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

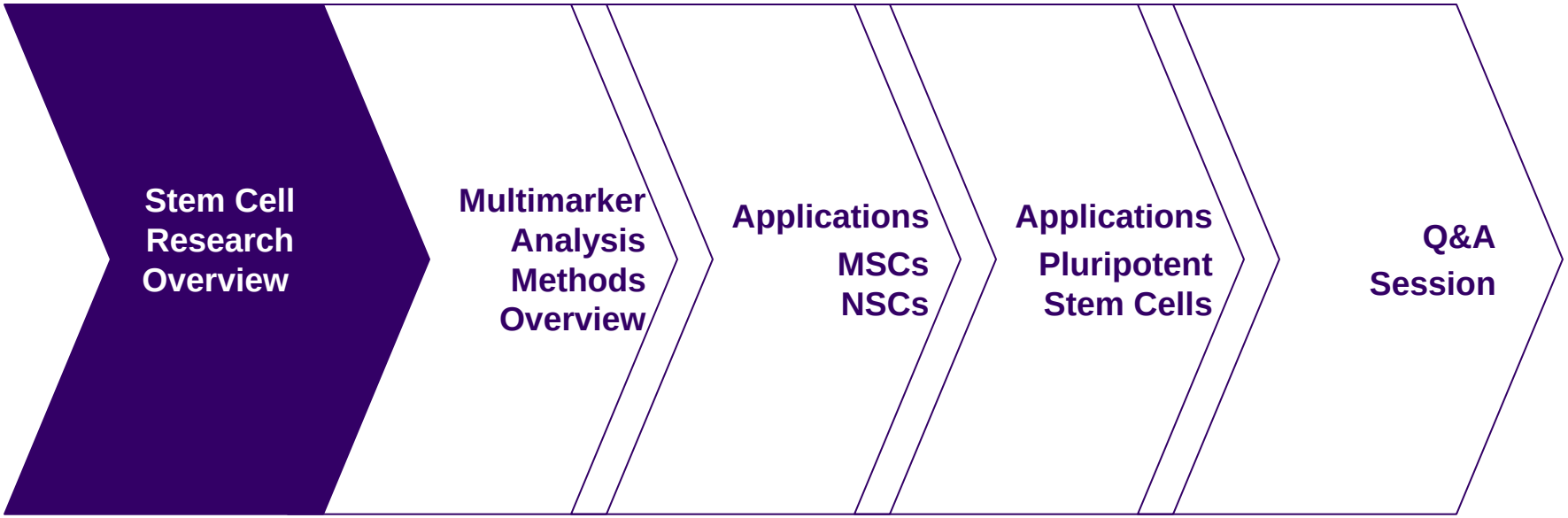
Christian Carson, PhD

BD Biosciences

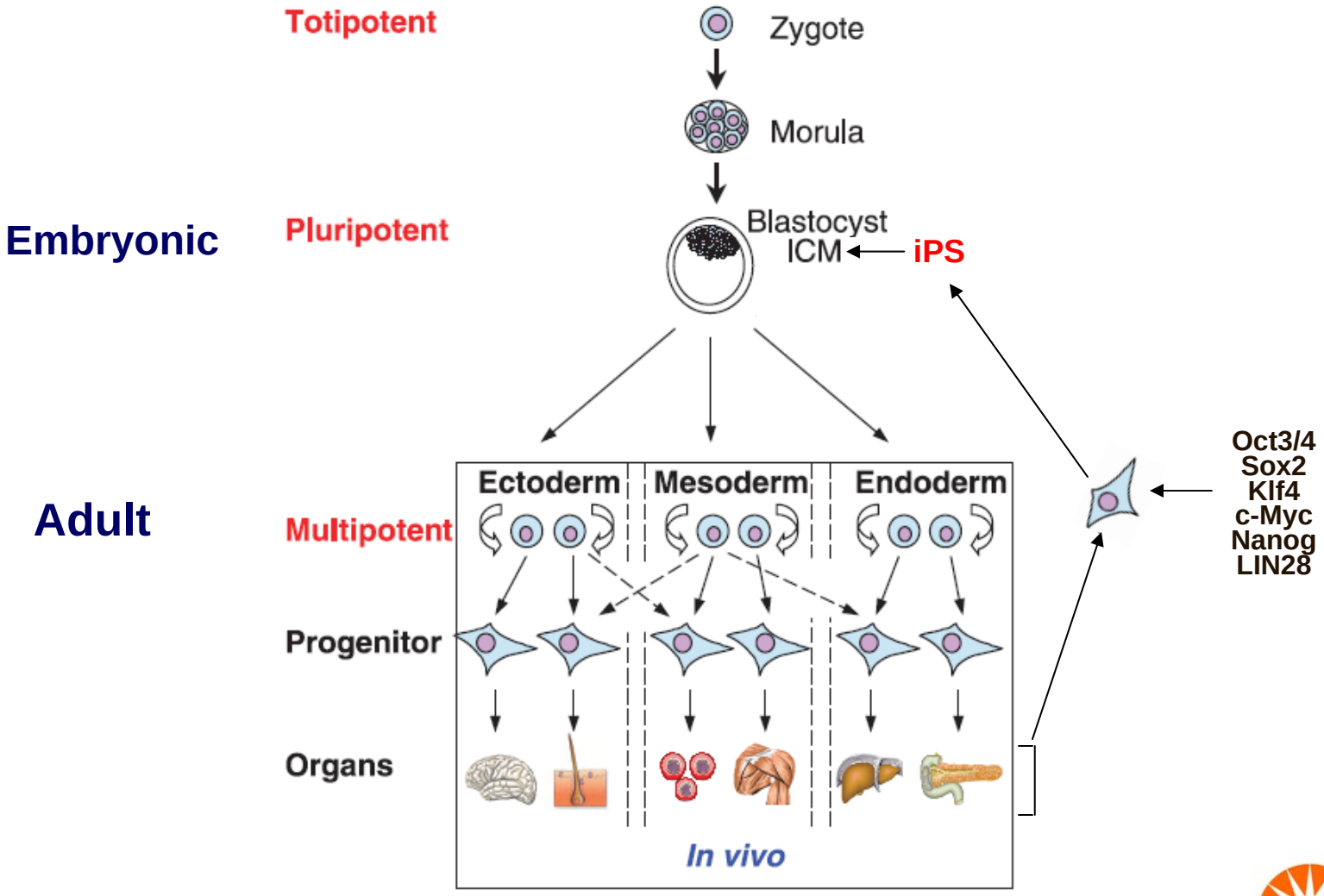
R&D Scientist

Stem Cell Research

23-11033-00

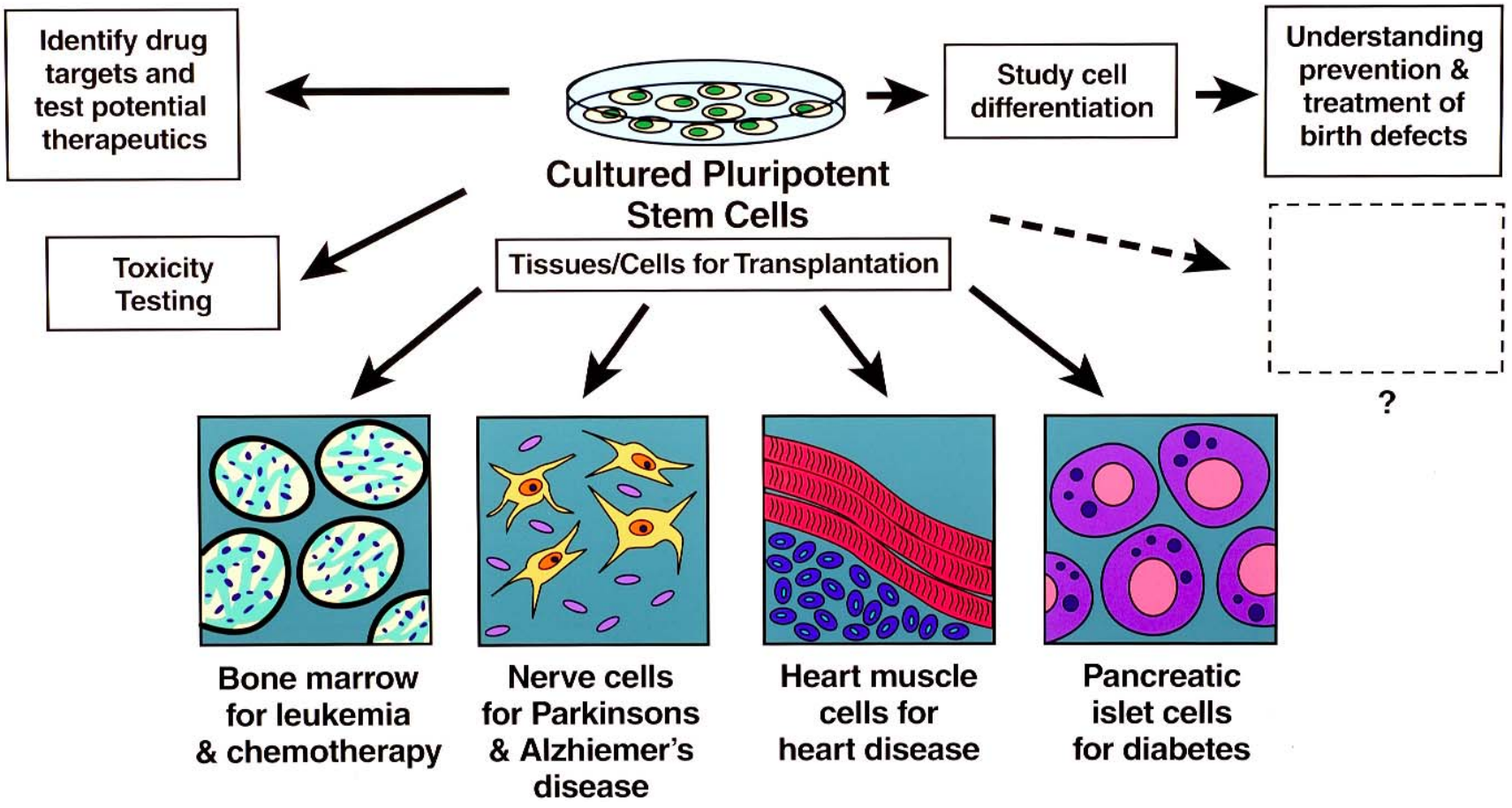


Stem Cells



Schematic adapted from Wobus AM and Boheler, KR. *Physiol. Rev. 2005;* 85:635-678.

