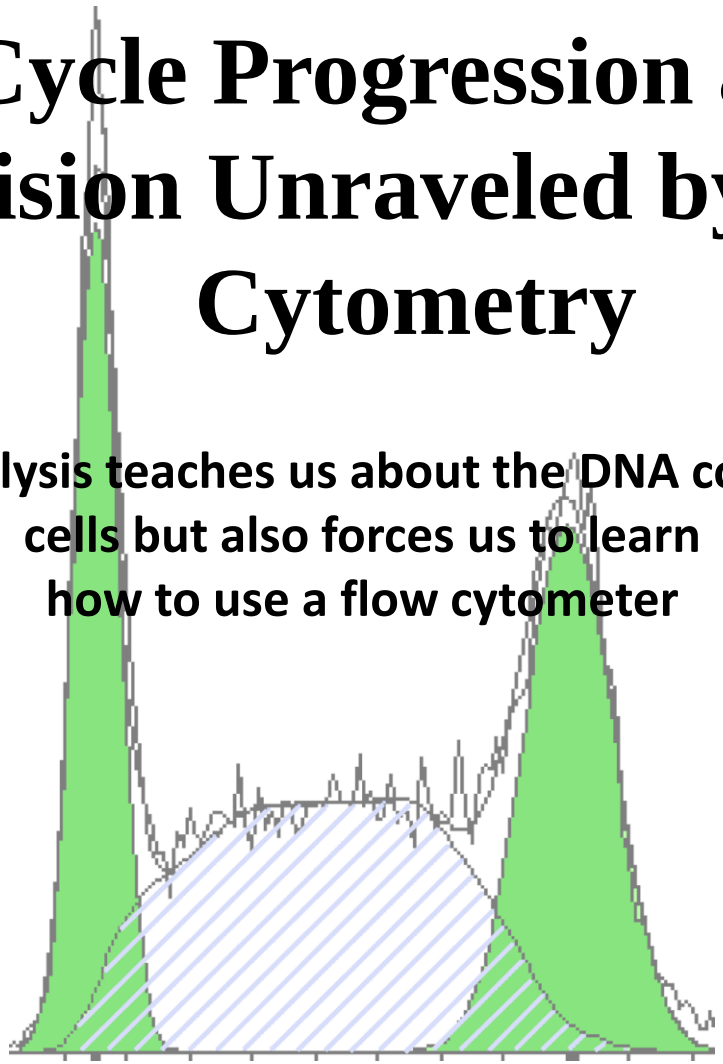
# Cell Cycle Progression and Cell Division Unraveled by Flow Cytometry

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



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ISAC International Society for Advancement of Cytometry
ISAC LETF
Prague Cytometry Workshop 2019
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# Acknowledgements


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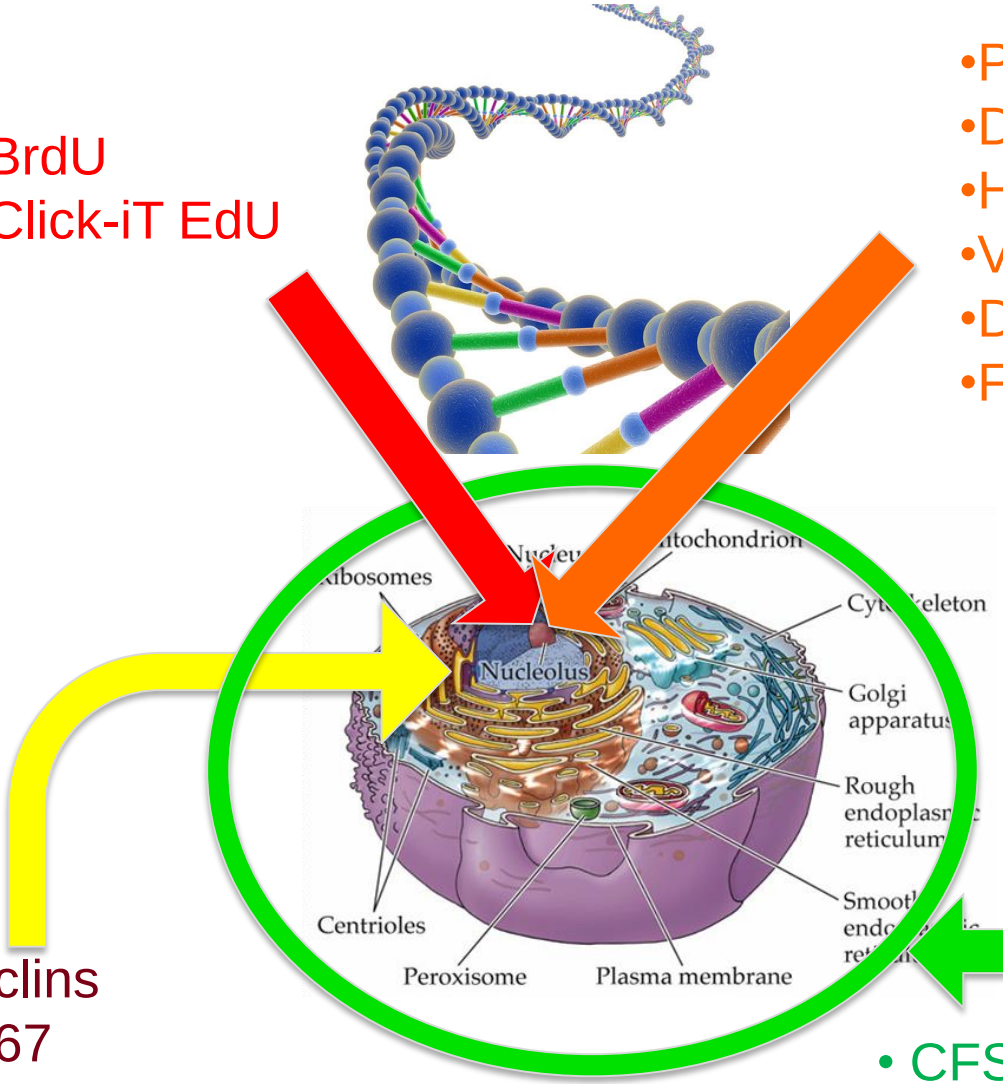
- Bruce Bagwell, Chris Bray, Mark Munson, Don Herbert (Verity)
- Alice Givan, Jan Fisher, Mary Waugh (Dartmouth)
- Lizanne Breslin, Brian Gray (PTI Research)
- Annual Course participants through the years

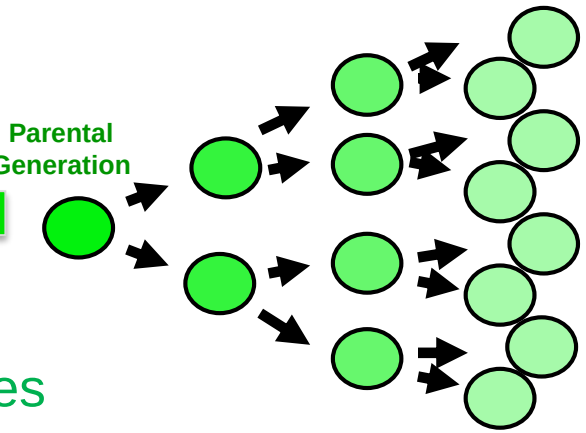

# How Can You Measure Cell Division by FCM?

- BrdU
- Click-iT EdU

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




- Cyclins
- Ki-67
- PCNA
- H3 Phosphorylation

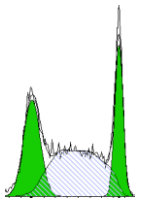



- CFSE
- Violet Dyes
- CellVue Dyes
- PKH Dyes

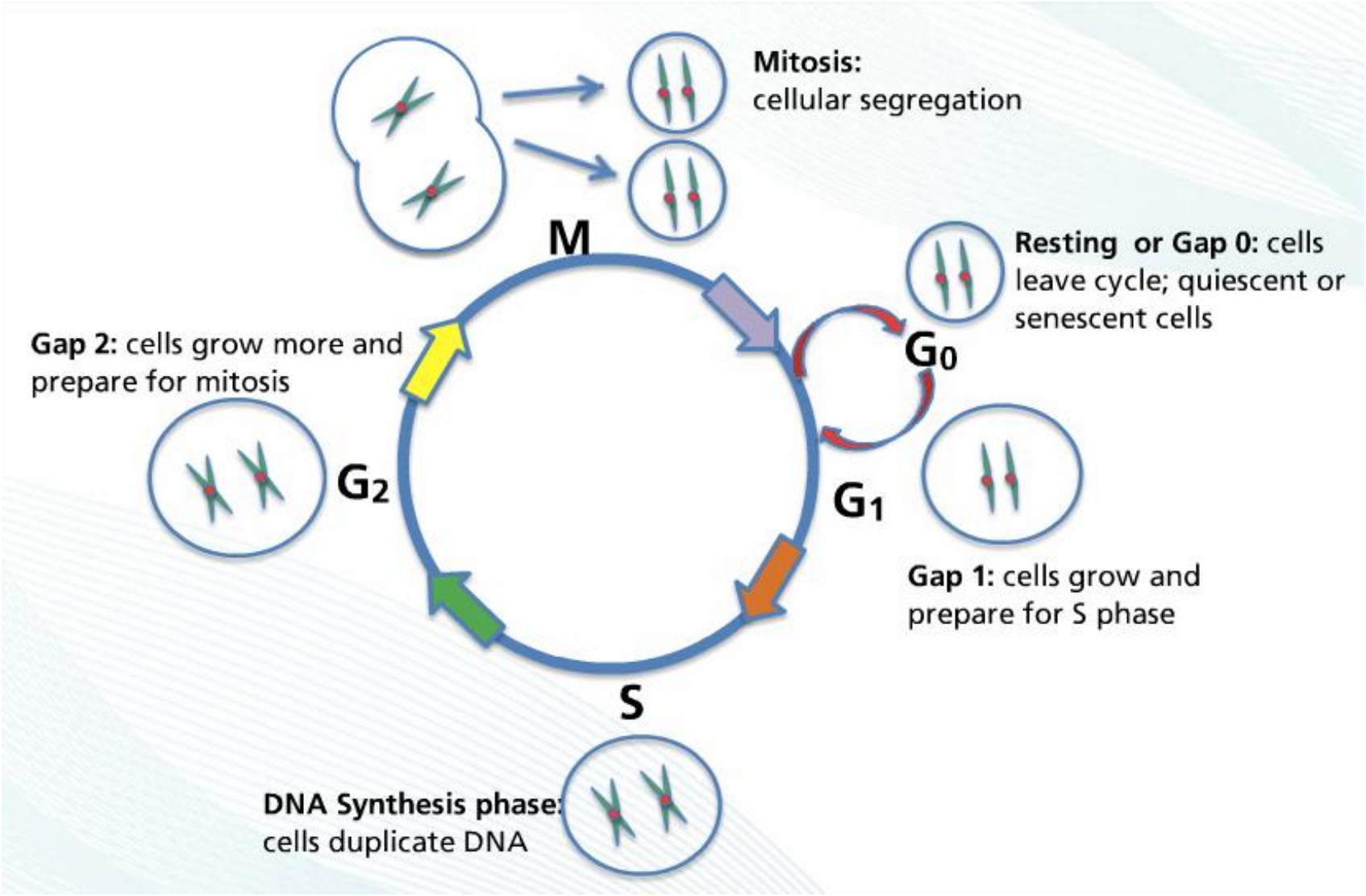
# THE PLAN

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





# Four Phases of The Cell Cycle



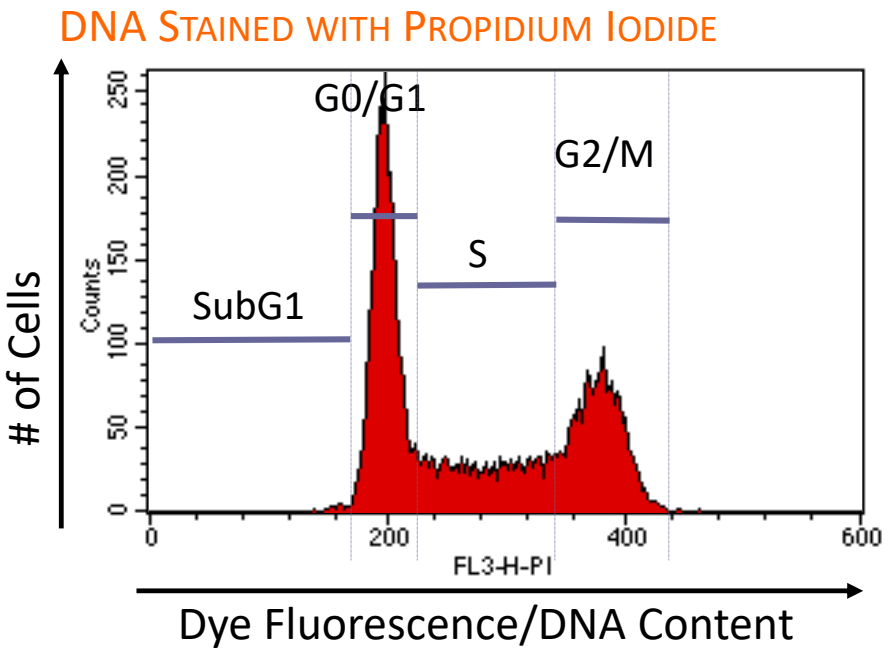
# DNA Content Changes During Cell Cycle

- 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)

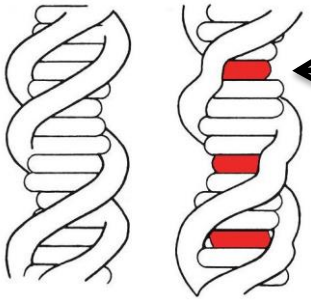
- Fluorescence data used to generate DNA histograms

- The DNA histogram gives a static picture of the proportion of cells in different phases of the cell cycle



