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

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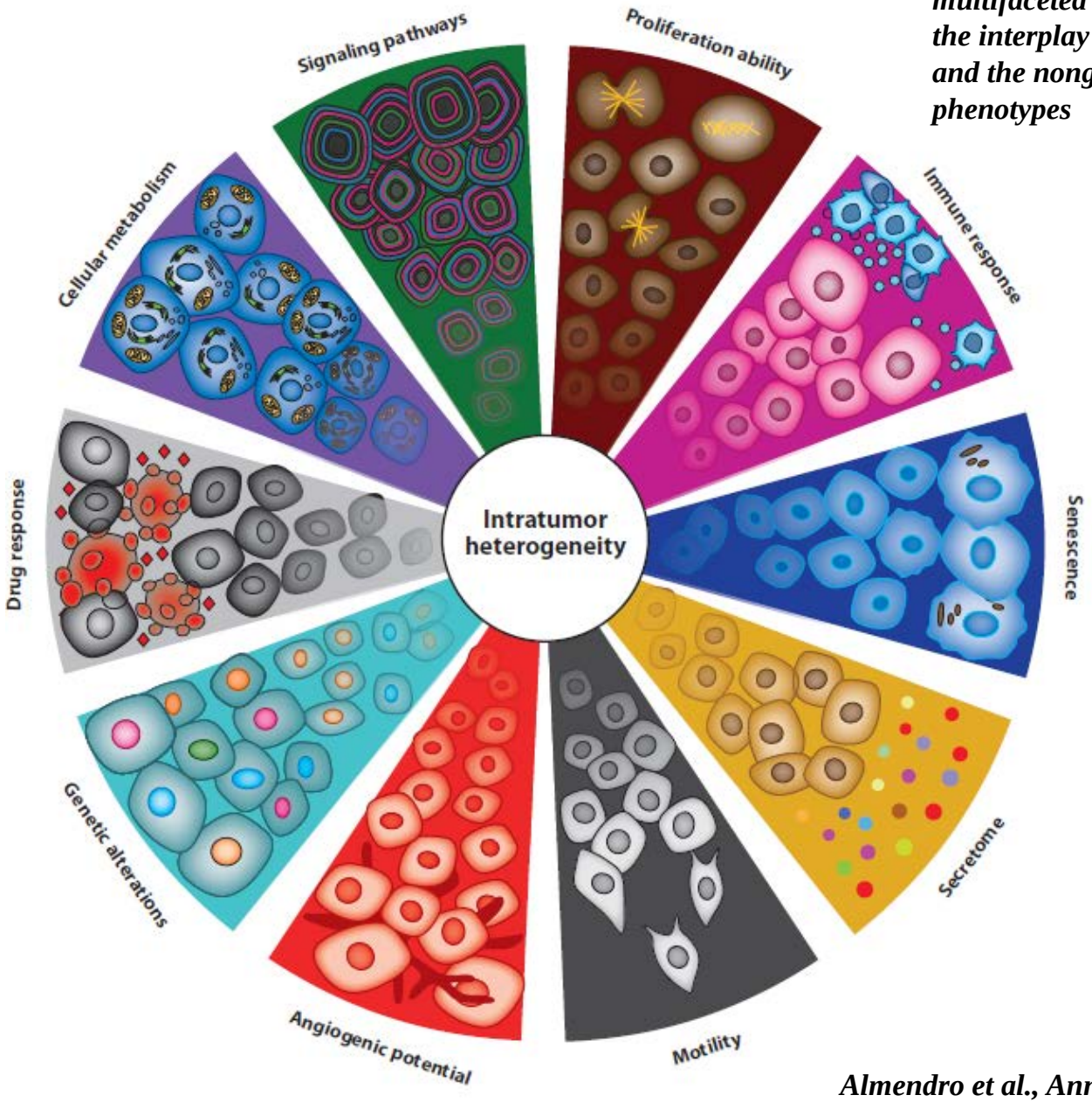
19.02.2013

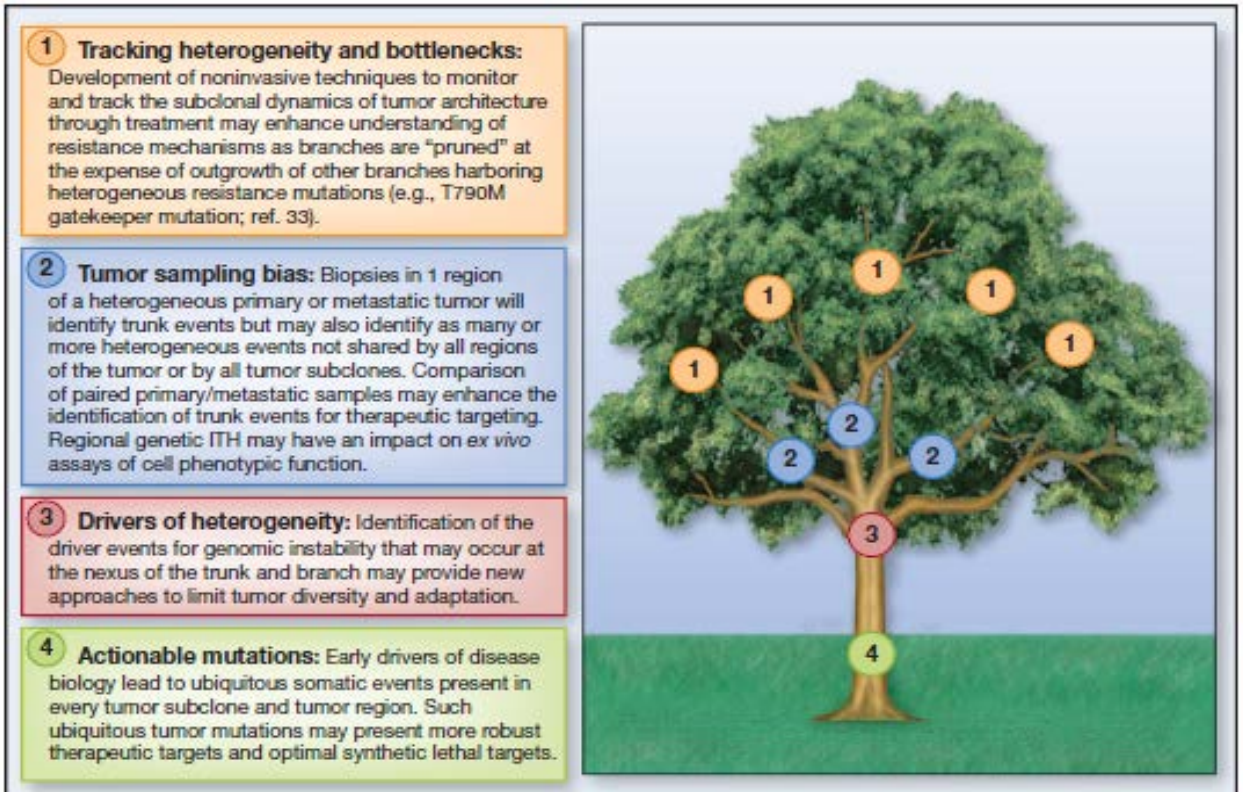
TUMOR HETEROGENEITY

Heterogeneity in tumor cell phenotypes is a multifaceted phenomenon that arises from the interplay between genetic heterogeneity and the nongenetic factors that shape cellular phenotypes



Almendro et al., Annu. Rev. Pathol. Mech. Dis. (2013)

Diversity in cancer cell populations



- TRUNKS OF THE TREE

Common or ubiquitous events in the tumor

- *BRANCHES and LEAVES*

Heterogeneous somatic events

Swanton, Cancer Research (2012)

