Published in final edited form as:

Curr Protoc Toxicol. ; 73: 11.23.1–11.23.28. doi:10.1002/cptx.29.

3D differentiation of LUHMES cell line to study recovery and delayed neurotoxic effects

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Abstract

Current neurotoxicity testing and study of molecular mechanisms in neurodegeneration, *in vitro*, usually focuses on acute exposures to compounds. 3D LUHMES allow long-term treatment or pulse exposure in combination with compound wash-out to study delayed neurotoxic effects as well as recovery and neurodegeneration pathways. In this unit we describe 3D LUHMES culture and characterization. Characterization of the model involved immunocytochemistry, flow cytometry and qPCR measurements. Studying the delayed effects of compounds is more relevant to human exposures and neurodegenerative diseases with a strong gene/environment component. Most assays for molecular endpoints have been developed for monolayer cell culture and therefore, need to be adapted for 3D models. In this unit, we further describe toxicological assays for molecular endpoints such as ATP levels, mitochondrial viability and neurite outgrowth, which have been adapted for use in 3D LUHMES cultures.

Keywords

LUHMES; 3D culture; wash-out; delayed cellular response; neurotoxicity

INTRODUCTION

Lund human mesencephalic (LUHMES) is a human, mesencephalon-derived cell line, immortalized by a tetracycline regulated *v-myc*-vector. These cells are increasingly used in *in vitro* research (Zhang et al., 2014; Tong et al., 2016; Pörtl et al., 2012; Noelker et al., 2015; Oliveira et al., 2015; Krug et al., 2014; Efrémova et al., 2015; Schildknecht et al., 2013), owing to their fast differentiation into homogeneous dopaminergic cell population (Scholz et al., 2011).

In vitro Toxicology is evolving towards the use of complex models, favoring human-derived cell lines and 3D cultures. It has been shown that 3D models show increased differentiation, signaling and cell-cell interactions, which better recapitulate tissue structure and function, when compared to traditionally used monolayer cultures (Pampaloni et al., 2007; Alépée et

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al., 2014). Fast growing technologies (bio-printing, cell culture scaffolds and bioreactors) have made 3D cultures possible (Knight and Przyborski, 2015). However, every model needs to be extensively characterized and molecular and toxicological endpoints (such as those described in this protocol) must be adapted to 3D cultures. For certain assays, 3D models can be treated similar to tissue samples due to their size, complexity and cell interactions.

During development of a new *in vitro* model, comparison to the existing model as well as characterization is important to understand its cellular composition and whether it can better answer a specific research question. We propose that immunostaining, flow cytometry and qPCR methods be used to identify and quantify expression of neuronal differentiation in the 3D LUHMES model (e.g. *NeuN*, *TH*, *DAT*, *MAP2*, *VMAT2*, β -*III-Tubulin*, *SYN1*) and proliferation markers (e.g. *Ki67*, *Nestin*). Controlling cellular composition and cell death (e.g. Annexin V, caspase 3/7 activation (for apoptosis), 7-AAD (for necrosis)) in 3D models is necessary to ensure that the results obtained are derived from differentiated cells and not undifferentiated or dead cells within aggregates.

As mentioned above, to apply 3D models for research, attention must be paid to aggregate shape and size, which must remain within 250-350 μ m, to avoid possible necrosis in the core of an aggregate (Mehta et al., 2012; Ou and Hosseinkhani, 2014; Zanoni et al., 2016). Maintaining the size below 350 μ m allows for all cells throughout the aggregate to receive the nutrients and necessary media components for differentiation (Smirnova et al., 2015a). In the case of LUHMES semi-confluent monolayer cultures, cells rapidly exit the cell cycle upon induction of differentiation (Scholz et al., 2011). In the 3D differentiation model, cell suspensions are placed on a gyratory shaker, which leads to formation of aggregates with tight cell-cell interactions. These interactions stimulate cell-to-cell signaling, and a significant percentage of the cells continue to proliferate. To inhibit LUHMES proliferation, treatment with the anti-proliferative drug, Taxol, during the early days of differentiation (days 3-5) was introduced in the 3D protocol, showing efficient inhibition of cell proliferation and generation of pure differentiated dopaminergic neuronal aggregates, which can be kept in culture for at least 21 days (Smirnova et al., 2015a).

This unit describes how to differentiate LUHMES in 3D (Basic Protocol 1) and ensure quality of the model (Support Protocols 1, 2 and 3). This 3D model was developed to recapitulate tissue-like cellular interactions, and prolong the shelf-life of differentiated cultures (> 2 weeks). It has previously been observed that medium exchanges and certain compound exposures can lead to disruption of neuro-differentiated monolayer culture morphology (Constantinescu et al., 2007; Ilieva and Dufva, 2013; Smirnova et al., 2015a). In this 3D model, aggregates are in suspension; neurite outgrowth arborization and cell connections within the floating aggregates protects these structures from the mechanical disruptions during medium changing and washing step. Furthermore, most toxicity testing to date involves measuring acute effects of single-dose exposures, however, 3D models allow measurement of chronic effects of low-dose long-term exposures, and delayed toxic effects and cellular adaptation after compound removal.

The second part of this unit describes the step-by-step protocol for compound exposure and wash-out using this 3D model (Basic Protocol 2). Protocol adaptations for 3D to measure

endpoints which can be used to assess acute and chronic effects (on viability, neurite outgrowth, mitochondrial function) of a single-dose exposure to the known dopaminergic toxicant, Rotenone, are described in Support Protocols 4, 5 and 6. With the development of *in vitro* 3D models, testing chronic or delayed effects of compounds as well as cellular adaptation and/or recovery after stress will provide data which is more relevant to human exposures leading to disease.

BASIC PROTOCOL 1

LUHMES NEURONAL DIFFERENTIATION IN 3D

LUHMES is a tetracycline-regulated v-myc-vector immortalized cell line. In the presence of tetracycline, cAMP and GDNF, the v-myc promotor is switched off and LUHMES rapidly exit the cell cycle and homogeneously differentiate to mature dopaminergic neurons (Lotharius et al., 2005; Scholz et al., 2011). LUHMES routine culture and neuronal differentiation in monolayer were extensively characterized (Krug et al., 2014; Scholz et al., 2011). This protocol will describe the adaptation of LUHMES neuronal differentiation into 3D cultures. We developed a 3D protocol in order to perform long-term exposures as well as to wash-out the compounds for cellular recovery and adaptation studies.

Basic protocol 1 describes LUHMES cell culture and differentiation in 3D. Support protocol 1 covers the characterization of differentiation by immunohistochemistry. Support protocol 2 describes the characterization of neuronal differentiation by flow cytometry with the emphasis in handling of spheroids for flow cytometry analysis. Support protocol 3 refers to the essential steps of characterization by RT-PCR.

Materials—See “*Reagents and Solutions*” for main recipes, and stock solution preparations used in this protocol.

- LUHMES ATCC® CRL-2927™
- Coating solution (see recipe, Table 1)
- Proliferation medium (see recipe, Table 2)
- Differentiation medium (see recipe, Table 2)
- Wash medium (see recipe, Table 2)
- Poly-L-Ornithine (Sigma-Aldrich, P-3655) (see recipe)
- Fibronectin solution (Sigma-Aldrich, F-1141) (see recipe)
- Advanced DMEM/F-12 medium (ThermoFisher Scientific, cat # 12634-010)
- N-2 supplement (ThermoFisher Scientific, cat # 17502048)
- L-Glutamine (Sigma-Aldrich, cat # G7513)
- Human basic fibroblast growth factor (bFGF, R&D Systems, cat # 4114-TC) (see recipe)
- Glia Derived Neurotrophic factor (GDNF, Gemini, cat # 300-121P) (see recipe)

- Tetracyclin (Sigma-Aldrich, cat # T-7660) (see recipe)
- cAMP (Santa Cruz, cat # 16980-89-5) (see recipe)
- TrypLE™ Express (ThermoFisher Scientific, cat # 12605036)
- Taxol (Sigma, cat # P4543) (see recipe)
- Dulbecco's Phosphate buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} (Quality Biological)
- TrypanBlue
- Double distilled water
- Sterile syringe filters, 0.20 μm (Corning)
- T75 and T175 Flasks (*NuncEasYFlask*, *Nunclon Delta Surface*, cat # for 75 cm^2 Flask 156472)
- 6-well plates (BD)
- Cell culture centrifuge
- Phase-contrast microscope
- Cell counter or hemocytometer
- Cell culture incubator suitable to accommodate a shaker
- Cell culture gyratory shaker. Model used in this protocol: ES-X Kuhner laboratory shaker, Tray size: 420×420 mm, orbital shaking motion with diameter 50 mm, speed 80 rpm.

