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

Alessandra Cesano MD, PhD
Nodality Inc.
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
Presentation Map

• Single Cell Network Profiling (SCNP) technology:
  – Principles
  – Industrialization

• Application to immune system profiling
SCNP foundation technology

- Proprietary phosphoflow signaling technology developed in Dr. Garry Nolan’s lab at Stanford University
Functional Pathway Analysis: Closer To Relevant Biology

Increasing insight into relevant biology & drug efficacy:

DNA → RNA → Proteins → Protein Function

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 vs. 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

1. Level of resolution: Single Cell Analysis

2. Type of assessment: Cell Function (baseline and modulated)

3. Type of measurement: Quantitative & Multiplexed

4. Assay characteristics: Highly repeatable & reproducible, suitable for regulatory submissions
Presentation Map

• Single Cell Network Profiling (SCNP) technology:
  – Principles
  – Industrialization

• Application to immune system profiling
A Complex Assay With Many Components & Dimensions To Track
SCNP assay “industrialization” for application to Clinical Medicine and Drug Development

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”

- 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 BMMMC 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

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

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
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

- 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

QC Layout Files using internal software

Cytometer

DIVA

Sample and Reagent inventory
Assay and Instrument controls on every plate

1. Reagents
2. Plate design
3. Assay controls

- 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

\[
\text{ERF} = a + b \times \text{MFI}
\]

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

\[
\begin{align*}
\text{Calibrated Readout} & \quad \text{Equivalent Reference Fluorescence (ERF)} \\
\text{Calculated Metric} & \quad \text{Quantified biological effect relative to basal state}
\end{align*}
\]

\[
\begin{align*}
\text{Magnitude of Cellular Response} & \quad \text{Fold Metric Class} \\
& \quad \text{Calculated as } \log_2 \text{Fold} \\
\text{Fraction of Cellular Response} & \quad \text{U Metric Class}
\end{align*}
\]

- \( \log_2 \text{Fold} \approx 0 \)  
  \( U_u = 0.5 \)

- \( \log_2 \text{Fold} > 0 \)  
  \( 0.5 < U_u < 1.0 \)

- \( \log_2 \text{Fold} > 1 \)  
  \( U_u = 1.0 \)

- \( \log_2 \text{Fold} >> 1 \)  
  \( U_u = 1.0 \)
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)

- Sample Inventory
- Plate Design
- Instrument setup
- LabPartner
  .Net & SQLServer
- Reagent Inventory

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

- Metrics
- Data Tracking
- Data Analysis/Algorithms
- Data Analysis Toolkit: Python, SQL, MySQL, R
- Visualisation
- Nodality Gating Tools

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

• Single Cell Network Profiling (SCNP) technology:
  – Principles
  – Industrialization

• 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**

**Phenotype, Receptors, Transporters**
- IgD
- IgG
- IgM
- CD5
- CD19
- CD20
- MHCII
- CD38
- CD27
- CD69
- ZAP-70
- CD133
- MDR1
- ABCG2

**DNA Damage & Apoptosis**
- Bendamustine
- Bortezomib, Fludarabine, Flavopiridol, PARPi, TMZ, Clofarabine, Mylotarg, Staurosporine, DNMTi’s, TKi’s

**BCR (IgM, IgD) / TCR Signaling**
- p-Chk2
- Bcl-2
- Cytochrome C
- cCaspase 3, 8
- cPARP
- Mcl1
- p-H2AX
- p-S3BP1
- p-ATM
- p-DNA-PKcs
- p-p53
- annexinV
- p-BRCA1
- DNMT1, 3a, 3b
- p-RPA2

**Differentiation, Maturation, Cytokine/Chemokine Responses**
- IFNα, IFNγ, IL-2, IL-4, IL-6, IL-10, IL-21, IL-27, G-CSF, GM-CSF, SDF1α,

**PKC, Ca++ Signaling**
- PMA, Ionomycin, Thapsigargin

**Survival, Proliferation / Cell cycle, Pattern Recognition Receptors**
- CD40L, R848, CpG, TNFα, LPS,

**Intracellular cytokines measured:**
- IL-2, IL-4, IL-6, IL-8, IL17A, IL-10, IFNγ, TNFα

**Current Nodes and Phenotypic Markers**
- CD3
- CD4
- CD8
- CD14
- CD16
- CD56
- CD25
- CD11b
- CD71
- CD235
- CD80
- CD86
- CD28
- CD123
- CD45RA/RO
- FOXP3
- HL-DR1
- CD123

