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Cytopathology Quantification in iPSCs with Harmony Software

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Yang Liu[1], Jessica Chedid[1], YuHong Fu[1], Glenda Halliday[1]

[1]University of Sydney

courtney.wright Wright
University of Sydney

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We use this protocol and it's working

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Abstract

The following protocol details microscopy analysis of cortical and midbrain dopaminergic differentiated iPSC's. Quantification of tau, phosphorylated tau, p62, alpha synuclein and phosphorylated alpha synuclein pathology is detailed using Harmony software (Perkin Elmer Health Sciences, now Revvity)

Materials

Harmony™ Software (PerkinElmer Health Sciences Canada Inc, Woodbridge, ON, now Revvity)

Troubleshooting

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Before start

Material: This analysis was performed on Digital scanned immunofluorescence images of iPSCs differentiated into cortical and dopaminergic neurons. The images analysed in this protocol were acquired using the Opera Phenix High-Content Screening System (PerkinElmer, now Revvity) with a 40X objective and a 10-plane Z stack. Quantification was performed across all planes. Acquired images can be exported as raw TIFF files.

1. Detection and Quantification of Neurons

1 Cell nuclei were detected and counted using the “Find Nuclei” block in the DAPI channel.

2 Calculate Morphology Properties” block was used to assess the nucleus area, ratio, and roundness for nuclei screening.

3 The “Selected Population” block was applied with the following conditions: “Nucleus Area” > 50 µm², “Nucleus Area” < 400 µm² “Nucleus Ratio Width to Length” > 0.3, and “Nucleus Roundness” > 0.7.

4 Differentiated cortical neurons were identified by MAP2 staining, and differentiated dopamine
neurons were identified by TH staining.

5 For cortical neurons, the “Calculate Morphology Properties” block was used to assess the MAP2 area. The “Select Population” block was then applied with “Image Region” > 200 to label cells as “MAP2+.”

6 For dopamine neurons, the “Select Cell Region” block was used for nuclei selected in previous steps. The region was resized by selecting “Nucleus Region,” with “Outer Border” set to -5 px and “Inner Border” set to +5 px. The output region was labelled as “Close Perinuclei.” The “Calculate Intensity Properties” block
was then used to assess “Close Perinuclei intensity”, with the “Select Population” block applied for “Close Perinuclei intensity” > 140, labelling cells as “TH+.”

7 The previously selected nuclei and masked populations of the “Image Region” were used to decide which cortical and dopamine neurons would be analyzed.

8 Neuron numbers and sizes were analyzed using the “Calculate Morphology Properties” block.

2. P62 Pathology Quantification

9 ***a. Proportion of P62 Expression***

- The “Calculate Intensity Properties” block was used to analyze the fluorescent intensities of P62 in the applied fluorescence channel (Alexa 488).
- The “Calculate Morphology Properties” block was used to assess total, mean, and relative P62 intensities.

- Histograms of P62 fluorescent intensity distribution were checked, and the top 10% of the control line's relative intensity was used as a threshold for the upregulated level of expression. New blocks were built for “Selection Population” using “P62 Intensity Total” > 4,000,000, “P62 Intensity Mean” > 600, and “P62 Intensity Relative” > 0.4, labelling cells as “P62 positive”.

10 ***b. Proportion of P62 Aggregation-Containing Cells***

- The “Find Spots” block was used with a detection sensitivity set to 0.10 and a splitting sensitivity set to 0.50 to detect P62 aggregation spots in the applied fluorescence channel (Alexa 488).
- The “Calculate Morphology Properties” block was used to assess total P62 spot intensity, mean P62 spot intensity, and relative P62 intensity in each region.
- New blocks were built for “Selection Population” using “P62 Spots number” > 0, “Relative Spot Intensity” > 0.02, and “Spot Size” > 2 μm^2, labelling cells as “P62 spot positive”.

11 ***c. Results Calculation***

- In the “Define Results” block, select the population of “MAP2+” or “TH+” cells and the number of objects. Then, select the populations of “P62 positive” and “P62 spot positive” cells, also by number of objects. Two “Formula Output” calculations were added: one for the percentage of P62 positive cells (100 * a/b, where a = number of P62 positive cells and b = number of MAP2+ or TH+ cells), and the other for the percentage of P62 spot positive cells (using the formula).

3. α-Synuclein and Phosphorylated α-Synuclein Pathology Quantification

12 ***a. Proportion of α-Synuclein and Phosphorylated α-Synuclein Expression***

- The “Calculate Intensity Properties” block was used to analyze the fluorescent intensities of α-synuclein and phosphorylated α-synuclein in each assigned channels (Alexa555 and Alexa 647 in this case).
- Separate “Calculate Morphology Properties” blocks were used for total, mean, and relative intensities of α-synuclein and phosphorylated α-synuclein.
- Histograms of fluorescent intensity distribution were checked, with the top 10% of the control line's relative intensity used as a threshold for the upregulated level of expression. New blocks were built for “Selection Population” using “α-synuclein

Intensity Total" > 1,000,000, "α-synuclein Intensity Mean" > 135, and labelling cells as "αsyn positive". Similarly, for phosphorylatedα-synuclein, "Intensity Total" > 1,500,000 and "Intensity Mean" > 135 were used to label cells as "P- αsyn positive".

