Fixation and imaging of HeLa cells after mitochondrial depolarization

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We use this protocol and it's working

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ABSTRACT

Ectopic expression of p62/SQSTM1 often induces puncta formation and poor cell health due to p62's proclivity to multimerize and form filaments. We used the cell fixation protocol described here in order to over-express fluorescently tagged p62 at low levels in p62-/- cells and amplify the signal with immuno-labeling. By this method, we were able to express a variety of tagged p62 mutations to determine their effects on NEMO recruitment to depolarized mitochondria without introducing toxic artifacts of p62 overexpression. We also note the use of a modified fixation protocol to visualize various endogenous ATG8 proteins, which are also difficult to over-express in our system. Thus, fixation techniques are a critical complement to studies in live cells.

ATTACHMENTS

470-982.pdf

GUIDELINES

- This protocol was adapted from a previous protocol for similar techniques (see dx.doi.org/10.17504/protocols.io.bujsnune)
- 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

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Keywords: Mitochondrial depolarization, Paraformaldehyde fixation, Confocal imaging, ASAPCRN

Materials

- 1.5 mL capped tubes **Merck MilliporeSigma (Sigma-Aldrich) Catalog #EP022364120**
- 10 ml conical tube **Corning Catalog #CLS430055**
- Aluminum foil (Reynolds)
- Jewelers forceps Dumont No. 5 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F6521-1EA**
- Kimtech Science™ Kimwipes™ Delicate Task Wipes **Kimberly-Clark Catalog #34155**
- PARAFILM® M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P7793-1EA**
- Tape
- Corning® tissue-culture treated culture dishes **Merck Millipore (EMD Millipore) Catalog #CLS430599-60EA**
- Fisherbrand™ Premium Frosted Microscope Slides Superfrost marking area **Fisher Scientific Catalog #12-544-2**
- VectaShield Plus **Vector Laboratories Catalog #H-1000-10**
- Nail polish or similar sealant

Reagents:

- Corning® 500 mL DMEM (Dulbecco's Modified Eagle's Medium) **Corning Catalog #10-017-CV**
- FBS (HyClone)
- GlutaMAX™ Supplement **Thermo Fisher Catalog #35050061**
- Dimethyl sulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D2650**
- Ethanol
- Antimycin A from Streptomyces sp. **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A8674**

and

- Oligomycin A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #75351**
- Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #158127**
- Phosphate buffered saline (PBS)

- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11332481001**
- Fisher BioReagents™ Bovine Serum Albumin Fraction V Cold-ethanol Precipitated **Fisher Scientific Catalog # BP1605100**
- Anti-HSP60 antibody produced in rabbit **Merck MilliporeSigma (Sigma-Aldrich) Catalog #SAB4501464**
- Anti-SQSTM1 / p62 antibody [2C11] - BSA and Azide free **Abcam Catalog #ab56416**
- Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody (A48254) in ICC/IF **Thermo Fisher Scientific Catalog #A48254**
- Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody (A-11030) in ICC/IF **Thermo Fisher Scientific Catalog #A-11030**

Equipment:

- Cell incubator to maintain 37 °C and 5% CO_2 atmosphere
- Vacuum apparatus
- 37 °C water bath
- Centrifuge, refrigerated
- Confocal microscope with 60X objective associated software

BEFORE START INSTRUCTIONS

- The start point for this protocol is after cells grown on glass coverslips in a 12-well plate have been transfected with Parkin, EGFP-NEMO, HaloOPTN, and

mCherry-p62 for 18- 24:00:00 and tagged with Halo ligand.

- Prepare [M] 45 millimolar (mM) stock of Antimycin A by suspending 50 mg solid AntA in 2 mL ethanol.
- Prepare [M] 10 millimolar (mM) stock Oligomycin A by suspending 5 mg solid OligA in 630 µL DMSO
- Prepare 4% PFA in PBS. Keep frozen at -20 °C.

Note

- Prepare fresh for day-of fixation or thaw directly before use.
- Will use 1 mL 4% PFA per well.

- Prepare Culture Media by making 10% FBS, 1% GlutaMAX solution in DMEM.
- Prepare 0.5% Triton X-100 in PBS (Permeabilization buffer).

Note

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

- Prepare 0.2% Triton X-100/3% BSA in PBS (Blocking buffer).

Note

- Prepare 0.75 mL Blocking buffer per well, store at 4 °C.
- Bring to Room temperature before use.

- Prepare a humidity chamber as previously described (dx.doi.org/10.17504/protocols.io.bujsnune)

AntA/OligA treatment

1

Prepare working AntA/OligA solution by transferring 0.5 mL conditioned media to a 1.5 mL tube and adding 0.25 µL [M] 10 millimolar (mM) OligA and 2 mL [M] 45 millimolar (mM) AntA.

2

Gently drop working AntA/OligA solution onto cells.

3

Incubate at 37 °C, 5% CO_2 for 00:55:00 - 01:05:00. 2h

Note

- Our protocol calls for ~ 01:00:00 mitochondrial damage since that was our time course of interest. Users may vary the time course in order to examine earlier or later effects of global mitochondrial damage.
- To carry out vehicle control experiment, use the equivalent amounts of ethanol and DMSO treatment.

Fixation

4

25 minutes before AntA/OligA treatment is complete, warm 4% PFA and 1X PBS to 37 °C.

5

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.

6 Quickly drop on 0.5 mL warmed 1X PBS.

7 Aspirate PBS.

8 Repeat warm PBS wash.

9 Drop on 1 mL warmed 4% PFA.

10 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.

