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TITLE:

Studying Pre-formed Fibril Induced α-Synuclein Accumulation in Primary Embryonic Mouse Midbrain Dopamine Neurons

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KEYWORDS:

primary embryonic dopamine neurons, α-synuclein, pre-formed fibrils, Lewy body, high-content image analysis, Parkinson's disease, synucleinopathy

SUMMARY:

Here, we present a detailed protocol to study neuronal α-synuclein accumulation in primary mouse dopamine neurons. Phosphorylated α-synuclein aggregates in neurons are induced with pre-formed α-synuclein fibrils. Automated imaging of immunofluorescently labeled cells and unbiased image analysis make this robust protocol suitable for medium-to-high throughput screening of drugs that inhibit α-synuclein accumulation.

ABSTRACT:

The goal of this protocol is to establish a robust and reproducible model of α-synuclein accumulation in primary dopamine neurons. Combined with immunostaining and unbiased automated image analysis, this model allows for the analysis of the effects of drugs and genetic manipulations on α-synuclein aggregation in neuronal cultures. Primary midbrain cultures provide a reliable source of bona fide embryonic dopamine neurons. In this protocol, the hallmark histopathology of Parkinson's disease, Lewy bodies (LB), is mimicked by the addition of α-synuclein pre-formed fibrils (PFFs) directly to neuronal culture media. Accumulation of endogenous phosphorylated α-synuclein in the soma of dopamine neurons is detected by immunostaining already at 7 days after the PFF addition. In vitro cell culture conditions are also

suitable for the application and evaluation of treatments preventing α-synuclein accumulation, such as small molecule drugs and neurotrophic factors, as well as lentivirus vectors for genetic manipulation (e.g., with CRISPR/Cas9). Culturing the neurons in 96 well plates increases the robustness and power of the experimental setups. At the end of the experiment, the cells are fixed with paraformaldehyde for immunocytochemistry and fluorescence microscopy imaging. Multispectral fluorescence images are obtained via automated microscopy of 96 well plates. These data are quantified (e.g., counting the number of phospho-α-synuclein-containing dopamine neurons per well) with the use of free software that provides a platform for unbiased high-content phenotype analysis. PFF-induced modeling of phosphorylated α-synuclein accumulation in primary dopamine neurons provides a reliable tool to study the underlying mechanisms mediating formation and elimination of α-synuclein inclusions, with the opportunity for high-throughput drug screening and cellular phenotype analysis.

INTRODUCTION:

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the death of the midbrain dopamine neurons in the substantia nigra (SN), subsequent loss of dopamine tone in basal ganglia, and consequent motor impairments[1,2]. A major histopathological feature in the brains of PD patients are intracellular protein/lipid aggregates found in neuronal soma, called Lewy bodies (LB), or in neurites, Lewy neurites (LN), collectively known as Lewy pathology[3]. Lewy pathology in the brain appears to progress with advancing PD resembling the spread of pathogenic factors through neuronal connections. Abundant Lewy pathology is found in dopamine neurons in the SN and cells in other areas affected by neurodegeneration[4]. However, during disease progression, spread and onset of protein aggregation do not always correlate with neuronal death and the exact contribution of Lewy pathology to neuronal death is still unclear[5].

LB and LN had been shown to consist of membranous and proteinaceous components[3]. The former are membrane fragments, vesicular structures (possibly lysosomes and autophagosomes) and mitochondria[3]. The latter consists of at least 300 different proteins[6]. A hallmark study by Spillantini et al.[7] demonstrated that the major protein component of Lewy pathology is α-synuclein. Highly expressed in neurons, and linked with membrane fusion and neurotransmitter release, α-synuclein in Lewy pathology is present mostly in misfolded, amyloid fibril form, the bulk of which is phosphorylated at Ser129 (pS129-αsyn)[4,8].

Importantly, it was also demonstrated that due to its prion-like properties, misfolded α-synuclein might have a causative role in Lewy pathology formation[4]. The prion-like properties of misfolded α-synuclein were shown with both midbrain extracts from patients and exogenously prepared α-synuclein preformed fibrils (PFFs) to induce α-synuclein aggregates in neurons in culture and in vivo[9,10]. PFFs present a reliable and robust model to study the progression of α-synuclein pathology in dopamine neurons. When PFFs are applied to cultured primary neurons or injected into the animal brain, they lead to the formation of α-synuclein-containing inclusions in neurites and cell soma[11] that recapitulate many features seen in Lewy pathology. Observed inclusions are detergent-insoluble in Triton X, ubiquitinated, stained with the amyloid specific dye Thioflavin S, and contain α-synuclein hyperphosphorylated at Ser129[11,12]. Importantly, these inclusions do not form in α-synuclein knockout animals[11], indicating the dependence of their formation on

endogenous α-synuclein.

Nonetheless, it is difficult to directly compare PFF-induced inclusions and Lewy pathology found in PD patients because human LBs and LNs are highly heterogeneous[3]. Observed heterogeneity of Lewy pathology might be caused by different stages of the formation, different anatomical location, or differences in the conformation of misfolded α-synuclein initiating the aggregation process. The same factors might influence PFF-induced pS129-αsyn positive inclusions. Indeed, recently it was demonstrated that PFF-induced pS129-αsyn positive inclusions in primary neuronal cultures represent very early stages of pathology that can mature to structures closely resembling LB after prolonged incubation period[12,13].

