Enabling a deeper understanding of what is on, in, and made by the cell
Analyzing what is on, in, and Made by the Cell

| Challenges:                                                                                   |
|                                                                                             |
| • Multiple methodologies commonly used in cell biology; consume time and materials            |
| • Unable to correlate expression of surface and intracellular molecules                       |
| • Difficult to quantitate the percentage of cells expressing markers of interest              |

<table>
<thead>
<tr>
<th>Cell Surface Markers</th>
<th>Methods of Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flow Cytometry</td>
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<tr>
<td></td>
<td>IF/IHC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intracellular Proteins</th>
<th>Methods of Analysis</th>
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<tbody>
<tr>
<td></td>
<td>IF/IHC</td>
</tr>
<tr>
<td></td>
<td>WB</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
</tr>
</tbody>
</table>

| Cytokines              | Methods of Analysis |
|                        | ELISA               |
|                        | PCR                 |

| Cellular Processes     | Methods of Analysis |
|                        | Multiple            |
Flow Cytometry: a Versatile Technology for Broad Applications

Microbiology
- Aquatic microbiome analysis
- Biofuel research
- Bacteria viability and concentration

Plant Biology
- DNA content

Cell Biology
- Apoptosis
- Cell signaling
- Immunophenotyping
- Cytokine analysis

Fluorescent Protein Analysis
- GFP, YFP
- mCherry, RFP
- mOrange, dTomato
Flow Cytometry and Cell Biology
Publications Using the Keyword “Flow Cytometry” from PubMed
The Relevance of Flow Cytometry in Cell Biology

Heterogeneous cells

Bulk Analyses
WB, PCR, Imaging

Cell Lysate
RNA Total Cells

Protein 1
Protein 2

1%

Protein 1
Protein 2

Average

vs

Single Cells

Undifferentiated
differentiated

Protein 1
Protein 2

1%

Protein 1
Protein 2

57%

Protein 1
Protein 2

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Flow Cytometry within Reach™

The BD Accuri™ C6 Personal Flow Cytometry Tour

Intranuclear Transcription factors

Cell Surface
Lineage marker
Activation marker
Cytokine and chemokine receptors

Cell Processes
Apoptosis
Cell viability
Proliferation

Antibodies and Functional Dyes

Quantification in supernatant, plasma, serum, or cell lysates:
cytokines, chemokines, immunoglobulins, or signaling proteins

Intracytoplasmic or Intranuclear
Cell signaling proteins

BD Phosflow™ technology

Intracytoplasmic
Cytokines, chemokines, various proteins

Intranuclear
Transcription factors

Antibodies and BD™ Cytometric Bead Array (CBA)

Analyzing a Cell by Flow Cytometry: More Than Surface Marker Analysis
Analyzing a Cell by Flow Cytometry: More Than Surface Marker Analysis

**Cell Surface**
- Lineage marker
- Activation marker
- Cytokine and chemokine receptors

**Cell Processes**
- Apoptosis
- Cell viability
- Proliferation

**Antibodies and Functional Dyes**

**Quantification** in supernatant, plasma, serum, or cell lysates:
- Cytokines, chemokines, immunoglobulins, or signaling proteins

**Intracytoplasmic or Intranuclear**
- Cell signaling proteins
  - *BD Phosflow technology*

**Intracytoplasmic**
- Cytokines, chemokines, various proteins

**Intranuclear**
- Transcription factors

*BD Biosciences*
Cell Function Assay Landscape

- **Cell Biology**
  - Viability
    - Live Cells
      - Apoptosis
        - Live Cells
          - Caspase Activity
          - Phosphatidyl Serine Exposure
        - Fixed Cells
          - Caspase Activity
          - Mitochondrial Membrane Potential
    - Fixed Cells
      - DNA Fragmentation
      - Esterase Activity
      - Membrane Integrity
  - Proliferation and Cell Cycle
    - Live Cells
      - DNA Content
      - Proliferation Dye Retention
      - Ki-67 Status
      - DNA Content
      - BrdU/EdU Incorporation
      - Cyclin Expression
      - Histone H3 Phosphorylation
    - Fixed Cells
      - DNA Content
The BD Accuri™ C6 Personal Flow Cytometer

