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Visualization and quantification of cardiac mitochondrial protein clusters with STED microscopy

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ABSTRACT

The visualization and quantification of mitochondria-associated proteins with high power microscopy methods is of particular interest to investigate protein architecture in this organelle. We report the usage of a custom-made STimulated Emission Depletion (STED) fluorescence nanoscope with ~30 nm lateral resolution for protein mapping of Percoll-purified viable mitochondria from murine heart. Using this approach, we were able to quantify and resolve distinct protein clusters within mitochondria; specifically, cytochrome c oxidase subunit 2 is distributed in clusters of ~28 nm; whereas the voltage dependent anion channel 1 displays three size distributions of ~33, ~55 and ~83 nm.

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1. Introduction

Mitochondria participate in key metabolic reactions, regulate Ca^{2+} signaling, and play a central role in apoptosis (Dimmer and Scorrano, 2006; Liu et al., 1996; Yang et al., 1997). In the heart, mitochondria can determine myocardium survival and protection, whilst mitochondria dysfunction contributes to heart disease (Baines, 2010). To understand the details of these mechanisms it is important to map mitochondria-associated proteins at nanometer resolution as localization is intimately related to functional performance.

STimulated Emission Depletion (STED) fluorescence nanoscopy with ~40–80 nm lateral resolution in biological samples offers the opportunity to scrutinize the distribution of proteins in subcellular compartments or organelles using common immunocytochemistry techniques and tagged proteins (Kellner et al., 2007; Neumann et al., 2010; Watanabe et al., 2011; Willig et al., 2006). STED has the advantage over electron microscopy that it reaches nanometer resolution while at the same time maintains the microscopic scale.

Only recently has STED been utilized to image a few mitochondrial proteins. Native hexokinase I was observed in mitochondria of an osteosarcoma cell line (U2OS) (Neumann et al., 2010); while ectopically expressed TOM20 was imaged delineating mitochondria periphery in a ring-like arrangement in *Caenorhabditis elegans* (Watanabe et al., 2011), and expressed voltage dependent anion channels (VDAC) 1–3 were visualized in U2OS cells with VDAC3 forming clusters of ~40 to 90 nm (Neumann et al., 2010).

The objective of this work was to map with STED nanoscopy two classical mitochondria proteins in cardiac mitochondria: VDAC1, an outer mitochondrial membrane protein that serves as an interface between cellular and mitochondrial metabolism (Shoshan-Barmatz and Ben-Hail, 2011); and cytochrome c oxidase (complex IV of the respiratory chain) located in the mitochondrial inner membrane and assembled by 13 subunits in humans. Specifically, we imaged cytochrome c oxidase's subunit 2 (Cox2) that forms part of the catalytic core of the enzyme (Brzezinski and Johansson, 2010; Mick et al., 2011).

2. Methods

2.1. Antibodies

Primary antibodies were used against VDAC1 (Ab14734, Abcam), Cox2 (A6407, Invitrogen), Cadherin (C1821, anti-Pan Cadherin

Non-standard abbreviations and acronyms: STED, STimulated Emission Depletion; VDAC1, Voltage Dependent Anion Channel 1; Cox2, Cytochrome c Oxidase subunit 2; SFKs, Src family of tyrosine kinases; PPI, protein proximity index; FWHM, Full Width Half Maximum; TDE, 2,2′-Thiodiethanol.

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antibody, Sigma), GM130 (ab1299, Abcam), Lamin B1 (ab16048, Abcam), GRP78 BiP (ab21685, Abcam), L-type Ca^{2+} channels (α1C) (ACC003, Alomone) and Src (sc-18, Santa Cruz). Secondary antibodies for Western blots were: Alexa Fluor® 680 goat anti-rabbit (A-21109, Invitrogen) and IRDye 800CW conjugated goat anti-mouse (926–32210, LI-COR); and for immunocytochemistry were: Atto 647 N goat anti-mouse (50185, Sigma), and Atto 647 N goat anti-rabbit (15048, Active Motif).

