Mass Cytometry Experiment Design: Signaling Focus

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Please ask questions!
Introduction & background

Experimental workflow

Optimizing & troubleshooting (experiments)
Vanderbilt University in Nashville
Overall Goal: Use Signaling Knowledge to Improve Therapies

1. Characterize signaling in major cell populations
2. Identify cancer cell subsets based on signaling
3. Model signaling networks to identify therapy opportunities

Molecular profiles guide therapy

Clinical outcome

Clinical signaling profile

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Quantitative Single Cell Signaling Network Profiling

Which cell?  How much?

- Can we spot rare, therapy resistant cells?
- Pre-transformation cells?

Translational opportunities

- What targetable signaling mechanisms enable cells to resist a particular therapy?
- By looking at the network (systems biology): Are there off-target effects of a drug?
- Can we detect circulating cells from the tumor or immune cells that encountered tumor?
- How do cancer cells interact with and alter the host microenvironment and immune system?
- Do patients that share responses share profiles? Can signaling guide therapy?
- Will more markers (high dimensional), measuring different features (signaling), or single cell resolution improve diagnosis?

Adapted from Krutzik et al., Clinical Immunol 2003
Mass Cytometry Phospho-Flow Overview

Adapted from Irish, Kotecha, and Nolan, *Nat Rev Cancer* 2006
Developing a Clinical Signaling Profile Begins with Optimizing Stimuli and Readouts

1. Identify signaling features relevant for the disease
2. Identify features that stratify clinical outcome
3. Test clinical model in blinded samples

(This is after optimizing & validating the instrument, core antibodies, tissue acquisition & storage protocols, etc.)
BCR Signaling across B cell Development

1. **μ** rearrangement, pre-BCR expression
2. **κ/λ** rearrangement, BCR expression
3. Lack of self-reactive BCR signaling
4. Continuous requirement for expression of a functional BCR in mature cells (tonic survival signaling)
5. Mutation & antigen affinity fine tune BCR signal
6. BCR class switch, ongoing mutation optimize BCR signaling

**Checkpoints in healthy B cells**

- Mature B (before antigen)
- Mature B (after antigen)
- Activated B
- Memory B
- Plasma B

**Cell Lineages**
- Hematopoietic stem cell
- Multipotent progenitor
  - Common myeloid progenitor
  - Common lymphoid progenitor
- ME progenitor
- GM progenitor
- Pro-B
- Pre-B
- Immature B
- Mature B (before antigen)
- Mature B (after antigen)
- Activated B
- Plasma B
- Red cells
- Platelets
- Macrophages
- Granulocytes
Mass Cytometry Detects Key Signaling Readouts

= Signaling proteins validated for mass cytometry

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Network View: Map Inputs x Readouts in Each Cell

= Signaling Inputs

- Unstim
- PMA + iono
- IL-2
- IL-4
- IL-6
- IL-7
- IL-10
- IL-13
- IL-15
- α-BCR 4'
- α-BCR 15'
- α-BCR 45'
- H₂O₂ 4'
- H₂O₂ 15'
- H₂O₂ 45'
- α-BCR + H₂O₂ 4'
- α-BCR + H₂O₂ 15'
- α-BCR + H₂O₂ 45'
- SDF-1α
- CD40-L
- IFN-γ
- IFN Type I
- CpG

= Phospho-protein readouts

- p-SFK (LCK, LYN; Y505)
- p-STAT5 (Y694)
- p-PLCγ2 (Y759)
- p-STAT3 (Y705)
- p-STAT6 (Y641)
- p-STAT1 (Y701)
- p-ERK (ERK1/2; T202/Y204)
- p-S6 (S235/236)
- p-STAT6 (Y641)
- p-SYK (SYK/ZAP70; Y352/Y319)
- p-BTK (BTK /ITK; Y551)
- p-p53 (S15)
- p-BLNK (Y84)
- p-p38 (T180/Y182)

