STANFORD
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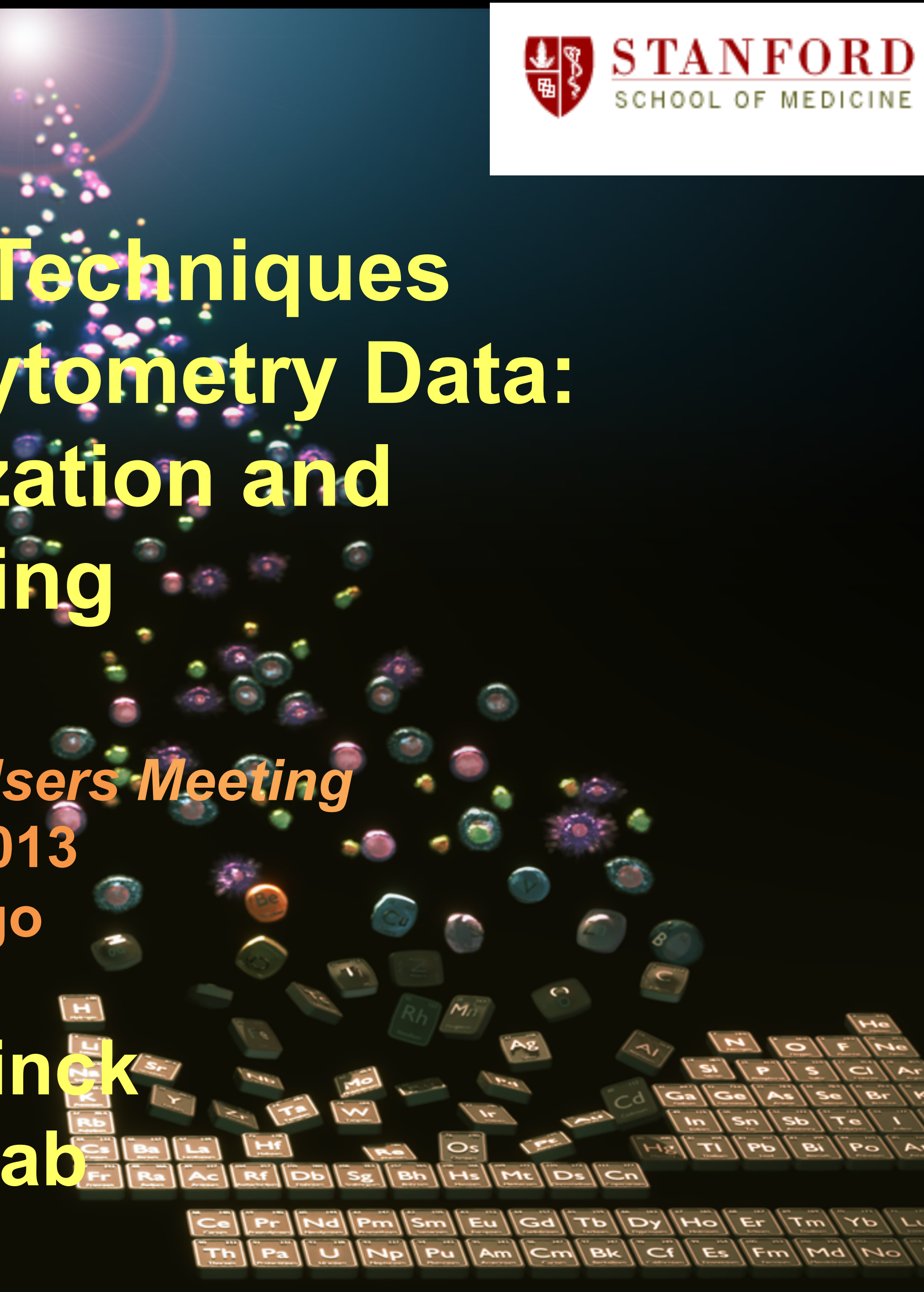


Computational Techniques Applied to Mass Cytometry Data: Bead Normalization and Barcoding

2nd Annual CyTOF Users Meeting
May 23, 2013
San Diego

Rachel Finck
Nolan Lab

Outline

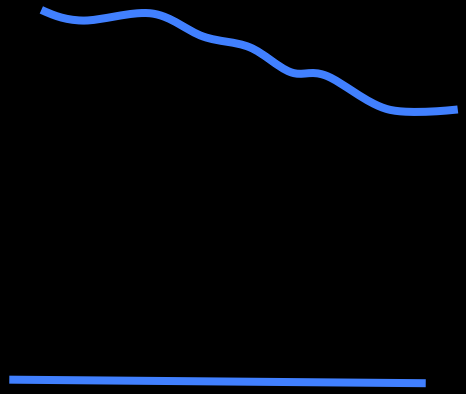
- Normalization of Mass Cytometry Data with Bead Standards
    - Review of normalization algorithm
    - Demonstration of normalization software with updated beads
- Mass-Tag Cellular Barcoding
    - Single-cell deconvolution of barcoded populations
    - “Doublet-free” barcoding scheme

Normalization of Mass Cytometry Data

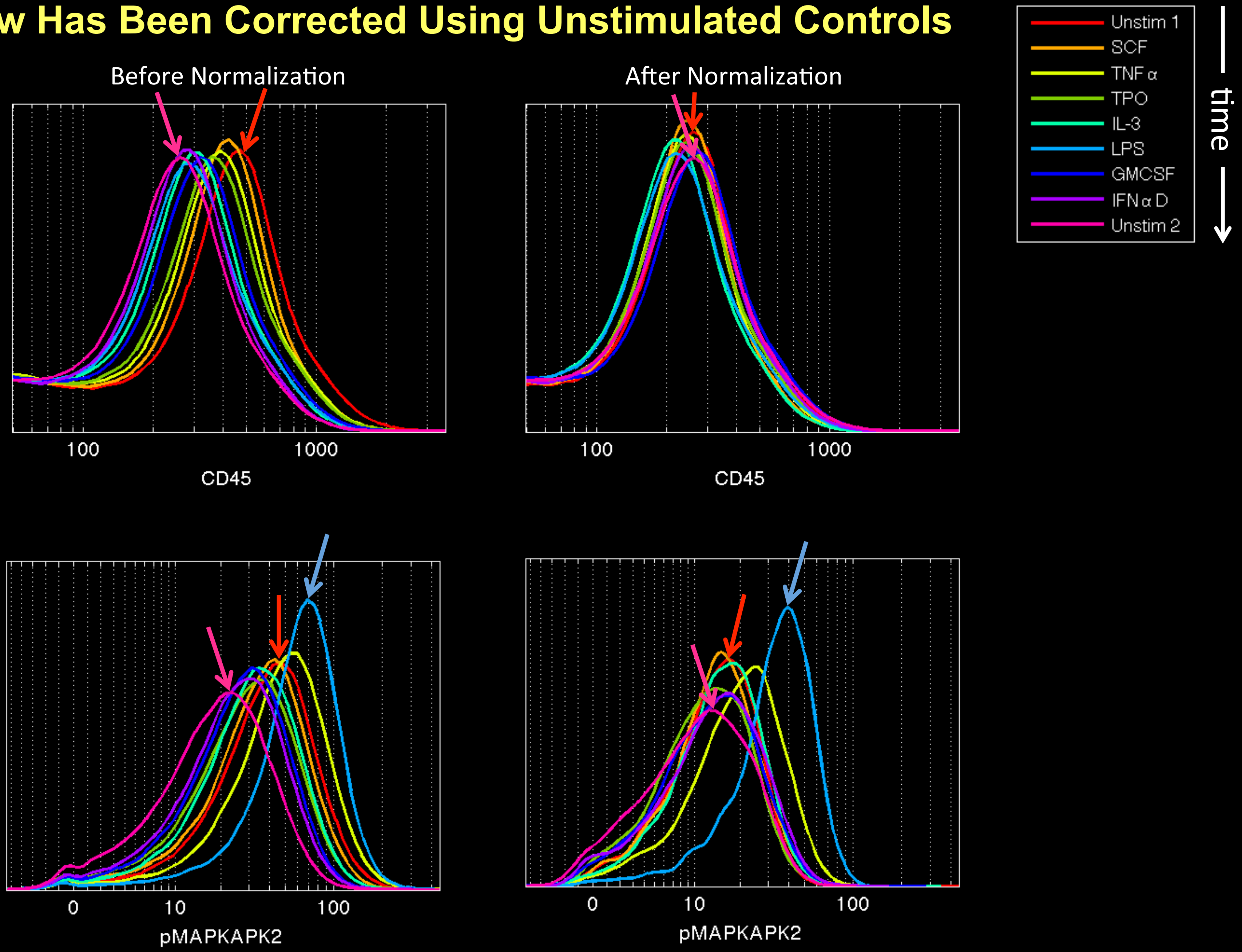
Goal: Reliably compare mass cytometry data across patients, conditions, tissues, etc.

Problem: Drifts in mass cytometry instrument sensitivity over time due to cellular debris, fluctuations in plasma temperature, and calibrations.

Solution: Normalization using internal bead standards measured concurrently with cell samples.



Signal Drift Across Multiple Conditions Measured in Human Bone Marrow Has Been Corrected Using Unstimulated Controls



Before Normalization

After Normalization

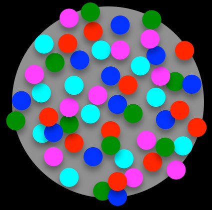
Bendall, Simonds, et al., *Science*, May 2011

Internal Bead Standards Generalize Normalization Capabilities

Desirable Features for Internal Standard

- Independent of biological sample
- Not subject to variable staining
- Can be directly mixed with sample to monitor instrument sensitivity at all times

Metal-embedded polystyrene bead

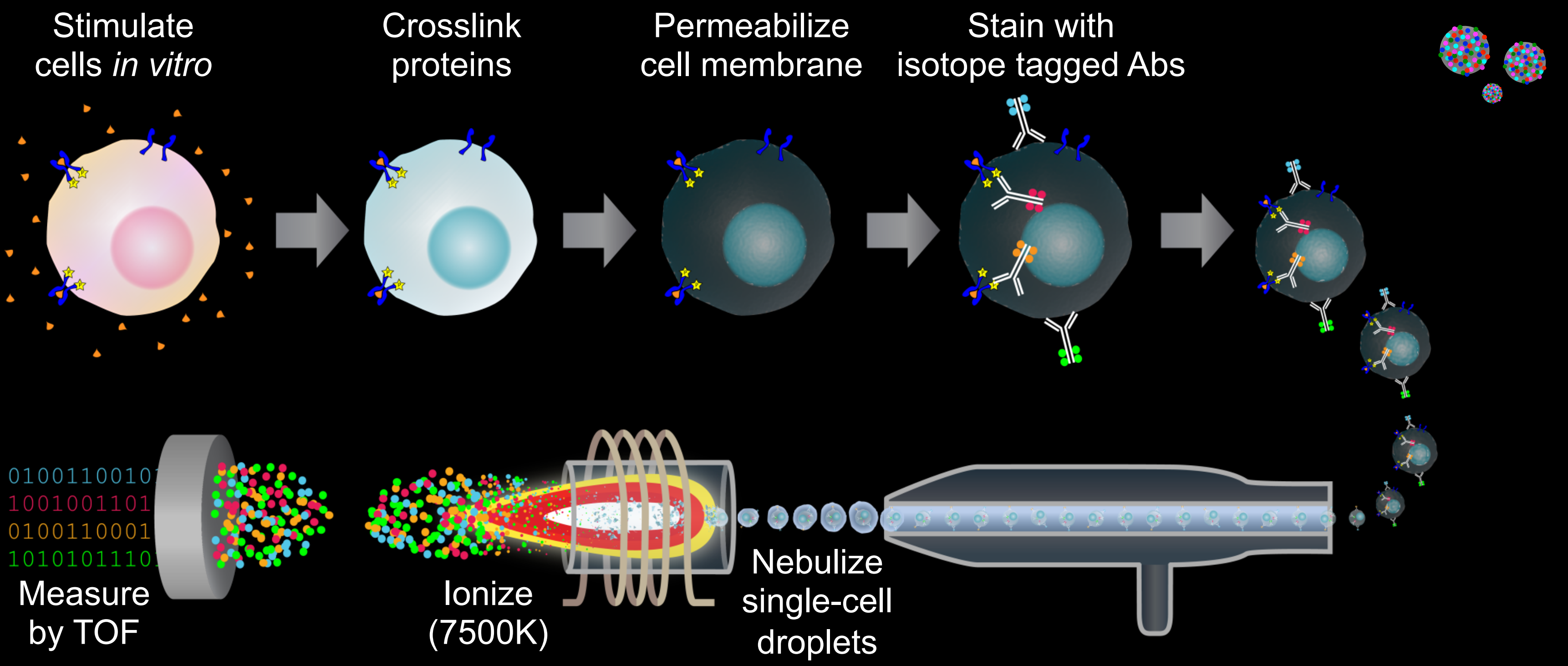


● La139		● Ce140
● Pr141		● Eu151
● Tb159	or	● Eu153
● Tm169		● Ho165
● Lu175		● Lu175

- Span instrument mass and dynamic ranges
- Unique 5-element signature allows antibodies to be tagged with bead elements

beads were gift from DVS Sciences

Beads can be spiked into standard mass cytometry protocol



Stimulate cells *in vitro*

Crosslink proteins

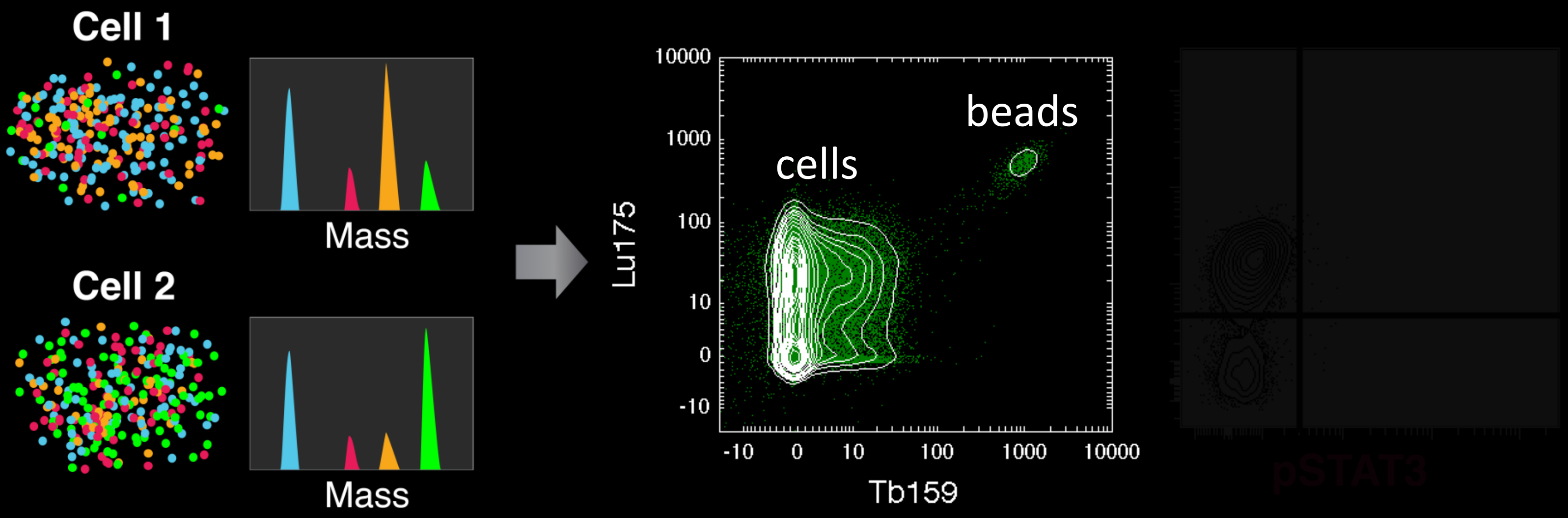
Permeabilize cell membrane

Stain with isotope tagged Abs

Nebulize single-cell droplets

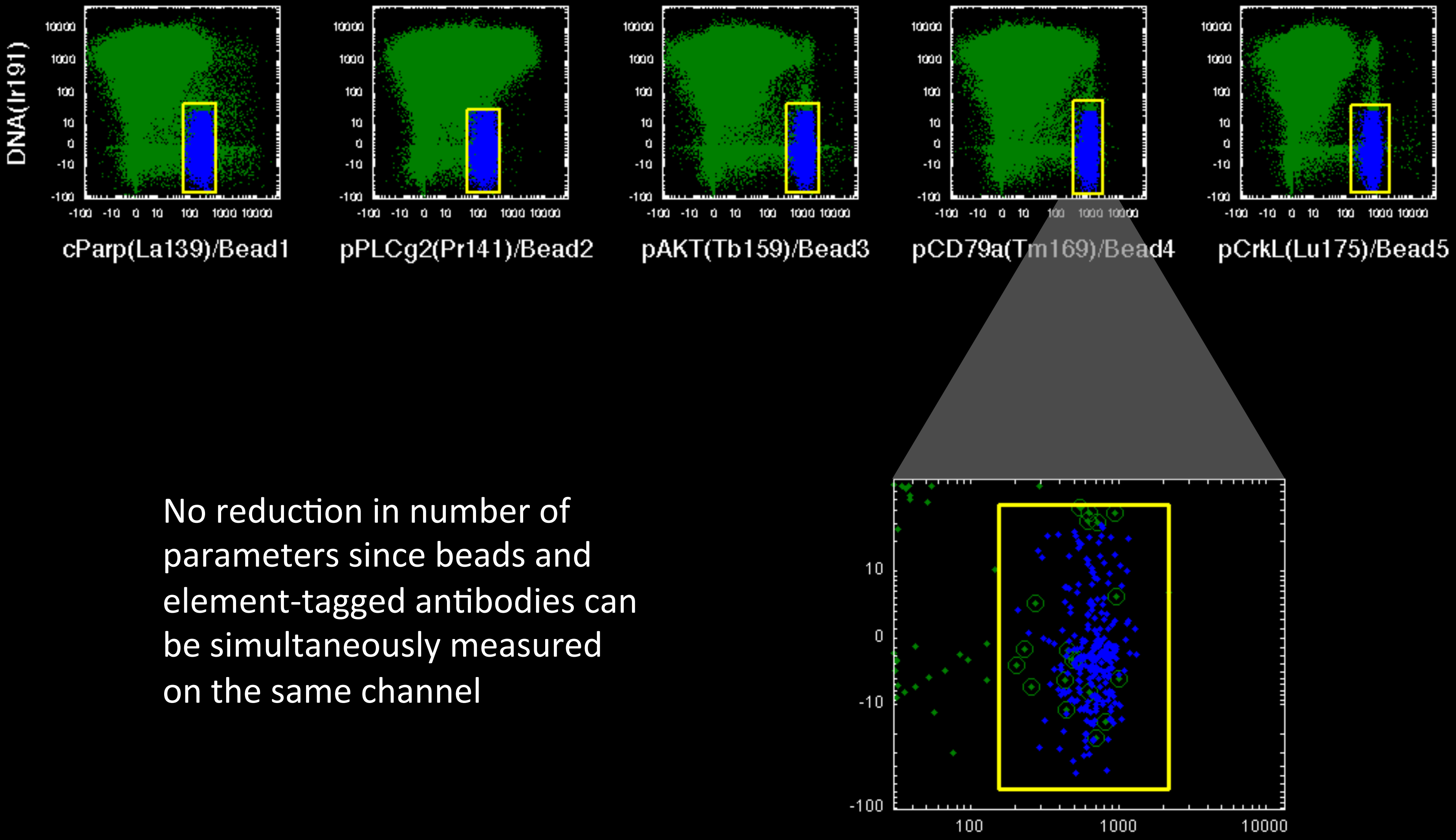
Ionize (7500K)

