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Zooming in on biological processes with fluorescence nanoscopy

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Fluorescence nanoscopy enables the study of biological phenomena at nanometer scale spatial resolution. Recent biological studies using fluorescence nanoscopy have showcased the ability of these techniques to directly observe protein organization, subcellular molecular interactions, structural dynamics, electrical signaling, and diffusion of cytosolic proteins at unprecedented spatial resolution. Super-resolution imaging techniques critically rely on bright fluorescent probes such as organic dyes or fluorescent proteins. Recently, these methods have been extended to live cells and multicolor, three-dimensional imaging, thereby providing exquisite spatiotemporal resolutions of the order of 10–20 nm and 1–2 s for subcellular imaging. Further improvements in image processing algorithms, labeling techniques, correlative microscopy, and development of advanced fluorescent probes will be required to achieve true molecular-scale resolution using these techniques.

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Introduction

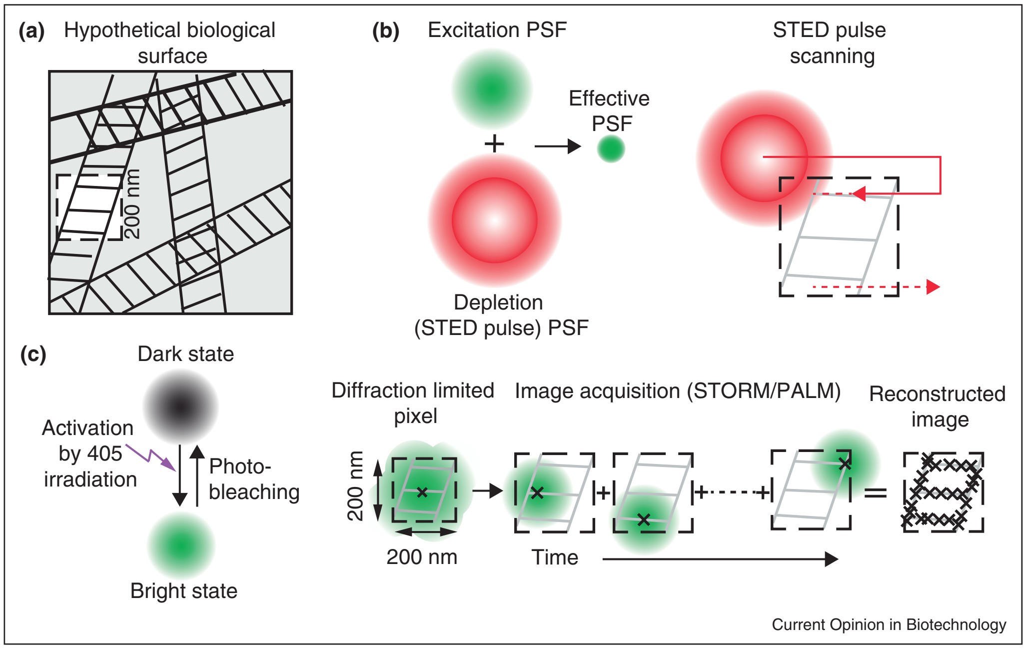
The spatial resolution of conventional optical microscopy is limited by the diffraction limit of light, which inhibits high-resolution imaging of subcellular structures and hinders a molecular-level understanding of cell structure and dynamics. Electron microscopy (EM) allows for imaging at molecular-scale resolution, but this approach has limited applicability due to complex staining procedures, inaccessibility and unspecificity of antigens (e.g. for immunocytochemical EM), and incompatibility with living systems. To circumvent these issues, recent advances in fluorescence nanoscopy have enabled imaging below the diffraction limit using optical microscopy.

The response of an imaging system to a point source of light is known as the point spread function (PSF), which governs the spatial resolution based on the Rayleigh criterion. Spatial resolution is typically limited to ≈200 nm in the lateral direction and ≈500 nm in the axial direction for diffraction-limited optics [1]. Over the past decade, it has been realized that biological systems can be effectively probed by breaking this diffraction limit using various super-resolution (SR) microscopy techniques, thereby facilitating direct visualization of biological processes (Figure 1). In general, SR techniques employ physical or chemical concepts to distinguish fluorescence emission from nearby probes in a diffraction-limited region. SR approaches can be classified into two broad categories: deterministic ensemble-level methods based on patterned illumination (such as stimulated emission depletion microscopy, STED), and single molecule-based stochastic methods employing photoswitching or other mechanisms to reduce the number of simultaneously active fluorophores (such as PALM or STORM).

