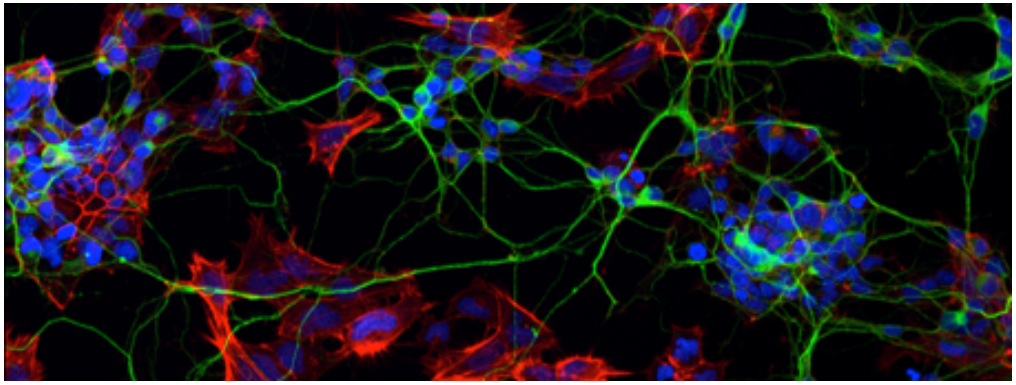
INTRACELLULAR FLOW CYTOMETRY

Validation and Optimization

Edward Verwayen



Cell Signaling TECHNOLOGY®

Agenda

Part 1:

Introduction to Cell Signaling Technology

What is intracellular Flow Cytometry

Antibody validation for Flow Cytometry

Fluorescent-conjugated antibodies

Part 2:

Protocol optimization for the detection of intracellular targets

Uses of intracellular Flow Cytometry

FAQ

Cell Signaling Technology (CST)

"To deliver the world's highest quality research products that accelerate biological understanding and enable personalized medicine"

Dedicated to making high quality products

Rigorous validation of products in several applications

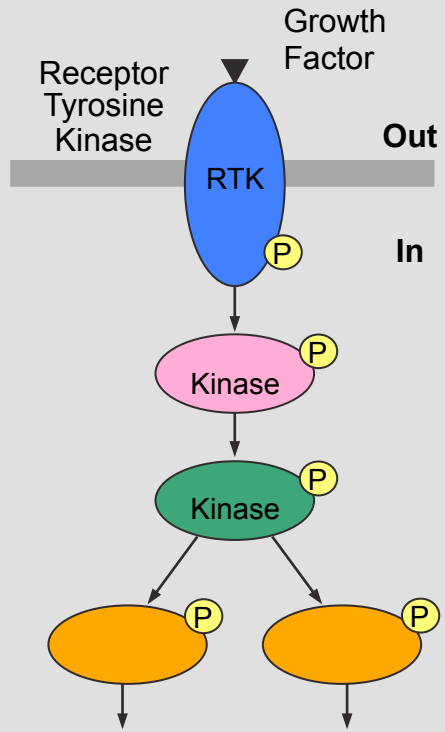
Technical support provided by CST scientists

Cell Signaling TECHNOLOGY®

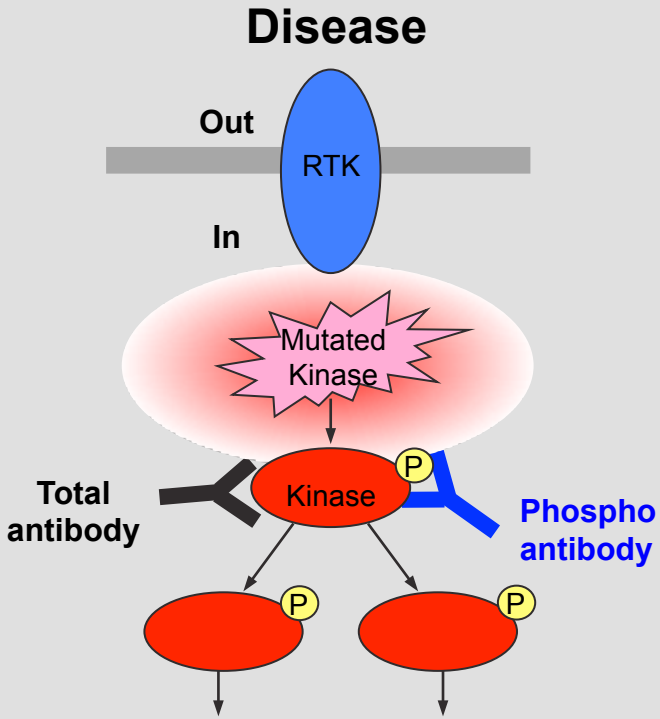
CST develops rabbit monoclonal antibodies

- Signal transduction targets are highly conserved in mice, rats and humans
- Rabbits elicit a stronger immune response
- Simpler structure:
  - No IgG subclasses
  - Fewer amino acids at the N-terminus and D-E loop
  - Extra light-chain disulfide bond gives greater stability
- Higher binding affinity: Increased sensitivity
- Robust reproduction

CST product expertise

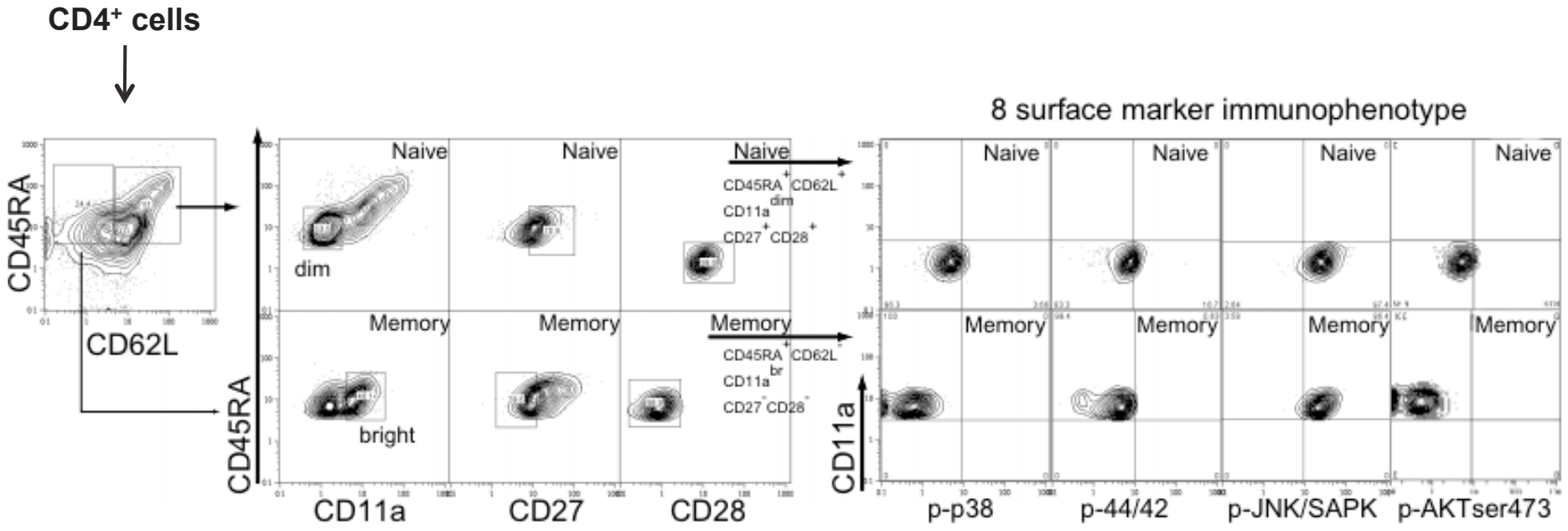


Growth, Proliferation, Gene Transcription, Protein Secretion…



Abnormal Growth, Proliferation, etc.

How does intracellular flow cytometry work?

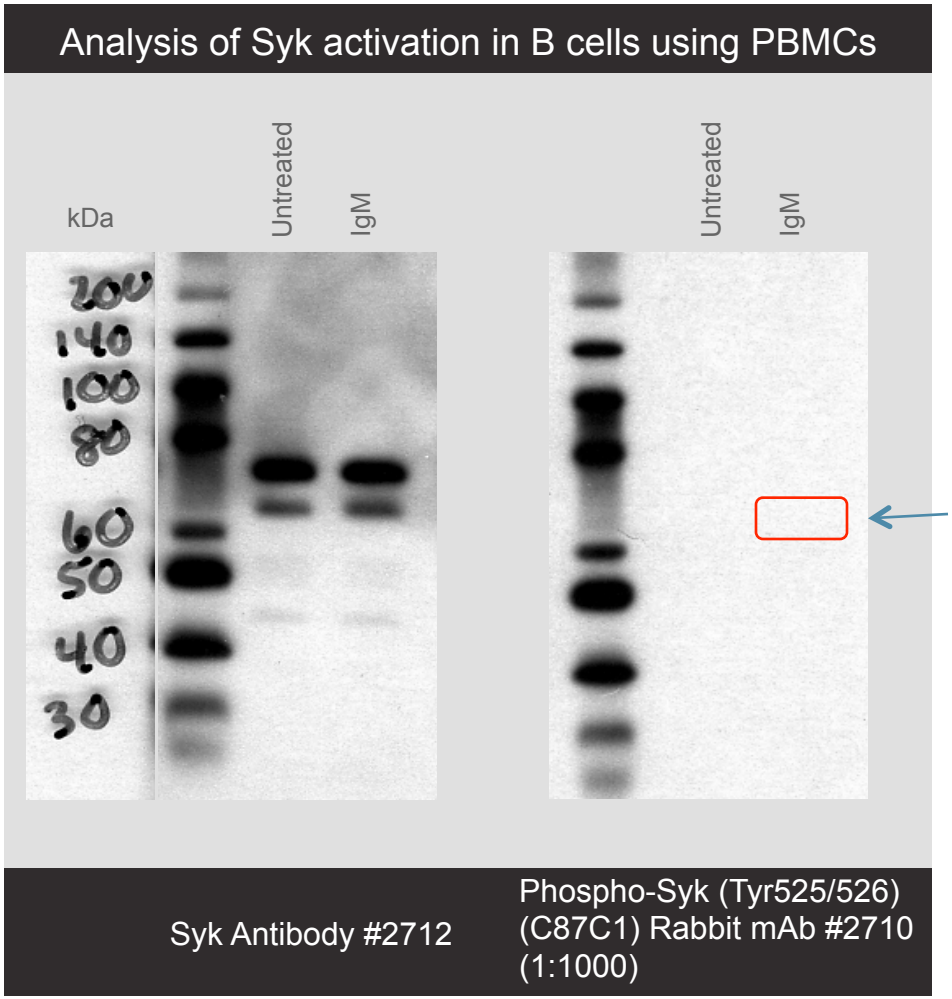


Perez & Nolan, 2002. Nat. Biotech.

Distinguish Th4 cell status

Memory and naïve Th4 cells display different protein activation status

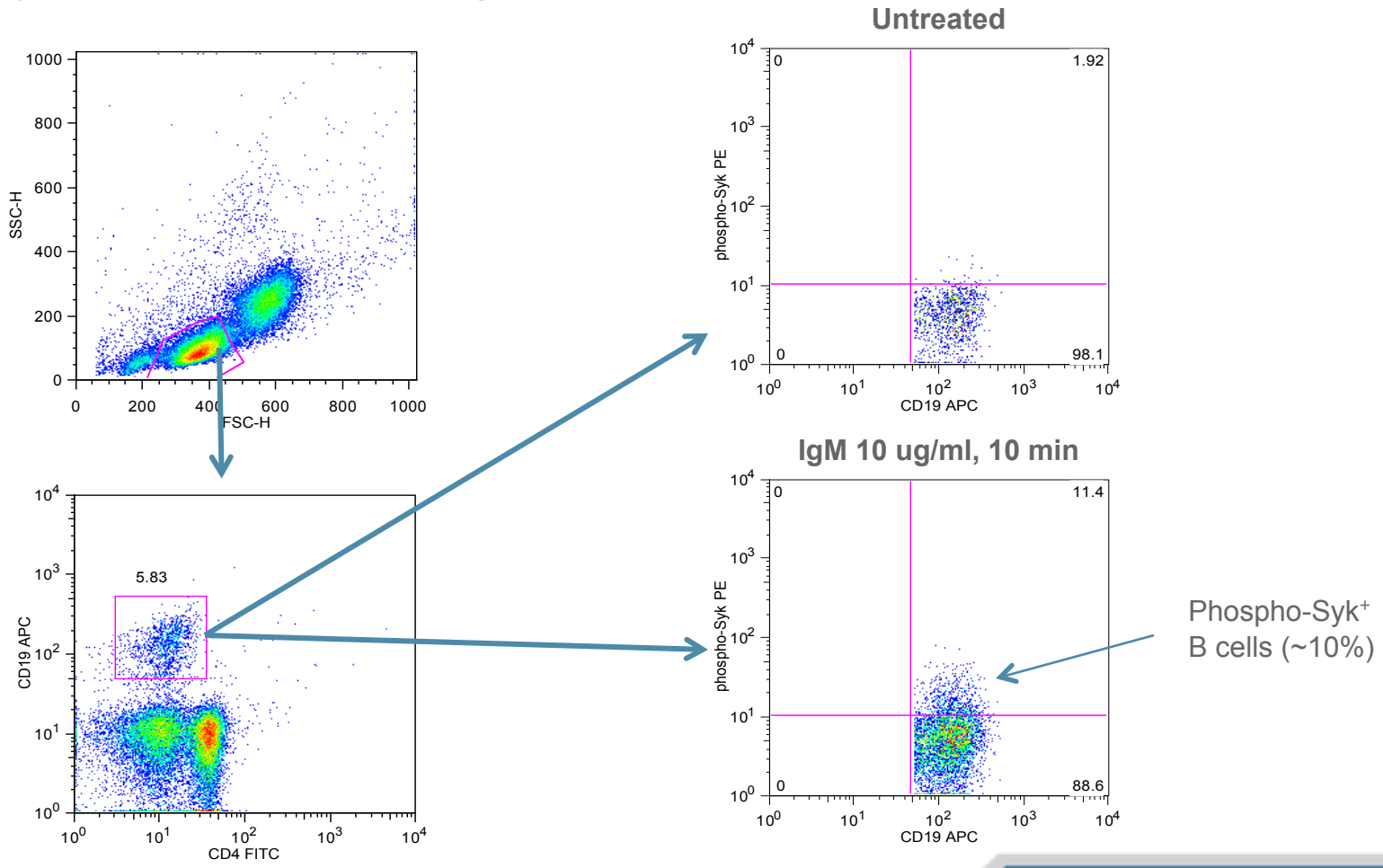
Analysis of cellular signaling by western blot and flow cytometry



- Ability to analyze rare cell subsets

Analysis of cellular signaling by western blot and flow cytometry

- Analysis of Syk activation in B cells using PBMCs



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Antibodies for Intracellular Flow Cytometry

