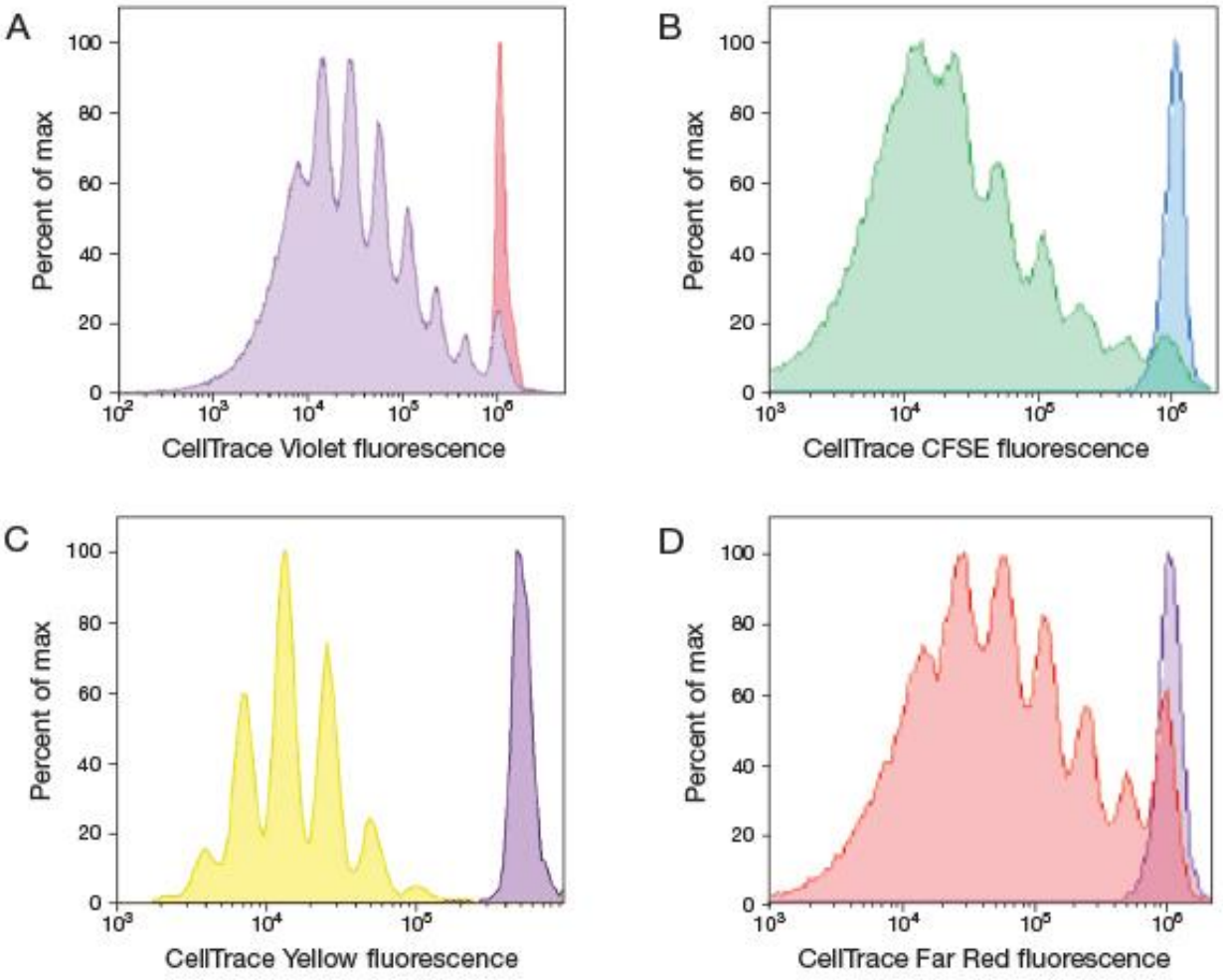


CellTrace™ Violet: Generational Analysis



Including a sample of cells that has been labeled with CellTrace™ Violet but not stimulated provides a valuable control for data analysis and is often required for proper proliferation analysis using modeling software. This control permits the identification of the parent generation of proliferating cells. Both figures represent human PBMCs labeled with and grown in culture for 7 days. **(A)** Cells stimulated with mouse anti-human CD3 and hIL-2 prior to the 7-day incubation. **(B)** Unstimulated cells (non-proliferating) labeled with 10 µM CellTrace™ Violet, provide a clear reference point for the fluorescence intensity of the parent generation of cells. Both of these figures were gated on live, CD4 positive cells. Data acquired on the Attune® Acoustic Focusing Cytometer using a 200 µL/min collection rate in Standard mode.

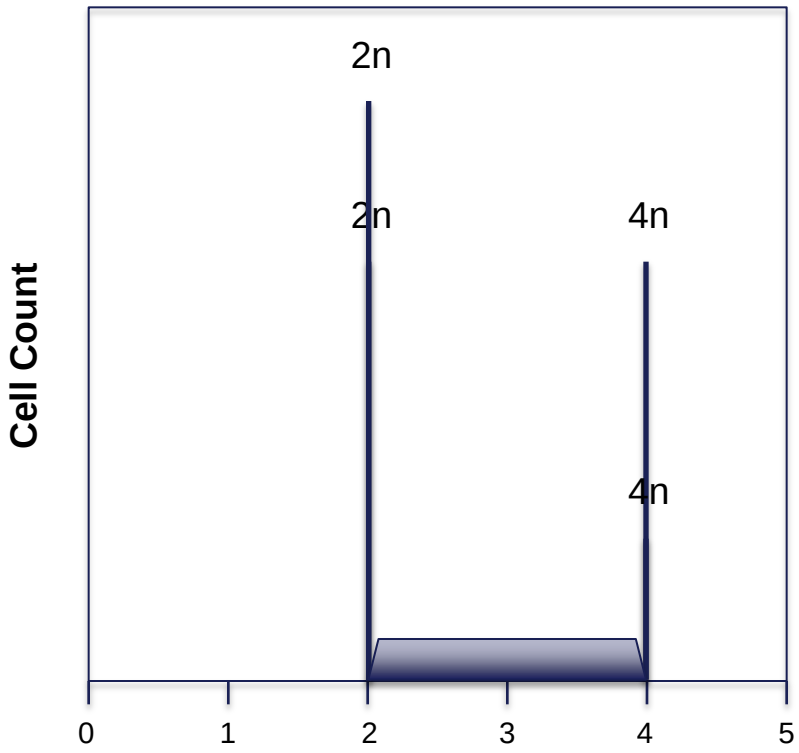
Expanding Choice for Cell Tracing Applications



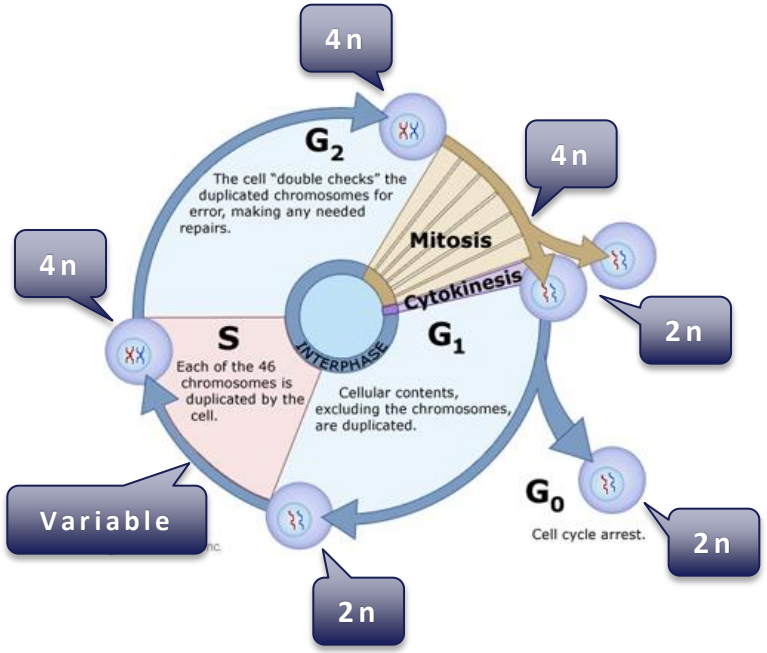
Proliferation:

DNA Content & Cell Cycle

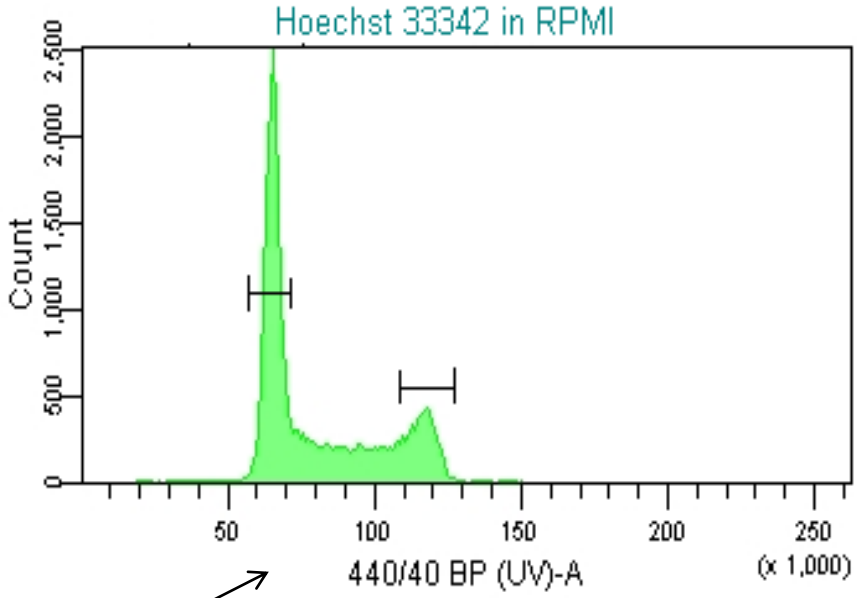
Fluorescent Signal ∝ DNA Content



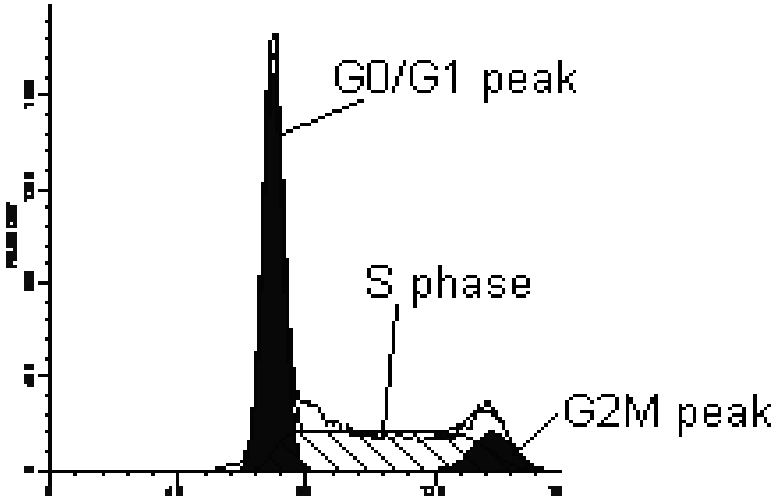
Vybrant™ DyeCycle™ Violet Fluorescence



Frequency Histogram showing DNA content distribution



Note linear scale



Live Jurkat cells stained with Hoechst 33342

Frequency distribution histogram & software deconvolution

Abundance of nucleic acid dyes

- Propdium iodide
- Ethidium Bromide
- 7-AAD
- Ethidium homodimers
- Monomeric cynanines (YO-PRO®-1, TO-PRO®-3)
- Dimeric cynanines (POPO™-1, YOYO® -1)
- SYTOX® dyes
- SYTO® dyes
- DAPI
- Hoechst dyes
- Vybrant® DyeCycle™ stains

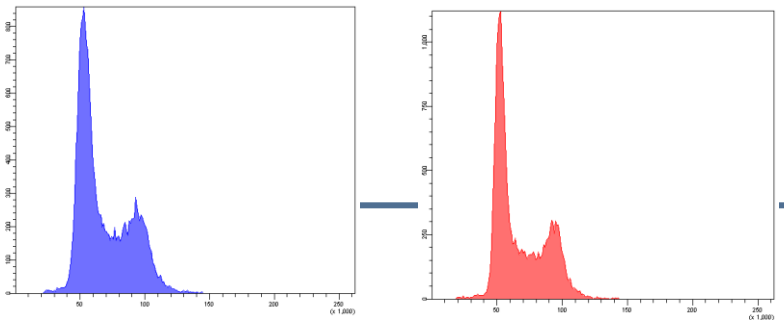
Abundance of nucleic acid dyes

- Propdium iodide
- Ethidium Bromide
- 7-AAD
- Ethidium homodimers
- Monomeric cynanines (YO-PRO®-1, TO-PRO®-3)
- Dimeric cynanines (POPO™-1, YOYO® -1)
- SYTOX® dyes
- SYTO® dyes
- DAPI
- Hoechst dyes
- Vybrant® DyeCycle™ stains

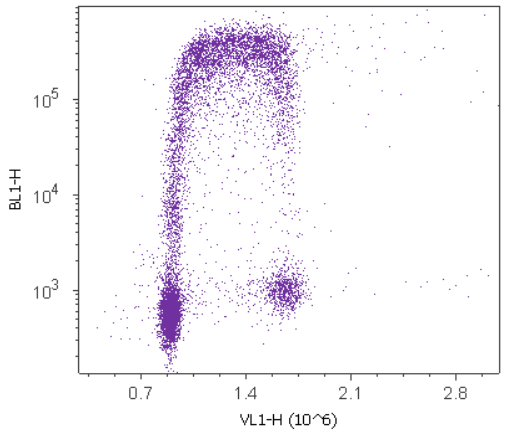
Cell-Permeant Nucleic Acid Dyes

- Dyes which have the ability to penetrate an intact cell membrane to stain nucleic acid
- Used for determining the DNA content of *viable* cells.
- Allows resolution of cell cycle information against the dynamic background of living cells

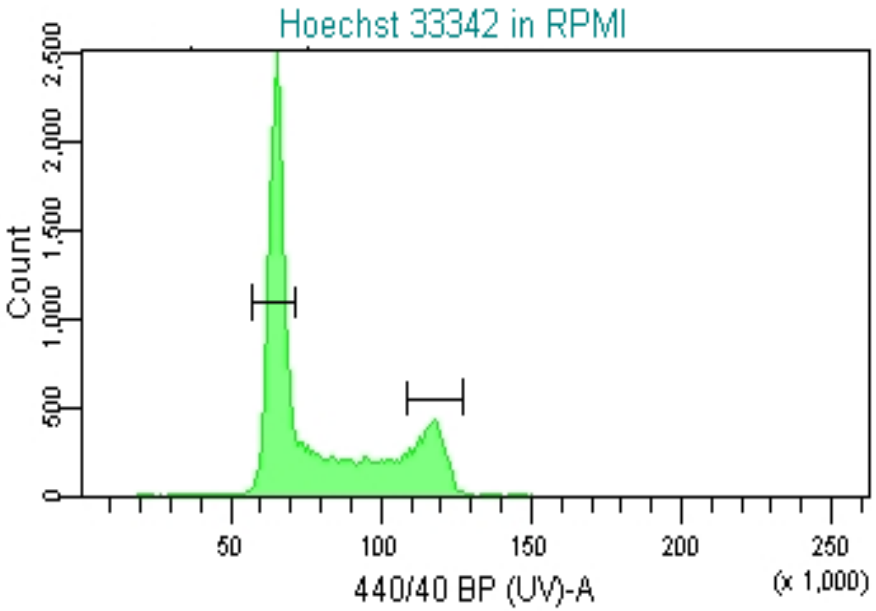
• **Hoechst dyes (UV ex)**	**dsDNA(A-T)**
• **Vybrant® DyeCycle™ Violet stain (UV, 405 ex)**	**dsDNA**
• **Vybrant® DyeCycle™ Green stain (488 ex)**	**dsDNA**
• **Vybrant® DyeCycle™ Orange stain (488 & 532/561)**	**dsDNA**
• **Vybrant® DyeCycle™ Ruby stain (488–633 ex)**	**dsDNA**