STED relies on shrinking the PSF by depleting the fluorescence emission in the periphery of a diffraction limited spot using stimulated emission (Figure 1b) [2]. The size of the nanometric focus scales inversely with the intensity of depletion beam, which suggests that the resolution of STED is theoretically diffraction-unlimited. Using STED, spatial resolutions down to 20 nm have been achieved on biological samples involving fluorophore tagged DNA on glass surfaces [3].

Single molecule-based SR methods function by stochastically activating individual fluorescent molecules in a diffraction-limited region and localizing their position. In this way, single fluorophores are stochastically ‘switched on’, localized, and ‘switched off’ over subsequent images (Figure 1c). An integrated SR image is reconstructed by repeating this cycle of activation, imaging, and bleaching to accumulate a sequence of images containing many single molecule localizations. Several point-localization SR techniques have been developed, including photoactivated localization microscopy (PALM) [4], fluorescence photoactivated localization microscopy (FPALM) [5], and stochastic optical reconstruction microscopy (STORM) [6]. For these methods, spatial resolution relies on high-precision localization of dyes (i.e. bright probes and a high signal-to-background ratio) [7] and a sufficiently large labeling density of fluorophores such that the average

Figure 1



Principle of super-resolution microscopy. **(a)** Hypothetical biological structure with a diffraction limited pixel size of 200 nm × 200 nm. **(b)** Principle of stimulated emission depletion microscopy (STED) demonstrated by an excitation PSF by labeled fluorophore combined with depletion pulse that converts fluorophores back to ground state, thereby resulting in an effective PSF with a higher resolution. Right panel demonstrates the STED imaging procedure, which involves scanning the whole surface with the STED pulse. **(c)** Principle of blinking in STORM is demonstrated using reversible photoactivation and photobleaching of a single fluorophore upon irradiation with 405 nm pulse and imaging laser, respectively. A diffraction-limited image of a single pixel using conventional optics is shown for reference. Right panel depicts the principle of single molecule localization microscopy (STORM/PALM) wherein multiple images acquired over time are used to reconstruct a final image with fluorophores separated both spatially and temporally. Each image consists of diffraction-limited spots whose position is determined by a fit, and crosses mark the center of the fit. PSF: Point spread function, STED: stimulated emission depletion, STORM: stochastic optical reconstruction microscopy, PALM: photoactivated localization microscopy.

dye spacing is no larger than approximately half the desired resolution (Nyquist criterion) [8]. Several recent reviews provide an excellent description of the implementation of various SR microscopy techniques [9–13,14•]. This review focuses on fluorescent probes for SR imaging, recent biological applications, and future advancements towards the wide-scale adaptation of these methods in biology, genetic engineering and nanomedicine.

