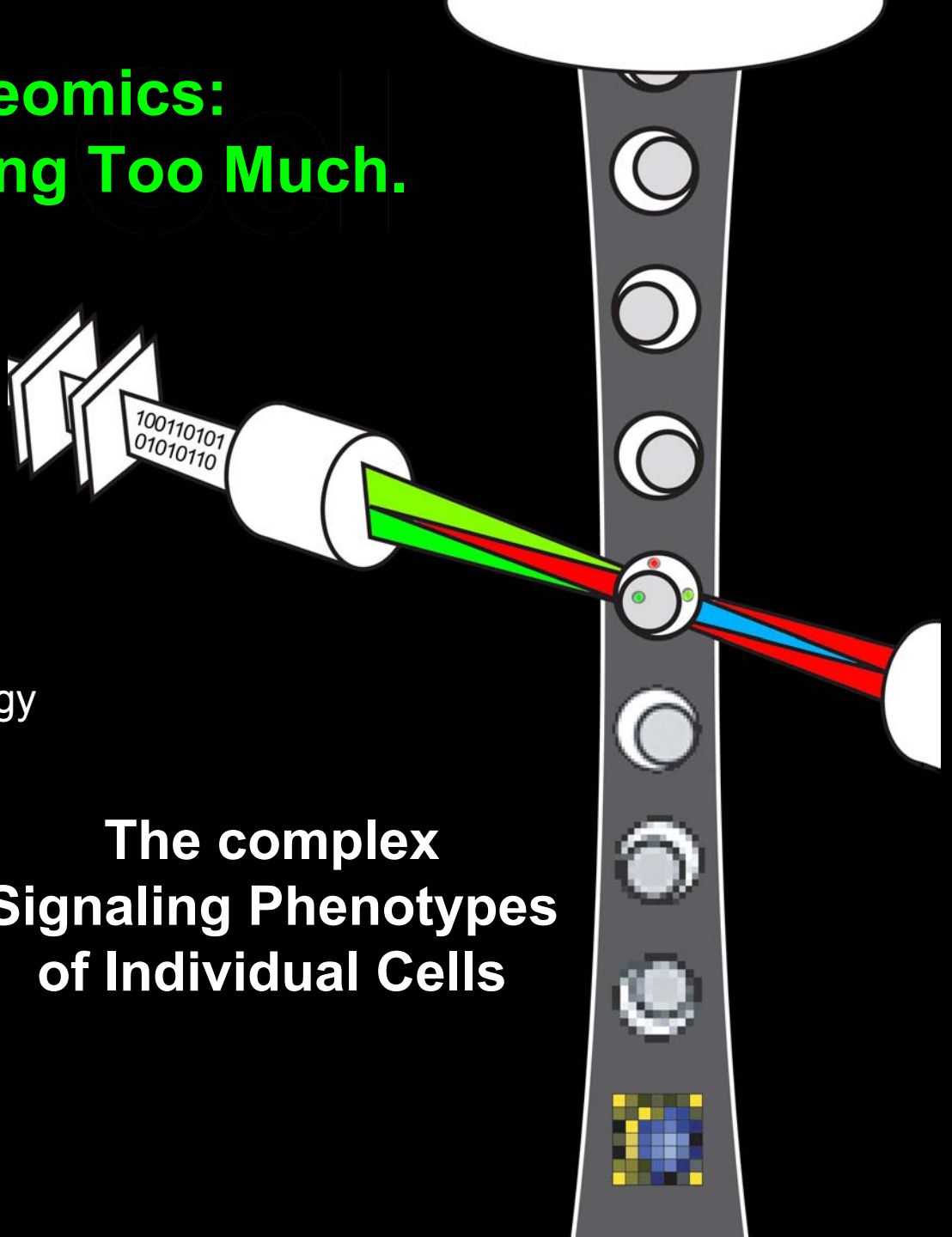
Single Cell Proteomics:
A Challenge of Knowing Too Much.



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Stanford University
Dept. of Microbiology & Immunology

The complex Signaling Phenotypes of Individual Cells

Early Single Cell Proteomics Innovators

Len Herzenberg - - Argon laser flow sorter 1972 - placed an argon laser onto their sorter and successfully did high speed sorting - Coined the term Fluorescence Activated Cell Sorting (FACS)

Mack Fulwyler - Coulter Electronics manufactured the TPS-1 (Two parameter sorter) in 1975 which could measure forward scatter and fluorescence using a 35mW argon laser.

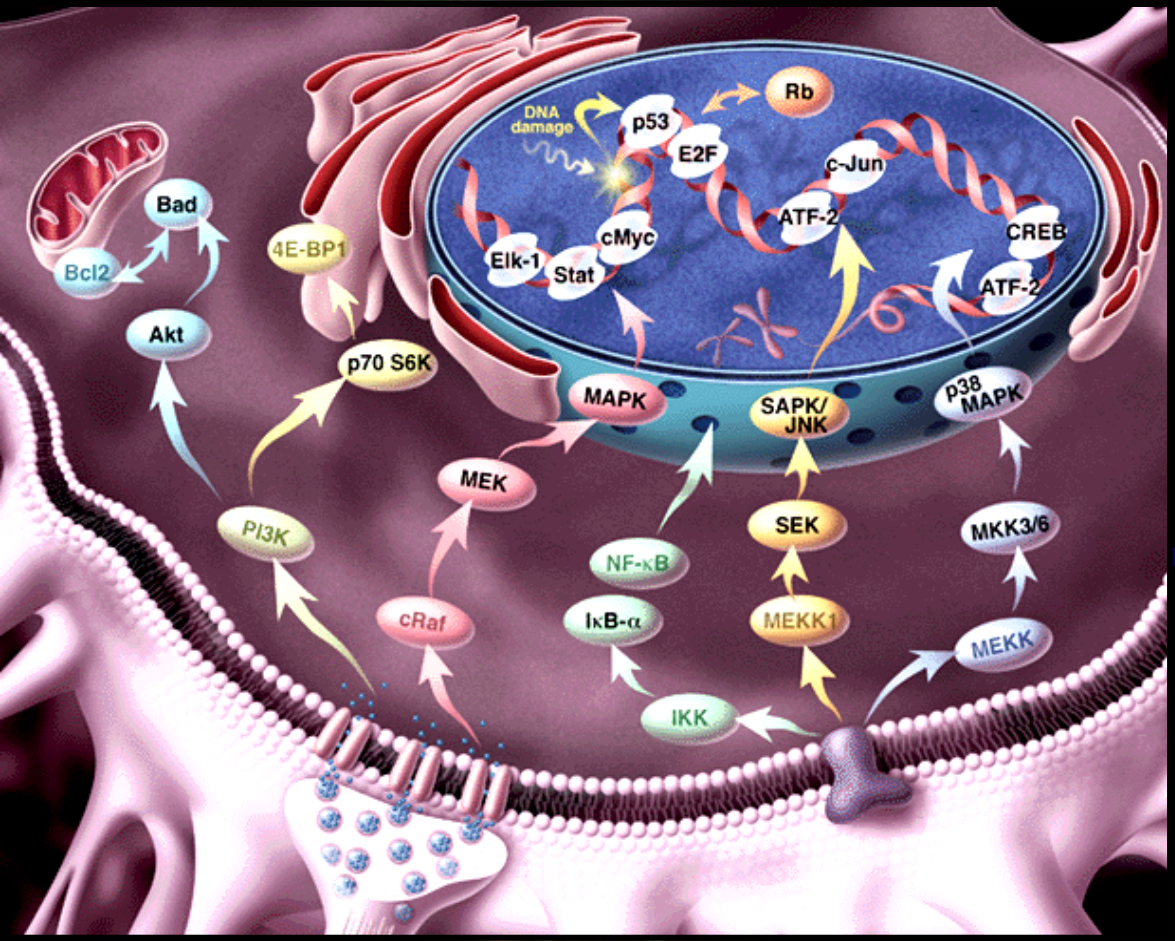
Howard Shapiro: Block instruments (1973-76) - a series of multibeam flow cytometers that did differentials and multiple fluorescence excitation and emission.

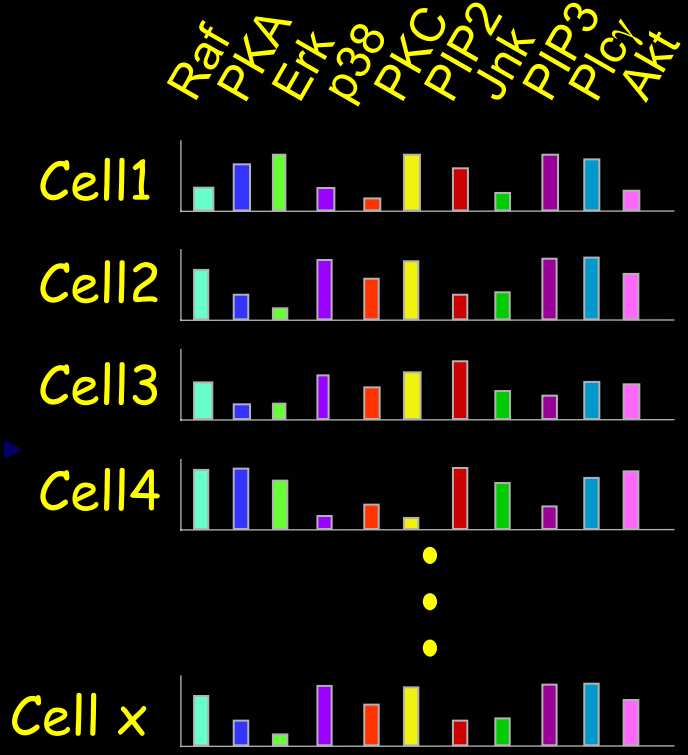
Multi-Color Flow Cytometry

- Combines complex immunophenotypic analysis with functional analysis (intracellular biochemical events)
  - Detailed characterization of rare subsets (e.g., antigen-specific T cells)
  - Identify new subsets more specifically associated with mechanism and clinical parameters
- Largely unappreciated in much of molecular biology– 11 parallel assays (classic) is NOT the same as 11 simultaneous measurements.

Single Cell Analysis in Drug Discovery

From phospho-molecular profiling to Signaling pathways





Signaling Pathways

Single Cell Standards Issues to consider

- # of simultaneous parameters to measure
- Absolute vs relative (qualitative vs quantitative)
- Baseline fluorescence standards (quantitative)

- How does one FIND an informational ‘blob’ in n-space (feature extraction)?
- How does one represent such a 20-dimensional object?

- How does one apply such knowledge from flow cytometry to 3D imaging (confocal, other cytometry)?
- How does one deal with solid tissue slice (tissue biopsies)

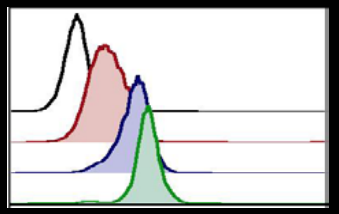
- SOPs for sample handling?

Why use Single Cells to Measure Cell events?

