Chapter 18

Quantifying Regulated Mitochondrial Fission in Macrophages

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Abstract

Mitochondria have co-evolved with eukaryotic cells for more than a billion years, becoming an important cog in their machinery. They are best known for being tasked with energy generation through the production of adenosine triphosphate, but they also have roles in several other cellular processes, for example, immune and inflammatory responses. Mitochondria have important functions in macrophages, key innate immune cells that detect pathogens and drive inflammation. Mitochondrial activity is influenced by the highly dynamic nature of the mitochondrial network, which alternates between interconnected tubular and fragmented forms. The dynamic balance between this interconnected fused network and fission-mediated mitochondrial fragmentation modulates inflammatory responses such as production of cytokines and mitochondrial reactive oxygen species. Here we describe methods to differentiate mouse bone marrow cells into macrophages and the use of light microscopy, electron microscopy, flow cytometry, and Western blotting to quantify regulated mitochondrial dynamics in these differentiated macrophages.

Key words Drp1, Fission, Fusion, Inflammation, Macrophages, Microscopy, Mitochondria, Mitochondrial dynamics

1 Introduction

Macrophages are key cellular components of the innate immune system, participating in pathogen detection, host defense, wound healing, and homeostasis. These cells use pattern recognition receptors to sense danger and environmental perturbations [1]. One family of these receptors, the toll-like receptors (TLRs), recognizes microbial products such as lipopolysaccharide (LPS) and lipoproteins, activating downstream inflammatory and antimicrobial gene expression programs [2, 3]. In addition to regulating inflammatory gene expression [4, 5] and cell metabolism [6, 7], TLR signaling also modulates mitochondrial functions, which in turn affects immune responses [8, 9].

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Mitochondria are derived from ancient bacterial ancestors, merging with eukaryotic cells more than a billion years ago [10]. Inside each cell, mitochondrial numbers are finely regulated by the processes of biogenesis and mitophagy (Fig. 1). Mitochondrial biogenesis is mediated by both nuclear and mitochondrial transcription regulators such as peroxisome proliferator-activated receptor gamma-1α and mitochondrial transcription factor A, respectively, enabling expression of genes required for both biogenesis and mitochondrial DNA replication [11]. Mitophagy involves the delivery of damaged mitochondria to autophagosomes for recycling, with the PTEN-induced kinase 1/PARKIN complex being critical in this process [12, 13]. In addition to biogenesis and mitophagy, the balance between an interconnected tubular state (fusion-skewed) and a fragmented state (fission-skewed) also regulates mitochondrial numbers. This dynamic is mediated by specific GTPases; dynamin-related protein 1 (DRP1) drives fission, while the mitofusins (MFN1, MFN2) and dominant optic atrophy (OPA1) support fusion [14]. Modulation of the mitochondrial network can be very rapid as the activation of these molecules is regulated by post-translational modifications (PTMs), for example, deacetylation of MFN1 [15] and phosphorylation/dephosphorylation of DRP1 [16].

Mitochondrial dynamics have been linked to many cellular processes, including macrophage inflammatory responses [17–20]. Bacterial components, such as LPS, modulate mitochondrial dynamics by skewing towards fission, with subsequent induction of inflammatory mediators in macrophages [21, 22]. LPS stimulation also rewires mitochondrial activity, resulting in increased mitochondrial reactive oxygen species (mitoROS) that contributes to the pro-inflammatory phenotype of LPS-activated macrophages [23]. Several reports have shown an association between mitochondrial fission and mitoROS production in an inflammatory context. For instance, pharmacological antagonism of fission abrogated mitoROS generation in osteoblasts [24]. Furthermore, a peptide-based inhibitor of fission attenuated mitoROS production in a Huntington's disease cell culture model [25]. It is worth noting that mitoROS generation and mitochondrial fission can also have a reciprocal relationship with mitoROS shown to skew mitochondrial dynamics towards fission in neurons [26]. Given this intertwined connection, there may be value in assessing mitoROS generation when characterizing regulated mitochondrial dynamics responses in macrophages.

Here we present in vitro methods to assess mitochondrial dynamics and mitoROS production in murine macrophages responding to inflammatory stimuli. We describe simple methods that can be used to visualize and quantify mitochondrial dynamics, as well as important specificity controls (Fig. 1). We focus on the use of both confocal imaging and electron microscopy to not only

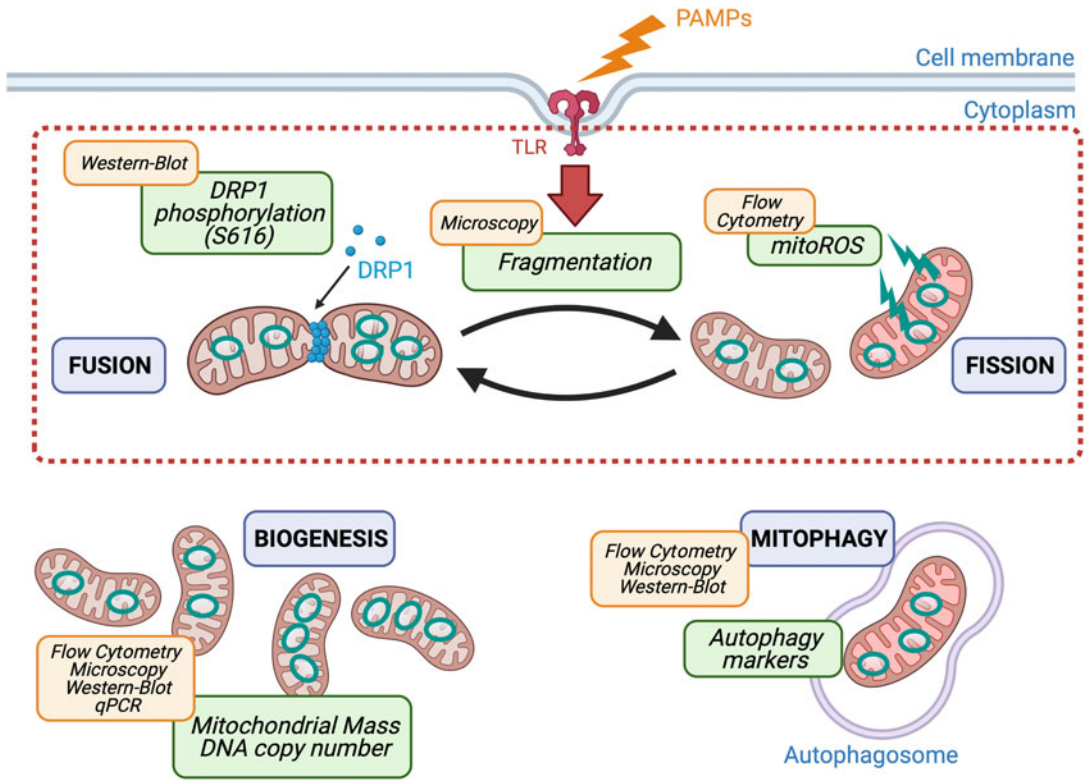

Fig. 1 TLR-inducible mitochondrial fission and methods to monitor this process. TLR stimulation alters the equilibrium between mitochondrial fusion and fission, skewing towards fragmentation of the mitochondrial network. Mitochondrial fission is accompanied by the acute phosphorylation of Drp1. Fragmented and stressed mitochondria release mitoROS and, if too damaged, are directed towards autophagy. Importantly, mitochondrial fission can be induced independently of mitochondrial biogenesis as mitochondria possess more than one copy of their DNA (small green circles). This chapter describes methods to assess inducible mitochondrial fission (in red dashed box) with specific methods (yellow boxes)

assess and enumerate mitochondrial morphology and regulated mitochondrial fission in macrophages but also outline additional approaches that can be used for further validation. These methods should be broadly applicable to other cell types and stimuli.

