Optimizing Intracellular Flow Cytometry:

Simultaneous Detection of Cytokines and Transcription Factors

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BD

Outline

- Introduction
  - Cytokines
  - Transcription factors
- Basic concepts of intracellular flow cytometry
  - Optimization examples
- Treg/Th17 cell analysis
  - Considerations
  - Examples

Cytokines

- Soluble polypeptides produced by most nucleated cells in the body
- Some potent producers include endothelial and epithelial cells and resident macrophages, especially near the interface with the external environment
- Critical to the development and functioning of both the innate and adaptive immune responses
- Promote cellular differentiation and proliferation
  - Example: IL-2 involved in T cell activation and maintenance of a Th1 response
- Work in either an autocrine or paracrine manner

Th17 Cells

- A subset of $CD4^+$ T helper cells
- Developmentally distinct from Th1 and Th2 cells
- Immunity against bacterial and fungal infectious
- Play a key role in autoimmune diseases (tissue injury)
- Controlling Th17 activity could aid in the treatment of autoimmune diseases
- TGF-β, IL-6, IL-21, IL-1β, and IL-23 appear to drive Th17 development
- Produce IL-17A, IL-17F; also IL-21, IL-22, IL-26, and less TNF and IL-6

Transcription Factors

- Proteins that bind to specific DNA sequences
- Control the transfer of genetic information from DNA to RNA
- Regulators of gene expression
- A single transcription factor can bind hundreds of promoters

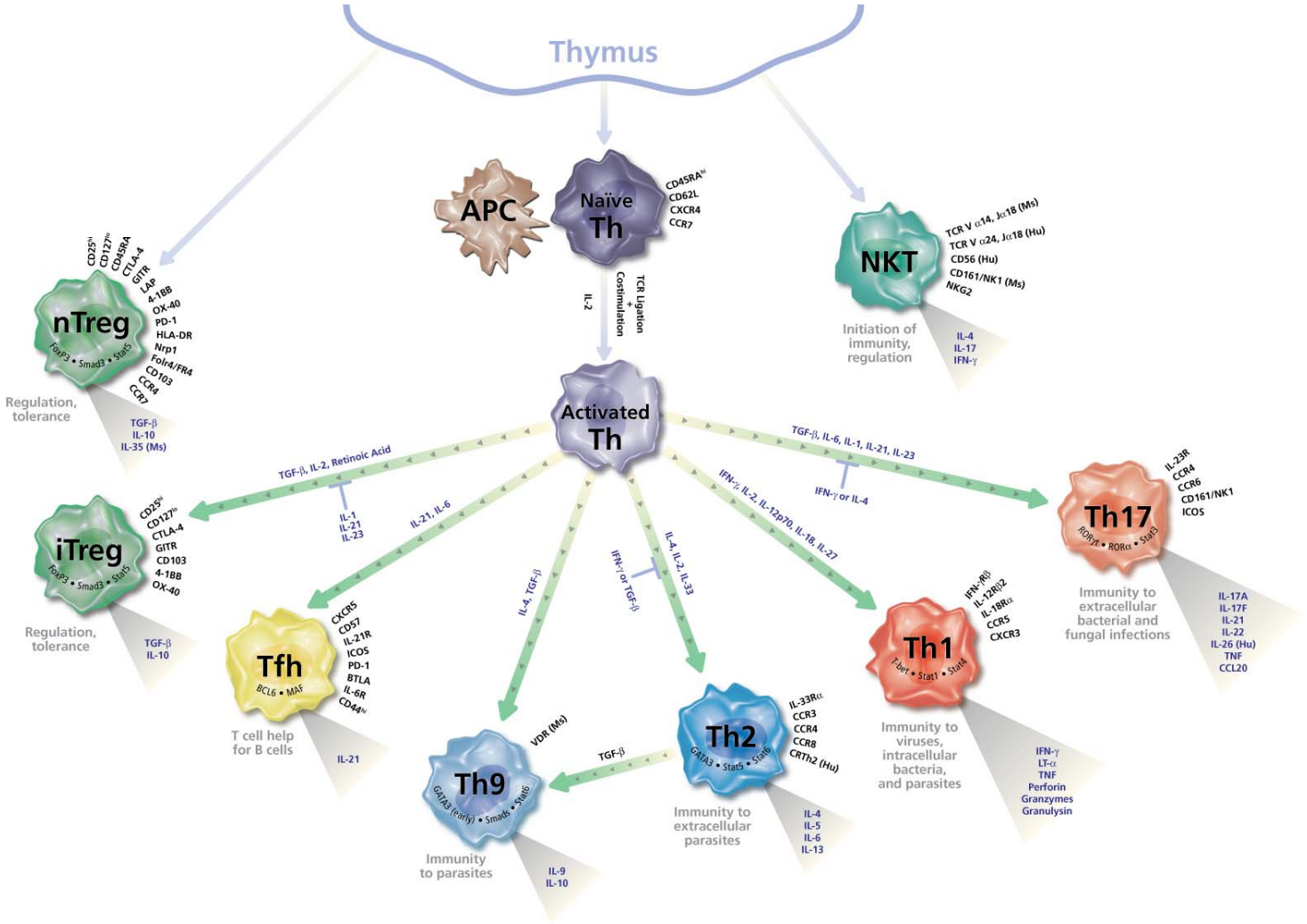
Regulatory T Cells

- Tregs = $CD4^+$ T regulatory cells
- Comprise ~ 1–3% of human PBMCs and ~ 4–8% of mouse spleen
- Actively suppress T cell proliferation
- Play a crucial role in T cell homeostasis
- nTreg develop in the thymus, iTreg require TGFβ, IL-2 and RA
- FoxP3, a forkhead family transcription factor, is a specific marker for Tregs
- FoxP3 is necessary for the development and function of Tregs

Regulatory T Cells, *cont'd*

- Produce TGFβ and IL-10 and express high levels of CD25 and low levels of CD127
- Diminish immune responses against cancers, allogeneic transplants, and infectious pathogens
- Dampening Treg activity could improve anti-tumor responses and responses to vaccinations and chronic infections
- Deficiencies contribute to the development of autoimmune diseases
- Boosting Treg activity could be useful in the treatment of T cell induced diseases

CD4+ T Cell Differentiation



What is Intracellular Flow Cytometry?

- Detection of:
  - Transcription factors
  - Intracellular signaling molecules
  - Cytokines
  - Structural proteins
  - Scaffold proteins
  - Pan and phospho-specific antigens

Considerations for Intracellular Flow Cytometry

- Must permeabilize a cell to access cell contents
- If a cell is permeabilized, then contents could “leak” out and the protein of interest could be lost
- Therefore, cells are fixed first, followed by permeabilization
- To detect secreted proteins, they must be “trapped” within the cell prior to fixation and permeabilization to increase the likelihood of detection

Considerations for Intracellular Flow, *cont'd*

- Protein transport inhibition
  - Monensin vs Brefeldin A (BD GolgiStop™ vs BD GolgiPlug™ inhibitor)
  - Optimal time for inhibition
  - Optimal concentration of inhibitor
- Fixation
  - Concentration (paraformaldehyde)
  - Time
  - Temperature
  - Compatibility with fluorochromes
  - Compatibility of cell surface markers

Considerations for Intracellular Flow, *cont'd.*

- Permeabilization
  - Perm agent (saponin, methanol, Tween® 20, Triton X-100™)
  - Concentration
  - Time
  - Temperature
  - Compatibility with fluorochromes
  - Compatibility of cell surface markers
- Different locations in cells are more difficult to access
- Types of proteins being identified, single or in a complex?

