

## Bright Solutions to Get Sharp Images: Confocal and Two-Photon Fluorescence Microscopy and the Pros and Cons of New Multifocal Approaches

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This chapter gives an overview on basic concepts of microscopical techniques that allow measuring time-dependent functional signals in biological tissue and compares the performance of these techniques with respect to temporal and spatial resolution. The application of conventional widefield, confocal and two-photon laser scanning microscopy to *in vivo* imaging is illustrated in one example, the registration of calcium signals during neuronal activity in the fly visual system. In particular, the capacity of a two-photon microscope equipped with a laser beamsplitter that enables simultaneous excitation in multiple focal spots is evaluated. By using multifocal excitation the acquisition rate can be raised considerably when it is necessary to register signals from numerous cellular structures that are far apart from each other.

**Keywords** calcium; confocal microscopy; multifocal; neuron; two-photon microscopy

### 1. Introduction

Confocal and two-photon laser-scanning microscopy (TPLSM) are nowadays widely used in biological imaging. The superior spatial resolution of these modern techniques compared to conventional wide-field fluorescence microscopy is beyond question. However, it is often neglected that the principle of these techniques makes it inevitable that sensitivity is to a certain extent traded for spatial resolution.

In confocal microscopy (see section 3) a large fraction of the fluorescence emitted from the sample is discarded because a pinhole blocks out-of-focus light. This is particularly critical in living biological tissue, which is easily damaged by excessive excitation light, and with fast bleaching fluorescence dyes. Although TPLSM (see section 4) provides an elegant solution to these problems by confining excitation to the focal spot, sensitivity often remains to be a problem because high energy is required in the sample to generate two-photon excitation. Whereas poor sensitivity is often not critical in structural imaging, it may hamper the resolution of fast temporal events, for example when imaging neuronal signals. Here, temporal resolution is directly influenced by the sensitivity of microscopical imaging, so that conventional wide-field fluorescence excitation may prove superior to sophisticated confocal and two-photon techniques.

In section 5 recent attempts are outlined that may help to find a good compromise between the high spatial resolution of laser microscopy and the high sensitivity reached with conventional wide-field excitation. In particular, one of these new approaches, multifocal TPLSM, is introduced. Multifocal TPLSM (see section 6) is based on a technique that allows to generate multiple focal points in the sample by a two-photon microscope provided with a laser beamsplitter. This principle raises temporal resolution considerably because signals from different cellular locations can be sampled simultaneously. However, in scattering tissue the excellent temporal resolution of multifocal two-photon imaging comes along with a certain loss in spatial resolution when compared to single-focus imaging. In section 7 classical wide-field microscopy, confocal microscopy, TPLSM and multifocal TPLSM are compared with respect to their performance in a challenging task, *in vivo* imaging of calcium concentration dynamics in single motion-sensitive visual interneurons of the fly nervous system.

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## 2. Fluorescence microscopy in biological samples

Microscopical techniques allow imaging of biological structures with high spatial resolution. The visualization of cellular structures of interest can be improved considerably by the use of specific fluorophores, which can be selectively excited and thus specifically visualized in the presence of myriads of non-fluorescent molecules. Tagging of cellular proteins with synthetic or genetically encoded fluorophores enables measuring their spatial distribution and dynamics. On the other hand, by the use of functional fluorescent dyes aspects of cellular activity can be monitored, such as changes in membrane potential, the process of endo- and exocytosis, and concentration changes in  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  or  $\text{Cl}^-$ . Particularly in neuroscience functional fluorescence microscopy has proven extremely valuable to provide spatially resolved information on activity-related processes in single cells and in cell assemblies [1]. However, imaging of live material is very demanding, since not only fluorescent dyes themselves may be toxic to living cells and limit tissue viability, but also the irradiation with excitation light may cause photodamage. Structures of interest often lie deep within compact tissue and proper cellular functioning is often not guaranteed when the tissue is cut into too thin slices. On the other hand, imaging structures deep inside a sample requires delivering sufficient excitation energy into deep tissue layers without causing excessive photodamage. Moreover, spatial resolution is limited by the strongly light-scattering nature of intact tissue.

