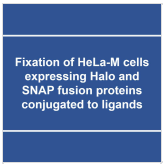

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Fixation of HeLa-M cells expressing Halo and SNAP fusion proteins conjugated to ligands

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ABSTRACT

Mitophagy is a tightly regulated mechanism in which components are sequentially recruited to a damaged mitochondrion in order to clear it by lysosomal degradation. Defects in mitophagy are thought to contribute to multiple human diseases, including neurodegenerative diseases; thus, further study of the pathway is imperative. Many of the molecular steps that comprise mitophagy were first investigated in the HeLa culture system before their translation to specific cell types. Here we developed a protocol for conjugating multiple mitophagy components exogenously expressed in HeLa cells to commercially available fluorescent ligands with the Halo (Promega) and SNAP (New England Biolabs) systems. We precisely control the time course of our investigation by fixing cells with paraformaldehyde after timed incubation with a mitochondrial poison that induces global mitophagy. After fixation, cells can be imaged for a variety of assays and quantifications.

ATTACHMENTS

Fixation of HeLa-M cells expressing Halo and SNAP fusion proteins conjugated to ligands.pdf

DOI

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PROTOCOL CITATION

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GUIDELINES

- This protocol was developed with the HeLa subtype, HeLa-M. HeLa-M cells are flatter than standard HeLa cells, making them easier to image. They also uptake siRNA better than standard HeLa. Regardless, the protocol would be easily adaptable to standard HeLa cells or other cell culture lines.
- This protocol was created in order to investigate Parkin-dependent mitophagy. Parkin and several other fluorescently-tagged mitophagy components are intended for use in the protocol.
- Here we use a primary antibody to a mitochondrial protein, HSP60, in order to identify mitochondria, instead of employing a genetically encoded fluorescent protein. While there are excellent mitochondrially-targeted fluorescent proteins, using anti-HSP60 allows us to demonstrate the versatility of the protocol to image both expressed, tagged proteins and immune-labeled structures.

MATERIALS TEXT

Materials:

- 1.5 ml capped tubes [Millipore Sigma, EP022364120]
- 10 ml conical tube [Corning, CLS430055]
- Aluminium foil [Reynolds]
- Jewelers forceps, Dumont #5 [Millipore Sigma, F6521-1EA]
- Kimwipes [Kimberly-Clark Professional, 34155]
- Parafilm [Millipore Sigma, P7793-1EA]
- Tape
- 15 cm cell culture dish [Millipore Sigma, CLS430599]
- Microscope slides [Fisher Scientific, 12-544-2]
- VectaShield Plus [Vector Laboratories, H-1000-10]
- Nail polish or similar sealant

Reagents:

- DMEM [Corning, 10-017-CV]
- FBS [HyClone]
- GlutaMAX glucose supplement [Gibco, 35050061]
- HaloTag ligand TMR [Promega, G8251]
- SNAP-Cell 647-SiR fluorescent ligand [New England Biolabs, S9102S]
- Dimethyl Sulfoxide (DMSO) [Sigma, D650]
- Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) [Millipore Sigma, C2759]
- Paraformaldehyde (PFA) [Millipore Sigma, 158127]
- Phosphate buffered saline (PBS)
- Triton X-100 [Millipore Sigma, 11332481001]
- Bovine serum albumin [Fisher BioReagents, BP1605-100]
- Anti-HSP60, produced in rabbit [Sigma, SAB4501464]
- Goat anit-rabbit lgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 405 [Thermo Fisher Scientific, A48254]

Equipment:

- Cell incubator to maintain 37°C and 5% CO_2 atmosphere
- Vacuum apparatus in biological safety hood
- 37°C water bath
- Centrifuge, refrigerated

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SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Before start 1d 18h

1 The start point for this protocol is after cells grown on a glass coverslip in a 12- well plate have been transfected with[1d] YFP-Parkin, Halo-OPTN, and SNAP-TBK1 for **18:00:00** - **24:00:00** .

2 Prepare **4 % PFA** in PBS. Keep frozen at **-20 °C** .

Prepare fresh for day-of fixation or thaw directly before use.