Measure by TOF



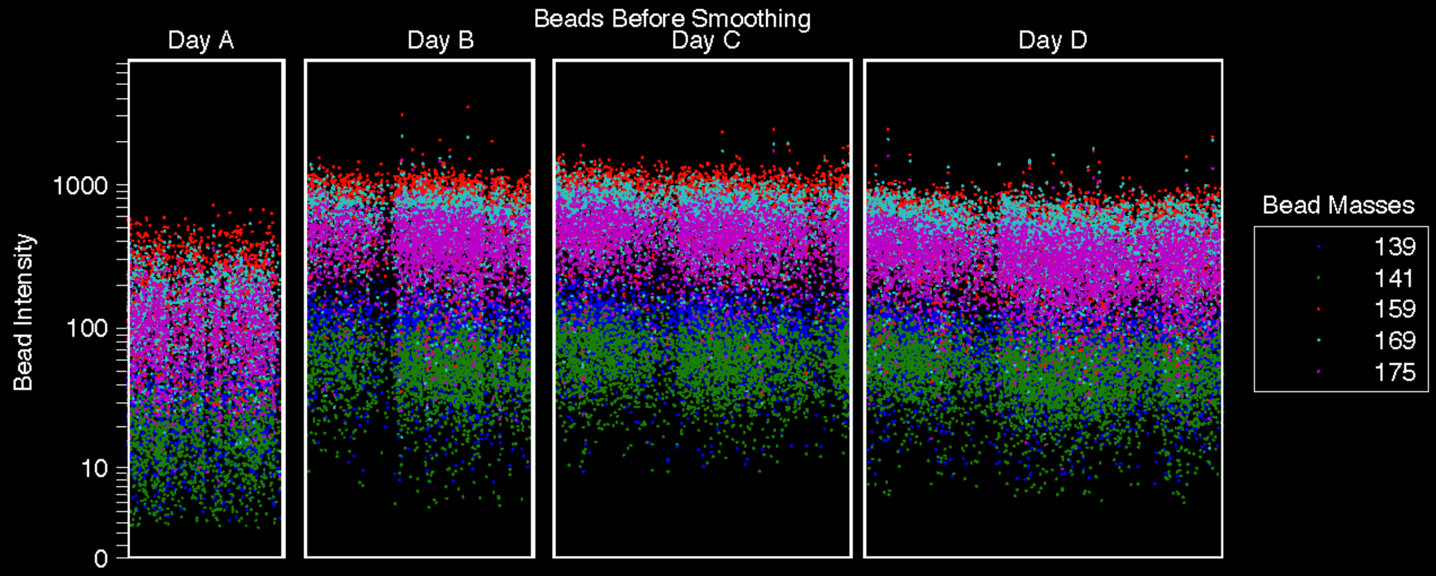
E. Simonds

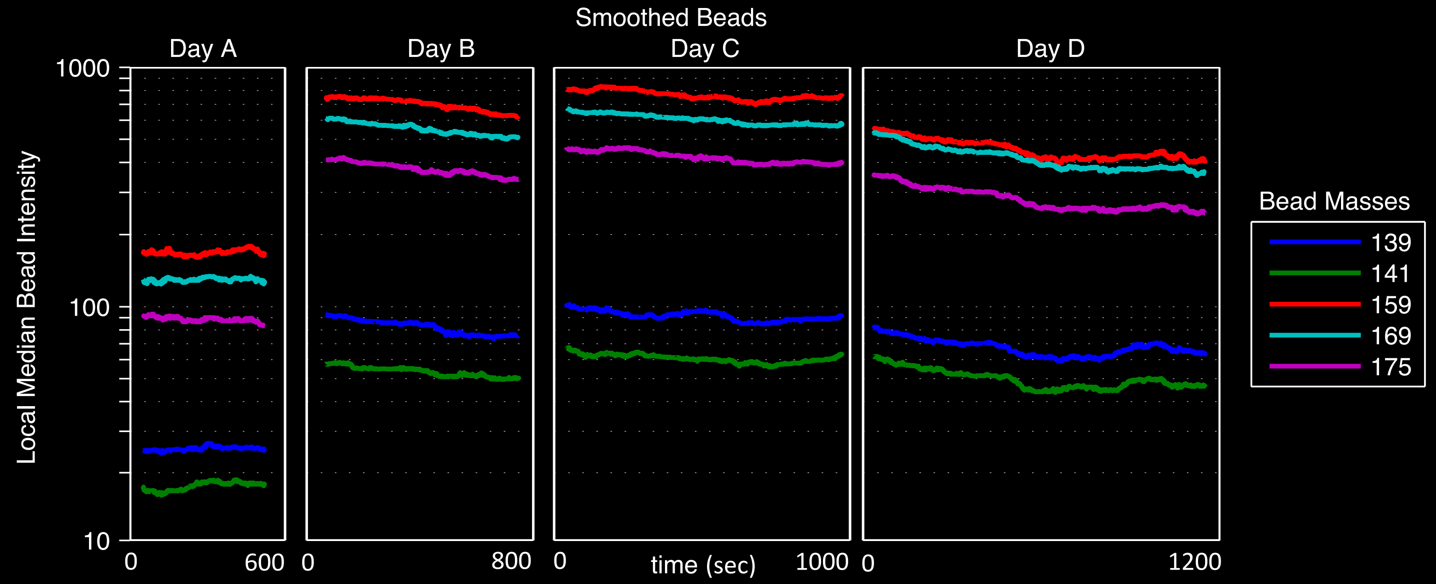
Beads are Identifiable from Cells Even When Measured on Overlapping Channels



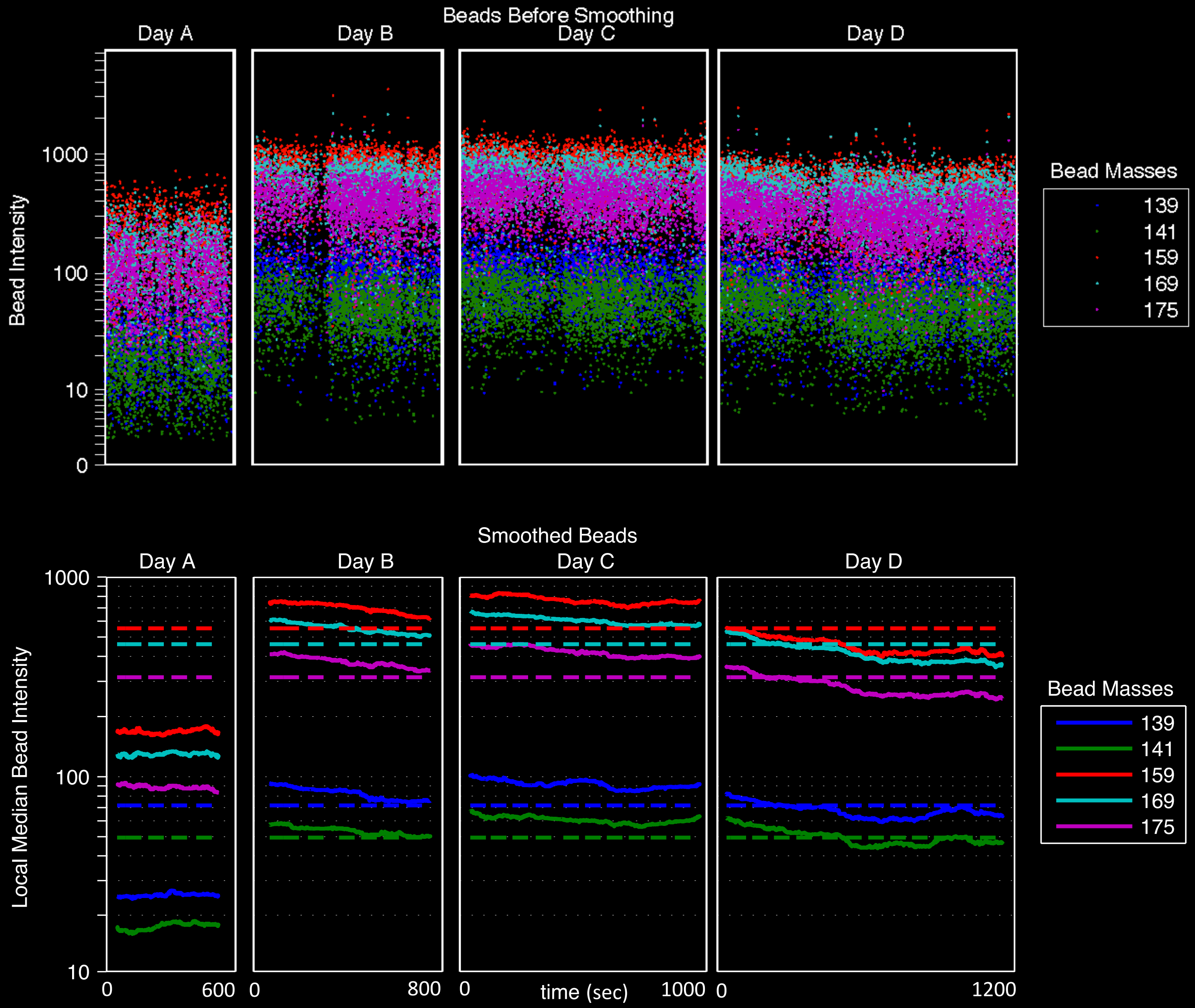
No reduction in number of parameters since beads and element-tagged antibodies can be simultaneously measured on the same channel

Bead Smoothing Removes Local Variance

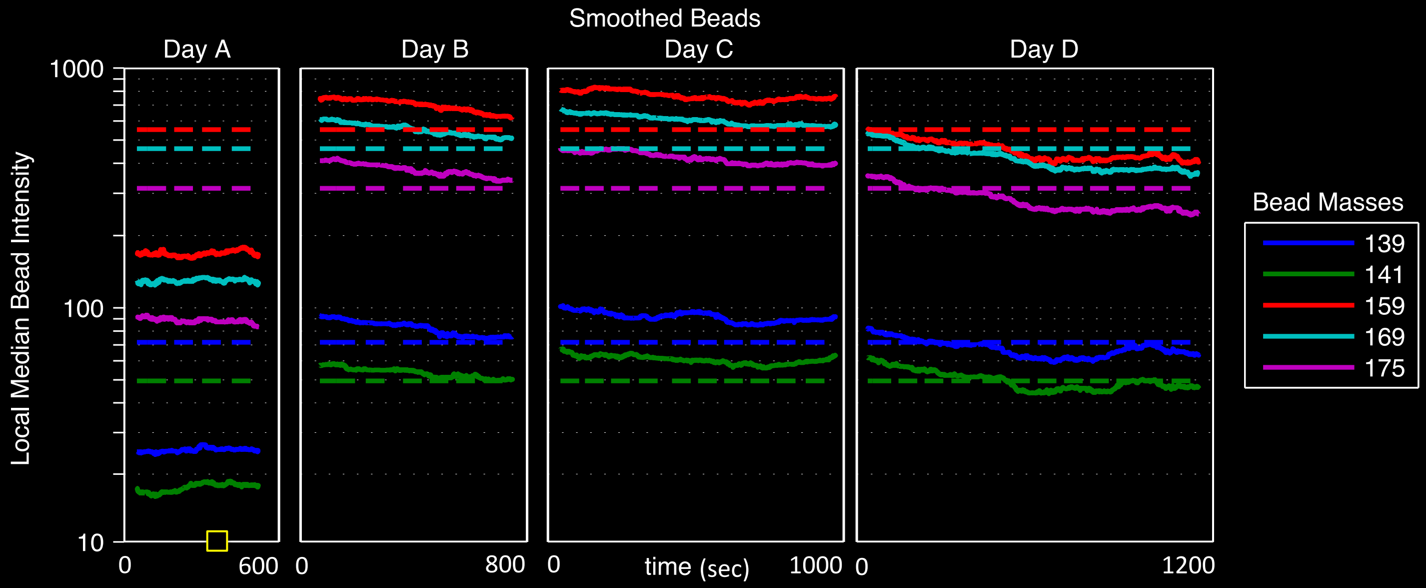

Beads Before Smoothing

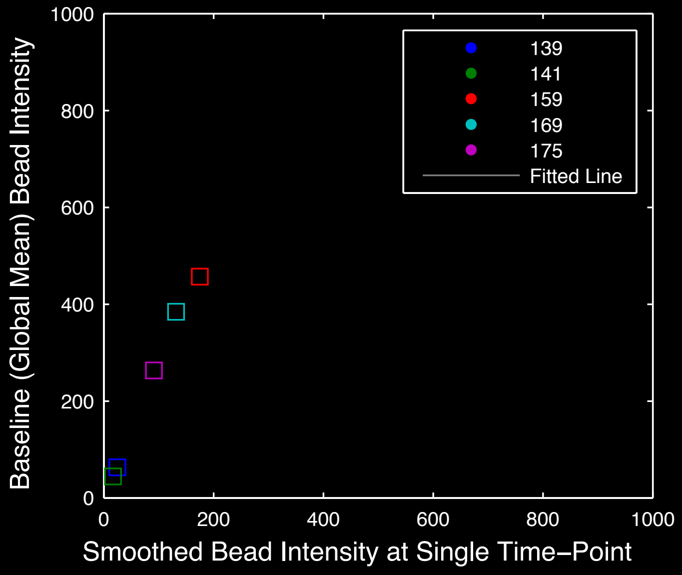

Smoothed Beads

Bead Smoothing Removes Local Variance

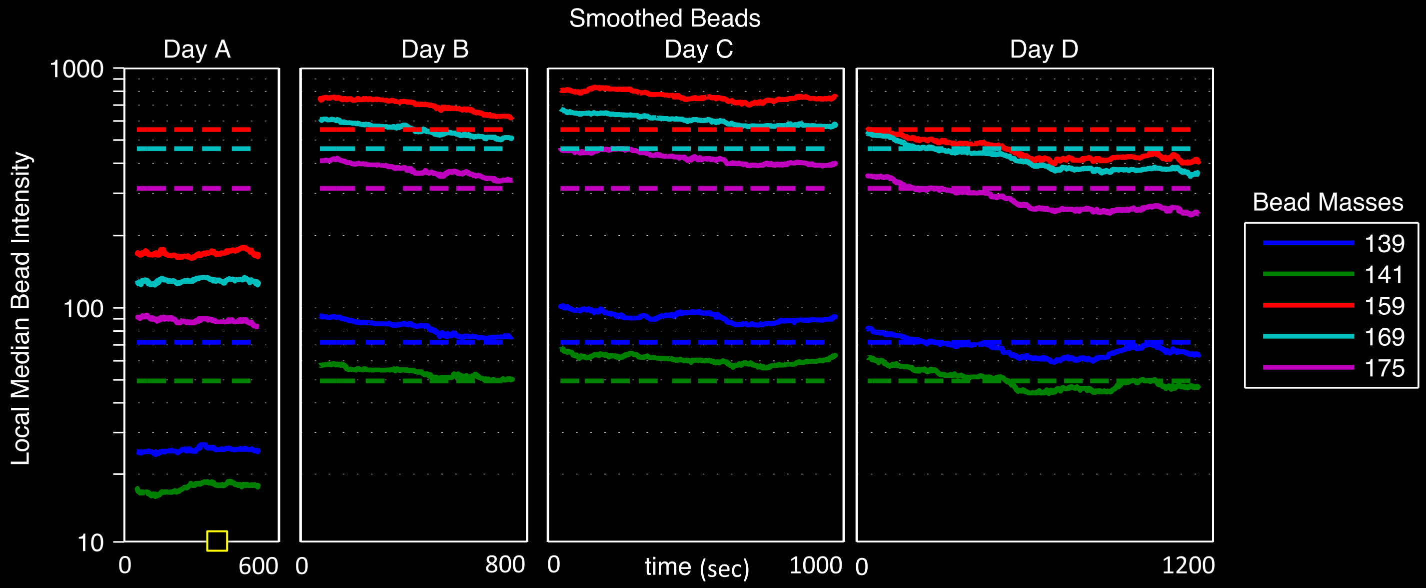


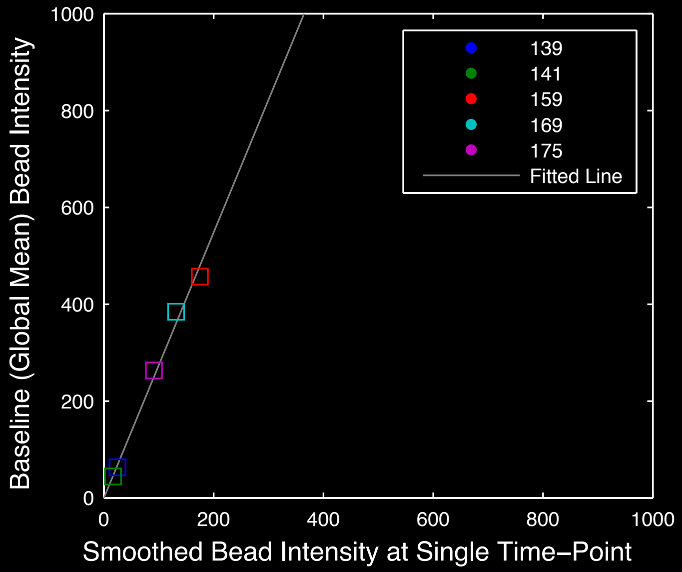
Fitted Slopes Define a Correction Function



