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# Inner membrane dynamics in mitochondria

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ABSTRACT

Combining the use of cells with sparse cristae marked with IMP–EGFP and short pulsed sub-saturating fluorescence excitation (non-saturation fluorescence microscopy/NSFM) revealed inhomogeneous fluorescence distribution along mitochondria in living cells. Also the matrix located TMRE was distributed non-uniformly and at least in part filling the gaps between the IMP–EGFP fluorescence: fluorescence intensities are modulated in space and time in part in an antidromic manner. The spatial modulations can be interpreted to represent cristae/matrix distributions. The temporal fluctuations of fluorescence vary within 0.3–3 s. Because most peak positions of IMP fluorescence remain stationary up to at least several minutes, temporal intensity modulations may result from varying emissions related to the degree of excitation and/or represent wobbling of cristae, i.e. lateral movements, bending or size changes. Modulations by noise and non-saturated excitation have been reduced by 3 steps of deconvolution followed by averaging 4 images. This allowed a final temporal resolution of 150 ms. Disappearance of cristae or formation of new ones takes place within a few seconds, but these are rare events. Thus position of cristae seems to be rather stable, but they regularly disassemble close to fission sites. Treatment with oligomycin strongly reduces "wobbling" activity.



## 1. Introduction

Mitochondria are well known for their dynamic structure, involving crista shape and shifts in matrix volume (Hackenbrock, 1972; Hackenbrock et al., 1971; Frey and Mannella, 2000; Mannella et al., 2001) as well as their abilities to fuse and divide, to branch and to locomote within a cell (Bereiter-Hahn and Jendrach, 2010). These abilities are essential for their functional maintenance. One good example is the rescue hypothesis, assuming that mitochondrial proteins damaged by their exposure to ROS become diluted within the chondriome by being spread via fusion processes. Indeed, fusion and fission provide a powerful mixing mechanism which distributes mitochondrial proteins throughout the whole chondriome within 2–5 h (Busch et al., 2006; Nunnari et al., 1997; Mouli et al., 2009; Muster et al., 2010). Shape changes and intracytoplasmic displacements of mitochondria can be assumed to comprise concerted activities of mitochondrial membranes and cytoskeleton based motor proteins acting as driving forces. Recently junctions of cristae to the inner boundary membrane were found to be aligned with a complex cytoskeletal superstructure facing the presynaptic membrane in cat axons (Perkins et al., 2010). These observations point to a crosstalk between the mitochondrial interior with its immediate cytoplasmic environment.

Formation or withdrawal of extensions or branches within a few seconds (with a speed in the range up to 30 μm/min) give an idea how fast cristae reorganization may occur, because all these shape changes are accompanied by formation or disintegration of cristae (Bereiter-Hahn, 1978). These findings have been based on correlative microscopy, but no direct observations of IMM dynamics have been reported because the size of the structures is beyond the resolution of diffraction limited fluorescence microscopy. Artificial membrane systems revealed the formation of invaginations resembling mitochondrial cristae within in a time scale comparable to mitochondrial shape changes. Schmidt et al. (2009) succeeded in visualizing mitochondrial cristae in well preserved PtK2 cells after antibody staining using isoSTED and Appelhans et al. (2012) showed single molecule movements within mitochondria using Halo-tagged proteins and high precision localisation. But until now high resolution light microscopy techniques as are e.g. stochastic optical reconstruction microscopy (STORM) or fluorescence photoactivation localization microscopy (FPALM)-related

*Abbreviations:* FPALM, fluorescence photoactivation localization microscopy; NSFM, non-saturation fluorescence microscopy; GFP, green fluorescent protein; IMM, inner mitochondrial membrane; IMP, inner mitochondrial membrane protein; $\Delta\psi$, mitochondrial membrane potential; OMM, outer mitochondrial membrane; px, pixel; ROS, reactive oxygen species; SOFI, super-resolution optical fluctuation imaging; STED, stimulated emission depletion – microscopy; TMRE, tetramethylrhodamine ethylester.

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procedures (Hess et al., 2006), or structured illumination, still are not sufficiently fast to visualize movements within less than a second (Shao et al., 2011; Zhu et al., 2012). A way out of this dilemma is to use a combination of non-saturation fluorescence microscopy (NSFM) similar to FPALM and cells with mitochondria containing only a few cristae which may be away from each other far enough to be distinguished from the adjacent matrix. HeLa cells represent such a cell type, and these allowed us to distinguish IMM structures (marked with GFP-labelled components of respiratory chain complexes) from the matrix compartment with sufficiently good temporal resolution (>40 ms). Recently SOFI-based super-resolution has been brought forward to high spatial resolution but temporal resolution still is in the range of 60 s (Dertinger et al., 2012) and therefore not suited for investigations of mitochondrial dynamics.

Because of the vivid motions of mitochondria in the thin periphery of cultured cells where mitochondria can be observed with high resolution, pretreatment of the cells with nocodazole or nocodazole plus cytochalasin D was applied to stop or at least to reduce these movements. After this pretreatment mitochondria show only minor movements and very rare fusion or fission events. The localisation of the brightest areas which are considered to represent cristae, remained constant within several minutes, and only occasionally continuous lateral movements, disappearance or appearance of new bright structures was observed.

