



Use of standardized
lyophilized reagents to
develop a functional T-
cell signature

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Assoc. Scientific Director
BD Biosciences



Functional T cell responses to tumor antigens in breast cancer patients have a distinct phenotype and cytokine signature

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- J Immun, 2007, 179: 2627-2633



http://maeckerlab.typepad.com/

Maeckerlab Weblog
A forum for issues in cytokine flow cytometry and immune function research. --> Looking for Julia's weblog? Click the link in the sidebar.

ABOUT

CATEGORIES

- Instrument setup
- Links to BD literature
- Literature reviews
- Maeckerlab protocols and tools
- Maeckerlab publications
- Miscellaneous
- Multicolor topics
- Protocol issues

RECENT POSTS

- June-July 2007 Reference Update
- Upcoming African Symposium and Workshop
- New multicolor workshop materials
- Subscribe to this blog's feed

USEFUL LINKS

Welcome to the Maeckerlab Weblog!

This weblog is intended to address current topics in cytokine flow cytometry, answer frequently asked questions, and introduce new ideas and technical data from our lab. Please use the Categories links at left to navigate to topics of interest to you.

The content presented here has not been officially reviewed by BD, and as such it reflects the views of the author only. Note that BD cannot provide support for the material provided here, so please address any questions by commenting to the weblog or e-mailing me at hmaecker@yahoo.com. Thanks for visiting!

Posted on 28 March 2006 | [Permalink](#) | [Comments \(1\)](#)

June-July 2007 Reference Update

This month, papers on Th17, Treg, and multifunctional T cells caught my eye:

Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639. [Link to PubMed abstract](#)

IL-17 production in humans is easily detected by ICS, and this paper shows phenotypic subsets of human T cells that produce IFN γ and/or IL-17 upon polyclonal TCR stimulation.

Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Fili, S. Ferri, F. Frosali, F. Giudici, P. Romagnani, P. Parronchi, F. Tonelli, E. Maggi, and S. Romagnani. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med*. [Link to PubMed abstract](#)

More on human Th17 cells, including ICS and comparison with IFN γ and IL-4 production, and relation to Crohn's disease.

Billerbeck, E., H. E. Blum, and R. Thimme. 2007. Parallel Expansion of Human Virus-Specific FoxP3- Effector Memory and De Novo-



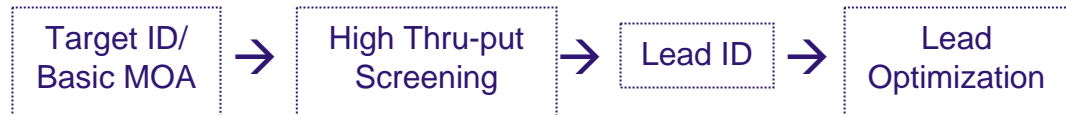
Cell Analysis in Drug Development Process - Step #1

1. NCE Selection

Preclinical activities aimed at screening, designing & optimizing the new chemical entity or biological agent that will interact with the drug target

Discovery & pre-clinical work to identify & narrow a field of potential therapeutic agents to a single new chemical entity (NCE) or biologic compound.

Usage commonly falls along the typical drug discovery workflow:



Cell analysis techniques are most commonly used to test binding specificities of agents and illuminate basic disease mechanisms of action (MOA)



Cell Analysis in Drug Development Process - Step #2

2. Pharmacodynamics

Preclinical and/or clinical studies of the biochemical & physiological effects of a drug, its mechanisms of action, and the relationship between drug dosage & effect on the body

Evaluates the interactions between an optimized drug candidate and a biological system.

Includes drug development activity as early in the pipeline as target validation and as late as dose-ranging & early efficacy.

Typical cell analysis applications in this area include cell signaling (e.g. Phosflow™ and Lyoplates)



Cell Analysis in Drug Development Process - Step #3

If successful, these clinical trials would demonstrate the safety and/or efficacy benefit of a “companion diagnostic” that pre-screens potential patients and/or monitors their progress during treatment.

These trials could be conducted both pre- and post-approval of the underlying therapeutic.

Cell analysis applications here are largely customized and are developed in partnership with the therapeutic company’s development organization

3. Patient Profiling

Clinical trials designed to determine if and how the phenotypes of individual patients or groups influence response to a given therapy. If successful, these trials would establish the need for new pre-treatment screening and/or therapeutic monitoring assays for this indication.



Cellular Analysis in Clinical Trials

This presentation will focus on addressing the need for;

- Applying the use of biomarkers in drug development
- Standardizing cellular assays across multiple sites
- Assay and data analysis criterion
- Monitoring immune function
- Cellular Biomarkers of Disease progression (Cancer and HIV)



Why collect and analyze blood?

- You can get it
- It shows immunological memory
- It is a systemic organ
- We can measure it in detail



Response Profiles Can Be Very Rich

B-cells		Monocytes	Basophils		Platelets			T-cells				Dendritic Cells
CD40 + IL4	PWM	LPS	fMLP	FceR1 Cross-linking	TRAP	Epinephrine	ACD	PMA + Iono	SEB + CD28	CD3 + CD28	CMV + CD28 + CD49d	LPS
CD95	CD95							TNF a	TNF a	TNF a		
CD25	CD25							IFN g	IFN g	IFN g		
CD71	CD71	IL-6			PAC-1	PAC-1	PAC-1	CD25	CD25	CD25	IFN g	
CD69	CD69	IL-8			AnxV	AnxV	AnxV	IL-4	IL-4	IL-4	TNF a	CD80
CD80	CD80	IL-1 b	IL-4	IL-4	CD62	CD62	CD62	IL-2	IL-2	IL-2	IL-2	CD86
CD86	CD86	TNF a	CD63	CD63	CD63	CD63	CD63	BrdU	BrdU	BrdU	CD69	TNF a

Leukocyte Response Profiles

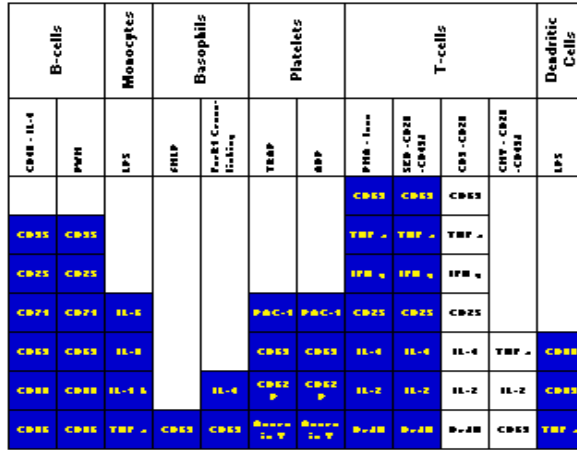
- **Describe drugs**
 - to characterize the effect of an inhibitor on a molecular pathway
 - to recognize the pleiotropy of that pathway
- **Describe people**
 - to recognize genetic or environmental polymorphisms
 - to characterize the summed effect of a complicated therapeutic regimen



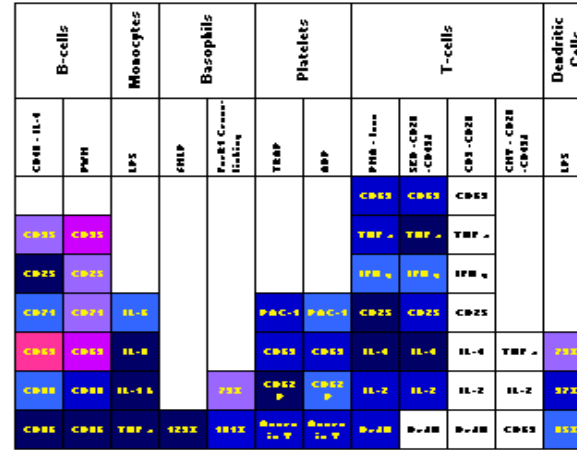
Typical Blood Profile

Herbimycin A

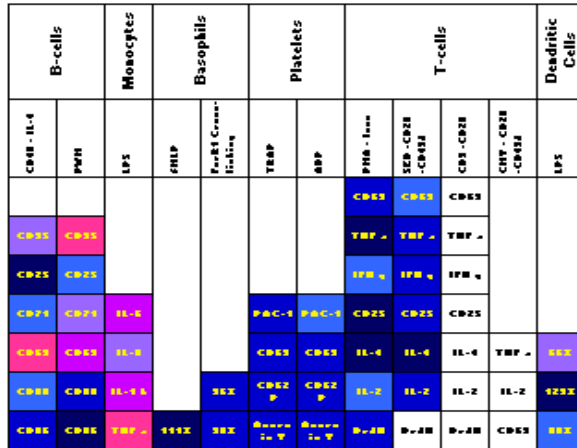
0 μ M



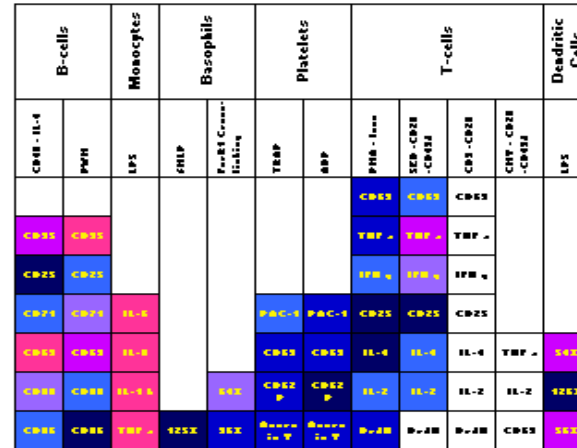
0.4 μ M



2 μ M



10 μ M



Effect of Coincubation of Two Immunosuppressive Drugs On T cell and B cell Markers

CD86 FITC
Total Median Fluorescence
% Difference from Zero Drug

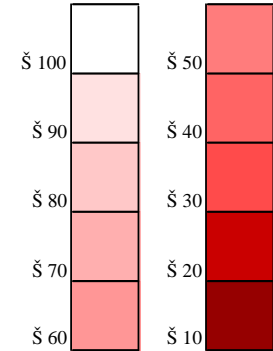
		Drug 1				
		0 μ M	0.1 μ M	0.2 μ M	0.4 μ M	1 μ M
Drug 2	0 ng	100%	75%	54%	40%	40%
	2 ng	95%	71%	47%	35%	28%
	6 ng	95%	85%	47%	34%	26%
	10 ng	92%	53%	43%	35%	27%
	30 ng	100%	68%	46%	34%	25%

CD95 APC
Total Median Fluorescence
% Difference from Zero Drug

		Drug 1				
		0 μ M	0.1 μ M	0.2 μ M	0.4 μ M	1 μ M
Drug 2	0 ng	100%	96%	86%	62%	59%
	2 ng	81%	79%	67%	54%	45%
	6 ng	81%	79%	69%	58%	41%
	10 ng	80%	71%	64%	56%	44%
	30 ng	86%	79%	70%	57%	40%

