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Imaging Dynamic Subcellular Organization at High Spatiotemporal Resolution

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Abstract

The spatiotemporal organization of intracellular compartments is fundamental to cellular function and to the understanding of the processes underpinning health and disease. Fluorescence microscopy offers a powerful means to observe organelle morphology and dynamics with high specificity. However, no single technique can capture the wide range of relevant spatiotemporal scales due to inherent trade-offs in resolution, speed, field of view, signal-to-noise ratio, and sample viability. In this review, we describe recent developments across high-resolution fluorescence microscopy techniques and associated computational methods, critically evaluating how these advances address key limitations. Through biological examples of organelle dynamics at different scales, we illustrate the impact of these technologies on our understanding of cellular organization and function. Finally, we discuss the current challenges and outline future directions for imaging-based research, highlighting the potential for further innovations to deepen insights into dynamic subcellular processes.

Contents

INTRODUCTION

Subcellular organization is dynamic, relying on the precise spatiotemporal coordination of membrane-bound organelles [such as endoplasmic reticulum (ER), mitochondria, and lysosomes] and membraneless organelles (biomolecular condensates) formed by phase separation of multivalent macromolecules (50, 108, 109). This coordination is central to cellular function, as these organelles orchestrate critical processes, including metabolism, protein synthesis, and signal transduction. Accurately capturing organelle dynamics within the crowded intracellular environment is therefore crucial to gain further insight into cell function and disease.

Fluorescence microscopy is a powerful method for studying the dynamics of organelles, enabling observation of their morphology and movement within live cells with high sensitivity and specificity. One can gain insights into dynamic events taking place both within and between organelles and into their responses to environmental stimuli. However, the wide range of spatiotemporal scales across which these processes occur cannot be captured by any single fluorescence microscopy technique: Each imaging method represents a compromise between spatial resolution, imaging speed, field of view (FOV), signal, and sample health.

On the spatial scale, resolution is restricted by the diffraction limit (1) and depends on the wavelength of light and the microscope numerical aperture (NA). High-resolution imaging requires the use of high-NA objectives, which leads to a shallow depth of field ($\propto 1/NA^2$). In conventional epifluorescence microscopy, this results in the detection of out-of-focus signal, which reduces image contrast. Optical sectioning techniques mitigate this problem either by rejecting light from out-of-focus planes or by not exciting fluorophores in them. This allows volumetric image stacks to be captured with higher contrast, containing information on the 3D organization within the sample

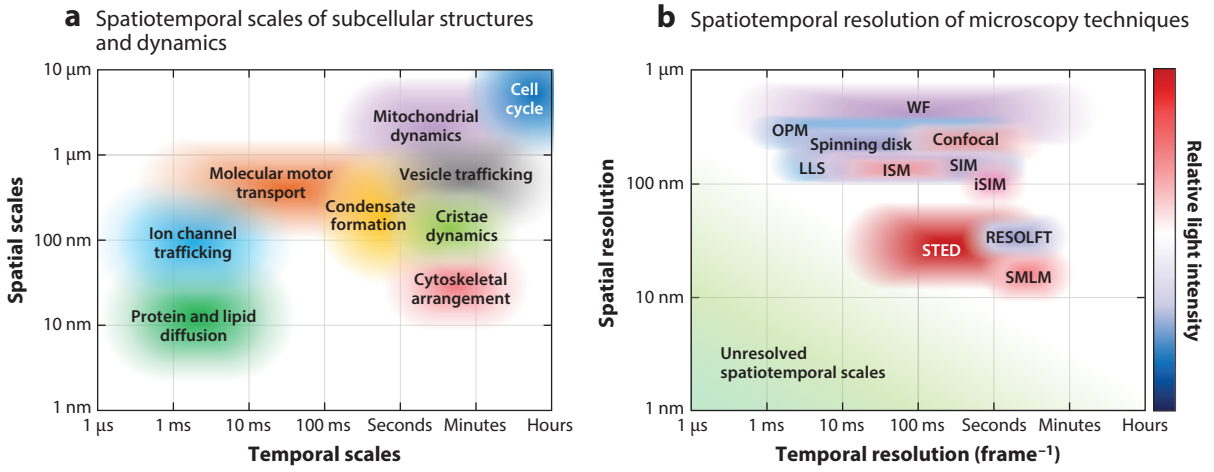

Figure 1

Microscopy techniques do not cover all spatiotemporal scales. (*a*) Intracellular dynamics occur over extensive length scales and timescales. (*b*) Range of spatiotemporal scales and relative illumination intensities associated with various fluorescence microscopy techniques. Abbreviations: iSIM, SIM variants with interferometric detection; ISM, image scanning microscopy; LLS, lattice light-sheet microscopy; OPM, oblique plane microscopy; RESOLFT, reversible saturable optical fluorescence transitions; SIM, structured illumination microscopy; SMLM, single-molecule localization microscopy; STED, stimulated emission depletion microscopy; WF, widefield. Spatial scales in panels *a* and *b* cover different ranges.

(2). However, even high-resolution optically sectioning microscopes can extract only limited spatial information from subcellular structures. Diffraction limits the resolution to ∼200 nm in the lateral direction and ∼500 nm in the axial direction for high-NA lenses, and the dimensions of many organelles and their substructure fall near or below this scale. For example, lysosomes range between 100 and 500 nm in diameter (122); ER tubule diameters range from ∼60 to 100 nm (97); and while mitochondria average between 1 and 2 µm in length, they contain internal structures that are separated by only tens of nanometers (92). Therefore, while certain phenomena can be investigated using diffraction-limited techniques (for example, the trafficking of vesicles), optical imaging of organelle topology requires the use of super-resolution (SR) methods.