Will use **1 mL 4% PFA** per well.

3 Prepare culture media by making a **10 % FBS** , **1 % GlutaMAX** solution in DMEM, place in **37 °C** water bath to warm.

Prepare ~ **3 mL media** per well.

4 Prepare Halo Dilution A by making a 1:200 dilution of stock Halo ligand in DMEM/FBS/GlutaMAX.

Will use **20 µl Halo Dilution A** per well.

5 Prepare **20 Milimolar (mM) CCCP stock** in DMSO. This stock can be stored at **-20 °C** . Thaw at **Room temperature** when needed.

Will use **1 µl 20 mM CCCP** per well.

6 Prepare **0.5 % Triton X-100** in PBS (Permeabilization buffer).

Prepare **0.75 mL Permeabilization buffer** per well, store at **4 °C** .

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Bring to **Room temperature** before use. This will provide less shock to cells, better preserving fixed structures.

Do not use Triton for permeabilization if LC3-autophagosomes are the structure of interest, since Triton is too harsh. Use ice cold methanol if imaging LC3 structures.

7 Prepare [M]**0.2 % Triton X-100/3% BSA** in PBS (Blocking buffer).

Prepare **0.75 mL Blocking buffer** per well, store at **4 °C** .

Bring to **Room temperature** before use.

8 Prepare a humidity chamber by covering a 15-cm cell culture dish with aluminum foil. Cut an area of Parafilm to cover the inside of the dish, and tape each side. (See Figure 1)

Figure 1: Humidity chamber

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HaloTag 30m

9 Prepare working ligand solution by transferring **366 µl conditioned media** from the well where cells are plated to a 1.5 mL tube and adding **20 µl Halo Dilution A** .

10 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.

11 Gently drop working ligand solution onto cells.

12 15m

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

This incubation step can be up to **02:00:00** , but a longer incubation introduces the risk of media evaporation.

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

14

Wash cells by gently dropping **300 µl DMEM/FBS/GlutaMAX** .

15

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

16 Drop on **300 µl conditioned media** .

17 15m

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

During this step, prepare working SNAP ligand solution.

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18 

Wash cells by gently dropping **300 µl DMEM/FBS/GlutaMAX** .

19 

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

SNAP Tag 1h 30m

20 Prepare working SNAP ligand solution by transferring **300 µl conditioned media** from the conical tube to a 1.5 mL tube and adding **0.75 µl SNAP-Cell 647-SiR** .

21 Gently drop working ligand solution onto cells.

22  1h

Incubate cells at **37 °C** , **5 % CO2** for at least **01:00:00** .

This incubation step can be up to **02:00:00** , but a longer incubation introduces the risk of media evaporation.

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

24 Wash cells by gently dropping **300 µl DMEM/FBS/GlutaMAX** .

25 

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

26 Drop on **300 µl conditioned media** .

27  30m

Replace plated cells in incubator and rest for **00:30:00** .

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During this step, prepare working CCCP solution

28 Wash cells by gently dropping **300 µl DMEM/FBS/GlutaMAX** .

29 

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

CCCP treatment 1h 30m

30 Prepare working CCCP solution by transferring **1 mL conditioned media** from the conical tube to a 1.5 mL tube and adding **1 µl 20 mM CCCP stock** .

31 Gently drop working CCCP solution onto cells.

32  1h 30m

Incubate at **37 °C** , **5 % CO2** for **01:30:00** .

Our protocol calls for 1.5 hours mitochondrial damage since that was our timecourse of interest. Users may vary the timecourse in order to examine earlier or later effects of global mitochondrial damage.

Fixation 38m

33 **00:25:00** before CCCP treatment is finished, warm **4 % PFA and 1X PBS** to **37 °C** . 25m

34 Remove cells from incubator and aspirate media.

When possible, keep cells covered with a sheet of aluminum foil, since fluorescent ligands are light-sensitive.

35 Quickly drop on **0.5 mL warmed 1X PBS** .

36 Aspirate PBS.

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37

Repeat warm PBS wash.