CD54 PE
Total Median Fluorescence
% Difference from Zero Drug

		Drug 1				
		0 μ M	0.1 μ M	0.2 μ M	0.4 μ M	1 μ M
Drug 2	0 ng	100%	85%	66%	48%	49%
	2 ng	82%	72%	55%	41%	31%
	6 ng	79%	82%	53%	40%	25%
	10 ng	74%	55%	48%	37%	28%
	30 ng	74%	66%	49%	37%	24%



IFN γ
Median Fluorescence of Positives
% Difference from Zero Drug

		Drug 1				
		0 μ M	0.1 μ M	0.2 μ M	0.4 μ M	1 μ M
Drug 2	0 ng	100%	83%	64%	37%	15%
	2 ng	80%	68%	51%	36%	17%
	6 ng	89%	71%	59%	35%	17%
	10 ng	82%	66%	50%	35%	14%
	30 ng	95%	75%	58%	39%	18%

IL-2
Median Fluorescence of Positives
% Difference from Zero Drug

		Drug 1				
		0 μ M	0.1 μ M	0.2 μ M	0.4 μ M	1 μ M
Drug 2	0 ng	100%	89%	77%	59%	43%
	2 ng	94%	88%	75%	57%	49%
	6 ng	96%	91%	77%	60%	46%
	10 ng	94%	90%	77%	63%	50%
	30 ng	104%	94%	78%	62%	41%

TNF α
Median Fluorescence of Positives
% Difference from Zero Drug

		Drug 1				
		0 μ M	0.1 μ M	0.2 μ M	0.4 μ M	1 μ M
Drug 2	0 ng	100%	67%	50%	27%	23%
	2 ng	81%	54%	43%	25%	13%
	6 ng	93%	61%	43%	26%	14%
	10 ng	75%	60%	38%	28%	16%
	30 ng	83%	63%	43%	28%	14%

Cytokine Flow Cytometry Baseline Study

Lyophilized
stimulation
plate

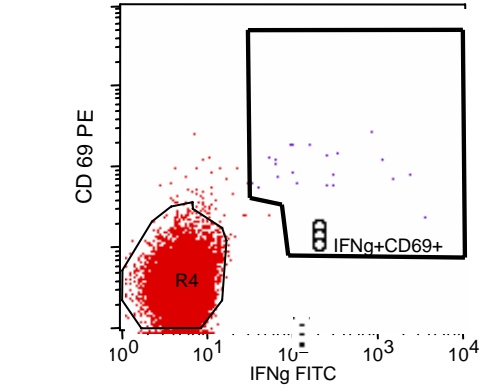
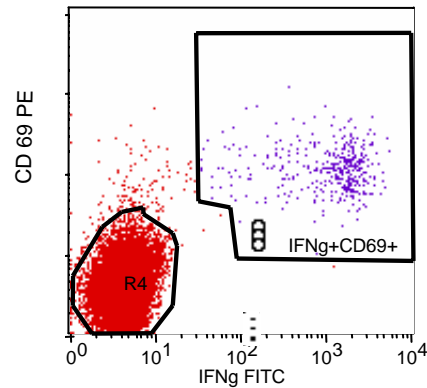
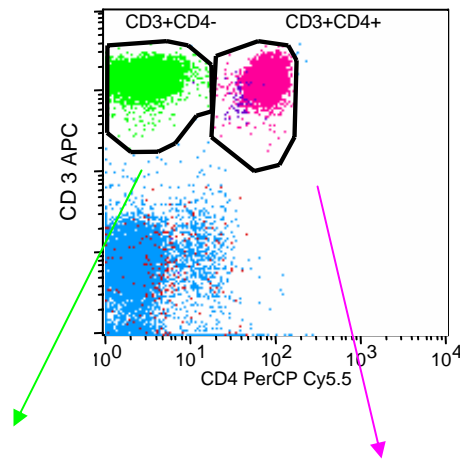
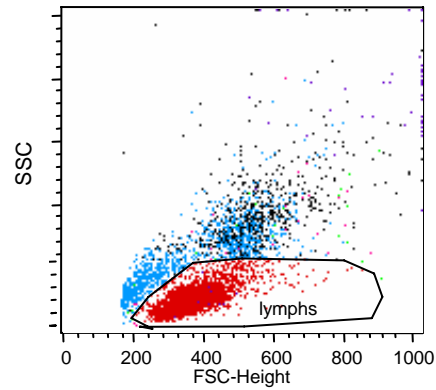
blank	CD28+49d 1 ug/ml ea	pp65 mix 3.4 ug/ml	IE mix 3.4 ug/ml	A2 CMV pep 1 ug/ml	B7 CMV pep 1 ug/ml	flu HA+M1 3.4 ug/ml	HIV gag mix 3.4 ug/ml	CEA mix 3.4 ug/ml	MAGE-3 mix 3.4 ug/ml	her2/neu ICD mix 3.4 ug/ml	CEF
BFA	CD28+49d 1 ug/ml ea	pp65 mix 1.7 ug/ml	IE mix 1.7 ug/ml	A2 CMV pep 0.2 ug/ml	B7 CMV pep 0.2 ug/ml	flu HA+M1 1.7 ug/ml	HIV gag mix 1.7 ug/ml	CEA mix 1.7 ug/ml	MAGE-3 mix 1.7 ug/ml	her2/neu ICD mix 1.7 ug/ml	CEF
DMSO cntl	BFA	pp65 mix 0.8 ug/ml	IE mix 0.8 ug/ml	A2 CMV pep 0.04 ug/ml	B7 CMV pep 0.04 ug/ml	flu HA+M1 0.8 ug/ml	HIV gag mix 0.8 ug/ml	CEA mix 0.8 ug/ml	MAGE-3 mix 0.8 ug/ml	her2/neu ICD mix 0.8 ug/ml	PMA 10 ng+ ion 1 ug/ml
DMSO cntl	CD28+49d 1 ug/ml ea	pp65 mix 0.4 ug/ml	IE mix 0.4 ug/ml	A2 CMV pep 0.008 ug/ml	B7 CMV pep 0.008 ug/ml	flu HA+M1 0.4 ug/ml	HIV gag mix 0.4 ug/ml	CEA mix 0.4 ug/ml	MAGE-3 mix 0.4 ug/ml	her2/neu ICD mix 0.4 ug/ml	SEB 1 ug/ml
blank	CD28+49d 1 ug/ml ea	pp65 mix 3.4 ug/ml	IE mix 3.4 ug/ml	A2 CMV pep 1 ug/ml	B7 CMV pep 1 ug/ml	flu HA+M1 3.4 ug/ml	HIV gag mix 3.4 ug/ml	CEA mix 3.4 ug/ml	MAGE-3 mix 3.4 ug/ml	her2/neu ICD mix 3.4 ug/ml	CEF
BFA	CD28+49d 1 ug/ml ea	pp65 mix 1.7 ug/ml	IE mix 1.7 ug/ml	A2 CMV pep 0.2 ug/ml	B7 CMV pep 0.2 ug/ml	flu HA+M1 1.7 ug/ml	HIV gag mix 1.7 ug/ml	CEA mix 1.7 ug/ml	MAGE-3 mix 1.7 ug/ml	her2/neu ICD mix 1.7 ug/ml	CEF
DMSO cntl	BFA	pp65 mix 0.8 ug/ml	IE mix 0.8 ug/ml	A2 CMV pep 0.04 ug/ml	B7 CMV pep 0.04 ug/ml	flu HA+M1 0.8 ug/ml	HIV gag mix 0.8 ug/ml	CEA mix 0.8 ug/ml	MAGE-3 mix 0.8 ug/ml	her2/neu ICD mix 0.8 ug/ml	PMA 10 ng+ ion 1 ug/ml
DMSO cntl	CD28+49d 1 ug/ml ea	pp65 mix 0.4 ug/ml	IE mix 0.4 ug/ml	A2 CMV pep 0.008 ug/ml	B7 CMV pep 0.008 ug/ml	flu HA+M1 0.4 ug/ml	HIV gag mix 0.4 ug/ml	CEA mix 0.4 ug/ml	MAGE-3 mix 0.4 ug/ml	her2/neu ICD mix 0.4 ug/ml	SEB 1 ug/ml

Lyophilized
staining
plate

IFN γ FITC / CD69 PE / CD4 PerCP-Cy5.5 / CD3 APC											
TNF α FITC / IL-2 PE / CD4 PerCP-Cy5.5 / CD3 APC											

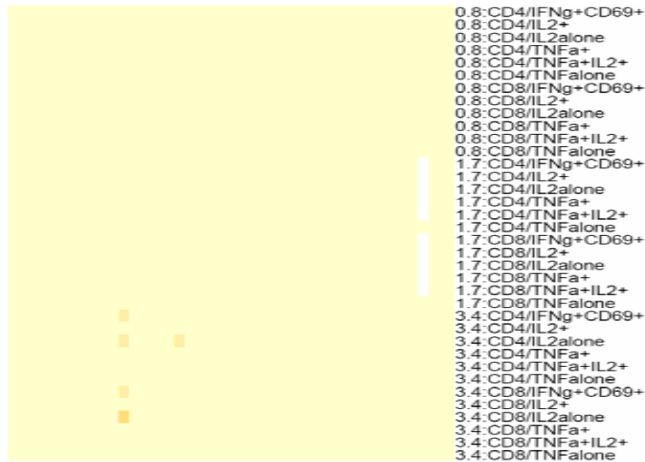


Cytokine Gating



Normal Donor Cytokine Responses - Controls

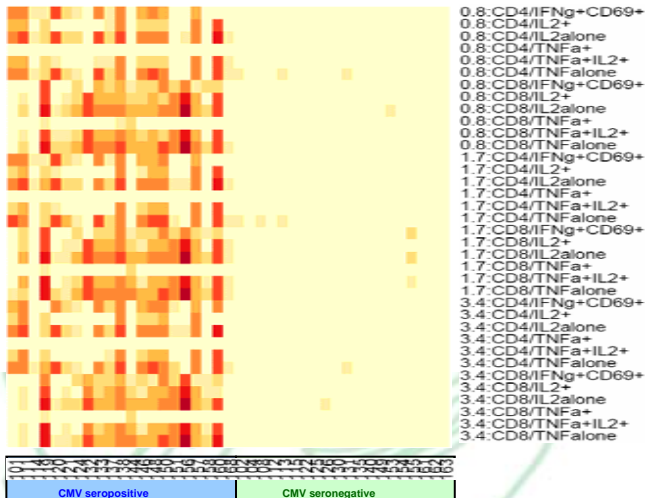
HIV mix (neg control)