The temporal scales over which subcellular dynamics take place are highly variable, ranging from milliseconds to days, and depend on the process under investigation (**Figure 1*a***). Fleeting processes can be captured using short exposure times to increase acquisition speed, at the cost of reduced signal. While signal loss can be compensated with higher illumination power, excessive photodoses damage the sample via photobleaching and phototoxicity, as energy transfer from exogenous fluorophores and naturally occurring organic molecules to molecular oxygen results in the formation of reactive oxygen species that react with lipids, proteins, and DNA (41). Not only can this cause permanent loss of fluorophore emission, it is also harmful to the cell (21). These effects are especially pronounced for SR techniques, which require the acquisition of multiple frames or the use of high illumination intensities (**Figure 1*b***), reducing their application potential for delicate and dynamic live samples.

To address these challenges and capture previously unresolvable organelle dynamics, advancements continue to be made in fluorescence microscopy. In this review, we discuss these developments and show how they enable novel research directions, offering unprecedented insights into the organization of life at the subcellular scale. We comment on the biological significance of these advancements using application examples involving both intra- and

interorganelle dynamics and discuss current technical limitations and opportunities. We conclude by identifying possible areas for future development.

RECENT ADVANCES IN HIGH-RESOLUTION MICROSCOPY

Developments in optical sectioning techniques have enabled faster and gentler 3D imaging, increasing their application potential for live-cell microscopy. Traditionally, optical sectioning has been achieved with confocal microscopy (72), where out-of-focus light is rejected using a pinhole, improving image contrast and resolution. This technique has established itself as a workhorse of biological imaging, facilitating numerous discoveries. However, as a confocal image is built up by scanning a single spot across the sample volume, the technique is too slow to capture fast dynamics. Additionally, the photodose delivered by the tightly focused laser beam is high, and only a fraction of the illuminated sample contributes to the signal. Variants such as line-scanning and spinning disk confocal microscopy (25) can improve imaging rates to hundreds of frames per second (fps). They achieve this by parallelizing the point excitation and detection either to a line (paired with slit detection) or to multiple spots (detection via a rapidly rotating disk with pinholes). However, these methods reduce optical sectioning efficiency and photodamage remains too high for many live-cell applications.

Light-Sheet Fluorescence Microscopy

Light-sheet fluorescence microscopy (LSFM), also known as selective plane illumination microscopy (SPIM) (40), addresses these challenges and achieves both fast and gentle volumetric imaging. When the sample illumination is confined to a single plane that is orthogonal to the detection axis, no fluorescence signal is generated from out-of-focus planes. This simultaneously achieves optical sectioning and a reduction in photodamage by avoiding unnecessary exposure of the sample to excitation light. As a consequence, the method is highly photon efficient, and since fluorescence is detected from all points in a slice at once, imaging rates up to ~1,000 fps are achievable, limited only by camera frame rates or signal levels. Originally developed for imaging larger volumetric samples, such as embryos, newer variants have specifically been adapted for the study of subcellular dynamics, enabling, for example, the study of calcium signaling in neurons (20) and cardiomyocytes (23) on millisecond timescales. Originally, light-sheet illumination and signal detection were achieved through two separate objectives arranged at right angles. However, the high-NA objectives required for subcellular imaging introduce geometrical constraints that complicate sample mounting. Oblique plane microscopy (OPM) (22) addresses this limitation through the generation of a tilted light-sheet and orthogonal fluorescence detection via a single high-NA objective. Because the image plane in an OPM system is tilted relative to the optical axis, remote refocusing (8) is required to map an isotropically magnified image onto the camera. This complicates the optical setup but combines the advantages of LSFM with conventional sample mounting, pushing the limits of spatiotemporal resolution in live-cell imaging (51).

Recent developments in optical sectioning methods have been driven by advances in optical technologies, image reconstruction, and analysis. For example, spinning disk microscopy has been implemented with iterative spectral unmixing to address the problem of spectral overlap when multiple fluorescent reporters are used to label different organelles. The method allowed the simultaneous imaging of nuclei, plasma membrane, mitochondria, Golgi apparatus, lysosomes, ER, and peroxisomes in live transfected U2OS cells (53). In OPM, the image quality has been improved using a custom-designed objective lens in the refocusing arrangement (71). This results in higher signal collection efficiency and resolution gain, as demonstrated in the study of clathrin-mediated endocytosis and of the dynamic reshaping of the ER and membrane structures (91). A

combination of OPM with label-free microscopy has enabled the simultaneous imaging of the physical and molecular composition of live cells, with parallel observation of mitochondria, lysosomes, and nuclei (42).

Structured Illumination Microscopy

Structured illumination microscopy (SIM) (29, 33) is an optical SR technique that is well-suited for imaging organelle dynamics beyond the diffraction limit. It achieves an increase in resolution by extracting high-frequency information from beat patterns generated when a fluorescent sample is illuminated by patterned excitation light. SR in the axial direction can also be achieved by using excitation patterns that vary along the optical axis (30). Typically, sinusoidal stripes are used as excitation patterns, as these maximize the encoding of subdiffraction frequencies, but it is possible to extract SR information from fluorescence modulated by any illumination pattern. Other common excitation patterns include lattices made up of multiple illumination spots. For instance, image scanning microscopy (ISM) (74) uses the excitation spot of a confocal or spinning disk microscope as the excitation pattern. When an array of point detectors is used, image resolution can be increased through iterative SIM reconstruction or photon reassignment (127).