2 Materials

2.1 Macrophage Culture

1. 100-mm square petri dishes, 6-well/24-well tissue culture plates, 3-cm petri dishes, tweezers, scissors, 50-mL conical tubes.
2. Complete medium: RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS), antibiotics (50-U/mL penicillin, 50-mg/mL streptomycin), and GlutaMAX (2 mM).

3. Recombinant human colony-stimulating factor-1 (rhCSF-1): Stock concentration is 15 μg/mL in RPMI 1640 complete medium (without antibiotics), stored at 4 °C (for 2–3 weeks, −80 °C for longer storage) and used at a final concentration of 150 ng/mL.
4. 10-mL syringe, 25G needle, 18G blunt needle.
5. Phosphate-buffered saline (PBS): Dulbecco's Phosphate Buffered Saline, [-] calcium chloride, [-] magnesium chloride.
6. Lipopolysaccharide from *Salmonella enterica* serotype minnesota (Sigma-Aldrich, L2137) or ultrapure lipopolysaccharide from *Salmonella minnesota* R595 (InvivoGen, tlrl-smlps).
7. Dimethyl sulfoxide (DMSO).
8. M1 (Sigma-Aldrich, #SML0629), reconstituted in DMSO, used at a final concentration of 20 μM, and stored at −20 °C in small aliquots to avoid repeated freeze-thaw cycles.
9. Mdivi-1 (Sigma-Aldrich, #M0199), reconstituted in DMSO, used at a final concentration of 10 μM, and stored at −20 °C in small aliquots to avoid repeated freeze-thaw cycles.
10. CO_2 incubator.
11. Centrifuge that can spin 50-mL falcon at 400 *g*.

2.2 Quantification of Mitochondrial Dynamics and mitoROS Production

1. 13-mm #1.5 coverslips, fine tweezers/forceps.
2. Mounting media (with a refractive index close to 1.4–5 when using with an oil objective): Gelvatol mounting medium (6-g polyvinyl acetate, 15-mL dH_2O, 30-mL Tris-HCl (pH 8.6), 15-g glycerol).
3. MitoSOX™ Red Mitochondrial Superoxide Indicator (Invitrogen, #M36008) abs/em ~510/580 nm.
4. MitoTracker Deep Red FM (*see* **Note 1**) (Invitrogen, #M22426) abs/em ~644/665 nm.
5. PBS-DAPI: 20 ng/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS; stock concentration is 1 μg/mL, stored at −20 °C.
6. Calcein Blue AM Fluorescent Dye (Invitrogen, #C1429), stored at −20 °C in small aliquots to avoid repeated freeze-thaw cycle.
7. Primary antibodies:

   Tom20 (Rabbit, Cell Signaling Technology, #42406S), diluted 1:200 and used at a final concentration of 0.26 μg/mL.

   DRP1 (Rabbit, Cell Signaling Technology, #8570S), diluted 1:100 and used at a final concentration of 3.66 μg/mL.

8. Secondary antibody: Goat anti-rabbit Alexa Fluor 488 (Invitrogen, #A-11008) diluted 1:400 and used at a final concentration of 5 μg/mL.
9. 0.1% Triton X-100 in PBS.
10. 0.5% bovine serum albumin (BSA) in PBS.
11. 8% paraformaldehyde (PFA) in PBS.
12. 2.5% glutaraldehyde in water.
13. Lift buffer: 1-mM EDTA, 0.1% sodium azide in PBS.
14. Confocal microscope: e.g., Carl Zeiss LSM 710 confocal scanner attached to an AxioExaminer Upright Stand using a 63 × 1.4 NA plan apochromat objective.
15. Software used for image analysis: Fiji, an open-source image-processing package based on ImageJ.
16. Flow cytometer with a blue laser (488 nm) and a detection filter set around 585 nm. We routinely use a Gallios Cytometer (Beckman Coulter), as well as its acquisition/analysis software Kaluza.

2.3 Quantification of DRP1 Phosphorylation by Western Blotting

1. Anti-DRP1 antibody (Mouse, Cell Signaling Technology, #14647S), diluted 1:1000 and used at a final concentration of 1.03 μg/mL.
2. Anti-phospho-DRP1 S616 antibody (Rabbit, Cell Signaling Technology, #3455S), diluted 1:1000 and used at a final concentration of 0.12 μg/mL.
3. Anti-rabbit IgG HRP-linked (Cell Signaling Technology, #7074S), diluted 1:2500 and used at a final concentration of 0.03 μg/mL.
4. Anti-mouse IgG HRP-linked (Cell Signaling Technology #7076S), diluted 1:2500 and used at a final concentration of 0.06 μg/mL.
5. PVDF or nitrocellulose membrane.
6. Whatman filter paper.
7. 1-mL syringes with a 25G needle.
8. Cell scrapers.
9. Phosphatase inhibitor tablets, PhosSTOP (Sigma-Aldrich, #4906837001).
10. cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich, #11873580001).
11. NuPAGE™ LDS sample buffer (Invitrogen, #NP0008).
12. NuPAGE™ sample reducing agent (Invitrogen, #NP0009).
13. Prestained Protein Standard.

14. Western ECL substrate (clarity western luminol/enhancer solution and peroxide solution).
15. Hydrogen peroxide 30%, aqueous solution.
16. *Optional*: Benzonase, diluted 1:10 in $MgSO_4$ and used at a final concentration of 25 units/μL RIPA buffer – 50-mM Tris-HCl pH 8.0, 1-mM EDTA, 1% Triton-X100, 10% Glycerol, 0.1% sodium deoxycholate, 150-mM NaCl.
17. Wash buffer: Tris-buffered saline (TBS) with Tween 20 (TBST) (10-mM Tris-HCl pH 8.0, 150-mM NaCl, 0.05% Tween 20).
18. Running buffer: 10X stock (30.3-g/L Tris, 144-g/L glycine, 50 mL of 20% sodium dodecyl sulfate solution (SDS) in 1-L ultrapure water).
19. Transfer buffer (1 L): 200 mL of Trans-Blot Turbo 5x Transfer Buffer, 600 mL of Ethanol, 200 mL ultrapure water.
20. Blocking buffer: 5% skim milk in 1X TBST.
21. Gel Imaging System.
22. Semidry Blot System.
23. Roller (to use during blot transfer preparation).
24. Heating block to boil samples for Western blotting.
25. Centrifuge that can spin 1.5-mL reaction tubes at 17,000 g.
26. Image Lab software from Bio-Rad.

