



Violet Laser Tools in Flow Cytometry

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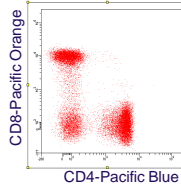
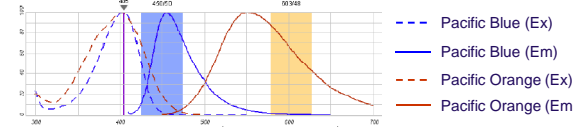
Agenda

- Traditional Dyes
 - Pacific Blue and Pacific Orange antibodies
- Qdot® Nanocrystal Conjugates
 - What are Qdots?
 - Considerations
- Cell Process Violet Dyes
 - Cell Proliferation
 - > CellTrace Violet
 - > Click-IT EdU
 - Cell Viability
 - > LIVE/DEAD Fixable Dead Cell Stain
 - > Nucleic Acid Stains
 - Cell Cycle
 - > Live cell cycle analysis (DyeCycles)
 - > Fixed cell analysis (FxCycle)
 - Multi-parameter Apoptosis



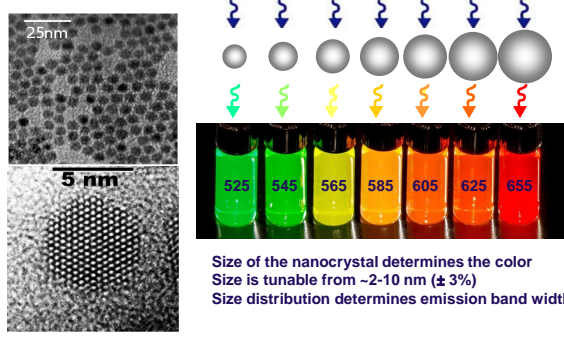
Pacific Blue™ & Pacific Orange™ Dyes

- First dual dye combination on violet laser
 - > Pacific Blue™ (455 nm emission)
 - > Pacific Orange™ (551 nm emission)
- Minimal compensation between dyes
- Pacific Orange uses PE filter (585/42)
- Over 50 human & mouse conjugates available





--- Pacific Blue (Ex)
— Pacific Blue (Em)
--- Pacific Orange (Ex)
— Pacific Orange (Em)

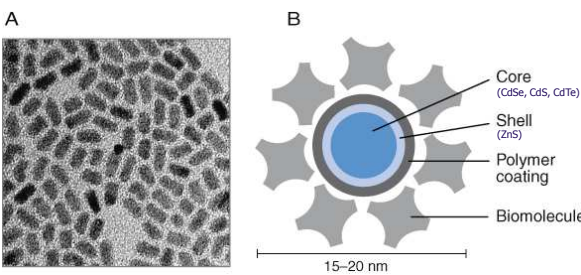
Impactful nanotechnology: Quantum Dots



Size of the nanocrystal determines the color
Size is tunable from ~2-10 nm (± 3%)
Size distribution determines emission band width




