Trends in fluorescence imaging and related techniques to unravel biological information

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Optical microscopy is among the most powerful tools that the physical sciences have ever provided biology. It is indispensable for basic lab work, as well as for cutting edge research, as the visual monitoring of life processes still belongs to the most compelling evidences for a multitude of biomedical applications. Along with the rapid development of new probes and methods for the analysis of laser induced fluorescence, optical microscopy over past years experienced a vast increase of both new techniques and novel combinations of established methods to study biological processes with unprecedented spatial and temporal precision. On the one hand, major technical advances have significantly improved spatial resolution. On the other hand, life scientists are moving toward three- and even four-dimensional cell biology and biophysics involving time as a crucial coordinate to quantitatively understand living specimen. Monitoring the whole cell or tissue in real time, rather than producing snap-shot-like two-dimensional projections, will enable more physiological and, thus, more clinically relevant experiments, whereas an increase in temporal resolution facilitates monitoring fast nonperiodic processes as well as the quantitative analysis of characteristic dynamics. [DOI: 10.2976/1.2778852]

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Optical microscopy dates back about 350 years when Hook developed the compound microscope and, although it has been greatly improved since then, the basic principle is still the same ([Hooke, 1664](#)). Indeed, it has become an indispensable tool for life scientists that allows seeing intact samples, including live cells and organisms, with the naked eye. This is not the case for other, more invasive microscopic techniques, e.g., electron or scanning probe microscopy, although they exhibit superior spatial resolution. One of the most important developments and major breakthroughs in optical microscopy, however, was probably the introduction of fluorescence microscopy. Since then, fluorescence microscopy has not only become the method of choice for a plethora of life-science applications, but also the foundation for many fluorescence-microscopy-based techniques. These

technical developments on the one side and the reductionist approach to study molecular mechanisms rather than whole species on the other side started a positive feedback loop aimed at distinguishing smaller and smaller structures faster and in more detail, even down to the molecular level.

The resolving power of a microscope is limited by the wave-like nature of electromagnetic radiation. Hence, the spatial resolution of a light microscope is limited to about half the wavelength of visible light, i.e., about 200 nm. Although the diffraction limit seems fundamental, finding new ways to circumvent it, and thus increase spatial resolution, has been both a successful and promising field of research for the past years, pushing the spatial resolution limit to below 30 nm. However, such a dramatic increase in resolution is currently only feasible for thin fixed samples or extremely long measure-

ment times. Therefore, alternative techniques concentrate on three-dimensional imaging — and rendering — of whole organisms, if possible in real time or even faster. To enhance the local temporal resolution, these imaging techniques can be combined with other methods based on single-point measurements, e.g., based on fluorescence resonance energy transfer (FRET), fluorescence lifetime, or fluorescence fluctuation correlation spectroscopy (FCS).

Most of the techniques discussed here are still restricted to the physics laboratory. Only time will tell which of them will be converted into commercial instruments, i.e., instruments that are both straightforward to use and understand and still give high-quality data for standard measurements in a biology laboratory or standard imaging facility.

INTRODUCTORY CONCEPTS

Fluorescence

Fluorescent molecules are capable of absorbing light at specific wavelengths to be excited to a higher electronic state and subsequently relax back to the ground state by emitting longer wavelength fluorescence light. The difference in wavelength between absorption and emission is known as Stokes shift. The phenomenon of fluorescence is highly selective because the transition of a molecule to a higher electronic state can only occur through absorption of a suitable photon with energies much larger than kT . This leads to good signal-to-noise ratios and high selectivity in fluorescence microscopy, compared to all other kinds of optical techniques.

The excited state lifetime of fluorescent molecules is another useful parameter that can be exploited for fluorescence imaging. Typical fluorescence lifetimes of autofluorescent proteins and commonly used synthetic dyes are between 1 and 5 ns. Moreover, the fluorescence lifetime is subject to environmental parameters because the more polar excited state is stabilized in a polar environment, resulting in longer lifetimes. However, all physical features of fluorescence dyes, i.e., the color, the excited state lifetime, and the emission intensity, can be used in fluorescence microscopy to study biological processes that would otherwise be inaccessible.

Optical resolution

Abbe was the first to formally describe the finite resolution of an optical lens (Abbe, 1873), which is caused by the diffraction of light at small structures, such as cells, that act like a grating on illuminating rays. A specific detail in the specimen can be resolved when the numerical aperture (NA) of the objective lens is large enough to capture the first-order diffraction pattern produced by the detail at the wavelength used.

