BECKMAN COULTER

We're better together

Cell death, transduction pathway and secretion tracked by flow cytometry

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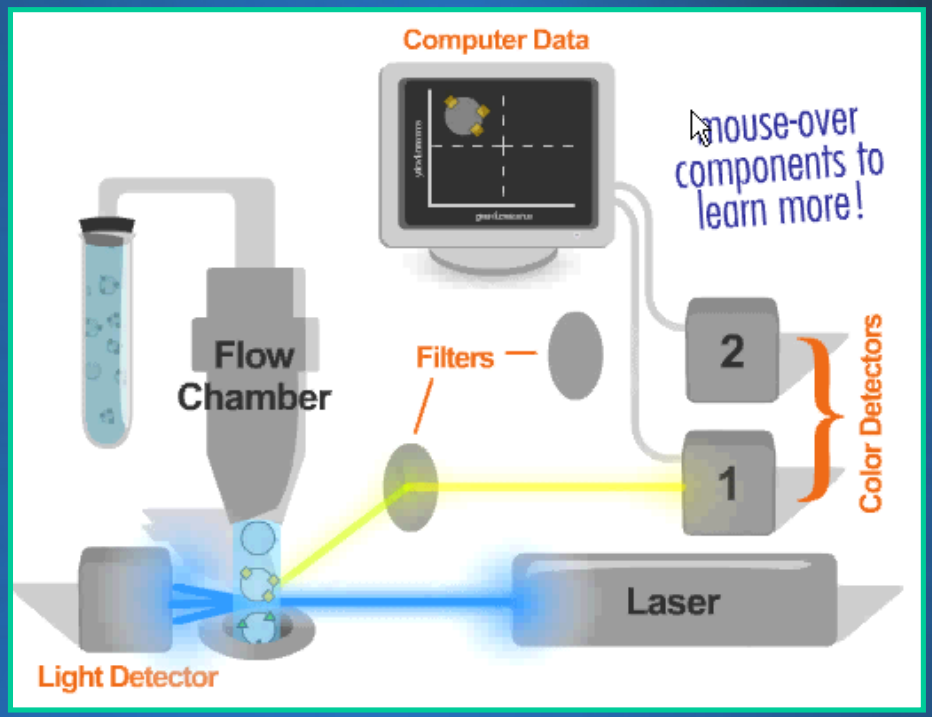
I.What is Flow Cytometry?

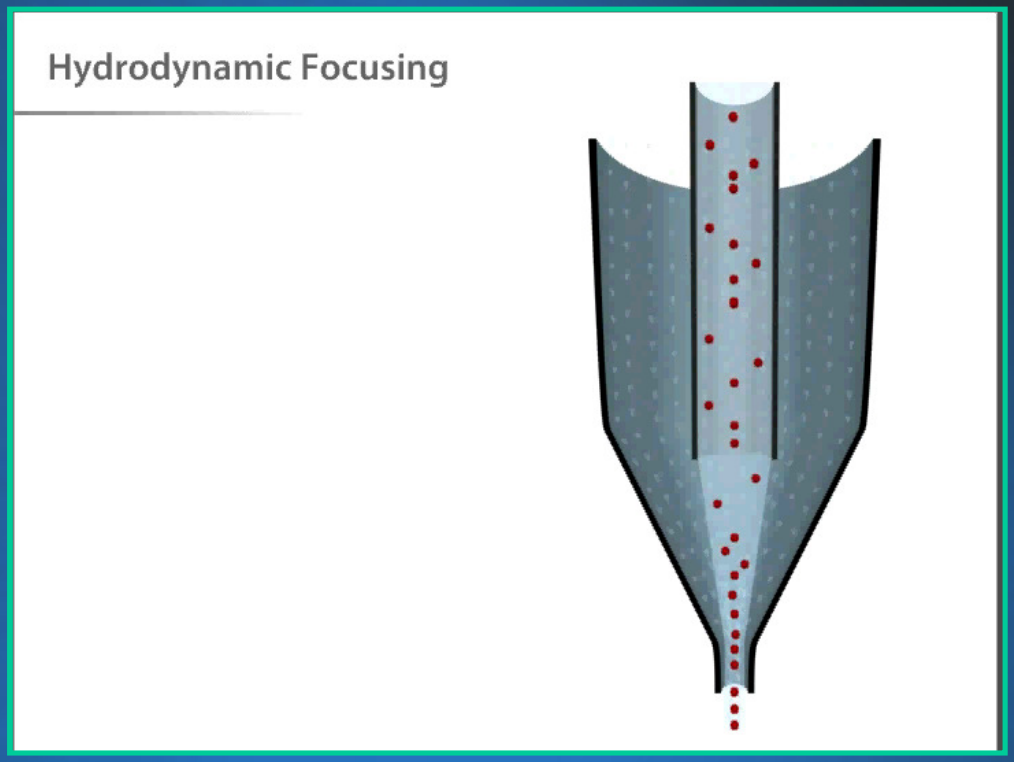
- It is a cellular measure of several parameters
- Based on flow
- Using electronic apparatus to measure



http://www.unsolvedmysteries.oregonstate.edu/flow_06

I.Critical point

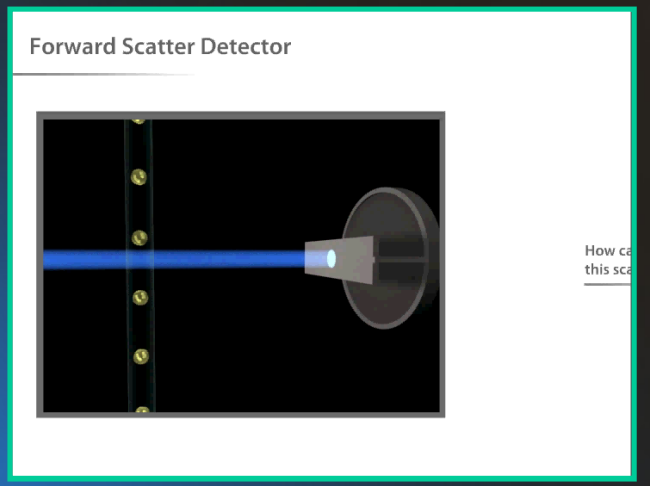
- Hydrodynamic focusing : one by one

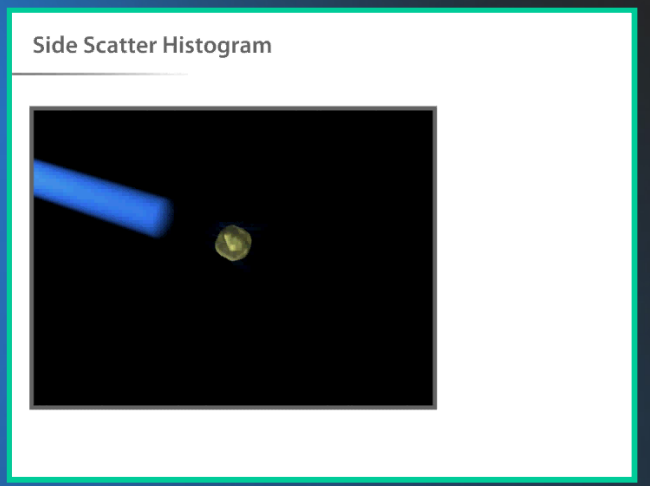


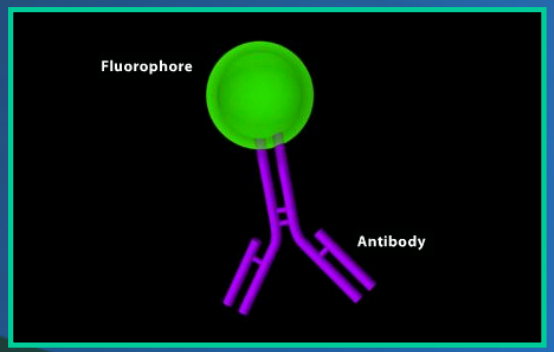
http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

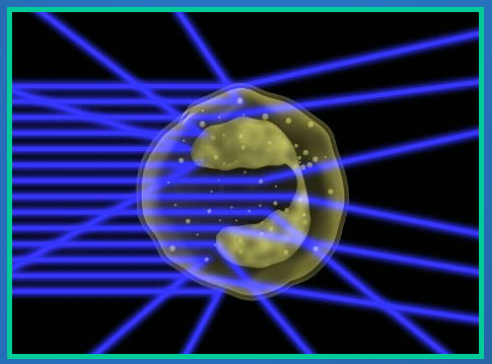
I.Which parameters are measured?

- Forward Scatter : FSC cell size
- Side Scatter : SSC cell granularity
- Fluorescences : probe linked to a cellular component





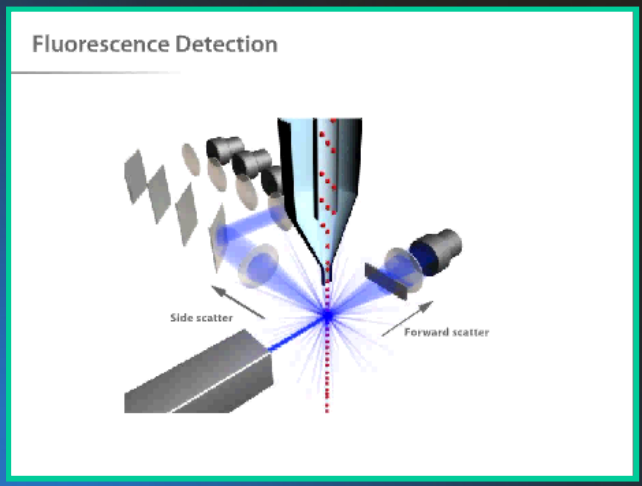


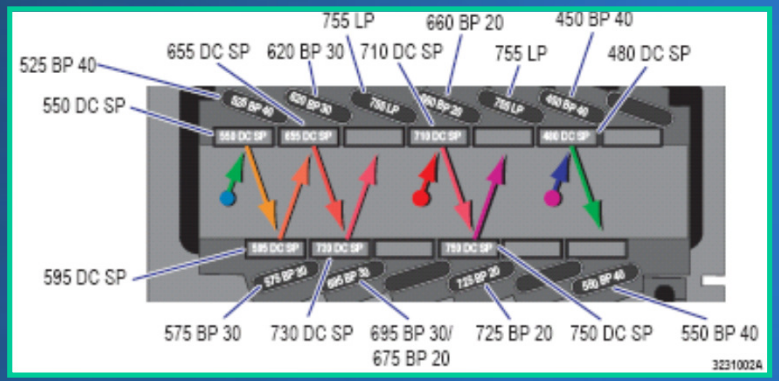


http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

I.Optical Bench

- Collects light from sample
- Separates light to have specific wavelenght

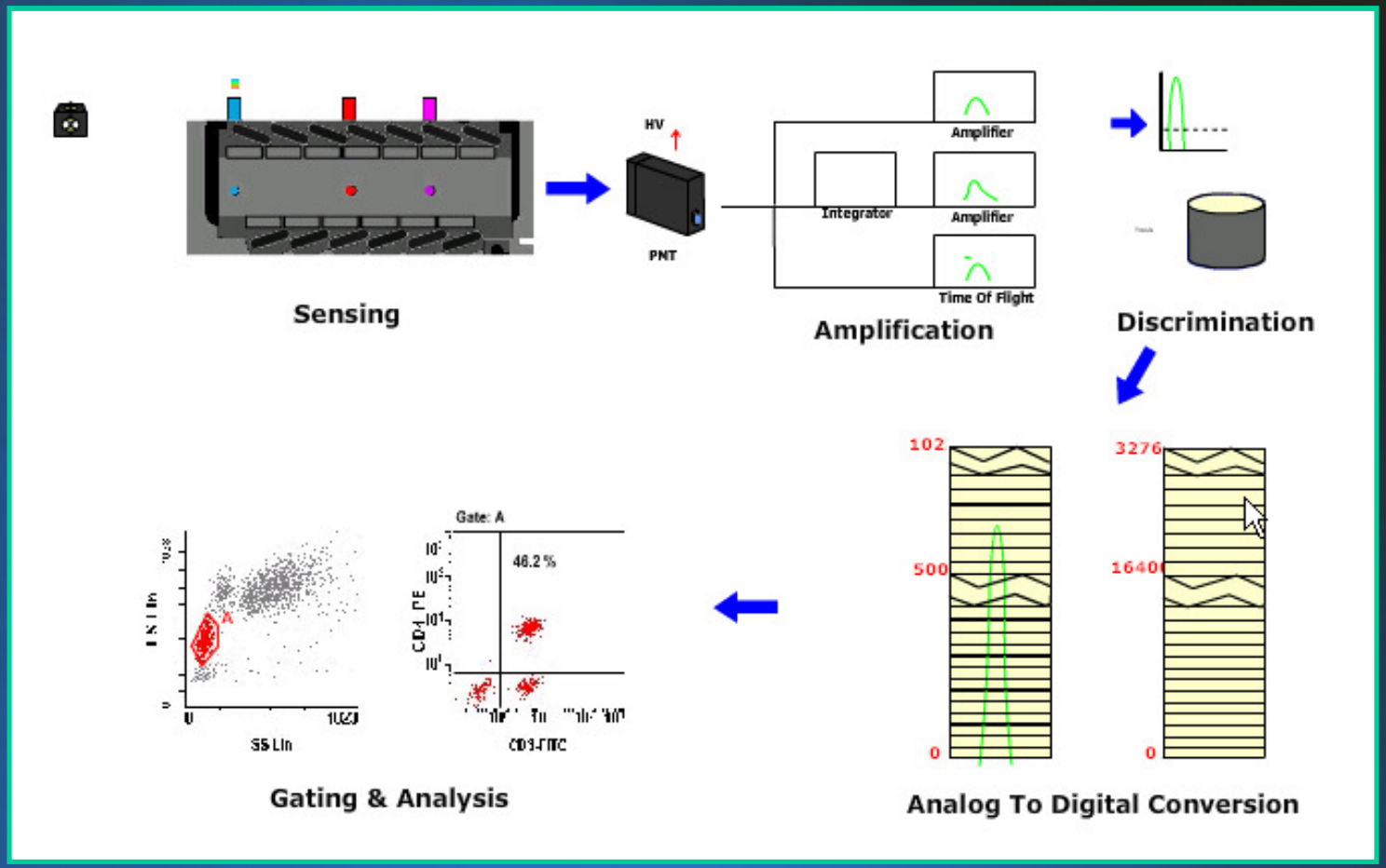




http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

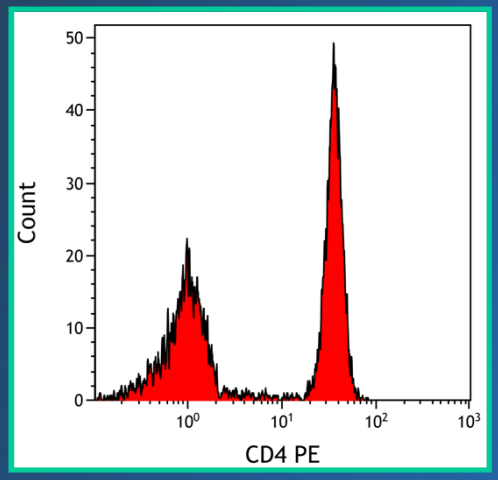
http://www.coulterflow.com/bciflow/instrumentsot.php

