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Review

Advancements and Practical Considerations for Biophysical Research: Navigating the Challenges and Future of Super-resolution Microscopy

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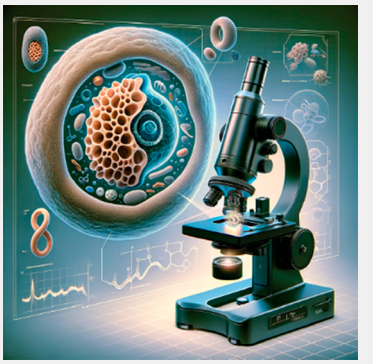
ABSTRACT: The introduction of super-resolution microscopy (SRM) has significantly advanced our understanding of cellular and molecular dynamics, offering a detailed view previously beyond our reach. Implementing SRM in biophysical research, however, presents numerous challenges. This review addresses the crucial aspects of utilizing SRM effectively, from selecting appropriate fluorophores and preparing samples to analyzing complex data sets. We explore recent technological advancements and methodological improvements that enhance the capabilities of SRM. Emphasizing the integration of SRM with other analytical methods, we aim to overcome inherent limitations and expand the scope of biological insights achievable. By providing a comprehensive guide for choosing the most suitable SRM methods based on specific research objectives, we aim to empower researchers to explore complex biological processes with enhanced precision and clarity, thereby advancing the frontiers of biophysical research.



KEYWORDS: *Super-resolution Microscopy, Single-molecule Approach, Protein Complexes, Cellular Assemblies, Biomolecular Structures, In Situ Analysis, Fluorescent Tags, Physiological Conditions, Imaging Penetration Depth*

1. INTRODUCTION

In the dynamic field of biological research, pursuing an understanding of the complexities of cellular processes and molecular interactions in cells is a relentless endeavor. The advent and continual refinement of super-resolution microscopy (SRM) have marked a transformative era in this quest.[1−5] This review focuses on the pivotal role of SRM techniques in deciphering complex biological problems, offering a window into the previously unseen world of molecular dynamics and interactions at the nanoscale.

The necessity to transcend beyond the conventional diffraction limit of light microscopy spearheaded the development of various SRM techniques, such as stimulated emission depletion (STED) microscopy,[6] structured illumination microscopy (SIM),[7,8] and single-molecule localization microscopy (SMLM).[9,10] These methodologies have unlocked new potentials in biological research, enabling the visualization and analysis of cellular components and molecular assemblies with unprecedented precision.

At the heart of SRM's impact is its ability to provide detailed insights into individual molecules' spatial organization, dynamics, and interactions within their native cellular environments. This granular view is crucial in understanding the fundamental processes of life at a molecular level, such as protein−protein interactions, protein oligomeric states, DNA-RNA transactions, the intricate workings of cellular organelles, and drug catalysis efficiency in the cells.[3,11−17] Moreover, the application of SRM has been instrumental in studying the pathophysiology of diseases, aiding in the visualization of disease progression at a molecular scale, and offering new avenues for therapeutic interventions.[12,18−34]

Despite its profound capabilities, SRM is not without challenges. Issues such as fluorophore stability, the potential interference of fluorescent tags with protein function, optimal sample preparation, and handling complex data sets have necessitated ongoing technological and methodological improvements. Additionally, integrating SRM with other imaging modalities and computational analysis tools has become increasingly important to address its inherent limitations and

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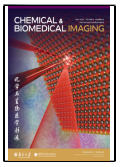

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Table 1. Comparison of SRM Techniques

			SMLM
Technique	STED	SIM	PALM and STORM/dSTORM
Principle	Exploits nonlinear properties of fluorophores	Patterned light and computational image processing	PALM: Stochastic activation of fluorophores and precise localization; STORM/dSTORM: stochastic blinking of fluorophores and precise localization
Lateral resolution	20−50 nm	100−130 nm	10−30 nm
Axial resolution	50−100 nm	300−400 nm	50−100 nm
Live-cell imaging	Yes	Yes	Yes, with optimized fluorophores and conditions
Fluorophores	Specific fluorophores with suitable photophysical properties; commonly used dyes: Atto, Alexa Fluor, Abberior STAR	Compatible with a wide range of samples and fluorophores	Requires fluorophores with specific photophysical properties to achieve single molecule detections
			PALM: photoactivatable/convertible fluorophores
			STORM: a pair of photoswitchable activator-reporter fluorophores
			dSTORM: a single fluorophore in specific buffers
Software program	Imspector	SIMcheck	QuickPALM, ThunderSTORM, custom software
Single-molecule imaging	Limited	Limited	Suitable
Limitations	Photobleaching, potential phototoxicity	Limited resolution improvement compared to other techniques	Requires specific fluorophores, slower imaging speed
Best for	Intermediate resolution, larger cellular structures	Wide compatibility, lower resolution requirements, cellular structures and organelles	Structural resolving: using highest resolution 3D SMLM
			Molecular dynamics: using sptPALM or SMT through stochastically switching, smFRET, smFA, smND
refs	36−39, 43	40−44	10, 43, 45−47

extract more comprehensive insights from the data. In this review, we will navigate through the advancements, applications, challenges, and future prospects of SRM in biological research. Our focus will be on how SRM has reshaped our understanding of cellular mechanisms and how it continues to propel the frontiers of biological sciences.

2. SUPER-RESOLUTION MICROSCOPY: UNVEILING FINE STRUCTURES AND MOLECULAR DYNAMICS IN BIOLOGICAL RESEARCH

SRM techniques, such as STED, SIM, and SMLM, have drastically advanced our capability to examine protein formations *in situ*. These techniques achieve this by surpassing the diffraction boundary of conventional light microscopy.[2,35] In this section, we comprehensively compare these widely used SRM techniques, discuss how these techniques can be integrated with single-molecule imaging, and suggest factors to consider while choosing the proper SRM based on the biological questions (Table 1).