Considerations for Intracellular Flow, *cont'd.*

- Antibody staining
  - Order
  - Concentration
  - Time
  - Temperature
  - Fluorochromes
- Storage conditions
  - Buffer
  - Time
- Matching one antibody protocol with another antibody protocol

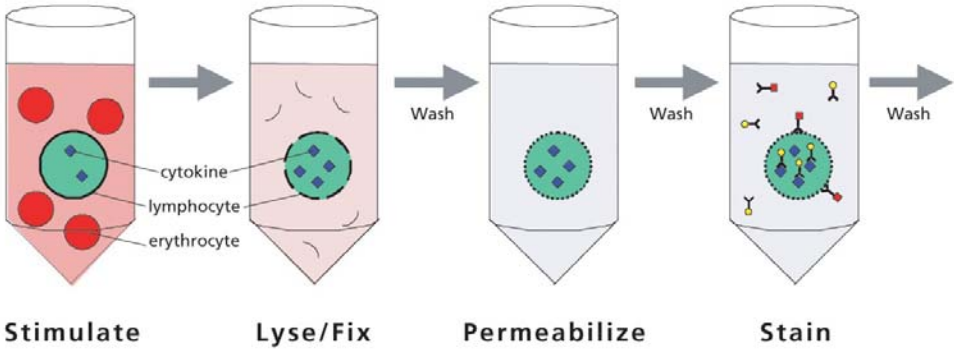
Buffer Choices

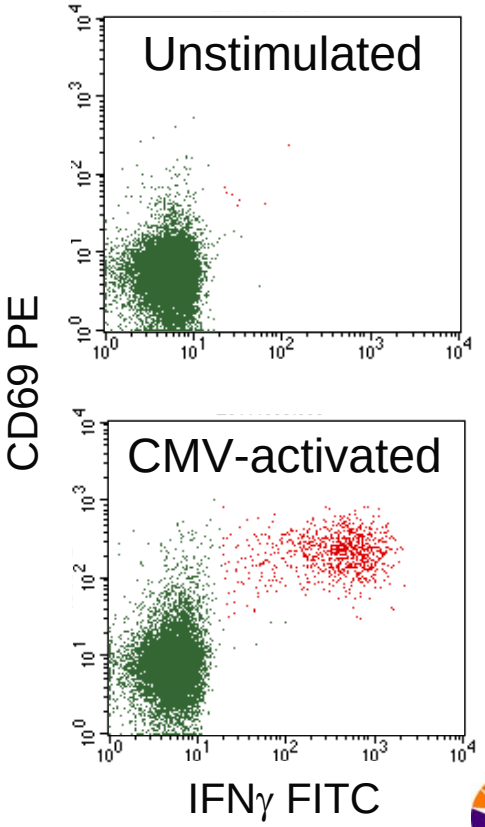
- Fixation buffer
- BD Cytofix/Cytoperm™ and BD™ Perm/Wash buffer
- BD Pharmingen™ FoxP3 buffer set (mouse or human)
- BD™ Phosflow Perm Buffer II
- BD™ Phosflow Perm Buffer III
- BD IntraSure™ kit

- BD FastImmune™ kits

BD FastImmune™ Kits

- Optimized kits containing antibodies and buffers for simultaneous detection of cell surface markers and cytokines from whole blood

