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Perspective

Surpassing the Diffraction Limit in Label-Free Optical Microscopy

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ABSTRACT: Super-resolution optical microscopy has enhanced our ability to visualize biological structures on the nanoscale. Fluorescence-based techniques are today irreplaceable in exploring the structure and dynamics of biological matter with high specificity and resolution. However, the fluorescence labeling concept narrows the range of observed interactions and fundamentally limits the spatiotemporal resolution. In contrast, emerging label-free imaging methods are not inherently limited by speed and have the potential to capture the entirety of complex biological processes and dynamics. While pushing a complex unlabeled microscopy image beyond the diffraction limit to single-molecule resolution and capturing dynamic processes at biomolecular time scales is widely regarded as unachievable, recent experimental strides suggest that elements of this vision might be already in place. These techniques derive signals directly from the sample using inherent optical phenomena, such as elastic and inelastic scattering, thereby enabling the measurement of additional properties, such as molecular mass, orientation, or chemical composition. This perspective aims to identify the cornerstones of future label-free super-resolution imaging techniques, discuss their practical applications and theoretical challenges, and explore directions that promise to enhance our understanding of complex biological systems through innovative optical advancements. Drawing on both traditional and emerging techniques, label-free super-resolution microscopy is evolving to offer detailed and dynamic imaging of living cells, surpassing the capabilities of conventional methods for visualizing biological complexities without the use of labels.

KEYWORDS: *super-resolution microscopy, interferometric scattering microscopy, iSCAT, coherent Raman scattering, label-free microscopy*

INTRODUCTION

Since its inception more than four centuries ago, optical microscopy has fueled many groundbreaking discoveries in all kinds of natural sciences. As the simplest and most natural method of operation, it relied first on absorption and elastic scattering of light in unlabeled samples. It was only until the 20th century when fluorescence microscopy started to be in many cases the preferred choice of microscopists[1] due to its major advantage to specifically address only parts of the specimen with fluorescent labels while reducing significantly the signal coming from the unlabeled matter. It was also fluorescence microscopy that enabled a recent revolution in the field with many new techniques[2−5] capable of surpassing the diffraction limit,[6] long thought to be unbreakable.

Most recently, methods based on specific labeling have enabled detailed characterizations of single biomolecule dynamics, interactions, and functions with remarkable precision.[7,8] While the specificity of label-based imaging is invaluable for targeted inquiries into biological systems, by design, it excludes sometimes valuable components of the studied system from the observation, making the interpretation of ubiquitous multicomponent molecular processes challenging or biased. We argue here that label-free techniques are getting into the spotlight again due to the progress in optical instrumentation and advanced microscopy methods that enable achieving single-molecule sensitivity, subdiffractional resolution, and high temporal resolution in order to observe processes that are either too complex, too fast, or simply not compatible with the labeling.

Interferometric scattering microscopy (iSCAT) has emerged as a powerful and straightforward strategy for retrieving super-resolution details of various samples, first by imaging plasmonic nanoparticles,[9] and later, in label-free contexts, demonstrating the feasibility of localizing single viruses[10,11] and unlabeled proteins.[12,13] While the detection and localization of individual unlabeled biomolecular species represent initial

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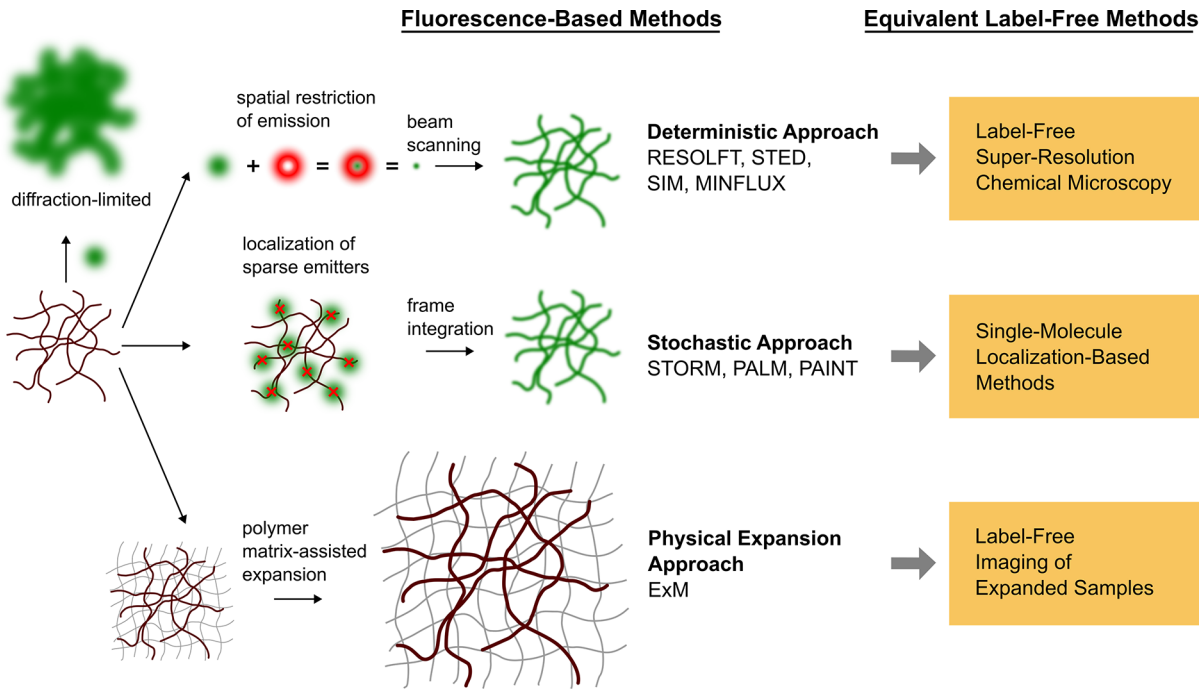

Figure 1. A scheme illustrating super-resolution microscopy techniques categorized into three distinct groups based on the approach used to overcome the diffraction limit. The generalized mechanisms and representative examples of the fluorescence-based techniques are shown on the left, while the corresponding label-free approaches are listed on the right.

steps toward super-resolution imaging of complex biomolecular or biological systems, the interpretation of data to portray their shape and dynamics with subdiffraction accuracy remains an active area of research.[14] Additionally, the combination of high sensitivity with the linear dependence of interferometric contrast on the biomolecule's mass[15] provides a novel level of specificity that complements traditional fluorescence label-based sorting.