The importance of validation

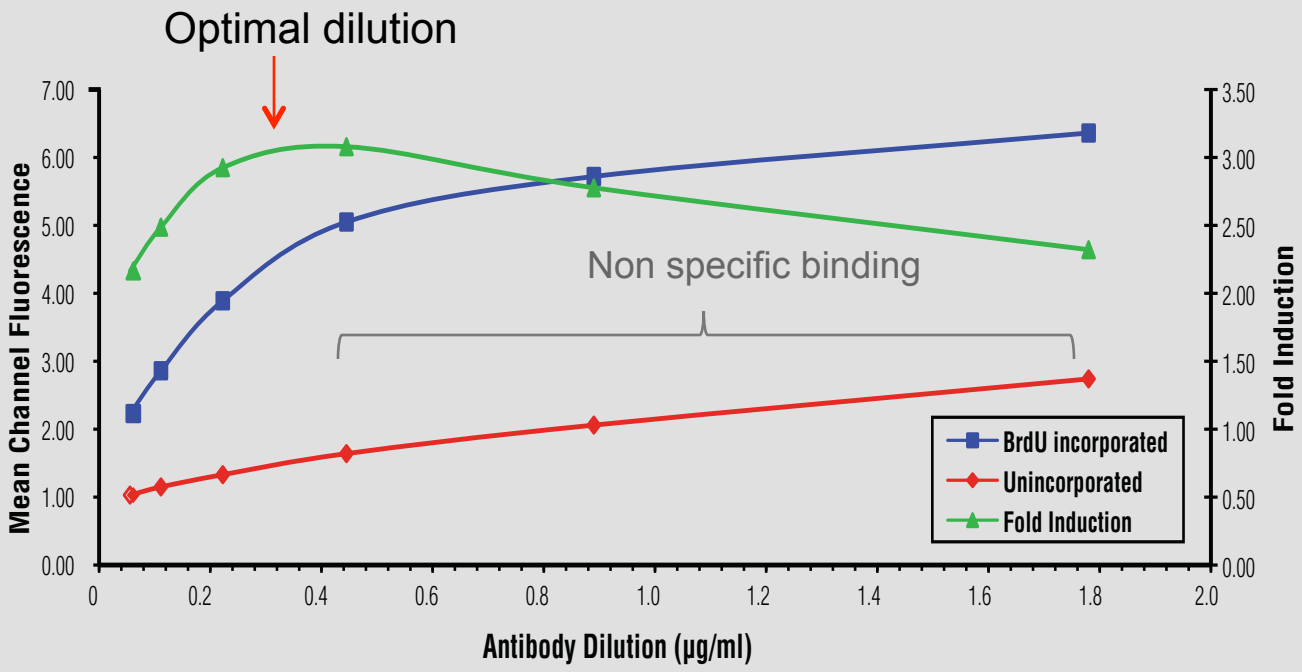
Antibody validation at CST

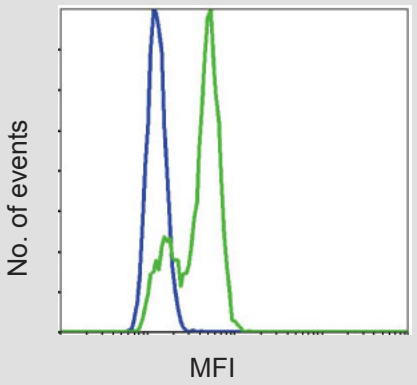
CST scientists follow a stringent validation protocol :

- Negative vs. positive control samples:
  - Expressing vs. non-expressing cell line
  - Modulated vs. un-modulated
  - Test antibody vs. isotype
- Signal to noise ratios
- Titration curve of S/N values to determine optimal working concentration
- Testing in multiple applications

Validation of flow cytometry antibodies: Performance

- Serial titration to determine optimal working dilution of each antibody





BrdU (Bu20a) Mouse mAb #5292

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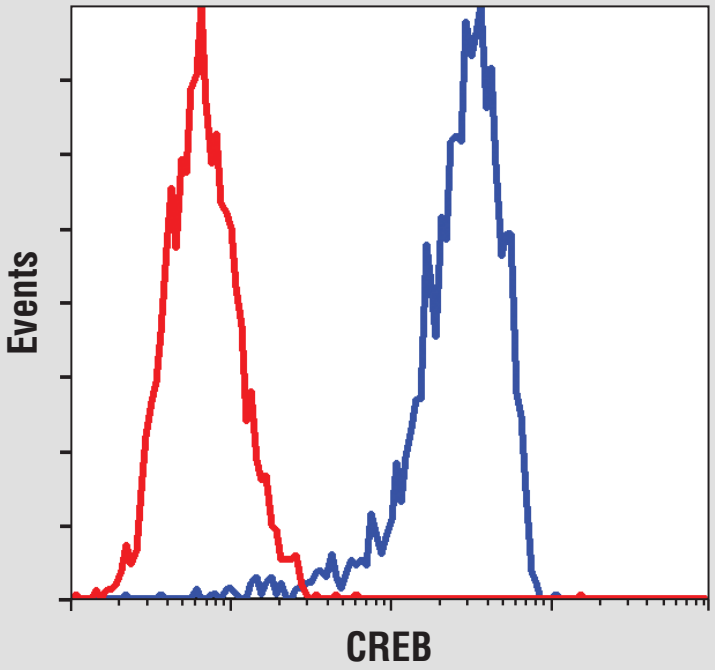
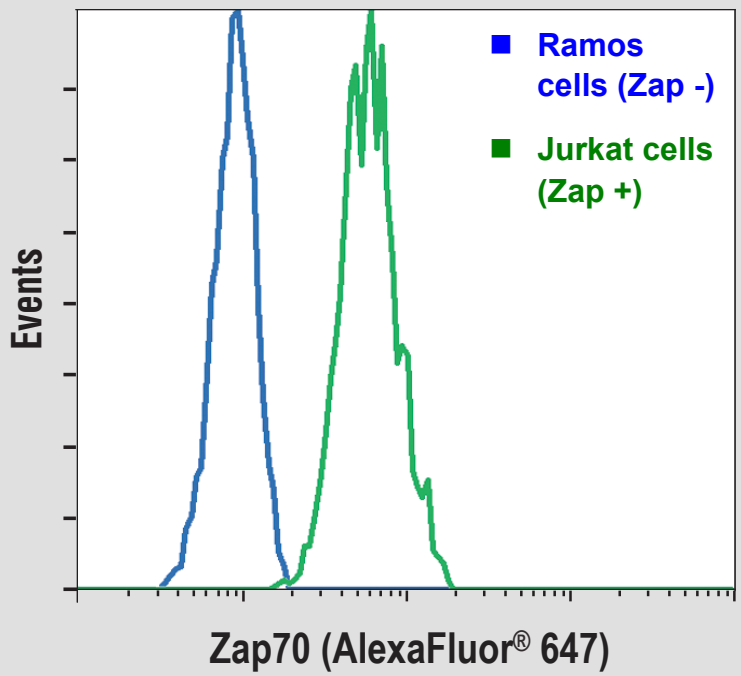
Validation of flow cytometry antibodies: Specificity

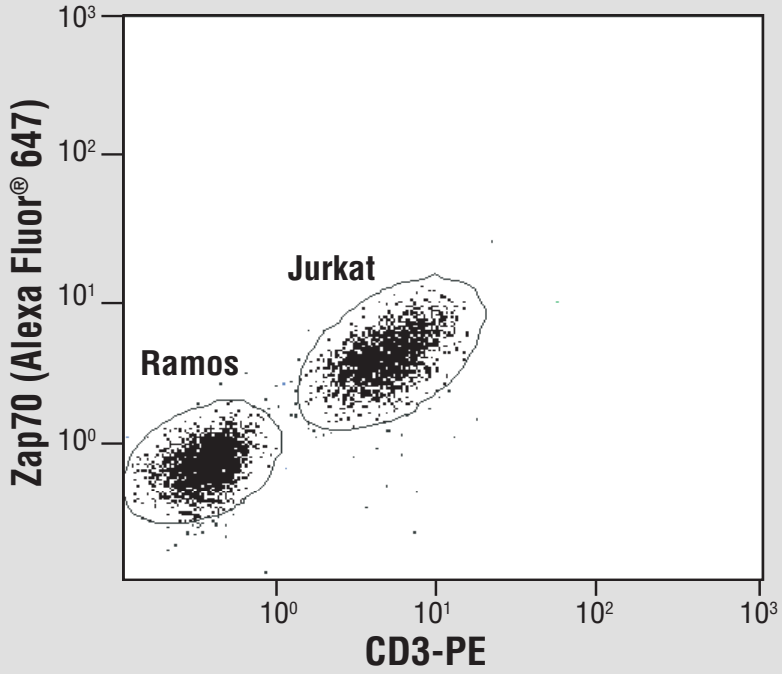
- Comparison with isotype control antibodies to distinguish specific from non-specific binding



Validation of flow cytometry antibodies: Specificity

- Positive and negative cell line analysis to verify antibody specificity

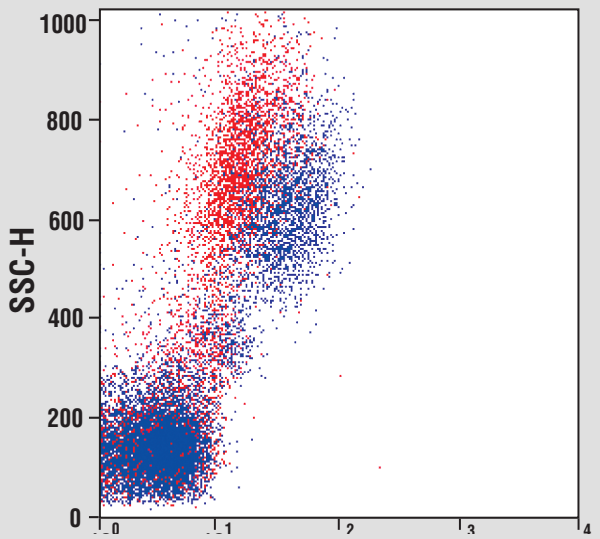




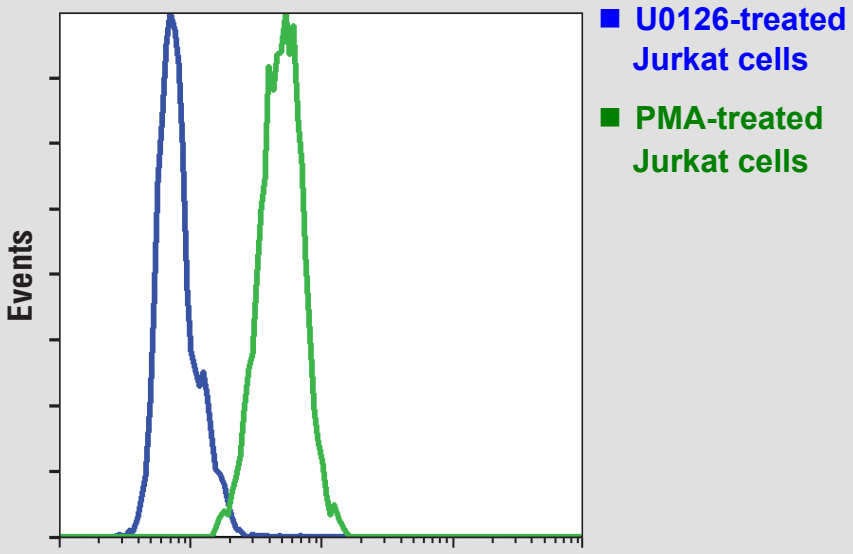
Zap-70 (136F12) Rabbit mAb (Alexa Fluor® 647 Conjugate) #2707

Validation of flow cytometry antibodies: Specificity

- Activation-state specificity testing using pathway-specific inhibitors or activators



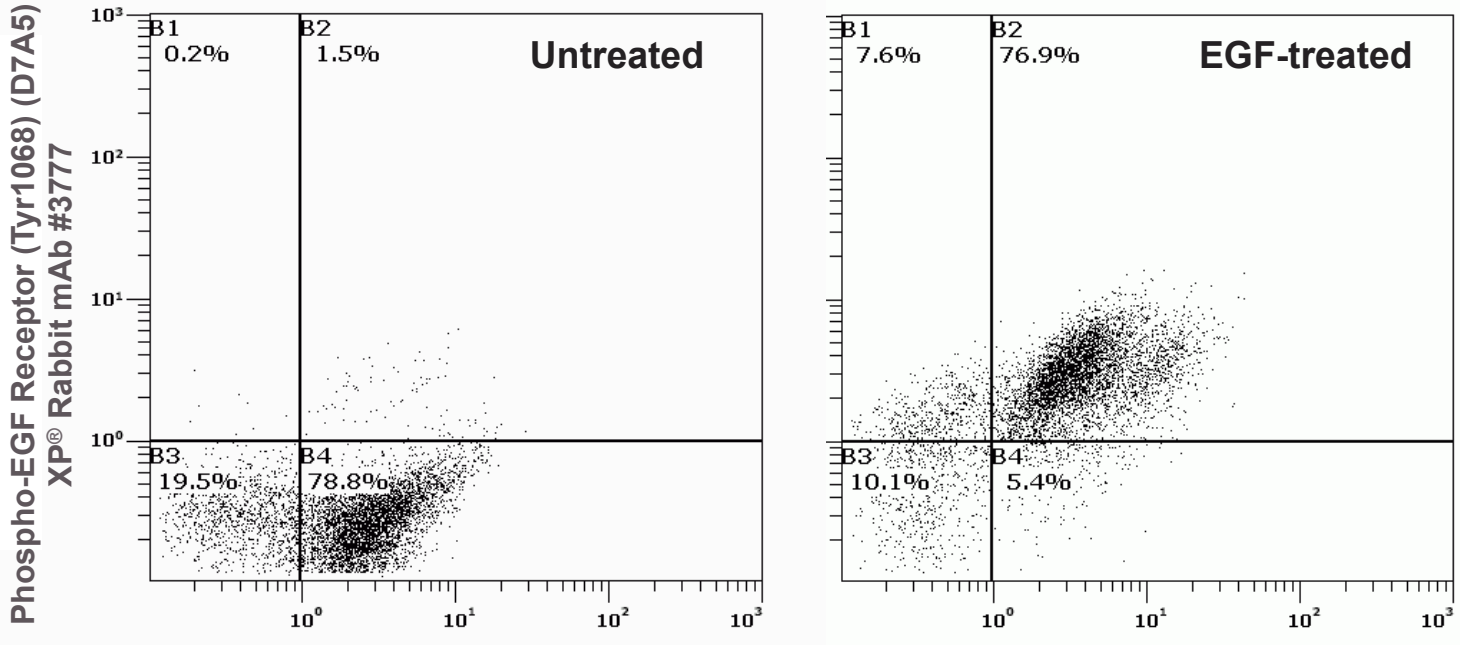
Phospho-Stat3 (Tyr705) XP® Rabbit mAb (Alexa Fluor® 647 Conjugate) #4324



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate) #4344

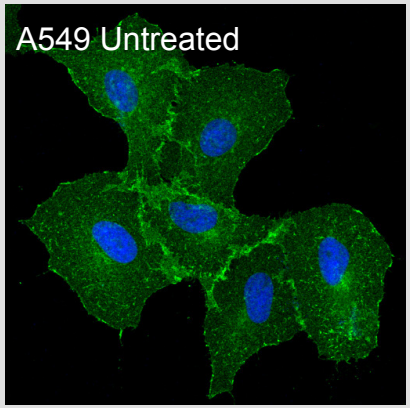
Validation in flow cytometry: Activation state-specific antibodies

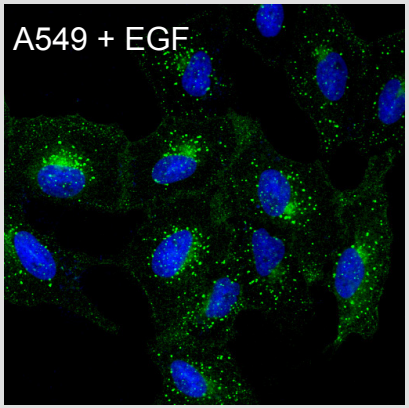
- Phospho-specificity verified by simultaneous analysis of total and phosphorylated protein expression



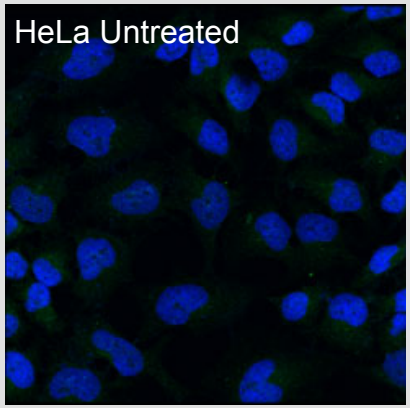
Validation in flow cytometry: activation state-specific antibodies

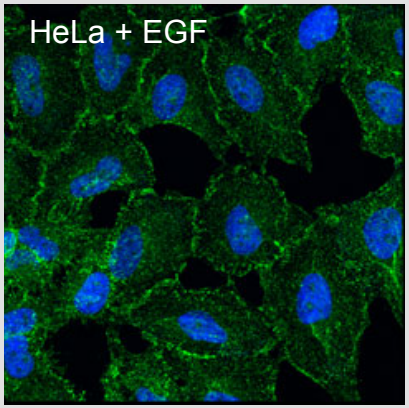
EGF Receptor (D38B1) XP® Rabbit mAb #4267





Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777





GREEN = antibody BLUE = DRAQ5® #4084 (nuclei)