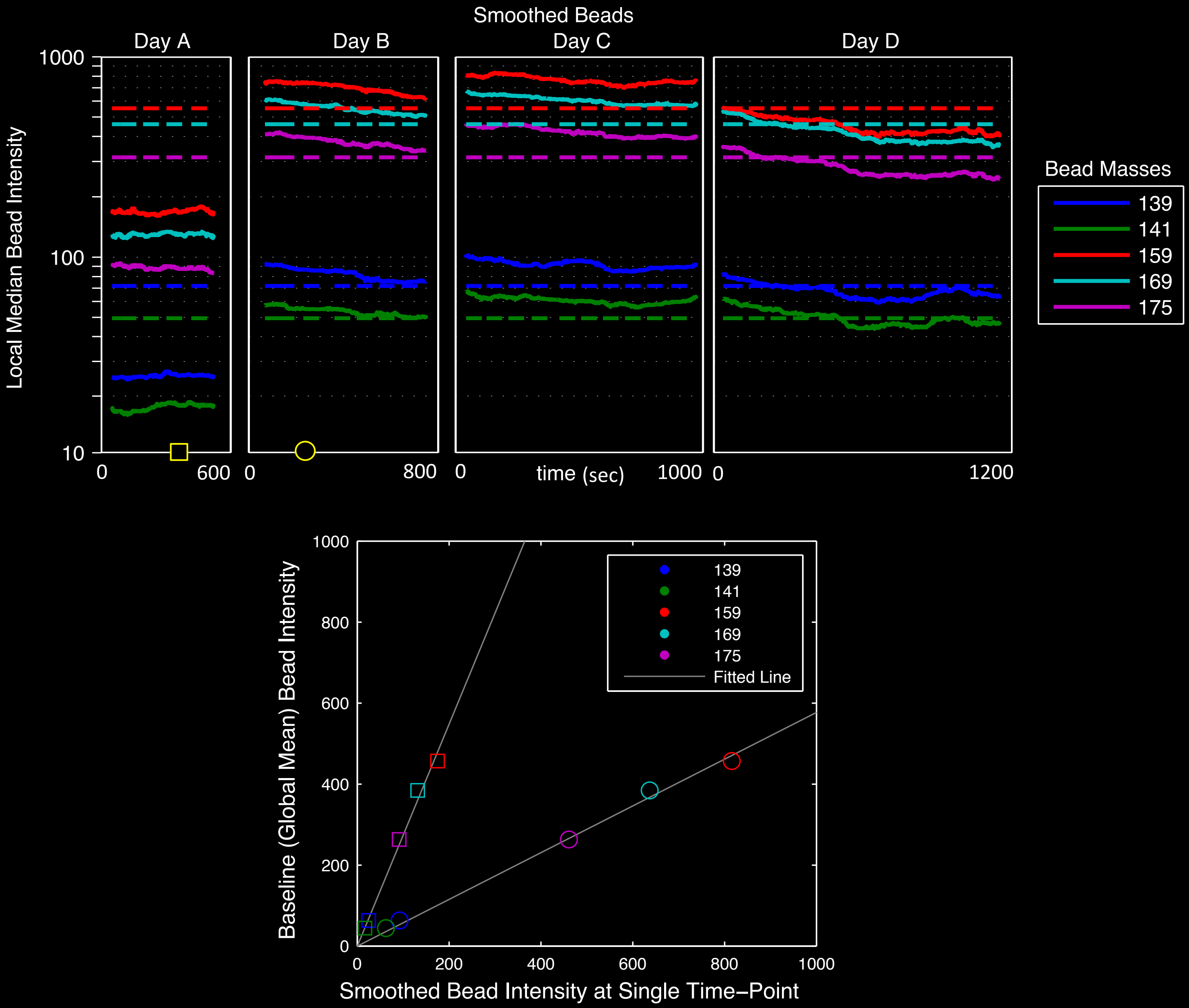


Fitted Slopes Define a Correction Function

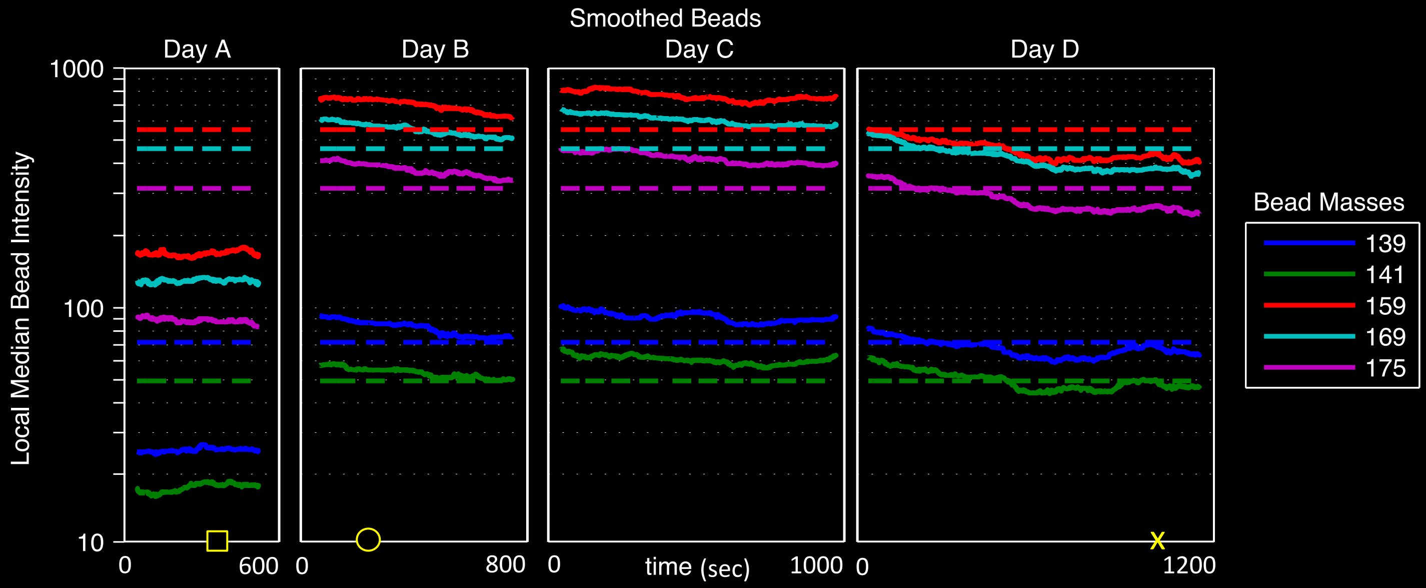


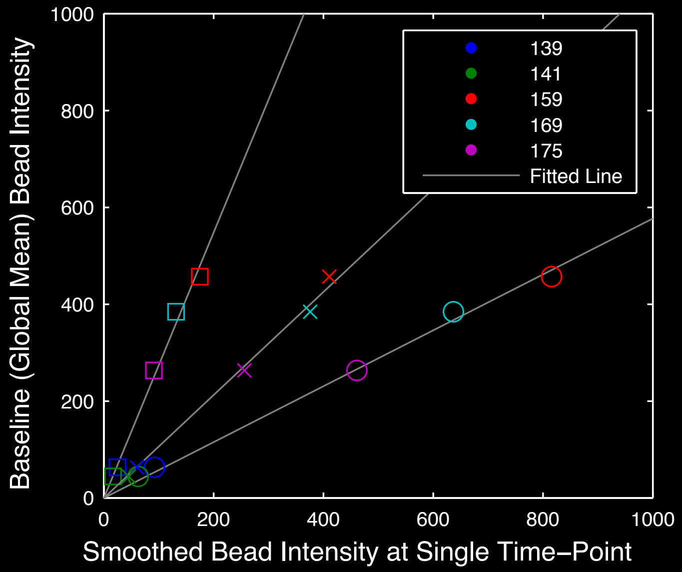


Fitted Slopes Define a Correction Function

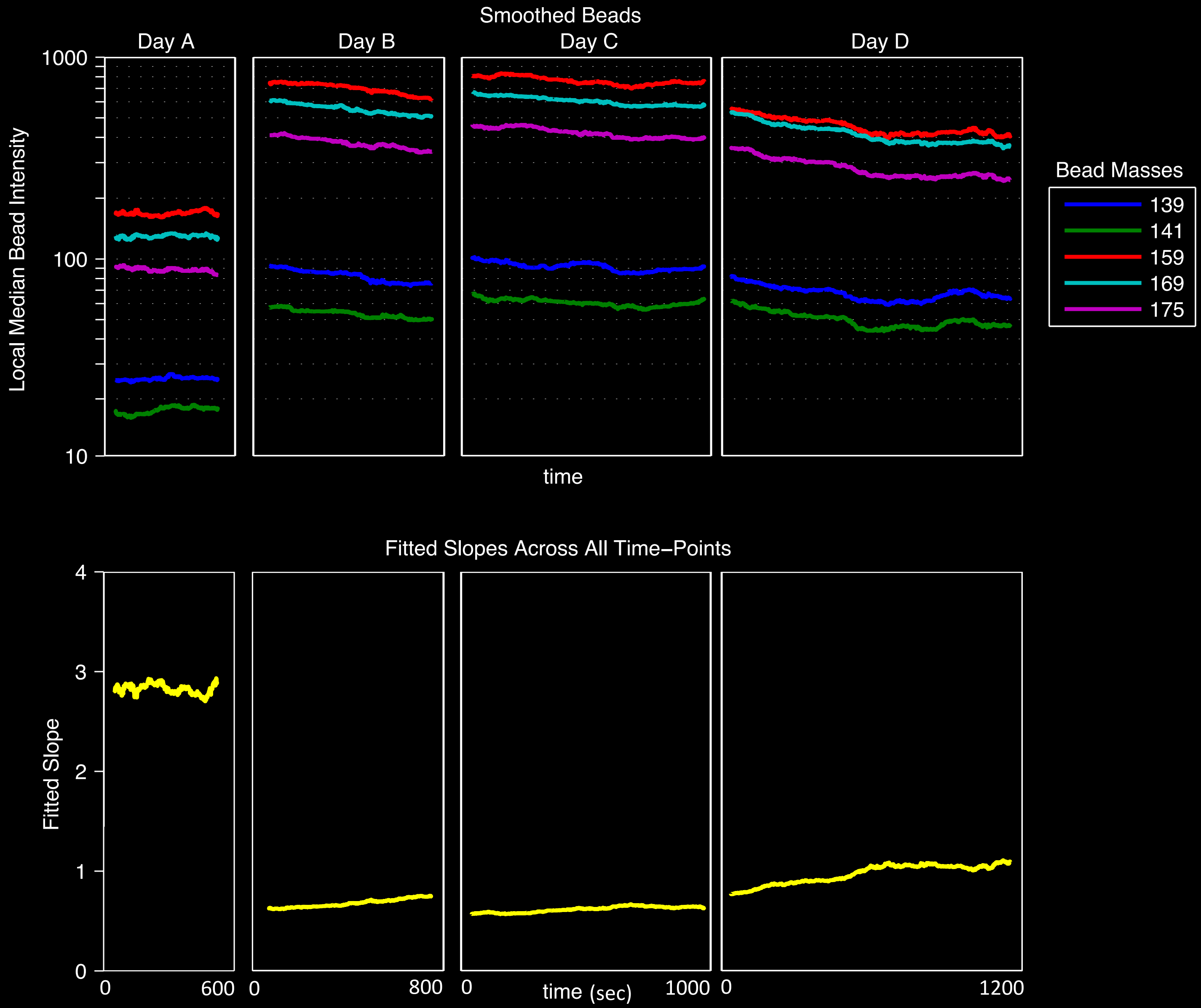


Fitted Slopes Define a Correction Function



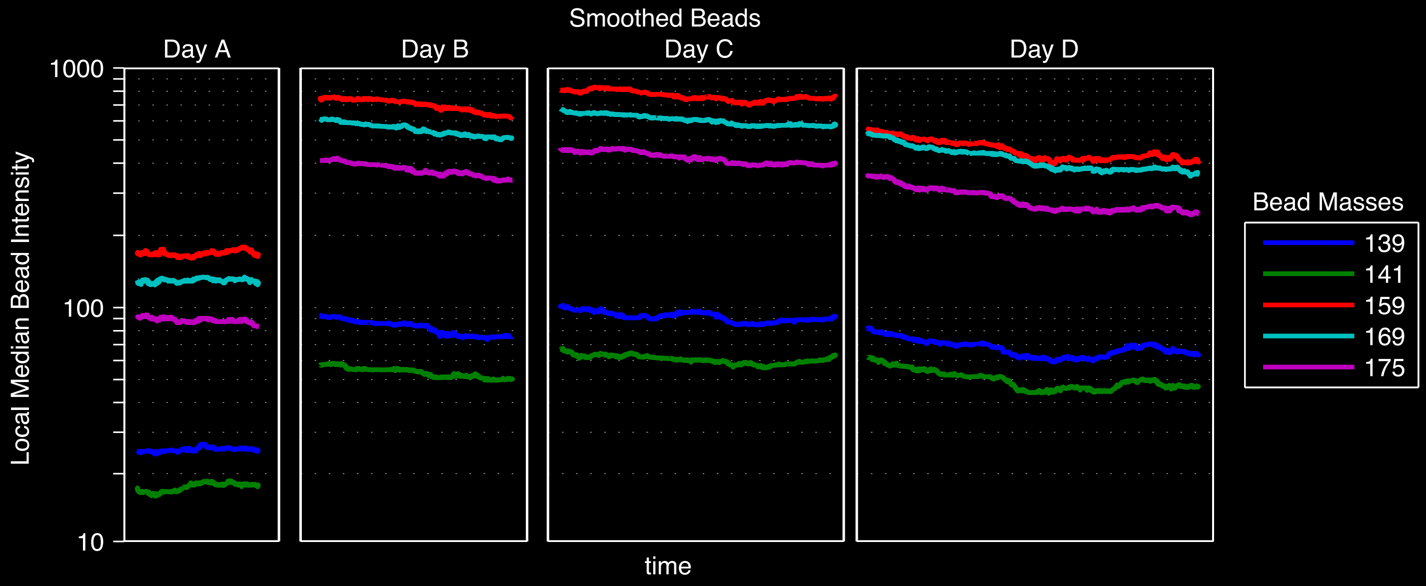


Fitted Slopes Define a Correction Function

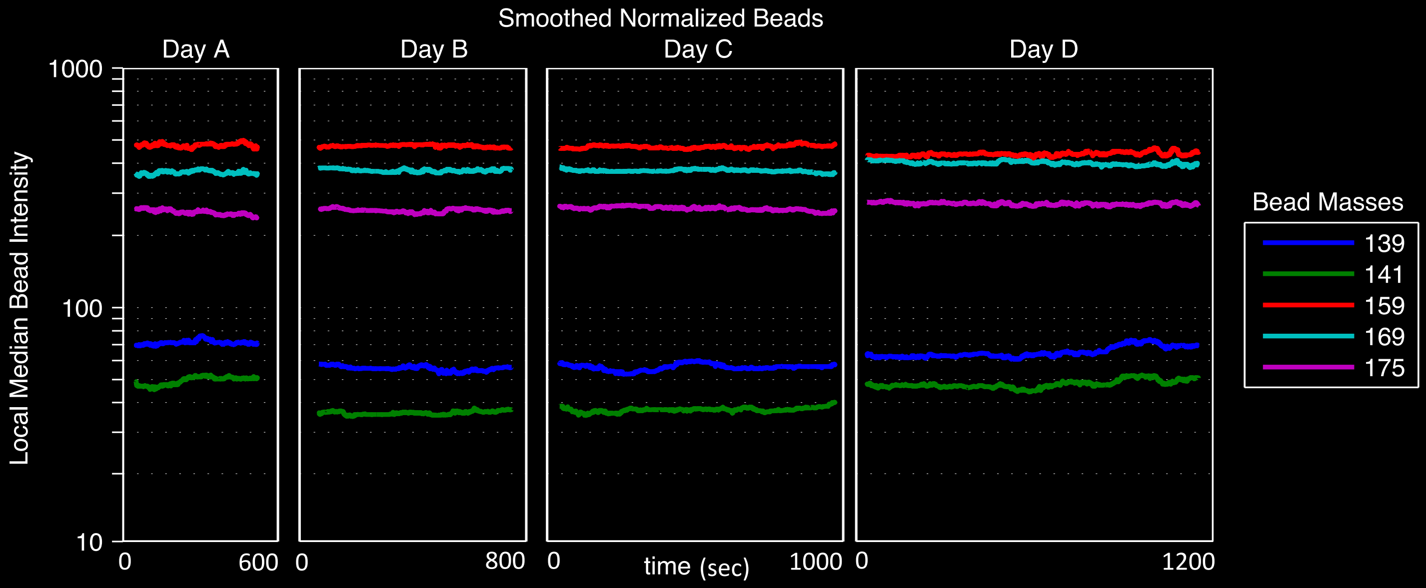


Normalization Reduces Bead Intensity Variation

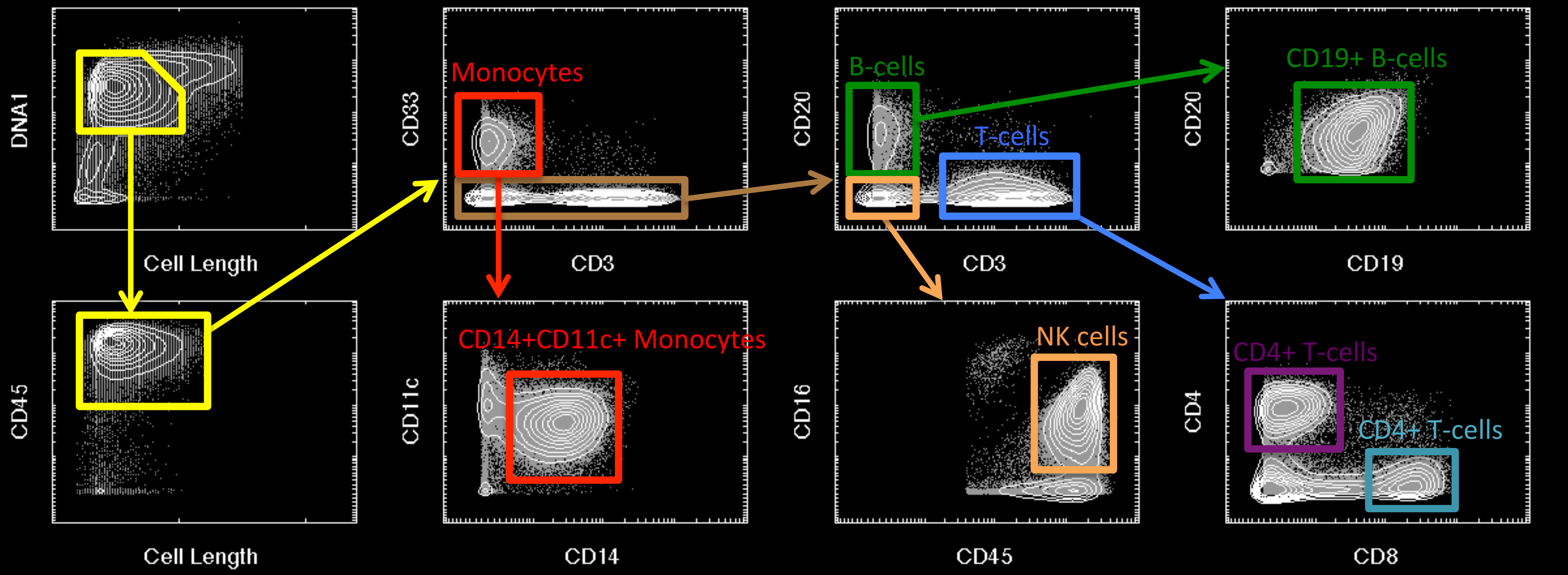
4.9-fold
mean range

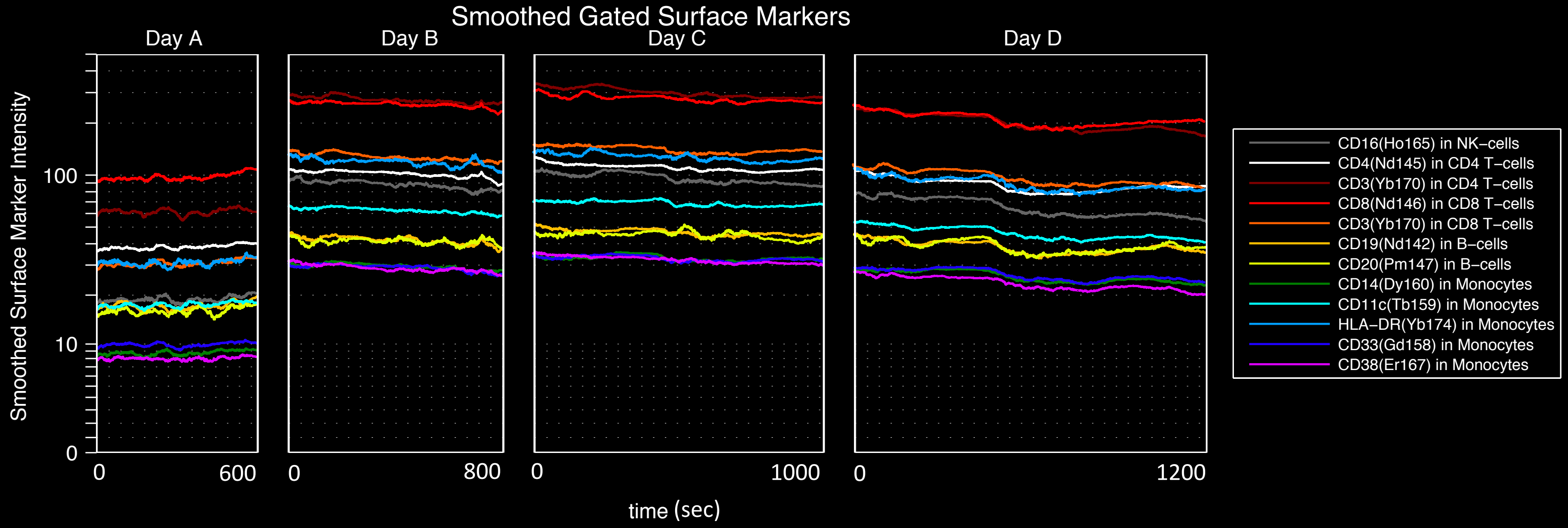


1.3-fold
mean range

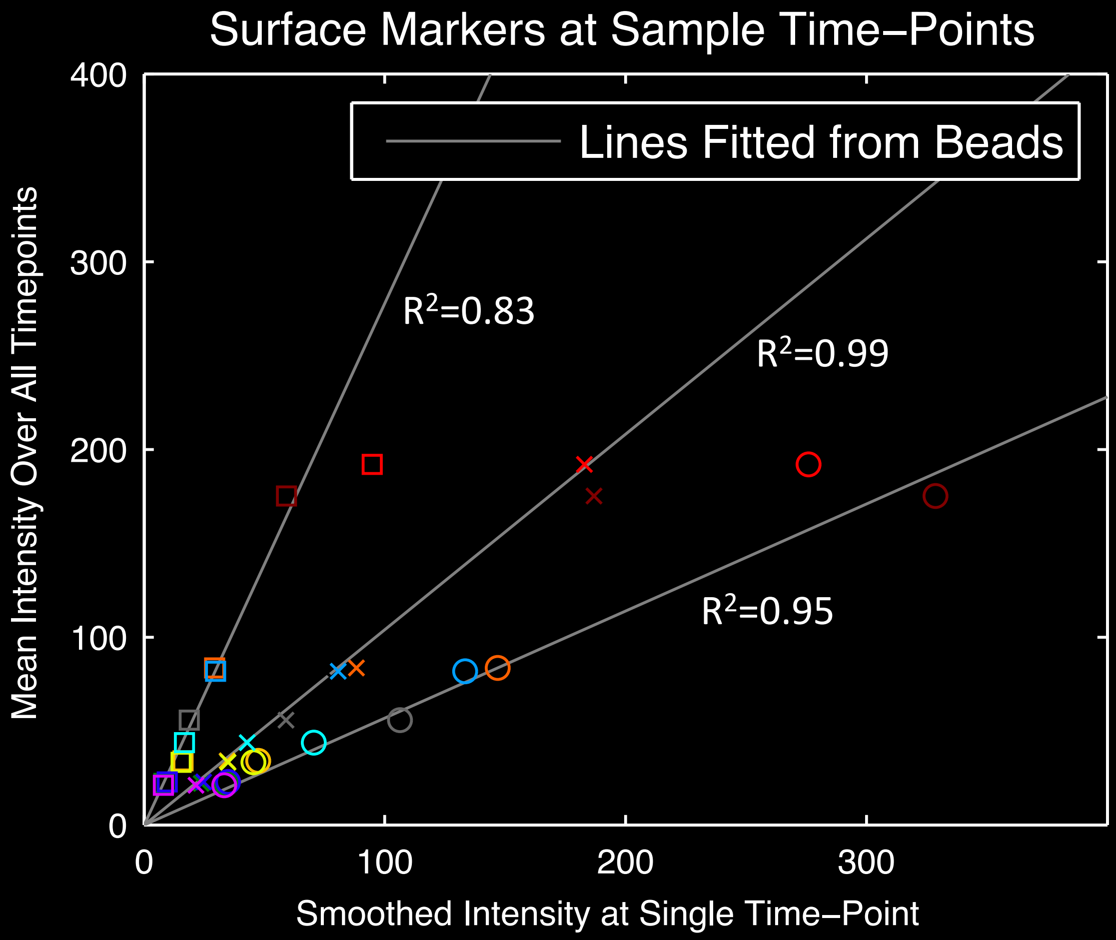


Gated PBMC Populations Validate Bead-Based Normalization

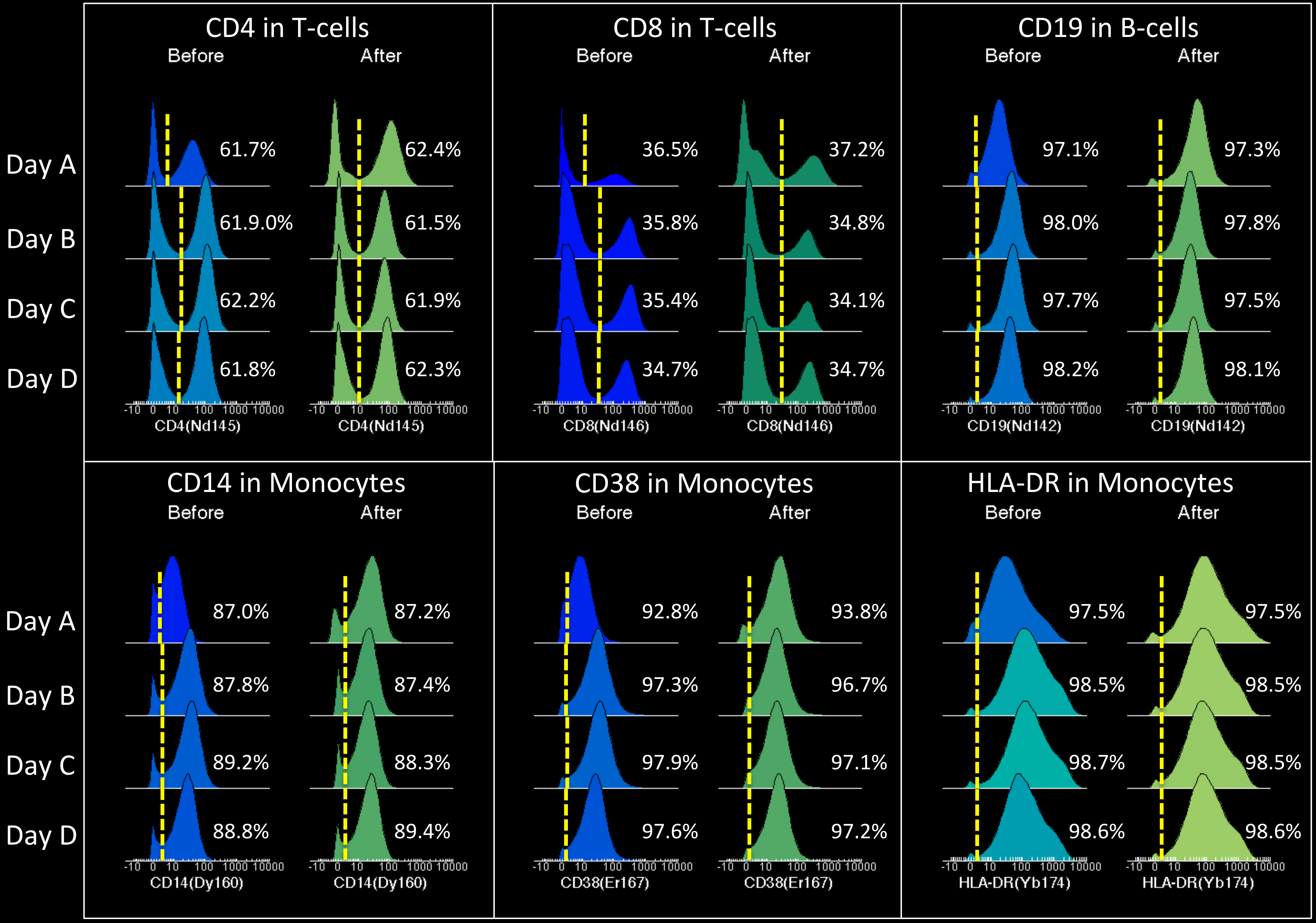



Smoothed Gated Surface Markers

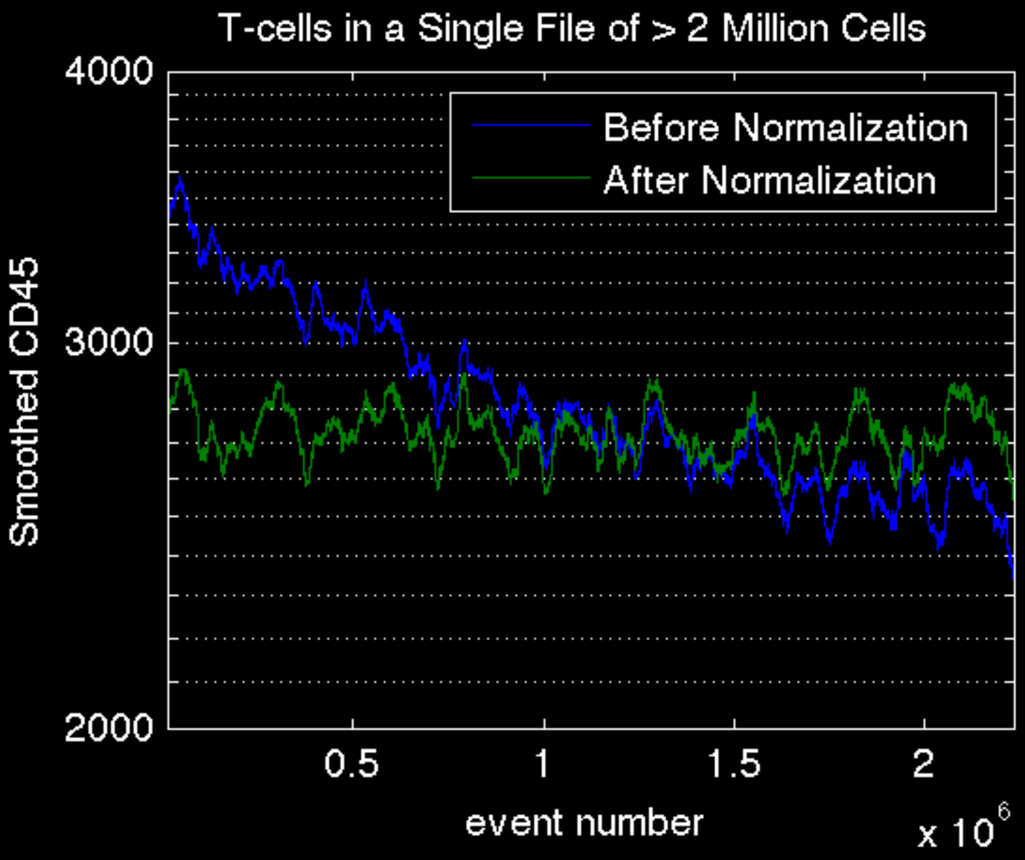
Surface Marker Intensities Agree With Bead Slopes



Normalization Aligns Surface Marker Histograms