2.1. Working Principles, Resolutions, and Image Analysis of SRM

STED microscopy utilizes the nonlinear characteristics of fluorophores to surpass the diffraction limit of traditional light microscopy.[36,37] It operates using two laser beams: an excitation beam that stimulates the fluorophores in the sample to emit fluorescence and a doughnut-shaped depletion beam that quenches the fluorophores via stimulated emission. By superimposing the excitation and depletion beams, STED microscopy effectively minimizes the effective point spread function (PSF) of the fluorophore, thereby augmenting its spatial resolution (Figure 1a). Typically, STED microscopy has a lateral resolution of 20−50 nm and an axial resolution of 50−100 nm.[37,38] Although early STED microscopes had relatively slow imaging speeds, progress in laser technology, fluorophore creation, and detector sensitivity has improved imaging speeds of up to several frames per second, making STED microscopy suitable for studying fast cellular processes.[37,39]

SIM is another SRM technique that surpasses the diffraction limit of conventional widefield fluorescence microscopy by using patterned light and computational image processing to obtain high-resolution images.[7,8,40] SIM illuminates the sample with a series of sinusoidal striped patterns. These patterns result from interfered light beams, whose frequency and orientation are controlled by an optical system (Figure 1b). The resulting images, known as Moiré fringes, carry both low-frequency and high-frequency (super-resolution) data. By capturing multiple images with varying pattern orientations and phases, the high-frequency data can be computationally isolated, and a high-resolution image can be reconstructed using a Fourier-based algorithm. SIM can achieve a lateral resolution of approximately 100−130 nm and an axial resolution of 300−400 nm.[40,41] While early SIM implementations were relatively slow, advances in camera technology and computational methods have significantly increased the imaging speed, allowing modern SIM systems to achieve frame rates up to several frames per second for live-cell imaging.[42]

SMLM techniques, such as Photoactivated Localization Microscopy (PALM),[9] Stochastic Optical Reconstruction Microscopy (STORM),[10] and direct STORM (dSTORM),[48,49] surpass the diffraction limit of conventional fluorescence microscopy by exploiting the stochastic presence of fluorophores and the precise localization of individual molecules. STORM typically requires an activator-reporter pair to achieve stochastic activation and subsequent imaging of individual fluorophores, whereas direct STORM, or dSTORM, simplifies

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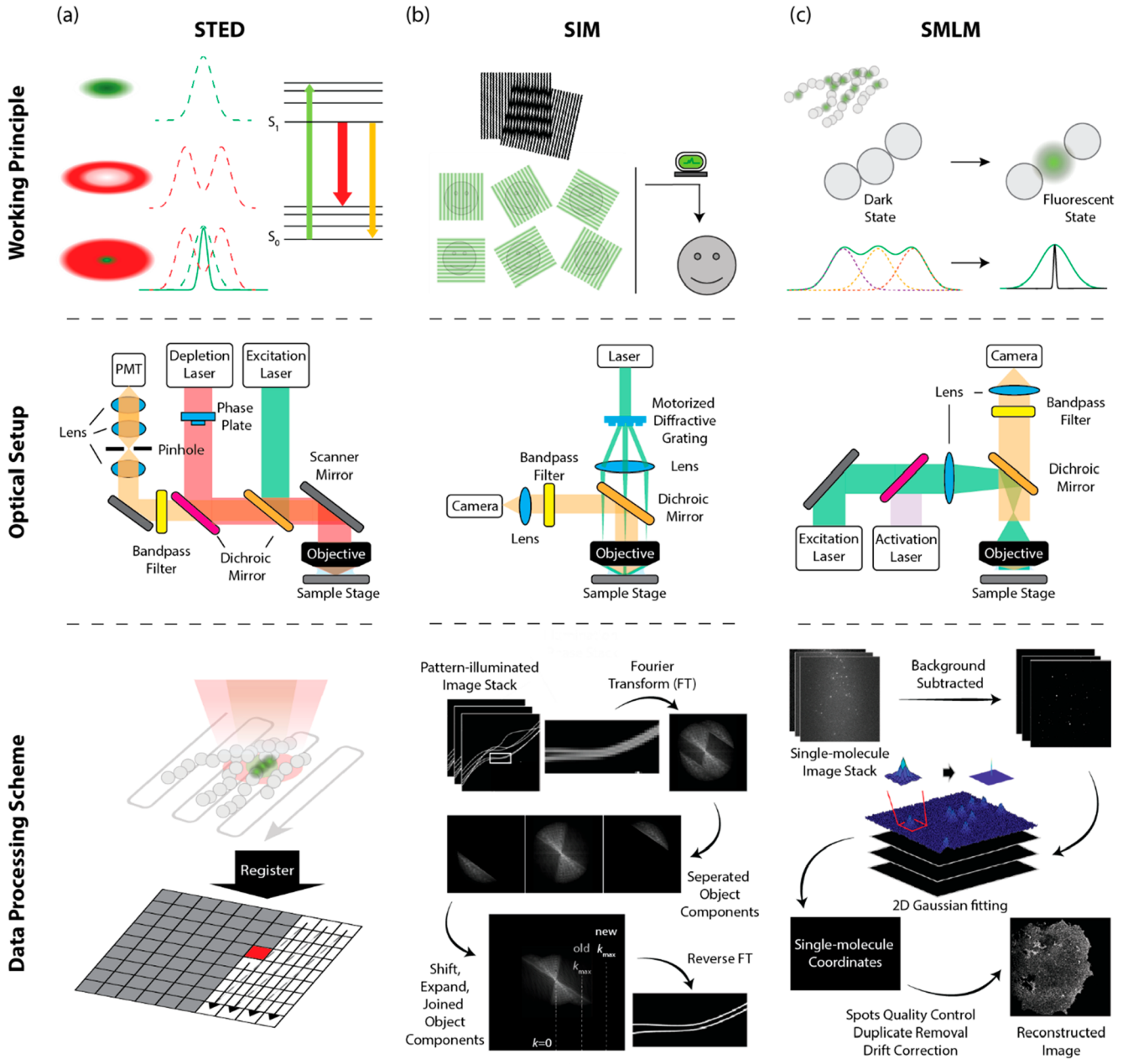

