**Supplementary Materials for Online**

### Cell culture and differentiation

SH-SY5Y cells (ECACC, cat. 94030304) were cultured in Dulbecco's modified Eagle's medium (DMEM, D5030, Sigma-Aldrich, USA) containing 25 mM glucose (G7021, Sigma-Aldrich), 6 mM L-glutamine (G3126, Sigma-Aldrich), 5 mM HEPES (H4024, Sigma-Aldrich), 44 mM sodium bicarbonate (S6014, Sigma-Aldrich), 1 mM sodium pyruvate (P2256, Sigma-Aldrich), 10% (v/v) fetal bovine serum (41F6445K, Gibco, Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (1772652 Thermo Fisher Scientific) in a humidified atmosphere (5% CO2, 37 °C). Cell media was changed every 2 to 3 days, and cells were split when reaching 90-100% confluency.

For cell differentiation, cells were seeded at the density of 3x104 cells/cm2 in low glucose (5 mM) media supplemented with 1% FBS and 10 µM retinoic acid (RA) (A6947 Panreac AppliChem ITW Reagents, Germany) for 3 days. Following differentiation, cells were treated with increasing concentration of 6-OHDA (H4381 Sigma-Aldrich) or rotenone (MKBS1062V, Sigma-Aldrich).

### ATP levels determination

Cell differentiation and treatments were accomplished in white, opaque-bottom, 96-well plates (136101, Thermo Fisher Scientific). At the end of cell treatments, the medium was removed and replaced by 50 µl of fresh medium. 50 µl of the Cell Titer-Glo reagent was added, and plates were agitated for 2 min on an orbital shaker to promote cell lysis. After 10-min incubation, the luminescent signal was recorded using Cytation™ 3 microplate reader (BioTek, USA).

### Immunocytochemistry and fluorescence microscopy

After cell differentiation and treatment, the cell culture medium was removed, cells were washed with warm phosphate buffer saline (PBS), fixed with 4% paraformaldehyde in PBS and stored at 4 ºC. The cells were then washed 3 times with PBS and permeabilized with 0.2% (v/v) Triton X-100 (AC327371000, Fisher Scientific) in PBS for 2 min. The cells were then washed 3 times with PBS, and incubated with the blocking solution (3% bovine serum albumin, BSA; A6003 Sigma-Aldrich) in PBS. The cells were washed 3 times with PBS containing 1% BSA and incubated overnight at 4 ºC with mouse anti-βIII tubulin (sc80005, Santa Cruz, Germany) at 1:200 dilution prepared in 3% BSA in PBS. This was followed by 90-min incubation with goat-anti-mouse Alexa Fluor 488 (A-11001, Cat. M7512, Invitrogen, Thermo Fisher Scientific, USA) at 1:1000 dilution in 3% BSA in PBS. Finally, cells were washed 3 times with 1% BSA in PBS and incubated with 1 µg/ml Hoechst 33342 (B2261, Sigma-Aldrich) in PBS for nuclei visualization.

Cell visualization was performed using an INCell Analyzer 2200 (GE Healthcare) cell imaging system. Images were acquired using a 20x objective (INCA ASAC 20 x/0.45, ELWD Plan Fluor). Image analysis was performed using the INCell Analyzer 1000 analysis software - Developer Toolbox. The image stack was uploaded by the software to identify our target set and to establish the respective parameters of area and number. The representative images shown in this work were visualized using ImageJ 1.52a (Wayne Rasband, National Instituted of Health, USA).

### ImageJ image pre-processing

Following the published protocol (Kandel, Chou et al. 2015), we pre-processed raw image files using ImageJ. Briefly, time-lapse images were first convolved using the 5×5 edge-detection, converted to the frequency domain using a Fast Fourier Transform, and then subjected to a bandpass filter ranging from 2 pixels (∼0.3 μm) to 100 pixels (∼16 μm). The resulting images were manually thresholded to eliminate the noise, and the results saved as a sequence of individual binary images.

### MATLAB Algorithm

The stacks of individual images were analyzed by an open source MATLAB algorithm ([www.github.com/kandelj/MitoSPT](http://www.github.com/kandelj/MitoSPT)) (Kandel, Chou et al. 2015). Briefly, the algorithm read each frame into MATLAB and used the built-in functions *bwconncomp* and *regionprops* to find the connected white objects and to measure their sizes, respectively. The image was then recreated to contain only objects with the area within the specified limits defined by the user. Each frame went through the same process. The current frame objects were labeled or re-labeled by comparing their pixel locations with the ones from the previous frame. After all objects were labeled/re-labeled, their locations were stored, and they were prepared to be compared with the next frame. After this process was repeated frame by frame, the collected centroid locations were used to calculate the total and net distances traveled by each object (Kandel, Chou et al. 2015). In addition, the software was adapted to output the raw trajectories of each individual mitochondria into a comma-separated values file (csv) for external analysis.