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Review

Fluorescent Probes for Nanoscopic Imaging of Mitochondria

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Studying the ultra-fine structure and functions of mitochondria at a nanoscale level has garnered tremendous attention from biologists. Mitochondria perform many more functions than merely generating adenosine triphosphate (ATP), and their functions can vary in different eukaryotic cells. In place of diffraction-limited conventional imaging techniques, advanced nanoscopic technologies have been devised in the past decades to explore the unknown aspects of mitochondrial dynamics and complex structures with a sub-diffraction resolution. The success of these super-resolution microscopy and nanoscopy techniques is complemented by the advancements in designing smart fluorescent probes that target mitochondria. Therefore, this review includes the comprehensive aspects of the recent progress in developing fluorogenic systems for nanoscopic imaging of mitochondria. The review also critically assesses the associated benefits and limitations of such fluorophores when they are employed in practical experiments. Future scope and challenges in developing suitable fluorophores for several nanoscopic techniques are also judiciously evaluated.

INTRODUCTION

Mitochondria, popularly termed the "powerhouses" of the cell, are one of the most important constituents of modern eukaryotic cells. Apart from playing a central role in adenosine triphosphate (ATP) production, mitochondria perform numerous additional functions within the cell.[1] Mitochondria have typical structural features such as a double membrane and a semi-isolated conformation, which play important roles in upholding their unique, complicated functions. However, mitochondrial functions may vary in different eukaryotic cells. It has been established that the mitochondrial proteins that are required for the generation of ATP through aerobic respiration are primarily encoded by the mitochondrial DNA (mtDNA).[2] Therefore, mutations in mtDNA often result in a number of complex mitochondrial diseases in humans, which ultimately lead to severe organ-specific ailments.[3] Recently, studies related to mutations in mtDNA also revealed that mtDNA-associated somatic stem cell defects could accelerate aging.[4] Since mtDNAs perform such important roles in the cells of living organisms, biologists have employed several means to study the fine structures and complex biological functions of mitochondria. The functions of organelles such as mitochondria can be mechanistically resolved by studying their molecular and structural composition and large-scale spatial arrangements, as well as closely monitoring the dynamic alterations occurring in the cells. Fluorescence microscopy (FM), a powerful tool for studying cellular dynamics, has been exploited over the years to study specific protein distribution in a single mitochondrion. However, because of the very small size of mitochondria, which falls under the limit of diffraction, conventional FM has failed to address several key issues pertaining to mitochondrial structures and functions at the nanoscale level.

The Bigger Picture

Accurate, detailed understanding of the biological events occurring in the sub-cellular organelles is important for countering critical physiological disorders and, hence, can promote the welfare and optimal health for mankind. In this regard, fluorescence bio-imaging has paved the way for studying cell biology in depth. However, subcellular organelles such as mitochondria, which perform numerous important functions in the cellular context, cannot be studied in detail because of their very small size, which falls under the diffraction limit. To overcome this issue, several nanoscopic microscopy techniques have come to light recently, and upon being coupled with appropriate fluorophores, these can facilitate the study of the structure and dynamic processes inside mitochondria with nanoscale resolution. In this review, we have carefully assessed both the technical advancements in various nanoscopic techniques and the progress in developing smart fluorophores for mitochondria-specific nanoscale imaging.

In this context, in the last decade, several super-resolution microscopy (SRM) techniques or nanoscopic imaging methods that allow researchers to observe the fluorescence image of the subcellular organelles beyond the diffraction limit have been developed.[5] In particular, commercially available far-field super-resolution optical microscopy techniques—such as structured illumination microscopy (SIM), stimulated emission depletion (STED), fluorescence photoactivation localization microscopy (FPALM), and stochastic optical reconstruction microscopy (STORM)—have put forth a broad impact on biological research. However, these nanoscopy techniques are highly susceptible to the availability of suitable fluorescent probes, and the designing of a practical SRM experiment could be inhibited because of the lack of adequate fluorophores. Because objects of interest are exposed to an intense laser beam in SRM optical systems, the key to studying subcellular organelles with high precision is to develop organelle-targeted fluorescent markers and probes that exhibit certain specialized features.

Therefore, there is an immediate need to articulate the recent progress in the use of SRM and nanoscopic microscopy systems in studying the fine structure and functions of mitochondria with sub-diffraction resolution. The key to employing SRM for investigating detailed mitochondrial structure and functions depends on developing outstanding fluorescent markers and probes with suitable qualities, and hence it is prudent to critically assess the recent progress in designing mitochondria-targeted probes and tags, particularly for SRM. However, despite the tremendous contribution of SRM in mitochondria-related research, no comprehensive review discusses the use of advanced optical techniques and devices, or the progress in rational probe design, pertaining to mitochondria-targeted imaging with sub-diffraction resolution. Recently, some review articles have deliberated upon mitochondria-targeted fluorescent probes in the context of organelle-specific sensing applications and/or bio-imaging.[6,7] However, neither are these articles comprehensive in describing the usefulness of SRM techniques, nor do they comment on the structural requisites of the fluorescent probes for nanoscopic imaging of mitochondria. On the other hand, most of the review articles about SRM in recent times are generally limited to discussions regarding the particular facets of SRM and mostly stress upon the conceptual basis of various nanoscopic techniques through summarizing the contemporary applications.[8–10] A review article by Jacobs et al. has provided a broad outline about the use of SRM for mitochondria.[11] However, it mainly restricted its discussion to the scope of utilizing SRM for realizing precise structural information of the proteins and nucleoids in the organelle and hardly deliberated upon the progress in designing newer fluorescent probes for mitochondria-specific SRM. In this context, a sole review that effectively covers both the advancements in the development of optical SRM techniques and the progress in designing appropriate fluorescent probes that are useful in highlighting the progress of biological events in mitochondria with sub-diffraction resolution holds tremendous potential to define the future directions in this active field of research. Hence, in the present review, the utility of different types of mitochondria-targeted fluorescent probes in studying the structure and the important biological functions of mitochondria by using various super-resolution microscopic methods has been comprehensively accentuated. Unlike other review articles, the present review article covers the inclusive aspects of the advancements in optical techniques, exploration of biological events occurring in mitochondria, and the progress in chemical probe design together in a single review (Scheme 1).

MITOCHONDRIAL STRUCTURES AND THEIR FUNCTIONS

Structurally, mitochondria are mainly composed of six different components: outer membrane, inner membrane, inter-membrane space, cristae, mitochondrial matrix,

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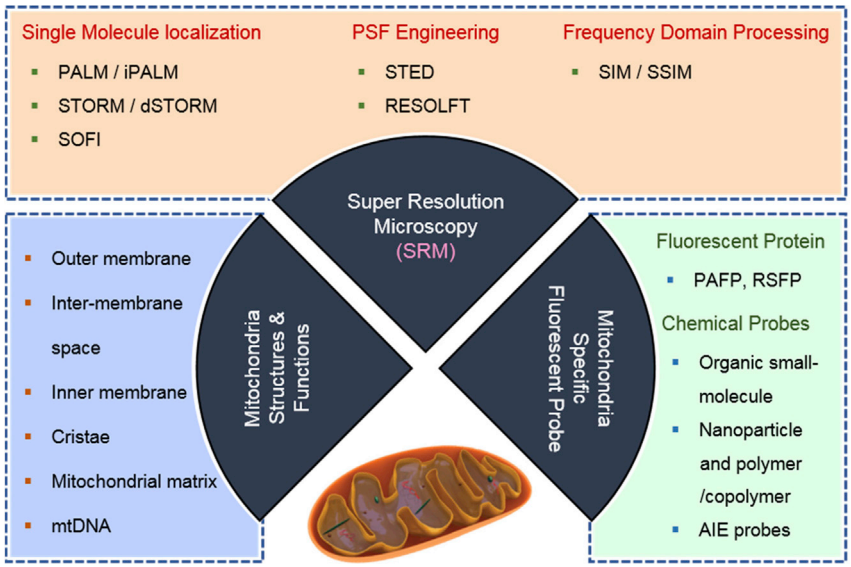


Scheme 1. Schematic Representation of the Structure of the Review at a Glance

and mtDNA, which have been schematically represented in Scheme 2. The basic structure and function of mitochondria along with diseases related to their dysfunction have been discussed in various literature.[3,12–14] Hence, only a brief description of the mitochondrial structure and related functions are included below.

Outer Membrane

The outer membrane of mitochondria that encircles the whole organelle is very much comparable to the plasma membrane given that both contain a similar protein-to-phospholipid ratio (1:1). Essentially, the outer membrane contains a large number of integral membrane proteins (known as porins) and has an average thickness of 60–75 angstroms (Å). In the outer membrane, these porins mainly form channels to monitor the molecular-weight-dependent diffusion wherein molecules with a molar mass of <5,000 daltons are allowed to enter freely except in special cases.

Inner Membrane

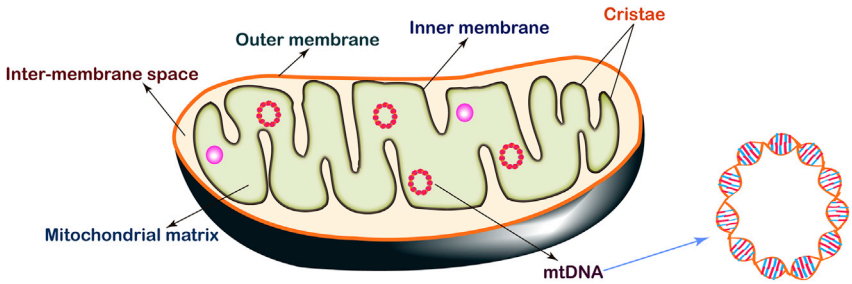
Unlike the outer membrane, the inner membrane contains a very high protein-to-phospholipid ratio (more than 3:1), and about 151 different polypeptides are found in it. The inner membrane is highly impermeable to most of the ions and molecules. It is only in the presence of special membrane transporters that it permits the entry of molecules and ions into the matrix.

Inter-membrane Space

The space between the outer and inner membrane of a mitochondrion is identified as inter-membrane space. Small molecules, such as ions and sugars, are abundant in this part of mitochondria, which is comparable to that of the cytosol because these are easily permeable through the outer membrane. However, large proteins need to adhere to a specific signaling sequence for being transported across the outer membrane.

Cristae

The inner mitochondrial membrane comprises numerous folds termed cristae. These cristae not only confer the characteristic wrinkled shape to the inner membrane



Scheme 2. Schematic Representation of the Mitochondrial Structure

structure but also increase the surface area, allowing many chemical reactions such as the production of ATP.

Mitochondrial Matrix

The inner membrane of a mitochondrion encloses the mitochondrial matrix that comprises about two-thirds of the overall mitochondrial proteins. The matrix not only hosts important chemical events such as the production of ATP, assisted by the inner membrane derived ATP synthase, but also contains several enzymes, special mitochondrial ribosomes, tRNA, and several copies of the mitochondrial genome.

mtDNA

mtDNA basically exists as a small circular chromosome inside the mitochondrial matrix. In humans, the mtDNA is mainly inherited from the mother, and it only represents a very small portion of the entire DNA.

NANOSCOPY FOR STUDYING MITOCHONDRIAL STRUCTURES AND FUNCTIONS

Conventional FM cannot overcome the diffraction limit, which makes it unsuitable for studying the fine structures and dynamics of mitochondria at the nanoscale level. Even though electron microscopy (EM) can overcome the diffraction limit to achieve fine structural imaging of mitochondria, it also has limitations in terms of performing live-cell imaging. In this regard, to circumvent these difficulties, various nanoscopy or SRM techniques—mainly based on spatial processing in wide-field imaging (single-molecule localization microscopy [SMLM]), point spread function (PSF) engineering, or frequency-domain-processing technology—have recently been devised.

Single-Molecule Localization Microscopy

The basic concept of every SMLM technique is to determine the position of single fluorophores with high precision by switching the fluorophore molecules sequentially rather than concurrently.[9,15] Thereafter, by collecting a large number of positional coordinates that basically represent the position of individual fluorophores, a super-resolution image can be constructed computationally through the superimposition of the collected frames. Eventually, the quality of an SMLM image becomes highly dependent on the localization precision, which basically corresponds to the number of detected photons with respect to the single fluorophore.[9] Even though the basic concept of all the SMLM techniques is very similar, there could be some unique features, particularly in the working principle of the probe, for individual SMLM techniques.