A growing number of label-free imaging techniques present a promising horizon. These methodologies, which span from single-molecule imaging to chemically specific vibrational imaging, offer a complement to fluorescence-based super-resolution by allowing the visualization of subdiffraction details with added details of the chemical constituents, insight into the molecular interplay, and without the need for labels. This perspective aims to outline the course toward novel imaging techniques that can provide comprehensive insights into the structure and dynamics of complex specimens, surpassing the limitations of the diffraction limit. We will focus primarily on far-field label-free methods that we believe are most promising in shaping the field of super-resolution optical microscopy in the future, while more thorough and complete reviews on the topic can be found elsewhere.[16−18] By drawing lessons from the successes of fluorescence-based super-resolution, we will explore emerging trends in label-free imaging with a particular emphasis on leveraging elastic and inelastic scattering, the most universal forms of light−matter interaction, for groundbreaking advancements in optical microscopy.

DIFFRACTION LIMIT

The spatial resolution of an imaging system is fundamentally constrained by the point spread function. In an aberration-free optical microscope, the size of the point spread function scales linearly with the wavelength of light and inversely with the numerical aperture (NA), which characterizes the angular distribution of light captured from the specimen. Historically, several criteria were used to determine the resolvability of two closely spaced objects at a distance d, based on the contrast drop between them. From the Sparrow criterion, where the contrast drop is nonexistent at $d \approx 0.47\lambda/\mathrm{NA}$, to the Rayleigh criterion, where the contrast drops by ≈20% and the two points are well resolvable by the human eye at $d \approx 0.61\lambda/\mathrm{NA}$. The diffraction limit then derives from the shortest length of spatial frequencies which can be transmitted from the object to the image plane.[19] For coherent illumination at normal incidence the diffraction limit yields $d = \lambda/\mathrm{NA}$ while for incoherent illumination is improved by a factor of 2 to $d = 0.5\lambda/\mathrm{NA}$.[20,21] Interestingly, already Erns Abbe considered a coherent illumination case in his seminal work[6] and argued that under oblique illumination, the diffraction limit can be improved by a factor of 2, matching the incoherent illumination case. The oblique illumination is exploited in recent advanced methods such as quantitative phase imaging.[22−24]

In experimental reality, the assessment of the resolution of the detected images is much more complex and has to include factors such as properties of the light source and sample, light collection and transmission capabilities of the optical system, and the sensitivity and noise properties of the detector.[19,25,26] In this perspective, we will provide only a basic quantitative comparison of the cited methods to Abbe's diffraction limit $d = 0.5\lambda/\mathrm{NA}$ and, where applicable, we express the reported resolution as the percentage of the diffraction limit.

LEARNING FROM FLUORESCENCE-BASED MICROSCOPY

The diffraction limit, a direct consequence of the wave nature of light, sets a ceiling on the clarity and detail that can be attained in images of microscopic specimens. Super-resolution microscopy includes methods that overcome this limit to

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reveal finer details than those the diffraction limit would normally allow. Among these methods, we will focus on those that can show real objects, like parts of cells, with detail finer than the light's diffraction limit.

For over three decades, extensive efforts have been dedicated to formulating the fundamental principles for surpassing the diffraction limit in the realm of fluorescence microscopy.[27−29] The general principles behind these fluorescence-based super-resolution methods can be coarsely divided into three groups, as shown in Figure 1.

The first group, deterministic methods, improves the resolution by controlling the emission pattern of the specimen and enhancing the image resolution based on the prior knowledge of this emission pattern. This category includes structured illumination microscopy (SIM)[30−32] and methods based on the reversible saturable optical fluorescence transitions (RESOLFT)[33,34] concept. SIM uses a periodic illuminating pattern to drive the incoherent[35] emission and the beating of object spatial frequencies with the spatial frequencies of the illumination creates a moiré pattern making the higher spatial frequencies of the sample resolvable. While the linear SIM can achieve a 2-fold improvement of the resolution, the nonlinear variants are not limited by the wavelength of the used light, but rather by the signal-to-noise properties.[31] Techniques under the RESOLFT umbrella, such as stimulated emission depletion (STED)[3,36,37] microscopy and saturated structured illumination microscopy (SSIM),[38] work by selectively turning off fluorescence in certain areas. This leaves a smaller area of emission at predetermined coordinates, allowing for the imaging at the higher resolution. The key benefit here is the ability to enhance resolution directly by modifying the light distribution without needing to detect or pinpoint individual fluorescent molecules.

Another group of techniques here denoted as stochastic relies on turning single fluorophores on and off randomly. High-resolution images are then assembled from many low-resolution images, each capturing a different set of molecules. This approach is based on the precise timing and localization of the fluorescent molecules as they light up and fade out. The underlying mechanism for this fluorescence tethering varies across different methods. For instance, stochastic optical reconstruction microscopy (STORM)[4,39] and photoactivated localization microscopy (PALM)[2,40] depend on the complex photophysics of the fluorophores employed. Point accumulation for imaging in nanoscale topography (PAINT) together with its variant DNA-PAINT driven by a controlled DNA hybridization[5,41] stand out among the fluorescence-based super-resolution approaches with its independence from fluorophore photophysics. These methods rely on the transient or permanent binding of single fluorescent molecules to the specimen to induce temporally separated blinking in the fluorescence signal. While the PAINT concept requires an abundance of unbound fluorophores in the system, the background signal can be effectively suppressed using probes that light up only upon binding to the structure of interest.[5,42]

Some methods, such as minimal photon flux (MINFLUX),[43,44] combine both of the above-mentioned principles as they rely on both, stochastic switching of the fluorescent emitters, as well as the deterministic shape of the emission pattern. MINFLUX utilizes a doughnut-shaped illumination beam to find the position of a single fluorophore when it coincides with the minimum of the illumination beam. This approach offers several benefits such as nanometer-level resolution or increased speed of single-molecule tracking.[45]

Expansion microscopy[46,47] (ExM) represents a unique strategy among the super-resolution methods. Instead of overcoming the diffraction limit by optical means, ExM increases the distances between the adjacent constituents of the specimen above the diffraction limit, so that the image of the specimen can be resolved with finer detail irrespective of the used imaging method. This is typically done by attaching the molecules of the specimen to a swellable hydrogel matrix, breaking the bonds between them and uniformly and isotropically expanding the specimen in all directions. The method can be combined with standard light microscopy to gain up to 20-fold linear expansion and comparable improvement in the resolution.[48] The combinations of ExM with fluorescent-based optical super-resolution methods have been recently reported to further improve the resolution by capitalizing on the synergistic effect of these two complementary methods.[49−52] The limiting factors remain sample fixation and chemical invasion of the system, typically involving protein denaturation or digestion and generation of free radicals during polymerization. The chemical reactions can highly reduce the yield of standard fluorescence probes surviving the expansion process which motivates research into label-retention approaches[53,54] or alternative imaging strategies.