3 Methods

In this chapter, we focus on methods that do not require specific cell lines or plasmid constructs (e.g., mito-Dendra2) but rather simple mitochondrial-staining dyes that are commercially available. The first step is to collect bone marrow progenitor cells from mice and then differentiate them for 7 days with the growth factor CSF-1 to generate bone marrow-derived macrophages (BMMs). After stimulation of these cells, mitochondria are stained with MitoTracker or mitochondria markers (e.g., anti-Tom20), and the mitochondrial network is then analyzed by microscopy using Fiji software [27] to determine various parameters, for example, mitochondrial numbers per cell. Specific controls that can be incorporated to confirm that changes observed relate to changes in mitochondrial dynamics are also described. Use of electron microscopy to assess mitochondrial morphology and the level of fragmentation is also outlined, as is phosphorylation of Drp1, an indirect readout of fission. The use of flow cytometry to quantify production of mitoROS, a well-known inflammatory mediator relevant to mitochondrial fission, is also detailed. Finally, we briefly

discuss the assessment of biogenesis via quantification of mitochondrial mass and mitochondrial DNA copy number, as well as mitophagy, as important controls that should be considered when quantifying mitochondrial fission (Fig. 1).

3.1 Differentiating Bone Marrow Progenitor Cells into BMMs

1. Euthanize a mouse (*see* **Note 2**), as per institutional animal ethics requirements, collect the two femurs and tibias (without breaking the bones), and place them in a small 3-cm dish filled with complete medium.
2. In a biosafety cabinet, use tweezers to remove any muscle and surrounding tissues from the bones. Soak the clean bones in 70% ethanol for 1 min and place in complete medium again (we normally place the bones in medium in a 6-well plate).
3. Carefully cut off the tips of the femurs and tibias using scissors and then flush the four bones with complete media using a 25G needle (10-mL syringe) into a 50-mL falcon tube. Continue the flushing until the bones are white in appearance. Usually, it takes 10- to 15-mL media to flush four bones.
4. Centrifuge bone marrow cells at 500 *g* for 5 min.
5. Resuspend the cell pellet in complete medium (*see* **Note 3**), split them into eight 100-mm square petri plates with 15-mL media per plate, and add recombinant human colony-stimulating factor-1 (rhCSF-1) to each plate at a final concentration of 10^4 U/mL. We use rhCSF-1 produced in a highly efficient baculovirus-insect cell expression system at the Protein Expression Facility, the University of Queensland, but commercial rhCSF-1 is also available. In both cases, we recommend optimizing the rhCSF-1 concentration needed for your own experimental conditions. Culture the cells for 6 days in a 37 °C CO_2 incubator (*see* **Note 4**).
6. On day 6, BMMs are harvested by removing media from the plates and adding 10-mL sterile PBS to each plate. Use a 10-mL syringe with an 18G blunt needle to harvest the cells from the plate, collecting the BMMs in a 50-mL falcon tube. Centrifuge the cells at 500 × *g* for 5 min, resuspend them in complete media, and count them. Be sure to add rhCSF-1 to the media when plating cells (final concentration of 10^4 U/mL), as this growth factor is essential for mouse BMM survival.

3.2 Measuring Mitochondrial Fission by Microscopy After LPS Stimulation

1. Place sterile coverslips into the wells of a 24-well tissue culture plate. Wash once with sterile PBS to clean the coverslip from any contaminants. Note that it is important to have at least duplicate coverslips for each condition.
2. Plate 2×10^5 BMMs per well in 1-mL complete media supplemented with rhCSF-1 (10^4 U/mL) and incubate overnight at 37 °C and 5% CO_2.

Table 1
list of siRNAs used to silence *Drp1* in mouse macrophages

siRNA target	Sequence (5′-3′)
mouse Drp1_1	GCCAUGCUGUCAAUUUGCUAGAUGU
mouse Drp1_2	CAGGCAACUGGAGAGGAAUGCUGAA
mouse Drp1_3	GGUGGUGCUAGGAUUUGUUAUAUUU

3. On the following day, add 100-ng/mL LPS to the designated well(s) and incubate for 6 h (or desired time points). As LPS does not solubilize very well, it is important to vortex the LPS before use for a minimum of 30 s. It is also important to note that LPS from distinct species and the methods of purification confer very different activities and may result in contamination with other bacterial products that also activate macrophages, so attention should be given to the source of LPS and the purification method used [28, 29]. Depending on the bacterial species from which it is purified, a concentration between 10 and 1000 ng/mL of LPS should be sufficient to achieve maximum activity in macrophages, whereas a concentration range of approximately 0.1 to 10 ng/mL is generally considered to be sub-maximal. For our studies, we use *Salmonella* LPS at 10 to 100 ng/mL. However, we find a similar fission response when using sub-maximal LPS concentrations (e.g., 0.5 to 1 ng/mL).
4. *Controls*: (a) BMMs can be pre-treated for 1 h with compounds that will inhibit mitochondrial fission before LPS stimulation. Mdivi-1 attenuates fission by inhibiting Drp1 [30] and M1 promotes fusion [31]. We use these compounds at a final concentration of 10 μM and 20 μM, respectively. At these concentrations, Mdivi-1 and M1 do not affect BMM viability in our hands, as assessed by lactate dehydrogenase release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assays. However, we note that Mdivi-1 can also have Drp1-independent effects [32], so additional controls are strongly recommended. (b) Genetic approaches such as siRNAs that target *Drp1* (Table 1) or *Drp1*-deficient cell lines can be used as controls to confirm LPS-inducible fission [22].

3.3 Staining with MitoTracker Red FM

1. After the required time of LPS stimulation (*see* **Note 5**), replace the medium from the wells with new medium containing MitoTracker Red FM at a final concentration of 150 nM.
2. Incubate at 37 °C for 30 min. Keep the plate in the dark as much as possible.

3. Wash three times with 1-mL PBS per well (place plate for 5 min in the incubator for each wash). Be careful while washing—do not pipette PBS directly on the coverslips; rather direct it towards the wall of the wells.
4. Add 300 μL per well of 4% cold PFA diluted in PBS (*see* **Note 6**) and incubate for a minimum of 15 min in the dark (e.g., covered with foil paper) at room temperature (RT) for fixation.
5. Wash three times with 1-mL PBS (5 min/wash at RT). At this point, coverslips can be stored at 4 °C for approximately 2 days, if necessary. At this stage, BMM can also be stained for mitochondria-related proteins of interest, such as Tom20 or Drp1 (*see* Subheading 3.4).
6. Remove PBS and add 300-μL PBS-DAPI (20 ng/mL) per well.
7. Keep for 30 min at RT in the dark.
8. Wash three times with 1-mL PBS (5 min/wash).
9. Pipette 5-μL mounting media on the slides for each cover slip. Carefully grab the cover slip from the well using forceps. Remove excess PBS by carefully tapping the side of the coverslip on a Kimwipe.
10. Place the coverslip onto the slide ensuring the side with the cells is in contact with the mounting media.
11. Keep the slides in the dark at RT overnight for drying off. On the following day, store at 4 °C in the dark until imaging is performed (*see* **Note 7**).
12. Image using a laser scanning confocal microscope with a 63 × 1.4NA plan apochromat objective.

