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ASSESSING MITOCHONDRIAL MORPHOLOGY AND DYNAMICS USING FLUORESCENCE WIDE-FIELD MICROSCOPY AND 3D IMAGE PROCESSING

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Abstract

Mitochondrial morphology and length change during fission and fusion and mitochondrial movement varies dependent upon the cell type and the physiological conditions. Here, we describe fundamental wide-field fluorescence microscopy and 3D imaging techniques to assess mitochondrial shape, number and length in various cell types including cancer cell lines, motor neurons and astrocytes. Furthermore, we illustrate how to assess mitochondrial fission and fusion events by 3D time-lapse imaging and to calculate mitochondrial length and numbers as a function of time. These imaging methods provide useful tools to investigate mitochondrial dynamics in health, aging and disease.

1. Introduction

The central role of mitochondria in cellular energy production has long been appreciated, but new research highlighting the dynamic nature of mitochondria has begun to clarify the importance of these organelles in health and disease. Mitochondria are dynamic organelles that migrate and undergo regular cycles of fission and fusion. Mitochondrial fission and fusion facilitates mixing of metabolites and mitochondrial DNA (mtDNA) and helps ensure bioenergetic functionality [1, 2]. Fission and fusion events also allow proliferation and distribution of mitochondria throughout long neuronal processes, which can extend up to one meter in motor neurons [3, 4]. Proper distribution and efficient functioning of

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mitochondria is particularly important in neurons, which rely heavily on mitochondrial oxidative phosphorylation to meet their high energy needs [2]. In addition, neuronal mitochondria are important players in Ca^{2+} regulation, plasma membrane potential maintenance, and release and re-uptake of neurotransmitters at synapses [1, 4, 5].

Large GTPases, which are related to dynamin, a protein involved in scission of vesicles in endocytosis, coordinate and direct mitochondrial fission and fusion [2, 6]. Because both fission and fusion are important for proper mitochondrial function, an imbalance between mitochondrial fission and fusion events can cause disease. For example, hereditary mutations in mitofusin 2 (MFN2), a protein involved in fusion of the mitochondrial outer membrane, cause Charcot-Marie-Tooth Type 2A, a peripheral neuropathy [7, 8], and mutations in optic atrophy 1 (OPA1), a protein involved in fusion of the mitochondrial inner membrane, cause autosomal dominant optic atrophy (ADOA), the most common form of optic atrophy characterized by progressive blindness and degeneration of retinal ganglion cells and the optic nerve [9–11]. In addition to these genetic diseases, recent studies have begun to suggest a possible role for defects in mitochondrial trafficking, fission, and fusion in sporadic neurodegenerative diseases such as Parkinson's disease [12], Huntington's disease [13], stroke [12] and Alzheimer's disease [12]. Blocking fission can prevent apoptosis and neuronal cell death [12, 14–18]. Because mitochondrial dynamics appears to play an important role in healthy and diseased cells (and neurons in particular), the ability to visualize changes in mitochondrial morphology is critical to achieving a better understanding of disease mechanisms. Here, we describe a method to assess mitochondrial morphology and dynamics using fluorescent wide-field microscopy and 3D imaging. We provide the basics of microscope set-up, preparation and fixation of samples, assessment of mitochondrial size and number, and time-lapse imaging of live samples.

2. Equipment

We used here a Zeiss Axiovert 100M inverted microscope with a motorized focus, a Plan Achromat 63×1.4 NA oil, and a Ludl MAC 5000/bioprecision 2 linear encoded XY-motorized stage. However, other systems might achieve similar results.

Our illumination system consists of a Sutter DG-4/Lambda 10–2 combo Xenon arc lamp source and filter-based wavelength selector. The detection system has a Sutter Lambda 10–2 emission filter wheel and Cooke Sensicam QE high sensitivity, slow scan, cooled digital CCD camera. Different filter sets and dichromatic mirrors enable fluorescence imaging of a variety of fluorophores simultaneously.

Additionally, our microscope is equipped with a thermo-controlled environment box, a humidification bottle and a CO_2 controller (Zeiss). This system heats the sample and the entire microscope, which is important for stable long-term time-lapse imaging and prevents drift. The microscope is controlled by MetaMorph 6.3 (Molecular Devices) for visualization and quantitation.

3. Sample preparation

3.1 Primary cell isolation

3.1.1 Motor neuron preparation—Motor neurons are isolated and prepared from rat E15 spinal cords as previously described [19] and seeded on an astrocyte monolayer.

3.1.2 Astrocyte preparation—Astrocytes are isolated from E18 rat spinal cords following the protocol described [20]. Alternatively, the cells can be electroporated (as described below in Section 3.2.2) with DsRed2-Mito or Mito-mPlum expression plasmids at the time of isolation and plated for imaging.

