Mass Cytometry Experiment Design: Signaling Focus

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Please ask questions!

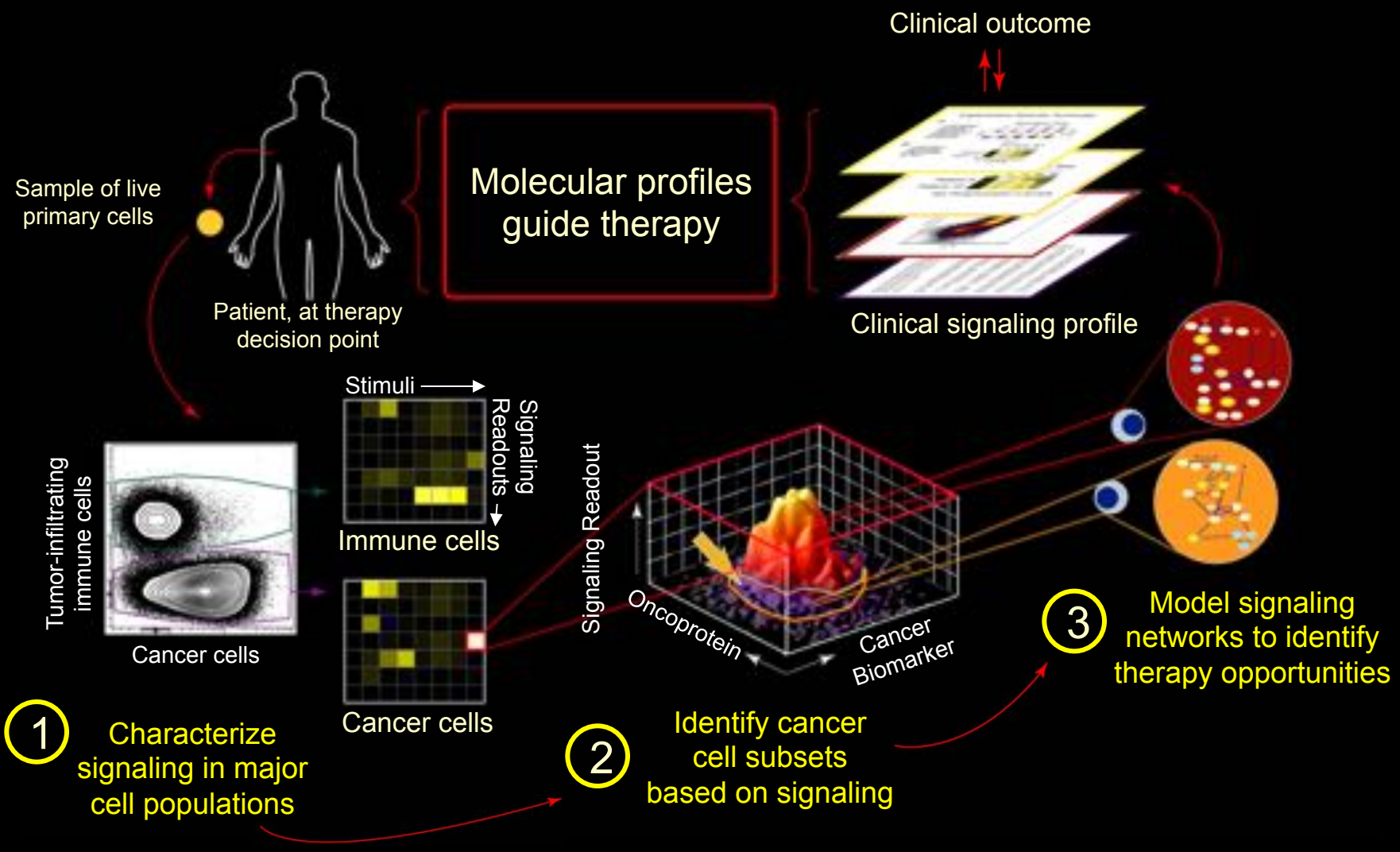
Introduction
& background

Experimental workflow

Optimizing & troubleshooting
(experiments)

Vanderbilt University in Nashville

Overall Goal: Use Signaling Knowledge to Improve Therapies



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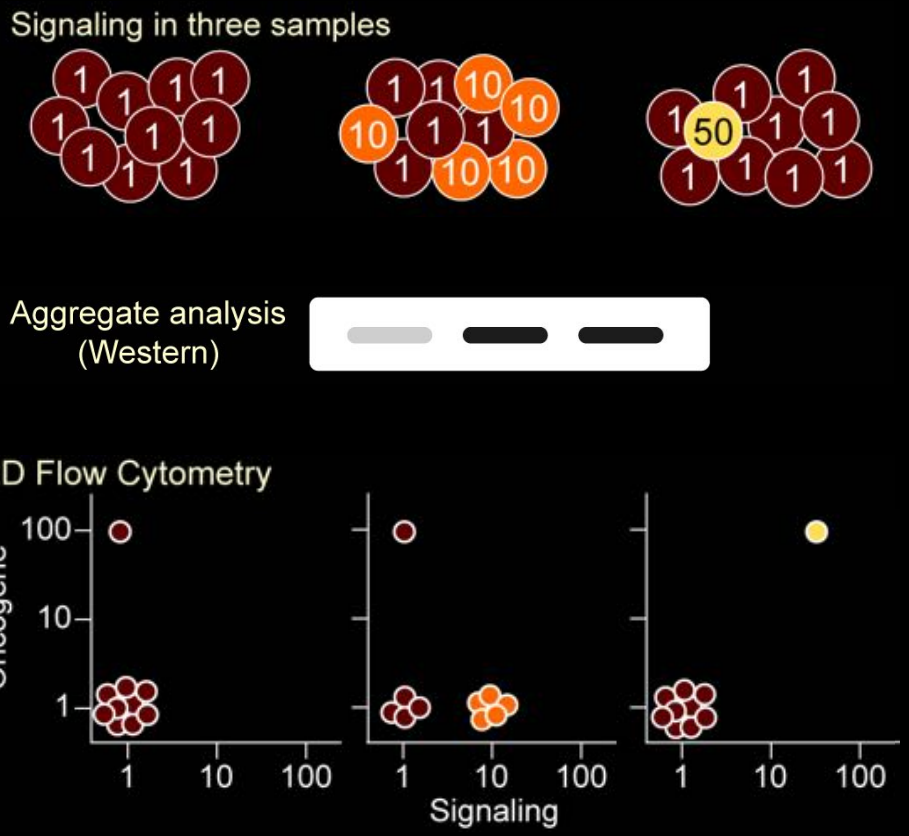
Quantitative Single Cell Signaling Network Profiling

Which cell? How much?

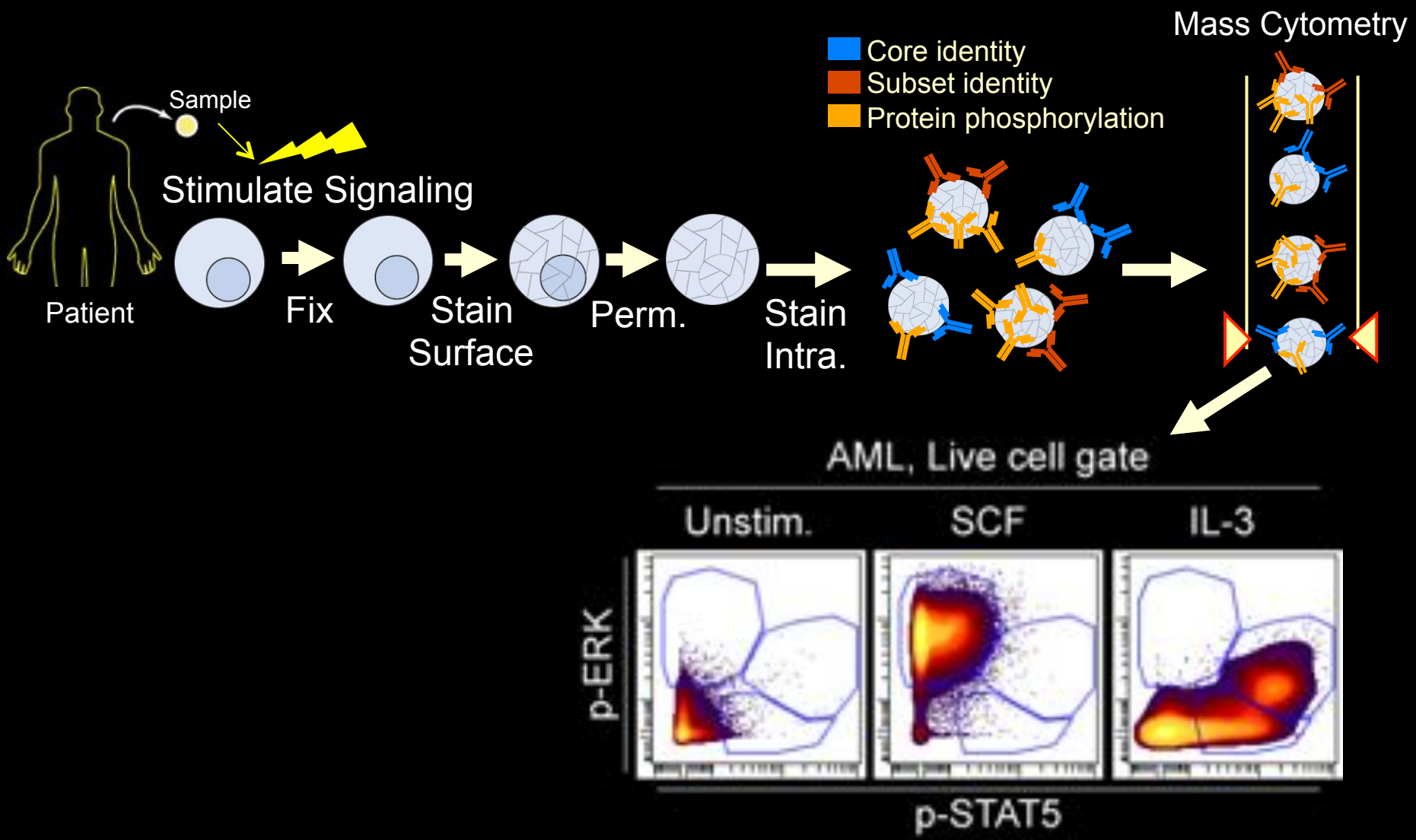


Translational opportunities

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

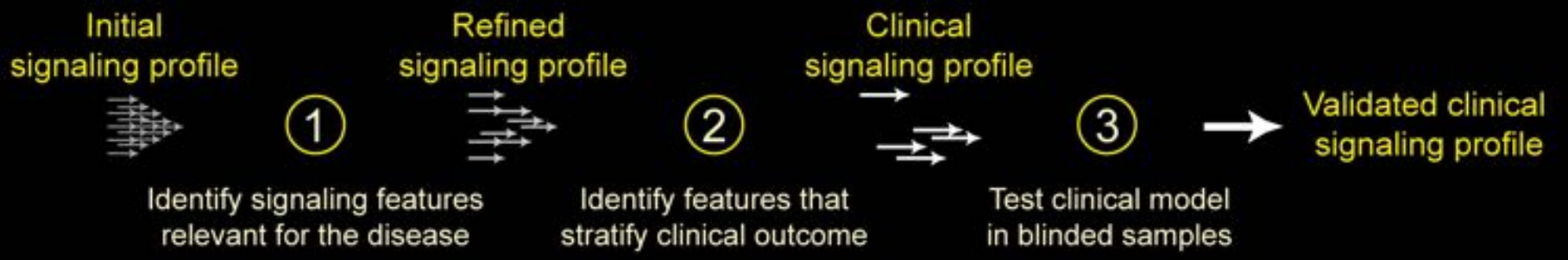
Mass Cytometry Phospho-Flow Overview



Adapted from Irish, Kotecha, and Nolan, *Nat Rev Cancer* 2006

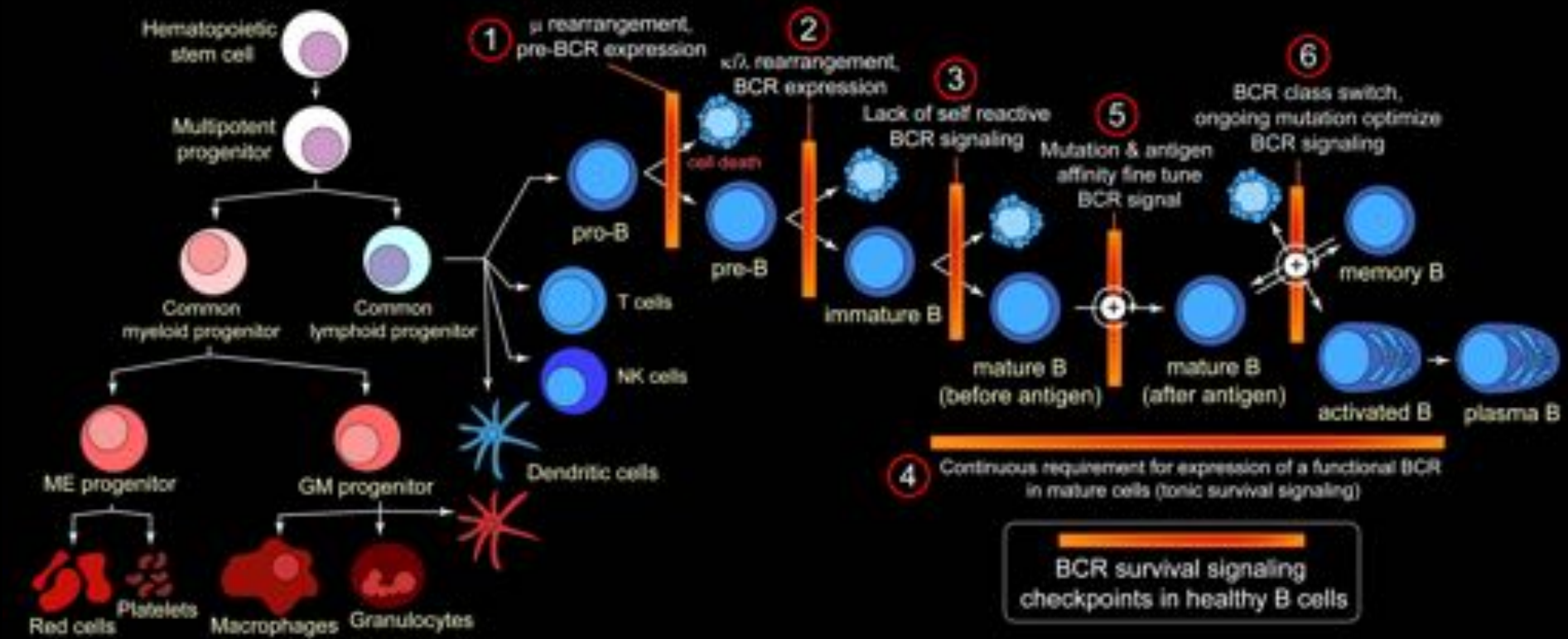
B. Ferrell, Irish Lab

Developing a Clinical Signaling Profile Begins with Optimizing Stimuli and Readouts

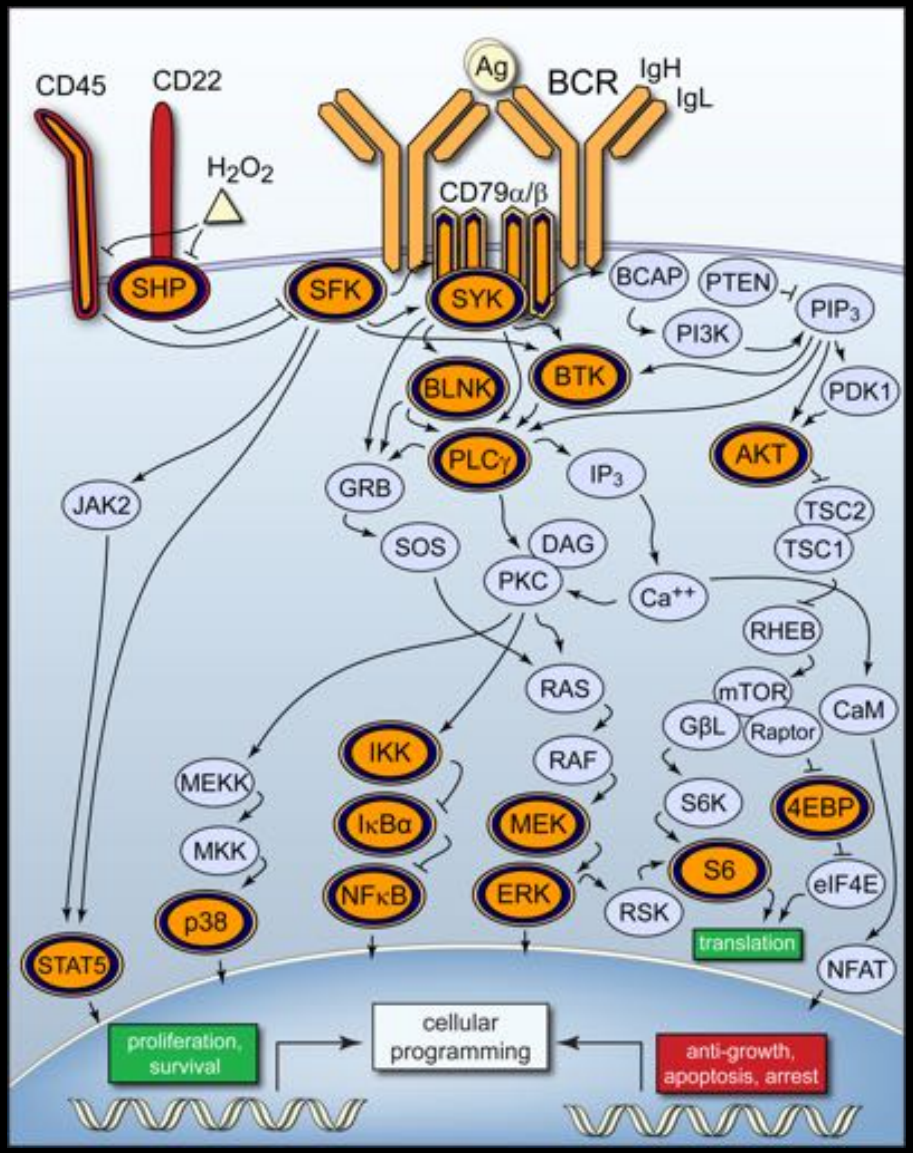


(This is after optimizing & validating the instrument, core antibodies, tissue acquisition & storage protocols, etc.)