3.4 Immunostaining for Mitochondrial Proteins (Drp1/Tom20)

1. Use PFA-fixed BMM from **Step 5** in Subheading 3.3 for the following steps.
2. Permeabilize cells by adding 500-μL 0.1% Triton X-100 in PBS per well for 5 min.
3. Wash three times with 1-mL PBS.
4. Block the cells by adding 100 μL of 0.5% bovine serum albumin (BSA) in PBS to each well. Incubate at RT for 1 h.
5. Place 30-μL droplets of diluted antibodies onto a large piece of parafilm attached inside a box, protected from light. We use anti-Drp1 and anti-Tom20 antibodies at final concentrations of 3.66 μg/mL and 0.26 μg/mL, respectively.
6. Carefully remove the coverslips from the wells using forceps, dry the edges of each coverslip on a Kimwipe, and then place coverslips cell side down on 30-μL droplets of anti-Drp1 or anti-Tom20. Be sure to place one coverslip on 30-μL droplets of 0.5% BSA in PBS without the primary antibody. This control coverslip will receive only the secondary antibody.

7. Incubate at RT for 1 h.
8. Using forceps, carefully flip the coverslips still in the box, dry the edges, and place them cell side up. Wash three times by gently adding 100-μL droplets of PBS on the coverslip (5 min per wash).
9. Stain with the secondary antibody and DAPI in a similar fashion to **Steps 5** and **6**. Prepare a master mix of secondary antibody and DAPI in 0.5% BSA in PBS.
   (a) Secondary antibody: goat anti-rabbit Alexa Fluor 488 diluted 1:400 (final concentration 5 μg/mL).
   (b) PBS-DAPI: 20 ng/mL final concentration.
10. Dab the edges of each coverslip gently on a Kimwipe and place cell side down on 30-μL droplets of the master mix.
11. Incubate in the dark at RT for 1 h.
12. Repeat **Step 8** and wash three times with 100-μL droplets of PBS for 5 min per wash (in the dark).
13. Dab the edges of each coverslip between washes to ensure excess secondary antibody and stain are removed.
14. Mount the coverslips onto slides as described previously. Nail polish can be used, but we recommend using mounting media that seals the coverslip.
15. Store at 4 °C in the dark until they are imaged (*see* **Note 7**).

3.5 Assessing Fission by Immunofluorescence: Image Acquisition

Fragmentation of the mitochondrial network can be quantified by different methods. Here we present two techniques we have routinely used in our studies (Fig. 2). Fluorescent images of fixed cells are acquired using a Carl Zeiss LSM 710 confocal scanner attached to an AxioExaminer Upright Stand using a 63 × 1.4 NA plan apochromat objective. It is extremely important that, once the acquisition settings are chosen, they are not altered. Because of this, we recommend sampling a control and an LPS-stimulated slide to ensure detected intensities don't increase above the detector range, saturating the signal. To have a full view of the mitochondrial network, acquisition of a z-stack may be useful. However, we have observed that, in macrophages in vitro, most of the mitochondrial network resides on a plane close to the basal membrane. Thus, a single high-definition scanning plane can be performed without the loss of significant data. This will allow for increased individual field of view and thus more cells at a high resolution for the duration of the imaging session. To increase the signal-to-noise ratio and the quality of the image, we recommend using a slower scanning speed and line averaging (×16).

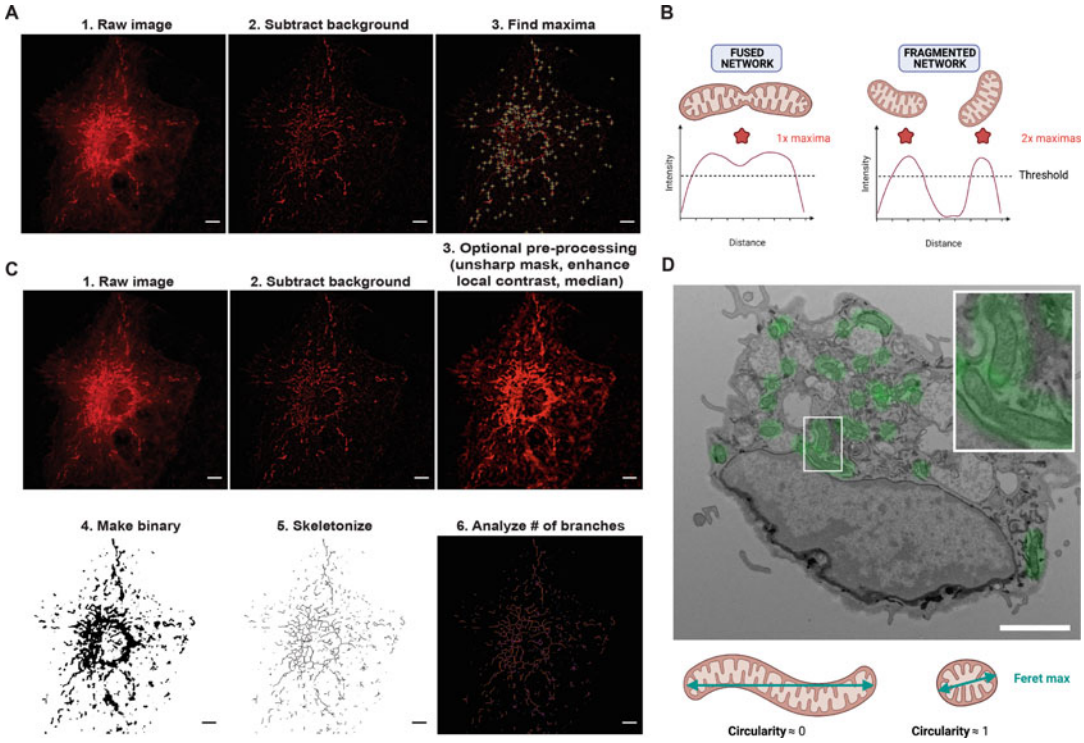

Fig. 2 Quantifying mitochondria fission by light and electron microscopy. (**a**) Maxima methods from a RAW image of a BMM, including processing using the "subtract background" and "find maxima" functions. (**b**) Schematic diagram of the maxima identification using intensity threshold. (**c**) Steps from the skeletal method to detect fragmentation of the mitochondrial network. (**d**) An electron micrograph of mitochondria in a macrophage artificially colored in green (upper). Morphology of mitochondria can be assessed using their circularity or ferret max number (lower). Scale bar 1 μm for light microscopy (**a**, **c**) and 2 μm for electron microscopy (**d**)

3.6 Assessing Fission by Microscopy: Image Quantification

We use Fiji, an open-source image-processing package based on ImageJ, for further image processing and quantification [27]. Two different toolsets in ImageJ are used to quantify regulated fission (*see* **Note 8**): "Find Maxima" and "Mitochondrial Network Analysis" (MiNA) [33], both of which are described below.

