

*Cell Cycle Profiles
Proliferation, arrest and
apoptosis workshop*

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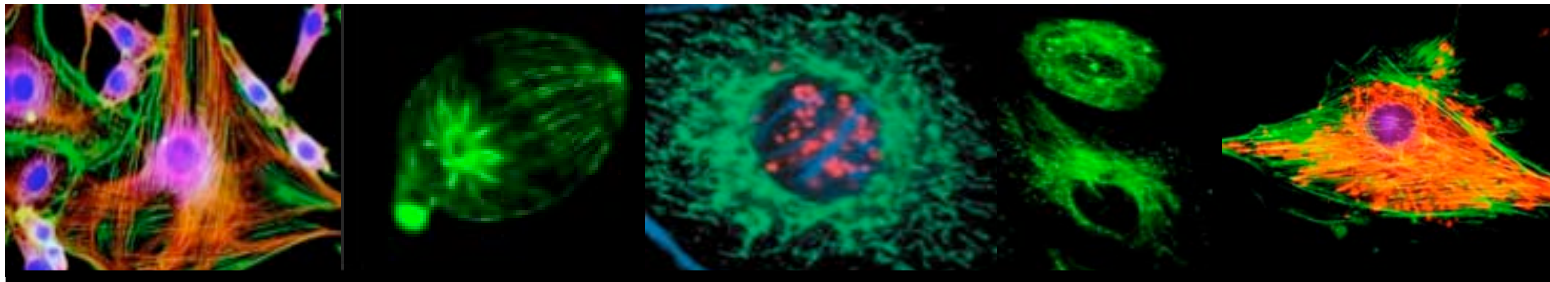
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BRISTOL-MYERS SQUIBB

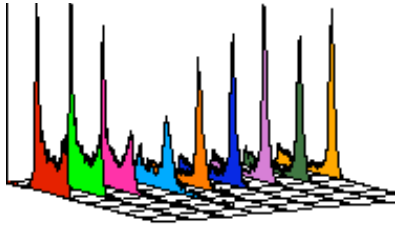
When We Study Cells

At the microscope we can visualize many structures inside the cells



FACS Answers presented on this workshop

- Proliferation: What When and How
- Arrest: Why “When” is a key feature
- Apoptosis: Dealing with the kinetics of a disappearing population



Proliferation

What?

A cycling phenomena regulated by check point controls
DNA duplication followed by segregation into two new entities

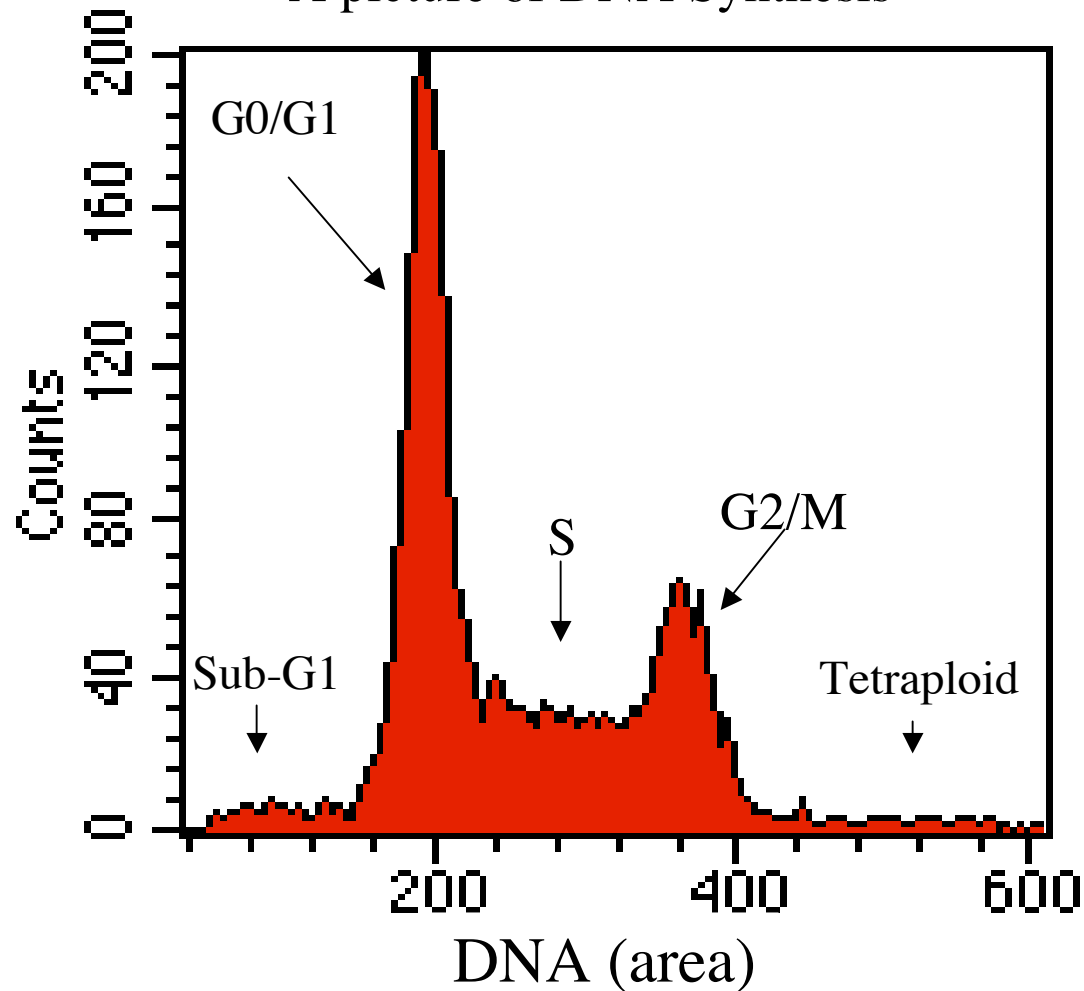
Proliferation

When do you measure it?

- Basic science research
 - signal transduction evaluation
- Pharmaceutical industry
 - biochemical to cellular assays
- Clinical follow up
 - cancer therapeutic efficiencies
 - inflammatory cascades identification
 - immune activation responses

Cell Cycle Profile Components

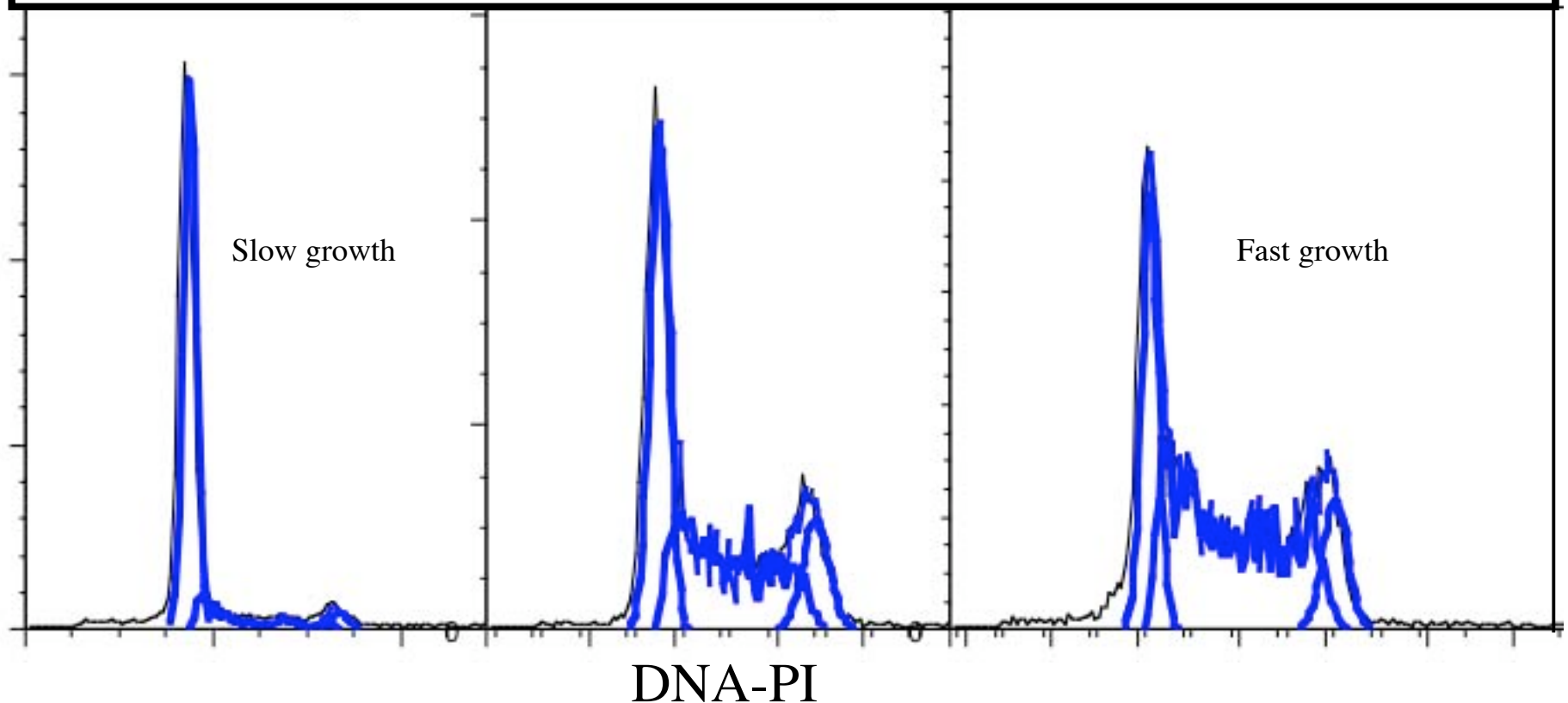
A picture of DNA Synthesis



Cell Quest
software

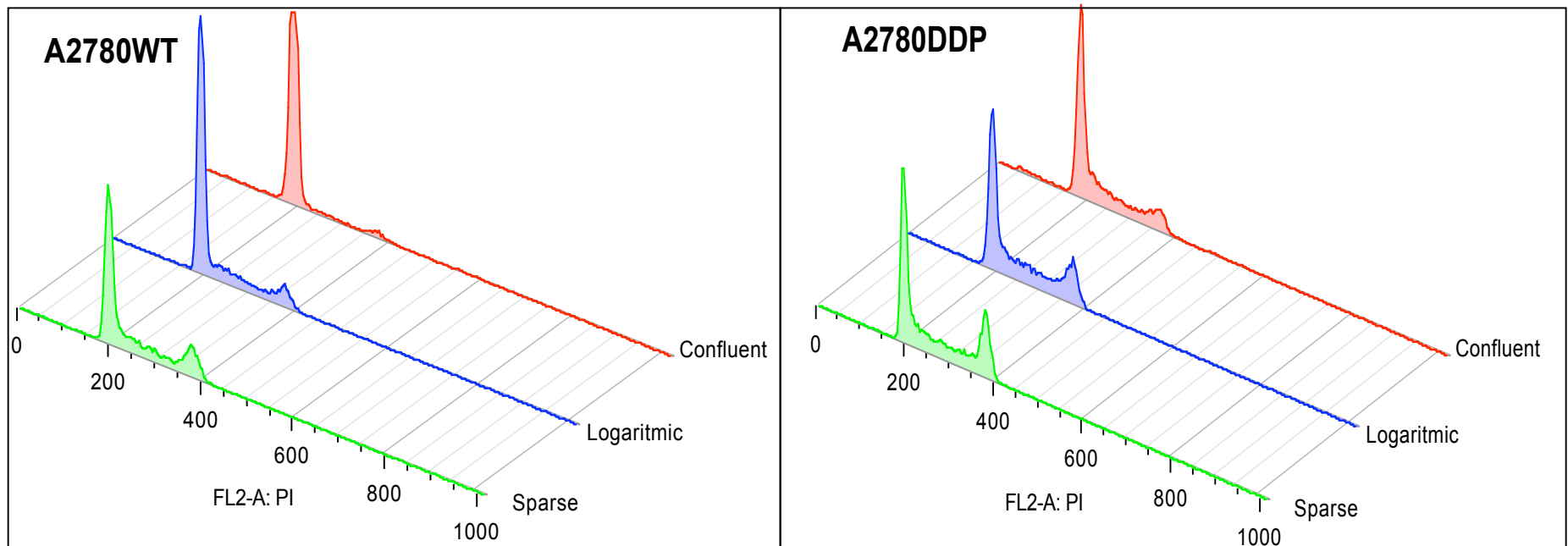
Cell Cycle Profiles: A Personal Signature of Cells

DNA histograms gives a first hint about cells proliferation potential



Proliferation Features

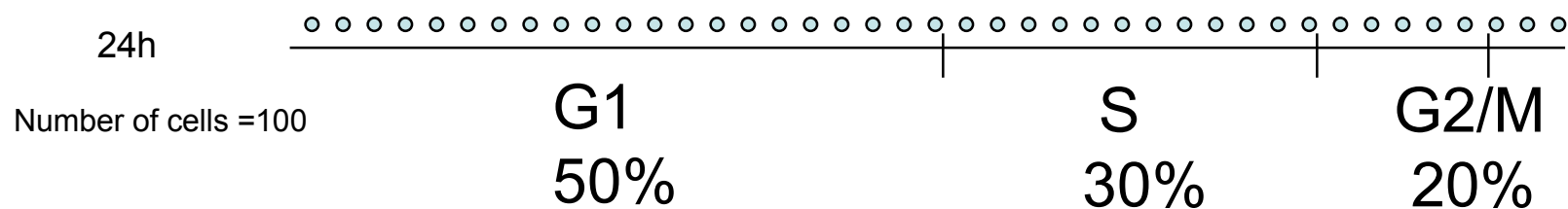
What does “Cell Cycle Profiles” really mean?