Anatomy of a Qdot Nanocrystal



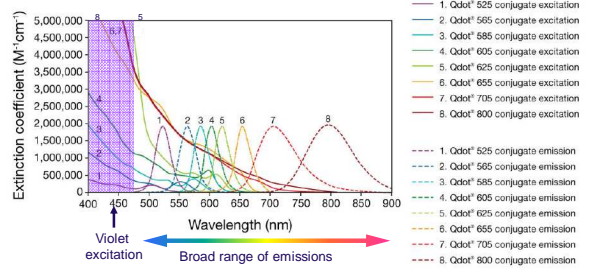
A **B**

- Core (CdSe, CdS, CdTe)
- Shell (ZnS)
- Polymer coating
- Biomolecule


15–20 nm

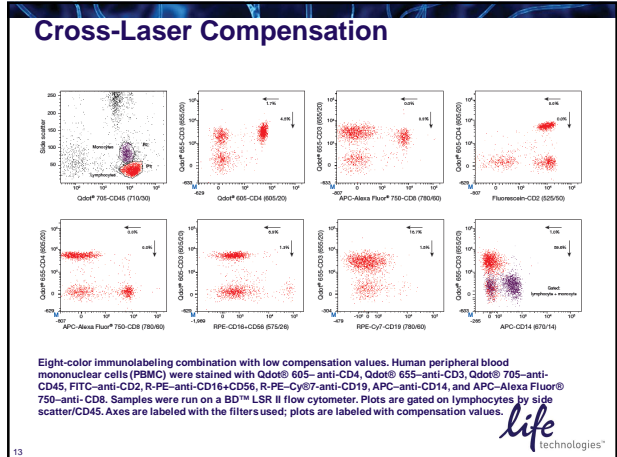


Qdot® nanocrystal optical properties



Single-color excitation, multicolor emission for easy multiplexing





LSR II filter configuration to detect selected Qdot® nanocrystals and conventional fluorophores.

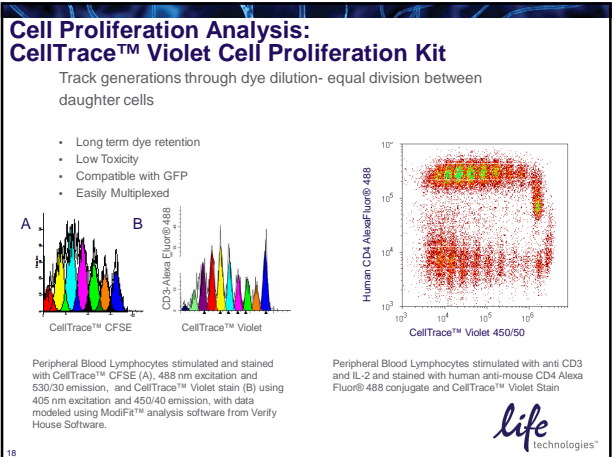
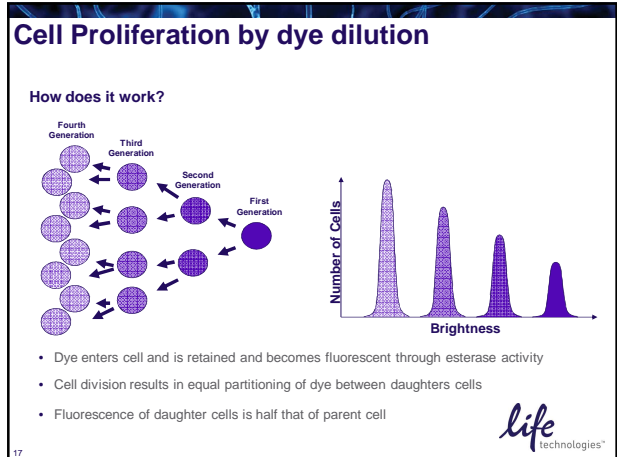
Fluorophore	Excitation laser (nm)	Emission maximum (nm)	LSR II filter configuration
Pacific Blue™ dye	405	565	555LP,565/20
Qdot® 565 nanocrystal	405	565	555LP,565/20
Pacific Orange™ dye*	405	551	555LP,575/26
Qdot® 605 nanocrystal	405	605	570LP,605/20
Qdot® 655 nanocrystal	405	655	640LP,655/20
Qdot® 705 nanocrystal	405	705	690LP,720/20
Qdot® 800 nanocrystal*	405	800	750LP,780/60
Alexa Fluor® 488 dye	488		525/50
R-PE	488		575/26
R-PE-Texas Red® dye*	488		620/10
R-PE-Alexa Fluor® 700 dye*	488		730/30
R-PE-Cy7	488		780/60
Alexa Fluor® 647 dye or APC	633/635		660/20 or 670/14
Alexa Fluor® 700 dye*	633/635		730/30
APC-Alexa Fluor® 750 dye	633/635		780/60

* Fluorophores with spectral overlap challenges.