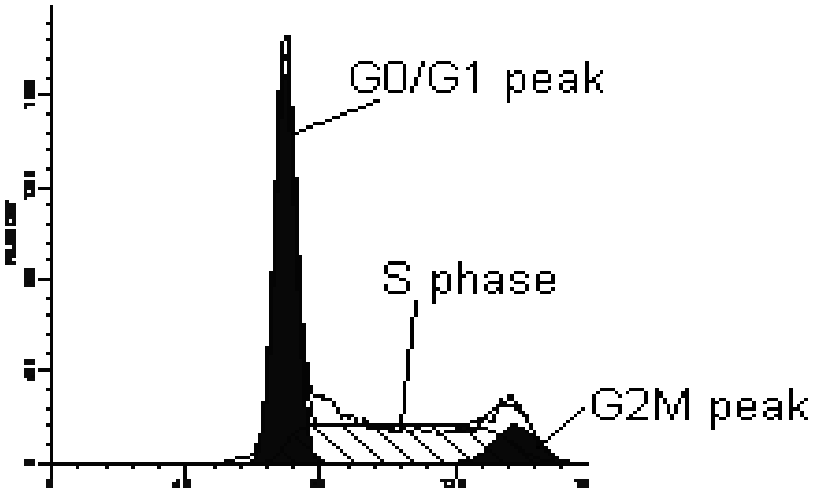


Proliferation: Click-iT® EdU

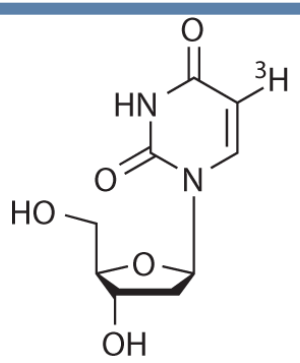


Frequency Histogram--DNA content distribution





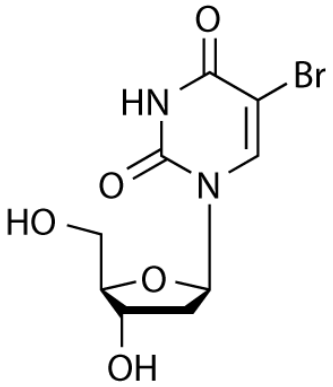
Thymidine Analogs for Measuring S-phase



^{3}H-thymidine

-Radioactive

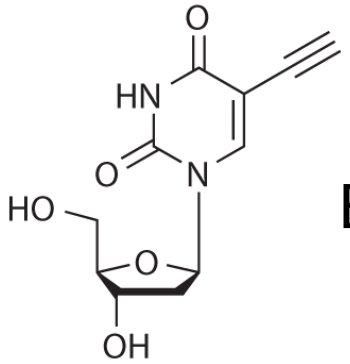
-Cannot multiplex



BrdU

-Requires DNA denaturation for detection with antibody

-Cell cycle stains require dsDNA

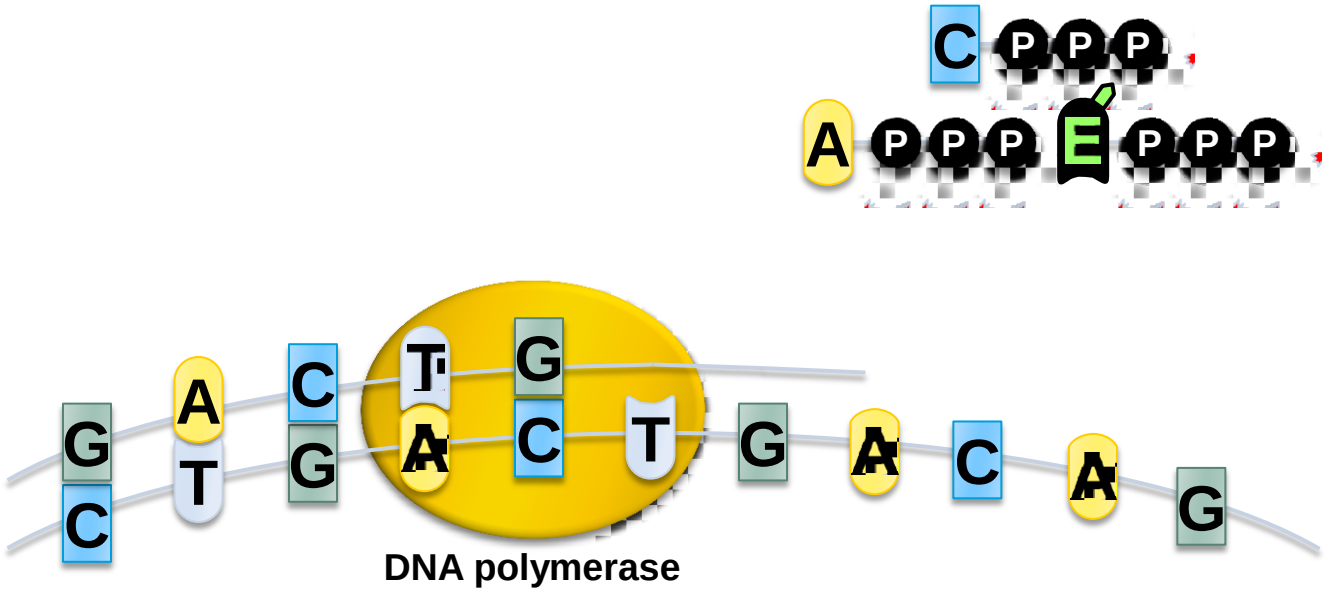


EdU

-No DNA denaturation required for detection

-Multiplex compatible – including antibodies and stains for cell cycle analysis

Metabolic labeling with Click-iT® Chemistry



Buck et al (2006) Biotechniques

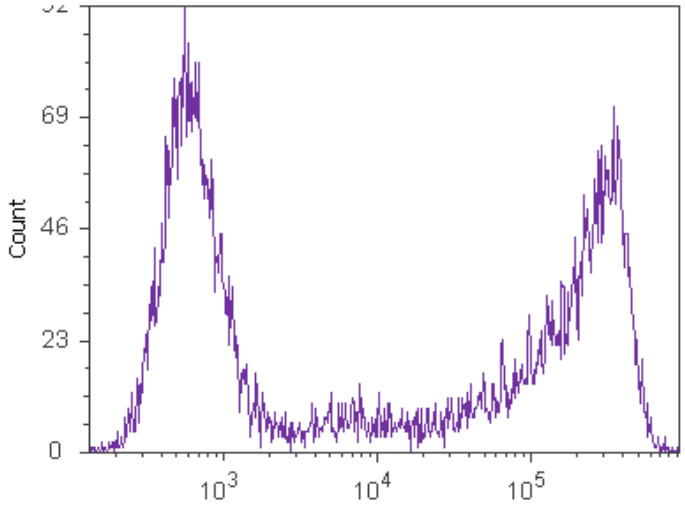
Click-iT® EdU cell proliferation: Flow Cytometry