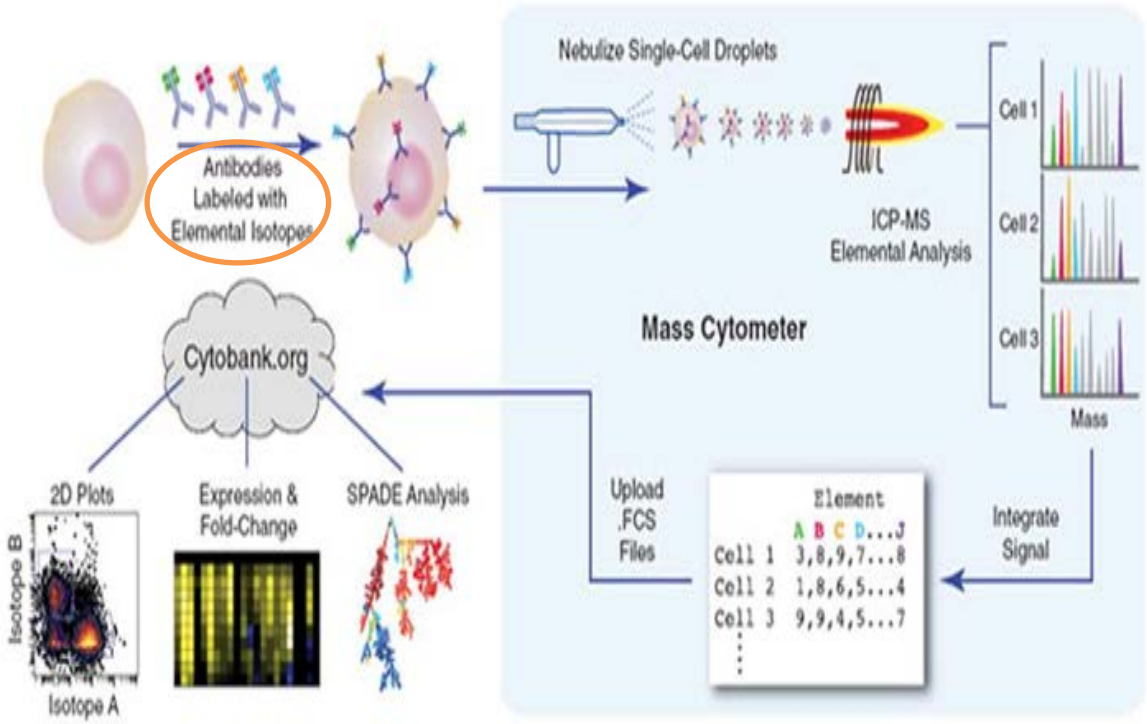
Table 1 Different approaches for analysis of tumors at the single-cell level

Technique	Sensitivity	Speed	Resolution	Pros	Cons
Flow cytometry	~500–10^7 molecules/cell	~25,000 cells/sec	Single cell	Up to 15 parameters/cell Many fluorescent probes for cellular biology Sorting of live cells	Cellular autofluorescence interference Emission spectra interference as multiplexing increases
Mass cytometry	~1,500–10^7 molecules/cell	~1,000 cells/sec	Single cell	Currently 42 parameters per cell; ~100 possible No autofluorescence or spectral overlap	For every new parameter, new chemistries are required to attach isotopes to reagents Currently, 70% of the cells ejected from the nebulizer do not maintain full integrity. After ionization, such subcellular fragments do not reach the detector as ionic clouds representing the constituents of a complete cell. A new cell nebulizer design is expected to reduce cell loss to only 30%
Single-cell sequencing	93% of complete genome	10 d/cell	Single cell	Most of genome can be sequenced Identification of mutations	Sequences prone to possible mutation during early PCR steps
Single-cell PCR of targeted transcripts	96 transcripts/well	96 cells/plate/4 h	Single cell	Possible 1,000 cells/day Relative quantification, Absolute quantification with controls	Only 96 transcripts per cell reported
Transcriptome	1 cellular exome or 10^7 transcripts	2–3 d/cell	Single cell	Quantitative sequence counting of all transcripts Splice-o-forms quantified Point mutations identified	Limited by exome selection method
MALDI-imaging[a]	~10^{-15} mols/µm^2	~1 sec/pixel	~50 µm pixel size	Theoretically hundreds of different molecular species (protein and small molecule) can be analyzed Tissue structure intact	Reporter masses <250 Da difficult to observe due to 'matrix effects' Fragmentation and molecular abducts complicate interpretation
SIMS/MIMS[a]	~10^{12}–10^{16} atoms/cm^2 for trace elements	~1 ms/pixel	~0.05 µm pixel size	Currently able to determine biologically labeled isotope ratios, such as N, C, O Subcellular resolution Tissue structure largely intact	Fragmentation and molecular abducts complicate interpretation Most instrument configurations limited to <10 analytes per scan
Laser ablation ICP mass spectrometry[a]	~10^2 ppb for lanthanides	~1 sec/pixel	4 µm pixel size	Tissue structure intact Relatively simple to interpret	Limited to the analysis of only elemental constituents and reporters (see mass cytometry)

SMIS, secondary ion mass spectrometry; MIMS, multi-isotope imaging mass spectrometry.
[a]Resolution, speed and sensitivity are interdependent. Sensitivity can be increased by increasing pixel size (lower resolution) and increasing scan dwell time (lower speed).

Bendall SC & Nolan GP, Nature Biotechnology (2012)

Work-flow summary of mass cytometry analysis



- Cells are stained with antibodies are labeled with isotopically pure metals
- Cells are nebulized into single-cell droplets
- elemental mass spectrum is acquired for each
- Analysis of the integrated elemental reporter signals for each cell

Bendall et al., Science (2011)

Table 1. Comparison of utility and performance of state of the art commercial fluorescence flow cytometry and mass cytometry single-cell analysis platforms.

Technology		Fluorescence flow cytometry	Mass cytometry
Measurement basis		Fluorescent probes	Stable mass isotope probes
Experimental design			
Max no. of measurements		20 (18 fluorescence)	37 (including DNA)
Theoretical no. of subsets[a]		2.6×10^5	1.4×10^{11}
Panel design complexity (no. of probes)	Easy	<8	37
	Moderate	8–12	
	Hard	12–18	
Sensitivity range for different probes[b]		0.1–10	1–2
Sample throughput			
Sampling efficiency		> 95%	< 30%
Measured cells/s		25 000	500–1000
Cells/h		25–60 million	2 million
Commercial reagent cost			
Per probe per test[c]		$2.00–$8.00	$1.50–$3.00

[a]Theoretical number of subsets is the number of distinct cell types determinable, assuming only on or off for each marker; that is, 2^{colors}.

[b]Sensitivity range is in arbitrary units, and compares the rough sensitivity for different probes (fluorescence or ICP-MS) to detect a given epitope on a cell by immunophenotyping.

[c]Estimated based on the price of commercially conjugated reagents or unconjugated antibodies and commercial conjugation kits.

Bendall et al. Trends in Immunology (2012)

- **high-dimensional, quantitative analysis at single-cell resolution**
- **lack of background signal (autofluorescence)**
- **the substantially greater number of parameters that can be simultaneously analyzed(45)**

an attractive platform currently available for highly multiplexed single-cell analysis

ARTICLES

nature biotechnology

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

Bernd Bodenmiller[1,5,6], Eli R Zunder[1,6], Rachel Finck[1,6], Tiffany J Chen[1–3], Erica S Savig[1,4], Robert V Bruggner[1,2], Erin F Simonds[1], Sean C Bendall[1], Karen Sachs[1], Peter O Krutzik[1] & Garry P Nolan[1]

- ***characterize human peripheral blood mononuclear cell (PBMC) signaling dynamics and cell-to-cell communication***

- ***signaling variability between PBMCs from eight human donors***

- ***the effects of 27 inhibitors on this system***

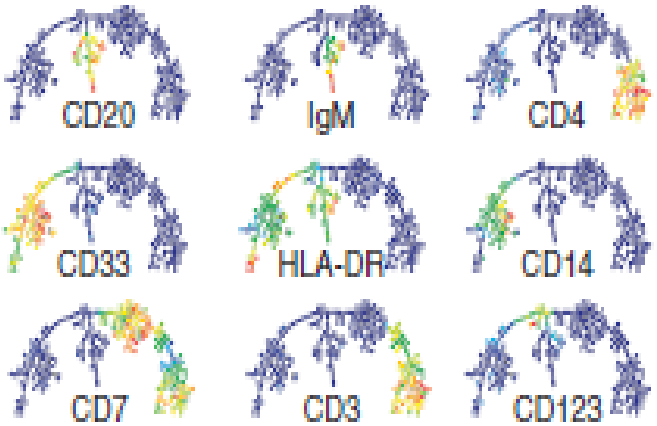
METHODS

EXPERIMENTs	***DATA ANALYSIS***
PBMC isolation, culture and stimulation	*SPADE*
Time-course	*PCA*
Inhibitor dose-response	*VISUALIZATION*
Mass cytometry analysis	
In vitro kinase assays	