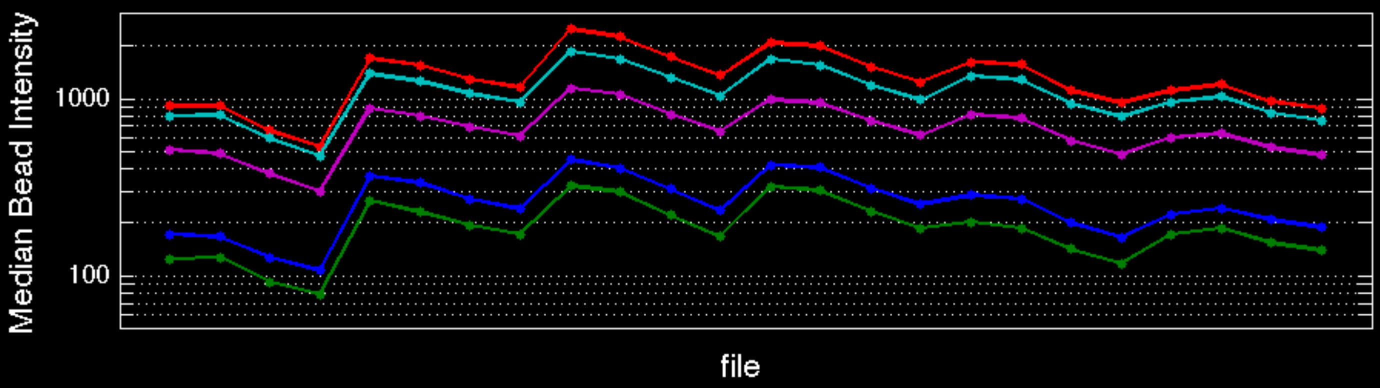
Internal Bead-based Normalization Corrects Intra-File Decay



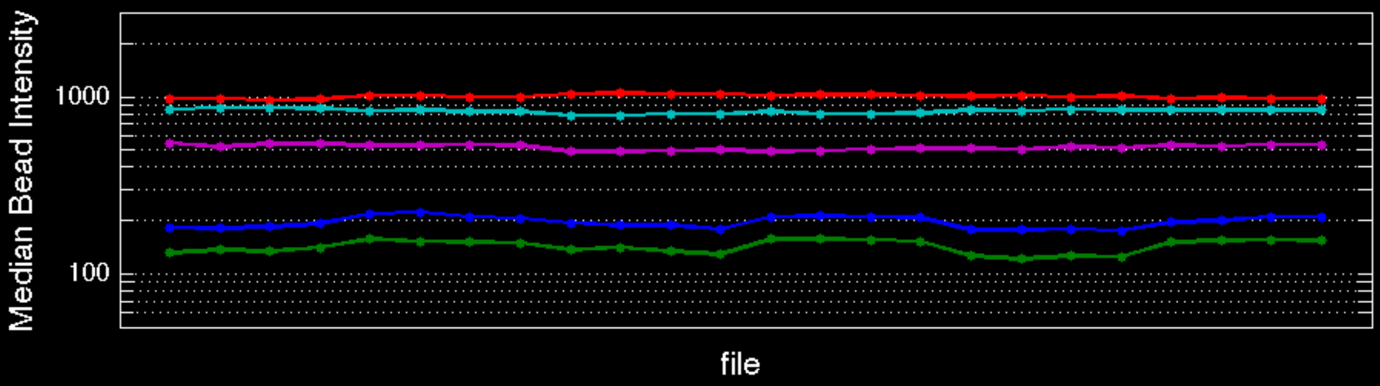
98% of initial intensity

73% of initial intensity

Normalization Reduced Non-Biological Variation in Primary Pediatric ALL Bone Marrow Samples

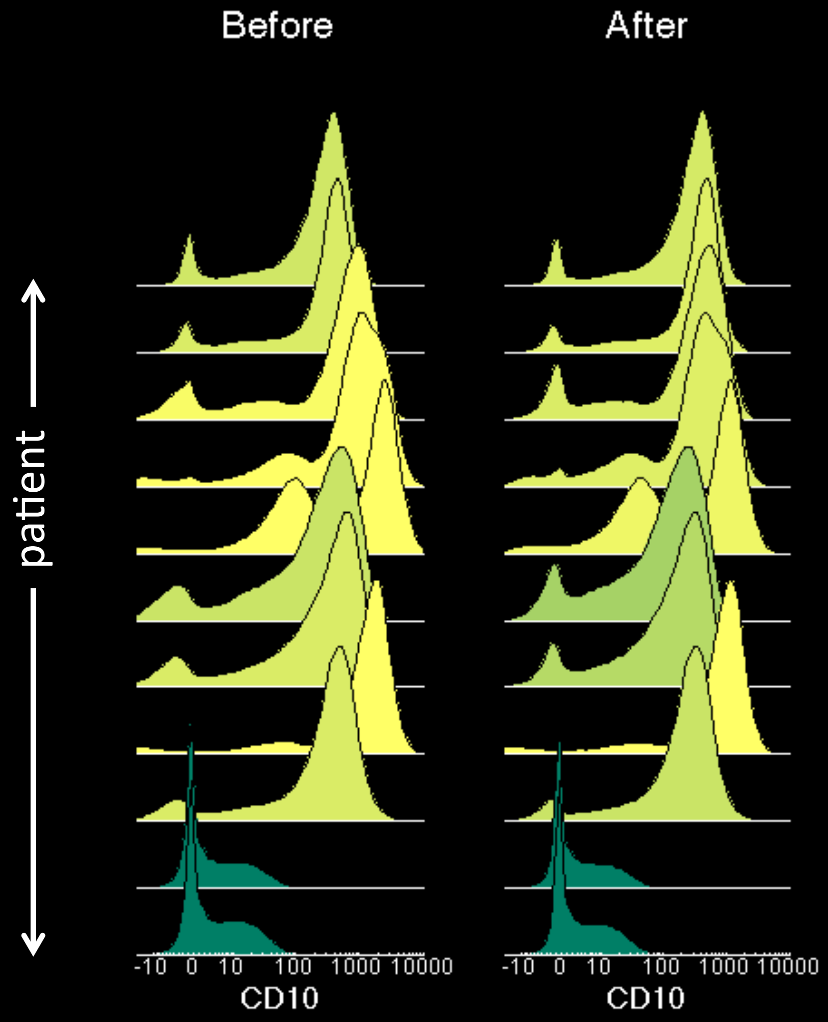

Before Normalization

4.1-fold mean range

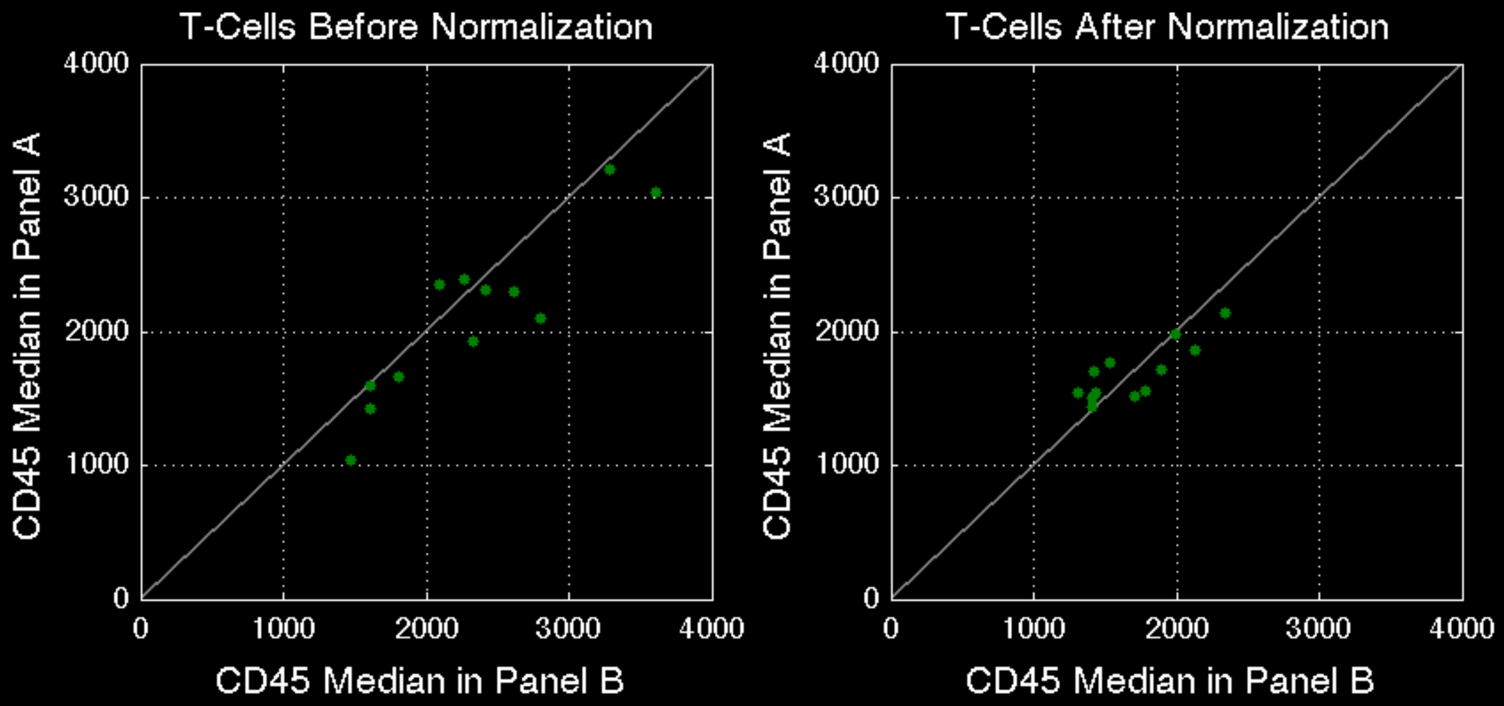

After Normalization

1.2-fold mean range

Normalization Reduced Non-Biological Variation in Primary Pediatric ALL Bone Marrow Samples

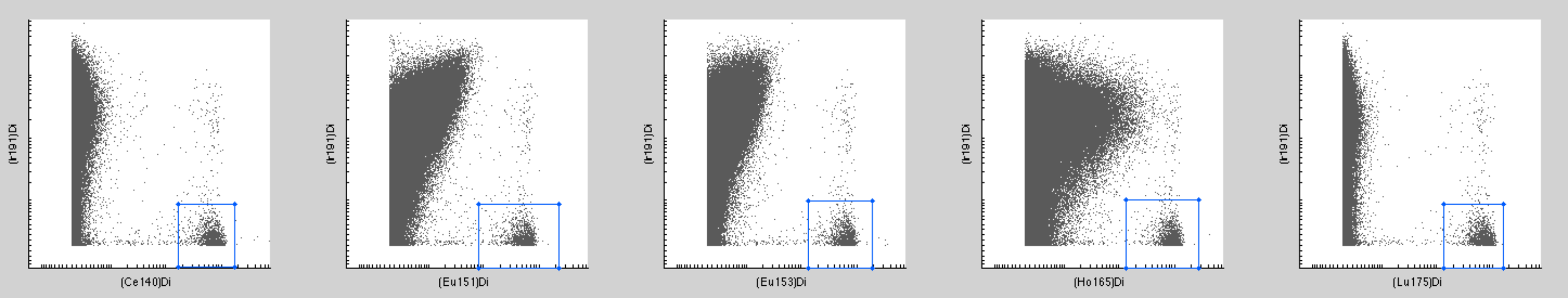


Normalization Reduced Non-Biological Variation in Primary Pediatric ALL Bone Marrow Samples

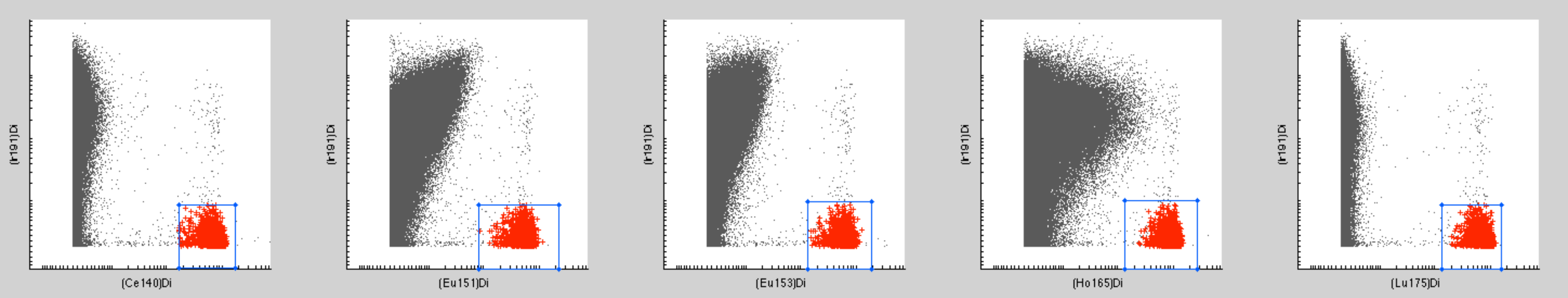


Normalization Software Example Using New Beads

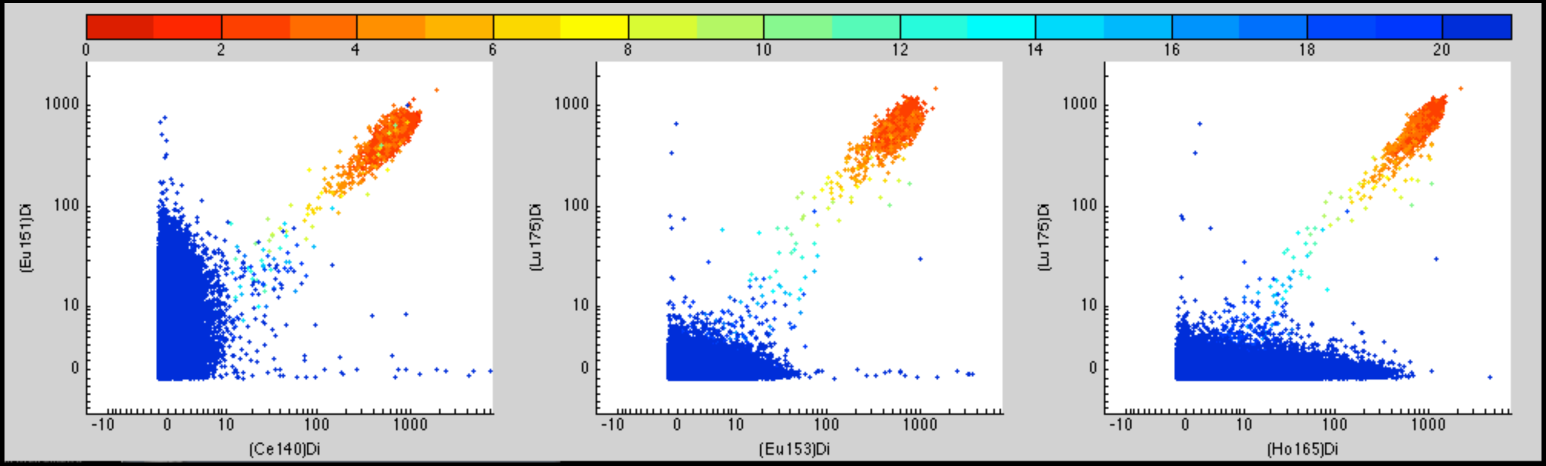
1. Adjust gates.



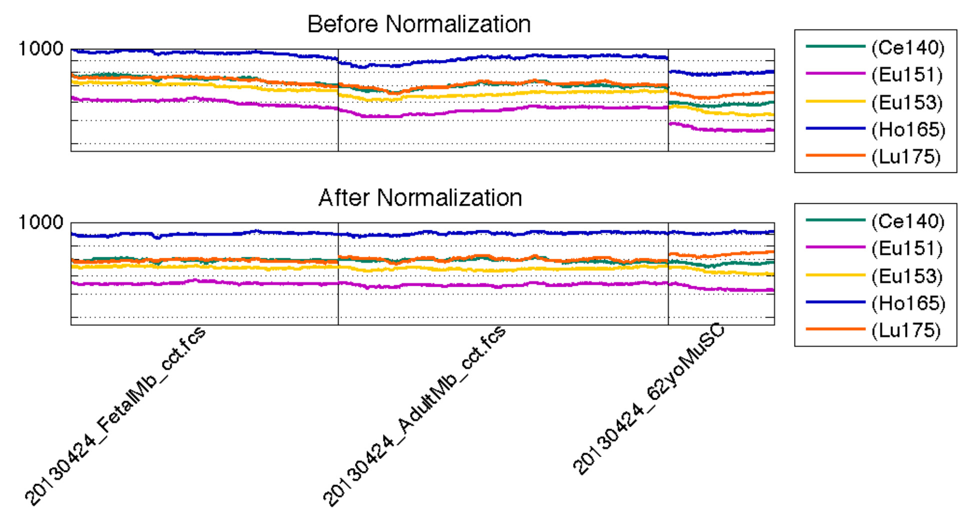
2. Approve gates.