While fluorescence-based super-resolution techniques have enabled imaging at the nanoscale, beyond the diffraction limit, they also come with limitations. These methods depend on fluorescence detection, requiring changes to the specimen and affecting the duration, speed, and accuracy of the observations. The specificity of fluorescent methods is of great advantage when targeting the structure or function of a tiny biomolecular subpopulation of an enormously complex biological system. However, it falls short of addressing the full complexity of the cellular machinery and molecular interplay. Avoiding fluorescent labels in super-resolution microscopy thus remains the major challenge with the potential to influence the future direction of the whole field.

BEYOND THE DIFFRACTION LIMIT WITHOUT THE USE OF LABELS

In optical microscopy, image formation is fundamentally governed by the interaction of electromagnetic radiation at optical frequencies with the specimen. When fluorescent or other labels are excluded, the interactions available for imaging are inherently weaker, including minimal autofluorescence, absorption, and both elastic and inelastic scattering. Among these, inelastic scattering can occur as either incoherent or coherent, encompassing either spontaneous or stimulated processes, respectively.

Label-free super-resolution techniques use these inherently weak interactions, without the need for external labels, to reveal the intricate details of the specimens. These methods draw upon the principles of manipulating light and matter interactions in a similar fashion as outlined in Figure 1 for fluorescence-based methods, albeit the nature of the light-matter interaction plays a crucial role in the choice of the approach. A compelling illustration is seen in the application of SIM within the realm of label-free imaging. The implementation of SIM with incoherent inelastic scattering, such as spontaneous Raman scattering, can indeed improve the resolution beyond the diffraction limit. Similarly to the

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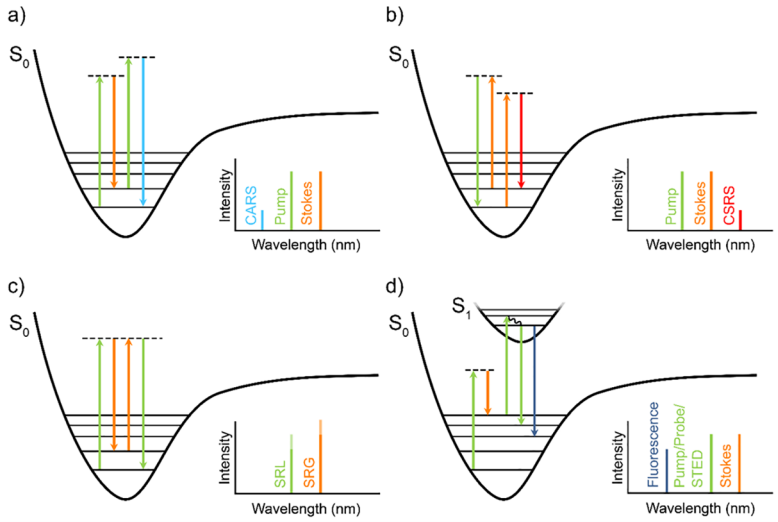

Figure 2. Schematic representation of energy diagrams and relative radiation wavelengths in (a) coherent anti-Stokes Raman scattering (CARS), (b) coherent Stokes Raman scattering (CSRS), (c) stimulated Raman scattering—stimulated Raman loss (SRL) and stimulated Raman gain (SRG), and (d) stimulated Raman excited fluorescence microscopy (SREF).

fluorescence-based techniques, the resolution can be improved by a factor as large as ~2, which has been demonstrated to reach the spatial resolution of 83 nm (532 nm, 1.7 NA, resolution at 53% of the diffraction limit).[31,55] This enhancement is not replicated with coherent (Rayleigh) scattering,[35] but can be even surpassed in coherent Raman scattering due to its nonlinear origin, as demonstrated by 157 nm spatial resolution (40% of the diffraction limit at 800 nm and 1.0 NA).[31,56,57] Such complexity underscores an interesting aspect of how the choice of interaction influences the success of a seemingly straightforward technique like SIM.

Historically, the exploration of high-resolution imaging through the scattering of light approached the boundaries of the diffraction limit. However, as the ambition for higher resolution intensified, the focus shifted toward nonlinear optical phenomena. Nonlinear absorption,[58] photothermal effects,[59] and resonant scattering phenomena[60] have been demonstrated as label-free super-resolution methods, however, remain restrictive on the analyte sample.[16]

An alternative approach to shaping the focal spot size in the far field is found in the phenomenon of optical super-oscillation[61] emerging from the mathematical concept of tailoring a finite-bandwidth function into having zero points at arbitrary positions. The interference of light within a limited field of illumination results in locally sharper spatial fluctuations than those in the diffraction-limited case. Multiple challenges in direct applicability, however, result from very specific and confined patterns comprising an excess of illumination in the vicinity of the narrow field of view. Fortunately, the problems with limited field of view and strong sidebands can be overcome when the superoscillatory hotspot is used as a point illumination source in a confocal, point-scanning microscope.[62] First demonstrations of super-resolution microscopes based on superoscillatory lenses have already been reported.[63,64]

LABEL-FREE SUPER-RESOLUTION CHEMICAL MICROSCOPY

Nonlinear interactions between light and the observed sample enable super-resolution imaging similar to deterministic approaches in fluorescence microscopy. Specifically, the saturation of electronic or vibrational states in specimens, or the mixing of multiple waves, enables the adjustment of the interaction spot's size in a manner akin to techniques that utilize fluorescence saturation. Advantageously, this method does not require special absorbers or fluorescence probes. Instead, it uses more general absorption or scattering processes that are intrinsic to the sample being imaged.[65] This adjustment enhances the ability to probe structural and material details, providing insight into the chemical composition of the specimen.

Pump—probe microscopy, employing transient absorption spectroscopy, shares a conceptual similarity with STED microscopy through the selective deactivation of excitation at the focal point. However, instead of depleting fluorescence, pump—probe microscopy achieves ground-state bleaching.[66] This is accomplished by integrating a nonmodulated pump beam, utilizing optical elements like vortex phase plate[67−69] or spatial light modulator[70] to form a doughnut-shaped bleaching spot. Such an arrangement achieves subdiffraction resolution, demonstrated to reach as low as 36 nm corresponding to only 12% of the diffraction limit at the wavelength of 902 nm using 1.49 NA imaging optics.[67]