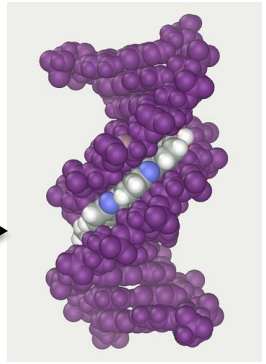
# DNA Binding Dye Characteristics

• **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

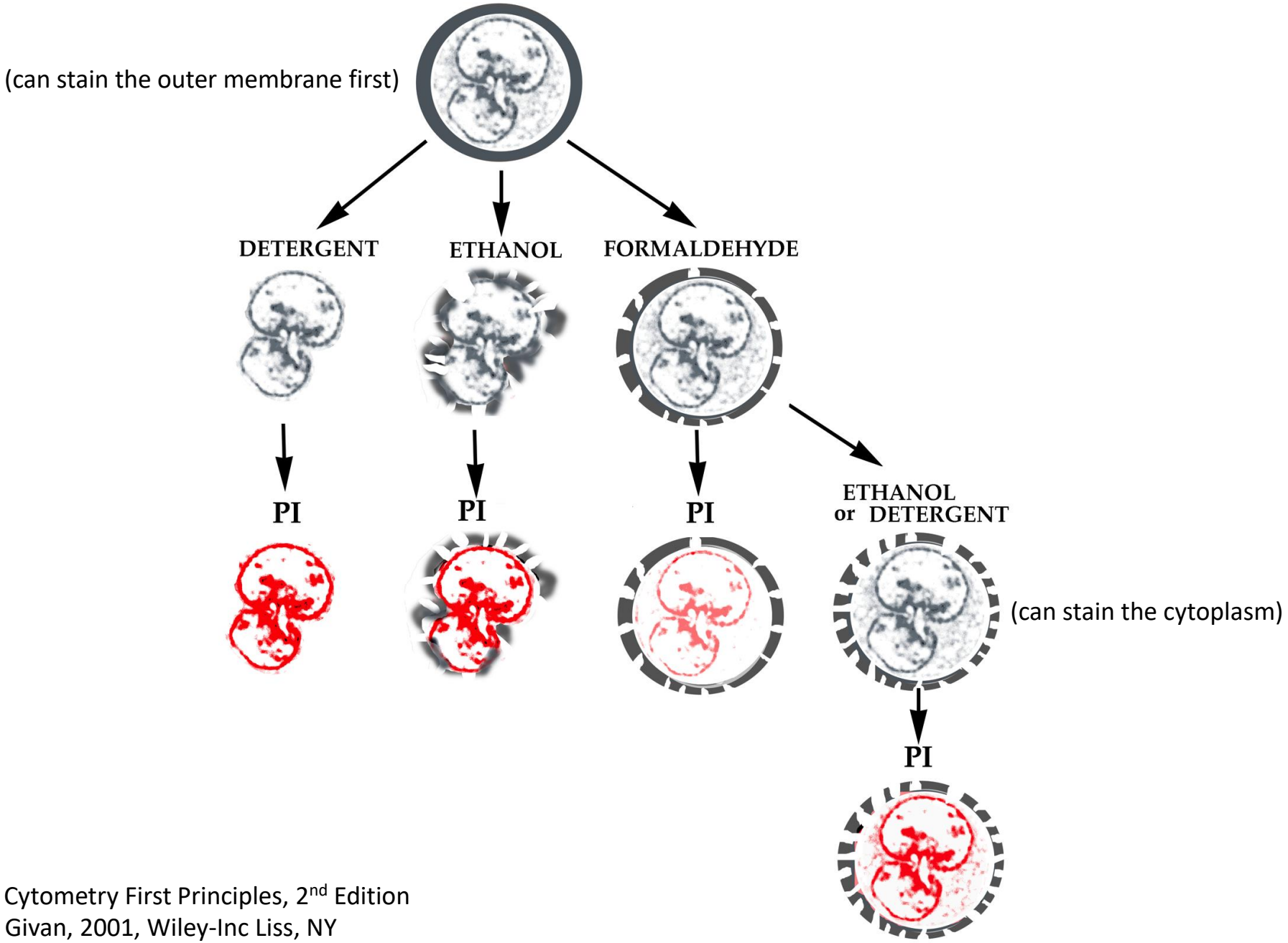
• **PERMEANT** (a.k.a. VITAL) – pass straight through intact cell membrane

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

• **NUCLEIC ACID SPECIFICITY:**

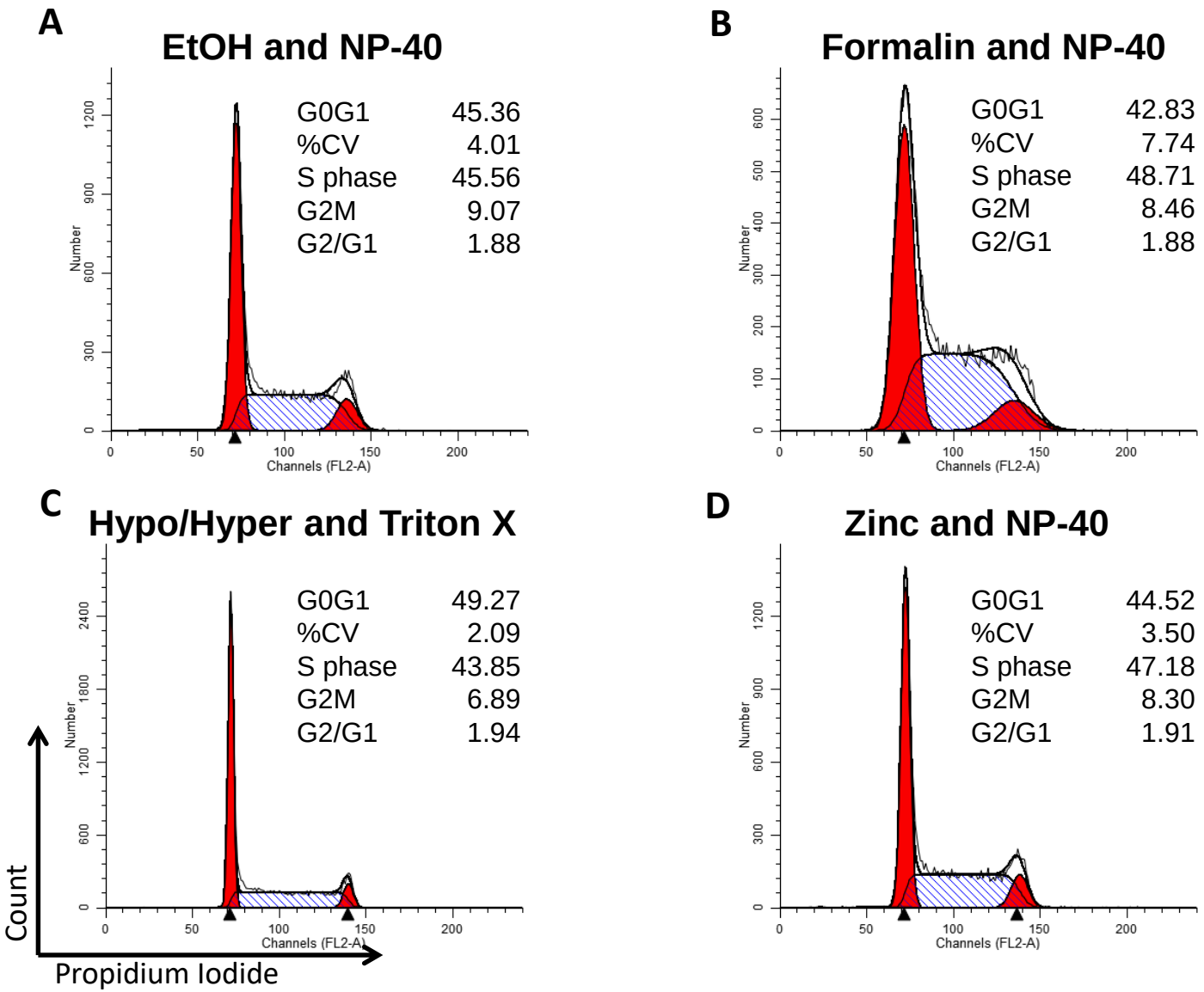
| **dsDNA and dsRNA** | **dsDNA only (nucleic acid preference)** |
|---|---|
| – Propidium Iodide (PI) | – 7-AAD (G-C) |
| – SYTOX® | – Hoechst 33342 (A-T) |
| – TOTO or TO-PRO | – DAPI (A-T) |
| – DRAQ 5 | – Vybrant ® DyeCycle |

# Fixation and Permeabilisation

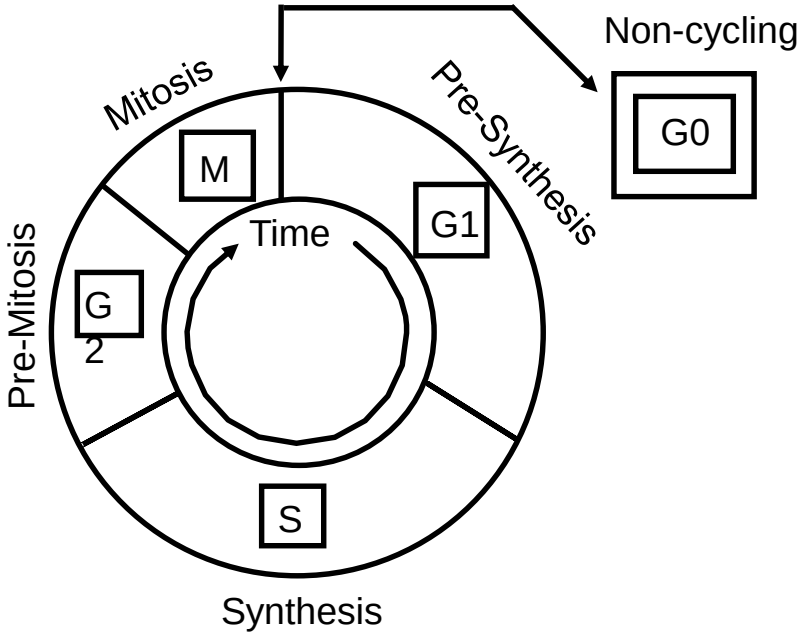


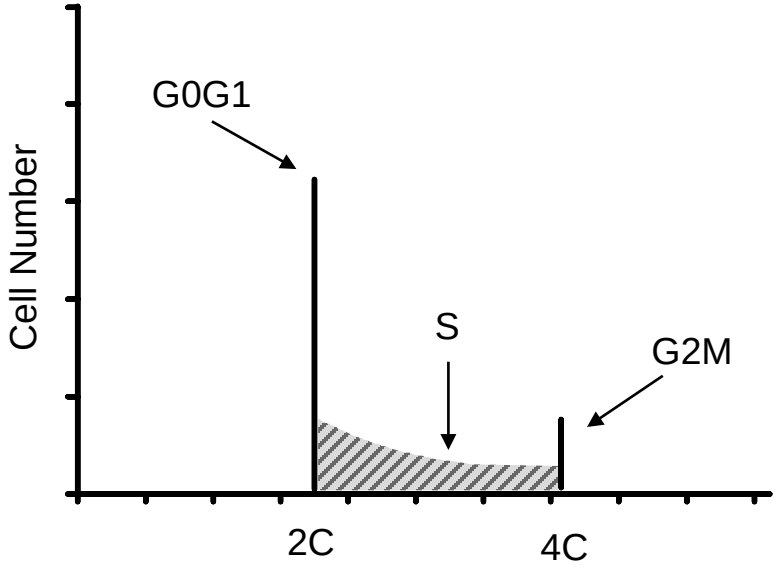

Flow Cytometry First Principles, 2nd Edition
Alice Givan, 2001, Wiley-Inc Liss, NY

