Multimarker Analysis Methods for the Rapid Characterization of Pluripotent, Multipotent, and Differentiating Stem Cells

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R&D Scientist
Stem Cell Research
Schematic adapted from Wobus AM and Boheler, KR. Physiol. Rev. 2005; 85:635-678.
**Stem Cell Research**

- Identify drug targets and test potential therapeutics
- Cultured Pluripotent Stem Cells
- Study cell differentiation
- Understanding prevention & treatment of birth defects
- Toxicity Testing
- Tissues/Cells for Transplantation
- Bone marrow for leukemia & chemotherapy
- Nerve cells for Parkinson's & Alzheimer's disease
- Heart muscle cells for heart disease
- Pancreatic islet cells for diabetes

Schematic adapted from http://stemcells.nih.gov/index.asp

For Research Use Only. Not for use in diagnostic or therapeutic procedures.
Challenges in Stem Cell Research

- Characterize cell types and stages of differentiation by identifying cell-type specific biomarkers
- Identify and isolate cells of interest from a heterogeneous pool
- Analyze cells for quality and purity
- Analyze cell function
Immunophenotyping is a cellular analysis method for the identification of biomarkers and their expression/co-expression profiles using directly- or indirectly-fluorochrome conjugated antibodies and an analyzer such as a flow cytometer or imaging system.

The identification of biomarkers using immunophenotyping methods facilitates the characterization of a cell type, its identification, and its isolation.

• **Identification of cell subpopulations:**
  – Cells suitable for transplantation
  – Tumor initiating cells

• **Isolation of pure cell populations for downstream assays:**
  – Arrays/sequencing
  – In vitro disease models
  – Biochemistry
  – Transplantation

• **Development of quality control assays for cell preparations:**
  – Assessing purity
  – Identification of contaminants
Tools for Immunophenotyping

**BD Lyoplate™ Screening Panels** support the rapid, cost-effective immunophenotyping of stem cells and stem cell–derived cells.

<table>
<thead>
<tr>
<th>BD Lyoplate Screening Panel</th>
<th>Contents</th>
<th>Applications</th>
<th>Cat. No.</th>
</tr>
</thead>
</table>
| Human Cell Surface Markers | • 242 CD Markers*  
 • Isotype Controls  
 • Alexa Fluor® 647 Second Step | • Flow Cytometry  
 • Bioimaging | Available September 2009 |
| Mouse Cell Surface Markers | • 200+ CD Markers  
 • Isotype Controls  
 • Alexa Fluor® 647 Second Step | • Flow Cytometry  
 • Bioimaging | Available Fall 2009 |

*CD and other cell surface molecules. One marker per well.

- Plate-based format is compatible with automation and multichannel pipetting
- The proprietary lyophilized format allows for room temperature storage, long shelf life
- Open wells permit the use of additional markers of choice
- Compatible with BFP, CFP, GFP, YFP, OFP, and RFP expressing cells

Alexa Fluor® is a registered trademark of Molecular Probes, Inc. The Alexa Fluor® dye antibody conjugates in this product are sold under license from Molecular Probes, Inc., for research use only, excluding use in combination with microarrays, or as analyte specific reagents.
Tools for Immunophenotyping

BD FACS™ CAP (Combinatorial Antibody Profile) is a custom multicolor immunophenotyping and analysis service. The in-depth analysis service provides an inventory of receptors and markers present on the cell surface, yielding an information-rich “fingerprint.”

- 212 fluorescently-labeled anti-human antibodies
- Multicolor cocktails in each well of a 96-well plate
- Flexibility to integrate researcher’s specific markers
- Analysis supported by proprietary software

In addition, the BD Custom Technology Team helps researchers to create multicolor antibody cocktails for in-house flow cytometry immunophenotyping. Optimized multicolor cocktails streamline sample preparation, acquisition and analysis, and improve standardization between experiments.
• Screening of MSCs
• Screening, isolation and analysis of hESC-derived NSCs, glia, and neurons
Mesenchymal Stem Cells (MSCs)

- MSCs are the progenitors of multiple mesenchymal lineages
  - Bone, cartilage, muscle, fat tissue, and marrow stroma
- MSCs can be isolated and cultured in vitro
- Promises of MSCs
  - Regenerative medicine
  - In vitro models of human diseases
    - Drug screening
    - Toxicology
    - Basic research
Characterization of MSCs

Objective: correlate CD marker profile to lineage capacity and multipotency

Collaboration with Paul Coffer and Koen Braat
University Medical Center Utrecht, Netherlands

Experimental results using a beta version of the BD Lyoplate™ Human Cell Surface Marker Screening Panel
Neural Stem Cells (NSCs)

- Found during embryonic development and in restricted regions of the adult brain
- NSCs can be isolated and cultured in vitro
  - Fetal and adult brain
  - Differentiated from hESCs
- Promises of NSCs
  - Transplantation therapy
  - In vitro models of human development
  - In vitro models of human diseases
    - Drug screening
    - Toxicology
    - Basic research
### Neural Stem Cell Research

