Analyzing Stem Cell Populations Using Flow Cytometry

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BD Biosciences

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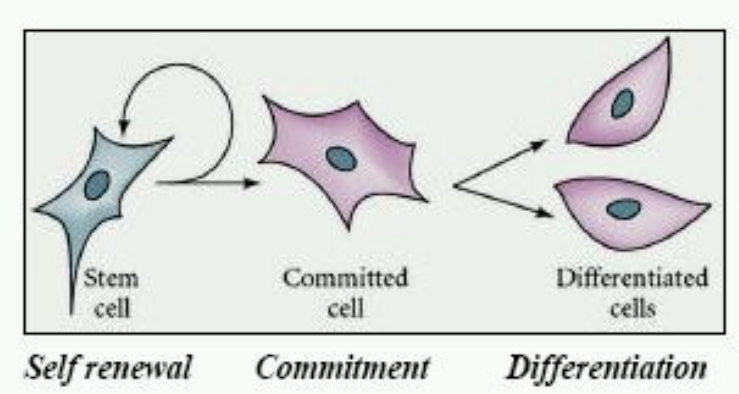
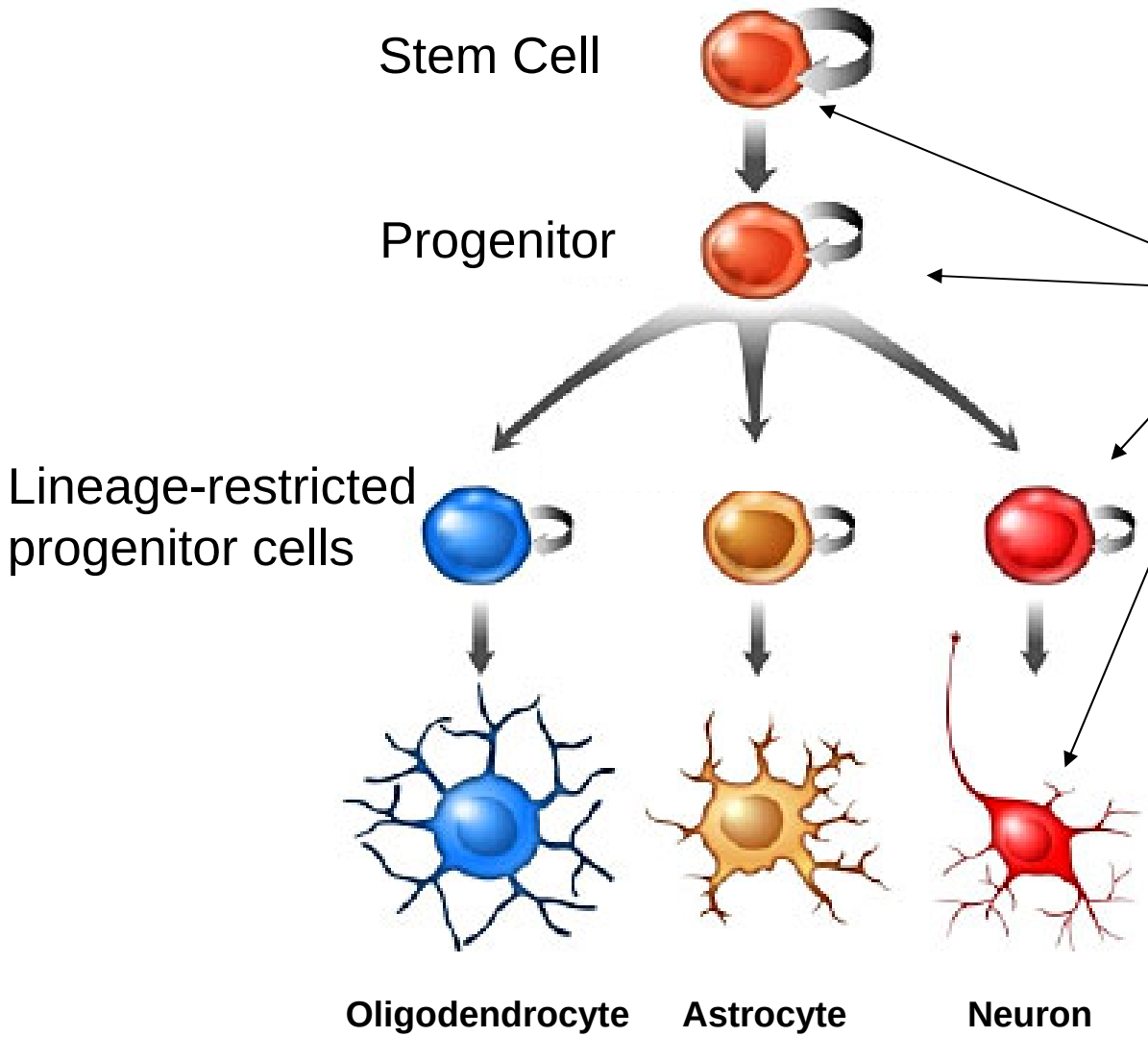
Stem Cell Research



- **What drives cell differentiation in a specific direction?**
  - How are stem cells programmed?
  - What are the environmental factors?
- **What are the characteristic features of a cell in a particular state of differentiation?**
  - Which biomarkers are expressed?
  - What is the function behind expression patterns?
- **What are the required factors for keeping a cell in a particular differentiation state?**

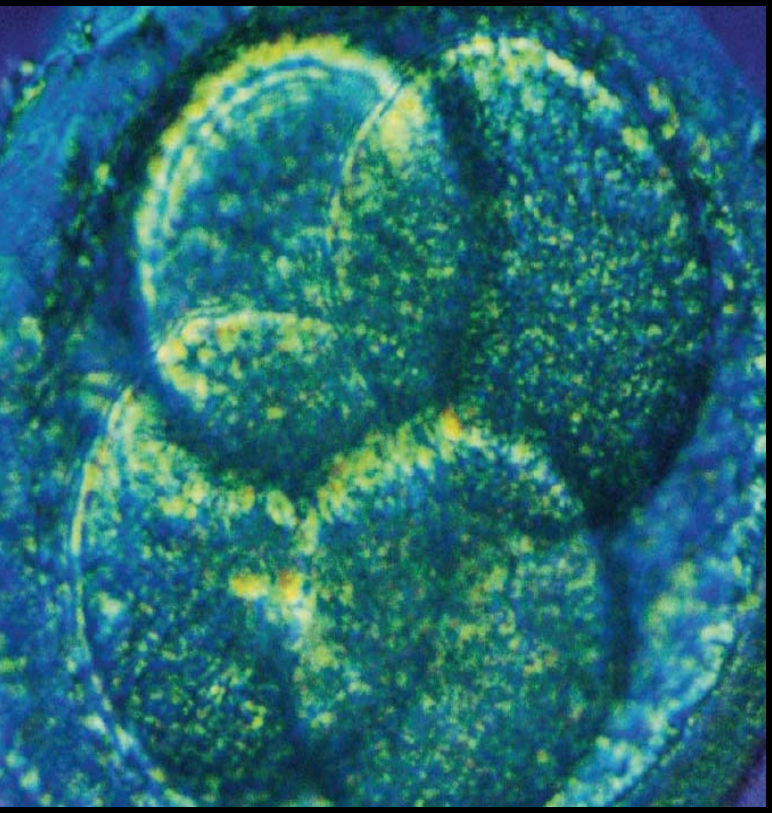


Immediate need for:

Cell surface markers specific to ESCs and differentiated progeny

Assays for fast qualification and quantification of stem cell cultures

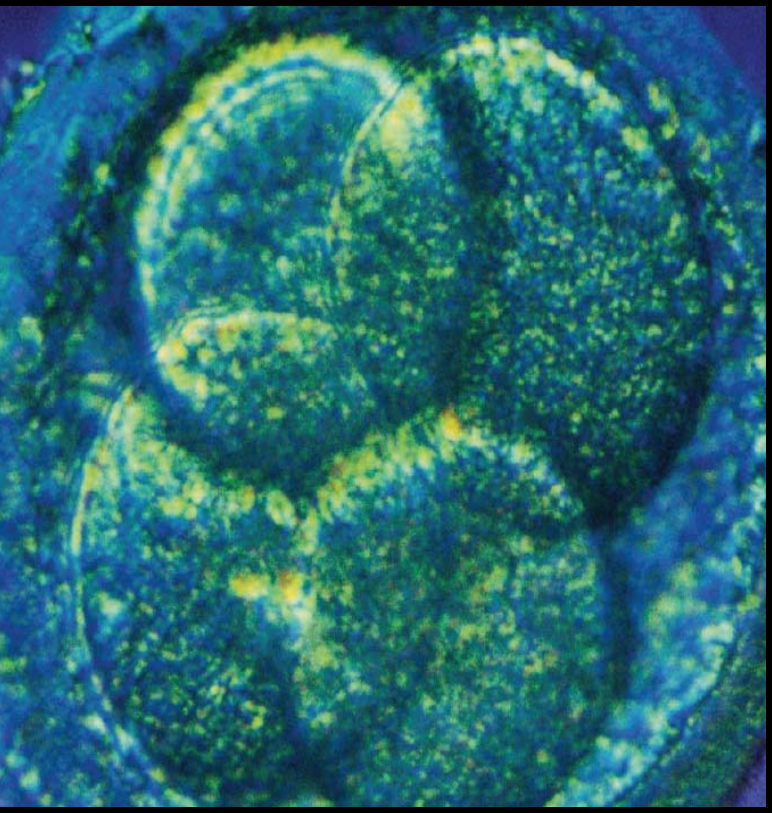
Stem Cell Subpopulations
Determining How Stem Cells Differentiate



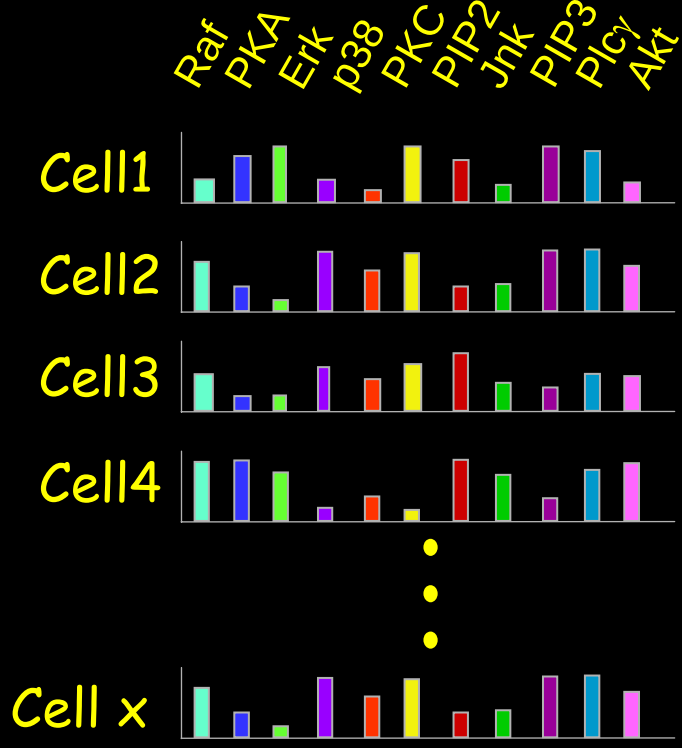
- Cells are computational devices
- Their inner workings (algorithms) cannot be determined by reading their steady-state values
- Cells must be interrogated to determine relationships of signaling components