13 ***b. Proportion of α-Synuclein and Phosphorylated α-Synuclein Aggregation-Containing Cells***

- The "Find Spots" block was used with detection sensitivity set to 0.10 and splitting sensitivity set to 0.50 to analyze α-synuclein and phosphorylated α-synuclein aggregation spots in each assigned channels (Alexa555 and Alexa 647 in this case).
- The "Calculate Morphology Properties" block was used to assess total and mean intensity of spots, and relative intensity in each region.
- New blocks were built for "Selection Population" using "Spots number" > 0, "Relative Spot Intensity" > 0.02, and "Spot Size" > 2 μm^2, labelling cells as "αsyn spot positive" and "P- αsyn spot positive".

14 ***c. Results Calculation***

- In the "Define Results" block, select the population of "MAP2+" or "TH+" cells and the number of objects. Then, select the populations of "αsyn positive," "P- αsyn positive," "αsyn spot positive," and "P- αsyn spot positive" cells by number of objects. Four "Formula Output" calculations were added: one for the percentage of α-synuclein positive cells (100 * a/b, where a = number of αSyn positive cells and b = number of MAP2+ or TH+ cells) and another for the percentage of α-synuclein spot-positive cells, and similarly, two additional formulas for phosphorylated α-synuclein positive and phosphorylated α-synuclein spot-positive cells.

4. Tau Pathology Quantification

15 ***a. Proportion of Tau Pathology Expression***

- The "Calculate Intensity Properties" block was used to analyze the fluorescent intensity of tau in the Alexa 555 channel.
- Separate "Calculate Morphology Properties" blocks were used for total, mean, and relative tau intensity.
- Histograms of tau fluorescent intensity distribution were checked, with the top 10% of the control line's relative intensity used as an upregulated threshold for abnormal expression. New blocks were built for "Selection Population" using "Tau Intensity Total" > 3,000,000, "Tau Intensity Mean" > 400, and labelling cells as "Tau positive".

16 ***b. Proportion of Tau Aggregation-Containing Cells***

- The “Find Spots” block was used with a detection sensitivity set to 0.10 and splitting sensitivity set to 0.50 to analyze tau aggregation spots in the Alexa 555 channel.
- The “Calculate Morphology Properties” block was used to assess the total and mean intensity of tau spots, as well as the relative intensity in each region.
- New blocks were built for “Selection Population” using “Spots number” > 0, “Relative Spot Intensity” > 0.02, and “Spot Size” > 2 μm^2, labelling cells as “Tau spot positive”.

17 ***c. Results Calculation***

- In the “Define Results” block, select the population of “MAP2+” or “TH+” cells and the number of objects. Then, select the populations of “Tau positive” and “Tau spot positive” cells by number of objects. Two “Formula Output” calculations were added: one for the percentage of Tau positive cells (100 * a/b) and another for Tau spot-positive cells.

5. Phosphorylated Tau Pathology Quantification

18 ***a. Proportion of Phosphorylated Tau Expression***

- The “Calculate Intensity Properties” block was used to analyze phosphorylated tau intensity in the Alexa 555 channel.
- Separate “Calculate Morphology Properties” blocks were used for total, mean, and relative phosphorylated tau intensity.
- Histograms of phosphorylated tau fluorescent intensity distribution were checked, with the top 10% of the control line's relative intensity used as a threshold for abnormal expression. New blocks were built for “Selection Population” using “Phosphorylated Tau Intensity Total” > 5,000,000, “Phosphorylated Tau Intensity Mean” > 220, and labelling cells as “P-tau positive”.

19 ***b. Proportion of Phosphorylated Tau Aggregation-Containing Cells***

- The “Find Spots” block was used with detection sensitivity set to 0.10 and splitting sensitivity set to 0.50 to analyze phosphorylated tau aggregation spots in the Alexa 555 channel.
- The “Calculate Morphology Properties” block was used to assess total and mean phosphorylated tau spots intensity, as well as the relative intensity in each region.

- New blocks were built for “Selection Population” using “Spots number” > 0, “Relative Spot Intensity” > 0.02, and “Spot Size” > 2 µm², labelling cells as “P-tau spot positive”.

20 ***c. Results Calculation***

- In the “Define Results” block, select the population of “MAP2+” or “TH+” cells and the number of objects. Then, select the populations of “P-tau positive” and “P-tau spot positive” cells by number of objects. Two “Formula Output” calculations were added: one for the percentage of P-tau positive cells (100 * a/b) and another for P-tau spot-positive cells.