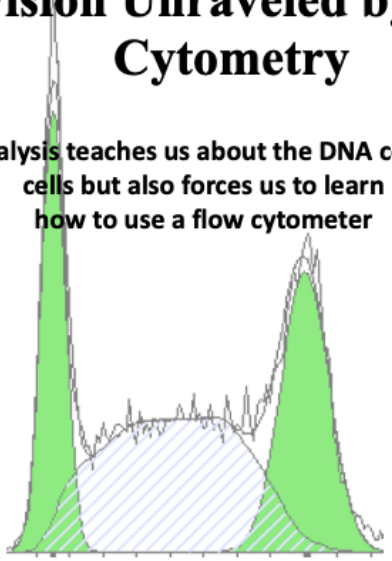
Cell Cycle Progression and Cell Division Unraveled by Flow Cytometry

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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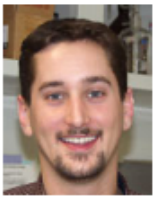

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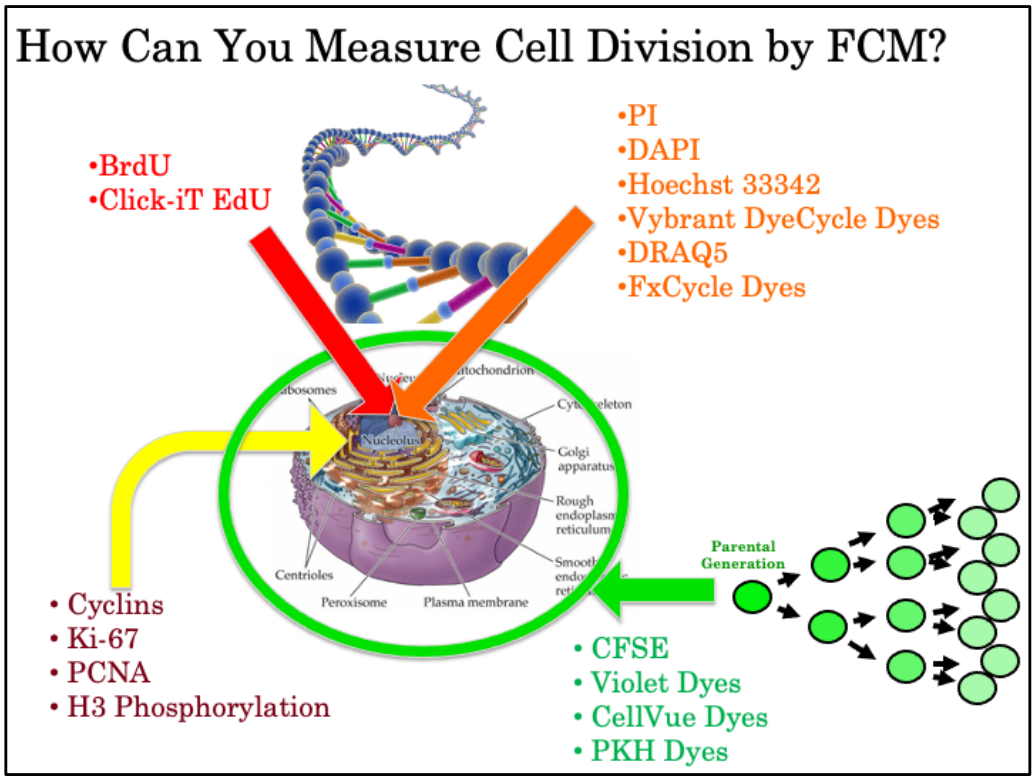
the work that I'll be discussing!

Special thanks to Kylie and the Malaghan for all the work in planning and hosting this workshorp and for travel support, and to the Infinity Foundation for accommodation support.



PI/DAPI IMPERMEANT
Hoechst…. PERMEANT

Remember to state I will be discussing all of these dyes in more detail……..

1. **Dye Dilution by General Protein/Membrane Labelling**
2. **Proliferation-Related Antigens**
3. **DNA synthesis using Thymidine Analogs**
4. **DNA binding dyes**

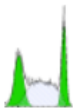
There are two approaches to studying cell proliferation. One is to observe changes in the cell cycle; the other is to follow the number of cell divisions over a period of time. In the first method, at the most, three cell divisions can be followed. In the second, five or six cell divisions may be observed but no information is obtained about the cell cycle. The method is most informative when applied to lymphocytes and is generally used in the study of immune responses. It can also be combined with an immunophenotypic analysis.

MANY MARKERS OF PROLIFERATION

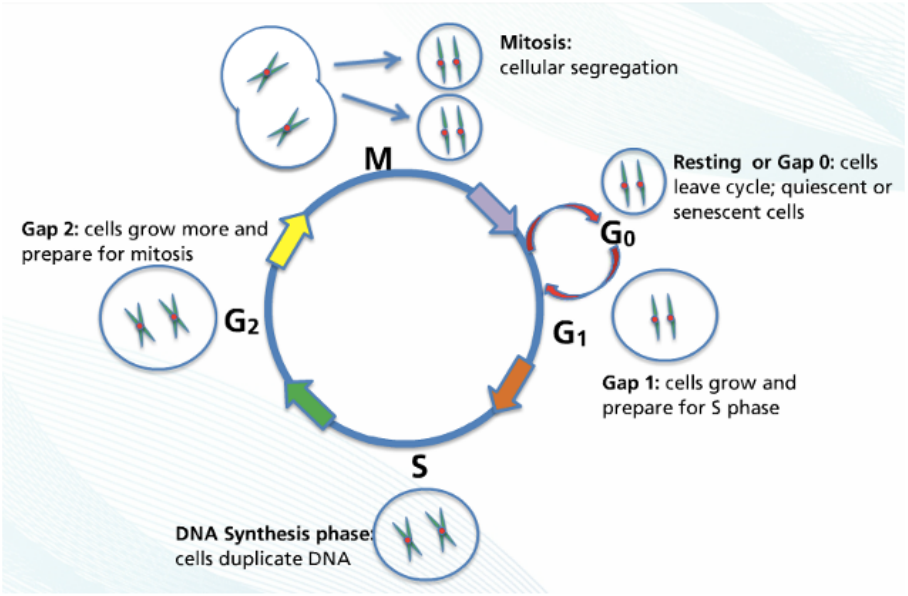
A eukaryotic cell cannot divide unless it replicates its genome (DNA) and then separates the duplicated genome. To achieve these tasks cells must perform **DNA synthesis** and **mitosis**. The cell cycle is an ordered set of events. The **G1** phase stands for "GAP-1" and is required for cell growth and preparation of DNA synthesis. The **S**-phase stands for "Synthesis" and replicates the genome. The **G2** phase is "GAP-2" and needed for cell growth and preparation for mitosis. The last phase is **M** and it stands for "Mitosis" in which cells segregate duplicated chromosomes.

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

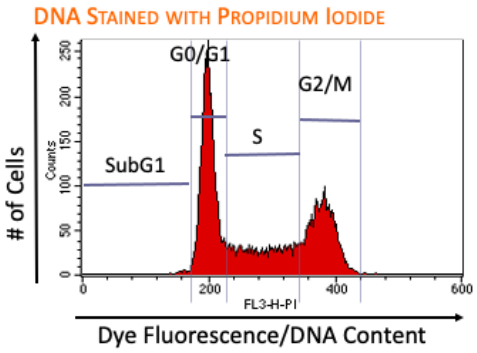


Four Phases of The Cell Cycle



DNA Content Changes During Cell Cycle

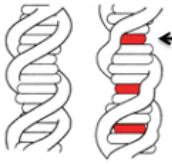
- DNA binding dyes bind stoichiometrically = Fluorescence intensity is proportional to the amount of DNA present within cell
- Used to quantitate amount of DNA and therefore position in the cell cycle (e.g., cells in G2 have 2x amount of DNA as cells in G1)
- Fluorescence data used to generate DNA histograms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



The DNA content of cells is measured by the ability of DNA binding dyes to bind stoichiometrically to DNA under appropriate staining conditions. The nuclei of these stained cells are evaluated individually for DNA content by flow cytometry. The results are displayed graphically as a histogram in which the fluorescence emitted by each nucleus is directly proportional to its DNA content.

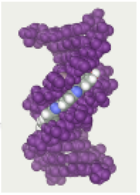
DNA Binding Dye Characteristics

• **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:**

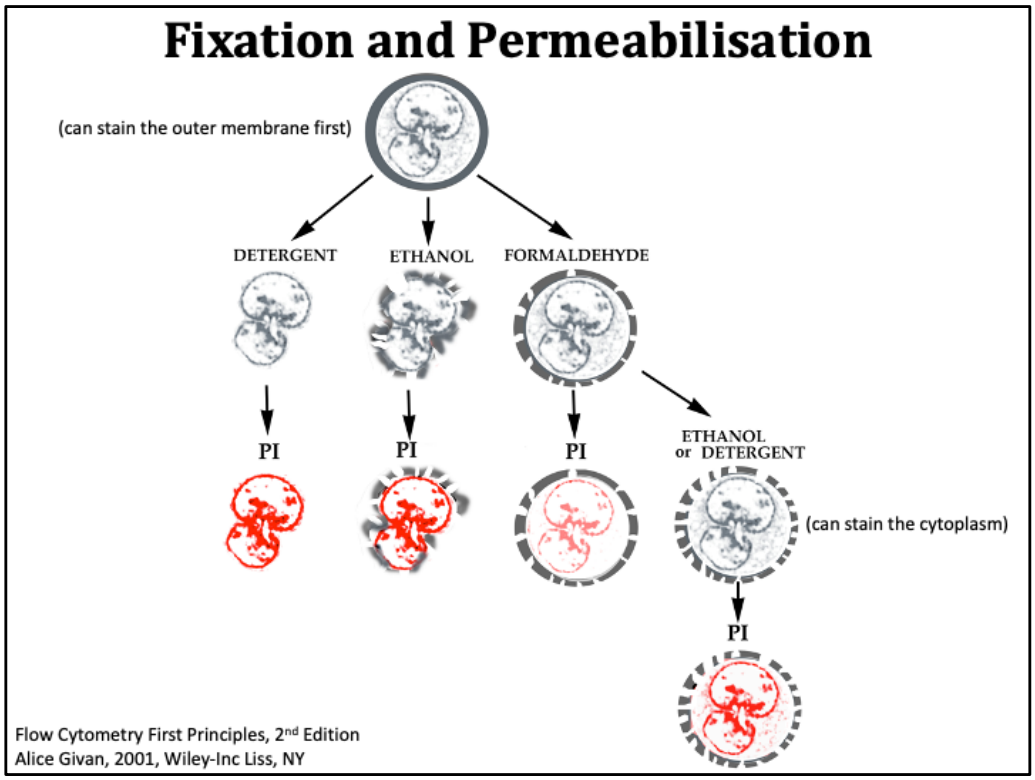
dsDNA and dsRNA	**dsDNA only (nucleic acid preference)**
– Propidium Iodide (PI)	– 7-AAD (G-C)
– SYTOX®	– Hoechst 33342 (A-T)
– TOTO or TO-PRO	– DAPI (A-T)
– DRAQ 5	– Vybrant ® DyeCycle

Images from: http://en.wikipedia.org/wiki/DNA

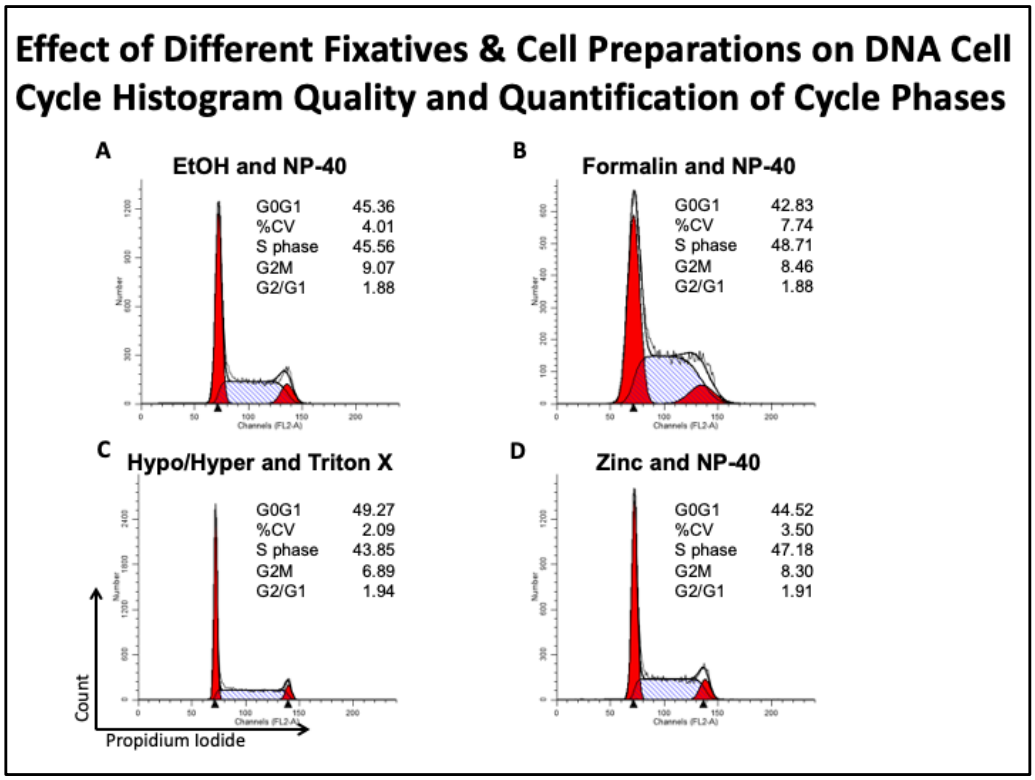
•Use DNA binding dyes to quantitate relative amount of DNA per cell

DAPI binds AT rich duplexes in the minor groove

Intercalation is when a molecule (dye/ligand) can bind in between the base pairs



There are a number of ways cells can be processed for DNA staining with a non-permeate dye like PI. As illustrated here bare nuclei can be made with detergents such as Triton X or NP-40. Ethanol fixation can also be used. Ethanol fixation has the advantage that once the cells are fixed they can be stored in the ethanol at -20°C for long periods of time. Then when you are ready, the cells are washed and stained. Formaldehyde or formalin alone are generally not recommended for DNA staining as this generally results in dim staining with a wide G0G1 CV. Alternatively, cells can be formalin fixed and then treated with either ethanol or detergent. The formalin followed by ethanol or detergent approach has the added advantage in that it can be combined with surface or intracellular staining with mAbs. For detailed procedures see the next slide and Current Protocols in Cytometry Unit 7.5 (DNA Content Measurement for DNA Ploidy and Cell Cycle Analysis. Z. Darzynkiewicz, G. Juan).