Stem Cell Subpopulations

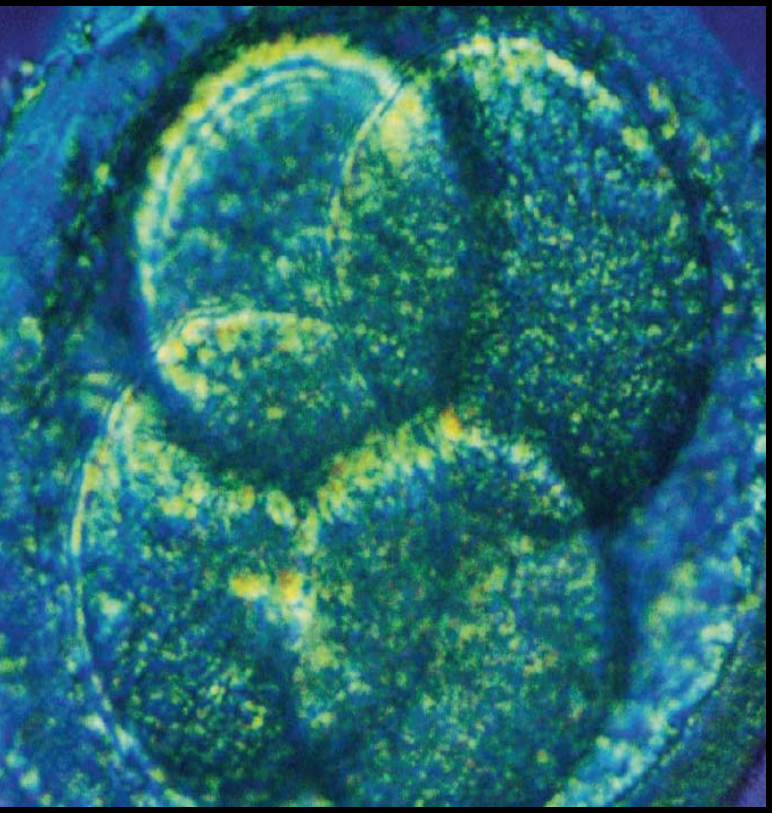
Determining How Stem Cells Differentiate



Flow cytometry



Stem Cell Subpopulations
Determining How Stem Cells Differentiate



Computational analysis

- Quantitative nature of flow data allows powerful statistical regimes to *derive* signaling maps.
- These inner relationships relate to
  - Differentiation state
  - Response to a given growth factor
  - Biosignatures and cell response profiles

Program Overview

- Gating strategies for analyzing stem cell populations
- Pluripotent cell analysis
- Neuronal progenitor cells
- Phosphorylation analysis

Gating Strategies for Stem Cell Populations Using Flow Cytometry

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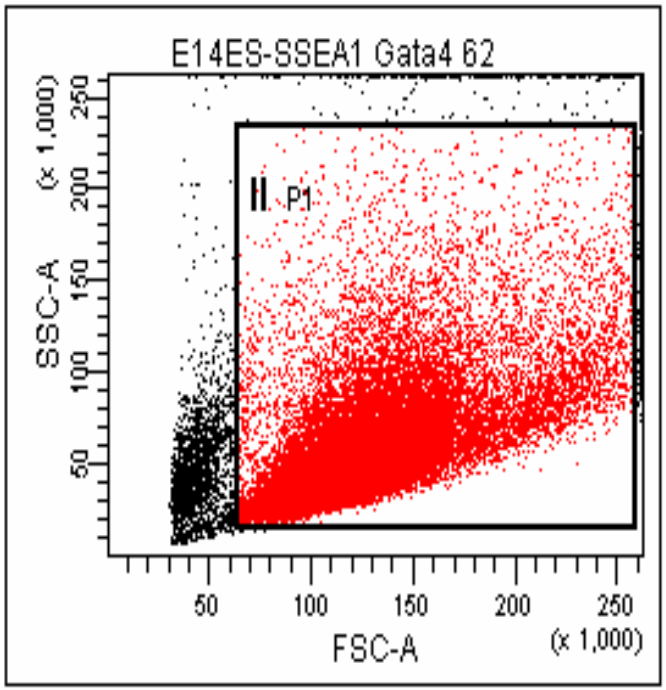
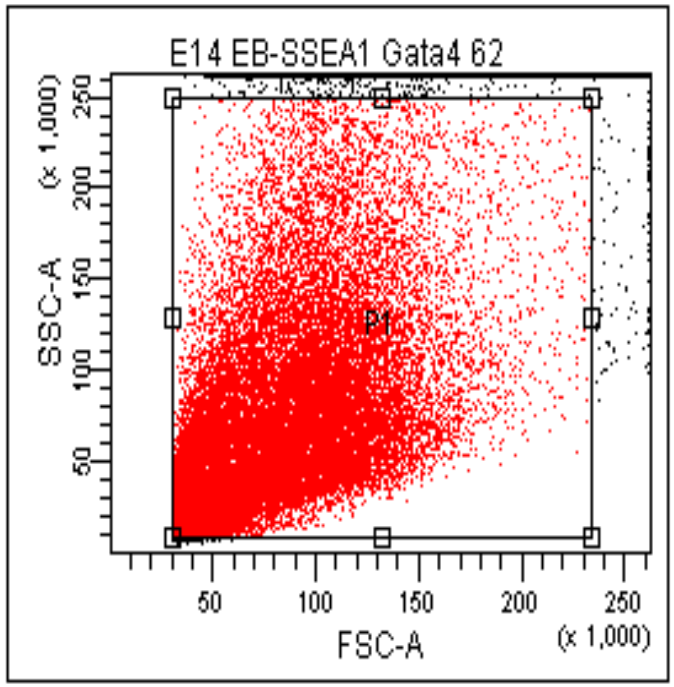
Gating Strategies for Analyzing Stem Cell Flow Data

- mES and mEB E14 cell populations were stained and analyzed with:
  - FITC-conjugated SSEA-1
  - PE GATA4
- Populations were gated in different areas of the scatter plot to determine if size and granularity could be used to provide additional information
- Gating strategies can be created for sorting of homogeneous subpopulations

Gating Strategies for Analyzing Stem Cell Flow Data



ES



EB

Can we find sub-populations of cells within each cell profile?

(Forward and side scatter patterns are different)

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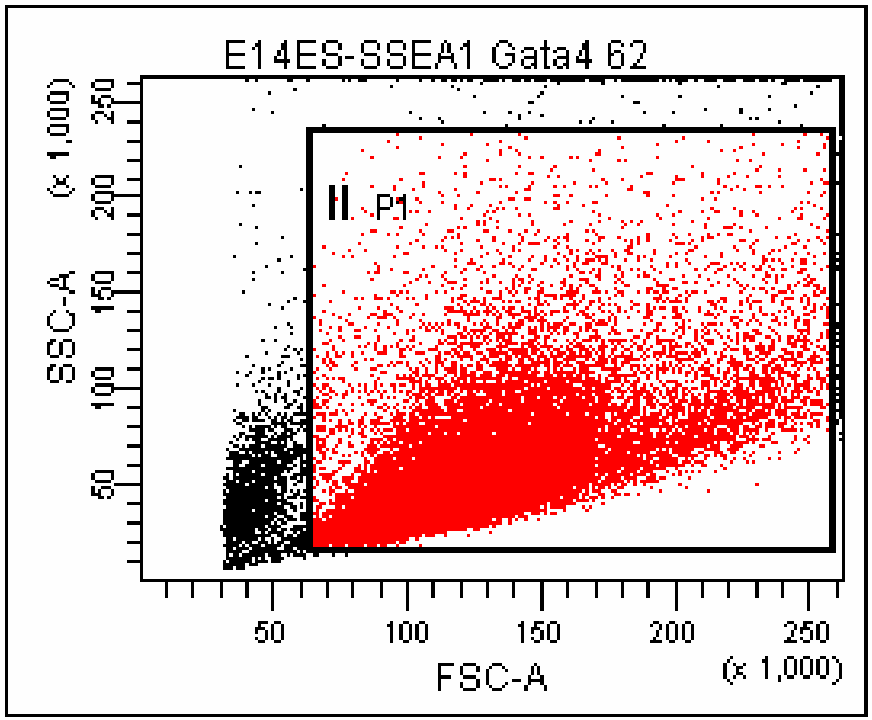
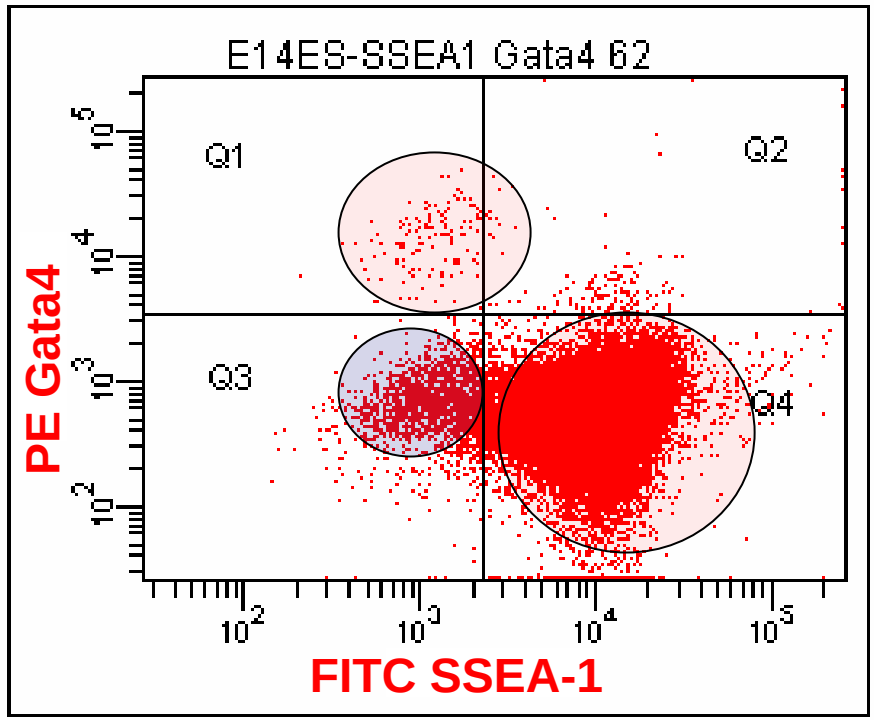
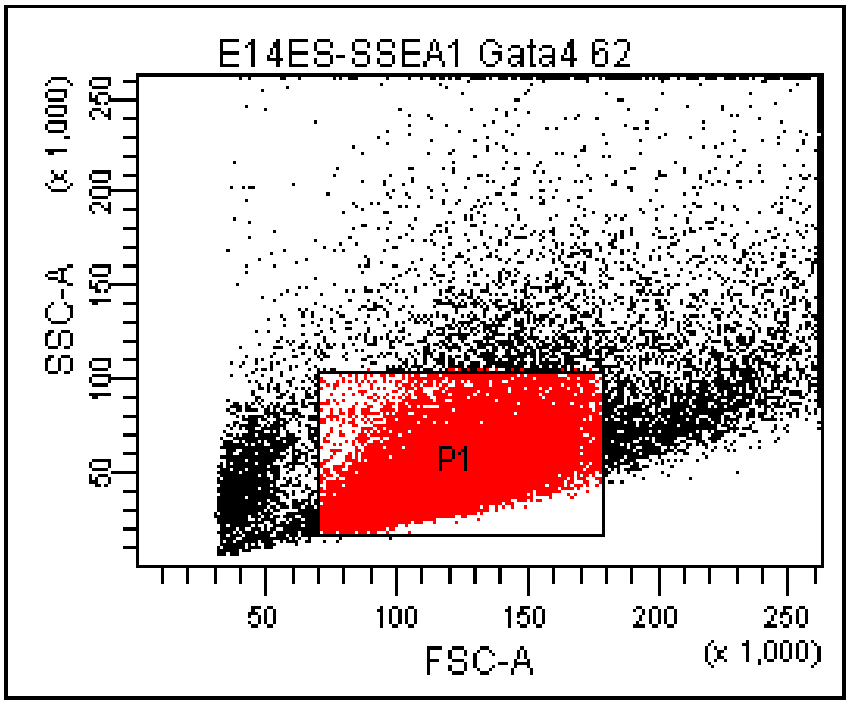
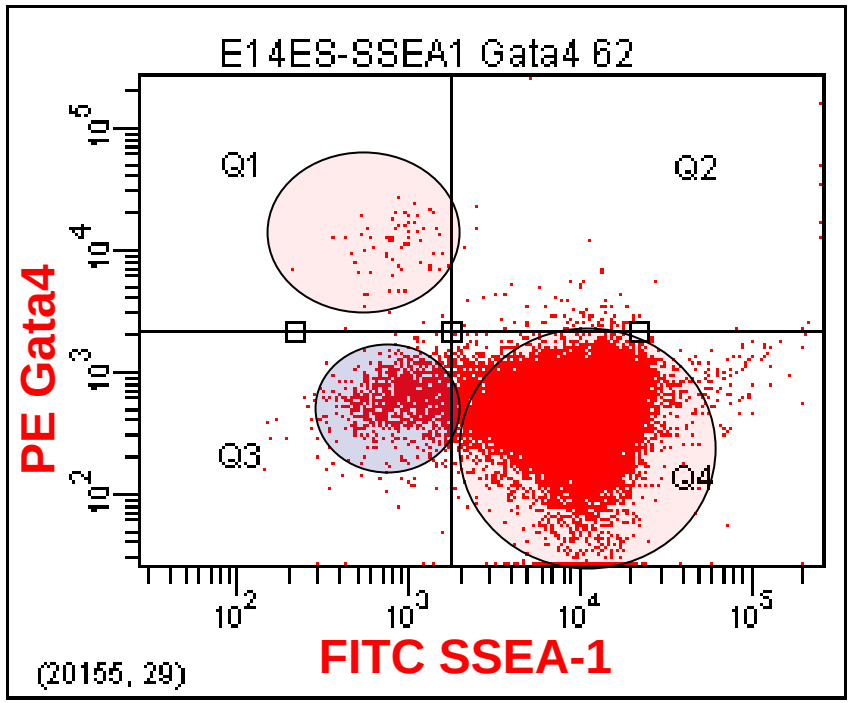
Analysis of SSEA-1 and GATA4 on mES E14





