A Single Cell Network Profiling (SCNP) view of the immune system

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Nodality Inc.

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WORKSHOP • PRIMER • ANNUAL MEETING

Disclosure Information

The following relationships exist related to this presentation:

Alessandra Cesano

Nodality Inc.: Salary, Shares, Full time Employee

Presentation Map

- Single Cell Network Profiling (SCNP) technology:
  - Principles
  - Industrialization

- Application to immune system profiling

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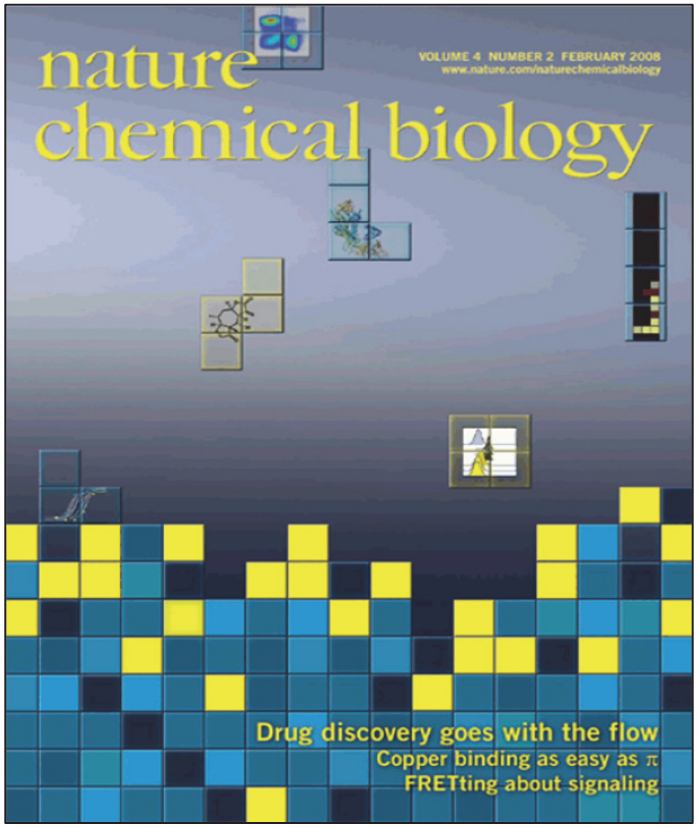
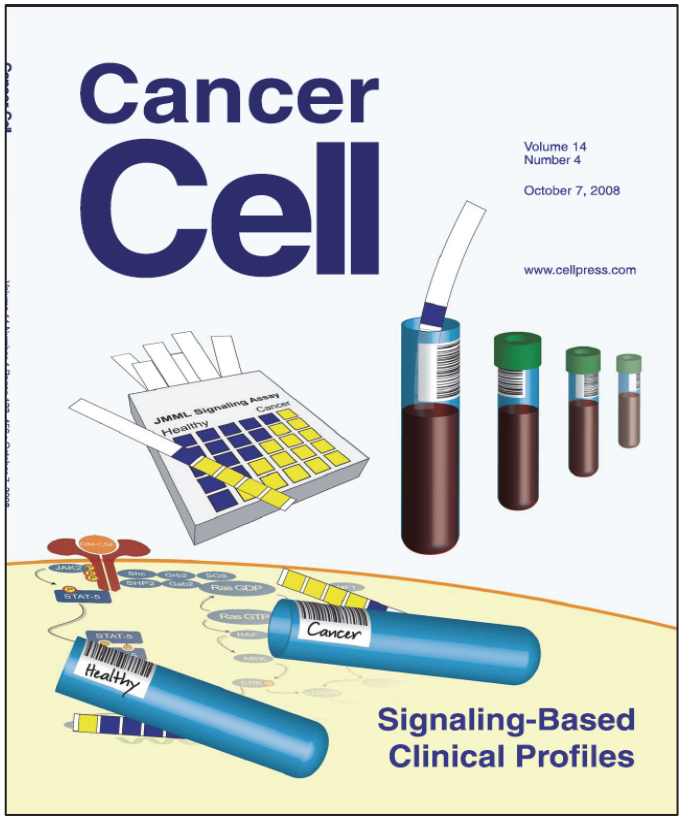
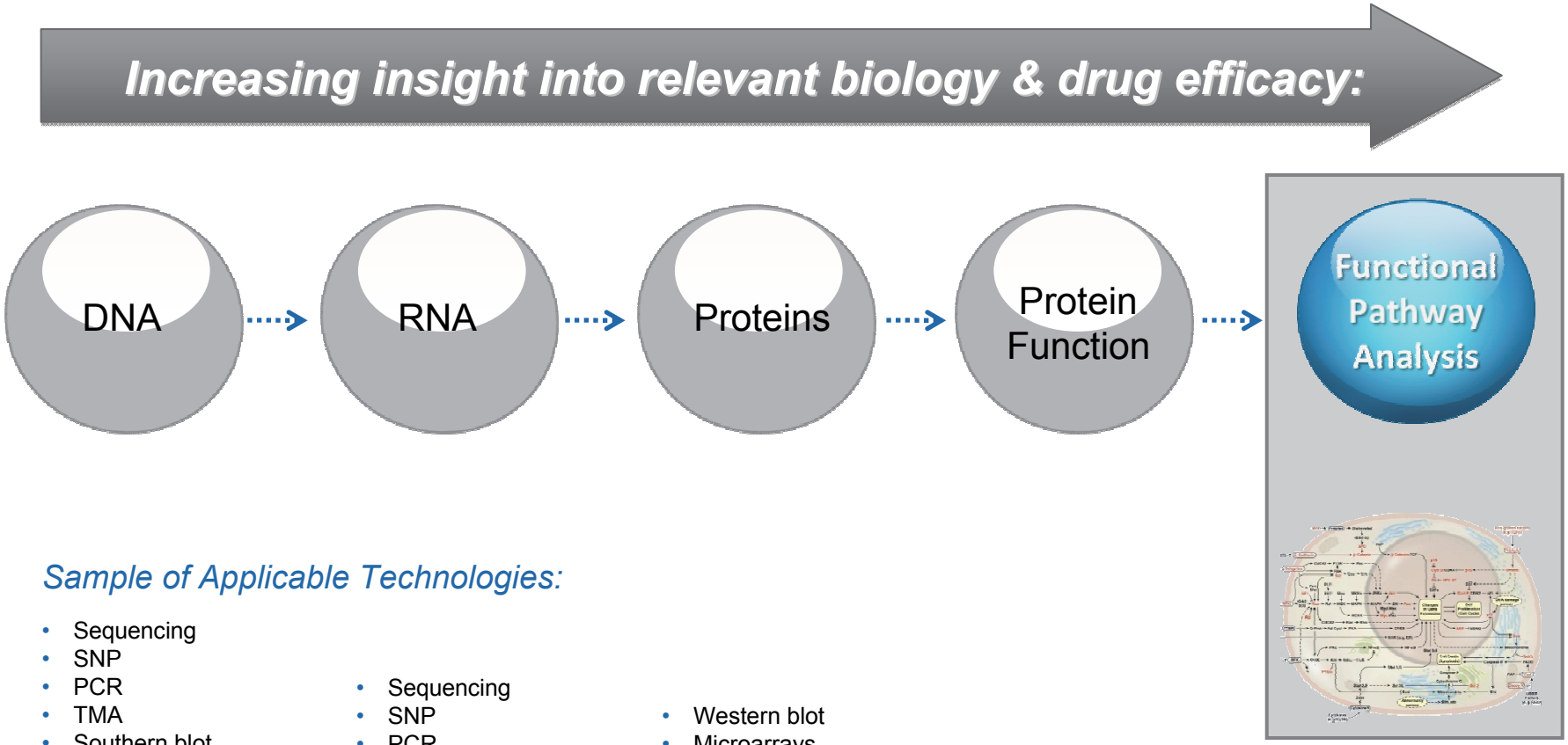
SCNP foundation technology

- Proprietary phosphoflow signaling technology developed in Dr. Garry Nolan's lab at Stanford University





Functional Pathway Analysis: Closer To Relevant Biology



Sample of Applicable Technologies:

- Sequencing
- SNP
- PCR
- TMA
- Southern blot
- RFLP
- STR
- Microarrays

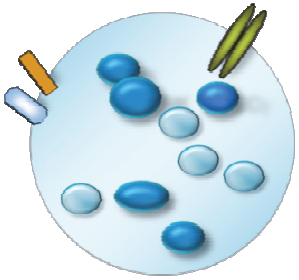
- Sequencing
- SNP
- PCR
- TMA
- Northern blot
- Microarrays

- Western blot
- Microarrays
- Mass spec
- IHC
- Immunoprecipitation

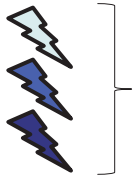
- IHC
- Mass spec
- Functional imaging

Key Steps In The SCNP Assay

1 *Bone Marrow or Blood Whole, Fractionated, or Cryopreserved*

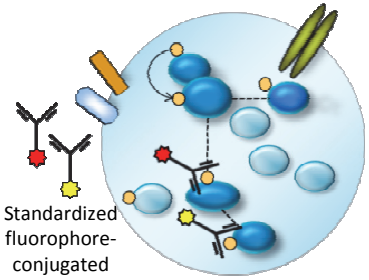


2 *Modulate Living Cells, Fix Cells to Stop Signaling, Permeabilize Cells*



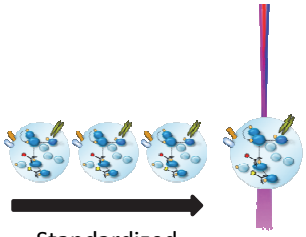
Modulate Factors, Inhibitors, Drugs

3 *Add Antibodies to Quantify Cellular Pathway Activity and Cellular Morphology*



Standardized fluorophore-conjugated antibodies

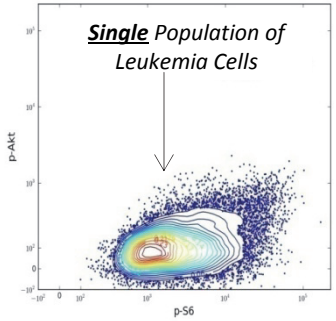
4 *Acquire Quantitative Data at the Single Cell Level*



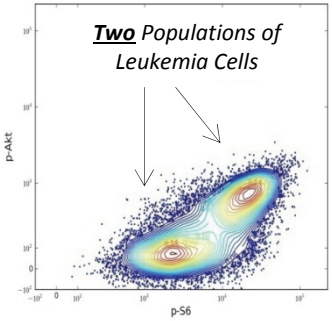
Standardized Instrumentation

5 *Quantify Intracellular Pathway Activity in Cell Subsets Identified by Gating on Surface Markers*

