STED and parallelized RESOLFT optical nanoscopy of the tubular endoplasmic reticulum and its mitochondrial contacts in neuronal cells

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ABSTRACT

The classic view of organelle cell biology is undergoing a constant revision fueled by the new insights unraveled by fluorescence nanoscopy, which enable sensitive, faster and gentler observation of specific proteins in situ. The endoplasmic reticulum (ER) is one of the most challenging structure to capture due the rapid and constant restructuring of fine sheets and tubules across the full 3D cell volume. Here we apply STED and parallelized 2D and 3D RESOLFT live imaging to uncover the tubular ER organization in the fine processes of neuronal cells with focus on mitochondria-ER contacts, which recently gained medical attention due to their role in neurodegeneration. Multi-color STED nanoscopy enables the simultaneous visualization of small transversal ER tubules crossing and constricting mitochondria all along axons and dendrites. Parallelized RESOLFT allows for dynamic studies of multiple contact sites within seconds and minutes with prolonged time-lapse imaging at ~50 nm spatial resolution. When operated in 3D super resolution mode it enables a new isotropic visualization of such contacts extending our understanding of the three-dimensional architecture of these packed structures in axons and dendrites.

1. Introduction

Neurons are polarized and three-dimensional cells whose firing activity is dynamically modulated by the local availability of energetic resources and ionic balance. The endoplasmic reticulum (ER), main component of the secretory pathway, extends from the cell soma to the periphery of the dendritic tree, spines and axon. Classically, it is functionally and morphologically subdivided in tubules and sheets/cisternae; sheets are the main site of polyribosomes docking and secre- tory protein production, whereas ER tubules are involved in lipid biogenesis (Jacquemyn et al., 2017), glucose homeostasis (Burchell et al., 1994), calcium storage (GL, 1990) and organelle shaping and transport (English and Voeltz, 2013). In neuronal processes, such as dendrites and axon, ER tubules predominate over sheets (Wu et al., 2017; Cataldo, 1983; Krijnse-Locker et al., 1995; Spacek and Harris, 1997). ER tubular functions are carried out via the interaction with several other cellular organelles including the Golgi apparatus, endosomes and mitochondria. The fundamental role of ER in neuronal functionality is made clear by several neurodegenerative diseases in which protein mutations affect the ER tubular organization and shape in axons, like hereditary spastic paraplegias (Depienne et al., 2007; Adami et al., 2019; Stoica et al., 2016). Even more, alterations in the mitochondria-ER contacts (MERCs or MAMs, mitochondrial associated-membranes) are identified as hallmarks in amyloidogenic pathologies like Alzheimer’s Disease, Parkinson’s Disease and Amyotrophic Lateral Sclerosis (Hedskog et al., 2013). In the interest of the complete understanding of neurodegeneration pathophysiology, it is fundamental to develop tools to study the ER organization and its interactome dynamics in living neurons. At present, most of the imaging-related ER studies in neurons have been performed with Electron Microscopy (EM) (Wu et al., 2017; Terasaki, 2018) which reaches Ångström resolution but it is limited to dead samples, which hamper dynamic studies and can be affected by sample preparation artifacts especially in fragile membrane-enclosed organelles. In non-neuronal cells the endoplasmic reticulum and its interactome dynamics have been extensively studied with live cell fluorescence microscopy techniques in combination with genetic labeling of proteins of interest. For instance, this led to the understanding of the ER role in mediating the first mitochondrial constriction which then triggers the mitochondrial fission by the dynamin-like protein DRP1 (Friedman et al., 2011). Conventional diffraction limited light microscopy, however, lacks the spatial resolution required to resolve the intricate tubular structure of neurite reticulum and its interaction