Figure 1. Working principle, optical setup, and data processing scheme for various SRM. (a) STED microscopy combines focused excitation and doughnut-shaped depletion beams to enhance spatial resolution. The optical setup uses a phase plate to shape the depletion laser, and the system employs a confocal scanning module and pinhole setup. Photomultipliers measure fluorescence intensity at predetermined grid locations, providing improved resolution compared to confocal microscopy. (b) SIM uses Moiré fringes and Fourier-based processing to produce high-resolution images. The optical setup directs an excitation laser through a phase plate, forming a sinusoidal pattern. Fluorescent images are captured and processed by separating, shifting, and joining object components. Finally, a reverse Fourier transformation yields images with better spatial resolution than confocal microscopy. (c) SMLM distinguishes fluorescent molecules through temporal and spatial separation using controlled activation, conversion, or switching. In the optical setup, the activation laser's power density is managed to maintain low molecular density, while the excitation laser stimulates single-molecule fluorescence, enabling precise molecule location (with tens of nanometers resolution) using Gaussian fitting.

this by using a single fluorophore, necessitating specific buffers to induce the requisite blinking behavior for super-resolution imaging. The working principle of SMLM relies on the controlled activation and detection of sparse subsets of fluorophores in the sample (Figure 1c). By ensuring that only a small fraction of fluorophores is emitting light at any given time, the centroid of each fluorophore's PSF can be pinpointed with nanometric accuracy. The process of activating, imaging, and localizing fluorophores is repeated numerous times, and the final high-resolution image is constructed by combining the localized positions of all detected fluorophores in the sample. SMLM can achieve a lateral resolution of approximately 10−30 nm and an axial resolution of 50−100 nm.[35] The detection speed of SMLM depends on several factors, including the blinking properties of the fluorophores, the imaging frame rate, and the total number of imaging cycles required to accumulate a sufficient number of localizations for constructing the final image. Typically, SMLM acquisition times can range from

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seconds to minutes per frame, with faster imaging speeds achievable using optimized fluorophores, camera technology, and computational methods.[47]

The rapid advancement of SRM opened new realms of understanding the structure and behavior of protein complexes within cells. However, the complexity of the resulting data requires refined computational methods to fully leverage their imaging power. The increasing quality and quantity of super-resolution microscopy data calls for creating high-level image analysis and quantification tools that can extract significant information from these data sets. Customized software and algorithms have been developed to process, analyze, and quantify the spatial organization, molecular interactions, and dynamic properties of protein complexes and assemblies in cells. There are several publicly available software programs and tools for analyzing super-resolution microscopy images, including those obtained from STED, SIM, PALM, and STORM/dSTORM techniques, such as Imspector, SIMcheck, QuickPALM, and ThunderSTORM, that offer a wide range of plugins and modules for processing and analyzing SRM data.[43−46,50]

2.2. Enhancing SRM through Single-Molecule Approaches

The integration of single-molecule imaging approaches with SRM further expands the power of these techniques, allowing researchers to visualize extended fine structures and probe dynamic protein interactions and molecular behaviors, offering a comprehensive way to explore biological complexity.[1−5,51,52]

A key advantage of single-molecule conditions in SRM is the ability for PSF engineering, utilizing techniques such as astigmatism and double-helix patterns to enhance three-dimensional (3D) resolutions.[53−56] Further integration of light sheet microscopy leverages a thin sheet of light to selectively illuminate and capture images of thin sample sections, enabling deep tissue imaging with excellent clarity.[57,58] Similarly, the incorporation of 4Pi microscopy, which employs two opposing objectives to focus on the same point from opposite sides of the specimen, significantly enhances axial resolution below 100 nm.[59] This technique increases the detail in three-dimensional structures at the molecular level by effectively doubling the numerical aperture in the axial direction. These advancements facilitate a detailed examination of protein complexes and subcellular structures.

Furthermore, the evolution of single-molecule tracking (SMT) methods, including single particle tracking PALM (sptPALM) and stochastic switching within STORM, extends SRM's utility to dynamic studies.[60−63] These adaptations allow for the exploration of protein behaviors and interactions within the cellular milieu, offering spatial resolutions down to 10−30 nm and temporal resolutions from milliseconds to seconds. A pivotal innovation in this field is the minimal photon fluxes (MINFLUX) technique, which combines the targeted illumination strategy of STED with the localization precision of PALM.[64−67] By minimizing photon use and precisely locating fluorophores with a donut-shaped excitation beam, MINFLUX and its derivative MINSTED achieve near-atomic localization precision (∼1 nm) and significantly enhances temporal resolution (∼100 μs). This method, and its derivative MINSTED, represents a leap forward in dynamic imaging, facilitating long-duration tracking of molecules with minimal photobleaching.[68−70]

Highlighting the power of single-molecule approaches in dynamic studies, our work contributes two new methodologies: single-molecule fluorescence anisotropy (smFA) and the single-molecule neighboring density (smND) assay.[15,16] smFA brings a new dimension to SRM by enabling the observation of intracellular viscosity through the measurement of rotational mobility of fluorescent proteins. On the other hand, the smND assay quantifies protein oligomeric states directly within cells by analyzing the spatial distribution and density of individual protein molecules.[71] Utilizing the probability of neighbor density model, which accounts for factors like protein concentrations and photoactivation efficiency, the smND assay can distinguish between various oligomeric states by comparing experimental data against simulated distributions of protein assemblies. This method offers a more nuanced and accurate assessment of protein behavior in vivo, enabling the detection of transient or less abundant oligomeric states that are often overlooked in traditional in vitro studies.