Protocol Steps—*All cell culture steps should be conducted in sterile laminar hood, biosafety 2. All solutions and media should be kept sterile.*

All culture reagents must be pre-warmed to 37 °C in a water bath.

LUHMES cells should only be used up to passage 25.

All plates and flasks for routine cultures must be NuncEasYFlask, Nunclon Delta Surface to avoid difficulties with neuronal cell attachment

For routine cell culture, thawing and freezing details refer to protocol described by (Krug et al., 2014; Scholz et al., 2011).

Flask Coating

1. Prepare coating solution according to Table 1.
2. Add coating solution to flasks, (T25 flask: 4 mL; T75 flask: 8 mL; T175 flask: 14 mL) and incubate overnight at 37 °C.
3. After incubation aspirate the coating-solution and wash twice with sterile distilled water.

4. Dry flasks under the laminar flow bench before use.

Routine Cell Culture and Preparation for Differentiation—*Follow the cell culture protocol as described by (Krug et al., 2014; Scholz et al., 2011). To prepare cells for differentiation, expand LUHMES culture as follows:*

- 5 Culture 2×10^6 LUHMES cells in a T75 pre-coated flask containing 12 mL proliferation medium. Incubate at 37 °C, 5% CO₂, 95% humidity.
- 6 When 70-80% confluency is reached, passage the cells by removing the medium and adding 2 mL TrypLE™ for 3 min at 37 °C, 5% CO₂, 95% humidity.
- 7 When the cells have detached, re-suspend the cells in 10 ml DMEM/F12 Advanced medium without supplements and pipette up and down several times to prepare a single cell suspension.
- 8 Transfer the cell suspension to a 50 mL falcon tube containing 10 mL DMEM/F12 Advanced medium without supplements and pipette up and down three more times.
- 9 Centrifuge the cells 5 min at $200 \times g$, room temperature.
- 10 Aspirate the medium, and gently re-suspend the pellet in 15 ml of proliferation medium.
- 11 Count the cell stock using a cell counter and trypan blue.
- 12 Fill the pre-coated culture flask with proliferation medium. Final volume for T75 flask is 12 mL, for T175 - 22 mL.
- 13 Passage 2×10^6 cells into T75 flask to passage cells or 4×10^6 cells into a T175 flask for differentiation.
- 14 Place flasks in an incubator at 37 °C, 5% CO₂, 95% humidity for 48 hours.

3D culture and differentiation

Day 0: Initiation of differentiation

- 15 Prepare necessary volume of differentiation medium (Table 2).
Prior to use, warm DMEM/F12 Advanced medium, differentiation medium and TrypLE™ solution to 37 °C in a water bath.
- 16 Un-package and label 6-well plates and place under sterile laminar hood.
Examine cell culture from step 11 by phase-contrast microscope, which should be 70-80% confluent.
- 17 Remove the medium, add 2-3 mL of pre-warmed TrypLE™ solution and incubate 3-5 min in incubator at 37 °C, 5% CO₂, 95% humidity.
- 18 When the cells have detached, follow the steps 7 to 9.
- 19 Aspirate the medium and gently re-suspend the pellet in 15 ml of differentiation medium.

- 20** Count the cell stock. Ideally this cell stock is between $2\text{--}3 \times 10^6$ viable cells per mL.

Note: one 80% confluent T175 flask contains sufficient cells for ~ 8 6-well plates.

- 21** Add 2 mL of differentiation medium to each well of desired number of 6 well plates.

- 22** Add the volume of cell suspension from step 19 required to reach a final concentration of 5.5×10^5 cells per well.

Cell density is an important parameter for differentiation. Too many cells can increase LUHMES proliferation rate during the later steps of the differentiation process. Make sure to have no more than 5.5×10^5 cell per well.

The humidified incubator containing the gyratory shaker is kept at 10 % CO₂ to ensure CO₂ diffusion to the inside of aggregates under constant gyratory movement.

- 23** Place 6-well plates on a shaker at 80 rpm (orbital diameter 50 mm) in an incubator at 37 °C, 10 % CO₂, 95% humidity.

Note: For all steps below involving medium exchange, compound treatment and washing out compounds, do not take more than two plates out from the incubator (shaker) at once and work quickly.

Make sure aggregates are not exposed to air for more than a few seconds. When fresh medium is added, gently shake the plate prior returning it to the incubator to make sure aggregates are floating as single aggregates and are not stuck together.

Day 3: Addition of anti-proliferation drug, Taxol: *Note: In monolayer cultures LUHMES rapidly exit cell cycle after induction of differentiation, however, due to increased cell-to-cell interaction within 3D aggregates, a portion of LUHMES cells continue to proliferate (observed in (Smirnova et al., 2015a)). To inhibit proliferation, ensure complete differentiation, and maintain the size of the aggregates below 350 µm; treatment with anti-proliferation drug, Taxol, was introduced into the 3D differentiation protocol.*

- 24** Prepare fresh anti-proliferation medium (Table 2).

For anti-proliferation medium, standard differentiation medium is supplemented with 2 µM Taxol stock to reach the Taxol concentration of 20 nM (see recipe).

To calculate total medium amount, count 1 mL of anti-proliferation medium per well.

Pre-warm the medium to 37 °C.

- 25** Transfer plates from the shaker to the laminar hood.

Examine the culture by phase-contrast microscope: Three days after cell seeding, aggregate formation is visible.

- 26 Shake plate in small circle motions to allow aggregates to collect in the middle of the well.
- 27 Tilt plate slightly, remove 800 μ L medium and add 1 mL of anti-proliferation medium to each well.
- Final Taxol concentration in each well is 10 nM. Aspiration of 800 μ L instead of 1 mL from each well takes in account evaporation during the incubation.*
- 28 Return plates back to the incubator on the shaker at 80 rpm at 37 °C, 10 % CO₂, 95% humidity.

Day 5: Taxol wash-out (Video 1)

- 29 Prepare the necessary volume of wash and differentiation media (Table 2). Pre-warm media to 37 °C.
- 30 Transfer plate from the shaker to the laminar hood.
- 31 Shake plate in small circle motions to allow aggregates to collect in the middle of the well.
- 32 Tilt plate slightly and remove the maximum possible volume of medium (leaving <200 μ L in the well). Add 2 mL of wash medium.
- 33 Repeat step 31 and 32 to remove wash medium and add 2 mL of differentiation medium to each well.
- 34 Return plates back to the incubator on the shaker at 80 rpm at 37 °C, 10 % CO₂, 95% humidity.

Day 8, 10, 12, 15, and 18: Medium exchanges

- 35 Shake plate in small circle motions to allow aggregates to collect in the middle of the well.
- 36 Tilt plate slightly and remove 1 mL medium from each well.
- 37 Add 1.2 mL differentiation medium to each well.
- 38 Cultures can be kept at least up to 21 days.

SUPPORT PROTOCOL 1

CHARACTERIZATION OF NEURONAL DIFFERENTIATION BY IMMUNOCYTOCHEMISTRY

Different antibodies can be used to visualize LUHMES differentiated cells (NeuN, MAP2, NF200 and SYN1) and proliferating cells (Ki67). Although methods such as flow cytometry are more quantitative, immunocytochemistry can provide details on aggregate structure, as well as cell and protein localization within aggregates. This protocol is modified from routine immunocytochemistry for monolayer cultures to enhance antibody penetration into aggregates.