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2. Materials and methods

2.1. Primary hippocampal neuron cultures

Primary hippocampal cultures were prepared from embryonic day 18 (E18) Sprague Dawley rat embryos. The pregnant mothers were sacrificed with CO₂ inhalation and aorta cut; brains were extracted from the embryos. Hippocampi were dissected and mechanically dissociated in Minimum Essential Medium, MEM, (Thermo Fisher Scientific, 21,090,022). 40 × 10⁶ cells per well were seeded in 12 well plates on a poly-D-ornithine (Sigma Aldrich, P8638) coated #1.5 18 mm glass coverslips (Marienfeld, 0117580) and let them attach in MEM with 10% Horse Serum (Thermo Fisher Scientific, 26,050,088), 2 mM L-Glutamine (Thermo Fisher Scientific, 25,030-024) and 1 mM Sodium pyruvate (Thermo Fisher Scientific, 11,360–070), at 37 °C at an approximate humidity of 95–98% with 5% CO₂. After 3 h the media was changed to Neurobasal Medium (Thermo Fisher Scientific, 21,103-049) supplemented with 2% B-27 (Thermo Fisher Scientific, 17,504-044), 2 mM L-Glutamine and 1% Penicillin-Streptomycin (Sigma Aldrich, P4333). The cultures were kept at 37 °C at an approximate humidity of 95–98% with 5% CO₂ up to 24 days. Medium was changed twice per week. The experiments were performed on cultures starting from DIV 6 up to DIV 10. To perturb the microtubule cytoskeleton, neurons were incubated either with 50 μM of nocodazole (Abcam, ab120630) for 1 h at 37 °C. After treatment, cells where washed three times and then images in ACSF buffer.

All experiments were performed in accordance with animal welfare guidelines set forth by Karolinska Institutet and were approved by Stockholm North Ethical Evaluation Board for Animal Research. Rats were housed with food and water available ad libitum in a 12 h light/dark environment.

2.2. Neuron transfection and live cell staining

For labelling organelles with Halo-Tag and SNAP-Tag technology, neurons were transfected with Halo-Omp25 and SNAP-Sec61β plasmids using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, 11,668,019), according to the instructions of the manufacturer. For labelling fusion proteins, 24 h after transfection, neurons were washed in Artificial Cerebrospinal Fluid (ACSF) and labeled with 5 μM of the SNAP and Halo substrates ((New England BioLabs, SNAP-Cell 647-SIR), 590-Halo ligand), for 1 h at 37 °C. Then, neurons were washed three times with ACSF for 5 min each time. For two-color imaging experiments, the cultures were incubated for 30 min with 2 μM of SiR-Actin (Spirochrome, SC001). The cultures were washed again in ACSF and then imaged in the same buffer at room temperature (RT). HALO-Omp25, SNAP-Sec61β and 590-Halo substrate were kindly gifted by the Dr. Bottanelli laboratory. The AIS was stained with anti-pan-neurofascin primary antibody (UC Davis/NIH NeuroMab Facility clone A12/18) and AlexaFluor488 secondary antibody (ab150113) following the procedure described in D Este et al. Cell Report 2015 (Sidenstein et al., 2016).

For labelling ER with the photo-switchable protein rsEGFP2, neurons were either transfected with the plasmid rsEGFP2-sec61β using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, 11,668,027) following the manufacturer’s instructions; or infected with a modified Semliki Forest Virus expressing the same construct rsEGFP2-sec61β by adding 1 μL of the virus to the culture medium 18 h before experiment. For mitochondria confocal imaging recording, MitoTracker CMXRos (ThermoFisher, M7512) and MitoTracker DeepRed FM (ThermoFisher, M22425) where used, following the manufacturer’s instructions.

2.3. STED imaging

STED nanoscopy has been performed at the Leica TCS SP8 3 × STED,
which is equipped with a HC PL APO 100×/1.40 Oil STED White objective. The images were recorded by exciting AF488, 590-Halo and 647SiR with 488, 590 nm and 650 nm laser lines respectively. A STED beam at 775 nm has been used to deplete 590 and 650 laser lines. Two-color STED images were recorded line-by-line, averaging over 4–16 lines. The spectral detection windows were adjusted to 510–560 for AF488, 600–645 nm for 590-Halo and to 665–750 nm for 647 SiR. The pixel size of the images was 30 or 35 nm. Raw images and movies were processed with Imspector. When necessary, images and movies were deconvolved using the Richardson-Lucy algorithm, implemented in Imspector. The PSF was modelled as a Gaussian function and the FWHM of iterations were varied depending on the quality of the output image. The regularization parameter was set as either $10^{-5}$ or $10^{-10}$, while the number of iterations was chosen up to a maximum of 10. After deconvolution, the intensity of the images was adjusted in order to reduce background noise. Movie S1 was corrected for bleaching using the “Bleach correction” and for drift using the “MultiStackReg” (based on “StackReg”) plugins in Fiji.

