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

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 We use this protocol and it's working

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

In 1 collection

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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](#)

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

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 anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 405 [Thermo Fisher Scientific, A48254]

Equipment:

- Cell incubator to maintain 37°C and 5% CO₂ atmosphere
- Vacuum apparatus in biological safety hood
- 37°C water bath
- Centrifuge, refrigerated

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 YFP-Parkin, Halo-OPTN, and SNAP-TBK1 for 18:00:00 - 24:00:00 . 1d

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

Note

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

Note

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.

Note

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.

Note

Will use 20 µL Halo Dilution A per well.

- 5 Prepare [1M] 20 millimolar (mM) CCCP stock in DMSO. This stock can be stored at -20°C . Thaw at Room temperature when needed.

Note

Will use $\text{1 } \mu\text{L}$ 20 mM CCCP per well.

- 6 Prepare [1M] 0.5 % Triton X-100 in PBS (Permeabilization buffer).

Note

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

Note

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

Note

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 [1M] 0.2 % Triton X-100/3% BSA in PBS (Blocking buffer).

Note

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

Note

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

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

15m

Note

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

15m

Note

During this step, prepare working SNAP ligand solution.

- 18 Wash cells by gently dropping 300 μ L DMEM/FBS/GlutaMAX .

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

SNAP Tag

1 h 30m

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

- 21 Gently drop working ligand solution onto cells.

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

1h

Note

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

30m

Note

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

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

1h 30m

Note

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.

Note

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.

37

Repeat warm PBS wash.

38 Drop on 1 mL warmed 4% PFA .

39 Incubate at 37 °C for 00:10:00 .

10m

Note

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

3m

Note

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 .

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

5m

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 μ L Blocking buffer onto the cells.

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

45m

Note

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

Note

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.

Note

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.

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

- 54 Incubate covered at 4 °C Overnight .

Note

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 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.

1d 18h

56 From the edge of the coverslip, pipet 100 μ L room temperature PBS onto the cells.

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

5m

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.

Note

Prepare 150 μ L secondary solution for each coverslip.

Note

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.

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

62

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

45m

Note

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

Note

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 μ L room temperature PBS onto the cells.

65

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

5m

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.

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

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.

Note

Store slides at 4 °C .