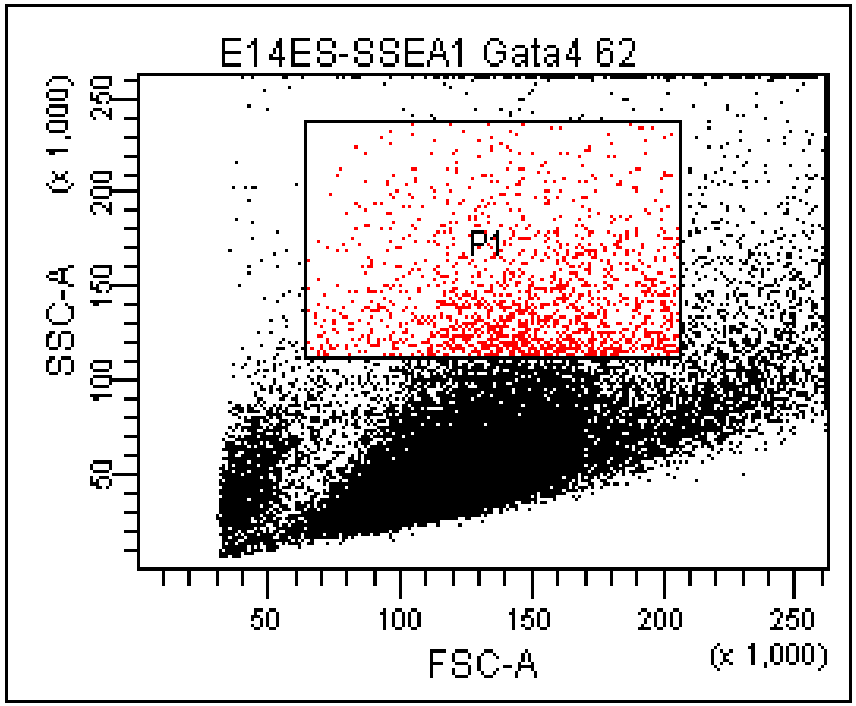
Analysis of SSEA-1 and GATA4 on mES E14

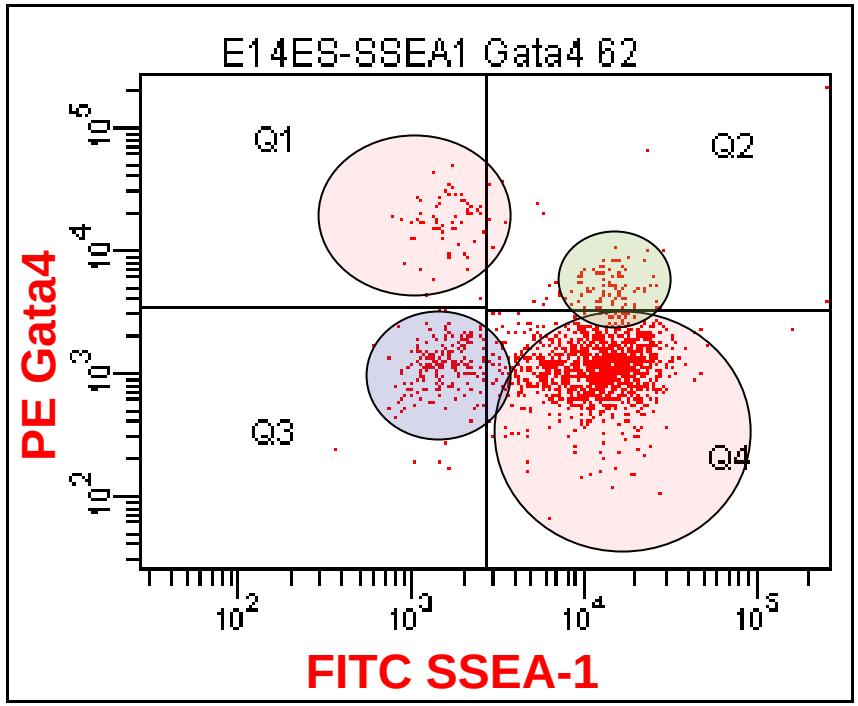




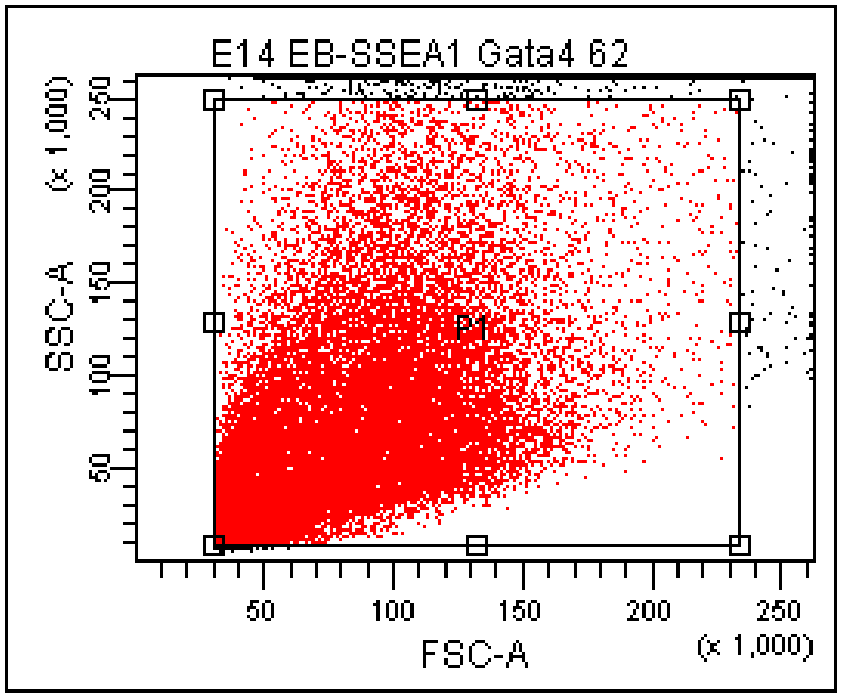
Analysis of SSEA-1 and GATA4 on mES E14

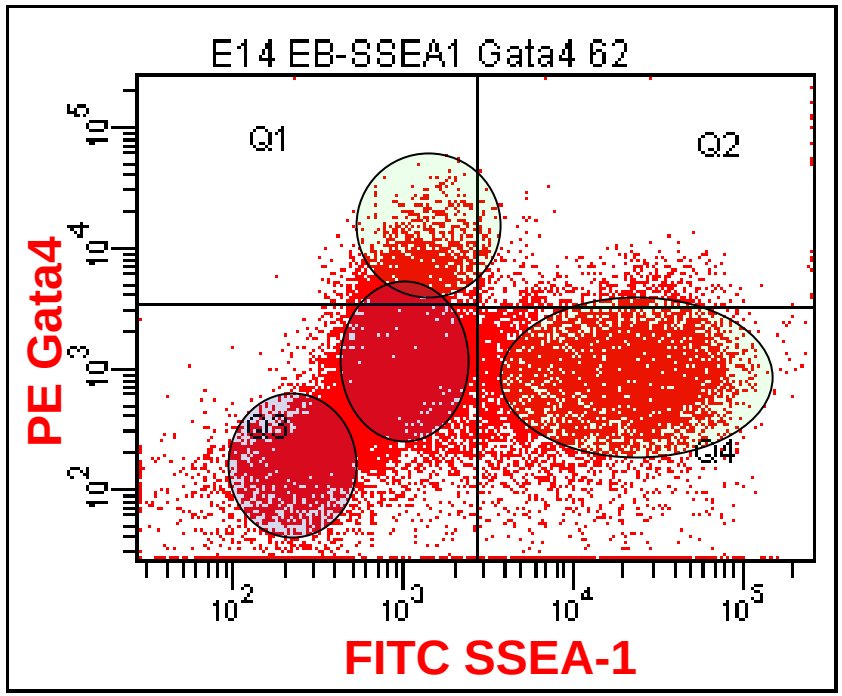
Gating subpopulations reveals SSEA-1-negative and GATA4-positive cells in naïve mES culture