Basal State



Modulated

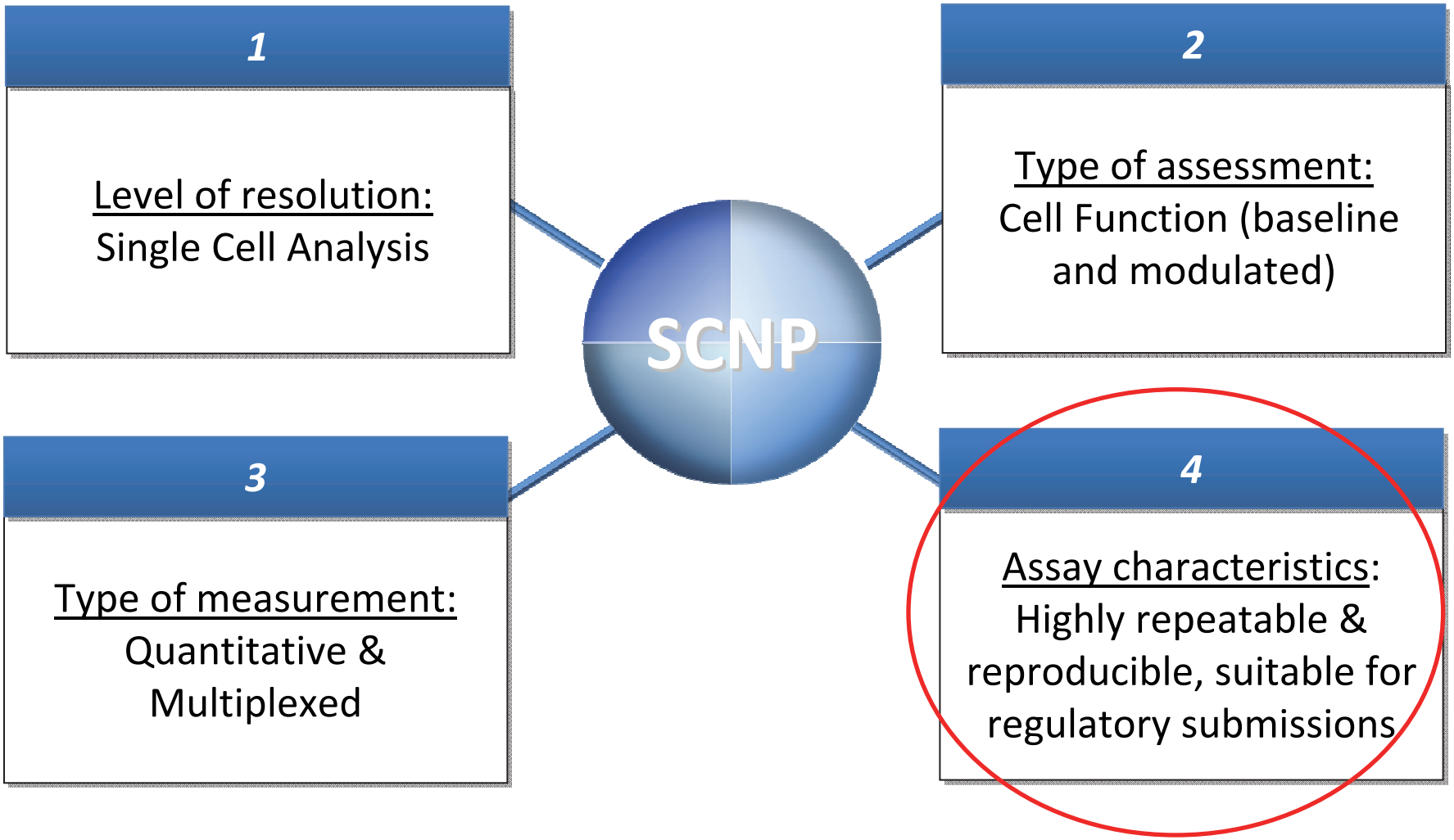


Correlate Pathway Biology to Efficacy & Outcomes

Node: **A signaling readout being measured under modulation in a cell to determine its contribution to pathway activity**

Node = (Modulator) → (Signaling Readout)

SCNP: Unique Combination of Functional & Customizable Characteristics in One Assay



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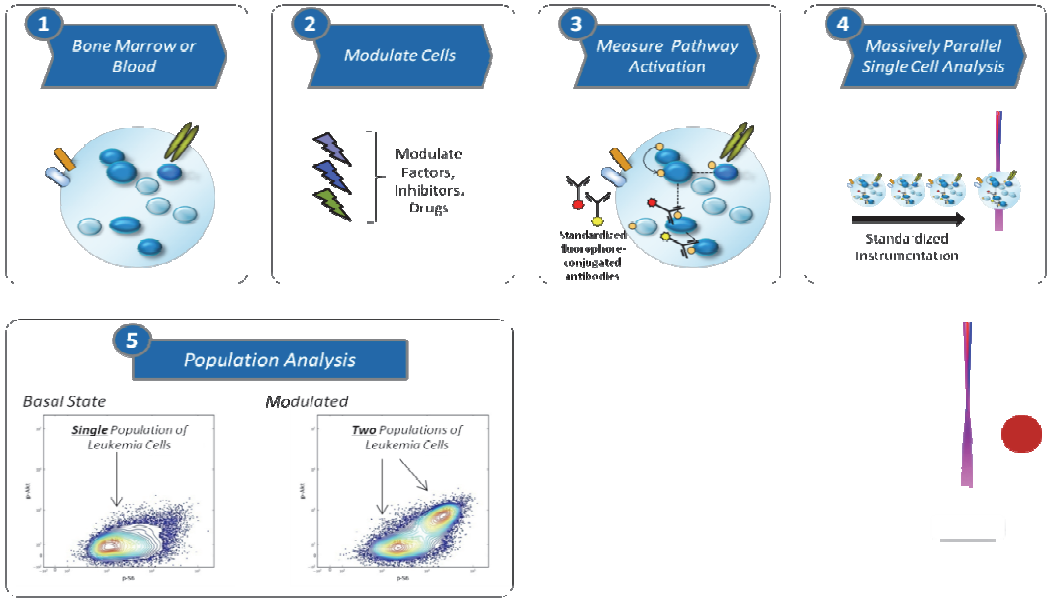
  - Principles
  - Industrialization
- Application to immune system profiling

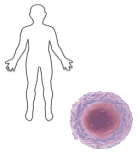
A Complex Assay With Many Components & Dimensions To Track



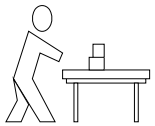
Instruments

Antibodies

Modulators

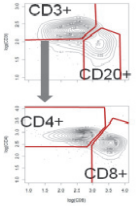


Patient Data



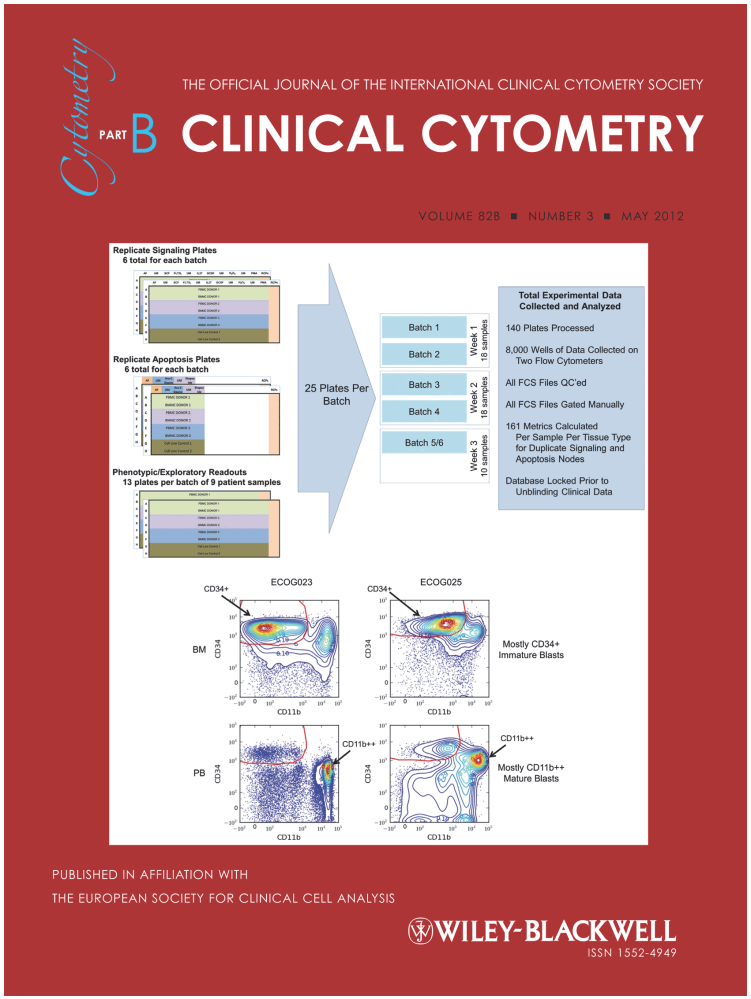
Operational Parameters

Data Files and Images



Cell Populations

SCNP assay “industrialization” for application to Clinical Medicine and Drug Development

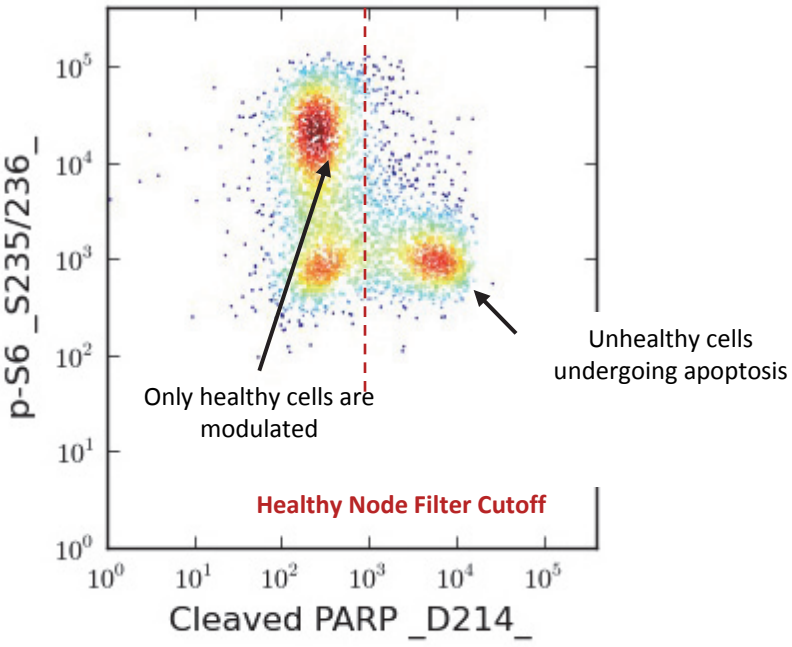


Cesano et al, Cytometry Part B **2012**; 82B: 158–172.

