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# Analysis of ER Flux in Cultured Induced Neurons using Keima ER reporters

Forked from Analysis of Lysophagic Flux in Cultured Induced Neurons using RFP-GFP-galectin3

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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). 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 with proteomic and computational tools to create a quantitative landscape of ER proteome remodeling via selective autophagy. Through analysis of single and combinatorial ER-phagy receptor mutants, we delineate the extent to which each receptor contributes to both magnitude and selectivity of ER clearance via autophagy for individual ER protein cargos. We define specific subsets of ER curvature-shaping proteins or lumenal proteins as preferred clients for distinct receptors. Using spatial sensors and flux reporters, we demonstrate receptor-specific autophagic capture of ER in axons, which correlates with aberrant ER accumulation in axons of ER-phagy receptor or autophagy-deficient neurons. This molecular inventory of ER proteome remodeling and versatile genetic toolkit provides a quantitative framework for understanding contributions of individual ER-phagy receptors for reshaping ER during cell state transitions.

MATERIALS

| A | B | C |
|---|---|---|
| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
| **Chemicals** | | |
| Dulbecco's MEM (DMEM), F12 | Thermo | 11320033 |
| Phosphate Buffered Saline 1X | Corning | 21-031-CV |
| 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 | |

| | A | B | C |
|---|---|---|---|
| | Hygromycin B | Life Technologies | 10687-010 |
| | Bafilomycin A1 | Cayman Chemical | 88899-55-2 |
| | Sar405 Selective ATP-competitive inhibitor of Vps34 | Apexbio | A8883 |
| | pAC150 Keima-RAMP4 | | Addgene 201929 |
| | pAC150 Keima-REEP5 | | Addgene 201929 |
| | pCMV-hyPBase hyperactive piggyBac vector | Yusa et al 2011 | Available upon request at the Sanger Institute Achives |
| | **Software** | | |
| | FlowJo Software | " Flowjo, v10.7 " | https://www.flowjo.com |
| | | | |
| | **Hardware** | | |
| | ThermoFisher Neon Electroporator & Kit | | |
| | AttuneNxT | Thermo Fisher Scientific, | Cat#A28993 |

## Electroporation of PB vectors. Use ThermoFisher kit and Th...

1

Add 10ml buffer R to a sterile 1.5ml tube. Add 0.5µg of pAC150 ER Keima vector and 0.5µg of pCMV-hyPBase hyperactive piggyBac vector. Pipet up and down to mix. Let it sit at RT for 10min. This is enough for 2 transfections (== one 6 well).

2

Individualize cells with Accutase. Neutralize Accutase with 5 times volume E8 with Rock inhibitor.

3 Count cells. You will need $2x10^5$ for each transfection.

4 Spin down cells. Let it sit for a while so all the residue media can go down to the bottom of the tube. If the residue media is too much, take it out with a P200 pipet.

5 Resuspend cells to a concentration of $2x10^5$ per 5 ml (ie $4x10^7$ per ml) using buffer R. You don't have to take all the residue media off but you will need to take into account the volume of residue media so you are not too much off.

6 Prepare a 6 well matrigel coated plate. Add 2mL of E8+ rock inhibitor (1:1000) to the wells you will use. Two transfections go into one well.

7 Wipe the Neon pipet station with EtOH and place it inside the hood.

8 Add 3ml of electrolytic buffer (buffer E) to the neon tube. Place the tube inside the station. You should feel a click before the tube is securely seated in the station.

9 Use program 13 from the optimization tab for electroporation parameter (Voltage: 1100. Pulse width: 20 Pulse number: 2 ). Program 9 should also work.

10 When everything is ready, mix 10-11ml of resuspended cells with the plasmid containing R buffer. The final volume should be in the range of 21-22ml.

11 Take up a neon tip, pipet 10ml of the cell protein mix and electroporate with program 13. It is important to pipet slowly to avoid air bubble formation. It is also important to insert the pipet slowly into the station, especially during the end of the insertion when you will feel a click. Help the pipet down slowly during the clicking so there is no sudden movement of the tip, which might

create tiny air bubbles.

12 If you see air bubble in the tip, take it out, push everything out of the tip and repipet the mixture.

13 If you see sparking during the electroporation, your efficiency will reduce significantly.

14 Once electroporation is complete, push everything into one well of a 6 well plate. Do not pipet up and down with Neon tip.

15 Repeat the same procedure with the same tip and the left over cell mixture. Place the second electroporated mixture into the same well.

16 Disperse cells evenly in the well and place cells in a low O2 incubator. 17. Put electroporated cells into low oxygen incubator for 2 days

17 Select cells with 50mg/mL hygromycin B 4 days post-electroporation. Grow cells in selection medium for 7-10 days until there is no longer any cell death and every cell has integration of the piggyBac Keima-ER construct. Also can sort for the same level of Keima in each ESC.

## Differentiation of Stable Cell ES H9 line expressing Keima-E..

18 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 Keima-ER via confocal microscopy

19 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 1x105 cells per 2mL dish).

20 At day 12 or any day during differentiation, image the iNeurons

21 5mL of fresh ND2/LLoMe is added to a final concentration of 500 mM of LLoMe in 2mL. Cells are treated for 1h.

22 At day 12 or any day during differentiation, image the iNeurons. 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 µm2 photodiode) with MetaMorph image acquisition software.

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

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

## Analysis of Keima-ER via flow cytometry

25 iNeurons can be treated throughout the differentiation with a vps34i (1 microM in ND2 each day you add media) to block the ER flux. On the day of flow cytometry 4hr to collection for analysis treat one set of each condition with BAFA (100nM in ND2).

26 Detach cells from plate (1 well of 6-well dish of iN is sufficient) using accutase, add ND2 media and spin down, and resuspend carefully with 1XPBS so they do not clump. If required, filtered through a cell strainer cap tube. Place in vessel of choice for cytometry, for example tubes or 96 well plates.

27 Use dual-excitation (440nm for ph7 and 561nm for pH3) and collect in 620 nm range. Analyze at least 10.000 single, healthy cells. Calculate of acidic:neutral Keima ratio on a per-cell basis in FlowJo Software.
Use BafA treated as normalization for sample.