Photoactivated Localization Microscopy

Photoactivated localization microscopy (PALM) and/or interferometric PALM (iPALM) is a typical SMLM wherein the used fluorogenic system (either fluorescent proteins [FPs] or organic fluorophores) needs to be photoconvertible or photoactivatable, such that it can endure photoactivation (for once or for several times) until it becomes permanently non-fluorescent as a result of photobleaching. Our previous review included the conceptual basis as well as the technical advancements of this SRM technique.[5] So here, we mainly focus on some applications of this SRM technique for obtaining resolved mitochondria-specific nanoscopic images along with the related advantages and drawbacks.

Brown et al. successfully achieved high-resolution imaging of mitochondrial proteins using PALM and similar methods.[16] Later, with the help of both two-dimensional and three-dimensional (3D) PALM (PALM and iPALM, respectively), Brown et al. were also able to study the relative locations and dimensions of mitochondrial nucleoids with an exceptionally high spatial resolution.[17] Compared to the previously used surface-limited EM technique, the iPALM technique better represented the true dimensions of the nucleoids. However, one limitation of the two-color PALM study[17] with respect to the detection of FPs remaining within the cryosections was its inability to determine the precise molecular locations in the z dimension. Recently, Jezek and co-workers were also able to study the alterations in the mitochondrial network and cristae morphology in HepG2 cells caused by hypoxia, using SMLM nanoscopy (PALM and STORM).[18] Hence, the SMLM technique, PALM, has the potential to shed light on many mitochondria-related unknown structural and functional problems at the nanoscale level. However, if the data points with poor localization are selectively excluded in PALM to obtain high localization precisions, it might compromise the molecular density, resulting in an unrecognizable image. The mentioned situation might often arise in the case of many FPs wherein the target molecule (FP) is minimally represented in the sample.

Stochastic Optical Reconstruction Microscopy

STORM is another type of SMLM wherein photoblinking or photoswitching plays a key role in determining the employability of a single fluorophore for imaging application. In our previous review, we thoroughly discussed the fundamentals of this super-resolution imaging technique.[19] In the recent past, the use of STORM imaging in resolving the ultra-fine mitochondrial structure as well as its functions in nanoscale level has fetched some important breakthrough in cell biology. For example, using multicolor 3D STORM, Zhuang's group visualized the interactions between the cellular structures with a sub-diffraction spatial resolution.[20] Eventually, they acquired the nanoscopic images of the entire mitochondrial network in fixed monkey kidney BS-C-1 cells by using 3D STORM to reveal the mitochondria-microtubule contacts that were not resolvable by conventional microscopy.[20] Recently, Ježek and co-workers also presented a reasonable bio-analytical tool to study the size and/or shape of mitochondrial cristae, even in the case of unresolved direct images, by using direct STORM (dSTORM), wherein the ATP-synthase F1 subunit α (F1α) clearly indicated the variations in cristae width and/or morphology.[21] Moreover, the super-resolution imaging method was also instrumental in resolving the sub-mitochondrial nucleoids of mtDNA.[21]

It must be pointed out here that STORM is generally advantageous over PALM in terms of obtaining a more precise localization of single molecules because it can handle higher photon yield from the activated single fluorophores and deals with

the reactivation of the same molecule again and again.[22] However, the larger size of antibodies (conjugated with dyes) and a lower density of used fluorophore could be inconvenient for STORM imaging. Moreover, dimerization and oligomerization of FPs can adversely affect the reconstruction of the image in stochastic SRM because there would be multiple fluorophores (instead of single) per each molecule of the localized fluorescent probe.

Super-resolution Optical Fluctuation Imaging

As discussed earlier, in SMLM, the PSF centroid position basically corresponds to the location of the single fluorescent molecule, and by sequentially determining the position of the isolated fluorophores (PSF centers) and plotting them into a position histogram, an image with sub-diffraction resolution can be achieved. However, fluorophores with specialized photo-physical characteristics, such as photoswitching and/or photoactivation, are only suitable for SMLM techniques such as STORM (or dSTORM) and PALM (or iPALM). Therefore, there is always the issue of fine-tuning the activation and deactivation or photoswitching behavior of the probe with regard to the single fluorophore. In this context, super-resolution optical fluctuation (SOFI) imaging has been introduced as an alternative to the commonly used single-molecule localization nanoscopies, such as STORM (or dSTORM) and PALM (or iPALM), wherein the requirements for the photoswitching kinetics can be much more flexible. Basically, SOFI has the advantage of accommodating the overlapping of images obtained from the fluorophores with reversible stochastic and independent intensity fluctuations.[22,23] In addition, the technique can endure inadequate on-off switching and not-so-prominent intensity fluctuations.[22,23] Therefore, in the near future, this method has tremendous potential to be used in fast live-cell experiments for the study of subcellular organelles, such as mitochondria, in depth. Theoretically, SOFI can provide a very high resolution of images; however, many practical issues, such as noise and non-uniform brightness of the sample, restrict the usage of a higher-order model. Generally, the resolution of SOFI cannot be improved markedly.

PSF Engineering-Based Nanoscopy

In confocal microscopy, the resolution of the microscope is diffraction limited. This basically depends on the size of the illumination spot, which is commonly defined as the PSF. However, if the effective PSF could be made sharper by engineering new methods, it could result in substantial enhancements in both resolution and contrast.[24] This constitutes the basic feature of a nanoscopic technique based on PSF engineering.

Stimulated Emission Depletion Microscopy

STED microscopy uses the fundamental concept of minimizing the effective PSF to break the diffraction limit. A second doughnut-shaped laser is used alongside the excitation laser to send back all the excited fluorophores to the ground state before they can emit, except a few molecules that remain inside the focus. Images can then be captured by scanning the excitation laser beam pixel by pixel. The working principle of STED microscopy has been effectively illustrated in our previous review.[5] STED nanoscopy has been utilized vastly to understand several mitochondrial functions. For instance, this technique can be employed to quantitatively determine the nanoscale distribution of Tom20, which is basically a subunit of translocase of the mitochondrial outer membrane (TOM) complex. Using STED nanoscopy, Hell and co-workers determined the localization of Tom20 in clusters, which has a direct connection with the mitochondrial membrane potential.[25] The distribution of the clusters of Tom20 and Tom22 was found to follow the inner-cellular gradient

from the perinuclear to the peripheral mitochondria.[25] With the help of super-resolution STED microscopy, Große et al. also revealed that in the case of Bax-mediated mitochondrial outer membrane permeabilization, the pore size plays a key role.[26] However, it should be mentioned here that the use of intense STED laser could be detrimental for live cells and can cause photodamage to both the fluorophores as well as the sample. In this regard, reversible saturable optical linear fluorescence transitions (RESOLFT) imaging approach can be used as an alternative wherein the slowly switched emitters are pertinent.

Reversible Saturable Optical Linear Fluorescence Transitions

Hell and co-workers have also introduced the concept of RESOLFT wherein the switchable or reversibly saturable fluorophores can be utilized to achieve the sub-diffraction resolution in far-field microscopy. Based on PSF engineering, RESOLFT can be described as the more generalized use of the concept of STED and ground state depletion (GSD) microscopy wherein saturated optical transition (depletion) between the different states of a fluorogenic probe is crucial for garnering nanoscale resolution. For example, Wang et al. were able to visualize the dynamics of the Bcl-xL and Bak complex on the mitochondrial membrane in live cells through parallelized RESOLFT microscopy.[27] In fact, very rare fluorophores can be used in RESOLFT, which also limits the usage of RESOLFT.

Frequency-Domain-Processing Technology

SIM is one of the most promising SRM techniques that use the frequency-domain-processing technology. The best part about this nanoscopy is that it allows versatility in the choice of fluorescent probes. There are no such hitches of the sophisticated sample preparations as in the other SRM methods. Furthermore, the SIM can also be applied to samples that are prepared for conventional FM. The widespread use of SIM in vast samples can be attributed to its simple working principle, which is basically an extension of the wide-field FM. Therefore, multicolor imaging and live-cell imaging can be well implemented in the SIM experiments.[28] For example, Li and co-workers utilized the 3D-SIM to get the nanoscopic information about sub-mitochondrial localization of proteins. With the dual-color 3D-SIM imaging analysis, they were able to validate that the mitochondrial kinase PINK1 exist mainly in the cristae membrane and intra-cristae space, but it cannot be found in the outer membrane of healthy mitochondria.[29] Recently, Ahluwalia and co-workers also studied the sub-mitochondrial structures with the help of 3D-SIM in live cells.[30] However, this microscopy technique has some obligations to using a very sophisticated computational procedure that needs to be simplified in the future. In addition, this imaging technique cannot be used to achieve ultra-high resolution similar to the SMLM (PALM and STORM). In our view, to resolve the issues related to the ultra-fine structure of mitochondria, SMLM techniques are still the best as they can afford extremely high resolution (~20 nm) even though there are concerns about the appropriate probe selections.

Airyscan Super-Resolution Microscopy

This microscopy technique basically represents advancements in the performance of confocal microscopy. In this newly devised concept, a different type of laser-scanning microscope (LSM) detector that can effectively enhance the resolution and signal-to-noise ratio (SNR) in comparison with the usual LSM has been introduced. Essentially, in Airyscan microscopy, a hexagonally packed detector is used in place of the physical confocal pinhole aperture and unitary detector so that it can collect additional information of a pinhole-plane image at every excitation scan position and result in improved spatial resolution.[31] In Airyscan microscopy, the selection

of fluorophores is highly convenient. However, compared to other SRMs, Airyscan is unable to provide a desirable high imaging resolution.

ADVANCES IN DESIGNING MITOCHONDRIA-TARGETED FLUORESCENT PROBES FOR SRM

With the technical advancements in SRM, the scope of monitoring subcellular structures such as mitochondria at a nanoscale level has become feasible. However, the choice of appropriate fluorescent tags and probes plays a crucial role in achieving a good quality nanoscopic image. Without employing a rationally designed fluorophore, it is difficult to access biological insights in subcellular matrices, even if an advanced SRM method is introduced. Each SRM technique requires fluorescent probes with different sets of specific criteria to process a super-resolution image as already discussed in the previous sections. Usually, biologists try to fuse FPs with mitochondrial proteins, which is helpful in studying specific genes (proteins). However, in recent years, small-molecule-based fluorescent probes have attracted great attention because of their beneficial attributes in both *in vitro* and *in vivo* labeling. Hence, it is prudent to gain insights into recent advancements in nanoscopic imaging of mitochondria using both FPs as well as chemical probes in the light of contemporary literature. Therefore, this section mainly summarizes the design, synthesis, and utility of various fluorescent probes (along with their associated merits and demerits), which have been utilized to unravel mitochondrial structure or functions via SRM. The discussion in each subsection, related to the selection of mitochondria-specific fluorescent probes for different SRM techniques, follows the same order as narrated in the previous sections (i.e., SMLM, PSF engineering, and frequency domain processing, chronologically) for the convenience of the readers.