Even if a point-like object is viewed through the microscope, its image is significantly broadened compared to its original dimensions. The intensity distribution of this image is called the point spread function (PSF). The PSF usually

exhibits radial symmetry laterally, whereas it is broadened along the optical axis. Lord Rayleigh was the first to derive the resolved distance of two identical, diffraction-limited point sources with the aid of Bessel functions in 1879. This separation arises when the peak of one Bessel function falls on the first zero point of the other function. This means that the resolution limit of a conventional light microscope in lateral direction is given by

$$d_{\text{lateral}} = \frac{0.61 \cdot \lambda}{NA} \quad \text{with } NA = n \cdot \sin \alpha, \quad (1)$$

whereas

$$d_{\text{axial}} = \frac{2 \cdot \lambda}{NA^2} \quad (2)$$

in axial direction. This is also known as the diffraction limit, which depends on the wavelength of the light, λ , and the NA of the objective lens, which in turn is given by the refractive index, n , of the medium between lens and sample and the sine of half the observation angle, α . In conventional wide field microscopy in the visible spectrum, the lateral resolution is, therefore, about 200 nm and the axial resolution about 1000 nm. However, alternative descriptions of the optical resolution include the Sparrow criterion, which depends on the property of the summed intensity profiles. To cut a long story short, the narrower the PSF of an optical setup, the higher the resolution that can be achieved. Various approaches and the possible resolution will be discussed below in more detail, for a schematic overview see Fig. 1.

Figure 1. A graphic summary of the best resolution obtained to date in lateral (x, y) and axial (z) direction for various optical microscopy methods. Methods to which the Abbe theory applies are represented as solid symbols whereas the other methods are represented by empty symbols and italic labels. For the surface-bound localization methods (STORM, etc.) no axial resolution is given. FIONA (semi-filled gray star) is, strictly speaking, no imaging technique. However, it is included here as ultimate limit for STORM and related localization-based techniques. (For the corresponding references please see the corresponding sections in the text.)

THREE-DIMENSIONAL (SPATIAL) INFORMATION

Conventional (wide-field) microscopy is the most common and omnipresent type of optical microscopy. The only required piece of equipment is a compound microscope, whose main parts are the objective lens and the eyepiece lens. For infinity-corrected objectives, an additional tube lens is required to realize flexible working distances. This design has been used successfully for more than three centuries, and the main improvements in the optical resolution resulted from higher-quality components. The correction of the objective lens is as crucial nowadays as it has been in the past to avoid aberrations.

Nearly all techniques described below rely on this kind of microscope — or at least the objective lenses — as instrumental basis. It is therefore important to understand that it is only worthwhile to bother with resolution enhancement if the classical means have been exploited, i.e., if the instrumental basis is an optimally corrected and diffraction-limited conventional optical microscope.

Wide-field microscopy

In the age of electronics, microscopic images are usually recorded digitally using charge-coupled device (CCD) cameras. These area detectors, which exhibit extremely high quantum efficiency, collect light from all parts of the field of view simultaneously. They are optimized for a wide range of different applications. Digital image processing, especially various deconvolution methods using the instrument's previously determined PSF (Le Puil *et al.*, 2006) are applied to enhance small details in two- and three-dimensional images taken on a conventional microscope. Due to its impressive simplicity, however, it will likely remain one of the key techniques employed by life scientists for many years to come.

Confocal microscopy

The principle of confocal imaging was patented in 1961 (Minsky, 1961), making this technique one of the earliest approaches for resolution enhancement. The principle of optical sectioning was combined with fluorescence microscopy more than 20 years later to increase the two-point resolution by more than a factor of 2 compared to conventional microscopy (Cox *et al.*, 1982). Today, with a large number of commercial instruments available, confocal fluorescence imaging has become one of the indispensable standard techniques in many laboratories, despite the drawback of requiring fluorescent labeling of the structures or molecules to be studied. In contrast to the wide-field illumination used in a conventional microscope, a confocal microscope uses point illumination by a laser and a pinhole in an optically conjugate plane in front of the (point-)detector to eliminate out-of-focus light. Two- or three-dimensional images are acquired by raster-scanning the excitation volume over the sample. An example for a three-color 2D image is shown in Fig. 2. With all improvements made in the past years regarding the speed

Figure 2. Three-color image of HeLa SS6 cells stained using immunofluorescence taken on a confocal scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany) using a 63x/1.2W objective lens. Microtubuli are stained in green (Alexa 488, top left) and P-bodies in red using RCK/p54 (Alexa 647, top right). DNA is shown in blue (Hoechst 33342, bottom left). An overlay of all three spectral channels is shown in the bottom right panel. The scale bar corresponds to 10 μm . (Courtesy of Thomas Ohrt, Biophysics Group.)

and pointing precision of scanners, this is still a rather slow process. Spinning-disk (Nipkow-disk) confocal microscopes are faster, because the single pinhole is replaced by a spinning disk with strategically placed arrays of tiny holes, but temporal resolution is increased at the cost of spatial resolution. This is also true for one of the more recent developments where line illumination is combined with a slit and a detector array (LSM 5 Live, Zeiss, Jena). Nevertheless, this instrument allows the recording of three-dimensional movies at still nearly-confocal resolution in real time.