BCR Signaling across B cell Development



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

= Phospho-protein readouts

- 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'
- H_2O_2 4'
- H_2O_2 15'
- H_2O_2 45'
- α-BCR + H_2O_2 4'
- α-BCR + H_2O_2 15'
- α-BCR + H_2O_2 45'
- SDF-1α
- CD40-L
- IFN-γ
- IFN Type I
- CpG

- p-SFK (LCK, LYN; Y505)
- p-STAT5 (Y694)
- p-ERK (ERK1/2; T202/Y204)
- p-SYK (SYK/ZAP70; Y352/Y319
- p-BTK (BTK /ITK; Y551)
- p-STAT3 (Y705)
- p-STAT6 (Y641)
- p-STAT1 (Y701)
- p-AKT1 (S473)
- p-NFκB p65 (S529)
- p-p38 (T180/Y182)
- p-CBL (Y700)
- p-PLCγ2 (Y759)
- p-S6 (S235/236)
- p-BLNK (Y84)
- p-p53 (S15)

Immunophenotyping

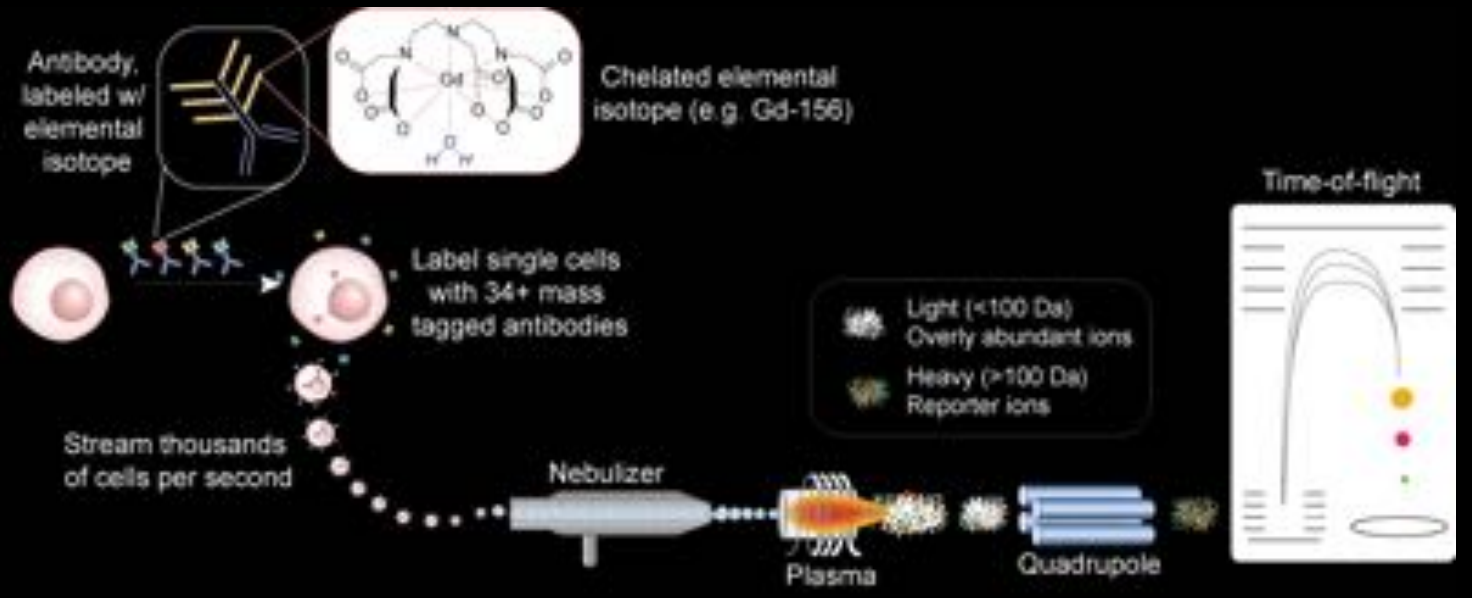
Identity markers in signaling panels	CD3 CD5 BCL2 CD20
'Side' identity markers	CD19 CD10 CD4 CD79b CD38 CD8 IgM CD81 CD25 IgG CD40 CD56 IgK CD137 CD3 IgL CD22 HLA-DR CD20 CD5 CD14

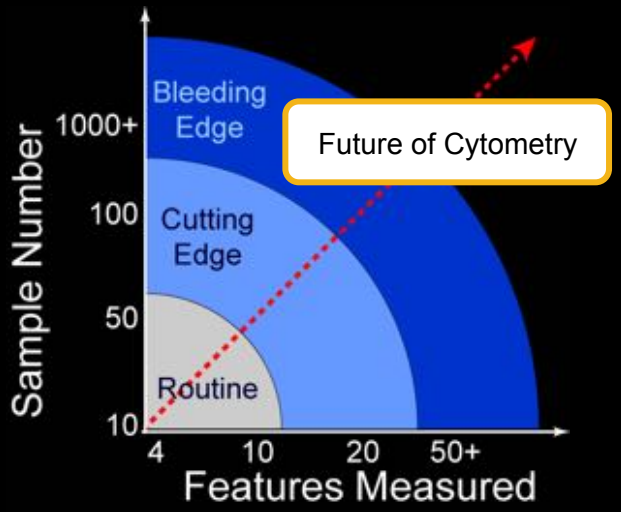
Focus on BCR and TIL T cell signaling

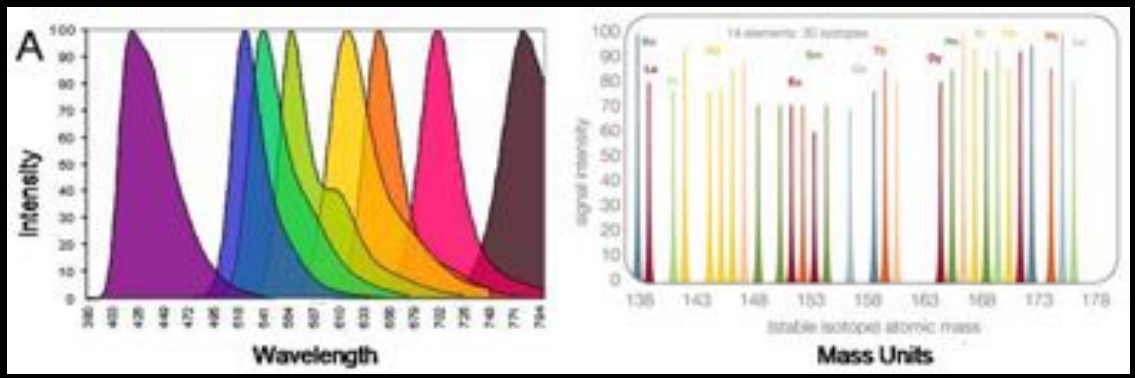
Irish et al., *PNAS* 2010

Mass Cytometry: Next-Gen 34+ Dimensional Single Cell Analysis

Vanderbilt Mass Cytometer







Adapted from Bendall et al., *Science* 2011, Cytobank, & DVS Sciences

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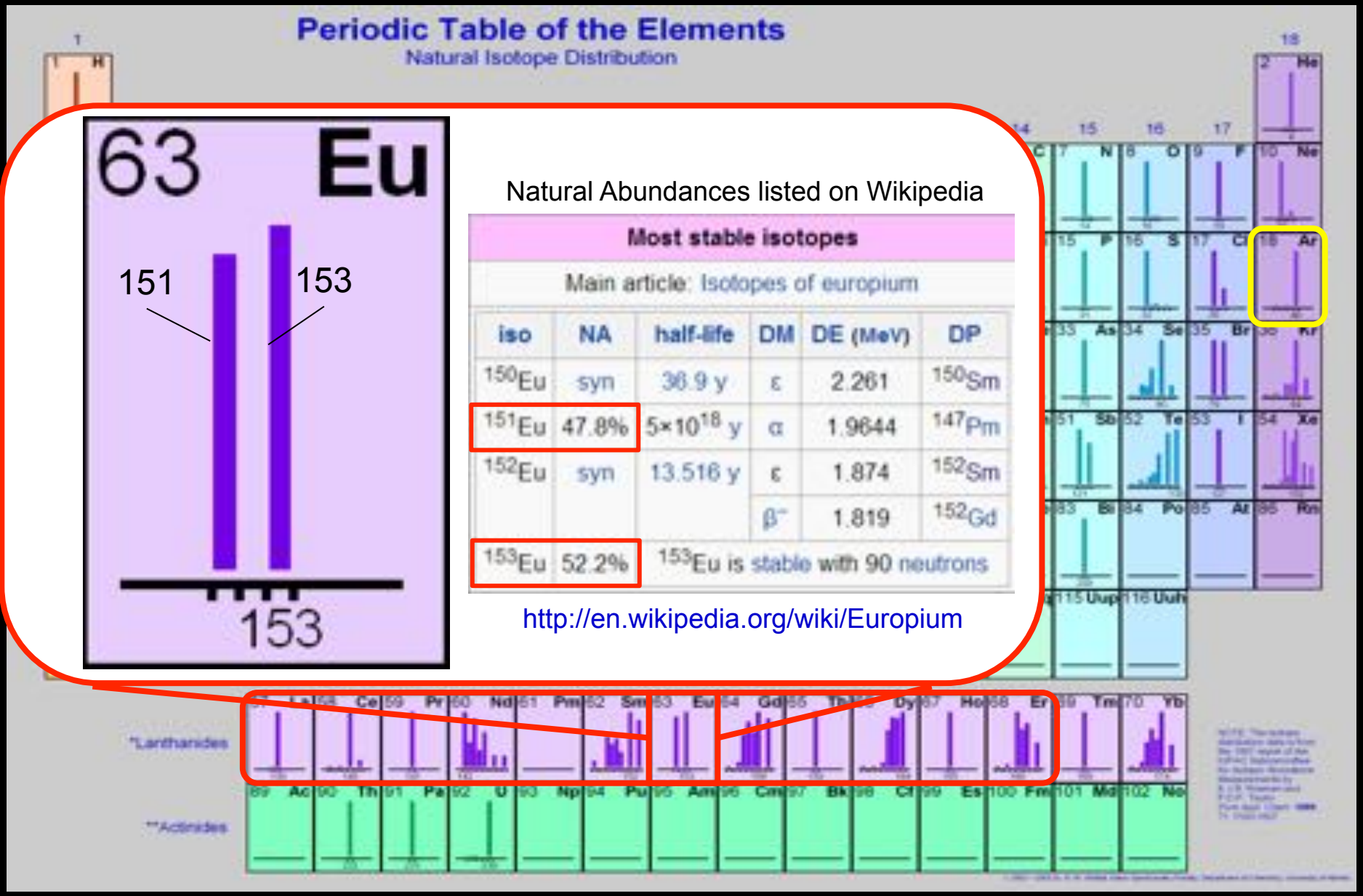
Elemental Isotopes (e.g. Lanthanides) are Used As Mass Tags



Natural Abundances listed on Wikipedia

Most stable isotopes

Main article: Isotopes of europium

iso	NA	half-life	DM	DE (MeV)	DP
^{150}Eu	syn	36.9 y	ε	2.261	^{150}Sm
^{151}Eu	47.8%	5×10^{18} y	α	1.9644	^{147}Pm
^{152}Eu	syn	13.516 y	ε	1.874	^{152}Sm
			β⁻	1.819	^{152}Gd
^{153}Eu	52.2%	^{153}Eu is stable with 90 neutrons			

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:
(~1.5 hours)

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 ^{o}C
3) Permeabilized in -20 ^{o}C MeOH, 10’
4) Labeled w/ natural Iridium based nucleic acid intercalator (191 & 193), 23 ^{o}C 15’
5) Counted & resuspended in ddH_2O 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

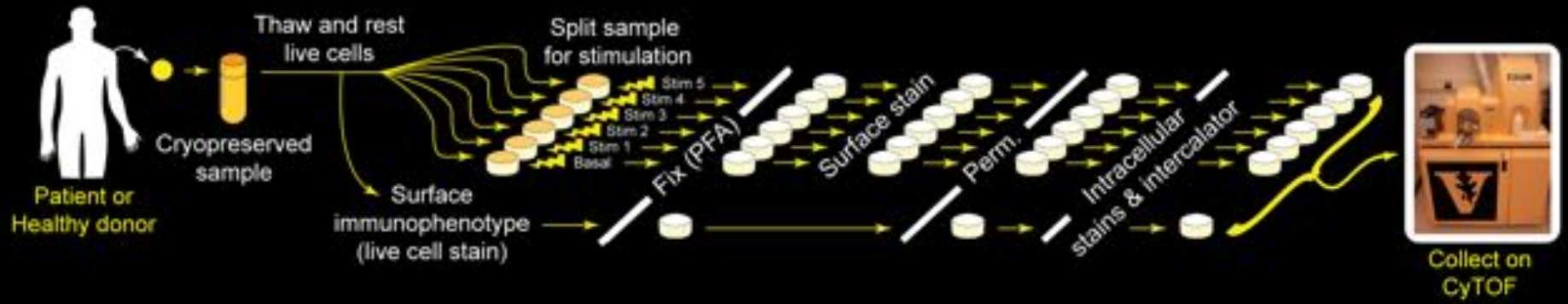
5 Signaling Readouts

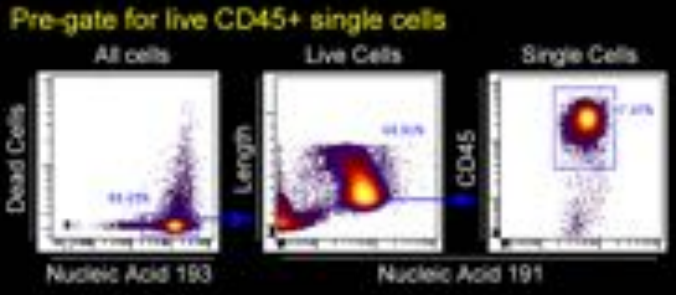
p-STAT5, p-STAT1, p-SFK, p-ERK, p-STAT6

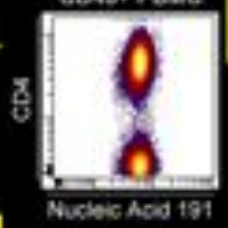
Protocol:
(~2 hours)