Improving SIM capability continues to be an active field of research. Using complex excitation arrangements for multidirectional illumination, and interferometric signal detection, multiple research groups have achieved an isotropic image resolution of ~100 nm in all directions (81, 94). However, their methods, termed I^5S or 4Pi-SIM, require multiple illumination objectives and sample mounting is challenging. An alternative approach to improve axial resolution is to retroflect the axial illumination beam in 3D SIM back through the sample (56, 68). In most cases, the performance gains achieved by these newer 3D SIM methods are offset by their technical complexities and so standard SIM geometries remain the methods of choice for dynamic subcellular imaging. However, this restricts applications to the imaging of thin structures (e.g., the ER in relatively flat cells). To improve the temporal resolution, achromatic methods for pattern generation have been introduced, for example, using interferometers (113) or optical fiber arrays (79). These methods allow SIM to be performed simultaneously in multiple color channels, removing temporal delays that afflict the investigation of organelle–organelle interactions.

SIM has also benefitted from advances in computational approaches (16). For example, SIM^2 uses a priori knowledge of the image formation model to further extend the resolution (62). Hessian SIM (39) makes use of the temporal continuity of sample dynamics to enhance denoising, allowing for faster image acquisition at low excitation powers, and thus improves live-cell compatibility. Combined with sparse deconvolution, the method can also be used to increase the resolution of SIM, although this requires significant parameter tuning to ensure reliable reconstructions (128).

Stimulated Emission Depletion Microscopy

Stimulated emission depletion (STED) microscopy is a SR variant of point-scanning confocal microscopy (34, 45). Through the depletion of excited fluorophores in the outer part of the excitation focus, STED achieves an increase in resolution by reducing the effective size of the excitation spot (46). Like SIM, STED is regularly applied to live-cell imaging, although the requirement for suitable fluorophores and the photodamage associated with the high-intensity depletion beam limit its application range (111). The principle of STED is generalized in reversible saturable optical fluorescence transition (RESOLFT) microscopy. Here, photoswitchable fluorophores provide an alternative to stimulated emission for generating the dark state needed to reduce the effective excitation spot size (36). While this approach struggles to achieve the same spatial resolution

as STED, it works with fluorescent proteins, and the absence of a high-power depletion beam improves live-cell compatibility.

The latest advances in STED have primarily aimed at improving its usability for live imaging by reducing the required depletion beam intensity. For example, use of the highly photostable HBmito Crimson dye for labeling the inner mitochondrial membrane rather than the more commonly used ATTO family of dyes achieves a comparable resolution at more than 70% reduction in depletion power (88). Because of their high photostability and brightness, fluorescent or luminescent nanoparticles offer promising alternatives to organic dyes and fluorescent proteins, although challenges around their biocompatibility and functionalization remain (84).

STED can be combined with other SR methods so that lower depletion powers can be used to achieve similar resolution enhancements. For example, STED has been used in conjunction with array detectors and photon reassignment to exploit the advantages of the ISM principle described above (106). This obviates the need for a pinhole, increasing resolution with minimal loss of signal. An alternative method makes use of the fact that the fluorescence decay is faster in regions exposed to the depletion beam; measuring photon arrival times thus allows the differentiation between fluorophores that lie either outside or within the depletion beam focus (102, 105).

Single-Molecule Localization Microscopy

Single-molecule localization microscopy (SMLM) and its latest variants, such as MINFLUX (132), can achieve the highest spatial resolution of all fluorescence imaging techniques. Here, the diffraction limit is circumvented by switching sample fluorophores on and off and imaging only a sparse subset at a time. An image is reconstructed from a map of all fluorophore localizations. Broadly, SMLM techniques differ only by how they achieve this on/off switching: through photoactivation (7), chemical switching (31, 90), or transient binding of engineered molecules such as DNA (44) or proteins (78). The need to acquire multiple frames of the same FOV means that imaging speeds are usually too slow for live-cell imaging applications; however, recent improvements in probe design (104) have enabled the mapping of the spatial distribution and directionality of the cytoskeleton in live cells. While often too slow for imaging, SMLM can be used for single-particle tracking in live cells. Here, fluorophores are localized and followed as they move through the sample, building up tracks of their locations and velocities (rather than an image). In live-cell imaging, these tracks provide rich information on the dynamics of the cellular environment and so have been used to observe phenomena such as flow in the lumen of the ER (37) and the movement of motor proteins along microtubules (117). However, as single-particle tracking does not generate images directly, it is beyond the scope of this review.

Combining SIM and LSFM for Improved Spatiotemporal Resolution

Integrating SR microscopy with LSFM combines the advantages of these modalities within a single configuration, opening up new spatiotemporal regimes for live-cell organelle imaging. Lattice light-sheet microscopy (LLS) (14) involves the generation of ultrathin 2D light lattices from Bessel beams. Dithering the lattice evens out intensity variations in the excitation plane, yielding an excitation slice thinner than that generated with a 2D Gaussian light-sheet. This improves the axial resolution and reduces phototoxicity. In the LLS-SIM variant, periodic stepping of the light lattice between camera exposures (rather than dithering during single exposures to even out the intensity pattern in the excitation slice) combines the advantages of SIM and LSFM. Since its first application, the technique has been used for the rapid high-resolution visualization of multiple organelles in live cells, including the ER, Golgi apparatus, lysosomes, peroxisomes, mitochondria, and lipid droplets in COS-7 cells (107). LLS has also been combined

with adaptive optics, a technique that involves the measurement and correction of sample- and optical path–induced wave front aberrations. This combination has enabled the observation of subcellular reorganization in living specimens, such as organelle remodelling during mitosis (59).

