SUPPLEMENTARY INFORMATION

Membrane bending energy and tension govern mitochondrial division

Lina Carlini[1*], Dora Mahecic[1*], Tatjana Kleele[1], Aurélien Roux[2,3], and Suliana Manley[1]

[1] Institute of Physics, École Polytechnique Fédérale de Lausanne (EPFL), Route Cantonale, 1015 Lausanne, Switzerland

[2] Department of Biochemistry, University of Geneva, Geneva, Switzerland
[3] Swiss National Centre for Competence in Research Programme Chemical Biology, Geneva, Switzerland

[*] These authors contributed equally to this work

Correspondence should be addressed to
S.M. (email: suliana.manley@epfl.ch)
Institute of Physics
École Polytechnique Fédérale de Lausanne
Lausanne, Switzerland

MATERIALS AND METHODS

Cell culture, transfection and drug treatment

Cos-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were plated on 25 mm, #1.5 glass coverslips (Menzel) 16-24 h prior to transfection at a confluency of ~10^5 cells per well. Dual transfections containing mCh-Drp1 (Addgene, plasmid #49152) and Mito-GFP (gift from Hari Shroff) were performed with either Lipofectamine 2000 (Life Technologies) or using electroporation (BioRad Xcell). Lipofectamine transfections were carried out in OPTI-MEM using 150 ng of mCh-Drp1, 150 ng of Mito-GFP and 1.5 µL of Lipofectamine 2000. All quantities listed are per well of cells containing 2 mL of culture medium. The Lipofectamine mixture sat for 20 min before its addition to cells. Electroporation was performed using salmon sperm as a delivery agent. Briefly, cells were pelleted by centrifugation and resuspended in optiMEM. Plasmids and sheared salmon sperm DNA were added to 200 µL of the cell suspension prior to electroporation using a Bio-Rad Gene Pulser (190 Ω and 950 µFD).Triple transfections containing mCh-Drp1 (Addgene, plasmid #49152), Mito-BFP (Addgene, plasmid #49151) and Dyn2-GFP (gift from Gia Voeltz) were performed with Lipofectamine 2000 (Life Technologies). Lipofectamine transfections were carried out in OPTI-MEM using 80 ng of mCh-Drp1, 100 ng of Dyn2-GFP and 80 ng of Mito-BFP and 1.5 µL of Lipofectamine 2000. All quantities listed are per well of cells containing 2 mL of culture medium. The Lipofectamine mixture sat for 20 min before its addition to cells.

For drug perturbations, cells were incubated with 10µM Nocodazole (Sigma-Aldrich) for 1h before imaging. Control cells were incubated with the equivalent volume of DMSO for 1h before imaging.

Microscopy

Structured Illumination Microscopy

Fast dual-color SIM imaging was performed at Janelia Farm with an inverted fluorescence microscope (AxioObserver ; Zeiss) using an SLM (SXGA-3DM; Fourth Dimension Displays) to create the illumination pattern and liquid crystal cell (SWIFT; Meadowlark) to control the polarization. Fluorescence was collected through a 100X 1.49 NA oil immersion objective and imaged onto a digital CMOS camera (ORCA-Flash4.0 v2 C11440; Hamamatsu). Time-lapse images were acquired every 1 s for 3-5 min, with 50 ms exposure time.

Dual-color SIM imaging for Nocodazole and Dyn2 experiments was performed on an inverted fluorescence microscope (Eclipse Ti; Nikon) equipped with an electron charge coupled device camera (iXon3 897; Andor Technologies). Fluorescence was collected with through a 100x 1.49 NA oil immersion objective (CFI Apochromat TIRF 100XC Oil; Nikon). Images were captured using NIS elements with SIM (Nikon) resulting in temporal resolution of 1 s for single-color and 6-8s for dual-color imaging. Images were acquired every 1 s for single color and every 6-8 s for dual color timelapse, with 50 ms exposure time. All imaging was performed at 37˚C with 5% CO2, in pre-warmed DMEM medium.

Confocal microscopy

Confocal imaging was performed on an inverted microscope (DMI 6000; Leica) equipped with hybrid photon counting detectors (HyD; Leica). Fluorescence was collected through a 63x 1.40 NA oil immersion objective (HC PL APO 63x/1.40 Oil CS2; Leica). Images were captured using the LAS X software (Leica). All imaging was performed at 37˚C in pre-warmed Leibovitz medium.