3.2 Transfection

Mitochondria are visualized by transfecting cells with mitochondrially targeted fluorescent proteins (DsRed2-Mito or Mito-mPlum).

3.2.1 Plasmids—The pDsRed2-Mito expression plasmid (Clontech) contains the gene coding for DsRed2 fused at the 3' end of the mitochondrial targeting sequence from subunit VIII of cytochrome *c* oxidase and driven by the immediate early promoter of cytomegalovirus. DsRed2-Mito has an excitation peak at 558 nm and emission at 583 nm. Expression of this vector results in red fluorescent protein labeled mitochondria.

We constructed the pMito-mPlum plasmid by replacing DsRed2 with mPlum [21] in the pDsRed2-Mito plasmid. We amplified the mPlum coding sequence by PCR using pBAD/mPlum as a template [21] and 5'-TTGGATCCTCCTGTAGCAACTATGGTGAGCAAGGGCGAG-3' and 5'-CTATCTAGATTAGGCGCCGGTGGAGTGG-3' as primers to create BamHI and XbaI restriction sites. The digested and gel-purified PCR product replaced the DsRed2 sequence in pDsRed2-Mito (CLONTECH) on the 3' side of the mitochondrial targeting sequence and preserved the open reading frame. Mito-mPlum has an excitation peak at 590 nm and an emission peak at 630 nm (far-red). Mito-mPlum lacks the 500–550 nm green emission observed with Mito-DsRed2 at 488 nm excitation.

All expression plasmids are purified with the High Purity Plasmid Purification System from Marligen Biosciences, Inc. High quality DNA is particularly important for primary culture transfection to prevent toxicity.

3.2.2 Motor neuron, astrocyte and HeLa cell transfection—Rat E15 motor neurons are electroporated with 4 µg of pDsRed-Mito DNA using the Rat Neuron Nucleofector Kit (DPG-1003 from Amaxa Biosystems) and the G-13 or O-03 program of the Amaxa Nucleofector I. Rat E18 astrocytes are transfected after 5–7 days in culture with 3 µg of pDsRed2-Mito DNA and the O-03 program of the Amaxa Nucleofector I. One to two million astrocytes are transfected with 100 µl of electroporation buffer 1 (12.5 mM NaCl, 123 mM KCl, 4.5 mM MgCl₂, 10 mM EGTA, 20 mM PIPES, pH 7.2 set with KOH), supplemented with 20% bovine calf serum (Tissue Culture Biologicals) in a 2 mm electrode gap cuvette (BioRad). HeLa cells (from ATCC) were stably transfected with 4 µg Mito-mPlum and 500 µg/ml Geneticin (Gibco) selection.

3.2.3 RNA interference—HeLa cells with stable expression of Mito-mPlum were transiently transfected one day after seeding, using Oligofectamine (Invitrogen) with OPA1 siRNA or scrambled non-targeting siRNA (Dharmacon), as previously described [22].

3.3 Fixation

Standard cell fixation protocols can alter mitochondrial morphology and induce fission. A reliable method to preserve mitochondrial morphology is a 20 minutes fixation at 37°C with pre-warmed 3.7% formaldehyde (F8775, Sigma), 5% sucrose in PBS (pH 7.4).

4. Assessing mitochondrial morphology in different cell types (for fixed samples)

4.1 Image acquisition

4.1.1 Experimental system—Here, we imaged mitochondria of HeLa cells, motor neurons, and astrocytes, expressing fluorescent proteins targeted to the mitochondrial matrix. HeLa cells were transfected with Mito-mPlum and motor neurons and astrocytes were transfected with DsRed2-Mito. Several cell types were used to demonstrate the applicability of the techniques. HeLa cells exhibit a fused perinuclear mitochondrial network with few elongated peripheral mitochondria (Figure 1, left panels). Astrocytes have a large number of peripheral mitochondria of tubular morphology (Figure 1, middle panels). As an example of an extreme case, motor neurons have many mitochondria of variable length lined up as single organelles along narrow neuronal projection (Figure 1, right panels). Fluorescent Mito-mPlum or DsRed2-Mito was visualized using a 555/28 excitation filter for both and a 685/40 or 617/73 emission filter, respectively.

4.1.2 Microscope setup to image fixed samples—To obtain high resolution images of mitochondria we used a Plan Achromat 63× oil immersion objective with a 1.4NA. Fixed sample imaging is done at room temperature using the standard Zeiss Immersol 518F oil.

4.1.3 Binning and exposure time selection for fixed samples—Selection of the correct exposure time and binning is critical for the acquisition of high resolution images. The settings vary based on sample brightness and the fluorophores.

