Supplementary Information

Experimental section

Isolation of mitochondria

Our method for mitochondrial preparations is a modification of that described by Pon[1] , which is based on the differential centrifugation procedure. Male Wistar rat weighing 150-250 g was killed by cervical dislocation. Approx. 1g liver was quickly removed and submerged in 20 mL solution A (0.22 M mannitol, 0.07 M sucrose, 0.02 M HEPES, 2 mM Tris–HCl, pH 7.2, 1 mM EDTA). Subsequently, the liver was minced with scissors and washed thrice in solution A containing 0.4% BSA to remove blood and connective tissue. The mince were suspended in 20 mL solution A containing 0.4% BSA and then homogenized in a prechilled Dounce homogenizer for 3 min with the loose-fitting pestle followed by 7 min with the tight-fitting pestle. The homogenate was centrifuged at 3000 × g for 2 min (Eppendorf 5804R). The pellet was decanted. The supernatant was centrifuged at 10000 × g for 10 min, and the pellet was washed in Solution A and Solution B (0.22 M mannitol, 0.07 M sucrose, 0.01 M Tris–HCl, pH 7.2, 1 mM EDTA) in succession then centrifuged at 10000 × g for 10 min. The final pellet was resuspended in 1 mL Solution B and stored at 4 °C for use. Protein concentration was determined as 20 mg/mL by the biuret method. All operations were performed at 0 – 4 °C.

Characterization of isolated mitochondria by transmission electron microscopy

The pellet of mitochondria or mitoplasts were fixed, sectioned, stained and observed as described [2].

APTES functionalized mica

The desiccator with two small containers at the bottom was purged with argon for 3 min. Mica sheets were stripped on one side until smooth and immediately placed into the desiccator. 50 µL APTES (3-aminopropyltriethoxysilane, 99%, Sigma-Aldrich, St. Louis, MO) and 15 µL N,N-diisopropylethylamine (99%, distilled, Sigma-Aldrich) were placed into the two small containers respectively [3]. The desiccator was purged with argon for another 3 min and then sealed off, leaving the mica exposed to APTES vapor for 4 h. After this exposure, the treated mica (APTES-mica) was stored in the sealed desiccator until needed.

Mitochondria preparation for AFM imaging

The crude mitochondria suspension was diluted 10 times by Tris buffer (10 mM Tris–HCl, 150 mM KCl, pH 7.4). The sucrose and mannitol was removed by centrifugation at 10000 rpm (Anke TGL-16B) for 10 minutes. Subsequently, 200 µL diluted mitochondria suspension was deposited onto APTES-mica. After 20 minutes, the unadsorbed mitochondria were washed away by Tris–HCl buffer. The prepared sample was mounted into the SPM liquid flow cell containing Tris–HCl buffer and imaged immediately.

Hypotonic treatment of mitochondria

Mitochondria were immobilized on the APTES-mica as above. After the surface was rinsed by Tris–HCl buffer, 200 µL 15 mM KCl was dropped onto the APTES-mica and the sample was kept on ice for 15 min, then washed and imaged. Alternatively, the mitochondria suspension was 10 times diluted by hypotonic buffer (15 mM KCl) in 1.5 mL eppendorf tube and kept on ice for 15 min.

Freeze-thaw mitochondria

The crude mitochondrial suspension was subjected to two successive freeze-thaw cycles at -20 °C, centrifuged to remove the fragments, and then immobilized on the APTES-mica for AFM imaging as above.

In-situ AFM imaging

AFM imaging was performed by 5500 AFM (Agilent Technologies, Chandler, AZ). The topographic images of mitochondria and mitochondrial membrane were acquired by Acouctic AC (AAC) mode AFM using oxide-sharpened microfabricated Si_3N_4 probes (Veeco, DNP-S) with a spring constant of 0.06 N/m (nominal) at a scan rate of 1.68 Hz. All the images were obtained at room temperature (21-25 °C) in Tris–HCl buffer and recorded with 512 × 512 pixels. Mitochondria dimension and membrane heights were measured using PicoScan 5.3.3 software (Agilent Technologies, Chandler, AZ). The membrane roughnesses were measured using Scanning Probe Image Processor (SPIP, Image Metrology A/S).

High resolution image of the outer surface of the intact mitochondria

To further reveal the details of the outer mitochondrial membrane, we tried to obtain the even higher resolution image. As shown in Fig.S1, still no apparent proteins were detected at the membrane surface. The average roughness of the entire membrane surface is measured to be 1.1 ± 0.1 nm, much less than that of membranes with obvious protein particles (Fig.4A-C).

