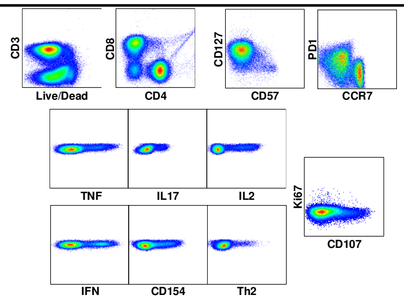
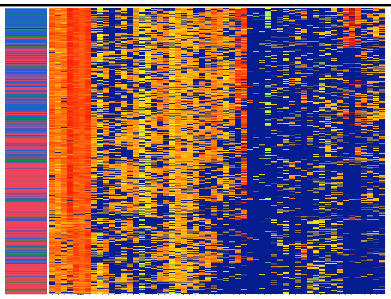




Integrating Flow Cytometry and Single Cell Transcriptomics: Instrumental Synergy

Mario Roederer

Vaccine Research Center

The National Institutes of Health

T Cell Functions

T cells are capable of a large repertoire of cellular functions:

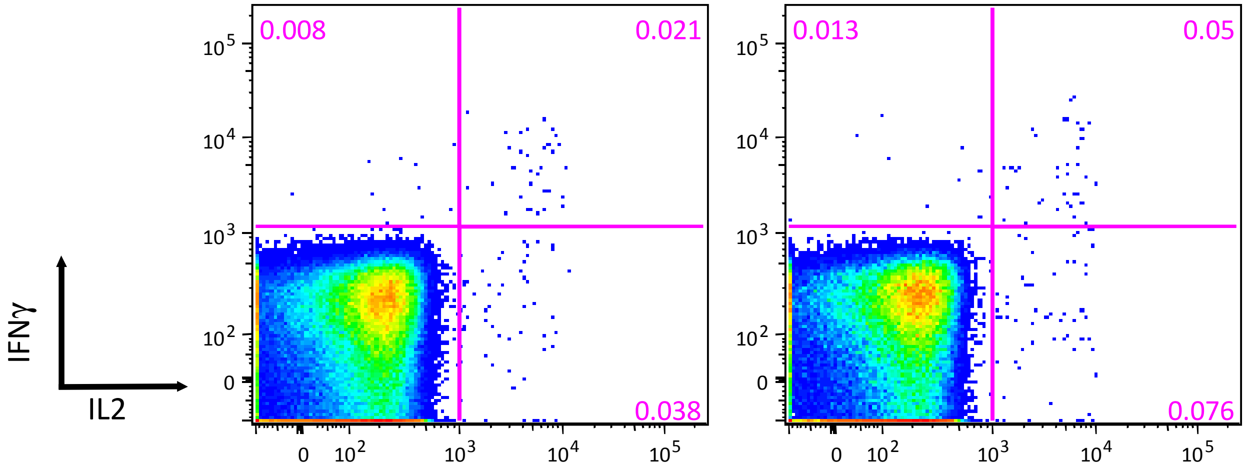
- Killing
- Proliferation
- Secreting Effector Molecules (cytokines)
  - Orchestrate immune responses
  - Induce inflammation
  - Kill target cells

Using flow cytometry, we can measure these on a *cell-by-cell* basis, to quantify the different types of effector T cells present.

One Function is Not Enough!

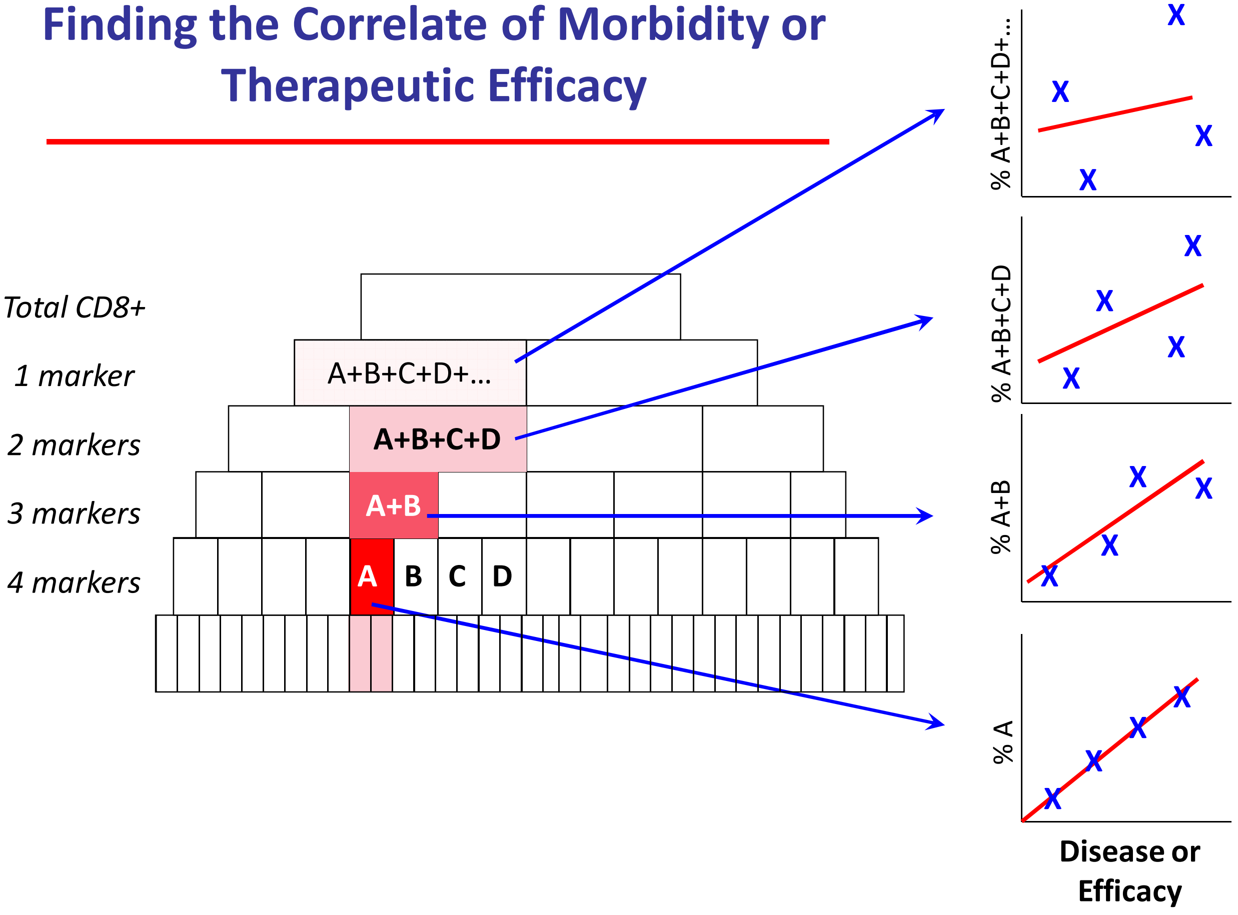
De Rosa et al., J. Immunology 173 (2004)

CD4 T cells



Total Response:	0.067	0.139
γIFN+	0.029 (43%)	0.063 (45%)
IL2+	0.059 (88%)	0.126 (91%)

Finding the Correlate of Morbidity or Therapeutic Efficacy



The Search for Immune Correlates

Antigen-specific lymphocytes display enormous heterogeneity:

- Differentiation stage (CD62L, CCR7, CD45Rx, CD95, CD28, CD27, CD57, CD11a…)
- Homing profile (a4b7, CD103, CCR9, CLA…)
- Regulatory molecules (PD1, TIM3, LAG3, KIRs, CTLA4, ICOS…)
- Stimulated effector functions (dozens of cytokines, chemokines, degranulation, proliferation…)

Protective responses almost certainly comprise cells expressing a *pattern* of *multiple functions.*

Even today's state-of-the-art immunophenotyping panels cannot fully interrogate potential subsets.

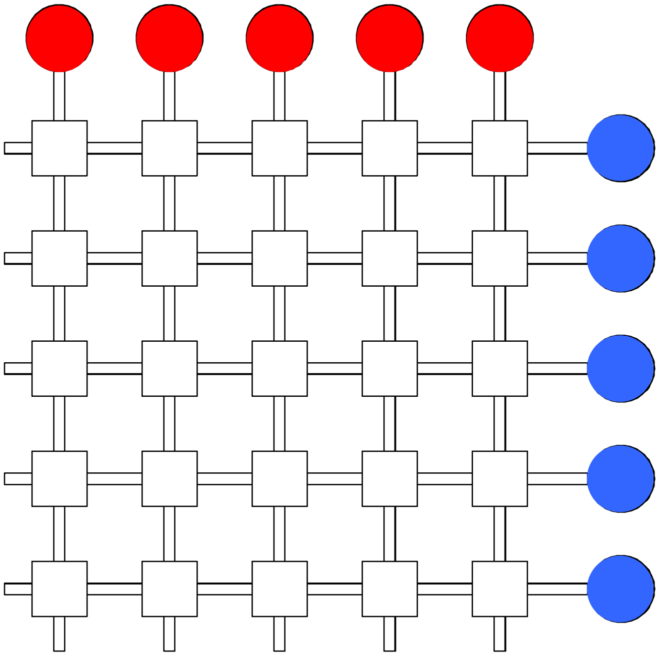
***Single-cell transcriptomics is part of the solution*.**

Fluidigm BioMark Technology

Dispense cDNA into sample vessels

Primers & probes into reagent vessels

Microfluidics Chip

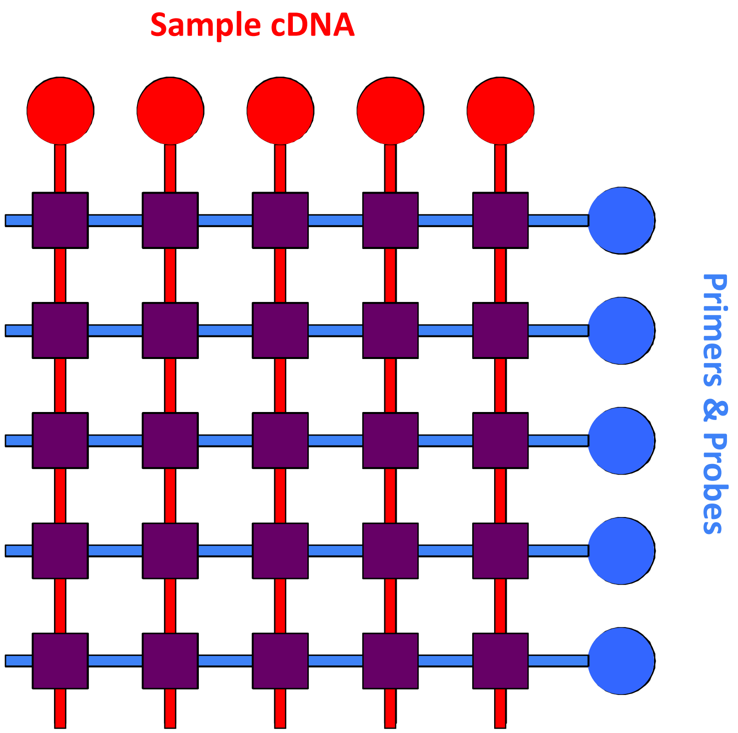


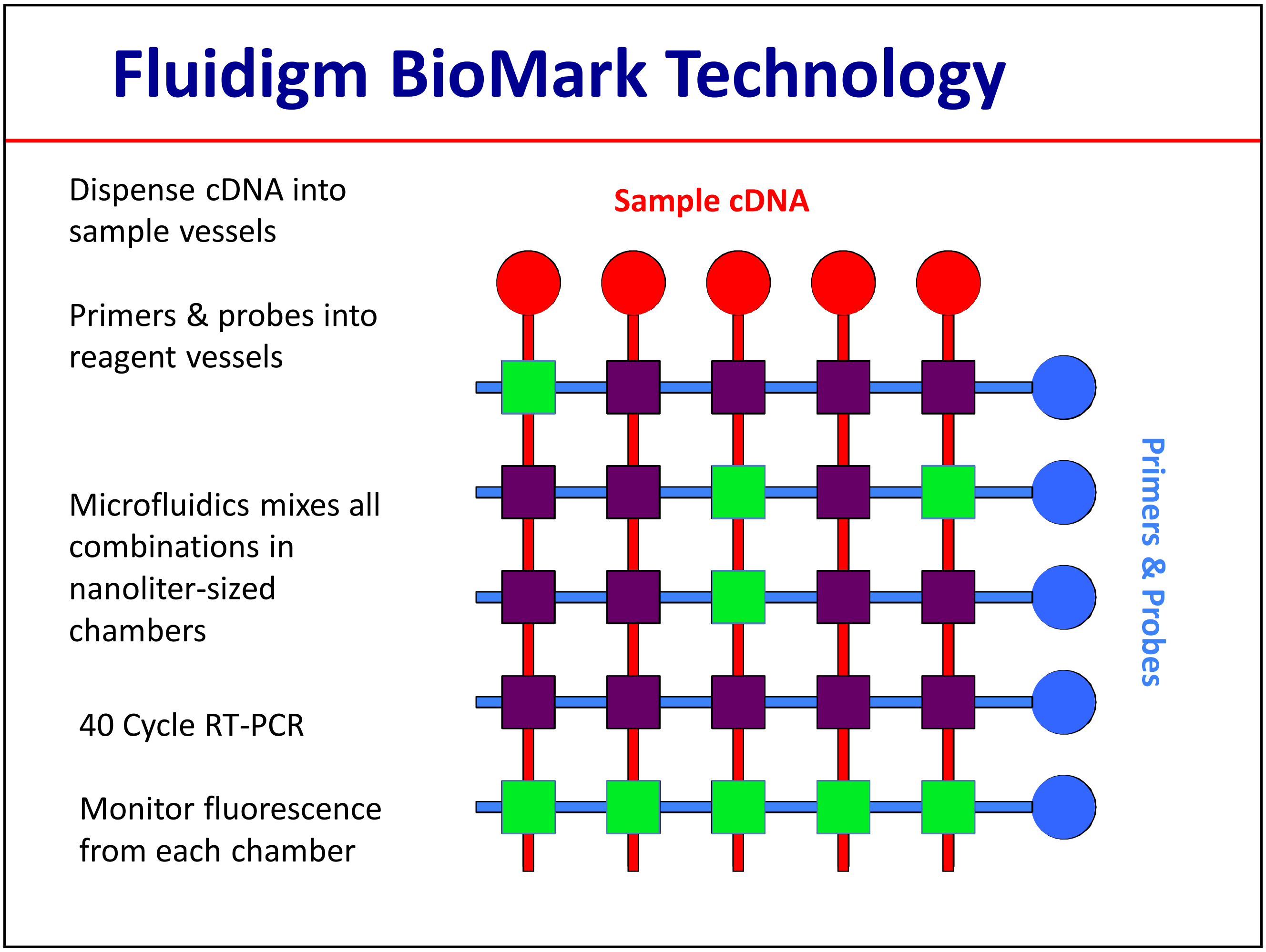
Fluidigm BioMark Technology

Dispense cDNA into sample vessels

Primers & probes into reagent vessels

Microfluidics mixes all combinations in nanoliter-sized chambers





Fluidigm BioMark Technology

Dispense cDNA into sample vessels

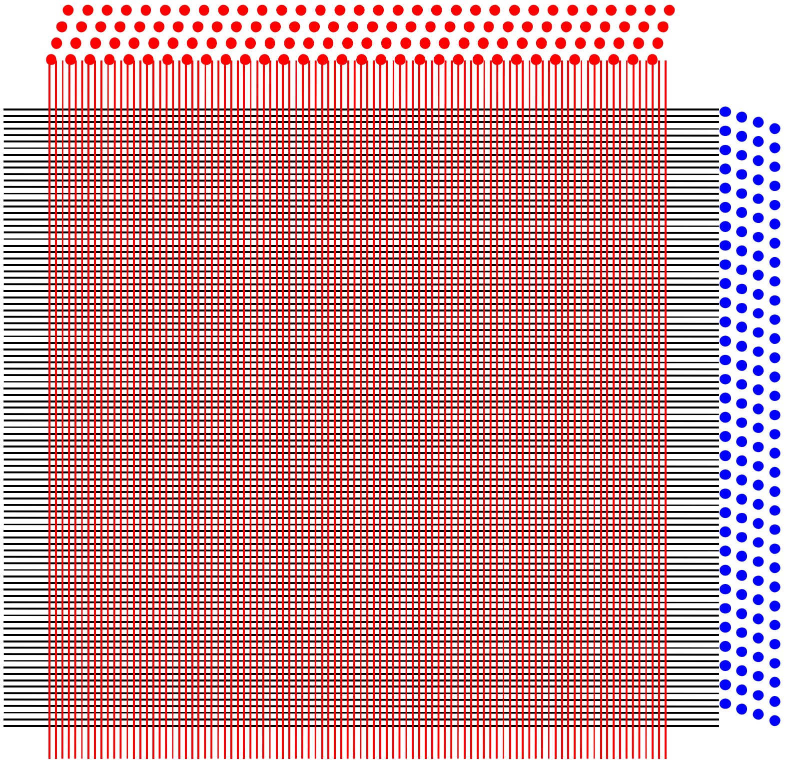
Primers & probes into reagent vessels

Microfluidics mixes all combinations in nanoliter-sized chambers

40 Cycle RT-PCR

Monitor fluorescence from each chamber

Fluidigm BioMark



Samples (96 cDNAs)

Primers & Probes
(96 different genes)

9,216 simultaneous
RT PCR reactions

Assessing Gene Expression

"Nanoarray"

Sort 50-5000 desired cells… Quantify gene expression of 96 selected genes (can multiplex plates for 192, 384, … genes)

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"Nanoarray"

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In principle, similar to microarray analysis… but:

- Highly directed vs. 40,000 genes
  - Disadvantage: Not interrogating the entire genome; gene selection bias
  - Advantage: Much smaller statistical penalty = more sensitive
- No pre-amplification; extremely sensitive (1 copy)
- Large dynamic range (RT PCR: ~10^4), linear, high degree of precision

Assessing Gene Expression

"Nanoarray"

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- Large dynamic range (RT PCR: ~10^4), linear, high degree of precision

Single cell

Sort 1 cell per well… Quantify gene expression of 96 selected genes

- No loss in sensitivity or specificity compared to Nanoarray

Single-Cell Expression Profiling

RV144:

Is there a signature of vaccine-elicited CD4 T cells associated with durable antibody responses (or protection)?