The SR capabilities of SIM and advantages of LSFM have also been combined in a single-objective configuration through oblique plane structured illumination microscopy (OPSIM) (13). The method permits conventional sample mounting and uses a mirror-based image rotator to change the structured illumination pattern orientation for 2D SIM. This implementation allowed the movement of clathrin-coated vesicles and mitochondria to be observed at rates of approximately 1 Hz. The temporal capabilities of this technique have been pushed further in combination with multi-angle projection imaging (11), which exploits optical shearing to capture image projections of rapidly moving biological structures from variable viewing angles. With this projective OPSIM approach, mitochondria and ER dynamics could be localized throughout the cell volume at rates of up to 2.7 Hz (12). The ability of OPM and LLS variants to access new resolution regimes in space and time with low photodoses holds promise for future discoveries across diverse biological systems (**Table 1**).

Machine Learning

Fluorescence microscopy methods have been greatly advanced by developments in machine learning (ML) (70, 83, 110). So far, ML has been used primarily in postacquisition image processing tasks. These tasks include deconvolution and denoising of raw data, reconstruction of SR images, and image segmentation and object classification (6, 54, 60, 70). Using ML for denoising images with low signal levels reduces the photodose to the sample and increases imaging speed. For SIM, this has allowed the observation of dynamic processes in live cells and up to a 30-fold increase in imaging duration over classical image deconvolution (43, 85). In SIM, ML offers an alternative to classical reconstruction and filtering algorithms. Not only does this enable parameter-free reconstructions, it also reduces artifacts from sample movement and noise, improving imaging capability for highly dynamic processes (17, 56, 93). In STED, ML-based denoising has advanced the live-cell capabilities of the technique, enabling imaging of mitochondrial structures with low

Table 1 Indicative performance characteristics for fluorescence microscopy techniques discussed in this review, comparing the lateral and axial resolutions as well as the acquisition speed

Technique	Lateral resolution (nm)	Axial resolution (nm)	Acquisition speed (fps)	Reference(s)
Widefield	250	600	>1,000	1
Confocal/spinning disk	250/175[a]	600/600	100[b]/>1,000	95, 126
ISM	125	350	25[b]	121
OPM	300	700	>1,000	22
3D SIM	120	300	>100	30
I^5S/4Pi-SIM	100	100	0.7	94
LLS/SR-mode	230/150	370/280	>100	14
3D STED	25	50	0.8[b]	46
RESOLFT	40	70	0.8[b]	36
SMLM	10	10	<0.01	7

[a]Resolution for spinning disk confocal microscope equipped with additional photon reassignment disk.

[b]Scanning time depends on the field of view used. The acquisition rates represent the expected imaging speed for a field of view equivalent to a single mitochondrion (5 μm^2) imaged with Nyquist-limited pixel sizes and an integrated pixel dwell time of 25 μs.

Abbreviations: fps, frames per second; ISM, image scanning microscopy; LLS, lattice light-sheet microscopy; OPM, oblique plane microscopy; RESOLFT, reversible saturable optical fluorescence transition; SIM, structured illumination microscopy; SMLM, single-molecule localization microscopy; SR, super-resolution.

photodamage at unprecedented speeds (24). For SMLM, ML enables reconstruction from samples with high active emitter densities, reducing the number of raw images required. This improves temporal resolution sufficiently to achieve live-cell imaging of dynamic structures, such as the cytoskeleton (75, 80, 99).

Furthermore, ML on its own can increase spatiotemporal resolution, irrespective of the imaging technique used to acquire data. In the spatial dimension, this has been applied in single-image SR microscopy (16, 101). In the temporal dimension, frame interpolation with ML allows one to increase the effective imaging speed by estimating images of the sample between sequentially acquired frames (82). These techniques, however, require a priori information about the samples that is learned during training and only provide estimates of missing information. As such, one has to take a cautionary approach: ML estimations are subject to the well-known problem of hallucinations (57), which can be visually convincing and are particularly common in the field of biological imaging, where ground truth data are limited.

CASE STUDY: VISUALIZATION OF INTRA-ORGANELLE COMPARTMENTS

Mitochondria are bioenergetic organelles with critical roles in cellular metabolism, biosynthesis, and signaling (103) and are therefore a key target for high-resolution imaging studies of subcellular organization. As mitochondria have an endosymbiotic origin (69), they feature an inner membrane and an outer membrane, as well as remnant genomes and associated transcriptional and translational machinery (28) (**Figure 2*a***). The inner membrane is the site of oxidative phosphorylation, in which a transmembrane electrochemical proton gradient is established to power ATP synthesis (73). This membrane has a highly invaginated and variable structure specialized for this function, consisting of stacked tubular and lamellar cristae separated by junction complexes and inner boundary regions (18), with localized protein complexes (19). Within the mitochondrial matrix, gene expression is spatially organized through compartment formation by mitochondrial DNA and RNA, likely via biomolecular condensation (5).

