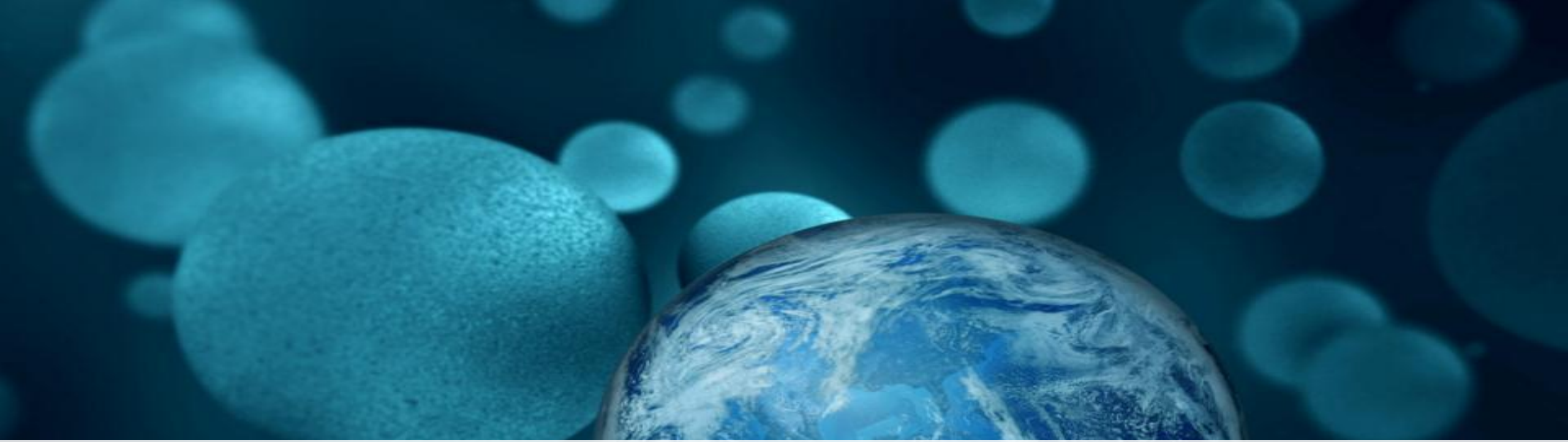

ThermoFisher SCIENTIFIC

Cell Health & Functional Assays for Flow Cytometry

Life Sciences Solutions: Part of Thermo Fisher Scientific

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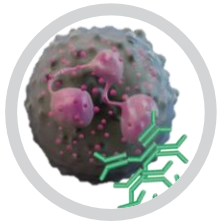
Goals for this presentation

- **High level introduction of different dyes/ chemistries and alternative flow cytometry assays**
- **Understanding of Thermo Fisher flow cytometry resources available (people, online tools, protocols, antibodies, cell health assays, comp beads/ buffers, analysis instrumentation, sorting instrumentation, spectral flow etc..)**
- **Direction and perhaps even some refreshed motivation for the next flow cytometry steps in your work**