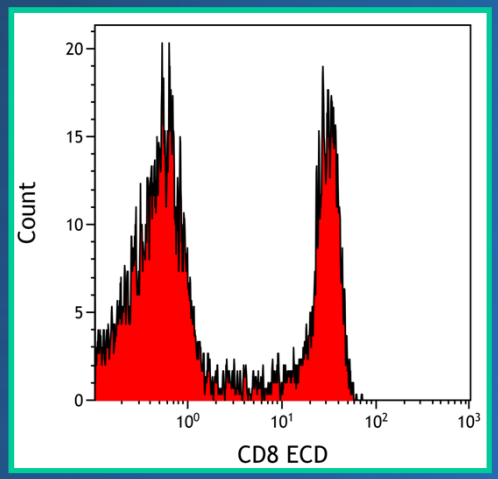
I.From light to data



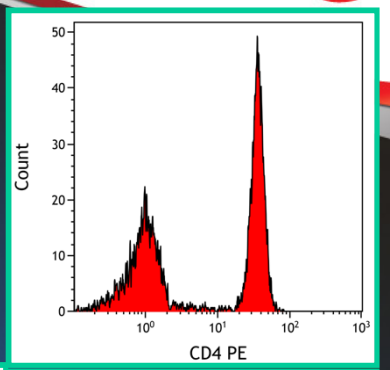
http://www.coulterflow.com/bciflow/

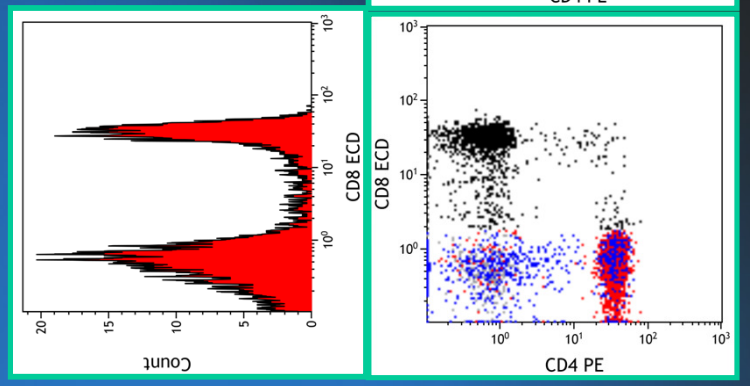
I.Data representation



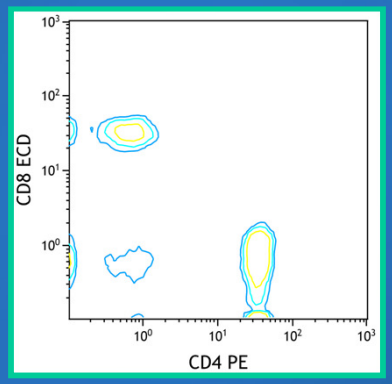


« Histogram Plot »

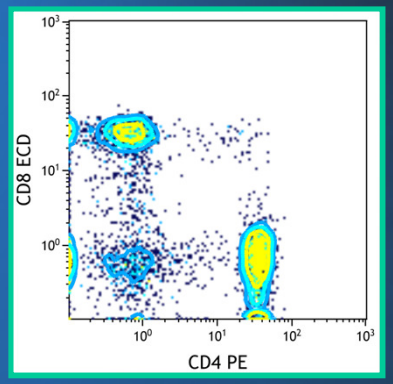




« Dot Plot »



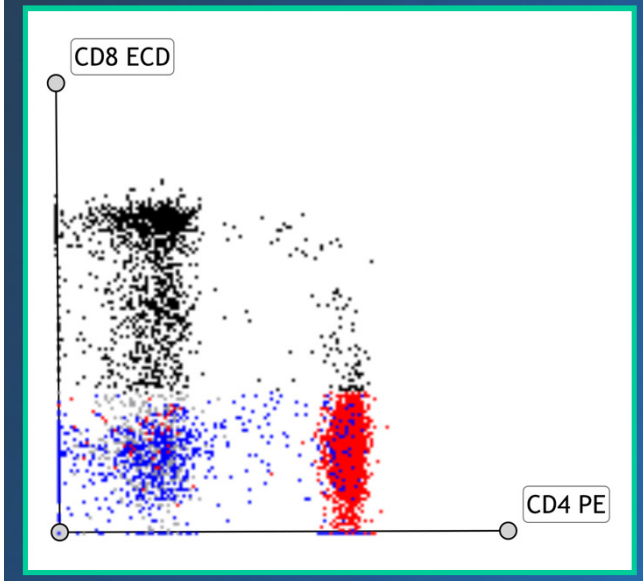
« Contour plot »

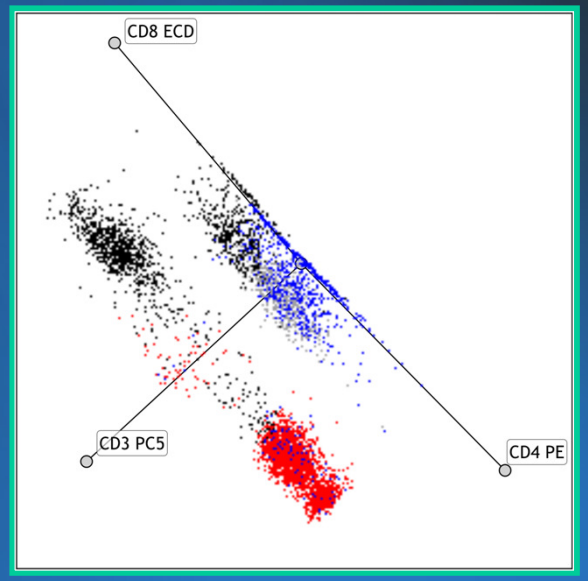


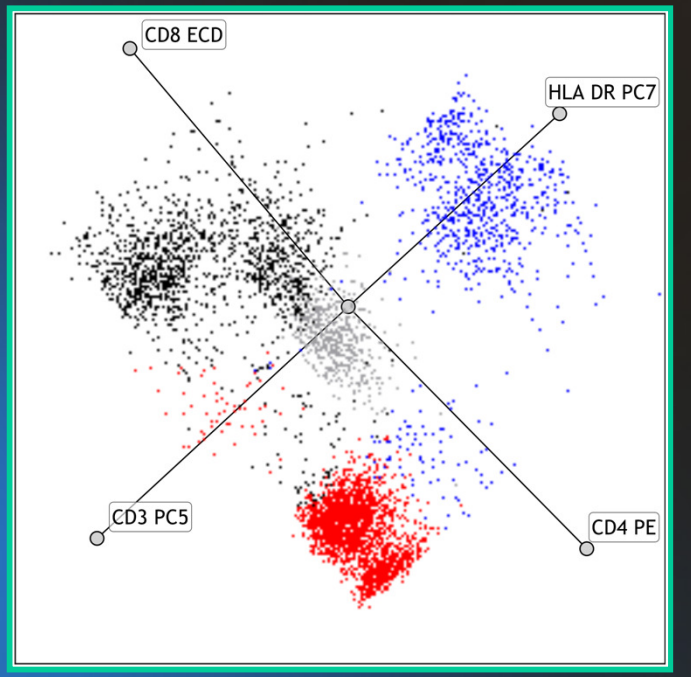
« Contour plot with density »

Images were made with Kaluza™ Software, BeckmanCoulter

I.Data representation : Radar Plot



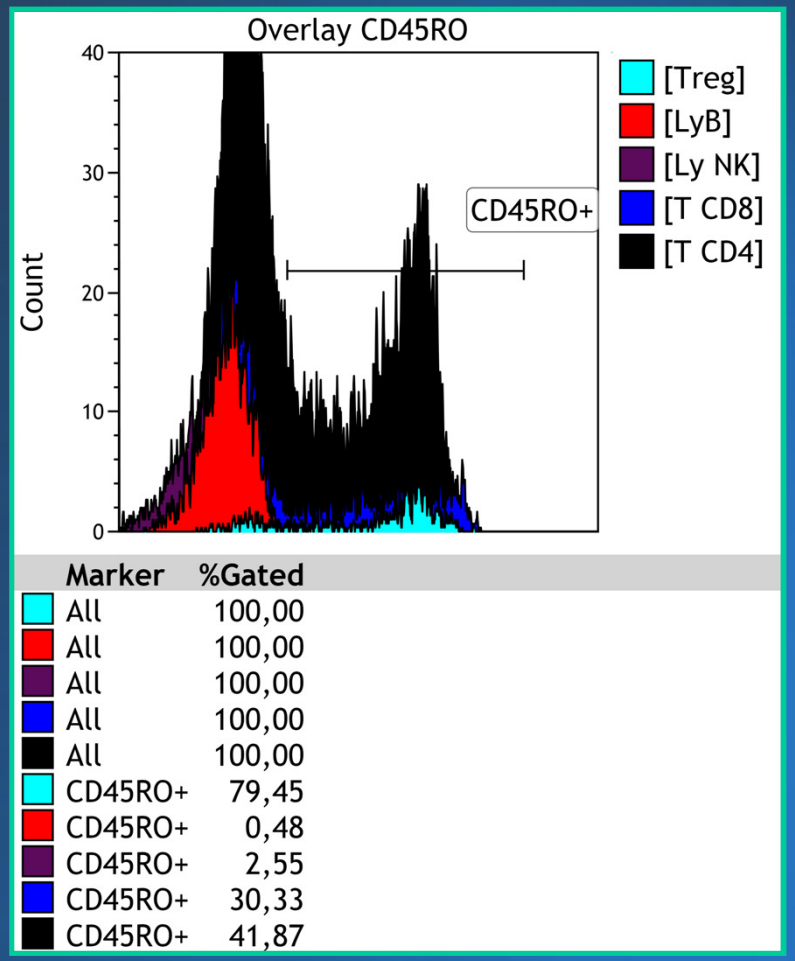




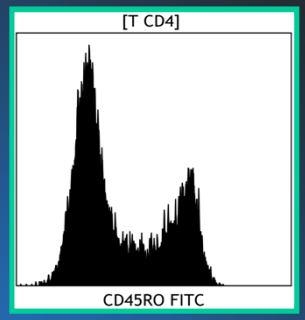
Multidimensional analysis

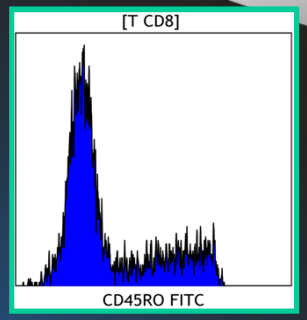
Images were made with Kaluza™ Software, BeckmanCoulter

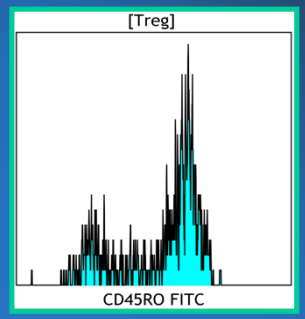
I.Data representation

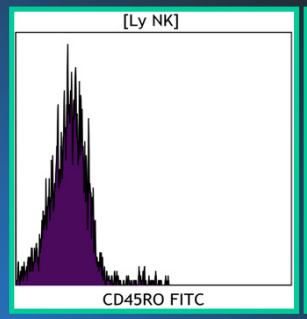


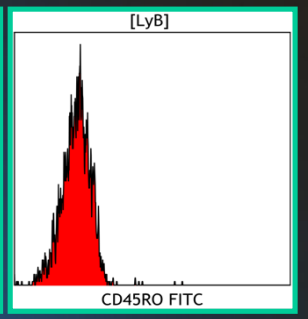
Marker	%Gated
All	100,00
All	100,00
All	100,00
All	100,00
All	100,00
CD45RO+	79,45
CD45RO+	0,48
CD45RO+	2,55
CD45RO+	30,33
CD45RO+	41,87