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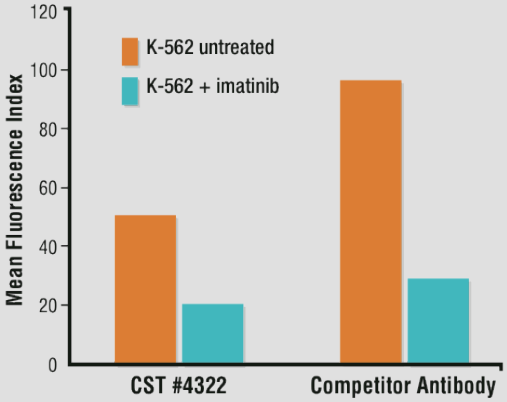
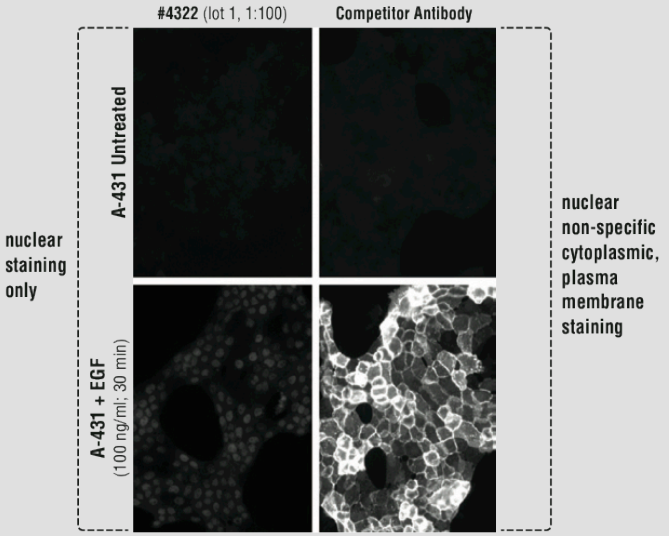
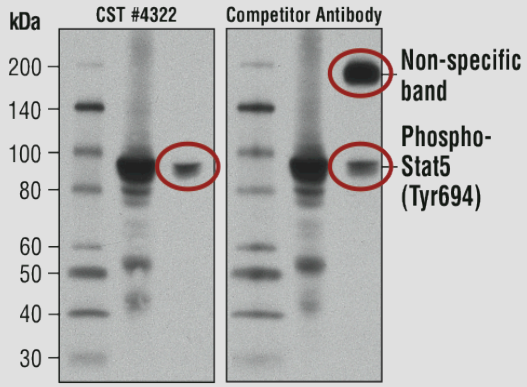
The importance of validating in multiple applications

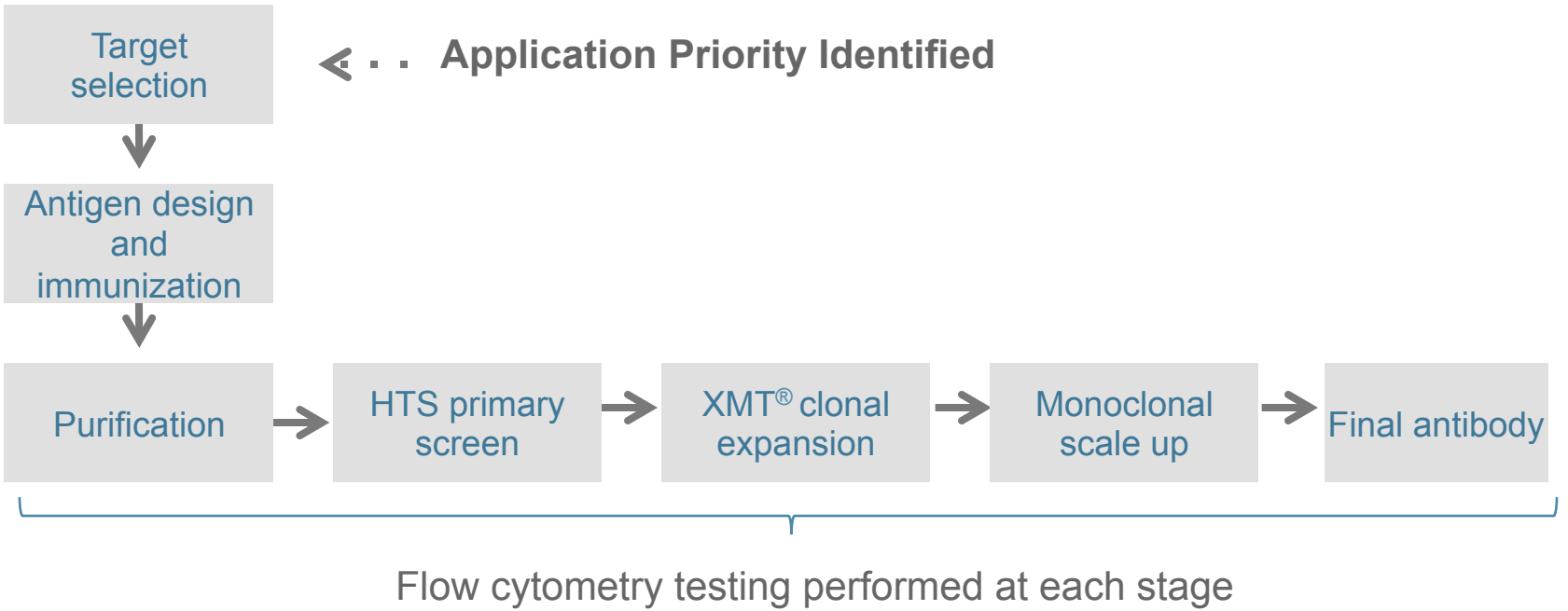






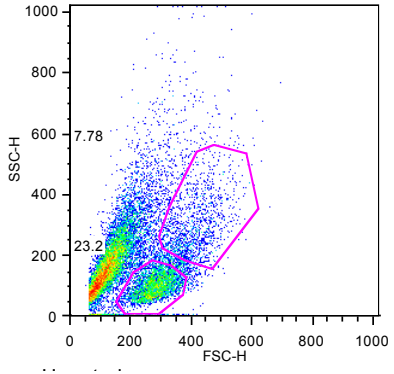
Phospho-Stat5 (Tyr694) (D47E7) XP® Rabbit mAb #4322 and competitor antibody

Antibody development and flow cytometry testing

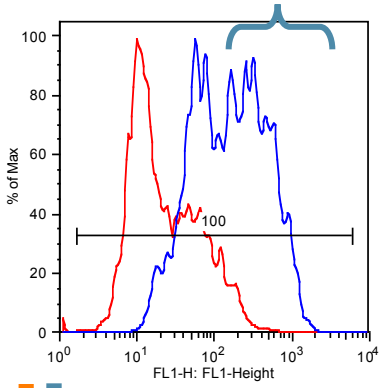


Flow cytometry screening during early antibody development

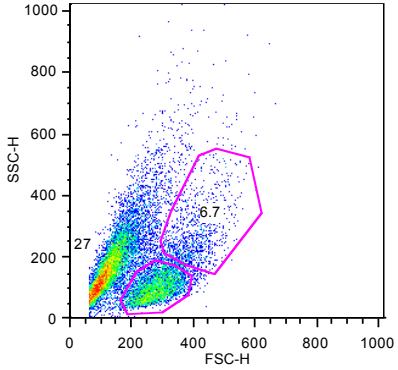
Clone 7F9



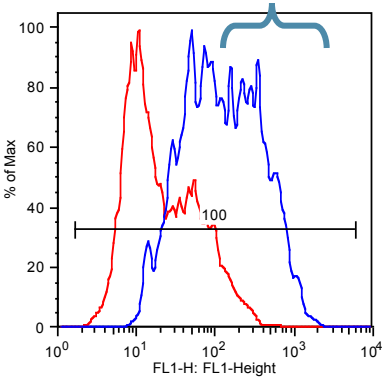
Positive cells



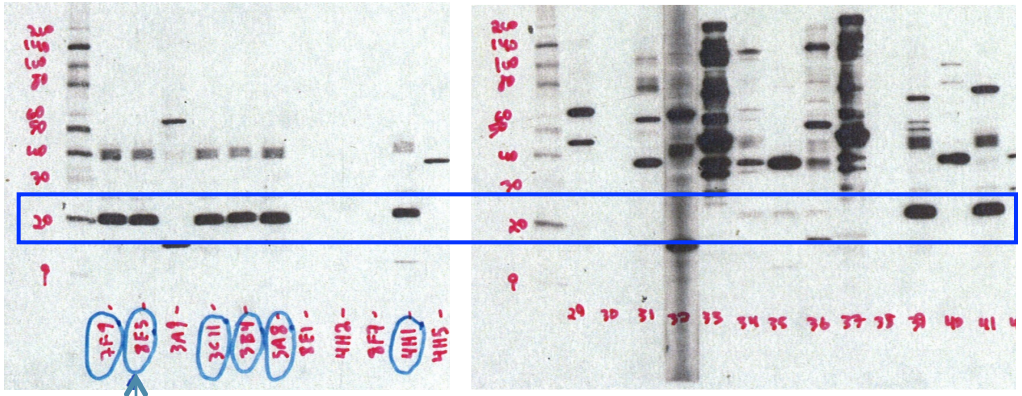
Clone 8E5



Positive cells



Western blot with flow cytometry positive clones



Western blot with ELISA positive clones

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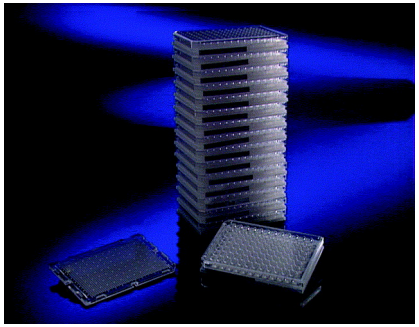
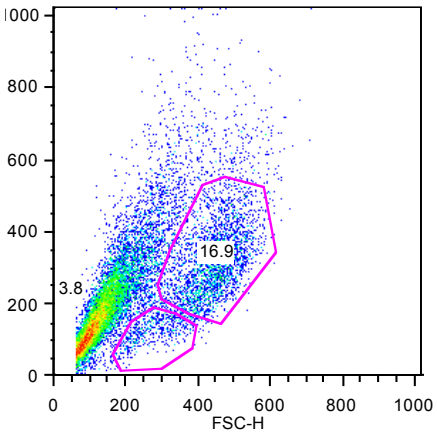
High throughput screen by flow cytometry

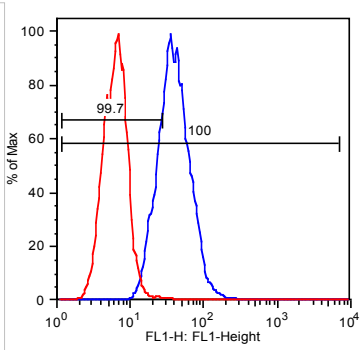




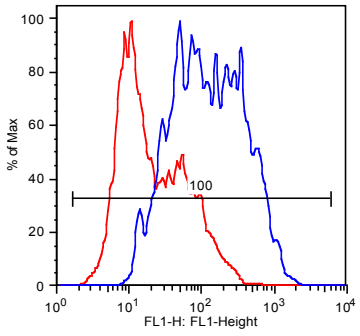
The image cannot be displayed. Your computer may not have enough memory to open the image, or the image may have been corrupted. Restart your computer, and then open the file again. If the red x still appears, you may have to delete the image and then insert it again.

DCs

LCs



Clone selected



Clone failed

Antibody validation at CST

CST scientists follow a stringent validation protocol to provide you with:

- Antibodies with guaranteed specificity
- Antibodies guaranteed to work in your application
- An optimized protocol for each antibody

Cell Signaling TECHNOLOGY®

Is my antibody validated for flow cytometry?

Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060

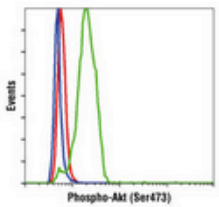
PRINT

All > Category: Primary Antibodies > Products

APPLICATIONS PREV. NEXT.

WB IP IHC IF F ChIP

REACTIVITY	SENSITIVITY	MW (kDa)	Isotype
H M R Hm Mk Dm Z B	Endogenous	60	Rabbit IgG



enlarge

Flow cytometric analysis of Jurkat cells, untreated (green) or treated with LY294002 #9901, wortmannin #9951 and U0126 #9903 (blue), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb compared to a nonspecific negative control antibody (red).

Learn more about how we got this image

Protocol collapse

Cell Signaling TECHNOLOGY®

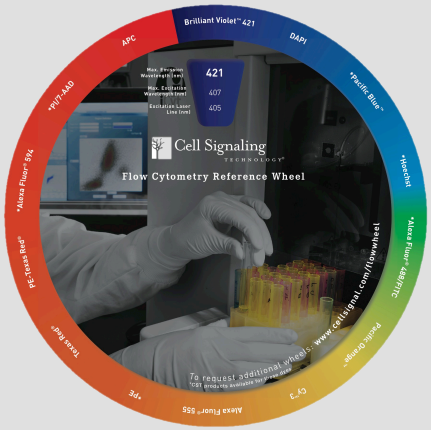
Antibodies for Flow Cytometry

Fluorescently-labeled antibodies

Conjugated antibodies at CST

Conjugated antibodies are required for multiplexing with flow cytometry

- CST™ antibodies plus:
  - AlexaFluor® 488, 555, 594, 647
  - Pacific Blue
  - R-Phycoerythrin (PE)
- Only antibodies validated for flow cytometry are conjugated
- Optimized in-house conjugation
- Tested and validated for fluorescent imaging and/or flow cytometry



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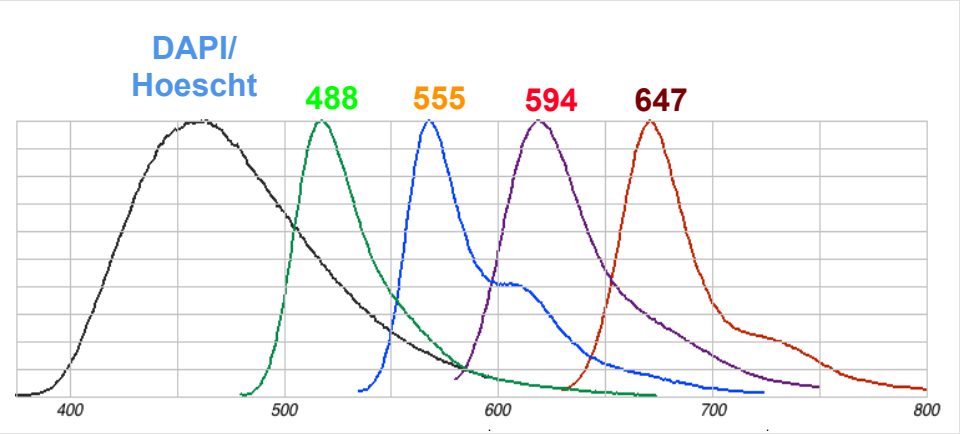
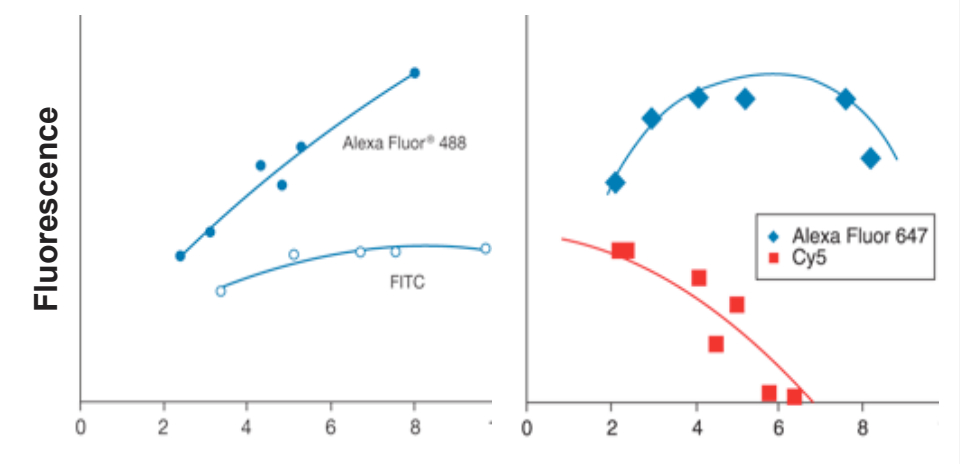
Benefits of intracellular flow cytometry with AlexaFluor®





- Full spectrum coverage
- Brighter fluorescence output than similar fluorochromes
- Highly photostable
- Smaller: easier cell penetration
- Alexa 555 and 594 not regularly used for flow cytometry (561nm laser required)
- ***Custom conjugations on request on flow validated antibodies***