(i) *MAXIMA:* The maxima method quantifies relative numbers of single mitochondria, with this varying depending upon the threshold of fluorescence chosen (Fig. 2a, b).

1. Open an image in ImageJ.
2. Select "Image" and then "color" and "split channels." For further processing, use mitochondria-stained channel.
3. You can run the "sharpen" function to preprocess the image (optional).
4. Use "Process/Subtract Background" to reduce and normalize background staining, as per your requirements (ensuring the radius is greater than the mitochondrial cross section).

The effect of this subtraction will vary between experiments due to the staining process and the microscopy acquisition settings.

5. Using the manual selection tools, isolate one macrophage. Run "Process/Find Maxima…." Set the "noise tolerance" to sampled intensities of your mitochondria (place the cursor over mitochondria and noting the intensity value displayed in the status bar of the main FIJI window). As a guide, we typically obtain around 60–100 maxima (mitochondria) per cell in BMM. However, this number can vary depending on staining, image quality, preprocessing steps, cell type, and treatments. Although variation is noted among differing experiments, it is critical that these settings should be fixed within one specific experiment and used across all samples.
6. A fully automated script has also been described [34]. If your sample size is not too large, you can manually select the cells and apply the post-acquisition steps. We usually measure maxima of 30–50 macrophages per condition per experiment to ensure that the inherent cell-to-cell variability of macrophage populations does not confound data interpretation. Each experiment is usually repeated at least three times independently.

(ii) *SKELETAL:* The skeletal method utilizes the Mitochondrial Network Analysis (MiNA) toolset, which is available in ImageJ. This approach allows a semi-automated quantification of mitochondrial networks in an in vitro cellular system (Fig. 2c).

1. Open an image in ImageJ.
2. Select "Image" and then "color" and "split channels." For further processing, use mitochondria-stained channel.
3. Preprocess the image using the following filters: Unsharp mask and enhance local contrast (CLAHE) and median. These preprocessing steps are optional; however, it allows to augment image quality before converting to binary image and producing morphological skeletons.
4. For analyzing images, you can either perform batch processing or select an individual cell. For our studies, we used individual cells for analysis. After selecting an individual cell, run the following commands: make binary and then skeletonize.
5. After skeletonizing, run the command "analyze skeleton," and then from the table of parameters, use "# branches" to calculate individual and networks of mitochondria. The sum of individual and networks gives the total number of mitochondria in one cell.
6. Repeat the same analysis for all other cells in that image, as well as for all other images from the same experiment.

3.7 Assessing Fission by Electron Microscopy (EM): Image Acquisition and Analysis

Fluorescence-based tools are a convenient way of quantifying mitochondrial numbers and assessing mitochondrial dynamics. However, EM is an important method to validate fluorescence-based imaging quantification and to provide quantitative ultrastructural information on mitochondrial morphology.

1. Seed day 6 BMM (from **Step 6**, Subheading 3.1) into two 3-cm petri dishes in complete media and leave them to rest overnight. Note that it is recommended to use a higher cell density for EM, by comparison to that used for light microscopy. We usually plate three million BMM in 3-mL complete media containing rhCSF-1 (final concentration of 10^4 U/mL).
2. On the following day, stimulate the BMMs with LPS (100 ng/mL final) or leave untreated.
3. After 4–6 h, wash cells twice with PBS and fix with PBS containing 2.5% glutaraldehyde for 10 min at RT. Remove the fixative agent and then wash the cells with PBS (*see* **Note 9**).
4. Process the samples for EM. Detailed methods for sample preparation for EM are described elsewhere [35, 36]. Collect sections on EM grids and transfer to the transmission EM.
5. Image grids at a suitable magnification (e.g., 10,000–25,000×) and capture micrographs in a random fashion across the section.
6. Image analysis: Ideally, this is performed on a set of images in a blinded fashion with the experimental condition indicated by a code. Identify mitochondria in each image (readily identified by their characteristic morphology, enclosed by a double membrane: an outer membrane and an inner membrane that is folding towards the inside of the organelle—the cristae, Fig. 2d, highlighted in green). Select the draw tool in ImageJ and highlight the circumference of each mitochondrion. Two measurements that are particularly useful for quantification, as indicated in Fig. 2d, are:
   (a) *Circularity*: ImageJ calculates a coefficient representing how close your selection is to a perfect circle (a circularity of 1 is a perfect circle). Mitochondria undergoing fission will show a higher circularity than a fused mitochondrial network.
   (b) *Feret max*: The ferret max is the longest line you can draw within your selected area. Mitochondrial fission will show a lower ferret max by comparison to the elongated mitochondria that are typically associated with fusion.
7. Manual annotation is possible, but, unfortunately, there is no easy way to automatically identify mitochondria with classic transmission EM to enable automated analyses of large data sets. However, some research groups have started to create

machine learning-based ImageJ scripts that recognize and quantify mitochondria [37, 38]. Note that, depending on your sample size, this method could take longer than conventional approaches to manually assess mitochondrial morphology.

4 Assessing Fission Response by Quantifying Drp1 Post-translational Modifications (PTM)

Drp1 activity is regulated by several PTMs, including phosphorylation, sumoylation, and nitrosylation [39]. Among these, serine phosphorylation has been intensively studied, particularly DRP1 activation by S616 phosphorylation (S635 in mouse Drp1) and S637 dephosphorylation (S656 in mouse Drp1). LPS treatment of BMM triggers rapid phosphorylation of Drp1 at S635 (within ~15–30 min). This acute response can be quantified by Western blotting using antibodies that detect phospho-S635 Drp1 in BMM (equivalent of human phospho-S616 DRP1) and total Drp1 (*see* **Note 10**).

1. Plate day 6 BMM (from **Step 6**, Subheading 3.1) at 2×10^6 cells per well of a 6-well plate in 2 mL of complete media with rhCSF-1 (10^4 U/mL) and incubate overnight at 37 °C and 5% CO2.
2. On the following day, add 100-ng/mL LPS to designated wells and incubate over a time course. Vortex the LPS before use for a minimum of 30 s.
3. After each time point, keep the plate on ice, wash the cells twice with 1-mL ice-cold PBS, and add 200 μL of RIPA buffer (including freshly added 1X phosphatase inhibitor and 1X protease inhibitor cocktail).
4. Using a cell scraper, lift all cells in RIPA buffer and transfer the lysate to a tube. Use a 1-mL syringe with a 25G needle to homogenize the lysate and to mechanically shear the DNA. Optional: If the lysate is too viscous, treat the lysates with 2-μL Benzonase per sample (diluted 1:10 in $MgSO_4$) for 20 min at RT. Then, centrifuge the samples at a high speed (~17,000 g) for 5 min. Collect the supernatant in a new tube. At this point, lysates can be frozen at −20 °C until Western blots are ready to be run.
5. If required, perform protein quantification assays of lysates (e.g., BCA or Bradford assay) to determine protein concentration and prepare your protein samples. We usually prepare 20 μL of sample for a 12-well blot (20 μL per well).
   (a) 1X NuPAGE™ LDS sample buffer (5 μL of the 4X stock solution).