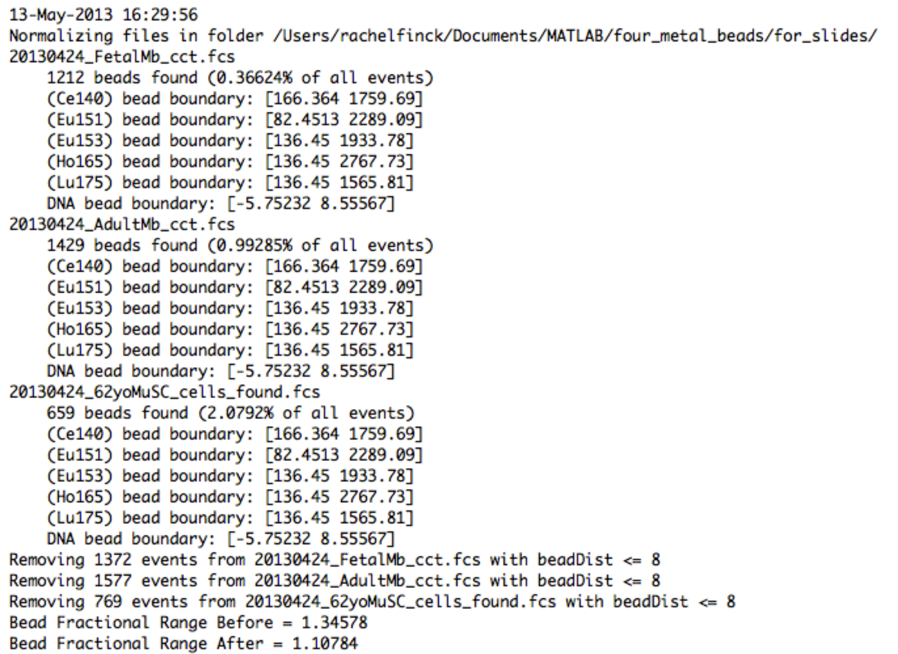


3. Set bead removal threshold.



Normalization Software Reports Bead Intensity Ranges





```
13-May-2013 16:29:56
Normalizing files in folder /Users/rachelfinck/Documents/MATLAB/four_metal_beads/for_slides/
20130424_FetalMb_cct.fcs
    1212 beads found (0.36624% of all events)
    (Ce140) bead boundary: [166.364 1759.69]
    (Eu151) bead boundary: [82.4513 2289.09]
    (Eu153) bead boundary: [136.45 1933.78]
    (Ho165) bead boundary: [136.45 2767.73]
    (Lu175) bead boundary: [136.45 1565.81]
    DNA bead boundary: [-5.75232 8.55567]
20130424_AdultMb_cct.fcs
    1429 beads found (0.99285% of all events)
    (Ce140) bead boundary: [166.364 1759.69]
    (Eu151) bead boundary: [82.4513 2289.09]
    (Eu153) bead boundary: [136.45 1933.78]
    (Ho165) bead boundary: [136.45 2767.73]
    (Lu175) bead boundary: [136.45 1565.81]
    DNA bead boundary: [-5.75232 8.55567]
20130424_62yoMuSC_cells_found.fcs
    659 beads found (2.0792% of all events)
    (Ce140) bead boundary: [166.364 1759.69]
    (Eu151) bead boundary: [82.4513 2289.09]
    (Eu153) bead boundary: [136.45 1933.78]
    (Ho165) bead boundary: [136.45 2767.73]
    (Lu175) bead boundary: [136.45 1565.81]
    DNA bead boundary: [-5.75232 8.55567]
Removing 1372 events from 20130424_FetalMb_cct.fcs with beadDist <= 8
Removing 1577 events from 20130424_AdultMb_cct.fcs with beadDist <= 8
Removing 769 events from 20130424_62yoMuSC_cells_found.fcs with beadDist <= 8
Bead Fractional Range Before = 1.34578
Bead Fractional Range After = 1.10784
```

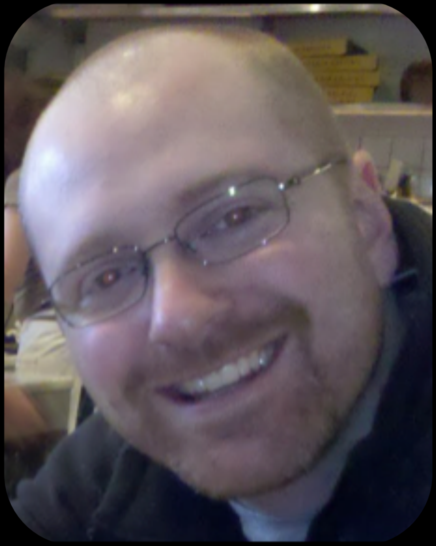
Normalization Summary

- the intensities of the normalization beads quantitatively monitor instrument sensitivity over acquisition time

- the effects of instrument variation on mass cytometry data are reduced by a correction derived from internal 5-metal bead standards

- normalization allows for the direct comparison of samples collected from multiple individuals and treated under different conditions

Bernd Bodenmiller



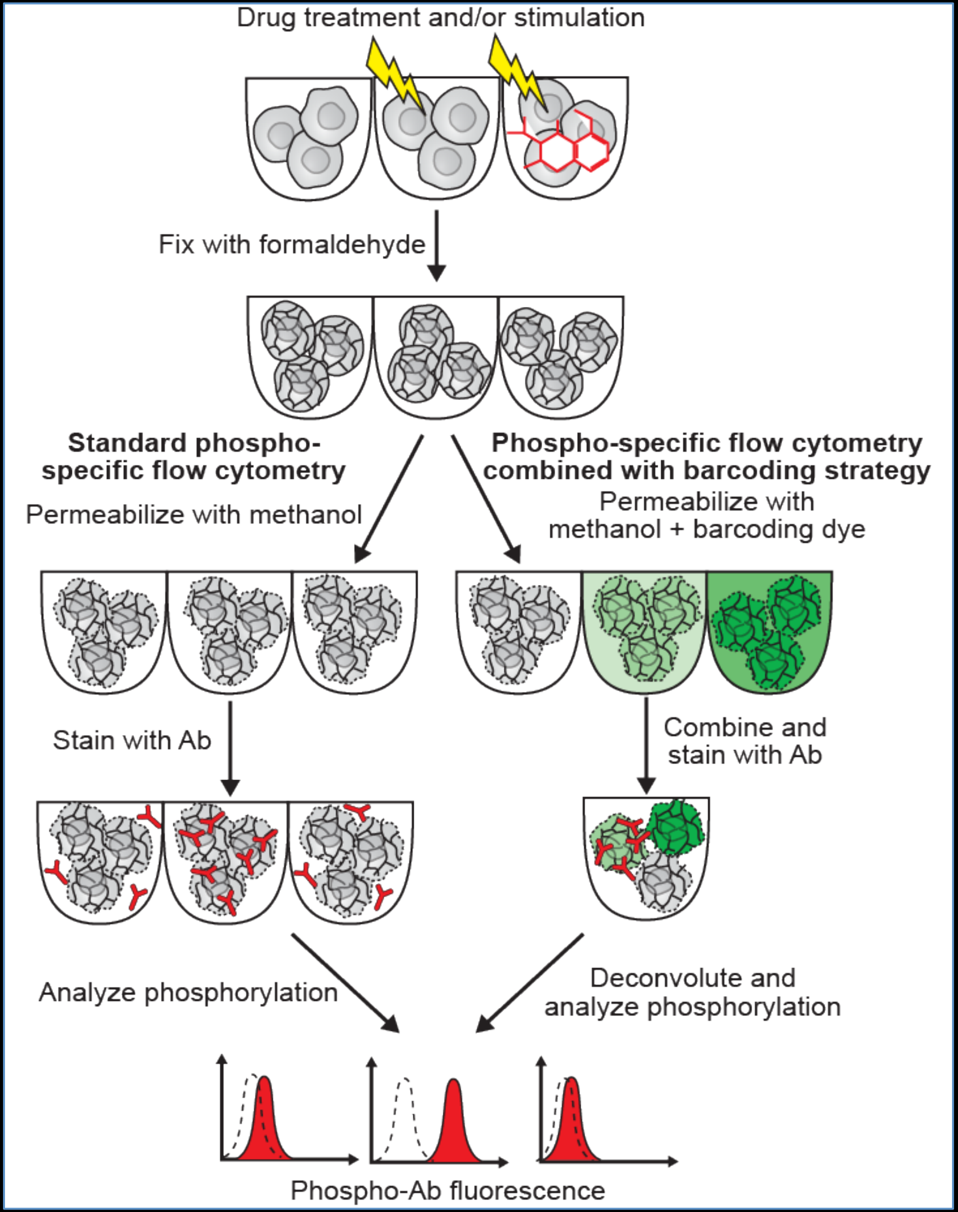
Eli Zunder

Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators

Bernd Bodenmiller*, ***Eli R. Zunder****, ***Rachel Finck****, *Tiffany J. Chen, Erica S. Savig, Robert V. Bruggner, Erin F. Simonds, Sean C. Bendall, Karen Sachs, Peter O. Krutzik and Garry P. Nolan*

Nature Biotechnology. 2012 September 10;30(9):858-67

Cell Multiplexing/Barcoding Overview

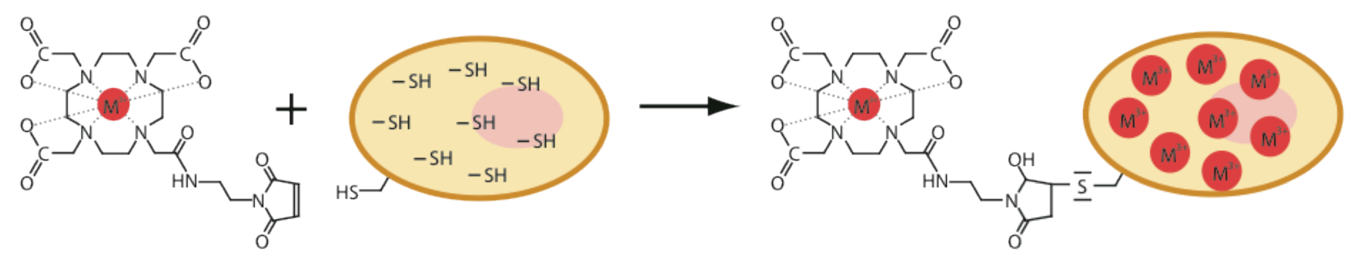


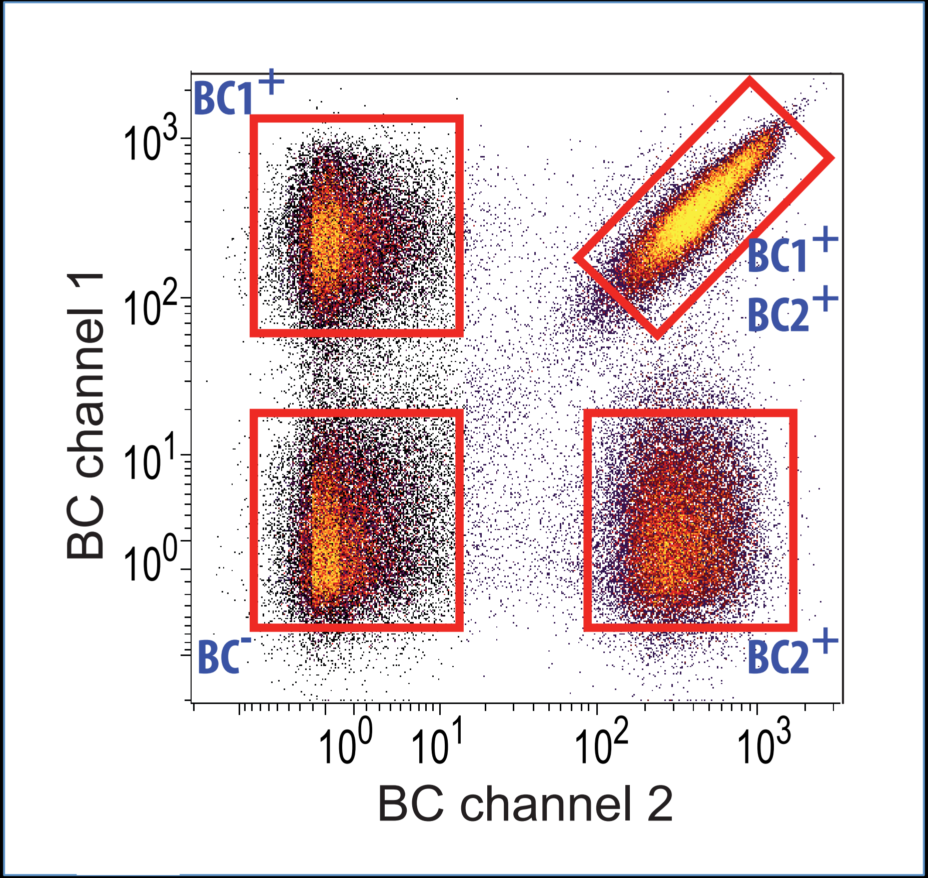
Advantages:

1. Uniform Staining
2. Reduced Antibody Consumption
3. Reduced Acquisition Time
4. Improved Singlet Detection

Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. Nat Methods. 2006 May;3(5):361-8.

Combinatorial Cell Labeling by DOTA-Maleimide Mass-Tag Cell Barcode (MCB) Reagents