Multi-photon excitation

Linear methods for improving the optical resolution rely either on directly improving the PSF, e.g., by increasing the numerical aperture of the objective, or manipulating it indirectly via interference, which will be discussed below in more detail. This is different for the so-called nonlinear optical methods, the oldest and most straightforward being multi-photon excitation (MPE). These techniques can achieve very small volumes from which fluorescence can be detected, thus also indirectly improving spatial resolution. Multi-photon microscopy provides major advantages for studying live cells and even biological tissue, because of the increased penetration depth for longer wavelengths. For two-photon (multi-photon) excitation, a fluorescent molecule has

to simultaneously (within $\sim 10^{-15}$ s) absorb two (or more) photons of longer wavelength than required for the corresponding one-photon excitation (OPE). For photons of the same wavelength, the transition probability is proportional to the square of the incident intensity (Göppert-Mayer, 1931). Thus, fluorophores are only efficiently excited close to the center of the focal region, which increases the spatial resolution. For the lateral resolution, this effect is balanced by the significantly longer wavelength of the incident photons, but the axial resolution is improved. MPE was first applied in laser scanning microscopy by the Webb group (Denk *et al.*, 1990).

As other prominent nonlinear methods, second harmonics generation (SHG) and coherent anti-stokes Raman scattering (CARS) should be mentioned. The former is especially suited for thick tissues, whereas the most prominent advantage of the latter is that it works without any fluorescent labels. More recent imaging-relevant nonlinear approaches like stimulated emission depletion (STED) and reversible saturable optical (fluorescence) transitions (RESOLFT) microscopy will be discussed below in more detail.

Light sheet illumination

The idea of illuminating samples from the side to observe small particles in a distinct plane within a specimen was first proposed more than 100 years ago (ultramicroscopy) (Siedentopf and Zsigmondy, 1903). This principle was extended to light sheet illumination in an imaging technique termed orthogonal-plane fluorescence optical sectioning (OPFOS) in 1993 (Voie *et al.*, 1993). The authors imaged the internal architecture of the cochlea with a lateral and axial resolution of 10 and 26 μm , respectively. A similar approach is used for single or selective plane illumination microscopy (SPIM) (Huisken *et al.*, 2004; Greger *et al.*, 2007). Here, a biological sample from a few micrometers up to several millimeters in size is illuminated with a “two-dimensional” light sheet rather than a point projection as in confocal microscopy, i.e., a laser beam that is collimated in one and focused in the other direction. Then the three-dimensional data set is recorded by moving the sample through the stationary light sheet while recording the fluorescence light perpendicular to the illumination plane with one or two CCD cameras. The lateral resolution of the subsequent image is comparable to a conventional fluorescence microscope and the axial resolution is improved up to a factor of 15. Due to the massively parallel approach, the temporal resolution is several orders of magnitude higher than for conventional confocal instruments (Greger *et al.*, 2007). Additionally, this method still provides inherent optical sectioning, because no fluorophores are excited outside the focal plane of the detectors. Combined with single-photon excitation and excitation powers in the μW range, this ensures reduced overall photobleaching of the fluorophores and, hence, lower phototox-

icity (Keller *et al.*, 2006). Combining light-sheet based ultramicroscopy with a special procedure to render fixed mouse brains transparent, Dodt and co-workers obtained 3D images with single-cell resolution (Dodt *et al.*, 2007). They could not only obtain anatomical detail when imaging whole fruit flies and mouse embryos using only autofluorescence, but they could also reconstruct 3D images of dendritic trees of GFP-labeled neurons in mouse hippocampi.

4Pi microscopy

The wave-like nature of electromagnetic radiation is responsible for the interference of different light rays, which can be seen in the form of a periodic illumination pattern. These patterns can be specifically created in the object plane of a microscope to excite fluorescent molecules so that the emitted light exhibits more spatial information than possible by conventional means. The most straightforward technical realization is based on two opposing objective lenses below and above the thin, transparent sample, one for each excitation beam. Alternatively, the fluorescent light can be collected by both objective lenses and combined to interfere in the focal plane of the CCD camera. A positive side effect of this geometry is an effective increase in the axial resolution by a factor of two, provided spherical illumination wave fronts are used (Freimann *et al.*, 1997; Nagorni and Hell, 2001).

The term 4Pi microscopy is rather suggestive: The principal idea is to get as close to illuminating and detecting from all sides of the sample, i.e., a spatial angle of 360° . However, for practical reasons a setup featuring two opposing high-numerical aperture objective lenses is used and combined with confocal illumination and detection. The excitation light beams create an interference pattern in the common focal volume (Hell and Stelzer, 1992a; Hell and Stelzer, 1992b; Hell *et al.*, 1994). The most pronounced resolution improvement is in axial direction, whereas the lateral resolution is only minimally better than a standard confocal setup. The acquisition times can be shortened by a parallel approach using multiple two-photon laser beams creating multiple focal volumes (Egner and Hell, 2000). This technique is termed multifocal multiphoton 4Pi confocal microscopy (MMM-4Pi). By deconvoluting the three-dimensional scanned data sets, spatial resolutions of about 100 nm are feasible (Egner *et al.*, 2002). 4Pi microscopy was implemented in a commercially available extension to a two-photon confocal microscope (Leica, Mannheim, Germany).