Modeling early spreading and accumulation of misfolded α-synuclein with PFFs is valuable for drug development, as Lewy pathology spread is considered one of the early-stage disease markers. Therefore, aggregation-preventive treatments may be promising for stopping or slowing down the progression of PD at very early stages. Several clinical trials aimed at slowing or stopping α-synuclein accumulation are ongoing[14]. For later-stage patients, transplantation of dopamine neuronal progenitors can be a better treatment alternative[15]. However, Lewy pathology was documented in transplanted embryonic neurons during the post-mortem analysis of PD patient brains[16,17], also indicating the need for protection against α-synuclein accumulation.

In vitro, α-synuclein PFFs are known to induce aggregation in immortalized cell lines, or more commonly, in rodent primary hippocampal or cortical neurons. Neither of these are close to recapitulating dopamine neurons[10]. Culturing these neurons requires dense plating of certain numbers of neurons in vitro[18]. To achieve high plating density with limited material (e.g., primary dopamine neurons), the micro island culturing method is commonly utilized. In micro island culturing, cells are initially plated in a small drop of medium (usually a few microliters) kept together by surface tension in the middle of a large well[18]. After the neurons attach, the entire well is filled with the medium while the cells remain confined at high density in the small plating area. In addition to achieving high plating density, micro islands also prevent plating near the edges of wells, where variations in cell density and survival are frequent. Micro islands are often utilized in relatively large wells or dishes; however, establishing midbrain neuronal cultures in micro islands in 96 well plate format enables the study of Lewy pathology in bona fide dopamine neurons with medium-to-high-throughput power. In vitro experiments with these neurons allowed us to discover the glial cell line-derived neurotrophic factor (GDNF), which promotes survival of mature dopamine neurons in vitro and in vivo[19-22] and also prevents the formation of α-synuclein aggregates in dopamine neurons[23]. Human patient-induced pluripotent stem cell-derived dopamine neurons constitute a more accurate model due to their human origin and longer survival time in vitro. However, induction of α-synuclein pathology in human neurons is observed after multiple months, compared to a week in mouse embryonic neurons, and/or with multiple stressors (e.g., combination of α-synuclein overexpression and PFFs)[24,25]. In addition, maintenance of human dopamine neurons is more costly and laborious when compared to primary embryonic neurons, essentially limiting their use in high-throughput applications.

Further, primary dopamine neuronal cultures can be genetically modified (e.g., with

CRISPR/Cas9) and/or treated with pharmacological agents[23]. They constitute a fast and reproducible platform for applications like molecular pathway dissection and drug library screening. Even though limited material can be obtained from these cultures, it is still possible to conduct small size genomics/proteomics analyses. Culturing primary neurons in 96 well format is better for immunocytochemistry and fluorescence microscopy techniques, followed by high-content phenotype analysis. Multispectral fluorescence images derived from automated imaging of 96 well plates can be converted into quantitative results (e.g., the number of LB-containing neurons per well). Such analyses can be done with free software, such as CellProfiler[26,27]. Overall, primary embryonic midbrain cultures plated in 96 well plates provide a robust and efficient platform to study dopamine neurons and α-synuclein aggregation with the opportunity for high-throughput phenotype screening.

PROTOCOL:

All animal experiments were approved by the Finnish National Board of Animal Experiments and were carried out according to the European legislation on the protection of animals used for scientific purposes.

1. Preparation

1.1. Prepare dopamine neuron medium (DPM) with 0.46% D-glucose, 1% L-glutamine, 1% N2, 0.2% primocin, completed with DMEM/F12. Filter the DPM after mixing the ingredients. Store DPM at 4 °C and warm each aliquot only once.

NOTE: DPM should not contain GDNF, as it will reduce α-synuclein accumulation in dopamine neurons[23].

1.2. Prepare siliconized glass pipettes that are extremely hydrophobic, thereby minimizing the attachment to the surface and loss of cells during the initial handling of embryonic neurons.

1.2.1. Add 10 mL of siliconizing fluid to 1 L of distilled water and mix by stirring in a 2 L vessel. Leave the glass pipettes immersed in the siliconizing solution for 15 min.

1.2.2. Rinse the pipettes 3–5x with distilled water. Dry the pipettes overnight at room temperature (RT) or for 1–2 h at 100-120 °C heated sterile space to speed up the drying.

1.2.3. Sterilize the pipettes by standard autoclaving in a sealed autoclave bag.

1.3. Prepare poly-L-ornithine (PO) coated 96 well plates with transparent bottoms by adding 60 µL of PO solution into the middle wells of the 96 well plate to be used for seeding of the neurons, leaving at least one row/column of wells at the edges of the plate to avoid edge effects. Keep the coated plate overnight at 4 °C or 4 h at RT.

1.4. Prior to plating the cells, aspirate PO completely and wash the cells thrice with 100 µL of 1x

PBS. Aspirate 1x PBS from the wells and keep the lid of the plate open for complete drying.

NOTE: It is possible to collect used PO and filter it for reusing. This can be repeated twice for the same PO solution.

1.5. Add 50 µL of DPM to previously coated wells. Aspirate DPM from the wells with a 100 µL plastic tip and simultaneously scratch the bottom of the well with circular movements to remove the coating at the perimeter of each well. A PO-coated island will remain in the middle of the well.