FlowJo
Software

Cell Cycle Times and Numbers

- DNA content and population distributions

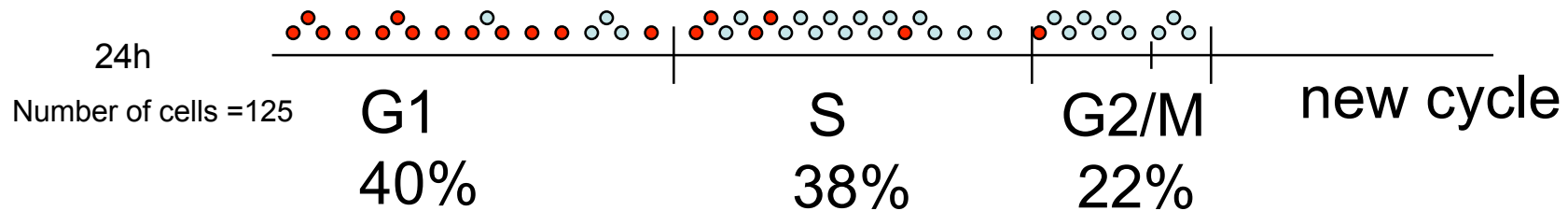


24h Cell cycle

12hrs

7.2hrs

4.8hrs



18h Cell cycle

7.2 hrs

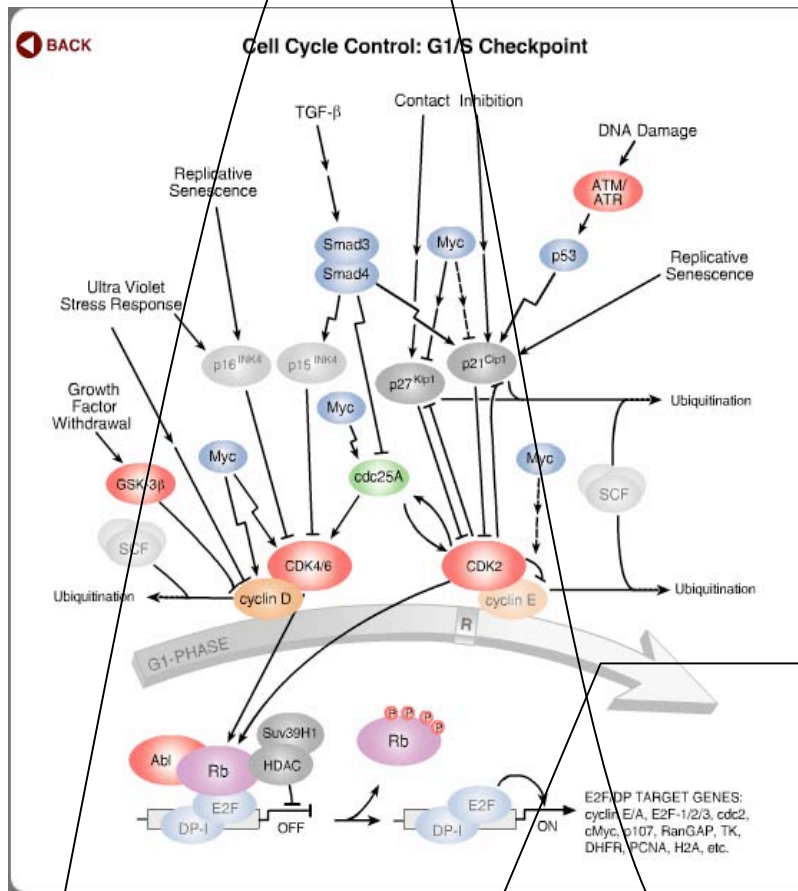
6.8 hrs

3.9hrs

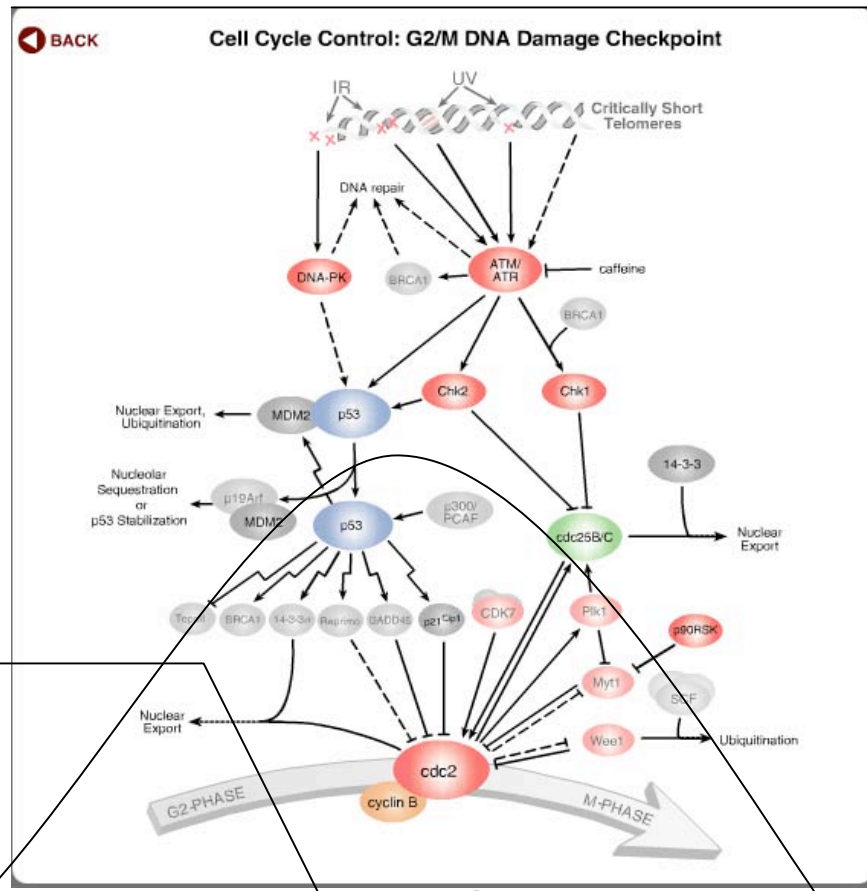
6h additional hours of growth

When A Cell Divides

Addressing cells one at a time



G1



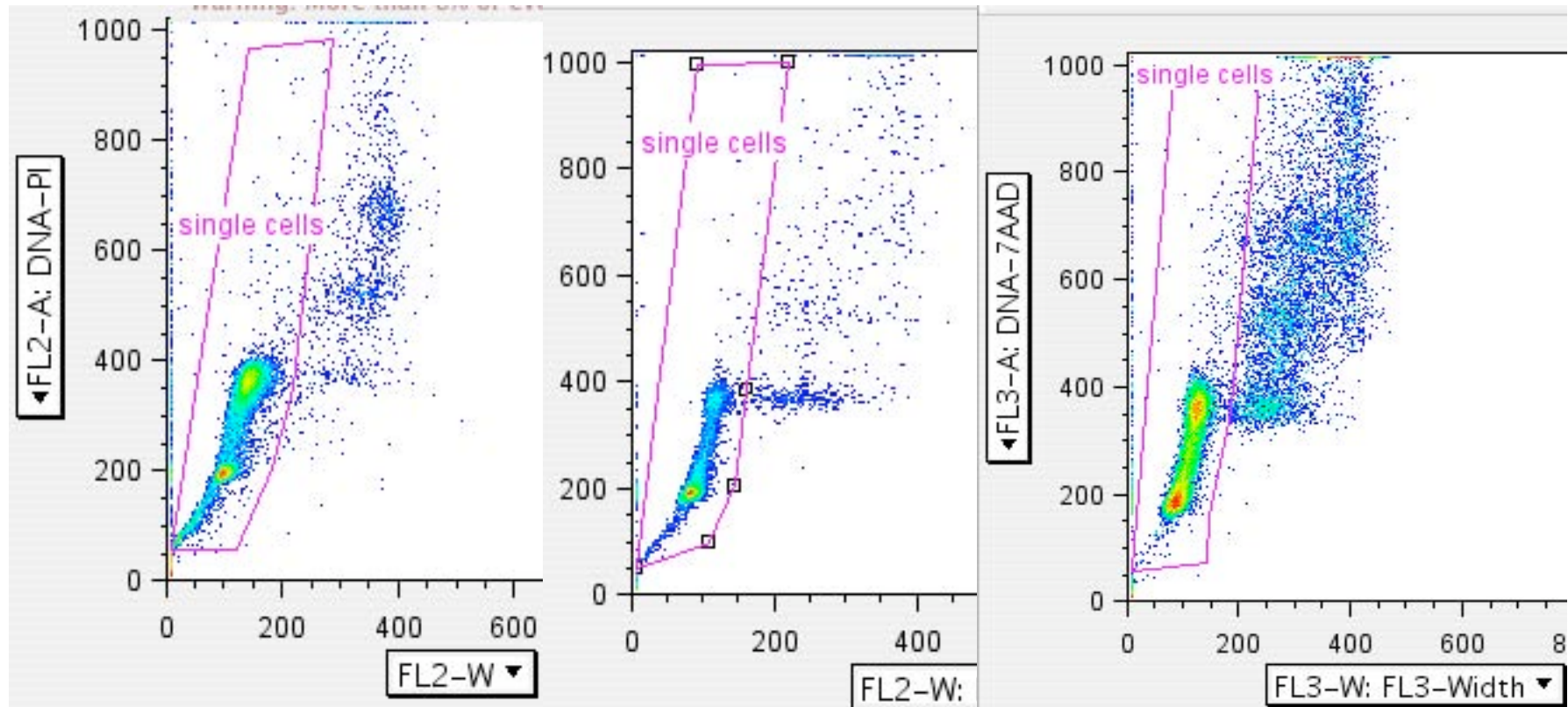
G2/M

Modified from Cell Signaling Web site

Getting The Components Right

The Question of Doublet Discrimination

FlowJo
Software

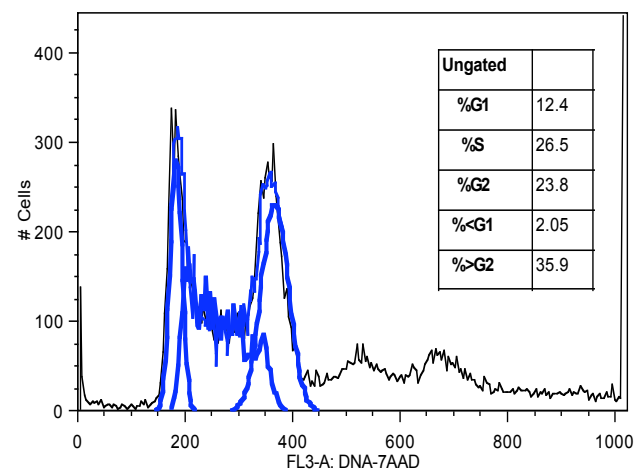
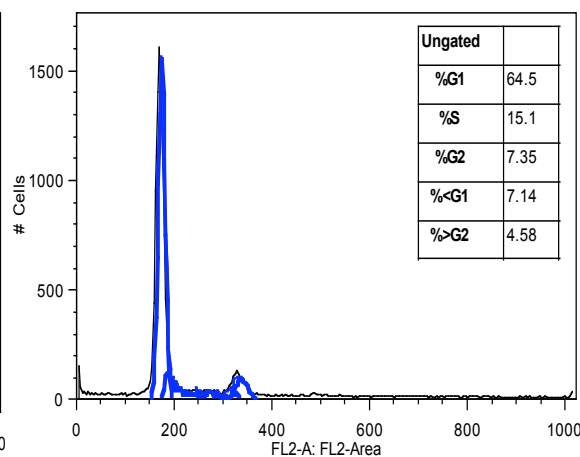
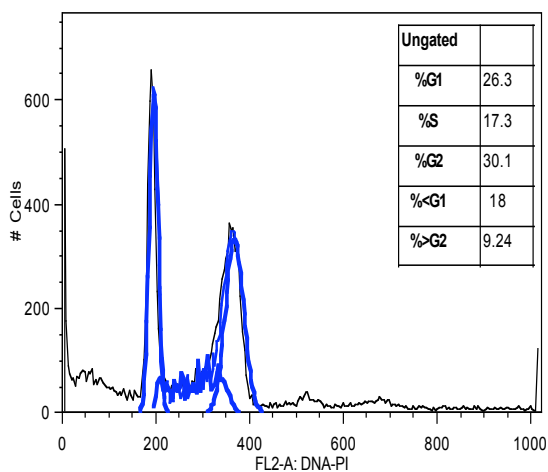
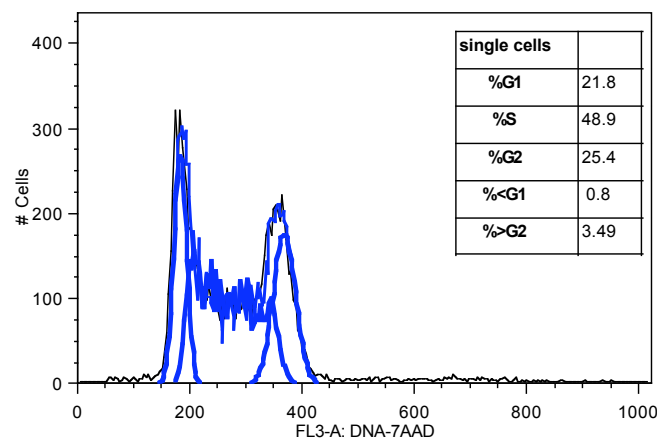
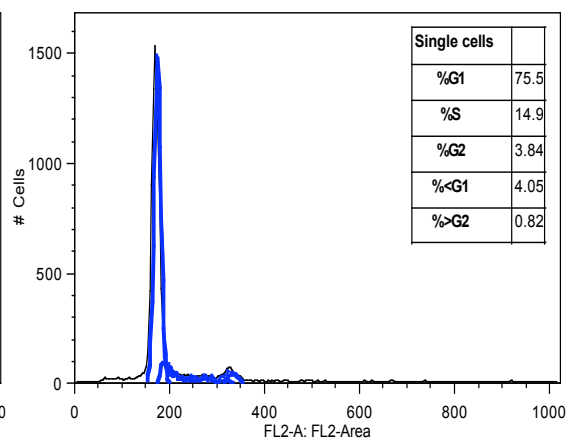
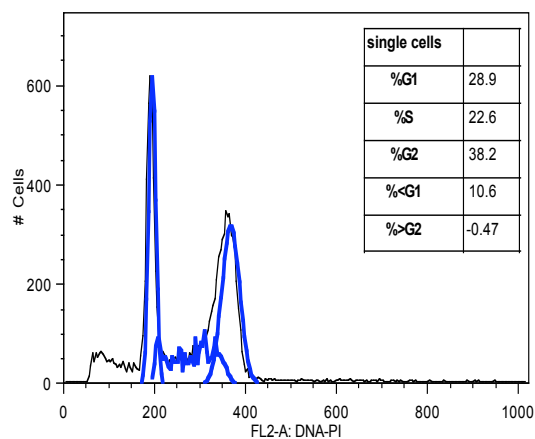


Alternatively Area vs. Height is also a way to discriminate doublets and higher clumps

Cell Cycle Profile of Single Cells

Gated vs. non gated populations Previous plots

FlowJo
Software



Nuclei vs. Whole Cells

What is better?

Nuclei

- Ploidy paraffin blocks
- Apoptosis problems
- Nuclear localization

Whole cells

- Ploidy fresh samples
- Apoptosis efficiency