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Protocol optimization

Detecting intracellular targets

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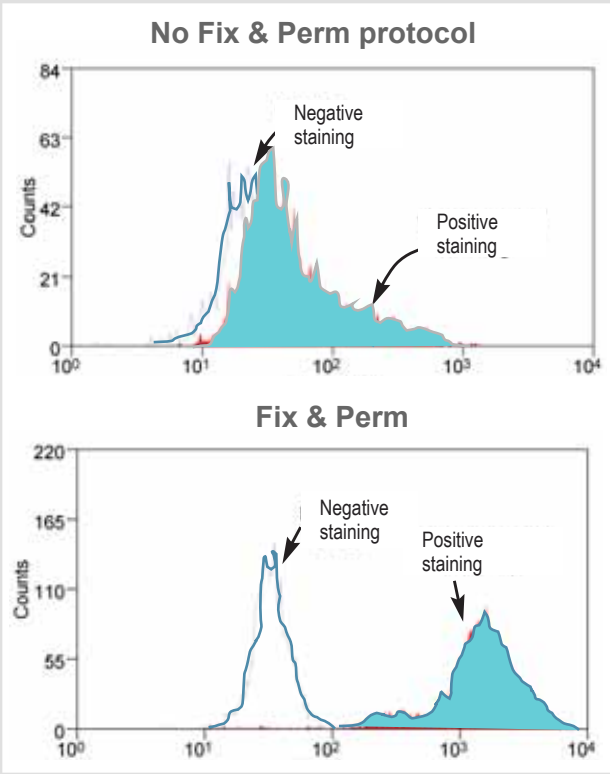
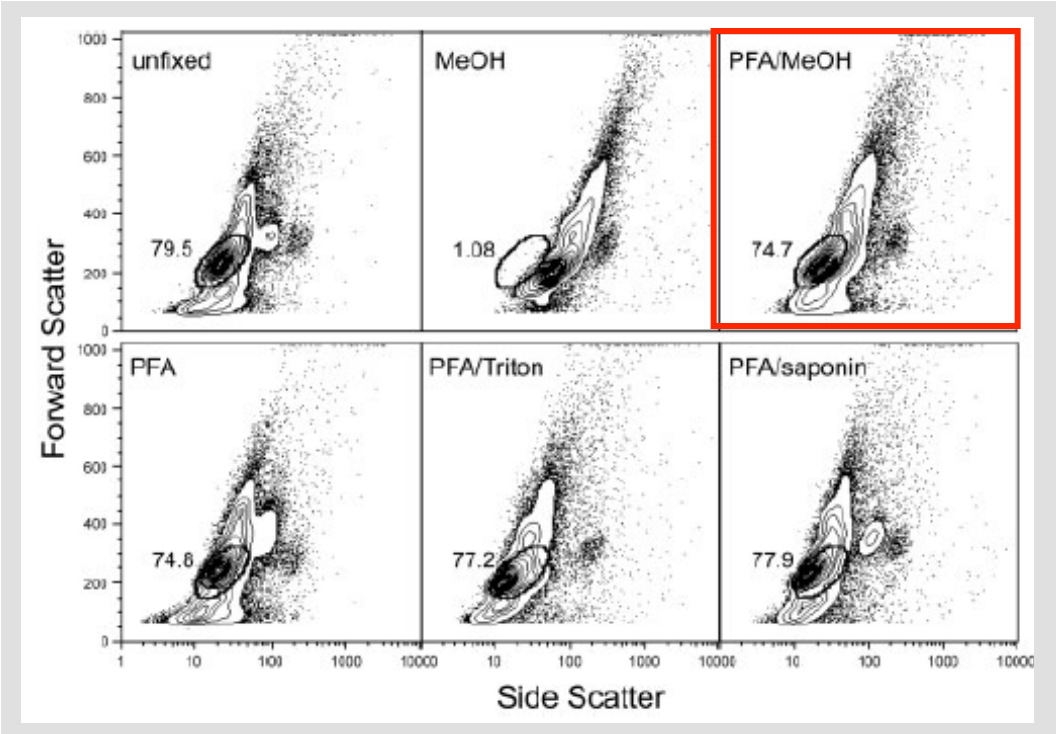
Preparing your samples for intracellular flow cytometry

Critical points for good flow cytometry

- Starting samples: cultured cells, adherent cells, blood, tissue-derived cells
- Cell manipulation
- Antibody specificity
- Cell ***fixation and permeabilization***

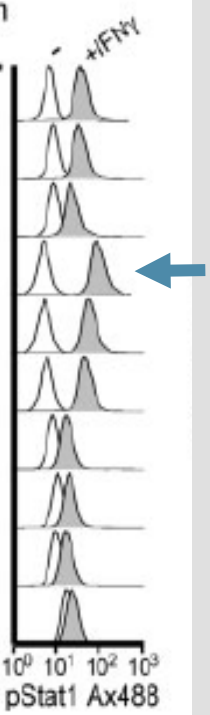


The importance of fixation and permeabilization



Forward/side scatter analysis

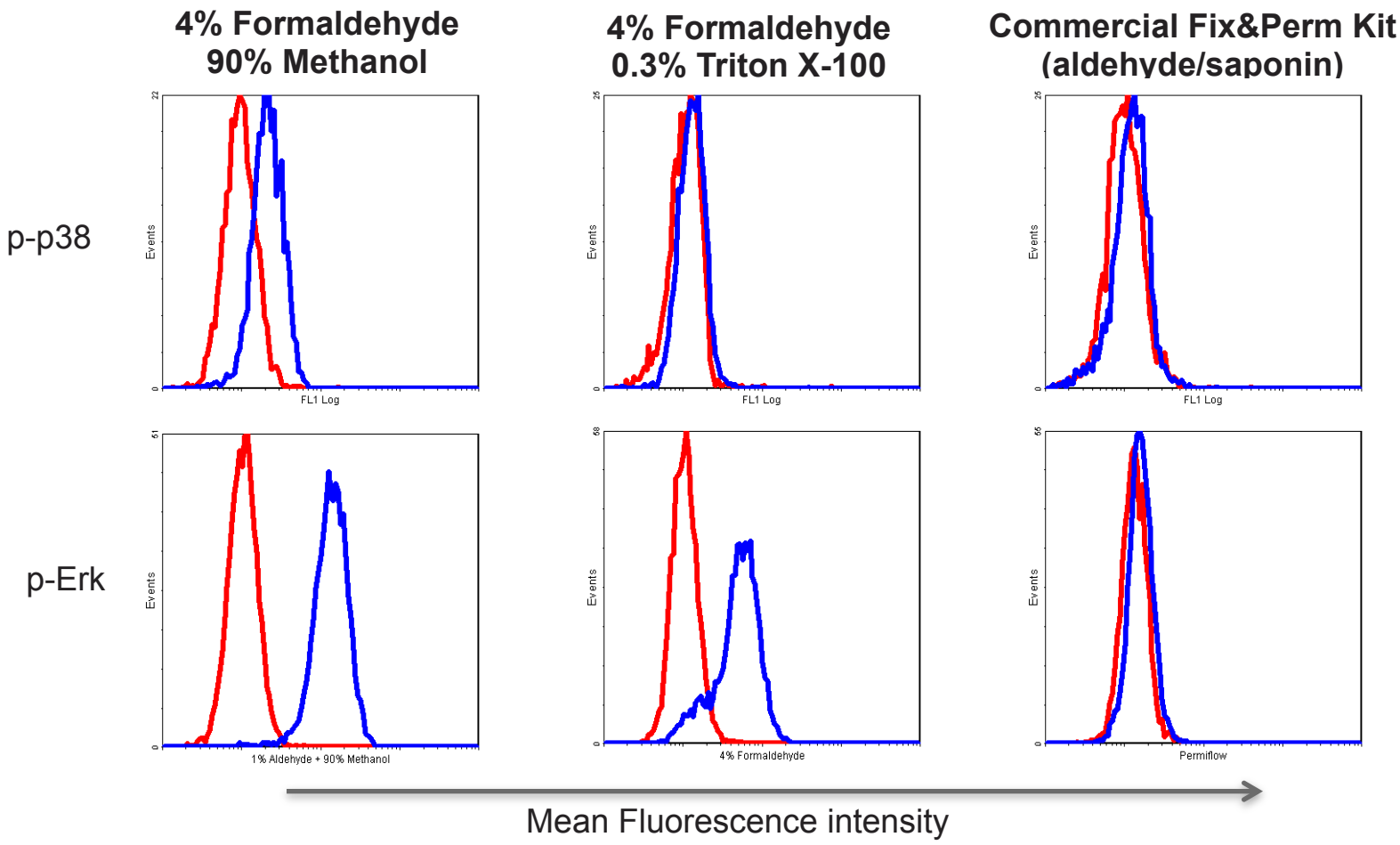
Perm Method	PFA Fix	Permeabilization Reagent
1	-	MeOH
2	-	EtOH
3	-	Acetone
4	+	MeOH
5	+	EtOH
6	+	Acetone
7	+	0.1% Triton
8	+	0.5% Triton
9	+	0.1% Saponin
10	+	0.5% Saponin



Analysis of phospho-Stat1

Krutzik and Nolan (2004) *Cytometry* 55A:61-70

The effect of fixation and permeabilization



Intracellular flow cytometry standard protocol

What is your starting material?

Cell treatment

Single cell suspension

Cell viability

→ Fixation → Permeabilization → Staining → Analyze

1ml 100% methanol drop-wise and cold (90% F.C.)

In flow buffer

4% Formaldehyde 10 min, R.T.

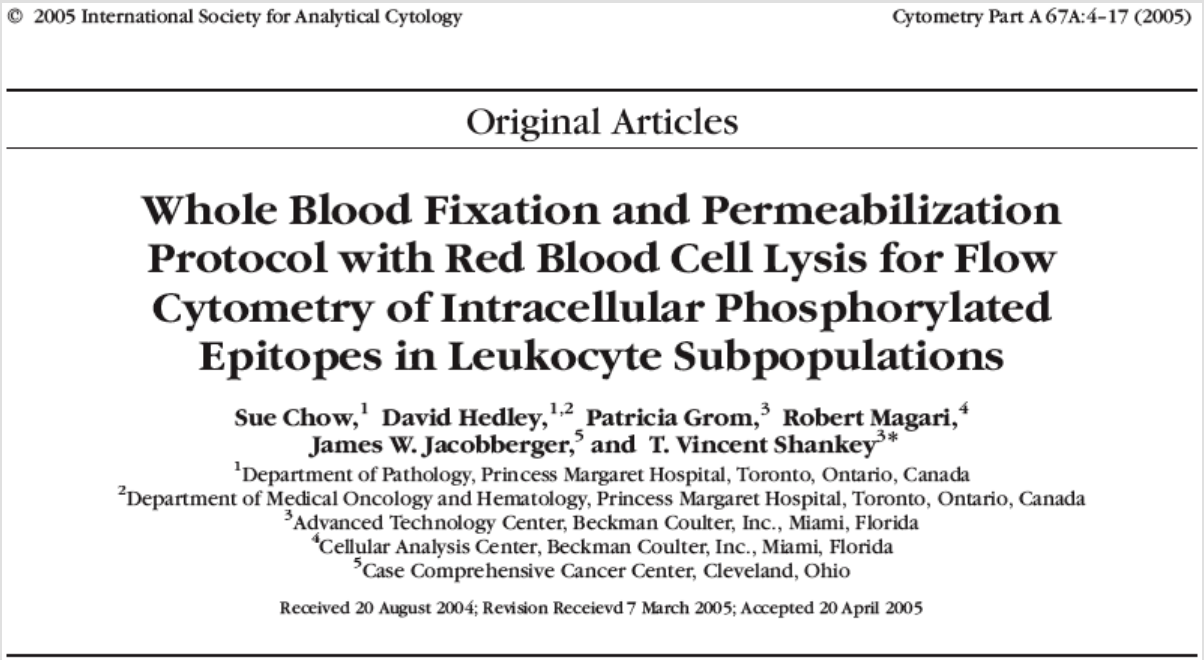
PBS/ 5% NGS + 10^5-10^6 cells/sample + Recommended antibody dilution

Many cell surface markers are sensitive to fix & perm and will not be recognized by the relevant antibodies after such procedures!

http://www.cellsignal.com/support/protocols

Alternate fix & perm protocol

A majority of surface markers are affected by methanol treatment: A different protocol is needed for the joint analysis of intracellular and surface markers



Cytometry Part A 67A:4–17 (2005)

Original Articles

Whole Blood Fixation and Permeabilization Protocol with Red Blood Cell Lysis for Flow Cytometry of Intracellular Phosphorylated Epitopes in Leukocyte Subpopulations

Sue Chow,[1] David Hedley,[1,2] Patricia Grom,[3] Robert Magari,[4] James W. Jacobberger,[5] and T. Vincent Shankey[3]*

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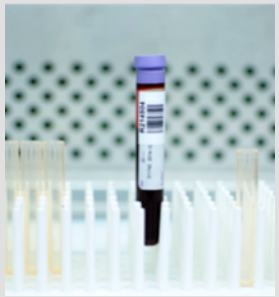
[5]Case Comprehensive Cancer Center, Cleveland, Ohio

Received 20 August 2004; Revision Receievd 7 March 2005; Accepted 20 April 2005

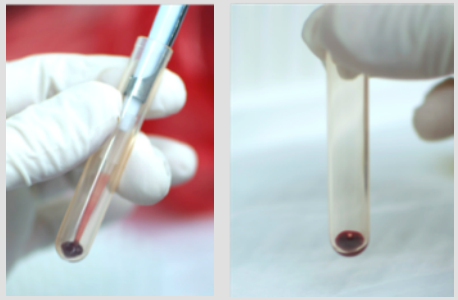
4% Formaldehyde + 0.1% Triton X-100 + 50% Methanol

Flow cytometry alternate protocol

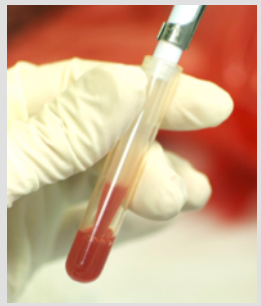


Collect blood in heparin or EDTA coated tubes

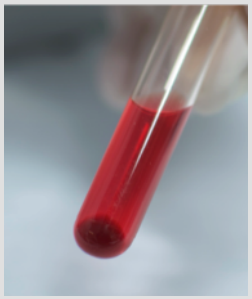
Aliquot 100ul of blood into a FACS tube; add treatment if necessary



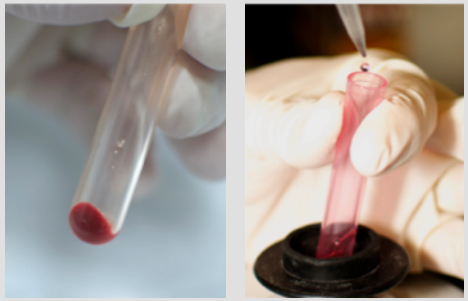
Add fixative; 15m, RT



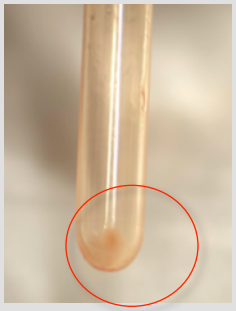
Add 1ml 0.1% Triton-X 100, 30m



Wash 2x (there may still be a pellet of RBCs)



Aspirate supernatant. Add ice-cold 50% methanol drop-wise, while gently vortexing; 10m

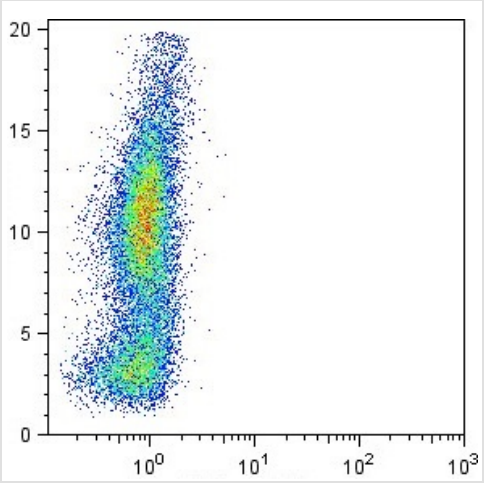


Add 1ml buffer and wash by centrifugation; remove supernatant
Add antibody diluted as recommended. Incubate 30m on ice, in the dark