The number of tubules per neurite width was measured tracing line profiles perpendicular to the filament orientation and averaged on 3 pixels on the raw images. The data were fitted with the software OriInLab. The neurite width was calculated measuring the peak-to-peak distance from the first to the last peak of actin fitting ($N_{\text{peak}} = 87$).

The mitochondrial constriction was calculated dividing the size of the mitochondrion at the ER contact site, measured tracing and fitting a line profile averaged on 3 pixels perpendicular to the ER tubules, by the size of the mitochondrion laterally measured tracing and fitting a line profile averaged on 3 pixels perpendicular to the ER tubules, less than 5 pixels, laterally to ER mito contact where the contact was not present ($N = 39$).

2.4. MoNaLISA for 2D parallelized RESOLFT time lapse imaging

The MoNaLISA setup used in this study was custom-built as reported in Masullo et al. (Masullo et al., 2018b). All the images have been recorded with a multifocci pattern of periodicity 625 nm coupled with an OFF pattern of 312.5 nm. The ON-switch is performed with 405 nm light 650 W/cm$^2$ for 0.5 ms, the OFF confinement with 1.5 ms of 488 nm light at 650 W/cm$^2$ and finally the Read Out with 240 kW/cm$^2$ of 488 nm for 1 ms. The step size is 35 nm, for a global dwell time of 5 ms and recording time of 1.9 s. The second confocal channel used for the recording of mitochondria has been imaged in a sequential manner using a 350 W/cm$^2$ of 590 nm light per 2 ms. The spectral interval of the second camera is 620/70 nm.

The interval between the super-resolved frames has been varied between 5 s and 1 min.

To quantify the number of junctions and the average branch length of the ER, images were analyzed with ImageJ v1.53c. A 0.65 gaussian filter was applied to each frame of timelapse, each frame was thresholded using Huang algorithm and then converted into a skeleton binary map. The skeleton was analyzed using the ImageJ plugin developed by Ignacio Arganda-Carreras et al. 2010 (Arganda-Carreras et al., 2010). To observe the movement of ER tubules and clusters along the mitochondria kymographs were generated following in time the ER along the mitochondria major axis. The kymograph has been analyzed through a custom written Matlab code. Starting from the deconvolved data, the image sequence has been time-filtered with a kernel of 2 frames. The number of peaks in the kymograph are automatically identify using the average of the first frames, subsequently the positions are calculated fitting the a number of identified peaks to each time point of the kymograph. Only time traces that span more than half the time window are further considered in the analysis. The same procedure is repeated backward starting from the last frame of the time series. The two set of traces are then compared and merged. The displacement is calculated as the distance between the minimum and maximum of the central coordinate of the peak fitted in all time points. The ER tubules traces are then sorted between in and outside the mitochondria using the signal in the other channel. The peak detection is defined by different parameters, like peak high, prominence and peak-to-peak distance. Similarly, in the fitting procedure the initialization of the fitting can be either link to the previous step or linked to the starting average peak positions. The set of parameters to guide the kymograph analysis has been done by manual supervision.

2.5. 3D pRESOLFT imaging

The 3D pRESOLFT images of the ER network and the mitochondria are acquired with the custom build microscope described in Boden et al (Boden et al., 2020). The ER data is acquired with all the three OFF-switching patterns active giving a maximum OFF-switching intensity of about 400 W/cm$^2$ and nearly isotropic confinement of emission. The ER data is acquired with a GFP detection filter (Chroma ET535/70 m). The mitochondria data is acquired without any OFF-switching illumination and using a red-shifted detection filter (Chroma ET620/60) giving a resolution comparable to a confocal microscope. Data is acquired using a Nikon 100 × 1.4NA Silicon Objective. Data is acquired with 50 nm step size and acquisition time for full volume is 31 s for both ER and Mitochondria data.

We applied bleaching correction between frames to compensate for switching fatigue. The images presented are deconvolved with a narrow Gaussian of 50 nm FWHM combined with a wider Gaussian of 175 nm FWHM accounting for 10% of the PSF amplitude, such a geometry take into account the properties of RSFPs, where a background signal due to a non-photoswitchable fraction of the molecules is expected. The final image is the result of 8 iterations of the Richardson-Lucy algorithm. Data shown in Fig. 4 has been 3D deconvolved using the approach described in our previous work (Boden et al., 2020) using 20 iterations for the ER data and 30 iterations for the mitochondria data. To assess whether a transversal ER contact was inducing a mitochondrial constriction, the 3D volume from raw data was Z-projected in a 2D image, a line profile was then traced along the main axis of the mitochondrion, a reduction in mitochondrial intensity represented a mitochondrial constriction (Voeltz et al., 2011).