Simplified Workflow

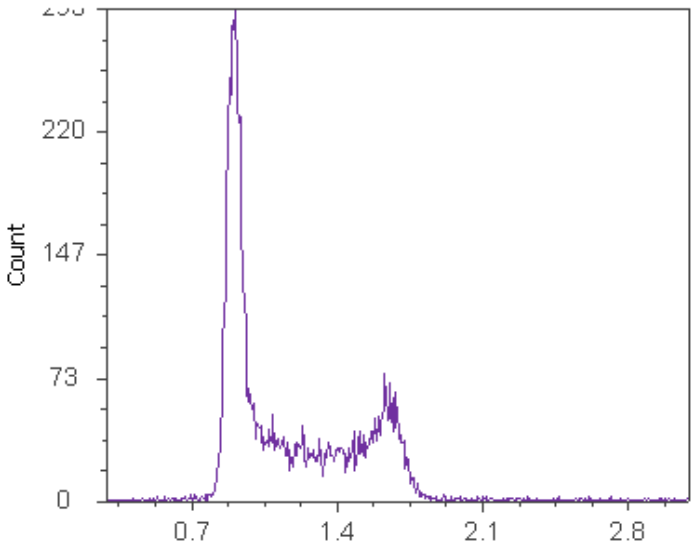
Click-iT® EdU follows a basic protocol of aldehyde fixation and detergent permeabilization

- **Fix for 15 minutes, wash**
- **Permeabilize for 30 minutes, wash**
- **Incubate in click labeling mixture for 30 minutes, wash**
- **Optional: Incubate with cell cycle stain for 15-30 minutes**
- **Analyze**

Attune® Acoustic Cytometer with Click-iT ® EdU Alexa Fluor® 488 and FxCycle™ Violet

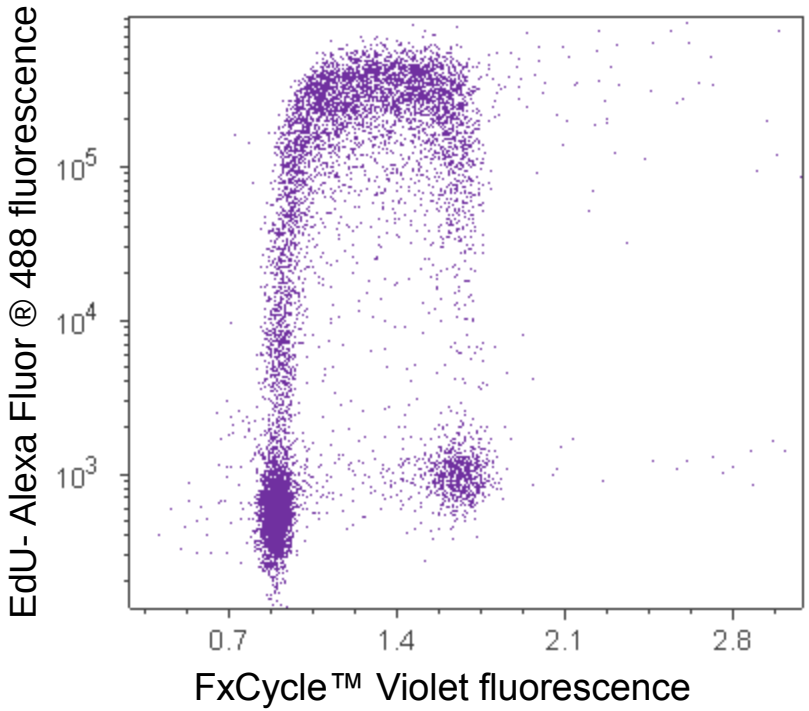


EdU- Alexa Fluor® 488 fluorescence



FxCycle™ Violet fluorescence

Collected at 100 µl/min



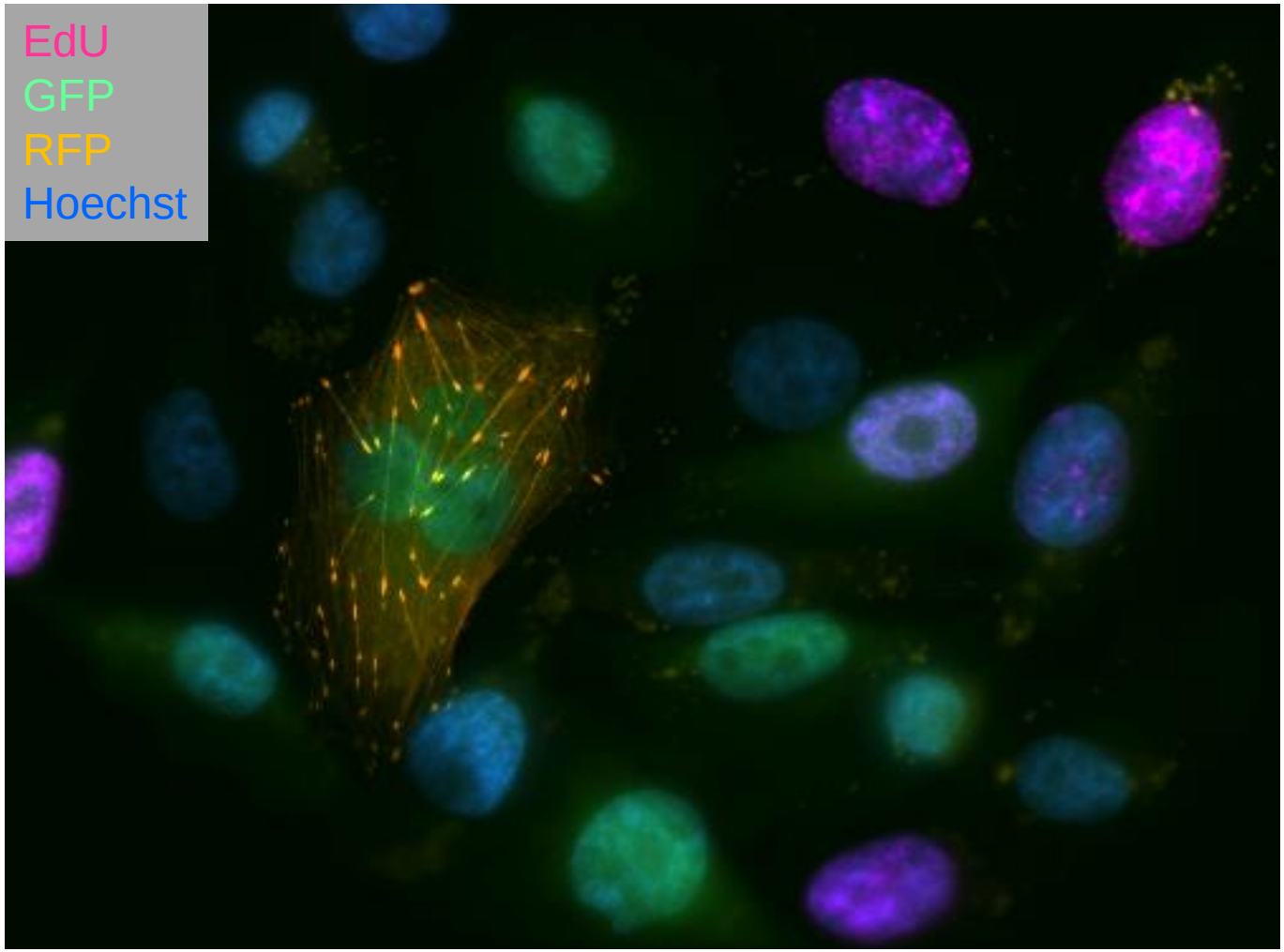
FxCycle™ Violet fluorescence

molecular probes® invitrogen

Click-iT® EdU Comparison

	Classic Click-iT	Click-iT Plus
Fluorophores	AF488, AF647, Pacific Blue azides	AF488, AF647, Pacific Blue picolyl azides
Signal intensity	Bright	Bright (AF647) or brighter (AF488 and Pacific Blue)
Reaction rate	Fast	As fast or faster
Workflow	~3 hours	<3 hours
Q-dot compatible	No	No
R-PE compatible	Post click staining	YES
R-PE tandem compatible	Post click staining	YES
PerCP compatible	YES	YES
APC compatible	YES	YES
GFP compatible	No	YES
mCherry	YES	YES
Brilliant Violet dyes	YES	YES