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

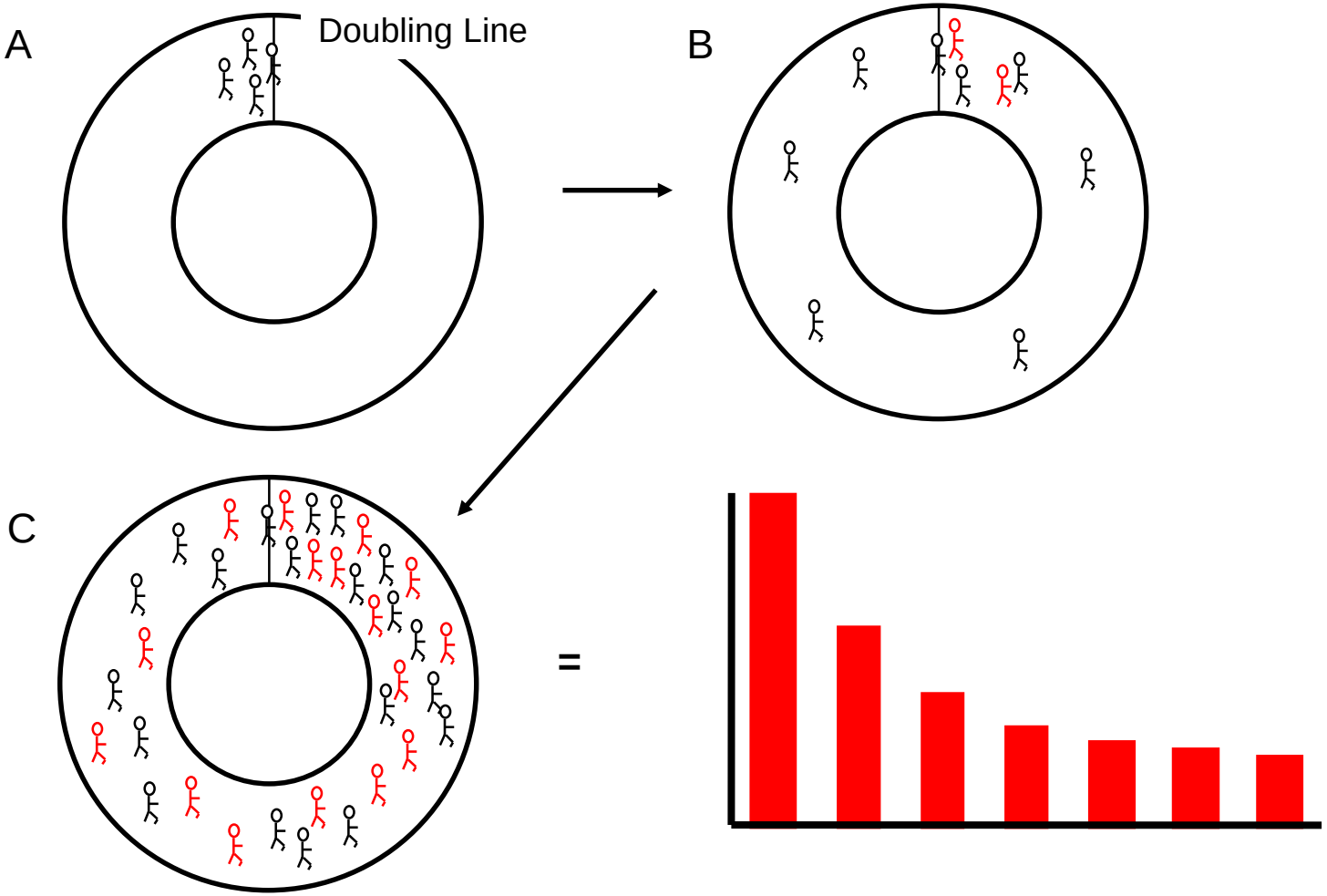


# CONCEPT 1: DNA VS TIME IN CYCLE



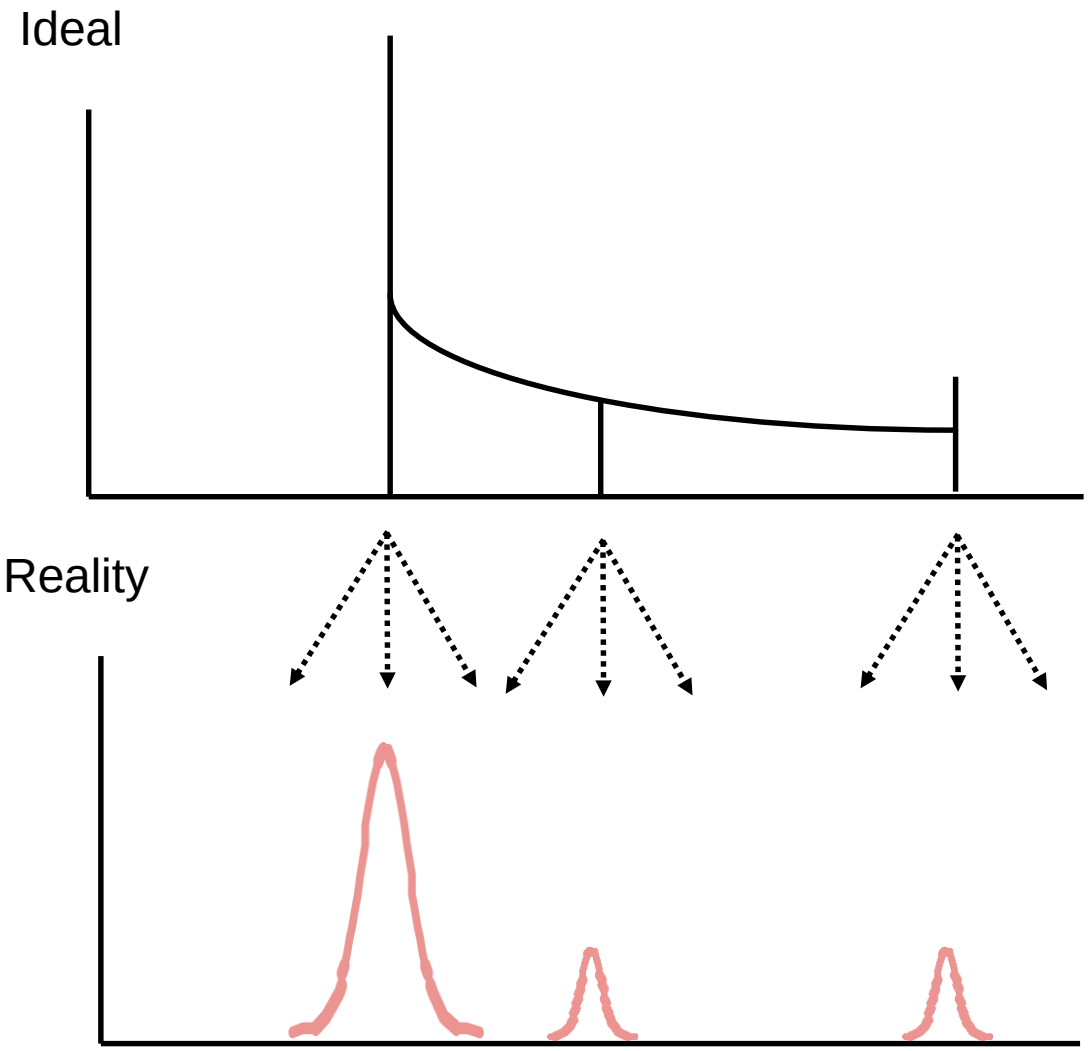



# CONCEPT 2: CELL NUMBER VS CELL AGE

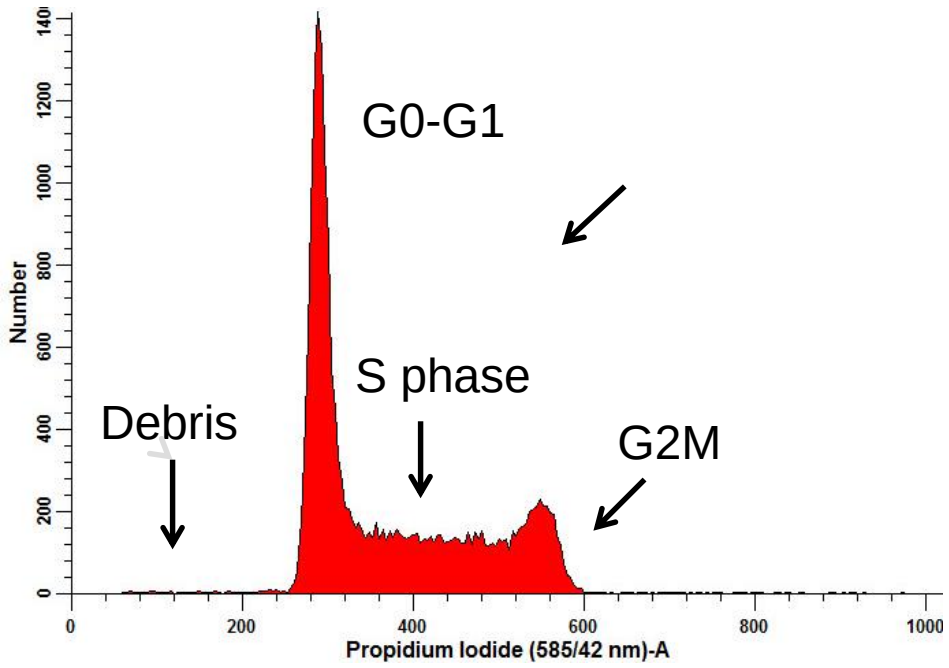



Bagwell, Bruce. Personal communications

# Concept 3: Signal Broadening



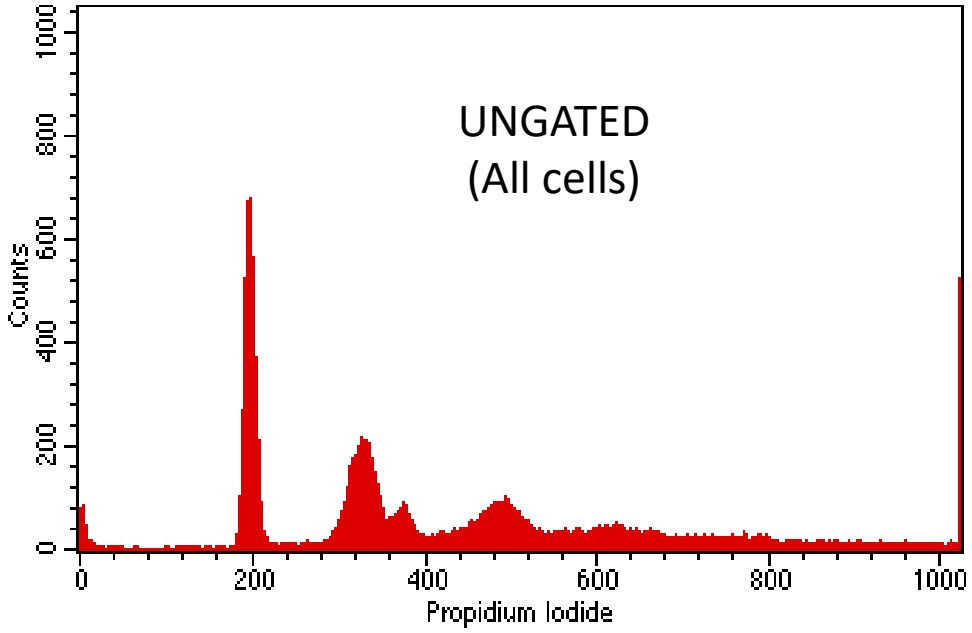
# The DNA Histogram

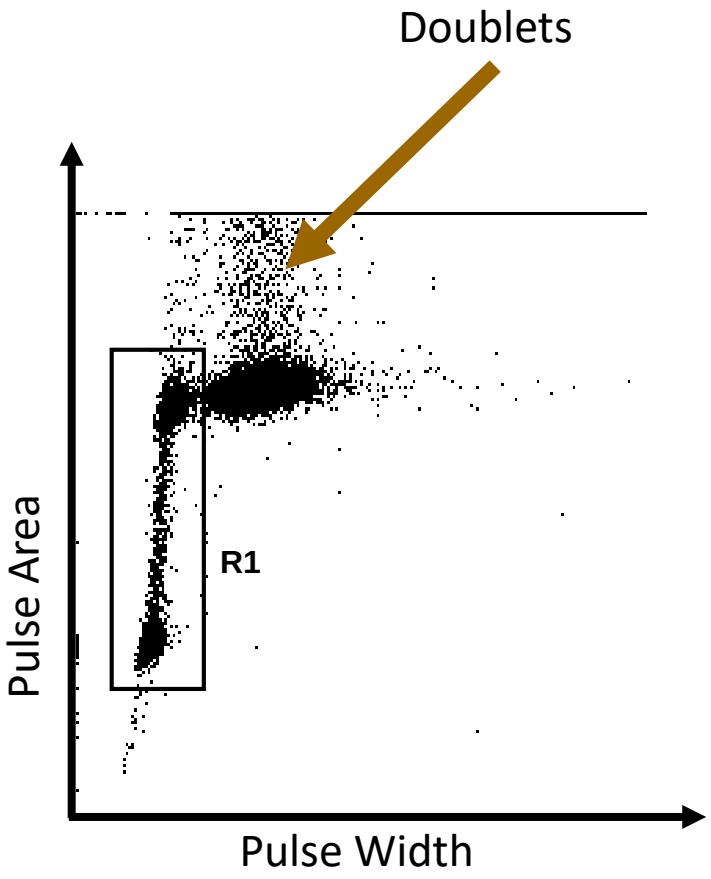



A product of:

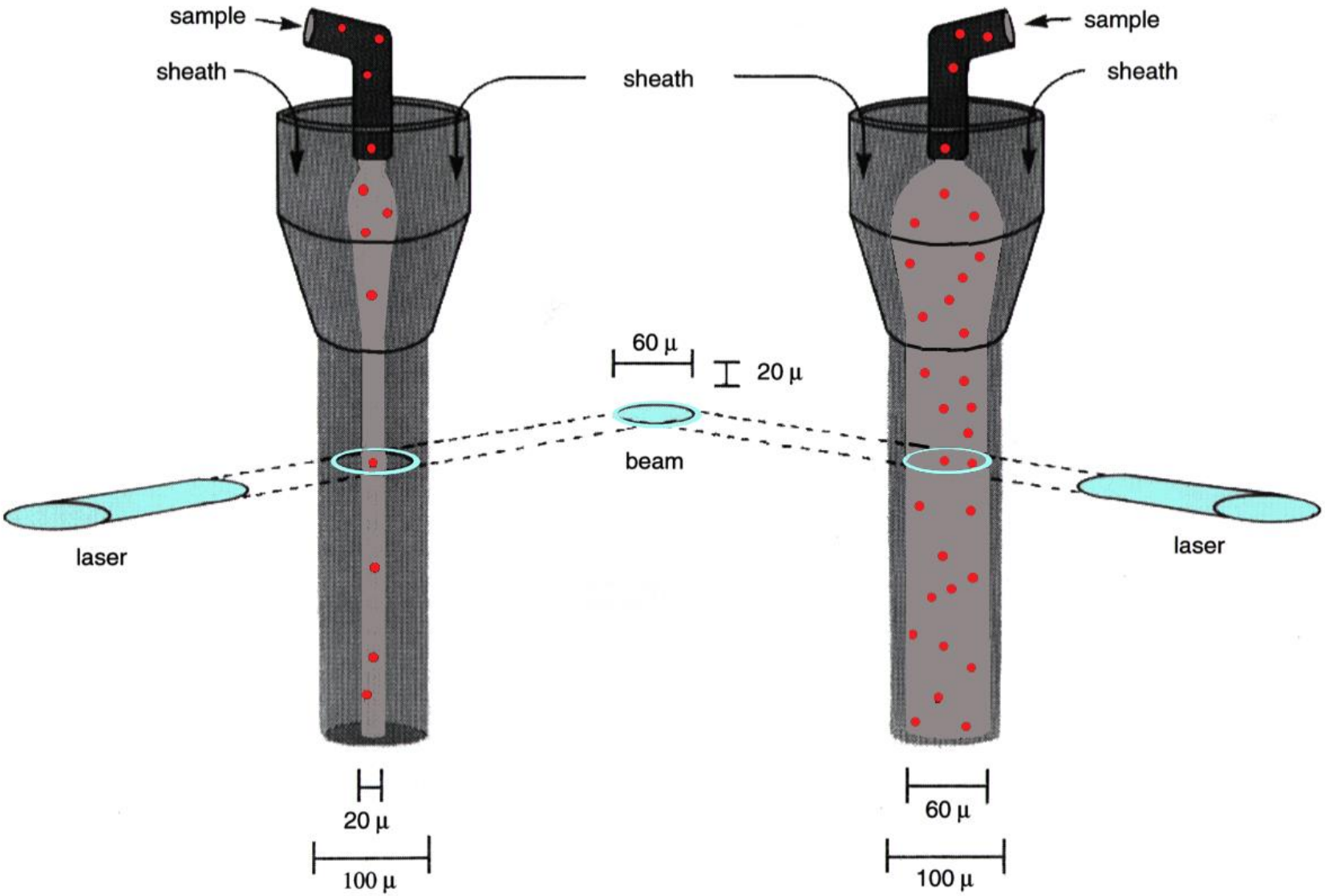
- DNA content per cell
- Number of cells present in each cell cycle stage
- Signal broadening due to staining and measurement variability

# What's Going on Here?

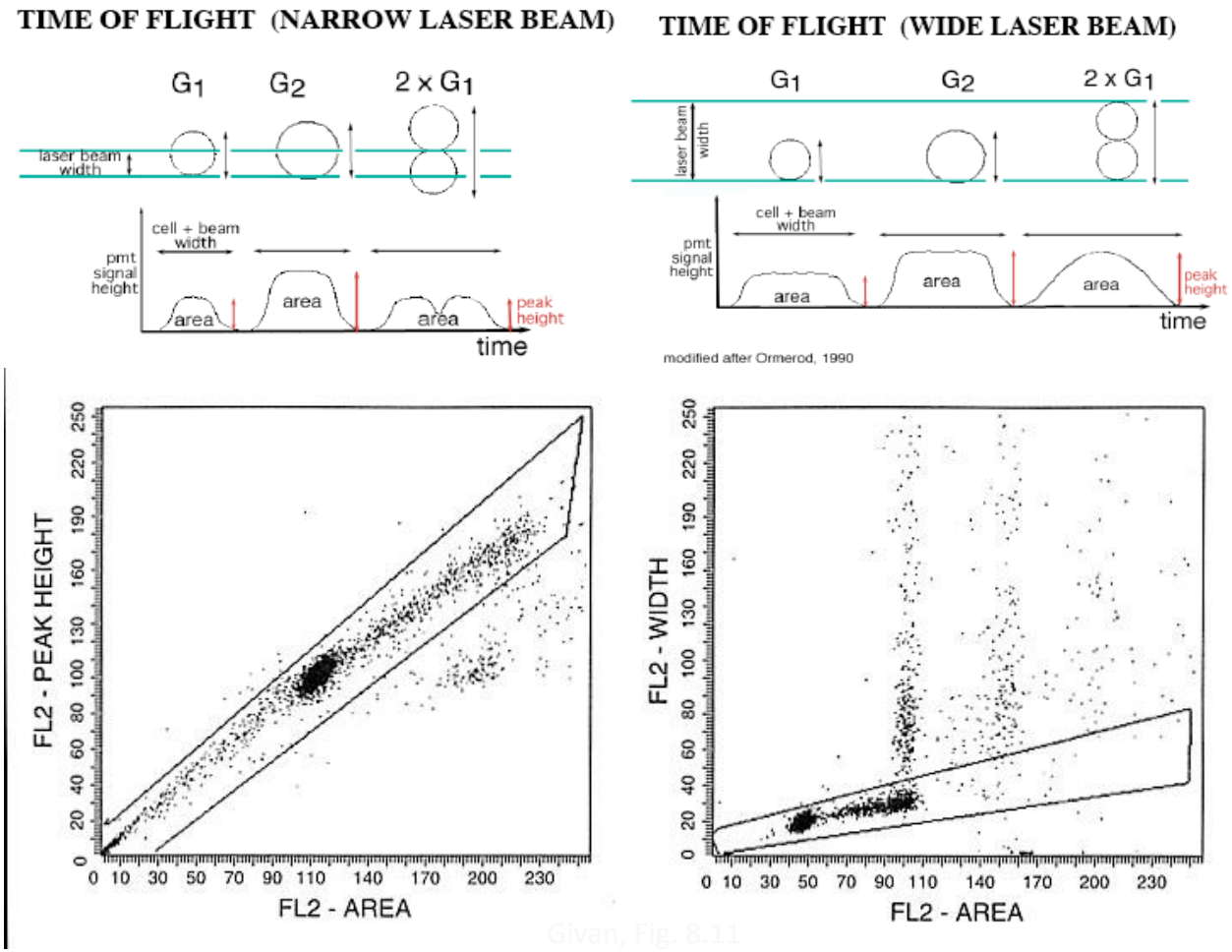


CELLS FLOWING THROUGH A LASER BEAM: with a wide core, the cells are not equally illuminated and multiple cells may coincide in the laser beam.