3. Results

3.1. The tubular ER organization in neurons is directly related to neurite width

We take advantage of three different fluorescent nanoscopy approaches to study multiple aspects of the tubular ER organization (Fig. 1A). Point scanning STED (Fig. 1B) with depletion at 775 nm is used together with permeable dyes to detect, with high sensitivity, multiple tags at the same time in living neurons for co-localization studies at ~40 nm resolution. Most of the recording are single screen-shot in living cells or short movies to avoid light induces photo-toxicity.

2D and 3D parallelized RESOLFT imaging with OFF switching at 488 nm (Fig. 1C) are instead used to access dynamics and 3D information while resolving the fine tubular organization of the ER in neurons with ~50 nm spatial resolution. Time-lapse imaging in extended sample regions can be performed within seconds and minutes.

The ER tubular organization was resolved applying two-color STED nanoscopy, the actin Membrane Periodic Skeleton (MPS) was used as a reference to define the neuronal sub-compartments dimension. Actin is involved in establishing the neuronal polarity, transport of cargos and stabilization of synaptic structures, it forms various structures along the processes of cultured hippocampal neurons, including bundles, patches and the MPS (Xu et al., 2013). The MPS, in optical fluorescence microscopy, can be uniquely explored in super resolution thanks to the nanoscale spatial resolution. The neurons were live stained for the actin cytoskeleton using SiR-Actin (Lukinavicius et al., 2014) and for the ER
via enzymatic labelling of the fusion protein Halo-Sec61β and the ATTO590-Halo ligand (Fig. 2A) (Bottanelli et al., 2016).

Results showed the intricate tubular network of ER along neurites clearly visible in the STED images but not in the corresponding confocal comparisons. The average width of the ER tubules distribution is 112 nm with some tubules as small as 60 nm (Fig. 2A, inset). We also found sheets located mainly in larger neurites close to the cell somata or in some varicosity, but their full description and characterization are outside the scope of this study (Supplementary Fig. S1).

We focused instead on thin ER tubules located in different sub-compartments of the neuron: a neurite, where the MPS is clearly visible (Fig. 2B, upper panel, 180 nm periodicity) and at a varicosity site where the MPS lost its organization (Fig. 2B, lower panel). At the varicosity, ER tubular network increased in complexity with a higher number of junctions and connections between tubules compared to the ER at the MPS.

To investigate whether these differences are related to the geometry or the specific function of the neuronal compartments we imaged the tubular ER in dendrites, axon initial segment (AIS) and axons, which are functionally different but that can also feature comparable tubular geometry and width throughout their length (Fig. 2C-F). By using the actin ring as the delimiting structure to approximate the diameter of the
Fig. 2. Tubular ER organization inside the membrane-associated periodic skeleton (MPS) is directly related to neurite width. (A) Two-color live STED image of the ER labeled with 590-Halo ligand through the membrane protein Sec61β, and the actin cytoskeleton labeled with SIR-Actin. The histogram shows the distribution of ER tubules diameter, measured over $N = 116$ tubules in $N = 6$ cells imaged in different days. (B) The upper Panel is the magnification of the ROI I in image A showing ER tubules along the MPS in STED and confocal mode. Two line' profiles, drawn across (1) and (2) show the ER tubules widths and the characteristic 180 nm spacing actin rings respectively. The lower panel is the magnification of the ROI II in image A, showing the ER network in a varicosity (3) and the partial loss of organization of the MPS quantified in plot (4). (C) Three colors image of ER (green) Actin (magenta) recorded in STED and Neurofascin (blue) to mark live the axon initial segment (AIS) in Confocal mode. (D) Magnification of the ROI III marked in image D shows dendrites of different diameter. Two line’ profiles, drawn across (5) and (6), show dendrites of 576 nm and 172 nm diameter holding three and one ER tubules respectively. (E) Magnification of the ROI IV marked in image D shows the AIS. Two line’ profiles drawn across (7) and (8) show that AIS of comparable diameter (583 nm and 552 nm) contain three and four ER tubules respectively. (F) A-STED image of axon with different diameters throughout its extension. Two line’ profiles, drawn across (9) and (10), show the axons’ diameters of 283 nm and 347 nm holding one and three ER tubules. (G) Box plot representing the average size of diameters for dendrite: pink, AIS: orange, and axon: blue, which contain from 1 to 5 ER tubules. Independently from the functionalization of the compartment, the number of ER tubules increases with the size of the neurite’s width. (N,tot = 87; N,den = 22; N,AIS = 24; N,axon = 41). (H) Box plot representing the ratio between the sum of ER tubules width (FHWM of each tubules) and the neurite diameter in dendrites (pink), in the AIS (orange) and in the axon (blue). The ratio is not significantly different in the three compartments (t Student $p$ values respectively 0.1187, 0.5501 and 0.08082, (N,tot = 87; N,den = 22; N,AIS = 24; N,axon = 41). (I) Scatter plot representing the correlation between the average branch length, marked in green, and the neurite’s diameter in pink ($N = 15$, Pearson’s coefficient, $r = -0.616$).
neurites (Vassilopoulos et al., 2019), we resolve and count individual ER tubules within neurites of different diameter. The AIS was live stained using a primary antibody designed against the extracellular domain of the protein Neurofascin (Evans et al., 2015). We report that, independently from the considered neuronal compartment, the number of ER tubules per neurite scales up with the width of the filaments (Fig. 2G). Neurites which are 100–200 nm wide contain only 1–2 ER tubules, which are hard to resolve with conventional confocal microscopy. The filaments are mostly organized along the process bifurcating and fusing multiple time. Also, fine tubular protrusions can be resolved transversally to the neurites interconnecting the longitudinal tubules.

