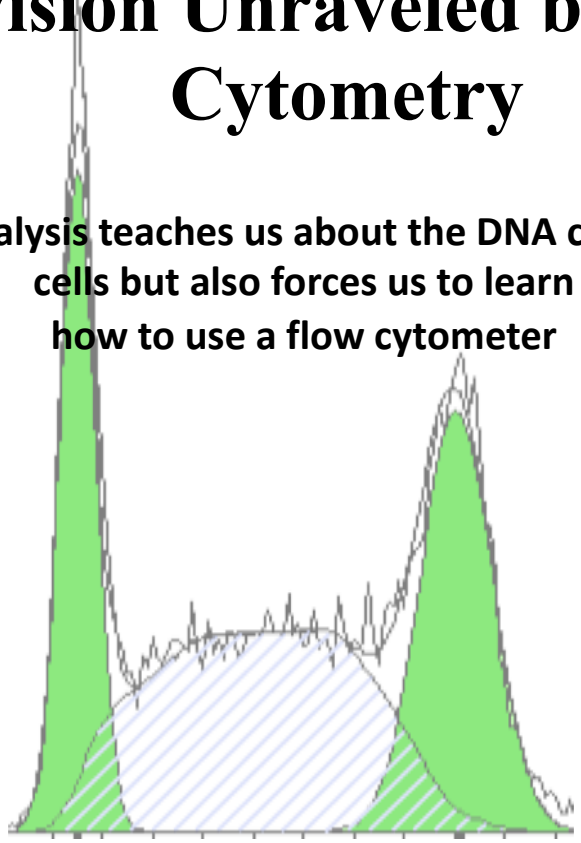
Cell Cycle Progression and Cell Division Unraveled by Flow Cytometry

DNA analysis teaches us about the DNA content of cells but also forces us to learn how to use a flow cytometer



Paul K Wallace
pkwallace@odobenus.org
Professor Emeritus
Roswell Park Comprehensive Cancer Center
Buffalo, NY 14263

Acknowledgements

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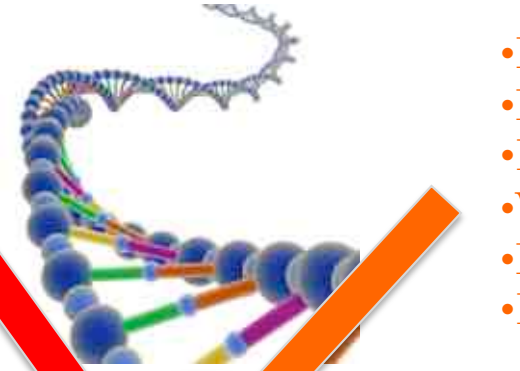
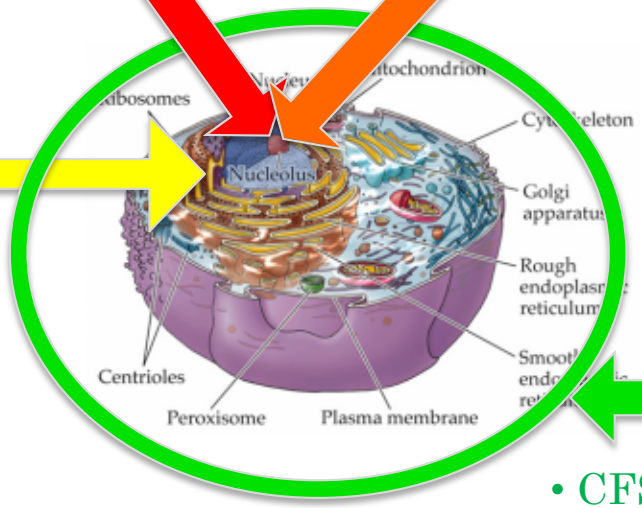
- Lizanne Breslin, Brian Gray (PTI Research)
- Kitty DeJong (RPCCC)
- Annual Course participants through the years

How Can You Measure Cell Division by FCM?

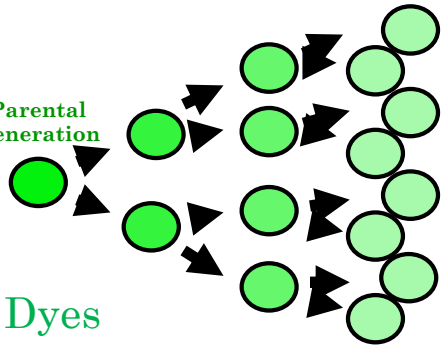
•BrdU
•Click-iT EdU

•PI
•DAPI
•Hoechst 33342
•Vybrant DyeCycle Dyes
•DRAQ5
•FxCycle Dyes





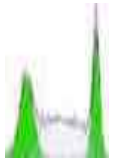
• Cyclins
• Ki-67
• PCNA
• H3 Phosphorylation



• CFSE
• CellTrace Dyes
• CellVue Dyes
• PKH Dyes

THE PLAN

- Cell Cycle, Dyes & Techniques
  - Doublets- slow is the way to go
  - Data Analysis
    - Simple analysis
    - Modelling
  - Clinical Significance of Aneuploidy & S phase
  - Modelling Synchronized Populations
- Tracking Dye Dilution
  - The dyes - many choices
  - Modelling Dye Dilution Data
  - Tracking Applications



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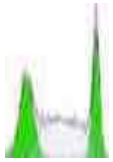
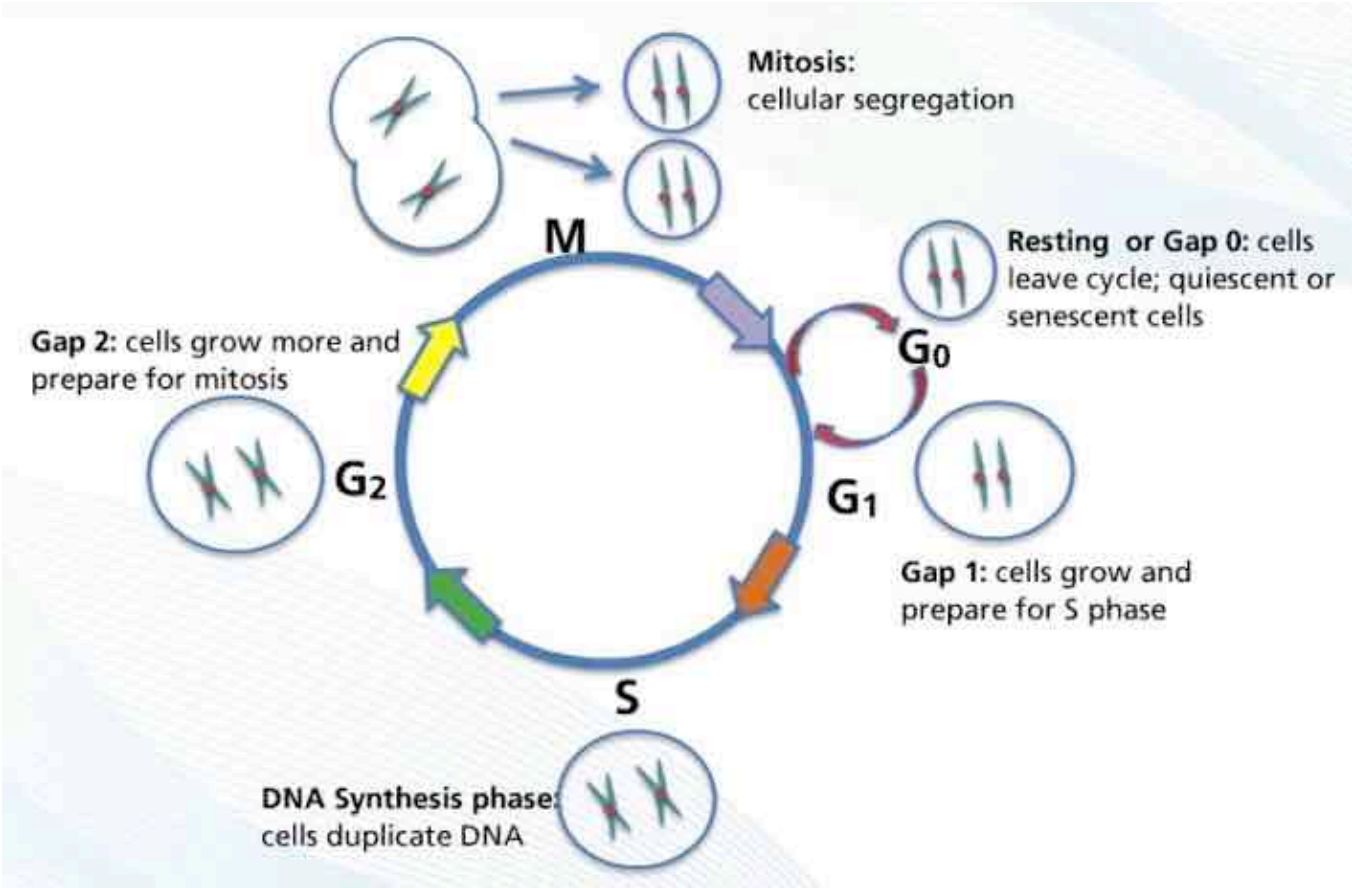
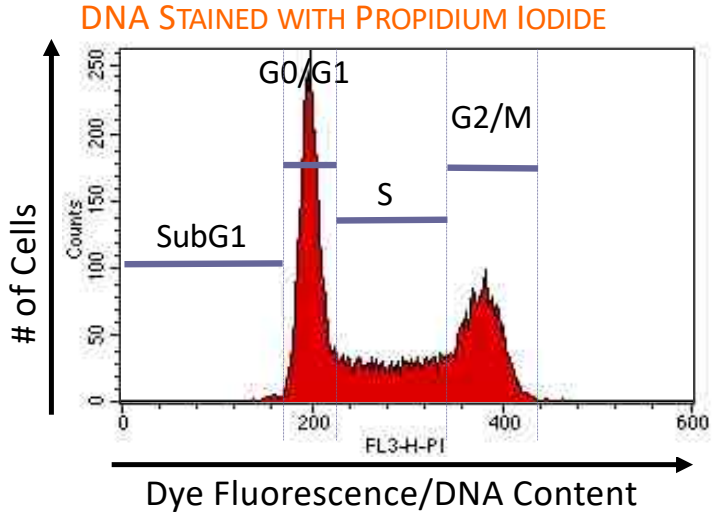


Four Phases of The Cell Cycle



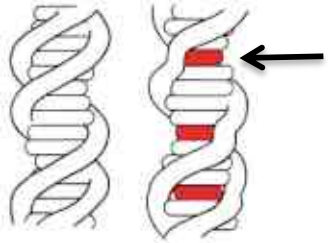
DNA Content Changes During Cell Cycle

- DNA binding dyes bind stoichiometrically = Fluorescence intensity is proportional to the amount of DNA present within cell
- Used to quantitate amount of DNA and therefore position in the cell cycle (e.g., cells in G2 have 2x amount of DNA as cells in G1)
- Fluorescence data used to generate DNA histograms
- The DNA histogram gives a static picture of the proportion of cells in different phases of the cell cycle



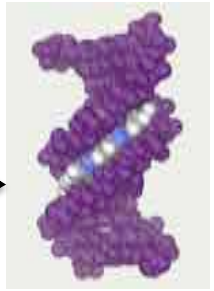
DNA Binding Dye Characteristics

• **WEAKLY-FLUORESCENT** – until bound to nucleic acids where fluorescence increases 100-1000x, once bound via:



• Intercalation (*e.g.,* PI)

• DNA minor groove (*e.g.,* DAPI)



•**IMPERMEANT** – cannot cross the cell membrane unless cells are fixed

OR – *e.g.*, PI, DAPI

• **PERMEANT** (a.k.a. VITAL) – pass straight through intact cell membrane

– *e.g.*, Hoechst 33342, DRAQ5, DyeCycle Dyes

• **NUCLEIC ACID SPECIFICITY:**

<u>dsDNA and dsRNA</u>

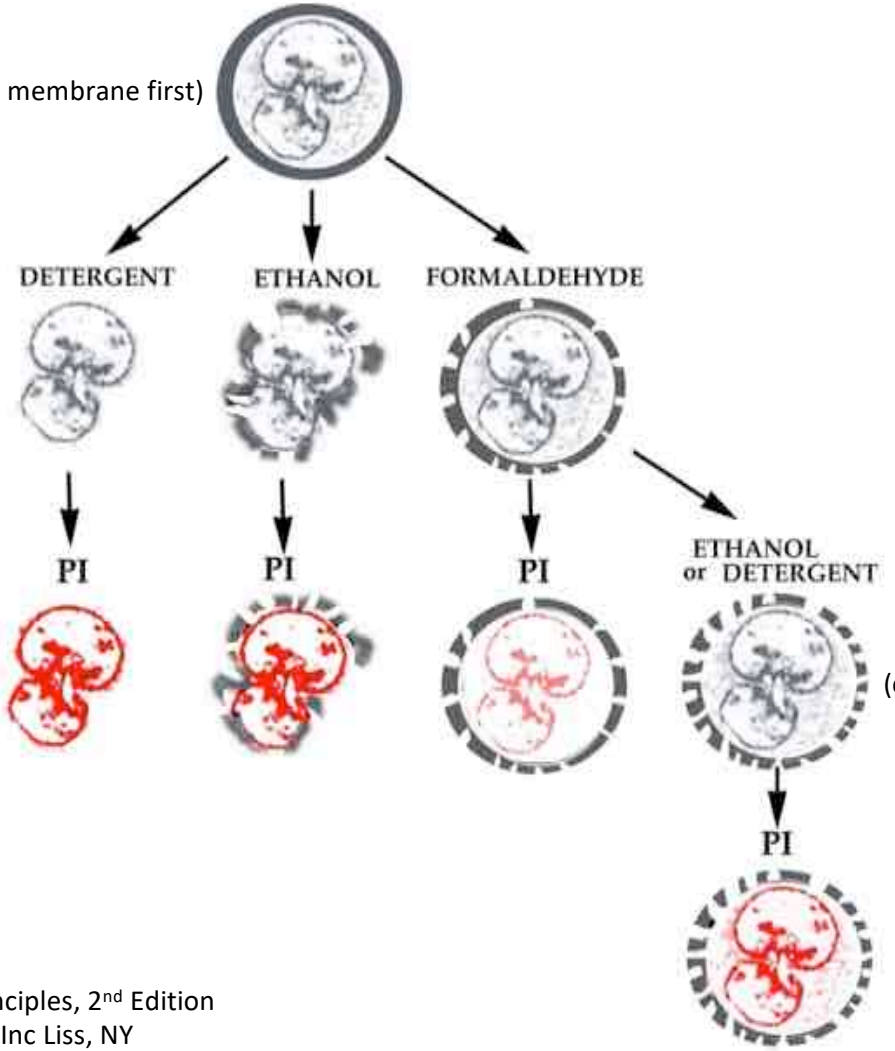
– Propidium Iodide (PI)
– SYTOX®
– TOTO or TO-PRO
– DRAQ 5