STORM

Prior to staining, cells were washed with PBS (Sigma). Cells were incubated with MitoTracker Red CMXRos at a concentration of 500 nM for 5 minutes, before washing again with PBS. STORM imaging was performed at room temperature in a glucose-oxidase/catalase (Glox) oxygen removal buffer described in Shim et al[1]. Briefly, a 2% glucose solution is prepared in DMEM (Gibco). Glucose oxidase (0.5 mg/mL) and catalase (40 μg/mL) were added to the glucose solution and the pH was left to drop for 30-60 min. After this time, the pH was adjusted to 7 yielding a final solution with 6.7% HEPES. Imaging was performed on an inverted microscope (IX71; Olympus) equipped with a 100x NA 1.4 oil immersion objective (UPlanSAPO100X; Olympus) using an electron multiplying CCD camera (iXon+; Andor Technologies), with a resulting pixel size of 100 nm. Laser intensities were between 1-5 $kWcm^{-2}$.

Image Reconstruction

SIM reconstruction

SIM images were reconstructed using a custom 2D linear SIM reconstruction software obtained at Janelia farm, as previously described [2,3]. Images were reconstructed using a generalized Weiner filter parameter value of 0.02-0.05 with background levels of ~100.

STORM reconstruction

Single molecules were localized using the RapidSTORM v3.3 software. Local signal-to-noise detection with a threshold value of 50 was used. Peaks with a width between 70-300 nm and at least 200 photons were rendered for the final STORM image.

Image analysis

Images were first segmented using the Weka Segmentation V3.2.17 ImageJ plugin with the resulting probability map used as the segmented image. Subsequent analysis was performed using a custom MATLAB functions which contoured the mitochondria and created a backbone with a mesh (Supplementary Fig. 3). This allowed us to measure the diameter and curvature along the constricted mitochondrion, and hence estimate the local bending energy. Tracking the position of the constriction site allowed us to measure the local Drp1 intensity. Tracking the leading edge of divided daughter mitochondria was used to estimate the membrane tension prior to fission.

Statistics

Datasets were tested for normal distribution using the D'Agostino-Pearson normality test using a significance value of 0.05[4]. If the datasets passed the test, then statistical significance was determined using a two-tailed t-tests. If datasets failed the normality test, then a nonparametric test was performed using the Mann-Whitney test with one or two-tailed distributions where appropriate[5]. $P<0.05$ were considered as significant and were marked by '*'; $P<0.01$ with '**' and $P<0.001$ by '***'.

Software description

The custom MATLAB package allowed us to perform the summarized steps (Supplementary Fig. 3):

1. ***mitoTrack***: Track mitochondria in the segmented image, and crop the mitochondrion of interest from both segmented and original time-lapse images for different channels
2. ***genContour***: Create contour of the mitochondrion of interest using a built-in matlab function based on Chan-Vese active contouring of the segmented[6]. A small Gaussian filter is applied prior to contouring. The generated contour is then smoothed over a length scale of ~ 170 nm, which was found to be optimal for eliminating noise without sacrificing envelope curvature sensitivity. Contour smoothing was performed using a third-party function based on least-squares smoothing for MATLAB[7].
3. ***mitoMesh***: Create a backbone of the mitochondrion of interest. The backbone is smoothed over a lengthscale of ~ 150 nm. Use generated backbone to divide mitochondrion into smaller segments, with the boundaries represented by a mesh[8] .
4. ***genCurv***: measure the curvature along the contour of the mitochondrion using a third party 'LineCurvature2D' function for MATLAB[9].
5. ***minDiameterSearch***: Use the diameters measured along the mesh to track the position of the constriction site, and measure its diameter.
6. ***measureBE***: Use mesh to measure dimensions of individual segments. For each segment, use the measured diameters and envelope curvatures to estimate the bending energy and bending energy density of that segment. Find the length scale that maximizes the local bending energy density.
7. ***genFWHM***: Generate FWHM based contour by fitting profiles plotted along the mesh with a Gaussian function. Connect the measured widths at along the mitochondrion and smooth at a length scale of ~ 170 nm to generate the FWHM contour.
8. Repeat *minDiameterSearch* and *measureBE* for FWHM contour.

9. ***measureDrp1***: Measure the local Drp1 intensity at the constriction site. Subtract background and bleach correct using a custom-written linear bleach correction function.
10. ***mitoPull***: Repeat *genContour* and *mitoMesh* for daughter mitochondria after fission. Track the leading edge retracting from the constriction site to estimate membrane tension.

To compare the snake and FWHM contours we simulated SIM images[10] of different shapes representing mitochondrial constriction sites at an SNR < 2, the typical value at mitochondrial constriction sites. We observed that although the measured diameters were comparable between the two contours, the snake contour was better at detecting high envelope curvatures (Supplementary Fig. 4). Hence the analysis was performed on the snake contour. All plots in this work were generated using a third-party function for MATLAB to generate shaded areas representing standard error[11].