Click-iT® Plus EdU Alexa Fluor® 647 compatible with GFP and RFP



ERK2 A375 GFP expressing cells (**green**)

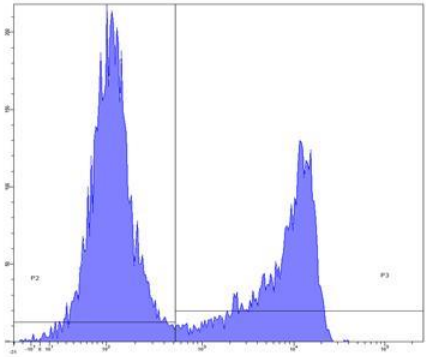
Click-iT® Plus EdU Alexa Fluor® 647 Imaging Kit (**pink**),

CellLight® Talin-RFP *BacMam 2.0* (C10611) (**orange**)

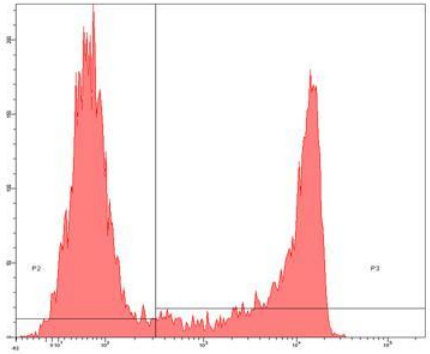
and Hoechst® 33342 (**blue**).

Click-iT® Plus EdU Detection Reagents

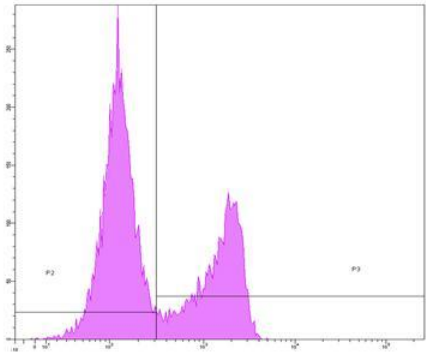
- Enables multicolor experiments



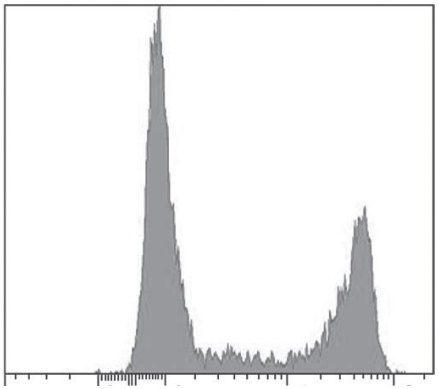
Alexa Fluor® 488 azide
Blue laser



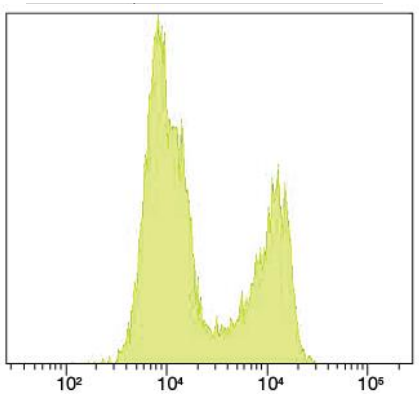
Alexa Fluor® 647 azide
Red laser



Pacific Blue™ azide
Violet laser



Alexa Fluor® 350 azide
UV laser

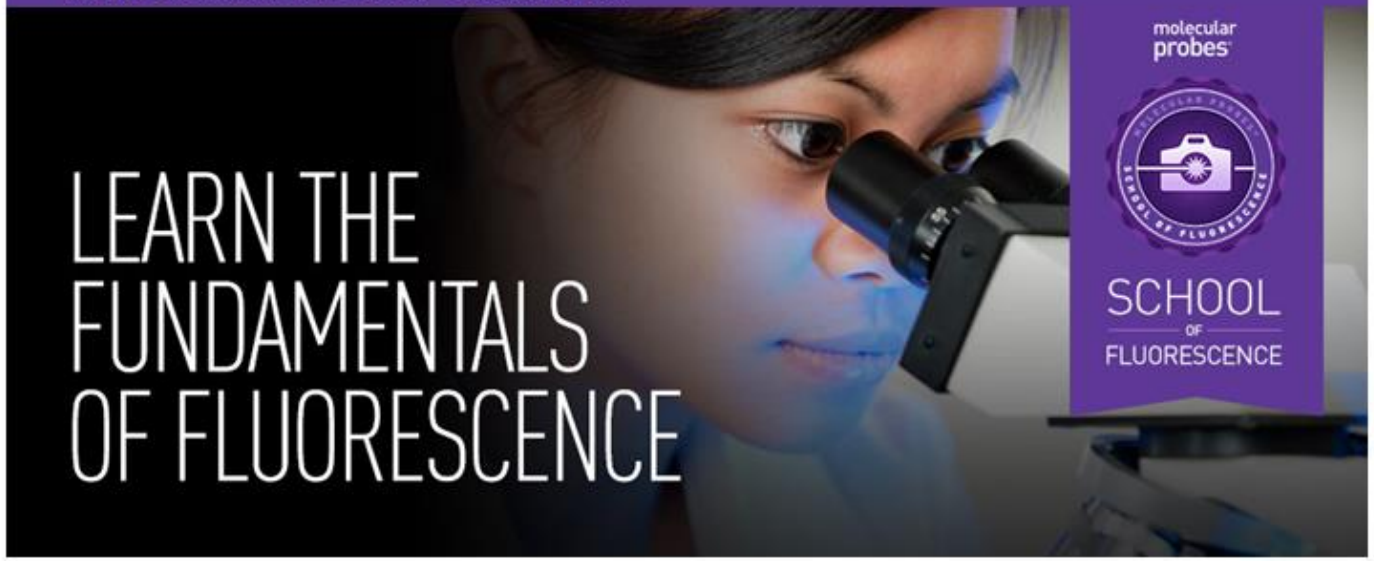


Alexa Fluor® 594 azide
Green or Yellow laser

Click-iT Plus EdU Compatibility

- ✓ Fluorescent proteins
- ✓ PE and PE tandems

Molecular Probes® School of Fluorescence



Fundamentals | Sample considerations | Labeling your samples | Capturing & analyzing samples | Protocols & troubleshooting

Fluorescence basics

- Understanding fluorescence
- Microscope basics
- Match fluorophores to filters
- Working with objectives

Preparing your samples

- Choosing a cell type
- Preparing fixed cells
- Live-cell imaging
- Choosing a vessel

Ways to label samples

- Intro to fluorescence labeling
- Labeling using fluorescence proteins
- Immunofluorescence labeling
- Choosing mounting media

How-tos & help

- Deciding your exposure time
- Image analysis & quantitation
- Imaging protocols
- Background, photobleaching, and other problems

Flow cytometry mobile app

Compatible with iPhone (requires iPhone 4 or 4S) and Android devices.

