MICROSCOPY

Single-molecule light absorption

It has long been thought that the detection of individual molecules in ambient conditions via their absorption signature was out of reach. Now, three independent research groups have developed three different methods that allow such a feat.

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In the natural sciences, insights on the molecular scale are often derived from bulk spectroscopic studies. Statistical analysis is used to relate these macroscopic, ensemble-averaged spectroscopic observations to the time-averaged pictures of individual molecules at the microscopic level. Such analysis, based on ergodic theory, masks static and dynamic heterogeneities and fails to describe complex, inhomogeneous systems.

There would of course be many benefits to being able to make direct observations of the dynamic behaviour of individual molecules. This has been the major incentive behind the advances in single-molecule studies over the past two decades. Whereas initial studies explored single molecules by probing the strong zero-phonon transitions at cryogenic temperatures[1,2], current room-temperature optical studies allow us to zoom in on the dynamic behaviour of individual molecules even in the smallest compartments of living systems, thus enhancing our understanding of the nanoscale organization of cells, tissues and other materials[3,4]. These *in vivo* and *in vitro* studies, however, solely rely on the detection of fluorescence and hence often require labelling with dedicated fluorophores as the majority of molecules under investigation have no or only weak fluorescence emission.

Now three new, non-fluorescence-based approaches have been developed independently that allow the observation of individual molecules through their absorption of visible light[5–8]. These methods open perspectives for single-molecule studies using a much larger group of molecules that absorb light but do not necessarily fluoresce. One of these new optical methods, developed by Celebrano *et al.*[8] and described in detail in *Nature Photonics*, allows for the direct imaging of individual molecules by monitoring the attenuation induced in probe light.

Direct observation of optical absorption by single molecules is heavily complicated by the very small light attenuation imparted to the probing light beam (Fig. 1a). Calculations with typical molecular absorption cross-sections, often smaller than a few square ångströms, and a tightly focused light beam, as used in optical microscopes, reveal that less than one photon in a million is absorbed. Given that inherent intensity fluctuations of light sources can easily be of the order of one per cent, it can be seen that the task of detecting these molecular induced light attenuations is especially challenging. At the heart of the solution that was developed by Celebrano and co-workers[6,8] is a dual-beam set-up that is also used in conventional UV–visible spectrometers: the incoming laser beam is split into two parts, one of which is tightly focused and probes the sample, and the other of which serves as an intensity reference. By balanced detection of both beams it is possible to virtually eliminate intensity fluctuations of the light source. When careful attention is paid to optimizing the signal read-out, intensity fluctuations down to the shot noise limit can be measured[6]. At this sensitivity, where the main source of signal variations is the statistical fluctuation of the number of photons, it becomes possible to measure single molecular light absorption events when signals are averaged over a sufficiently large time window. Whereas in their first report[6], absorption by individual fluorophores was only possible after locating them first using their fluorescence

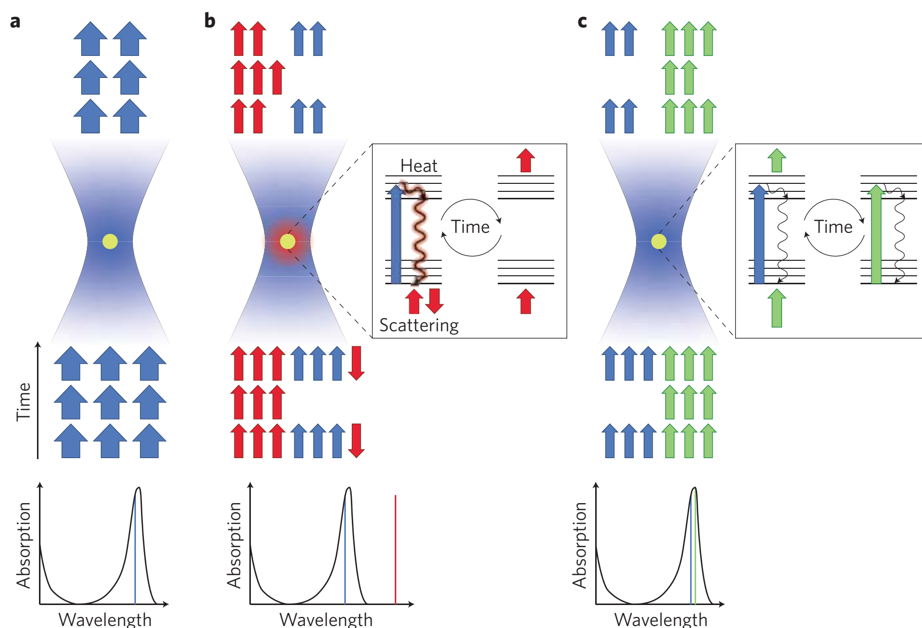

Figure 1 | Three different methods that allow detection of light absorption by single molecules (yellow dot) in the focus of a light beam (blue). **a**, Direct observation of a single molecule by measuring its attenuation of a light beam[6,8]. **b**, The local heating induced by light absorption results in tiny changes in the local refractive index, which can be probed by the enhanced backscattering of a second, probe, beam[5] (red). Fast intensity modulation of the absorbed blue beam results in measureable modulations in backscattering intensity with the same frequency. **c**, Modulation of the blue beam influences the absorption of the green beam by depleting the ground state. These fast intensity modulations in the green beam can be measured in transmission mode[7].