Estimating bending energy

To determine the area over which to measure the bending energy, we searched for the length scale that maximized the local bending energy density. This was done by considering areas composed of increased numbers of segments around the constriction site (Supplementary Fig. 6). The appropriate length scale was then selected by finding the area at which the bending energy density (ratio of bending energy and area) was maximal. The bending energy was then considered at this length scale. The length scales did not differ significantly between fissions and reversals (Supplementary Fig. 6C).

The following equation was then integrated over the area corresponding to the appropriate length scale, to find the bending energy[12] E_B:

$$E_B = \frac{\kappa_B}{2} \int J^2 dA \qquad (1)$$

Where κ_B is the bending rigidity, J the average curvature and A the area. The bending rigidity taken to be ~20 kT, as previously reported for lipid bilayers[13]. Since we consider a double-membrane system, we take the value of κ_B= 40 kT.

Estimating membrane tension

We used a visco-elastic model of the retraction of the mitochondrial membrane after fission to estimate the local membrane tension. The same model was used previously to estimate the tension in the intercellular bridge during cytokinesis[14].

$$\sigma(t) = \int_{-\infty}^{t} \frac{du}{dt}(t = t^*) \times E(t - t^*)dt^* \approx \int_{0}^{t_0} \frac{du}{dt}(t = t_0) \times E(0)dt \approx \frac{v(t_0)}{l_0} \times \eta \quad (2)$$

Where σ is the stress, u the strain, E the relaxation modulus. $E(0)$ is approximated as the effective viscosity η at the moment of the break. $v(t_0)$ is the retraction speed at the moment of the break and l_0 the deformation length. The viscosity was taken as $3.2 \cdot 10^{-2}$ kg m^{-1} s^{-1} [15]. The deformation length was measured by comparing the shape of mitochondria before and after fission.

From there, we find the force F by multiplying the stress by the cross-sectional area A:

$$F = \sigma A \cong \sigma \pi r^2 \quad (3)$$

Where r is the constriction radius. The membrane tension τ is then estimated by dividing the pulling force by the circumference at the constriction site:

$$\tau = \frac{F}{2\pi r} \quad (4)$$

Measuring fission time

To estimate the time of fission, we examined the evolution of the constriction site diameter and local Drp1 intensity (Supplementary Fig. 7). The fission time was then taken as the offset of the final increase of Drp1 and decrease in constriction diameter, which should correspond to the constriction time before fission.

Estimating energy contribution of membrane tension

To estimate the contribution of membrane tension to overcoming the energy barrier, we compared the difference in mean bending energies and mean tensions between control and nocodazole treated cells. Since membrane tension scales linearly with tension, we estimated the average contribution of membrane tension E_τ to fission as:

$$E_\tau = \left|\frac{\Delta E}{\Delta \tau}\right| \tau_{ctrl} = \left|\frac{E_{noco} - E_{ctrl}}{\tau_{noco} - \tau_{ctrl}}\right| \tau_{ctrl} \qquad (5)$$

Where E_{noco}, E_{ctrl} are bending energies for nocodazole-treated and control cells respectively, τ_{noco}, τ_{ctrl} the tensions for nocodazole-treated and control cells respectively. This gives a value of ~40 kT.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Examples of fission events

(a,c,e) Time-lapse SIM imaging of COS-7 cells transiently transfected with mito-GFP (grey) and mCh-Drp1 (red). (b,d,f) Corresponding measurements of the constriction diameter dynamics (blue, left axis) and evolution of the normalized integrated intensity of Drp1 at the constriction site (red, right axis). Scale bars represent 1 μm in each panel.

Supplementary Figure 2: Examples of reversal events

(a,c,e) Time-lapse SIM imaging of COS-7 cells transiently transfected with mito-GFP (grey) and mCh-Drp1 (red). (b,d,f) Corresponding measurements of the constriction diameter dynamics (blue, left axis) and evolution of the normalized integrated intensity of Drp1 at the constriction site (red, right axis). Scale bars represent 1 μm in each panel.

Supplementary Figure 3: MitoWorks analysis workflow

Schematic representation of the MitoWorks analysis workflow. Two raw channels and a segmented image are chosen as inputs. The mitochondria were then tracked, and a mitochondrion of interest chosen by the user. This mitochondrion is then contoured, from which MitoWorks creates a mesh and a skeleton. This allows the shape and energetics of the constriction to be analysed over time. Finally, the Drp1 signal at the constriction site is measured.

Supplementary Figure 4: Contour comparison

(a) Simulated SIM image[10] of a constricted mitochondrion was created based on a ground truth (yellow outline) to test the performance of the snake (blue outline) and FWHM (red outline) contours. (b)

Measurement of constriction diameters (top) and envelope curvatures (bottom) for Snake and FWHM contours, compared to the ground truth structure (yellow line). (c) Comparison of snake and FWHM contour from real microscopy data.