In wide-field fluorescence microscopy the entire sample is illuminated via the microscope optics with a specific band of wavelengths that matches the excitation optimum of the fluorophore. The emitted fluorescence is separated from the excitation light by a dichroic mirror and specific filtering and sent to a detector, usually a charged coupled device (CCD) camera. The separation of emission from excitation light is possible because the emission spectrum of an excited fluorophore is usually shifted to longer wavelengths when compared to the excitation spectrum. This phenomenon, known as Stokes shift, is a consequence of energy dissipation during the excited-state lifetime and correspondingly lower energy of the photon that is emitted upon relaxation of the fluorophore from the excited state back to the ground state. Fluorophores with large Stokes shifts facilitate the separation of emission from excitation light. Since the excitation light is usually much brighter than the emitted fluorescence the filter settings of the microscope critically determine the signal-to-background intensity of the image, and thus the sensitivity and the spatial resolution of the measurement.

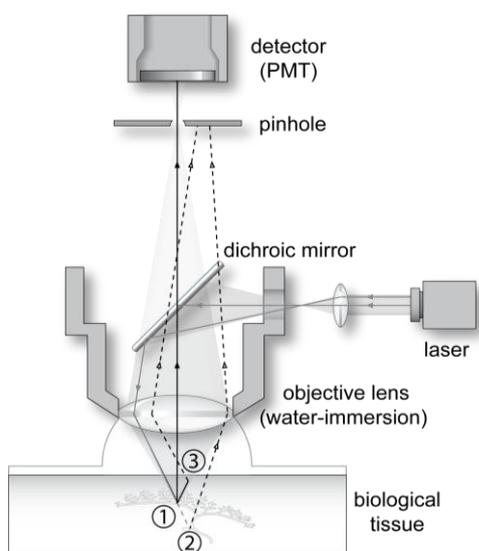
In wide-field microscopy, fluorophore excitation is not restricted to the plane of focus. Both above and below this plane fluorophores reach the excited state, which causes image blur and lowering of contrast. This is particularly problematic if the structures of interest lie deep below the tissue surface or if stained structures are stacked over one another, as is e.g. often the case in neurons, which form extended arborizations. But even the image of an isolated structure that is restricted to the focal plane may become blurred in wide-field fluorescence microscopy, because photons emanating from the focus may become scattered on their way through the tissue to the objective lens. Such scattered photons will degrade resolution when hitting the detector at locations displaced from their original destination.

## 3. Confocal laser-scanning microscopy

In confocal laser-scanning microscopy [2, 3] widefield illumination is replaced by the light from a laser, which is focused through the microscope lens to a single spot in the focal plane (see Fig. 1). The emission light generated in the sample at the focal position is collected by a fast detector, usually a photomultiplier tube (PMT) and the laser beam is consecutively shifted by scan mirrors to a large number of raster positions. An entire image of the specimen at a certain focal plane can be reconstructed from the time-course of the recorded signal and the predefined path of the laser focus. Three-dimensional reconstructions can be formed when stacks of data from different focal planes is combined. The scanning principle in itself does not raise spatial resolution over that of conventional fluorescence microscopy, because similar to wide-field illumination, excitation is produced in the entire beam path of the laser in the sample, i.e. both above and below the focal plane. However, with point illumination it is possible to place a pinhole in front of the detector to prevent light originating from above or below the focal plane in the

sample from reaching the detector. This elimination of out-of-focus emission by a confocal pinhole requires redirecting the emission light to the scan mirrors that also deflect the excitation laser beam (not illustrated in Fig. 1). This descanned detection mode is necessary to prevent the emission light from being shifted away from the pinhole when the position of the laser focus in the sample is changed.

The elimination of out-of-focus background by a confocal pinhole leads to excellent spatial resolution and enables acquisition of unblurred optical sections from within thick samples. However, during live imaging a major drawback of confocal microscopy is the fact that a large proportion of light emitted from the sample is discarded either because it is generated outside the laser focus or because photons emitted from the focus are scattered on their way to the detector and blocked by the pinhole. Thus, confocal microscopy reaches a sufficiently high sensitivity only with strong excitation light. Although much of this excitation light does not contribute to image formation it inevitably causes tissue photodamage and dye bleaching. Therefore, the ratio between excitation intensity and signal photons may become unacceptable in deep tissue layers, particularly in strongly scattering specimen, such as brain slices or intact brain preparations.