- Easy to use
- Two lasers, six parameters
- Fixed voltages
- Cell counting
- Continuous sampling
- Kits and templates
Apoposis/Viability Assay

- Live: Annexin V⁻/PI⁻
- Apoptotic: Annexin V⁺/PI⁻
- Dead: Annexin V⁺/PI⁺
MDA-MB-231 cells were treated for 48 hours with varying doses of camptothecin (0.1–100 µM).

Cells were stained with the BD™ Annexin V Apoptosis Detection Kit and analyzed using the kit template.
Cell Function Assay Landscape

- Cell Biology
  - Viability
    - Live Cells
      - Apoptosis
        - Live Cells
          - Caspase Activity
          - Phosphatidyl Serine Exposure
        - Fixed Cells
          - Mitochondrial Membrane Potential
            - Caspase Activity
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      - Ki-67 Status
      - DNA Content
      - BrdU/EdU Incorporation
      - Cyclin Expression
      - Histone H3 Phosphorylation

- Live Cell Caspase Probe
- Annexin V
- TMRE
- 7-AAD

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Multiparameter Apoptosis/Viability Analysis on Live Cells

<table>
<thead>
<tr>
<th>Function</th>
<th>Target</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Caspase Activity</td>
<td>Blue Live Cell Caspase (FL1)</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Mitochondrial Membrane Potential</td>
<td>TMRE (FL2)</td>
</tr>
<tr>
<td>Viability</td>
<td>Membrane Integrity</td>
<td>7-AAD (FL3)</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Phosphatidyl Serine Exposure</td>
<td>Annexin V (FL4)</td>
</tr>
</tbody>
</table>

MDA-MB-231 cells were treated for 48 hours with 10 μM of camptothecin.
### Designing Multiparameter Cell Function Panels

<table>
<thead>
<tr>
<th>Function</th>
<th>Target</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>BrdU Incorporation</td>
<td>α-BrdU antibody (FL1)</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>Cyclin-B</td>
<td>α-Cyclin-B antibody (FL2)</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>DNA Content</td>
<td>7-AAD (FL3)</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>Phosphorylated Histone 3</td>
<td>α-pH3 antibody (FL4)</td>
</tr>
</tbody>
</table>

### DNA Damage/Proliferation

<table>
<thead>
<tr>
<th>Function</th>
<th>Target</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Cleaved PARP</td>
<td>α-PARP cleaved form antibody (FL2)</td>
</tr>
<tr>
<td>Proliferation</td>
<td>BrdU Incorporation</td>
<td>α-BrdU antibody (FL3)</td>
</tr>
<tr>
<td>DNA Damage</td>
<td>Phosphorylated H2AX Histone</td>
<td>α-pH2AX (FL4)</td>
</tr>
</tbody>
</table>

### Cell Cycle/Viability/Apoptosis

<table>
<thead>
<tr>
<th>Function</th>
<th>Target</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>Proliferation</td>
<td>BrdU Incorporation</td>
<td>α-BrdU antibody (FL1)</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Caspase</td>
<td>α-Caspase 3 antibody (FL2)</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>DNA Content</td>
<td>7-AAD (FL3)</td>
</tr>
<tr>
<td>Viability</td>
<td>Membrane Integrity</td>
<td>FVS660 (FL4)</td>
</tr>
</tbody>
</table>

- Multiparameter panels can be designed to analyze different cell functions simultaneously.
- Cell function reagents are offered in a variety of colors for increased panel design flexibility.
- BD Horizon™ fixable viability stains allow for simultaneous analysis of viability and intracellular molecule expression.
Analyzing a Cell by Flow Cytometry: More Than Surface Marker Analysis

Cell Surface
- Lineage marker
- Activation marker
- Cytokine and chemokine receptors

