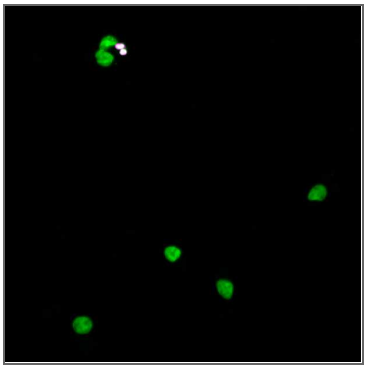

NOV 06, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.j8nlko6e1v5r/v1

Protocol Citation: Melissa Hoyer, Harper JW 2023. Analysis of nuclei integrity in cultured induced neurons by fluorescence microscopy. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlko6e1v5r/v1

Protocol status: Working
We use this protocol and it's working

Created: Nov 06, 2023

Analysis of nuclei integrity in cultured induced neurons by fluorescence microscopy

Melissa Hoyer[1,2], Harper JW[1,2]

[1]Harvard Medical School; [2]Aligning Science Across Parkinson's

ASAP Collaborative Research Network | Harper

Melissa Hoyer

ABSTRACT

The endoplasmic reticulum (ER) has a vast proteomic landscape to preform many diverse functions including protein and
lipid synthesis, calcium ion flux, and inter-organelle communication. The ER proteome is remodeled in part through membrane-embedded receptors linking ER to degradative autophagy machinery (selective ER-phagy). A refined tubular ER network is formed in neurons within highly polarized dendrites and axons. Autophagy-deficient neurons *in vivo*display axonal ER accumulation within synaptic ER boutons, and the ER-phagy receptor FAM134B has been genetically linked with human sensory and autonomic neuropathy. However, mechanisms and receptor selectivity underlying ER remodeling by autophagy in neurons is limited. Here, we combine a genetically tractable induced neuron (iNeuron) system for monitoring extensive ER remodeling during differentiation. To verify iNeuron health in various knockout out cell lines, we fixed iNeuron cultures, performed a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay and stained with DAPI. We then imaged these via confocal fluorescence microscopy, and quantified intact, nonapoptotic nuclei.

MATERIALS

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell line		

Last Modified: Nov 06, 2023

PROTOCOL integer ID: 90497

Keywords: ASAPCRN

Funders Acknowledgement:
ASAP
Grant ID: ASAP-000282

NGN2 inducible embryonic stem cells	See protocol dx.doi.org/10.17504/protocols.io.br9em93e	CVCL_9773 (modified from this source line)
Chemicals		
Dulbecco's MEM (DMEM), F12		
Phosphate Buffered Saline 1X	Corning	21-031-CV
16% Paraformaldehyde, Electron-Microscopy Grade	Electron Microscopy Science	15710
Triton-X	Sigma	T8787
Click-iT‰ Plus TUNEL Assay Kits for In Situ Apoptosis Detection , Alexa Fluor 488	Thermo Fisher Scientific	C10617
DAPI	Thermo Fisher Scientific	D1306
E8 components	See protocol dx.doi.org/10.17504/protocols.io.bsacnaaw	
ND1 and ND2 components	See protocol dx.doi.org/10.17504/protocols.io.br9em93e	
Software		
Nikon Imaging Software Elements	5.21.3 (Build 1489)	https://cellprofiler.org/ SCR_014329
Cell Profiler	CellProfiler v4.0.6	https://cellprofiler.org/ SCR_007358
Fiji	ImageJ V.2.0.0	https://imagej.net/software/fiji/ SCR_002285
Hardware		
Thermo Neon‰ Transfection System	Thermo Fisher Scientific	MPK5000
Yokogawa CSU-X1 spinning disk confocal on a Nikon Ti-E inverted microscope		

1

Genetic editing of Ngn2-inducible ES cells is done using the following protocol "Electroporation of Cas9 protein into human pluripotent stem cells" (dx.doi.org/10.17504/protocols.io.br87m9zn)

Differentiation of Stable Cell ES H9 line to induced neurons (i...

2 Differentiation to iNeurons (iN) is done by following the protocol "Neural differentiation of AAVS1-TREG3-NGN2 pluripotent stem cells (dx.doi.org/10.17504/protocols.io.br9em93e)

Induced neuron fixation, TUNEL assay, and DAPI nuclei stainin...