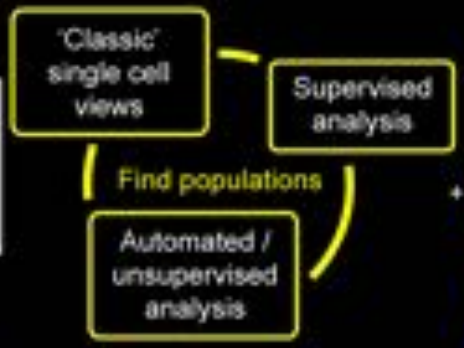
1) Stimulate Human PBMC 15' @ 37 °C
2) Fix in 1.6% formaldehyde 5' @ 23 °C
3) Label w/ surface antibodies 20' @ 23 °C
4) Permeabilize (Saponin | MeOH) 10' @ -20 °C
5) Label w/ Ir nucleic acid intercalator (191 & 193) 15' @ 23 °C
6) Count & resuspend in ddH_2O

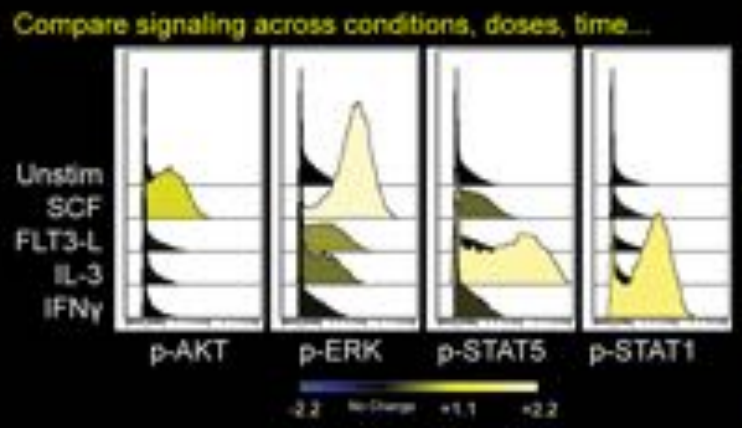
Mapping Signaling in Every Cell using Mass Cytometry

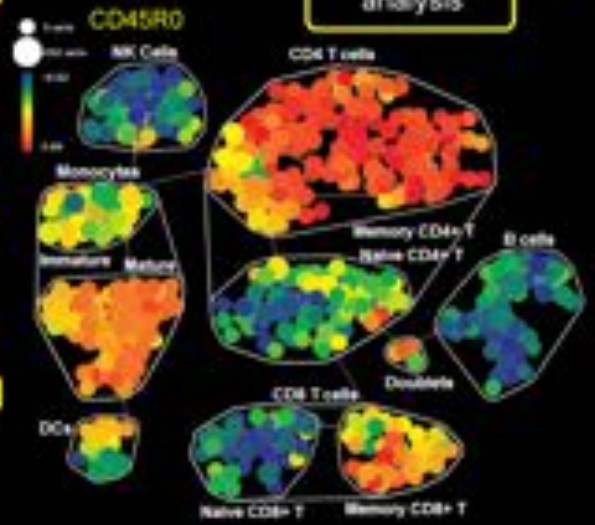


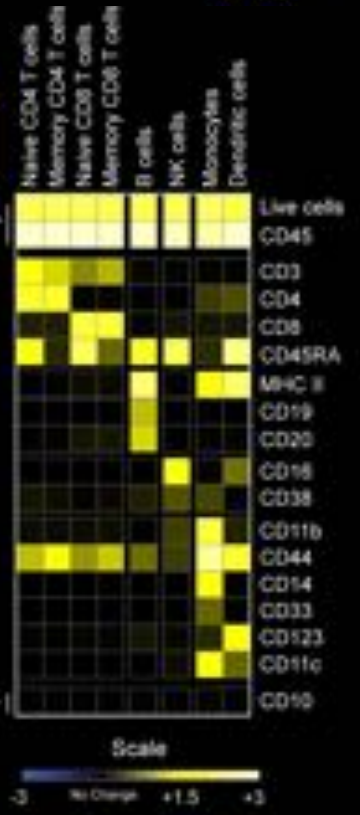












Introduction
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Optimizing & troubleshooting
(experiments)

Summary: Measuring and Dealing with Variation

Rule #1: Treat all samples as equally as possible

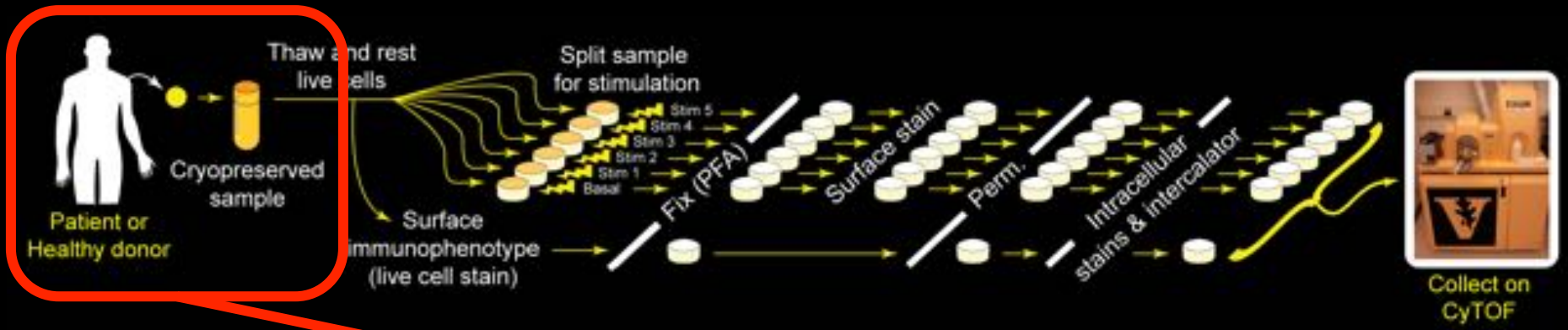
Sources of Variation

- 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

Optimizing the Experimental Workflow

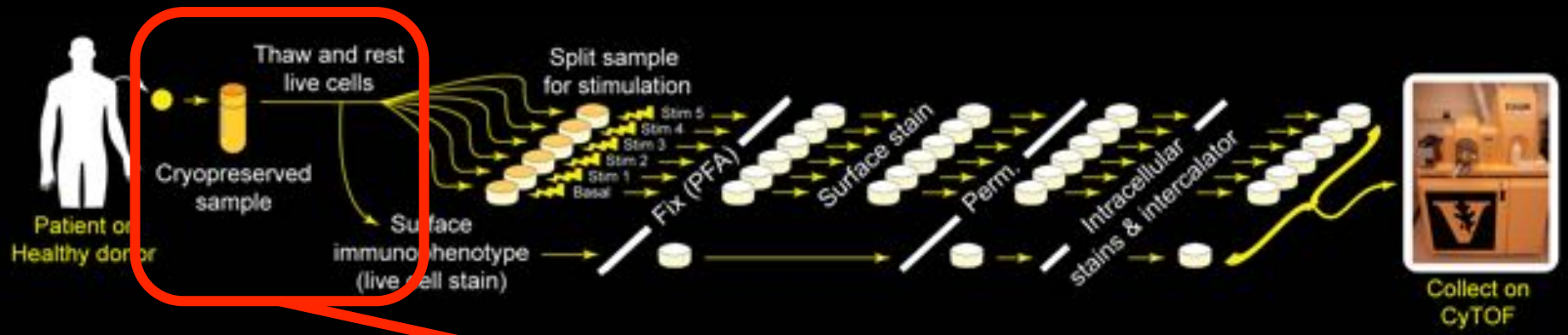


Individual variation

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)

Optimizing the Experimental Workflow

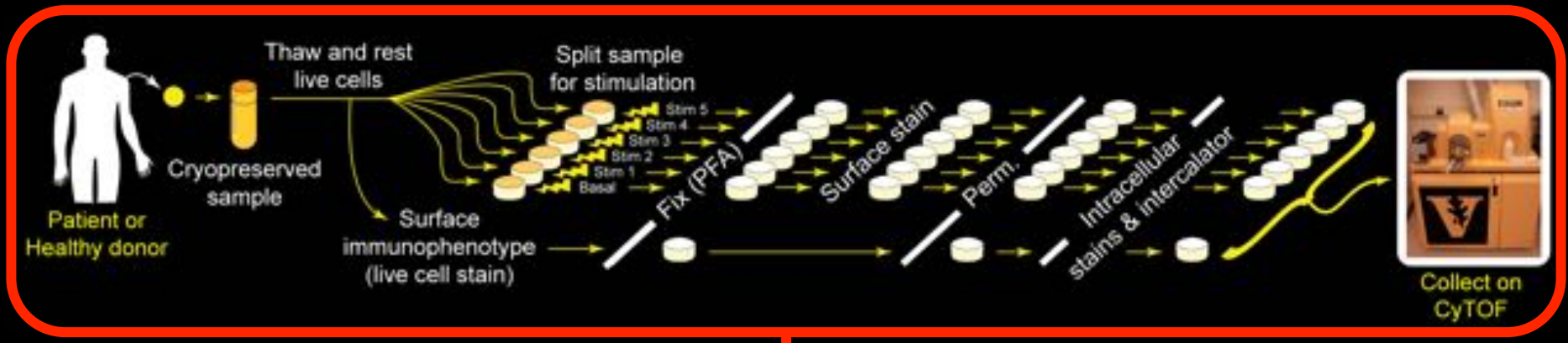


Sample Preparation & Storage

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

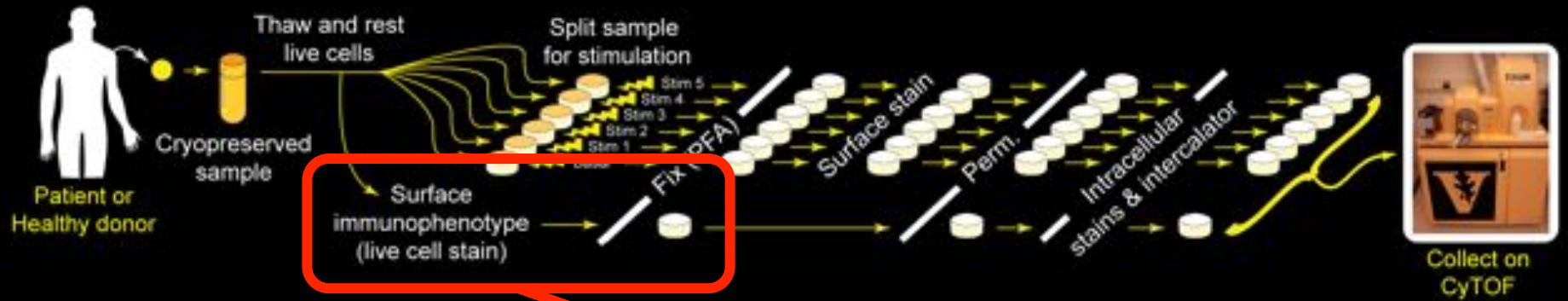


Assay Variation

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



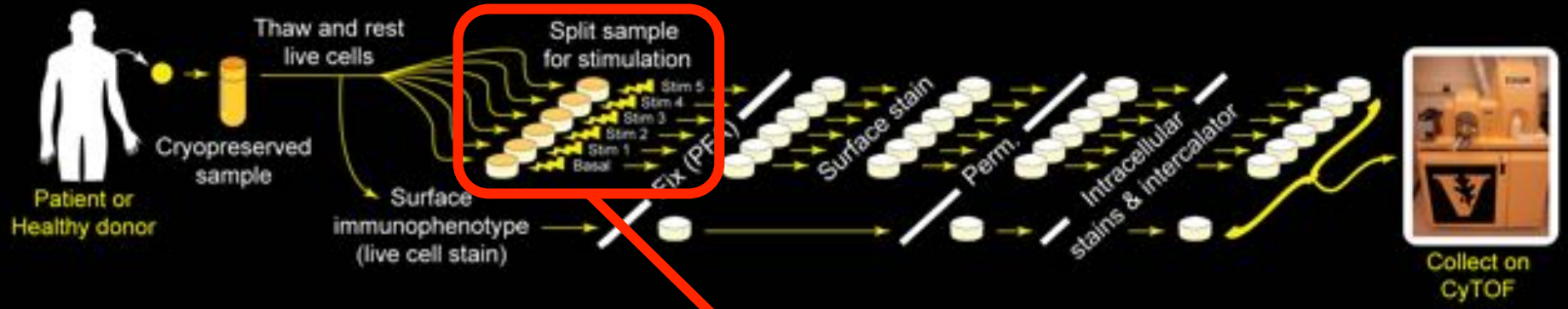
‘Side’ Immunophenotype Panel

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

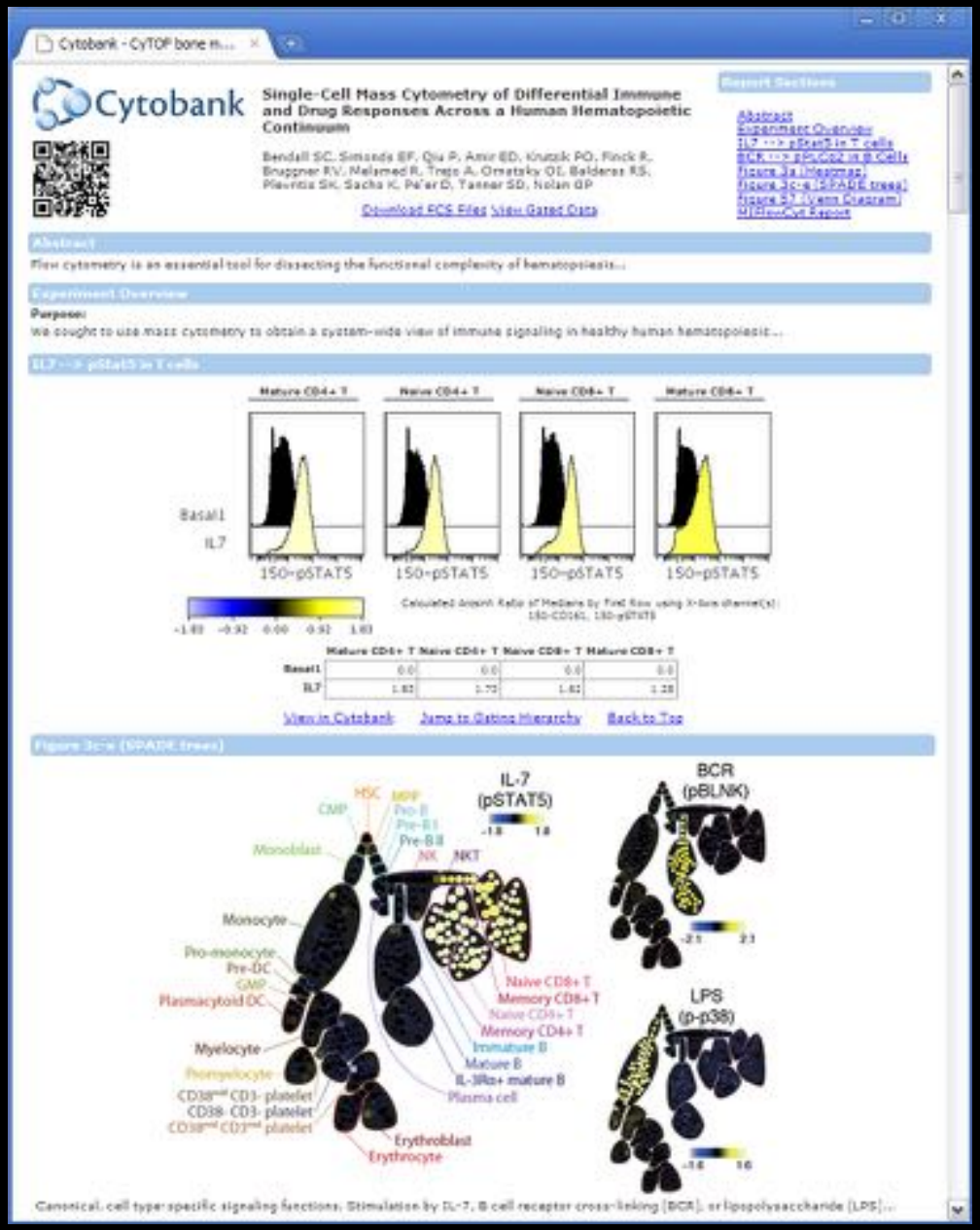


Picking Stimulation Conditions

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



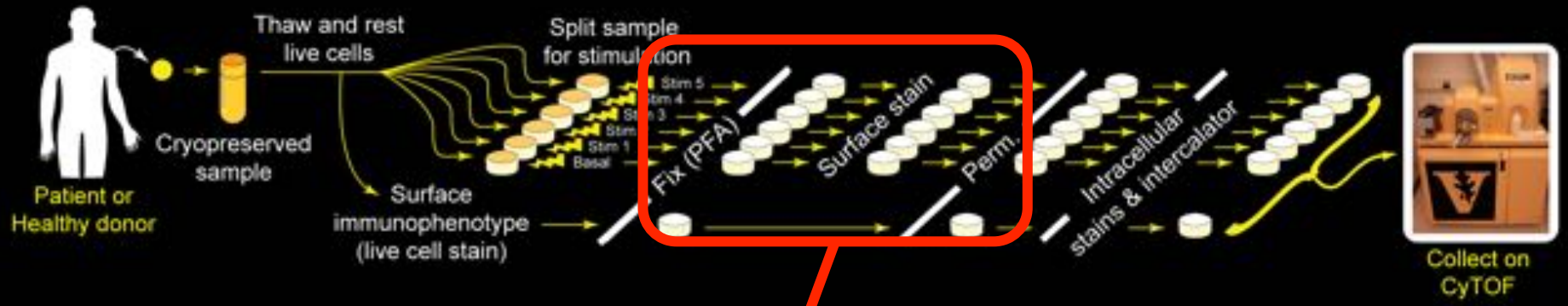
Nolan lab mass cytometry dataset
www.cytobank.org/nolanlab