#### Differentiation of hESCs into neural stem cells

<table>
<thead>
<tr>
<th>Day 0</th>
<th>7</th>
<th>17</th>
<th>20</th>
<th>35</th>
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</thead>
<tbody>
<tr>
<td>hESCs</td>
<td>EBs</td>
<td>Rosettes</td>
<td>NSCs</td>
<td>Neurons, Glia</td>
</tr>
<tr>
<td>mTeSR™1</td>
<td>withdraw bFGF</td>
<td>N2</td>
<td>N2, B27, bFGF</td>
<td>N2, B27, dbcAMP</td>
</tr>
</tbody>
</table>

The field needs:

- Robust standardized methods for isolating NSCs to eliminate batch-to-batch variability
- Isolation of pure populations of NSCs, glia, and neurons for downstream assays: arrays/sequencing, biochemistry, in vitro assays
Objective

- Define cell surface signatures of hESC, hESC-derived NSCs, glia, and neurons
- Develop standardized methods for isolating these cell types by flow cytometry

Collaboration with Larry Goldstein
University of California, San Diego (UCSD) / Howard Hughes Medical Institute (HHMI)
Materials and Methods

Identify markers
Cells were screened using a beta version of the BD Lyoplate Human Cell Surface Marker Screening Panel by flow cytometry and bioimaging to identify a unique cell surface signature for neurons. Signatures were validated by multicolor flow cytometric analysis.

Detect and isolate cells of interest from heterogeneous pool
Cell were isolated using a BD FACSARia II cell sorter.

Analyze for purity of sorted cells
Expression of cell-type specific markers was confirmed by multicolor flow cytometric analysis.

Cell Surface Marker Screen to Identify Markers for Sorting Neurons and Glia from Differentiating NSCs
Multicolor Analysis of hESC-derived NSCs

Sox2  Nestin  Ki67  Hoechst

Sox2 PE

Oct3/4 Alexa Fluor® 488

99%
Q3-3  Q4-3

Nestin Alexa Fluor® 647

99%
Q1-2  Q2-2
Q3-2  Q4-2
Characterization of Naïve and Differentiated hESCs

Experimental results using a beta version of the BD Lyoplate™ Human Cell Surface Marker Screening Panel

hESC (H9)  EB 1  EB 2  EB 3  NSCs

CD marker “A”

CD marker “B”

CD marker “C”

CD marker “D”
Isolation of Neurons by Flow Cytometry

NSCs were differentiated 2 weeks prior to sorting
BD FACSARia II sorter, 20 psi, 100-µm nozzle

Presort
Ki-67 Nestin Map2b Hoechst

Sort gating

Sorted
Ki-67 Nestin Map2b Hoechst

NSC, Glia

Neurons
NSCs were differentiated 2 weeks prior to sorting.

BD FACS Aria II sorter, 20 psi, 100-µm nozzle.

- Presort:
  - Nestin Alexa Fluor® 647: 46%
  - Ki-67 Alexa Fluor® 488: 45%

- Sort gating:
  - Nestin Alexa Fluor® 647:
    - NSC, Glia: 5%
    - Neurons: 90%
  - Ki-67 Alexa Fluor® 488:
    - NSC, Glia: 8%
    - Neurons: 94%
Further defined the cell surface signature for hESCs, NSCs, neurons, and glia using flow cytometry and imaging

Used signatures to develop methods for identifying and isolating these cell types by flow cytometry

These methods will enable:
  • More standardized and robust isolation of hESC-derived neural stem cells
  • Downstream applications requiring consistent or pure cell populations

Immunophenotyping can be applied to address similar problems in other stem cell fields
- Intracellular and cell surface marker analysis of pluripotent stem cells
- Methods for sorting hESCs
Challenges of Sorting Pluripotent Stem Cells

• Are sorted hESCs viable?

• Do sorted cells still express markers of pluripotency?

• Are sorted cells capable of further differentiation?
Multicolor Flow Cytometric Immunophenotyping Kits

BD StemFlow™ Kits are comprehensive, ready-to-use systems designed to increase productivity and minimize assay-to-assay variability.