2.3. General Guide to Choosing Appropriate SRM

While all SRM techniques provide resolutions beyond the diffraction limit, each has unique advantages and limitations (Table 1). STED microscopy offers higher spatial resolution than SIM, but its reliance on specialized fluorophores and higher laser intensities might pose challenges for some biological samples.[36−38] On the other hand, SIM is compatible with a wider range of biological samples and fluorophores, but it offers relatively lower spatial resolution compared to STED and SMLM.[40,41] SMLM techniques offer the highest spatial resolution among the three techniques but have relatively slower imaging speeds and require more complex data processing.[10,35] Additionally, SMLM techniques are sensitive to the blinking properties of fluorophores, which might affect the final image quality.[47]

Choosing the appropriate super-resolution microscopy method for studying biological processes or protein complexes depends on several factors, including the desired spatial resolution, imaging speed, sample preparation requirements, and the specific biological question being addressed. Below, we provide general guidelines on choosing the right SRM. (i) Spatial Resolution: If the study requires the highest possible spatial resolution, SMLM techniques are the most suitable options. (ii) Imaging Speed: For studies involving dynamic cellular processes or live-cell imaging, STED microscopy and SIM have faster imaging speeds, with frame rates of up to several frames per second, making them suitable for capturing dynamic cellular events. However, if the goal is to study dynamic interactions between proteins, combining SMLM with single-molecule imaging approaches provides the best spatial and temporal resolution. (iii) Sample Preparation and Compatibility: SIM is compatible with a wide range of biological samples and fluorophores, as it does not require specialized fluorophores or complex sample preparation procedures. STED microscopy and SMLM techniques often require specific fluorophores with suitable photophysical properties, which may limit their applicability to particular samples or experimental conditions. (iv) Biological Question: For studies focused on protein−protein interactions, molecular dynamics, or single-molecule behavior, SMLM techniques may be more appropriate. In contrast, for studies investigating larger cellular structures or organelles, STED or SIM may be more suitable.

3. CASE STUDIES: SUPER-RESOLUTION MICROSCOPY IN ACTION

SRM enables researchers to study protein complexes and assemblies in cells with unprecedented detail. In this section, we

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summarize key SRM applications to understand the roles of protein complexes and assemblies in the structural organization of organelles, expression regulation, cellular functions, and disease mechanisms (Table 2).

3.1. Structure Organization

The nanoscale architectures of organelles significantly govern protein function and cellular processes. Techniques such as STED, SIM, and SMLM, integral to SRM, have brought forth a nuanced understanding of the complex organization of chromosomes and membraneless organelles. They have unveiled the higher-order chromatin structures, offering fresh perspectives into the functional organization of the genome. These techniques have challenged traditional views, exposing the heterogeneity in nucleosome organization and presenting a three-dimensional view of chromatin loops (Figure 2a), both of which have significant implications for gene regulation.[72−75] Similarly, SRM techniques have also been employed to study the nanoscale organization of stress granules, processing bodies, and Cajal bodies. These membraneless organelles[78] are formed through liquid−liquid phase separation and play critical roles in cellular processes, including mRNA regulation, ribosome biogenesis, and stress responses. Stress granules are formed in response to cellular stress and have been suggested to act as hubs for mRNA regulation. Findings from these studies, including the core−shell structure of stress granules and the spatial organization of the protein Coilin within Cajal bodies, have provided novel insights into the assembly of these organelles, emphasizing the role of specific protein−protein interactions.[76,77,79]

3.2. Expression Regulation

SRM techniques also shed light on key aspects of cellular function, such as mRNA processing and localization, transcription regulation, and protein−DNA interactions. PALM, SIM, and STED has enabled researchers to visualize the spatial organization of the nuclear pore complex and the regulation of RNA polymerase II (Figure 2b), which play a crucial role in mRNA export, transcription initiation, and elongation.[24,31,80−83] SRM offers insights into the spatial organization and dynamics of transcription factors. These findings, including the discovery of segregated clusters of nucleosomes and RNA polymerase II, have brought new understanding to the operation of the transcription machinery.[12,72,84,85]

3.3. Cellular Functions

Protein assemblies play a critical role in cellular function. These complexes enable cells to coordinate and integrate a variety of signals, ensuring that cellular responses are tightly regulated and adapted to the changing environment. SRM techniques have been widely used to understand the working mechanisms of these protein assemblies.[13,118] For example, they have been used to study the organization and role of integrins at focal adhesions and synaptic adhesion proteins at synapses (Figure 2c). These investigations have yielded insights into their roles in adhesion, signaling, synapse formation, and function.[86−88] SRM has also made considerable contributions to understanding signaling networks and how they respond to external stimuli. Techniques such as PALM have been used to study receptor activation, dimerization, trafficking, and interactions (Figure 2d), which has enriched our understanding of molecular mechanisms underpinning receptor-mediated signal transduction and activation.[22,27,89−100]

Table 2. SRM Applications in Biophysical Research

Application	Biosystem	Protein Complex/Assemblies	Key Findings	ref.
Structure organization	Chromosome organization	Nucleosomes, cohesin complex, HP1	Revealed higher-order chromatin structures and heterogeneity in nucleosome organization	72−75
	Membraneless organelles	Stress granules, coilin, processing bodies, cajal bodies	Identified core−shell structure of stress granules; discovered nanoscale organization of coilin in cajal bodies	76−79
Expression regulation	mRNA processing and localization	RNA polymerase II, nuclear pore complex	Uncovered organization and dynamics of RNA polymerase II; visualized spatial organization of nuclear pore complex; found distinct domains within nuclear speckles	24, 31, and 80−83
	Transcription regulation	Mediator complex, nucleosomes, glucocorticoid receptor	Elucidated spatial organization and dynamics of transcription factors and regulatory proteins; studied the kinetics and spatial organization of TF binding	12, 72, 84, and 85
Cellular functions	Protein−protein interactions at cell adhesions	Integrins, synaptic adhesion proteins	Unveiled distinct nanoscale organizations of integrin subtypes; revealed the organization of synaptic adhesion proteins	86−88
	Signaling networks and responses	G-protein-coupled receptors, epidermal growth factor receptors, T-cell receptor	Visualized receptor activation, dimerization, nanoscale organization, trafficking, and interactions with other proteins	22, 27, 89, and 88−100
Disease mechanism	Alzheimer's disease	Amyloid-beta aggregates, tau protein, amyloid precursor protein processing machinery	Unveiled kinetics of Aβ aggregation and tau mislocalization; provided structural insights into the formation of Aβ plaques and neurofibrillary tangles	30 and 101−111
	Cancer	Epidermal growth factor receptor, nuclear chromatin	Investigated nanoscale organization of epidermal growth factor receptors in cancer cells; examined nuclear chromatin architecture of cancer cells	27, 52, 112, and 113
	Viral infections	HIV Gag protein, HSV-1	Visualized the organization of the HIV Gag protein; provided a detailed understanding of the HSV-1 replication process	21, 25, 29, and 114−117