Analysis of SSEA-1 and GATA4 on mES E14-derived Embryoid Bodies





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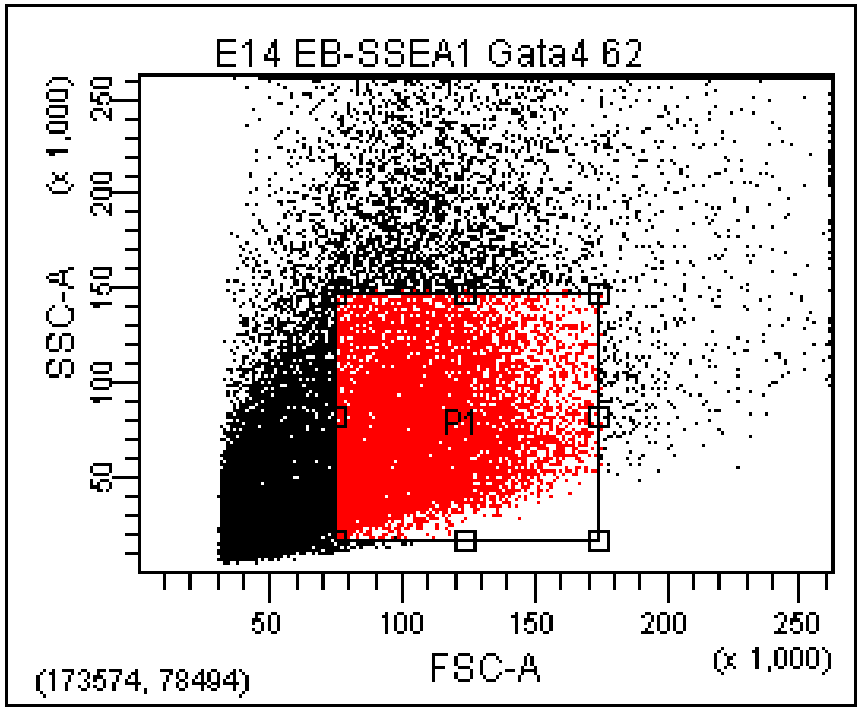
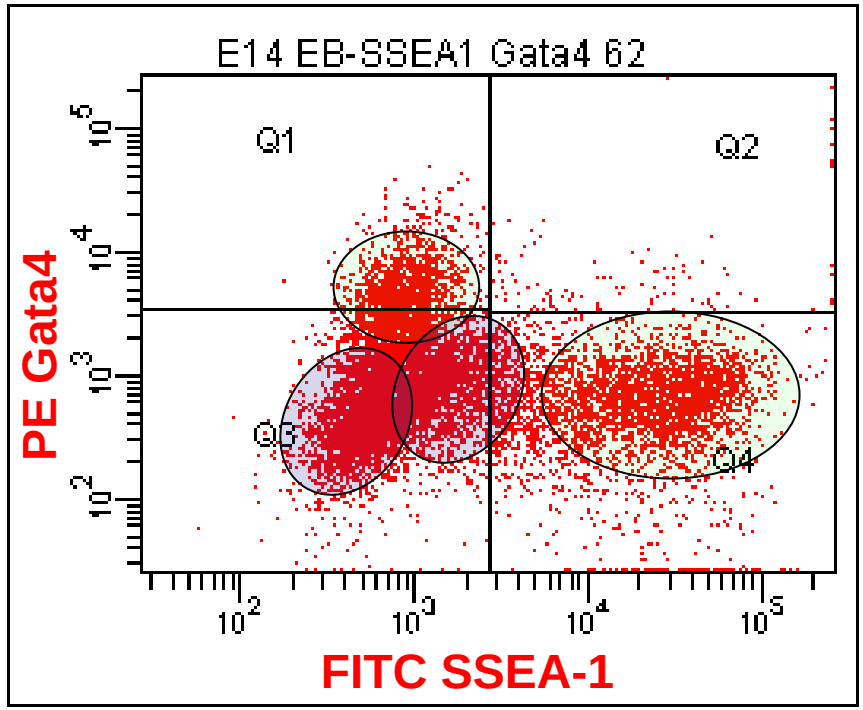
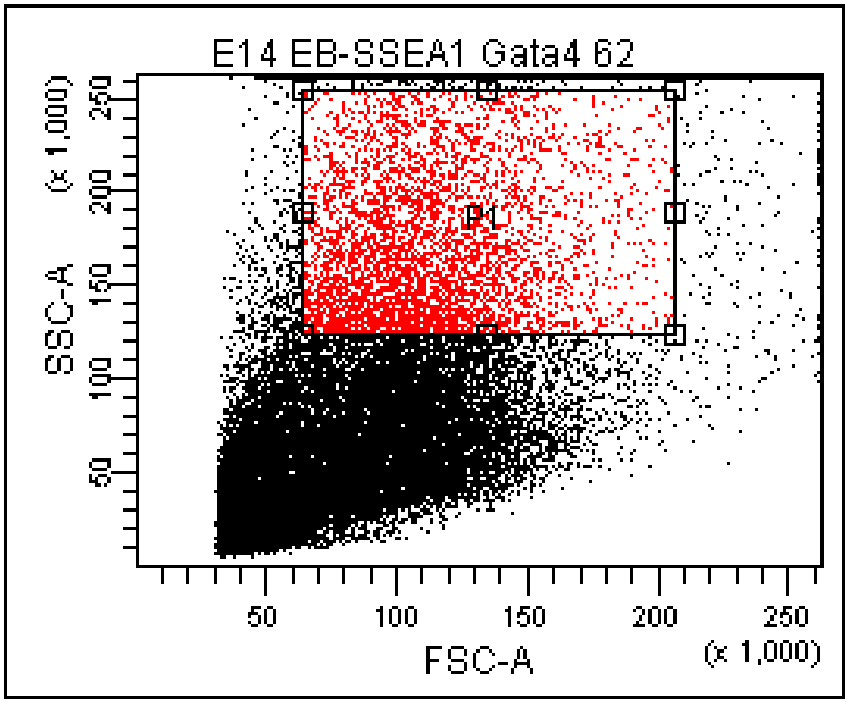
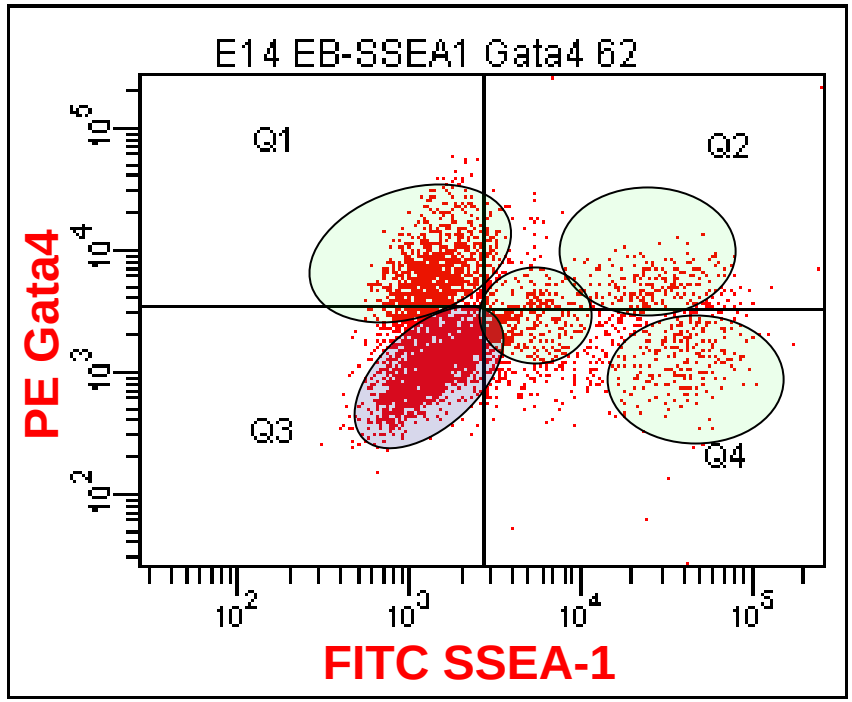




Analysis of SSEA-1 and GATA4 on mES E14-derived Embryoid Bodies

Gating reveals subpopulations of GATA4-positive cells in embryoid bodies





Pluripotent Cell Analysis

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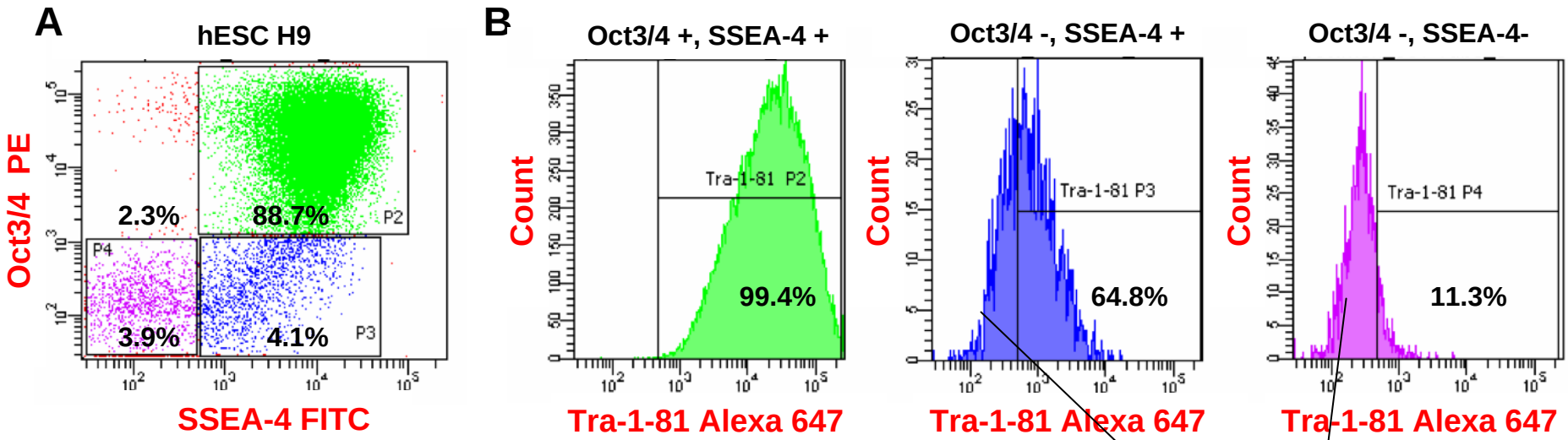
Markers of Pluripotency

Cell surface markers
SSEA-3
SSEA-4
Tra-1-60
Tra-1-81
CD9
CD81
SSEA-1/CD15 (neg)
CD30 (neg/low)

Intracellular markers
Sox2
Oct3/4
Nanog

Three-color flow-cytometric analysis of human embryonic stem cells using fluorochrome-conjugated antibodies to pluripotent stem cell markers

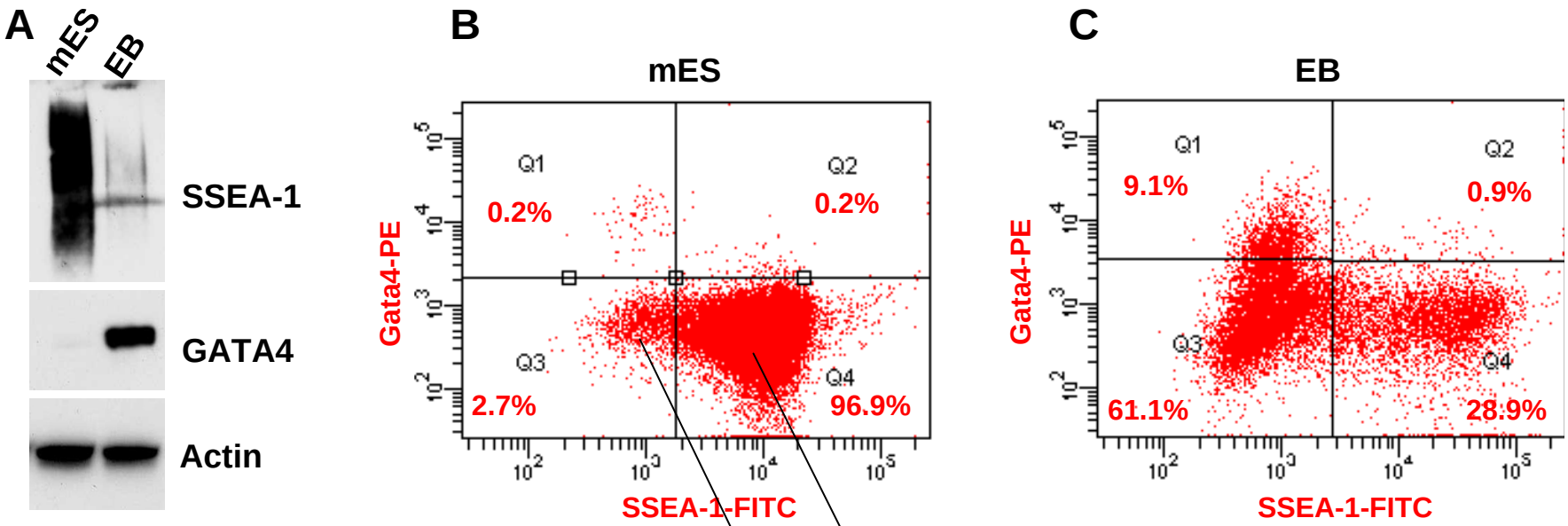


-Human ESC H9 grown on mouse embryonic fibroblast feeder layer

-Cells dissociated into a single cell suspension

Differentiated Cells

Analysis of GATA4 expression during differentiation of mouse embryonic stem cells (mESs) into embryoid bodies (EBs)



Majority of cells are undifferentiated

SSEA-1: mES pluripotent marker

GATA4: marker of cardiac mesoderm

Differentiated cells

Neuronal Progenitor Cells

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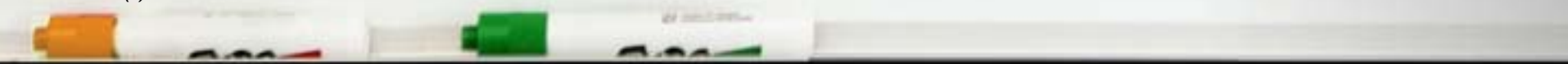