HIV/SIV-Productive Infection:

Is there a signature of productively or latently-infected cells?

RV144: HIV Vaccine Trial

Analysis of RV144 showed that antibodies were a correlate… but responses waned.

Total T cell responses (which were weak) did not correlate. *However*, a secondary analysis reveals that IL10 and IL13 production by PBMC following T cell stimulation *may be* a correlate.

Can we identify CD4 T cell responses that predict protection and/or durable humoral responses?

Fluidigm & RV144

We applied the nanoarray and single-cell profiling to samples from RV144 to characterize the functions of vaccine-elicited CD4 T cells.

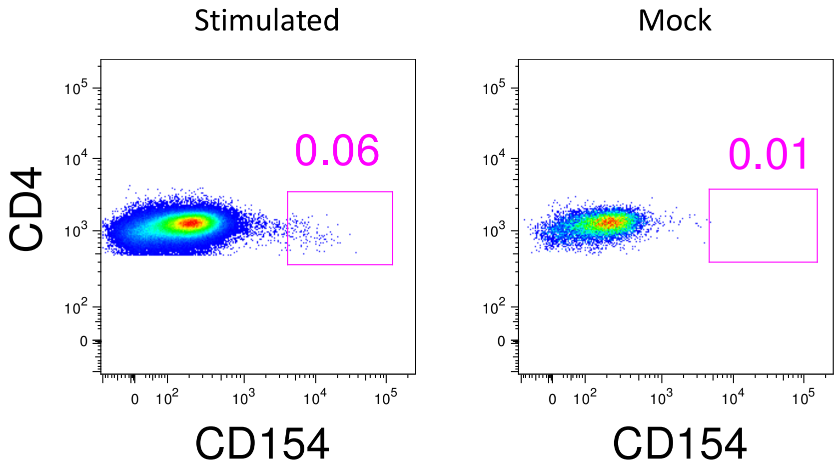
RV144 visit 8 samples (n=50: 40 vaccinees, 10 placebo)

PBMC were stimulated with HIV env peptides; the CD154 assay (5 hour stimulation) was used to sort live vaccine-specific CD4 T cells.

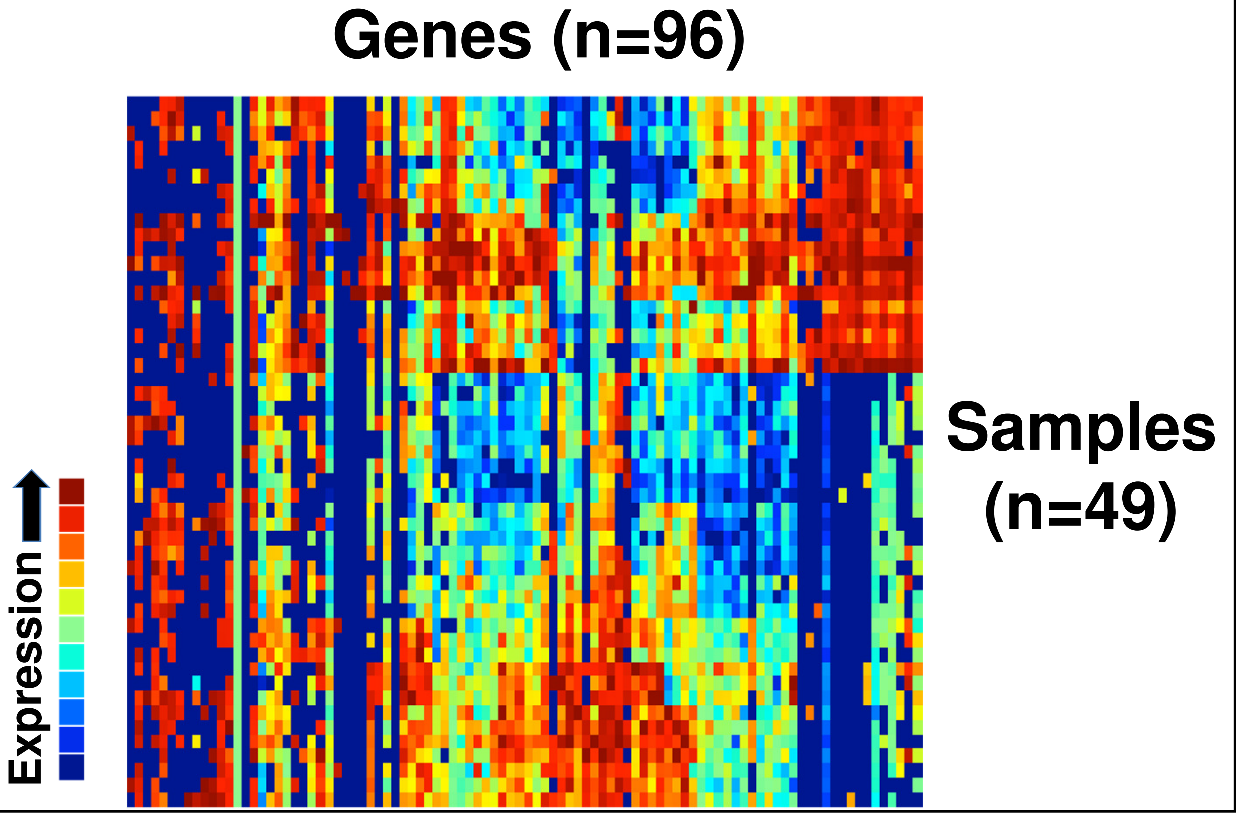
Fluidigm & RV144

Vaccine-elicited HIV-specific CD4 T cells are very rare!

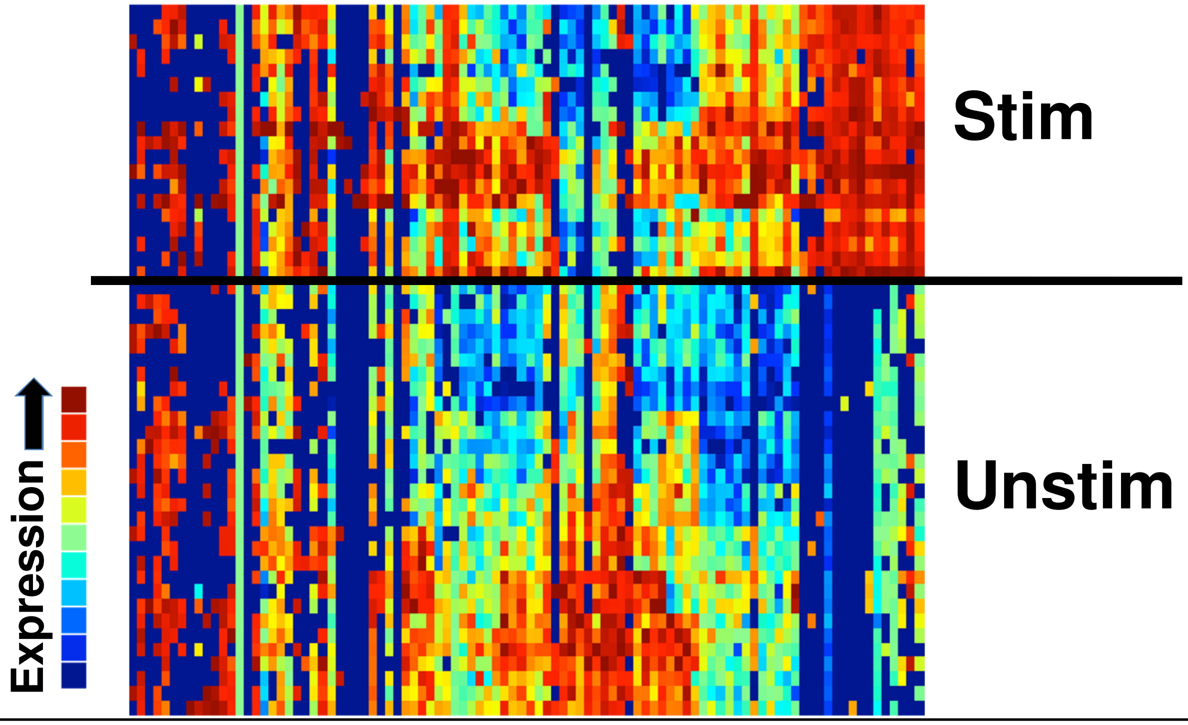
Range: 0.03 – 0.08% (**mean 0.05%**)
>4x above background (~20% "nonspecific" cell contamination)



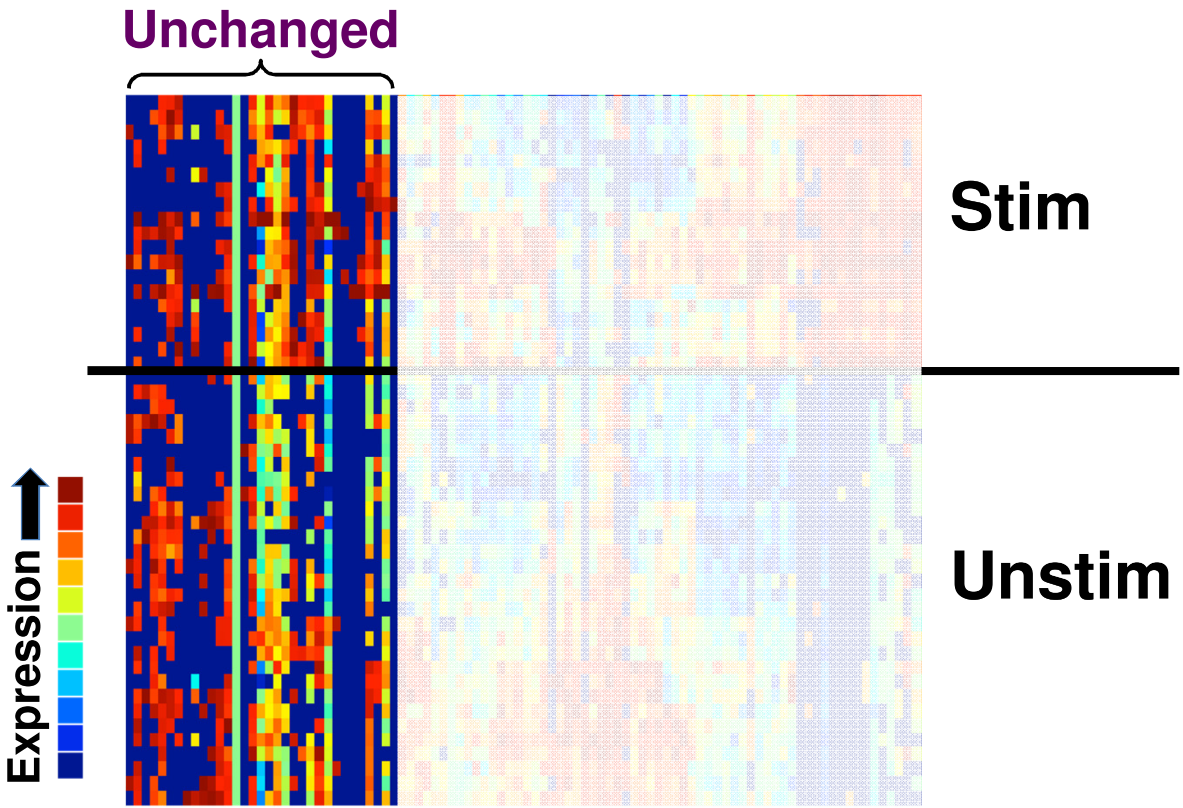
Nanoarray Analysis



Unsupervised Clustering



Stimulation-regulated Genes



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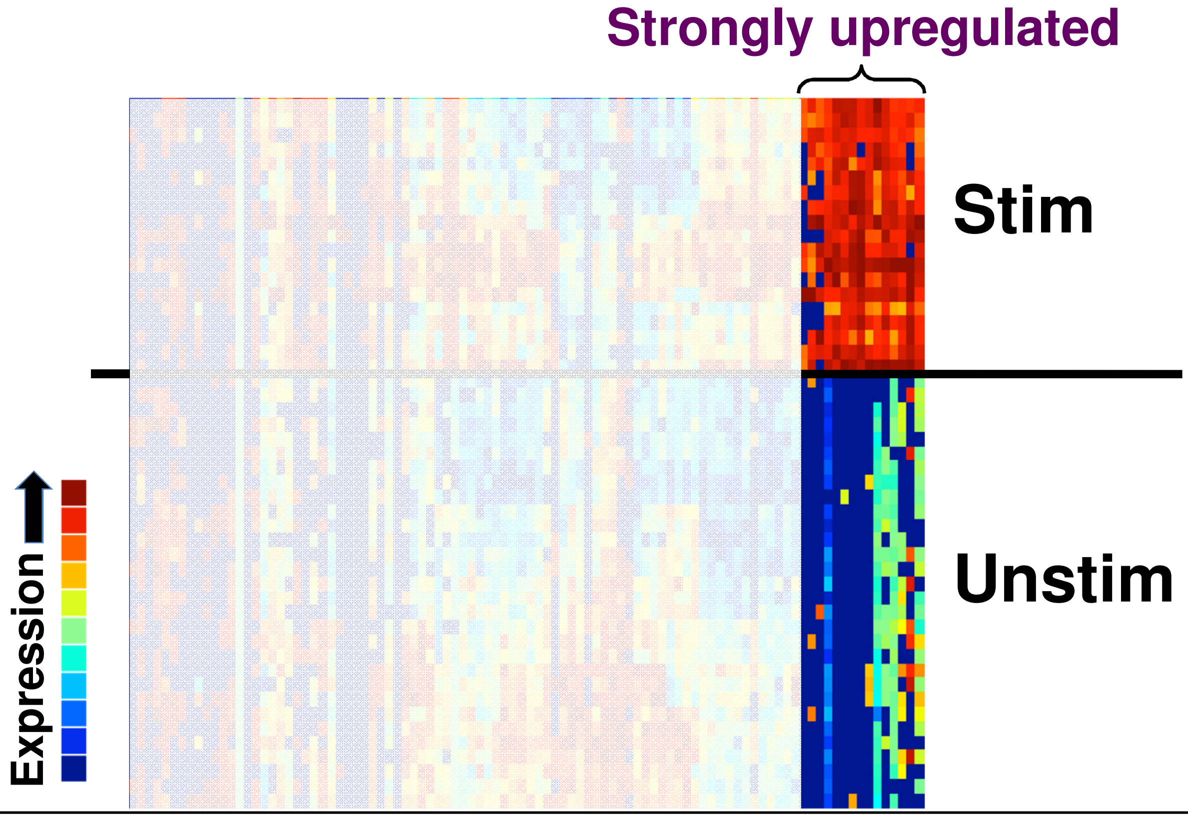
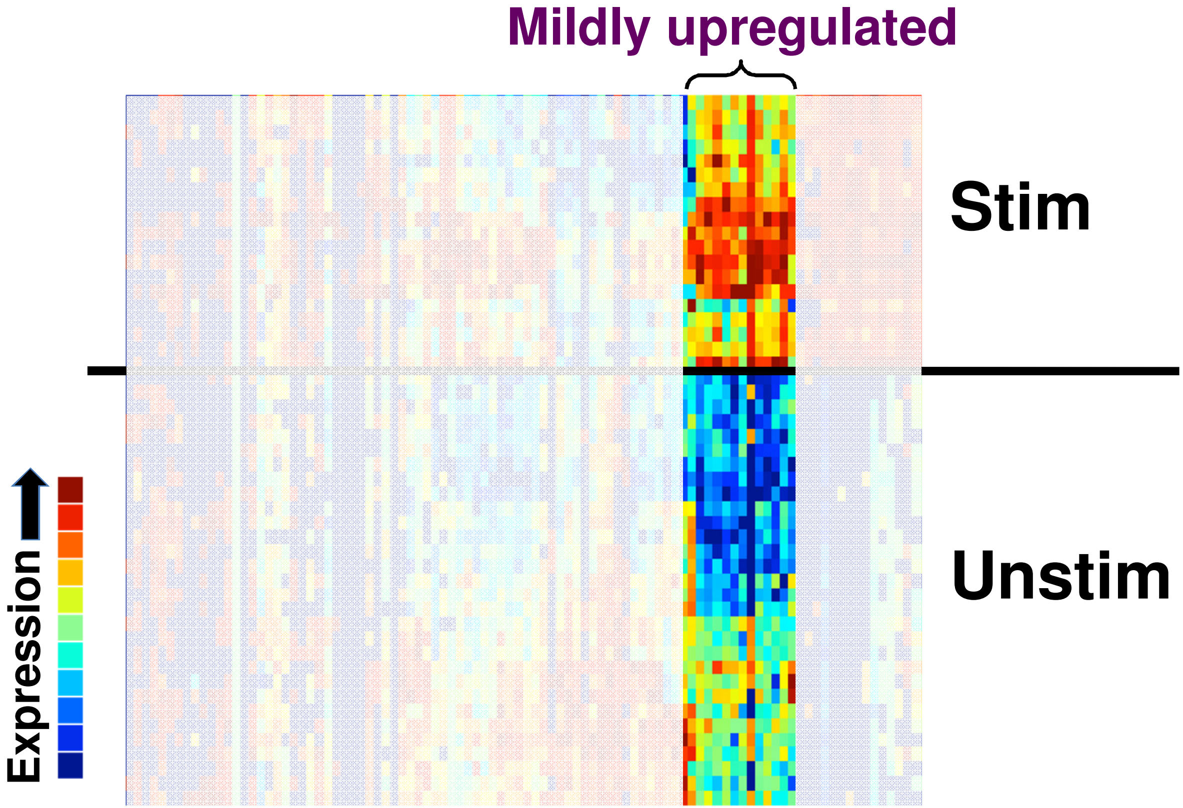
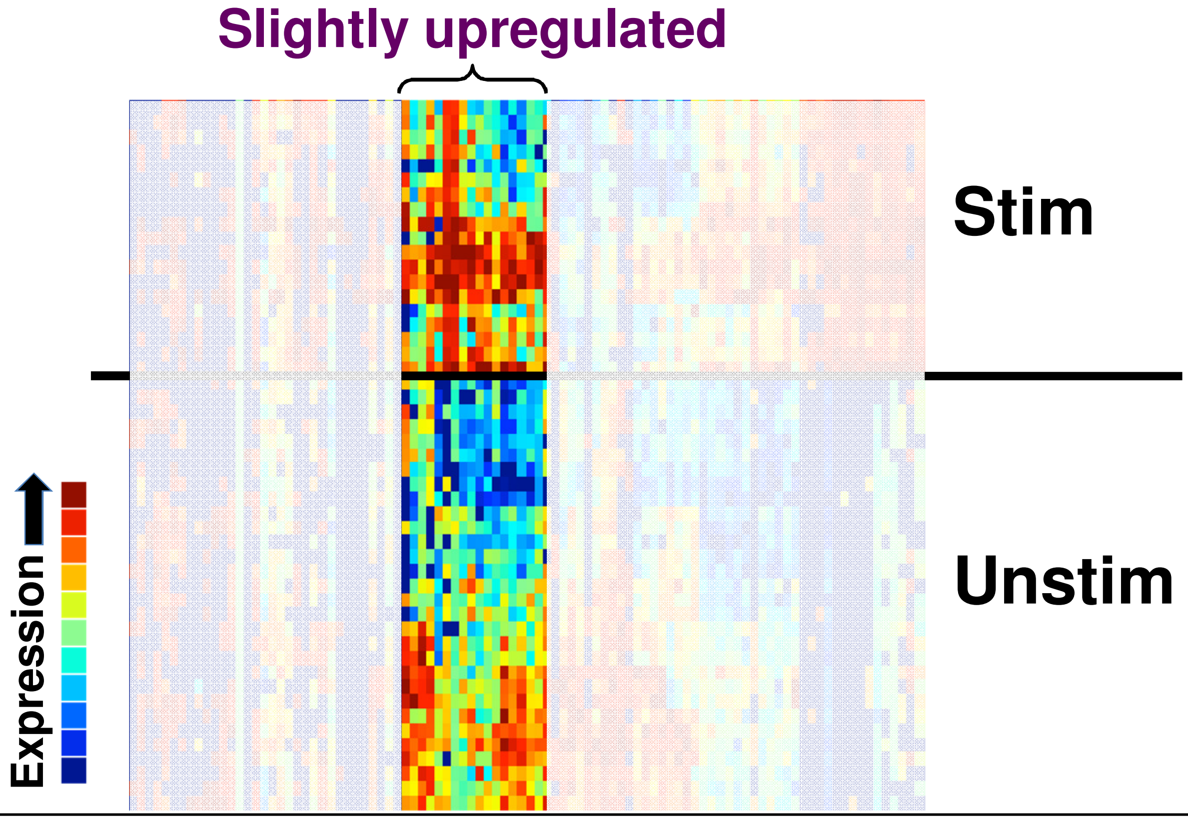