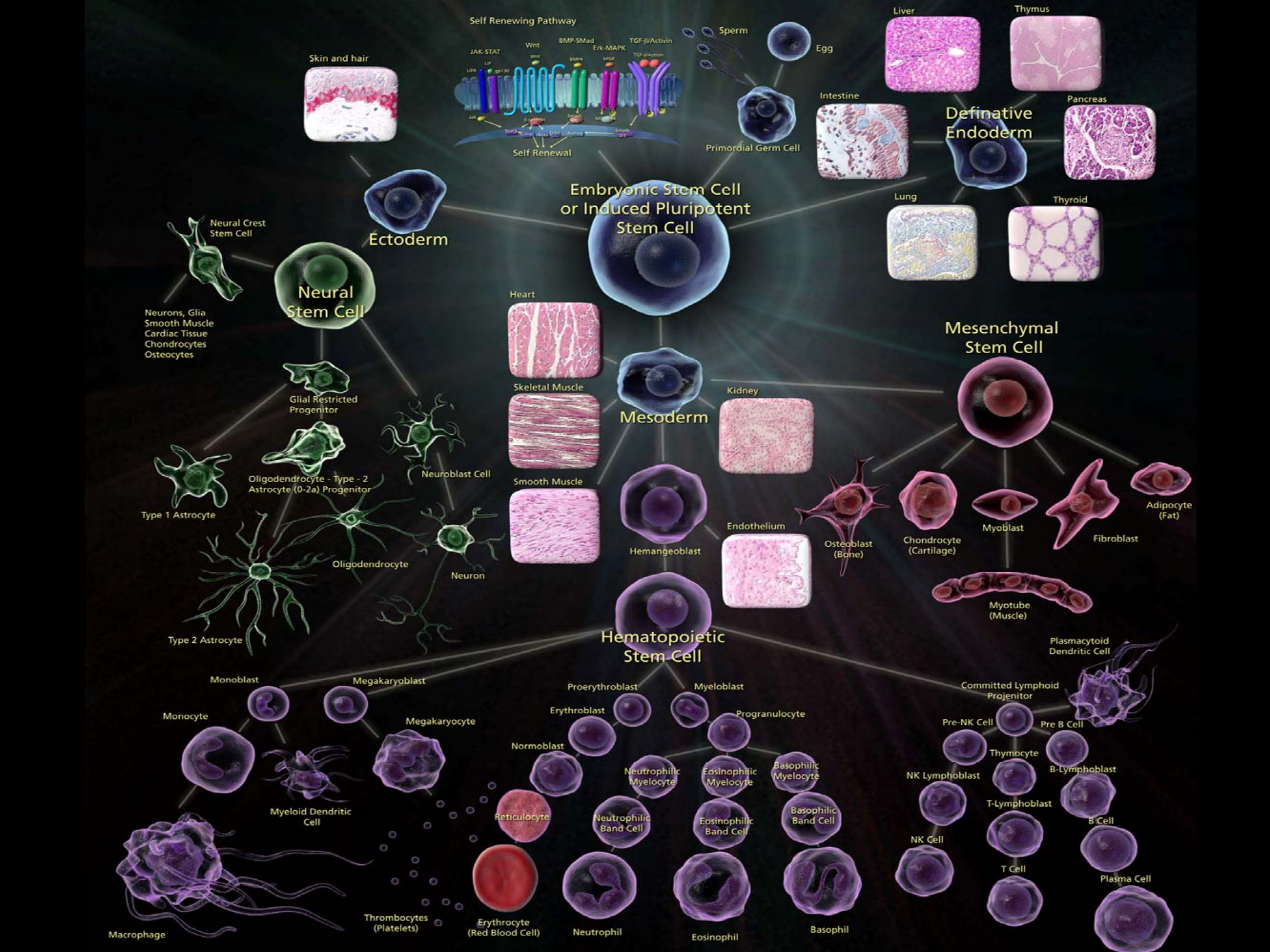
Stem Cell Research

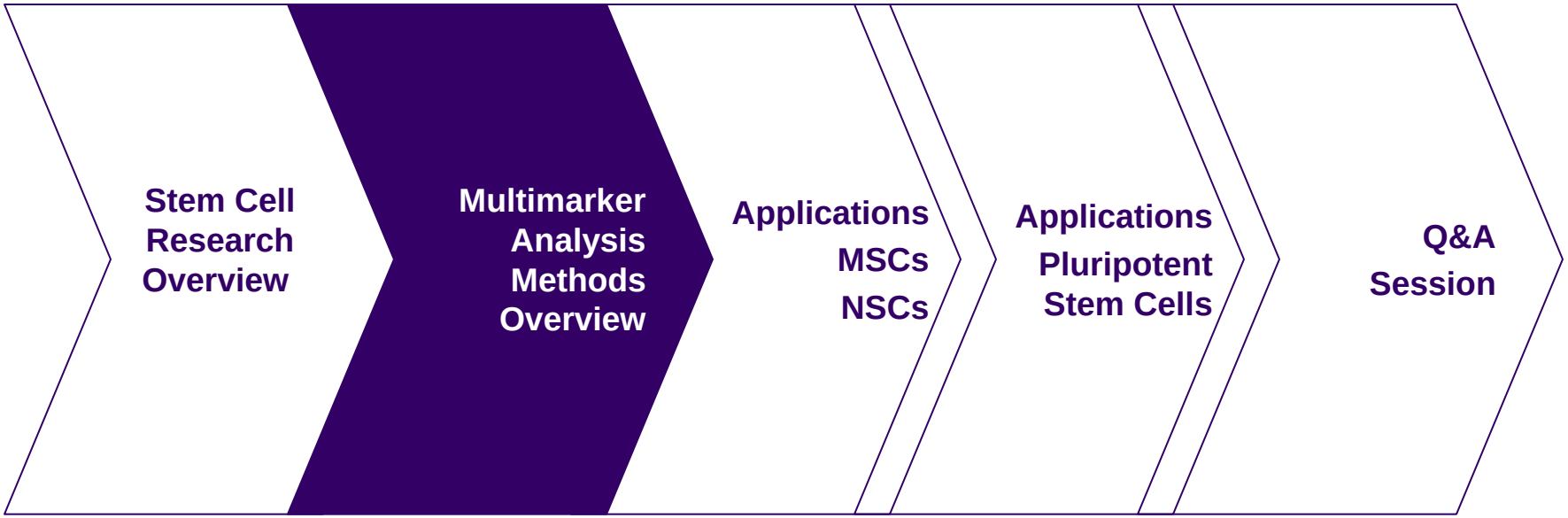


Schematic adapted from http://stemcells.nih.gov/index.asp

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


Self Renewing Pathway
JAK-STAT
Wnt
BMP-SMad
Erk-MAPK
TGF-β/Activin
Self Renewal
Skin and hair
Sperm
Egg
Primordial Germ Cell
Liver
Thymus
Intestine
Definative Endoderm
Pancreas
Lung
Thyroid
Embryonic Stem Cell or Induced Pluripotent Stem Cell
Ectoderm
Neural Crest Stem Cell
Neurons, Glia
Smooth Muscle
Cardiac Tissue
Chondrocytes
Osteocytes
Neural Stem Cell
Glial Restricted Progenitor
Oligodendrocyte - Type - 2 Astrocyte (0-2a) Progenitor
Neuroblast Cell
Type 1 Astrocyte
Oligodendrocyte
Neuron
Type 2 Astrocyte
Heart
Skeletal Muscle
Smooth Muscle
Mesoderm
Kidney
Hemangeoblast
Endothelium
Mesenchymal Stem Cell
Osteoblast (Bone)
Chondrocyte (Cartilage)
Myoblast
Fibroblast
Adipocyte (Fat)
Myotube (Muscle)
Hematopoietic Stem Cell
Monoblast
Megakaryoblast
Monocyte
Megakaryocyte
Myeloid Dendritic Cell
Macrophage
Thrombocytes (Platelets)
Proerythroblast
Myeloblast
Erythroblast
Progranulocyte
Normoblast
Neutrophilic Myelocyte
Eosinophilic Myelocyte
Basophilic Myelocyte
Reticulocyte
Neutrophilic Band Cell
Eosinophilic Band Cell
Basophilic Band Cell
Erythrocyte (Red Blood Cell)
Neutrophil
Eosinophil
Basophil
Plasmacytoid Dendritic Cell
Committed Lymphoid Projenitor
Pre-NK Cell
Pre B Cell
Thymocyte
NK Lymphoblast
B-Lymphoblast
T-Lymphoblast
B Cell
NK Cell
T Cell
Plasma Cell



Immunophenotyping

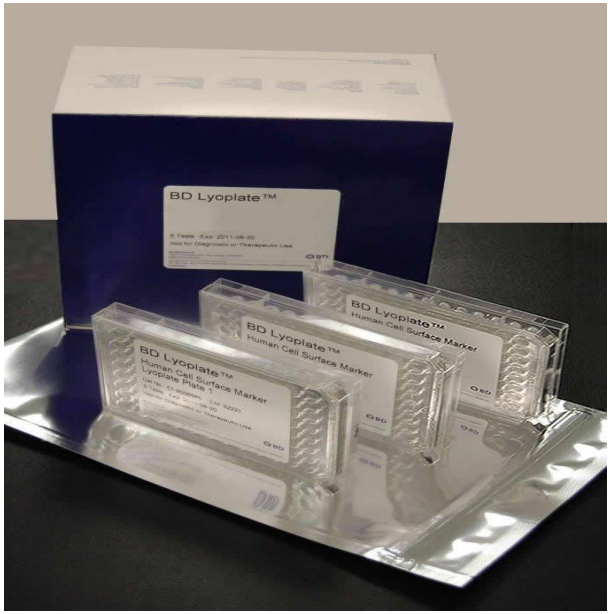
Immunophenotyping is a cellular analysis method for the identification of biomarkers and their expression/co-expression profiles using directly- or indirectly-fluorochrome conjugated antibodies and an analyzer such as a flow cytometer or imaging system.

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 stems cells and stem cell–derived cells.



BD Lyoplate Screening Panel	Contents	Applications	Cat. No.
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

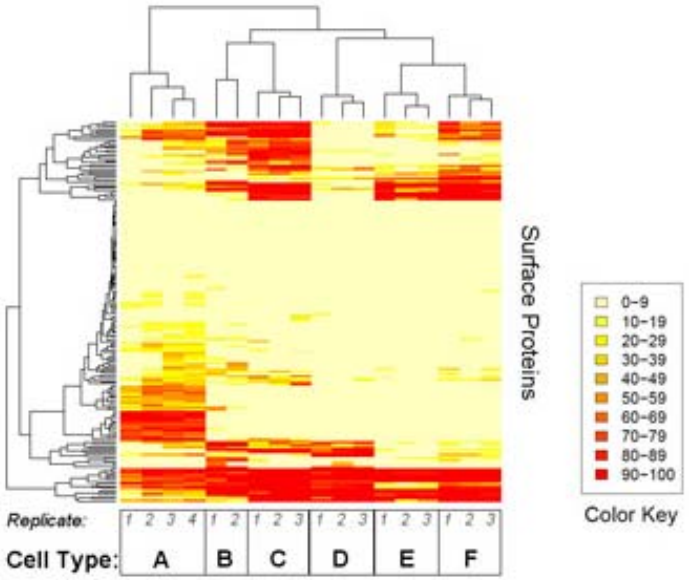
*CD and other cell surface molecules. One marker per well.