Note: Antibody dilutions suggested in Table 3 will vary if antibodies from different provider/clone are used.

Materials

- Paraformaldehyde (PFA) 4%
- Clearing solution (see recipe, Table 4)
- Blocking solution (see recipe, Table 4)
- Wash solutions I and II (see recipe, Table 4)
- Dulbecco's Phosphate buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} (Quality Biological)
- Antibodies (see Table 3)
- Hoechst 33342
- Immu-Mount mounting medium (Fisher Scientific)
- 1.5 mL Eppendorf tubes (Fisher Scientific)
- Glass slides (Fisher Scientific)
- Glass cover slip (Fisher Scientific)
- Nail polish
- Eppendorf tubes
- 24-well plates
- Microplate Shaker
- Fluorescence microscope (Nikon)
- Zeiss LSM 510 Confocal III confocal microscope (Zeiss)
- ZEN imaging software (Zeiss)
- ImageJ imaging software (<https://imagej.nih.gov/ij/>)

Protocol Steps

1. Prepare wash solutions I and II, optical clearing solution, blocking solution (see recipe). Keep the solutions on ice.
2. Transfer plate from the shaker to the laminar hood.
3. Shake plate in small circle motions to allow aggregates to collect in the middle of the well.
4. Collect ~ 20-40 aggregates per condition in 1.5 mL Eppendorf tubes.

The number of aggregates does not have to be exact, but similar for all conditions.

5. Allow aggregates to sink to the bottom of the tube and wash once with 500 μ L cold PBS.
6. Allow aggregates to sink to the bottom of the tube (3-5 min), aspirate PBS and add 500 μ L of cold wash solution I and 500 μ L 4% PFA. Incubate for 60 min at 4°C.
Look at the tube against light every time prior to washing to determine whether aggregates have completely sunk to the bottom of the tube.
7. Wash fixed aggregates twice with 500 μ L wash solution I as described in step 5. Aspirate wash solution I and add 500 μ L Optical Clearing Solution. Incubate for optical clearing 48 hours at 4°C on the shaker.
Note: this and the following steps can be performed in Eppendorf tubes or 24-well plates. Working with 24-well plates (200 μ L per well) will give a more homogeneous staining throughout aggregate, however lower volumes can be used with Eppendorf tubes (50 μ L per tube).
8. Remove optical clearing solution and add 500 μ L blocking solution and place tubes/plate on a shaker for 1 hour at 4 °C.
9. Aspirate blocking solution.
10. Incubate aggregates in 200 μ L primary antibody diluted in blocking solution for 48 h at 4°C on a shaker (see Table 3 for dilutions and list of antibodies).
11. Aspirate primary antibody and wash aggregates three times for 15 min each with wash solution II.
12. Remove wash solution II and incubate aggregates in 200 μ L secondary antibody diluted in blocking solution (see Table 3) for 24 h at 4 °C on a shaker. Keep samples protected from light.
13. Aspirate secondary antibody and wash aggregates three times for 15 min each with wash solution II and incubate with Hoechst 33342 nuclear stain diluted in PBS (1:10,000) for 1 h at room temperature on a shaker.
14. Wash aggregates twice with wash solution I, and once with PBS. Keep aggregates in PBS.
15. Label glass slides and place a drop of mounting medium to mount the aggregates.
16. Cut off the end of a 200 μ L tip and transfer aggregates from the Eppendorf tube/plate to the glass slide. Ensure mounting medium is covering aggregates and place a coverslip over slide.
Note: Avoid air bubble and keep slides protected from light.
17. Use nail polish to seal coverslip, allow slides to dry and preserve slide at 4 °C, protected from light.

18. Image with fluorescence or confocal microscope following the manufacturer's instructions.

SUPPORT PROTOCOL 2

CHARACTERIZATION OF NEURONAL DIFFERENTIATION BY FLOW CYTOMETRY

Flow cytometry can be used to quantify percentage of proliferating cells within the aggregates and to assess apoptotic and necrotic cells. The flow cytometry protocol is extensively described in *Current Protocols in Toxicology unit 20.9, supporting protocol 1* (Smirnova et al., 2015b). In this protocol, only modification steps for 3D cultures will be described.

Materials

- PBS-EDTA (see recipe, Table 4)
- EDTA 0.5 M (Sigma)
- TrypLE™ Express (ThermoFisher Scientific, cat # 12605036)
- RNase-free DNase 1500 Kunitz units (Qiagen)
- 4% PFA
- Blocking solution (see recipe, Table 4)
- Wash solution I and II (see recipe, Table 4)
- Dulbecco's Phosphate buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} (Quality Biological)
- Alexa Fluor® 647 Mouse anti-Human Ki-67 antibody (BD, cat # 561126)
- Alexa Fluor® 647 Mouse IgG1 κ Isotype control (BD, cat # 557783)
- PE Annexin V Apoptosis Detection Kit I (BD, cat # 559763)
- 1.5 and 2 mL Eppendorf tubes
- 1 mL BD Luer-Lok™ syringe with 20 G $\times$ 1 in. BD PrecisionGlide™ needle (BD)
- Falcon® 5 mL round bottom polystyrene test tubes with cell strainer snap cap (BD Biosciences)
- Centrifuge
- Flow cytometer (BD Biosciences)

Protocol steps

1. Retrieve differentiated aggregates from the incubator.
2. Remove medium from the cultures in 6 -well plates. Rinse cells with 3 mL of PBS-EDTA.

3. Add 1 mL dissociation solution (TrypLE™ Express containing 4 units/mL DNase).
4. Return plates to the shaker in the incubator for 30 min.
TrypLE™ Express, a recombinant trypsin, is very gentle and effective solution and should be used for dissociation to achieve better cell survival.
5. Dissociate the aggregates by gently pipetting up and down using a 200 µL pipette.
If aggregates are not dissociated, return the plate to the shaker in the incubator for another 10-15 min. Use a 1 mL syringe with 20 G × 1 in. needle to generate single cell suspension by passing un-dissociated aggregates through the needle 2-3 times. This form of dissociation is more aggressive and should be performed carefully.
6. Collect the entire cell suspension into a 2-mL centrifuge tube containing ice-cold PBS/EDTA solution.
7. Count a sample of the cell suspension.
Choose a method that guarantees to calculate the amount of viable cells before fixing the cells (e.g., trypan blue staining). Viability should be no lower than 80%.
8. Centrifuge the cells 3 min at 1,500 g, 4 °C.
All subsequent steps should be performed on ice.
9. For Annexin staining, re-suspend the pellet in 1× Annexin V binding buffer and follow the manufacturer's protocol for further steps (<http://www.bdbiosciences.com/ds/pm/tds/559763.pdf>).
10. For intracellular antibody staining re-suspend the pellet in 500 µL wash solution I, add 500 µL 4% PFA and fix the cells 20 min at 4 °C.
11. Follow the *Current Protocols in Toxicology Support Protocol 1, Unit 20.9* for washing and blocking steps (Smirnova et al., 2015b).
12. Dilute anti-Ki-67 and corresponding isotype control antibodies in blocking solution (dilution determined by manufacturer).
13. Add 100 µL pre-diluted antibody solution to 1×10⁶ cells and incubate 45 min on ice protected from light. Include unstained cells as additional control.
14. Wash twice with wash solution II and once with wash solution I. Re-suspend in 300 µL wash solution I.
15. Transfer the cells into the 5 mL Falcon flow cytometry test tube, passing the cell strainer, and proceed to measurement, following flow cytometer manufacturer's instructions and *Current Protocols in Toxicology Support Protocol 1, Unit 20.9* (Smirnova et al., 2015b).