Fluorescent-Protein-Based Probes

Structurally, typical FPs have many similarities with analogous synthetic dyes.[32] However, the photophysical properties of the actual FPs often differ significantly from those of their model chromophores in solution because of the presence of a protein barrel in FPs, which ultimately restricts the influence of several external factors on the fluorophore.[32] FPs such as green fluorescent protein (GFP) are used extensively for various imaging applications. However, in many GFP-like chromoproteins, the fluorescence quantum yield might become very low ($\sim 10^{-4}$–10^{-5}) mainly because of the predominance of thermal relaxation instead of the normal fluorescence. Still, FPs such as eGFP and eYFP have reasonable quantum yields (Φ_f = 0.6). Some of the other FPs with higher quantum yields are Citrine (Φ_f = 0.76), Ypet (Φ_f = 0.77), Dronpa (Φ_f = 0.85), mEOS2 (Φ_f = 0.84), and mTurquoise2 (Φ_f = 0.93).[33] Krylov and co-workers and Lukyanov and co-workers have summarized a large number of FPs with photophysical properties that can be used in various bio-imaging applications, though they did not specifically deliberate upon the significant aspects of choosing the appropriate FPs for mitochondria-specific super-resolution imaging.[32,33]

The mitochondria-specific localization of FPs is generally accomplished by fusing the gene of the FP to that of the gene encoding the appropriate mitochondrial protein.[34] It is worth noting that one such frequently used mitochondrial targeting sequence, which preferentially localizes to the mitochondria is subunit VIII of cytochrome c oxidase. Various mitochondrial targeting sequences can be selected according to the requirements of the imaging experiments by means of software prediction algorithms.[34]

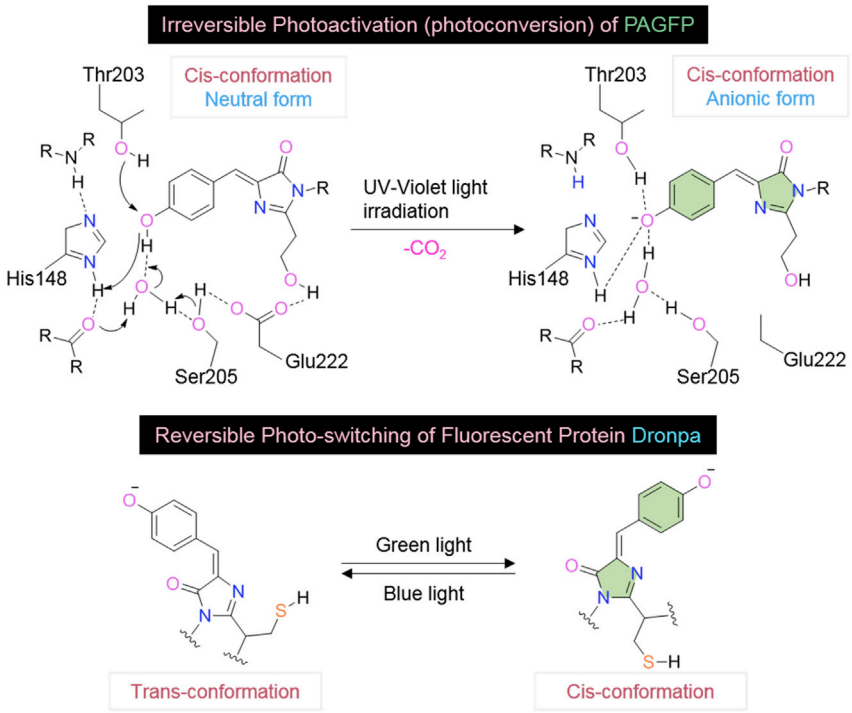


Figure 1. Schematic Representation of the Irreversible Photoactivation or Photoconversion (Promoted by Decarboxylation) of PA-GFP (Up) and Reversible Photoswitching of Dronpa (Down)

After attaining the mitochondria targeting ability, the chosen fluorescent-protein-based probe requires to be modified in such a way that it fulfills the essential photophysical criteria of the particular SRM experiment. Different SRM techniques demand different sets of criteria, which need to be fine-tuned with the chosen FP and the experimental setup to achieve a high-quality image with sub-diffraction spatial resolution. For instance, single-molecule localization and/or switching behavior are the key to using photoactivatable fluorescent proteins (PAFPs) in super-resolution imaging. The emission profile of a PAFP can be changed with the excitation at a particular wavelength, which might lead to a permanent photochemical modification of the FP. This is termed irreversible photoactivation or photoconversion, wherein the non-fluorescent (dark) state is irreversibly converted to a fluorescent state.[32,35]

However, isomerization between the two conformers of a FP can result in reversible photo-activation, which is mainly known as photoswitching. Figure 1 illustrates the photoactivation (photoconversion) of PAGFP and the photoswitching process in Dronpa as representative examples.[35] It is important to highlight that the quality of the super-resolution images depends on several other fundamental photophysical and photochemical aspects. For example, the localization precision of a single molecule is highly dependent on the number of photons emitted. Similarly, the quality of an image could be compromised because of the dimerization of FPs, which might subsequently lead to unsolicited aggregation of target proteins. Moreover, the low contrast ratio, which basically signifies the ratio of the rate constants for on- and off-switching, can impede the attainable localization density. In this context, Zhuang and co-workers have assessed such photophysical and photochemical performances of twelve common PAFPs.[36] They observed that even though the FP

Table 1. Properties of Some of the Fluorescent Proteins Discussed in This Review in the Context of Nanoscopic Imaging of Mitochondria

Fluorescent Protein	Excitation or Absorbance Maximum (nm)	Emission (nm)	Quantum Yield	Photo-stability	Additional Information
mMaple	489	505	0.74	good	prone to form a dimer; hence, it was modified as mMaple2 and mMaple3, which exist mainly as monomers
	566	583	0.56		
Dronpa	503	515	0.85	poor	photo-activatable; reversibly photo-switchable; good choice for PALM and STORM imaging
PAmCherry1	564	595	0.46	medium	red emission; exists as monomer
mEos2	573	584	0.66	good	monomeric form; suitable for SRM
	490	507	0.50	medium	
Dendra 2	553	573	0.55	good	photo-switchable monomeric FP
RsKame (DronpaV157L)	503	517	0.86	medium	a variant of Dronpa slows down the rate of the switching process
PA-GFP	504	517	0.79	poor	photo-activatable; can be modulated to target mitochondria
Mito-RFP	555	584	0.48	medium	monomeric; fast maturation; high pH stability

mMaple exhibited good photophysical properties, it had a notorious dimerization tendency. Therefore, they modified mMaple to develop a couple of new PAFPs designated as mMaple2 and mMaple3, which revealed least dimerization tendencies while maintaining other photophysical qualities of mMaple. Some of the frequently used FPs that have been discussed in this review, particularly in the context of nanoscopic imaging of mitochondria, are included in Table 1.

PAFPs are, in principle, eligible for PALM or FPALM. However, for single-color PALM or FPALM imaging, PAFPs are not favored because of their initial non-fluorescent nature, which precludes locating the precise area of interest inside the cell. Hence, these PAFPs are commonly employed in two-color or multicolor PALM instead of single-color PALM. However, some suitable monomeric photoswitchable FPs can be employed in single-color PALM or FPALM imaging. Dendra2 is one such monomeric photoswitchable FP that can undergo UV-induced transformation from the green fluorescent form to the red fluorescent form. Recently, in this context, Weatherly et al. were the first to use FPALM SRM technique in toxicological studies to demonstrate the antimicrobial agent triclosan (TCS)-meditated disruption of mitochondrial nanostructure in various cells, including mast cells.[37] To perform super-resolution imaging of mitochondria using the FPALM microscopy, the outer membrane of the mitochondria was labeled with Dendra2-TOM20 in NIH-3T3 cells.

Another photoswitchable, GFP Dronpa, is commonly chosen as a fluorescent marker for PALM. For instance, Vaziri et al. successfully achieved the super-resolution imaging of the mitochondrial matrix in fixed HFF1-cells using the FP Dronpa.[38] The mitochondrial targeting sequence, subunit VIII of cytochrome *c* oxidase, was attached to Dronpa to cause the FP-mediated mitochondrial expression. However, the photoactivation of a single molecule of Dronpa is often liable to rapid deactivation when strong excitation light is used. In this regard, Bustamante and co-workers evaluated the photoswitching properties of Dronpa.[39] They found that illumination with strong excitation light truly deactivates the single molecule of Dronpa soon after photoactivation, adversely affecting temporal separation and compromising the resolution of the image. Hence, they used rsKame, a variant of Dronpa, which has a V157L amino acid substitution adjacent to the chromophore, to resolve the issue by slowing down the switching process, wherein the photoactivation from the dark (or off) state to the fluorescent (or on) state was attenuated by an increase in the steric

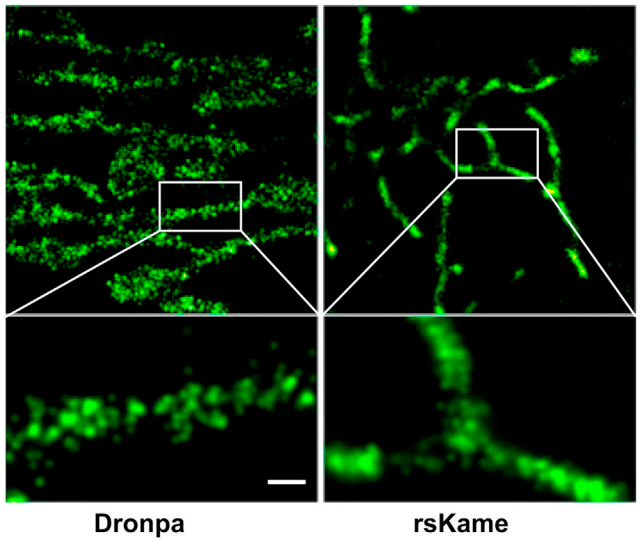


Figure 2. Comparison between the Efficacy of Labeling with Dronpa and rsKame to Procuring Super-resolution and Nanoscopic Images of Inner Mitochondrial Membranes in HeLa Cells
Scale bar, 200 nm. Reproduced with permission from Rosenbloom et al.[39]

hindrance caused by the assimilation of the substitution.[39] Eventually, to perceive the inner and outer membrane structures of mitochondria (Figure 2) through two-color PALM imaging, they combined rsKame with PAmCherry1, which assisted the selective labeling of dynamin-related protein 1 (Drp1). It is worth mentioning here that the combination of PAmCherry1 and rsKame worked as an outstanding pair for two-color PALM because of the large difference in their photoactivation rates, which helped in determining the diameter and the length of Drp1 helical rings.[39]

It should be noted that to achieve successful two-color nanoscopic imaging, choosing an appropriate pair of PAFPs is vital. Shroff et al. used Eos and Dronpa as one such pair, wherein Eos was used initially to collect positional information because there was a transformation in its emission from green to red upon photoactivation.[40] As soon as Eos was photobleached completely, Dronpa was utilized subsequently for collecting positional information. However, it is worth mentioning that PAFPs such as Eos, Dendra2, and KikGR (in which the shift in emission occurs from green to red during photoactivation) need to be completely photobleached before using the second protein for two-color PALM imaging. The use of PA-mCherry could be helpful in this context because upon photo-activation, it does not have any green emissive state that could interfere with the emission profile of Dronpa or PAGFP.[40] In our view, for studying mitochondrial dynamics using photoswitchable FPs, particularly in the case of SMLM, Dronpa and/or its variants could be the preferred options. On the other hand, among PAFPs, variants of Dendra and Eos are decent choices for multicolor SMLM studies because of their several, apposite photophysical properties such as lower duty cycles, pertinent contrast ratio, and high photon detection per localization.

Besides SMLM, reversibly switchable fluorescent proteins (RSFPs) have also been explored for RESOLFT nanoscopy. For instance, to achieve long-term live-cell super-resolution imaging, Sun and co-workers have reported a new class of monomeric RSFPs termed GMars-Q (green form Mars), which are particularly suitable for long-term RESOLFT nanoscopy.[41] Mutation of M168A to mMaple3, a monomeric

variant of mMaple was termed by them as Mars and GMars, essentially denoting green-form Mars. Basically, in this case, long-term imaging can be attributed to the good resistance of the probe to photobleaching and minimal residual fluorescence (in the off state) of the monomeric bright and photostable RSFP. Eventually, they used Mito-GMars-Q (targeting the mitochondria) to image mitochondria and track real-time mitochondrial dynamics by capturing the videos of live fission and fusion processes in mitochondria.[41] Very recently, the research group also developed another GMars-based monomeric bright green RSFP described as GMars-T that can be utilized for live-cell imaging with RESOLFT and photochromic SOFI (pcSOFI).[42] To visualize various protein-protein interactions in different subcellular compartments, they engineered a biosensor based on bimolecular fluorescence complementation (BiFC) of GMars-T. The specific localization of the Bcl-XL-Bak interaction in the mitochondrial membrane was obtained with high spatial resolution when RESOLFT nanoscopy was performed. Therefore, these variants of mMaple with outstanding photophysical qualities can serve as good candidates for mitochondrial RESOLFT nanoscopic studies.

On the other hand, Hess and co-workers described a new method wherein a delicate PAFP was utilized to label the target in SRM and EM. They obtained SRM images and "colorized" detailed EM images of the mitochondrion with the combination of interferometric photoactivatable localization microscopy and scanning EM.[43] Mitochondrial nucleoids were labeled using mtDNA-binding protein TFAM, which was subsequently attached to the FP mEos2, and imaging was performed. Using this approach, they observed 3D organization of the nucleoids inside the mitochondria. The results revealed that within the mitochondria, there were complex, non-uniform organizations of nucleoids having diverse sizes.[43] However, using STED microscopy, Kukat et al. had earlier reported that nucleoids within the mitochondria possess a uniform mean diameter of approximately 70 nm.[44] This anomaly might be attributed to the use of antibody labeling compared to that of protein fusion.

