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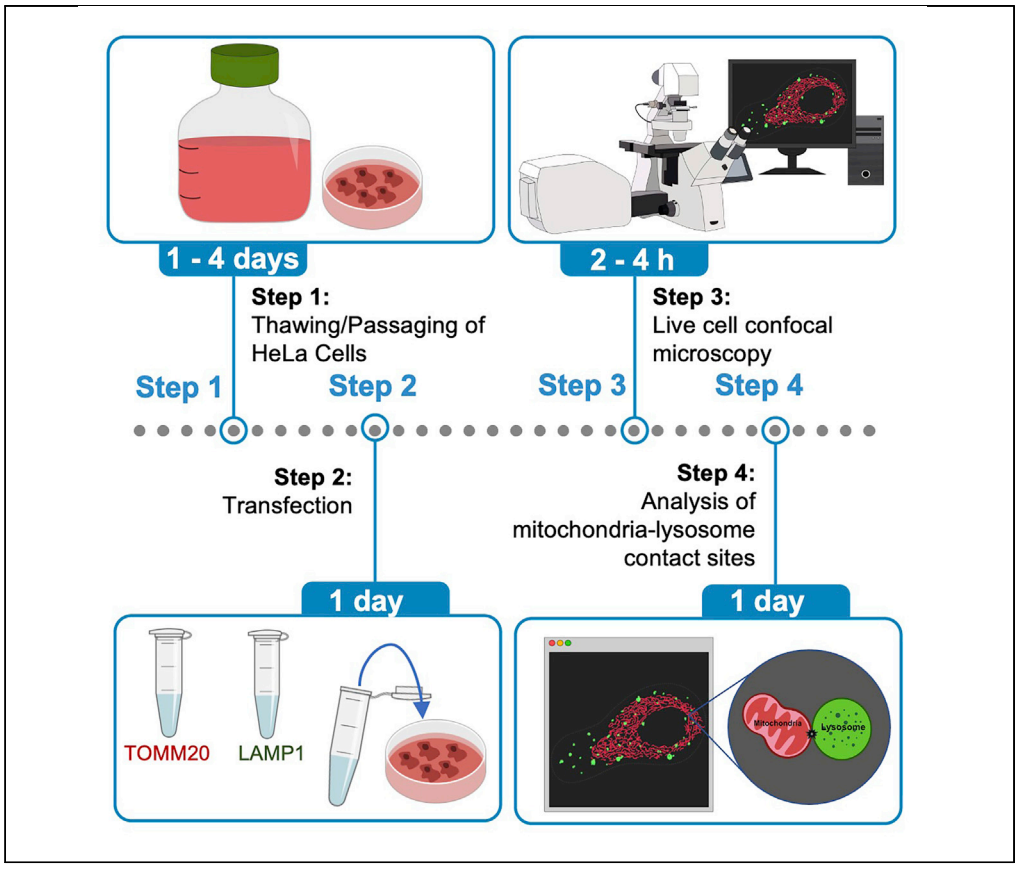
Live cell microscopy of mitochondria-lysosome contact site formation and tethering dynamics



Mitochondria-lysosome contact sites are critical for maintaining cellular homeostasis by regulating mitochondrial and lysosomal network dynamics and mediating metabolite exchange. Here, we present a protocol to quantitatively analyze the formation and tethering duration of mitochondria-lysosome contact sites by using time-lapse live confocal microscopy of LAMP1 and TOMM20. Although this protocol focuses on mammalian HeLa cells, it can be applied to other cell types for further studies on mitochondria-lysosome contact regulation and function, and elucidation of their role in human disorders.

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Highlights

Visualization of mitochondrial and lysosomal dynamics by confocal microscopy

Imaging of mitochondria-lysosome contact sites in live HeLa cells

Quantification of contact site dynamics using TOMM20/LAMP1

Analysis of contact site tethering duration over time

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Live cell microscopy of mitochondria-lysosome contact site formation and tethering dynamics

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SUMMARY

Mitochondria-lysosome contact sites are critical for maintaining cellular homeostasis by regulating mitochondrial and lysosomal network dynamics and mediating metabolite exchange. Here, we present a protocol to quantitatively analyze the formation and tethering duration of mitochondria-lysosome contact sites by using time-lapse live confocal microscopy of LAMP1 and TOMM20. Although this protocol focuses on mammalian HeLa cells, it can be applied to other cell types for further studies on mitochondria-lysosome contact regulation and function, and elucidation of their role in human disorders.

For complete details on the use and execution of this protocol, please refer to Wong et al. (2018) and Wong et al. (2019b).