Supplementary Figure 5: Fissions and reversals show no distinguishable features with STORM, or the presence of dynamin

(a,b) Time-lapse live-cell STORM imaging of COS-7 cells labelled with MitoTracker Red CMXRos showing a (a) fission and a (b) reversal event. (c) Distribution of minimum constriction diameters for fissions (N=13) and reversals (N=10) measured with STORM. Horizontal line represents the mean. (d) Time-lapse confocal and SIM imaging of mitochondria, Drp1 (top row only) and dynamin-2. (e) Pie charts indicating the fraction of fissions (N=30) and reversals (N=33) observed in the presence of Dyn-2 and Drp1. Scale bar represents 500 nm in each image.

Supplementary Figure 6: Estimating the constriction site length scale

(a) Cartoon illustrating how the length scale was determined by considering increasing areas around the constriction site. (b) The length scale was chosen by maximising the local bending energy density. (c) Distributions of measured length scales for fissions (N=61) and reversals (N=38). Statistical significance calculated by a paired t-test: n.s. indicated $p \geq 0.05$.

Supplementary Figure 7: Estimating fission time

(a) Example of an individual fission event showing how the time of the onset of constriction was chosen. Fission time was estimated as the onset of the rapid constriction coupled with an increase in Drp1 intensity at the constriction site (dotted red line).

Supplementary Figure 8: Tension distribution

(a) Normalized histogram showing the distribution of estimated tensions for fissions observed in control cells (N = 33). Fit curve represents an exponential distribution with mean $\mu = 6.4406 \cdot 10^{-9}$ N/m with confidence interval $[4.73 \cdot 10^{-9}, 9.41 \cdot 10^{-9}]$ N/m.

Supplementary Movie 1: Time-lapse SIM movie of a fission event

Time-lapse SIM imaging of a dividing mitochondrion (labelled with MitoGFP, grey, see Figure 1A) transiently transfected with Drp-mCherry (red). Time= 0 s is defined as the time frame before fission. Scale bar represents 1 μm.

Supplementary Movie 1: Time-lapse SIM movie of a reversal event

Time-lapse SIM imaging of a reversal event (mitochondria labelled with MitoGFP, grey, see Figure 1B) transiently transfected with Drp-mCherry (red). Time= 0 s is defined as the time frame with the minimal constriction diameter before reversing. Scale bar represents 1 μm.

REFERENCES

1 Shim, S. H. *et al.* Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes. *Proc Natl Acad Sci U S A* **109**, 13978-13983, (2012).

2 Gustafsson, M. G. L. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *Journal of Microscopy* **198**, 82-87, doi:10.1046/j.1365-2818.2000.00710.x (2000).

3 Gustafsson, M. G. L. *et al.* Three-Dimensional Resolution Doubling in Wide-Field Fluorescence Microscopy by Structured Illumination. *Biophysical Journal* **94**, 4957-4970, doi:10.1529/biophysj.107.120345 (2008).

4 Trujillo-Ortiz, A. DagosPtest - File Exchange - MATLAB Central. (2015). Retrieved October 13, 2017.

5 Cardillo, G. mwwtest(x1,x2) - File Exchange - MATLAB Central. (2015). Retrieved October 13, 2017.

6 Chan, T. F. & Vese, L. A. Active contours without edges. *IEEE Transactions on Image Processing* **10**, 266-277, (2001).

7 Jim. Least-Squares Smoother - File Exchange - MATLAB Central. (2015). Retrieved May 14, 2016.

8 NS. Curve intersections - File Exchange - MATLAB Central. (2010). Retrieved May 14, 2016.

9 Kroon, D.-J. 2D Line Curvature and Normals - File Exchange - MATLAB Central. (2011). Retrieved August 31, 2016.

10 Alharbi, K. A. Image fusion superresolution in structured illumination microscopy. *California State University Thesis* (2013).

11 Rooney, J. errorbarxy - File Exchange - MATLAB Central. (2003). Retrieved January 7, 2016.

12 Helfrich, W. Elastic properties of lipid bilayers: theory and possible experiments. *Z Naturforsch C* **28**, 693-703 (1973).

13 Niggemann, G., Kummrow, M. & Helfrich, W. The Bending Rigidity of Phosphatidylcholine Bilayers: Dependences on Experimental Method, Sample Cell Sealing and Temperature. *JOURNAL DE PHYSIQUE II* (1995).

14 Lafaurie-Janvore, J. *et al.* ESCRT-III Assembly and Cytokinetic Abscission Are Induced by Tension Release in the Intercellular Bridge. *Science* **339**, 1625-1629, (2013).

15 Hayashi, K. *et al.* Viscosity and drag force involved in organelle transport: Investigation of the fluctuation dissipation theorem. *The European Physical Journal E* **36**, 136, (2013).