<table>
<thead>
<tr>
<th>Species</th>
<th>Antibodies</th>
<th>Cell Surface Analysis</th>
<th>Intracellular Analysis</th>
<th>Sorting</th>
<th>Drop-ins</th>
<th>GFP</th>
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</thead>
<tbody>
<tr>
<td>Human Pluripotent Stem Cell Sorting and Analysis Kit (Cat. No. 560461)</td>
<td>Hu</td>
<td>SSEA-1 FITC</td>
<td></td>
<td>✓</td>
<td>✓</td>
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<tr>
<td></td>
<td></td>
<td>SSEA-3 PE</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Tra-1-81 Alexa Fluor® 647</td>
<td></td>
<td>✓</td>
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<tr>
<td>Human and Mouse Pluripotent Stem Cell Analysis Kit (Cat. No. 560477)</td>
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<td>✓</td>
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<td></td>
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<td>Oct3/4 PerCP-Cy™5.5</td>
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<tr>
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<td>Human Pluripotent Stem Cell Transcription Factor Analysis Kit (Cat. No. 560589)</td>
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<td>hNanog PE</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Sox2 Alexa Fluor® 647</td>
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<td>Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (Cat. No. 560585)</td>
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<td>mNanog PE</td>
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<tr>
<td></td>
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<td>Oct3/4 PerCP-Cy™5.5</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Sox2 Alexa Fluor® 647</td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- All kits contain BD™ CompBead Plus compensation particles, matched isotype controls, and verified protocols
- Intracellular analysis kits contain fix and perm buffers
- 50 tests
Materials and Methods

- Cell surface markers (BD StemFlow Human Pluripotent Stem Cell Sorting and Analysis Kit)
  - SSEA-1 negative (differentiation)
  - SSEA-3 positive (pluripotency)
  - Tra-1-81 positive (pluripotency)

- Cell sorting with BD FACSARia II system
  - 20 psi, 100-µm nozzle

<table>
<thead>
<tr>
<th>hESC</th>
<th>Media</th>
<th>Surface</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>H9</td>
<td>mTeSR™1</td>
<td>BD Matrigel™</td>
<td>Accutase or Dispase</td>
</tr>
<tr>
<td>H9</td>
<td>KOSR</td>
<td>MEFs</td>
<td>Collagenase IV</td>
</tr>
<tr>
<td>H7</td>
<td>KOSR</td>
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<tr>
<td>HUES9</td>
<td>HUES</td>
<td>MEFs</td>
<td>Trypsin</td>
</tr>
</tbody>
</table>

Accutase used to dissociate cells in single-cell suspension

mTeSR is a trademark of StemCell Technologies.
KnockOut™ Serum Replacement is a trademark of Invitrogen.
Sorting is Possible Under Feeder and Feeder-Free Culturing Conditions

H9 day 3 post-sort
mTeSR™1, BD Matrigel

HUER9 day 4 post-sort
HUER, MEF
Goldstein Lab, UCSD

H7 day 10 post-sort
KOSR, MEF
Sorted hESCs Express Pluripotency Markers

Experimental results using the BD StemFlow™ Human and Mouse Pluripotent Stem Cell Analysis Kit

H9 P6 post-sort

TRA1-81 SSEA-1 Hoechst

SSEA-4 Oct-3/4 SSEA-1 Hoechst

Sorted hESCs Express Pluripotency Markers

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Sorted hESCs Retain Differentiation Potential

H9 P7 post-sort

Mesoderm  Ectoderm  Endoderm

GATA4 Hoechst  Sox1 Hoechst  FOXA2 Hoechst
Conclusions

• Sorted hESCs are viable and recoverable

• Sorted cells express markers of pluripotency, are able to differentiate, and maintain normal karyotype

• Standardized method for sorting hESCs by flow cytometry
Spectral Overlap

- Always need a positive-stained control
- Wastes cells
- Cumbersome when assaying for markers that might or might not be expressed on cells
- Solution = compensation beads
- Standard BD CompBead particles work on small cells found in blood, but are not ideal for larger cells
BD CompBead Plus

Overlays of unstained cells and cells and beads stained with SSEA-4 conjugates

- Autofluorescence of beads tracks hESCs
- Facilitates scatter setup
- Compensation for any mouse or rat antibody
- Negative Control (BSA) particles included

BD CompBead Plus Anti-Mouse Ig Set | Catalog No. 560497
BD CompBead Plus Anti-Rat Ig Set | Catalog No. 560499
• Extracellular Matrices (eg, BD Matrigel)
• Media and media supplements
• Cell cultureware, growth factors, cytokines

• Validated antibodies and kits
  – Flow cytometry
  – Imaging
  – Western blot

• Flow cytometry systems
  – Cell sorting (live cells)
  – Cell analysis (fixed cells)

• Automated high-content imaging systems
  – Cell analysis (fixed cells and live cells)

• Custom media, surfaces, reagents, instruments
Acknowledgments

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Rhiannon Nolan
Shauna Yuan

R&D Cytometry Lab
Andrea Nguyen
Dennis Sasaki

TAS
Sue Reynolds
Stem Cell Research Overview
Multimarker Analysis Methods Overview
Applications MSCs NSCs
Applications Pluripotent Stem Cells
Q&A Session
To alert the presenter you have a question press 1, then 0.

If you have further questions:

• Contact your BD Biosciences Reagent Sales Account Manager or email Applications Support
• ResearchApplications@bd.com

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