7-metal Binary Cell Labeling Scheme for 96-well MCB Multiplexing

$2^1=2$

$2^2=4$

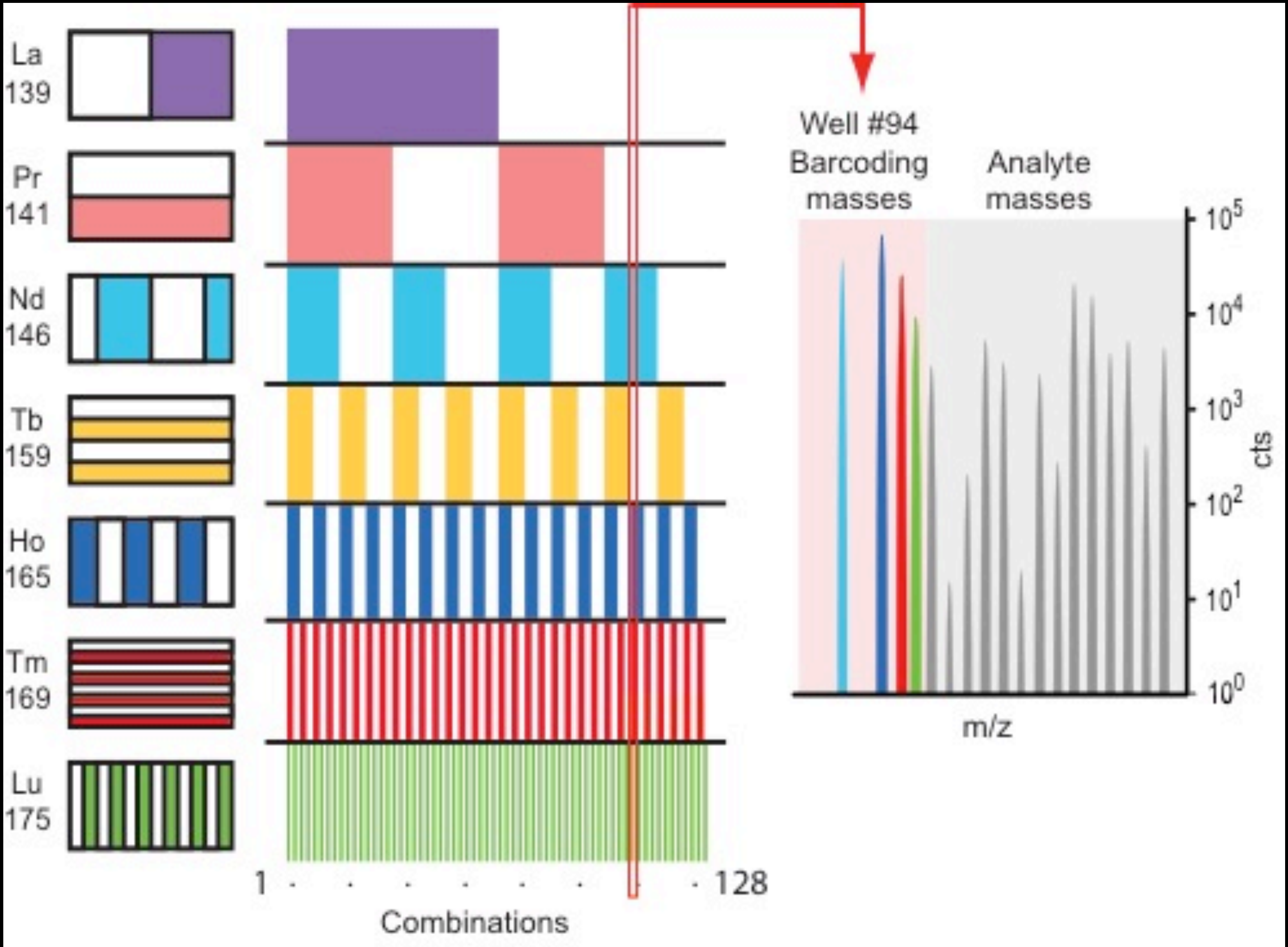
$2^3=8$

$2^4=16$

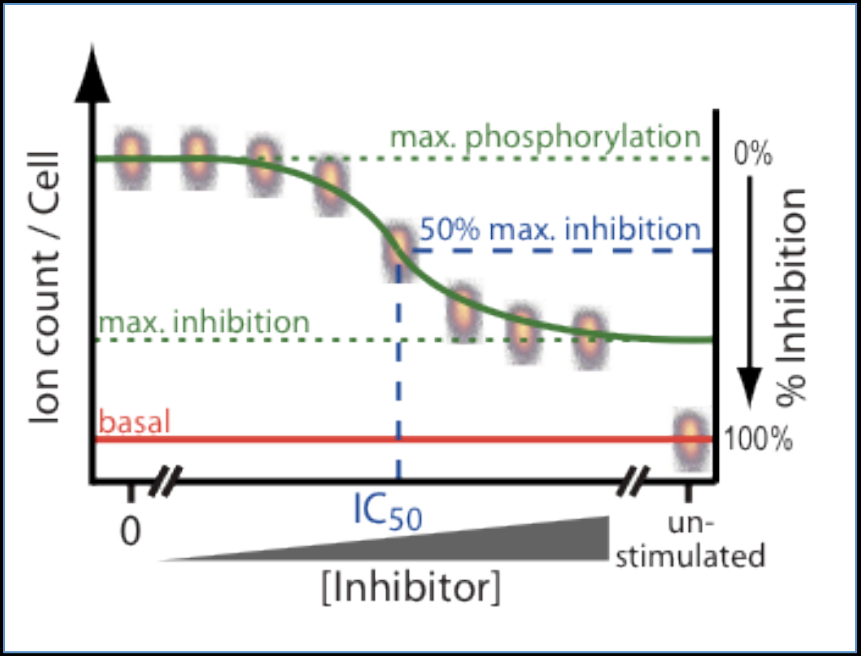
$2^5=32$

$2^6=64$

$2^7=128$

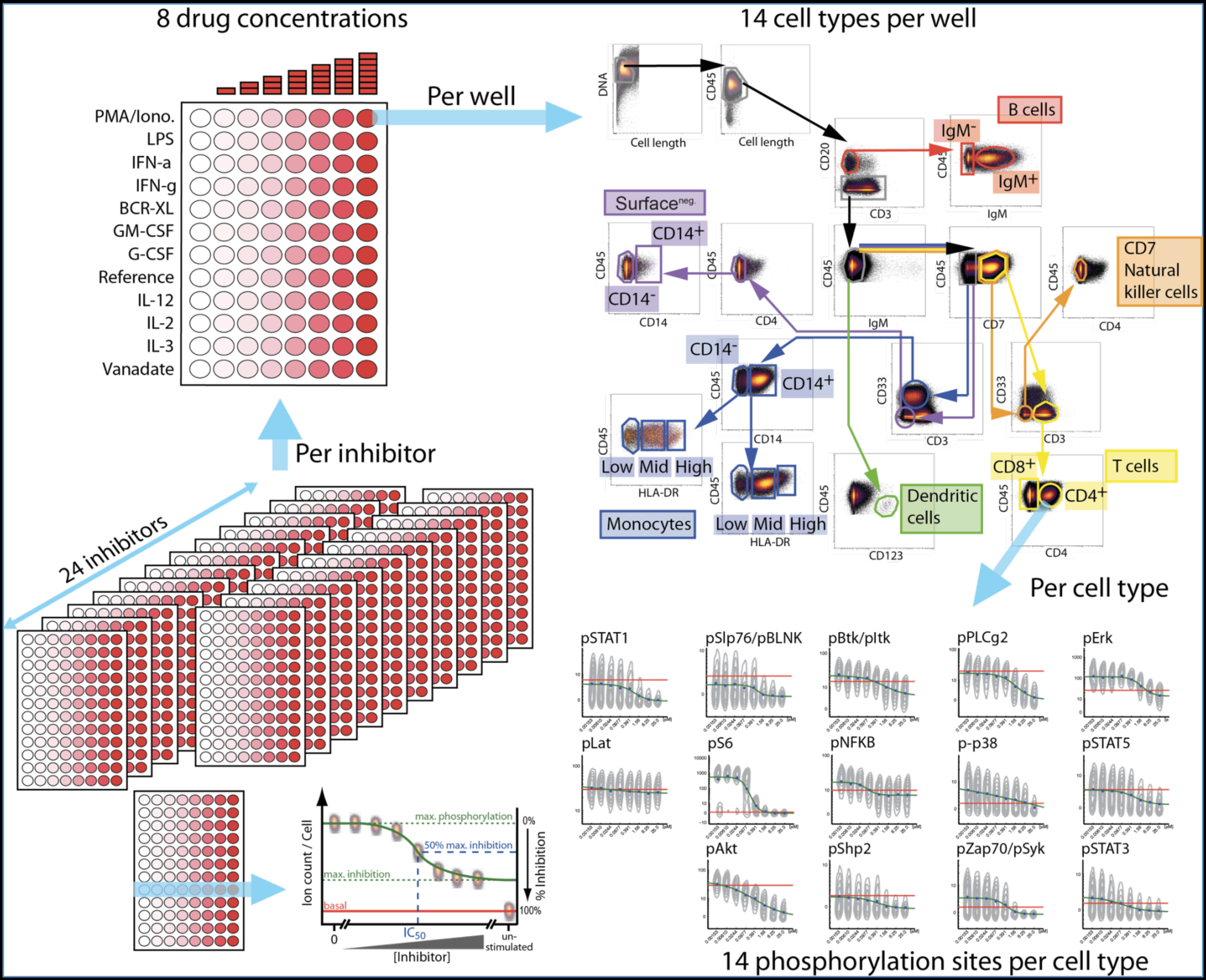


Small Molecule Kinase Inhibitor Profiling

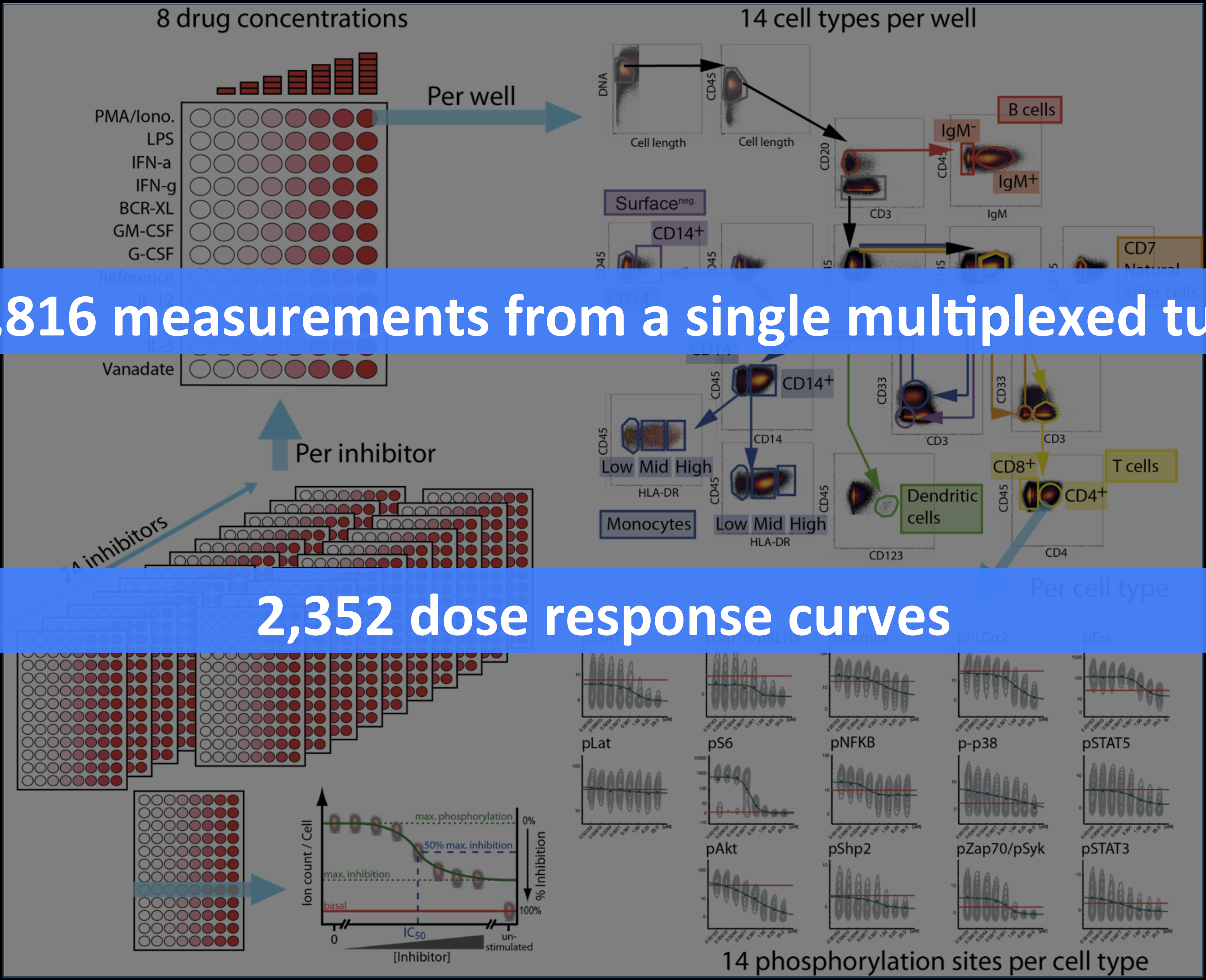


Calculation of phosphorylation site dose responses to inhibitors after various stimulations in multiple cell types.

Small Molecule Kinase Inhibitor Profiling



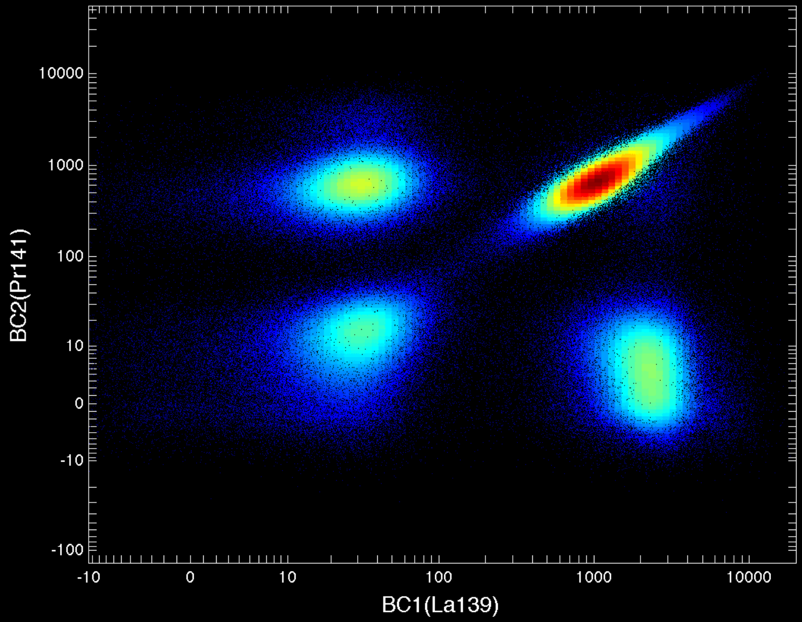
Small Molecule Kinase Inhibitor Profiling



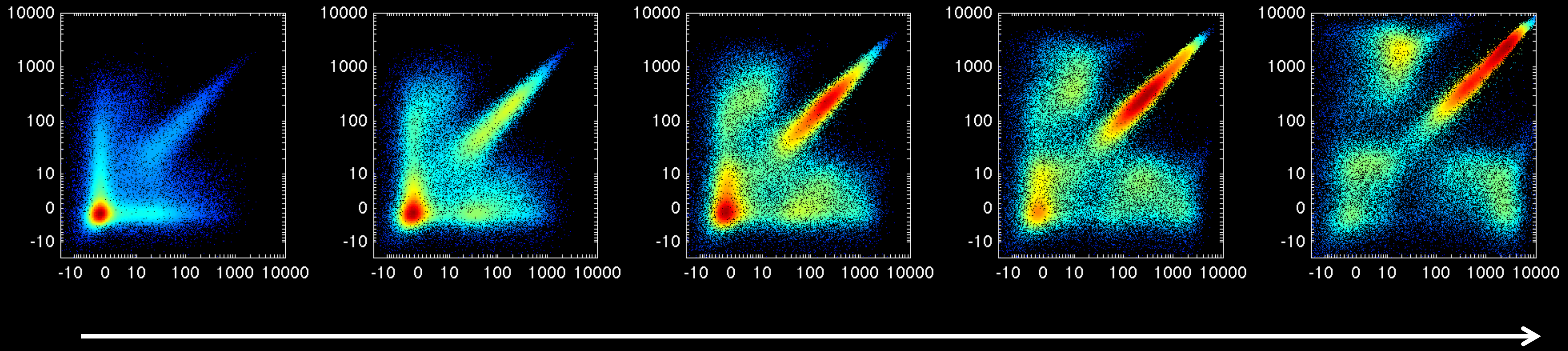
18,816 measurements from a single multiplexed tube

2,352 dose response curves

Advances in MCB: Single-cell debarcoding and doublet-free barcoding schemes

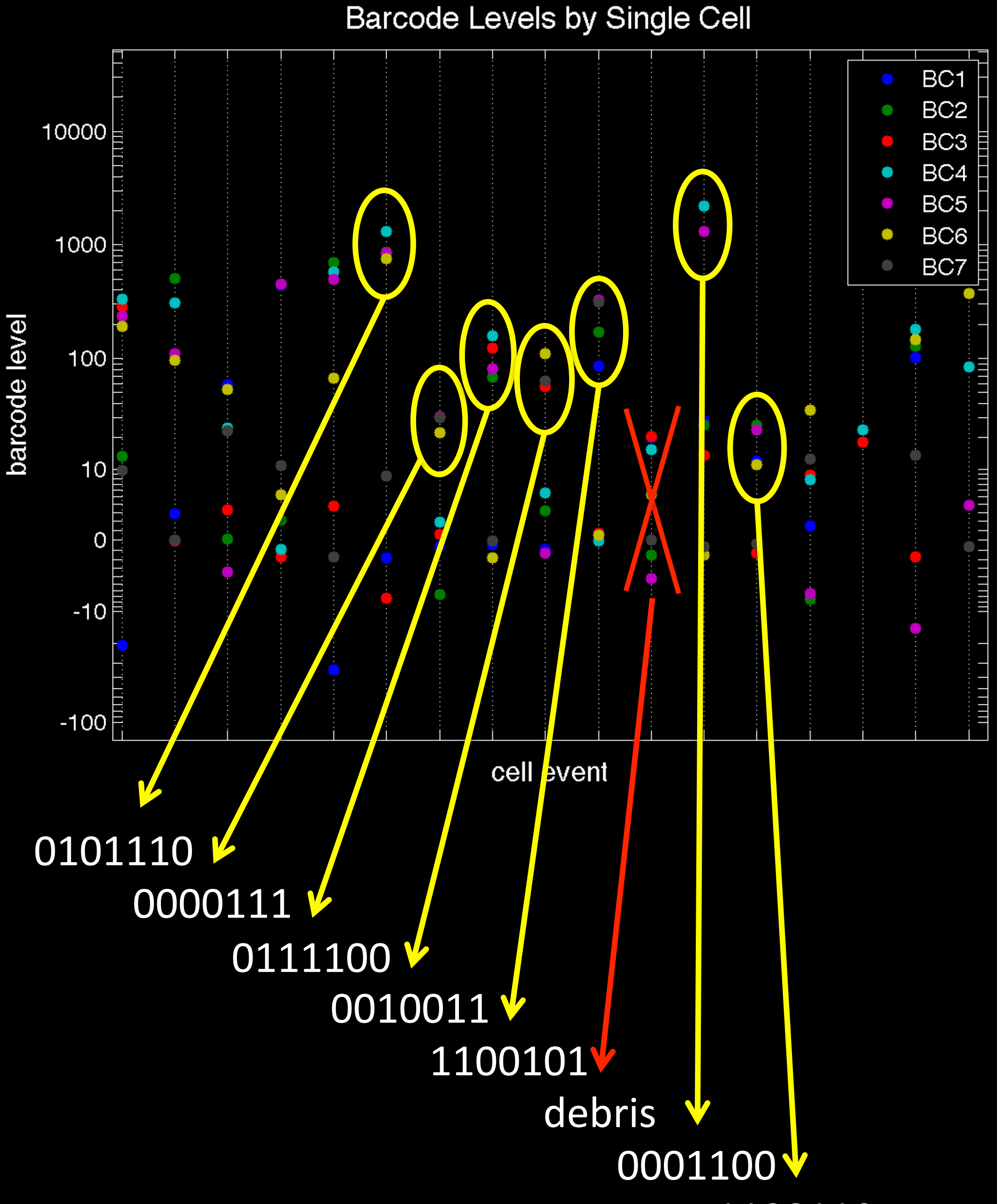


Barcodes Binned by Cell Length



cell length, incremented by 20% of the total cells

Single-Cell Debarcoding



0101110

0000111

0111100

0010011

1100101

debris

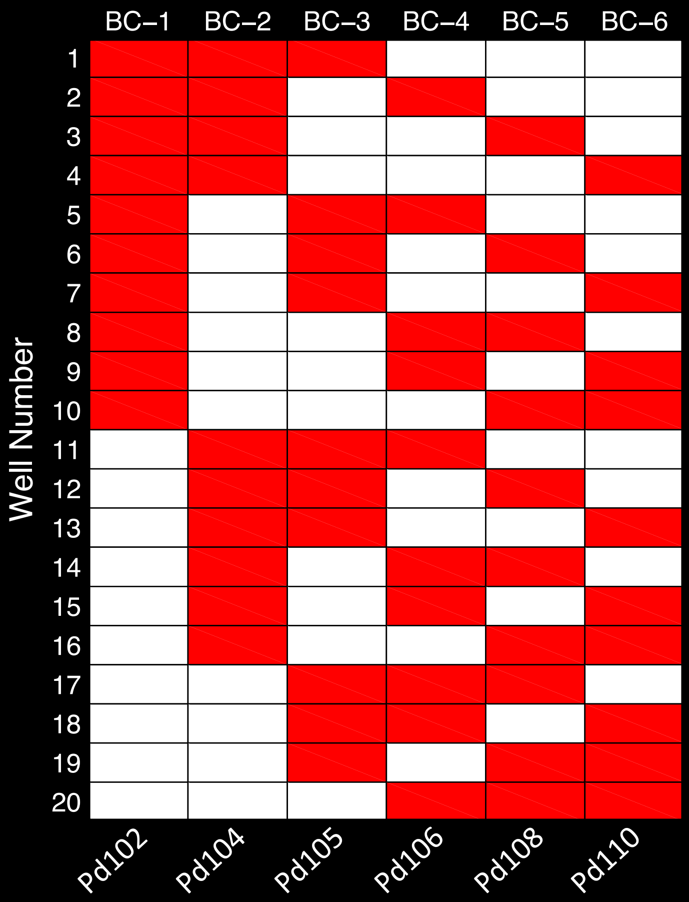
0001100

1100110

For each cell:

1. Sort signal intensity of barcode channels
2. Find largest separation in each cell
3. Check that signal intensity of first barcode above the largest gap is above a chosen cutoff
4. Check that largest gap is greater than a chosen fold change cutoff
5. Assign cell a barcode, or discard it

“Doublet-Free” Barcoding Schemes Simplify Single-Cell Debarcoding

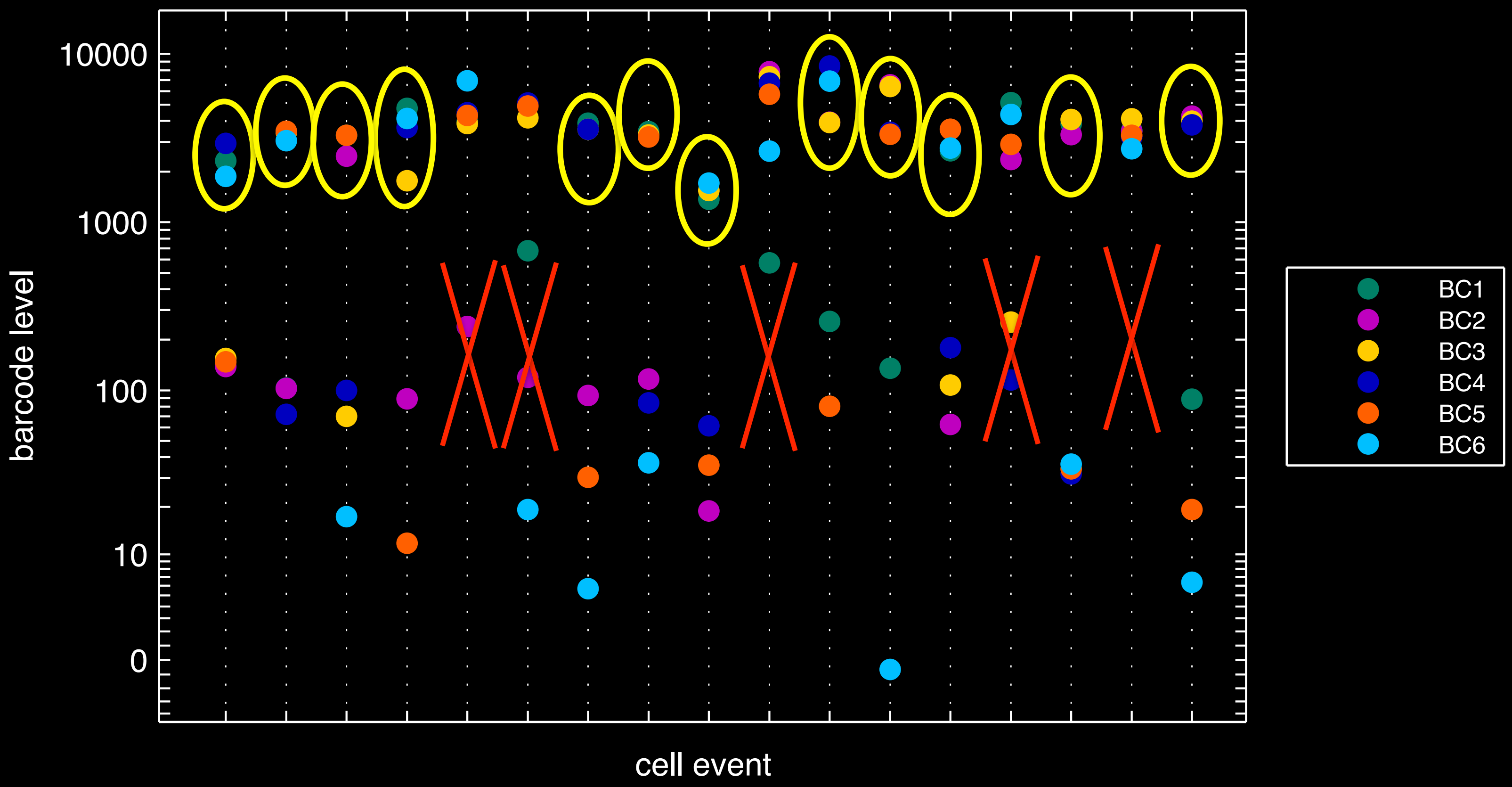


Well Number	BC–1	BC–2	BC–3	BC–4	BC–5	BC–6
1	🟥	🟥	🟥	⬜	⬜	⬜
2	🟥	🟥	⬜	🟥	⬜	⬜
3	🟥	🟥	⬜	⬜	🟥	⬜
4	🟥	🟥	⬜	⬜	⬜	🟥
5	🟥	⬜	🟥	🟥	⬜	⬜
6	🟥	⬜	🟥	⬜	🟥	⬜
7	🟥	⬜	🟥	⬜	⬜	🟥
8	🟥	⬜	⬜	🟥	🟥	⬜
9	🟥	⬜	⬜	🟥	⬜	🟥
10	🟥	⬜	⬜	⬜	🟥	🟥
11	⬜	🟥	🟥	🟥	⬜	⬜
12	⬜	🟥	🟥	⬜	🟥	⬜
13	⬜	🟥	🟥	⬜	⬜	🟥
14	⬜	🟥	⬜	🟥	🟥	⬜
15	⬜	🟥	⬜	🟥	⬜	🟥
16	⬜	🟥	⬜	⬜	🟥	🟥
17	⬜	⬜	🟥	🟥	🟥	⬜
18	⬜	⬜	🟥	🟥	⬜	🟥
19	⬜	⬜	🟥	⬜	🟥	🟥
20	⬜	⬜	⬜	🟥	🟥	🟥
	Pd102	Pd104	Pd105	Pd106	Pd108	Pd110

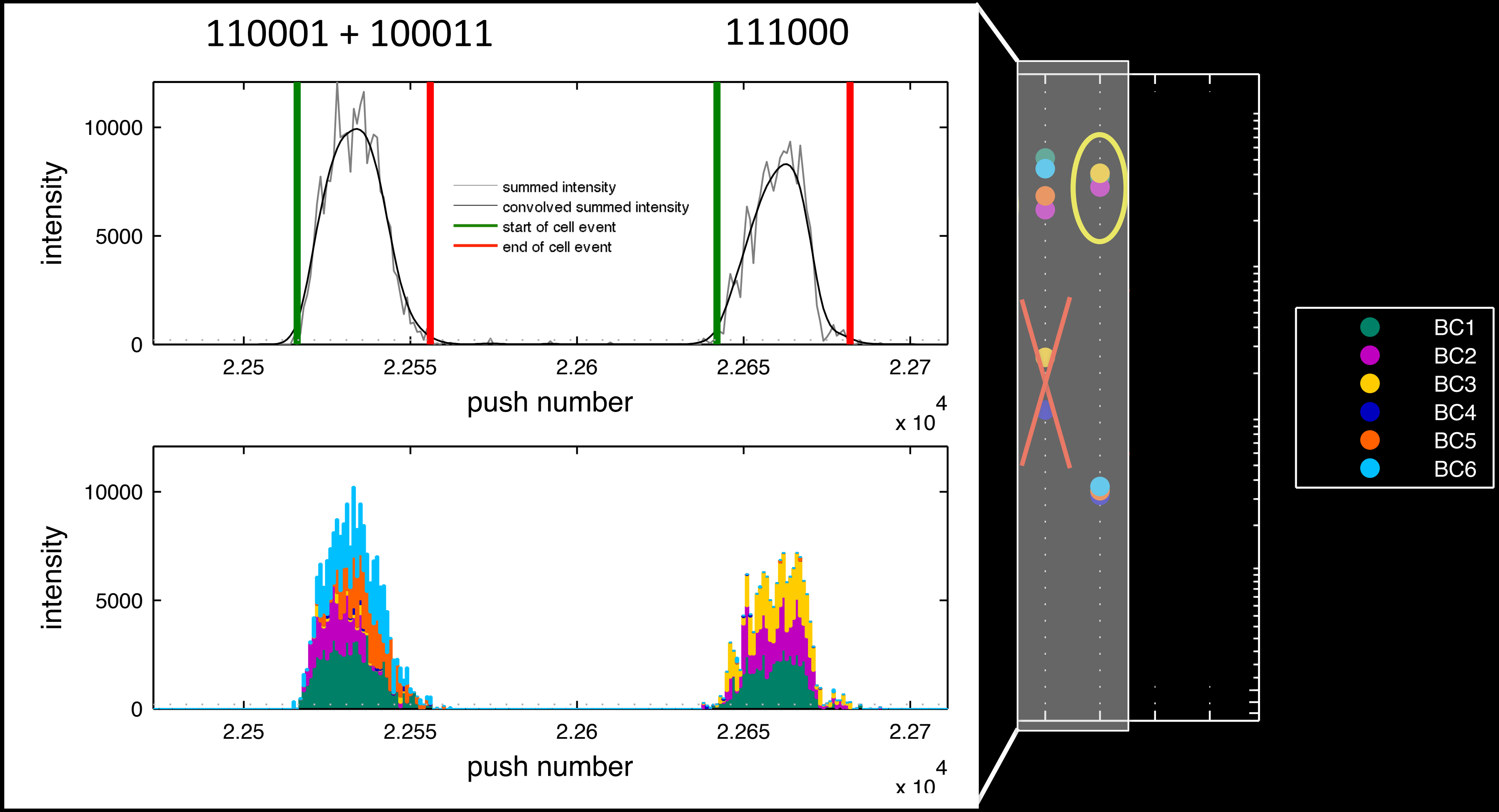
- Every well is labeled with exactly three barcode metals
- The combination of any two (or more) different barcodes is an “illegal” barcode
- Selecting only the events with legal barcodes filters out most doublets

Palladium barcode labeling does not overlap with antibody channels!

