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Super-resolution microscopy of mitochondria☆

Stefan Jakobs[1,2] and Christian A Wurm[1]

Mitochondria, the powerhouses of the cell, are essential organelles in eukaryotic cells. With their complex inner architecture featuring a smooth outer and a highly convoluted inner membrane, they are challenging objects for microscopy. The diameter of mitochondria is generally close to the resolution limit of conventional light microscopy, rendering diffraction-unlimited super-resolution light microscopy (nanoscopy) for imaging submitochondrial protein distributions often mandatory. In this review, we discuss what can be expected when imaging mitochondria with conventional diffraction-limited and diffraction-unlimited microscopy. We provide an overview on recent studies using super-resolution microscopy to investigate mitochondria and discuss further developments and challenges in mitochondrial biology that might by addressed with these technologies in the future.

Addresses
[1] Department of NanoBiophotonics, Max Planck Institute for Biophysical Chemistry, 37070 Göttingen, Germany
[2] Department of Neurology, University of Göttingen Medical School, 37073 Göttingen, Germany

Corresponding author: Jakobs, Stefan (sjakobs@gwdg.de)

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Mitochondria

Mitochondria usually exist within cells as an extended, dynamic, interconnected network of tubules that is intimately integrated with other cellular compartments [1]. An outer membrane and a highly folded inner membrane constitute the intricate inner architecture of mitochondria. The invaginations of the inner membrane, called cristae, are not simply random wide infolds. Rather they are topologically complicated and their shape and number is adapted to the cellular requirements. The inner membrane hosts the oxidative phosphorylation system (OXPHOS). This system facilitates energy conversion resulting in the production of ATP, which makes mitochondria indispensable 'power plants' of eukaryotic cells.

Since the 1950s, various forms of electron microscopy (EM) have provided a detailed view on the membrane architecture of these organelles (reviewed, for example, in [2,3]). EM is exquisitely suited for the investigation of membrane structures, but is generally less capable of determining the distribution of individual proteins. Typically, quantitative immunogold EM requires the decoration of sections with antibodies, resulting in relatively few gold particles per decorated section. To determine the suborganellar distribution of a specific protein with this approach, numerous individual gold localizations are recorded on many images and an average protein localization is determined [4,5]. Hence immunogold EM is usually not suited to study protein distribution in individual mitochondria.

Fluorescence microscopy is arguably the most suitable approach to study the distribution of proteins in single mitochondria [6]. However, studies using conventional fluorescence microscopy to investigate protein localizations in these organelles ultimately face the challenge that mitochondria are small; the width of mitochondrial tubules is typically between 250 and 500 nm [7–9]. In conventional (confocal) microscopes diffraction limits the achievable resolution to $\geq$200 nm in the lateral plane and to $\geq$500 nm in the axial direction [10]. Hence the size of most mitochondria is just at the resolution limit of optical microscopy making the analysis of submitochondrial protein distributions always challenging and often entirely impossible using diffraction limited optical microscopes [11–15].

Over the last decade several super-resolution microscopy (nanoscopy) concepts have been devised that allow diffraction-unlimited optical resolution. All concepts that fundamentally overcome the diffraction limit exploit a transition between two fluorophore states, usually a fluorescent (on-) and a non-fluorescent (off-) state in order to discriminate adjacent features. Depending on how the transition is implemented, the current super-resolution methods may be assigned to one of two classes, namely coordinate-targeted (prominent approaches: STED [16,17], SPEM/SSIM [18,19] and RESOLFT [20–22]) and coordinate-stochastic approaches (PALM [23], STORM [24], FPALM [25], GSDIM [26], dSTORM [27], and others). The various methods routinely provide optical resolution well below 50 nm (i.e. they fundamentally overcome the diffraction barrier), have been implemented with more than one color, and 3D versions are available. The underlying physical concepts as well as the

practical differences between the approaches have been expertly reviewed elsewhere [28•,29•,30].