- **Traditional**

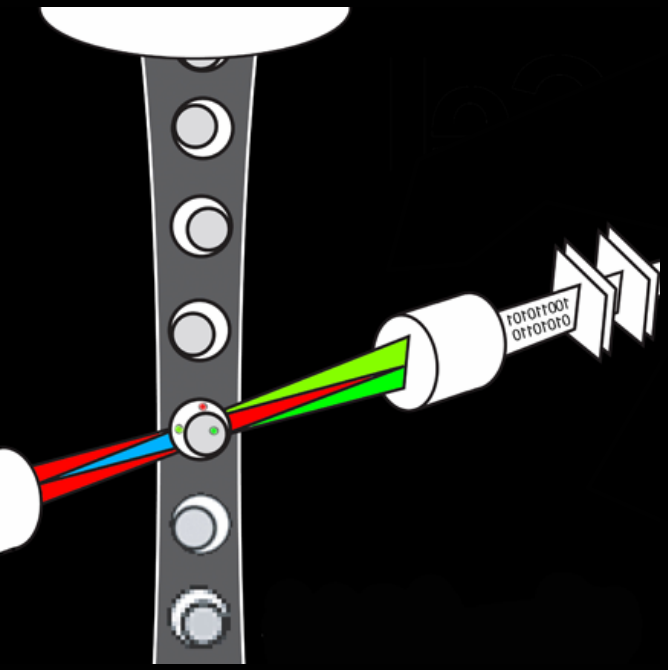
- Population analysis
- Homogeneous cell type: sorting, depletion

- **Flow Cytometry**



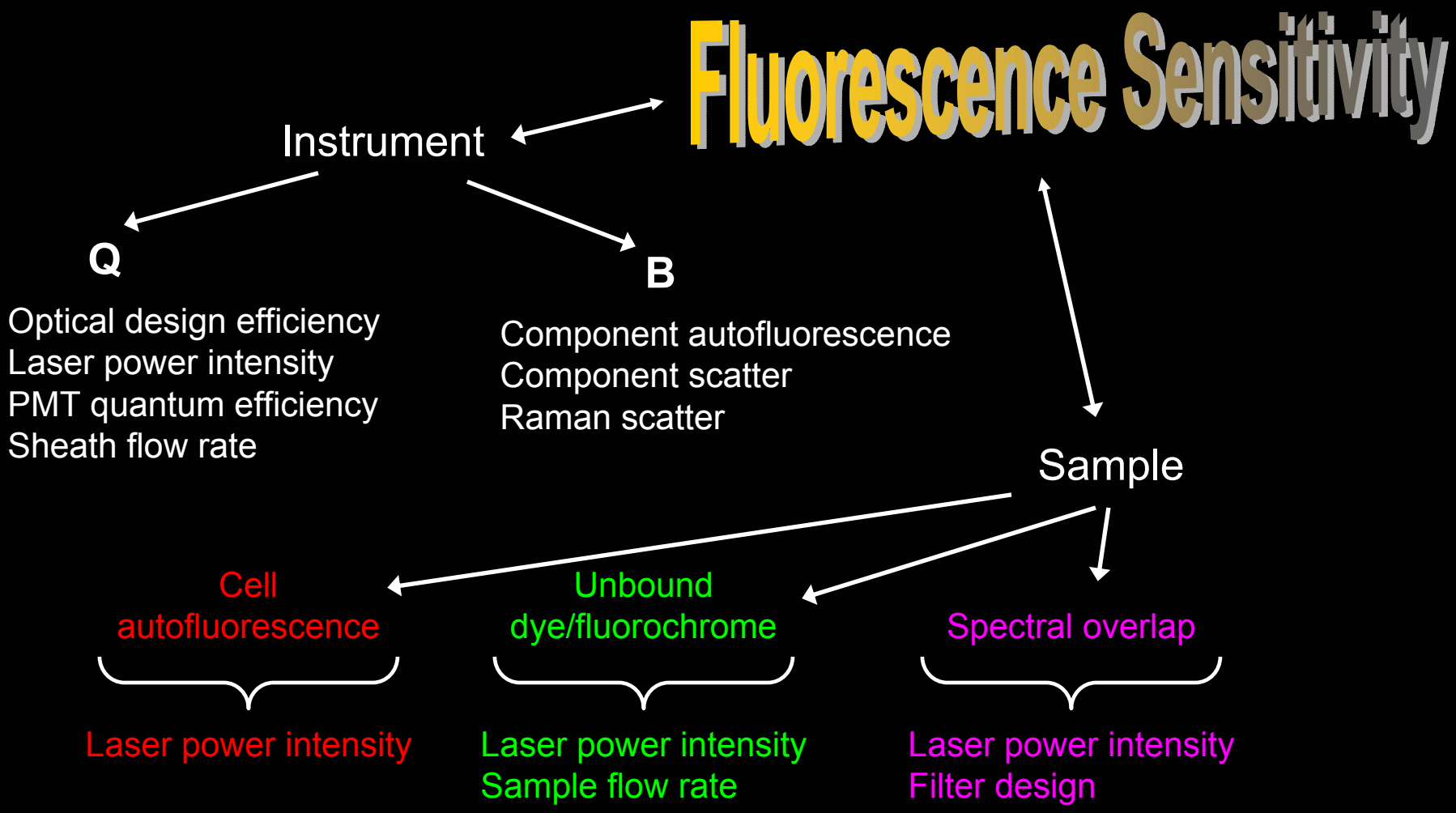
- Single cell analysis
- Heterogeneous populations can be separated via surface

Pulses to Numbers

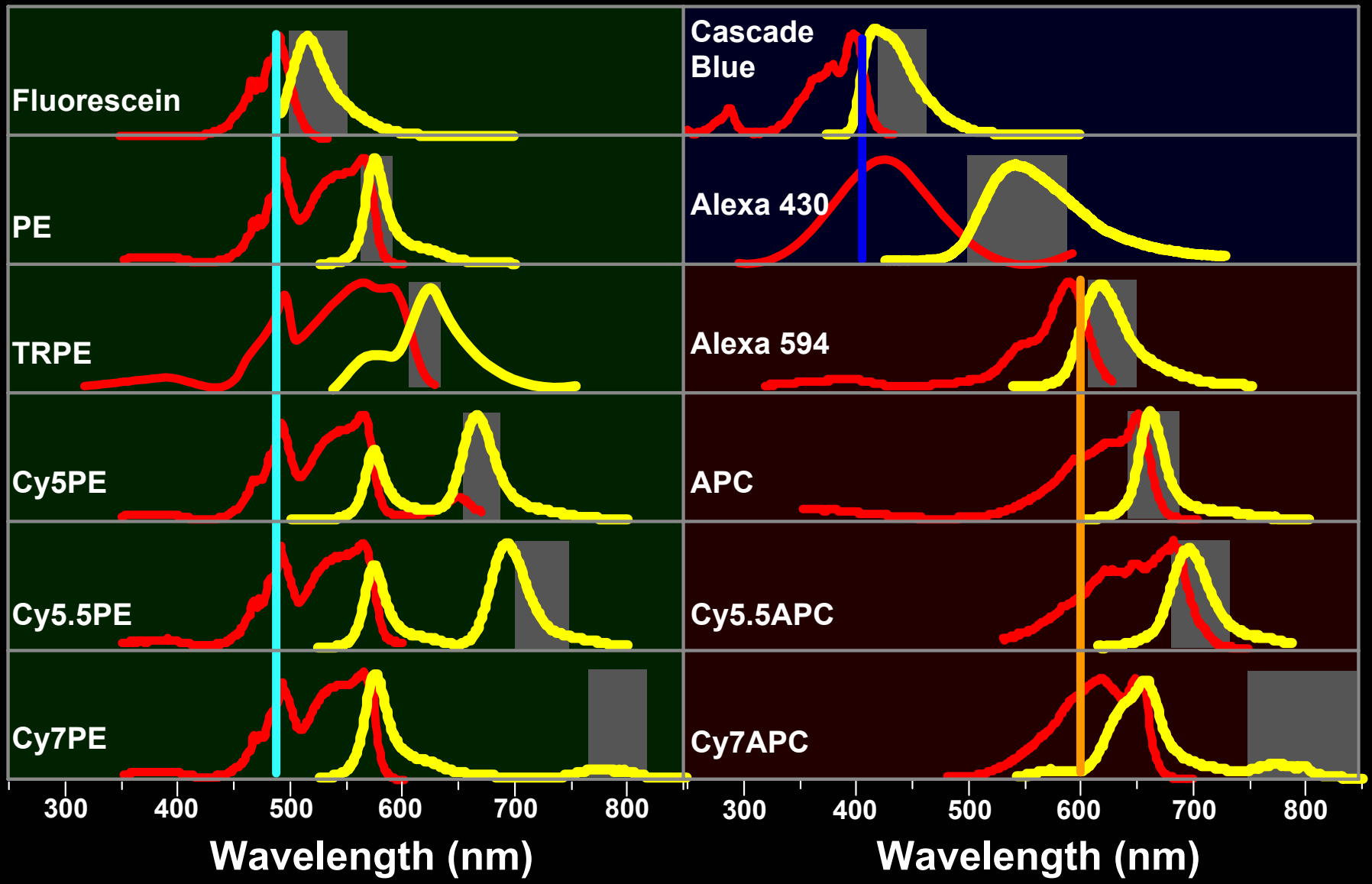


- Advantages of new digital processing over Analog:
  - Highly accurate (acquisition time enhanced)
  - Can correct for dye spillover (matrix algebra for n colors)
  - Obtain pulse geometry metrics and time
  - Perform statistics on raw linear data

Stuff that impacts sensitivity

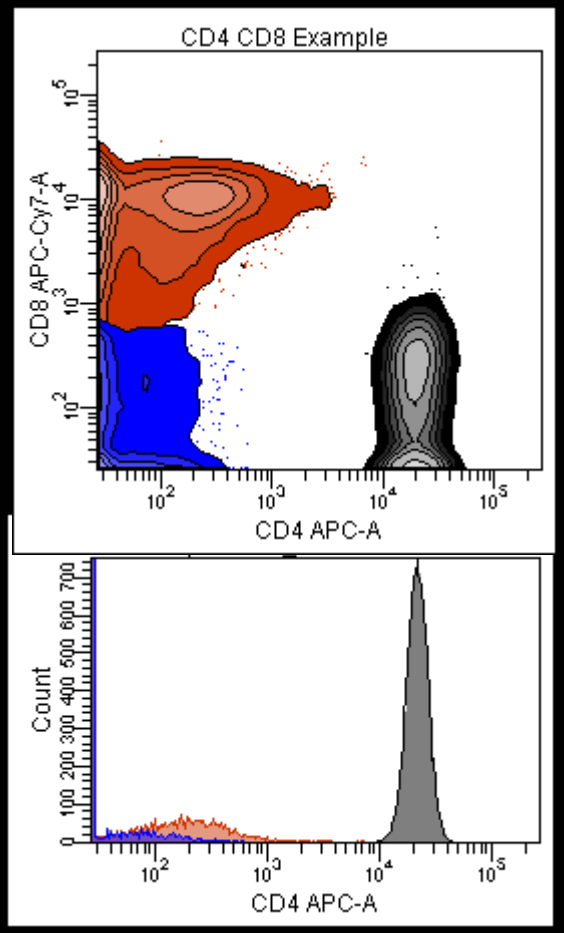


Multi-color FACS: Spectral Overlap

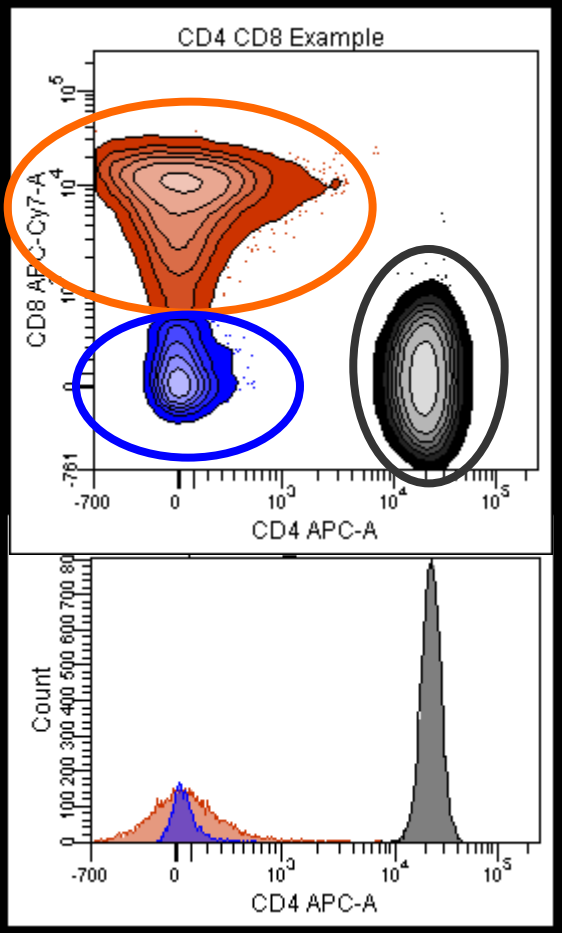


Stanford Biexponential Display (Logicle)