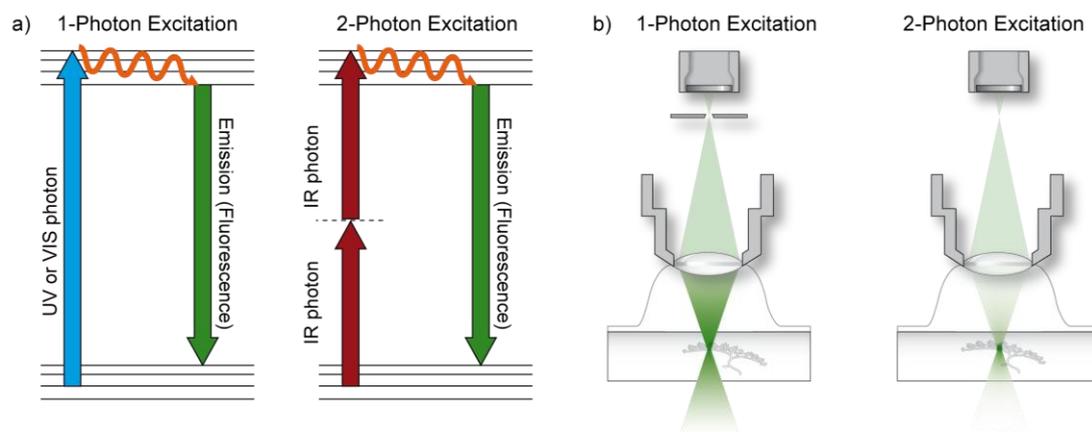
**Fig. 1** Schematic diagram of confocal microscopy. Excitation light from a laser is focused into the sample. Only emission photons that emanate from the focus and travel through tissue without being scattered pass the pinhole and reach the detector (1). Both emission from outside the focal plane (2) and photons that are scattered on their way from the laser focus to the objective lens (3) are blocked by the pinhole.

#### 4. Two-photon laser-scanning microscopy

As detailed above confocal microscopy can overcome the limitations in spatial resolution encountered when using wide-field fluorescence microscopy. However, the rejection of scattered signal photons by a detector pinhole lowers sensitivity. Moreover, photodamage and dye bleaching occur throughout the sample whereas signal is obtained only from the focal plane. These problems can be solved by the use of TPLSM [3-5].

TPLSM makes use of the principle that excitation of a fluorophore can be achieved by the nearly simultaneous absorption of two photons of about twice the wavelength required for single-photon excitation (see Fig. 2a). Due to the fact that the probability of two-photon absorption is quadratically dependent on incident light intensity, fluorophore excitation is elicited almost exclusively in the focus of a laser which generates high power during ultrashort pulses (see Fig. 2b). Thus, spatial resolution is nearly identical to the tiny focal volume, so that TPLSM can be performed without the need of a pinhole, since unlike confocal (one-photon) microscopy blocking out-of-focus excitation is not relevant. In particular for imaging in intact preparations TPLSM is often superior to conventional wide-field and confocal microscopy. One major reason for this is that the longer illumination wavelengths used in TPLSM scatter

less and penetrate deeper in compact living tissue. Due to its excitation principle, in TPLSM photodamage and photobleaching of the fluorescent dye are restricted to the focal volume. Moreover, the long wavelengths used in TPLSM are generally less phototoxic to living tissue, although this is to some extent countered by the high light intensities required for sufficient excitation. When studying the visual system an additional advantage of infrared excitation light is the absence of unintended visual stimulation of photoreceptors. In contrast, when using one-photon excitation such problems have to be accommodated with sophisticated experimental paradigms.



**Fig. 2** a) Principle of one-photon versus two-photon excitation. b) A quadratic dependency of excitation probability on laser intensity arises from the nonlinear nature of two-photon excitation. This confines the occurrence of excitation events in TPLSM to the laser focus.

