High content autophagy analysis in iPSC-derived neurons using immuno-fluorescence

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Abstract

Autophagy is the process by which cellular proteins and organelles are degraded and recycled and is essential to the survival of cells. Defective autophagic degradation has been linked to many neurodegenerative diseases and in particular lysosomal storage diseases. Here we describe a high content assay to detect defects in the autophagy pathway in induced pluripotent stem cell-derived neurons. This assay utilizes immunofluorescence to stain autophagosomes and uses automated image analysis to measure changes in autophagosome levels in response to modulators of autophagy.

Keywords

1. Introduction

Autophagy is a critical cellular process that results in the degradation and recycling of proteins [1]. In macroautophagy, referred to hereafter as simply autophagy, the process starts with the packaging of ubiquitinated proteins within a double-membraned structure called an autophagosome. The autophagosome is transported to and fuses with a lysosome, resulting in the degradation of the protein cargo by lysosomal enzymes. Many

neurodegenerative diseases are associated with defective autophagy, including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and multiple lysosomal storage diseases [2-9]. Here we use iPSC-derived neuronal cells, which provide an excellent model of neurodegenerative disorders, as they are human cells that can be generated from patients with a specific disease [10]. iPSC-derived neurons are thus an attractive model for identifying or testing potential therapeutics for neurodegenerative diseases [11], and this assay could be used to test modulators of autophagy as a potential therapeutic target for neurodegenerative diseases [12].

This assay utilizes high content image analysis principles to identify changes in LC3B-labelled autophagosomes within iPSC-derived neurons. Firstly, cells are exposed to control compounds: Torin to induce autophagy [13], Bafilomycin A1 to inhibit lysosomal function [14], and a combination of the two. Cells are then fixed and stained for Tubulin β3 to identify neuronal cells (and exclude non-neuronal cells from analysis), and LC3B, to identify autophagosomes (Fig. 1). The stained cells are then imaged and the images are analysed (Fig. 2).

LC3B is lipidated from cytoplasmic LC3B-I to LC3B-II when recruited from the cytoplasm to the membrane of the forming autophagosome [15]. LC3B-II then decorates the autophagosome membrane as it is transported to and fuses with a lysosome. By segmenting the bright LC3B-II puncta from the fainter cytoplasmic LC3B-I staining, autophagosomes can be identified and

measured. Torin is used to induce the formation of new autophagosomes, resulting in an increase in LC3B puncta. Bafilomycin A1 is used to inhibit lysosomal function by increasing lysosomal pH, causing a reduction in autophagosome turnover and thus an increase in LC3B puncta. By combining the two control compounds a greater increase in the amount of autophagosomes is detected (Fig. 3). In addition to demonstrating the efficacy of the image analysis, these compounds can also help identify where in this pathway a defect may be occurring in a disease model. For example, if Torin doesn't increase the number of punctae then formation of autophagosomes is disrupted. If Bafilomycin A1 doesn't increase the number of punctae there is either a problem with formation or autophagosomes, or lysosome function is already inhibited.

An image analysis pipeline using CellProfiler software [16] to segment neuronal cells and measure autophagosomes is outlined in this protocol (Fig. 3). Essentially, nuclei are identified from Hoechst staining, then the associated cytoplasm is identified from neuronal specific Tubulin β3 staining, any nuclei without Tubulin β3 staining are filtered from further analysis (Fig. 1). The proportion of neuronal cells can be used as a measure of cell viability, alongside the total number of cells identified. Autophagosomes are identified from LC3B staining using a fixed threshold and a variety of measures are taken for each cell. To measure changes in autophagy the total number of LC3 puncta per cell is measured (Fig. 2, a), as well as the total area of LC3 puncta normalized to cell area (Fig. 2, b) and the mean area of each LC3 puncta (Fig. 2, c). Accurate LC3 puncta identification is dependent on setting

the correct threshold for segmentation. Two measurements of intensity, upper quartile intensity (Fig. 2, d) and standard deviation in intensity (Fig. 2, e) of LC3 staining within FilteredNeurons can be measured to avoid the necessity of segmenting LC3 puncta while still revealing changes upon exposure to control compounds.