BEFORE YOU BEGIN

Cellular homeostasis depends on the proper regulation of both mitochondria and lysosomal function and dynamics over time. Mitochondria-lysosome contact sites were recently identified as a dynamic pathway for maintaining organelle homeostasis in multiple cell types, and are implicated in various human diseases (Kim et al., 2021; Peng et al., 2020; Wong et al., 2018, 2019b). These contacts serve as a pathway for crosstalk between both organelles through the bidirectional regulation of mitochondrial and lysosomal network dynamics, and metabolite transfer such as the exchange of calcium, iron, cholesterol, and other lipids (Wong et al., 2019a). As a result of the crucial role these sites play in organelle dynamics and maintaining normal metabolite composition within the cell, many human diseases have recently been linked to dysfunction in the formation and regulation of mitochondria-lysosome contacts (Cisneros et al., 2022). Consequently, investigating mitochondria-lysosome contact site dynamics may provide important insights into cellular function as well as defects occurring in disease.

In this protocol, we establish a method for visualizing and analyzing mitochondria-lysosome contact sites within HeLa cells in real time. Importantly, high spatial and temporal resolution live confocal imaging of the lysosomal membrane and the outer mitochondrial membrane permits the analysis of mitochondria-lysosome contact sites in living cells, and allows for further investigation into how they influence cellular homeostasis. Here, we describe the culturing of HeLa cells to provide viable cells for transfection and live imaging, and subsequent live cell confocal microscopy and analysis of mitochondria-lysosome contact formation and tethering dynamics. Together, this protocol provides a reliable, quantitative method for analyzing mitochondria-lysosome contact site dynamics in living cells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Lamp1-mGFP	(Falcon-Perez et al., 2005)	Addgene Plasmid # 34831
mApple-TOMM20	Michael Davidson	Addgene Plasmid # 54955
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium (DMEM), high glucose, pyruvate	Gibco™	Catalog # 11995-065
Penicillin-Streptomycin (10,000 U/mL)	Gibco™	Catalog # 15140122
Fetal Bovine Serum, qualified, heat inactivated, US origin	Gibco™	Catalog # 16140071
Dimethyl sulfoxide	Sigma-Aldrich	Catalog # D2650-100 mL
TrypLE™ Express Enzyme (1×), phenol red	Gibco™	Catalog # 12605-010
PBS, pH 7.4	Gibco™	Catalog # 10010-049
Opti-MEM™ I Reduced Serum Medium	Gibco™	Catalog # 31-985-070
Lipofectamine® 2000 Transfection Reagent	Invitrogen™	Catalog # 11-668-019
Experimental models: Cell lines		
HeLa Cells	ATCC	ATCC # CCL-2
Software and algorithms		
NIS-Elements	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer
Image J	National Institutes of Health (NIH)	https://imagej.nih.gov/ij/
Other		
Corning® 100 mm TC-treated Culture Dish	Corning	Catalog # 430167
35 mm glass bottom dish (No. 1.5 Coverslip | 14 mm Glass Diameter | Uncoated)	MatTek	Catalog # P35G-1.5-14-C
Nikon A1R laser scanning confocal microscope with GaAsp detectors and Plan Apo λ 100× 1.45 NA oil immersion objective	Nikon	N/A
Immersion oil Immersol 518 F for 30°C fluorescence free, oiler 20 mL	Zeiss	Item # 444970-9000-000
Lens Cleaning Spray	Zeiss	N/A
Lens Cleaning Paper (300 sheets/box)	Zeiss	Item # 462975-0000-000

MATERIALS AND EQUIPMENT

Preparation of HeLa Media		
Reagent	Final concentration	Amount
DMEM	n/a	445 mL
Penicillin-Streptomycin	1%	5 mL
FBS, heat inactivated	10%	50 mL
Total	**n/a**	**500 mL**

Note: HeLa media can be prepared prior to passaging and stored for weeks at 4°C. Media should be warmed to 37°C prior to use.

STEP-BY-STEP METHOD DETAILS

Thawing HeLa cells

Timing: 1 h

The first phase of the protocol describes how to prepare HeLa cell cultures for subsequent passaging, transfection and imaging.

1. Warm 9 mL of HeLa media in 50 mL conical tube in 37°C bath.
2. Take out frozen vial of HeLa cells (1 mL) stored in liquid nitrogen tank.
   a. Hold and thaw vial in 37°C bath (for ~1 min).

   Alternatives: Alternative mammalian cell lines can also be used to visualize and analyze mitochondria-lysosome contact dynamics such as HEK293 and HCT116 cells (Wong et al., 2018).

3. Immediately add thawed HeLa cells to 9 mL of warm HeLa media.
4. Spin down cells at 200 × *g* for 3 min in order to remove any DMSO from the media.