Image interference microscopy

In image interference microscopy (I^2M), a fluorescently labeled thin sample is mounted between two objective lenses facing each other and having a common focal plane within the sample. The two magnified images obtained are combined and superimposed onto a CCD camera. In this kind of setup, each chromophore gives rise to two light beams that interfere with each other on the camera chip after having

traveled the same optical path length. This interference pattern contains high-resolution information about the axial position of the molecules. Instead of using wide-field illumination through only one objective lens, excitation light from an “incoherent” light source (e.g., a standard arc or mercury lamp emitting light with extremely short coherence length) can also be split into two light beams impinging onto the sample through both objective lenses. These interfere to create an axially varying illumination pattern similar to standing wave microscopy (Bailey *et al.*, 1993). This technique is termed incoherent image interference microscopy (I²M). I²M and I³M are independent, but both of these approaches suffer from gaps in the axial optical transfer function and, thus, are severely limited in practice. Nevertheless, a combination of I²M, I³M, spherical rather than flat illumination wave fronts and deconvolution methods yields a working microscopic method with an increased axial resolution of about 100 nm (I⁵M) (Gustafsson *et al.*, 1999). A three-dimensional stack of images is collected by scanning the common focal plane through the thin sample.

Stimulated emission depletion

STED was introduced by Hell and co-workers more than a decade ago and has recently shown its enormous potential for biological applications despite the impressive complexity of the experimental setup (Hell and Wichmann, 1994; Willig *et al.*, 2006). This technique is using a modified confocal setup in which the pulsed excitation laser beam is focused to a diffraction limited spot. Superimposed on this first laser is a second one, the so-called STED beam that is phase-modulated to form a focal doughnut. The wavelength of the latter is chosen to optimally deplete the excited state of the chromophores and return them to the ground state. In this way, the volume in which fluorescence is possible is confined to the center of the doughnut, and only from this sub-diffraction sized spot can fluorescence emission be detected. Images with a resolution below 30 nm have been published, although there is no fundamental limitation to the size of the STED volume except the number of detectable photons. Even two-color applications have been demonstrated (Donnert *et al.*, 2007b). To date, the radiation damage imposed on the sensitive live biological sample is still a major issue, but recent developments are promising.

Structured-illumination microscopy

Structured light illumination *per se* can already provide enhanced axial and lateral resolution. This method was first introduced in 1997 by Neil and co-workers (Neil *et al.*, 1997) and has been implemented commercially by Zeiss (Apo-tome, Zeiss, Goettingen, Germany). Based on a conventional light microscope, it exhibits the same axial resolution as a confocal microscope and a better signal-to-noise ratio. Gustafsson and co-workers refined this approach to saturated structured-illumination microscopy (SSIM) with an axial

resolution of below 50 nm (Gustafsson *et al.*, 1999). Briefly, SSIM is exploiting the inverted principle of STED. The corresponding theory was established by Heintzmann and colleagues (Heintzmann *et al.*, 2002; Heintzmann, 2003). Here, two high-power interfering laser beams are focused onto the same sample spot for structured illumination. This means that the chromophores located within the illuminated sample regions saturate, leaving only a small fraction of unsaturated fluorophores at their edges. With increasing light intensity, these regions become smaller than any classical PSF. Although the required hardware is significantly cheaper than for some of the other resolution-enhancing techniques described below, the need for extremely photostable fluorescent dyes and/or specially treated fixed samples still precludes some of the usefulness of this technique.

Reversible saturable optical (fluorescence) transitions microscopy

However, STED microscopy is just the first example of a more general principle dubbed reversible saturable optical (fluorescence) transitions (RESOLFT) microscopy (Hell, 2003; Schwentker *et al.*, 2007; Keller *et al.*, 2007). This encompasses any reversible light-driven and saturable transition between two distinct molecular states, one of which is fluorescent. RESOLFT-type microscopy is not inherently diffraction limited and it can be combined both with confocal and wide-field illumination. The diffraction limited spatial illumination pattern is chosen to include zero-intensity points or lines. Along those, no molecules are transferred from one state to the other. If the transition is saturated, the spatial extension of the zero-intensity regions decrease to below the diffraction limit, theoretically even down to molecular scales. Collecting the fluorescence emission from these (scanned) regions allows reconstructing an image with spatial resolution below the diffraction limit. Novel photoswitchable autofluorescent proteins can efficiently be transferred from one fluorescent state to either a dark or a spectrally distinct state by illumination at certain wavelengths. These are extremely promising candidates to investigate the principle of RESOLFT microscopy in biologically relevant samples.

Dark-state relaxation microscopy

This is actually not a microscopic technique *per se*, but a recently developed and very efficient illumination scheme to extend the “lifetimes” and, thus, the number of emitted fluorescence photons per chromophores before photobleaching (Donnert *et al.*, 2007a). It can be combined with nearly all of the microscopic techniques described here.

To maximize the emission intensity, often high excitation intensities are applied, especially when using multi-photon excitation (see below). This causes both an enhanced population of molecules in the triplet state and increased photobleaching and, thus, actually reduces the fluorescence signal. However, if molecules trapped in a long-lived absorbing

dark state (lifetime $> 1 \mu\text{s}$) such as the triplet state have time to relax to the ground state before being re-excited, the amount of photobleaching is reduced dramatically and the same photon yield from the same sample is up to 25-fold higher (Donnert *et al.*, 2007a). This can be realized by pulsed excitation with a temporal pulse separation of longer than the lifetime of the relevant dark state, e.g., $1 \mu\text{s}$, thus allowing for dark or triplet state relaxation (D-Rex or T-Rex). This increase in image brightness with the same overall excitation power per pixel can be verified both for one- and multi-photon excitation. Hence, the total measurement time per frame is increased due to the additional relaxation times.