Available on the App Store

Download for Android

Cell Imaging mobile app

3D Cell mobile app

SpectraViewer mobile app

Flow Cytometry Resource Center

Fluorescence Spectraviewer

Webinars on Flow and Imaging Cytometry

Flow Cytometry Tutorials

Connect with Molecular Probes®

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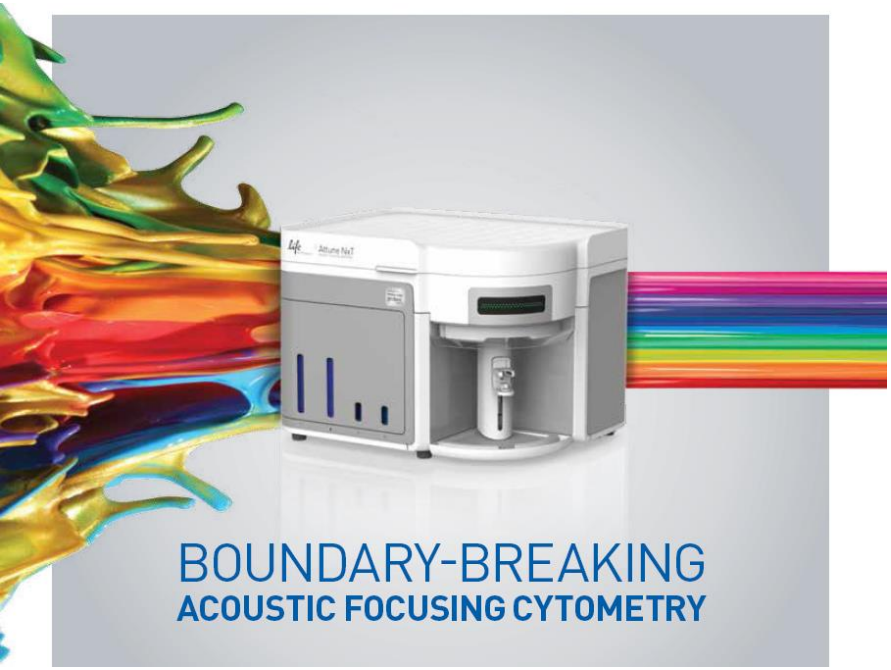
Follow us on Twitter

Watch us on YouTube



Attune NxT
Acoustic Focusing Cytometer
And
No-Lyse no-Wash Assays

Attune® NxT Acoustic Focusing Cytometer

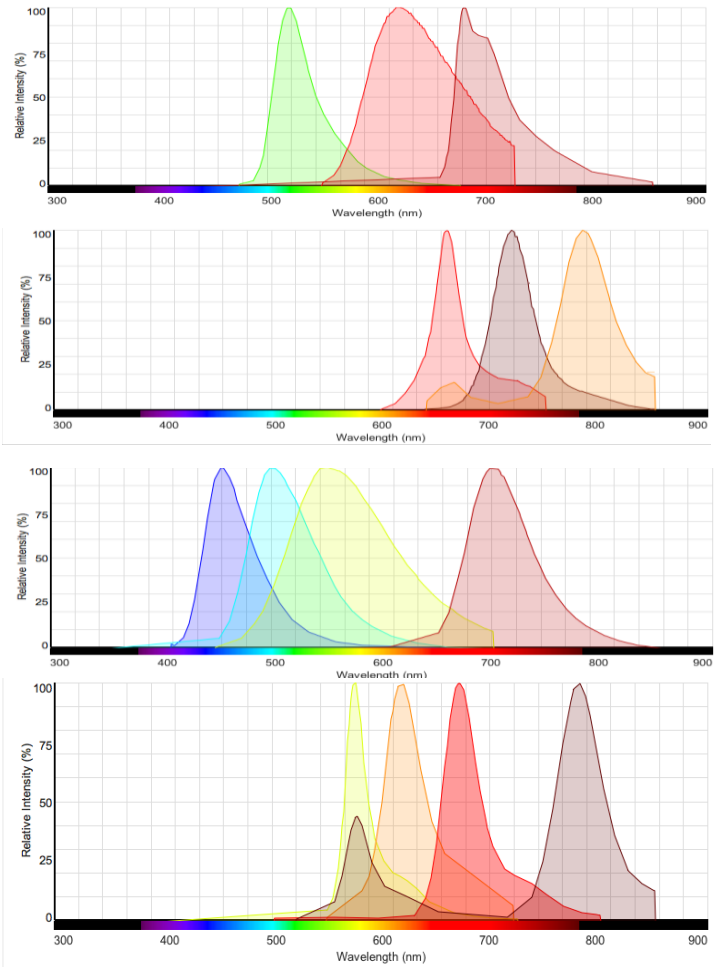
Small in Size/Big in Performance

- **Footprint (H x W x D):**
  - 16 in × 23 in × 17 in
  - 40 cm × 58 cm × 43 cm
- **Weight:**
  - 29 kg (64 lb)
- **Electrical requirements:**
  - 100–240 VAC, 50/60 Hz, <150 W

Expanded Access to Reagents

Yellow Laser Present

Laser	Intended Dye
Blue	FSC
	SSC
	FITC
	PI
	PerCP-Cy™5.5
Red	APC
	Alexa Fluor® 700
	APC-Alexa Fluor®750
Violet	Pacific Blue™
	Pacific Green™
	Pacific Orange™
	Qdot® 705
Yellow	PE
	PE-Texas Red™
	PE-Cy™5.5
	PE-Cy™7



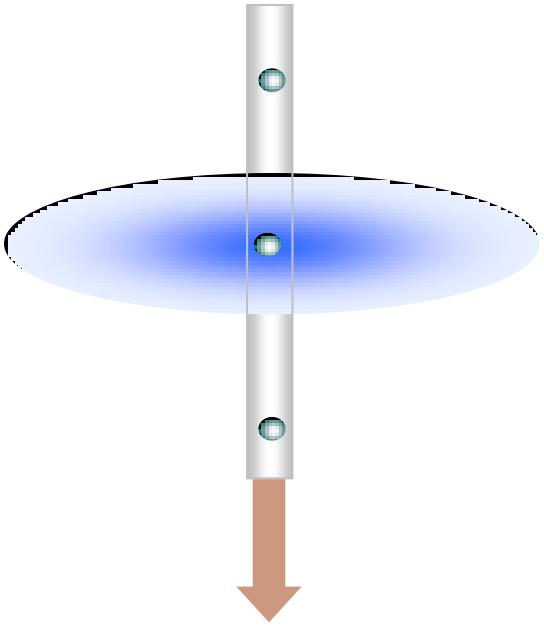
Attune® NxT Acoustic Focusing Cytometer

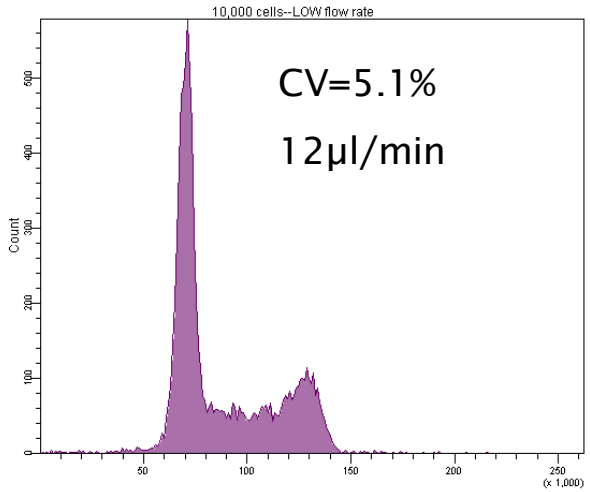
Modular Design

- **Lasers:**
  - Choose from 1-4 Lasers
- **Detection Channels:**
  - FSC, SSC
  - Up to 16 Detection Channels
- **Autosampler**
  - 96 well/384 well capabilities
  - Walk-away automation