Focus on BCR and TIL T cell signaling

Immunophenotyping

<table>
<thead>
<tr>
<th>Identity markers in signaling panels</th>
<th>CD3</th>
<th>CD5</th>
<th>BCL2</th>
<th>CD20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD19</td>
<td>CD10</td>
<td>CD4</td>
<td>CD8</td>
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<tr>
<td></td>
<td>CD79b</td>
<td>CD38</td>
<td>CD2</td>
<td>CD14</td>
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<tr>
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<td>IgM</td>
<td>CD81</td>
<td>CD25</td>
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<td>CD40</td>
<td>CD5</td>
<td>CD3</td>
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<td>IgK</td>
<td>CD137</td>
<td>HLA-DR</td>
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<tr>
<td></td>
<td>IgL</td>
<td>CD22</td>
<td>CD2</td>
<td>CD14</td>
</tr>
<tr>
<td></td>
<td>CD20</td>
<td>CD5</td>
<td>CD14</td>
<td></td>
</tr>
</tbody>
</table>
Mass Cytometry: Next-Gen 34+ Dimensional Single Cell Analysis

Antibody labeled with elemental isotope

Chelated elemental isotope (e.g., Gd-156)

Label single cells with 34+ mass tagged antibodies

Stream thousands of cells per second

Nebulizer

Plasma

Quadrupole

Time-of-flight

Future of Cytometry

Adapted from Bendall et al., Science 2011, Cytobank, & DVS Sciences
Elemental Isotopes (e.g. Lanthanides) are Used As Mass Tags

Natural Abundances listed on Wikipedia

http://en.wikipedia.org/wiki/Europium
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‘Simple Mass Cytometry Experiment’ Design

26 Markers:

Nucleic Acid (Ir), CD19, CD117, CD11b, CD4, CD8a, CD20, CD34, CD61, CD123, CD45RA, CD45, CD10, CD33, CD11c, CD14, CD69, CD15, CD16, CD44, CD38, CD25, CD3, CD66, IgM, HLA-DR, CD56

All available commercially from DVS Sciences:
http://www.dvssciences.com/conjugated-antibodies.html

Protocol:

1) Human PBMC (2 x 10^6, Ficoll, cryo.)
2) Labeled w/ antibodies, 1 µL each in 100 µL total PBS + 1% BSA, 20’ @ 23 °C
3) Permeabilized in -20 ºC MeOH, 10’
4) Labeled w/ natural Iridium based nucleic acid intercalator (191 & 193), 23 °C 15’
5) Counted & resuspended in ddH₂O at 0.75 x 10^6 cells per mL for analysis
30 Dimensional View of Healthy Human Blood Subsets & Signaling

25 Identity Markers
- CD45, CD3, CD5, CD4, CD8a, HLA-DR, CD19, CD20, CD33, CD16, CD57, CD56, NA-191, NA-193, Event Length
- CD25, CD107a, CD28, CD45R0, CD44, CCR4, CCR5, CCR6, CXCR3, CXCR5, CCR7

5 Signaling Readouts
- p-STAT5, p-STAT1, p-SFK, p-ERK, p-STAT6

Protocol: (~2 hours)
1) Stimulate Human PBMC 15’ @ 37 °C
2) Fix in 1.6% formaldehyde 5’ @ 23 °C
3) Label w/ surface antibodies 20’ @ 23 °C
4) Permeabilize (Saponin | MeOH) 10’ @ -20 °C
5) Label w/ Ir nucleic acid intercalator (191 & 193) 15’ @ 23 °C
6) Count & resuspend in ddH₂O

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Mapping Signaling in Every Cell using Mass Cytometry
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Summary: Measuring and Dealing with Variation

Rule #1: Treat all samples as equally as possible

Sources of Variation

• Biological Differences
• Individual Variation
• Sample Composition
• Sample Preparation
  – cell isolation / suspension
  – freeze / thaw
  – ‘rest’ before stimulation
• Signaling Assay Execution
• Unnoted Protocol Differences

Phospho-Flow Profiling

• Streamline the assay, titrate everything
• Use cell subsets as internal controls
• Measure individual variation across samples of healthy primary cells
• Measure variation on the same sample assayed over multiple days
• Consider barcoding & beads to minimize staining & instrument variation
• Ideally: Profile an aliquot of a known control sample every day a new sample is profiled

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Optimizing the Experimental Workflow

Individual variation

This might be interesting biology or a confounder. Either way you want to track it.

