Video Article

In Vitro and *In Vivo* Detection of Mitophagy in Human Cells, *C. Elegans*, and Mice

Evandro F. Fang[1,6], Konstantinos Palikaras[2], Nuo Sun[3], Elayne M. Fivenson[1], Ryan D. Spangler[4], Jesse S. Kerr[1], Stephanie A. Cordonnier[1], Yujun Hou[1], Eszter Dombi[5], Henok Kassahun[6], Nektarios Tavernarakis[2,7], Joanna Poulton[5], Hilde Nilsen[6], Vilhelm A. Bohr[1,8]

[1]Laboratory of Molecular Gerontology, National Institute on Aging, National Institutes of Health

[2]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology - Hellas

[3]Center for Molecular Medicine, National Heart Lung and Blood Institute, National Institutes of Health

[4]Laboratory of Neurosciences, National Institute on Aging, National Institutes of Health

[5]Nuffield Department of Obstetrics and Gynaecology, University of Oxford

[6]Department of Clinical Molecular Biology, University of Oslo and Akershus University Hospital

[7]Department of Basic Sciences, Faculty of Medicine, University of Crete

[8]Danish Center for Healthy Aging, University of Copenhagen

Correspondence to: Evandro F. Fang at evandrofeifang@yahoo.com; e.f.fang@medisin.uio.no, Vilhelm A. Bohr at BohrV@grc.nia.nih.gov

URL: https://www.jove.com/video/56301
DOI: doi:10.3791/56301

Keywords: Medicine, Issue 129, Mitophagy, aging, *C. elegans*, mouse, autophagy, rosella, Alzheimer's disease, Parkinson's disease, longevity

Date Published: 11/22/2017

Citation: Fang, E.F., Palikaras, K., Sun, N., Fivenson, E.M., Spangler, R.D., Kerr, J.S., Cordonnier, S.A., Hou, Y., Dombi, E., Kassahun, H., Tavernarakis, N., Poulton, J., Nilsen, H., Bohr, V.A. *In Vitro* and *In Vivo* Detection of Mitophagy in Human Cells, *C. Elegans*, and Mice. *J. Vis. Exp.* (129), e56301, doi:10.3791/56301 (2017).

Abstract

Mitochondria are the powerhouses of cells and produce cellular energy in the form of ATP. Mitochondrial dysfunction contributes to biological aging and a wide variety of disorders including metabolic diseases, premature aging syndromes, and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Maintenance of mitochondrial health depends on mitochondrial biogenesis and the efficient clearance of dysfunctional mitochondria through mitophagy. Experimental methods to accurately detect autophagy/mitophagy, especially in animal models, have been challenging to develop. Recent progress towards the understanding of the molecular mechanisms of mitophagy has enabled the development of novel mitophagy detection techniques. Here, we introduce several versatile techniques to monitor mitophagy in human cells, *Caenorhabditis elegans* (*e.g.*, Rosella and DCT-1/ LGG-1 strains), and mice (mt-Keima). A combination of these mitophagy detection techniques, including cross-species evaluation, will improve the accuracy of mitophagy measurements and lead to a better understanding of the role of mitophagy in health and disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/56301/

Introduction

Mitophagy is essential for mitochondrial maintenance. Mitochondria intersect multiple cell signaling pathways and are universal sub-cellular organelles responsible for cellular energy production, cell metabolism, and calcium homeostasis[1,2,3,4]. Mitochondria constantly experience challenges from endogenous and exogenous sources, such as reactive oxygen species (ROS) and mitochondrial toxicants, respectively, which lead to the generation of "aged" and dysfunctional mitochondria. Accumulation of damaged mitochondria decreases the efficiency of ATP production while increasing the amount of harmful ROS, and has been linked to age-related diseases such as metabolic diseases, AD, and PD[1,5,6]. To prevent mitochondria induced cellular dysfunction, cells need to specifically recognize damaged mitochondria and efficiently remove them through a cellular process termed mitochondrial autophagy (mitophagy). This demonstrated importance of mitophagy in health and disease illustrates the need for accurate and efficient methods to detect mitophagy both *in vitro* and *in vivo*.

Mitophagy is a multiple-step process involving many proteins and protein complexes[5,7,8]. In brief, a damaged mitochondrion is first recognized and engulfed by a double-membraned phagophore, which can originate from the plasma membrane, endoplasmic reticulum, Golgi complex, nucleus, or mitochondrion itself[9,10]. The spherical phagophore elongates and eventually seals the mitochondria inside, constituting the mitochondrial autophagosome (mitophagosome). The mitophagosome then fuses with the lysosome for degradation, forming an autolysosome in which the damaged mitochondrion is degraded and recycled[7,8]. Major autophagic proteins also involved in mitophagy include: Autophagy Related 7 (ATG7) and Beclin1 (initiation), Microtubule-Associated Protein 1A/1B-Light Chain 3 (LC3-II) (LGG-1 in *C. elegans*) and p62 (components of phagophore), and lysosomal-associated membrane glycoprotein 2 (LAMP2)[6,7]. In addition, there are several essential proteins unique to mitophagy, including PTEN-induced Putative Kinase 1 (PINK-1), Parkin1, Nuclear Dot Protein 52 kDa (NDP52), optineurin, BCL2 Interacting Protein 3 Like (NIX/BNIP3L) (DCT-1 in *C. elegans*), among others[5,6,11].

Copyright © 2017 Journal of Visualized Experiments

A common method to detect changes in levels of autophagy is by the ratio of LC3-II/LC3-I or LC3-II/actin. However, this method is nonspecific, as an increase in this ratio may reflect either an increased initiation or an impaired fusion of mitophagosome to lysosome[12]. Another method is to evaluate the colocalization between an autophagy marker (*e.g.*, LC3) and a mitochondrial protein (*e.g.*, Translocase of Outer Mitochondrial Membrane 20 (TOMM20, which could be degraded by proteasomes)). However, this can only indicate changes in total mitophagy levels and cannot distinguish the step(s) at which blockage occurs. This can be clarified by using lysosomal inhibitors (*e.g.*, E64d+Pepstatin A, termed EP) in parallel to cause the accumulation of mitophagosomes. The difference between the number of mitophagosomes at baseline and the number of mitophagosomes following treatment with EP can indicate mitophagy. These limitations have prompted the development of novel mitophagy detection techniques. In view of the increasing relevance of mitophagy in a wide spectrum of human diseases, we present several robust mitophagy detection techniques which may be useful for researchers. We cover both *in vitro* and *in vivo* techniques and recommend combining multiple techniques to verify changes of mitophagy.