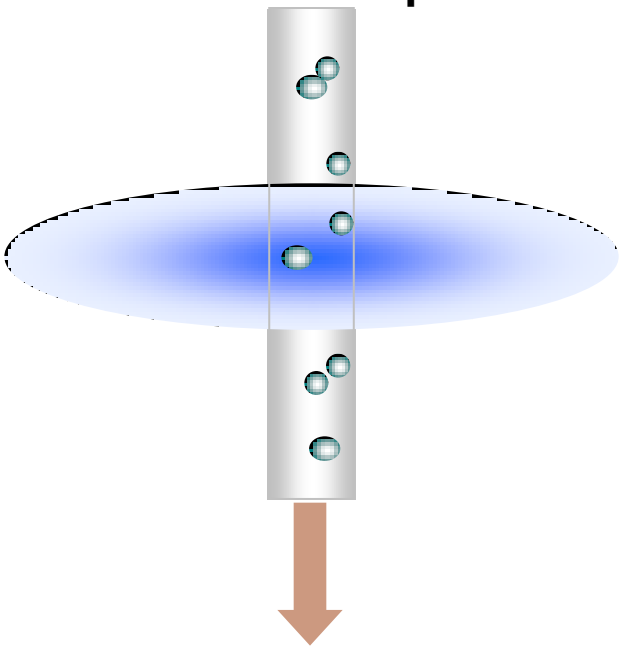
Flow Rate: Hydrodynamic Focusing

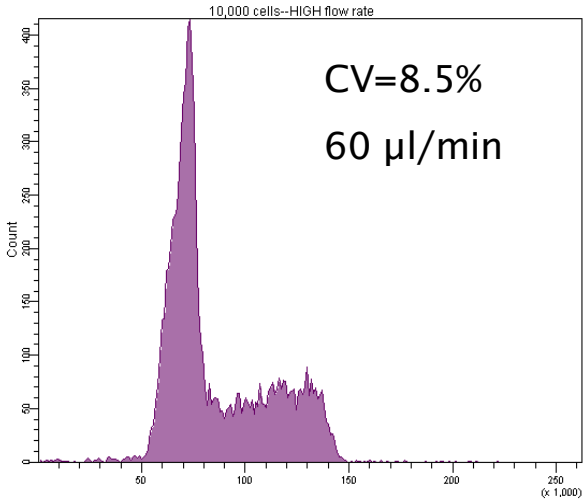
Narrow Sample Stream: Low Flow Rate-12 µL/min





Wide Sample Stream: High Flow Rate 60 µL/min





Traditional Hydrodynamic Focusing

Particle positioning in laser is important

Low Sample Flow Rate (e.g. 12 ul/min)

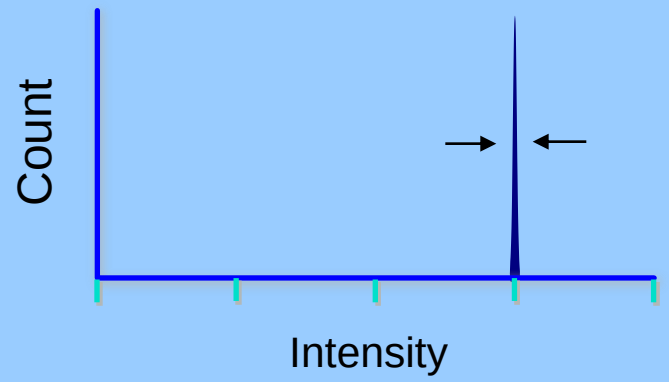
Hydrodynamic core

Focused laser

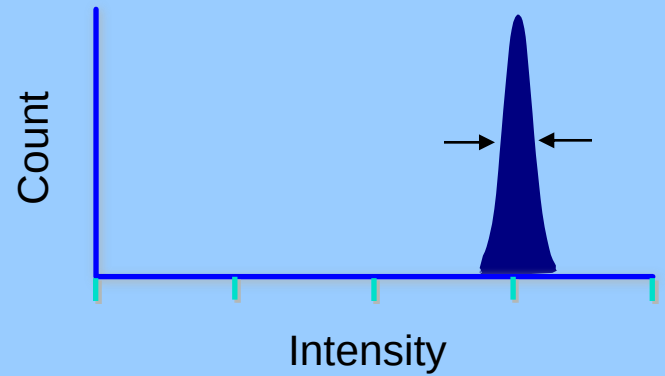
High Sample Flow Rate (e.g. 200 ul/min)