To minimize photo-bleaching, long exposure times must be avoided. Additionally, increasing exposure times causes a proportional increase in the signal-to-noise ratio and thus does not increase the image quality the way binning does. Binning is the combination of information from adjacent pixels into one pixel and it may be used to increase signal-to-noise ratio. A 2×2 or 3×3 binning is used for dim samples. For bright samples, a 1×1 binning can be used for optimal resolution. Ultimately, increasing the signal-to-noise ratio versus the resolution must be weighed when choosing the appropriate binning factor and exposure times.

The right exposure time is determined by scanning the field and selecting the brightest and the dimmest objects. The majority of pixels in the dim object should have an intensity that is

at least 4× the black current of the camera (the area that does not contain any cells) and less than 1% of the pixels in the bright object should be above 4095 intensity units (the saturation point of the camera). If the dim object is too dim to image using a reasonable exposure time, then a higher binning is selected and the process of exposure time selection is repeated. The exposure times are selected separately for each fluorophore, however the binning must be the same. Images with different binning would be different sizes and cannot be merged. Exposure times between 25–1000 msec and binning between 1–3× are generally appropriate for fixed samples.

4.1.4 Acquiring z-stacks—We use a motorized z-focus to obtain 3D images spanning the entire height of the specimen. Mitochondrial filaments span multiple focal planes. Thus, to capture the entire architecture of the mitochondrial network the entire height of the cell needs to be imaged. This is done by serial z-sectioning. Z sectioning by wide-field microscopy causes less photo-bleaching than confocal microscopy. The disadvantage, however, is that it collects more out-of-focus light. However, this out-of-focus light, seen as blur or haze in the image series, can be eliminated by applying mathematical algorithms (section 4.2). Last, the z-planes can be merged into a single image, generating the projection image.

The z-step size should be chosen based on the cell type and application. A 0.2 μm step size is optimal for fixed samples. The number of z-planes is calculated based on the sample thickness. To determine the sample height, we focus through the cell in the *show live* dialog box of MetaMorph and log the stage positions at which the bottom and top of the cell becomes out of focus. Given this distance and the step size selected, the MetaMorph software calculates the number of steps needed. Most fixed cell culture samples are 6–12 μm thick and require 30–60 z-planes at 0.2 μm step size.

4.1.5 Step-by step procedure for fixed sample image acquisition in MetaMorph

- 1) Open the Multi-dimensional acquisition (MDA) dialogue from the “Apps” drop down menu.
- 2) In the MDA, check-mark “Multiple Wavelengths” and “Z Series” (leave “Timelapse” and “Multiple Stage Positions” unchecked for fixed samples).
- 3) Select the saving location by choosing an appropriate file folder and base name for saving the images.
- 4) Select the exposure time for each wavelength to be imaged, using the strategy described above (Section 4.1.3).
- 5) “Z Series” tab.
 - A. Input the Z slice size.
 - B. Click “Show Live” and focus through the cell in the downward direction until the cell is completely out of focus.
 - C. Click “Set as Bottom”.
 - D. Focus through to the top of the cell and click “Set as Top”.

- E. MetaMorph will calculate the number of z-slices needed; check to make sure this is appropriate for your application.

- 6) Click “Acquire”.

4.2 Image processing and presentation

4.2.1 Image presentation: z-stack projection—We use a ‘maximum projection’ (the maximum intensity from each (x,y) pixel position) to combine z-planes into a single image. Depending on the desired visual results, other types of projections can be generated, such as an ‘average projection’ or a ‘minimum projection’. A z-stack acquired with a wide-field microscope contains a significant amount of out-of-focus light and therefore a maximum projection of the raw data appears hazy (Fig. 1A). In order to produce a sharp maximum projection, the out-of-focus light must be removed.

4.2.2 Image filtering: Out-of-focus light removal—Out-of-focus light can be removed by several techniques including Fourier transformation (high pass filtering) and median filter subtraction. The theoretical basis for both Fourier transformation and median filter subtraction is that out-of-focus objects in an image can be distinguished from in focus objects based on the spatial frequency of their components. Gray scale images can be represented as a series of two-dimensional (2D) sine waves of varying frequency. Out-of-focus objects contain fewer high frequency components than do in-focus objects. Fourier filtering separates these two components of an image leaving mostly the in-focus light. [23] A Fourier transformation of a maximum projected z-stack results in crisp mitochondrial visualization (Fig. 1B). Median filter subtraction uses a median filter to extract the out-of-focus light from each plane of the z-planes prior to maximum projection and also produces a sharp image (Fig. 1C), with a slightly different appearance than the Fourier transformed image.

4.2.3 Image display—Scaling, gamma correction, brightness and contrast adjustments are used to produce a final image in which all objects of interest are clearly visible when in print. This is critical because a printed format has a smaller dynamic range than does a recorded image. The image scaling can be set to include the pixel intensities of interest. Gamma correction provides a non-linear scaling. An elevated gamma correction (1.3 – 1.6) allows visualization of dim objects without a corresponding increase in bright objects. Brightness and contrast can be adjusted to achieve the desired visual effect.