   ⚠ CRITICAL: In order to avoid contamination, all manipulation of HeLa cells and preparation of solutions should be performed in a tissue culture hood. Cell vials, cell culture plates and all solutions should never be opened outside the sterilized hood. Sterilize the inside of the tissue culture hood with 70% EtOH to ensure adequate sterilization of the work area before using. Spray hands and all materials that are brought into the hood with 70% EtOH before each experiment.

5. Aspirate off supernatant and resuspend pellet. First, resuspend in 5 mL of HeLa media and once mixture appears homogeneous, resuspend cells again into a final total volume of 20 mL of HeLa media.
6. Plate HeLa cells into two 10 cm plates (10 mL of media per plate) and grow cells in an incubator at 37°C for 2–3 days.

Passaging of HeLa cells

Timing: 1 h

⚠ CRITICAL: Warm HeLa media for at least an hour prior to passaging to keep HeLa cells at a near optimal temperature during passaging. Before passaging, it is important to also examine cells under a cell culture microscope to ensure normal cell morphology, optimal confluency (80%–100%), and the absence of contamination.

7. From a 10 cm dish of confluent HeLa cells (80%–100%), aspirate off HeLa media with a glass Pasteur pipet attached to the vacuum.
8. Wash cells with 5 mL of sterile PBS (use at 20°C) and aspirate. Then add 2 mL of TrypLE Express (stored at 4°C, use at 20°C) to detach cells from the 10 cm cell plate.
9. Place cells in TrypLE Express in a CO_2 incubator for 3 min to ensure complete detachment of cells from the plate's surface.

   Note: Depending on the type of mammalian cells used in the protocol, the incubation time of cells with TrypLE Express in the CO_2 incubator can be extended to increase detachment of cells.

10. Add 5 mL of HeLa media to the plate containing TrypLE Express and resuspend media in the plate several times while rotating the cell dish to remove remaining cells from the plate.
11. Once the majority of cells have detached, collect resuspended cells (~7 mL total) into a 50 mL conical tube, and centrifuge cells at 200 × *g* at 20°C for 3 min.
12. Aspirate off media and TrypLE Express. Immediately add 5 mL of HeLa media to the same conical tube and resuspend.

⚠ CRITICAL: Do not disturb the cell pellet. Tilting the conical tube while aspirating helps avoid this.

13. Once the mixture appears homogeneous, dilute cell suspension into a final volume of 40 mL of HeLa media.
14. Add resuspended HeLa cells to 10 cm dishes for maintaining cell culture, and to 35 mm MatTek dishes for live cell microscopy:
    a. For 10 cm dish: Add 10 mL media/dish (approximately 2.2×10^6 cells/10 mL).
    b. For 35 mm MatTek dishes: Add 1.5 mL media/dish (approximately 0.3×10^6 cells/1.5 mL).
15. Cells should be grown until ~70% confluency before continuing to the next step.

Pause point: Incubate cells at 37°C and allow growth for at least 24 h prior to transfection.

⚠ CRITICAL: Avoid introducing bubbles into the resuspended media as well as cell dishes to avoid contamination. In addition, excessive movement of cell plates after initial passaging should be avoided to mitigate unnecessary clumping of cells.

Note: One confluent 10 cm dish of HeLa cells can be split into two to four 10 cm dishes during passaging, which will become confluent after 2–3 days depending on the rate of cell growth.

Transfection of HeLa cells for live cell imaging

Timing: 45 min

This step describes how to transfect plasmids into HeLa cells for visualization of mitochondria and lysosomes by confocal microscopy. Cells will express Lamp1-mGFP to label the lysosomal membrane in green, and mApple-TOMM20 to label the outer mitochondrial membrane in red.

⚠ CRITICAL: Cells should be examined under a cell culture microscope prior to transfection to check for confluency. Transfection reagents can be cytotoxic, and transfection of dishes with <60% confluency may result in decreased transfection efficiency and/or cell viability.

16. Mix 100 µL Optimem and 0.5 µg DNA of each plasmid (Lamp1-mGFP and mApple-TOMM20) in a sterile Eppendorf tube. Resuspend 5–6 times to ensure proper mixing.

    Alternatives: The outer mitochondrial membrane can also be labeled in red using mCherry-TOMM20-N-10 (Addgene Plasmid #55146) or in green using mEmerald-TOMM20-C-10 (Addgene Plasmid #54281). The lysosomal membrane can also be labeled in green using Emerald-Lysosomes-20 (Addgene Plasmid #56476) or in red using mApple-Lysosomes-20 (Addgene Plasmid #54921) Mitochondrial and lysosomal markers used must have different emission/excitation spectra to differentiate each marker during acquisition.