Bendall et al., *Science* 2011 data hosted by Cytobank

Find Examples in Annotated Online Datasets

DVS Cytobank: dvs.cytobank.org

Optimizing the Experimental Workflow



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

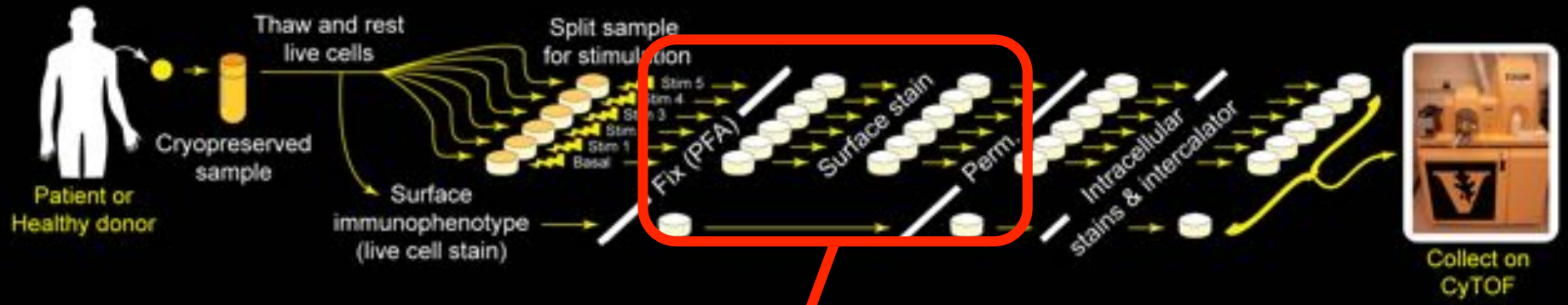
1b) Fixing cells to stop signaling hurts staining (minor)

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



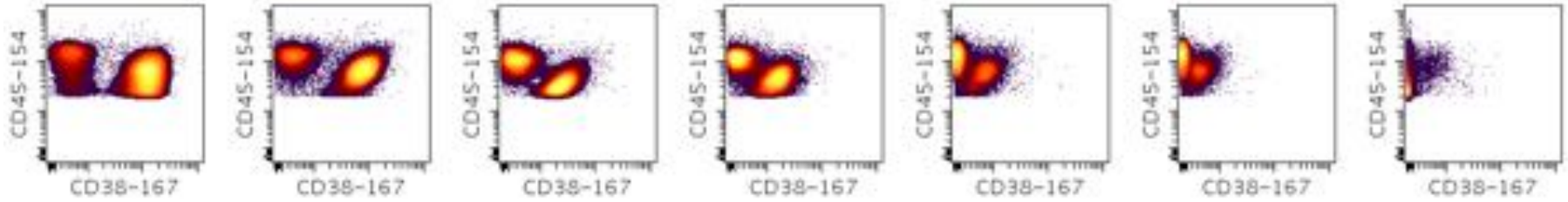
Titrating Multiple Markers

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



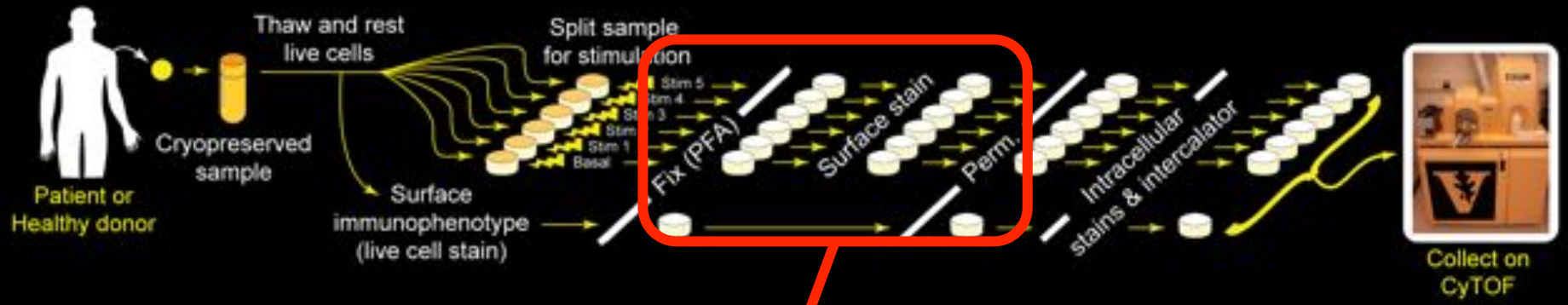
Calculated Raw values of Percentile Distance (5 to 95) using X-Axis channel(s): Use Panel/Channel Values

	Titration 1	Titration 2	Titration 3	Titration 4	Titration 5	Titration 6	Titration 7
CD38-167 - Panel 1	4.29	3.95	3.23	2.7	1.84	1.45	0.84

Statistic: 5th to the 95th percentile distance
(log like arcsinh scale)

B Ferrell, Irish Lab

Optimizing the Experimental Workflow

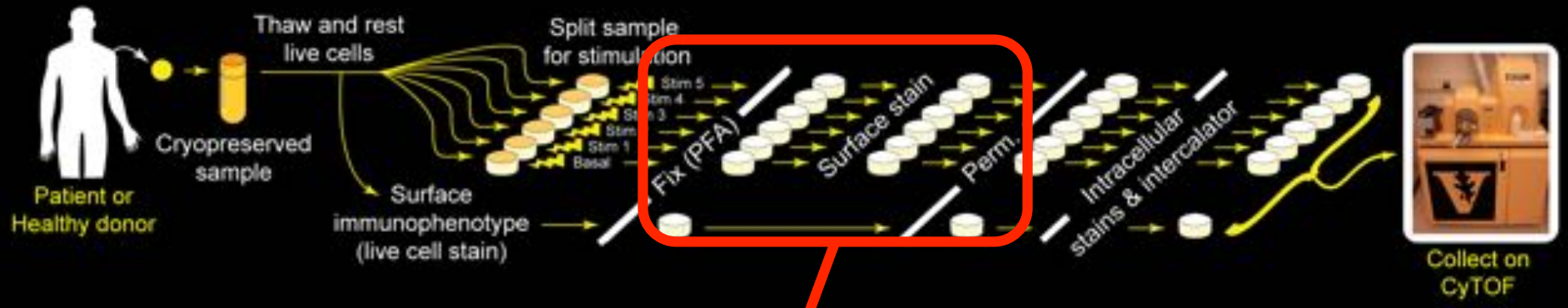


Testing Metal Labeled Antibodies

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

Can also run antibody in solution.

Optimizing the Experimental Workflow



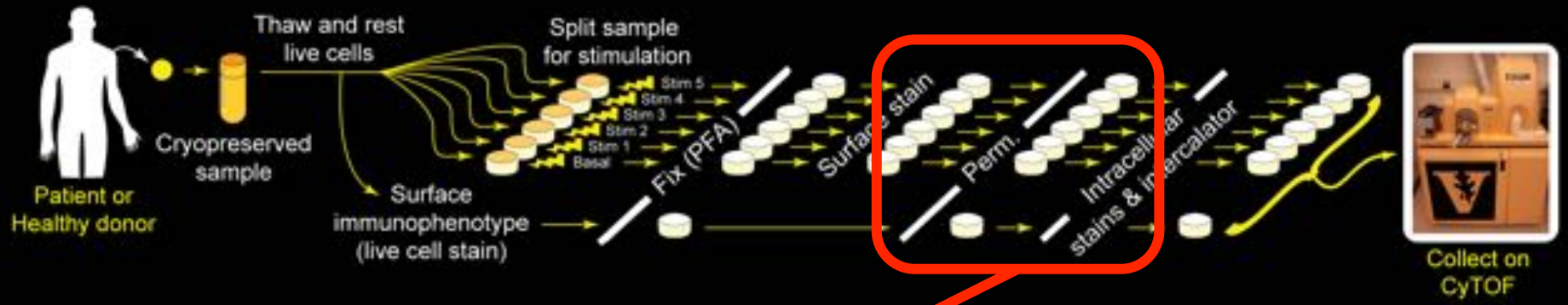
Marking Dead Cells

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

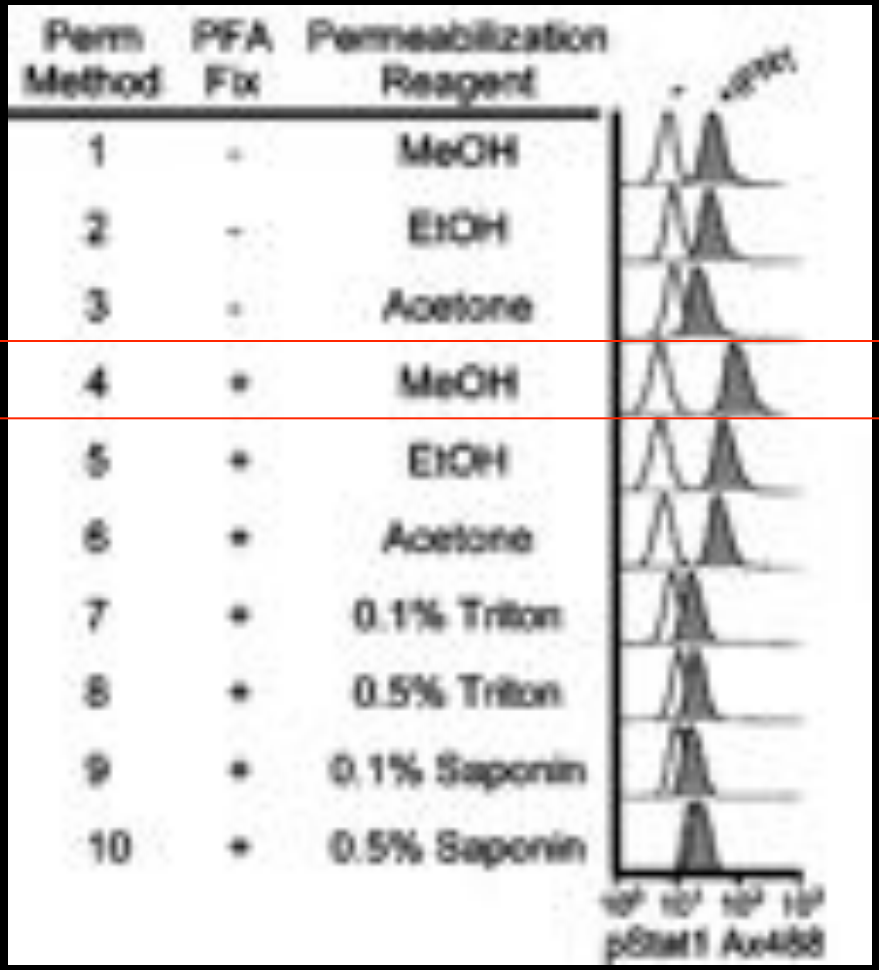


Permeabilization Options

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

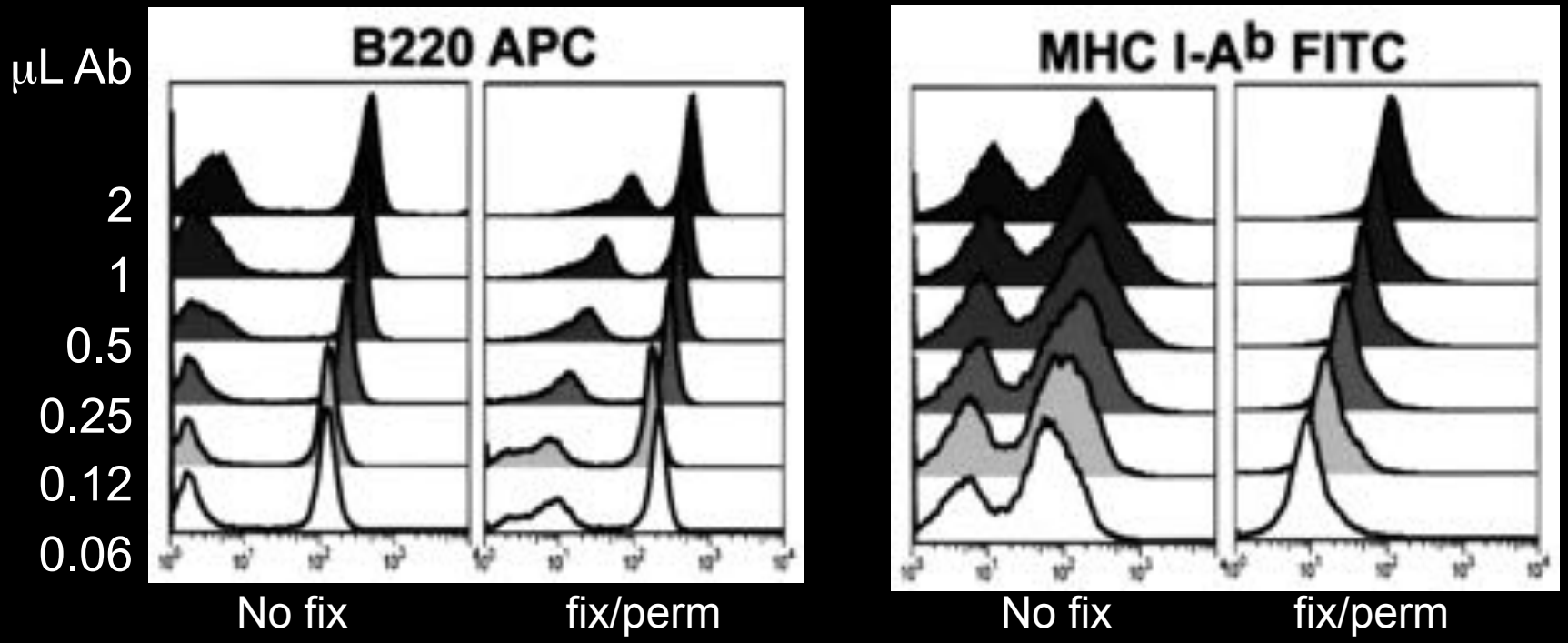
Potentially: new ‘one step’ perm reagents that work for many/all target epitopes (e.g. Foxp3 and p-STAT5)

Fixation in PFA and Permeabilization in Methanol is Ideal for Many Phospho-Epitopes, Especially p-STATs