## 5. New methods to improve temporal resolution in laser-scanning microscopy

The investigation of dynamic processes, e.g. calcium concentration changes in single nerve cells and in neuronal circuits, often requires spatially resolved imaging of activity-related signals with high temporal resolution. Unfortunately, the point illumination principle used in both confocal microscopy and TPLSM limits temporal resolution. This is mainly due to the inertia of the scan mirrors used to deflect the laser beam. Imaging speed can be raised to the kHz-level with laser microscopy, but only in line scans, i.e. when the laser is scanned repeatedly along a single axis. In contrast, scanning entire images or even image stacks in the z-plane is basically more time consuming than conventional wide-field imaging. This bears considerable problems, because it is often required to compare functional signals between distant locations in the specimen. For example, when imaging the activation of dendritic spines, the functional input structures of cortical neurons thought to be key structures in mediating neuronal plasticity [6, 7], it is important to assess to what extent the input activation remains restricted to individual spines. Scanning large areas, however, is usually too slow to record the fast dynamics of intracellular processes in full detail. On the other hand, it is hard to cover all the relevant structures in a single scan line, i.e. spine head, spine neck and 'parent dendrite', or even several neighbouring spines.

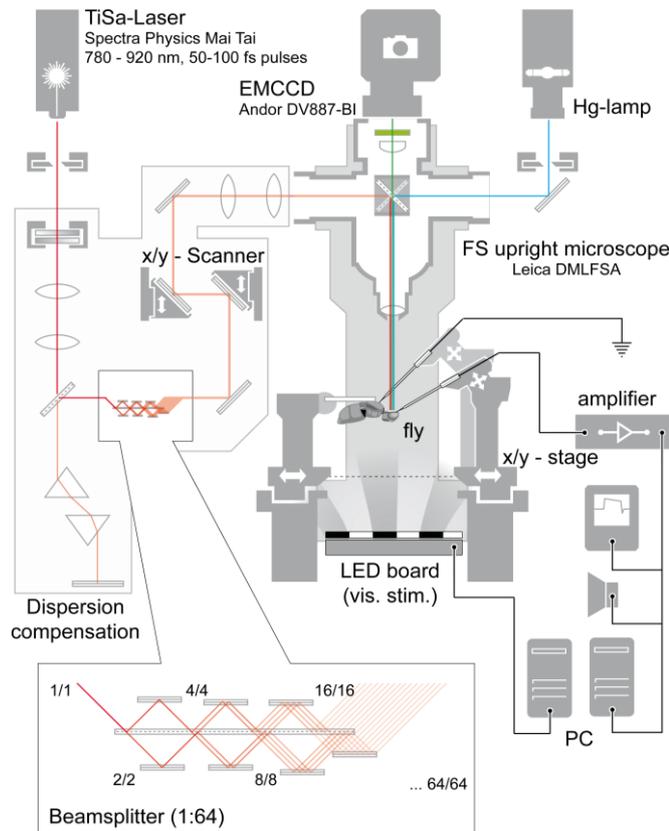
One approach to increase scan speed in laser microscopy is the use resonant galvanometer scanners, which minimize inertia effects [8-10]. However, with this scan mode image reconstruction is more problematic than with conventional scanners because laser dwell times are position dependent [9]. In general, scanning speed is fixed with resonant scanners and repeated illuminations may be required due to short laser dwell times, which might cause signal-to-noise problems in weakly fluorescent samples. On the other hand, short laser dwell times are beneficial with respect to avoidance of triplet state excitation, which leads to destruction of the fluorophores [10]. Recently, acousto-optical deflectors (AODs) have

been used in TPLSM as an entirely inertia-free device to position the laser focus in a discontinuous scan mode [11]. Thus, fast recordings from user-selected sites of interest in the sample can be performed. Unfortunately, the ultrashort laser pulses used in TPLSM are strongly distorted by AODs, which makes a compensation with additional diffractive elements necessary. In confocal microscopy the use of AODs is problematic, because their diffraction efficiency is limited, which would cause substantial fluorescence loss during descanning. This problem has recently been circumvented by the combination of AODs with a micro-mirror array functioning as an addressable spatial filter to achieve confocality without the need of descanning [12].