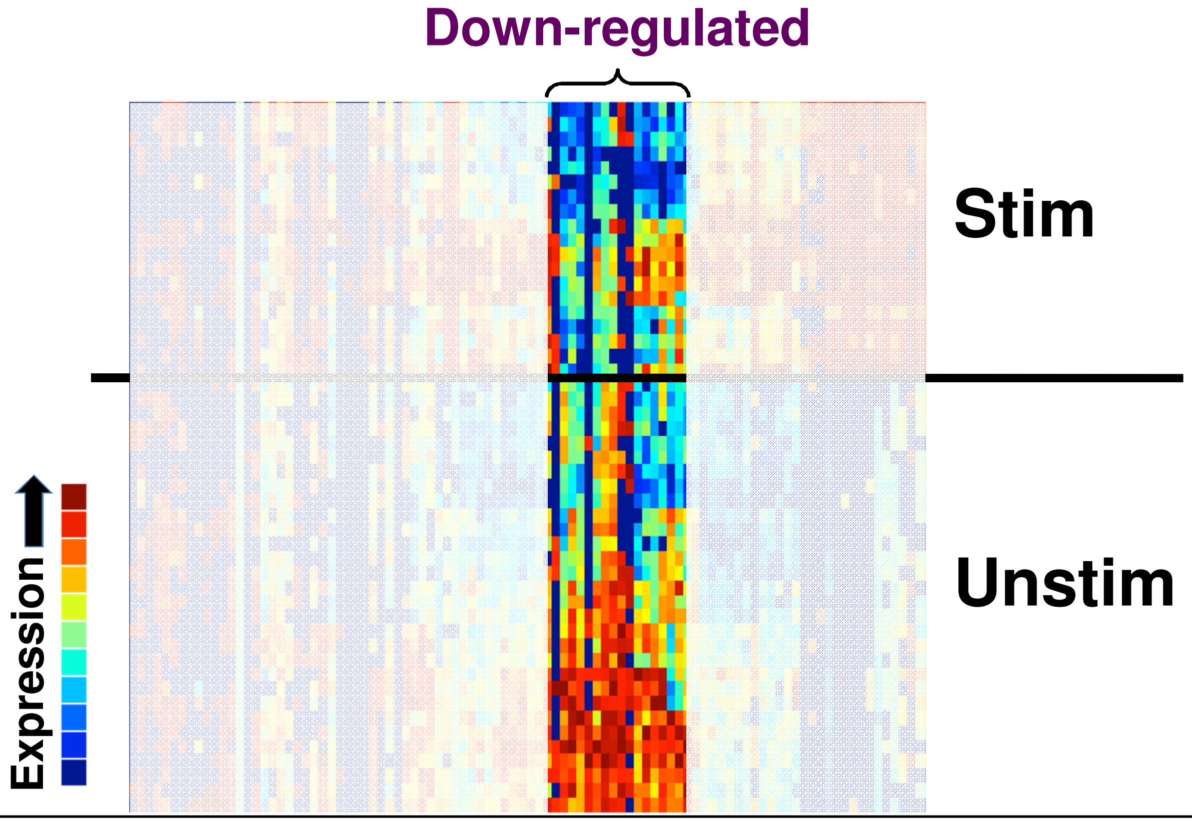
Stimulation-regulated Genes

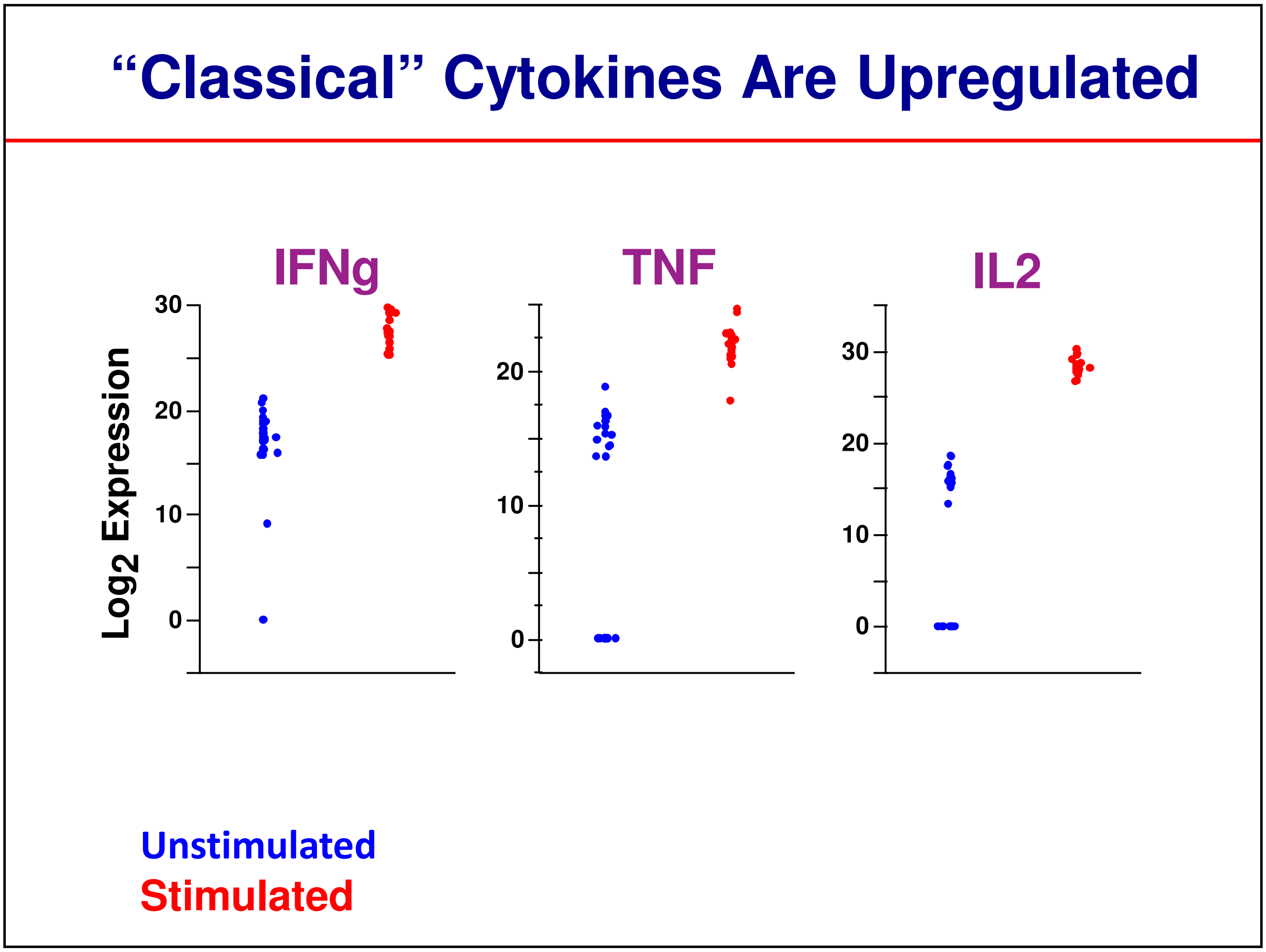


Stimulation-Regulated Genes



Stimulation-regulated Genes

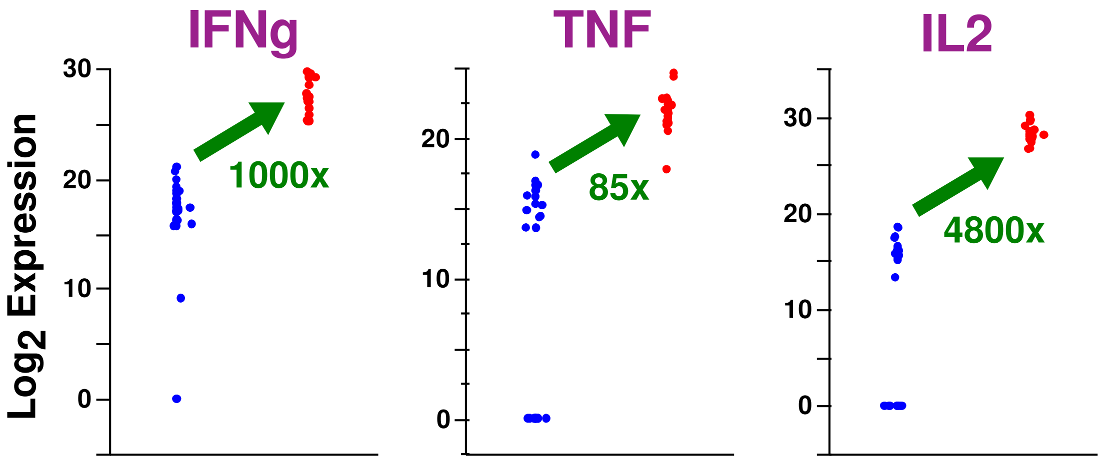




“Classical” Cytokines Are Upregulated

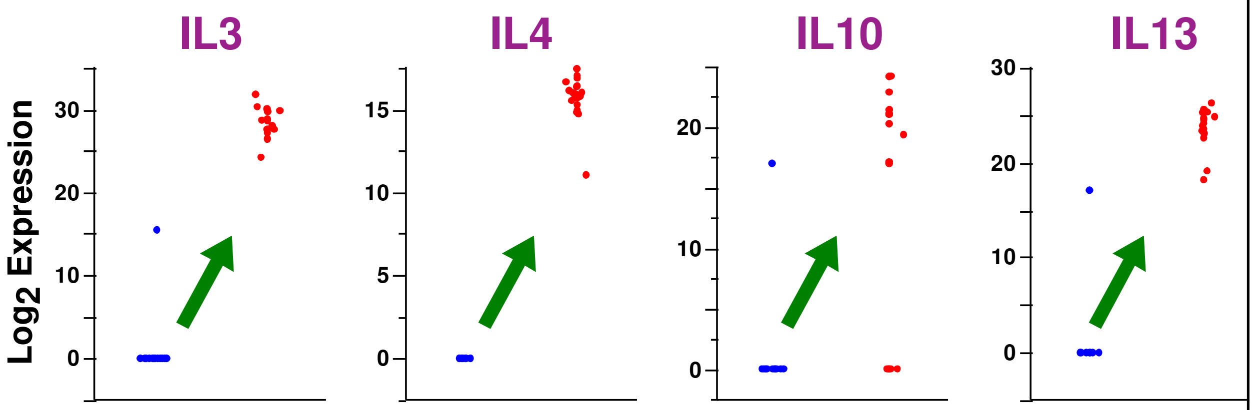
Unstimulated
Stimulated

“Classical” Cytokines Are Upregulated

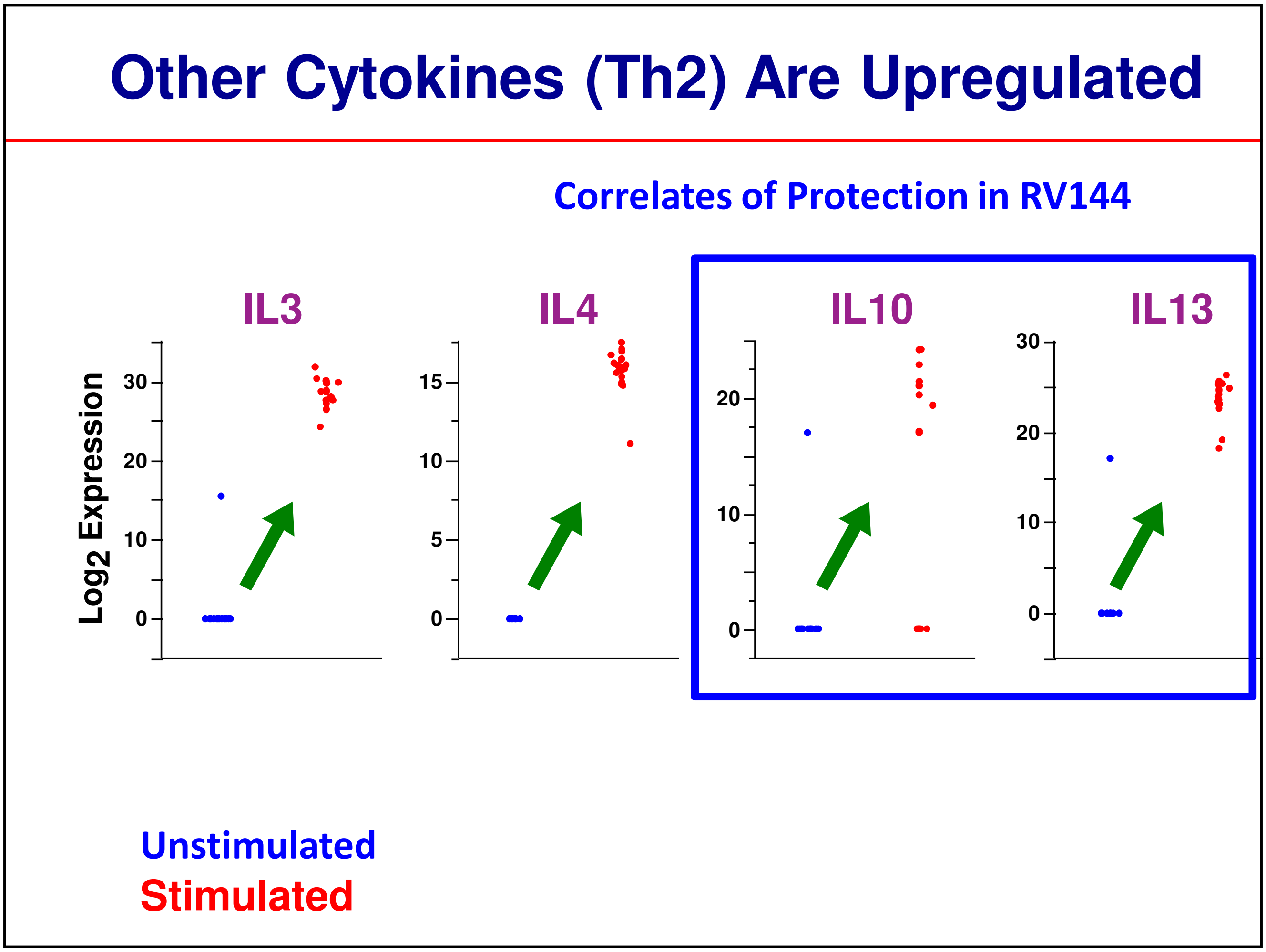


Unstimulated
Stimulated

Other Cytokines (Th2) Are Upregulated



Unstimulated
Stimulated



Other Cytokines (Th2) Are Upregulated

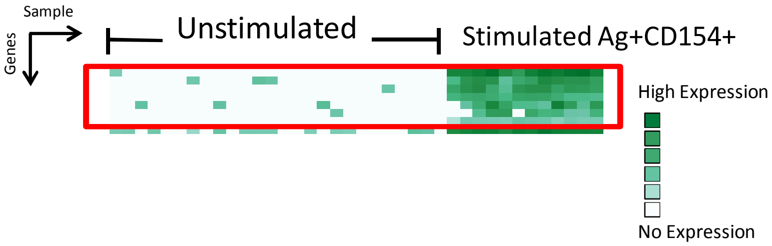
Correlates of Protection in RV144

Unstimulated

Stimulated

Genes Co-Regulated by Stimulation

Turn on

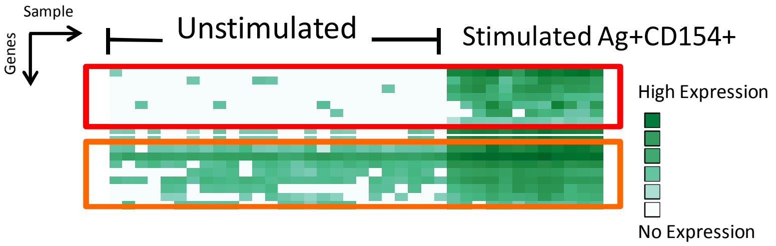


CYTOKINE/CHEMOKINE
GMCSF (CSF2)
MIP1α (CCL3)
LIF
IL3
IL4
IL10
IL13

Genes Co-Regulated by Stimulation

Turn on

Strongly Upregulated



CYTOKINE/CHEMOKINE	
GMCSF (CSF2)	IL2
MIP1α (CCL3)	IFNγ
LIF	TNFα
IL3	
IL4	
IL10	
IL13	

ACTIVATION
CD109
CD154 (CD40LG)
TNFRSF9 (CD137)