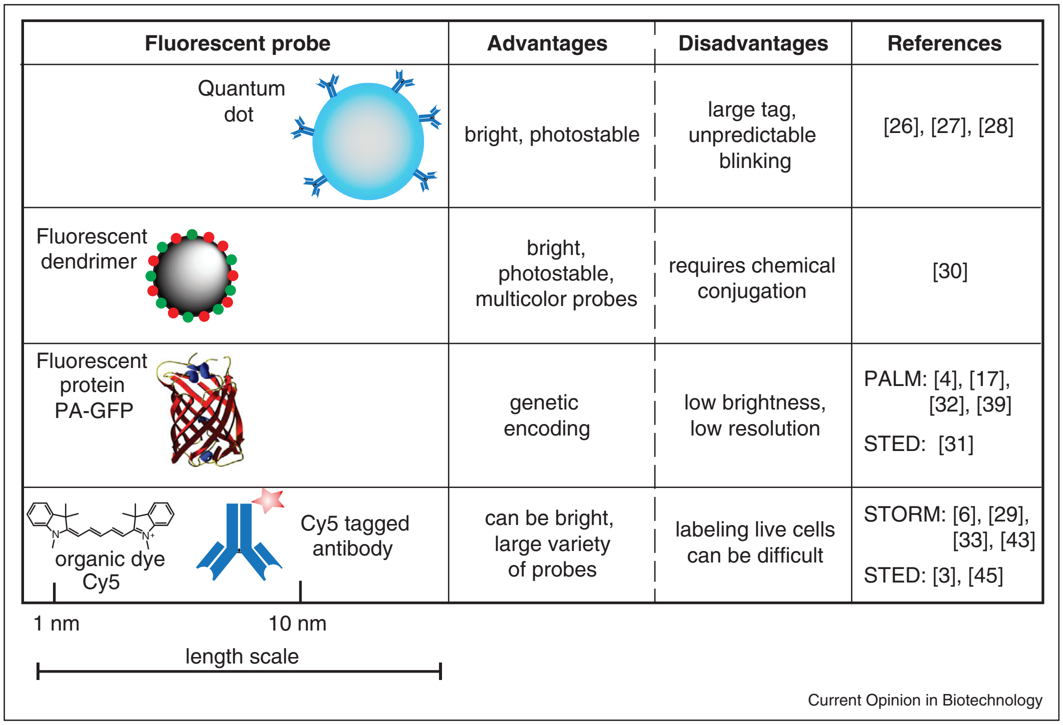
Fluorescent probes for SR imaging

For any imaging experiment, the SR imaging method dictates the selection of fluorescent probes. STED can utilize a wide range of photostable conventional fluorophores, but generally requires complex instrumentation to generate the imaging and depletion beams. On the other hand, stochastic SR imaging can be achieved with a comparatively simpler total internal reflection fluorescence microscopy (TIRF-M) setup, albeit with special requirements on the type of fluorescent probes, generally requiring the ability for photoactivation or photoswitching. Three primary types of fluorescent probes are most commonly used for SR imaging: fluorescent proteins, synthetic organic fluorophores, and semiconductor nanocrystals (quantum dots or QDs) (Figure 2). Probe brightness, photostability, and photoswitching properties are key aspects to consider before embarking on an SR imaging experiment, and the relative importance of each property can help dictate which probe should be used.

Fluorescent proteins can be genetically encoded, which is a major advantage over synthetic organic dyes, thereby allowing direct labeling of many proteins in a living cell. In addition to photoactivatable green fluorescent protein (PA-GFP) [15], recently developed proteins such as mEos2 [16] and PA-mCherry [17] have enabled increased localization precision and multicolor PALM experiments. PA-mCherry was used in conjunction with PA-GFP for dual-color imaging of clathrin-coated pits [17].

Fluorescent proteins tend to have a lower brightness [10] compared to synthetic fluorophores (e.g. Alexa, Atto and cyanine dyes) which exhibit enhanced brightness (via larger quantum yields or absorption, or both), thereby allowing for higher localization precision. The cyanine dye Cy5 can be efficiently and reversibly switched between bright and dark states in the presence of Cy3 [18] or

Figure 2



Fluorescent probe	Advantages	Disadvantages	References
Quantum dot	bright, photostable	large tag, unpredictable blinking	[26], [27], [28]
Fluorescent dendrimer	bright, photostable, multicolor probes	requires chemical conjugation	[30]
Fluorescent protein PA-GFP	genetic encoding	low brightness, low resolution	PALM: [4], [17], [32], [39] STED: [31]
organic dye Cy5 Cy5 tagged antibody	can be bright, large variety of probes	labeling live cells can be difficult	STORM: [6], [29], [33], [43] STED: [3], [45]

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Super-resolution imaging probes: synthetic organic dyes (e.g. Cy3 or Cy5), fluorescent proteins (PA-GFP), fluorescent dendrimer probes, and quantum dots. Relevant advantages and disadvantages for each probe are given. A partial list of references associated with each probe is shown.

in isolation [19]. Synthetic fluorophores are typically conjugated to primary or secondary antibodies (~150 kDa) to specifically target cellular components, which effectively increases the size of the probe and can add 10–20 nm in localization uncertainty [13]. However, Rizzoli and coworkers recently used aptamers (~15–30 kDa) for fluorophore labeling and cell component targeting, thereby resulting in higher resolution images [20•]. In another study, smaller ~13 kDa anti-GFP nanobodies tagged with organic dyes were developed to enhance brightness and specificity, thereby leveraging the existing library of GFP constructs [21].