1.6. Under a laminar hood, add 10 µL of DPM to the middle of each coated island to create micro islands.

NOTE: A plate with DPM-covered micro islands can be kept under the laminar flow hood for 1–2 h during the isolation of cells.

2. Isolation of the ventral midbrain floor from E13.5 mouse embryos

NOTE: Refer to **Figure 1** for midbrain floor dissection steps.

2.1. Prior to dissection, fill a 10 cm Petri dish with Dulbecco's buffer and keep it on ice.

2.2. Euthanize a E13.5 pregnant female mouse according to the institution's guidelines. Place the mouse flat on its back and spray the anterior body with 70% ethanol. Lift the skin above the womb with forceps and make an incision with surgical scissors to expose the uterus.

2.3. Carefully remove the uterus and place it into the previously prepared Petri dish on ice.

2.4. Using surgical scissors under the laminar hood at RT, carefully remove the embryos from the uterus. Remove all placental residue from the embryos with forceps and place them into a new 10 cm Petri dish filled with Dulbecco's buffer.

2.5. Using dissection forceps or needles, cut off the hindquarter of the head from the places marked with black arrows in **Figure 1A**. Take the cut piece away from the rest of the embryo (**Figure 1B**).

2.6. Place the posterior of the cut piece towards the observer (**Figure 1C**) and gently cut it open from caudal to cranial (**Figure 1D**). From 0.5 mm below the cranial opening, cut a 2 mm^2–3 mm^2 region, shown in **Figure 1E**.

2.7. Collect the ventral midbrain floor (see **Figure 1F**) in an empty 1.5 mL microcentrifuge tube. Keep the microcentrifuge tube on ice until all midbrain floors are collected in it.

NOTE: Alternatively, the midbrain floors can be collected with a 1 mL micropipette after

dissection of all embryo brains.

[Place **Figure 1** here]

3. Establishing primary embryonic midbrain cultures from E13.5 mouse embryos in 96 well plate format

3.1. After the collection of midbrain floors from all embryos in the same 1.5 mL tube, remove the residual Dulbecco's buffer and wash the tissue pieces thrice with 500 µL of Ca^{2+}, Mg^{2+}-free Hank's Balanced Salt Solution (HBSS).

3.2. Remove HBSS and add 500 µL of 0.5% trypsin to the tube. Incubate it at 37 °C for 30 min.

3.3. During incubation, warm 1.5 mL of fetal bovine serum (FBS) at 37 °C, add 30 µL of DNase I to the FBS, and mix. Also, fire-polish the tip of a siliconized glass pipette. Make sure that the hole has no sharp edges and is around the same size as a 1 mL micropipette tip.

NOTE: As an alternative, a low adhesion 1 mL micropipette tip can be used for trituration. However, siliconized glass pipettes seem to give the best results.

3.4. As soon as the incubation in step 3.2 ends, add 500 µL of the FBS/DNase mix to the partially digested tissue. Use the glass pipette to triturate the tissue in the FBS/trypsin mix. Triturate until tissues dissociate into tiny, barely visible particles. Avoid bubbles during trituration.

3.5. Let the leftover particles precipitate at the bottom of the microcentrifuge tube by gravity. Without pipetting the precipitate at the bottom, collect the supernatant into an empty 15 mL conical polypropylene tube.

3.6. Dilute FBS/DNase I from step 3.3 (98:2) with 1,000 µL of HBSS to obtain FBS/DNase-I/HBSS (49:1:50). Mix by pipetting up and down. Add 1,000 µL of the new mix to the leftover particles in the microcentrifuge tube. Triturate again and repeat step 3.5.

3.7. Repeat the previous step once more to use up all FBS/DNase-I/HBSS (49:1:50).

3.8. Once all the supernatant is collected inside the 15 mL tube (from steps 3.5, 3.7, and 3.8), use a tabletop centrifuge to spin down the supernatant (~3 mL) at 100 x *g*, for 5 min. Remove the supernatant without touching the pelleted cells at the bottom.

3.9. Wash the cell pellet by adding 2 mL of DPM to the tube and spin it down at 100 x *g* for 5 min. Remove the supernatant and repeat the washing 2x to minimize the debris in the pelleted cells.

NOTE**:** Always use fresh, warmed DPM for the cultured neurons. For the washing steps, DPM does not have to be fresh, but should be prewarmed to 37 °C.

3.10. Dilute the cells with fresh, warm DPM and transfer them to a microcentrifuge tube. The amount of DPM for dilution depends on the number of embryos used for tissue dissection. For example, use 150 µL of DPM to dilute the cells obtained from ten embryos.

3.11. Transfer 10 µL of cells in DPM to a microcentrifuge tube. Mix them with 10 µL of 0.4% Trypan blue stain. Count live (i.e., Trypan blue negative) cells using a hemocytometer or an automated cell counter.

NOTE**:** Use 30,000 cells for plating per well to obtain ~1,000 dopamine neurons per well. If the cell density is higher than ~30,000 cells per 6 µL, further dilute the cells with DPM before plating so that the seeding volume is no less than 6 µL.

3.12. Without touching the bottom of the wells, remove the DPM from the micro islands created at step 1.6.

3.13. In order to obtain reproducible cell density at each well, mix the cells by gentle pipetting prior to plating in the well. With a 1–10 µL micropipette, add 6 µL of cells to the middle of the well, at the location of each former micro island.