APOPTOSIS
CD95LG (FASLG)

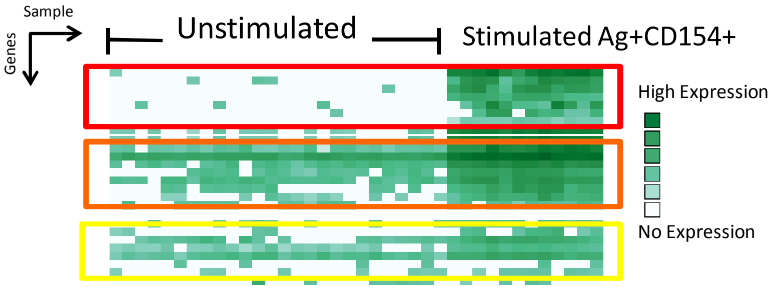
IMMUNE REGULATOR
LIGHT (TNFSF14)

Genes Co-Regulated by Stimulation

Turn on

Strongly Upregulated

Upregulated



CYTOKINE/CHEMOKINE	
GMCSF (CSF2)	IL2
MIP1α (CCL3)	IFNγ
LIF	TNFα
IL3	
IL4	
IL10	
IL13	

ACTIVATION
CD109
CD154 (CD40LG)
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APOPTOSIS
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LIGHT (TNFSF14)
BAMBI (NMA)
PD1
CTLA4

SIGNALLING
SLAMF1
CD84 (SLAMF5)
SHP2 (PTPN11)

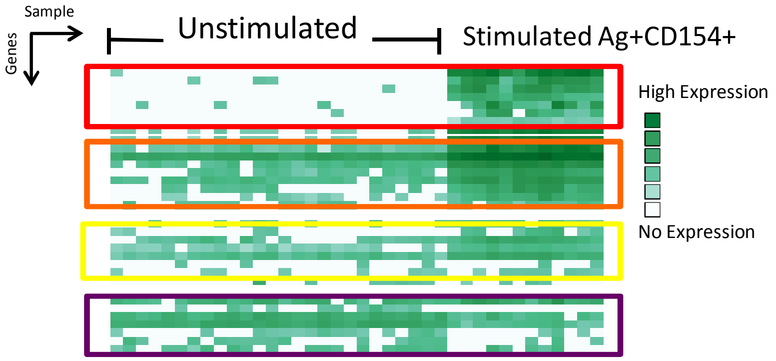
Genes Co-Regulated by Stimulation

Turn on

Strongly Upregulated

Upregulated

Turn off/ Downregulated



CYTOKINE/CHEMOKINE

GMCSF (CSF2)
MIP1α (CCL3)
LIF
IL3
IL4
IL10
IL13

IL2
IFNγ
TNFα

LCF (IL16)

ACTIVATION

CD109
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APOPTOSIS

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IMMUNE REGULATOR

LIGHT (TNFSF14)

BAMBI (NMA)
PD1
CTLA4

CD31 (PECAM1)
CD38

SIGNALLING

SLAMF1
CD84 (SLAMF5)
SHP2 (PTPN11)

PHENOTYPE

BAFF **DC**
CD94 (KLRD1) **NK**
FCRL3 **Treg**

Gene Family Regulation

Genes associated with T cell responses showed large changes (≥3 orders of magnitude) with extreme significance.

Expression of ~40 (of 96) genes is altered by stimulation of vaccine-specific cells.

… Many targets as possible correlates for protection.

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Expression of ~40 (of 96) genes is altered by stimulation of vaccine-specific cells.

… Many targets as possible correlates for protection.

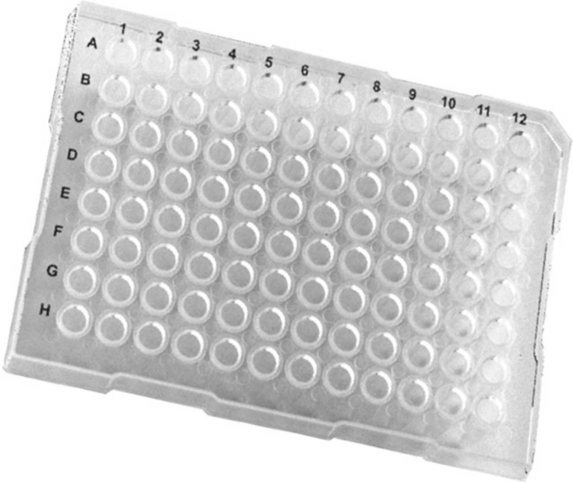
But this is still a bulk approach! What is the heterogeneity of the T cell response?

Can we do Fluidigm on single cells?

Single-Cell Genomics

Standard Flow Procedure

- Short Stimulation
- Cell Staining



Single-Cell Genomics

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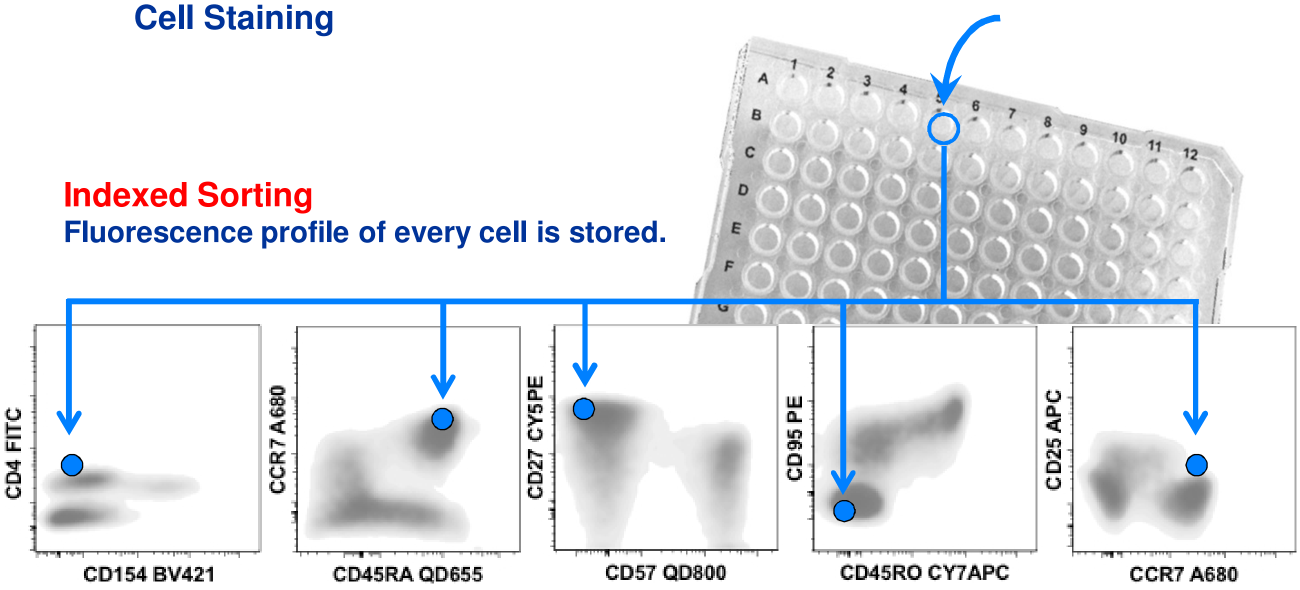
Indexed Sorting

Fluorescence profile of every cell is stored.



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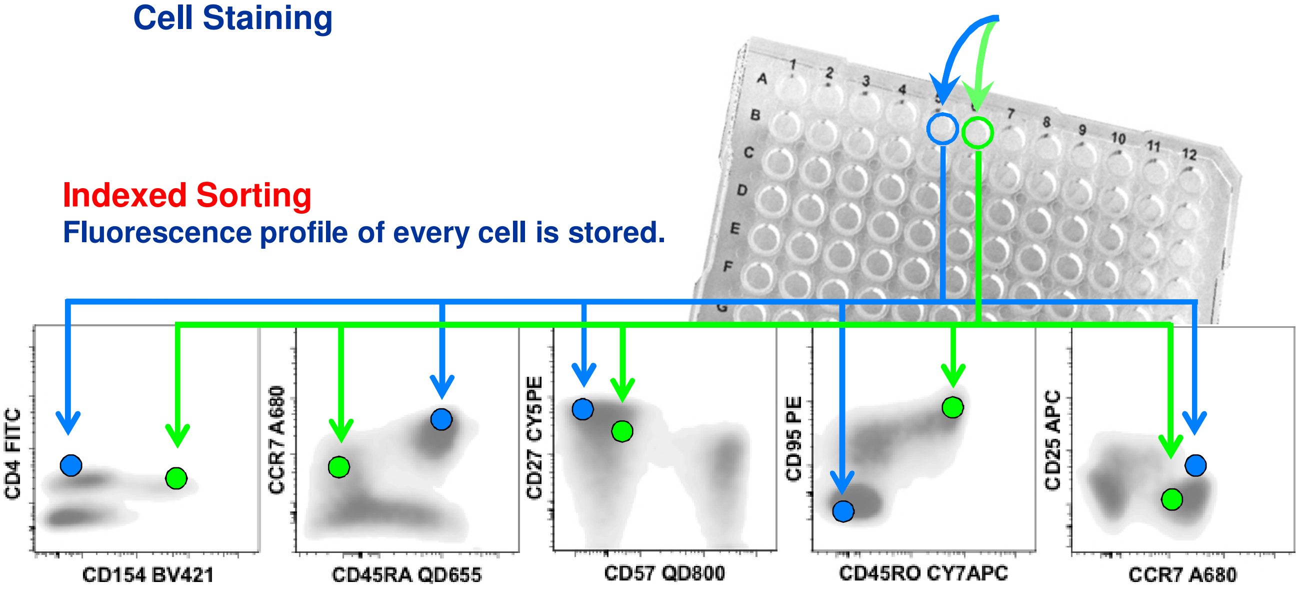

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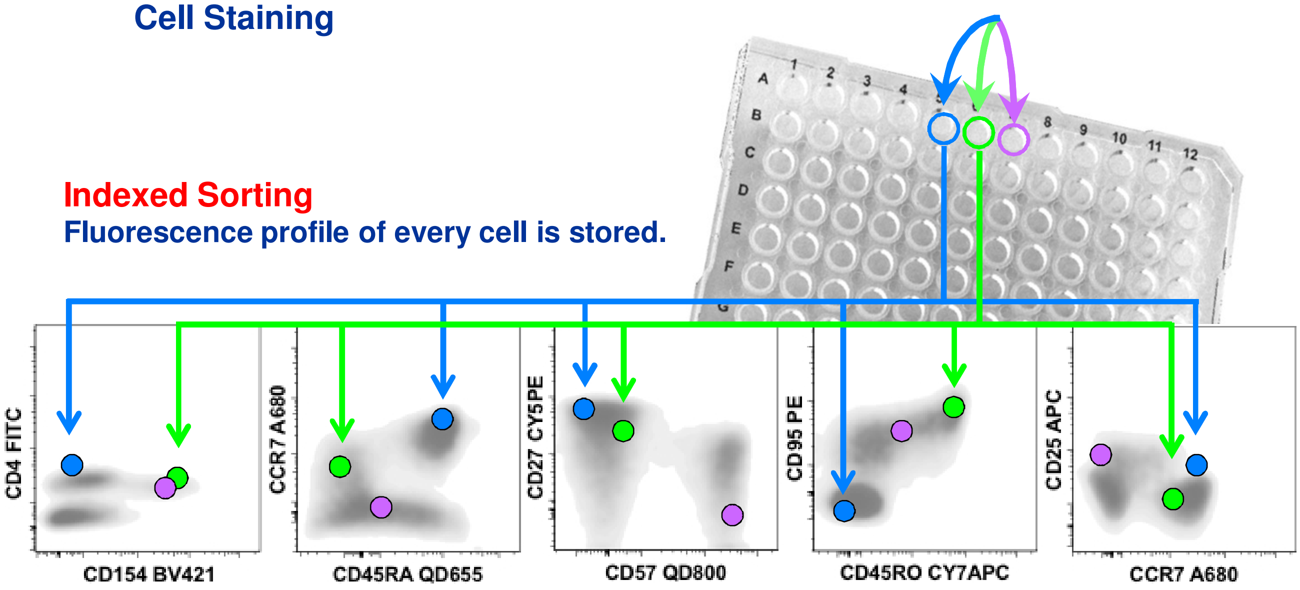
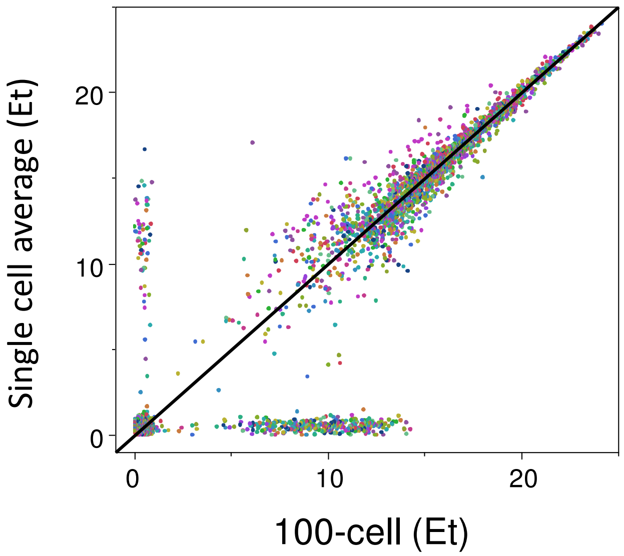


Single Cell Accuracy

10 stimulated CD4 PBMC samples were sorted for nanoarray (3 x 100 cells) or single cell (150 x 1 cell).



The average 100 cell signal (normalized to 1 cell) is graphed against the average single cell signal for all 10 samples x 96 genes.

Et = Log_2(Expression)

Single Cell Accuracy

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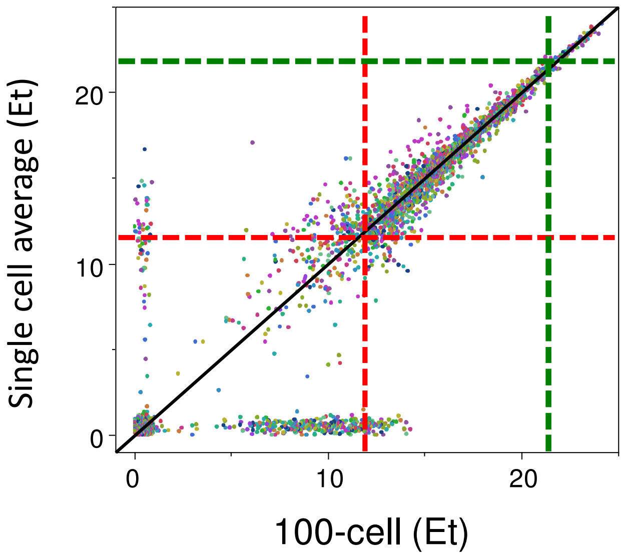
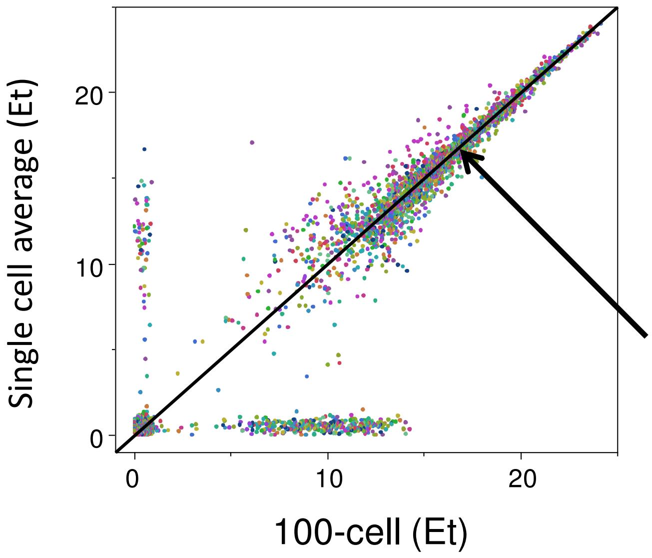


~1000 mRNA

~1 mRNA

Single Cell Accuracy

10 stimulated CD4 PBMC samples were sorted for nanoarray (3 x 100 cells) or single cell (150 x 1 cell).



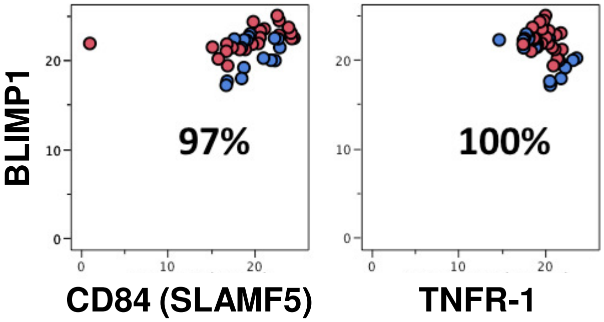
Excellent correlation: there is no loss in sensitivity/accuracy at the single cell level

Single Cell Genomics

- Just like flow cytometry, this technology provides us with two independent pieces of information:
  - **How many** cells express a gene?
  - **How much** do these cells express?
- Standard (bulk) analysis confounds these two measurements to generate an average
- Single cell analysis allows us then to answer another question:
  - What is the **co-expression** of genes?

Why is Single Cell Important?