2.2. Animals

Protocols received institutional approval. Male 3 mo old C57BL/6NCrL mice were injected (*i.p.*) with heparin (200 IU/kg). After 20 min, animals were anesthetized with sodium pentobarbital (70 mg/kg, *i.p.*). The heart was surgically removed and rapidly arrested in ice-cold phosphate buffer saline (PBS) (in mM): 2.7 KCl, 1.5 KH_2PO_4, 138 NaCl, 8.1 Na_2HPO_4, pH 7.4.

2.3. Isolation and purification of mitochondria

The entire procedure was at 4 °C and lasted approximately 75 min. One mouse heart ventricles were finely minced and homogenized in isolation buffer A (in mM): 70 sucrose, 210 mannitol, 1 EDTA-Na_2, 50 Tris–HCl, pH 7.4 using a Potter-Elvejem homogenizer (10 rapid strokes). The homogenate was transferred into a 2.0 ml Eppendorf tube and centrifuged at 1,300 × *g* for 3 min. The supernatant was carefully transferred into a clean 1.5 ml Eppendorf tube and centrifuged at 10,000 × *g* for 10 min. The pellet containing crude mitochondria was resuspended in 55 µl of isolation buffer A.

The crude mitochondria preparation was carefully overlaid on 3 ml of 30% (*v*/*v*) Percoll (Graham, 2001) in buffer B (in mM): 250 sucrose, 10 HEPES-Na, 1 EDTA-Na_2, pH 7.4. The sample was centrifuged in a fixed angle rotor at 50,000 × *g* for 45 min. After ultracentrifugation, three clear layers were observed which were labeled as M1, M2 and M3 (Fig. 1A). The M1, M2 and M3 fractions were carefully isolated and resuspended in 1 ml of isolation buffer A. Samples were centrifuged at 12,000 × *g* for 5 min, mitochondrial pellets were resuspended, and washed two times with 1 ml isolation buffer A. All the fractions were resuspended in buffer A for organelle immunochemistry and in buffer B for functional assays. Purified mitochondria were used within 2 h after isolation.

2.4. Western blot analysis

Isolated mitochondrial pellets from the different fractions were resuspended in lysis buffer (in mM): 50 Tris–HCl, 150 NaCl, 1 EDTA-Na_2, 1 EGTA-Na_4, 1% (*v*/*v*) NP-40 Alternative (Calbiochem), 0.5% (*w*/*v*) Na-deoxycholate and 0.1% (*w*/*v*) SDS, pH 7.4 supplemented with protease inhibitors (1 tablet/50 ml, Roche), and incubated for 1 hour with shaking. Samples were centrifuged at 10,000 × *g* for 5 min, the supernatants were collected, and the protein concentration was measured. Equal amounts of protein (50 µg) were loaded for 10% SDS-PAGE, and transferred to nitrocellulose membranes. Proper loading was corroborated with Ponceau S staining. Membranes were blocked with 5% (*w*/*v*) milk in TBS (150 mM NaCl, 20 mM Tris-HCl pH 7.4,) for 60 min at room temperature. Membranes were washed three times with TBS (5 min at room temperature) and incubated overnight with primary antibodies at 4 °C. Primary antibodies were used at the following concentrations: for plasma membrane (Cadherin, 19.4 µg/ml), endoplasmic reticulum (GRP78 BiP, 0.2 µg/ml), nuclear envelope (Lamin B1, 0.2 µg/ml), Golgi (GM130, 0.5 µg/ml), mitochondria (VDAC1, 0.2 µg/ml and Cox2, 0.1 µg/ml), T-tubules (α1C, 0.45 µg/ml) and for a signaling tyrosine kinase family (Src, 0.04 µg/ml). After washing three times with TBS (5 min, room temperature) membranes were incubated with 0.01 µg/ml secondary antibodies (anti-mouse/rabbit) for 1 h at room temperature, and washed again three times with TBS for 5 min. Signals were visualized using an infrared fluorescence system (Odyssey™ Imaging System, Li-Cor). The intensity of the bands was measured with Metamorph (Molecular Devices) and normalized to loaded protein using Ponceau S signals.