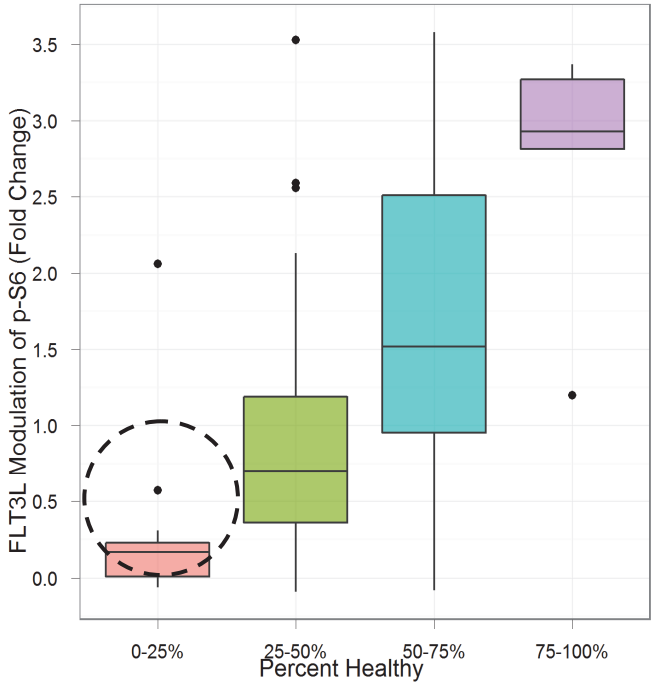
Industrialization of SCNP assay has been achieved through development of several controls, processes, and infrastructure:

1. Standardization of lab processes and instruments
2. Reagent manufacture and/or qualification
3. Study workflows
4. Built ad hoc informatics infrastructure
5. Pre-analytic sample quality and source tissue specifications
6. Repeatability and reproducibility
7. Flexible for pathway discovery & optimization of molecular/companion diagnostic constituents
8. Rigorously designed to meet CLIA & FDA requirements

Evaluation Of Sample Quality: Defining “Healthy Cells”



- Healthy cell % cutoffs are established for each disease/cell subtypes



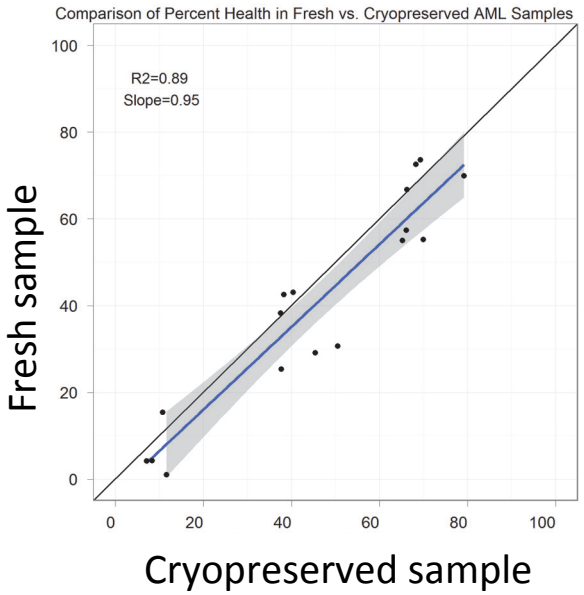
- Samples are tested for quality and alignment with predefined, disease-specific cutoffs
  - Cell health is defined as the fraction of cells in a given well that are not undergoing apoptosis as defined by amine aqua stain and cPARP levels

•Sample evaluability criteria are used to exclude these samples from further analysis

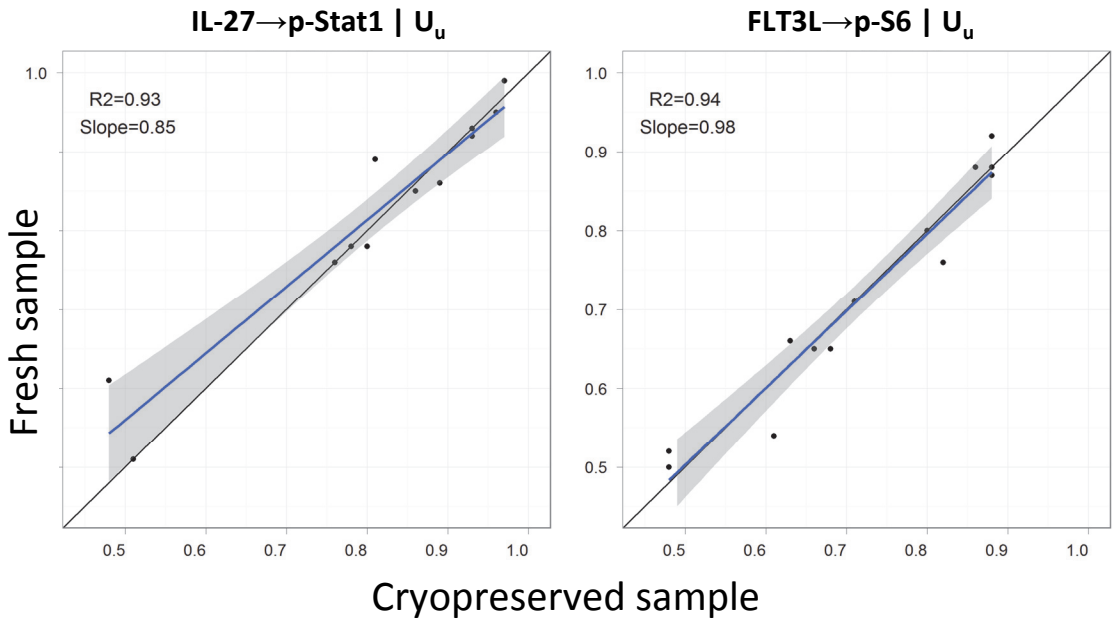
Robust Repeatability & Reproducibility Of SCNP Assay Following Sample Cryopreservation

Paired Fresh vs cryopreserved PBMC and BMMC samples from AML patients

Cell Health is not compromised by cryopreservation

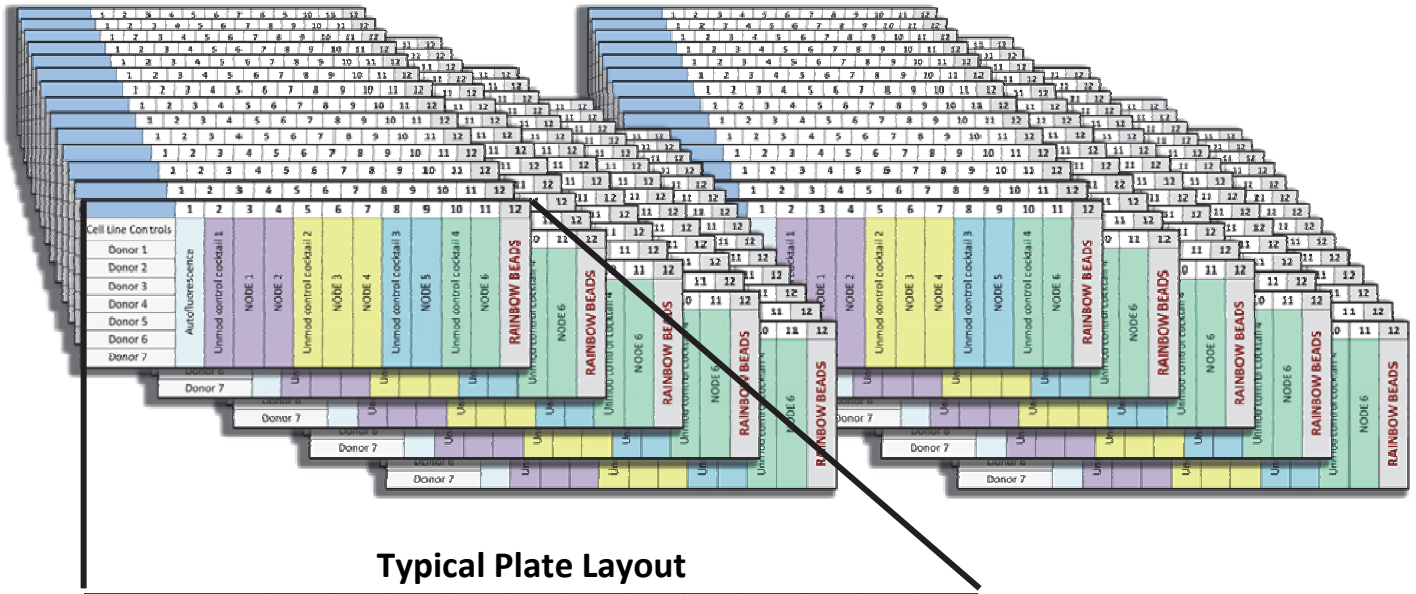


SCNP assay results are unaffected by sample cryopreservation



- ***SCNP data is highly concordant between fresh and cryopreserved samples***
- ***15 of the 19 nodes showed R > 0.80 for both tissue types***

High-Throughput, High-Content Assays With Many Internal Relationships



Typical Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
Cell Line Controls	Autofluorescence	Unmod control cocktail 1	NODE 1	NODE 2	Unmod control cocktail 2	NODE 3	NODE 4	Unmod control cocktail 3	NODE 5	Unmod control cocktail 4	NODE 6	RAINBOW BEADS
Donor 1												
Donor 2												
Donor 3												
Donor 4												
Donor 5												
Donor 6												
Donor 7												

Multiparametric analysis expands array of node options beyond the number of available fluorophores

Flexible format for different needs, such as IC_{50}s and kinetic studies

Example of Experimental Data Collected and Analyzed

92 Patient samples

140 plates processed

>8,000 wells of data collected on two flow cytometers

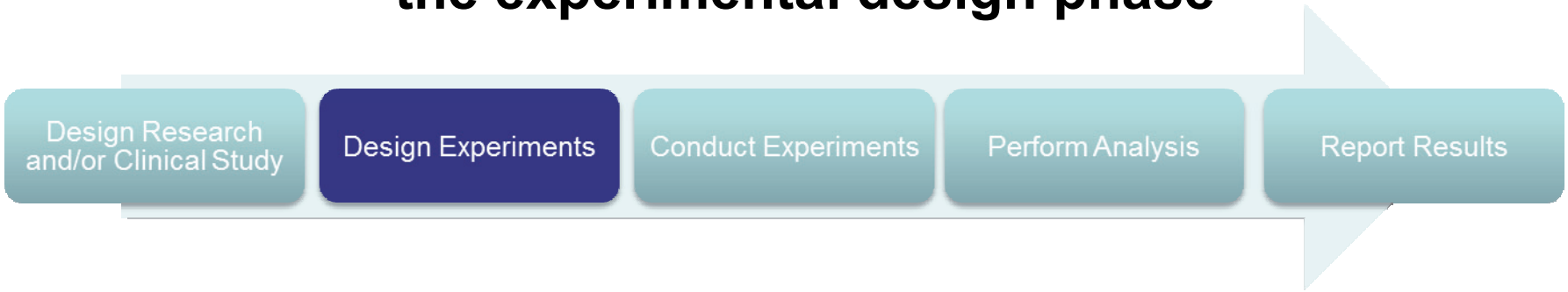
All FCS Files QC'ed

All FCS Files Gated manually

189 Metrics Calculated per sample per tissue type

Database Locked Prior to Unblinding Clinical Data

Reagents, Plate layouts and Controls are finalized in the experimental design phase



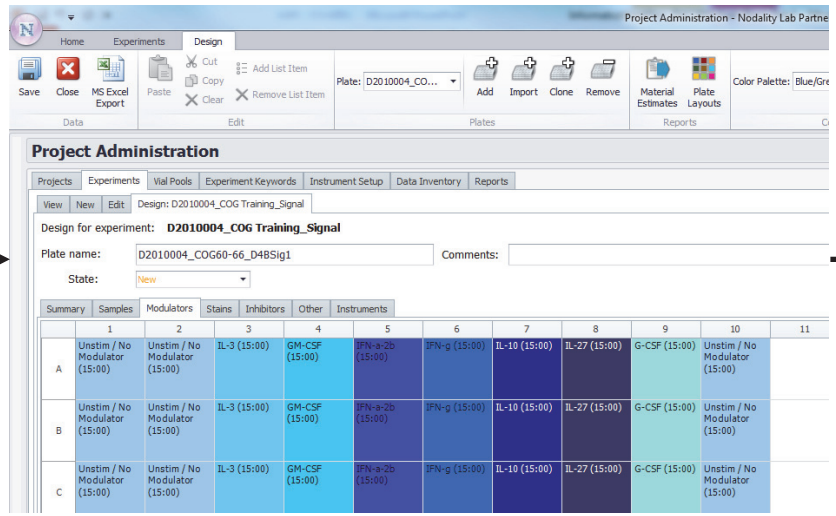
1. **Reagents**
   - **Antibody cocktails**
   - **Modulators**
2. Plate design
3. Assay controls

- Most studies use GMP reagents
  - Internal reagent qualification following standard SOP
  - Longitudinal reproducibility of the data
  - Reagent stability program
- Antibodies are often combined into cocktails and qualified
  - Biologically meaningful combinations (e.g. same pathway)
    - What data is needed at a single cell level vs. at an aggregate level
    - Are the kinetics the same for all components of the cocktail
  - Fluorophore combinations
  - Gating markers