The challenge of imaging mitochondria

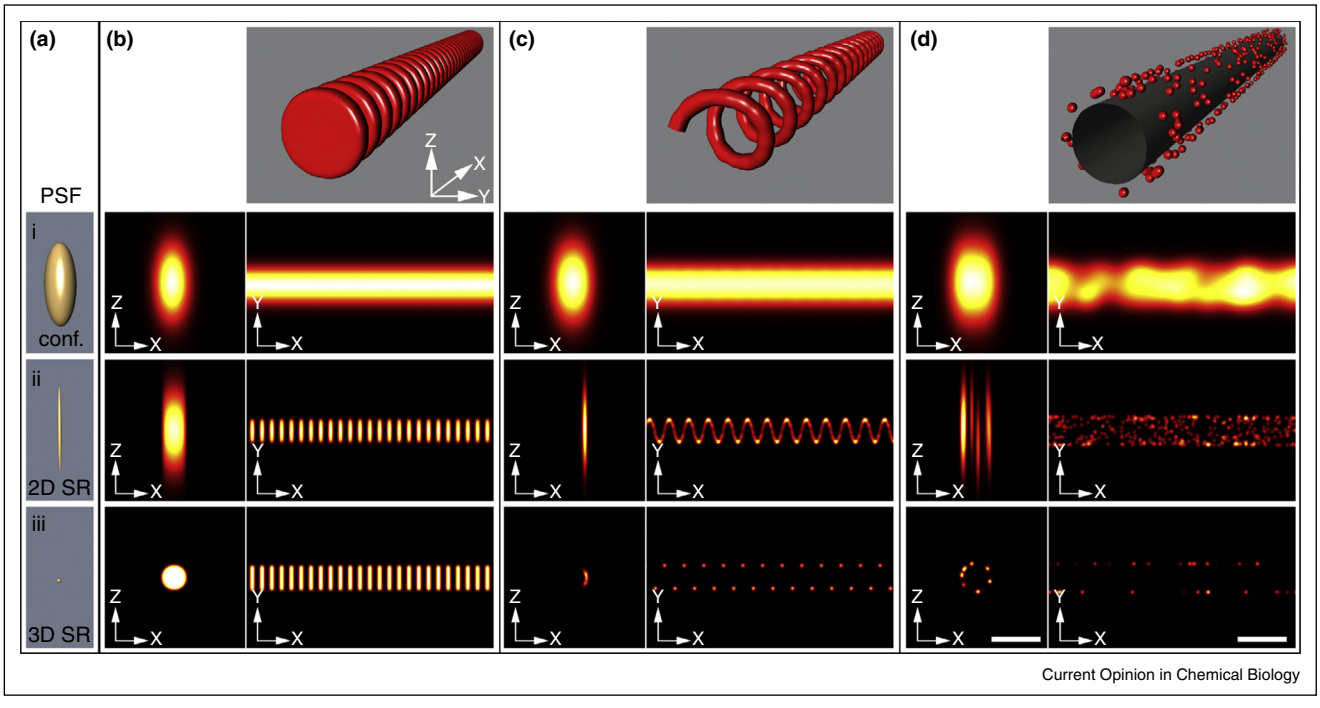
To evaluate what can be expected when imaging mitochondria with conventional diffraction-limited microscopy or diffraction-unlimited nanoscopy, we simulated three simplified models that should reflect differently labeled mitochondria (Figure 1): a mitochondrion with regularly stacked cristae (crista to crista separation is 100 nm), as often seen in EM images [31••] where only the cristae are labeled (Figure 1b). A helical structure circumventing the matrix, which might resemble a postulated mitoskeletal element [15] (Figure 1c). Randomly distributed proteins in the outer membrane (Figure 1d). We simulated how the labeled mitochondria would appear in, firstly, a conventional confocal microscope (i), secondly, a microscope enabling a resolution of 30 nm in the lateral plane and diffraction limited resolution along the optical axis (ii) and finally, a microscope enabling an isotropic 30 nm resolution (iii). These simulations purposely represent an ideal situation with a bright signal, no background and no noise. Hence, in reality, the obtainable images may look worse, but not better.