2. Materials

1. Neuronal cell culture medium
   a. Dependent on neuronal differentiation protocol used to generate iPSC-derived neurons

2. Control compounds
   a. Torin 1 in DMSO, used at 100nM final concentration
   b. Bafilomycin A1 in DMSO, used at 20nM final concentration
   c. DMSO

3. Fixation and immunofluorescence
   a. Methanol 100%, chilled
   b. 0.1% triton in PBS
   c. Goat serum
   d. Anti-LC3B antibody raised in rabbit (Sigma, L7543)
   e. Anti-Tubulin β3 antibody raised in mouse (Biolegend, 801201)
   f. Goat anti rabbit Alexa Fluor 488 antibody (ThermoFisher A-11034)

g. Goat anti mouse Alexa Fluor 546 antibody (ThermoFisher A-11031)

h. Hoechst-33342, 1ug/ml final concentration

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 8 wells are needed to use the control compounds plus DMSO in duplicate.

    c. Incubate for 7 days, changing media every 2 days.

2. Addition of control compounds

    a. Prepare enough neuronal cell culture medium for all wells and divide into 4 tubes. To one tube add Torin (100nM final concentration), to the second tube add Bafilomycin A1 (20nM final concentration), to the third tube add both Torin and Bafilomycin A1 (100nM and 20nM final concentration respectively) and to the final tube add an equivalent amount of DMSO.

    b. Aspirate media from cells and add control solutions to two wells each.

    c. Incubate for 24 hours (see note 2).

3. Fixation

   a. Aspirate media from all wells and add PBS to each well.

   b. Aspirate PBS immediately and add ice cold 100% Methanol to each well.

   c. Incubate at -20°C for 8 minutes.

   d. Aspirate methanol and add PBS.

4. Immunofluorescence

   a. Remove PBS and add blocking solution of 0.1% Triton in PBS with 5% goat serum.

   b. Incubate for 1 hour at room temperature.

   c. Prepare primary antibody solution; to PBS with 5% goat serum add anti-LC3 antibody and anti-Tubulin β3 antibody at a dilution of 1:1000 for both.

   d. Remove blocking solution and add primary antibody solution.

   e. Incubate for 2 hours at room temperature or overnight at 4°C.

   f. Aspirate primary antibody solution and add PBS, incubate for 10 minutes then remove and repeat a further two times.

   i. Prepare secondary antibody solution of goat anti rabbit Alexa Fluor 488 antibody and goat anti mouse Alexa Fluor 546, both diluted 1:500 in PBS.

   j. Remove PBS and add secondary antibody solution, incubate at room temperature for 1 hour.

g. Aspirate secondary antibody solution and add PBS with Hoechst-33342 (1ug/ml final concentration) and incubate for 10 minutes.

k. Remove Hoechst solution and add PBS, incubate for 10 minutes then remove and repeat.

4. Fluorescence microscopy

a. Configure microscope to image LC3 (Alexa Fluor 488, Ex/Em), Tubulin β3 (Ex/Em) and Hoechst 33342 (Ex350/Em461).

b. 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.

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. ⌞SEP⌟

3. In the NamesAndTypes module assign names to the different channels using either file name or metadata. ⌞SEP⌟

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 neuronal cell soma from Tubulin β3 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 of non-neuronal cells.

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 Cells without Tubulin β3

staining, i.e. non-neuronal cells, the remaining objects are named FilteredNeurons.

6. IdentifyPrimaryObjects – Identify LC3 puncta from LC3 image, these objects are named LC3Objects. 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 5. Then determine the median threshold used in test images (excluding outliers) and apply a manual threshold to analyse images.

7. ReassignObjectNumbers – Classifies all LC3Objects within a FileredNeuron as one object, called UnifyLC3Objects, this enables the measurement of the total area of LC3Objects in each cell.