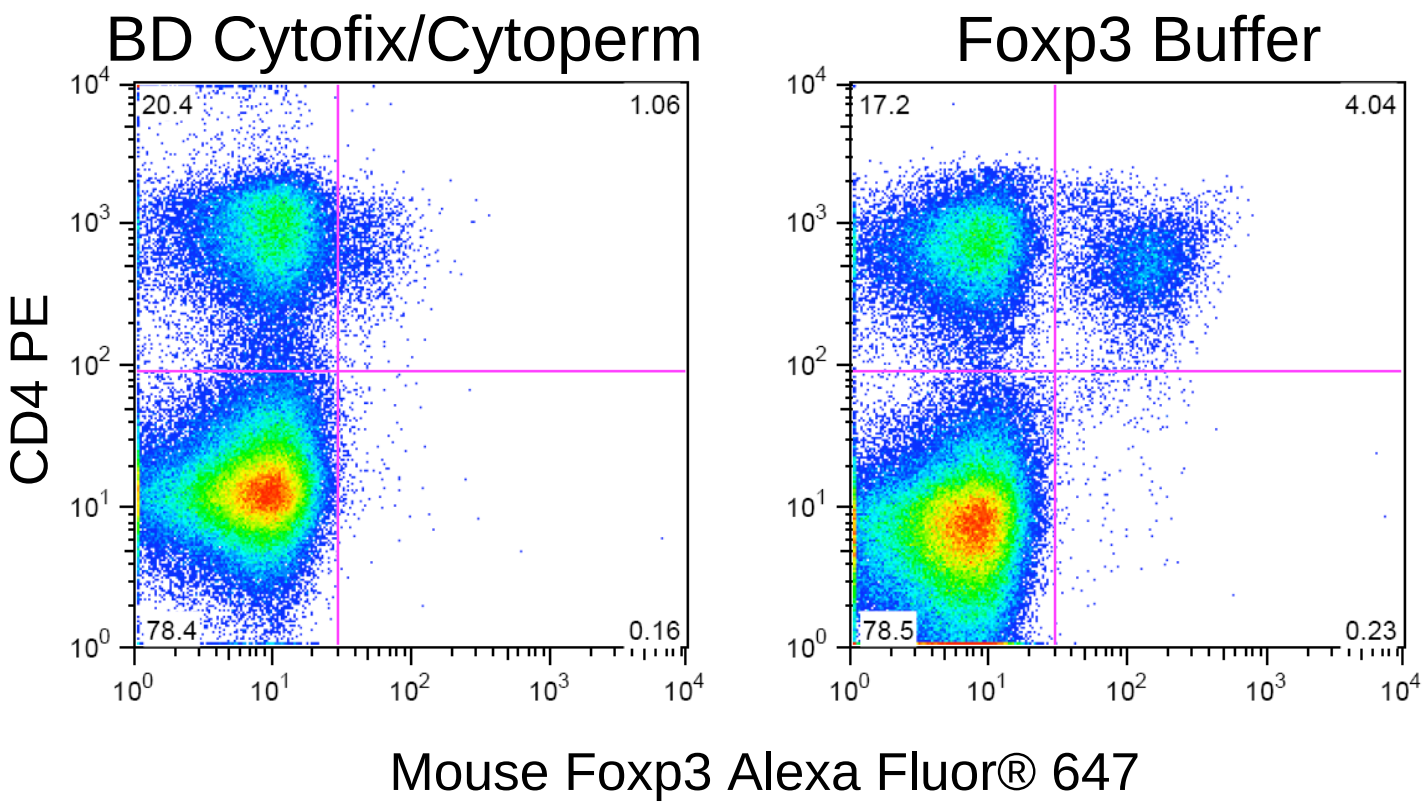




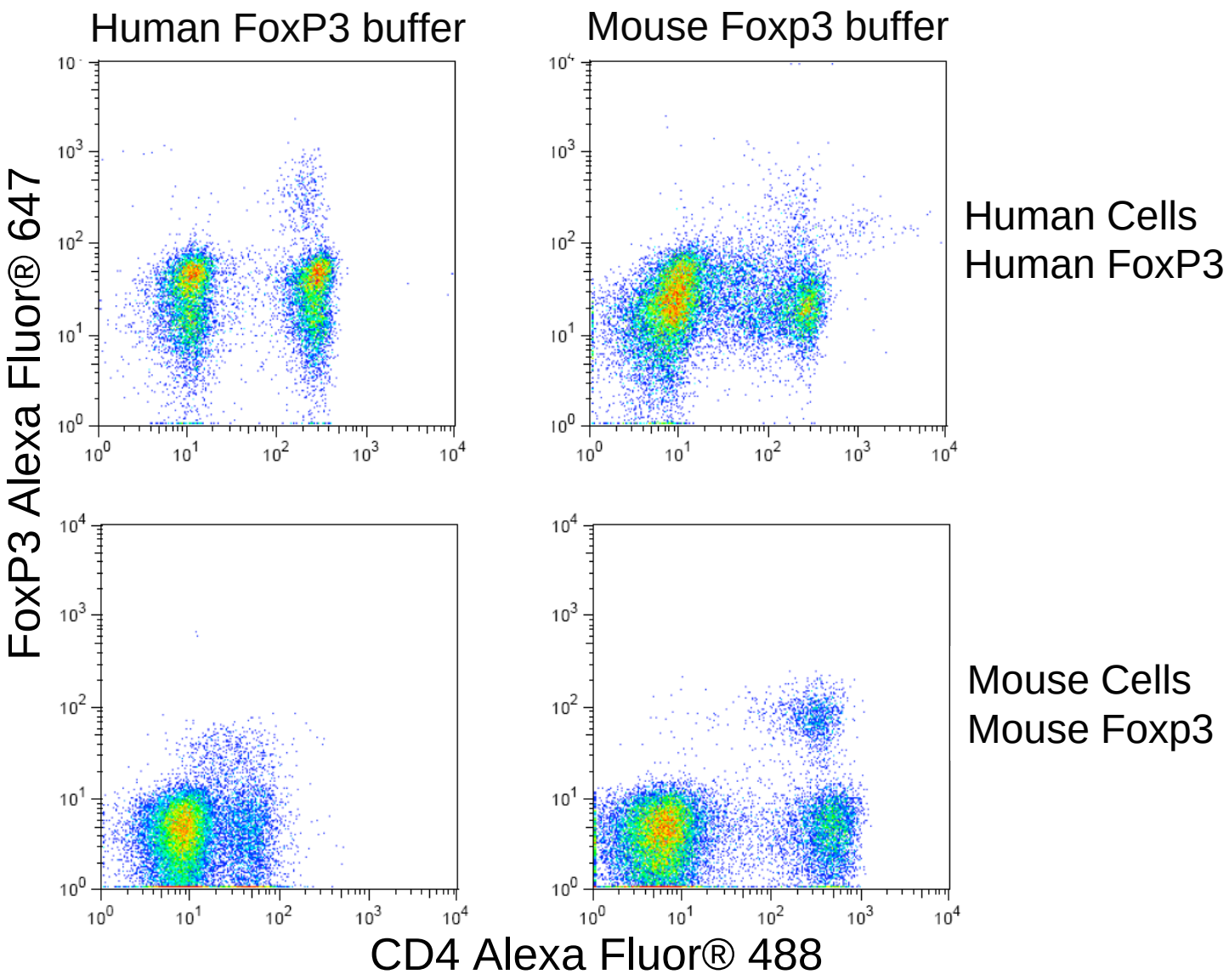
Case Study

- The study of Treg and Th17 cells
- Requires the need to detect both FoxP3 and IL-17 in the same sample
- Unique protocols for both mouse and human FoxP3 staining
- Questions are:
  - How well does IL-17 staining work in the FoxP3 buffer system?
  - How well do other intracellular and surface markers work with the FoxP3 buffer system?
- Examples of FoxP3 optimization followed by addition of other markers

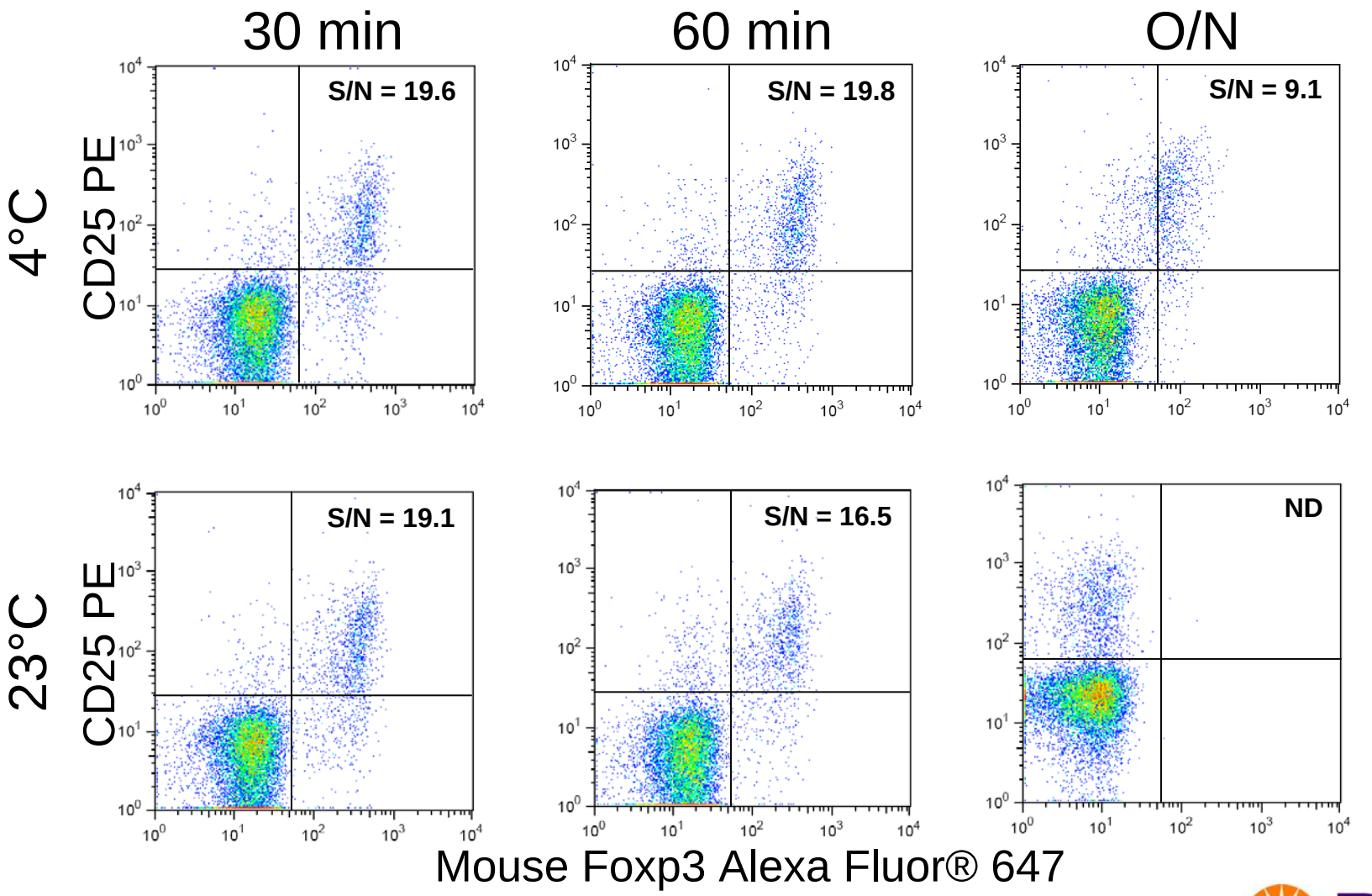
Effect of BD Cytofix/Cytoperm Buffer on Mouse Foxp3 Staining