3.14. Fill the empty wells at the edges of the plate with 150 µL of water or 1x PBS to minimize evaporation from the wells containing neuronal cultures. Incubate the plate in an incubator at 37 °C, 5% CO_2 for 1 h.

3.15. After 1 h, remove the plate from the incubator, add 100 µL of DPM into each well with cells and place it back in the incubator.

3.16. Two days after plating (day in vitro 2, or DIV2), remove 25 µL and add 75 µL of fresh DPM to bring the final media volume to 150 µL and avoid evaporation as much as possible.

3.17. Exchange half of the medium with fresh DPM (i.e., remove 75 µL and add 75 µL fresh DPM) at DIV5. Do not perform any media changes after DIV5.

4. Induction of α-synuclein aggregates in primary embryonic dopamine neurons by seeding with preformed fibrils

NOTE: Protocols for obtaining and validation of PFFs had been meticulously described and discussed in several recent publications[11,28-30]. Following any work with PFFs, clean the laminar hood or any equipment that might have contacted the PFFs with 1% SDS, then with 70% ethanol[31].

4.1. Prior to the experiment, dilute the PFFs with 1x PBS to a final concentration of 100 µg/mL. Sonicate the diluted PFFs in microcentrifuge tubes with a bath sonicator at high power with water bath cooling at 4 °C for 10 cycles, 30 s ON/30 s OFF.

NOTE: It is critical that the fibrils be properly sonicated to generate fragments ~50 nm long. The size of sonicated PFFs can be measured directly from transmission electron microscope images of PFFs stained as described by Patterson et al.[30]. Sonication can be achieved as described above in a high power bath sonicator. Alternatively, a tip sonicator can be used[30]. Sonicated PFFs can be stored at -80 °C in small aliquots to avoid multiple freezing/thawing cycles.

4.2. On DIV8, add 3.75 µL of 100 µg/mL of PFFs per well to the 150 µL of medium in the well to a final concentration of 2.5 µg/mL. Use the same amount of 1x PBS for the control group.

4.3. Prepare 4% paraformaldehyde (PFA) in 1x PBS and store the aliquots at -20 °C. To do so, follow the steps below.

NOTE: PFA is toxic; wear a mask and gloves during preparation, work always under a laminar hood, and dispose of all solid and liquid PFA waste according to the institution's directions.

4.3.1. Warm 500 mL of 1x PBS in a 1 L vessel. Put a stir bar in the vessel and put the vessel on a magnetic stirrer with a heating function. Adjust the temperature between 40–60 °C to prevent boiling while keeping the solution warm.

4.3.2. Measure 20 g of PFA powder under the hood in a disposable plastic measuring container. Carefully, add the PFA powder into the vessel filled with 1x PBS. Start stirring the solution.

4.3.3. Add 200 µL of 5 M sodium hydroxide into the solution and continue stirring for ~15 min, until the PFA dissolves completely.

4.3.4. After the solution appears homogenous, add 168 µL of 5 M hydrogen chloride to balance the pH to ~7. Check the pH with disposable color-fixed pH indicator strips.

4.3.5. Remove the vessel from the heater and allow it to cool down to RT. Filter the solution and aliquot for storage at -20 °C. Thaw the aliquots at RT before the use and do not refreeze afterwards.

4.4. On DIV15, remove all media from the wells by pipetting. Add 50 µL of 4% PFA to each well to fix the cells and incubate for 20 min at RT. After incubation remove the PFA from the wells and add 100 µL of 1x PBS to each well to wash the cells. Remove 1x PBS and wash 2x more.

4.5. Leave 100 µL of 1x PBS in each well to avoid drying. Store the plate at 4 °C until immunochemistry is performed.

5. Immunofluorescent staining and automated imaging of primary embryonic dopamine neurons in 96 well plates

5.1. Remove 1x PBS and permeabilize the cells by adding 100 µL of 0.2% Triton X-100 in PBS (PBST) per well and incubating at RT for 15 min.

5.2. Remove PBST and add 50 µL of 5% normal horse serum (NHS) per well to the PBST. To block the unspecific antigen activity, incubate at RT for 1 h.

5.3. Dilute the primary antibodies against TH and pS129-αsyn (1:2,000) in 5% NHS in PBST. Add 50 µL of diluted antibodies to each well and incubate overnight at 4 °C.

5.4. Remove antibodies and add 100 µL of 1x PBS to each well to wash the cells. Remove 1x PBS and repeat washing 2x.

5.5. To prevent the bleaching of fluorescent molecules, start working under minimum light conditions. Dilute the secondary fluorescently labeled antibodies (1:400) in PBST. Add 50 µL of diluted antibodies to each well and incubate at RT for 1 h.

5.6. Remove the antibody solution and add 100 µL of 1x PBS to each well to wash the cells. Remove 1x PBS and repeat washing 2x.

5.7. Remove 1x PBS, add 50 µL of 200 ng/mL 4',6-diamidino-2-phenylindole (DAPI) per well to stain the nuclei of the cultured cells and incubate at RT for 10 min.

5.8. Wash cells 3x with 100 µL of 1x PBS for 5 min each. Keep 100 µL of 1x PBS in each well after the last wash. Cover the plate with aluminum foil and store it at 4 °C until imaging.