In the three examples, confocal microscopy would fail to extract any submitochondrial protein distributions. As expected, isotropic super-resolution would give the most faithful representation of the starting structure. However because of their relative complex construction, microscopes providing true isotropic super-resolution are currently accessible only in a few specialized labs [32–34]. As shown by the simulations, already an improvement in the lateral resolution allows detailed additional insight. Hence currently for many applications 2D super-resolution microscopy is preferred by many researchers.

Super-resolution microscopy of mitochondria

A number of studies using light microscopy with increased, albeit not diffraction unlimited resolution, demonstrated the advantages of improved resolution when imaging mitochondria. 4Pi-microscopy, which increases the resolution along the z-axis to ~100 nm, allowed better representations of the overall structure of the mitochondrial network both in living yeast cells [9,35], as well as in chemically fixed human cells lines [36–38]. Likewise, 2D and 3D structured illumination, which can improve the resolution by a factor of about two, has been used to better represent mitochondrial networks

Figure 1



Simulations. (**a**) Point spread functions (PSFs) drawn to scale of (i) a confocal microscope (diffraction limited), (ii) a super-resolution (SR) microscope enabling a resolution of 30 nm in the optical plane and conventional resolution along the z-axis, and (iii) a super-resolution microscope enabling isotropic resolution of 30 nm. This resolution was chosen because it can be attained with various instruments and because antibody labeling using a primary and a dye labeled secondary antibody would increase any structure to about this size [69,70]. (**b**–**d**) Simulations on how different labeled mitochondrial structures would appear in the microscopic image. To this end, the simulated data (top: 3D rendering of the data) were convolved with the respective PSFs. Shown are xy and xz sections. The simulated mitochondria have a diameter of 220 nm (b,c; inner membrane) and 280 nm (d; outer membrane). Scale bars: 500 nm.

in living cells [39,40]. Although these methods improve the resolution compared to confocal microscopy, they do not allow substantially better resolution than ~100 nm. These methods have thus not established as routine tools to study submitochondrial protein distributions.

Cells with labeled mitochondria have been used in several early implementations of super-resolution microscopy, including the first manuscript using PALM microscopy [23] and the first manuscript demonstrating two-color STED microscopy [41]. isoSTED microscopy enabling isotropic 3D resolution of 30–40 nm was used to reveal the distributions of several proteins within the organelle and allowed the visualization of individual cristae [32,42]. Utilizing STORM, Shim *et al.* succeeded in visualizing mitochondrial inner membrane dynamics in living cells using MitoTracker Red, a photoswitchable membrane probe [43].