# Elimination of Cell Aggregates Using Pulse Shape




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

Flow Cytometry First Principles, 2nd Edition
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# THE PLAN

- Cell Cycle, Dyes & Techniques
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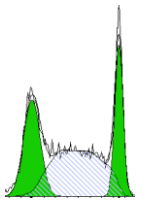
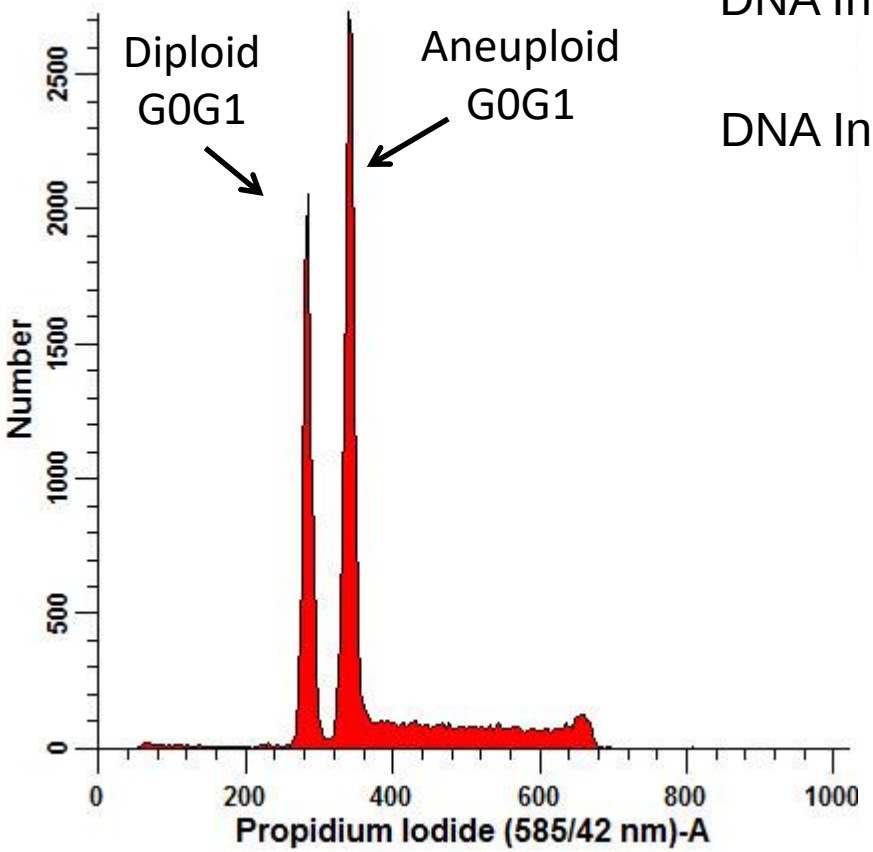


# DNA Diploid *vs.* DNA Aneuploid



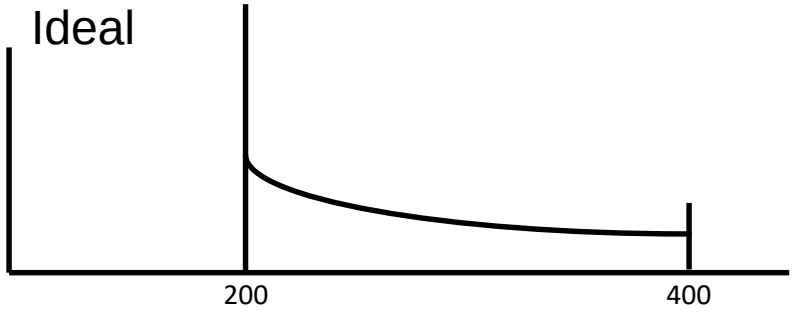

$$\text{DNA Index} = \frac{\text{Aneuploid G0G1 Peak Position}}{\text{Diploid G0G1 Peak Position}}$$

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

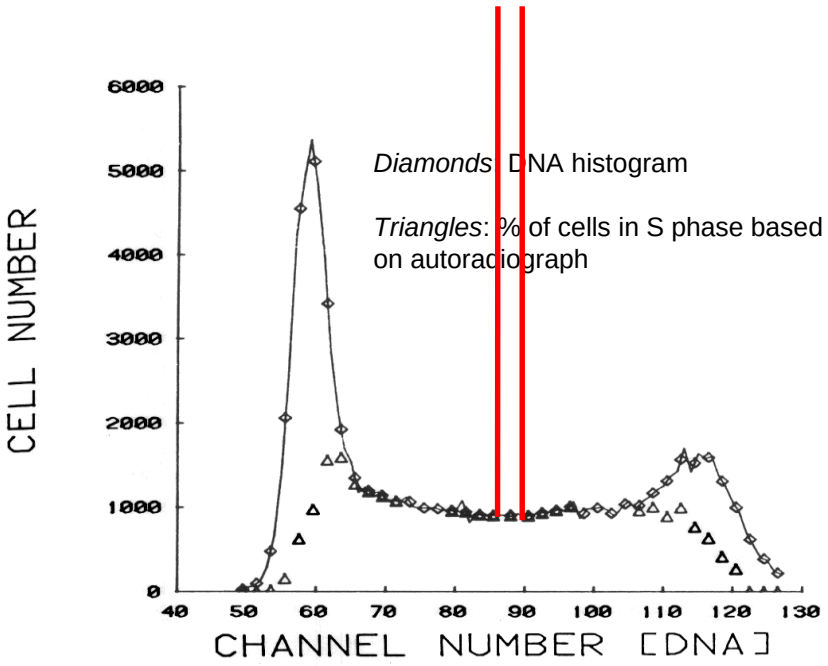
Tumor samples may contain multiple cell types:

- 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

# 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

**Experimental Setup**

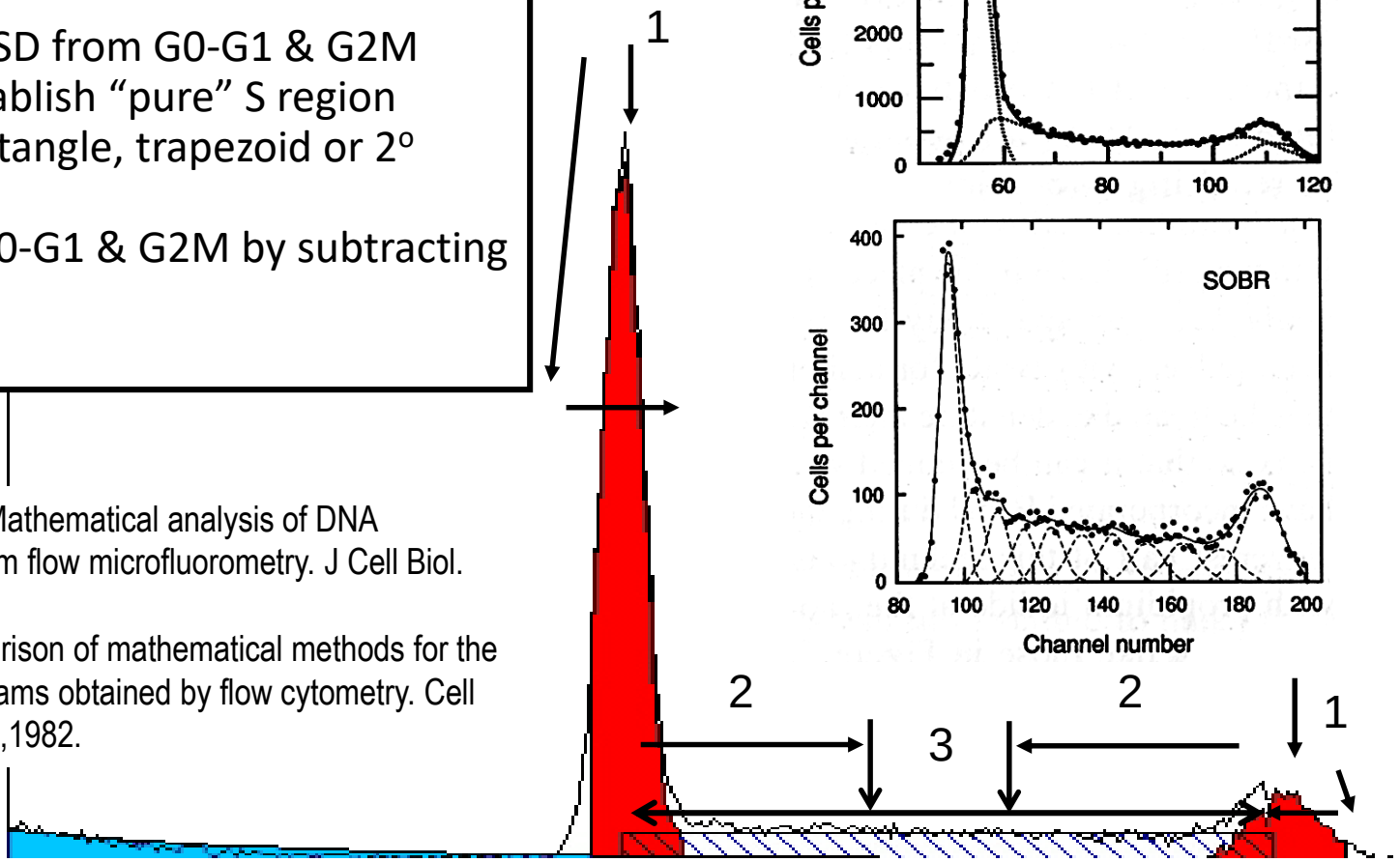
- Cells were labeled in culture for 10 min with $^{3}$H-thymidine
- Stained with mithramycin
- Sorted based on DNA content (2-channels per fraction)
- Perform autoradiography on each fraction and count the number of cells that had taken up $^{3}$H-thymidine (*i.e.* were in S phase)

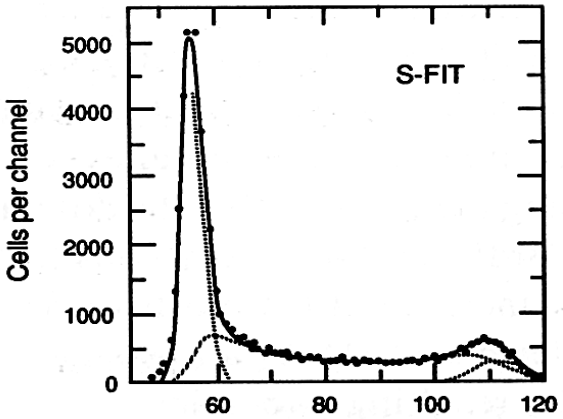
Sheck LE, *et. al.,* (1980). Cytometry. *1:*109

# 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



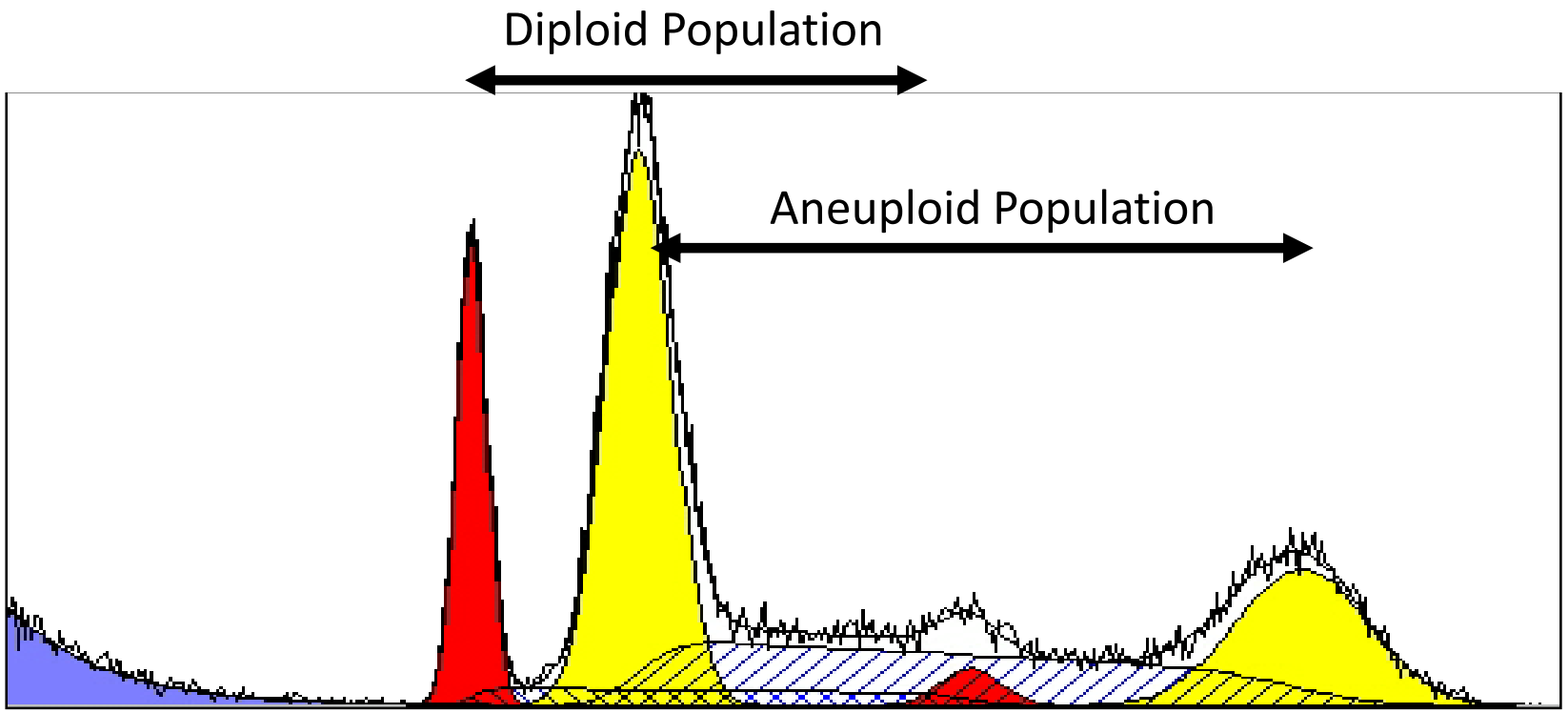





Dean PN and Jett JH. Mathematical analysis of DNA distributions derived from flow microfluorometry. J Cell Biol. *60*:523-7, 1974.

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.