Vibrational spectroscopy-based techniques stand out for their label-free nature and chemical specificity, deriving from individual molecular structures' responses. Despite the low cross-section of incoherent spontaneous Raman scattering compared to fluorescence, it has been advanced toward subdiffraction imaging.[71,72] Infrared (IR) absorption offers higher cross sections but operates in the micrometer wavelength range, which limits the diffraction-limited optical resolution. To bring the resolution back to the visible range, techniques like fluorescence-encoded IR[73] and bond-selective fluorescence-detected infrared-excited[74] spectro-microscopy incorporate fluorescent molecules that absorb visible light when in an excited vibrational state, resulting in spatial resolutions of 340 and 600 nm (corresponding to the diffraction limit at the fluorescence wavelength), respectively. While these studies did not reach beyond the diffraction limit, they demonstrated the means to encode the IR absorption

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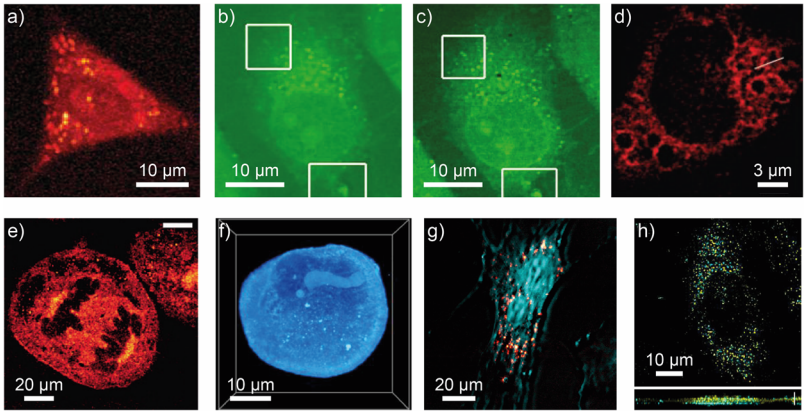

Figure 3. Chemical microscopy images of cells with an enhanced resolution. (a) Stimulated Raman scattering (human lung-cancer). Reprinted with permission from ref 82. Copyright 2008 AAAS (reprinted with permission from AAAS). (b, c) CARS and six-wave mixing CARS, respectively (HeLa). Reprinted with permission from ref 60 Copyright 2020 SNCSC (reproduced with permission from SNCSC). (d) Saturated stimulated Raman scattering (HeLa). Reprinted with permission from ref 85. Copyright 2019 American Physical Society (copyright (2019) by the American Physical Society). (e) Vibrational imaging of swelled tissues and analysis (HeLa, reprinted and adapted with permission under the Creative Commons CC-BY 4.0 from ref 91). (f) Molecule anchorable gel-enabled nanoscale imaging (HeLa). Adapted with permission from ref 92. Copyright 2022 Wiley. (g) Vibrational photothermal relaxation localization (chondroblast). Reproduced with permission from ref 59. Copyright 2023 SNCSC (reproduced with permission from SNCSC). (h) Stimulated Raman scattering with a deconvolution algorithm (HeLa). Reproduced with permission from ref 86. Copyright 2023 SNCSC (reproduced with permission from SNCSC).

image into visible wavelengths to propagate through the optical system. Another step in this direction is photothermal IR[17,75] and its advanced version, vibrational photothermal relaxation localization.[59,76] In Figure 3g the authors used IR to heat the sample without using any labels and probed dynamic changes in the refractive index with visible light. Different temporal behavior of heat dissipation in the center and edges of the beam improved the spatial resolution, reaching resolutions down to 120 nm (approximately 70% of the diffraction limit at 405 nm and 1.2 NA).

Coherent Raman scattering, including coherent anti-Stokes Raman scattering (CARS, Figure 2a), coherent Stokes Raman scattering (CSRS, Figure 2b), and stimulated Raman scattering (Figure 2c) amplifies signals by matching energy differences between vibrational levels to the energy difference between two pulsed lasers.[65,77,78] Since its adoption in microscopy in 1982,[79] CARS has been gaining increasing attention while it remained challenged by nonresonant background signals. High-Order CARS addresses this issue by improving the signal-to-background ratio and enhancing the spatial resolution. A principal improvement in the resolution is achieved by reducing the excitation volume through mixing of multiple waves. While a four-wave mixing process such as CARS involves overlapping two pump photons with one Stokes photon (Figure 3b), six-wave mixing CARS involves combining three pump and two Stokes photons in a spatial overlap (Figure 3c). This advanced technique has demonstrated a higher resolution, achieving 196 nm, which is 65% of the diffraction limit at 846 nm using 1.4 NA optics.[60] CSRS remained largely underexplored mainly due to the challenging suppression of the fluorescence background. However, recent studies indicate, that despite technological challenges, spatial separation of UV-excited CSRS offers numerous advantages such as compatibility with epi illumination and the potential to excel in resolution among other coherent Raman imaging techniques owing to the red-shifted nature of the detected beam.[77]

Stimulated Raman scattering detects signal variation (either loss in the probe beam or gain in the Stokes beam) at the incident laser wavelengths,[80−82] and rather recently it found its way among microscopy techniques (Figure 3a).[82] Similar to CARS, early super-resolution approaches employed doughnut-shaped beams to saturate the signal aiming to enhance the spatial resolution.[83,84] However, cascaded transitions to overtone states hinder signal saturation. Consequently, to achieve saturation, laser power as high as 200 GW/cm^2 may be required[60,85] due to low cross sections and ineffective saturation. The main limitation of these techniques is therefore linked with a potential photodamage.[17] Nevertheless, methods like virtual sinusoidal modulation[85] have shown a potential to partially overcome these limitations and offer advancements of the resolution below the diffraction limit, with spatial resolutions around 255 nm at 846 nm wavelength and 1.4 NA, which corresponds to 85% of the diffraction limit (Figure 3d).

Despite lacking single-molecule sensitivity, the development of deconvolution methods in stimulated Raman scattering has refined the achievable resolution to below 60 nm (20% of the diffraction limit at 797 nm and 1.4 NA), see Figure 3h.[86] Additionally, stimulated Raman scattering excitation linked to fluorescence, as in stimulated Raman-excited fluorescence (SREF[87] and STED-SREF,[88] Figure 2d), and reversible saturable optical Raman transitions[89] demonstrates super-resolution capabilities, with resolutions of 180 nm (50% of diffraction limit at 839 nm and 1.2 NA) and 151 nm (40% of diffraction limit at 839 nm and 1.2 NA), albeit requiring fluorescent labels.[90] Stimulated Raman-excited fluorescence in principle offers a single-molecule sensitivity[87] opening the potential to implement stochastic single-molecule localization approaches to super-resolution chemical microscopy which, however, remains to be explored.

The concept of expansion microscopy offers a uniquely straightforward path to accessing finer details within specimens, essentially by physically enlarging the sample itself. This approach is notably less reliant on the specific imaging technique or the nuances of the light-matter interaction. Yet, the introduction of a physical expansion matrix requires increased specificity in targeting the intended imaging subjects.