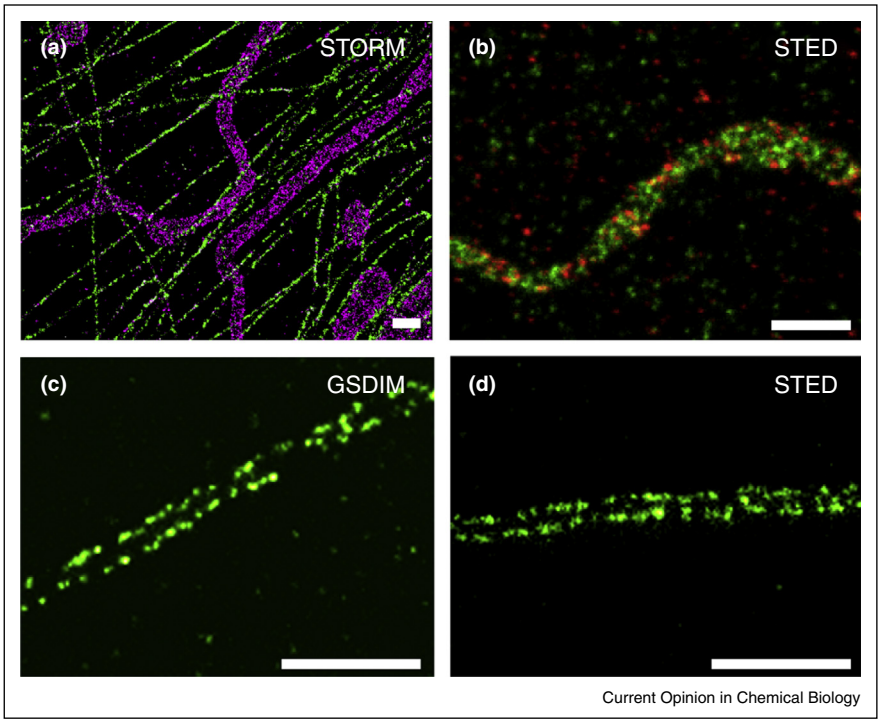
Outer membrane proteins

Tom20 is a subunit of the translocase of the mitochondrial outer membrane (TOM) complex, which is the major import gate for nuclear encoded proteins into mitochondria. Several studies have been using antisera against Tom20 to highlight the outer membrane or to study the distribution of the TOM complex itself [32,41,44•,45••]. Conclusively, these studies showed clustered distribution of the TOM complexes, which is concealed in diffraction-limited imaging, essentially as can be seen in the simulated image data (Figure 1d).

Huang and colleagues imaged cells immunolabeled for Tom20 and beta-tubulin by multicolor 3D STORM and provided detailed view of the intricate morphology of the entire mitochondrial network in chemically fixed monkey cells [45••]. This study provided detailed insights into the nanoscale spatial arrangement between mitochondria and the microtubule cytoskeleton. Interestingly, some mitochondria that appeared to co-align with microtubules when imaged with conventional microscopy were shown to have distinct interaction sites which were spaced by stretches of noncontact regions (Figure 2a).

In a high-throughput STED study involving more than 1000 cells we demonstrated that the clustering of the TOM complexes in the outer membrane is adjusted to the cellular growth conditions [44•]. Differences in the density of the clusters in the outer membrane were observed in cell lines having different growth rates. Likewise, a difference was recorded for cells forming a small colony of 20–30 cells: The clusters were sparser in the cells in the center of the colony than at its rim. Somewhat unexpectedly, this study also revealed that the density of TOM clusters followed an inner-cellular gradient from

Figure 2



Super-resolution microscopy of protein distributions in mammalian mitochondria. (**a**) Two-color STORM images showing the interaction between mitochondria (magenta) and microtubules (green) [45••]. (**b**) Two-color STED microscopy of hVDAC3 and hexokinase-I [47]. (**c,d**) GSDIM and STED imaging of Mic60 (mitofilin) showing the peculiar ordered arrangement of the MICOS clusters [31••]. Scale bars: 1000 nm.

the perinuclear to the peripheral mitochondria. Altogether, the reported findings showed a correlation of the metabolic activity of the cells and the nanoscale clustering of TOM. This suggests that the control of the distribution of TOM might be a mechanism to regulate protein import into mitochondria.

The voltage-dependent anion channel (VDAC, also known as mitochondrial porin) is the major transport channel mediating the transport of metabolites, including ATP, across the outer membrane [46]. In humans, three isoforms (hVDAC1, hVDAC2, hVDAC3) exist which are suggested to bind the cytosolic protein hexokinase-I. Dual-color STED microscopy of immunolabelled U2OS cells showed that the extent of colocalization between the hexokinase-I and hVDAC is isoform-specific (Figure 2b). This observation suggests functional differences between the three VDAC isoforms [47].

Inner membrane proteins

The inner membrane exhibits two structural domains, the inner boundary membrane that is parallel to the outer membrane and the cristae membrane. Only recently it was nonambiguously demonstrated that the cristae membrane and the inner boundary membrane have different protein compositions [4,5,48–50].