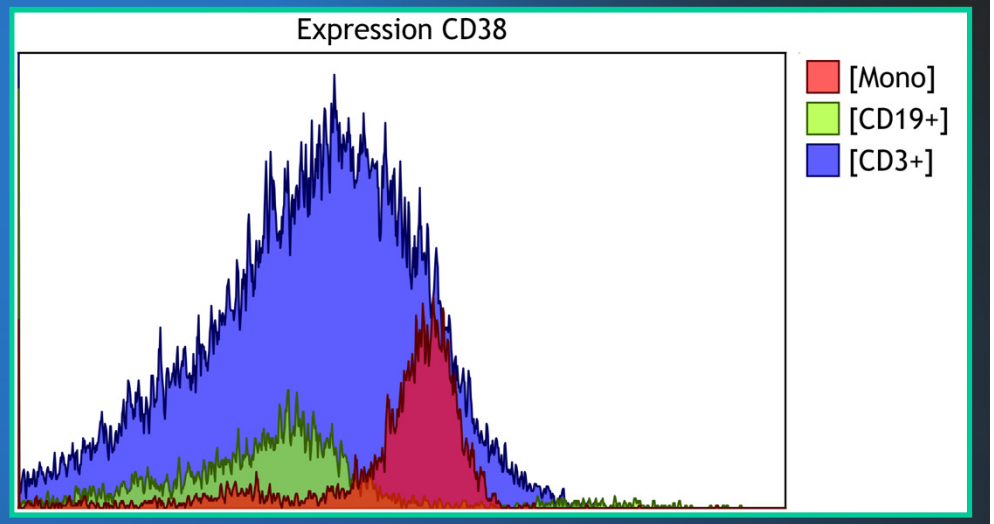












Images were made with Kaluza™ Software, BeckmanCoulter

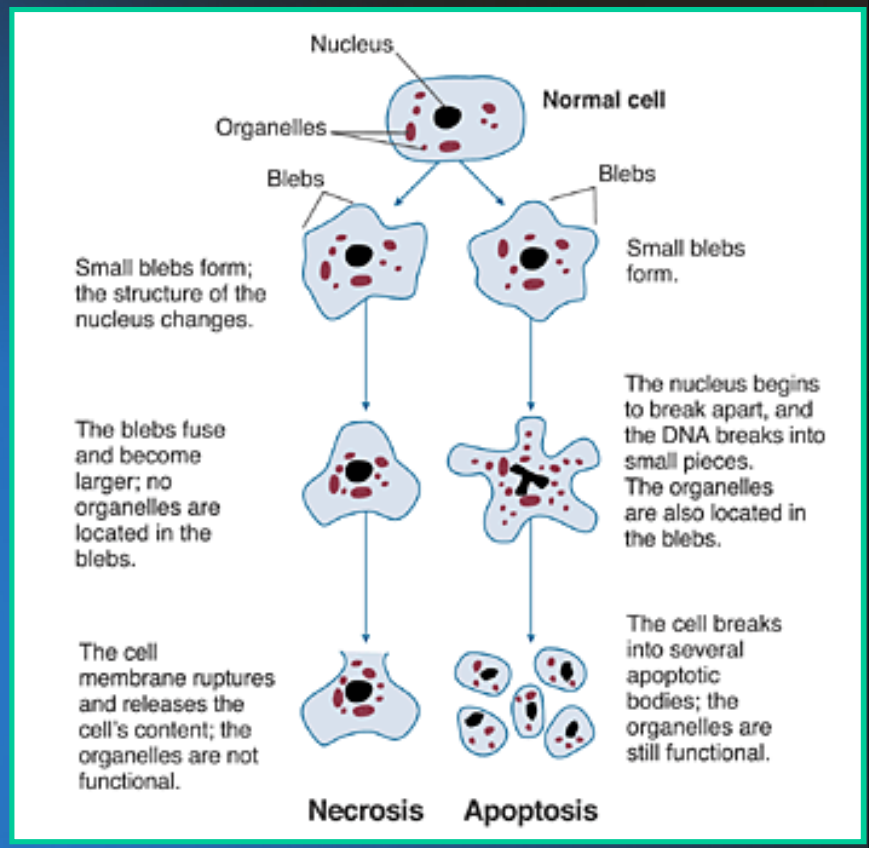
II.Cell death

- Two principal ways of death
- Apoptosis and necrosis
- Lot of modifications

- Apoptosis : Chromatin condensation, DNA fragmentation, cell size decrease, granularity increase, reduction of metabolism, mithochondrial events, caspases activation, exposition of phosphatidylserine

- Necrosis : Cell size increase due to loss of membrane integrity, and finally cell burst

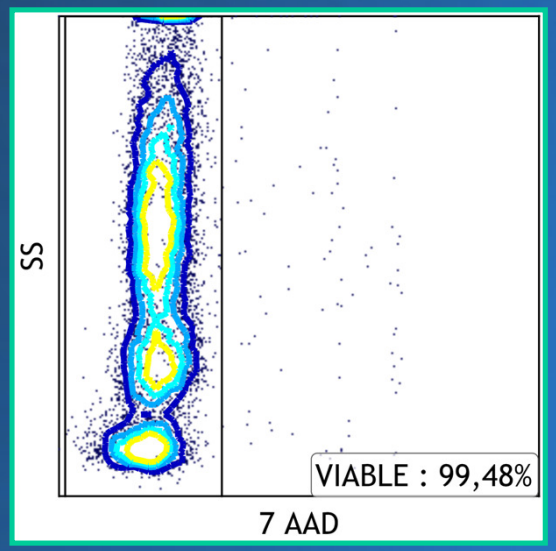
- Lot of ways to measure cell death

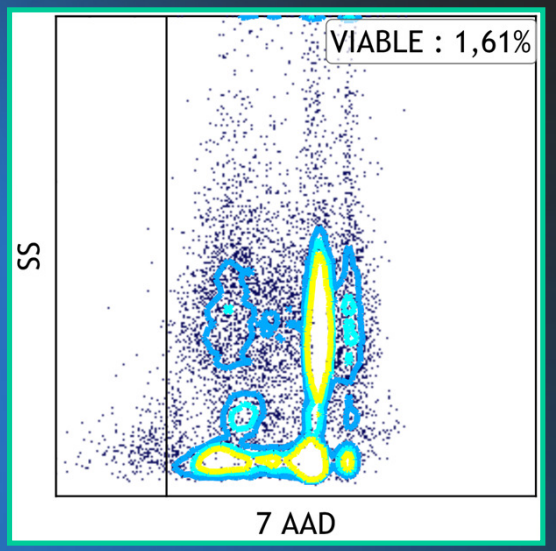


http://www.niaaa.nih.gov/Resources/GraphicsGallery/Liver/changes.htm

II.Basic death analysis

- Membrane permability tests : PI, 7AAD, Sytox, Ethidium Bromide, YOPRO…





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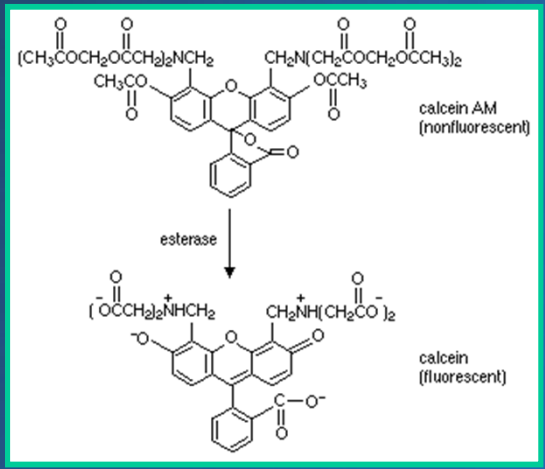
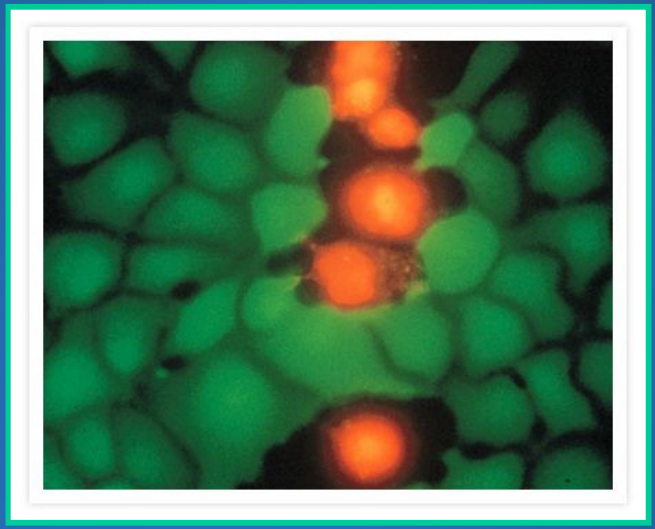
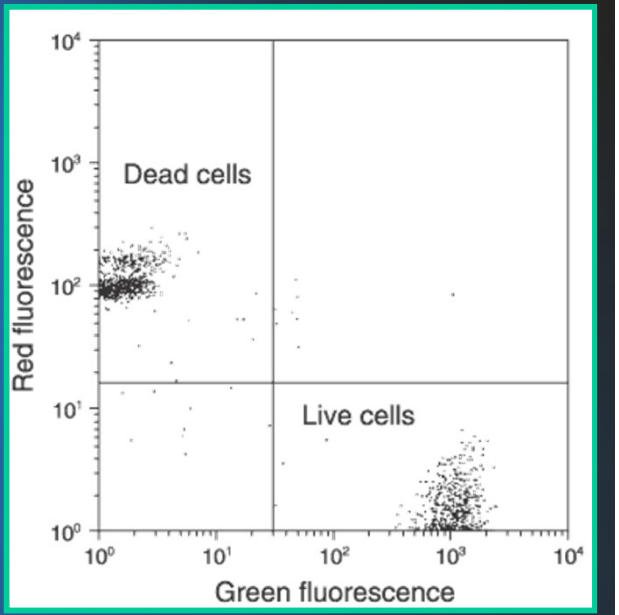
II.Basic death analysis

- Retention tests : Calcein-AM, CFDA-AM, Vital Dye
- Probe passively enters the cell, keeps in only in live cells





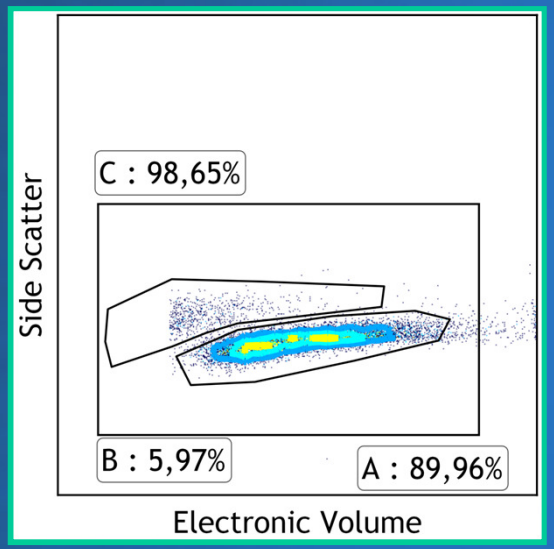


http://www.currentprotocols.com/protocol/cy0404
http://www.invitrogen.com

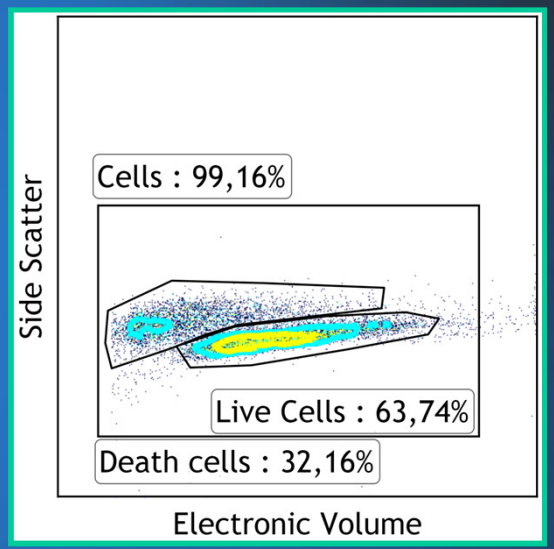
II.Apoptosis analysis

- Annexin V FITC : phosphatidylserine
- Propidium Iodide or 7-AAD : permeabilized membrane
- Jurkat cells treated by Thapsigargin, SERCA pump inhibitor (Eckstein-Ludwig U and al, 2003, *Nature* 424 (6951): 957–61)

Untreated



Treated

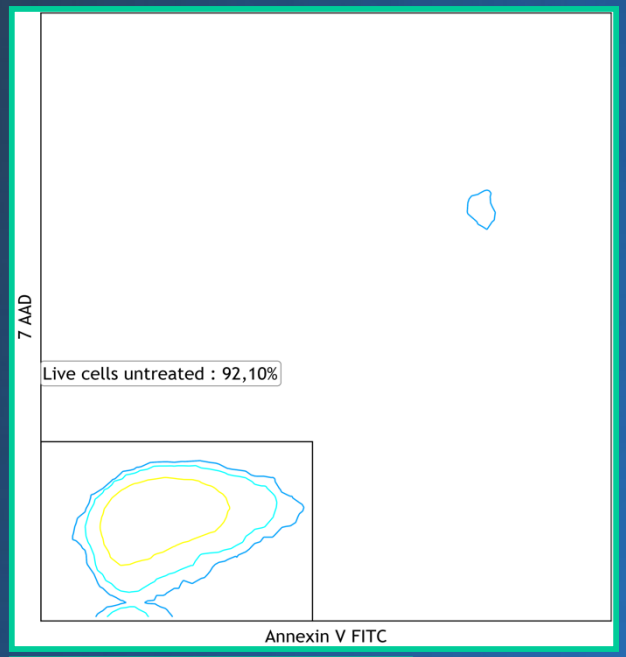


Data collected on Cell Lab QUANTA SC™, Beckman Coulter

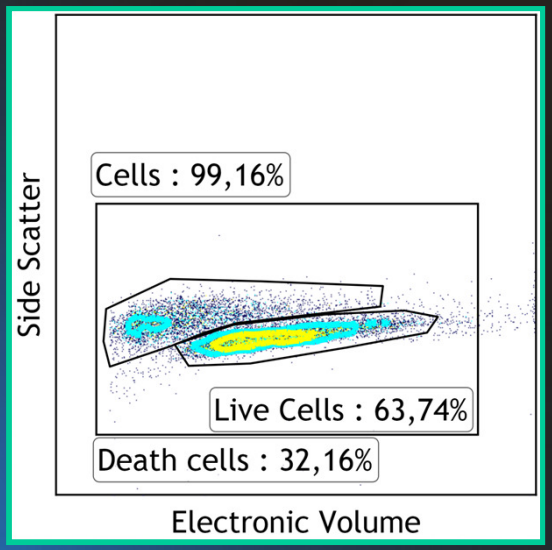
Images were made with Kaluza™ Software, Beckman Coulter