8. MeasureObjectIntensity – Measures intensity of LC3 image in FilteredNeurons, and UnifyLC3Objects.

9. MeasureObjectSizeShape – Measures size and shape of FilteredNeuron, LC3Objects and UnifyLC3Objects.

10. CalculateMath – Divide area (in AreaShape category) of UnifyLC3, by area (in AreaShape category) of FilteredNeurons to give the proportion of the area of each cell occupied by mitochondria.

11. RelateObjects – Relates Nuclei with associated FilteredCells and calculates per cell means for Nuclei

size/shape measurements from module 3.

12. RelateObjects – Relates LC3Objects with associated FilteredCells and calculates per cell means for LC3Object intensity and size/shape measurements from module 8 and 9.

13. RelateObjects – Relates UnifyLC3Objects with associated FilteredCells and calculates per cell means for UnifyLC3Object intensity and size/shape measurements from module 8 and 9.

14. 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 FilteredNeurons category; LC3Objects count (in the Children subcategory), the proportional area of LC3Objects (in the Math subcategory), LC3Objects area (in the LC3Objects subcategory) and StdIntensity and UpperQuartileIntensity for LC3 (in the Intensity subcategory). Nuclei characteristics such as area, compactness and eccentricity can be measured to assess cytotoxicity. Within the Image category make sure to select appropriate metadata and final threshold of Nuclei, Cells and LC3Objects. Also select count for Nuclei and

FilteredNeurons to calculate the number of cells identified per well and the proportion of neuronal cells in the culture.

    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. Control compounds can be incubated for a shorter period of time such as 8 or 16 hours.
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 perform the image analysis on projections of the stacks.

5. References

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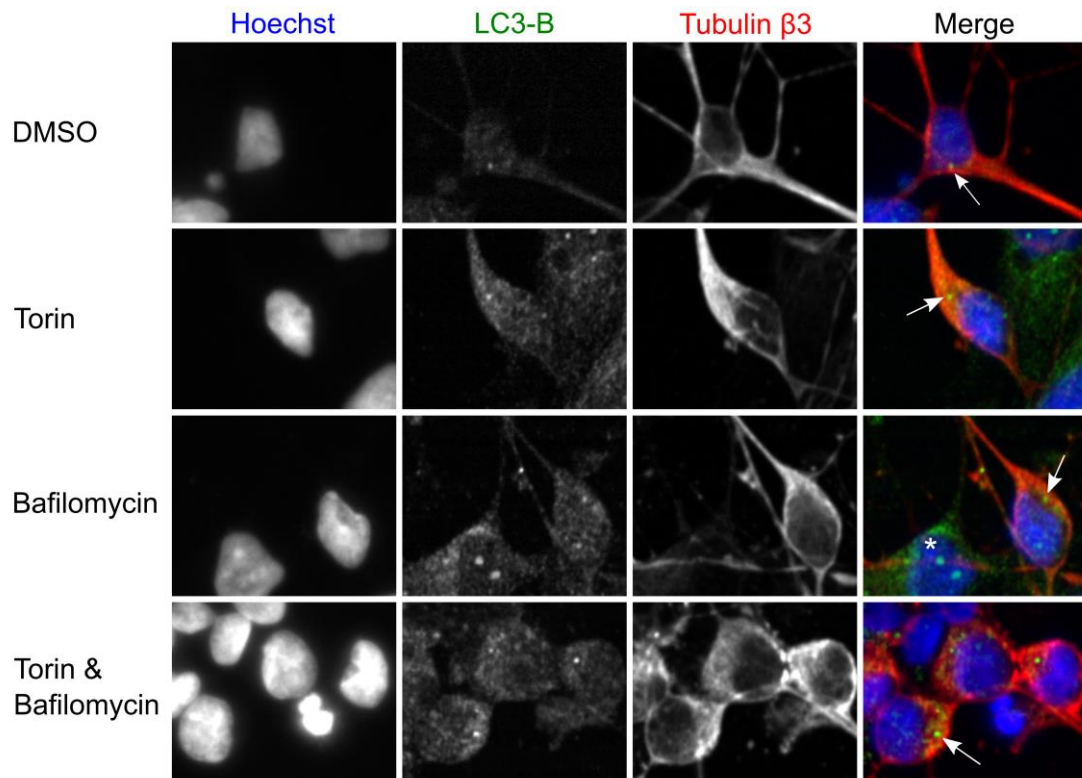
Figures