Krutzik et al., *Cytometry* 2003

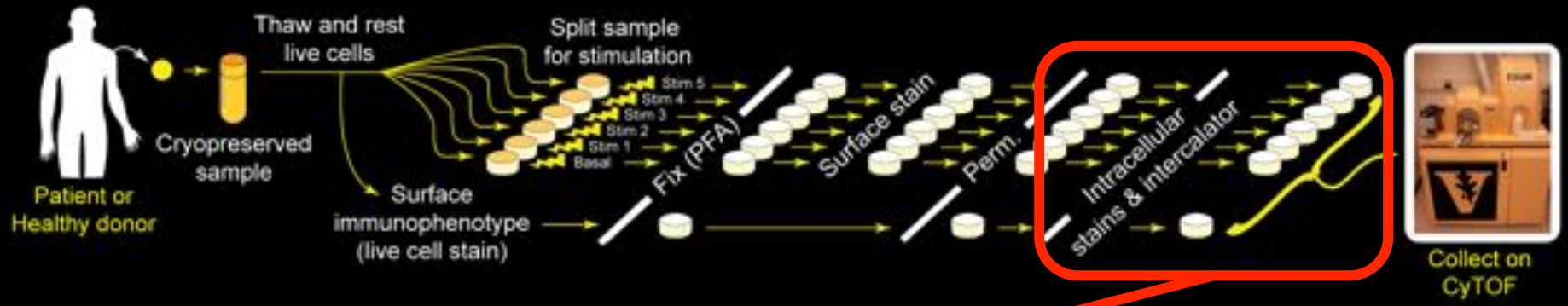
Not All Traditional Flow Antibodies Work Well in All Fix/Perm Buffer Conditions (Especially Methanol)



(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

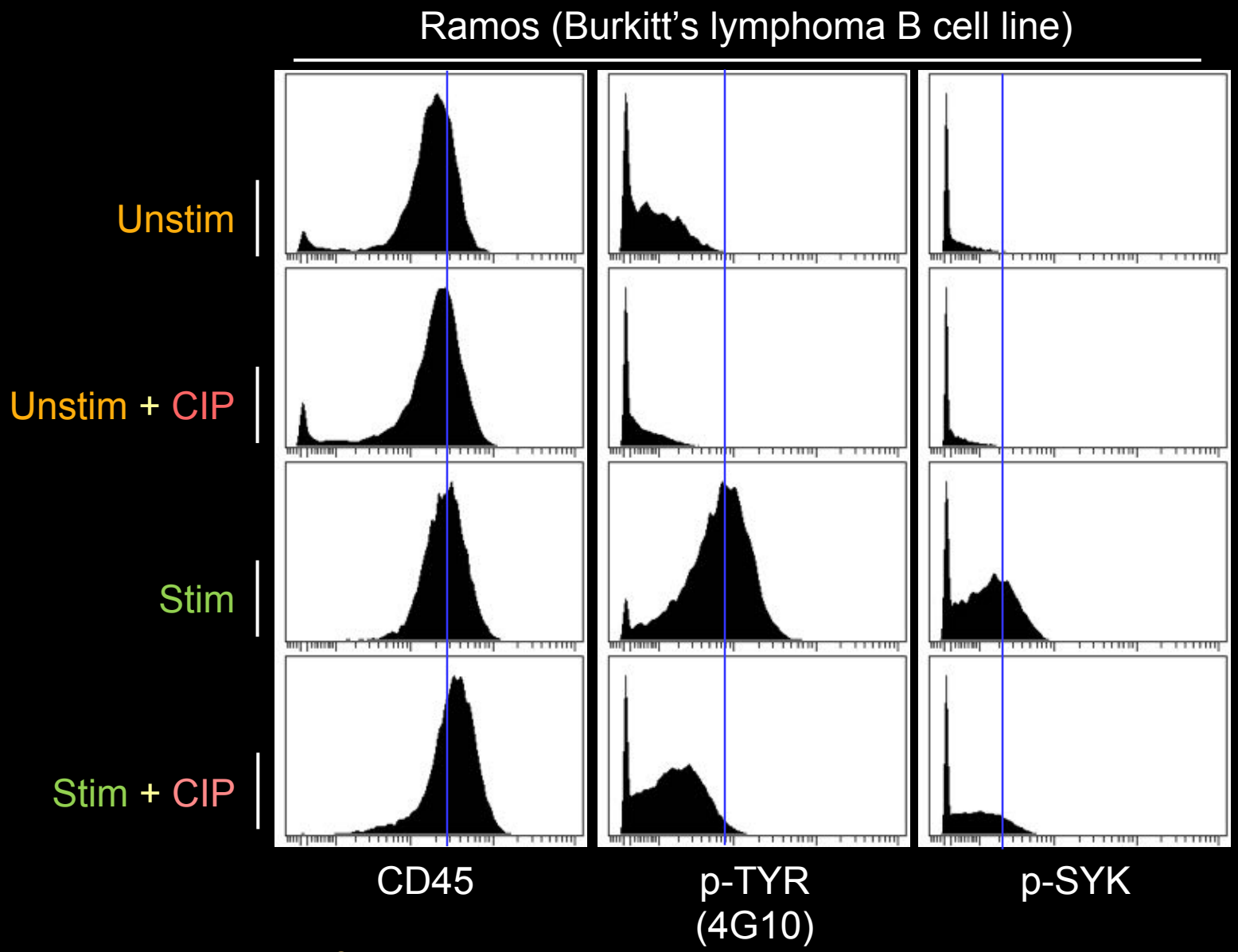


Titrating Phospho-Specific Antibodies

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.

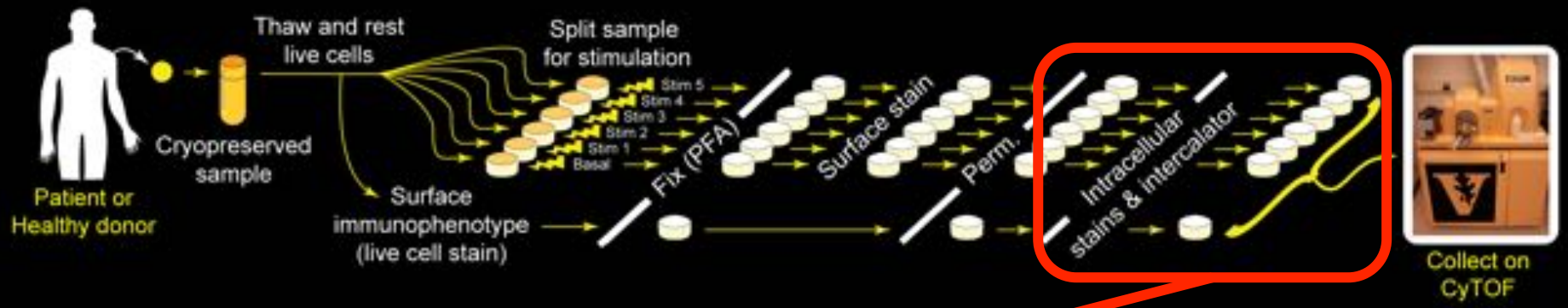
Phosphatase (CIP) Treatment Can Reveal High Basal Signaling



CIP approach: Khalil AM et al., *Science* 2012

H. Polikowsky, Irish Lab

Optimizing the Experimental Workflow

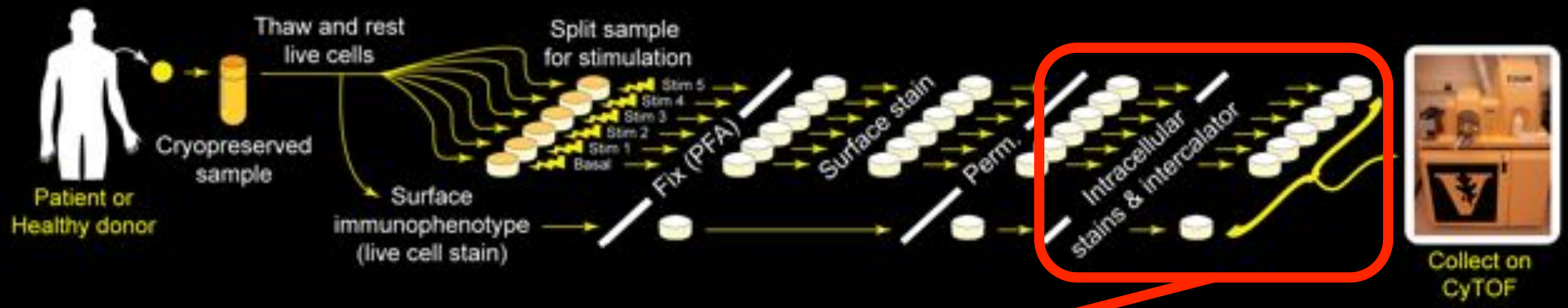


Picking Doses and Timepoints

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

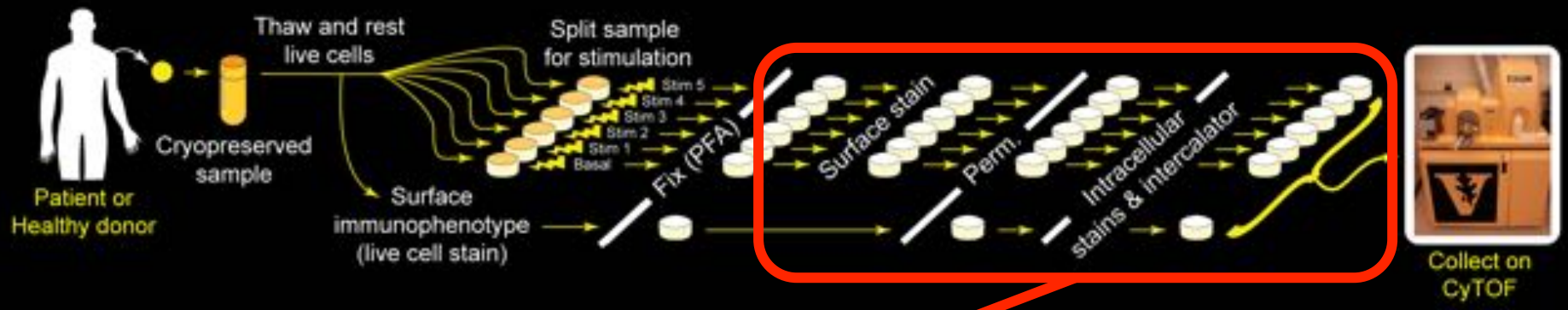


Nucleic Acid Intercalator

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)

Optimizing the Experimental Workflow



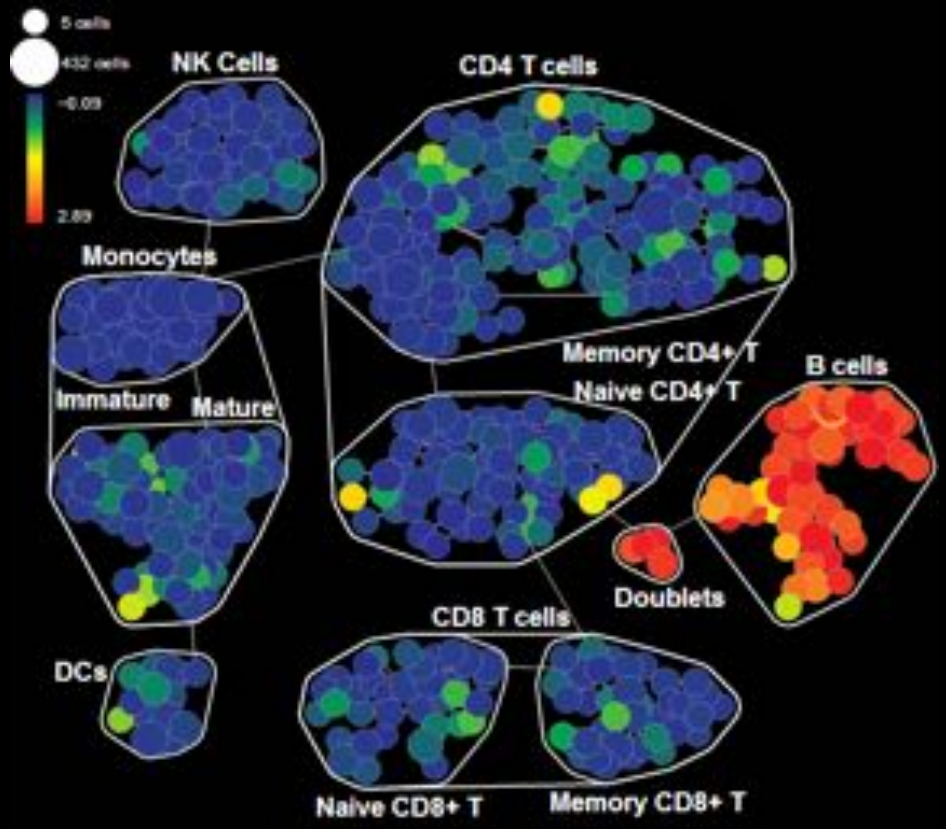
Panel Design

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

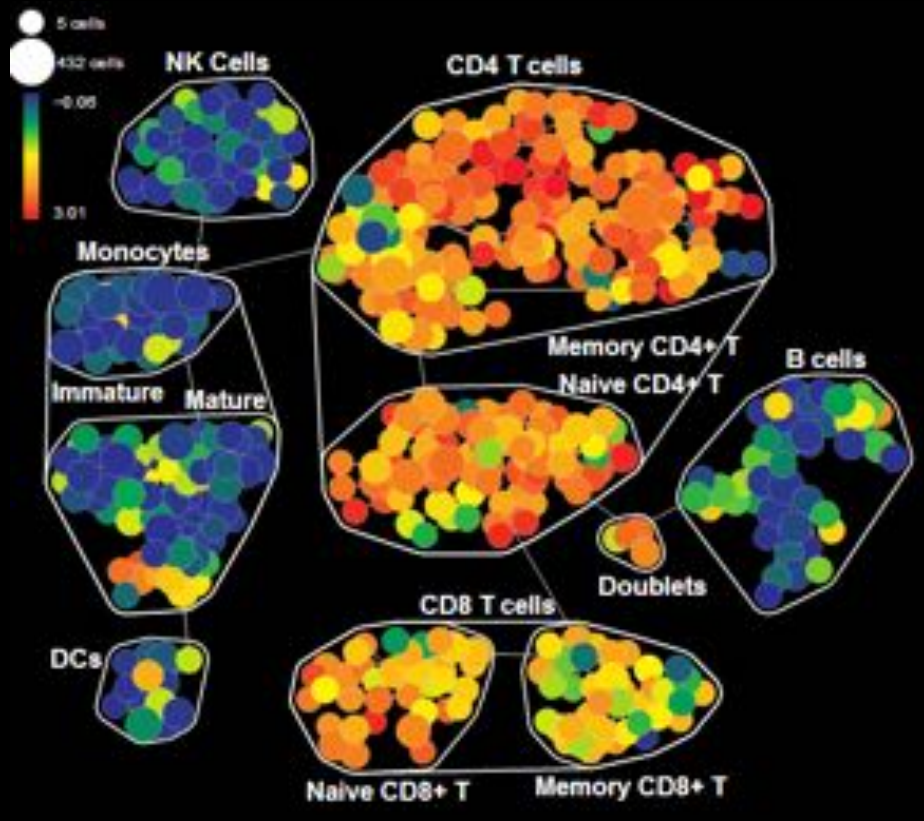
Use beads, titrations, ‘MMO’ controls, known biology.

Primary samples: use internal control cell subsets.