Internal software used to layout plates at user's desk: Maximize cytometer usage and minimize errors

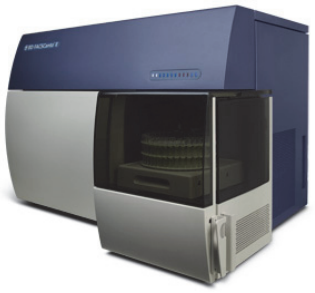
1. Reagents
2. **Plate design**
3. Assay controls

Sample and Reagent inventory



QC Layout Files using internal software

DIVA



Cytometer

- Pull down all key components from pre-defined inventory item
  - No manual typing
- Templating and rapid cloning
- Export all meta-data to layout files
  - Antibodies, modulators, samples
  - Cytometer settings
  - Detector voltages and compensation

Assay and Instrument controls on every plate

1. Reagents
2. Plate design
3. **Assay controls**

Typical Plate Layout

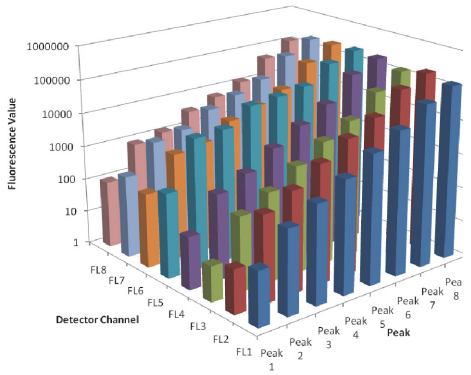
	1	2	3	4	5	6	7	8	9	10	11	12
Cell Line Controls	Autofluorescence	Unmod control cocktail 1	NODE 1	NODE 2	Unmod control cocktail 2	NODE 3	NODE 4	Unmod control cocktail 3	NODE 5	Unmod control cocktail 4	NODE 6	RAINBOW BEADS
Donor 1												
Donor 2												
Donor 3												
Donor 4												
Donor 5												
Donor 6												
Donor 7												

- Cell line controls undergo all steps of the SCNP process
  - Thawing
  - Modulation
  - Staining
  - Acquisition
- Typically used as positive controls

- Daily QC of instrument
- Rainbow beads are used as instrument controls and to calibrate the raw intensity value

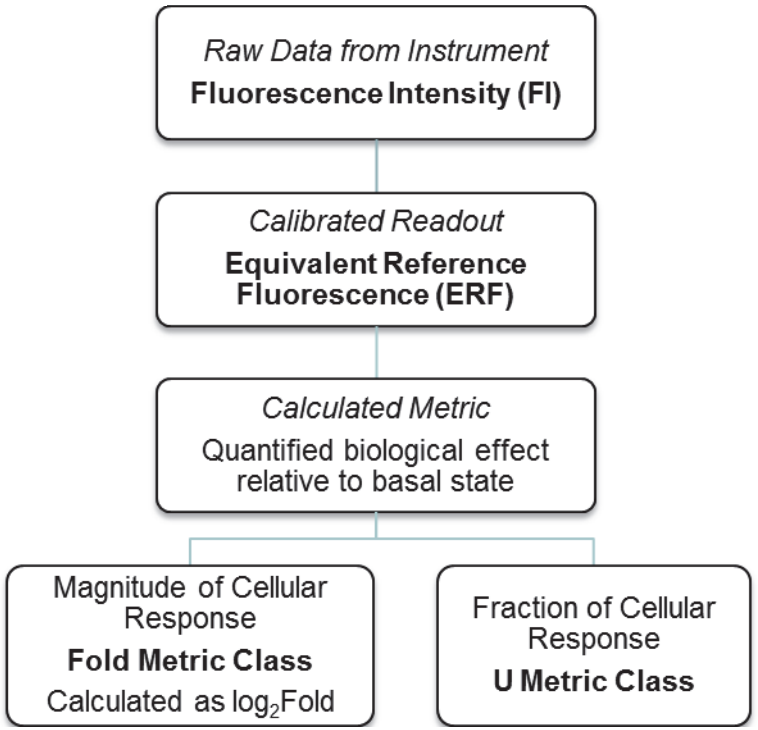
$$ERF = a + b*MFI$$

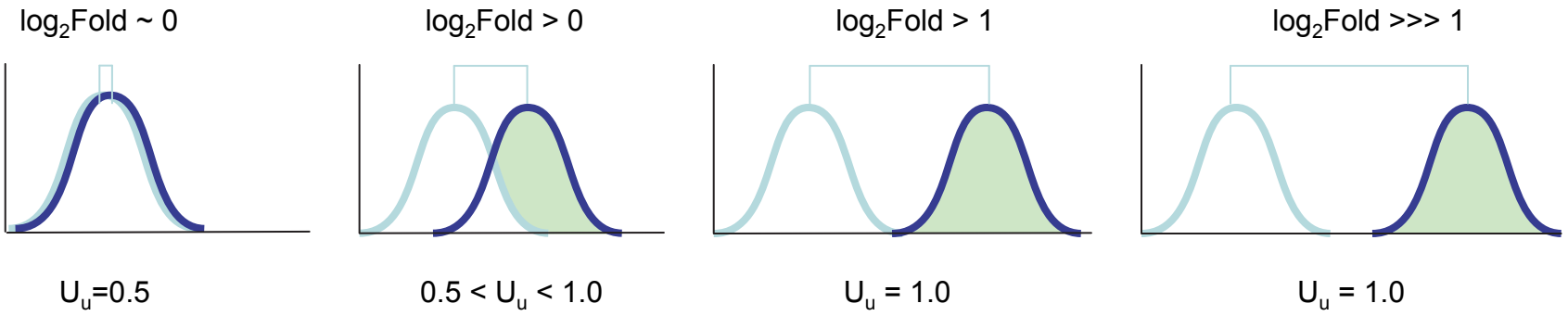
Linear calibration derived for each plate and color



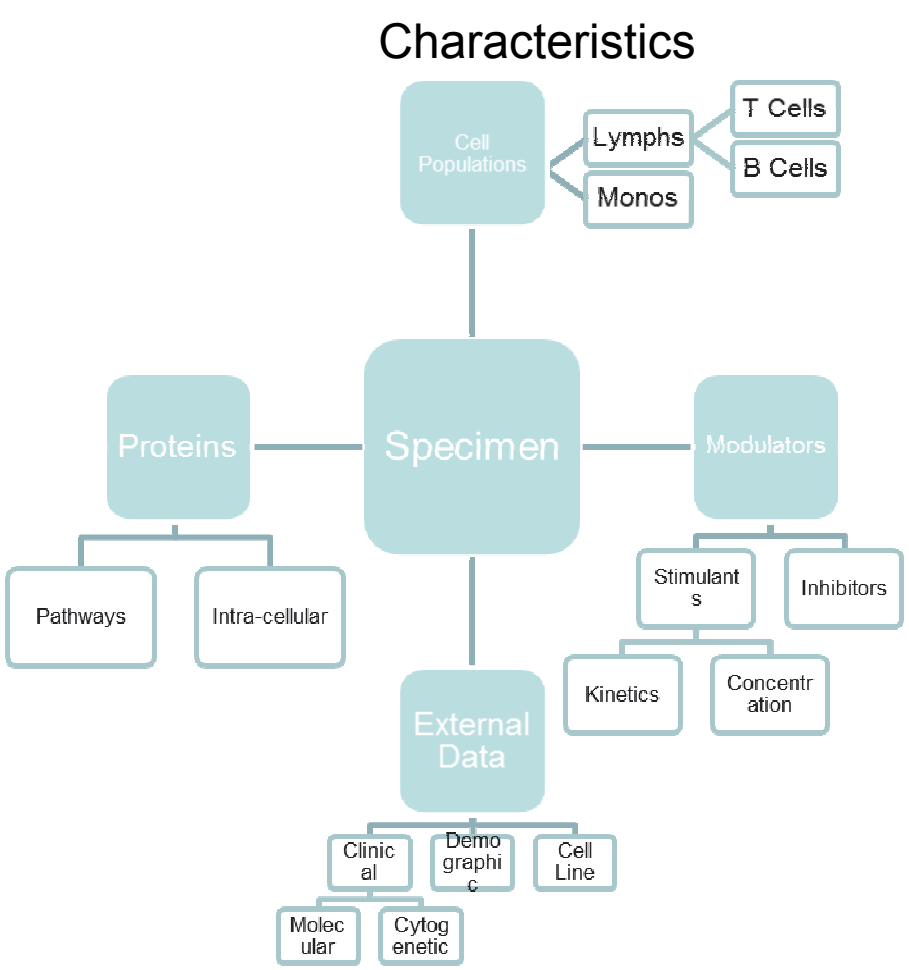
Functional Signaling Requires Appropriate Metrics That Are Different From Those Used To Quantify Surface/Static Markers

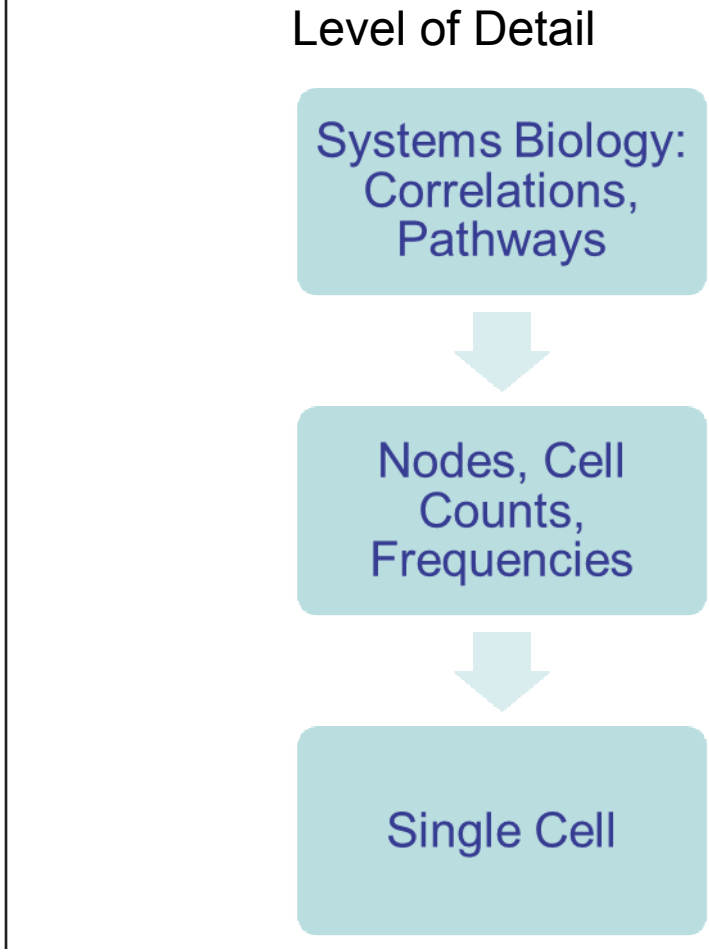
- Calibrated instruments, standardized reagents, rigorous data tracking allow for robust biological interpretations
- Specific biological interpretations require different functional readouts