11 Drop on 0.5 mL warmed PBS.

12 Aspirate PBS.

13 Drop on 0.5 mL warmed PBS.

14 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

15 Aspirate PBS.

16 Drop on 0.75 mL Permeabilization buffer.

17 Incubate covered at Room temperature for 00:05:00. 5m

Note

If fixation protocol is used to examine autophagosomal structures, do not use buffer with Triton, as this will destroy the vesicles. Instead, use ice cold methanol permeabilization buffer and goat serum/BSA blocking buffer.

Blocking

18

Add ~ 250 µL Blocking buffer to each well.

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

Note

- Blocking step can be up to 01:00:00.
- Prepare primary antibody dilution during this step.

20 Aspirate Blocking buffer.

Primary antibody

21

Prepare 200 µL Primary antibody dilution per coverslip, with 1:250 antiHSP60 and 1:500 anti-p62 in Blocking buffer.

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.
- While mCherry-p62 was transfected in these cells, anti-p62 antibody boosts the signal.

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

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

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

25 Incubate covered at 4 °C Overnight . 5m

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.

26 18- 24 hours 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.

27 From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.

28 Incubate covered at Room temperature for 00:05:00 . 5m

29 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.

30 Repeat previous three steps for a total of four washes.

30.1 Wash (1/4): 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

30.2 Wash (2/4): 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

30.3 Wash (3/4): 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

30.4 Wash (4/4): 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

Secondary antibody

31

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

Note

- Prepare 125 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.

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

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

Note

- Secondary incubation can be up to 01:00:00.
- Warm VectaShield to Room temperature during this step.

34 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.

35 From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.

36 Incubate covered at Room temperature for 00:05:00. 5m

37 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.

38 Repeat previous three steps for a total of four washes.

38.1 Wash (1/4) 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

38.2 Wash (2/4) 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

38.3 Wash (3/4) 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

38.4 Wash (4/4) 5m

- From the edge of the coverslip, pipet 100 µL Room temperature PBS onto the cells.
- Incubate covered at Room temperature for 00:05:00.
- 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.

Mounting

39 Pipet 12.5 µL Room temperature VectaShield onto a microscope slide.

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

41 Lay coverslip cell-side down onto VectaShield drop.

42 Seal edges of coverslip with nail polish.

43 Lay flat until set.

Note

Store slides at 4 °C.

Imaging

44 Using 60X objective and RFP epifluorescence, find the focal plane of fixed cells by looking for dsRed2-labeled mitochondria.

Note

We find that 60X is sufficient magnification to collect several cells in each field of view with enough resolution to perform analysis and quantification. Other magnifications may be appropriate for various applications.

45 Configure 405, 488, 561, and 647 lasers and accompanying exposure times to maximize dynamic range of each signal.

Note

Since the cells are fixed, there is less downside to photo bleaching the samples.

46 Configure collection parameters to record a >2 um Z stack of images.

Note

On our system, 0.15 um is the minimum step size, thus 21 sections is sufficient.

47 Collect images from fields of view with multiple cells exhibiting all visible tags. Collect volume from the 50th to 75th percentile of the cell (see Figure 1). If mitochondria are depolarized, can use OPTN signal (recruitment to
fragmented mitochondria) to determine whether Parkin is expressed.

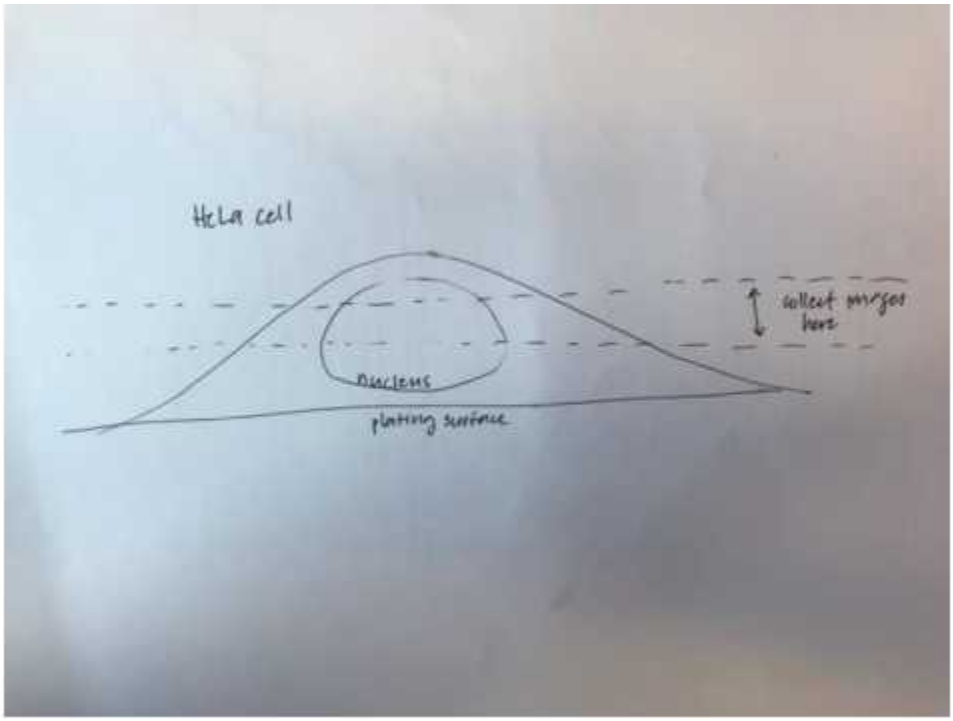

Figure 1. Schematic of HeLa cell volume. Dotted lines indicate target volume to image.

48 Collect images in order to record 10-20 cells per condition, depending on the quality of the transfection and cells. Collect images from diverse areas of the coverslip.