100
Cell



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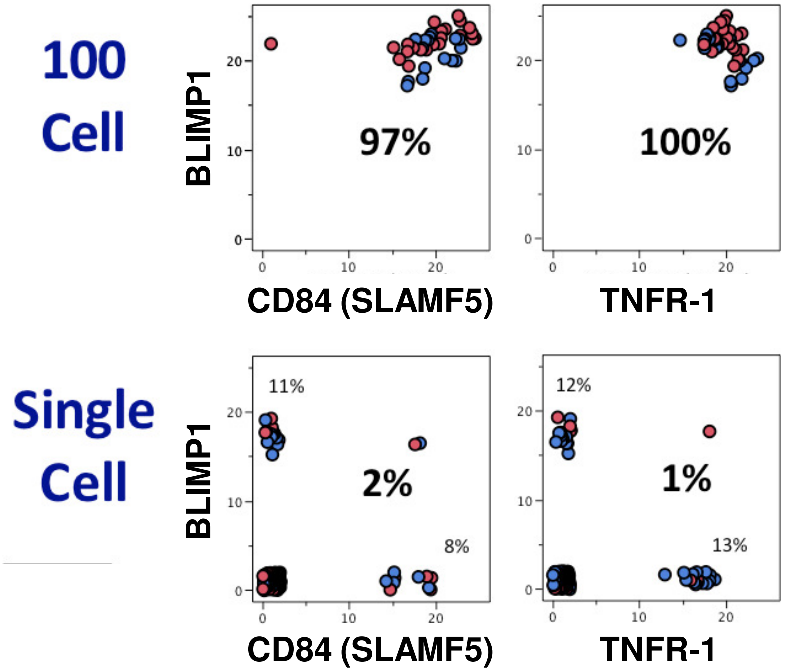
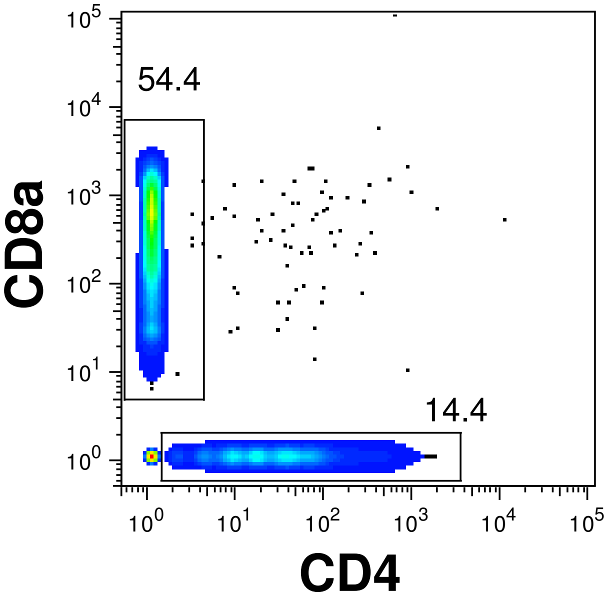


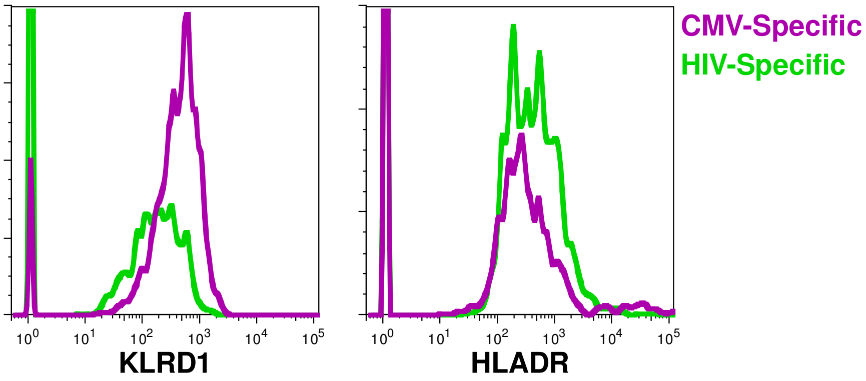
Single cell analysis reveals a *completely different picture* of regulation of these genes!

Standard Subset Identification



Gene Regulation Examples

Different by Antigen Specificity



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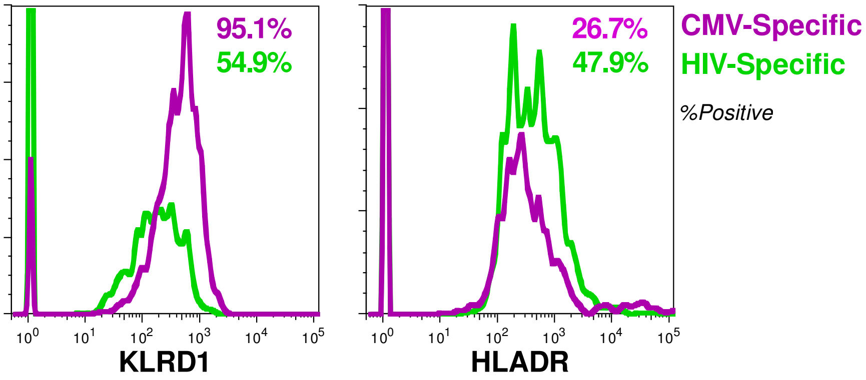

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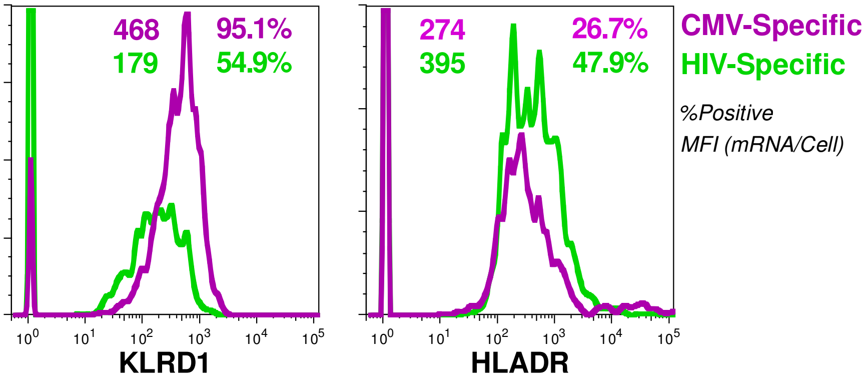
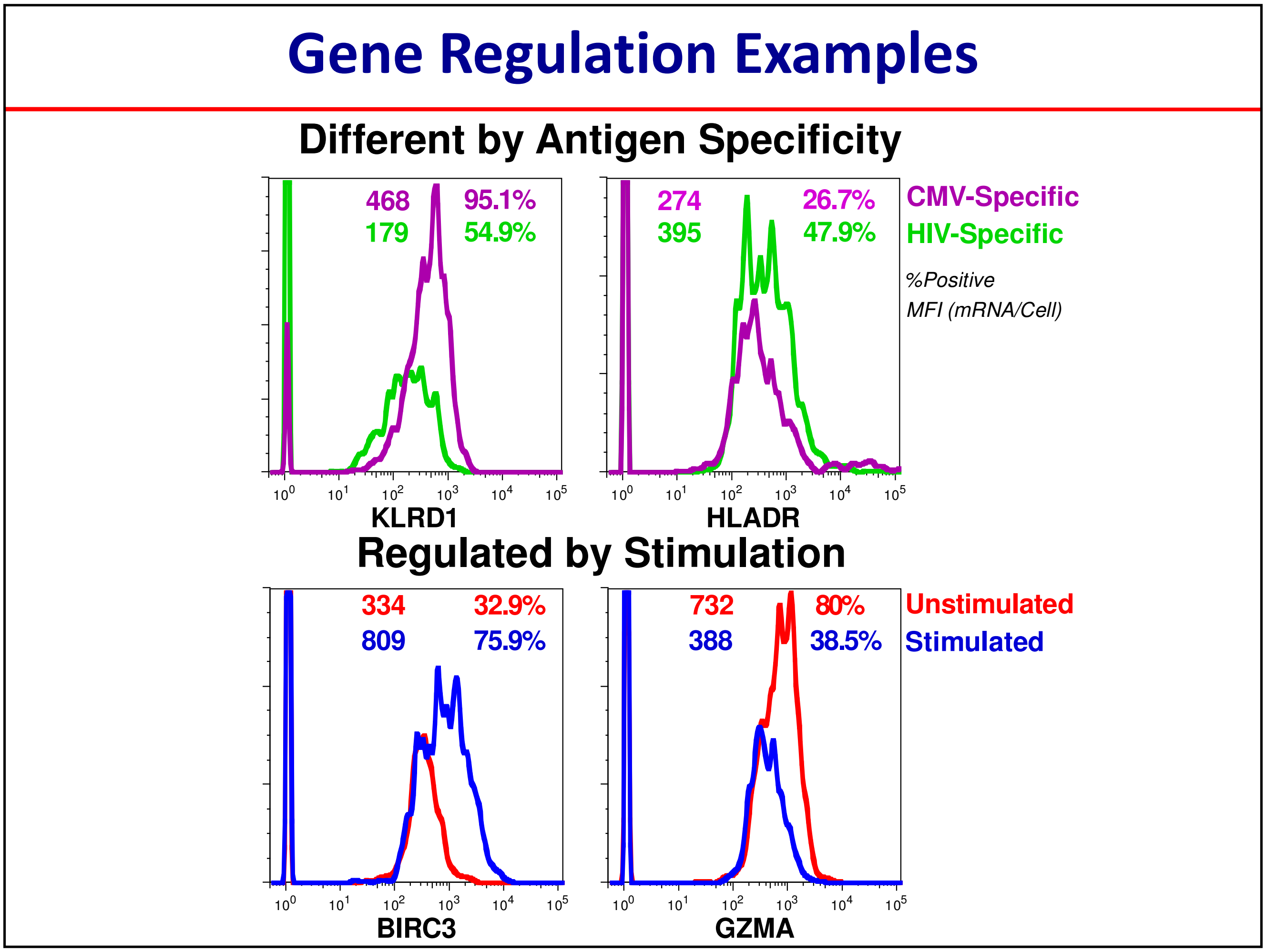




Gene Regulation Examples

Different by Antigen Specificity

CMV-Specific

HIV-Specific

%Positive

MFI (mRNA/Cell)

Regulated by Stimulation

Unstimulated

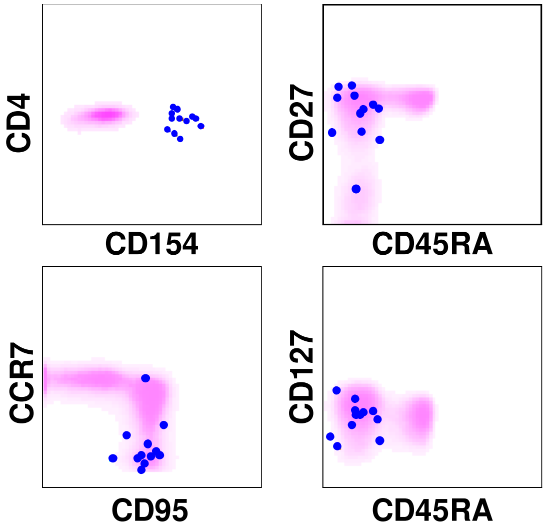
Stimulated

Single-Cell Analysis of RV144

Between 100 and 200 CD154+ CD4 memory T cells were sorted from stimulated cultures from each of 10 vaccinees

A total of 1,289 cells were sorted

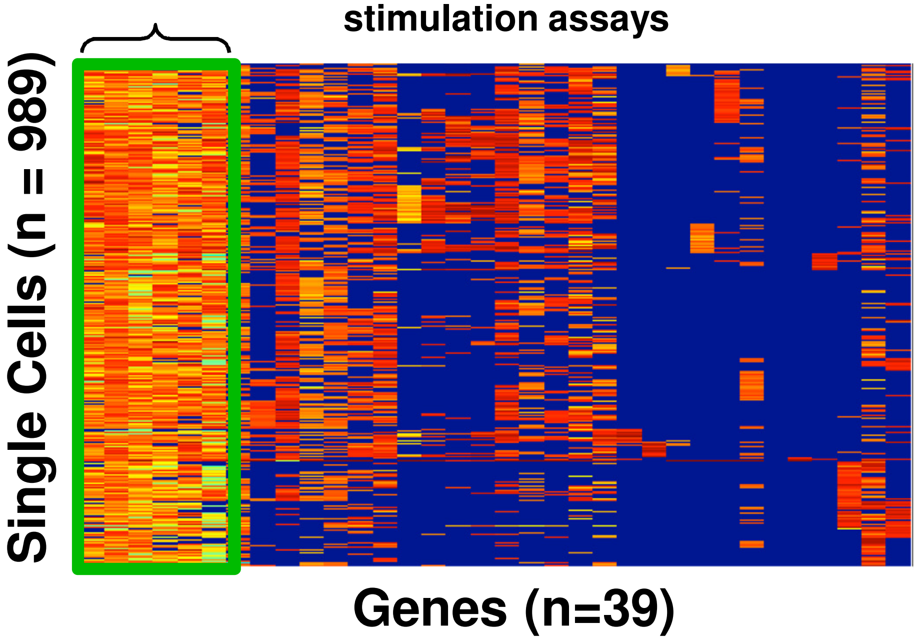
The phenotype of every cell is known



Gene Profiling of Single Cells

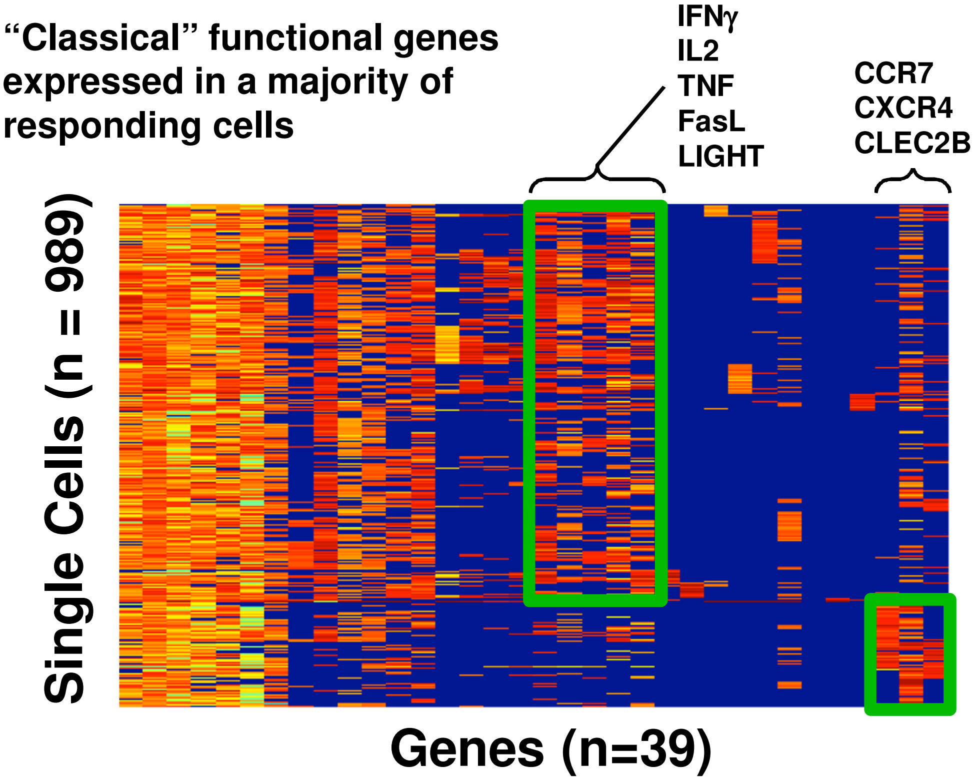
BIRC3 **CD69**
IL2Rγ **CD154**
TGFβ1 **ICOS**

“Core” set of 6 genes expressed by nearly all responding cells; many are not routinely measured in antigen stimulation assays



