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Soft Matter Nanoscopy

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Abstract

Super-resolution microscopy (SRM), also known as nanoscopy, has gained ground in the past decade as a valuable imaging and characterization tool to investigate sub-μm architectures. Widespread is its usage in cell biology and biophysics, while visualization of the inner life of man-made materials is still in its infancy. In this contribution, we review pioneering studies in this emerging application area focusing on the SRM imaging of mesoscale structures within soft nanomaterials, and its impact on the rational design of their functional properties. These studies on (supramolecular) polymers, colloidal particles, emulsions, foams and association colloids showcase the broad application perspective of nanoscopy owing to its high spatial resolution, low invasiveness, high penetration depth, high sensitivity, chemical specificity, and straightforward sample preparation.
Introduction

Super-resolution microscopy (SRM) refers to a family of fluorescence-based imaging techniques capable of resolving features smaller than the excitation wavelength. Since its first introduction roughly 20 years ago,\textsuperscript{[1]} SRM has opened up an unprecedented \textit{vista} on sub-\(\mu\)m structures \textit{in vitro}, in live,\textsuperscript{[2]} fixed,\textsuperscript{[3]} and vitrified cells.\textsuperscript{[4]} Stunning pictures based on tens of thousands of single dye molecules portraying the internal structures of mitochondria,\textsuperscript{[2]} cellular uptake mechanisms,\textsuperscript{[3]} and precise location of proteins in their native cellular environments\textsuperscript{[4]} demonstrated the power of this suite of techniques.

Contrasting starkly with the widespread application in biology and biophysics, utilisation of nanoscopy for high-resolution imaging of mesoscale features in man-made (soft) materials has only recently gained ground. Nanoscopy is well suited for this purpose owing to its high spatial resolution, low invasiveness, high penetration depth, high sensitivity, chemical specificity, and straightforward sample preparation. Indeed, these advantages are commensurate with the key requirements for successful visualization of soft matter, which is intrinsically responsive and often composed of multiple building blocks arranged in an hierarchical fashion on various length scales. Clearly, the spatial resolution of more conventional, powerful methods like (cryogenic) transmission electron microscopy and atomic force microscopy is unparalleled,\textsuperscript{[5-7]} enabling atomic reconstruction of the 3D structure of proteins,\textsuperscript{[8]} nanocrystals,\textsuperscript{[9]} small metal nanoparticles,\textsuperscript{[10]} and even single molecules.\textsuperscript{[11]} But, these are not well suited to identify chemical heterogeneities in multi-component (bio)organic materials. Nanoscopy on the other hand readily offers access to nanometric spatial resolution and chemically specific
information via covalent tagging of objects of interest with suitable fluorescent probes.

In this contribution we discuss recent literature on nanoscopy and its application to soft matter. For an in-depth perspective on SRM studies in biology and biophysics,[12–14] plasmonics[15] and catalysis,[16] we refer the interested reader to excellent recent accounts by others. This review is organized as follows. We start off with a broad overview of SRM techniques based on the localization of single molecules or on the engineering of the illuminating volume. Hereafter, these are referred to as single-molecule localization microscopy (SMLM) and reversible saturable, or switchable, optical fluorescence transitions (RESOLFT), respectively. Next, we discuss application of SRM in supramolecular chemistry and soft matter, showcasing e.g., recent advances in our fundamental understanding of monomer exchange processes, fiber growth dynamics, molecular arrangements and morphological transitions within association colloids. Two short sections follow hereafter on SRM dye development and on correlative approaches aiming to probe the exact same material by SRM and e.g., spectroscopy, electron microscopy, and atomic force microscopy. We conclude with a perspective on future directions and grand challenges wherein SRM can play a decisive role.

The principle of super-resolution microscopy
Life science has profited for centuries from the improved visualization of (multi-)cellular structures using visible light and lenses. Fluorescence microscopy combines the magnifying properties of optical microscopy with the visualization through fluorescence of purposely selected targets carrying a suitable label fluorescence. Disadvantageously however, the diffraction limit of light impedes sub-μm imaging with chemical specificity
as it limits the resolving power of fluorescence microscopy to the size of the so-called point spread function (PSF) being \( \sim 200 \) nm (fig. 1a-b). In recent years, two distinct approaches have been developed to circumvent this limitation: coordinate-stochastic methods employ the temporal separation of the emission of fluorophores (fig. 1c-g),\(^{[17]}\) while coordinate-targeted methods modulate the effective point spread function (PSF) of the microscope to shrink the illumination volume (fig 1h-k).\(^{[17]}\) Coordinate-stochastic methods such as (f)PALM,\(^{[18, 19]}\) (d)STORM,\(^{[20-22]}\) and PAINT\(^{[23]}\) are collectively referred to as single-molecule localization microscopy (SMLM) methods. Coordinate-targeted methods are also known as reversible saturable (or switchable) optical fluorescence transitions (RESOLFT), which include STED,\(^{[24]}\) GSD,\(^{[25, 26]}\) and SSIM.\(^{[27-31]}\)