- **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**

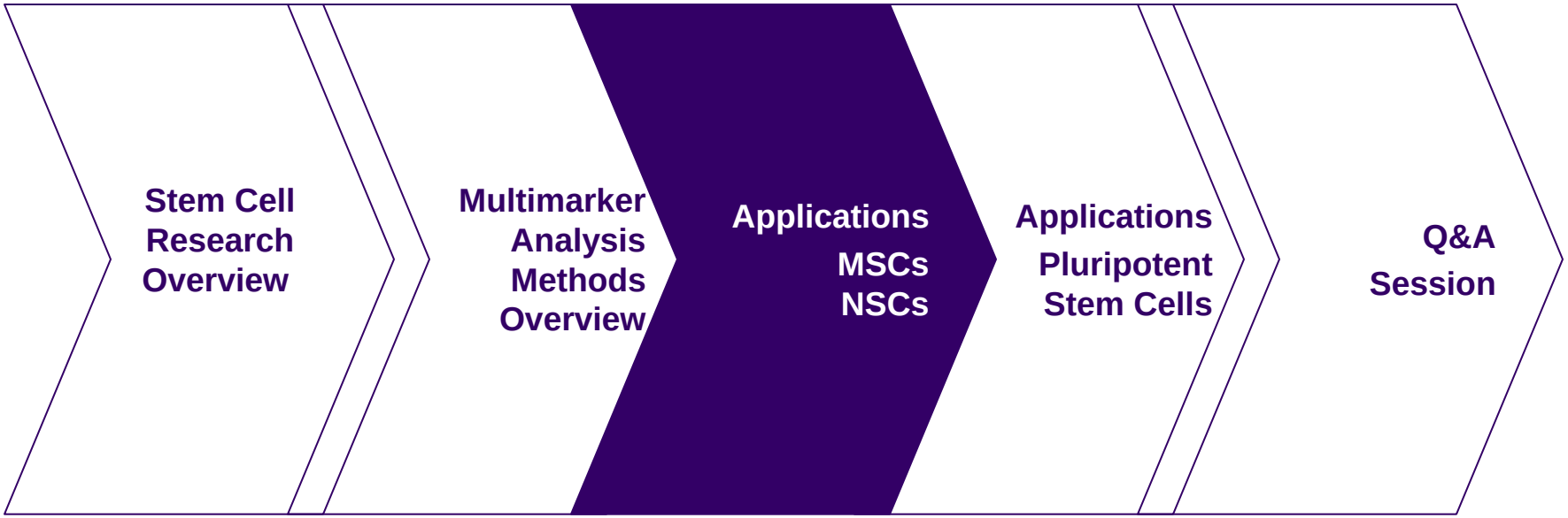
Tools for Immunophenotyping

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

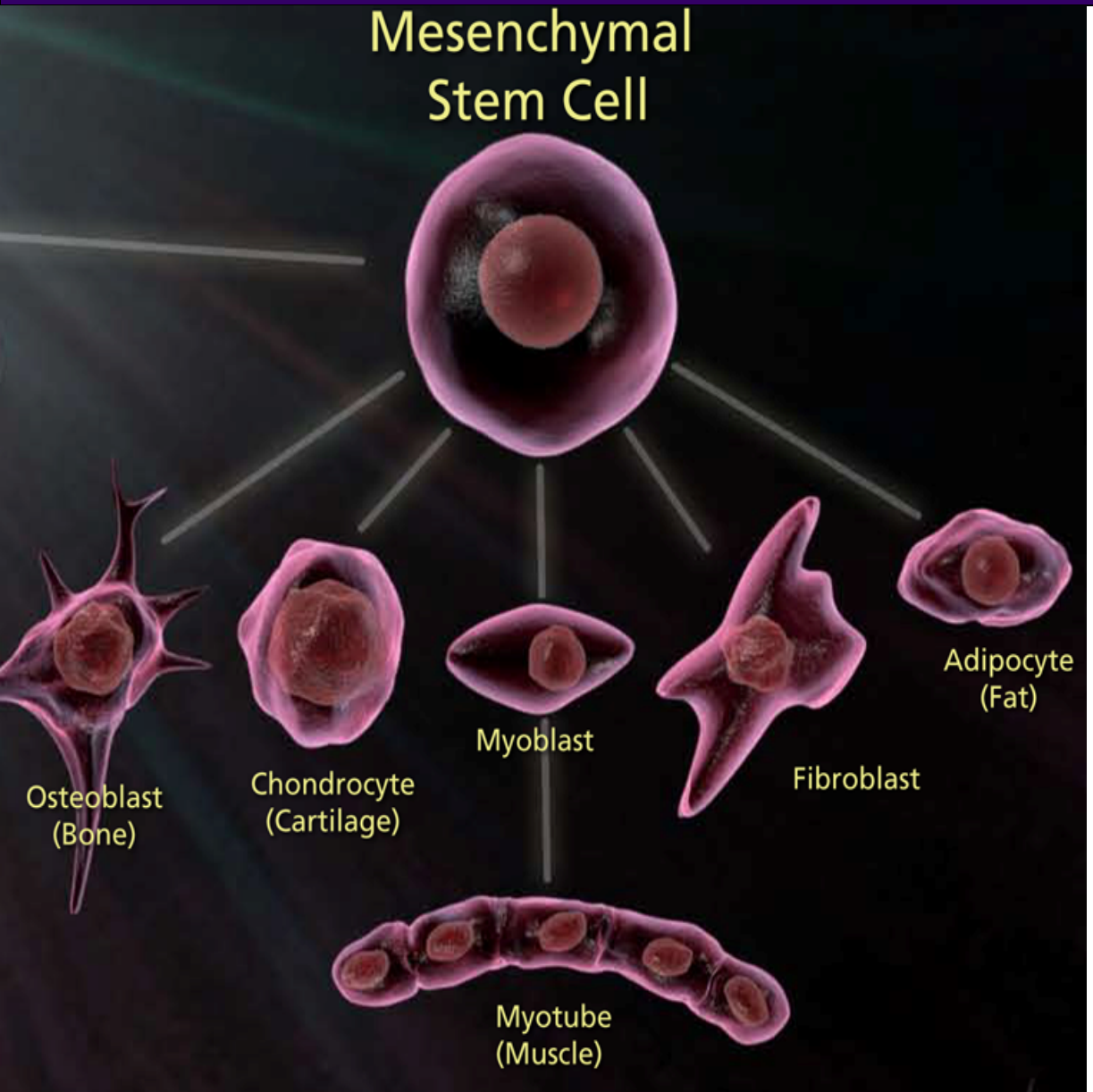


In addition, the BD Custom Technology Team helps researchers to create multicolor antibody cocktails for in-house flow cytometry immunophenotyping. Optimized multicolor cocktails streamline sample preparation, acquisition and analysis, and improve standardization between experiments.



- **Screening of MSCs**
- **Screening, isolation and analysis of hESC-derived NSCs, glia, and neurons**

Mesenchymal Stem Cells (MSCs)

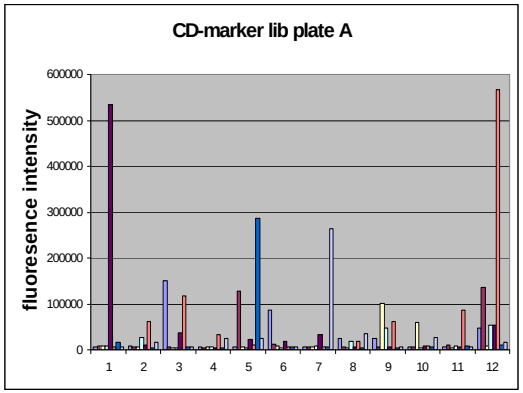


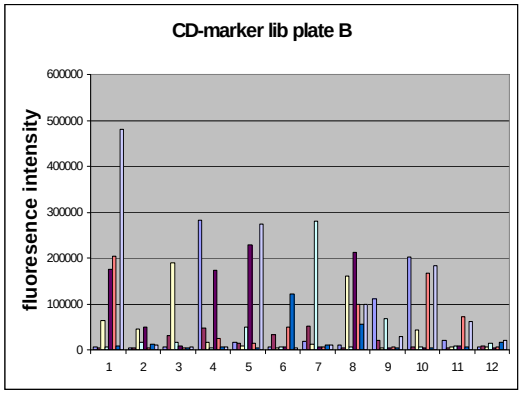
- **MSCs are the progenitors of multiple mesenchymal lineages**
  - Bone, cartilage, muscle, fat tissue, and marrow stroma
- **MSCs can be isolated and cultured in vitro**
- **Promises of MSCs**
  - Regenerative medicine
  - In vitro models of human diseases
    - Drug screening
    - Toxicology
   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

BD

Characterization of MSCs

Objective: correlate CD marker profile to lineage capacity and multipotency





A

buffer	CD1a	CD1b	CD1d	CD2	CD3	CD4	CD5	CD6	CD7	CD8	CD9
CD10	CD11a	CD11b	CD11c	CD13	CD14	CD15	CD15s	CD16	CD18	CD19	CD20
CD21	CD22	CD23	CD24	CD25	CD26	CD27	CD28	CD29	CD30	CD31	CD32
CD33	CD34	CD35	CD36	CD37	CD38	CD40	CD41a	CD41b	CD42a	CD42b	CD43
CD44	CD45	CD45RA	CD45RB	CD45RO	CD46	CD47	CD48	CD49a	CD49b	CD49b	CD49c
CD49d	CD49e	CD49e	CD50	CD51/61	CD53	CD54	CD55	CD56	CD57	CD58	CD59
CD61	CD62E	CD62L	CD62P	CD63	CD64	CD66	CD66b	CD66f	CD69	CD70	CD71
CD72	CD73	CD74	CD77	CD79b	CD80	CD81	CD83	CD84	CD85j	CD86	CD87

B

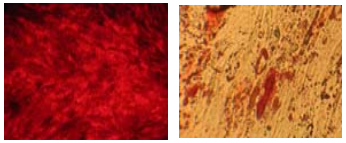
buffer	CD88	CD89	CD90	CD91	CD94	CD95	CD97	CD98	CD99	CD99R	CD100
CD103	CD104	CD106	CD108	CD109	CD110	CD117	CD123	CD130	CD134	CD135	CD137
CD137 L	CD138	CD140a	CD140b	CD141	CD142	CD146	CD147	CD150	CD151	CD152	CD153
CD154	CD158a	CD158b	CD161	CD162	CD163	CD164	CD165	CD166	CD172b	CD177	CD180
CD181	CD182	CD183	CD184	CD195	CD200	CD206	CD209	CD221	CD226	CD227	CD229
CD235a	CD244	CLIP	CMRF-44	CMRF-56	EGFR	F11 R	fMLPr	gdTCR	HLA-ABC	HLA-A2	HLA-DQ
HLA-DR	HLA-DR	InvNKT	LAIR-1	CD220	mu-Calp	MUC2	NGFR	NKB1	NKG2D	NKP46	NPM-ALK
P-glycop	Siglec-6	Siglec-7	Vbeta 5	Vbeta 8	INTb7	PRR2	ABCG2	abTCR	B7-H2	beta 2-m	Bd Grp A

<7000 >7000 >20000 >50000 >100000 >200000 >300000 >400000 >500000

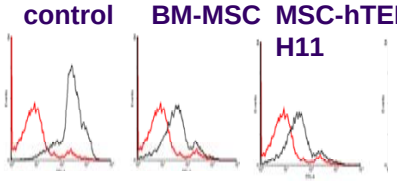
Collaboration with Paul Coffer and Koen Braat
University Medical Center Utrecht, Netherlands

Functional characterization

Multipotency?
Osteogenic capacity?

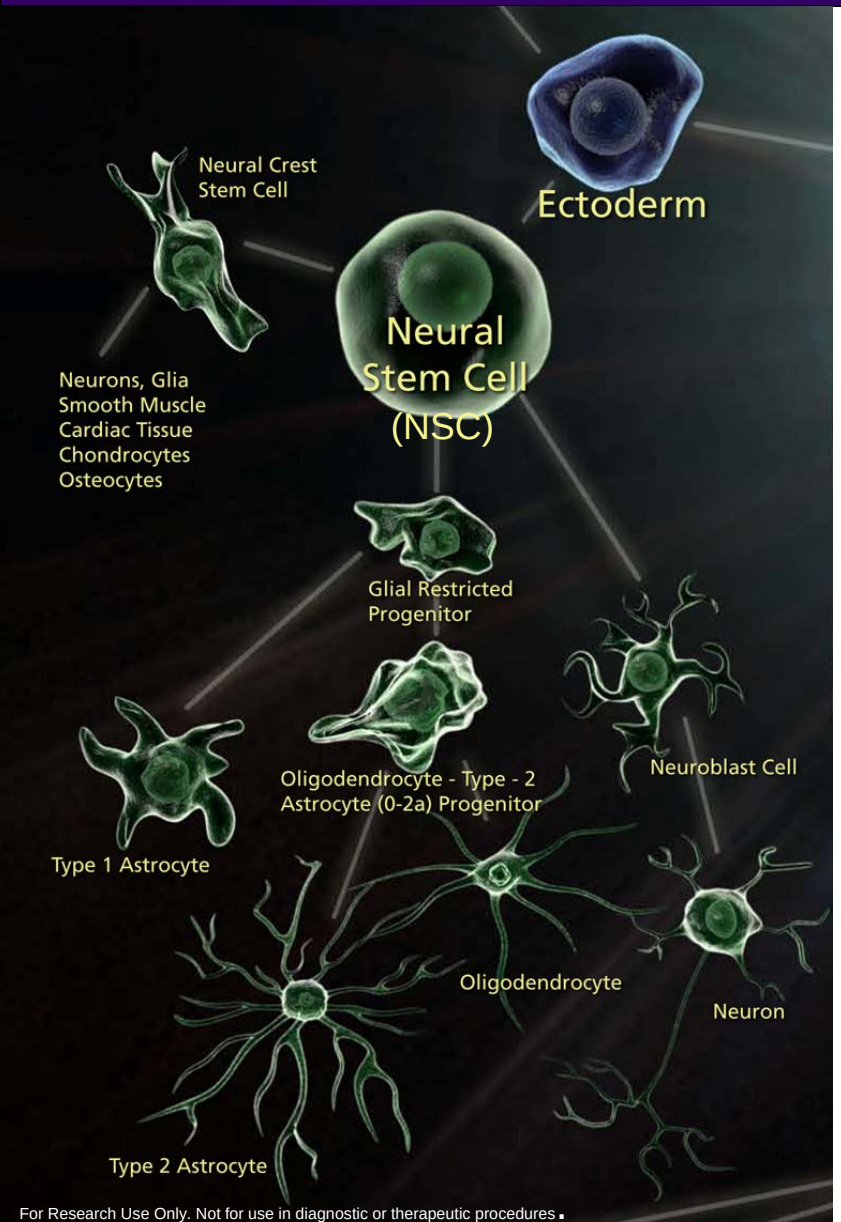


Immunosuppression?