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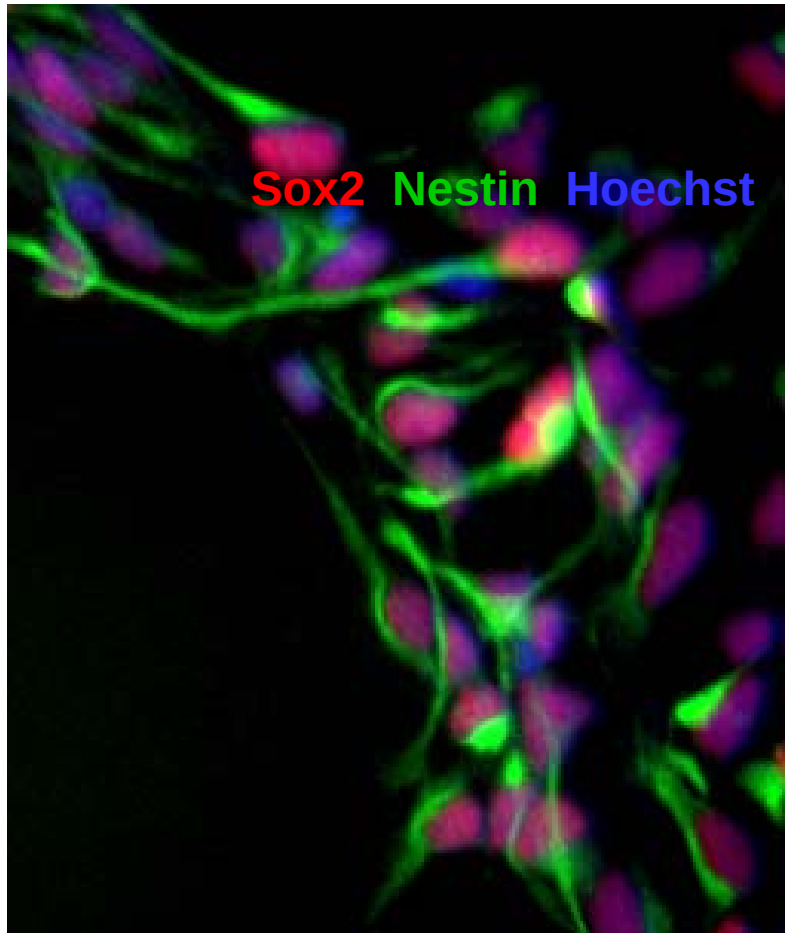
Analysis of neural differentiation of human embryonic stem cells

- Neural stem cells (NSCs) can be differentiated from hESCs.
- NSCs can self-renew for many passages and can be induced to differentiate into a variety of post-mitotic neural cell types.
- Assessing purity of NSCs is crucial to control for batch-to-batch variability.
- Identifying cell surface molecular signatures of NSCs and neurons will allow for rapid purification of specific neural subtypes from heterogeneous populations.

hESC (H9)–derived neural stem cells



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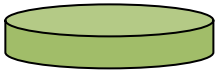
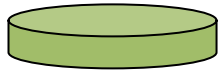


hESC H9

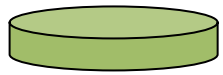
Remove cells from plate and differentiate into EBs

Media w/o FGF, 7 days

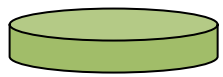


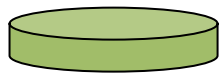
EBs

Plate EBs on gelatin coated plate, 2 days



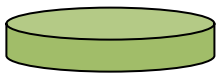
Change media into a neural stem cell growth promoting media (N2, transferin and selenium, w/o FGF)





Isolate the neuro ectotderm cells with a pipet and transfer

Dissociate cells with acutase

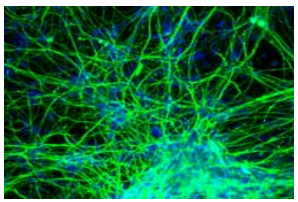


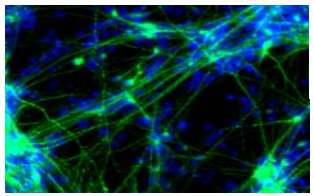
Neuronal Stem Cells

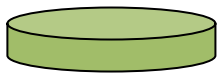
Transfer cells to media with N2, B27, and FGF. Plate coated with poly-l-ornithine

2 weeks

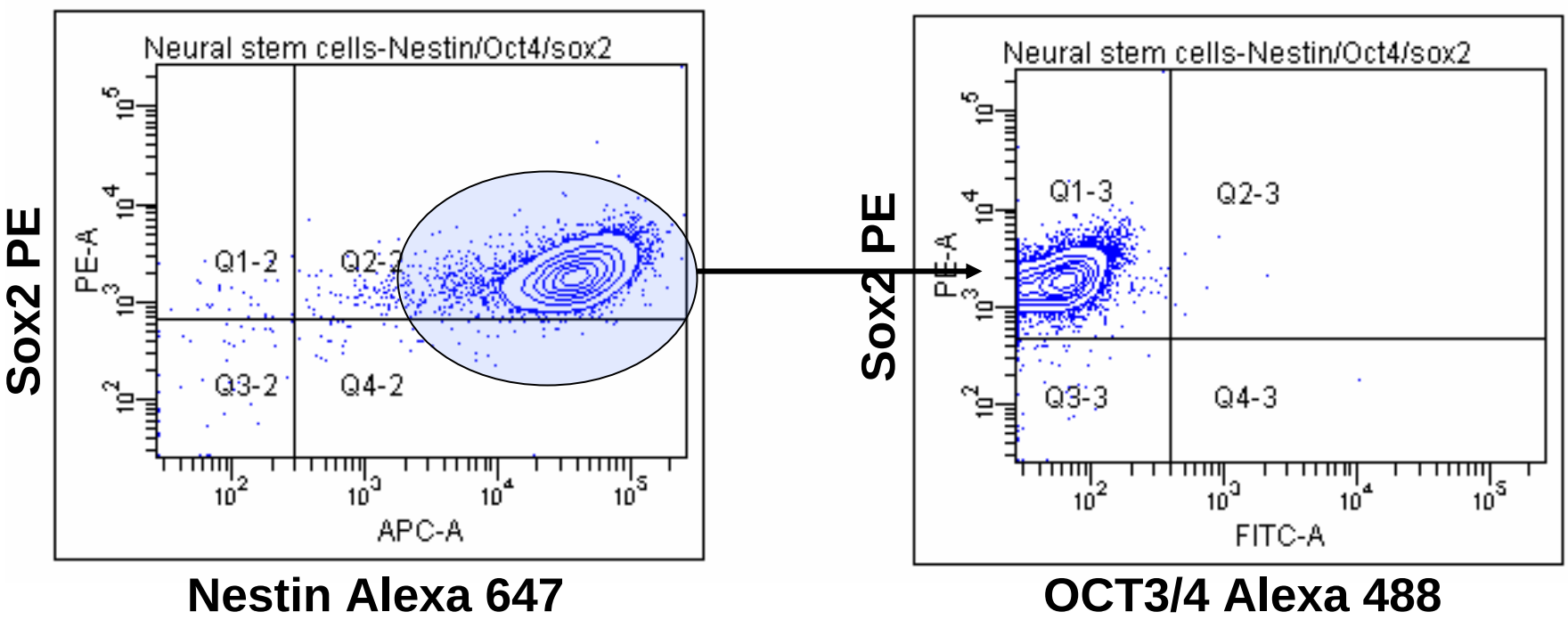
Transfer to plate coated with laminin/polyorn., w/o FGF and add different factors





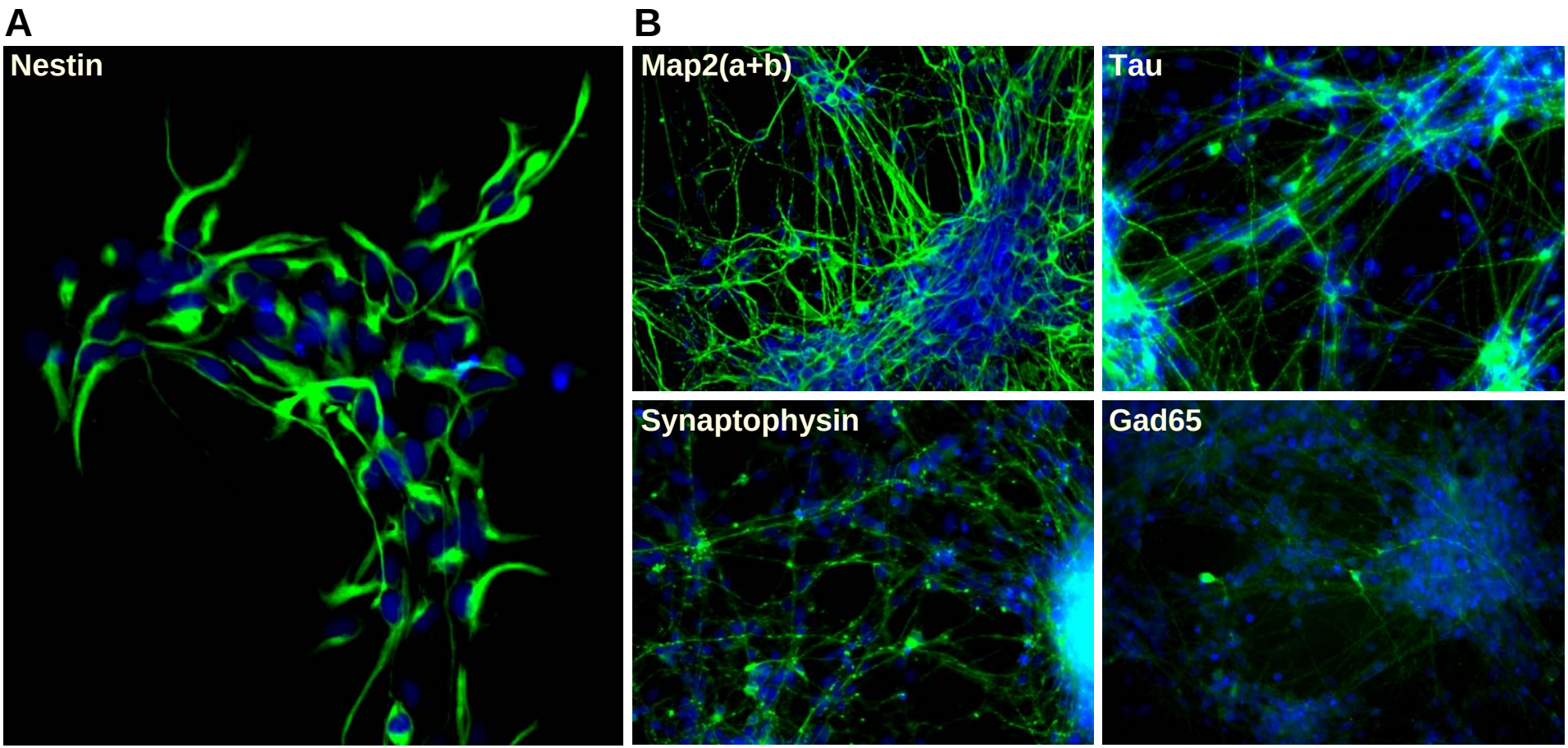


Flow cytometric quantification of hESC-derived neural stem cells



We are able to generate a near-pure population of neural stem cells expressing Sox2 and Nestin

Human ESC-derived neural stem cells can also be differentiated into neurons



A. hESCs (H9) were grown on hESC-qualified BD Matrigel™ and mTeSR1 media (Stem Cell Technologies). hESCs were differentiated into neural stem cells similarly as described by Yeo, et al. (2007)

B. Differentiation of H9-derived neural progenitors into neurons (2 weeks)

Finding New Stem Cell Markers using Flow Cytometry

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CD marker analysis of hESC (H9) and neural stem cells by flow cytometry

- Pilot screen of 21 CD markers was performed (partial analysis of >250 cell surface markers)
- Goal is to define cell surface molecular signatures of both cell types and potentially identify subpopulations
- Next step is to include differentiated neurons

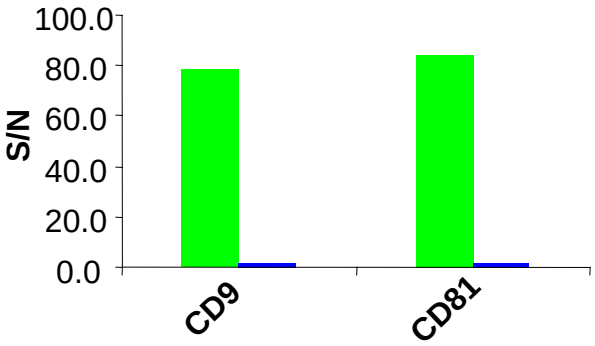
CD marker analysis of hESC (H9) and neural stem cells by flow cytometry

