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Label-free, ultrasensitive, ultrahigh-speed scattering-based interferometric imaging

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ABSTRACT

Label-free microscope imaging techniques allow direct visualization of biological substances in their most native forms. This review article provides an overview of recent advancements of scattering-based, interferometric laser microscopy and their applications to ultrasensitive and ultrahigh-speed biological imaging. In particular, common-path, widefield interferometric laser microscopy, namely interferometric scattering (iSCAT) microscopy and coherent brightfield (COBRI) microscopy, are discussed in details. Using these simple yet powerful optical techniques, single proteins and individual endogenous biological nanoparticles can be imaged and tracked without any label at ultrahigh spatiotemporal resolution. The development of ultrasensitive and ultrahigh-speed scattering-based interferometric microscope imaging enables investigation of biophysical and biochemical processes with minimal perturbation and unprecedented clarity.

1. Introduction

Biological processes start from interactions between individual molecules at the nanoscale. To elucidate the origins of cell functions and their underlying principles, it is useful to observe the dynamics of single molecules. Optical microscope imaging is a unique tool that can reveal dynamics in live cells down to the single-molecule level. Single-molecule imaging has been heavily reliant on fluorescence [1]. The targeted molecule of interest is tagged by a fluorescent probe, (*e.g.*, nanoparticle, protein or dye molecule), which emits a red-shifted fluorescence signal under excitation. When the surrounding environment does not have significant background fluorescence, fluorescence microscope imaging provides a convenient method for seeing single molecules. Modern scientific cameras and detectors offer sensitivity even to single photons. As a result, seeing single fluorescent molecules has become routine in laboratories. In addition, proper fluorescence labeling achieves high imaging specificity. Although single-molecule fluorescence imaging and detection have enabled many valuable studies over the past two decades [2–4], fluorescence methods have encountered several challenges and fundamental limitations. For example, it is always a concern whether the labeling could possibly perturb the native behavior of the targeted biological substance [5,6]. Furthermore, a limited fluorescence photon budget due to photobleaching restricts the total observation time. Saturation of the fluorescence signal as a result of fluorescence lifetime sets the maximum number of fluorescence photons per unit time, which leads to limited signal flux even if the fluorescent probe is highly resistant to photobleaching. Therefore, there has been a consistent demand to develop alternative detection and imaging methods that provide a high sensitivity at a high data acquisition rate without the need for labeling.

Detecting linear scattered light from an object of interest could be a solution to the aforementioned problems. When no label is required, no labeling artifact is present. A linear scattering signal is stable and indefinite, facilitating long-term observation. The strength of a linear scattering signal can be increased by raising illumination intensity. However, seeing a small, nano-sized object via scattering is still challenging. When an object of interest is small in size (*e.g.*, a nano-sized biological particle or a protein molecule), its scattering signal is easily overwhelmed by the background scattering that is created by the complexity of the sample or the imperfections of the optical system.

This review summarizes the most recent advances in widefield interferometric laser microscopy techniques that detect linear scattered light from very small objects (*i.e.*, single native biological nanoparticles and biomolecules) without labeling. Although several interferometric microscopy techniques work with low-coherence light sources [7–13], the use of highly coherent laser sources makes it possible to have sufficient illumination intensity and optical contrast for ultrasensitive and ultrahigh-speed measurement. In particular, this review focuses on two closely related techniques of scattering-based interferometric laser microscopy: interferometric scattering (iSCAT) microscopy [14,15] and coherent brightfield (COBRI) microscopy [16,17]. The combination

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of scattering-based interferometric laser microscopy and proper background correction [18] enables state-of-the-art sensitivity and temporal resolution for label-free imaging.

The content of this review is arranged as follows: first, the concept of scattering-based interferometric laser microscopy is explained. Next, the optical setups of iSCAT and COBRI are introduced and compared. The following two experimental sections demonstrate the two decisive advantages of scattering-based interferometric laser microscopy, sensitivity and speed. Finally, the opportunities and challenges of scattering-based interferometric laser microscopy are discussed.

2. Concepts of scattering-based interferometric laser microscopy

Seeing a small, nano-sized object via scattering has multiple difficulties. One technical difficulty is the presence of electronic noise of the detector. In darkfield microscope measurements where the scattering intensity is measured [19], electronic noise is present, and the signal must be stronger than the electronic noise to be detected. For very small objects, strong illumination is required to generate adequate signals above the electronic readout noise level. Interferometric detection offers a solution to improve the detection sensitivity beyond the limit set by detector noise. Rather than measuring the scattered intensity, interferometric detection measures the interference between the scattered signal and a strong coherent reference beam. Interferometric detection pushes the detection sensitivity to a shot-noise limited regime where the signal-to-noise ratio (SNR) is only determined by the number of detected signal photons, independent of the detector noise (information on the shot-noise limited sensitivity of interferometric imaging can be found in Ref. [20]). This property becomes especially advantageous in ultrasensitive and ultrahigh-speed measurements where the number of signal photons is limited; high-speed electronics inevitably possess considerable electronic noise.

