

Improving the stem cell characterization workflow: confirming differentiation potential via multi-color detection of germ layer markers

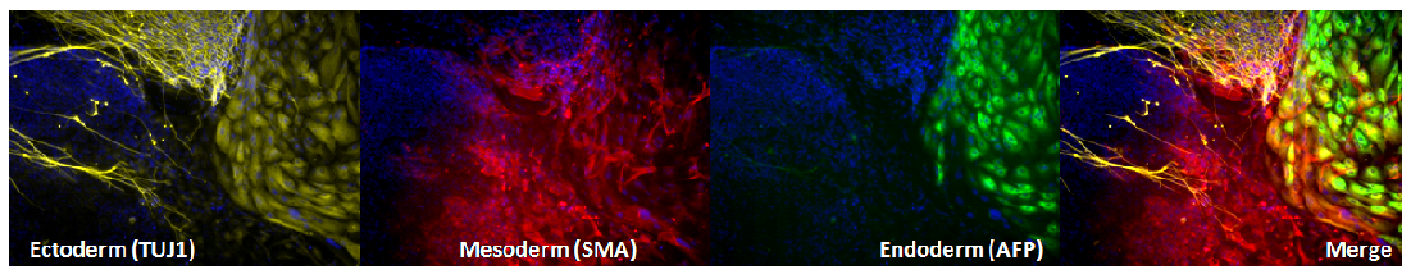


Figure 1 – Embryoid bodies were generated from H9 stem cells and allowed to spontaneously differentiate for 23 days prior to co-staining them with marker antibodies for the 3 germ layers: beta-III tubulin (TUJ1, yellow) for ectoderm, smooth muscle actin (SMA, red) for mesoderm, and alpha-fetoprotein (AFP, green) for endoderm. DAPI nuclear DNA counterstaining (blue) was also performed.

Reprogramming somatic cells such as fibroblasts or blood cells into undifferentiated cells with the potential to give rise to many distinct cell types requires multiple characterization steps in order to confirm the successful derivation of bona fide pluripotent cells. One important step to confirm pluripotency involves testing the ability of reprogrammed cells to form embryoid-like bodies that can be differentiated into cell types corresponding to each of the three embryonic germ layers. The 3-Germ Layer Immunocytochemistry Kit enables optimal image-based analysis of spontaneously differentiated embryoid bodies derived from human pluripotent stem cells. It detects widely accepted markers characteristic of the three embryonic germ layers: beta-III tubulin (TUJ1) for ectoderm, smooth muscle actin (SMA) for mesoderm, and alpha-fetoprotein (AFP) for endoderm (**Figure 1**, **Table 1**). Immunofluorescence using this set of germ layer markers is particularly advantageous in that each of these markers is not only preferentially expressed in certain cell lineages, but each marker also displays a distinguishing cellular localization pattern that serves as an extra measure of visual confirmation (**Figure 2**). For example, anti-TUJ1 staining is typically brightest for elongated cells with characteristic neuronal-like projections. Cells that robustly express SMA display lined patterns of actin filament staining, which cells are often found in large layered groupings or occasionally as isolated, large “fish gill”-looking cells. Anti-AFP staining commonly identifies tight clusters of cells, each with a pronounced “ring”-like staining pattern around the nucleus. This high performance immunocytochemistry (ICC) kit includes a complete set of primary and secondary antibodies, a nuclear DNA stain, and premade buffers for an optimized staining experiment. To obtain more information per sample this flexible kit was designed to enable the simultaneous detection of up to three germ layer markers per sample, depending on the multi-color capabilities of the imaging instrumentation (**Figures 2 – 4**).

Table 1 – Description of the markers detected by the Molecular Probes® 3-Germ Layer Immunocytochemistry Kit (Cat. No. [A25538](#)).

Marker	Description	Detection Options
Beta-III Tubulin (TUJ1 / TUBB3) – ectoderm marker	Class III beta-tubulin is a useful ectoderm marker since it is highly expressed in cells of neural lineage. Tubulins are essential building blocks that make up microtubules, which in turn support the formation and maintenance of the cytoskeleton.	Green / Alexa Fluor® 488 (FITC filter) or Far-red / Alexa Fluor® 647 (Cy5 filter)
Smooth Muscle Actin (SMA / ACTA2) – mesoderm marker	The alpha isoform of smooth muscle actin is commonly used as a mesoderm marker. Actin filaments help make up the cytoskeleton and the contractile apparatus in muscle cells.	Orange / Alexa Fluor® 555 (Cy3 / TRITC filter) or Red / Alexa Fluor® 594 (Texas Red® filter)
Alpha fetoprotein (AFP) – endoderm marker	Alpha fetoprotein is a widely used endoderm marker due to its characteristic expression in primitive endoderm cells.	Green / Alexa Fluor® 488 (FITC filter)

Reference protocol for fixed-cell immunofluorescence staining

For a complete protocol, please see the following link:

http://tools.lifetechnologies.com/content/sfs/manuals/A25538_3Germ_Layer_Immuncyto_Kit_PI.pdf

1. Remove media from the cells.
2. Add Fixative Solution and incubate for 15 minutes at room temperature.
3. Remove Fixative Solution.

Optional stopping point: After removing Fixative, add Wash Buffer, parafilm the sample to prevent it from drying out, and store at 4°C for up to 1 month.

4. Add Permeabilization Solution and incubate 15 minutes at room temperature.
5. Remove Permeabilization Solution.
6. Add Blocking Solution and incubate 30 minutes at room temperature.
7. Add desired primary antibody directly to the Blocking Solution covering the cells to yield a 1X final dilution, mix gently, and incubate overnight at 4°C.
8. Remove the solution. Add Wash Buffer and wait for 2–3 minutes. Repeat the wash procedure 2 more times so that the cells are washed a total of 3 times.
9. Remove the third Wash Buffer and add the appropriate Secondary Antibody and incubate for 1 hour at room temperature.
10. Remove the solution. Add Wash Buffer and wait for 2–3 minutes. Repeat the wash procedure 2 more times so that the cells are washed a total of 3 times.

Optional: Add 1–2 drops/mL of NucBlue® Fixed Cell Stain (DAPI) into the last wash step and incubate for 5 minutes.

11. Image the cells immediately or store the cells at 4°C in the dark, wrapped with parafilm to prevent the samples from drying out, for up to 1 month. Alternatively, for prolonged storage, apply a suitable antifade mounting medium, such as ProLong® Diamond Antifade Mountant, to the sample.

Differentiated EB preparation notes:

The cells photographed here were from cultures of spontaneously differentiated embryoid bodies (EBs) generated from human pluripotent stem cells (H9 embryonic stem cells or reprogrammed fibroblasts / induced pluripotent stem cells [iPSCs]). Using the medium conditions detailed below, ectoderm marker TUJ1 and mesoderm marker SMA could be detected within ~10 days whereas endoderm marker AFP required at least 14 days for robust immunofluorescence. Microscopy was performed using a Zeiss® Axiovert® 25 CFL.

EB Medium (recipe to prepare 100 ml)

DMEM-F12 (Cat. No. 10565-018)	79 ml
KSR (Cat. No. 10828-028)	20 ml
NEAA (Cat. No. 11140-050)	1 ml
2-Mercaptoethanol (Cat. No. 21985-023)	100 µl

Store EB Medium for up to 14 days at 4°C.

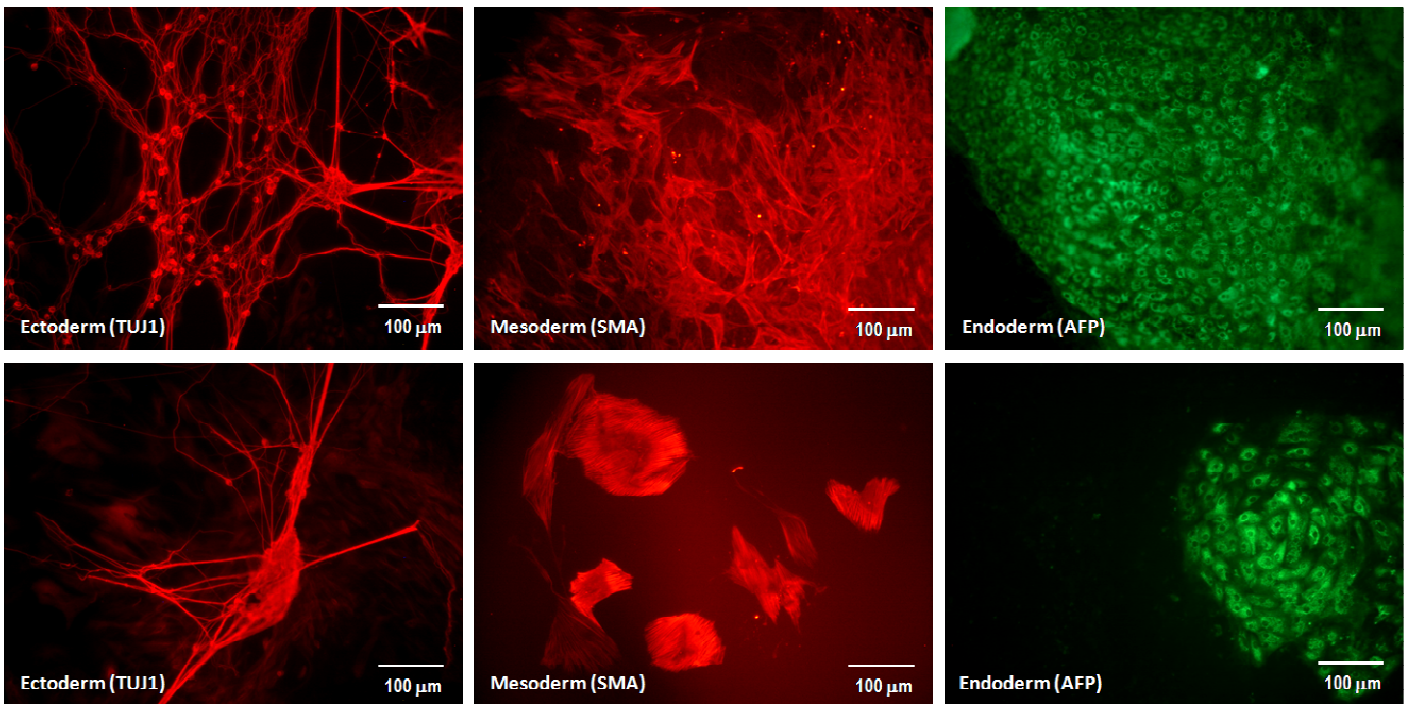


Figure 2 – Image examples showing one germ layer marker per sample. Embryoid bodies were generated from H9 stem cells and allowed to spontaneously differentiate for 24 days prior to staining them with marker antibodies corresponding to one of the three germ layers: beta-III tubulin (TUJ1, red) for ectoderm, smooth muscle actin (SMA, red) for mesoderm, or alpha-fetoprotein (AFP, green) for endoderm.

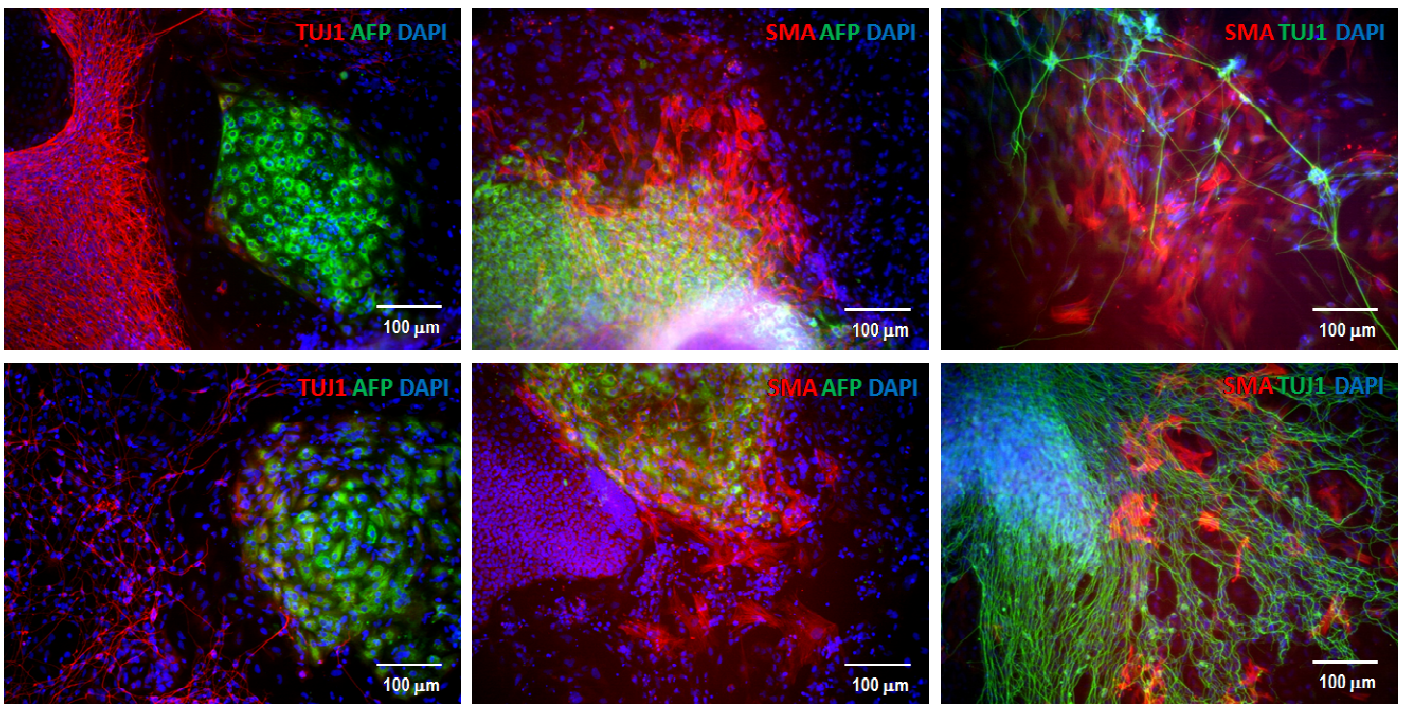


Figure 3 – Composite image examples demonstrating detection of two germ layer markers per sample. Embryoid bodies were generated from H9 stem cells and allowed to spontaneously differentiate for 20 – 23 days prior to co-staining them with marker antibodies corresponding to two out of the three germ layers: beta-III tubulin (TUJ1, red [left panel] or green [right panel]) for ectoderm, smooth muscle actin (SMA, red) for mesoderm, or alpha-fetoprotein (AFP, green) for endoderm. DAPI nuclear DNA counterstaining (blue) was also performed.

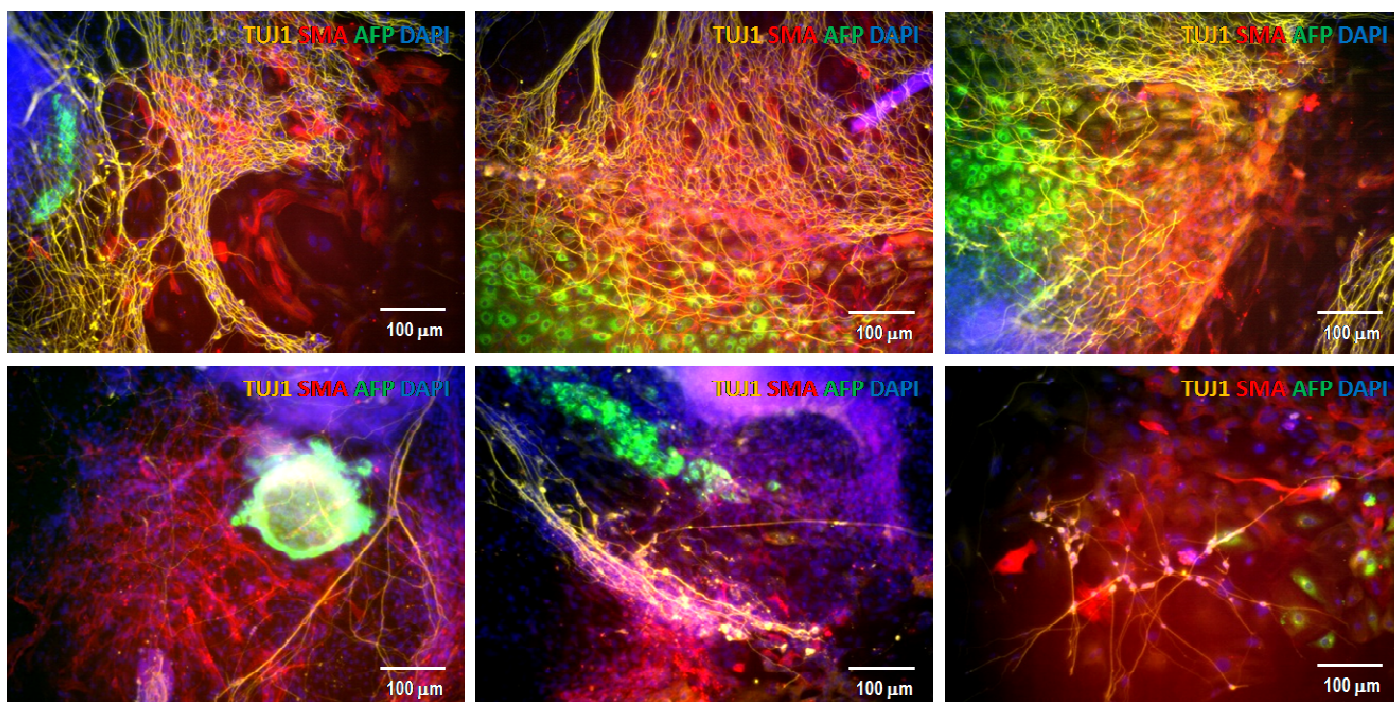


Figure 4 – Composite image examples demonstrating detection of all three germ layer markers from the same sample. Embryoid bodies were generated from H9 stem cells (top panel) or induced pluripotent stem cells (iPSC-derived EBs, bottom panel) and allowed to spontaneously differentiate for 19 – 23 days prior to co-staining them with marker antibodies corresponding to all three germ layers: beta-III tubulin (TUJ1, far-red staining was pseudo-colored yellow) for ectoderm, smooth muscle actin (SMA, red) for mesoderm, and alpha-fetoprotein (AFP, green) for endoderm. DAPI nuclear DNA counterstaining (blue) was also performed.

Technical Support

Visit www.Lifetechnologies.com/support or email probestech@invitrogen.com or phone (800) 438-2209 or (541) 335-0353.