Comparing both fix and perm protocols

4% formaldehyde RT 10m

90% ice cold MeOH -20ºC 10m

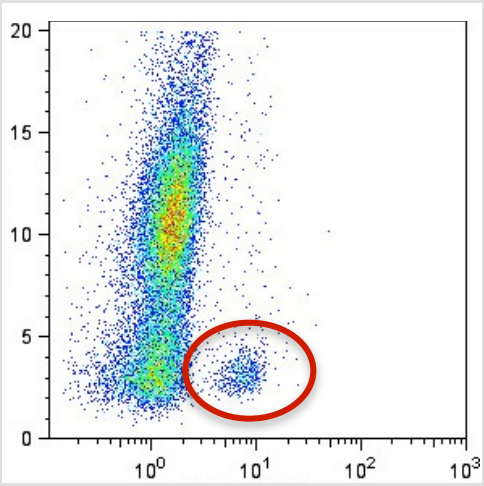


CD19 (FITC)

4% formaldehyde RT 10m

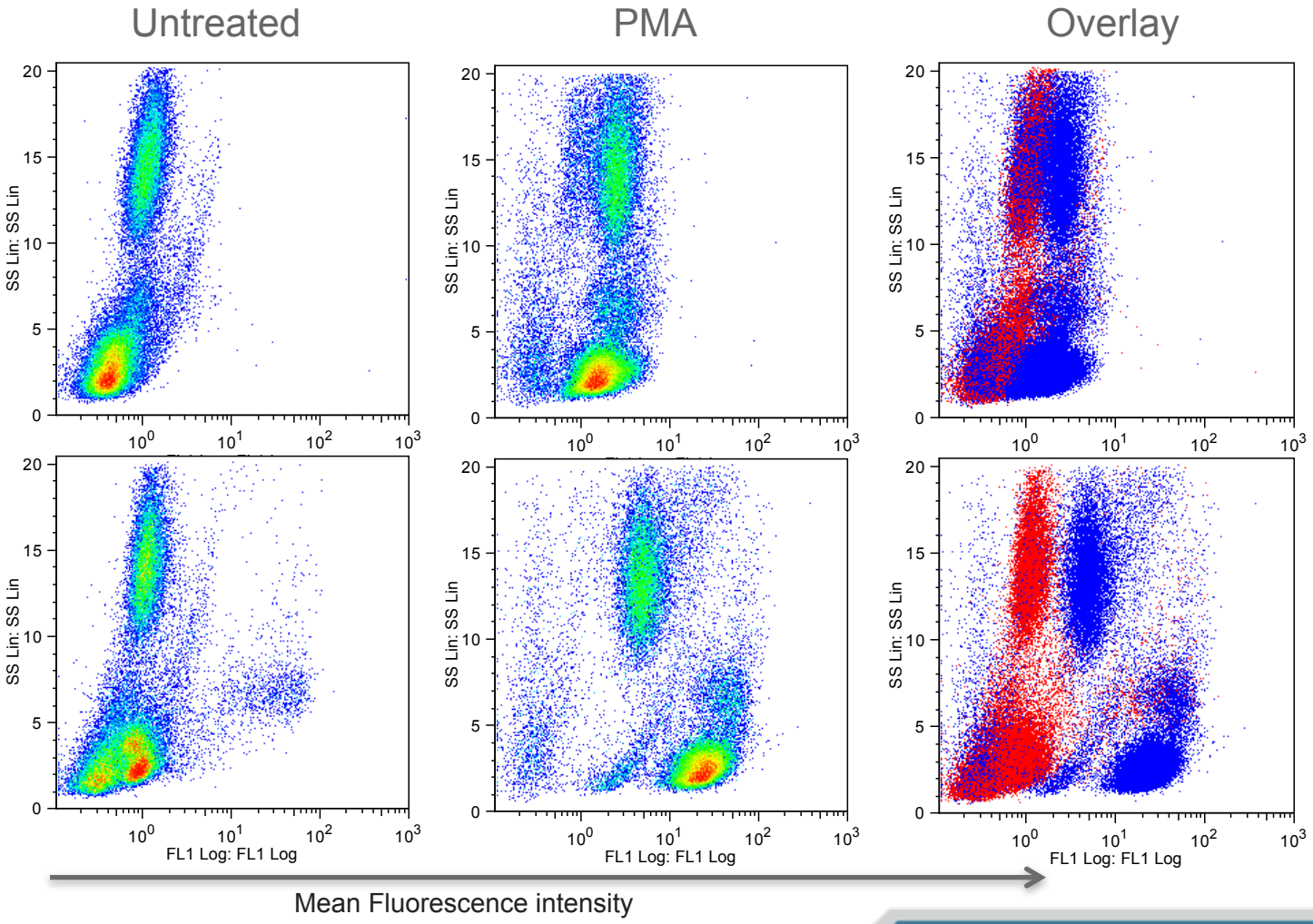
0.1% Triton X-100 RT 30m

50% ice cold MeOH at -20ºC10m



CD19 (FITC)

Alternate protocol preserves phospho-epitopes and dynamic range



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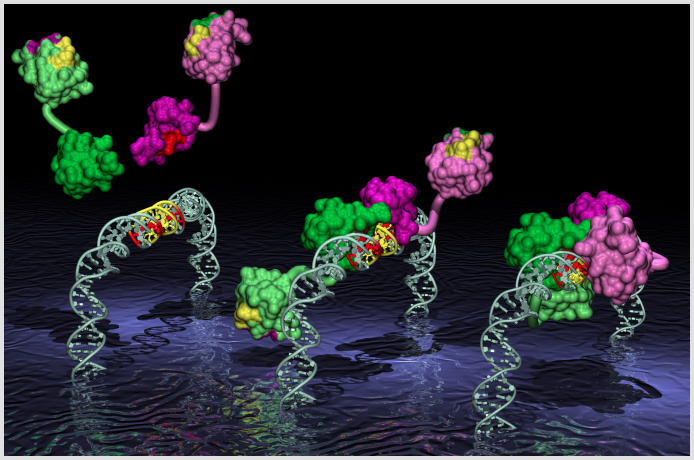
Fixation/Permeabilization protocol for transcription factors

- Some transcription factors are less accessible to antibodies
- Cross-linking of amine groups by 4% formaldehyde may be making the epitope inaccessible.
- **Modification of fix/perm protocol may be necessary:**
  - Fixation: 2% formaldehyde final concentration, 15 min. at room temp.
  - Permeabilization: 0.1% Triton X-100, 30min at room temp

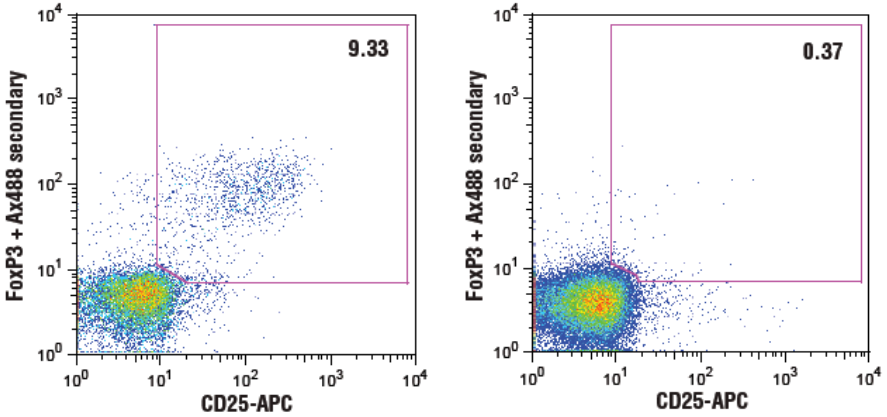


Ansgar Phillipsen, University of Basel

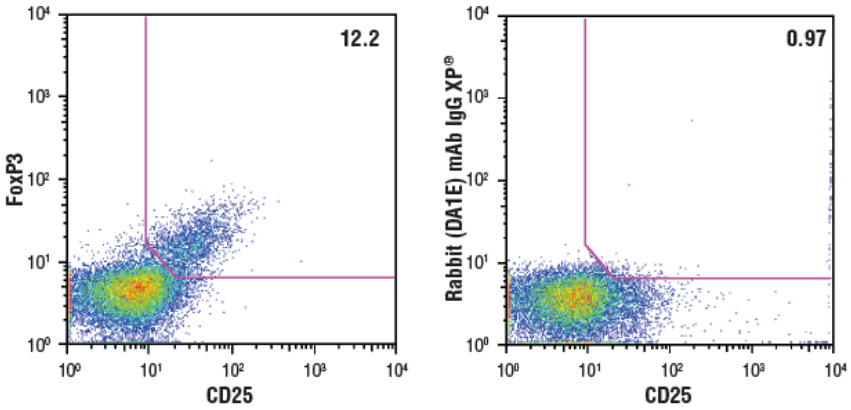
FoxP3+ T Regulatory cells (T-Regs)

- CD4+T Reg characterized by expression of forkhead transcription factor FoxP3

Mouse cells #12653



Human cells #12632



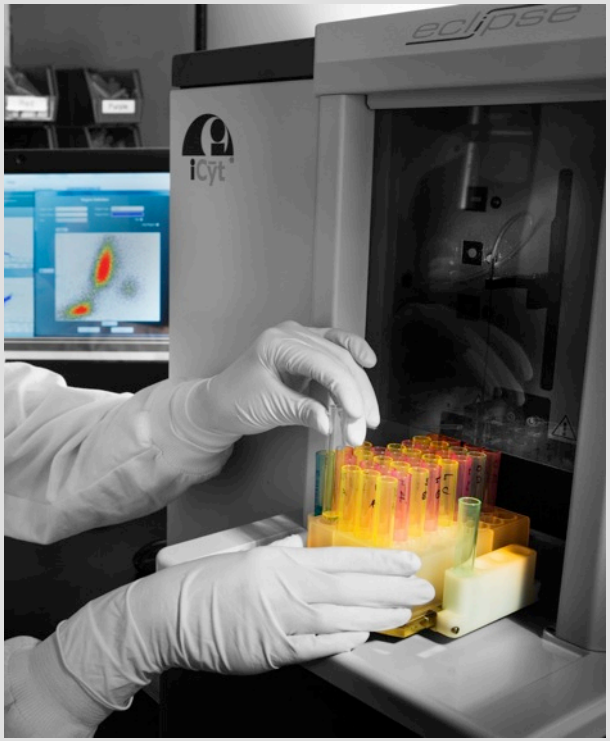
Cell Signaling TECHNOLOGY®

Uses of Intracellular Flow Cytometry

From the membrane to the nucleus

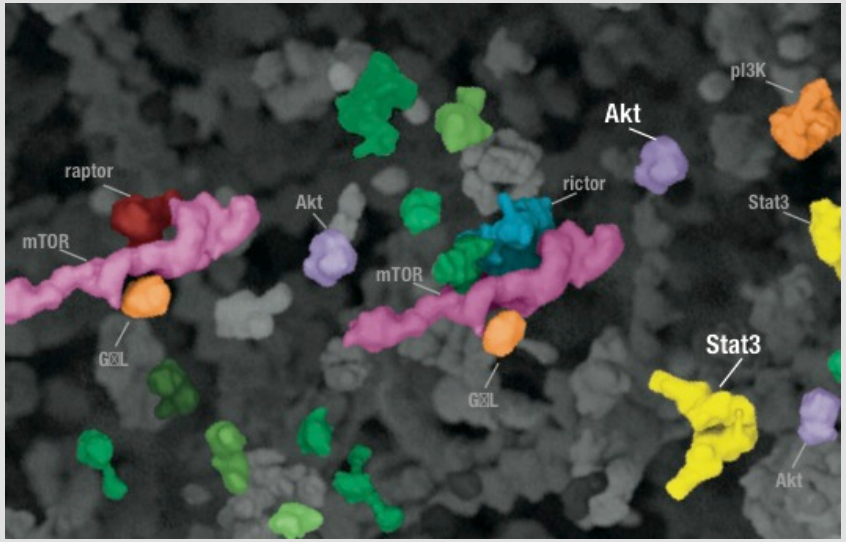
Uses of intracellular flow cytometry

- Active kinase states in cell subsets
- Identification of unique signaling profiles
- Analysis of cell cycle, apoptosis and autophagy
- Pluripotency and stem cells
- Metabolic responses



Intracellular flow cytometry and signaling

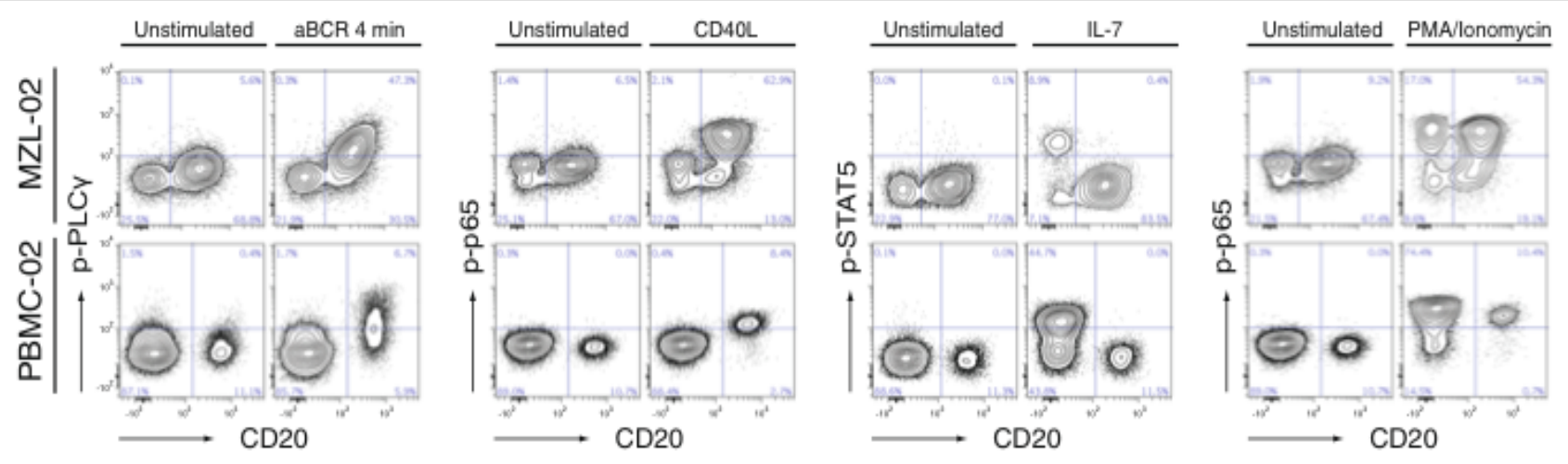
A combination of flow cytometry and activation-state specific antibodies enables the multi parametric analysis of intracellular complex biological processes in heterogeneous samples



Intracellular flow cytometry and cell signaling

Phospho-specific flow cytometry identifies aberrant signaling in indolent B-cell lymphoma

Egil S Blix[1,2*], Jonathan M Irish[3,4], Anne Husebekk[5], Jan Delabie[6], Lise Forfang[7], Anne M Tierens[6], June H Myklebust[7†] and Arne Kolstad[8†]

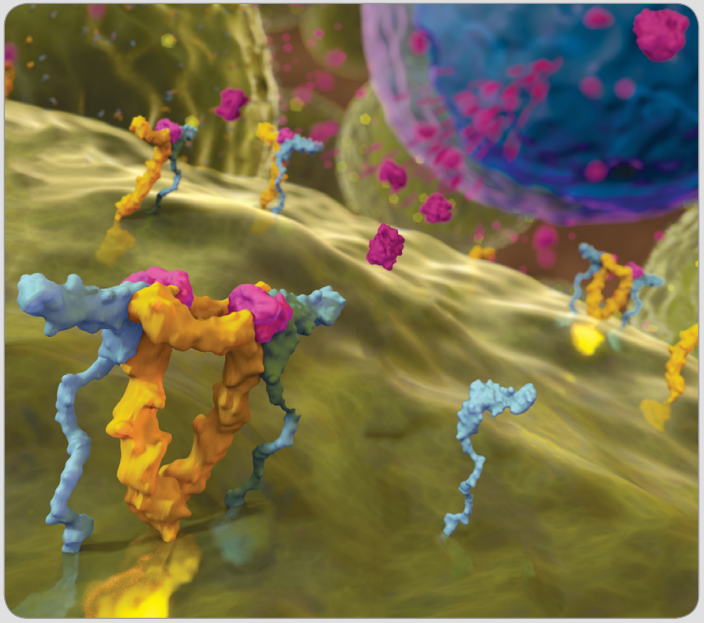


Basal and activation induced signaling samples from lymphoma patients and healthy donors, *BMC Cancer, 2012*