SCNP Data Has Various Characteristics at Various Levels of Detail

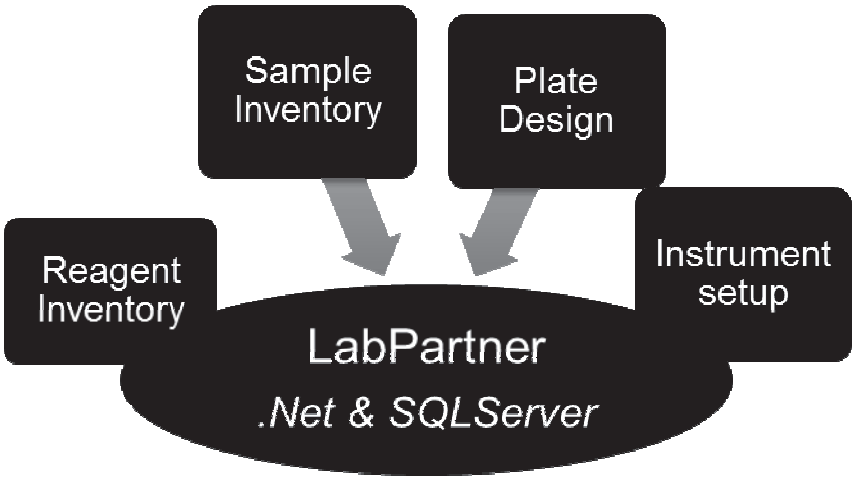




Analysis and visualization of SCNP data typically involves comparing data from one part of the characteristics space with another at an appropriate level of detail

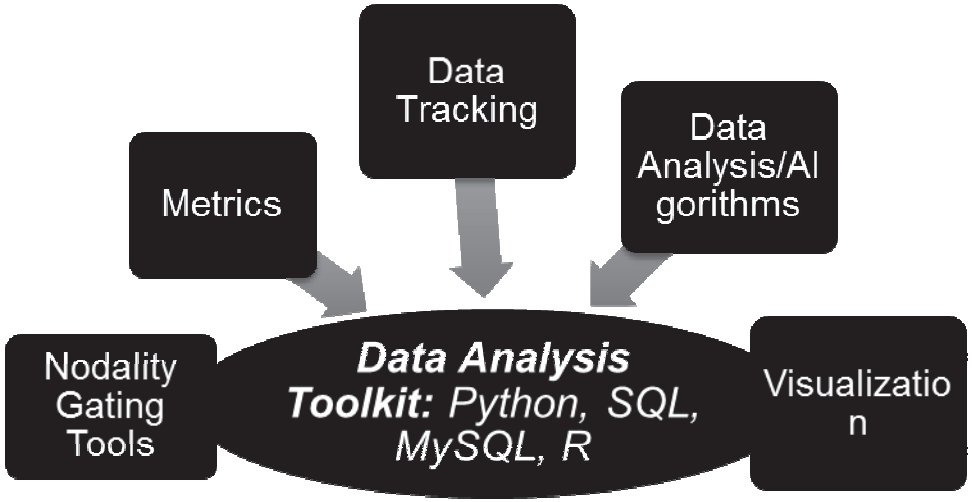
SCNP Platform Is Supported By A Robust Informatics Infrastructure

Front end user application with a rich User Interface (UI)



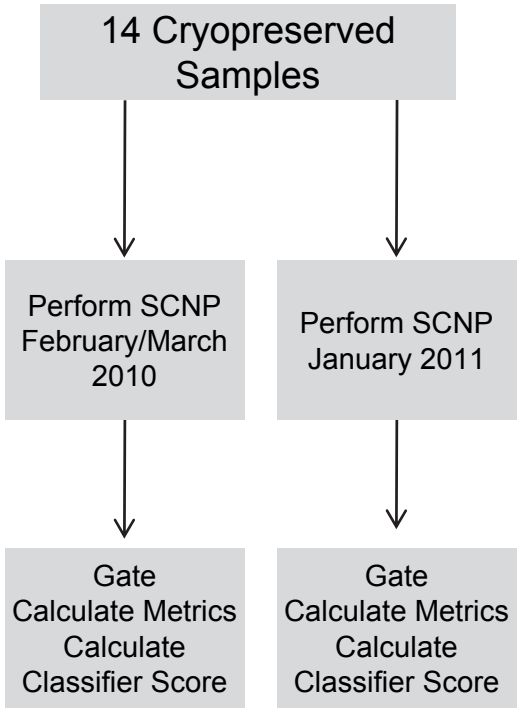
- Flexible, instrument agnostic experimental design tool
- Exploit rich UI framework in .NET
- Integration with sharepoint and report generation

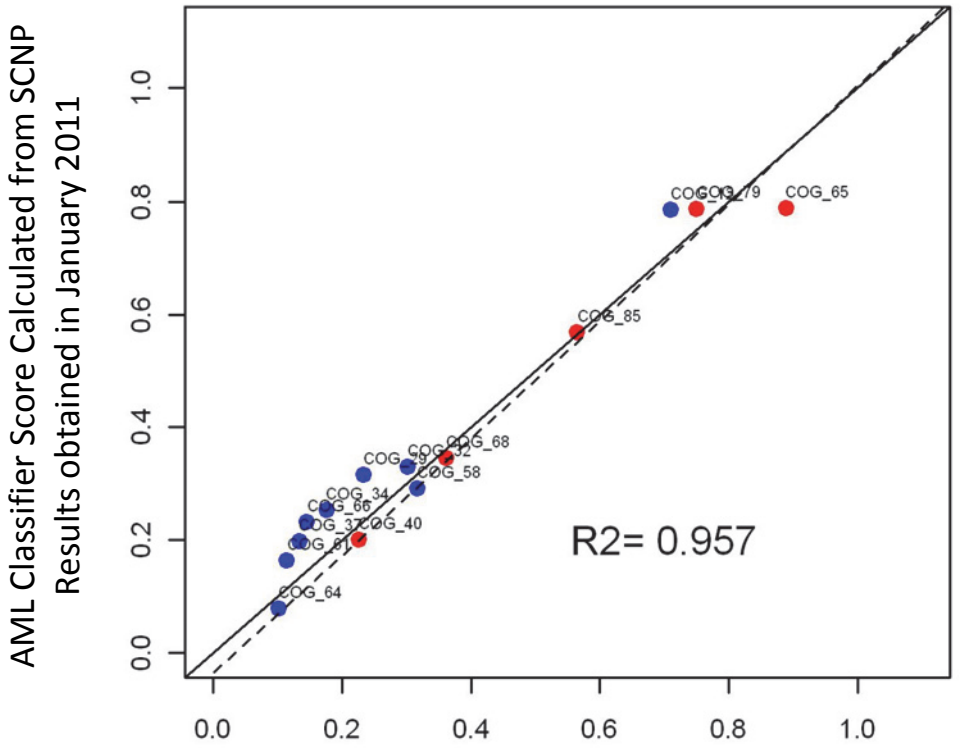
Back end Toolkit for flexibility with custom works flow and data analytics



- SCNP specific data management
- Data analytics on clinical studies
- Use Python as a glue
  - Numerics, R, plotting, office tools
- FCS file, WinList and FlowJo file parsing

SCNP Assay Results Are Reproducible Longitudinally





Results incorporate variance due to instruments, assays, reagents, operators, laboratory sites, sample storage

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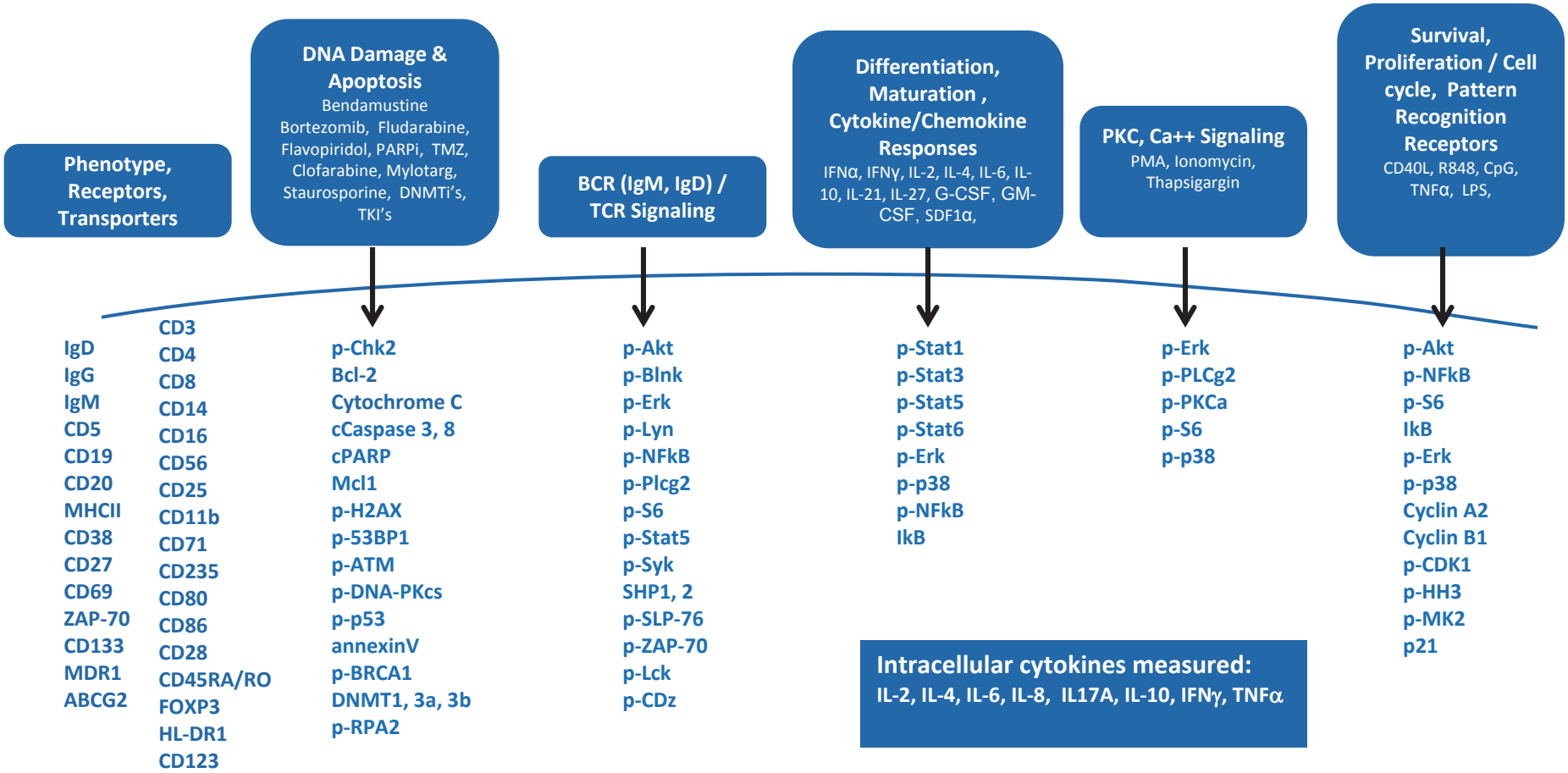
- Application to immune system profiling

SCNP-based Tools Developed To date to Monitor Immune Signaling Biology - Repertoire Is Constantly Expanding

- **Examination of healthy and disease-associated signaling**
  - Healthy immune landscaping studies in various demographic groups
  - Rheumatoid arthritis and systemic lupus erythematosus longitudinal studies
  - Cancer Immunotherapy

CURRENTLY ESTABLISHED NODES AND PHENOTYPIC MARKERS



Healthy Immune Landscape Network Map:
Establishing Baseline Signaling For Disease/Therapeutic Characterization

This information is current as of January 17, 2012

Single-Cell Network Profiling of Peripheral Blood Mononuclear Cells from Healthy Donors Reveals Age- and Race-Associated Differences in Immune Signaling Pathway Activation

Diane M. Longo, Brent Louie, Santosh Putta, Erik Evensen, Jason Ptacek, James Cordeiro, Ena Wang, Zoltan Pos, Rachael E. Hawtin, Francesco M. Marincola and Alessandra Cesano

