Novel Molecular Probes®
Reagents to Analyze
Cellular Function

Gayle Buller
Sr. Product Development Manager
Flow Cytometry Systems
Overview

UTILIZE THE POWER OF FLOW----

Reagents that detect cellular functions are easily incorporated into multi-color experiments

• Apoptosis
• Reactive Oxygen Identification
• Cell Proliferation
• Cell Cycle Analysis
• Viability
• Phagocytosis
Necrosis vs Apoptosis

Violet Fluorescent Biomembrane Probe- Ratiometric Detection of Apoptosis

Probe incorporates into plasma membrane

- Excitable with Violet laser, easily multiplexed
- Probe exhibits Excited State Intramolecule proton transfer—dual fluorescence at 530 and 585 nm
- Apoptosis modifies the surface charge of outer leaflet of plasma membrane
- Monitors changes in surface charges through the change in relative intensity of the two emission bands.
- Flow Cytometry Ratio parameter can be used
Apoptosis Assay for Adherent Cells

Less affected by cell membrane damage found during physical or chemical removal steps when assaying adherent cells

Hela (1 uM Staurosporine)
NIH/3T3 (10 uM Staurosporine)
A549 (10 uM Actinomycin D)

Control DMSO
Drug Treated
Comparison of F2N12S to Caspase 3 and 7, mitochondrial membrane potential indicators DiIC(5) and JC-1

- Comparable to other hallmark changes in apoptosis
- No special buffer required
- No Wash Assay
- Less affected by cell membrane damage found during physical or chemical removal steps when assaying adherent cells
CellEvent™
caspase-3/7 green detection
**CellEvent™ Caspase 3/7 Green reagent for apoptosis**

- Fluorogenic Caspase 3/7 Substrate
- A nucleic acid dye conjugated to a DEVD peptide
- Non-fluorescent until active caspase 3/7 cleaves the DEVD peptide and the free nucleic acid dye binds to DNA.

**ADVANTAGES:**
- Live cell amenable
- May be added to complete growth media
- No-wash protocol
- Retained after fixation and permeabilization
- May be multiplexed with other live or fixed cell probes

**CellEvent™ Reagent Workflow**

1. Add diluted CellEvent™ reagent to cells
2. Incubate 30 minutes
3. Measure fluorescence

**Active Caspase-3/7 Enzyme**

- **DEVD**
- DNA dye

**Bound DNA dye**

**Non-fluorescent No DNA binding**
Effect of Washes on Apoptotic Cells

Washes wash away the fragile apoptotic cells as illustrated here.

U2OS cells were treated with 0.375 µM staurosporine for 23 hours. Cells were then labeled with 5 µM CellEvent and Hoechst 33342 in complete media for 30 min. at 37°C. Cells were then imaged immediately, no wash. Cells were washed with DPBS and imaged after each wash for 4 washes. % positive (% of cells positive for CellEvent) were plotted.
Jurkat cells (1x10^6 cells/mL) were induced with 10µM camptothecin for 4 hours prior to harvest. Cells were washed 1x in PBS and stained using 4µM CellEvent™ and Fixable Aqua. Following a 30 minute incubation at room temperature protected from light, cells were washed with 1xPBS and analyzed on the Attune® Acoustic Focusing Cytometer using a collection rate of Standard 100µL/min. Each dual parameter plot indicates Fixable Aqua and CellEvent™ fluorescence from the gated population of parent cells (excluding debris). Treatment with camptothecin increases the percent of apoptotic (A, blue) cells while decreasing the percent of live (L, green) cells. The number of necrotic (N, red) cells remains fairly constant over the course of this experiment.
ROS Probes
• Under Oxidative Stress- ROS is dramatically increased.

• Reactive Oxygen Species can alter membrane lipids, proteins, and nucleic acids.

• Specifically Detects general reactive oxygen species (ROS) or Oxidative stress.