Cell Processes
- Apoptosis
- Cell viability
- Proliferation

Antibodies and Functional Dyes

Intracytoplasmic or intranuclear
- Cell signaling proteins
  - BD Phosflow technology

Intracytoplasmic
- Cytokines, chemokines, various proteins

Intranuclear
- Transcription factors

Quantification in supernatant, plasma, serum, or cell lysates:
- Cytokines, chemokines, immunoglobulins, or signaling proteins

Antibodies and BD Cytometric Bead Array (CBA)
Advantages of Phosphorylation Analysis by Flow Cytometry

• Single-cell analysis
• Rapid assay
• Reduced number of cells per test
• Quantitative
• Multiparametric
• Increased throughput
Standard Protocol for Analyzing Protein Phosphorylation by Flow Cytometry
Protocol Considerations

• Stimulating conditions

• Kinetics

• Assay controls

• Cell culture conditions (suspension vs adherent)

• Fix and permeabilization buffer selection
Protocol Considerations

- Stimulating conditions
- Kinetics
- Assay controls
- Cell culture conditions (suspension vs adherent)
- Fix and permeabilization buffer selection
Analysis of Stat Phosphorylation on the BD Accuri C6: Dose Response

- Human lymphoma U-937 cells were stimulated with increasing doses of IFN-γ for 15 minutes.
- Stat1 and Stat6 phosphorylation was assessed by WB or flow cytometry on the BD Accuri C6.

**Western Blot**

- 30 min: Lyse Cells
- 30 min: Quantify Proteins
- 1 h: Separate Proteins
- 10 min–2 h: Transfer Proteins
- 30 min–1 h: Block Membrane
- 1 h–O/N: 1° Antibody
- 1 h: 2° Antibody
- Detection

**Flow Cytometry**

- 10 min: Fix Cells
- 30 min: Perm Cells
- 30 min–1h: Conjugated 1° Antibody
- Analysis

**Graphs**

- Untreated
- 1 pg/mL
- 10 pg/mL
- 100 pg/mL

- FL2 pStat1 PE-A
- FL2 pStat6 PE-A
Analysis of Stat Phosphorylation on the BD Accuri C6: Time Course

- Human lymphoma U-937 cells were stimulated with 10 pg/mL of IFN-γ for 30, 15, and 5 minutes.
- Each tube was individually stained with pStat-1 PE antibody and analyzed on the BD Accuri C6.
How can we track populations of cells?

- Fluorescent markers (example, GFP, mCherry)
- Cellular dyes (example, CFSE)
- Genetic barcoding

Fluorescent cell barcoding for flow cytometry

- Increases throughput
- Enables larger screens/profiles
- Improves robustness of assays
- Decreases acquisition times
Barcoding: Kinetic Assay in One Tube

- Fixed and permeabilized cells were individually color coded using BD Horizon fixable viability stains.
- Cells were then mixed and stained with a single aliquot of anti-pStat1 PE antibody.

Mix and Stain

Deconvolute Barcoding

Analysis

 Untreated 30' 15' 5'

No FVS FVS520 FVS520 FVS660

pStat1 PE

• Fixed and permeabilized cells were individually color coded using BD Horizon fixable viability stains.
• Cells were then mixed and stained with a single aliquot of anti-pStat1 PE antibody.
Barcoding: Kinetic Assay in One Tube

- Only one tube containing all four samples was analyzed on the BD Accuri C6.

Deconvolute Barcoding

FL1 FVS520-A

FL4 FVS660-A

FL2 pStat1 PE-A

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Analyzing a Cell by Flow Cytometry: More Than Surface Marker Analysis

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**Antibodies and Functional Dyes**

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- cytokines, chemokines, immunoglobulins, or signaling proteins

**Intracytoplasmic or Intranuclear**
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  - *BD Phosflow technology*

**Intranuclear**
- Transcription factors

**Intracytoplasmic**
- Cytokines, chemokines, various proteins

*Antibodies and BD Cytometric Bead Array (CBA)*
Multiple Methods to Analyze Cytokine Expression