Agenda

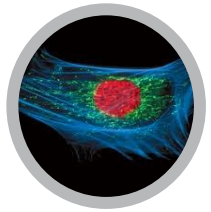
Stimulation

Activating and neutralizing antibodies



Cell health

Apoptosis, proliferation, viability

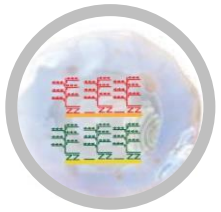


Antibodies

Conjugated antibodies for flow cytometry

RNA flow

Invitrogen™ PrimeFlow™ assays



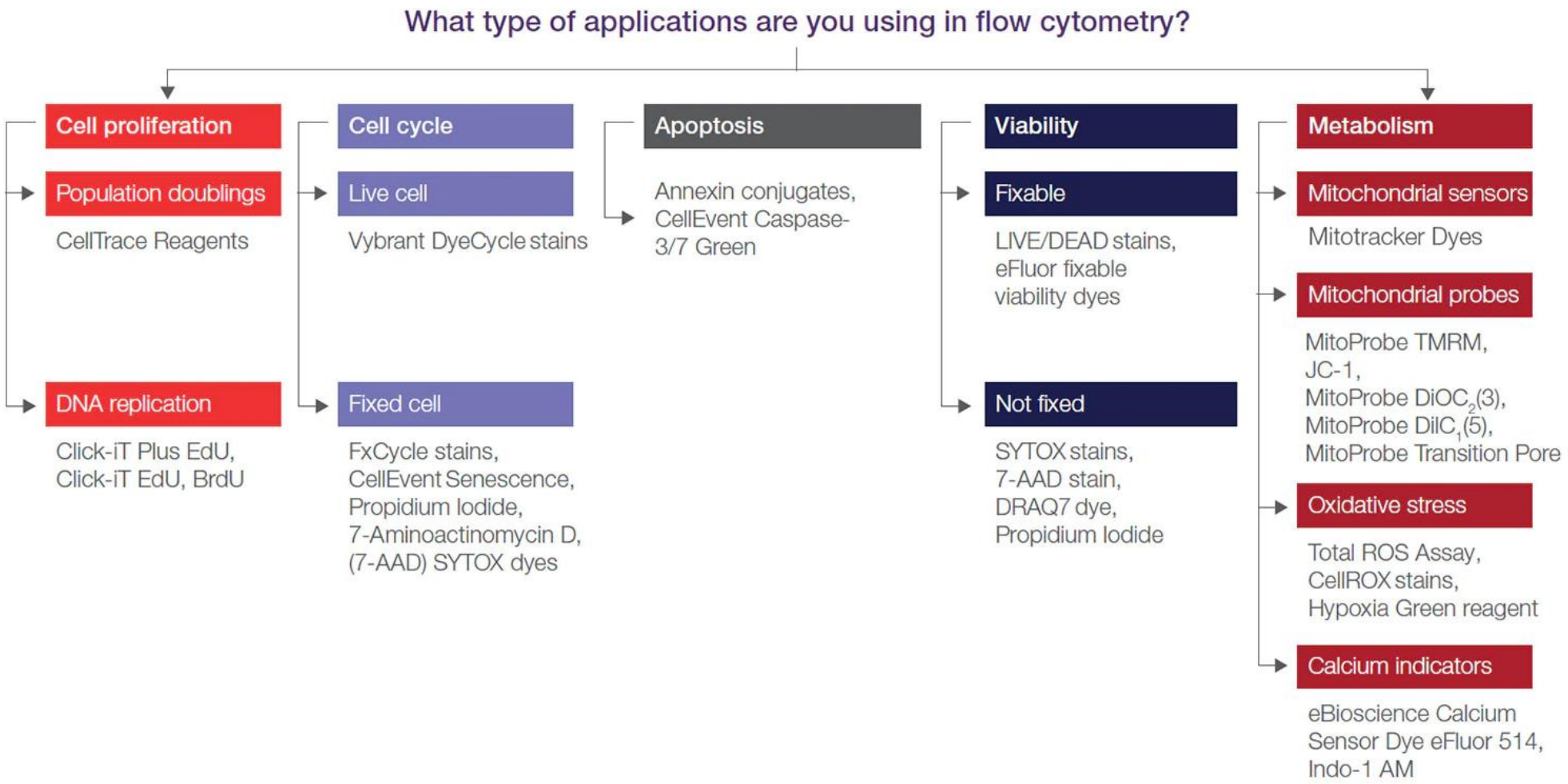
Everyday essentials

Beads, buffers, controls

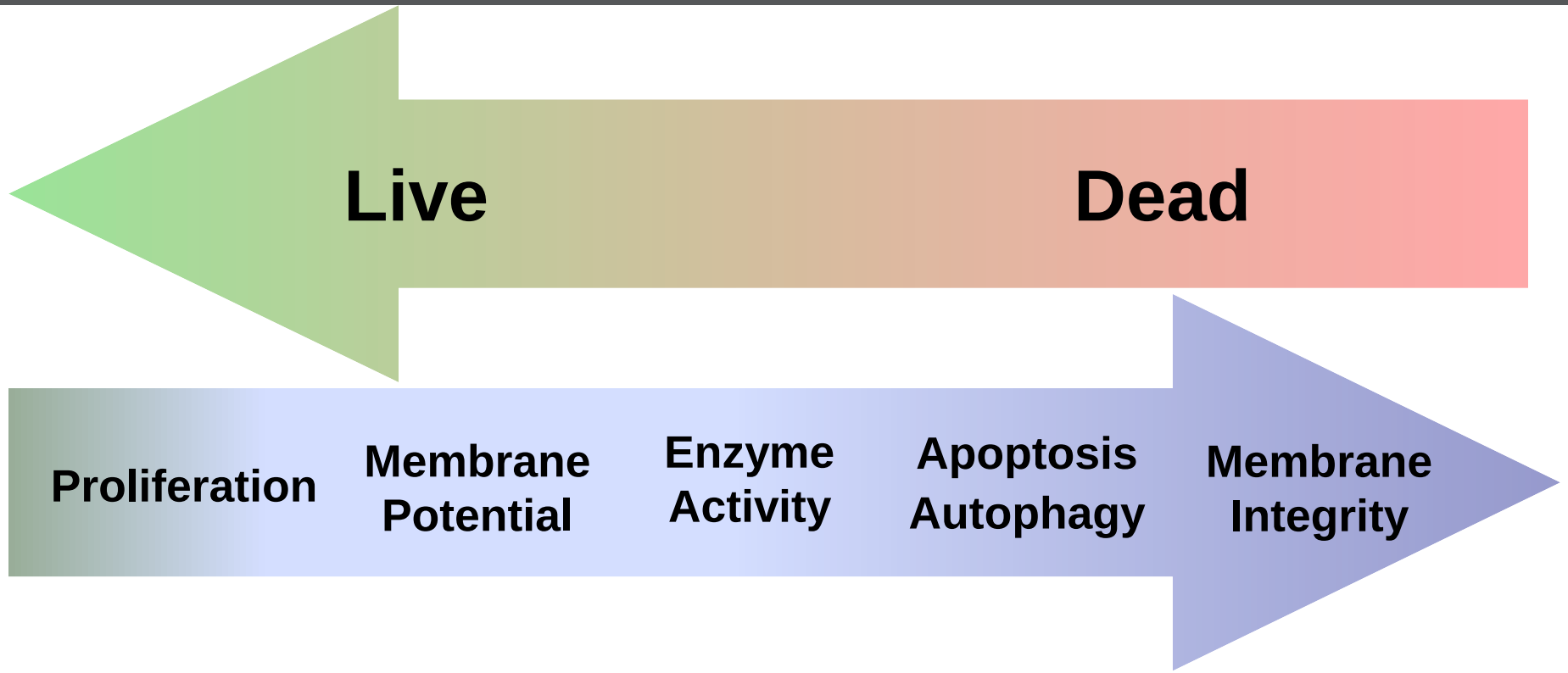
Instrumentation

Attune NxT Flow Cytometer with Autosampler

Assay Options



Viability

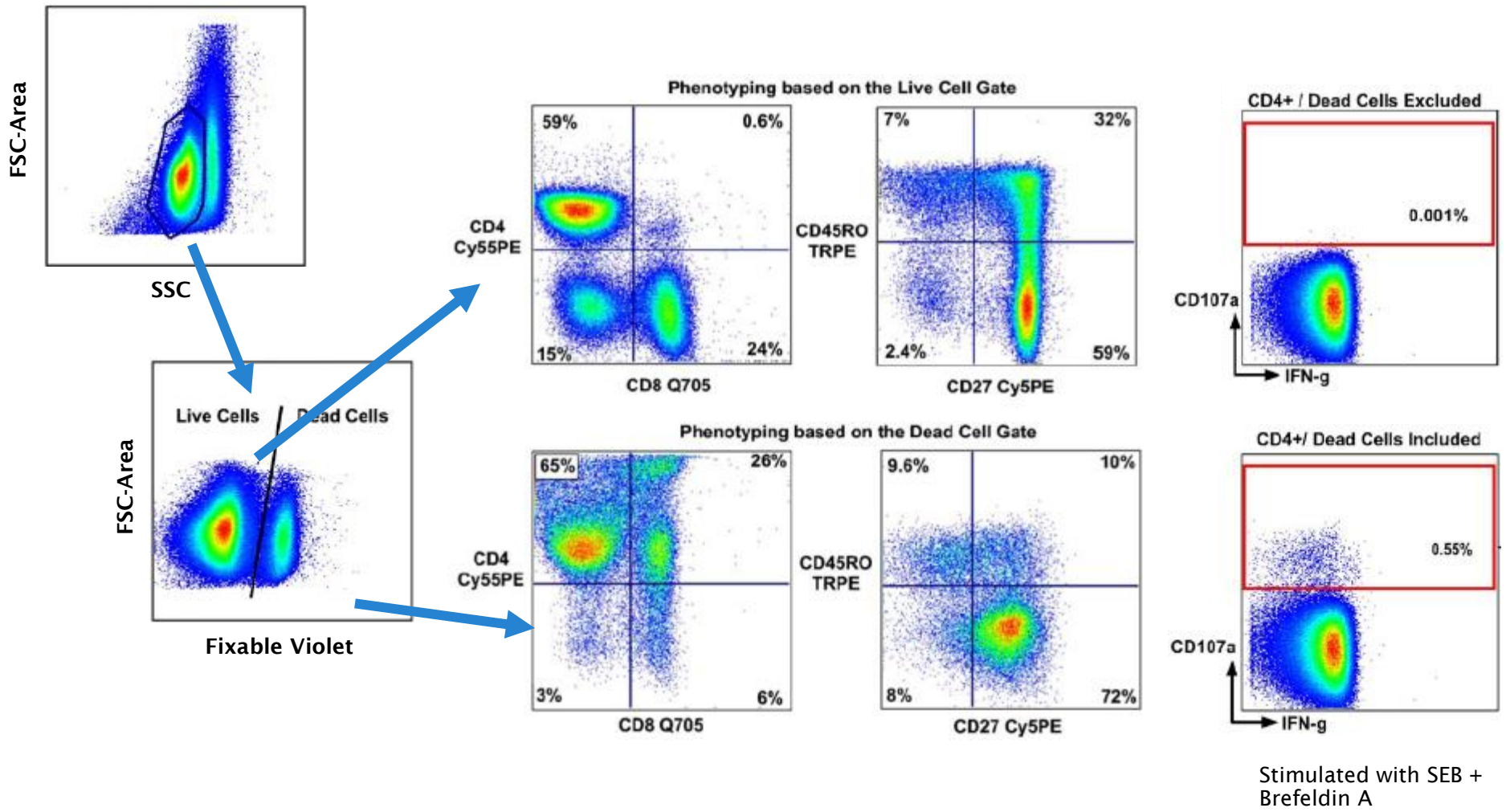


Cells exist anywhere on a continuum between healthy and dead

Why is viability important for flow cytometry experiments?

- Dead cells non-specifically bind antibodies, and look like viable cells
- Dead cells bind all kinds of markers
- Potentially lead to erroneous results
- Excluding dead cells improves accuracy of results

Why use a viability indicator?



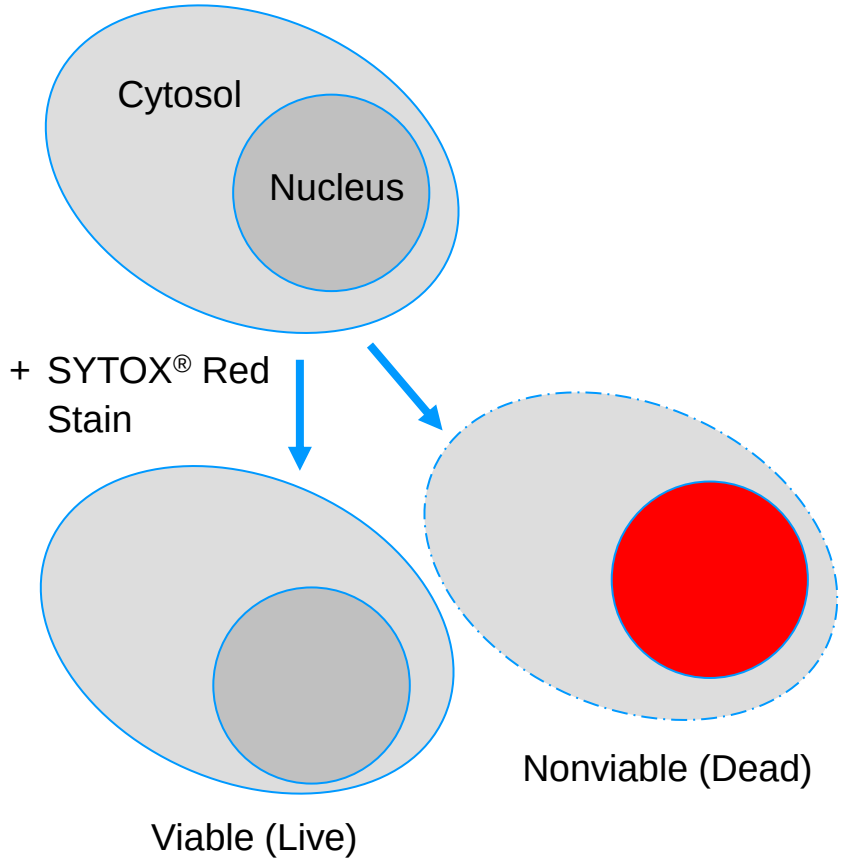
Stimulated with SEB + Brefeldin A

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

Viability: Impermeant nucleic acid-binding dyes

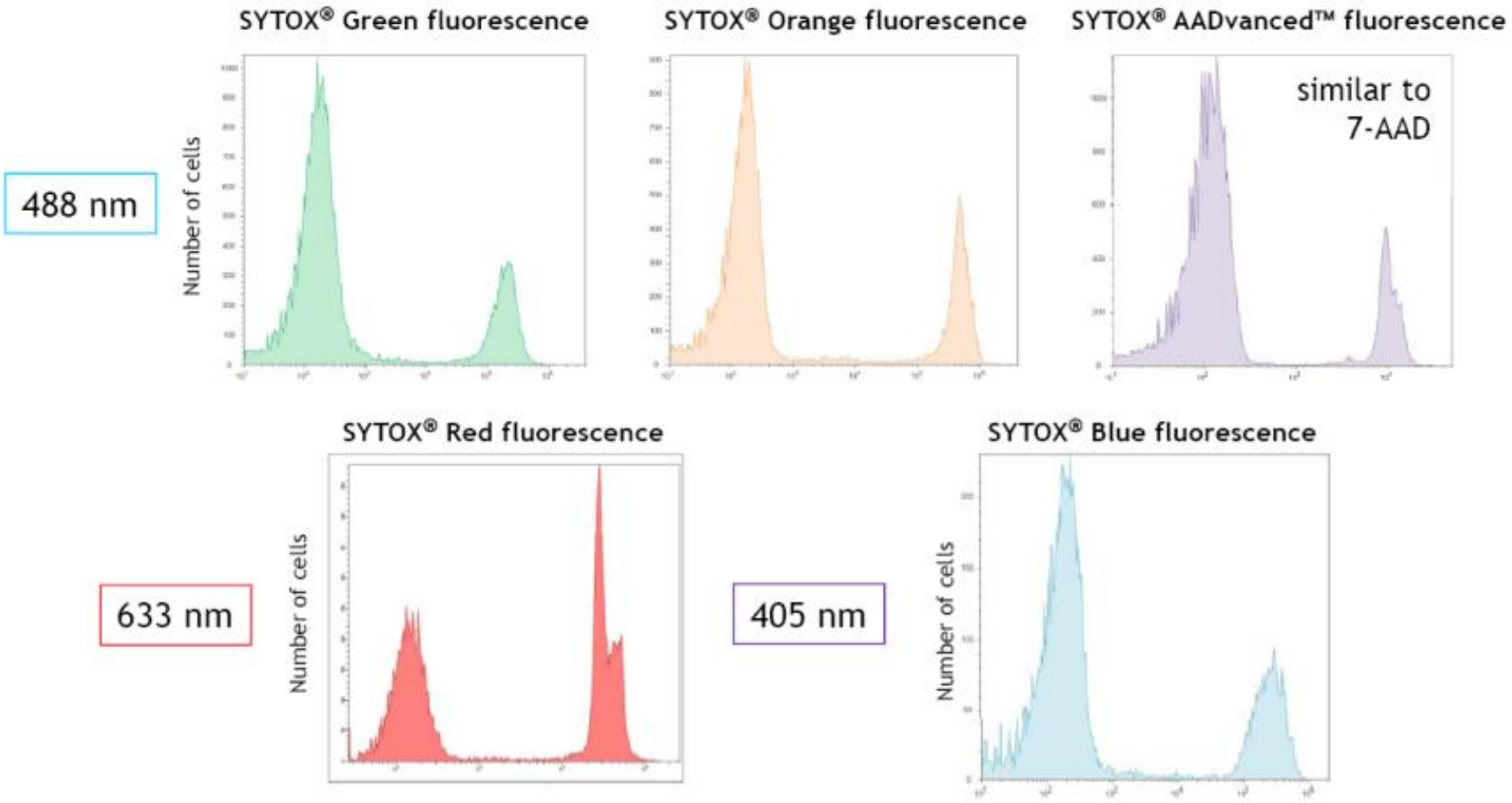
Viability:

Integrity of plasma membrane



Viability: SYTOX® Dead Cell Stains

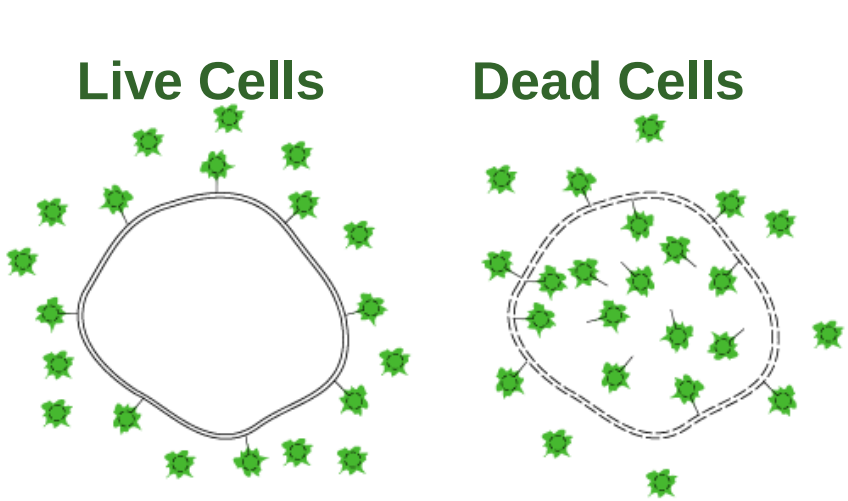
Five different colors for flexibility in multicolor panels