Few studies have investigated the nanoscale distribution of proteins in the mitochondrial inner membrane with light microscopy [23,32,51,52•] and mainly concentrated on proteins in OXPHOS, presumably because of the abundance and the relative ease of labeling of these proteins. One exception is a study using EM and STED microscopy to investigate the submitochondrial distribution of a protein complex named MICOS (mitochondrial contact site and cristae organizing system; previously also called MINOS or MitOS) [31••]. MICOS is a large hetero-oligomeric protein complex that has crucial roles in the maintenance of cristae junctions, inner membrane architecture and in the formation of contact sites to the outer membrane [53]. STED as well as GSDIM imaging of primary human fibroblasts decorated with antibodies against the MICOS core component Mic60 (according to a recently unified nomenclature [54]; previous name: mitofilin) showed that MICOS forms distinct clusters (Figure 2c,d). Unexpectedly, these clusters were arranged in a regularly spaced array in parallel to the cell growth surface. By quantitative immunogold EM we demonstrated that Mic60 is preferentially enriched at the cristae junctions [31••]. Furthermore, electron tomography showed a horizontal arrangement of cristae junctions in many mitochondria. Altogether this demonstrates that at least in the peripheral mitochondria of human fibroblasts the inner-mitochondrial localization of MICOS is correlated to the orientation of the cellular growth surface, suggesting an unexpected level of regulation of inner mitochondrial architecture.

Nucleoids

Mitochondria contain their own genome (mtDNA), which is packaged into nucleoprotein complexes (nucleoids) located in the innermost mitochondrial compartment, the aqueous matrix [55,56]. The nucleoids are distributed throughout the mitochondrial network. In humans, the mtDNA encodes 13 proteins, which are essential for the function of OXPHOS. An important and still not conclusively answered question is whether a single nucleoid contains one or multiple mtDNA copies [57].

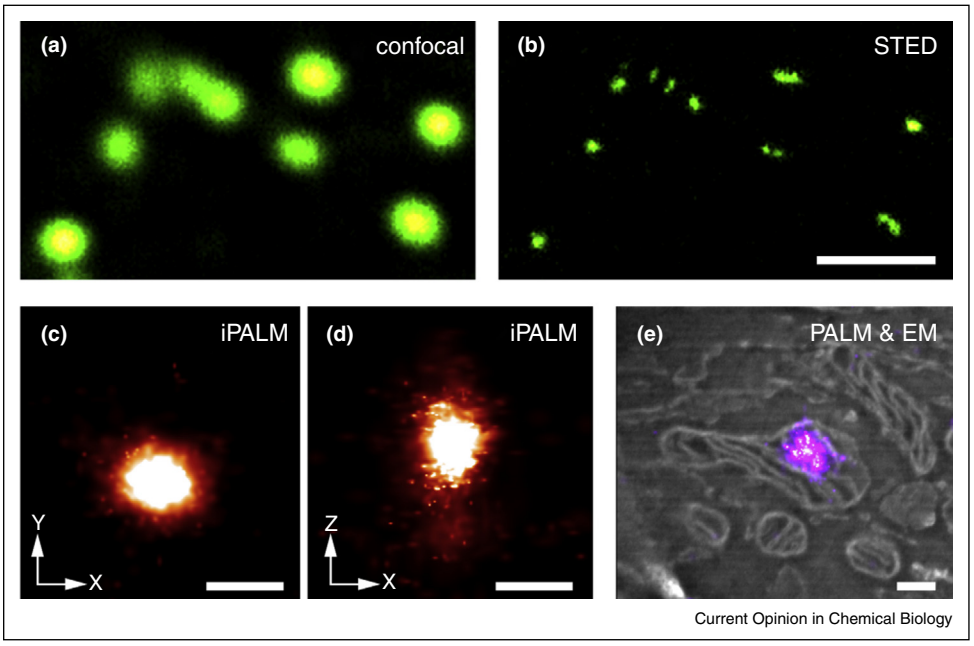
This issue has been addressed by a number of studies using different experimental procedures, which came to rather inconclusive estimates, ranging from on average 1.4–10 mtDNA molecules per individual nucleoid (reviewed in [55,56]). STED microscopy allowed to visualize ~1.6 times more nucleoids per human fibroblast than confocal microscopy (Figure 3a,b). Using automated image analysis we found 1883 ± 106 nucleoids per primary human fibroblast [58]. Based on this data combined with the average number of mtDNAs per cell as determined by molecular biology, the average number of mtDNA molecules per nucleoid was calculated as ~1.4. This number is smaller than most other estimates, which may be due to the fact that super-resolution microscopy allowed to count the number of nucleoids more precisely. In addition, there might be differences in the number of mtDNAs per nucleoid in different cell lines or tissues. Hence more studies are required to allow a definite answer on the average number of mtDNAs per nucleoid.

