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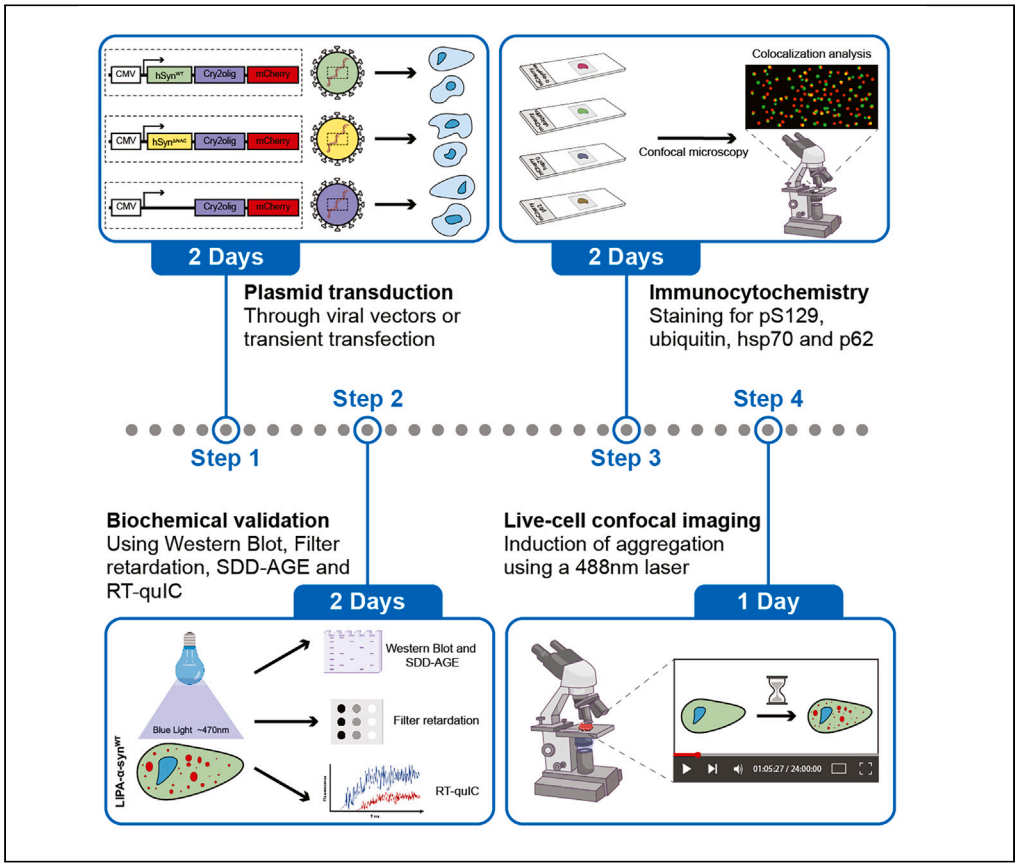
Optogenetic-mediated induction and monitoring of α-synuclein aggregation in cellular models of Parkinson's disease



Studying Parkinson's disease (PD) is complex due to a lack of cellular models mimicking key aspects of protein pathology. Here, we present a protocol for inducing and monitoring α-synuclein aggregation in living cells using optogenetics. We describe steps for plasmid transduction, biochemical validation, immunocytochemistry, and live-cell confocal imaging. These induced aggregates fulfill the cardinal features of authentic protein inclusions observed in PD-diseased brains and offer a tool to study the role of protein aggregation in neurodegeneration.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Optogenetic-mediated α-synuclein aggregation with high spatiotemporal resolution

Generation of α-synuclein inclusions mimicking key features of authentic Lewy bodies

Real-time monitoring of α-synuclein inclusion formation in living cells

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Optogenetic-mediated induction and monitoring of α-synuclein aggregation in cellular models of Parkinson's disease

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SUMMARY

Studying Parkinson's disease (PD) is complex due to a lack of cellular models mimicking key aspects of protein pathology. Here, we present a protocol for inducing and monitoring α-synuclein aggregation in living cells using optogenetics. We describe steps for plasmid transduction, biochemical validation, immunocytochemistry, and live-cell confocal imaging. These induced aggregates fulfill the cardinal features of authentic protein inclusions observed in PD-diseased brains and offer a tool to study the role of protein aggregation in neurodegeneration.

For complete details on the use and execution of this protocol, please refer to Bérard et al.[1]

BEFORE YOU BEGIN

The following protocol outlines the induction and monitoring of α-synuclein (α-syn) aggregation using optogenetics in cell culture. Our approach utilizes a modified variant of Arabidopsis thaliana's cryptochrome protein 2 (CRY2olig)[2] to create a construct that rapidly clusters upon exposure to blue light and reverts when the light is removed. By fusing this construct with α-syn, we initiate protein aggregation and generate Lewy body-like inclusions within living cells. This engineered system is referred to as the Light-Inducible Protein Aggregation (LIPA) system. To ensure uniform protein expression, the DNA quantity for each LIPA construct was adjusted. We assessed mCherry expression levels through western blot analysis and included control constructs, namely LIPA-Empty and LIPA-α-syn$^{\Delta NAC}$, lacking the non-amyloid-β component (NAC) region.

The characterization of optogenetically induced α-syn aggregates encompasses a series of techniques, including (1) Western blot validation of the distinct smear pattern of LIPA-α-syn, (2) a filter retardation assay, (3) semi-denaturing detergent–agarose gel electrophoresis (SDD-AGE) to confirm insolubility and SDS resistance, (4) *in vitro* seeding experiment using RT-QuIC (real-time quaking-induced conversion) and (5) immunocytochemistry using key markers of Lewy bodies (pS129, Ubiquitin, Hsp70, and p62). Additionally, we detail the protocol for real-time monitoring of LIPA-α-syn aggregation using live-cell imaging with a confocal microscope.

Lastly, we provide two supplemental protocols. The first outlines the transduction of human dopaminergic neurons derived from induced pluripotent stem cells (iPSCs). The second explains how to establish mammalian cell lines stably expressing LIPA constructs, enabling the generation of diverse cell lines for the study of LIPA-α-syn aggregation.4

The protocol described here is based on cell culture procedures that require a strictly controlled environment for cell growth. Sterile distilled water should be used in incubators, in addition to the preparation of sterile buffers described in the protocol.

Additionally, it is necessary to always carefully wipe down media bottles, buffers and cryopreserved vials with 70% ethanol before their transfer to a cell culture hood.

It is also essential to ensure good quality requirements for all material and reagents used throughout the protocol. All material and reagents should be purchased from well-established sources to ensure the integrity and reliability of the protocol for the induction and monitoring of α-syn aggregation using optogenetics.

Institutional permission

If hiPSCs are obtained from patients, collection should be performed in accordance with the relevant ethical guidelines. The hiPSCs utilized in this study were approved by the CHU de Quebec Research Center (#2022-6079).

Coverslips coating with Poly-L-Lysine (PLL)

Timing: 3 h

Note: Ensure using aseptic techniques to ensure the sterility of all material used under a cell culture hood.

Note: Prepare 0.1 mg/mL aliquots of PLL by reconstituting the PLL in dH_2O, filter the stock solution with a 0.22-μm-pore filter. Aliquot the diluted PLL and keep the aliquots in −20°C for up to 4 months.

1. Under a sterile hood, carefully place sterilized 12-mm glass coverslips into the individual wells of a 24-well plate.
2. Add 100 μL of 0.1 mg/mL PLL directly on the glass coverslips.
3. Incubate the glass coverslips for 15 min at 20°C–22°C under the hood.
4. Aspirate the PLL, and thoroughly wash the wells 4 times with large volumes of ddH_2O.
5. Aspirate the last ddH_2O wash and allow the plate to air dry for 2 h under the sterile hood.
6. After 2 h, cells can be plated on the coated glass coverslips.

Note: Dried PLL coated coverslips in plates can be sealed with parafilm and stored at 4°C for up to 2 weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse IgG polyclonal (IRDye 800CW); 1:10,000 (WB)	LI-COR Biosciences	926-32210
Anti-mouse IgG polyclonal (IRDye 680RD); 1:10,000 (WB)	LI-COR Biosciences	926-68070
Anti-mouse IgG (H + L), Alexa Fluor 488; 1:400 (ICC)	Fisher Scientific	A11034
Anti-mouse IgG (H + L), Alexa Fluor 633; 1:400 (CC)	Fisher Scientific	A21052
Anti-rabbit IgG polyclonal (IRDye 800CW); 1:10,000 (WB)	LI-COR Biosciences	926-32211
Anti-rabbit IgG polyclonal (IRDye 680RD); 1:10,000 (WB)	LI-COR Biosciences	926-68071
Anti-rabbit IgG (H + L), Alexa Fluor 488; 1:400 (ICC)	Fisher Scientific	A11034
Anti-rabbit IgG (H + L), Alexa Fluor 633; 1:400 (ICC)	Fisher Scientific	A21071
Mouse, anti-alpha-synuclein, clone: 42; 1:1,000 (WB/ICC)	BD	610787