Flu HA+M1 mix

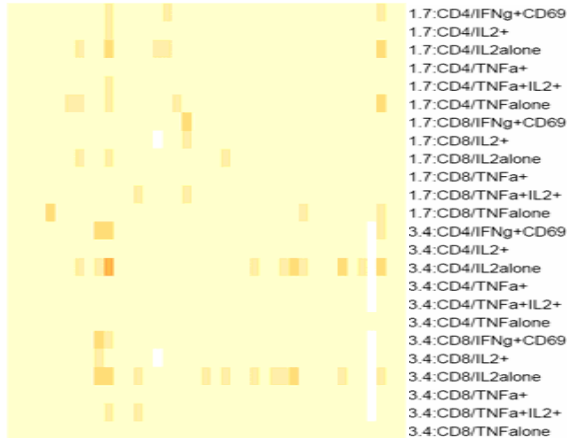


CMV pp65 mix



Normal Donor Cytokine Responses to Cancer Antigen

Her2/neu mix



MAGE-3 mix



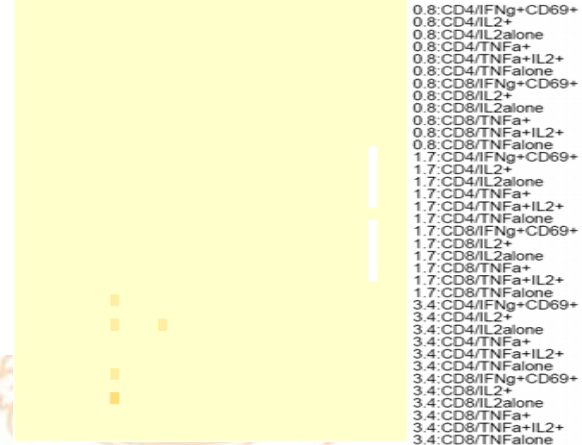
CEA mix



CMV seropositive

CMV seronegative

HIV mix (neg control)



CMV seropositive

CMV seronegative



Summary CFC Baseline Study

Cytokine flow cytometry offers quantitative assessments of T-cell function

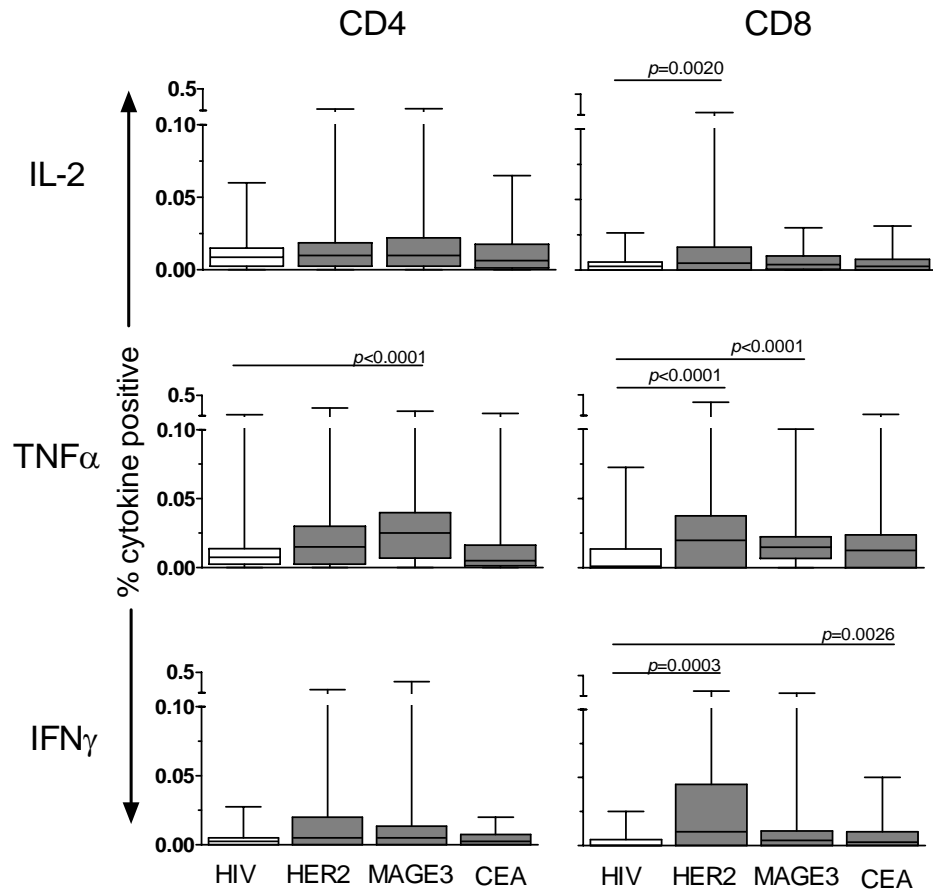
Healthy donors demonstrate correlation between
CMV serology and CFC response to CMV peptides.

TNF α responses in CD4 and CD4- cells to Her2/neu and
MAGE-3 were low but significantly different from
neg control antigen.

IFN γ , TNF α and IL-2 responses to CEA was not significantly
different from negative controls in healthy donors.



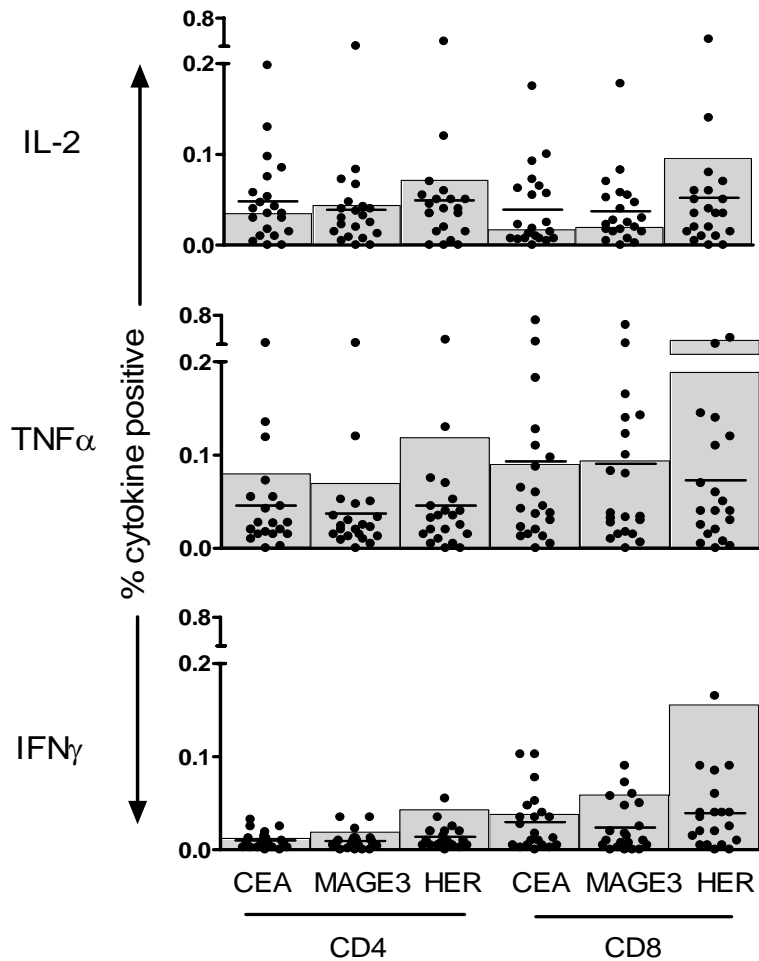
Healthy Donor Analysis of TAA by Flow



Percentage of cytokine producing cells in T-cell subsets



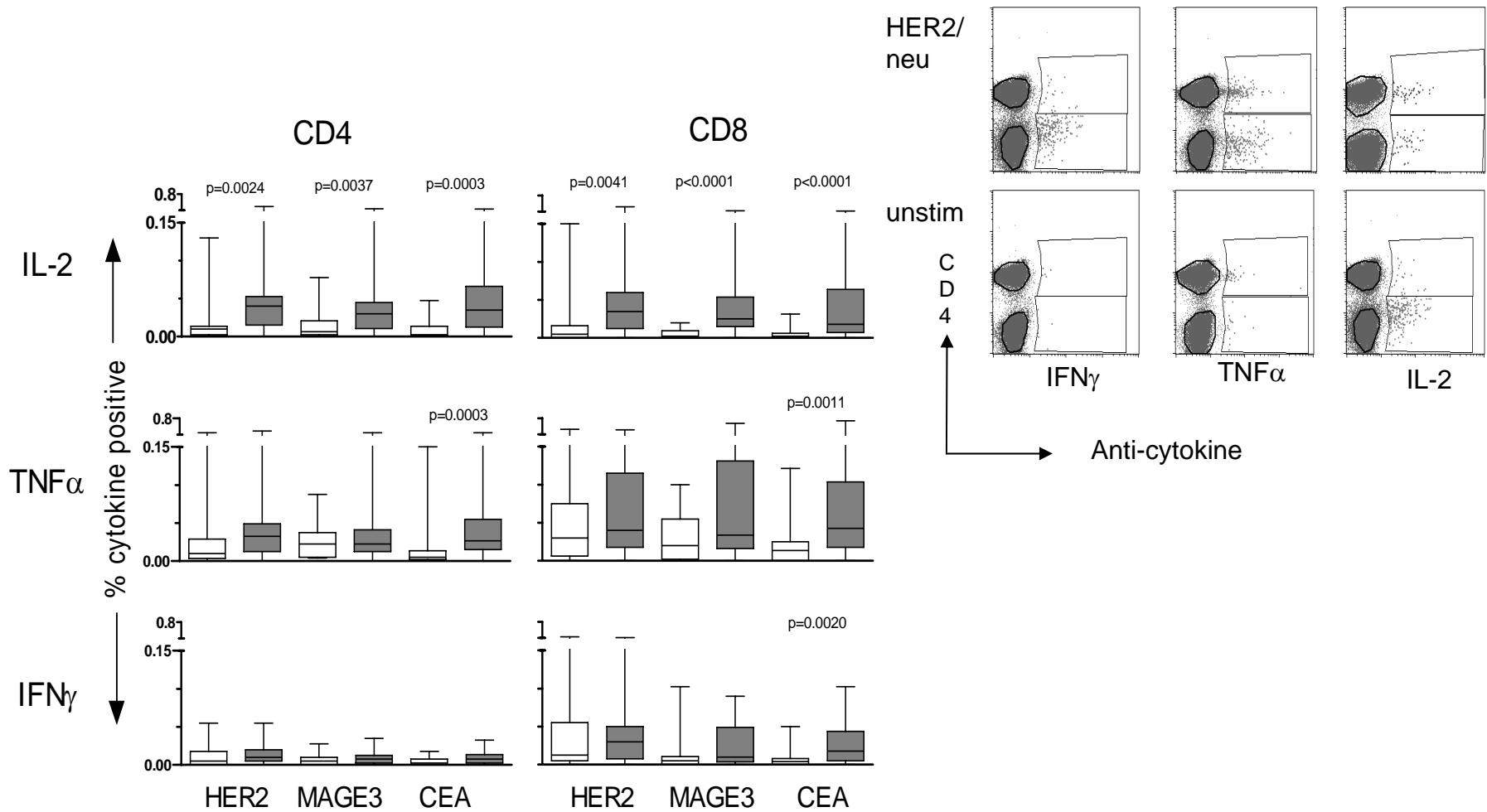
Cytokine Responses in Breast Cancer Patients