Comparison of different DNA cell preparation methods. For additional detailed procedures see Current Protocols in Cytometry, Unit 7.5 (DNA Content Measurement for DNA Ploidy and Cell Cycle Analysis. Z. Darzynkiewicz, G. Juan).

A. EtOH and NP-40.
1. Cells are fixed by slowly resuspending a loose pellet of cells (drop by drop with constant mixing while on ice) in ice cold 70% EtOH. Final concentration 2- 5 x 10^7 cells/mL. Cells once fixed can be stored for years at -20^{o}C.

2. Pipet 1 x 10^6 cells fixed in 70% EtOH into a flow tube
3. Wash 2 times with FCM buffer (PBS, 0.5% BSA, 0.1% Na Azide)
4. Centrifuge, blot and resuspend in 0.5 mL of Krishan buffer
5. Incubate at 4°C for >1 hour but <24 hours before acquisition on cytometer

B. Formaldehyde and NP-40
1. Cells are fixed by resuspending a pellet of cells in 2% formalin overnight or longer. Final concentration ~2 x 10^7 cells/mL.
2. Pipet 1 x 10^6 formalin fixed cells into a flow tube and follow steps 3 - 5 of the EtOH and NP-40 procedure

C. Hypo/Hyper and Triton X
1. Pipet 1 x 10^6 washed cells into a flow tube, centrifuge and blot
2. Resuspend the pellet in 250µL of Hypotonic Stain Buffer
3. Add 25µL of RNase A
4. Mix and incubate at 37°C for 15-45 min depending on cells
5. Add 250µL of Hypertonic Stop buffer
6. Incubate at 4°C for >1 hour, but <48 hours before acquisition on cytometer

D. Zinc and NP-40
1. Pipet 1 x 10^6 washed cells into a flow tube, centrifuge, blot and resuspend cells in 100µl of PBS.
2. Add 1ml ZBF buffer while vortexing
3. Incubate at 4^{o}C overnight
4, Stain following steps 3 - 5 of the EtOH and NP-40 procedure
Note: the zinc and NP-40 method is recommended when also acquiring fluorescent proteins (*i.e.* GFP, RFP *etc.*) as the other methods may destroy their fluorescence

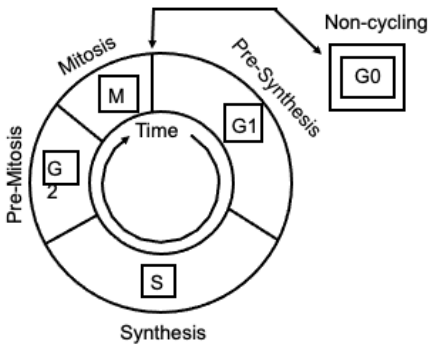
Krishan (NP-40) buffer
Sodium citrate 0.1%
Propidium iodide 0.05mg/mL
NP-40 0.2%
RNAse A 800U/mL
HCl 1N 1 drop/100mL

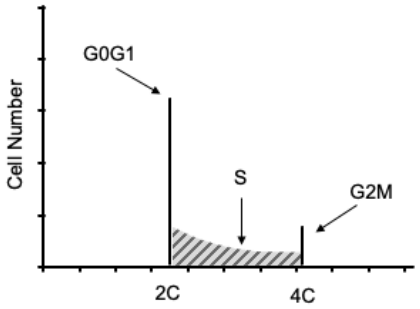
Hypotonic Stain buffer
Sodium citrate 3.6mM, pH 7.8
Propidium iodide 50µg/mL
PEG 4000 3%
Triton X-100 0.1%
Rnase A 800U/mL

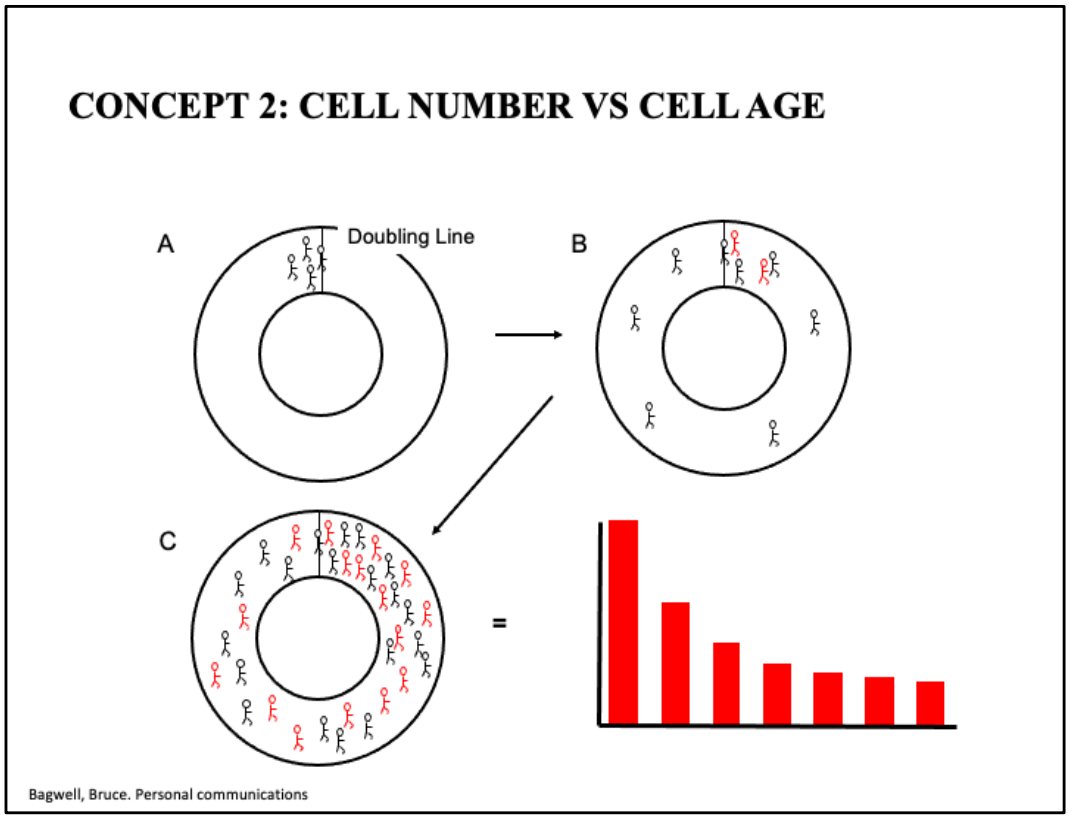
Hypertonic Stop buffer
NaCl 0.375M
Propidium iodide 50µg/mL
PEG 4000 3%
Triton X-100 0.1%

ZBF buffer:
Tris-HCl 0.1M Tris-HCl pH 7.8
Calcium acetate 0.05%
Zinc acetate 0.05%
Zinc chloride 0.05%
Note the ZBF buffer has a significant precipitant which can be removed by filtering or allowed to settle

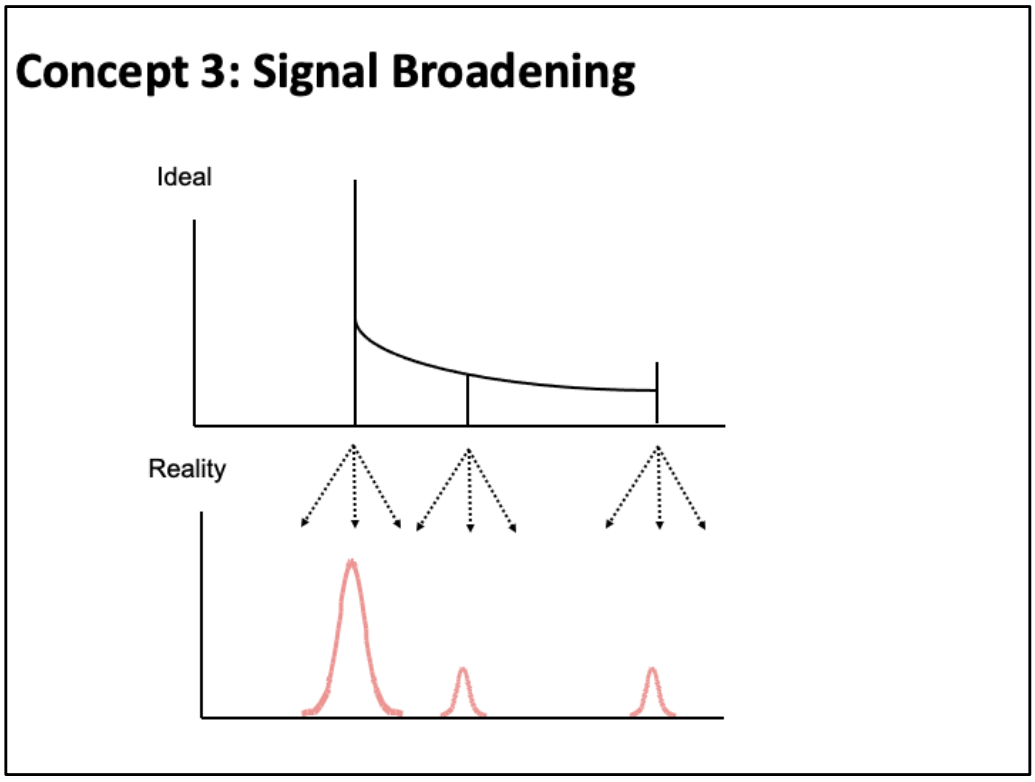
CONCEPT 1: DNA VS TIME IN CYCLE







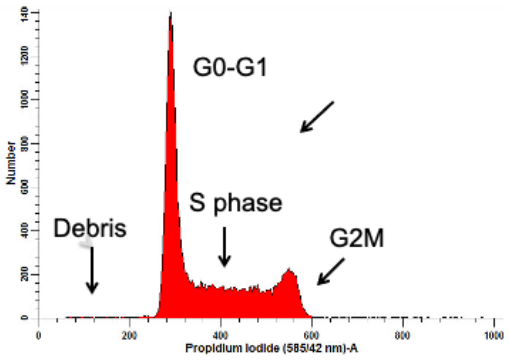
Concept 2: Cell number versus cell age. The shape of S-phase in an actively growing culture of cells is often like the image shown in the lower right of this slide. To better understand where this shape comes from think of a race where the runners are going around a track. As each runner crosses the start/finish line they double. If the runners continue to circle the track and double each time they cross the line there will naturally always be more runners at the start of the race then anywhere else along the course.
If cells are actively growing, with no or few detours into G0, they will behave much like our runners above. rapidly passing through G1 and entering S phase after just having undergone division in G2M.



Concept 3: Signal Broadening.
Since all cells in G0G1 have identical amounts of DNA, they should all pile up in just one channel of our DNA histogram. The same would be true for G2M. Only cells in S phase would have a continuous distribution. If this were the case all DNA histograms would look like the conceptualized "ideal" histogram shown above and it would be very easy to enumerate the numbers of events in G0G1, S and G2M.

However in the real world the signal from each cell is subject to broadening from a number of things. Factors causing signal broadening range from the biological to the instrumentation. For example the amount of DNA dye bound per cell can be affected by DNA condensation and its tertiary structure, which can inhibit access of the dye to the DNA. In addition, our instrument measurement may not be identical for each cell. For example cells flowing through different regions of the sample core as they pass through the laser will receive different amounts of illumination. Photon counting variables will also affect difference in our measurements and each measurement error contributes to a broadening of our final measurement. Thus the final recorded measurement will not fall in the single idealized channel but will be recorded in a range of channels which should mimic the Gaussian distributions shown above. A major goal when measuring DNA content is to minimize variability to get as close to the ideal histogram as possible.