**Single-molecule localization microscopy**

In SMLM, the distance, \( d \), between two dyes emitting at the same time should be larger than the 5 times diffraction limit to be properly localized (fig. 1c).\(^{[32]}\) This is accomplished using external triggers, such as UV light, solvatochromic effects, conformational changes, *et cetera*, to switch the dyes between ON (emitting) and OFF (dark, non-fluorescent) states. The dyes are exclusively localized in the ON state and remain invisible in the dark state. Whereas in conventional fluorescence microscopy many dyes are excited and detected simultaneously, in SMLM only a few ‘activated’ dyes are simultaneously emitting and their signals collected within the same acquisition frame. Many of such pointillistic frames are summed up to obtain a super-resolved image, which reconstructs with high precision the labeled object of interest (fig. 1c). To determine the precise location of each individual dye, the profile of the diffraction limited spot of each single molecule (fig. 1d-e) is analyzed. The measured intensity profile (fig. 1f) is
modeled with a 2D-Gaussian function to obtain its centroid, which corresponds to the most probable location of the molecule (fig. 1g). This enables >10-fold higher localization precision of emitters compared to the diffraction-limited image. The advantage of SMLM methods resides in the high labeling density achievable, such that nanostructures can be visualized with high details.

PALM microscopy relies on a so-called photo-activation step prior to photo-excitation, which constitutes a UV laser light triggering an irreversible conformational change of the organic dye or fluorescent protein to control the density of fluorophores in the ON state. All emitters start in a so-called ‘caged’, dark state and can be photo-activated into an ‘uncaged’, dark state, ready to be excited into the ON state by visible laser light.[18, 33]

(d)STORM employs fluorophores which can be switched reversibly between a fluorescent (ON state) and a non-fluorescent state (OFF triplet state). Intersystem crossing to bring the fluorophore in the dark triplet state is typically achieved upon addition into the sample of an oxygen scavenger e.g., glucose oxidase and a reducing agent, such as β-mercaptoethylamine. Continuous illumination with a high laser power pushes the dyes back in the ON state. In this manner, a record spatial resolution of tens of nanometers has been achieved in 2D and 3D (d)STORM due to the stochastic blinking of the fluorophores.[34]

PAINT exploits transient (a)specific binding of fluorescent dyes to the structure under investigation to realize a spatial resolution of 20 nm in the lateral direction. In a pioneering study published in 2006, Sharonov et al. exploited the aspecific binding of a solvatochromic dye, Nile red, which emits fluorescence signals only in rather
hydrophobic domains, to visualize large unilamellar vesicles. Specific binding to suitable ligands in cell membranes or complementary DNA strands on e.g., DNA origami scaffolds (DNA-PAINT) further enables the visualization with high precision of specific interaction partners and direct quantification of the relevant targets. PAINT thus exploits for SRM imaging the (in)ability to precisely localize a dye when (un)bound to the target structure due to slow (resp. fast) diffusion when temporarily immobilized (resp. free in solution).

STORM and PAINT excel in the attainable spatial resolution, due to the high fluorescence yield of STORM dyes and the prolonged exposure time in PAINT, which leads to a higher number of photons detected when compared to fluorescent proteins used for PALM. Owing to the highly specific hybridization of complementary DNA strands, DNA-PAINT is the most exploited technique for localization of single macromolecules with (sub-)nanometer resolution. By contrast, the irreversibility of the photo-activation process in PALM ensures that each dye is localized only once. Theoretically, this enables molecular counting, but this exciting goal is yet to be accomplished as quantitative, stoichiometric labelling is challenging. Another advantage of PALM is the possibility to precise control the density of photo-activated fluorophores by external triggers. In an attempt to profit from the aforementioned advantages of PALM and PAINT, our group recently developed a new strategy, which we coined as iPAINT, in which the reversible, aspecific binding of photo-activatable PALM dyes is utilized for high-resolution imaging of soft matter. Owing to the transient interfacial adsorption of short poly(ethylene glycol) chains end-functionalized with a photo-activatable moiety and their continuous exchange with unbound polymers, interfaces...
(solid-liquid, liquid-liquid, and vapour-liquid) as well as nanostructures have been visualized in 3D, both in aqueous and organic milieu.\cite{43,44}

The spatial resolution in coordinate-stochastic methods depends on the localization accuracy (\(\sigma\)) with which each single molecule is localized. This \(\sigma\) is influenced by the size of the PSF, the signal-to-noise ratio and the photon flux of the emitter. All these factors are included in the standard deviation of the distribution function used as fitting model. In case of an isotropic emitter, and a high signal-to-noise ratio (i.e., only the shot noise is contributing to the background signal), \(\sigma\) can be approximated as inversely proportional to the squared root of the number of photons emitted, \(\sqrt{N}\).\cite{45} The appeal of coordinate-stochastic methods to a broad audience is their straightforward implementation and low cost, as ‘conventional’ microscopes can be used and various dedicated fluorophores, both genetically encoded as well as organic dyes, are commercially available. Disadvantageously, artifacts may arise from numerical data treatment.\cite{38} These include molecule over-counting, i.e., the same fluorophore is localized multiple times, and under-counting, as different emitters can fluoresce simultaneously within the diffraction limited volume. Furthermore, the labelling density must be optimized to properly resolve the object of interest.\cite{46} The attainable spatial resolution is impacted by various optical aberrations, such as chromatic, dipole orientations, drift, etc., which are taken into account in a recent model which is universally applicable to all coordinate-stochastic methods.\cite{38}
Figure 1 | Principles of super-resolution microscopy.