5.9. Image primary embryonic dopamine neurons in a 96 well view plate with a high-content plate scanner (see **Table of Materials**) fitted with a 10x objective.

5.10. Adjust the settings based on the specifications of the 96 well plate, such as plate type, manufacturer, size, distance between wells, as well as type and amount of medium.

5.11. Select the imaging area of the well to cover all the cells in a micro island. Pick an example well to adjust the autofocus. Base the initial focus on DAPI.

5.12. Calibrate the acquisition time for each fluorescent channel, based on the intensity of the staining in control wells. Adjust the parameters so that in PFF-treated control wells one can clearly distinguish dopamine cells harboring pS129-αsyn aggregates in cell soma allowing for unambiguous quantification of pS129-αsyn positive and pS129-αsyn negative cells.

NOTE: Wells that do not contain PFFs should not have any staining for pS129-αsyn; therefore, these wells can be used as negative control for adjusting pS129-αsyn intensity.

5.13. Image all the selected wells with a 10x objective simultaneously for all channels with immunofluorescence staining with exactly the same parameters.

5.14. Optionally, label α-synuclein inclusions in a subset of the wells with antibodies specific for

filamentous α-synuclein to confirm that changes in the number of pS129-αsyn-positive inclusions reflect the reduction in protein accumulation rather than inhibition of phosphorylation or dephosphorylation of pS129-αsyn.

5.15. Repeat step 5.3 substituting pS129-αsyn antibody with α-synuclein filament antibody (1:2,000). Image the stained aggregated α-synuclein as in step 5.13.

6. High-content image analysis

NOTE: This step is performed with open access software CellProfiler version 3.15 and CellProfiler Analyst version 2.2.1.[27,32]. However, with some experience, the analogous image analysis pipelines could be set in a different version or similar software. Please refer to the software page for a detailed explanation (see **Table of Materials**).

6.1. Download and install CellProfiler and CellProfiler Analyst software.

6.2. Open CellProfiler. Select **File|Import|Pipeline from file** and load the example pipeline provided, **TH_LB_V1.cpipe** file (see the **Supplementary Files**).

NOTE: The example pipelines will require specific adjustments depending on properties of the acquired images and the image acquisition platform. **Example_Images** attached as supplementary files can be used for the initial trial of the software.

6.3. Load images to be analyzed by dragging them into **Images module**. Use filter options to select only image files from the loaded folder.

6.3.1. Use **Metadata module** to extract well, field of view, and channel information from the image file name. Click on the **magnifying glass** symbol and enter regular expression to extract Plate, Well, Imaging Site and Channel information from file names.

NOTE: Regular expression will depend on file naming convention of plate microscope. Clicking **question mark** next to **magnifying glass** will provide details of the syntax.

6.3.2. Under **NamesAndTypes module,** select correct channel numbers for DAPI, TH, and pS129-αsyn staining (**default channels 1**, **2**, and **3**). In **Groups module,** select "**No**".

6.4. Use **IdentifyPrimaryObjects modules** to segment dopamine neurons using TH staining of cell soma.

NOTE: Specific values will require initial optimization based on how plates are stained and imaged. If subsequent plates are processed similarly, none or minimal further adjustments shall be needed.

6.5. Use **MeasureObjectIntensity module** to acquire fluorescence intensity information from TH

and DAPI channels.

6.6. Use **MeasureObjectSizeShape module** to measure size and shape features of segment dopamine neurons.

6.7. Use **MeasureTexture module** to measure texture feature information from TH channel from segmented dopamine neurons.

6.8. Use **ExportToDatabase module** to save measurements into database.

6.8.1. Name database file according to the experiment naming schema (e.g., ExperimentNumber001_PlateNumber1_databaseFile1.db). Select **Output Folder** for the database file. The database file can be several gigabytes large and should be saved preferably in the parent folder of the image files.

6.9. Open CellProfiler Analyst and select the **V1_THCells.properties** file created at step 6.8. Open **Tools|Classifier.**

6.10. Sort segmented cells into two categories: positive (i.e., correctly segmented dopamine neuron cell bodies) and negative (i.e., segmentation and staining artifacts) See **Figure 2A** and **2B**.

6.10.1. Set the number of fetched cells to **50 random** cells and click **Fetch** (this loads images of the cells segmented in step 6.4). Sort at least **30 cells** in each bin by dragging them to the corresponding bin at the bottom of the window. Fetch more cells as necessary.

6.10.2. In the drop-down menu select **Use Fast Gentle Boosting** with **50 max rules** and click **Train**.

6.10.3. Set "**Fetch**" to **50 positive** cells and press **Fetch** to get TH positive cells according to the classifier (**Figure 2A**). Use the obtained result to evaluate the quality of the trained classifier.

6.10.4. Repeat steps 6.10.1–6.10.3, adding new example cells for training the classifier until the results are satisfactory.

6.10.5. Select **Advanced|Edit rules…** and in a new window **select all text** (**Ctrl+a**) and **copy** it (**Ctrl-c**) to **notepad** (**Ctrl-v**). Save as **TH_rules.txt file**.