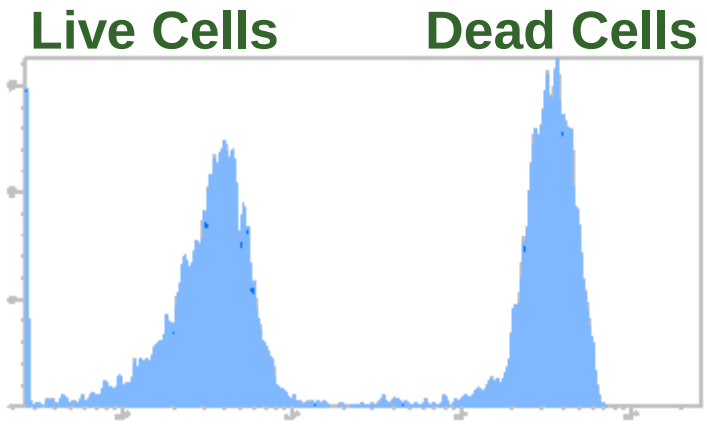


Amine reactive dyes for dead cell identification

LIVE/DEAD™ Fixable Dead Cell Stain Kit

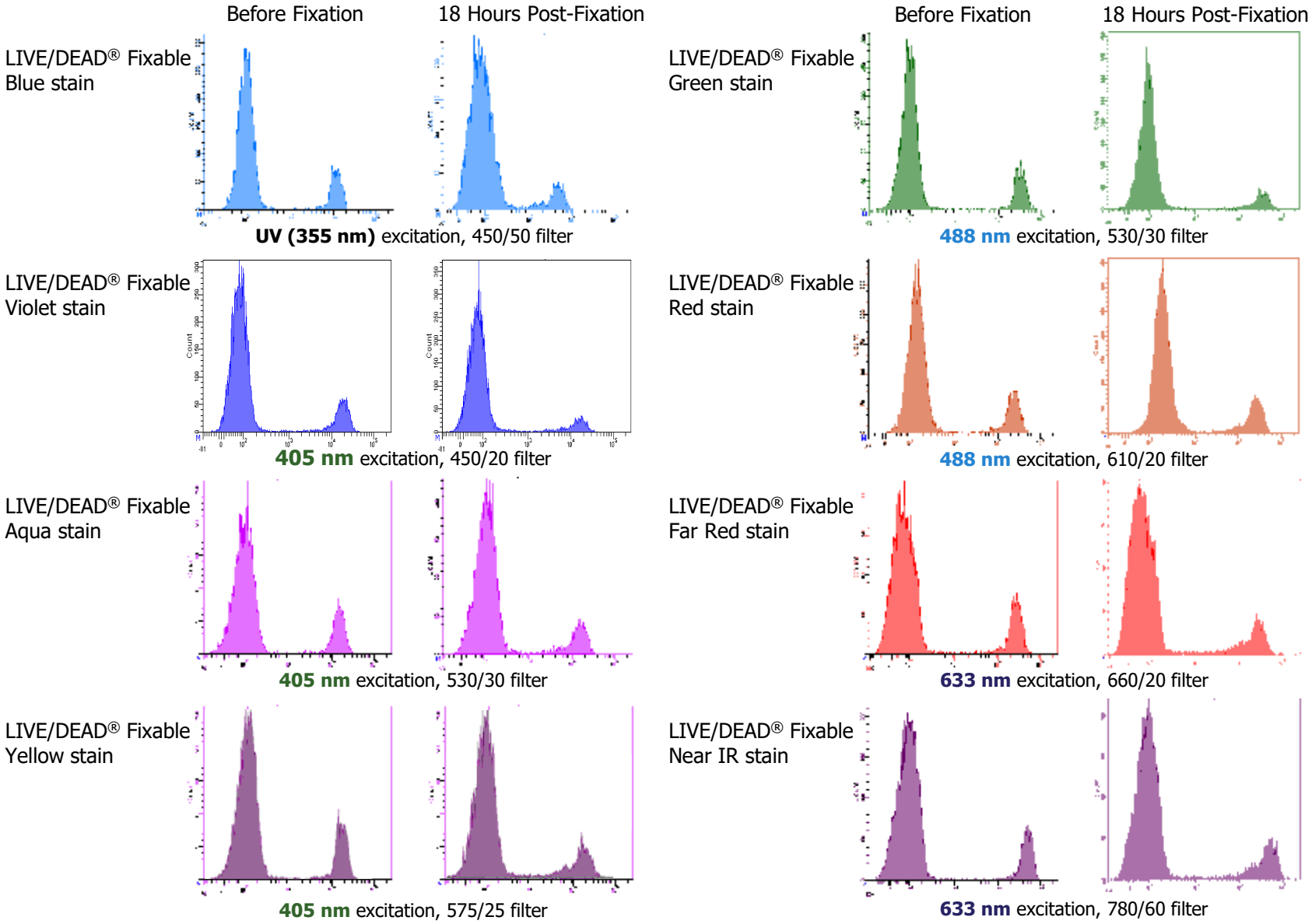
eBioscience™ Fixable Viability Dyes



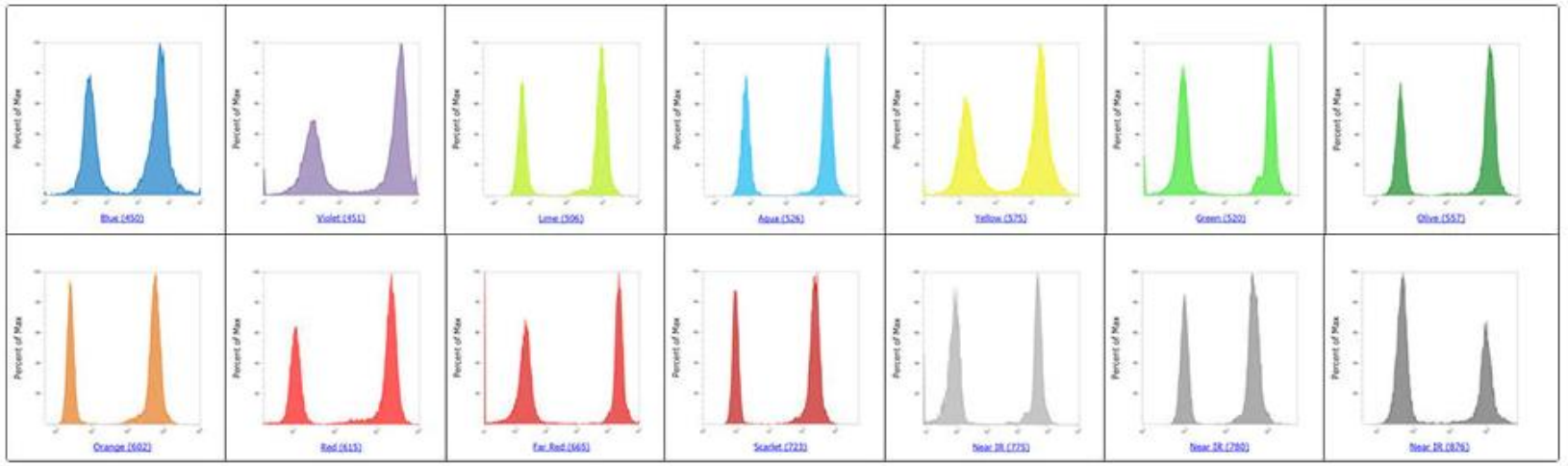


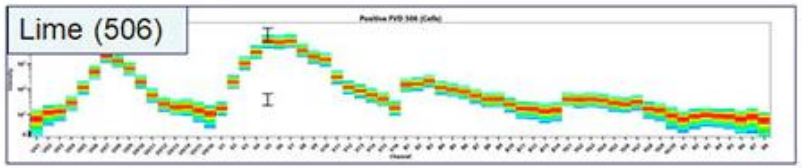
Aqua-fluorescent reactive dye

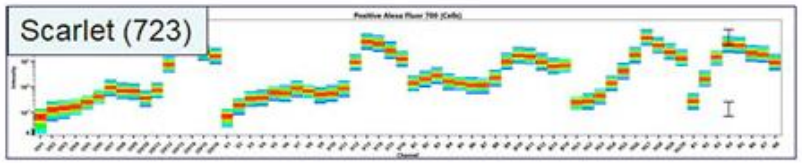
Viability: LIVE/DEAD® Fixable Dead Cell Stains – amine reactive

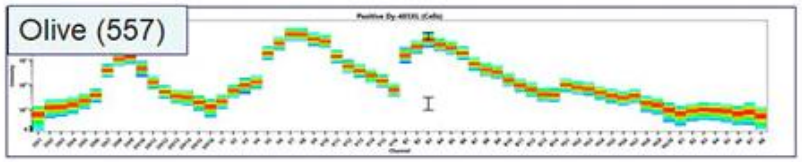


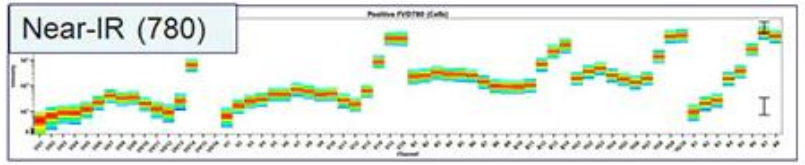
14 different dyes now available

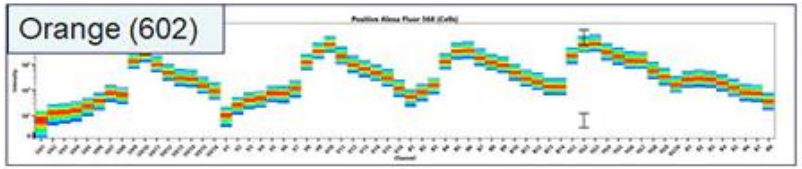


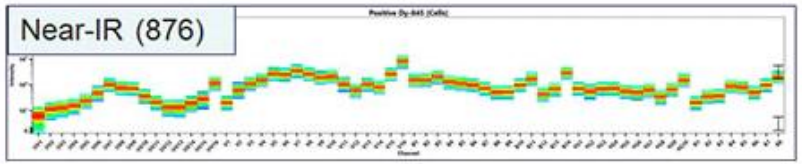












Viability – tips and tricks

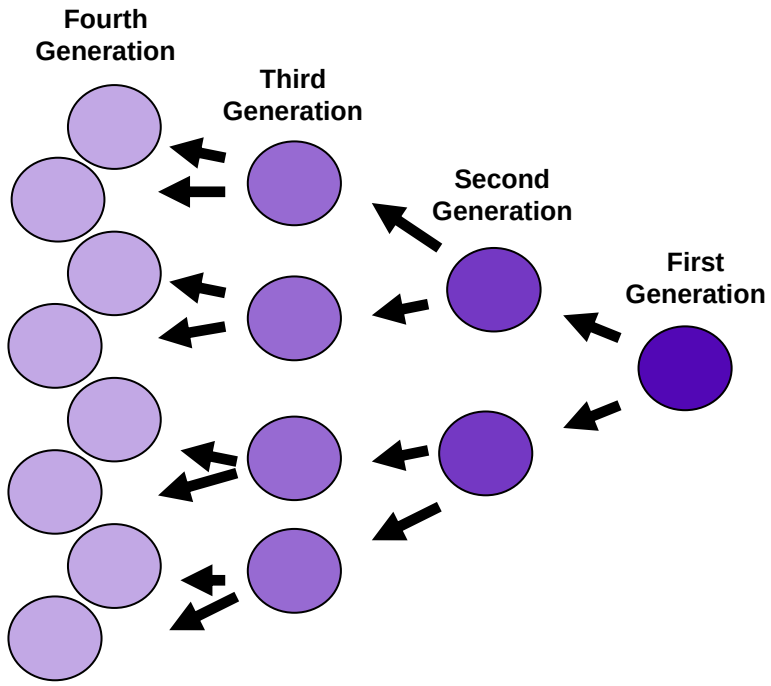
- **Impermeant DNA Dyes**
  - Add at final step, do not wash out
  - Emission is broad, consider for multicolor applications
  - Dead/Fixed cells can be used for compensation control

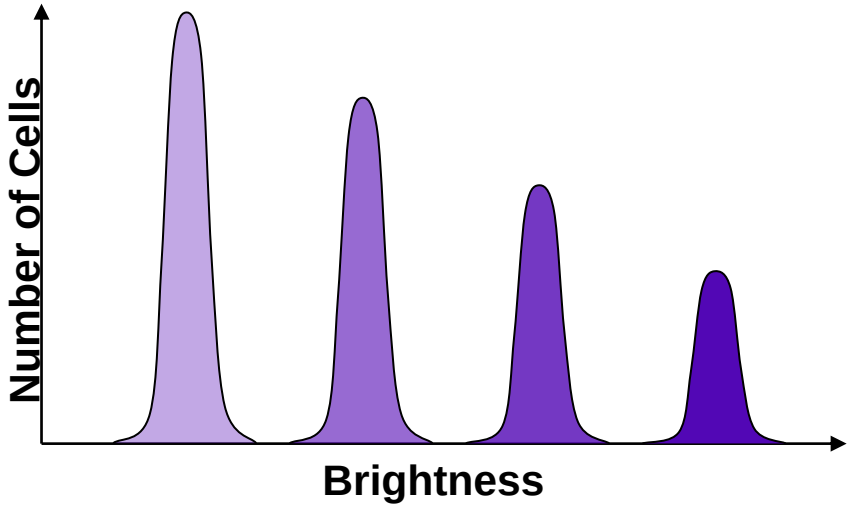
- **Amine-reactive Dyes**
  - Do not use protein in buffers
  - Live cells have dim fluorescence
  - Use with -aldehyde fixatives
  - Can be used without fixing cells too
  - ArC™ compensation beads useful