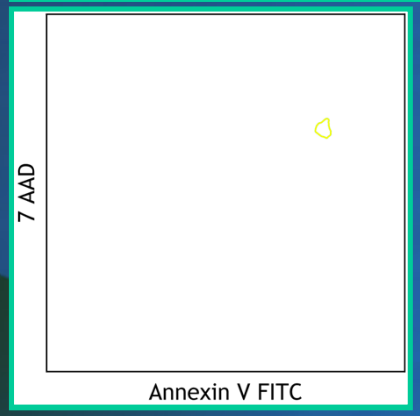
II.Apoptosis analysis

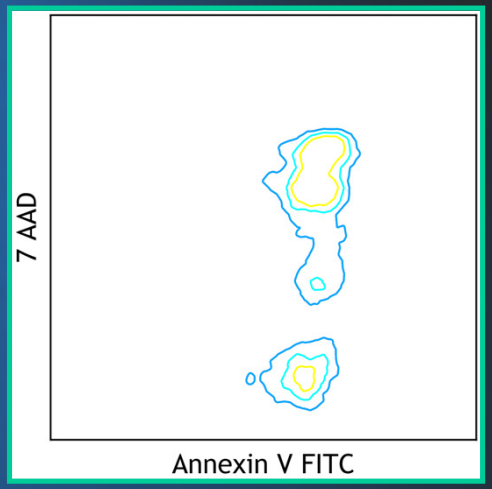
Untreated



Treated



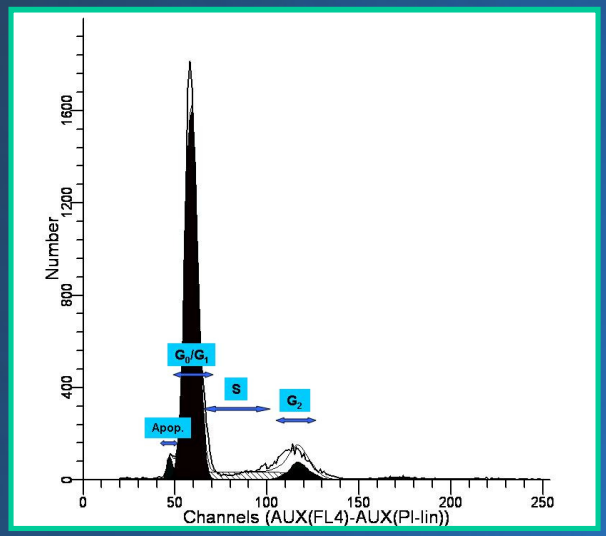


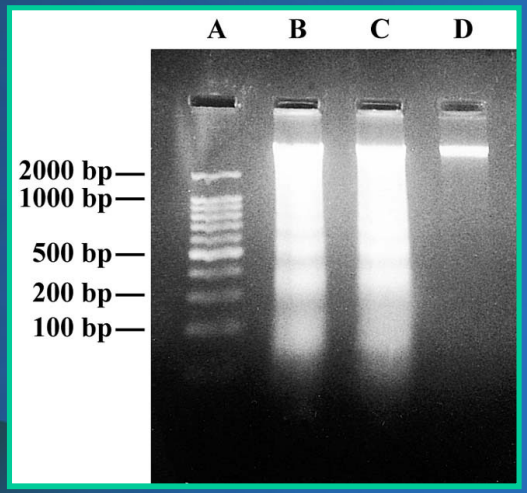


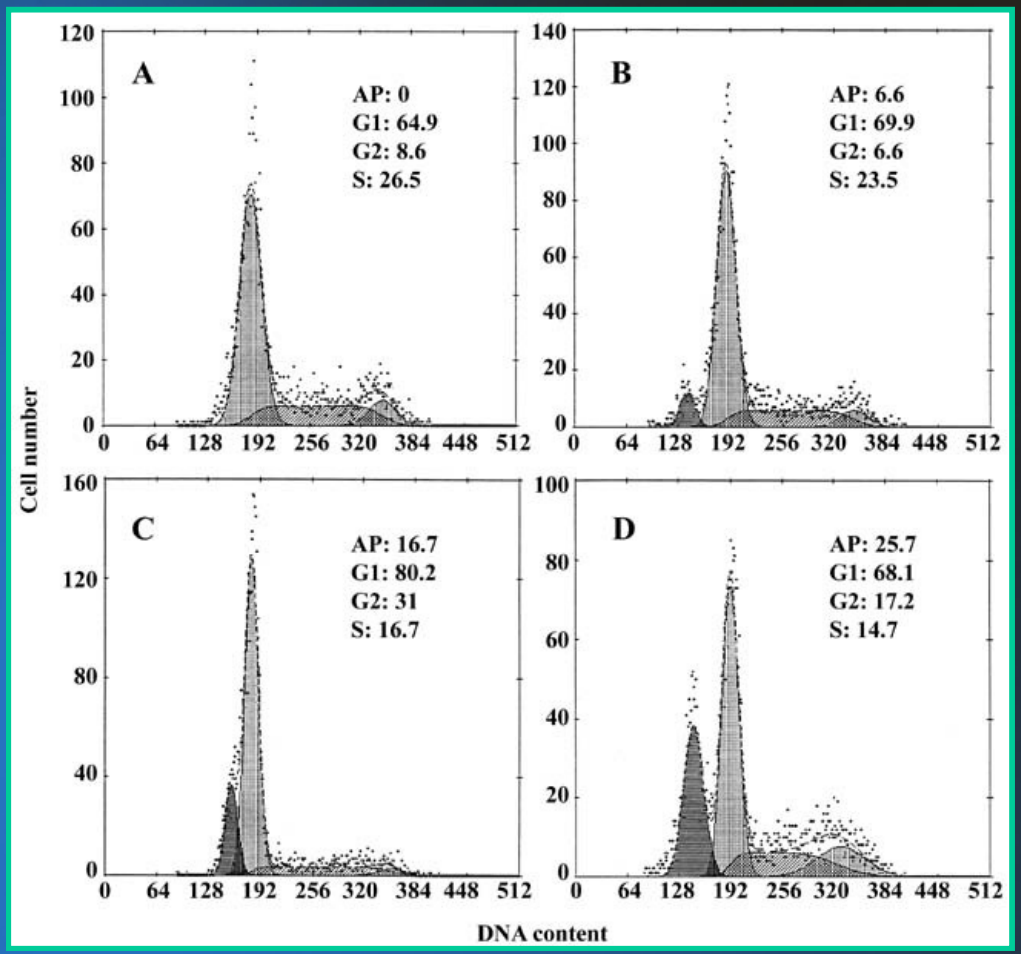
Images were made with Kaluza™ Software, Beckman Coulter

II.Apoptosis analysis

- DNA fragmentation : Sub G1 peak analysis (Propidium Iodide or 7AAD)



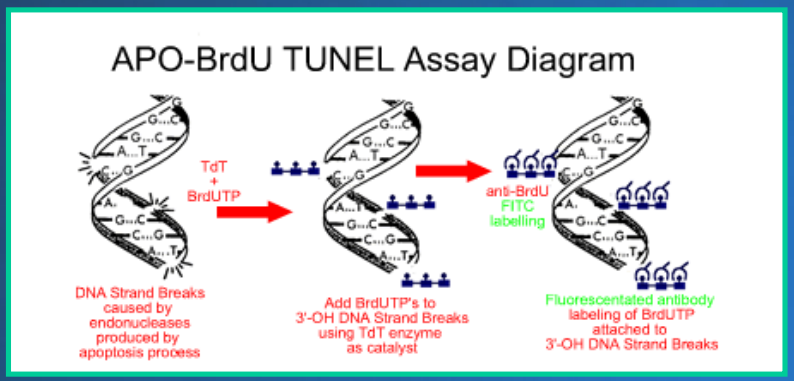


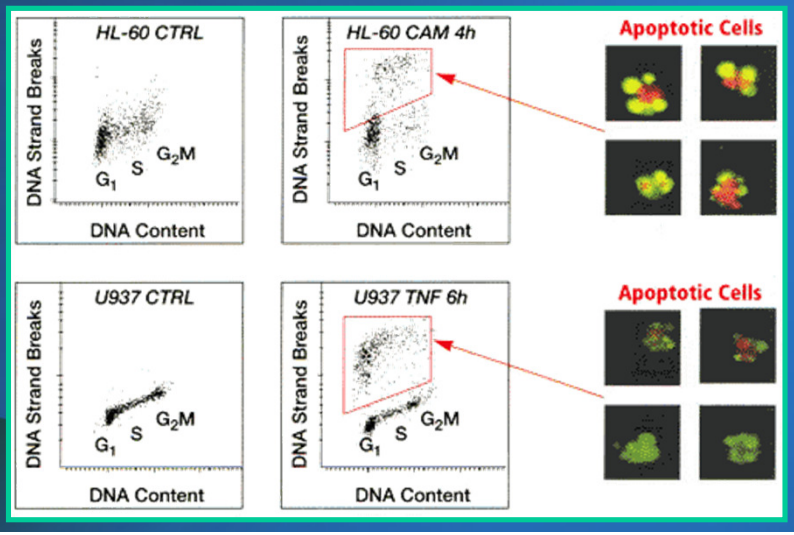


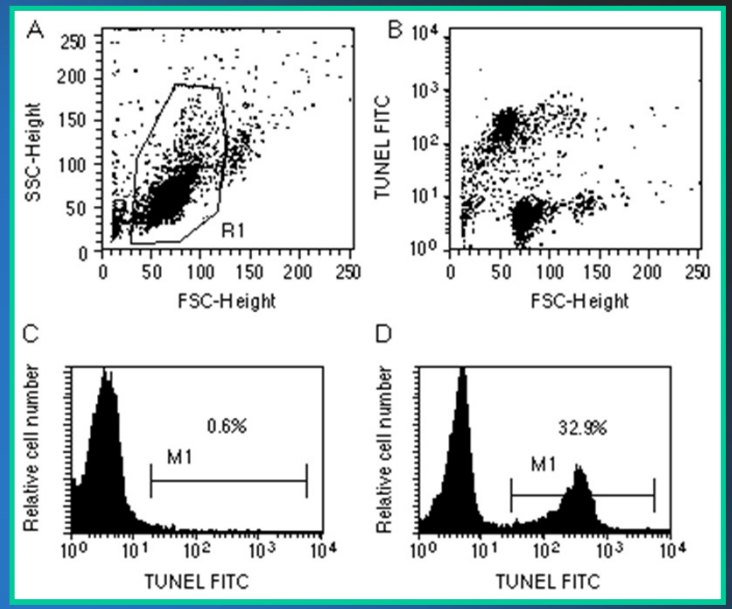
Hao LL et al *Acta Pharmacol Sin* 2004 Nov; 25 (11): 1509-1514

II.Apoptosis analysis

- DNA fragmentation : TUNEL Assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling)
- BrdU is added to DNA break by TdT, anti-BrdU-FITC/PI
- dUTP-FITC directly







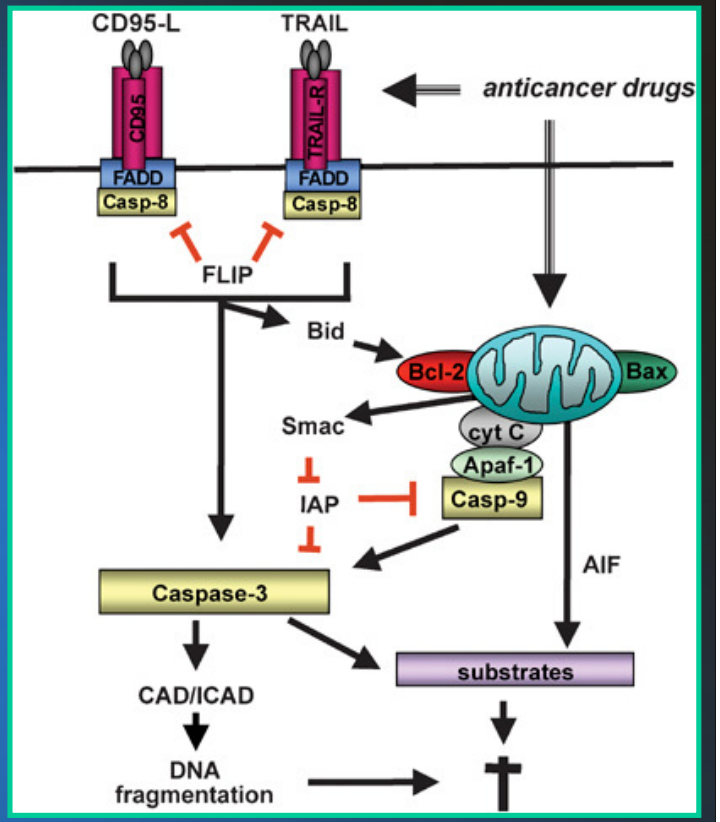
Martin and al, *Current Protocols in molecular biology* ; 2001

www.phoenixflow.com

www.Compucyte.com

II.Apoptosis analysis

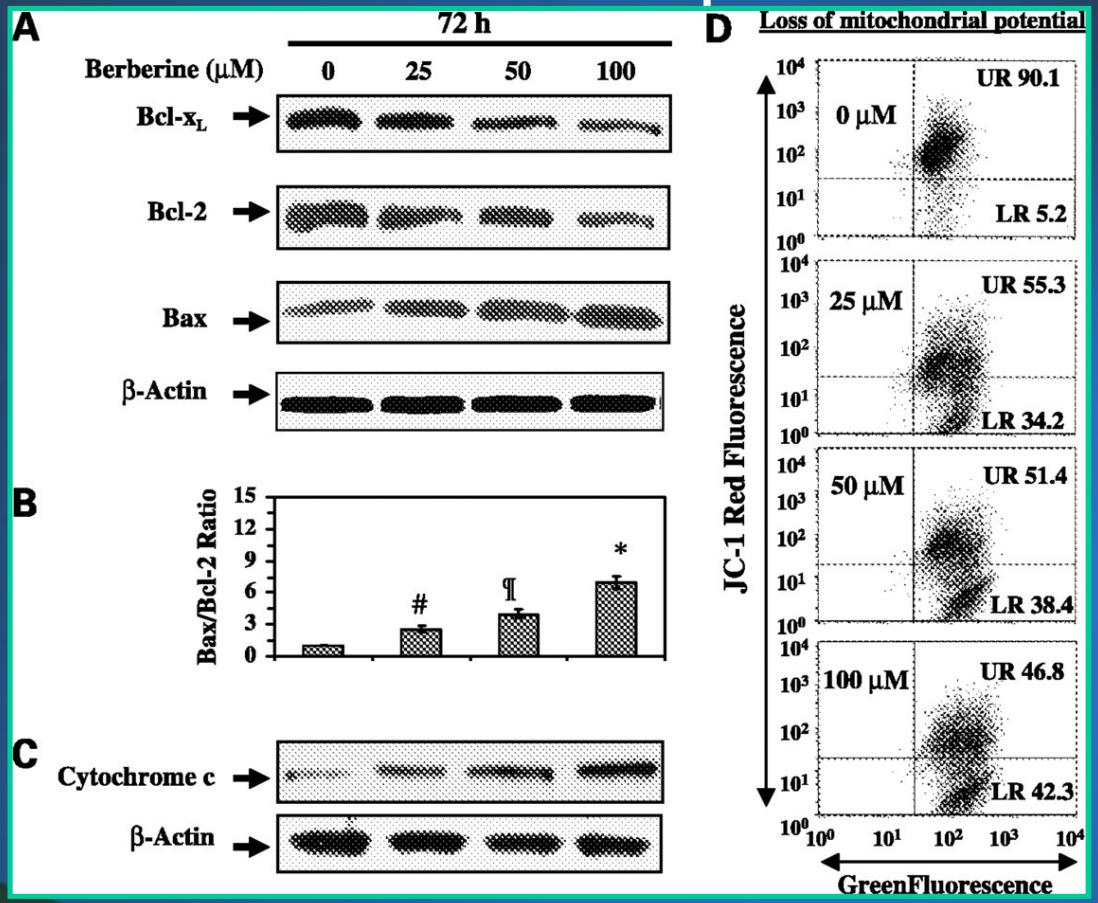
- Mitochondrial events :
  - ✓APO2.7 protein detection
  - ✓Membrane depolarisation JC-1/DiOC(6)/Mitotracker...
  - ✓Bax/Bad/Bcl-2/Bcl-xL detection
  - ✓Caspase global or specific activity