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse, anti-alpha-synuclein (pS129) pSyn#64 1:2,000 (ICC)	Fujifilm (Wako)	010-26481
Mouse, anti-beta-actin monoclonal; 1:5,000 (WB)	Applied Biological Materials	G043
Mouse, anti-Hsp70 (5A5); 1:50 (ICC)	Abcam	ab2787
Mouse, anti-SQSTM1 (p62); 1:1,000 (ICC)	Santa Cruz	Sc-28359
Mouse, anti-ubiquitin monoclonal (Ubi-1); 1:20 (ICC)	Thermo Fisher Scientific	13-1600
Rabbit, anti-alpha-synuclein (pS129) (EP1536Y); 1:2,000 (ICC)	Abcam	ab51253
Rabbit, anti-mCherry polyclonal; 1:2,000 (WB)	Abcam	AB167453
Recombinant DNA		
pcDNA 3.1-CMV-LIPA-α-syn	Available by direct request from Dr. A. Oueslati lab	N/A
pcDNA 3.1-CMV-LIPA-α-syn$^{\Delta NAC}$	Available by direct request from Dr. A. Oueslati lab	N/A
pcDNA 3.1-CMV-LIPA-empty	Available by direct request from Dr. A. Oueslati lab	N/A
pLenti Blast-CMV-LIPA-α-syn	Available by direct request from Dr. A. Oueslati lab	N/A
pLenti Blast-CMV-LIPA-α-syn$^{\Delta NAC}$	Available by direct request from Dr. A. Oueslati lab	N/A
pLenti Blast-CMV-LIPA-empty	Available by direct request from Dr. A. Oueslati lab	N/A
pLenti SIN-PGK-LIPA-α-syn	Available by direct request from Dr. A. Oueslati lab	N/A
pLenti SIN-PGK-LIPA-α-syn$^{\Delta NAC}$	Available by direct request from Dr. A. Oueslati lab	N/A
pLenti SIN-PGK-LIPA-empty	Available by direct request from Dr. A. Oueslati lab	N/A
Chemicals, peptides, and recombinant proteins		
2-Mercaptoethanol	Sigma-Aldrich	M3148-100ML
4%–20% Mini-PROTEAN TGX precast protein gels	Bio-Rad	4561096
40% Acrylamide/Bis solution	Bio-Rad	1610148
Molecular biology grade agarose	FroggaBio	A87-500G
Acetic acid, glacial	Thermo Fisher Scientific	A35-500
Ammonium persulfate	Sigma-Aldrich	A3678-25G
Bovine serum albumin (BSA)	BioShop	ALB001.500
Bromophenol blue	Sigma-Aldrich	B0126-25G
CalPhos Mammalian Transfection Kit	Takara	631312
Chameleon Duo pre-stained protein ladder	LI-COR Biosciences	92860000
DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride)	Thermo Fisher Scientific	D1306
Dextrose (D-glucose) anhydrous	Fisher Scientific	D16-1
Dithiothreitol (DTT)	Thermo Fisher Scientific	R0861
DPBS calcium magnesium (Ca^{+}/Mg^{+})	Thermo Fisher Scientific	14040182
Dulbecco's modified Eagle's medium (DMEM) - high glucose	Sigma-Aldrich	D5796-500ML
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	EX0534-1
Fetal bovine serum (FBS)	Sigma-Aldrich	F1051
FluoroBrite DMEM	Fisher Scientific	A1896701
Fluoromount-G mounting medium	Thermo Fisher Scientific	00-4958-02
Gelatin from cold water fish skin, 40%–50% in H_2O	Sigma-Aldrich	G7765-1L
GlutaMAX supplement	Thermo Fisher Scientific	35050061
Glycerol	Fisher Scientific	BP2291
Glycine	Sigma-Aldrich	G7126-5KG
HEPES	Sigma-Aldrich	H6147-100G
Isopropanol	Sigma-Aldrich	I9516-500ML
Lipofectamine 2000	Thermo Fisher Scientific	11668019

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant monomeric α-syn	Provided by Dr. Edward A. Fon's and Dr. Thomas M. Ducran's teams. ***Note:*** Recombinant monomeric α-syn can also be purchased from commercial sources such as: StressMarq SPR-321	N/A
Neurobasal medium, minus phenol red	Thermo Scientific	12348017
Normal goat serum (NGS)	Wisent Bioproducts	053-110
Paraformaldehyde, granular	Electron Microscopy Sciences	19210
PBS 10× solution	Fisher Scientific	BP399-20
Penicillin/Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15-140-122
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich	P7626-5G
Phosphatase inhibitor cocktail II	Sigma-Aldrich	P5726
Phosphatase inhibitor cocktail III	Sigma-Aldrich	P0044
Pierce TEMED	Thermo Scientific	17919
Polybrene	Millipore	TR-1003-G
Poly-L-lysine	R&D Systems	3438-100-01
Potassium chloride (KCl)	Sigma-Aldrich	P5405-250G
Ponceau S	Sigma-Aldrich	P3504-10G
Protease inhibitor cocktail	Sigma-Aldrich	P8340
Puromycin 2HCl	Wisent Bioproducts	400-160-EM
RIPA lysis and extraction buffer	Thermo Fisher Scientific	89900
Sodium chloride (NaCl)	Sigma-Aldrich	S7653-1KG
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L3771-1KG
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich	S0876-100G
STEMdiff Midbrain Neuron Maturation Kit	STEMCELL	100-0041
Sucrose	Sigma-Aldrich	S9378-1KG
Thioflavin-T (TH-T)	Sigma-Aldrich	T3516-5G
Trans-Blot Turbo RTA Midi Nitrocellulose Transfer Kit	Bio-Rad	1704271
Tris(hydroxymethyl)aminomethane	Sigma-Aldrich	252859-100G
Triton X-100	Sigma-Aldrich	T8787-100ML
Trizma hydrochloride solution (Tris-HCl)	Sigma-Aldrich	T2663-1L
Tween 20	Fisher Scientific	BP337-500
Experimental models: Cell lines		
Human-induced pluripotent stem cells (hiPSC), cell line: AIW002-02. Approved by the CHU de Quebec Research Center (#2022-6079)	Obtained through the C-BIG Repository at the Montreal Neurological Institute (The Neuro)	N/A
HEK293T	ATCC	CRL-3216
COS-7	Obtained from Dr. Chantal Guillemette	N/A
Neuro-2a (N-2a)	ATCC	CCL-131
Software and algorithms		
ImageJ	N/A	https://imagej.nih.gov/ij/
GraphPad Prism 9.0	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/
Other		
μ-Slide 8 well high glass bottom	ibidi	80807
24-Well TC plate	Sarstedt	83.3922
35-mm TC dish	Sarstedt	83.3900
35 mm dish | No. 1.5 coverslip | Poly-D-lysine coated	MatTek	P35GC-1.5-14-C
Cell scrapers	Fisher Scientific	08-100-241
Cellulose acetate membrane filter 0.2 μm	Sterlitech Corporation	CA022005
CO_2 incubator	Panasonic	N/A
Cytiva Whatman 3MM Chr chromatography paper	Fisher Scientific	05-716-3R
German glass coverslips #1.5 (8 mm)	Electron Microscopy Sciences	72296-08
German glass coverslips #1.5 (12 mm)	Electron Microscopy Sciences	72290-04
HC PL APO 63×/1.40 Oil CS2 (microscope objective)	Leica Microsystems	11506350

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Leica SP8 STED 3× WLL confocal laser scanning microscopy	Leica Microsystems	N/A
Lens heater with TPi controller	Tokai Hit	TPIE-LH
Light power meter LPM-100	Amuza	SKU: E00.170.00
Live-imaging chamber Leica SP8/SP5 Super Z Galvo stage	Tokai Hit	GSI2X
Microtube, polypropylene 1.5 mL	Sarstedt	72.706
Microplate reader, BioTek Cytation 5 multi-mode readers	Agilent	N/A
Nitrocellulose membrane 0.2 µm	Bio-Rad	1620112
Odyssey CLx infrared imaging system	LI-COR Biosciences	N/A
Sonicator model 505 Fisherbrand	Fisher Scientific	FB505110
Stage heater controller	Tokai Hit	STXG
T75, TC flask vent. cap	Sarstedt	83.3911.002
Trans-Blot Turbo transfer system	Bio-Rad	N/A
UHP-T-DI-LED series ultra-high power LEDs (blue light)	Prizmatix	UHP-T-460-DI
Ultracentrifuge – Sorvall MTX 150 micro-ultracentrifuge	Thermo Scientific	50135641
Vacuum manifold dot blot hybridization system	Fisher Scientific	11799545

MATERIALS AND EQUIPMENT

10× Running Buffer

Reagent	Final concentration	Amount
SDS	35 mM	100 g
Tris	247 mM	300 g
Glycine	1.9 M	1441 g
ddH_2O	N/A	Qsp. 10 L
Total	**N/A**	**10 L**

Store at 20°C–22°C for up to 6 months.

2× HEPES Buffered Saline (HeBS)

Reagent	Final concentration	Amount
ddH_2O	N/A	450 mL
NaCl	270 mM	8.0 g
KCl	10 mM	0.38 g
Na_2HPO_4 (M.W: 141.96)	1.50 mM	106.5 mg
Dextrose	11 mM	1.0 g
HEPES	42 mM	5.0 g
Total	**N/A**	**500 mL**

Adjust the pH to 7.05 with NaOH. Filter the stock solution with a 0.22-µm-pore filter before use. Store the filtrate solution in 50 mL aliquots at −20°C for up to 1 year. Once thawed, the HeBS solution can be kept at 4°C for several weeks without a significant effect on the transfection efficiency.

⚠ CRITICAL: It is critical to accurately adjust the pH of the 2× HEPES Buffered Saline. A pH lower than 6.95 prevents the DNA/buffer precipitate from forming, whereas a pH higher than 7.05 induces the creation of a coarse precipitate, consequently impacting the transfection efficiency.

2× Laemmli Buffer

Reagent	Final concentration	Amount
0.5 M Tris-HCl pH 6.8%/0.4% SDS	0.06 M	12 mL
Glycerol	20% v/v	20 mL
20% SDS	3.6% v/v	18 mL
Bromophenol blue	0.005% w/v	4 mg

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Reagent	Final concentration	Amount
2-mercaptoethanol	5% v/v	5 mL
ddH_2O	N/A	qsp. 100 mL
Total	**N/A**	**100 mL**

Store 2-mL aliquots at −20°C for at least 1 year.

Lysis buffer (Filter retardation/SDD-AGE)

Reagent	Final concentration	Amount
Triton X-100	0.05% v/v	5 µL
Phosphatase inhib. II 100×	1×	100 µL
Phosphatase inhib. III 100×	1×	100 µL
Protease inhib. 100×	1×	100 µL
100 mM PMSF	1 mM	100 µL
1× PBS	N/A	9.595 mL
Total	**N/A**	**10 mL**

The Lysis buffer should be prepared fresh before each experiment. The Lysis buffer can be kept on ice for 2–4 h.

10% SDS-PAGE gel (for 2 gels of 1 mm spacer thickness)

Reagent	Final concentration	Amount
Separation gel solution (10%)		
40% Acrylamide	10% v/v	2.50 mL
1.5 M Tris pH 8.8/0.4% SDS	0.38 M	2.50 mL
ddH_2O	N/A	4.90 mL
10% APS	0.03% v/v	30 µL
TEMED	N/A	12 µL
Total	**N/A**	**10 mL**
Loading gel solution (7%)		
40% Acrylamide	7% v/v	600 µL
0.5 M Tris pH 6.8/0.4% SDS	0.1 M	1 mL
ddH_2O	N/A	3 mL
10% APS	0.03% v/v	15 µL
TEMED	N/A	6 µL
Total	**N/A**	**4.6 mL**

Gels should be prepared fresh on the day of the experiment.

