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

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

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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.

ATTACHMENTS

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Keywords: tissue culture, transfection, fluorescent ligands, ASAPCRN

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 study this process.
- We use HeLa-M cells, HeLa OPTN-/- cells, and HeLa p62-/- cells in the study corresponding to this protocol.

MATERIALS

Materials:

- Countess slides **Thermo Fisher Scientific Catalog #C10228**
- 10 ml conical tube **Corning Catalog #CLS430055**
- 1.5 mL capped tubes **Merck MilliporeSigma (Sigma-Aldrich) Catalog #EP022364120**
- 35 mm glass-bottomed dishes **MatTek Corporation Catalog #P35G-1.5-20-C**

Reagents:

- Trypsin **Thermo Fisher Scientific Catalog #R001100**
- Trypan blue **Thermo Fisher Scientific Catalog #T10282**
- DMEM **Corning Catalog #10-017-CV**
- FBS (HyClone)
- GlutaMAX glucose supplement **Gibco - Thermo Fischer Catalog #35050061**
- Opti-MEM **Thermo Fisher Scientific Catalog #3198507**
- Plasmid DNA

1. Untagged Parkin (subcloned from YFP-Parkin, a gift from R. Youle, NIH, Bethesda, MD)
2. Mito-DsRed2 (kindly provided by. T. Schwartz, Harvard Medical School, Boston)
3. Mito-sBFP2 (Wong and Holzbaur, PNAS, 2014)
4. EGFP-NEM(kindly provided by E. Laplantine, Institut Pasteur, Paris),
5. EGFP-NEMOD304N (generated by site-directed mutagenesis of EGFPNEMO),
6. Halo-NEM(subcloned from EGFP-NEMO);
7. Halo-OPTN (subcloned from EGFP-OPTN kindly provided from I. Dikic, Goethe University, Frankfurt, ta pHaloTag vector, Promega);
8. 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)

9. IKK2-EGFP (Plasmid #111195) **addgene Catalog #111195**
10. pCellFree_G03 TFAM **addgene Catalog #67064**
11. mRFP-Ub **addgene Catalog #11935**

Equipment:

Equipment	
Countess II	NAME
Life Technologies	BRAND
AMQAX1000	SKU

- mini centrifuge (Southern Labware, MLX-106)
- Compound microscope

BEFORE START INSTRUCTIONS

- 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.

Day 1: Plating

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

Day 2: Transfection

1d 18h 30m 4s

2 Examine cells by compound microscope 18:00:00 - 24:00:00 after plating to confirm 80-90% confluence. 1d 18h

Note

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

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

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

- 200 µL Opti-mem
- 0.2 µg mito-dsRed
- 0.5 µg Parkin
- 0.2 µg EGFP-NEMO

Note

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 µg Halo-OPTN

3.2 Tube 2 (Lipofectamine 2000)

- 200 µL Opti-mem
- 1.5 µL Lipofectamine 2000

Note

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.

4 Invert tubes 8 times to distribute the contents.

5 Incubate 00:05:00 - 00:10:00 at Room temperature. 15m

6 Spin 00:00:02 in a mini centrifuge. 2s

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

8 Incubate 00:05:00 - 00:10:00 at Room temperature. 15m

9 Spin 00:00:02 in a mini centrifuge. 2s

10 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

30m

11 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
- Can freeze Dilution A at -20 for up to several months

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

13 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.

14 Gently drop Dilution A onto cells.

15 Incubate cells at 37 °C, 5% CO2 for at least 00:15:00.

15m

Note

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

16 Remove the cells from the incubator and aspirate ligand solution with vacuum.

17 Wash cells gently with ~ 200 µL conditioned media.

18 Aspirate wash media and repeat for a total of 2 washes.

19 Add 300 µL conditioned media.

20 Replace plated cells in incubator and rest for 00:15:00 . 15m

21 Wash cells gently with ~ 200 µL conditioned media.

22 Aspirate wash media and repeat for a total of 2 washes.

23 Cells are prepared for fixation or imaging.