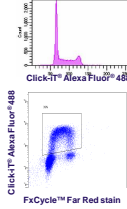
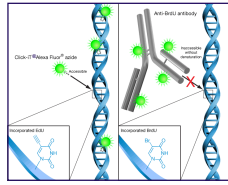
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- ### Factors for Multiplexing
- Fluorophore choice dependent upon:
1. Relative abundance of the targets
 2. Photostability and FRET efficiency with tandems
 3. Spectral separation of the dyes (minimal compensation)
 4. Specificity of dye for target/ minimal background
 5. INSTRUMENTATION!!! Know your instrument
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- ### Agenda
- Traditional Dyes
 - Pacific Blue and Pacific Orange antibodies
 - Qdot® Nanocrystal Conjugates
 - What are they?
 - How to best use them?
 - Cell Process Violet Dyes
 - Cell Proliferation
 - CellTrace Violet
 - Click-iT EdU
 - Cell Viability
 - LIVE/DEAD Fixable Dead Cell Stain
 - Nucleic Acid Stains
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 - Multi-parameter Apoptosis
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Click-iT Cell Proliferation Assay



- Click-iT® Edu steps**
- 1) Add Edu to cells/animal
 - 2) Add detection reagent
 - 3) Analyze

- **Simplified protocol**
 - > No DNA denaturation required
- **Highly specific reaction with minimal background**
 - > Reaction not found in biological systems
- **Easier multiplexing**
 - > Multiple colors options for all common lasers
 - > No harsh treatments makes combined phenotyping more accurate

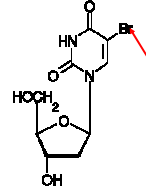
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Edu compared to BrdU

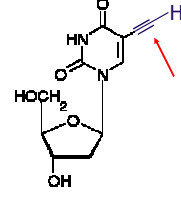
Both are nucleoside analogs of thymidine
and are incorporated into actively synthesizing DNA

BrdU



5-Bromo-2'-deoxyuridine

Edu



5-Ethynyl-2'-deoxyuridine

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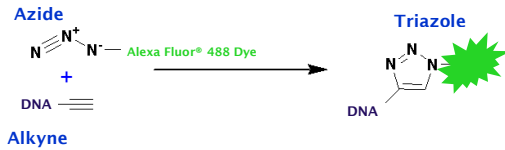
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Just what is "click" chemistry?

Click chemistry is a concept introduced by K. Barry Sharpless in 2001 and describes chemistry tailored to generate substances quickly and reliably by joining small units together as nature does.

Click chemistry-based labeling and detection

Copper catalyzed azide-alkyne cycloaddition



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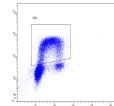
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Click-iT™ Edu Cell Proliferation: Flow Cytometry

Simplified Workflow

Click-iT™ Edu: Basic protocol of aldehyde fixation and detergent permeabilization

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

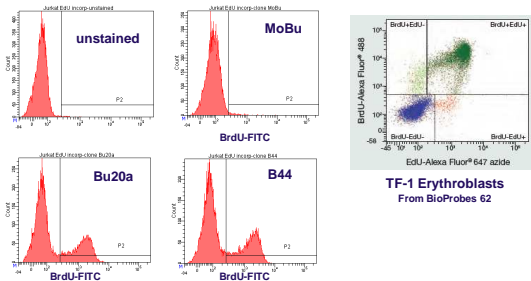


2 Hour Assay Time!

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Edu and BrdU dual labeling



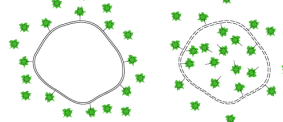
- MoBu clone specific to BrdU, does not react to Edu
- Allows pulse labeling of cells with Edu and BrdU
- MoBu antibody available unconjugated and conjugated to: Pacific Blue™, Alexa Fluor® 488, and Alexa Fluor® 647