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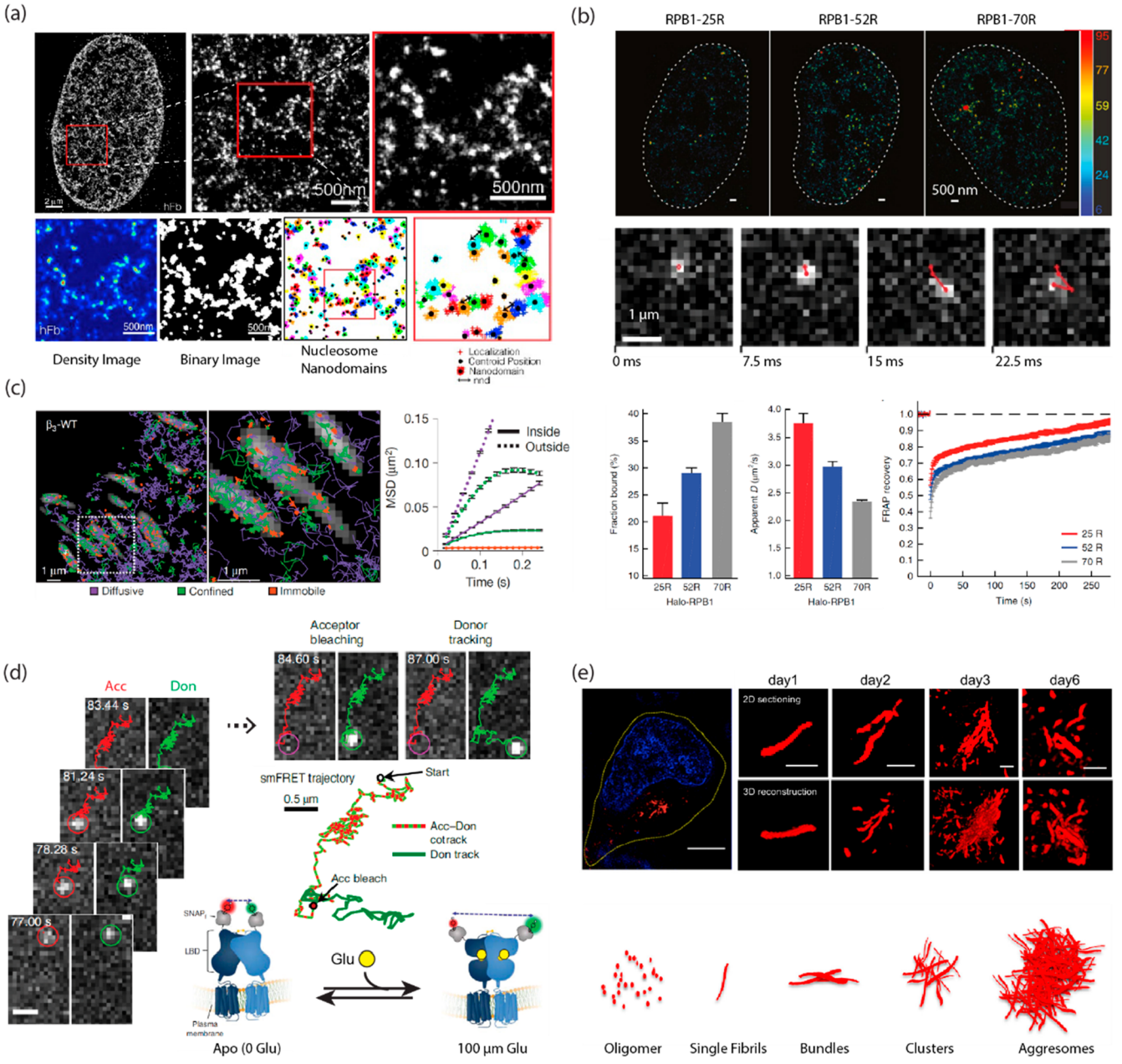

Figure 2. SRM Applications in Biophysical Research. (a) Top: STORM images display nucleosome organization in human fibroblast nuclei, revealing clustered nanodomains. Bottom: Density images highlight areas with varying H2B concentrations. Binary images are created after thresholding, with white regions representing H2B presence. Cluster identification algorithms group localizations into nanodomains based on proximity. Reproduced with permission from ref 72. Copyright 2015 Elsevier. (b) Top: 3D-PALM reconstruction images revealed the spatial densities of RNA polymerase (Pol) II catalytic subunit (RPB1) with various domains (25R, 52R, 70R) in the nucleus. Live-cell single-particle tracking assesses the molecular mobility of RPB1, including bound fraction, diffusion coefficients, and FRAP recovery rate. Reproduced with permission from ref 83. Copyright 2018 Springer Nature. (c) sptPALM imaging tracks β3-integrins in MEF cells, revealing focal adhesion dynamics. Mean square displacement analysis identifies three diffusion modes: diffusive, confined, and immobile. Reproduced with permission from ref 88. Copyright 2012 Springer Nature. (d) Using smFRET to analyze receptor dimerization upon ligand binding. It is demonstrated by tracking Sf-mGluR2 receptors labeled with donor (Don) and acceptor (Acc) fluorophores. Reproduced with permission from ref 100. Copyright 2021 Springer Nature. (e) SIM images depict the development of intracellular Aβ42(E22G) aggregates over time, illustrating five progression phases. Reproduced with permission from ref 107. Copyright 2019 American Society for Biochemistry and Molecular Biology.