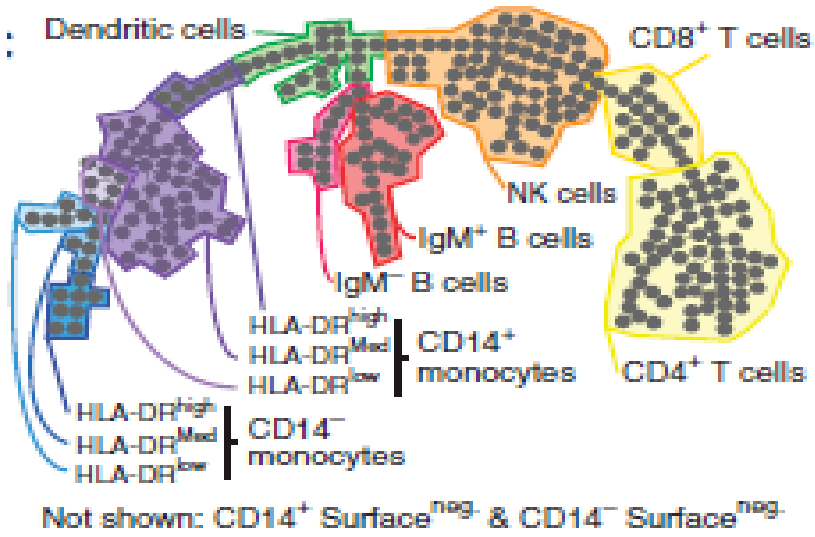
Spanning-tree Progression Analysis of Density-normalized Events (SPADE)

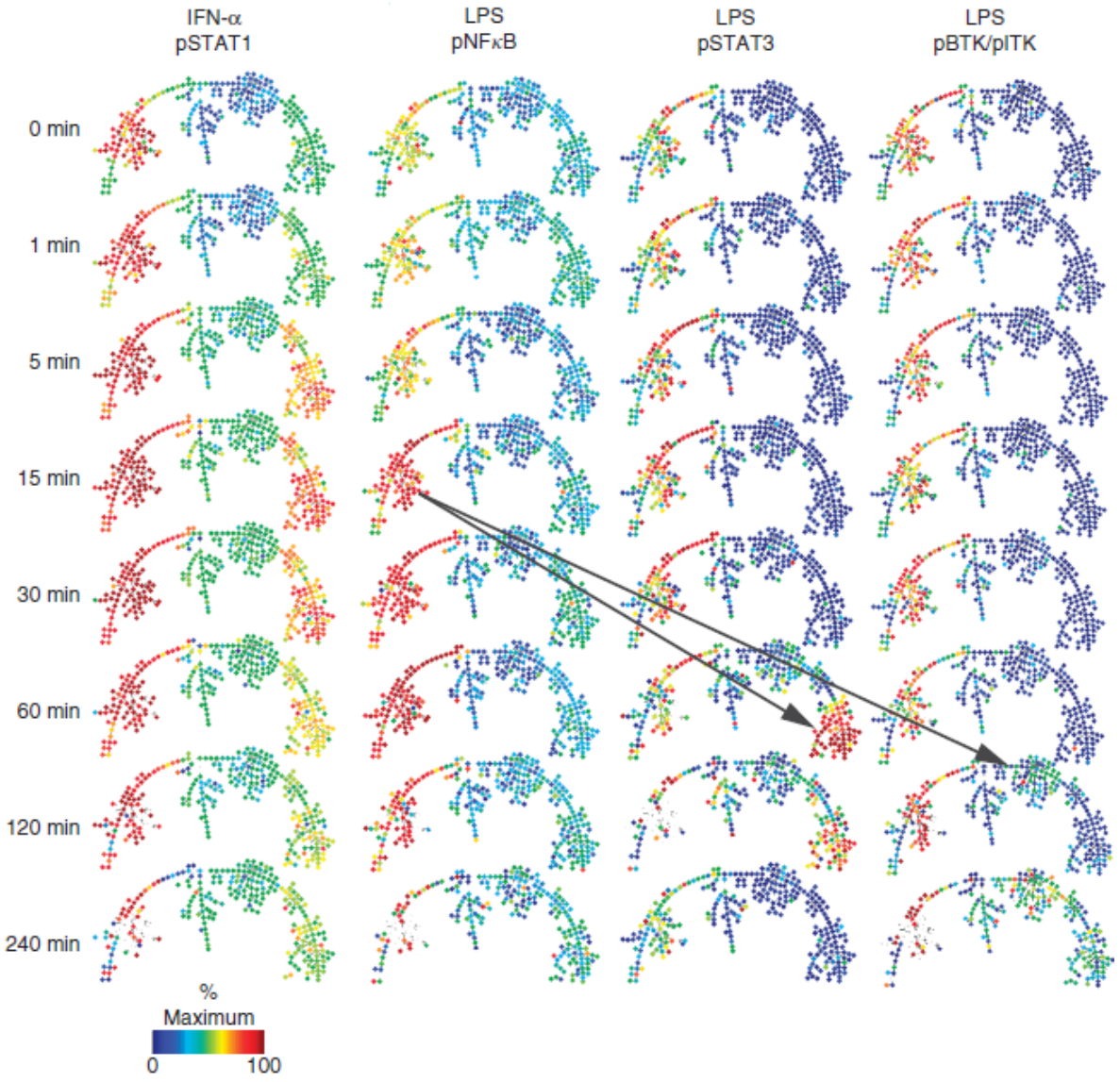
- computational tool
- hierarchically clusters high-dimensional single-cell data
- connects clusters of cells by a minimum spanning tree for two-dimensional visualization

- The cell clusters were generate using 9 cell surface markers, CD33, CD20, CD3, CD4, CD7, CD123, CD14, IgM, and HLA-DR



- The cell surface marker expression levels of these trees were used to define 14 immune cell populations within the PBMC cellular hierarchy





SPADE-generated minimum spanning tree can also be used to

- display the levels of the signaling molecules over the entire 4-hour stimulation time course, revealing subpopulation-specific signaling states and the signaling network dynamics of each cell type and subpopulation

PCA (Principal component analysis)

- used to visualize the differences between various groups in the data, including all cell types, as well as the differences between all inhibitors