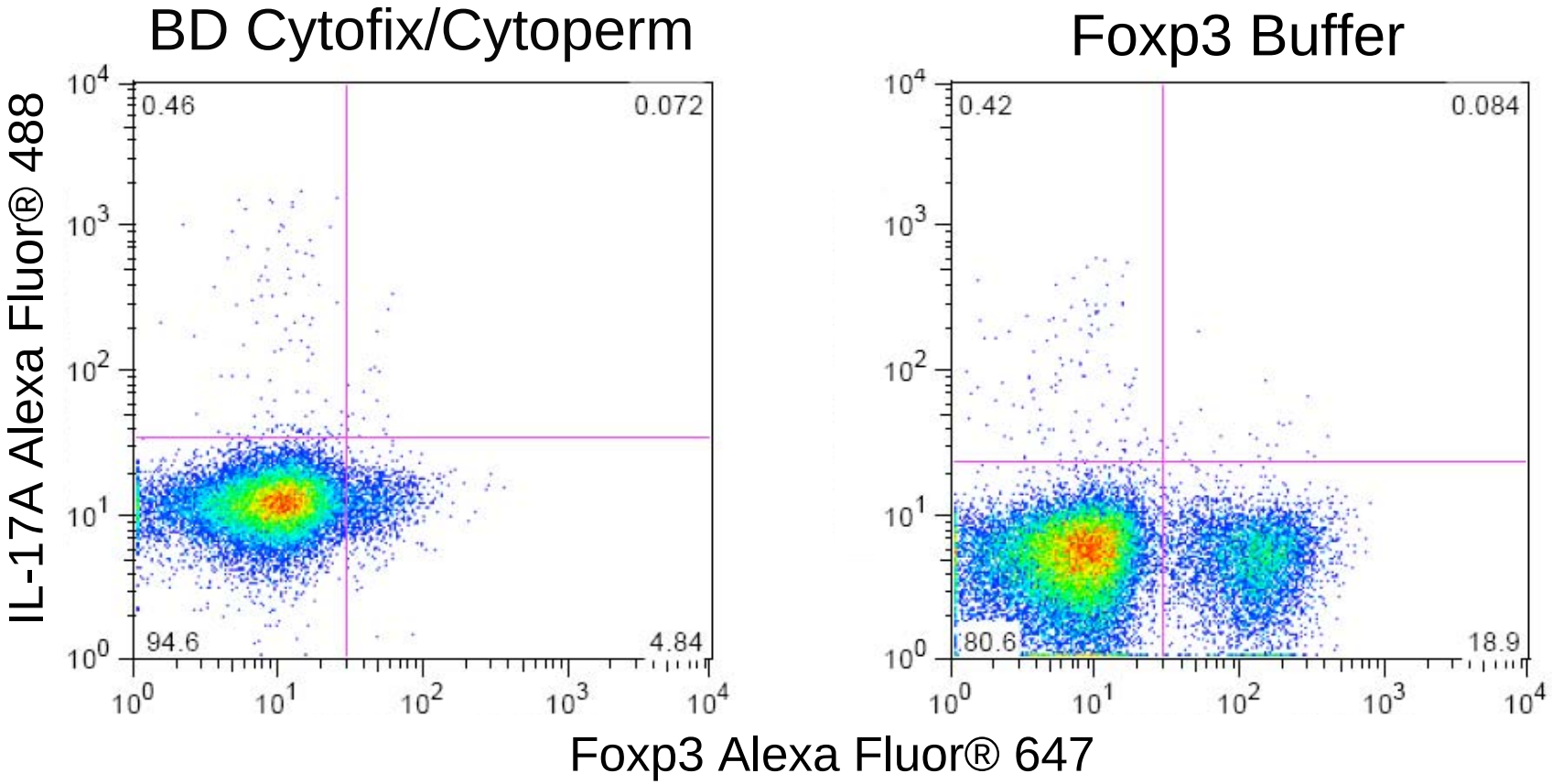
Effect of Human FoxP3 Buffer System on Mouse Foxp3 Staining



