

Protocol: HeLa culture, transfection, and labeling of Halo-fusion proteins

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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](https://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 study this process.
- We use HeLa-M cells, HeLa *OPTN*^{-/-} cells, and HeLa *p62*^{-/-} cells in the study corresponding to this protocol

Materials

- Countess slides (ThermoFisher Scientific, C10228)
- 10 mL conical tube (Corning CLS430055)
- 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-NEMO^{D304N} (generated by site-directed mutagenesis of EGFP-NEMO),
 - 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, Δ PB1, PB1^{AA}, Δ UBA, and LIR^{AAAA}, were kindly provided by S. Martens, University of Vienna, Austria, and TIR^{AAA} was generated by site-directed mutagenesis of mCherry-p62^{WT})
 - o IKK β (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](https://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
 - o **Note: Will use 20 uL Dilution A per dish**
 - o **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% CO₂ for at least 15 min
 - o **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.