17. In a separate sterile Eppendorf tube, mix 100 µL Optimem and 3 µL Lipofectamine 2000 (use 1.5 µL/plasmid). Resuspend 5–6 times to ensure proper mixing.

    Alternatives: Mammalian cells can also be efficiently transfected using FuGENE® 6 Transfection Reagent (Promega) instead of Lipofectamine 2000.

18. Mix each tube individually and wait 5 min. Combine the solutions from both Eppendorf tubes and wait 20 min. Resuspend 5–6 times to ensure proper mixing.
19. Add the combined solution to cells plated in a 35 mm dish for live cell microscopy.
20. Cells should be transfected for 24–32 h prior to imaging, to ensure efficient expression of plasmids.

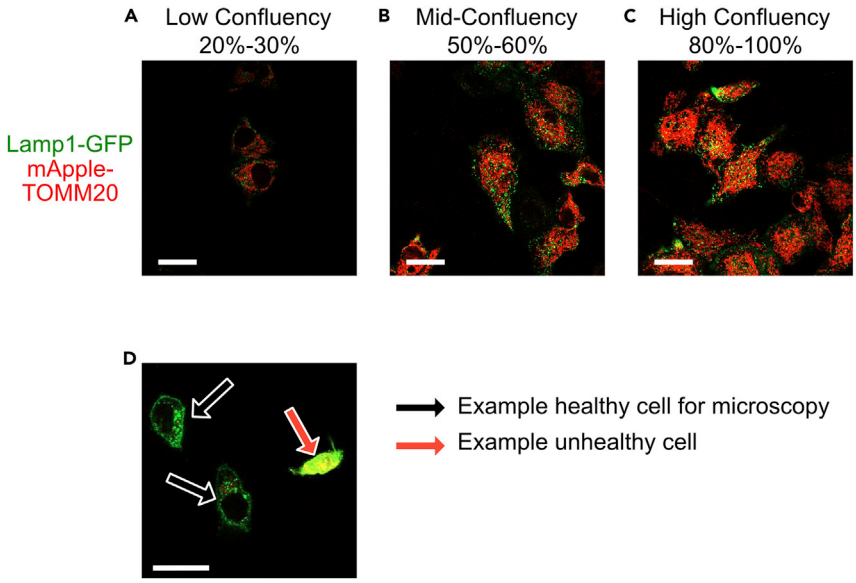

Figure 1. Representative images of appropriate cell confluency and health for live imaging of HeLa cells

(A–C) Live HeLa cells were transfected with LAMP1-mGFP (lysosomal membrane, green) and mApple-TOMM20 (outer mitochondrial membrane, red) and imaged by live cell confocal microscopy. These representative images demonstrate the variability in cell confluency (from low to high) that can be obtained during imaging.

(D) Representative image of live HeLa cells transfected with LAMP1-mGFP (lysosomal membrane, green) and mApple-TOMM20 (outer mitochondrial membrane, red). Black arrows indicate a good, healthy cell that is ideal for microscopy analysis whereas the red arrow indicates an unhealthy cell that should not be used during analysis.

Scale bars, 10 μm (A–D).

Pause point: Live cell plates must be kept at 37°C in an incubator for at least 24 h before proceeding to live cell imaging, and cell confluency should be ~90% at the point of imaging. It is also important to ensure that cells are properly transfected by evaluating the percent of cells expressing fluorescent protein which should be ~60%–80% of adherent cells (Figures 1A–1C) (see troubleshooting 1, 2, and 3).

Live cell confocal microscopy of mitochondria-lysosome contact sites

Timing: 40 min (per live cell plate)

This protocol describes how to obtain high resolution live cell confocal movies of mitochondria-lysosome contacts in HeLa cells using a Nikon A1R laser scanning confocal microscope with a 100× objective.

⚠ CRITICAL: To avoid contamination, sterilize the work area by spraying the microscope buttons and desk area with 70% EtOH. Prior to imaging, examine cells under a cell culture microscope to check for cell health and general morphology.

21. To ensure the optimal environment for cell health during imaging, the microscope CO_2 levels should be set to 5% CO_2, and the heater set to 37°C for at least 30 min before imaging to allow for the conditions to stabilize.
22. Turn on the Nikon A1R laser scanning confocal microscope (with GaAsP PMT detector and a Nikon Plan Apo λ 100× 1.45 NA oil immersion objective). Imaging will be conducted using the microscope's software (NIS-Elements (Nikon)).