- Cytoplasmic markers.

Cell Cycle Software Packages

Getting Numbers to Fit Your Data

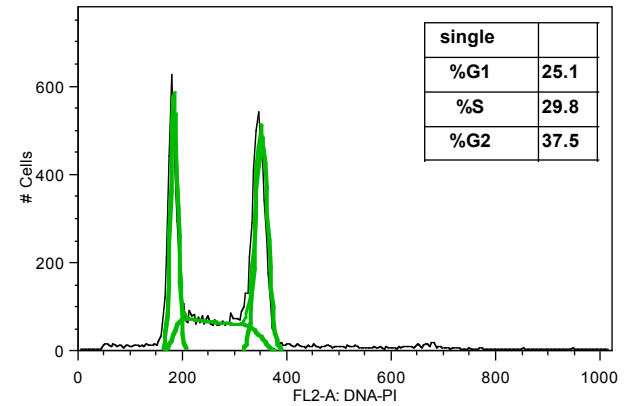
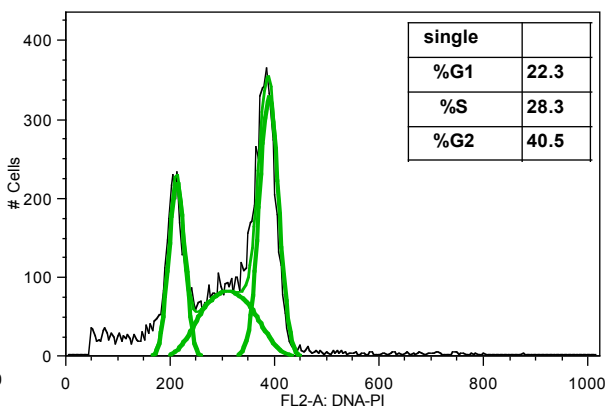
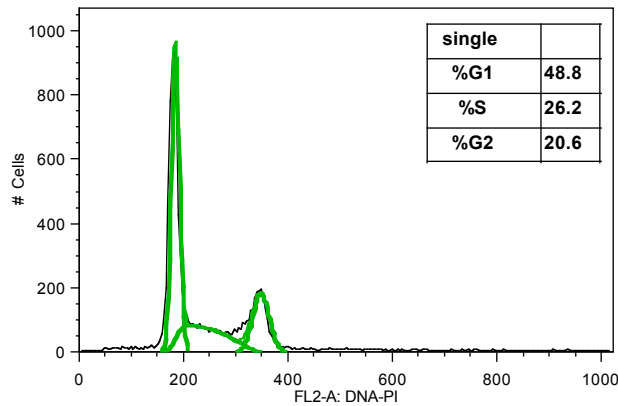
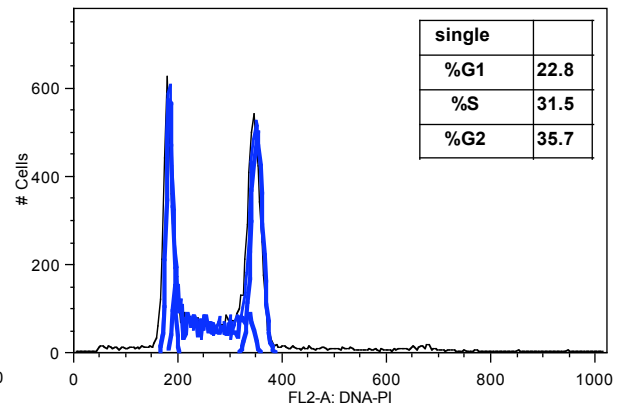
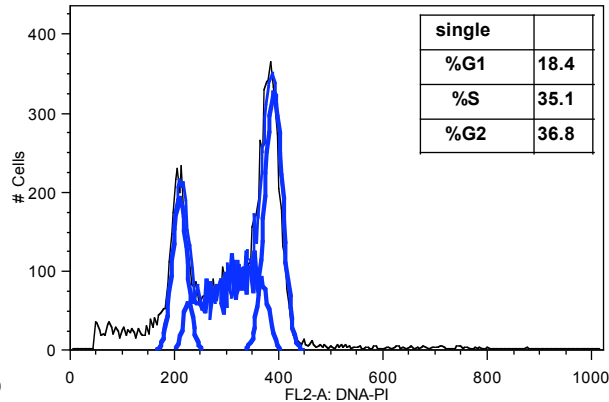
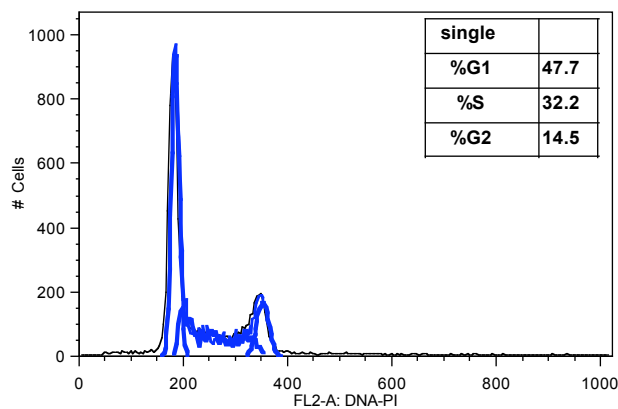
- Multicycle
- Modfit
- FlowJo

All are packages that contain algorithms with different restrictions bound to their calculations.

Unfortunately, no algorithms are consistently reliable in fitting all the distributions you may encounter. You may have to experiment with different options and constraints in finding your best results. (copied FlowJo quote)

Getting To See The Numbers

Watson Pragmatic vs. Dean Jett Fox in FlowJo software



Alice Givan previously showed the Mod Fit variations on their selective options

Precautions-Tissue Culture Monolayers

- Learn the metabonomics of your cells
 - Doubling times, density optimization
- Cell Culture Stocks Maintenance
 - Rigid protocol for split times/avoid overgrowth
- Protocol for Experimental Tests
 - Standardized number of cells & scheduling
 - Efficiently trypsinize to a unicellular suspension