(b) 1X NuPAGE™ sample reducing agent (2 μL of the 10X stock solution).

(c) 10-μg protein.

(d) Water (adjust for a final volume of 20 μL).

6. Boil the samples at 100 °C for 10 min, spin down the samples, and leave them on ice while preparing Bio-Rad gels for loading. Do not forget to remove the sticky strip from the bottom of the gel. Add 1× SDS running buffer to fill the internal space and the external space, as required. Wash and readjust the wells before loading. Load 5 μL of protein marker and the samples and then run at desired voltage and time (e.g., 200 V for approximately 30 min).
7. For transferring, we use the Trans-Blot Turbo transfer system from Bio-Rad:
   (a) Cut a piece of nitrocellulose or PVDF membrane to the size of blotted gel. Nitrocellulose can be used without preparation, whereas PVDF membrane must be immersed in methanol for 1 min before use to increase hydrophilicity.
   (b) Prepare Turbo transfer buffer, as described in the materials.
   (c) Soak the gel, 8× filter paper, and membrane in transfer buffer and arrange onto the cassette in the following order: 4× filter paper (bottom), membrane, gel, 4× filter paper (top). Remove any bubbles and excess buffer using a roller. Soak off excess buffer from the cassette using tissue paper.
   (d) Run at 25 V for 9 min.
   (e) Rinse the cassette in water after use and clean the turbo lid using wet tissue paper.
8. Block the membrane for 1 h at RT or overnight at 4 °C with blocking buffer on a shaker.
9. Remove blocking buffer, wash once with 1× TBST, and then incubate the membrane with primary anti-phospho-S635 Drp1 rabbit antibody (diluted 1:1000) in 5% BSA in TBST overnight at 4 °C on a shaker.
10. On the following day, wash the membrane three times with TBST on a shaker (5 min for each wash) and incubate with the HRP-conjugated anti-rabbit secondary antibody (diluted 1: 2500) in 5% milk in TBST for 1 h at RT.
11. Wash three times with TBST (5 min at RT for each wash).
12. Set up 1.6-mL ECL reagent (50% from each solution) on parafilm. Blot the membrane on tissue to remove as much liquid as possible and place the blot protein side down on to

the ECL reagent. Leave for 1 min. Remove liquid from membrane as earlier, put on transparent plastic wrap, and image the blot using a sensitive camera system or scanner.

13. After imaging, inactivate the membrane using 30% H_2O_2 (just enough volume to cover the blot) keeping in a shaking incubator (37 °C) for approx. 30 min.
14. Wash the membrane once with TBST, add the next primary antibody (mouse anti-Drp1) diluted 1:1000 in 5% milk in TBST, and incubate overnight, as before (*see* **Note 11**).
15. Follow **Steps 10** to **13** but use HRP-conjugated anti-mouse secondary antibody (diluted 1:2500).
16. After exporting the image from the camera/scanner system, you can quantify the band volume using ImageLab (Software available from Bio-Rad). Quantify the volume of p-Drp1 S635 and total Drp1 bands for each lane to calculate relative levels of p-Drp1 S635/total Drp1.
17. You can also probe for α-tubulin and the mitochondrial outer membrane protein Tom20 as additional controls for total protein content and mitochondrial mass, respectively. Details of these antibodies are provided in materials.

5 Measuring mitoROS Production After LPS Stimulation

Reactive oxygen species play an important role in immune responses. mitoROS oxidizes and irreversibly damages intracellular microbes and promotes inflammatory responses [40]. Several studies have also linked mitochondrial fission to mitoROS production [41], so mitoROS production is often quantified in parallel with mitochondrial fission. To assess mitoROS production, we stain macrophages with MitoSOX (a dye that stains mitochondrial ROS) and quantify relative levels by flow cytometry.

1. Plate 5×10^5 of BMM (day 6) in 1 mL of complete media per well in a 24-well plate and leave them overnight. Plate enough cells to perform at least duplicates for each condition. You will also need unstained control cells (without MitoSOX) as a control.
2. On the following day, check the cells for healthy morphology using a microscope, add 100 ng/mL LPS (*see* **Note 12**) to designated wells, and incubate for 6 h (or desired time points). Vortex the LPS before use for a minimum of 30 s.
3. After 6 h of stimulation, remove the media and wash twice with PBS. Reconstitute a vial of MitoSOX in 13 μL of DMSO (5-mM stock). Dilute MitoSOX 1:1000 in lift buffer (e.g., 5 μL in 5 mL) to a final concentration of 5 μM. You will need 0.5 mL of diluted working stock per well.

4. Add 0.5 mL of MitoSOX in lift buffer (*see* Subheading 2.2, **item 13**) to the respective wells and incubate at 37 °C for 20 min. Add 0.5-mL lift buffer alone to the unstained control sample.
5. Leave plates on ice in the dark, then collect cells by pipetting up and down and transfer into FACS tubes. Keep cells on ice. Run the cells through a flow cytometer using a blue laser with the detection filter set around 585 nm.
6. Gating technique (using the acquisition software, e.g., Kaluza): First gate cells on size and granularity (SSC-A x FSC-A) and then remove any doublets (FSC-A x FSC-H). You can also exclude dead cells by using dyes such as Calcein Blue AM Fluorescent Dye when staining with MitoSOX (Calcein-AM can be used at a final concentration of 1 μM). Live cells will take up and hydrolase the Calcein-AM, leading to its intracellular accumulation. Other dyes such as 7-AAD or PI can be problematic as their emission wavelength is close to that of MitoSOX. Once the gating is configured, run your samples and measure median fluorescence intensity (MFI).

6 Controls to Assess Mitochondrial Fission Versus Mitochondrial Biogenesis and/or Mitophagy

Although this chapter focuses on mitochondrial dynamics, the quantification of biogenesis and mitophagy may also be important for appropriate interpretation of findings. Mitochondrial fission can be initiated independently of biogenesis. Individual mitochondria can contain several copies of the mitochondrial genome; thus mitochondrial DNA replication is not a prerequisite for fragmentation [22, 42]. However, any changes in mitochondrial numbers that are observed could reflect alterations in biogenesis and/or mitophagy. For these reasons, it is preferable to have controls for biogenesis and mitophagy when assessing mitochondrial fission. Several methods can be used to quantify biogenesis and total mitochondrial mass. For example, the number of copies of mitochondrial DNA can be determined by using qPCR to quantify a mtDNA-specific region versus that of a nuclear-encoded gene such as beta-2-microglobulin (B2M) [22, 43]. Alternatively, mitochondrial mass per cell can be estimated using confocal microscopy or flow cytometry through quantifying fluorescence of MitoTracker-stained cells. Levels of mitochondrial proteins such as Tom20 can also be quantified by Western blotting as an indirect read-out of mitochondrial mass [22]. Contrary to biogenesis, macrophages can discard damaged mitochondria through mitophagy, a specific autophagy pathway [44]. Mitophagy can be challenging to assess as it may be difficult to discriminate from regular autophagy. Nonetheless,

traditionally mitophagy is assessed by monitoring the colocalization of LC3-positive autophagosomes and mitochondria (stained with MitoTracker or with an antibody against Tom20) [45]. Flow cytometry can also be used as a quantitative approach to measure mitophagy flux, utilizing MitoTracker Deep Red in combination with both lysosomal and mitophagy inhibitors [46].