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Fixable Dead Cell Stains

LIVE/DEAD® Reduced Biohazard Cell Viability Kit #2
Principal

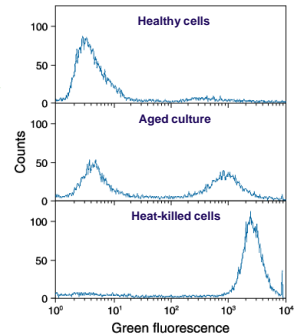


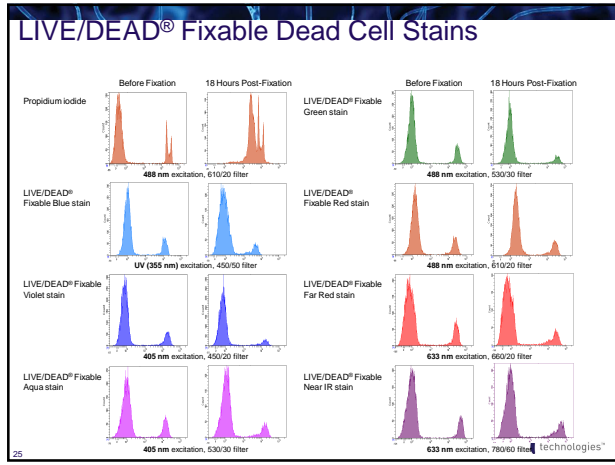
Live cells (left) react with the kit's fluorescent reactive dye only on their surface to yield weakly fluorescent cells.

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

In both cases, the excess reactive dye is subsequently washed away.

LIVE/DEAD® Fixable Green Dead Cell Stain Kit (L23101)



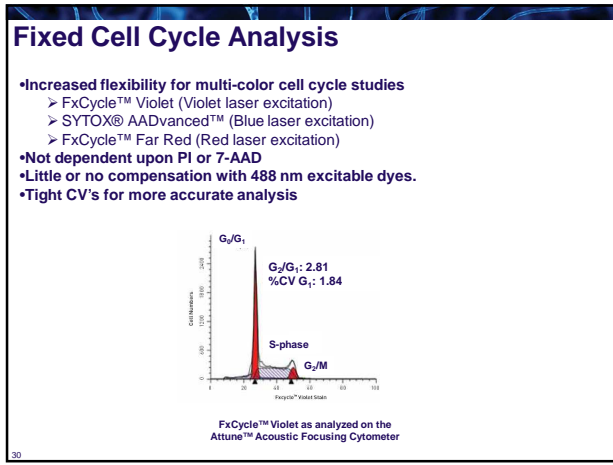
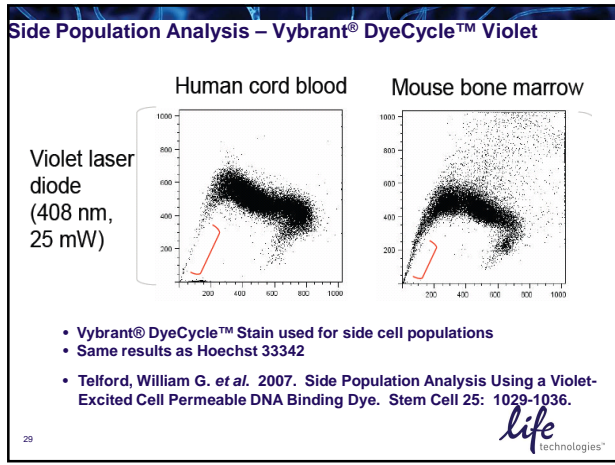
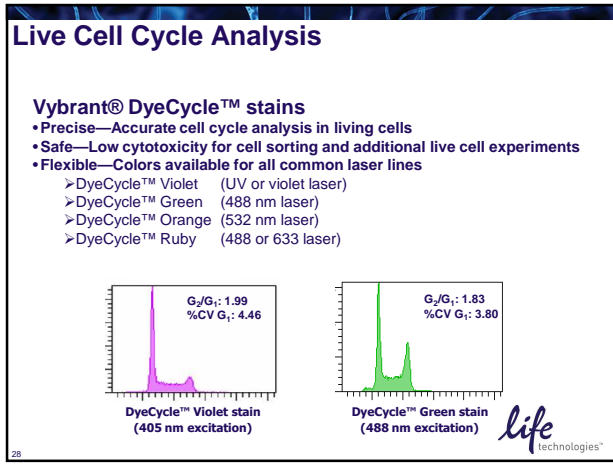
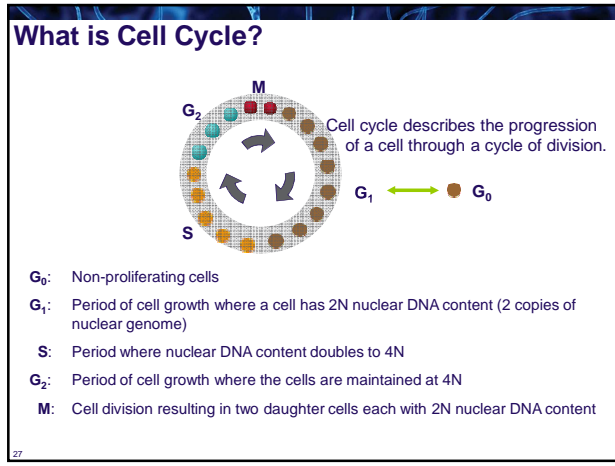