Protocol

Animals (male and female mice) were born and bred in an accredited animal facility, in accordance and approval of the NIH Animal Care and Use Committee. Euthanasia methods must be consistent with all national and institutional regulations.

1. Detection of Mitophagy in Human Cells

1. **Detection of mitophagy using mt-Keima plasmid**
   NOTE: The Keima protein, fused to a mitochondria target signal, simplified as mt-Keima, has proved useful for *in vivo* mitophagy detection. mt-Keima is a ratiometric pH-sensitive fluorescent protein which exhibits green fluorescence (excitation 458 nm) in basic or neutral conditions and red fluorescence (excitation 561 nm) in acidic conditions. Healthy mitochondria thrive in basic or neutral conditions (pH 7-8) and are indicated by green fluorescence when transfected with mt-Keima. When mitochondria undergo mitophagy and fuse with acidic lysosomes, the pH drops to 4.5 and mitochondria containing mt-Keima exhibit red fluorescence[6,13,14]. Importantly, mt-Keima is resistant to lysosomal proteases, enabling evaluation of mitophagy over longer periods. The protocol below is designed for 6-well plates.
    1. Day 1: Use either primary human cells (*e.g.*, fibroblasts) or immortalized human cells (*e.g.*, Hela Cells or U2OS cells). Seed approximately 0.3-1 × 10^6 cells/well (depending on the cell growth rate to allow cells to reach 70% confluency on the next day) in a 6-well plate. Maintain the cells in complete cell culture medium (Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% FBS, and 1% mixed solution of penicillin-streptomycin), and incubate in a cell culture incubator at 37 °C (20% O_2, 5% CO_2). Add 1 mL complete cell culture medium/well in a 6-well plate.
       NOTE: Gene overexpression/knockdown or drug treatments can be done at this stage[6].
    2. Day 2: Mix mt-Keima plasmid DNA[6] and DNA transfection reagent (the mixture for one well of a 6-well plate).
        1. Dilute 15 µL of a DNA transfection reagent with 150 µL of serum-free medium according to the manufacturer's protocol.
        2. Dilute 2-5 µg of mt-Keima plasmid DNA with 150 µL of serum-free medium.
        3. Add diluted mt-Keima plasmid to diluted DNA transfection reagent, vortex for 10 s, and incubate for 10 min at room temperature.
           NOTE: Optimization of DNA transfection conditions may be necessary depending on the plate size (see the manufacturers' protocols for details).
        4. Add DNA/transfection reagent mixture dropwise to cells, then shake the plates gently for 5-10 s until the solution is completely mixed.
    3. Day 3: Change the media by replacing the DNA transfection reagent-containing medium with fresh complete cell culture medium (1 mL/well).
    4. Day 4: Image the cells using confocal microscopy[6]. Use two imaging channels: green channel (excitation 458 nm, emission 570-695 nm) to visualize normal mitochondria and red channel (excitation 561 nm, emission 570-695 nm) to visualize mitochondria which are undergoing mitophagy. Randomly image 20 areas under 40x magnification to cover a total of 100-200 cells in every well.
    5. Perform data analysis. Calculate the "mitophagy index" (percentage of mitochondria undergoing mitophagy), using image analysis software to quantify the amount of fluorescence in both the red and green channels: take the ratio of red fluorescence to red + green fluorescence, and multiply by 100[6].
       NOTE: Alternatives to transfection include using mt-Keima stably expressed HeLa cell lines[14]. Since HeLa cells do not express Parkin, Parkin must be stably expressed to study Parkin-dependent mitophagy in this cell line.

2. **Detection of mitophagy using the colocalization between Cytochrome C oxidase subunit II (COXII) and LAMP2**
   NOTE: COXII is a mitochondrial genome-encoded inner membrane protein. The 'mitophagy index' equals the ratio of COXII colocalized with LAMP2 expressed as a percentage of the total amount of COXII.
    1. Seed HeLa cells (or other cells of interest mentioned in step 1.1.1) in a 4-well chamber slide (1-5 × 10^4 cells in 1 mL complete cell culture medium/well) in a cell culture incubator at 37 °C (20% O_2, 5% CO_2) overnight.
    2. Add 3.0 µL of 10 mM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and incubate in the cell culture incubator (step 1.2.1) for 3 h.
       NOTE: Other drugs affecting mitophagy may be used.
    3. Remove the culture medium using a 1 mL pipette, wash the cells twice with 1 mL/well of cold (0-4 °C) 1x PBS. Let the solution sit for 10-30 s before the next wash. Then add 0.5 mL 3.7% paraformaldehyde (PFA) in 1x PBS to each well and incubate for 10 min on ice to fix the cells.
       Caution: PFA is toxin. Work in a fume hood when using PFA.
    4. Discard the PFA solution using a 1 mL pipette, wash the cells twice with cold 1X PBS (1 mL/well; let solution sit for 10 s before the next wash), and permeabilize the cells with 1 mL/well of 0.25% detergent (in 1x PBS) for 10 min on ice.
    5. Discard the permeabilizing buffer, gently wash the cells twice with cold 1x PBS and discard (let the solution sit for 10 s between washes). Block the cells with 1 mL/well of 5% FBS in 1x PBS overnight at 4 °C. Cover the chambers to prevent moisture loss.
    6. Prepare 0.5 mL/well of a mixed antibody solution with COXII antibody (mouse, 1:250 dilution) and LAMP2 antibody (rabbit, 1:20-1:50 dilution) in 1x PBS with 5% FBS, and store on ice for 0.5-1 h.