Alternatives: Mitochondria-lysosome contact sites can also be imaged using a Zeiss LSM 980 confocal microscope with GaAsp detectors using an α Plan-Apochromat 100× 1.46 Oil DIC

immersion objective (Zeiss) with Zen Blue (Zeiss). It is also possible to perform the experiment with a 63× oil objective with increased zoom factor, but organelles will be visualized at a lower resolution than with a 100× oil objective.

⚠ CRITICAL: Ensure sufficient amount of Immersol immersion oil is added to the objective prior to adding cell plate. As initial changes in oil temperature may affect the z-plane of imaging, ensure that the final imaging plane allows for mitochondria and lysosomes to be clearly visualized in live cells.

23. Carefully place the live cell imaging plate into the heated microscope chamber.
24. Turn on the 488 nm laser and adjust objective depth until the fluorescent plane is visible through the eyepiece.

⚠ CRITICAL: Remember to turn off the laser immediately after finding cells of interest in order to limit light dose on sample. To mitigate this issue, it is helpful to use the channel with the most photostable fluorophore when looking for cells for prolonged periods of time (see troubleshooting 4).

25. Once the correct focal plane is found, search for 10–15 viable cells to be used for live cell confocal movies. Cells will be acquired from a single z-plane (see troubleshooting 5).

⚠ CRITICAL: Avoid imaging cells that are not adherent to the plate, or that produce a high level of autofluorescence (Figure 1D). These cells are likely floating dead cells and will not produce useful or reliable information on the behavior of mitochondria-lysosome contacts (see troubleshooting 2). Also avoid imaging cells with excessively high levels of fluorescent plasmid expression (see troubleshooting 6).

26. Analyze previously selected cells and first take a confocal image of each cell (Figure 2A).
27. For each cell, acquire a laser scanning confocal time-lapse movie (Figure 2B), visualizing lysosomes (Lamp1-mGFP) and mitochondria (mApple-TOMM20) with Green 488 nm and Red 561 nm lasers respectively.
    a. Obtain movies at a minimum rate of 1 frame/2 s for 3 min to visualize mitochondria-lysosome contact site formation and dynamics.
    b. Use the following recommended settings:
        i. Green 488 nm: Excitation wavelength: 488 nm, emission wavelength: 525 nm, laser power: 3.0, PMT HV (gain): 25, PMT offset: 0.
        ii. Red 561 nm: Excitation wavelength: 561 nm, emission wavelength: 595 nm, laser power: 3.0, PMT HV (gain): 15, PMT offset: 0.

⚠ CRITICAL: While imaging, it is important that the laser intensity, gain, and offset setting are standardized across cells and between plates to ensure consistency during analysis.

Note: Mitochondria-lysosome contact sites are defined as sites at which mitochondria and lysosome remain stably tethered to one another for at least 10 s (Wong et al., 2018).

⚠ CRITICAL: Live cell imaging plates should not be imaged for more than 1 h per plate. Longer imaging may lead to unwanted phototoxicity-induced events and photobleaching (see troubleshooting 4).

28. Save all confocal images and time-lapse movies acquired.
29. Carefully remove the live cell imaging plate from the microscope chamber.
30. Turn off $C0_2$, microscope heater, and the Nikon A1R laser scanning confocal microscope.

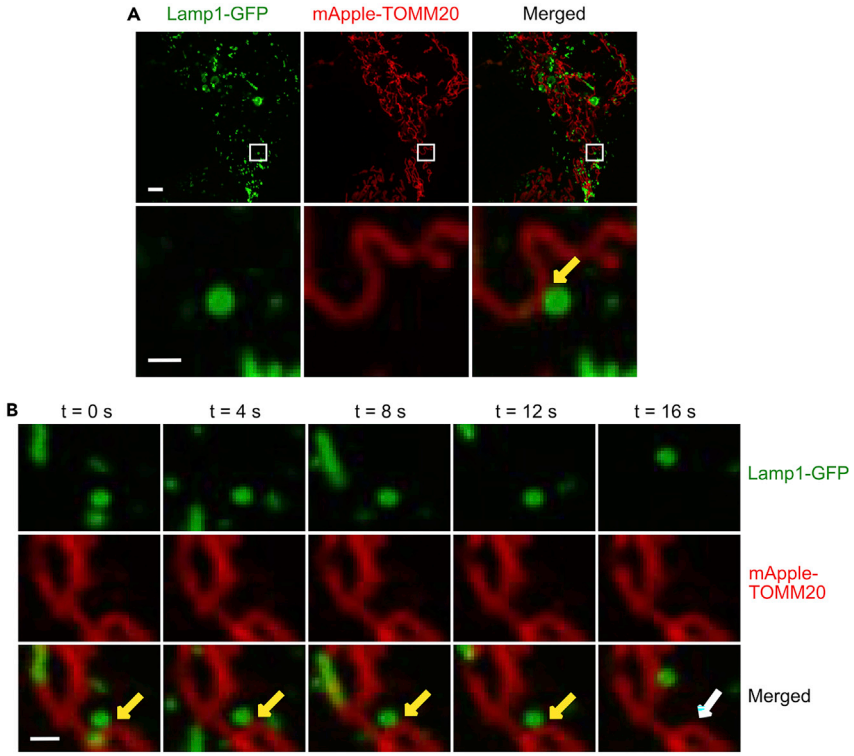

Figure 2. Representative live cell confocal microscopy images of mitochondria-lysosome contacts in live HeLa cells