Figure 1. Immunofluorescence for markers of autophagosomes and neurons. iPSC-derived neurons stained for autophagosome marker LC3-B (green), neuronal specific Tubulin B3 (red) and DNA stain Hoechst-33342 (blue), following exposure to either DMSO, Torin, Bafilomycin or Torin and Bafilomycin. White arrows highlight LC3 puncta, the white asterisk highlights a non-neuronal cell which is excluded from analysis.
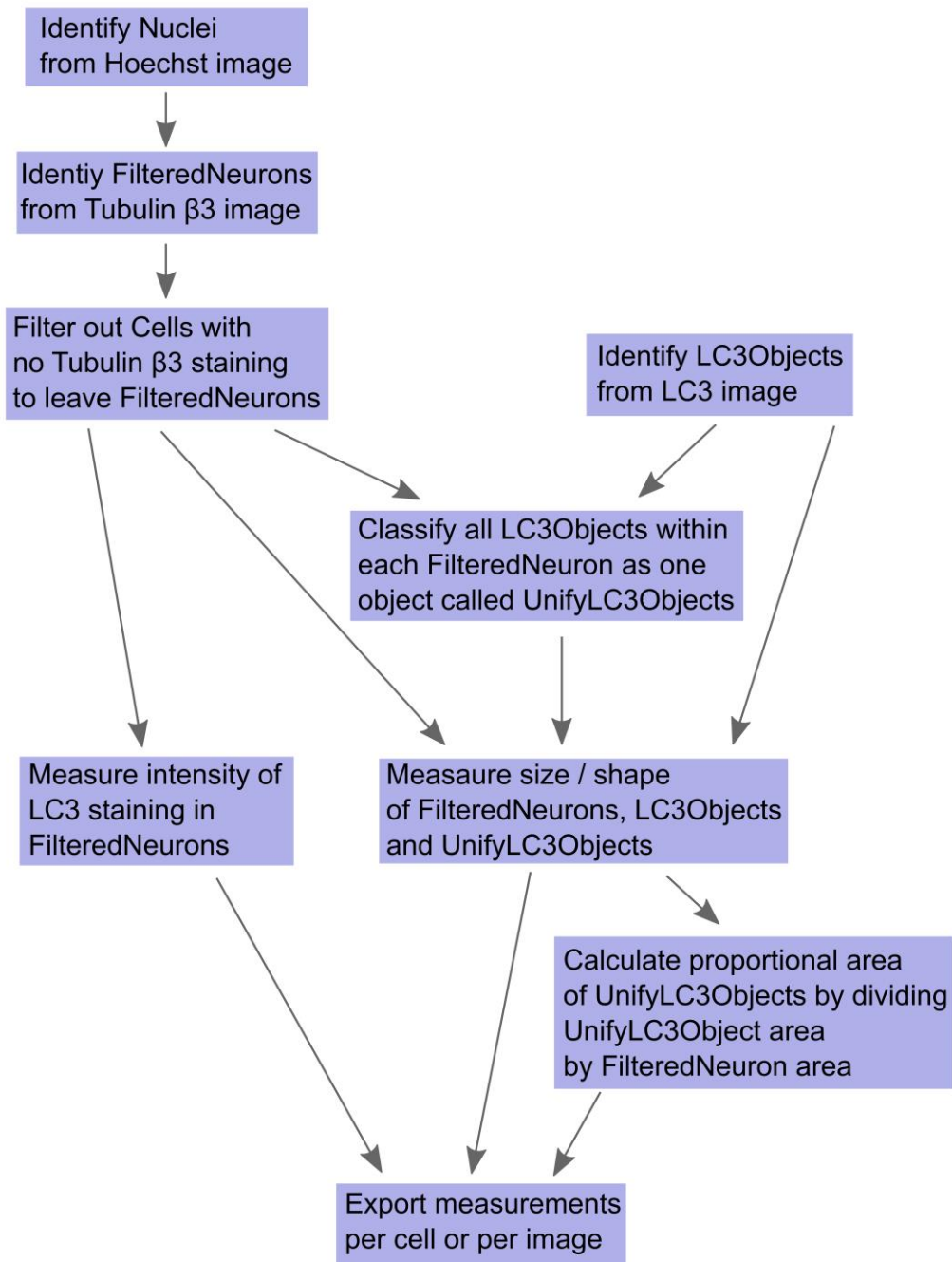
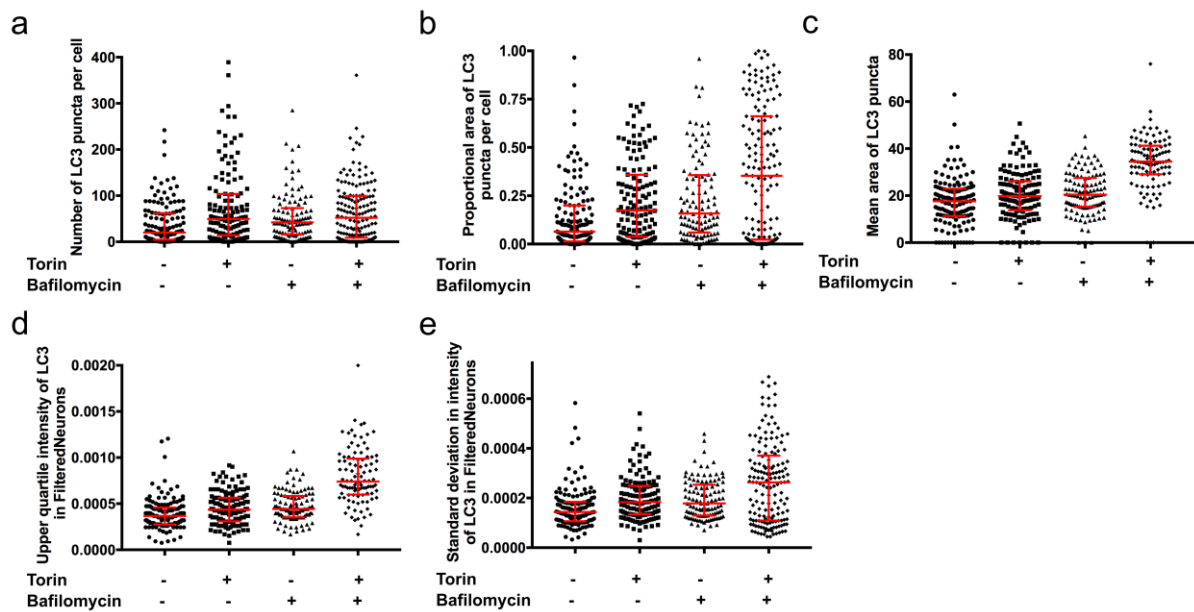Figure 2. Flow chart showing outline of image analysis pipeline.

Figure 3. Effect of control compounds on autophagy by different measures.
Three measures based upon segmentation of LC3 puncta were taken; the
number of puncta per cell (a), the proportional area of LC3 puncta (b) and the
mean area of LC3 puncta (c). Two measures independent of LC3
segmentation were also taken; the upper quartile intensity of LC3 staining (d)
and the standard deviation in intensity of LC3 staining (e) within each
FilteredNeuron. All measures are able to detect changes in autophagy
following exposure to control compounds. Each dot represents one cell, bars
represent median ± interquartile range.