Nucleic Acid Stains for viability

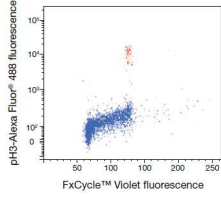
	Name	Ex. Max.	Em. Max	Ex. Laser	Filter	Notes
Traditional Dyes (DNA Dyes)	SYTOX® Blue stain	445 nm	470 nm	405 nm	450/40	*Violet laser NA viability stain
	SYTOX® Green stain	504 nm	523 nm	488 nm	530/30	
	Propidium iodide	535 nm	617 nm	488 nm	610/20	*Commonly Used *Uses two emission channels
	7-AAD	546 nm	647 nm	488 nm	650BP or 670BP	*Requires 30 minute staining
	SYTOX® AADvanced™ stain	546 nm	647 nm	488 nm	650BP or 670BP	*7-AAD Replacement *Requires 5 minute staining
	SYTOX® Red stain	640 nm	650 nm	633 nm	660/20	*Replacement for PI (frees 488 laser) *Uses APC channel!

Principle:
Dyes can enter cells with compromised plasma membranes (dead cells) and bind to nuclear dsDNA

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Multiplexability with FxCycle

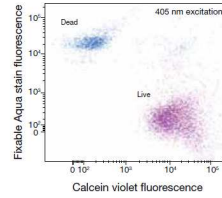


- TF-1 erythroblast cells were alcohol-fixed overnight, washed, and then suspended in 0.1% Triton® X-100/PBS/1% BSA before staining with anti-histone H3[pS10] purified antibody (Cat. No. 441190G) complexed with Zenon® Alexa Fluor® 488 Rabbit IgG labeling reagent and FxCycle™ Violet Stain. The pH3 signal (red) identifies cells that are in mitosis.



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Live/Dead Viability/Vitality Kit



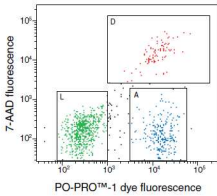
- Two color assay to check Viability and Vitality on the 405 laser
- Calcein Violet AM-vitality
- Live/Dead Fixable Aqua-viability

- Combination of esterase substrates with dead cell dyes using mixtures of heat-treated and untreated cells. Chinese hamster ovary (CHO) cells were stained according to the protocol in the LIVE/DEAD® Viability/Vitality Kit (calcein violet and fixable violet dead cell stain). Cells were analyzed by flow cytometry using 405 nm excitation.



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Violet-excitable DNA Stain for Apoptosis



- Monomeric Cyanine dye that labels nucleic acid
- Selectively passes through the plasma membranes of apoptotic cells and labels them
- Po-Pro™-1 for apoptotic cells
- 7-AAD for dead cells

Jurkat cells were treated with 10 μ M camptothecin for 4 hr and then stained with the reagents in the Violet Membrane Permeability/Dead Cell Apoptosis Kit. Cells were analyzed by flow cytometry using 405 nm and 488 nm excitation. Apoptotic cells (A) are clearly distinguished from live cells (L) and dead cells (D).



