High content analysis of mitochondrial function in iPSC-derived neurons

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Abstract

Mitochondrial dysfunction is linked to many neurological diseases; therefore the ability to measure mitochondrial function is of great use for researching disease and testing potential therapeutics. Here we describe a high content assay to simultaneously measure mitochondrial membrane potential, morphology and cell viability in iPSC-derived neurons. Neurons are seeded into plates suitable for fluorescent microscopy, stained with the mitochondrial membrane potential-dependent dye TMRM, cytoplasmic dye Calcein-AM and nuclear stain Hoechst-33342. Images are acquired in live cells and analysed using automated image analysis software.

Keywords

Mitochondria, induced pluripotent stem cells, neurons, high content screening, image analysis

1. Introduction

Mitochondrial dysfunction has been implicated in many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's

disease and motor neuron disease [1-4]. Here we present a method of analyzing mitochondrial function in iPSC-derived neurons in a manner that is compatible with high-content screening methods and could be used to test potential therapeutics for neurodegenerative diseases. Being derived from patients, neurons differentiated from iPSCs are felt to provide accurate disease models that can be used to identify or test potential therapeutics for neurodegenerative diseases [5,6].

This method utilizes the dye tetramethylrhodamine (TMRM) to measure mitochondrial membrane potential and morphology and utilizes the dyes Calcein-AM and Hoechst-33342 to aid cell segmentation and measure cell viability (Fig. 1). TMRM has been widely used to measure mitochondrial membrane potential and has helped to implicate mitochondrial dysfunction as a mechanism of various neurodegenerative diseases [7-10]. The presence of the mitochondrial membrane potential ($\Delta\psi m$) is essential for TMRM to be taken up by mitochondria; when $\Delta\psi m$ is reduced, fluorescence intensity decreases when used in non-quench mode as it is here. Reduced $\Delta\psi m$ equates to reduced capacity for ATP generation because ATP generation is dependent on the electrochemical proton gradient across the mitochondrial inner membrane [11-13]. Mitochondrial function is directly related to morphology, because mitochondria must move, divide and fuse in response to cellular demands [14,2,15], which is particularly important in neuronal cells [16].

An image analysis pipeline using CellProfiler software [17,18] to segment cells and measure features is outlined in this protocol (Fig. 2). Essentially, nuclei are identified from Hoechst staining, then the associated cytoplasm is identified from Calcein staining. Any nuclei without cytoplasmic staining are classed as apoptotic and filtered from further analysis (Fig. 3). The proportion of apoptotic cells can be used as a measure of cell viability, alongside the number of filtered cells identified (Fig. 4). Mitochondria are identified from TMRM staining using a fixed threshold following image enhancement using a white tophat filter to reduce local background, used previously [19]. Touching mitochondria are classed as one object for morphology measurements, and mitochondria are also grouped as one object per cell for some measurements.

To measure mitochondrial membrane potential the mean intensity of the TMRM fluorescence can be measured within whole cells or averaged across all identified mitochondria within each cell. In this assay, morphology measurements only include functioning mitochondria due to the mode of action of TMRM described above. CCCP is not used as a control for morphology measurements as very few mitochondria are identified. Mitochondrial morphology measurements used include mean area, aspect ratio and major axis length. The total area of functioning mitochondria is also measured, normalized to the total area of the cell to give the proportional area of mitochondria. Data is collected per cell and can be averaged per image if desired, as shown here (Fig. 5). For $\Delta\psi m$ measurements, TMRM fluorescence intensity should be reduced in cells exposed to rotenone, and almost completely absent in CCCP wells (Fig. 5 a & b). The proportional area

of mitochondria should also be reduced in cells exposed to rotenone (Fig. 5 c). Reduction in the mean area of mitochondria following exposure to rotenone suggests fragmentation of mitochondria (Fig. 5 d), which should be associated with reduced major axis length and increased aspect ratio of mitochondria as they become rounder (Fig. 5 e & f).