Alternative to improving the speed or the flexibility of the scan path of a single laser focus acquisition rate can be increased by recording fluorescence emission simultaneously from multiple excitation spots. In confocal microscopy this principle can be implemented by a Nipkow “spinning disk” design [13]. In its most advanced form a spinning disk with a pattern of micro-lenses is used to generate multiple foci in one plane of the sample [14, 15]. The confocal principle is implemented by moving in conjunction with the micro-lens disk a second disk with a corresponding pattern of pinholes. Particularly in thick samples spatial resolution of spinning-disk confocal microscopy is compromised by cross talk between adjacent excitation spots. A further disadvantage of spinning disk confocal microscopes is the fixed pinhole size. In an alternative concept a laser beam is formed into a line, which is scanned across the object plane by a galvanometer scanner [16]. This principle requires to replace the conventional arrangement of a pinhole and a PMT detector by an adjustable slit and an array detector. Slit detection partially violates the confocal principle and results in a reduced spatial resolution along one axis and in a weaker depth discrimination as compared to point scanning confocal detection. Thus, line illumination and related detection principles make it inevitable that the gain in temporal resolution comes at the expense of spatial resolution. This is to a certain extent also the case in multifocal TPLSM, which will be described in detail in the following section.

## 6. Multifocal TPLSM

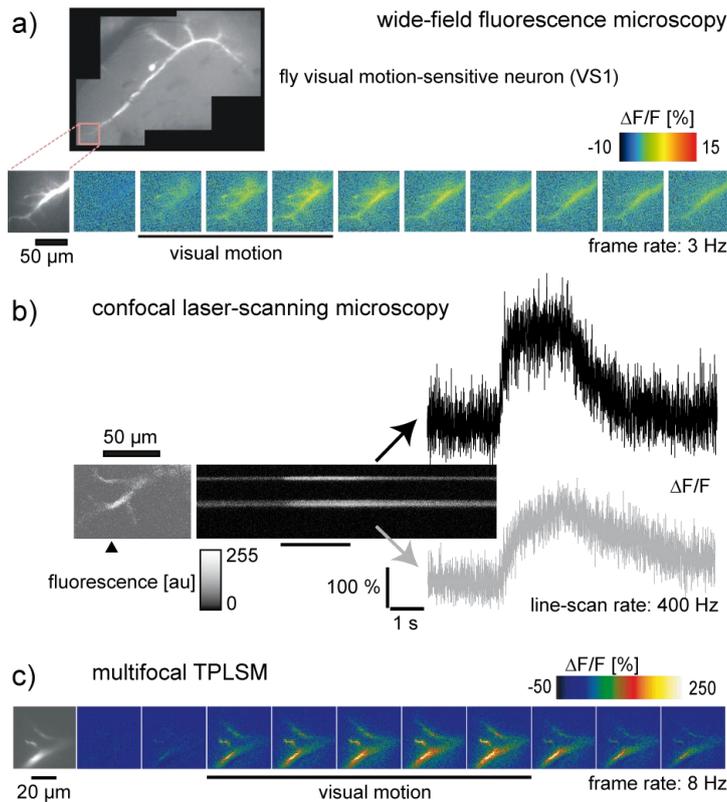
In order to combine high temporal resolution with the ability to perform spatially resolved imaging over extended areas a setup for multifocal TPLSM was constructed (see Fig. 3). The basic concept of this approach is to split a single excitation laser beam into multiple beams, in order to illuminate several points in the sample simultaneously [17]. A mirror-based beamsplitter is used to create multiple laser foci [18]. The operating principle of this beamsplitter is to divide a laser beam by a 50 % mirror and to send back the separated beams to this mirror by full reflectivity mirrors. By repeating this procedure several times an array of up to 64 unitary beams can be generated. This beam array is directed to the sample via the scan mirrors and the optics of the microscope. Signal detection, i.e. detection of photons emitted from the laser foci, is performed by the use of a fast and highly-sensitive electron-multiplying charged-coupled device (EMCCD) camera [19]. When synchronizing CCD read-out to one or multiples of sweeps of the laser beams across the sample simultaneous multiple line scans are collected when the scan movement is restricted to one dimension (‘multiple line scan mode’). This allows simultaneous recording of functional signals at high temporal resolution, e.g. changes in the fluorescence of a calcium indicator dye, from several cellular structures which are too far apart from each other or too awkwardly arranged to be easily hit by a single scan line. In addition, by scanning in two dimensions (‘frame scan mode’) it is possible to acquire entire images at a much higher speed than is possible with a single laser focus.



**Fig. 3** Set up for simultaneous multifocal TPLSM and electrophysiological recording. See main text for details.

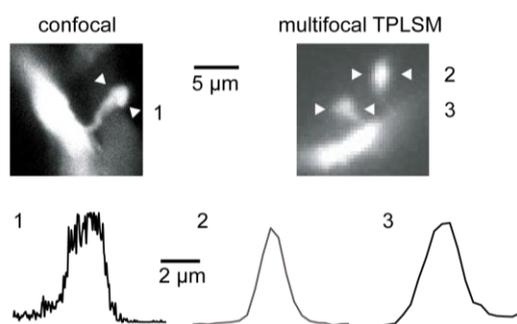
## 7. Spatio-temporal resolution of multifocal TPLSM in comparison with wide-field, confocal and single-focus TPLSM

Figure 4a shows a spatio-temporal measurement with conventional wide-field fluorescence microscopy of calcium concentration changes in a single visual motion-sensitive neuron in the fly brain [20, 21]. Spatial resolution and contrast of the images are degraded by the fact that excitation is not limited to the focal plane and by strong scattering of emitted photons in intact brain tissue. These problems are to some extent solved by confocal microscopy. Due to the rejection of signals emanating from out-of-focus regions the cellular structures in the focal plane appear to be less affected by scattering compared to wide-field microscopy (see Fig. 4b). However, depending on the intensity of fluorescence staining and imaging depth, the temporal resolution of confocal microscopy is degraded either by low sensitivity, due to the rejection of a large fraction of the emitted fluorescence by the pinhole, or by the relatively slow speed of the scan mirrors. In the preparation shown in Figure 4b the rate at which entire images could be recorded (2.6 s per frame) was too slow to monitor calcium dynamics. Therefore, instead of recording entire images repeated scanning of a single line at a high rate (400 Hz) was used.



**Fig. 4** Fluorescence imaging of changes of cytosolic calcium concentration in the axonal output region of visual motion-sensitive neurons of the fly (*Calliphora vicina*) brain. a) By wide-field fluorescence microscopy the changes in intensity of a calcium-sensitive dye (fura-2) were recorded with a CCD camera [22]. The color-coded image series shows the spatio-temporal course of the fluorescence signal and indicates an increase in cytosolic calcium concentration during presentation of a visual motion stimulus. b) Confocal microscopy (Leica TCS SP2) was used in line-scan mode (position of the line indicated by arrowhead in the image) to monitor changes in fluorescence of the calcium dye Oregon-Green 488 BAPTA-1 [17]. c) Calcium signal monitored with multifocal TPLSM in 'frame scan mode' ([17], see main text for details). The calcium dyes Oregon-Green 488 BAPTA-1 and fluo-4 were used in combination. All microscopes were equipped with long-distance water-immersion 40x/0.8 NA objectives.

By the use of multifocal TPLSM image series can be taken at frame rates comparable to those of wide-field fluorescence microscopy (see Fig. 4c). Much higher acquisition rates (up to 100 Hz) can be achieved when using multifocal TPLSM in the 'multiple line scan mode' (not shown). Both with confocal microscopy and with multifocal TPLSM, small neuritic spine-like protrusions in the axonal output region of the neurons could be detected (see Fig. 5). However, the spatial resolution of multifocal TPLSM seems lower than that of confocal microscopy and intensity profiles of spatial transections across spine-like structures exhibit steeper flanks in the confocal image than in the image taken with multifocal TPLSM.

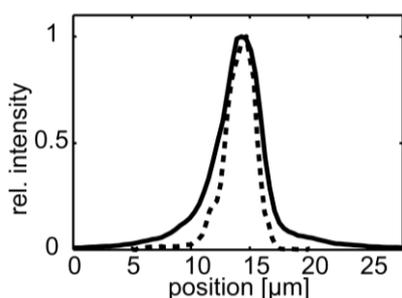


**Fig. 5** Spine-like protrusions in the axonal output region of a fly visual motion-sensitive neuron imaged with confocal microscopy (Leica TCS SP2) and multifocal TPLSM. Both microscopes were equipped with long-distance water-immersion 40x/0.8 NA objectives. The line plots show intensity profiles along transections as indicated by the arrowheads in the images.