(A) Live HeLa cells were transfected with LAMP1-mGFP (lysosomal membrane, green) and mApple-TOMM20 (outer mitochondrial membrane, red) and imaged by live cell confocal microscopy as shown in the top panel. The inset (bottom) demonstrates a mitochondria-lysosome contact site (yellow arrow). These representative images show the level of contrast, intensity, and background that should appear in each of the fluorescent channels during imaging of mitochondria and lysosomes.

(B) Representative time-lapse images from live HeLa cells transfected with LAMP1-mGFP (lysosomal membrane, green) and mApple-TOMM20 (outer mitochondrial membrane, red), demonstrating a dynamic mitochondria-lysosome contact site (yellow arrows) that remains tethered for 12 s before subsequently untethering (white arrow). Scale bars, 5 μm (A, top); 1 μm (A, bottom); 1 μm (B).

Alternatives: Contact sites can also be visualized in fixed mammalian cells using immunofluorescent staining of endogenous LAMP1 and TOMM20 to label lysosomal and mitochondrial membranes respectively (ex. LAMP1 rabbit antibody (Sigma-Aldrich, L1418); TOMM20 mouse antibody (BD biosciences, 612278)), followed by confocal microscopy with a 100× oil immersion objective (Wong et al., 2018).

EXPECTED OUTCOMES

Prior to imaging, HeLa media in live cell plate appears clear without an excess of floating cells. At time of imaging, cell confluency is expected to be 80%–100%, with normal morphology (Figures 1A–1C). During confocal imaging, cells have normal morphology and demonstrate bright fluorescently labeled mitochondria and lysosomes which are clearly distinguishable from any autofluorescence, background fluorescence, or noise (Figure 1D). This indicates appropriate transfection efficiency and fluorophore localization. However, some cells may have an abnormally high amount of autofluorescence with indistinguishable organelle structures. These cells are likely dead, containing static mitochondria and lysosomes, and should not be included in the analysis (Figure 1D). Mitochondria and lysosomes within healthy properly transfected cells are highly mobile and visibly form mitochondria-lysosome contact sites that remain stably tethered over time (~60 s on average) before untethering from each other, over the course of the movie. Time-lapse acquisitions should be

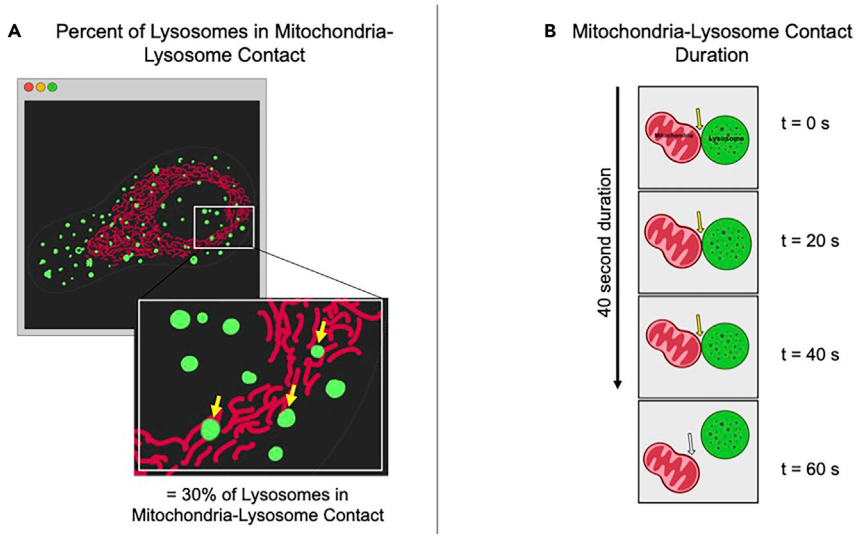

Figure 3. Analysis of mitochondria-lysosome contact site formation and tethering dynamics

(A) Example analysis of mitochondria-lysosome contact site formation, measured as the percent of lysosomes in contact with a mitochondria in a given frame, such that all contacts last at least 10 s. In the example, 3 lysosomes are in contact with mitochondria (yellow arrows) out of 10 lysosomes in the region of interest, resulting in a final quantification of 30% lysosomes tethered in mitochondria-lysosome contacts.