Percentage of cytokine producing cells in T-cell subsets

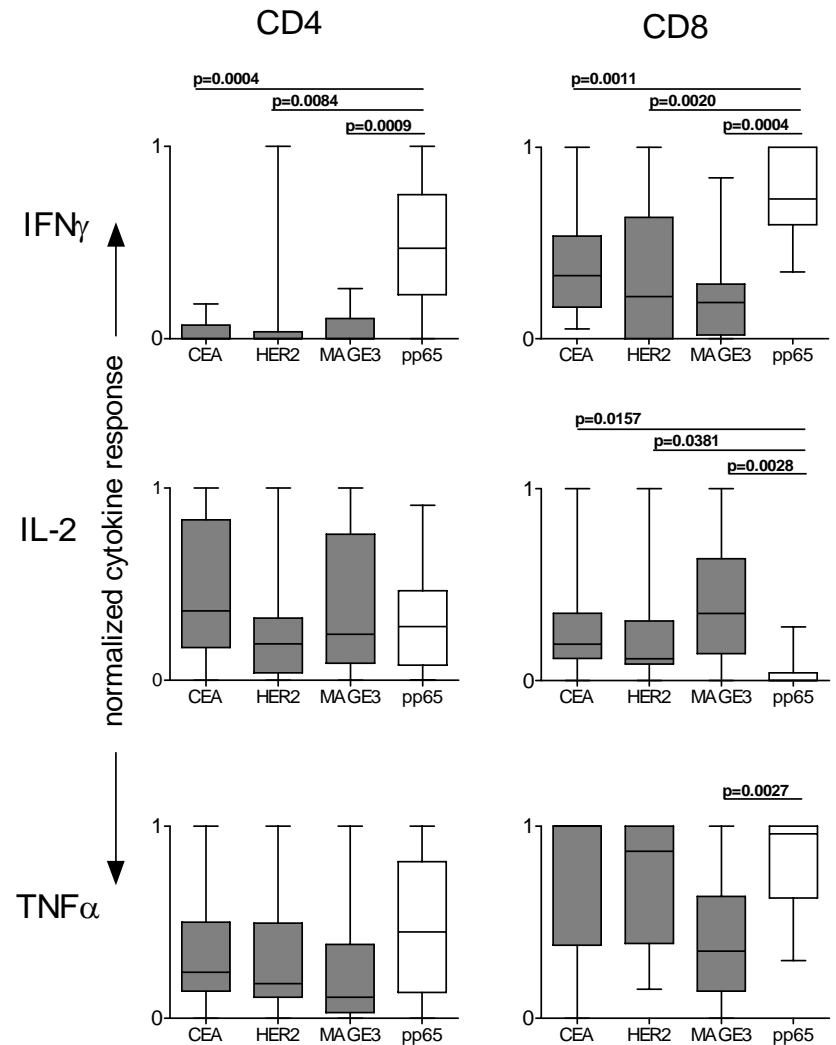


Cytokine Responses in Breast Cancer Patients



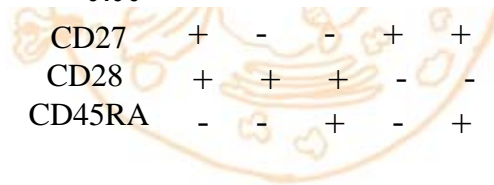
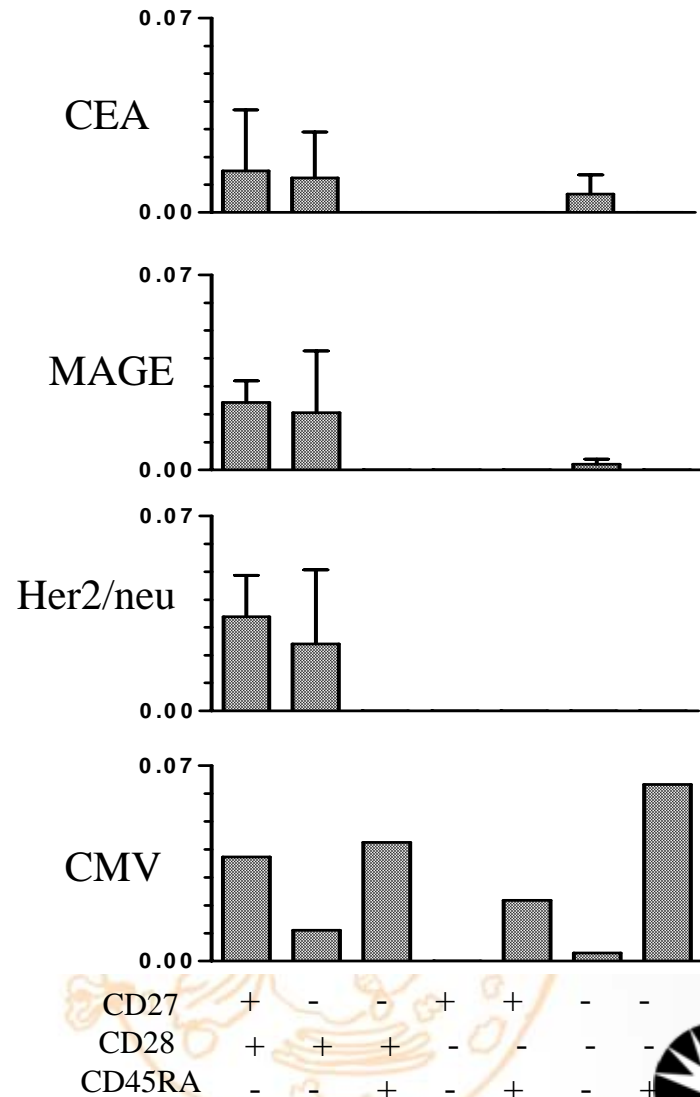
Cytokine T-cell Responses to Antigenic Challenge

- Responses to CMV antigen in CMV seropositive without clinical disease
- Unsuccessful response to self antigen (TAA in breast cancer patients)

