Journal of Visualized Experiments

Flow cytometric analysis of multiple mitochondrial parameters in human induced pluripotent stem cells and their neural and glial derivatives

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63116R2
Full Title:	Flow cytometric analysis of multiple mitochondrial parameters in human induced pluripotent stem cells and their neural and glial derivatives
Corresponding Author:	Kristina Xiao Liang Bergen University: Universitetet i Bergen Bergen, Hordaland NORWAY
Corresponding Author's Institution:	Bergen University: Universitetet i Bergen
Corresponding Author E-Mail:	xiao.liang@uib.no
Order of Authors:	Kristina Xiao Liang
	Anbin Chen
	Cecilie Kristiansen
	Laurence A. Bindoff
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Neuroscience
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bergen, Hordaland, Norway
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

Flow Cytometric Analysis of Multiple Mitochondrial Parameters in Human Induced Pluripotent Stem Cells and Their Neural and Glial Derivatives

AUTHORS AND AFFILIATIONS:

Kristina Xiao Liang[1,2*], Anbin Chen[1,2,3,4*], Cecilie Katrin Kristiansen[1,2], Laurence A. Bindoff[1,2]

[1]Department of Clinical Medicine (K1), University of Bergen, Jonas Lies vei 87, P. O. Box 7804, 5021 Bergen, Norway

[2]Neuro-SysMed, Center of Excellence for Clinical Research in Neurological Diseases, Haukeland University Hospital, Jonas Lies vei 87, P. O. Box 7804, 5021 Bergen, Norway

[3]Department of Neurosurgery, Qilu Hospital and Institute of Brain and Brain-Inspired Science, Cheeloo College of Medicine, Shandong University, 107 Wenhua Xi Road, Jinan, 250012, Shandong Province, China

[4]Shandong Key Laboratory of Brain Function Remodeling, 107 Wenhua Xi Road, Jinan, 250012, Shandong Province, China

*These first authors contributed equally.

Email addresses of co-authors:

Anbin Chen (championabc1235@gmail.com)

Cecilie Katrin Kristiansen (cecilie_2001@hotmail.com)

Corresponding authors:

Kristina Xiao Liang (Xiao.Liang@uib.no)

Laurence A. Bindoff (Laurence.Bindoff@uib.no)

SUMMARY:

This study reports a novel approach to measure multiple mitochondrial functional parameters based on flow cytometry and double staining with two fluorescent reporters or antibodies to detect changes in mitochondrial volume, mitochondrial membrane potential, reactive oxygen species level, mitochondrial respiratory chain composition, and mitochondrial DNA.

ABSTRACT:

Mitochondria are important in the pathophysiology of many neurodegenerative diseases. Changes in mitochondrial volume, mitochondrial membrane potential (MMP), mitochondrial production of reactive oxygen species (ROS), and mitochondrial DNA (mtDNA) copy number are often features of these processes. This report details a novel flow cytometry-based approach to measure multiple mitochondrial parameters in different cell types, including human induced pluripotent stem cells (iPSCs) and iPSC-derived neural and glial cells. This flow-based strategy uses live cells to measure mitochondrial volume, MMP, and ROS levels, as well as fixed cells to estimate components of the mitochondrial respiratory chain (MRC) and mtDNA-associated proteins such as mitochondrial transcription factor A (TFAM).

By co-staining with fluorescent reporters, including MitoTracker Green (MTG), tetramethylrhodamine ethyl ester (TMRE), and MitoSox Red, changes in mitochondrial volume,

MMP, and mitochondrial ROS can be quantified and related to mitochondrial content. Double staining with antibodies against MRC complex subunits and translocase of outer mitochondrial membrane 20 (TOMM20) permits the assessment of MRC subunit expression. As the amount of TFAM is proportional to mtDNA copy number, the measurement of TFAM per TOMM20 gives an indirect measurement of mtDNA per mitochondrial volume. The entire protocol can be carried out within 2–3 h. Importantly, these protocols allow the measurement of mitochondrial parameters, both at the total level and the specific level per mitochondrial volume, using flow cytometry.

INTRODUCTION:

Mitochondria are essential organelles present in almost all eukaryotic cells. Mitochondria are responsible for energy supply by producing adenosine triphosphate (ATP) via oxidative phosphorylation and act as metabolic intermediaries for biosynthesis and metabolism. Mitochondria are deeply involved in many other important cellular processes, such as ROS generation, cell death, and intracellular Ca^{2+} regulation. Mitochondrial dysfunction has been associated with various neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), Friedreich's ataxia (FRDA), and amyotrophic lateral sclerosis (ALS)[1]. Increased mitochondrial dysfunction and mtDNA abnormality are also thought to contribute to human aging[2,3].

Various types of mitochondrial dysfunction occur in neurodegenerative diseases, and changes in mitochondrial volume, MMP depolarization, production of ROS, and alterations in mtDNA copy number are common[4-7]. Therefore, the ability to measure these and other mitochondrial functions is of great importance when studying disease mechanisms and testing potential therapeutic agents. Moreover, in view of the lack of animal models that faithfully replicate human neurodegenerative diseases, establishing suitable *in vitro* model systems that recapitulate the human disease in brain cells is an important step toward a greater understanding of these diseases and the development of new therapies[2,3,8,9].

Human iPSCs can be used to generate various brain cells, including neuronal and non-neuronal cells (i.e., glial cells), and mitochondrial damage associated with neurodegenerative disease has been found in both cell types[3,10-13]. Appropriate methods for iPSC differentiation into neural and glial lineages are available[14-16]. These cells provide a unique human/patient platform for *in vitro* disease modeling and drug screening. Further, as these are derived from patients, iPSC-derived neurons and glial cells provide disease models that reflect what is happening in humans more accurately.

To date, few convenient and reliable methods for measuring multiple mitochondrial functional parameters in iPSCs, particularly living neurons and glial cells, are available. The use of flow cytometry provides the scientist with a powerful tool for measuring biological parameters, including mitochondrial function, in single cells. This protocol provides details for the generation of different types of brain cells, including neural stem cells (NSCs), neurons, and glial astrocytes from iPSCs, as well as novel flow cytometry-based approaches to measure multiple mitochondrial parameters in different cell types, including iPSCs and iPSC-derived neural and glial cells. The

protocol also provides a co-staining strategy for using flow cytometry to measure mitochondrial volume, MMP, mitochondrial ROS level, MRC complexes, and TFAM. By incorporating measures of mitochondrial volume or mass, these protocols also allow the measurement of both total level and specific level per mitochondrial unit.

PROTOCOL:

NOTE: See the **Table of Materials** and the **Supplemental Table S1** for recipes of all media and solutions used in this protocol.

1. Differentiation of human iPSCs into NCSs, dopaminergic (DA) neurons, and astrocytes

1.1. Prepare matrix-coated plates.

1.1.1. Thaw a vial of 5 mL of matrix on ice overnight. Dilute 1 mL of matrix with 99 mL of cold Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (Advanced DMEM/F12) (1% final concentration). Make 1 mL aliquots and store them at -20 °C.

1.1.2. Thaw the matrix solution at 4 °C (keep it cold) and coat 6 wells (1 mL per well in a 6-well plate).

1.1.3. Place the matrix-coated plate in a humidified 5% CO_2/95% air incubator at 37 °C for 1 h. Take the plate out of the incubator and let it equilibrate to room temperature (RT).

NOTE: It is recommended to use the plate within 3 days of coating. However, the coated plate can be stored for up to 2 weeks at 4 °C. Just remember to take it out and let it warm up to RT before use. For longtime storage, add 1 mL of iPSC culture medium to the coated plate to avoid drying of the matrix.

1.2. Thawing iPSCs

1.2.1. Prewarm the matrix-coated plates at RT or in the incubator at 37 °C for 20–30 min. Prewarm the required amount of iPSC culture medium at RT.

1.2.2. Mix 6 mL of prewarmed iPSC culture medium with 12 µL of Y-27632 ROCK inhibitor to obtain a final concentration of 10 µM.

1.2.3. Partially thaw the frozen vial of iPSCs at 37 °C in a water bath until a small piece of ice remains. Transfer the liquid content of the vial with iPSCs dropwise into one well of a 6-well precoated plate using a 5 mL pipette.

1.2.4. Slowly add the prewarmed iPSC culture medium with 12 µL of ROCK inhibitor dropwise to the cells.

1.2.5. Move the plate in perpendicular directions to mix the well contents and return the plate to the incubator. Change the iPSC culture medium after 24 h after washing with Dulbecco's phosphate-buffered saline (DPBS) (1x) (Ca^{2+}/Mg^{2+}-free) (4 mL per well in a 6-well plate).

NOTE: Do not add ROCK inhibitor to subsequent feedings. Change the iPSC culture medium daily.

1.3. Subculturing of iPSCs

1.3.4. Prewarm the matrix-coated plates at RT or in the incubator at 37 °C for 20–30 min. Prewarm the required amount of iPSC culture medium at RT.

1.3.2. Aspirate the culture medium from the plates containing the cells. Rinse the iPSCs with DPBS (1x) (Ca^{2+}/Mg^{2+}-free) (4 mL per well in a 6-well plate).

1.3.3. Add EDTA (0.5 mM) (1 mL per well in a 6-well plate). Incubate the plate at 37 °C until the edges of the colonies start to lift from the well (usually 3-5 min). Aspirate the EDTA.

1.3.4. Add prewarmed iPSC culture medium (4 mL per well in a 6-well plate) and forcefully detach the iPSC colonies using a 10 mL sterile pipette once. Do not pipette up and down as this may break cell clumps into single cells.

1.3.5. Transfer the contents of each well into two individual wells in a matrix-coated 6-well plate (2 mL per well in a 6-well plate) and incubate at 37 °C. Do not generate bubbles in the suspension while pipetting.

NOTE: Shake the plate gently before keeping it in the incubator. The split ratio can be 1:2 (one well into 2 new wells) to 1:4 (one well into 4 new wells).

1.3.6. Replace the medium daily until the colonies reach 60% confluence with good size and connections.

1.4. Neural induction and neural progenitor generation

1.4.1. Prepare 500 mL of Chemically Defined Medium (CDM), 500 mL of Neural Induction Medium (NIM), and 500 mL of Neural Stem Cell Serum-free (NSC SF) medium.

1.4.2. Rinse the cells with DPBS (1x) (Ca^{2+}/Mg^{2+}-free) (4 mL per well in a 6-well plate) and add NIM (3 mL per well in a 6-well plate). Set up as Day 0.

1.4.3. Replace the NIM (3 mL per well in a 6-well plate) on Day 1, Day 3, and Day 5 and observe under the microscopy daily.

1.4.4. On Day 6, detach the neural rosettes into suspension culture as described below.

1.4.4.1. Wash once gently with DPBS (1x) (Ca^{2+}/Mg^{2+}-free) (4 mL per well in a 6-well plate). Add collagenase IV (1 mL per well in a 6-well plate) and keep in an incubator for 1 min. Aspirate the collagenase IV and wash once with DPBS (1x) (4 mL per well in a 6-well plate) gently.

1.4.4.2. Add 2 mL of NSC SF Medium per well to a 6-well plate. Detach the cells by scraping the bottoms of the wells by drawing grids using a 200 µL pipette tip.

1.4.4.3. Collect the cell suspension from the 6-well plate into a 10 cm non-adherent dish. Make up the volume to 12 mL with NSC SF Medium.

1.4.4.4. Shake the non-adherent dish at 65–85 rpm on an orbital shaker in an incubator to prevent aggregation.

1.5. DA neuron differentiation

1.5.1. On Day 7, add 12 mL of CDM supplemented with 100 ng/mL fibroblast growth factor-8b (FGF-8b) and place the dish on the orbital shaker in the incubator.

1.5.2. On Days 8–13, change the medium every 2 days and observe under the microscopy daily.

1.5.3. On Day 14, add 12 mL of CDM supplemented with 100 ng/mL FGF-8b and 1 µM purmorphamine (PM). Place the dish on the orbital shaker in the incubator.

1.5.4. On Days 15–20, change the medium every 2 days and observe the cells under the microscopy daily.

1.5.5. Mechanically passage the spheres by using 1000 µL tips to break up the larger spheres.

NOTE: The ratio can be 1:2 (one well into 2 new wells).

1.6. Termination of differentiation

1.6.1. Coat a 6-well plate or coverslips with Poly-L-Ornithine (PLO) and laminin as described below.

1.6.1.1. Coat a 6-well plate with 1 mL of PLO/well, and incubate the plate at 37 °C for 20 min. Aspirate the PLO solution.

1.6.1.2. Sterilize the plate under UV for ~20 min. Rinse the wells twice with DPBS (1x) (4 mL per well in a 6-well plate).

1.6.1.3. Add 5 µg/mL laminin solution (1 mL per well in a 6-well plate) to the well and incubate at 37 °C for 2 h. Aspirate the laminin and wash the wells briefly with DPBS (1x) (4 mL per well in a 6-well plate) once before plating.

1.6.2. Collect all spheres (from step 1.5.5) in 15 mL tubes and top up with DPBS (1x). Spin at 300 × *g* for 5 min. Aspirate the supernatant.

1.6.3. Incubate with 2 mL of cell dissociation reagent (see the **Table of Materials**) for 10 min at 37 °C in a water bath followed by gentle trituration with a 200 µL pipette (20–50 times depending on the size of the spheres, avoiding bubble formation).

1.6.4. Neutralize the cell dissociation reagent with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and centrifuge the 15 mL conical tube containing the cells at 300 × *g* for 5 min at RT. Aspirate the supernatant.

1.6.5. Add 1 mL of CDM supplemented with 10 ng/mL Brain-Derived Neurotrophic Factor (BDNF) and 10 ng/mL Glial cell line-derived neurotrophic factor (GDNF) to resuspend the cell pellets by gently pipetting up and down to obtain single-cell suspensions.

1.6.6. Aspirate the PLO and laminin solution from the plate (step 1.6.1.3), wash briefly with DPBS (1x) (4 mL per well in a 6-well plate), and seed the cells (from step 1.6.5) in the precoated plates or coverslips in 3 mL of CDM supplemented with 10 ng/mL BDNF and 10 ng/mL GDNF. Feed the cells every 4 days.

NOTE: Differentiating cultures can be maintained for many weeks up to 3 months. The neural morphology usually appears after 2 weeks of termination and can be used for downstream analyses from that point onwards. BDNF and GDNF are not necessary for culturing for longer maintenance (up to 2 months).

1.7. NSC generation

1.7.1. Coat matrix plates.

1.7.2. Collect all neural spheres (generated from step 1.4) in 15 mL tubes and top up with DPBS (1x) (Ca^{2+}/Mg^{2+}-free). Spin at 300 × *g* for 5 min. Aspirate the supernatants.

1.7.3. Incubate the pellets with 2 mL of cell dissociation reagent for 10 min at 37 °C in a water bath, followed by gentle trituration with a 200 µL pipette (20–50 times depending on the size of the spheres, avoiding bubble formation).

1.7.4. Neutralize with 2 mL of DMEM with 10% FBS and centrifuge the 15 mL conical tubes containing the cells at 300 × *g* for 5 min at RT. Aspirate the supernatants. Resuspend the cell pellets by gently pipetting up and down to obtain single-cell suspensions.