Copyright © 2017 Journal of Visualized Experiments

7. Take out the 4-well chamber slide from the cold room and discard the blocking solution with a 1 mL pipette. Gently wash the cells twice with 1 mL/well of cold 1x PBS and discard (let the solution sit for 10 s between washes), then add 0.5 mL/well of mixed antibody solution. Incubate at 37 °C for 1 h on a shaker at low speed.
8. Discard the first antibody solution using a 1 mL pipette and gently wash the cells three times with 1 mL/well of cold 1x PBS and discard. Let the solution sits for 10 s before the next wash. Add 1 mL/well of fluorescent secondary antibodies (*e.g.*, goat anti-rabbit with wavelength 568 nm of red fluorescent protein (RFP) for LAMP2; and goat anti-mouse with wavelength 488 nm of green fluorescent protein (GFP) for COXII) in 1:250 dilution with 5% FBS in 1x PBS. Cover the 4-well chamber slide with aluminum foil and incubate at 37 °C for 1 h on a shaker at low speed.
9. Discard the second antibody solution with a 1 mL pipette. Gently wash the cells six times with 1 mL/well of cold 1x PBS. Let the solution sit for 1 min before the next wash.
10. Mount the cells with 50 µL/well of antifade mounting medium with DAPI, and add cover slips.
11. Image the cells under a confocal microscope with 66x magnification and immersion oil (Tile scans/ mosaics may be used to increase the number of cells imaged by the software). Use the same settings such as channel exposure time, digital gain, digital offset, *etc.* throughout all imaging processes (see software instructions for details)[15].
12. Use colocalization analysis software to quantitatively analyze at least 20 random images of 100 cells[15]. Use the "Closed Bezier" function to select entire individual cells. Compare the Pearson's colocalization coefficient of GFP to RFP, either weighted or unweighted, to determine mitophagy levels in analyzed cells. Maintain values for horizontal and vertical crosshairs of the colocalization analysis software throughout analysis. To accurately set these crosshairs, prepare two extra sets of cells and label one COXII and the other LAMP2. Then, set crosshairs directly above the pixel distribution for each channel[15] (see software instructions for details).

3. **A high throughput imaging measurement for mitophagy screening**
NOTE: Performing high throughput screens of mitophagy-inducing or mitophagy-inhibiting compounds from large compound libraries is technically demanding. An imaging technique based on the colocalization of mitochondria with autophagosomes is a more simplistic, yet highly sensitive alternative for mitophagy screening[16].
    1. Day 1: Seed cells (*e.g.*, human primary fibroblasts at 5,000 cells/100 mL cell culture media/well) in a 96-well plate (see step 1.1.1).
    2. Day 2: Treat the cells from the second day with FCCP or other compounds of interest for designated incubation time (see step 1.2.2).
    3. Follow steps 1.2.2-1.2.9 using the primary antibodies for COXII (mouse, 1:250 dilution with 5% FBS in 1x PBS) and LC3B (rabbit, 1:100-1:200 dilution with 5% FBS in 1x PBS). Alternatively, primary antibodies for COXII and LAMP2 may be used.
    4. Image the cells using a fluorescent reader[16]. Collect images in randomly placed fields and with a minimum 500 cells/well.
    5. Analyze data using a cell analyzer customized protocol[16].

2. Detection of Mitophagy in *C. elegans*

NOTE: The nematode *C. elegans* provides a platform to assay mitophagy at the organismal level. Two strains can be used to monitor mitophagy: (1) mitochondria-targeted Rosella (mtRosella) or (2) mitophagy receptor DCT-1 fused with GFP along with autophagosomal marker LGG-1 fused with Discosoma sp. red fluorescent protein (DsRed)[5,17].

1. **Mitophagy monitoring using Rosella biosensor**
NOTE: Rosella is a molecular biosensor that combines a pH-insensitive DsRed fused to a pH-sensitive GFP variant. The Rosella biosensor takes advantage of the pH differences between the acidic lysosome (pH ~4.5) and other cellular compartments. This biosensor has been used to successfully monitor mitophagy in *Saccharomyces cerevisiae* and several mammalian cell lines, such as HeLa, HEK293, and HCT116[18,19]. We adapted this versatile dual-fluorescent reporter and generated transgenic *C. elegans* nematodes expressing mitochondria-targeted Rosella (mtRosella) in body-wall muscle cells. Mitophagy induction is indicated by a reduced GFP/DsRed ratio of mtRosella fluorescence.
    1. Day 1: Pick L4 larvae of worms expressing mitochondria-targeted Rosella (mtRosella) in body-wall muscle cells onto a Nematode Growth Media (NGM) plate seeded with *E. coli* (OP50) using a dissection microscope[5]. Place 10-20 worms/plate on at least three 3.5 cm plates. Incubate the nematodes at the standard temperature of 20 °C[5].
    NOTE: See reference for the worm anatomy, including identification of L4 larvae[20], and the way to make a pick which is used to transfer worms from one location to another[21].
    2. Day 5: Synchronize nematodes by selecting 15-20 L4 transgenic larvae and transferring them onto fresh OP50 seeded NGM plate. Use at least five plates per experimental condition.
    3. Day 7: Prepare vehicle plates for mitophagy-affecting drugs of interest or positive controls.
        1. Kill *E. coli* (OP50) bacteria seeded by exposing NGM plates for 15 min with 222 $\mu W/cm^2$ (intensity) of UV light to ensure that mitophagy-inducing compounds are not metabolized by bacteria. Plates must be exposed with 2,000 J/m^2.
        NOTE: Seconds of exposure needed = 2,000/((intensity in $\mu W/cm^2$)*100).
        2. Add compound(s) of interest to the top of seeded NGM plates. Add an equivalent amount of compound vehicle (solvent used to dissolve compound, such as dimethyl sulfoxide (DMSO)) to the vehicle plates as a negative control. For positive control of mitophagy induction, a mitochondrial toxicant, paraquat (8 mM final concentration) was used. Add a solution containing 10 µL of 8 M paraquat stock with 190 µL of ddH_2O on the top of the agar plate for the treatment group. For the vehicle group, add a solution containing 10 µL of DMSO with 190 µL of ddH_2O on the top of the agar plate.
        NOTE: The paraquat-working solution may be kept for 1 month at 4 °C.
        3. Gently swirl the plates until the drug or vehicle coats the entire surface. Allow the plates to dry with the lids closed at room temperature for at least 1 h before transferring the worms.
        NOTE: Drug plates can also be prepared by adding drugs in the liquid NGM before it solidifies.
    4. Day 7: Transfer 10-20 of 2-day-old adult transgenic animals prepared in step 2.1.2 to plates containing either paraquat, compound(s) of interest, or vehicle (DMSO). Incubate the transgenic animals at 20 °C for 2 days.
    5. Day 9: Prepare 2% agarose pads (see **Supplementary Material**). Then add one droplet (10 µL) of 20 mM levamisole in M9 per pad. Immobilize the transgenic animals for imaging, by placing them in the M9-levamisole drop. Gently place a coverslip on the top.