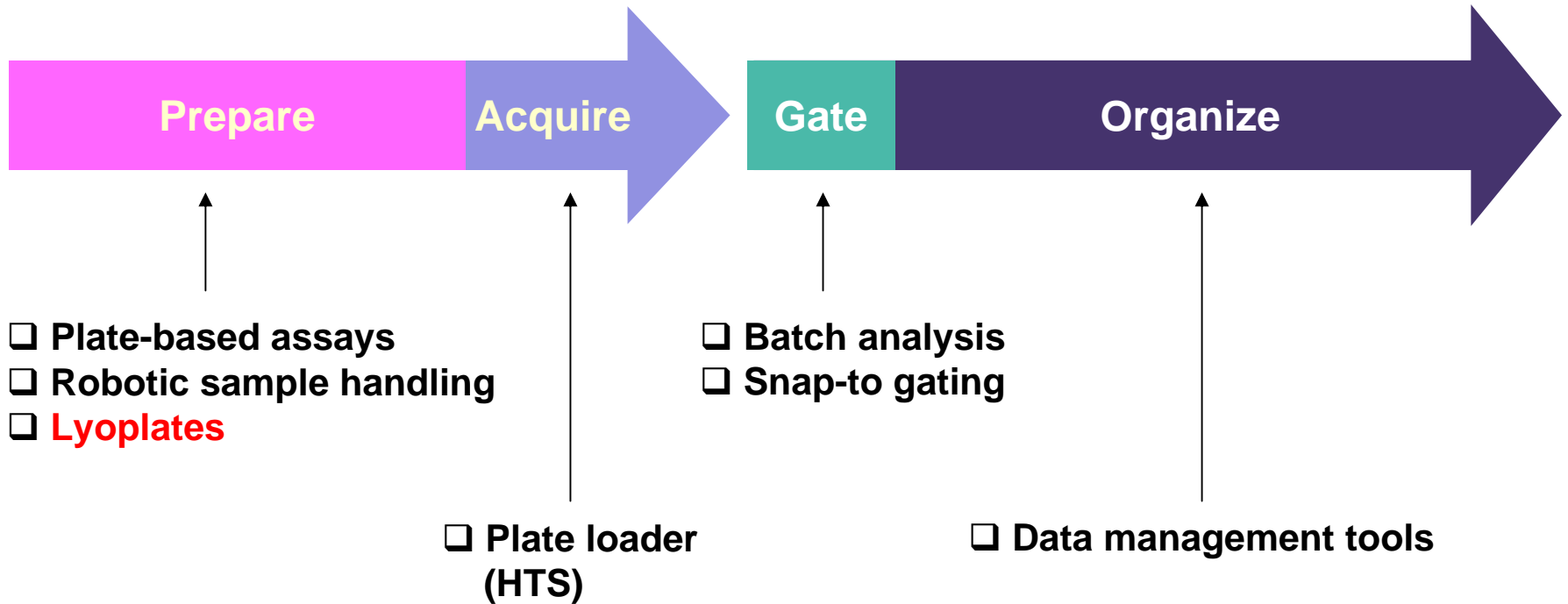


Effector and Memory T-cell Distribution in Cancer Patients

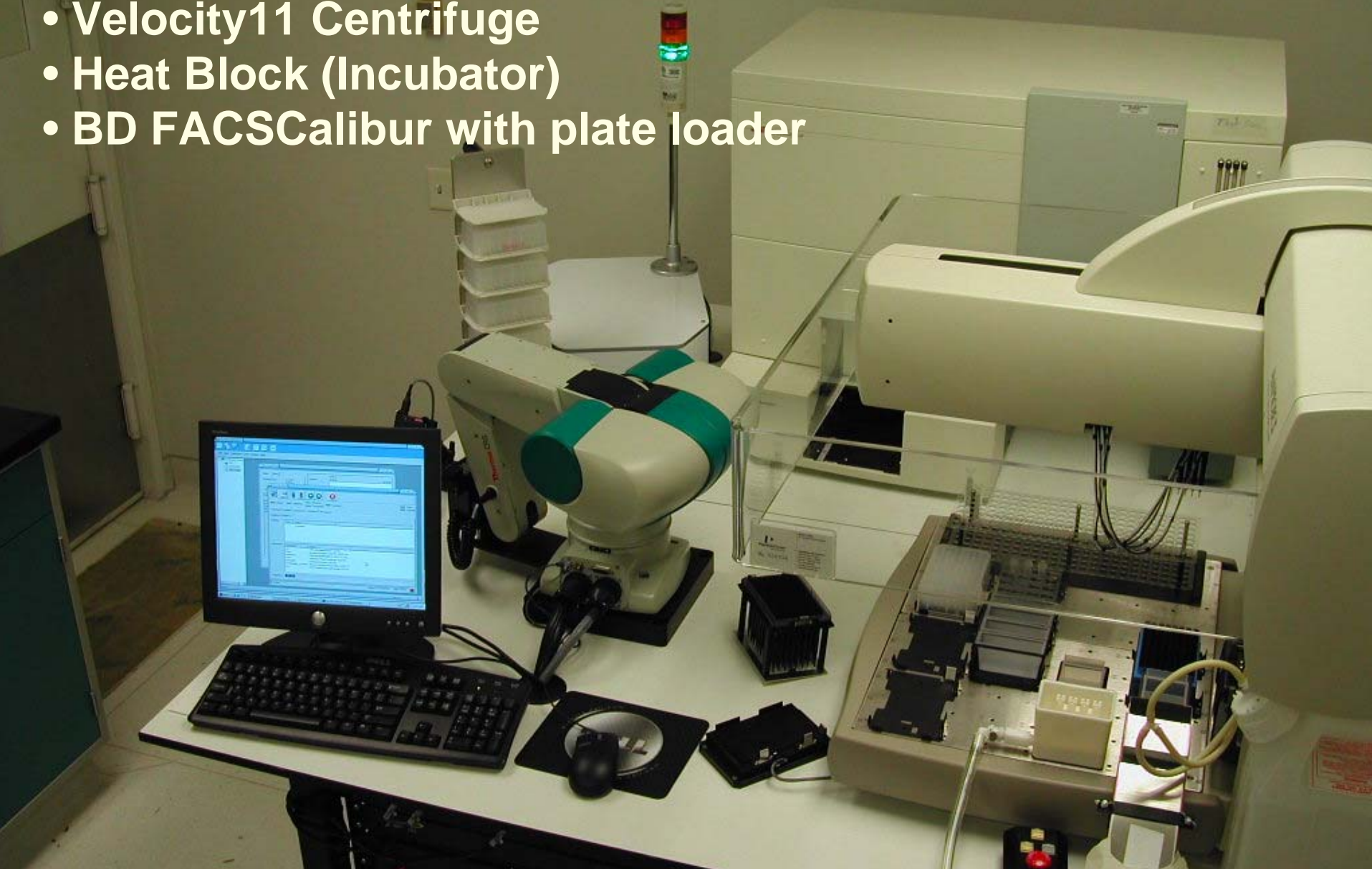
- Immunophenotyping of T-cells responding to tumor associated antigens compared to CMV
- Expression of IFN γ and/or TNF α
- Data arranged from central memory to terminal effector



Consider the entire workflow



- ThermoCRS Robotic Arm
- Packard Pipetting Station
- Velocity11 Centrifuge
- Heat Block (Incubator)
- BD FACSCalibur with plate loader



Lyophilized Reagent CFC Protocol

PBMC or whole blood

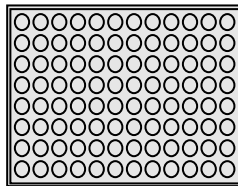


Plate with lyophilized Ag+BFA

6 h @ 37°C, EDTA, BD FACS Lyse, BD FACS Perm

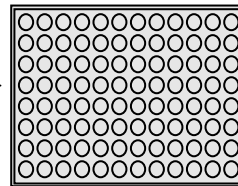


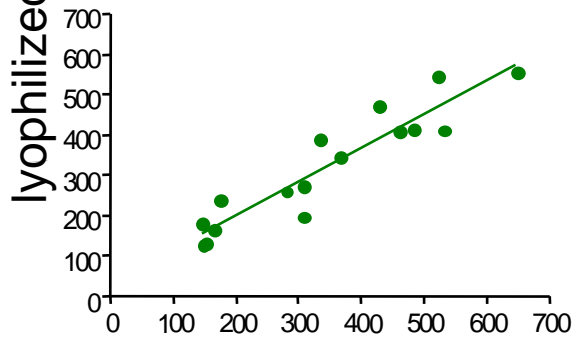
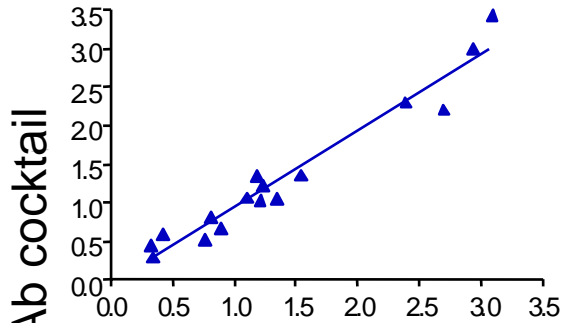
Plate with lyophilized Ab

to Flow cytometer

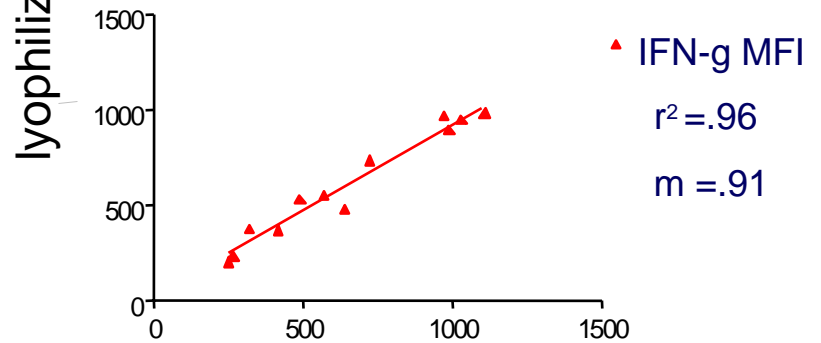
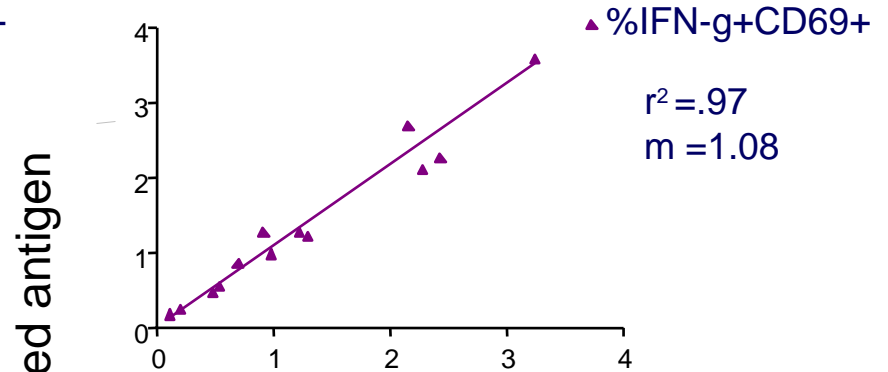


Liquid vs Lyophilized Reagents

CMV pp65-mix activated blood



liquid mAb cocktail



liquid antigen

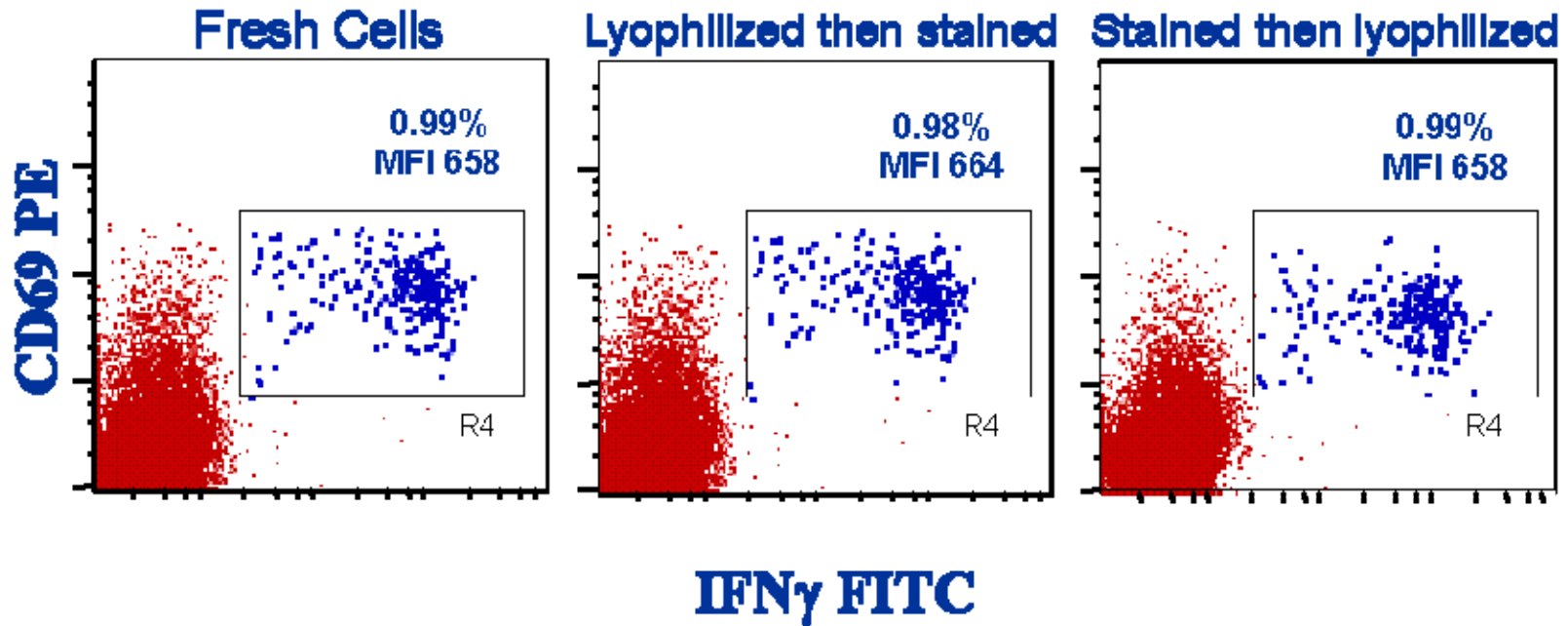


Multi-Site Data with BD Lyoplates

Assay Type	Fixed Activated Blood	Shipped Whole Blood	Cryopreserved PBMC	Cryopreserved PBMC with Lyoplates
Number of samples	12	12	24	9
Mean % CV	24%	31%	23%	18%



