**Cell culture, transfection, immunocytochemistry, and imaging**

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**Abstract**

This protocol describes

1. the maintenance, transfection, immunocytochemistry, and imaging of RPE1.

2. transfection, immunocytochemistry, and imaging of iPSCs, i3Neurons and DA neurons.

**Keywords**

Transfection, LipoSTEM, lipofectamine, RPE1, iPSCs, i3Neurons, DA neurons, SoRa, microscopy.

**Protocol**

**A. General cell culture for RPE1**

1. hTERT-RPE1 cells were grown in DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific), 1% glutaMAX, 1% penicillin-streptomycin. Cells were kept at 37°C with 5% CO2 in an enclosed incubator.

Note: For general maintenance, when cells reached 80-90% confluency, they were detached from the

dish with Trypsin and diluted 1:10-20 in a new dish.

**B. Cell transfection for RPE1**

1. For live-cell imaging experiments, cells were seeded on glass-bottom dishes (MatTek; 35mm) at the concentrations ranging from 1-2 x 105 cells.

2. For RPE1: cells were allowed to adhere for 8-24 hours before being transiently transfected using 4 μl Lipofectamine™ 2000 Transfection Reagent (Invitrogen) in Opti-MEM media, mixed with the respective plasmids (1-2 μg) and visualized after 48 hours.

3. For cilia generation, cells were serum-starved in DMEM/F12 media (without FBS) for 48 hours.

**B. Cell transfection for iPSCs, i3Neurons and DA Neurons**

1. For live-cell imaging experiments, cells were seeded on glass-bottom dishes (MatTek; 35mm) at the concentrations ranging from 3-5 x 105 cells.

2. 4 μl of Lipofectamine™ Stem Transfection Reagent (Invitrogen) in Opti-MEM media was used with respective plasmids (1-3 μg) and visualized at least 48 hours later.

**C. Immunocytochemistry of RPE1, iPSCs, i3Neurons and DA Neurons**

1. For fixed-cell imaging experiments, cells were seeded on glass-bottom dishes (MatTek; 35mm) at the concentrations ranging from 1-3 x 105 cells.

For immunofluorescence visualization, cells, were fixed with 4% (v/v) paraformaldehyde (Electron Microscopy Sciences) in 1x phosphate-buffered saline (PBS) for 20 mins.

2. Cells were washed thrice in PBS.

3. Cell extraction was performed with 0.25-0.5% (v/v) Triton X-100 in PBS for 10 mins.

4. Cells were washed thrice in PBS.

5. For removal of free aldehyde groups, cells were quenched with fresh 1 mg/ml sodium borohydride (Sigma-Aldrich) in PBS for 7 mins, and then washed thrice in PBS.

6. Cells were further blocked for 30 min in 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS and then incubated overnight at 4 °C with the respective antibodies listed in Table S1.

7. Cells were washed with PBS thrice the following day and incubated with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature.

8. Cells were washed thrice in 1xPBS.

9. DAPI (Thermo Fisher Scientific) was used for nuclear staining, when necessary.

**D. Imaging**

For live imaging, cells were maintained in a caged incubator with humidified atmosphere (5% CO2) at 37°C. The Yokogawa spinning disk field scanning confocal system with microlensing (CSU-W1 SoRa, Nikon) controlled by NIS elements (Nikon) software was used for imaging. Excitation wavelengths between 405-640 nm, CFI SR Plan ApoIR 60XC WI objective lens and SoRa lens-switched light path at 1x, 2.8x or 4x were used. SoRa images were deconvolved using the Batch Deconvolution (Nikon) software.