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

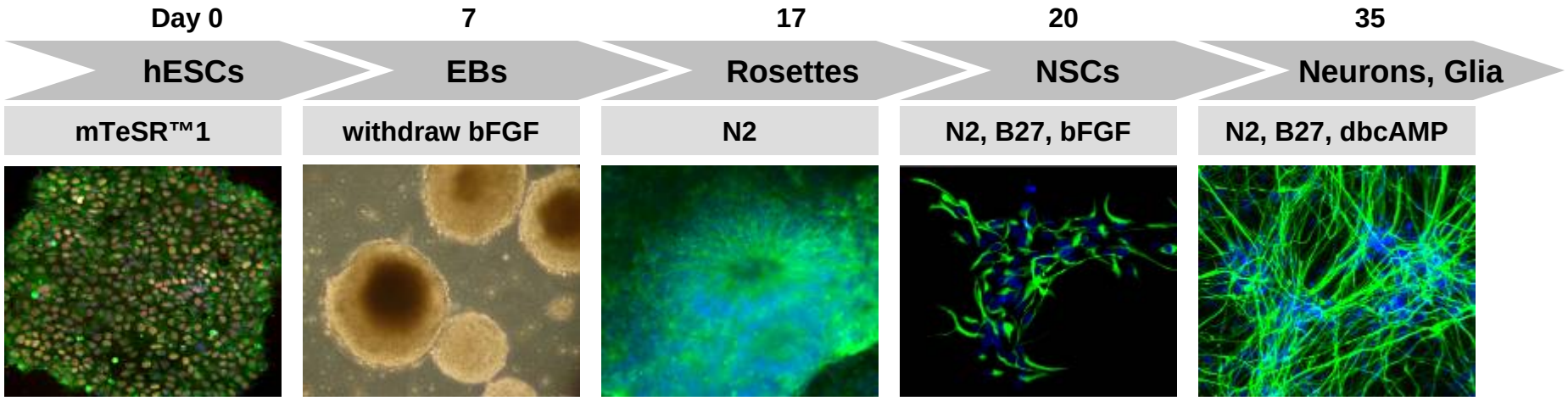
Neural Stem Cells (NSCs)



- **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

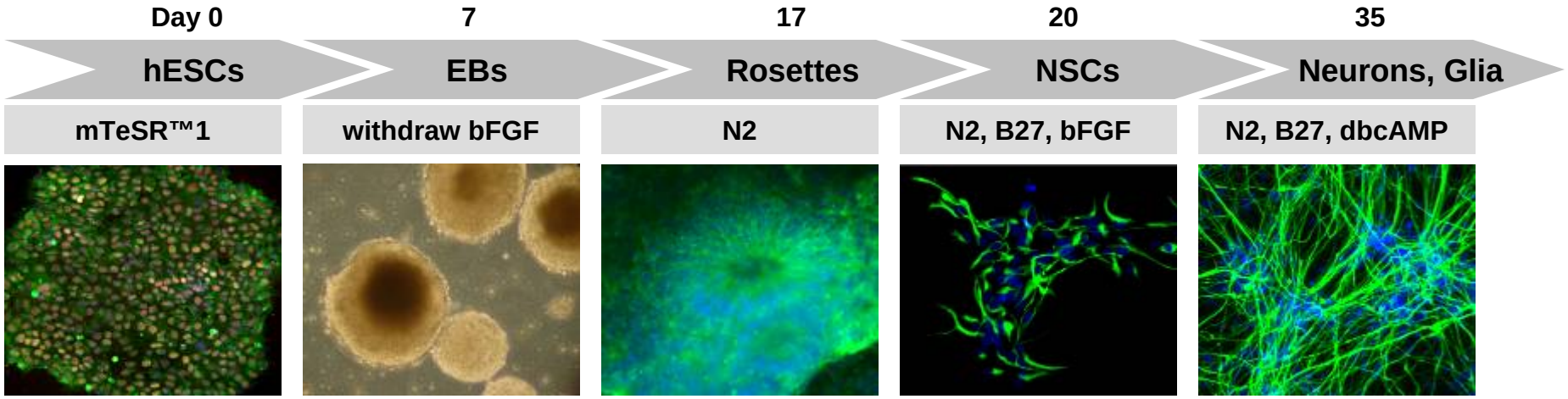
Differentiation of hESCs into neural stem cells



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**

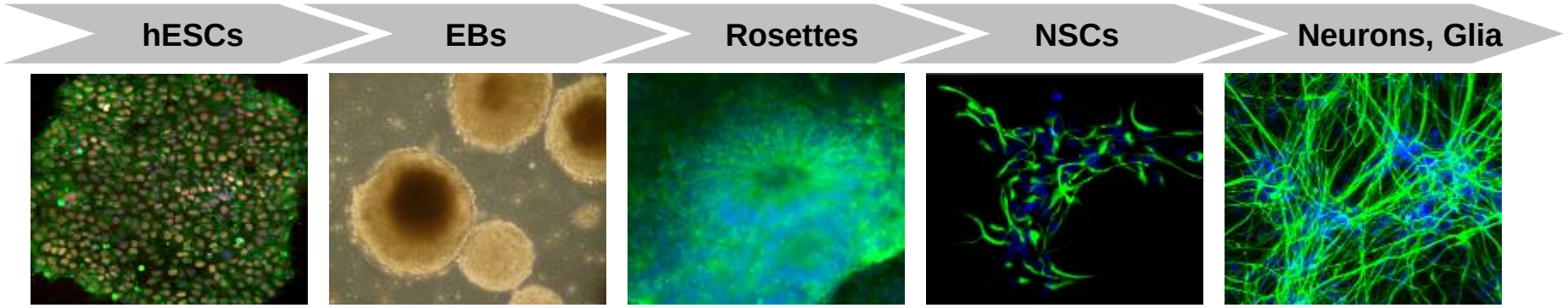
Objective



- **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



Cell Surface Marker Screen to Identify Markers for Sorting Neurons and Glia from Differentiating NSCs

Generate hESC-derived NSCs, glia, and neurons

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.

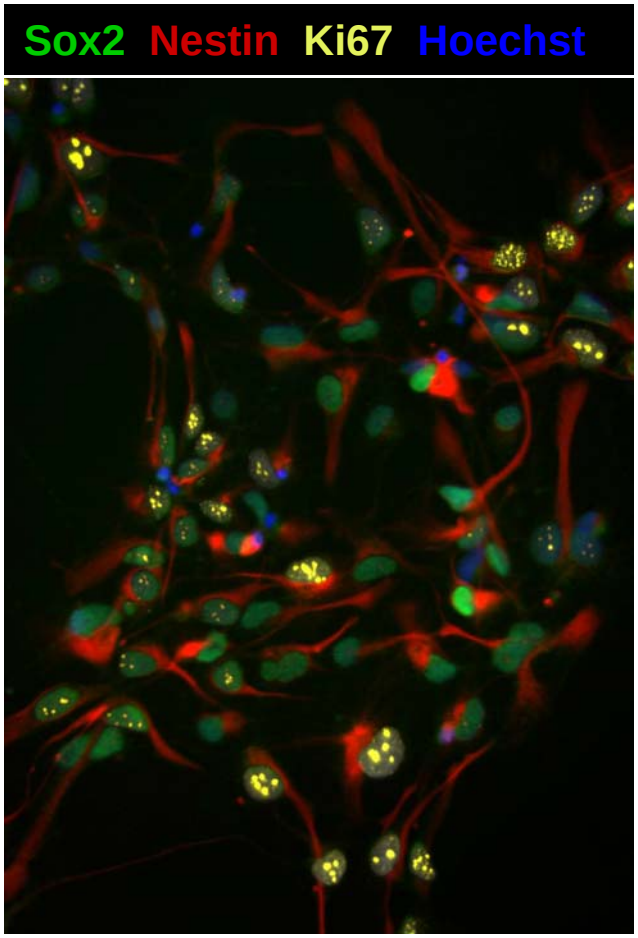
Detect and isolate cells of interest from heterogeneous pool

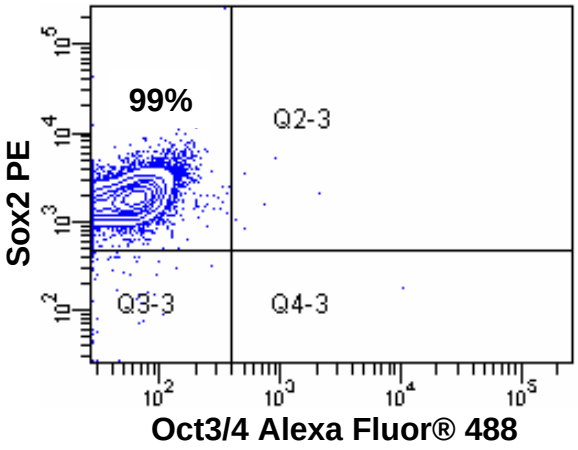
Cell were isolated using a BD FACSAria™ II cell sorter.

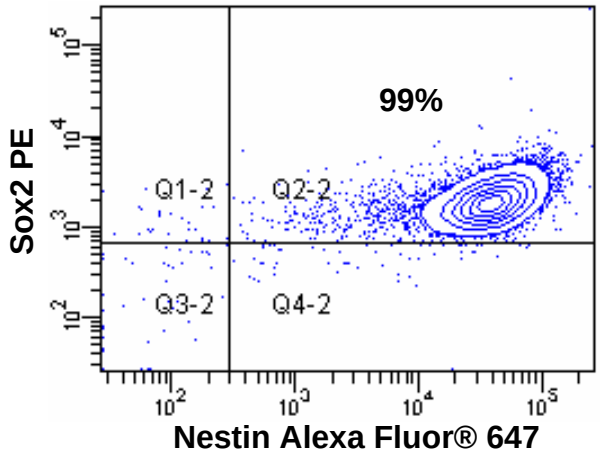
Analyze for purity of sorted cells

Expression of cell-type specific markers was confirmed by multicolor flow cytometric analysis.