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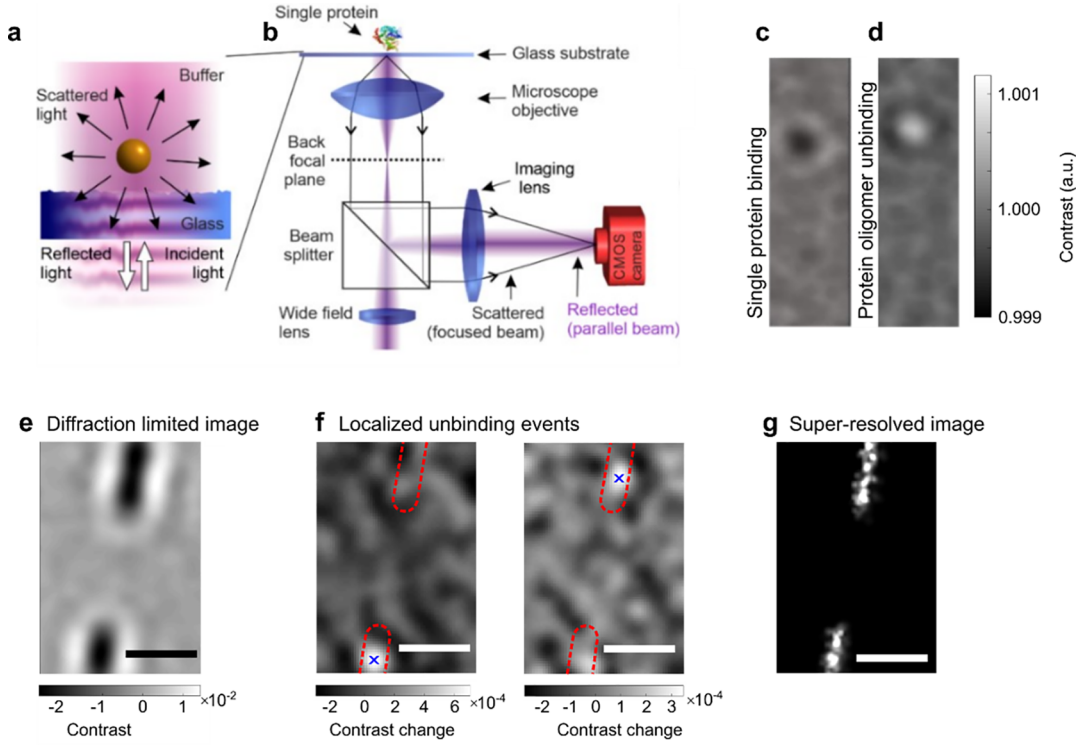

Figure 4. Principle of label-free super-resolution imaging based on molecular turnover and interferometric scattering microscopy. (a) Subwavelength particle and optical fields involved in the interferometric detection of the light scattered by it and (b) optical layout of the microscope with interferometric detection of scattering. Examples of the detected interferometric images corresponding to (c) the binding of single protein to and (d) unbinding of protein oligomer from the imaged sample. Reconstruction of a super-resolved image of a disassembled microtubule. (e) Diffraction-limited interferometric image obtained by an iSCAT microscope during the microtubule disassembly, (f) two examples of incremental images highlighting the localization of two distinct unbinding events, and (g) super-resolved image obtained by overlaying localized positions of detected and localized unbinding events. Scale bars in (e)−(g) correspond to 1 μm. (a, b) Illustration by the author adapted with permission from ref 12. Copyright 2014 Springer Nature. (c−g) Adapted with permission from the published data in ref 14 by the author.

Recent integrations of expansion microscopy with stimulated Raman scattering have demonstrated significant advancements, bypassing some of the method's intrinsic limitations. Vibrational imaging strategy in expanded samples has been demonstrated by Vibrational Imaging of Swelled Tissues and Analysis (VISTA, Figure 3e)[91] achieving a spatial resolution of 65 nm (20% of the diffraction limit at 784 nm and 1.2 NA). The implementation of newer expansion microscopy variants[93] has increased the sample compatibility and improved the spatial resolution down to 41 nm (12% of the diffraction limit at 803 nm and 1.2 NA, Figure 3f).[92] This approach demonstrates nearly 100% protein retention, and together with direct visualization without any label probes, it minimizes the signal loss during the expansion process.

SINGLE-MOLECULE LOCALIZATION-BASED METHODS

The stochastic approach to label-free imaging requires imaging sensitivity at the single-molecule level. Over the past decade, there has been significant progress in detecting and localizing elastically scattered light from single unlabeled molecules, especially proteins, DNAs, and other biomolecules,[12,13,94,95] broadening the scope for label-free bioanalytic and imaging applications.[96] Already the pioneering study in interferometric scattering microscopy (iSCAT) with single-protein sensitivity demonstrated the potential to render super-resolution images of spatial distributions of binding sites by localizing bindings to a functionalized surface with a localization precision of 5 nm (4% of the diffraction limit at 405 nm and 1.46 NA).[12] At the heart of the iSCAT technique is the interference between light scattered by the sample and a coherent reference beam, typically a reflected beam (Figure 4a),[9,13,97] transmitted beam,[98,99] or a beam scattered on a reference object,[94,100] typically in a common-path interferometer arrangement. The scattered light is collected by a high-numerical-aperture oil-immersion objective and is imaged on a camera where it interferes with the reference light (Figure 4b).

The high sensitivity of the interferometric detection enables precise imaging and localization of single biomolecules by distinguishing those transitioning between diffusive and confined states (Figure 4c,d) or tracking their displacement in highly diluted solutions. The localization precision $\sqrt{\langle (x)^2 \rangle}$ of a single scattering object, such as a molecule, is dependent on the amplitude of the corresponding signal and the noise. In the case of iSCAT measurement and the approximation of a weak signal dominated by the shot noise of the bright background, the localization precision yields[101,102]

$$\sqrt{\langle (x)^2 \rangle} = \frac{\sigma_a}{\mathrm{SNR}}, \quad \sigma_a^2 = \sigma^2 + \frac{a^2}{12} \tag{1}$$

where σ is the standard deviation of the point-spread function (approximately half of the Abbe limit $\sigma \approx \lambda/4NA$), σ_a encompasses the correction for the pixelization, a denotes the pixel size and SNR is the signal-to-noise ratio. In a shot-noise-limited regime, the SNR can reach the maximum value of

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SNR $\leq 2\sqrt{N_{scat}}$, where N_{scat} is the number of scattered photons detected in each frame.[103] A localization precision of <2 nm is typically achievable for small scattering labels such as 20 nm large gold nanoparticles, and a precision of 5 nm has been achieved with single unlabeled proteins at SNR = 10.[12]