• Absorption/emission maxima: ~644/665 nm,

• Compatible with formaldehyde-based fixation methods, facilitating convenient multi-parameter analyses
Simple Work flow for CellROX™ for Multiple platforms

1. Plate cells (suspension cells, if using flow cytometry, are grown in flasks)
2. Drug treatment
3. Add CellROX™ Deep Red reagent & Hoechst 33342 (incubate for 30 mins)
4. Wash cells 3X in PBS (Suspension cells are washed by centrifugation)
5. Analyze

Validated with all Major platforms
Flow Cytometer

5 uM Far Red ROS Sensor

Control 100uM Menadione 500nM PMA

Count

U2-OS cells

5 fold change in signal intensity in menadione treated cells compared to controls
CellTrace™ Violet Stain:
An alternative to CellTrace™ CSFE
Cell Proliferation Analysis by Dye Dilution

- Cell division results in equal partitioning of dye between daughter cells.
- Fluorescence of daughter cells is half that of parent cell.
Proliferation Analysis with CFSE

Carboxyfluorescein Diacetate Succinimidyl Ester
CFSE: Occupies a Popular Channel

Fluorophore
1: fluorescein (antibody conjugate)
2: Alexa Fluor 488 (antibody conjugate)
3: GFP
4: Vybrant DyeCycle Green stain
5: fluo-4

Excitation
Laser (nm): 488
Filter / Bandpass

Emission
Filter / Bandpass
525 / 50
585 / 42
695 / 40
780 / 60
CellTrace™ Violet Analyzed with Proliferation Modeling Software

Human CD8+ T lymphocytes stained with 10µM CellTrace™ Violet and incubated in OpTmizer T-cell Expansion Medium at 37°C for 7 days. (A) Unstimulated cells. (B) Cells stimulated with 200ng mouse anti-human CD3 antibody and 100ng Interleukin-2 per milliliter cells.
Peripheral blood mononuclear cells were isolated from whole blood, stained with 10µM CellTrace™ Violet, and stimulated with mouse anti-human CD3 and Interleukin-2 for 7 days in culture. Cells were stained with mouse anti-human CD4 Alexa Fluor® 488 and SYTOX® AADvanced™ dead cell stain immediately prior to analysis.

**Multiplexed Analysis on the Attune™ Acoustic Focusing Cytometer**

**Experiment Name:** 022510 CellTrace - CLL  
**Specimen Name:** Stained Lymphs  
**Tube Name:** ST25

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<th>% Total</th>
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<td>100.00%</td>
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<tr>
<td>Live</td>
<td>99,806</td>
<td>70.145%</td>
<td>70.145%</td>
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<tr>
<td>Cells</td>
<td>28,532</td>
<td>28.587%</td>
<td>20.053%</td>
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<tr>
<td>CD4 AF488</td>
<td>16,263</td>
<td>56.999%</td>
<td>11.430%</td>
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Multiplexed Analysis on the Attune® Acoustic Focusing Cytometer

Several generations of live CD4+ T Cells as visualized with the Attune® Acoustic Focusing Cytometer.

Several generations of live CD4+ and CD4- T Cells as visualized with the Attune® Acoustic Focusing Cytometer.
DNA Content Analysis: Options for live or fixed cells
Cell Cycle in brief

\[ G_0: \text{Non-proliferating cells} \]
\[ G_1: \text{Cell have 2N nuclear DNA content} \]
\[ S: \text{Nuclear DNA content doubles to 4N} \]
\[ G_2: \text{Cells are maintained at 4N} \]
\[ M: \text{Cell division results in two cells each with 2N DNA content} \]
DNA Content Analysis in Proliferating Cells

U2OS osteosarcoma cells transduced with Cellular Lights™ Tubulin and MAP4
Technical Considerations for Cell Cycle Staining

• Instrument
  – Know your instrument (lasers/emission filters)
  – Know your dye (excitation/emission)
  – Proper maintenance and careful optical alignment
  – Verify instrument linearity

• Sample Prep
  – Single cell suspension
  – Cell concentration and dye concentration
  – Optimize for cell type, medium or buffer used, time of incubation, temperature of incubation.

• Acquisition and Analysis
  – Acquire sample in low flow rate
  – Total number of cells acquired
  – Gating strategies
  – Software analysis
Narrow Sample Stream: Low Flow Rate - 12µL/min

Wide Sample Stream: High Flow Rate - 60µL/min

Hydrodynamic Focusing

CV = 5.1%

CV = 8.5%
Hydrodynamic Focusing: DNA Content Cell Cycle Data

Low - 12 µl/min

Fixed Jurkat cells stained with Propidium Iodide

As Sample Rates Increase

Note overt degradation of CV values and changes in data

Medium - 35 µl/min

G0G1: 40.16%  Mean: 50.00
- CV: 6.12%
G2M: 20.89%  Mean: 98.00
- G2/G1: 1.96
S-Phase: 38.95% Mean: 73.19