2. Materials

1. Cell culture medium for fluorescence microscopy
    a. Neurobasal medium without phenol red.

2. Dyes for fluorescence microscopy
    a. TMRM, 25nM final concentration
    b. Calcein-AM, 5μM final concentration
    c. Hoechst-33342, 1μg/ml final concentration

3. Control Compounds
    a. CCCP, stock made in DMSO, diluted to 10μM final concentration in fluorescent microscopy medium
    b. Rotenone, stock made in DMSO, diluted to 500nM final concentration in neuronal medium and fluorescent microscopy medium
    c. DMSO

3. Methods

1. Seeding cells
    a. Seed iPSC-derived neurons into plates suitable for live fluorescent microscopy at a density of 120,000 cells / cm^2 by preferred protocol (see note 1).
    b. At least 6 wells are needed to use the two control compounds plus DMSO in duplicate.
    c. Incubate for 7 days, changing media every 2 days.

2. Addition of control compound rotenone
    a. Prepare neuronal medium in 2 tubes, to one tube add rotenone and to the other add DMSO to the equivalent amount as added to the rotenone tube.
    b. Remove media from 4 wells and add either rotenone or DMSO solutions to 2 well each. Leave two wells for CCCP.
    c. Incubate for 24 hours (see note 2).

3. Addition of dyes and CCCP
    a. Make up imaging media – to phenol red-free neurobasal medium add TMRM (25nM), Calcein-AM (5uM) and Hoechst 33342 (5ug/ml)
    b. Divide imaging media into three tubes, to tube 1 add CCCP (10uM), to tube 2 add rotenone (500nM), to tube 3 add equivalent amount of DMSO.

c. Remove neuronal growth media from wells and add the solution containing CCCP to the wells that weren't used the day before, add rotenone and DMSO-containing solutions to the same wells as the previous day.
d. Incubate at 37°C for 40 minutes.

4. Fluorescence microscopy
   a. Remove cells from incubator and transfer to fluorescent microscope for live imaging, ensure microscope is equipped with temperature control set to 37°C and 5% CO_2.
   b. Configure microscope to image TMRM (Ex552/Em578), Calcein-AM (Ex495/Em516) and Hoechst 33342 (Ex350/Em461), and acquire images from multiple sites per well at 40x magnification (see note 3).

5. Image analysis using CellProfiler Software
   a. Settings such as diameter of objects and threshold correction factor will vary depending on image acquisition parameters. Below are suggested settings only.
   b. Configure input modules:
      1. On the Images module, drag images you wish to analyse into the File List box.
      2. Metadata can be extracted from file or folder names to extract information describing the images. Regular expression code can be entered to tell the software how

the image names relate to information describing the images.

3. In the NamesAndTypes module, assign names to the different channels using either file name or metadata.
4. Groups can be used to split a list of images into subsets.

c. Configure analysis modules:
   1. IdentifyPrimaryObjects – Identifies nuclei from Hoechst image, these objects are named Nuclei. Use Otsu thresholding with three-class thresholding, minimize weighted variance, assign pixels in middle intensity class to foreground, automatic smoothing for thresholding with a threshold correction factor of 3. Distinguish clumped objects by shape and draw dividing lines between clumped objects by intensity. Lower and upper bounds on threshold can be adjusted to avoid incorrect segmentation.
   2. IdentifySecondaryObjects – Identifies cell soma from Calcein image by propagation from nuclei identified in module 1, these objects are names Cells. Use two-class thresholding, minimize weighted variance with automatic thresholding, threshold correction factor of 3.5. Lower and upper bounds on threshold can be adjusted to avoid incorrect segmentation.
   3. MeasureObjectSizeShape – Measures size of Nuclei and

Cells identified in modules 1 and 2.