Signal to Noise over Isotype control

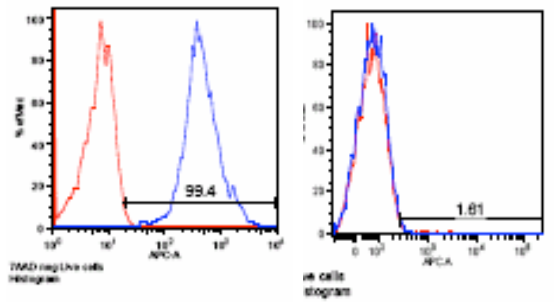
Representative data

Significance

High in hESC, negative in NSC



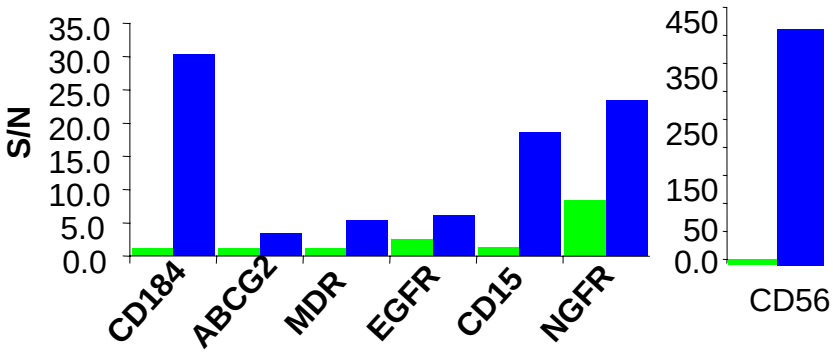
H9 **NSC**

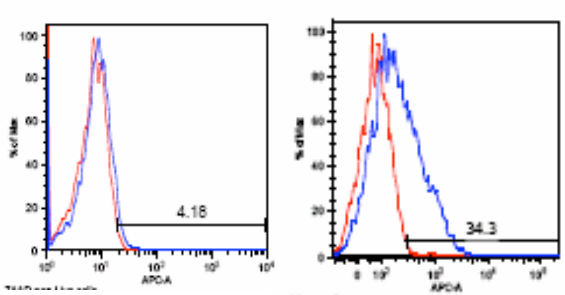


CD81

Potential markers of pluripotency

High in NSC, negative to low in hESC





ABCG2

Potential markers of neuroectoderm, NSC or of adult SC but not of pluripotent stem cells

Phosphorylation Studies

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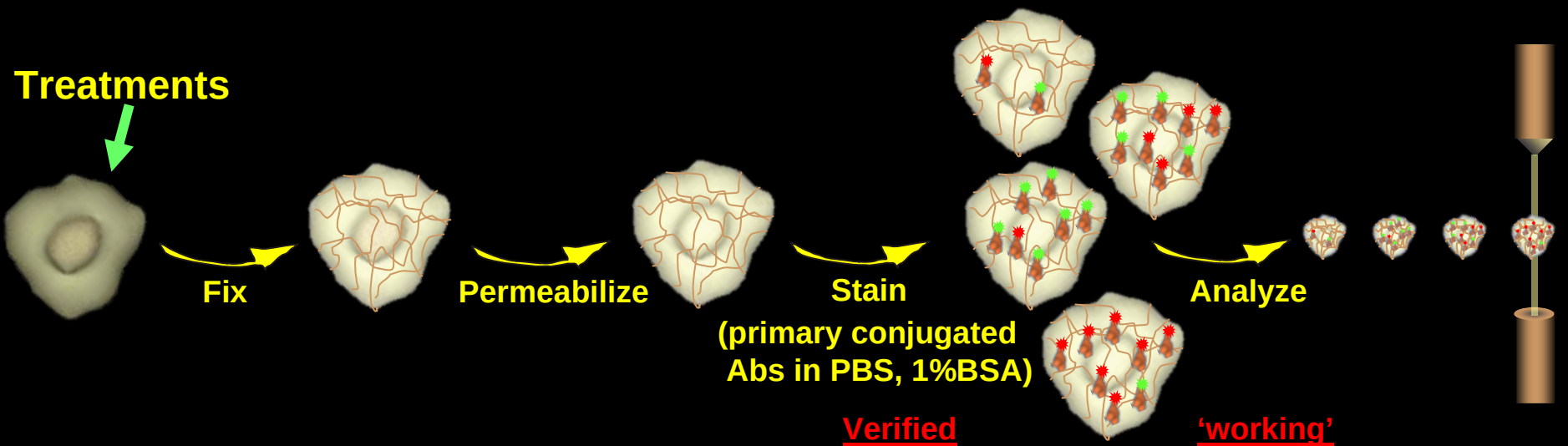
Cell Surface and Intracellular Staining for Flow Cytometry



Thorough development of fixation protocols for cell lines and whole blood (immediately out of fresh samples).

Verified

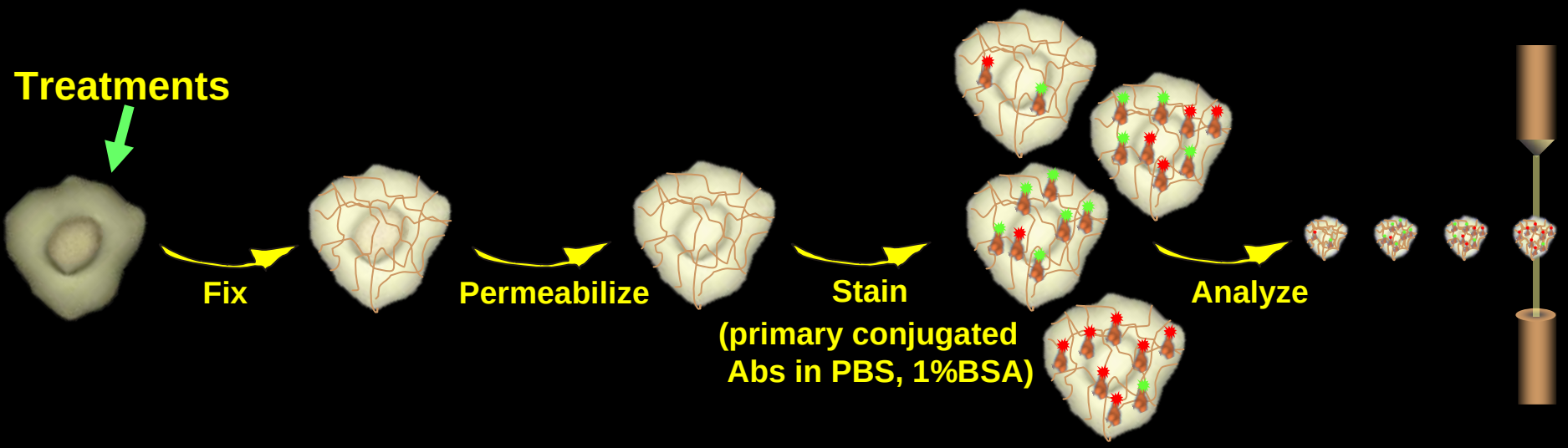
p38 MAPK
JNK, cJun
AKT, PIP2, PIP3,
PKCα/β/θ/δ, Rsk
Raf, Mek, ERK, ELK
Rsk, Creb,
STATs, SRC
CREB, cJUN, IKKα
p53 s15, s20 s37, s392
Pyk2, Shc, Fak, Src
Slp76, Zap70, Syk, Lat, Vav,
Lck, PLCγ
Beta-integrins

>80 specificities

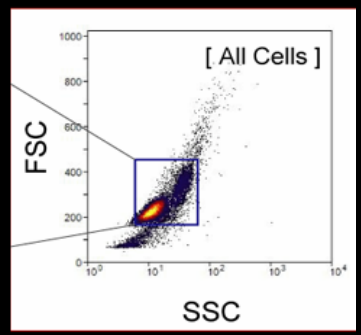
'working'

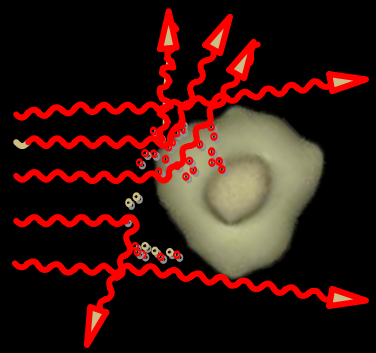
EGFR
PDGFR
cKit
VEGFR
PKA
RB
NFAT
NF-κB p65
Caveolin
Paxillin
FLT3
MEKS

Cell Surface and Intracellular Staining for Flow Cytometry



1. State-specific antibodies: phospho-specific antibodies and others
2. Adopting entirely new fluorophores
3. Generation of efficient conjugation, purification, and testing protocols

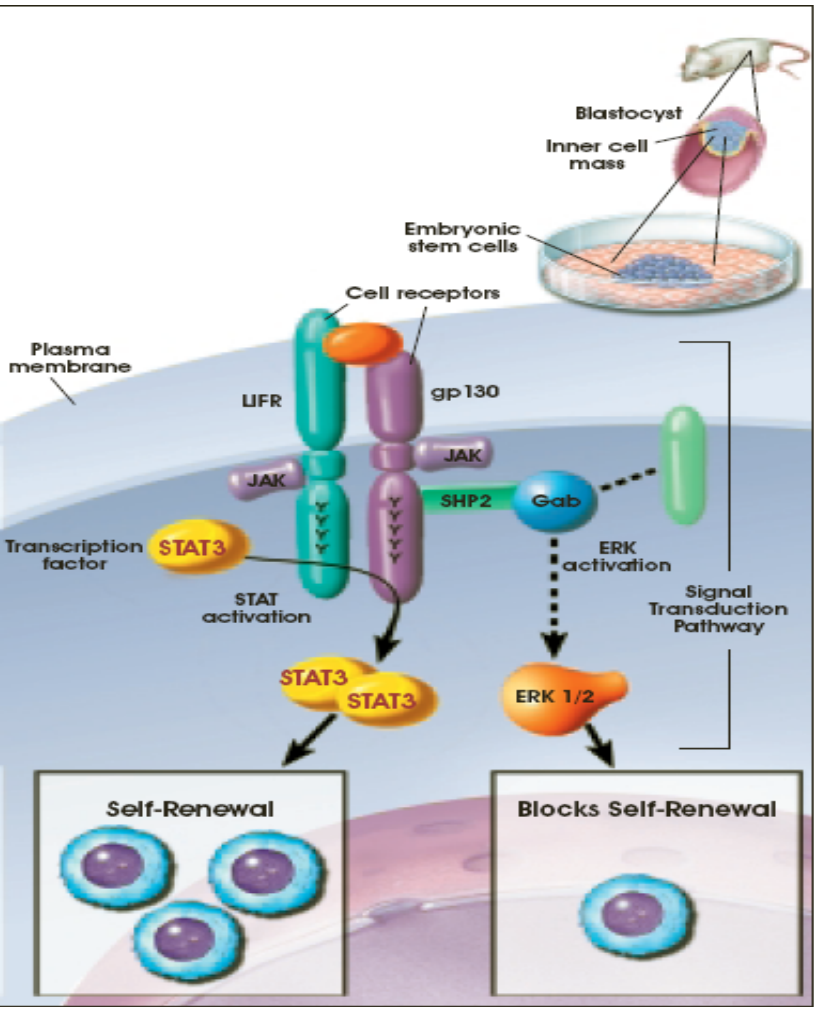




Analysis Steps:

1. Identify live cells
2. Identify cell type
3. Evaluate cell signal

Activation of STAT3 is Essential for Self-Renewal of Mouse Embryonic Stem Cells



- Mouse ES cells can be maintained in a proliferative undifferentiated state in vitro with the addition of LIF into the culture medium

- LIF binds to a two-part receptor complex composed of the LIF receptor and the gp130 receptor

