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Optical Nanoscopy for Elucidating Nano–Bio Interactions: A One Health Perspective

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Advances in nanotechnology have led to an increased adoption of nanoengineered materials in several areas (including, but not limited to, medicine, food, cosmetics, electronics, and energy), implying their direct or indirect contact with the human body or their dispersion in the environment. In the "Era of One Health", the concept of exposome is becoming central, thereby demanding for a deeper understanding of the relationships and interactions of "stressors" in the environment and their biological effects. The fast evolution of super-resolution microscopy (also called optical nanoscopy) greatly contributes to the characterization of biological structures and cellular dynamics at the nanometer scale, helping to unveil the intricate nature of the interactions occurring between nanomaterials and biological systems (nano–bio interactions), which need to be elucidated in view of a safe and sustainable application of nanotechnology. In this review, the contribution of optical nanoscopy (with a focus on far-field fluorescence-based techniques) to the characterization and understanding of nanomaterial interactions with biological systems at the single-particle and molecular scale is enlightened, with an accent on their impact on human and ecosystem health.

1. Introduction

In recent years, the "nanotechnology revolution" has led to new engineered nanomaterials for applications in many different fields including medicine, agriculture, consumer goods, food production, electronics, industrial additives, and fuels,[1–10] which have attracted significant financial and research investments.[11] At present, however, there is still limited knowledge on the interactions between nanomaterials and biological systems (nano–bio interactions) and on the short- and long-term impact of nanomaterials on human health. Indeed, concerns have been raised regarding the negative consequences on human and ecosystem health from unwanted or unexpected exposure to nanomaterials.[12–14] In the current "Era of One Heath",[15] where greater attention is posed to human, animal, and environmental safety as a whole, it is fundamental to deepen the knowledge on engineered nanomaterials and their derivatives, and it is likewise essential to provide a mechanistic understanding of nano–bio interactions at a systemic, cellular, and molecular level, in view of a safe and sustainable design of nanomaterials, as advocated by the so-called "safe-by-design" paradigm (SbD).[16] SbD has emerged as a cornerstone of nanomaterials design, requiring potential toxicity risks to be

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evaluated earlier in the innovation cycle. Therefore, the development of innovative nanoscale research methods capable of investigating nanomaterials–biosystems interactions is essential and represents a scientific priority.

As the reduction in size from the micro- to the nano-scale can substantially change or enhance the physical, chemical, and biological properties of a material, great emphasis is now placed on systematically assessing and understanding the relationship between nanomaterials structure and their biological activity.[17–20] These studies are relevant not only in the nanomedicine field for the development of diagnostic tools, targeted drug delivery systems, and regenerative medicine approaches, but also in other fields involving the direct contact of nanomaterials with the human body and the environment. Aside the conventional analytical methods for the characterization of the physicochemical properties of newly synthesized materials,[21] several imaging-based methods are being routinely used for the characterization of nano–bio interactions. These include dark field scattering microscopy,[22] high-resolution label-free imaging microscopy,[23,24] (including atomic force microscopy, AFM,[25–27] and electron microscopy, EM[28,29]) and multicolor widefield and confocal light scanning microscopy (CLSM).[30,31] Despite the extensive use of fluorescence microscopy for fast and real-time imaging of dynamic processes, diffraction-limited resolution hampers its use for the investigation of sub-diffraction architectures and nano–bio interactions at the molecular scale. On the other hand, the ultra-high resolution of AFM and EM can unveil details on nano–bio interactions inaccessible to other methods. Although transmission electron microscopy (TEM) continues to represent the gold standard for the imaging of nanomaterials in a biological setting, particularly for what concerns the association of nanoparticles (NPs) to subcellular structures, the technique lacks throughput and is poorly informative of dynamic processes, as it is disruptive for the sample and requires labor-intensive sample preparation.

Recent years have witnessed an increased application of super-resolution microscopy (SRM), also known as optical nanoscopy, for the investigation of the structure, dynamics, and function of advanced materials. Taking advantage of the considerably high spatial resolution achievable by optical nanoscopy, far below the diffraction limit (down to tens of nanometers), it is possible to assess nanoscale structural features of nanomaterials, undetectable with diffraction-limited optical fluorescence microscopy, and only accessible to scanning probe and EM techniques. Although near-field optical nanoscopy is mostly applied in the field of materials science for its great potential in the characterization of optical properties and sub-diffraction features of advanced materials, its use in the label-free imaging of biological samples at the nanoscale is increasing, and very recent literature reports its expansion toward the imaging of nano–bio interactions in aqueous environment and in living biological samples.[32] On the other hand, optical nanoscopy in the far-field regime has showcased its potential in the investigation of materials properties (polymers, DNA origami, lipid-based and self-assembled materials, etc.)[33] and in the quantitative analysis of structure–activity relationships at the nanometric scale.[34] Moreover, the multicolor ability and molecular specificity of nanoscopy labeling, along with the single molecule detection sensitivity of nanoscopic fluorescent imaging, has enabled the accurate visualization of labeled cellular structures and the characterization of their dynamics within their physiological context at high spatio-temporal resolution, both in vitro and in vivo. Taken together, the progress in sub-diffraction imaging of both nanomaterials and biological samples has opened the way to the study of the fundamentals of nano–bio interactions at the molecular scale, thus unveiling the journey of nanomaterials within living organisms, including their intracellular fate and biodegradation.

The purpose of this work is to critically review the recent literature that has contributed to elucidate the mechanisms of nanomaterials–biosystems interactions with far-field fluorescence nanoscopy approaches, focusing on nanomaterials that enter in direct contact with the human body, as those employed in biomedicine. Reference will also be made to nanoscale materials proliferated into consumer goods, in light of the raising concerns for their potential detrimental impact on human and environmental health.[35] An overview of the most relevant studies performed over the last decade is presented in **Table 1**.

2. SR Techniques: An Outlook

In order to get super-resolution bio-imaging of both fixed and living cells and organisms, a number of fluorescence nanoscopy systems have emerged,[36–38] pushing the resolution limit down to a few tens of nanometers. Far-field techniques represent the most widely applied family of microscopies for the characterization of biological systems, including: i) the deterministic functional SRM techniques, such as stimulated emission depletion (STED),[39] ground state depletion (GSD),[40] reversible saturable optical fluorescence transitions (RESOLFT),[41] structured illumination microscopy (SIM);[42] and ii) the stochastic single-molecule localization microscopy (SMLM) techniques[43,44] including stochastic optical reconstruction microscopy (STORM),[45] photoactivation localization microscopy (PALM),[46] point accumulation for imaging in nanoscale topography (PAINT),[47] and super-resolution optical fluctuation imaging (SOFI)[48] (**Figure 1**). This classification is challenged by the newly developed minimal fluorescence photon fluxes nanoscopy (MINFLUX)[49] and MINSTED[50] techniques, which can be regarded as hybrid systems combining the stochastic and deterministic nature of the basic techniques.

Label-free super-resolution approaches (LFSR), including reflection-based imaging methods (i.e., reflectance-SIM[51]), subtraction approaches (i.e., intensity-weighted subtraction microscopy (IWS)[52]), pump–probe microscopy[53,54] and photoacoustic imaging (PAM),[55] have emerged as prominent techniques for imaging sub-diffraction features of naïve molecules and nanomaterials, as extensively reviewed by Friedrich et al.[56]

Last, reference has to be made to image scanning microscopy (ISM)[57,58] and re-scan confocal microscopy (RCM).[59] While only offering a moderate enhancement in lateral resolution by effectively halving the diffraction limit, they significantly improve the signal-to-noise ratio (SNR) and sensitivity with minimal modifications to conventional confocal microscopy systems. These techniques have been applied in both fluorescence and label-free imaging, expanding their applicability in a variety of imaging scenarios.[60–62] Furthermore, their versatility makes them valuable as integrative approaches to enhance the performance of conventional nanoscopy techniques, as demonstrated by the

Table 1. Salient applications of optical nanoscopy for the characterization of nano–bio interactions.

		Nanosystem type	Technique	Notes	Refs.
Diagnosis and therapy	Bio-nano sensors	Ultrasmall fluorescent core–shell aluminosilicate NPs	TIRFM, STORM	Ratiometric pH sensor maps at a resolution above the optical diffraction limit	[106]
		DNA-based molecular switch	STORM	Visualization of the specific molecular interactions between a DNA-based molecular switch and transcription factor protein NF-κB in the cytosol	[109]
		Chimeric-locked nucleic acid-DNA sensor	ExM	Imaging of cell-nanosensor interaction at a resolution down to 70 nm	[111]
		Dual-color core–shell silica nanosystems	SIM with SIM^2 processing	SIM^2 imaging of ultrabright dual fluorescent core–shell silica NPs within the highly heterogeneous cellular environment	[107]
		Caspase-sensitive nanoaggregation fluorescent probe	3D-SIM	Visualization of in situ self-assembling of nanoaggregates to be used as small-molecule probes for imaging caspase activity in vivo	[110]
	Nanosized gene/drug delivery systems	Polystyrene (PS) beads	Two-color dSTORM	STORM-based technology to probe NP interaction with the cellular machinery to obtain quantitative data on the internalized nanoparticle size, number, and location	[112]
		Single-layer graphene (SLG)	STORM	SMLM and cluster analysis to quantitatively analyze the mechanisms of cell adhesion on graphene substrates	[122]
		Exosomes	Simultaneous PALM/STORM	Observation of the interaction between cancer-derived exosomes and normal cells with a resolution of exosomes down to 70 nm	[113]
		Exosomes	STORM	Nano-sized tracking of exosomes and exosomal content dynamics in living recipient cells at a resolution down to 20–50 nm	[114]
		Polyplexes	Two-color dSTORM	Imaging of structure, molecular composition, and stability of single oligonucleotide polyplexes with nanometer accuracy	[115]
		Nanostructured lipid carriers	dSTORM	Study of the composition of the biomolecular layer on the surface of nanostructured lipid carriers, before and after the crossing of an *in vitro* model of the blood–brain barrier	[144]
	Nanomaterials for regenerative medicine	Nanopatterned Ti- or AuPD- lines	PALM	Study of the influence of the geometric organization in modular integrin cluster formation and cell adhesion	[117]
		RGD functionalized PEG-based hydrogels	dSTORM	Analysis of how RGD concentration in hydrogels affects the individual cell surface receptors (integrin $\alpha5\beta1$) localization and clustering	[118]
		Peptide amphiphile (PA) nanofibers	STORM	Study of the exchange mechanism involving monomers or small clusters of molecules in peptide amphiphile nanofibers	[121]
	Nanomaterials with antimicrobial activity	Au NPs	SIM, STORM	Ultrasmall NP uptake by bacteria (*E.coli*)	[127]
		Ag NPs	SMLM	Reorganization of H-NS proteins in *E.coli* induced by Ag NPs	[128]

(*Continued*)

Table 1. (Continued)

	Nanosystem type	Technique	Notes	Refs.
Nanomaterials for theragnostics	Fluorescent carbon dots (FCDs)	STED	Sub-resolution visualization of highly biocompatible fluorescent cDots at a resolution down to 30 nm within the intracellular environment in live cell STED imaging	[133]
	Core–shell ND@MSN	STED	Study of drug release within cells by STED microscopy	[134]
Nanotoxicity and bioaccumulation	Silica NPs	3D STED	Study of NP delivery (administered vs. internalized dose) and NP toxicological profile	[151]
	Nanoceria	SIM/STED	Investigation of nanoceria neurotoxicity on neural stem cells	[155]
	Nanoplastics	STED	Detection of nanoplastics of different shapes and compositions in whole animal tissues, down to 50 nm resolution, without compromising sample integrity	[161]

combination of ISM with STED microscopy to improve STED capabilities for the imaging of live and thick specimens.[63]

The present review will restrict the focus to far-field fluorescence nanoscopy. A detailed explanation of the physics underlying such methodologies can be found in the comprehensive reviews by S. Hell,[41,64,65] Requejo-Isidro et al.,[66] and E. Betzig.[67] In the following, we will instead detail the application of such methodologies to dissect the interactions of nanosized materials with biological matter and their potential accumulation in biological systems.

Several SRM approaches have been validated for the investigation of synthetic nanomaterials.[68–75] Indeed, SRM can be regarded as a robust family of microscopy techniques applied to chemistry for the characterization of nanomaterials in vitro in terms of size, functionality, composition, and surface chemistry to determine multiparametric inter-intraparticle

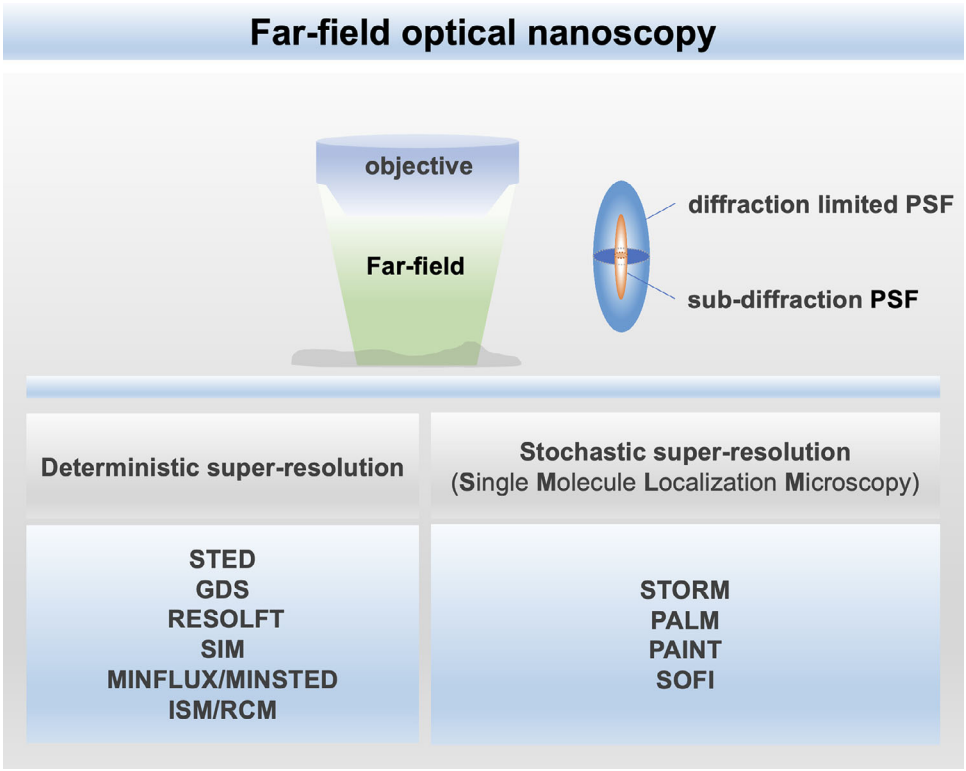

Figure 1. Graphical representation of optical nanoscopy techniques in the far-field regime.

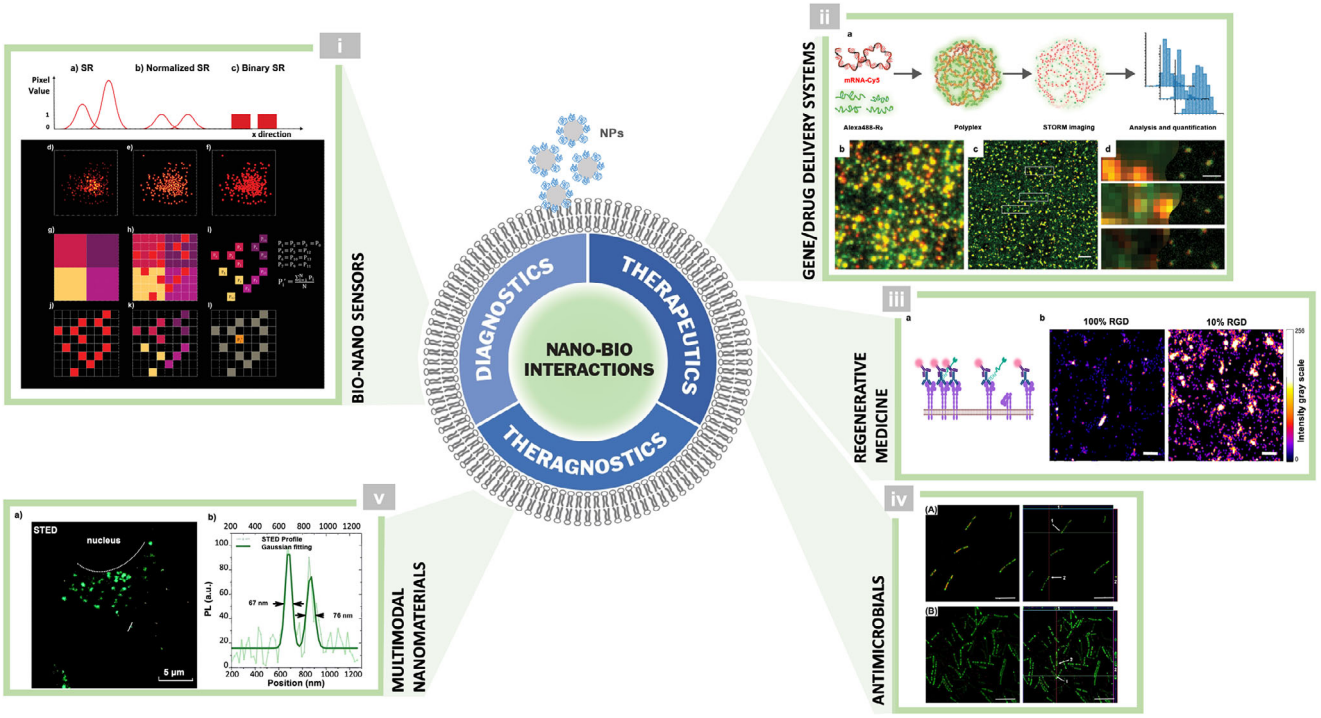

Figure 2. SRM approaches to characterize nanomaterial-based diagnostic, therapeutic, and theragnostic tools. i) Bio-nano sensors. Imaging of ultrasmall fluorescent core–shell aluminosilicate NPs (pH sensors) by a combination of TIRF imaging and STORM-based localizations. Adapted with permission.[106] 2021, Wiley. ii) Gene/drug delivery systems. Imaging of polyplexes by dSTORM. Adapted with permission.[115] 2019, American Chemical Society. iii) Regenerative medicine. Imaging of integrin $\alpha5\beta1$ clusters on the surface of hMSCs bound to RGD hydrogels by dSTORM. Adapted with permission.[118] 2020, American Chemical Society. iv) Antimicrobials. Imaging of the uptake of red fluorescent Au NPs in green fluorescent *E. coli* by SIM. Adapted with permission.[127] 2022, Wiley. v) Theragnostic systems. STED live cell imaging of carbon dots. Adapted with permission.[133] 2014, Royal Society of Chemistry.

heterogeneity. Additionally, they can be successfully applied to a biological setting to study, for example, the target biomarkers of such synthetic nanomaterials in a biological environment, as well as to analyze the interactions at the boundary between synthetic matter and the biological milieu, with the ultimate aim of optimizing the design and effectiveness of nanosystems. As such, SRM can complement conventional analytical[76] and biochemical techniques[77] available for nanomaterials characterization, and work synergistically with other imaging techniques including confocal microscopy,[78] scanning probe microscopies (e.g., AFM[79]) and EM[80–82] to track nanomaterials intracellular localization and their bio-interaction within cells.

3. SRM Imaging for Applications in Biomedicine

A wide variety of nanosystems have found increasing relevance in biomedicine, with applications including diagnostics (with emphasis on intracellular sensing), therapeutics (e.g., gene/drug delivery systems, nanomaterials for regenerative medicine and nanoantimicrobials), as well as novel systems combining diagnostic and therapeutic features (theragnostics) (as reported in **Figure 2**). While referring the reader to dedicated reviews concerning the physicochemical properties of polymeric,[83] ceramic,[84] and metal nanosystems for biomedical applications,[85,86] in the present Section, we will focus on the use of SRM for characterizing nanomaterials at the interface with biological matter. In such a setting, the contribution of physisorbed biomolecules (the so-called protein corona) to nanomaterials surface cannot be neglected, as it deeply influences recognition mechanisms by the host, ultimately affecting nanomaterials performance. Hence, in the following, the use of SRM to investigate the protein corona will also be reviewed.

3.1. SRM for Imaging Nanodiagnostic Tools

The use of nanostructured materials in sensor manufacturing[87–89] has aided the development of sensing devices which find application in the food industry (food safety and quality check[90–92] for the detection of food additives or the presence of microorganisms[93]), for environmental monitoring and protection (to measure, monitor and remove pollutants in the environment[94–97]), and for medical and biological applications (containment of infections,[98] viral propagation[99,100] and in vitro and in vivo diagnostics[101]).

Nanoscale sensors are nanotechnological tools designed to detect and respond to specific physical, chemical, or biological stimuli at the nanometric scale. Although optical, magnetic, and electrochemical nanosensors are the prevalent type of nanosensors used in medical diagnostics, fluorescent nanosensors are emerging technologies that play a significant role for the early

detection and diagnosis of several diseases, including cancer, viral infections, arthritis, cardiovascular and inflammatory diseases, as well as for therapeutic drug monitoring.[102] They enable the optical sensing of biochemical parameters and the detection of disease-specific biomarkers in real-time. In light of the fast evolution of the field of intracellular bio-nanosensors, it is becoming increasingly important to validate their biocompatibility, functionality, and dynamics at the nanoscale, directly within the cellular environment, where the conditions are considerably different from those found in vitro.[103] Some recent works demonstrate the potential of SRM for the characterization of biosensing interactions at the single molecule level, enabling the detection of chemical reactions and rare events inaccessible to ensemble measurements.[104,105] Indeed, SRM approaches have been employed to validate the activity of both nanomaterial- and biomolecule-based nanosensors for diagnostic aims directly within cells.

Several nanomaterial-based sensors have shown promise in early cancer detection, with the final aim to enhance patient long-term survival. Among these, nanosensors that monitor biologically relevant metabolic parameters, like pH, oxygen, and temperature, play a key role. In a recent work, Lee et al. developed pH-sensing probes, merging the information from total internal reflection fluorescence microscopy (TIRFM) based ratiometric sensing with that provided by STORM imaging (number of NPs per vesicle, vesicle size, and vesicular motion), thereby extending the ratiometric pH sensor maps resolution above the optical diffraction limit (Figure 2i). This approach efficiently returned spatial and functional information of NPs, such as their intracellular mapping and the study of their spatio-temporal processing in living cells, in view of their application in nanomedicine.[106] In a similar fashion, Lattice SIM with SIM^2 processing (an image reconstruction algorithm doubling the resolution of classical SIM), was successfully adopted by Ramirez-Morales and colleagues to obtain sub-diffraction details of the intracellular spatial distribution of ultrabright dual fluorescent core–shell silica NPs, designed as potential nanoscale intracellular pH indicators of metabolic parameters directly within cells.[107]

Inflammation and apoptosis represent other targets of interest for nano-enabled diagnostics. The nanometric resolution of STORM imaging has been exploited by Glab et al. to dissect the intracellular functionality and fate of DNA-based nanosensors, which represent a powerful tool for early disease detection.[108] The authors demonstrated that STORM enables the direct visualization of the specific molecular interactions between a DNA-based molecular switch (Nanoswitch$_{\text{NF-}\kappa\text{B}}$) and transcription factor protein NF-κB (a known player in inflammation development and progression) in the cytosol, confirming the evidence obtained with Förster resonance energy ransfer (FRET) microscopy that the Nanoswitch$_{\text{NF-}\kappa\text{B}}$ molecules deployed in the cytosol effectively target NF-κB.[109] The activity of caspases, highly conserved proteins involved in cell death and inflammation, has also been investigated by SRM. 3D-SIM has been used to study the in situ self-assembling of nanoaggregates, designed as nanoprobes to image the in vivo activity of caspases. 3D-SIM revealed the nanoaggregation of fluorescent probes in situ in both apoptotic cells and *ex vivo* tumor samples, demonstrating the in vivo applicability of such self-assembled NPs for monitoring the tumor response to chemotherapy.[110]

SRM has also been proven to be effective in the context of the diagnosis of infective diseases. Amodio et al. used super-resolution expansion microscopy (ExM) for the real-time detection and nanoscale imaging of human immunodeficiency virus type 1 ribonucleic acid (HIV-1 RNA) infection within cells, using an engineered chimeric locked nucleic acid (LNA)-DNA sensor, selectively targeting a 20-bases sequence transcribed from the HIV-1 promoter.[111] Their study demonstrates the potential of SRM to perform nanoscale resolution imaging of the interactions of the biomolecule-based nanosensors with the cell to dissect the still poorly elucidated mechanisms of intracellular trafficking and functionality.

Even though the potential of SRM for imaging of diagnostic tools at the nanometric level is still at its early steps, these literature studies sharply highlight the contribution of sub-diffraction resolved imaging in deciphering nano–bio interactions, a pivotal step for the functional characterization of nanosensor performance within the native biological framework.

3.2. SRM for Imaging Nanostructured Therapeutics

3.2.1. Nanosized Drug Delivery Systems

The application of SRM to the study of nanomaterials for drug delivery has accelerated the understanding of the mechanisms underlying nano–bio interactions for a wide class of nanomaterials, including NPs, dendrimers, liposomes, micelles, and many other nano-vesicular carriers. Therefore, an extensive study on the mechanisms dictating the interaction of nanocarriers with living matter, such as nanomaterial uptake, transport, intracellular localization, and fate is essential to improve their diagnostic and therapeutic potential and to reduce off-target effects. By covering the gap between confocal and EM, multicolor SRM is now more frequently being adopted to monitor multiple interactions between combinatorial nanosized delivery systems, such as nanomedicines and cellular structures.

In this regard, a comprehensive analysis of different NPs commonly used as nanomedicine carriers demonstrated that the nanometric axial sectioning of nanoparticle clusters, obtained by two-color direct STORM (dSTORM) imaging, could probe NP interactions with the cellular machinery. These include the effective size, the number, and the precise positioning within clusters of internalized NPs, details otherwise unresolvable with conventional confocal microscopy, where NPs appear larger due to the diffraction limit.[112]

Another relevant class of nanomaterials is represented by natural vesicular nanocarriers, also known as exosomes, which are being widely investigated for personalized medicine applications. Their small size ranging from 30 to 150 nm in diameter and their unique physicochemical/biochemical properties make them promising nanotechnology tools for the design of advanced nano-theragnostic platforms for targeted drug/gene delivery. Also, exosomes may overcome some of the limitations of artificial NPs (i.e., toxicity, immunogenicity, and short-term circulation) and can carry and deliver nucleic acids and drugs, as well as strategically incorporate other NPs for diagnosis, drug delivery, imaging, and photothermal therapy. The nanoscale resolution imaging of cell–exosome interactions was explored by Chen

et al., in the perspective of elucidating the mechanisms underlying exosome-mediated cancer metastasis, an essential step toward the design of targeted exosome-based strategies.[113] Simultaneous dual-color PALM/STORM imaging of two receptors labeled with photo-switchable probes on the exosome membrane captured the interaction between cancer-derived exosomes and normal cells with a resolution down to 70 nm (far below the organelle level) and resolved closely packed exosomes, indistinguishable with conventional TIRFM, thereby obtaining structural information on exosome dynamics, content (i.e., DNA, proteins, and miRNAs) and function in tumoral pathogenesis. The same authors adopted single and dual-color SMLM for the nanosized tracking of exosomes and exosomal content dynamics in living recipient cells, demonstrating the motion of internalized exosomes into intercellular filamentous structures to transfer cellular contents between cells and the mechanism of cargo encapsulation as a way to prevent its enzymatic degradation.[114] The achieved level of imaging accuracy is expected to be instrumental for the future application of engineered exosomes in targeted drug delivery scenarios.

Multicomponent nanometric assemblies, such as polyplexes, represent another promising class of gene delivery systems. The poor knowledge at nanoscale precision of structural information (composition and modifications) that greatly influence polyplex activity has long limited their rational design. By using super-resolution dual-color dSTORM, analysis of the structure and molecular composition of individual CPP-mRNA polyplexes on a single particle basis could be achieved (Figure 2ii). Since dSTORM accesses single-molecule localization, quantitative data on stoichiometry and distribution of mRNA within the polyplexes could be acquired, representing a milestone in the field of gene delivery.[115]

3.2.2. Regenerative Medicine

Understanding the complex interplay between cells and biomaterial substrates is crucial in the development of novel tissue engineering and regenerative medicine strategies. By understanding the dynamic interactions between cells and scaffolds at the nanoscale, researchers can develop biomaterials that best support tissue regeneration, by providing cells with optimal biochemical and mechanical stimuli, that is, via functionalization with biomolecules that mimic the components of the native extracellular matrix (ECM) to facilitate cell adhesion and migration.[116]

Cell adhesion is mediated by the formation of focal adhesion sites, a complex network of proteins that regulate cell adhesion and function and which, in turn, are affected by surface topography. SRM techniques give an accurate and deep understanding on how cells interact with surface nanotopography. As an example, Changede et al. demonstrated that cellular processes are influenced by the geometry and rigidity of ECM. The authors applied the PALM technique to visualize several integrin nanoclusters depending on RGD-patterned surfaces (with different line widths and arrangements), evidencing a width minimum threshold of 40 nm as permissive of cell spreading and focal adhesion formation.[117]

Besides topography, ligand density is another factor affecting the cell-material interface. dSTORM technique was applied to precisely determine the molecular-scale interaction between functionalized hydrogels (with varying amounts of RGD moieties) and integrin $\alpha 5\beta 1$ receptors (Figure 2iii).[118] Results showed that the amount of membrane integrin clusters was indirectly proportional to the amount of RGD binding sites and that the density of the ligands partially influenced cell adhesion and migration. Abuzineh et al. proposed a microfluidics-based SRM platform to investigate hematopoietic stem cells (HPSC) homing under shear stress, which is mediated by the interaction between selectin ligands expressed on HPSCs (i.e., CD44) and selectin expressed on endothelial cells.[119] This approach enabled the capture and quantification of the spatial reorganization of selectin ligands that result from the rolling of cells over E-selectin in the presence of external shear force, suggesting a complex and dynamic behavior that challenges the relatively simple model of selectin-ligand interactions used to explain HPSC homing. SRM was also applied to an in vitro model of osteoclastic bone resorption. By applying STED to osteoclasts cultured on a nanosized bone-like surface, it was possible to capture the early stages of bone resorption, visualize actin micro-domains above resorption pits, and establish a vesicular transport mechanism utilizing polymerization of branched actin.[120]

SRM was also applied to the characterization of nanostructured scaffolds,[121] as in the case of peptide amphiphile (PA) nanofibers, a biomaterial used in several tissue engineering applications (i.e., bone, cartilage, neuronal regeneration) as a bioactive artificial ECM for cell signaling. Spatial details of fluorescently labeled individual nanofibers were analyzed by STORM, successfully capturing the transfer mechanism of monomers and small clusters, with results matching those obtained by cryoTEM. Also in the case of graphene, a nanomaterial finding increasing application in tissue engineering, SMLM, and cluster analysis were applied to quantitatively analyze the mechanisms governing the affinity and the preferential adhesion of cells on graphene substrates, elucidating the molecular events at the cell/material interface.[122]

3.2.3. Nano-Antimicrobials

With the rise of antibiotic-resistant bacteria posing a significant threat to public health,[123] there has been growing interest in exploring alternatives to antibiotics, particularly those involving noble metals.[124,125] While the mechanism of action of antibiotics is well-established, the interaction of bacteria with antimicrobic nanomaterials remains less understood. Most nanomaterials act by membrane disruption (both physical and chemical), but they can also induce the arrest of cell division (as for Ag NPs), catalytic or ionic killing (for several inorganic NPs), and the disruption of the electron transport chain (e.g., for alkaline magnesium oxide films). The antibacterial effect is generally attributed more to the release of antibacterial ions rather than NP uptake, largely due to the structural resistance provided by the bacterial cell wall.[126] Given the small size of bacteria, traditional diffraction-limited microscopy techniques are insufficient for studying the bacteria–nanomaterials interface, necessitating higher-resolution methods like SRM and EM. However, while EM is a well-established technique in this field, fewer studies report the application of SRM. This could be related to challenges in sample preparation

(observing nanoscale interactions between antimicrobial materials and bacteria requires specific preparation protocols to preserve biological structures and minimize artifacts), and to the fact that antimicrobial mechanisms involve various factors (oxidative stress, membrane damage, interactions with proteins and DNA), making it difficult to capture them using a single technique. In an interesting study by Bialas and colleagues, the uptake of ultrasmall gold NPs and their cytosolic distribution in Gram-negative *E. coli* was analyzed using a combination of different optical microscopic techniques (CLSM, SIM, STORM) and integrating imaging data with those obtained by flow cytometry analysis that is characterized by a more robust statistical assessment, enabling the examination of thousands of cells from the same sample in a single run.[127] SRM was also used by Alqahtany et al. to investigate and quantify the antimicrobial effect of Ag NPs at the molecular level, revealing that Ag NPs cause a nanoscale reorganization of H-NS proteins in *E. coli*, forming denser clusters at the bacterial center. The work also highlighted the role of Ag^+ release in the overall antimicrobic effect. Reported differences in H-NS reorganization for different Ag NP coatings also suggested particle-specific effects.[128] Further investigations into the intracellular effects of nanoparticles exploiting SRM would offer promising avenues for understanding the full spectrum of nanomaterial-induced bacterial responses.

3.3. SRM as a Tool for Nanotheragnostics

Theragnostics holds promise to couple, in a single vector, diagnostic/imaging properties with the release of therapeutic agents. Several theragnostic platforms have been developed using nanomaterials, including inorganic NPs (silica and metallic), carbon-based nanomaterials, liposomes, polymeric NPs, and quantum dots (QDs) for the treatment of cancer and other diseases, such as autoimmune, cardiovascular and neurodegenerative diseases.[129–131]

Carbon nanostructures, like fullerenes, carbon nanotubes, graphene, and carbon dots are emerging as promising theragnostic and imaging agents. Due to their small size, highly biocompatible profile, and ease of functionalization, fluorescent carbon dots (FCDs) have found extensive use in nano-theragnostics as delivery agents for chemotherapeutics in cancer treatment and diagnosis,[132] as well as in bioimaging. STED nanoscopy was applied to examine the intracellular localization of intrinsically fluorescent carbon dots at a resolution down to 30 nm—a sixfold resolution improvement compared to conventional confocal microscopy within living cells—suggesting that these nanomaterials can be employed as probes for the detailed visualization of cellular events over long periods of time and as nanocarriers in in vivo drug delivery experiments (Figure 2v).[133]

Silica-based materials represent another class of potential theragnostic platforms. As an example, Prabhakar et al. designed a composite material integrating the effective drug-carrying capabilities of mesoporous silica nanoparticles (MSNs) with the distinctive optical properties of photoluminescent nanodiamonds (NDs). NDs can be imaged with fluorescence and reflectance microscopy, but are also suitable for STED microscopy, enabling long-term imaging and tracking of cellular processes in living cells.[134]

Complex multimodal NPs with theragnostic capabilities encounter several biological barriers when interacting with biological systems. Therefore, they must be finely engineered to cross these barriers and reach the target site. SRM can help to study the mechanisms at the root of such interactions, helping the design of nanomedicines with the expected theragnostic efficacy. One salient example is represented by the blood brain barrier (BBB), a microvascular network that acts as a barrier between the central nervous system (CNS) and the peripheral blood circulation, significantly restricting the application of theragnostic strategies for CNS diseases. Liposome-based therapeutics have attracted large attention due to the ease of production, biocompatibility, flexibility in drug encapsulation, and prolonged circulation upon surface modification. Also, the possibility to decorate liposomes with a wide spectrum of molecules, diagnostic agents, and in vivo imaging probes for optical imaging, magnetic resonance imaging, positron emission tomography, and single photon emission computed tomography, makes them one of the most promising platforms for theragnostic approaches in CNS diseases. Using multiscale and multimodal optical imaging, including STED nanoscopy, May et al. have studied the penetration and accumulation of two different prototype drug delivery systems (namely, 10 nm-sized pHPMA polymers and 100 nm-sized PEGylated liposomes) deep into mouse brains upon the opening of the BBB by sonopermeation treatment.[135] These findings expand the knowledge of the mechanisms by which nanomaterials can cross biological barriers and help to improve the development of nanomedicine-based therapeutic approaches for CNS diseases. SRM has also been applied to investigate the interaction of liposomes with another important biological barrier: the skin. Using a combination of STED and raster image correlation spectroscopy on ex vivo skin specimens, liposomes were characterized for transdermal drug delivery, demonstrating that no intact liposomes were able to carry their payload across the skin barrier; conversely, they fused with the outer lipid layers of the stratum corneum.[136]

3.4. Surface Rearrangements of Nanomaterials: The Protein Corona

An important aspect modulating the behavior of nanosystems and significantly influencing their performance in vivo (including toxicity, bioactivity, efficacy, circulation time, and biodistribution) is their interaction with the biomolecules present in biological fluids. These molecules form a (bio)molecular corona, also referred as protein corona, on the surface of the nanomaterials, which undergo significant alteration in their surface chemistry and acquire a "biological identity" that drives their uptake, transport, fate, and behavior when passing through biological barriers.[137–140] Recent studies report the application of SRM to the study of nanomaterial surface chemistry, with a focus on the composition of the protein corona. The molecular scale understanding of the surface composition before and after nanomaterials interaction with the biological environment, as well as the dynamics of adsorption and distribution of proteins or other biomolecules on nanomaterials surface, is relevant for a rational design of nanotherapeutics. Multicolor STORM and STED have been used to complement high-resolution imaging and

proteomic analysis and to determine particle-to-particle differences in protein corona formation/composition, spatial organization, and dynamic composition changes. Wang et al. resolved the structural features of protein (bio)molecular corona on single particles using multi-color STED. By simulating the coating of NPs with the three main blood plasma proteins (BSA, transferrin, and IgGs) labeled with STED-compatible dyes, the authors determined the composition of the protein corona at the single particle level and correlated it with nanoparticle size and geometry, demonstrating the major role played by NP roughness and porosity on corona composition. Protein corona was also visualized on single particles in cells upon internalization, evidencing the considerable potential of STED in elucidating nanomaterial dynamics occurring on NPs within biological systems.[141] Similarly, dSTORM was used to visualize and quantify the proteins adsorbed onto MSNs,[142] a class of NPs which has been widely studied for drug delivery applications, thanks to their great versatility and efficient conveyance of a wide variety of therapeutic agents.[143] The increase in resolution achieved with dSTORM enabled researchers not only to visualize the protein layer surrounding individual NPs and to quantify its size but also to estimate the number of biomolecules on single NPs, highlighting the heterogeneity in protein absorption. Moreover, dSTORM imaging allowed the evolution of protein corona composition to be characterized, demonstrating a dynamic change in protein corona arrangement over time that involved an interplay between fast- and strongly adsorbing proteins. The evolution of the protein corona on the surface of nanostructured carriers was also investigated in a recent study by Battaglini et al., where a complete analysis of (bio)molecular corona formation and evolution was performed, from the first contact with biological media to the interaction with their therapeutic target. The authors combined confocal microscopy, dSTORM, and proteomic analysis to study the composition of the biomolecular layer on the surface of nanostructured lipid carriers, selected for their potential as drug delivery systems in the treatment of glioblastoma, before and after crossing an in vitro simulated BBB. Results demonstrated that the composition of the biomolecular corona is heterogeneous and dependent on exposure time and that the composition changes as the nanostructures interact with biological fluids and cell barriers. This evidence points at the biomolecular corona as one of the key aspects to be elucidated in order to achieve the desired nanocarrier-mediated targeting and to avoid undesired cell-nanostructure interactions.[144] Compared to dense particles, the study of protein adsorption on porous NPs is more complex due to protein penetration within the porous material. In virtue of their enhanced resolution, SRM techniques are also suitable to explore the inner portion of NPs. The information derived from stochastic optical reconstruction was used to develop a non-invasive quantitative method to study the absorption and penetration of proteins within MSNs, supporting the evidence for different patterns of protein adsorption on porous versus dense NPs.[145]

Overall, the reported examples support the key role of SRM in the dissection of those mechanisms intervening at the nanoscale once a nanomaterial interacts with a biosystem, generating new knowledge for the rational design of nano-enabled diagnostic, therapeutic and theragnostic tools, maximizing their clinical translation potential.

4. SRM as a Tool to Address the Environmental Impact of Nanomaterials

The introduction of nanomaterials in several industrial and commercial products is progressively increasing and, along with it, the presence in the environment of several types of incidental or engineered NPs[146,147] (as schematized in **Figure 3**). The study of the penetration of nanosized particles across cell and tissue barriers is relevant to predict the short- and long-term risks associated with exposure to nanomaterials. Several nano-sized particles are used in food packaging, cosmetics (e.g., sunscreens), and in the textile industry: examples include silicon dioxide (SiO_2) and metal oxide NPs, such as titanium dioxide (TiO_2) and zinc oxide (ZnO) for their UV protective[148] and/or antimicrobial activity.[149] However, the effects of exposure to nanophases (by oral ingestion, skin contact, etc.) are still largely unknown, raising concerns about their potential negative effects on health. There are pieces of evidence that SRM is a useful method for the analysis of many aspects associated with the biocompatibility/cytotoxicity of nanomaterials,[150] such as their agglomeration, the extent of cellular uptake, routes of internalization, and their biodegradation. As an example, Peuschel et al. have proposed 3D STED imaging of whole cells as a model to quantify the internalization of fluorescently labeled silica NPs of different sizes to study NP delivery (administered vs internalized dose) and their toxicological profile. By complementing STED imaging with other in vitro assays, the authors assessed the cytocompatibility of silica NPs and correlated it to both NP size and protein content in the surrounding medium. The high-resolution segmentation and reconstruction of the whole cell volume allowed the efficiency of NP internalization to be determined, distinguishing between truly internalized NPs versus NPs attached to the cell surface, paving the way for the design of safe nanomaterials with effective delivery performances.[151]

The efficacy of nanoscopy techniques including STORM, SIM, and STED over standard methods (i.e., mass spectrometry and cytometry) has also been documented by Andrian et al. to gain an understanding of NPs endosomal escape mechanism, which is essential for their successful cytosolic transport/delivery and is impeded by NP endosomal entrapment and degradation, which reduce their intracellular bioavailability and the efficacy of nanoformulations.[152]

Looking at nanomaterials with a potential impact on the environment and human health, nanoceria and nano/micro-plastics appear of great interest.

Nanoceria (cerium oxide NPs, CeO_2-NPs) is an attractive material for therapeutics, and abundant literature proves its antioxidant and cytoprotective effect both in vitro and in vivo.[153] On the other side, concern has been raised on the potential toxicity of this nanomaterial following inadvertent human exposure to CeO_2-NPs in the environment. It is thought that the main source of CeO_2-NPs entering the natural environment resides in diesel fuel additives for road and aquatic transportation, which represent a potential ecological concern.[154] Water draining from road surfaces is predicted to contain the highest levels of CeO_2-NPs, which can be released into the aquatic habitat representing a relevant risk for the marine environment. To investigate the potential neurotoxicity of nanoceria, Gliga et al. used SIM and STED nanoscopy to unveil its effects on neuronal differentiation,

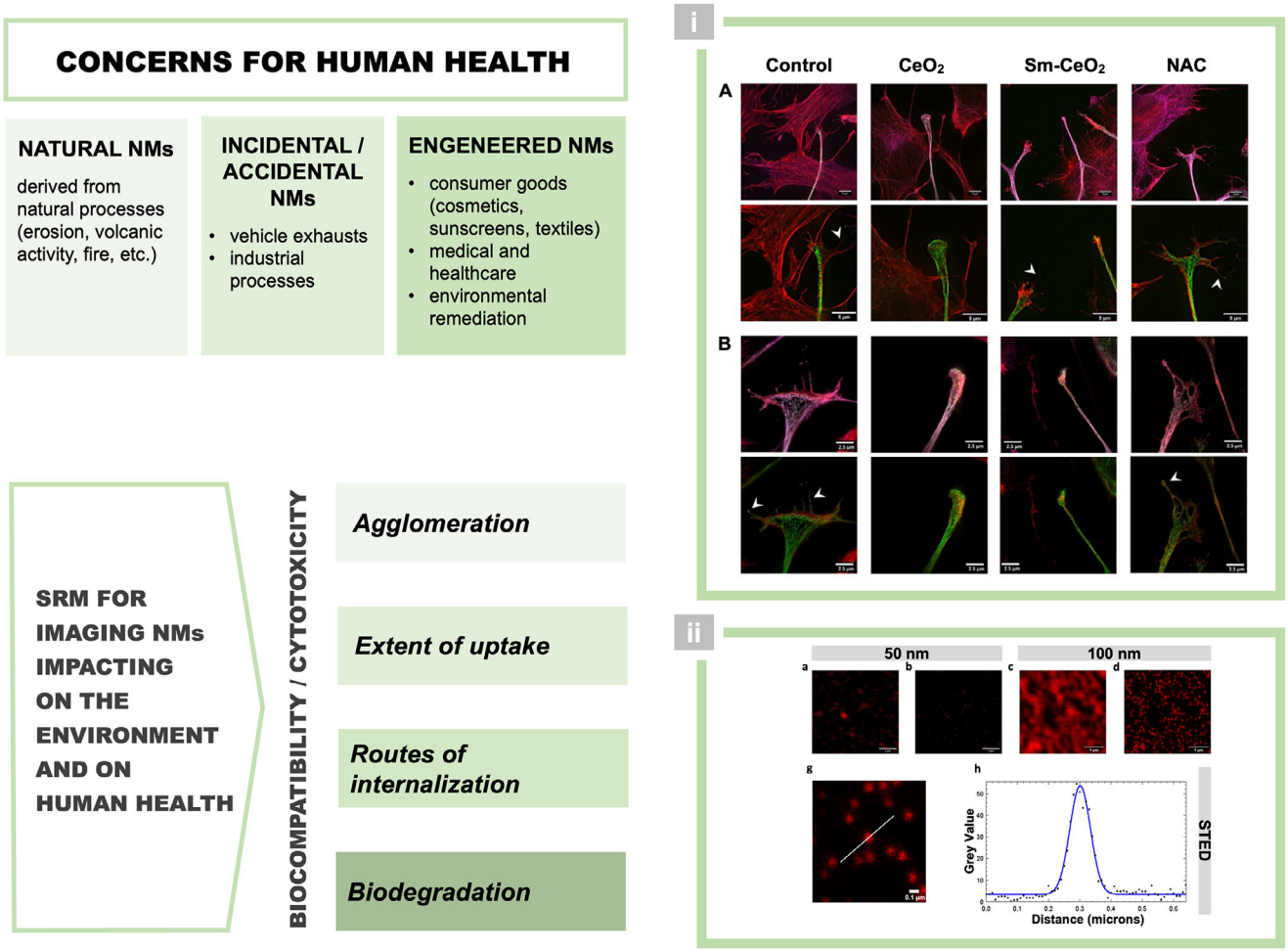

Figure 3. SRM for nanomaterials of environmental concern. Graphical representation of the sources of nanomaterials (NMs) potentially hazarding human and environmental health, enlisting potential applications of SRM. i) Ceria NPs. Imaging of filopodia by SIM and STED microscopy. Adapted with permission.[155] 2017, Springer-Nature. ii) Nanoplastics. Imaging of nanoplastics with STED microscopy. Adapted with permission.[161] 2022, American Chemical Society.

complementing the information obtained by other molecular and computational analyses (Figure 3i). The SR imaging of growth cones stained with diverse cytoskeletal proteins, including markers of neuronal differentiation, demonstrated that nanoceria inhibited the development of neural progenitor cells by interfering with cytoskeletal organization and altering the structure of neural growth cones, suggesting that nanoceria may pose developmental neurotoxicity hazards.[155]

Hence, monitoring the exposure to nanoceria originating from pharmaceutical or industrial sectors is becoming of primary importance, with a particular focus on the food industry, where it is being introduced as a multifunctional filler in food packaging.[156]

In recent years, also micro- and nanoplastics are gaining increasing attention due to their potential effects on human and environmental health. However, their small size hampers their detection within tissues and organs. Widefield and CLSM have been used to track and identify micro and nanoplastics in biological samples, including biological tissues in exposure studies.[157–159] Also, dark-field microscopy, combined to spectroscopy, has been demonstrated to be an effective tool for the identification of label-free micro- and nanoplastics in vivo, even differentiating among chemically different materials.[160] However, diffraction-limited approaches lack the resolution to localize nanoplastics in complex matrices (like native tissue) and hinder their quantification in environmental samples. Therefore, SRM approaches have been used to achieve the required nanoscale optical resolution, and SR-based techniques have been developed to resolve nanoplastics with single-particle resolution. Interestingly, Nguyen and Tufenkji developed a labeling technique combined with STED microscopy to detect nanoplastics of different shapes and compositions in whole animal tissues, without compromising the integrity of specimens, down to 50 nm resolution (Figure 3ii).[161]

The above-reported examples emphasize growing attention to the healthcare and environmental hurdles associated with the increasing presence of nanomaterials in consumer goods, including food, with an increasing risk of bioaccumulation in humans. SRM represents a valuable tool to gain detailed insight on their fate in the environment and in biological systems.

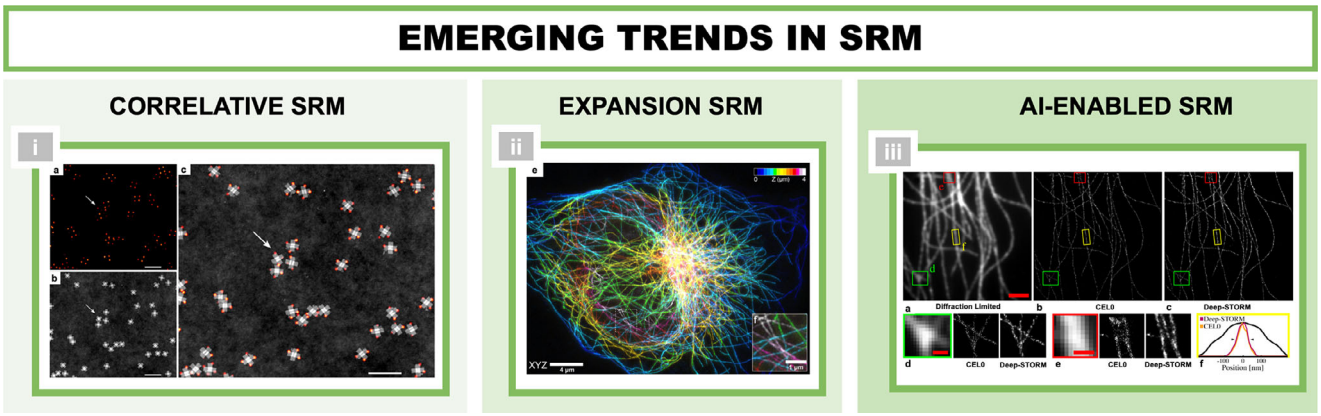

Figure 4. Emerging trends in SRM. Graphical representation of the emerging approaches in SRM. i) Correlative SRM. Correlative DNA-PAINT and AFM imaging of DNA origami. Adapted with permission.[172] 2021, American Chemical Society. ii) Expansion microscopy. Imaging of a whole cell using 3D ExSTED. Adapted with permission.[177] 2021, Elsevier. iii) AI-enabled SRM. Reconstruction of microtubules via Deep-STORM algorithm. Adapted with permission.[182] 2018, Optica Publishing Group.

5. Current Trends and Future Outlooks

As outlined in the former sections, SRM represents a powerful tool to investigate nano–bio interactions in applicative scenarios which encompass the biomedical and environmental ones. However, SRM techniques also show some pitfalls, still hampering their widespread diffusion. These are related to the quest for dedicated optical systems, labor-intensive or dedicated sample preparation routes. Importantly, SRM deals with contrasting requirements: spatial resolution often comes at the expense of temporal resolution (as in the case of many SMLM techniques); at the same time, the laser power required to achieve a good signal-to-noise ratio can result detrimental for the sample in terms of photobleaching and phototoxicity.

In the following, we will detail some of the current research trends in SRM (as schematized in **Figure 4**), underlying how they are expected to positively impact on its applicability for the study of nano–bio interactions and discuss the perspective of in vivo application of SRM and their migration to the clinic.

5.1. Correlative and Multimodal Workflows

The fast growth of the SRM field is offering new opportunities for the development of correlated multimodal imaging approaches. The correlation of SRM with other light microscopy techniques (confocal/SRM,[162] time-lapse microscopy/SRM,[163] SRM/SRM[164]), with scanning probe microscopies (e.g., AFM[165]), EMs,[46,166] and different types of spectroscopies,[167] also referred as correlative nanoscopy, has demonstrated its potential by unraveling cellular and molecular processes within their complex native environment.[168] SRM is often combined with EM and TIRFM for the study of intracellular trafficking, to unveil how cells interact and ingest extracellular material. The coupling of AFM and optical SRM, as demonstrated in the pioneering study of Harke et al.,[169] gives unique opportunities to investigate dynamic biological processes taking advantage of the topographic and nanomechanical information deriving from AFM and the capability of SRM to identify individual elements in heterogeneous samples with high specificity and resolution. Materials science has also benefited from correlative nanoscopy approaches, enabling the characterization of advanced materials and chemical strategies, with relevant applications in the study of single-particle catalysis.[167] However, despite its potential, correlative nanoscopy suffers from experimental complexity and high costs, which still limit the spread of this emerging technology in biology and materials science, albeit integrated commercial hybrid correlative systems are currently available. Most recent reports show that, although still at a seminal level, also bio-nano interactions can be characterized with techniques that superimpose SRM to other microscopies commonly used for nanoparticle imaging, such as AFM, TEM, and scanning electron microscopy. The integration of highly spatial and temporal resolution imaging of fluorescently labeled cellular structures or nanomaterials, achievable with multi-color, live, 3D SRM, with other label-free high-resolution microscopy techniques, enables their accurate localization within complex environments and the correlation of nano-mechanical parameters with specific parameters of nanomaterials, like their size, structure, surface functionalization, subcellular localization, etc.

A correlative nanoscopy approach, referred to as super-resCLEM, has been conceived to perform the advanced structural characterization of nanomaterials of interest for drug delivery applications. It consists of a hybrid imaging system combining TEM and the SMLM variant DNA-PAINT to simultaneously investigate at the single nanoparticle level both NPs size and ligand number, two important parameters that significantly affect the fate and biological responses of ligand-functionalized NPs designed for nanomedical applications. This approach shows great promise for the optimization of cell targeting strategies in nanomedicine, as it provides researchers with information not achievable by single-technique analyses.[170]

Interestingly, a similar correlative approach, combining DNA-PAINT super-resolution and AFM, has been used for the nanometric characterization of DNA nanostructures, namely DNA origami, which are emerging as promising tools for drug delivery, biosensing, nanofabrication, and bioimaging.[171] The authors used this correlative technique to characterize, at nanometric precision, addressable site defects on DNA origami (Figure 4i).