Resolving topological features at the suborganelle scale, such as cristae and mitochondrial condensates, is at the limit of what can be achieved with current methods in live cells (**Figure 1*b***) and typically requires STED or ISM (48). On the spatial scale, cristae are both small and densely packed: Crista-to-crista separation can be under 100 nm (58, 100), and cristae's tubular connections to the inner membrane have a diameter of only ~30–40 nm (67). Therefore, SIM can distinguish only well-separated cristae, but STED can resolve those more closely arranged. Mitochondrial condensates are similarly small: Mitochondrial DNA-based condensates (nucleoids) are predicted to be ~70 nm in size from antibody-based STED (52) [though more variable shapes and sizes are identified from correlative SMLM/electron microscopy studies (49)]. Mitochondrial RNA condensates are larger but still measure only ~130 nm in diameter (89). Both SIM and STED can be used to identify these structures, but STED is required to resolve their size and interactions. On the temporal scale, cristae position and shape are remodeled on a timescale of seconds (38, 47), and condensates interact with and are confined by cristae. Therefore, imaging of these compartments does not require imaging speeds greater than ~2–3 fps, which is compatible with STED imaging across small FOVs.

Cristae Dynamics

SR imaging of live cristae has revealed that they are constantly reorganized and respond to changes in overall mitochondrial morphology. Hessian SIM allowed monitoring of the effects of mitochondrial fission/fusion on cristae, as well as monitoring of intramitochondrial cristae fusion (39).

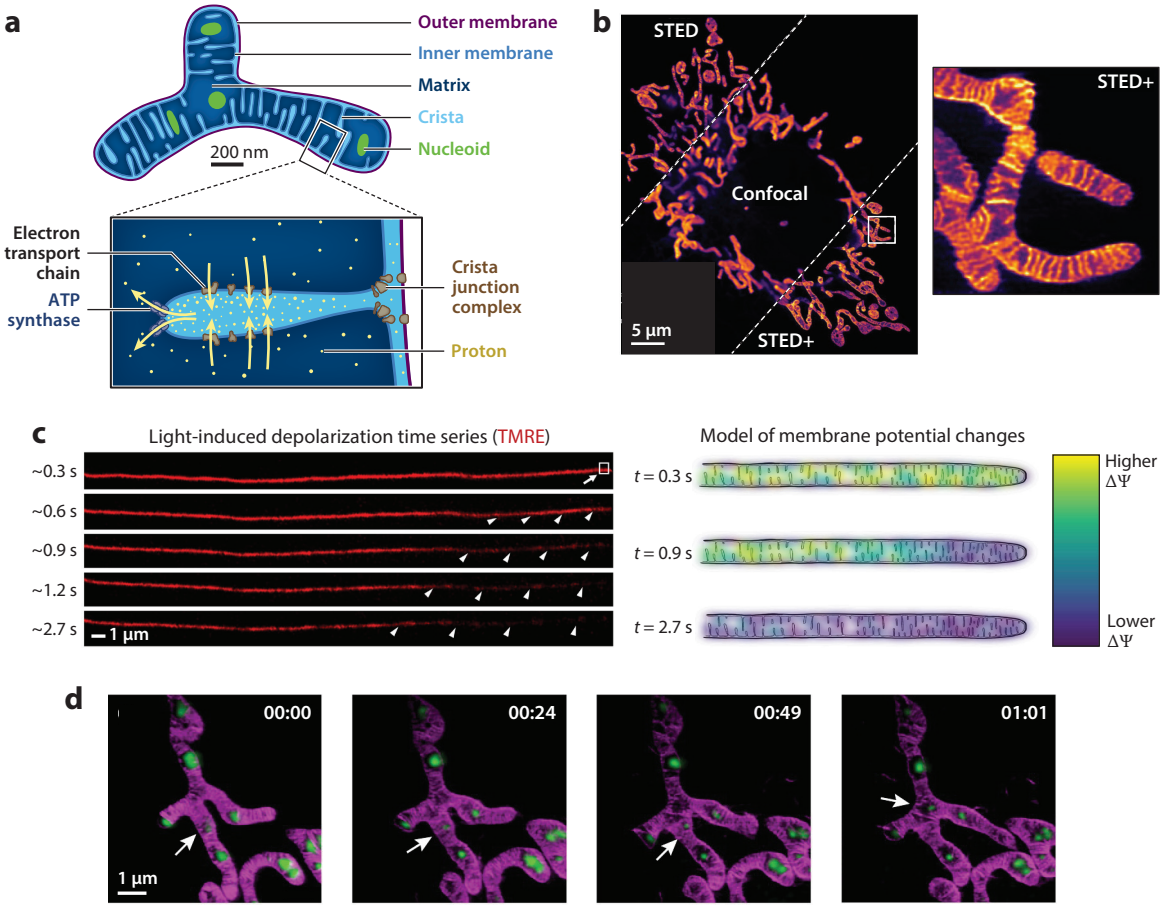

Figure 2

The dynamics of mitochondrial subcompartments can be resolved using super-resolution microscopy. (*a*) Mitochondria have a double-membrane organization with internal compartments. Scales are approximate. (*Inset*) Oxidative phosphorylation is enabled by cristae-bound protein complexes (not to scale). (*b*) Cristae are not resolved by confocal imaging but can be visualized using STED or STED+. (*c*) As shown by image scanning microscopy, the loss of mitochondrial inner membrane potential ($\Delta\Psi$) upon local laser ablation gradually spreads. Cristae dynamics are omitted from the diagram for clarity. Left images generated using data from Reference 118. (*d*) As shown using STED, nucleoids (*green*) interact with cristae (*magenta*), for instance, migrating into newly formed voids. Abbreviations: STED, stimulated emission depletion; STED+, STED with deconvolution; TMRE, tetramethylrhodamine ethyl ester. Panels *b* and *d* adapted from Reference 88.