S Fulda and K-M Debatin ***Oncogene,* 2006 : 25**, 4798-4811

II.Apoptosis analysis

- Membrane depolarisation



JC-1 monomeric form : green

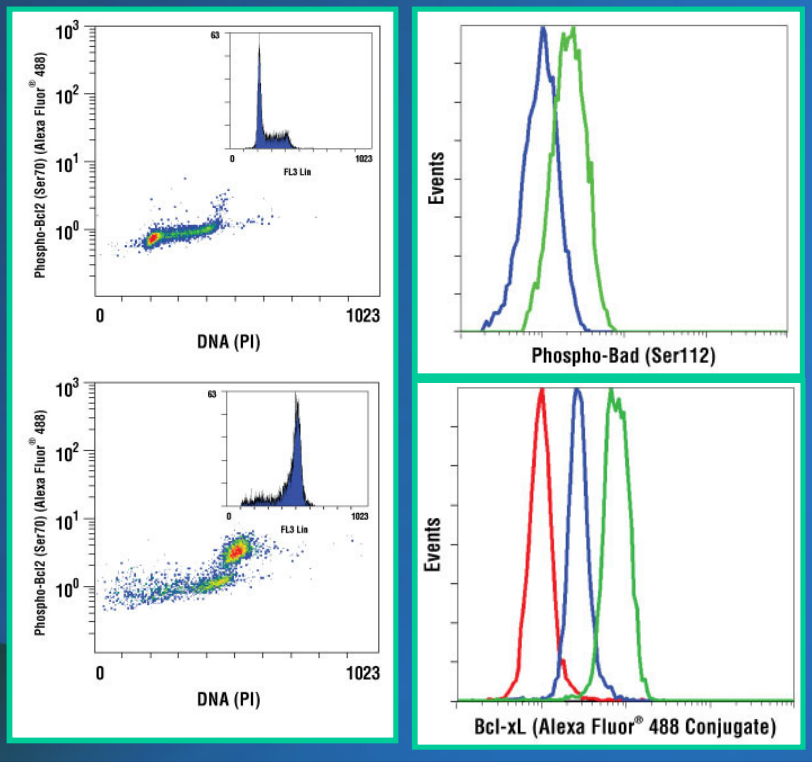
JC-1 agregats : red

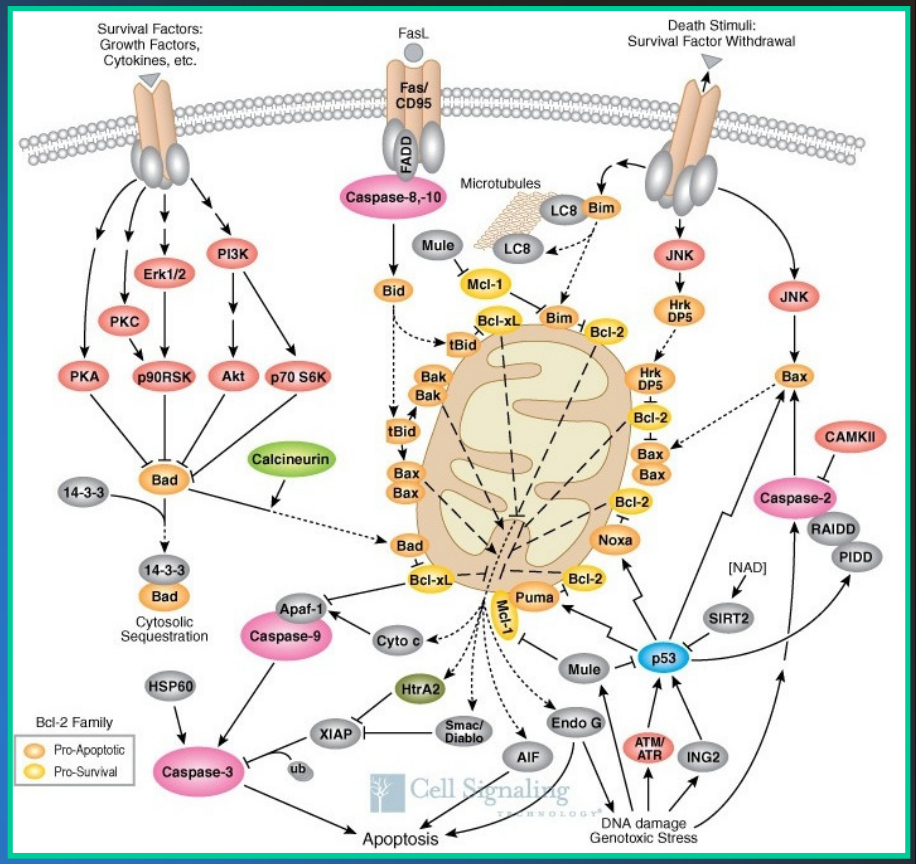
Changing form is dependant of mitochondrial potential

Mantena S.K and al, *Mol Cancer Ther,* 2006;5:296-308

II.Apoptosis analysis

- Bax/Bcl-2 expression
- Other protein expression, phosphoprotein



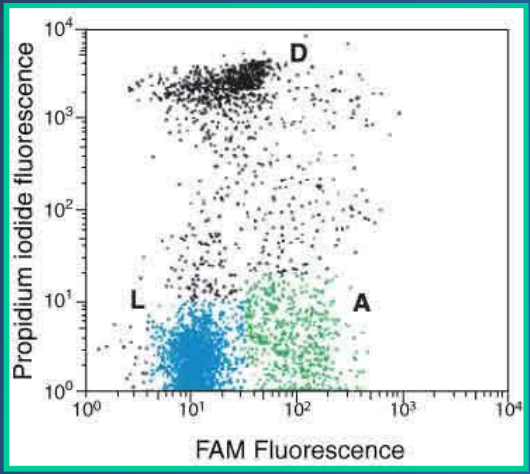


Difficult but precise state of proteins that really act

Images from Cell Signaling Technology/Beckman Coulter

II.Apoptosis analysis

- Global or specific caspase activity : FLICA (FLuorescent Inhibitor of CAspase)



Substrate reacts with all active caspases, covalently bound to caspase cystein

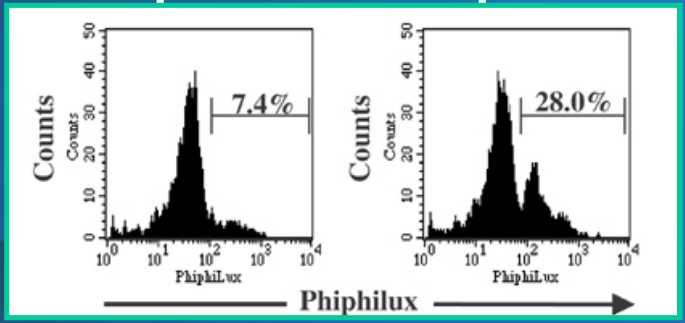
VAD sequence specific, (DEVD 3,7; LETD 8)

Reporter emits green light

Non covalently bound molecules leave the cell

Image from www.invitrogen.comc

- Caspase 3 specific substrate : PhiPhiLux

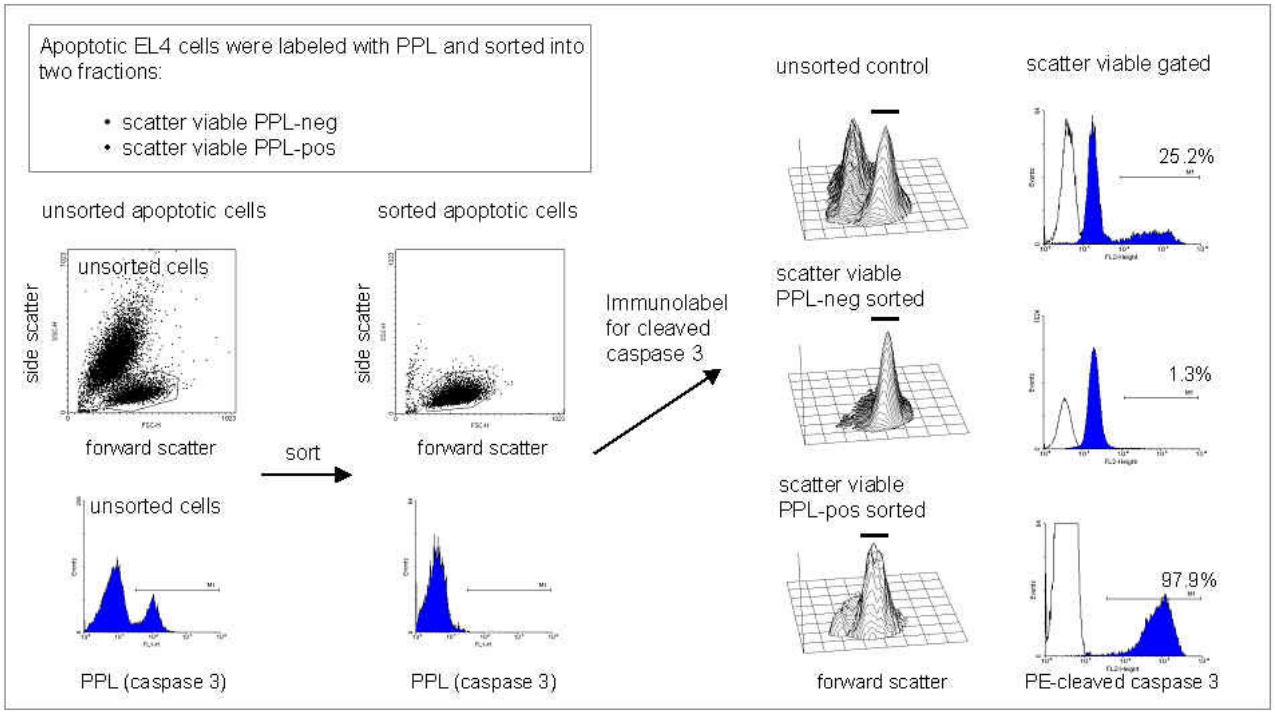


Arnoult D and al, *Cell Death and Differentiation*, 2003;10:1240-1252

www.PhiPhiLux.com

II.Apoptosis analysis

- Active form of caspase 3



http://home.ncifcrf.gov/ccr/flowcore/caspase3.htm

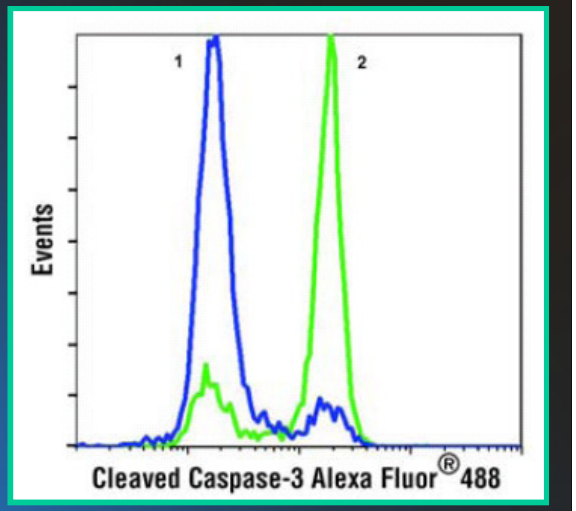


Image from Beckman Coulter

II.Apoptosis analysis

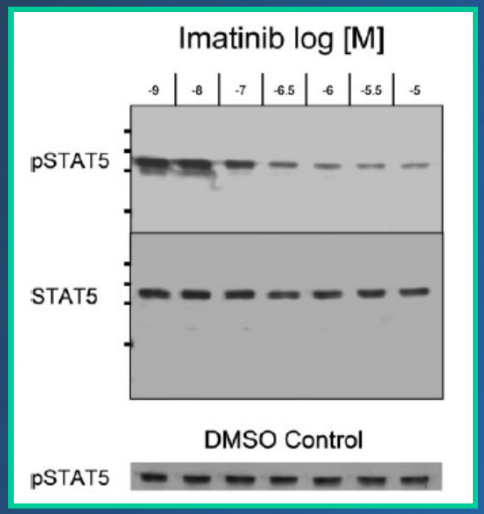
- To resume :
  - ✓PS : Annexin V/7AAD or PI
  - ✓DNA fragmentation : Sub G1 peak/TUNEL
  - ✓Mitochondrial depolarisation : JC-1/DiOC(6)…
  - ✓Mitochondrial events : APO2.7/BcL-2/Bad…
  - ✓Caspase activity : FLICA/PhiPhilux
  - ✓Caspase active state : anti-cleaved caspase

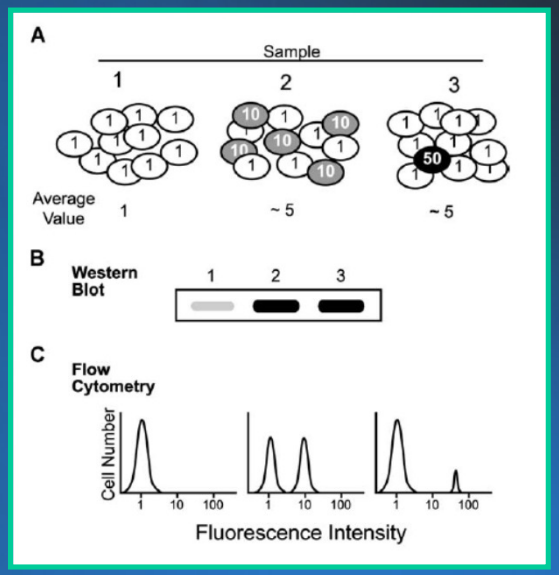
- Not only one, more you have more proofs you give…