Multicolor Analysis of hESC-derived NSCs

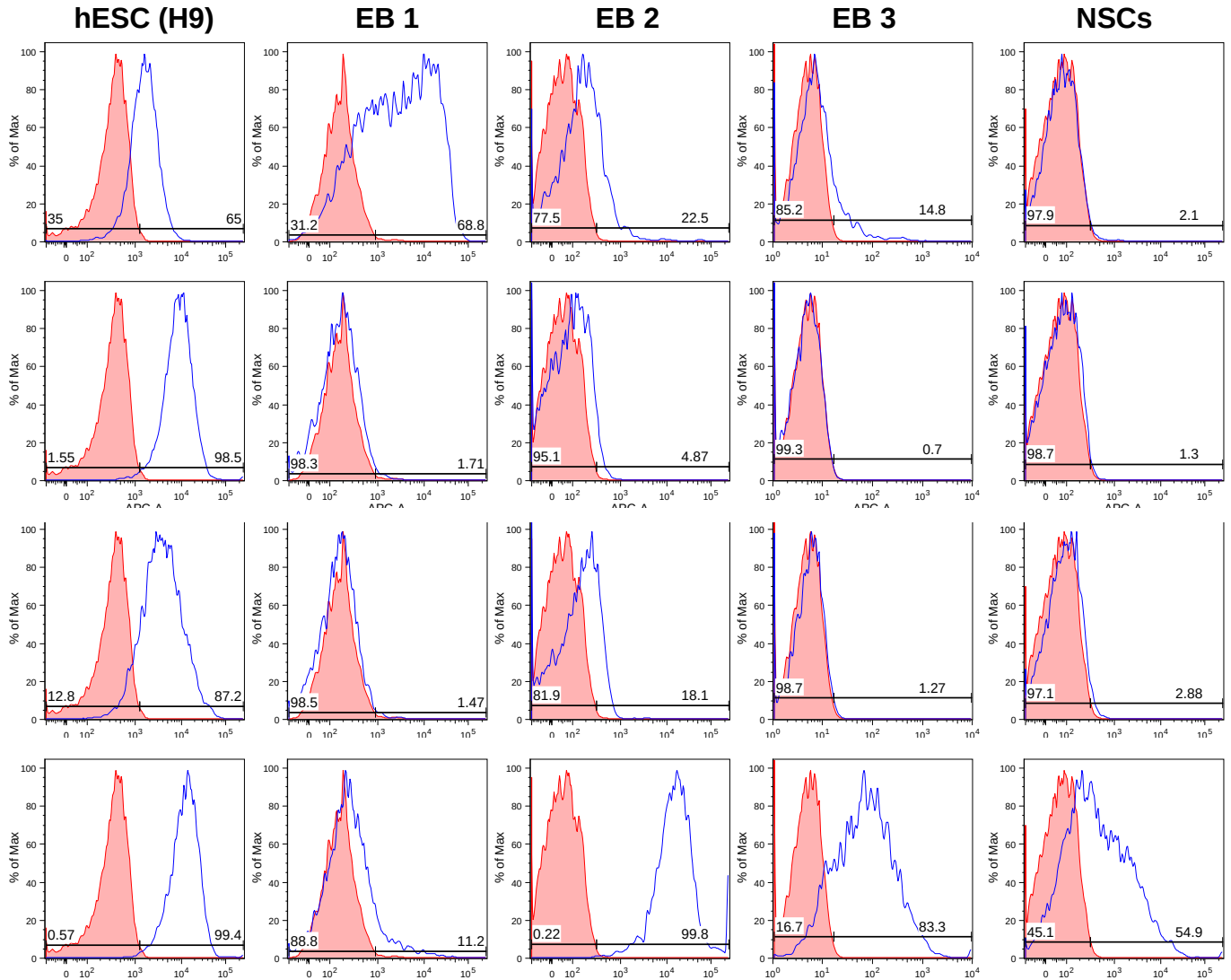






Characterization of Naïve and Differentiated hESCs

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



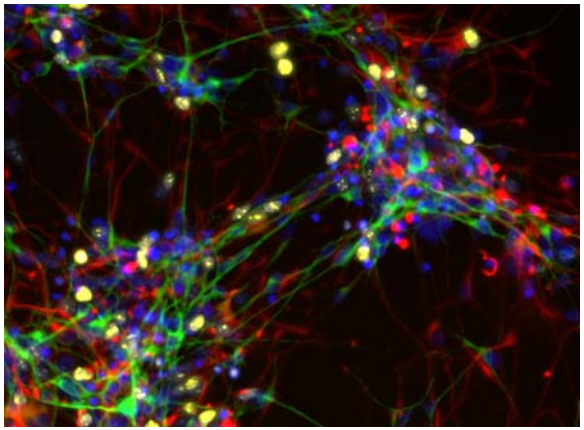
Isolation of Neurons by Flow Cytometry

NSCs were differentiated 2 weeks prior to sorting

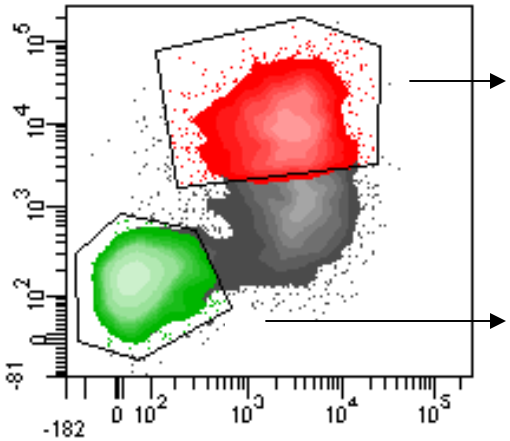
BD FACSAria II sorter, 20 psi, 100-µm nozzle