6. Capture single transgenic animals using a camera attached to an epifluorescence microscope. Acquire fluorescent images of whole transgenic nematodes expressing mtRosella in body-wall muscle cells at 10X magnification[5]. Save the collected images.
7. Process the acquired images with ImageJ software. Analyze the body wall muscle cells located in the head of the worm to avoid autofluorescence in the intestinal tissue. Measure the average pixel intensity value and total area for each fluorescent image only of the head region of each animal. To analyze the specific area of interest:
    1. Select 'split channel' under the 'image' and 'color' drop-down menu to convert images .
    2. Utilize the 'freehand selection' tool to capture the fluorescent area of interest.
    3. Select the 'measurement' command under the 'analyze' drop-down menu and perform pixel intensity analysis.
    4. Pixel intensity should be normalized by dividing intensity by the total area analyzed. Upon normalization, calculate the GFP to DsRed ratio[22].
8. Use statistical analysis software to either conduct student *t*-test (comparison between two groups) or ANOVA (comparison among multiple groups) for statistical analysis with $p < 0.05$ as significant[5,17].

2. **Assessing mitophagy using co-localization between DCT-1 and LGG-1**
NOTE: DCT-1 is an outer mitochondrial membrane protein that acts as a mitophagy receptor in response to stress conditions, similar to its putative orthologues BNIP3 and NIX/BNIP3L in mammals[5]. Thus, mitophagy initiation is assessed by co-localization between DCT-1 mitophagy receptor and autophagosomal membrane protein LGG-1 (homolog of the mammalian LC3).
    1. Day 1: Pick L4 larvae of transgenic animals expressing both DCT-1::GFP and DsRed::LGG-1 in body-wall muscle cells[5] onto an OP50 seeded NGM plate. Place 5-10 worms/3.5 cm plate, and use at least three plates. Incubate the nematodes at 20 °C.
    NOTE: The transgenes are located on separate plasmids and they are not integrated in the genome. Pick up nematodes carrying both *rol-6(su1006)* and p_{myo-2} GFP contransformation markers. Only transgenic worms with both contransformation markers express both DCT-1::GFP and DsRed::LGG-1 in body-wall muscle cells.
    2. Follow the steps from 2.1.2-2.1.5.
    3. Image single body-wall muscle cells using a camera attached to a confocal microscope[5,23]. Detect the borders of entire body-wall muscle cells and take z-stack images under 63x magnification. Higher magnification can also be used.
    4. Open and process images acquired with the confocal software. Initiation of mitophagy is indicated by co-localization of the mitophagy receptor (DCT-1::GFP) to the autophagosomal marker (DsRed::LGG-1). Manually measure co-localization events in each stack of body-wall muscle cell[5]. It is essential to keep all microscope and camera settings (lens and magnifier, filters, exposure time, resolution, laser intensity, gain, *etc.*) consistent throughout experiments. All settings should be noted.
    5. Use statistical analysis software to either conduct student *t*-test (comparison between two groups) or ANOVA (comparison among multiple groups) for statistical analysis with $p < 0.05$ as significant[5,17]. For two-way comparisons use the Student's *t*-test ($p < 0.05$ is considered significant). For multiple comparisons use the single factor (ANOVA) variance analysis, corrected by the *post hoc* Bonferroni test. We recommend analyzing at least 50-70 animals or 30-50 body-wall muscle cells for each experimental condition. Repeat the experiment at least three times.

3. Detection of Mitophagy in Mice

NOTE: Previous methods to detect mitophagy in mice were cumbersome, insensitive, and difficult to quantify. A transgenic mouse model expressing the mitochondrial-targeted form of the fluorescent reporter Keima (mt-Keima) can now be utilized to assess levels of mitophagy in a wide range of physiological and pathophysiological conditions.

1. Euthanize mice using a protocol approved by the Institutional Animal Care and Use Committee[14].
2. Secure the mouse (ventral side up) to the working platform using pins in the limbs. Make an incision in the lower abdomen using microsurgery scissors and forceps to expose the liver[24]. Cut a piece of liver (*e.g.*, a piece of right lobe at 1 × 1 × 1 cm) using microsurgery scissors and forceps.
NOTE: Detection of mitophagy in mice is complicated and requires knowledge on mouse anatomy and highly skillful mouse handling and mouse surgery. This protocol provides basic, key steps on this method, and a more detailed method is available[24].
3. Place the liver sample on a metal plate on ice to rapidly cool the tissue. Keep the tissue on the cold metal plate and process as quickly as possible. Optimally, use within 1 h of dissection.
4. Lift the liver sample using curved forceps and rinse with 5 mL of ice-cold 1x PBS.
5. Transfer the liver to a Petri dish (Ø 100 mm) on ice using the spoon end of a spatula.
6. Cut the liver sample by hand into 0.5-1 mm thick sections using single-edge blades.
7. Carefully transfer the tissue sections to a glass bottom dish using microsurgery forceps.
8. Carefully set the liver section with forceps to flatten on the bottom.
9. Cover the sliced liver with 3-5 drops of cold 1x PBS.
NOTE: DAPI staining is recommended to identify the region of interest in certain tissues (*e.g.*, brain)[24]. To validate co-localization of the red mt-Keima signal with the lysosome, lysosomal dyes, such as dextran cascade blue and lysosensor, can be used[24].
10. Image the tissue sections under confocal microscopy via two sequential excitations[24]. Set two imaging channels: "green" mt-Keima channel (excitation 458 nm, emission 570-695 nm range) and "red" mt-Keima (excitation 561 nm, emission 570-695 nm range). Maintain imaging settings between experimental conditions. For more efficient imaging, analyze two animals at once.