TWO-DIMENSIONAL (SURFACE) INFORMATION

Surface methods

There are also a few methods that only allow intrinsically very high-resolution two-dimensional measurements of surfaces or structures extremely close to surfaces. Particularly in the study of cell membranes, such techniques can be favorable due to their efficient discrimination of background light emanating from deeper layers of the observed cell or tissue which are not supposed to contribute to the observed processes and phenomena.

Scanning near-field optical microscopy

First demonstrated for microwaves in 1972 (Ash and Nicholls, 1972), scanning near-field or near-field scanning optical microscopy (SNOM, NSOM) is currently one of the best commercially available options for ultra-high optical resolution. Although the detection elements of an NSOM system are similar to that of wide-field microscopes discussed above, the excitation strategy is quite different. The laser light is directly coupled into a metal-coated optical fiber with a tip aperture of less than half the wavelength of the incident light, typically 20–200 nm. Hence, this light gives rise to an evanescent field, a phenomenon that cannot be explained within the framework of classical physics. The volume illuminated by the optical near-field has the tip diameter and a depth of a few nanometers. The tip is then scanned across the sample at a distance of about 10–50 nm. Images with nanometer-scale resolution are possible, as shown for membrane domains or single nuclear pore complexes (Hoppener *et al.*, 2003; Hoppener *et al.*, 2005).

Total internal reflection fluorescence microscopy

Total internal reflection fluorescence microscopy (TIRFM) is also based on an evanescent field, which is created by an exciting laser beam incident at a high angle onto a glass coverslip (objective-type TIRF with a high-NA objective) or a prism (prism-type TIRF) (Axelrod, 2001). Snell's law describes how light is reflected or refracted at the interface between two different media as a function of their respective refractive indices and the incident angle. At a specific critical angle, the light ray is totally reflected from the glass/water

interface. It can no longer propagate into the optically thinner sample medium. However, an evanescent field is generated within the aqueous medium. This can be used to excite fluorescent molecules only in an extremely thin ($\sim 100 \text{ nm}$) layer on the surface, thus enhancing axial resolution. The emission is collected with an objective lens. Recent applications include determining the step size of molecular motor proteins by combining this technique with pattern illumination to also enhance lateral resolution (Cappello *et al.*, 2003). Commercial add-ons to conventional light microscopes are offered by most major microscope manufacturers.

Fluorophore-based methods

The methods described so far are neutral insofar as they try to enhance the spatial resolution irrespective of the sample. However, it is also possible to exploit prior knowledge about the system under investigation or its special physical properties to enhance resolution where necessary. Particularly interesting are the approaches which utilize the detection of single fluorescent molecules. The fast progress in dye development (including the release of GFP and related genetically encoded probes), along with the rapid improvement in detector and laser technology, has promoted single molecule methods from selected physical chemistry labs to almost all larger imaging facilities, where nowadays EMCCD cameras or avalanche photomultipliers capable of single photon counting are state of the art equipment, being combined with either wide-field or confocal laser illumination.

Fluorescence resonance energy transfer

Förster (or fluorescence) resonance energy transfer (FRET) is a physical concept rather than a special microscopic method *per se*, but it is unique in generating fluorescence signals sensitive to molecular conformation, association, and separation in the 1–10 nm range. Thus, FRET is capable of resolving distances far below the diffraction limit, yet it is not only compatible with conventional microscopic methods but also with most of the super-resolution techniques. In FRET, energy is transferred nonradiatively, via long-range induced dipole-dipole coupling, from an excited fluorescent donor molecule to another molecule serving as acceptor. The latter may also be nonfluorescent. The transfer rate k_t and, hence, the transfer efficiency E , varies inversely with the sixth power of the intermolecular distance R_{DA} , as first described by Theodor Förster in 1948 and experimentally shown more than 40 years ago (Forster, 1948; Stryer and Haugland, 1967):

$$E = (1 + (R_{\text{DA}}/R_0)^6)^{-1} = 1 - (I_{\text{DA}}/I_{\text{D}}). \quad (3)$$

The Förster radius R_0 is a coefficient depending on the donor quantum efficiency, the orientation of the dipole moments, and the spectral characteristics of the dye molecules and the surrounding medium for a given molecular system. I_i are the fluorescence intensities measured in presence or absence of

the donor molecule, respectively. The natural donor fluorescence lifetime τ_0 is then shortened to

$$\tau^{-1} = \tau_0^{-1} + k_t. \quad (4)$$

For more detailed information see, for example, [van der Meer *et al.* \(1994\)](#), and [Clegg \(2002\)](#). In well-defined single-donor, single-acceptor systems, ratiometric fluorescence measurements involving the spectrally distinct donor and acceptor signals can be calibrated to yield the FRET efficiency. However, they require an accurate reference for the acceptor-free donor signals and are difficult to achieve in practice. The determination of fluorescence lifetime, either in the time or in the frequency domain, is one of the most straightforward measures of FRET. A comprehensive review of FRET imaging techniques can be found in [Jares-Erijman and Jovin \(2003\)](#).