1.8% agarose gel (for SDD-AGE assay)

Reagent	Final concentration	Amount
Molecular Biology Grade Agarose	1.8% w/v	1.8 g
1 M Tris	20 mM Tris	2 mL
10 M glycine	0.2 M glycine	2 mL
1% SDS	0.02% v/v SDS	2 mL
ddH_2O	N/A	94 mL
Total	**N/A**	**100 mL**

Gels volumes prepared here are estimated for the exact thickness of 0.7 cm in a 10 × 15 cm casting tray. Adjust the thickness according to the tray size.

RT-QuIC reaction mixture

Reagent	Final concentration	Amount
5 mg/mL of recombinant monomeric α-syn	20 µg/mL	19.2 µL
336 mM EDTA	1 mM	14.3 µL
100 mM SDS	70 µM	3.36 µL

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Continued

Reagent	Final concentration	Amount
10 mM Th-T	10 μM	4.8 μL
150 mM NaCl	N/A	4.758 mL
Total	**N/A**	**4.8 mL**

The RT-QuIC reaction mixture recipe is listed for volumes of 200 μL per well for a total of 24 wells of a 96-well plate. Adjust the volumes accordingly.

Note: The reaction mixture should always be protected from light.

- **0.5 M $CaCl_2$ solution:** Prepare 0.5 M of $CaCl_2$ by adding 3.67 g of $CaCl_2$-$2H_2O$ in 50 mL of ddH_2O. Filter the stock solution with a 0.22-μm-pore filter before use.

Store the filtrate solution in 10 mL aliquots at −20°C for up to 1 year.

- **10× Transfer Buffer (wet transfer):** Prepare 1 L of 10× transfer buffer by adding 30 g of Tris and 144 g of Glycine to 1 L of water under constant stirring.

Store the solution at 20°C–22°C for up to 2 years.

- **1× Transfer Buffer (wet transfer):** Prepare 1 L of 1× transfer buffer by adding 100 mL of 10× transfer buffer and 200 mL of anhydrous ethanol (100% Et-OH) to 700 mL of ddH_2O. Store the solution at 4°C for up to 1 week.
- **Blocking Buffer (for WB):** Prepare 500 mL of blocking buffer (PBS-Tween 0.1%, 3% Fish Gelatin) by adding 33 mL of 40%–50% Fish Gelatin and 500 μL of Tween 20–467 mL of ddH_2O while stirring on a hot plate. Store 50-mL aliquots at −20°C for up to 6 months.
- **Blocking Buffer (for ICC):** Prepare 50 mL of blocking buffer (PBS 5% normal goat serum (NGS), 2% BSA, 0.1% Triton X-100) by adding 2.5 mL NGS, 1 g BSA and 50 μL of Triton X-100 to 47.5 mL of 1× PBS. Solution can be stored at 4°C for the duration of the experiment.
- **Buffer G:** Prepare 1 L of Buffer G by adding Tris (20 mM final concentration) and glycine (0.2 M final concentration) to ddH_2O. Store the solution at RT for up to 1 month.
- **Washing Buffer (for WB):** Prepare 1 L of washing buffer (PBS-Tween 0.1%) by adding 1 mL of Tween 20 to 1 L of ddH_2O.

Store buffer at 20°C–22°C for up to 1 week. Concentrated solutions (20×) can be prepared and stored at RT for up to 2 years.

- **100 mM (PMSF):** Prepare 10 mL of 100 mM PMSF by adding 174 mg of PMSF to 10 mL of isopropanol. Store 50 μL aliquots of PMSF at −20°C for up to 1 year.
- **Ponceau red solution (0.1% w/v):** Prepare 500 mL of Ponceau red solution by adding 0.5 g of Ponceau S and 25 mL of Glacial Acetic Acid to 475 mL of ddH_2O. Store 50 mL aliquots at 4°C protected from light in for up to 1 year.

CAUTION: Always open glacial acetic acid bottle under a chemical fume hood.

- **150 mM NaCl:** Prepare 50 mL of 150 mM NaCl by adding 0.438 g of NaCl to 50 mL of 1× PBS, adjust the pH of the solution with NaOH to reach a pH of 7.1. Store the solution at 20°C–22°C for up to 1 year.
- **336 mM EDTA:** Prepare 50 mL of 336 mM of EDTA by adding 4.9 g of EDTA to 50 mL of ddH_2O. Adjust the pH of the solution with NaOH to reach a pH of 8.0. Store the solution at 4°C for up to 1 year.

- **10 mM of Thioflavin-T (TH-T):** Prepare 10 mL of 10 mM Th-T, by adding 0.036 g of Th-T in 10 mL of ddH_2O and vortex the mixture for 30 s. Filter-sterilize the 10 mM Th-T solution using a 0.22-μM filter. Store the 10 mM Th-T buffer stocks in the dark at 20°C–22°C for up to 3 months.

STEP-BY-STEP METHOD DETAILS

Transfection and illumination of mammalian cells overexpressing LIPA plasmids

Timing: 2 days

This step will focus on explaining how to transfect HEK293T cells using the calcium phosphate technique in 35-mm dishes to reach more than 90% of the cells expressing the LIPA constructs. In the described protocol, we use homemade solutions, but it is also possible to order the CalPhos kit from Takara that performs as well. If transfecting other cell lines, you can alternatively use Lipofectamine 2000 or other lipofection-based methods following the manufacturer's instructions (https://www.thermofisher.com/ca/en/home/references/protocols/cell-culture/transfection-protocol/lipofectamine-2000.html - :~:text = Add the oligomer-Lipofectamine 2000, changed after 4–6 h) and the same plasmid concentration as described below. If focusing on neuronal cells, we are highly recommending using lentiviral particles with the phosphoglycerate kinase (PGK) promoter (supplemental protocol). In addition to PGK, alternative promoters capable of sustaining transgene expression in both iPSCs and neurons, such as enhancer/chicken β-actin (CAG) and human elongation factor-1α (EF-1α), could also be employed.[3]

Optional: Immortalized cell lines can be transduced using lentiviral particles obtained from the plasmid Cytomegalovirus (pCMV) blast harboring a puromycin resistance cassette allowing to create stable cell lines (supplemental protocol).

1. Plate 0.6×10^5 cells/cm^2 of HEK293T cells in a 35-mm plate in 1 mL of DMEM with 10% FBS, 1% Penicillin/Streptomycin (P/S) and incubate 16 h at 37°C and 5% CO_2.

⚠ CRITICAL: The greatest transfection efficiency was observed when cell confluency reached 60%.

2. After 16 h incubation, replace media with fresh DMEM with 10% FBS, 1% P/S 2 h prior to transfection.
3. Prepare three individual transfection mixes (LIPA-α-syn, LIPA-α-syn$^{\Delta NAC}$ and LIPA-empty) in 1.5-mL Eppendorf tubes by adding the following reagents in the defined order:
   a. Add plasmid DNA to the tube (LIPA-α-syn: 1 μg or LIPA-α-syn$^{\Delta NAC}$: 1.6 μg or LIPA-empty: 0.8 μg).

Note: In order to achieve uniform protein expression levels across the various LIPA constructs, we adjusted the quantity of DNA utilized for transfection of each plasmid and subsequently confirmed equal protein levels by Western blot, as we previously reported.[1]

   b. Add 25 μL of sterile ddH_2O.
   c. Add 25 μL of 0.5 M $CaCl_2$ (materials and equipment) and mix well by pipetting.
   d. While vortexing, add 50 μL of 2× HeBS (materials and equipment) dropwise.

⚠ CRITICAL: Use a high-speed shaking vortex to ensure efficient DNA-calcium phosphate co-precipitation.

4. Leave the precipitate from step 3 at 20°C–22°C for at least 10 min, and do not exceed an incubation time of 15 min.

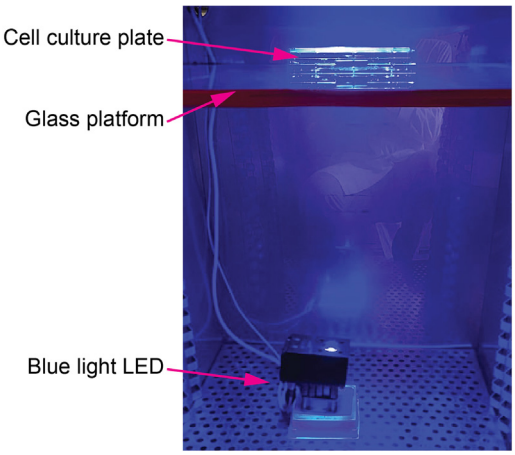

Figure 1. Set-up of blue light stimulation placed below the cell culture plate in the incubator

5. Add the mix from step 4 dropwise to the cells (100 µL/dish). Mix by gently swirling the plate until the medium color goes back to a uniform red color.

   Note: The total volume of added mix should not exceed 10% of the total volume of the culture media in the well.

6. Incubate the plate with the transfection mix for a minimum of 5 h or for a maximum of 16 h.

   ⚠ CRITICAL: For both optimal transfection efficacy and the maintenance of cell health, it is advisable to incubate cells with the transfection mix for a duration ranging from 5 h to 6 h. However, it is crucial to emphasize that while incubation for up to 16 h is permissible, any incubation period exceeding this limit is not recommended, as prolonged exposure to the transfection mix may potentially result in cell damage.

7. Following an incubation time of 5 h, aspirate the medium, and slowly add fresh medium to the plate.
8. Twenty-four hours post-transfection, 95% of the cells are expected to express the LIPA constructs and are ready for the light stimulation.
9. Illuminate the cells under a blue light (469 nm) at 0.8 mW/mm^2 for (0, 6, 12 and 24 h).

   Note: Blue light can be used at an intensity between 0.3 mW/mm^2 and 0.8 mW/mm^2 with time ranges starting from 1 min to 48 h, using UHP-T-DI-LED series Ultra High-Power LEDs (materials and equipment) (Figure 1).

   Note: Light intensity is measured at of the culture plate bottom, using the light power meter LPM-100 (materials and equipment).