Copyright © 2017 Journal of Visualized Experiments

Representative Results

Detection of Mitophagy in Human Cells:

Using the procedure presented here, human HeLa cells were transfected with mt-Keima plasmid. Healthy cells demonstrated a well-organized mitochondrial network (GFP, 488 nm) with few incidences of mitophagy (RFP, 561 nm). However, cells pretreated with a mitochondrial uncoupler FCCP (30 µM for 3 h) exhibited a profound increase in mitophagy incidence (**Figure 1A**). Mitophagy was also measured using the co-localization between LAMP2 and COXII (**Figure 1B**).

Detection of Mitophagy in *C. Elegans*

We examined the body-wall muscle cells of transgenic nematodes expressing DsRed fused with LGG-1 and either mtRosella or DCT-1 fused with GFP under normal and mitophagy-inducing conditions such as oxidative stress. Transgenic animals were exposed to paraquat (8 mM for 2 days). Mitophagy induction is indicated by the decreased GFP/DsRed ratio of mtRosella fluorescence (**Figure 2A**). Additionally, the elevated number of co-localization events between DCT-1::GFP and DsRed::LGG-1 signals signifies mitophagy stimulation in response to oxidative stress (**Figure 2B**).

Detection of Mitophagy Using the mt-Keima Mice:

Imaging analysis of *in vivo* mitophagy provides insight into mitophagy in normal and pathophysiological conditions. Using the protocol described above, mitophagy occurrence in different organ tissues, such as liver and cerebellum (**Figure 3**), can be visualized with confocal mitophagy.

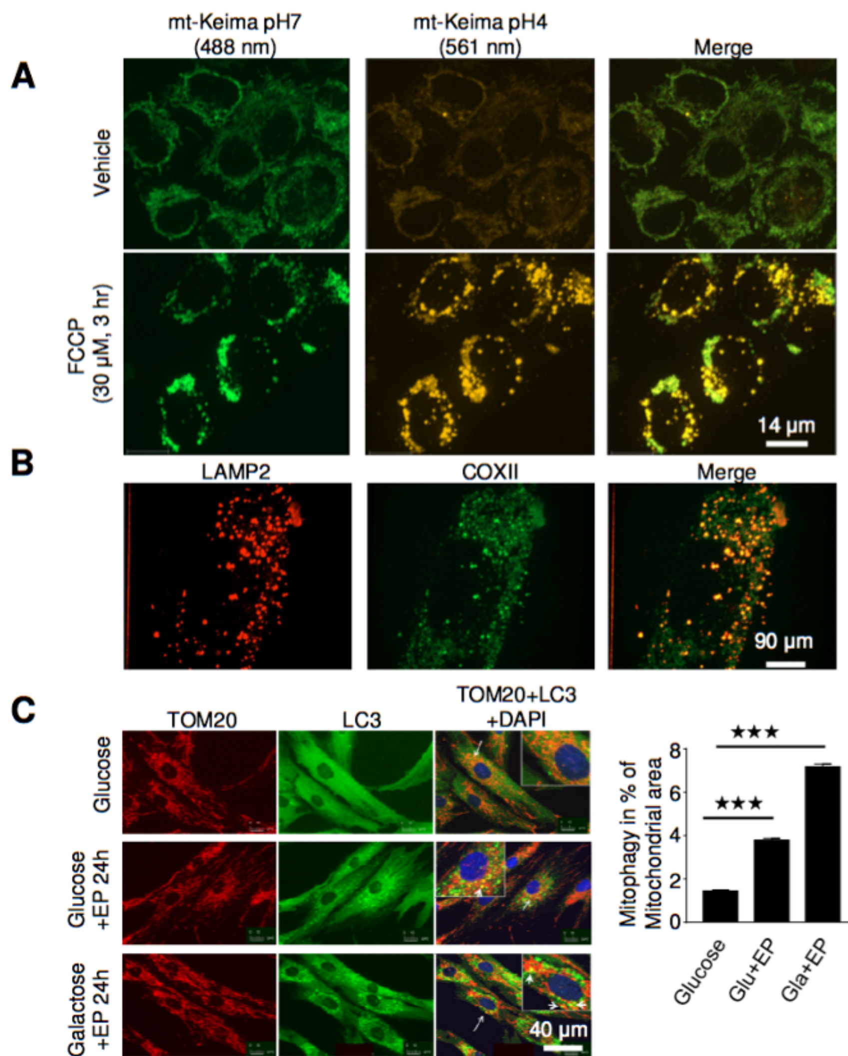

Figure 1. Different methods to evaluate mitophagy in human cells. (**A**) Human HeLa cells were transfected with mt-Keima plasmid for three days, followed by imaging at both 488 nm and 561 nm. As a positive control, ceflls were treated with FCCP (30 µM) 3 h prior to imaging. (**B**) Images of human primary fibroblasts stained with LAMP2 (RFP) and COXII (GFP). (**C**) Images of human fibroblasts stained with anti-TOM20 (red) and anti-LC3 (green) antibodies. The bars on the right show the readout of mitochondria co-localizing with autophagosomes. Co-localization is increased by energetic stress (glucose-free galactose media) and the addition of lysosomal inhibitors E64d and Pepstatin A (EP) for 24 h. Representative scale bars for (**A**), (**B**), and (**C**) are labeled on the last figure of each panel, independently. Quantitative data are shown in mean ± S.E.M. ***$p < 0.001$; *t*-test. Please click here to view a larger version of this figure.