1.7.5. Aspirate the matrix solution from a precoated plate, wash briefly with DPBS (1x) (4 mL per well in a 6-well plate) (Ca^{2+}/Mg^{2+}-free), and seed the cells in the precoated plates in NSC SF Medium (3 mL per well in a 6-well plate). Feed the cells every 2–3 days and split the cells when

confluent.

NOTE: From this stage onwards, NSCs can be expanded and frozen down.

1.8. Glia astrocyte differentiation

1.8.1. Astrocyte differentiation from NSCs

1.8.1.1. Prepare 500 mL of Astrocyte Differentiation Medium.

1.8.1.2. Seed NSCs on Poly-D-Lysine (PDL)-coated plates/coverslips with NSC SF Medium. Change the medium every other day until the cells reach 60% confluency.

1.8.1.3. Rinse the cells with DPBS (1x) (Ca^{2+}/Mg^{2+}-free) (4 mL per well in a 6-well plate) and add Astrocyte Differentiation Medium (3 mL per well in a 6-well plate). Set up as Day 0.

1.8.1.4. Observe the NSCs under the microscope daily and replace the Astrocyte Differentiation Medium (3 mL per well in a 6-well plate) every 2 days from Days 1 to 27.

1.8.2. Astrocyte maturation

1.8.2.1. Prepare Astrocyte Maturation Medium.

1.8.2.2. On Day 28, rinse the cells with DPBS (1x) (4 mL per well in a 6 well plate) and add Astrocyte Maturation Medium (3 mL per well in a 6-well plate).

1.8.2.3. On day 29 and onwards, observe the cells under the microscope daily and replace the Astrocyte Maturation Medium (3 mL per well in a 6-well plate) every 2 days.

1.8.2.4. After one month of maturation, expand the cells and cryopreserve them from this stage onwards.

NOTE: During this phase, the number of cells will increase. When splitting the cells, PDL-coated coverslips are not necessary for culturing.

2. Cell characterization by immunocytochemistry and immunofluorescence staining

2.1. At the end of the culture period, transfer the coverslips with the cells to a 12-well plate. Rinse the cells with phosphate-buffered saline (PBS) (1x) two times and incubate for 10 min in 4% Paraformaldehyde (PFA) (0.5 mL per well in a 12-well plate) at RT.

NOTE: The fixed sample can be covered with 2 mL of PBS (1x) and stored at 4 °C until required for immunostaining. PFA is toxic and is suspected of causing cancer. Prevent skin and eye exposure, and work under a chemical fume hood.

2.2. Permeabilize the cells and incubate with blocking buffer containing PBS (1x), 0.3% Triton X-100, and 10% normal goat serum for 1 h at RT.

2.3. Incubate with primary antibodies in blocking buffer overnight at 4 °C: stain iPSCs with anti-octamer-binding transcription factor 4 (Oct4) and anti-stage-specific embryonic antigen-4 (SSEA4), NSCs with anti-sex-determining region Y box-2 (Sox2) and anti-Nestin, neural spheres with anti-paired box-6 (Pax6) and anti-Nestin, astrocytes with anti-glial fibrillary acidic protein (GFAP) and anti-S100 calcium-binding protein β (S100β), and DA neurons with anti-tyrosine hydroxylase (TH), anti-B III Tubulin (Tuj 1), anti-Synaptophysin, and anti-PSD-95 (0.5 mL of primary antibody solution per well in a 12-well plate; see the **Table of Materials** for details).

2.4. Wash the samples with PBS (1x) three times for 10 min each with gentle rocking.

2.5. Incubate with secondary antibody solution (1:800 in blocking buffer, 0.5 mL of Alexa Fluor secondary antibody solution per well in a 12-well plate) for 1 h at RT with gentle rocking.

2.6. Incubate the cells with Hoechst 33342 (1:5,000, 0.5 mL per well in a 12-well plate) in PBS (1x) for 15 min at RT to label the nuclei.

2.7. Mount the cells with mounting medium and dry overnight at RT for imaging under a fluorescence microscope in the dark. See **Supplemental Figure S1** for the microscope settings and parameters.

3. Flow cytometry measurement of mitochondrial volume, MMP, and mitochondrial ROS in live cells

3.1. Seed the cells separately into 4 wells in a 6-well plate until the cells reach 50%–60% confluency. Label these four wells as #1, #2, #3, and #4.

3.2. At the end of the culture period, prepare 5 individual staining solutions (500 µL per well in a 6-well plate) as follows: #1,only culture medium (to well #1 containing only cells for control); #2-1 containing FCCP (100 µM); #2-2 containing FCCP (100 µM) + TMRE (100 nM) + MTG (150 nM) in culture medium; #3 containing TMRE (100 nM) + MTG (150 nM) in culture medium; #4 containing MitoSox Red (10 µM) + MTG (150 nM) in PBS (1x) with 10% FBS. See **Figure 1A, Supplemental Table S2**, and the **Table of Materials** for details about these compounds and flow cytometry setup.

NOTE: Use the culture medium to prepare the staining solution. Warm up the medium and PBS (1x) at RT before using. FCCP is toxic; prevent skin and eye exposure and work under a chemical fume hood.

3.3. Aspirate the medium from the #2 well and add #2-1 solution (FCCP only). Incubate the cells at 37 °C for 10 min.

3.4. Aspirate the medium from #2 and #3 wells and add #2-2 solution (FCCP + TMRE + MTG) in the #2 well and #3 solution (TMRE + MTG) in #3. Incubate the cells at 37 °C for 45 min.

3.5. Aspirate the medium from the #4 well and add #4 solution (MitoSox Red + MTG). Incubate the cells at 37 °C for 15 min.

3.6. Aspirate the medium from all wells. Wash with PBS (1x) (4 mL per well in a 6-well plate). Detach the cells using 1 mL of cell dissociation reagent (1 mL per well in a 6-well plate) at 37 °C for 5 min. Neutralize the cell dissociation reagent in 1 mL of DMEM with 10% FBS (2 mL per well in a 6-well plate).

3.7. Collect the contents of all the wells in 15 mL conical tubes. Centrifuge the tubes at 300 × *g* for 5 min. Wash the pellets with PBS (1x) once or twice.

3.8. Aspirate the supernatants but leave approximately 100 µL in the tubes. Resuspend the cell pellets in 300 µL of PBS (1x). Transfer the cells to 1.5 mL microcentrifuge tubes. Keep the tubes in the dark at RT.

3.9. Analyze the cells using a flow cytometer (with a 3 blue and 1 red laser configuration). Detect MTG in filter 1 (FL1) using a 530/30 bandpass filter, TMRE in filter 2 (FL2) using the bandpass filter 585/40, and MitoSox Red in filter 3 (FL3) using a 510/580 bandpass filter.

4. Flow cytometry measurement of MRC complex subunits and TFAM in fixed cells

4.1. At the end of the culture period, detach the cells (~10^6) by adding the cell dissociation reagent; then, pellet and collect the cells in a 15 mL tube.

4.2. Fix the cells in 1.6% PFA (1 mL of 1.6% PFA in a 15 mL tube) at RT for 10 min.

4.3. Permeabilize the cells with ice-cold 90% methanol (1 mL of 90% methanol in a 15 mL tube) at -20 °C for 20 min.

4.4. Block the samples in blocking buffer containing 0.3 M glycine, 5% goat serum, and 1% bovine serum albumin (BSA) - Fraction V in PBS (1x) (1 mL of blocking buffer in a 15 mL tube). Wash the cells by centrifugation with PBS (1x) twice (as in step 3.7).

4.5. Incubate the cells with the following primary antibodies for 30 min: anti-NDUFB10 (1:1,000) for measurement of complex I subunit, anti-succinate dehydrogenase complex flavoprotein subunit A (SDHA, 1:1,000) for measurement of complex II subunit and anti-COX IV (1:1,000) for measurement of complex IV subunit, and anti-TFAM antibody conjugated with Alexa Fluor 488 (1:400). Stain the same number of cells separately with anti-TOMM20 antibody conjugated with Alexa Fluor 488 (1:400) for 30 min (1 mL of primary antibody solution in a 15 mL tube; see the **Table of Materials** for details about the antibodies).

4.6. Wash the cells with PBS (1x) once with centrifugation at 300 × *g* for 5 min. Add secondary antibody (1:400) into tubes of NDUFB10, SDHA, and COX IV and incubate the cells with these solutions for 30 min, followed by immediate analysis on the flow cytometer.

4.7. Wash the cells with PBS (1x) once by centrifuging at 300 × *g* for 5 min. Aspirate the supernatants, leaving approximately 100 µL in the tubes. Resuspend the cell pellets in 300 µL of PBS (1x). Transfer the cells to 1.5 mL microcentrifuge tubes kept in the dark on ice.

4.8. Analyze the cells on the flow cytometer (with a 3 blue and 1 red laser configuration). Detect signals in filter 1 (FL1) using a 530/30 bandpass filter. See **Supplemental Figure S2** for the microscope settings and parameters.

5. Flow cytometry acquisition and analysis

5.1. Use the non-stained control tube to set the forward scatter area (FSC-A) and side scatter area (SSC-A) scatter plots based on the size and complexity of the cell population analyzed. See **Supplemental Figure S2** for the microscope settings and parameters.

NOTE: Set up non-stained controls for individual cell types.

5.2. Use the non-stain control tubes to select the positive gates, and use single-color control tubes to compensate for the fluorescence spectral overlap between MTG (fluorophore-1 [FL-1]) and TMRE (FL-2) in multicolor flow cytometry. Use isotype control for negative control to monitor background staining in MRC and TFAM samples. Use the FCCP tube as a depolarization control for TMRE staining.

5.3. Gate out extraneous debris to select live cells and the main gating from the forward and side scatter plot (**Figure 2A**). Gate out doublets using a forward scatter height (FSC-H) versus (vs.) FSC-A density plot to exclude doublets and also construct a side scatter height (SSC-H) vs. SSC-A plot (**Figure 2A**).

5.4. Data acquisition (flow cytometer)

5.4.1. Using the unstained or isotype samples as a negative control, create a gate above the main population of the single-cell events while viewing SSC-A and the various filters (FL1, FL2, FL3, FL4) (**Figure 1B** and **Figure 2B**).

5.5. Data analysis (CFlow software)

5.5.1. Copy the position of the gates onto the stained cell samples, and record the amount of positively stained cells for the positive staining.

5.5.2. For each cell subpopulation, select a histogram plot and analyze the median fluorescence

intensity (MFI) of the different filter channels (FL1, FL2, FL3, FL4) (x-axis).

5.5.2.1. Calculate the TMRE levels by subtracting the MFI of FL2 of #2FCCP-treated cells from the MFI of FL2 from #3 TMRE-stained samples in a histogram, as in Eq (**1**) below.

TMRE levels = MFI of FL2 from #3 TMRE-stained samples – MFI of FL2 of #2 FCCP-treated cells (**1**)

5.5.2.2. Calculate the specific values for MMP and ROS by MFI in TMRE or MitoSox Red, dividing the mitochondrial volume indicator MTG.

5.5.2.3. Calculate the specific value for complex subunit and TFAM by using MFI in complex expression or TFAM, dividing the mitochondrial volume indicator TOMM20.

REPRESENTATIVE RESULTS:

A schematic description of the differentiation method and flow cytometric strategies is shown in **Figure 3**. Human iPSCs are differentiated into neural rosettes and then lifted into suspension culture for differentiation into neural spheres. Neural spheres are further differentiated and matured into DA neurons. Neural spheres are dissociated into single cells to generate glial astrocytes, replated in monolayers as NSCs, and then differentiated into astrocytes. This protocol provides the strategies needed for acquiring and analyzing the samples by flow cytometry for the measurement of MMP, mitochondrial volume, ROS levels, expression levels of MRC complex subunits and TFAM—an indirect measurement of relative mtDNA copy number. Specifically, co-staining with fluorescent reporters, TMRE and MTG, was used to detect and quantify changes in mitochondrial volume and MMP. Co-staining with MitoSox Red and MTG permits measurement of mitochondrial ROS production in live cells. Double staining with antibodies against MRC complex subunits together with TOMM20 permits the assessment of the MRC and double staining of TFAM and TOMM20 for indirect assessment of mtDNA. Importantly, MTG and TOMM20 allow the measurement per mitochondrial volume, counteracting the influence of mitochondrial volume on these parameters.

DA neurons are generated from iPSCs through dual SMAD inhibition and exposed toFGF-8b and the Sonic hedgehog (SHH) agonist PM, as shown in **Figure 4A**. Human iPSCs are seeded in iPSC culture medium on matrix-coated plates. When the cells reach 50%–80% confluency, the medium is changed to NIM using a CDM supplemented with SB431542, AMPK inhibitor, Compound C, and *N*-acetylcysteine for 5 days. After 5 days, the iPSCs (**Figure 4B, a**) progress to a neural epithelial stage exhibiting clear neural rosette structures (**Figure 4B, b**). On day 5, neural spheres are generated by lifting the neural epithelium into suspension culture and culturing them in NSC SF medium on an orbital shaker inside the incubator. Round, well-defined spheres are shown in **Figure 4B, c**. On day 7, the medium is changed into CDM supplemented with 100 ng/mL FGF-8b. On day 14, the medium is changed into the CDM supplemented with 100 ng/mL FGF-8b and 1 µM PM. On day 21, the spheres are dissociated into DA neurons in a monolayer by dissociating them into single cells and culturing them in CDM supplemented with 10 ng/mL BDNF and 10 ng/mL GDNF in PLO and laminin-coated plates/coverslips. Neurons (**Figure 4B, e**) matured for

15–30 days are further used for mitochondrial functional measurements.

NSCs are produced by dissociating neural spheres into single cells and then replating them in monolayers to generate astrocytes. These NSCs show a classic neural progenitor appearance (**Figure 4B, d).** NSCs at this stage can readily be expanded and banked for further use. To initiate the astrocyte differentiation, NSCs are plated on PDL-coated coverslips in NSC SF medium. The following day, the medium is changed into astrocyte differentiation medium for 28 days. After 28 days, the differentiated astrocytes are further matured in astrocyte maturation medium. At this stage, astrocytes should display star-shaped morphology (**Figure 4B, f**), and these cells can be expanded and banked for further use, including mitochondrial functional assessment.

During differentiation, cell identity is confirmed using immunofluorescence staining. In **Figure 5A**, immunostaining shows that the iPSCs express the specific pluripotent markers, SSEA4 and Oct4. **Figure 5B** shows that neural spheres exhibit positive staining of Nestin and Pax6, while **Figure 5C** shows that iPSC-derived NSCs in monolayers exhibit positive staining of Nestin and Sox2. To identify DA neurons, cells are stained with the neural marker B III Tubulin (Tuj 1) and the DA neuronal marker tyrosine hydroxylase (TH) (**Figure 6B)**. In addition, DA neurons show staining for the synaptic markers, synaptophysin and PSD-95, confirming their functional synaptic connections (**Figure 5B)**. Immunostaining of iPSC-derived astrocytes shows the expression of the astrocyte markers, glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein β (S100β).
The investigation of mitochondrial function in differentiated neurons and astrocytes using flow cytometry is performed as described above in protocol sections 3 and 4. A flow cytometer was used for data acquisition and CFlow Sampler for data analysis, as shown in **Figure 1B**.