4. CalculateMath – Divides area of each Nuclei by area of each related Cell to give CellNucleiRatio.
5. FilterObjects – Removes "Cells" with a CellNucleiRatio lower than 1.1 to remove any objects without cytoplasmic staining i.e. dead cells, the remaining objects are named FilteredCells.
6. EnhanceOrSuppressFeatures – Applies tophat filter to TMRM image to enhance mitochondria staining and reduce background, based on object size, the resulting image is called EnhancedMito (see note 4).
7. IdentifyPrimaryObjects – Identify mitochondria from EnhancedMito image, these objects are named Mitochondria. First analyse a selection of control images using three-class Otsu thresholding, minimize weighted variance, assign pixels in middle intensity class to background, with automatic smoothing and a threshold correction factor of 2. Then determine the median threshold used in test images and apply a manual threshold to analyse images. Lower and upper bounds on threshold can be adjusted to avoid incorrect segmentation.
8. ReassignObjectNumbers – Classifies mitochondria with 0 pixels between objects as a single object, to make objects that are touching one object, these objects are

called JoinedMito. This improves mitochondrial morphology measurements.

9. MeasureObjectSizeShape – Measures size and shape of JoinedMito objects.
10. CalculateMath – Divide MinorAxisLength (in AreaShape category) of JoinedMito by MajorAxisLength (in AreaShape category) to give the aspect ratio of JoinedMito.
11. RelateObjects – Relates JoinedMito with associated FilteredCells and calculates per cell means for measurements from module 9 and 10.
12. ReassignObjectNumbers – Classifies all mitochondria within a cell as one object per cell, called UnifyMito, this enables measurment of the total area of mitochondria, and the average intensity of all mitochondria, in each cell.
13. MeasureObjectIntensity – Measures intensity of TMRM image in Cell and UnifyMito objects.
14. MeasureObjectSizeShape – Measures size and shape of UnifyMito objects.
15. CalculateMath – Divide area (in AreaShape category) of UnifyMito, by area (in AreaShape category) of FilteredCells to give the proportion of the area of each cell occupied by mitochondria.
16. RelateObjects – Relates UnifyMito with associated FilteredCells and calculates per cell means for

measurements from module 13 and 14.

17. ExportToSpreadsheet – exports data from previous modules to spreadsheets for each object and per image. Add image metadata columns to object data file and calculate per-image mean values for object measurements. Select measurements to export in particular within the FilteredCells category: mean intensity, mean JoinedMito area measurements, area and major axis length, mean UnifyMito mean intensity and measurements from the calculate math modules. Within the Image category make sure to select appropriate metadata and final threshold of Nuclei, Cells and Mitochondria. Also select count for nuclei and filtered cells to calculate the number of cells identified per well and the proportion of excluded cells (Nuclei count minus FilteredCells count) to determine cell viability.

d. Determine correct segmentation parameters for each object identification module using test mode. OverlayOutlines and SaveImages modules can be used to check accuracy of segmentation by saving an outline of the segmentation on top of the relevant image. Check settings using a subset of control images then run analysis on all images with manual thresholding for mitochondria identification.

4. Notes

1. It is recommended to seed iPSC-derived neurons at approximately 45 days from start of neuronal induction.
2. Rotenone is incubated for 24 hours to cause an effect on mitochondrial morphology as well as on membrane potential. It may be added at the same time as the dyes to detect the effect on mitochondrial membrane potential alone.
3. Z-stacks can be acquired to ensure the full extent of each cell is captured but this is not essential for the accuracy of the assay. If stacks are acquired then perform the image analysis on projections of the stacks.
4. Image enhancement uses a white tophat filter that reduces low intensity staining around brighter objects of a defined size. TMRM image enhancement is optional, depending on the acquired images and may not be necessary for z-stack projections.