However, this does not allow contact points between cristae to be fully resolved, which requires live-cell STED. This involves the use of STED-compatible, photostable dyes to label the inner membrane, via SNAP-tag protein fusion (47, 100) or mitochondrially targeted dyes (58, 88, 98, 112, 125, 131). Live-cell STED has permitted the tracking of both cristae junction dynamics (47) and cristae group dynamics during mitochondrial fission (100). It has been suggested that these dynamics are critical for a range of processes, such as regulation of oxidative phosphorylation and metabolite exchange with the cytosol (48).

Membrane Potential

With the use of the membrane potential–sensitive dyes tetramethylrhodamine methyl ester (TMRM) and tetramethylrhodamine ethyl ester (TMRE), cristae dynamics can be linked to

mitochondrial function. Using ISM and STED, Wolf et al. (118) showed that cristae junctions provide electrical insulation: Distinct cristae maintained different membrane potentials, as reported by varying TMRE intensities (**Figure 2*c***). Furthermore, light-induced membrane depolarization took several seconds to spread down mitochondria, which suggests the phenomenon is mediated by transport of a biochemical signal rather than via electrical depolarization in an electrically continuous system (118). This finding indicates cristae are biochemically distinct compartments. In a subsequent STED study, it was shown that TMRM can be locally redistributed due to cristae membrane dynamics, indicating that these affect local membrane potential (47). This compartmentalization of membrane potential may be important to prevent spread of depolarization following a breach of membrane integrity of a single crista, particularly within highly branched mitochondria, and may enable different cristae to function primarily in ATP synthesis or reactive oxygen species signaling, depending on their potential (118).

Condensate Dynamics

In addition, a link between cristae and condensate dynamics has been revealed using SR imaging. Using a SNAP-based label with STED, Stephan et al. (100) found that nucleoids occupy most spaces in the matrix that are devoid of cristae. They are therefore nonrandomly positioned, being found most often at tubule tips and branch points, which contain unique cristae topologies (88). Furthermore, when new mitochondrial tubules form near nucleoids, cristae are rapidly remodeled to maintain a perpendicular orientation with respect to tubule axes, resulting in a void at the branch point into which nucleoids can migrate (88), as shown using STED (**Figure 2*d***). For mitochondrial RNA-based condensates (RNA granules), SIM has been used to demonstrate that they are liquid-like compartments stably associated with the inner membrane and that their fusion often coincides with mitochondrial rearrangements like fusion or fission (89). This is significant, as their dynamics and compaction may influence gene expression (28).

CASE STUDY: INTERORGANELLE INTERACTIONS

Regulation by the ER Through Contacts

The ER is the largest organelle (77) and has a highly complex and dynamic morphology, making it another key target for studies of subcellular organization. It extends throughout the cytoplasm as a mesh of interconnected tubules, stacked sheets, and ribosome-studded domains (**Figure 3*a***). Different ER domains function to support different critical processes, such as protein synthesis, lipid metabolism, and calcium signaling (116). In particular, its interconnected tubules can rapidly rearrange, enabling responsiveness to metabolic and signaling demands. Understanding this dynamic restructuring is important for comprehending the ER's role in pathological conditions, such as in viral infections; for example, Zika virus induces formation of tubular matrices that facilitate viral replication (61).

Resolving ER tubule structure and dynamics requires SR imaging. ER tubule diameters are below the diffraction limit, varying between 125 nm in some cultured animal cells (32, 35) and 10 nm within plant plasmodesmata (120). Furthermore, ER tubule structure is highly complex, as revealed by SIM: Regions previously identified as ER sheets have now been shown to consist of dense ER tubules (76), and tubular ER can be embedded in sheet domains (63). These structures form, extend, fuse, and retract on the timescale of seconds, as ER tubules extend via sliding and hitchhiking processes that are limited by the speed of molecular motors (~1 μm/s) (77, 115). A temporal resolution of 1 to 2 Hz is therefore sufficient to record the dynamics of the ER. However, the size of the organelle requires large FOVs, of the order of the cellular dimensions. In practice,

