Highlights

3D Nanoscopy: Bringing Biological Nanostructures into Sharp Focus

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*U*nderstanding the nanometer-scale organization of living cells, tissues, and materials is an essential goal of present and future research efforts, and yet zooming into the small compartments of life remains one of the great challenges of science. Even though microscopy techniques such as electron microscopy can help us, these approaches are usually limited by the elaborate and destructive sample preparation and measurement, or by the inability to study details beyond the surface of the sample.

Fluorescence microscopy, on the other hand, has proven to be one of the most convenient and widespread tools to study key issues in the life sciences, though the problems that it can address are still limited. As with any microscopy technique, there is a lower limit to the sizes of the details that can be discerned. This finite resolution is a direct consequence of the wavelike character of light, known as the diffraction limit, which is about 250 nm in optical microscopy. This means that it is not possible to discriminate between two objects that are separated by less than that distance, and thus it seems that the nanostructuring of cells and materials is not accessible by direct optical means. More specifically, (part of) the reason that the resolution is limited is because a fluorescent "point object" of negligible size (such as a single molecule) does not appear as a point after imaging through the microscope but rather appears as a three-dimensional shape of much larger dimensions (Figure 1). Because the distributions from adjacent molecules in close proximity overlap we lose the ability to distinguish between molecules that are close together, and hence to visualize very small details of the sample.

Several schemes recently developed to address these limitations have succeeded in providing images with unprecedented detail.[1] In particular, several papers appeared in the last half of 2006 that converged into a similar approach, which has become known as photoactivation–localization microscopy (PALM).[2–4] PALM relies on the insight that individual, well-isolated emitters can be localized with nanometer precision simply by determining the centroid of the
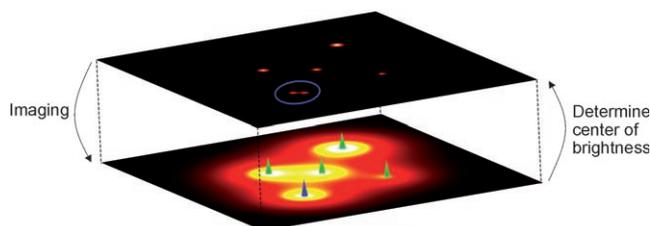

Figure 1. The effect of diffraction: the original pointlike fluorescence emitters appear as a blurred distribution after imaging through the microscope. The positions of the isolated emitters can be recovered by determining the center-of-brightness of the resulting spots (indicated by the markers), although molecules that are spaced closer together than the diffraction limit cannot be separated (the blue marker and blue circle).

emission distribution (Figure 1). If we could determine the position of all the emitters in this way then we could construct an image of the sample with nanometer resolution by carefully processing the fluorescence image. Unfortunately, the obtained data is meaningful only if we are indeed looking at the emission of a single isolated emitter (Figure 1), yet if we are interested in imaging beyond the diffraction limit then our sample likely contains many fluorophores within nanometers of each other. As a result, the only way to apply this localization procedure to practical samples is to separate the emission of the fluorophores in time, so that only a single fluorophore emits within a diffraction-limited region at any instant.

In PALM this is achieved by making use of photoactivation: the stochastic "on" switching of the fluorescence emission at the single-molecule level followed by subsequent photobleaching or other deactivation. Hence PALM measurements are based on repeating cycles of on/off switching events, where fluorescence can be registered only for those molecules that are in the "on" state during a particular cycle (Figure 2). Because the activation of the fluorescence is a stochastic process, different molecules will be activated in each cycle, and if these molecules are spaced sufficiently apart then we can reconstruct the high-resolution picture. The need to control the fluorescence emission of the sample, down to the single-molecule level, through photoirradiation has recently inspired many studies and developments of photoswitchable fluorescent proteins[5–9] and other compounds,[10–12] though the method was applied mainly to fluorescent proteins

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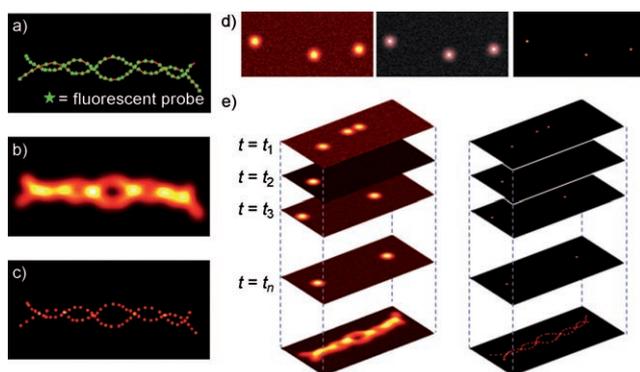E-mail: johan.hofkens@chem.kuleuven.be

Figure 2. The principle behind PALM measurements. a) A target object labeled with photoactivatable fluorescent tags. b) Diffraction-limited fluorescence image. c) A reconstructed image resulting from the PALM approach. d) Principle of nanometer localization by means of 2D Gaussian fitting: left: charge-coupled device (CCD) image of activated fluorescent molecules; center: 2D Gaussian fitting; right: image reconstruction by using the Gaussian centroids of the activated molecules. e) Photoactivation–localization cycles: CCD images recorded at different time intervals (left) show the emission from different molecules that are individually localized with nanometer precision (right), followed by the reconstruction of the complete image.

until now.[2,4,13] In fact, the key concept exploited in PALM and related techniques is the stochastic nature of all single-molecule processes combined with the presence of a non-fluorescent and a fluorescent state. As a result, fluorescence photoswitching is ideally suited to these measurements, as was pointed out by Hell et al. in 2003,[14] though other phenomena, such as diffusion followed by docking[15] and individual turnovers in chemical reactions,[16] can also be used.