(B) Example analysis of mitochondria-lysosome contact tethering duration, measured as the minimum duration of mitochondria and lysosomes in contact with one another before untethering. Mitochondria and lysosome should appear directly beside each other throughout the duration of contact tethering. Once the two organelles separate from each other (distance between organelles is >0.1 μm), the mitochondria and lysosome are no longer considered to be in contact. In the example, the mitochondria-lysosome contact remains tethered for a 40 s duration.

free of significant photobleaching for at least 3 min to ensure appropriate subsequent analysis of contact sites.

QUANTIFICATION AND STATISTICAL ANALYSIS

The number of mitochondria-lysosome contact sites can be determined using Image J (with Bio-Formats plugin) or NIS-Elements (Nikon) software. Mitochondria-lysosome contact sites are defined as sites at which mitochondria and lysosome remain stably tethered to one another for at least 10 s (Wong et al., 2018).

1. Download and open the confocal microscopy movies (the .ND2 file format for Nikon is used in this procedure) by drag and drop into Image J.
2. In the Bio-Formats import options screen:
   a. In the "Stack viewing" section, select to view stack with "Data Browser"
   b. In the "Color options" section, select the color mode "Composite"
   c. Uncheck all other boxes.
3. Select the number of series to view.
4. Once the movie window appears, use the frame number in the top left and the xy location provided in the toolbar of ImageJ to analyze mitochondria-lysosome contact dynamics.
   a. Analysis of the percentage of lysosomes in mitochondria-lysosome contact (Figure 3A):
      i. Define a region of interest in the cell which contains at least 10 lysosomes. If possible, the entire cell can be analyzed rather than a specific region of interest.
      ii. Count the total number of lysosomes in that region at frame 1 (t=0 s).
      iii. Identify which lysosomes are dynamically tethered to mitochondria (within 0.1 μm of one another throughout the duration of contact) in a mitochondria-lysosome contact at frame 1 (t=0 s) and which remain tethered together for >10 s. Thus, for each contact visualized at

t=0 s, confirm that the contact remains tethered for at least 10 s (i.e., still remains in contact at t=10 s) (Figure 3B).

iv. The percentage of lysosomes in mitochondria-lysosome contacts is calculated as the number of lysosomes in mitochondria-lysosome contact divided by the total number of lysosomes in the region of interest.

b. Analysis of mitochondria-lysosome contact duration (Figure 3B):
   i. Identify a mitochondria-lysosome contact that has already tethered at frame 1 (t=0 s).
   ii. For each contact visualized at t=0 s, confirm that the contact remains tethered for at least 10 s (i.e., still remains in contact at t=10 s).
   iii. Record the x,y location at t=0 s of the lysosome in the mitochondria-lysosome contact.
   iv. Track the mitochondria-lysosome contact throughout the movie until the mitochondria and lysosome clearly visually untether from one another (distance between organelles is >0.1 μm).
   v. Record the time of the last frame that the mitochondria-lysosome contact remains tethered before undergoing an untethering event. This is defined as the mitochondria-lysosome contact minimum duration.

Note: Users should be blind to experimental conditions during image analysis when possible, in order to minimize bias and subjectivity during analysis of mitochondria-lysosome contact formation and tethering dynamics. Additional super-resolution microscopy imaging techniques may further help to identify and validate the formation and tethering of membrane contact sites between mitochondria and lysosomes (see troubleshooting 7).

LIMITATIONS

Lysosomes are highly mobile in healthy cells and can cross paths with other lysosomes during the movie. This may make it difficult to clearly track the movement and location of one lysosome and its contact tethering dynamics throughout the movie. If the lysosome being analyzed significantly overlaps with other cellular components, it is recommended to exclude it from the analysis and to track another lysosome instead. It is also advisable to take note of the average lysosome size within the cell type used to aid in distinguishing between one or multiple lysosomes. For the cell type used in this experiment, lysosomes had an average diameter of 0.3–0.6 μm. Any lysosomes which were grouped together in clusters significantly larger than this diameter were not considered as a single lysosome. In addition, while the cells used in this protocol are HeLa cells, other cell types can be used for analysis with alternative plasmids and conditions of interests, depending on the research question. Experimental results may also depend on cell health and size. Thus, it may be challenging to find contact sites or obtain a representative measure of their contact percentage and duration if cells are unhealthy or at low confluency at the time of imaging.

TROUBLESHOOTING

Problem 1

Difficulty in finding cells during live cell imaging.