<u>dsDNA only (nucleic acid preference)</u>

– 7-AAD (G-C)
– Hoechst 33342 (A-T)
– DAPI (A-T)
– Vybrant ® DyeCycle

Fixation and Permeabilisation

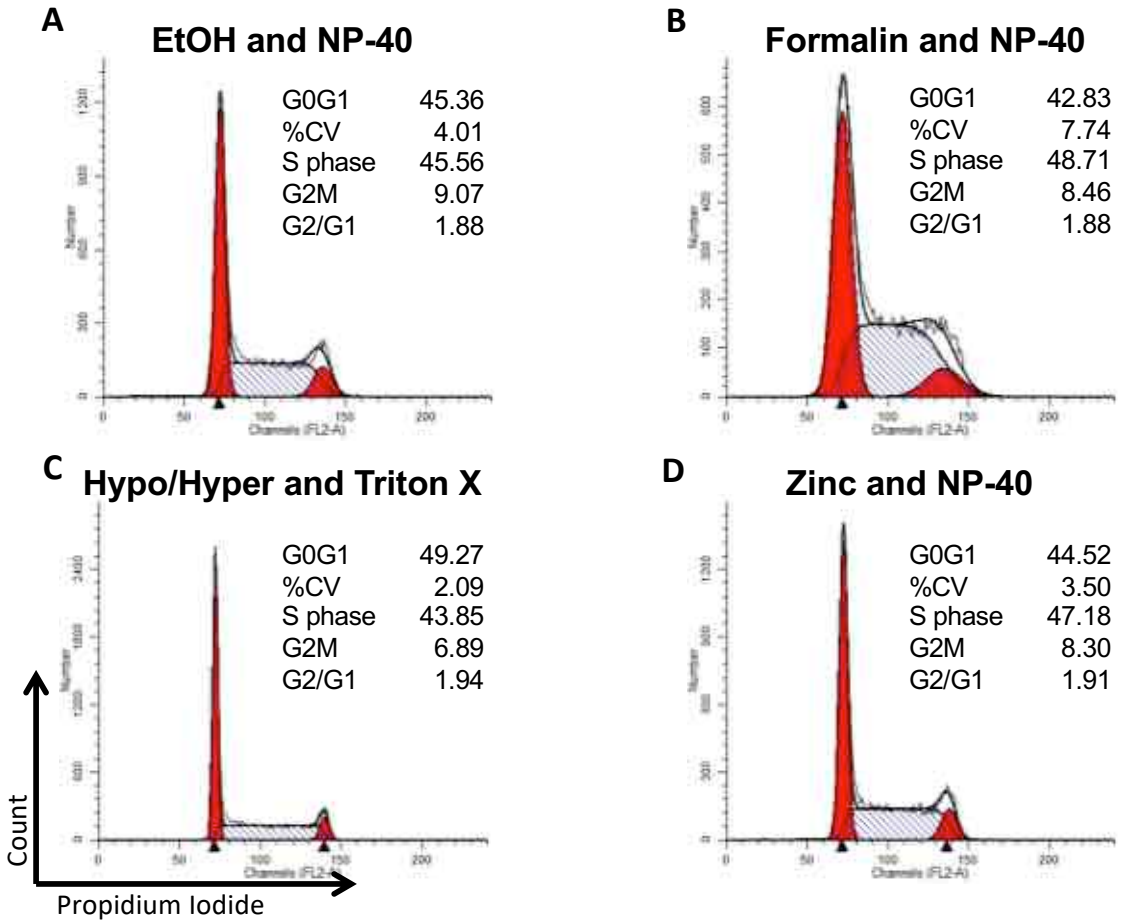
(can stain the outer membrane first)



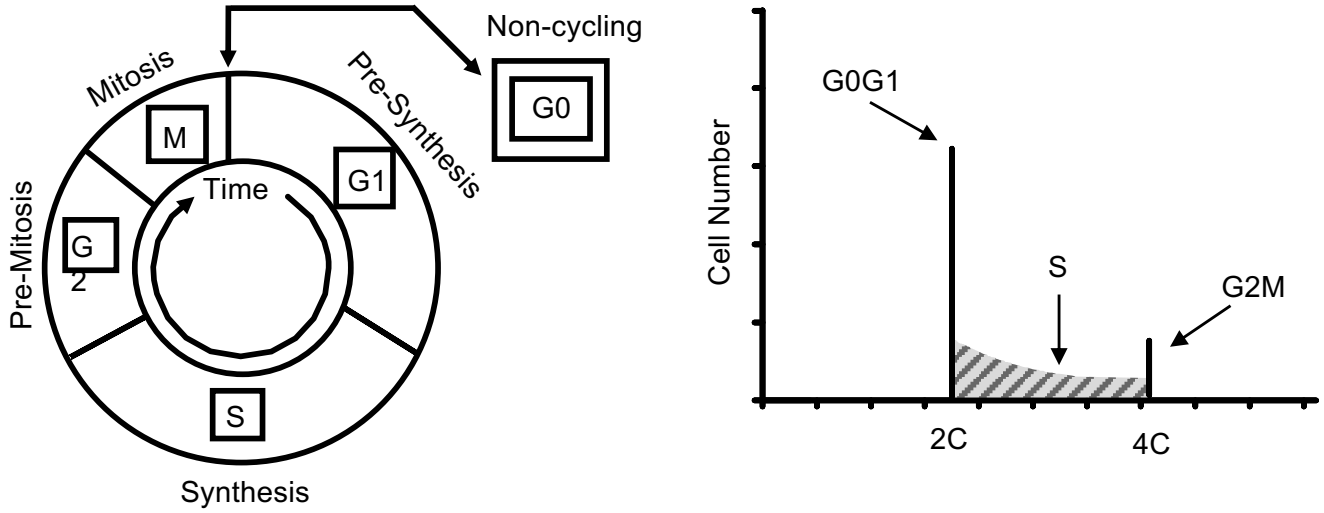
(can stain the cytoplasm)

Flow Cytometry First Principles, 2nd Edition
Alice Givan, 2001, Wiley-Inc Liss, NY

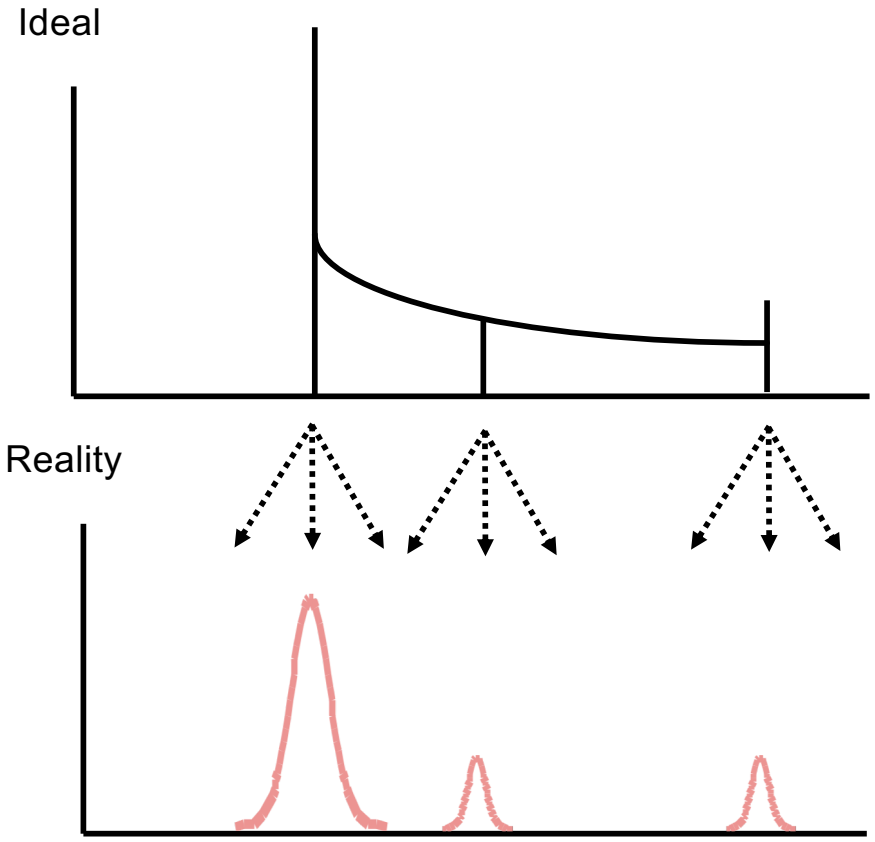
Effect of Different Fixatives & Cell Preparations on DNA Cell Cycle Histogram Quality and Quantification of Cycle Phases



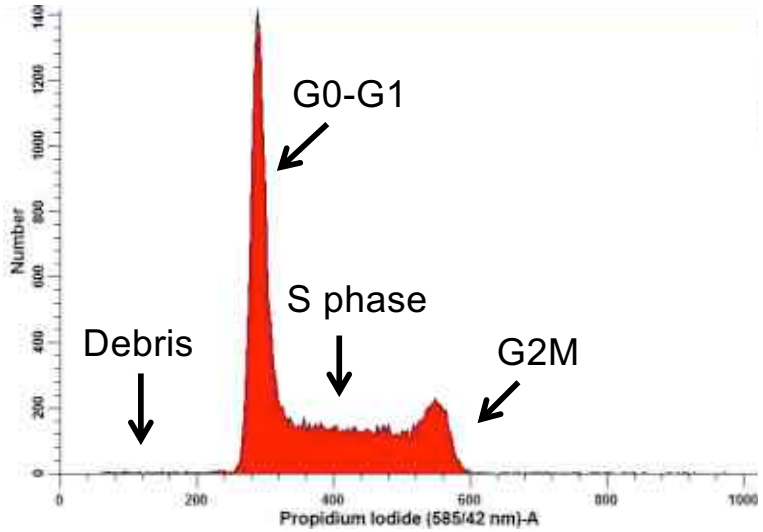
CONCEPT 1: DNA VS TIME IN CYCLE



Concept 3: Signal Broadening



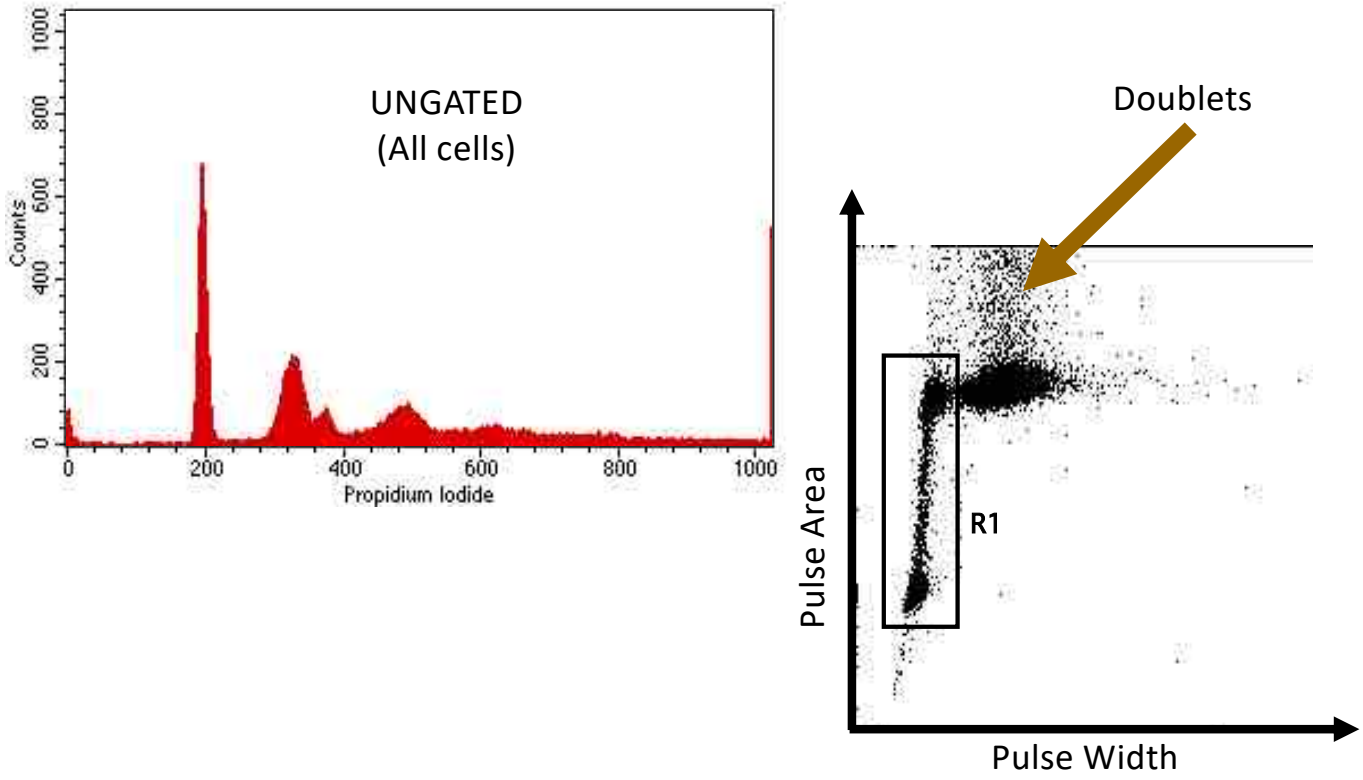
The DNA Histogram



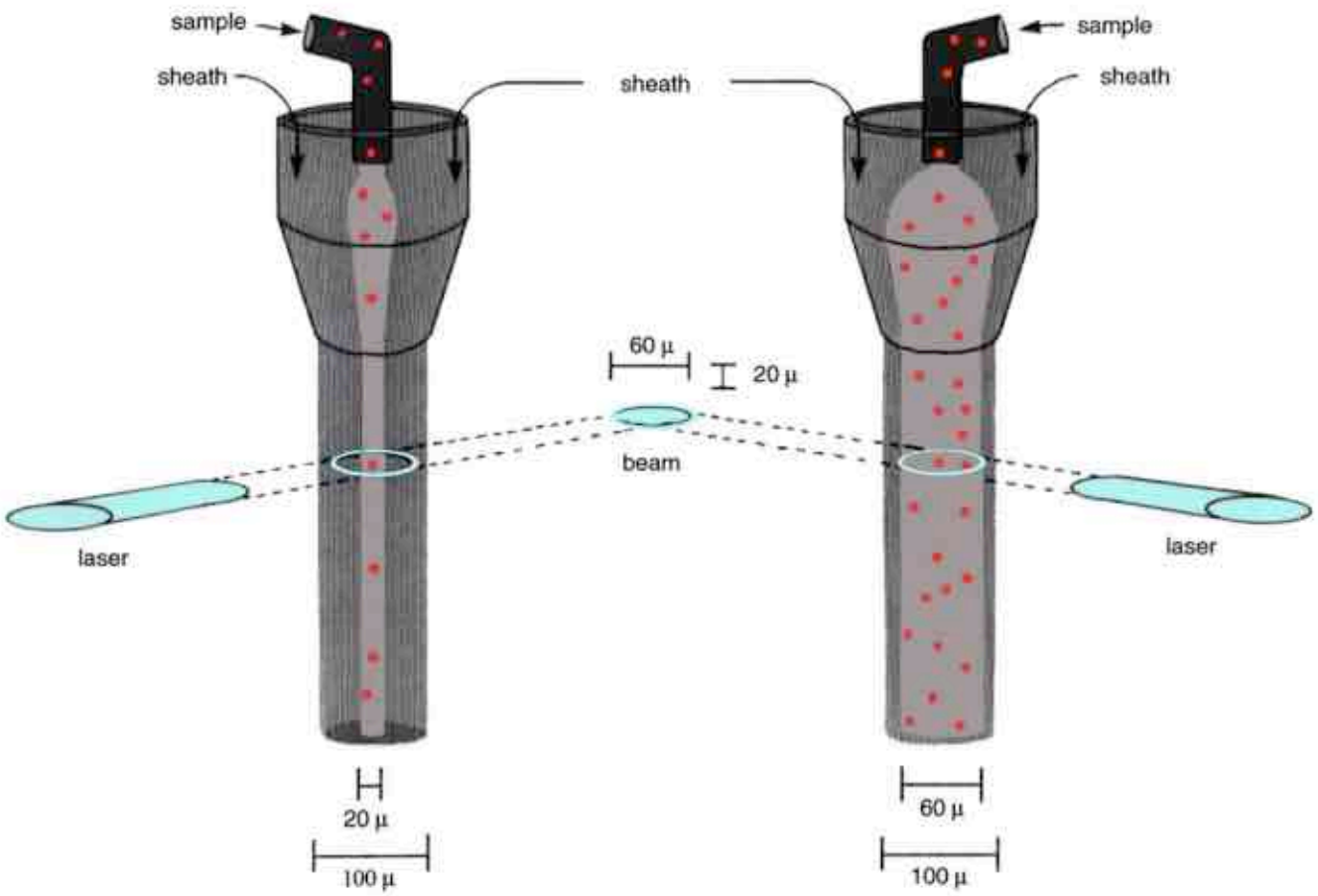
A product of:

- DNA content per cell
- Number of cells present in each cell cycle stage
- Signal broadening due to staining and measurement variability

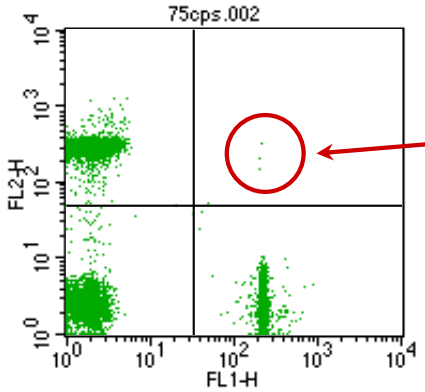
What's Going on Here?