4.2.4 Step-by-step procedure for image processing and presentation in MetaMorph

Procedure 1: Fourier transformation

- 1) Create a Maximum projection of the original stack.
 - A. Open the “Stack Arithmetic” window in the “Process” dropdown menu.
 - B. Choose the “Maximum” option, then “Apply”.
- 2) Fourier transform the Maximum projection.

- A. Open the “FFT” window in the “Process” dropdown menu.
 - B. Choose the sensitivity (usually start with 40 and try other settings if the desired visual effect is not achieved).
 - C. Select the image to be filtered, then “Apply”.
- 3) Visual adjustment of the Fourier transformed image.
- A. Open the “Scale Image” window in the “Display” dropdown menu.
 - B. Make sure “Auto scale” is not checked.
 - C. Start at the far left and move the left scale slider toward the right side until the background becomes black or close to black.
 - D. Move the right slider toward the left slider until the objects of interest (the mitochondria) are the desired brightness.
 - E. Additional visual adjustment can be made using the “Adjust Digital Contrast” window found in the “Display” dropdown menu, setting the gamma correction to 1.3 – 1.6.

Procedure 2: Median filter subtraction

- 1) Create a median filtered stack.
 - A. Open the “Basic Filters” window in the “Process” dropdown menu.
 - B. Choose the “median” filter option.
 - C. Select the image stack to be filtered and the “All Planes” option, then “Apply”.
- 2) Subtract the median filtered stack from the original stack.
 - A. Open the “Arithmetic” window in “Process” dropdown menu.
 - B. Choose the “subtract” option in the “Operation” section.
 - C. Choose the original unfiltered stack as source image 1 (use all planes).
 - D. Choose the median filtered stack as source image 2 (use all planes), then “Apply”.
- 3) Maximum project the resulting stack.
 - A. Open the “Stack Arithmetic” window in the “Process” dropdown menu.
 - B. Choose the “Maximum” option, then “Apply”.
- 4) Image display
 - A. Open the “Scale Image” function from the Display dropdown menu.
 - B. Move the left slider to the right until the background looks dark gray-almost black and move the right slider to the right until the objects of interest have the desired brightness.

- C. Open the “Adjust Digital Contrast”.
- D. Set the gamma correction between 1.3 – 1.6 to prevent over-saturation of the brightest objects in the image.
- E. Brightness and contrast can be left at 50 in most cases.

5. Measuring mitochondrial length and number

5.1 Experimental system

HeLa cells are used in this study as a model to determine mitochondrial length and number. They were first transfected with pMito-mPlum to visualize mitochondria by fluorescence microscopy. In a second step, HeLa cells were transfected with either control (scrambled) siRNA or OPA-1 siRNA. OPA-1 is essential for mitochondrial fusion [9–11]. Thus OPA1 protein decrease by RNAi generates multiple fragmented mitochondria (Fig. 2B). This is in contrast with the long, tubular, partially interconnected mitochondria that are present in control HeLa cells transfected with scrambled siRNA (Fig. 2A).

5.2 Image acquisition

To obtain quantitative data, a large number of cells are typically imaged. To decrease the time and memory required for this task, an average of 10 z-planes of 0.5–1.0 μm are acquired instead of the smaller z-steps described above. A region of interest (ROI) smaller than the entire field of view can also be selected to further limit the memory required. The stacks are acquired with the Multi-Dimensional Acquisition (MDA) module of MetaMorph 6.3 as described above (Sections 4.1.4 and 4.1.5).

5.3 Image processing and presentation

Median filter subtraction is used to remove the out-of-focus information and to present the data in a single plane using maximum projection (4.2.4; procedure 2).

5.4 Mitochondrial length and number quantification

To measure mitochondrial length and number, dehazed maximum projection images are first ‘skeletonized’. The objects in the image become a single pixel wide and thus the number of pixels that makes up a single skeleton is equal to its length. The thresholding function in MetaMorph helps to select the objects to be skeletonized based on their fluorescence intensity. A black and white, binary image of the selected objects is created as a template for skeletonization. The skeleton length and number are measured for each frame of selected ROI. Quantification of mitochondrial length and number can be performed both on fixed samples (Fig. 2) and for time-lapse series (Section 6 and Fig. 3).

5.4.1 Object selection: Thresholding—Thresholding identifies objects of interest based on their fluorescence intensity. Proper threshold selection is critical for accurate mitochondrial length measurement because a threshold that is too low will merge objects that should be separate and will split apart objects that should be together if it is too high. One must set the same threshold for all images for a given comparative analysis or in a time-lapse series, but one can re-adjust the threshold for each new experiment.