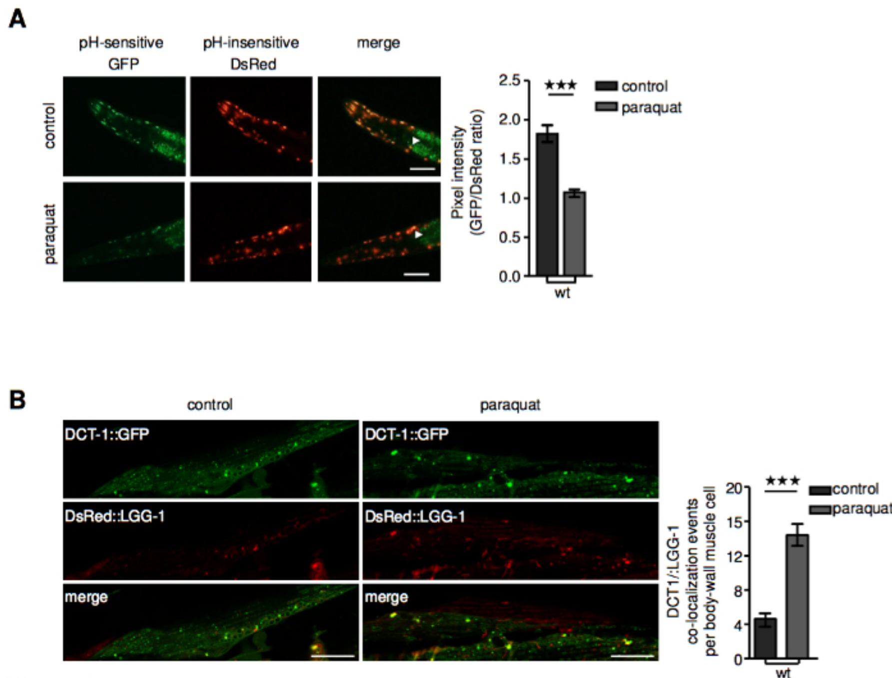

Figure 2. *In vivo* detection of mitophagy in *C. elegans*. (**A**) Transgenic nematodes expressing mtRosella in body-wall muscle cells were treated with 8 mM paraquat. Mitophagy stimulation is signified by the decreased ratio between pH-sensitive GFP to pH-insensitive DsRed (n = 50; ***$p < 0.001$; *t*-test). Arrowheads point out intestinal autofluorescence. Scale bars denote 20 µm. Acquisition details: Exposure time, 200 ms; Contrast, medium. Images were acquired using a 10X objective lens. Quantitative data are shown in mean ± S.E.M. values. (**B**) Transgenic nematodes co-expressing mitophagy receptor DCT-1 fused with GFP together with the autophagosomal protein LGG-1 fused with DsRed in body-wall muscle cells were exposed to 8 mM paraquat. Mitophagy induction is signified by co-localization of GFP and DsRed signals (for each group of images DCT-1 is shown in green on top, autophagosomes in red below, and a merged image at the bottom; n = 30; ***$p < 0.001$; *t*-test). Sale bars denote 20 µm. Acquisition details: Resolution, 1,024 X 1,024; Master gain, Track1: 562 and Track2: 730; Emission filters, Track1 Channel1: 639 nm and Track2 Channel2: 519 nm; Laser intensity, Track1 (543 nm): 12.9% and Track2 (488 nm): 39.4%. Images were acquired using a 40X objective lens. Quantitative data are shown in mean ± S.E.M. Please click here to view a larger version of this figure.

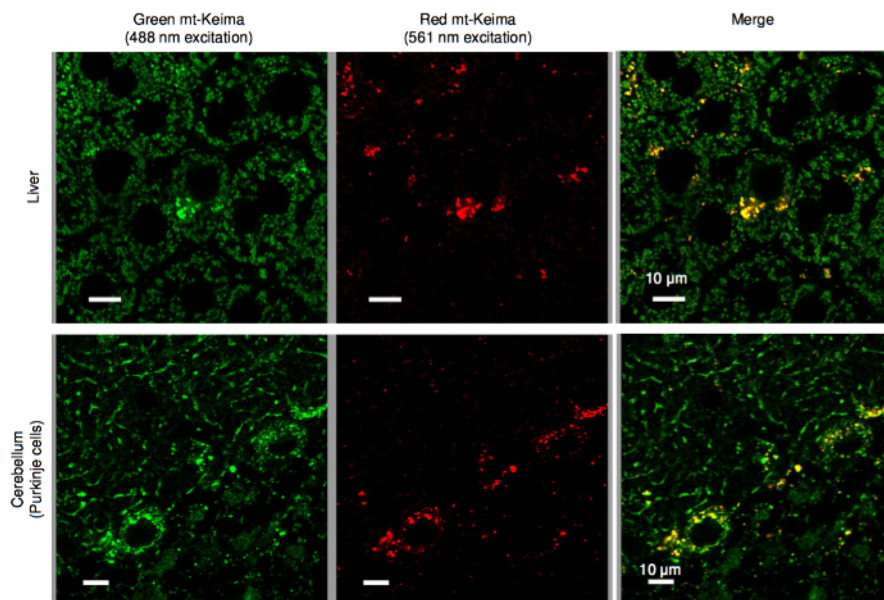

Figure 3. *In vivo* detection of mitophagy in mt-Keima transgenic mice. Representative images of mt-Keima signals in the liver (upper) and cerebellum area (including Purkinje cells, lower panel) of a mt-Keima mouse (458 nm, green; 561 nm, red). Scale bar denotes 10 µm. Please click here to view a larger version of this figure.

Discussion

Accurate measurement of mitophagy is technically demanding. Here, we have presented several robust techniques which allow for both qualitative detection of mitophagy and quantification of mitophagy levels in the most common laboratory experimental models.