Figure 6 demonstrates the method for gating live single cells. Dead cells and cell debris are excluded using an FSC vs. SSC plot (**Figure 2A, a**). Cell doublets are excluded using an FSC-H vs. FSC-A plot (**Figure 2A, b**) followed by an SSC-H vs. SSC-A plot (**Figure 2A, c**). Background fluorescence is properly assessed if the negative population of a particular cell type is compared with the positive population within that same cell type (**Figure 2B, a**). For MMP samples, treating the cells with FCCP eliminates interference from mitochondrial membrane potential and TMRE staining (**Figure 2B, b**).

These flow cytometric approaches have been used to study DA neurons generated from the human iPSCs carrying mutation(s) in the catalytic subunit of mitochondrial DNA polymerase, POLG (W748S), and compare them with disease-free samples generated from Detroit 551 fibroblasts. As reported previously[4], this study also demonstrated decreased MMP and increased specific ROS levels in POLG DA neurons (**Figure 7**). However, the mitochondrial volume, total MMP, and ROS level were unchanged. In **Figure 8,** the results show a decrease in the specific complex I levels, lower total and specific TFAM levels, but similar specific complex II levels in mutant DA neurons compared to controls.

This approach was also used to study astrocytes generated from the same iPSC lines. As reported previously[7] and shown in **Figure 9**, the results show that POLG-mutated astrocytes had lower

total and specific MMP but similar mitochondrial volume and ROS compared with controls, as well as decreased levels of specific complexes I and IV (**Figure 10**). However, there were no changes in the total levels of complexes I and IV and no change in the total and specific levels of complex II in POLG astrocytes. Overall, these data suggest that flow cytometric analysis of multiple mitochondrial parameters provides a first-step approximation that is valuable in evaluating mitochondrial function in cells such as iPSCs and their neural and glial derivatives.

FIGURE AND TABLE LEGENDS:

Figure 1: Setup for flow cytometry. (**A**) MMP, mitochondrial volume, and ROS staining; (**B**) an example of data acquisition in a C6 flow cytometer. Abbreviations: MMP = mitochondrial membrane potential; ROS = reactive oxygen species; FCCP = carbonyl cyanide p-trifluoromethoxyphenylhydrazone; TMRE = tetramethylrhodamine ethyl ester; MTG = MitoTracker Green. Also, see **Supplemental Table S2**.

Figure 2: Gating strategies. (**A**) Data acquisition; (**B**) the histograms of the fluorescence with MTG and TMRE staining in live cells. Abbreviations: SSC-A = side scatter area; FSC-A = forward scatter area; PI = propidium iodide; SSC-H = side scatter height; FSC-H = forward scatter height; FL#-A = fluorophore # area; MTG = MitoTracker Green; FCCP = carbonyl cyanide p-trifluoromethoxyphenylhydrazone; TMRE = tetramethylrhodamine ethyl ester.

Figure 3: Schematic representation of the protocol workflow. Abbreviations: DA = dopaminergic; MMP = mitochondrial membrane potential; ROS = reactive oxygen species; NDUFB 10 = NADH: Ubiquinone oxidoreductase subunit 10; SDHA = succinate dehydrogenase complex flavoprotein subunit A; COX IV = cytochrome c oxidase complex IV; mtDNA = mitochondrial DNA; TFAM = mitochondrial transcription factor A.

Figure 4: iPSC differentiation. (**A**) Flow chart and (**B**) representative images for the cells from different stages during the differentiation, including iPSCs (a), neural rosette (b), neural spheres (c), NSCs (d), DA neurons (e), and astrocytes (f). Scale bars = 25 µm. Abbreviations: iPSC = induced pluripotent stem cell; NSC = neural stem cell; DA = dopaminergic; SFM = serum-free medium; CDM = chemically defined medium; FGF-8b = fibroblast growth factor-8b; PM = purmorphamine; BDNF = brain-derived neurotrophic factor; GDNF = glial cell line-derived neurotrophic factor; PLO = poly-L-ornithine.

Figure 5: Representative confocal images of the iPSCs. Immunostaining of SSEA4 (red) and Oct4 (green) (**A**), neuron spheres (**B**) for immunostaining of Nestin (red) and Pax6 (green), and iPSC-derived NSCs (**C**) for immunostaining of Nestin (red) and Sox2 (green). Nuclei are stained with DAPI (blue). Scale bars = 50 µm. Abbreviations: iPSCs = induced pluripotent stem cells; NSCs = neural stem cells; SSEA4 = stage-specific embryonic antigen-4; Oct4 = octamer-binding transcription factor 4; Pax6 = paired box-6; Sox2 = sex-determining region Y box-2; DAPI = 4',6-diamidino-2-phenylindole.

Figure 6: Representative confocal images of the iPSC-derived astrocytes. Immunostaining GFAP

(red) and S100β (green) (**A**) and iPSC-derived DA neurons (**B**) for neural lineage marker TH (green), Tuj 1 (green, red), and neural functional markers, Synaptophysin (green) and PSD-95 (red). Nuclei are stained with DAPI (blue). Scale bars = 25 µm. Abbreviations: iPSCs = induced pluripotent stem cells; GFAP = glial fibrillary acidic protein; S100β = S100 calcium-binding protein β; Tuj 1 = B III Tubulin; PSD-95 = postsynaptic density protein 95; DA = dopaminergic; DAPI = 4',6-diamidino-2-phenylindole.

Figure 7: Flow cytometric analysis for DA neurons derived from one clone of POLG patient iPSCs and one clone of Detroit 551 control iPSCs. (**A**) Mitochondrial volume measured by MTG, (**B**) total MMP measured by TMRE, (**C**) specific MMP level calculated by total TMRE/MTG, (**D**) total ROS measured by MitoSox Red, and (**E**) specific ROS level calculated by total MitoSox Red/MTG. Data presented as mean ± standard error of the mean (SEM) for the number of samples (n ≥ 3 per clone). Data analyzed and produced using GraphPad Prism software. Mann-Whitney *U* test was used to assess statistical significance for variables. Significance is denoted for $P < 0.05$. *$P < 0.05$; ns, not significant. Abbreviations: DA = dopaminergic; POLG = DNA polymerase subunit gamma; iPSCs = induced pluripotent stem cells; MMP = mitochondrial membrane potential; ROS = reactive oxygen species; MTG = MitoTracker Green; TMRE = tetramethylrhodamine ethyl ester.

Figure 8: Flow cytometric analysis for astrocytes derived from one clone of POLG patient iPSCs and one clone of Detroit 551 control iPSCs. (**A**) Mitochondrial volume measured by MTG, (**B**) total MMP measured by TMRE, (**C**) specific MMP level calculated by total TMRE/MTG, (**D**) total ROS measured by MitoSox Red, and (**E**) specific ROS level calculated by total MitoSox Red/MTG. Data presented as mean ± standard error of the mean (SEM) for the number of samples (n ≥ 3 per clone). Data analyzed and produced using GraphPad Prism software. Mann-Whitney *U* test was used to assess statistical significance for variables. Significance is denoted for $P < 0.05$. *$P < 0.05$; ns, not significant. Abbreviations: POLG = DNA polymerase subunit gamma; iPSCs = induced pluripotent stem cells; MMP = mitochondrial membrane potential; ROS = reactive oxygen species; MTG = MitoTracker Green; TMRE = tetramethylrhodamine ethyl ester.

Figure 9: Flow cytometric analysis for DA neurons derived from one clone of POLG patient iPSCs and one clone of Detroit 551 control iPSCs. (**A**) Total complex I measured by NDUFB10, (**B**) specific complex I level calculated by total NDUFB10/TOMM20, (**C**) total complex II measured by SDHA, (**D**) specific complex II level calculated by total SDHA/TOMM20, (**E**) total TFAM measured by TFAM, and (**F**) specific TFAM level calculated by total TFAM/TOMM20. Data presented as mean ± standard error of the mean (SEM) for the number of samples (n ≥ 3 per clone). Data analyzed and produced using GraphPad Prism software. Mann-Whitney *U* test used to assess statistical significance for variables. Significance is denoted for $P < 0.05$. *$P < 0.05$; ns, not significant. Abbreviations: DA = dopaminergic; POLG = DNA polymerase subunit gamma; iPSCs = induced pluripotent stem cells; NDUFB10 = NADH: Ubiquinone oxidoreductase subunit 10; TOMM20 = translocase of outer mitochondrial membrane 20; SDHA = succinate dehydrogenase complex flavoprotein subunit A; TFAM = mitochondrial transcription factor A.

Figure 10: Flow cytometric analysis for astrocytes derived from one clone of POLG patient iPSCs

and one clone of Detroit 551 control iPSCs. (**A**) Total complex I measured by NDUFB10, (**B**) specific complex I level calculated by total NDUFB10/TOMM20, (**C**) total complex II measured by SDHA, (**D**) specific complex II level calculated by total SDHA/TOMM20, (**E**) total complex IV measured by COX IV, (**F**) specific complex IV level calculated by COX IV/TOMM20, (**G**) total TFAM measured by TFAM, and (**H**) specific TFAM level calculated by total TFAM/TOMM20. Data presented as mean ± standard error of the mean (SEM) for the number of samples (n ≥ 3 per clone). Data analyzed and produced using GraphPad Prism software. Mann-Whitney *U* test was used to assess statistical significance for variables. Significance is denoted for $P < 0.05$. *$P < 0.05$; ** $P < 0.01$; ns, not significant. Abbreviations: POLG = DNA polymerase subunit gamma; iPSCs = induced pluripotent stem cells; NDUFB10 = NADH: Ubiquinone oxidoreductase subunit 10; TOMM20 = translocase of outer mitochondrial membrane 20; SDHA = succinate dehydrogenase complex flavoprotein subunit A; TFAM = mitochondrial transcription factor A; COX IV = cytochrome c oxidase complex IV.

Supplemental Figure S1: Settings of the confocal laser scanning fluorescence microscope and steps for taking images. (**A**) Choose the **Configuration** tool and select the correct laser type from **Current available laser** and set its power. (**B**) Choose **Acquire-Acquisition Mode-SEQ** and select the corresponding fluorescence wavelength photo mode from the database. (**C**) Choose **Sequential Scan-Load** and import corresponding mode. (**D**) Choose 40x objective lens and add dropwise. (**E**) Specific setting parameters for taking photos at different wavelengths. (**F**) Set photo parameters, preview, and save the photo.

Supplemental Figure S2: Steps and settings for flow cytometry. (**A**) Open Cflow software, choose the **File** tool, and select **Open CFlow file or template**. (**B**) Set up **40000 events** and select the gate containing only the live single cells in the **Run Limits** panel. Choose **Medium** speed in the **Fluidics** panel. (**C**) Choose **FSC-A vs. FSC-A plot** (a) for setting up the main gating. Choose **FSC-A vs. FSC-H plot** (b) and **SSC-A vs. SSC-H plot** (c) to exclude doublets. Choose the corresponding filters, such as FL1 or FL2, and use **FL1-A vs. FSC-A plot** (d) or **FL2-A vs. FSC-A plot** to draw the positive events when running the unstained cells. (**D**) Set up the same parameters, preview, run the stained samples and save the photo. Also, see **Supplemental Table S2**.

Supplemental Table S1: Media and solution recipes.

Supplemental Table S2: Setup for flow cytometric staining.

DISCUSSION:

Herein are protocols for generating iPSC−derived neurons and astrocytes and evaluating multiple aspects of mitochondrial function using flow cytometry. These protocols allow efficient conversion of human iPSCs into both neurons and glial astrocytes and the detailed characterization of mitochondrial function, mostly in living cells. The protocols also provide a co-staining flow cytometry-based strategy for acquiring and analyzing multiple mitochondrial functions, including volume, MMP, and ROS levels in live cells and MRC complexes and TFAM in fixed cells. Specifically, these protocols permit the estimation of both total and specific levels per mitochondrial volume. While this strategy detects mitochondrial dysfunction in a known

mitochondrial disease (POLG) in DA neurons and astrocytes, these techniques are applicable to any type of cell and disease. Moreover, the protocol is robust and reproducible. Several previous studies have successfully applied this protocol to analyze the mitochondrial changes in fibroblasts, iPSCs, NSCs, DA neurons, and astrocytes[2,3,13,17].

There are some critical points to consider while executing this protocol. To ensure consistent and high-efficiency differentiation, it is critical to initiate the conversion with high-quality iPSCs (cells containing <5% differentiated cells). Although other commercially available defined media can be valid alternatives, this study did not address the alternatives. As medium composition and clonal differences of iPSC lines can influence both proliferation of the starting cell population and differentiation efficiency, adapting this protocol to other maintenance media will likely require optimization.

The relationship between MTG and MMP fluorescence has been studied previously[3]. This is important as MTG fluorescence is reported to be both independent of[18] and sensitive to MMP[19,20]. In previous studies in which iPSCs were titrated with different concentrations of TMRE and co-stained with 150 nM MTG, the MTG level remained the same at lower concentrations of TMRE (5–100 nM), whereas a decreased MTG signal was observed for higher TMRE concentrations (over 100 nM). Therefore, 100 nM TMRE and 150 nM MTG were chosen to measure the specific MMP. As this relationship may be cell-specific, the correlation between MTG and MMP fluorescence must be assessed before using MTG and TMRE dual staining to measure MMP.

Cell density can also influence mitochondrial function and cell metabolism. In this study, cell-density-dependent changes in MMP were observed, which has also been shown in other studies[21]. Therefore, it is important to choose a similar cell density in all samples—not too high or low—to minimize variation when establishing the co-staining protocol for different cell types. Compared with other microscopy-based assays, flow cytometry has the advantages of speed and reproducibility when analyzing large numbers of cells. In the analysis of microscopic images, the bias of researchers will distort the results to a certain extent, which is not a problem when using flow cytometry. In addition, flow cytometry analysis requires less than one million cells, and analysis of one sample only takes a few minutes, which means that dozens of samples can be analyzed in 1–2 h. This technique can also be applied to a wide variety of cell types, including those from other neurodegenerative diseases, and should therefore be useful for understanding mechanisms and testing potential therapeutics in different neurodegenerative diseases.

ACKNOWLEDGMENT:
We kindly thank the Molecular Imaging Centre and the Flow Cytometry Core Facility at the University of Bergen in Norway. This work was supported by funding from the Norwegian Research Council (Grant number: 229652) and Rakel og Otto Kr.Bruuns legat.

DISCLOSURES:
The authors have no conflicts of interest to disclose.

REFERENCES:

1 Wang, Y., Xu, E., Musich, P. R., Lin, F. Mitochondrial dysfunction in neurodegenerative diseases and the potential countermeasure. *CNS Neuroscience & Therapeutics.* **25** (7), 816–824 (2019).

2 Chen, A. et al. Nicotinamide riboside and metformin ameliorate mitophagy defect in induced pluripotent stem cell-derived astrocytes with POLG mutations. *Frontiers in Cell and Developmental Biology.* **9**, 737304 (2021).

3 Liang, K. X. et al. Disease-specific phenotypes in iPSC-derived neural stem cells with POLG mutations. *EMBO Molecular Medicine.* **12** (10), e12146 (2020).