Presentation overview

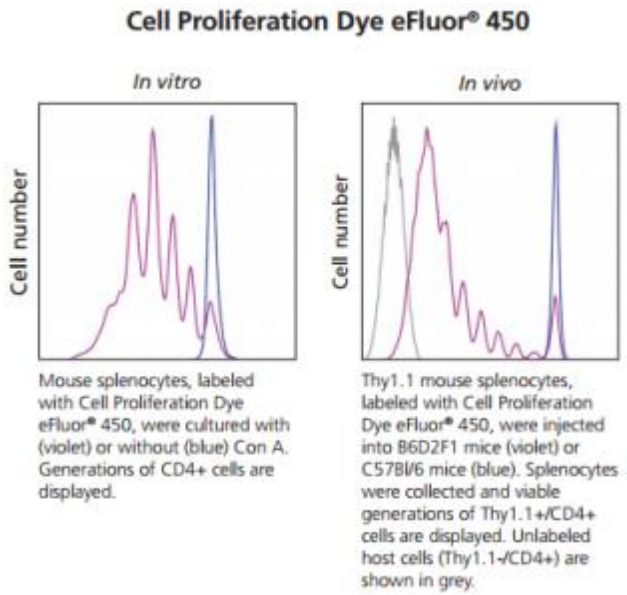
Cell proliferation analysis by dye dilution

- Cell permeant, cleaved by intracellular esterase giving 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





Cell proliferation dye

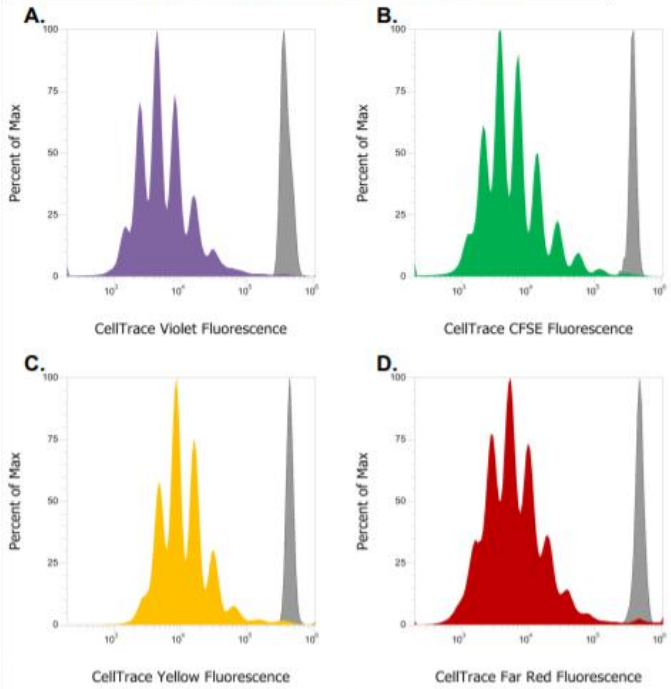


Mouse splenocytes, labeled with Cell Proliferation Dye eFluor® 450, were cultured with (violet) or without (blue) Con A. Generations of CD4+ cells are displayed.

Thy1.1 mouse splenocytes, labeled with Cell Proliferation Dye eFluor® 450, were injected into B6D2F1 mice (violet) or C57Bl/6 mice (blue). Splenocytes were collected and viable generations of Thy1.1+/CD4+ cells are displayed. Unlabeled host cells (Thy1.1-/CD4+) are shown in grey.

Cell Proliferation Dyes				
Description	**Excitation**	**Emission**	**Size**	**Cat. No.**
Violet Laser				
Cell Proliferation Dye eFluor® 450	409 nm	450 nm	500 µg	65-0842-85
			4 x 500 µg	65-0842-90
Blue Laser				
CFSE	488 nm	521 nm	5 x 500 µg	65-0850-84
Red Laser				
Cell Proliferation Dye eFluor® 670	633 nm	670 nm	500 µg	65-0840-85
			4 x 500 µg	65-0840-90

Figure 1. CellTrace Violet, CFSE, Yellow, and Far Red

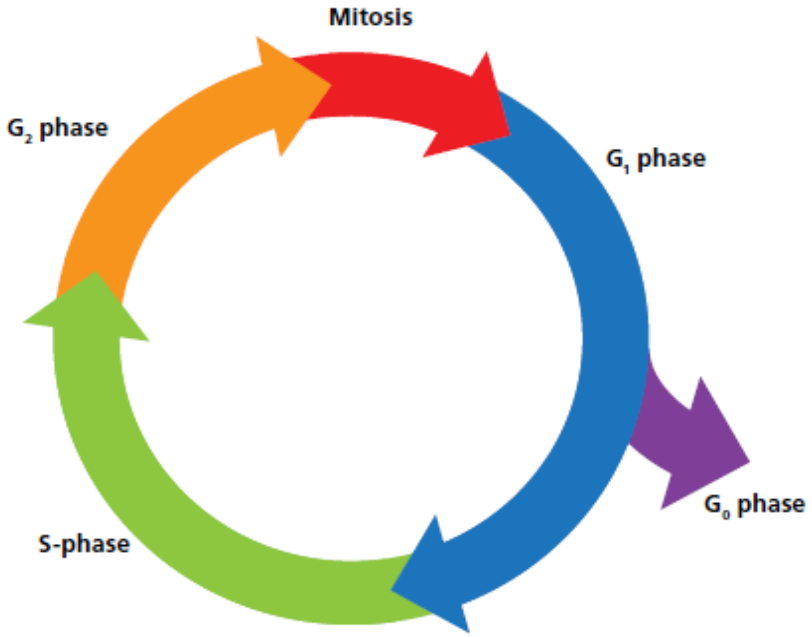


PBMC's were stained with (A) 5 µM CellTrace Violet, (B) 2 µM CellTrace CFSE, (C) 10 µM CellTrace Yellow, and (D) 2 µM CellTrace Far Red. Dynabeads Human T-Activator CD3/CD28 were used for T cell expansion and activation. Samples were incubated in OpTmizer T-cell Expansion Medium at 37° C / 5% CO_2 for 7 days. Samples were analyzed using SYTOX Green or SYTOX Red dead cell stains to gate on live cells and mouse anti-human CD4 Pacific Blue or CD4 FITC were used to gate on proliferating cells. The gray peaks represents unstimulated control cells (parent generation) and the peaks to the left of each gray peak represent individual generations of cells that proliferated during the course of the experiment.

Fluorescent label	CellTrace Blue	CellTrace Violet	CellTrace CFSE	CellTrace Yellow	CellTrace Far Red
Laser	UV	405	488	532, 561	633/635
Ex/Em (nm)	355 or 375/410	405/450	495/519	546/579	630/661
Multiplexable	✓	✓	✓	✓	✓
20 tests	C34574	C34571	C34570	C34573	C34572
180 tests	C34568	C34557	C34554	C34567	C34564

ThermoFisher SCIENTIFIC

Cell cycle and proliferation by flow



Cell cycle analysis

G_0 phase: Resting cells have zero growth

G_1 phase: Enzyme synthesis is required for DNA replication

S-phase: DNA replication producing two identical sets of chromosomes

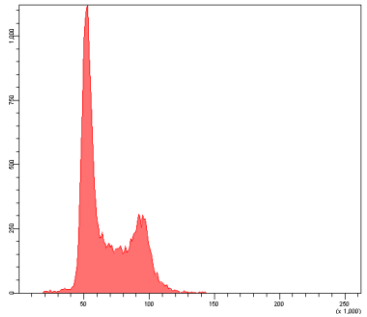
G_2 phase: Protein synthesis occurs

Mitosis: The nucleus and cell divide

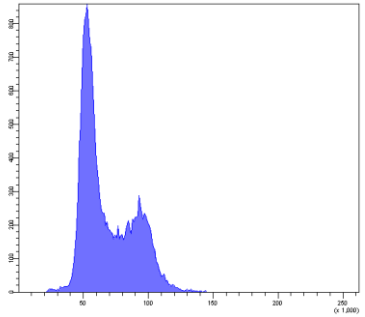
Methods to Evaluate Cell Proliferation			
BrdU	**Ki-67**	**PCNA**	**Proliferation Dyes**
Measures cells in S-phase only	Measures proliferating cells at any cell cycle stage except G_0	Measures S-phase but also includes late G_1 phase	Measures generational proliferation
Pulse-labeling common to avoid cytotoxicity	BrdU is a subset of Ki-67 positive cells	Data supports IHC applications Not as robust for flow cytometry	Long-term labeling assay. Does not require fixation
In long-term culture, BrdU can be pulse-labeled and washed out Dividing cells will not incorporate BrdU so toxicity is diluted	Ki-67 and BrdU are used together in both IHC and flow cytometry		Cannot distinguish cell cycle phases of daughter cells

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 ex)** **dsDNA**
- **Vybrant® DyeCycle™ Ruby stain (488–633 ex)** **dsDNA**



Impermeant nucleic acid dyes

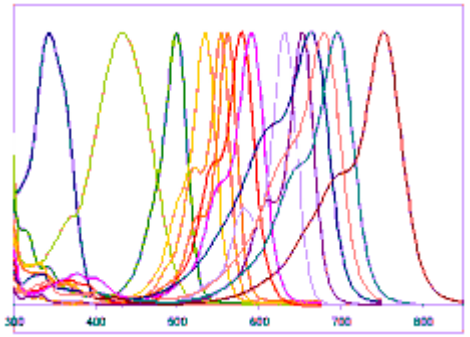
- Dyes which penetrate cells with a compromised cell membrane to stain nucleic acids, but do not cross the membranes of live cells
  - >**Can be used to identify dead cells in a population**
  - >**Can be used to quantitate DNA content in fixed cells**

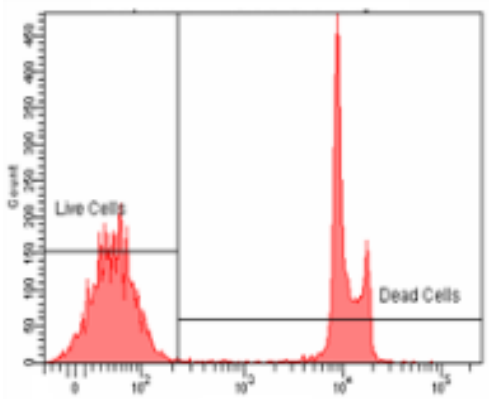
- Propidium Iodide (488 nm ex)
- 7-AAD (488 nm ex)
- SYTOX® AADvanced™ dead cell stain (488 nm ex)
- SYTOX® Green dead cell stain (488 nm ex)
- SYTOX® Orange dead cell stain (488 /532/561 ex)
- SYTOX® Blue dead cell stain (405 nm ex)
- SYTOX® Red dead cell stain (633 nm ex)

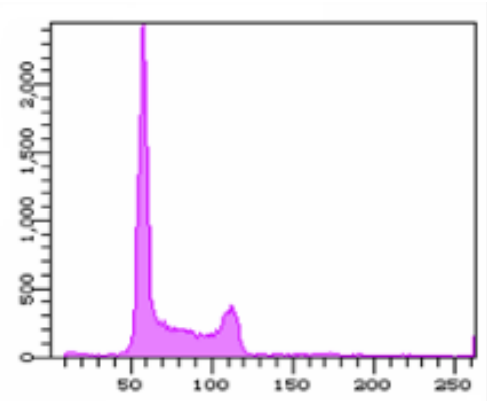
Which dye to use for cell cycle analysis?