1) Same individual, repeated regularly (assay variation)
2) New individuals (to capture healthy variation)
Sample Preparation & Storage

Removal of RBCs & platelets (ACK lysis) or Ficoll Prep can significantly clean up samples.

Cryopreservation helps lower variation. Can be a flag for grant reviews, so compare fresh vs. frozen.
Optimizing the Experimental Workflow

Assay Variation

Streamline the assay. Consider human nature.

Do experiments the same way as someone else first.
Then only change one thing at a time.
Optimizing the Experimental Workflow

‘Side’ Immunophenotype Panel

Even when signaling is your focus, run a ‘surface only’ immunophenotype. Advantages include:
1) better staining (pre-fix)
2) more markers to clarify subset identity
Optimizing the Experimental Workflow

Picking Stimulation Conditions

Consider ‘combo stims’ to minimize sample number.

Find a strong activator upstream of each readout.
Phospho-flow > IF > IHC > Western
Find Examples in Annotated Online Datasets

Nolan lab mass cytometry dataset
www.cytobank.org/nolanlab

Bendall et al., Science 2011 data hosted by Cytobank
Find Examples in Annotated Online Datasets

DVS Cytobank:  dvs.cytobank.org
Finding Subsets of Interest

Stain for surface markers after fix.

**Pro:** Can’t artificially stimulate fixed cells.

**Con:** Mildly reduced staining conditions (90%).

Note: with mass cytometry, loss of signal from perm is a non-issue.
Intracellular vs. Surface Staining Challenges

1a) Staining the cell surface can trigger signaling

1b) Fixing cells to stop signaling hurts staining (minor)

2a) Permeabilizing cells denatures many surface markers
    (so lineage staining after perm can fail; antibody clone dependent)

2b) Protein fluorophores can be hurt by permeabilization
    (so staining before perm can fail; protein fluorophore dependent)

These differences can affect the quality / shape of the data
Optimizing the Experimental Workflow

Titrating Multiple Markers

Can sometimes titrate multiple markers simultaneously.

In this case it is critical to use constant identity markers (for gating) and to make sure subset proportions are maintained across the titration.
Heterogeneous Populations Are Ideal for Titrations

Titration of anti-CD38-167 on a mix of two cell lines

<table>
<thead>
<tr>
<th>Titration 1</th>
<th>Titration 2</th>
<th>Titration 3</th>
<th>Titration 4</th>
<th>Titration 5</th>
<th>Titration 6</th>
<th>Titration 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38-167 - Panel 1</td>
<td>4.29</td>
<td>3.95</td>
<td>3.23</td>
<td>2.7</td>
<td>1.84</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Statistic: 5th to the 95th percentile distance (log like arcsinh scale)
Testing Metal Labeled Antibodies

Antibody capture beads: great for testing spillover; gives a discrete signal for FCS export

Can also run antibody in solution.
Optimizing the Experimental Workflow

Marking Dead Cells

Exclusion test, any time before permeabilization.
(Cisplatin for live/dead by Fienberg HG et al., Cytometry 2012)

Some fix conditions also perm,
1.6% PFA for 5-10 min typically does not.

Can be convenient to do exclusion test before fix.
Optimizing the Experimental Workflow

Permeabilization Options

Detergents (Saponin, Triton), not ideal for STATs
Alcohol (EtOH, MeOH), tend to be harsh

Potentially: new ‘one step’ perm reagents that work for many/all target epitopes (e.g. Foxp3 and p-STAT5)
Fixation in PFA and Permeabilization in Methanol is Ideal for Many Phospho-Epitopes, Especially p-STATs

Krutzik et al., *Cytometry* 2003
Not All Traditional Flow Antibodies Work Well in All Fix/Perm Buffer Conditions (Especially Methanol)