5.4.2 Conversion of selected objects into skeletons—In order to create a template for skeletonization, the thresholded image is converted from a gray scale image to a black and white image, by 'binarization'. All pixels above the chosen threshold intensity (objects of interest) are converted to white or "1" and the lower ones are converted to black or "0". In order to remove some noise and artifacts, the binarized image is eroded, meaning that pixels which have few other pixels around them are removed. Skeletonization converts the width of each binary object to one pixel. After skeletonization of the image, single pixels (i.e., any pixel with no neighbors) are removed to further reduce noise and artifacts.

5.4.3 Logging mitochondrial length and number—Pixels can be easily converted to μm in MetaMorph by calibrating the microscope based on the objective used. Using the "Region Measurements" or "Show Region Statistics" function in MetaMorph, the "distance" or the "perimeter" of the objects can be directly logged into an Excel spreadsheet. These measures are $2\times$ the skeletal length, because the skeletons are only one pixel thick.

5.5 Step-by-step procedure for quantification of mitochondrial length and number

5.5.1 Image thresholding and skeletonization

- 1) Open a filtered, maximum projected image.
- 2) Open the "Binary operations" window from the "Process" dropdown menu.
- 3) Adjust the threshold with the cursor in the "Parameter" area of the "binary operations" window.
- 4) In the "Operation" section, choose the "Binarize" option to exclude all objects with a intensity lower than the threshold and click "Apply".
- 5) Choose "Erode" with parameters set on 6 neighbors and 1 pixel in the "Operation" section and click "Apply".
- 6) Choose "Skeletonize" in the "Operation" section and click "Apply".
- 7) Choose "Remove single pixels" in the "Operation" section and click "Apply".

5.5.2 Selection of regions of interest (ROIs)—Choose a region tool by selecting the corresponding icon in the menu task bar and select one or several ROIs. We usually do not select the mitochondria that are part of the complex network surrounding the nucleus because it is difficult to determine their precise shapes and dimensions of single mitochondria due to the high density of mitochondria in the perinuclear region (Fig. 2 A, B).

5.5.3 Measurements

- 1) In the "Region" menu, select "Create region around objects".
- 2) Open the "Region Measurements" function from the "Measure" dropdown menu and go to "Open Log" to create an Excel spreadsheet window.
- 3) Log all the regions in a selected image by clicking the "Log Data" button, which appears once you open the log. One can configure the region measurements log to include one's choice of parameters and image identification information. The

mitochondrial length is calculated by selecting the “perimeter” parameter and dividing the corresponding values by two.

5.5.4 Analysis and results—The length and number of mitochondria in two cell populations (scrambled or OPA1 siRNA transfected HeLa cells) can be presented as averages (Fig. 2A and 2B) or as histogram (2C). In Figure 2C, the mitochondrial populations are categorized into six groups (bins) according to their sizes. One can use the “Data Analysis tool pack” in Excel (Microsoft Office) to convert the data into a histogram, by using the “Add-Ins” (in the “Tools” menu). The two cell populations ($n=10$) do not have statistically different numbers of mitochondria in the median range ($0.5-5\ \mu\text{m}$). In contrast, the histogram indicates that OPA1 siRNA treated cells have a significantly larger percentage of small mitochondria ($<0.5\ \mu\text{m}$) and fewer long mitochondria (between $5-10\ \mu\text{m}$). Figures 2D and 2E represent the average total number and length of mitochondria in OPA1 siRNA and scrambled siRNA cell populations ($n=10$), respectively. The OPA1 siRNA transfected cells have significantly shorter mitochondria than the control cells (Fig. 2E). Although the average number of mitochondria is higher after OPA1 siRNA transfection, this difference was not statistically significant at $n=10$ due to the large variations within each cell population (Fig. 2D).

6. Measuring mitochondrial dynamics by time-lapse imaging

6.1 Experimental system and set-up

To illustrate mitochondrial dynamics by live cell imaging, we triggered mitochondrial fission in motor neurons by removing neurotrophic factors. Astrocytes were grown on poly-L-lysine coated LabTek II chamber dishes (#1.5 glass) (Nalgen Nunc) (Section 3.1). Spinal cord motor neurons were then isolated, transfected with DsRed2-Mito by electroporation, and seeded on top of the astrocyte monolayer. LabTek II dishes have a 4-well format that allows simultaneous imaging of controls and treatments and using the same neuronal preparation.

Auto-fluorescence from the culture medium must be minimized for live-cell imaging. For this purpose, cells are either grown in a custom-made DMEM (Hyclone) medium lacking phenol red, riboflavin and folic acid. Alternatively, the medium can be replaced with Hibernate-E_{lf} (BrainBits, Inc.) medium before the experiment. The latter method was used here. Two hours before image acquisition the culture medium was replaced with Hibernate-E_{lf} containing no serum or neurotrophic factors. The culture was then imaged for 3 hours to monitor mitochondrial dynamics. Hibernate-E_{lf} may be supplemented with 10% bovine calf serum (CS) and penicillin/streptomycin for long-term time-lapse imaging.