NOTE: Depending on the density of the neuronal culture and the quality of the staining and imaging, this step may not be necessary, as it might be possible to set parameters in step 6.4 to segment only TH positive cells with high accuracy. If this is the case, an entire **TH_LB_V1.cpipe** run is not necessary, and the correct parameters of **IdentifyPrimaryObjects modules** should be put directly into the corresponding module in **TH_LB_V2.cpipe**.

[Place **Figure 2** here].

6.11. Open CellProfiler and select **File|Import|Pipeline from file** and load **TH_LB_V2.cpipe** file. Repeat steps 6.3–6.7. This part of the pipeline should be identical to **TH_LB_V1.cpipe.**

6.12. Use **FilterObjects module** to pass only true TH positive cells for further analysis.

6.12.1. Set **select filtering mode** to **Rules**. In **Rules** or **classifier file name** select the **TH_rules.txt** file created in step 6.10.5.

6.12.2. Set **Class number** field to **1** if TH positive cells were sorted to bottom left window.

6.13. Use **MeasureObjectIntensity module** to acquire fluorescence intensity information from pS129-αsyn channel.

6.14. Use **MeasureTexture module** to measure texture feature information from TH channel from filtered cells.

6.15. Use **MeasureObjectSizeShape module** to measure size and shape features of filtered cells.

6.16. Use **ExportToDatabase module** to save measurements into database.

6.16.1. Name database file accordingly with your experiment naming schema (e.g., ExperimentNumber001_PlateNumber1_databaseFile2.db). Select output folder for database file.

6.17. Open CellProfiler Analyst and select **V2_THpos.properties** file.

6.17.1. Sort segmented cells into two categories – pS129-αsyn positive and pS129-αsyn negative cells (**Figure 2C**,**D**).

6.17.2. Set the number of fetched cells to **50 random** cells and click **Fetch** (this loads images of cells segmented in step 4). Sort at least **30 cells** in each bin by dragging them to the corresponding bin at the bottom of the window.

6.17.3. In the **drop-down** menu, select **Use Fast Gentle Boosting** with **50 max rules** or **Random Forest** classifiers. Click **Train**.

6.17.4. Set "**Fetch**" to **50 positive** cells and press **Fetch** to get pS129-αsyn positive cells according to classifier (**Figure 2C**). Set "**Fetch**" to **50 negative** cells and press **Fetch** to get pS129-αsyn negative cells according to classifier (**Figure 2D**). Evaluate the quality of the trained classifier.

6.17.5. Repeat steps 6.17.2–6.17.4, adding new example cells to train the classifier until the results are satisfactory.

6.18. Click **Score All** to get results table summarizing number of pS129-αsyn positive and negative

dopamine neurons in each well.

REPRESENTATIVE RESULTS:

A few days after the plating (DIV1–DIV3), bright-field microscopy was done to assess the health and homogenous spread of the cultured cells, and uniformity of these conditions at the individual wells (**Figure 3**). Cultured midbrain cells were spread homogenously within the micro island created before the plating (**Figure 3A**,**B**). Primary neurons had settled on the coated ground homogenously and established neuronal projections (**Figure 3B**). A small clump of cells (diameter smaller than 150 μm) was observed at the well and shown as an example (**Figure 3C**).

[Place **Figure 3** here].

Primary mouse midbrain cultures were immunostained with anti-TH and anti-pS129-αsyn antibodies and imaged with an automated microscope after 15 days in vitro. Coated micro islands provided restricted area for the attachment of cells in the middle of wells (**Figure 4A**,**B**). Dopamine neurons immunolabeled with TH marker were spread around the micro island in a monolayer, separated from each other, without any clumping (**Figure 4A'** and **4B'**).

[Place **Figure 4** here].

While cultures without PFF treatment did not have any pS129- αsyn signal (**Figure 4A'** and **4A''**), cultures treated with α-synuclein PFFs developed pS129-αsyn positive inclusions (**Figure 4B'** and **4B''**). In vitro PFF treatment for 7 days did not cause any significant decrease in numbers of TH-positive neurons, compared to other experimental groups (**Figure 4C**). PFF-treated cultures had a population of ~40% of pS129-αsyn positive TH-positive dopamine neurons. Treatment with positive control, GDNF, reduced the percentage of TH-positive dopamine neurons with pS129-αsyn positive inclusions (**Figure 4D**, see also the raw data and example images in the **Supplementary Files**).

FIGURE AND TABLE LEGENDS:

Figure 1: Dissection of midbrain floor from E13.5 mouse embryo. (**A**) Cutting locations at the hindquarter of the head is marked with black arrows and white dashed lines. (**B**) The piece was removed from the rest of the embryo. The removed piece is circled. (**C**) The piece was turned 90° to face the posterior towards the observer. (**D**) The piece was opened from the black arrows, from caudal to cranial (marked with white dashed line). (**E**) From 0.5 mm–1 mm below the opening, the 2 mm^2–3 mm^2 region was cut (marked with black lines). (**F**) The ventral midbrain floor was isolated (marked with black dashed square). Scale bars = 1 mm.

Figure 2: Quantification of dopamine neurons and pS129-αsyn positive dopamine neurons with CellProfiler Analyst software based on DAPI, TH, and pS129-αsyn immunofluorescence. (**A**) TH cells in the positive bin were selected based on DAPI staining (blue) marked with a small square at the first cell selected at the image and the surrounding TH staining (gray) at soma. (**B**) Non-cell artifacts were placed in the negative bin. (**C**) pS129-αsyn positive TH neurons were selected based on large inclusion of pS129-αsyn staining (red) marked with a small square at the first cell selected

at the image, surrounding the nuclei or at cell soma. (**D**) TH cells without such pS129-αsyn inclusions were placed in the negative bin. Scale bars = 10 μm.