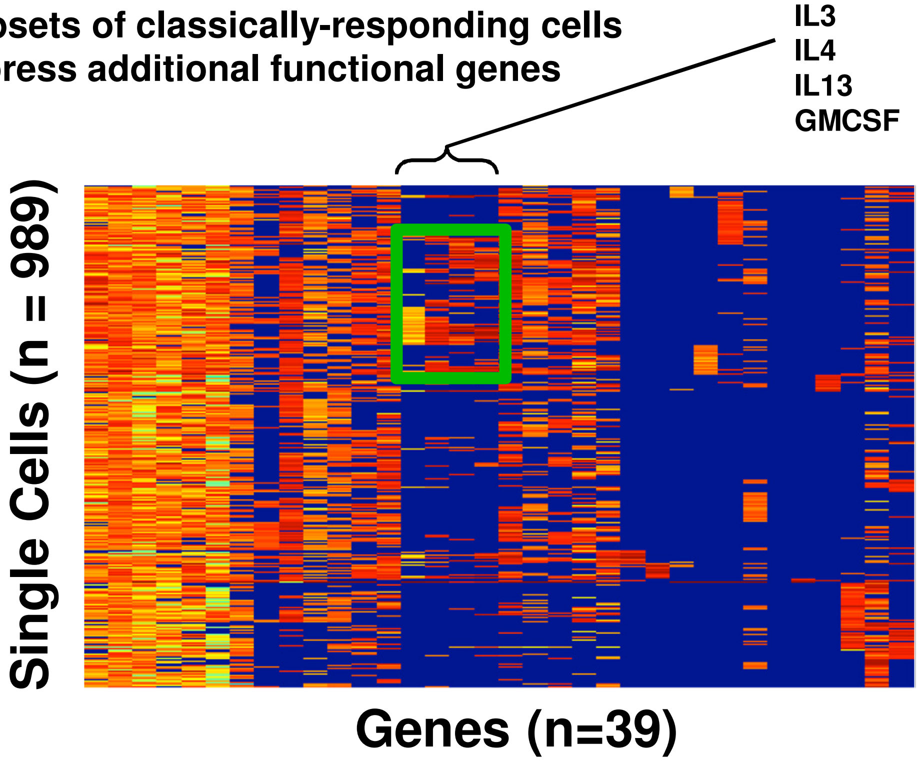
Gene Profiling of Single Cells

"Classical" functional genes expressed in a majority of responding cells



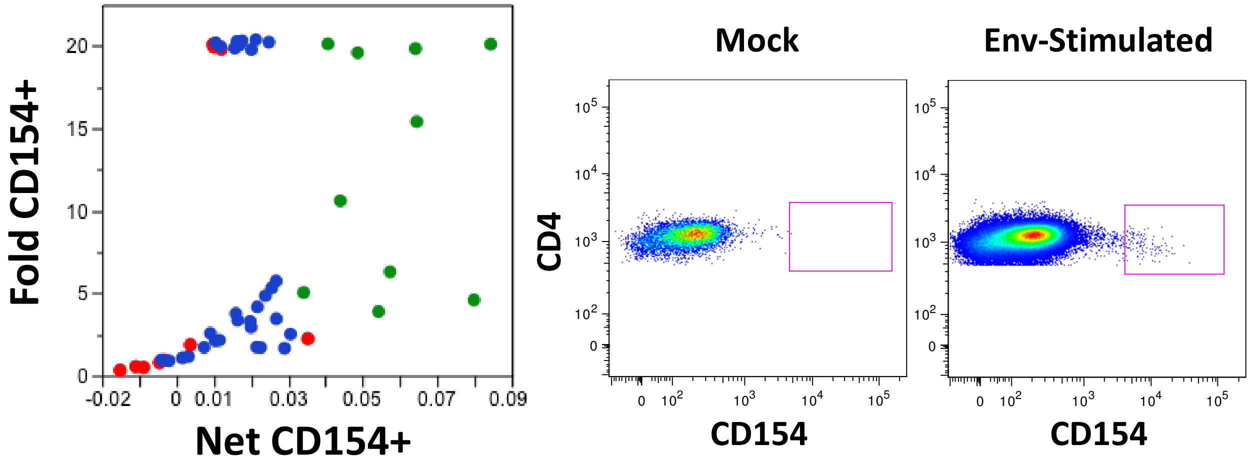
Gene Profiling of Single Cells

Subsets of classically-responding cells express additional functional genes



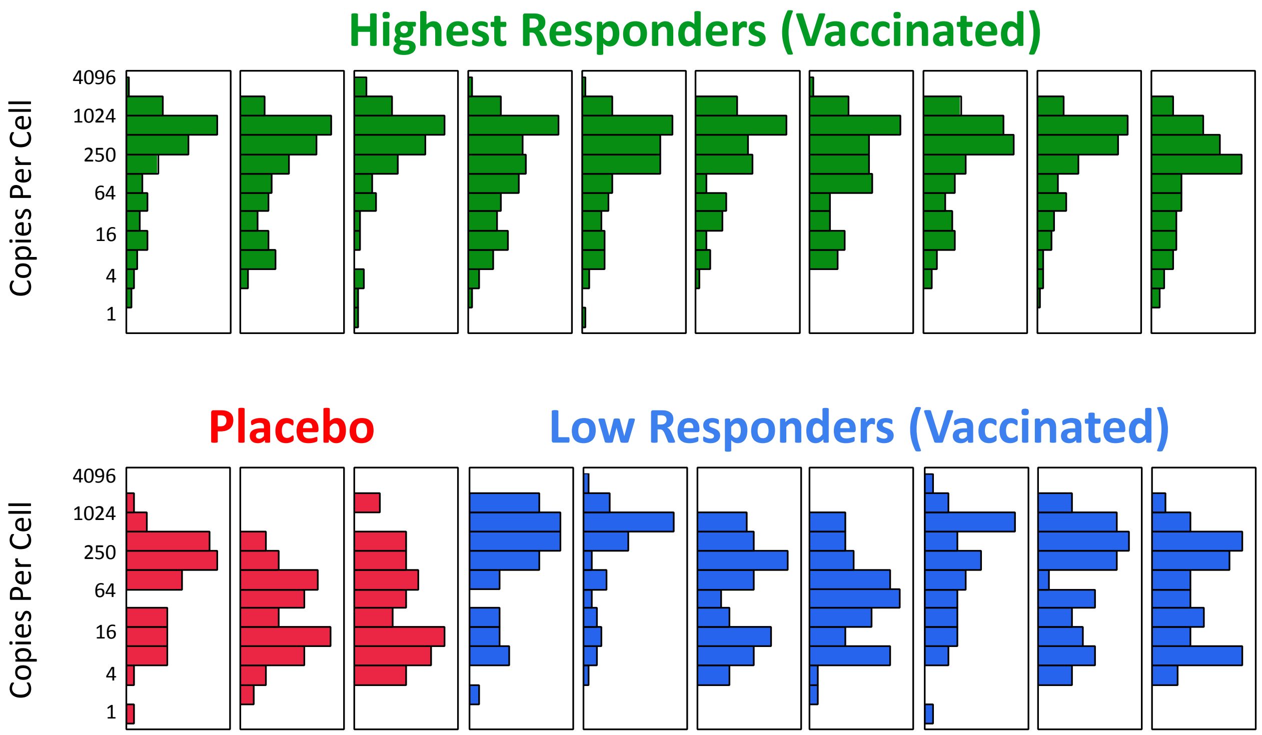
Can We Identify a Vaccine-Specific Signature?

20 Individuals
80-150 **Single CD154+** CD4 T cells
96 Genes

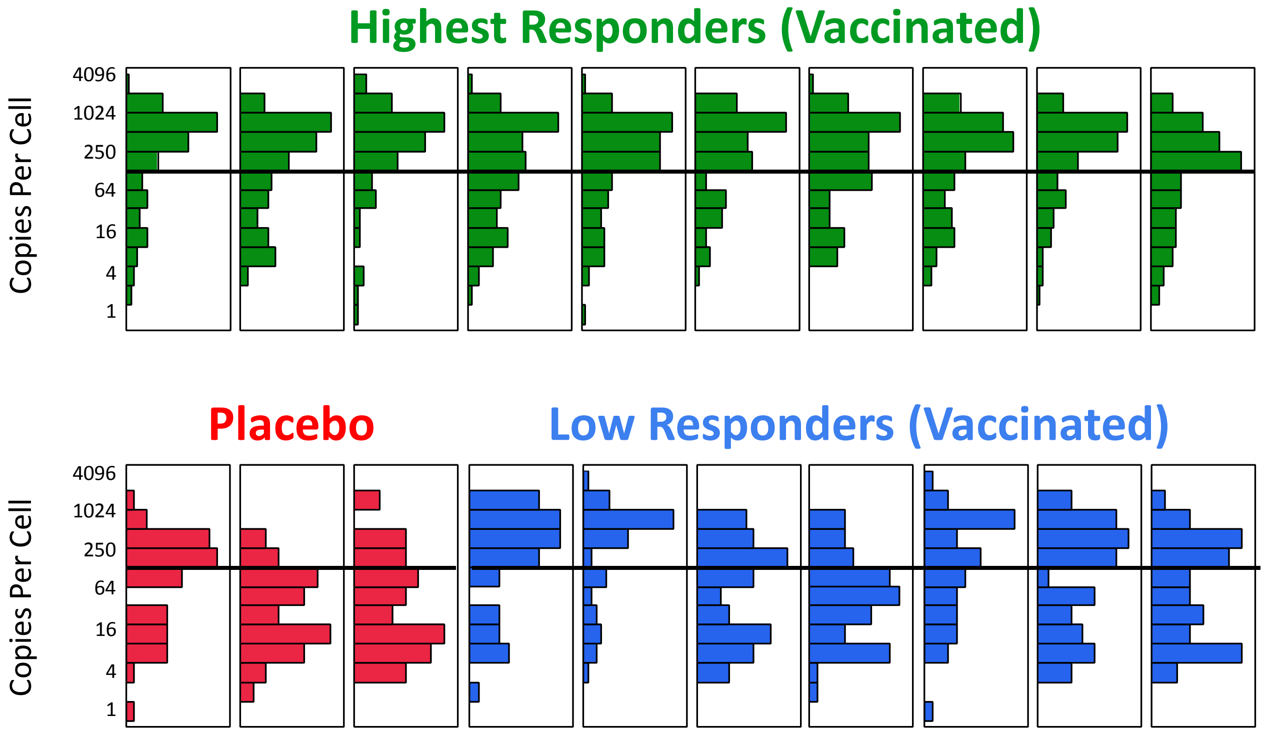


🟩 n=10 High Responders (Vaccinated)
🟦 n=7 Low Responders (Vaccinated)
🟥 n=3 Placebo

Single-Cell Gene Expression of CD154



Single-Cell Gene Expression of CD154



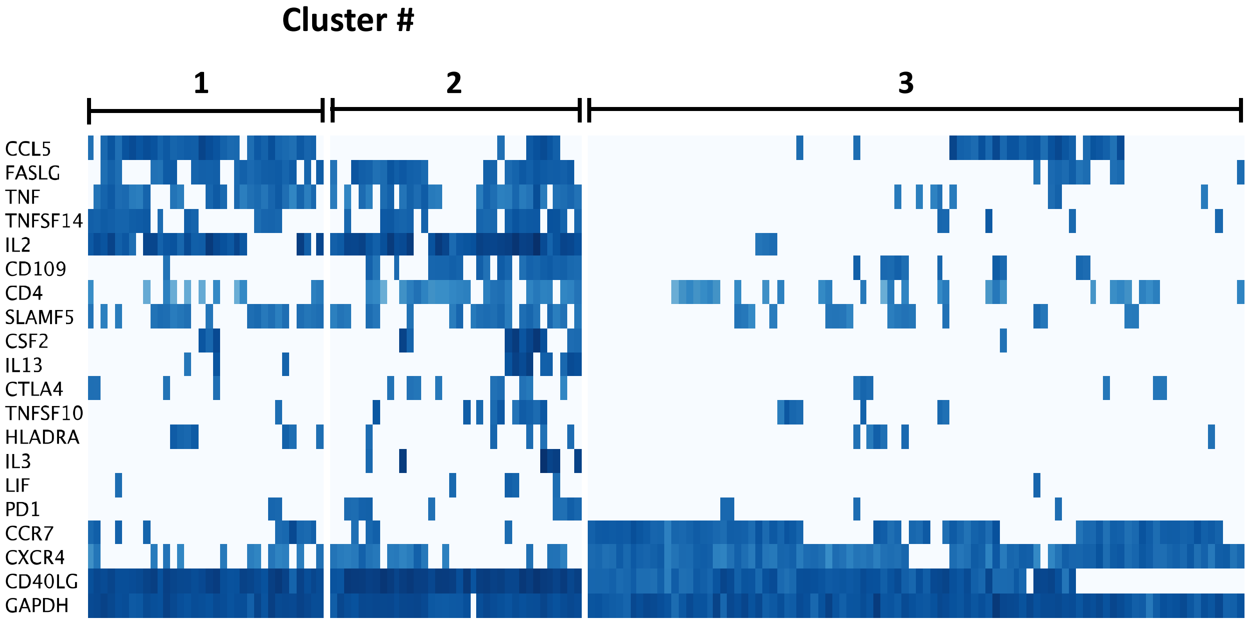
Highest Responders (Vaccinated)

Placebo

Low Responders (Vaccinated)

Signature of Vaccine-Specific Response

Based on the Single-Cell Expression of only 20 Genes



Signature of Vaccine-Specific Response

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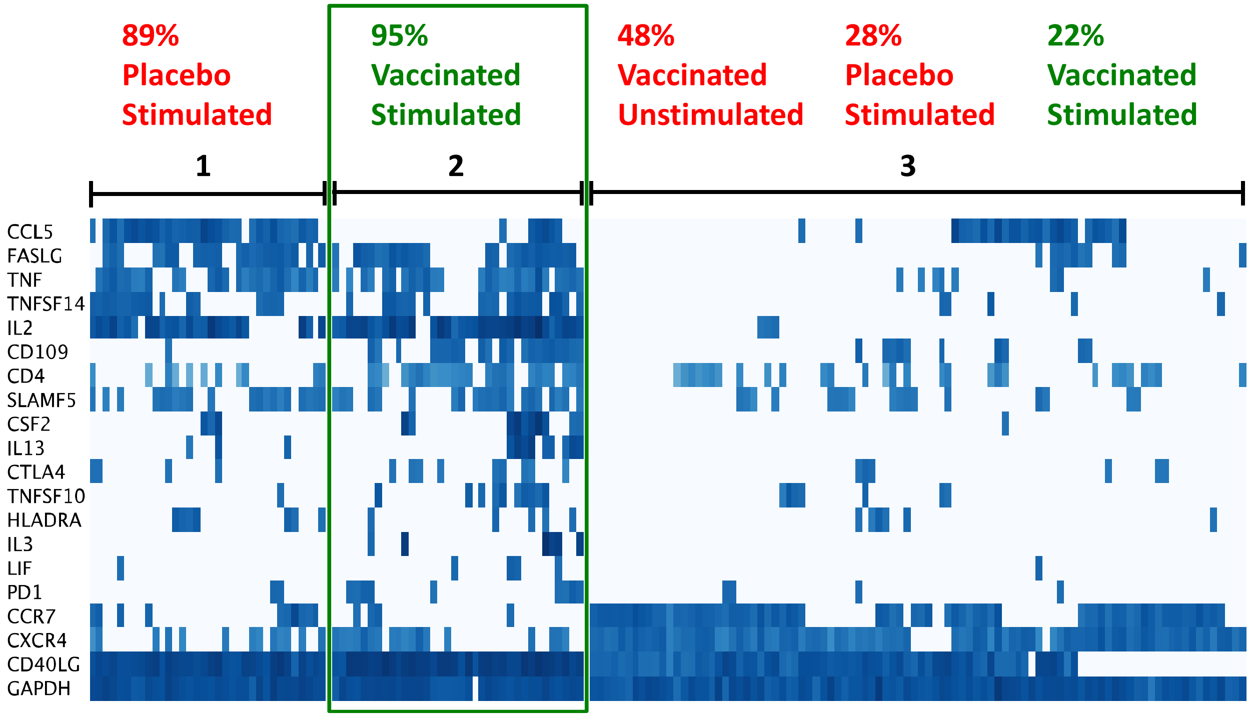

89%
Placebo
Stimulated

95%
Vaccinated
Stimulated

48%
Vaccinated
Unstimulated

28%
Placebo
Stimulated

22%
Vaccinated
Stimulated

Single-Cell Transcriptomics for Vaccine Evaluation

We identified ~40 genes whose expression is modulated by stimulation of vaccine specific CD4 T cells

Gene expression patterns identify subsets of CD4 T cells within the vaccine-specific response

These can be further evaluated as potential correlates of durably humoral responses and/or protection

Vaccine-specific signatures can be identified and serve as comparators to other (protective) vaccines

Gene expression analysis of SIV productively infected CD4$^+$ T-cells

What is the **phenotype** and **gene expression** profile of the rare CD4$^+$ T cells that produce HIV/SIV *in vivo*?

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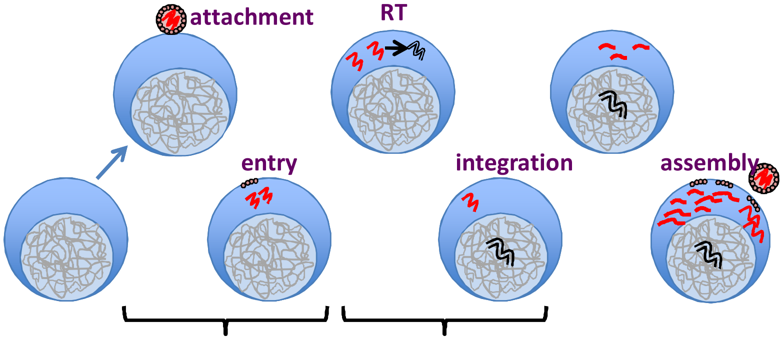
What is the **phenotype** and **gene expression** profile of the rare CD4$^+$ T cells that produce HIV/SIV *in vivo*?

We take advantage of the kinetics of SIV gene expression to distinguish parts of the viral life-cycle.



gag/LTR RNA:	-	+	+	+	+++
gag/LTR DNA:	-	-	+	+	+
tat/rev RNA:	-	-	-	+	++
env RNA:	-	-	-	+/-	+

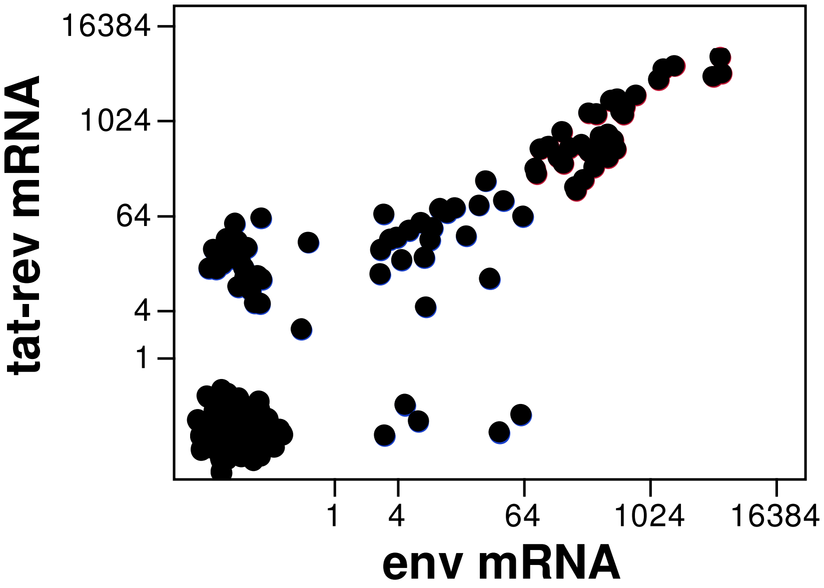
Gene expression analysis of SIV productively infected $CD4^+$ T-cells

We sorted CD4 memory T cells from an acutely-infected NHP

Fluidigm analysis was performed for gag, LTR, env, and tat-rev gene products, as well as 92 rhesus genes.