Unlike fluorescence-based techniques, where sample complexity can be adjusted through labeling efficiency and specificity, label-free microscopy must enhance the imaging speed and detection sensitivity to match the intrinsic dynamics of biological systems. By capturing natural fluctuations at single-biomolecule sensitivity, we can reveal details of the structures with subdiffractional clarity. Direct rendering of complex biomolecular structures from fluctuations attributed to the turnover of single-molecules closely resembles the course of PAINT super-resolution microscopy[5,41,104,105] on temporal separation of single-molecule interactions without the reliance on the intrinsically blinking fluorophores. In the label-free alternative, PAINT derives the visibility of the imaged structure from the contrast difference between the sharply focused scattering probes attached to the structure and defocused and motion blurred images of those diffusing in the volume. The label-free PAINT approach was demonstrated in vitro on microtubules, where a super-resolved image was rendered from the localizations of proteins detaching from the disassembling microtubule tip with a localization precision around 20 nm (10% of the diffraction limit at 660 nm and 1.3 NA, at SNR $\geq$ 3),[14] as shown in Figure 4e−g. An iSCAT microscope, operating at 3000 frames per second, was sensitive enough to detect unbinding tubulin oligomers with a > 50% detection yield, allowing for a detailed reconstruction of the super-resolution image of the microtubule (Figure 4g) and the dynamics of its disassembly. In combination with polarization-sensitive iSCAT,[106] the technique discerned the subdiffractional shape and orientation of the macromolecular structures based on their anisotropy.[14]

Interferometric scattering microscopy stands out for its sensitivity and precision in single-molecule localization, achieving accuracies down to a few nanometers.[12,13] Its suitability for quantifying the mass of detected biomolecules,[15] as evidenced by its linear relationship with interferometric contrast, has been validated for DNA,[107] lipids,[15] and proteins ranging from 40 kDa to 5 MDa, catalyzing a whole new field of mass photometry.[108−110] This quantitative capability introduces an additional level of specificity to label-free super-resolution imaging, complementing the chemical specificity derived from inelastic scattering-based microscopy. Beyond detecting and quantifying single molecular species, tracking the diffusion and interactions of single biomolecules and complexes enriches our understanding of nanoscopic biological mechanisms, such as lipid membrane-associated protein interactions and aggregations,[111−113] demonstrated with diluted samples. When pushing the label-free super-resolution microscopy such as the label-free PAINT toward more complex specimens, we can expect the mass-specificity to become increasingly important in discerning different biomolecular species.

Another important aspect of super-resolution microscopy, particularly in its mission toward the complex environment of living cells, is its compatibility with three-dimensional samples. Introducing the third dimension to 2-D projections, nanoscopic quantitative phase imaging has been developed. By manipulating the phase shift between the scattered and reference field[114,115] or encoding phase information into the point-spread-function shape,[116] the scattering amplitude and phase of single scatterers can be simultaneously discerned, enhancing precision and the localization readout. Among recent innovations is single-protein optical holography,[117] merging total internal reflection illumination with optical quadrature microscopy. Though distinct from common-path interferometry, this method's separation of the reference beam allows comprehensive phase control and simplifies amplitude and phase measurements of nanoparticles and single biomolecules, thus allowing for quantification of their complex polarizabilities or axial localization.

While the applicability of iSCAT-based super-resolution concepts remains to be explored and validated in biology and biophysics we take the opportunity to highlight its perspective as a general tool in biological systems. In one example, high-speed (30000 fps) monitoring of individual cell vesicles in live cells in 3D with approximately a nanometer localization (<1% of the diffraction limit at 532 nm and 1.4 NA) precision has been demonstrated to gain insight into the molecular dynamics in living cells.[118] Nevertheless, it is important to note that the prevalence of single-biomolecule interaction studies based on iSCAT detection has been limited to in vitro studies. While super-resolution microscopy is supposed to find its major strength in increasingly complex specimens, where the resolved details offer new knowledge, whole-cell imaging with legible details in iSCAT has been challenging for the past decade. The basis for this conceptual challenge is found in the principle of iSCAT image formation involving only two optical modes of light interfering at the detector, thus resulting in a pure interferogram of highly quantitative information content. As sample complexity increases, more optical fields overlap on the detector, leading to intricate images with unclear details.[119] Recent studies have shown, that the background scattering contribution can be effectively minimized in a confocal arrangement of the experiment where only a diffraction-limited segment of the specimen is illuminated.[99,120]

Nanofluidic scattering microscopy, another extension of interferometric scattering microscopy principles, offers label-free detection of freely diffusing molecules confined within a nanochannel.[100] This technique overcomes the need for surface binding and enables real-time imaging of single biological nanoparticles as small as tens of kDa, providing insights into their molecular weight and diffusivity. Although the requirement for a nanofluidic arrangement differentiates this technique from the super-resolution methods reviewed in this discussion, it introduces unifying concepts that enhance the sensitivity and spatiotemporal resolution in the detection of unlabeled single molecules.

COMPUTATIONAL-ENHANCEMENT MICROSCOPY

In addition to super-resolution techniques derived from fluorescence-based methods, a growing number of strategies employ advanced image processing and computational signal interpretation. The field of computational microscopy has continually aimed to enhance the quality of images obtained through label-free techniques, dating back to the 1970s with developments in computational methods for image enhancement and denoising.[121−123] Recently, advancements in machine learning, especially the integration of artificial intelligence (AI), have opened new avenues for computational image enhancements in label-free microscopy.

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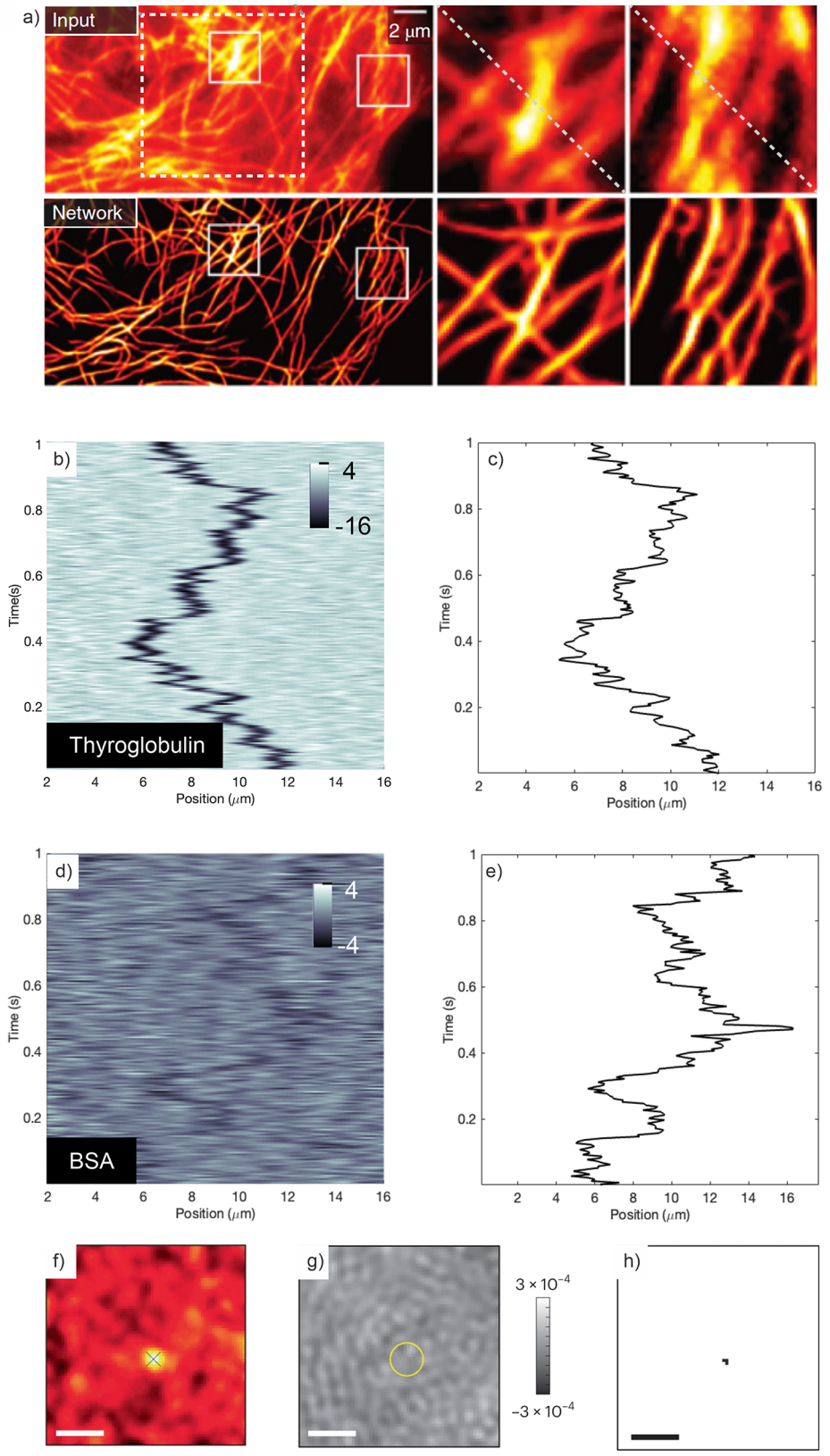

Figure 5. Computationally enhanced feature detection. (a) Raw image of GFP-tagged microtubules in HeLa cells and the CARE network restored image with cropped details of the fine structure, reproduced with permission from ref 144. Copyright 2018 SNCSC. (b and d) Nanofluidic-scattering-microscopy kymographs of one-dimensional trajectories of single (b) thyroglobulin and (d) albumin (BSA) confined in a nanochannel; (c and e) corresponding single biomolecule trajectory identified using a neural network. Kindly adapted with permission from the published data in ref 100 by the author. Detection of a 9 kDa protein in (f) fluorescence and, simultaneously, in elastic scattering using a (g) differential rolling

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Figure 5. continued

average algorithm and (h) results of the isolation forest classification based on probability maps. Color bar: binary classification. (f−h) Scale bars, 1.5 μm. Reprinted and adapted with permission under the Creative Commons CC-BY 4.0 from ref 140.

Convolutional neural networks, originally successful in digitally enhanced photography,[124] have inspired further exploration into their applicability across various medical imaging modalities, including magnetic resonance imaging,[125] computed tomography,[126] and phase retrieval.[127] In fluorescence microscopy, deep-learning algorithms have helped the visualization of intracellular dynamics with enhanced details.[128] These computational algorithms significantly reduce acquisition times[129,130] and improve the throughput of fluorescent microscopes,[131] enabling faster and more efficient data collection without the need for hardware modifications.[132,133] Utilizing the temporal evolution of images, the super-resolution radial fluctuations algorithm has improved real-time imaging of microtubules within cells.[134,135] Broadly, deep learning methods have proven compatible with single-molecule localization microscopy techniques, both in labeled[136−138] and label-free contexts.[139−143]

These methods often extend beyond mere signal enhancement based on presumed characteristics of the specimen such as temporal or spatial continuity. For example, Figure 5a demonstrates the restoration of a diffraction-limited fluorescence image of GFP-labeled microtubules in He−La cells using a content-aware image restoration (CARE) network trained on fully synthetic data of continuous filament structures.[144] Although the specificity of fluorescent labeling is highly effective for identifying a single type of structure within complex specimens, deep-learning strategies are increasingly being applied to enhance resolution in label-free modalities, such as dark-field microscopy,[139] and to refine the interpretation of quantitative imaging techniques involving phase and polarization.[143] Object reconstruction via neural networks, which are trained using numerous scattering events, has been successfully demonstrated in label-free subwavelength optical microscopy.[141,142] Additionally, a synergistic approach that combines deep learning with adaptive optics has been developed to correct aberrations in both the microscope and the specimen.[145,146]

The next example of interconnecting spatial and temporal information in biological imaging is demonstrated by researchers using a novel real-time deep learning denoising method.[147] They effectively denoised data with an SNR < 5, achieving a 10-fold increase in imaging SNR beyond the shot-noise limit in real time. This enhancement facilitates high-sensitivity observations under photon-limited conditions, allowing for the precise visualization of rapid biological processes such as brain activity in vivo.

Another case, depicted in Figure 5b−e, illustrates the effective 1D tracking of extremely low-contrast single-molecule scattering signals in nanofluidic scattering microscopy, leveraging their temporal continuity.[100] The kymographs in Figures 5b and d display the profile of the scattering contrast over time. Specifically, for the larger molecule (Thyroglobulin) in Figure 5b, the data enable straightforward localization of one-dimensional displacement, showcasing the trajectory with a localization precision of less than 2 nm (wavelength range 450−750 nm, NA = 1.27, SNR > 10). Conversely, for the smaller molecule (BSA in Figure 5d), the signal contrast is frequently masked by noise over multiple consecutive frames. Despite these challenges, the trained network consistently produces continuous trajectories and provides quantitative data on contrast and diffusivity (Figure 5e).

