Perspective

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Unleashing the potential: super-resolution microscopy as the key to advanced mitochondrial research

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Abstract: Investigating the fine structure of mitochondria and their dynamic interactions with other organelles is crucial for unraveling the mechanisms underlying mitochondrial-related diseases. The development of super-resolution techniques has provided powerful visualization tools for mitochondrial research, which is significant for investigating mitochondrial cristae structure, the localization of mitochondrial-related protein complex, and the interactions between mitochondria and other organelles. In this perspective, we introduce several advanced super-resolution techniques and their applications in mitochondrial research, and discuss the potential roles these techniques may play in future studies of mitochondria.

Keywords: super resolution microscopy; mitochondria; fluorescence microscopy

As the powerhouse of the cell, mitochondria provide the majority of required ATP through oxidative phosphorylation. In addition to supplying energy to cells, mitochondria are also involved in the synthesis of fatty acids, hemoglobin, amino acids, and iron-sulfur clusters, and play roles in a broad array of functions like cellular calcium homeostasis, cell differentiation, signal transduction, apoptosis, and possess the ability to modulate cell growth and cell cycle [1].

Being highly dynamic double-membraned organelles, mitochondria undergo rapid processes of fission and fusion. Mitochondria are sensitive to phototoxicity and the distance between mitochondrial cristae is typically less than 100 nm. While the resolution of electron microscopy meets the imaging requirements for mitochondria, it lacks the specificity and dynamic imaging capabilities of fluorescence microscopy. In recent years, the rapid development of super-resolution optical microscopy techniques has revealed their potential to unveil the fine structures, dynamics and functions within cells, particularly in mitochondrial-related research. Here, we will review the super-resolution microscopy commonly focused on mitochondria and introduce the latest advancements in super-resolution research.

Stimulated emission depletion (STED)

Stimulated emission depletion (STED) [2], similar to confocal microscopy in terms of hardware, incorporates an additional depletion light source in addition to the excitation light source. STED light forms a “doughnut” pattern on the sample, concentric with the excitation spot. Fluorophores in the region illuminated by STED light undergo stimulated emission, suppressing spontaneous emission processes. Only the central fluorescence signal is ultimately collected. STED achieves super-resolution through optical modulation and does not require subsequent algorithmic processing, providing direct super-resolved images. Higher-power STED illumination improves spatial resolution but requires more photostable fluorescent dyes for live cell imaging.

In earlier years, due to the lack of fluorescent probes with sufficient photostability and cell membrane permeability, STED microscopy was only employed for observing fixed samples. In 2013, a study used STED and immunogold microscopy to investigate the submitochondrial distribution of a conserved large hetero-oligomeric protein complex in the mitochondrial inner membrane called the mitochondrial inner membrane organizing system (MINOS) in fixed cells [3]. In recent years, various mitochondrial-targeted probes have been developed for live-cell imaging on STED microscopy. Wang et al. developed a specific probe based on a structurally reinforced naphthophosphole fluorophore that was conjugated with an electron-donating diphenylamino group,

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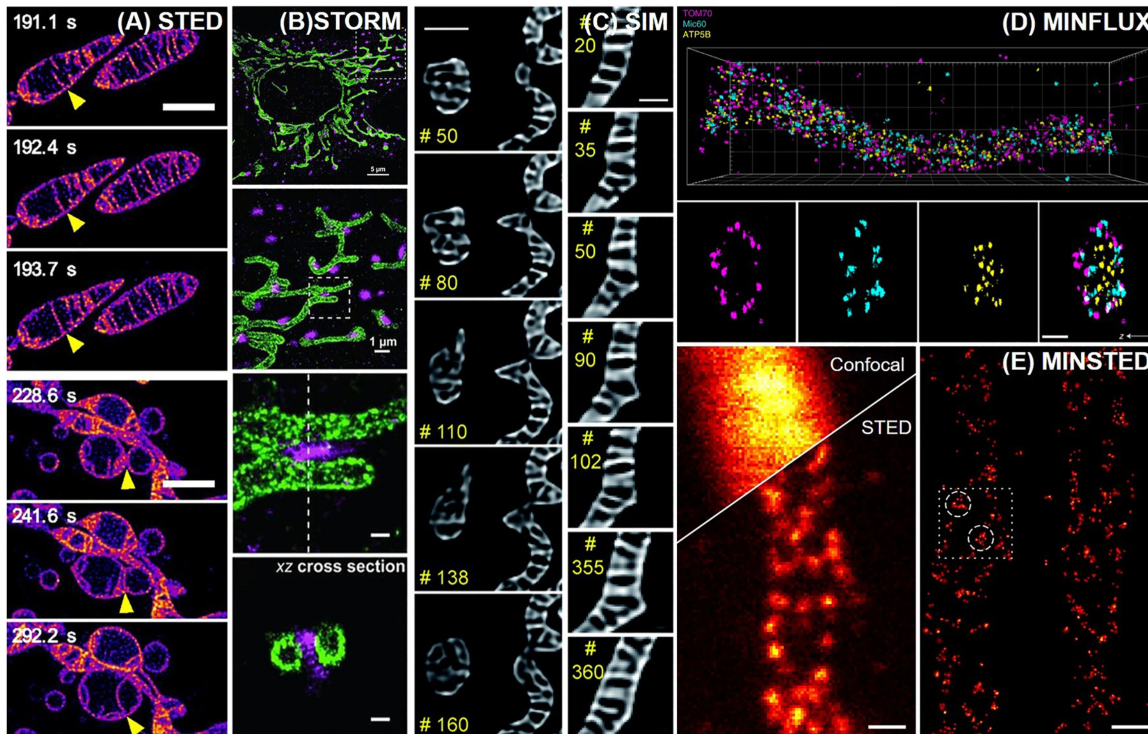