III. Transduction pathway analysis

- Apoptosis is a transduction pathway
- Phospho (S70) BcL-2 more informative than BcL-2 for sensoring apoptotic death
- A lot of transduction can be monitored by flow cytometry
- Why?
- Monitoring of cell pathway more easily, and more quickly than Western Blotting
- Agonist/antagonist studies
- Pharmacological studies

III.Transduction pathway analysis





Western blot	Flow cytometry
Population analysis Obtain average value of multiple cells	**Single cell analysis** Collects data for each individual cell
Homogeneous sample Limited to cultured or purified cells	**Heterogeneous cell types** Complex primary samples, that is, immune cells
One parameter Obtain data sets individually	**Multiparameter** Correlate multiple markers simultaneously
Large number of cells Requires in vitro derived cultures of rare cells	**Small number rare subsets** Direct analysis of rare cell types (i.e., DC)
Time consuming for large sample sets Not amenable to large screening efforts	**Rapid and scalable** Performed in 96-well plates in parallel
Protein size and Ab specificity Ab selectivity for target is clearly visible	**Ab must be validated** Ab must have high affinity and selectivity

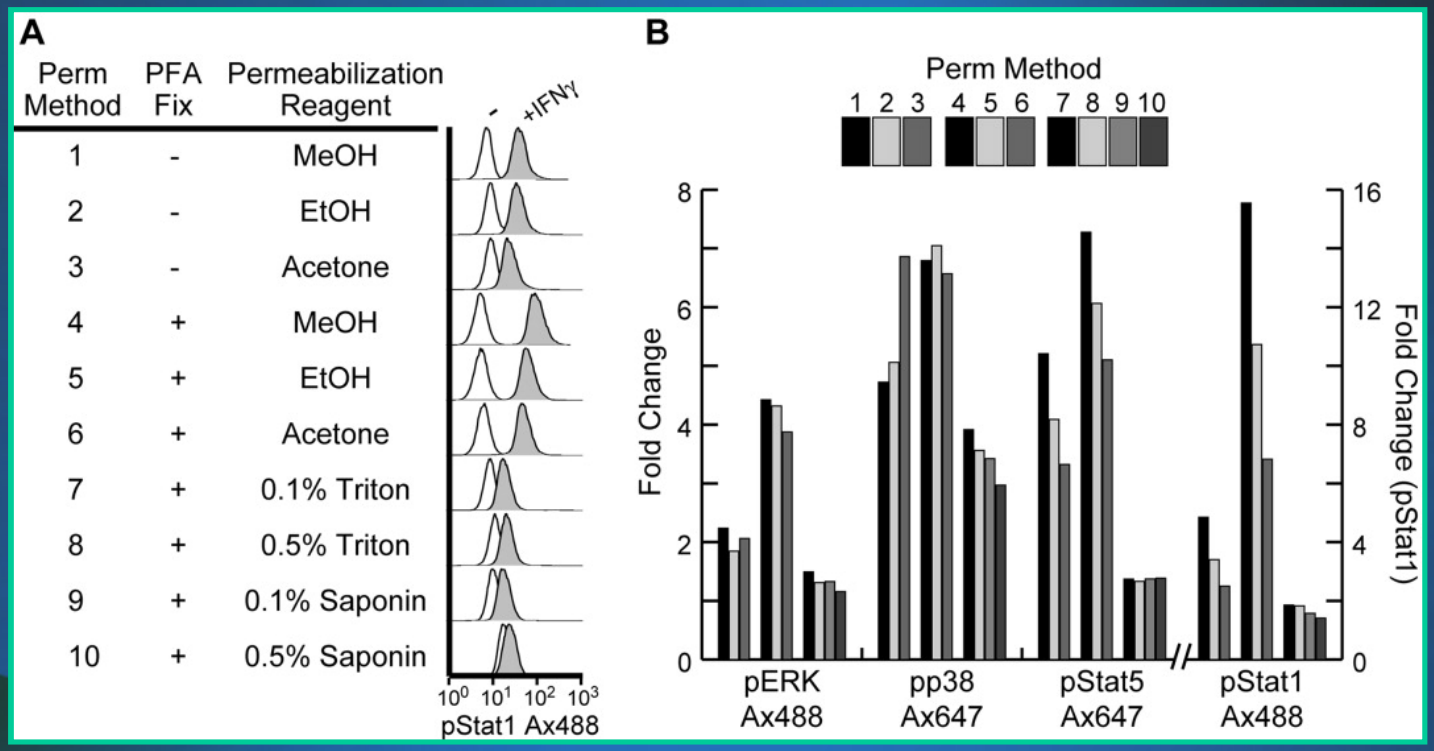
Jacobberger and al, *Cytometry*, 2003;54A:75-88

Krutzik and al, *Clin. Immunol*, 2004;110:206-21

- Analysis of phosphoprotein is not simple
- Need optimisation, comparison, validation vs WB
- Flow Cytometry is the only one to provide you informations on each cell
- Multiparametric analysis cell by cell
- No need to culture, native sample
- Technique is more easy and faster
- More stats

III.Transduction pathway analysis

- Accessibility to the antigen?
- Protein complex, fixator and permeabilisator effect on sample?

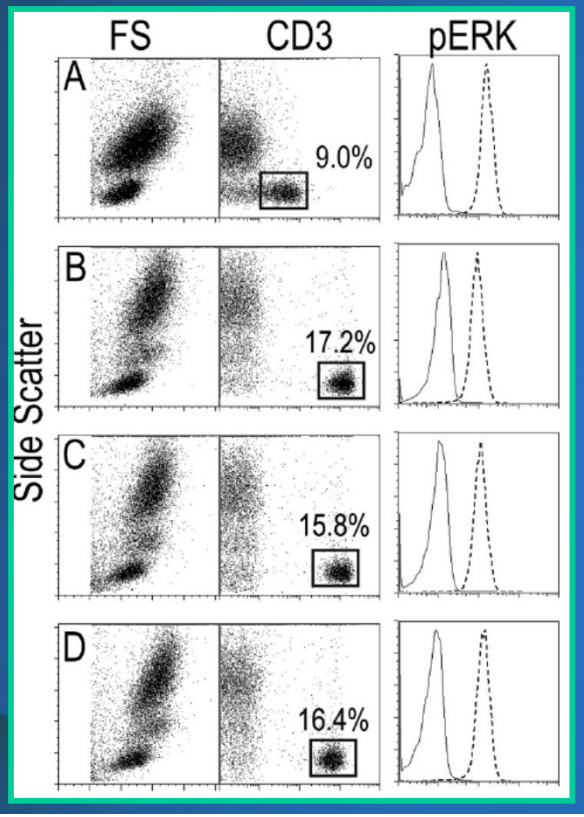


- Formaldehyde (1,5%) fixation and MeOH (90%) permeabilisation

Krutzik and al, *Clin. Immunol*, 2004;110:206-21

III.Transduction pathway analysis

- What i need? Best separation between neg and pos populations
- Scatters? FSC and SSC?
- Gating marker? Depends of your question…

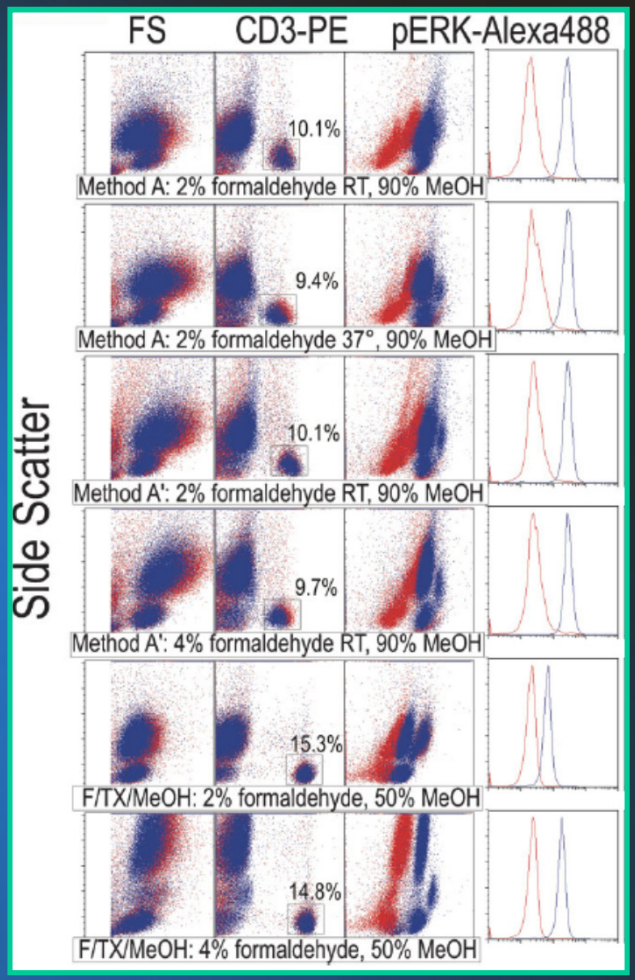


H_20
2% For
100% MeOH

4% For
0,1% Triton X-100

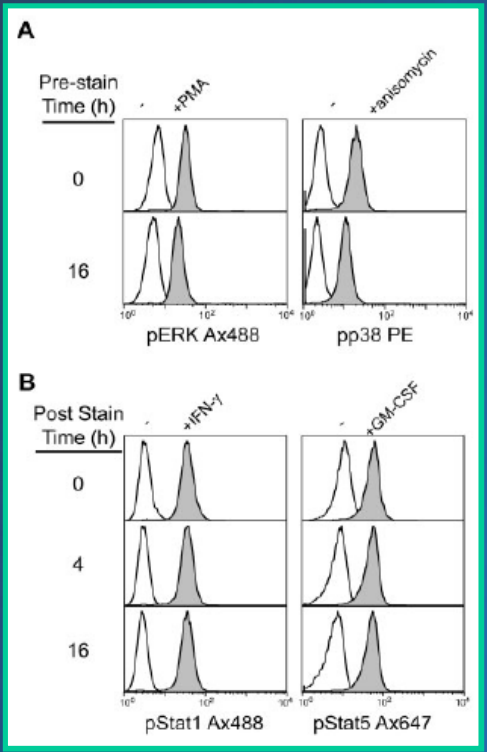
4% For
0,1% Triton X-100
50% MeOH

4% For
0,1% Triton X-100
50% EtOH



Chow et col, *Cytometry*, 2005;67A:4-17

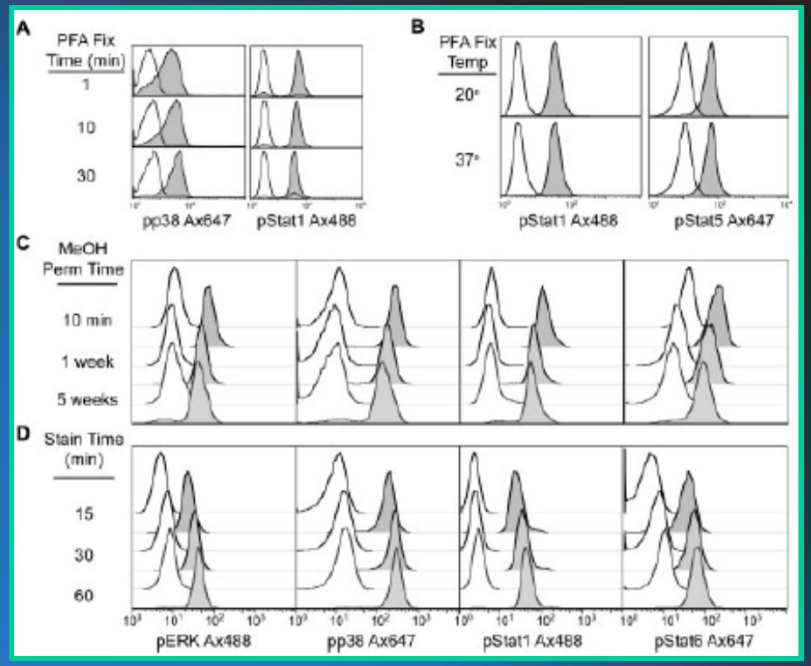
III.Transduction pathway analysis



- Samples and staining are stable

Table 1
Alexa 647 Antibody Conjugates Give Larger Fold Changes Than Alexa 488 Conjugates