Things to think about:

- Excitation source
- Emission of dye
- Instrumentation
- Application
- Specificity
- Live DNA content analysis
- Fixed DNA content analysis







Presentation overview

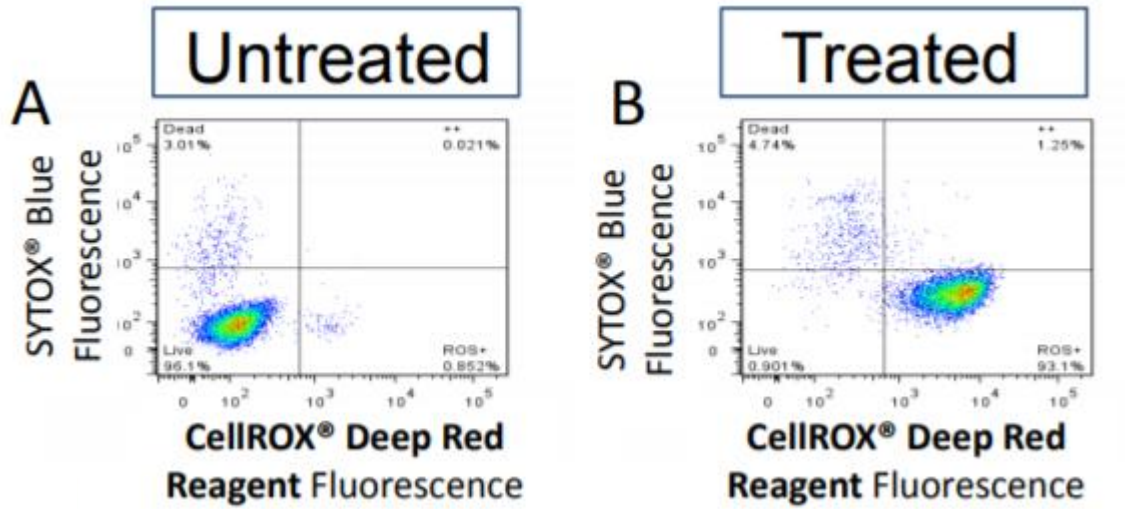
Oxidative stress

- Oxidative stress – imbalanced production of reactive oxygen species (ROS) and the ability of the cell to scavenge them
- ROS react with nucleic acids, proteins and lipids causing cell and tissue damage
- Can be measured using ROS indicators.

	CellROX Deep Red Reagent	CellROX Orange Reagent	CellROX Green Reagent	CM-H_2-DCFDA
Common filter set	Cy5	RFP	FITC	FITC
Reporter	CellROX Deep Red Reagent	CellROX Orange Reagent	CellROX Green Reagent	H_2-DCFDA
Ex/Em (nm)	640/665	545/565	485/520	495/527
Cat. No.	C10422	C10443	C10444	C6827

Oxidative stress - CellROX

- Fluorogenic probes for measuring generalized oxidative stress in cells
- Detection and quantitation of reactive oxygen species (ROS)
- Live cell compatible (cell permeable)
- Nonfluorescent in a reduced state and fluoresce upon oxidation by ROS
- Some are fixable/detergent resistant for multiplexing
- Reagent can be applied to cells in complete growth media



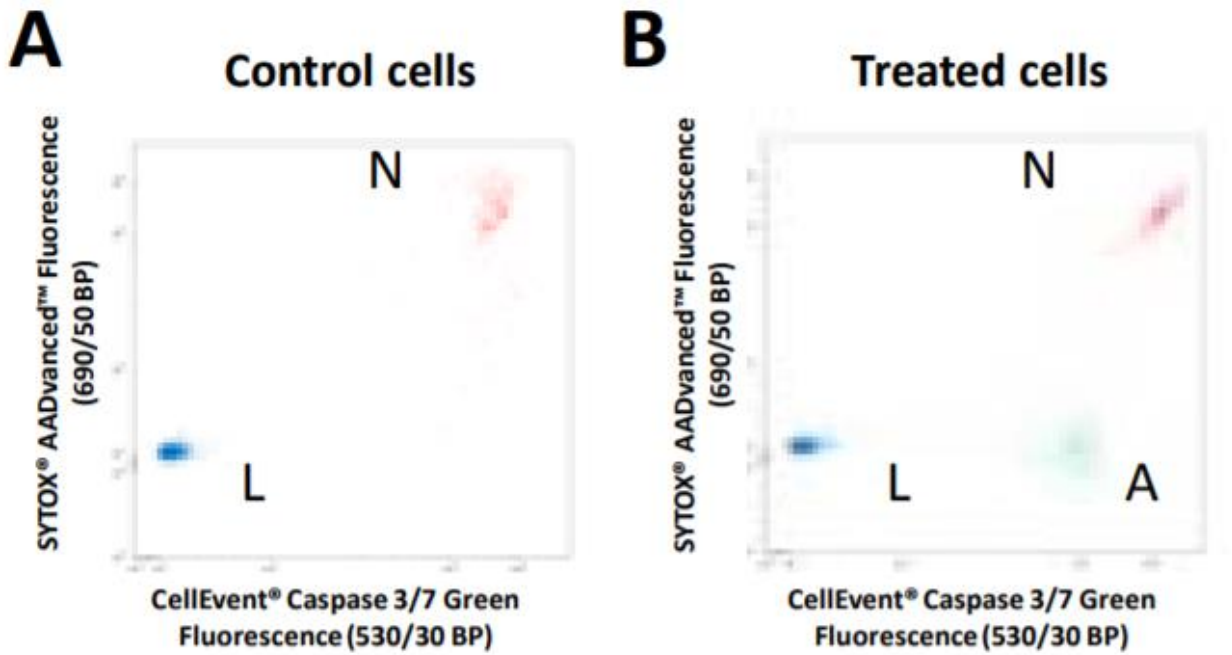
Presentation overview

Apoptosis assays for flow cytometry

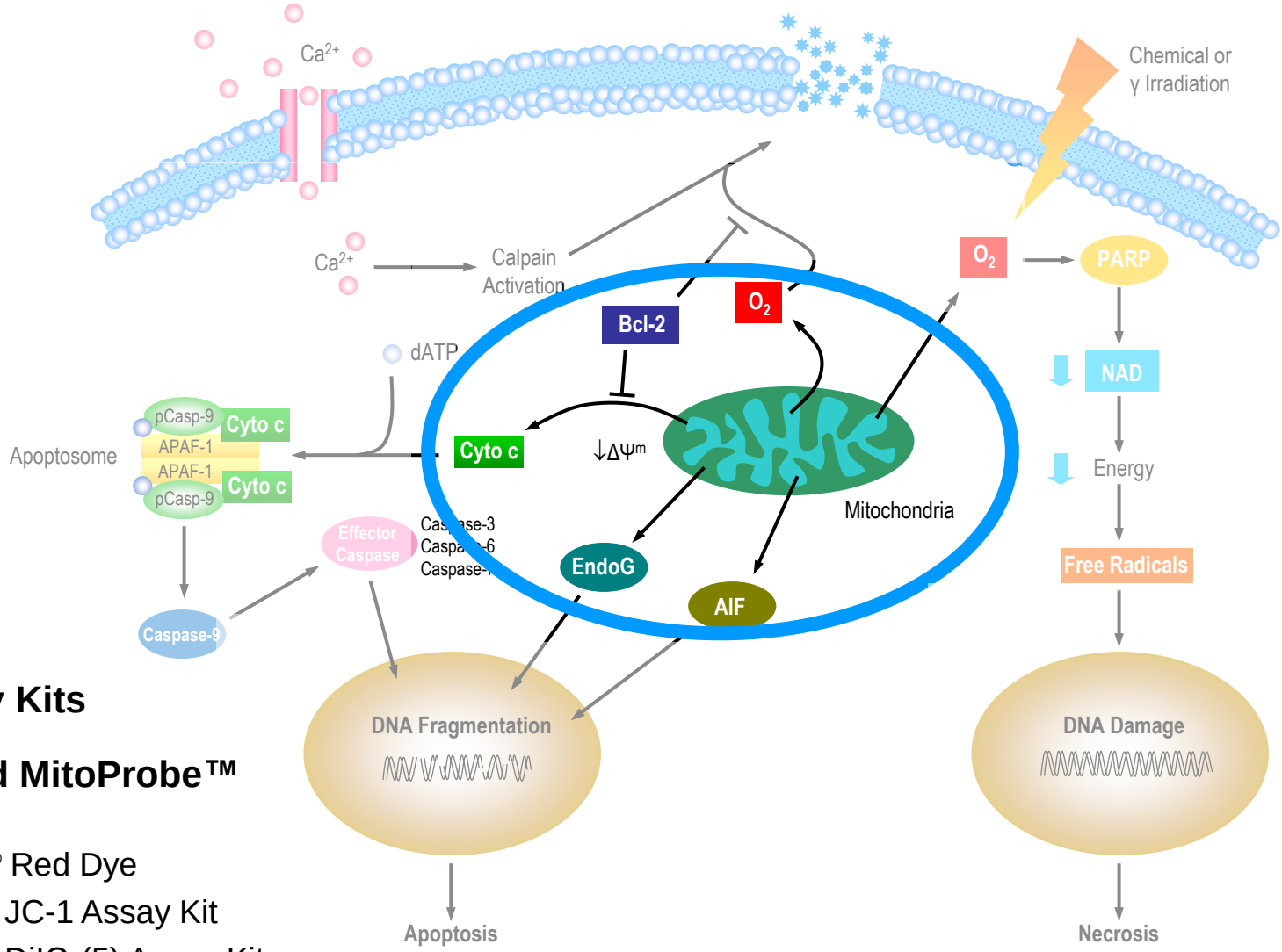
- Plasma membrane changes
  - Annexin V
  - Membrane permeability
  - Membrane asymmetry
- Nucleus
  - Chromatin condensation
  - DNA fragmentation
- Mitochondrial membrane changes
  - Membrane potential
  - Mitochondrial transition pore
- Cytoplasm
  - Caspase activity

Apoptosis - caspase detection

- CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (C10740, C10427)
  - Detection of activated caspases 3 and 7
  - Cell-permeant reagent conjugated to nucleic acid binding dye
  - Reagent contains caspase-3/7 recognition sequence
  - Fluorescent detection upon cleavage and DNA binding