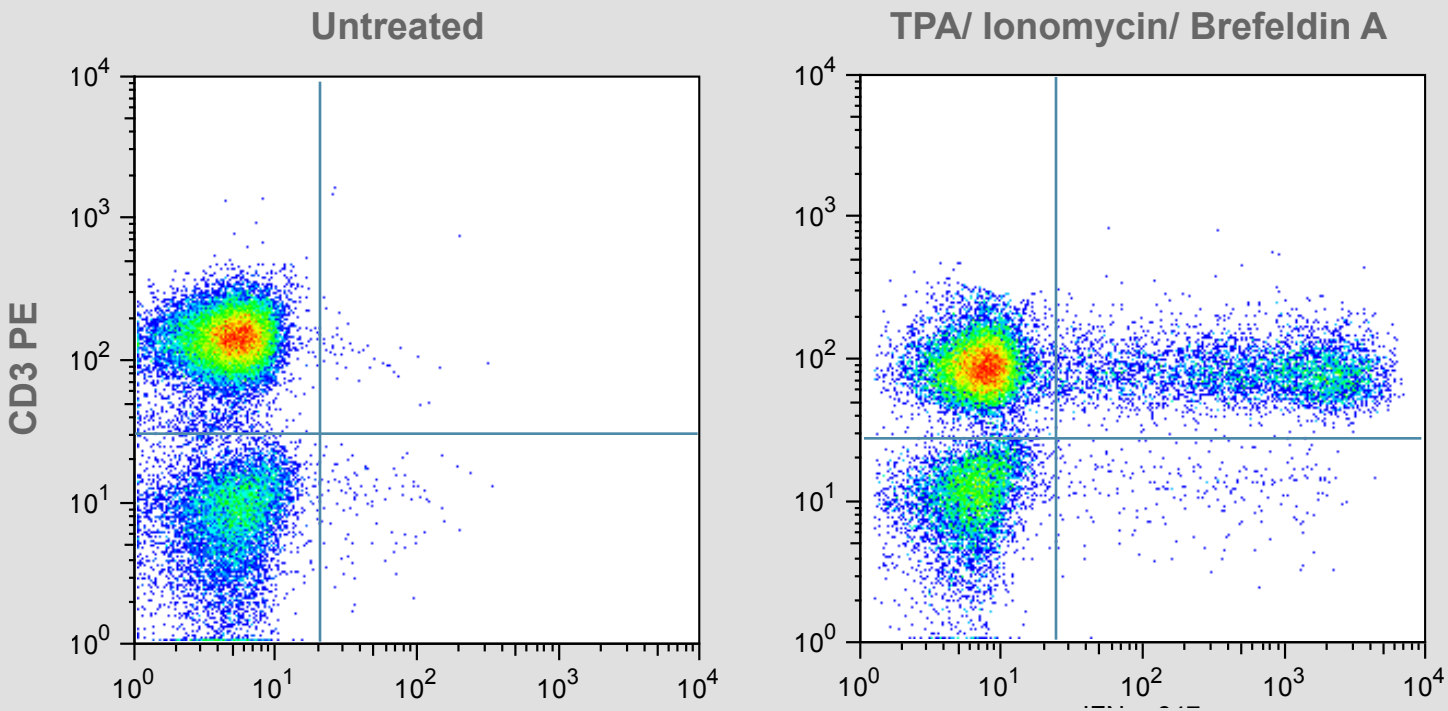
- Differences in signaling properties between B and T cell subsets from SLL/CLL and MZL patient samples
- Opportunity to personalize inhibitor treatment in B cell Lymphoma cells

Intracellular flow cytometry in immunology

Analysis of changes in the level of cytokines secreted by immune cells provides information about inflammatory responses



Immunology: Analysis of inflammatory responses

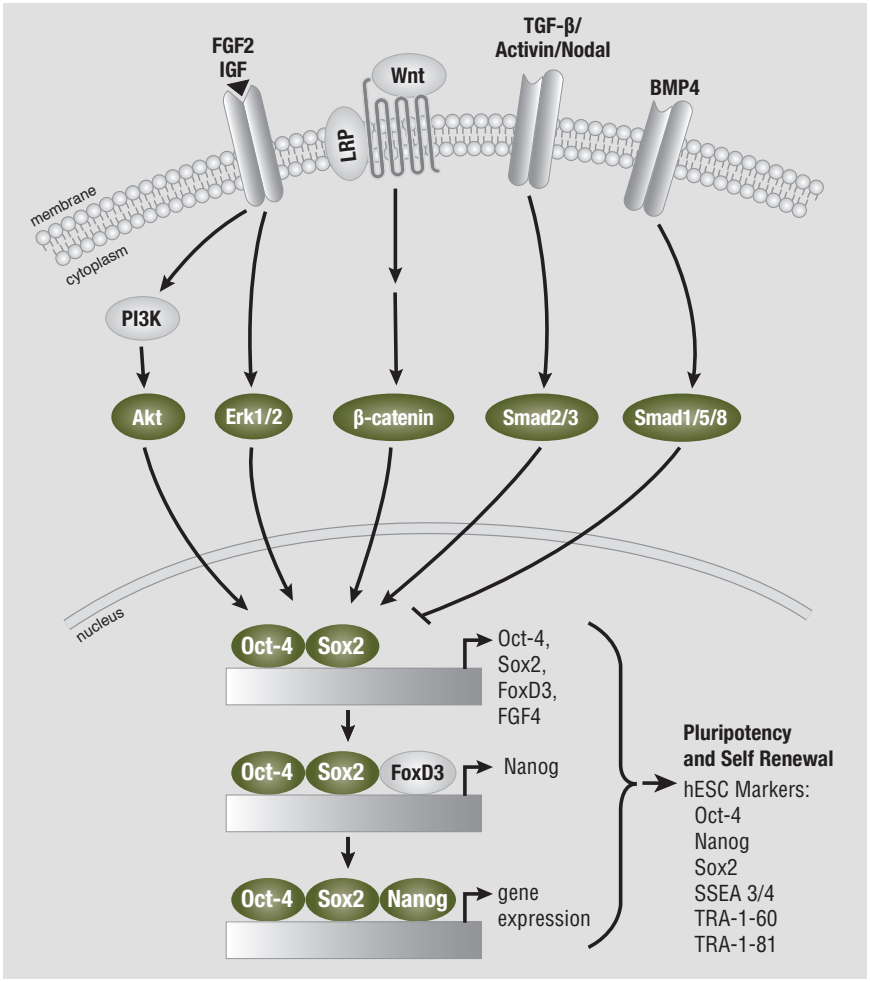


IFN-γ (D3H2) XP® Rabbit mAb #8455

Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) #4414

Stem cell analysis by intracellular flow cytometry

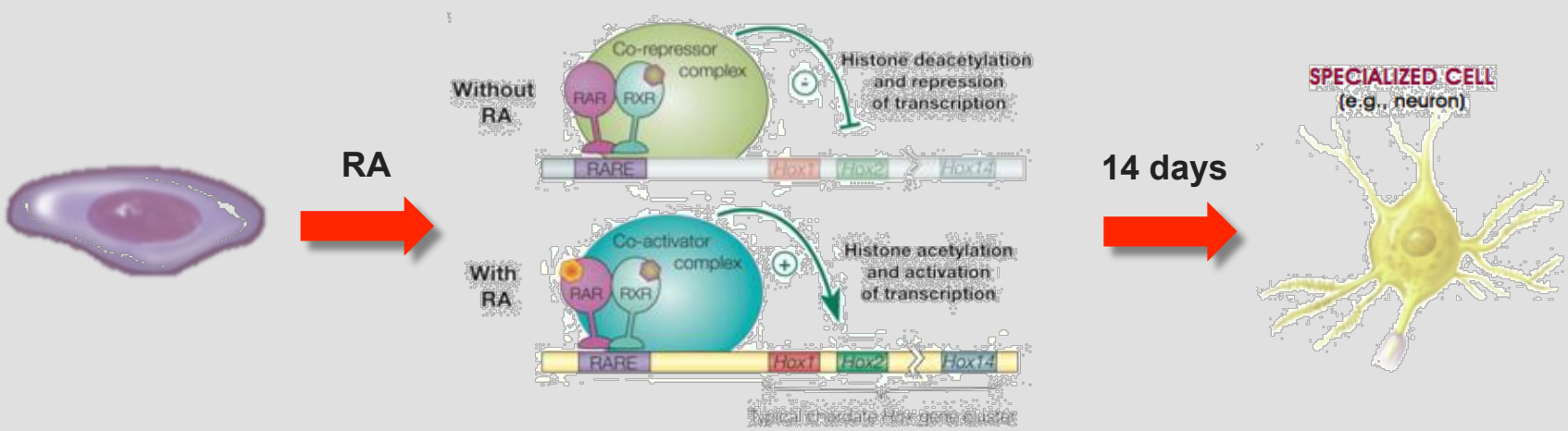
The transcription factors Oct-4, Sox2 and Nanog are commonly used as markers for human embryonic stem cells



NTERA Differentiation Assay

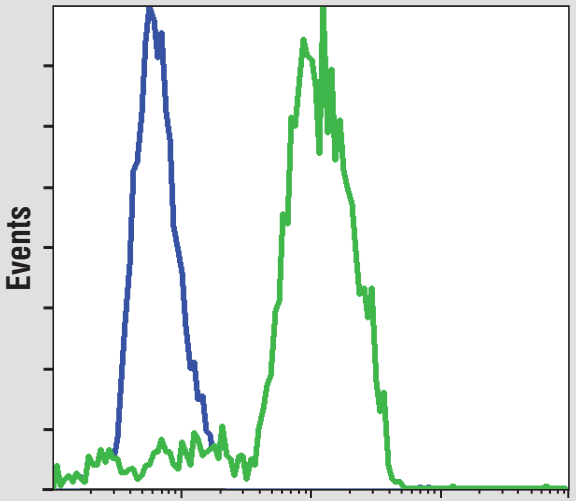
NTERA 2 cells:

- Pluripotent human embryonal carcinoma cell; can give rise to teratocarcinomas
- Developmental and biochemical properties similar to early embryonic stem cells
- Differentiates in response to retinoic acid (RA); is a model for neuronal cell differentiation
  (Lee et al., The Journal of Neuroscience, February 1986, 6(2): 514-521)

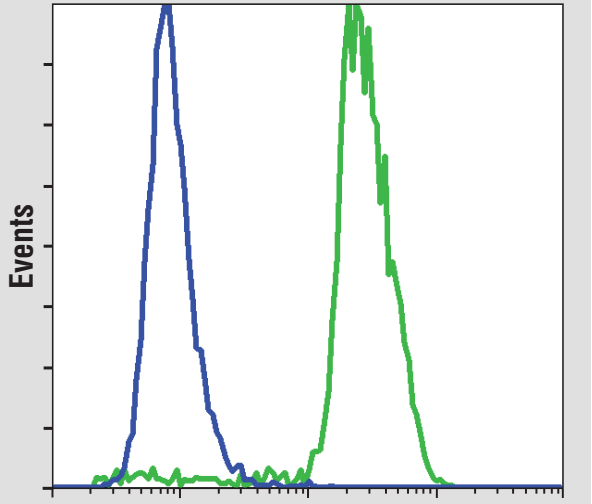


Marletaz et al.,,International Journal of Biological Sciences, Int J Biol Sci 2006; 2(2):38-47

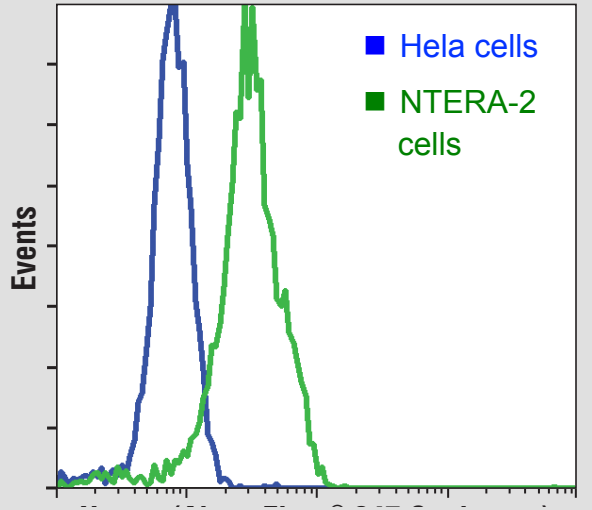
Analysis of pluripotency by flow cytometry



Oct-4A (C30A3)
Rabbit mAb (Alexa Fluor® 488 Conjugate)#5177



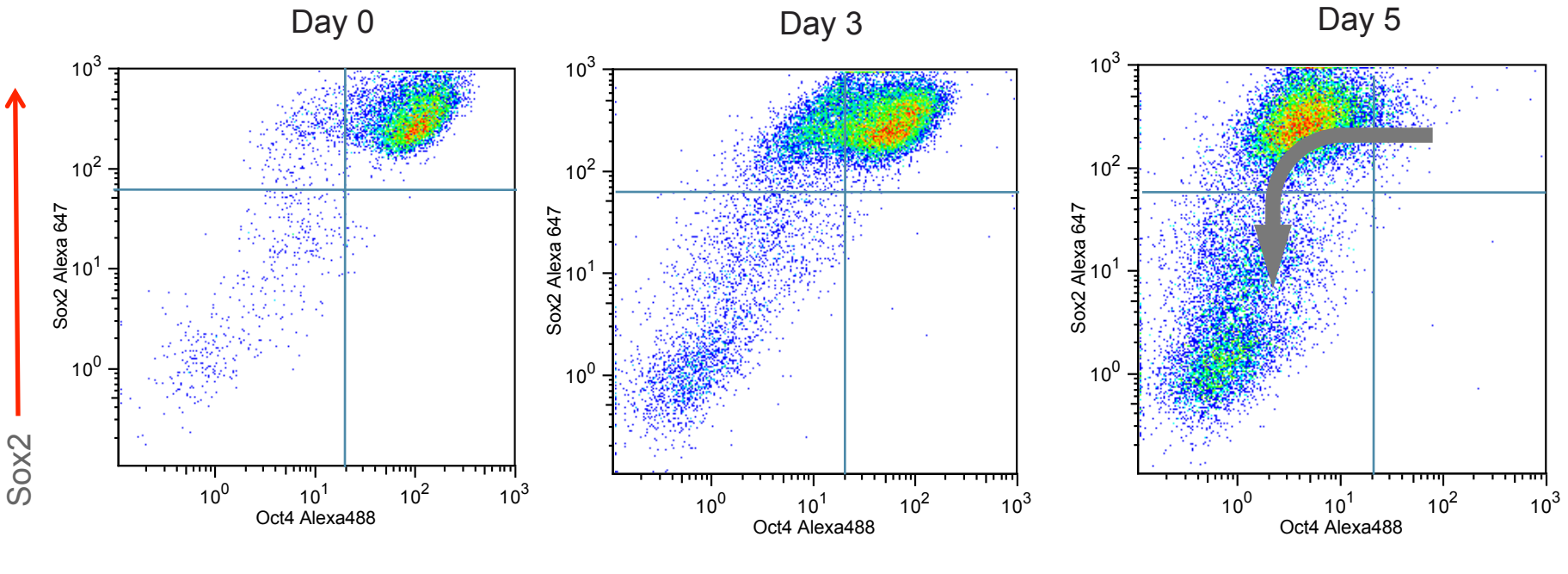
Sox2 (D6D9)
XP® Rabbit mAb (Alexa Fluor® 647 conjugate)#5067