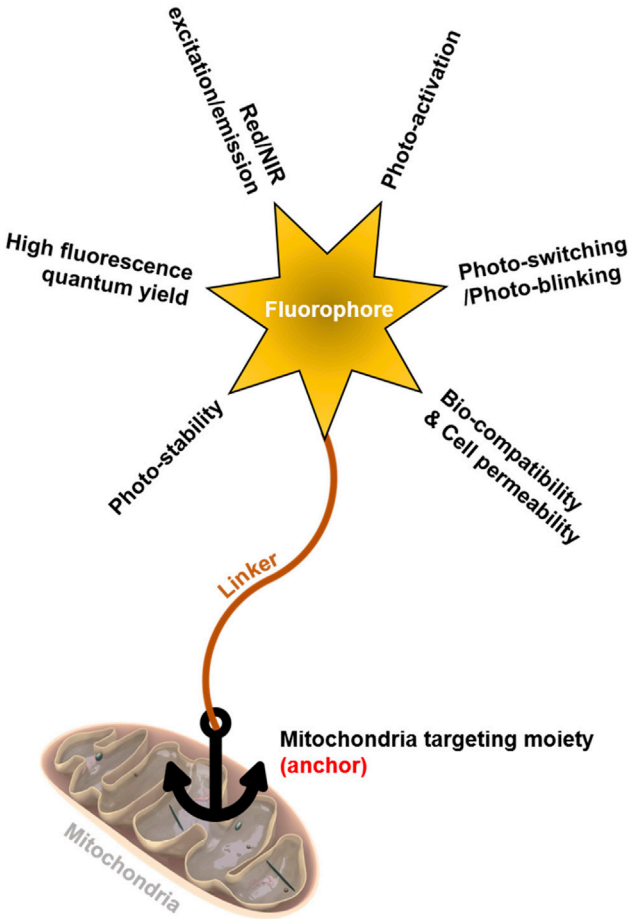
Commercially available mitochondrial marker FP CellLight Mitochondria-RFP (Mito-RFP) is very useful to fluorescently tag mitochondria, especially in SIM-based imaging applications. For example, Sitcheran and co-workers used CellLight Mito-RFP-labeled BT25 cells and performed SIM to reveal that nuclear factor κB (NF-κB)-inducing kinase (NIK) has a proactive role in the regulation of mitochondrial dynamics and motility and essentially promotes cell invasion.[45] It should be highlighted here that the relevance of NIK in cancer besides its well-recognized role in immunity is an emerging topic. The important takeaway from this study was that the mitochondria in BT25 control cells were fragmented and existed as elongated tracks (extended toward the periphery of the cell) all over the cytoplasm. On the contrary, it was observed that mitochondria in the BT25-sgNIK cells showed a phenotype comprising more tangled, fused, and condensed mitochondria around the nucleus, which could be restored by the overexpression of murine NIK (mNIK). Interestingly, lack of NIK (BT25-sgNIK cells) resulted in increased mitochondrial size and a decrease in the number of mitochondria, whereas overexpression of it revealed just the opposite trend. Basically, it was observed that the inhibition of NIK and Drp1 could attenuate mitochondrial dysfunction (which is important for tumor pathogenesis) and therefore could be useful in developing new therapeutic strategies.[45] Hence, this nanoscopic mitochondrial study has undoubtedly provided an outstanding breakthrough in cancer biology.

With the help of SIM, Yu and co-workers also reported a new mechanism for the formation of mitochondrial networks based on the dynamic tubulation of mitochondria,

which is mediated by KIF5B, instead of the usual, individual mitochondrial fusion pathway.[46] They were able to observe thin tubules extending from mitochondria, which were difficult to observe using light FM or EM. They used the mitochondrial marker TOM20-GFP for imaging NPK cells, which were effectively visualized through N-SIM microscopy. Using time-lapse imaging and stable mitochondrial markers such as TOM20-GFP and Mito-YFP, they found that the tubules were highly dynamic in nature and possessed a very different character from the regular tubular mitochondria. These highly dynamic tubules are pulled out of mitochondria by KIF5B, and the mitofusin-mediated fusion of these dynamic tubules leads to the formation of the mitochondrial network. Moreover, they also demonstrated that mitochondrial network formation might be solely governed by dynamic tubulation and fusion and that KIF5B controls network formation only in the cell periphery.[46] In our view, commercially available FP-based mitochondrial tracker dyes such as Mito-RFP and TOM20-GFP are best suited to perform SIM nanoscopy of mitochondria. However, the choice of FP should be made according to the specific requirements of the experiment and desired wavelengths of the acquired signals.

It is evident from the above discussion that FPs can be conveniently engineered to perform high-quality nanoscopic imaging of mitochondria. However, some critical issues related to the utilization of FPs should be considered while assessing their potential application in SRM. More specifically, one should take note of the fact that compared to small-molecule organic fluorophores, FPs are extremely less emissive. The fluorogenic behavior of some FPs is highly environmentally sensitive and can be influenced greatly by the dynamic experimental condition of SRM.[33,34] Moreover, it is very important to monitor the expression of FPs in a controlled manner so that the overexpression of these proteins does not lead to any additional issues in terms of protein aggregation or unregulated localization.[47] Sometimes, the biological activity of the target (tag) can also be adversely affected by the steric influence of the large size of FPs.

It is also worth mentioning here that covalent labeling techniques of proteins in living cells involving SNAP-tag, Halo-Tag, and CLIP-tag fusion proteins[48] are also very useful in various SRM experiments. These relatively newer labeling technologies basically combine the features of organic dyes with those of genetically encoded proteins to obtain better imaging outcomes. As these techniques offer the scope of functionalizing various proteins with organic chemical fluorescent probes, this approach has certain advantages over common FPs. For instance, in two-color live-cell STED imaging, STED compatible organic fluorophores (e.g., ATTO dyes) having outstanding photostability and brightness can be introduced into the system using this technique. In the past, Bewersdorf and co-workers used EGF-CLIP$_f$ with ATTO647N to reduce the non-specific binding of the probe when staining mitochondria.[49] Very recently, Bewersdorf and co-workers also used the membrane-permeable organic dyes SiR and ATTO590, conjugated with Halo and SNAP tags, respectively, to label the endoplasmic reticulum (ER) and mitochondria.[50] This combination of Halo- and SNAP-tag-based fluorescent probes provided a breakthrough for observing the dynamic live events of the contact between ER tubules and mitochondria in unprecedented detail.[50] To understand the dynamic structural and functional changes of the cytosolic phosphatase and tensin homolog PINK1 during the proteolytic process inside mitochondria, Busch and co-workers performed live-cell triple-color SRM by using a combination of FPALM and tracking and localization microscopy (TALM) wherein the localization of PINK1 in mitochondria was studied by fusing the Halo-Tag to the C terminus of PINK1 (PINK1-Halo-tag); Tom20 and respiratory complex I (CI) were used to monitor the localizations of the reference proteins



Scheme 3. Design of a Common Chemical Fluorescent Probe for Nanoscopic Mitochondrial Imaging

in the outer mitochondrial membrane and the inner mitochondrial membrane, respectively.[51] Essentially, inner mitochondrial membrane tag CI was fused to a photoactivatable GFP (PAGFP), whereas to perform dual-color TALM, Tom20 was attached with a C-terminal SNAPf-tag (TOM20-SNAPf).[51] Hence, these techniques can effectively pave the way for resolving employability issues of various FPs and small-molecule fluorophores in SRM studies by combining protein and organic probes. However, in our view, the major drawback of this combination could be the high background from the unbound fluorescent probe that could eventually lower the contrast of the image.

Chemical-Compound-Based Probes

FP-based probes are useful for studying a specific protein distribution in a single mitochondrion. However, their photophysical properties are not suitable for long-term live imaging. Hence, various chemical compounds have recently been judiciously designed to achieve mitochondria-targeted SRM imaging. A schematic representation of designing an ideal chemical fluorescent probe for the nanoscopic imaging of mitochondria is shown in Scheme 3. Commercially available Mito-Tracker dyes (which are extensively used for mitochondrial staining) can be specified in this category as Mito-Tracker probes and basically comprise a rosamine or cyanine scaffold having a chloromethyl moiety that acts as a thiol-reactive site and hence serves

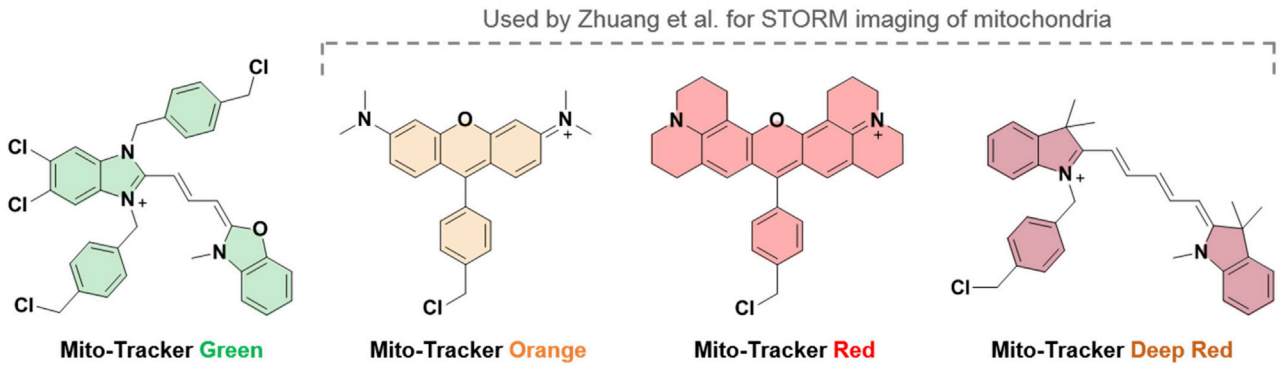


Figure 3. Structures of the Various Commercially Available Mito-Tracker Probes

as an anchor for mitochondrial proteins. However, recently, several organic small-molecule-based mitochondria-targeting fluorescent probes (including luminogens with aggregation-induced emission [AIEgens]) as well as some nanoparticle (NP) and polymer- and copolymer-based probes have been reported and can be efficiently employed in the nanoscopic imaging of mitochondria.

Organic Small-Molecule Probes

The characteristic negative membrane potential of mitochondria has served as a key to design various small-molecule-based mitochondria-targeting probes. Hence, most of the probes developed to label mitochondria are cationic in nature so that they can easily accumulate in the mitochondrial environment having negative membrane potential. The cationic lipophilic mitochondria-targeting probes might also be used often to monitor the changes in mitochondrial membrane potential.[52] However, it should be considered that the cationic probes, which mainly accumulate in mitochondria across the membrane in a Nernstian fashion to maintain the equilibrium of the membrane potential, could translocate back into the cytosol as well as to the extracellular medium when the membrane potential is altered. On the other hand, mitochondrial membrane-potential-responsive mitochondria targeting fluorescent probes, which usually bind strongly with mitochondrial proteins and form stable covalent bonds, could offer more reliable staining and labeling. Figure 3 represents the chemical structures of the commonly used, commercially available membrane-potential-based mitochondria-targeting probes known as Mito-tracker dyes. Compared with unbound probes, these probes form a strong covalent bond with mitochondrial proteins. The mitochondrial trackers that bind to the mitochondrial proteins also have certain drawbacks. Some of these probes show respiration-inhibitory activity with prolonged incubation and at higher concentrations.[52] One such example is MitoTracker Orange (chloromethyltetramethyl rosamine), which causes a change in mitochondrial permeability and complex I inhibition even at very low (micromolar) concentrations.[52] Therefore, if a minimum physiological change in studying the characteristics of mitochondria is requisite, one should choose a fluorescent probe wherein the specificity to bind to mitochondria is solely governed by the membrane potential rather than covalent binding of the probe to any protein. On the contrary, when studying some dynamic physiological process involving significant alteration of mitochondrial potential wherein the system demands reliable and stable staining of the mitochondria, then the commercially available Mito-Tracker dyes are suitable choices because they anchor to mitochondrial proteins and eventually lead to steady mitochondrial localization (Figure 3).