Presort

Ki-67 Nestin Map2b Hoechst

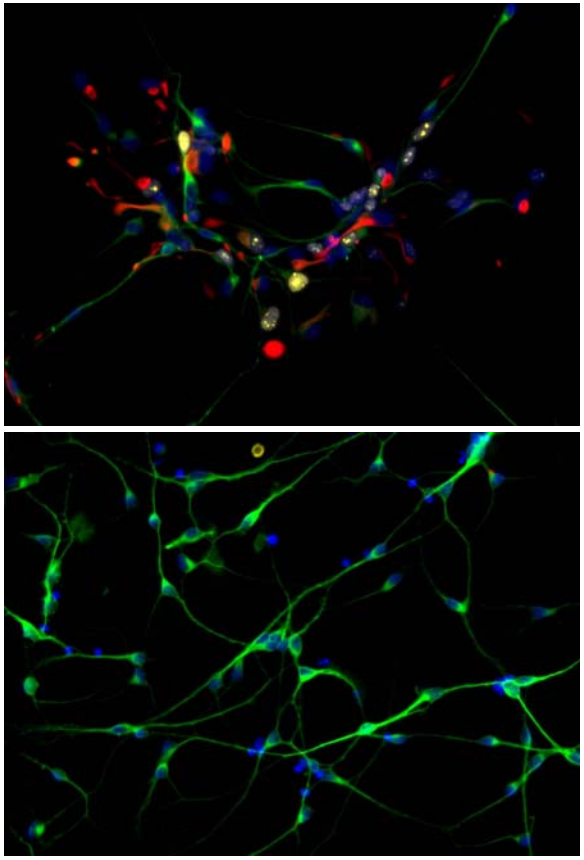


Sort gating



Sorted

Ki-67 Nestin Map2b Hoechst



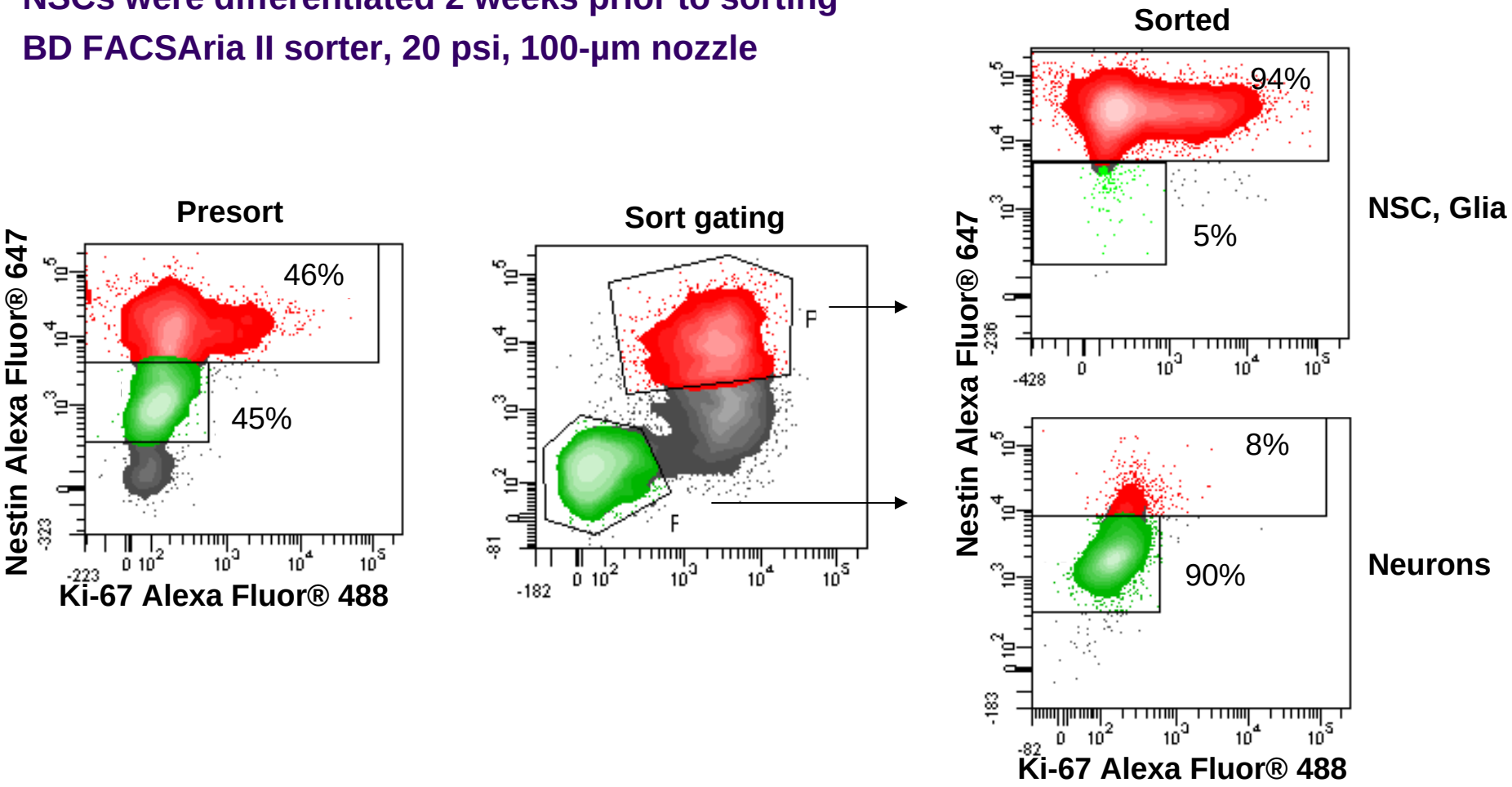
NSC, Glia

Neurons

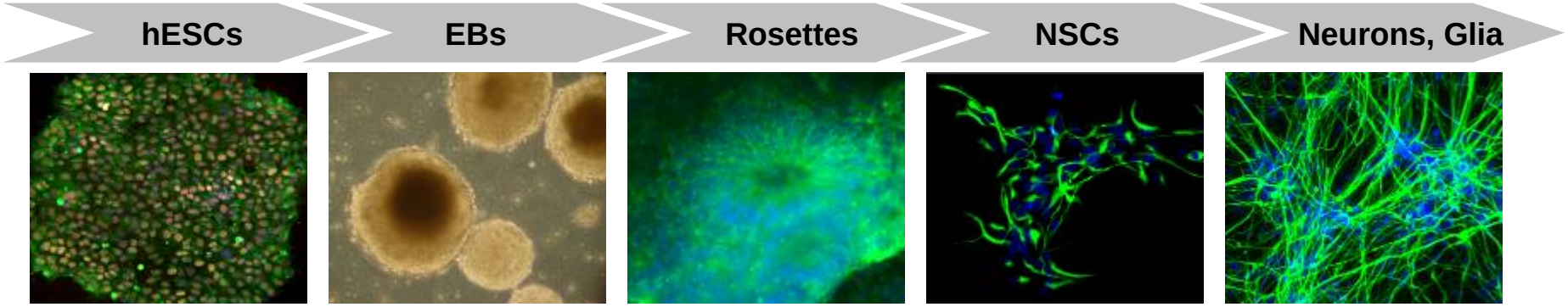
Isolation of Neurons by Flow Cytometry

NSCs were differentiated 2 weeks prior to sorting

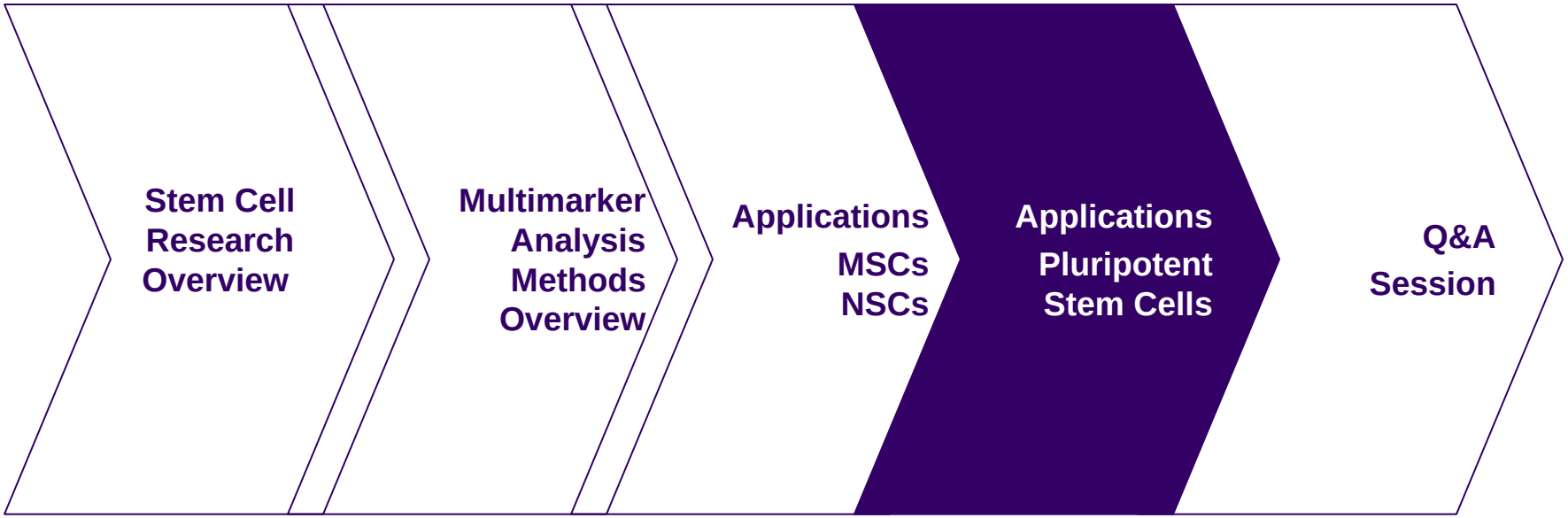
BD FACSAria II sorter, 20 psi, 100-µm nozzle



Conclusions



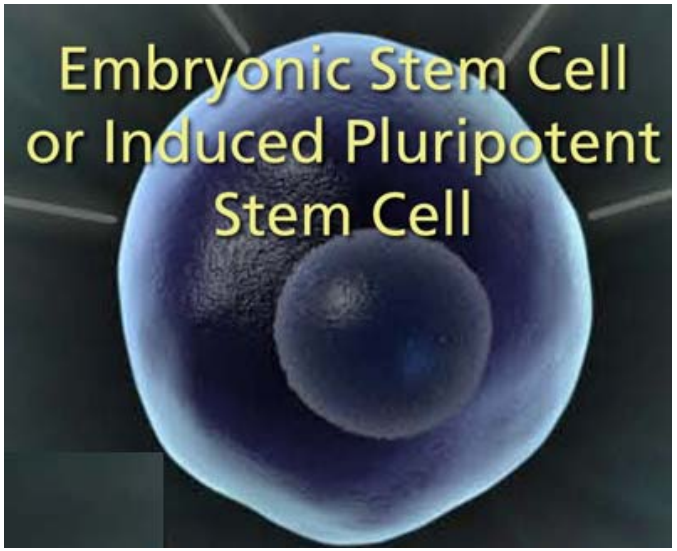
- Further defined the cell surface signature for hESCs, NSCs, neurons, and glia using flow cytometry and imaging
- Used signatures to develop methods for identifying and isolating these cell types by flow cytometry
- These methods will enable:
  - More standardized and robust isolation of hESC-derived 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?
  - Fong, et al. *Stem Cell Rev.* 2009
  - Nicholas, et al. *Stem Cells Dev*. 2007
  - Sidhu, et al. *Stem Cells Dev.* 2006
- 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.

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

- 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

hESC	Media	Surface	Enzyme
H9	mTeSR™1	BD Matrigel™	Accutase or Dispase
H9	KOSR	MEFs	Collagenase IV
H7	KOSR	MEFs	Collagenase IV
HUES9	HUES	MEFs	Trypsin

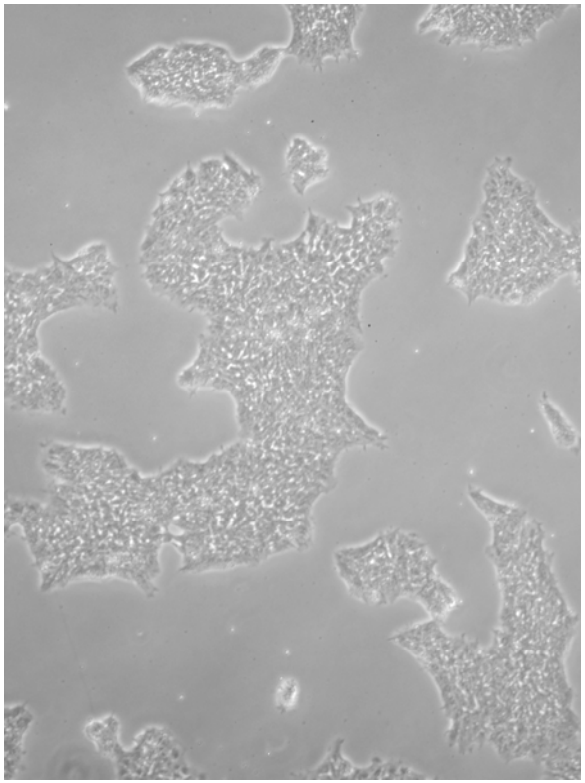
- Accutase used to dissociate cells in single-cell suspension

mTeSR is a trademark of StemCell Technologies.

KnockOut™ Serum Replacement is a trademark of Invitrogen.

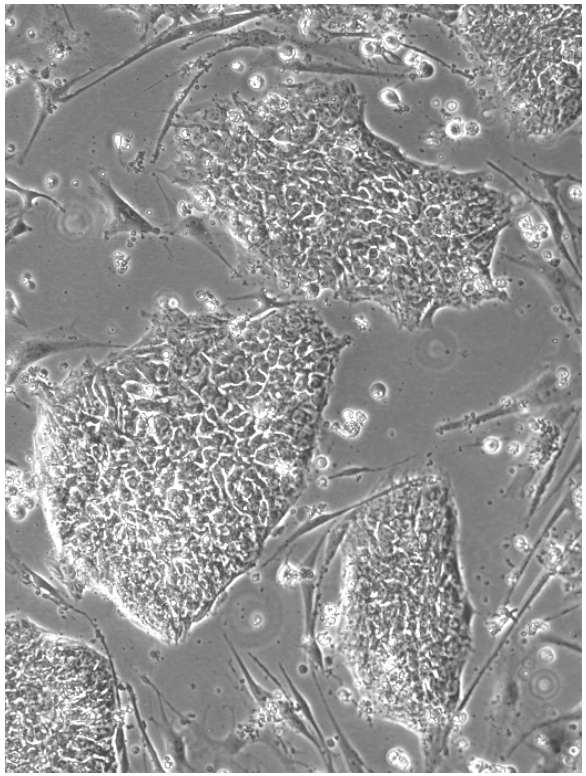
Sorting is Possible Under Feeder and Feeder-Free Culturing Conditions

H9 day 3 post-sort



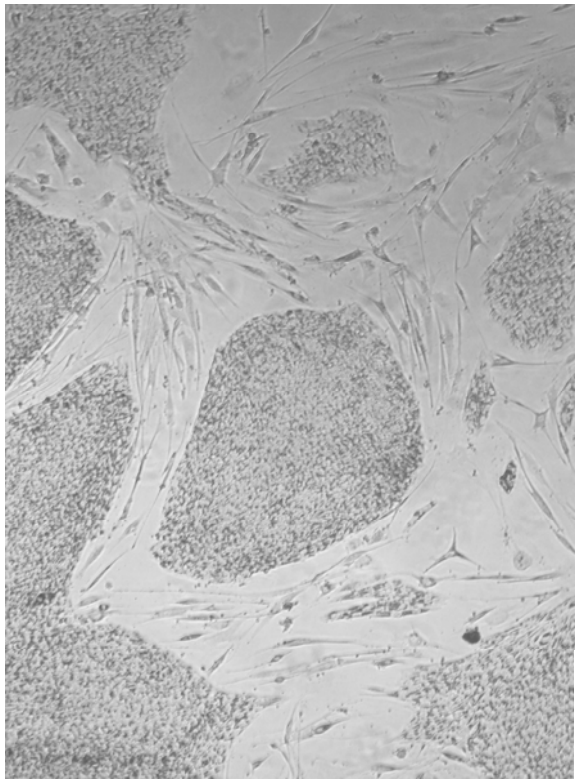
mTeSR™1, BD Matrigel

HUES9 day 4 post-sort



HUES, MEF
Goldstein Lab, UCSD

H7 day 10 post-sort



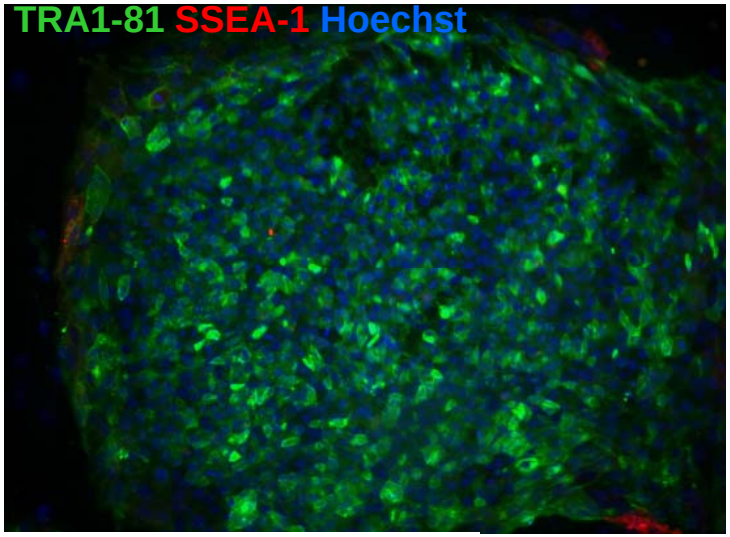
KOSR, MEF

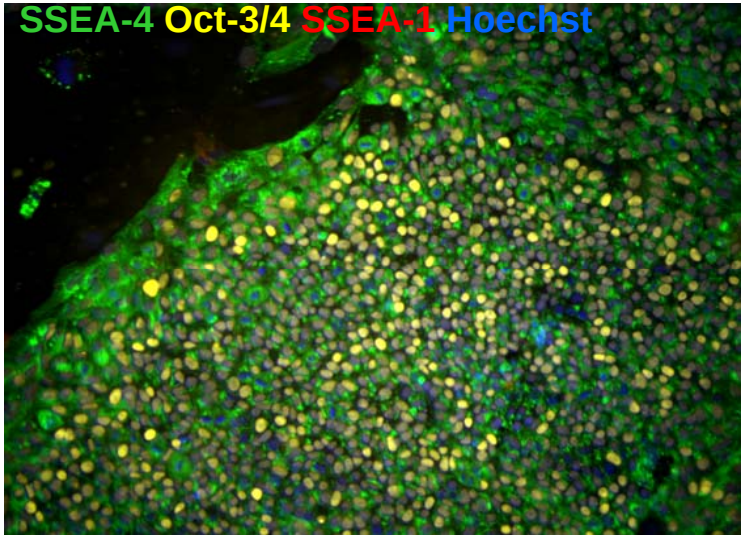
KnockOut™ Serum Replacement is a trademark of Invitrogen.