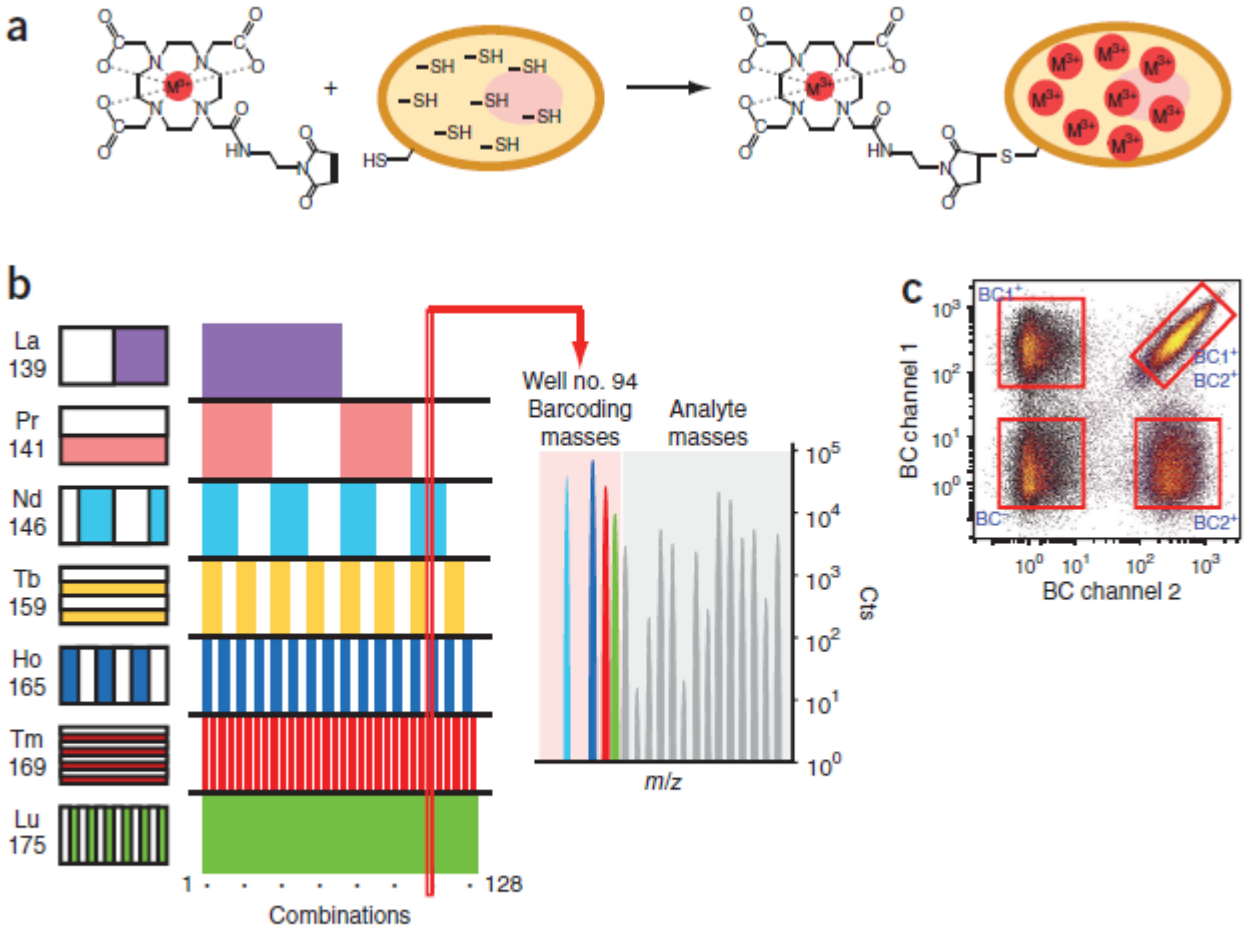
- run on data stratified by various subconditions, including stimulation conditions

Data visualization

- all cell density plots and heat maps were created in *Cytobank* (http://www.cytobank.org/, Cytobank, Inc.)

