Optimizing Intracellular Flow Cytometry:

Simultaneous Detection of Cytokines and Transcription Factors

An encore presentation by
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Outline

• Introduction
  – Cytokines
  – Transcription factors
• Basic concepts of intracellular flow cytometry
  – Optimization examples
• CD4 T helper cell polarization analyses
  – 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-$\beta$, IL-6, IL-21, IL-1$\beta$, 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 (Treg)

- Actively suppress T cell proliferation, crucial for T cell homeostasis
- FoxP3, transcription factor is a specific marker for Treg
- FoxP3 is necessary for both development and function of Treg
- nTreg develop in the thymus, iTreg require TGFβ, IL-2 & RA
- Produce TGFβ and IL-10 and express high levels of CD25 and low levels of CD127
- Dampening Treg activity could improve anti-tumor responses and responses to vaccinations and chronic infections
- Boosting Treg activity could be useful in the treatment of T cell induced diseases
CD4⁺ T Cell Differentiation

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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, 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™ & 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.

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Studying T cell Polarization

- Requires the need to detect both transcription factors, surface markers and cytokines in the same sample
- If detecting FoxP3 then have a unique protocol for both mouse and human FoxP3 staining
- Questions:
  - How well does cytokine staining work in the FoxP3 buffer system or vice versa?
  - 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

BD Cytofix/Cytoperm

Foxp3 Buffer

Mouse Foxp3 Alexa Fluor® 647
Effect of Human FoxP3 Buffer System on Mouse Foxp3 Staining

Human FoxP3 buffer

Mouse Foxp3 buffer

Human Cells
Human FoxP3

Mouse Cells
Mouse Foxp3

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Effect of Fixation Time and Temperature on Mouse Foxp3 Staining

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Effect of FoxP3 Buffer on Mouse IL-17 Staining

Gated on CD4\(^+\) lymphocytes
Effect of BD Cytofix/Cytoperm Buffer on Human Foxp3 Staining

Unstimulated

Stimulated

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Effect of FoxP3 Buffer on Human IFN$\gamma$ Staining
Optimizing Cell Surface Staining Example
Human CD4, Clone: RPA-T4

Live Cell Stain

FITC          A488           PE         PE-Cy5      PE-Cy7     PCP-Cy5.5    APC          A647    APC-Cy7     PB           V450          A700

1x

1/4x

1/16x

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Optimizing Cell Surface Staining Example, cont.

BD Phosflow Lyse/Fix Buffer and BD Perm/Wash buffer

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Optimizing Cell Surface Staining Example, cont’d.

BD Phosflow Lyse/Fix Buffer and BD Phosflow Perm Buffer III

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Optimizing Cell Surface Staining – Unstimulated

Clone SK3

0.5 μg

Unstimulated CD4 PerCP-Cy™5.5

Clone L200

0.125 μg

BD Cytofix/Cytoperm Staining Conditions

Unstimulated PBMC
Optimizing Cell Surface Staining – Stimulated

Clone SK3

Clone L200

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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 BD 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

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Stimulated PBMC

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Cytokine and FoxP3 detection in differentially polarized human Thelper Cells

PBMC stimulated with plate-bound anti-CD3 and soluble anti-CD28 plus recombinant cytokine IL-2 in the presence of additional cytokines and neutralizing mAbs as follows:

- **TH1:** IL-12, neutralizing anti-IL-4
- **TH2:** IL-4, neutralizing anti-IFN-γ
- **TH9:** IL-4, TGF-β, neutralizing anti-IFN-γ
- **TH17:** IL-6, IL-1β, IL-23, TGF-β, neutralizing anti-IFN-γ and anti-IL-4

Cells were cultured for 4-14 days and then washed and restimulated for 5 hours with PMA and Ionomycin in the presence of BD GolgiStop protein transport inhibitor before intracellular staining.
Cytokine Expression on Polarized Th Cells

BD Cytofix/Cytoperm Staining Conditions

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TGF-β Regulates hIL-17A, hIL-9 Secretion

BD Cytofix/Cytoperm Staining Conditions

w/o TGF-β

w/TGF-β

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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

No TGFβ

+TGFβ

BD Cytofix/Cytoperm  
FoxP3 Buffer

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Differentiated CD4+ T cells, cont’d.

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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
• If cell surface markers do not stain post fixation/permeabilization:
  – Try stain surface markers post fixation but prior to permeabilization
  – Try stain surface markers prior to fixation and permeabilization
• Once optimal conditions have been determined for your particular needs, proceed with experiments
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If you have further questions:

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