(a) Hypothetical arrangement of fluorophores in a labeled structure. Scale bar represents 200 nm. (b)
Fluorescence emission of all dyes at the same time in a conventional wide-field image. The diffraction-limited spots of each single molecule overlap, creating a so-called diffraction limited image of (a) on the camera. The details of the underlying structure are lost; in other words, the underlying structure may be any of the six patterns depicted in (b). (c-g) Principle of coordinate-stochastic methods. (c) Separation in time of single molecule localizations to achieve a super-resolved image. (d) Diffraction limited image of a single molecule and (e) its contour. (f) Intensity profile of the diffraction limited image of a single molecule. (g) 2D Gaussian function fit of the diffraction limited image of a single molecule to determine its centroid (indicated by a red arrow), which corresponds to the most probable location of the emitter. (h-k) Principle of coordinate-targeted methods. (h) Jablonski diagram for a stimulated emission process in STED microscopy. (j) Scheme illustrating the raster scanning employed in STED microscopy to image a structure of interest. (k) Zoom-in on the two coaxially aligned laser beams used to shrink the PSF.

Reversible saturable (or switchable) optical fluorescence transitions

Patterned illumination is the key innovation to enhance spatial resolution in coordinate-targeted approaches like STED,\textsuperscript{[24]} GSD,\textsuperscript{[25, 26]} and (S)SIM.\textsuperscript{[27-31]} In STED microscopy,\textsuperscript{[24, 47, 48]} Abbe’s limit is circumvented by excitation with one laser at $h\nu_1$, and subsequent depletion of the surrounding area with another, donut-shaped, laser beam at $h\nu_2$ (fig. 1h). The two lasers with $\nu_1 > \nu_2$ are coaxially aligned, such that the excitation laser (a common diffraction limited Gaussian beam) excites the fluorescent molecules in its focal volume, some of which are switched OFF by the second donut-shaped incoming beam through a process called stimulated emission. In this process, the electrons populating the excited state of the fluorophore are forced to decay into a higher vibrational state of the ground non-fluorescent state, thereby emitting red-shifted radiation compared to spontaneous fluorescence. The red-shifted photons are blocked by a spectral filter, and hence remain

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undetected. Thus, only the molecules residing in the hole of the donut emit and are subsequently detected. This reduces the size of the effective PSF resulting in super-resolved images with a spatial resolution of a few tens of nanometers\cite{24} upon scanning of the sample with the coaxially aligned beams (fig. 1j). A major drawback of STED microscopy is the high intensity of the depletion beam, which causes photodamage to the sample and substantially reduces the lifetime of the fluorophores pushed into the OFF-state. Time-gated detection and pulsed excitation have recently been introduced to circumvent this limitation without loss of spatial resolution by effectively shrinking the Gaussian beam and reducing the intensity of the donut beam by 2/3 compared to conventional STED microscopy.\cite{49}

Another widely applied means to shrink the effective PSF is ground state depletion (GSD) microscopy. In this approach, fluorophores are temporarily kept in a non-fluorescent triplet state using a second excitation beam. Since the dark triplet state is much longer-lived (microseconds - seconds) than the singlet state (nanoseconds) exploited in STED, GSD necessitates much lower laser intensity, which reduces photodamage significantly. The Moiré effect is exploited in saturated structured illumination microscopy (SSIM) to achieve spatial resolution beyond the diffraction of light.\cite{50} Herein, the specimen is illuminated with a striped pattern using an optical grating. Subsequently, the pattern arising from the interference between the fluorescent signal and the striped excitation is recorded in the focal plane. As the optical grating is rotated and the phase shift between the excitation beam and fluorescence signal is varied, striped illumination with different orientations is applied resulting in diverse beating patterns. A Fourier transform analysis of the superimposed beating patterns reveals fine
structures on the order of 100 nanometers, hence, smaller than the diffraction limit of light. Based on the working principle of SIM, alternative approaches (e.g., image scanning microscopy - ISM, re-scan confocal microscopy – RCM) have been developed recently to ensure a two-fold gain in resolution without resorting to high laser power.\textsuperscript{[51, 52]}

**Nanoscropy of soft matter**

Inspired by the architecture and functionality of complex nanostructures in Nature, chemists developed a wide array of synthetic self-assembling building blocks to fabricate hierarchical materials with interesting optical, mechanical and catalytic properties. Rational design of the performance of these materials requires a detailed understanding of the relation between their structure and function. In the following sections we showcase specific examples where SRM imaging has advanced our fundamental understanding of the mesoscale organization of self-assembled ranging from one-dimensional fibers to multi-dimensional colloids.

**Imaging the structure, dynamics, and formation pathways of supramolecular fibers**

One-dimensional supramolecular fibers may form upon self-assembly of protein-polymers,\textsuperscript{[53]} peptides,\textsuperscript{[54-56]} peptide amphiphiles,\textsuperscript{[57]} and small molecules\textsuperscript{[39, 43, 44, 58-61]} due to directional, non-covalent interactions between the constituent (macro)molecules. Several recent SRM studies on supramolecular fibers elucidate their steady-state morphologies and assembly pathways to understand how these are related to the chemical structure of the building blocks and impacted by their physical environment. These first
accounts clearly illustrate the potential of SRM to visualize the (time-evolution of the) chemical heterogeneity of such one-dimensional aggregates in their native environment and thereby shed light on important characteristics such as fiber composition, persistence, growth direction, stability, exchange dynamics, and the efficiency of cargo incorporation.