MitoProbe™ Reagents: Detecting mitochondrial changes



Apoptosis Assay Kits

MitoTracker® and MitoProbe™ Assay Kits

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

Apoptosis - annexin V

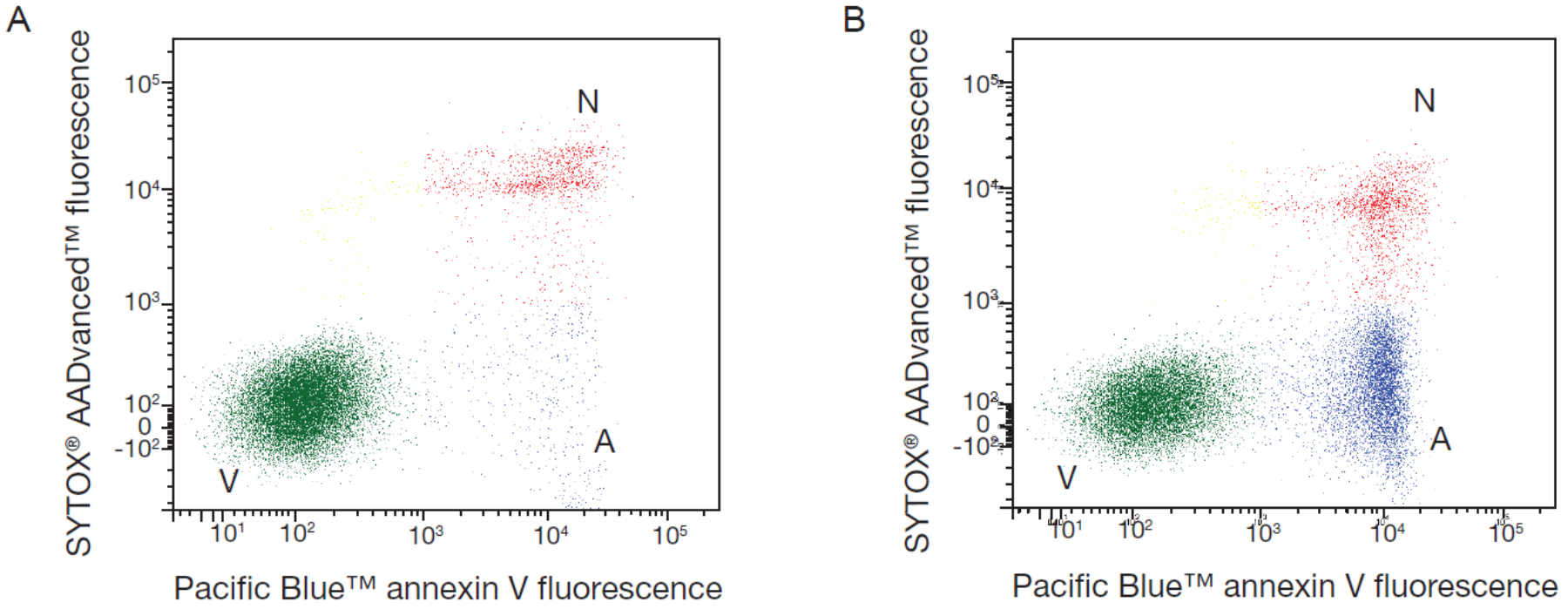


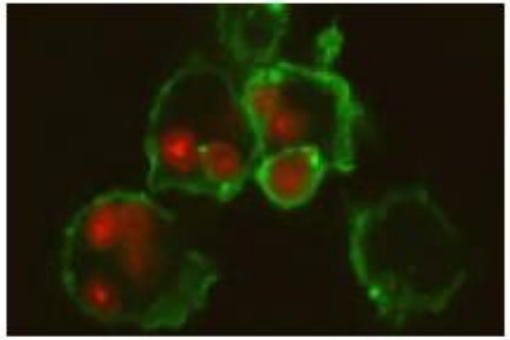
Figure 1. Jurkat cells (T-cell leukemia, human) treated with 10 µM camptothecin for four hours (panel B) or untreated control (panel A). Cells were treated with the reagents in the kit and analyzed by flow cytometry using 405 nm and 488 nm excitation. Note that the camptothecin-treated cells have a higher 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.

Presentation overview

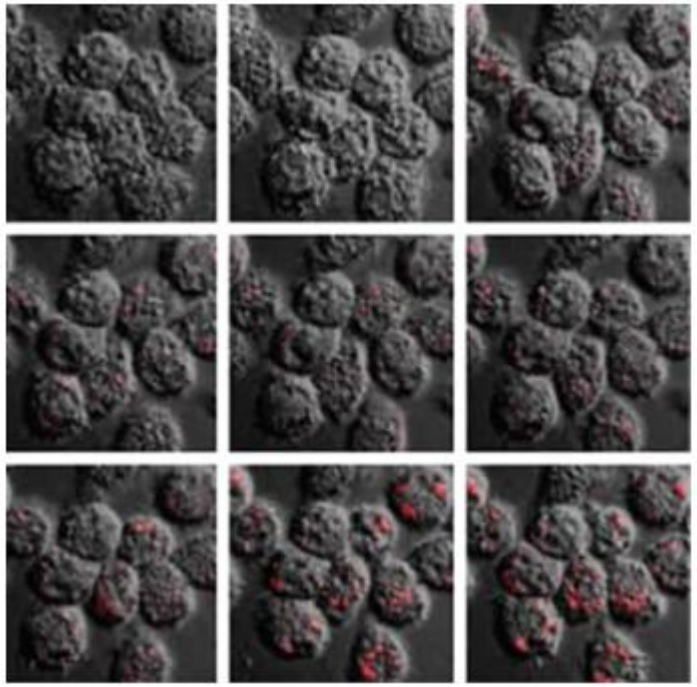
Phagocytosis, Endocytosis, Receptor Internalization

- Cells internalize particulate matter such as microorganisms, and this process is important for immune responses and during the clearance of apoptotic cells
- Tracking phagocytosis using a quench/wash-based assay can report on simple uptake, or a pH indicator can be used monitor stages in the pathway.

- pHrodo dyes are essentially non-fluorescent at neutral pH and exhibit increasing signal with a red or green readout respectively as the pH decreases. The increase in fluorescent signal can be used to monitor progression in the phagocytic pathway.



MMM macrophage cells incubated with Zymosan A (*S. cerevisiae*) BioParticles, Alexa Fluor 594 Conjugate and washed in Live Cell Imaging Solution before imaging.

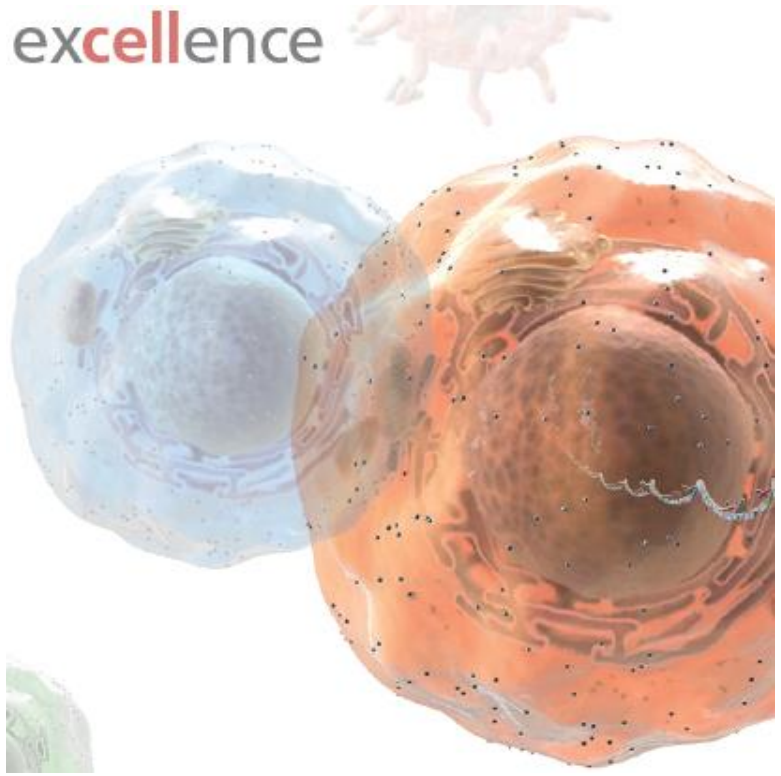


pHrodo Bioparticles for Phagocytosis

	pHrodo Red *E. coli* BioParticles Phagocytosis Kit for Flow Cytometry	pHrodo Red Phagocytosis Particle Labeling Kit for Flow Cytometry	pHrodo Green *E. coli* BioParticles Phagocytosis Kit for Flow Cytometry	pHrodo Green *S. aureus* Bioparticles Phagocytosis Kit for Flow Cytometry
Readout	Measures phagocytic activity in whole blood samples by flow cytometry			
Range	Monitors phagosome formation			
Vehicle or Method	*E. coli*	Label your own particles	*E. coli*	*S. aureus*
Common filter set	TRITC		FITC	
Labels	pHrodo Red		pHrodo Green	
Ex/Em (nm)	500/585		509/533	

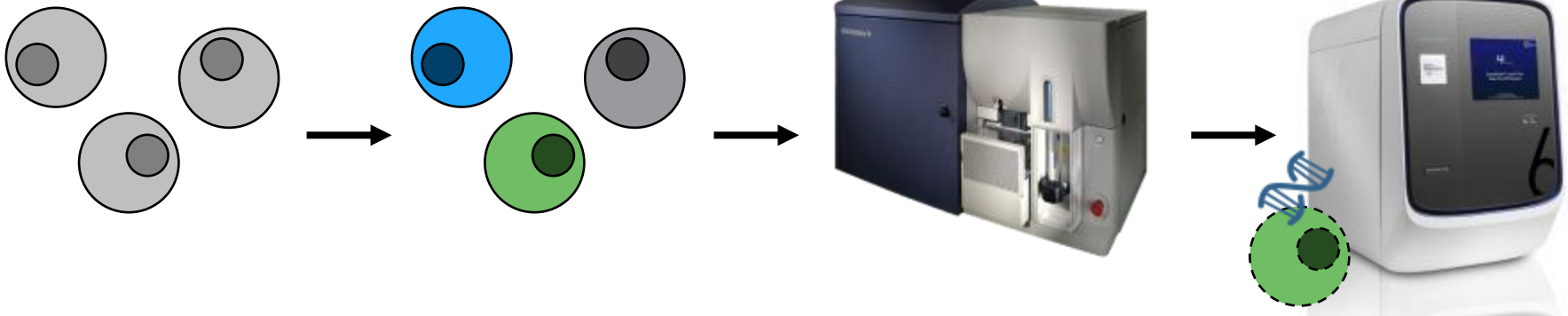
Presentation overview

What is PrimeFlow® RNA assay?



- **RNA** AND **protein** expression in millions of single cells
- Novel *in situ* hybridization assay: Simultaneous detection of **4 RNA (mRNA, lncRNA, vRNA & miRNA!)** using your flow cytometer
- **Combine** with your antibodies: **Cell surface** and **IC proteins**