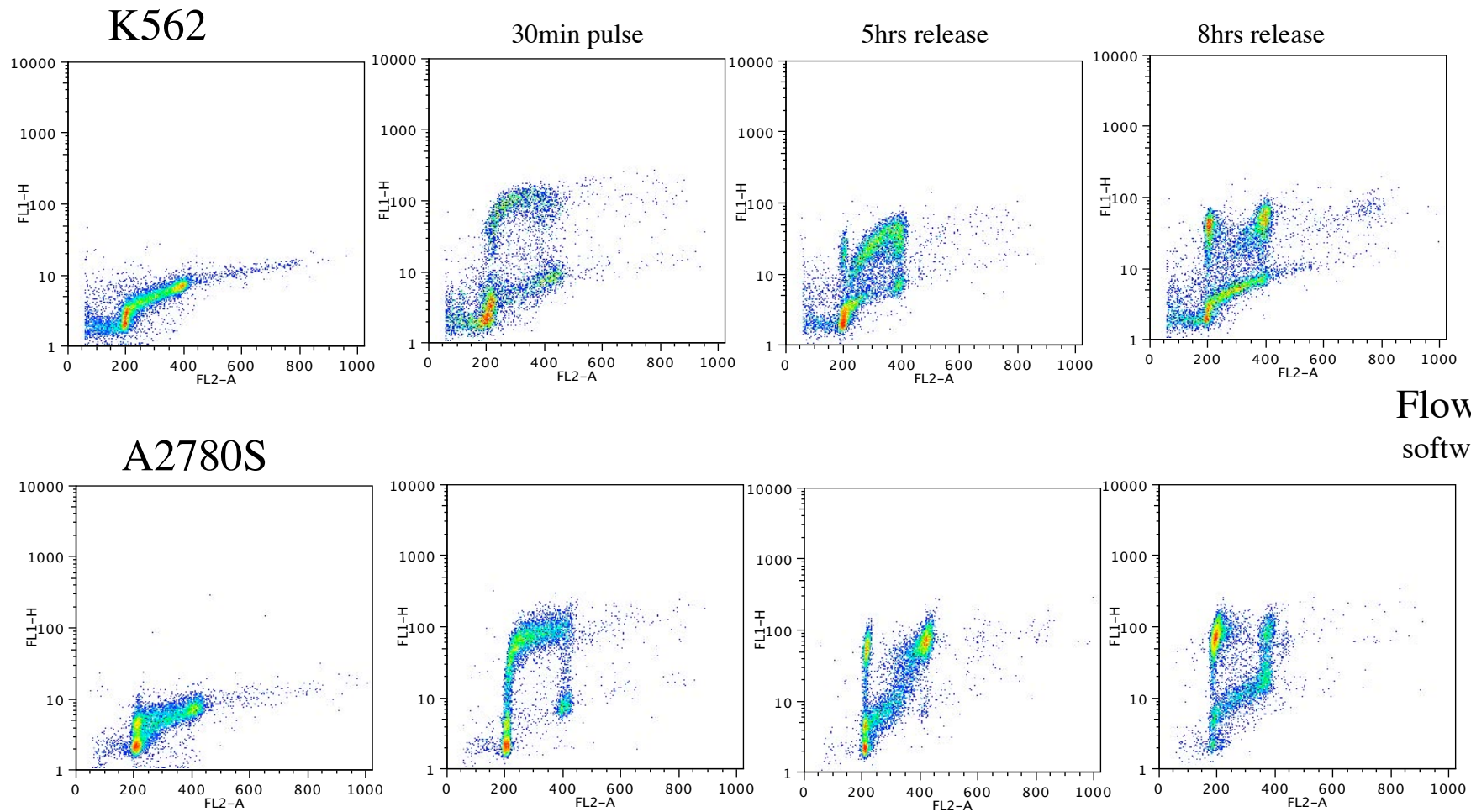
Beyond DNA Quantitative Analysis

How to add more significance to your data?

- Adding a second marker to your cells
 - Cell identification markers (CD's or signal transduction probes)
 - DNA doubling features, BrdU incorporation
- Adding a third or more markers to your cells
 - When preset configuration is fixed in your instrument
 - Search for probes that allow to you the best combinations on added color
 - When you are able to set up your own configuration
 - First pick up different lasers then change your dichroics and band filters

Proliferation and BrdU

S phase and Doubling Times



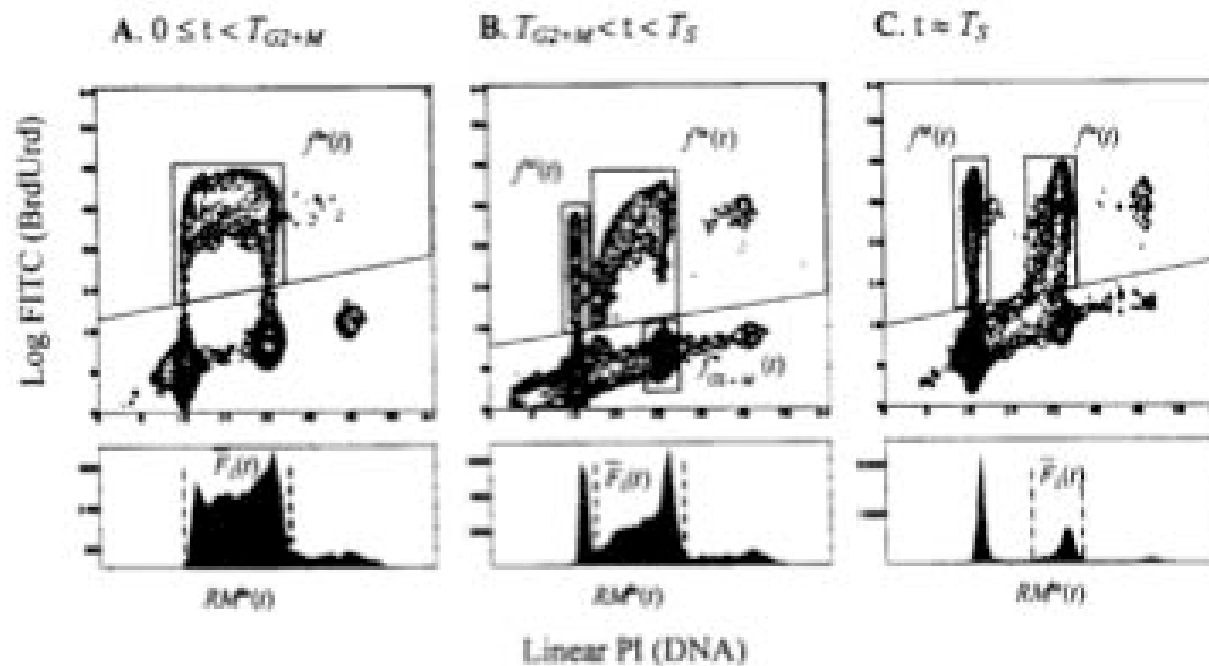
FlowJo
software

Calculating the Numbers

Getting the mathematics to work for you

Cell Cycle Kinetics by BrdUrd Incorporation

365



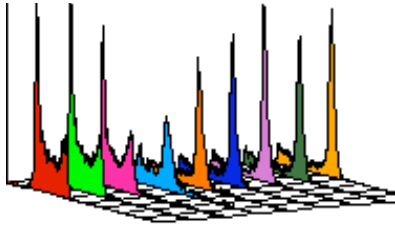
From: Cell Cycle Kinetics Estimated by Analysis of Bromodeoxyuridine Incorporation.
Terry N.H.A., and White A. Methods in cell Biology 63:355-374 (1994)

Overall messages

- **When to do DNA?**
 - Maybe every time you address cells
- **Watch out! Single cells exclusively**
 - Cell preparation. Avoid clumping
- **How to address specific questions?**
 - Cell culture conditions. Use tight protocols
 - Most appropriate software. Up to you

References

- **Solid Tumor dissociation and storage (nuclei)**
 - Cerra.R., Zarbo R.J., and Crissman. JD.,1990 Dissociation of cells from Solid Tumors. *Methods in Cell biology* 33: 1-12
 - Vindelov.L.L.,Cristensen.IJ. An integrated set of methods for routine flow cytometry DNA Analysis. 1990. *Methods in Cell biology* 33: 127-137
- **Cell Lines (whole cells)**
 - Pozarowwski.P., Darzynkiewiicz.Z. Analysis of Cell Cycle by Flow Cytometry. 2004. *Methods Mol Biol* 281:301-311.
 - Traganos.F.,Juan.G.,Darzynkiewiicz.Z. Cell Cycle Analysis of Drug treated Cells 2001. *Methods Mol Biol* 95:229-240.
- **Overall**
 - Rabinovitch.P.S. Practical considerations for DNA Content and Cell Cycle Analysis in Clinical Flow Cytometry. *Principles and applications*. Williams and Wilkins-1993
 - Pham.NA., Jaccobberger.JW.,Schimmer.A.D.,Cao.P.,Gronda.M.,Hedley.D.W. 2004. The dietary isothiocyanate sulforaphane targets pathways of apoptosis,cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. *Mol.Cancer.Ther.*3:1239-1248
 - Bagwell.B. et al. 2004. Optimizing Flow Cytometric DNA ploidy and S phase Fraction as nNegative prognostic Markers for Node-Negative Breast Cancer Specimens *Cytometry* 46:121-135



Proliferation Disturbances

Arrest

A Way to Cope with Stress

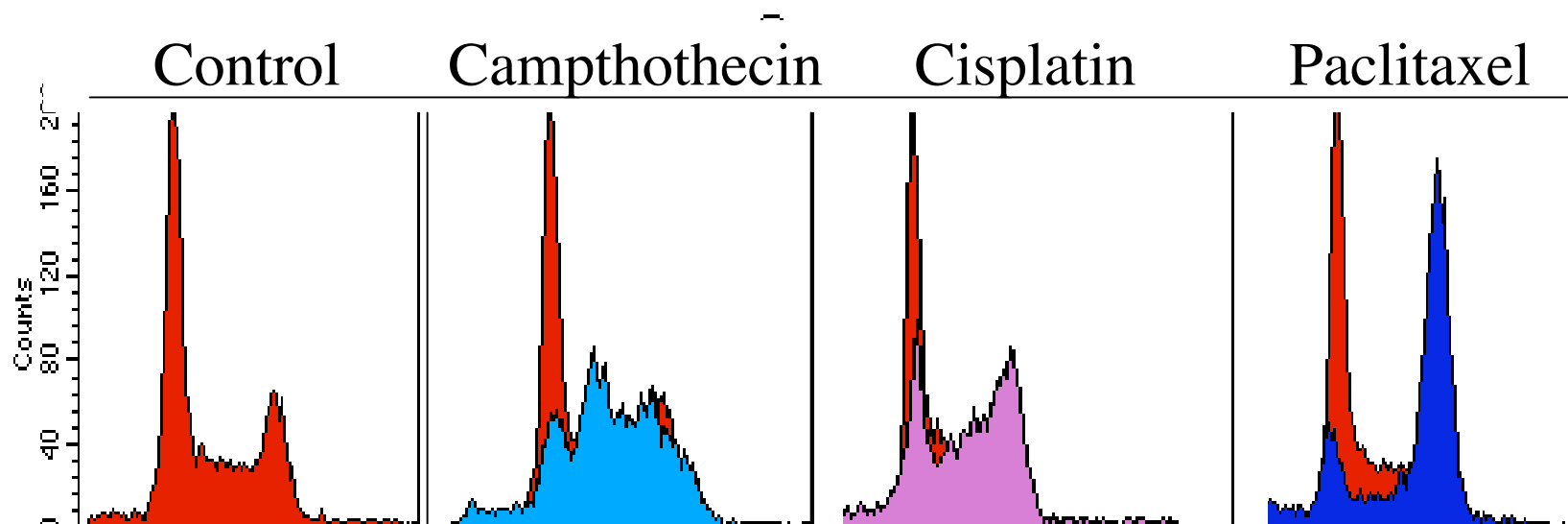
ARREST

What is it?

- A slowdown in cell cycle progression?
- A reversible or irreversible block in one phase of the cycle?
- An induced effect of stress or toxic insult?
- A response of the cell genetic makeup?
- All of the above?

The Meaning of Cell Cycle Arrest

How Do You Identify Specific Growth Arrest?