Recently, self-supervised machine learning has advanced the sensitivity in label-free detection of single proteins to below the 10 kDa threshold.[140] The detectability of single molecules in an interferometric scattering microscope, as seen in Figure 4c is limited by the photon noise and temporal instabilities of background speckles. With the decreasing size of detected molecules, the interferometric signal gets fainter, however, the arrival of the molecule is characterized by a step-like fluctuation in the normalized image. For a sufficiently small molecule, the contrast of the fluctuation becomes too low to be identified in a simple differential image (or a sequence of differential rolling averages) shown in Figure 5g; however, the temporal signature of the arriving molecule within a diffraction-limited radius differs from random fluctuation. Therefore, unsupervised machine learning using the isolation forest algorithm has detected highly probable anomalies (Figure 5h) closely aligned with the positive control detection in a fluorescence channel (Figure 5f), thereby enhancing the mass sensitivity limit by a factor of 4.

Although the field of deep learning for super-resolution microscopy is rapidly evolving, it is essential to be cautious of the potential for AI-generated artifacts.[144] Researchers are actively developing solutions to this issue, and techniques to mitigate these risks are increasingly available.[148,149]

PERSPECTIVE AND OUTLOOK

As we explore the evolving landscape of label-free super-resolution microscopy, it is clear that transformative advancements are already taking shape. Deterministic super-resolution approaches now offer astonishing chemical specificity, capable of rendering images of whole living cells in action. Similarly, stochastic super-resolution techniques discretize biomolecular systems to their most fundamental components, capturing the turnover of single proteins within dynamic organelles. Meanwhile, expansion microscopy is progressing beyond the limitations of fluorescent labeling while preserving the precise chemical specificity offered by vibrational imaging. By examining these developments in the context of their practical applications and inherent limitations, we can foresee a course for the next generation of label-free imaging technologies that will deepen our understanding of complex biological systems.

Nonetheless, significant challenges remain. Label-free methods analogous to STED, which utilize coherent Raman scattering, continue to struggle with low inelastic scattering efficiency and, consequently, limited signal levels. This results in modest resolutions and speeds that are difficult to improve. Although achieving single-molecule resolution may range from challenging to impossible with inelastic scattering concepts, the specificity of imaging in highly complex systems provides unparalleled benefits with each incremental improvement in resolution. Conversely, the detection speed and sensitivity of stochastic label-free approaches, which focus on the localization of single molecules through elastic scattering, are rapidly evolving, from seconds[12] to milliseconds,[14] for

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detecting a single-molecule fluctuation. This trend, although promising, faces substantial hurdles in matching the dynamic complexity of living systems. Without the benefit of sparse labeling, stochastic label-free methods must capture all single-molecule fluctuations at their native rates, dealing with the high molecular density typical in cellular environments.

We can highlight the experimental challenges related to the native density of molecular fluctuations through the concept of label-free PAINT, underscoring its potential as a universal imaging tool for biological systems, a potential that still requires thorough exploration and validation. It is reasonable to expect molecular traffic in all living systems, whether incoming, outgoing, or translocating within the structure. However, detecting this traffic on a molecule-by-molecule basis presents significant challenges. The molecules or other scattering probes must be sufficiently large and have long enough residence times on the structure to be detectable. Additionally, the background signal, namely, the image of the structure itself, must remain relatively static compared to the scattering probes that diffuse in and out, allowing for effective separation of their scattering signals. Recent advancements aimed at increasing detection sensitivity and speed are addressing these issues: background fluctuations diminish at higher acquisition rates, and with microsecond integration times, we can reasonably expect to be feasible to distinguish hundreds of discrete interactions within each millisecond. Nonetheless, improvements in sensitivity and detection rates inherently compete, as both require increased illumination intensity and scattering rates, necessitating careful consideration of potential photodamage to the sample.

The advantages of high-speed single-protein studies are well recognized, and the integration of small scattering labels[150] with single-biomolecule dynamics has demonstrated its value in describing complex biological details that go beyond their simple subdiffraction shapes. Techniques like these, which probe the dynamics of epidermal growth factor receptors (EGFRs) on HeLa cell membranes[151] or ASE-1 motor proteins interacting with microtubules,[8] highlight the need for further innovation in high-speed, label-free imaging. While the challenges are predominantly technical and not fundamentally impossible, they underscore the need for continuous technological advancements.

Looking ahead, avoiding labels, especially fluorescent ones, in super-resolution studies not only simplifies specimen preparation but also preserves the native state during the investigation. Observing individual proteins in their natural environment is crucial for a thorough understanding of biological functions. Label-free super-resolution techniques open a new level of insight into the dynamics of single-molecule processes and offer a more complete view of biomolecular activity in living systems. Exploring both elastic and inelastic scattering allows for the rapid capture of nearly continuous single-molecule trajectories, essential for matching the time scales of biomolecular interactions. Additionally, the continuous nature of single-molecule fluctuations enhances the utility of advanced data processing and deep-learning algorithms, aiding in the interpretation of highly dynamic systems. By the merging of high-speed imaging with label-free single-protein analysis, there is significant potential to revolutionize our understanding of the intricate, dynamic world of biomolecular assemblies and living systems.

The upcoming generation of super-resolution techniques may transform how researchers analyze their data. While fluorescence-based microscopy has enabled the dissection of specimens into individual contributions from various biomolecular species, providing enhanced detail, we now aspire to capture the complete interplay among these species. This includes identifying them by their spectral fingerprints or masses and discerning their mechanisms in an almost continuous manner. We envision future recordings that reveal the comprehensive molecular machinery within a segment of a living cell, illustrating the activity of each biomolecule. The trajectories and forces at play, colorfully mapped based on molecular mass or interaction probabilities, promise to illuminate the once obscure complexity of biological systems at the molecular level (Figure 6). This would not be merely a shift in perspective, it would be a leap toward decoding the dynamic symphony of life itself.

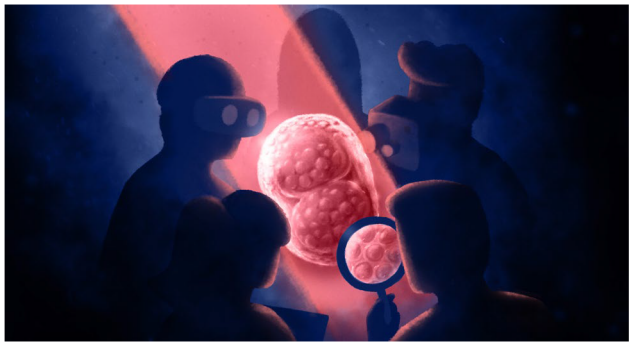

Figure 6. Illustration portrays the diversity of optical imaging techniques used to analyze dynamic biological entities. Illustration by Pavel Trávníček.

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Notes

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