4 Chen, H., Chan, D. C. Mitochondrial dynamics--fusion, fission, movement, and mitophagy--in neurodegenerative diseases. *Human Molecular Genetics.* **18** (R2), R169–R176 (2009).

5 Lin, M. T., Beal, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature.* **443** (7113), 787–795 (2006).

6 Singh, A., Kukreti, R., Saso, L., Kukreti, S. Oxidative stress: A key modulator in neurodegenerative diseases. *Molecules (Basel, Switzerland).* **24** (8), 1583 (2019).

7 Kondadi, A. K., Anand, R., Reichert, A. S. Functional interplay between cristae biogenesis, mitochondrial dynamics and mitochondrial DNA integrity. *International Journal of Molecular Sciences.* **20** (17), 4311 (2019).

8 Sterneckert, J. L., Reinhardt, P., Schöler, H. R. Investigating human disease using stem cell models. *Nature Reviews. Genetics.* **15** (9), 625–639 (2014).

9 Patani, R. Human stem cell models of disease and the prognosis of academic medicine. *Nature Medicine.* **26** (4), 449 (2020).

10 Liang, K. X. et al. N-acetylcysteine amide ameliorates mitochondrial dysfunction and reduces oxidative stress in hiPSC-derived dopaminergic neurons with POLG mutation. *Experimental Neurology.* **337**, 113536 (2021).

11 Kikuchi, T. et al. Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature.* **548** (7669), 592–596 (2017).

12 Juopperi, T. A. et al. Astrocytes generated from patient induced pluripotent stem cells recapitulate features of Huntington's disease patient cells. *Molecular Brain.* **5**, 17 (2012).

13 Liang, K. X. et al. Stem cell derived astrocytes with POLG mutations and mitochondrial dysfunction including abnormal NAD+ metabolism is toxic for neurons. *bioRxiv.* doi:10.1101/2020.12.20.423652 (2020).

14 Liu, Q. et al. Human neural crest stem cells derived from human ESCs and induced pluripotent stem cells: induction, maintenance, and differentiation into functional schwann cells. *Stem Cells Translational Medicine.* **1** (4), 266–278 (2012).

15 Hong, Y. J., Do, J. T. Neural lineage differentiation from pluripotent stem cells to mimic human brain tissues. *Frontiers in Bioengineering and Biotechnology.* **7**, 400 (2019).

16 Lundin, A. et al. Human iPS-derived astroglia from a stable neural precursor state show improved functionality compared with conventional astrocytic models. *Stem Cell Reports.* **10** (3), 1030–1045 (2018).

17 Liang, K. X. et al. N-acetylcysteine amide ameliorates mitochondrial dysfunction and reduces oxidative stress in hiPSC-derived dopaminergic neurons with POLG mutation. *Experimental Neurology.* **337**, 113536 (2021).

18 Pendergrass, W., Wolf, N., Poot, M. Efficacy of MitoTracker Green™ and CMXrosamine to

measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry. Part A.* **61** (2), 162–169 (2004).

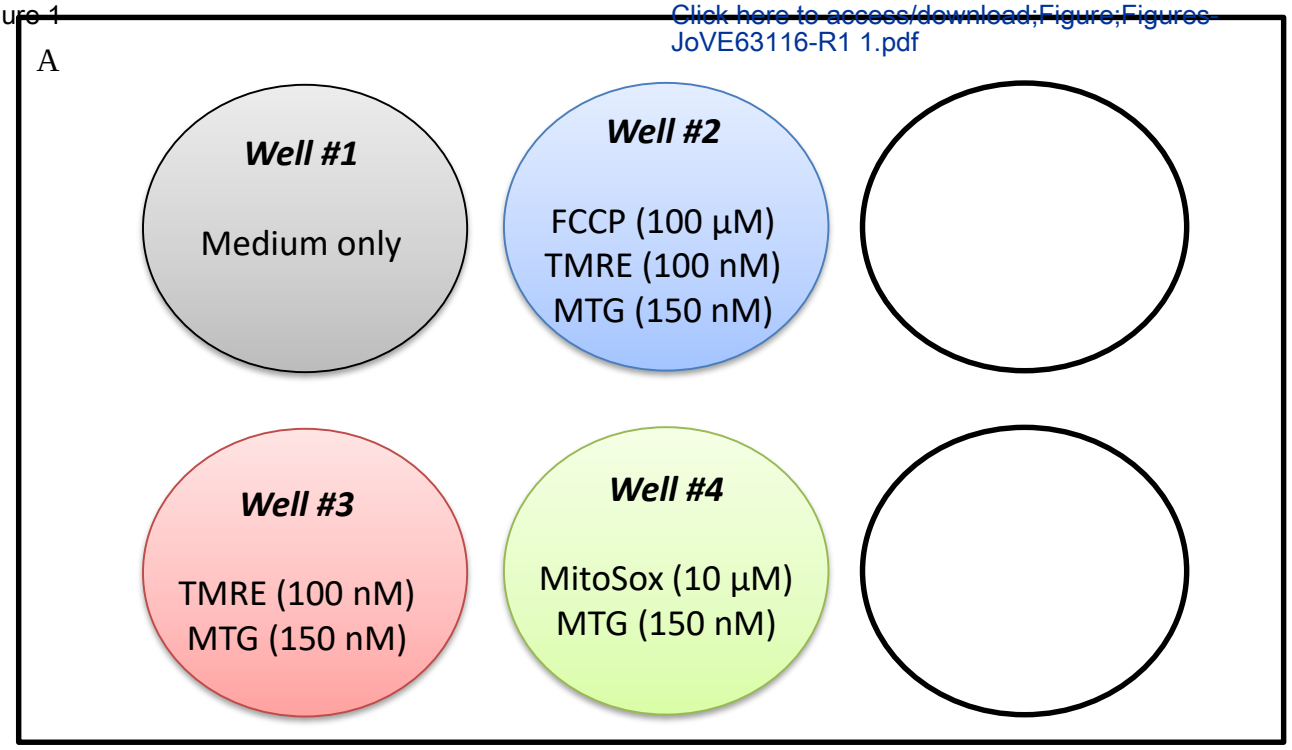
19 Keij, J. F., Bell-Prince, C., Steinkamp, J. A. Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker Green is affected by mitochondrial membrane potential altering drugs. *Cytometry.* **39** (3), 203–210 (2000).

20 Buckman, J. F. et al. MitoTracker labeling in primary neuronal and astrocytic cultures: influence of mitochondrial membrane potential and oxidants. *Journal of Neuroscience Methods*. **104** (2), 165–176 (2001).

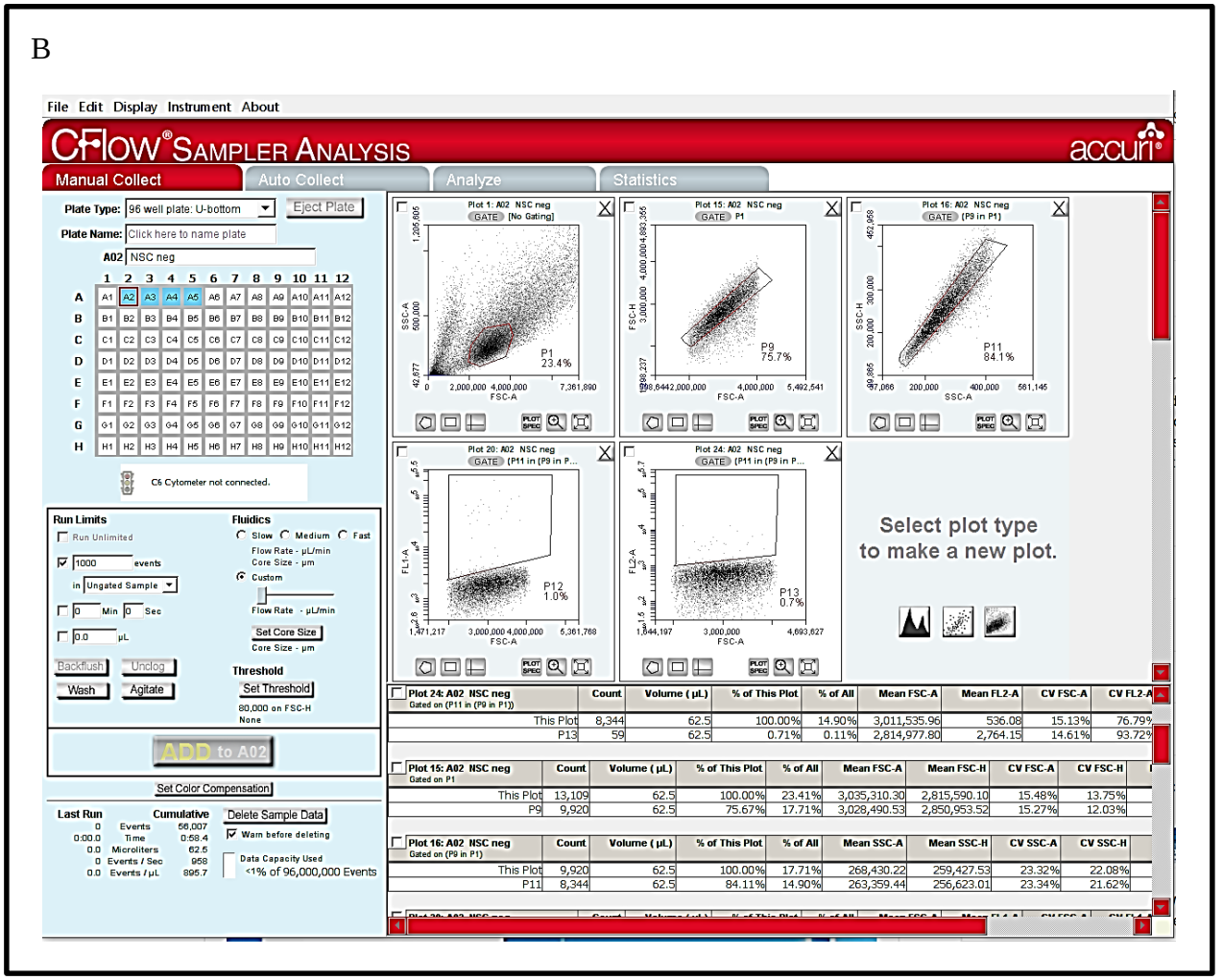
21 Zanchetta, L. M., Kirk, D., Lyng, F., Walsh, J., Murphy, J. E. Cell-density-dependent changes in mitochondrial membrane potential and reactive oxygen species production in human skin cells post sunlight exposure. *Photodermatology, Photoimmunology & Photomedicine.* **26** (6), 311–317 (2010).