6.2 Image acquisition

6.2.1 Microscope setup for live cell imaging—Live cell image acquisition is performed at 37°C under humidified conditions in a 5% CO_2 atmosphere when phenol red-free DMEM is used. Hibernate-E_{lf} medium does not require a CO_2 atmosphere. The microscope temperature is equilibrated 4 hours prior image acquisition to avoid image drift due to temperature changes. A high viscosity oil (Cargille immersion oil type 37, cat.

#16237, Cargille Laboratories) is used to avoid oil loss from the objective during long-term imaging. Z-stacks are acquired with the MDA module of MetaMorph 6.3 as described in 4.1.4 for fixed samples.

6.2.2 Binning and exposure time selection for live cell imaging—For time-lapse imaging, it is critical to minimize the exposure time to prevent photo-toxicity and photo-bleaching during repeated revisiting of the same cells rather than maintaining high resolution. The exposure time and binning are selected as described in section 4.1.3. Typically, 2×2 binning and 100–200 msec exposure times work well for live-cell imaging.

6.2.3 Selecting cells and setting stage coordinates for time-lapse imaging—Here we selected 20 x-y stage positions were selected and each motor neuron was revisited every 10 minutes for 3 hours. Revisiting multiple x-y stage positions over time allows the user to compare mitochondrial parameters with other cellular events [12].

6.2.4 Z-steps and time intervals—For live-cell imaging, a course z-slicing (9–11 planes) is used to minimize memory requirements and photo-damage. The “Range Around Current” option in the z-slice tab is selected to ensure inclusion of the best in-focus slice for each cell.

6.2.5 Step by step procedure for stage position selection

- 1) Open the Multi-dimensional acquisition (MDA) dialogue from the “Apps” drop down menu.
- 2) In the MDA main tab, check “Timelapse”, “Multiple Stage Positions”, “Multiple Wavelengths” and “Z Series”.
- 3) In the “Save” tab, select the saving location by choosing an appropriate file folder and base name for saving the images.
- 4) In the “Wavelength” tab, select the appropriate light source and exposure time for each wavelength to be imaged, using the strategy described above (Section 4.1.3).
- 5) In the “Timelapse” tab, input the number of time points and the time interval. (The software can automatically calculate the total duration based on the number you input and give you an estimated minimum interval based on the exposure time).
- 6) “Z Series” tab
 - A. Input the Z slice size.
 - B. Check “range around current” and input the range you want to acquire around the z-position.
 - C. MetaMorph will calculate the number of z-slices needed; check to make sure this is appropriate for your application.

- 7) In the stage position tab of the MDA menu, clear all stage positions from previous experiments using the red x button.
- 8) Scan the 4-well chamber slide using the eyepiece or directly on the computer using the “Show Live” option.
- 9) Adjust manually the focus at the best in-focus position, using the “Show Live” option in the MDA menu.
- 10) Type a new identification number for the selected stage position in the “Position label”.
- 11) Input the new stage position name into the stage position list using the arrow button (each time you put the stage position, make sure the position name in the “Position label” panel is not the same as the ones already in the list to prevent overlap).
- 12) Repeat steps 2–5 for all stage positions to be imaged for the current experiment.
- 13) After selecting all cells to be imaged, the stage positions are saved as a file that can be reload into the stage selection window if a computer error should occur during setup or acquisition.
- 14) Start the acquisition by clicking the “Acquire” button.

6.3 Reviewing acquired time-lapse data

When the time course is complete, each time-point of each x-y stage position has been automatically saved as a separate z-stack into the folder specified in the “Saving” tab of the MDA menu at the start of the experiment. The “Review Multi Dimensional Data” function in the “Apps” dropdown menu is used to view automatically the maximum projections of all time-points of a given stage position. This function allows one to quickly evaluate the time-lapse series and to choose representative time-points of a given x-y stage position and cell (Fig.3). In the selected motor neuron, fragmentation of the mitochondria begins around the 80-minute time-point and continues until the 190-minute time-point (Fig. 3A). The arrows in the images point to sites of mitochondrial fission in comparison with the previous image.

To make a movie from a time-lapse series, a new stack containing the maximum projected time-points for each stage position is created, using the “add plane” function in the “stack” dropdown menu. Each stack, corresponding to a single stage position, can be converted to an avi movie file or a Quicktime file using the “make movie” function in the “stack” dropdown menu. To insert a movie into a PowerPoint presentation, go to the “Insert” dropdown menu in Powerpoint and click “add movie from file”. The movie file will be inserted into your PowerPoint presentation.