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



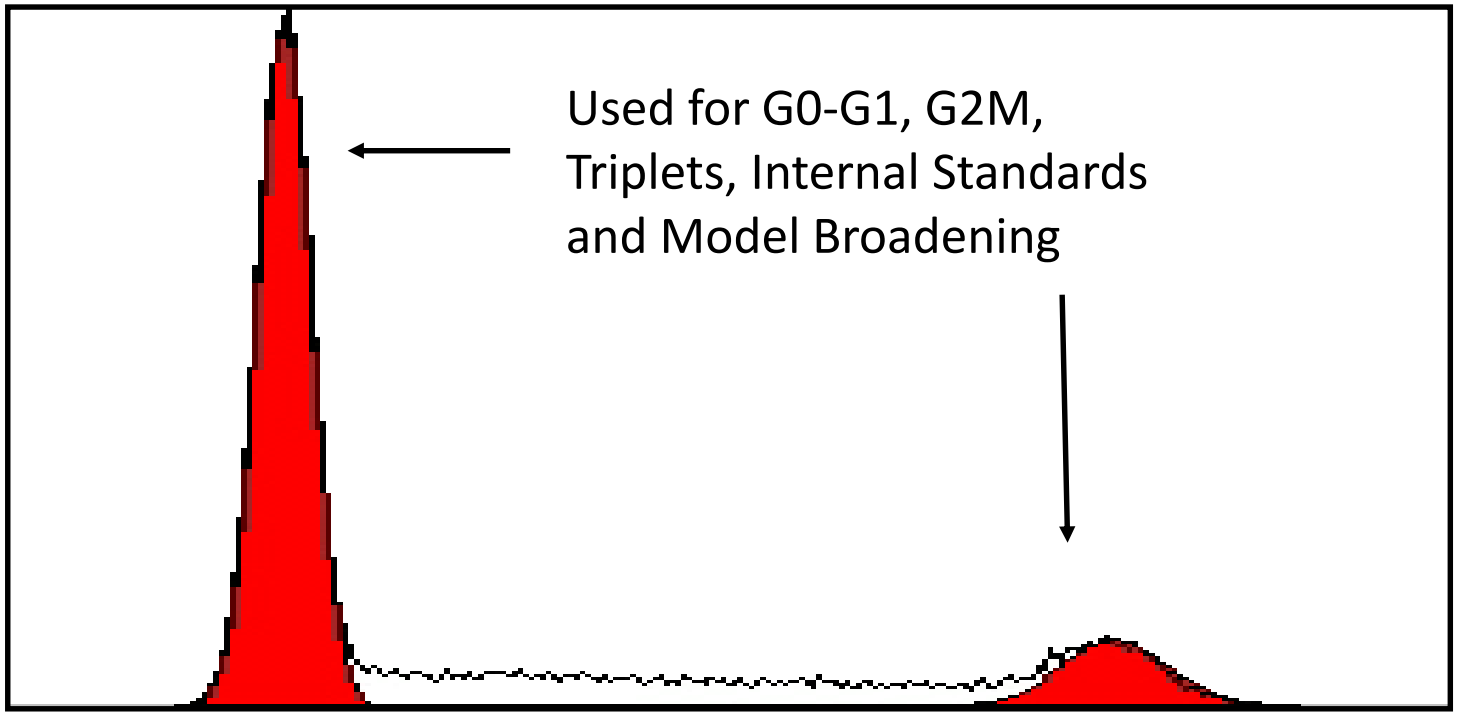
# Model Components

Model Component:

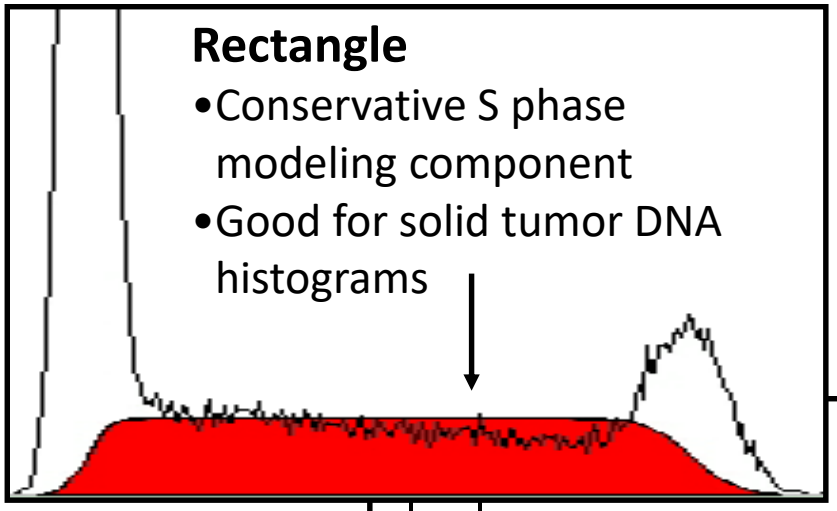
A mathematical construct that simulates some physical or biological process

- 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

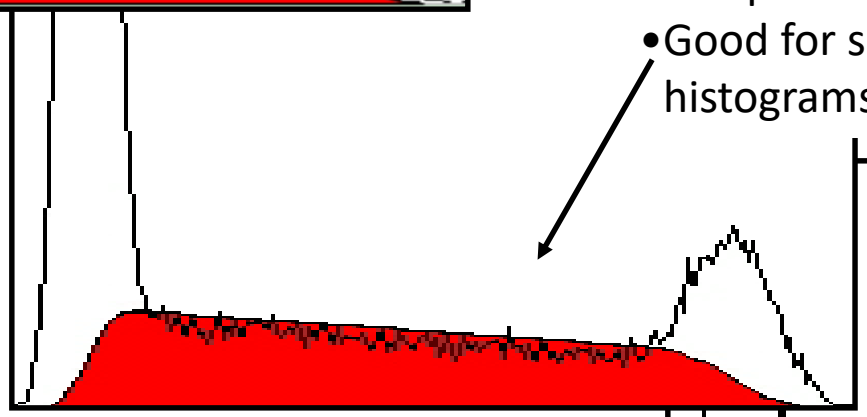


**Rectangle**

- Conservative S phase modeling component
- Good for solid tumor DNA histograms

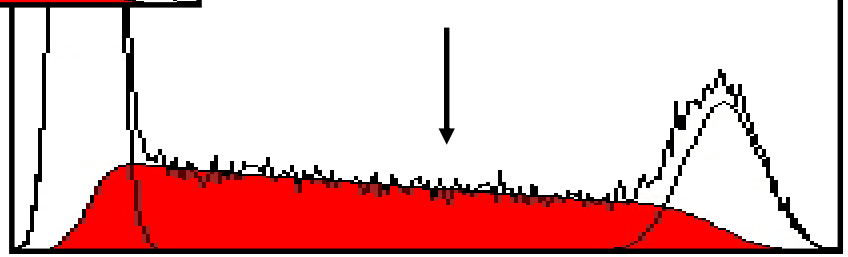
**Trapezoid**

- Also a good conservative S phase modeling component
- Good for solid tumor DNA histograms

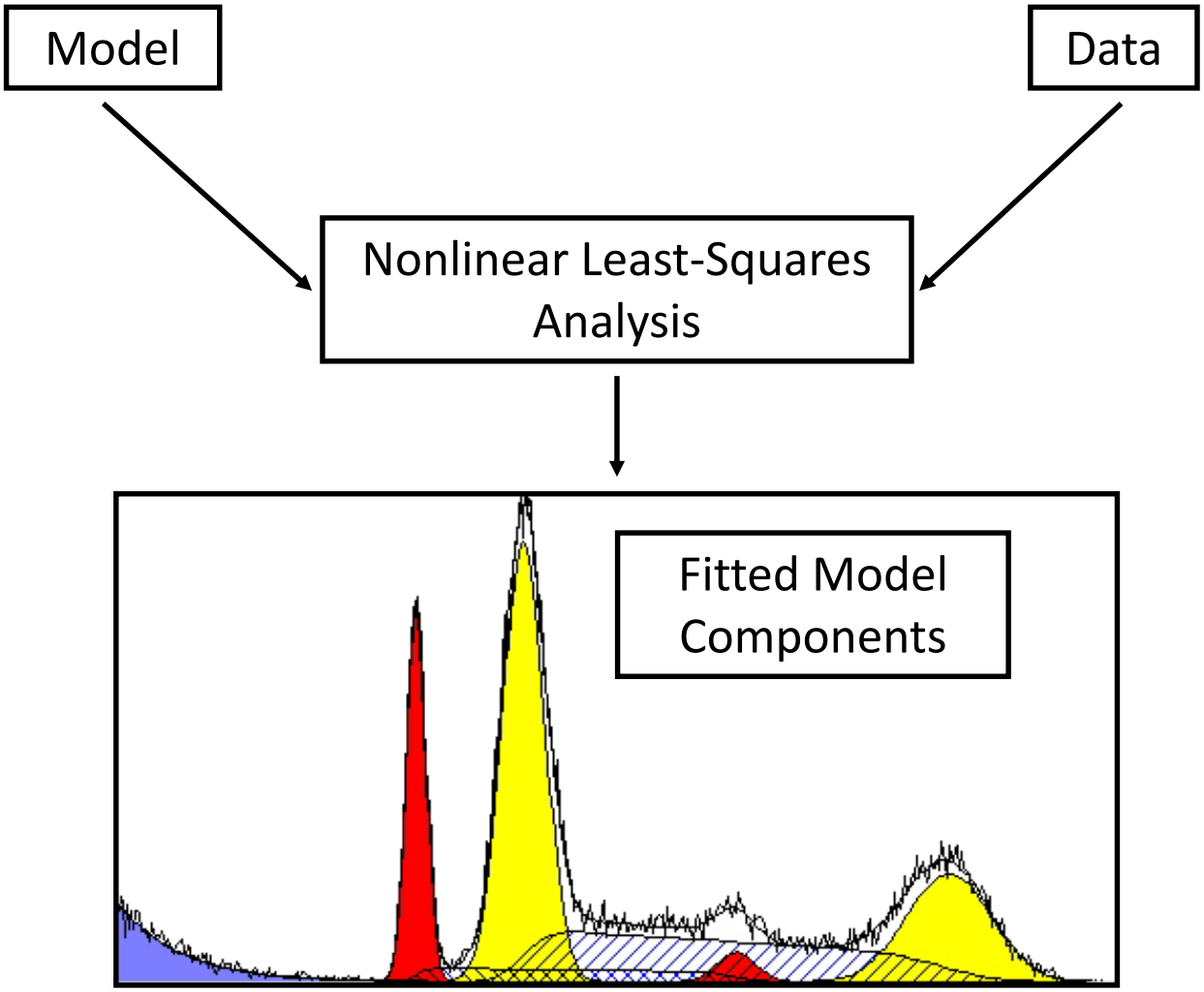


**Polynomial**

Good for “well behaved” DNA histograms such as those from tissue culture lines

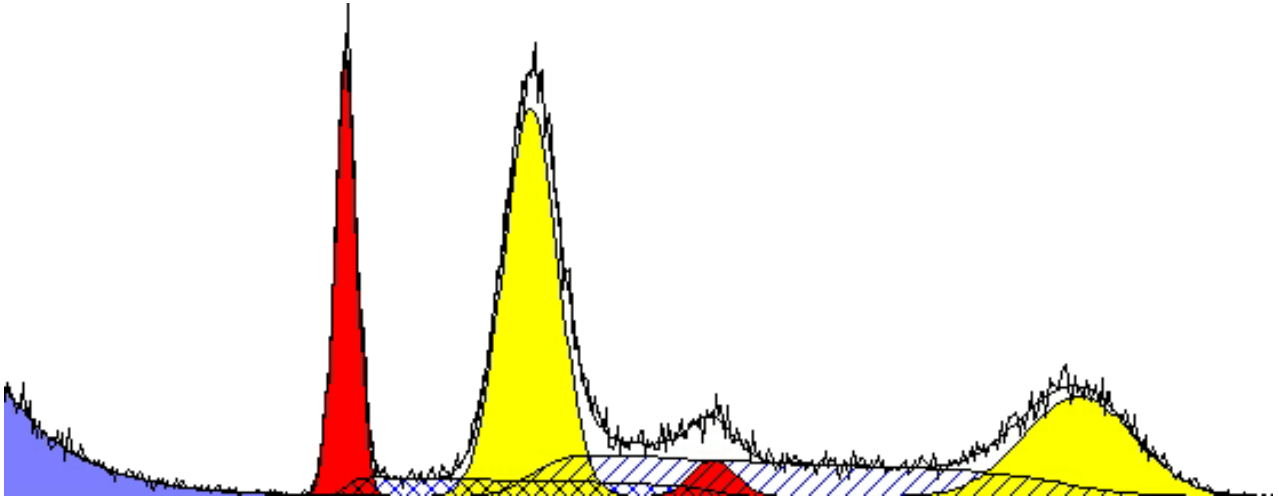


# Non-Linear Least-Squares Analysis



# 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]

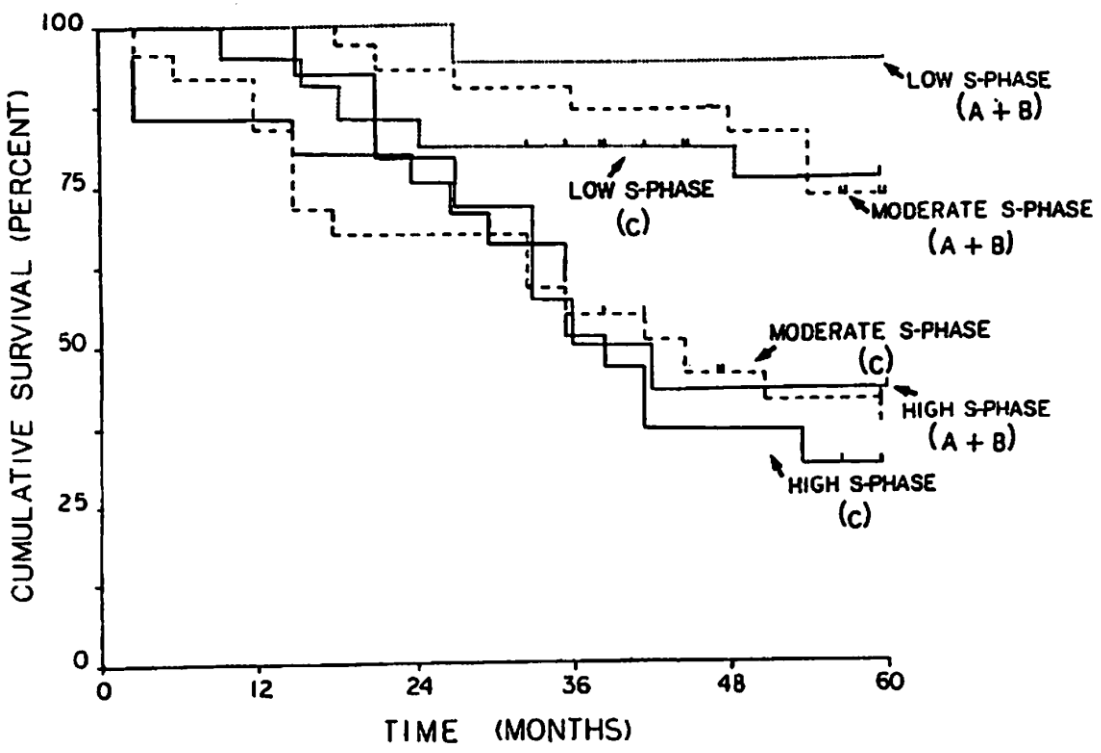


# Is DNA Ploidy or %S Phase Prognostic in Colorectal Neoplasia? Some Say "Yes", Others "No"

**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



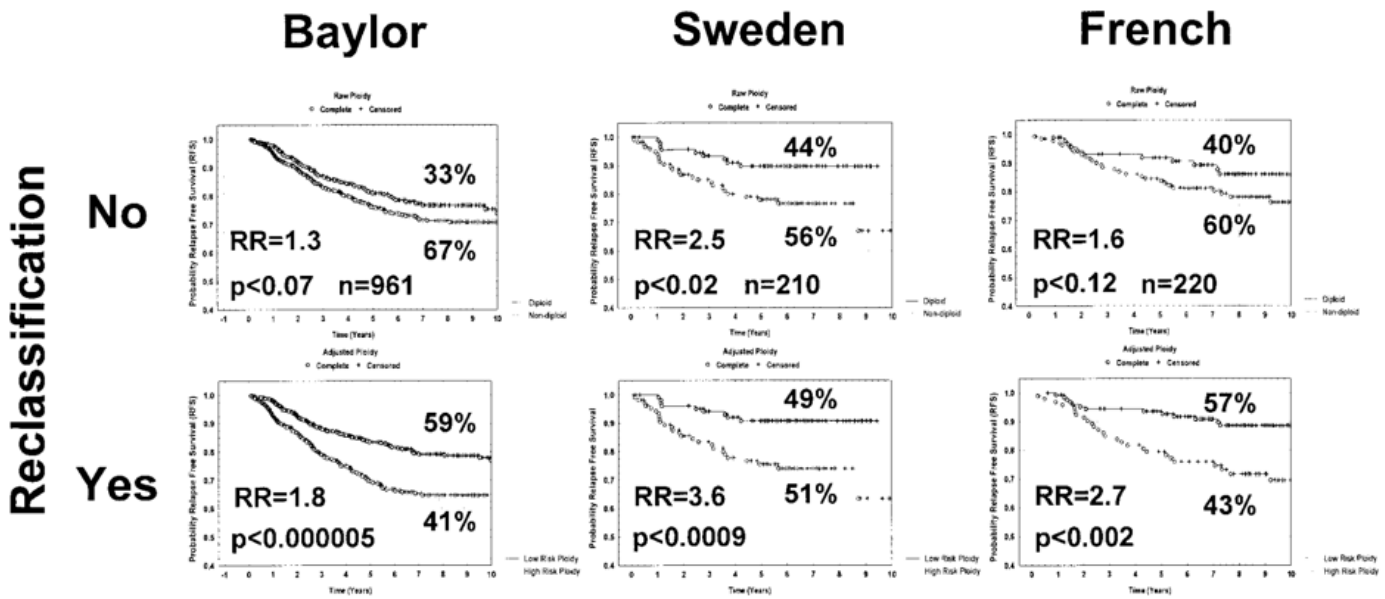

Summary data from 15 studies evaluating the importance of DNA ploidy in colorectal cancer