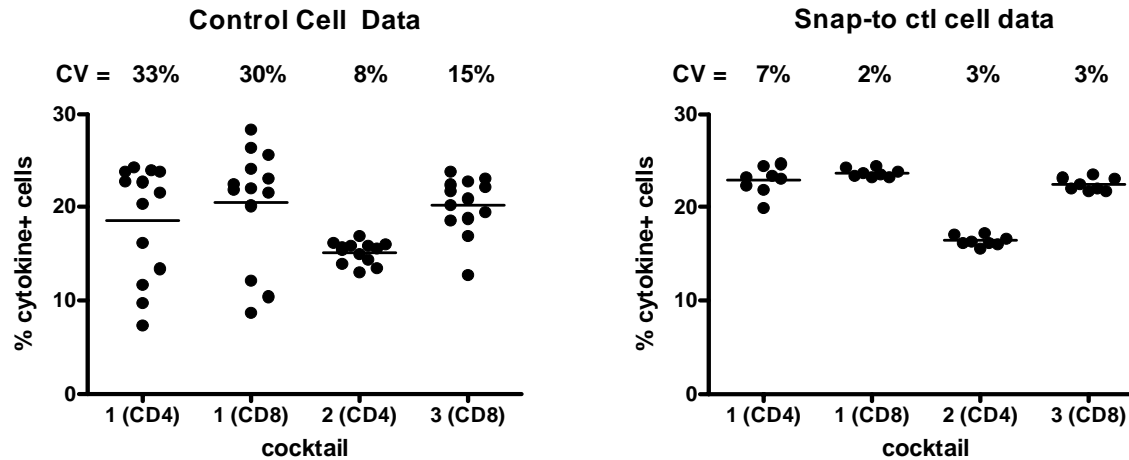
Cell Lyophilization for use as Controls



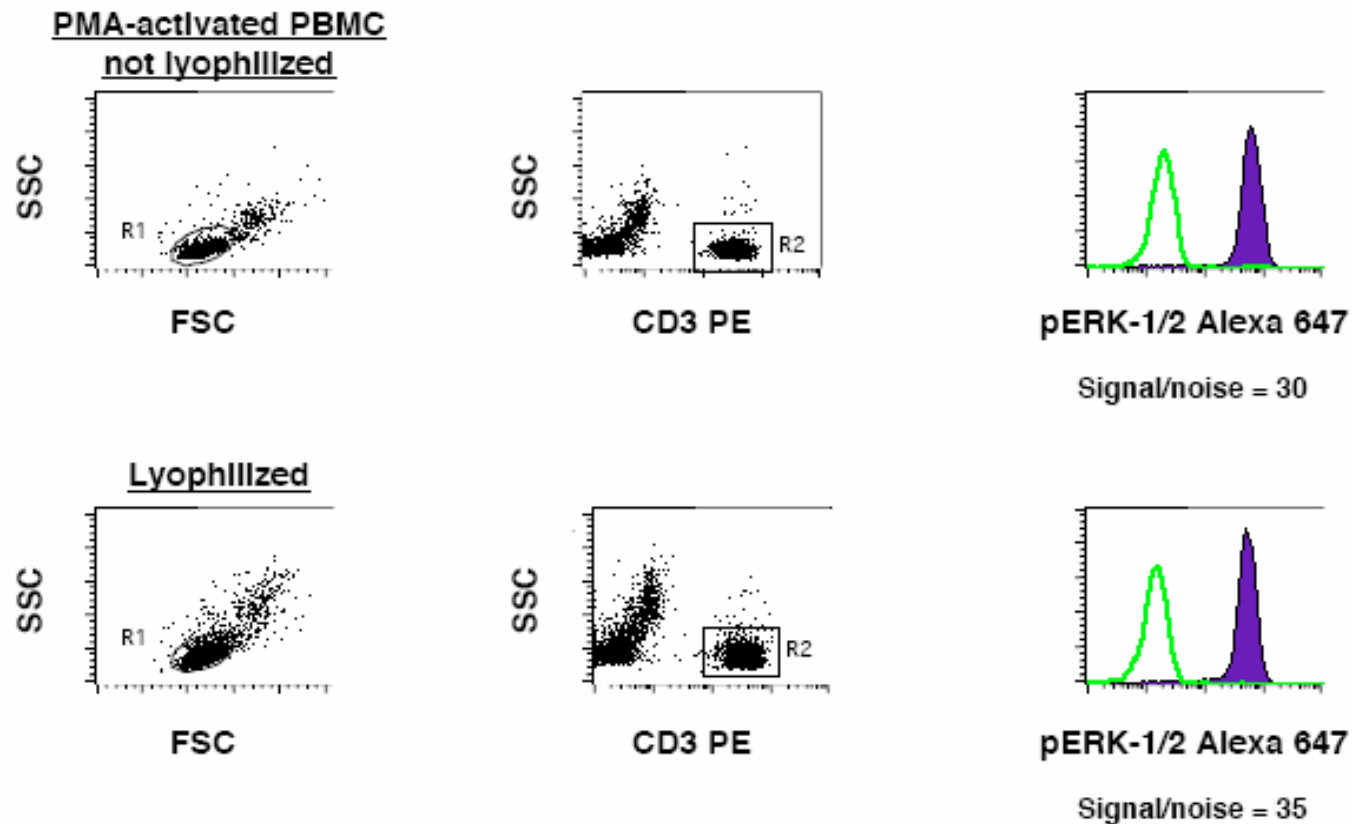
Data Management: Lyophilized Cells in a Multi Site Study

This is to cover

The title below

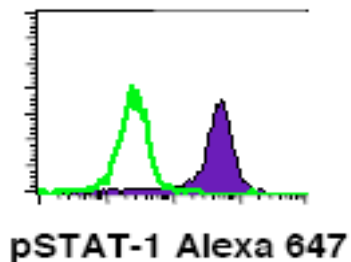
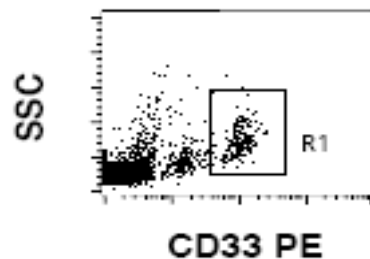


Lyophilized BD Phosflow Control Cells



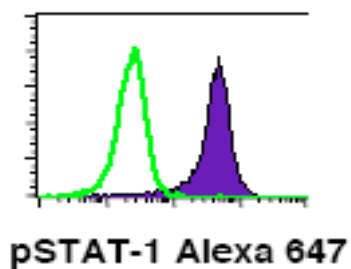
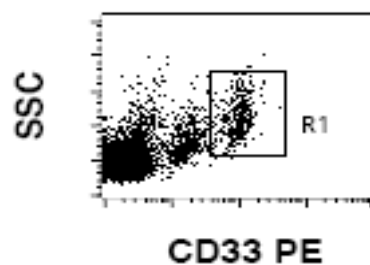
Lyophilized BD Phosflow Control Cells

rIFN γ -activated PBMC
not lyophilized



Signal/noise = 19

Lyophilized

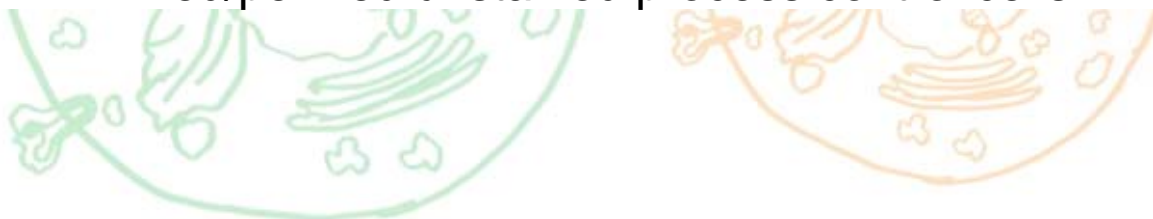


Signal/noise = 19

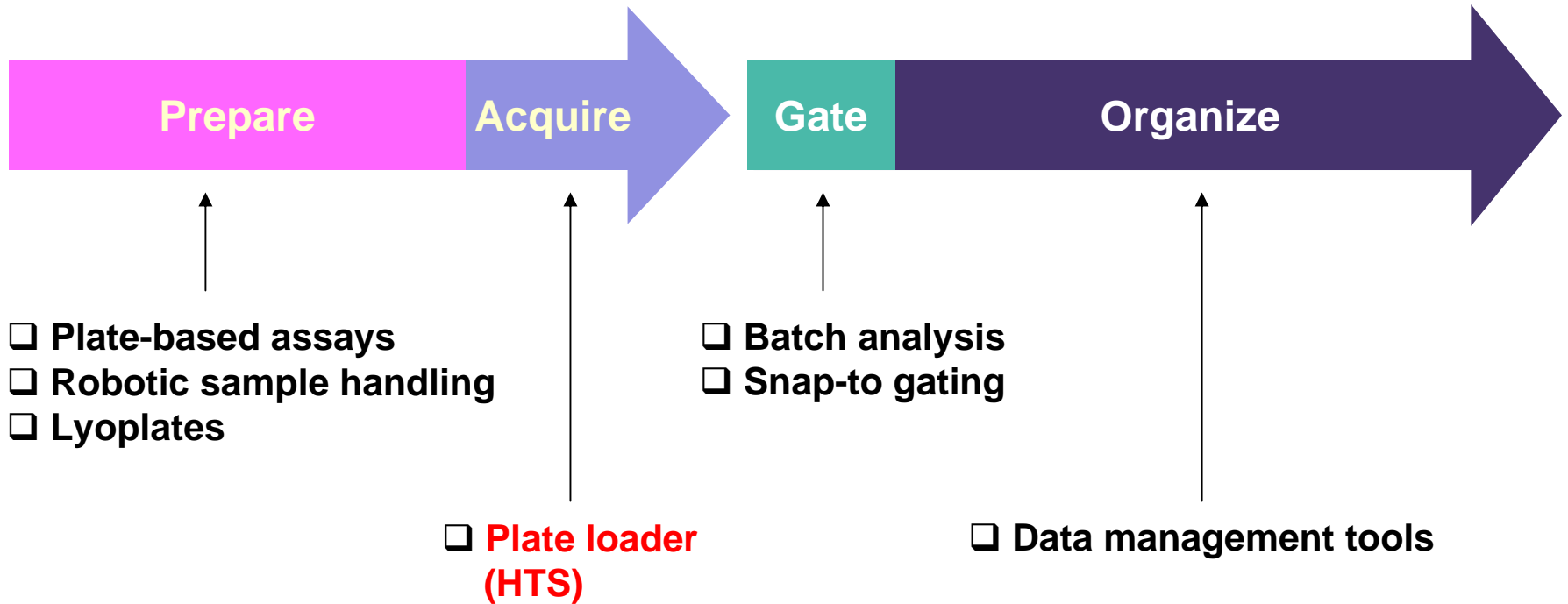


Custom BD Lyoplate Products

- Vessel configurations
 - Standard 96 well, polypropylene or polystyrene
 - 8-well strip plates, polystyrene
 - Deep well 96 polypropylene plates
 - Deep well 96 polypropylene individual 1ml vials
- Contents
 - Fluorochrome antibody conjugate cocktails
 - BD CBA beads
 - Peptide antigen cocktails
 - Polyclonal protein mitogens
 - Pharmacophores
 - Fixed/permed/stained instrument/data control cells
 - Fixed/permed unstained process control cells