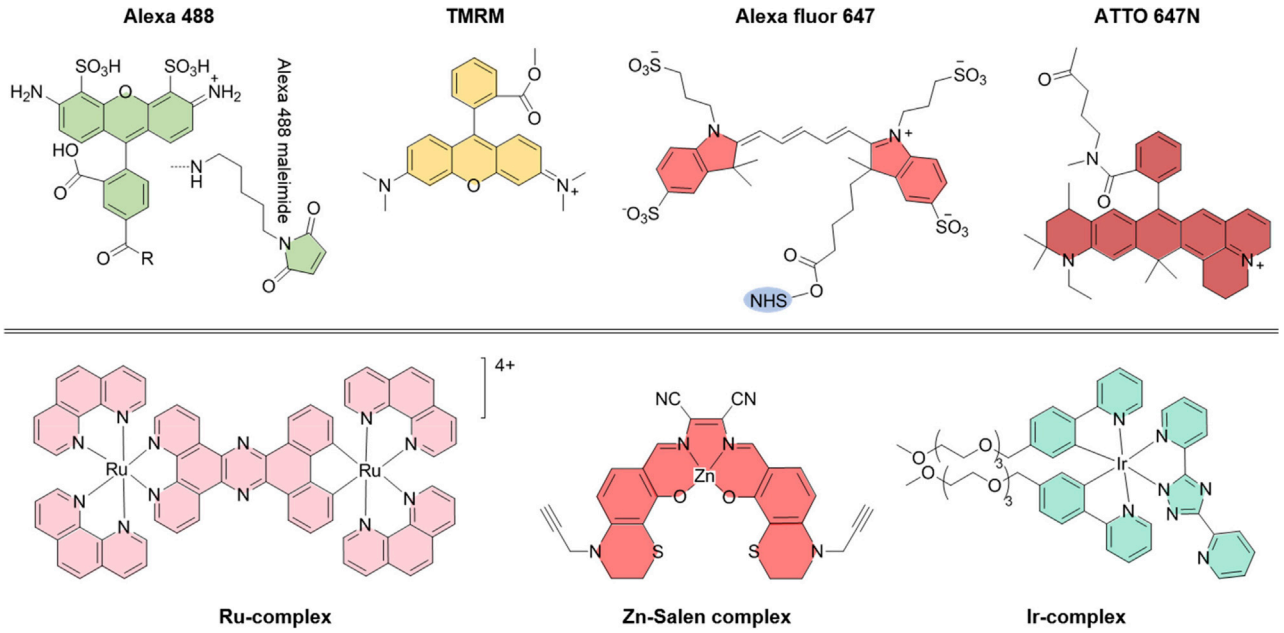


Figure 4. Chemical Structures of Different Mitochondria Targeting Fluorophores for Nanoscopy Discussed in This Review

In this context, Zhuang and co-workers assessed the photoswitching capabilities of these MitoTracker dyes and noted that MitoTracker Orange and MitoTracker Red (which belong to the category of cationic rosamine dyes), as well as MitoTracker Deep Red (which is a cationic carbocyanine dye), are capable of showing good photoswitching behavior.[53] It should be noted that photoswitching or photoblinking constitutes one of the essential criteria for a fluorescent probe to be utilized in the SMLM-based super-resolution imaging technique STORM. Therefore, the authors used all three photoswitchable MitoTracker dyes for STORM imaging of mitochondria. Moreover, since the emission profiles of MitoTracker Red and ER-Tracker Red revealed the distinct emission maxima separated by 16 nm, they utilized these probes to achieve two-color super-resolution STORM images of mitochondria and the ER. Ultimately, by labeling the mitochondria with the mentioned Mito-Tracker dyes, they were able to capture the details of the dynamic morphological changes during mitochondrial fusion and fission by using STORM imaging.[53] It is important to highlight that the photoswitching behavior of the cyanine-based Alexa-Fluor-647 dye (Figure 4) is well recognized and is suitable for STORM imaging. Especially, Alexa-Fluor-647-coupled antibodies are found to be good candidates for dSTORM-based super-resolution imaging. Zhuang and co-workers have presented a common protocol to prepare such antibodies conjugated with photoswitchable fluorophores for performing effective STORM imaging.[54] In this context, Ježek and co-workers have observed the alterations in mitochondrial cristae and studied the size and distribution of mtDNA nucleoid using Alexa-Fluor-647 (Figure 4)-based labeling and STORM technique.[21] Klotzsch et al. recently utilized the dSTORM super-resolution technique to comprehend various mitochondrial proteins in mouse primary neurons. They basically used antibodies conjugated to Alexa 488 and Alexa 647 dyes (Figure 4) for staining different proteins.[55] Geissbuehler et al. have also reported certain technical advancements in achieving 3D SOFI image of the mitochondrial network in fixed C2C12 cells. They also have chosen the frequently used blinking fluorophore Alexa 647, with deep red emission to image mitochondria in fixed, immunostained cells.[56] Hence, from the practical usage point of view, Alexa 647 dye, which is the

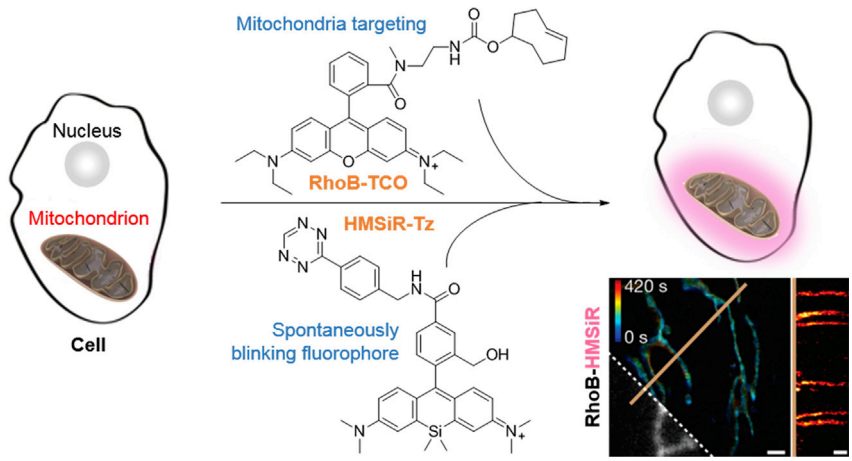


Figure 5. Strategy for Achieving SRM Imaging of Mitochondria by Using High-Density Environmentally Sensitive Membrane Probes

Scale bar, 1 μm. Reproduced in parts with certain modifications from Thompson et al.[57] Copyright 2017 American Chemical Society.

close structural analog of Cy 5 dye, still remains the best choice for SMLM (especially in STORM)-based nanoscopy of mitochondria. The optimum balance of the key features (such as brightness, switching cycle, contrast ratio, survival fraction, and others) makes Alexa 647 an obvious choice for SMLM-based nanoscopic studies of mitochondria.

However, the development of new fluorophores for single-molecule switching (SMS)-based super-resolution techniques, such as PALM, FPALM, STORM, dSTORM, and ground-state depletion microscopy followed by individual molecule return (GSDIM), has gained tremendous interest. Recently, a high-density environmentally sensitive (HIDE) membrane probe based on cationic Rhodamine B derivative, termed RhoB-TCO, was synthesized by Schepartz and co-workers (Figure 5). The probe enables live-cell SMS-based super-resolution imaging of mitochondria by targeting HMSiR-Tz to the mitochondrial membrane. This new strategy is illustrated in Figure 5.[57] Cosa and co-workers have also designed a fluorogenic boron-dipyrromethene (BODIPY)-acrolein probe, AcroB, that can undergo a dramatic enhancement in its fluorescence intensity upon formation of protein adduct through mitochondrial alkylation of the probe in cell milieu (Figure 6). With the help of super-resolution imaging and the newly designed fluorogenic probe (AcroB), they were able to study the chemical reactions within the cell lipid milieu, particularly in mitochondria.[58] However, these new probes require further studies to establish their applicability in a broad range of SRM imaging studies.

Some articles[52,59] have recently summarized the vast array of fluorescent probes that can target mitochondria, but only a few of these probes are suitable for the purpose of super-resolution imaging because the chosen mitochondria-targeting fluorescent probes for a particular SRM technique should satisfy some additional, essential criteria apart from being specific to mitochondrial localization. Therefore, nanoscopic mitochondria targeting small-molecule-based organic chemical probes must be composed of a mitochondrial-targeting moiety as well as a fluorogenic system that fulfills the required conditions to be employed in the particular SRM method, as already illustrated in Scheme 3. Other important features of a common fluorescent probe such as cell permeability, aqueous solubility, near-infrared (NIR),

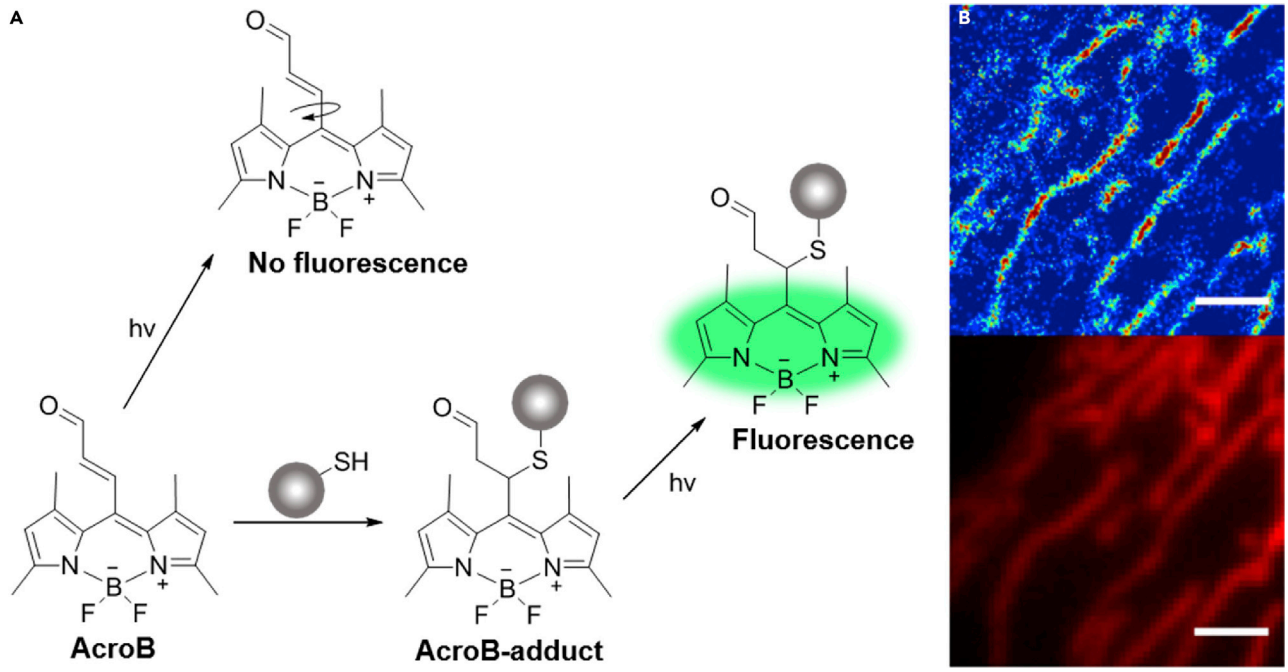


Figure 6. Mitochondria Alkylation and Cellular Trafficking Tracked by Super-resolution Imaging Using a Fluorogenic BODIPY-Acrolein Probe
(A) The switch-on mechanism of the BODIPY-Acrolein fluorogenic probe AcroB upon formation of adduct with protein.
(B) Intense AcroB-adduct (top) formation in mitochondria, confirmed by the co-localization with MitoTracker Deep Red (bottom). Scale bars, 2 μm.
Reproduced with permission from Cosa and co-workers.[58] Copyright 2017 American Chemical Society.

or red emission, and many others can be subsequently incorporated in the probe design according to the experimental requirements through molecular engineering.