+1 Bleed Comparison: CD19 into CD5 (no issue)



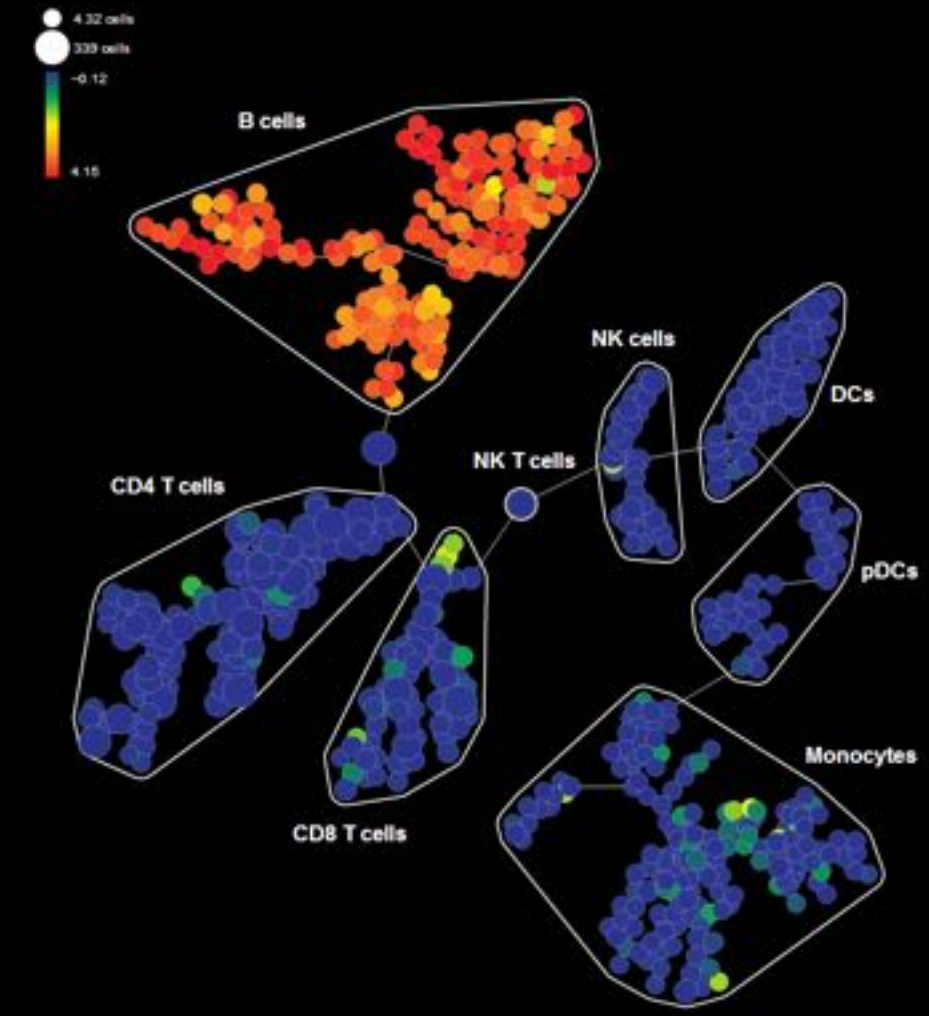
CD19-142: 2.9 dynamic range



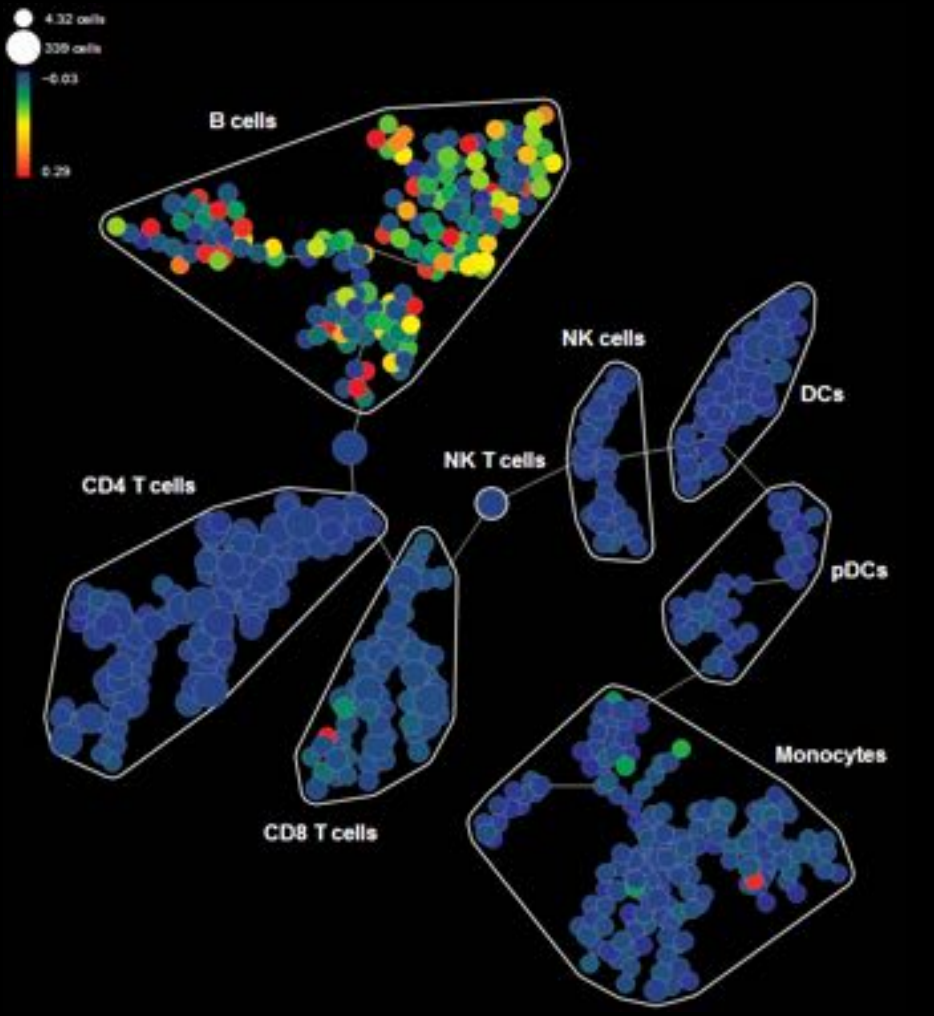
CD5-143: 3.0 dynamic range

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

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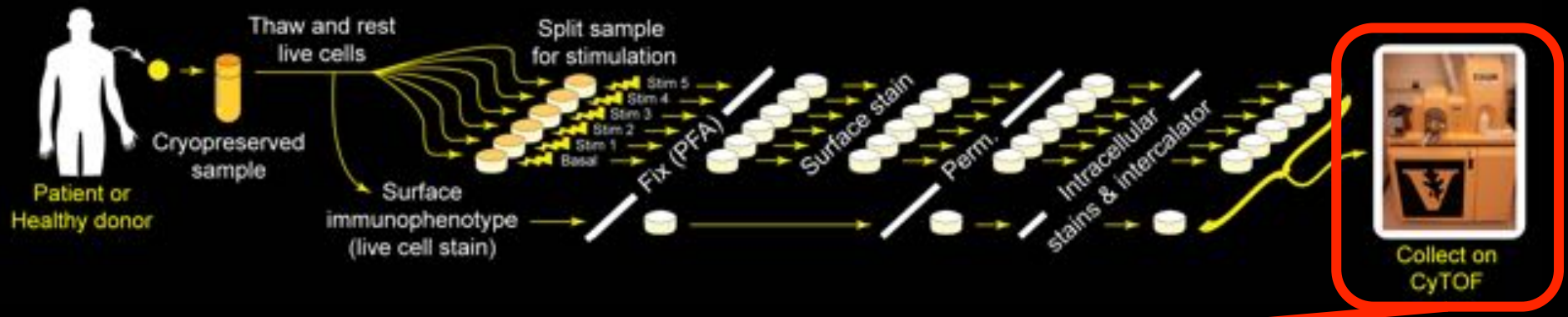
Optimizing the Experimental Workflow



Collection

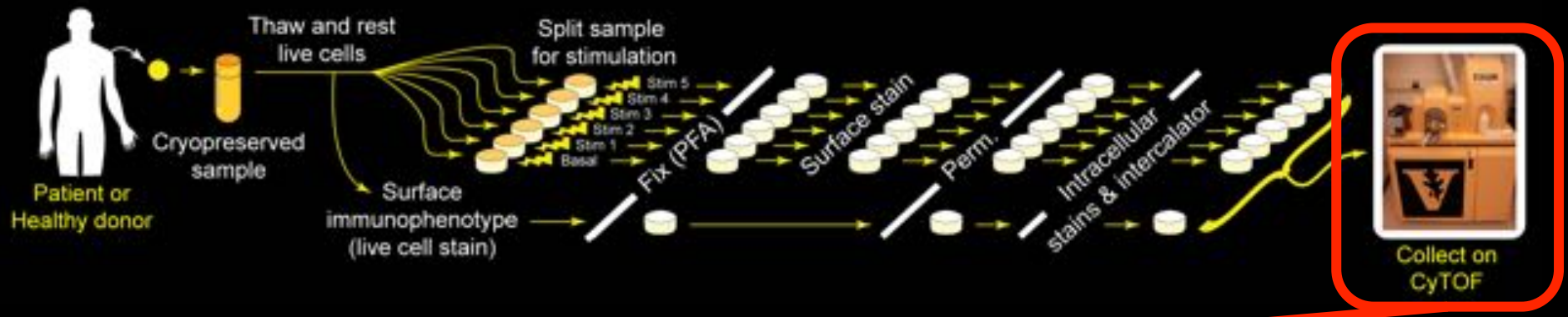
Keep carrier fluid running (software & physical).

30-40 sec delay following sample introduction.

Wash between samples. Run ¼ diluted control first.

Collect open channels.

Optimizing the Experimental Workflow



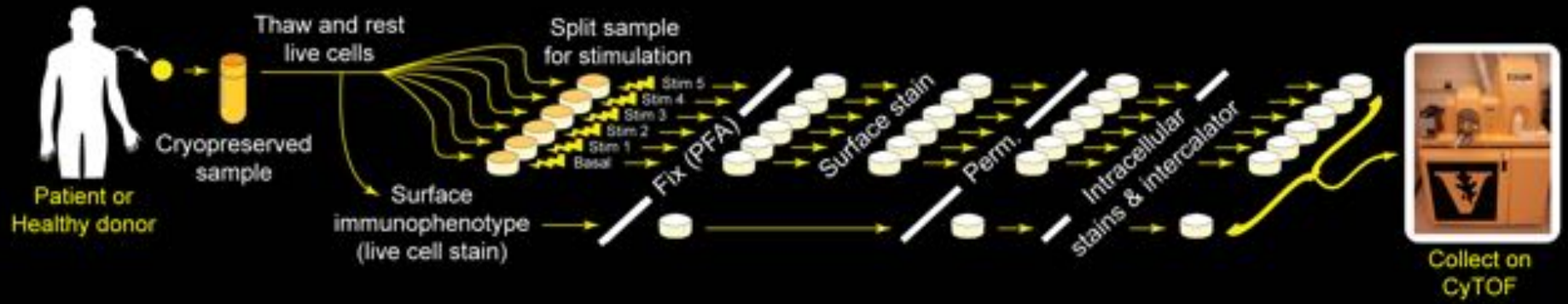
Collection

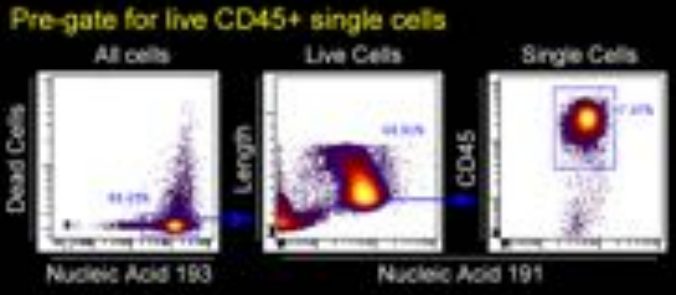
Run standardization controls before and after sample (at minimum). Tuning solution, beads, standard cells.

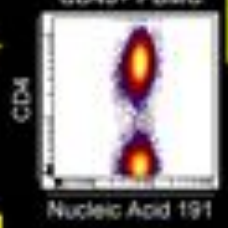
Ideally run internal beads for continuous normalization. (Finck R et al., *Cytometry* 2013)

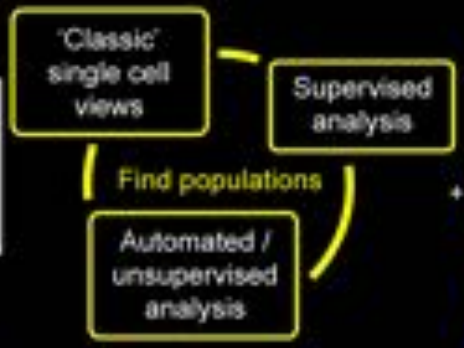
Putting it all together…

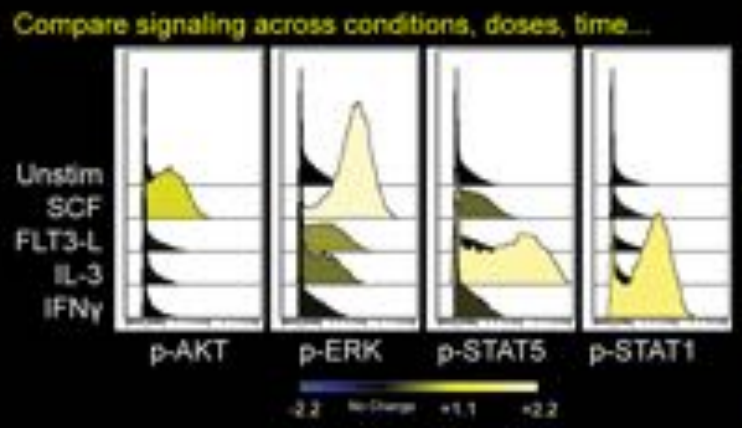
Mapping Signaling in Every Cell using Mass Cytometry

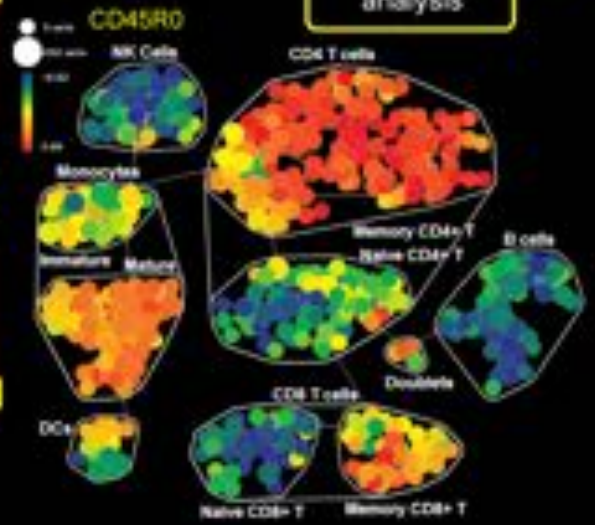


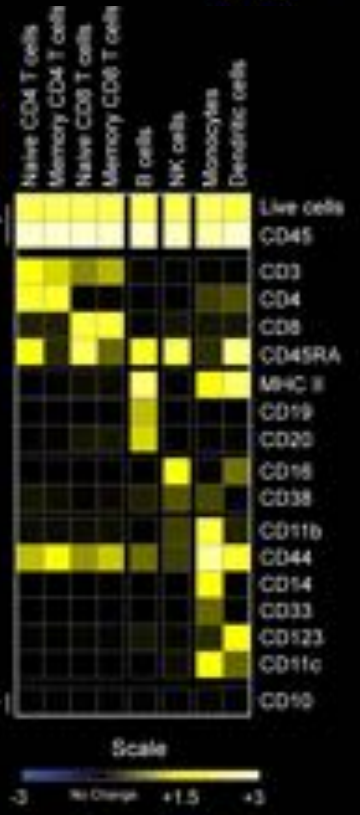












30 Dimensional View of Healthy Human Blood Subsets & Signaling

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

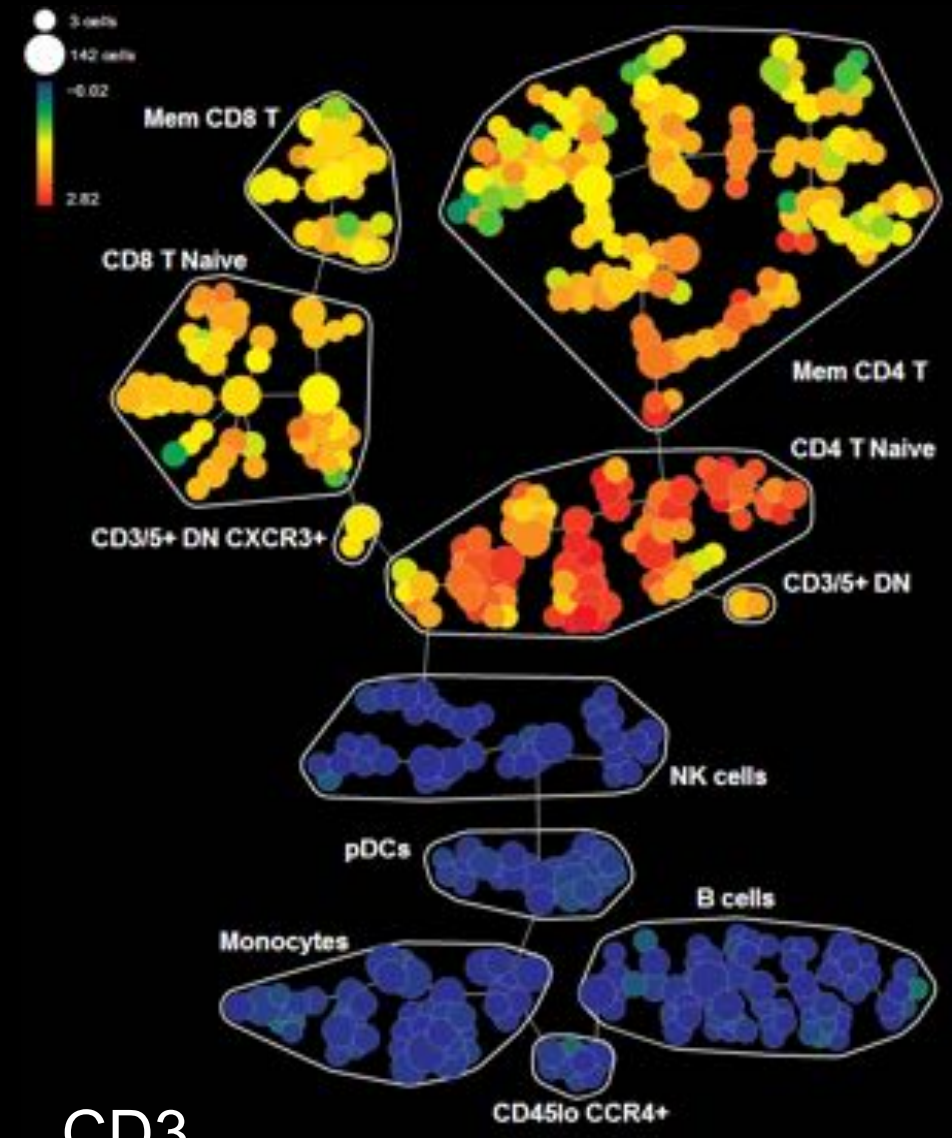
p-STAT5, p-STAT1, p-SFK, p-ERK, p-STAT6

Protocol:
(~2 hours)