(Note: It is critical to have multiple populations during titration; looking for best separation between known + and -)

Krutzik et al., *J Immunol* 2005
Optimizing the Experimental Workflow

Titrating Phospho-Specific Antibodies

Create an artificial mix of + and –
(plus separate + and – alone as controls)

Many + control stimuli listed in published papers; phosphatase inhibitors can be useful, but are not specific.
Phosphatase (CIP) Treatment Can Reveal High Basal Signaling

Ramos (Burkitt’s lymphoma B cell line)

Unstim

Unstim + CIP

Stim

Stim + CIP

CD45

p-TYR (4G10)

p-SYK

CIP approach: Khalil AM et al., Science 2012

H. Polikowsky, Irish Lab
Optimizing the Experimental Workflow

Picking Doses and Timepoints

Start with high doses.
Cytokines typically ‘max out’ at 2 ng/mL.
Antibody stimuli ~10 µL/mL

Many pathways ~15 min is great.
Anywhere from 30s to 2h can work well, though.
Optimizing the Experimental Workflow

Nucleic Acid Intercalator

Can fix it in place; otherwise it can wash out over time.

We have found Ir (191/193) reliable; dropping Rh103 allows us to track Pb206 (CyTOF v1.0)
Panel Design

Don’t place very low abundance targets +1 from high abundance targets; look for issues using biology.

Use beads, titrations, ‘MMO’ controls, known biology.

Primary samples: use internal control cell subsets.
+1 Bleed Comparison: CD19 into CD5 (no issue)

CD19-142: 2.9 dynamic range

CD5-143: 3.0 dynamic range

https://irishlab.cytobank.org/cytobank/experiments/645/spade/236
+1 Bleed Comparison: CD19 into CD117 (minor issue)

CD19-142: 4.2 dynamic range

CD117-143: 0.3 dynamic range

https://irishlab.cytobank.org/cytobank/experiments/382/spade/148
Collection

Keep carrier fluid running (software & physical).

30-40 sec delay following sample introduction.

Wash between samples. Run ¼ diluted control first.

Collect open channels.
Run standardization controls before and after sample (at minimum). Tuning solution, beads, standard cells.

Ideally run internal beads for continuous normalization. (Finck R et al., Cytometry 2013)
Putting it all together…
Mapping Signaling in Every Cell using Mass Cytometry

Pre-gate for live CD45+ single cells

- All cells
- Live Cells
- Single Cells

Compare signaling across conditions, doses, time...

- Unstim
- SCF
- FLT3-L
- IL-3
- IFNγ

\[
\text{p-AKT} \quad \text{p-ERK} \quad \text{p-STAT5} \quad \text{p-STAT1}
\]

-2.2 No Change +1.1 +2.2

'Classic' single cell views

- Supervised analysis

Find populations

- Automated / unsupervised analysis

CD45R0

- NK Cells
- Memory CD4+ T cells
- B cells
- Monocytes
- DCS
- CD8 T cells
- Doublets
- CD4 T cells
- Memory CD4+ T
- Native CD8+ T
- CD8+ T
- CD4+ T
- CD11c

Scale

-3 No Change +3

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6) Count & resuspend in ddH₂O

(Note: this was a rotation student’s first experiment)
Mass Cytometry: APCs & T cells in Healthy PBMC

CD3

HLA-DR

https://irishlab.cytobank.org/cytobank/experiments/641/spade/234
Mass Cytometry: Healthy CD4+ and CD8+ T cells

https://irishlab.cytobank.org/cytobank/experiments/641/spade/234
Mass Cytometry: Healthy B Cells (CD19+ CD20+)

https://irishlab.cytobank.org/cytobank/experiments/641/spade/234
Mass Cytometry: phospho-STAT1 IFNα Response

Unstim

IFNα

https://irishlab.cytobank.org/cytobank/experiments/641/spade/234
Mass Cytometry: p-ERK H$_2$O$_2$ Response

Unstim

https://irishlab.cytobank.org/cytobank/experiments/641/spade/234

H$_2$O$_2$

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