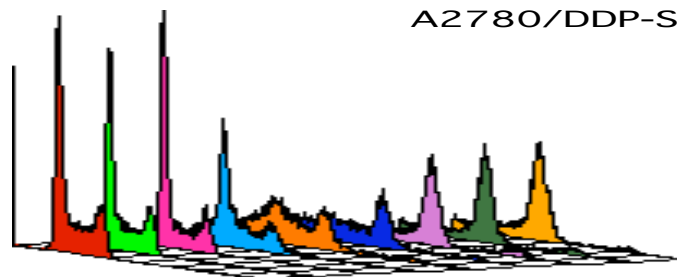


Cell growth arrest induced by stress or chemical compounds are efficiently identified when cells are growing at their optimal logarithmic rates and inducers are at an equilibrium concentration where cells are halted to activate repair mechanisms or apoptosis

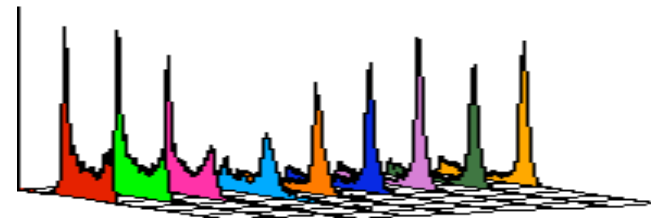
Identification of Arrest

Paclitaxel a model drug for G2/M arrest

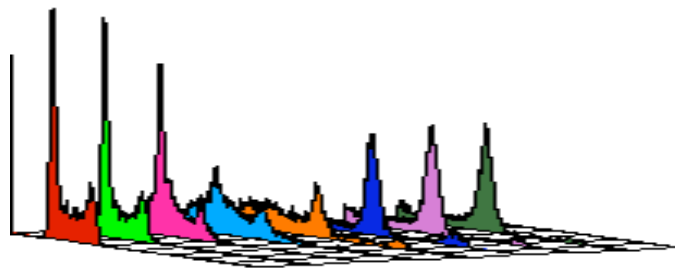
Taxol



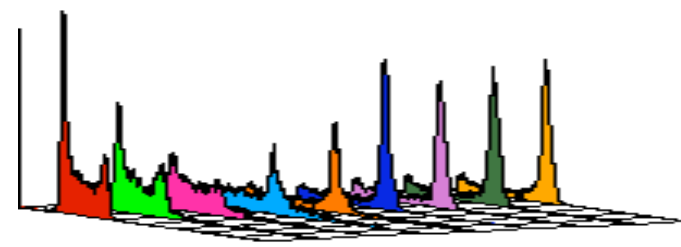
A2780/DDP-R



Taxotere



Titration



Control 2.5 5.0 10 20 40 80 160 320nM

Control 2.5 5.0 10 20 40 80 160 320nM

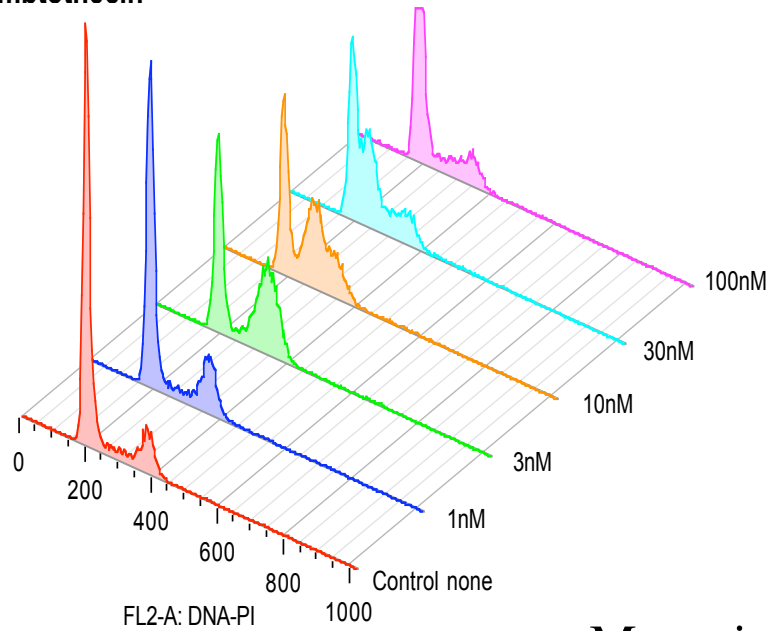
Laidlaw, J., Raventos-Suarez, C., Fairchild, C.R., Peterson, R.W., and Menendez, A.T.
Taxol and taxotere have similar potency in cytotoxicity assays, cell selectivity, bcl-2 phosphorylation, G2/M arrest and induction of apoptosis. 91st Annual meeting of the AACR. San Francisco, April 1-5. 2000.

CellQuest
software

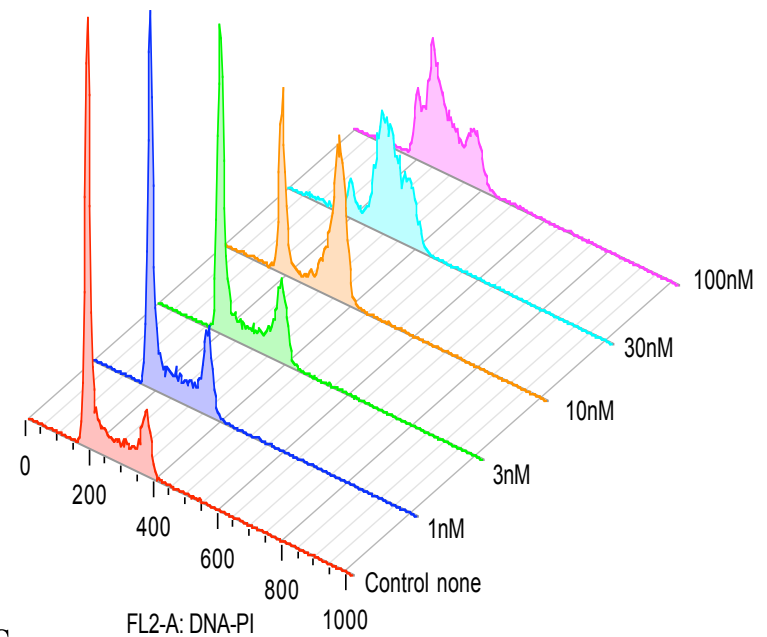
Identification of Arrest

Camptothecin a Model drug for S arrest?

LnCaP
Camptothecin



A2780
Camptothecin



More titrations

FlowJo
Software

How to Evaluate a Compound

- Preliminary Requirements
 - Cytotoxic tests provide the best tool to identify dosage
- Cell Cycle Titration Curves
 - Cell cycle profiles need to be address one cycle of growth at a time
 - Preliminary titration curves done at a 24h time point provide good hints of activity

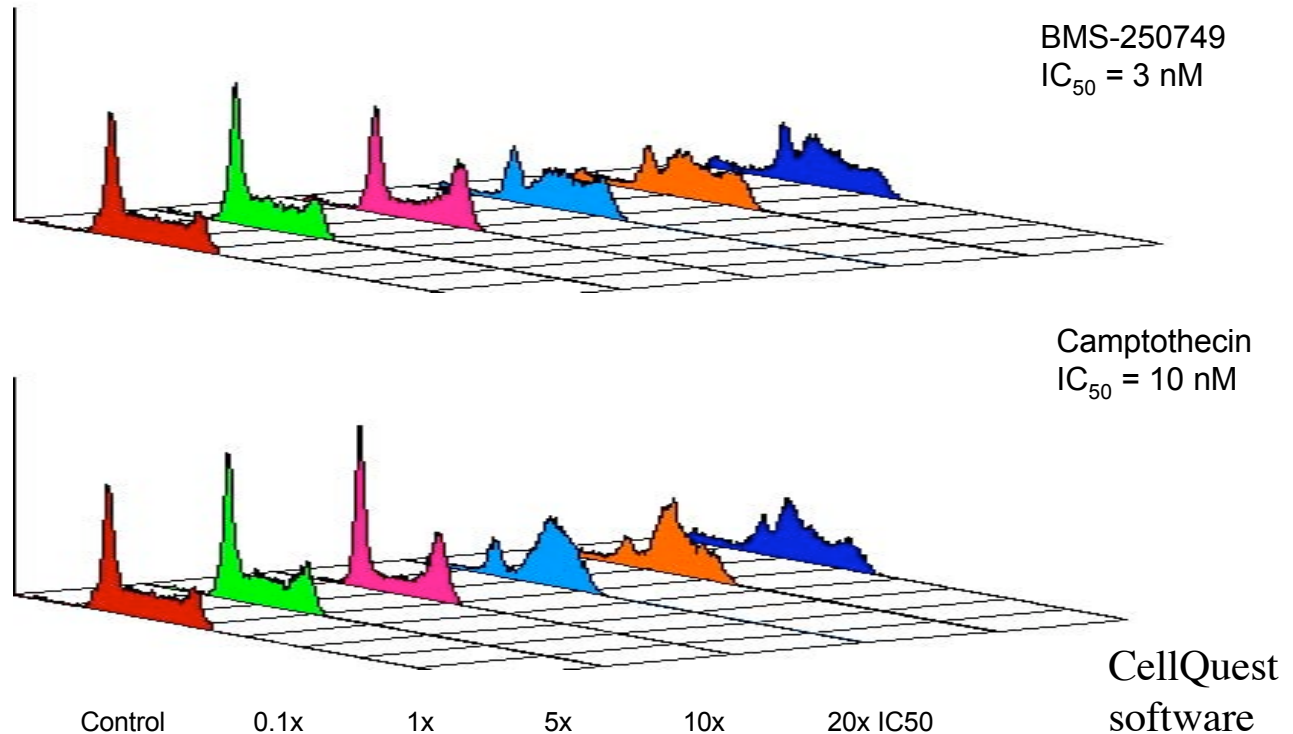
Arrest As An Assay

Mechanism of Action

- The Story of BMS-250497
 - BMS-250497 is the first non camptothecin compound described to be specific for Topoisomerase I inhibition.
 - Because of the side effects of camptothecin another efficient compound with this kind of activity have been actively pursued by the pharmaceutical industry
 - Biochemical assays required confirmation at the cellular level to reinforce the value of this compound as a candidate for the clinic

Results on BMS-250749

- Flow cytometry analysis of A2780 cells with wt p53



- Effects of BMS-250749 and camptothecin

-Raventos-Suarez,C., Class. K., Buczek,J,L.,Balasubramanian,N., Long, B., and Menendez, A,T. 2001 Topoisomerase I is the Target Responsible for Cytotoxicity of BMS-250749: Confirmation by Flow Cytometry 92th Annual Meeting, New Orleans March 24-28; Proc. AACR 2001 42:103 Abs 562

-Raventos-Suarez,C., Class, K., Wild, R., Menendez, A., Long, B. 004 *IN VITRO* Specificity Profiling of Cellular Topoisomerase Activities Using FACS Analyses.2ISAC XXII International Congress on Analytical Cytology. May22-27 2004 Montpellier, France. Cytometry 58A: p115 Abst# 96207

In Arrest Profiles

A second parameter reinforced your observations

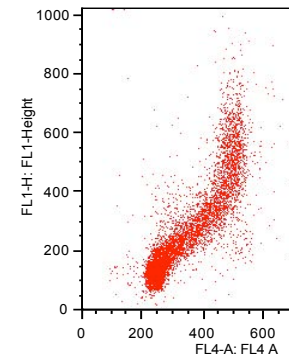
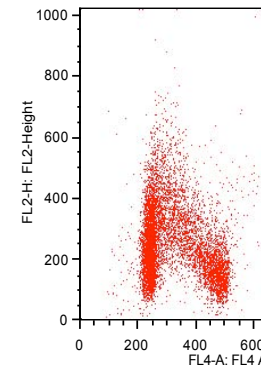
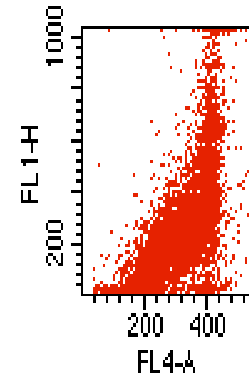
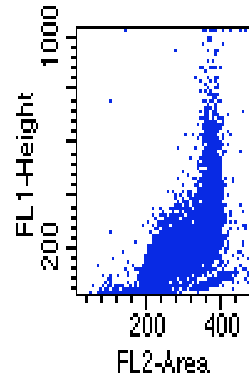
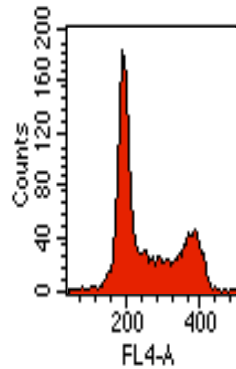
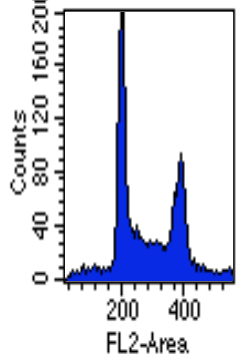
DNA