High - 60 µl/min

G0G1: 44.60%  Mean: 49.97
- CV: 7.76%
G2M: 29.23%  Mean: 95.13
- G2/G1: 1.90
S-Phase: 26.17% Mean: 69.12
FxCycle™ Stains: for DNA content measurement in FIXED cells

Easily add DNA content to multicolor experiments

**FxCycle™ Violet stain**

- Violet 405 nm excitation
- ~ 450 nm emission

**FxCycle™ Far Red stain**

- Red 633 nm excitation
- ~ 660 nm emission
Identify Mitotic Cells with FxCycle™ Violet and FxCycle™ Far Red dyes combined with pH3 + Zenon® Alexa® Fluor 488

BioSource Rabbit polyclonal anti-Histone H3 [pS10] Complexed with Zenon® Alexa Fluor® 488 Rabbit IgG
DNA Content Analysis in Live Cells

- Cell-permeant nucleic acid dyes can penetrate an intact cell membrane
- Can be 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 ex)  
  dsDNA
- Vybrant® DyeCycle™ Ruby stain (488 - 633 ex)  
  dsDNA
Vybrant® DyeCycle™ Ruby stain
for DNA content in LIVE cells

488 nm laser

633 nm laser

Vybrant®DyeCycle™ Ruby fluorescence
Jurkat cell growth eight days after sorting

- Control
- Hoechst 33342
- Vybrant® DyeCycle™ Ruby stain
- DRAQ5®
Re-Growth After Sorting

Data Courtesy of: Tina Luke and Derek Davies, London Research Institute, Cancer Research UK

File analyzed: Specimen_002_HL-60.fcs
Date analyzed: 5-Jun-2009
Model: 1nn0n_DSF
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 55.52 % at 51.37
Dip G2: 1.41 % at 91.98
Dip S: 43.07 % G2/G1: 1.79
%CV: 11.46

Total S-Phase: 43.07 %
Total B.A.D.: 0.00 % no debris no aggs

Debris: %
Aggregates: 0.00 %
Modeled events: 8199
All cycle events: 8199
Cycle events per channel: 197
RCS: 1.067

HL-60 cell count

Day post sort
Cell count/ml x 10e5
0
1
2
3
4
5
6
7
8
1 2 3 4 5

Percentage viable

Days post sort
HL-60
0
20
40
60
80
100
0
20
40
60
80
100
1 2 3 4 5
Premo™ FUCCI Cell Cycle Sensor

- **Fluorescent Ubiquitination-based Cell Cycle Indicator**
  - A fluorescent protein-based sensor using a red (RFP) and a green (GFP) fluorescent protein fused to different regulators of the cell cycle: Cdt1 and geminin
  - In G1 phase, geminin is broken down and only Cdt1 tagged with RFP may be visualized. Cells in the G1 phase have red fluorescent nuclei.
  - In S, G2, and M phases, Cdt1 is degraded and only geminin tagged with GFP remains. Cells in these phases have green fluorescent nuclei.
Premo™ FUCCI Cell Cycle Sensor

U2OS cells transduced with Premo™ FUCCI Cell Cycle Sensor and allowed to divide. Images were collected over 15 hours.
Limbal Stem Cell Side Population

Limbal stem cells give rise to cells that migrate to the central cornea. To identify corneal epithelial progenitor populations in cell cultures derived from human limbal tissue, DyeCycle™ Violet is used, here shown with a pump blocker. Useful to quantify stem cells in expanded limbal cell cultures.
Fluorescence Histogram of DNA content distribution

*Where is the S-phase?*

G1: 21.98%
G2: 62.92%
S: 15.10%

G1: 1.77%
G2: 82.74%
S: 15.49%
Click-iT® EdU Cell Proliferation Assay: Reliable S-Phase Analysis
TRADITIONALLY
1960’s - (³H-thymidine)

Tritiated (³H) thymidine
1970’s - (BrdU)

BrdU (5-bromo-2’-deoxyuridine)
BrdU incorporated in dsDNA is inaccessible to the BrdU antibody
- BrdU antibody requires DNA denaturation for detection

- Numerous protocols: acid, heat, or nuclease for DNA denaturation

*Acid or DNase*
• Denatured DNA is required for antibody detection of BrdU
New in 2007-EdU

EdU (5-ethynyl-2’-deoxyuridine)
Click-iT® EdU detection

Click labeling does not require DNA denaturation

Dye azide reacts with the alkyne on double stranded DNA
Attune® Acoustic Cytometer with Click-iT® EdU: FxCycle™ Violet with Alexa Fluor® 488 azide