   ⚠ CRITICAL: Light stimulation duration and intensity has been tested. Stimulating the cells using blue light at an intensity of 0.8 mW/mm^2 for up to 48 h, is below the phototoxicity threshold as confirmed in the study by Bérard et al.[1]

   ⚠ CRITICAL: The aggregation rate and the inclusions size are positively correlated to the light intensity and the duration of light stimulation. Both parameters play a role on the kinetics, reversibility and maturation of the aggregates and must be optimized. For instance, when illuminated at an intensity of 0.8 mW/mm^2, inclusion formation occurs significantly faster compared to an intensity of 0.3 mW/mm^2. Furthermore, illumination at 0.8 mW/mm^2 for up to 6 h results in reversible aggregates, while continued illumination

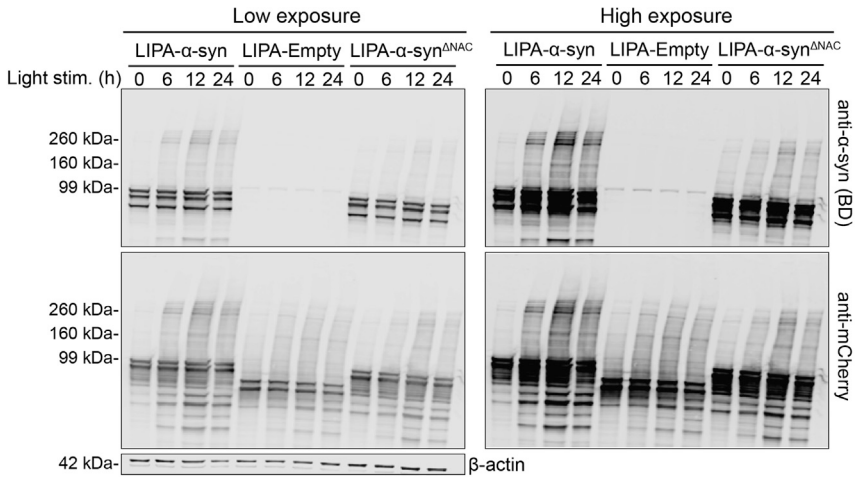

Figure 2. Time course formation of high-molecular LIPA aggregates using Western blot

WB analysis of aggregate formation of the LIPA constructs (LIPA-α-syn, LIPA-empty and LIPA-α-syn$^{\Delta NAC}$) at the different time points post-stimulation (0 h, 6 h, 12 h, and 24 h) with blue light (0.8 mW/mm^2) using anti-α-syn and anti-mCherry antibodies respectively. β-actin was used as the loading control.

for 24 h yields mature aggregates displaying key biochemical features akin to authentic Lewy bodies. Our optimization experiments in mammalian cells and iPSC-derived neurons demonstrated that employing a light intensity of 0.3 mW/mm^2 was sufficient to induce LIPA-α-syn inclusions in cells for up to 48 h without causing significant toxicity.

Biochemical characterization of the LIPA-α-syn aggregates – Western blot

Timing: 2 days

After induction of LIPA-α-syn aggregates, we proceed with the biochemical characterization of the light-induced α-syn inclusions. We start with the detection of the high molecular weight (HMW) α-syn aggregated species (Figure 2), which appear as a protein smear spanning approximately from 180 to 370 kDa in the Western blot and correspond to different α-syn oligomeric forms.[1,4]

Here we describe the steps of a quantitative Western blotting procedure using a LI-COR Odyssey Imager. The protocol could also be applied to any other standard Western blot procedure.

10. Remove the media of cells exposed to the blue light (from step 9) and wash them twice with 2 mL of ice-cold dPBS (Ca^+/Mg^+).

⚠ CRITICAL: The use of cold dPBS is recommended to minimize possible destabilization of LIPA-α-syn aggregates. Moreover, the addition of (Ca^+/Mg^+) is used to preserve cell-matrix interaction and avoid cell loss during the washing process.

11. Cells are directly lysed in 2× Laemmli buffer, by adding 250 µL of 2× Laemmli Buffer to a 35 mm plate (materials and equipment).
    a. Scrape the cells and collect the cells lysate and transfer into a microcentrifuge tube.

Alternatives: Other lysis buffers such as Radioimmunoprecipitation Assay (RIPA) buffer can be used for protein extraction from cells. The RIPA buffer can be stored at 4°C for up to 6 months. It is recommended to add protease and phosphatase inhibitors, phenylmethylsulfonyl fluoride (PMSF), as well as 1 mM of dithiothreitol (DTT) prior to cell lysis to minimize protein degradation and prevent the oxidation damage. Moreover, proceed with cell lysis on ice

and use gentle cell trituration to help minimize aggregates destabilization. When collecting in RIPA buffer, heat the samples at 70°C for 10 min prior to loading into Western blot gel to denature proteins for a proper migration in the gel.

Pause point: Although we always recommend the use of freshly collected cell lysate, protein samples can be stored for 7 days at −20°C after a snap freeze on dry ice. For longer storage periods, store the samples at −80°C.

12. Use 4%–20% precast polyacrylamide gradient gels for proper visualization of LIPA-α-syn HMW species.

Alternatives: Home-made 10% acrylamide SDS-PAGE gels can also be used to visualize LIPA-α-syn aggregates (materials and equipment). Troubleshooting 1.

Alternatives: Alternative membrane types, such as PVDF, can also be employed for Western blot detection of LIPA aggregates. However, we recommend the use of nitrocellulose membranes or FL-PVDF membranes to minimize background noise when using a LI-COR Odyssey Imager.

13. Heat the samples for 15 min at 95°C to help denature the proteins as well as DNA.
14. Load 5–10 μL (corresponding to 10–20 μg of protein) per well of each sample and allow the samples to migrate for 45 min at 160 V in 1× Running Buffer (materials and equipment).

Note: Quantifying the exact total protein amount in Laemmli buffer can be challenging. An alternative approach involves using Ponceau red staining to quantify dot blots with known increasing concentrations of BSA, generating standard curves and estimate the protein concentration in our samples. Moreover, normalization to a housekeeping protein, such as β-actin, is also required.

   a. Use a protein molecular weight marker (i.e., Chameleon Duo ladder) to estimate the molecular weight of proteins of interest and to monitor the progress of electrophoresis.

15. After the SDS-PAGE gel electrophoresis, follow a wet-transfer protein transfer protocol to transfer the protein from the gel to a 0.2 μm pore nitrocellulose membrane.

Alternatives: The wet transfer protocol is more appropriate for transferring larger proteins (>100 kDa), making it the recommended choice for effectively visualizing high molecular weight (HMW) α-syn oligomers. Alternatively, semi-dry transfer or Bio-Rad's Turbo transfer can be used but optimizing transfer time and voltage might be necessary. Troubleshooting 1.

16. All the components (Whatman filter papers, sponges, and membranes) are mounted and soaked in a cold 1× transfer buffer (materials and equipment):
   a. To assemble the transfer "sandwich" in a transfer cassette, the pre-wet nitrocellulose membrane and gel are packed together inside one pre-wet sponge followed by one Whatman filter paper. The gel needs to face the negative electrode while the nitrocellulose membrane faces the cathode.
   b. After assembling the transfer stack components, remove air bubbles with a roller or pipette.
   c. Place the transfer cassette inside the transfer tank.
      i. Add a magnet to the bottom of the apparatus to allow for constant stirring at low speed to maintain an even buffer temperature buffer distribution in the tank.
      ii. Place an ice block in the transfer tank to maintain an even and cold buffer temperature.
      iii. Put the transfer tank in a bucket and add ice to ensure that the tank temperature does not increase during the transfer protocol.
      iv. Perform the transfer protocol at 4°C.

Alternatives: PVDF membranes can be used, membranes must be wet in methanol. However, methanol-free transfer buffers can be used downstream.

Alternatives: Transfer of the protein can be performed at 4°C, on ice, overnight at 90 mA.

17. Run the transfer for 2 h at 350 mA.
18. After the transfer, rinse the membrane in 1× PBS.
19. Heat the membrane in a microwave by submerging it in a microwave-safe container filled with approximately 50 mL of PBS.
    a. The container containing the membrane and PBS is then irradiated using a household microwave oven set at 500 W for a duration of 1 min.
    b. This step will result in unfolding the proteins on the membrane, thus allowing for the proper exposure of epitopes and a better detection of the protein of interest.

⚠ CRITICAL: Avoid overheating the membranes by going over the 1 min duration suggested. Prolonged heating can lead to damage to the nitrocellulose membrane, thereby impeding proper protein detection.

20. Allow the membrane to cool down to 20°C–22°C before proceeding with the blocking step.

Pause point: Membranes can be stored for 16 h at 4°C in 1× PBS, protected from light.

21. Block the membranes for 1 h at 20°C–22°C in WB blocking buffer (materials and equipment) under constant rocking.
22. After blocking, incubate the membranes for a minimum of 2 h at 20°C–22°C with anti-α-syn antibody (BD Lab) at 1:1000 diluted in WB blocking buffer (materials and equipment).

Pause point: Membranes can be incubated with primary antibody for 16 h at 4°C under constant rocking.

23. Wash the membrane with WB washing buffer (materials and equipment) 3 times for 5 min each under constant rocking.
24. Incubate the membrane for a minimum of 1 h with an anti-mouse 680RD LI-COR secondary antibody diluted at 1:10000 in WB blocking buffer (materials and equipment).
25. Wash the membrane with WB washing buffer (materials and equipment) 3 times for 5 min each under constant rocking.
26. Detect the protein bands using an Odyssey CLx Infrared Imaging System using the IR680 channel.

⚠ CRITICAL: Always image both channels (IR680 and 800) to visualize all the bands of the molecular weight marker.

27. Repeat steps 22–23 using the anti-mCherry antibody as primary antibody, diluted at 1:2000.

Use an anti-rabbit 800CW LI-COR secondary antibody diluted at 1:10000 in WB blocking buffer (materials and equipment).

28. Evaluate and quantify the smear corresponding to the LIPA-α-syn aggregates using the Image Studio Software or the Empiria Studio Software from LI-COR.

Alternatives: Alternatively, we can use an antibody raised against the pathological form of α-syn phosphorylated at the residue serine 129 (pS129) diluted at 1:2000 (materials and equipment).

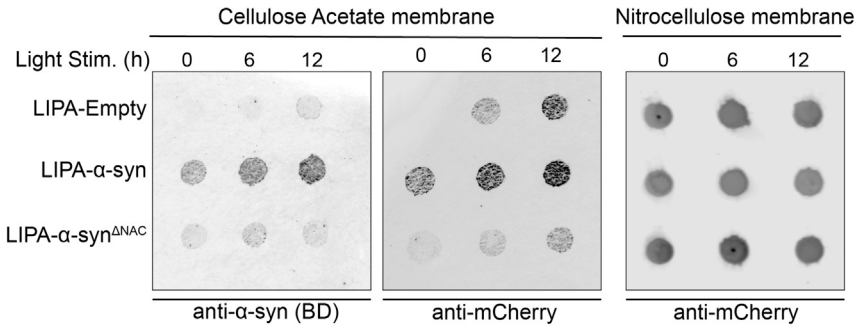

Figure 3. Time course formation of high-molecular LIPA aggregates using filter retardation assay

Filter retardation assay on cellulose acetate membrane showing the time course of the LIPA aggregation formation at the different time points post-stimulation (0 h, 12 h, and 24 h), as examined using anti-α-syn and anti-mCherry antibodies respectively. Prior to introducing SDS, samples were filtered through a nitrocellulose membrane for loading control. This step enables the retention of both monomeric and aggregate proteins, facilitating the assessment of total protein levels in each experimental condition. Membrane was examined using the anti-mCherry antibody.