Small molecular discotics, such as 1,3,5-benzenetricarboxamide (BTA) derivatives, and other hydrogen-bonding synthons, such as ureidopyrimidinone (Upy), represent an important class of self-assembling moieties that undergo one-dimensional assembly into supramolecular fibers both in aqueous and organic milieu. Baker et al. studied whether BTA chirality impacts the structure and dynamics of nm-wide, µm-long supramolecular BTA fibers in a comprehensive experimental and computational study. Interestingly, STORM microscopy revealed that the (a)chiral discotics formed fibers of similar shape and dimensions with a persistence length of several hundreds of nanometers. The fibers did differ markedly in equilibrium dynamics, which was attributed to differences in their internal order (i.e., BTA stacking).

Quantifying the relative abundance of coexisting morphologies is notoriously challenging at the ensemble level. At the single-molecule level, it is time-consuming, but feasible. Albertazzi and co-workers monitored by PAINT using cyanine-5 the coexistence between µm long fibers and small, spherical aggregates (~60 nm in diameter) formed upon self-assembly of diphenylalanine peptides in phosphate buffer. Based on these experiments, a step-wise assembly pathway was proposed, in which the dipeptides initially form spherical aggregates, which subsequently aggregate to yield fibers (fig. 2a). Onogi et al. monitored the self-sorting into two distinct fiber types of a
short peptide-gelator tagged with Oregon Green dye and an amphiphilic phosphate-gelator tagged with Alexa546.\textsuperscript{55} Individual supramolecular fibers of 80-100 nm in diameter were first detected by STED microscopy after which their self-sorting was quantified using the Pearson correlation coefficient as a metric for (absence of) co-localization of the two dyes within one fiber.\textsuperscript{55}

Figure 2 | Examples of single-molecule localization microscopy on one-dimensional synthetic aggregates. (a) PAINT microscopy reveals diphenylalanine peptide assembly into spherical aggregates and fibers. Figure adapted from \textsuperscript{56}. (b) PALM imaging of BTA fibers with 5% Cage-552 labeled BTA acquired in cyclohexane with 2\% v/v isopropanol. Figure adapted from \textsuperscript{43}. (c) Unidirectional growth through end-attachment of Alexa-488 (green) monomers onto pre-formed, Alexa-647 (red) tagged fibrils. Figure adapted from \textsuperscript{53}. (d) Exchange pathway of supramolecular µm-long fibers unveiled by STORM imaging. Figure adapted from \textsuperscript{61}. Scale bar represents 1 µm in (a), (c) and (d) and 2 µm in (b).
Other aspects of supramolecular polymerization wherein SRM has been particularly illuminating are exchange dynamics and growth kinetics. Profiting from the accessibility of high-resolution, real-space information on the time-evolution of the fiber composition under both steady-state and out-of-equilibrium conditions, monomer exchange,[54, 57, 59, 61] directional fiber growth,[53] and cargo incorporation[54] have been studied in-depth. Albertazzi et al. monitored the exchange dynamics of water-soluble, triple hydrogen-bond forming BTA discotics by STORM. Unexpectedly, a homogeneous migration was observed of fluorescently tagged BTA monomers between fibers initially stained exclusively with either Cyanine3 or Cyanine5.[61] This was attributed by the team to the presence of weak bonds along the fiber backbone offering easy access to free monomers (fig. 2d). Interestingly, the findings contrast sharply with the hitherto consensus views of fragmentation-recombination and end-attachment mechanisms dominating exchange dynamics and thus call for detailed studies on other supramolecular fibers. Following-up on the pioneering study by Meijer and co-workers, monomer exchange between peptide-functionalized BTA,[54] peptide amphiphile[57] and Upy-polymer[59] nanofibers was probed. Aiming to understand whether the introduction of bioactivity impacts the morphology and exchange dynamics of an otherwise completely synthetic system, Baker et al. probed the insertion of a peptide-functionalized BTA into pristine BTA fibers.[54] The team first confirmed by STORM imaging the insertion of peptide cargo into the BTA fibers. Next, they demonstrated that the peptide-functionalized monomers act as catalyst for the monomer exchange among fibers, which was significantly accelerated in comparison to pristine BTA fibers without peptides.[54] Hendrikse et al. compared the monomer exchange rates of nanofibers of monovalent and
bivalent ureidopyrimidinone (Upy) based polymers using STORM microscopy.\textsuperscript{[59]} The monovalent Upy scaffolds appeared less prone to monomer exchange, resulting in a quasi-static, well-ordered packing. Conversely, bivalent Upy polymers assembled into shorter and more dynamic fibers exhibiting a fast monomer exchange rate. Stupp and co-workers investigated by time-lapse STORM imaging the relation between monomer exchange and compositional variations in peptide amphiphiles nanofibers pre-stained before mixing with either Cyanine3 or Cyanine5.\textsuperscript{[57]} This revealed the spatially heterogeneous character of the nanofibers exhibiting domains of hundreds of nanometers of fast monomer exchange starkly contrasting with sub-µm long regions where no exchange took place during as long 24 hours. Aiming to elucidate whether both ends of µm-long protein-polymer nanofibers remained ‘living’ (once polymerization has ceased) and whether growth proceeds in one or two directions (i.e., from one or both fiber ends), Beun \textit{et al.} utilized STORM. First, a well-known fiber forming triblock protein-polymer was selected, comprising a self-assembling silk-like domain (containing six repeats of a pH responsive octapeptide with glutamine, asparagine, and histidine residues) flanked by two lateral water-soluble collagen-like domains (being 99-amino-acid-long polypeptides rich in glutamine, asparagine, and serine residues). Next, nanofibers were allowed to form in aqueous solutions of triblocks bearing Alexa-647 (red), after which Alexa-488 (green) labeled triblock protein-polymer were added and time-lapse SRM imaging commenced.\textsuperscript{[53]} Interestingly, the added triblocks were found to attach to only one of the two fiber ends, resulting in the formation of green-red diblock fibers (fig. 2c), evidencing uni-directional growth.

