Jun 21, 2022

HeLa culture, transfection, and labeling of Halo-fusion proteins (Provisional unformatted)

OLIVIA HARDING[1], holzbaur [1]

[1]University of Pennsylvania

protocol .

Liv

OLIVIA HARDING

High-throughput, predictable systems that are easily modulated are ideal for the study of cell biology. Here we developed a protocol to investigate the role of the Nuclear Factor kappa-B Effector Molecule (NEMO) in Parkin-dependent mitochondrial clearance. Transient transfection of fluorescent constructs allowed us to visualize subcellular structures and dynamics while maintaining flexibility in a consistent model system. The EGFP-NEMO plasmid was repeatedly employed to study NEMO interactions during mitophagy, and we were also able to edit the construct to create both a NEMO point mutation and a Halo-tagged NEMO construct, which we readily expressed in HeLa cells. Halo-fusion constructs, including NEMO and OPTN used in our study, allowed us to visualize the exogenously expressed proteins conjugated to chemical ligands in a variety of colors. This and the accompanying protocols were critical to our characterization of NEMO's involvement in mitophagy.

OLIVIA HARDING, holzbaur 2022. HeLa culture, transfection, and labeling of Halo-fusion proteins (Provisional unformatted) . **protocols.io** https://protocols.io/view/hela-culture-transfection-and-labeling-of-halo-fus-cbrbsm2n

protocol ,

Jun 21, 2022

Jun 21, 2022

65027

1 Olivia Harding, Erika L.F. Holzbaur
Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104
Abstract
High-throughput, predictable systems that are easily modulated are ideal for the study of cell biology. Here we developed a protocol to investigate the role of the Nuclear Factor kappa-B Effector Molecule (NEMO) in Parkin-dependent mitochondrial clearance. Transient transfection of fluorescent constructs allowed us to visualize subcellular structures and dynamics while maintaining flexibility in a consistent model system. The EGFP-NEMO plasmid was repeatedly employed to study NEMO interactions during mitophagy, and we were also able to edit the construct to create both a NEMO point mutation and a Halo-tagged NEMO construct, which we readily expressed in HeLa cells. Halo-fusion constructs, including NEMO and OPTN used in our study, allowed us to visualize the exogenously expressed proteins conjugated to chemical ligands in a variety of colors. This and the accompanying protocols were critical to our characterization of NEMO’s involvement in mitophagy.
Keywords
tissue culture | transfection | fluorescent ligands
Guidelines
This protocol was adapted from a previous protocol for similar techniques see
dx.doi.org/10.17504/protocols.io.bt7wnrpe
In order to investigate Parkin dependent mitophagy, we over express Parkin,
which is not endogenously expressed in HeLa cells. We employ an untagged

Parkin construct, however there are many permutations of fluorescently
conjugated proteins that could be used to stu dy this process.
We use HeLa M cells, HeLa OPTN cells, and HeLa p62 cells in the study
corresponding to this protocol
Materials

2 Countess slides (ThermoFisher Scientific, C10228

3 10 mL conical tube Corning CLS430055)

4 1.5 mL capped tubes (Millipore Sigma, EP022364120)
35 mm glass bottomed dishes MatTek, P35G 1.5 20 C
Reagents Trypsin (ThermoFisher Scientific R001100)
Trypan blue (ThermoFisher Scientific, T10282)
DMEM (Corning, 10-017-CV)
FBS (HyClone)
GlutaMAX glucose supplement (Gibco, 35050061)
Opti-MEM (ThermoFisher Scientific, 3198507)
Plasmid DNA
o untagged Parkin (subcloned from YFP-Parkin, a gift from R. Youle, NIH,
Bethesda, MD)
o Mito-DsRed2 (kindly provided by. T. Schwartz, Harvard Medical School,
Boston)
o Mito-sBFP2 (Wong and Holzbaur, PNAS, 2014)
o EGFP-NEMO (kindly provided by E. Laplantine, Institut Pasteur, Paris),