Note: For loading control, repeat steps 22–23 using the same membrane, and incubate with anti-β-actin antibody, diluted at 1:5000. Use an anti-mouse 800CW LI-COR antibody diluted at 1:10000 in WB blocking buffer (materials and equipment).

Alternatives: β-actin is a popular choice as a Western blot loading control due to its high and ubiquitous expression, as well as its stability under various experimental conditions. Alternative loading controls can be beta tubulin and GAPDH since they are constitutively expressed in high quantities in all model systems.

Biochemical characterization of the LIPA-α-syn aggregates – Filter retardation assay

Timing: 2 days

Another characterization step to validate the insoluble status of the generated aggregates is the filter retardation assay. We describe below the steps that allow for the detection and differentiation of LIPA-α-syn aggregates from empty (control) aggregates and the non-aggregatable form LIPA-α-syn$^{\Delta NAC}$ (Figure 3).

29. Remove the media of cells exposed to the blue light (from step 9) and wash them twice with 2 mL of cold dPBS (Ca^{+}/Mg^{+}).
30. To a 35-mm plate add 250 μL of Lysis buffer (materials and equipment).

Using a cell scrapper, scrape cells off the dish and transfer the cell suspension into a microcentrifuge tube.

Pause point: Samples can be stored at −80°C for 2–4 days after a snap freeze on dry ice.

Note: It is always recommended to use fresh samples and avoid freeze/thaw cycles.

31. Lyse the cells using a Dremel tissue homogenizer.

Optional: Cells can be lysed using a water bath sonicator at 20% Amplitude (30 s with pulses every 2 s (1 s on/off).

32. Centrifuge the samples at 500 × *g* for 5 min to pellet the nuclear fraction. LIPA-α-syn aggregates should remain in the supernatant (soluble fraction).

33. Collect the supernatant and centrifuge at 1000 × *g* for 10 min to clarify the fraction.
34. Collect the supernatant and place it on ice.
35. Mount the vacuum manifold (materials and equipment) using two thin Whatman filter papers pre-soaked with 1× PBS, 1.5% SDS and assemble them on the vacuum manifold.
36. On top of the filter paper, place a 0.2 μm pore size acetate membrane pre-soaked in 1× PBS containing 1.5% SDS.
37. Tightly close the manifold and apply the vacuum to check for the proper tightening of the vacuum manifold. Turn off the vacuum.
38. Prepare the samples:
    a. Put aside 20% of the volume of cell lysate collected from each experimental condition (step 34), which will be used as follow:
        i. Loading control: Half of this volume will be run in Western blot and analyzed on a nitrocellulose membrane (step 45) to evaluate the total amount of the LIPA protein in the starting material.
        ii. Negative control: The other half of the volume will be depleted from the LIPA-α-syn aggregates by adding SDS to the sample to reach 1.5% SDS final concentration. Heat this sample for 15 min at 95°C to denature the protein.
    b. Add SDS to the samples from step 34 to reach 1.5% SDS final concentration and incubate for 10 min at 20°C–22°C.
39. While the samples are incubating in SDS, load 100 μL of 1.5% SDS solution in each well and apply vacuum until the buffer passes through the filter. Wait until the filtration is complete.

CAUTION: Ensure that the wells are not entirely dried before loading the sample. Complete drying can lead to an uneven sample distribution, causing halos formation during protein detection.

40. Quickly load the samples from step 34 into the manifold (samples incubated with SDS at 20°C–22°C and the negative controls) into each well (50 μL is the recommended volume per sample).

    Note: Load the samples as duplicates or triplicates for proper quantification.

    ⚠ CRITICAL: Fill the empty wells with a 1.5% SDS solution. This step is crucial for maintaining consistent vacuum pressure across the entire membrane.

41. Apply the vacuum to filter the samples until complete passage. This step lasts typically from 30 s to 2 min.
42. Load 100 μL of 1.5% SDS solution into the wells and apply vacuum again to filter the solution. Wait until complete passage.
    a. Immediately turn the vacuum off to prevent excessive drying. Repeat this washing step at least 1 time.

    Note: This wash step may be repeated as necessary when the background signal may appear to be too high in the control samples. This high background is often dependent on the cell type, the expression levels of the LIPA mCherry aggregates, and the number of aggregates Troubleshooting 2.

43. Unmount the vacuum manifold, take the acetate membrane and rinse it in Milli-Q water.
44. Place the membrane in PBS at 4°C while running the loading controls.
45. To run the loading controls, follow steps 41 to 45 but instead:
    a. Use a 0.2 μm pore Nitrocellulose membrane. This membrane will capture the total protein fraction, offering insights into loading control for each experimental condition.
    b. Use PBS without SDS as buffer for all the subsequent steps 38–42.

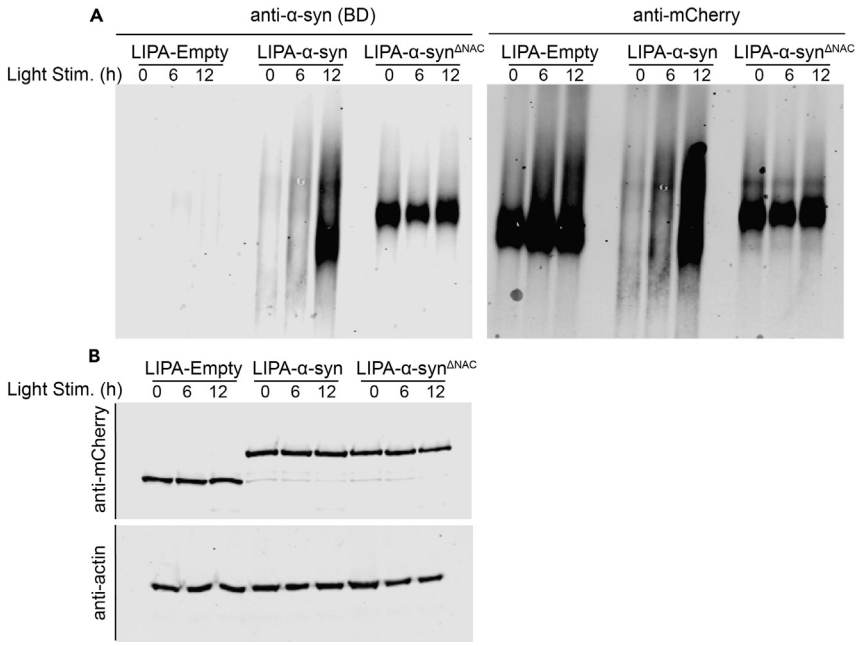

Figure 4. Time course formation of high-molecular LIPA aggregates using SDD-AGE

(A) SDD-Age analysis showing the formation of high-molecular LIPA-Empty and LIPA-α-syn aggregates at different times points post-stimulation (0 h, 6 h, and 12 h) with blue light (0.8 mW/mm^2), as examined using anti-α-syn and anti-mCherry antibodies respectively.

(B) For loading control, samples were run on WB and protein expression was examined using anti-mCherry and anti-β-actin antibodies respectively.

46. For the acetate membrane containing the samples and the negative control from steps 41–45, perform the blocking and incubation with antibodies as described in the Western Blot protocol (step 19 to step 26).
47. For the Nitrocellulose membrane from step 45 used for the loading controls, reveal for the total protein using a Ponceau red solution (materials and equipment):
    a. Place the membrane into a flat-bottom container, protein side up.
    b. Stain the membrane in Ponceau red solution and place on a rocker.

    Note: Nitrocellulose membranes saturate in around 5 min.

    c. Quickly rinse the membrane with distilled H_2O (dH_2O).
    d. Reduce the Ponceau red staining through repeated washes in 1× PBS until achieving a clear total protein stain.
    e. Image the membrane and assess total protein levels in each condition.
48. Visualize and quantify the filter retardation signal using the LI-COR Odyssey Imager and Image Studio Software.

Biochemical characterization of the LIPA-α-syn aggregates– SDD-AGE

Timing: 2 days

Another significant step in the characterization of LIPA-α-syn aggregates involves employing the semi-denaturing detergent–agarose gel electrophoresis (SDD-AGE) technique to provide additional confirmation of the presence of insoluble high molecular weight (HMW) α-syn aggregate species[5–7] (Figure 4).

49. Remove the media of cells exposed to the blue light (from step 9) and wash them twice with 2 mL of cold dPBS Ca^{+} Mg^{+}.
50. Follow the lysis and centrifugation steps as described for the filter retardation (steps 29–34).

Note: Keep 10% of the sample volume for the detection of the total protein levels by Western blot.

51. From the remaining samples, determine the volume of the supernatant and mix the supernatant with the SDD-AGE sample buffer (materials and equipment).
    a. Incubate the sample mix at 20°C–22°C for 7 min.

Note: A 7 min incubation period is necessary to observe the smearing bands for both α-syn and mCherry signals. This duration proved to be the optimal time for achieving the desired smearing effect in the samples. Using shorter incubation times may not yield the desired smearing effect. While some protocols have recommended incubating samples for up to 10 min, it's important to note that we have not tested this specific incubation time in the context of this protocol.

52. Load the samples onto a 1.8% agarose gel (materials and equipment) prepared in buffer G (materials and equipment) containing 0.02% SDS.

Note: Allow the liquid agarose to cool down to a temperature ranging between 60°C–65°C before slowly adding the SDS while avoiding solidification or air bubble formation.

53. Run the SDD-AGE in buffer G containing 0.01% SDS at 12V/cm at 20°C–22°C for 2 h until the dye front reaches the end of the gel. Troubleshooting 3.

Alternatives: Gels can be run at 4°C to minimize the effect of the heat generated from the high voltage.

54. Transfer the gel into a nitrocellulose membrane using the Turbo transfer method provided from Bio-Rad.

Note: Here, we suggest utilizing the Turbo transfer method from Bio-Rad, benefiting from the machine's reliable and compact membrane stacking. This approach ensures effective protein transfer onto the membrane.