Figure 1

A



B



1

Figure 2

Click here to access/download;Figure;Figures-JoVE63116-R1 2.pdf

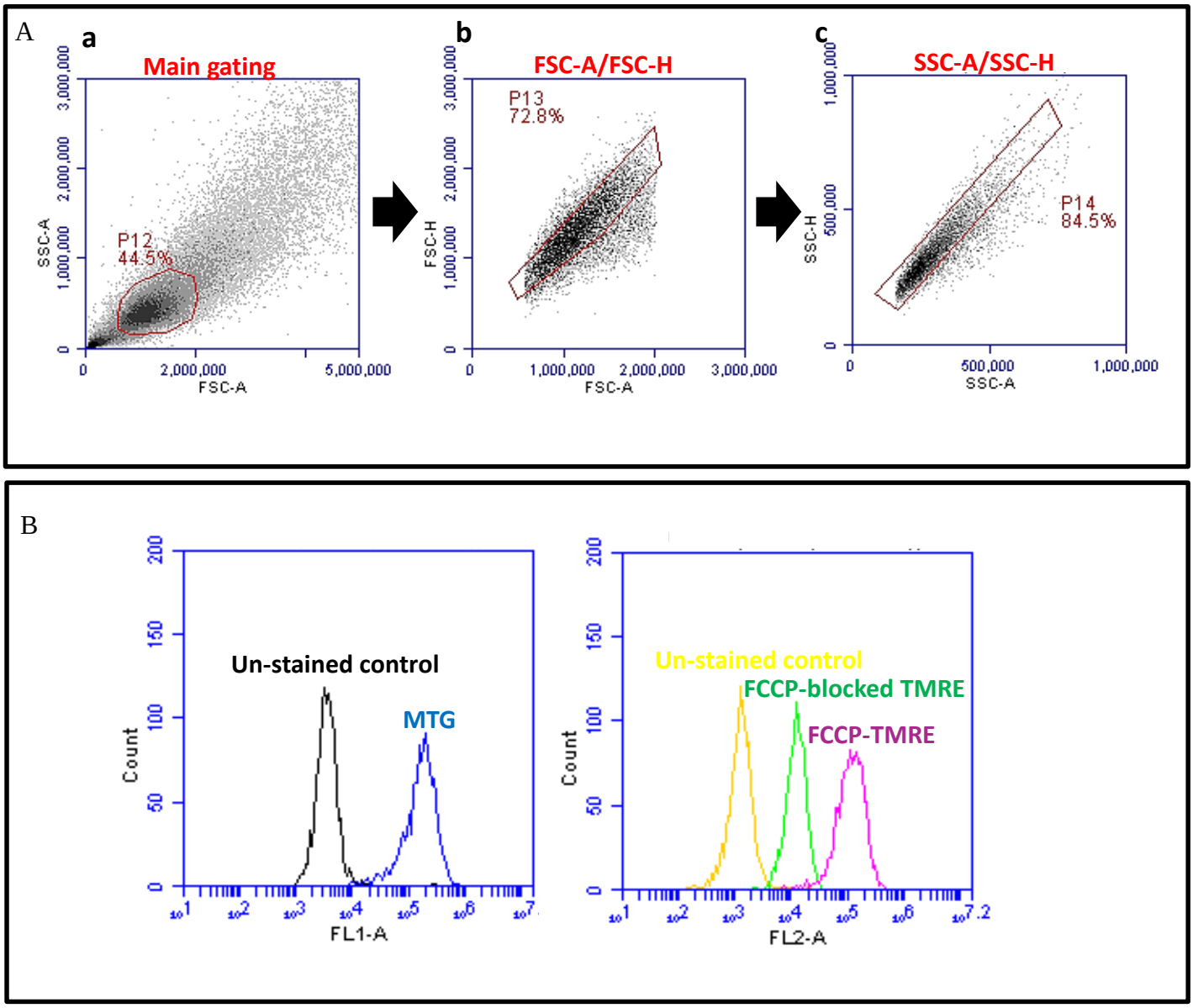


Figure 3

Click here to access/download;Figure;Figures-JoVE63116-R1 3.pdf

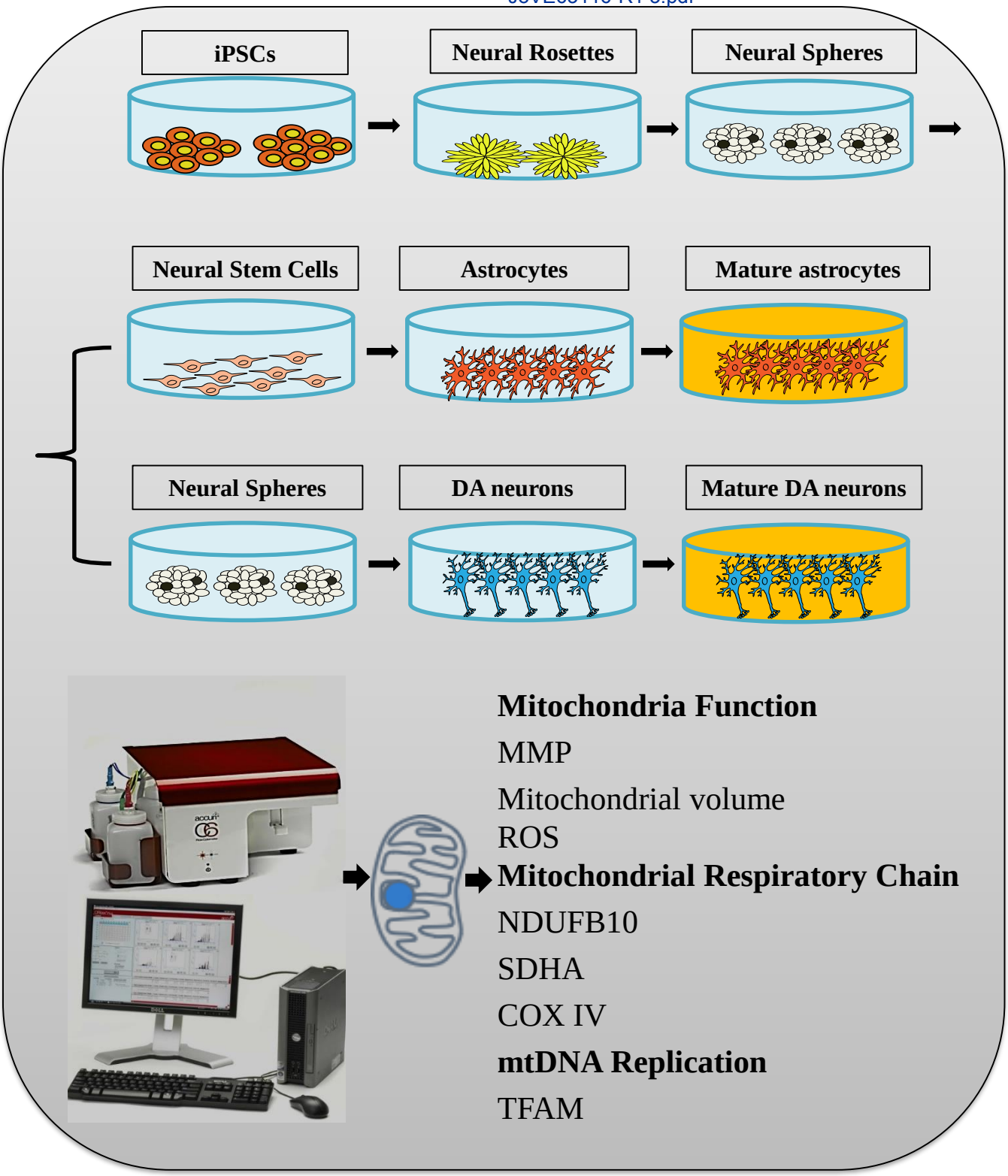


Figure 4

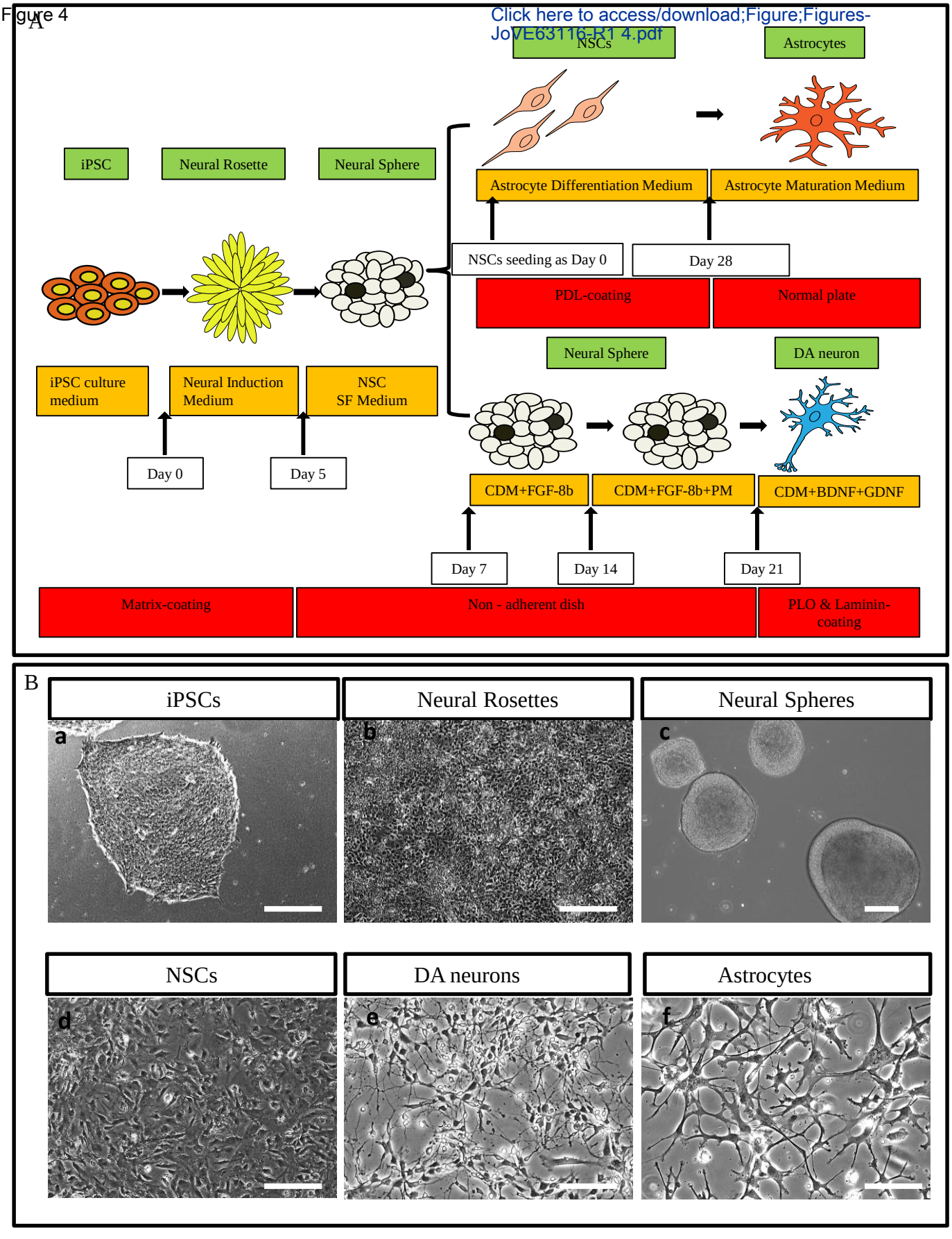


Figure 5

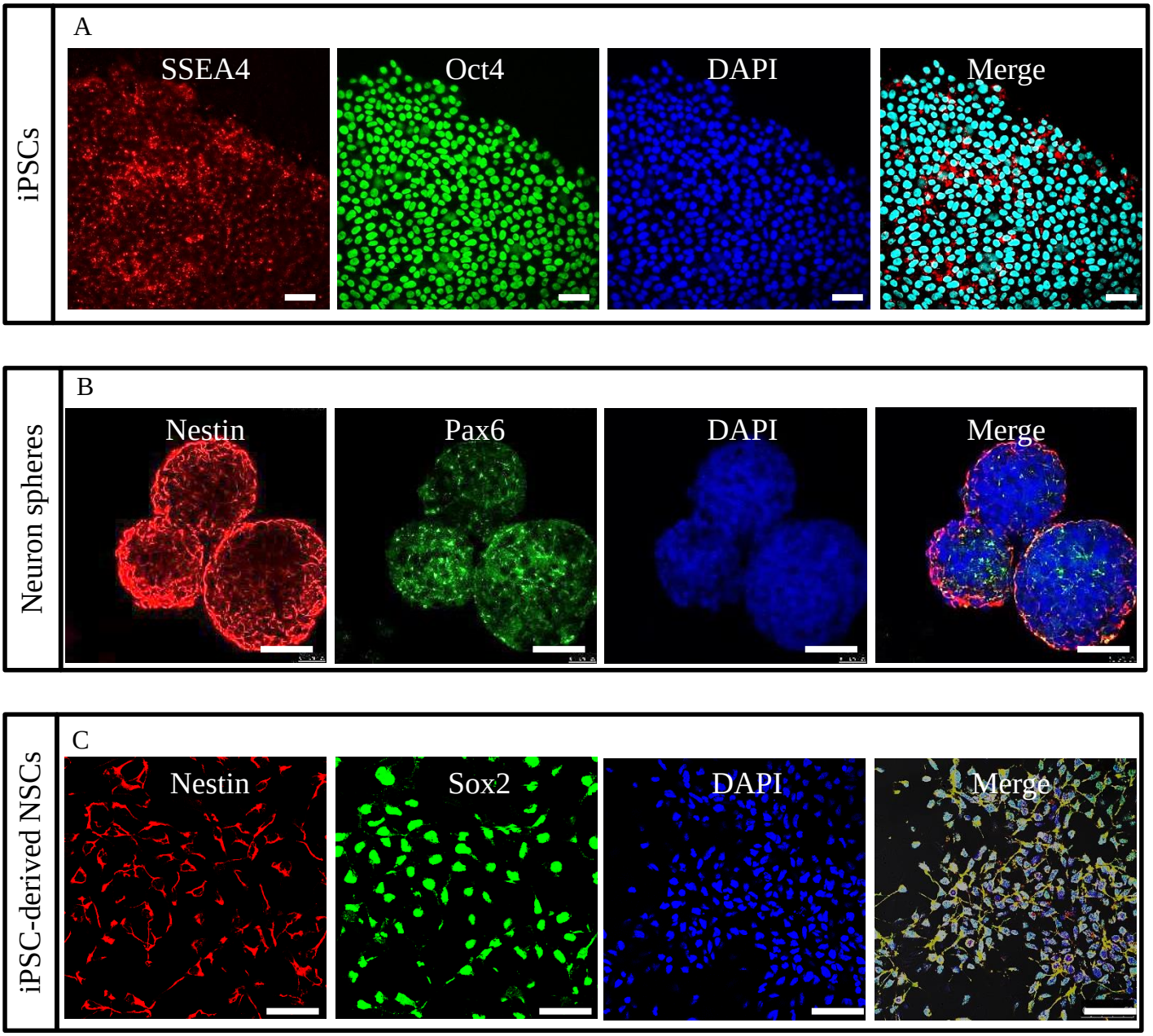
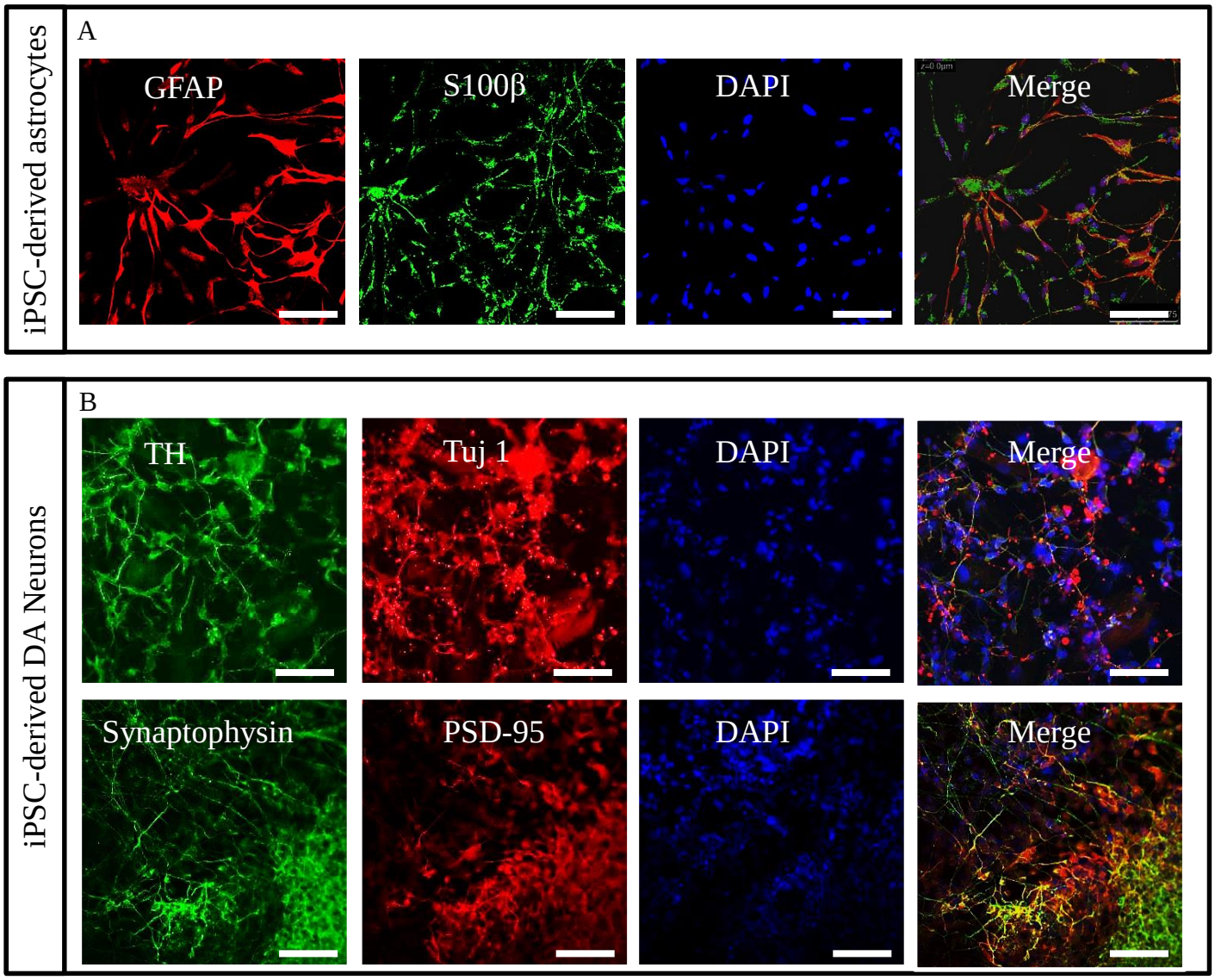