3.4. Disease Mechanism

By investigating the assembly, aggregation, and localization of disease-related proteins using super-resolution microscopy, scientists can uncover crucial insights into pathological processes in various diseases, including neurodegenerative disorders, cancer, and viral infections.[33,34,119] One of the most studied protein complexes in neurodegenerative diseases is amyloid-beta aggregates. SRM techniques have been used to understand the kinetics of amyloid-beta aggregation and the organization of amyloid precursor protein processing machinery (Figure 2e).[30,101−111] In cancer research, SRM has offered insights into the nanoscale organization of the epidermal growth factor receptor, linking receptor clustering to activation. It has also provided insights into chromatin organization alterations

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associated with malignant transformation.[27,52,112,113] In terms of viral infections, SRM techniques have been applied to study the assembly and organization of viral protein complexes in viruses such as HIV and HSV-1, offering a more comprehensive understanding of the viral replication process.[21,25,29,114−117]

4. CURRENT CHALLENGES AND FUTURE DIRECTIONS

SRM techniques have revolutionized the field of cell biology by breaking the diffraction limit of light and allowing visualization of cellular structures and protein complexes at the nanometer scale. Despite these techniques' significant advancements and insights, several limitations persist in applying SRM to *in situ* studies. This essay discusses the major limitations of SRM, including fluorophore stability, effects of fluorescent tags on protein function, appropriate biological sample setup and physiological conditions, penetration depth, and the complexity and size of the data sets.

4.1. Fluorophore Stability and Effects of Fluorescent Tags on Perturbing Targeted Protein Function

In SRM, the choice of labeling strategy and fluorophore significantly influences the data qualities of *in situ* studies (Table 3). The choice between fluorescent proteins (FPs) or synthetic dyes requires a careful evaluation of their photophysical properties and impacts for the biological systems or processes.

Labeling methods, either through genetic or nongenetic engineering, affect the study's specificity and the potential impact on the protein function under examination. Genetic methods, employing FPs or tags like SNAP and Halo, provide precise targeting at the cost of possible protein disruption. Using non-natural amino acids (NAA) offers a potential solution to the limitations. These NAA can be site-specifically incorporated into proteins via genetic code expansion, allowing for covalent attachment of small and photostable synthetic dyes.[120] This strategy enables the direct labeling of proteins with minimal perturbation to their structure and functions. However, several challenges are associated with using NAA for SRM. First, the incorporation efficiency of NAA can be lower than that of natural amino acids, leading to incomplete labeling and potential misinterpretation of results.[121] Second, introducing NAA requires genetic manipulation, which can be more difficult in certain cell types or organisms. Third, the availability and cost of NAA and their corresponding synthetic fluorophores can limit widespread adoption. Despite these challenges, using NAA is promising to improve the specificity and minimal perturbation of protein labeling, ultimately enabling a more accurate investigation of protein function and interactions *in situ*.

Nongenetic engineering, including various immunolabeling strategies, offer flexibility and specificity, though challenges such as antibody availability can arise.[122,123] Direct immunolabeling involves attaching synthetic dyes directly to antibodies, ensuring high specificity and stability, but can be limited by the availability and quality of specific antibodies. Indirect immunolabeling, on the other hand, uses unlabeled primary antibodies and dye-coupled secondary antibodies, amplifying the fluorescent signal and allowing for greater flexibility in dye selection. However, this method introduces potential complexities, such as nonspecific binding and cross-reactivity, which can impact the accuracy of the results.[124,125] Furthermore, the PAINT (points accumulation for imaging in nanoscale topography) techniques, such as DNA-PAINT, offer advanced super-resolution imaging capabilities.[126−130] These methods use transiently binding fluo-

Table 3. Advantages and Limitations of Labeling Strategies

Labeling consideration				Fluorophore consideration			
Engineering type		Function perturbation	Specificity	Fluorophore	Brightness/photostability	Pros	Cons
Genetic	Genetic labeling	perturb	high	FPs	Lower/lower	Directly labeled on target	Require correct protein folding and maturation
	SNAP/Halo tags	perturb	high but may suffer incomplete dye conjugation	Synthetic dyes	Higher/higher	Directly labeled on target; great flexibility in dye selection	May encounter false signals from trace dye, need extensive cleaning
	NAA	less perturbation	high but may suffer incomplete dye conjugation	Synthetic dyes	Higher/higher	Directly labeled on target	Limited dye availability and SMLM compatibility
Nongenetic	Immunolabeling	Direct	No	Varies, determined by the antibody used	Synthetic dyes	Higher/higher	Can detect endogenous targets	Limited qualified specific antibodies; potential nonspecific binding and cross-reactivity; not compatible for live imaging
		Indirect	No	Varies, determined by the antibody used	Synthetic dyes	Higher/higher	Can detect endogenous targets; greater flexibility in dye selection	Higher potential of non-specific binding and cross-reactivity; not compatible for live imaging
	PAINT		No	High	Synthetic dyes	Higher/higher	Can detect endogenous targets; greater flexibility in dye selection; can repeatedly and selectively label the same target	Need appropriate binding affinity for the target structure

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rescent molecules, providing high precision and resolution suitable for visualizing structures at the nanoscale. DNA-PAINT, in particular, allows for multiplexed imaging using different DNA sequences but may involve slower imaging speeds and more complex sample preparation.