6.4 Time-lapse image processing and mitochondrial length/number quantification

Image processing for time-lapse experiments is the same as for fixed samples except that due to the large quantity of data, it is critical to automate the image processing by writing journals.

6.4.1 Writing/Recording Journals—Journals are written using the “Start Recording” function in the “Journal” drop down menu of Metamorph. Thresholding is described in the first journal below. The threshold settings needed to highlight the mitochondria will vary from experiment to experiment; thus, this journal must be edited for each new experiment. It is useful to load the appropriate journals onto a ‘Taskbar’. This is performed by using the “Create Taskbar” function from the “Journal” dropdown menu and by dragging and dropping the journals of interest into the Taskbar.

Journal 1: filtering and skeletonization

1. Open the first image stack of a time-lapse series.
2. Click “Start Recording” in the “Journal” dropdown menu.
3. Open the “Basic Filters” function in the “Process” dropdown menu.
 - A. Select “Median” in the “Operation” section of the window.
 - B. In the “Source Image” choose section “Select on Playback” (select the original image when prompted in the dialog box) and then “Apply”.
4. Open the “Arithmetic” function in the “Process” dropdown menu.
 - A. In “source image 1” choose “Select On Playback”.
 - B. In “source image 2” choose “Last Result”.
 - C. In the “Operation” choose section “Subtract” and then “Apply”.
5. Open the “Stack Arithmetic” function in the “Process” dropdown menu.
 - A. In the “Source Image” select “Last Result”.
 - B. In the “Operations” select “Maximum” and then “Apply”.
6. Open the “Binary Operations” function in the “Process” dropdown menu.
 - A. In the “Operation” select “Binarize”.
 - B. In the “Source Image” choose “Last Result”.
 - C. In the “Parameters” select the appropriate threshold setting. The setting should highlight the mitochondria and omit the background and is good for all time-points and x-y stage positions for a given experiment.
 - D. Click “Apply”.
 - E. In the “Operation” select “Erode” (use 1 pixel, 6 neighbors).
 - F. In the “Source Image” choose “Last Result” and then “Apply”.
 - G. In the “Operation” select “Skeletonize”.
 - H. In the “Source Image” choose “Last Result” and then “Apply”.
 - I. In the “Operation” select “Remove Single Pixels”.
 - J. In the “Source Image” choose section “Last Result” and then “Apply”.

7. In the “File” dropdown menu select “Save as”.
8. In the “File” menu select “Close All” and select the “Close without saving open images” option in the window that opens.
9. In the “Journal” dropdown menu select “Stop Recording”.
 - A. Save the journal.
 - B. Then add the journal to a taskbar.
10. Click yes and drag it to the taskbar that appears.

Journal 2: Logging mitochondrial length

1. Open the first two skeletons of the stage position of interest.
2. Open the “Region Measurements” tool in the “Measure” dropdown menu.
 - A. Click the “Open Log” button and select “Dynamic Data Exchange (DDE).”
 - B. Ensure “Microsoft Excel” is the application selected in the window that appears and click “OK”.
3. In the “Regions” menu, select “Region Tools” and choose an icon from the taskbar to draw a region of interest, excluding the soma.
4. In the “Journal” dropdown menu select “Start Recording”.
5. In the “Regions” dropdown menu select the “Transfer Region” function.
 - A. In the “Source Image” window choose “Select On Playback”.
 - B. In the “Destination Image” window choose “Select On Playback”.
 - C. In the “Region selection” section of the window select “Active”.
6. In the “Journal” dropdown menu choose the “Select image” tool contained within the “Recording Tools” submenu and “Select On Playback”.
7. In the “Regions” dropdown menu choose “Create Regions Around Objects”.
8. In the “Journal” dropdown menu choose the “Select Image” tool contained within the “Recording Tools” submenu and “Select On Playback”.
9. In the “Region Measurements” window (which was opened before recording this journal) click the “Log Data” button.
10. In the “Journal” dropdown menu select “Stop Recording”.
 - A. Save the journal.
 - B. Then add the journal to a Taskbar. Click yes and drag it to the Taskbar.

6.4.2 Running Journals

Running the Filtering and Skeletonization Journal: In the “Journal” dropdown menu select the “Loop journal for all Images in Directory” function within the “Loop” submenu.