Effect of Fixation Time and Temperature on Mouse Foxp3 Staining

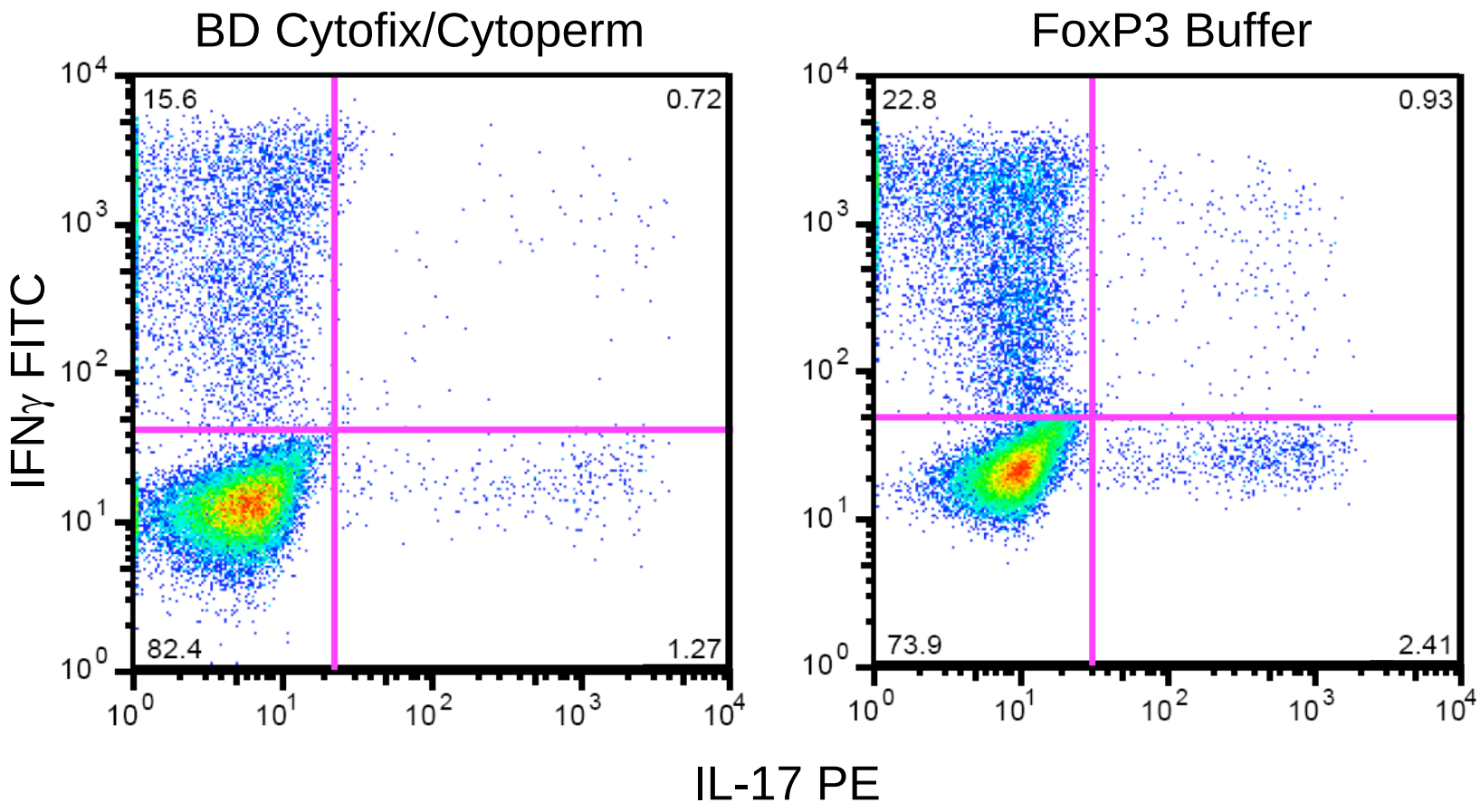


Effect of FoxP3 Buffer on Mouse IL-17 Staining

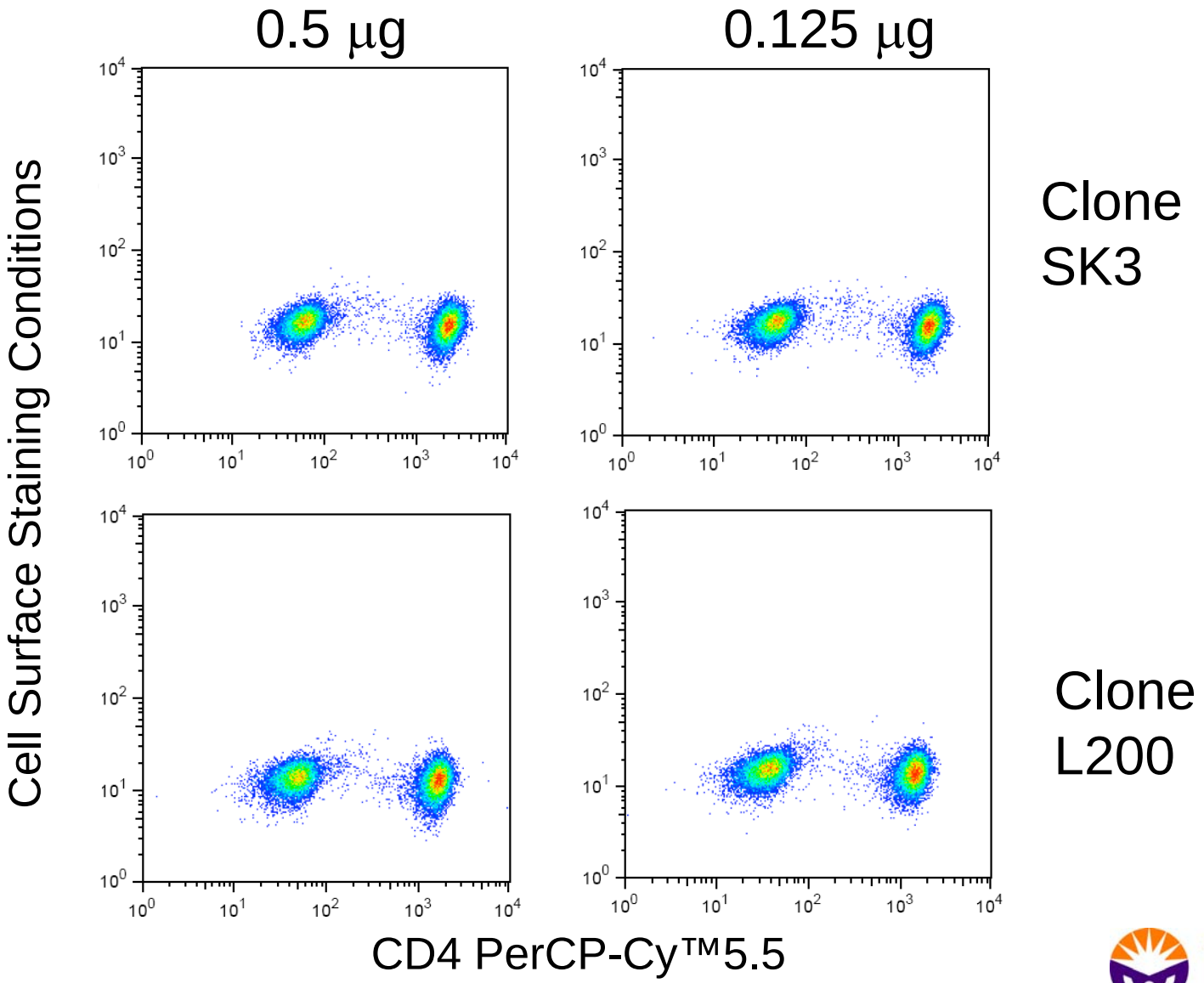


Gated on $CD4^+$ lymphocytes

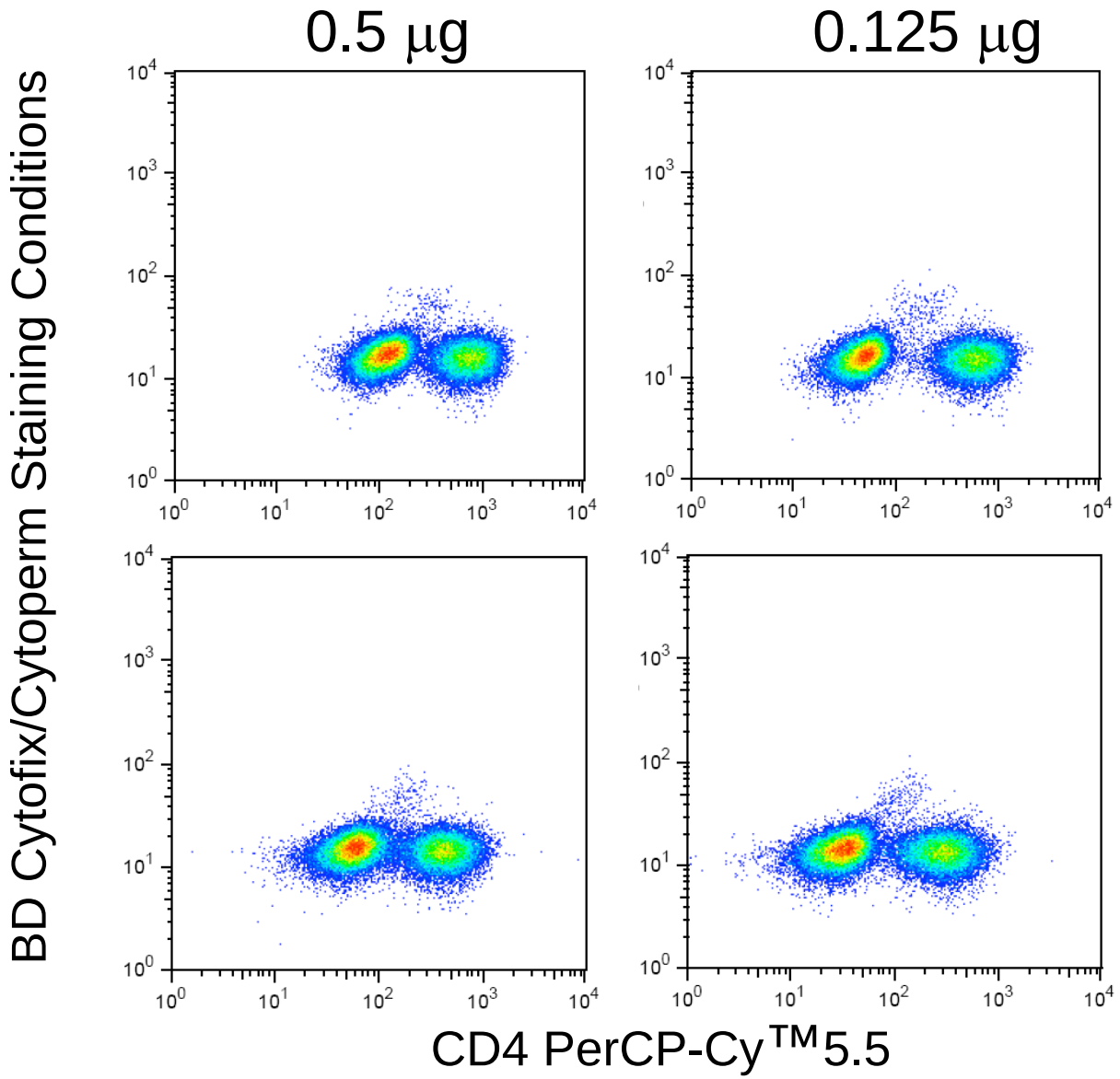
Effect of FoxP3 Buffer on Human Cytokine Staining



