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Analysis of ER structures in Cultured Induced Neuron axons

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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)[1,2]. A refined tubular ER network[3,4] is formed in neurons within highly polarized dendrites and axons[5,6]. Autophagy-deficient neurons *in vivo* display axonal ER accumulation within synaptic ER boutons,[7] and the ER-phagy receptor FAM134B has been genetically linked with human sensory and autonomic neuropathy[8,9]. 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. With this system, we imaged fixed iNeuron cultures, imaged these via confocal fluorescence microscopy, and quantified ER structures in axons.

	A	B	C
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
	Cell line		
	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
	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		

Genetically modify Ngn2-inducible embryonic stem (ES) cel...

1 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...

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

Analysis of endoplasmic reticulum (ER) in iN via confocal m...

3 Fixation and immunofluorescence is next done to label the ER, axons and dendrites of the iNs

3.1 1)To help get spread out iNeurons for imaging, when cells get 90 percent confluent at any point in the day 5-7 range, cells 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 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 cells for a resulting 4% paraformaldehyde solution. iN are incubated with this solution at 37C for 15min.

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

3.4 4)Fixed iN are solubilized with 0.1% triton-X in PBS for 5 min at RT.

3.5 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 PBS)

3.6 6)Primary antibodies for Endoplasmic reticulum (ER), axons and dendrites are added to blocking buffer at 1:300 and then spun down for 1min at 10,000 x g. The blocking buffer is completely removed and then the antibody in blocking buffer is applied to the cells (100mL applied to the center of the well where the glass coverslip is attached) for 1h at RT.

3.7 7)Cells are washed 4 times with PBS (5min for each wash).

3.8 8)Fluorescently conjugated secondary antibodies are added to blocking buffer at 1:300 and then spun down for 1min at 10,000 x g. The blocking buffer is completely removed and then the antibody in blocking buffer is applied to the cells (100mL applied to the center of the well where the glass coverslip is attached) for 1h at RT.

3.9 9)Cells are washed 4 times with PBS (5min for each wash) and left in 1XPBS. Dapi at 1:10,000 is added in the second 1XPBS wash.

4 iN are imaged via confocal fluorescence microscopy and ER, axons and dendrites of the iNs are measured.

4.1 1)Cells 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. 488nm (100mW) and 561nm (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.

4.2 2)Z series are displayed as maximum z-projections and saved using Fiji software.

4.3 3)The ER positive structures are detected using Cell Profiler with the same pipeline applied for each condition (see attached CellProfiler pipeline for specific thresholding details). Cell nuclei are detected using a “identify primary objects” module with 50-to-125-pixel units min and max and a global minimum cross-entropy thresholding method. Both axon and dendrite

area are identified using a “identify primary objects” module with 10-to-40-pixel units min and max using default thresholding method. Each ER structure is identified using a “identify primary objects” module with 5-to-50-pixel units min and max and a global robust background thresholding method. The number of ER structures masked specifically within the axonal regions is related to the number of nuclei in each field of view to yield the number of ER structures in axons per nuclei readout. Object size and shape is measured, and these values are also exported.

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