CELLS FLOWING THROUGH A LASER BEAM: with a wide core, the cells are not equally illuminated and multiple cells may coincide in the laser beam.



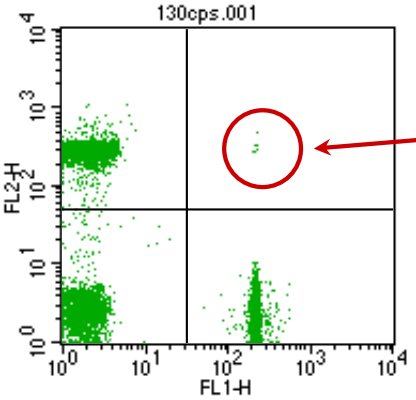
HOW TO MAKE DOUBLE POSITIVES LESS RARE: CREATE PSEUDO-AGGREGATES



File: 75cps.002

Quad	% Gated
UL	42.84
UR	0.04
LL	21.92
LR	35.20

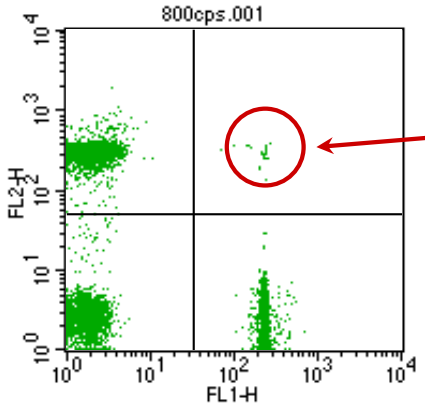
75 cps



File: 130cps.001

Quad	% Gated
UL	42.87
UR	0.07
LL	23.13
LR	33.93

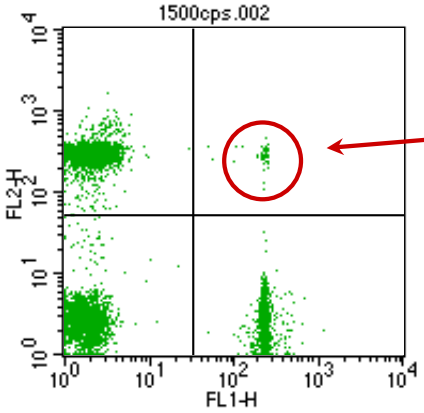
130 cps



File: 800cps.001

Quad	% Gated
UL	43.01
UR	0.23
LL	21.23
LR	35.53

800 cps



File: 1500cps.002

Quad	% Gated
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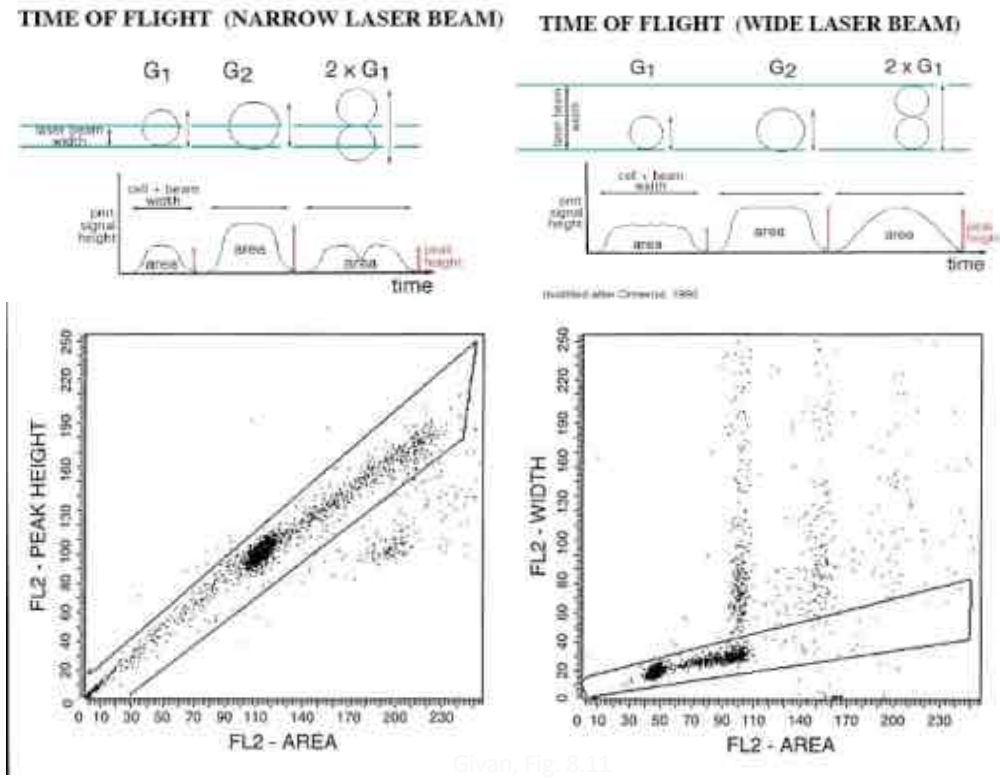
UR	0.59
LL	21.85
LR	34.76

1500 cps

Elimination of Cell Aggregates Using Pulse Shape



Givan, Fig. 8.11

- Area vs. Height for narrow beam
- Area vs. Width for wide beam

Flow Cytometry First Principles, 2nd Edition
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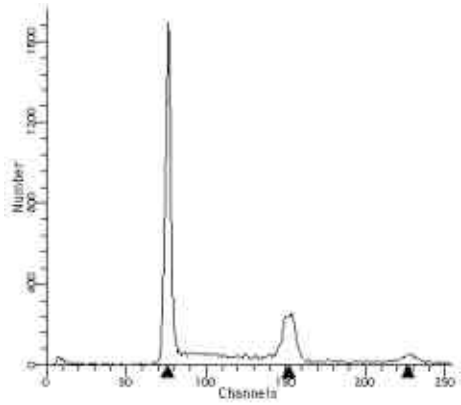
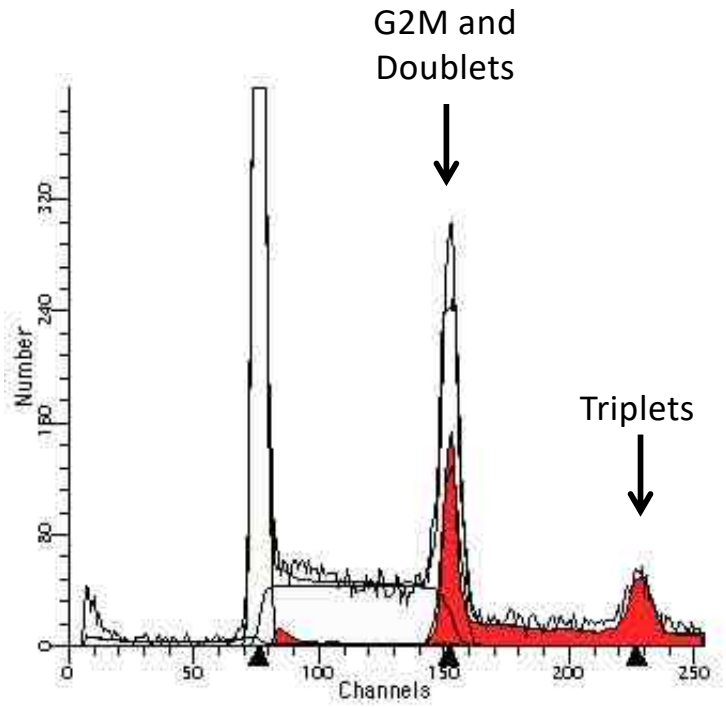
MATHEMATICAL MODELING TO "REMOVE" AGGREGATES



By knowing the number of G0G1 singlets and the number of G0G1 triplets, the number of doublets can be mathematically estimated.



Slide courtesy of Alice Givan

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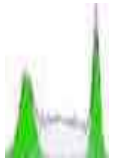
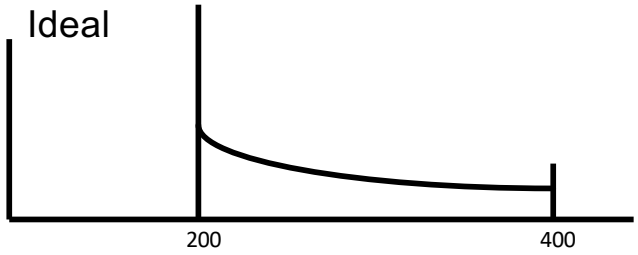
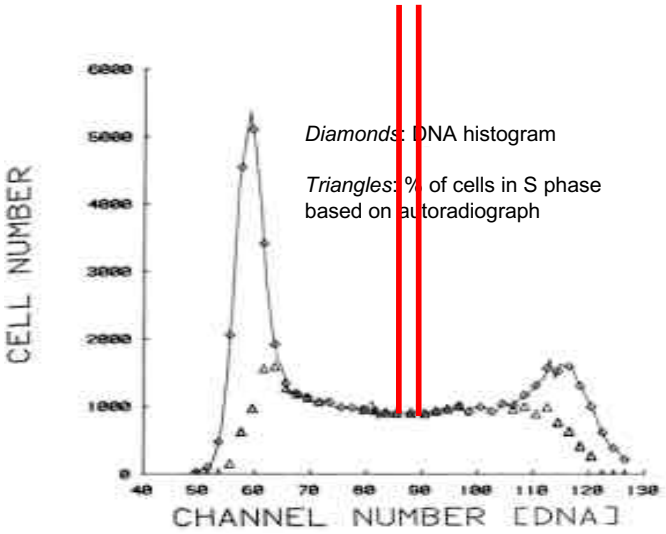


Why Model DNA Content?



Real



Ideal

- Identical DNA content => identical # DNA dyes/cell
- Identical # DNA dyes/cell => identical fluorescence
- 2X # of DNA dyes => 2X fluorescence intensity

Real

- 1-5% variation in # DNA dyes/cell for cells with identical DNA content
- 1-5% variation in fluorescence intensity for cells with identical # DNA dyes per cell

⇒ Boundaries between cell cycle phases blur

⇒ Goal is to minimize staining and analysis-related variability

?? How does variability effect our analysis

Experimental Setup

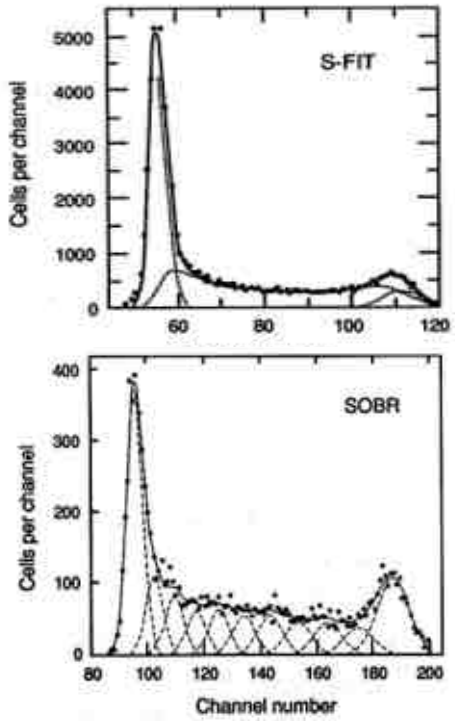
- Cells were labeled in culture for 10 min with ^{3}H-thymidine
- Stained with mithramycin
- Sorted based on DNA content (2-channels per fraction)
- Perform autoradiography on each fraction and count the number of cells that had taken up ^{3}H-thymidine (*i.e.* were in S phase)

Sheck LE, *et. al.,* (1980). Cytometry. *1:*109

Simple Analysis:
Inside Out or S-FIT Method

Inside Out:

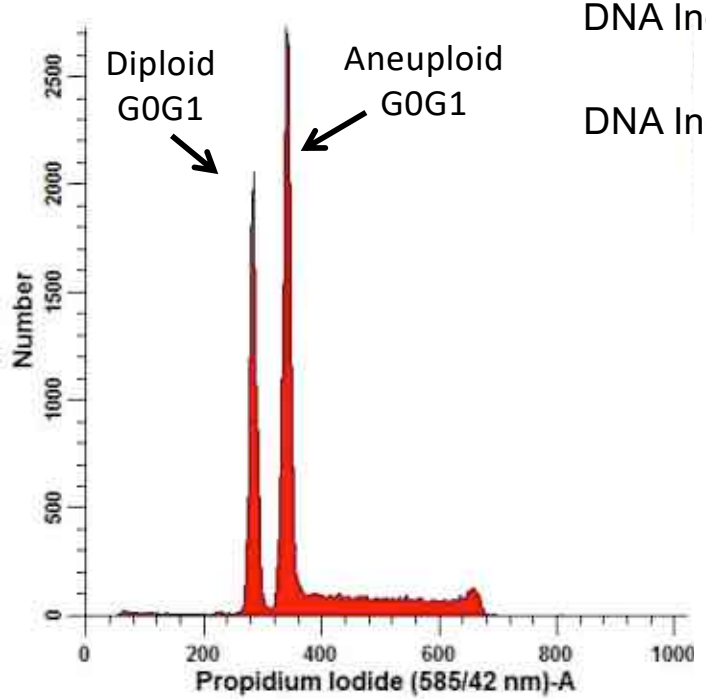
1. Find G0-G1 & G2M peaks. Estimate SD of each
2. Move 2 or 3 SD from G0-G1 & G2M peaks to establish "pure" S region
3. Fit S with rectangle, trapezoid or 2° polynomial
4. Determine G0-G1 & G2M by subtracting fitted S



Dean PN and Jett JH. Mathematical analysis of DNA distributions derived from flow microfluorometry. J Cell Biol. *60*:523-7, 1974.

Baisch H *et al*. A comparison of mathematical methods for the analysis of DNA histograms obtained by flow cytometry. Cell Tissue Kinet. *15*:235-49,1982.

DNA Diploid *vs.* DNA Aneuploid



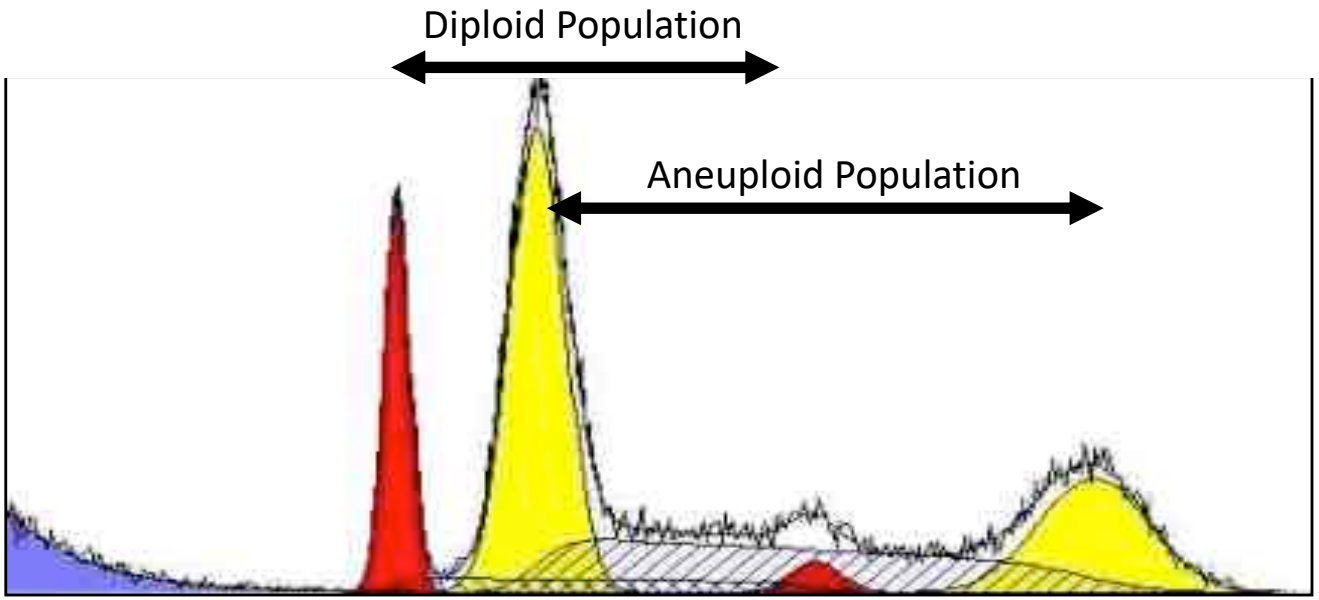
$$\text{DNA Index} = \frac{\text{Aneuploid G0G1 Peak Position}}{\text{Diploid G0G1 Peak Position}}$$

$$\text{DNA Index} = \frac{291.8}{222.4} = 1.31$$

Tumor samples may contain multiple cell types:

- A diploid stromal cell population with normal DNA content (may be cycling or not)
- One or more aneuploid neoplastic populations with abnormal DNA content
- The challenge is to determine which of the G0G1 peaks is tumor vs. normal cells

Add a Little More Complexity: Non-Linear Least Squares Analysis



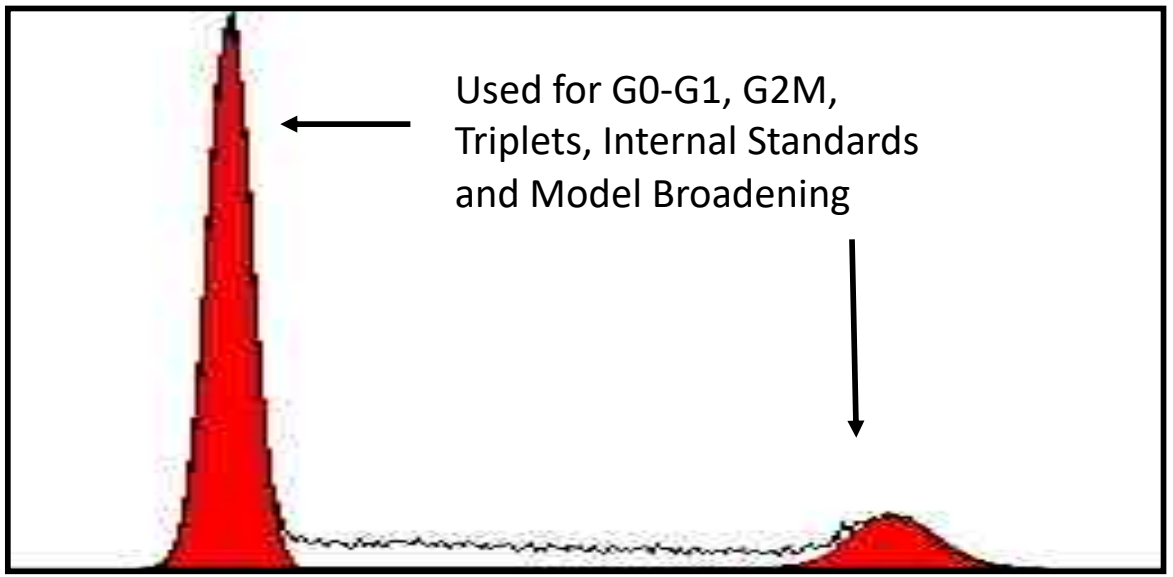
Model Components

Model Component:

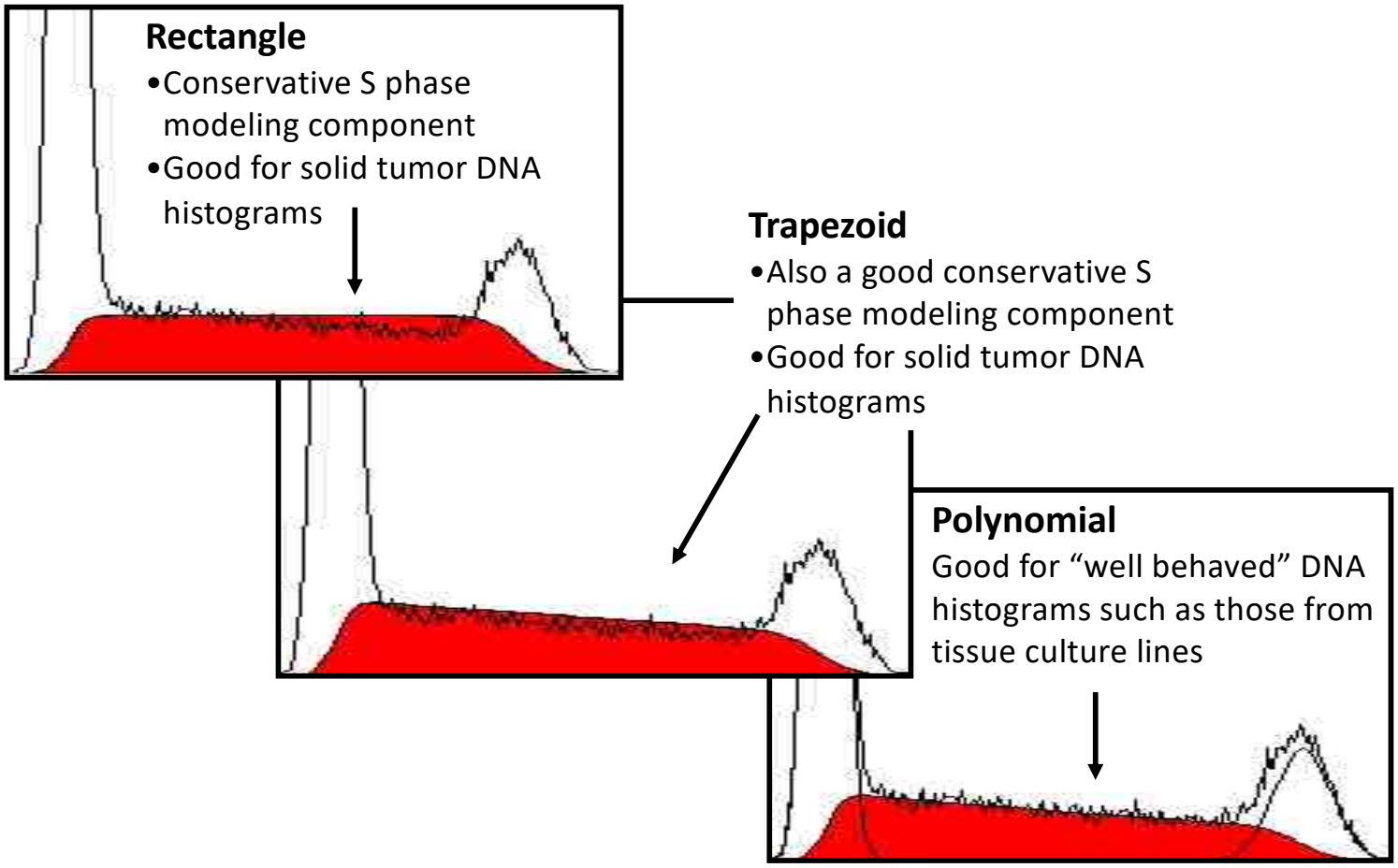
A mathematical construct that simulates some physical or biological process

- Gaussian
- Broadened Rectangle(s)
- Broadened Trapezoid(s)
- Broadened Polynomial
- Debris Fit (Exponential, Single Cut, Multiple-cut)
- Aggregate Compensation

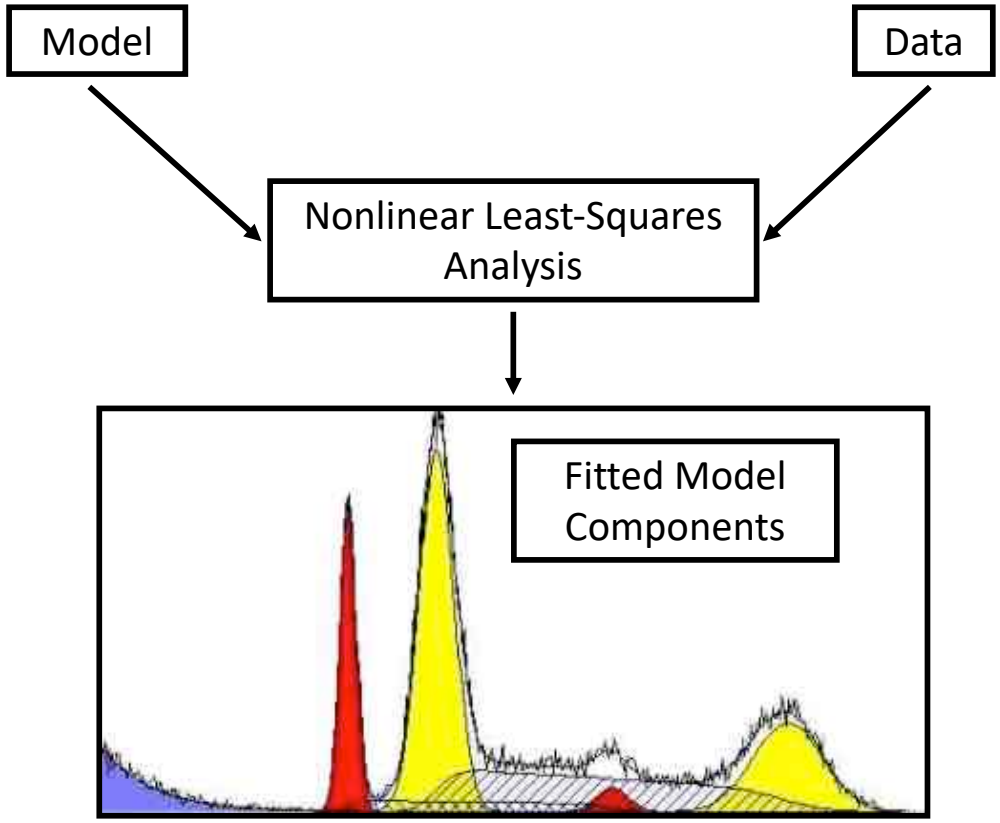
Model Component: Gaussian



Model Components Used to Fit S Phase

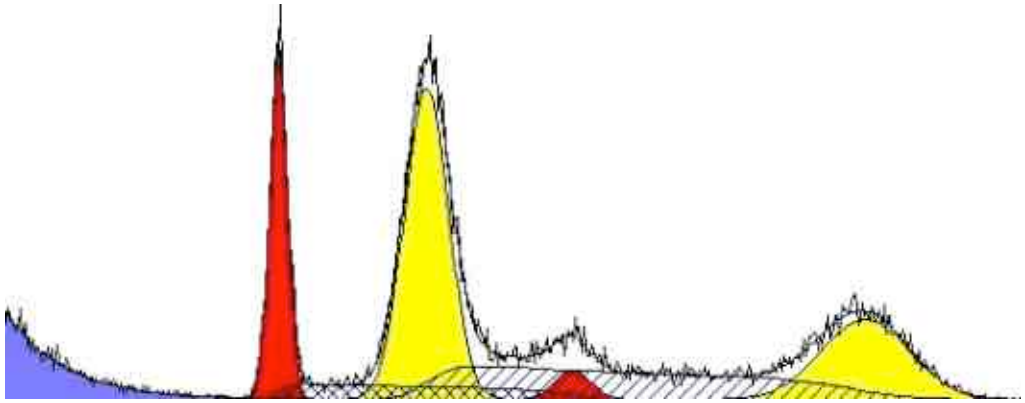


Non-Linear Least-Squares Analysis



Advantages and Disadvantages of DNA Modeling

- **Advantages**
  - Accurate
  - Reproducible
  - Efficient
  - Conducive to graphical reporting
- **Disadvantages**
  - Problems choosing the appropriate model
  - Different modeling algorithms in different programs will give slightly different results
  - Accurate modeling requires sufficient events to avoid fitting noise[1]

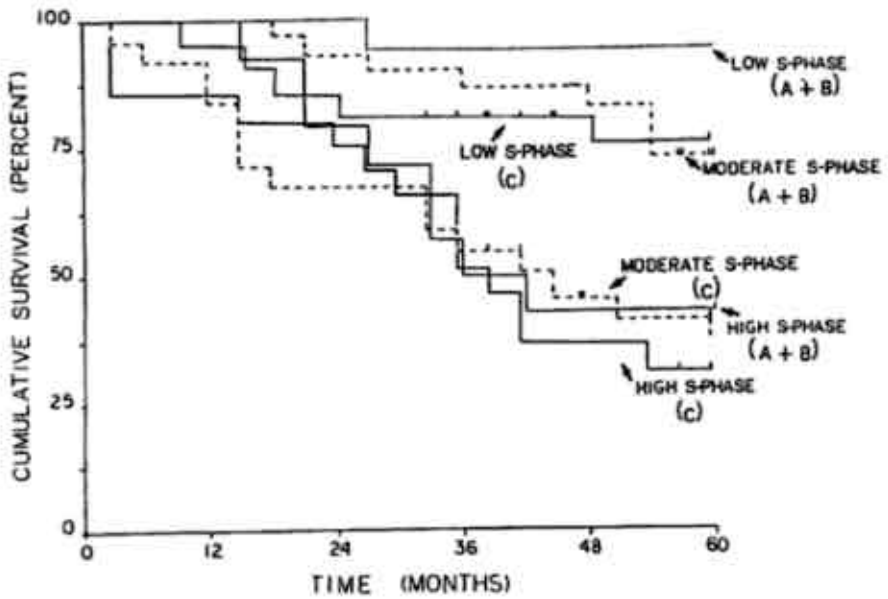


Is DNA Ploidy or %S Phase Prognostic in Colorectal Neoplasia? Some Say "Yes", Others "No"

Prognostic significance of DNA Ploidy in Colorectal Cancer in Relation to Risk for Recurrence or Overall Survival

Reference	Number of Cases	Probability
Witzig et al.	694*	0.001 (multivariate)
Harlow et al.	69	0.2 (multivariate)
Scott et al.	264	0.003 (multivariate)
Halvorsen et al.	149	ns[a] (multivariate)
Melamed et al.	33	ns
Schutte et al.	279	0.07 (univariable)
Emdin et al.	37	0.007 (univariable)
Bauer et al.	97	0.1 (multivariate)
Wolley et al.	33	nr[b]
Giaretti et al.	115	0.005 (multivariate)
Wiggers et al.	350	0.12 (univariable)
Quirke et al.	125	0.02 (univariable)
Rognum et al.	100	0.04 (multivariate)
Armitage et al.	326	ns (multivariate)
Kokal et al.	77	0.004 (univariable)

[a]ns = not significant; bnr = not reported; [c]colonic cancer only
[d]rectal cancer only



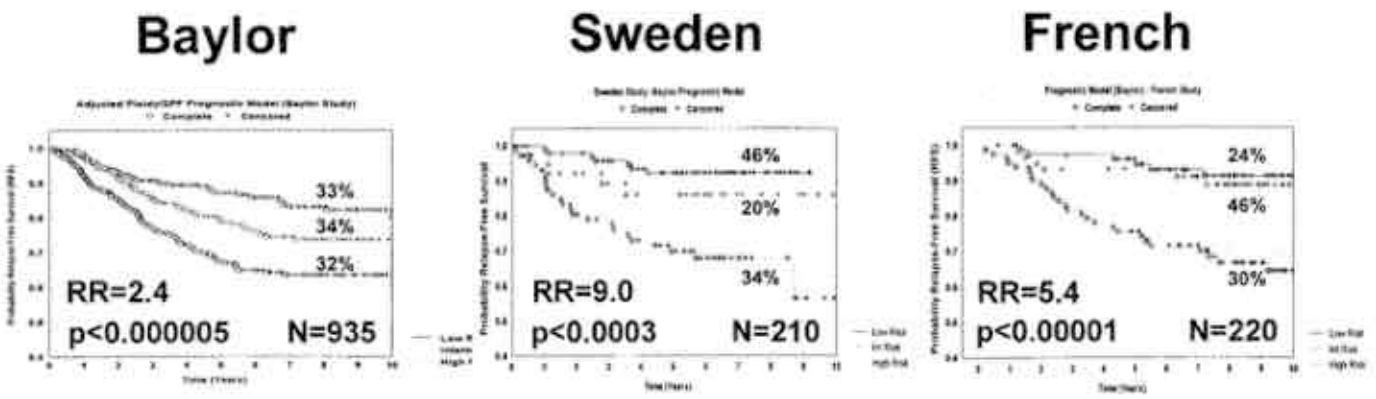
Summary data from 15 studies evaluating the importance of DNA ploidy in colorectal cancer

- 7/15 (47%) found no significant correlation ($p>0.05$)
- The rest found a high degree of correlation with either recurrence or overall survival

Survival curves for colon cancer cases stratified by S phase fraction and tumor grade showed a significant difference between low, moderate and high proliferative activity and survival

Bauer, K.D. (1993) Colorectal Neoplasia. In: Clinical flow cytometry, principles and applications. Edited by Bauer K.D. et. al. Williams & Wilkins, Baltimore. pp 307-317.

Prognostic Significance of S-Phase Fraction in Node-Negative Breast Cancer



- If the S phases are not adjusted for both the aneuploid fraction and the diploid normal dilution effects, there is a significant correlation between DNA ploidy and S phase fraction, 0.42, and a modest *p*-value
- Statisticians would normally either drop the S phase or the DNA ploidy in the analysis due to this correlation (usually the DNA ploidy was dropped)
- If DNA ploidy and S phase adjustment are applied, the correlation between DNA ploidy and S phase is reduced and the *p*-value becomes very significant

Bagwell et al., Cytometry, 46: 121-134, 2001
Updated at www.vsh.com/Presentations/BreastCAPrognosis.pdf

%S Phase for Prognosis in Node-Negative Breast Cancer? Conflicting Literature

Bagwell *et. al.* also tested 3 rules to the calculation of tumor %S phase fraction to improve the prognostic utility of this measurement in node negative breast cancer patients

Rule 1

Aneuploid fraction[1] effect: As the aneuploid fraction approaches zero there is a strong tendency for modeling software to over-estimate the aneuploid S phase fraction

- Assuming the reason for this bias is due to signal-to-noise, this rule uses a mathematical function to calculate the adjusted %S phase fraction in aneuploid histograms

Rule 2

When the percent aneuploid fraction is less than or equal to 5%, the adjusted %S phase fraction cannot be determined and the data is excluded from the relative risk assessment

- Diploid rescaling: in a DNA diploid histograms, the total %S phase calculated is a mixture of both normal and tumor %S phase

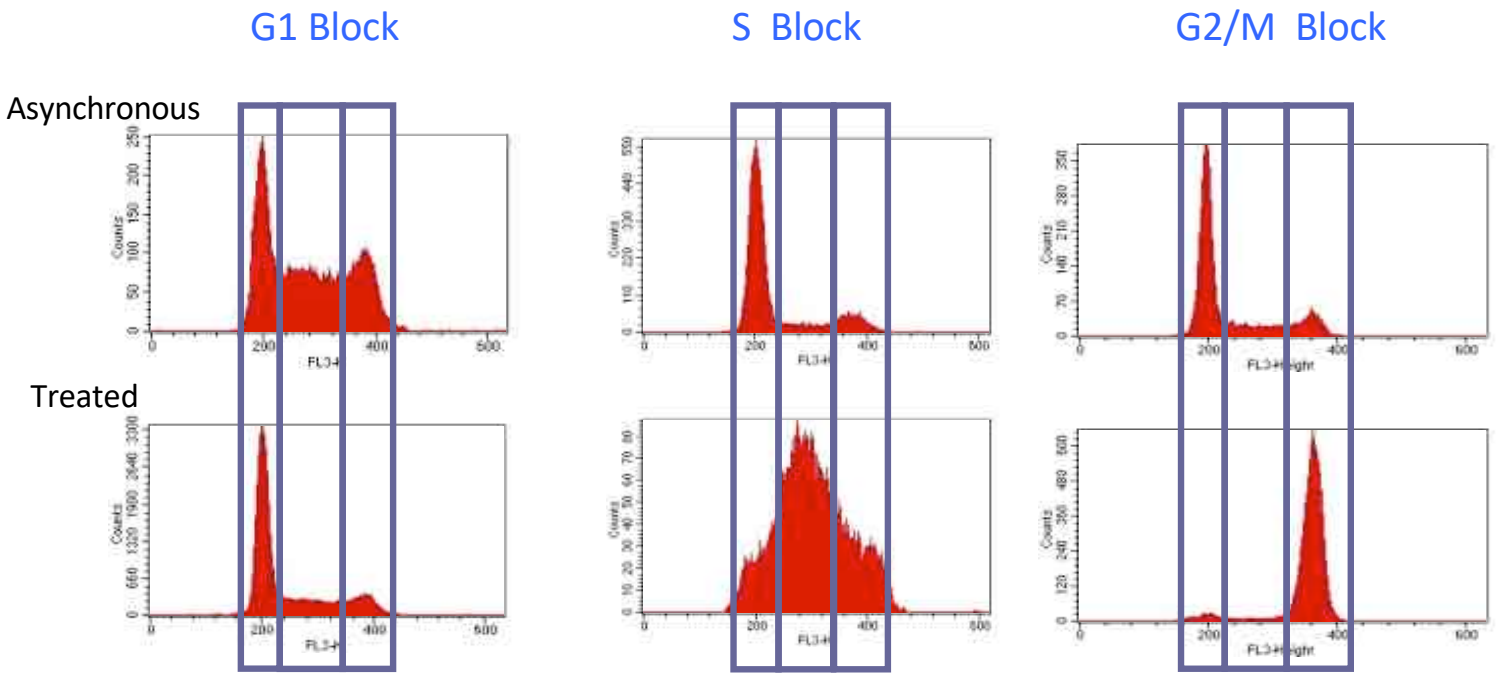
Rule 3

To estimate %S phase for a diploid tumor, observed %S phase (combined result for tumor plus stromal cells) is divided by the tumor fraction, if known from an independent marker

- If tumor fraction is not known, it can be crudely estimated as 50%

Bagwell et al., Cytometry, 46: 121-134, 2001. Updated at www.vsh.com/Presentations/BreastCAPrognosis.pdf

Visualising Synchronisation by FCM

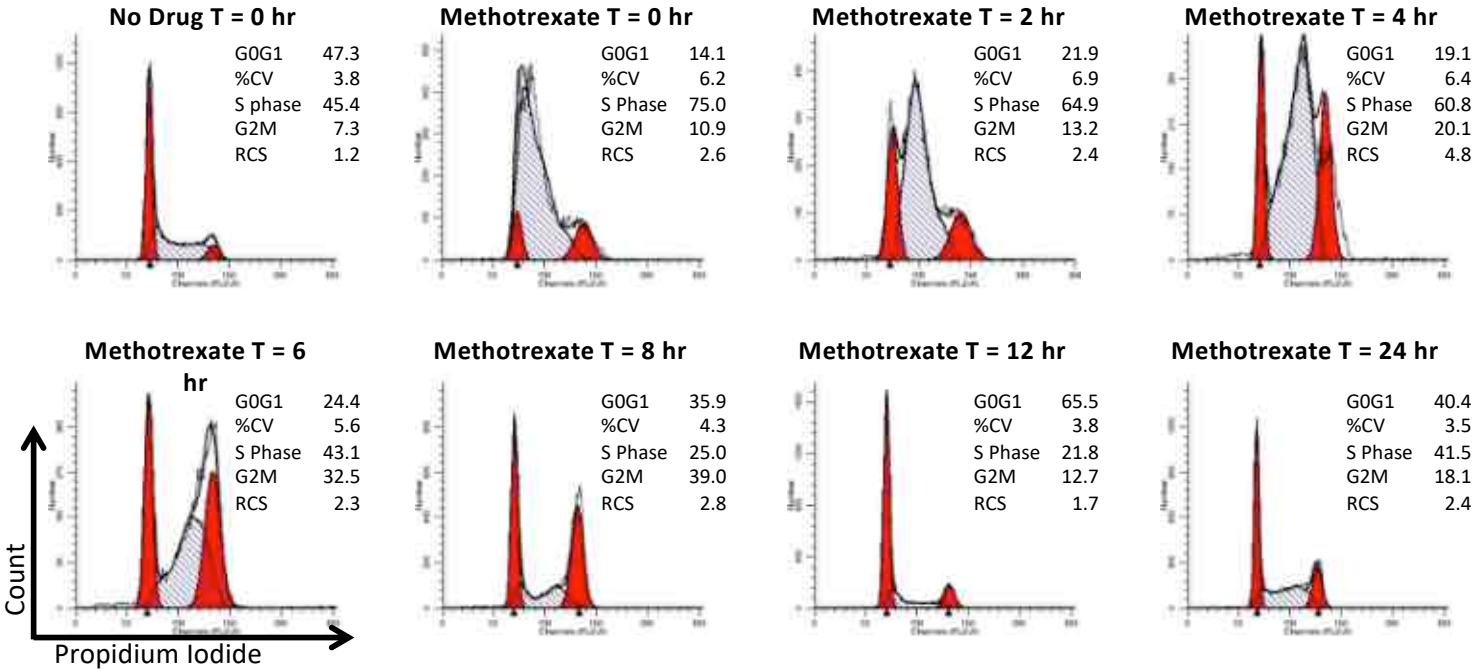


Cells stained with PI to visualise DNA content

Data courtesy of Derek Davies, London Research Institute

Modeling Synchronized Cell Distributions

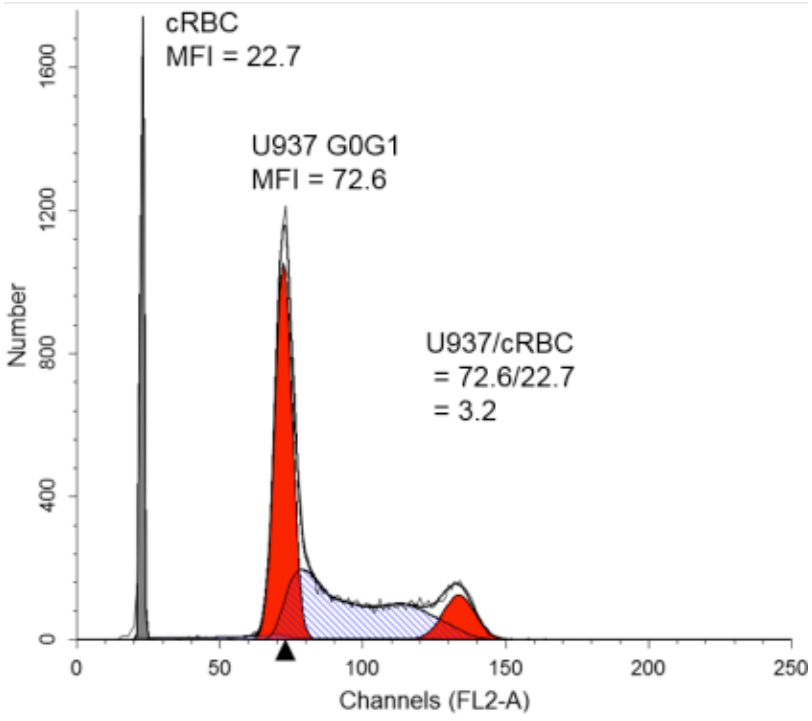
- U937 cells were synchronized by incubation with methotrexate for 16 hours. The cells were then washed with complete medium and sampled at the indicated time points pos-synchronization.
- The G0G1 peak position was fixed at the position seen in the untreated control (assumes tube to tube consistency)
- The G2/G0G1 ratio was predetermined using normally cycling control material and fixed at 1.89
- Using the ModFit's Synchronization wizard:
  - The G0G1 position and SD were allowed to float
  - For fitting flexibility 5 equally spaced rectangles were used to model S phase