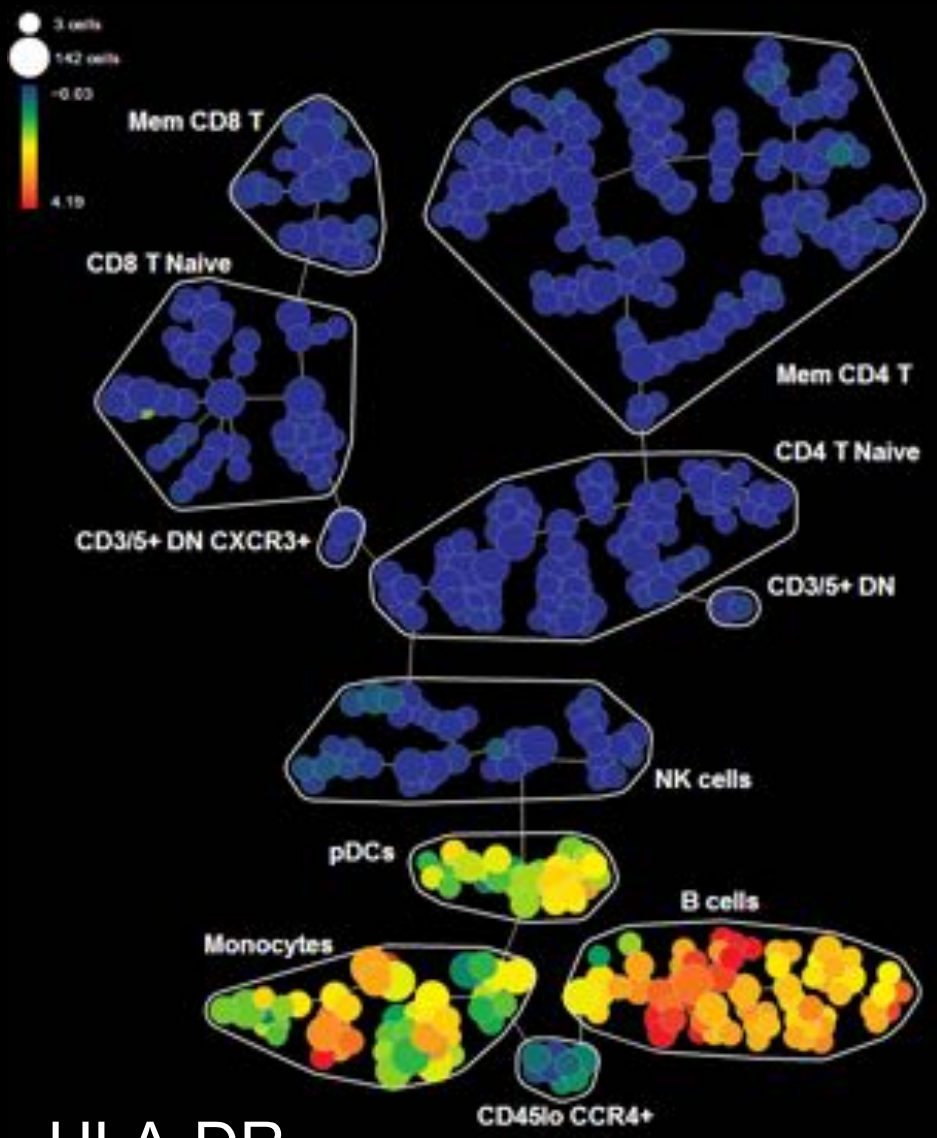
1) Stimulate Human PBMC 15' @ 37 °C
2) Fix in 1.6% formaldehyde 5' @ 23 °C
3) Label w/ surface antibodies 20' @ 23 °C
4) Permeabilize (Saponin | MeOH) 10' @ -20 °C
5) Label w/ Ir nucleic acid intercalator (191 & 193) 15' @ 23 °C
6) Count & resuspend in ddH_2O

(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

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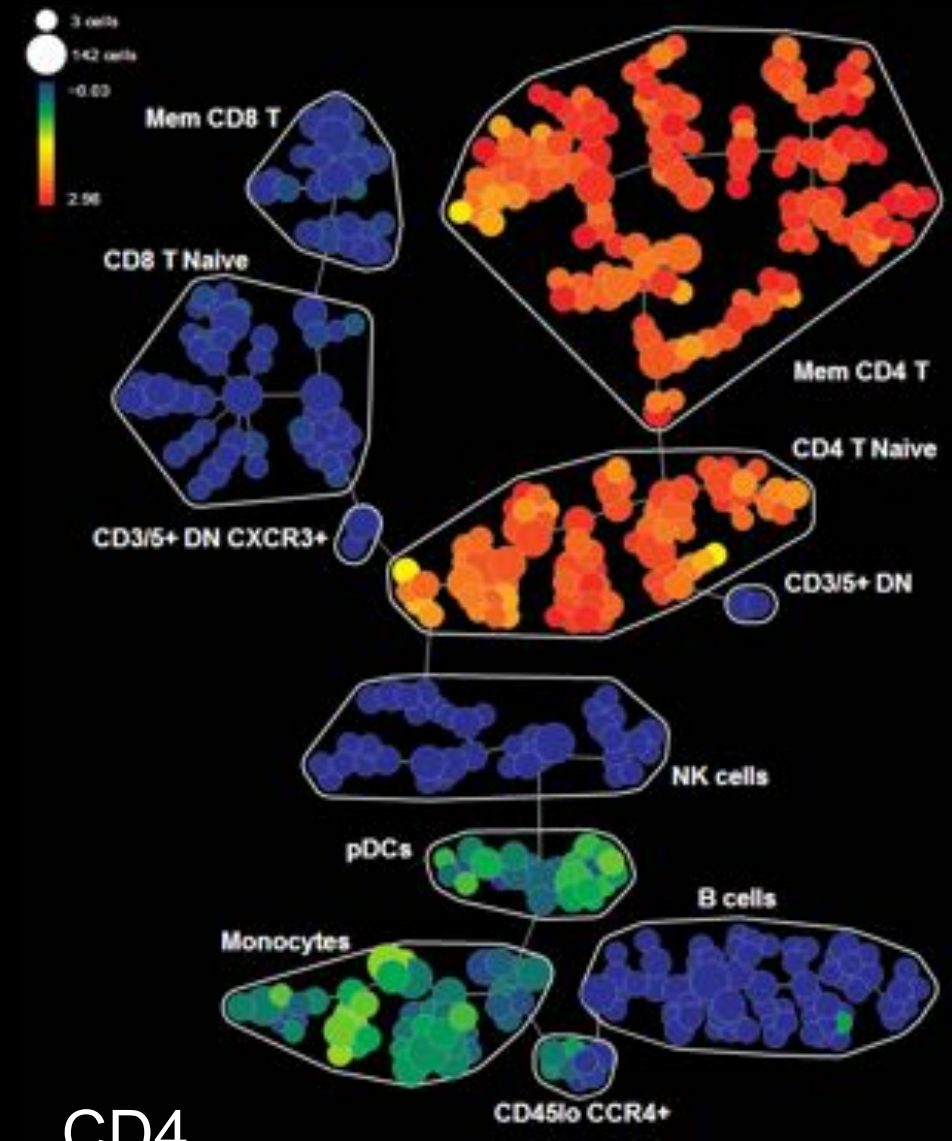
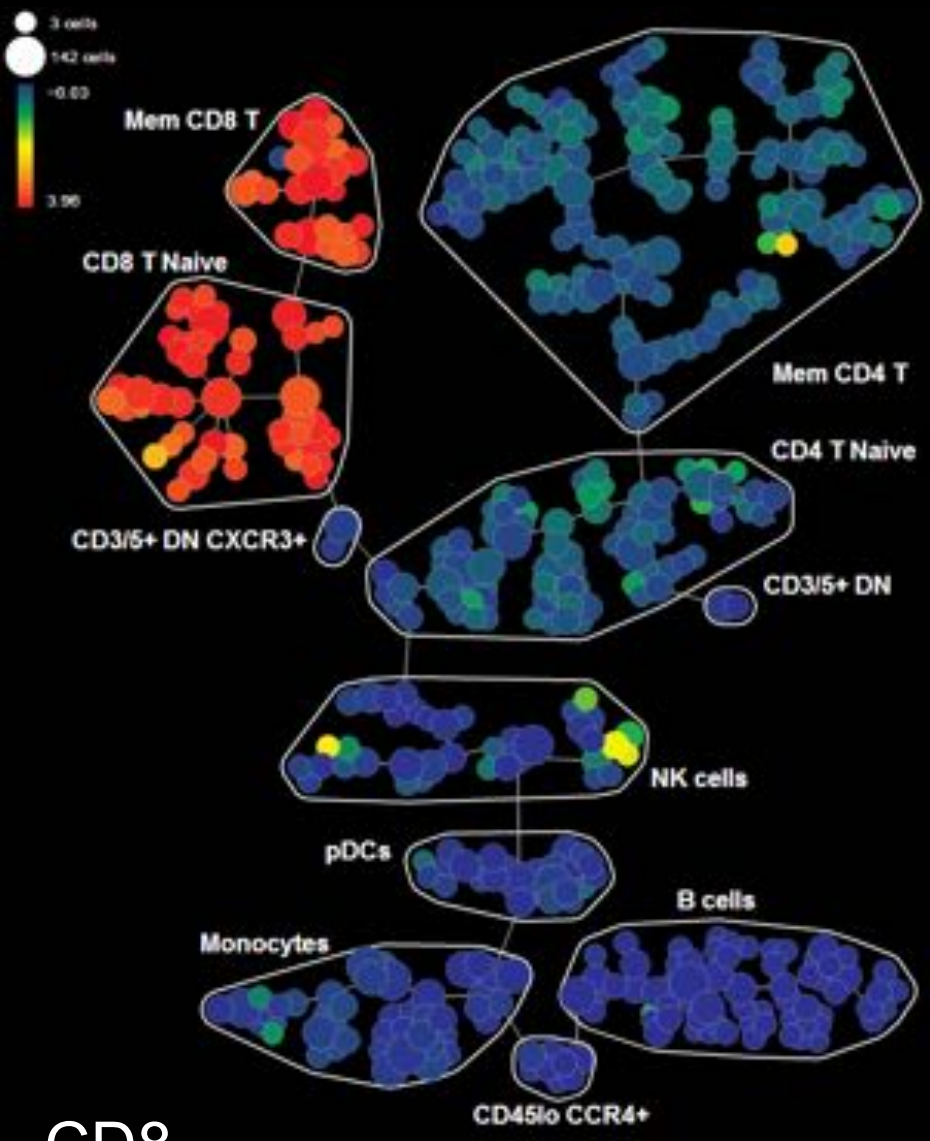
Mass Cytometry: Healthy CD4+ and CD8+ T cells



CD4



CD8

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

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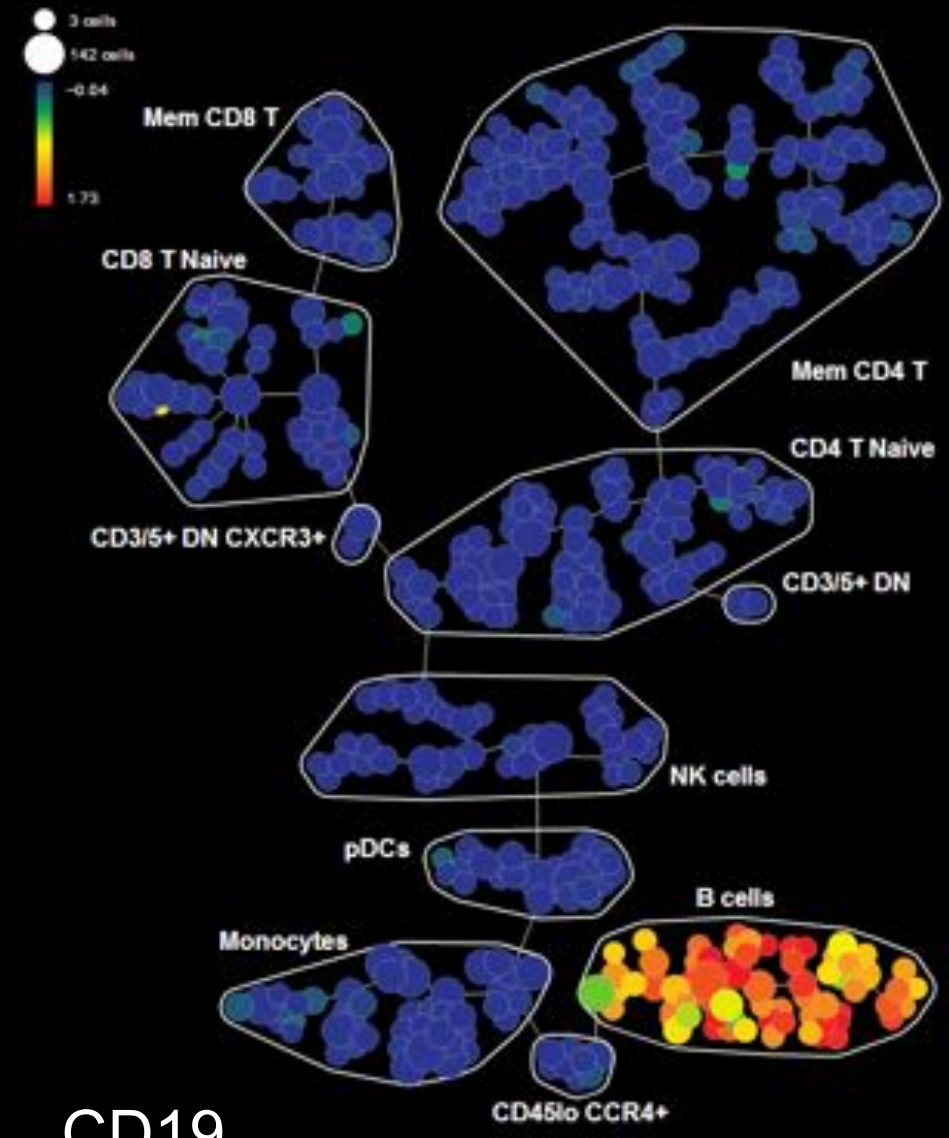
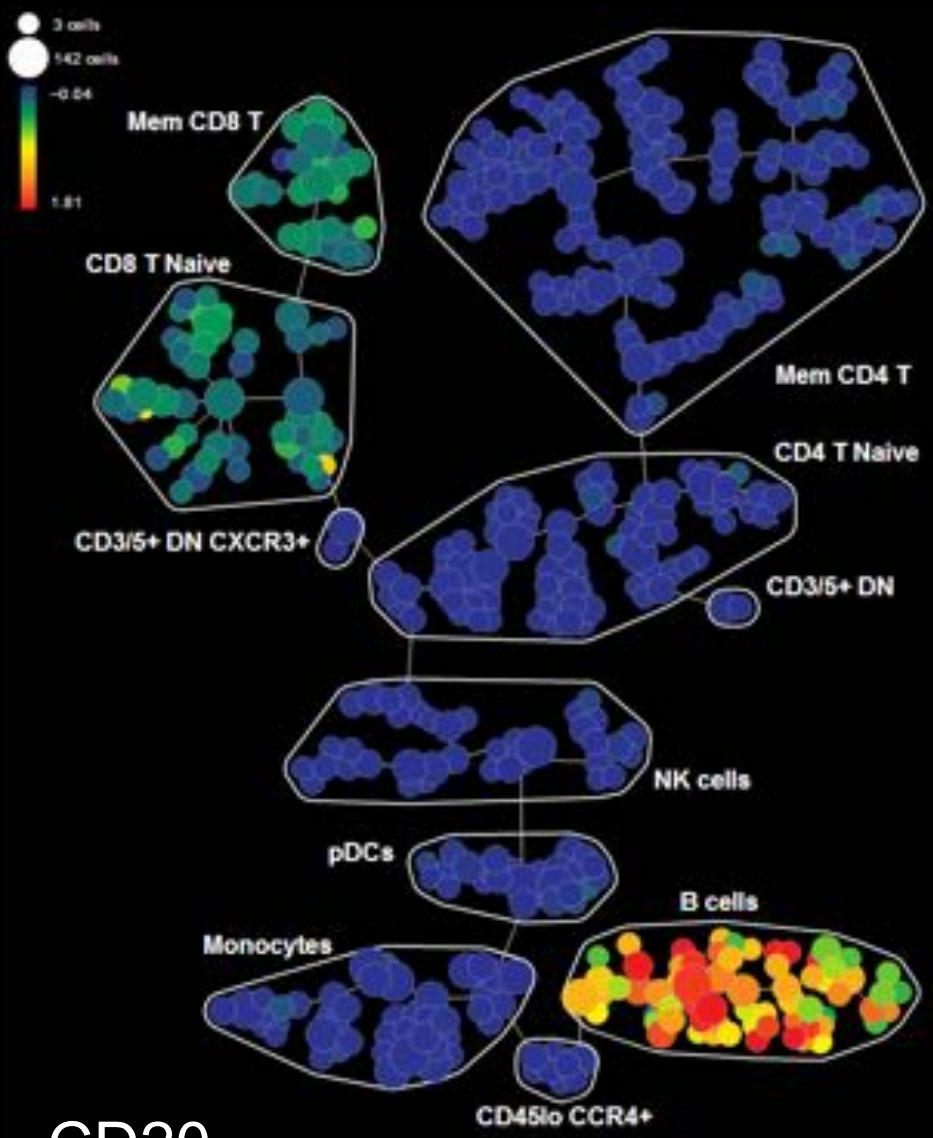
Mass Cytometry: Healthy B Cells (CD19+ CD20+)