## 2. Materials and methods

### 2.1. Cell culture and staining of mitochondria

Human umbilical vein endothelial cells (HUVEC) were purchased from Promocell (Heidelberg) and cultivated in Endothelial Cell Growth Medium (Invitrogen, Carlsbad) at 37 °C in flasks that had been coated with 0.2% gelatin (Sigma, St. Louis).

HeLa cells were kept in Minimal Essential Medium with Earle's salts (Invitrogen), supplied with 10% FCS, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 1% non-essential amino acids (Invitrogen).

Cell fixation was performed in growth medium supplied with 4% formaldehyde (Applichem, Darmstadt) for 20 min.

5 μg/mL oligomycin (Sigma) was used for blocking the $F_o$ part of ATP-synthase. Disruption of the microtubule network and longitudinal immobilization of the mitochondria was achieved by at least 30 min exposure to 30 μM nocodazole (Sigma) prepared by 1:500 dilution form DMSO stock solution. Actin fibrils were disassembled by exposure to 2 μg/mL cytochalasin D (Applichem) in culture medium. Stock solution of CD was prepared in DMSO and diluted 1:1000.

Mitochondrial matrix staining was performed in growth medium supplemented with 100 nM tetramethyl-rhodaminylester (TMRE) (Invitrogen). TMRE is a membrane potential sensitive dye which translocates into mitochondria but easily is released when mitochondria are disturbed and their membrane potential diminished (e.g. Perry et al., 2011).

### 2.2. Transfection

Both HeLa cells and HUVEC were transiently transfected with the pcDNA 3.1 vectors using Effectene (Qiagen, Venlo) on gelatin pre-coated glass cover slips (Marienfeld, Lauda Königshofen) in 3.5 cm Petri dishes (Sigma) according to the manufacturer's instructions. Experimental observations started 24 h after transfection.

#### 2.2.1. Constructs

pcDNA 3.1 mtDsRed was obtained from Clontech (Mountain View). Matrix targeted GFP (mtGFP) used here, as was described previously (Mai et al., 2010). Mito-mCherry was derived by exchanging the CFP sequence with mCherry via the BamHI and NotI restriction sites from the pcDNA3.1 mtCFP vector (Clontech).

The EGFP labelling of the 30 kDa subunit of the NADH dehydrogenase (CI-EGFP) was performed according to (Busch et al., 2006). The ORFs encoding subunit cox8a of the human cytochrome c oxidase (Complex IV) was amplified from HUVEC cDNA and cloned into the EcoRI and KpnI sites of vector mDsRed-N1 (Clontech). From the resulting constructs cox8a-mDsRed, DsRed was exchanged with mCherry and EGFP via the BamHI and NotI restriction sites resulting in vectors cox8a-mCherry (CIV-mCherry) and cox8a-EGFP (CIV-EGFP).

The constructs were verified by sequencing.

### 2.3. Microscopy, image processing and image analysis

All measurements were performed in a temperature-controlled environment at 37 °C (temperature control system by Life Imaging Services, Basel, Switzerland) and constant $CO_2$ flow. Time series of mitochondrial behaviour were taken with a Leica SP5 (Wetzlar, Germany) confocal microscope, equipped with a HCX PL Apo CS 100× 1.46 oil or Plan Apo 63 N.A. 1.4 lens. Pinhole setting corresponded to 1 Airy or larger.

To avoid crosstalk, EGFP was excited at 458 nm and emission was measured within 493–538 nm, TMRE fluorescence was excited simultaneously at 561 nm and emission was measured within 593–663 nm. The microscope was operated with a resonance scanner at 8000 Hz. Each scan line was 1024 px long, thus single pixel illumination lasted 122 nsec. Excitation intensity was chosen far below saturation, i.e. the single frames were extremely noisy and structures became visible only by image averaging and intensity accumulations. Depending on the averaging (mostly 4× line averaging, sometimes plus 4 frame accumulations) and the number of lines (64–128 lines) the time required for a single image lasted from 37 to 160 ms. Further image processing required deconvolution which was done using the AutoQuant Deconvolution Software (Media Cybernetics, Rockville) with medium noise settings and 3 iterations for noise reduction without major influence of the overall impression of the raw images (Supplementary Fig. 1).

ImageJ was used for further image processing. Pixel intensities either in raw image stacks or deconvolved stacks were averaged for 4 frames, thus the time covered by a single frame used for final evaluation was 148–640 ms. Zoom settings resulted in a single pixel size corresponding to about 30 nm in *x* and *y* direction. For visualization image contrast was increased and pictures were pseudocoloured, but measurements were performed on the full grey level images: total intensity was plotted along a rectangle drawn over the mitochondrial area of interest. Thus the intensity distributions shown for instance in Fig. 3 represent intensity courses along a mitochondrion disregarding the lateral distribution. Because diameters of many mitochondria are >0.5 μm accumulating all *y*-values related to one *x*-value represent the whole cross section on a mitochondrion (point spread function [PSF] in *z*-direction is about 4× the extension of the lateral PSF).

#### 2.3.1. Limitations and possibilities of NSFM

The intention of this study was investigate the dynamics of intramitochondrial compartments. This requires super-resolution microscopy. However, the super-resolution systems available so far do not provide sufficient temporal resolution, therefore we used NSFM, combining non saturation fluorescence excitation with a commercially available confocal microscope with high scanning speed (8 kHz), deconvolution and contrast enhancement by