5. References

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Figures

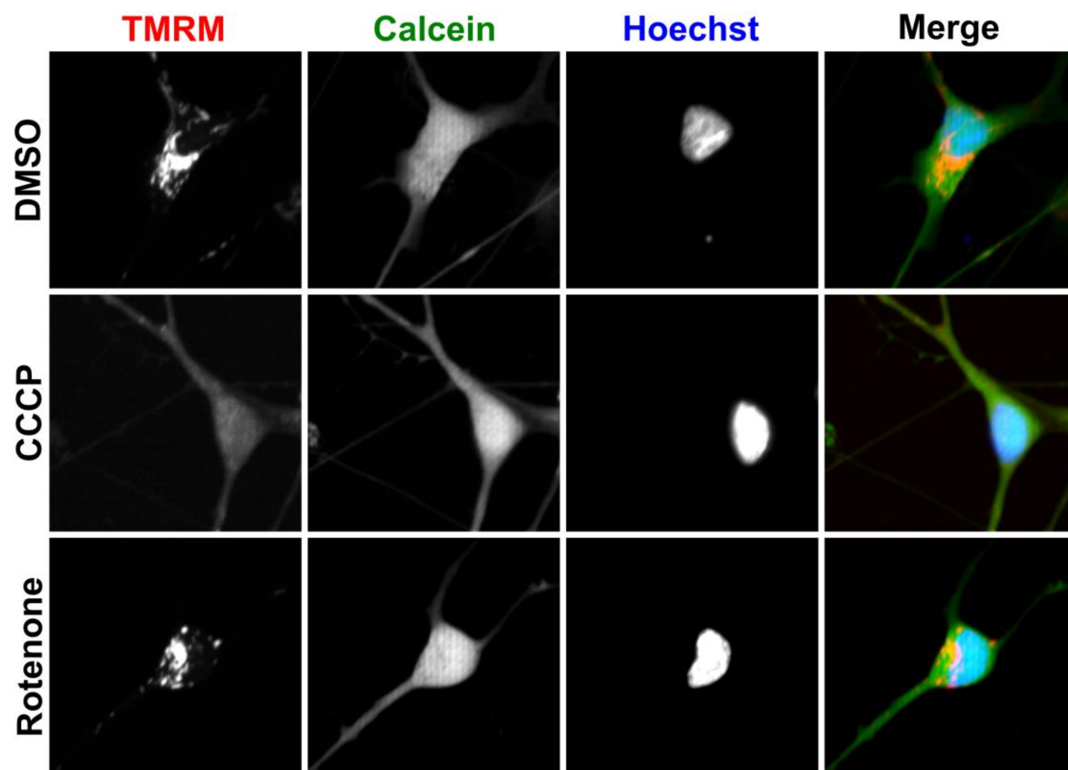

Figure 1. Example of stains and effect of control compounds

iPSC-derived dopaminergic neurons stained with TMRM (red in merge), Calcein (green in merge) and Hoechst (blue in merge) exposed to DMSO, CCCP or rotenone.

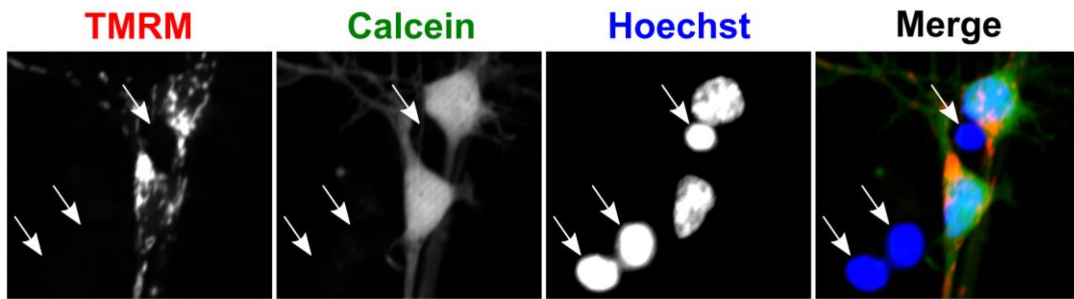

Figure 2. Apoptotic cells used in cell viability assessment

In this assay apoptotic cells are classed as cells with Hoechst staining but no Calcein staining (white arrows), these are filtered from mitochondrial function analysis. The proportion of these apoptotic cells, compared to the total number of nuclei, can be used as a measure of cell viability.