J Immunol; Prepublished online 13 January 2012;

Longo *et al. Journal of Translational Medicine* 2012, **10**:113
http://www.translational-medicine.com/content/10/1/113

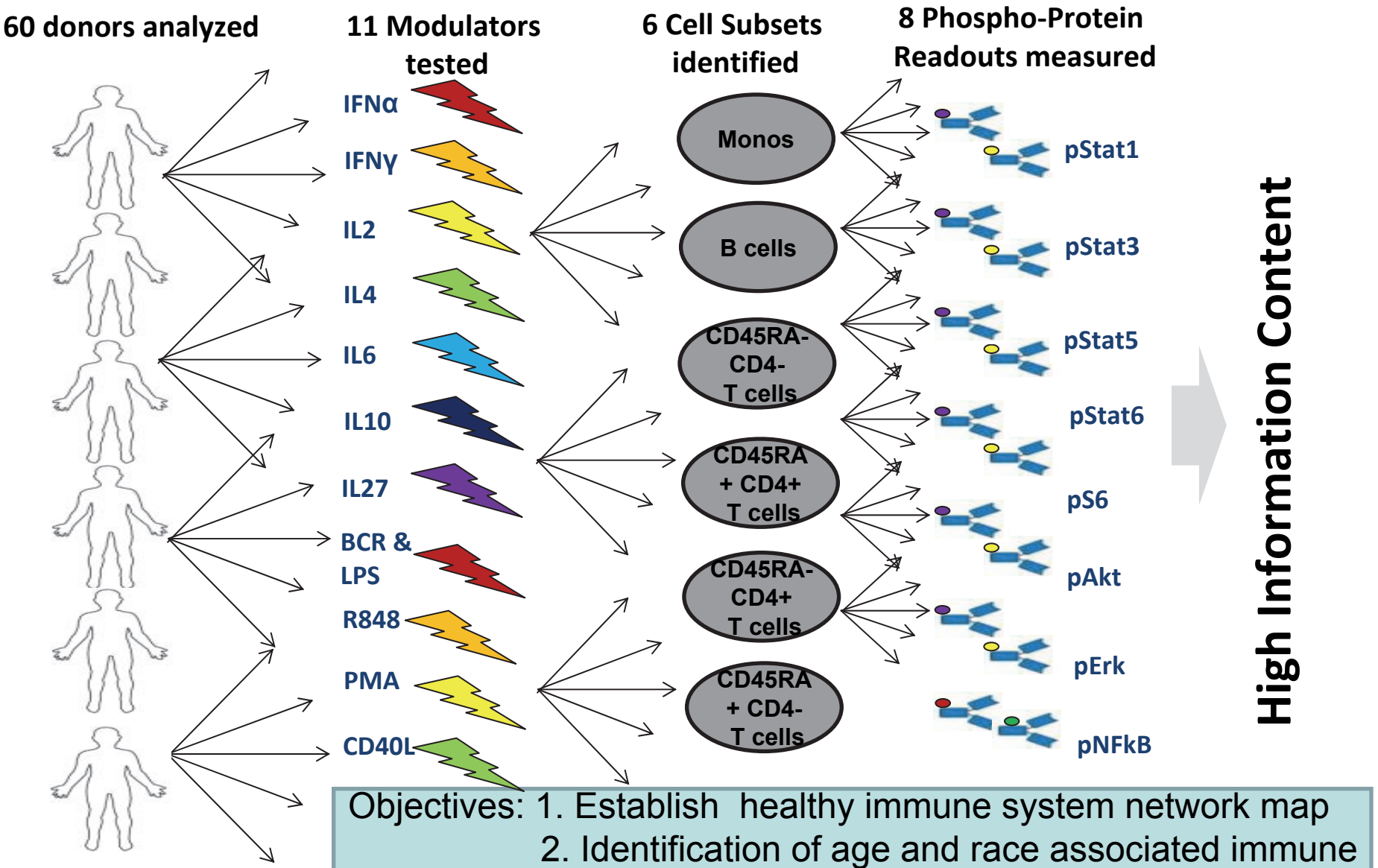
JOURNAL OF TRANSLATIONAL MEDICINE

RESEARCH Open Access

Racial differences in B cell receptor signaling pathway activation

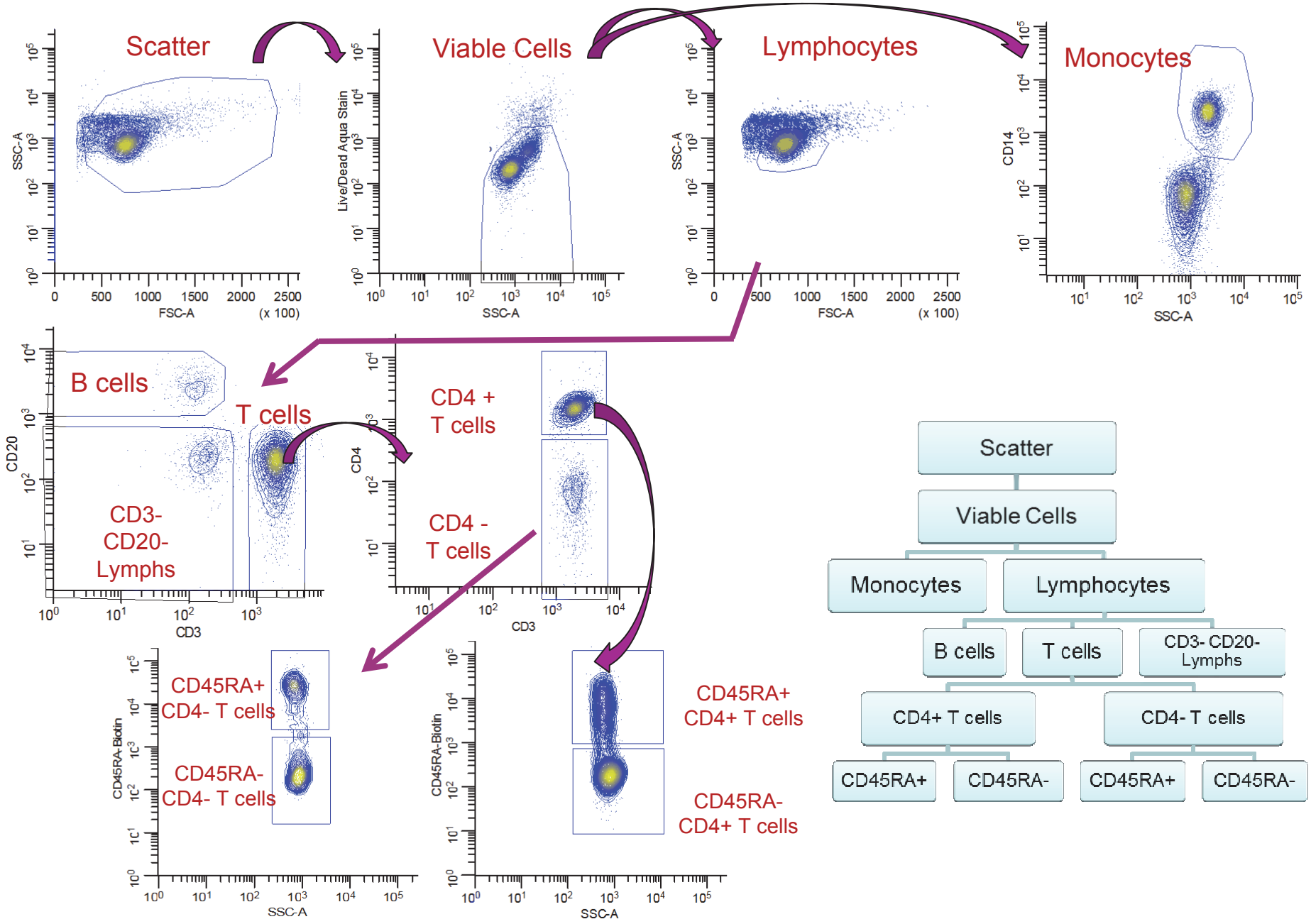
Diane M Longo[1*], Brent Louie[1], Kavita Mathi[2], Zoltan Pos[3], Ena Wang[4], Rachael E Hawtin[1], Francesco M Marincola[4] and Alessandra Cesano[1]

Experimental Design For Healthy Immune Landscape Study

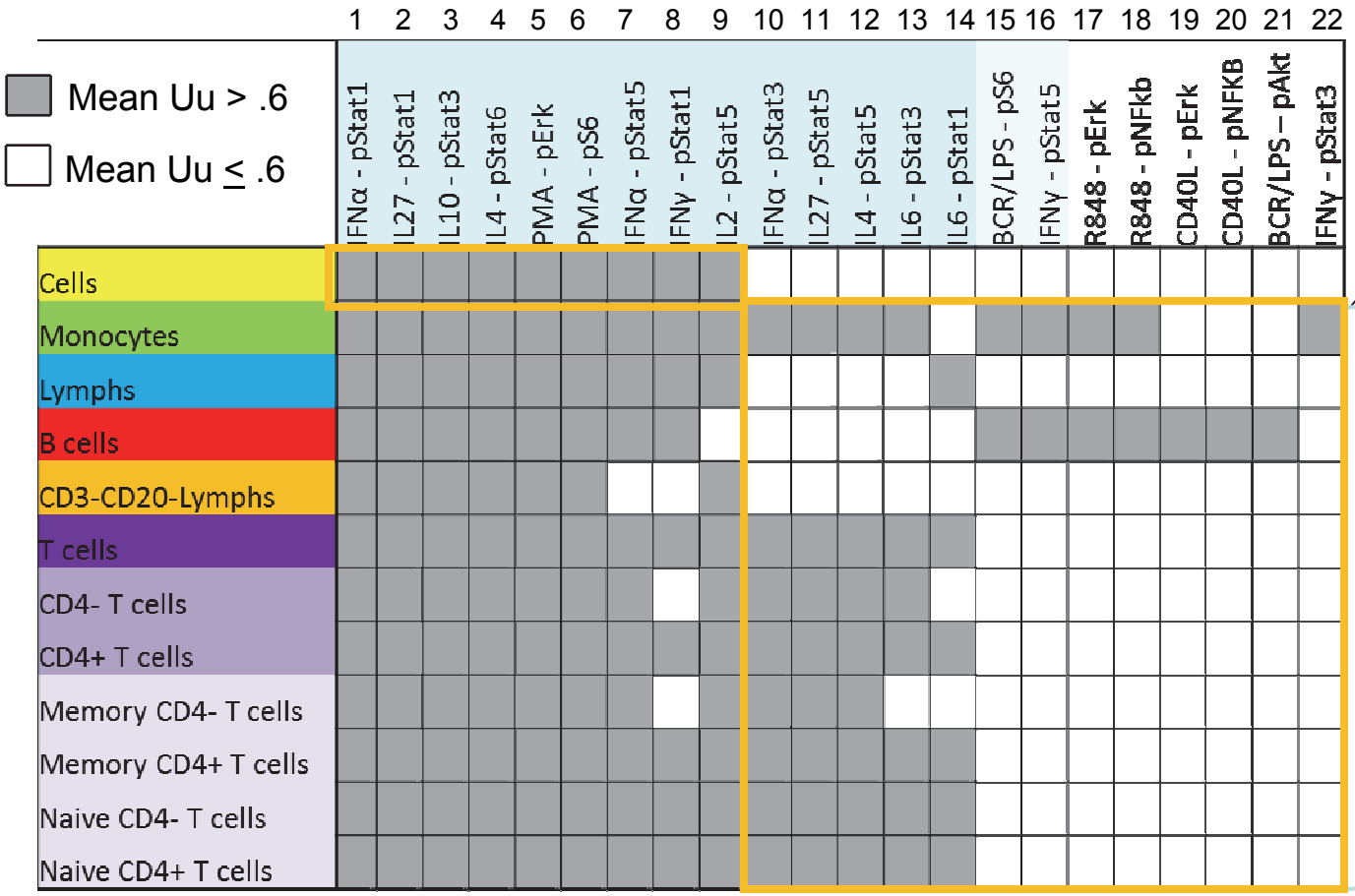


Objectives: 1. Establish healthy immune system network map
2. Identification of age and race associated immune signaling responses