38 Drop on **1 mL warmed 4% PFA** .

39 10m

Incubate at **37 °C** for **00:10:00** .

> Warmed PBS and 4% PFA need not be sterile. Thus, if cells are no longer sterile, incubate in a non-sterile **37 °C** environment.

40 Drop on **0.5 mL warmed PBS** .

41 Aspirate PBS.

42 Drop on **0.5 mL warmed PBS** .

43 3m

Incubate covered at **Room temperature** for **00:03:00** .

> Cells can be covered and stored at **4 °C** for up to several days after this step. If you choose to pause at this step, add at least 1.5 mL PBS to wells to prevent evaporation.

Permeabilization 5m

44 Aspirate PBS.

45 Drop on **0.75 mL Permeabilization buffer** .

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46 5m

Incubate covered at **Room temperature** for **00:05:00** .

Blocking 45m

47 Use sharp forceps to carefully lift the coverslip out of the well and dab excess Permeabilization buffer on a Kimwipe.

48 Place slip cell-side up on Parafilm in the humidity chamber.

49

From the edge of the coverslip, pipet **150 µl Blocking buffer** onto the cells.

50 45m

Incubate covered at **Room temperature** for **00:45:00** .

Blocking step can be up to **01:00:00** .

Prepare primary antibody dilution during this step.

51 Aspirate Blocking buffer from the edge of the coverslip, gently lifting one side of the slip with forceps if necessary to allow buffer to slide off.

Primary antibody 1d 18h 10m

52 Prepare **200 µl anti-HSP60** at 1:125 in Blocking buffer for each coverslip.

Before use, spin primary antibody at top speed in a refrigerated centrifuge for **00:05:00** . Pipet from top of solution to ensure that no aggregates are present.

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53 From the edge of the coverslip, pipet primary antibody dilution onto the cells.

54 

Incubate covered at **4 °C** **Overnight** .

Twist two Kimwipes and wet with water so that they are more than damp but not dripping. Pack the wet Kimwipes into the edges of the humidity chamber in order to prevent evaporation of antibody solution during the incubation.

55 1d 18h

18:00:00 - **24:00:00** later, aspirate antibody dilution from the edge of the coverslip, gently lifting one side of the slip with forceps if necessary to allow buffer to slide off.

56 

From the edge of the coverslip, pipet **100 µl room temperature PBS** onto the cells.

57 5m

Incubate covered at **Room temperature** for **00:05:00** .

58 Aspirate PBS from the edge of the coverslip, gently lifting one side of the slip with forceps if necessary to allow buffer to slide off.

59 

Repeat previous three steps for a total of four washes.

Secondary antibody 50m

60 Prepare secondary antibody solution by diluting goat anti-rabbit 405 1:200 in Blocking buffer.

Prepare **150 µl secondary solution** for each coverslip.

Before use, spin primary antibody at top speed in a refrigerated centrifuge for **00:05:00** . Pipet from top of solution to ensure that no aggregates are present.

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61 From the edge of the coverslip, pipet secondary antibody dilution onto the cells.

62 

45m

Incubate covered at **Room temperature** for **00:45:00** .

Secondary incubation can be up to **01:00:00** .

Warm VectaShield to **Room temperature** during this step.

63 Aspirate antibody dilution from the edge of the coverslip, gently lifting one side of the slip with forceps if necessary to allow buffer to slide off.

64 

From the edge of the coverslip, pipet **100 µl room temperature PBS** onto the cells.

65 

5m

Incubate covered at **Room temperature** for **00:05:00** .

66 Aspirate PBS from the edge of the coverslip, gently lifting one side of the slip with forceps if necessary to allow buffer to slide off.

67 

Repeat previous three steps for a total of four washes.

Mounting

68 Pipet **12.5 µl room temperature VectaShield** onto a microscope slide.

Pick up coverslip with forceps and dab excess PBS onto a Kimwipe.

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69

70 Lay coverslip cell-side down onto VectaShield drop.

71 Seal edges of coverslip with nail polish.

72 Lay flat until set.

73 Samples can now be imaged.

Store slides at **4 °C** .

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