- 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



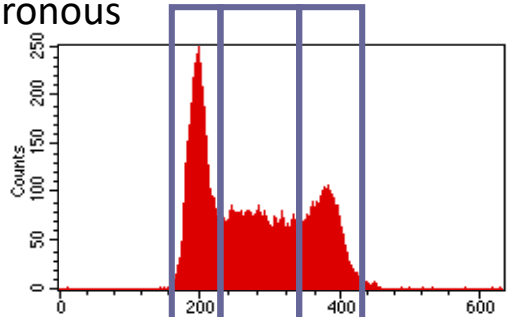

- Rule 1 Aneuploid fraction1 effect: As the aneuploid fraction approaches zero there is a strong tendency over-estimate the aneuploid S phase fraction
- Rule 2 When the percent aneuploid fraction is less than or equal to 5%, the adjusted %S phase fraction cannot be
- Rule 3 To estimate %S phase for a diploid tumor, observed %S phase is divided by the tumor fraction, if known from an independent marker

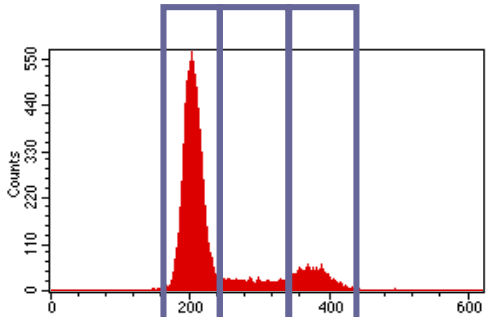
Bagwell et al., Cytometry, 46: 121-134, 2001
Updated at www.vsh.com/Presentations/BreastCAPrognosis.pdf

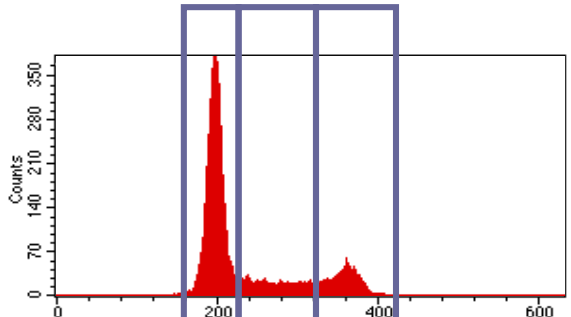
# Visualising Synchronisation by FCM

G1 Block | S Block | G2/M Block

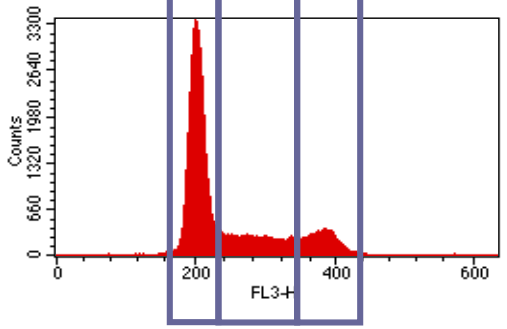
Asynchronous

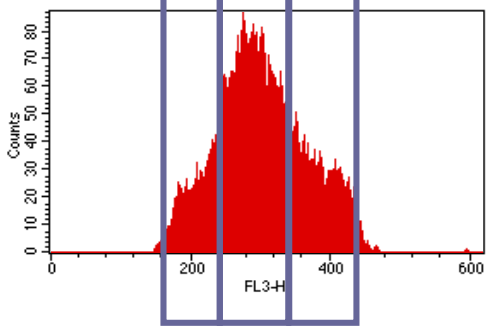


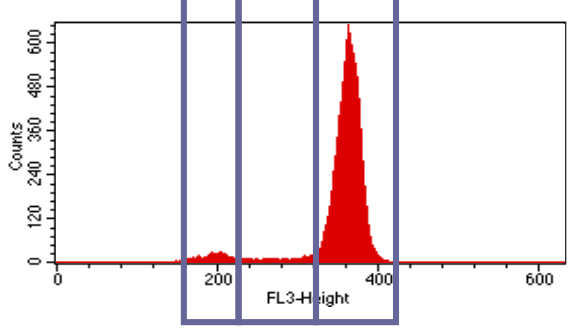




Treated




G1 S G2/M | G1 S G2/M | G1 S G2/M

Serum Starvation | Hydroxyurea | Nocodazole

Cells stained with PI to visualise DNA content



Data courtesy of Derek Davies, London Research Institute

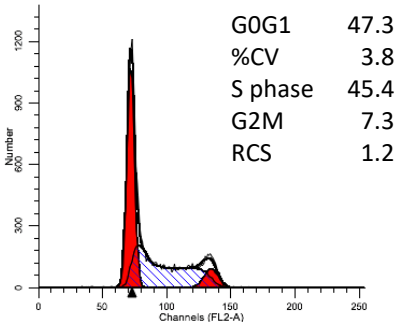
# Modeling Synchronized Cell Distributions

- 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:

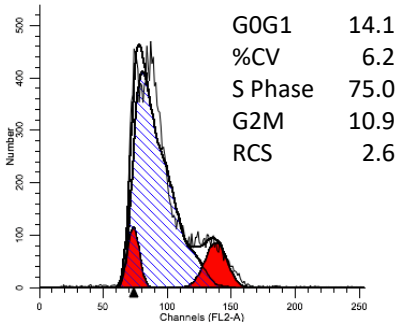
  The G0G1 position and SD were allowed to float

  For fitting flexibility 5 equally spaced rectangles were used to model S phase

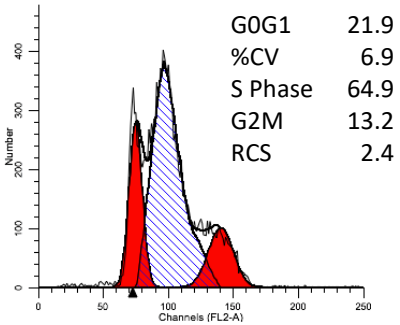
**No Drug T = 0 hr**



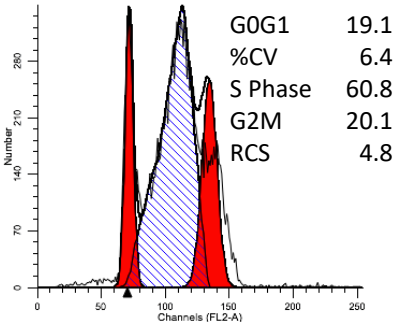

**Methotrexate T = 0 hr**



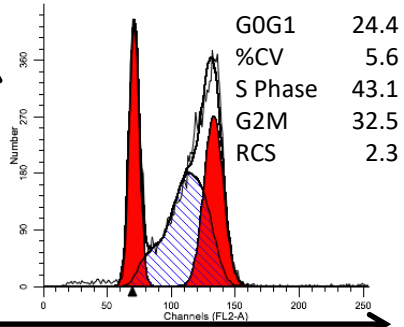

**Methotrexate T = 2 hr**



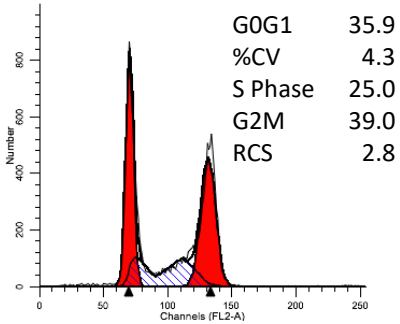

**Methotrexate T = 4 hr**



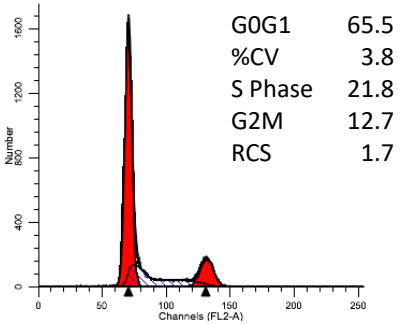

**Methotrexate T = 6 hr**



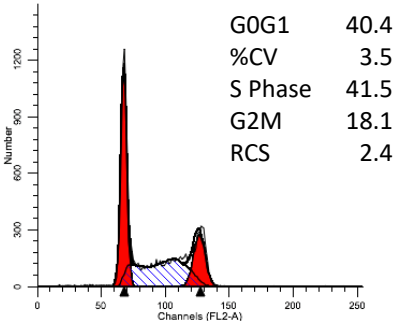

**Methotrexate T = 8 hr**




**Methotrexate T = 12 hr**




**Methotrexate T = 24 hr**

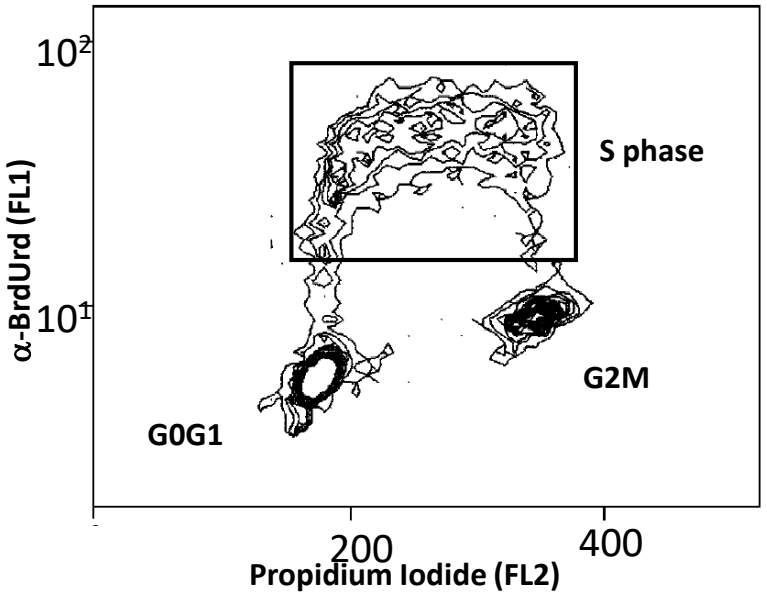



Count

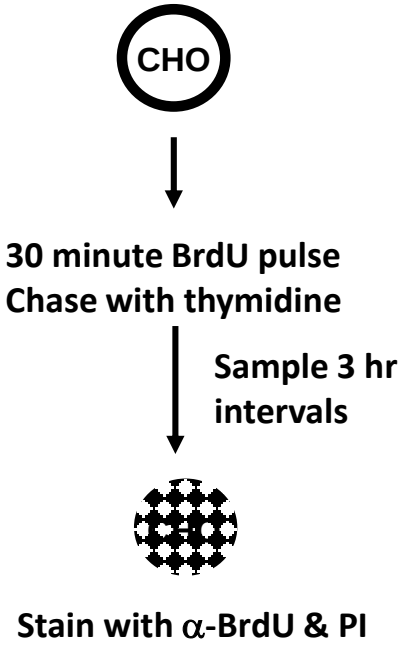
Propidium Iodide

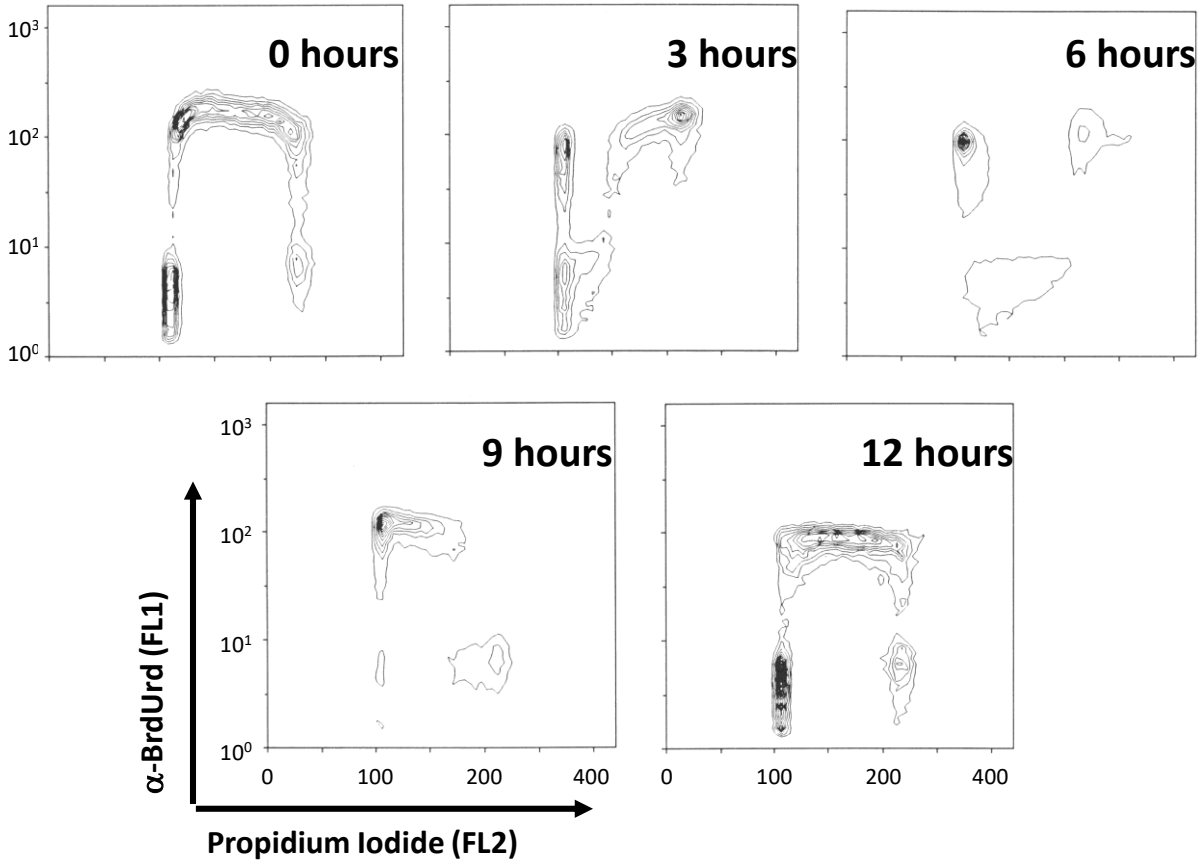
# Dynamic Indices: Combining PI with BrdU