To acquire replicable data, an experimental design with at least three biological repeats is necessary. All researchers involved in experimentation and analysis must be blinded to experimental group identities. Furthermore, imaging fields must be randomly chosen during image acquisition. For cell culture and *C. elegans* studies, at least three biological repeats should be performed. For mt-Keima mouse studies, it is recommended to use a sufficient number of mice to achieve statistical significance. For mammalian cells, analyze 30-100 cells for each experiment, and run at least 3 different experiments. Quality control at these steps enables replicable results. Detection of mitophagy in yeast has recently been summarized elsewhere[25].

There are several critical steps in the *in vitro* mitophagy detection techniques. Transfection efficiency, which depends on the quality of reagents and the DNA used, is vital during transfection of the mt-Keima protein. Thus, optimization of the transfection efficiency in different conditions (*e.g.*, using different cell lines) is necessary. For the LAMP2/COXII method, antibody specificity and optimal primary antibody dilution fold affect the quality of the images and should be tested before beginning experiments. The same applies to the high-content imaging method, where antibody quality and dilution are crucial. The automated microscopy system and the customized analysis protocol allows for the imaging and analysis of at least 1,500 cells/condition, which makes the results extremely robust compared to other *in vivo* imaging based methods. Additionally, the analysis protocol provides data on further mitochondrial parameters such as length and total area on a cell by cell basis, which is highly useful in mitophagy research.

In some cases, it is not recommended to assay the colocalization of LAMP2 with mitochondrial outer membrane proteins such as TOMM20. The very early stages of mitophagy involve Parkin-mediated ubiquitination of mitochondrial outer membrane proteins, including TOMM20 and Mitofusin 1 (MFN1). Parkin conjugates multiple different ubiquitin chain linkages on these proteins including K48-linked ubiquitin chains, which promote degradation of the protein by the 26S proteasome. Therefore, these ubiquitinated mitochondrial outer membrane proteins can still be degraded even though mitophagy is blocked[6]. This can skew the data, resulting in false positives in overall data interpretation. In addition, elimination of mitochondrial DNA (mtDNA nucleoids) is a second indicator of mitophagy, and it can be quantified by immunofluorescence using an anti-DNA antibody[6].

Some critical steps for monitoring *in vivo* mitophagy in *C. elegans* are listed below:

1. Transfer of transgenic animals to new plates every 24–48 h is recommended to avoid the outgrowth of progeny, which leads to a mixed population or starvation, which itself may trigger mitophagy. Therefore, non-starved nematodes should be used.

2. Levamisole is a mild anesthetic which is used to immobilize nematodes. Avoid anesthetics that could affect mitochondrial activity, such as sodium azide. Sodium azide blocks components of the mitochondrial respiratory chain and perturbs energy generation, leading to mitochondrial and oxidative stress. Thus, sodium azide is likely to trigger mitophagy.

3. Specimens should not be allowed to dry out during microscopic examination. Therefore, the use of M9 buffer instead of water is recommended to ensure favorable osmotic conditions.

4. Intestinal autofluorescence increases with age in *C. elegans*. Thus, body-wall muscle cells close to the intestine should be avoided during microscopic examination.

5. If paraquat fails to induce mitophagy: **a.** increase paraquat exposure time on worms, **b.** increase paraquat concentration, or **c.** prepare a fresh working solution of paraquat since its efficiency declines over time.

6. If increased matricidal hatching is observed in worms exposed to paraquat, either: **a.** reduce the period of paraquat treatment, **b.** reduce paraquat concentration, **c.** supplement plates with fluorodeoxyuridine (FUdR) (final concentration of 100-400 µM), an inhibitor of DNA synthesis that prevents egg hatching, or **d.** conduct the experiment in older worms (*e.g.*, 4-day-old worms).

This procedure describes the steps for imaging mitophagy in the liver using the mt-Keima transgenic mice, as shown in **Figure 3**. Tissues other than the brain and liver have been analyzed using the mt-Keima system[14]. Dual-excitation ratio imaging in liver tissues is obtained via two sequential excitations. All images must be obtained from freshly excised organs. Tissues examined should be kept cold and processed as quickly as possible. Liver slices can be obtained at any age. When imaging mitophagy, optimize laser powers and exposure times for each organ during microscopic analysis. Laser power should be set at the lowest output that allows clear visualization of the mt-Keima signal[14]. However, there are some limitations for the mt-Keima transgenic mice. Due to the instability of Keima as well as the loss of a pH gradient across the lysosomal membrane under fixation, this mouse model is not suitable for cryo-sectioning and immunohistochemistry. Another limitation is that mt-Keima, or matrix aggregates of this protein, may affect unknown mitochondrial or cellular functions, even though it does not change mitochondrial oxygen consumption rate[14]. Furthermore, the time and cost associated with mt-Keima mice render it less desirable for high-throughput analysis than the aforementioned cell culture and *C. elegans* methods. Besides the methods mentioned here, other ways to quantitatively study mitophagy in mt-Keima mice include Western blot or FACS. To note, in addition to the mt-Keima transgenic mice, there is another mitophagy mouse model, the "*mito*-QC", a transgenic mouse model with a pH-sensitive fluorescent mitochondrial signal[26]. Due to the complexity and dynamics of mitophagy, we recommend combining the aforementioned fluorescence methods with other common mitophagy detection methods, such as electron microscopy and Western blotting, to strengthen the findings.

The development of new mitophagy detection techniques such as those mentioned here will have broad applications, from mechanistic studies of mitophagy to high throughput drug screens. It should be noted that mitophagy is easily affected by both endogenous and exogenous fluctuations (*e.g.*, cell culture conditions such as cell density, starvation, hypoxia, *etc.*)[1,5,8]. Therefore, it is paramount that the same experimental conditions are maintained for all experiments. It is important to use a combination of different mitophagy detection methods in both *in vitro* and

Copyright © 2017 Journal of Visualized Experiments

in vivo studies to verify the correct interpretation of mitophagy status. Further understanding of the mechanisms of mitophagy, achieved through techniques mentioned herein, will allow for the development of new, more precise methods of mitophagy detection.