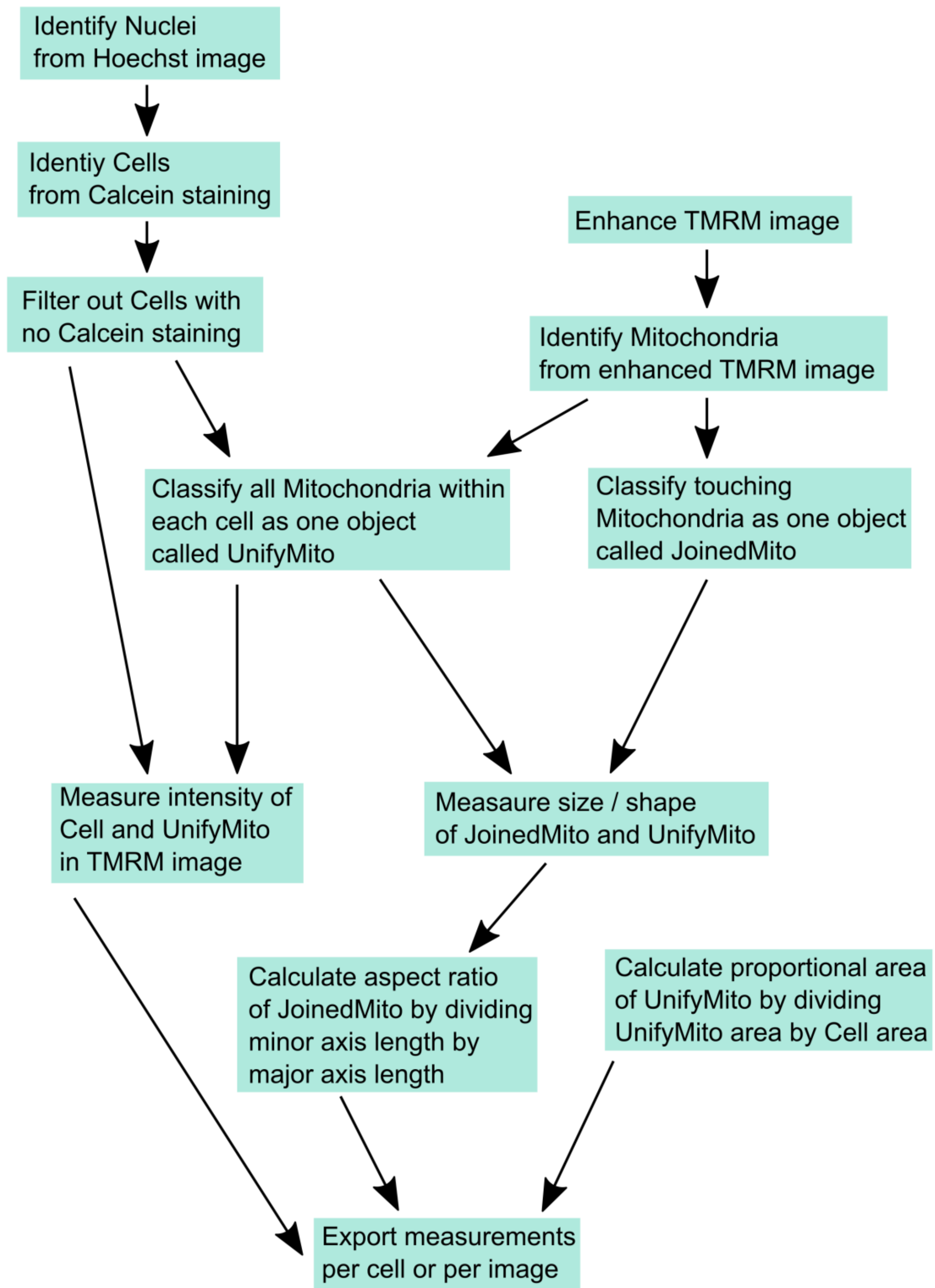

Figure 3. Flow chart showing outline of image analysis pipeline

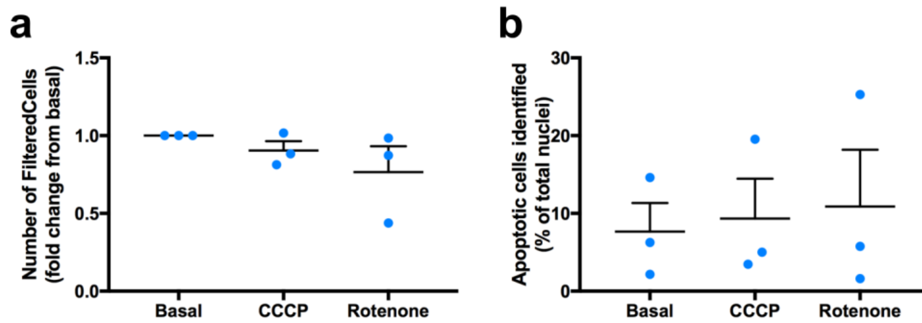

Figure 4. Cell viability assessment.

Cell viability is assessed by the number of filtered cells identified for each condition (a) and the proportion of apoptotic cells identified. Each dot represents data for one experiment, error bars represent mean + SEM.

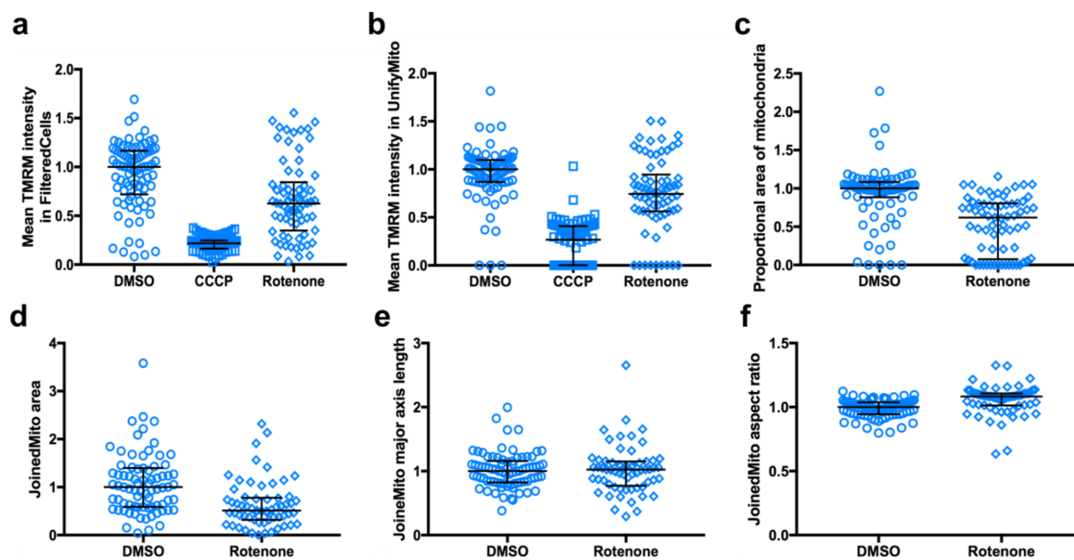

Figure 5. Expected results of control compounds by different measures. TMRM intensity measurements used are mean TMRM intensity per Cell (a) and mean intensity in UnifyMito (b). Mitochondrial morphology measurements used are proportional area of mitochondria (c), calculated by dividing area of UnifyMito by area of Cell, JoinedMito area (d), JoinedMito major axis length (e), and JoinedMito major axis length (f). Measurements averaged per cell

then per image for cells exposed to DMSO, CCCP or Rotenone, data represents fold change from DMSO. Each dot represents mean data for one image, error bars represent median ± interquartile range, n=3 independent experiments.