Cyclin B1

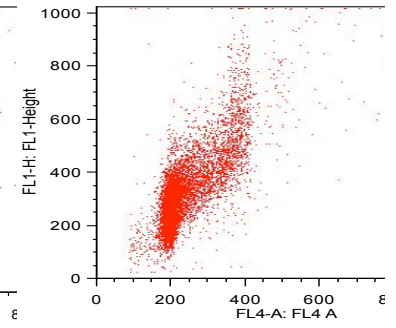
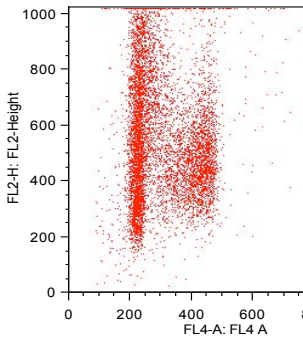
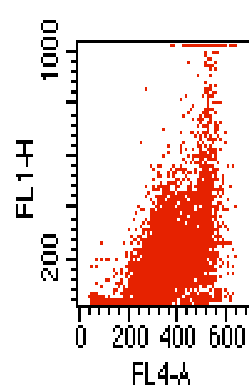
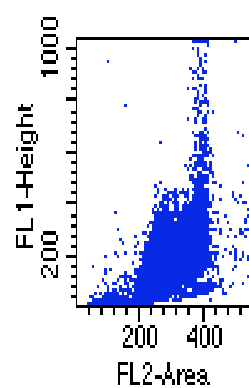
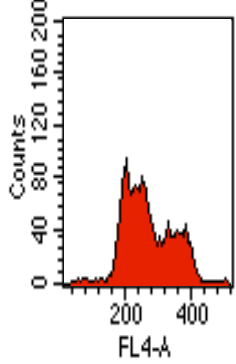
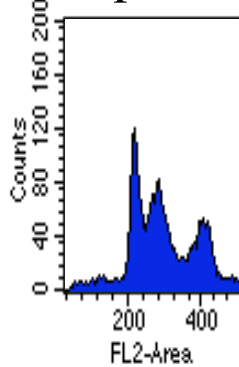
Cyclin E

Cyclin A

Control cells



Camptothecin treated

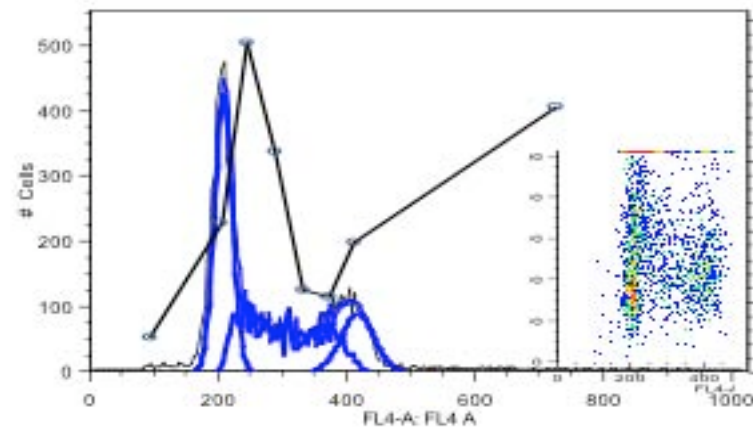


Comparison between PI and ToPro3

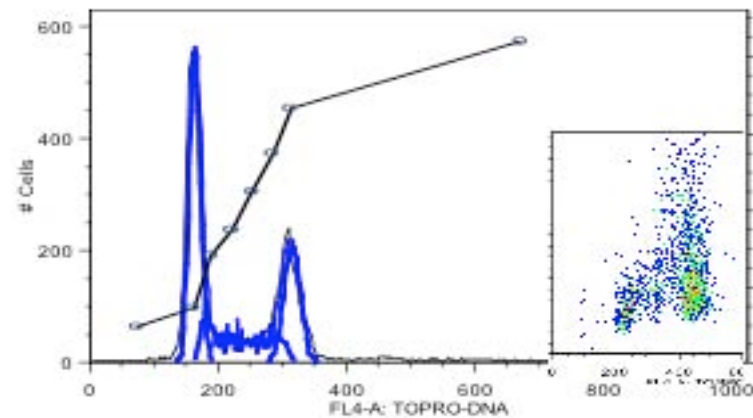
Blue= 488nm Red=633nm

FlowJo Allows Line Graphs Over DNA Profiles

A second parameter always reinforced your observations



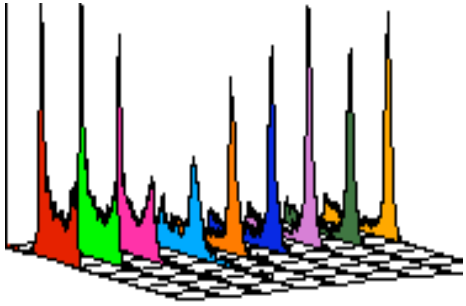
Cyclin E



Cyclin A

Results on BMS-250749

- We used a panel of 3 paired cell lines: sensitive and resistant to identify activity
- It takes several comparisons to efficiently confirm at the cellular level effects of known activities from a biochemical assay



Arrest Leads into Apoptosis

Apoptosis

An active metabolic process
devised to dismiss stressed cells
unable to cope with the insult

An active act of disappearance

Morphology of Apoptosis

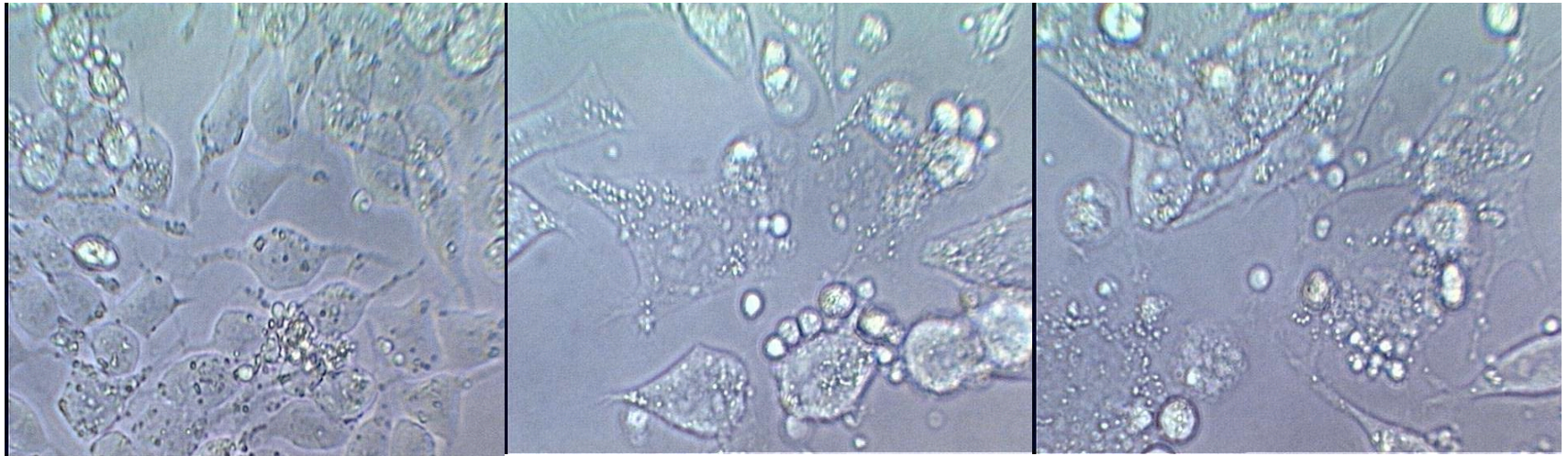
Control



Apoptotic



Apoptosis Pictures



Apoptosis a disappearing population

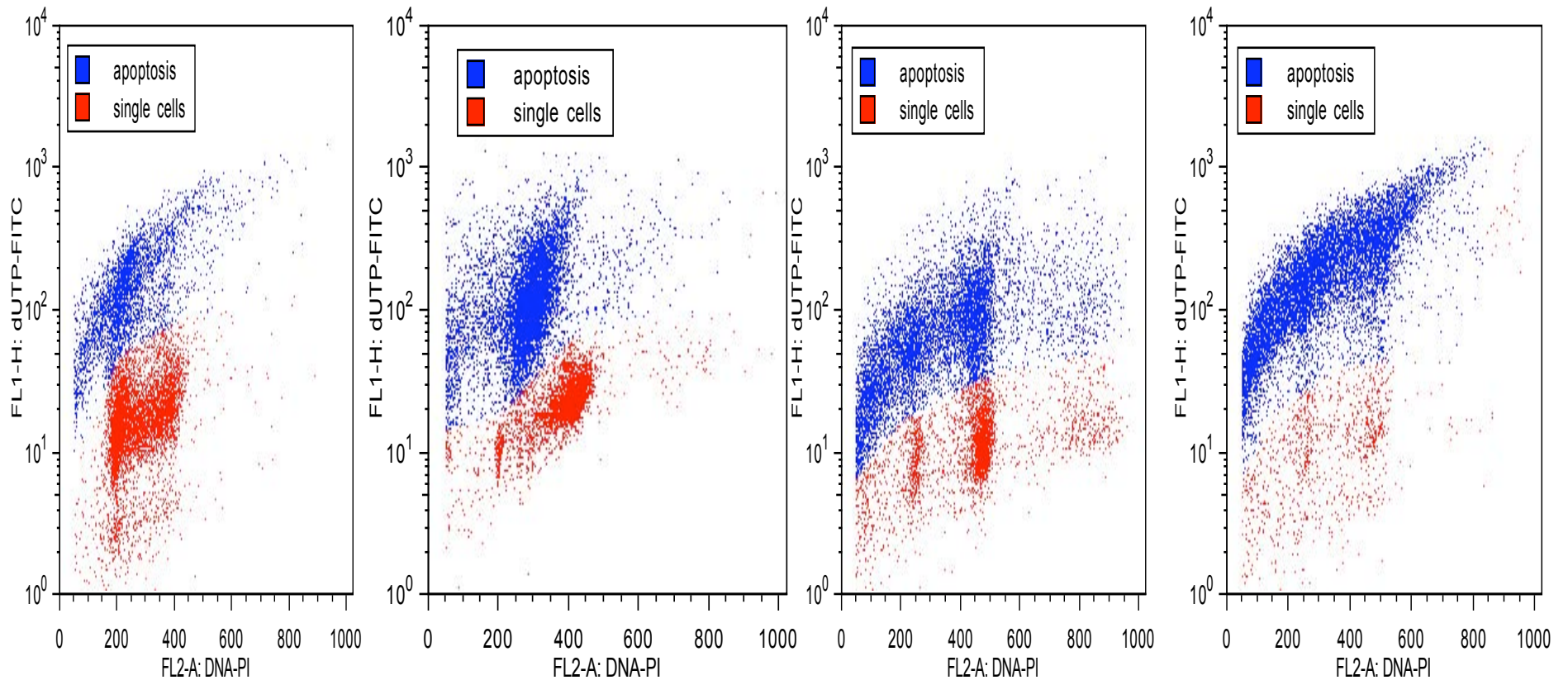
Evaluation Methods

Best When Linked to DNA profiles

- Annexin V
- TdT Tunel
- p85PARP
- Caspase 3
- Many other caspases
- Other approaches- The Sub G1 fraction