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Apoptosis Assay for Adherent Cells

- Violet Fluorescent Biomembrane Probe-Ratiometric Detection of Apoptosis
- 4'-N,N-diethylamino-6-(N-dodecyl-N-methyl-N-(3-sulfopropyl)ammoniomethyl-2-hydroxyflavone - F2N12S for short
- Uses Excited State Intramolecular Photon Transfer
 - 530=dead/apoptotic
 - 585=live
- Probe incorporates into plasma membrane
- Correlates well with other indicators of apoptosis i.e. annexin V



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Staining with F2N12S

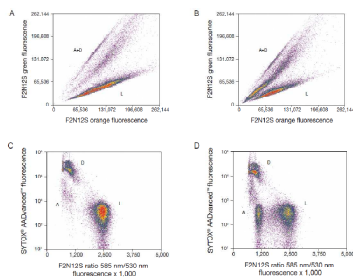


Figure 3. Jurkat cells (7.4x10⁶) were fixed overnight with 1% paraformaldehyde for four hours (panels B and D) or not treated overnight (panels A and C). Cells were stained according to the protocol. Samples were analyzed as shown (excitation with 405 nm excitation using 488 nm and 530 nm bandpass filters for F2N12S and 488 nm excitation for SYTOX™ Advanced™). Dead cell stain using 405 nm bandpass filter. In panels A and C, living cells can be discriminated from apoptotic and dead cells by the relative intensity of the two emission bands from F2N12S. In panels B and D, SYTOX™ Advanced™ dead cell stain fluorescence is plotted against a derived ratio parameter from the two emission bands (excitation of 488 nm, 530 nm) to reveal cells in the cells in the dead cells.



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F2N12S, Sytox Advanced, and MitoProbe DiIC₁₍₅₎

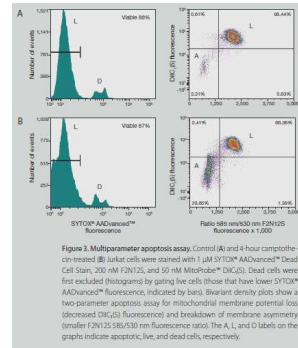
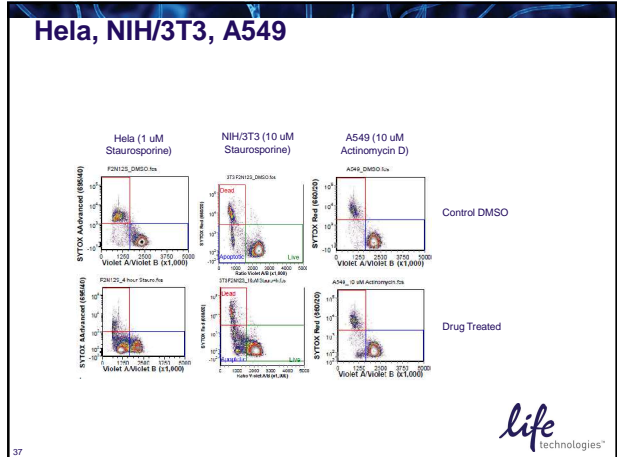


Figure 3. Multiparameter apoptosis assay. Control (A) and 4-hour camptothecin-treated (B) Jurkat cells were stained with 1 μ M SYTOX™ Advanced™ Dead Cell Stain, 200 nM F2N12S, and 50 nM MitoProbe DiIC₁₍₅₎. Dead cells were first excluded (histograms) by gating live cells (those that have lower SYTOX™ Advanced™ fluorescence, indicated by bars). Bivariate density plots show a two-parameter apoptosis assay for mitochondrial membrane potential loss (decreased DiIC₁₍₅₎ fluorescence) and breakdown of membrane asymmetry (smaller F2N12S 585/530 nm fluorescence ratio). The A, L, and D labels on the graphs indicate apoptotic, live, and dead cells, respectively.



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Online Resources

Livia.Vo@lifetech.com

www.invitrogen.com/flowcytometry

www.invitrogen.com/qdotsinflow

www.invitrogen.com/linnea **LINNEA™ Guide to Antibodies**
pursue every angle

Fluorescence & Flow Cytometry Tutorials

Spectraviewer

BD FACS Calibur

Flow Instrumentation Guide

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