Nanog (D73G4)
XP® Rabbit mAb (Alexa Fluor® 647 Conjugate)#5448

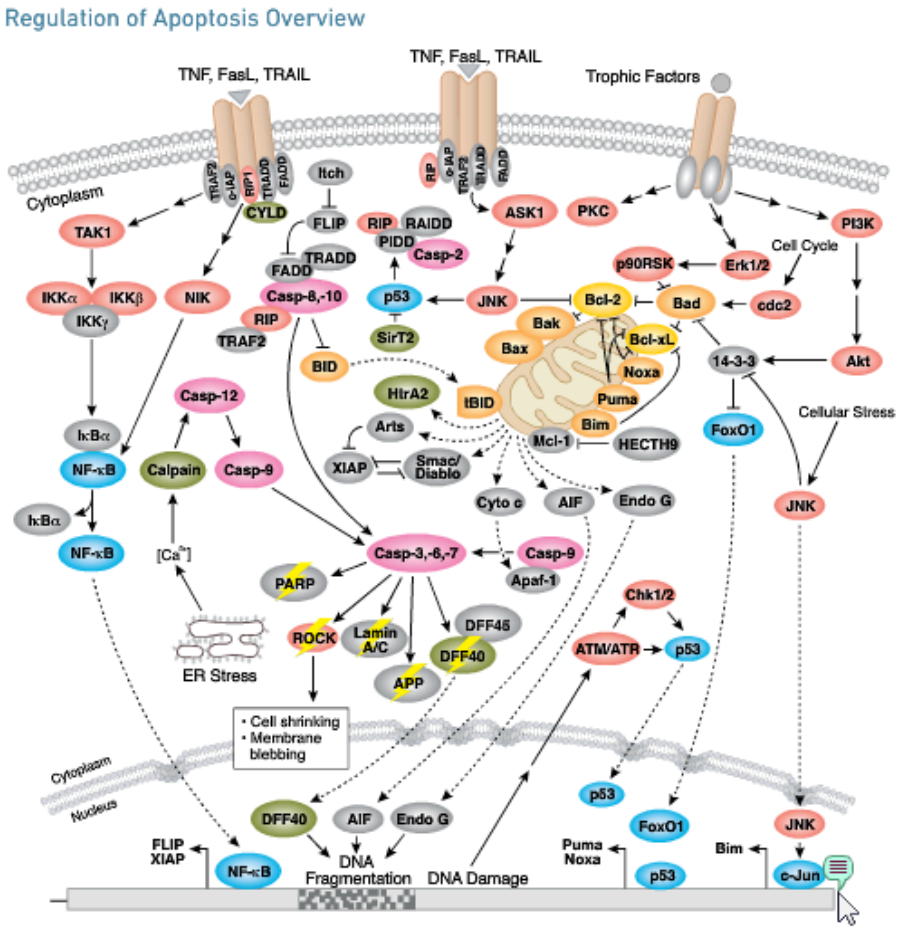
NTERA Differentiation Assay

- Oct4 and Sox2 levels decrease significantly after 5 Day RA induction



5 day retinoic acid differentiation

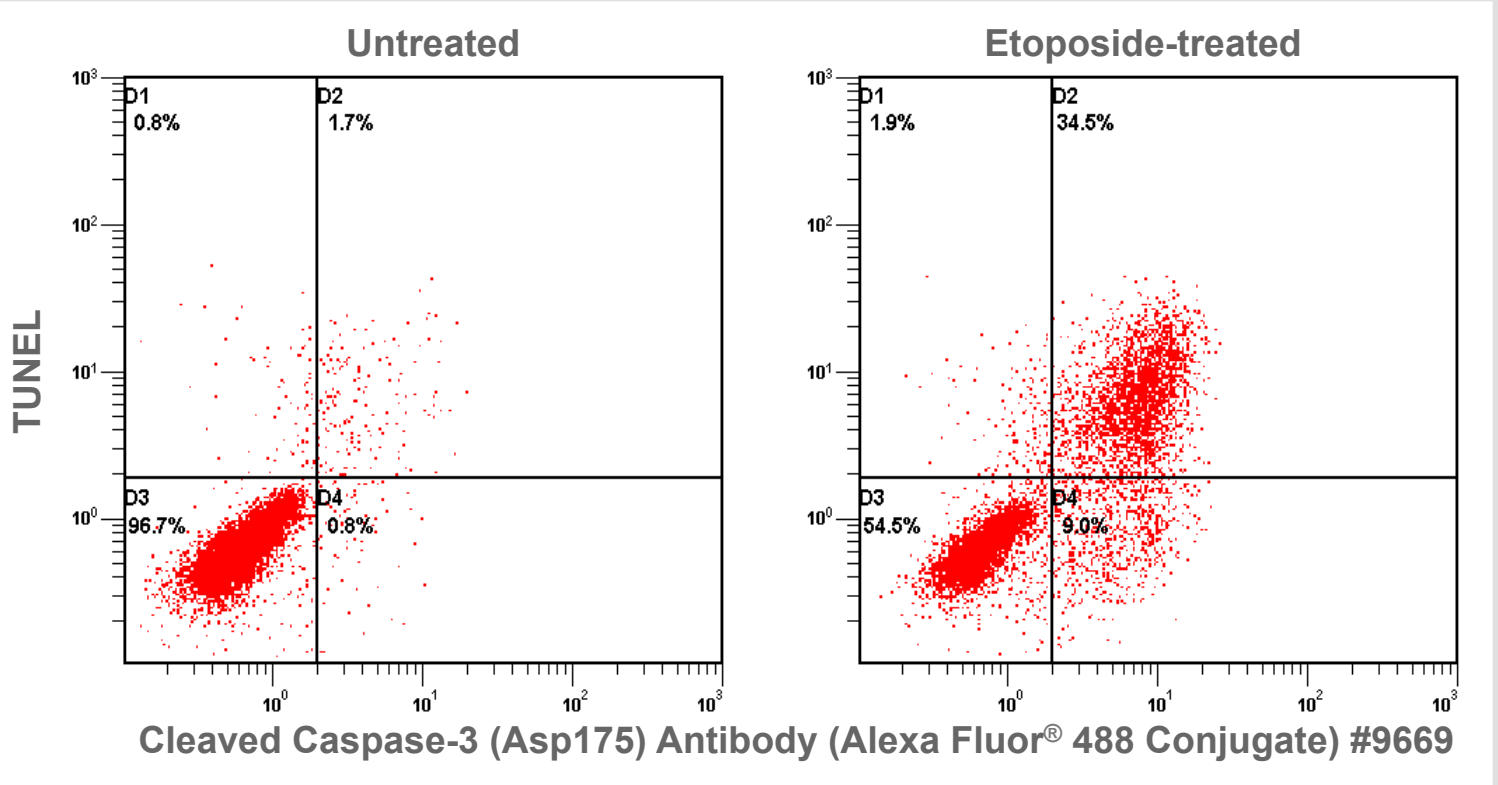
Analysis of apoptosis by flow cytometry



Apoptosis is a complex process that needs a multiparametric approached to be fully defined

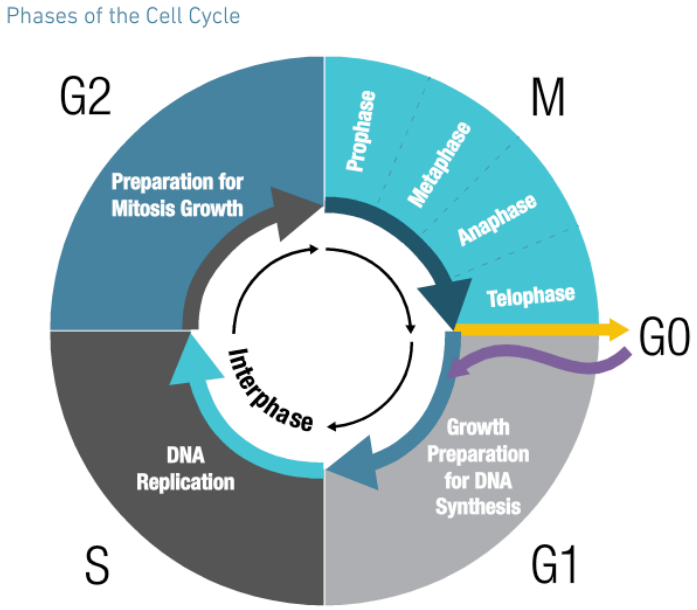
Analysis of apoptosis by flow cytometry

- Caspase 3 activation represents one of the earliest and easily measurable markers of apoptosis



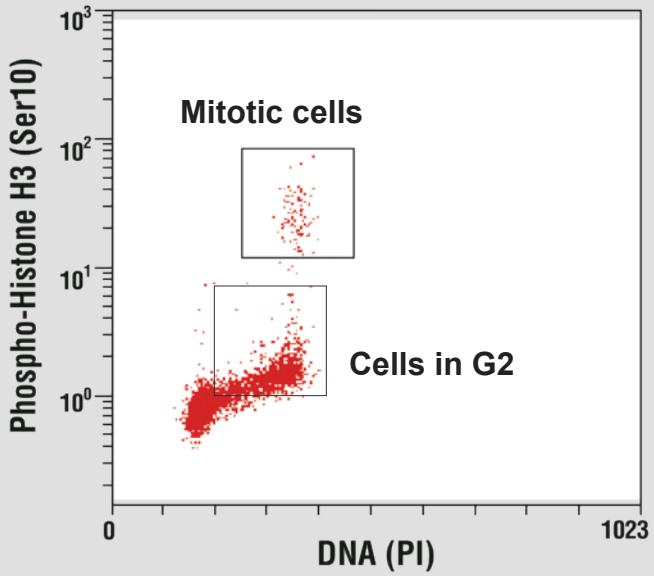
Analysis of cell cycle phase-specific proteins

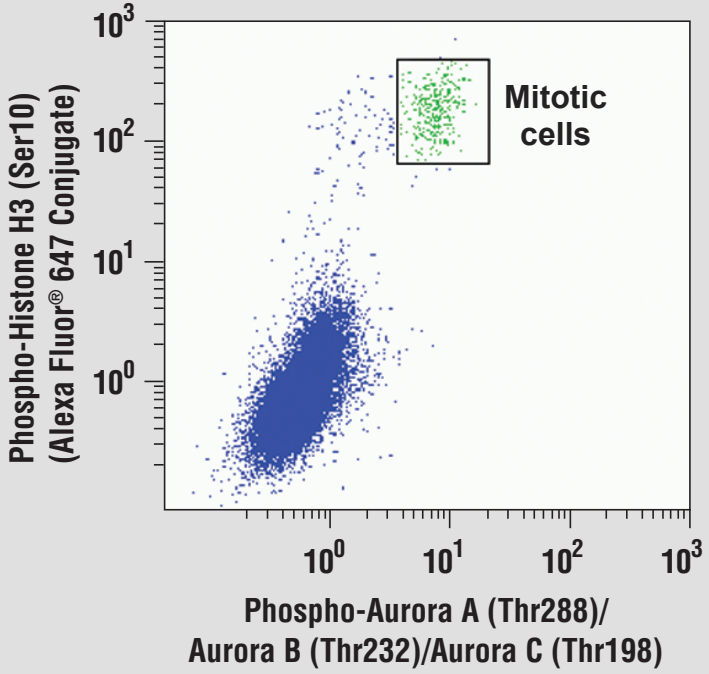
Cell-cycle analysis employs flow cytometry to distinguish cells in different phases of the cell cycle



Using phospho-Histone H3 to study cell cycle

- Phosphorylation of Histone H3 is tightly correlated with chromosome condensation during both mitosis and meiosis



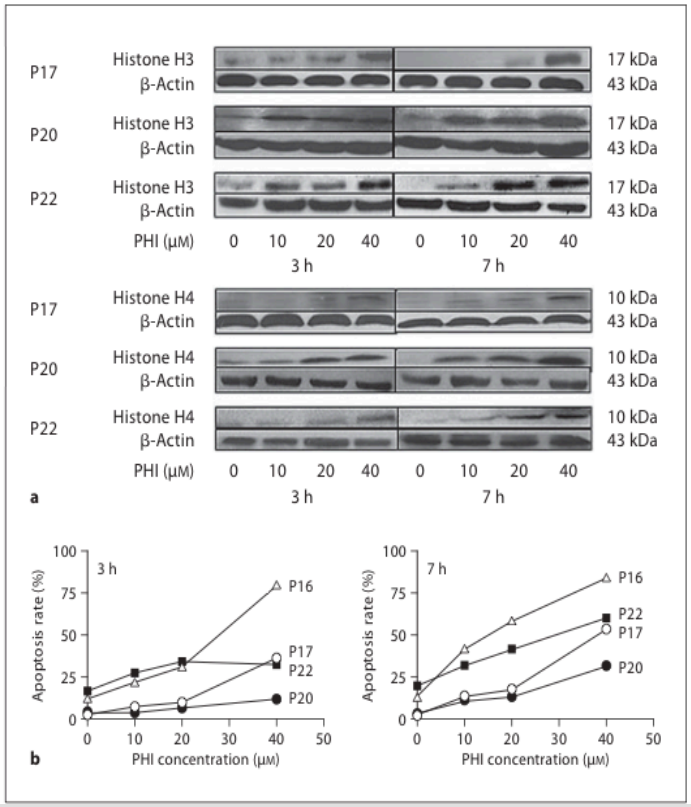


P-Histone H3 (Ser10) (D2C8) XP® Rabbit mAb (Alexa Fluor® 647 Conjugate) #3458
Propidium Iodide (PI)/RNase Staining Solution #4087
P-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (D13A11) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate) #8525

Epigenetics flow cytometry

Study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence

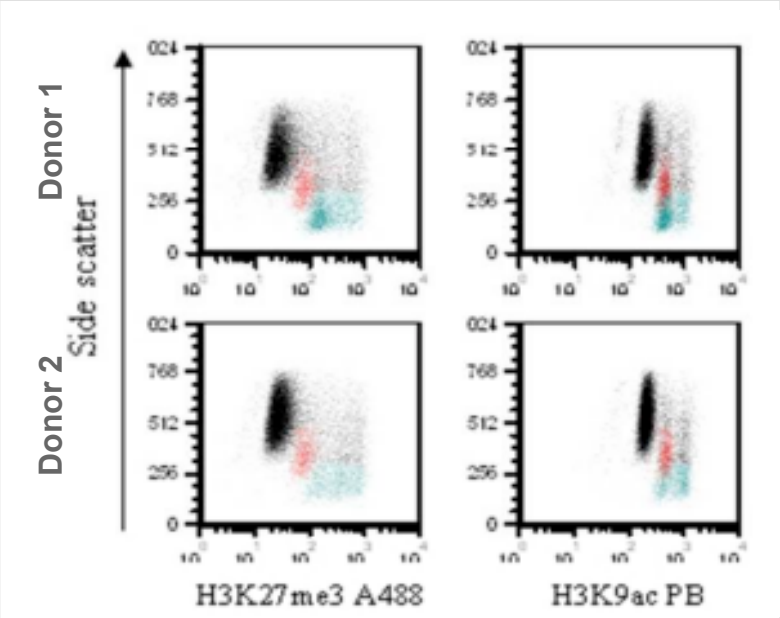
- Epigenetic modification include: methylation of DNA, modification of histones, modification of transcription factors
- Epigenetic aberrations well established in many diseases
- Epigenetic changes are reversible
- Current methods to study epigenetic changes
  - Western blot
  - Chromatin Immunoprecipitation (ChIP)



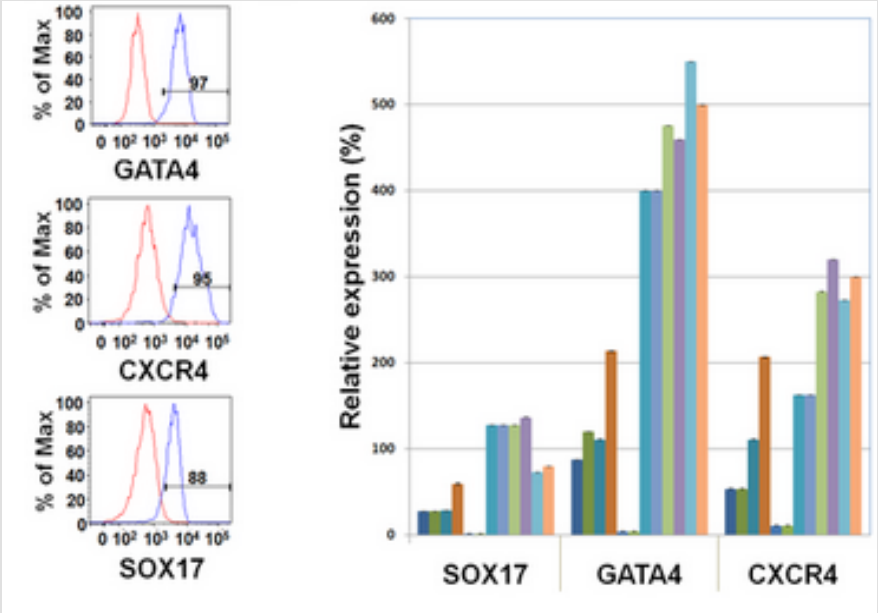
Deficient Histone Acetylation in Acute Leukemia...
Acta Haematologica, 2010, 123

Flow cytometry used to study epigenetic status

- Expression levels of histone marks (methylation, acetylation)
  - **Watson *et al*, Cytometry 2013** - Provides fix and perm optimizations!