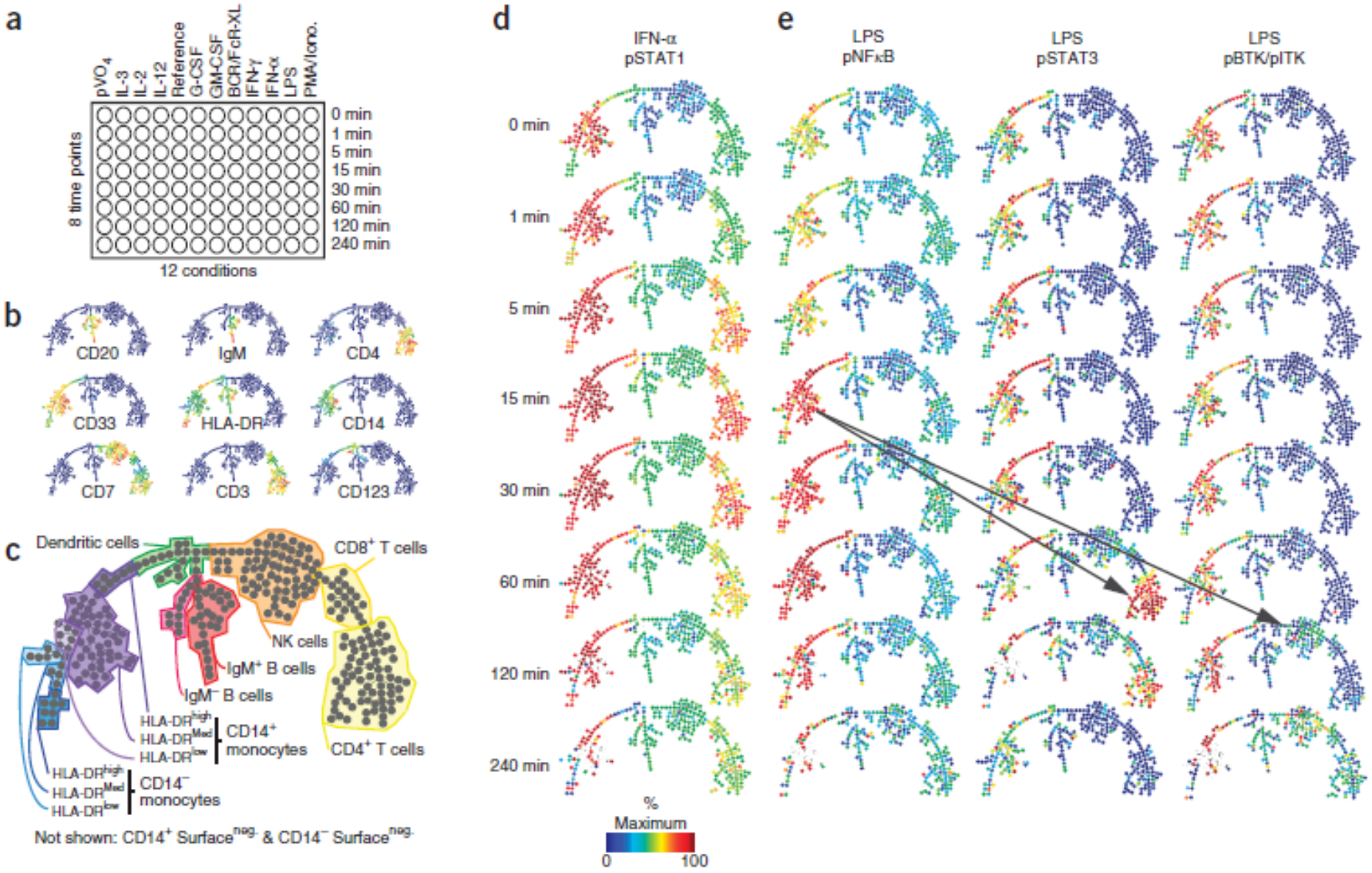
RESULTS

- **Mass-tag cellular multiplexing**

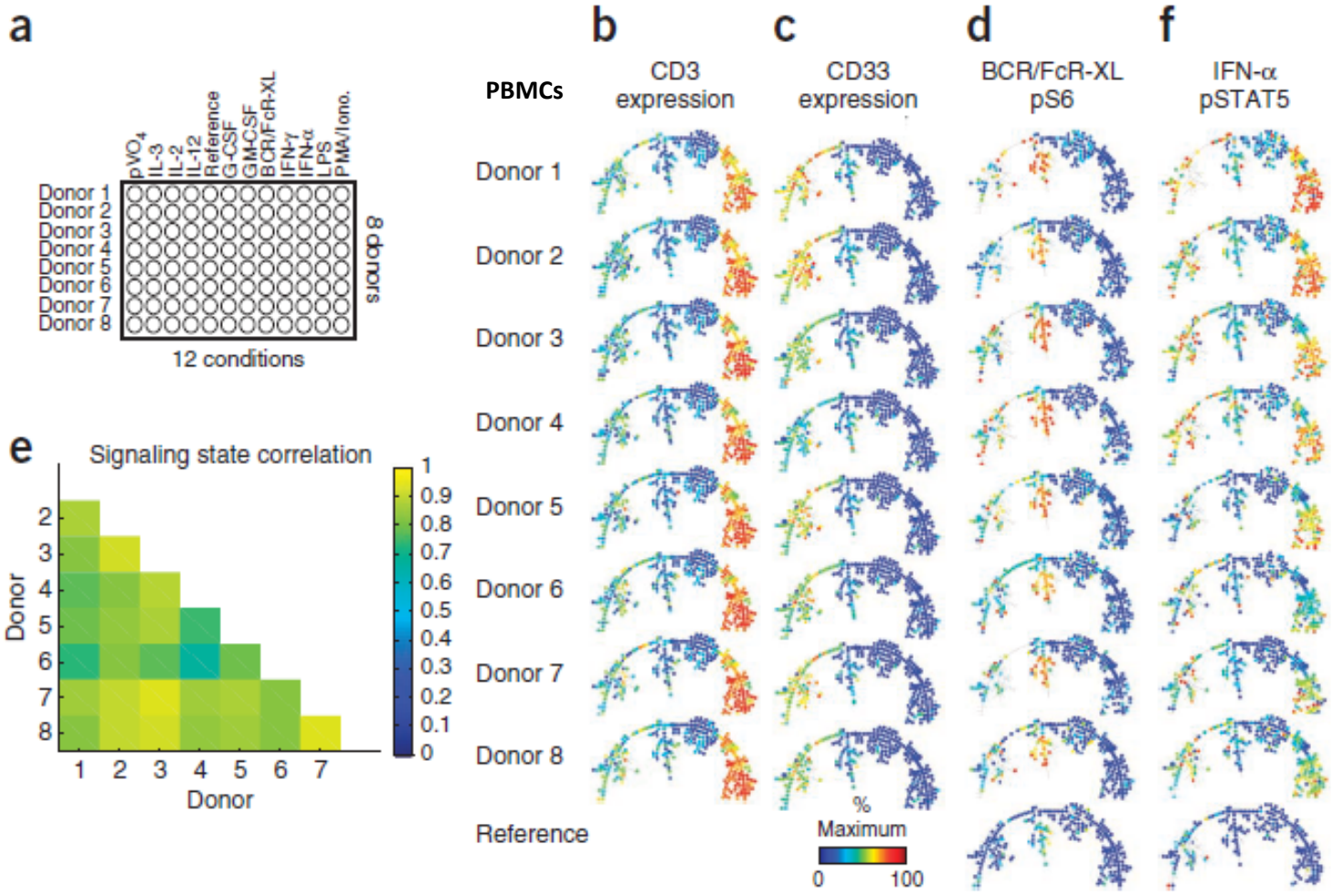


- Cells were covalently labeled with a bifunctional compound, maleimido-mono-amide-DOTA (mDOTA). This compound can be loaded with a lanthanide(III) isotope ion, and reacts covalently with cellular thiol groups through the maleimide moiety.
- Seven unique lanthanide isotopes were used to generate 128 combinations, enough to barcode each sample in a 96-well plate
- A density dot plot of barcoded cells is shown with the *y*-axis and *x*-axis plot showing barcoding (BC) channel 1 (lanthanum 139) versus barcoding channel 2 (praseodymium 141).

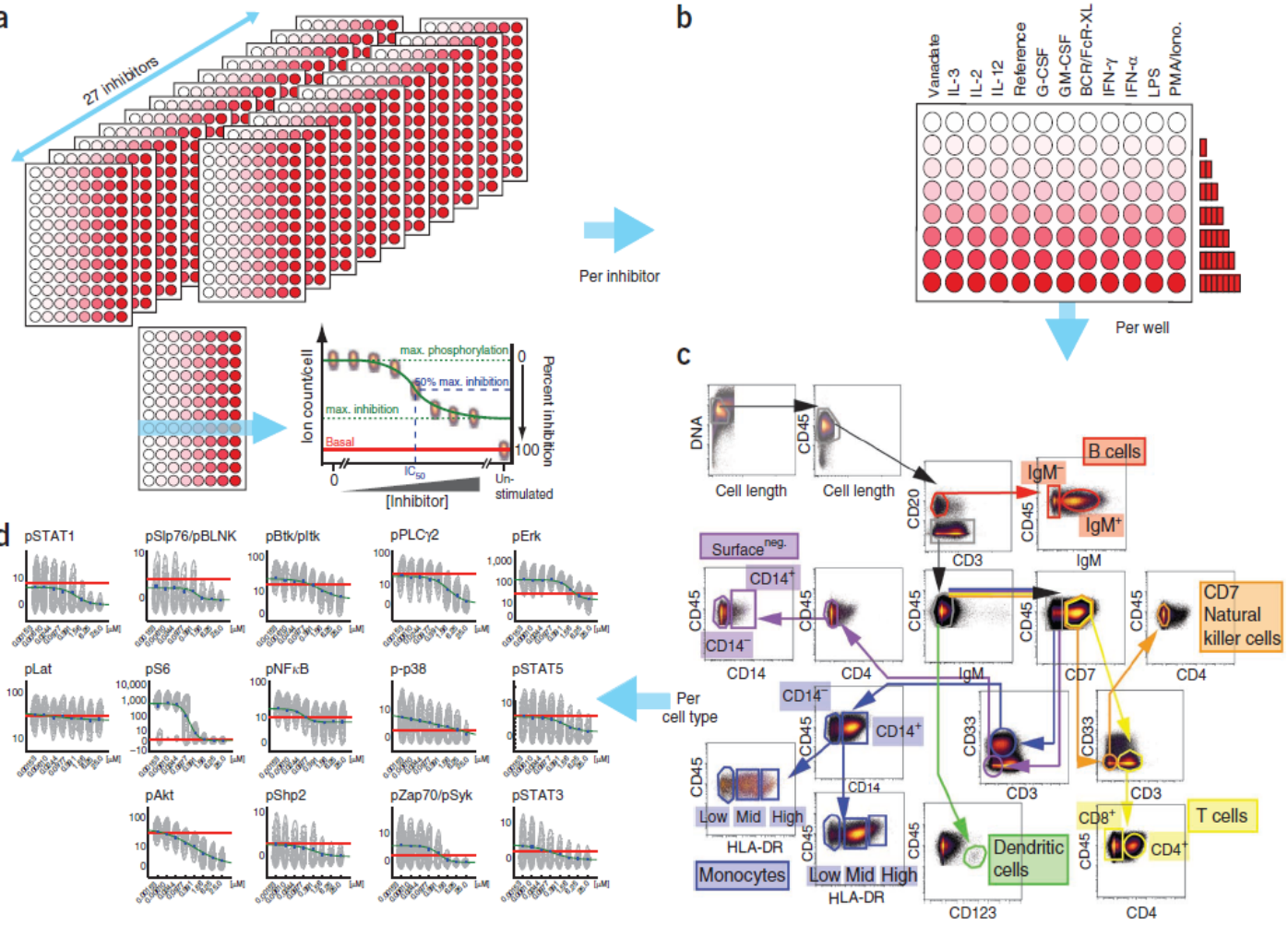
- **Time course analysis of PBMC signaling**



- **Comparison of signaling response in PBMCs from multiple donors**



- **Systematic quantification of PBMC response to kinase inhibition**



- ***18,816 quantified phosphorylation site levels per inhibitor from each multiplexed sample (12 stimuli × 8 doses × 14 cell types × 14 phosphorylation sites)***

