Materials and Methods

Plasmid constructs

Plasmid cassettes with different fluorescent fusion proteins were generated by substitution of hAGT into the pSEMS-26m vector from @Covalys as described (1). Then, the ORF from different mitochondrial proteins was set between the multiple cloning sites such as the protein of interest was N-terminally placed. The ORF’s were obtained from a selfmade cDNA library from HeLa cells by PCR amplification with the adequate primers. Complex I was labelled at its 30 kDa subunit as described earlier (2). For spreading experiments, paGFP was fused to CI and TFAM, respectively, at their C-termini. Plasmid cassettes with HaloTag7® fusion proteins were generated by substitution of hAGT in the pSEMS-26m vector from ®Covalys Biosciences. HaloTag7 (3) was amplified by PCR from pFC17A-HaloTag (pFC17A HaloTag® CMVd3 Flexi® Vector purchased from Promega). The HaloTag® has been shown to react very quickly and specifically with tetramethylrhodamin (TMR) conjugated to the HaloTag ligand (HTL-TMR) (3). TFAM was cloned N-terminally to HaloTag7 into the pSEMS-26m-HaloTag vector.

Cell culture

HeLa Cells were grown in MEM’s Earle’s with stable Glutamine (Biochrom AG, FG0325) medium supplied with 10% foetal bovine serum superior(FBS) (Biochrom AG, S0615), 1% MEM non-essential amino acids (PAA laboratories GmbH M11003) and 1% 2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonacid HEPES buffer at 37°C with 5% CO2. 3T3 cells were grown in DMEM with 10% FBS superior (Biochrom AG, S0615), 3% Glutamin (Biochrom AG, K0302) and 1% HEPES buffer at 37°C with 5% CO2. For transfection of cells the calcium phosphate method (4) was used.

Single molecule tracking

Before single molecule tracking measurements, cell expressing HaloTagged TFAM were incubated with 0.5 nM HTL-TMR (haloTag-ligand-tetramethylrhodamin) for 30 min. TMR is a bright and photostable dye and substoichiometric labelling allows the recording of single
molecules (5). To remove unbound HTL-TMR, cells were then incubated for another 20 min in incubation medium without HTL-TMR. During this time, medium wash changed twice. For single molecule (SM) recording, an inverse microscope (IX71, Olympus) equipped with a TIRF-condensor (Olympus), and a back-illuminated EMCCD camera (Andor iXON 897) was used. For excitation of TMR, a solid state laser (561nm, 200 mW, CrystaLaser) was focused onto 700 µm multi-mode-optical polarization maintaining monomode fiber (KineFlex, Pointsource) and transmitted via the rear illumination port of the microscope. For excitation, the HiLo mode – illumination with a highly inclined and thin beam - was used (6). The focal drift was negligible (5). A digitally synchronized mechanical shutter controlled exposure times. Laser light reflected from a dichroic mirror (OBS-U-M3TIR 405/488/561, Semrock) passed through a high-numerical aperture objective (150x TIRF objective N.A. 1.45, Olympus, UAPO). Fluorescence emission from TMR was passed through a bandpass filter (Semrock brightline FF01-523/610-25), and was collected by a back-illuminated EMCCD camera. Under this magnification, an image pixel size of 107 nm was obtained. Fluorophores were excited for 15 ms per single image and usually a time series with 1000-3000 images was recorded with 59 Hz and a readout time of 17 ms per single image. The full frame was 512 by 512 pixels with no additional pixel binning, however, due to HiLo excitation, only 375 by 375 pixels were optimally illuminated and further processed by TALM. For localization of single emitters, a modified 2D Gaussian mask for approximation was used (7, 8). Since the mitochondrial diameter in average is 500 nm and the diffraction limited resolution is about 500 nm in z, all signals assigned to a mitochondrion derived from the same mitochondrion. To further confine the focal position without 3D equipment, exclusively single emitters with a signal of 8 times above the mean background were accepted, yielding an excellent calculated localization precision of 12-15 nm for in focus spots. The position of each molecule was then plotted as a Gaussian blurred spot with amplitude proportional to the observed intensity and width equal to the estimated localization precision. Between 300 and 3000 images were analyzed per image series. In parallel, single molecules were tracked using the multiple-target tracing algorithm (9). Multiple deflation loops were performed to ensure identification of all particles. Particle trajectories were recovered based on maximum likelihood estimators (9). At maximum the 24 nearest neighbours were analysed for each trajectory elongation. The two major factors for particle selection are the observed particle displacement and particle intensity. The PDF for observed displacements was modelled as a weighted Gaussian mixture composed of the local diffusion coefficient calculated from the past 6 frames and an expected diffusion coefficient
set to an empirically optimized value between 0.2 and 0.3 \( \mu \text{m}^2/s \) (local and expected diffusion equally weighted). For particle intensity the PDF is made of the weighted sum of a Gaussian function modelling the photon statistics of the particle trajectory under observation and a uniform distribution accounting for the possibility of photon bursts or jumps along the z-axis (weight of Gaussian distributed intensity set to 0.9 owing to the complex photophysics of TMR) (10, 11). In order to recover the entire trajectory a detection gap between successive frames of 1 missing point was closed. For trajectory analysis, only trajectories observed for at least 8 consecutive steps were used, and the analysis window for displacement was set to 5 frames according to short range diffusion. We describe the diffusion constants as to be apparent diffusion constants (\( D_{\text{app}} \)), since we have to deal with the problems of 2D projection.