On the fluorophore side, FPs and synthetic dyes are the two major classes of fluorophores used in SRM.[131−133] Regarding fluorophore photophysical properties, synthetic dyes generally exhibit higher brightness, superior photostability, and narrower emission spectra, resulting in better signal-to-noise ratios and improved resolution in SRM than the FPs.[133−135] However, unlike FPs that naturally fluoresce after maturation within cells, synthetic dyes require extra conjugation steps via conjugating tags, such as SNAP and Halo tags, or antibody-based staining. This process can introduce challenges, including the need for thorough postlabeling cleanup.[136,137] The selection of the most suitable fluorophores is influenced by the specific requirements of the biological system, the labeling technique, and the inherent properties of the fluorophores themselves. For those delving into SRM, numerous reviews offer in-depth analyses of fluorophores' photophysical attributes, guiding researchers to identify the optimal dye for their experiments.[138,139]

Considering these factors, appropriate control experiments must be conducted to extract reliable scientific insights into protein function from super-resolution microscopy data. These may include (i) comparing the localization and function of the labeled protein to an unlabeled counterpart to assess the impact of the fluorescent tag on protein function and (ii) performing colocalization studies with known marker proteins or organelles to verify the subcellular localization of the labeled protein.

4.2. Biological Sample Setup and Physiological Conditions

Having a suitable biological sample setup and maintaining the physiological conditions of target proteins are crucial for obtaining reliable and biologically relevant data from SRM studies. One common challenge in SRM studies is balancing the need for a large number of detections while maintaining the native physiological environment. Overexpression of a fluorescently tagged protein can result in high data output, which is advantageous for reaching statistically significant conclusions. However, overexpression may also lead to artifacts, such as protein aggregation, mislocalization, or alterations in cellular processes due to an unnaturally high concentration of the protein of interest.[140] Endogenous expression of the protein of interest is more biologically relevant, as it ensures that the protein is expressed at physiological levels, avoiding the potential artifacts associated with overexpression. However, endogenous expression levels may be too low to yield a sufficient signal for super-resolution microscopy, making it difficult to visualize and analyze the protein of interest.[48] Thus, careful consideration must be given to the choice between overexpression and endogenous expression when designing SRM experiments.

To overcome the hurdle of obtaining SRM-applicable sample formats while maintaining them under their closest physiological setup, induced pluripotent stem cells (iPSCs) provide a promising option for SRM studies. iPSCs are adult cells reprogrammed to exhibit pluripotency, allowing them to differentiate into various cell types.[141] iPSCs offer several advantages for SRM studies. First, they can be generated from patient-derived cells, allowing for studying proteins in a disease-relevant genetic background.[142] This is particularly important when investigating the role of protein dysfunction in disease, as iPSCs can be used to model the disease *in situ*, providing a more physiologically relevant environment. Second, in combination with genome editing methods such as CRISPR, iPSCs can be genetically modified to express fluorescently tagged proteins at endogenous levels, ensuring that the protein of interest is present at physiological concentrations. This enables researchers to study proteins in their native cellular context without needing overexpression or ectopic expression systems.[143−145] This approach circumvents the potential artifacts associated with overexpression while still providing a detectable signal for super-resolution microscopy. Finally, iPSCs can be differentiated into various cell types, allowing researchers to study protein localization and function in various cellular contexts. This is particularly valuable when investigating proteins that play a role in multiple cell types or examining cell type-specific differences in protein function.

Choosing a proper biological sample setup and physiological conditions is critical for obtaining biologically relevant data from super-resolution microscopy studies. Balancing the advantages and limitations of overexpression and endogenous expression is essential, and iPSCs offer a promising avenue for addressing these challenges. Using iPSCs and ensuring physiological conditions, researchers can obtain more accurate and meaningful insights into protein function and localization using super-resolution microscopy techniques.[146]

4.3. Imaging Penetration Depth and Processing

The limited penetration depth of SRM techniques is a significant obstacle for *in situ* studies of thick biological samples, such as tissues or whole organisms. The imaging depth of STED or SIM is often restricted to a few micrometers, limiting their applicability for deep tissue imaging.[35] To overcome this limitation, researchers are developing new imaging modalities that can achieve super-resolution at greater depths. One such technique is lattice light-sheet microscopy (LLSM), which uses ultrathin light sheets to illuminate the sample, minimizing photobleaching and photodamage while allowing for high-speed, three-dimensional imaging.[57] This technique has been successfully applied to image various biological processes, such as cell division and neuronal activity, in thick samples and even living organisms. LLSM has the potential to provide crucial insights into protein interactions and functions in a more physiologically relevant context.[147,148] Adaptive optics (AO) is another approach being explored to overcome depth limitations in super-resolution microscopy. AO uses deformable mirrors or spatial light modulators to correct for optical aberrations caused by the inhomogeneous refractive index of biological samples, thus improving image quality and resolution at greater depths.[149] AO has been combined with various super-resolution microscopy techniques, such as STED and SMLM, to image cellular structures and protein distributions deep within tissues.[150]

Efforts are also being made to increase the imaging speed of super-resolution microscopy techniques, enabling the capture of dynamic processes and interactions in real time.[151] For instance, recent advances in high-speed cameras and image acquisition methods have allowed researchers to capture rapid cellular events and molecular interactions at the nanoscale level, providing new insights into cellular components' spatiotemporal organization and dynamics.[152] The large and complex data sets generated by SRM also pose significant challenges for downstream data analysis and interpretation. Future directions in this field should focus on the development of advanced

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computational tools and machine learning algorithms to automate image processing, segmentation, and analysis tasks.[153] These tools will facilitate the extraction of biologically relevant information from super-resolution microscopy data, accelerating the discovery of novel protein interactions and functions.