Taking advantage of the simultaneous combination of the topographic and structural information of DNA nanostructures, achievable with AFM, and the direct characterization of addressable sites, attained with DNA-PAINT SRM, it was possible to precisely distinguish between active, inactive, and unincorporated sites, whose identification is relevant to improve DNA nanostructural fidelity, since DNA nanotechnologies in healthcare must meet tight requirements for product purity and functionality.[172]

Also, amyloid-like hybrid nanomaterials composed by βLG (β-lactoglobulin) fibers functionalized with carboxyl-coated Cd/Se quantum dots has been investigated by the combination of complementary techniques (AFM and SMLM), extracting details on topography and composition.[173]

To date, very few works in the literature report the use of multimodal imaging to study the cellular uptake (i.e., the nanoscale interactions with cellular membrane) and trafficking of nanomaterials. A notable example is represented by the work of Beldman et al., who combined two-color super-resolution GSD microscopy and EM to track hyaluronan NPs in the microenvironment of the atherosclerotic lesions, elucidating the trafficking pathway of these NPs during atherosclerotic progression and metabolic therapy.[174]

Together, the reported studies enlighten how the combination of complementary approaches for characterizing bio-nano interactions can further increase the amount of information extractable from imaging datasets.

5.2. Expansion SRM

When first introduced, expansion microscopy was demonstrated to achieve 70 nm lateral resolution in combination with a conventional confocal microscope, thereby enabling the observation of sub-diffraction features on diffraction-limited optical systems. As such, ExM has attracted research interests also for the imaging of cell–material interfaces, and expansion protocols have been specifically adapted to preserve the nano–bio interface also in the presence of material substrates not readily compatible with hydrogel expansion,[175] reaching a fourfold resolution increase on confocal systems. As a further advancement, ExM was combined with SRM techniques to push the resolution even further, as in the case of SIM (ExSIM)[176] (30 nm lateral resolution), STED (ExSTED, Figure 4ii),[177] and SOFI (ExSOFI)[178] (<10 nm lateral resolution). These resolution levels, almost matching those of EM, strengthen the leading role of SRM for nano–bio interface studies.

5.3. AI-Enabled SRM

Deep learning (DL)[179] encompasses a series of artificial intelligence methodologies that use a multi-layer artificial neural network to perform computational tasks. DL has rapidly revolutionized modern image processing approaches, including the analysis of microscopy datasets. DL has found increasing application also in SRM pursuing two strategies: on the one side, with the objective of increasing the performances of existing super-resolution systems; on the other side, with the aim to extract sub-diffraction features from diffraction-limited micrographs.[180,181] DL algorithms have been used to improve signal-to-noise ratio, augment spatio-temporal resolution, and reduce photobleaching/phototoxicity of available SRM techniques. As an example, DL was demonstrated to enhance single molecule localization in 2D STORM, overcoming conventional point spread function (PSF) fitting under high fluorophore density (a condition resulting in localization inaccuracy) and low SNR (Figure 4iii).[182] The approach was further extended to 3D STORM by combining PSF engineering with DL-based single-molecule localization and PSF pattern recognition.[183] Noise reduction represents another salient application of DL, as software denoising is an effective and non-invasive alternative to increased laser power, which can be detrimental for the sample. DL-based denoising has demonstrated superior performances in combination with SIM,[184] STED,[185] and SMLM.[186] The transformative performance advancement introduced by DL-based methods is expected to impact the characterization of nano–bio interfaces, especially for those experimental settings in which high spatio-temporal resolution is required together with reduced phototoxicity, as in the evaluation of live dynamic intracellular processes.

5.4. In Vivo Nanoscale Imaging of Nano–Bio Interactions

Research on nano–bio interactions cannot disregard to investigate (nano)reactions in a physiological environment, and the increasing effort of microscopy developers is now put on conceiving imaging methods supporting these approaches with enhanced spatiotemporal resolution. This represents a new challenge especially in the medical field, enabling the study of new synthetic materials in vivo and streamlining the clinical translation of nanomedicines. Here, the comprehension of how nanomaterials distribute at the cellular level within tissues and how they trigger cellular responses is an essential aspect to understand their global effect on the entire organism. However, in vivo, imaging of thick (ex vivo and in vivo) tissues is hindered by several factors, that is, tissue-induced background, aberration, and light scattering. Most biological tissues scatter light, making it difficult for traditional fluorescence microscopy, confocal fluorescence microscopy, and the newly developed super-resolution techniques, to achieve high-resolution imaging at greater depths. To date, in fact, the application of super-resolution microscopy to thick structures in vivo is still limited and is mainly addressed to neuroscience studies, where STED is actually the most suitable technique due to its optical sectioning capability and compatibility with standard fluorescent proteins.[187–190] Conversely, SIM and SMLM are less suitable for live in vivo imaging and their use is restricted to the imaging of tissue slices and live imaging of dissociated cell cultures or small multicellular organisms.

To extend the application of optical nanoscopy in vivo, several hybrid platforms for intravital nanoscale imaging have been developed, integrating super-resolution microscopy approaches (whether of mild or high resolving power) with nonlinear optical microscopy, particularly multiphoton microscopy capable to achieve nanoscale resolution at high contrast and penetration depth. These specialized custom-made microscopes exploit the low tissue absorption of MP excitation to facilitate imaging in various tissues of living animals at depths of hundreds of microns

below the surface, with minimal phototoxicity and photodamage, and the nanoscale resolution of SR methods.[191–194]

To date, the field of in vivo SRM of nanomaterials, their structural rearrangements, and modifications at the interface with the biological environment is still at its early stages. Intravital imaging is only possible on superficial tissues and higher temporal resolution would be needed to grasp fast processes occurring at the interface between biological systems and nanomaterials. For these reasons, the challenge remains in going beyond the barrier of static in vitro or *ex vivo* imaging. The application of nanoimaging systems with high spatial and temporal resolution, such as the recently developed MINFLUX, could lead to significant advancements in the field of intravital imaging and the characterization of nano–bio interactions in vivo.

5.5. Migration of Far-Field Optical Nanoscopy Techniques to the Clinic

Beyond basic scientific research, super-resolution techniques developed in recent years are fueling growing interest in the preclinical and clinical settings for diagnostics, therapy, and clinical monitoring. This is particularly true for those SR techniques achieving a twofold increase of resolution, such as SIM and evolutions of confocal microscopy techniques, like ISM, as they do not require significant changes to the labeling protocols used for confocal microscopy and they are compatible with a wide range of commercial fluorescent markers. Therefore, their simplicity and versatility make them suitable for analyzing various types of samples and for routine investigations conducted by non-expert users. Although more complex and less user-friendly, even the more advanced optical nanoscopy techniques are making their way into diagnostic and clinical applications.[195] These techniques would provide clinicians with high-resolution visualization of biological structures and pathological processes, facilitating early and accurate diagnosis, evaluation of the effectiveness of nanotherapies, monitoring of the distribution and targeting of nanomedicines, and planning of personalized treatments. In the near future, it is not difficult to imagine nanometer-resolution SRM equipment in medical diagnostic laboratories for the daily analysis of clinical samples and in which the integration of high-resolution imaging, digitalization, and automation work together to enhance diagnostic capabilities.

6. Conclusion

The ever-growing production of nano-enabled goods is increasing the potential impact of disperse nanophases. In this scenario, the One Health approach is emerging as a multiscale model underpinning how human, animal, and environmental health are inextricably linked. For this reason, we are witnessing the push of regulatory agencies (e.g., EU Green Deal Chemicals Strategy for Sustainability) toward a more rigorous implementation of safe (and sustainable) by design strategies.

This review sought to highlight the significant role that SRM plays in clarifying the nano–bio interactions of nanoscale materials. Any nanotechnological approach that involves the application of nanomaterials must be finely characterized employing techniques capable of reaching the scale of these materials. Thanks to its relatively simple implementation, coupled to its high spatial and axial sub-diffraction resolution, SRM is a good fit to address this need. Notably, the integration of SRM with other nanoscopic techniques—including AFM and EM—and the advent of AI-based software tools optimally trading-off between spatiotemporal resolution and low invasiveness to the sample represent two founding pillars enabling an unprecedented level of detail on the processes occurring at the nanoscale. As such, SRM is expected to boost the design of next-generation nano-enabled goods fully responding to the most stringent safety and sustainability requirements.

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Conflict of Interest

The authors declare no conflict of interest.

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