In this regard, modification of fluorescent probes with lipophilic triphenylphosphonium moiety (TPP) to facilitate mitochondria targeting ability is a well-known, commonly used strategy (Figure 7A). Kalyanaraman and co-workers have commendably outlined the design, synthesis, and utilities of several triphenylphosphonium-based mitochondria-targeting probes. The use of TPP to assimilate mitochondria targeting ability in the probe design is advantageous over other approaches because the balance of lipophilic and hydrophilic character of it not only offers a stable environment for mitochondrial localization but also does not impede other intracellular processes because of its less reactive nature (Figure 7B). Moreover, TPP does not interfere in the absorption or fluorescence in the visible or NIR region and generally exerts minimal toxicity (for example, the case of Mito-Q) (Figure 7B). However, judiciously attaching a mitochondrial-targeting TPP moiety to a fluorogenic system with appropriate SRM criteria is not very easy. In this context, our group has recently reported a BODIPY-based fluorescent probe having mitochondria targeting TPP moiety for nanoscopic STED imaging of vicinal-dithiol containing proteins (VDPs) on mitochondrial membrane.[60] In the probe design, the triphenylphosphonium group as a typical mitochondrial targeting unit has been attached to the BODIPY fluorophore via an alkyl spacer, which is important for the membrane localization of the probe by adjusting the lipophilic character (Figure 7C). Moreover, the fluorogenic system is conjugated to phenylarsenicate to ensure its VDP binding and labeling potential. Ultimately the highly photostable probe (tolerant to the high STED laser illumination) was subjected to STED super-resolution imaging, which revealed the nanoscopic images of the fluorescently labeled VDPs (Figure 7C) on the

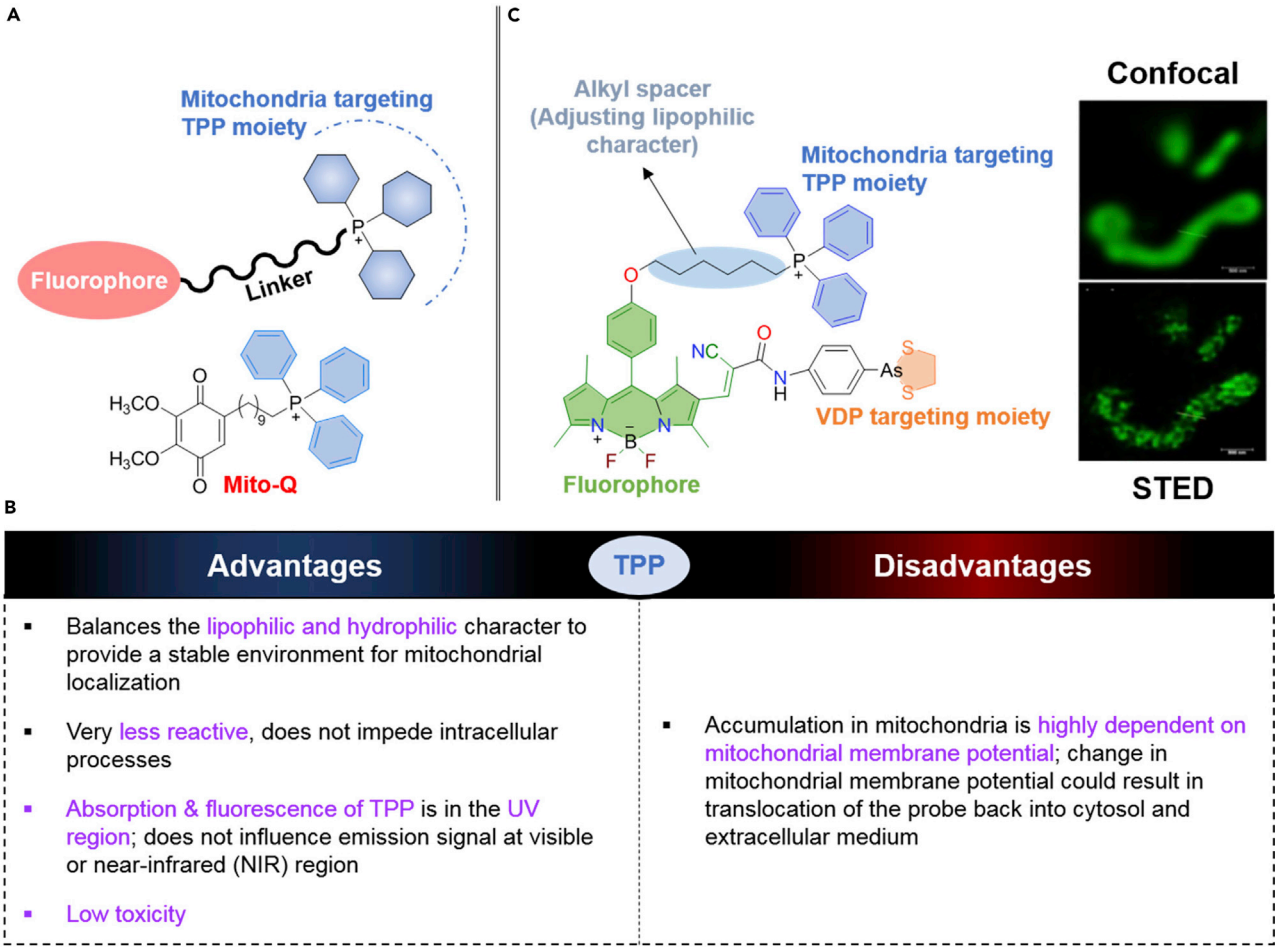


Figure 7. Triphenylphosphonium Moiety as a Mitochondria-Targeting Unit

(A) Schematic design of a common triphenylphosphonium (TPP)-based mitochondria-targeting probe and the structure of Mito-Q.

(B) Advantages and disadvantages of incorporating TPP moiety in the probe design to target mitochondria.

(C) BODIPY-TPP-based fluorogenic probe reported by our group to achieve successful nanoscopic STED imaging of mitochondrial VDPs.[60] Scale bar, 500 nm.

Reproduced in parts with permission from Yang et al.[60] Copyright 2018 American Chemical Society.

mitochondrial membrane.[60] Hell and co-workers have also recently designed a new type of cell-permeable fluorescent dye (Figure 8A) with emission in the red region by using 9-iminoanthrone, 9-imino-10-silaxanthone, and 9- imino-10-germaxanthone as the basic fluorophore cores.[61] They engineered the probes to selectively target mitochondria, lysosomes, and F-actin. Particularly, to introduce mitochondria targeting ability, they attached TPP moiety to the fluorophore cores (Figure 8A). The probes were found to be less cytotoxic in nature and revealed the organelle-specific sub-structures when used in single-color live-cell STED nanoscopy as well as multi-color live-cell STED nanoscopy.[61] Ohsawa and co-workers also studied the details of sub-mitochondrial structures with a sub-diffraction resolution by performing STED imaging.[62] They used tetramethylrhodamine methyl ester (TMRM), a common cationic orange-red fluorescent dye to stain the mitochondria (Figure 4). The high-resolution STED imaging revealed that the cationic dye TMRM, which could be easily sequestered by mitochondria, neither co-localized with the mitochondrial proteins in the matrix nor with the outer membrane. Rather, it was found to be preferentially localized to the nucleoid.[62] Kehrein et al. also used antibodies labeled with Alexa

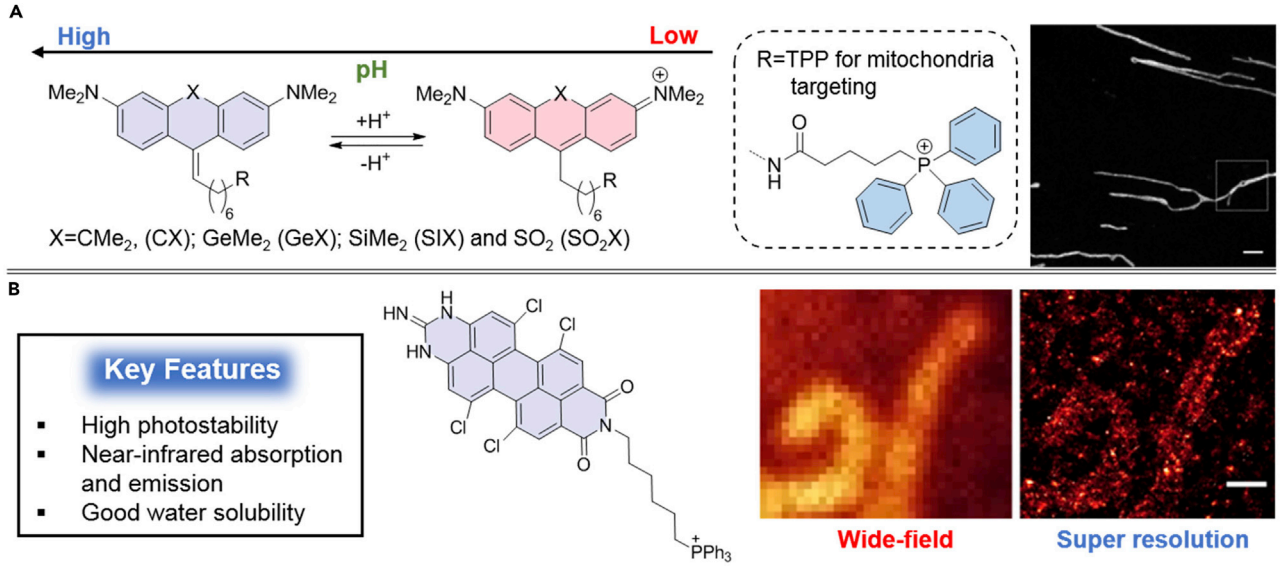


Figure 8. Integration of TPP Moiety in the Probe Design to Achieve Mitochondria-Targeting Super-resolution Imaging

(A) Protonation and deprotonation behavior of the anthrone- or xanthone-core-based fluorophores and the nanoscopic STED imaging of mitochondria with the SiX-TPP probe. Scale bar, 1 μm. Reproduced in parts with permission from Butkevich et al.[61] Copyright 2017 American Chemical Society.

(B) Structure and key features of the rylene chromophore utilized for GSDIM nanoscopic imaging of mitochondria compared with the wide-field imaging. Scale bar, 500 nm. Reproduced in parts with permission from Kaloyanova et al.[64] Copyright 2016 American Chemical Society.

Fluor 594 (directed against GFP-tagged proteins) to observe the precise sub-mitochondrial localization of the mitochondrial organization of gene expression (MIOREX) complexes through super-resolution imaging.[63] The breakthrough of this study was that the MIOREX complexes were found to be evenly dispersed across the mitochondrial network wherein the mitochondrial ribosomes (represented by Mrpl4-GFP) were mainly localized in evenly distributed clusters throughout the mitochondrial tubules. This SRM experiment also revealed that a subset of adjacent ribosome clusters co-localized with mtDNA, which indicated the presence of nucleoid-MIOREX complexes that store the whole spectrum of organellar gene expression.[63] Hence, in our view, to achieve effective STED imaging of mitochondria, the designed and synthesized fluorophore could be chosen from diverse structural families provided that it contains a mitochondria-targeting moiety (potential dependent or covalent binding triggered) and maintains good photostability, high brightness, improved SNR, and good resistance to photobleaching. Moreover, it is recommended to go through the excitation and emission wavelengths of such dyes before selecting them for a particular STED experiment as the availability of a STED laser is crucial for the successful acquisition of images.

Less phototoxicity of NIR-emitting fluorescent dyes in the biological milieu makes them ideal choices for live-cell imaging purposes. Therefore, developing biocompatible, organic small-molecule NIR fluorescent probes for organelle-specific super-resolution imaging is desirable, but it is challenging at the same time. In this context, Peneva and co-workers have reported a couple of highly photostable peri-guanidine-fused perylenemonoimide-based probes (Figure 8B) that can exhibit NIR absorption and emission.[64] Particularly, the NIR probe is highly water soluble, and it can selectively localize in the mitochondrial compartment with a low background signal in both live cells and fixed cells. The NIR probe can be additionally modified in a one-step reaction with functional groups for covalent labeling of proteins. The low cytotoxicity allows long-time exposure of the live cells to the dyes

without the necessity of washing. The probe was successfully employed to reveal the nanoscopic images of mitochondria by (GSDIM) SRM in the absence of any reducing or oxidizing agents.[64]

To utilize a dye in SIM imaging, it requires having some essential photophysical qualities such as high fluorescence intensity and brilliant photostability. In this regard, Atto 647N (Figure 4) is an excellent fluorescent dye with optimal photophysical and chemical requirements to be utilized in SIM. Hence, Zhang and co-workers employed the dye Atto 647N NHS ester to perform super-resolution imaging of mitochondria wherein the co-localization experiment with Mito-Tracker Green confirmed the mitochondrial staining ability of the Atto dye by demonstrating a reasonable Pearson's coefficient (0.885).[65] They also performed some experiments to highlight that, apart from its tendency to localize in the mitochondrial compartment having negative membrane potential, the cationic carbopyronine dye containing an NHS moiety might possibly bind to the mitochondrial membrane proteins. Therefore, its localization in the mitochondrial matrix is not weak, as observed in the case of many other Mito-probes that are dependent solely on mitochondrial potential. As a result, even after cell fixation, the Atto 647N NHS ester probe was able to stain mitochondria.[65] This study also provided a breakthrough in identifying the lysosome-mitochondrion interaction with nanoscale resolution.