In a careful study employing 2D and 3D photoactivated localization microscopy (PALM and iPALM, respectively), Brown *et al.* [59] demonstrated that nucleoids often adopt an ellipsoidal shape (Figure 3c,d), although their shape may vary strongly and may depend on the interaction of the nucleoid with the inner membrane. They determined the mean dimensions of an ellipsoidal nucleoid as 85 nm × 108 nm × 146 nm. From these data they concluded that the mtDNA is extraordinarily condensed, similar to the situation in a papillomavirus capsid [56,59].

In an independent STED study, Kukat *et al.* semi-automatically analyzed the size of >35 000 nucleoids in seven different cells lines [58]. Fully in line with the study by Brown *et al.*, this study also demonstrated a large variability in the shape and size of the nucleoids. Assuming the simplified model of a spherical nucleoid, it was determined that the antibody decorated nucleoids had a diameter of ~100 nm, also in good agreement with the study be Brown *et al.* Interestingly, the mean diameter was well conserved across several cultured mammalian cell lines.

In a technical tour de force, Kopek *et al.* correlated 3D super-resolution (iPALM) images of mitochondrial nucleoids with 3D EM data [60•]. Using a modified Tokuyasu cryosectioning protocol for fixation and freezing,

Figure 3



Super-resolution microscopy of mitochondrial nucleoids. (**a,b**) Nucleoids were labeled with antibodies against dsDNA and imaged with a confocal or a STED microscope [58]. (**c,d**) Ellipsoidal nucleoid projections recorded with iPALM [59]. (**e**) Correlated image of TFAM-mEos2 PALM data with electron micrograph [63]. Scale bars: 1000 nm (a,b), 150 nm (c), and 200 nm (d,e).

they prepared 500–750 nm thick slices of cells expressing TFAM fused to the photoconvertible protein mEOS2. These slices were imaged with iPALM [33] to record the 3D distribution of TFAM in the slice. Next, 3D EM images were obtained with focused ion beam block-face ablation followed by scanning EM imaging. Using this approach Kopek *et al.* observed a variety of nucleoid sizes and shapes. In some cases cristae and nucleoids appeared to be intertwined in a complex manner. Understanding the biological relevance of these observations would require a lot more image recordings, which presumably would be a considerable challenge given the technical complexity of the chosen approach. However, technically less demanding 2D approaches correlating various super-resolution microscopy approaches with EM have been developed by the same group and others (Figure 3e), opening up this technology to a wider community [23,61–63].

Conclusions and perspectives

Given the tremendous benefits that super-resolution offers for imaging mitochondria, we are undoubtedly going to see many more studies using these technologies to tackle fundamental problems in mitochondrial biology in the near future. There are many issues where super-resolution is needed; some of those that we feel are amongst the most current ones are highlighted in Figure 4.

Answering these questions will require further progress in (semi-)automated super-resolution microscopy and image analyses to evaluate the heterogeneity on the nanoscale [44•,64,65], in analyzing protein movements [52•,66], as well as in counting the number of molecules [67,68]. Each of these tasks represents a formidable challenge, but the first important steps have been taken and given the impressive progress that super-resolution has made over the last decade, the challenges seem surmountable.

Figure 4

Mitochondrial architecture
How are cristae established, shaped and maintained?
Do mitochondria have structural elements corresponding to a skeleton?

Heterogeneity of mitochondria
Are all mitochondria / nucleoids alike in a single cell?
What is the level of heterogeneity between cells?

Mitochondrial inner membrane
How is the heterogeneity of the inner membrane established and maintained? How do proteins move within the various mitochondrial compartments? Do cristae junctions act as barriers?

Assembly of OXPHOS supercomplexes
Where are the nuclear and mitochondrial encoded subunits of OXPHOS supercomplexes inserted? Where and when is assembly of the supercomplexes taking place?

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Future challenges.

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