SR imaging of live cells can be particularly difficult to achieve using synthetic fluorophores, mainly due to challenges associated with labeling and the requirement for non-perturbative imaging conditions. In 2012, the Manley group used a cyanine based dye (PicoGreen) that binds to DNA to achieve the first SR image of DNA in live cells [22•]. Live cell imaging of genomic DNA can be very useful for understanding the dynamics of DNA organization, particularly during processes such as cell division. Direct conjugation of chemical tags to proteins can be used for live cell labeling and imaging without antibodies. In one approach, the high affinity of trimethoprim (TMP) for the *Escherichia coli* enzyme dihydrofolate reductase (eDHFR) enabled the imaging of the human histone protein H2B at 20 nm resolution [23]. Similar approaches utilize covalent attachment of a synthetic fluorophore to a DNA transferase that can be genetically encoded to a protein of interest, commercially known as the SNAP tag [24] and CLIP tag [25].

Finally, QDs have also been used for SR imaging, although they are much less common in applications due to their large size, requirement for surface passivation, and unpredictable blinking properties [26,27]. The superior brightness of the QDs allows for short acquisition times, which is advantageous for cell imaging to capture events over biologically relevant timescales. Using this approach, a localization precision of ~10 nm of microtubules in Chinese hamster ovary cells was achieved [28].

Future directions in the development of improved probes should be aimed at methods to increase probe brightness and/or photostability. In 2012, the Zhuang group demonstrated a reductive caging effect on synthetic organic dyes by employing sodium borohydride in the imaging buffer, which increased photon output per switching cycle and enabled localization precision as low as 1 nm [29••]. A promising new class of probes based on multi-dye conjugated dendritic molecules can offer the potential for increased brightness, which was recently demonstrated by Schroeder and co-workers [30]. In this work, multiple

Cy3 or Cy5 dyes were linked to a dendritic molecular scaffold, thereby achieving a ~4× improvement in brightness compared to a single dye molecule and improved probe photostability.

Biological applications of fluorescence nanoscopy

The unique nanoscale view conferred by fluorescence nanoscopy holds the potential to improve our understanding of signal transduction pathways, gene expression dynamics, mechanisms of cellular machines such as ribosomes and organelles, and molecular drug targets.

Virions

Human immunodeficiency virus-1 (HIV-1) virions have a diameter of ~120 nm, which is below the diffraction limit. Recently, direct visualization of virions using STED revealed redistribution profile of envelope proteins (Env) as single or multiple foci during viral maturation [31••]. In this work, SR microscopy also provided mechanistic insights on 'inside-out signaling', which correlates the rearrangement of the inner protein lattice with surface alterations for viral infectivity (Figure 3a). In another study, the size and shape of the viral complex was measured at ~30 nm resolution during infection by labeling the integrase enzyme of HIV with FlAsH [32]. These studies illustrate tremendous potential of fluorescence nanoscopy in the field of drug discovery, including drug screening and evaluation of mechanistic sites of action.

Neurons

The actin cytoskeleton was imaged in neurons using 3D STORM, which enabled a <10 nm lateral resolution and <20 nm axial resolution using a dual-objective astigmatism-imaging scheme [33]. In this way, dual-objective imaging allows for the collection of more photons (thereby increasing resolution), and a cylindrical lens is used to induce asymmetry in spots varying in z-direction, thereby providing axial resolution [34]. Similar multicolor colocalization studies of other proteins such as adducin and spectrin with actin in axons revealed a striking periodicity in structure [35]. Distinct synaptic structures were resolved via STORM imaging of presynaptic and postsynaptic scaffolding proteins [36]. Recently, the dynamics of voltage gated sodium channels in neuritic spines was demonstrated in real time using live STORM with small molecule (STX-CY5) probes [37•]. Such studies can assist in designing novel pharmacological agents that can potentially combat pain and cure nerve disorders.