SUPPORT PROTOCOL 3

CHARACTERIZATION OF NEURONAL DIFFERENTIATION BY qRT-PCR

To ensure LUHMES differentiation in 3D aggregates, expression of specific neuronal markers should be assed as quality control of the cultures. The protocols for RNA extraction, cDNA synthesis and qRT-PCR is no different to monolayer cultures, and are described in *Current Protocols in Toxicology, Unit 20.9* (Smirnova et al., 2015b) and TaqMan gene expression assay protocol (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf). To assess the quality of the cultures, differentiation markers such as tyrosine hydroxylase (*TH*), Dopamine transporter (*DAT*), β -III-tubulin and Synapsin 1 (*SYN1*) should be checked for significant increase in expression by day 3, compared to Day 0. Similarly, strong down-regulation of proliferation marker *Ki-67* (lower than 5-10%) and neoroprogenitor marker Nestin (*NES*) should be observed by day 6 to ensure complete differentiation and verify that no expansion of proliferating cell populations is present (refer to Figure 5 in (Smirnova et al., 2015a) for details). TaqMan specific primers for these genes are presented in Table 5.

BASIC PROTOCOL 2

COMPOUND TREATMENT AND WASH-OUT

The following protocol describes compound exposure scheme for 3D LUHMES in 6-well and 24-well formats. 3D cultures are particularly useful to study delayed cellular response after compound removal. Basic protocol 2 describes how to wash out compounds to study long-term effects, cellular recovery and adaptation pathways. Measurement of the compound concentration in the medium prior to and after washing steps is recommended (but not described in this protocol), as different compounds have different degradation and diffusion rates within the aggregates. The number of washing steps may need to be modified for different compounds.

Materials

- Differentiated 3D LUHMES (from Basic Protocol 1)
- Rotenone 100 mM (see recipe)
- Dimethyl sulfoxide (DMSO) HPLC grade, 99.9% (Sigma-Aldrich)
- Dulbecco's Phosphate buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} (Quality Biological)
- Differentiation medium (Table 2)
- Wash medium (see recipe, Table 2)
- 24-well plates (BD Biosciences)
- 6-well plates (BD Biosciences)
- 15 mL tubes (BD Biosciences)

Protocol Steps

A. Treatment in 24-well plate: For concentration range finder experiments and dose-response curves, a 24-well plate format can be used and is described below. The differentiating cultures are dynamic and changing over the time. To minimize variability between samples due to differentiation, apply reverse treatment scheme (Figure 1) and collect all samples at the same time/stage of differentiation.

1. On the day of treatment defrost rotenone stock (100 mM), or compound of interest, stored at -20 °C.
2. For treatment, pre-dilute 100 mM Rotenone in differentiation medium (1:10,000) to obtain a 10 µM stock.

When adding volume of 100 mM rotenone to medium, a white precipitation is observed. Vortex the solution for 30 seconds to ensure that the compound has fully dissolved and no white precipitate remains.

3. Dilute DMSO (1:10,000) and vortex for 30 seconds.
4. Prepare serial dilutions of stock compound (Figure 2).
5. Retrieve the plate with differentiated aggregates from the incubator. Shake plate in small circle motions to allow aggregates to collect in the middle of the wells.
6. Collect all aggregates using a 1000 µL pipette. Pool aggregates from 6 wells together into a 15 mL tube.
7. Allow aggregates to sink to the bottom of the tube.
8. Remove medium from 15 mL tubes and re-suspend aggregates in 6 mL differentiation medium.
9. Pipette up and down carefully and transfer 250 µL of medium with aggregates into each well of 24-well plate.

Aggregates must be distributed evenly across wells. Ensure to pipette up and down carefully every time 250 µL are transferred to another well.

10. Add 250 µL of compound serial dilutions from step 4 to designated wells (see Figure 3 for plate layout).

Note: The serial dilutions are prepared 2× the final concentration desired in the well.

11. Gently tilt plate to ensure that aggregates are not collecting in the middle of the wells to avoid agglomeration.
12. Place 24-well plate in a cell culture incubator at 37 °C, 5 % CO₂, 95% humidity for desired compound treatment time (up to 48 h).

Note: Handle the plate with caution, to avoid aggregates accumulation in the middle of the plate and formation of single aggregate.

B. Treatment in 6-well plate

1. Differentiate 3D LUHMES cells in 6- well plates as described in Basic Protocol 1.
2. On the day of treatment defrost rotenone stock (100 mM), or compound of interest, stored at -20 °C.
3. For treatment, pre-dilute 100 mM rotenone stock in differentiation medium (1:10,000) to obtain a 10 µM rotenone solution.

It is recommended to store rotenone at high concentrated stock solution and perform high dilution, since the compound is not soluble in water or medium.

When adding volume of 100 mM rotenone to medium, a white precipitation is observed. Vortex the solution for 30 seconds to ensure that the compound has fully dissolved and no white precipitate remains.

4. Dilute DMSO (1:10,000) in differentiation medium and vortex for 30 seconds.
5. Add volume of 10 µM rotenone solution, required to reach desired final concentration in 6-well plate containing 2 mL medium per well. Add the same amount of 1:10,000 diluted DMSO solution to vehicle control wells.

The necessary volume to achieve the desired final concentration can be calculated as follows:

$$V_2 = \frac{C_1 \times V_1}{C_2}$$

where,

C₁ = desired final concentration in µM

C₂ = 10 µM rotenone

V₁ = 2000 µL volume of medium in well

V₂ = required volume of 10 µM rotenone solution in µL

6. Place 6-well plate on gyratory shaker at 80 rpm, in an incubator at 37 °C, 10 % CO₂, 95% humidity.
7. Incubate LUHMES aggregates for the experimental exposure time.
8. Collect the aggregates for end-point measurement (described in Support protocols 4, 5 and 6) or proceed to compound wash-out steps of this protocol.

C. Compound wash-out (Video 1): If experimental design foresees studies of delayed compound effects after compound withdrawal, wash-out steps are introduced to the protocol. In order to avoid exposure to compound, which may be retained and released by the plastic of 6-well plates, this protocol involves washing aggregates and transferring them into new culture plates.

1. After desired exposure duration, retrieve 2 plates from the incubator at a time.
2. Shake plate in small circle motions to allow aggregates to collect in the middle of the wells.
3. Tilt plate and using a 1 mL pipette, remove medium from each well leaving 200 μ L to avoid aggregate exposure to air.
4. Add 2 mL wash medium to each well.
These steps must be performed quickly to avoid aggregate exposure to air and clumping (sticking together) during washing.
5. Add 2 mL differentiation medium per well in new 6-well plates.
6. Shake plate containing aggregates in wash medium in small circle motions to allow aggregates to collect in the middle of the well. Tilt plate slightly and collect aggregates and as little medium as possible using a 200 μ L pipette.
7. Transfer the aggregates into the new plate containing 2 mL differentiation medium (prepared in step 5).
8. Return plates to shaker at 80 rpm, in an incubator at 37 °C, 5 % CO₂, 95% humidity.

SUPPORT PROTOCOL 4

VIABILITY ASSAY AFTER COMPOUND TREATMENT

In this protocol two assays to study cell viability based on mitochondrial integrity and function are described. The ability of mitochondria to uptake and irreversibly reduce resazurin informs about the aerobic respiration capacity of mitochondria (metabolic activity). The ATP assay gives total cellular ATP content and informs about cellular respiration. ATP levels will vary depending on the number of cells in the sample therefore a surrogate measure of cell number such as total protein concentration must be conducted in parallel to normalize these results.