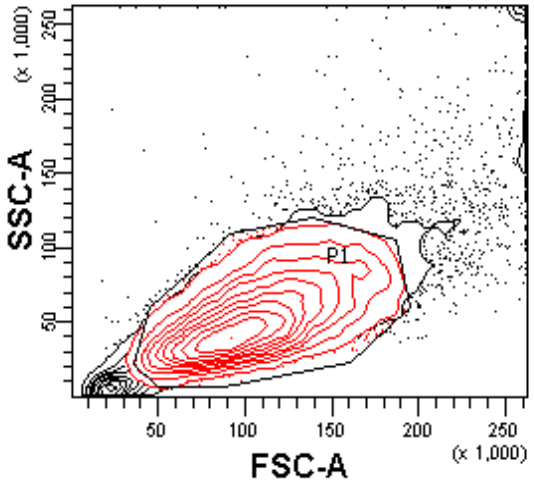
Sorted hESCs Express Pluripotency Markers

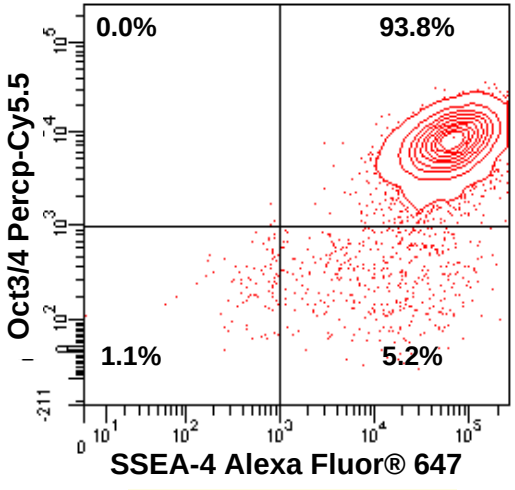
Experimental results using the BD StemFlow™ Human and Mouse Pluripotent Stem Cell Analysis Kit

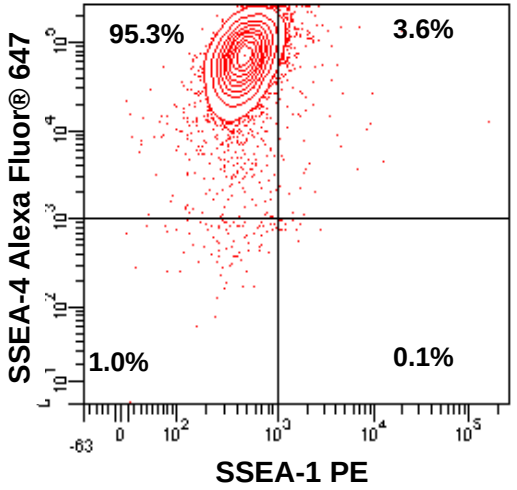
H9 P6 post-sort





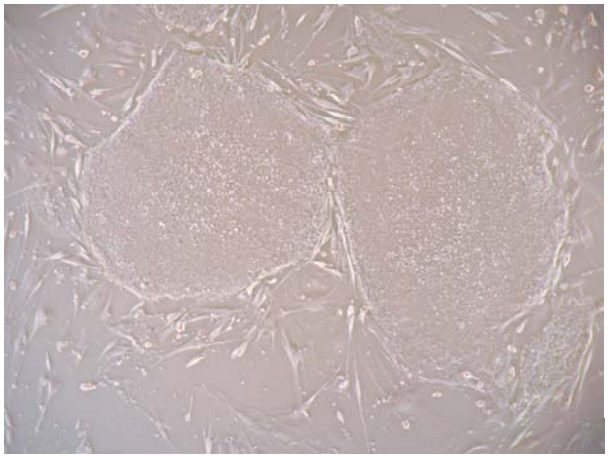


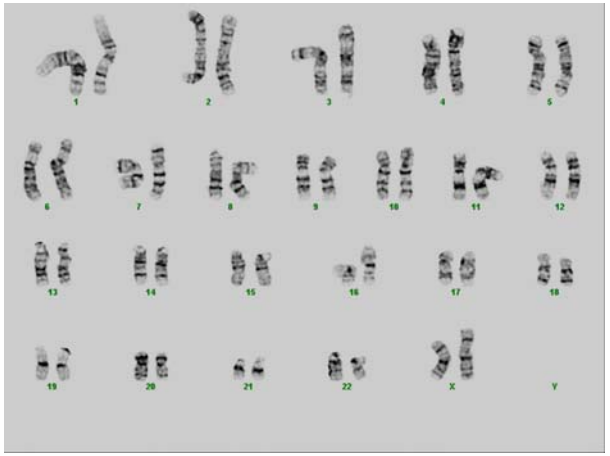




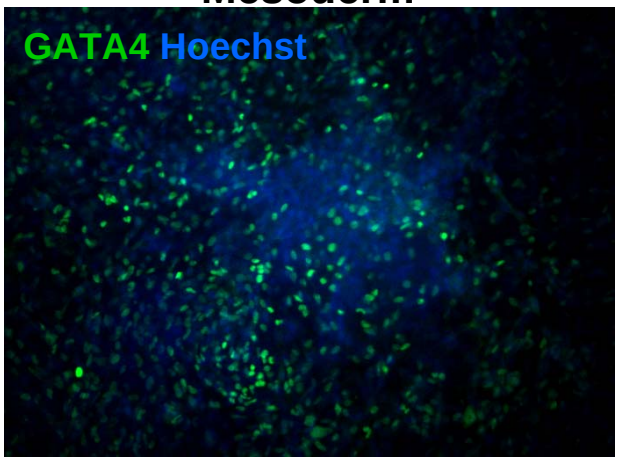
Sorted hESCs Retain Differentiation Potential

H9 P7 post-sort

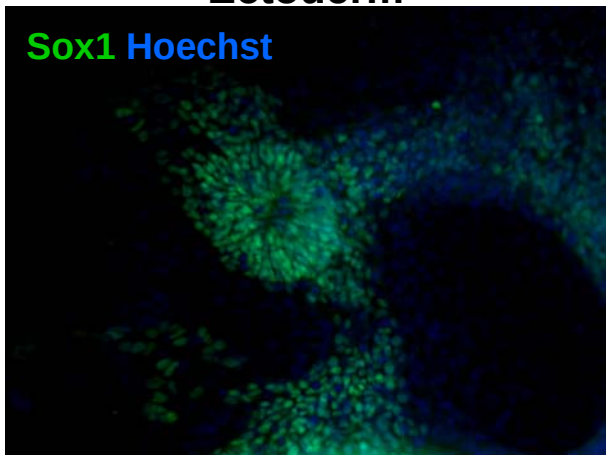




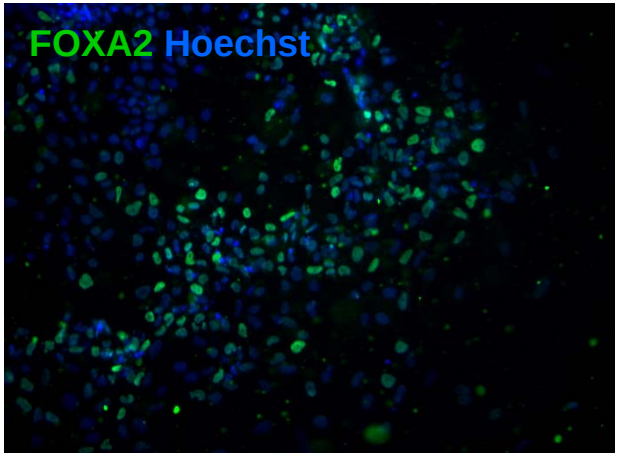
Mesoderm



Ectoderm



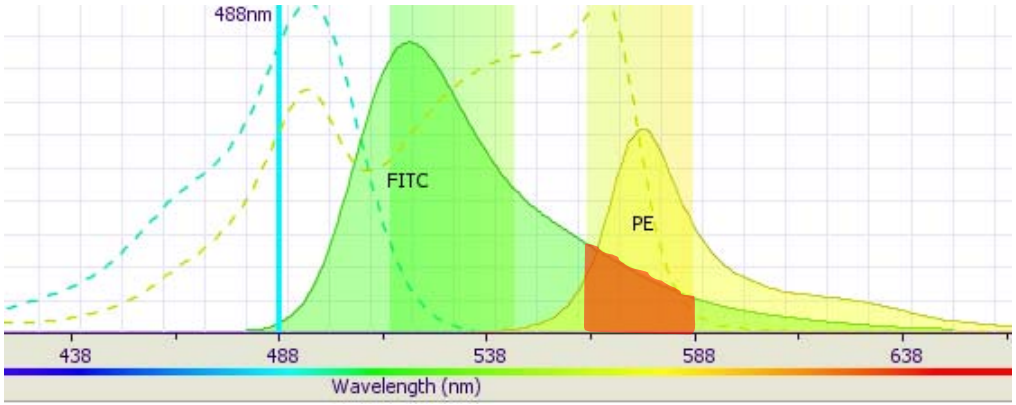
Endoderm

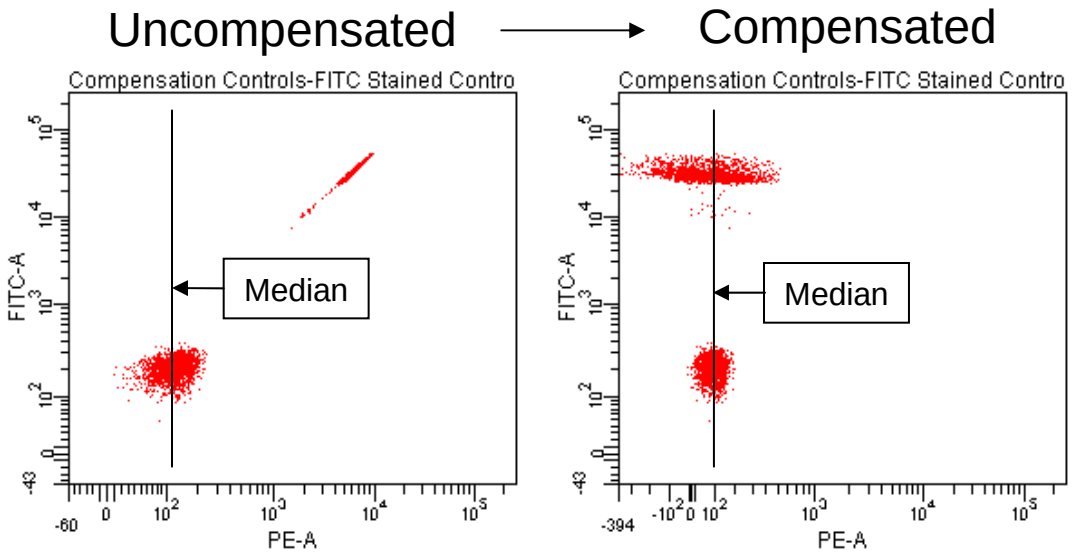


Conclusions

- 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

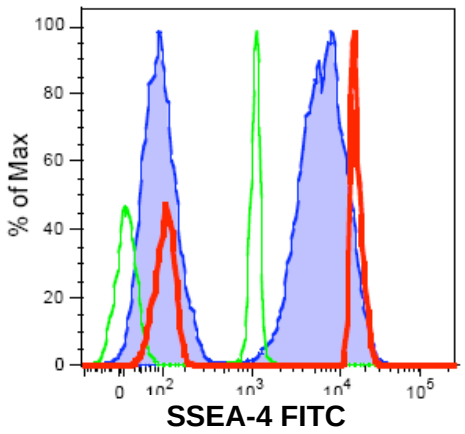


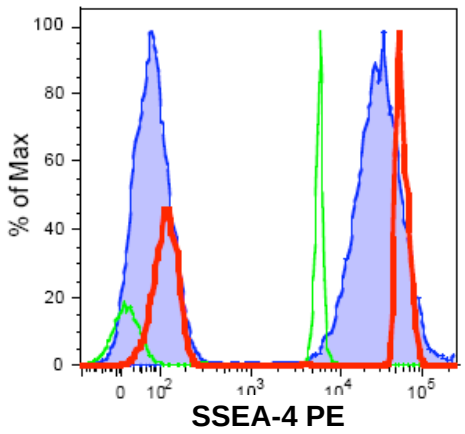


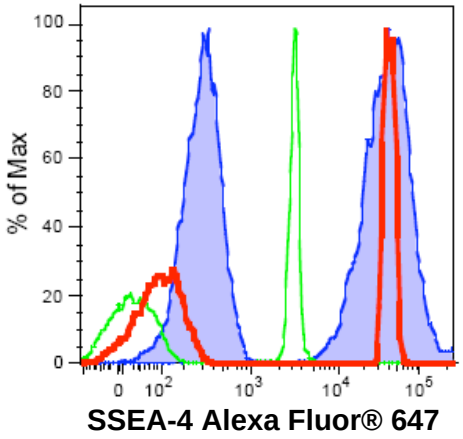
- Always need a positive-stained control
- Wastes cells
- Cumbersome when assaying for markers that might or might not be expressed on cells
- Solution = compensation beads
- Standard BD CompBead particles work on small cells found in blood, but are not ideal for larger cells