Fluorescence imaging with one-nanometer accuracy

Because the position of a single bright particle can be determined extremely accurately, single-molecule detection offers alternative possibilities to obtain ultra-high spatial resolution. Selvin and colleagues developed this already-existing approach further to fluorescence imaging with one-nanometer accuracy (FIONA) in order to monitor the step size of molecular motors at work ([Yildiz *et al.*, 2003](#)). This fluorescence-based real-time localization technique, with 1 nm spatial resolution and down to 1 ms temporal resolution, uses TIRF illumination to reduce noise and a novel oxygen scavenging system to render the fluorophores brighter and more stable. Imaged with a CCD camera, the PSF of the single molecules can be fitted very well to a 2D Gaussian, and their peak position can be localized to within ± 1.3 nm ([Kural *et al.*, 2005](#)). Unfortunately, the localization accuracy of FIONA is not directly related to the spatial resolution required to resolve multiple emitters in close proximity. This problem can be addressed by utilizing the digital photobleaching of single chromophores, but resolving more than five emitters within a diffraction limited spot still remains challenging.

Photoactivation or photoswitching to enhance localization

A sufficiently bright single molecule can be localized very accurately following these procedures of spatial reconstruction. However, in biological samples, single molecules are usually not isolated but rather clustered in close proximity on labeled biological structures such as organelles. Many approaches have recently been provided to isolate these many single molecules temporally, using photoactivation or photoswitching to separate their individual excitation and emission behavior, in so-called photoactivated localization microscopy (PALM or FPALM), or stochastic optical reconstruction microscopy (STORM) ([Rust *et al.*, 2006](#); [Hess *et al.*, 2006](#); [Betzig *et al.*, 2006](#)). They are using chro-

mophores that are switched on and off using the light of two distinct wavelengths and multiple imaging cycles. Only a fraction of all molecules present in the field of view is activated in each imaging cycle, thus allowing their positions to be determined with nanometer precision. An overall high-resolution image can be reconstructed from the various positions determined during a series of imaging cycles, each containing a stochastically different subset of molecules. Using TIRF illumination, a spatial resolution of 20 nm can be achieved ([Rust *et al.*, 2006](#)). These approaches are so far quite time-consuming and limited to immobile samples such as fixed cells or single DNA molecules immobilized on surfaces. Thus it is questionable whether they will be versatile enough to be employed in cutting edge cell biology. However, they are easy to implement in standard fluorescence microscopes without the need for extensive new technology, and may thus be able to fill important resolution gaps in standard confocal measurements particularly on fixed specimens.

TEMPORAL RESOLUTION TECHNIQUES

Fluorescence lifetime imaging

Fluorescence lifetime microscopy (FLIM) is recording changes if fluorescence lifetime rather than intensities or colors ([Tinnefeld *et al.*, 2001](#)). These changes can be induced simply by different environmental conditions, but they also may be due to FRET. As mentioned above, donor fluorescence is quenched and acceptor fluorescence is increased (sensitized) upon energy transfer, resulting in a decrease in donor excitation lifetime. Indeed, one chief application of FLIM is fluorescence resonance energy transfer (FRET) microscopy ([Cremazy *et al.*, 2005](#)). In principle, there are two main approaches to perform FLIM: time-domain and frequency domain measurements. For the former, a scanning microscope is used to illuminate the sample with ultrashort laser pulses. Then the fluorescence decay characteristics is determined for each pixel. This method is more expensive but also more precise if multiple decay components are to be resolved. Alternatively, the excitation light intensity can be varied in a sinusoidal fashion. Then the fluorescence lifetime of the chromophores can be determined from the phase shift between excitation light and the detected fluorescence. In both cases, the data are analyzed by computer to calculate the lifetime image. The combination of FRET and FLIM has, e.g., proven useful for analyzing protein-protein interactions with high spatial and temporal specificity. Another application was investigating the interaction of a DNA-binding GFP-fusion protein and DNA in the cell nucleus, using FRET and FLIM as a reliable tool to detect protein in contact with DNA ([Cremazy *et al.*, 2005](#)). More recently the interactions of Cdt1 with its inhibitor, Geminin, within living cells was investigated ([Xouri *et al.*, 2007](#)).