Figure 1: Super-resolution microscopy techniques applied in mitochondrial research. (A) Time-lapse STED imaging of mitochondrial cristae dynamics in MitoPB yellow-labeled Hela cells. Scale bars, 2 μm. Panel adapted with permission from Ref. [4]. (B) The spatial colocalization between mitochondria and purinosomes in HeLa cells imaged by STORM. Magenta: mEos2 fused to a purinosome protein FGAMS; green: immunolabeled against TOM20. Scale bars: 250 nm. Panel adapted with permission from Ref. [8]. (C) Time-lapse Hessian-SIM imaging of mitochondria labeled by PK Mito Red in COS-7 cells. Scale bars: 1 μm (left), 500 nm (right). Panel adapted with permission from Ref. [13]. (D) Distribution of three mitochondrial related protein under MINFLUX. TOM70, Mic60 and ATP5B are displayed in magenta, cyan and yellow, respectively in U2OS cells. Scale bar: 100 nm. Panel adapted with permission from Ref. [17]. (E) Comparing MINSTED nanoscopy with confocal and STED images in U2OS cells with ONB-2SiR-labeled primary anti-Mic60 antibodies. Scale bars: 200 nm. Panel adapted with permission from Ref. [18].

termed MitoPB Yellow. In combination with atime-gated-STED microscopy, they captured the ultrastructures of the mitochondrial cristae with a resolution of 60 nm and successfully observed a rapid intercristae mergence in a single mitochondrion [4] (Figure 1A).

Single-molecule localization microscopy (SMLM)

Single-molecule localization microscopy (SMLM) transcends the diffraction limit, achieving spatial resolutions of 10–70 nm. SMLM does not illuminate the entire sample simultaneously. Instead, it randomly activates individual fluorophores, precisely localizing each based on fitting and calculation. The most common techniques within SMLM are Photoactivated Localization Microscopy (PALM) [5] and Stochastic Optical Reconstruction Microscopy (STORM) [6]. PALM typically utilizes fluorescent proteins, while STORM uses commercially available probes conjugated with antibodies. High-precision localization requires specific fluorophore labeling, intense laser excitation, and prolonged data acquisition, making it challenging for dynamic tracking of live cells.

In the visualization study of mitochondria in fixed cells, 3D-STORM demonstrates its powerful advantages, with a spatial resolution of 20–30 nm and 60–70 nm in the lateral and axial dimensions, respectively [7]. Researchers utilized 3D-STORM to unveil the spatial colocalization characteristics between mitochondria and purinosomes, and suggest an mTOR-mediated link between purinosomes and mitochondria [8] (Figure 1B). In 2020, a STORM probe was developed to conduct mitochondrial dynamics imaging in live cells by covalently binding to VDPs in the mitochondrial outer membrane, demonstrating mitochondrial dynamics such as fusion, fission, and tubulation [9].

Structured illumination microscopy (SIM)

Structured illumination microscopy (SIM) [10] employs high spatial frequency interference patterns to illuminate biological samples, generating Moiré fringes with fine subcellular structures. By reconstructing images, SIM enhances resolution two-fold in both lateral and axial dimensions, compared to traditional wide-field imaging. It operates at high speed, covering a large field of view and capturing rapid changes in cellular dynamics. In 2018, Hessian SIM first achieved capturing cristae membrane movement and intercristae merging [11]. Later, a study proposed the sparse deconvolution algorithm, which integrates both sparsity and continuity as prior knowledge to guide the iterative deconvolution process, further enhancing the resolution of super-resolution microscopy [12]. In 2020, Yang et al. reported the development of a fluorogenic probe named PK Mito Red for mitochondrial imaging via mitochondrial membrane potential (MMP). This probe exhibited low phototoxicity and good photostability during imaging, reducing reactive oxygen species (ROS) generation and mitigating cyanine phototoxicity. Moreover, it enabled time-lapse imaging at 2,000 frames per second using Hessian SIM, revealing intricate mitochondrial dynamics [13] (Figure 1C).