3 **Induced neuron fixation, TUNEL assay, and DAPI nuclei staining**

3.1 To help get spread out iN for imaging, when iN get 90 percent confluent at any point in the day 5-7 range, iN are plated into onto 35 mm-glass bottom dishes (No. 1.5, 14 mm glass diameter, MatTek) at a low confluency (approximately $1x10^5$cells per 2mL dish).

3.2 At day 20, iN are fixed. phosphate buffered saline (1xPBS) with 8% paraformaldehyde is prewarmed to 37C and then slowly added to an equal volume of media on the iN for a resulting 4% paraformaldehyde solution. iN are incubated with this solution at 37C for 15min.

3.3 Fixed iN are washed one time with room temperature 1xPBS

3.4 Fixed iN are solubilized with 0.1% triton-X in 1xPBS for 5 min at Room Temperature.

3.5 Fixed iN are then blocked for 30 min at RT with
sterile filtered blocking buffer (1% bovine serum albumin, 0.1% triton-X in 1xPBS)

3.6 Fixed iN are then treated as directed in the Click-iT Plus TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay (Invitrogen C10617, Alexa Fluor 488), which detects DNA breaks formed when DNA fragmentation occurs at the end of apoptosis.

a. Perform a TdT reaction. In this reaction, the TdT enzyme takes EdUTP (a dUTP modified with a small, bio-orthogonal alkyne moiety) and incorporates it at the 3'-OH ends of fragmented DNA.

b. Perform the click reaction (Breinbauer and Köhn, 2003; Wang et al., 2003; Rostovtsev et al.,
2002; Kolb et al., 2001), where a copper catalyzed covalent reaction occurs between the Alexa Fluor™ picolyl azide dye and an alkyne. DNA break detection is based on Alexa Fluor signal at that site.

3.7 iN are incubated with 1xPBS containing DAPI (1:10,000) for 5 min.

3.8 iN are washed 2 times with PBS (5min for each wash).

Imaging of DAPI nuclei and TUNEL stain via confocal microsco...

4 iN are imaged at RT using a Yokogawa CSU-X1 spinning disk confocal on a Nikon Ti-E inverted microscope at the Nikon Imaging Center in Harvard Medical School. Nikon Perfect Focus System was used to maintain cell focus over time. The microscope is equipped with a Nikon Plan Apo 40x/1.30 N.A or 100x/1.40 N.A objective lens. 405nm (100mW) and 488nm (100mW) laser lines are controlled by AOTF. All images are collected with a Hamamatsu ORCA-ER cooled CCD camera (6.45 μm^2 photodiode) with Nikon Imaging Elements. Z-series image sets were acquired.

Analysis and visual representation of nuclei and TUNEL stain ...

5 **Analysis and visual representation of nuclei and TUNEL stain via confocal microscopy**

5.1 Z series are displayed as maximum z-projections and saved using Fiji software.

5.2 Nuclei structures are detected using Cell Profiler with the same pipeline applied for each condition (see attached CellProfiler pipelines for specific thresholding details).

a. Analysis of cell nuclei using size. Cell profiler pipeline file to be used: 'WTvATG12vpentarintactnucleianalysis.cpproj'. The DAPI channel images for all genetic backgrounds was thresholded in the same way with the following pipeline: two different 'identify primary objects' modules were used to find and count nuclei structures. In one, only larger "intact" nuclei were selected and counted (as was done previously for the analysis of ER structures in axons to get ER structures per nuclei). In the second, smaller fragmented nuclei were included in the thresholding method. The ratio of intact to total DAPI-positive nuclei structures was calculated and reported for each condition.

b. Analysis of cell nuclei using TUNEL. Cell profiler pipeline file to be used: 'dapi_withtunel.cpproj'. The TUNEL channel images for all genetic backgrounds was thresholded in the same way using an 'identify primary objects' modules to find and count all damaged DNA structures. For the corresponding DAPI channel images, two different 'identify primary objects' modules were used to find and count DAPI structures. In one, only larger "intact" DAPI-positive nuclei were selected. In the second, smaller fragmented DAPI-positive nuclei were included in the thresholding method. To calculate a total nuclei number, the number of damaged TUNEL-positive DNA structures was added to the number of intact DAPI nuclei. In the final analysis, the ratio of intact DAPI-positive nuclei structures to total nuclei (damaged TUNEL positive nuclei plus intact) was calculated and reported for each condition.

5.3 Each channel z series are brightness and contrast adjusted equally and then converted to RGB for publication using FIJI software.