Consider the entire workflow

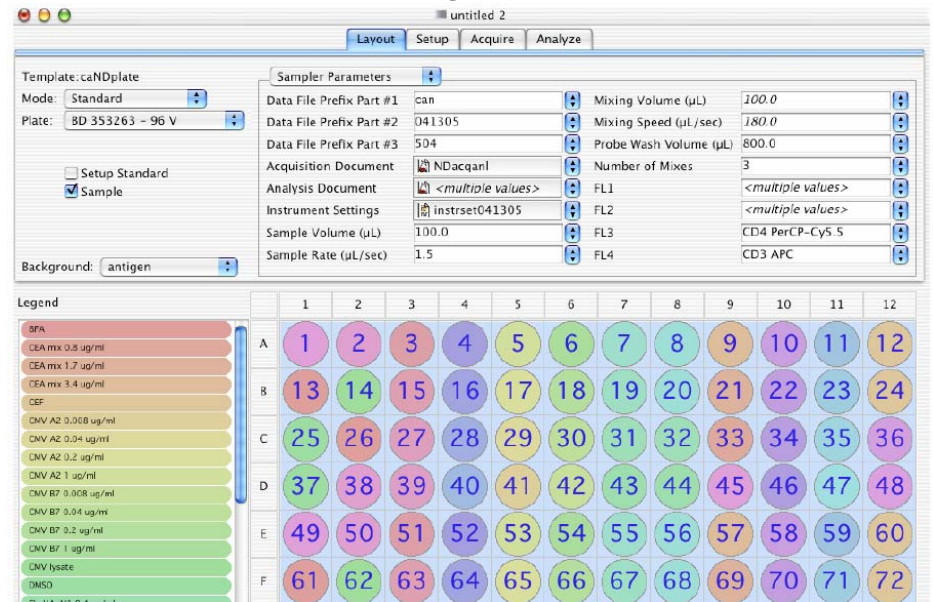


High Throughput Sampler

High Throughput Sampler on BD FACSCanto II



Plate manager software



The screenshot shows the 'Plate manager software' interface with the following parameters:

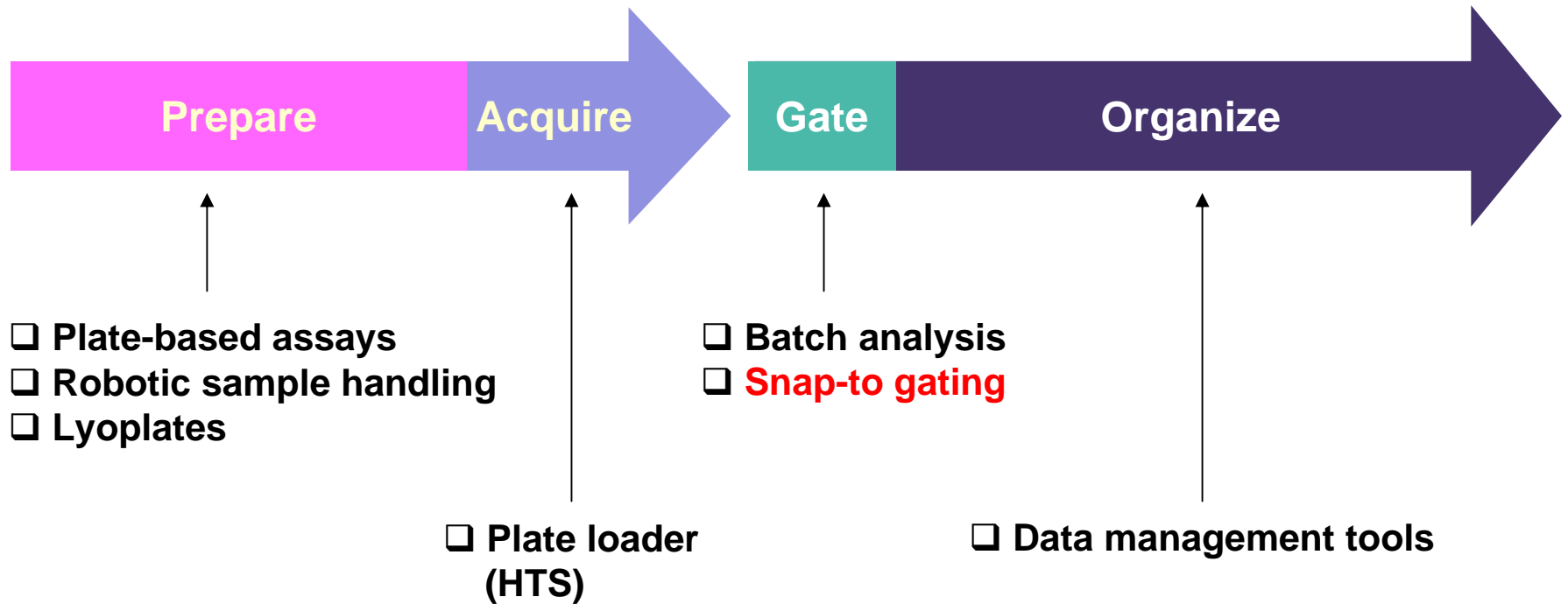
Parameter	Value
Template	caNDplate
Mode	Standard
Plate	BD 353263 - 96 V
Setup Standard	<input type="checkbox"/>
Sample	<input checked="" type="checkbox"/>
Background	antigen
Data File Prefix Part #1	can
Data File Prefix Part #2	041305
Data File Prefix Part #3	504
Acquisition Document	NDacqanl
Analysis Document	<multiple values>
Instrument Settings	instrset041305
Sample Volume (µL)	100.0
Sample Rate (µL/Sec)	1.5
Mixing Volume (µL)	100.0
Mixing Speed (µL/sec)	180.0
Probe Wash Volume (µL)	800.0
Number of Mixes	3
FL1	<multiple values>
FL2	<multiple values>
FL3	CD4 PerCP-Cy5 5
FL4	CD3 APC

The 'Legend' section shows a 6x12 grid of wells (A-F, 1-12) with colored circles representing different samples:

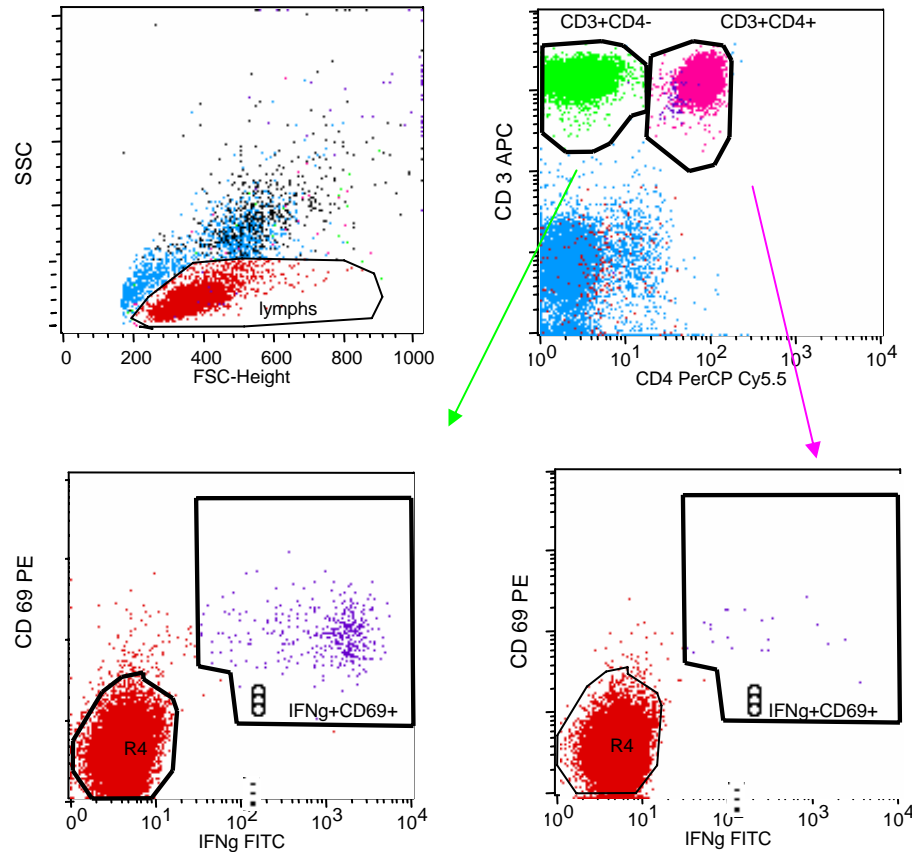
	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72



Consider the entire workflow



Automated Snap-to Gating



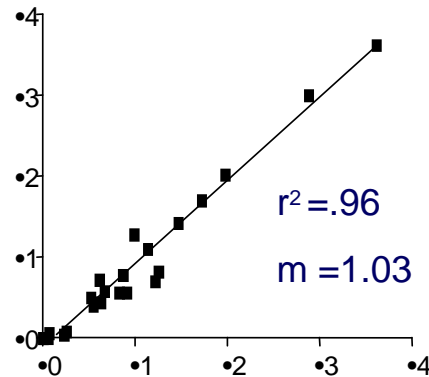
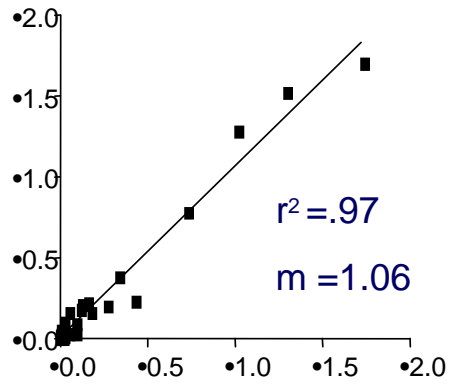
Automated vs Manual Gating