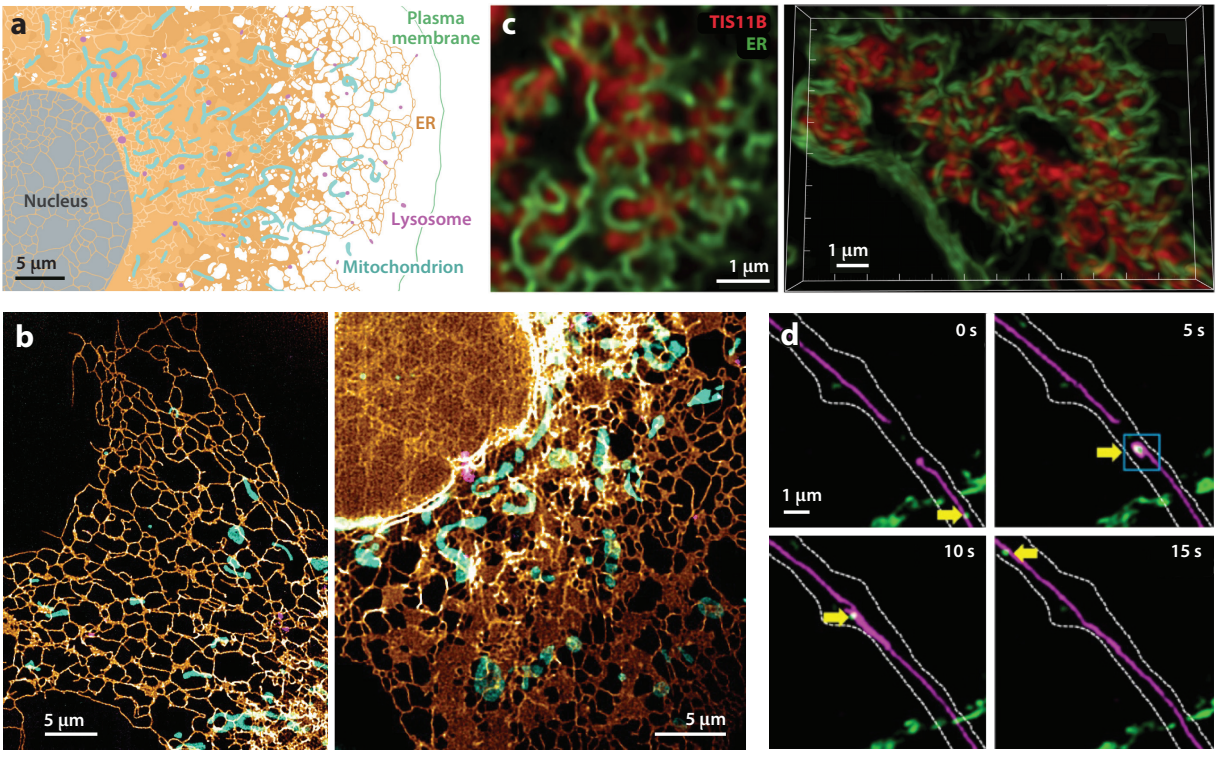

Figure 3

Super-resolution imaging reveals the structure and dynamics of ER–organelle contacts. (*a*) The ER (*gold*) forms an extensive network throughout the cell, contacting most other organelles, including mitochondria (*cyan*) and lysosomes (*magenta*). (*b*) A comprehensive overview of the ER (*gold*) and its contacts with mitochondria (*cyan*) and lysosomes (*magenta*) can be obtained using 2D SIM (F.W. van Tartwijk & C.F. Kaminski, unpublished data). (*c*) The ER (*green*) is interwoven with a reticulated condensate, including the protein TIS11B (*red*), as shown using image scanning microscopy. Panel adapted with permission from Reference 65. (*d*) Lysosomes (*green*) aid the extension and reconnection of ER tubules (*magenta*) in axons through direct interaction, as shown using 2D SIM. Panel adapted from Reference 64 (CC BY 4.0). Abbreviations: ER, endoplasmic reticulum; SIM, structured illumination microscopy.

this often makes SIM or ISM the best technique to record not only the structural dynamics of the ER but also its interactions with other organelles (**Figure 3*b–d***).

Through its large surface area, the ER can form a wide range of contacts with other organelles, regulating their function. These contacts are functionally highly significant. For example, using grazing-incidence SIM, Guo et al. (27) imaged a region near the basal cell cortex at a high spatiotemporal resolution (97 nm at 266 fps), revealing that ER contacts not only mark the sites of mitochondrial division but also play a role in their stabilization. Further increases in resolution using 3D SIM provided support for a mechanistic model explaining this: The ER–mitochondrion contact area was shown not to correlate strongly with mitochondrial constriction, indicating ER tubules act as platforms to recruit fission machinery rather than mechanically promoting fission (129). These ER–mitochondrion contacts also influence mitochondrial nucleoid transport, as shown by grazing-incidence SIM, by acting as platforms for the interaction of nucleoids, the mitochondrial proteins MICOS and Miro1, and the motor protein KIF5B (86).

The ER also interacts with condensates to regulate their function. Again, ER tubules can regulate morphology: ISM revealed dynamic tethering between ER tubules and P-bodies and stress granules, with fission events occurring at ER contact sites (55). However, the regulatory

significance of these interactions is much broader. For instance, calcium transients on the ER surface trigger biomolecular condensation of the autophagosome-initiating FIP200 complex, as shown by SIM (130). Furthermore, using ISM, Ma & Mayr (65) discovered that the broadly expressed RNA-binding protein TIS11B condenses into a so-called TIS granule, which forms a reticular meshwork intertwined with the ER (**Figure 3*c***). This compartmentalization affects a range of cellular proteins: mRNAs with alternatively spliced 3′-untranslated regions are enriched in this granule, which causes proteins translated from them to have different interaction partners than mRNA variants without these sequences, altering protein activity (65). These findings, among others, establish the ER as a mechanical and regulatory component in membraneless organelle dynamics.