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signals, in their new work Celebrano *et al.*[8] directly image the absorption of individual chromophores by scanning the sample. Three methods that can further enhance the contrast, namely subtraction of the background found after photobleaching or use of the polarization-sensitive or wavelength-sensitive absorption response of a molecule.

Because the measured attenuation of the probe light beam is not only induced by absorption processes but also by scattering in the sample, it is important to minimize surface roughness and match the refractive index throughout the sample to achieve single-molecule sensitivity. These restrictions, together with the limited time resolution, of around 1 s per molecule, are all areas that have to be improved for this approach to be applicable to more heterogeneous samples such as cells, tissues or other multicomponent materials.

Interestingly, two alternative single-molecule detection schemes have also been described in recent literature by researchers that are well recognized for their pioneering work in optical microscopy: Michel Orrit[5] and Sunney Xie[7]. These approaches both make use of a two-colour pump–probe scheme and fast modulation of the undetected beam to reduce the measurement noise.

The scheme developed by Orrit and co-workers[5] detects the local heating of the sample induced by light absorption of molecules in the microscope's focus (Fig. 1b). After excitation, molecules will lose some of the absorbed energy through non-radiative decay processes[5]. As a result, heat is released into the molecule's close environment. The set-up developed by Orrit and co-workers probes this photothermal effect with a second laser beam, to which the sample is transparent, via the enhanced scattering of this locally heated zone with slightly altered refractive index. To detect intensity fluctuations of the probe beam, a fast modulation of the pump (or heating) beam was incorporated. Because the locally induced heat dissipates into the surrounding medium, this modulation can also be retrieved in the second probe beam, from its temporarily enhanced backscattering. Even though this method allows imaging of non-fluorescent molecules with a very good signal-to-noise ratio, the method crucially depends on heat-induced changes in the refractive index and the level of heat conduction. For some liquids, such as glycerol, which was used in the reported study[5], the heat-induced changes in refractive index close to room temperature are sufficiently large to detect individual chromophores. However, in aqueous environments room-temperature measurements are problematic as small temperature changes result only in minute changes in refractive index. Hence, single-molecule absorption measurements in a biological context remain a challenge with this approach.

The method developed by Xie and co-workers[7] also relies on two different laser beams and a fast modulation scheme, but a different molecular process, ground-state depletion, is probed. The sample is illuminated by two tightly focused laser beams (a pump and a probe) that have different wavelengths but are both within the molecular absorption band of the molecule under study. The first, pump, beam excites a molecule such that it resides in its excited state and, hence, photons from the second probe beam cannot be absorbed. Fast on–off modulation of a strong, saturating pump beam therefore results in the modulation of the transmitted probe beam with the same modulation frequency. With this approach Xie and co-workers imaged single chromophores by simultaneously monitoring the probe beam attenuation and recording the fluorescence signal from the same spot. After localizing an individual molecule in the fluorescence channel, the absorption signal of this molecule was imaged repeatedly using line scans. By averaging over many of these line scans, a clear increase in probe beam transmission at the location of the individual molecule could be observed.

Because these experiments rely on the saturation of the electronic transition, this scheme requires relatively intense excitation powers. The fact that chromophores can withstand the photon fluxes required for repeated saturation of electronic transitions was demonstrated before as it lies at the heart of super-resolution stimulated emission depletion microscopy, pioneered by Stefan Hell[9], and can be rationalized on the basis of the photophysics of the molecules involved[10]. A challenge that still remains is applying the detection scheme of Xie and co-workers[7] to non-fluorescent dyes with short, picosecond, excited-state lifetimes.

Whereas single-molecule fluorescence techniques owe their success to the fact that the fluorescence signal can be detected in a wide variety of settings, these newly developed single-molecule absorption methods heavily rely on specific experimental conditions. In only 15 years single-molecule fluorescence has evolved from a curiosity into a technique that affects many research fields. Hence, a frenzy of research activities to improve these absorption schemes and make them more flexible and user friendly can be anticipated. Exciting times in optical microscopy lie ahead. ❐

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