Figure 3: A few days after the plating (e.g., DIV3), the condition of primary midbrain cultures were observed with bright-field microscopy. (**A**) Cultured cells spread across the middle of the well within the PO-coated micro island with an approximate radius of 4.4 mm (shown with white arrows) created by scratching PO from approximately 1 mm well perimeter (shown with black arrows). (**B**) Cultured primary neurons homogenously spread within the area and neuronal projections (marked with red arrowheads) were observed. (**C**) A cell clump with a diameter smaller than 150 μm is marked with blue arrowheads. Scale bars = 100 μm.

Figure 4: At DIV15, 800–1,000 dopamine cells were quantified from each micro island, with or without PFF treatment. Representative images of embryonic midbrain cultures immonustained with anti-TH and anti-pS129-αsyn antibodies. (**A**, **A'**, **A''**) Control cells without PFF treatment. (**B**, **B'**, **B''**) PFF-treated cells with pS129-αsyn inclusions. (**C**) Quantification of TH-positive cell numbers in wells treated with vehicle (control), PFFs, or PFFs with 50 ng/mL GDNF. (**D**) Quantification of pS129-αsyn aggregates in TH-positive cells treated with vehicle (control), PFFs, or PFFs with 50 ng/mL GDNF. Statistical significance was calculated with random block design ANOVA. **$p < 0.01$, n = 4 individual plates (biological replicates), each with 3–6 wells per treatment group (technical repeats). Scale bars = 300 μm for **A**, **B**; 50 μm for **A'**, **B'**; 25 μm for **A''**, **B''**.

Supplementary Files: Example pipelines for high-content image analysis with CellProfiler and the CellAnalyst software packages, example images, and raw data for **Figure 4E**, **F**. (**1-9**) Example_Images. Open these images with ImageJ or CellProfiler. (**10**) Fig_4_raw_data_Er_et_al.xlxs. (**11**) TH_LB_V1.cpipe (Steps 6.2–6.8), (**12**) TH_LB_V2.cpipe (Steps 6.11–6.18)

DISCUSSION:

Spreading Lewy pathology, of which pS129-αsyn is a major constituent, is a histopathological hallmark of PD. Stopping or slowing down the accumulation of aggregated pS129-αsyn may slow down the degeneration of dopamine neurons and the progression of alpha-synucleinopathy. However, a mechanistic understanding of how pS129-αsyn aggregation contributes to the demise of dopamine neurons still has to be established. Evidence from human postmortem studies on brain samples from patients at different stages of the disease as well as observation of pS129-αsyn positive inclusion in transplanted fetal neurons strongly suggests the spreading of Lewy pathology between cells[16,17,33]. Consequently, prion-like spreading of pS129-αsyn was recently recapitulated by using α-synuclein PFFs[9,10]. Establishing a robust, cost-effective, and relatively high- or medium-throughput model of pS129-αsyn spreading and accumulation, specifically in dopamine neurons, can considerably speed up the search for novel treatments and compounds modifying this process.

Because loss of dopamine neurons is the main cause of motor symptoms in PD and these cells possess many unique properties[2,34,35], modeling of prion-like spreading of pS129-αsyn in

dopamine neurons is the most relevant type of model from the translational perspective. Protocols utilizing micro island cultures of embryonic midbrain neurons on 4 well plates and semiautomatic quantification have been described previously[18]. The protocol described here was adapted to 96 well plates and provides less laborious preparation of micro islands, allowing for the preparation of up to four plates containing 60 wells each by an experienced researcher during one workday. Culturing dopamine neurons in 96 well plates allows for testing drugs at lower amounts and enables high transduction rates with lentivirus vectors. It is also possible to combine different treatments to perform more complex experiments.

Before applying any treatments (including PFFs), the quality of the culturing should be checked with bright-field microscopy. If the microscopy system does not utilize a CO_2 chamber with heating, the cells should not be kept outside of the incubator for more than couple of minutes, because primary mouse dopamine neurons are delicate and easily stressed. For the same reason, it is advised that the first imaging should be done after 24 h of incubation (between DIV1-DIV3). The cells should appear to be alive with present cell bodies and homogenously spread inside the micro island. Primary neurons would have settled on the PO-coated ground and started to establish neuronal projections. It is possible to observe small clump of cells (i.e., diameter smaller than 150–200 µm) that can be formed if the trituration process is not done properly or plating density is higher than recommended. These small clumps would not affect the experiment, unless they are more than a few per well and/or larger. Clumped cells make it very difficult to identify immunohistochemical markers and individual cells during the image analysis. It is essential to avoid such clumps by careful coating, triturating, and controlling plating density. If the uniformity of these conditions cannot be observed at certain wells, do not include these defective wells in the experiment. Such exclusion should be done before the execution of any treatments.