- The binding of LIF triggers the activation of STAT3

- NiwaH. et al. *Gene & Dev.* 12, 2048, 1998
- Raz R. et al. *PNAS,* 96, 2846, 1999
- Matsuda T. et al. *EMBO J.* 18, 4261, 1999

http://stemcells.nih.gov/staticresources/info/scireport/PDFs/appendixb.pdf

LIF-Dependent STAT3 Signaling Can Be Measured Using BD™ Phosflow Technology

Mouse ES + LIF

↓

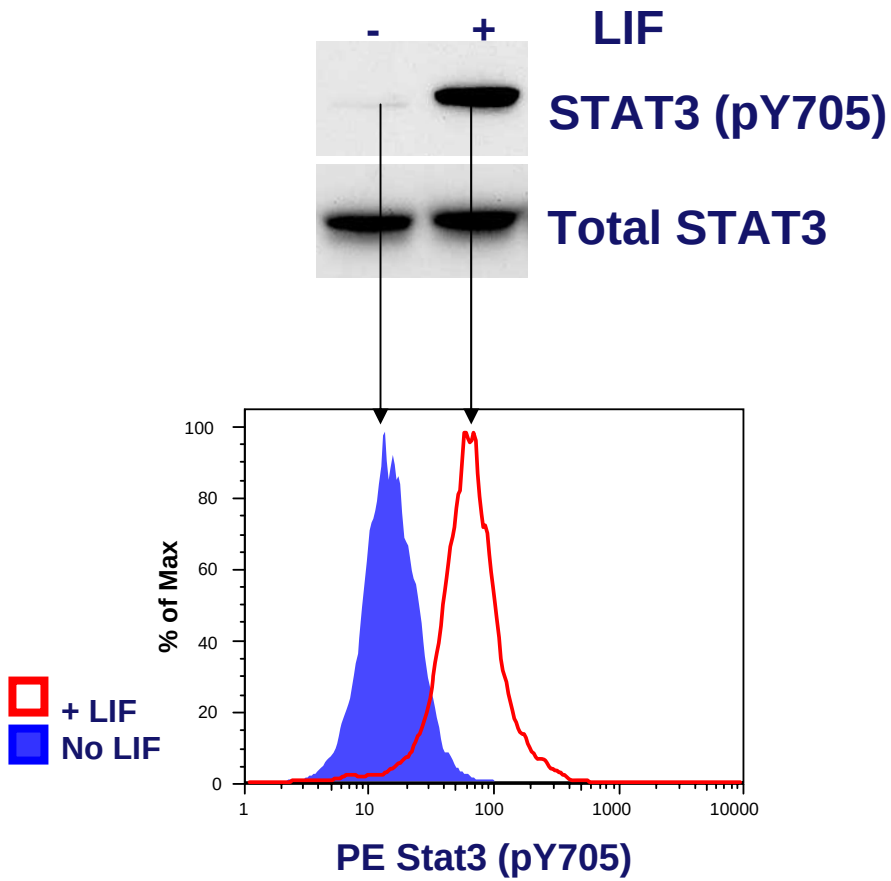
Starve 12h - LIF

↓

-/+ LIF 30 min

↙ ↘

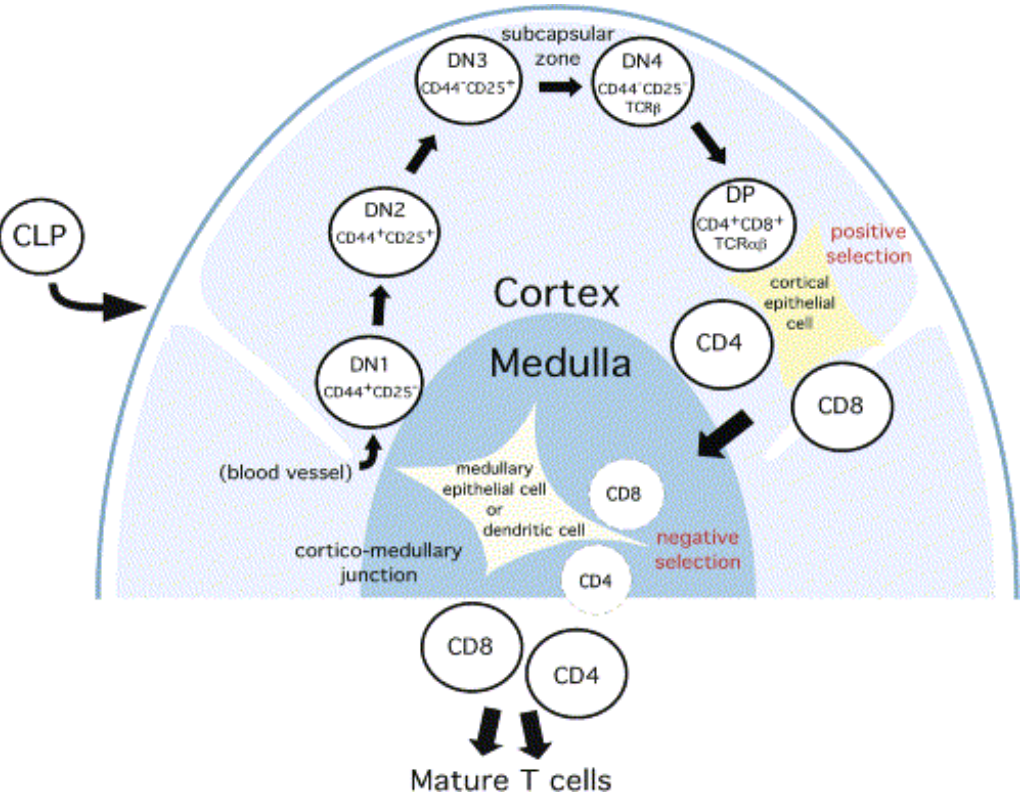
Western Blot | Phosflow



Intrathymic T-cell Development

BD

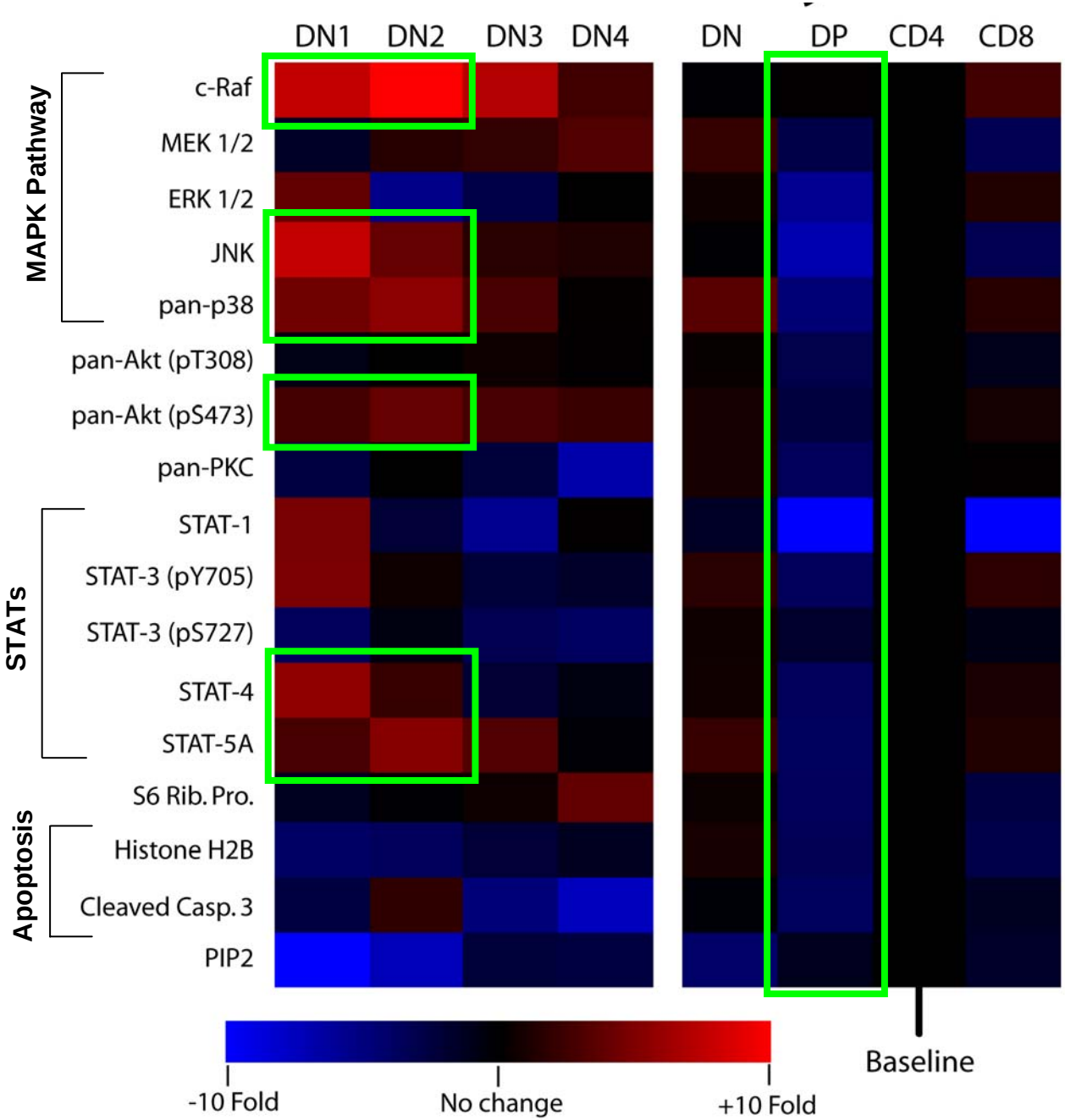
Intrathymic T-Cell Development



- **Commitment to T lineage: Earliest stage is ETP (Sca-1^{+} Lin^{-} Thy1.1^{-}c-kit ^{hi} CD62L^{+})**
- **Migration of maturing T cells in the thymic cortex: DN1-4, DP, positive selection of DP cells**
- **Medulla: Negative selection of SP cells**
- **Migration of mature SP cells from the thymus**

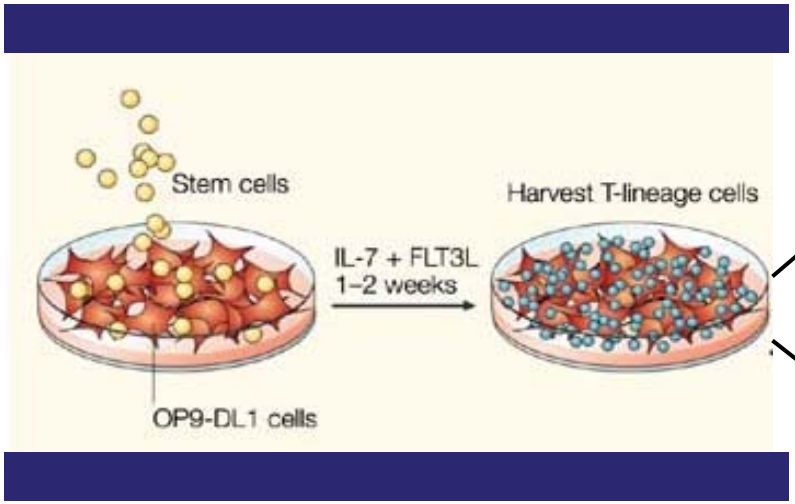
Source: Marcel van den Brink

Normal B6 Thymus



T-Cell Development on OP9-DL1 Cells

- OP9-DL1: Murine BM stromal cell line that has been retrovirally transduced to express the Notch 1 receptor ligand Delta-like 1 (DL1)
- Culture of HSC on OP9-DL1 cells in the presence of IL-7 and FLT3L induces T-cell differentiation