Disclosures

The Bohr laboratory has CRADA arrangements with ChromaDex and GlaxoSmithKline.

Acknowledgements

We thank Dr. Atsushi Miyawaki and Dr. Richard J. Youle for sharing the mt-Keima plasmid and mt-Keima integrated Hela cells. We thank Raghavendra A. Shamanna and Dr. Deborah L. Croteau for the critical reading of the manuscript. This research was supported by the Intramural Research Program of the NIH (VAB), National Institute on Ageing, as well as a 2014-2015 and a 2016-2017 NIA intra-laboratory grant (EFF, VAB). EFF was supported by HELSE SOR-OST RHF (Project No: 2017056) and the Research Council of Norway (Project No: 262175).

AUTHOR CONTRIBUTIONS:

EFF designed the manuscript and prepared the draft; KP, NS, EMF, RDS, JSK, SAC, YH, and ED wrote different sections of the paper; NT, JP, HN, and VAB revised the manuscript and provided expertise.

References

1. Fang, E.F.*, et al.* Nuclear DNA damage signalling to mitochondria in ageing. *Nat Rev Mol Cell Biol.* **17**(5), 308-321 (2016).
2. Scheibye-Knudsen, M., Fang, E.F., Croteau, D.L., Wilson, D.M., 3rd, Bohr, V.A. Protecting the mitochondrial powerhouse. *Trends Cell Biol.* **25** (3), 158-170 (2015).
3. Sun, N., Youle, R.J., Finkel, T. The Mitochondrial Basis of Aging. *Mol Cell.* **61** (5), 654-666 (2016).
4. Shadel, G.S., Horvath, T.L. Mitochondrial ROS signaling in organismal homeostasis. *Cell.* **163** (3), 560-569 (2015).
5. Palikaras, K., Lionaki, E., Tavernarakis, N. Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature.* **521** (7553), 525-528 (2015).
6. Lazarou, M.*, et al.* The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature.* **524** (7565), 309-314 (2015).
7. Kerr, J.S.*, et al.* Mitophagy and Alzheimer's Disease: Cellular and Molecular Mechanisms. *Trends neurosci.* **40** (3), 151-166 (2017).
8. Fivenson, E.M.*, et al.* Mitophagy in neurodegeneration and aging. *Neurochem Int.* (2017).
9. Menzies, F.M., Fleming, A., Rubinsztein, D.C. Compromised autophagy and neurodegenerative diseases. *Nat Rev Neurosci.* **16** (6), 345-357 (2015).
10. Rubinsztein, D.C., Shpilka, T., Elazar, Z. Mechanisms of autophagosome biogenesis. *Curr Biol.* **22** (1), R29-34 (2012).
11. Kerr, J.S.*, et al.* Mitophagy and Alzheimer's disease: cellular and molecular mechanisms. *Trends neurosci.* **40** (3), 151-166 (2017).
12. Menzies, F.M., Moreau, K., Puri, C., Renna, M., Rubinsztein, D.C. Measurement of autophagic activity in mammalian cells. *Curr Protoc Cell Biol.* **Chapter 15**, Unit 15. 16 (2012).
13. Katayama, H., Kogure, T., Mizushima, N., Yoshimori, T., Miyawaki, A. A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem Biol.* **18** (8), 1042-1052 (2011).
14. Sun, N.*, et al.* Measuring In Vivo Mitophagy. *Mol Cell.* **60** (4), 685-696 (2015).
15. Fang, E.F.*, et al.* Defective mitophagy in XPA via PARP-1 hyperactivation and NAD(+)/SIRT1 reduction. *Cell.* **157** (4), 882-896 (2014).
16. Diot, A.*, et al.* A novel quantitative assay of mitophagy: Combining high content fluorescence microscopy and mitochondrial DNA load to quantify mitophagy and identify novel pharmacological tools against pathogenic heteroplasmic mtDNA. *Pharmacol Res.* **100**, 24-35 (2015).
17. Schiavi, A.*, et al.* Iron-Starvation-Induced Mitophagy Mediates Lifespan Extension upon Mitochondrial Stress in C. elegans. *Curr Biol.* **25** (14), 1810-1822 (2015).
18. Rosado, C.J., Mijaljica, D., Hatzinisiriou, I., Prescott, M., Devenish, R.J. Rosella: a fluorescent pH-biosensor for reporting vacuolar turnover of cytosol and organelles in yeast. *Autophagy.* **4** (2), 205-213, (2008).
19. Sargsyan, A.*, et al.* Rapid parallel measurements of macroautophagy and mitophagy in mammalian cells using a single fluorescent biosensor. *Sci Rep.* **5**, 12397 (2015).
20. Altun, Z.F., Hall, D.H. Introduction to C. elegans anatomy. in *In WormAtlas.* (2009).
21. Chaudhuri, J., Parihar, M., Pires-daSilva, A. An introduction to worm lab: from culturing worms to mutagenesis. *J Vis Exp.* (47), e2293 (2011).
22. Palikaras, K., Tavernarakis, N. In vivo Mitophagy Monitoring in Caenorhabditis elegans to Determine Mitochondrial Homeostasis. *Bio Protoc.* **7** (7), e2215 (2017).
23. Palikaras, K., Tavernarakis, N. Assessing Mitochondrial Selective Autophagy in the Nematode Caenorhabditis elegans. *Methods Mol Biol.* **1567**, 349-361 (2017).
24. Sun, N., et al. A fluorescence-based imaging method to measure in vitro and in vivo mitophagy using mt-Keima. *Nat Protoc.* **12** (8), 1576-1587 (2017).
25. Eiyama, A., Okamoto, K. Assays for Mitophagy in Yeast. *Methods Mol Biol.* **1567**, 337-347 (2017).
26. McWilliams, T.G., et al. mito-QC illuminates mitophagy and mitochondrial architecture in vivo. *J Cell Biol.* **214** (3), 333-345 (2016).

Copyright © 2017 Journal of Visualized Experiments