4.4. Integration with Other Imaging Modalities for Advancing Biophysical Studies

To fully leverage the advantages of SRM in biophysical studies, it is vital to blend these methods with other imaging techniques. Integrating SRM data with molecular simulations and structural models is a powerful approach to bridging the gap between structural information and *in situ* cellular context. Computational techniques, such as molecular dynamics simulations, can provide atomistic insights into the structural dynamics and interactions of protein complexes and assemblies.[154] Combining this information with experimental data from super-resolution microscopy allows researchers to generate detailed models of macromolecular structures and their dynamic behavior in the cellular environment.[15,155−157] Moreover, integrating super-resolution data with electron microscopy and X-ray crystallography-derived structures can help to refine the spatial organization of protein complexes *in situ* and reveal conformational changes associated with their cellular functions.[82,158] Machine learning (ML) and artificial intelligence (AI) have emerged as powerful tools for analyzing and interpreting complex biological data sets, including super-resolution microscopy data. ML and AI algorithms can be employed to automate image segmentation, feature extraction, and classification tasks, thus significantly reducing the time and effort required for data analysis.[159,160] Furthermore, deep learning approaches, such as convolutional neural networks (CNNs), have been successfully applied to denoise and enhance the resolution of SRM images, enabling the visualization of finer structural details and improving the accuracy of downstream analyses.[153,159,161] ML and AI can be integrated with other computational approaches, such as molecular simulations and structural modeling, to generate more accurate and comprehensive models of protein complexes and assemblies in their native cellular contexts. This integration holds great promise for advancing our understanding of the molecular mechanisms underlying cellular functions and disease states.

This fusion not only enriches the informational value of the data but also optimizes the opportunity to create more thorough models of protein complexes and their dynamic actions within the cell environment.[71,162] Recent progress in correlative light and electron microscopy has made it possible to visualize fluorescently labeled proteins and cellular ultrastructure concurrently, hence offering a potent tool for exploring the structure−function relationships of macromolecular complexes within their natural cellular environments.[163] Similarly, the integration of SRM with cryo-electron tomography can connect high-resolution structural data acquired from isolated protein complexes with the functional context that *in situ* imaging provides.[164] X-ray crystallography and nuclear magnetic resonance spectroscopy are two synergistic techniques that are particularly effective in demonstrating protein complexes' conformational dynamics and functional states *in situ*.[165] Raman imaging is another powerful technique that can be integrated with SRM to provide additional information on the chemical composition and molecular interactions within cells.[166] By integrating data from these diverse imaging techniques with SRM, it is possible to achieve a more holistic view of the spatial arrangement, conformational shifts, and functional states of protein complexes in their natural setting, hence supporting comprehensive biophysical studies.

5. CONCLUSION

Single-molecule biophysics, a blossoming field, marries super-resolution microscopy techniques with sophisticated computational methods to examine protein complexes and their spatial arrangement within cells. By directly observing and measuring protein interactions and structural organization, scientists can delve deeper into cellular operations, molecular mechanisms, and the functional effects of protein assemblies.

Despite the significant progress in super-resolution microscopy and single-molecule imaging, several obstacles remain. One major challenge is the development of robust and versatile fluorophores with improved photophysical properties, such as enhanced brightness, photostability, and switching properties, for the quicker and more precise imaging of dynamic cellular activities. Creating novel imaging modalities and computational strategies to simultaneously access various cellular components and pathways is necessary, enabling a comprehensive understanding of protein complexes and assemblies.

Another hurdle involves improving sample preparation and labeling techniques to reduce potential artifacts and disturbances to the natural cellular environment while boosting the signal-to-noise ratio and imaging resolution. This involves devising new genetically encoded tags and labeling methods to enable specific and efficient labeling of target proteins without disrupting their natural structure or function.

Finally, with the ever-increasing volume and complexity of super-resolution microscopy and single-molecule imaging data, new computational tools and algorithms will be necessary to glean meaningful insights from these data sets. This includes creating machine learning and AI-based methods for automated image analysis, segmentation, and quantification, as well as integrating super-resolution microscopy data with other omics data sets like transcriptomics, proteomics, and metabolomics. This would enable a more comprehensive understanding of cellular processes and functions, laying the groundwork for developing innovative therapeutic and diagnostic tools for various diseases.

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Author Contributions

H.C., G.Y., and M.-H.W. contributed equally as first author. All authors contributed to the content and revisions of the review. H.C., G.Y., M.-H.W., K.N.B., Y.Z., and P.-S.H. prepared the figures and provided fruitful discussions and helpful feedback. H.C., G.Y., M.-H.W., K.N.B., and T.-Y.C. wrote the manuscript.

Notes

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■ VOCABULARY TERMS

smFA, single-molecule fluorescence anisotropy is a technique that measures the rotational mobility of fluorescent molecules at the single-molecule level, providing insights into molecular dynamics, interactions, and conformations; smND, single-molecule neighboring density is a method that quantifies the spatial distribution and density of individual protein molecules within cells, enabling the identification of different oligomeric states based on their localization patterns; it leverages super-resolution microscopy to offer a precise analysis of protein−protein interactions and assembly processes in situ; LLSM, lattice light-sheet microscopy is an advanced imaging technique that uses a thin sheet of light to illuminate a small section of the specimen at a time, minimizing photodamage while capturing high-resolution, three-dimensional images of living cells; MINFLUX, MINFLUX is a super-resolution microscopy technique that combines the principles of STED microscopy with the localization accuracy of single-molecule techniques, achieving nanometer-scale resolution. It minimizes the number of photons required to precisely localize fluorescent molecules, enabling detailed imaging of molecular dynamics within cells; 4Pi microscopy, 4Pi microscopy is a super-resolution imaging technique that significantly improves axial resolution by using two opposing objectives to simultaneously focus light on the same spot from both sides of the specimen. This configuration effectively doubles the numerical aperture in the axial direction, enhancing the detail of three-dimensional structures; NAA, non-natural amino acids are artificially synthesized amino acids that can be incorporated into proteins at specific sites, allowing for the precise attachment of functional groups or fluorophores; iPSC, induced pluripotent stem cells are a type of pluripotent stem cell generated directly from adult cells through the introduction of specific genes, enabling them to differentiate into a wide range of cell types. They are a powerful tool for studying disease mechanisms, drug development, and the potential for regenerative medicine.

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