Time-correlated single photon counting

Almost all time-domain lifetime measurements rely on time-correlated single-photon counting (TCSPC). Having been an extremely complicated research tool for photo-physicists in the early 1980s (Zimmermann *et al.*, 1974; O'Connor and Phillips, 1984), TCSPC is a standard technique for recording time-resolved fluorescence data. Using highly-sensitive detectors and pico- or femtosecond pulsed excitation sources, the photon arrival times relative to the excitation pulse for different input channels are registered by a special computer card and then stored on the hard disk of a PC (Becker *et al.*, 1999). Hence, e.g., the fluorescence lifetime can be determined. Even multi-channel measurements with more than one detector are possible. As the number of channels, the quantities measured therein (polarization or spectral range), and the subsequent data evaluation are completely user-defined (i.e., they only depend on the chosen setup), this technique has proven extremely versatile and provides maximum information on any fluorescent system. Among the standard applications are measurements of fluorescent life-

time, time-resolved anisotropy and fluorescence correlation spectroscopy (FCS), but especially on the single-molecule level, other applications like burst-integrated fluorescence lifetime (BIFL) studies have become predominant (Eggeling *et al.*, 2001; Antonik *et al.*, 2006). (Fig. 3)

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a powerful technique based on a statistical analysis of fluorescence fluctuations of highly dilute fluorescent molecules in a small detection volume in thermal equilibrium to determine fluctuation-related parameters of the system under investigation. These include photophysical characteristics of the chromophores (fluorescence lifetimes, triplet life times), but also rotational and translational diffusion constants or even conformational changes of biomolecules (Aragón and Pecora, 1975; Felekyan *et al.*, 2005). In principle, FCS covers an impressive temporal range from nanoseconds to several seconds, therefore allowing both a high temporal resolution and long-time measurements. (Fig. 4)

Figure 3. FLIM image of a HeLa SS6 cell and GFP-tagged P-bodies (bright spots). The intensity image (top left) and the false color lifetime image were recorded on a custom-built setup using TCSPC-based time-domain measurements (SPC830, Becker&Hickl, Berlin, Germany) and two-photon excitation. The panel on the bottom left shows the distribution of fluorescence lifetimes and the color coding used in the top left figure. Although significantly brighter than the surrounding cytosol, the P-bodies do not exhibit a significant change in fluorescence lifetime, which is corroborated by the less better quality fluorescence decay curves recorded at single focus positions within a P-body and the cytosol, respectively (bottom right). The difference in calculated fluorescence lifetimes between the FLIM image and the point measurements is due to a better signal-to-noise ratio for the latter and a more elaborate data analysis. However, GFP-labeled proteins in the nucleus seem to exhibit on average a slightly longer lifetime. (Courtesy of Thomas Ohrt and Zdenek Petrasek, Biophysics Group.)

Figure 4. Simulated auto-correlation curve showing four different kinetic processes. The maximum length of the time-window is limited by diffusion (red). Fast conformational fluctuations or photophysical reactions, e.g., triplet blinking (green), give rise to a second step in the correlation curve. From the amplitudes, the total average number of all molecules or only the number of bright molecules may be determined. On even faster time-scales, rotational diffusion (blue) and fluorescence antibunching (orange) can be seen. The latter process is actually anti-correlated and occurs on the time-scale of the fluorescence lifetime. The decay times of these processes reflect their characteristic time scales, i.e., the diffusion time, the triplet lifetime, average rotational correlation time, and fluorescence lifetime.

First introduced in 1972 by Webb and co-workers to measure the diffusion and binding of ethidium bromide onto double-stranded DNA (Elson and Magde, 1974; Magde *et al.*, 1974; Magde *et al.*, 1972), the technique has been undergoing a renaissance since Rigler and colleagues combined the technique with a confocal microscope for enhanced background reduction (Rigler and Widengren, 1990; Rigler *et al.*, 1993). Since the relative fluctuations become smaller with increasing numbers of measured particles, it is important to minimize the number of molecules in the focal volume. Roughly, the temporal average of the particle number should be between 0.1 and 1000, which corresponds to concentrations between subnanomolar ($\sim 10^{-10}$ M) and (sub-)micromolar ($\sim 10^{-6}$ M) for a focal volume of about one femtoliter.

The dual-color analog, fluorescence cross-correlation spectroscopy (FCCS), has been introduced by us in 1997 (Schwille *et al.*, 1997). It is extremely useful for determining molecular interactions and colocalization between two or more species emitting at different wavelengths, and raises many hopes to carry out *in situ* proteomics at the single molecule level. Recently, dual color cross-correlation spectroscopy has even enabled us to reveal the complex 1:12 binding stoichiometry of CaM kinase with calmodulin at different calcium levels in living cells (Kim *et al.*, 2004; Kim *et al.*, 2005). FCS can be combined with multi-photon excitation as

well as most other techniques mentioned above (Heinze *et al.*, 2000; Heinze *et al.*, 2004). This possibility, together with its prime measurement parameters: concentrations and distribution coefficients, renders FCS potentially very attractive for future quantitative cell differentiation studies, e.g., in developmental biology.

The first commercial FCS instrument was introduced in 1996, offering only autocorrelation capabilities but nevertheless triggering the evolution of FCS into a standard technique. Later, these instruments were extended to cross-correlation, combined with laser-scanning microscopes (LSMs), and, recently, even equipped with optional two-photon excitation (Weisshart *et al.*, 2004). At present, intracellular FCS applications probing the *in situ* dynamics of fluorescent probes experience a rapidly growing popularity. But the increasing complexity of the biological systems under investigation also causes a virtual explosion in the development and refinement of different methods that still belong to the generic concept of FCS. For a detailed review of this technique and potential applications, see, e.g., Bacia *et al.* (2006), Haustein and Schwille (2007).