The DNA Histogram



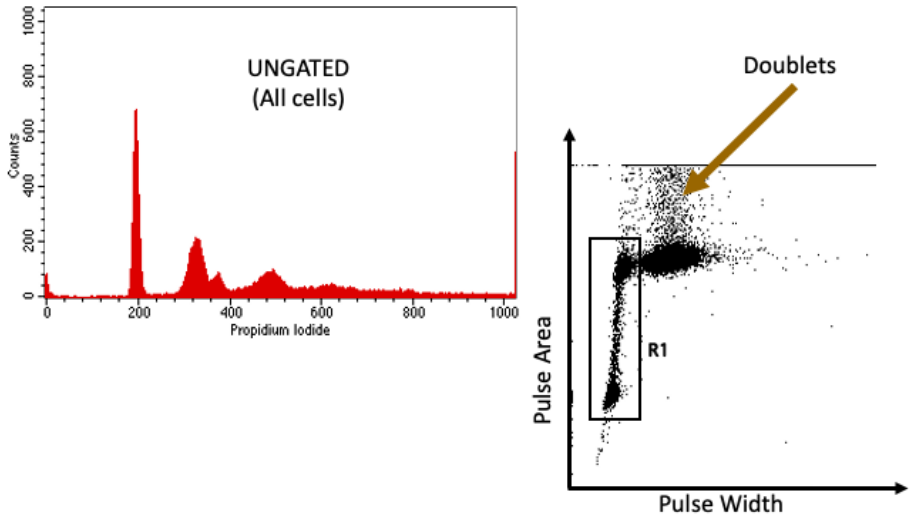
A product of:

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

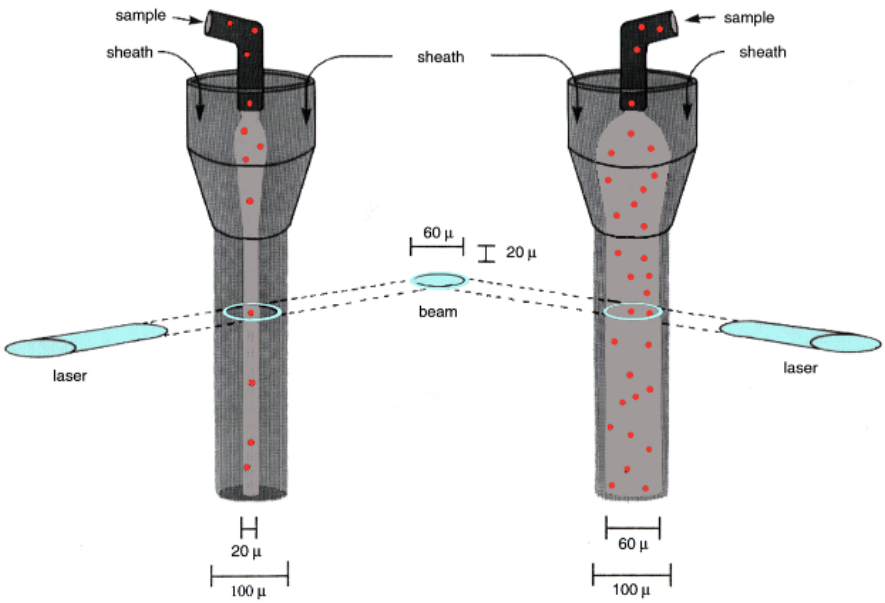
The shape of the final DNA histogram is the result of:

1. DNA content of each cell (dependent on cell cycle stage; see slide 24)
2. Relative number of cells at each stage of the cell cycle (see slides 24 - 25)
3. Signal broadening during staining and measurement (see slide 26)

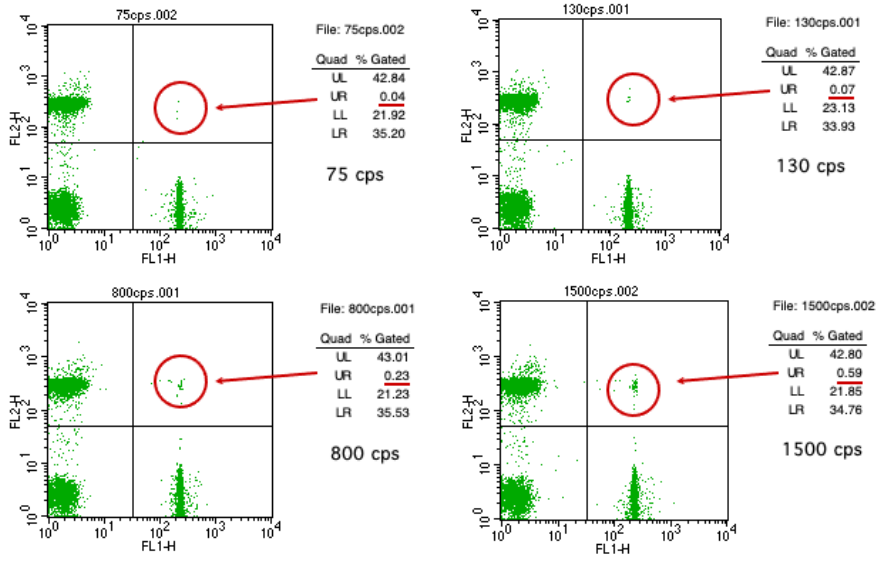
What's Going on Here?



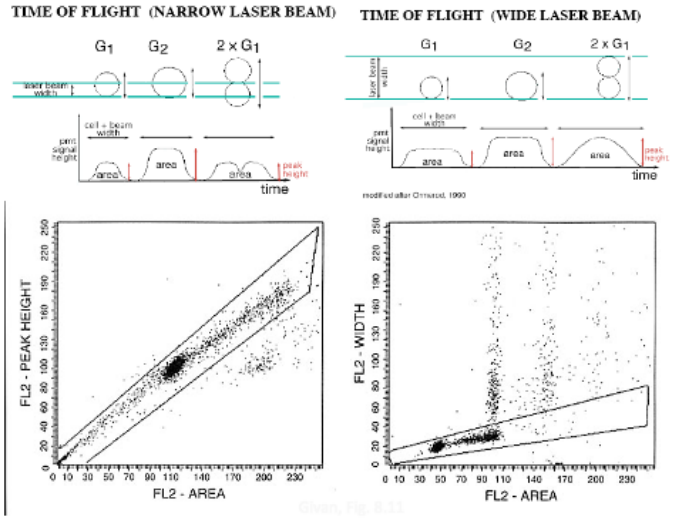
CELLS FLOWING THROUGH A LASER BEAM: with a wide core, the cells are not equally illuminated and multiple cells may coincide in the laser beam.



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



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
Alice Givan, 2001, Wiley-Inc Liss, NY

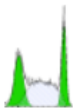
Doublets can be "eliminated" via gating (as shown in this slide) or via the software used for analysis (slide 97) since all of the modeling programs have doublet and debris algorithms.

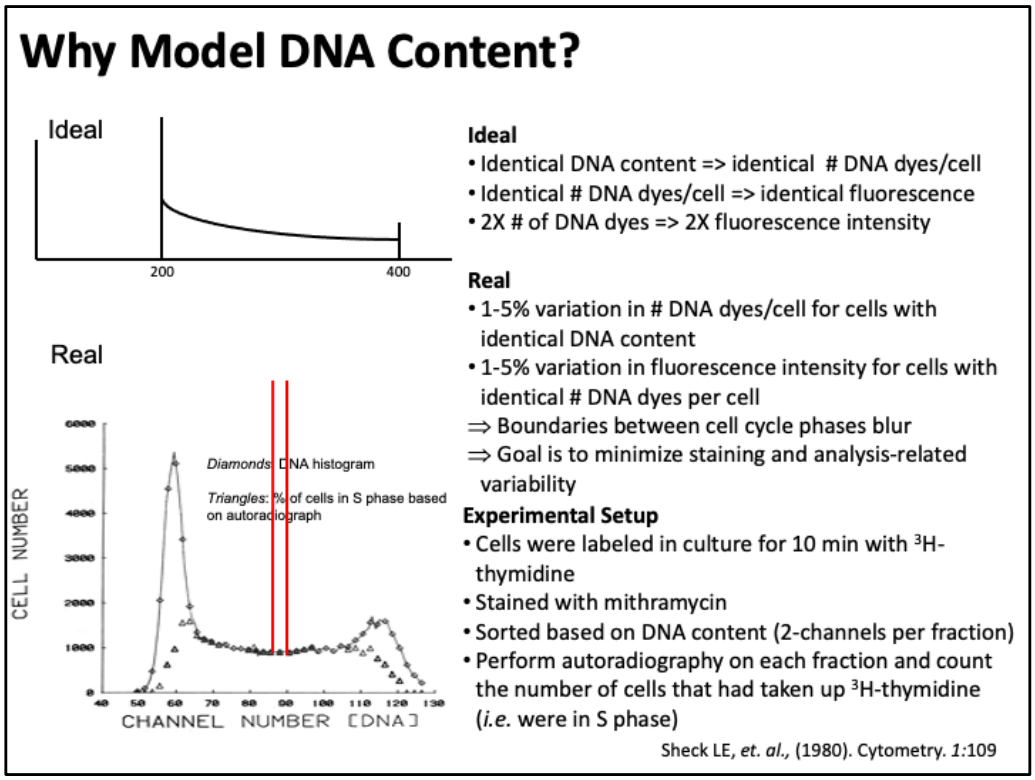
As each cell or event passes through the laser (upper row) a pulse is detected by the PMT (middle row) which is processed by the electronics. This includes a measure of the signal's width (the time it takes for the cell to pass through the laser beam), its height (the maximum fluorescence during this passage), and its area (the total fluorescence emitted during this passage). Normally fluorescence from cells stained with a DNA dye is analyzed using area as this is the most precise measurement of DNA content, and the other parameters can be used to eliminate most doublets. One large G2M nucleus will pass through the laser beam more quickly then will two smaller aggregated G0G1 nuclei even though they have the same total DNA content. With proper hydrodynamc focusing, the doublets will normally pass oriented perpendicularly to the beam and therefore their pulse height will be similar to a singlet on a narrow beam cytometer. Because of these characteristics pulse area *vs*. pulse height can be used to eliminate most doublets on a narrow beam cytometer and pulse area *vs*. pulse width can be used to eliminate most doublets on a wide beam cytometer (bottom row). The left dot plot was acquired on a Beckman Coulter cytometer and the right dotplot was acquired on a Becton Dickinson cytometer.

Eliminating doublets using pulse shape analysis *vs*. an alternative approach is a matter of personal preference. Doublets can also be eliminated using a software algorithm as described in slide 97. The software approach is more reproducible but **in practice neither approach eliminates all doublets**. It is critical, however, that these techniques never be combined as the data eliminated by the pulse shape method is required by the software approach to estimate doublets.

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





Cell sorting and ^{3}H-thymidine auto-radiography were used to define the distribution of S phase cells in a flow cytometric histogram. In the "real" histogram (bottom left) note the overlap of S phase cells based on autoradiography into regions that would be generally considered to be either G0G1 or G2M. For this reason simply drawing straight lines to bisect the G0G1 and S regions or the G2M and S regions would underestimate the actual number of events in S phase. Any software used to calculate the % S phase should take into consideration this overlap. This experiment verified that the Dean and Jett (S-Fit) approach for the analysis of DNA histograms (see slide 33) correctly estimated S phase.

In this experiment, mouse lymphoma cells (L5178Y) with a doubling time of 9.5 hours were pulse labeled for ten minutes in medium containing ^{3}H-thymidine. The labeling period was terminated by the addition of 10 mM excess cold thymidine. Cells were immediately washed and then fixed in 95% ethanol. After fixation the cells were washed and then stained with 100 ug/mL mithramycin in 0.85% NaCl and 15 mM MgCl at a concentration of 1 x l0^{6} cells/mL. The 457 nm argon ion laser line with 200 mw power output was used to excite the mithramycin and a a 515 nm long pass filter was used for detection. The DNA histogram was displayed on a 128 channel pulse height analyzer.

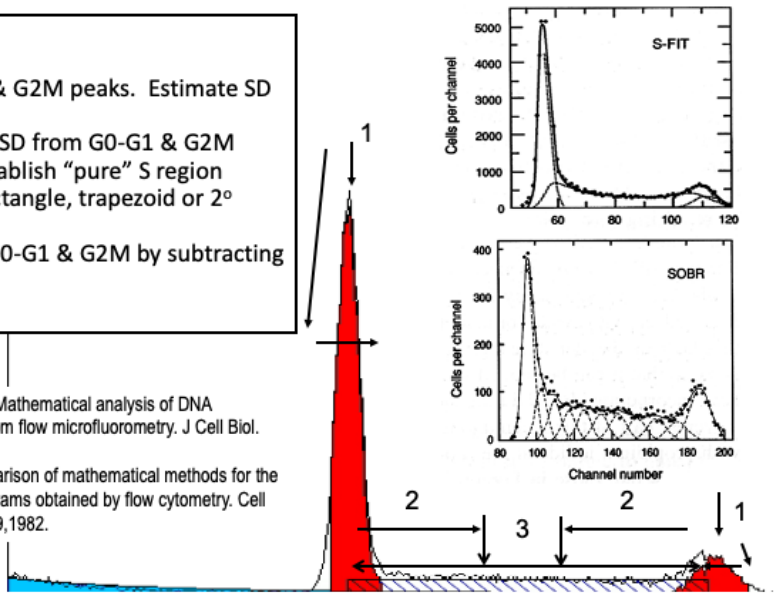
Cells were analyzed for DNA content and simultaneously sorted onto glass slides in successive 2-channel windows from G0G1 to G2M regions of the histogram. The slides were then air dried, fixed with ethanol, dipped into an emulsion and developed 7-9 days later. Each slide was blindly counted (500 cells/slide) and the number of cells with 3 or more grains scored as positive.

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

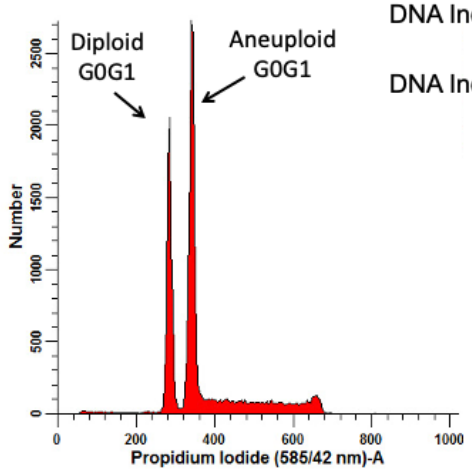
Dean PN and Jett JH. Mathematical analysis of DNA distributions derived from flow microfluorometry. J Cell Biol. *60*:523-7, 1974.
Baisch H *et al*. A comparison of mathematical methods for the analysis of DNA histograms obtained by flow cytometry. Cell Tissue Kinet. *15*:235-49,1982.



Similar to the Outside In method (slide 32), the Inside Out method is an older method used when computer processors were much slower. It starts by finding the G0G1 and G2M peaks usually with the assistance of the operator. Then like the Outside In approach it calculates the ½ height SD for each. From here the methods diverge. The Inside Out approach then moves 2 or 3 of the G0G1 widths (SD's) to the right of G0G1 and drops down an anchor point. It does the same for G2M, moving 2 or 3 of G2M SD's to the left of the G2M peak and dropping down a second anchor. Each anchor point is assumed to represent a region of pure S and these are used to fit the S phase fraction. In the model shown above a rectangle is drawn intersecting the top of S phase at the two anchor point. It stops at the G0G1 peak position on the left and at the G2M peak position on the right. Everything within in the box is set to represent S phase cells. What remains to the left is considered G0G1 and to the right G2M events.

Besides a rectangle (large figure), trapezoids, broadened 2nd degree polynomials (S-FIT, upper right) and multiple broadened rectangles (SOBR, middle right) have been used in this approach to fit S phase.

DNA Diploid *vs.* DNA Aneuploid



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

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

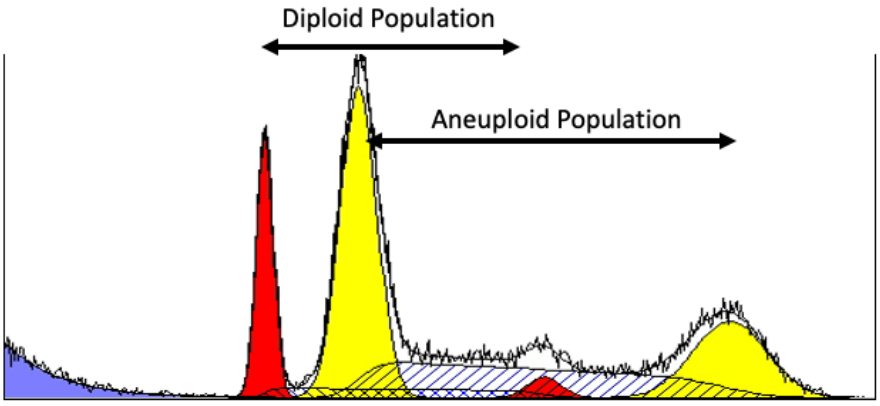
Tumor samples may contain multiple cell types:

- 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

To complicate matters even more some clinical samples come from tumors with an aberrant (aneuploid) amount of DNA. Such samples usually include both the tumor and some normal diploid stromal cells as illustrated above. It was determined that in this sample the G0G1 diploid stromal cells are the large peak on the left and the G0G1 aneuploid tumor cells are the large peak on the right. We can quantify the degree of abnormality in the tumor DNA content by dividing the peak position of the G0G1 tumor cells by the peak position of the G0G1 diploid cells. This is usually referred to as the DNA Index (DI) which in this case is 291.8 / 222.4 = 1.31.

The resulting DNA histogram is composed of a complex set of peaks and continuous distributions some of which are overlaid upon each other, making it very difficult to figure out proportions of all the cell fractions that make up this DNA histogram.

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



The simple models discussed so far work reasonably well for diploid cell lines and asynchronously cycling cell populations but are unable to handle more complex situation like the mixed diploid and aneuploid cycling populations shown here. To address these more complex situations a modeling software should be used. The first step is to define the correct mathematical model that is most appropriate for the data to be analyzed. Often these models can be quite complex and most times there are numerous decisions to be made about the interpretation of the data. For an excellent discussion of DNA modeling with the nonlinear least squares method see Bagwell, C.B. Theoretical aspects of flow cytometry data analysis. *In* Clinical flow cytometry principles and application, Edited by Bauer, K.D., Duque, R.E., and Shankey, T.V. (1993) pp. 41-61.

A model is a list of mathematically derived components that are bound together by a number of dependencies. An example of dependency is that the G2M peak position should theoretically be twice the position of the G0G1 peak. Similarly the standard deviation of the G2M component should be twice that of the G0G1standard deviation. Another dependency is that the position where S phase begins is the same as the G0G1 peak position and S phase ends at the G2M peak position. There are many dependencies that can be used to accurately model a complex DNA histogram. These can be used as set (fixed) dependencies or they can be used to provide initial estimates for the model component.

The second major part of DNA modeling is the nonlinear least squares algorithm. This attempts to find the best set of parameters that control the shape position in area of all the model components. The goal is that when combined, the model components best match the actual data. The goodness of fit is generally evaluated using a chi-square test which is the summation of each (observed minus the calculated value)^2/ observed value. The higher the chi-square value the poor the fit. Consider a G0 G1 population. The model components are the peak position and the width or standard deviation. In a nonlinear least squares analysis the position and width of the model component are repeatedly changed until the model component best fits the observed data as evaluated by a chi-square test. This process is used to fit all of the model components.

There are a number of model components that are used in the analysis of DNA histograms and these will be defined in the next series of slides.

For additional details see: http://vsh.com/Documentation/ModFitLT/mf40userguide.pdf
Rabinovitch, P. Practical considerations for DNA content and cell cycle analysis. *In:* Clinical flow cytometry: principals and application. Bauer, KD, Duque, RE, Shankey, TV. 1993 Williams & Wilkins, Balitomore, MD. Pp. 117-142.

Model Components

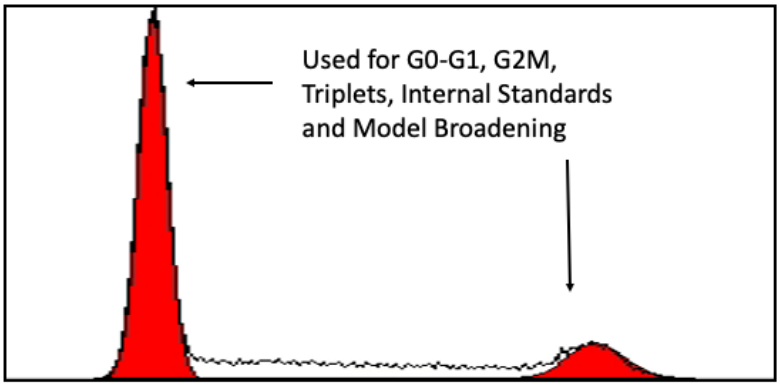
Model Component:

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

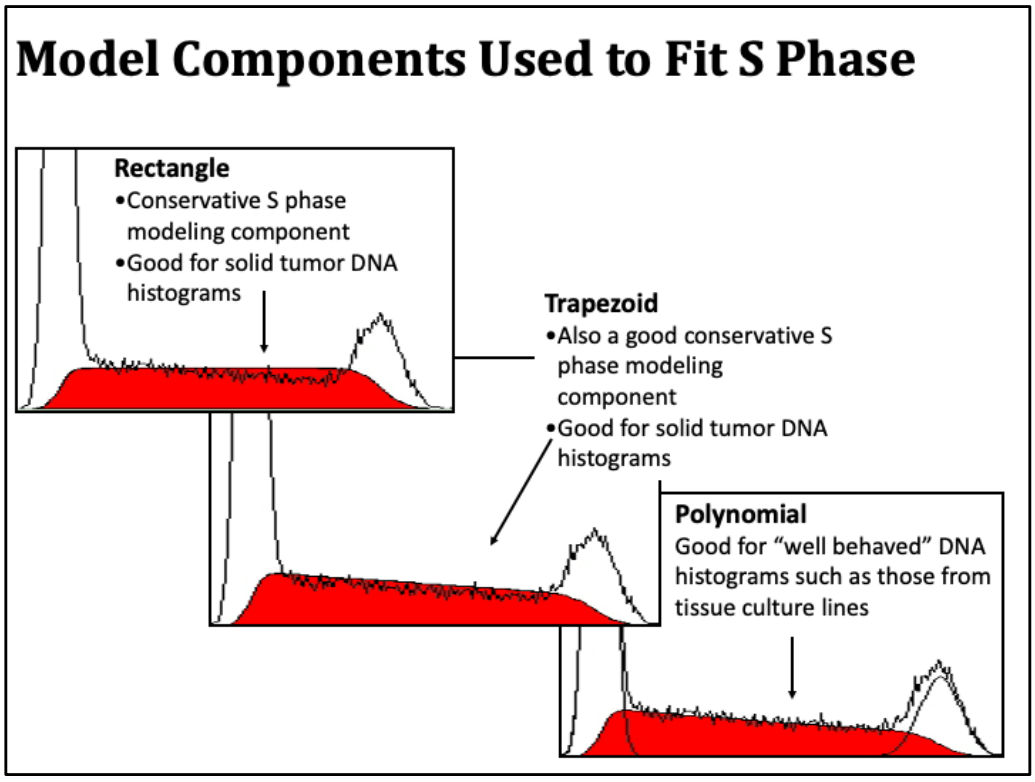
To model a DNA histogram various geometric shapes are used to fit each of the cell cycle phases (G0G1, S and G2M) and associated noise (doublets, debris etc.). The size and shape of each component is iteratively adjusted until the best fit of the real data is accomplished. The relative area for each individual shape is used to calculate its percentage of the total histogram.

Model Component: Gaussian



Gaussian's are always used to fit G0G1, G2M. They are also used to fit internal standards such as chick red blood cells (cRBC), trout erythrocytes (tRBC) and even non-cycling lymphocyte populations. A series of Gaussian's has also been proposed to represent S phase, although in general practice it is better to use the model components discussed in the next slide.

A Gaussian has a mean or peak position and width or standard deviation that can be varied during the nonlinear least squares analysis. It's not uncommon to set the G2 M peak position to be dependent on the G0G1 peak position by a factor between 1.95 and 2 .00. Similarly, G2 M with can be fixed to the G0G1 with times a multiplier between 1.95 and 2.00.

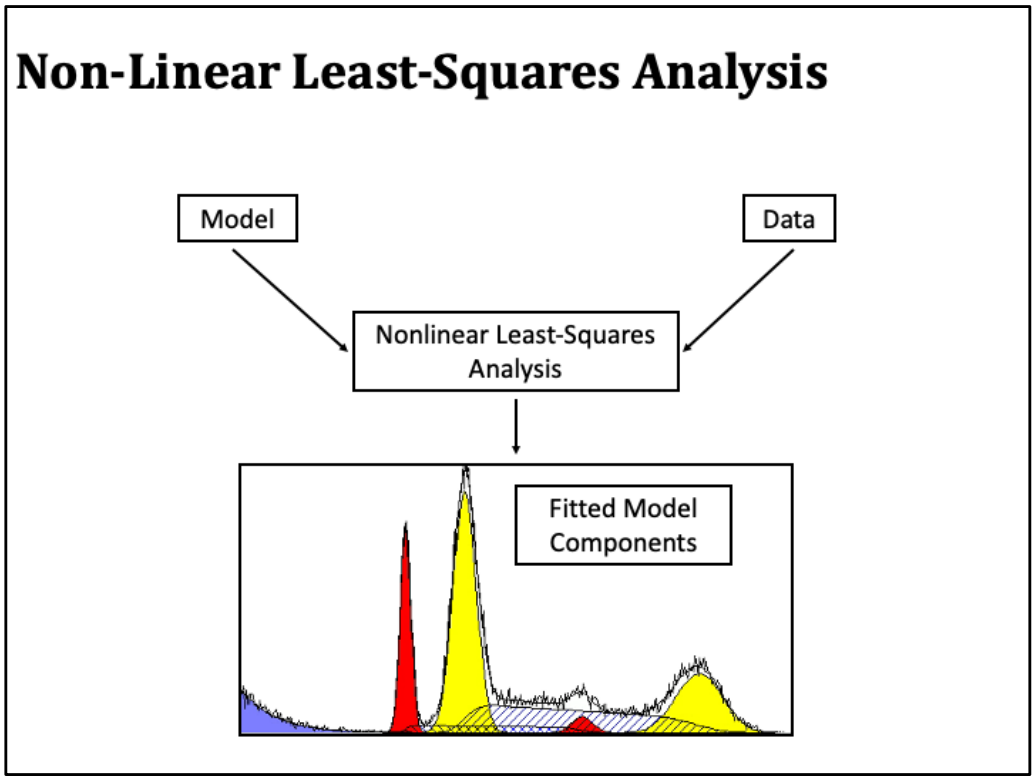


The rectangle, trapezoid, and 2nd degree polynomial are the most common shapes used to fit S phase.

The broadened rectangle is the most conservative model component used to fit S Phase. This is generally a good choice when fitting solid tumors as minor variations in the raw S phase data will have very little impact on the final S phase calculation. The beginning of S phase is usually set at the G0G1 peak position and it terminates at the G2 M peak position. A broadening function is usually applied at each end and is basically a half Gaussian used to broaden its shape. Multiple broadened rectangles can be used to fit S phase to give a little bit more shape (slide 33, SOBR plot). There is no correct number of rectangles to use, although in practice for the most conservative modeling a single rectangle is used and if multiple rectangles are chosen three is a convenient number since it splits S phase in the early middle and late compartments.

The broadened trapezoid is is also a relatively conservative model component. It is particularly useful for fitting an exponentially growing cell population such as cells in culture. Like a rectangle, it's beginning is set at the G0G1 peak position and its terminus at the G2M peak position. Broadening is applied and multiple trapezoid's can be used to fit S phase.

The broadened polynomial is the S phase component used in the original Dean and Jett DNA model. The broadened 2nd degree polynomial has 3 degrees of freedom and thus can take on numerous shapes. It is often used to fit tissue culture cell lines but is not recommended for DNA analysis of tissue samples. Because of its increased flexibility, the 2nd polynomial can give poor results for tissue samples if there is a lot of debris or if either the G0G1 or the G2M populations are skewed. See: Dean, P.N. and Jett, J.H. Mathematical analysis of DNA distribution derived from flow microfluorimetry., J Cell Biology 60: 523, 1974.

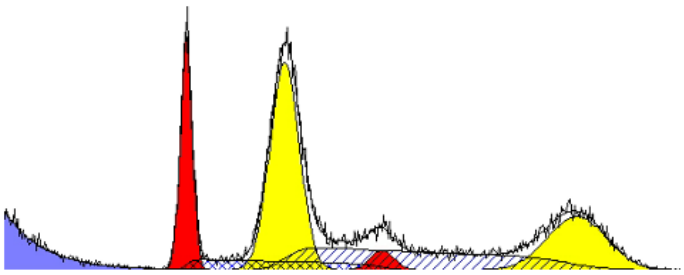


In nonlinear least squares analysis a series of appropriate model components are combined and used to fit the actual data. In the example here, four Gaussian's were used to fit the diploid and aneuploid G0G1and G2M, respectively. Two broadened trapezoid's were used to fit the diploid and aneuploid S phase. A multi-cut model component was used to fit the debris. Because the G2 M population is relatively well resolved the location and width of this Gaussian were allowed to float. In contrast, because the diploid G2M population is disguised by the aneuploid S phase the diploid G2M's Gaussian peak location and width were made dependent, by a factor of 1.98, on the diploid G0G1's position and width. The beginning and terminus of the S phase trapezoid's were made dependent on the peak location of G0G1 and G2M for both the diploid and aneuploid model components.

As mentioned earlier, the initial estimates for each model component comes from one of the simple fits (either the Outside In or Inside Out fit discussed previously). The program then iteratively fits the model components to the data calculating a reduced chi-square each time that on the next iteration systematically making minor changes to the model parameters and recalculating the chi-square value. The iterations terminate when any further adjustments to the parameter values results in an increased reduce chi-square.

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]



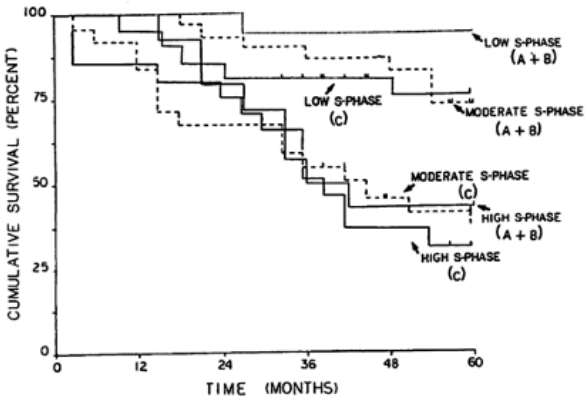
[1]An average minimum of 100 events per histogram channel over the channels of interest is suggested by Verity Software House

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

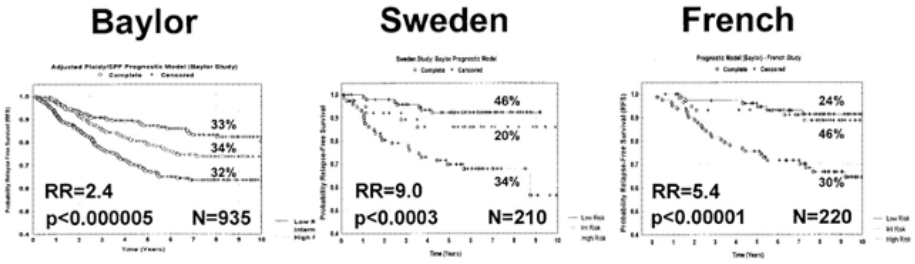
Bauer, K.D. (1993) Colorectal Neoplasia. In: Clinical flow cytometry, principles and applications. Edited by Bauer K.D. et. al. Williams & Wilkins, Baltimore. pp 307-317.

Both DNA ploidy and S phase fraction have been extensively studied in colorectal cancer. As shown in the table at lower left, some of these studies have found significant prognostic value in determining DNA ploidy, while others have not. Similar discordance was seen for S phase fraction. While the Bauer study (lower right graph) shows a significant survival advantage for colorectal cancers with a low S phase, other investigators have found no significance. These discrepancies are perhaps due to technical difficulties, such as eliminating debris from S phase calculations, the presence of overlapping cycling populations and the varied analysis strategies available. For example Chen, Y.T. et al. found that the proliferation marker, Ki-67, was significantly associated with disease recurrence whereas in the same study DNA index and S phase fraction were not. This discordance between studies led the American Society of Clinical Oncology to conclude "the data are insufficient to recommend the routine use of DNA index and DNA flow cytometric proliferation analysis" for colorectal cancers.

Chen YT, Henk MJ, Carney KJ, Wong WD, Rothenberger DA, Zheng T, Feygin M, and Madoff RD. (1997) Prognostic significance of tumor markers in colorectal cancer patients: DNA index, S phase fraction, p53 expression, and Ki-67 index. J Gastrointest Surg. *1*:266-72.

American Society of Clinical Oncology. Clinical practice guidelines for the use of tumor markers in breast and colorectal cancer. (1996) J Clin Oncol. *14*:2843-77

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

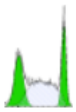


- If the S phases are not adjusted for both the aneuploid fraction and the diploid normal dilution effects, there is a significant correlation between DNA ploidy and S phase fraction, 0.42, and a modest *p*-value
- Statisticians would normally either drop the S phase or the DNA ploidy in the analysis 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

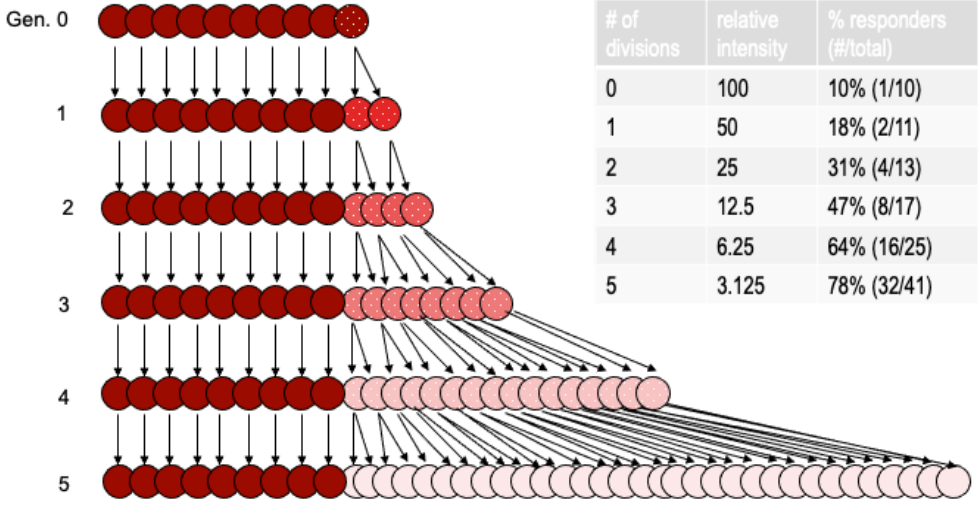
THE PLAN

- 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)

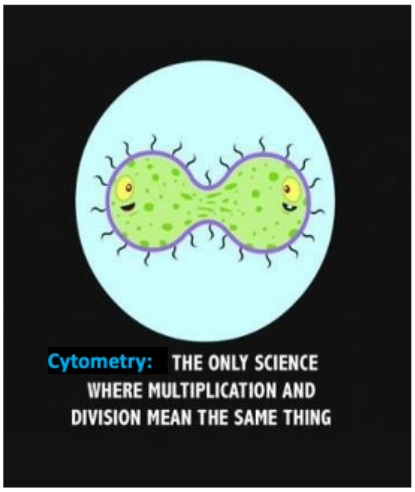
Adapted from Givan *et al.* (2004) Methods Mol Biol, 263: 109–124

This type of assay allows the extent of proliferation for a given cell subset within a complex population can be monitored without having to isolate and count the cells of interest. Stable "proliferation tracking dyes" that distribute approximately equally between daughter cells at each division can be combined with antibodies, tetramers/multimers or other probes to analytically or physically isolate the cell subset(s) of interest.

This cartoon illustrates what happens to the distribution of intensities for a population of cells stained at T0 with a proliferation tracking dye when 1 cell in 10 in the original population responds to a stimulus by proliferating:

- Responders outgrow non-responders (if 10% of the original cells respond and continue to divide, the number of responding cells almost equals the number of non-responders after 3 rounds of cell division, and by the 5th daughter generation, responders outnumber the quiescent cells by almost 4:1)
- Responders become progressively less bright compared with non-responders (cells in the 5th daughter generation are 1/32 as bright as the cells that haven't undergone any division)

Key Assumptions for Cell Division Monitoring based on Dye Dilution

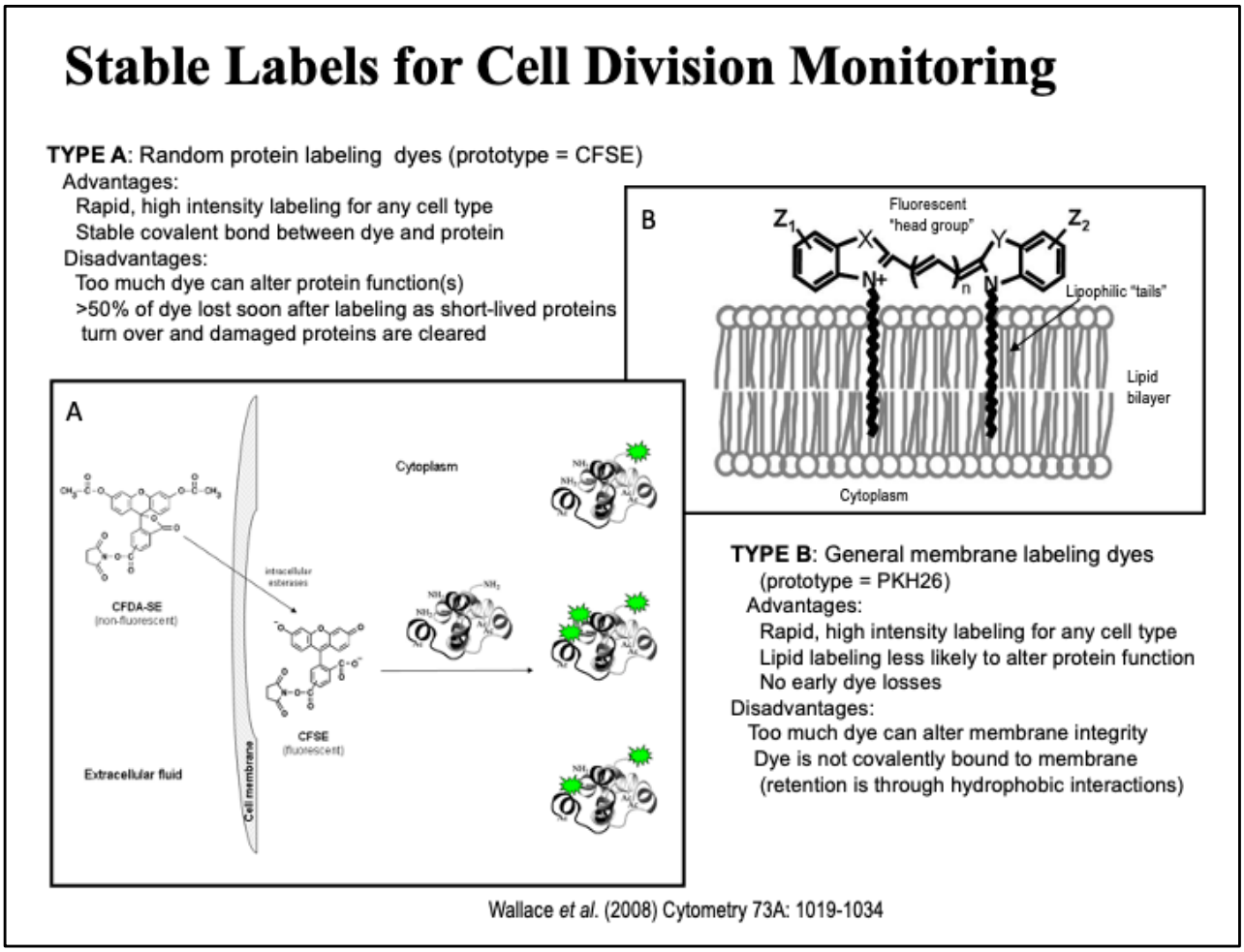


- Decrease in fluorescence intensity is proportional to increase in cell number

  $\Rightarrow$ 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

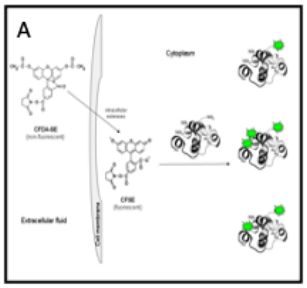


Commercially available cell tracking dyes vary widely in their chemistries and fluorescence properties but the great majority fall into one of two classes based on their mechanism of cell labeling:
A) "Protein dyes", typified by CFSE, are amino-reactive dyes that form stable covalent bonds with cell proteins;
B) "Membrane dyes", typified by PKH26, are highly lipophilic dyes that partition stably but non-covalently into cell membranes.

Each class has its own advantages and limitations, which are discussed in the next several slides. The key to their successful use is therefore to understand the critical issues enabling optimal use of each class, particularly in multicolor studies where multiple dyes are used to track different cell types. For detailed labeling protocols and typical assay setups and controls, see the following references:

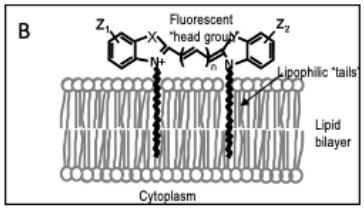
Givan A.L. Meth. Mol. B iol. 263, 109-24 (2007)
Quah *et al*. Nature Protocols 2, 2049-56 (2007)
Wallace *et al*. Cytometry 73A, 1019-1034 (2008)
Quah & Parish J. Vis. Exp. 44, e2259 doi:10.3791/2259 (2010)
Tario *et al*. Meth. Mol. Biol. 699, 119-164 (2011)
Quah & Parish. J. Imm. Meth. 379, 1-14(2012)
Tario *et al*. J. Vis. Exp. 70, e4287, doi:10.3791/4287 (2012)

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

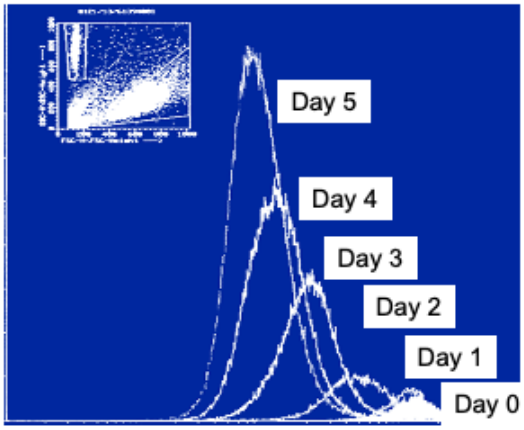
General Membrane Labeling Dyes



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

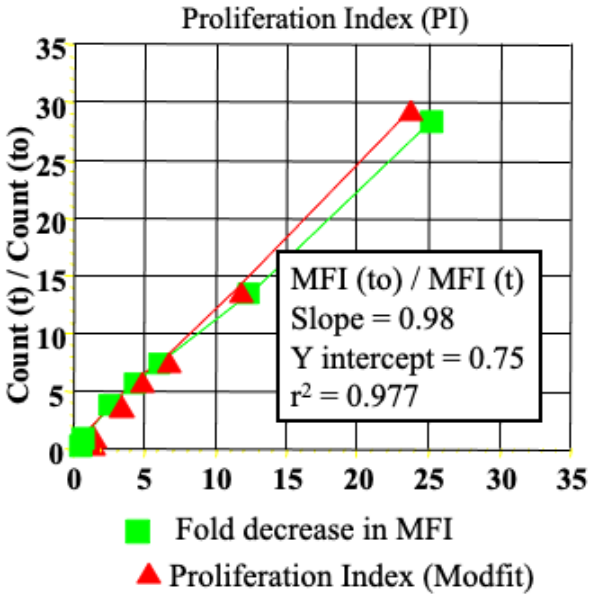
Does PKH26 Dye Dilution Track Increase in Cell Number in Simple Systems? YES

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)

To be useful for proliferation monitoring, a tracking dye should not only exhibit stable cell association. It also needs to:

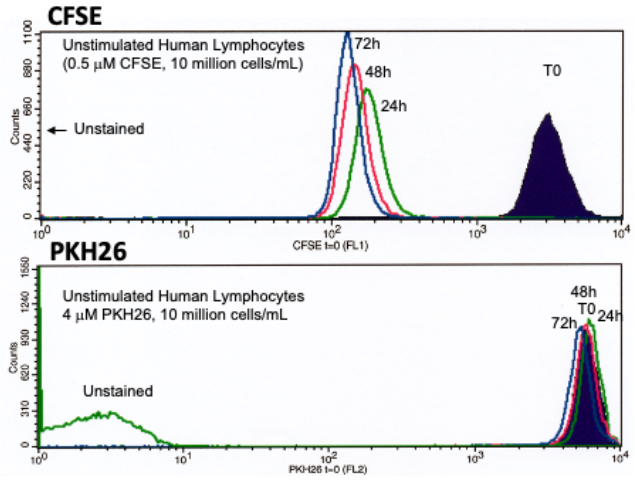
1) Be known to behave as shown in a simple system where a single cell type is growing logarithmically (i.e., dye dilution -- fold decrease in tracking dye intensity -- should correlate linearly with fold increase in cell count).
2) Have no effect on viability, growth rate or other functions when cells are labeled at initial intensities high enough to follow the desired number of cell divisions.

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	

Overall there are more similarities than differences in the critical issues required for successful use of each class of cell proliferation dyes. The major differences between the two classes lie in the techniques required for achieving optimal labeling and how soon after labeling cell division monitoring can be started.

Critical Controls:
Dye Positive but Unstimulated



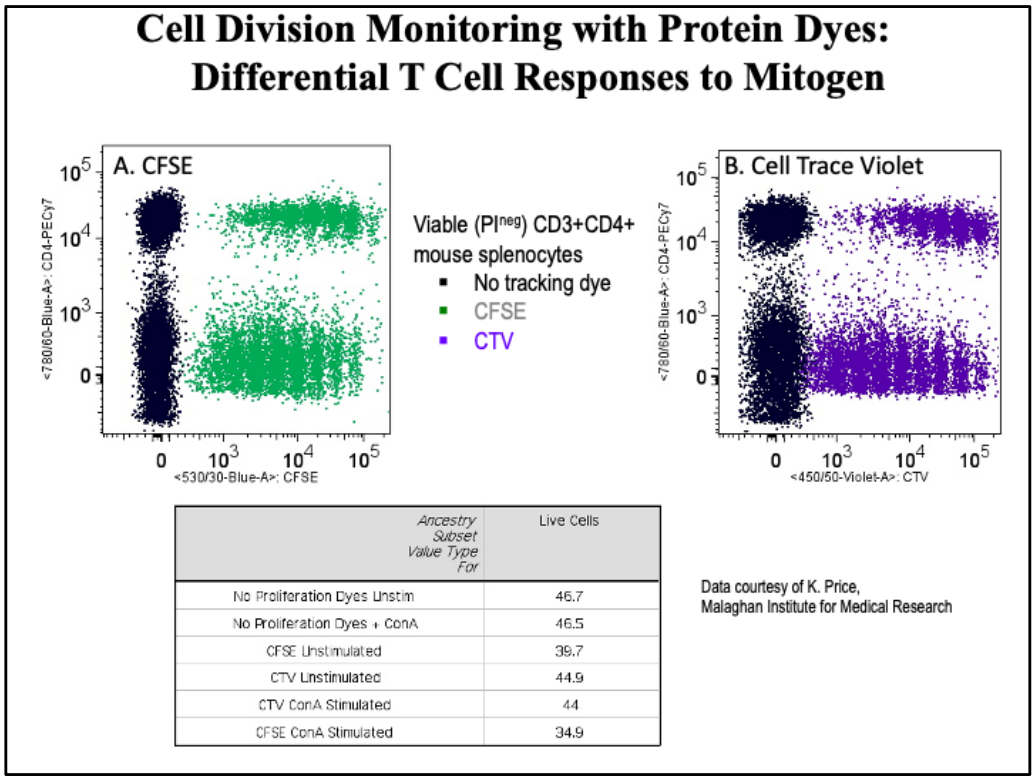
Unstimulated cells stained with proliferation tracking dye are used for:

1) Instrument setup (brightest sample*)
2) Color compensation (brightest sample*)
3) Biological negative control (confirm intensity of non-responders)

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

Self Test: Which of the unstimulated samples shown in the upper panel is the best compensation control for a proliferation assay based on dye dilution?
Answer: Usually the brightest sample (here T0) would be chosen as the single color control to set compensation. For a protein dye like CFSE, however, that is not appropriate. The T0 cells are much brighter than any undivided cells that would be present in test samples post-stimulation because dye intensity drops dramatically during the first 24 hours post-labeling even in the absence of stimulation (compare T0 and T24 data shown). Detector voltage would then have to be increased to place negative controls for T24 and later times at the upper end of the intensity scale, and changing detector voltage would require different compensation settings. The best choice for the single color compensation control in this case would therefore be the unstimulated but CFSE stained control from the same time point in the assay as the stimulated sample being analyzed.

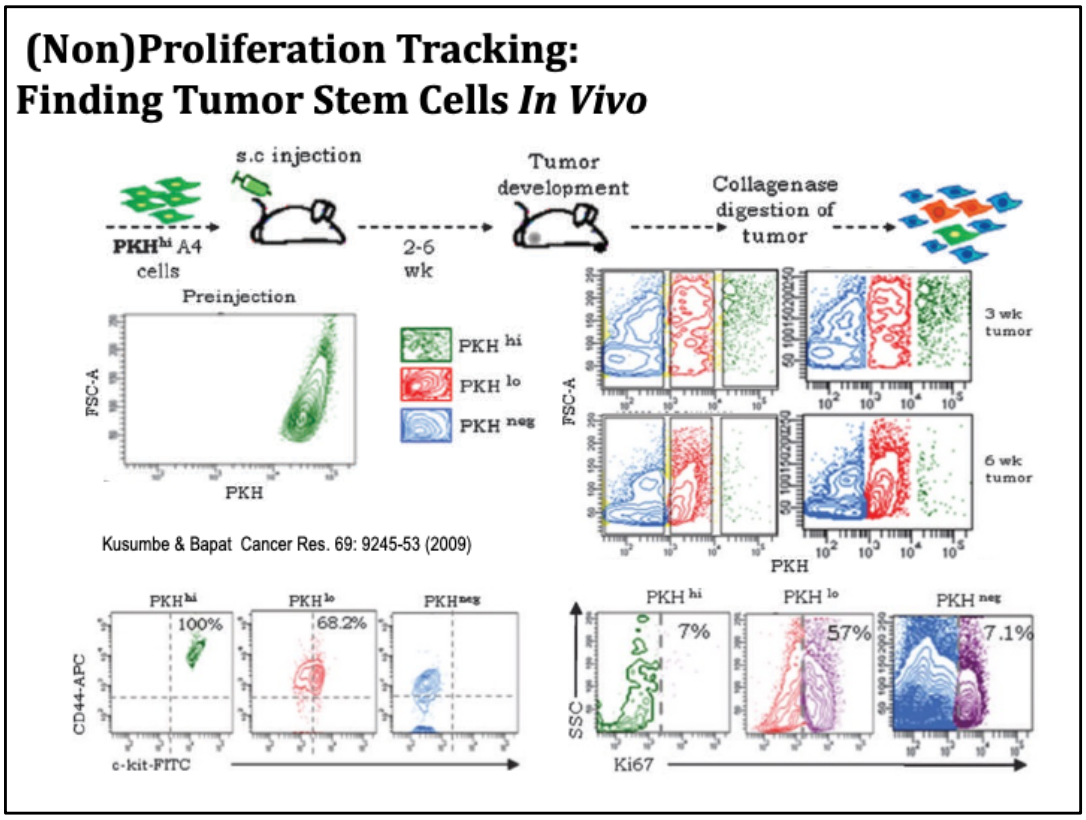
Self Test: Which of the unstimulated samples shown in the lower panel is the best compensation control for a proliferation assay based on dye dilution?
Answer: The best choice would again be the unstimulated but PKH26 stained control from the same time point in the assay as the stimulated sample being analyzed. In this case, however, it would also be possible to use the unstimulated controls one of the other time points since membrane dyes do not typically exhibit early intensity losses unrelated to cell division and all of the negative controls can be run at the same instrument settings.



Proliferation dye labeling at T0 followed by harvest and counterstaining with subset-specific probes at selected time points post-stimulation allows assessment of differential proliferative responses within complex populations where it would be difficult or impossible to quantitatively recover and directly count the subset(s) of interest. In this example, murine splenocytes (1x10^7cells/mL) were stained at T0 with 1.2uM CellTrace CFSE or 5 uM Cell Trace Violet (CTV) and placed in culture for 4 days with the T cell mitogen Concanavalin A (1.5 ug/mL). Cells were then harvested and counterstained with anti-mouse CD3e-APC (eBiosciences #17-0032-82, clone 17A2, 0.5µg/mL), PI, and anti-mouse CD4-PE-Cy7 (BD Pharmingen #552775, clone RM4-5, 1µg/mL) and analyzed on an FACSCanto II.

Data shown in panels A (CFSE) and B (CTV) were gated on viable (PI Negative) CD3 positive cells with lymphocyte FSC and SSC characteristics.

- For both proliferation dyes, extent of cell division was clearly less in the $CD4^{pos}$ population than in the $CD4^{neg}$ population (upper panels) and as evidenced by the presence of more cells with intensities of $10^4 – 10^5$ and fewer cells with intensities of $10^3 – 10^4$.
- No cell toxicity was seen due to proliferation dye labeling as indicated by similar viabilities for stained vs. unstained cells and unstimulated vs. stimulated cells (lower middle panel)
- For With this strong polyclonal stimulus up to 8 daughter generations could be visualized before the intensity of highly divided cells began to overlap with that of controls not stained with a proliferation dye (black dots)



Isolation and characterization of rare non-proliferating cells from tumors grown *in vivo* suggests that these cells have stem-like properties.

Experimental Strategy

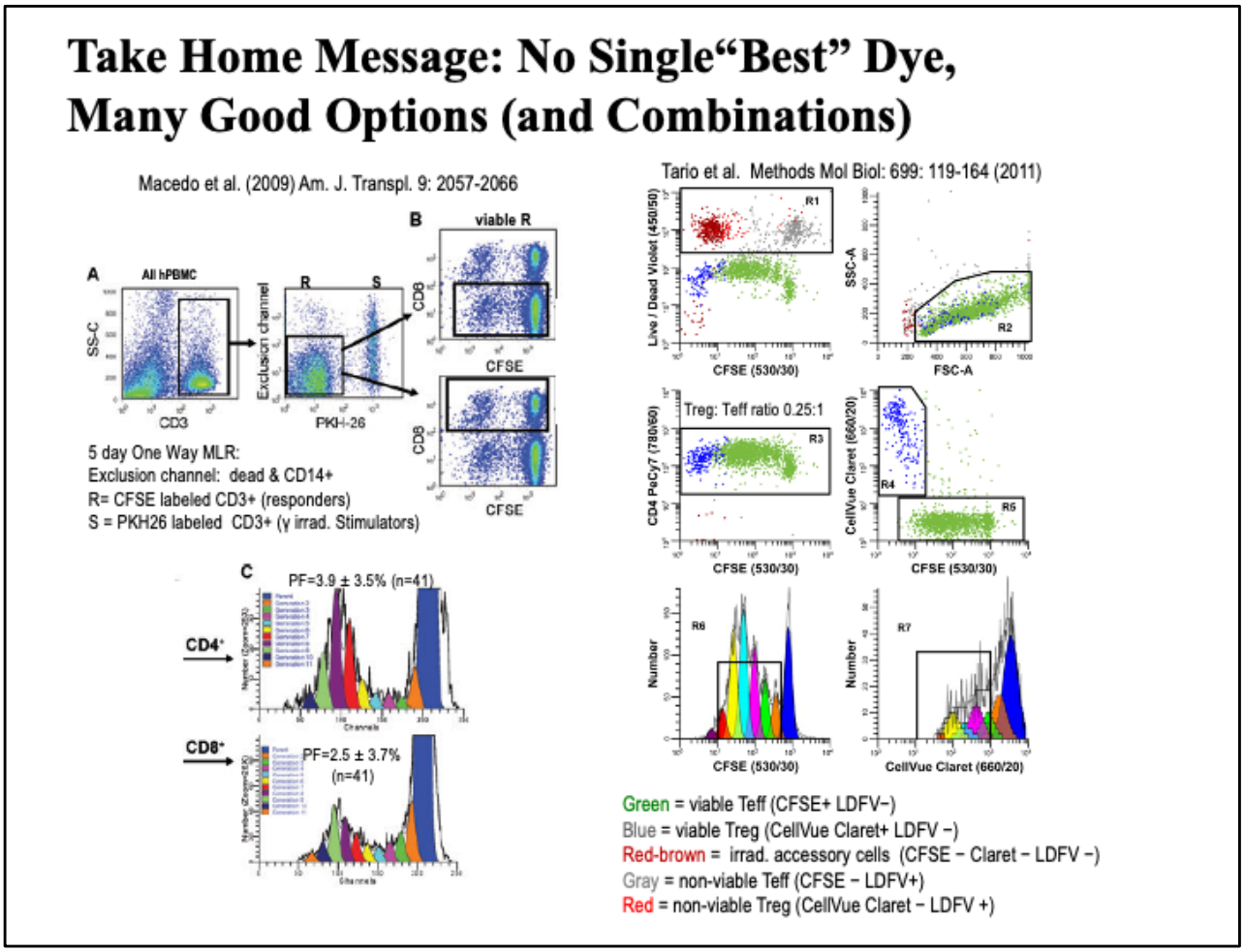
- Implant PKH67 (or PKH26) labeled A4 human ovarian tumor cells in NOD/SCID mice
- Harvest tumors, prepare single cell suspensions and analyze frequency of PKH^{hi} (slow-cycling/quiescent), PKH^{low} (limited divisions) and PKH^{neg} (multiple rapid divisions) populations
- Counterstain for tumor stem cell markers
- Sort for *in vitro* colony formation and *in vivo* tumorigenicity determination

Note: Although not indicated on the headers for the panel C contour plots, PKH26 was used for the study in panel C where cells were counterstained with c-kit-FITC (personal communication, S. Bapat).

Conclusions

Sorted PKH^{hi} tumor cells showed properties consistent with those expected for ovarian tumor stem cells

- Increased clonogenicity and tumorigenicity (not shown)
- Increased expression of Nanog and Oct4 by semiquantitative PCR (not shown) compared with PKH^{med} or PKH^{neg} cells
- Elevated expression of the stem cell markers CD44 and c-kit compared with PKH^{med} or PKH^{neg} cells
- Proliferative quiescence as indicated by lack of expression of Ki67



The answer, as with everything in flow cytometry (or science), is "It Depends". Now you know some of the variables to consider as you choose (and use) a dye for cell division monitoring. Paul Wallace will now describe some further considerations, in particular those related to choice of analysis method to be used in quantifying and comparing extent of proliferation among different populations or samples.

MACEDO details:
Precursor frequency can also serve as an alternative to ^{3}H-TdR for quantifying T cell responses to transplantation antigens present on lymphocytes from different individuals. Using PKH26 to tag the stimulator cells in a mixed lymphocyte reaction and CFSE to tag the responder cells, Macedo and colleagues found that CD4 and CD8 T cells both exhibited low but reproducible alloreactive precursor frequencies (something not previously detected by traditional ^{3}H-TdR MLR).

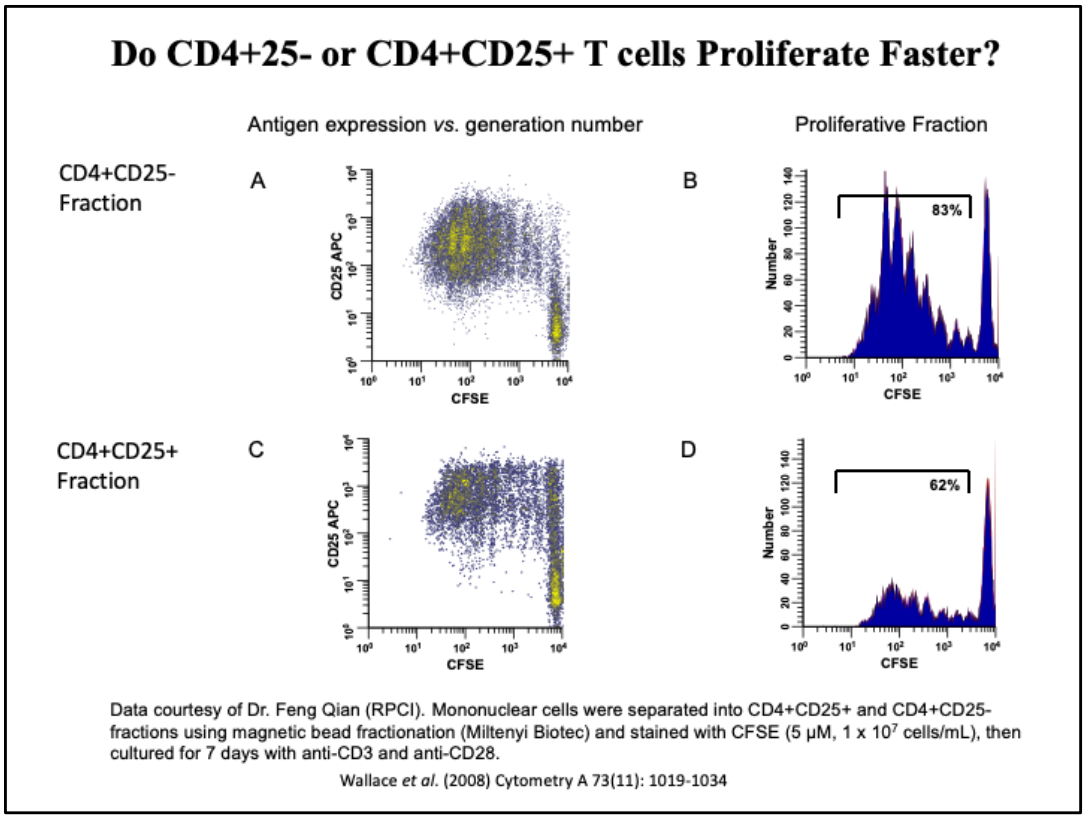
TARIO details:
Regulatory CD4 T cells (Treg) down-modulate immune responses and are functionally defined by their ability to suppress the proliferation of effector CD4 T cells (Teff). Flow cytometric suppression assays in which Teff are labeled with a tracking dye to monitor proliferation have increasingly replaced ^{3}H-TdR assays. However, in the flow assay it can be difficult to reliably distinguish highly proliferated tracking dye^{dim} Teff from unlabeled Treg, since both populations express similar levels of CD4.
Data from Tario and colleagues illustrate how use of a 2nd tracking dye simplifies discrimination between Treg (here labeled with CellVue Claret) and highly proliferated Teff (here labeled with CFSE) – compare center left with center right dot plots Labeling conditions for Treg were verified not to interfere with ability of the Treg to inhibit Teff proliferation (not shown) and use of the 2nd tracking dye also allowed assessment of both the effect of Treg on Teff prolferation (lower left) and the effect of Teff on Treg proliferation (lower right).
NOTE: Clearly it would have been desirable to accumulate more cells for the Treg proliferation profile to reduce the counting related error

"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

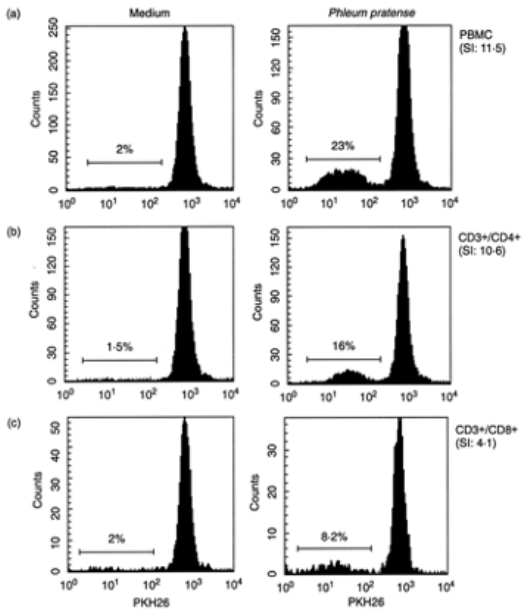
Numerous ways have been devised to compare the extent of proliferation based on dye dilution profiles and these can generally be divided into two groups: eyeball methods which are descriptive or semi-quantitative and quantitative methods that rely on modeling. Each of these will be discussed in the following slides.



Descriptive Analysis. Describing trends in antigen expression across generations is an easy and perfectly satisfactory approach to analyzing how expression of surface antigens or cytokines changes across daughter generations. Panels A and C can illustrate this approach in a study where CD4+ cells were separated into CD25+ and CD25− fractions, stained with CFSE, and then stimulated with anti-CD3 and CD28 for 7 days. Qualitative examination of this figure reveals that in the CD4+CD25− fraction (Panel A), non-proliferating cells are CD25 negative but CD25 expression is seen as early as the first generation of daughter cells and is retained through subsequent daughter generations. In contrast, in the CD4+CD25+ fraction (Panel C) nearly half of the non-proliferating cells lack CD25 expression and overall extent of proliferation is less than in the CD4+CD25− fraction.

Proliferative fraction. In this approach the results are simply expressed as percent of cells that have undergone one or more divisions (panels B and D). The stained but unstimulated control (not shown) is used to set the upper boundary for enumeration of daughter cells. If the test sample contains unstained cells (e.g., accessory cells, allogeneic stimulators, etc.), an unstained control containing these cells, but treated similarly to the stained sample, is used to the define lower boundary for enumeration of proliferating cells. The Proliferative Fraction is then defined as the percentage of proliferating cells with fluorescence intensity less than the stained but unstimulated control and more than the unstained control. Using this approach gives a Proliferative Fraction of 83% for the CD4+CD25− sample after 7 days in culture (Panel B) vs. 62% for the CD4+CD25+ sample (Panel D), confirming reduced proliferation in the latter population.

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

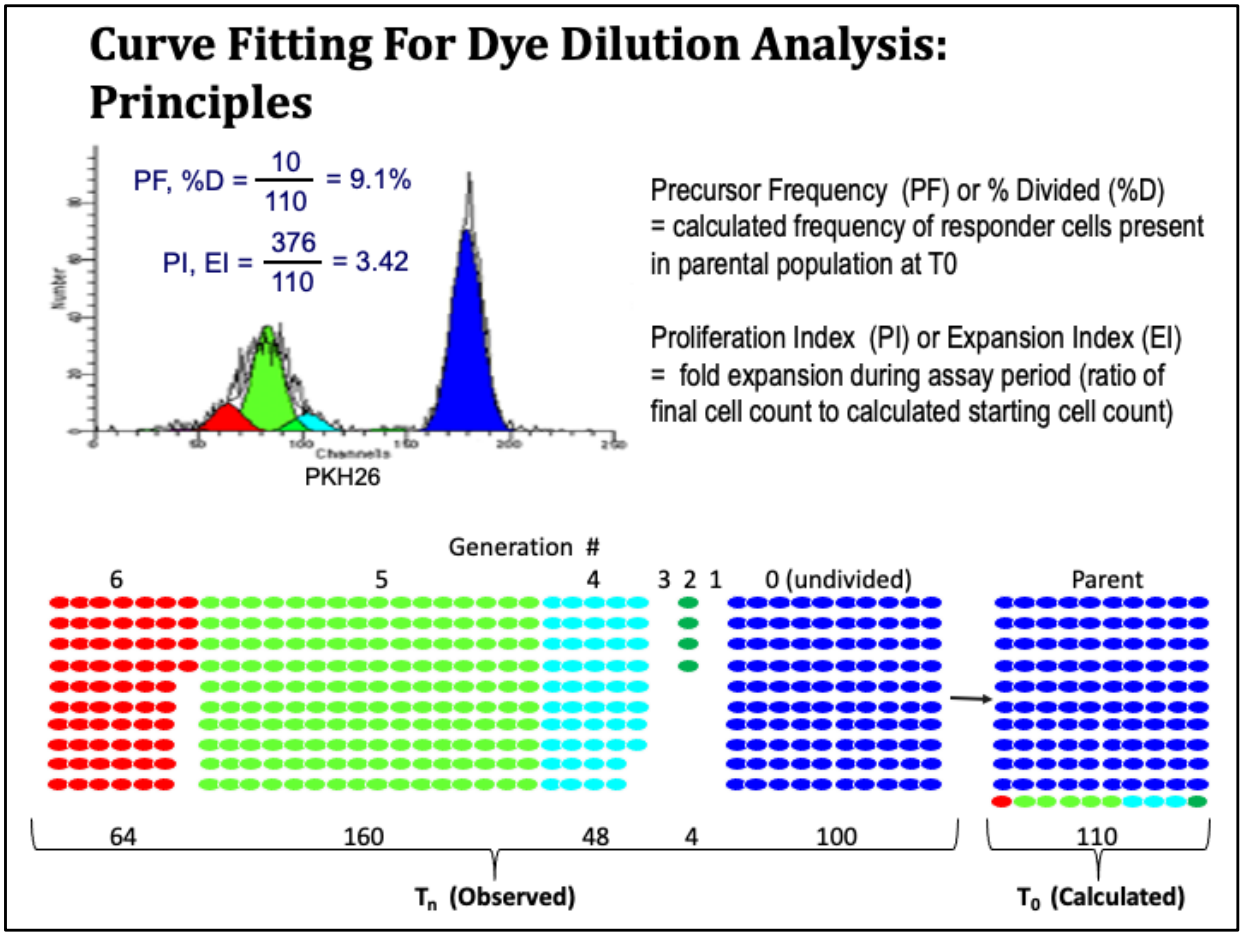
SI = % proliferating cells (+ stim.) / % proliferating cells (unstim.)

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

In this experiment PBMCs were stained with PKH26 and cultured with with *P. pratense* (an abundant perennial grass also known as Timothy grass) for 11 days. The proliferative fraction was then calculated as described in the previous slide for both the stimulated and the unstimulated control culture. To calculate the stimulation index (SI) the proliferative fraction (*i.e.* % proliferating cells) from the *P. pratense* culture was divided by the control's proliferative fraction.

In this example, both CD4 and CD8 cells responded to with *P. pratense*, however most of the proliferating cells in the PBMC were CD3+ CD4+. Compare the total PBMC SI of 11.5 to the CD3+ CD4+ SI of 10.6 vs. the CD3+ CD8+ SI of 4.1.

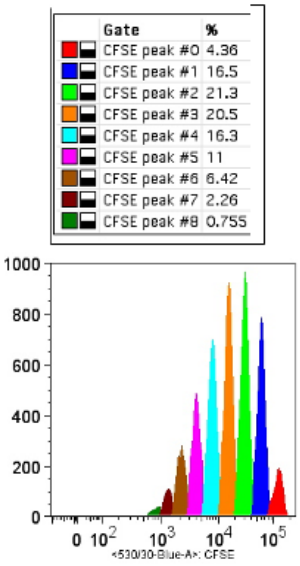


Modeling the number of cells in each generational peak allows calculation of how many cells in the original population must have gone on to divide how many times in order to generate the observed fluorescence intensity profile. Fold increase in cell number during the stimulation period (Proliferation Index in Modfit LT and FCS Express, % Divided in FlowJo), can then be calculated.

Precursor Frequency (ModfitLT) or % Divided (FlowJo, FCS Express) gives an estimate of the fraction of cells initially present that went on to respond to stimulation by proliferating. In this illustration, there were theoretically 64 events in generation 6 at the time this dataset was collected, they must have arisen from 32 events in generation 5, which must have arisen from 16 events in generation 4, which must have arisen from 8 events in generation 3, which must have arisen from 4 events in generation 2, which must have arisen from 2 events in generation 1, which must have arisen from 1 responder cell in the starting (parent) population. Similarly, the 160 events in generation 5 are calculated to have arisen from 5 responder cells in the parent population, the 48 events in generation 4 from 3 responder cells in the parent population, etc. In this hypothetical example 10 cells out of the calculated original population of 110 cells went onto proliferate,(*i.e.*, the precursor frequency or % divided was 9.1%), giving rise to a total of 64+160+48+4+100 = 376 cells at the end of the assay period, *i.e.* a proliferation index or expansion index of 376/110 =3.42.

Proliferation Modeling Statistics

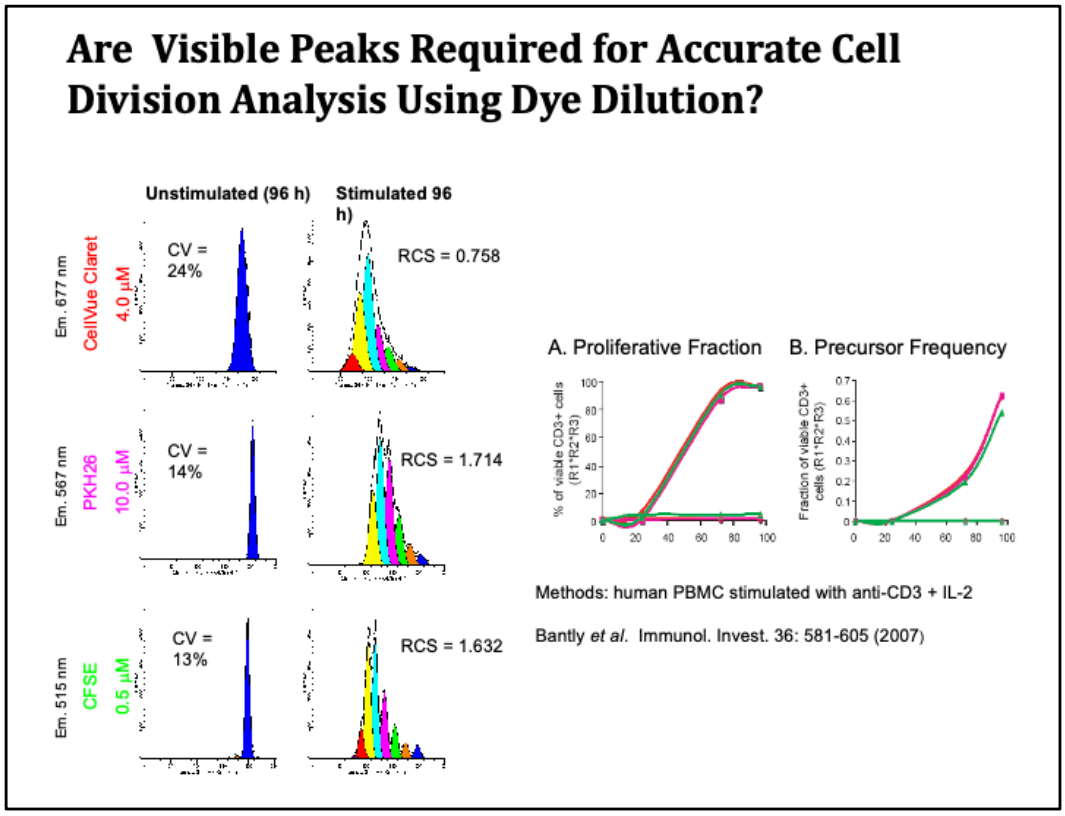
Statistic	Software	Interpretation
Precursor Frequency	ModFit FCS Express FlowJo	Probability that a cell will divide at least once
Proliferation Index Expansion Index	ModFit FCS Express FlowJo	Fold expansion during culture (ratio of final cell count to starting cell count)
Division Index Replication Index	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)

For additional statistics and a more detailed description of each and their proper use see Mario Roederer's manuscript in Cytometry A. 79: 95-101, (2011) entitled "Interpretation of cellular proliferation data: avoid the panglossian".

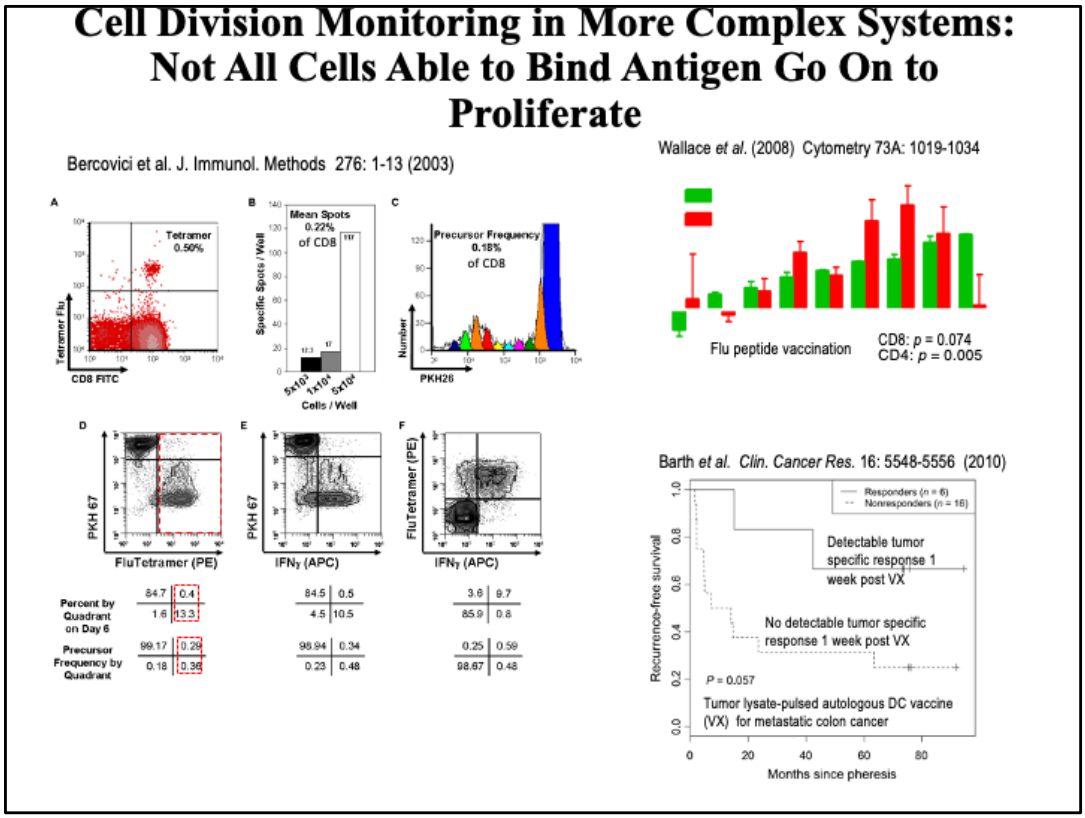
There are several statistics that may be calculated to characterize a cellular proliferation experiment. By far, the most commonly-reported statistic is the proliferative fraction or percent of cells in the final culture that have divided, however, this statistic has significant limitations. Statistics provided by software modeling programs such as ModFit, FCS Express and FlowJo provide a rich characterization of the biological response, however, different software use different names to refer to the same statistic.



The proliferative fraction and precursor frequency for CellVue Claret, PKH26 and CFSE-labeled lymphocytes. PBMC from the same donor were labeled with each dye and cultured for 96 hours with anti-CD3 and IL-2. Proliferation profiles of 30,000 viable (7-AAD^{neg}) CD3^{pos} cells were collected at 24, 72 and 96 hours.

Left Panel: 96 hour proliferation profiles analyzed in Modfit LT 3.0, using the floating proliferation model and the unstimulated control to provide a first estimate of the position of the parental peak in the stimulated sample. CV's for the unstimulated control peak and reduced chi square for the best fit model to each proliferation profile are shown.

Right Panel A. Proliferative fractions were calculated for each dye at the 24, 72 and 96 hour time points.
Right Panel B. Calculated precursor frequencies for the same data set as in Panel A. Importantly, note that both the proliferative fraction and precursor frequency for the different dyes and even different classes of dyes give very similar results.
Also note that using these approaches to analyze data, discrete separation between generational peaks is not required. The 96 hour stimulated proliferation profiles (left panel) CFSE has some separation between the generational peaks, PKH26 has less and no separation is seen between fitted peaks for CellVue Claret. Nevertheless, the results from each are similar (right panels A & B).



A series of studies by Wallace and colleagues at Dartmouth and IDM (France) illustrate the power of using dye dilution and precursor frequency to monitor responses to vaccination. Their ultimate goal was a therapeutic anti-tumor vaccine, but to test the method they used 3 different assays to look at change in precursor frequency (PF) among CD8 T cells after vaccination against seasonal flu:

1) Ability to bind antigen (flu peptide) as measured by % of cells able to bind flu-tetramer reagent (Panel A).
2) Ability to make and secrete interferon-gamma after in vitro restimulation with flu peptide (ELISpot assay; Panel B).
3) Ability to proliferate after in vitro restimulation with flu peptide (dye dilution assay; Panel C) .

Their data consistently showed that about twice as many cells bound tetramer as those that responded by producing IFNγ or by proliferating. The cause of this discrepancy became clear when looking at how tetramer binding cells broke down in terms of extent of proliferation after restimulation with flu peptide for 6 days (panel D). At the end of the culture period, responding cells that were Tet+ (defined as cells having undergone at least 2 cell divisions, 13.3%) greatly outnumbered non-responding cells that were Tet+ (0.4%). However, the precursor frequencies (i.e., calculated % of the parent population that went on to respond) were very similar for both Tet+ populations, suggesting that ~50% of Tet+ cells in the original population did not go on to proliferate.

Most volunteers showed increases in precursor frequency to flu peptide post-vaccination in both CD4 and CD8 T cells, with the most robust response in the CD4 population (bar graph, upper right). A paired analysis of pre-and post-vaccination samples found change in precursor frequency to be highly significant for the CD4 T cells (p=0.005) and nearly significant (p=0.074) for CD8 T cells.

Barth and colleagues asked whether tumor-specific T cell proliferative responses after autologous anti-tumor vaccine therapy predicted improved recurrence free survival in individuals with metastatic colon carcinoma (lower right graph). A PKH67 dye dilution assay was used to determine CD4 and CD8 T cell precursor frequencies before and after bilateral inguinal node immunization with an autologous tumor-lysate pulsed dendritic cell vaccine. Triplicate samples stained with PKH67 were run and the result for a given patient at a given time point considered positive if the mean value obtained after stimulation with tumor lysate–pulsed DCs was significantly greater (by t test) than the mean value obtained after stimulation with unpulsed DCs. The 6 individuals showing a detectable increase in tumor-specific T-cell proliferative response at 1

week post-vaccination (mean precursor frequencies of 1.3% and 1.6% for CD4 and CD8 T cells, respectively) showed substantially improved recurrence free survival at 5 years compared with those who showed no increase at 1 week (67% vs 31%; log rank P = 0.057).

Roswell Park Cancer Institute