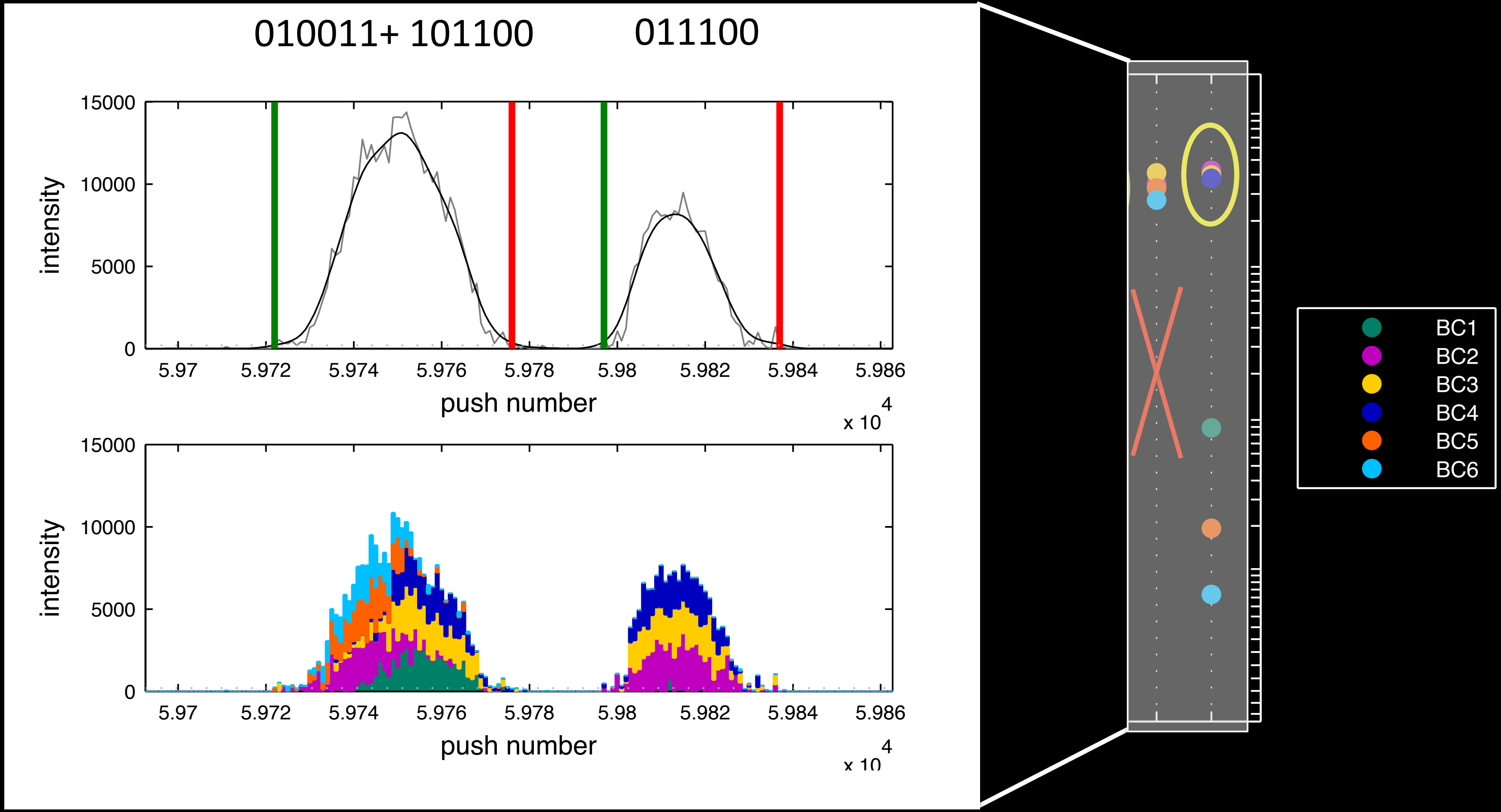
Single-Cell Debarcoding With a "Doublet-Free" Scheme



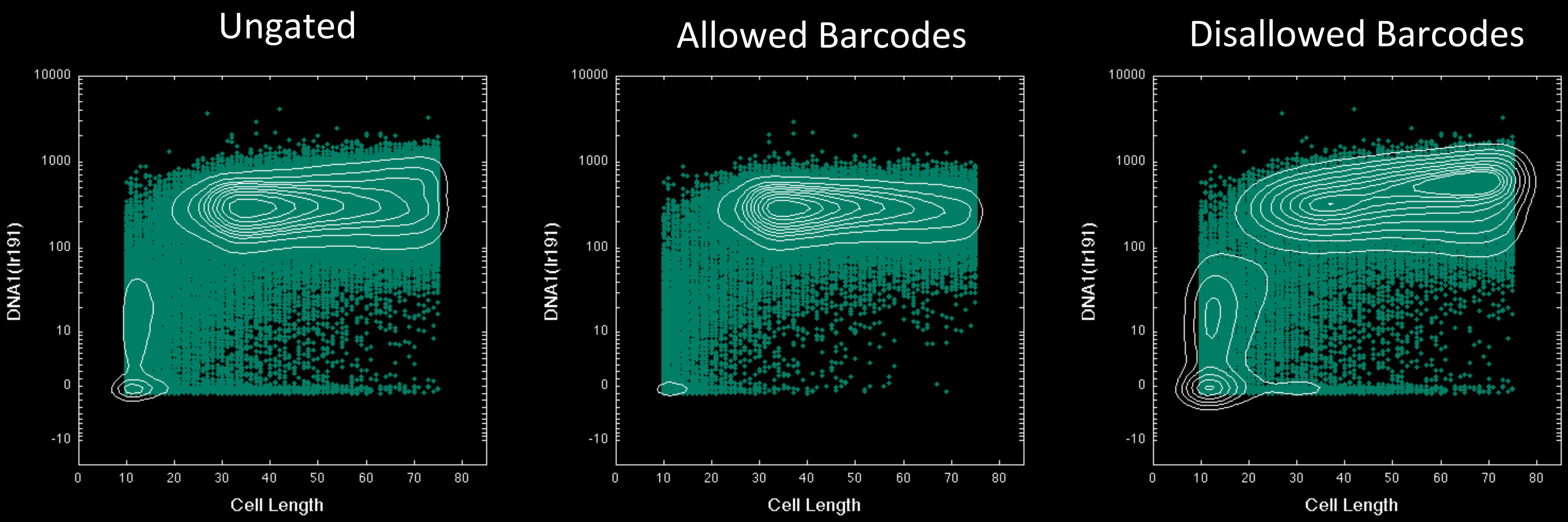
Single-Cell Debarcoding With a “Doublet-Free” Scheme



Single-Cell Debarcoding With a "Doublet-Free" Scheme



Doublets Removed via Debarcoding



Ungated

Allowed Barcodes

Disallowed Barcodes

Barcoding Update Summary

- Single-cell debarcoding decreases cell size bias

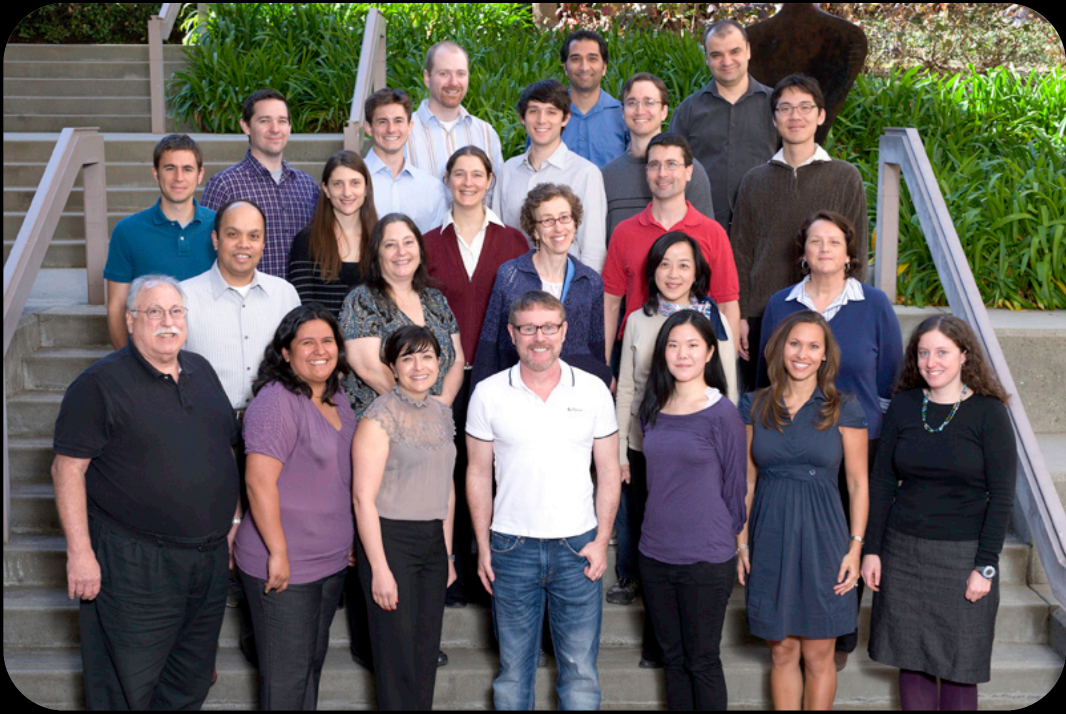
- Doublet-free barcoding scheme improves accuracy of barcode deconvolution and singlet gating

- Palladium barcode labeling does not affect the number of available antibody channels

STANFORD
SCHOOL OF MEDICINE

Prof. Garry P. Nolan

www.stanford.edu/group/nolan



Sean Bendall
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Greg Behbehani

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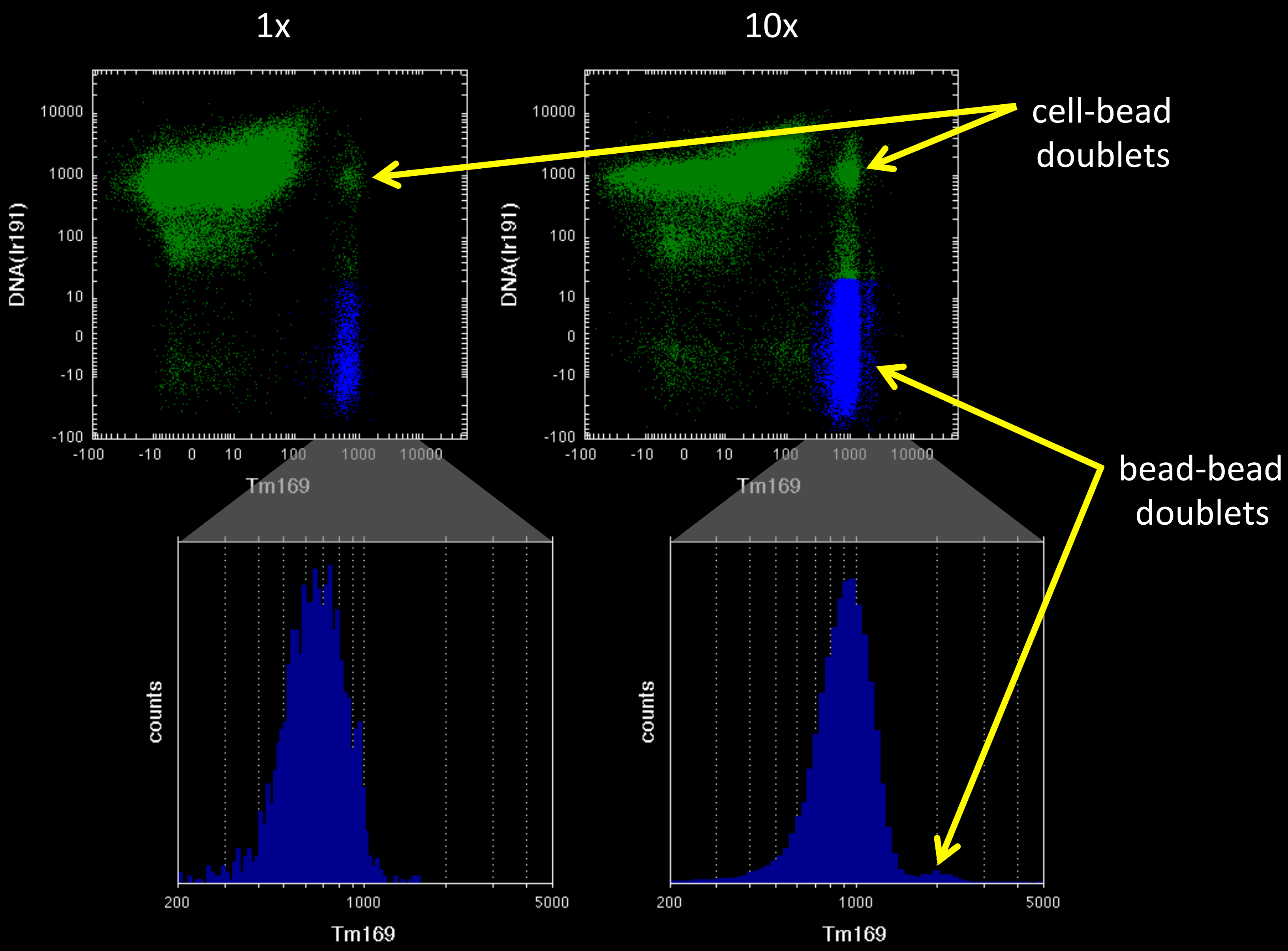
Dana Pe’er
El-ad David Amir

U. Toronto / DVS Sciences
Scott Tanner
Olga Ornatsky
Vladimir Baranov
Dmitry Bandura
Tad George

Extra Slides

Bead Doublets Are Few



1x

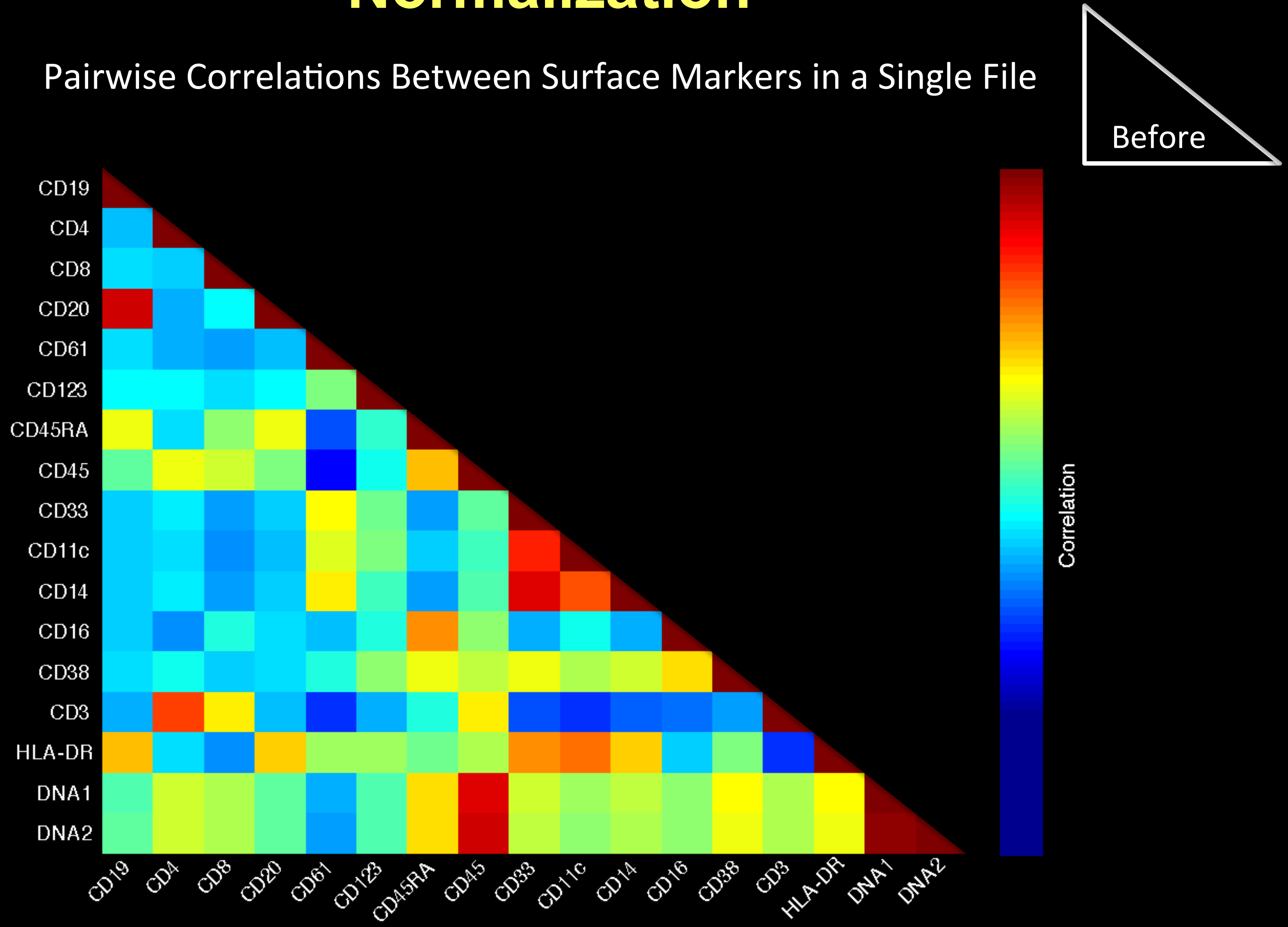
10x

cell-bead doublets

bead-bead doublets

Multivariate Relationships are Preserved by Normalization

Pairwise Correlations Between Surface Markers in a Single File



Peripheral Blood Mononuclear Cell (PBMC) MCB Staining Panel

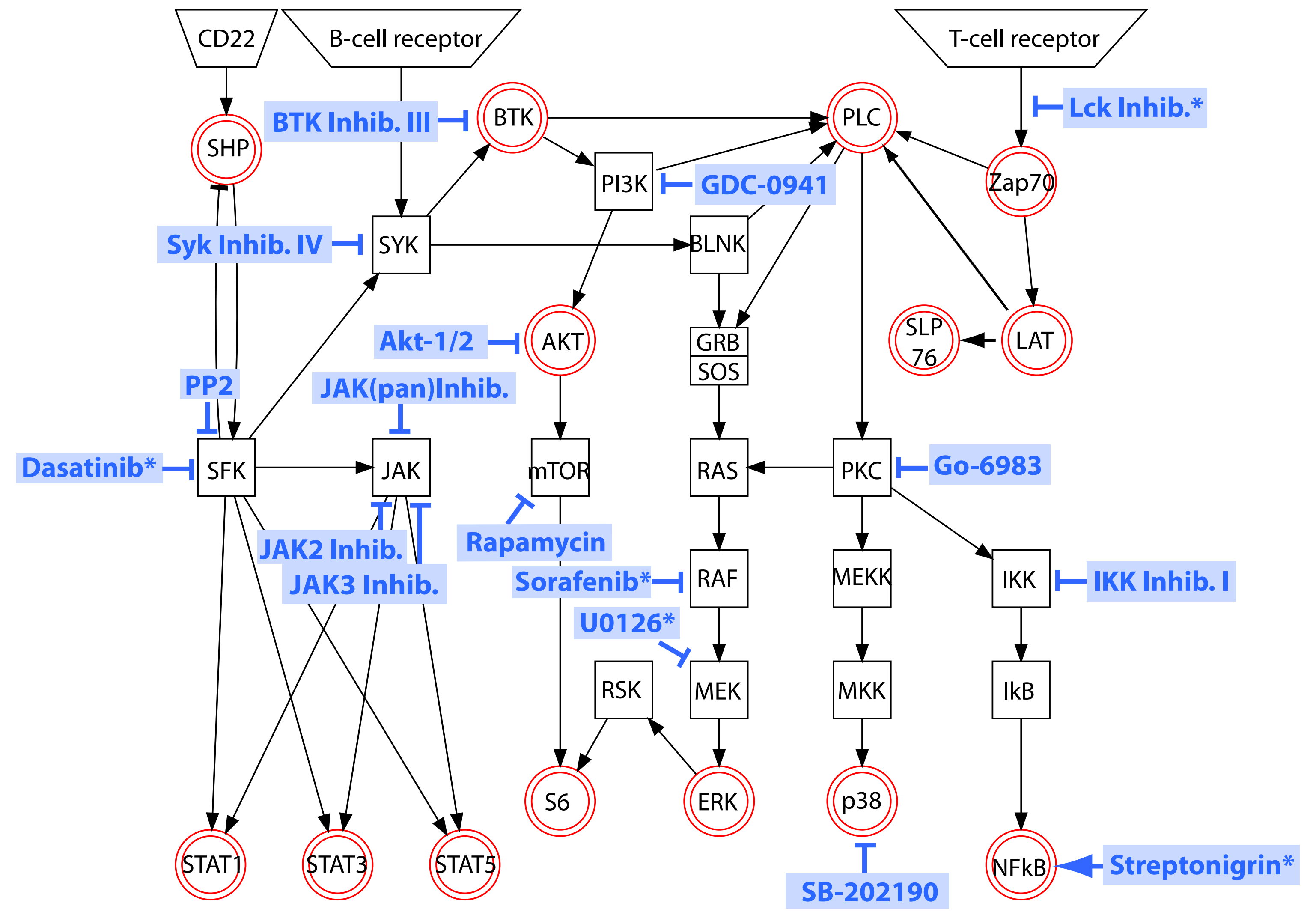
Surface Markers

CD45
CD20
IgM
HLA-DR
CD3
CD4
CD7
CD33
CD14
CD123

Phospho-proteins

Stat1 (pY701)
Stat3 (pY705)
Stat5 (pY694)
SHP2 (pY580)
Zap70/Syk (pY319/pY352)
Slp76 (pY128)/BLNK (pY 72)
BTK (pY551/pY511)/Itk (pY511)
LAT (pY226)
PLCγ (pY759)
Akt (pT308)
S6 (pS235/pS236)
Erk1/2 (pT202/ pY204)
p38 (pT180/pY182)
NFKB (pS529)