Solvents other than water are virtually irrelevant in biology, but organic media are
widespread in other areas of science and technology. Independently, The Voets and
Manners groups developed a facile strategy enabling SRM imaging in organic solvents
using commercially available dyes.\cite{43, 44} Simple addition of 2% v/v of a protic solvent
was found sufficient to achieve photo-activation of caged dyes in non-polar media. Voets
and co-workers illustrated this achievement with the PALM imaging of hitherto elusive
BTA fibers in methyl cyclohexane (fig. 2b).\cite{43} Manners and his team performed STED
imaging of cylindrical micelles comprising block copolymers of poly(ferrocenyldimethylsilane) and polydimethylsiloxane (PFS<sub>56</sub>-<i>b</i>-PDMS<sub>775</sub>) in
hexane.\cite{44}

**Visualizing the morphology and internal structure of self-assembled polymeric
nanomaterials**

Super-resolution microscopy has been utilized to image the morphology of self-assembled polymer materials comprising polymer amphiphiles,\cite{60} hydrophilic (block co)polymers,\cite{64} and block copolymers with crystallizable core-forming blocks.\cite{65-70}

Complex coacervate core micelles (C3Ms) originate from the electrostatically-driven co-assembly of block copolymers with oppositely charged species. The architectures adopted by C3Ms are often an important determinant of their functionalities, hence, the investigation of their morphologies is crucial. Aloi et al. resorted to nanoscopy to study the morphological evolution of C3Ms made of a stiff, conjugated anionic derivative of polyfluorene (PF<sub>22</sub>) and a flexible ionic-neutral copolymer poly(N-methyl-2-vinyl pyridinium chloride)-<i>b</i>-poly(ethylene oxide) (P2MVP<sub>29</sub>-<i>b</i>-PEO<sub>204</sub>).\cite{64} The authors observed a gradual increase in the micellar anisotropy upon an increase in concentration
(fig. 3a). At concentrations as low as 0.04 mM, the C3Ms form spherical core-shell structures, which gradually become more elliptical up to an apparent maximal aspect ratio of 2.3 at concentrations larger than 0.14 mM. Yan et al. imaged the transition from cylindrical micelles to polymersomes of poly(styrene-block-ethylene oxide) (PSt-b-PEO). Wormlike micelles transformed into branched and open architectures as the concentration of the polymeric surfactant in solution was reduced. To this end, spiropyrans were incorporated in the hydrophobic, glassy-like poly(styrene) core of the PSt-b-PEO micelles formed in water, which were subsequently cast into thin polymer films. Profiting from the stochastic blinking of spiropyrans in hydrophobic, solid-like environments, the spiropyran-containing microphases were readily imaged yielding reconstructions of both the cores of cylindrical micelles and the bilayer walls of the polymersomes with nanometer resolution (fig. 3b).

Upon co-assembly of (co)polymers containing crystallizable blocks, uniform, patchy and hollow rectangular micelles were prepared by the Manners group. Cylindrical micelles were first prepared from seeds of polydimethylsiloxane (PDMS) which formed lenticular platelet micelles upon reaction with poly(ferrocenyldimethylsilane)-block-polydimethylsiloxane (PFS-b-PDMS) copolymer micelles with a PFS core and PDMS corona. The same approach was used to obtain poly(ferrocenyldimethylsilane)-block-poly(2-vinylpyridine) (PFS-b-P2VP) copolymer micelles. Rectangular alternating multi-block platelets of PFS-b-PDMS and PFS-b-P2VP micelles were targeted by sequential addition of P2VP into solutions of PFS-b-PDMS formed platelets in a mixture of hexane and isopropanol. Qiu et al. dye-functionalized the PDMS with BODIPY dyes and observed by SIM microscopy a
concentric arrangement of rectangular platelets (fig. 3d), demonstrating the feasibility of a tunable platform to achieve planar functional materials.

Insight in the internal structure of micellar and vesicular architectures is valuable information complementary to their morphological and dimensional characterization. In a first attempt to quantitatively assess encapsulation and compartmentalization in nanostructured lipid carriers (NLC), Boreham et al. combined STORM imaging and single-particle tracking approaches to measure the size and shape of the NLC nanocompartments loaded with ATTO-Oxa12 fluorescent dyes with 6 nm spatial resolution. This allowed to monitor the precise distribution of drugs inside the NLCs, showing the existence of small (Ø ~70 nm) and big (Ø 120-130 nm) drug-loaded nanocompartments filling the NLCs up to ~50% of their volume. Vicent and co-workers took a step further, and investigated the exchange rate among star-shaped polyglutamates carriers exclusively labelled with either Cyanine3 or Cyanine5 using dual-colour STORM imaging. The authors for the first time reported on the slow self-assembly of nanocarriers induced by the attraction of polyions at low salt content. This ‘extraordinary’ charge-like aggregation mechanism was theoretically predicted for such disordered soft materials, but had not yet been observed experimentally. Gramlich et al. used STORM microscopy to visualize the nanostructures within polystyrene and polymethyl methacrylate blends (fig. 3c). A correlation between nanodomain size and sample thickness was found, namely thicker samples gave rise to with larger microphases. This was attributed to slower solvent evaporation from thicker samples, such that coarsening could proceed longer. STORM imaging further revealed morphological differences in the nanoscale domains. In ~50 nm thin films the domains
appeared spherical and homogeneous. Conversely, films of \( \sim 150 \) nm thick showed more irregular domains.

**Figure 3** | Examples of super-resolution imaging of 2D and 3D polymeric nanostructures. (a) Co-assembly of oppositely charged copolymers into complex coacervate core micelles (C3Ms) imaged by iPAINT microscopy. Scale bars represent 100 nm. Figure adapted from [64]. (b) Visualization of poly(styrene-block-ethylene oxide) micelles stained with spiropyans, which exhibit stochastic ON-OFF fluorescence upon UV irradiation. Scale bars represent 4 \( \mu \)m. Figure adapted from [60]. (c) Thin slices
nm (left) and 150 nm (right) thick of polymer blends showing nanoscale domains imaged by STORM. Figure adapted from [74]. (d) SIM imaging of rectangular platelet micelles formed by living crystallization-driven self-assembly of PFS$_{36}$-b-P2VP$_{60}$/PFS$_{20}$ blends. Scale bars represent 5 μm. Figure adapted from [65]. (e) DNA-PAINT imaging of the conformation adopted by an APPV-DNA polymer upon immobilization on an instructive 3D DNA nanoscaffold. Figure adapted from [75].

Super-resolution microscopy has also been employed to evaluate whether three-dimensional DNA-based materials, also known as DNA origami, can be used as templates to program the configuration of individual polymer chains. To this end, DNA-origami scaffolds of approximately 50 by 20 nm were first manufactured as guides for complementary DNA strands to adopt U-shaped, wave, staircase, circular, and right-handed helical configurations (fig. 3e). Individual DNA graft copolymers comprising a conjugated polymer backbone of (2,5-dialkoxy)paraphenylene vinylene (APPV) carrying short oligonucleotide grafts, were subsequently allowed to adsorb onto their template DNA-origami. Next, 3D DNA-PAINT experiments were performed to detect whether the DNA-origami scaffolds successfully instructed the APPV-g-DNA bottlebrush polymers to adopt the pre-designed configurations upon template-induced folding. Gratifyingly, Knudsen et al. observed with high resolution single polymer chains wrapped around their DNA scaffold in the desired patterns, demonstrating that macromolecular conformations can be manipulated on demand.[75]

Characterizing the size, shape, and functionalization of colloidal particles and microgels
Microgel particles represent an important class of colloidal particles, responsive to temperature, pH and solvent composition, with application perspective in coating technology, biomedicine and sensing. Conley et al. studied the swelling and de-swelling of poly(N-isopropylacrylamide) (pNIPAM) particles in aqueous-methanolic mixtures with up to 30% of alcohol by dSTORM.\textsuperscript{[76]} The density profiles of the microgel particles were determined as a function of solvent composition, which revealed that the pNIPAM particles shrank in radius by roughly 20% upon an increase in methanol content from 0% v/v to 30% v/v (fig. 4a). Gau et al. used dSTORM microscopy to assess the distribution of epoxy groups in Poly(N-vinylcaprolactam) (VCL) microgels with different percentages of glycidyl methacrylate (GMA).\textsuperscript{[77]} From the obtained density profiles the authors were able to localize the epoxy groups in the microgel shells, and demonstrate their accessibility for further surface modifications. Gellisen et al. developed a versatile labeling strategy to interrogate the internal structure of inhomogeneous (e.g., core-shell) pNIPAM particles. To tag either the core or the shell of the microgels, the authors introduced primary amines in either one of these microcompartments for subsequent labeling with a suitable dye. Detailed reconstructions of the 3D density profiles from the super-resolved STORM images enabled Wöll and co-workers to identify and size the different microcompartments.\textsuperscript{[78]} Clear differences in the spatial distribution of STORM dyes are visible in the 3D reconstructions of homogeneously (i.e., core and shell) labeled particles (fig. 4b top), core-labeled particles (fig. 4b middle), and shell-labeled particles (fig. 4 bottom) with core radii of ~145 nm and shell thickness of ~135 nm. The same strategy may be utilized in the future for a systematic analysis of more complex (core-shell) colloids, such as multi-layered lipo- or polymersomes and patchy particles.
Figure 4 | Examples of super-resolution imaging of colloids and interfaces. (a) 3D dSTORM imaging of pNIPAM particles reveals (top) the de-swelling of microgel particles upon addition of different percentages of methanol and (bottom) the corresponding density profile in the swollen state in water. Scale bars represent 200 nm. Figure adapted from [76]. (b) pNIPAM-based core/shell microgels with a cationic core and an anionic shell imaged by dSTORM. Scale bars represent 500 nm. Figure adapted from [78]. (c) iPAINT microscopy on (top) a dispersion of silica colloids of ~300 nm in radius; scale bar represents 1 μm, (middle) oil and water nanodroplets in emulsions; scale bars represent 2 μm, and (bottom) two nanobubbles nucleated on the microscope coverslip. Figure adapted from [42].