Alternatives: Wet transfer methods can be performed as described by Dr. Lindquist group.[8]

55. Following the transfer step, follow the steps described in Western blot protocol for the visualization and quantification of the aggregates (steps 19–26).

In vitro seeding experiment using RT-QuIC (real-time quaking-induced conversion)

Timing: 7 days

One of the prominent features of α-syn aggregates is their ability to self-propagate through the recruitment of monomeric counterparts, a phenomenon referred to as seeding capacity. Here, we evaluate the seeding capacity of LIPA-α-syn aggregates, using the RT-QuIC assay. This assay allows for the monitoring of the formation of seeded aggregates from recombinant monomeric α-syn through a template-seeded aggregation process using LIPA-α-syn aggregates as the seeding template material. As the newly formed aggregates develop, they interact with an amyloid-sensitive dye called thioflavin T (ThT), leading to an observable increase in fluorescence signal. The RT-QuIC

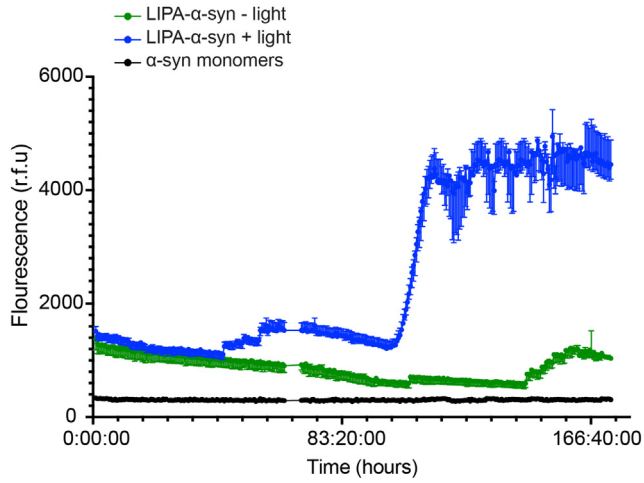

Figure 5. Real-time quaking-induced conversion (RT-QuIC) analysis for the validation of the seeding capacity of purified LIPA-α-syn aggregates *in vitro*

RT-QuIC analysis illustrating the kinetics of recombinant monomeric α-syn aggregation in the presence of purified LIPA-α-syn aggregates (+light), and monomeric LIPA-α-syn (−light). The average Th-T fluorescence intensity was plotted against time, data are presented of triplicate wells. The data are presented as the means ± SEM.

protocol we outline here offers the advantage of facilitating high-throughput assessment of the kinetics of LIPA- α-syn aggregate seeding capacity in real-time (Figure 5).

56. Remove the media of cells exposed to the blue light (from step 9) and wash them twice with 2 mL of cold dPBS Ca^{+} Mg^{+}.
    a. As a negative control, cells not exposed to the blue light can be used for validation of the seeding capacity of the LIPA- α-syn aggregates.
57. Follow the lysis and centrifugation steps as described in steps 29–34.
58. The clarified lysate from step 57 is then subjected ultracentrifugation at 150,000 × *g* for 1 h at 4°C.
59. The pellet from step 58 gets resuspended in 150 μL of PBS.
60. The pellet from step 59 is then subjected to sonication at: 30% intensity, 1 s on and 1 s off, for 45 s using a Fisherbrand Model 505 sonicator.

Note: If the pellet remains visible in the tube, you may repeat the sonication step up to two times until the pellet becomes no longer visible by eye. It may be necessary to perform additional pipetting to confirm the absence of the pellet.

⚠ CRITICAL: It is important not to add any additional lysis buffer to prevent excessive sample dilution.

61. Determine the protein concentration of the cell pellet using colorimetric assay kits such as: Pierce BCA Protein Assay Kits.
    a. Dilute the samples to reach a final concentration of 2 μg/mL.
62. In a black clear bottom 96-well plate add 200 μL of the RT-QuIC reaction mixture (materials and equipment) containing monomeric α-syn.
    a. To the mixture add 2 μg/mL of LIPA-α-syn aggregates by carefully pipetting the sample into each well.

Note: The volume of the sample at 2 μg/mL should not exceed 10% of the total reaction volume.

Table 1. RT-QuIC plate reader program details

Plate type	96 well plate clear bottom black side
Discontinuous kinetics	Runtime 7:00:00 (D:HH:MM), Interval 0:00:30, 350 reads
Set temperature	Setpoint 41°C, Gradient 0°C
	Preheat before moving to next step
Shake	Orbital: 1:00 (MM:SS)
	Frequency: 425 cpm (3 mm)
Delay	0:02:00 (HH:MM:SS)
Read	Fluorescence end point
	Full plate
	Filter Set 1
	Excitation: 450/10, Emission: 480/10
	Optics: Bottom, Gain: extended
	Light source: Xenon flash, Lamp energy: High

CPM, cycle per minute.

b. For the negative control condition, carefully pipette 2 μg/mL of LIPA-α-syn monomers purified from the cells that were not exposed to the blue light.
c. Another experimental control will include wells of the RT-QuIC reaction mixture (materials and equipment) containing recombinant monomeric α-syn alone.

63. Plates are covered with sealing film, and creases are removed manually.
64. Plates are placed in a plate reader and the program is setup as described in Table 1.

Note: The preheat option of the plate must be set to allow the plate to reach the optimal temperature (41°C) before starting the experiment.

Alternatives: Alternative plate readers, such as the FLUOstar OMEGA plate reader (BMG Labtech, Germany), can also be used for the RT-QuIC experiment.

⚠ CRITICAL: The kinetics of LIPA-α-syn aggregation using RT-QuIC can be influenced by various factors, including the temperature used for the plate reader, the shaking time, and the resting period. Additionally, the chemical factors of the reaction mixture such as the pH, the concentration of monomeric α-syn, and the percentage of the detergent such as SDS need to be followed based on the optimized protocol we describe here.

Alternatives: Silica beads can be added to the plates, which have been suggested to help shorten the assay incubation period. However, in the present protocol we did not test the addition of beads to the plates.

65. Allow the program used in step 64 to run for up to 1 week.
66. The Th-T fluorescence is measured (excitation 435 nm; emission 485 nm) every 30 min using (BioTek Cytation 5 Multi-Mode Readers) plate reader.
67. Fluorescence is recorded and graphed against time in h, and depicts a typical exponential curve characterized by a lag phase and a plateau. Troubleshooting 4.
68. For standardizing the data, a positive RT-QuIC seeding reaction is recorded when the observed TH-T fluorescence intensity is equal to or higher than a predefined threshold.
    a. The threshold can be set at three times the fluorescence intensity observed after the initial amplification phase recorded at time 0 h.

Note: RT-QuIC, experiments need to be performed at least 3 times with 3 replicates per experiment.

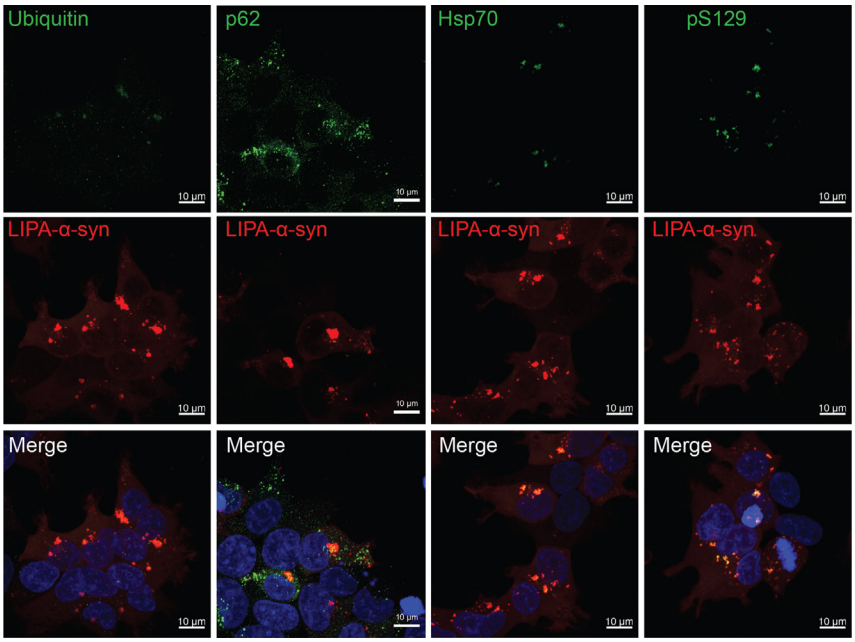

Figure 6. LIPA-α-syn inclusions are positive to authentic Lewy body (LB) markers

Confocal images of HEK293T cells overexpressing LIPA-α-syn exposed to blue light for 12 h (0.8 mW/mm^2) and stained with LB markers: ubiquitin, p62, Hsp70, and phosphorylated α-syn at S129 (pS129). (Scale bar = 10 μm).

Note: The Rt-QuIC assay was performed in at least 3 independent experiments with 3 technical replicas per experiment. The average ThT fluorescence intensity was plotted against time (n = 3). The data are presented as the means ± SEM.

Detection of the key biochemical markers of authentic Lewy bodies using immunocytochemistry approach

Timing: 2 days

Subsequently, we explored if light-induced LIPA-α-syn inclusions could replicate essential biochemical characteristics of genuine LBs. This was achieved through the application of a range of LB markers identified in PD-affected brains, namely phosphorylation of α-syn at serine 129 (pS129), ubiquitin, SQSTM1 (p62) and hsp70 (Figure 6).

69. Plate 0.8 × 10^5 cells/cm^2 of HEK293T cells on poly-L-lysine-coated 12-mm coverslips (see before beginning) in a 24-well plate in DMEM 10% FBS 1% P/S. Incubate for 16 h at 37°C 5% CO_2.
70. Transfect the cells following the previously described method (step 2 to step 8), using 0.25 μg LIPA-α-syn, 0.40 μg LIPA-α-syn$^{\Delta NAC}$ and 0.20 μg LIPA-empty. DNA was adjusted for each LIPA construct to obtain equal protein expression levels in the different conditions.
71. Illuminate the cells for 16 h under the blue light, at the intensity of 0.8 mW/mm^2, to induce LIPA aggregation.
72. Following illumination, fix the cells for 15 min at 20°C–22°C in a solution of 4% (w/v) paraformaldehyde (PFA) and 3% (w/v) sucrose.
73. Wash the cells three times with 1× PBS for 5 min each.
74. Permeabilize the cells using a solution of 0.3% (v/v) Triton X-100 in 1× PBS for 10 min at 20°C–22°C.
75. Rinse the cells once with 1× PBS to remove any remaining Triton.