To achieve the strongest interference contrast (and thus the highest SNR), a highly coherent light source (*i.e.*, a laser) is preferred. When using a coherent light source for illumination, speckles and interference fringes caused by the multi-reflections of optical components in the beam path (*e.g.*, lens, beamsplitters, microscope objectives, and the cover window of the camera) usually create a non-uniform background that complicates the detection of a weak scattering signal. In the most sensitive measurements, any imperfections of the sample (*e.g.*, impurities in the buffer solution, roughness of the sample coverglass) can provide a heterogeneous background that is markedly stronger than the signal. In theory, the background could be measured in advance and removed from the raw image. In practice, the background is often time-varying due to the fluctuating optical path difference between the signal and the reference, making precise background correction challenging. In general, common-path interferometry provides the most stable background because optical path difference between the signal and the reference beam is minimal. This technical advantage becomes indispensable in ultrasensitive, label-free imaging where high-quality background correction is necessary.

3. iSCAT and COBRI microscopy

Recently, several ultrasensitive and ultrahigh-speed label-free measurements have been demonstrated by common-path, scattering-based interferometric laser microscope techniques. Sandoghdar, Kukura, and their coworkers conceived of iSCAT microscopy, where a sample is illuminated by laser light through a microscope objective and the back-scattered light is collected by the same objective, and subsequently detected via interference (see Fig. 1a) [21–24]. In iSCAT, the reference beam is created by the reflection at the interface between the supporting coverglass and the aqueous solution [15]. iSCAT microscopy can be considered a specialized interference reflection microscopy [25] that uses a highly coherent illumination light source.

The other approach to common-path interferometric laser microscopy is to collect the forward-scattered light as the signal where the non-scattered transmitted light serves as the reference. Such an arrangement is essentially a brightfield microscope that uses a laser for illumination. It is thus termed COBRI (coherent brightfield) microscopy (see Fig. 1b) [16]. More experimental details of iSCAT and COBRI microscopy can be found in Ref. [15] and Ref. [16], respectively. Because both iSCAT and COBRI microscopy record interference between a signal and a reference, they have sometimes been referred to as holographic microscopy [26]. However, iSCAT and COBRI microscopy take images of objects at the focal plane of the microscope objective and thus no holographic reconstruction step is involved, which makes them distinct from typical holographic microscopy.

The iSCAT and COBRI microscopy share many common features. Both offer shot-noise limited sensitivity where the SNR is determined by the number of detected signal photons. Therefore, their detection sensitivities are identical under the same illumination intensity. Nevertheless, in practice, iSCAT and COBRI are operated at very different illumination intensities. iSCAT microscopy usually allows 100 times stronger illumination than COBRI microscopy; in iSCAT, only a small portion of illumination is reflected at the water–coverglass interface, serving as the reference beam, whereas in COBRI, most of the illumination is transmitted and arrives on the camera. As a result, given the same camera operated at the same image acquisition rate, iSCAT microscopy can excite a sample with more light (before saturating the camera), leading to more scattered signal photons from the object. The typical illumination intensities for iSCAT and COBRI microscopy at an acquisition rate of 1000 frames per second (fps) are on the order of 1 kW/cm^2 and 0.01 kW/cm^2, respectively.

In addition to the distinct operating light intensity, iSCAT and COBRI microscopy have another major difference. The reflection geometry of iSCAT microscopy makes it sensitive to partially reflecting interfaces of the sample. Taking advantage of its high sensitivity to interfaces, iSCAT microscopy was recently demonstrated to be capable of determining the number of atomic layers of graphene (interference reflection microscopy, IRM, is essentially iSCAT microscopy with a low-coherence light source) [27]. For the same reason, cell plasma membranes become apparent under iSCAT microscopy. Depending on the application, the plasma membrane signals in an iSCAT image may greatly complicate the observation of dynamics of nano-sized objects in cellular environments. Conversely, COBRI microscopy of transmission geometry is insensitive to the reflective interfaces of a sample. Therefore, the cell plasma membrane is nearly transparent under COBRI microscopy, which usually enables more convenient observation of cellular dynamics.

4. Ultrasensitive imaging and tracking of native single biological molecules

In interferometric measurements where shot-noise limited sensitivity is achieved (either by iSCAT or COBRI), every detected signal photon contributes to perceptible information above the noise level. iSCAT microscopy is sensitive and can be extremely fast: it can detect single 20 nm gold nanoparticle with 2 nm spatial precision at 500,000 fps [20], single native microtubules with sub-nanometer spatial precision at 1000 fps [28], phase-separated lipid nanodomains as small as 50 nm at 500 fps [29], and orientation and DNA content of an unlabeled bacteriophage (λ) at 100 fps [30].

Given that the measurement is shot-noise limited, the strategy of improving sensitivity is to collect more signal photons from the small objects. Intuitively, stronger illumination leads to more signal photons. Technically, a maximal illumination intensity exists for successful measurements: the number of detected photons of the reference beam must be smaller than the full well capacity of the camera, otherwise the camera becomes saturated. To push the boundaries of the detection sensitivity, several experimental arrangements have been reported which allows more light to illuminate the sample without saturating the detector. Two main strategies have been used: one operates through image accumulation using post-processing [31–33], the other operates through hardware modification [34–37].