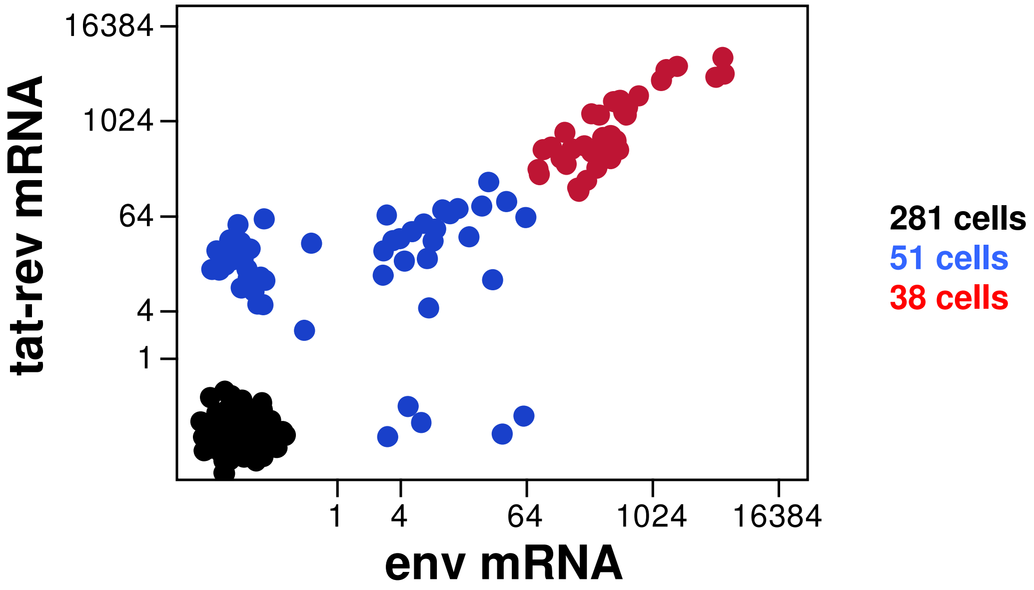
6% of cells contain tat-rev mRNA (previously: ~10% of gag+ cells produce virus)

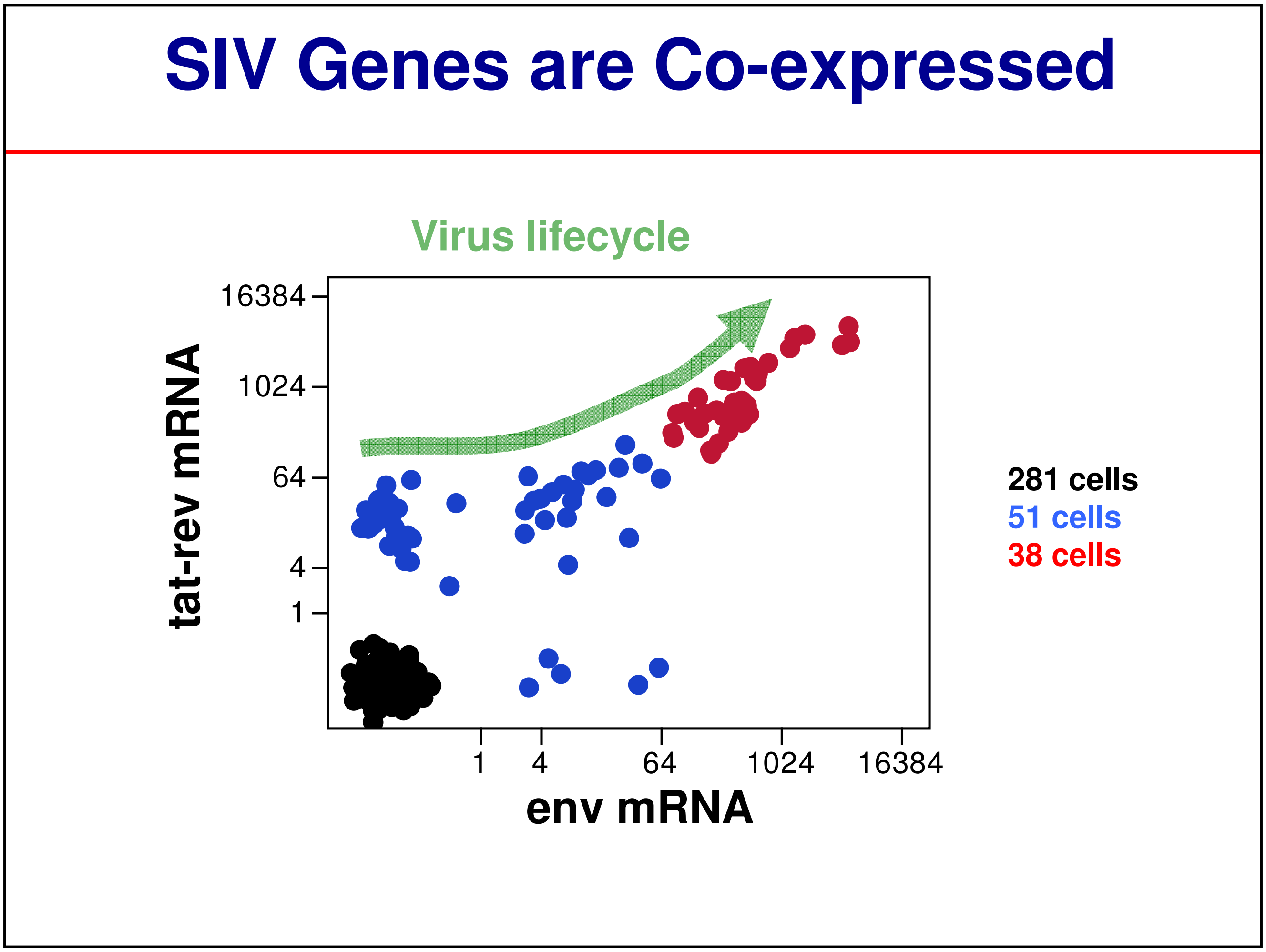
SIV Genes are Co-expressed



370 cells

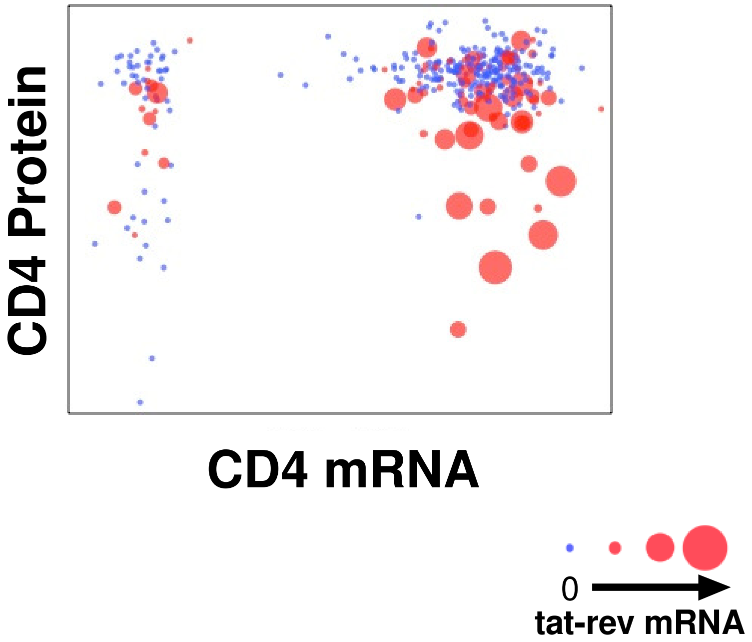
SIV Genes are Co-expressed



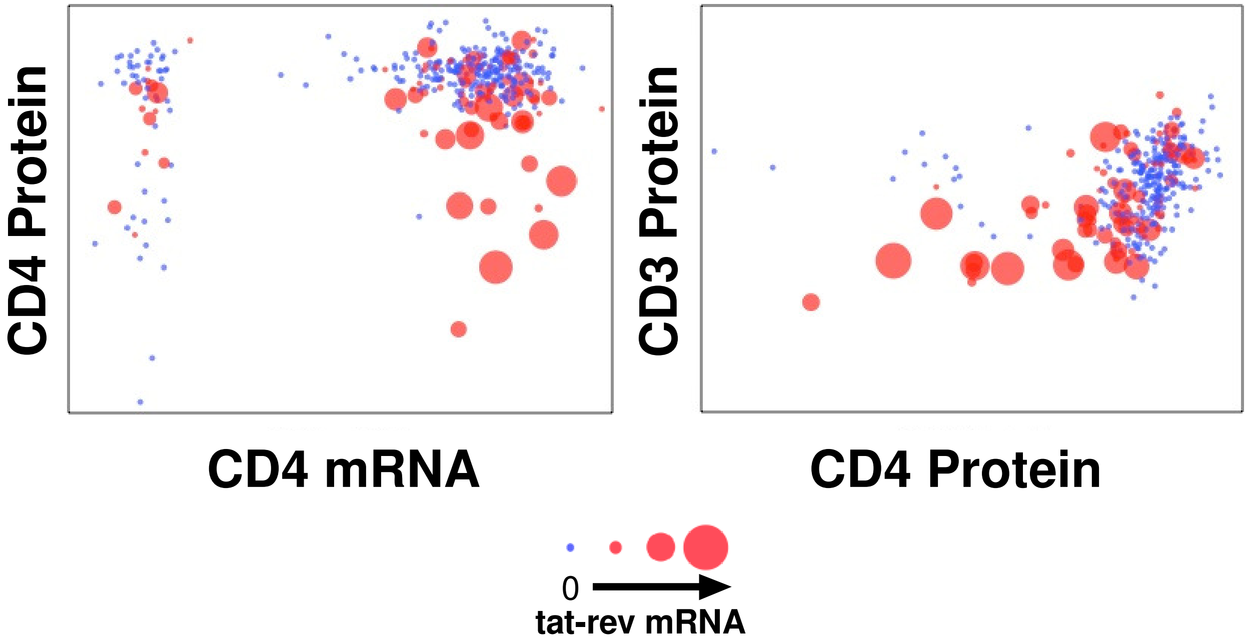


SIV Genes are Co-expressed

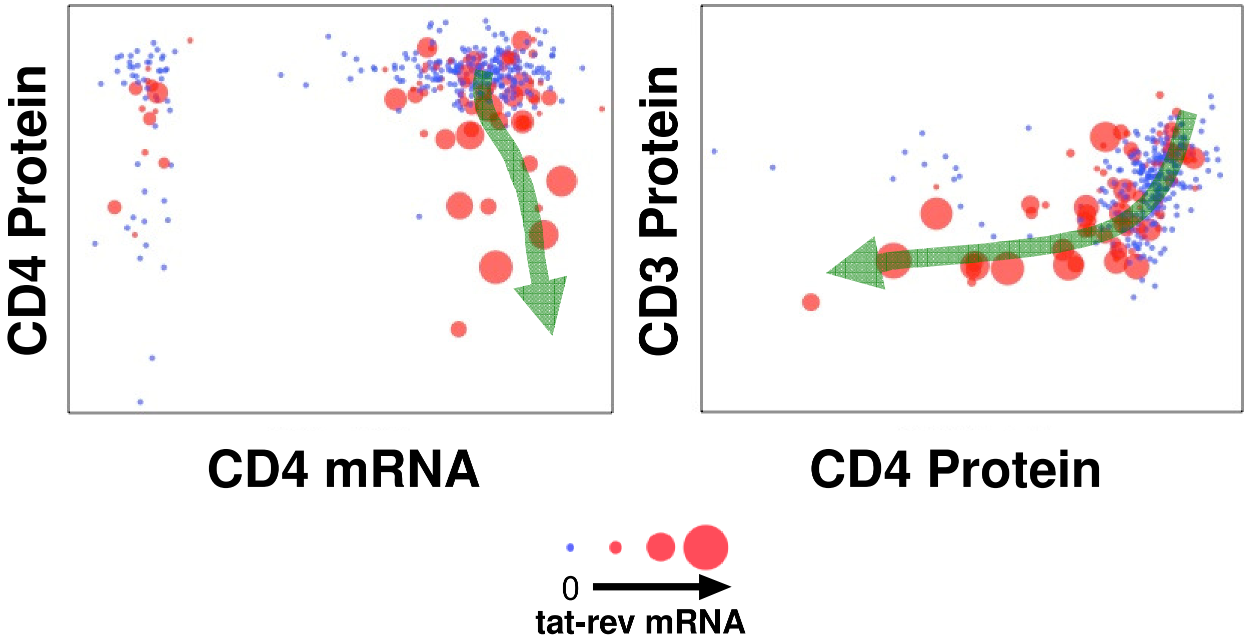
Host Gene Expression During the SIV Lifecycle



Host Gene Expression During the SIV Lifecycle



Host Gene Expression During the SIV Lifecycle

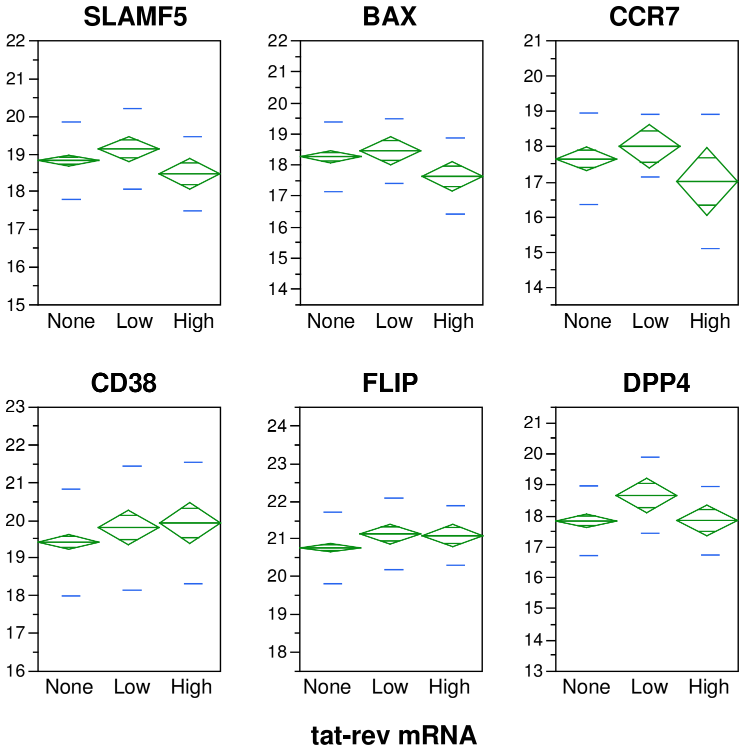


Host Protein Expression in Productively SIV-Infected Cells

Surface protein expression differences associated with SIV infection

	p value	
CD3	<0.001	Decreased
CD4	<0.001	Decreased
ICOS	0.003	Increased
CD69	0.04	Increased
CD45RA	0.01	Decreased

Patterns of Host Gene Expression Associated with SIV Lifecycle



Host Gene Expression in Productively SIV-Infected Cells

Decreased

	p value
CD62L	0.005
LEF1	0.01
TCF7	0.003

Increased

	p value
Bax	0.04
CCR5	0.04
CCR6	0.03
CDK1	0.005
CTLA4	0.0005
FOXP3	0.02
ICOS	0.04
LAG3	0.02
MKI67	0.03

Consistent with productive infection occurring more frequently in differentiated & activated cells

Gene Expression & SIV Infection

A number of genes are differentially expressed by SIV-infected and productive cells

Differences can be measured at the mRNA and protein level – and reveal post-transcriptional regulation

These data do not distinguish between an impact of SIV on host gene regulation vs. a requirement of cell activation state for viral lifecycle progression

Integrating FACS & Transcriptomics

Nanoarray: Quantify 96+ genes from 50-5000 cells

Interrogate *large* numbers of *small* samples for gene expression

GENE EXPRESSION as potential correlates

Single Cell: Identify subsets *within* vaccine-specific T cells

Patterns of gene (co-) expression

CELL SUBSETS as potential correlates

Regulation of Expression

Correlate protein & mRNA expression at a single-cell level

POST-TRANSCRIPTIONAL regulation

This approach is the stepping stone to deep sequencing.

Bioinformatics Techniques

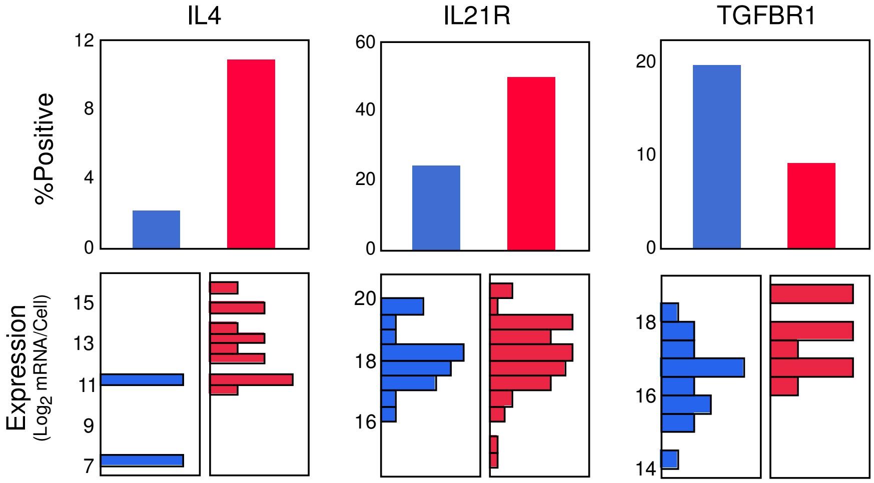
Single-Cell analysis generates two types of data

1.) Frequency of Expression (Bimodal/On-Off)

Is the Gene Expressed?

2.) Magnitude of Expression (C_T)

For Expressed Genes, How Many Copies per Cell?