Tunel Assay

Provides Specificity of Phase

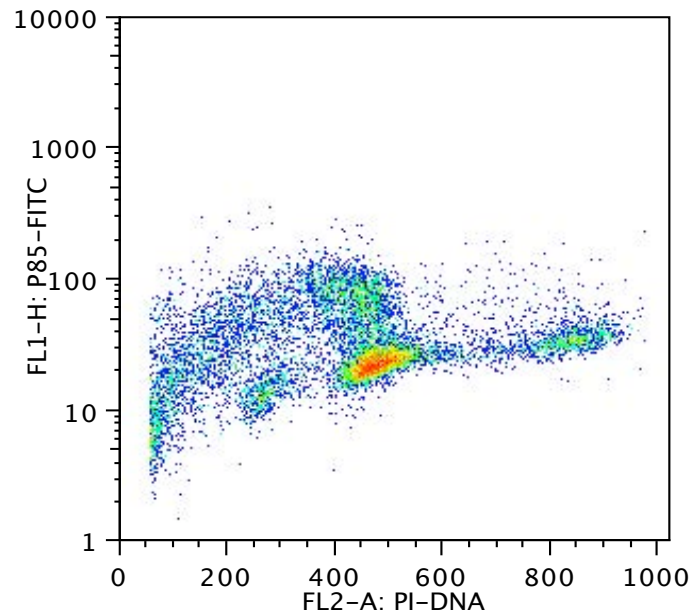


Dot Plot Overlays

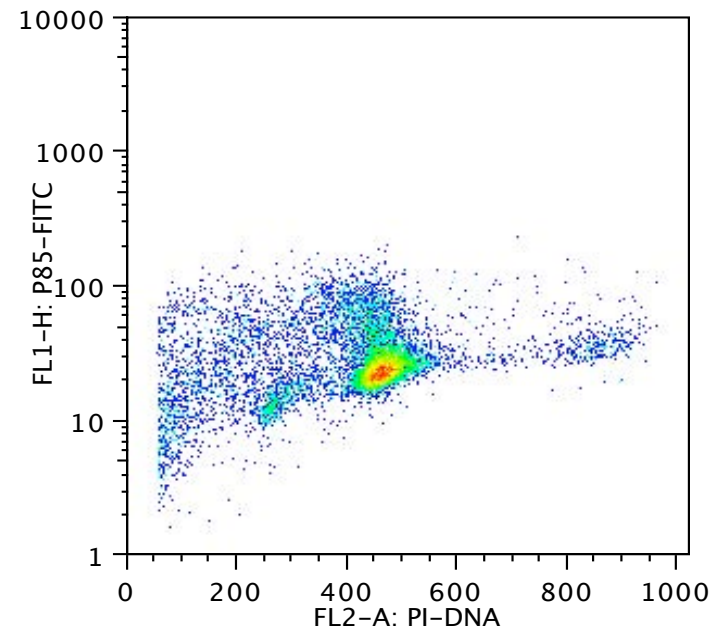
p85PARP on Drug Effects

Localization of populations engaged in apoptosis

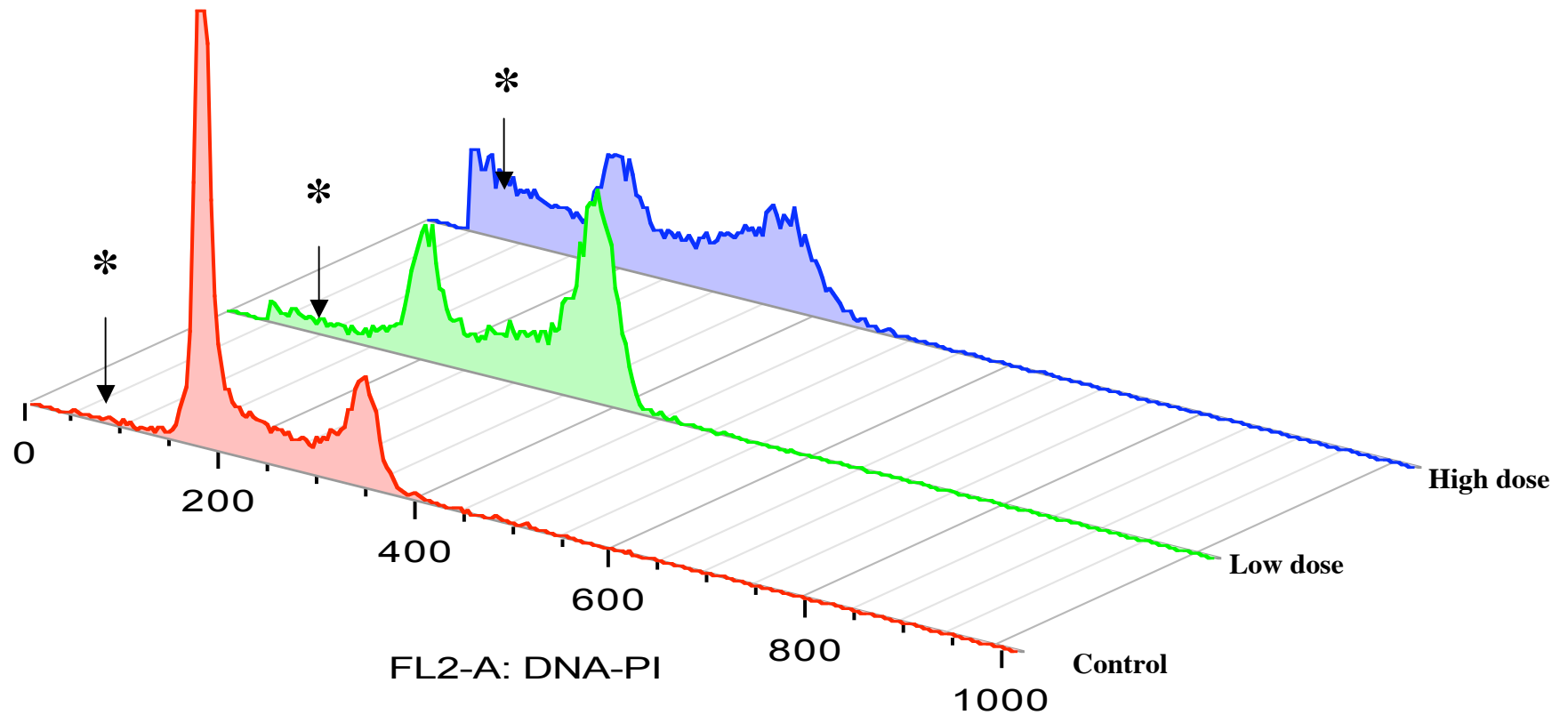
Vinblastin 5nM



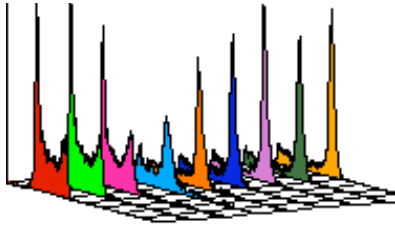
Colchicine 10nM



The Sub-G1 Fraction



* Apoptosis by Sub-G1, an increasingly growing population



When Proliferation, Arrest and Apoptosis Meet

The Story of BMS-214662

- BMS-214662 is a farnesyl transferase inhibitor with high apoptosis induction capabilities.
- Targets on the farnesyl transferase cascade are not expected to produce any apoptosis in short periods of time but this compound did it
- An assay needed to be devised to stain for proliferation and apoptosis simultaneously to directly answer this question

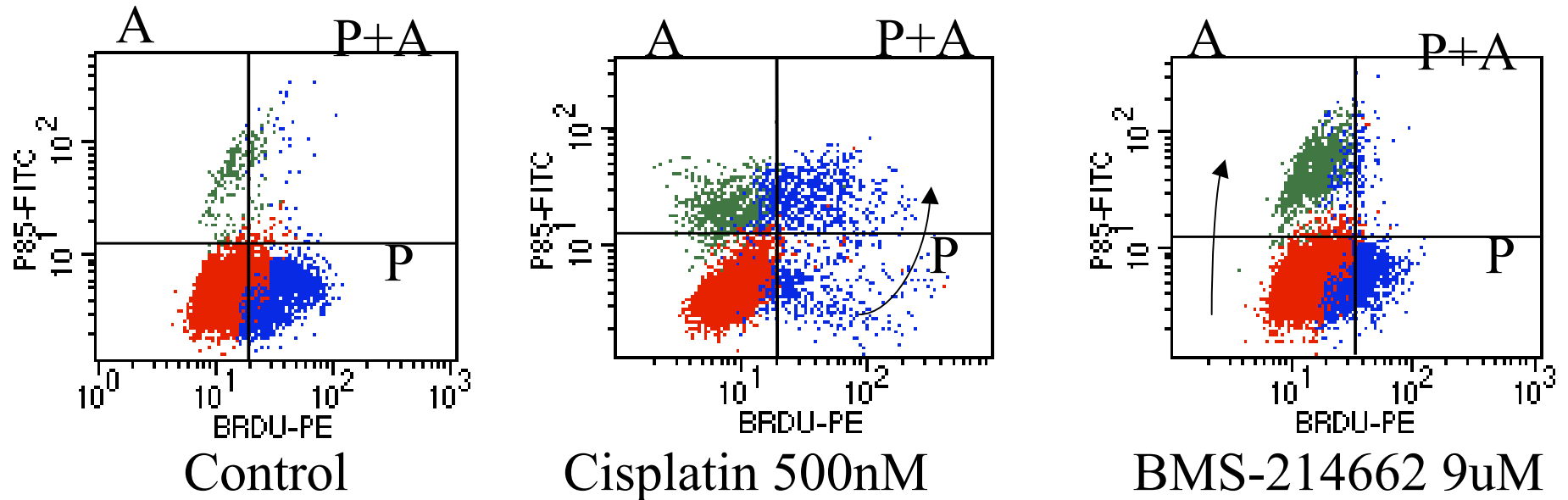
-Raventos-Suarez,C., Class. K. and Lee F. 2002 The pro-apoptotic FT-inhibitor BMS-214662 selectively targets non-proliferating tumor cells. Demonstration by a new flow cytometric method. ISAC XXI International Congress on Analytical Cytology .May 2002 San Diego, California. Cytometry supp11: p72

Building An Assay

- Proliferation by BrdU Uptake and evaluation by Dnase treatment: modify the BrdU Flow Kit from BD cat#552598”
- Cell cycle profiles by DNA 7AAD stain
- Apoptosis by p85 PARP
“use the Anti-PARP p85 fragment Ab from Promega” cat#G734A”

Proliferation and Apoptosis

Mechanism of Action of BMS-214662



Red = non proliferating
Blue = proliferating
Green = apoptotic non proliferating
P+A = proliferating cells engaged in apoptosis

Take Home Messages

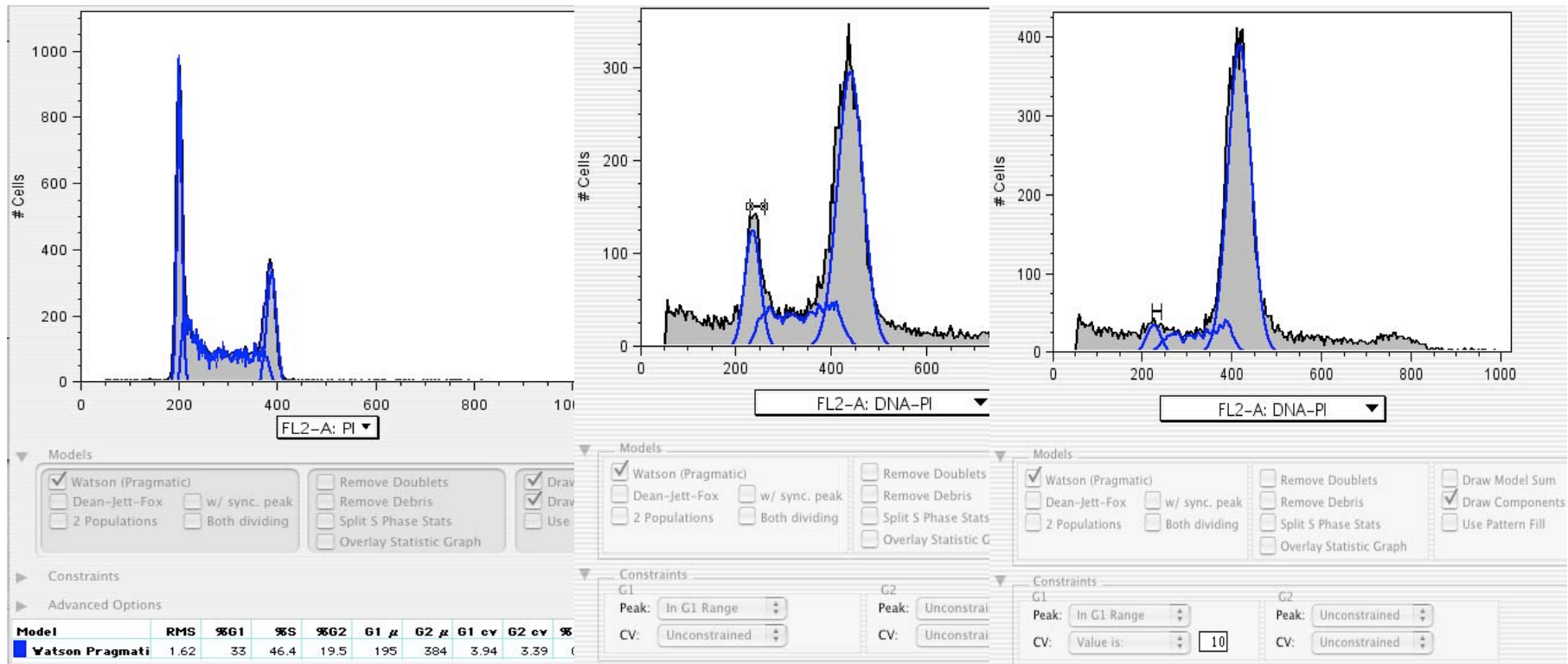
- **Cell Cycle Profiles are the best handle in addressing cell behavior, capacity to respond to an stimulus and possible engagement in apoptosis**
- **Careful preparation of cells is essential**
- **Multiparameter analysis linked to cell cycle profiles provide a more complete picture of cellular activities**