A. Resazurin assay for 3D Culture

Materials

- Differentiated 3D LUHMES (after compound treatment, Basic Protocol 2)
- Resazurin solution 1 mg/mL (see recipe)
- 6-well, 24-well plates
- Black 96-well multi-well plates (BD Biosciences)
- Fluorescence Microplate Reader (PerSeptive Biosystems CytoFluor II)

Protocol Steps

1. Differentiate cells following Basic Protocol 1.

2. Treat aggregates according to Basic Protocol 2 either in 24- or 6-well plate format.
3. Prepare 1 mg/mL resazurin solution (see recipe).
4. Retrieve the plates from the incubator and place them under laminar hood.
5. Remove half of the medium from each well.
6. Add 10 % volume 1 mg/mL resazurin solution to each well (i.e. add 100 μ L 1 mg/mL resazurin solution to 1 mL medium in each well of 6-well plate or 25 μ L to 250 μ L medium in 24-well plates).
7. Return the cell culture plate to the incubator at 37 °C, 5 % CO₂, 95% humidity.
8. Prepare a blank solution with 90 % differentiation medium and 10 % 1 mg/mL resazurin solution.
9. Incubate for 1 – 4 hours until a visible color change from blue to purple is observed in control wells.
10. To measure, carefully pipette 100 μ L from each well into 96-well plate in triplicates per condition.
Note: avoid air bubble formation, since this may interfere with the measurement.
11. Read fluorescence emission at 580-610 nm.
12. Calculate % cell viability by comparing relative fluorescence units (RFU) of treated vs. vehicle control samples using following formula:

$$Viability (\%) = \frac{RFU(treated) - RFU(blank)}{RFU(control) - RFU(blank)} \times 100$$

B. ATP assay for 3D Culture

Materials

- Differentiated 3D LUHMES (after compound treatment, Basic Protocol 2)
- ATP determination kit (ThermoFisher Scientific, Cat # A22066) Dulbecco's Phosphate buffered saline (PBS) solution without Ca²⁺ and Mg²⁺ (Quality Biological)
- Whole cell lysis buffer (see recipe)
- Pierce BCA Protein Assay Kit
- 1.5 mL Eppendorf tubes (ThermoFisher Scientific)
- White flat bottom 96-well plates (BD Biosciences)
- GloMax® 96 Microplate Luminometer (Promega)
- Spectrophotometer (Molecular Devices)

Protocol steps

1. Defrost ATP assay components.
2. After compound treatment retrieve the 6-well plates with differentiated cells from the incubator and place them under laminar hood.
3. Shake plate in small circle motions to allow aggregates to collect in the middle of the well.
4. Tilt plate slightly and collect 1 well of aggregates into a 1.5 mL Eppendorf, using a 200 μ L pipette.
5. Allow aggregates to sink, remove the medium.
6. Wash aggregates in each sample tube by adding 1 mL PBS.
7. Wait for aggregates to sink to the bottom of the Eppendorf tube and aspirate as much volume as possible.
Make sure aggregates have sunk to the bottom to not lose part of your sample.
8. Add 50 μ L whole cell lysis buffer (see recipe) to each sample.
9. Pipette up and down carefully 5 - 10 times and place for 30 min on ice until aggregates have dissolved.
Note: When pipetting aggregates up and down, avoid bubble formation.
For larger sample size, incubate for up to 1 hour on ice, and increase lysis buffer volume to 100 μ L if aggregates have not dissolved.
10. Follow manufacturer's instructions to prepare reaction buffer and reaction solution (<https://tools.thermofisher.com/content/sfs/manuals/mp22066.pdf>).
Prepare fresh reaction solution immediately prior use and do not vortex once prepared. Reaction solution cannot be stored and should be discarded after use.
11. Label white flat bottom 96-well plates.
12. Add 100 μ L of reaction solution to each well (each condition in triplicates + 3 blanks (lysis buffer only)).
13. Add 10 μ L of cell lysate from step 9 to each well in triplicates. Save the rest of the samples on ice for protein measurement. Alternatively, freeze lysates at -20 °C for later protein quantification.
14. Shake plate gently and incubate 15 min at room temperature protected from light.
15. If using GloMax® 96 Microplate Luminometer, click "activate PMT" (5 minutes).
16. Measure luminescence using GloMax® 96 Microplate Luminometer immediately.

When testing different conditions/treatments which may affect cell viability within aggregates, normalization to number of cells is necessary. In this protocol, total protein concentration is used to normalize ATP measurements to cell number.

17. Prepare Pierce™ BCA Protein working reagent (1:50) by following manufacturer's instructions (https://tools.thermofisher.com/content/sfs/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf).
18. Pipette 200 µL BCA working reagent per well in 96-well plate (for each sample in duplicate, blank in duplicate and wells desired for BSA standard curve).
19. For each sample, transfer 10 µL of cell lysate from step 9 (in duplicates) to wells containing 200 µL BCA working reagent.
20. For blank measurements, transfer 10 µL lysis buffer (in duplicate) to wells containing 200 µL BCA working reagent.
21. Add 10 µL bovine serum albumin (BSA) standard protein concentrations (in duplicate) to wells containing 200 µL BCA working reagent.
Recommended concentrations are 0, 0.25, 0.5, 0.75, 1, 1.5, 1.75 and 2 mg/ml BSA.
22. Incubate plate 30 min at 37 °C.
23. Measure absorbance at 563 nm using spectrophotometer.
24. Generate a standard curve by plotting average blank-corrected absorption values of each BSA standard vs. its concentration in mg/mL.
25. Calculate the protein concentration for each sample using the protein standard curve by following protocol described in (<https://tools.thermofisher.com/content/sfs/brochures/TR0057-Read-std-curves.pdf>)
26. Normalize ATP relative luminescence values (RLU) of each sample (measured in step 16) to its protein concentration (mg/mL, calculated in step 25). Present normalized ATP luminescence for each condition as a % of vehicle control:

$$ATP (\%) = \frac{(RLU(treated) - RLU(blank)) / Protein(treated)}{(RLU(control) - RLU(blank)) / Protein(control)} \times 100$$

SUPPORT PROTOCOL 5

MEASURING MITOCHONDRIAL MEMBRANE POTENTIAL IN 3D CULTURES

To assess the effect of compounds on mitochondrial membrane potential, a MitoTracker probe, which enters and accumulates in active mitochondria, is used. As for most assays, manufacturer's protocols provide experimental steps for monolayer cultures or cells in single-suspension. This support protocol provides the experimental steps to measure total mitochondrial membrane potential in aggregates after compound exposure.

Materials

- Differentiation medium (see recipe, Table 2)
- Differentiated 3D LUHMES (after compound treatment, Basic Protocol 2)
- MitoTracker ® Red CMXRos (Thermofisher Scientific)
- 4% PFA
- Dulbecco's Phosphate buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} (Quality Biological)
- Immu-Mount mounting medium (Fisher Scientific)
- 24-well plates (BD Biosciences)
- Glass slides (Fisher Scientific)
- Glass cover slip (Fisher Scientific)
- Nail polish
- Fluorescence microscope (Nikon)
- ImageJ software (<https://imagej.nih.gov/ij/>)

Protocol steps

1. Differentiate cells following Basic Protocol 1.
Note: If using any fluorescence LUHMES reporter cell lines, make sure this wavelength does not interfere with that of the fluorescent dye used in the MitoTracker® assay.
2. Prepare 1 mM MitoTracker® stock solution by adding 94.1 μL DMSO to tube provided by manufacturer (protect from light and store at -20°C for up to 6 months) (<https://tools.thermofisher.com/content/sfs/manuals/mp07510.pdf>).
3. On the day of sample collection, add 500 μL differentiation medium to desired number of wells in a 24-well plate.
4. Retrieve 6-well plates with aggregates from the incubator.
5. Shake plate in small circle motions to allow aggregates to collect in the middle of the 6-well plate.
6. Collect ~ 20 aggregates per condition/replicate and pipette into 24-well plate (1 well per condition/replicate). Final volume 500 μL .
7. Add 0.5 μL of 1 mM MitoTracker® stock and 0.5 μL of 1:10 pre-diluted Hoechst 33342 to each well containing 500 μL medium.
8. Protect plate from light and incubate 45 min at 37°C , 5 % CO_2 , 95% humidity. Give plate a slight shake every 15 minutes to avoid aggregates clumping together.
9. After incubation, tilt the plate to allow aggregates to sink and remove medium containing MitoTracker® and Hoechst 33342.