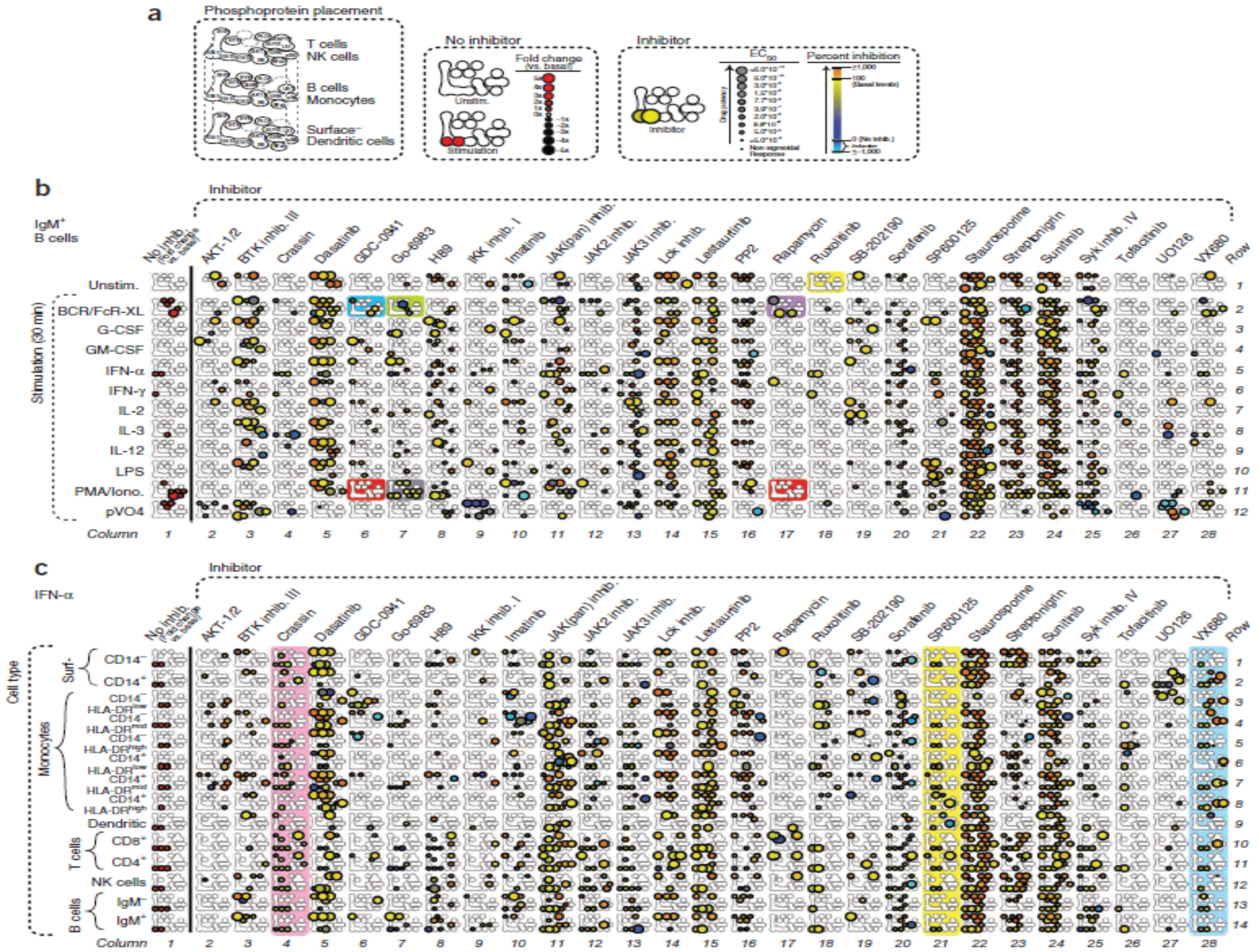
- *[Total 65,504 dose tritations]*

Inhibitor selectivity

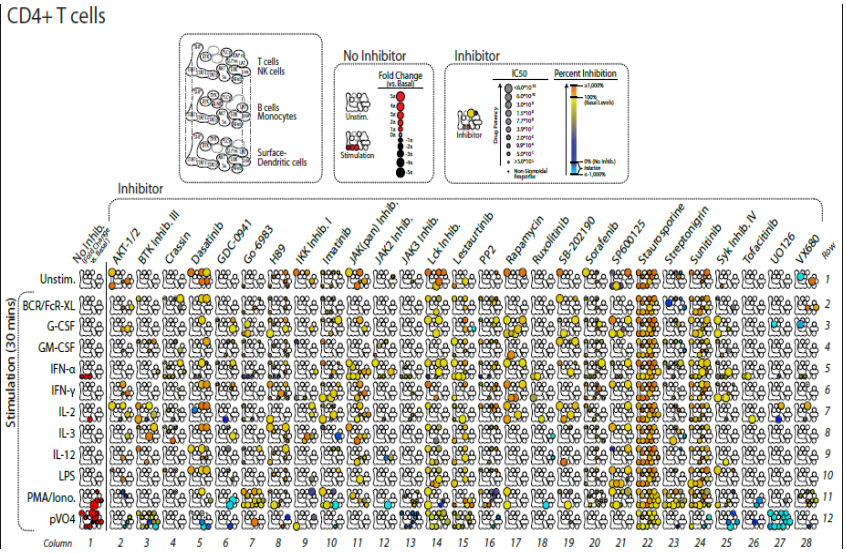
- inhibitors of the JAK-STAT pathway:
- ruxolitinib
- tofacitinib → STAT phosphorylation
- lestauritinib
- JAK2 inhibitor III
- JAK3 inhibitor VI
- pan-JAK inhibitor I
- → agreement with *in vitro kinase inhibition profile*

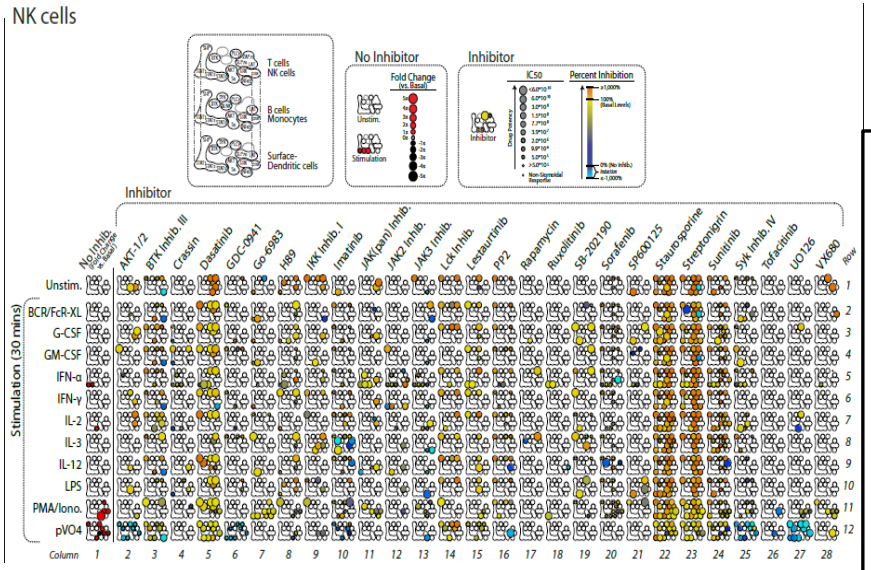
↓

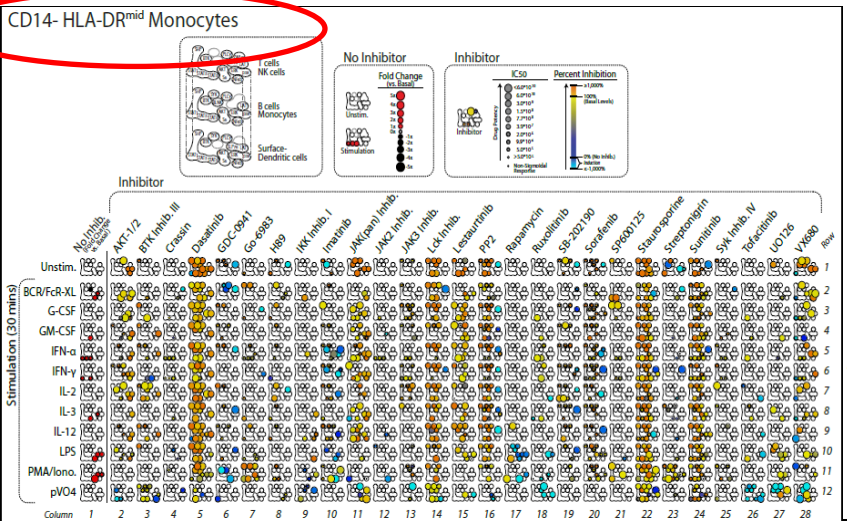
Generation of a cellular inhibitor "fingerprinting"