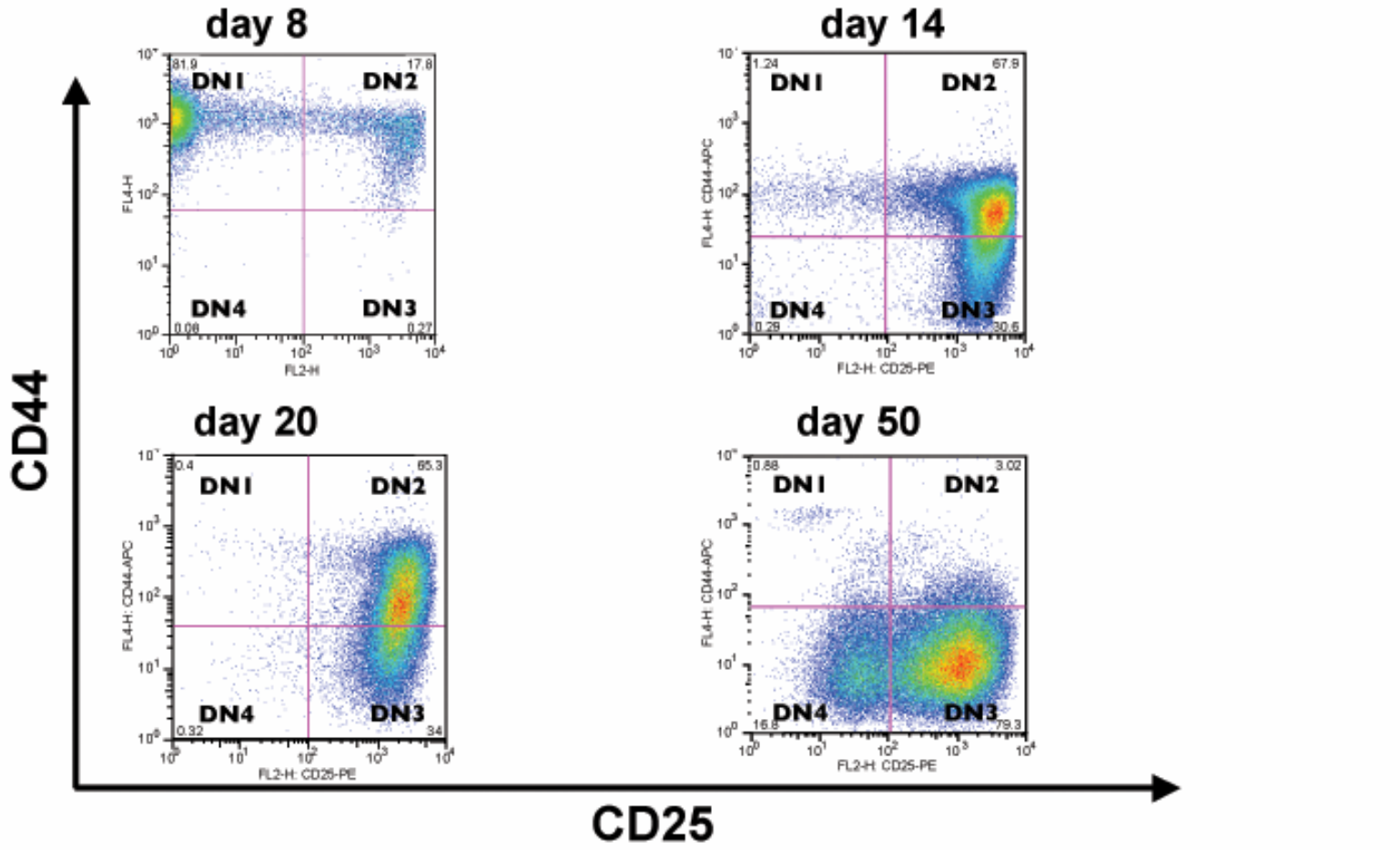
In vitro analysis

Adoptive transfer into allogeneic HSCT recipients

Marcel van der Brink, Sydney Lu

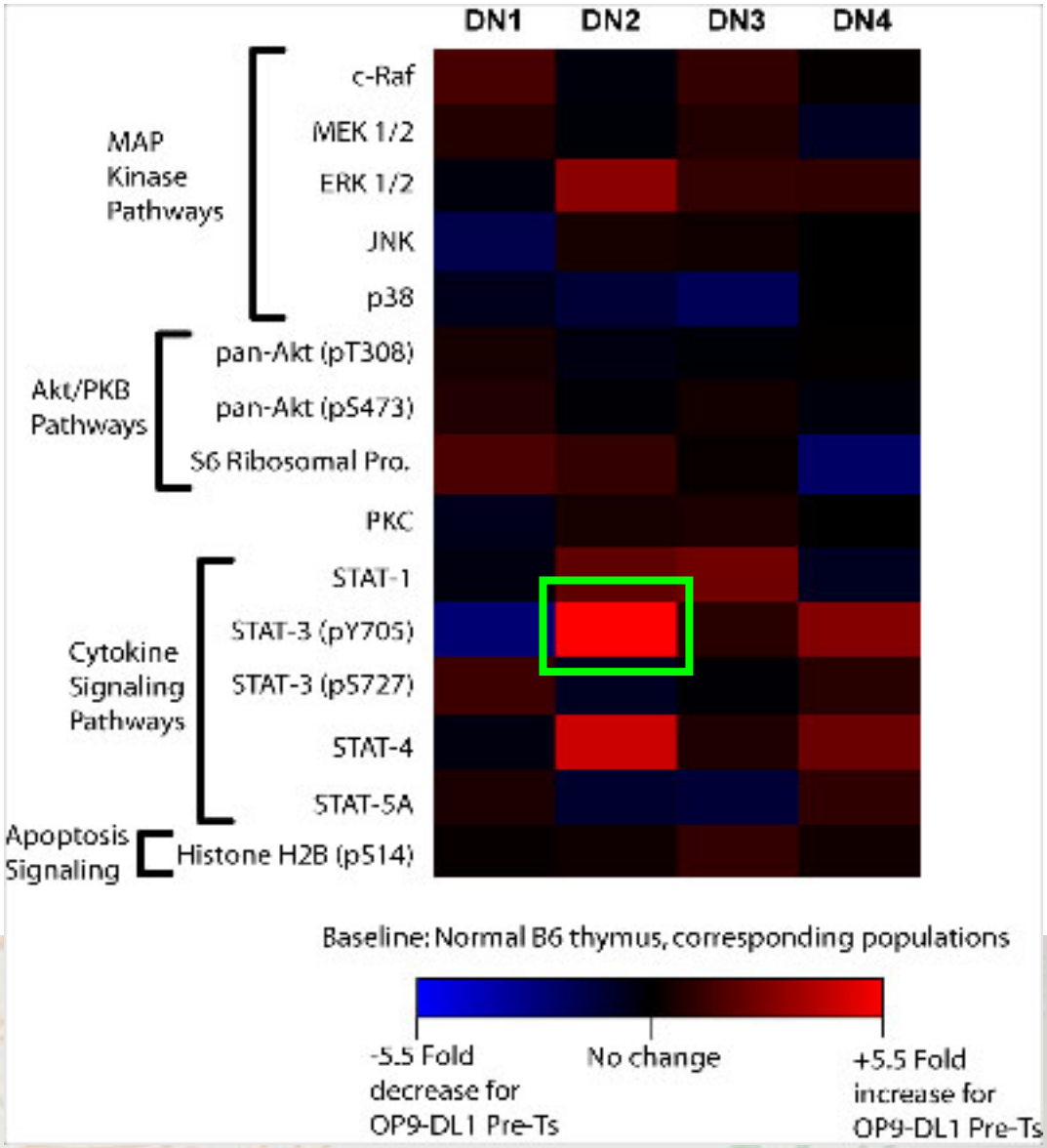
Zúñiga-Pflücker JC.
Nat Rev Immunol 2004;4:67-72.

T-Cell Development from Adult Murine BM Derived HSC In Vitro Using OP9-DL1 Cells Generates Primarily DN2-DN3 Precursors



Marcel van der Brink, Sydney Lu

OP9-DL1 Derived T-Cell Precursors



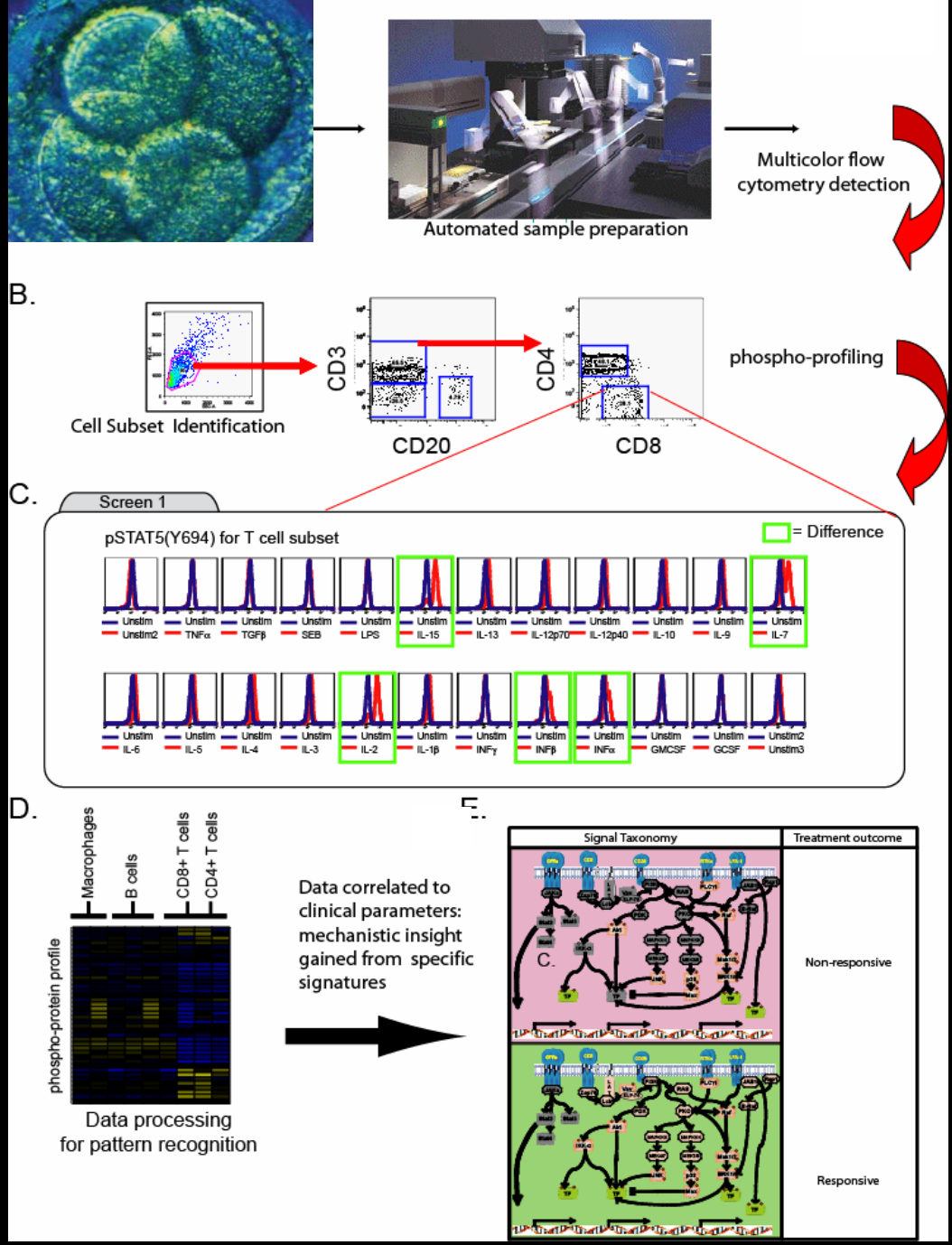
Summary of Findings for the Thymus

- DP thymocytes: Low signaling profile
- DN1/DN2 thymocytes: Increased Raf, Jnk, p38, Akt, and STAT-4 and -5 signaling
- DN and SP thymocytes phosphorylate STAT-5A in response to challenge with IL-7

1

2

3


Automated sample preparation
Multicolor flow cytometry detection
B.
Cell Subset Identification
CD3
CD20
CD4
CD8
phospho-profiling
C.
Screen 1
pSTAT5(Y694) for T cell subset
= Difference
Unstim
Unstim2
TNFα
TGFβ
SEB
LPS
IL-15
IL-13
IL-12p70
IL-12p40
IL-10
IL-9
IL-7
IL-6
IL-5
IL-4
IL-3
IL-2
IL-1β
INFγ
INFβ
INFα
GMCSF
GCSF
Unstim3
D.
Macrophages
B cells
CD8+ T cells
CD4+ T cells
phospho-protein profile
Data processing for pattern recognition
Data correlated to clinical parameters: mechanistic insight gained from specific signatures
E.
Signal Taxonomy
Treatment outcome
C.
Non-responsive
Responsive

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- Garry Nolan

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