Log Display



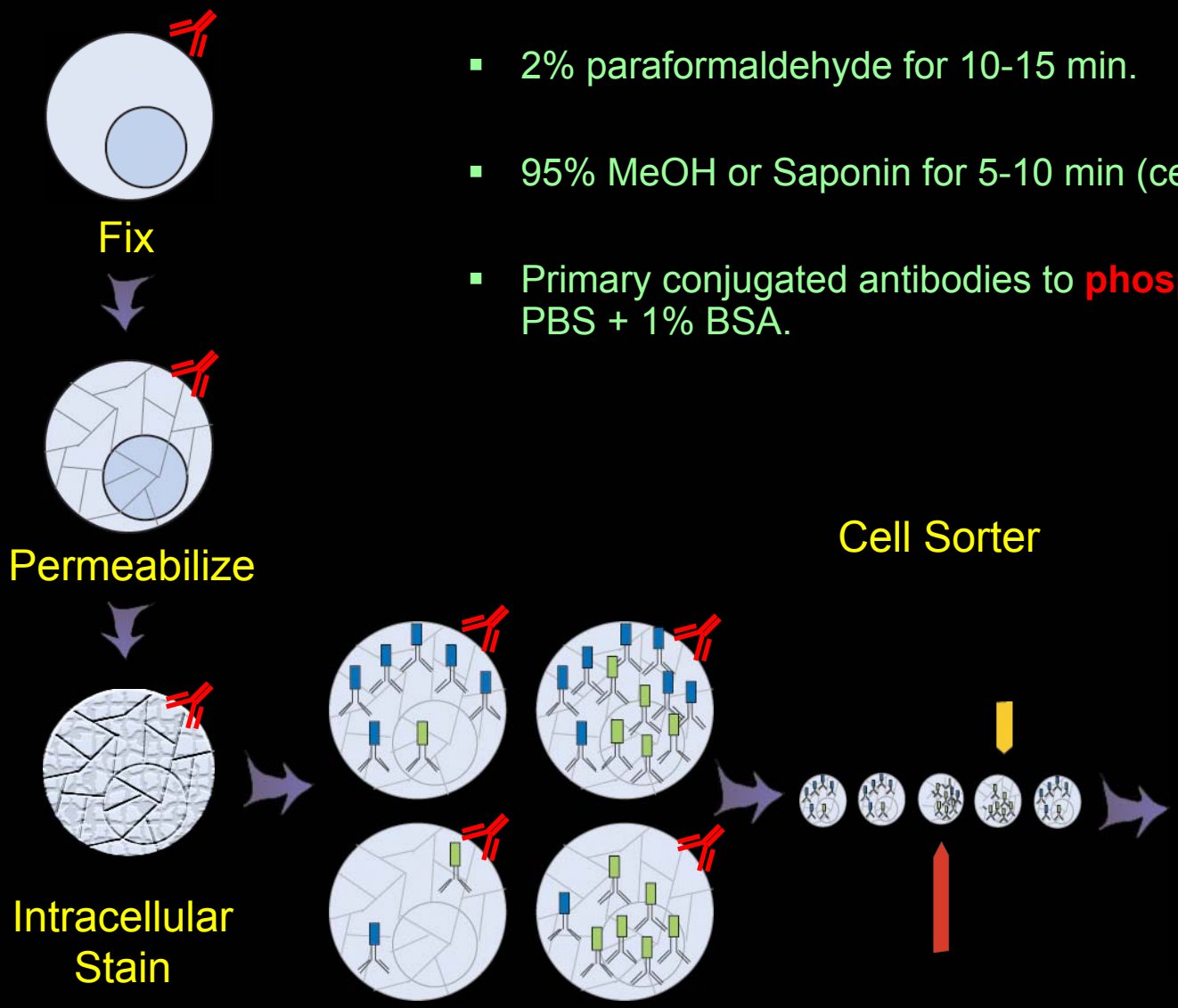
Biexponential



BD
BD Biosciences

Intracellular Flow Cytometry Technique

- Stain with antibodies to surface proteins
- 2% paraformaldehyde for 10-15 min.
- 95% MeOH or Saponin for 5-10 min (cell type dependent).
- Primary conjugated antibodies to **phospho-epitopes** in PBS + 1% BSA.



Increasing Phospho Ab Repertoire

- Phospho Antibodies
- p38 MAPK
- JNK, cJun
- AKT, PIP2, PIP3,
- PKC$\alpha/\beta/\theta/\delta$, Rsk
- Raf, Mek, ERK, ELK
- Rsk, Creb,
- STAT1,3,5,6, c-Src
- CREB, cJUN, IKKα
- p53 s15, s20 s37, s392
- Pyk2, Shc, Fak, src
- Slp76, Zap70, Syk, Lat, Vav,
- Lck, PLCγ
- Beta-integrins

Every new antibody increases the potential of discovering entirely new correlations for disease processes (targets and diagnostics) as well as utility in drug design and development

Phospho Antibodies

- EGFR
- PDGFR
- cKit
- VEGFR
- PKA

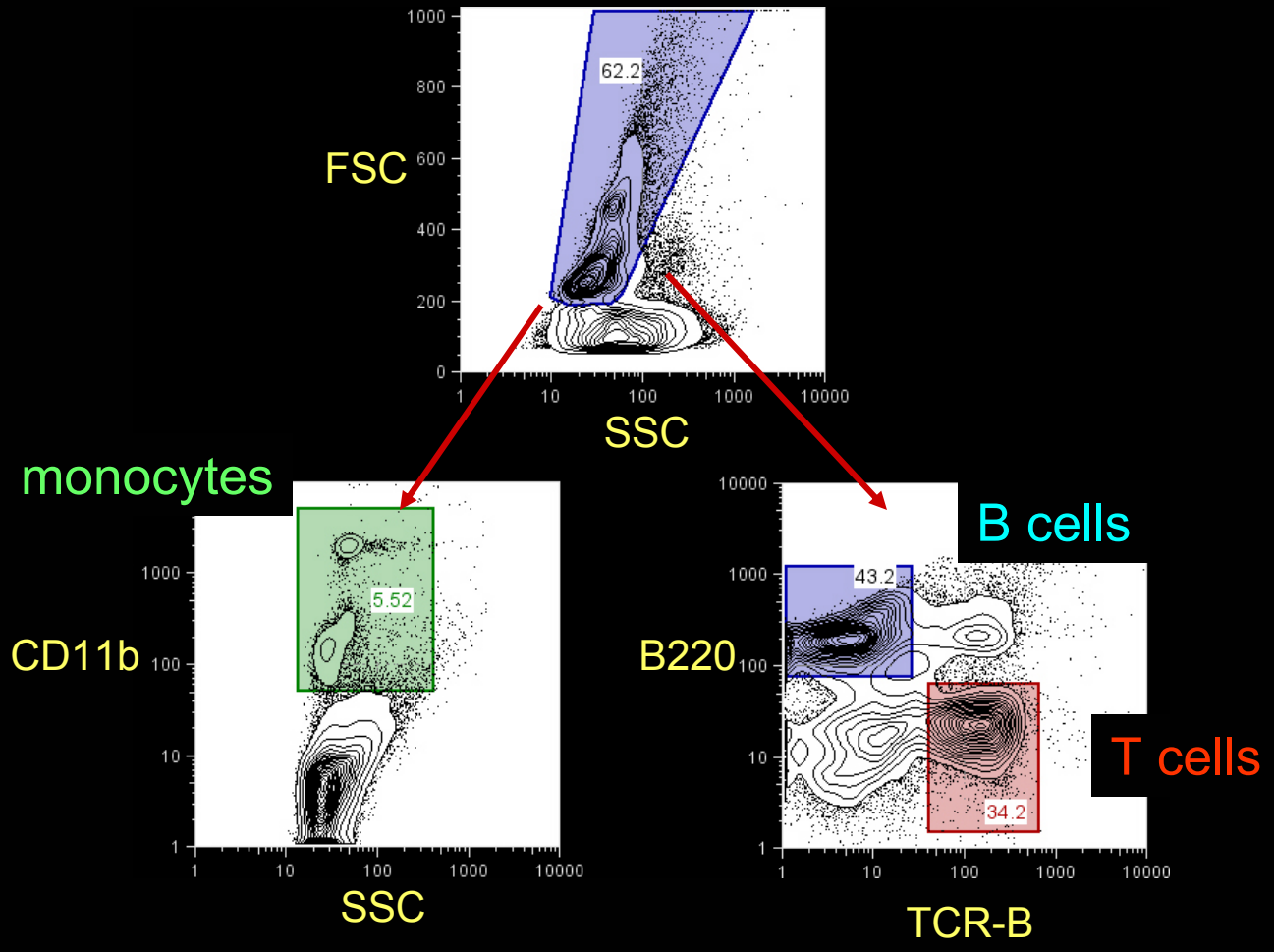
Pkg
RB
NFAT
NFKB
Caveolin
Paxillin

Stimulation of Murine Splenocytes
Dendritic Cell Subpopulation Analysis ($B220^{-}$ $CD8^{-}$ $CD11c^{+}$)

Collect Splenic cells 10 Minutes post-injection of IFNγ (in vivo)