To compare spatial resolution between the confocal microscope and the setup for multifocal TPLSM in a more quantitative way the point-spread-functions (PSFs) of small fluorescent particles (e.g., quantum dots) were mapped [17]. If these particles are small enough to be regarded point-like light sources it is possible to take their full-width at half-maximum (FWHM) in a microscope image as a direct measure of the maximally attainable spatial resolution of the microscope setup. The FWHM of PSFs characterizing the spatial resolution of the confocal and the multifocal TPLSM setups equipped with 40x/0.8 NA long-distance water-immersion objective lenses were 560 nm and 420 nm, respectively. Thus, the spatial resolution limits differ only slightly between the two techniques. One reason for the difference is the use of a CCD detector in multifocal TPLSM, because the effective pixel resolution of the CCD is 400 nm.

The small difference in the resolution limit between confocal microscopy and multifocal TPLSM can only to some extent explain the differences in resolution encountered when the two techniques are applied to imaging of structures deep inside living tissue. The reason for this is that in the measurement described above spatial resolution was determined in non-scattering medium. Scattered emission photons do not degrade image quality in confocal and single-focus TPLSM, but they do so in multifocal TPLSM. This is due to the fact that imaging detection with a CCD is used in multifocal TPLSM instead of a point-scanning detection mode. With the latter, scattered emission photons can be collected and reassigned to the focus during image reconstruction. This is possible because the position of the laser focus at each time point is known and due to the nonlinear nature of two-photon excitation the vast majority of emission photons emanates from the laser focus. In contrast, a similar procedure is impossible when imaging detection with a CCD is applied as in the multifocal TPLSM setup. Therefore, scattering of photons on their way from the laser foci to the detector will degrade spatial resolution to some extent. Important to note, one major advantage of TPLSM, namely confinement of excitation to the laser focus, remains unaffected by the detection concept used. Moreover, in the 'multiple line scan' mode of multifocal TPLSM only resolution along lines, but not lateral resolution is affected, if the spacing between individual lines is large enough to avoid overlap of the signals.

In order to determine directly in how far the spatial resolution of multifocal TPLSM is degraded by scattering of emission photons in compact brain tissue, a point-scanning detection mode was implemented with the multifocal TPLSM setup (for details see [17]).



**Fig. 6** Comparison of spatial resolution between point-scanning and imaging detection. Both detection modes were implemented at the set up for multifocal TPLSM. Spatial intensity profiles across a small neurite were extracted from one and the same data set according to the point-scanning (broken line) and the imaging detection mode (solid line), respectively.

Spatial intensity profiles of small-diameter neurites appeared slightly narrower and had steeper flanks, when the point-scanning detection mode was applied compared to imaging detection. In the example shown in Figure 6, the FWHM of the normalized intensity profile was 2.80  $\mu\text{m}$  for point-scanning detection (broken line). For imaging detection the FWHM of the corresponding profile was 3.95  $\mu\text{m}$  (solid line). The absolute difference between the FWHM of spatial intensity profiles determined with the two detection modes provides a relative measure of the difference in spatial resolution. This difference was determined in several neurons at different small neurites located 30 - 60  $\mu\text{m}$  below tissue surface. It was consistently in a range of about 0.4 - 1.2  $\mu\text{m}$ , indicating a considerable advantage of point-scanning detection over imaging detection in strongly scattering brain tissue. In the future, a strict implementation of a point-scanning detection mode in multifocal TPLSM could help to design fast multifocal TPLSM with a spatial resolution similar to single-focus TPLSM. This would require recording the fluorescence emitted from individual laser foci separately, e.g. by using a fast and sensitive photodiode array.

## 8. Conclusions

Functional fluorescence imaging in biological tissue poses strong demands on microscopic techniques. The choice of the technique best suited to solve a scientific question depends on many factors, among them the vulnerability of the preparation to photodamage, the required imaging depth and the significance of high spatial resolution on the one hand and high temporal resolution on the other. New technical approaches in laser-scanning microscopy, such as line illumination in confocal microscopy and multifocal TPLSM, help increasing temporal resolution considerably (see also [23, 24]). Although a certain loss in spatial resolution relative to conventional confocal and single-focus TPLSM is inevitable in these techniques, they will present the method of choice in many *in vivo* applications in which wide-field fluorescence microscopy provides insufficient spatial resolution and conventional laser-scanning microscopy is too slow.

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