While some impressive results have been demonstrated so far, the technique is not without limitations. In particular, the need for many photoactivation cycles to determine the positions of a large amount of molecules often leads to very long measurement times (up to hours). Moreover, the technique does not provide inherent optical sectioning (the positioning is precise only in the sample plane, that is, perpendicular to the optical axis of the microscope), and as a result the samples either have to be very thin or have to be illuminated using a total internal reflection (TIRF) geometry. Additionally, spontaneous activation of the dye molecules or a rather low contrast between the "on" and "off" state can lead to a significant amount of fluorescence background, which renders the analysis more difficult.

Recent work published by Fölling et al.[17] provides important and significant steps towards overcoming these limitations. In their extension of the technique, which they term PALMIRA (PALM with independently running acquisition), this is achieved by a dual approach, involving the rational design and synthesis of a novel photoactivatable compound while simultaneously extending the experimental procedure to include two-photon activation of the fluorescence.

The novel photoactivatable compound, termed SpaRh, is a rhodamine-based photochromic system that can be switched from a nonfluorescent to a fluorescent state through photo-irradiation, while the spontaneous formation of the fluores-

cent state is very slow. While this photoactivation requires ultraviolet light, the bright state of the chromophore absorbs green light and emits red light. The large separation between these absorption bands means that the problem of fluorescence background due to residual emission from molecules in the "off" state can be nearly completely avoided, and in combination with the slow thermal activation of the fluorescent form a denser labeling of the sample is possible. The back reaction to the dark state is thermally induced, with a rate constant that is dependent on the environment, and with lower survival times in polar surroundings (milliseconds). Moreover, the fact that this dye is a rhodamine derivative is advantageous to some measurements, as not all cellular components can be labeled with a genetically encoded fluorescent protein in a straightforward way, and direct chemical labeling is required in these cases.

With this compound in hand, the authors set out to demonstrate its applicability to PALM-based high-resolution microscopy. They show that individual dye molecules provide enough photons (ranging from over 300 photons in 2 ms to about 900 photons in 20 ms) to allow their localization with a high precision (11–14 nm) and requiring very short integration times (2–20 ms). These results further show that the limited survival time of the bright state of SpaRh is not a disadvantage, but rather it allows for very fast measurement cycles, drastically reducing the required measurement duration. While previous PALM measurements required measurement times of over two hours,[4] Fölling et al. demonstrate the acquisition of meaningful images in only 1–3 minutes. Hence the use of this dye brings the possibility of dynamic photoactivation–localization microscopy one step closer to reality. Moreover, these dynamic measurements would be facilitated if the single molecules could be activated and localized more than once, limited only by photobleaching. This is, in principle, similarly provided for by the short survival time of the bright state in aqueous environments, as the fast recovery can deactivate the molecules to the non-fluorescent state before photobleaching takes place. In this way the molecules can be reactivated in subsequent imaging cycles.

The experiment described in the last section of the article is perhaps the most innovative contribution in the publication and makes use of the inherent optical sectioning provided by two-photon absorption. By activating the dye molecules in a thin layer with picosecond pulses of red light, the authors can effectively restrict the fluorescence to that plane only, leading to a depth resolution similar to that of a confocal microscope. When different planes of the sample are measured, the resulting slices can then be arranged together to obtain a fully three-dimensional high-resolution image (Figure 3). In a different but related study Hell and co-workers expanded the use of PALM microscopy to the simultaneous detection and analysis of two different labels,[18] which further extends the possibilities of this technique to include the study of molecular interactions.

Owing to its relative simplicity, we believe that PALM-based microscopy will become an essential and highly popular tool in the future. Certainly, 3D imaging and fast acquisition times were on the "wish list" for PALM microscopy, and
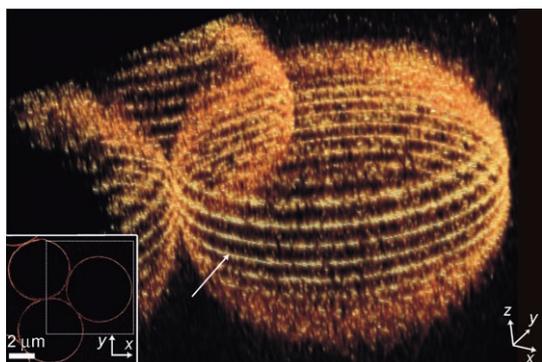
Figure 3. A reconstructed three-dimensional PALM image of 5-µm silica beads coated with SpaRh, as recorded by Fölling et al.

consequently the developments reported by Fölling et al. represent key steps for the application of the technique to intracellular imaging. Altogether, we can say that this technique is presenting us with a new window into the fascinating world of biological nanostructures.

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