Then select the directory containing only your time-lapse image files (The .nd file which is automatically created by MDA must be removed from the folder prior to looping the journal.) and the journal you wish to run. Choose the “Filtering and skeletonization” journal written above (Section 6.4.1). Click the “Run” button. While the journal is running, one has to select images to be processed. First, one has to select the source image for the basic filter (the popup window will say “jnlBasicFilter.imSource:Basic Filters”). Then, one chooses the image stack corresponding to the first time-point. Next, one has to choose the first source image for the image arithmetic (the popup window will say: “arithmeticJNL.imSource1:Arithmetic”). Choose the same image that was selected in the basic filter window (the current unfiltered image). Finally, one has to decide on the saving location for the skeletonized image just created. The next image stack in the time course will open automatically and one will follow the same procedure as for first image stack. This should be the file that just opened automatically. If it is not, one might have to rename the files in the image folder such that when they are sorted by name they are in chronological order. The journal will stop automatically when the last image stack of the time course has been filtered, skeletonized and saved.

Running the Logging Mitochondrial Length Journal: Prior to starting the journal one need to open the Excel sheet as described in Journal 2. In the “Configure” tab of the “Region Measurements” window, check-mark the image name, distance, x-y stage position, and acquisition date. Open the first skeletonized image of the x-y stage position and draw a region of interest. With these windows open, the “Loop journal for all Images in Directory” function must be selected from the “Journal” dropdown menu within the “Loop” submenu. Then one has to select the source image for the transfer region function (the popup window will say “TransferRegionsJNL.imSourceImage”). Then one picks the skeletonized image corresponding to the first time-point and each time one is asked for the source image throughout the run. One also has to select the destination image for the active region (the popup window will say “TransferRegionsJNL.imDestImage”). For the first run, the source and destination image will be the same. For each subsequent run, one chooses the next skeletonized image for the destination image. The data will be automatically logged into the open Excel file.

6.5 Data Analysis for time-lapse series

The logged Excel data contains the “distance” of each skeleton (a measure equal to twice the skeletal length of each object) as well as the time-point and x-y stage position of each skeletonized image. The data also contains measurements of the region of interest drawn to exclude the soma in the first line of each time-point. This line should be deleted to simplify data analysis.

Motor neurons undergoing neurotrophic factor withdrawal undergo mitochondrial fission (Fig. 3A). This event coincides with a decrease in mitochondrial length calculated with this method (Fig. 3B). A decrease in the mitochondrial length starts at 80–90 minutes and reaches a minimum length of 1.5 μm at 140–150 minutes. As the length of the mitochondria decreases, their numbers increases (Fig. 3C). This indicates that mitochondria indeed

underwent fission instead of simply changing their morphology from elongated to round (Fig. 3A).

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Figure 1. Mitochondrial morphology in different cell types

Mitochondrial morphology of HeLa cells (left panels), astrocytes (middle panels), and motor neurons (right panels). HeLa cells express Mito-mPlum to visualize mitochondria. Astrocytes and motor neurons express DsRed2-Mito. Images were acquired by collecting 10 z-planes of 1 μm spacing. (A) Maximum projection of unfiltered, raw images. (B) Maximum projections of Fourier transformed images. (C) Maximum projections of median filter subtracted images. Scale bar: 25 μm .

Figure 2. Quantitation of mitochondrial fission by OPA1 siRNA transfection in Mito-mPlum transfected HeLa cells
Representative images of Mito-mPlum expressing HeLa cells transfected with (A) scrambled siRNA or (B) OPA1 siRNA after median filter subtraction, following an acquisition of 15 z-planes of 0.2 μm spacing. Mitochondria in (A) exhibit elongated and tubular morphology, while mitochondria in (B) exhibit fragmented morphology. Dashed lines indicate selected regions of interest (ROI). Scale bar: 10 μm. (C) Comparison of mitochondrial length distribution in OPA1 siRNA and scrambled siRNA transfected HeLa cells (n=10). * indicates significant differences between the two cell populations in ROI (p<0.01; Student's t-test, two-sided). (D) Average number of mitochondria in OPA1 and scrambled siRNA transfected HeLa cells (n=10) in ROI, excluding perinuclear and nuclear area. (E) Average mitochondrial length (μm) in ROI (excluding perinuclear and nuclear area) of OPA1 and scrambled siRNA transfected HeLa cells (n=10). ** indicates significant difference between the two cell populations (P<0.01; Student's t-test, two-sided).

Figure 3. Mitochondrial fission in a motor neuron addressed by 3D time-lapse imaging

(A) Representative images of a 190-minute time-lapse series of a motor neuron transfected with DsRed2-Mito undergoing mitochondrial fission due to neurotrophic factor withdrawal. Images were maximum projected and medium filtered. The inset is a 3 $\times$ zoom of the indicated ROI showing a single mitochondrion undergoing fission over time. Scale bar: 25 μm . The arrows indicate fission of selected mitochondrial filaments. The arrowheads indicates condensation. (B) The mean mitochondrial length of the motor neuron shown in (A) is plotted over time. (C) Mitochondrial numbers within the ROI of the same motor neuron is plotted over time. An increase mitochondrial numbers occurs at ~ 100 minutes concomitant with a decrease in size (B).