o EGFP-NEMOD304N (generated by site-directed mutagenesis of EGFPNEMO),
o Halo-NEMO (subcloned from EGFP-NEMO);
o Halo-OPTN (subcloned from EGFP-OPTN kindly provided from I. Dikic,
Goethe University, Frankfurt, to a pHaloTag vector, Promega);
o mCherry constructs (vector, WT, ΔPB1, PB1AA, ΔUBA, and LIRAAAA, were
kindly provided by S. Martens, University of Vienna, Austria, and TIRAAA
was generated by site-directed mutagenesis of mCherry-p62WT)
o IKKβ (aka IKK2)-EGFP (Addgene #111195)
o TFAM-SNAPf (Addgene #67064, TFAM was subcloned into pSNAPf
Vector, New England Biolabs); and
o EGFP-Ubiquitin (Addgene #11935 Ubiquitin subcloned into pEGFP-C1
Vector, Clontech).
Lipofectamine 2000 (ThermoFisher Scientific, 11668027)
Halo ligand suspended in DMSO at 200X (Promega, JaneliaFluor 646, GA112A)

Equipment

Countess automated cell counter (Invitrogen, AMQAX1000)
mini centrifuge (Southern Labware, MLX-106)
Compound microscope

Before start

HeLa-M cells are best transfected before passage 30; KO cells are best utilized
before passage 15 and may grow slower than WT cells.
Prepare Culture Media by making a 10% FBS, 1% GlutaMAX solution in DMEM.

Store at 4 C and warm to 37 C before use

Step-by-step

Day 1: Plating

Follow plating protocol as described in dx.doi.org/10.17504/protocols.io.bt7wnrpe

Day 2: Transfection

Examine cells by compound microscope 18-24 hr after plating to confirm 80-90% confluence

o Note: If cells are not at 80-90% confluence, do not transfect. Wait until they reach 80-90%.

For each dish, prepare the mixture of desired plasmids in 1.5 mL tubes

o For example, to characterize percent of mitochondria that recruit NEMO after depolarization, to Tube 1 (nucleic acids) add

■ 200 uL Opti-mem

■ 0.2 ug mito-dsRed

■ 0.5 ug Parkin

■ 0.2 ug EGFP-NEMO

• Note: NEMO over-expression must be kept to low levels in order to avoid activating cell response pathways. In our preliminary work, we established that 0.2 ug or less is ideal to transfect for a 35 mm dish.

■ 0.5 ug Halo-OPTN

o Tube 2 (Lipofectamine 2000)

■ 200 uL Opti-mem

■ 1.5 uL Lipofectamine 2000

• Note: The Lipofectamine 2000 volume needed is less than

previously reported and less than recommended on the product datasheet. Using greater volumes of this reagent is toxic to cells.

Invert tubes 8 times to distribute the contents

Incubate 5-10 min at room temperature

spin 2 sec in a mini centrifuge

Add Tube 2 to Tube 1 and invert 8 times to mix.

Incubate 5-10 minutes at room temperature.

Spin 2 sec in a mini centrifuge.

Add entire volume (~ >400 uL) to the cells dropwise, distributing the drops mostly in the center of the dish (where the imaging window is).

Day 3: Labeling with fluorescent Halo ligands

Prepare Halo Dilution A by making a 1:200 dilution of stock Halo ligand in Culture Media

- Note: Will use 20 uL Dilution A per dish
- Note: Can freeze Dilution A at -20 for up to several months

Prepare working ligand solution by transferring 280 uL conditioned media from the dish where cells are plated to a 1.5 mL tube and adding 20 uL Halo Dilution A

Transfer the rest of the conditioned media in the well (~2 mL) to a 10 mL conical tube and store in a 37 C water bath

Gently drop Dilution A onto cells

Incubate cells at 37 C, 5% CO_2 for at least 15 min

- Note: this incubation step can be up to 2 hr, but a longer incubation introduces the risk of media evaporation

Remove the cells from the incubator and aspirate ligand

solution with vacuum
Wash cells gently with ~200 uL conditioned media
Aspirate wash media and repeat for a total of 2 washes
Add 300 uL conditioned media
Replace plated cells in incubator and rest for 15 min
Wash cells gently with ~200 uL conditioned media
Aspirate wash media and repeat for a total of 2 washes
Cells are prepared for fixation or imaging.