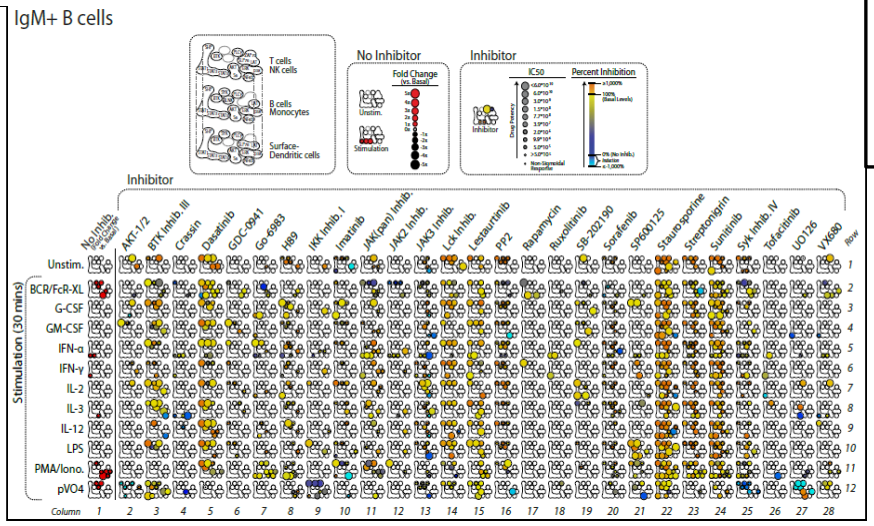

a
Phosphoprotein placement
T cells
NK cells
B cells
Monocytes
Surface⁻
Dendritic cells
No inhibitor
Unstim.
Stimulation
Fold change (vs. basal)
Inhibitor
Inhibitor
EC50
Percent inhibition
Drug potency
Non-sigmoidal Responses
b
Inhibitor
IgM+ B cells
No inhib. (fold change vs. basal)
AKT-1/2
BTK inhib. III
Crassin
Dasatinib
GDC-0941
Go-6983
H89
IKK inhib. I
Imatinib
JAK(pan) inhib.
JAK2 inhib.
JAK3 inhib.
Lck inhib.
Lestaurtinib
PP2
Rapamycin
Ruxolitinib
SB-202190
Sorafenib
SP600125
Staurosporine
Streptonigrin
Sunitinib
Syk inhib. IV
Tofacitinib
UO126
VX680
Row
Stimulation (30 min)
Unstim.
BCR/FcR-XL
G-CSF
GM-CSF
IFN-α
IFN-γ
IL-2
IL-3
IL-12
LPS
PMA/Iono.
pVO4
Column
c
Inhibitor
IFN-α
Cell type
Surf-
CD14⁻
CD14⁺
Monocytes
CD14⁻ HLA-DRlow
CD14⁻ HLA-DRmid
CD14⁻ HLA-DRhigh
CD14⁺ HLA-DRlow
CD14⁺ HLA-DRmid
CD14⁺ HLA-DRhigh
Dendritic
T cells
CD8⁺
CD4⁺
NK cells
B cells
IgM⁻
IgM⁺

Cell type selectivity



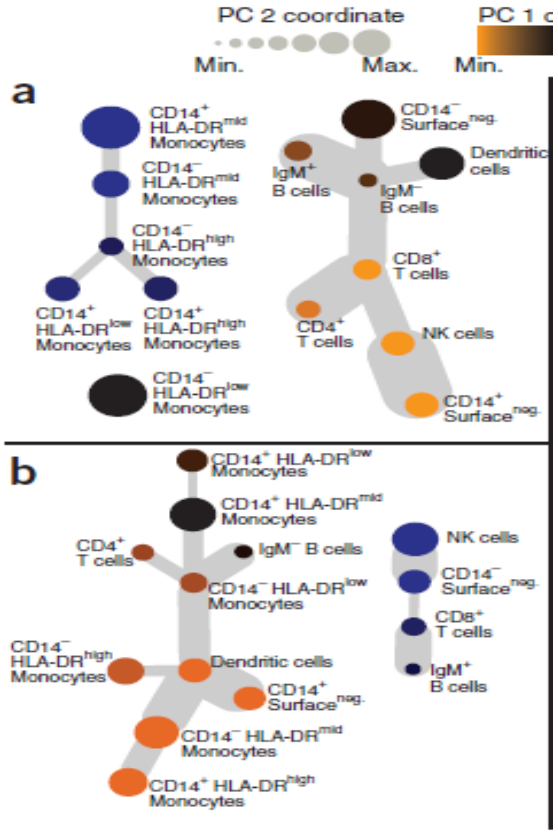






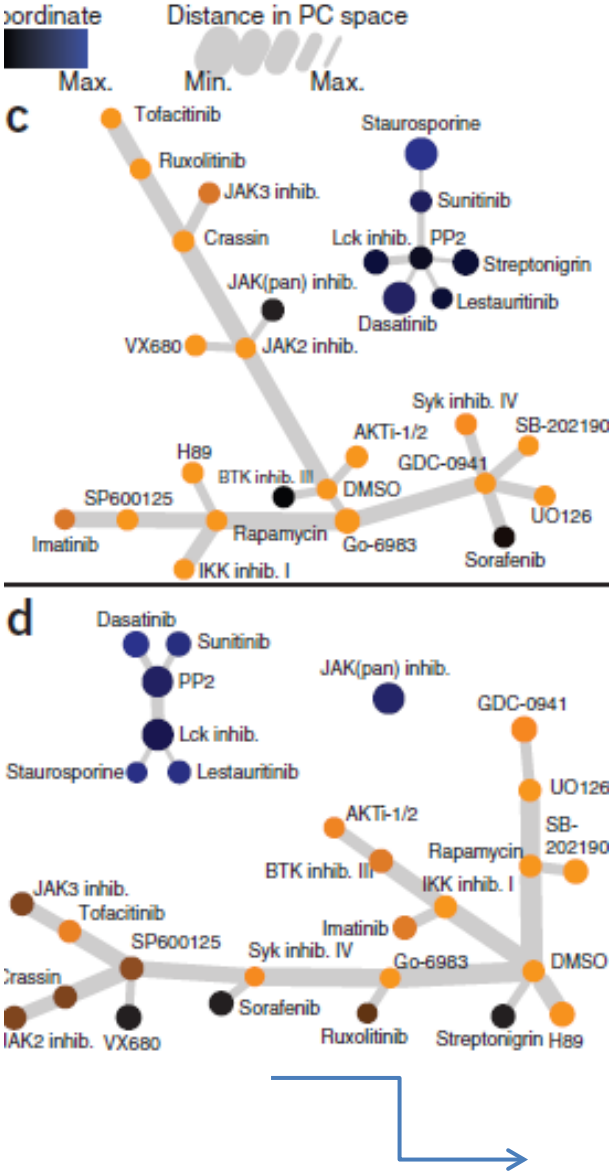
can be used to reveal how different cell types and their underlying signaling network states are uniquely affected by given inhibitors