Use of an In-tube Staining Standard to Assess Consistency of G0G1 Peak Position

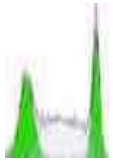
<u>Internal Controls</u>
Chick RBCs
Trout RBCs
Normal Lymphocytes

DNA Content
Human Diploid cells ~6 pg
Trout RBCs ~5.5 pg
Chick RBCs ~2.5 pg
E. coli ~0.02 pg



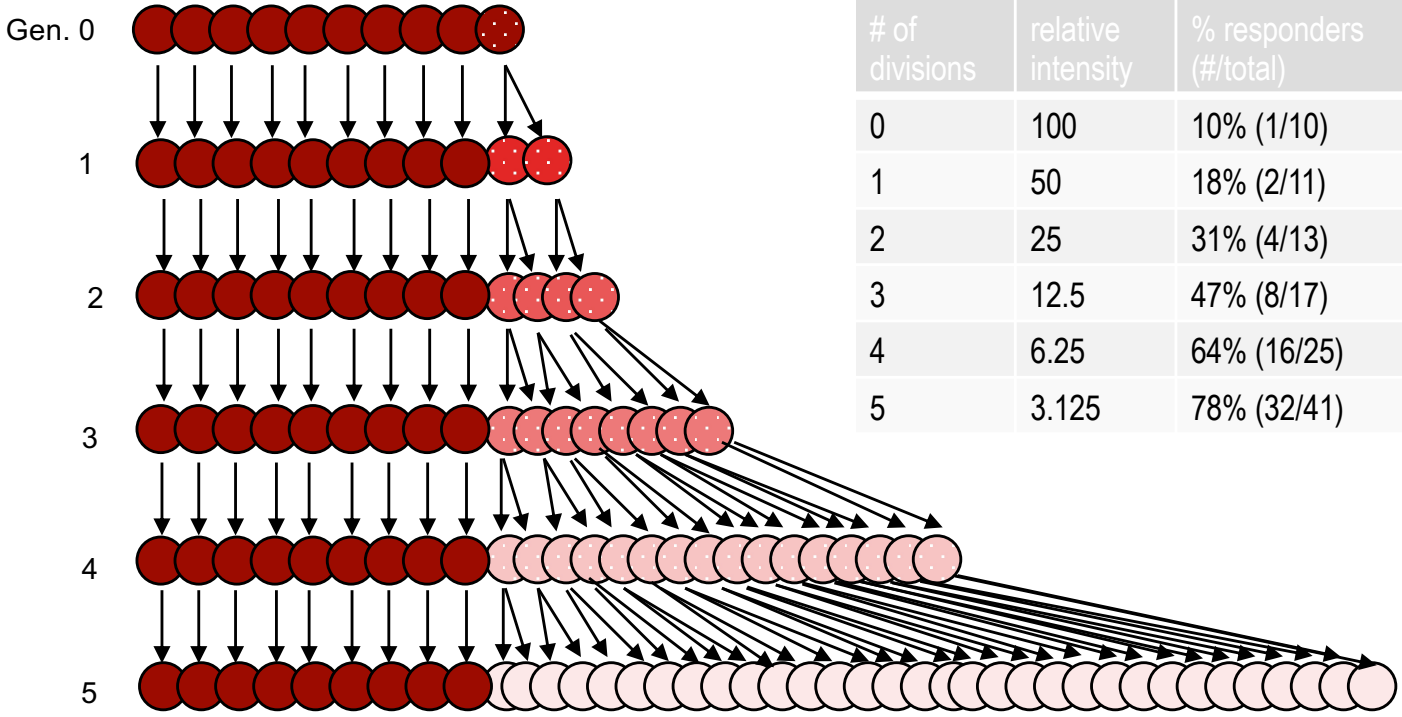
THE PLAN

- Cell Cycle, Dyes & Techniques
  - Doublets- slow is the way to go
  - Data Analysis
    - Simple analysis
    - Modelling
  - Clinical Significance of Aneuploidy & S phase
  - Modelling Synchronized Populations
- Tracking Dye Dilution
  - The dyes - many choices
  - Modelling Dye Dilution Data
  - Tracking Applications



Dye Dilution Proliferation Assay: Principles

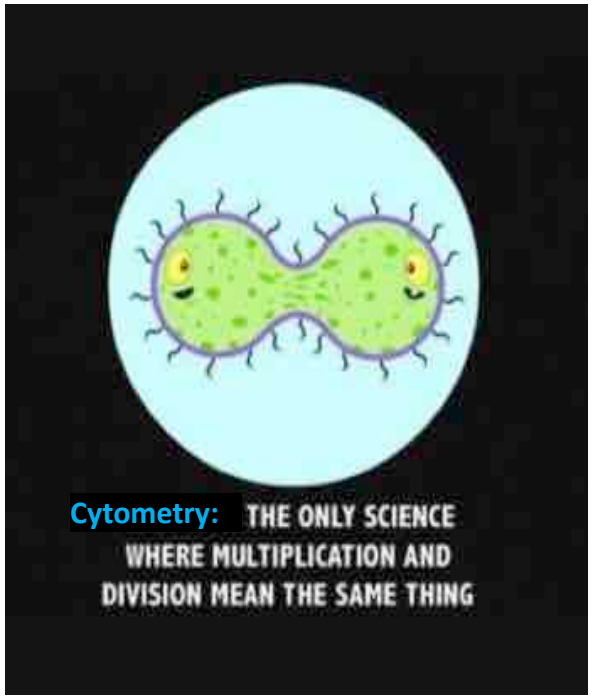
- Label starting population with bright, stable, non-toxic dye that distributes approximately equally between daughter cells at each division
- Monitor dye intensity profile at later time(s) to estimate extent of cell division based on 1) proportion of cells with decreased fluorescence intensity and 2) extent of intensity decrease



# of divisions	relative intensity	% responders (#/total)
0	100	10% (1/10)
1	50	18% (2/11)
2	25	31% (4/13)
3	12.5	47% (8/17)
4	6.25	64% (16/25)
5	3.125	78% (32/41)

Adapted from Givan *et al.* (2004) Methods Mol Biol, 263: 109–124

Key Assumptions for Cell Division Monitoring based on Dye Dilution



- Decrease in fluorescence intensity is proportional to increase in cell number

  $\Rightarrow$ constant intensity ratio from generation to generation (ideal = 0.5)
- Decrease in fluorescence intensity reflects only cell division

  $\Rightarrow$ loss of dye due to other biological processes (e.g., apoptosis, necrosis, protein turnover or export, membrane transfer) must be excluded when analyzing dye dilution

Stable Labels for Cell Division Monitoring

TYPE A: Random protein labeling dyes (prototype = CFSE)

Advantages:

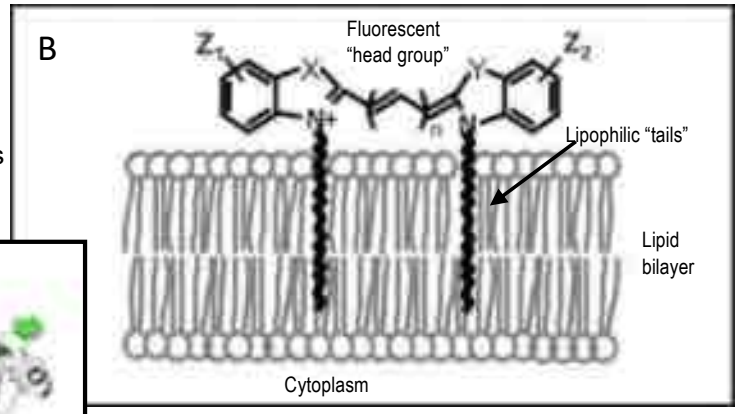
Rapid, high intensity labeling for any cell type

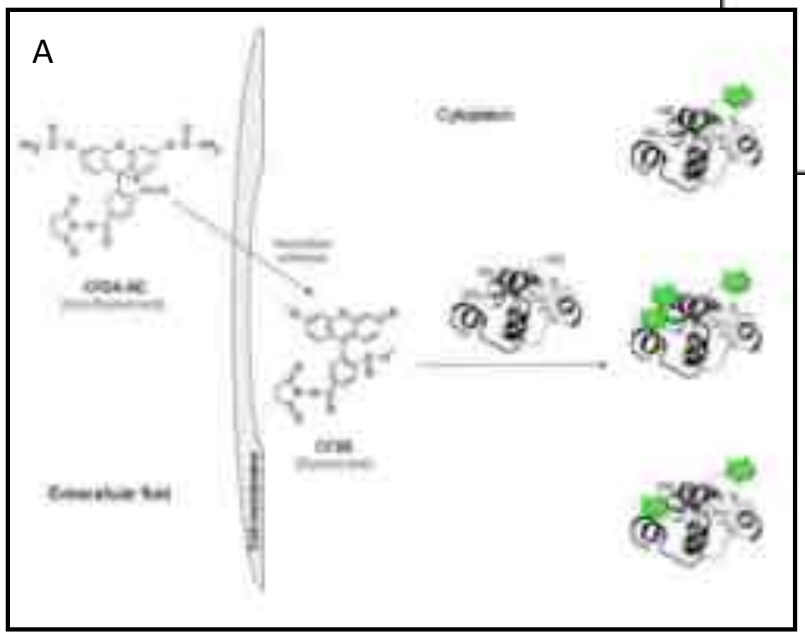
Stable covalent bond between dye and protein

Disadvantages:

Too much dye can alter protein function(s)

>50% of dye lost soon after labeling as short-lived proteins turn over and damaged proteins are cleared





TYPE B: General membrane labeling dyes (prototype = PKH26)

Advantages:

Rapid, high intensity labeling for any cell type

Lipid labeling less likely to alter protein function

No early dye losses

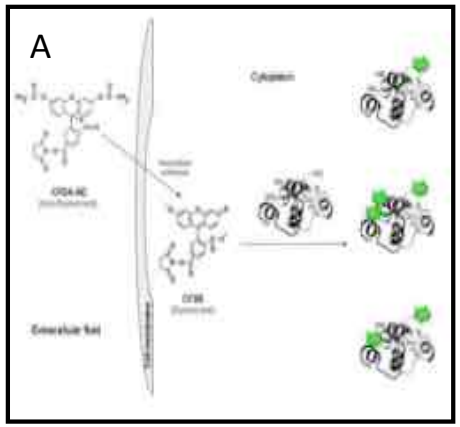
Disadvantages:

Too much dye can alter membrane integrity

Dye is not covalently bound to membrane (retention is through hydrophobic interactions)

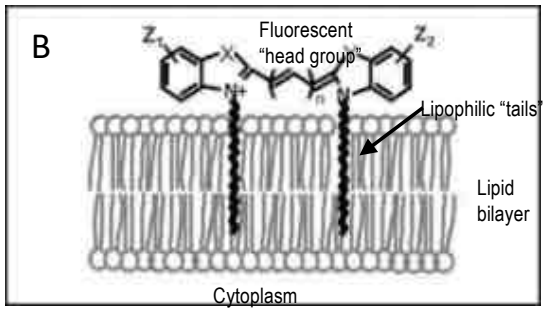
Wallace *et al*. (2008) Cytometry 73A: 1019-1034

General Protein Labeling Dyes



Dye	Emission max., nm	Useful laser lines, nm
Fully characterized in published studies		
CellTrace™ Violet	450	405
CFSE	525	488
CPD eFluor® 670	670	633 - 647
Emerging/preliminary studies		
CytoPainter Blue	454	405
CytoTell™ Blue	450	405
CytoTrack™ Blue	454	405
CPD eFluor® 450	450	405
VPD™ 450	450	405
CytoTell™ Green	525	488
CytoTrack™ Green	525	488
Oregon Green SE	518	488
CellTrace™ Far Red DDAO-SE	659	633 - 647
CellTrace™ Far Red	661	633 - 647

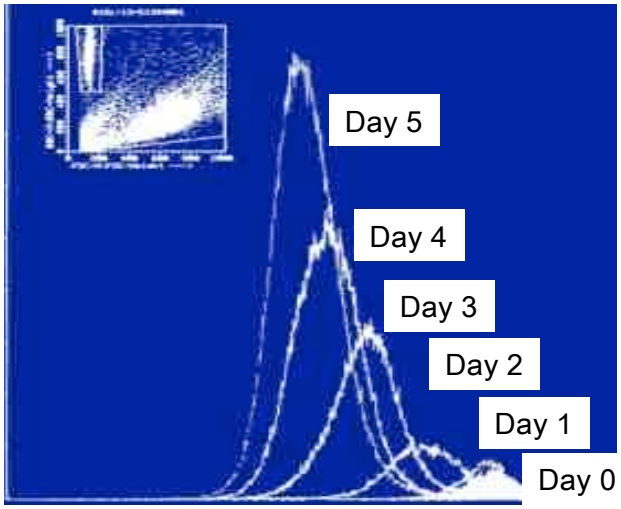
General Membrane Labeling Dyes



Dye	Emission max., nm	Useful laser lines, nm
Fully characterized in published studies		
CellVue® Lavendar	461	405
PKH2	504	488
PKH67	502	488
PKH26	567	488, 514, 543
CellVue® Plum	671	633 - 647
CellVue® Claret	675	633 - 647
CellVue® NIR780	776	780
CellVue® NIR815	814	780
Emerging/ preliminary studies		
CellVue® Lilac	460	405
CytoID Green	527	355, 488
CytoID Red	583	457, 561

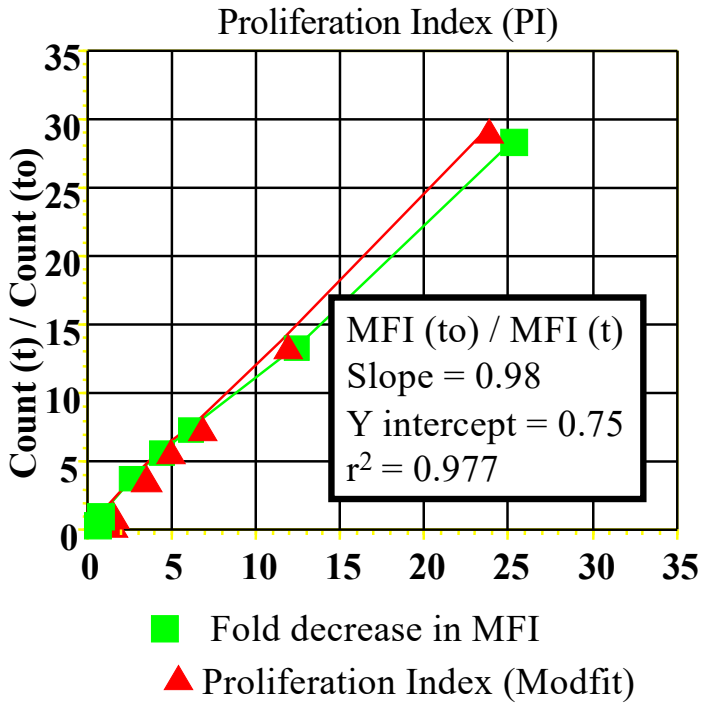
Does PKH26 Dye Dilution Track Increase in Cell Number in Simple Systems? YES