Figure 6

Click here to access/download;Figure;Figures-JoVE63116-R1 6.pdf



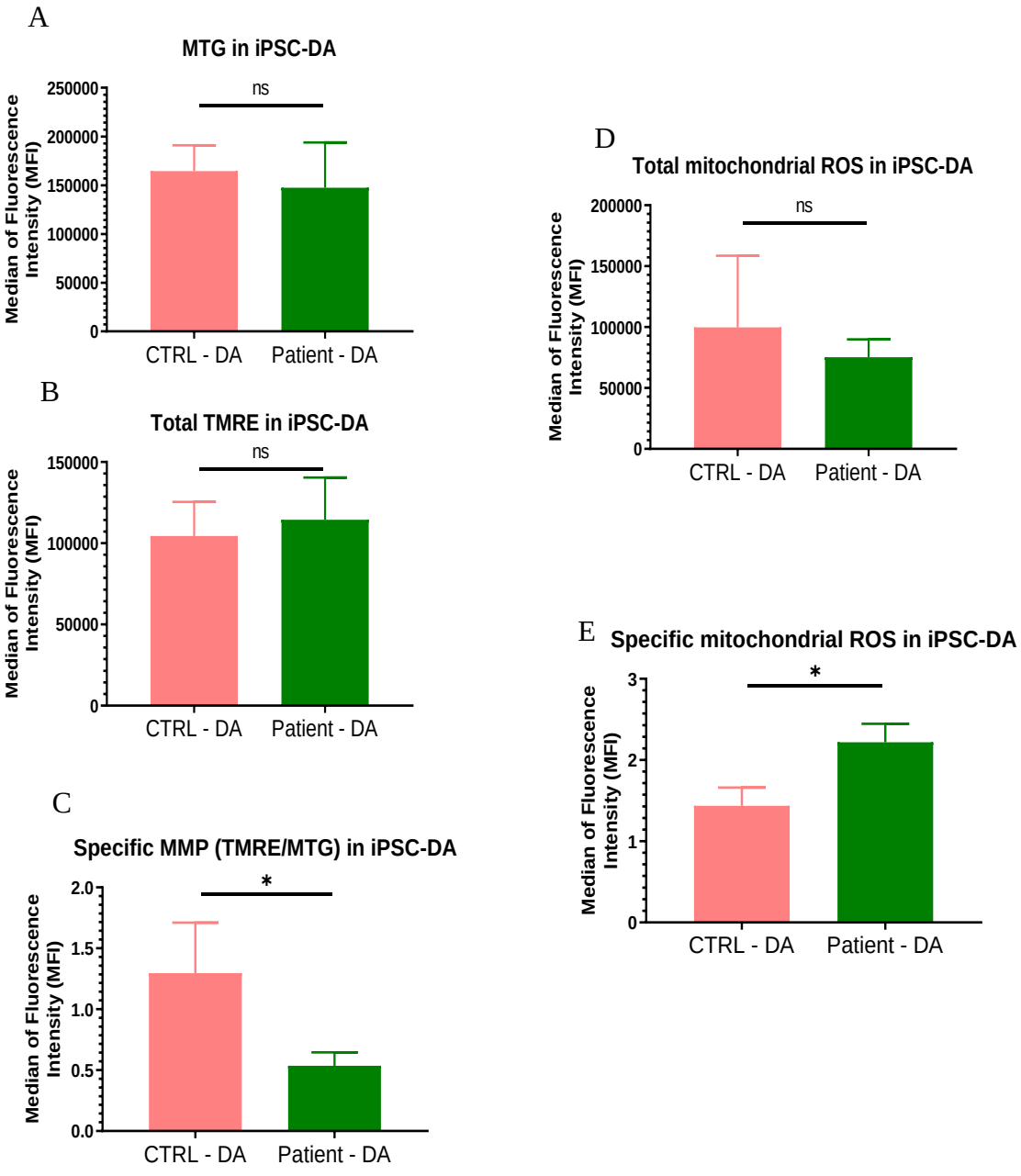
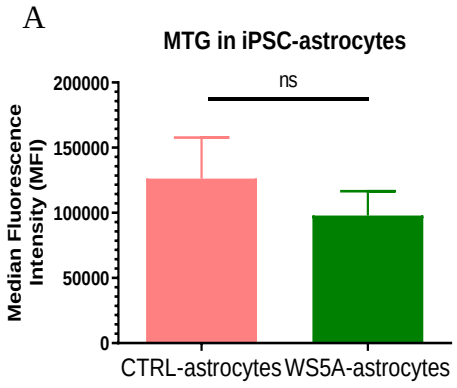
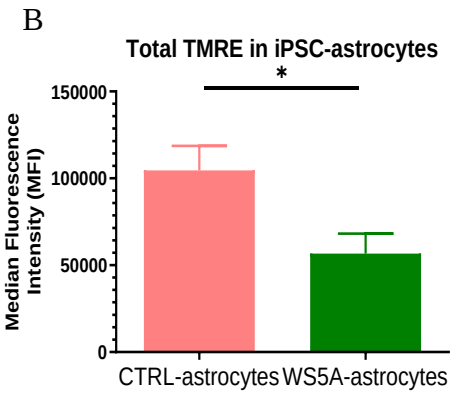
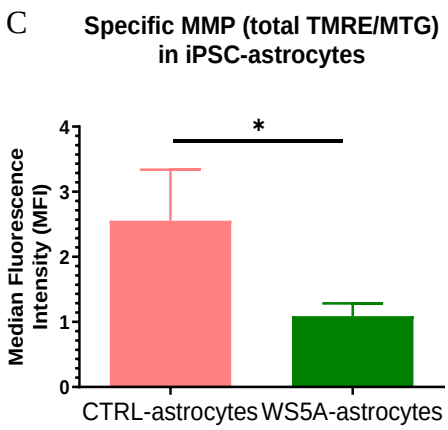


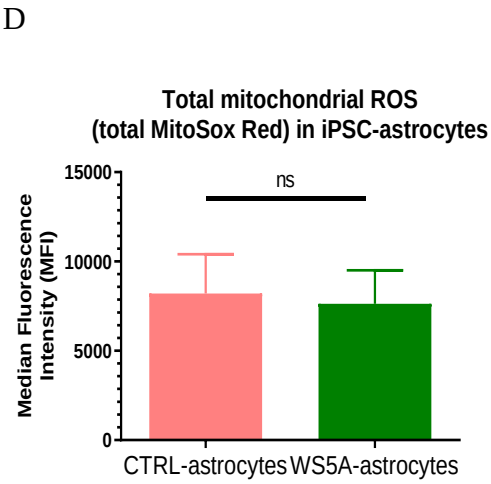
Figure 8

Click here to access/download;Figure;Figures-JoVE63116-R1 8.pdf









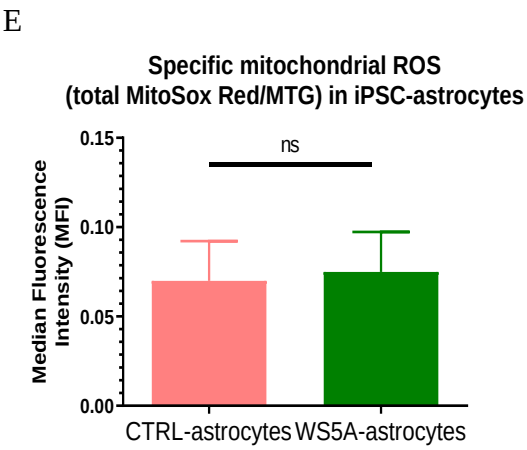


Figure 9

Click here to access/download;Figure;Figures-JoVE63116-R1 9.pdf

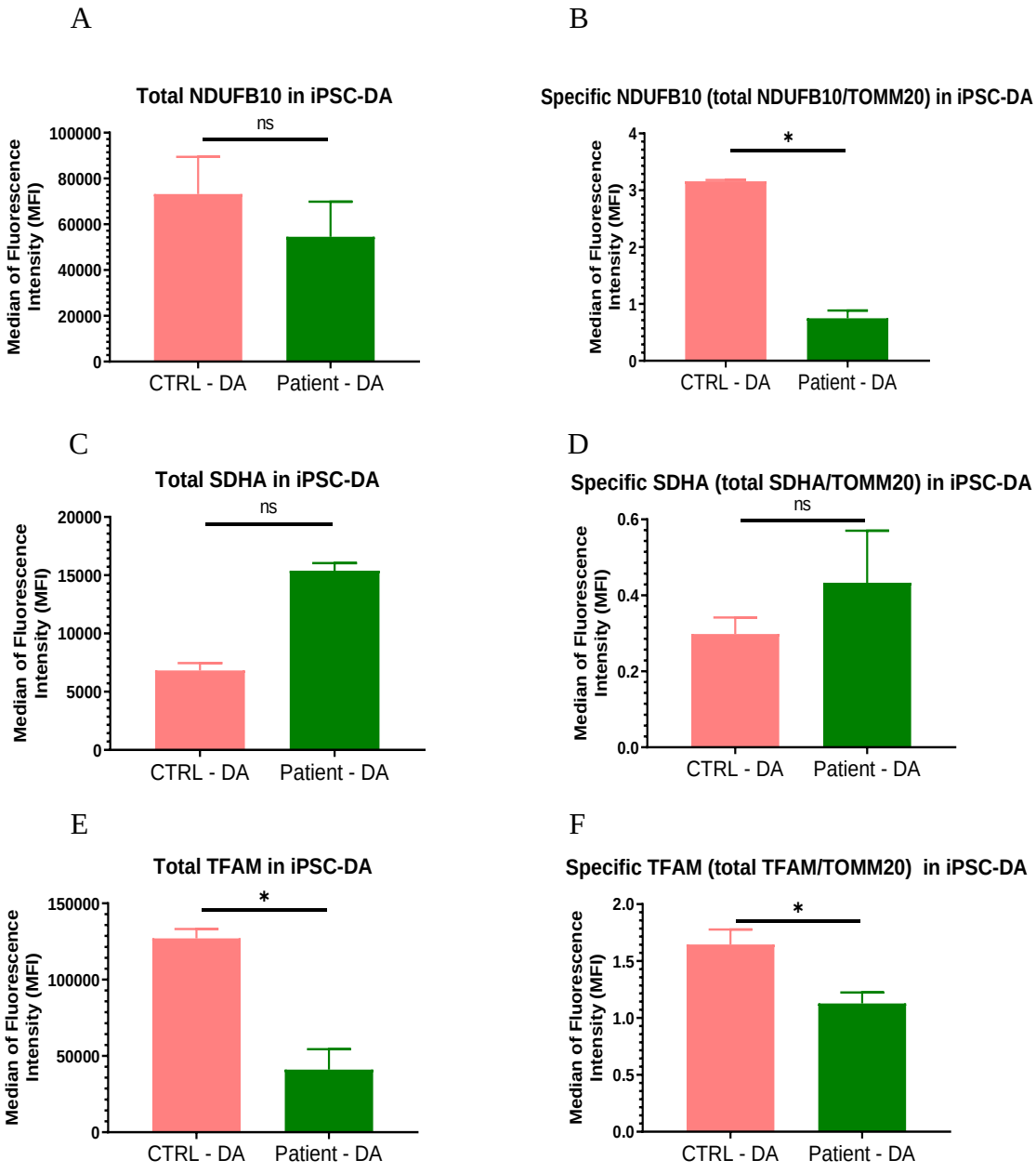
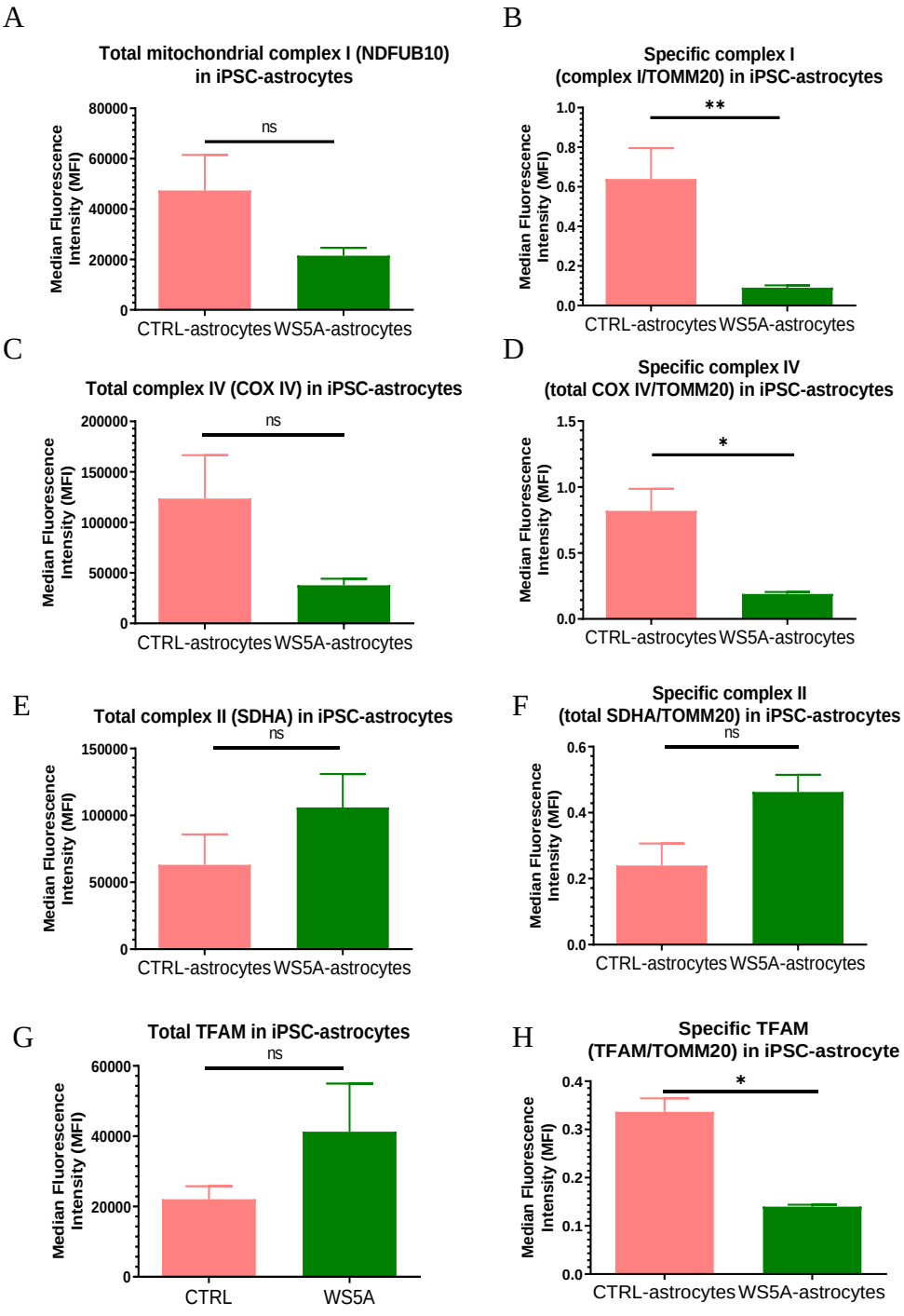


Figure 10

Click here to access/download;Figure;Figures-JoVE63116-R1 10.pdf



Dear Review Editor Amit Krishnan,

Thank you very much for the review of our manuscript entitled: “Flow cytometric analysis of multiple mitochondrial parameters in human induced pluripotent stem cells and their neural and glial derivatives.” (JoVE63116). We sincerely appreciate all valuable comments and suggestions, which helped us to improve the quality of the article

We have modified the manuscript according to the changes indicated. And provided answers to the questions raised by the reviewers. We believe that the comments have resulted in a much better manuscript, and we hope it is now finally acceptable for publication in your journal.

We enclose a point-by-point response to the reviewers’ comments typed in bold in blue with the original reviewer comments typed in regular typeface.