7 Notes

1. We find that *MitoTracker Deep Red FM* works well in our studies. Because it uses the far-red channel, it also allows the simultaneous use of other dyes or antibodies. *MitoTracker* ***Green*** is also a mitochondrial-selective probe, but we find that its performance is affected by fixation. Tetramethylrhodamine, methyl ester (TMRM) is also widely used.
2. We usually use male or female 8–12-week-old C57Bl/6 mice. Older mice can also be used but may result in lower BMM yields because of an altered composition of their bone marrow [47].
3. At this stage, bone marrow cells can also be frozen in 90% FCS/10% DMSO for later use.
4. Depending on the activity of the rhCSF1 and the density at which bone marrow cells are initially plated, we recommend topping up cultures with 5-mL rhCSF-1-containing media per plate on the afternoon or evening of day 4 or day 5.
5. We find that LPS-inducible fission in BMM occurs rapidly and is sustained for up to 24 h; however, we routinely assess this response at 6 h post-LPS stimulation. We note that inducible fission can vary, depending on the stimuli and/or cell type. For example, MEF cells require overnight FCS starvation and a longer incubation with LPS for a maximal fission response.
6. 8% PFA can be stored at −20 °C for several months. We usually do not keep 4% PFA (diluted in PBS) at 4 °C for more than a week.
7. Don't forget to bring the slides to RT at least 30 min before imaging them, as temperature can affect image acquisition.
8. We have performed both methods on the same images to directly compare and find that they give similar data, but the maxima method is a little less variable in our hands [22].
9. At this stage, cells can be kept at 4 °C for a couple of days, if necessary, before further processing.
10. The antibody directed against phospho-DRP1 (*Cell Signaling Technology*) does not perform well for microscopy in our hands; thus, it is challenging to visualize the localization of phosphorylated Drp1 in BMM using confocal microscopy.

11. If you are using rabbit anti-Drp1, you must strip the membrane instead of using H_2O_2 for HRP inactivation. For stripping, incubate the membrane on shaker at RT with 1X antibody stripping solution in distilled water for 15 min. Then, remove the stripping solution and block the membrane for 1 h at RT with blocking buffer on a shaker before adding another antibody.
12. Bacterial infection is usually a better stimulus than LPS for mitoROS production.

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References

1. Kawasaki T, Kawai T (2014) Toll-like receptor signaling pathways. Front Immunol 5:461. https://doi.org/10.3389/fimmu.2014.00461
2. Stocks CJ, Schembri MA, Sweet MJ, Kapetanovic R (2018) For when bacterial infections persist: Toll-like receptor-inducible direct antimicrobial pathways in macrophages. J Leukoc Biol 103(1):35–51. https://doi.org/10.1002/JLB.4RI0917-358R
3. Satoh T, Akira S (2016) Toll-Like Receptor signaling and its inducible proteins. Microbiol Spectrum 4(6). https://doi.org/10.1128/microbiolspec.MCHD-0040-2016
4. Schroder K, Irvine KM, Taylor MS, Bokil NJ, Le Cao KA, Masterman KA et al (2012) Conservation and divergence in Toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. Proc Natl Acad Sci U S A 109(16):E944–E953. https://doi.org/10.1073/pnas.1110156109
5. Perkins DJ, Patel MC, Blanco JC, Vogel SN (2016) Epigenetic mechanisms governing innate inflammatory responses. J Interf Cytokine Res 36(7):454–461. https://doi.org/10.1089/jir.2016.0003
6. Palsson-McDermott EM, O'Neill LA (2013) The Warburg effect then and now: from cancer to inflammatory diseases. BioEssays: news and reviews in molecular, cellular and developmental biology 35(11):965–973. https://doi.org/10.1002/bies.201300084

7. O'Neill LA, Pearce EJ (2016) Immunometabolism governs dendritic cell and macrophage function. J Exp Med 213(1):15–23. https://doi.org/10.1084/jem.20151570
8. West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P et al (2011) TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. Nature 472(7344):476–480. https://doi.org/10.1038/nature09973
9. Banoth B, Cassel SL (2018) Mitochondria in innate immune signaling. Transl Res J Lab Clin Med 202:52–68. https://doi.org/10.1016/j.trsl.2018.07.014
10. Roger AJ, Munoz-Gomez SA, Kamikawa R (2017) The origin and diversification of mitochondria. Current Biol 27(21): R1177–R1R92. https://doi.org/10.1016/j.cub.2017.09.015
11. Popov LD (2020) Mitochondrial biogenesis: an update. J Cell Mol Med 24(9):4892–4899. https://doi.org/10.1111/jcmm.15194
12. Youle RJ, Narendra DP (2011) Mechanisms of mitophagy. Nat Rev Mol Cell Biol 12(1):9–14. https://doi.org/10.1038/nrm3028
13. Eiyama A, Okamoto K (2015) PINK1/Parkin-mediated mitophagy in mammalian cells. Curr Opin Cell Biol 33:95–101. https://doi.org/10.1016/j.ceb.2015.01.002
14. Giacomello M, Pyakurel A, Glytsou C, Scorrano L (2020) The cell biology of mitochondrial membrane dynamics. Nat Rev Mol Cell Biol 21(4):204–224. https://doi.org/10.1038/s41580-020-0210-7
15. Lee JY, Kapur M, Li M, Choi MC, Choi S, Kim HJ et al (2014) MFN1 deacetylation activates adaptive mitochondrial fusion and protects metabolically challenged mitochondria. J Cell Sci 127(Pt 22):4954–4963. https://doi.org/10.1242/jcs.157321
16. Chang CR, Blackstone C (2010) Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. Ann N Y Acad Sci 1201:34–39. https://doi.org/10.1111/j.1749-6632.2010.05629.x
17. Park S, Won JH, Hwang I, Hong S, Lee HK, Yu JW (2015) Defective mitochondrial fission augments NLRP3 inflammasome activation. Sci Rep 5:15489. https://doi.org/10.1038/srep15489
18. Katoh M, Wu B, Nguyen HB, Thai TQ, Yamasaki R, Lu H et al (2017) Polymorphic regulation of mitochondrial fission and fusion modifies phenotypes of microglia in neuroinflammation. Sci Rep 7(1):4942. https://doi.org/10.1038/s41598-017-05232-0
19. Nair S, Sobotka KS, Joshi P, Gressens P, Fleiss B, Thornton C et al (2019) Lipopolysaccharide-induced alteration of mitochondrial morphology induces a metabolic shift in microglia modulating the inflammatory response in vitro and in vivo. Glia 67(6): 1047–1061. https://doi.org/10.1002/glia.23587
20. Gao F, Reynolds MB, Passalacqua KD, Sexton JZ, Abuaita BH, O'Riordan MXD (2020) The mitochondrial fission regulator DRP1 controls post-transcriptional regulation of TNF-alpha. Front Cell Infect Microbiol 10:593805. https://doi.org/10.3389/fcimb.2020.593805
21. Park J, Choi H, Min JS, Park SJ, Kim JH, Park HJ et al (2013) Mitochondrial dynamics modulate the expression of pro-inflammatory mediators in microglial cells. J Neurochem 127(2): 221–232. https://doi.org/10.1111/jnc.12361
22. Kapetanovic R, Afroz SF, Ramnath D, Lawrence GM, Okada T, Curson JE et al (2020) Lipopolysaccharide promotes Drp1-dependent mitochondrial fission and associated inflammatory responses in macrophages. Immunol Cell Biol 98(7):528–539. https://doi.org/10.1111/imcb.12363
23. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE et al (2016) Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. Cell 167(2):457–70 e13. https://doi.org/10.1016/j.cell.2016.08.064
24. Zhang L, Gan X, He Y, Zhu Z, Zhu J, Yu H (2017) Drp1-dependent mitochondrial fission mediates osteogenic dysfunction in inflammation through elevated production of reactive oxygen species. PLoS One 12(4):e0175262. https://doi.org/10.1371/journal.pone.0175262
25. Guo X, Disatnik MH, Monbureau M, Shamloo M, Mochly-Rosen D, Qi X (2013) Inhibition of mitochondrial fragmentation diminishes Huntington's disease-associated neurodegeneration. J Clin Invest 123(12): 5371–5388. https://doi.org/10.1172/JCI70911
26. Hung CH, Cheng SS, Cheung YT, Wuwongse S, Zhang NQ, Ho YS et al (2018) A reciprocal relationship between reactive oxygen species and mitochondrial dynamics in neurodegeneration. Redox Biol 14:7–19. https://doi.org/10.1016/j.redox.2017.08.010
27. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T et al (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9(7): 676–682. https://doi.org/10.1038/nmeth.2019

