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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.

Photo ©2000 – J.P. Robinson
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

- **Target ID/Validation**
  - Pathway mapping
  - Target Validation

- **Hit Discovery**
  - Primary screening assays
    - cell-based
    - primary cells

- **Pre-Clinical Lead Development**
  - Secondary Assays
    - Specificity Assays
    - Animal models

- **Clinical Trials**
  - Ph1
  - Ph2
  - Ph3
  - Novel biomarkers
  - Patient stratification

Drugs
From phospho-molecular profiling to 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 markers
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

**Fluorescence Sensitivity**

- Instrument
  - Q
    - Optical design efficiency
    - Laser power intensity
    - PMT quantum efficiency
    - Sheath flow rate
  - B
    - Component autofluorescence
    - Component scatter
    - Raman scatter

- Sample
  - Cell autofluorescence
    - Laser power intensity
  - Unbound dye/fluorochromes
    - Laser power intensity
    - Sample flow rate
  - Spectral overlap
    - Laser power intensity
    - Filter design
Multi-color FACS: Spectral Overlap

Fluorescein

PE

TRPE

Cy5PE

Cy5.5PE

Cy7PE

Cascade Blue

Alexa 430

Alexa 594

APC

Cy5.5APC

Cy7APC

Wavelength (nm)
Stanford Biexponential Display (Logicle)

Log Display

Biexponential
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\(\alpha/\beta/\gamma/\delta\), Rsk
- Raf, Mek, ERK, ELK
- Rsk, Creb,
- STAT1,3,5,6, c-Src
- CREB, cJUN, IKK\(\alpha\)
- p53 s15, s20 s37, s392
- Pyk2, Shc, Fak, src
- Slp76, Zap70, Syk, Lat, Vav,
- Lck, PLC\(\gamma\)
- 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 Pkg
- PDGFR RB
- cKit NFAT
- VEGFR NFKB
- PKA Caveolin
- PKA 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 Pharmacodynamics in Vivo

Cell Subset Specific IFN$\gamma$ Sensitivity across a titration

**IFN$\gamma$ Dose Response Curves**

- T Cells
- B Cells
- Monocytes

- 0.5 U
- 6 U
- 3 U
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

Phosphorylation Scale

-3 Fold  No Change  +3 Fold

log₂ [ stimulated / unstimulated ]
Cytokine Responses of Normal and Tumor Cells

Irish et al, Cell, 2004
Clustering of Biosignature, Clinical Significance

Irish et al, Cell, 2004
SC-P2 (Flt3 mutant, chemotherapy insensitive)

SC-P2 Composite Profile
Array Overview of Lymphoma Signaling

- Various mAb Panels
  - (250,000 cells / well)
  - Staining of Stimulated Biopsy Cells

- Control
- IL-4
- IL-6
- IL-7
- IL-10
- IFN-α
- IFN-γ
- TNF-α
- PMA-comp.
- eGF-HM
- eCD20

- p-Stat6
- p-Stat1
- p-Stat3
- p-Stat5
- p-p38
- p-Erk1/2
- p-Lck
- p-Syk
- p-PLCγ1

- FL-P01 [B Cells]
- FL-P01 [Lymphocytes]
- FL-P01 [TIL T Cells]

- log2 scale
- Stimulated / Control
- 83% B
- 17% T

- J. Irish

+2.5 fold
- no change
- -2.5 fold

Cancer Patient
Tumor Biopsy

[Diagram with color-coded heat maps and scatter plots showing cellular signaling pathways and percentages of B and T cells]
New Approaches To Representing Single Cell Data Present New Problems, but suggest Interesting possibilities.

Classic

Heatmap

Multi-D Single Cell

Macrophage

B cells

CD4+ T cells
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

Datasets of cells
- condition 'a'
- condition 'b'
- condition... 'n'

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

- 9 phosphoproteins, 2 phospholipids
- 600 cells per condition
  - 5400 data-points
T-Lymphocyte Data

Influence diagram of measured variables

Datasets of cells
- condition 'a'
- condition 'b'
- condition... 'n'

Bayesian Network Analysis

Conditions (96 well format)

perturbation a

perturbation b

perturbation n

11 Color Flow Cytometry

Raf Mek1/2 Erk p38 PKA PKC Jnk PIP2 PIP3 Plc γ Akt
A T cell signaling map *ab initio* from multiparameter data by Bayesian Inference.

- 14/17 Classic
- 16/17 Reported
- 1 Unexplained
- 4 Missed
Interventions are Required for Directionality

Dataset: 1200 samples:
- 2 conditions
- no interventions
Simulated Westerns Diminish Network Integrity

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

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

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