10. Add 500 μ L fresh differentiation medium and incubate 1 hour at 37 °C, 5 % CO₂, 95% humidity. Give plate a slight shake every 15 minutes to avoid aggregates clumping together.

*Note: this step is **not** in the manufacturer's protocol (<https://tools.thermofisher.com/content/sfs/manuals/mp07510.pdf>) and must be included to effectively wash out excess MitoTracker® from 3D cultures and obtain more accurate results. Cells with disrupted mitochondria cannot retain the red fluorescent dye, however, the dye will remain in the aggregate if not washed sufficiently.*

11. Tilt the plate to allow aggregates to sink and remove medium.
12. Wash aggregates twice for 3 min with pre-warmed PBS.
13. Aggregates can be imaged live (proceed to step 17), or fixed for later imaging.
14. To fix, remove 300 μ L PBS and add 200 μ L PFA 4 % (2 % PFA final).
15. Incubate for 45-60 min at room temperature.
16. Wash twice with PBS and transfer aggregates to a micro centrifuge tube for storage at 4°C.
17. Place a drop of mounting medium on glass slides.
18. Cut off the end of the yellow tip and transfer aggregates from the plate or Eppendorf tube to the glass slide. Pipette directly in the middle of mounting medium drop. Avoid bubbles.
19. Ensure mounting medium is covering aggregates and place a coverslip over slide.
20. Allow the slides to dry at least 4 hours at 4 °C protected from light.
21. Use nail polish to seal coverslip and preserve slide at 4 °C, protected from light until imaging.
22. Take individual images of aggregates using a fluorescence microscope. Adjust exposure time to ensure that aggregates are not over-exposed. Configure the settings by checking negative and positive controls (or highest compound concentration).

Any over-exposed regions will interfere with accurate quantification of fluorescence intensity.

Make sure the exposure time is consistent throughout the experiments for all conditions.

23. Quantify images using ImageJ open source software (Figure 4).
24. From the dropdown menu chose “Analysis” – “Set Measurements”. Activate area, min & max gray area and mean grey area.
25. Use “oval” selection to select aggregate area. From the dropdown menu chose “Analysis” - “Measure” or use key combination Ctrl+M (Command+M) to

quantify mean intensity (mean grey value) in each aggregates. The table with the results will be generated automatically.

26. Measure mean intensity for 15-20 aggregates per condition.
27. Save results table as text or excel format.
28. Normalize the intensities (mean grey values) in treated samples to the intensity of the vehicle control. Use median with interquartile range and scatter dot plot to present the results.

SUPPORT PROTOCOL 6

NEURITE OUTGROWTH QUANTIFICATION

This protocol involves LUHMES 3D aggregates, stably expressing Red Fluorescence Protein (RFP, (Schildknecht et al., 2013)) in order to enable easy and fast visualization of the neurites without additional antibody staining. After differentiation and compound treatment in 3D, LUHMES aggregates are seeded onto matrigel-coated 96-well plates. Clear radial neurite outgrowth occurs from aggregates within 24 hours after seeding, which can be then imaged and quantified using automated high content imaging by measuring parameters such as number and density of neurites and neurite total length per aggregate.

Materials

- Differentiated aggregates (Basic Protocol 1) from LUHMES stably transfected with RFP reporter (kindly provided by Prof. Dr. Leist, Konstanz, Germany).
- BD Matrigel hESC-qualified Matrix 5 mL (BD Biosciences Research #354277)
- Differentiation medium (see recipe, Table 2)
- 4% PFA
- 1.5 mL Eppendorf tubes (Fisher Scientific)
- Black 96-well plate, clear glass bottom (ThermoScientific)
- ArrayScan Confocal Module ArrayScan™ XTI (Thermo Scientific™)

Protocol steps

1. Differentiate RFP-LUHMES cells following Basic Protocol 1.
2. Treat the cells following Basic Protocol 2.
3. 24 hours prior to sample collection, defrost an aliquot of matrigel on ice.
Note: Allow at least 4 hours for defrosting. Defrosting must be on ice. Keep matrigel cold, since it starts to polymerize at room temperature.
4. Dilute 125 μ L matrigel in 10 mL DMEM/F12 advanced medium. Add 70 μ L of diluted matrigel to each well of a black/clear bottom 96-well plate. Avoid air bubbles formation. Incubate overnight in cell culture incubator.

Note: Ensure there is sufficient matrigel and that it does not evaporate from the wells prior to seeding.

5. On the day of sample collection, aspirate matrigel from 96-well plate.
6. Add 50 μ L differentiation medium per well of the 96-well plate.
7. Retrieve the 6-well plate with differentiated aggregates from the incubator and place under laminar hood.
8. Shake plate in small circle motions to allow aggregates to collect in the middle of the well. Collect aggregates using a 200 μ L pipette. Collect one well of aggregates for each condition into a 1.5 mL Eppendorf tube.
9. Allow aggregates to sink to the bottom of the tube.
10. Remove medium from Eppendorf tubes and re-suspend aggregates in 200 μ L of fresh differentiation medium.
11. Pipette up and down carefully and transfer 50 μ L of aggregates into each well of 96-well plate from step 6. This is sufficient volume for 4 technical replicates (4 wells).

Note: Handle the plate with caution and avoid shaking. Visually check that aggregates have not clumped together in the center of the well. Aggregates should be dispersed as evenly as possible. Gently pipetting 20 μ L up and down can help disperse aggregates. Individual aggregates will allow for better identification, imaging and neurite quantification.

12. Incubate the plate 24 hours in the incubator at 37°C, 5 % CO₂, 95% humidity.
13. Next day, remove 50 μ L medium and add 50 μ L of 4% PFA with Hoechst 33342 (1:5000) to all wells.

Plate must be handled with care to avoid disrupting neurites during fixation. When adding or removing solutions do so slowly and carefully. Don't remove the medium or wash solution completely to avoid detachment of the aggregates from the plate.

14. Incubate the plate 1 hour at room temperature, protected from light.
15. Wash wells three times by removing 50 μ L and adding 50 μ L PBS to the corner of the wells. Each time incubate for 10 min.
16. Store plate at 4 °C protected from light.
17. For confocal High Content Analysis (HCA) follow steps as described by Thermo Scientific Cellomics® *Neuronal Profiling V4 Bio Application Guide* (http://www.med.cam.ac.uk/wp-content/uploads/2016/02/NeuronalProfiling_V4_LC06190800.pdf).
18. Briefly, set a mask to detect nuclear stain, which will outline attached aggregates (excluding neurites) and focus on object to obtain images (Figure 5).

19. Then, work with a second mask to identify individual neurites. Settings may need to be adapted for better neurite identification. Configure these settings by checking negative and positive controls (Figure 5).

Due to RFP expression in these cells, the aggregate “body” will be much brighter than individual neurites. Configure settings to ensure neurites are bright enough to be quantified. Overexposure of the aggregate bodies does not affect neurite measurements and is acceptable for this endpoint.

20. Once mask settings are configured, set machine to search and image each well upon aggregate detection.

Keep the settings the same for all samples within experiment.

21. Once images are obtained, apply masks to detect and quantify neurites.

22. Following manufacturer’s instructions, use data to obtain total neurite length or neurite number per aggregate.

REAGENTS AND SOLUTIONS

Use milli-Q-purified or double distilled and autoclaved water in all recipes and protocol steps. Avoid repeated thawing/freezing for all medium supplements. Once defrosted store all medium supplements at 4 °C for no longer than one week.

Poly-L-Ornithine (PLO) (1mg/ml)—Dissolve 50 mg directly in the container in 5 mL of sterile double distilled water. Sterile filter, put this stock into a 50 ml Falcon, filled with 45 mL of sterile double distilled water. Aliquot. Store 5 mL aliquots at -20 °C.

Fibronectin (1mg/ml)—Aliquot. Store at 4°C.

N-2 supplement (100X)—Aliquot. Store at -20 °C.

L-Glutamine (200 mM)—Thaw in water bath, thoroughly mix, store aliquots of 5-7 ml in falcon tubes at -20 °C.