Bioinformatics Techniques

Bimodal (On/Off)

Percent Positive

🟥🟥🟥⬛⬛⬛⬛🟩🟩🟩🟩🟩

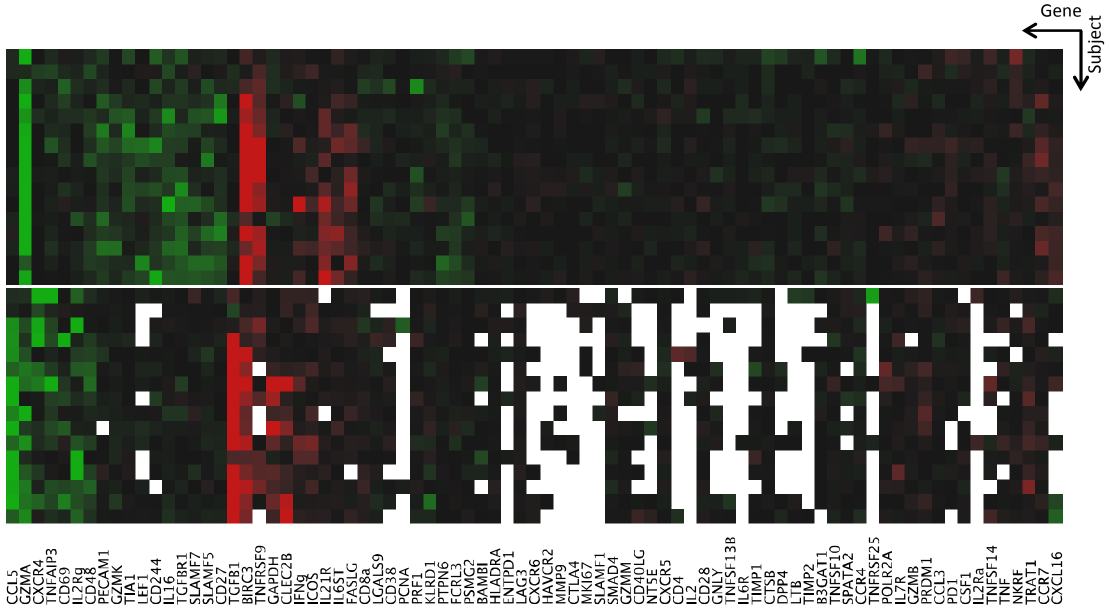
Low High

Continuous

C_t

🟥🟥🟥⬛⬛⬛⬛🟩🟩🟩🟩🟩

Low High



Bioinformatics Techniques

Bimodal (On/Off)

Percent Positive

Low High

Continuous

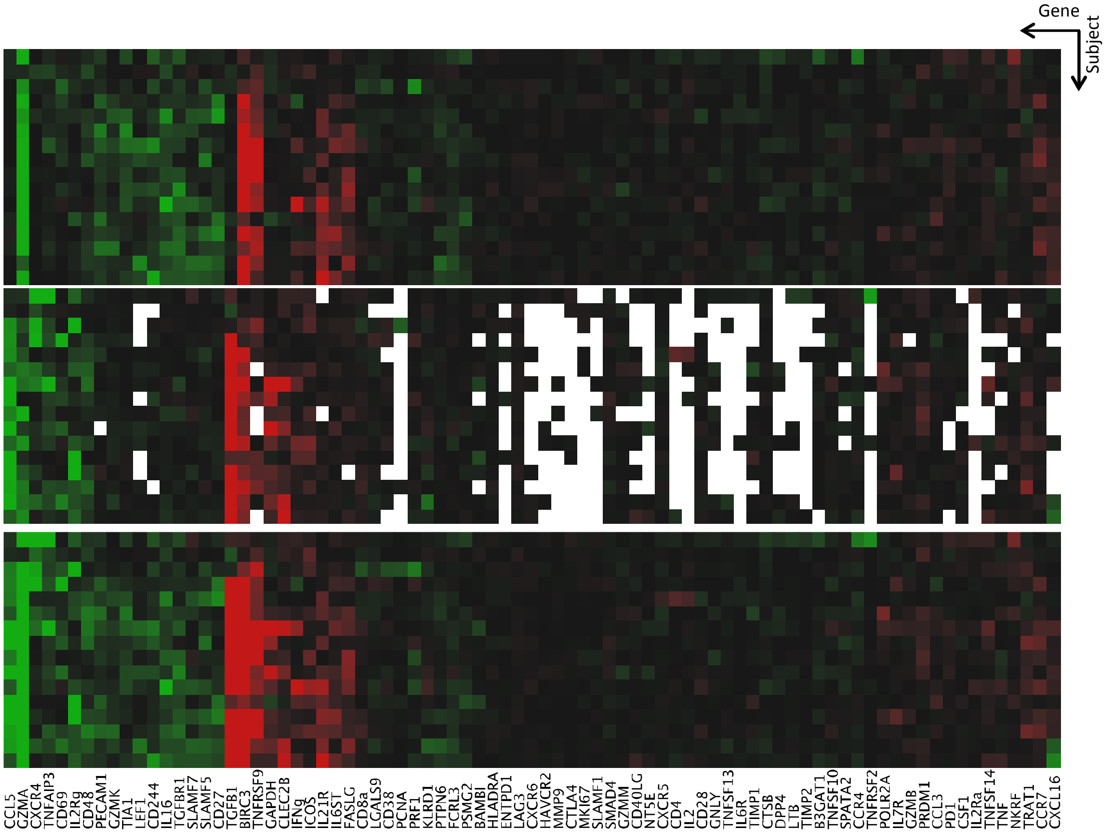
C_t

Low High

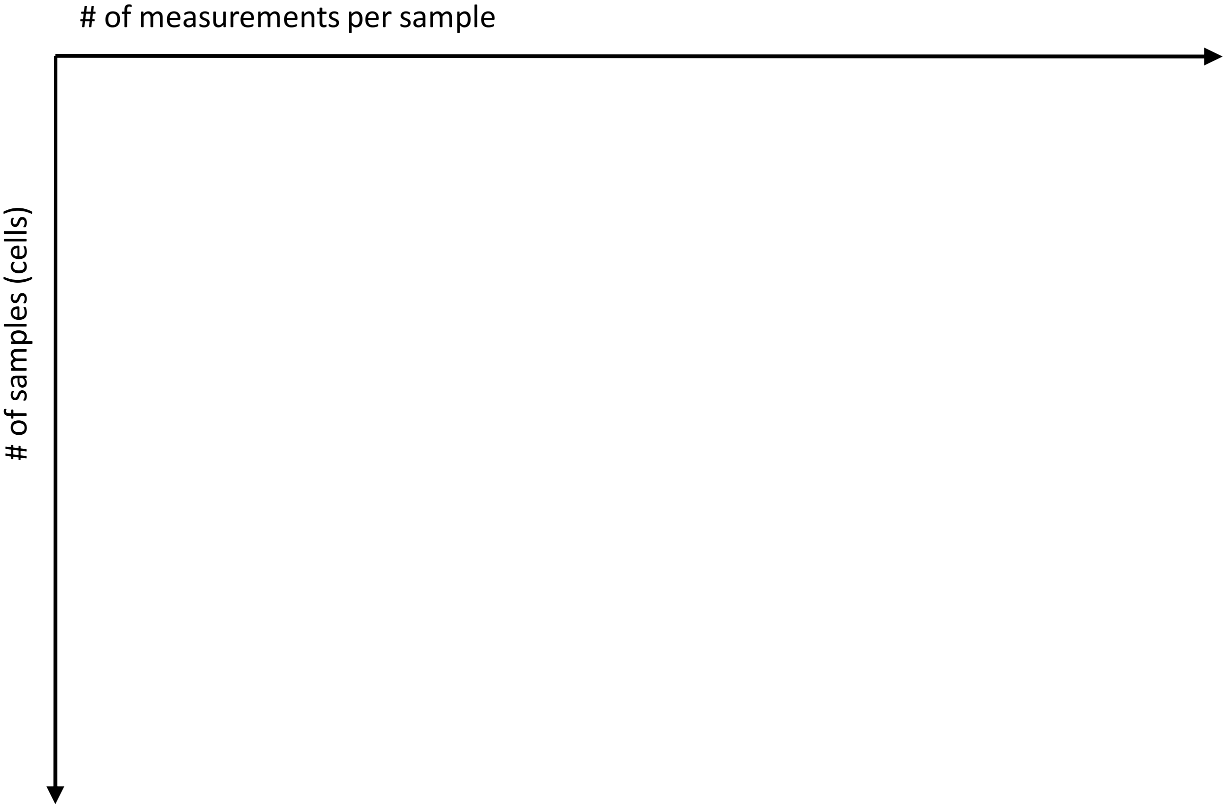
Combined

Expression

Low High



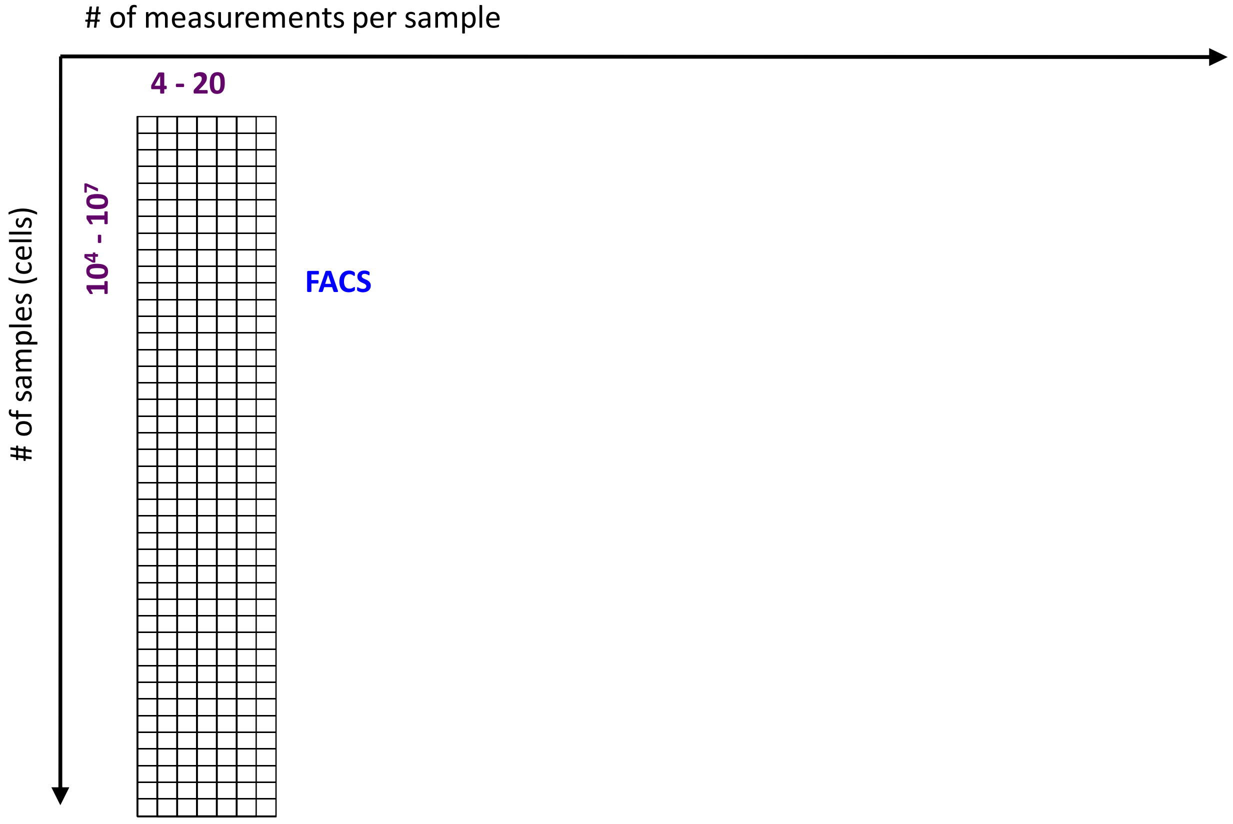
Data Analysis Demands



# of measurements per sample

# of samples (cells)

Data Analysis Demands



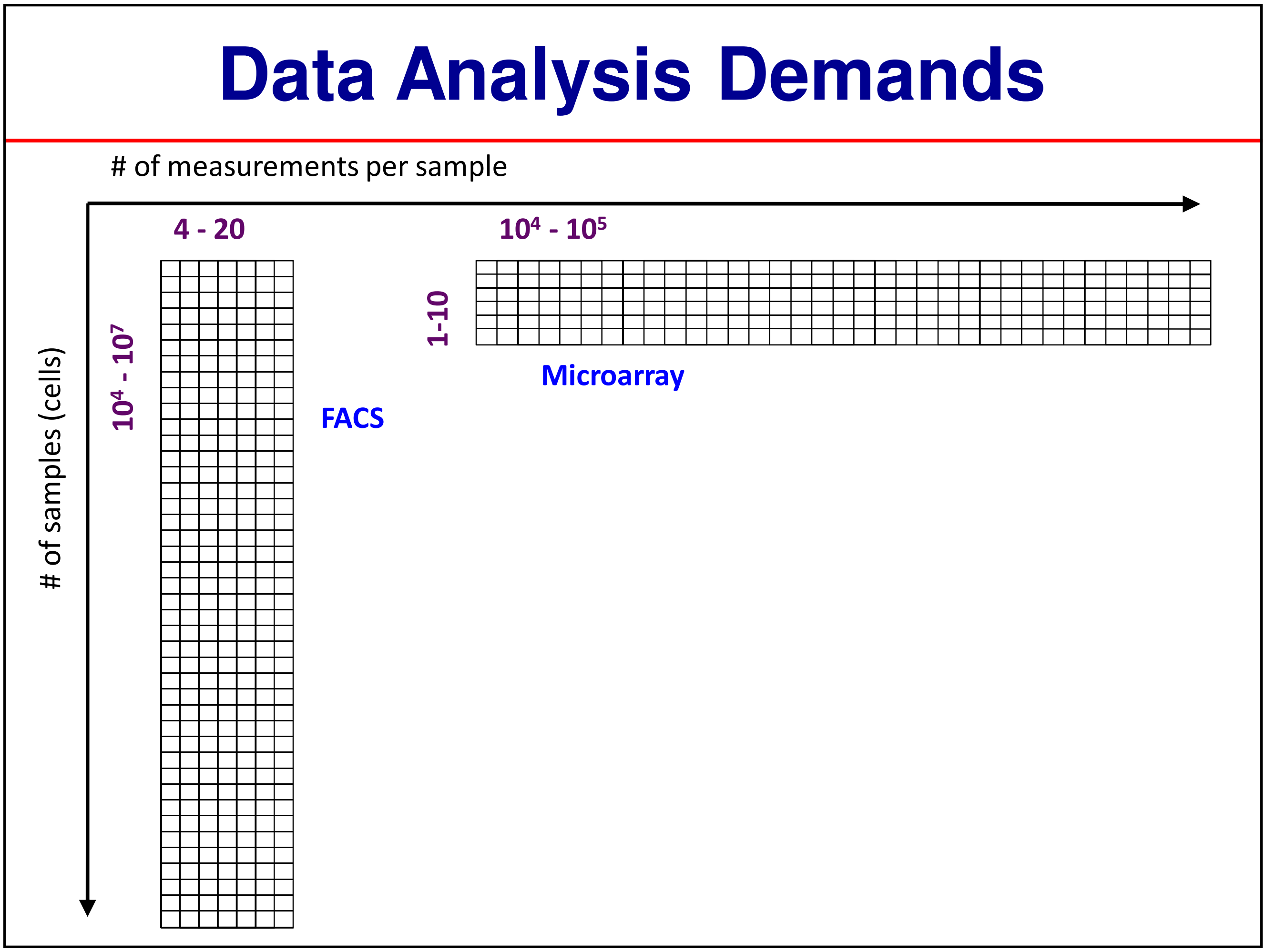
# of measurements per sample

4 - 20

# of samples (cells)

10^4 - 10^7

FACS



Data Analysis Demands

of measurements per sample

4 - 20

10^4 - 10^5

# of samples (cells)

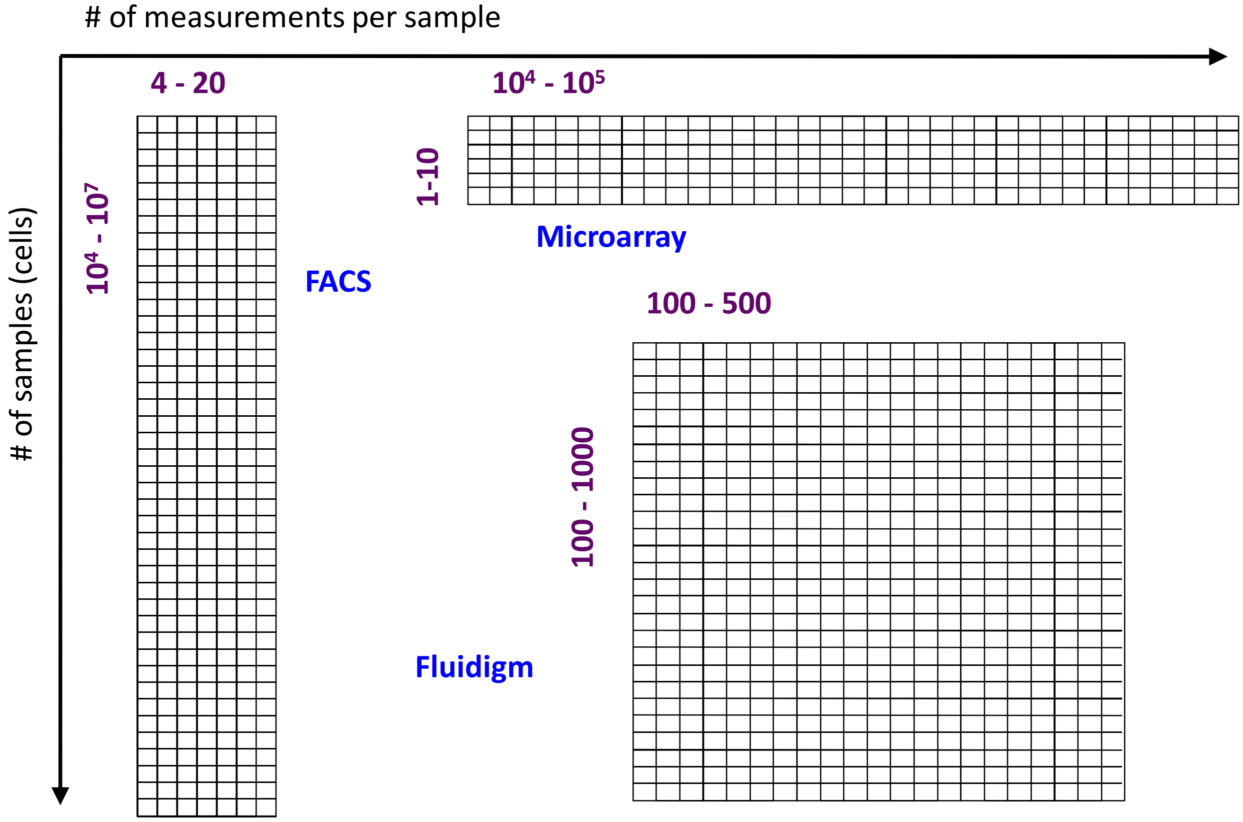
10^4 - 10^7

1-10

Microarray

FACS

Data Analysis Demands



# of measurements per sample

# of samples (cells)

4 - 20

$10^4 - 10^7$

FACS

$10^4 - 10^5$

1-10

Microarray

100 - 500

100 - 1000

Fluidigm

Integrating Transcriptomics and Flow Cytometry

- Quantitative single-cell transcriptomics is possible
    - Fairly laborious
    - Often requires significant pre-enrichment

- Indexed sorting is a key component
    - Rare event selection
    - Phenotyping

- Integrating technologies provides new information
    - Correlate gene & protein expression

A powerful tool for identifying new correlates of protection or disease

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Turning fog…

… into rainbows