Moreover, utilization of 96 well plates allows for convenient multichannel pipette use during staining procedures and direct visualization with automatic plate microscopes, further increasing throughput. Utilization of automated image quantification is indispensable for the analysis of the data from high-content imaging platforms. In addition to the capability to process thousands of images obtained from each experiment, it ensures unbiased, identical quantification of all treatment groups. The workflow proposed for the image analysis is based on simple principles of segmenting dopamine neurons, filtering correctly segmented cells by supervised machine learning, and subsequently quantification of phenotypes (pS129-αsyn positive and pS129-αsyn negative), again by supervised machine learning. Although several different approaches for this task can be envisioned, we have found the combination of segmentation with machine learning to be the most robust for dopamine cultures due to high plating density, the diverse shapes of dopamine neurons, and the presence of strongly stained neurites. The proposed image analysis algorithm was implemented in CellProfiler and CellProfiler Analyst, open source, freely available high-content image analysis software[26,27]. The algorithm could also be implemented with other image analysis software, either open source (e.g., ImageJ/FIJI, KNIME) or proprietary. However, in our experience these often sacrifice customization capabilities for ease of use, and therefore might not perform well in complicated analyses. We have found that the CellProfiler and CellProfiler Analyst software packages give particularly reliable results by combining a substantial number of implemented algorithms, extreme flexibility in designing workflow, and

simultaneously handling and efficiently processing of high-content imaging data.

The described protocol could also be adapted for quantification of other cellular phenotypes characterized by immunostaining with different antibodies, such as markers of other neuronal populations (e.g., DAT, GAD67, 5-HT etc.) and protein aggregates (e.g., phospo-Tau, ubiquitin). Multiple fluorescent markers could also be combined to distinguish multiple phenotypes (e.g., cells with inclusions at different stages of maturation). Automated classification of multiple phenotypes should also be easy to implement in the described image analysis pipelines by merely adding a channel containing immunofluorescent images of additional markers to measurement steps and sorting cells into multiple bins. Utilization of multiple markers at the same time would, however, require the optimization of immunostaining and imaging conditions. Additionally, for better quality in immunofluorescence imaging, the use of special black-walled 96 well plates explicitly designed for the fluorescent microscope is recommended. However, these can be considerably more expensive than standard cell culture plates, which are sufficient for the analysis described in our protocol.

The type and quality of utilized PFFs are critical for the outcome of the experiments. PFFs can both affect the robustness of the assay and the interpretation of results. Preparation conditions might affect the seeding efficiency of PFFs and, indeed, PFF "strains" with different physiological properties have been reported[36]. Nonetheless, the preparation and validation of PFFs are beyond the scope of this article and have been described in several publications[11,28-30]. In addition to the preparation protocol, the species of origin of α-synuclein in PFFs (e.g., mouse, human) and the usage of wild type or mutated protein (e.g., human A53T α-synuclein) should be considered, depending on the particular experimental conditions. Induction of pS129-αsyn accumulation by PFFs was shown to be dependent on age of the culture (i.e., days in vitro), with more mature cultures showing more pronounced induction[11]. This is probably due to the increased number of neuronal connections in more mature cultures, and increased α-synuclein protein levels. In our hands, treatment with PFFs at DIV8 gave the most robust results, with pronounced accumulation of pS129-αsyn in dopamine neuron soma, while not compromising neuronal survival. The described protocol is well suited to study treatments modifying early events leading to aggregation of endogenous α-synuclein because we quantify pS129-αsyn positive inclusions at a relatively early time point, 7 days after inoculation with PFFs. At this time point, intrasomal inclusions are present in a significant fraction of cells and can be easily distinguished by immunostaining while no PFF-induced cell death is observed, simplifying the interpretation of the results. Importantly, as the morphology and composition of PFF-induced inclusions can change over time[12,13], the described protocol could, in principle, be modified to study more mature inclusions by fixation and immunostaining at later time points. However, keeping dopamine neurons in culture for longer than 15 days requires extreme care, and might induce additional variation because of cells failing to survive independently from PFF inoculation. Additionally, more extended cultures complicate drug treatment schedules. Many compounds have limited or not poorly characterized stability in the cell culture medium, and replenishment of a drug is not trivial because complete exchange of medium compromises the survival of the dopamine cultures.

Phosphorylation of α-synuclein at Ser129 is consistently reported in PFF-based models of α-synuclein aggregation and colocalizes with markers of misfolding and aggregation such as Thioflavin S, ubiquitin, or conformation-specific antibodies[11,12]. In our hands, immunostaining for pS129-αsyn also gives the strongest signal with the lowest background and is most straightforward to analyze, giving robust results when multiple treatments are screened. Importantly, immunostaining with pS129-αsyn antibody does not detect PFFs that remain outside of the cells, significantly reducing the background. However, it is important to remember that Ser129 phosphorylation is probably one of the earliest processes linked with the misfolding of α-synuclein and might be differently regulated under specific conditions. Therefore, any findings that show positive effects on pS129-αsyn should be confirmed by other markers.

Statistical analysis should be tailored correspondingly to experimental design. It is essential to perform experiments in at least three independent biological replicates (i.e., separate primary neuronal cultures). These replicates should be plated on different plates and treated independently. We analyze the data obtained from replicates on different plates with random block design ANOVA[37] to take into account the pairing of data for different experimental plates.

Altogether, the described model allows for robust, medium-to-high throughput analysis of seeded pS129-αsyn accumulation specifically in dopamine neurons while limiting costs and laboriousness.

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DISCLOSURES:

The authors have nothing to disclose.

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