SIM has the flexibility of using several other dyes too. Shibata et al. have isolated mitochondria by permeabilizing the cell membrane using a pore-forming protein and visualized the conformational changes due to the isolation process using SIM. They used MitoTracker Red to label inner mitochondrial membranes of the isolated mitochondria but visualized the outer membrane by labeling it with Alexa Fluor 488 dye conjugated to a secondary antibody.[66] They observed that even though the isolated mitochondria retained outer and inner membrane structure and were capable of producing ATP, the elongated portion of mitochondria had diminished with concurrent increase in the spherical content in mitochondria.

Monitoring mitochondrial glutathione (GSH) is a difficult task. Wang and co-workers have synthesized a mitochondria-specific GSH probe designated as Mito-RealThiol (MitoRT), which can be utilized to track live-cell GSH dynamics of mitochondria in real-time, based on a Michael addition reaction (Figure 9A). The probe was subsequently used successfully to achieve mitochondria-specific super-resolution imaging via SIM.[67] Similarly, Yang and co-workers recently developed a new nanoscopic nitric oxide (NO) donor (NOD550) fluorescent probe, which can be utilized to monitor the dynamics of mitochondrial morphology during the photo-triggered release of NO at the nanoscale level (Figure 9B).[68]

Besides the organic, small-molecule probes, several metal complexes have also been developed for SRM that can perform nanoscopic imaging of mitochondria. For example, Thomas and co-workers have recently developed a dinuclear Ru (II) complex (Figure 4), which exhibited excellent cell-membrane permeability at lower concentrations. The metal complex-based probe was found to be localized in the mitochondria.[69] The highly photostable probe was found to be suitable for SIM and STED imaging. Hence, the probe was utilized to perform super-resolved organelle-specific imaging of mitochondria in fixed and live cells. It should be mentioned here that at higher concentrations, the probe is preferentially localized in nuclear DNA.

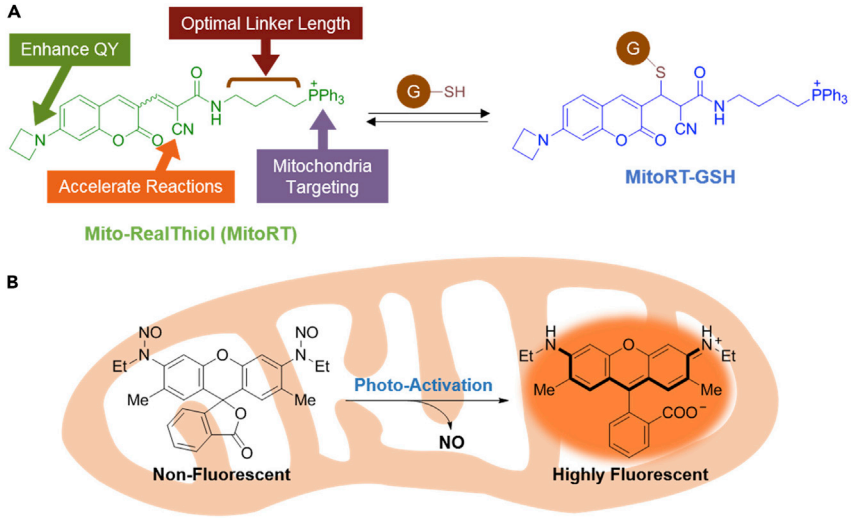


Figure 9. Probes to Monitor Real-Time Mitochondrial Dynamics with Super-resolution Microscopy

(A) Sensing mechanism of the GSH sensitive probe Mito-RealThiol (MitoRT). Reproduced with permission from Wang and co-workers.[67] Copyright 2017 American Chemical Society.

(B) Photo-triggered activation of the NO donor probe NOD550. Reproduced with permission from Yang and co-workers.[68] Copyright 2018 American Chemical Society.

Zhang and co-workers have also reported a new photoactivatable Zn-salen complex (J-S-Alk) (Figure 4) that can be utilized to achieve specific mitochondria-targeted super-resolution imaging in live cells.[70] The probe has the advantage that it can act well without being treated with a photocleavable quencher or additives such as redox chemicals or oxygen scavengers. The potential of using luminescent metal complexes for live-cell super-resolution imaging was also demonstrated by Edward and co-workers. Basically, Edward's and Ward's group utilized a brightly emissive photostable Ir-complex (Figure 4) to obtain super-resolution images of mitochondria using 3D-SIM by specifically staining the mitochondrial compartment of HeLa cells.[71] Even though there is some significant progress in the nanoscopic imaging of mitochondria using metal complexes as highlighted above, controlling the photo-physical performances of these complexes still require further studies to implement them in more substantial imaging applications.

There is no doubt about the fact that small-molecule-based organic fluorophores, capable of displaying photoactivation and/or photoswitching abilities have been overwhelmingly utilized in several nanoscopy imaging applications because of their attractive photophysical and photochemical properties such as bright and stable emission, specific targeting ability, and many more.[19,34,72] However, before employing these dyes in SRM experiments, one should carefully consider the fact that most of the commonly used fluorophores (such as Alexa Fluor 647) exhibit poor cell permeability, which might restrict them from practical usage in many live-cell imaging applications. Moreover, the use of special imaging buffers and/or external additives (in STORM imaging) comprising thiols (β-mercaptoethylamine, 2-mercaptoethanol, and GSH), ascorbic acid, and oxygen-scavenging agents to introduce effective blinking property, specifically in cyanine dyes and its closely related analogs (e.g., Cy 5, Alexa Fluor 647), might adversely affect the biocompatibility and eventually lead to cytotoxicity-related issues in long-term live-cell imaging. Even though some smart fluorescent probes such as azido-dicyanomethylenedihydrofuran fluorophores and hydroxymethyl silicon-rhodamine derivatives have the

flexibility to avoid the influence of external additives, they often require to be coupled with protein tags to be utilized in an SRM experiment. It should also be highlighted that even though the synthetic chemical fluorescent probe has certain advantages over the FPs in terms of brightness and photostability, in some cases, it lacks the superiority over FPs because of poor specificity and targeting and high background signal compared with that of FPs. Hence, these aspects should be judiciously deliberated upon before developing newer, elegant fluorescent systems for mitochondria-specific SRM.

Nanoparticle and Polymer- and Copolymer-Based Probes

In addition to organic compounds, some NP and polymer- and copolymer-based fluorescent probes have also been explored for mitochondria-specific SRM in recent years.[73] Developing highly photostable fluorogenic systems (with better photostablility than organic fluorophores) with a minimal rate of photobleaching is highly desired, and this can then be coupled with other important photophysical features such as good photoblinking properties, high brightness, and others to acquire long-term, high-quality SRM images. In this context, NP-based probes have shown great potential to be utilized in various nanoscopic bio-imaging applications as they possess several outstanding properties such as good fluorescence quantum yield, broad-band optical absorption, brilliant biocompatibility, and cell permeability.[73] It should be highlighted that compared to the inorganic semiconductor quantum dots (Qdots), composed of toxic heavy metals, carbon dots are highly preferable candidates for bio-imaging because of their eco-friendly properties. In this context, Haynes and co-workers recently developed a new malic acid carbon dot probe, capable of exhibiting outstanding photoblinking property, to capture high resolution images of intracellular compartments in fixed cells.[74] The malic acid carbon dot probe demonstrated adequate photoblinking property even in the absence of any special buffer and hence highlights its tremendous potential to be utilized in long-term live-cell nanoscopic imaging. Basically, depending upon the excitations, the carbon dot probe revealed two different types of localizations. Upon illumination of green-to-yellow light excitations (488, 514, and 561 nm, respectively), the carbon dots revealed high-resolution filament-like distribution inside live cells by localizing to the mitochondrial compartment, as confirmed by a co-localization experiment.[74] Besides, it provided the opportunity to capture the directional movement of the carbon-dot-labeled mitochondria in live cells with a sub-diffraction resolution, which showed the dynamic transport of mitochondria-associated carbon dots.

Besides carbon dots, polymer dots (Pdots) have higher brightness and better biocompatibility and photostability than Qdots, organic dyes, and FPs, making them decent choices for bio-imaging applications. Sun and co-workers have reported a couple of small photoblinking Pdots, termed blue PFO and carmine PFTBT5 (Figure 10A), with sharp distinct colors with narrow emission spectral range, which could be used in multicolor statistical super-resolution imaging.[75] Especially, the ability of Pdots to demonstrate good multicolor blinking apart from ensuring specific subcellular (mitochondria) targeting capability facilitated multicolor SOFI imaging. However, there is still scope for advancement in developing superior Pdots, which can address some critical issues such as (1) decreasing the size of Pdots virtually to the size of FPs (5 nm) and (2) increasing blinking rate to capture fast, live-cell dynamics. Sun and co-workers also developed two small semiconducting Pdots with good photoblinking properties that could be utilized for super-resolution imaging (Figure 10B).[76] The new Pdots were mainly prepared through a modified nanoprecipitation method wherein the small-sized Pdots exhibited high photostability,

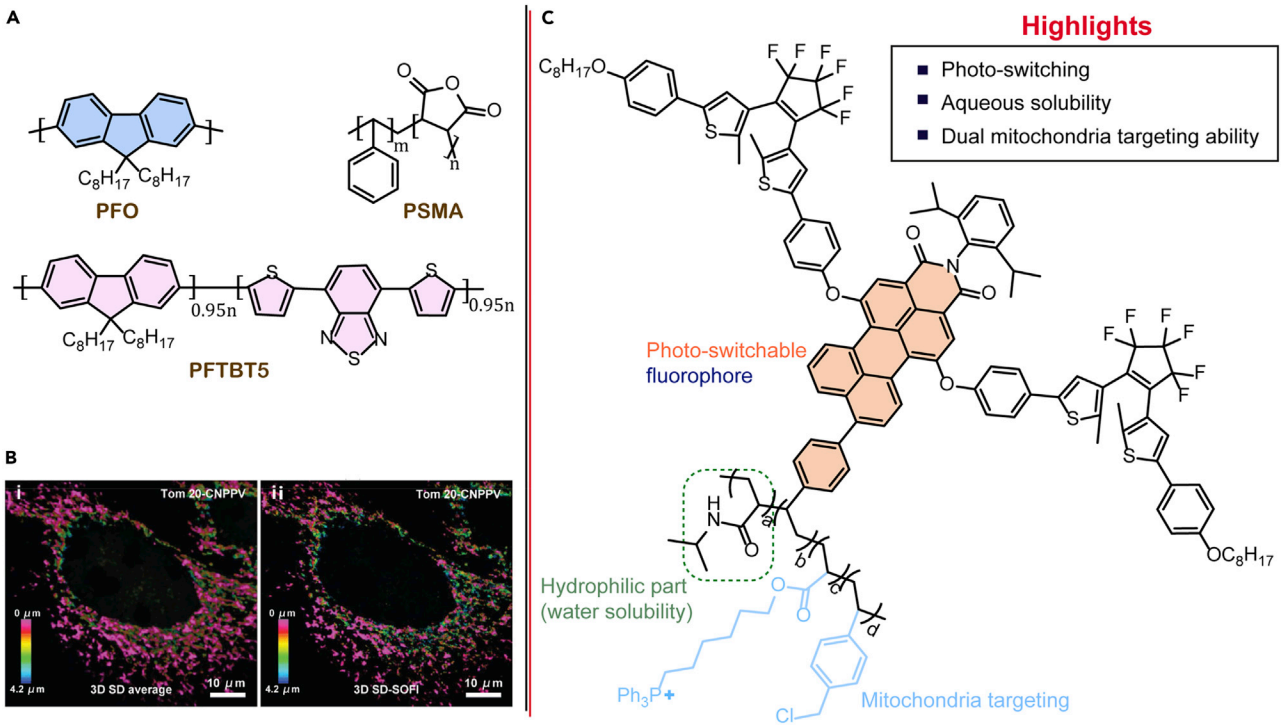


Figure 10. Polymer dots (Pdots) and Polymer-Based Fluorogenic Probes for Super-resolution Imaging of Mitochondria

(A) Structures of the semiconductor polymers utilized for the synthesis of Pdots by Sun and co-workers.[75]

(B) Spinning disk (i) confocal and (ii) SOFI imaging of the mitochondria using Pdots. Scale bar, 10 μm. Reprinted with permission from Sun and co-workers.[76] Copyright 2016 Wiley-VCH Verlag GmbH & Co. KGaA.

(C) Design of the polymer-based fluorogenic probe used to procure nanoscopic mitochondrial images by Zhu and co-workers.[77]

excellent brightness, and good biocompatibility apart from demonstrating adequate photoblinking properties. These Pdots were used to study long-term mitochondrial dynamics at a nanoscale level via SOFI imaging (Figure 10B).