Other Regulatory Organelle Contacts

While the ER dominates the cellular interaction landscape, regulation through organelle contacts is not unidirectional, and smaller organelles also engage in biologically significant contacts that reinforce the same homeostatic network, including by regulating the ER. As for ER, ISM and especially SIM are the preferred techniques to study contacts made by smaller organelles, as interaction events are again distributed through the whole cytoplasm. In this category, lysosomes are of particular interest: They are highly motile degradative hubs, which continuously undergo repositioning to match metabolic demand (4), enabling them to regulate the distribution of other organelles. For instance, lysosomes have been shown by SIM to pull ER tubules into the cell periphery in response to changes in nutritional status, retracting ER tubules back to the cell center under stress (64). This ER-housekeeping role of lysosomes is crucial to maintain the structural integrity of the ER: The growth of axons in *Xenopus laevis* retinal ganglion cells was shown to depend on intact ER–lysosome interactions, indicating that their dysfunction may be relevant to multiple neurological disorders (64) (**Figure 3*d***).

However, like the ER, lysosomes are in turn regulated by organelle contacts. SIM was used to show that lysosomal network dynamics are modulated by interlysosomal tethering and that mitochondrial contacts promote untethering of these interlysosomal contacts (119). This mitochondrion-mediated untethering was compromised by mutations in the mitochondrial Mid51–Fis1 complex potentially linked to Parkinson's disease, suggesting that disruption of organelle interaction networks may be a factor in this pathology (119). The potential of SIM for studying lysosome–mitochondrion contacts is being further extended through probe development, with the dual-color probe Coupa being used to track mitophagy and detect local viscosity changes at contact sites (15).

DISCUSSION AND FUTURE DIRECTIONS

High-resolution fluorescence microscopy techniques represent the current state of the art for observing organelle dynamics at the nanoscale. These methods—and their evolving variants—have seen remarkable progress, continuously enabling new biological insights to be made and uncovering functional relationships between organelle structure and activity. For example, the morphology and dynamics of mitochondrial cristae have been linked to local membrane potential and therefore energetic performance. These methods have also clarified mechanisms of organelle compartmentalization, such as the spatial organization of transcription hubs. One of their greatest contributions is enabling the contextualization of organelle dynamics with overall cell behavior. For example, ER contact points serve as hubs for mitochondrial division and nucleoid segregation, while lysosomes coordinate ER repair and repositioning during stress. Many of these transient phenomena occur on subsecond timescales and throughout the cytoplasm, necessitating high resolution over a large FOV, which has only recently become possible.

However, challenges remain. A major bottleneck is the limited range of fluorophores that are both bright and photostable while retaining biocompatibility and targeting specificity. Imaging complex 3D cell arrangements is still a challenge. Key hurdles include ensuring biological viability, achieving good optical access, and maintaining practicality. Techniques like multiobjective SIM offer remarkable potential for observing organelle dynamics but remain highly complex and often impractical for live samples. For now, such methods are confined largely to a few highly specialized laboratories. At the highest spatiotemporal resolutions, ML is increasingly used, though it comes with large risks of reconstruction artifacts. Below, we outline directions for future developments.

Whole-Cell Reorganization

Major questions that can potentially be addressed with SR methods are how internal cues (e.g., metabolic stress) or external triggers (e.g., pathogen invasion) can induce whole-cell remodelling. Capturing these responses requires the imaging of whole-cell volumes with SR, repeatedly and at subsecond speeds: While global cellular reorganization often unfolds over minutes, organelles can move on subsecond timescales. Hybrid techniques like OPSIM and LLS-SIM may be suitable to tackle this challenge. In the future, they could enable detailed studies of how organelle contacts shift during cell-wide remodelling. More broadly, integrated platforms like MOSAIC (26) allow researchers to flexibly combine multiple imaging modalities into a single system, adapting to the demands of diverse biological applications.

Automation of Imaging Tasks

There is substantial potential in automating microscopic imaging with ML (114). For STED, event-driven imaging (3) and task-assisted generative adversarial networks (9) have been used to reduce photodamage by triggering high-resolution acquisition only when rare events occur. Similar advances have been made in SIM, with event-driven implementations improving live-cell compatibility (66). Newer LLS designs also incorporate artificial intelligence (AI)-based instrument control to switch between imaging modalities, allowing multicolor 4D imaging to study features that are rare within a cell population, such as kinetochore dynamics during mitosis (96).

Multimodal Data Integration

Looking ahead, the future of imaging likely lies in the integration with multimodal data to gain deeper insights into cellular function. Although not yet fully realized, merging high-resolution imaging with genomics, transcriptomics, proteomics, and metabolomics could drastically expand mechanistic insights across cellular length scales (10). AI models—particularly multimodal deep learning frameworks (87)—are especially promising for this task. This will involve encoding imaging and ‘omics data into compatible representations, which can then be linked via cross-modal transformers (123) or variational graph autoencoders (124) to correlate morphological features (e.g., ER sheet thickness, lysosome motility) with functional states (e.g., unfolded protein response, metabolic rewiring). Once trained, such models could serve as generative digital twins, for instance, forecasting cellular responses to genetic perturbations or identifying structural hallmarks of disease. Realizing this vision will require robust data standards, precise spatial registration across scales, and interpretable AI architectures.

CONCLUSION

As high-resolution and SR fluorescence microscopy techniques continue to evolve, their successful application to live-cell imaging hinges on thoughtful experimental design, that is, careful selection of sample preparation strategy and imaging modality. As these technologies increasingly integrate

with computational tools and multimodal platforms, they hold unprecedented potential for transforming our understanding of cell biology, transitioning from descriptive visualization toward predictive integrated models of cellular function.

DISCLOSURE STATEMENT

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