Multiple Cell Subsets Are Simultaneously Examined In The Same Sample



Cellular Subsets Reveal Signaling Responses Not Seen In The Entire Cell Population

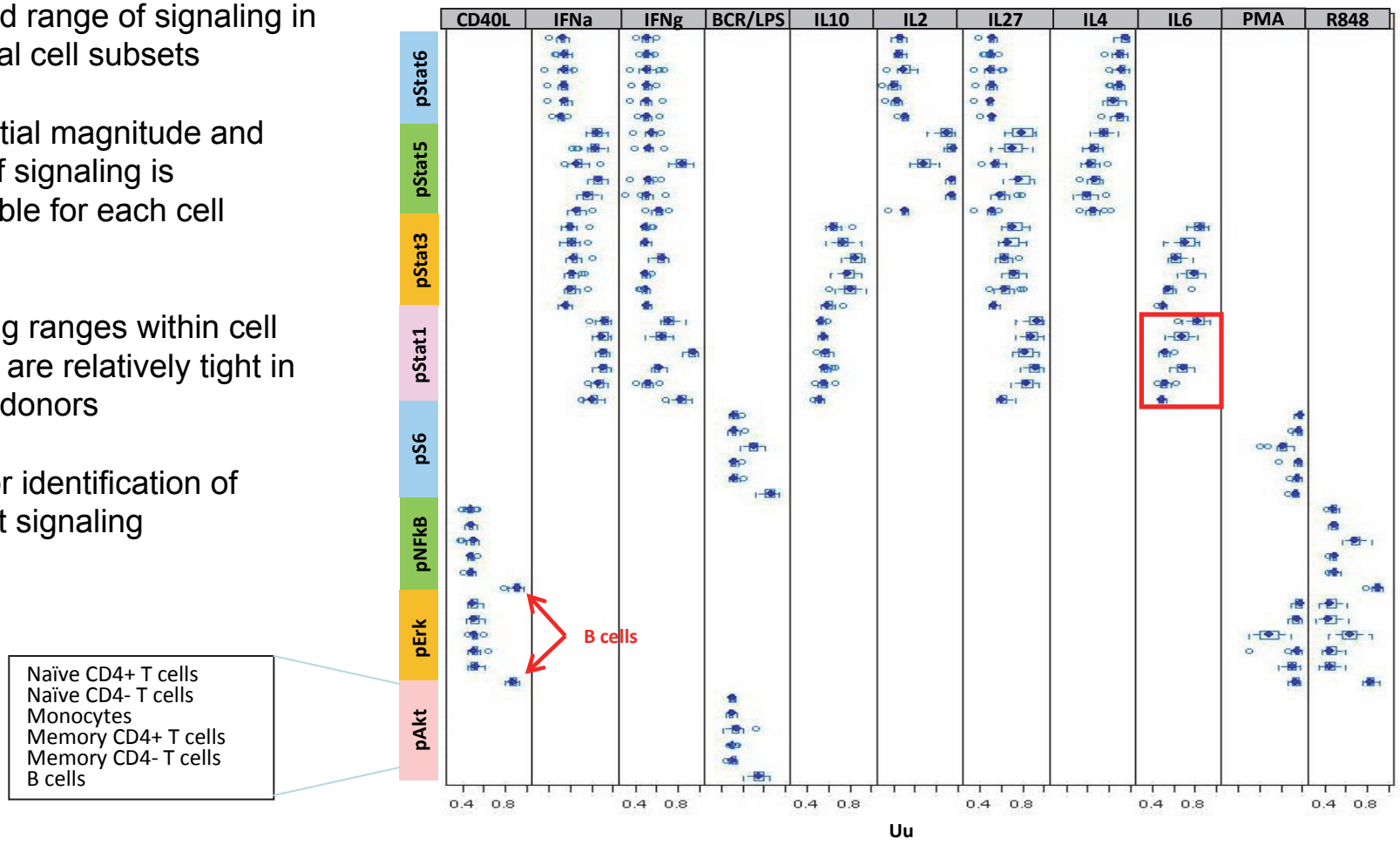


- First 9 nodes show modulation when PBMC evaluated as cell type
- Last 13 nodes show the significance of subtype analysis

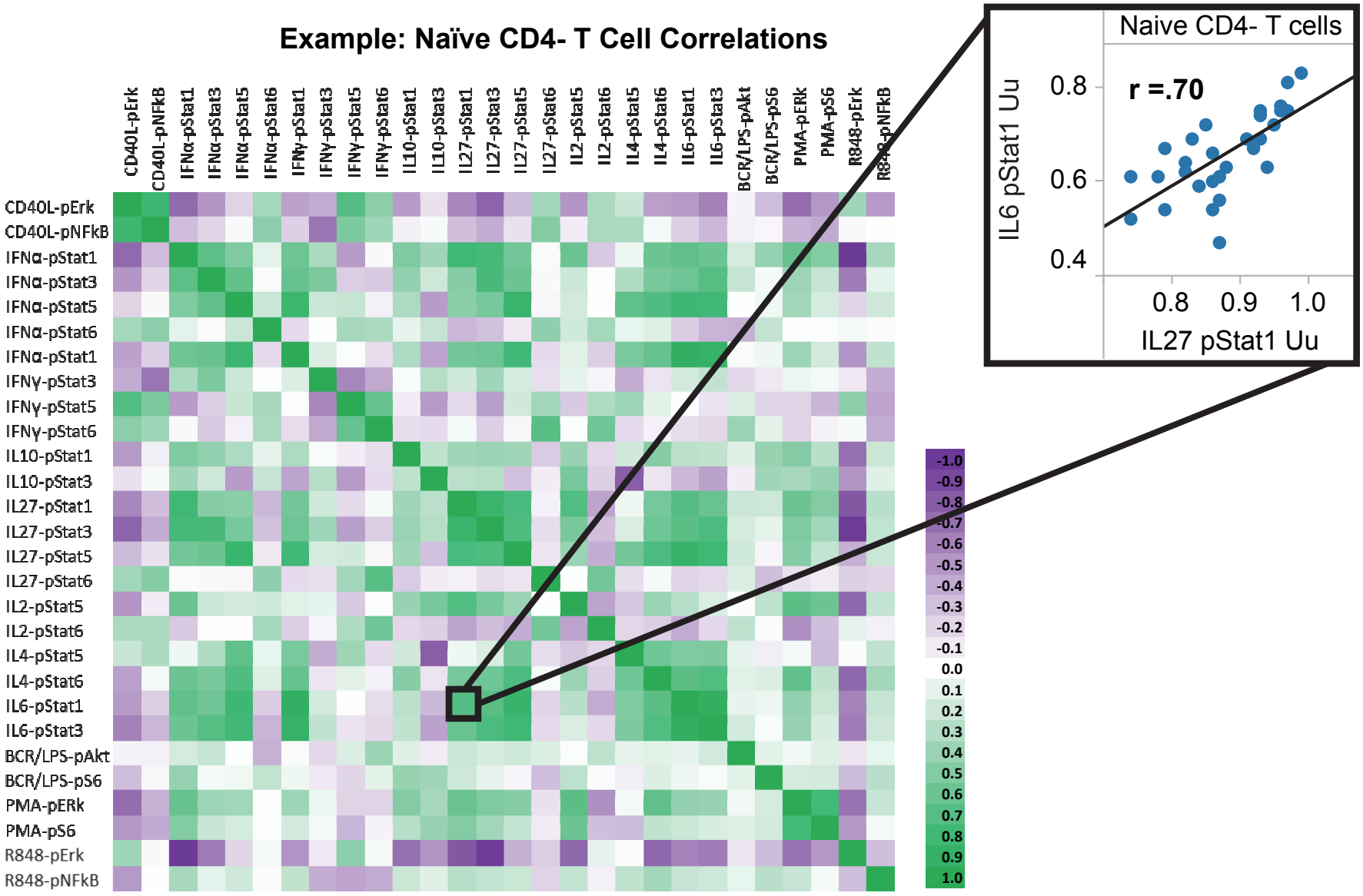
Most responses would not be detected in the total cell population

Cell Subtype-Specific Signaling Dynamics For Multiple Siganling Nodes Across 60 Healthy Donors

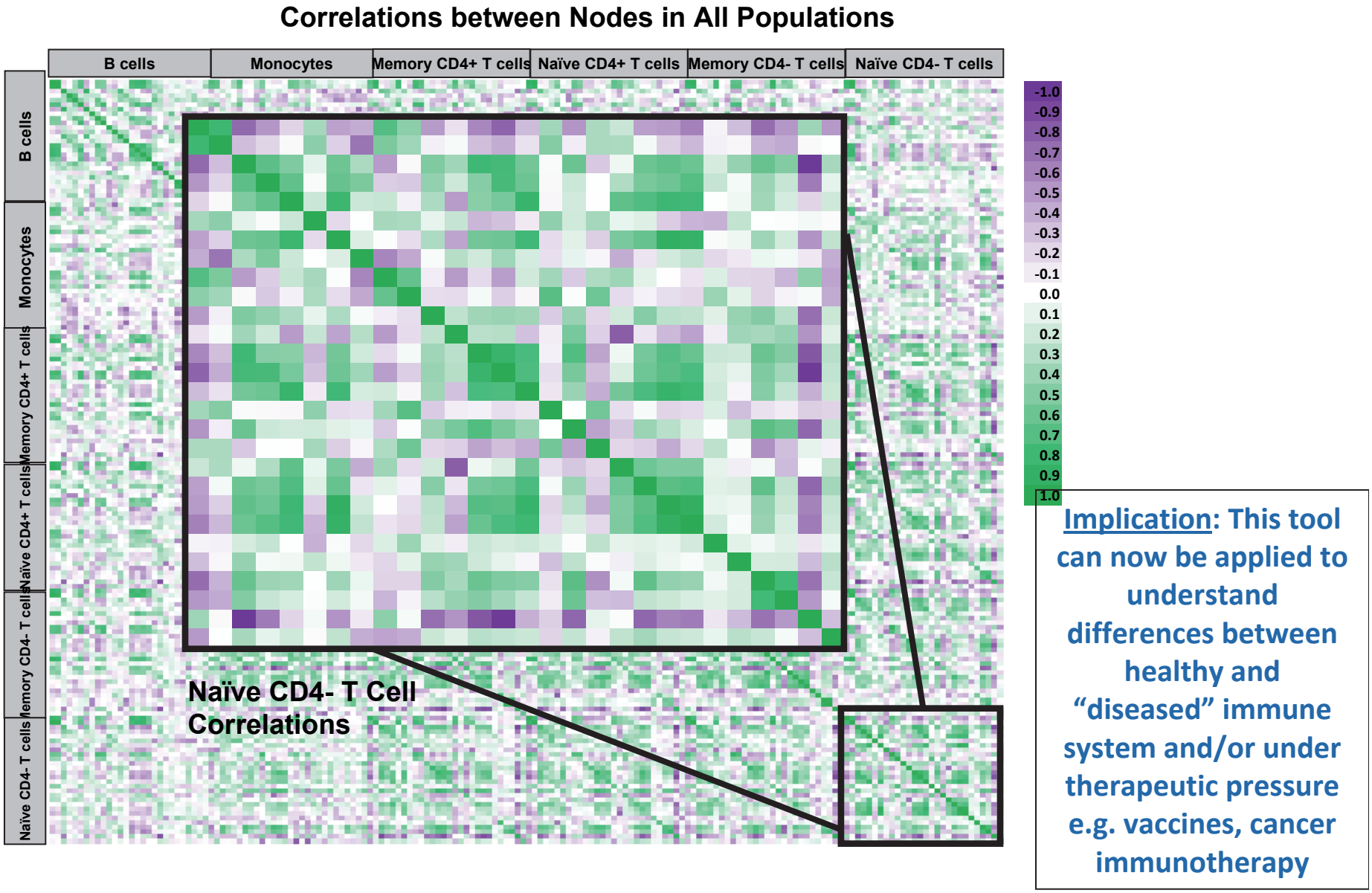
- Identified range of signaling in individual cell subsets
- Differential magnitude and range of signaling is identifiable for each cell subset
- Signaling ranges within cell subsets are relatively tight in healthy donors
- Basis for identification of aberrant signaling