Illuminating interfaces in soft matter

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Interfaces are ubiquitous in the colloidal domain, where these are critical determinants of phase behaviour and functionality. This motivated our group to develop a versatile strategy based on PALM and PAINT for single-molecule localization microscopy imaging of nanostructures and interfaces, in particular those that cannot be labeled (readily) in a covalent fashion. The new strategy, which was coined as ‘iPAINT’, exploits the natural propensity of polymer chains to adsorb at interfaces to ‘stain’ the interface instead in a non-covalent fashion.\cite{42} To this end, Aloi \textit{et al.} equipped poly(ethylene glycol) with photo-activatable moieties like Cage-552 (yielding PEG-552) to localize the bound probes, one macromolecule at a time. The fast diffusion of PEG-552 in solution hinders its localization, whereas when adsorbed onto interfaces, the molecules can be individually detected. Continuous cycles of PEG-552 adsorption, localization, photo-bleaching and exchange with unbound PEG-552 ensure long acquisition times up to 30 minutes enabling detailed reconstructions of interfaces with <20 nm spatial resolution.

The applicability of iPAINT to visualize the topology of liquid-liquid, solid-liquid, and air-liquid interfaces was demonstrated by imaging R = 330 and R = 110 nm silica nanoparticles, oil-in-water and water-in-oil emulsions, and air nanobubbles yielding particle radii of 350±15 nm and 118±14 nm, and contact angles of 13°±0.7° and 35°±2.6°, in good agreement with results from conventional methods like scanning electron microscopy (SEM) and atomic force microscopy (fig. 4c). Future work will focus on interfacial co-localization of specific targets using aspecific physical adsorption of interface markers, like PEG-552, to identify the interface and other ligand-tethered photo-activatable dyes to identify the interfacial abundance and spatial distribution of the specific, interface-bound target (macro)molecules.
Synthetic efforts to develop brighter photo-switchable dyes in organic solvents

Many approaches in nanoscopy rely on the controlled photo-switching of organic dyes or fluorescent proteins. This inspired various exciting developments in fluorophore synthesis aiming to synthesize brand new probes with high fluorescence yield, fast ON-OFF switching and minimal photo-bleaching or to optimize existing organic dyes for application under physiological as well as other environmental conditions (e.g., low solvent polarity, high pH). Recently, the diarylethene (DE) family has been in the spotlight as a promising alternative to traditional SRM dyes for their photochromism, achievable in apolar solvents without resorting to co-solvents, as discussed. Although this compound class exhibits a high quantum yield and fast, repetitive and prolonged ON-OFF switching, it is next-to-impossible to switch OFF most fluorophores at the onset of an SRM imaging experiment, which results in a remnant, background fluorescence due to the presence of the unconverted open form of DE. To profit from the high photo-switching stability of dithienylethenes and the bright fluorescence of perylenemonoimides, Li et al. bridged a perylenemonoimide dyad through an oxygen with three dithienylethenes. The resultant fluorophore could be cycled rapidly between its ON and OFF states with a high fluorescence ON-OFF ratio and utilized as imaging probe to visualize polystyrene-\textit{b}-poly-(ethylene oxide) vesicles with ~30 nm resolution. Wöll and co-workers improved the fluorescence yield of the closed form of diarylethene-based photoswitchers and put these to work imaging block copolymer structures (polystyrene and polyethylene oxide) on a molecular basis. Here, they used a combination of UV and visible lasers to repeatedly switch between the open, dark form
and the closed, fluorescent form of a dye. Miyasaka and co-workers modified the core structure of diarylethene derivatives to control their activation, excitation and deactivation by means of a low power, single wavelength laser. Here, a laser with $\lambda = 532$ nm is used to continuously promote the ON-OFF transition and the reverse process through a photo-cycloreversion reaction. Gong et al. exploited the fast photo-bleaching, spontaneous fluorescence recovery, and high quantum yield of napthalimide-hexaarylbimidazole conjugates (NI-N-HABI) to visualize amphiphilic block polymers with an optical resolution of $\sim 50$ nm.

Ideally, one would perform super-resolution microscopy without any manipulation of the sample by exploiting the intrinsic fluorescence properties of synthetic polymers. The feasibility of this idea was evaluated by Urban et al., who were able to exploit the sparse blinking from long-chain poly(methyl methacrylate) (PMMA) polymers when irradiated with a $\lambda = 532$ nm laser. Zhang and co-workers nanopatterned a glass substrate with PMMA stripes at increasingly narrow pitches (i.e., inter-stripe distances), and reconstructed their super-resolved images in dry and wet conditions with 45 nm resolution. The intrinsic blinking of several polymers (PMMA, etc.) here exploited offers the great advantage of label-free imaging, which is extremely valuable for the non-invasive characterization of the nanopatterns.