Instead, wider filaments (diameter of 1–1.2 um) contain up to 5 longitudinal tubules. The ratio of the summed ER tubules widths against the neurite diameter is not significantly different in the three compartments (Fig. 2H). However, the average branch length, defined as the length of the ER tubules in between two junctions and calculated using a skeletonization algorithm (Arganda-Carreras et al., 2010), is directly correlated (Pearson’s coefficient r = −0.616) to the diameter of neurite (Fig. 2I), extended 2D area haveha have shorter branches and significantly longer branches are found in fine axons and dendrites.

We showed that the complexity of the ER network in terms of number of tubules, junctions and tubules branch length are therefore related to the size of the neurite width rather than the specific function of the compartment. Based on these quantifications we conclude that the tubular three-dimensional geometry of the neuronal arbors affects the spatial organization of the ER network in order to ensure and optimize the allocation of resources.

3.2. ER tubules constrict and shape mitochondria along neuronal processes

Many functions are associated with the ER contacting mitochondria, among them, calcium exchange, lipid biogenesis and ER mediated mitochondrial fission are the most well studied (Tatsuta et al., 2014) (Friedman et al., 2011). We applied two-color STED time-lapse imaging to resolve the ER tubules packed across mitochondria inside neurites of living neurons (Fig. 3). The neurons were live stained for ER and mitochondria via enzymatic labelling of the fusion protein SNAP-Sec61β with SiR-647-SNAP ligand and Halo-Omp25 with ATTO590-Halo ligand (Fig. 3A). The live staining was crucial to preserve the membranes and avoid inhomogeneity and shrinking artifacts. The interactions between the two organelles showed the rapid and continued motion of the ER tubules around mitochondria. In Fig. 3B and Movie S1 an example of membrane contacts between the two organelles is shown, where six ER tubules are spaced 120–500 nm apart. The contact sites are not visible in conventional images due to the lack of spatial resolution (Fig. 3A, inset).

We focused on mitochondria-ER transversal contacts, which are the ER tubules that cross transversally the main axis of the mitochondrion (Friedman et al., 2011). We imaged and quantified the mitochondria diameter in proximity of the ER tubules (Fig. 3C). Two-color STED is required here to resolve the outer membrane of the mitochondrion and therefore to infer the mitochondrial diameter at the level of the potential contact. We observed that not all the contact sites lead to a mitochondria constriction (Fig. 3G-H). We, then, questioned if such variability is just partial or claw like (marked with an asterisk in Fig. 4C, Fig. 4C inset) or can extend across the entire 3D space, another one even if it is just partial or claw like (marked with an asterisk in Fig. 4C, Fig. 4C inset and Supplementary Fig. S2) can cause a mitochondria constriction.