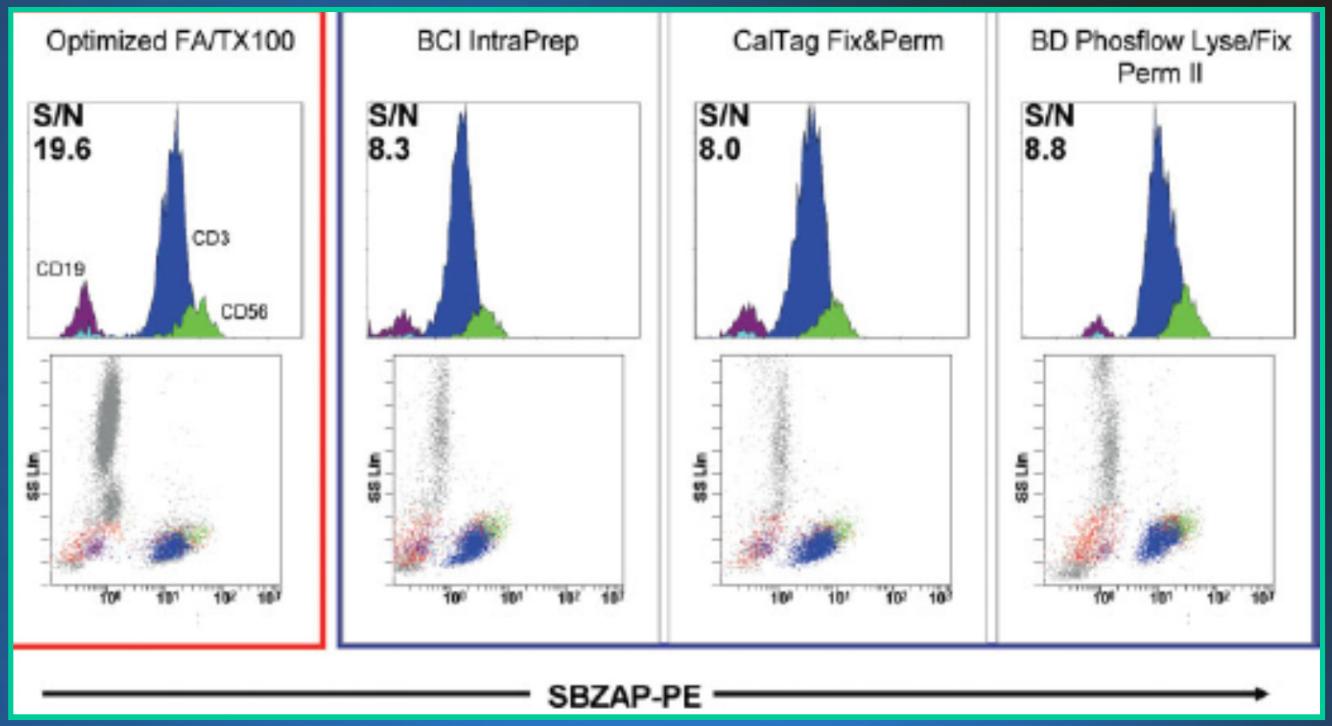
	Fold change[a]	
	Alexa 488	Alexa 647
pERK	4.6 ± 0.9[b]	5.5 ± 1.2
pp38	8.1 ± 1.3	17.1 ± 3.0[c]
pStat1	12.4 ± 2.5	42 ± 7.8[c]
pStat5	3.3 ± 0.2	5.7 ± 1.5
pStat6	4.7 ± 0.6	6.8 ± 0.4



- Increasing fixation is not need
- Fix Temp room or 37℃ : same
- Storage @ -20℃ up to 5 weeks
- Staining time : 15 min
- Alexa Fluor 647 better, but depends of F/P ratio and cytometer characterisitcs

Krutzik and al, *Clin. Immunol*, 2004;110:206-21

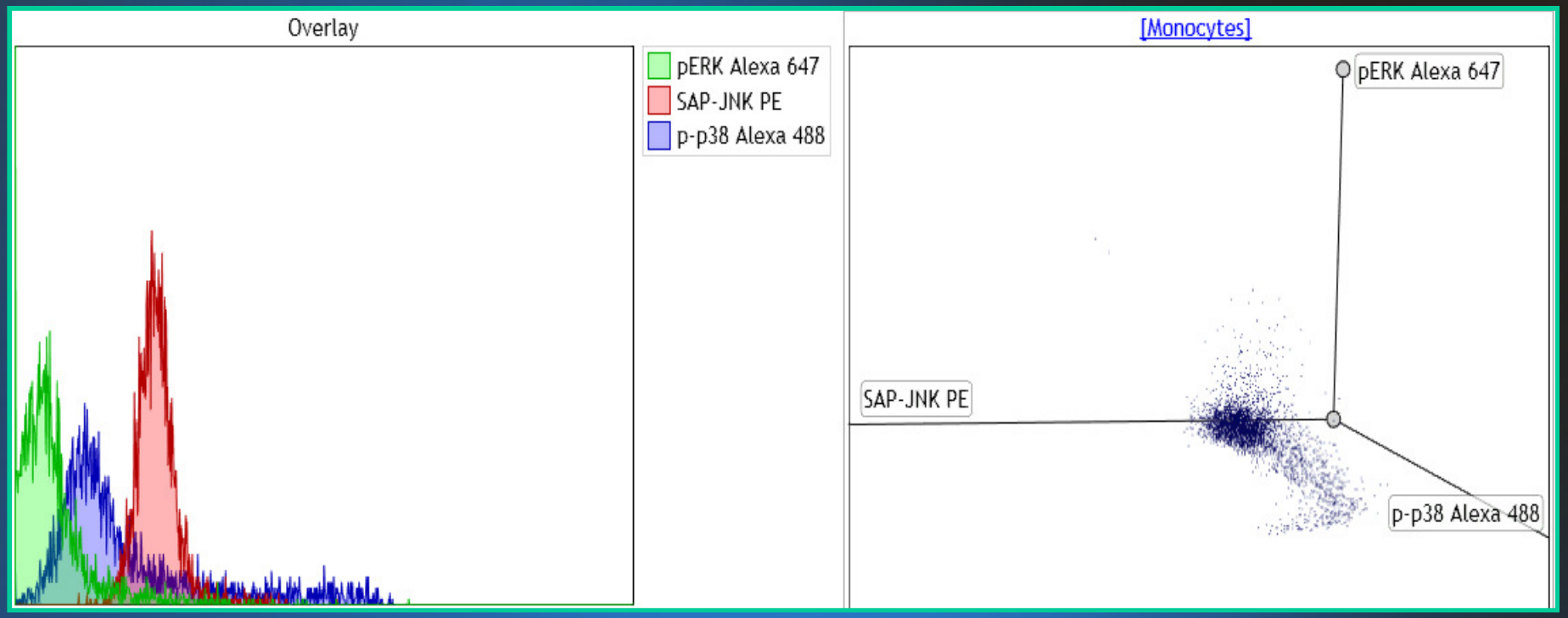
III.Transduction pathway analysis



- Differents methods For/Saponine, For/MeOH
- Good, but not the best
- For (2-4%)/Triton X-100 (0,1%) best for fresh blood samples : Best separation, good gating, scatters maintened

Shankey et col, *Cytometry*, 2006;70B:259-69

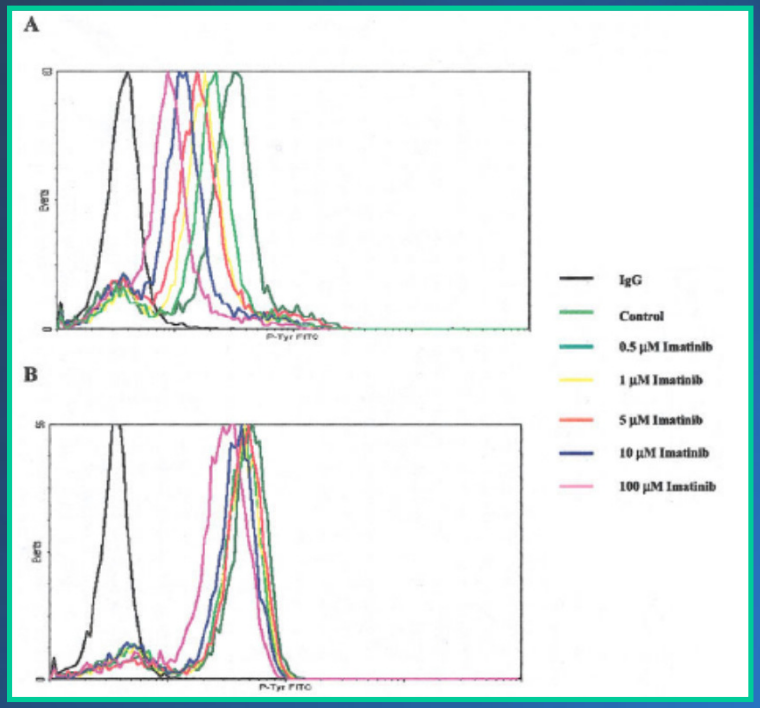
III.Transduction pathway analysis

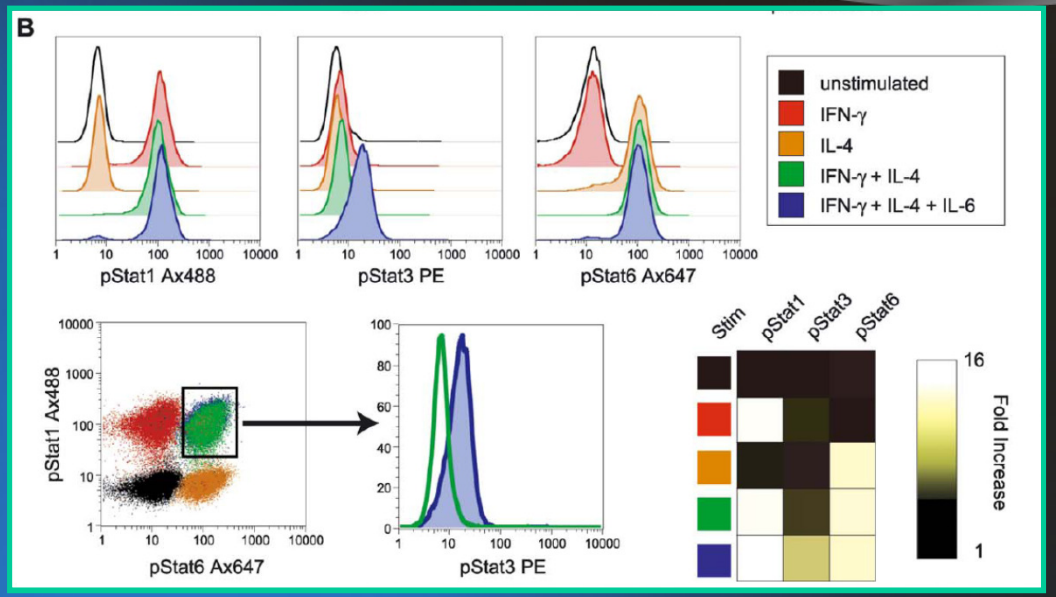


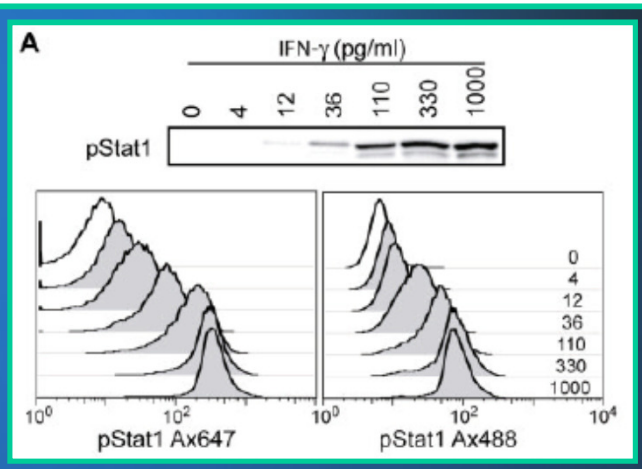
- Monocytes Stimulated by LPS for different times
- Stained with anti-CD14, fixed, permeabilized and stained for phospho ERK, SAP-JNK and p38

Images were made with Kaluza™ Software, Beckman Coulter

III.Transduction pathway analysis







- Evaluation of cancer cells sensitivity to chemotherapy
- Appreciate cytokines effects
- Titration of an agonist

Desplat and al, *Cytometry*, 2004;62A:35-45

Krutzik and al, *Cytometry*, 2003;55A:61-70

Krutzik and al, *Clin. Immunol*, 2004;110:206-21

III. Transduction pathway analysis

Table 3
Clinical applications of phospho-specific flow cytometry

Immune system characterization

Immune cell development: monitoring phospho-signature of developing T, B, or other lineage specific cells to correlate intracellular activities with stages of cellular differentiation.

Disease state profiling: combining tetramer staining with intracellular signal assessment to study antigen-specific T cells in viral or bacterial infections. This offers the potential to monitor lymphocyte subsets for responses under acute and chronic infections.

Monitoring lymphocyte populations in disease murine models or patients, such as blood borne leukemias, or autoimmune diseases, such as rheumatoid arthritis, to correlate phospho-signatures with disease manifestation.

Biochemical signatures of rare cell populations (dendritic cells, naive and memory effector cells, stem cells) that cannot be analyzed by conventional biochemical techniques.

Multidimensional assessment of cell signaling networks to understand cell function. Identification of signaling thresholds and connections among disparate signaling cascades.

Monitoring virally infected cells for altered function and intracellular signaling.

Characterizing immune cell response patterns to cytokines and extracellular stimuli.

Pharmacodynamic monitoring and drug screening

Intracellular kinase screens for rapid identification of specific inhibitors or modulators of target kinases.

Drug screening in primary cells to determine subset-specific efficacy and side effects.

Target validation of compound specificity by analyzing multiple intracellular pathways simultaneously.

Clinical trials: monitoring particular compounds for their effects during drug treatment on cellular populations of interest.

Identification of phospho-epitopes on kinases as diagnostic indicators of disease progression by correlating intracellular biochemical differences with additional clinical parameters.

Phospho-epitope analysis during vaccination protocols to monitor efficacy at the cellular level.