Focused laser

Narrow particle focus = Narrow distribution

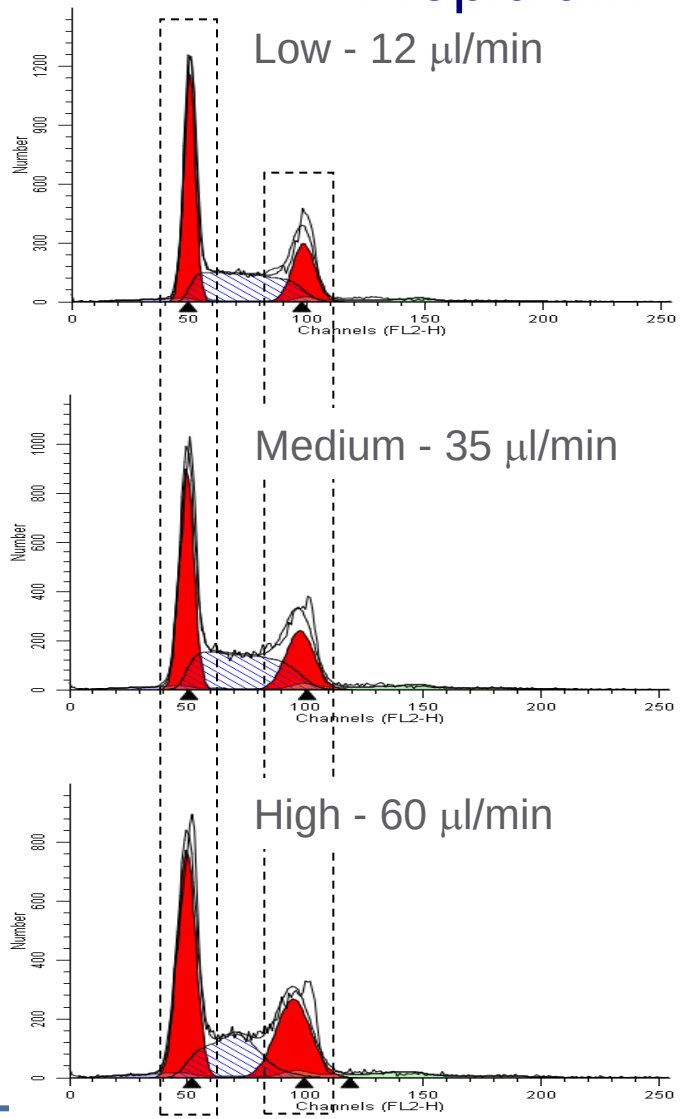


Broad particle focus = Broad distribution



Hydrodynamic Focusing Instrument Cell Cycle Data

Propidium Iodide labeled Jurkat cells



As Sample Rates Increase

Increase of CV and changes in data

G0G1: 41.73%
- **CV: 4.83%**

G2M: 20.44%
- **G2/G1: 1.96**

S-Phase: 37.83%

G0G1: 40.16%
- **CV: 6.12%**

G2M: 20.89%
- **G2/G1: 1.96**

S-Phase: 38.95%

G0G1: 44.60%
- **CV: 7.76%**

G2M: 29.23%
- **G2/G1: 1.90**

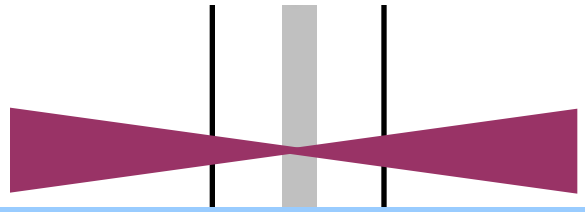
S-Phase: 26.17%

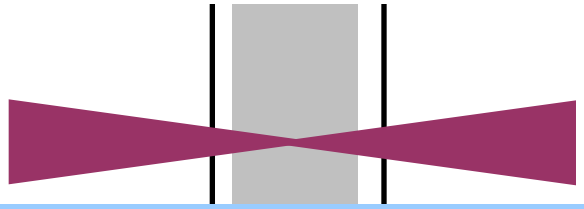
Acoustic Focusing

High sample input flow rates allow for more sample flexibility

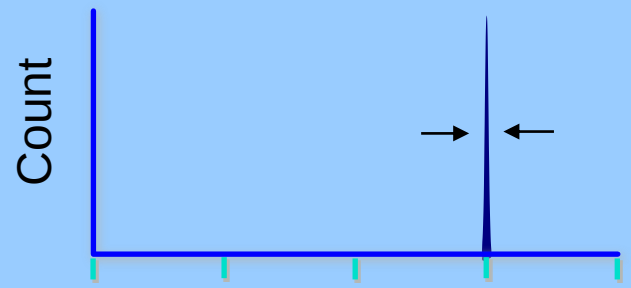
12.5 µL/min

1000 µL/min

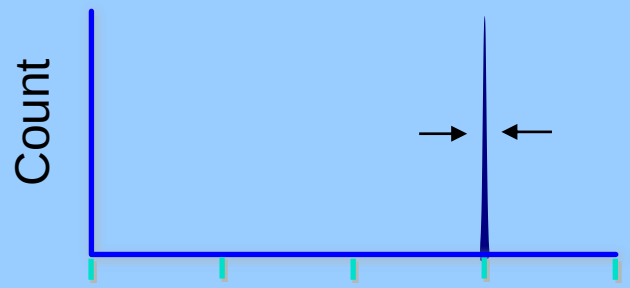


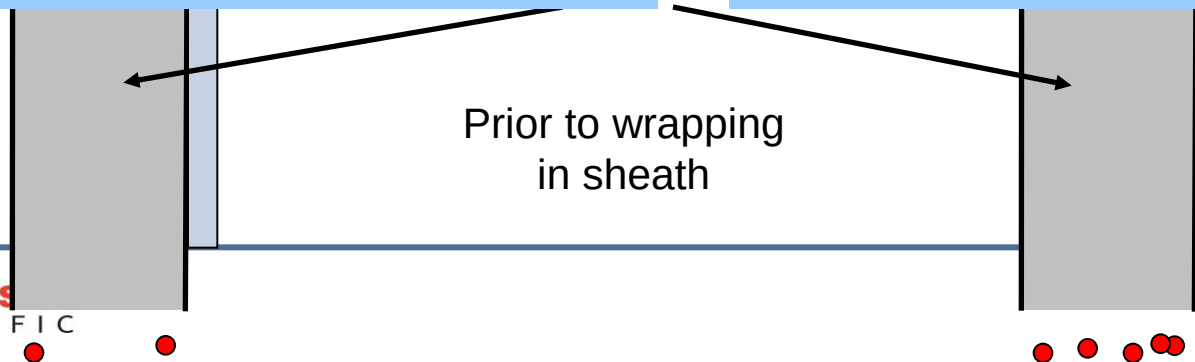


Narrow particle focus = Narrow distribution



Narrow particle focus = Narrow distribution

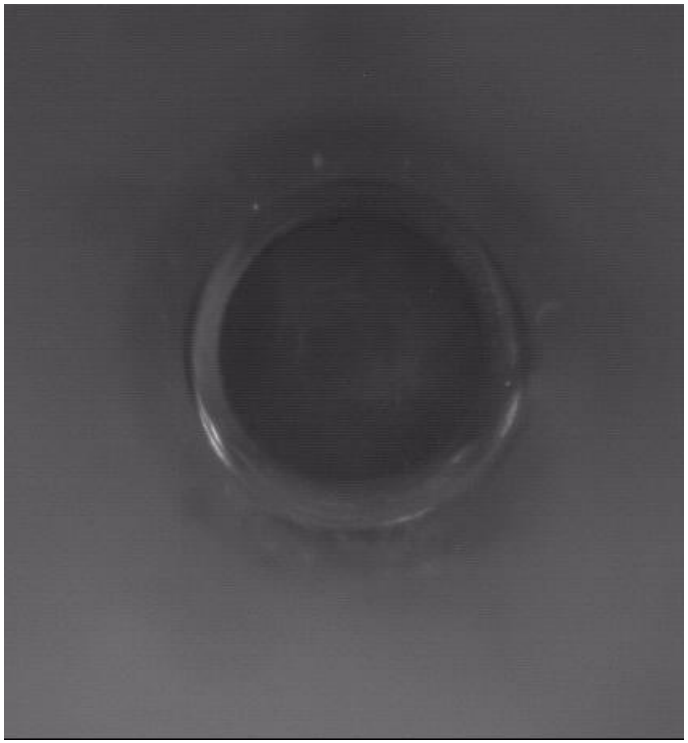




Prior to wrapping in sheath

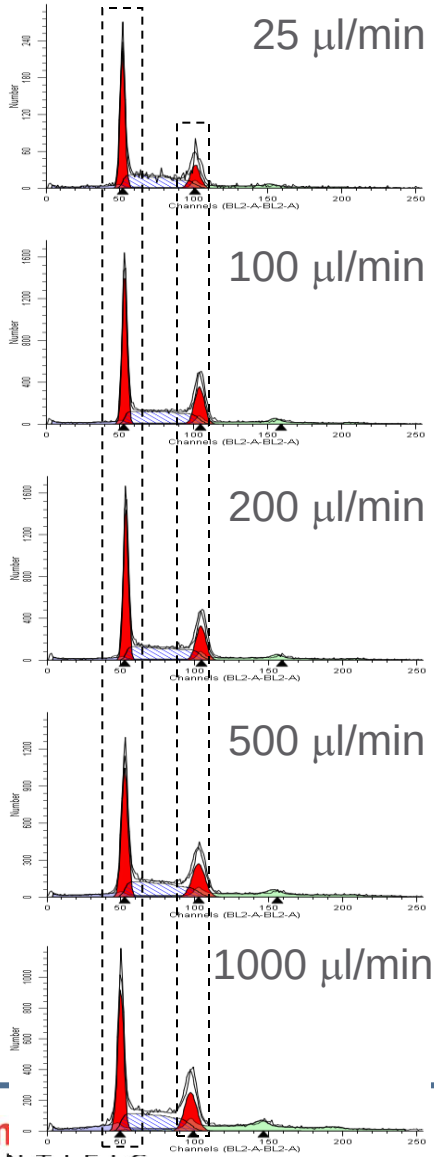
Acoustic Focusing

End-on view of capillary



Attune® Acoustic Cytometer Cell Cycle Data

Propidium Iodide labeled Jurkat cells



As collection rates Increase:

Consistent CV

Consistent data quality

Much higher collection speeds

G0G1: 45.20 %
- CV: 3.22 %
G2M: 14.51 %
- G2/G1: 1.94
S-Phase: 40.29 %

G0G1: 41.97 %
- CV: 3.16 %
G2M: 19.86 %
- G2/G1: 1.94
S-Phase: 38.18 %

G0G1: 42.81 %
- CV: 3.17 %
G2M: 18.47 %
- G2/G1: 1.94
S-Phase: 38.72 %

G0G1: 41.54 %
- CV: 4.16 %
G2M: 20.79 %
- G2/G1: 1.94
S-Phase: 37.67 %

G0G1: 40.25 %
- CV: 4.21 %
G2M: 21.20 %
- G2/G1: 1.94
S-Phase: 38.55 %

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molecular probes® invitrogen