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Immunofluorescencent Staining of A-synuclein, TFEB, GCase and LAMP1 in treated cultured cells

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We use this protocol and it's working

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Abstract

This protocol can be used to detect the amount and localization of endogenous a-synuclein, TFEB, GCase and LAMP1 by confocal immunofluorescence. In our hands, we performed the same protocol in undifferentiated SH-SHY5Y parental cell line and in SH-SY5Y expressing a mutant form of the SNCA gene which leads to a rapid aggregation of a-synuclein protein following its interaction with lipid-membrane organelles. In this mutant model, the familiar PD mutation E46K was “amplified” by introducing two analogous extra-lysine mutations into the nearby KTKEGV repetitive motive in positions E35K, E46K, and E61K. Subsequently, we applied a differentiation protocol both to the SH-SY5Y parental cell line and the 3K-SNCA overexpressing ones. In differentiated cells, the same immunostaining protocol was again conducted.

Materials

Cell lines:

SH-SY5Y cells were used as parental control cells (#91396-88-2, Atcc,UK). SH-SY5Y cells constitutively expressing the 3K-SNCA gene were a kind gift from Dr. Ulf Dettmer laboratory (Harvard, USA).

Compounds:

Compounds YM201636 (#13576, Cayman Chemical, Ann Arbour, US) and ambroxol hydrochloride (#A9797, Merck, London, UK) were diluted in DMSO to obtain a 10mM stock solution. Subsequent dilutions were all made in sterile PBS (Gibco® REF 14190-144) and stored at -80C until usage.

- DMEM, high glucose, GlutaMAX™ Supplement **Thermo Fisher Catalog #**61965026
- Neurobasal Medium (1x) **Gibco - Thermo Fisher Scientific Catalog #**21103049
- Retinoic acid, 97% **Thermo Fisher Scientific Catalog #**044540.04
- B-27™ Supplement (50X), serum free **Gibco - Thermo Fisher Scientific Catalog #**17504044
- Glutamax **Gibco - Thermo Fisher Scientific Catalog #**35050-061
- Human Brain-Derived Neurotrophic Factor (BDNF) **Cell Signaling Technology Catalog #**3897
- Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix **Thermo Fisher Catalog #**A1413202
- DMEM/F-12, GlutaMAX™ Supplement **Thermo Fisher Catalog #**31331028
- YM-201636 **Cayman Chemical Company Catalog #**13576
- Ambroxol hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A9797
- Phosphate buffered saline (PBS) without Ca/Mg **Thermo Fisher Scientific Catalog #**14190144
- DMEM/F-12, GlutaMAX™ Supplement **Thermo Fisher Catalog #**31331028
- Fetal Bovine Serum **Gibco - Thermo Fisher Scientific Catalog #**10270106
- Penicillin-Streptomycin **Gibco - Thermo Fisher Scientific Catalog #**15140122
- Trypsin (2.5%), no phenol red **Thermo Fisher Catalog #**15090046
- Versene Solution **Thermo Fisher Catalog #**15040066
- Normal Goat Serum **Abcam Catalog #**ab7481
- Recombinant Anti-Alpha-synuclein antibody **Abcam Catalog #**ab138501
- TFEB (D2O7D) Rabbit mAb **Cell Signaling Technology Catalog #**37785
- Purified Mouse Anti-Human Lamp-1 **BD Biosciences Catalog #**611042
- Anti-Alpha-synuclein antibody [4D6] - BSA and Azide free **Abcam Catalog #**ab1903

F(ab)2-Goat anti-Mouse IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 **Invitrogen - Thermo Fisher Catalog #**A-11017

Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 **Invitrogen - Thermo Fisher Catalog #**A-11011

4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #**D1306

Cell differentiation protocol

1 Differentiate the wild-type and 3K-SNCA overexpressing SH-SY5Y into neurons using retinoic acid-based protocol.

2 Detach the 70% confluent non-differentiated cells using trypsin and Versene® (Gibco® REF 61965-026) and resuspend in Neurobasal media (Gibco® REF 21103-049) supplemented with 30μM retinoic acid (ThermoFisher® # 044540.04), 1X vitamin B-27 (Gibco® REF 17504-044), 1X Glutamax® (Gibco® REF 35050-061), and 5ng/ml brain-derived neurotrophic factor (BDNF) (Cell Signaling® REF 3897S).

3 Replace the differentiation medium every 48 hours, with a neuronal morphology appearing after 4 days of culture.

4 Use the differentiated neurons for downstream analysis after 10 days in culture.

5 Seed the cells in Geltrex® (Gibco® REF A1413202) Matrix solution diluted in DMEM/F-12 medium (Gibco® REF 31331-028) (1:100) coated plates at 1.5×10^5 cells/ml.