28. Xiao X, Sankaranarayanan K, Khosla C (2017) Biosynthesis and structure-activity relationships of the lipid a family of glycolipids. Curr Opin Chem Biol 40:127–137. https://doi.org/10.1016/j.cbpa.2017.07.008
29. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ (2000) Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 165(2):618–622. https://doi.org/10.4049/jimmunol.165.2.618
30. Manczak M, Kandimalla R, Yin X, Reddy PH (2019) Mitochondrial division inhibitor 1 reduces dynamin-related protein 1 and mitochondrial fission activity. Hum Mol Genet 28(2):177–199. https://doi.org/10.1093/hmg/ddy335
31. Wang D, Wang J, Bonamy GM, Meeusen S, Brusch RG, Turk C et al (2012) A small molecule promotes mitochondrial fusion in mammalian cells. Angew Chem Int Ed Engl 51(37):9302–9305. https://doi.org/10.1002/anie.201204589
32. Smith G, Gallo G (2017) To mdivi-1 or not to mdivi-1: is that the question? Dev Neurobiol 77(11):1260–1268. https://doi.org/10.1002/dneu.22519
33. Valente AJ, Maddalena LA, Robb EL, Moradi F, Stuart JA (2017) A simple ImageJ macro tool for analyzing mitochondrial network morphology in mammalian cell culture. Acta Histochem 119(3):315–326. https://doi.org/10.1016/j.acthis.2017.03.001
34. Bosch A, Calvo M (2019) Automated quantitative analysis of mitochondrial morphology. Methods Mol Biol 2040:99–115. https://doi.org/10.1007/978-1-4939-9686-5_6
35. Graham L, Orenstein JM (2007) Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat Protoc 2(10):2439–2450. https://doi.org/10.1038/nprot.2007.304
36. Takasato M, Er PX, Chiu HS, Maier B, Baillie GJ, Ferguson C et al (2015) Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature 526(7574):564–568. https://doi.org/10.1038/nature15695
37. Liu J, Li L, Yang Y, Hong B, Chen X, Xie Q et al (2020) Automatic reconstruction of mitochondria and endoplasmic reticulum in electron microscopy volumes by deep learning. Front Neurosci 14:599. https://doi.org/10.3389/fnins.2020.00599
38. Li R, Zeng X, Sigmund SE, Lin R, Zhou B, Liu C et al (2019) Automatic localization and identification of mitochondria in cellular electron cryo-tomography using faster-RCNN. BMC Bioinform 20(Suppl 3):132. https://doi.org/10.1186/s12859-019-2650-7
39. Adaniya SM, J OU, Cypress MW, Kusakari Y, Jhun BS (2019) Posttranslational modifications of mitochondrial fission and fusion proteins in cardiac physiology and pathophysiology. Am J Physiol Cell Physiol 316(5):C583–C604. https://doi.org/10.1152/ajpcell.00523.2018
40. Shekhova E (2020) Mitochondrial reactive oxygen species as major effectors of antimicrobial immunity. PLoS Pathog 16(5):e1008470. https://doi.org/10.1371/journal.ppat.1008470
41. Jezek J, Cooper KF, Strich R (2018) Reactive oxygen species and mitochondrial dynamics: the Yin and Yang of mitochondrial dysfunction and cancer progression. Antioxidants (Basel) 7(1). https://doi.org/10.3390/antiox7010013
42. Waters LR, Ahsan FM, Wolf DM, Shirihai O, Teitell MA (2018) Initial B cell activation induces metabolic reprogramming and mitochondrial remodeling. iScience 5:99–109. https://doi.org/10.1016/j.isci.2018.07.005
43. Sun X, Lee W, Vaghjiani V, St John JC (2016) Analysis of mitochondrial DNA copy number and its regulation through DNA methylation of POLGA. Methods Mol Biol 1351:131–141. https://doi.org/10.1007/978-1-4939-3040-1_10
44. Pickles S, Vigie P, Youle RJ (2018) Mitophagy and quality control mechanisms in mitochondrial maintenance. Curr Biol 28(4):R170–RR85. https://doi.org/10.1016/j.cub.2018.01.004
45. Zhu J, Dagda RK, Chu CT (2011) Monitoring mitophagy in neuronal cell cultures. Methods Mol Biol 793:325–339. https://doi.org/10.1007/978-1-61779-328-8_21
46. Mauro-Lizcano M, Esteban-Martinez L, Seco E, Serrano-Puebla A, Garcia-Ledo L, Figueiredo-Pereira C et al (2015) New method to assess mitophagy flux by flow cytometry. Autophagy 11(5):833–843. https://doi.org/10.1080/15548627.2015.1034403
47. Gibon E, Loi F, Cordova LA, Pajarinen J, Lin T, Lu L et al (2016) Aging affects bone marrow macrophage polarization: relevance to bone healing. Regen Eng Transl Med 2(2):98–104. https://doi.org/10.1007/s40883-016-0016-5