Yours sincerely,
Kristina Xiao Liang
Xiao.Liang@uib.no

Klinisk institutt 1, Universitetet i Bergen
Postboks 7804, 5020 BERGEN
post@k1.uib.no
+47 55 58 54 31

Responses for the comments:

Comments from editor,

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have invited an English Native Speaking professor to revise our manuscript to improve the English language. We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

Response: We have provided an email address for each author.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have revised the text to avoid the use of any personal pronouns.

4. Line 24: Repeat term mitochondrial volume.

Response: We have deleted the repeated texts.

5. Please revise the following lines to avoid previously published work: 46-51,65-67,103-106,108-111,352-354,389-390,428-429,440-441, 450-454. Please refer to the iThenticate report attached.

Response: We have revised the texts to avoid previously published work.

6. Please define all abbreviations upon first use. For example, TFAM, TMRE, TOMM20, DPBS, SF, FGF, FBS, DMEM, BDNF, GDNF, PFA, RT, etc.

Response: We have defined all abbreviations upon first use.

7. Please provide references (wherever appropriate) for lines 56-63, 66-83.

Response: We have provided references for lines 56-63 and 66-83.

8. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Response: We have removed all commercial language from your manuscript and used generic terms instead. For example: "Geltrex" into "matrix", "Essential 8" into "iPSCs culture medium", deleted "StemPro", "BD Accuri" into "Flow Cytometer", etc.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2

if necessary.
Response: We have adjusted the numbering of the Protocol to follow the JoVE Instructions for Authors.

10. Please include all safety procedures and use of hoods, etc.
Response: We have included all safety procedures and use of hoods, etc.

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Response: We have added some more details to our protocol steps.

12. 1.1.1: What was the final volume of the solution? What was the volume in each aliquot? Please specify.
Response: We have added the final volume of the solution and specified the volume in each aliquot.

13. 1.1.2: How many total wells were used? Please specify.
Response: We have specified the number of the wells in step 1.1.2.

14. 1.2: How much media was prepared? Please specify.
Response: We have moved the step of preparation of E8 medium into the supplemental files, where we have described the medium preparation.

15. 1.3.3: How much DPBS was used for rinsing(should all 4ML) and what was the DPSB concentration? Please specify.
Response: We used 4 mL DPBS with a concentration of 1X. We have specified this information.

16. 1.3.4: At which step were the iPSCs inoculated in the plate? Please describe iPSC inoculation in detail along with growth and colony formation.
Response: We have described such details as suggested by reviewer.

17. 1.3.6: How much pressure was applied? Please specify.
Response: We have specified how much pressure we applied.

18. 1.3.7 Note: What does the split ratio stand for? Please mention.
Response: We have mentioned what the split ratio stands for in the steps.

19. 1.4.2: What is the concentration of NIM used? Please specify.
Response: We have described the concentration of NIM used in the supplemental files.

20. 1.4.3: Please describe the microscope settings and parameters.
Response: We have added Figure S1 to describe the microscope settings and parameters.

21. 1.6.2: How much DPBS was added? Please specify.
Response: We have added the amount of DPBS.

22. 1.6.7: What was the solution used for re-suspension? Please mention.
Response: We have described solution used for re-suspension.

23. 1.6.8: What was the volume of PLO and laminin added? At what step were they added, please specify.
Response: We have described the volume of PLO and laminin used and the steps when they were added.

24. 1.6.4, 1.7.4: What was the water bath temperature? Please specify.
Response: We have described the water bath temperature.

25. 1.8.1.2: How much PDL was used to coat each well? Please specify.
Response: We have specified the amount of PDL used to coat in each well.

26. 2.1, 2.2, 2.4: Please mention the volume of PBS, PFA, blocking solution.
Response: We have described the volume of PBS, PFA, blocking solution.

27. 2.3: What was the concentration of primary antibody used?
Response: We have described the concentration of primary antibody used in the Table of Materials.

28. 3.15, 4.8, 4.12: Please provide all instrument settings and parameters.
Response: We have provided the all-instrument settings and parameters in Figure S1 and S2.

29. 4: Please provide volume used for PFA, methanol, block buffer, PBS, primary antibodies, staining solution, secondary antibody.
Response: We have provided the volume used for PFA, methanol, block buffer, PBS, primary antibodies, staining solution, secondary antibody in the manuscript.

30. 5: Please include all the button clicks, command lines, etc. in the software and instrument. If using long scripts and long command, please include as supplementary file. We need actions to show how the software and instrument

are used.
Response: We have provided all the button clicks, command lines, etc. in the software and instrument in Figure S1 and S2.

31. Please include a single line space between all the steps and ensure that the highlight is up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: We have highlighted the essential steps of the protocol for the video and ensure that the highlight is up to 3 pages of the Protocol.

32. Please remove the titles and Figure Legends from the uploaded figures. Please provide this information in the Figure Legends section after the Representative Results in the manuscript.
Response: We have removed the titles and Figure Legends from the uploaded figures and provided this information in the Figure Legends section after the Representative Results.

33. All figures should be uploaded separately to your Editorial Manager account.
Response: We have uploaded all figures separately to the Editorial Manager account.

34. Please do not abbreviate journal names in references.
Response: We have changed into the full journal names in references.

35. Please arrange the Table of Materials alphabetically.
Response: We have arranged the Table of Materials alphabetically.

36. Figure 3/4: Please include scale bars in all the images of the panel.
Response: We have included the scale bars in all the images of the panel.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript "Flow cytometric analysis of multiple……" provides an interesting perspective on the entire process from generating specific neuronal cell types to analysing mitochondrial function. The protocols presented are comprehensive and interesting but are set out in a way that are difficult to follow in a linear fashion. This is complicated by poor grammar and stylistic issues, and lack of explanation of some parameters before they are used in the protocol. Everything seems to be there, it is just really difficult to follow. A longer, clearer, and more detailed introduction would be helpful to

communicate the specific issues, or splitting the protocol in two parts; namely generation of the specific cell types followed by analysis of mitochondrial function.

Summary
"mitochondrial volume" is repeated.
Response: We have deleted the repeated text.

Abstract
1) Neurodegenerative diseases and changes in mitochondrial volume are not a conjunction. Consider splitting this sentence for clarity.
Response: We have splitted this sentence into two in order to clarity.

2) Be careful to define mitochondrial membrane potential as distinct from membrane potential. This is important because the plasma membrane also has a potential that is also detected by staining with TMRE, the author refers to the acronym MMP to acknowledge this later, and is also why FCCP is used later as a control.
Response: We agree with the review that the mitochondrial membrane potential is distinct from other membrane potential such as plasma membrane. We have defined the mitochondrial membrane potential in our paper.

3) "are often the feature of these processes" should be " are often a feature of these processes"
Response: We have revised this sentence.

4) The part of the sentence " …and ROS levels and fixed cells" would be better defined using a semi colon "and ROS levels; and fixed cells"
Response: We have revised this sentence.

5) The abstract gives an indication that using TMRE and MTG measure ROS. This is clearly not possible but Mitox-Red is not mentioned until the discussion.
Response: We have described using MitoSox Red for ROS measurement in the abstract.

6) A similar issue arises with the description of TFAM and TOMM20, which suggests that these are used to measure MRC. Only much later is there any indication that these do not measure the MRC, and that the protocol uses a group of antibodies to detect components of each subunit of the electron transport chain. Using specific examples in the abstract therefore may confuse the message. One way around this is to refer to "outer membrane"
Response: To avoid the confusion of the MRC subunit measurement and TFAM assessment. We have revised the sentences in abstract into

“Double staining with antibodies against MRC complex subunits together with translocase of outer mitochondrial membrane 20 (TOMM20) permits assessment of the MRC subunit expressions. Since the amount of TFAM is proportional to mtDNA copy number, measurement of TFAM per TOMM20 gives an indirect measurement of mtDNA per mitochondrial volume.”

Introduction
The use of appropriate commas to delineate lists vs conjunctions is pervasive
1) It is important to be accurate and careful with grammar and intent. For example, Mitochondria may be responsible for producing 80% of total ATP, but they are responsible for producing 100% of the ATP by oxidative phosphorylation.
Response: In order to be accurate and careful with the grammar and intent, we have revised the sentences in abstract into “Mitochondria re responsible for energy supply by producing adenosine triphosphate (ATP) via oxidative phosphorylation and act as metabolic intermediator for biosynthesis and metabolism.”

2) Remove any superfluous definite article "the", such as "the regulation of cell death" or "the production of reactive oxygen species"
Response: We have removed the superfluous definite article "the".

3) Delete "findings" on line 58
Response: We have deleted "findings" on line 58.

4) Line 59. "the ability to measure these, and other' mitochondrial functions is therefore of greet use (this allows future implications of measuring actual respiration as an important function)
Response: We have revised this sentence.

5) Line 60. The statement that animal models do not replicate faithfully human disease is a bit strong and would be best tempered with "some". For example, "Failure of animal models to faithfully replicate some human disease" or "failure of animal models to faithfully replicate human neurodegenerative disease.
Response: We have revised this sentence.

6) Line 74. "multiple" should be replaced with "different types of"
Response: We have replaced "multiple" with "different types of".

Protocol
Part 1 - Subheading 1
1) Thaw a vial (replace "the" with "a")

2) Geltrex (Gibco) (add vendor - if specific)
3) Dilute 1:100 (delete "into")
4) 1:100 is 1% of a stock, and explanation is repetitive unless qualified by v/v or w/v.
5) Store "at" -20oC instead of "in" -20oC
6) Similarly use "at" 4oC
7) "wells" should not be plural for "a 6 wells plate" as the plate is singular
8) "Coated plate" is not a noun and should have a small "c"
9) "recommended to use in 3 days" is not correct English
10) "To avoid gel dry out" should be "To avoid the gel drying out"
Response: We have revised accordingly as the reviewer suggested.

Subheading 2
11) Preparation "of" Essential 8. This does not make sense as a step.
Response: We have removed this step.

Subheading 3
12) Use " at RT or in an incubator at 37^{o}C"
Response: We have changed into " at RT or in an incubator at 37^{o}C".

13) "6 well" not "6 wells"
Response: We have changed into " 6-well plate".

14) There are too many references to "Table of Materials" to provide a continuous protocol.
Response: We have removed some unnecessary references to "Table of Materials"

15) Step 5. "aspirate the gentle dissociation medium" is confusing. Is the gentle dissociation medium the 0.5 mM EDTA.
Response: We mean the EDTA is a dissociation medium that is "gentler" on cells and avoid the potentially damaging. We have revised this into "5 mM EDTA" to avoid confusion.

16) "Gentle shake before putting in the incubator" should be "Shake gently before ..."
Response: We have revised into "Shake gently before ..."

Subheading 4
17) "Preparation" should be "Prepare"
18) Add "(NIM)" after Neural Induction Medium in step 1 as it is not defined before in step 2.
19) Replace Neural Induction Medium with NIM in Step 3.
20) "Observe" is not noun. Use lower case "o".

Response: We have made corresponding changes to these contents based on the reviewers' suggestions.

Part 3

21) MitoSox Red appears out of nowhere.

Response: We have made MitoSox Red in the manuscript.

22) Step 15. Which dye is excited off which laser in this combination.

Response: We have described the filter related to each dye in section 3.15, as described in the paper "3.15 Analyze on Flow Cytometer (with a 3 blue and 1 red laser configuration). MTG is detected in filter 1 (FL1) by use of a 530/30 bandpass filter, while TMRE is detected in filter 2 (FL2) using the bandpass filter 585/40. MitoSox Red is detected in filter 3 (FL3) by use of a 510/580 bandpass filter."

Part 4

23) Wash in PBS "once or twice" is not very scientific

Response: We have clarified the washing step.

24) Primary antibodies "NDUFB10, SDHA, and CoxIV come out of nowhere.

Response: We have added the description of "NDUFB10, SDHA, and Cox IV" in section 4.6.

25) Were all of the antibodies conjugated with alexa 488and mixed together in the same tube at the same time.

Response: All the sample were stained in individual tubes with single staining. Then they were measured individually for their median fluorescence intensity, referred as the total level of the protein expression (after substrate with the MFI of negative control). The specific level is calculated with total level per mitochondrial volume measurement of TOMM20, for example, specific TFAM = total TFAM/TOMM20.

26) What were the second antibodies conjugated to. Were they alexa 488 as they were all detected in the 530/30 filter. What is the reason for for using 4 antibodies here. Does this raise the overall fluorescence, or average out the indirect measure of volume.

Response: We have descripted the detailed information of second antibodies in Tables of Materials. The NDUFB10, SDHA, and COX IV are all conjugated with Alexa 488 using second antibodies and detected in the 530/30 filter. The TFAM and TOMM20 are conjugated with Alexa 488 and in the 530/30 filter.

Part 5

27) The staining protocols do not mention the IgG control or single colour controls, or which is which.

Response: We have added the detailed description of the IgG control in Table of Materials.

Representation of results

28) The author becomes lax, using "mito-volume".

29) Line 340 MRC complex subunit expression should be "expression levels of MRC complex subunits"

30) "TFAM" should be "TFAM;"

Response: We have made corresponding changes to these contents based on the reviewers' suggestions.

Reviewer #2:

Manuscript Summary:

Authors report a novel approach to measure multiple mitochondrial function parameters based on flow cytometry and double staining with two fluorescent reporters or two antibodies, which allows detection of changes in mitochondrial volume, mitochondrial volume, membrane potential, reactive oxygen species level, mitochondrial respiratory chain and mitochondrial DNA.

The present manuscript developed a flow cytometry-based approach measuring multiple mitochondrial parameters in various cell types. This approach has a certain novelty in understanding mitochondrial changes in different diseases. The results were convincing, and the protocols were described clearly. My only concern is how consistent they will be when compared to the commonly used methods. It might be more solid if the author could include some traditional methods and compare those parameters measured by different systems.

Response: We thank the reviewers for the positive response of our manuscript and agree that it may be more reliable, including some traditional methods and comparing the parameters measured by different systems. In our manuscript, we have discussed the comparison of our methods with the traditional microscope-based analysis in the last paragraph of discussion section, as described in the paper "Compared with other microscopy-based assays, flow cytometry has the advantages of speed and reproducibility when analyzing large numbers of cells. In the analysis of microscope photos, the bias of researchers will distort the results to a certain extent, which is not a problem when using flow cytometry. In addition, Flow cytometry analysis requires less than one million cells, and analysis of one sample only takes a few minutes, which means that dozens of samples can be analyzed in one to two hours. This technique can also be applied to a wide variety of cell types including those from other neurodegenerative diseases and should therefore be useful for understanding mechanisms."