Continuously dividing 8E5LAV cell line



Yamamura *et al*. (1995)
Cell. Mol. Biol. 41 (Suppl. 1): S121-132

Continuously dividing U937 cell line

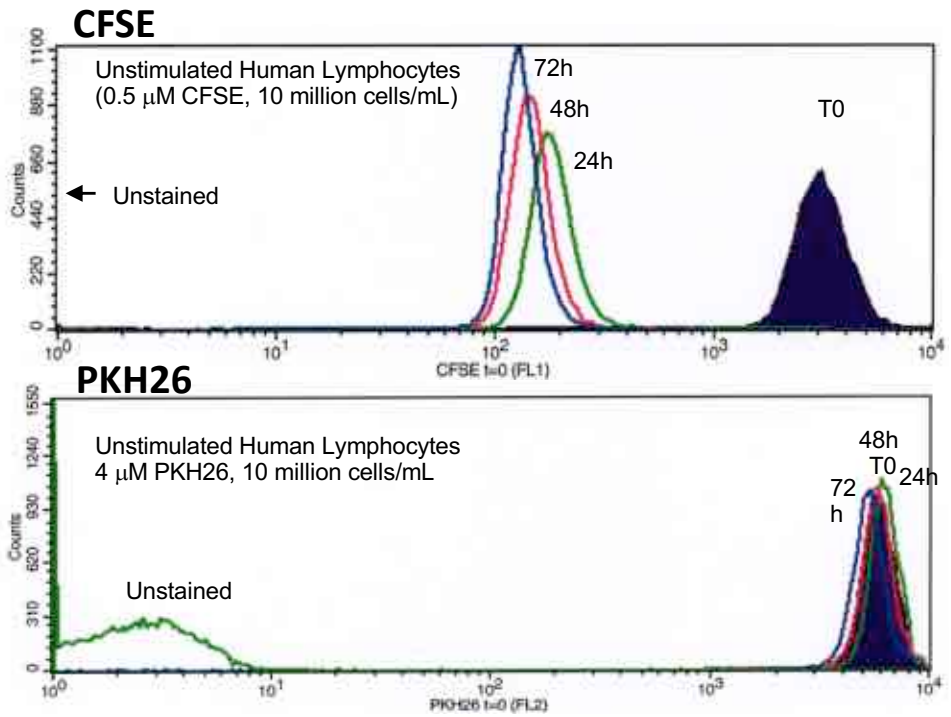


Data collected during 2001 Annual Course on Clinical Applications of Cytometry (Dartmouth Medical School)

Critical Issues for Dye Dilution Assays: Overview

Goal	Protein-reactive dyes (e.g., CFSE)	Membrane dyes (e.g., PKH26)
	DIFFERENCES	
Bright, homogeneous, stable staining of parent population	Rapid mixing matters Dye uptake affected by cell size and/or esterase activity Allow ~24h for intensity stabilization; T=0 NOT a good biological or compensation control	Rapid mixing is even more important than with protein dyes Dye uptake affected by cell size Stable initial intensity; T=0 OK as biological or compensation control
	SIMILARITIES	
No effect on cell function(s)	Must be verified for each system	
Appropriate Instrument setup	Linearity of Intensity scale Color compensation	
Data acquisition/gating	Exclude dead cells and contaminating cell types (e.g. monocytes) Accumulate enough cells	
Data analysis matched to study goals	% (non-)proliferating Proliferation or Stimulation Index Precursor Frequency	

Critical Controls:
Dye Positive but Unstimulated



Wallace & Muirhead Immunol.Invest. 36:527–561 (2007)
(Data courtesy of D. Bantly and J. Moore, Univ. of Pennsylvania)

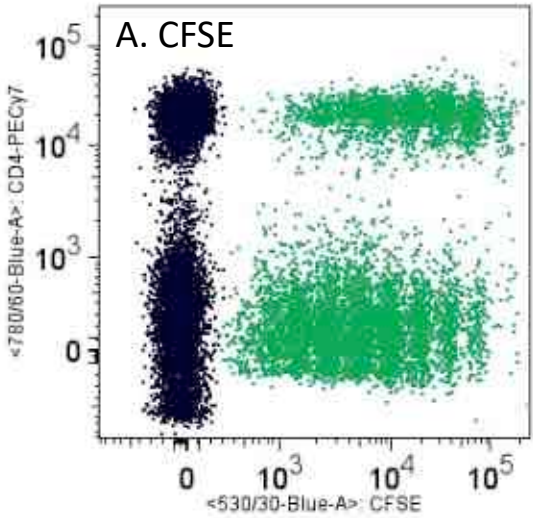
Unstimulated cells stained with proliferation tracking dye are used for:

1) Instrument setup (brightest sample*)

2) Color compensation (brightest sample*)

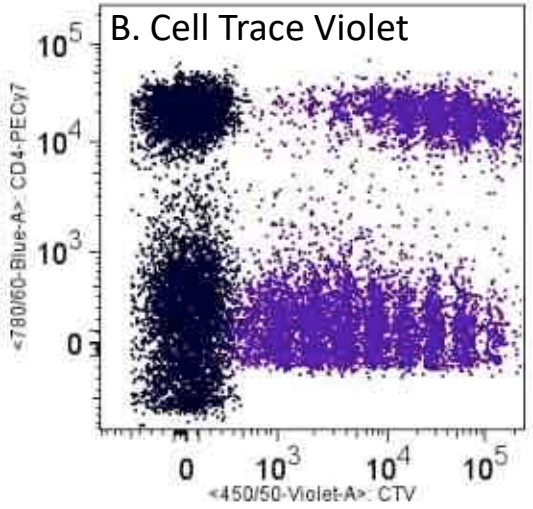
3) Biological negative control (confirm intensity of non-responders)

Cell Division Monitoring with Protein Dyes: Differential T Cell Responses to Mitogen (Con-A)



Viable (PI^{neg}) CD3+CD4+ mouse splenocytes

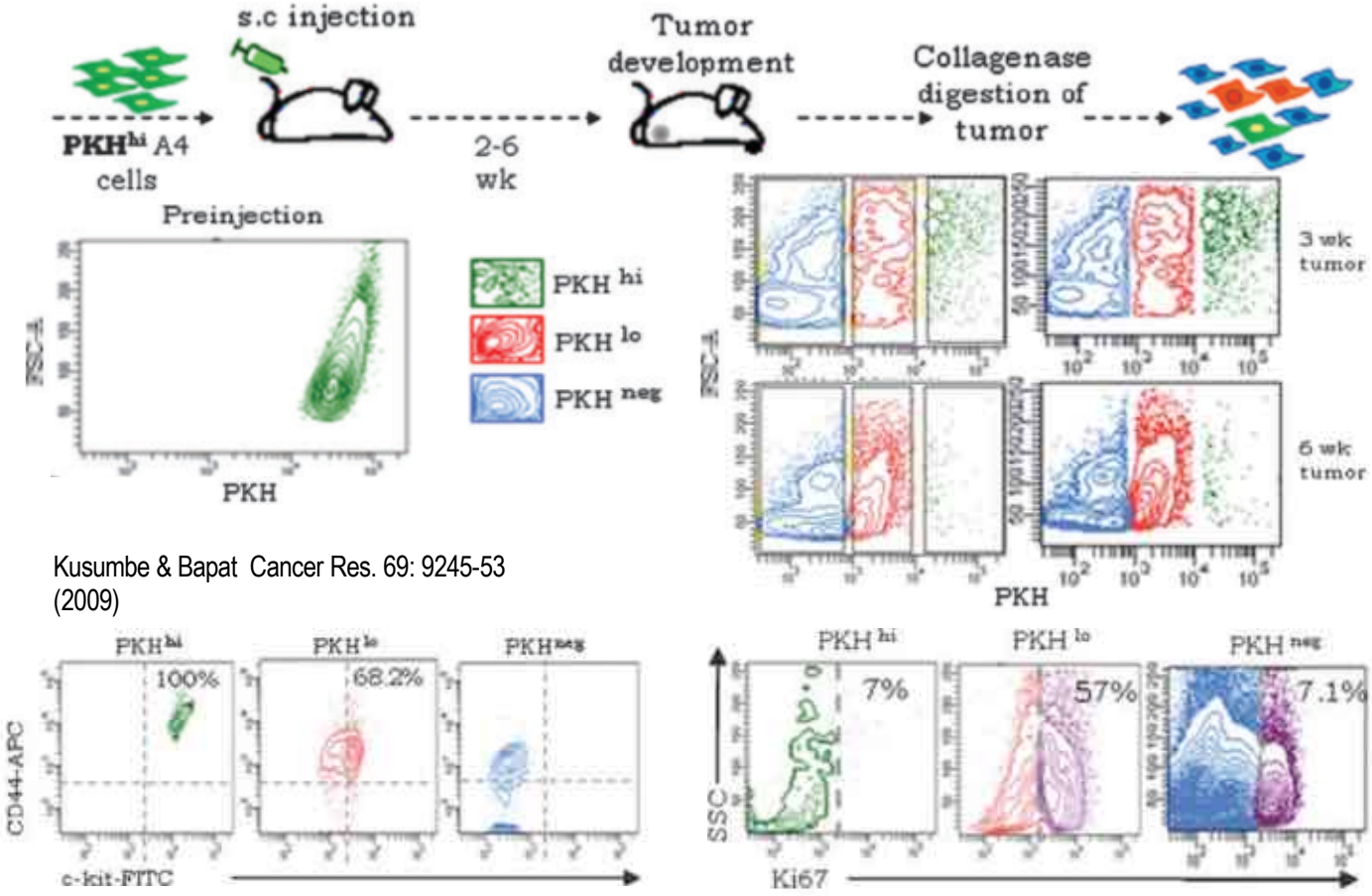
- No tracking dye
- CFSE
- CTV



Ancestry Subset Value Type For	Live Cells
No Proliferation Dyes Unstim	46.7
No Proliferation Dyes + ConA	46.5
CFSE Unstimulated	39.7
CTV Unstimulated	44.9
CTV ConA Stimulated	44
CFSE ConA Stimulated	34.9

Data courtesy of K. Price,
Malaghan Institute for Medical Research

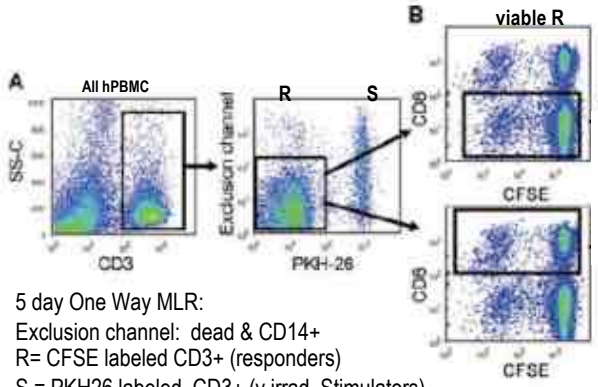
(Non)Proliferation Tracking: Finding Tumor Stem Cells *In Vivo*



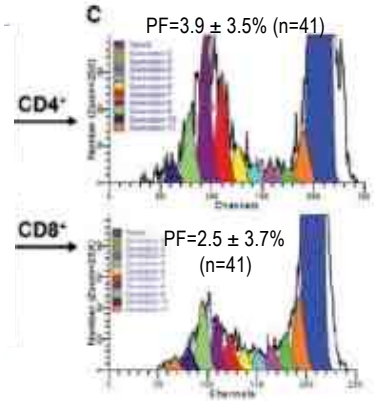
Kusumbe & Bapat Cancer Res. 69: 9245-53 (2009)

Take Home Message: No Single“Best” Dye, Many Good Options (and Combinations)

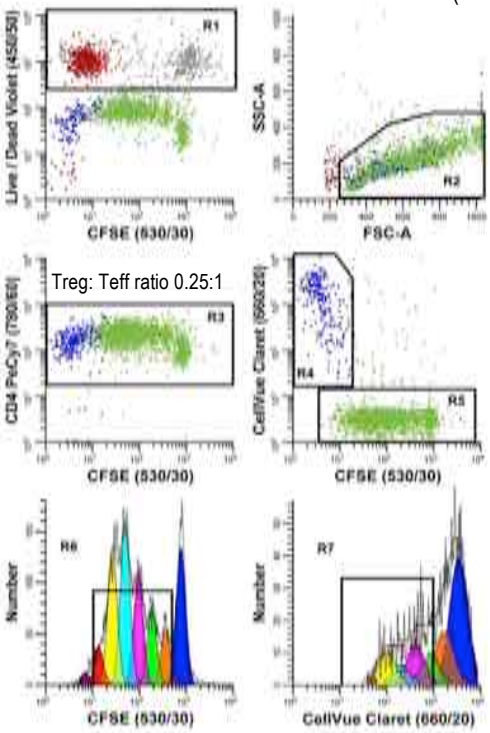
Macedo et al. (2009) Am. J. Transpl. 9: 2057-2066



5 day One Way MLR:
Exclusion channel: dead & CD14+
R= CFSE labeled CD3+ (responders)
S = PKH26 labeled CD3+ (γ irrad. Stimulators)



Tario et al. Methods Mol Biol: 699: 119-164 (2011)



Green = viable Teff (CFSE+ LDFV−)
Blue = viable Treg (CellVue Claret+ LDFV −)
Red-brown = irrad. accessory cells (CFSE − Claret − LDFV −)
Gray = non-viable Teff (CFSE − LDFV+)
Red = non-viable Treg (CellVue Claret − LDFV +)

“Let Us Count the Ways…”

Methods for reporting/comparing extent of proliferation based on dye dilution profiles:

Type 1 -- “Eyeball” methods

a) Trend(s) in antigen expression across generations
b) % (non-)proliferating cells
c) Stimulation Index

Type 2 -- Proliferation Profile deconvolution

a) Proliferation index
b) Precursor frequency

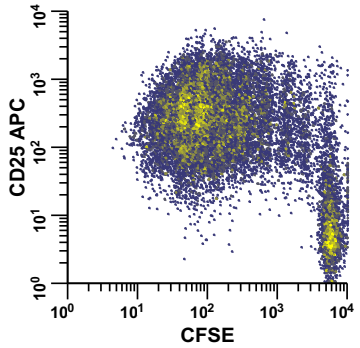
Do CD4+25- or CD4+CD25+ T cells Proliferate Faster?