Read out Stat1 transcription factor activation via its phosphorylation

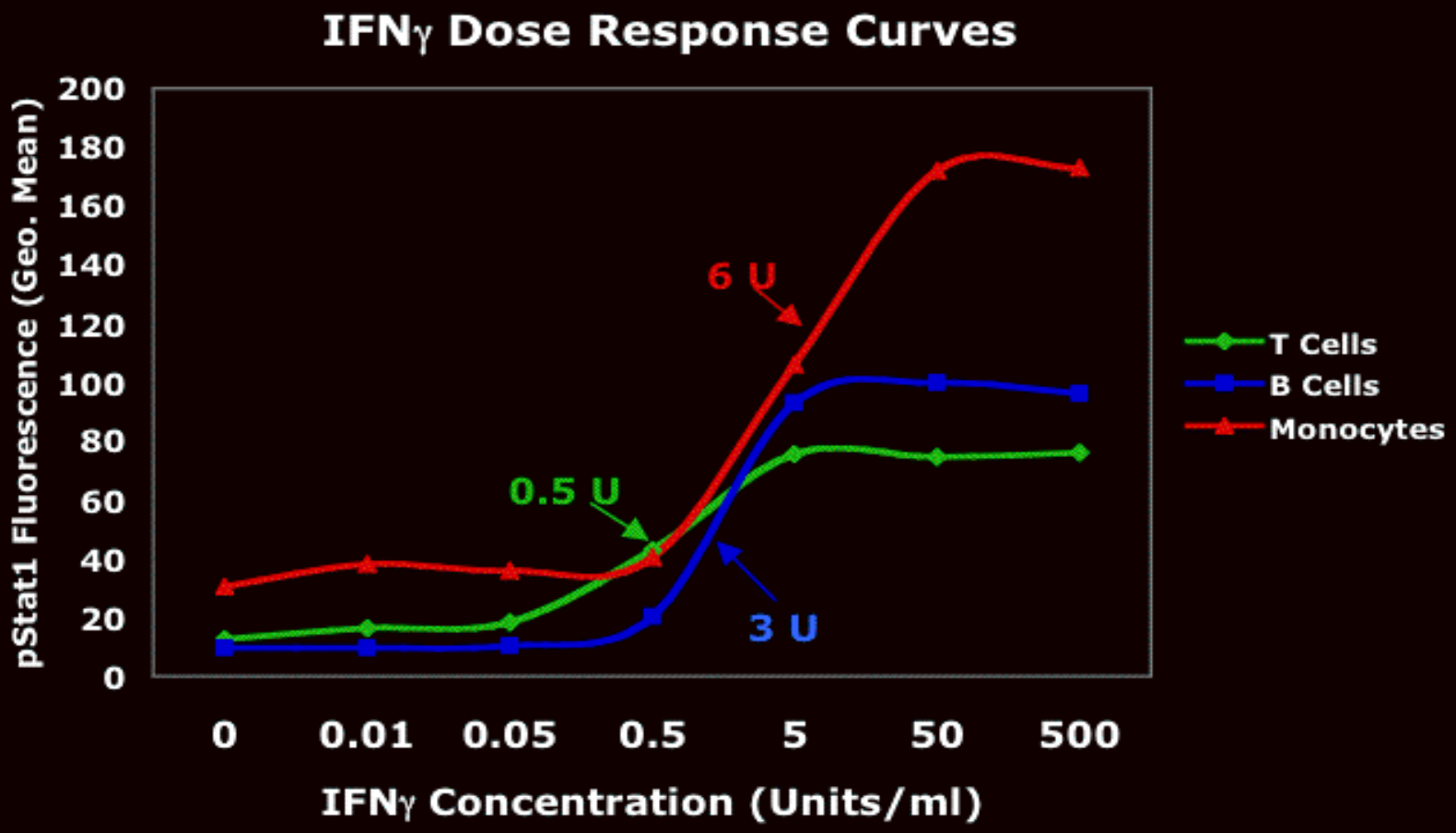
Murine Splenocytes - Gating



Matt Hale, Nolan Lab

Phospho-FACS allows for Pharmacodymics in Vivo

Cell Subset Specific IFNγ Sensitivity across a titration

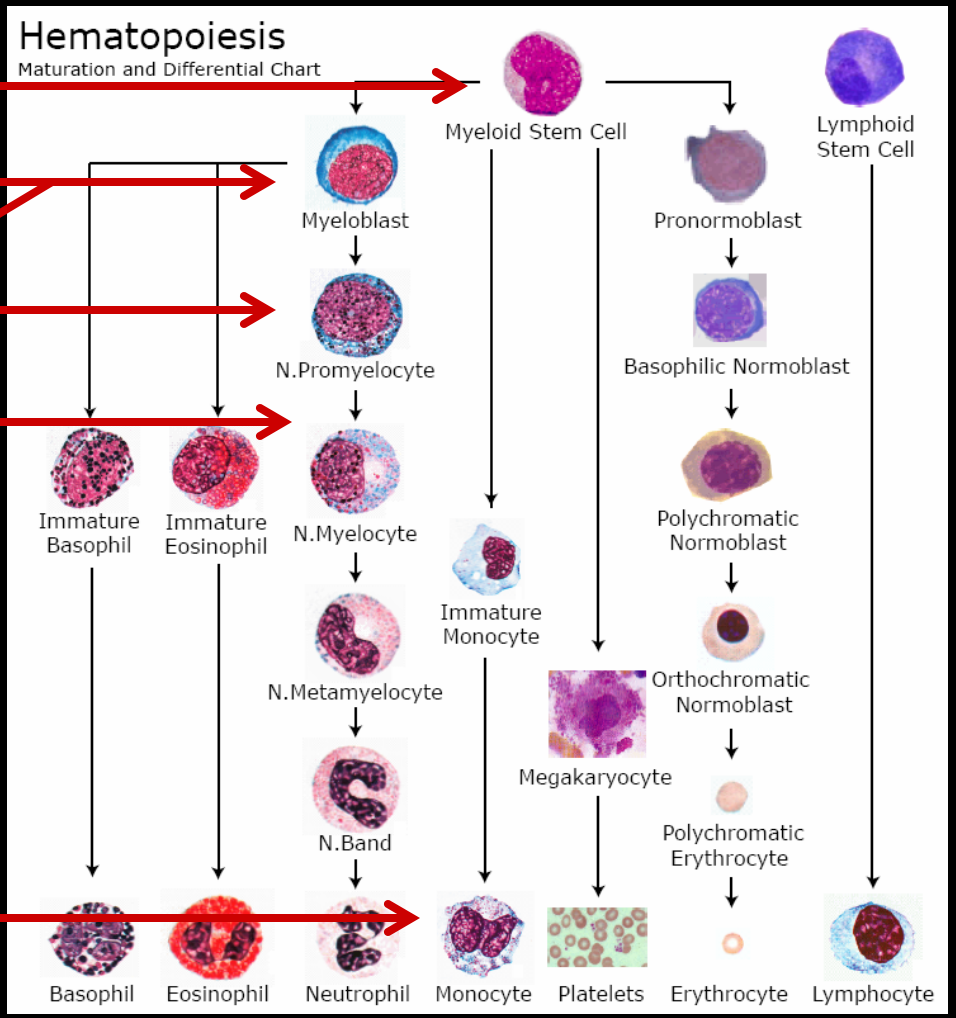


Leukemia (AML) Classification by Differentiation

- M0 – undifferentiated AML
- M1 – myeloblastic, immature
- M2 – myeloblastic, mature
- M3 – promyelocytic
- M4 – myelomonocytic
- M5 – monocytic

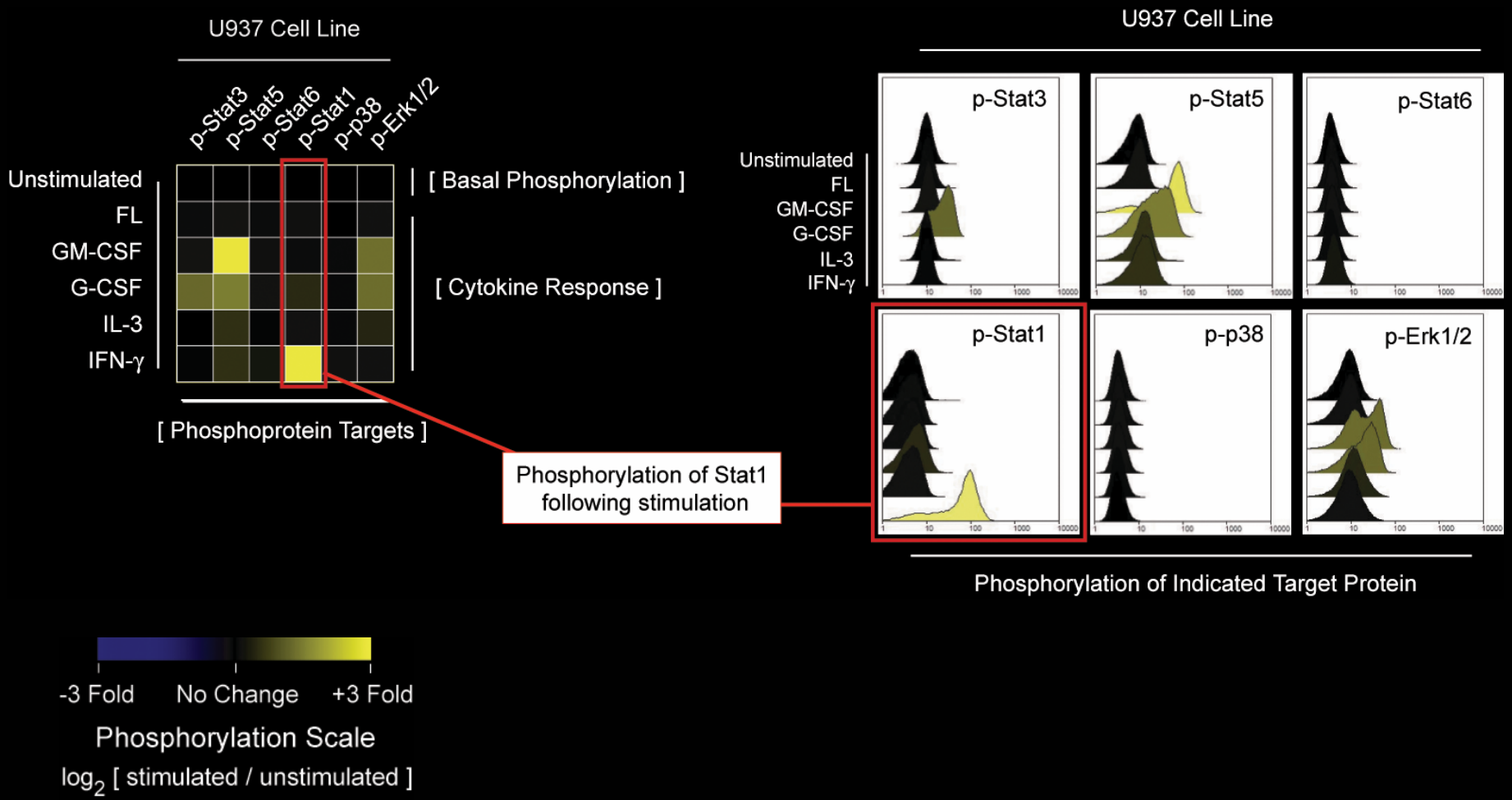
CD34 marker can be found on AMLs from all FAB classes

(lymphohematopoietic stem/progenitor cell marker)



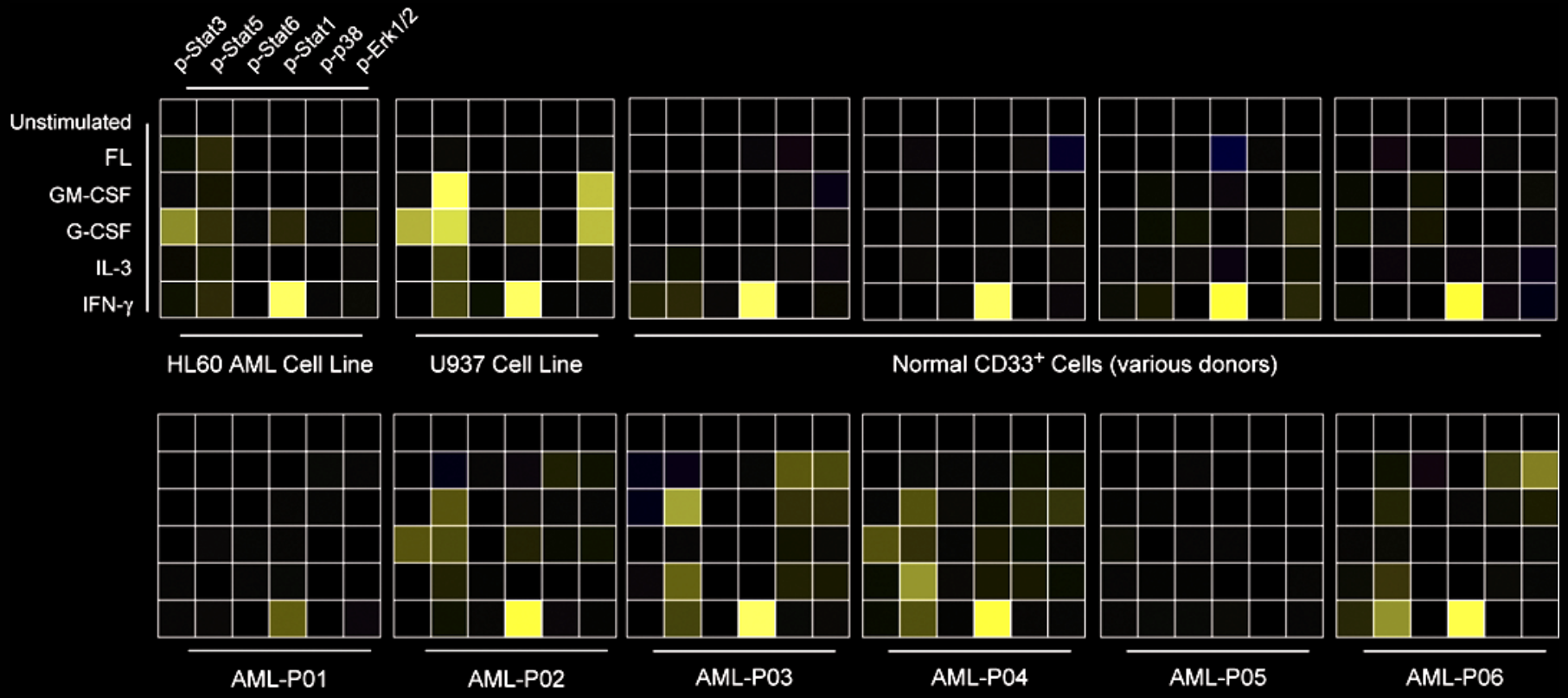
Could provoking cells to respond to external stimuli, such as cytokines, differentiate AML blasts with altered signal transduction networks?