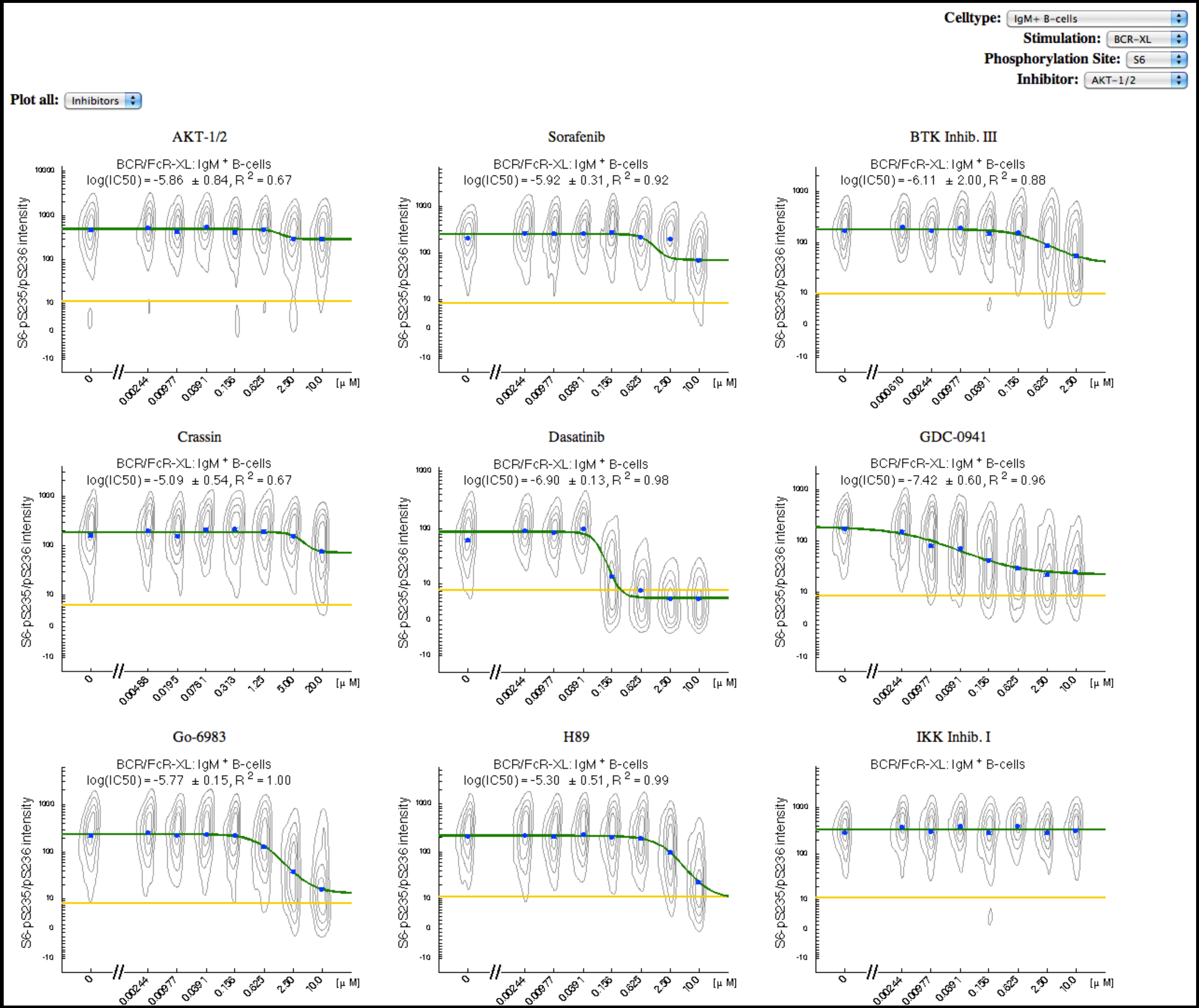
Kinase Inhibitors Screened: Approved Drugs, Pre-clinical Compounds, and Chemical Tools



CD22
B-cell receptor
T-cell receptor
BTK Inhib. III
BTK
PLC
Lck Inhib.*
SHP
PI3K
GDC-0941
Zap70
Syk Inhib. IV
SYK
BLNK
Akt-1/2
AKT
GRB
SOS
SLP 76
LAT
PP2
JAK(pan)Inhib.
Dasatinib*
SFK
JAK
mTOR
RAS
PKC
Go-6983
JAK2 Inhib.
JAK3 Inhib.
Rapamycin
Sorafenib*
RAF
MEKK
IKK
IKK Inhib. I
U0126*
RSK
MEK
MKK
IkB
S6
ERK
p38
STAT1
STAT3
STAT5
NFkB
Streptonigrin*
SB-202190

Crassin, H89 (PKA), Imatinib* (BCR-ABL), SP600125 (JNK), Staurosporine (broad), Sunitinib (RTKs), VX-680* (Aurora kinase)

Dose Response Curve Website



Celltype: IgM+ B-cells

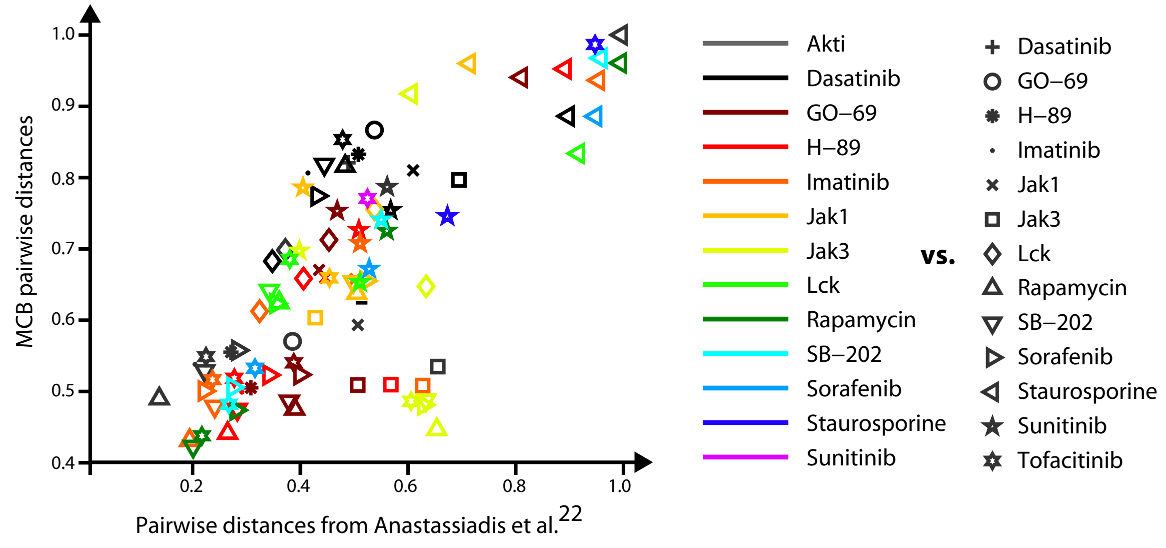
Stimulation: BCR-XL

Phosphorylation Site: S6

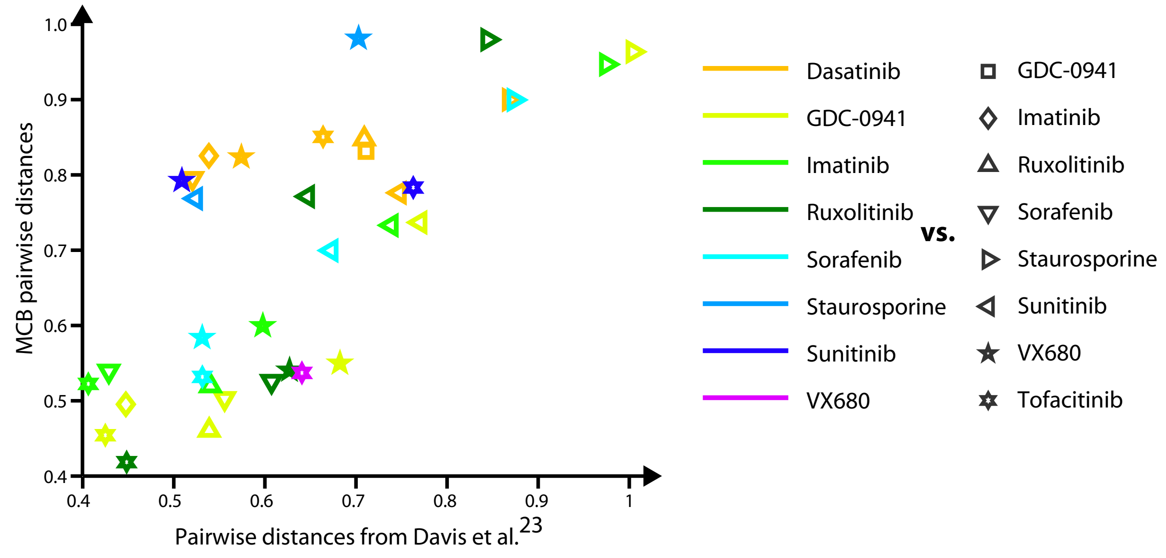
Inhibitor: AKT-1/2

Plot all: Inhibitors

PBMC In Vivo IC50s Strongly Correlate with In Vitro Percent Inhibition Values

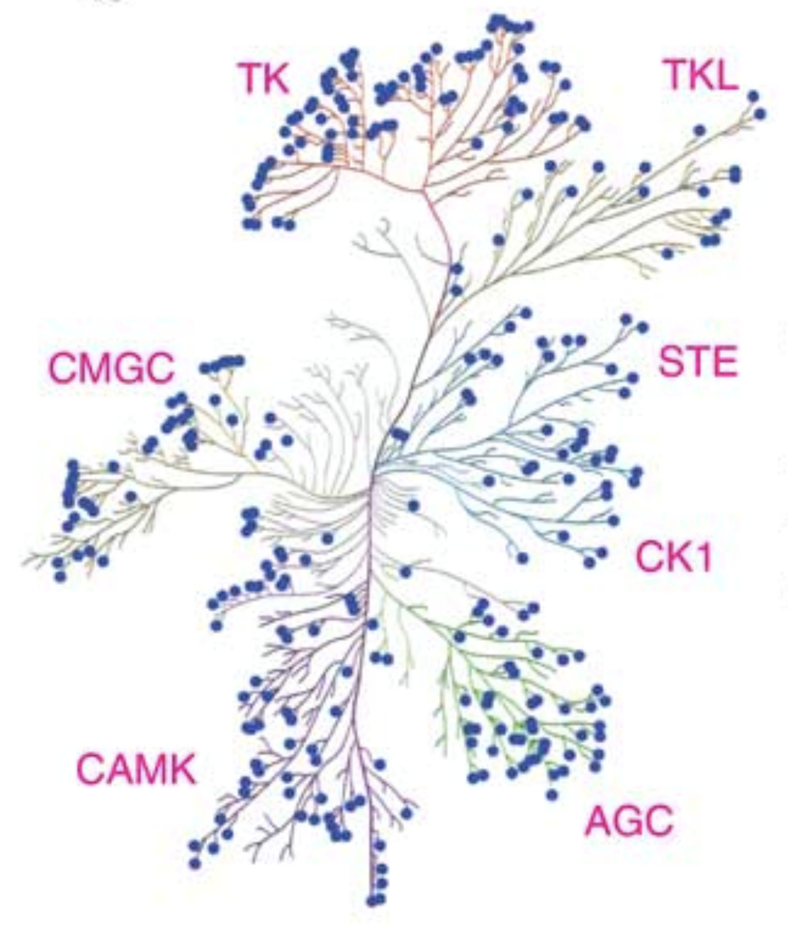


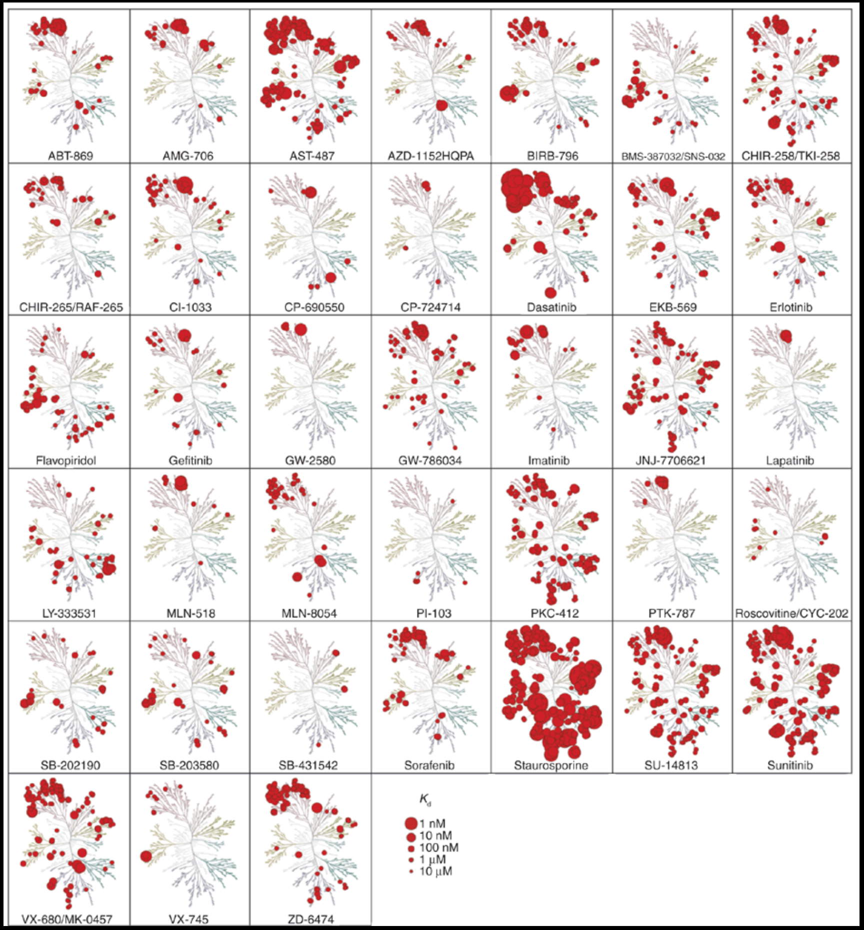
Anastassiadis et al. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. Nat Biotechnol. 2011 Oct 30;29(11):1039-45.



Davis et al. Comprehensive analysis of kinase inhibitor selectivity.
Nat Biotechnol. 2011 Oct 30;29(11): 1046-51.

How Does Our 96-well MCB In Vivo Analysis Compare to In Vitro Kinase Inhibition Assays?

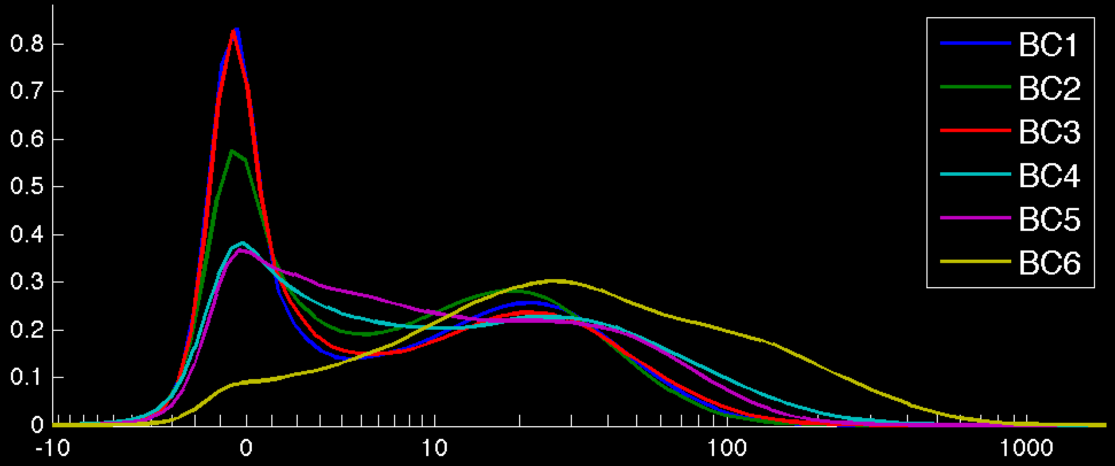




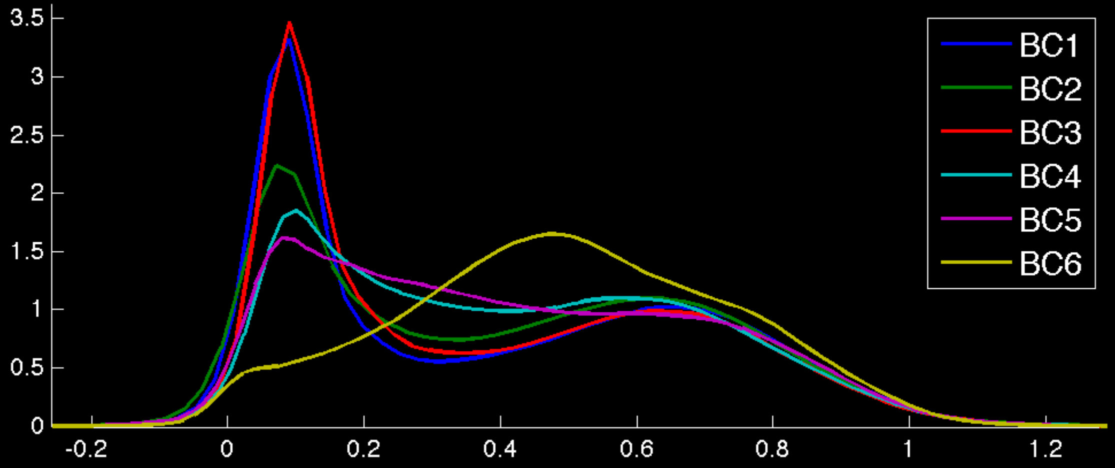
Anastassiadis et al. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. Nat Biotechnol. 2011 Oct 30;29(11):1039-45.

Re-Scaling of Barcode Channels

We were assuming that barcode channels were on comparable scales, but what if they're not?

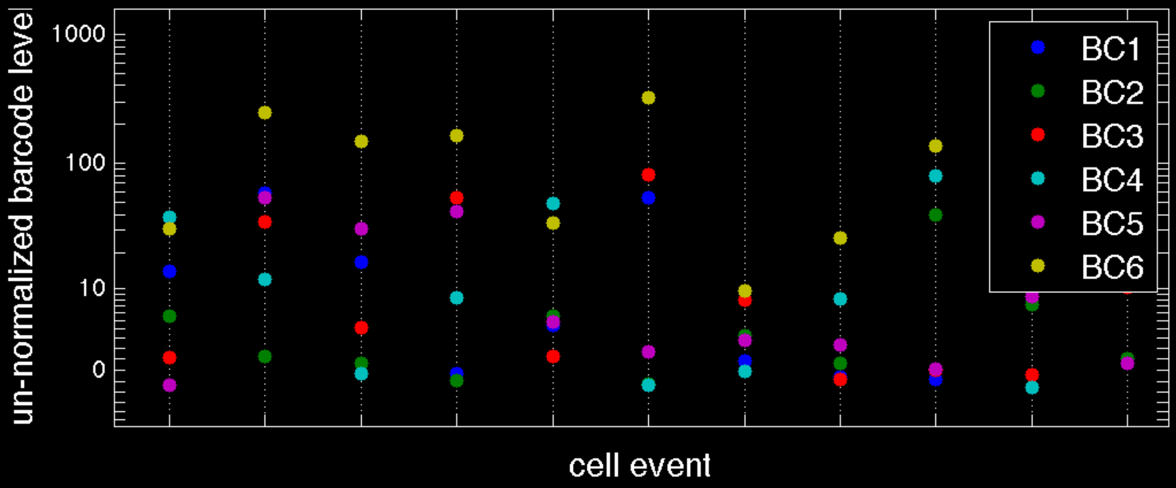


Normalize:



Re-Scaling of Barcode Channels

Before Normalization:



After Normalization: