Standard operating procedure

Trilineage differentiation on matrigel coated coverslips for IF staining

Materials:

- Matrigel Aliquot
- DMEM/F12 (Gibco/31331-028)
- TeSR-plus (iPS_SOP_0099.1)
- Fasudil (10mM stock) (LC/F4660)
- Gentle Cell DR (SCT/07174)
- STEMdiff Trilineage Kit (SCT/05230)
  - Ectoderm Medium
  - Mesoderm Medium
  - Endoderm Medium
- 24-well plate (Greiner/662 160)
- Coverslips 14mm Ø, autoclaved Menzel-Glaeser) (Thermo Scientific/
- DPBS (Gibco/14190-094)
- 100% EtOH (EMSURE/1.00983.250)
- 4% PFA (iPS_SOP_0097)
- Centrifuge (Eppendorf/5702)

Note:

Before beginning trilineage differentiation mention the following in the lab journal:

- Name of hiPSC line and clone number
- Passage number of hiPSC line
- Date of cell seeding on coverslips
- Amount of cells seeded on coverslip
- Date of fixation
- Lot number of media used

Method:

Required: At least 1x10^6 hiPSC's, recommended to use 2 full 6-wells on day 7 post last split.

Matrigel coating of coverslips:

- □ Dip coverslips in 100% EtOH.
- □ Put coverslips in 24-well culture plate.
  - Use at least 3 coverslips/hiPSC line, one per lineage

- □ Prepare Matrigel coating according to SOP0013.10
- □ Make sure the coverslips are completely dry.
- □ Coat coverslips with 333 µl diluted Matrigel per coverslip. Make sure the entire coverslip is submerged in the coating solution.
- □ Leave at 37°C for at least 1 hour before use. Coated plates can be kept at 4°C for 1 month if not used immediately.

Day 0:

- □ Prepare Single cell plating medium. Needed for each clone (equals 3 coverslips):
  - ➢ Ectoderm plating medium:
    add 0.5 µl Fasudil (10mM) to 0.5 ml of StemDiff Ectoderm medium.
  - ➢ Endoderm/Mesoderm plating medium:
    add 1.5 µl Fasudil (10mM) to 1.5 ml TeSR-plus.

- □ Remove differentiated parts of the hiPSC colonies with a pipet tip.
- □ Remove medium and wash once with DPBS.
- □ Add 1 ml/well of Gentle Cell Dissociation Reagent.
- □ Incubate for 8 minutes at 37°C.
- □ Add 1 ml of RT DMEM/F12/well and dislodge cells by gently pipetting up and down with 1 ml pipet. Ensure that all cell aggregates are broken up into single cells.
- □ Transfer cells to 15-ml tube and rinse well twice with 1 ml DMEM/F12 to collect remaining cells.
- □ Centrifuge tube at 300 xg for 3 minutes. Aspirate supernatant.
- □ Re-suspend cells in 250 µl of Endoderm/Mesoderm plating medium. Count live cells with CountessII or LUNA Fx7 according to SOP74.1 or SOP111.1 and calculate cell suspension plating volume according to the equations below.

Cell Count (CC)	**Ectoderm: 400,000/CC**	**Mesoderm: 100,000/CC**	**Endoderm: 400,000/CC**

- □ Aspirate Matrigel coating from coverslips. Add 0.5 ml of indicated Single-cell plating medium to coverslips.
- □ Add calculated plating volume of cell-suspension to the coverslips.
  - ➢ Cell-suspension volume added should not exceed 100 µl
- □ Distribute cells evenly in well by gently rocking the plate. Place plate at 37°C.

Day 1-6 (once a day):

- □ *Anticipated cell density: Cells plated for Ectoderm and Endoderm differentiation should be close to confluency whereas cells for Mesoderm differentiation are sub confluent*.
- □ Aspirate media from wells.
- □ Add 1 ml of the appropriate Trilineage medium to each well.
- □ Refresh every day. Endoderm and Mesoderm until day 4 and Ectoderm until day 6
- □ If necessary the feeding of day 2 or 3 can be replaced with a double feed. If a double feed is done make a note in the lab journal.

Fixation:

Note: Fix Endoderm and Mesoderm on day 5.
Fix Ectoderm on day 7.
PFA is harmful to stem cells. Fix samples when all other cell culture has been finished. Switch on UV afterwards.

- □ Anticipated results at the day of fixation: Mesoderm and Endoderm differentiation: Can be sub- or completely confluent; Ectoderm differentiation: confluent monolayer occasionally with island-like 3D structures; neural rosettes are often visible

- □ Wash coverslips 1x with DPBS.
- □ Fix with 1 ml 4% PFA per coverslip for 30 min at RT.
- □ After adding the PFA transfer the plates in a transport box to the chemical hood in room L2.04 for incubation and following steps
- □ Remove 4% PFA and wash coverslips 1x with DPBS.
  (*Note: collect PFA and DPBS in tube, don't use the suction system)*

- □ Add 1 ml DPBS per coverslip.
- □ Wrap the plate with Parafilm to prevent dehydration and store at +4ºC in the cold room for a maximum of 6 months.