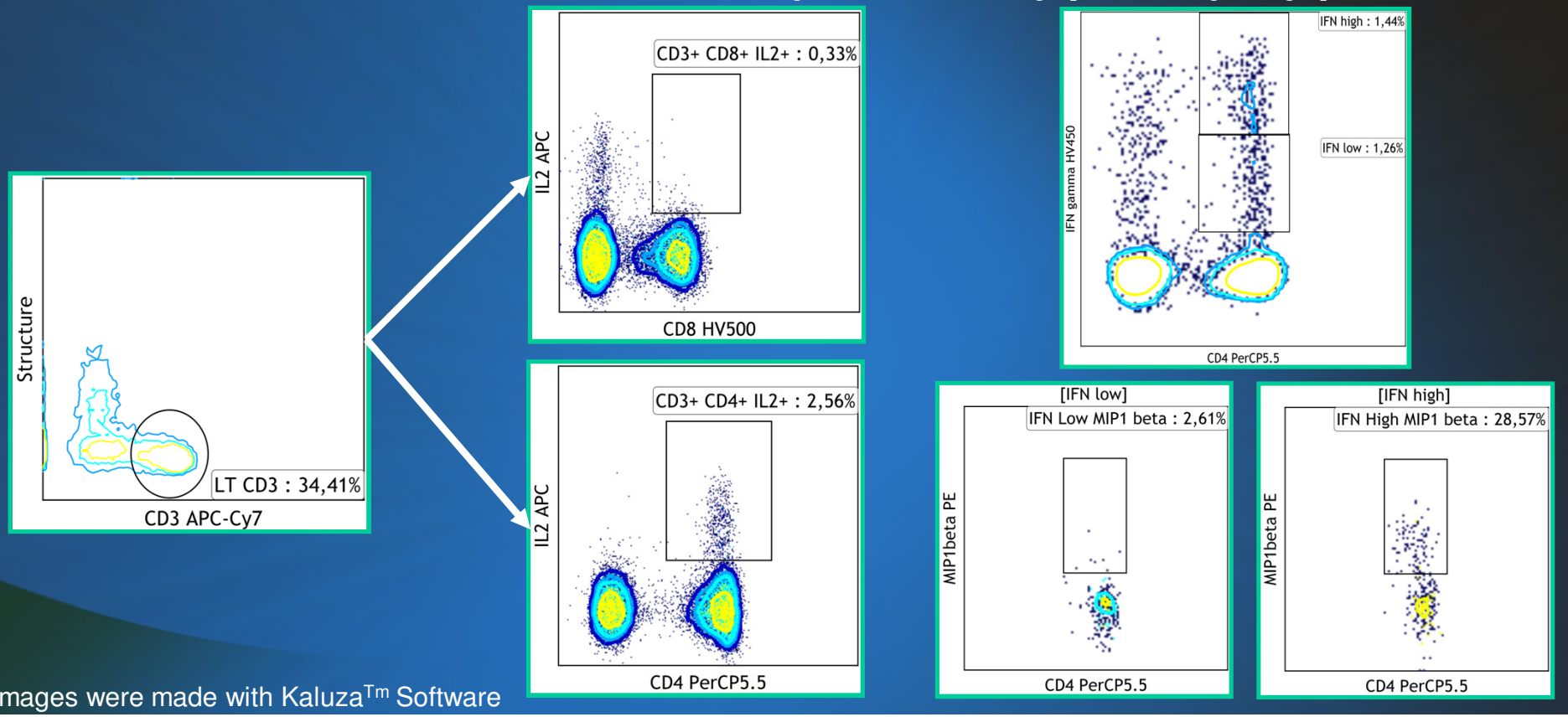
Krutzik and al, *Clin. Immunol*, 2004;110:206-21

- Immunology : Following activation of cells after stimulation with cytokines, GF, tetramers, vaccination trial
- Hematology/pharmacology : Following activation status of cancer cells, pathological cells (auto-immune disease), treatment effectiveness
- Discovery of signaling pathways
- Discovery of pathway blocking or activating molecules
- Prognostic marker in disease

- Limits :
  - ✓ No localization informations unlike fluorescence microscopy
  - ✓ Accessibility to the antigen depends of protocol
  - ✓ Sensitivity is higher than WB, no enzyme amplification
  - ✓ Quantity of data, need time to analyse

IV.Cytokine secretion analysis

- Combine with results from ELISA in supernatant : bulk
- Have a result cell by cell / type by type



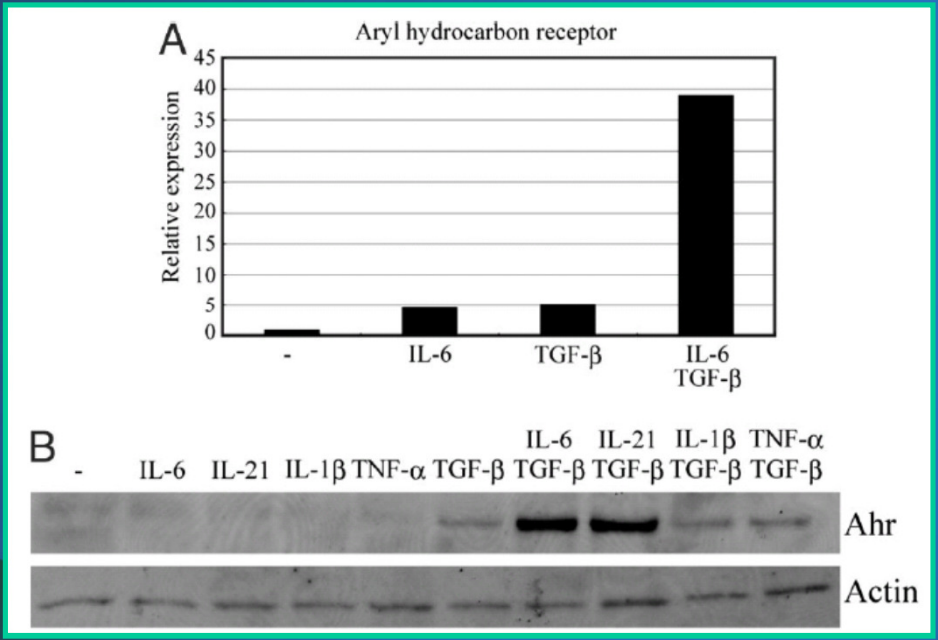
Images were made with Kaluza™ Software

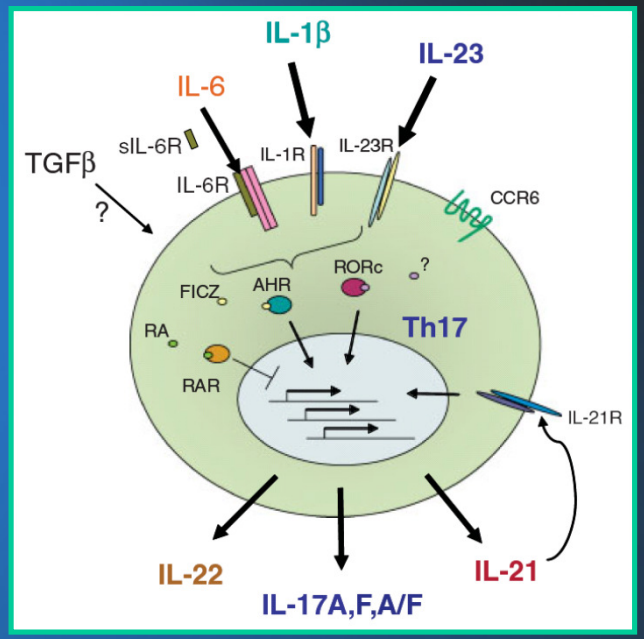
IV.Cytokine secretion analysis

- How to do it?
- Stimulate cells or not
- Block secretion pathway : Brefeldin A…
- Stain membrane marker(s)
- Fix and permeabilise cells : For/MeOH or Triton X-100
- Stain intracellular cytokine/phosphoprotein/intracellular protein
- Acquire on flow cytometer

IV.Cytokine secretion analysis

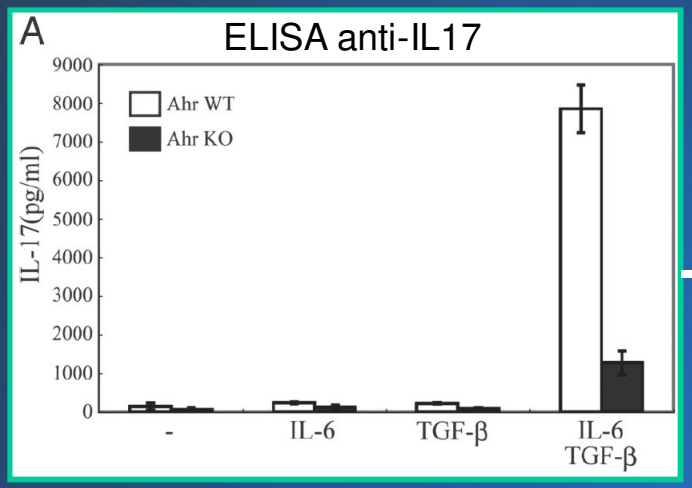
- Exemple : Effect of polluant on T cells?
- Th17 cells are T cells $CD3^+$ $CD4^+$ $IL17^+$ $ROR\gamma t^+$ $CCR6^+$ Ouyang W and al, *Eur J Immunol*, 2009;39:634-675
- Express the AHR Kimura and al, *Proc Natl Acad Sci USA*, 2008;28:9721-26





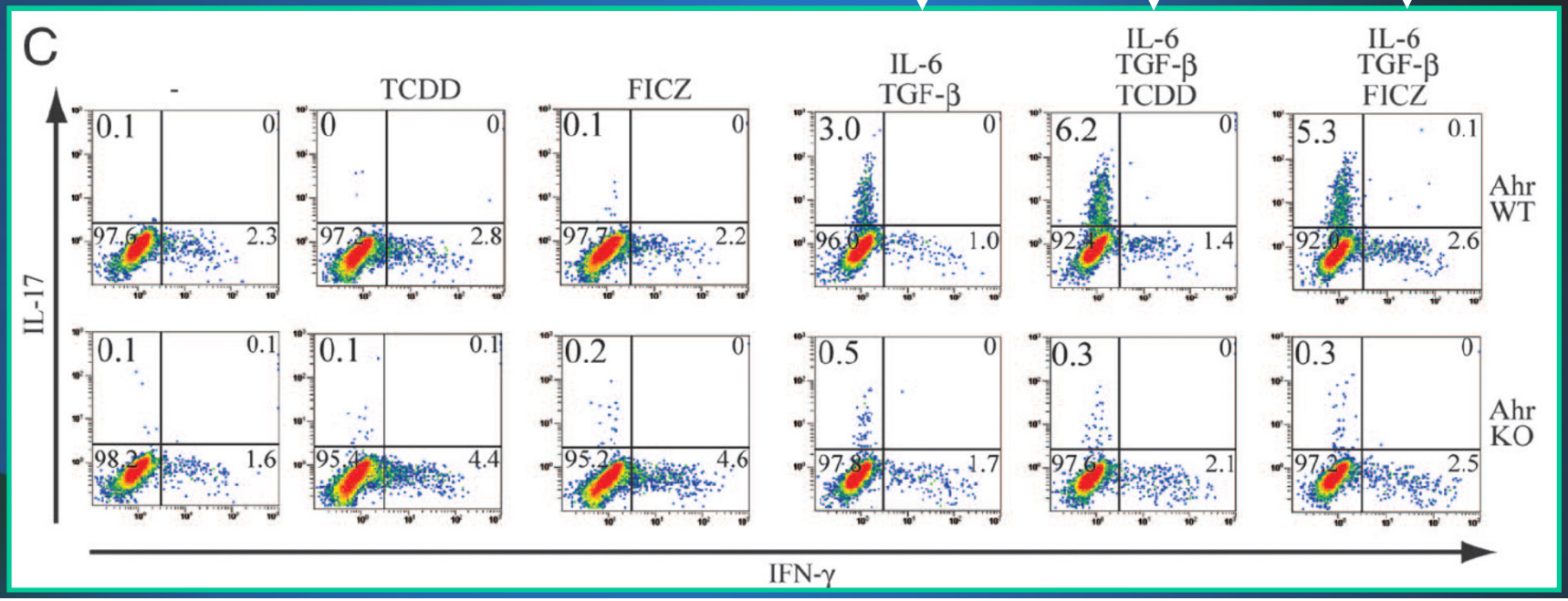
IV.Cytokine secretion analysis

Kimura and al, *Proc Natl Acad Sci USA*, 2008;28:9721-26



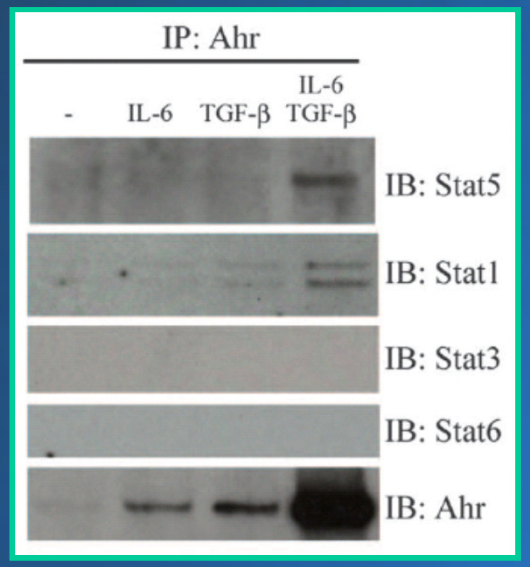
+ Dioxine

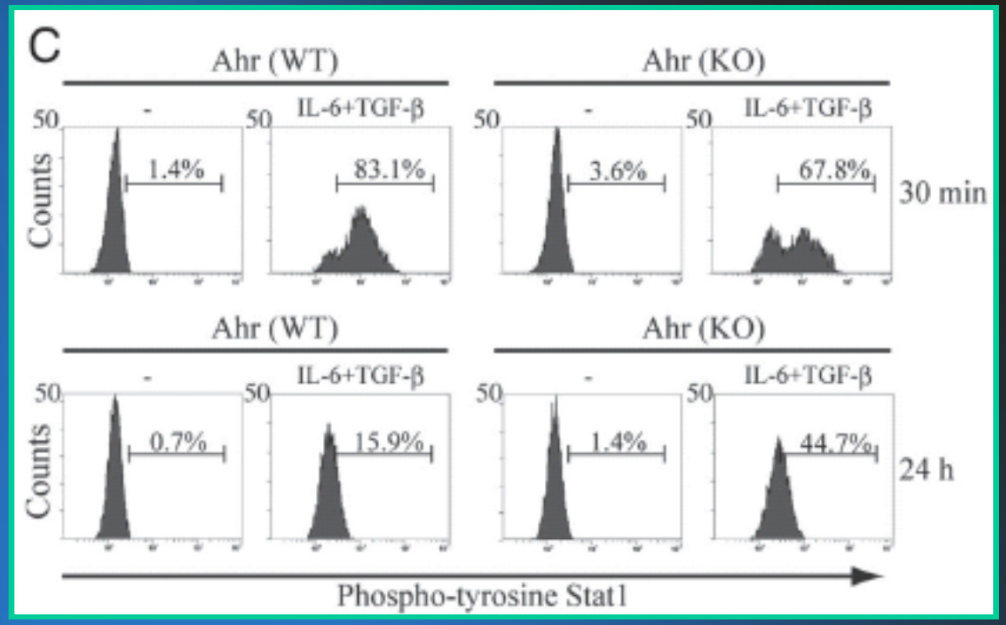
+ Carbazole



IV.Cytokine secretion analysis

- How AhR works?





Inhibition of pStat1

Kimura and al, *Proc Natl Acad Sci USA*, 2008;28:9721-26

V.Conclusion

- Flow cytometry is a powerfull analytical tool, polyvalent

- Cell death
- Transduction pathway
- Secretions

- All you want if you have liquid sample monodispersed elements, which emit light…