Bacteria

Bacteria present unique challenges for studying subcellular structure and dynamics due to their small size and crowded intracellular environments. 3D astigmatic SR imaging in *C. crescentus* revealed the depolymerization time of FtsZ (midplane protein) to be under 100 ms, significantly faster than the previously known estimates of 8 s [38]. Precise single-molecule tracking of a protein, RelA, in live *E. coli* cells revealed a mechanism whereby bacteria respond to stress and starvation (Figure 3b). This study successfully overcame the challenges of the sheer number (7000–50 000) and speed (temporal resolution of 4 ms) of freely diffusing cytosolic entities, thereby providing a general tool for the investigation of intracellular kinetics in living cells [39••].

Quantification methods for counting protein numbers or interaction events can be particularly challenging in biology. Importantly, fluorescence nanoscopy can provide quantitative information on protein clustering, such as the number of molecules, density, cluster shape and size, and pair correlation functions. Chemotaxis proteins in *E. coli* including Tar receptors were studied using PALM with a precision of 15 nm [40•]. This investigation apparently solved a long-standing mystery by attributing chemotaxis cluster formation to a stochastic-nucleation model. In another study, pair correlation analysis coupled with PALM (PC-PALM) presented an ideal tool to study protein reorganization, heterogeneity and function during various physiological processes without determination of absolute protein numbers [41]. Direct visualization of living *V. cholerae* biofilms using dual color 3D SR imaging revealed unique biofilm characteristics by providing spatial information on clustering and interaction of essential matrix constituents [42•]. Such information may prove handy for biotechnological processes enabling nucleation or disruption of biofilms (Figure 3c).

Epithelial cells and yeast

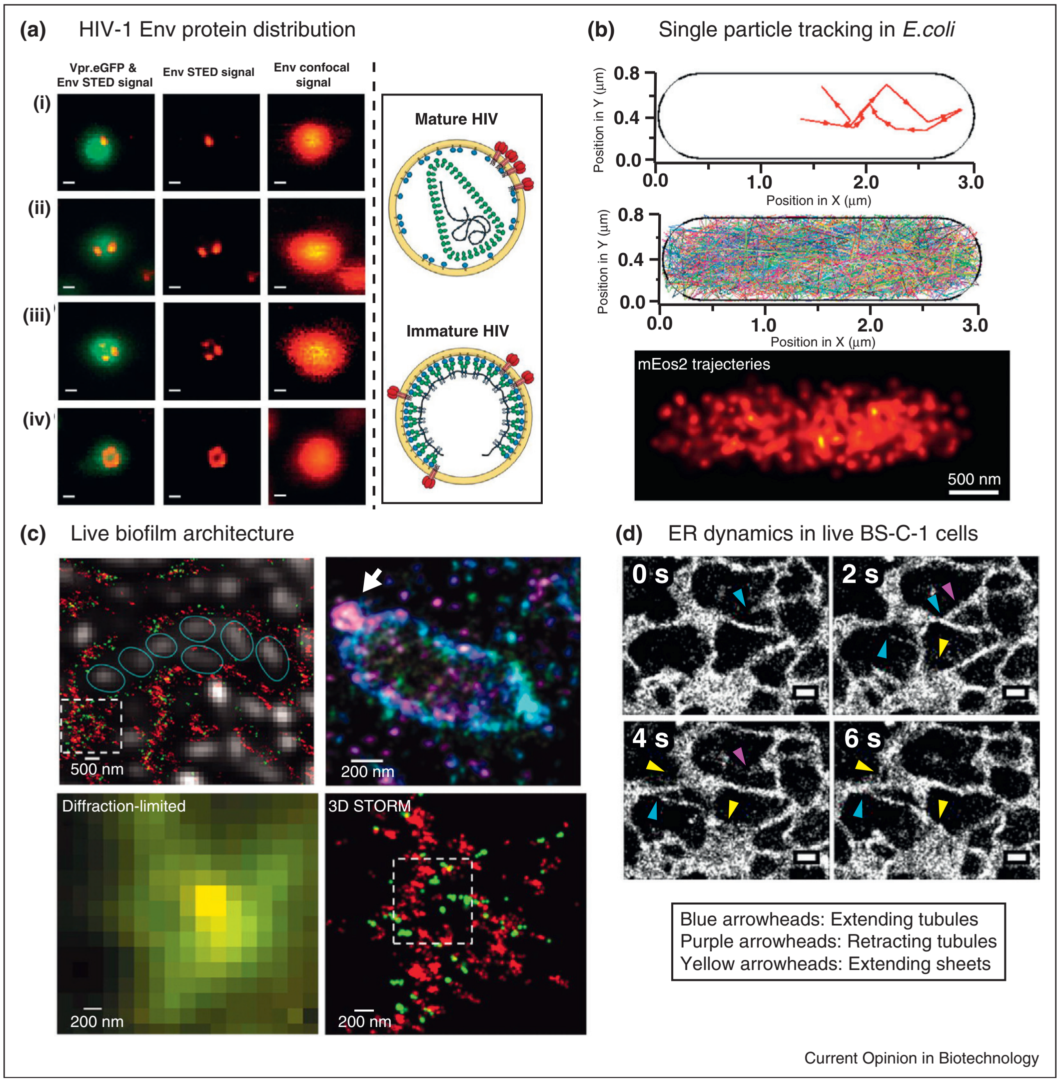
Dynamic STORM based on photoswitchable small molecule probes was used to study organelles in living cells with 30–60 nm spatial resolution and 1–2 s temporal resolution (Figure 3d). Time-resolved STORM images revealed extension and retraction of dendritic spines, morphological dynamics of plasma membrane, and molecular motion within the membrane, thereby showing that these probes are ideal candidates to study ultrastructural dynamics of organelles [43•].