Signaling Correlations Across All Examined Nodes In different cell subsets can be studied

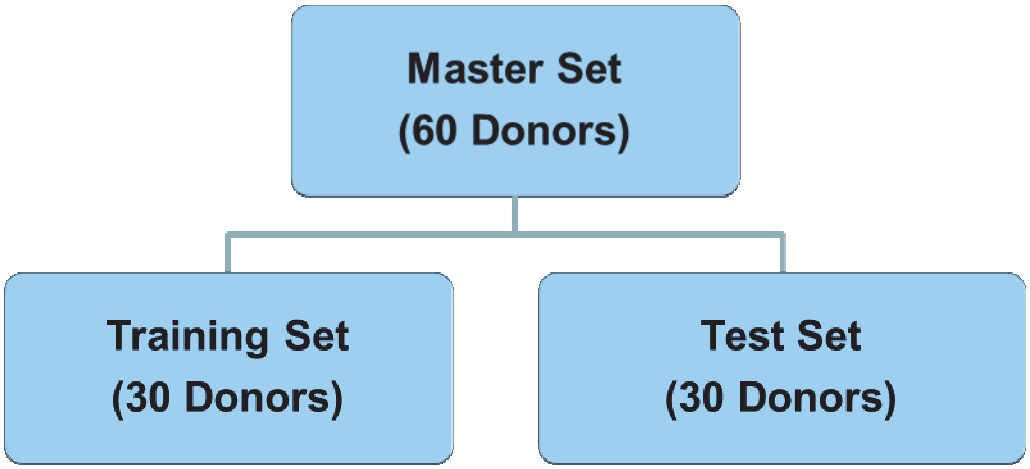


Signaling Correlations Between All Examined Nodes Within Multiple Immune Cell Subsets: An Immune System Signaling Network Map



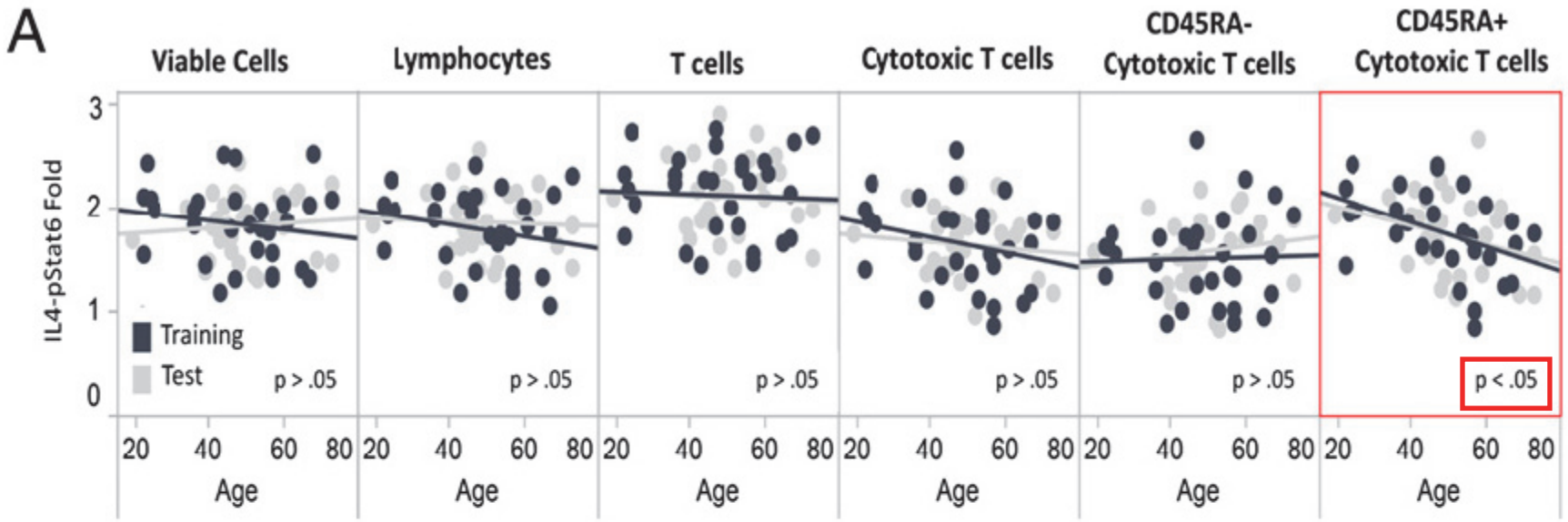
Implication: This tool can now be applied to understand differences between healthy and "diseased" immune system and/or under therapeutic pressure e.g. vaccines, cancer immunotherapy

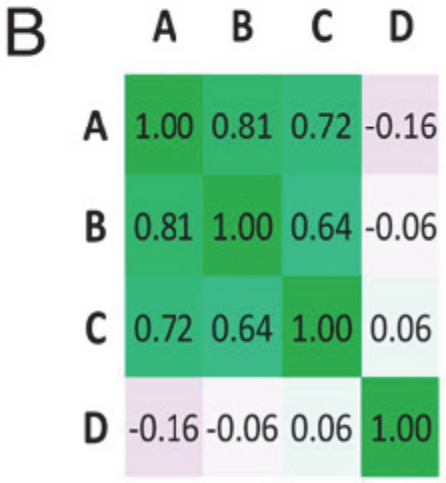
Master Donor Set Subdivided Into Training & Test Sets



	Master	Training	Test
Number of Donors	60	30	30
Mean Age	48.9	47.9	49.8
Gender	12 Female 48 Male	5 Female 25 Male	7 Female 23 Male
Race	25 African American 34 European American 1 Hispanic	10 African American 19 European American 1 Hispanic	15 African American 15 European American 0 Hispanic

Age-Associated Immune-Signaling Responses Identified In Immune Cell Subsets





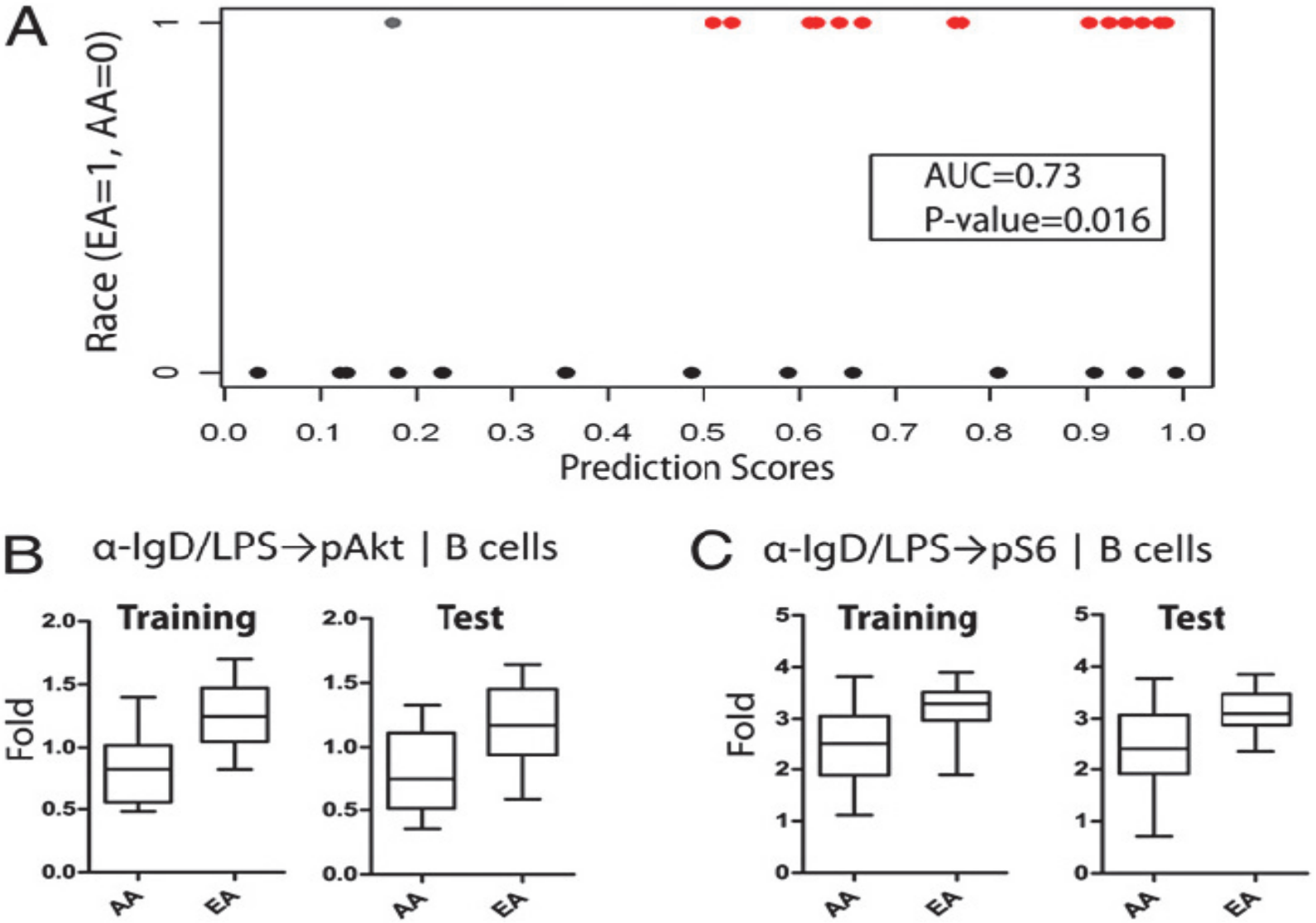
A) IFNα→pStat5 | CD45RA+ cytotoxic T cells

B) IL27→pStat5 | CD45RA+ cytotoxic T cells

C) IL4→pStat6 | CD45RA+ cytotoxic T cells

D) IL2→pStat5 | CD45RA+ helper T cells

Race-Associated Immune-Signaling Responses Identified



Summary Of Healthy Immune Landscaping

- SCNP allows for a systems biology view of signaling of immune system in healthy and disease conditions and under therapeutic pressure (immunomonitoring)
- SCNP Immune system "Landscaping" is being applied to:
  - Define the healthy parameters around key signaling nodes in specific immune cell subsets that are re-routed in autoimmune diseases or cancer (i.e. disease profiling)
  - Define the impact of therapeutics on immune signaling networks (e.g. in cancer immunotherapy and autoimmune disease drugs) – **See Poster N. 92 Titled**: ***CTLA-4 defines distinct T cell signaling populations in healthy donors and metastatic melanoma patients.*** **Presenter: Drew Hotson**
  - Create the basis for patient stratification tools and PD assays

Team NODALITY