When cells are not grown at a high confluency or appear unhealthy, it can be difficult to find healthy cells for confocal microscopy imaging (see Protocol step 20).

Potential solution

Prior to confocal imaging, it is important to ensure that the live cell plates are at an appropriate confluency. If cells remain at a low confluency even after passaging, it is possible that a new cell batch may be required in order to address this issue.

Problem 2

Live cell plates with high degree of floating cells.

High rates of cell death in live cell plates can result in a large amount of floating dead cells within the live cell plate. It is important that this is avoided because it will significantly decrease the ability to find large, unobstructed, healthy cells. Large numbers of dead cells may also increase fluorescent background (see Protocol steps 20 and 25).

Potential solution

Incomplete resuspension, vigorous handling, or contamination may all result in decreased cell viability. For this reason, gentle and sterile handling of cells is essential during cell culture, as well as thorough resuspension and distribution. If these problems persist after re-passaging, it may be helpful to thaw a new batch of cells.

Problem 3

Dim or absent fluorescent signal.

If the transfection efficiency is not sufficient or too little time has passed since transfection, it is possible that the fluorescent signal may appear dim or be completely absent (see Protocol step 20).

Potential solution

It is important to allow at least 24 h (but not more than 36) to pass between transfection and imaging to ensure high levels of fluorophore expression. It is also critical to occasionally check the concentration of the plasmids used during transfection to ensure that an adequate amount of DNA is being used for transfection.

Problem 4

Fluorescence bleaching during live cell imaging.

Depending on the fluorophores used and the desired duration of imaging, photobleaching may occur with increasing light dose (see Protocol steps 24 and 27).

Potential solution

To limit light exposure on your sample, adjust the laser power to the minimum point at which a clear signal can still be observed. Generally this occupies half of the available gray levels in an image. It may also be helpful to work quickly and diligently while locating cells for acquisition in order to avoid unnecessary bleaching. It is also helpful to use the channel with the slowest bleaching fluorophore when searching for cells to decrease photobleaching prior to time-lapse acquisitions.

Problem 5

High background fluorescence.

High levels of background fluorescence may be a result of low fluorescent protein expression (resulting in a low signal to noise ratio) or high cell density while imaging (see Protocol step 25).

Potential solution

First confirm the appropriate expression levels of the chosen fluorescent proteins. If the expression is consistent with successful image acquisition, check the cell confluence with a low magnification objective. If cell confluency is >95%, consider repeating this protocol plating at an initial lower cell density. Additionally, imaging in Fluorobrite™ DMEM may also help limit background fluorescence for sparse fluorophores.

Problem 6

High levels of fluorophore clustering.

Many fluorescent proteins may form oligomers when expression levels are too high. Regions of highly clustered and unusually bright signal may represent oligomerization which may affect downstream analysis (see Protocol step 25).

Potential solution

Perform a new imaging experiment with conditions that include different plasmid dilutions during transfection. Alternatively, it is also possible to examine expression levels of the chosen fluorescent proteins by immunoblot analysis to determine if levels are significantly higher (ex. more than two times) than the endogenous levels of that protein. If expression is too high, repeat the protocol with a lower amount of plasmid DNA. If the expression is appropriate but clustering is still observed, repeat the protocol with alternative fluorescent fusion proteins which are monomeric and do not form dimers or oligomers.

Problem 7

Validation of membrane contact sites during analysis of live cell movies.

The ability to identify mitochondria and lysosomes tethered at a membrane contact site may be difficult using standard 100× objectives on conventional confocal systems (see Quantification and statistical analysis).

Potential solution

As conventional confocal imaging resolution is physically limited by the diffraction barrier to ~200 nm, while membrane contacts often span junctions of ~10–30 nm, identifying contact sites may be difficult in certain scenarios. In addition, sub-optimal imaging conditions may further convolute images, resulting in resolutions closer to 300 nm. Thus, utilization of super-resolution imaging methods such as structured illumination microscopy (SIM) or Airyscan which achieve resolutions as small as 80–120 nm and 120–180 nm respectively may be helpful. Importantly, super-resolution microscopy may help to dramatically enhance image quality, making determination and tracking of organelle contacts easier and more accurate (Wong et al., 2018). Alternatively, software-based approaches to super resolution such as point-scanning super resolution (PSSR) (Fang et al., 2021) may also help to enhance image resolution when access to super resolution systems are limited.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yvette C. Wong, PhD (yvette.wong@northwestern.edu).

Materials availability

All of the reagents described here are commercially available through the indicated vendors.

Data and code availability

This study did not generate or analyze any datasets or codes.

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AUTHOR CONTRIBUTIONS

This manuscript was written by T.B.B., E.D.L., J.C., and Y.C.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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