Run at Standard collection

1000 µl/min

EdU- Alexa Fluor® 488 fluorescence

FxCycle™ Violet fluorescence
One hour EdU then one hour BrdU

EdU
• Incorporated into cells replicating DNA

Add BrdU
• Cells that have already completed DNA synthesis will not incorporate BrdU and will express EdU only
• Cells newly entering S-phase will incorporate BrdU and express BrdU only
• Cells in S-phase during both pulses will express BrdU and EdU
Jurkat cells: Edu then BrdU

![Fluorescence dot plots for Edu Alexa Fluor® 647 and BrdU FITC with DNA content axes.](image-url)
### Selection of BrdU clone

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<th>BrdU Clone</th>
<th>react with EdU</th>
<th>react with BrdU</th>
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<tr>
<td>3D4</td>
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<tr>
<td>PRB-1</td>
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<td>yes</td>
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<tr>
<td>B331</td>
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<td>no</td>
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<tr>
<td>Bu20a</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>B44</td>
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<td>yes</td>
</tr>
<tr>
<td>MoBu</td>
<td>no</td>
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There are many more clones of BrdU....
Click-iT® EdU is compatible with multiplex fluorescence labeling across species (and Kingdoms)


Medicago sativa (alfalfa) suspension cultures labeled with Click-iT® EdU. Image courtesy of Ferhan Ayaydin, Cellular Imaging Laboratory, Biological Research Center, Szeged, Hungary

Image courtesy of Sarah Cheesman, University of Oregon

E. coli

Image courtesy of Julian P.S. Smith III, Winthrop Microscopy Facility, Winthrop University

marine flatworm

zebrafish larva
Tools for Determination of Cell Viability
Common Nucleic Acid Dyes for Flow

Dead cell identification based on membrane integrity

- Propidium Iodide (488 nm ex)
- 7AAD (488 nm ex)
- SYTOX® Green dead cell stain (488 nm ex)
- SYTOX® Blue dead cell stain (405 nm ex)
- SYTOX® Red dead cell stain (633 nm ex)

SYTOX® AADvanced™ dead cell stain

- Uses common 488 nm laser
- Spectra similar to 7AAD
- Efficient staining, only 5 minutes
- Can easily use with FITC & PE channels
7AAD vs SYTOX® AADvanced™

Comparison with 5 minutes incubation time

SYTOX® AADvanced™ stain
MFI ratio of Dead:Live = 102

7-AAD
MFI ratio of Dead:Live = 56
Alternatives for Dead Cell Identification

- Amine-reactive fluorescent dyes in a range of colors
- Easy discrimination of live and dead cells

- **Live cells** react with the 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.
Fixable Dead Cell Stains

Same population discrimination pattern before and after fixation.

Jurkat cells were mixed 50% live: 50% dead and stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain.
Effect of Fixation on Dead Cell Dyes

Propidium iodide

Before Fixation

After Fixation

488 nm excitation, 610/20 filter

LIVE/DEAD® Fixable Red stain

Before Fixation

After Fixation

488 nm excitation, 610/20 filter
LIVE/DEAD® Fixable Dead Cell Stains: amine-reactive dyes

LIVE/DEAD® Fixable Blue stain
- Before Fixation
- 18 Hours Post-Fixation
- UV (355 nm) excitation, 450/50 filter

LIVE/DEAD® Fixable Violet stain
- 405 nm excitation, 530/30 filter

LIVE/DEAD® Fixable Aqua stain
- 405 nm excitation, 575/25 filter

LIVE/DEAD® Fixable Yellow stain
- 405 nm excitation, 530/30 filter

LIVE/DEAD® Fixable Green stain
- Before Fixation
- 18 Hours Post-Fixation
- 488 nm excitation, 530/30 filter

LIVE/DEAD® Fixable Red stain
- 488 nm excitation, 610/20 filter

LIVE/DEAD® Fixable Far Red stain
- 633 nm excitation, 780/60 filter

LIVE/DEAD® Fixable Near IR stain
- 633 nm excitation, 660/20 filter
Lysed human peripheral blood was labeled with CD4-Pacific Blue™ and CD8-Pacific Orange™ direct conjugates, and then labeled with SYTOX® Orange dead cell stain before acquisition using 488 nm excitation with a 575/24 BP for detection of SYTOX® Orange and 405 nm excitation with 450/40 BP for Pacific Blue™ and 603/48 BP for Pacific Orange™ detection.