3.3. Dynamics of the tubular endoplasmic reticulum in soma and neurites with parallelized RESOLFT nanoscopy

The ER tubular network constantly rearranges via the formation of new tubules, the retraction of existing ones and the rapid wobbling/collapse/expansion of the tubular polygonal net (Friedman et al., 2011; Friedman et al., 2010; Voeltz et al., 2006). The highly dynamic ER rearrangement relies primarily on the interplay between ER shaping proteins and microtubule cytoskeleton (Voeltz et al., 2006). ER elongating and retracting tubules slide along existing microtubules transported by motor proteins or they grow/retract attached to the tip of the growing (+) end of microtubules (Tip Attachment Complex, TAC) (Salmon, 1998). The ER tubular reorganization occurs in the time window of seconds (Guo et al., 2018). We employed RESOLFT nanoscopy to investigate the ER dynamics in different neuronal compartments. Parallelized RESOLFT time-lapse imaging at 0.2 Hz allowed us to image a 40-by-40 μm² field of view and quantify simultaneously ER dynamics in distinct regions of the somata and primary neurites of DIV6–7 hippocampal neurons genetically encoding rsEGFP2-Sec61β (Fig. 5).

The complexity of the ER tubular polygonal network of neuronal cell soma, clearly visible in the RESOLFT image, is not equally identifiable in the corresponding confocal image (Fig. 5A, ROI I and histogram in Fig. 5C). We quantified the tubular ER reshaping as the variation in time of the number of junctions and the average branch length. The complexity of ER reshaping is, then, visualized via the temporal color-coded skeleton (Fig. 5D-F). In seconds time scale, we observed specific ER dynamics such as the elongation/retraction dynamics of an ER tubule (Fig. 5A, ROI II and Fig. 5D left panel) and the contraction of the ER tubular polygon (Fig. 5A, ROI III and Fig. 5D left panel). Quantifying a
larger field allows us to observe a more complex ER reshaping behavior in which fast wobbling/collapse/expansion of the polygonal net occurs at different timescales in distinct sub regions, and tubular growth extends along multiple polygons over 2 min (Fig. 5 E-F, Movie S2). The different types of tubular rearrangement and dynamics described here, in neurons at diffraction unlimited spatiotemporal resolution is in accordance with the second time scale ER dynamics previously reported in mammalian cells (Guo et al., 2018).

The photodamage reduction of RESOLFT enabled to record prolonged time lapse over ten minutes of the same region of the neuron and to resolve and track the complexity of the tubular ER network reshaping, in neurites (Fig. 5 B and F). The temporal color-coded skeleton and the quantification of the tubular ER reshaping for two regions of interest (Fig. 3F) exemplify the substantial reorganization of the tubular network. In ROI V of Fig. 5B, the average tubular length oscillates from 433 to 658 nm over 10 min, and the ER spans the entire diameter of the neurite.

In accordance with STED quantification in axon, dendrite e AIS, in flat and 2D regions of the cell body, the branch length is shorter than in thin filaments. The average branch length measured in 3 regions of the somata in two different neurons, normalized over 2 min of imaging is 342 nm, whereas the average branch length measured in 2 neurites of 450 nm width, belonging to two different cells, is 480 nm.
Fig. 5. Dynamics of ER network imaged via parallelized RESOLFT nanoscopy for different cellular compartment and across different time scale. (A, B) Representative super resolved images of hippocampal neuron encoding for sec61β tagged with rsEGFP2 for RESOLFT imaging. The two areas show different cellular compartments, closer to the soma (A) or projected toward the branches (B). In the inset a zoom of ROI I comparing confocal and RESOLFT and highlighting the higher level of detail provided by the second in following the ER network. (C) Estimation of the diameter of ER tubules in MoNaLISA live cell imaging, giving a mean value of 107 ± 23 nm (mean ± std) on N = 7 different images. (D) Examples of elongation of a tubule (left) and contraction of the network (right) from ROI II and ROI III respectively. The orange arrow indicates the process, while the panels on the bottom are the corresponding skeleton images of the images above. (E, F) Color coded maximum projection of the skeletonized images of different ROIs representative of the ER in the proximity of the soma (4 cell bodies) (first and third panels) and in filaments (9 neurites from 9 different neurons); second and fourth panels (E) integrate the ER movements over 120 s (with a sampling of 5 frames/s), while (F) over 10 min (with a sampling of 1 frame/min). The number of three-way junctions and the mean branch length over time for each image are reported below. Scale bars, 5 μm (A, B) and 500 nm (A inset, D, E, F).
3.4. Transversal mitochondria-ER contacts dynamics reveals distinct persistency in seconds and minutes time windows, as well as in microtubules-depolymerizing condition