COMBINED TECHNIQUES

In the future, a trend that has been already noticeable over the past few years is likely to become even more pronounced: Designing the experiment in the most efficient way to simul-

taneously apply as many different techniques as possible to gain additional “dimensions.” For example, commercial add-on instruments are available to allow TCSPC and FLIM measurements on an LSM setup, but also FCS, FRET, and fluorescence lifetime measurements can be combined with each other, e.g., by performing TCSPC measurements and the corresponding subsequent data analysis, and with various imaging approaches. Some of these combinations will be discussed in the last section of this paper using FCS as an example technique because, in recent years, these rather traditional basic FCS concepts have been extended to a vast family of FCS-related methods in a confocal setup with single molecule sensitivity.

Spatially resolved multi-channel measurements

Increasing the number of spatial channels is not quite as straightforward as increasing the number of spectral channels for FCS measurements because the number of individual laser foci and detectors monitoring them is finite. Successful experiments were performed with up to four focal volumes and array detectors were used (Gosch *et al.*, 2005). To cover larger areas, epifluorescence or TIRF illumination combined with highly sensitive CCD cameras (Burkhardt and Schwille, 2006; Kannan *et al.*, 2006) have been shown to yield better results. Here, the individual pixels act as confocal pinholes. Digman *et al.* present another approach to obtain temporal and spatial information. Using a standard laser scanning microscope, they exploit the hidden time structure of the scan method, i.e., the microsecond time delay between adjacent pixels to record processes on time-scales of microsecond to seconds (Digman *et al.*, 2005). Using this method called raster image correlation spectroscopy (RICS), they can access the temporal resolution of single-point FCS while retaining the spatial information provided by the imaging technique. First experimental results show the spatially resolved diffusions of paxillin-EGFP in CHO-K1 cells.

Resolution enhancements and FCS

Currently, there are two main reasons to enhance the spatial resolution for FCS applications. First, the size of the observation volume and the sensitivity of FCS restrict the method to dye concentrations in the sub-micromolar range. Frequently, however, equilibrium binding constants of biochemical reactions dictate much higher concentrations. To keep the average number of molecules in the focal volume within a reasonable range, the volume has to shrink. Alternatively, heterogeneities caused by subwavelength structures like membrane rafts are simply averaged out if the resolution is not increased.

For example, the illuminated region can be restricted by total internal reflection (TIR) (Thompson *et al.*, 1981), but more recently also by stimulated emission depletion (STED) (Hell, 2003). Whereas the latter offers a fivefold compaction of the focal volume (Kastrup *et al.*, 2005), attoliter detection

volumes have been reported with TIRFM, i.e., a reduction by almost two orders of magnitude (Ruckstuhl and Seeger, 2004). Established by Thompson *et al.* in 1981, TIR-FCS lay dormant for nearly two decades after some promising initial applications on surface-binding rates of biomolecules (Thompson *et al.*, 1981). It was successfully applied to receptor-ligand interactions, even with nonfluorescent competitors (Lieto *et al.*, 2003; Lieto and Thompson, 2004). For this kind of application, potential hydrodynamic surface effects should be kept in mind. The diffusion coefficients of large biomolecules very close to supported planar membranes decrease more strongly than expected from literature values for freely diffusing particles (Pero *et al.*, 2006). Nevertheless, TIR-FCS, as TIRFM, is restricted to surface measurements with a maximum illumination depth in the range of 200 nm. This means that only the axial resolution is increased, whereas lateral resolution is enforced by pinholes. STED causes a more uniform reduction of the illuminated region in all three dimensions, and it can be applied in solution (Kastrup *et al.*, 2005). Thus, intracellular measurements seem feasible in spite of the rather high laser intensities required.

CONCLUSIONS

Throughout the centuries new developments in light microscopy have promoted the biological sciences and catalyzed many breakthroughs, and this development is far from coming to an end. Particularly in combination with fluorescence, cellular processes can nowadays be elucidated and unraveled down to the molecular level, allowing us to tackle the overwhelming complexity of cellular networks and interaction patterns with, so far, unprecedented temporal and spatial resolution. For a very long time, people believed that optical microscopy was intrinsically limited to a spatial resolution of ~200 nm in lateral and ~600 nm in axial direction. Major developments during the last two decades, but especially during the last few years, have shown that this can be improved by at least a factor of ten and maybe more. At the same time, time resolved techniques will provide us with detailed information about the dynamics of living systems rather than delivering mere stationary snap-shots at selected times. The nonlinear methods seem to be the most promising ones among these novel techniques, both with regard to biological applications and to other versatility when combined with other highly-sensitive single-molecule techniques. However, the ultimate proof of acceptance by the scientific community, the widespread availability as commercial instruments, is bound to take some time and also depend on the development of brighter and more photostable chromophores to fully appreciate the potential of these methods, in order to unravel at least some of the still remaining biological mysteries.

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