FGF (160 µg/ml)—Dissolve in 0.1% BSA/PBS. Sterile filter. Aliquot into 50 µL aliquots. Store at -20 °C.

GDNF (20 µg/ml)—Dissolve GDNF in 0.1% BSA/PBS and sterile filter. Aliquot into 20 µL aliquots. Store at -20 °C.

Tetracycline (2 mg/ml)—Weigh reasonable amount and dissolve in sterile double distilled water, sterile filter and aliquot into 500 µL aliquots. Store in the dark at -20 °C.

cAMP (150 mM)—Dissolve 500 mg in 5 mL of sterile double distilled water directly in the container; add into 50 mL Falcon filled with 5.2 mL of sterile double distilled water. Sterile filter. Aliquot 1mL in black eppendorf tubes. Store in the dark at -20 °C.

0.1% BSA/PBS—Dissolve 100mg BSA in 100 mL PBS.

Taxol (Paclitaxel, 10 mM)—Weigh a reasonable amount (~15 mg) of Taxol and dissolve in the necessary volume of sterile DMSO to reach 10 mM concentration. Vortex until the powder has completely dissolved. Aliquot 5 µL of 10 mM stock into sterile 100 µL Eppendorf tubes. Store at -20 °C for up to six months. Before use predilute the 10 mM stock 1:1000 in PBS (working solution). Dilute the working solution 1:100 in differentiation medium to the final concentration 20 nM. Once defrosted discard the rest.

Rotenone (100 mM)—Weigh a reasonable amount (~ 80 mg) of rotenone powder and dissolve in the necessary volume of sterile DMSO to reach 100 mM concentration. Vortex until the powder has visibly dissolved. Aliquot 5 µL of 100 mM stock into sterile 100 µL Eppendorf tubes. Store at -20 °C for up to six months. Once defrosted, discard the remaining volume.

Matrigel—Defrost on ice and aliquot 125 µL matrigel in 0.5 mL Eppendorf tubes. Store at -20 °C for up to six months.

LUMES culture and differentiation media—See Table 2 for medium composition. Defrost components, which are stored at -20 °C. Always prepare medium fresh on the day of use. Prior to use, cell culture medium should be pre-warmed to 37 °C in a water bath.

Blocking solution, wash solution I and II, optical clearing solution—See Table 4 for clearing, blocking and wash solutions for immunocytochemistry and flow cytometry endpoints.

COMMENTARY

Background Information

A limited number of compounds have been tested for neurotoxicity. Current animal tests cost \$700,000 to \$1 million per compound, are time consuming and have limited prediction for human health due to interspecies differences. At the same time evidence shows that early life or life long exposures to certain pesticides or industrial emissions (metals/PCBs) can potentially increase the risk of neurodegenerative diseases, such as Parkinson's and Alzheimer's (Chin-Chan et al., 2015; Hatcher-Martin et al., 2012; Tanner et al., 2011; Wang et al., 2011).

The development of 3D organotypic models opens a new venture into studying tissue complexity *in vitro* (Ou and Hosseinkhani, 2014; Knight and Przyborski, 2015). Although *in vitro* assays are more time and cost effective than *in vivo* experiments, most assays to date have focused on acute effects of high-dose, exposures. Differentiation of neuronal cell types in monolayer cultures has presented challenges for repeat-dose chronic studies due to their limited shelf-life and easy destructible morphology due to experimental manipulations. In particular, monolayer cultures have limited applications for studies where delayed effects after compound withdrawal are of interest. Adherent neuronal cultures cannot be properly washed and transferred to a new culture plate which ensures complete compound removal. There is a need for models which allow for compound treatment and removal to study

cellular adaptation, recovery and delayed (chronic) effects. Data from these experiments could be better correlated to animal studies and human population data.

Dopaminergic cells comprise <1 % of neurons in the brain and are involved in motor function and the reward system. This neuronal type, which comprises the Substantia Nigra, is prone damage by oxidative stress. Compounds such as Paraquat, MPP+ and rotenone induce selective dopaminergic neurodegeneration (Langston et al., 1984; Gao et al., 2002; McCormack et al., 2002). The gene-environment interactions which increase susceptibility to dopaminergic neurodegeneration are obscure; therefore a better understanding on how compounds in our environment may affect this cell type is needed. Moreover, specifically low-dose, long-term effects must be studied *in vitro*. The 3D LUHMES model was developed to allow exposure and removal of compound to study reversible, delayed and permanent molecular changes. Although more complex models exist (Pamies et al., 2016; Lancaster et al., 2013), this model can isolate molecular and cellular changes pertinent to this neuronal cell type.

Critical Parameters and Troubleshooting

Basic Protocol 1 was developed for wild-type and RFP-expressing 3D LUHMES. When establishing this protocol in a new laboratory, quality control of the differentiated 3D LUHMES must be carried out routinely (e.g. by RT-PCR of neural markers, flow cytometry, and immunocytochemistry). Especially, the level of proliferation after induction of differentiation should be monitored since proliferating cells may strongly affect the results of toxicological endpoints.

Compound penetration

Existing assays need to be modified for application to 3D cultures. In most cases, dye or antibody penetration will be slower; therefore incubation times need to be increased. In the same way, wash steps also need to be longer to allow for dissolution of the dye/antibody.

Size and chemical properties of compounds influence the penetration rate and speed. Some compounds may lack the ability to penetrate the aggregate fully. The use of fluorescently labeled compounds can help visualize compound penetration. For example, Hoechst 33324 takes at least one hour to fully penetrate LUHMES aggregates (Smirnova et al., 2015a).

Cell number

At day 0 of induction of differentiation, careful attention must be paid to seeding: cell number per well should not exceed 6×10^5 , with optimal cell count between $5-5.5 \times 10^5$ cells/well. With a higher number of cells per well, the effect of the anti-proliferative compound Taxol is less efficient. This can lead to irregular aggregate shape and size as well as increased proliferation leading to a non-homogeneous cell population throughout differentiation. For reproducible results, cell number is a key step to ensure complete differentiation.

Wash steps

During wash steps, prolonged plate standing outside the incubator without shaking and aggregate exposure to air can lead to aggregate clumping or floating on the medium surface. Enough medium must be left in the well to cover aggregates and steps must be performed quickly to reduce time plates are kept outside.

Optical clearing and antibody incubation

Due to the compactness of cells within aggregates and 3D tissue-like structure, incubation in optical clearing solution is required prior to antibody staining to optimize visualization of staining by confocal microscopy. This solution was published by (Hama et al., 2011) to clear mouse brain tissue for staining.

Assay endpoint normalization

If compounds decrease cell number due to cytotoxicity, this may be visible by phase contrast. Endpoints such as gene expression or protein expression should be normalized to a housekeeping gene or protein, respectively. Molecular endpoints such as ATP release, ROS production or GSH depletion should be normalized to cell number (can be measured by quantifying cell, protein or DNA content). Lack of normalization can lead to false positives or false negatives results.

Resazurin reduction assay, RNA isolation and flow cytometry

During 3D LUHMES differentiation, it sometimes occurs that a few larger aggregates (>300 μm diameter) form. These are visible by eye and should be removed from the plate before taking samples for RNA extraction or flow cytometry. However, these larger aggregates must remain in the plate if a cell number-dependent assay is being performed, such as resazurin reduction assay for cell viability after compound treatment.

Assay interference

When performing cell viability measurements (resazurin reduction, LDH release assays), pipette medium carefully from the surface to avoid the accidental transfer of the aggregates into measurement plate. Aggregates will strongly interfere with measurements and influence final results. When selecting other endpoints, one must take into consideration potential aggregate interference with assay and consult with the manufacturer on whether this may be an issue.