Optimizing Cell Surface Staining – Surface Stain



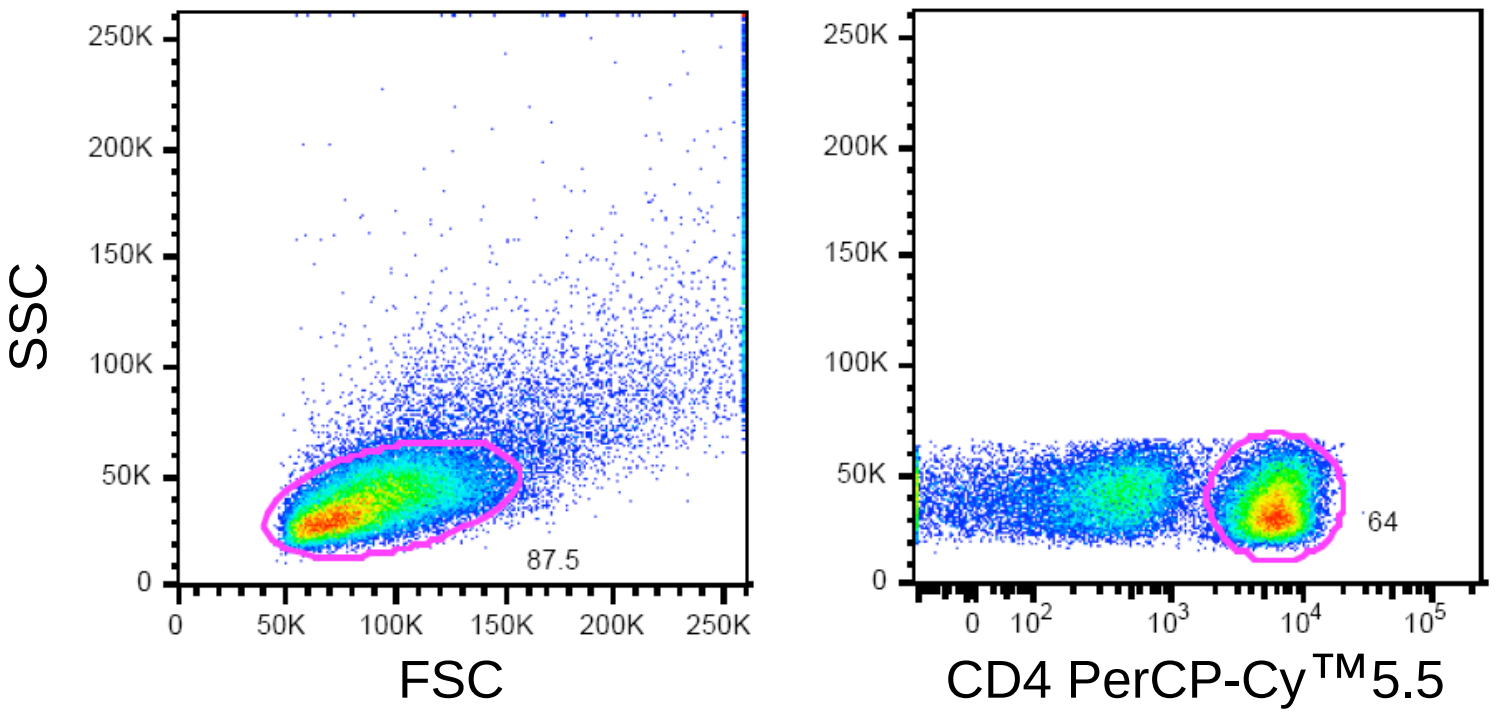
Optimizing Cell Surface Staining – BD Cytofix/Cytoperm Stain



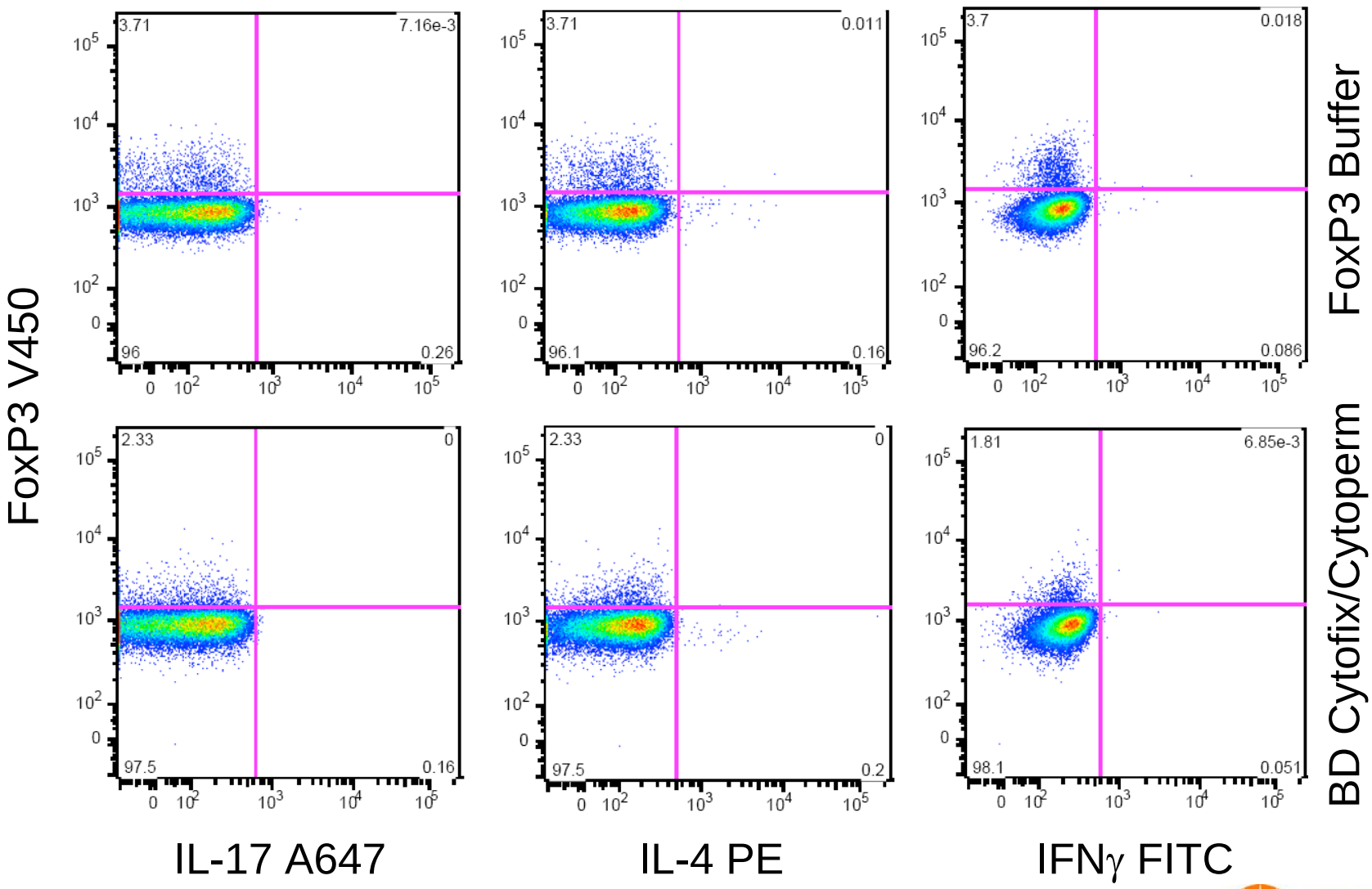
Example: Simultaneous detection of human FoxP3, IL-17, IL-4, and IFNγ in CD4⁺ T cells.