Cell culture of non-differentiated cells

6 Culture the SH-SY5Y cells and 3K-SNCA overexpressing cells in 1:1 DMEM/F12 media (Gibco® REF 31331-028) with Glutamax® (Gibco®, REF 35050-061) supplemented with 10% fetal bovine serum (FBS) (Gibco® REF 10270-106) and 1X Penicillin/Streptomycin (Gibco® REF 15140-122). Passeage the cells appropriately to maintain a confluency between 50-70%.

7 Subsequently, detach the cells using Trypsin 10x (Gibco® REF 15090-046) into Versene 1X (Gibco® REF 15040-066) (1:1), count, and seed in a new 96-well plate. In order to acquire images with the Opera Phenix® Plus High-Content Screening System, seed the cells in a specific type of 96 well plate, the Phenoplate®-96 TC (PerkinElmer®, #39269097).

8 Seed the 3*104 cells into each well, avoiding placing them into borders, rows and columns to prevent plate-edge effects.

9 24 hours post seeding, discard the medium and replace it with 100 µL of medium containing the appropriate compound or vehicle.

10 Left the cells to incubate for 24 hours and subsequently use it for downstream analysis.

Cell culture of differentiated cells

1d

11 After 10 days in the cell culture described above (see Cell differentiation protocol section), use the differentiated neurons for downstream analysis.

12 Detach the differentiated cells using Trypsin 10x (Gibco® REF 15090-046) into Versene 1X (Gibco® REF 15040-066) (1:1), count, and seed in a new 96-well plate. In order to acquire images with the Opera Phenix® Plus High-Content Screening System, seed the cells in a specific type of 96 well plate, the Phenoplate®-96 TC (PerkinElmer®, #39269097).

13 Seed the 3*104 cells into each well, avoiding placing them into borders, rows and columns to prevent plate-edge effects.

14 24 hours post seeding, discard the medium and replace it with 100 µL of differentiation medium (see Cell differentiation protocol section) containing the appropriate compound or vehicle.

15 Left the cells to incubate for 24:00:00 and subsequently used for downstream analysis.

1d

Cell Staining

4h 50m

16 Following compound treatment for 24 hours, remove the cell culture medium and fix the cells with 100 µL of 4% para-formaldehyde (PFA) (Severn Biotech Ltd. REF 40-7401-05) for 00:20:00 at Room temperature.

20m

17 Subsequently, remove the PFA and add the 100 µL of ice-cold methanol to each well for 00:20:00 .

20m

18 Discard the methanol and was each well 3 times with 1X PBS (Gibco® REF 14190-144).

19 Block the cells with 100 µL 5% normal goat serum (NGS) (ab7481, Abcam®, Cambridge, UK) for 01:00:00 at Room temperature.

1h

20 Add the primary antibodies according to the experiment: (i) monoclonal rabbit anti-synuclein 1:750 (#ab138501, Abcam®, Cambridge UK), (ii) monoclonal mouse anti-GBA 1:1000 (Anti-GBA mAb (2E2), #AP1140-100UG, Calbiochem®), (iii) monoclonal rabbit anti-TFEB 1:500 (Anti-TFEB D207D Rabbit mAb #37785 Cell Signalling®), (iv) monoclonal mouse anti-LAMP1 1:1000 (#611042, BD® Transduction Lab), (v) monoclonal mouse anti-synclein 1:750 (#ab1903, Abcam®, Cambridge, UK).

21 Upon addition of primary antibody, left the cells to incubate Overnight at 4 °C. 1h

22 Wash the cells 3 times with PBS and incubated for 02:00:00 at Room temperature with secondary antibody according to the experiment: AlexaFluor 488 1:2000 (Invitrogen® #A11017), AlexaFluor 568 1:2000 (Invitrogen® #A11011), AlexaFluor 647 1:2000 (Invitrogen® #A21244). 2h

23 Subsequently, wash the cells 3 times with 1X PBS, and incubate them for 00:10:00 with 100 µL of DAPI (Invitrogen® #D1306) solution (5 µL). 10m

24 Wash each well 3 times with 1X PBS and fill with 200 µL of PBS. Store the plates at 4 °C avoiding light until analysis.

Image acquisition

25 Perform the cell imaging using the Opera Phenix® Plus High-Content Screening System (PerkinElmer®, Waltham US) using confocal mode. Acquire each channel separately to avoid overlap signal.

25.1 Acquire DAPI with laser power at 30% with exposure set at 100ms.

25.2 Acquire 488 channel with laser power at 70% with exposure at 100ms.

25.3 Acquire 564 channel acquired with laser power at 70% with exposure at 100ms.

25.4 Acquire 647 channel with a laser power at 80% with exposure at 100ms.

25.5 Acquire z-stack of 2 um for each image.