Anticipated Results

The expected acute, reversible and delayed effects (measured on day 8 and day 15) from 3D LUHMES exposure to rotenone following this protocol unit, are described in (Smirnova et al., 2015a). As a known mitochondrial and dopaminergic toxicant, 24 hours exposure to rotenone decreases LUHMES cell viability (measured by resazurin reduction assay) at concentrations above 100 nM. Rotenone (100 nM, 24 h) is anticipated to decrease mitochondrial activity, ATP levels and neurite outgrowth acutely. Effects before and after compound removal (acute vs. chronic) can be measured by gene expression profiling. When

the compound is washed-out, some effects on gene expression are reversible while others are irreversible.

Time Considerations

Figures 6 and 7 summarize time frames needed to conduct the protocols described in this unit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the International Foundation for Ethical Research (IFER) graduate fellowship and d by NIH NCATS (grant U18TR000547 “A 3D Model of Human Brain Development for Studying Gene/Environment Interactions”, PI Hartung). Authors would like to acknowledge Prof. Marcel Leist’s group (University of Konstanz, Konstanz, Germany). In particular, Prof. Leist, Dr. Krug, Dr. Schildknecht and Dr. Scholz for providing LUHMES cells and support in establishing the LUHMES culture in the laboratory at Johns Hopkins Bloomberg School of Public Health. The video published with this paper was produced by Dr. Pamies (Johns Hopkins University, Baltimore, USA). Authors would like to acknowledge Dr. Berlinicke for her support with HCA.

INTERNET RESOURCES (optional)

Technical data sheet for PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™) <http://www.bdbiosciences.com/ds/pm/tds/559763.pdf>

Taqman® Gene Expression Assays Protocol (Applied Biosystems) http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf

Product information for ATP Determination Kit (Molecular Probes™) <https://tools.thermofisher.com/content/sfs/manuals/mp22066.pdf>

Pierce BCA Protein Assay Kit instructions (ThermoScientific) https://tools.thermofisher.com/content/sfs/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf

How to use a protein assay standard curve (ThermoScientific) <https://tools.thermofisher.com/content/sfs/brochures/TR0057-Read-std-curves.pdf>

Thermo Scientific Cellomics ® Neuronal Profiling V4 BioApplication Guide http://www.med.cam.ac.uk/wp-content/uploads/2016/02/NeuronalProfiling_V4_LC06190800.pdf

Image Processing and Analysis in Java (ImageJ) website <https://imagej.nih.gov/ij/>

MitoTracker® Mitochondrion-Selective Probes (Molecular Probes®) manual <https://tools.thermofisher.com/content/sfs/manuals/mp07510.pdf>

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Significance Statement

Over the last decade, *in vitro* Toxicology has started moving away from traditional monolayer cultures toward 3D organotypic models. 3D cultures have several advantages over 2D cultures, such as increased cell survival, cell-to-cell interactions and more efficient differentiation. In neurotoxicology, the most promising organotypic models are based on induced pluripotent stem cells, which pose challenges due to their complex cellular composition (neurons, astrocytes, oligodendrocytes). It is intricate to derive molecular signatures of exposure in multi-cellular systems. Therefore, simple homogeneous models are also needed to answer specific biological questions. We have developed a protocol for 3D differentiation of the LUHMES cell line, a homogeneous 3D model, consisting of pure dopaminergic neuronal cells. The 3D model can be applied to the study of acute and chronic effects by exposing to and washing out compounds as necessary. We further adapted multiple protocols currently employed for monolayer *in vitro* assays to measure endpoints using the 3D model.

Figure 1.

Suggested treatment scheme for sample collection on day 8. In this scheme, 48 h treatment takes place on day 6 of differentiation, while 24, 12 and 6 h treatments take place on day 7 of differentiation. All treated and control samples are collected at the same time on day 8 at the same stage of differentiation.

A : 6.32 mL differentiation medium containing 2x highest concentration of test compound
B: 4.32 mL differentiation medium

Figure 2.
Pipetting scheme for serial dilution of test compound.

Figure 3.

Plate layout for concentration range finder experiments and dose-response curves. Vehicle control (DMSO) and compound concentrations are tested across four replicates, where C1 is the lowest and C5 is the highest compound concentration.

Figure 4. Screenshot for ImageJ Software with uploaded fluorescence image of an aggregate. Oval shape selection is used to select aggregate area for measurement of mean grey values (Analysis – Measure (Ctrl+M)).

Figure 5.

High content analysis (HCA) mask settings to identify aggregates (blue circle) and individual neurite outgrowth (purple lines) observed after 24 h incubation in matrigel-coated wells.

Figure 6.

Time consideration scheme for the Basic Protocols 1 and 2: LUHMES neuronal differentiation in 3D, compound treatment and wash-out steps.

Figure 7.

Time consideration scheme for the toxicological endpoints described in Support Protocols: ATP assay, neurite outgrowth quantification, MitoTracker, flow cytometry, immunostaining.

Table 1

Flask coating solution

Components	Coating (20 mL)
Milli Q H ₂ O	19 mL
PLO (1 mg/ml)	1 mL
Fibronectin (1 mg/ml)	20 µL

Table 2

LUHMES medium composition

Components	Proliferation Medium (20 mL)	Anti- Proliferation Medium (50 mL)	Differentiation Medium (100 mL)	Wash Medium (100 mL)
Advanced DMEM/F12 Medium	19.6 mL	49.5 mL	97 mL	98 mL
N2 Supplement (100×)	200 µL	500 µL	1 mL	1 mL
Glutamine (1 mg/mL)	200 µL	500 µL	1 mL	1 mL
FGF (160 µg/mL)	5 µL			
cAMP (100 mM)		500 µL	1 mL	
Tetracycline (2 mg/mL)		50 µL	100 µL	100 µL
GDNF (20 µg/mL)		5 uL	10 µL	
Taxol (2 uM)		500 µL		

Table 3

Antibody dilutions for immunocytochemistry

Antibody	Clone	Catalogue #	Host	Dilution
Ki67 (H-300) (Santa Cruz)	Polyclonal	sc15402	Rabbit	1:100
MAP2a+b (Sigma)	AP-20	M1406	Mouse	1:200
NeuN (Millipore)	A60	MAB377	Mouse	1:200
NF200 (Sigma)	Polyclonal	N4142	Rabbit	1:200
SYP (Millipore)	SY38	MAB5258	Mouse	1:200
Goat-anti-Mouse Alexa 488 (ThermoFisher Scientific)	Polyclonal	A-21202	Goat	1:500
Goat-anti-Rabbit Alexa 568 (ThermoFisher Scientific)	Polyclonal	A-11011	Goat	1:500

Table 4

Solutions for immunocytochemistry and flow cytometry

Components	PBS/EDTA (1 mM) Solution	Optical Clearing Solution (Hama et al., 2011)	Blocking Solution	Wash Solution I	Wash Solution II
PFA (4%) (R&D Systems)					
Urea (8M) (Sigma)		10 mL			
Triton 1 % (Sigma)		2 mL			
Glycerol (Sigma)		2 mL			
H ₂ O (Distilled)		6 mL			
PBS (1×)	50 mL		25 mL	100 mL	200 mL
Goat Serum (Sigma)			2.5 mL		
Bovine Serum Albumin (BSA) (Sigma)			250 mg	1 g	2 g
Saponin (Sigma)			37.5 mg		300 mg
EDTA (0.5 M) (Sigma)	100 µL				

Table 5

Marker genes for 3D differentiation quality control

Gene Symbol	Gene Name	TaqMan® Probe #	Catalogue #
<i>TH</i>	Tyrosine Hydroxylase	Hs00165941_m1	4331182
<i>DAT (SLC6A3)</i>	Dopamine Transporter	Hs00997374_m1	4331182
<i>βIII-Tub</i>	Class III β-tubulin	Hs00801390_s1	4331182
<i>Syn1</i>	Synapsin I	Hs00199577_m1	4331182
<i>NES</i>	Nestin	Hs04187831_g1	4331182
<i>MKI67</i>	Antigen Ki-67	Hs01032443_m1	4331182
<i>18S</i>	Eukaryotic 18S rRNA	Hs99999901_s1	4331182