In two-color STED time-lapse imaging we demonstrated that there is no correlation between the ER tubule diameter and the deformation of the mitochondrion at the site of the contact. We hypothesize that the size of the ER tubule in contact with the mitochondrion might not be a defining factor for the function of the contacts. We questioned, then, if the dynamics and the persistence of the contact, in addition to its size, could be used to distinguish different subpopulations of mitochondria-ER contacts.

We used parallelized RESOLFT nanoscopy to image at 0.2 Hz the dynamics of transversal mitochondria-ER contacts. Mitochondria were live stained with MitoTracker (Red CMXRos or Deep-Red FM) in hippocampal neurons genetically encoding rsEGFP2-Sec61β (Fig. 6).

Mitochondria were imaged in confocal modality while ER was super resolved to discern mitochondria-ER traversal contacts (Fig. 6B). For each mitochondrion a line profile was traced along its longitudinal axis (Fig. 6D). By analyzing the derived kymographs in each contact, descriptors of the dynamics, along with the dimension of the tubules, can be identified: the velocity (nm²/s) and the displacement (Fig. 6E-H). The distributions of velocities extracted from sampling in second and in the minute time windows are significantly different (p < 0.05, KS p value). In particular, we observe a spreading of the distribution toward higher values of velocity when sampling on the second time window, this reflects the presence of faster movements that either results from directional displacement that can’t be sampled on the minute time scale or a

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Fig. 6. Dynamics of the ER tubules across the mitochondria with parallelized RESOLFT time-lapse imaging. (A) Representative dual color imaging showing the super resolved MoNALISA image of ER-rsEGFP2 and the confocal image of mitochondria labeled with MitoTracker Red on hippocampal neuron DIV6/5. Overview of the neuron in the inset. (B) Zoom of ROI I where the confocal and RESOLFT image of the same area is reported as comparison. (C) The color code projection of the skeleton for the same ROI helps to visualize the different extend of movements that characterized the ER filaments that orthogonally intercept the mitochondrion along its length. To help the visualization of the movement inside vs outside the mitochondria a different shadow has been applied to these two regions. (D) Line profile along the mitochondrion following the magenta arrows for two time points, the starting frame and after 10 min. (E) Analysis of the movement of ER on the mitochondria at the second timescale (5 s/frame). From left to right, representative zoom on the ER and mitochondria, RESOLFT and confocal imaging. Outline of the mitochondrion on the first frame of the ER network. Overlay of the ER skeleton for different time points only inside the mitochondria area to highlight the different mobility of the ER that can be captured at different recording speed. Line profile and kymograph in the area of the mitochondria along the direction defined by the magenta arrows in the previous panel. Filaments that are inside the mitochondria area are shown as solid lines while traces outside are presented as dashed grey lines. The triangles on top of the line profile identify the peaks recognized by the algorithm. (F) Similar analysis on time-lapses recorded at one-minute interval between the frames. Scale bar, 5 μm (A) and 500 nm (B, C, E, F). (G) Velocity of the ER tubules movements in nm/s for the recording at 5 s intervals (blue) and one-minute interval (orange). (H) Displacement of the ER tubules traces along the major axis of the mitochondria for different timescale, seconds in blue and minutes in orange, the effect on the displacement introduced by nocodazole is shown in magenta. The graph collects the behavior of n = 4 for the different conditions. KS test: ns > 0.05, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
partial sampling of a more randomized motion which is only partially detectable on the second time scale. The displacement has a broad distribution ranging from 50 to 600 nm with the mean centered at 134 nm. Since a significant sub-distribution of tubules showed a minimal displacement we further investigate the dynamics across minutes (Fig. 6A-F, Movie S3). With similar quantification we found that the mean displacement of transversal mitochondria-ER contacts in the minute time scale is 165 nm with some contacts which still persist with a minimal displacement of 50 nm and other spanning 200–300 nm across the mitochondria. The mitochondria-ER contacts’ distribution of displacements across minutes is significantly different from the displacement measured in seconds (KS test p value 0.0001) (Fig. 6H), maintaining a component at around 100 nm but also having a broader tail toward bigger displacements.