Recently, STORM was used to obtain quantitative in situ data estimating the levels of mRNA in yeast cells via combinatorial labeling [44••]. The SR barcoding technique employed in this work was based on hybridized fluorophore-labeled probes using two strategies: spatial ordering of probes and spectral coding using a combination of colors. This labeling technology is scalable and makes STORM amenable for high-throughput single-cell systems biology.

Conclusion and future perspective

SR imaging techniques are poised to revolutionize our understanding of biology. However, the inherent tradeoff between temporal and spatial resolution in current SR

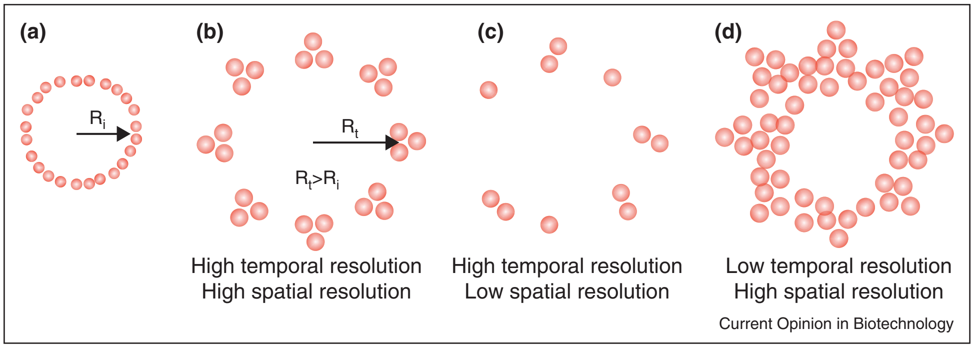
Figure 3



Role of fluorescence nanoscopy in quantitative, structural, and dynamical studies of biological systems. **(a)** STED imaging of Env (orange) protein distribution profiles in HIV-1 (green) particles displaying single Env focus or multiple Env foci. Scale bars: 100 nm [31••]. Cartoon corresponds to variation of Env (red) clustering based on HIV-1 maturation. **(b)** Single molecule tracking of mEos2 in *E. coli* cytosol showing free diffusion [39••]. Single experimentally obtained trajectory and an overlay of 1355 trajectories in an individual cell. Lower panel corresponds to overlay of 500 positions of single molecule trajectories in single cell. **(c)** Three-dimensional two-color STORM imaging (200-nm z-section) of *V. cholerae* biofilm components – cells (white with blue outline), Vibrio polysaccharide, VPS (red) and RbmC matrix protein (green) [42•]. Lower panel corresponds to the enlarged boxed region from the top left panel. White arrow in the top right panel corresponds to early stage VPS organization with a color scale indicating height: −300 nm (violet) to +300 nm (red). **(d)** STORM imaging containing 2-s snapshots of ER dynamics in live BSC-1 cells using photoswitchable membrane probes [43•]. Reproduced with permission from [31••,39••,42•,43•]. HIV: human immunodeficiency virus, Env: envelope proteins, ER: endoplasmic reticulum.

techniques presents a critical bottleneck to achieve true molecular-scale visualization with high temporal resolution (Figure 4). In order to image dynamic events, it is imperative that the image acquisition rate is faster than target mobility. However, faster acquisition rates generally result in lower numbers of detected photons, which can lower spatial resolution. Recently, STED achieved a focal spot size of $\sim$62 nm with frame rates up to 28 Hz [45], albeit over a small area. For single molecule localization methods such as STORM and PALM, higher spatiotemporal resolution can be achieved by using an optimal labeling density [46] of complex structures (e.g. $\sim 10^6$ fluorophores/μm^3 for $\sim$20 nm spatial resolution [47]) and increased photoswitching rates of fluorophores. A spatial resolution of $\sim$20 nm and a temporal resolution of $\sim$0.5 s was demonstrated using 2D/3D STORM while imaging live cells [48]. In the next few years, development of high sensitivity cameras and powerful image processing