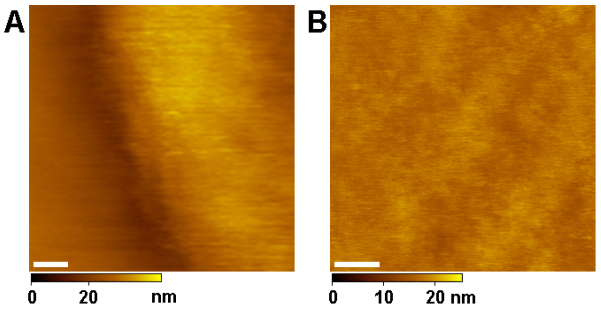

Fig.S1 High resolution image of the outer surface of the intact mitochondria. (A) shows the edge of the mitochondrion with the substrate visible; (B) is the image of the small square area on the mitochondrion. Scale bar: 30 nm.

Isolated outer mitochondrial membrane

By combining topography (Figure.S2A) and phase images (Figure.S2B), we further demonstrate the exsiting of protein praticles underneath the upper smooth membrane layer. In Figure.S2B, the protein particles can be clearly distinguished from the lipid membrane because of different stiffness, elasticity etc [4].

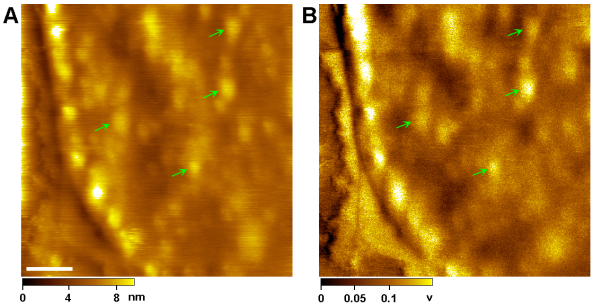

Fig.S2 (A) AFM topographic image of isolated outer mitochondrial membrane. (B) Corresponding AFM phase image. Arrows point to protein particles underneath the lipid membrane. Scale bar: 200 nm.

References

1. L. A. Pon and E. A. Schon, Methods in Cell Biology. Mitochondria, 2001,**80**, 17-25.
2. V. Petronilli, J. Sileikyte, A. Zulian, F. Dabbeni-Sala, G. Jori, S. Gobbo, G. Tognon, P. Nikolov, P. Bernardi and F. Ricchelli, Biochimica Et Biophysica Acta-Bioenergetics, 2009, **1787**, 897-904.
3. Y. Lyubchenko, L. Shlyakhtenko, R. Harrington, P. Oden and S. Lindsay, Proceedings of the National Academy of Sciences of the United States of America, 1993, **90**, 2137-2140.
4. R. Garcia, R. Magerle and R. Perez, Nature Materials, 2007, **6**, 405-411.

OM	IMS	MS	Mitoplast	Mitochondria
1.1859	0.3603	1.5137	14.2903	0.9558
1.5386	0.3505	1.4528	11.579	1.2145
1.1595	0.4391	1.3957	8.2361	1.1826
1.1723	0.3194	1.4909	8.2426	1.1149
1.4368	0.405	1.8558	8.0867	1.11695
1.2423	0.7514	1.9471	8.7723	0.11518
1.2613	0.6149	1.3789	11.7089	
0.9089	0.5462	0.9309	10.13084	Average
0.9627	0.5063	1.1932	2.238245	Stdev
1.0527	0.5982	1.3534		
1.0406	0.5659	1.2757		
1.2429	0.4468	1.1989		
1.4172	0.617	1.9561		
1.1752	0.6112	1.7308		
1.1768	0.5657	1.738		
0.9079	0.6075	1.1474		
1.7124	0.5796	1.6205		
0.9078	0.4133	1.6375		
1.5347	0.5559	0.6		
0.7536	0.8587	0.6651		
0.8006	0.7861	2.3643		
1.0224	1.2627	2.2081		
0.8333	1.073	1.458196		Average
0.961	0.529	0.374695		Stdev
0.6793	0.5192			
0.6908	0.668			
0.6114	0.5783			
0.1365	0.4428			
0.7076	0.4535			
0.5556	0.6189			
0.6764	0.5706			
0.8725	0.587581			Average
0.6785	0.198697			Stdev
0.6622				
0.6221				
1.1316				
1.711				
1.3467				
0.7039				
0.7851				
1.1759				
1.4019				
0.8474				
0.8329				
0.8808				
0.6675				
0.6345				
1.282				
1.5446				
1.6576				
1.7146				
1.3361				
0.8705				
0.8715				
1.2105				
0.78				
0.9106				
1.0604				
0.8068				
0.8329				
0.8462				
1.3174				
1.1533				
1.0164				
0.5484				
0.5266				

0.8542				
0.681				
0.7551				
0.6037				
0.5886				
0.7031				
1.1622				
1.1269				
0.962683				Average
0.273344				Stdev

Table 1. The whole data for the roughness measurements. For roughness measurement on the membranes, several units with equal size (usually 200-300 nm) are chosen as the representatives and all these roughness values are averaged. OM: the outer mitochondrial membrane; IMS: the intermembrane space surface; MS: the matrix side; Mitoplast: the outer surface of the mitoplast ; Mitochondria: the outer surface of mitochondria.