Medium recipes

Component	Source	catalog no.
Essential 8 Medium (iPSC culture medium)		
Essential 8 Basal Medium	Thermo Fisher Scientific	A1516901
Essential 8 Supplement (50x) 2% (v/v)	Thermo Fisher Scientific	A1517101
Store at 4 °C and warm up to RT before use.		
Commercial Defined Medium (CDM)		
IMDM	Thermo Fisher Scientific	21980032
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	31765027
Bovine Serum Albumin 5 mg/mL	Europa Bioproducts	EQBAH62-1000
Chemically Defined Lipid Concentrate 1% (v/v)	Thermo Fisher Scientific	11905031
Monothioglycerol 450 µM	Sigma	M6145
Insulin 7 µg/mL	Roche	1376497
Transferrin 15 mg/mL	Roche	652202
Optional: Antibiotic-Antimycotic 1% (v/v)	Thermo Fisher Scientific	15240062
Filter-sterilize through a 0.22 µm filter membrane. Store for up to 4 weeks in		
Neural Induction Medium (NIM)		
CDM (above)		
SB 431542 10 µM	Tocris Bioscience	TB1614-GMP
InSolution AMPK Inhibitor, Compound C 2 µM	Sigma-Aldrich	171261
N-Acetyl-L-cysteine 0.5 mM	Sigma-Aldrich	A7250
Filter-sterilize through a 0.22 µm filter membrane. Store for up to 2 weeks in the dark at 4 °C.		
StemPro NSC SF Medium		
KnockOut DMEM/F-12 medium	Thermo Fisher Scientific	12660012
StemPro Neural Supplement 2% (v/v)	Thermo Fisher Scientific	A10508-01
FGF-basic (AA 10–155) Recombinant Human Protein 20 ng/mL	Thermo Fisher Scientific	PHG0024
EGF Recombinant Human Protein 20 ng/mL	Thermo Fisher Scientific	HG0314
GlutaMAX Supplement 1% (v/v)	Thermo Fisher Scientific	35050061

Filter-sterilize through a 0.22 µm filter membrane. Store for up to 2 weeks in the dark at 4 °C.

Astrocyte Differentiation Medium

DMEM/F-12, GlutaMAX supplement	Thermo Fisher Scientific	10565018
N-2 Supplement 1% (v/v)	Thermo Fisher Scientific	17502048
B-27 Supplement 1% (v/v)	Thermo Fisher Scientific	17504044
FGF-basic 8 ng/mL	PeproTech	100-18B
Heregulin beta -1 human 10 ng/mL	Sigma-Aldrich	SRP3055
Activin A 10 ng/mL	PeproTech	120-14E
Insulin-like Growth Factor-I human 200 ng/mL	Sigma-Aldrich	I3769
Fetal Bovine Serum 1% (v/v)	Sigma-Aldrich	12103C

Filter-sterilize through a 0.22 µm filter membrane. Store for up to 2 weeks in the dark at 4 °C.

Astrocyte Maturation Medium

ABM Basal Medium 500 mL	Lonza Bioscience	CC-3187
AGM TM Astrocyte Growth Medium BulletKit TM	Lonza Bioscience	CC-4123
FBS, 15.0 mL	Lonza Bioscience	CC-4123
L-Glutamine, 5.00 mL	Lonza Bioscience	CC-4123
GA-1000, 0.50 mL	Lonza Bioscience	CC-4123
Ascorbic Acid, 0.50 mL	Lonza Bioscience	CC-4123
Human EGF, 0.50 mL	Lonza Bioscience	CC-4123
Insulin, 1.25 mL	Lonza Bioscience	CC-4123

Store for up to 14 days at 4 °C.

Solution recipes

MTG 0.5 mM

Add 149 µL of DMSO to the 50 µg MitoTracker Green to reach to the final concentration of 0.5 mM. Prepare 20 µL aliquots and store in the dark at -20 °C.

FCCP 100 mM

Add 393.4 µL of DMSO to the 10 mg FCCP tube to obtain the final concentration of 100 mM. Prepare 20 µL aliquots and store in the dark at -20 °C.

CAUTION: FCCP is toxic; avoid contact with skin and eyes and handle under a chemical fume hood.

MitoSox Red 5 mM

Add 13 µL of DMSO to 50 µg of MitoSox Red in a tube to obtain the final concentration of 5 mM. Prepare 20 µL aliquots and store in the dark at -20 °C.

Geltrex 1%

Thaw the vial of Geltrex on ice overnight. Aliquot into 20 µL per vial and store at -20 °C.
When coating the plate, dilute the aliquot 1:100 in ice-cold Advanced DMEM/F12.

EDTA 0.5 mM

Dilute EDTA 5mM into 1:1,000 in DPBS (Ca^{2+}/Mg^{2+}-free), filter-sterilize through a 0.22 µm filter membrane, and store at RT.

Collagenase IV 200 unit/mL

Dissolve 1 g of collagenase IV powder in 1,300 mL of Advanced DMEM/F-12 to make the final concentration of 200 U/mL. Filter through a 0.22 µm filter. Protect from light. The solution can be stored at -20 °C, but the activity is best when freshly prepared.

Laminin 5 µg/mL

Dissolve 1 mg of laminin in 200 mL of sterilized water to obtain the final concentration of 5 µg/mL. Filter through a 0.22 µm filter. The solution can be stored at -20 °C.

PFA 4%

Dissolve 12.5 mL of 16% PFA aqueous solution, EM Grade with 37.5 mL of PBS. The solution can be stored at 4 °C, protected from light.

PFA 1.6%

Dissolve 5 mL of 16% PFA aqueous solution, EM Grade with 45 mL of PBS. The solution can be stored at 4 °C, protected from light.

SB 431542 10 mM

Dissolve 10 mg of SB 431542 with 2601.5 µL of DMSO, prepare 100 µL aliquots, and keep it in the dark at -20 °C.
NOTE: It is important to recalculate the amount of DMSO due to the interbatch variation in molecular weight.

InSolution AMPK Inhibitor, Compound C 10 mM

Dissolve 1 mg of AMPK Inhibitor, Compound C, with 250 µL of DMSO, prepare 100 µL aliquots, and keep it in the dark at -20 °C.

Insulin 10 mg/mL

Resuspend 100 mg of insulin in 10 mL of sterile water, aliquot into 1 mL, and store at -20 °C.

N-Acetyl-L-cysteine 500 mM

Dissolve 1630 mg of N-acetyl-L-cysteine with 20 mL of sterilized water, store in 1 mL aliquots at -20 °C.

FGF-8b 25 µg/mL

Dissolve 25 µg of FGF-8b with 1 mL of sterilized PBS containing 0.1% BSA. Prepare 40 µL aliquots and store them at −80 °C for up to 6 months. Once thawed, aliquots can be kept at 4 °C for a week.

Purmorphamine (PM) 10 mM

Dissolve 5 mg of PM with 960 µL of DMSO, mix thoroughly and store in 20 µL aliquots at -20 °C for up to 6 months. Once thawed, use within 7 days, and protect from light.

BDNF 10 µg/mL

Dissolve 2 µg of BDNF with 200 µL of cold, sterile 0.2% BSA/DPBS solution and mix well, but gently, to avoid foaming. Make 20 µL aliquots and store up to 6 months at - 80 °C.

GDNF 10 µg/mL

Dissolve 2 µg of GDNF with 200 µL of cold, sterile 0.2% BSA/DPBS solution and mix well, but gently, to avoid foaming. Make 20 µL aliquots and store up to 6 months days at - 80 °C.

Heregulin beta -1 human 0.1 mg/mL

Dissolve 50 ng of heregulin beta -1 human with 500 µL of sterile water. Make 55 µL aliquots and store at -20 °C.

Activin A 0.1 mg/mL

Dissolve 10 µg of Activin A with 110 µL of sterile water. Store at -20 °C.

Insulin-like Growth Factor-I human 0.1 mg/mL

Dissolve 100 µg of Activin A with 100 µL of 0.2% acetic acid solution; dilute it further by adding 900 µL of sterile water. Store at -20 °C.

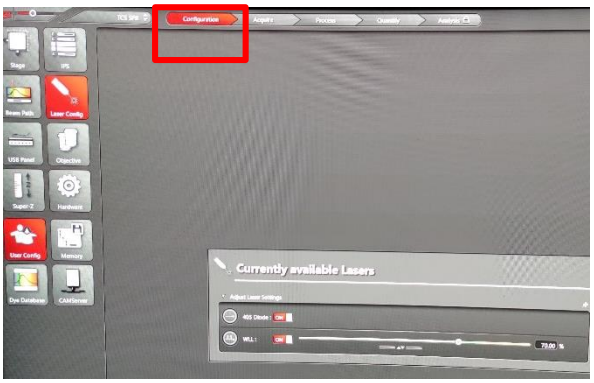
Setup for Flow Cytometric Staining

Measurements	Dye or antibody	Filter in flow	Parameter measurement
Complex I	1:1000 anti-NDUFB10	FL1	Total complex I (total NDUFB10)
	1:200 anti-TOMM20		Specific complex I (total NDUFB10/TOMM20)
Complex II	1:1000 anti-SDHA	FL1	Total complex II (total SDHA)
	1:400 anti-TOMM20		Specific complex II (total SDHA/TOMM20)
Complex IV	1:1000 anti-COX IV	FL1	Total complex IV (total COX IV)
	1:400 anti-TOMM20		Specific complex IV (total COX IV/TOMM20)
MMP	100 nm TMRE	FL1-MTG	Mitochondrial volume (total MTG)
	150 nM MTG	FL2-TMRE	Total MMP (total TMRE)
	100 µM FCCP		Specific MMP (total TMRE/MTG)
ROS	10 µM MitoSox Red	FL3-MitoSox Red	Total ROS (total MitoSox Red)
	150 nM MTG	FL1-MTG	Specific ROS (total MitoSox Red/MTG)
TFAM	1:400 anti-TFAM	FL1	Total TFAM (total TFAM)
	1:400 anti-TOMM20		Specific ROS (total TFAM/TOMM20)

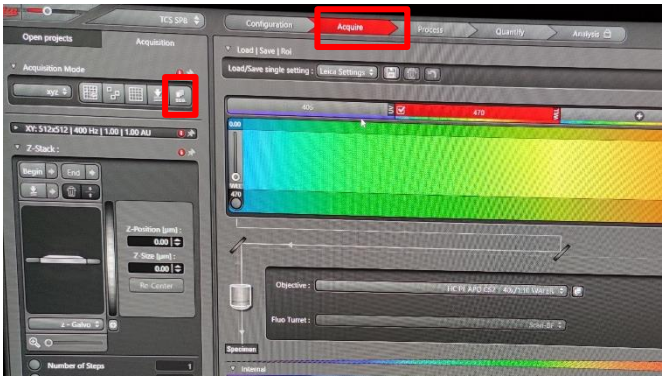
Supplemental Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figures-

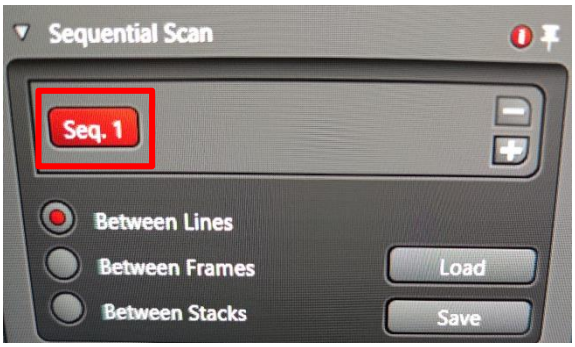
A



B

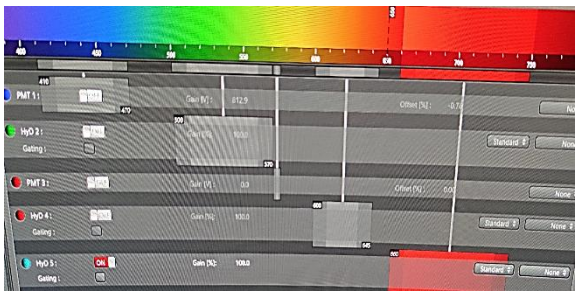


C

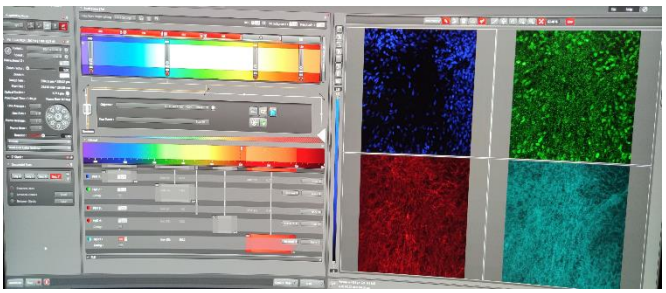


D

E



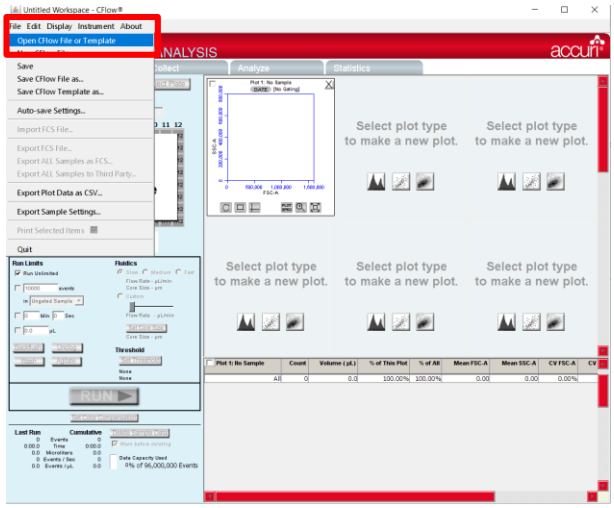
F



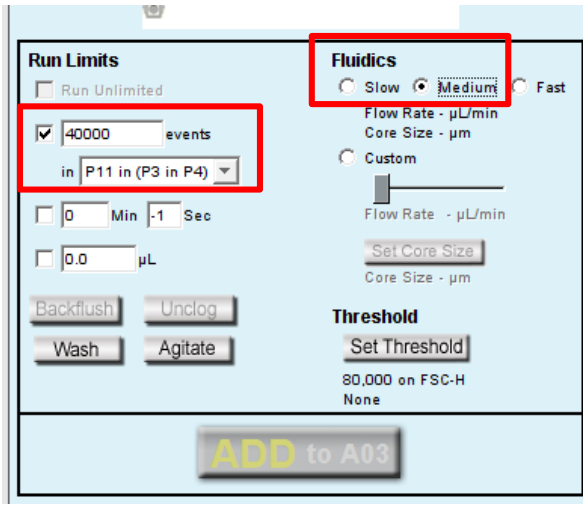
Supplemental Figure S2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figures-

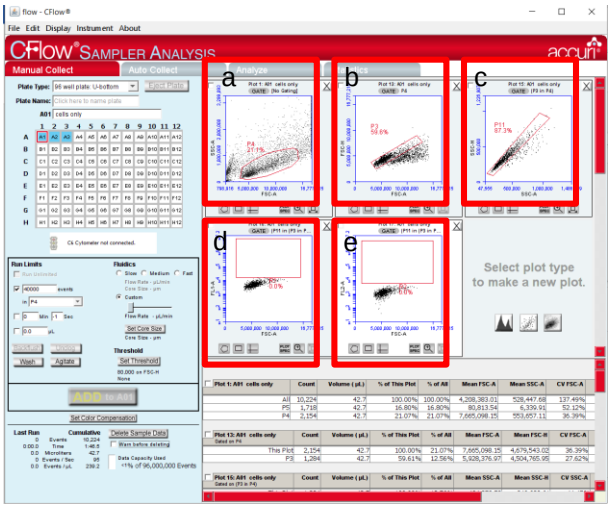
A



B



C



D