Antibodies will bind nonspecifically to dead cells. Gating on live cells will improve the accuracy of results.
**LIVE/DEAD® Fixable Dead Cell Stain Sampler Kit**

**SYTOX® Dead Cell Stain Sampler Kit**

### Standard Workflow
1. Resuspend 1 x 10⁶ cells in 1 mL cell suspension buffer.
2. Add 1 μL dye.
3. Incubate at room temperature: SYTOX® AADvanced™: 5 min; SYTOX® Blue: 15 min; SYTOX® Green: 20 min; SYTOX® Orange: 20 min; SYTOX® Red: 15 min.
4. Analyze.

### SYTOX® Dead Cell Stain Sampler Kit for Flow Cytometry

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<tr>
<th>SYTOX® dead cell stain</th>
<th>Excitation Maximum (nm)</th>
<th>Emission Maximum (nm)</th>
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<th>Attune Channel; bandpass filter</th>
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<td>SYTOX® AADvanced™</td>
<td>546</td>
<td>647</td>
<td>488</td>
<td>BL3; 640LP</td>
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<tr>
<td>SYTOX® Blue</td>
<td>444</td>
<td>480</td>
<td>405</td>
<td>VL1; 450/40</td>
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<tr>
<td>SYTOX® Green</td>
<td>504</td>
<td>523</td>
<td>488</td>
<td>BL1; 530/30</td>
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<tr>
<td>SYTOX® Orange</td>
<td>547</td>
<td>570</td>
<td>532 or 488</td>
<td>BL2; 575/24</td>
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<td>SYTOX® Red</td>
<td>640</td>
<td>658</td>
<td>633/635</td>
<td>n/a; 660/20</td>
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**SYTOX® fluorescence Count**

For research use only. Not intended for any animal or human therapeutic or diagnostic use, unless otherwise stated.

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Tools for Compensation

ArC™ Bead Kit: **Amine- Reactive Compensation**

**Protocol:**
- Stain one drop of reactive beads with 1 µl Live/Dead™ Fixable Stain
- Incubate 15 minutes
- Wash
- Add one drop of negative beads
- Ready to Run

**Why Use them?**
- Eliminates the hassle of heat-treating cells
- Fast and simple bead based compensation
- Save precious cell sample
- Auto-compensation compatible
ArC™ Bead Kit

Amine Reactive Compensation Beads

ArC™ Beads stained with Live/Dead™ fixable Blue, Green, and Near IR dyes
Phagocytosis Studies
**pHrodo™ BioParticles® for Phagocytosis**

**pHrodo™: pH-sensitive fluorescence**

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**Figure 1. Analysis of phagocytosis in a granulocyte population.** Whole human blood was incubated with pHrodo™ *E. coli* Bioparticles® either on ice (4°C) or at 37°C for 15 minutes before red blood cell lysis and wash. Samples were analyzed using the Attune® Acoustic Focusing Cytometer equipped with 488 nm laser for pHrodo™ dye excitation. Samples were run at a collection rate of Standard 25 µL/min and fluorescence emission was detected using a 575/24 bandpass filter. Histogram (A) and dot plot (B) overlays gated from a granulocyte population demonstrate the difference in *E. coli* Bioparticles fluorescence upon phagocytosis. Phagocytosis is inhibited by incubation on ice (4°C), whereas incubation at 37°C permits efficient phagocytosis.
pHrodo™ BioParticles® for Phagocytosis
Life Technologies - Reagents and Instruments

Attune® Acoustic Focusing Cytometer
Innovative New Technology to Align Cells for flow analysis
Ideal for rare cell analyses, precious samples

Tali™ Image-based Cytometer
2 Channel Fluorescent Image Cytometer
Ideal for GFP /RFP Enumeration
Simple Cell Assays

Countess® Automated Cell Counter
Ideal for cell counting /viability
Promotions

• 15% off the featured Flow technologies & Countess
• A free box of slides when you order the Tali™
• Coupon to save $15K when you order both the Tali™ & Attune®
Contacts
Kris Zuraitis - Consumable Sales Representative
kris.zuraitis@lifetech.com
Sara Staggs - Flow Technical Sales Specialist
sara.staggs@lifetech.com
Russ Bennett - Technical Specialist - Drug Discovery and Cellular Analysis
russ.bennett@lifetech.com

Questions???????????