We question then, if these distinct displacements might be linked to different ER shaping mechanisms that depend on microtubules and that are known, as well, to occur at different velocity and to affect mitochondria-ER contacts. These two mechanisms are the TAC mechanism and the sliding. TAC, relies on polymerization and depolymerization of microtubules and its slower, the sliding, instead, is faster and it is dependent on motor protein sliding over acylated microtubules. Mitochondria-ER contacts co-localize with acylated microtubules in COS-7 cells (Friedman et al., 2010) and non-acylated microtubules depolymerization significantly reduce the number of mitochondria-ER contacts in Jurkat cells (Bantug et al., 2018). We exposed neurons to nocodazole treatment (50 μM, 1 h), a drug that depolymerizes primarily the non-acylated component of microtubules (Friedman et al., 2010; Salmon, 1998) and, therefore, affects more the TAC mechanism. We quantified the mitochondria-ER contact displacement in the minute time window after nocodazole treatment: the displacement is significantly lower than the control condition (KS test p value 0.002), with an average displacement of 132 nm. This suggests that long-range displacement of mitochondria-ER contacts in the minute time range are mainly controlled by mechanisms that rely on microtubules polymerization/depolymerization, whereas short-range displacements are, instead, dependent on nocodazole resistant microtubular components.

4. Conclusions

In this study we apply two-color live STED and parallelized 2D and 3D pRESOLFT live imaging to look at the tubular ER organization, dynamics and mitochondria-ER contacts in DIV6–7 hippocampal neurons. We inspect the complexity of the ER tubular network and its occupancy in highly polarized cells such as neurons. We found that its organization in branches and junctions is related to the diameter of the compartment rather than on its specialization. Lower number of ER tubules with less junctions and longer branches characterizes thinner axons and dendrites.

The rapid and pro-longed parallelized RESOLFT time lapse imaging of large portion of the neurons at ~50 nm resolution allowed us to access the dynamic behavior of the ER tubular network in neurons while resolving individual ER tubule. Across seconds we observed tubular elongations/retraction and rapid wobbling of the polygonal net which can be related to sliding or TAC tubules growth mechanisms. Over several minutes the ER reorganization is massive, especially for tubules branch length in neurites. Overall, the ER tubular network dynamics are heterogeneous within the different neuronal compartments.

ER function is strictly related to its interplay with other organelles, especially mitochondria, and a complete description of ER organization and dynamics can’t exclude mitochondria-ER contacts. Mitochondria-ER contacts has been extensively studied since their alterations has been reported as a trait in neurodegenerations. In neurons though, in live cell super resolution imaging, a proper characterization of mitochondria-ER contacts and their dynamics is missing. Here, we apply two-color live STED to depict mitochondria-ER transversal tubules and the mitochondrial constriction at the site of the ER transversal tubules.

We found that the dimension of the ER transversal tubule does not correlate with the mitochondria deformation. A single and thin ER tubule can induce a constriction in the mitochondrion as well as larger multi-tubular assemblies. With 3D pRESOLFT super resolution imaging we showed that in a neuronal filament the mitochondria-ER contacts extend three-dimensionally wrapping around the mitochondrion at different extents, and even an incomplete, claw like contact can cause a mitochondria constriction. Furthermore, mitochondria-ER interactions are highly dynamic within seconds, they travel along the main axis of the mitochondrion with short-range (~50–100 nm) or long-range (up to 400 nm) displacements. Long-range displacements are driven by the nocodazole-resistant component of microtubules.

Taken together, the results of our work generated with multi-color STED for short term live cell imaging and parallelized 2D and 3D pRESOLFT for pro-longed time lapse and volumetric imaging respectively, allow to reveal, for the first time in a living neuron at adequate spatio-temporal resolution, the complex tubular ER organization and its heterogeneous dynamics in contact with mitochondria.

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Author contributions

I.T. designed and supervised the project. M.D. carried out the cloning, neuronal preparation and imaging. M.D. and G.C. performed the treatments and STED imaging. M.D. and F.P. performed the MoNaLISA imaging and data analysis. M.D. and A.B. performed the 3D pRESOLFT imaging and data reconstruction. I.T. and M.D. wrote the manuscript with assistance from all the authors.

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