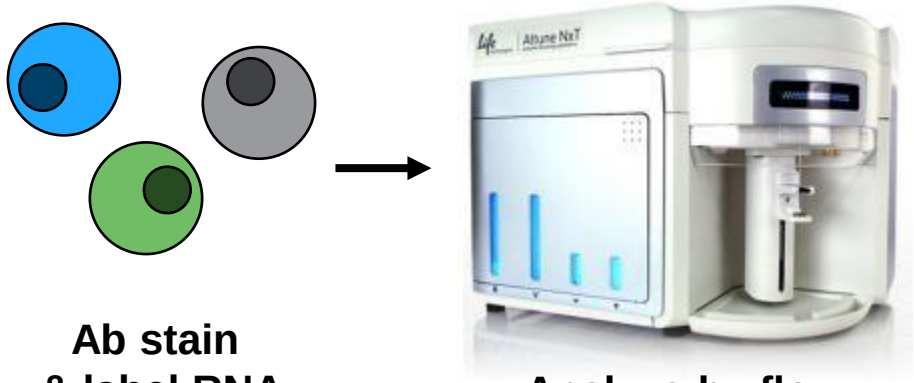
Transcript characterization… until now



Culture cells → Ab stain → FACS sort → Purify RNA, RT for cDNA, qPCR

REPEAT

VERSUS



Ab stain & label RNA → Analyze by flow

Transcript characterization… until now

SENSITIVE

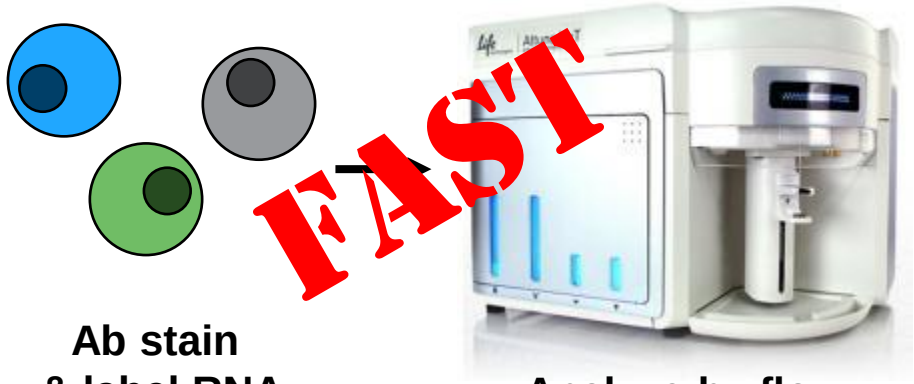
Culture cells

Ab stain

FACS sort

Purify RNA
RT for cDNA
qPCR

REPEAT



FAST

Ab stain
& label RNA

Analyze by flow

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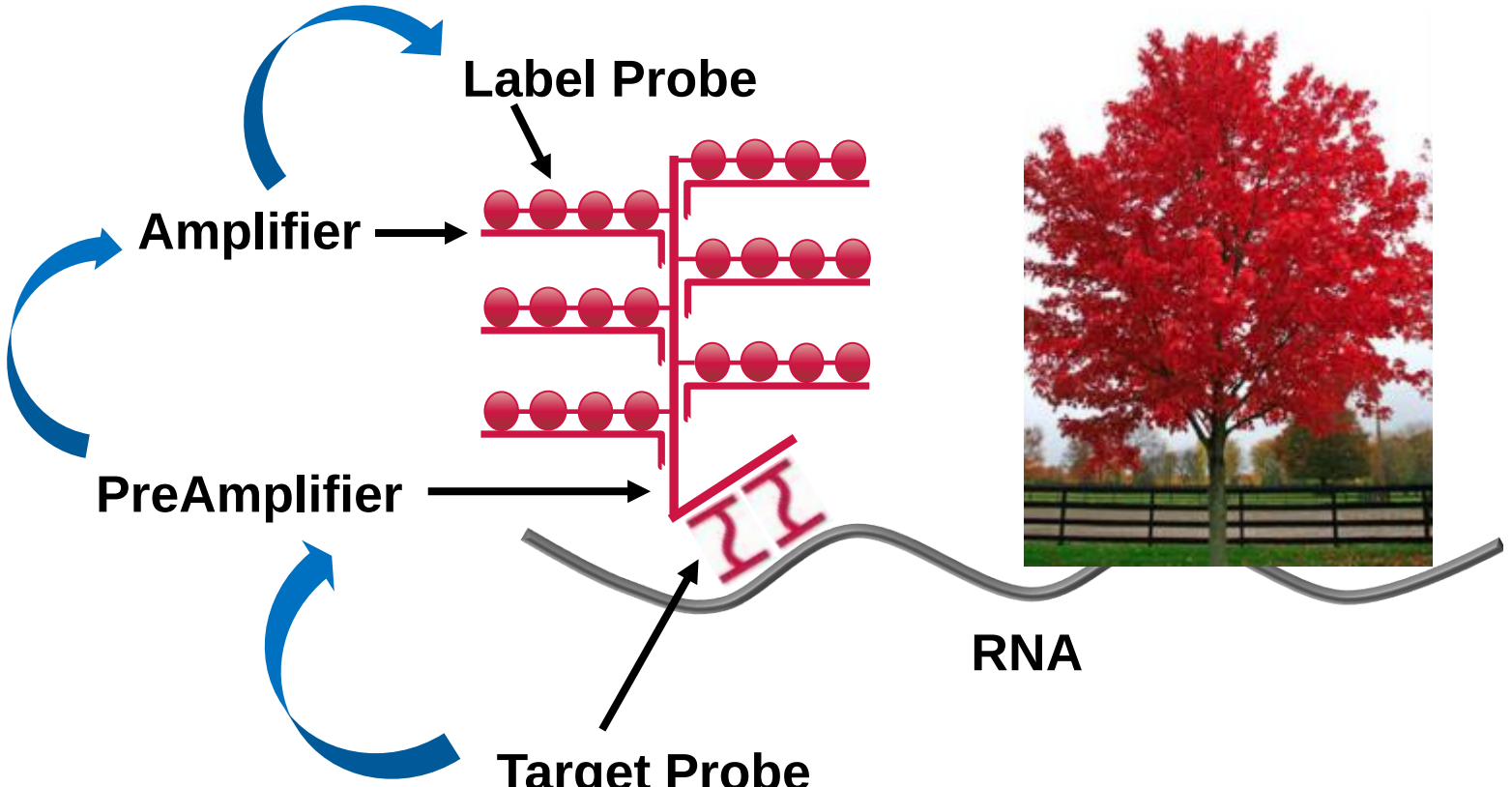
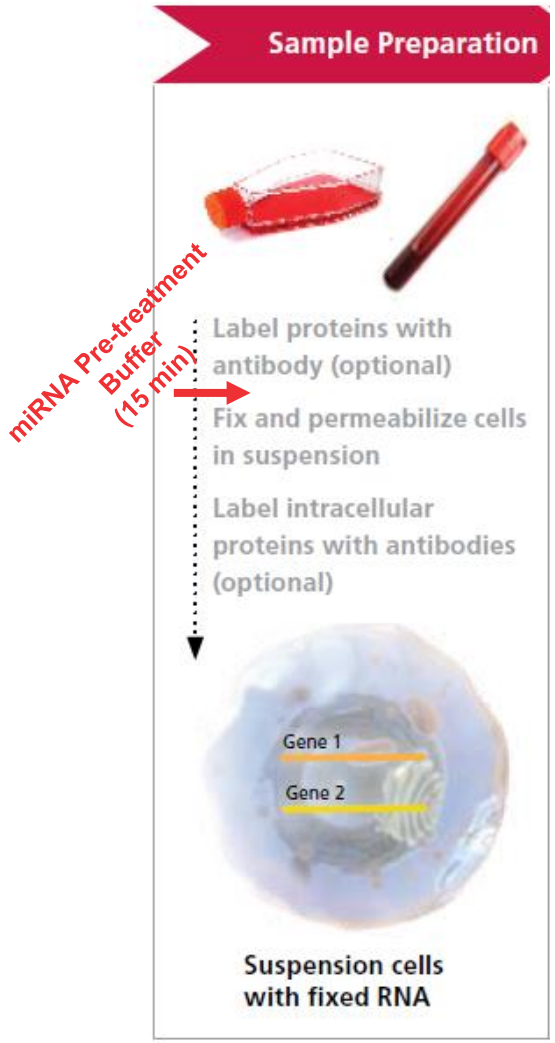
Branched DNA: How does it work?



Target Probe

- Custom oligos binding to specific RNA target region
- Each oligo pair secures one bDNA “tree”

Workflow overview



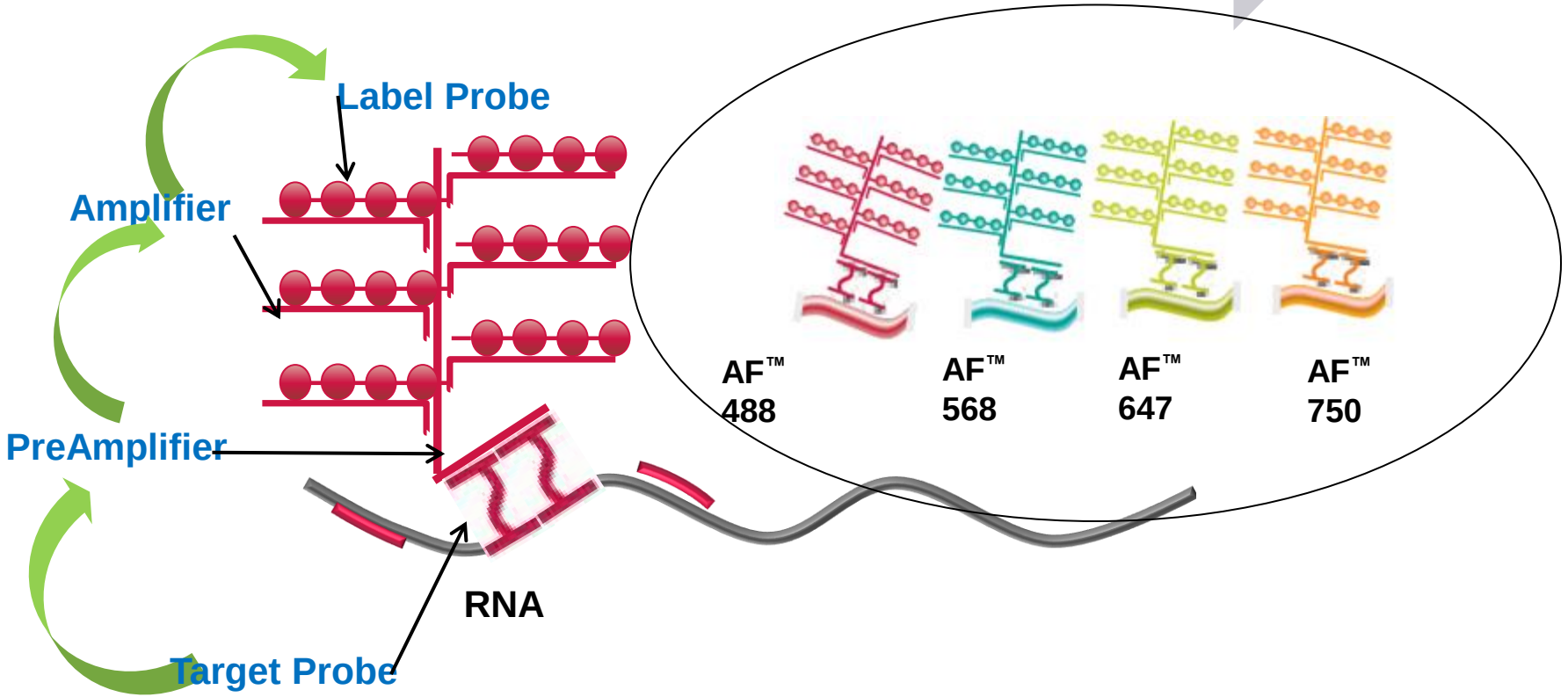
PrimeFlow RNA assay allows detection of (mi)RNA by Flow

probe design

bDNA Signal amplification

multiplex 4 RNA transcripts/ miRNA

Surface and intracellular protein staining

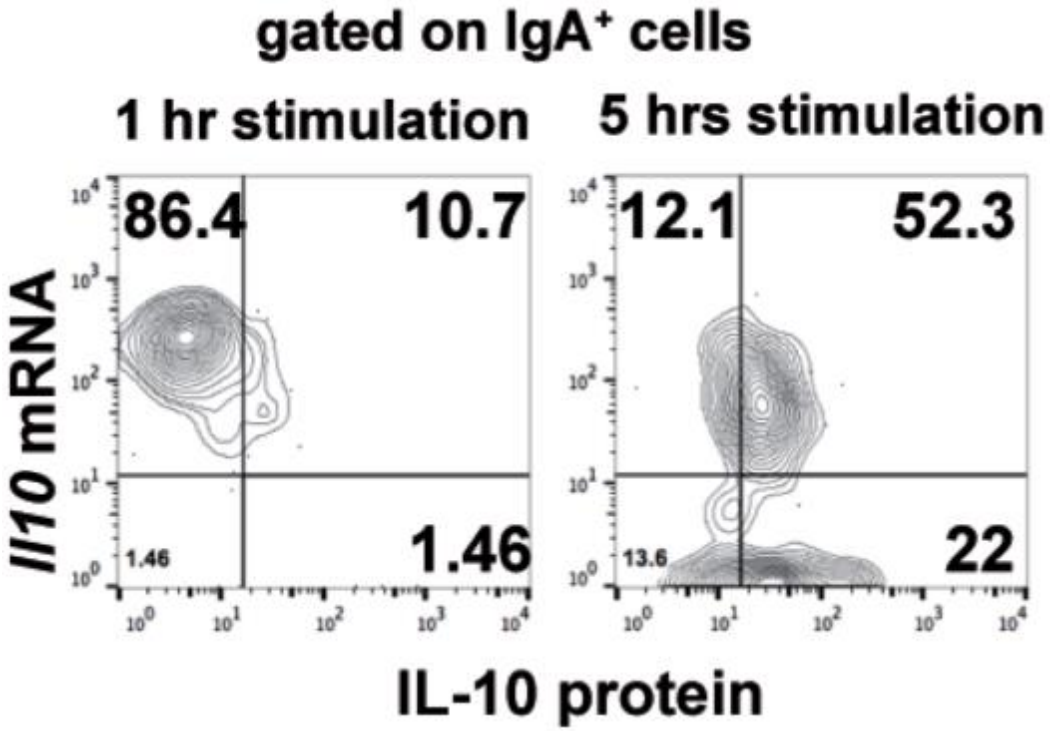


Why PrimeFlow?

- Gene expression heterogeneity
  RARE POPULATIONS
- Simultaneous transcription + translation studies
  Cytokines
- Viral immunology
  Replication and latency (RARE POPULATIONS!)
- No antibody? No problem!