- Freshly isolated PBMC
- Either stimulated or not
  - PMA/Ionomycin with GolgiStop™
  - 5 hours 37°C
- Fix (2 ways) and stored O/N in stain buffer
- Perm (2 ways) and stain 40 minutes
  - CD4 PerCP-Cy5.5
  - FoxP3 V450
  - IL-17 Alexa Fluor® 647
  - IFNγ FITC
  - IL-4 PE
- Acquire and analyze

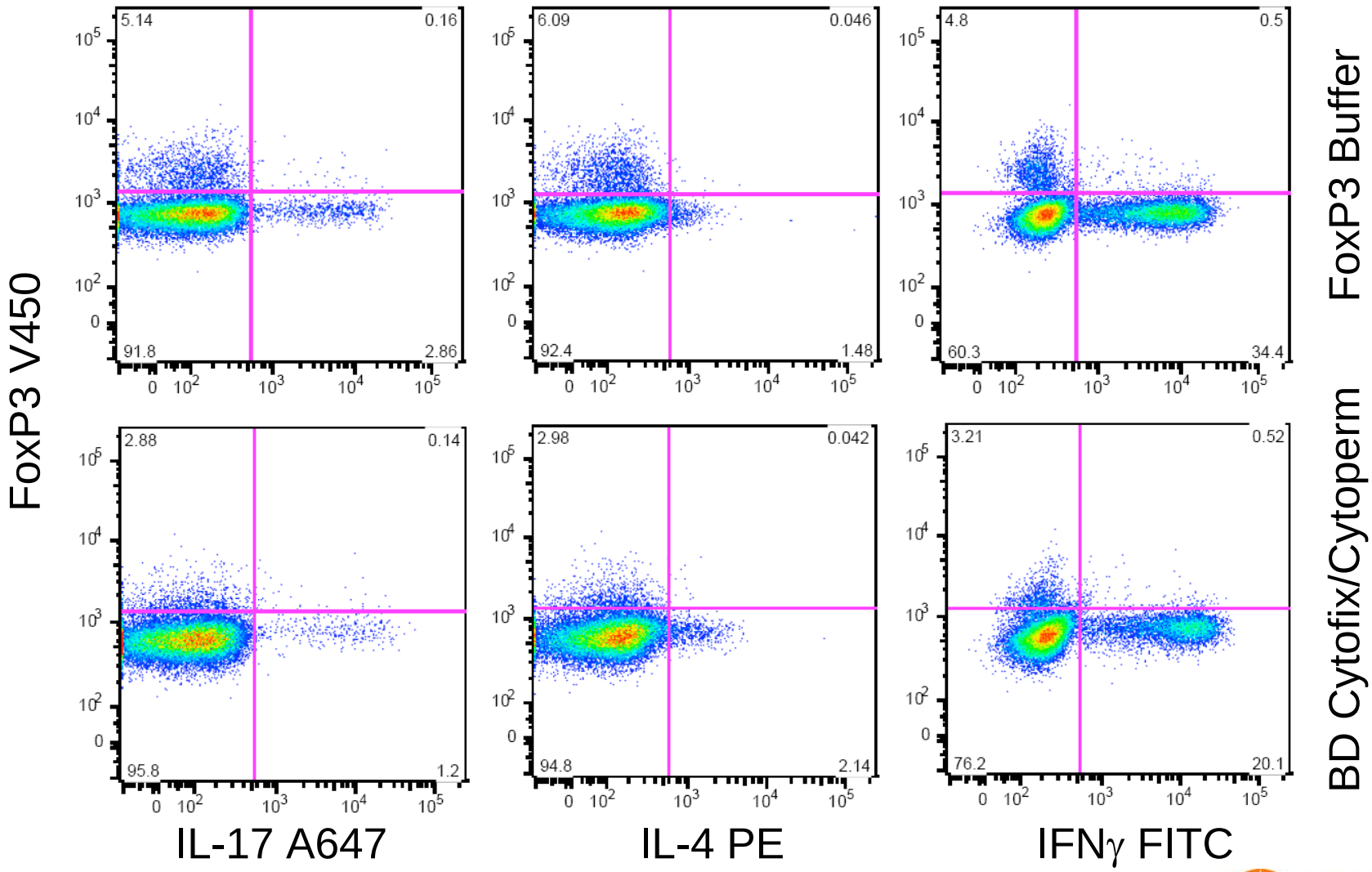
Setting the CD4⁺ gate



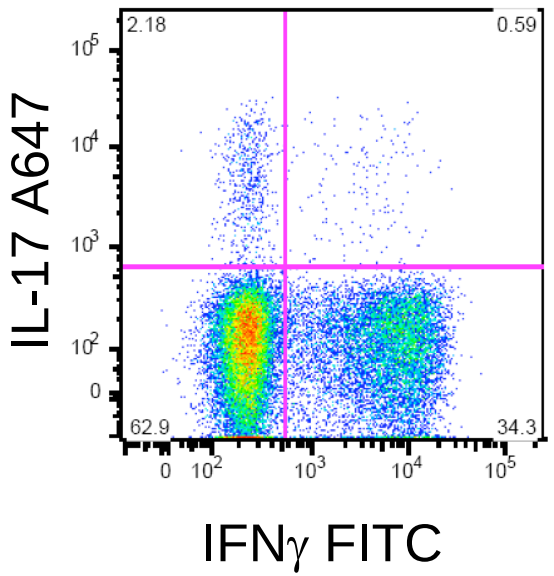
Unstimulated PBMC

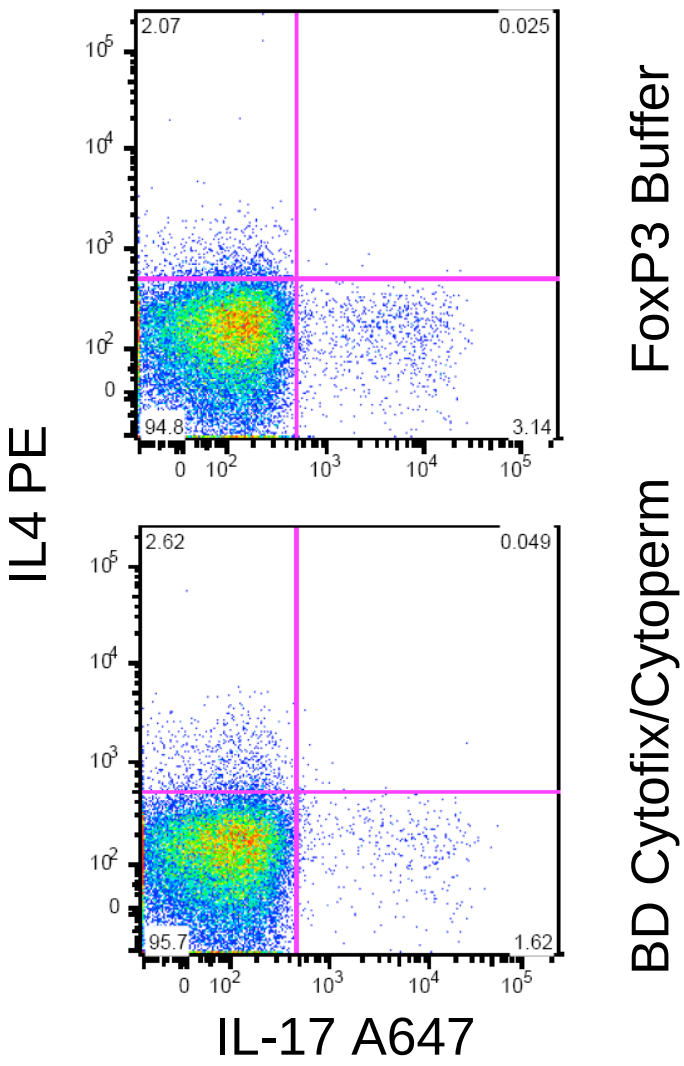


Stimulated PBMC



Stimulated PBMC, *cont'd.*

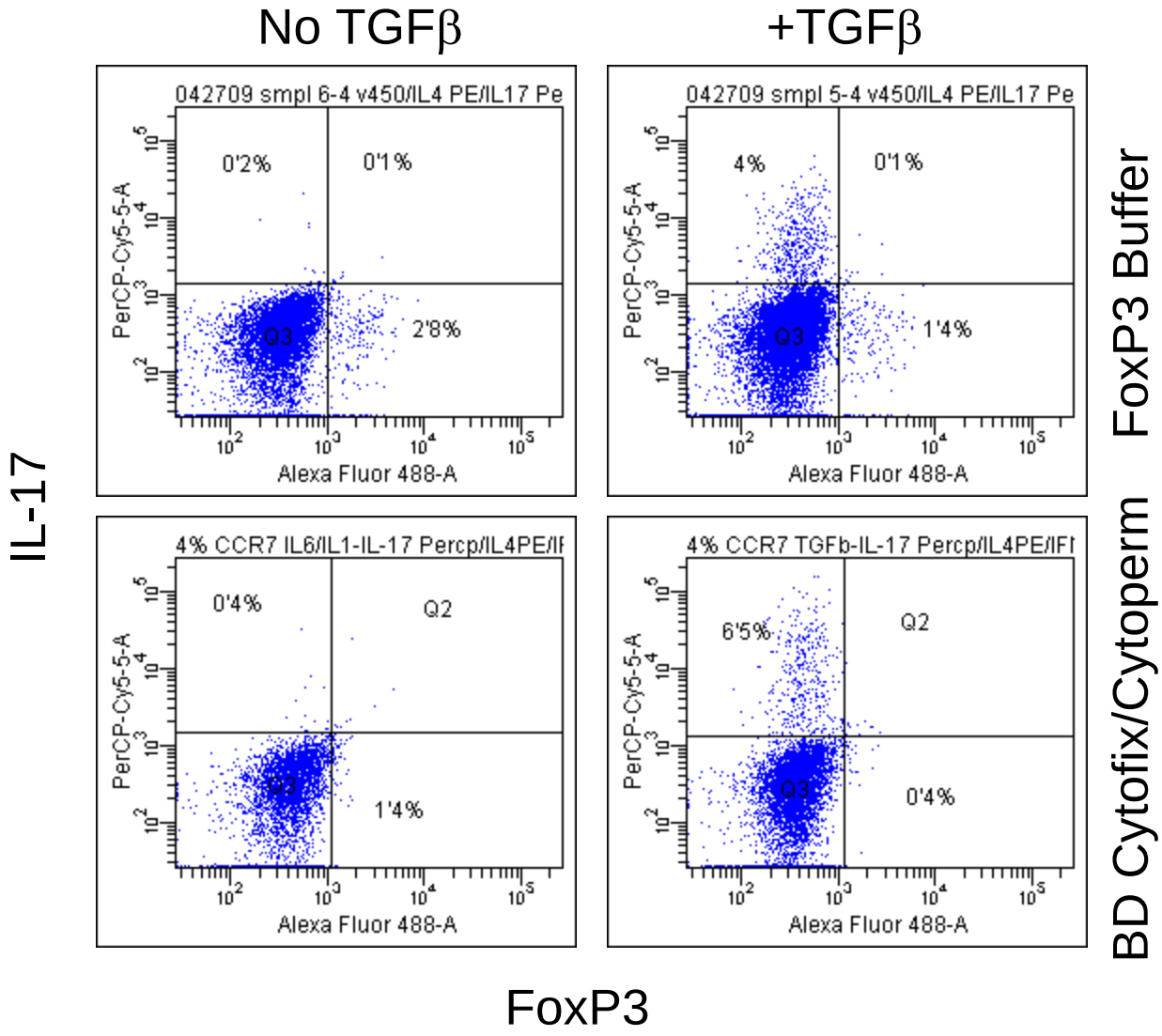




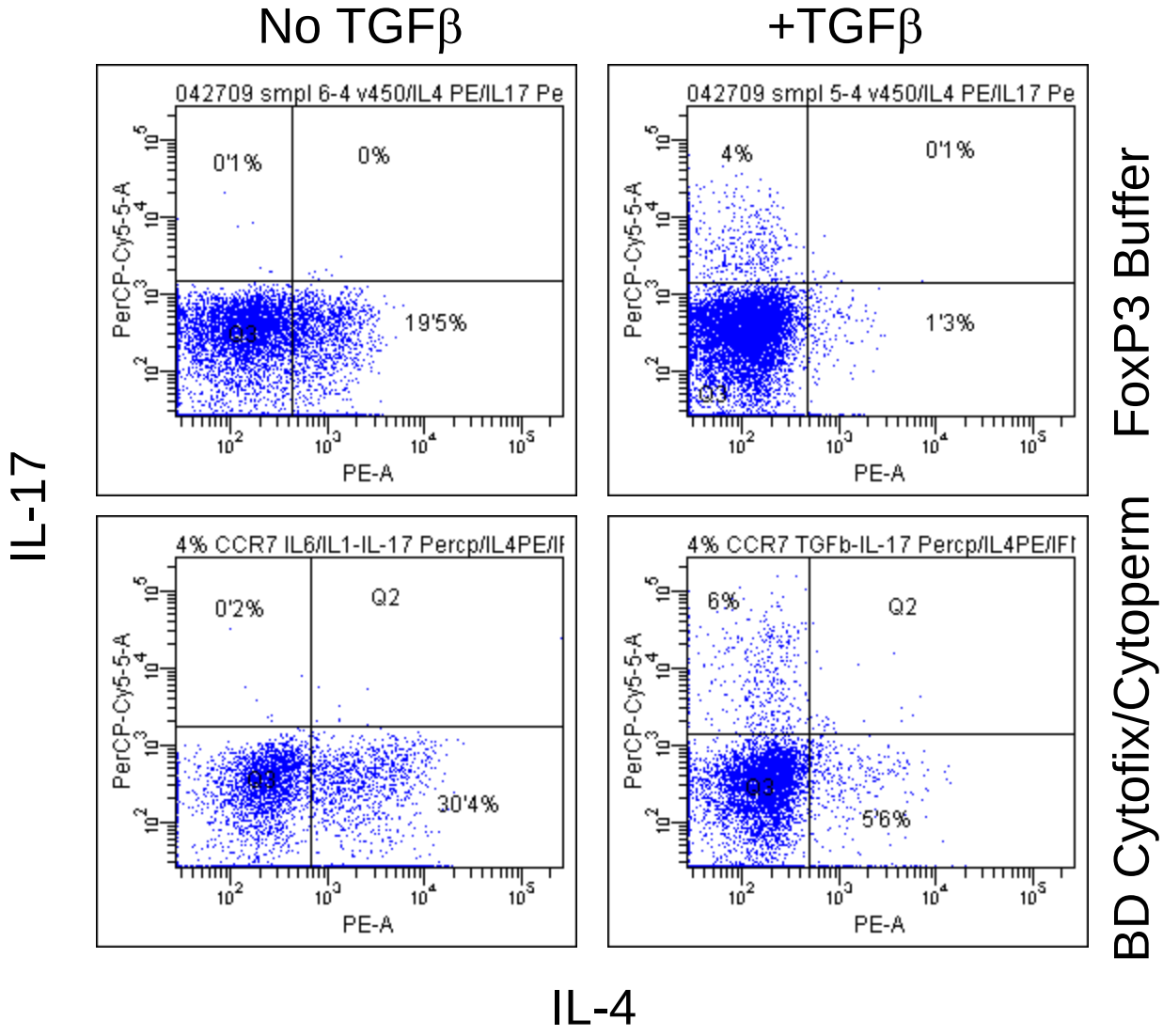
Example: Requirement of TGFβ for the differentiation of mouse Th17 CD4+ T cells.

- Freshly isolated spleen
- Purify CD4+ T cells by panning
- Polarize T cells on anti-CD3 coated plates in the presence of CD28, IL-6 and IL1β either with or without TGFβ
- After 4 days harvest the cells and stimulate with PMA/Ionomycin with GolgiStop™ for 5 hours
- Fix (2 ways) and store O/N in stain buffer
- Perm (2 ways) and stain 40 minutes
  - CD4 V450
  - FoxP3 Alexa Fluor® 488
  - IL-17 PerCP-Cy™5.5
  - IL-4 PE
- Acquire and analyze

Differentiated CD4+ T cells



Differentiated CD4+ T cells, *cont'd.*



Summary

- Determine marker combination(s) for your experiment
- Pair the brightest dye with dimmest marker
- Determine optimal buffers for your antibodies
- Begin cross testing antibodies in different buffers
  - Typically optimize conditions for intracellular staining first and then determine what works best for your chosen cell surface markers
  - Understand what compromises can be made
- Once optimal conditions have been determined for your particular needs, proceed with experiments

Acknowledgements

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If you have further questions:

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