**PLCγ2**
- p-PLCγ2

**PKCa**
- p-PKCa

**p38**
- p-p38

**Stat5**
- p-Stat5

**Bcl-2**
- p-Bcl-2

**ERK**
- p-ERK

**S6**
- p-S6

**Stat1**
- p-Stat1

**Stat3**
- p-Stat3

**Stat5**
- p-Stat5

**Stat6**
- p-Stat6

**SHP1, 2**
- p-SHP1, 2

**ATM**
- p-ATM

**DNA**
- p-DNA

**DD**
- p-DD

**Apoptosis**
- p-Annexin V

**DNA Damage & Apoptosis**
- p-Bax
- p-Bak

**p53**
- p-p53

**IKK**
- p-IKK

**NFκB**
- p-NFκB

**IkB**
- p-IκB

**MAPK**
- p-MAPK

**PKcs**
- p-PKcs

**S6K**
- p-S6K

**Stat1**
- p-Stat1

**Stat3**
- p-Stat3

**Stat5**
- p-Stat5

**Stat6**
- p-Stat6
Healthy Immune Landscape Network Map: Establishing Baseline Signaling For Disease/Therapeutic Characterization

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:

Racial differences in B cell receptor signaling pathway activation

Diane M Longo¹, Brent Louie¹, Kavita Mathi², Zoltan Pos³, Ena Wang⁴, Rachael E Hawtin¹, Francesco M Marincola⁴ and Alessandra Cesano¹
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

60 donors analyzed
11 Modulators tested
- IFNα
- IFNY
- IL2
- IL4
- IL6
- IL10
- IL27
- BCR & LPS
- R848
- PMA
- CD40L

6 Cell Subsets identified
- Monos
- B cells
- CD45RA-CD4- T cells
- CD45RA+CD4- T cells
- CD45RA-CD4+ T cells
- CD45RA+CD4+ T cells

8 Phospho-Protein Readouts measured
- pStat1
- pStat3
- pStat5
- pStat6
- pS6
- pAkt
- pErk
- pNFkB
Multiple Cell Subsets Are Simultaneously Examined In The Same Sample

- Viable Cells
- Lymphocytes
- Monocytes
- Scatter

- CD3- CD20- Lymphs
- CD3+ CD20+ T cells
- CD45RA+ CD4- T cells
- CD45RA- CD4- T cells
- CD45RA+ CD4+ T cells
- CD45RA- CD4+ T cells
- B cells
- T cells
Cellular Subsets Reveal Signaling Responses Not Seen In The Entire Cell Population

<table>
<thead>
<tr>
<th>Cells</th>
<th>Monocytes</th>
<th>Lymphs</th>
<th>B cells</th>
<th>CD3-CD20-Lymphs</th>
<th>CD4- T cells</th>
<th>CD4+ T cells</th>
<th>Memory CD4- T cells</th>
<th>Memory CD4+ T cells</th>
<th>Naive CD4- T cells</th>
<th>Naive CD4+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Uu &gt; .6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Uu &lt; .6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Most responses would not be detected in the total cell population

- First 9 nodes show modulation when PBMC evaluated as cell type
- Last 13 nodes show the significance of subtype analysis
Cell Subtype-Specific Signaling Dynamics For Multiple Signaling 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

![Graph showing signaling dynamics across different cell subsets and signaling nodes with markers for B cells, Naïve CD4+ T cells, Naïve CD4- T cells, Monocytes, Memory CD4+ T cells, and Memory CD4- T cells]
Signaling Correlations Across All Examined Nodes In different cell subsets can be studied
Correlations between Nodes in All Populations

<table>
<thead>
<tr>
<th>B cells</th>
<th>Monocytes</th>
<th>Memory CD4+ T cells</th>
<th>Naïve CD4+ T cells</th>
<th>Memory CD4- T cells</th>
<th>Naïve CD4- T cells</th>
</tr>
</thead>
</table>

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

The master donor set consists of 60 donors, which is subdivided into a training set of 30 donors and a test set of 30 donors. The table below provides a comparison of the number of donors, mean age, gender, and race in each set.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Master</th>
<th>Training</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Donors</td>
<td>60</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean Age</td>
<td>48.9</td>
<td>47.9</td>
<td>49.8</td>
</tr>
<tr>
<td>Gender</td>
<td>12 Female, 48 Male</td>
<td>5 Female, 25 Male</td>
<td>7 Female, 23 Male</td>
</tr>
<tr>
<td>Race</td>
<td>25 African American, 34 European American, 1 Hispanic</td>
<td>10 African American, 19 European American, 1 Hispanic</td>
<td>15 African American, 15 European American, 0 Hispanic</td>
</tr>
</tbody>
</table>
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

A

Race (EA=1, AA=0)

Prediction Scores

AUC=0.73
P-value=0.016

B

α-IgD/LPS → pAkt | B cells

C

α-IgD/LPS → pS6 | B cells
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