Figure 4



High temporal resolution
High spatial resolution

High temporal resolution
Low spatial resolution

Low temporal resolution
High spatial resolution

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Cartoon demonstrating inherent trade-off between spatial and temporal resolution in current fluorescence nanoscopy techniques. **(a)** Initial state of a hypothetical biological assembly with radius R_i. The final state (radius R_t) is shown in **(b)**, **(c)** and **(d)** with different imaging scenarios. (b) Ideal single molecule localization imaging scenario (currently difficult to achieve) where image acquisition is performed under quasi steady state conditions. The biological assembly expands to a radius R_t without translation, but with a redistribution of surface proteins. (c) Final state imaging with faster image acquisition rate providing improved temporal resolution but low spatial resolution. This obscures accurate structural determination. (d) Final state image obtained with high acquisition time resulting in increased localizations, with some fluorophores being counted repeatedly, while compromising the temporal resolution. This violates the quasi steady state approximation as biological assembly continues to expand while image is being acquired and results in false positives and inaccurate structure determination.

algorithms capable of handling multiple active fluorophores per frame such as compressed sensing [49], Bayesian analysis [50], and DAOSTORM [51] will significantly reduce image acquisition times, thereby enabling imaging of dynamic biological processes occurring over a faster time scale. These algorithms have revealed high association/dissociation dynamics in podosomes [50] and have a potential to be pervasive in the field of biomedical imaging and even fluorescence imaging-based DNA sequencing [51,52].

The field of fluorescence nanoscopy continues to advance with improvements in the design of commercial microscopes, the emergence of user-friendly software packages, and the development of bright and photostable fluorescent probes. Combination of fluorescence nanoscopy with other techniques such as fluorescence correlation spectroscopy (STED-FCS) [53] and electron microscopy micrographs [54] will immensely supplement biological imaging data. Using such combination approaches, deep tissue penetration was achieved by combining STED with two photon excitation [55], and a separation of ventral and dorsal plasma membranes in mammalian cells was observed with sub 20 nm axial resolution using 3D STORM with interferometry [56]. Given the current pace of technology development, fluorescence nanoscopy will soon progress to a routinely used method to image biological systems with subcellular resolution, to design or evaluate potential drug therapies, and to quantify genetic data, all of which will have a profound impact on the field of biotechnology.

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- •• of outstanding interest

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