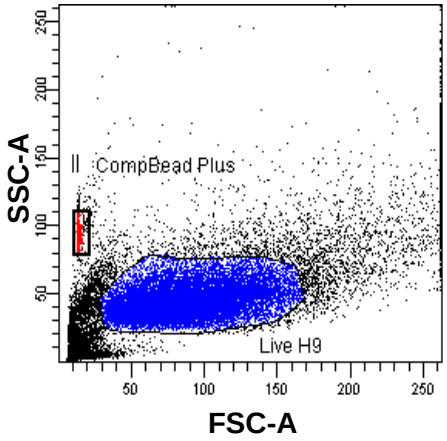
BD CompBead Plus

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



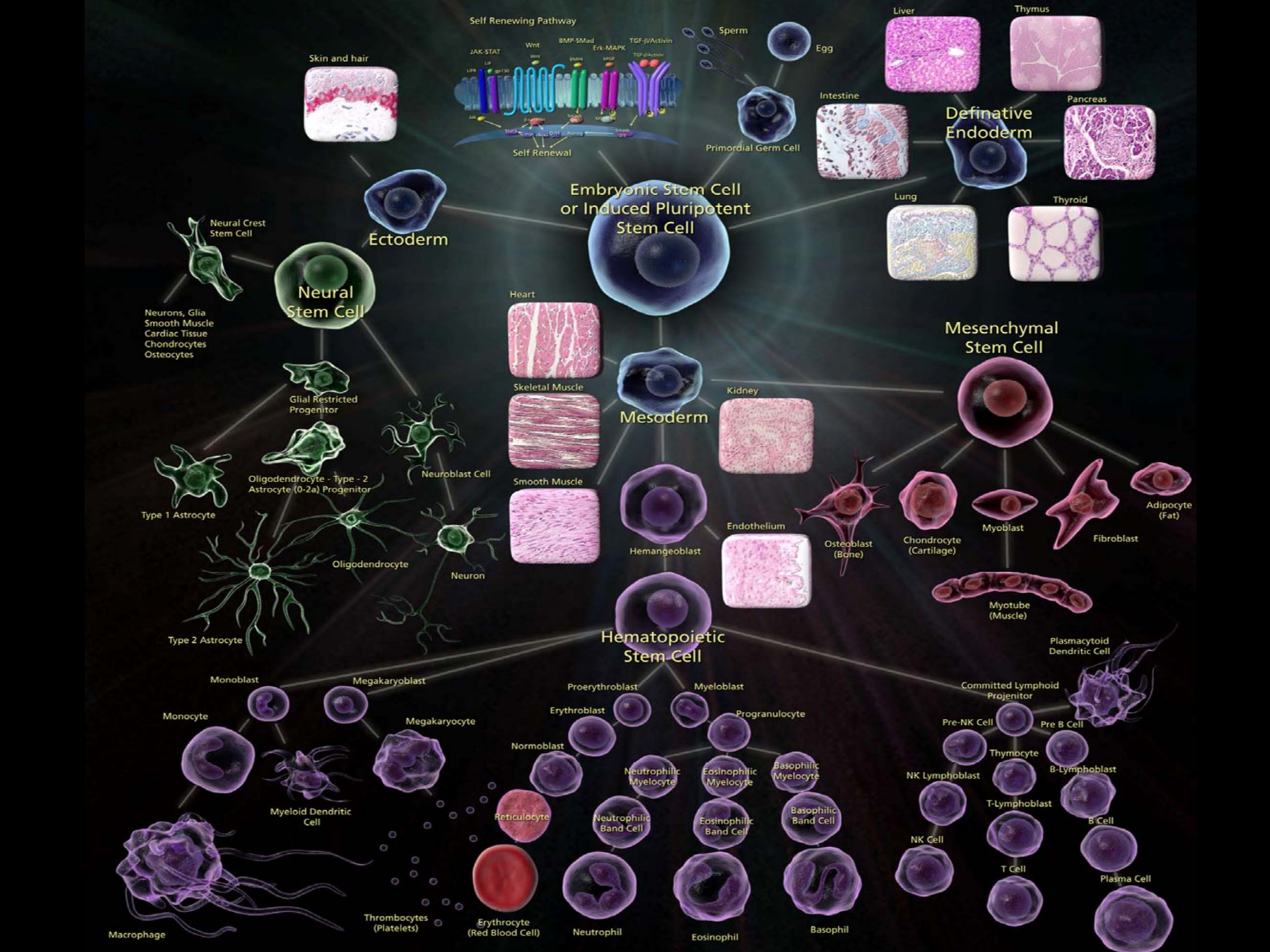






- **Autofluorescence of beads tracks hESCs**
- **Facilitates scatter setup**
- **Compensation for any mouse or rat antibody**
- **Negative Control (BSA) particles included**

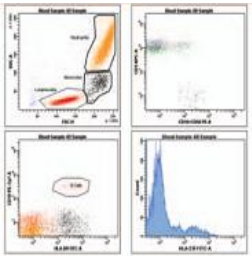
BD CompBead Plus Anti-Mouse Ig Set	Catalog No. 560497
BD CompBead Plus Anti-Rat Ig Set	Catalog No. 560499

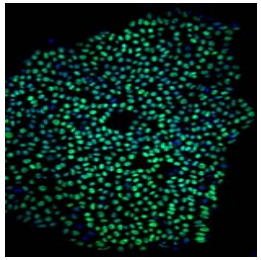

Self Renewing Pathway
JAK-STAT
Wnt
BMP-SMad
Erk-MAPK
TGF-β/Activin
Self Renewal
Skin and hair
Sperm
Egg
Primordial Germ Cell
Liver
Thymus
Intestine
Definative Endoderm
Pancreas
Lung
Thyroid
Embryonic Stem Cell or Induced Pluripotent Stem Cell
Ectoderm
Neural Crest Stem Cell
Neurons, Glia
Smooth Muscle
Cardiac Tissue
Chondrocytes
Osteocytes
Neural Stem Cell
Glial Restricted Progenitor
Oligodendrocyte - Type - 2 Astrocyte (0-2a) Progenitor
Neuroblast Cell
Type 1 Astrocyte
Oligodendrocyte
Neuron
Type 2 Astrocyte
Heart
Skeletal Muscle
Smooth Muscle
Mesoderm
Kidney
Hemangeoblast
Endothelium
Mesenchymal Stem Cell
Osteoblast (Bone)
Chondrocyte (Cartilage)
Myoblast
Fibroblast
Adipocyte (Fat)
Myotube (Muscle)
Hematopoietic Stem Cell
Monoblast
Megakaryoblast
Monocyte
Megakaryocyte
Myeloid Dendritic Cell
Macrophage
Thrombocytes (Platelets)
Proerythroblast
Myeloblast
Erythroblast
Progranulocyte
Normoblast
Neutrophilic Myelocyte
Eosinophilic Myelocyte
Basophilic Myelocyte
Reticulocyte
Neutrophilic Band Cell
Eosinophilic Band Cell
Basophilic Band Cell
Erythrocyte (Red Blood Cell)
Neutrophil
Eosinophil
Basophil
Plasmacytoid Dendritic Cell
Committed Lymphoid Projenitor
Pre-NK Cell
Pre B Cell
Thymocyte
NK Lymphoblast
B-Lymphoblast
T-Lymphoblast
B Cell
NK Cell
T Cell
Plasma Cell

BD Biosciences Technologies to Support Stem Cell Research Applications

- Extracellular Matrices (eg, BD Matrigel)
- Media and media supplements
- Cell cultureware, growth factors, cytokines

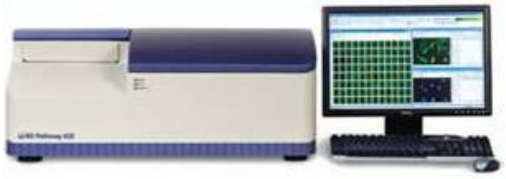
- Validated antibodies and kits
  - Flow cytometry
  - Imaging
  - Western blot





- 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

BD

Acknowledgments

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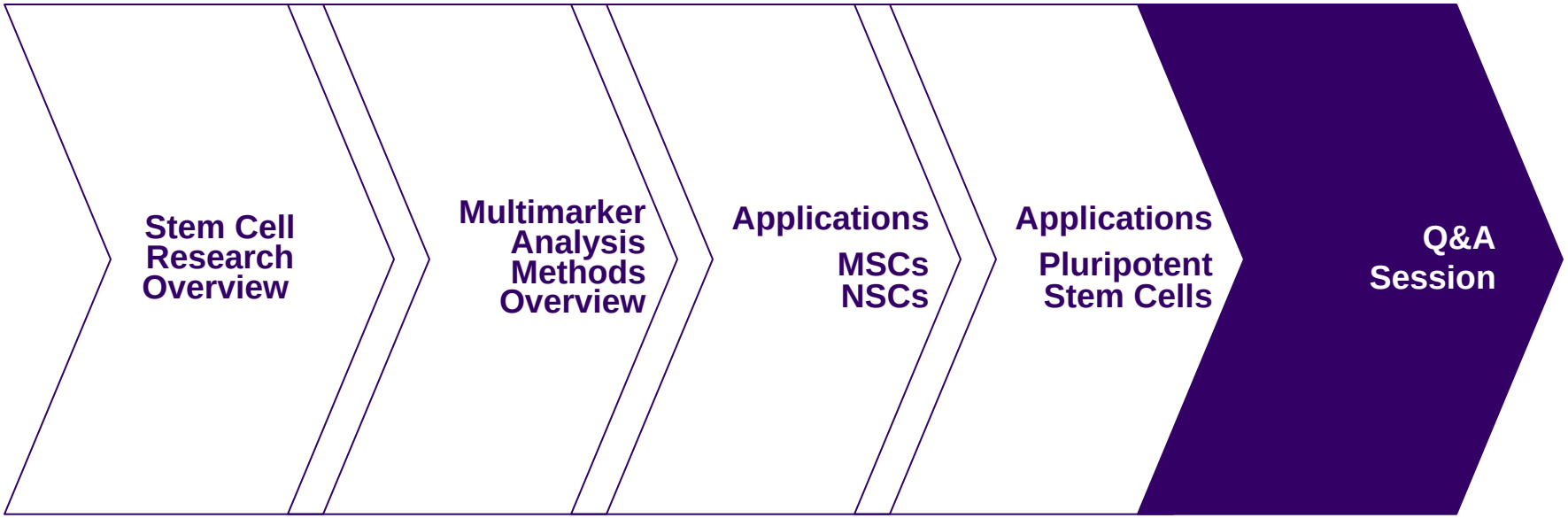
Shauna Yuan

R&D Cytometry Lab
Andrea Nguyen
Dennis Sasaki



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or email Applications Support

- **ResearchApplications@bd.com**

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