- True proliferation measurements need a time componet
  - Ts: S phase duration
  - Tpot: Potential doubling time
- Halogenated pyrimidines
  - Bromodeoxyuridine (BrdUrd)
  - Iododeoxyuridine (IdUrd)



# Cell Kinetics



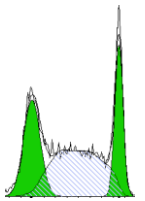




Dolbeare, F, et al., (1990) Methods in Cell Bio. 33:207

# THE PLAN

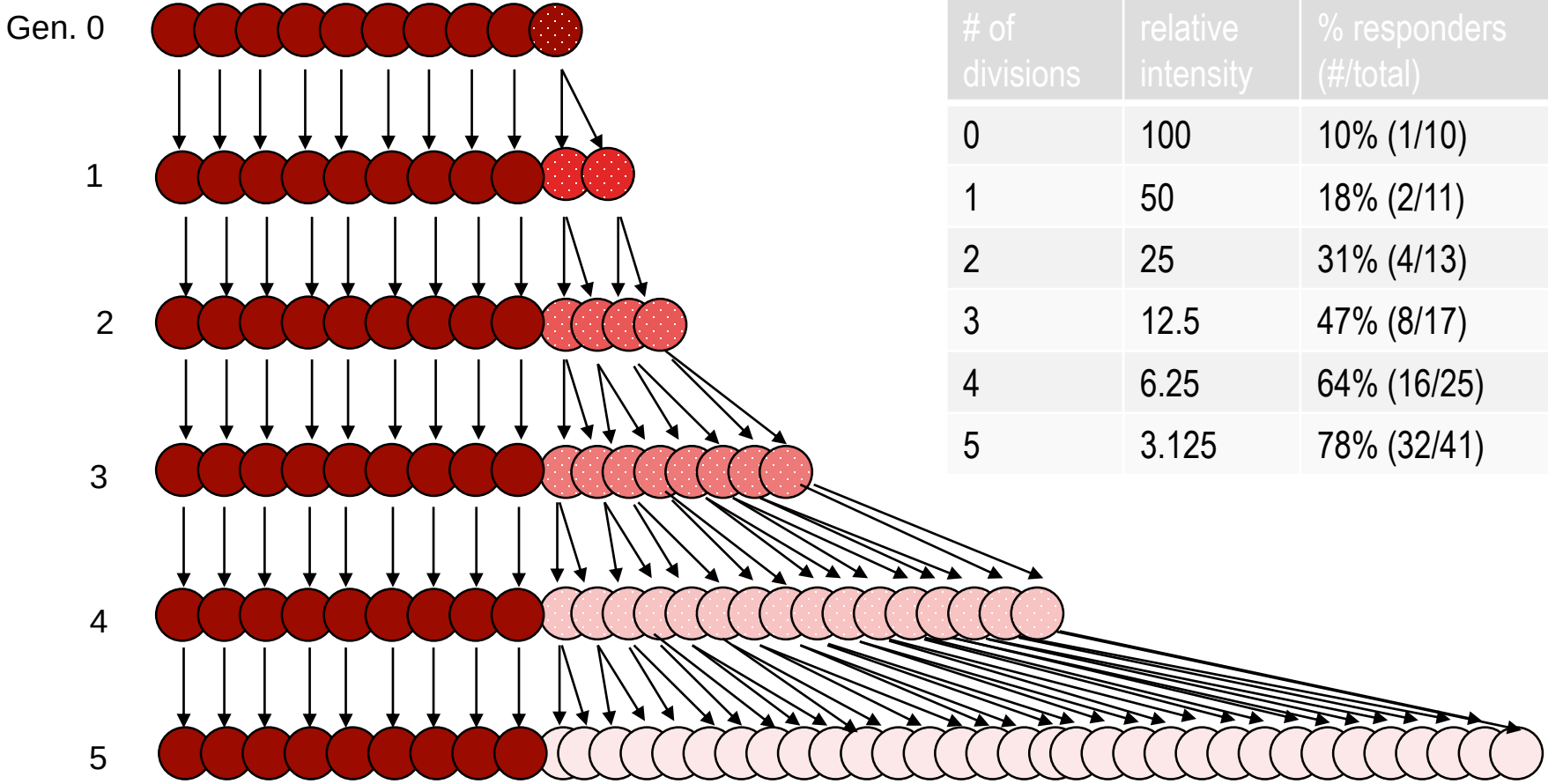
- Cell Cycle, Dyes & Techniques
  - Doublets- slow is the way to go
  - Data Analysis
    - Simple analysis
    - Modelling
  - Clinical Significance of Aneuploidy & S phase
  - Applications
- Tracking Dye Dilution
  - The dyes - many choices
  - Tracking Applications





# Dye Dilution Proliferation Assay: Principles

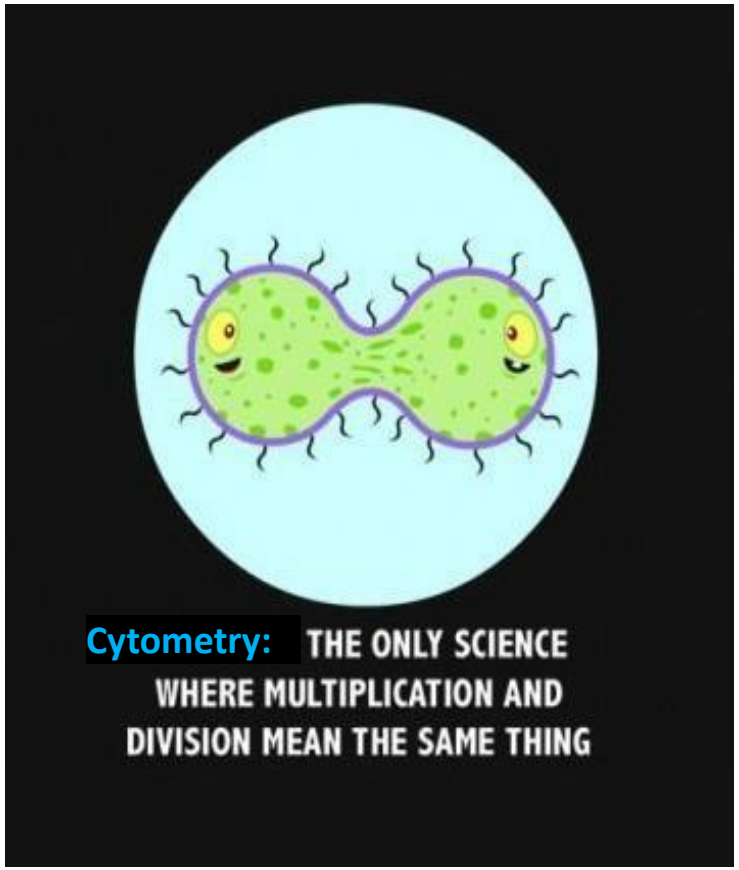
- Label starting population with bright, stable, non-toxic dye that distributes approximately equally between daughter cells at each division
- Monitor dye intensity profile at later time(s) to estimate extent of cell division based on 1) proportion of cells with decreased fluorescence intensity and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

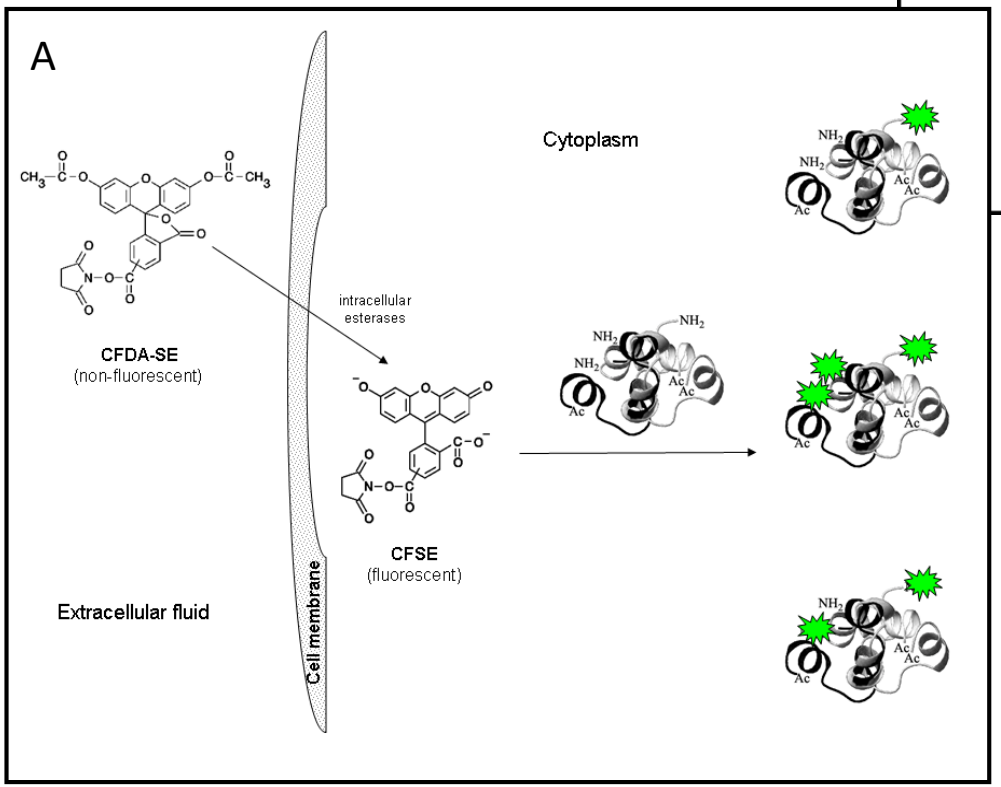
Rapid, high intensity labeling for any cell type

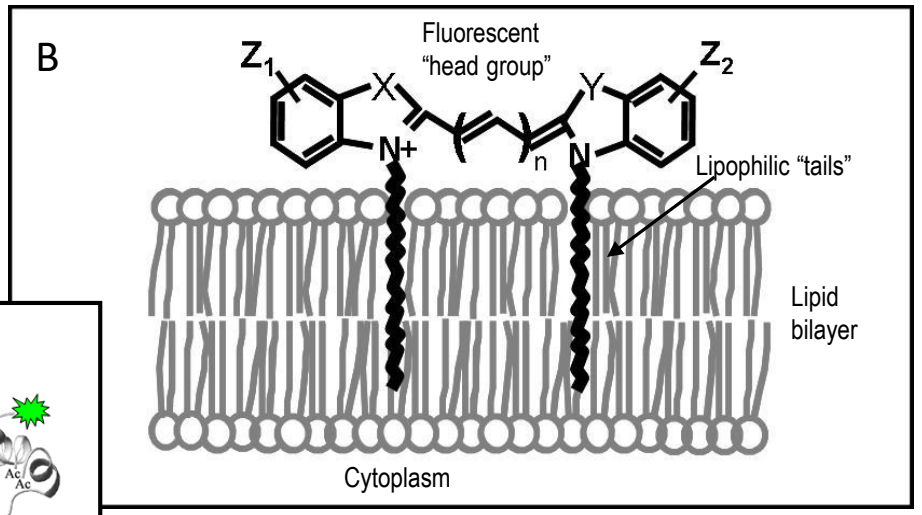
Stable covalent bond between dye and protein

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

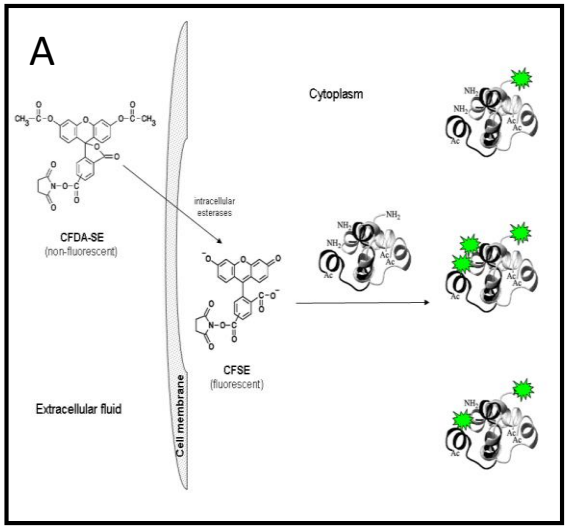
Disadvantages:

Too much dye can alter membrane integrity

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

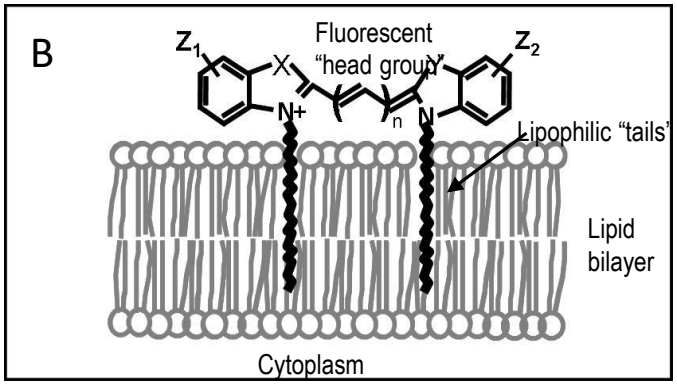
Wallace *et al*. (2008) Cytometry 73A: 1019-1034