Back up data

Cell Cycle Profile of Cells

Getting to see the numbers

When algorithms don't seem to fit you can force constrains

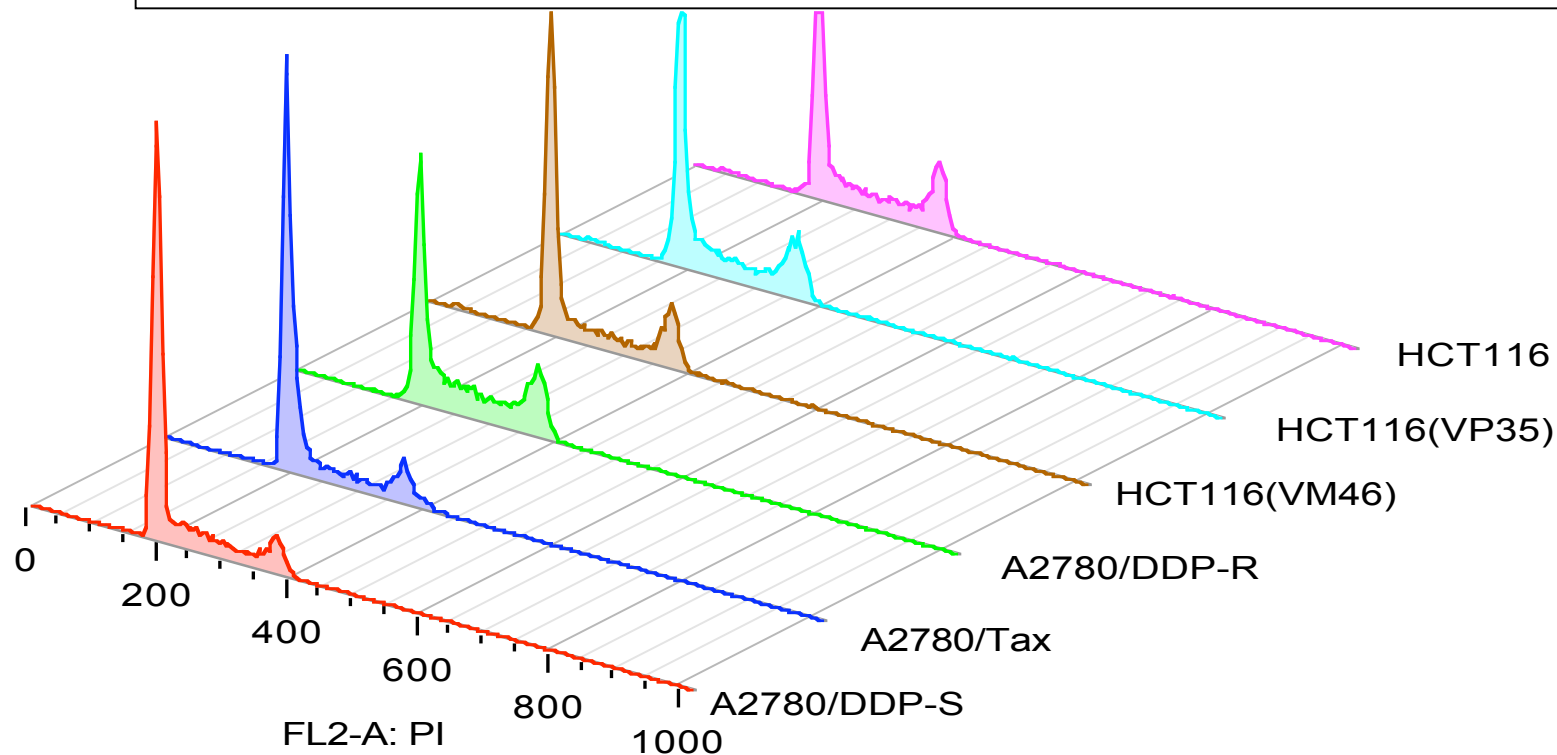


Cell Cycle Profile of Cells

A cell specific signature

FlowJo
Software

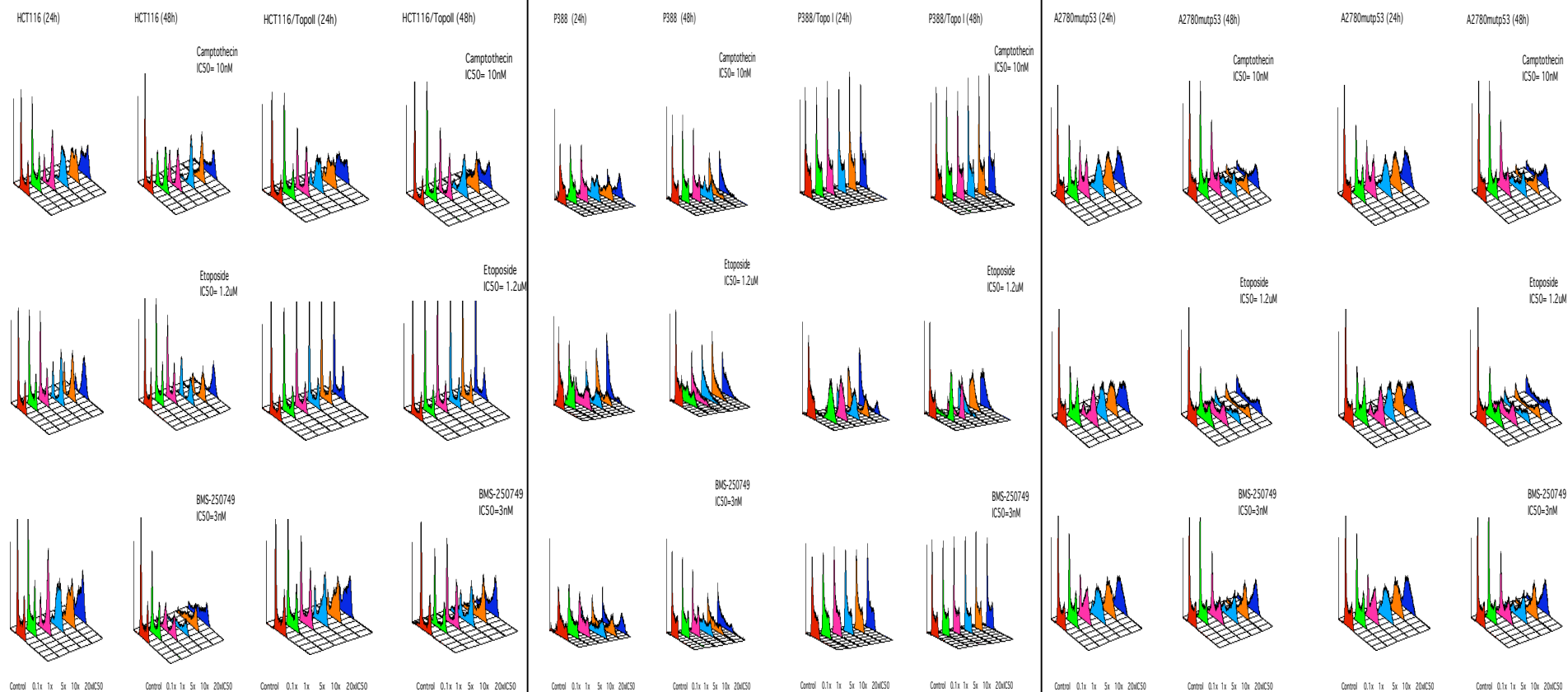
Panel of cell lines growing at logarithmic growth rates



Why are times at logarithmic rate of growth taken as optimal?

Results on BMS-250749

Six cell lines two time points plus two control drugs



It takes a panel of comparisons to address population distribution effects
Shown are Cells in 3 pairs:sensitive/resistant (TopoII, TopoI and p53)

Proliferation What and When

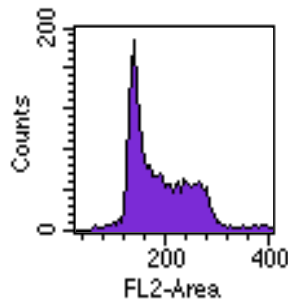
Beyond DNA quantitative analysis

- Proliferation activities require more than DNA measurements to account for disturbances
 - BrdU assay
 - will identify the S phase and determine doubling times.
 - Mitotic markers:
 - will allow discrimination of G2 and S phases
 - Cyclins:
 - will determine how far into a cell cycle phase cells have been able to go before stop growing
 - Specific altered functions by induced by resistance or mutation mechanisms

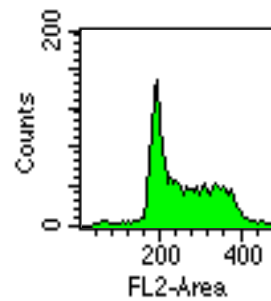
More on Mechanism of Action

Specificity on Topo II

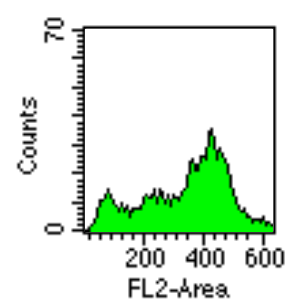
A Camptothecin
Resistant
Cell line



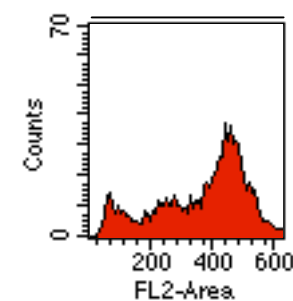
Control



Camptothecin

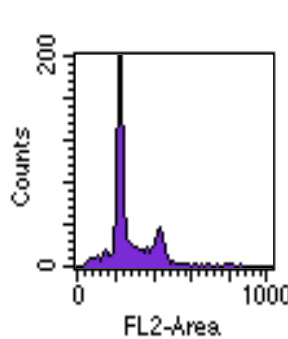


Etoposide

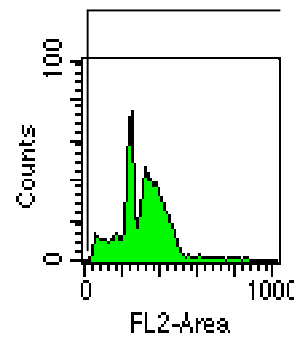


TAS-103

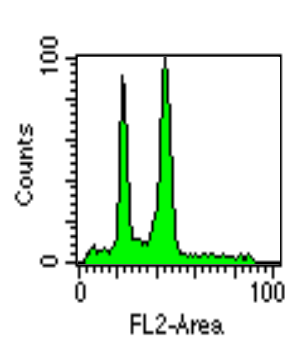
A Camptothecin
Sensitive
Cell line



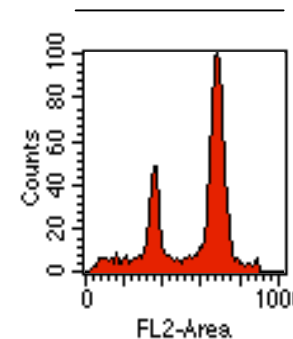
Control



Camptothecin



Etoposide



TAS-103

Long, B.H., Fairchild, C.R., Raventos-Suarez, C., Cornell, L., and Menendez, A.T.
Cytotoxic mechanism of TAS-103 is related to topoisomerase II mediated DNA cleavage.
91st Annual meeting of the AACR. San Francisco, April 1-5, 2000.