- **Systematic analysis of cell type and inhibitor similarity**



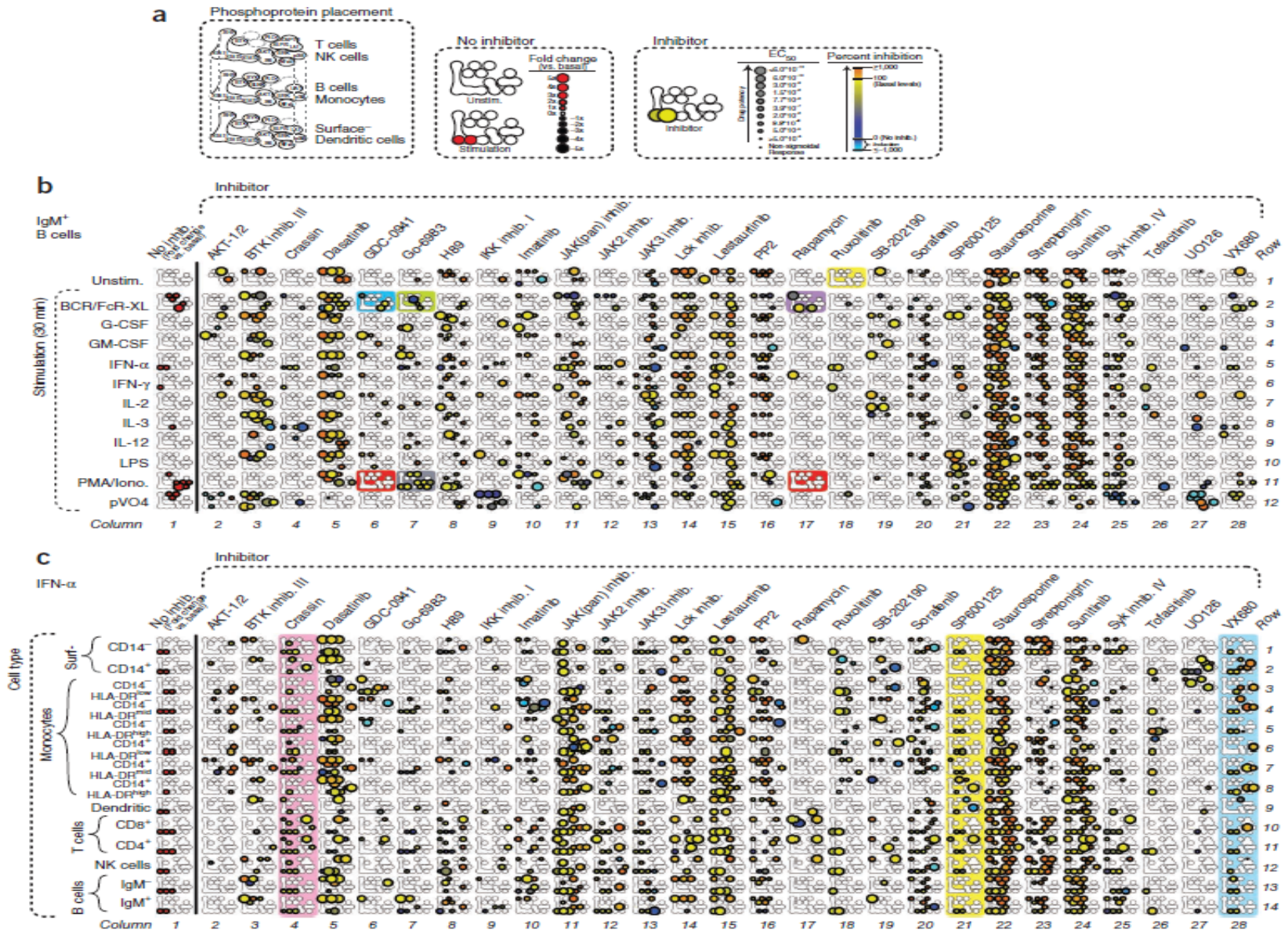
STREPTONIGRIN

Inhibitor similarity

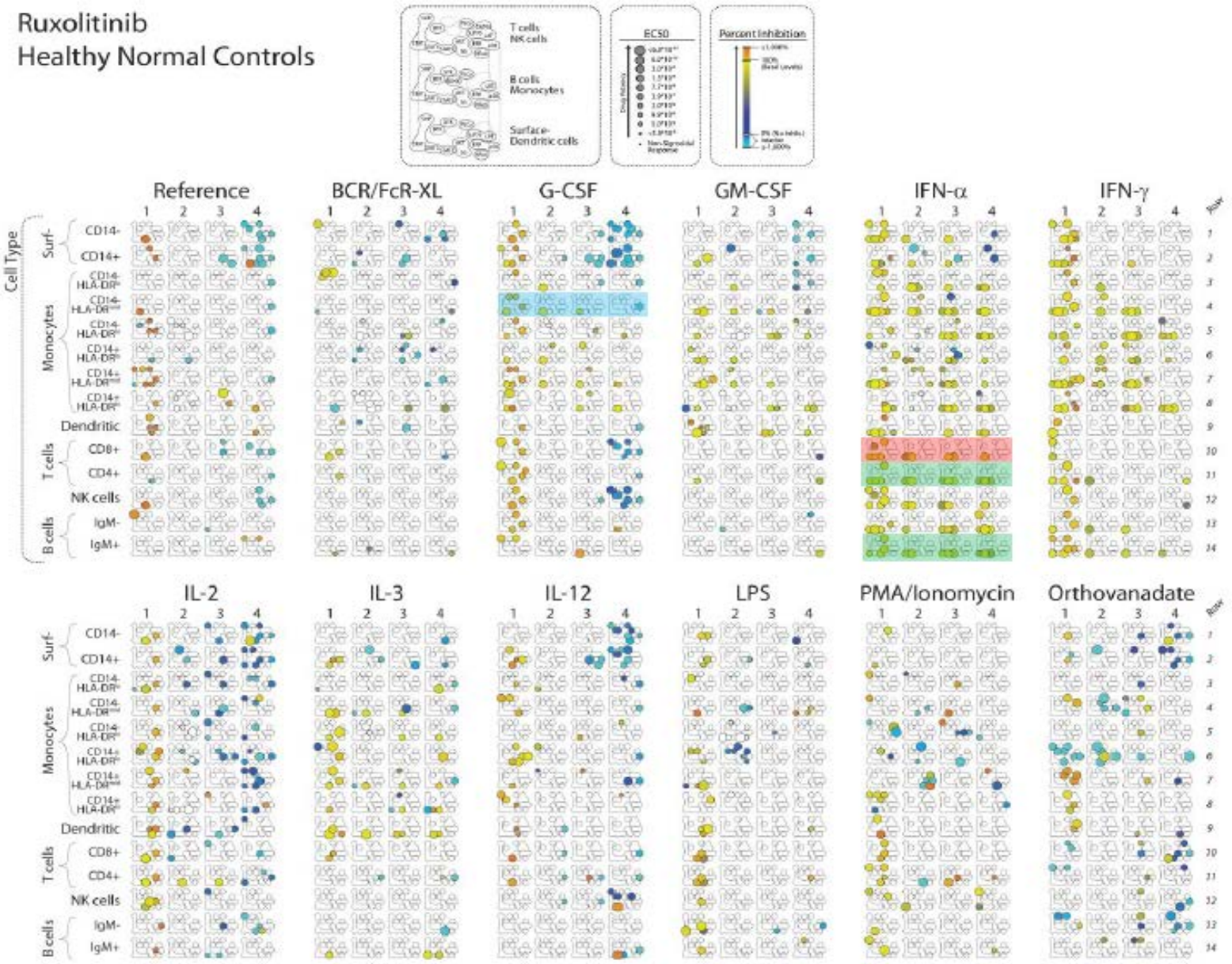


•rapid classification of inhibitors based on their profiles at a given drug exposure or in a given experimental condition(inhibitors SP600125 and VX680)

•characterization and identification of similar cell type responses to a given inhibitor


a
Phosphoprotein placement
T cells
NK cells
B cells
Monocytes
Surface⁻
Dendritic cells
No inhibitor
Unstim.
Stimulation
Fold change (vs. basal)
Inhibitor
Inhibitor
EC50
Percent inhibition
b
Inhibitor
IgM+ B cells
No inhib. (+ stim.)
AKT-1/2
BTK inhib. III
Crassin
Dasatinib
GDC-0941
Go-6983
H89
IKK inhib. I
Imatinib
JAK(pan) inhib.
JAK2 inhib.
JAK3 inhib.
Lck inhib.
Lestaurtinib
PP2
Rapamycin
Ruxolitinib
SB-202190
Sorafenib
SP600125
Staurosporine
Streptonigrin
Sunitinib
Syk inhib. IV
Tofacitinib
UO126
VX680
Row
Stimulation (30 min)
Unstim.
BCR/FcR-XL
G-CSF
GM-CSF
IFN-α
IFN-γ
IL-2
IL-3
IL-12
LPS
PMA/Iono.
pVO4
Column
c
Inhibitor
IFN-α
Cell type
Surf-
CD14⁻
CD14⁺
Monocytes
CD14⁻ HLA-DR^neg
CD14⁻ HLA-DR^mid
CD14⁻ HLA-DR^high
CD14⁺ HLA-DR^low
CD14⁺ HLA-DR^mid
CD14⁺ HLA-DR^high
Dendritic
T cells
CD8⁺
CD4⁺
NK cells
B cells
IgM⁻
IgM⁺
Column

• **Comparison of inhibition response in PBMCs from multiple donors**



CONCLUSIONS

• MCB makes possible high-throughput experiments that are impractical to do using FBFC or mass cytometry alone

• allows analyses that span from the systems-level down to single pathways and molecules

• provides an opportunity to study the connectivity of signaling pathways, the effects of inhibitors on feedback signaling, and intercellular communication

• could be used to categorize drug effects or drug combinations, to eventually guide therapeutic strategies based on discrete knowledge of a patient's cellular phenotypes and genotypes

• could be used directly as a tool for personalized medicine, with the pathway activation and drug response of a patient's in vivo or ex vivo tissue samples used to guide therapy decisions

THANK YOU FOR YOUR ATTENTION