Antigen expression *vs*. generation number

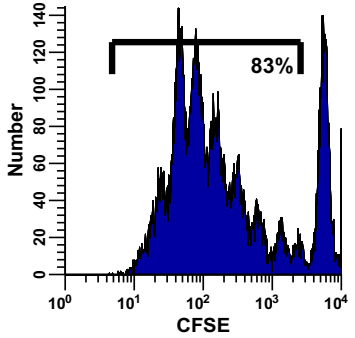
Proliferative Fraction

CD4+CD25- Fraction

A

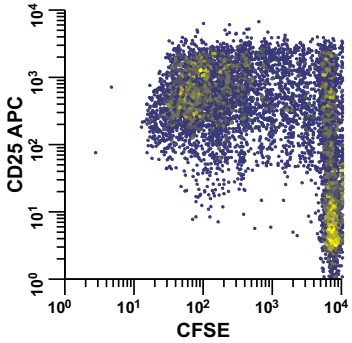


B

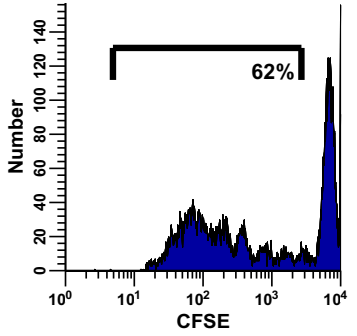


CD4+CD25+ Fraction

C



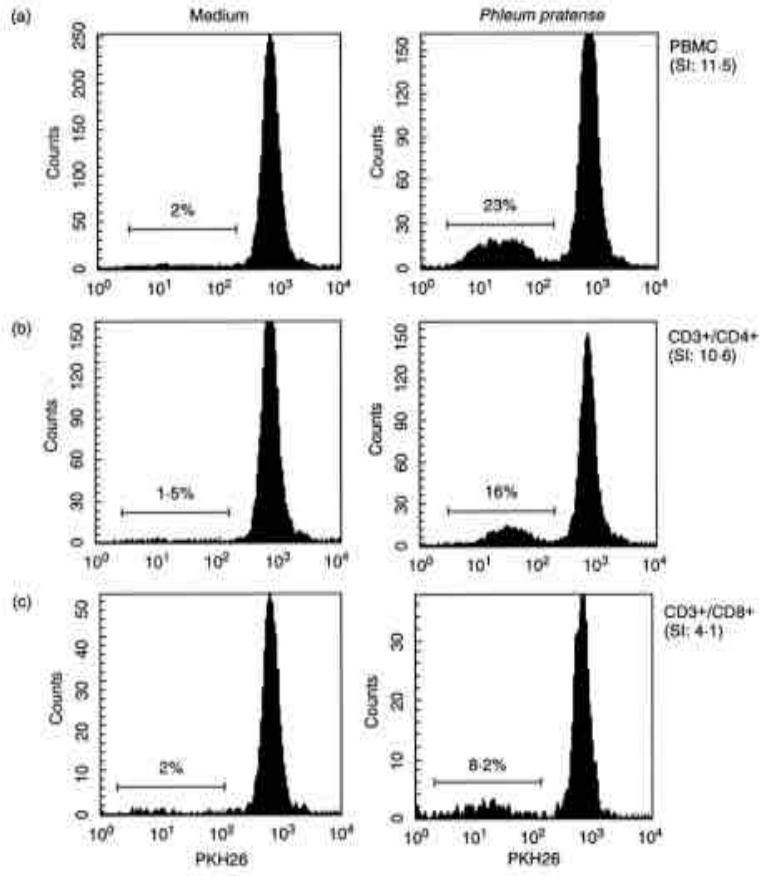
D



Data courtesy of Dr. Feng Qian (RPCI). Mononuclear cells were separated into CD4+CD25+ and CD4+CD25- fractions using magnetic bead fractionation (Miltenyi Biotec) and stained with CFSE (5 μM, 1 x 10^7 cells/mL), then cultured for 7 days with anti-CD3 and anti-CD28.

Wallace *et al*. (2008) Cytometry A 73(11): 1019-1034

Tracking Antigen Driven Responses by Subset: Stimulation Index



Methods:

Stain 10^6 PBMC/mL with 1.25 µM PKH26

- Culture 10^5/well for 11 days with *P. pratense*, PPD or TT (medium change at day 5-7)
- Counterstain with FITC-CD3 and PC5-CD4 or PC5-CD8

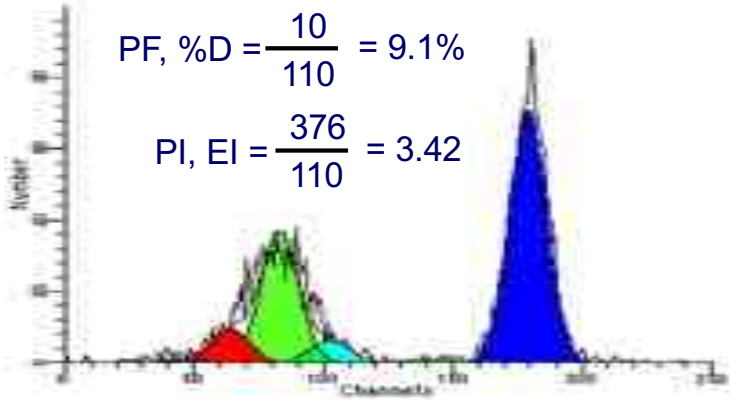
SI = % proliferating cells (+ stim.) / % proliferating cells (unstim.)

Conclusions:

- Increased SI in CD3+CD4+ cells from grass pollen sensitive atopic patients than non-allergic controls or non-grass pollen sensitive atopics
- Increased SI in CD3+CD8+ cells in only half of grass pollen sensitive pts.
- CD3+CD4+ precursor frequencies similar for *P pratense,* PPD and TT

Rimaniol *et al.* (2003)
Clin. Exp. Immunol. 132: 76-80

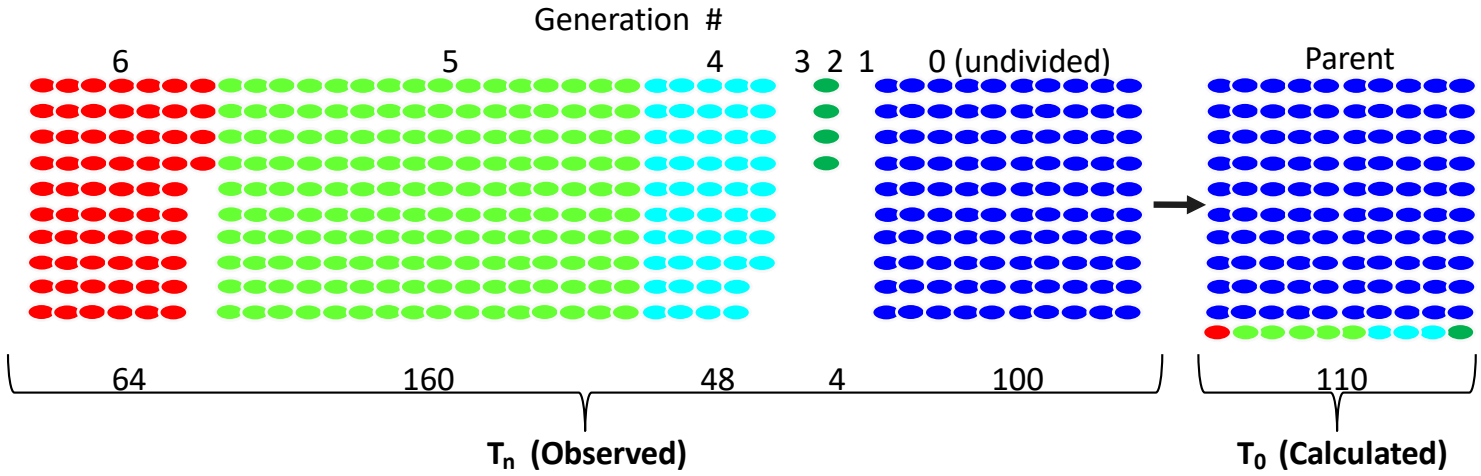
Curve Fitting For Dye Dilution Analysis: Principles



PKH26

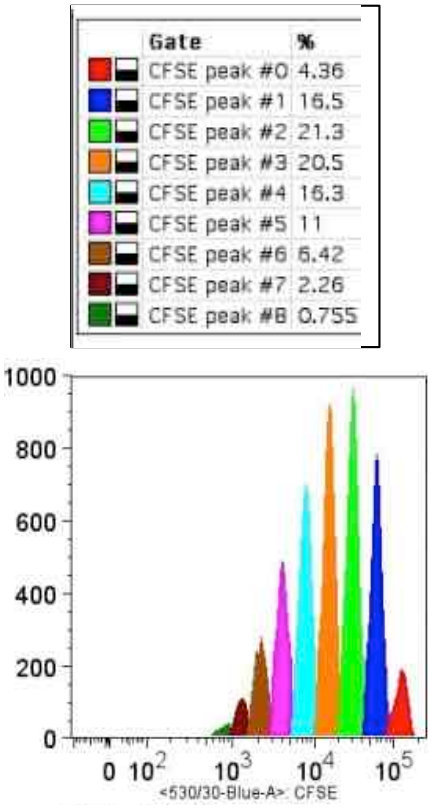
Precursor Frequency (PF) or % Divided (%D) = calculated frequency of responder cells present in parental population at T0

Proliferation Index (PI) or Expansion Index (EI) = fold expansion during assay period (ratio of final cell count to calculated starting cell count)



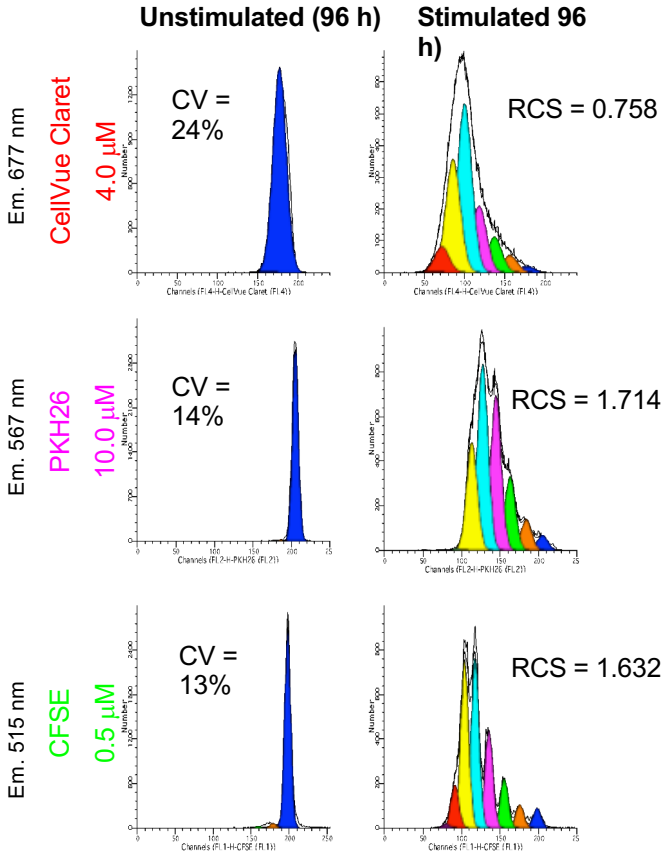
Proliferation Modeling Statistics

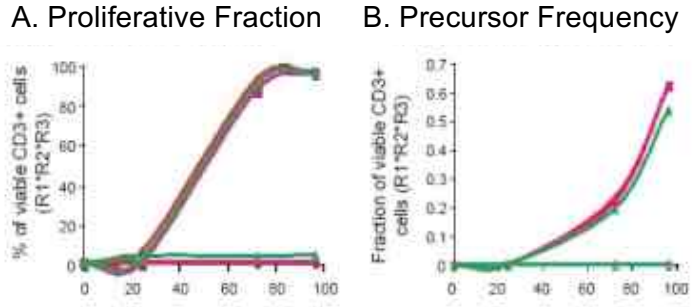
Statistic	Software	Interpretation
Precursor Frequency	ModFit FCS Express FlowJo	Probability that a cell will divide at least once
Proliferation Index Expansion Index	ModFit FCS Express FlowJo	Fold expansion during culture (ratio of final cell count to starting cell count)
Division Index Replication Index	FCS Express FlowJo	For the responding cells the fold expansion during culture
Proliferation Index	FlowJo	For the responding cells, the average number of divisions they will undergo



Adapted from: Roederer, M. Cytometry A. 79: 95-101, (2011)

Are Visible Peaks Required for Accurate Cell Division Analysis Using Dye Dilution?



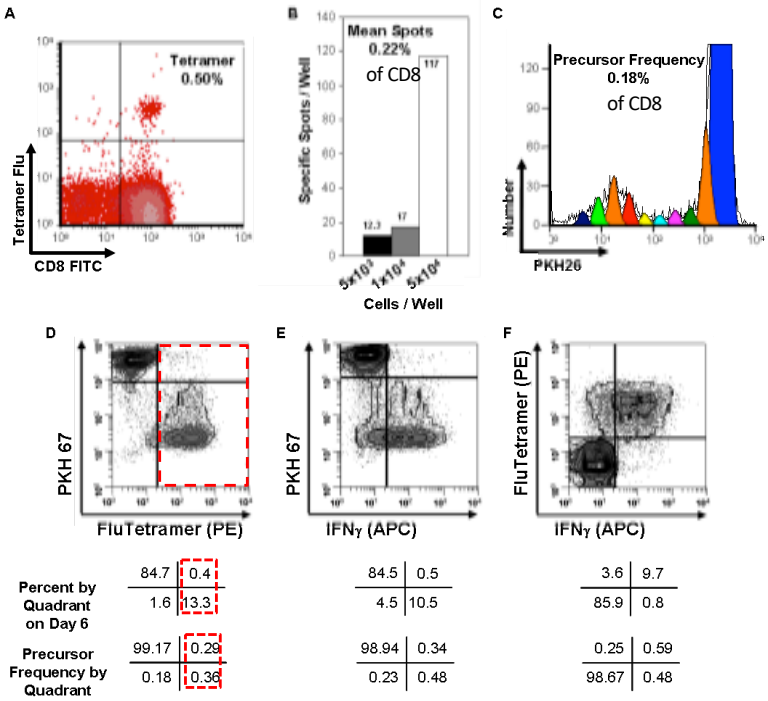


Methods: human PBMC stimulated with anti-CD3 + IL-2

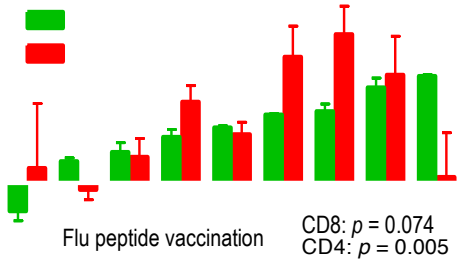
Bantly *et al*. Immunol. Invest. 36: 581-605 (2007)

Cell Division Monitoring in More Complex Systems: Not All Cells Able to Bind Antigen Go On to Proliferate

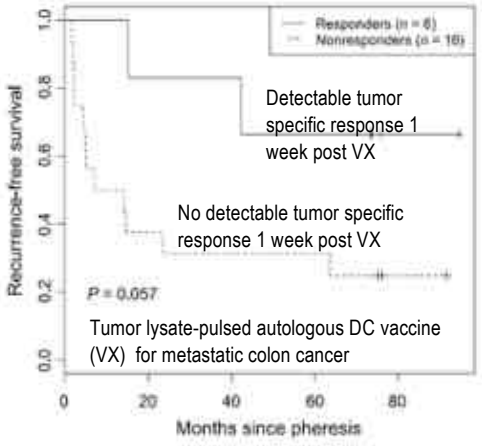
Bercovici et al. J. Immunol. Methods 276: 1-13 (2003)



Wallace *et al*. (2008) Cytometry 73A: 1019-1034



Barth *et al*. *Clin. Cancer Res.* 16: 5548-5556 (2010)



Roswell Park Cancer Institute