Model: Cytokine Response of U937 Cells



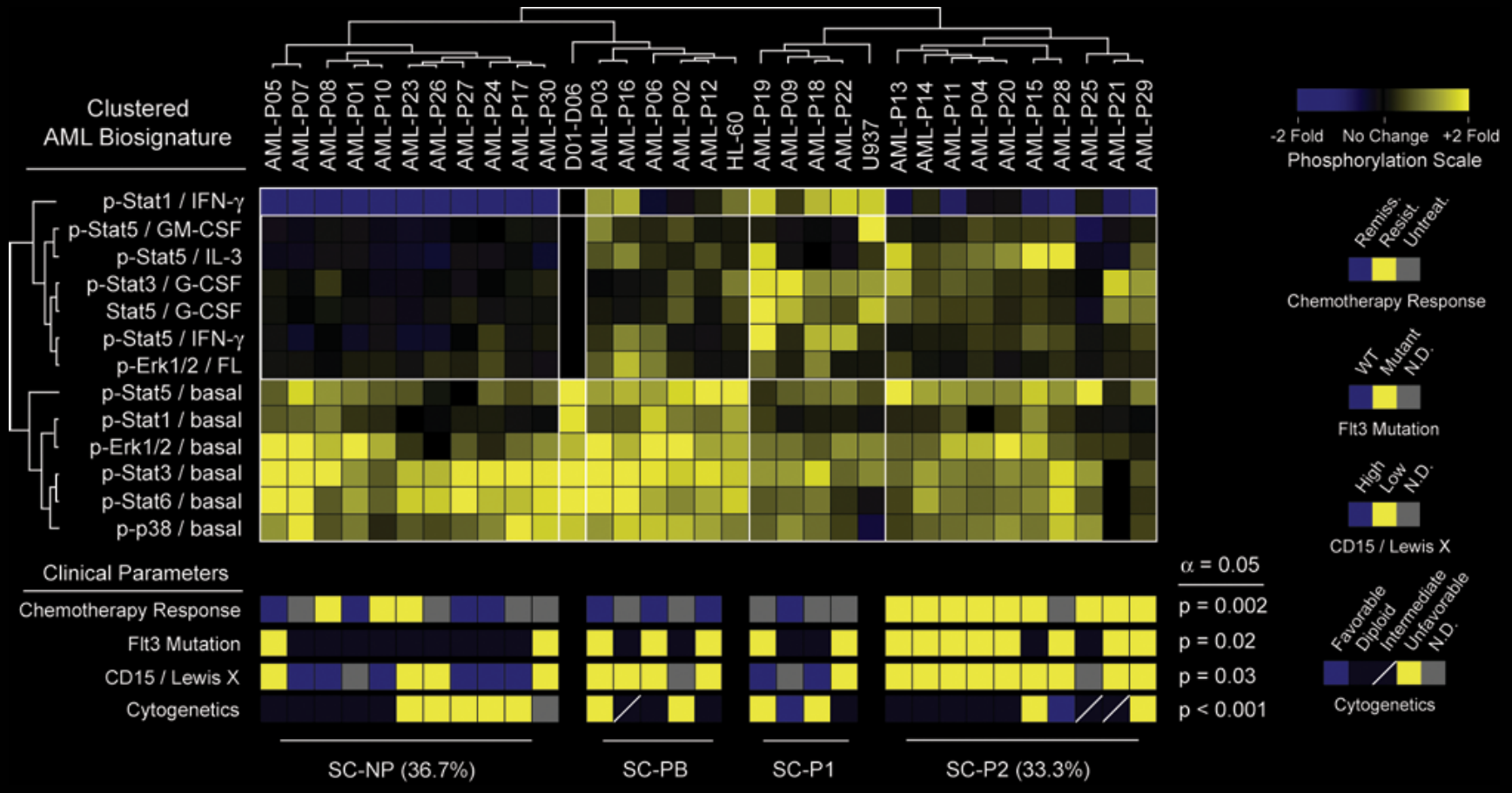
Irish et al, Cell, 2004

Cytokine Responses of Normal and Tumor Cells



Irish et al, Cell, 2004

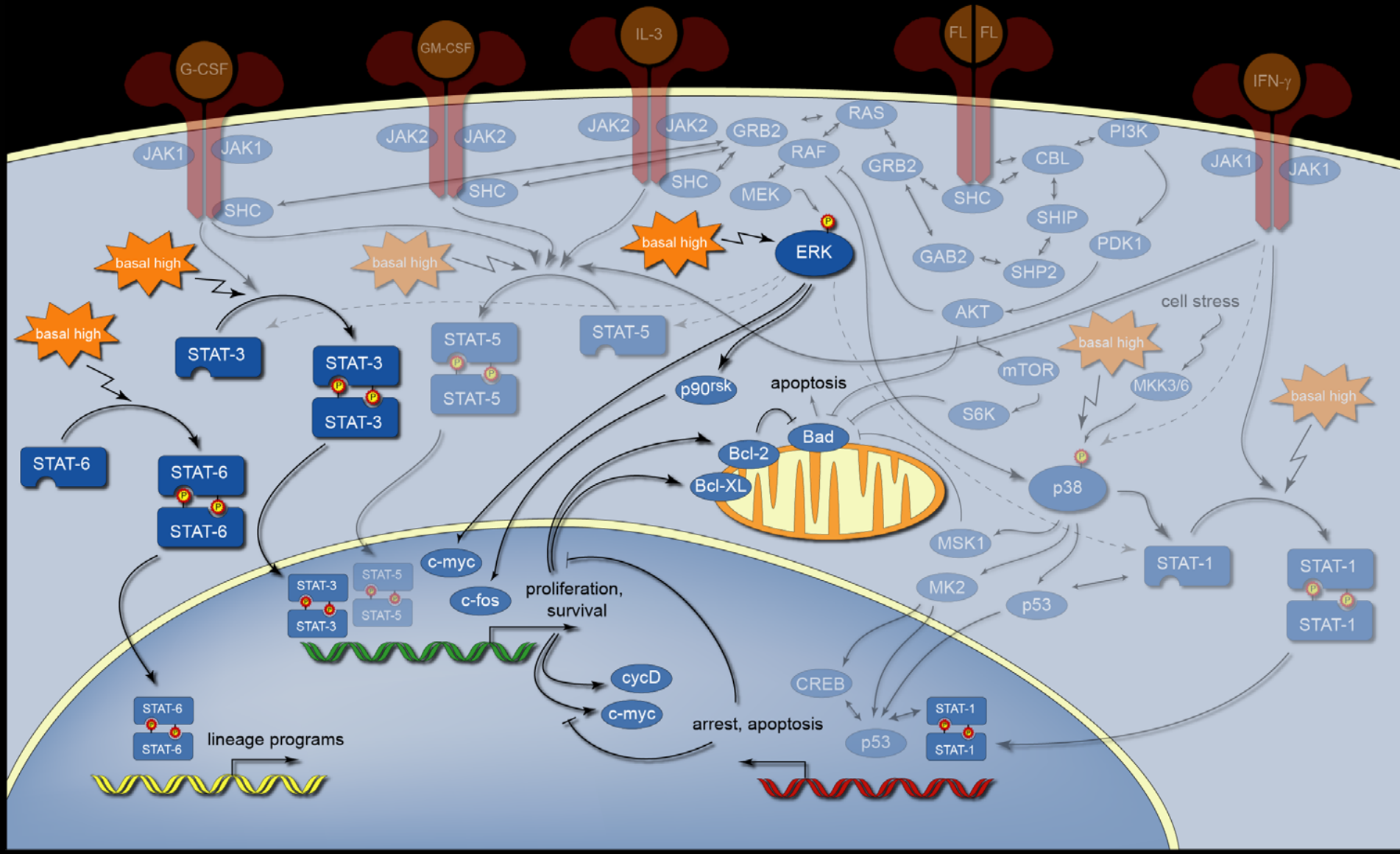
Clustering of Biosignature, Clinical Significance



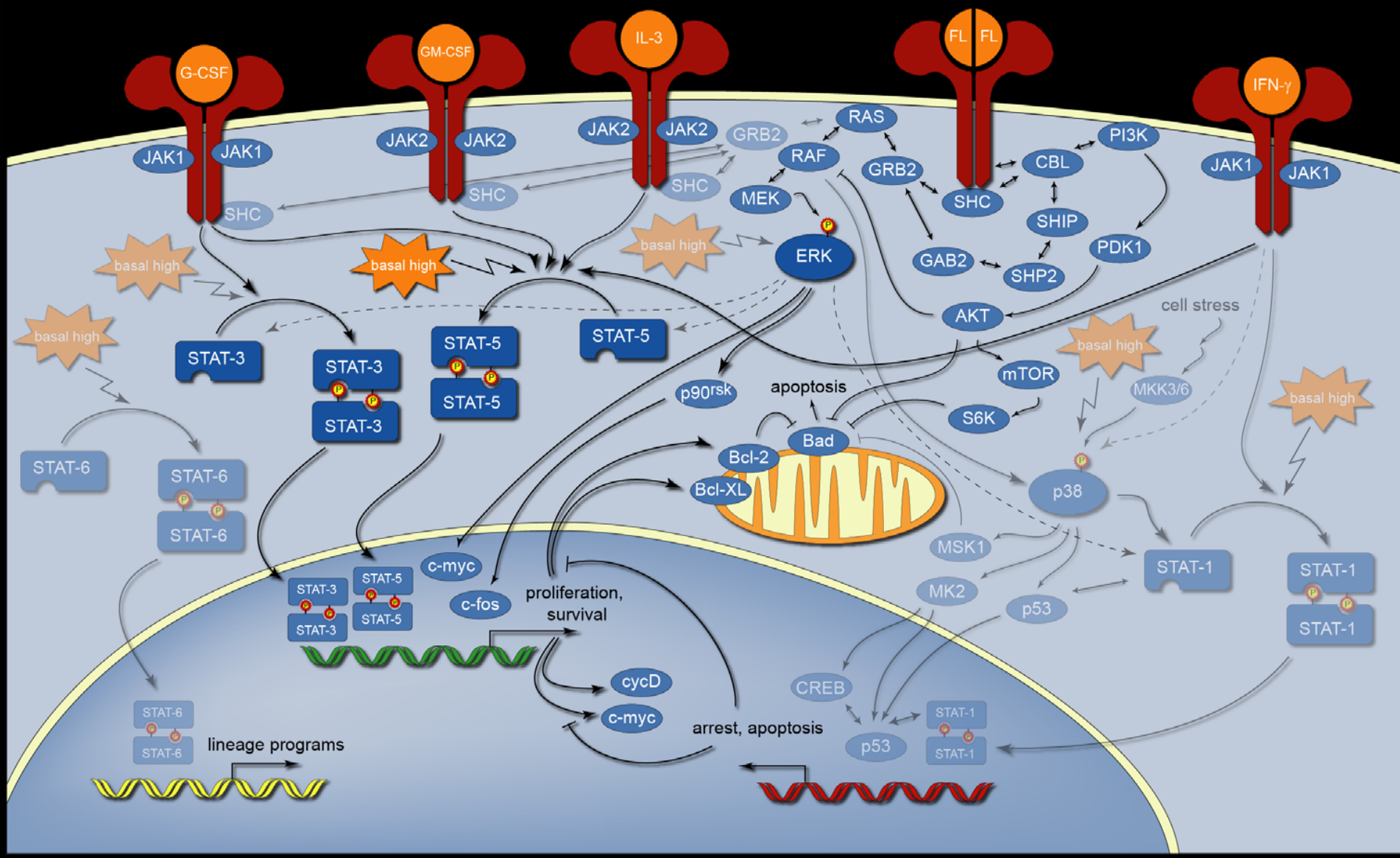
Irish et al, Cell, 2004

SC-NP (standard chemotherapy responses)

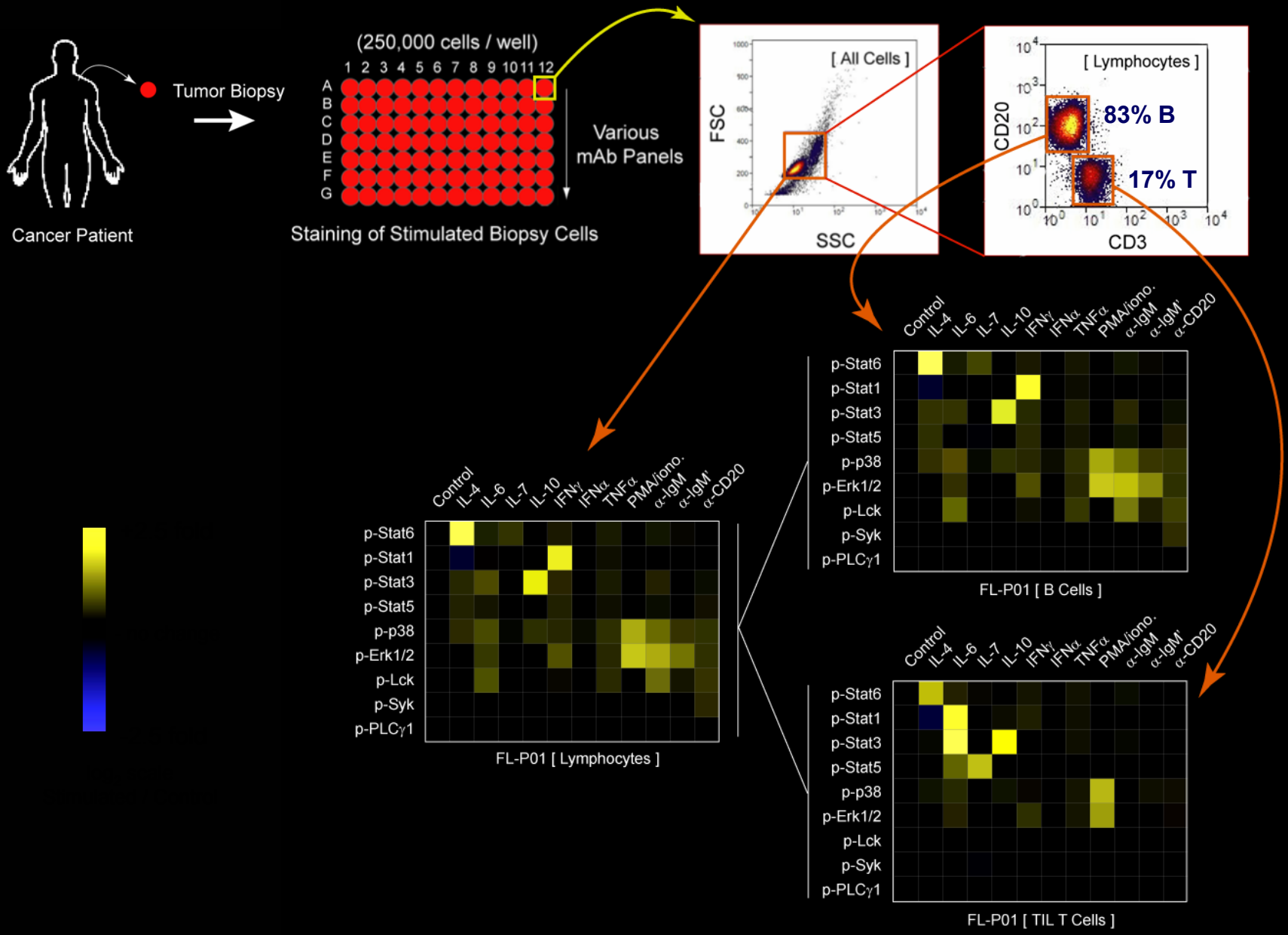
SC-NP Composite Profile



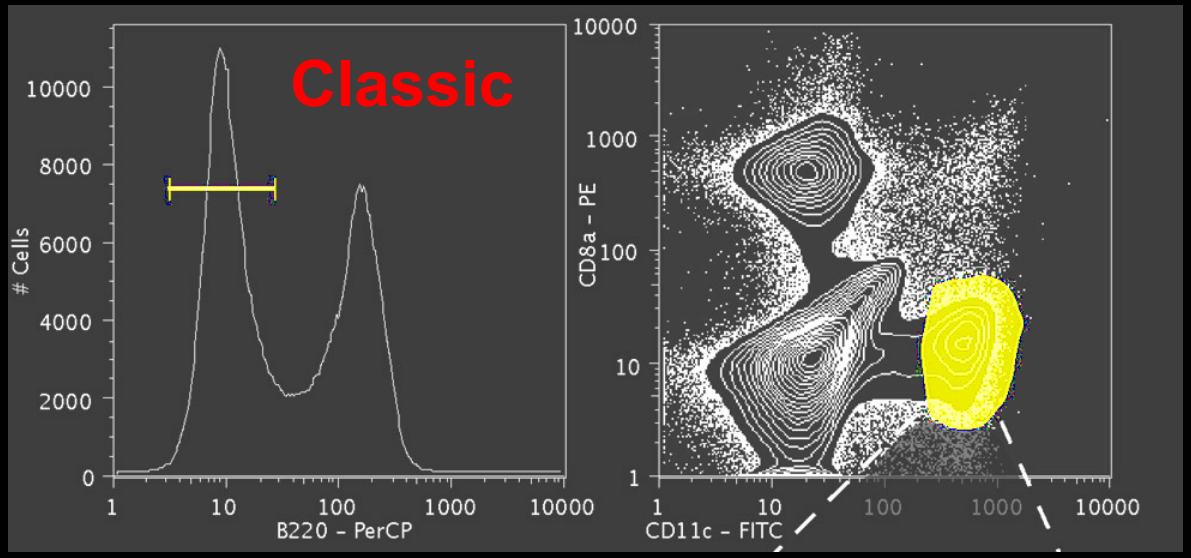
SC-P2 (FIt3 mutant, chemotherapy insensitive)

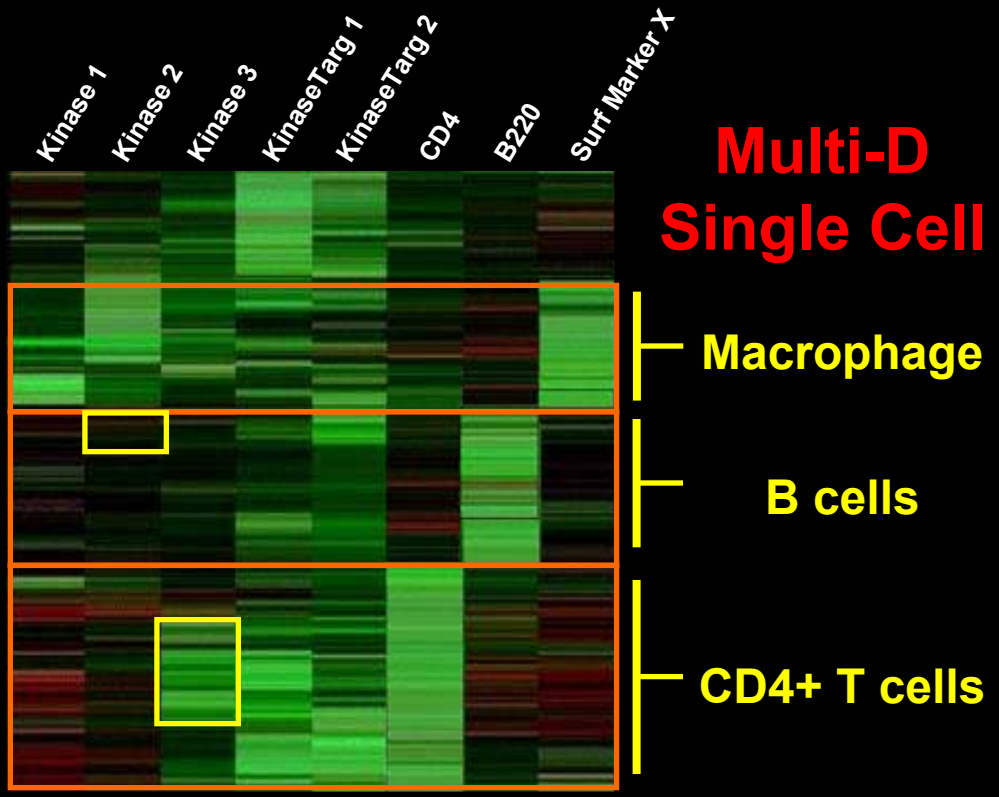


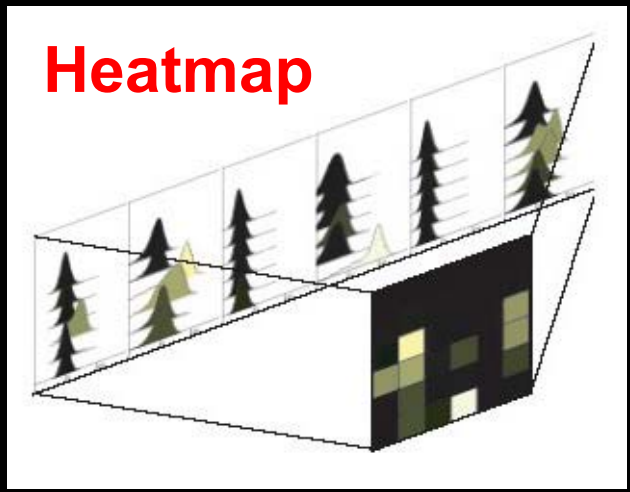
Array Overview of Lymphoma Signaling



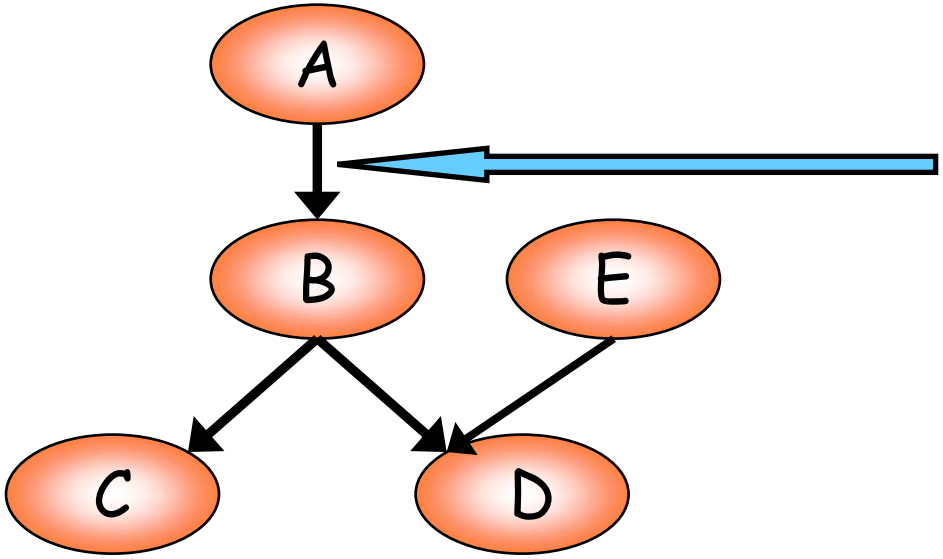
New Approaches To Representing Single Cell Data Present New Problems, but suggest Interesting possibilities

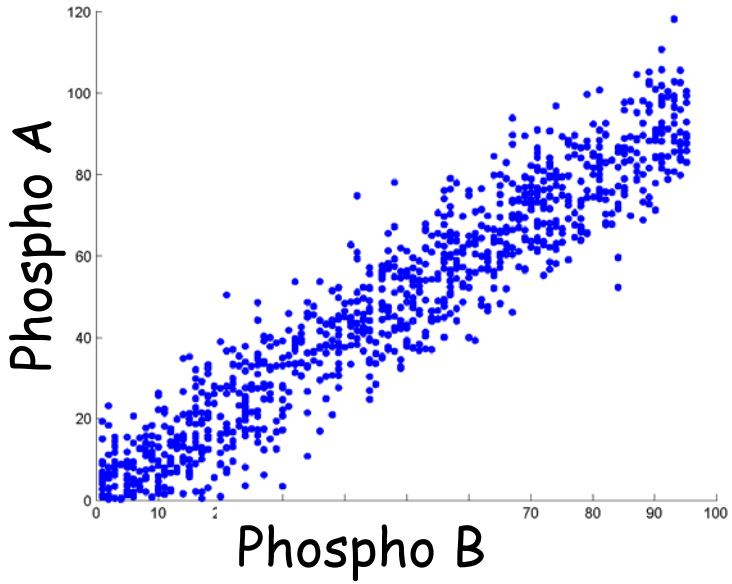






What is a Bayesian Network?



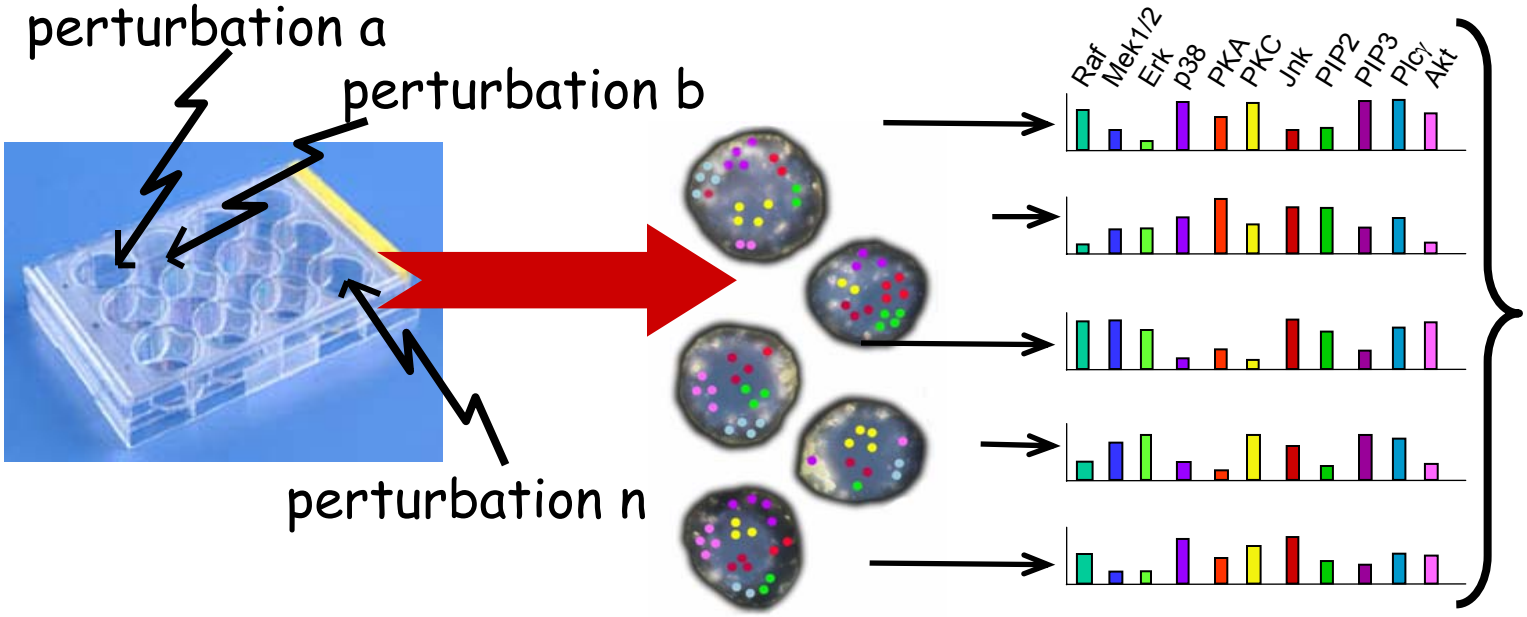


+ A Mathematical (probabilistic) description of the connections in the graph ...

T-Lymphocyte Data

Conditions (96 well format)

11 Color Flow Cytometry



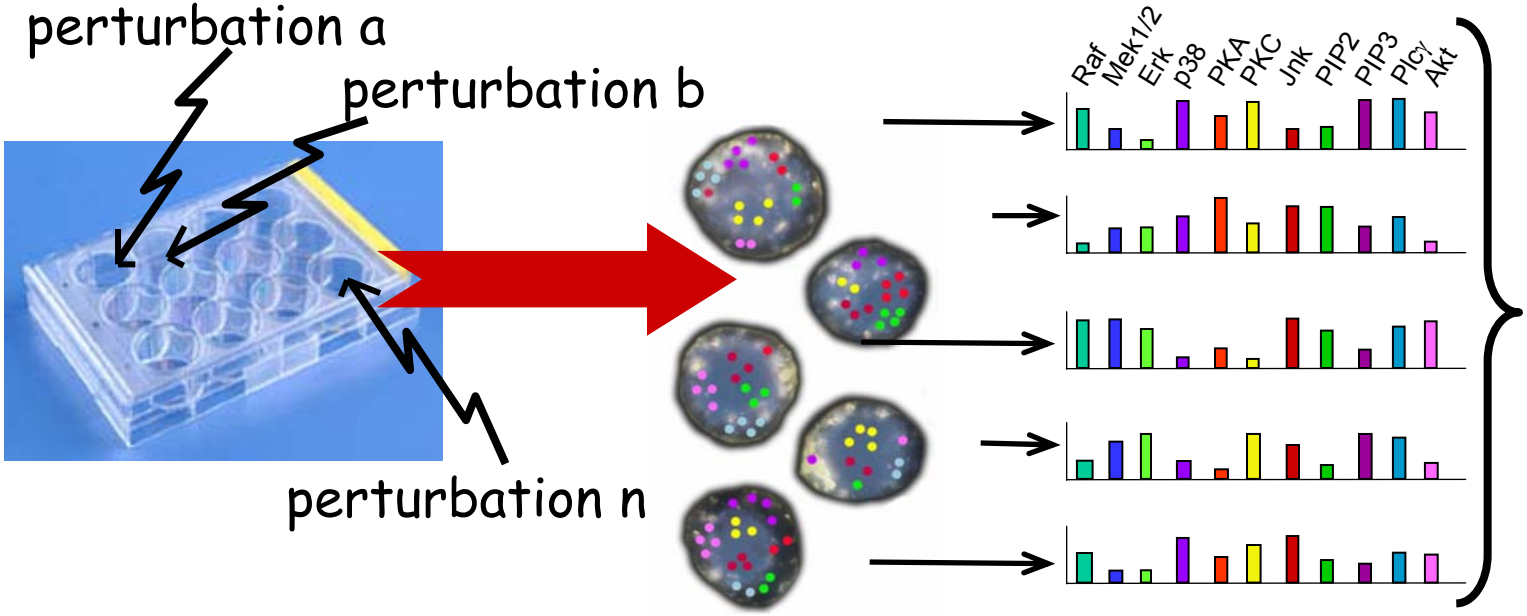
- Primary human T-Cells
- 9 conditions
  - (6 **Specific** interventions)

- 9 phosphoproteins, 2 phospolipids
- 600 cells per condition
  - 5400 data-points

T-Lymphocyte Data

Conditions (96 well format)

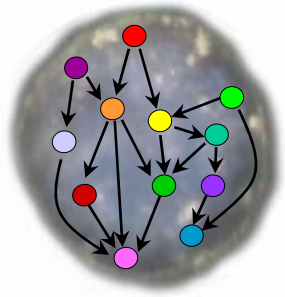
11 Color Flow Cytometry



Datasets of cells

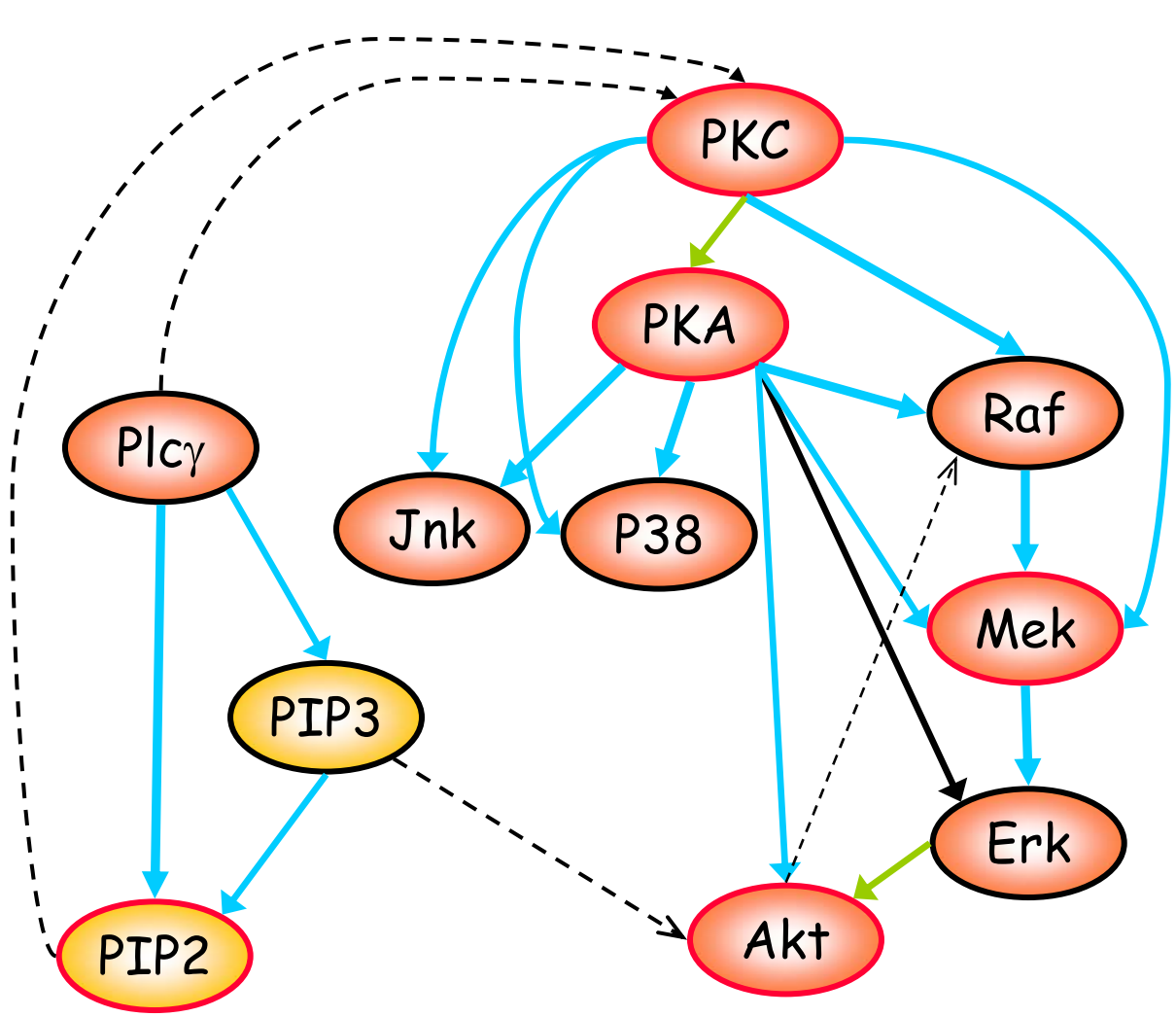
- *condition 'a'*
- *condition 'b'*
- *condition...'n'*

Bayesian Network Analysis



Influence diagram of measured variables

A T cell signaling map *ab initio* from multiparameter data by Bayesian Inference.

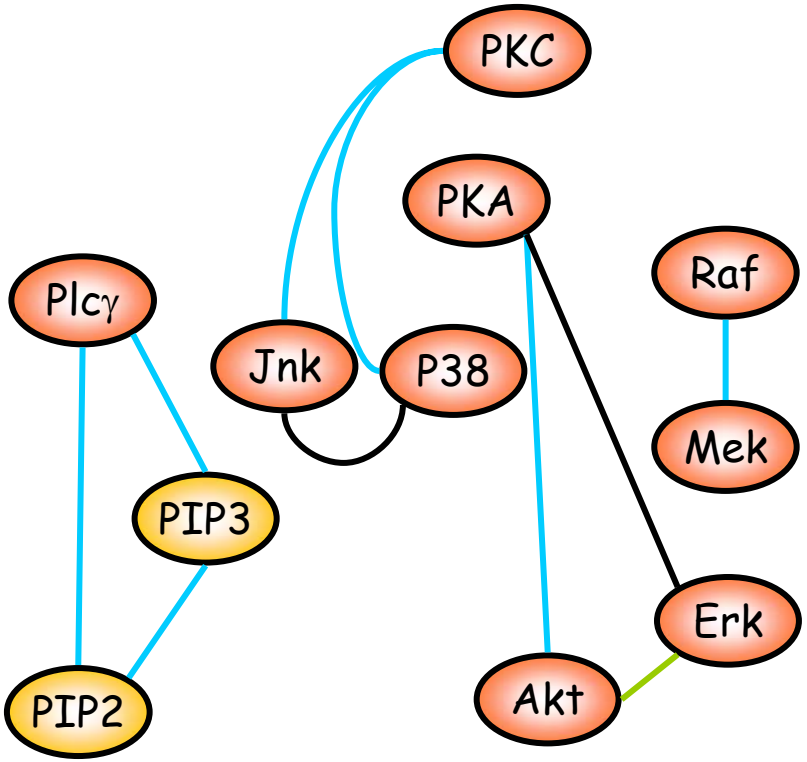


Phospho-Proteins
Phospho-Lipids
Perturbed in data

Expected Pathway
Reported
Unexplained
Reversed
Missed

- 14/17 Classic
- 16/17 Reported
- 1 Unexplained
- 4 Missed

Interventions are Required for Directionality

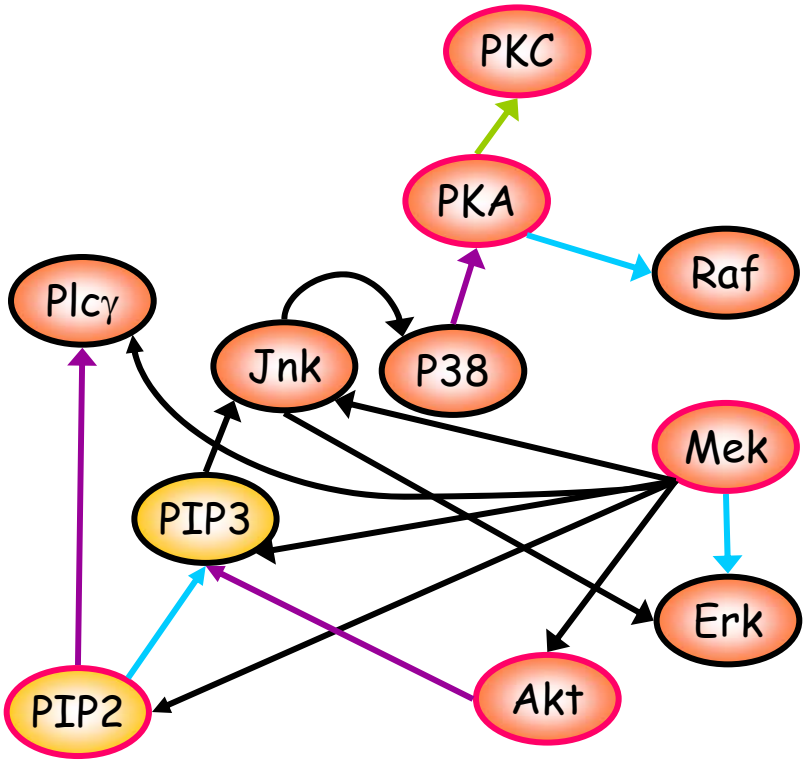


	Lacking Intervention data	Complete Dataset
Expected	7/10	14/17
Reported	1/10	2/17
Reversed	N/A	1
Unexplained	2	1
Missed	11	4

Dataset: 1200 samples:

- 2 conditions
- no interventions

Simulated Westerns Diminish Network Integrity



	"Western blot"	Complete Dataset
Expected	6/16	14/17
Reported	1/16	2/17
Reversed	3	1
Unexplained	8	1
Missed	12	4

Simulated western blot: 420 samples:

- 14 conditions
- Each point average of 20 random cells

Huge Problem with complex instrumentation

- Setting up the machine to ensure valid output.
- Setting up complex experiments in an automated fashion.
- 'Forcing' students/technical staff to conform.

*FacsXpert** and the *Libris DataStore*

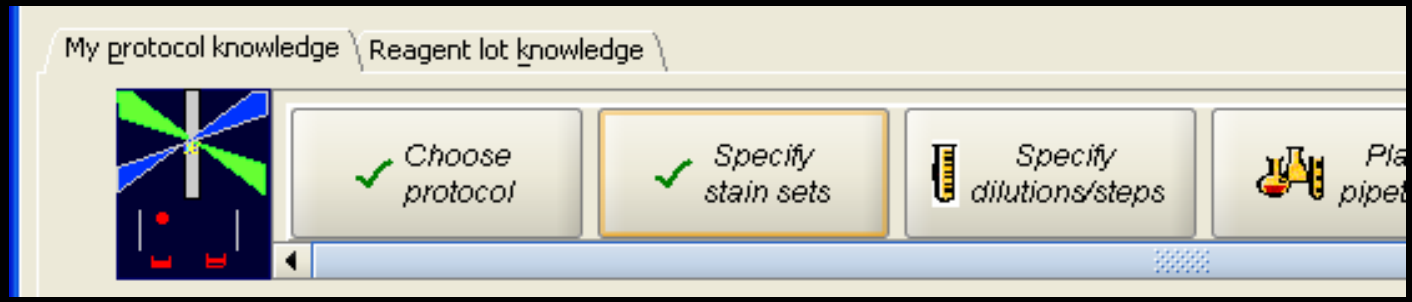
Designed to help researchers:

- Cope with this complexity when designing and executing FACS experiments
- Comply and with demanding requirements for long-term recoverability of FACS and other large data sets (Collaborative Electronic Notebook standards Association (CENSA)), US 21 CFAR part 11

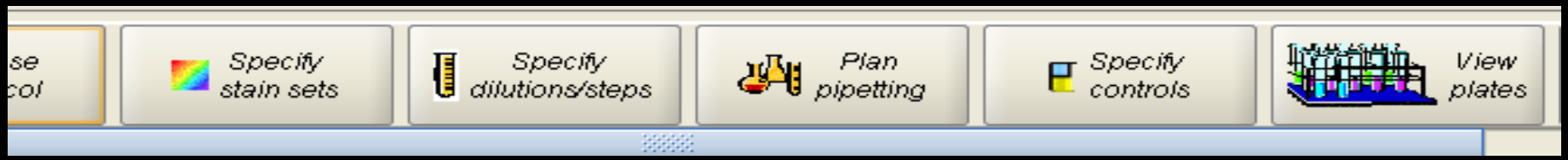
****a knowledge-based system, Herzenberg laboratory (sold by ScienceXperts, Inc.)***

Start by choosing a new/existing protocol, specify

- Study and experiment name, Subject species, Cell source (tissue)

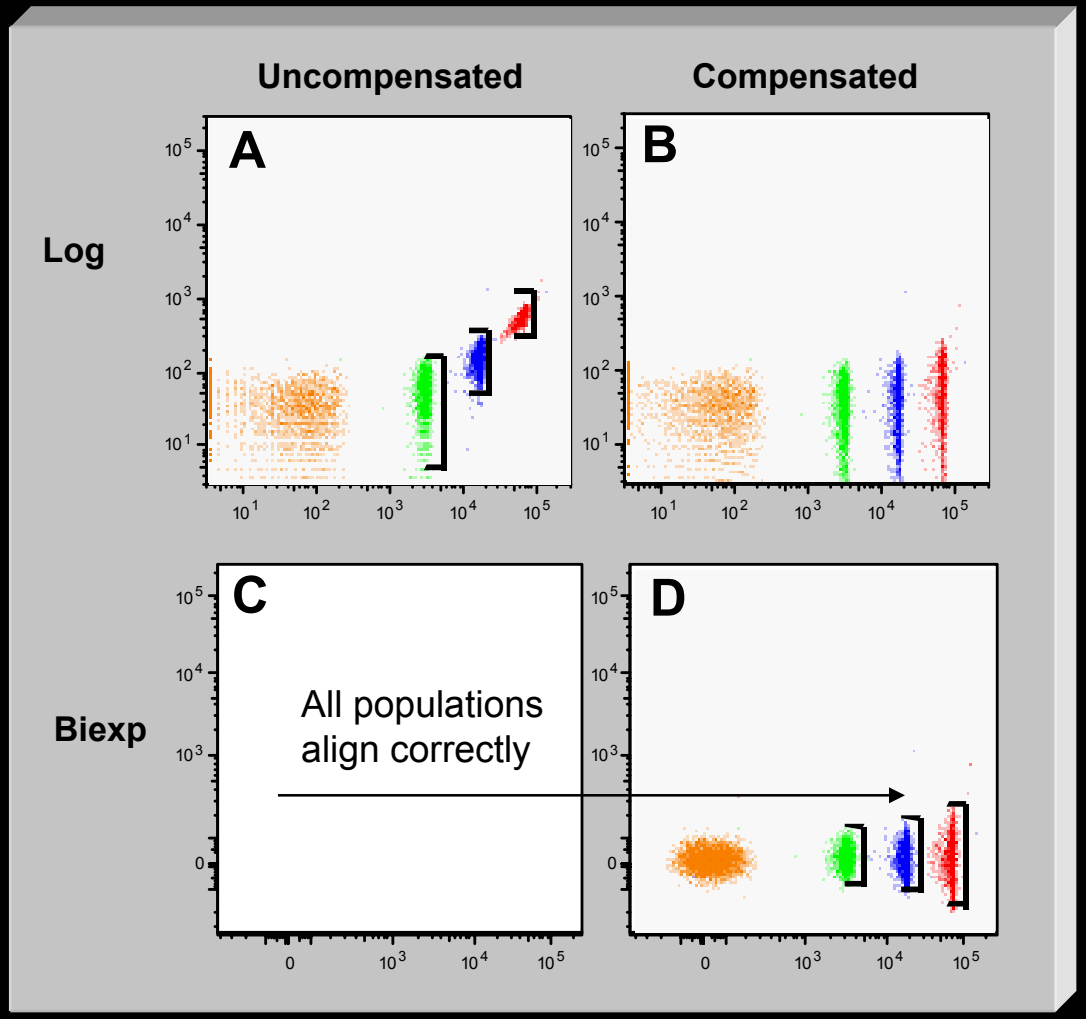


Take the individual through the experimental planning



Carry out experiment, collect data, store, analyze

Important to validate instrument setup in an automated manner



Antibody capture beads stained with 3 levels of an APC reagent

The transformed display shows aligned populations In the APC-Cy7 dimension

Single Cells are an Unparalleled Information Resource… but…

- Common standards needed for instrument setup, runs.
- Automated experiment setup/protocols
  - *intelligent notebooks*

- Standards for representation of multi-D populations.
  - what is a population and what is the biological inference?
  - Cluster analysis
- Support (i.e. $$) for new visualization of multi-D

Acknowledgements


Jonathan Irish


Peter Krutzik


Omar Perez


Matt Hale

Nolan Lab / Stanford University
NHLBI: National Proteomics Center
Kinase Signaling and IC FACS Group

Bjørn Tore Gjertsen Nina Ånensen
Randi Hovland Øystein Bruserud

Collaborators in Bergen, Norway

Karen Sachs Dana Pe'er (Harvard)
Douglas Lauffenberger
MIT

- **Bob Hoffman**
- **Dave Parks**
- **Marty Bigos**
- **Wayne Moore**
- **Diether Recktenwald**
- **Joe Trotter**

- **BD-BioSciences**

Publications

- Perez O.D. et al. (2002). **Immunity**. 16:51-65.
- Perez O.D. and Nolan G.P. (2002) **Nature Biotechnology** 20:155-162.
- Krutzik PO, Irish J, Nolan GP, and Perez OD, Analysis of Phospho-Proteins by Flow Cytometry: Techniques and Clinical Applications. **Clinical Immunology Reviews**, 2003, December.
- Perez OD and Nolan GP. Flow cytometric analysis of kinase signaling cascades. **Methods in Flow Cytometry**. Humana Press. Editor: Howard Shapiro, 2004, March.
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http://proteomics.stanford.edu

http://www.stanford.edu/group/nolan