**Fluorescence Microscopy**

TFAM-paGFP and CI-paGFP spreading were recorded by an inverted confocal laser scanning microscope (Olympus Fluoview FV1000). The cLSM was equipped with a 60x oil immersion objective (UPLSAPO oil, NA 1.35, \( \infty/0.17/FN26.5 \)) and two spectral detectors. In part of the cell (usually 10-20\% of the mitochondria), paGFP fusion proteins were photoactivated by a single scan with 405 nm excitation, paGFP was then excited with 488 nm. Measurements were performed at 37°C in a heated incubation chamber. For the spreading experiment, z-stacks of cells were taken every 2-5 min. Fluorescence for red and green (fusion-) fluorescent proteins was recorded in a sequential mode to avoid cross-talk.

**Electron microscopy**

Sample preparation and electron microscopy was performed as described earlier (12). In short, cultures were fixed using 2.5 \% glutaraldehyde, postfixed with 1\% OsO\textsubscript{4}, scraped cells in 2 \% agar embedded, cubed block stained with 1\% uranyl acetate, dehydrated in a graded series of ethanol, embedded in LV Resin (Agar Scientific) and polymerized for 16 h at 65°C. 90 nm sections were mounted on 100-mesh nickel grids supported by formvar film and stained with 2 \% uranyl acetate for 2 min and Reynolds’ lead citrate for 1 min at R.T.

**Single molecule tracking microscopy**

For single molecule (SM) recording, an inverse microscope (IX71, Olympus) equipped with a TIRF-condensor (Olympus), and a back-illuminated EMCCD camera (Andor iXON 897) was
used. For excitation of TMR, a solid state laser (561 nm, 200 mW, CrystaLaser) was focused onto 700 µm multi-mode-optical polarization maintaining monomode fiber (KineFlex, Pointsource) and transmitted via the rear illumination port of the microscope. For excitation, the HiLo mode – illumination with a highly inclined and thin beam – was used (6). Laser light reflected from a dichroic mirror (OBS-U-M3TIR 405/488/561, Semrock) passed through a high-numerical aperture objective (150x TIRF objective N.A. 1.45, Olympus, UAPO). Fluorescence emission from TMR was passed through a bandpass filter (Semrock BrightLine FF01-523/610-25), and was projected on top a back-illuminated EMCCD camera yielding an image pixel size of 107 nm. The Camera was operated continuously using the CCDs’ Frame Transfer Mode allowing full frame acquisition at 60-100 Hz. For localization of single emitters, a modified 2D Gaussian mask for approximation was used (7, 8). Single molecules were tracked using the multiple-target tracing algorithm (9). Multiple deflation loops were performed to ensure identification of all particles. Particle trajectories were recovered based on maximum likelihood estimators (9). For more details see Appelhans et al., 2012.

References


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Suppl. Figure 1: Ambiguous localization of Twinkle-RFP (TwRFP) in mitochondria. (a) Good but incomplete co-localization of TwRFP (red) with picogreen-stained mtDNA (green) depicted in yellow spots. (b) Unfastened distribution of TwRFP (red) in mitochondria with only partial co-localization with mtDNA (green), most TwRFP has no association with mtDNA. RFP: monomeric red fluorescent protein.