- Transcription factor-based cell sorting followed by DNA/RNA analysis
  - **Pan *et al*., PLOSOne, 2011**

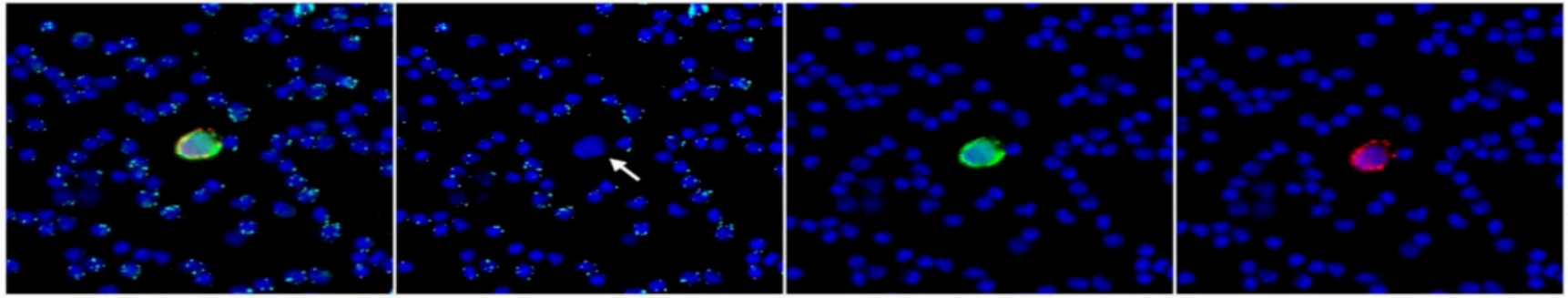


New Techniques!

ACD: CTCScope

- Detection of circulating tumor cells

The four-color assay system:



panCTC (Alexa488) - for CTC identification.

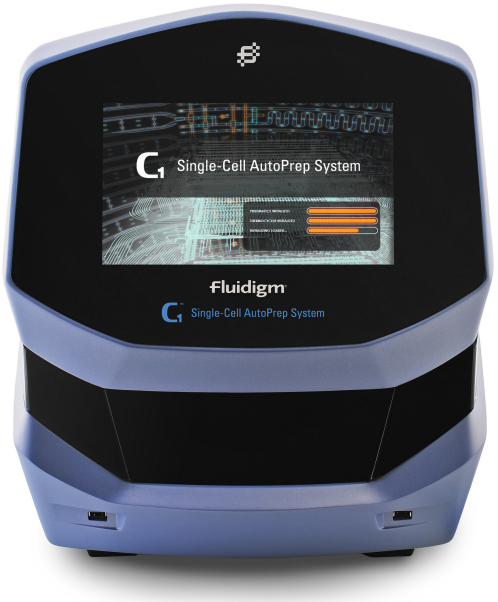
CD45 (Alexa647) - blood cell marker as negative selection marker and assay control

EGFR or HER2 (Alexa546) - for CTC profiling.

DAPI - Nuclei stain

Fluidigm: C1

- Enabling Single-Cell Gene Expression Analysis in Rare Events Combining FACS and the C1Fluidigm System

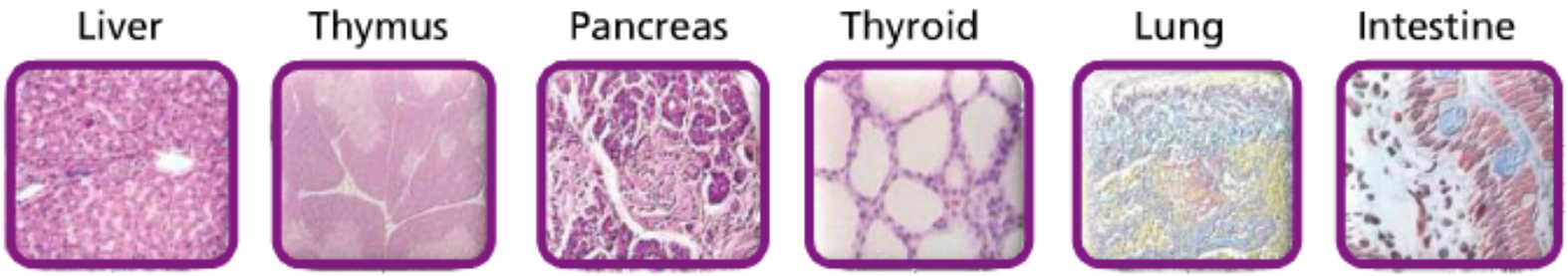


FAQs

Your questions answered

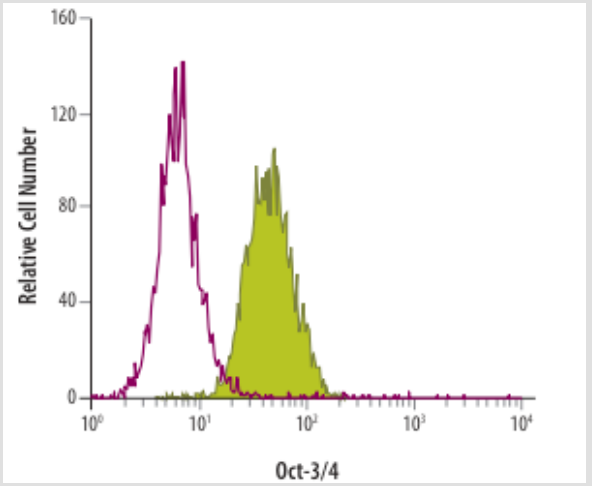
Sample preparation for intracellular flow cytometry

- All samples for flow cytometry must be in liquid suspension
- Tissues must be disaggregated into single cell suspension
  - Mechanical disaggregation : Syringe plunger, cell strainer, scalpel blade
  - Density separation: fycol gradient
  - Chemical disaggregation: trypsin



Can I do intracellular-flow in adherent cells? Will detachment methods alter significantly signaling events?

- Intracellular flow can be successfully performed in adherent cells
- An internal control is necessary
- Detach cells prior to fix and perm
- PBS/EDTA preferred to trypsin or mechanical detachment methods
  - Accutase - more specific for extracellular matrix molecules
  - Commercial, enzyme-free cell dissociation buffers
- Remove dead cells prior to fix and perm and staining



Human embryonic stem cell line BG01W stained with Oct 3/4

What fluorochromes do I use for intracellular targets?

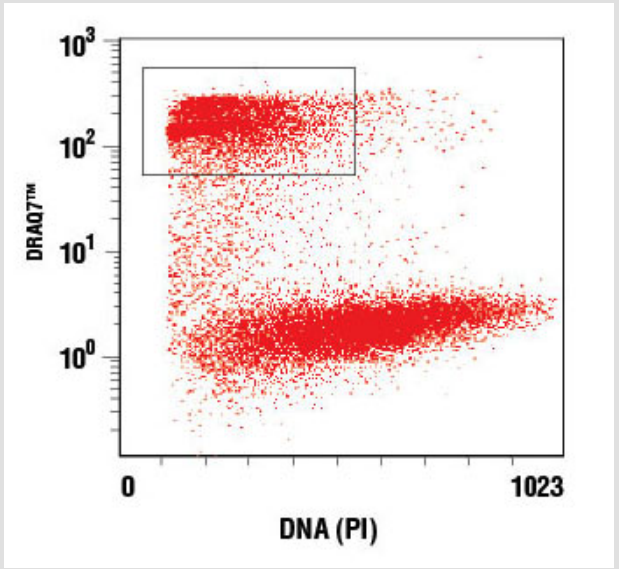
- Fluorochromes compatible with your cytometer (FACSCalibur A: blue, green, red and violet laser)
- Minimum Spectral Overlap – Avoid high compensation values
- Use the brightest fluorochrome for the lowest expressed protein – Stain index
- More stable and resistant to fixation fluorochrome for surface markers

Fluorochrome	Stain Index
PE-Cy5	353
PE	302
APC	278
Alexa Fluor® 647	214
PE-Cy7	139
PerCP-Cy5.5	107
Pacific Blue™	80
Alexa Fluor® 488	73
Alexa Fluor® 700	61
FITC	56
APC-Cy7	37
PerCP	37
AmCyan	25

Maecker & Trotter, 2008

What can I use as a viability marker?

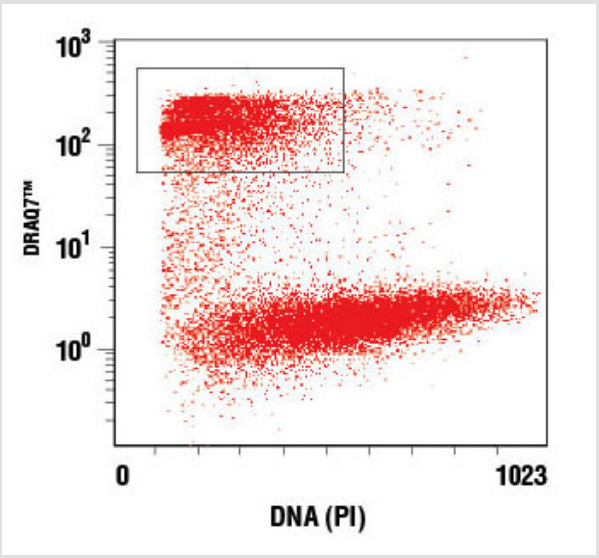
- DRAQ7™ #7406: far-red fluorescent DNA dye
- Only stains membrane-comprised, dead or permeabilized cells
- Can be used in combination with GFP, AlexaFluor® 488 and PE (excitation: blue-to-red, emission: >675nm)
- Highly photo-stable
- Unique for real-time viability:
  - Smith et al., *Cytometry A*. 2013, 83
  - Akagi et al., *Cytometry A*. 2013, 83
- Unlike DAPI or PI does not need compensation



Live Jurkat cells treated with Staurosporine #9953. Gated population represents DRAQ7™-positive apoptotic cells.

What can I use as a viability marker?

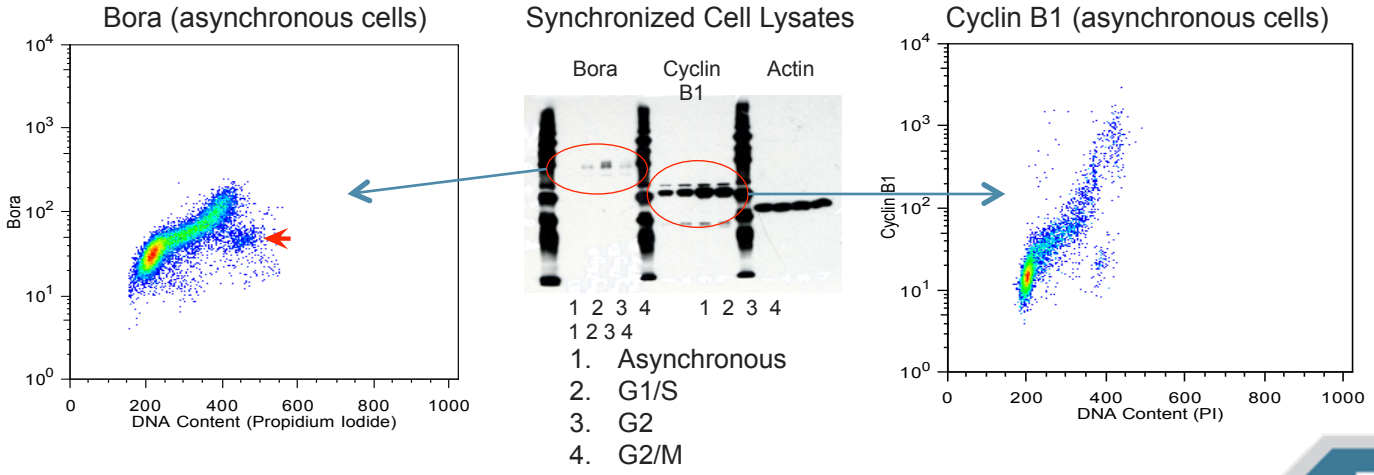
Viability marker	Channel occupation	*Emission max*
DRAQ7™	Allows all 4 major colors	694
7-AAD	Occludes AlexaFluor 647 (partially)	647
PI	Occludes PE, APC	617
TOTO-3	Accludes APC	642
DAPI	Occludes GFP	458



Live Jurkat cells treated with Staurosporine #9953. Gated population represents DRAQ7™-positive apoptotic cells.

How do I analyze cell cycle data?

- Use of cyclins: cyclin A is expressed in late S and G2 phase and degraded during mitosis. Cyclin B1 is expressed during G1 phase
- Phospho-Histone H3: phosphorylation correlated to both mitosis and meiosis
- Bora: peak expression in G2 phase
- All antibodies can be plotted against each other or against DNA dyes (PI/DAPI)
- Jacobberger et al., 2011. Flow cytometry protocols, Methods in Molecular Biology, Gong et al., 2008 Cell proliferation



Technical support

Our help to you

Cell Signaling TECHNOLOGY®

Technical support

The same scientists who develop and validate all CST™ antibodies are available as technical resources to help you at any stage in your research

Application support provided by the Flow Cytometry group

- Protocol-fixation/perm/staining
- Antibody concentrations
- Has antibody been tested/validated for flow cytometry?
- Recommendations for the best antibody for your experiment when multiple options are available

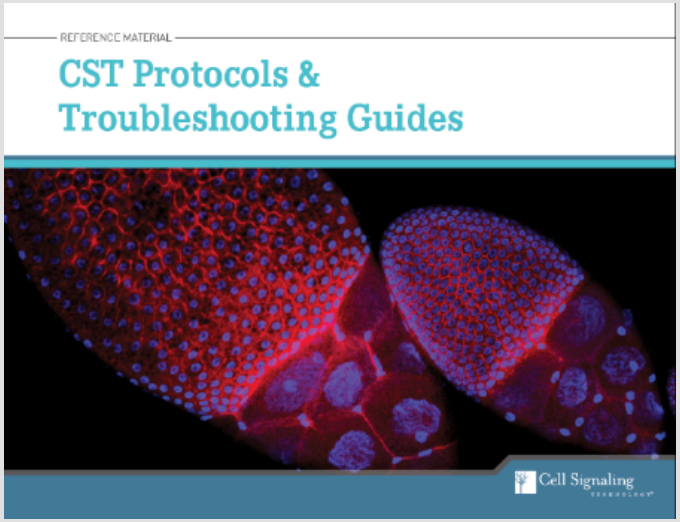
Contact us at:
eusupport@cellsignal.eu

Cell Signaling TECHNOLOGY®

Optimized protocols available

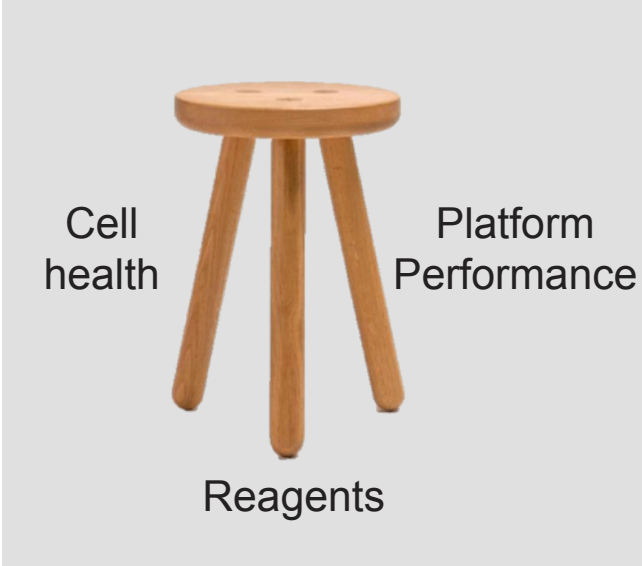
The most common troubleshooting cases can be solved using CST optimized protocols

- We test our products to obtain the best possible results
- We strongly recommend using our optimized application-specific protocols for each product
- These guarantee accurate and reproducible results



Take-home message

- Antibody-antigen interactions are complex - No protocol fits all
- Your assay is only as good as your reagents
- Don't assume any commercial antibody will work with your assay: Carefully review validation procedures
- CST validates each antibody and optimizes protocols for flow cytometry



When in doubt, ask us!

eusupport@cellsignal.com

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Thanks for listening!

Founded by research scientists in 1999, Cell Signaling Technology (CST) is a private, family-owned company with over 400 employees worldwide. Active in the field of applied systems biology research, particularly as it relates to cancer, CST understands the importance of using antibodies with high levels of specificity and lot-to-lot consistency. It's why we produce all of our antibodies in house, and perform painstaking validations for multiple applications. And the same CST scientists who produce our antibodies also provide technical support for customers, helping them design experiments, troubleshoot, and achieve reliable results. We do this because that's what we'd want if we were in the lab. Because, actually, we are.

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