Likewise, NP-based probes and the polymer- and copolymer-based fluorogenic probes with suitable functionalities can also be explored to achieve effective nanoscopic imaging of mitochondria. For example, Zhu and co-workers have developed a new perylenemonoimide-dithienylethene (PMI-DTE) conjugate-based photoswitchable poly-fluorophore (Figure 10C) that can be utilized for super-resolution imaging purpose.[77] The polymer-based probe was modified with the incorporation of mitochondria-targeting functionalities, such as triphenylphosphonium and benzyl chloride, to ensure its mitochondria-targeting ability (Figure 10C). It should be mentioned that the lipophilic TPP cation basically facilitates the mitochondrial-membrane-potential-dependent localization of the probe, whereas the inclusion of benzyl chloride moiety can act as an anchor to the mitochondrial protein and form a stable covalent bond as soon as the probe approaches the mitochondria. On the other hand, the PMI unit of the poly-fluorophore acts as a strong emitting fluorophore, whereas two attached DTE units basically serve as the photoswitching quencher. Moreover, issues related to the aqueous solubility of the polymer-based probe were addressed by the integration of *N*-isopropylacrylamide (NIPAM) in the probe design, which conveniently resulted in good hydrophilicity.[77] The good photoswitching behavior that is essential for achieving successful super-resolution imaging could be attributed to the sequestration or isolation of the two consecutive

fluorophore units by NIPAM across the polymer chains. Essentially, the newly designed poly-fluorophore was successfully employed to obtain mitochondrial-targeting super-resolution images with about 30 nm spatial resolution.

Very recently, Marks and co-workers have also used a hydrophilic pluronic block copolymer F68, coupled with nanoscopic STORM imaging methods, to reassess the mechanism by which F68 could effectively decrease the dysfunction of mitochondria and inhibit the mitochondria-dependent death pathways in a model of neuronal injury.[78] Essentially, F-68 has the ability to target the mitochondrial dysfunction and thereby rescue neurons from acute injury.

Hence, there is no doubt about the fact that NP-based probes or polymer- and copolymer-based probes have the potential to play a significant role in mitochondria-specific SRM. However, the development of these types of fluorescent probes is not easy and the endeavor to develop ideal NP-based probes is still being researched. For example, targeting the ability of NPs toward specific biomolecules within the cell can be modulated by immunostaining using antibodies. However, in general, the approach works satisfactorily in fixed-cell studies only. Therefore, it is highly important to optimize the NP size and surface functionalization to tailor the probe aptly for utilizing it in living systems.[73] Moreover, the photostability of Qdots is exceedingly higher than the several known fluorophores (synthetic fluorophores as well as FPs), which is problematic for STORM imaging because of the difficulties in attaining photoswitchable behavior. Introducing specific organelle-targeting capability also remains a challenging task for Qdots.

Aggregation-Induced-Emission-Based Probes

Recently, AIEgens have represented an important part of photo-biological research because of their tolerant and interesting photophysical properties and high fluorescence quantum yields.[79] Unlike simple fluorogenic systems, aggregation-induced-emission (AIE)-based probes become highly fluorescent when aggregated, which might be attributed to the unique structural features of these probes that facilitate the restriction in intramolecular rotation upon aggregation and ceases the non-radiative decay.[80] Therefore, the role of AIE-based fluorescent probes in acquiring bright, fluorogenic signals in high concentration, complex biological systems with the propensity for aggregate formation is highly commendable. Recently, there has been some significant progress in exploring the scope of utilizing AIE-based fluorescent probes for mitochondria-targeted SRM imaging. For instance, Tang and co-workers have recently reported a novel photoactivatable bio-probe, *o*-TPE-ON+ (Figure 11), comprising a typical AIEgen tetraphenylethylene (TPE) unit that could exhibit photoactivation through a unique photo-cyclo-dehydrogenation mechanism.[81]

Interestingly, the AIE-based probe *o*-TPE-ON+ could demonstrate spontaneous blinking property in the absence of any imaging buffer or additives. This might be attributed to the oxygen-promoted, novel photo-activation behavior of *o*-TPE-ON+ through photo-cyclo-dehydrogenation (Figure 11). Interestingly, the newly formed cyclized product is visible light (>500 nm) excitable, and its photophysical properties do not get significantly influenced by changes in the external environment, including pH.[81] Besides, the probe has good cell permeability and outstanding biocompatibility, which makes it an excellent choice for SLM. The probe demonstrated mitochondria-specific localization both in fixed cells as well as in live cells, which might be attributed to the negative-membrane-potential-based accumulation of the positively charged probe on the mitochondrial outer membrane.

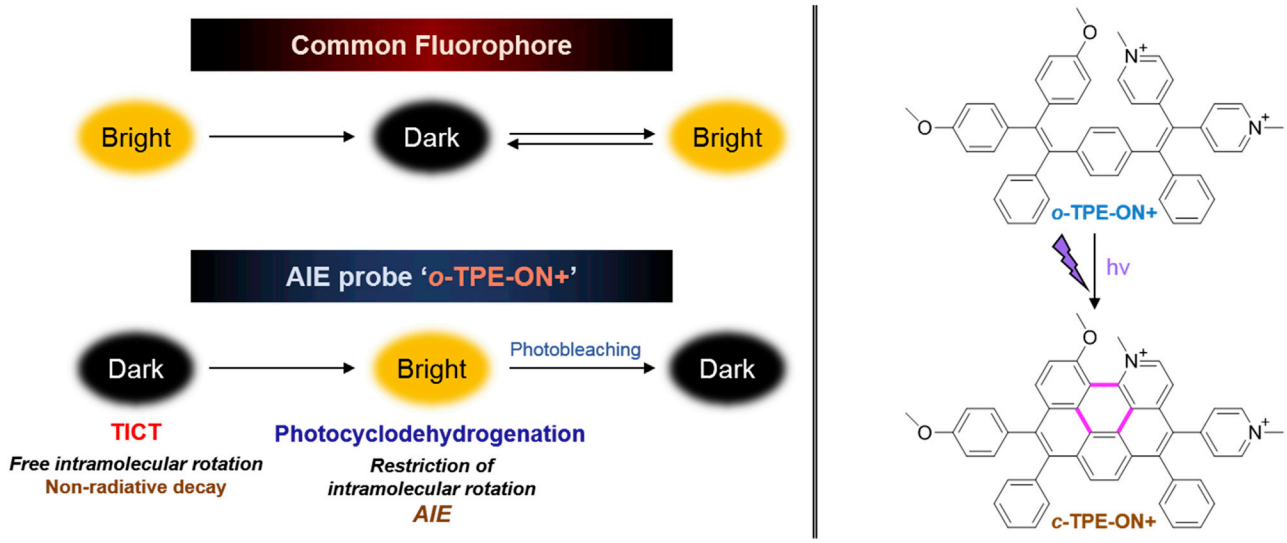


Figure 11. Mechanism of Photoactivation of the AIE-Based Probe *o*-TPE-ON+
Reconstructed with slight modifications from Tang and co-workers.[81]

Eventually, the photoactivable probe *o*-TPE-ON+ was utilized to obtain high-resolution nanoscopic imaging of mitochondria, and subsequently, with the help of this SRM, it became possible to monitor the dynamic alterations of mitochondria.[81]

Elgass and co-workers also utilized the well-known mitochondria targeting AIE-based fluorescent probe MitoRed-AIE (Figure 12; left) to perform dSTORM super-resolution imaging of mitochondria in live cells.[82] The probe MitoRed-AIE has some outstanding photophysical properties such as high photostability and excellent biocompatibility that enabled successful long-term super-resolved live-cell imaging (Figure 12; left). The cyanine core of the probe was the key to induce good blinking property in the presence of special imaging buffers, which is essential for achieving successful STORM imaging. Eventually, using AIE-based probe and single-molecule localization-based STORM imaging, they were able to track the dynamic mitochondrial changes in high resolution.[82]

Recently, Qian and co-workers have also developed a new AIE-based fluorescent probe (Figure 12; right) that can be utilized to monitor the organelle-specific dynamic changes in live cancer cells via STED nanoscopy.[83] Essentially, the newly designed synthetic AIE-probe TPA-T-CyP (Figure 12; right) exhibited strong red or NIR emission when spontaneously aggregated in the mitochondrial compartment with high specificity and thereby negated the complications of introducing any structural modification in the probe to ensure organelle-specific accumulation.[83] In the aggregated form of the probe, STED efficiency as high as 80% was achieved by the authors, which accelerated the super-resolution live-cell imaging of mitochondria in HeLa cells resulting in the visualization of the dynamic mitochondrial motion, fusion, and fission at a nanoscale level (lateral spatial resolution of 74 nm).

Therefore, the scope of utilizing AIEgen-based probes to procure organelle-specific super-resolution images and visualizing live intracellular dynamics at a nanoscale level could become an indispensable tool for bio-diagnostics and biomedical studies in the near future.

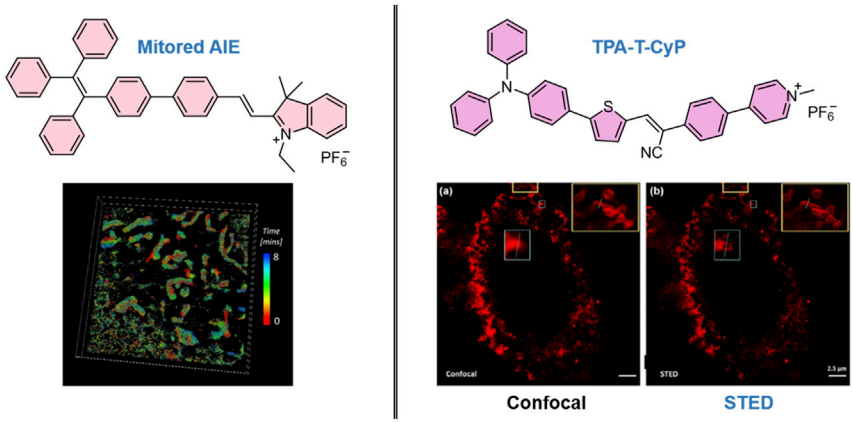


Figure 12. Nanoscopic Imaging of Mitochondria Using AIE Probes
(Left) Mitored AIE. Reproduced in parts from Elgass and co-workers.[82]
(Right) TPA-T-CyP. Scale bars, 2.5 μm. Reproduced in parts with permission from Qian and co-workers.[83] Copyright 2018 Springer Nature.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Despite the young age of this field, the advancements in SRM techniques have provided an ideal opportunity to study the details of subcellular structures as well as complex biological processes at a nanoscale level. The perfect combination of suitable fluorescent probes and nanoscopic techniques make it possible to visualize the fine structures of mitochondria with sub-diffraction resolution. Nanoscopy holds tremendous potential to resolve physiological ailments pertaining to mitochondrial dysfunctions. Therefore, this review basically covers the emerging aspects of the technical progress in developing modern nanoscopic techniques as well as the advancements in designing tailor-made fluorophores for procuring mitochondria-specific images with nanoscale resolution. Especially, the detailed structural features of successfully utilized mitochondria targeting fluorescent probes for various nanoscopy techniques have been judiciously assessed to expedite the scope of future probe design. A diverse category of fluorescent probes, including FPs, organic small molecules, NPs, polymer-copolymer complexes, and AIE-based probes have been discussed with regard to their potential benefits and drawbacks to lay down the basis of choosing proper fluorescent probes for mitochondria-specific nanoscopic studies. However, though there has been significant development in the nanoscopic imaging of mitochondria, there are still numerous areas for active progression. For instance, the development of irreversible FPs for multicolor PALM, improved STORM buffers for long-term imaging setup, and new strategies for *in vivo* labeling are some of the areas that have tremendous scope for improvement. It is expected that with a rapid development in nanoscopic techniques in recent years, several new SRM-compatible fluorescent labeling methods will be developed, which will ultimately help to circumvent the usual complications that arise during organelle-specific nanoscopic imaging. It is now envisaged that the nanoscopic imaging of mitochondria can be successfully utilized for medical diagnosis to acquire exact nanoscale information on specific expression profiles and, therefore, reveal new dimensions to personalized therapies in the near future.

AUTHOR CONTRIBUTIONS

Z.Y. proposed the topic of the review. S.S., Y.H., and W.P. investigated the literature and wrote the manuscript. J.K. and A.S. revised the figures. Z.Y., J.Q., and J.S.K.

guided the overall structure and content of the review. J.L. and A.S. revised several sections of the manuscript.

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