Compared to SMLM, SIM requires lower power illumination, which minimizes photobleaching and phototoxicity and makes it suitable for long-term imaging, especially for observing the morphology and movement of mitochondria and studying the dynamic interactions between mitochondria and various organelles. Wong's study revealed that the mitochondria-lysosome interaction is subject to bidirectional regulation by both organelles in live cells using super-resolution N-SIM [14]. In addition, the dynamic process of mitochondrial division and fusion mediated by endoplasmic reticulum-mitochondria interaction sites was captured by GI-SIM [15].

Minimal emission fluxes (MINFLUX)

Minimal emission fluxes (MINFLUX) [16] randomly excites fluorescent molecules like SMLM but overcomes photobleaching and phototoxicity issues. It employs ring-shaped light similar to STED light. The fluorophore in the ring region is excited while the fluorescent molecule in the center of the ring is not. The closer the center of the ring light is to the fluorescent molecule, the weaker the fluorescence signal. By calculating the position of minimal emission flux, MINFLUX achieves sub-5 nm resolution. Its advantage lies in not requiring prolonged exposure to collect numerous photons, enabling sub-millisecond temporal resolution. In 2022, with DNA-PAINT MINFLUX nanoscopy imaging, researchers achieved accurate localization of three target proteins within mitochondria across three channels, with a three-dimensional localization precision of approximately 5.4 and 3.1 nm [17] (Figure 1D).

Minimal stimulated emission depletion (MINSTED)

Similar to MINFLUX, Minimal stimulated emission depletion (MINSTED) iteratively locates molecules by collecting photon information from each position. It utilizes STED's laser illumination, continuously shifting ring-shaped suppression light and central excitation light. As the center of the ring approaches the fluorescent molecule, STED suppression diminishes, allowing for increased fluorescence emission, ultimately locating the fluorescent molecule at the position with the highest photon count. Recent reports indicate MINSTED achieving precision as low as 4.7 Å, measuring the position of individual molecules. Two-dimensional MINSTED data provided valuable insights about the nanoscale distribution of Mic60 in mitochondria [18] (Figure 1E). Recent reports indicate MINSTED achieving precision as low as 4.7 Å, measuring the position of individual molecules [19].

Currently, the applications of MINFLUX and MINSTED in cellular structure imaging are relatively limited. However, as the development of these techniques and the applications in biological studies, they hold the potential for breakthroughs in cell biology research, given their high temporal resolution and precise spatial localization.

The super-resolution microscopy we introduced above covers three conventional techniques commonly applied in mitochondrial research and two emerging techniques with great potential. The spatial and temporal resolution, advantages and limitations of these five techniques are summarized in Table 1. Besides, we have listed some excellent techniques that have improved upon them. It should be noted that the spatial and temporal resolutions in the table only represent the approximate range of spatial and temporal resolutions of these microscopy techniques in general cases.

Super-resolution techniques involve a delicate balance and compromise between temporal resolution, spatial resolution, field of view, and light power. SIM, STED, and STORM techniques have their own strengths and weaknesses in mitochondrial research. SIM offers rapid imaging and multicolor capabilities but lacks the resolution achieved by other super-resolution techniques. STED microscopy

Table 1: Super-resolution microscopy techniques for mitochondrial research.

Super-resolution microscopy technique	Spatial resolution		Temporal resolution	Advantages	Limitations	Technique improvement
	XY	Z				
STED	50 nm	500 nm	s	Super-resolved images directly obtained; high spatial resolution	Slow imaging speed; phototoxicity; multicolor imaging limited	3DSTED, isoSTED, RESOLFT, 4Pi RESOLFT, ExSTED
SMLM	20 nm	50 nm	s-min	High position accuracy	Low throughout; phototoxicity; post-processing required	PALM, STORM, iPALM, DNA-PAINT
SIM	120 nm	400 nm	ms-s	High imaging speed; low phototoxicity; suitable for live-cell imaging; available for multicolor imaging	Post-processing required; demanding reconstruction process; sensitive to aberrations	TIRF-SIM, 3D SIM, Hessian-SIM, NL-SIM
MINFLUX	5 nm	5 nm	ms	Super-high spatial resolution; low phototoxicity	Post-processing required	DNA-PAINT MINFLUX
MINSTED	0.5 nm	0.5 nm	s	SNR (Signal to Noise Ratio) better than MINFLUX; super-high spatial resolution; low phototoxicity	Post-processing required	–

achieves ultra-high resolution but may be limited by photobleaching and phototoxicity. SMLM provides single-molecule localization but can be time-consuming and technically challenging. Selecting an appropriate technique is crucial when addressing different biological questions.

Furthermore, as technology advances, super-resolution microscopy techniques are evolving to enhance various aspects, aiming to better fulfill research requirements. For instance, MINFLUX has enhanced time resolution by 100 times while significantly reducing photodamage to cells compared to SMLM. Although these new techniques have not yet led to groundbreaking discoveries in mitochondrial biology, they hold immense potential to become powerful tools in the future, unraveling disease mechanisms at the cellular organelle scale.

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