2.5. Mitochondrial H_2O_2 measurement

Mitochondrial reactive oxygen species (ROS) generation was measured spectrophotometrically (560 nm excitation and 590 nm emission) by incubating 250 µg of mitochondrial protein in a solution containing (in mM): 20 Tris–HCl, 250 sucrose, 1 EGTA-Na_4, 1 EDTA-Na_2, and 0.15% (*w*/*v*) bovine serum albumin, pH 7.4 at 25 °C with 10 µM Amplex Red (a H_2O_2 sensitive-dye, Invitrogen) and continuous stirring. Sodium succinate (3 mM) was used to activate complex II.

2.6. Labeling of mitochondrial proteins

Purified mitochondria were incubated with 500 nM Mitotracker® Red CMXRos (Invitrogen) for 60 min with shaking, plated drop-wise onto coverslips and incubated at 4 °C for 1 h. Coverslips (0.17 mm thickness; Warner Instruments, Cat no. 64–0712) were coated with 0.1% Poly-L-Lysine (Sigma-Aldrich) for 2 h at room temperature and washed with PBS before use. Attached mitochondria were then washed with PBS once and fixed with 4% (*w*/*v*) paraformaldehyde in PBS for 10 min at room temperature. After three washes with PBS, mitochondria were permeabilized with 0.5% (*v*/*v*) Triton-X 100 in PBS for 10 min at room temperature. Mitochondria were further colabeled with specific antibodies (in 1% (*w*/*v*) normal goat serum (NGS), 0.2% (*v*/*v*) Triton X-100 in PBS) for mitochondrial (0.2 µg/ml anti-VDAC1 and 0.2 µg/ml anti-Cox2 monoclonal antibodies) and nuclear envelope (0.2 µg/ml anti-Lamin B1 polyclonal antibody) markers. ATTO 647 N-labelled secondary antibodies (1 µg/ml anti-mouse and anti-rabbit) in 1% (*w*/*v*) NGS, 0.2% (*v*/*v*) Triton X-100 in PBS were used. Some mitochondria were only incubated with secondary antibodies as controls. Preparations were sequentially treated with 10, 25, 50 and 97% (*v*/*v*) 2,2′-Thiodiethanol (TDE, Sigma-Aldrich) solutions in PBS (pH 7.5) for 5 min each at room temperature (Staudt et al., 2007). TDE solutions were added and aspirated gently to prevent uplifting of mitochondria from the coverslips. Coverslips were mounted onto slides and sealed with regular nail polish. Samples used only for regular confocal microscopy were mounted using ProLong®Gold (P36934, Invitrogen) instead of TDE. Confocal images were acquired with an Olympus confocal microscope using a 60X oil immersion objective with 1.42 NA (PlanAppoN) at a scanning resolution of 0.0575 µm per pixel. STED images were collected as described below.

2.7. STED microscopy

STED images were acquired with a custom-made STED system using an oil immersion objective (HCX PL APO CS 100x/1.40–0.70 OIL, Leica Germany). A 635 nm pulsed diode laser (LDH-D-C-635, PicoQuant GmbH) was used for excitation. The pulses for STED depletion were delivered by a tunable Ti:sapphire laser (Mai Tai HP, Spectra Physics) set at 780 nm. Fluorescence emission from ATTO 647 N-labeled secondary antibodies was collected through a Semrock BrightLine FF01-685/40–25 nm band pass filter in front of a photomultiplier (H7422PA-40, Hamamatsu Photonics K.K.). Images (955 × 960 pixels) were acquired with a 16 kHz line frequency (resonant mirror of 8 kHz) and summed 256 times. Pixel size was ~9.575 nm × 9.575 nm. For a fair comparison between conventional confocal images and STED images, all imaging parameters were kept identical except for the number of summations which was 64 when recording confocal images. For comparison, confocal images were acquired for the same field prior STED imaging.

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