# 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 |

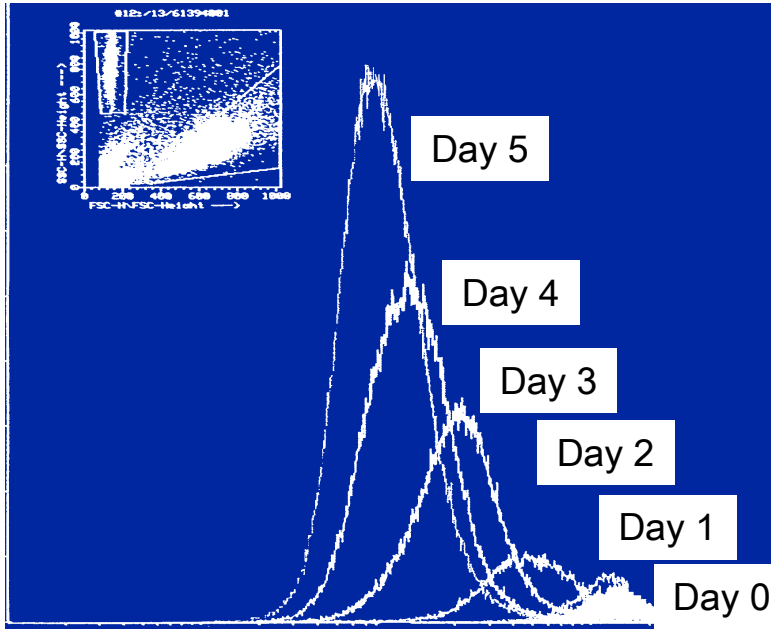
# General Membrane Labeling Dyes




| 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 |

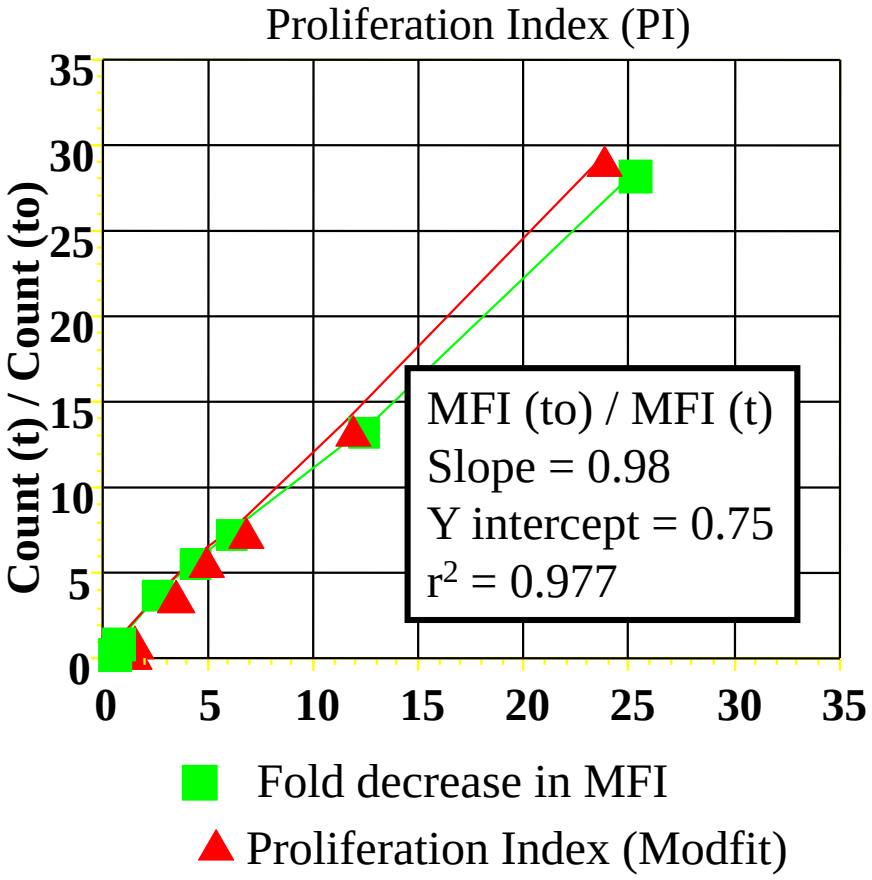
# Does PKH26 Dye Dilution Track Increase in Cell Number in Simple Systems? YES

Continuously dividing 8E5LAV cell line



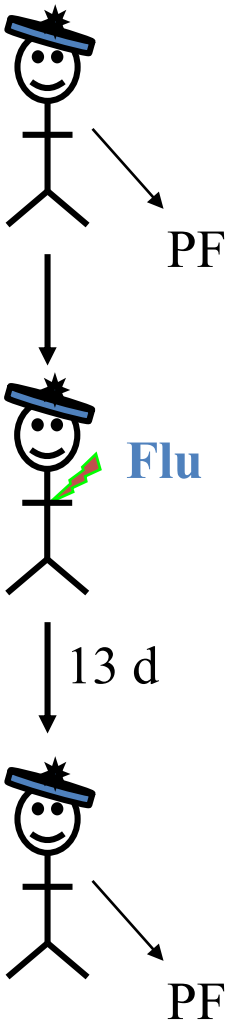

Yamamura *et al*. (1995)
Cell. Mol. Biol. 41 (Suppl. 1): S121-132

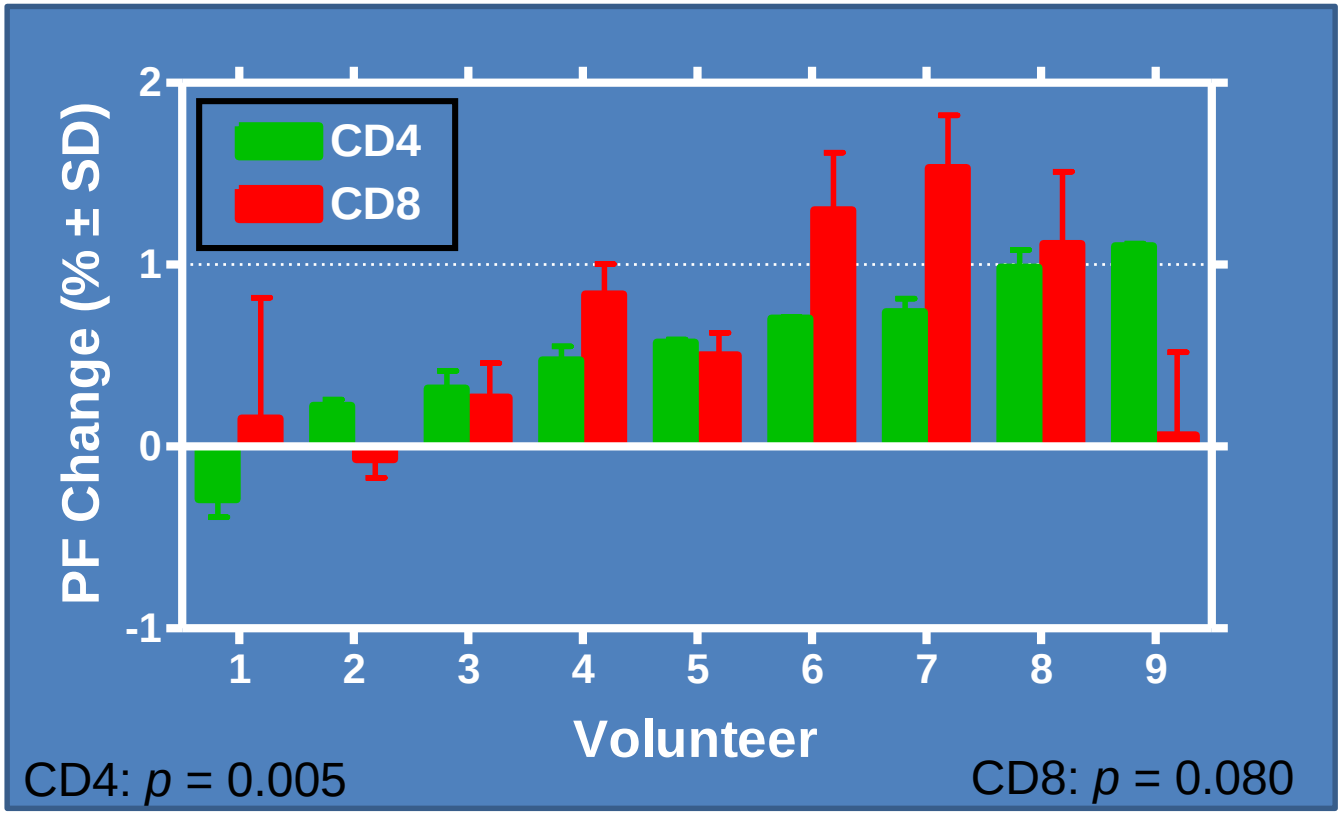
Continuously dividing U937 cell line




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

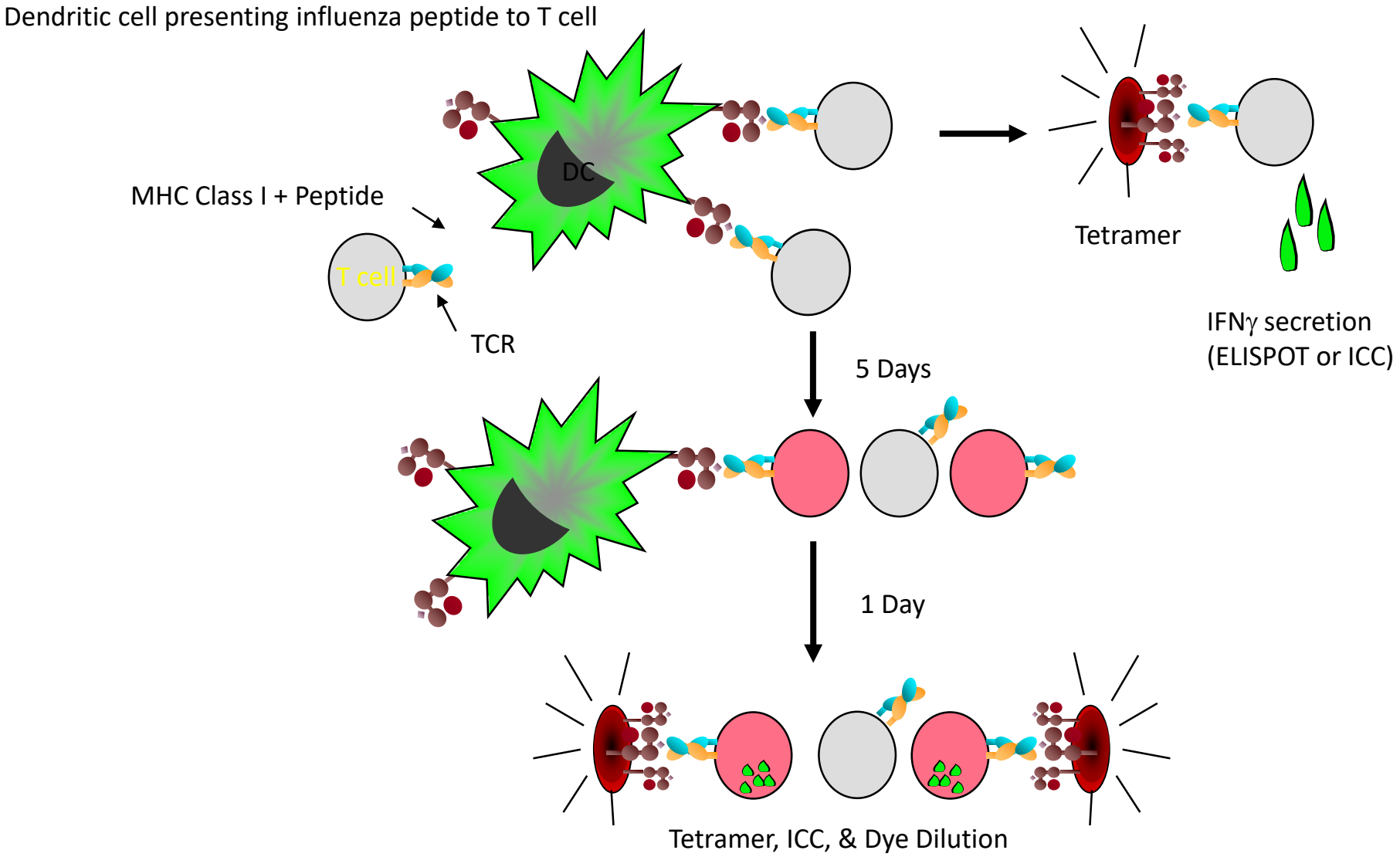
# Change in Precursor Frequency to Influenza Following Vaccination



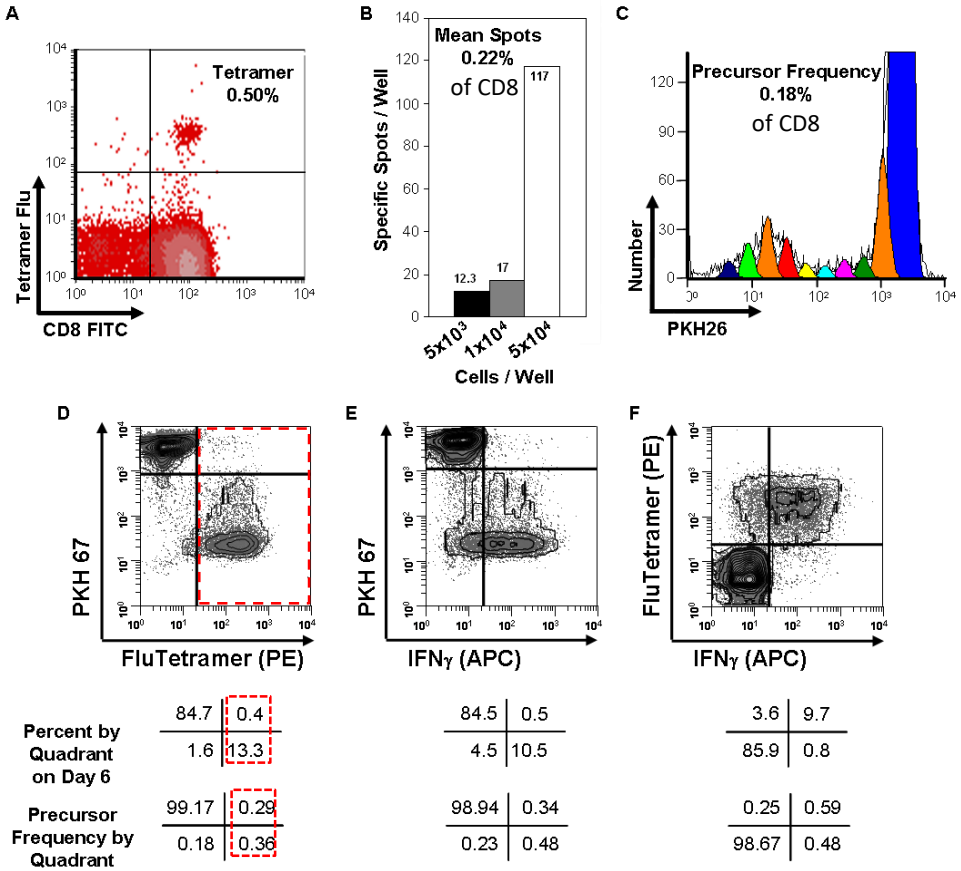

Wallace *et al*. (2018) Cytometry A 73(11): 1019-1034

# Putting it All Together: Multi-parameter Immune Monitoring Protocol

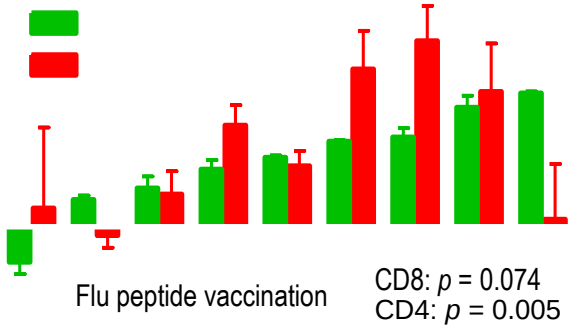


# Cell Division Monitoring in More Complex Systems: Not All Cells Able to Bind Antigen Go On to Proliferate

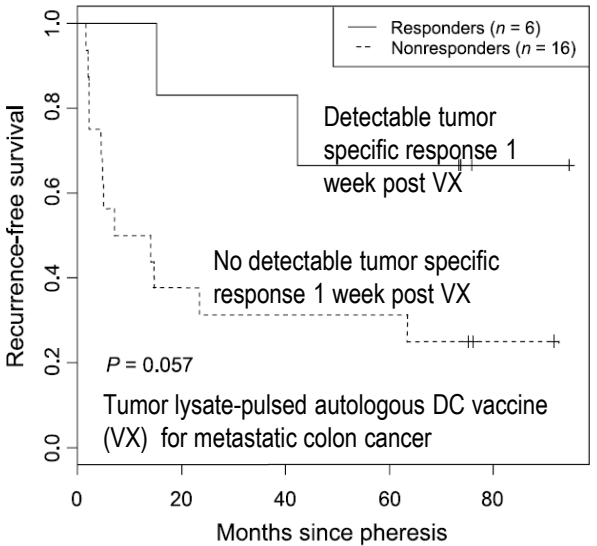
Bercovici et al. J. Immunol. Methods 276: 1-13 (2003)




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




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



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