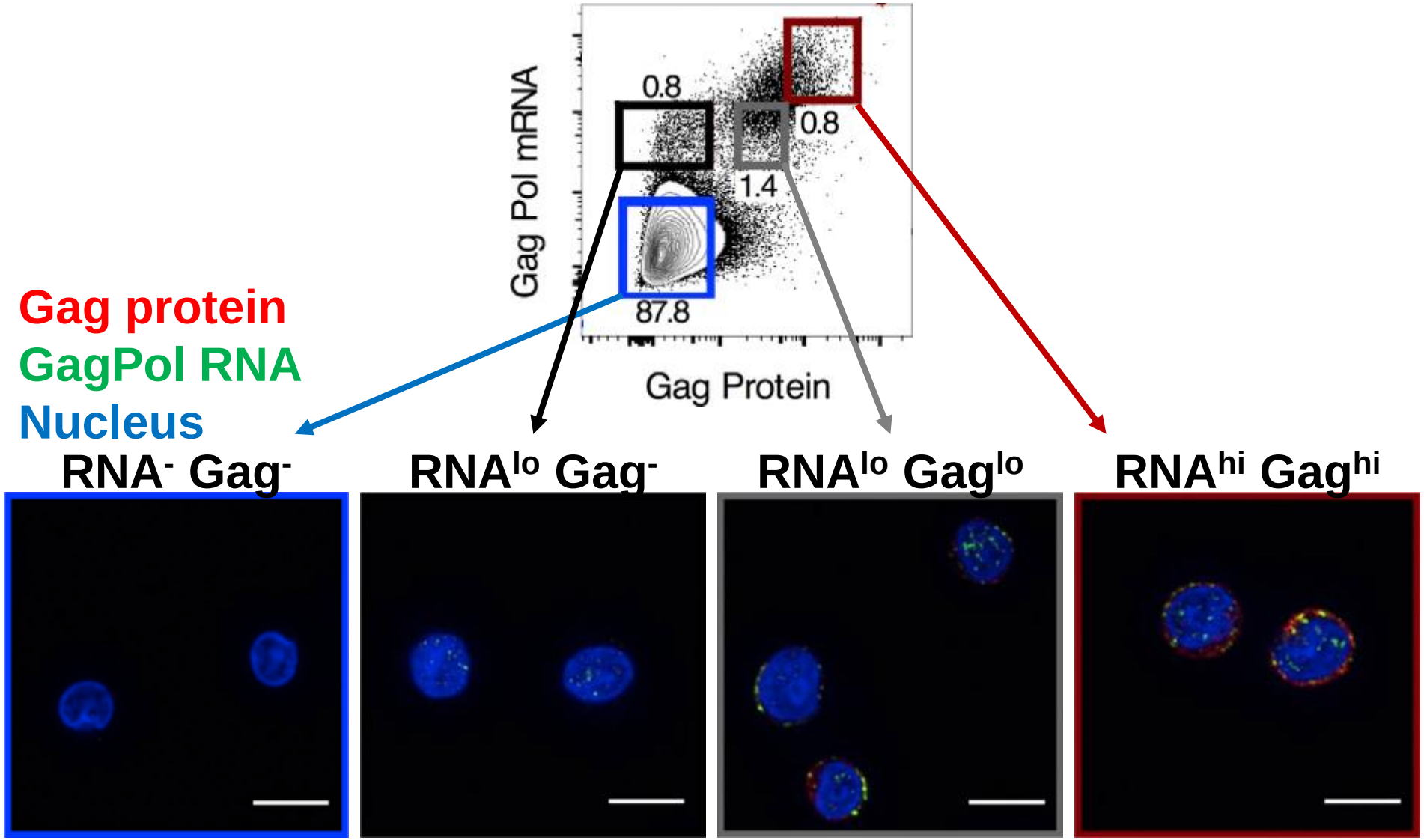
IgGA^{hi} B cells regulate tumor-infiltrating CD8 T cells

Mouse prostate cancer model



Shalapour *et al*., 2015, *Nature*

Visualizing HIV protein & RNA

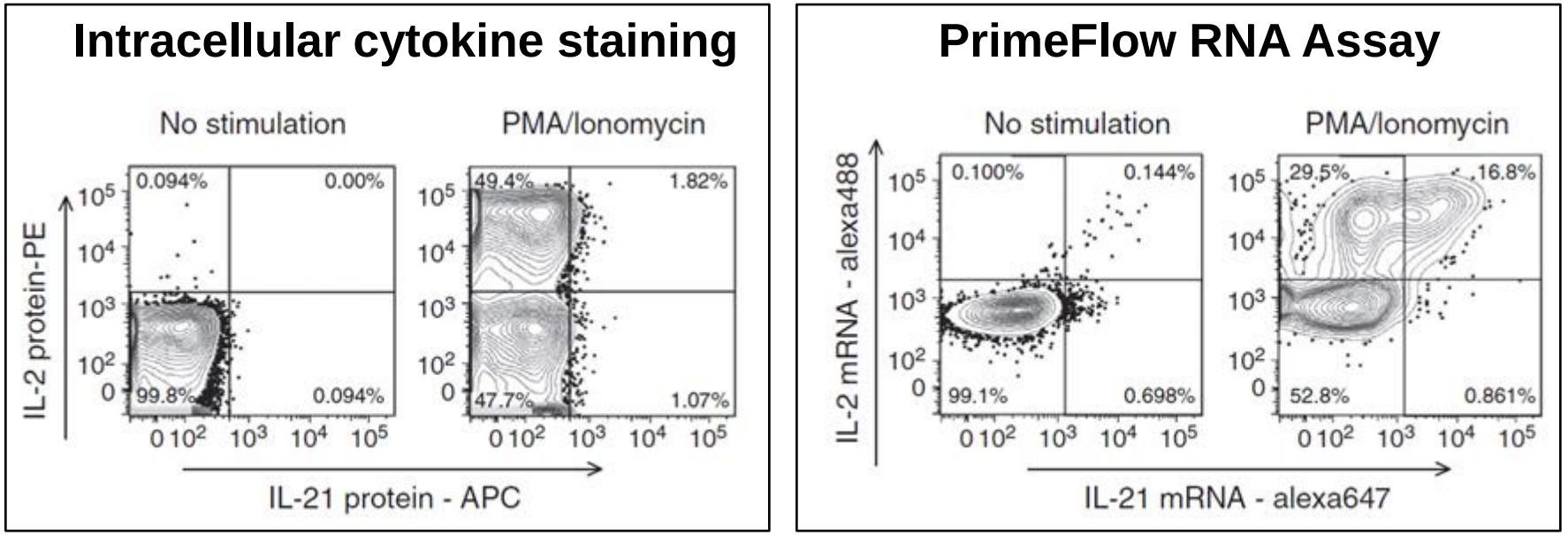


Baxter *et al*., 2016, *Cell Host & Microbe*

No antibody? No problem!

IL-21 in Idiopathic thrombocytopenic purpura (ITP)

No good Ab for IL-21!



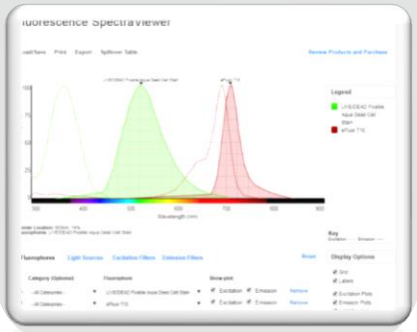
Porichis *et al*., 2014, *Nature Communications*

Summary

Stimulation	Cell health	Antibodies	RNA flow	Everyday essentials	Instrumentation
Activating and neutralizing antibodies	Apoptosis, proliferation, viability	Conjugated antibodies for flow cytometry	Invitrogen™ PrimeFlow™ assays	Beads, buffers, controls	Attune NxT Flow Cytometer with Autosampler

Antibody and Flow Cytometry Experimental Resources

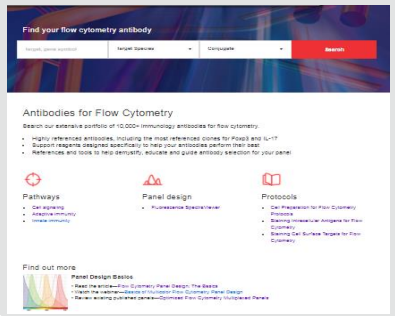
Fluorescence SpectraViewer



Multicolor panel building

Learn more at:
thermofisher.com/spectraviewer

Antibody Center



Antibody education and purchase

Learn more at:
thermofisher.com/flowantibodies

Invitrogen™ eBioscience™ reagents

Immunology antibody education and purchase

Learn more at:
thermofisher.com/antibodies/ebioscience

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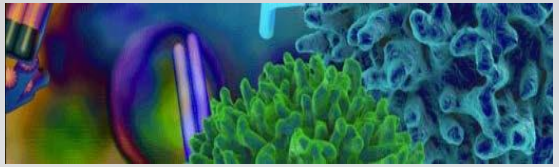
Flow Cytometry Learning Center



Flow Cytometry Learning Center
Learn more about flow cytometry applications, techniques, and basic principles.

Molecular Probes™ School of Fluorescence—Flow Cytometry Basics
Learn how a flow cytometer works including the fluidics, optics and electronics. This is a free resource to help you get started with flow cytometry, which can be a complex and challenging application.

Flow Cytometry Resource Library
Curated collection of scientific application notes, publications, videos, webinars, and scientific posters for flow cytometry.

- **Flow Cytometry Application Notes, Scientific Posters, and BioProbes Articles** Various applications, providing the conditions and reagents used to achieve the results. Scientific posters presented by our R&D scientists at key conferences. Articles from BioProbes Journal.
- **T Cell Stimulation and Proliferation eLearning Course** Modular, animated, and narrated eLearning course on T cell activation and the methods used to measure T cell function. Knowledge checks and a practical application session.
- **Flow Cytometry Educational Videos & Webinars** Media for researchers interested in flow cytometry.
- **Flow Cytometry Research Tools** Fluorescence SpectraViewer, flow cytometry panel design tool, antibodies search tool, mobile apps and more.
- **Flow Cytometry Protocols** Step-by-step instructions for successful fluorescence-based assays to measure cell proliferation, viability, and vitality using your flow cytometer.
- **The Molecular Probes™ Handbook—A Guide to Fluorescent Probes and Labeling Technologies** Extensive references and technical notes. Contains 3,000 technology solutions representing a wide range of biomolecular labeling and detection reagents.

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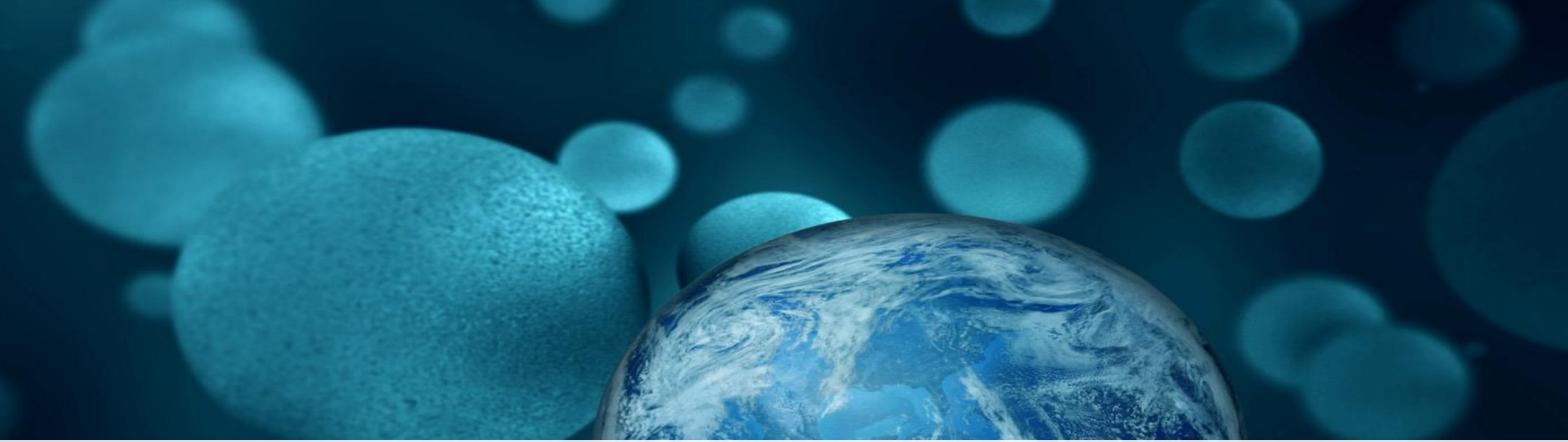

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Questions?

Thank you!