•%IFN-g+ CD69+

•CD4

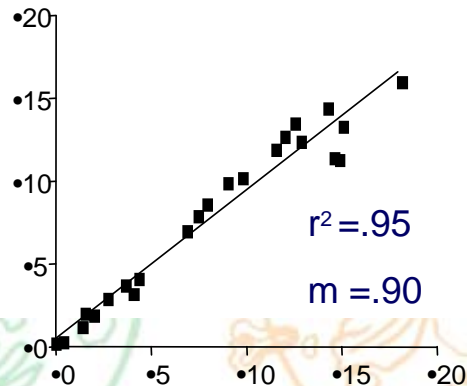
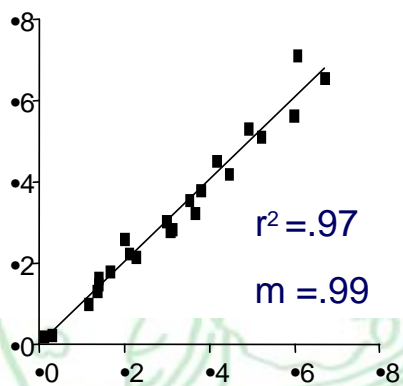
•CD8

CMV
pp65



Auto
gating

SEB

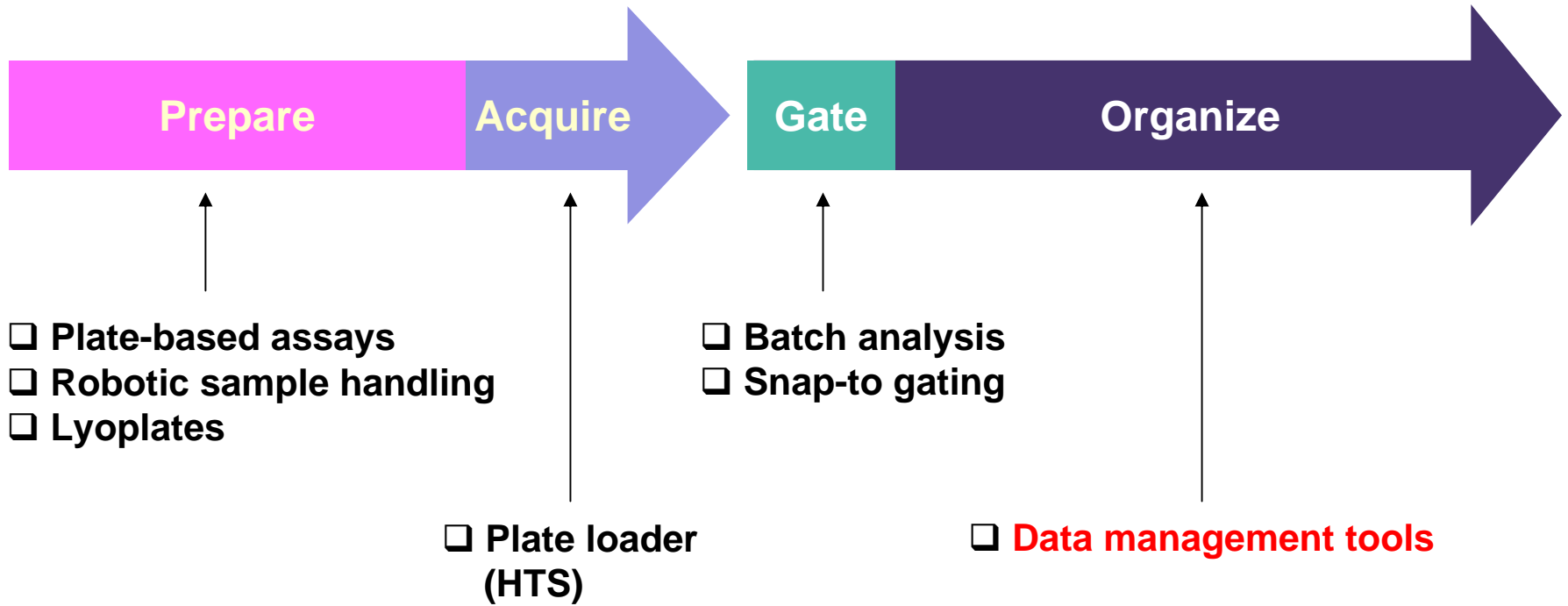


Manual gating

- Expert algorithm development
- Increased reproducibility
- Speed
- Ease-of-use

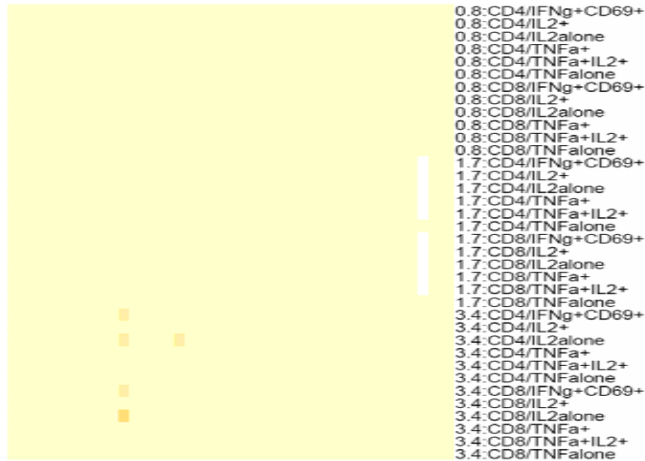


Consider the entire workflow



Normal donor cytokine responses, controls

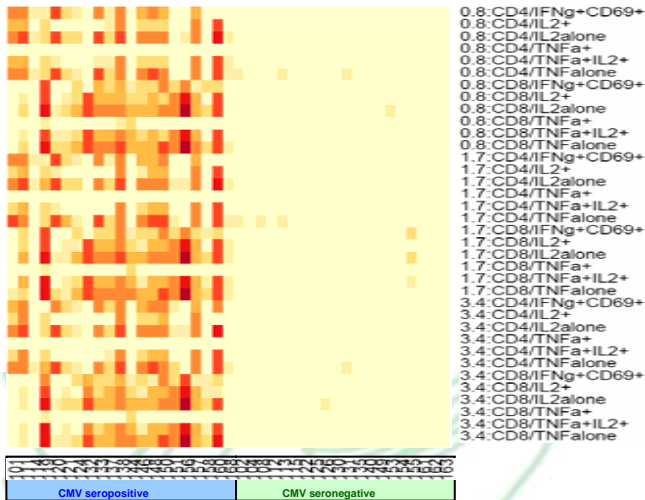
HIV mix (neg control)



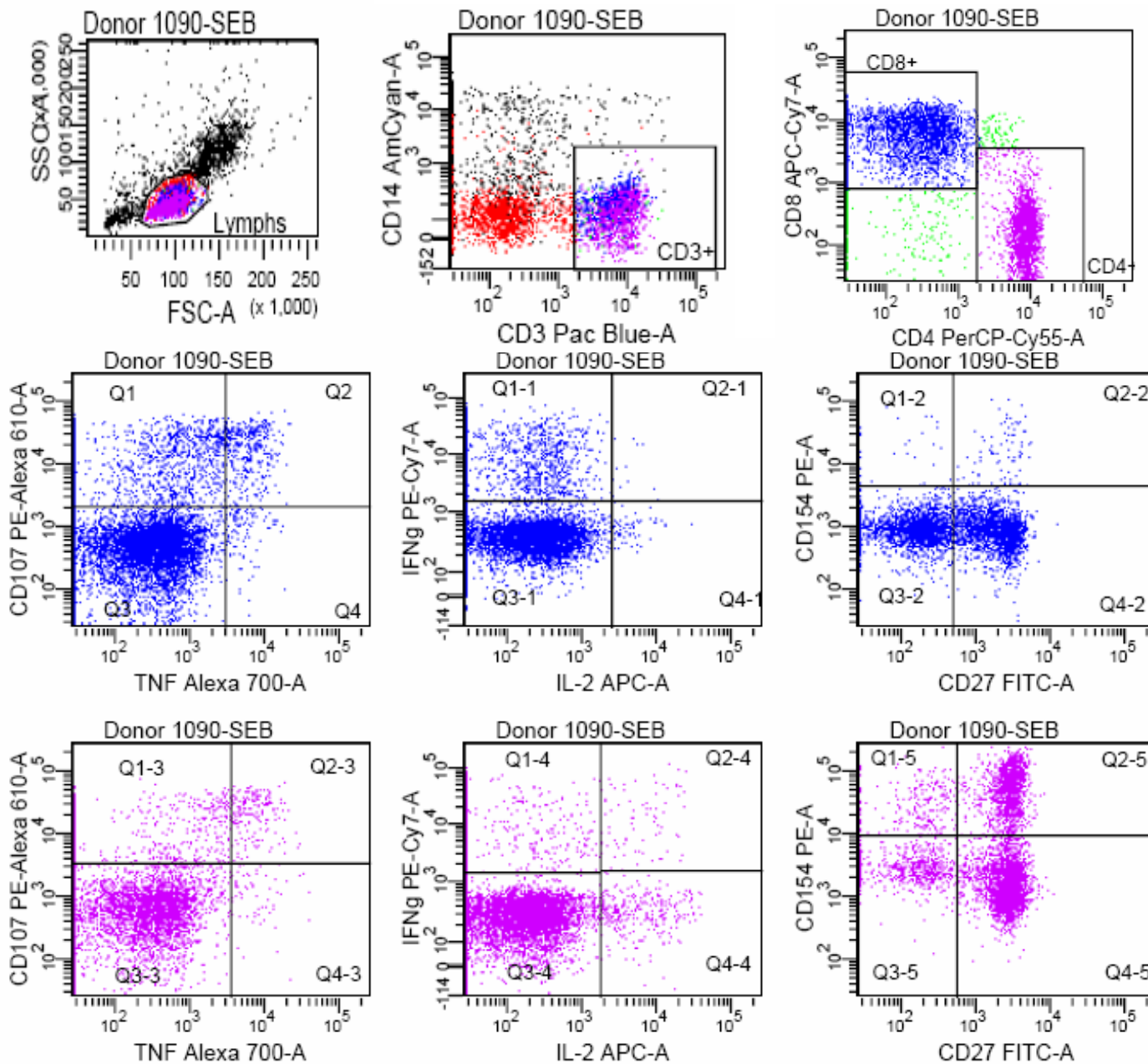
Flu HA+M1 mix



CMV pp65 mix



10 Color BD Lyoplates



BD

Technology Summary

Lyophilized stimulation and staining reagents in a 96-well format provides standardization of assays.

Flow cytometer with 96-well plate loader reduces operator hands-on time.

Dynamic gating in flow analysis software reduces variability in data analysis.

Data management tools allow for manipulation of large data sets.



Applying Cell Analysis to Clinical Trials

- Cellular analysis is a very important tool in understanding drug response
- Monitor Immune function via blood is applicable to numerous disease states
- Flow Cytometry is a viable tool for clinical trials
- Assay standardization reduces CVs
- Centralized data analysis reduces assay variability
- May be applicable to pre-clinical, clinical trial and patient profiling



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