**Soluble Proteins**
- ELISA
- ELISPOT
- BD CBA Array

**Intracellular Proteins**
- Flow Cytometry
- Western Blot
- Immunohistochemistry

Two distinct flow cytometry assays to analyze cytokine expression
- Bead-based immunoassay (BD CBA)
- Intracellular flow cytometry
Advantages of Cytokine Analysis Using Flow Cytometry

• **BD CBA:**
  - Quantitative and sensitive
  - Analysis of multiple cytokines simultaneously
  - Reduced sample volume requirement
  - Requires less sample dilution

• **Intracellular flow cytometry:**
  - Cytokine analysis at the single cell level
  - Compatible with simultaneous surface marker analysis

*BD CBA and intracellular flow cytometry can be used as complementary techniques for a more comprehensive cytokine analysis.*
BD CBA Assay

- More than 20 analytes can be detected.
- The BD CBA array is like running multiple ELISA assays in one single tube.
Cytokine Analysis in Cancer Cells

- Pro-inflammatory stimuli induce expression of cytokines involved in cancer progression.
- Breast cancer cell lines MDA-MB-231 and MDA-MB-468 were stimulated with TNF.
- Cytokine expression was evaluated on the same sample using BD CBA and intracellular flow cytometry.

+/- TNF

24, 48, 72 h

• Collect supernatant aliquot
• Dilute as needed
• Store at -20°C

• Add BD GolgiStop™
• Incubate for 6 h

• Collect cells
• Surface marker stain
• Fix and perm
• Intracellular cytokine stain
Quantification of IL-6 in Cancer Cell Cultures using a BD CBA Array

- The more aggressive cell line MD-MB-231 expressed a higher basal level of IL-6.
- Upon TNF stimulation, MDA-MB-231 responded by robustly increasing IL-6 secretion.

### IL-6 Concentration (pg/mL)

<table>
<thead>
<tr>
<th>Sample</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>75.7</td>
<td>165</td>
<td>429.7</td>
</tr>
<tr>
<td>MDA-MB-231 +TNF</td>
<td>791.2</td>
<td>4,564.5</td>
<td>258,805</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.26</td>
<td>0.54</td>
<td>1.3</td>
</tr>
<tr>
<td>MDA-MB-468 +TNF</td>
<td>15.5</td>
<td>42.4</td>
<td>82.7</td>
</tr>
</tbody>
</table>
Combining Surface and Intracellular Stain for Single Cell Cytokine Analysis

- Treat with BD GolgiStop™ inhibitor for six hours to block cytokine secretion.
- Detach with BD Accutase™.
- Stain for surface markers CD24 and CD44.
- Fix and perm.
- Stain with antibodies against IL-6.
MDA-MB-231 cells displayed a CD44^+CD24^- cancer stem cell signature correlating with a more aggressive cancer phenotype.
Surface and Intracellular Analysis: CD44 and IL-6

Intracellular flow cytometry results correlate with BD CBA data.
Cytokine Analysis Summary

- The BD CBA assay allowed us to test multiple cytokines simultaneously and to identify IL-6 as a cytokine regulated by TNF treatment.

- The combination of surface and intracellular flow cytometry allowed us to:
  - Confirm the different phenotype of the two cell lines tested
  - Determine that only a discrete subset of MDA-MB-231 cells express IL-6 upon TNF stimulation
Analyzing what is on, in, and Made by the Cell

- One rapid methodology for broad cell biology applications
- Ability to correlate expression of surface and intracellular molecules
- Ability to quantitate the percentage of cells expressing markers of interest and the amount of secreted cytokines

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<tr>
<td>Cytokines</td>
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<tr>
<td>Cellular Processes</td>
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</tbody>
</table>

• Flow Cytometry
Research Solutions for Cell Biology

- Free Downloadable Templates
- Broad Reagent Portfolio
- Product Information Sheets
- Technical Documents
- Webinars
- BD Accuri News
Acknowledgments

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- David Draper

San Jose
- Ranga Partha
- Andy Wang
Questions?

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