**Correlative approaches**

A complete picture of the formation, structure, dynamics and function of a material can only be obtained by combining information harvested from several techniques, ideally, on exactly the same specimen to enable direct correlation of the results. Exciting
advances in this direction have been reviewed by Hauser et al. covering the development in correlating super-resolution microscopy with (cryo-)electron microscopy, light microscopy, atomic force microscopy, fluorescence, Raman, IR, mass and single-molecule force spectroscopy on biological samples.\[85\] To visualize non-biological samples, super-resolution microscopy has been correlated to AFM,\[83, 84\] EM\[85-87\] and spectral analysis.\[88-92\] Correlative SRM and spectroscopy has been employed to develop the SRM technique,\[88,89\] or as readout results.\[80-82\] For example, stimulated Raman scattering\[93\] has been utilized to develop label-free super-resolution imaging methods,\[88\] and to investigate the role of active sites in zeolites to improve their catalytic properties,\[94\] while correlative SRM and surface- and tip-enhanced Raman spectroscopy have been used to harvest chemical information at the nanoscale\[89\] and to increase SRM imaging selectivity and sensitivity.\[90\] Exciting is the work of Bongiovanni et al., who used the spectral domain to gain more insight in the local environment of the sample.\[91\]

The solvatochromic and fluorogenic properties of the probe Nile Red were exploited to map the surface hydrophobicity of fiber-like aggregates of (macro)molecule based on the changes in the spectral fingerprints of the dye in the super-resolved images. Taking advantage of the spectral information contained in the emission of single molecules, Kim et al. unveiled the reaction pathway of the cis-trans isomerization of spiropyran.\[92\] To this end, spin-coated molecules were imaged by spectrally resolved-STORM in several solvents ranging from low to high polarity, namely n-hexane, acetone, ethanol, methanol, and water. The spectral fingerprints of the spiropyrans showed two subpopulations, which were attributed to the two isomers. The authors were also able to address the role of solvent polarity on the isomerization in each solvent, recording a sharp difference in
the statistics of single-molecules.

Correlative super-resolution microscopy and AFM or S/TEM allows to investigate the relation between the topological, mechanical and optical properties of soft matter. Flors and co-workers probed the morphology, mechanics and optical properties of hybrid organic-inorganic materials comprising amyloid-like fibrils functionalized with quantum dots (QDs). The topological analysis carried out by AFM quantified the filaments composing the fibers and probed the twisting of the latter. The mechanical properties were then correlated with the optical one resorting to STORM imaging, which offered clear insights on the emissive properties of different QDs (blinking rates, brightness and non-emissive dark fraction). The Roeffaers and Hofkens groups performed correlative SRM/SEM experiments to investigate the dynamics of charge trapping-detrapping processes in single organometal halide perovskite crystals of several hundreds of nanometers. By localizing the photoluminescence blinking of methylammonium lead iodide nanostructures, the team unveiled the relation between detrapping processes and surface reactions. Sambur et al. correlated SMLM with EM to map the surface heterogeneities of photoanodes with 30 nm resolution during photoelectrocatalytic activities on titanium oxide single nanorods. Using the non-fluorescent Amplex Red and the weakly fluorescent Resazurin, the authors monitored the photogeneration of holes and electrons, respectively, following the production of highly fluorescent Resorufin. The mapping unveiled strong heterogeneities of the reactive sites along the crystals, while most of their surface remained inactive. These results pave the way for a high-precision functional instead of structural evaluation of success of interventions aiming to block the active sites, where the photocurrent is low.
Conclusions and outlook

The last few years have seen a significant increase in the use of nanoscopy to tackle arduous challenges in the fields of soft matter and material science. Circumventing the problem of light diffraction, SRM brought the advantages of chemical specificity, multi-target labeling, low invasiveness and high selectivity in high-resolution imaging. Even though nanoscopy was originally developed to unveil the mysteries of the biological world, it is becoming increasingly apparent that SRM can likewise advance insight into structure-property relations in man-made materials as polymers, colloids, foams, microgels and self-assembled systems.

In the future, we envision a widespread employment of SRM in supramolecular chemistry, material science and soft matter. The design of new fluorophores with brighter emission, higher photo-stability, smaller size and compatibility with organic solvents will remain a focal point and key enabling technology for further advances in the field. For example, SRM dyes capable of reporting on local changes in viscosity, hydrophobicity, pH, concentration, and stiffness would open up a wealth of information which is virtually unattainable to date on nanoscopic length scales.

Other exciting recent developments are innovative strategies aiming to push the temporal resolution towards milliseconds and the emergence of correlative approaches, wherein super-resolution microscopy is utilized side-by-side with EM, AFM, spectroscopy and/or light microscopy. This offers unprecedented topological,
mechanical, and optical information on the exact same region of interest within a sample revealing not only the topology, but also the compositional and functional heterogeneity of fine structures and interfaces within materials. We anticipate exciting developments in correlative cryo-TEM with single molecule detection in the near future,\cite{101,102} and we look forward to novel insights in soft matter from spectrally resolved super-resolution microscopy.

To summarize, nanoscopy, with its resolving power far beyond the wavelength of the excitation laser light, has become an established research tool complementing the spectrum of advanced imaging modalities including (cryo-)S/TEM and AFM. Beneficial traits of SRM are undoubtedly low invasiveness, high penetration depth, chemical specificity, and multi-colour labeling, all of which can be achieved in-situ. Exciting developments are plentiful signaling many further advances in the years to come before the culmination of the “imaging revolution”, which started off in biology and biophysics, but has now also gained solid ground in supramolecular chemistry, materials science and soft matter. We hope to see many more contributions of SRM to advance our fundamental insight in the structure-function relations of nanomaterials, from hybrid systems for energy harvesting to bio-compatible coatings, and self-organizing systems.

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Highlights:

- Nanoscopy unveils sub-μm structure and function details in man-made materials.

- Basic and innovative approaches to carry out super-resolution imaging are described.

- Soft and dynamic interfaces are imaged with nanometric resolution.

- New photo-switchable dyes for super-resolution microscopy in organic solvents.

- Spectroscopy, EM and AFM correlate with nanoscopy to harvest complete information.
Graphical abstract