76. Incubate cells for 1 h at 20°C–22°C in ICC blocking buffer (materials and equipment).
77. Incubate cells with primary antibodies raised against pS129, ubiquitin, p62 or hsp70 diluted in blocking solution (materials and equipment) at 4°C for 16 h.

Optional: Incubation with the primary antibodies can be shortened to 2 h at 20°C–22°C.

78. Perform three 10-min washes with fresh blocking buffer (materials and equipment).
79. Incubate cells in secondary antibodies (Alexa Fluor 488 or 633) diluted at 1:400 for 1 h at 20°C–22°C.
80. Perform two 10-min washes with 1× PBS.
81. Stain the cell nuclei with DAPI at 1:5000 to reach 1 μg/mL in 1× PBS for 2 min.
82. Perform two quick washes with 1× PBS.
83. Before mounting, quickly rinse the coverslips with dH_2O to remove any residual salt.
84. Mount the 12-mm coverslips onto a microscope slide using 4.5 μL of mounting media.
85. Image LB-markers staining using confocal microscope, as we previously reported.[1]

Real-time monitoring of LIPA-α-syn inclusion formation – Live-cell imaging

Timing: 2 days

A key benefit of optogenetics is its ability to provide precise spatiotemporal resolution in light stimulation, enabling real-time monitoring of LIPA-α-syn aggregation process. Here, we present a live imaging protocol allowing for the continuous observation of LIPA-α-syn inclusion formation using confocal microscopy (Methods video S1).

Note: The following protocol has been optimized on a confocal Stimulated emission depletion (STED) microscope Leica Dmi8 TCS SP8 STED 3D X using a x63 objective (1.40 NA). Moreover, the microscope is equipped with a White Light laser (WLL) that can be set at any wavelength, set at 70% power (input power), and then used in a range of 5%–30% power (software end laser power).

86. Plate 0.8 × 10^5 cells/cm^2 of HEK293T cells on a pre-coated glass-bottom imaging chamber such as a 35-mm MatTek dish.

Alternatives: If multiple conditions are required, cells can be plated in a chamber containing multiple wells (ibidi chambers), and any glass-bottom fitting your microscope setup would work.

87. Transfect the cells following steps 2–8.
88. One hour prior to the start of the experiment replace the cell culture media with pre-warmed (37°C) live-cell imaging medium (DMEM FluoroBrite, 10% FBS and GlutaMAX).

Note: If working with primary neurons or iPSC-derived neurons, we recommend using Neurobasal medium without phenol red.

89. While the cells are incubating, prepare the microscope live-imaging setup:
    a. Mount the stage-top incubator on top of the Super Z Galvo stage.
    b. Fill the stage-top incubator with 15 mL of sterile ddH_2O.
    c. Open the CO_2 cylinder, linked to the heating and gas mixing controller, and adjust the percentage to 5% CO_2. Wait for 10 min for CO_2 stabilization.
    d. Turn-on the stage heating and top heating. Set the stage at 40°C and the top at 43°C.

Note: When using the above suggested parameters, the sample temperature will be maintained at 37°C. Depending on the system used and the environment, these values can be adjusted once the sample temperature has been measured.

Optional: Attach the lens heater to the objective and turn-on the controller to allow it to reach 37°C.

Note: The use of a lens heater will stabilize the system temperature, thus maintaining the immersion oil at 37°C, avoiding any Z-drift during imaging.

e. Place a drop of immersion oil (type F) on the lens and immediately place the live-cell chamber inside the holders of the stage-top incubator.
f. Close the stage-top incubator and use the cursor to get the object into focus.

⚠ CRITICAL: At this point, do not start the imaging session since the system is not yet stabilized. Instead, wait for 15–30 min and redo the focus (the sample would have drifted away of the immersion oil, through a phenomenon called thermal drift).

90. To induce and image the aggregation in real time:
    a. Set one sequence of image acquisition meant to stimulate and record the mCherry signal corresponding to the LIPA.
        i. Set the WLL at 587 nm.
        ii. Open a HyD detector to capture signals ranging from 595 to 650 nm.
    b. During the same image acquisition sequence, add a WLL laser at 470 nm to stimulate the LIPA aggregation.

Note: If there is no WLL laser available to be set at exactly 470 nm, it is possible to use a 488 nm laser. We do not recommend using argon laser (405 nm) since this wavelength is toxic. Troubleshooting 5.

c. Choose a cell that is expressing a high level of mCherry and start an image trial, playing with the laser power at 470 nm.

Note: A laser power of 5% or less will induce a slow aggregation pattern, while a 30% laser power induces robust aggregation with aggregates appearing as early as the stimulation starts. Troubleshooting 6.

d. Start acquiring a Z-stack overtime, using a scan speed between 400 and 8000 Hz and a resolution adapted to the optical zoom used.

⚠ CRITICAL: Imaging should be done continuously since LIPA-α-syn aggregates are reversible at early time points. The imaging parameters should be optimized to obtain a good resolution, while minimizing the phototoxicity during imaging.

Note: We recommend starting with 700 Hz scan speed. A resonant scanner at 8000 Hz is more adapted to track quick interactions between the aggregates and organelles for example inside the cell.

Note: Using the above-described protocol, we were able to induce and monitor LIPA-α-syn aggregation in HEK293T and COS-7 cells, as well as in human iPSC-derived dopaminergic neurons. We were also able to perform up 12 h of continuous live imaging without inducing cell death, by using high-speed laser scanning microscopy (resonant scanner).

Note: If a GFP-like reporter is present in the green channel, it is possible to acquire both channels simultaneously, minimizing the crosstalk. For this purpose, use HyD detectors while narrowing the band pass. If the microscope is not set up to acquire the two wavelengths

simultaneously, create two sequences of imaging and make a sequential Z-stack at high-speed scanning.

Supplement protocol to transduce cells with the LIPA plasmids

Transduction of hiPSC-derived dopaminergic neurons

Timing: 7–10 days

While immortalized mammalian cells are prevalent for α-syn aggregation studies, employing iPSC-derived human neurons present distinct advantages. These neurons closely mimic human neuronal physiology, enhancing the fidelity of neurological disorder investigations. However, transfecting iPSCs is challenging, necessitating lentivirus-mediated transgene overexpression. Here, we outline the sequential stages involved in transducing iPSC-derived neurons with lentiviruses carrying LIPA constructs.

Note: Using plasmids with a phosphoglycerate kinase 1 (PGK) promoter is crucial for the proper transduction of iPSC-derived neurons, as CMV tends to be repressed as hiPSC are differentiated towards neurons.[9,10]

Note: For a complete and detailed protocol on how to differentiate iPSCs into mature dopaminergic neurons, please follow our recently published protocol.[11]

91. Following the protocol of 9 days *in vitro* (DIV) culture that allows for the differentiation of iPSCs to immature dopaminergic neurons, thaw a lentiviral tube from −80°C, and place it on ice.

Note: Lentiviral particles are generated from HEK293T following the protocol from Tiscornia et al.[12] Viral particles are titrated using HIV-1-based qPCR kits ABM (ABM; cat. no. LV900).[11]

92. Prepare a master mix of cell culture medium (STEMdiff Maturation media) coupled with lentiviral particles to reach a multiplicity of infection of 10 (MOI = 10).

Note: The suggested dilution is only an example, it may be necessary to experiment with higher or lower dilutions. These dilutions can be adjusted based on the viral titration results obtained.

93. Slowly add the maturation media containing the viral particles to the neurons.
94. Incubate for 7–10 days, changing half the media every 4 days.

⚠ CRITICAL: Seven days post infection, check the cells every day for expression detection. The viral expression peak should be reached 2 days after the first expression of the mCherry signal.

Note: Analysis should start when the peak of expression is observed (usually 2 days after the first expression of the mCherry signal appears) . Do not wait for longer than 4 days, as cells will be stressed and stress granules might appear and preclude the detection and observation of LIPA aggregates.

Creation and use of stable cell lines expressing the LIPA backbones

Timing: 10–15 days

Opting for a stable expression of the LIPA backbone in immortalized cell lines, instead of transient transfection, is advised for efficiency, cost effectiveness, and enhanced data reproducibility. The

forthcoming protocol elucidates the procedure for establishing, sustaining, and utilizing stable cell lines that express the LIPA constructs.

Note: We use the pCMV lentivirus which allows for a high level of expression in mammalian cell lines. Cells with stable transgene expression can be selected by applying puromycin selection pressure.

Note: Prior to starting an experiment for the creation of a stable line, it is necessary to establish the ideal dosage of puromycin for the specific target cell line. This can be achieved by subjecting the target cells to a series of puromycin doses and identifying the lowest dose that effectively kills off all the cells.

FACS sorting can also be used in parallel following puromycin selection to enrich and collect for the highest expressing mCherry cells.

Alternatives: The SIN-PGK lentiviral backbone can be used. However, it lacks a puromycin resistance cassette for the selection pressure and the use of FACS to select cells with stable transgene expression is necessary.

95. Plate cells at 20% confluency in 6-well plate and incubate them at 37°C and 5% CO_2 for 16 h.
96. Thaw a tube of lentiviral particles from −80°C on ice.
97. The next day add the following in the cell culture media with:
    a. Polybrene, to reach 10 μg/mL (1000-fold dilution from the stock prepared at 10 mg/mL) final concentration.
    b. Lentiviral particles diluted to reach a MOI of 1, 10 or 100.

Note: The suggested dilutions are only examples, it may be necessary to experiment with higher or lower dilutions. These dilutions can be adjusted based on the viral titration results obtained.

98. Incubate the cells with the medium from step 97 for 3 days at 37°C and 5% CO_2.
99. After 3 days, split each confluent well into 6 wells of a 6-well plate in complete cell culture medium, and incubate at 37°C and 5% CO_2 for 16 h.
100. The next day, expose the cells to increasing concentrations of puromycin (1–1.5–2–2.5–3–5 μg/mL).
101. After 3–5 days, when cells reach confluency, sort the cells using a flow cytometer for mCherry expressing cells, while keeping the 30% highly expressing cells.

Note: Steps 95–101 describe a protocol that will allow for the selection of a polyclonal cell line, but the mCherry will remain unequal between cells. A high expression of the LIPA backbone is necessary to allow for an efficient Cry2olig oligomerization under blue light stimulation.