CD19



CD20

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

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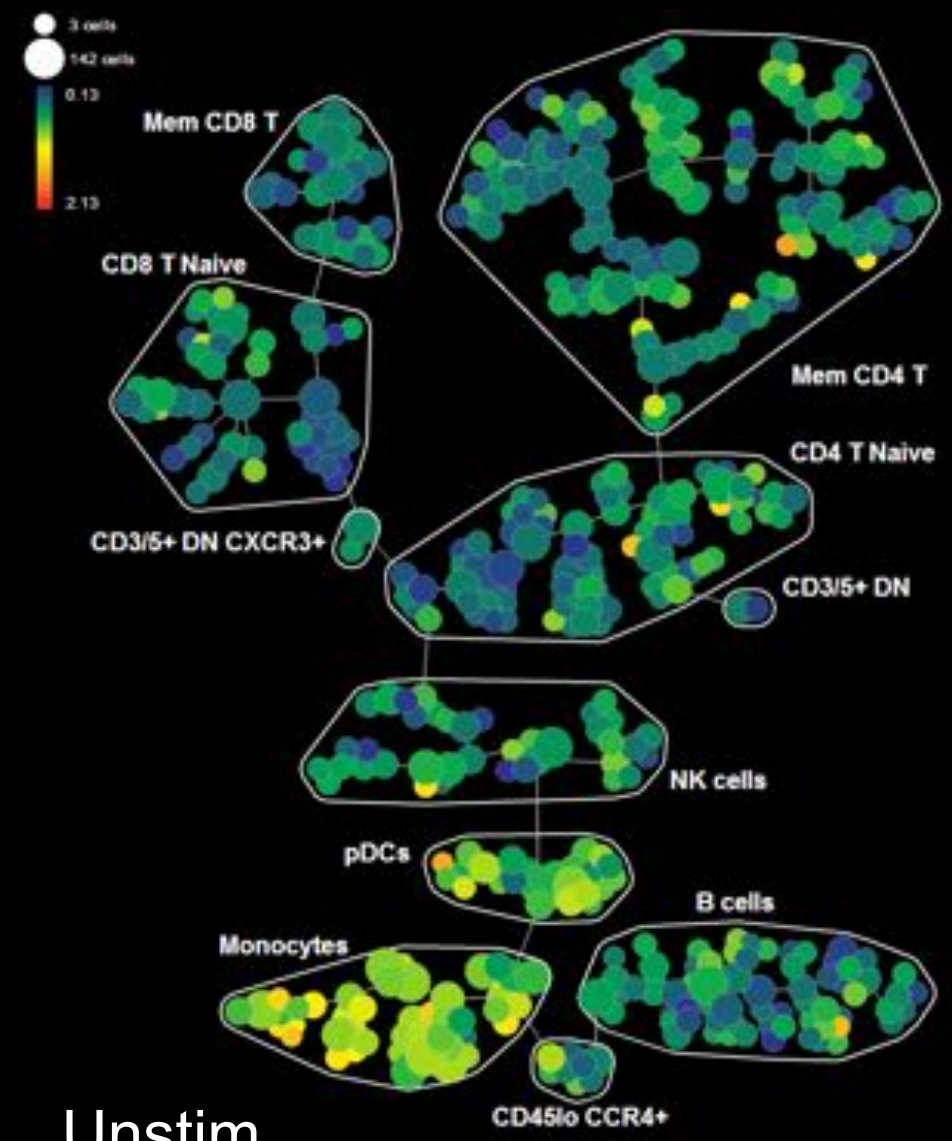
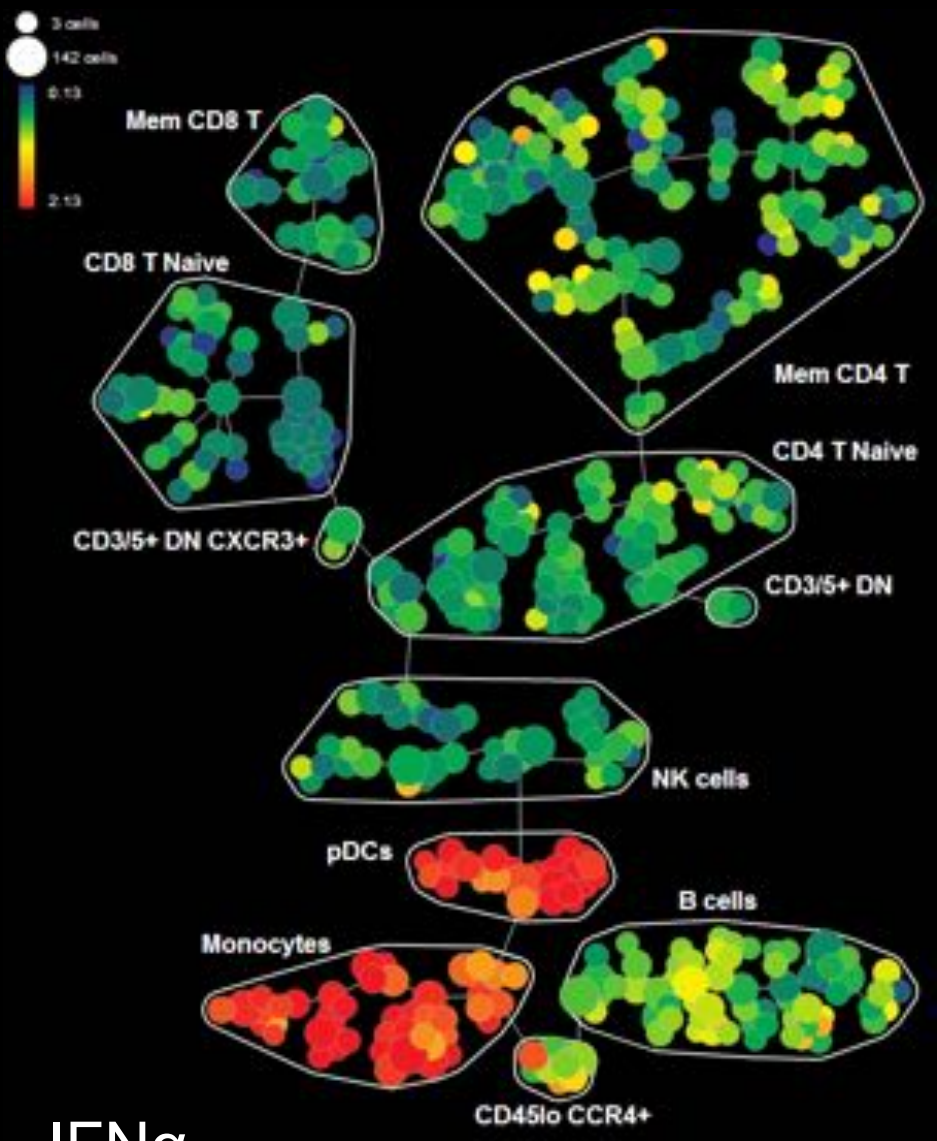
Mass Cytometry: phospho-STAT1 IFNα Response



Unstim



IFNα

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

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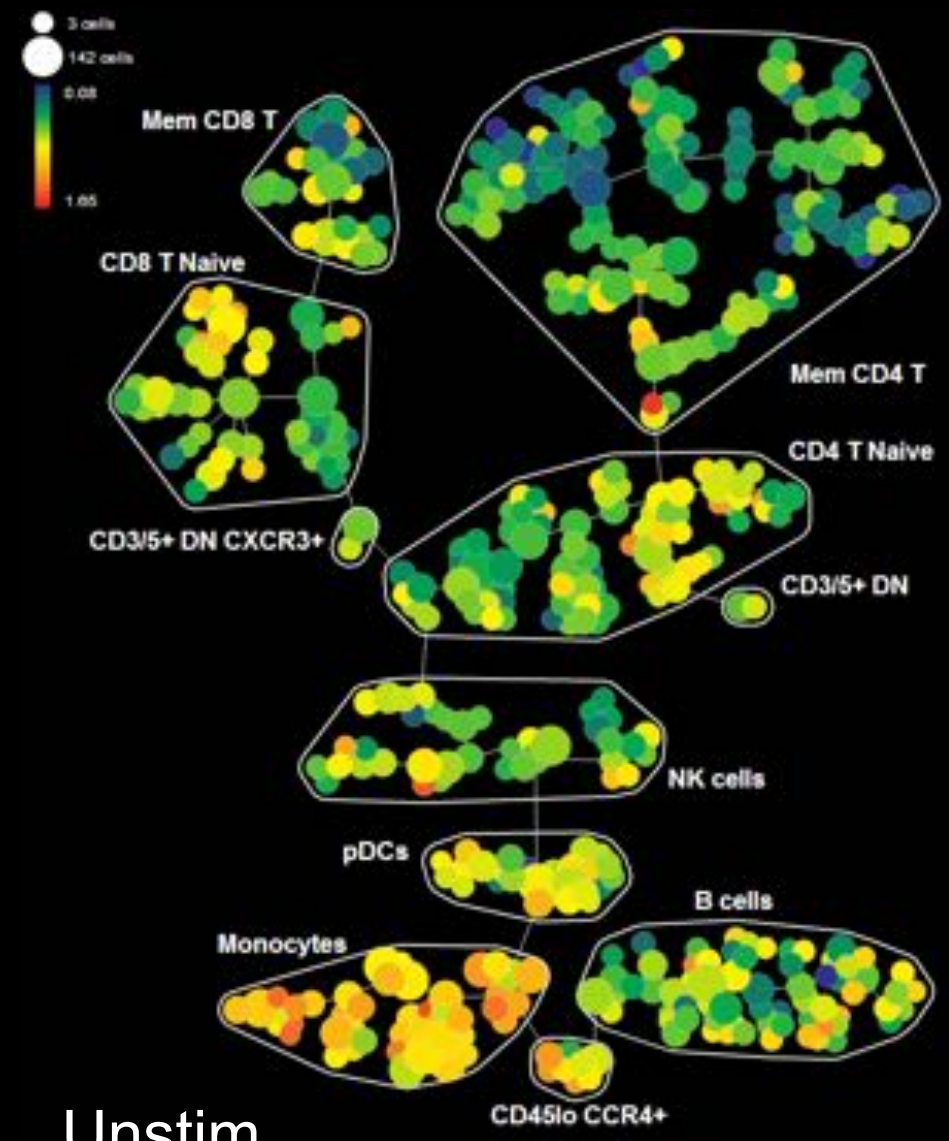
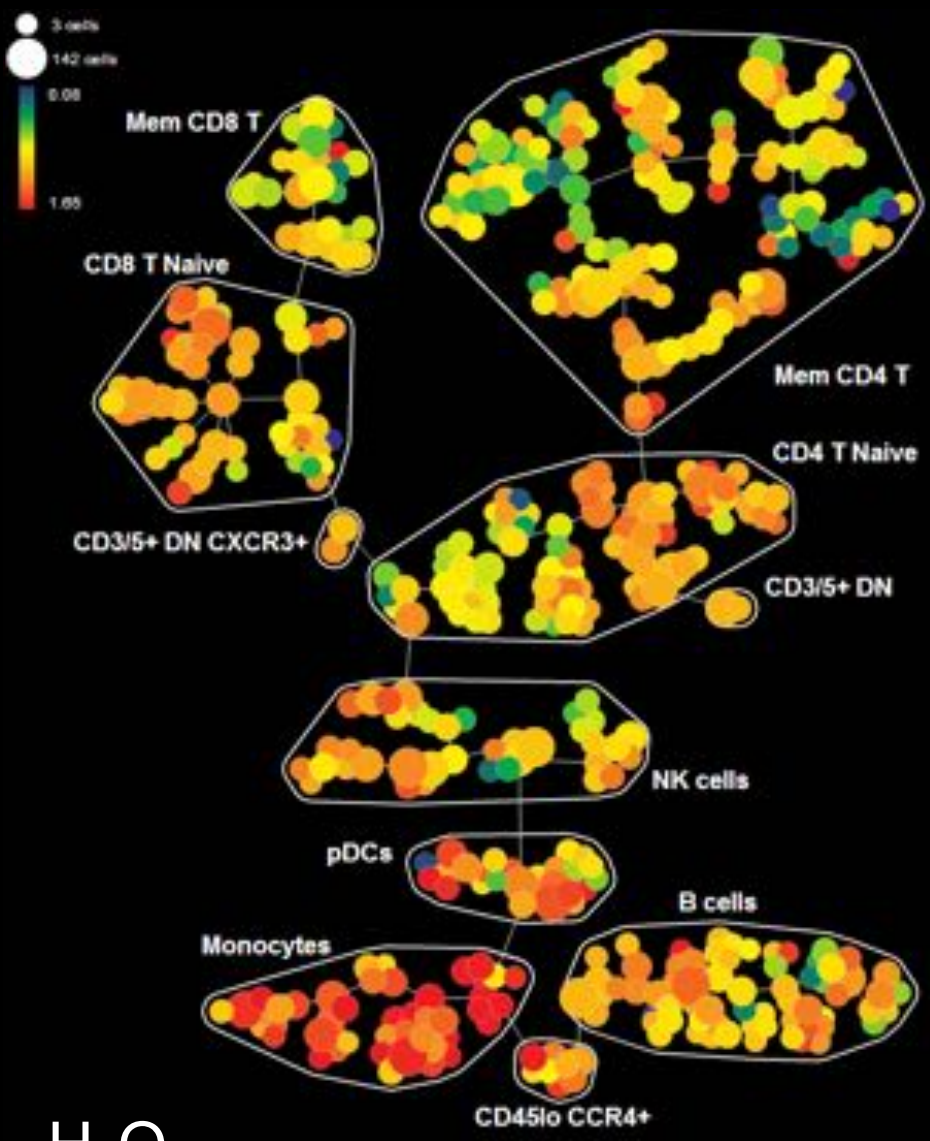
Mass Cytometry: p-ERK H_2O_2 Response



Unstim



H_2O_2

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

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