102. Plate the sorted cells in two T25 flasks; One will be used for cryopreservation, and the second one will be used for monoclonal selection.

Note: To ensure stability of the line created, it is advisable to generate monoclonal cell lines from the polyclonal pool at the early stages of the protocol.

103. When the T25 flask is 70% confluent, passage the cells to plate 100 cells in a 10-cm dish. Incubate for 3–5 days at 37°C and 5% CO_2 to allow the colonies to grow.
104. Under a fluorescent microscope, look for colonies with the highest expression of mCherry that are well spaced to ensure no contamination from surrounding colonies. When a desired colony is found, circle the colony with a pen.

105. Using a 20 μL pipette set to a volume of 15 μL, scrape the colony with the pipette tip and then aspirate the colony.

Note: The colony should be visible inside the tip.

106. Transfer the selected colony to an empty well of a 96-well plate.
    a. Add 150 μL of medium to the well of a 96-well plate.
    b. Repeat this step until at least 20 colonies have been selected from the 10-cm plate.
107. Incubate the 96-well plate for 5–7 days at 37°C and 5% CO_2 until the cells recover, changing media every 3 days.
108. When the cells reach confluency, expand the culture, and cryopreserve the cells as soon as 70% confluency is reached in a T75 flask.

⚠ CRITICAL: Once the monoclonal LIPA clones are isolated and grown, it is important to keep the cells protected from ambient white light since it may trigger Cry2olig oligomerization. We recommend turning off the direct white light in the sterile hood while maintaining and plating the cells.

⚠ CRITICAL: Cells should never reach 100% confluency and medium should never turn yellow. We observed that when cells become overconfluent, or when the cell culture media turns yellow, this trigger self-aggregation of the LIPA-α-syn construct. Therefore, we recommend passaging the cells every 3–4 days.

109. Cells stably expressing the LIPA backbones should be plated 12–16 h before being exposed to blue light.

⚠ CRITICAL: To obtain the best levels of aggregation, it is important to illuminate cells 12 h post plating and no more than 16 h to avoid the cell division event. Indeed, we suspect that cell division might interfere with the aggregation process, reducing the aggregation propensity and kinetics.

EXPECTED OUTCOMES

The outlined protocols have undergone validation across various immortalized cell lines (HEK293T, COS-7, and N-2a) using transient transfection or in stable cell lines overexpressing the LIPA system. Furthermore, we have successfully validated these methodologies with different human iPSC lines and various types of derived neurons, including both glutamatergic and dopaminergic neurons. Utilizing the LIPA system enables rapid generation of α-syn aggregates within a matter of seconds. As these aggregates mature, they undergo physiological changes that fully mimic authentic-Lewy bodies' biochemical features.

In addition to the notable resemblances between the optogenetically induced α-syn aggregates and the pathological LB observed in the brains of individuals with PD, the successful induction of LIPA-α-syn aggregates has been employed to effectively model PD in mice. This modeling approach resulted in selective loss of dopaminergic neurons in the midbrain and the emergence of Parkinsonian-like symptoms.[1] Importantly, the spatial and temporal precision of the LIPA system, coupled with intravital real-time imaging utilizing calcium imaging techniques, has enabled the ground-breaking achievement of monitoring dopaminergic synaptic dysfunction in response to protein aggregation during the early stages of PD.[1] This represents the first instance of real-time observation of such synaptic dysfunction.

LIMITATIONS

The primary limitation of the LIPA system stems from the substantial size of the construct, wherein α-syn is fused with CRY2olig and mCherry proteins. While it may raise questions regarding the

potential impact of the fusion construct on α-syn's aggregation properties, our findings unequivocally established that α-syn not only sustained its aggregation and seeding capacity but also retained its aggregation patterns and kinetics within the context of the CRY2-mCherry fusion construct. This assertion is grounded in the following experimental evidence: the presence of α-syn expedited light-induced LIPA aggregation, whereas the inclusion formation was prevented in the presence of the non-aggregating form of α-syn$^{\Delta NAC}$.

LIPA-induced α-synuclein inclusions had a remarkable seeding capacity, thereby perpetuating the aggregation process. Taken together, these observations substantiate that the size of the LIPA constructs does not interfere with α-syn's behavior, including its aggregation process within living cells. Consequently, this supports the reliability of this model for investigating protein aggregation and Lewy body formation.

TROUBLESHOOTING

Problem 1

HMW bands are not properly visualized on the membrane after Western blot transfer, related to step 12 and step 15.

Potential solution

- Make sure to choose the right gel percentage and the right gel type.
- For efficient targeting of HMW proteins during Western blot transfer, it is recommended to use a Tris-acetate gel, or a low-percentage Tris-glycine gel.
- Optimize the transfer conditions, new transfer devices have allowed for rapid Western blot transfers, however certain parameters need to be optimized for the visualization of HMW protein bands. Therefore, we recommend the application of wet transfer techniques since it is the preferred technique for detecting HMW proteins.
- Regardless of the chosen transfer technique, it is common to encounter a significant amount of protein that remains in the gel after the transfer process. The efficiency of the transfer may be influenced by the transfer time. When staining the gel after the transfer, if a large amount of protein appears to remain on the gel, try adjusting your transfer time. Follow our recommendations suggested here for efficient protein transfer: Transfer at 4°C for 2 h at 350 mA or at 4°C, on ice, for 16 h at 90 mA.

Problem 2

Weak signal of a sample of interest or high background signal detected in the wells, related to the filter retardation assay (steps 42–46.).

Potential solution

- A weak signal in protein detection can result from inadequate aggregation of proteins. For better detection using the filter retardation assay, ensure the optimal level of aggregation by conducting an mCherry visual check to validate proper aggregate formation.
- To enhance the protein signal, another option would be to increase the loading volume of the protein material but ensure not to overload the membrane which may cause leakage of the sample.
- The protein signal maybe masked if the blocking buffer is used at excessively high concentrations, or due to long incubation times in the blocking buffer. To mitigate this, test different dilutions of the buffer and incubation times.
- No or weak signal detection maybe due to low concentrations of the antibodies used. Increase the concentration of primary and/or secondary antibody. Optimizing the signal-to-noise ratio is best achieved through titrating antibody concentrations .
- To help reduce the background signal, consider adding more washing steps during the filtration assay.

Problem 3

Sample signal appears weak, diffused, and very spread out, related to the SDD-AGE technique (step 53).

Potential solution

- Minimize the heat induced by the high voltage by either:
  - Adjusting the thickness of the gel, by following our recommendation of a 0.7 cm thickness in 10 × 15 casting tray. The thinner the gel, the less resistance it has to the voltage applied.
  - Run the gel at 4°C to minimize the heat and sharpen the signal.
- Use 1.8% gels, and do not go lower than 1.5%. A smaller gel pore size will cause a diffused sample spread, weakening the detection of the sample.
- Possible poor contact between the gel and membrane: optimize and ensure proper capillary action in the transfer cassette.

Problem 4

- No amplification observed in the presence of the purified LIPA-α-syn aggregates, related to steps 66–68 from RT-QuIC assay.
- False positive amplification in the absence of the purified LIPA-α-syn aggregates, related to steps 66–68 from RT-QuIC assay.
- One out of the 3 triplicates show an amplification increase, related to steps 66–68 from RT-QuIC assay.
- High amplification observed at 0 h, related to steps 66–68 from RT-QuIC assay.

Potential solution

- Perform quality control checks using Western blot analysis as described in steps 10–28 to validate the adequate expression of the LIPA-α-syn aggregates in the sample.
- Check for the recombinant monomeric α-syn, to ensure that he starting material is adequate for the RT-QuIC test. Western blot can be performed to check that the monomeric α-syn has not changed conformation. Otherwise, it is also highly recommended to follow the steps suggested in this publication.[13]
- Make sure to properly handle and store the recombinant monomeric α-syn protein as well as the purified LIPA- α-syn aggregates. Recombinant monomeric α-syn should always be stored at −80°C, and purified aggregates should always be kept on ice during the preparation of the experiment and prior to the RT-QuIC experimental setup.
- Avoid freeze/thaw cycles, since repeated freeze-thaw cycles can lead to a range of physical and chemical alterations in the recombinant protein, including structural changes and potential protein degradation.
- Ensure that the 96-well plate is properly sealed throughout the amplification experiment, since evaporated wells can show up as false positive amplifications. Evaporation can also cause for amplifications to be detected too early during the process.

Problem 5

The cells placed in the live-imaging chamber are not aggregating when using a laser between 470 and 488 nm, related to step 90 from live-cell imaging.

Potential solution

- If the cells used are cells used after transient transfection, the incubation time post-transfections can be increased to up to 48 h depending on the cell type (e.g.,: iPSC-derived neurons) in order to allow for a higher and more stable plasmid expression.

- Increase the level of expression by increasing the concentration of the transfected constructs.
- If working with stable cell lines expressing the LIPA-α-syn construct, try to use cells that have been freshly plated for the experiment (16–24 h prior to performing the live-imaging experiment).
- Make sure that the laser power is high enough to induce aggregation. If increasing the laser power to the higher levels (with a moderate scan speed), ensure to perform the required optimization not to induce any cellular toxicity.

Problem 6

Prior to the light stimulation, few cells placed in the live-imaging chamber might exhibit small dots within the cytosol, thus precluding the initiation of live imaging from free-noise background, related to step 90 from live-cell imaging.

Potential solution

- The tiny dots observed may represent stress granules if the cells are stressed or imaged after a long period in culture. Therefore, it is recommended to use healthy cells in culture for no more than 48 h.
- The propensity for LIPA aggregation is directly linked to the levels of LIPA expression. Elevated protein expression can result in inclusion formation, even in the presence of ambient light. Hence, it is advisable to choose stable cell lines that demonstrate LIPA expression without any spontaneous inclusions. If employing transient transfection, use a DNA concentration that permits detectable mCherry levels without the occurrence of spontaneous inclusions. Moreover, as a precaution, it is advisable to work in low ambient light conditions and cover the plates with aluminum foil during transfers.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Abid Oueslati (abid.oueslati.1@ulaval.ca).

Materials availability

All the plasmids used in this protocol are available up-on request to Dr. Oueslati (abid.oueslati.1@ulaval.ca).

Data and code availability

This study did not generate/analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102738.

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AUTHOR CONTRIBUTIONS

M.T., R.S., and W.I.: collection and analysis of the data. M.T., W.I., and R.S.: figure preparation. M.T.: graphical abstract realization. A.O. and M.T.: conceptualization, data analysis, and manuscript preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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