Excitation beyond the monochromatic laser limit: simultaneous 3-D confocal and multiphoton microscopy with a tapered fiber as white-light laser source

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Abstract. Confocal and multiphoton microscopy are essential tools in modern life sciences. They allow fast and highly resolved imaging of a steadily growing number of fluorescent markers, ranging from fluorescent proteins to quantum dots and other fluorophores, used for the localization of molecules and the quantitative detection of molecular properties within living cells and organisms. Up to now, only one physical limitation seemed to be unavoidable. Both confocal and multiphoton microscopy rely on lasers as excitation sources, and their monochromatic radiation allows only a limited number of simultaneously usable dyes, which depends on the specific number of laser lines available in the used microscope. We have overcome this limitation by successfully replacing all excitation lasers in a standard confocal microscope with pulsed white light ranging from 430 to 1300 nm generated in a tapered silica fiber. With this easily reproducible method, simultaneous confocal and multiphoton microscopy was demonstrated. By developing a coherent and intense laser source with spectral width comparable to a mercury lamp, we provide the flexibility to excite any desired fluorophore combination. [DOI: 10.1117/1.2114788]

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1 Introduction

Microscopy in general has revolutionized biological sciences over the last century. Confocal laser scanning microscopy (CLSM) in particular has important advantages over traditional microscopy as it combines high-intensity single-point illumination with the ability to suppress out-of-focus and scattered light,[1] leading to three-dimensional image acquisition with diffraction-limited resolution of fluorescently labeled samples. Even extremely weak labeling can reliably be detected, and unnecessary bleaching is significantly reduced due to point illumination.[2] To further decrease the photodamage and bleaching rate of CLSM, multiphoton microscopy can be used.[3–5] Unfortunately, the abilities of these important imaging techniques are physically restricted, since modern CLSM still lacks an intense excitation laser source with broad spectral properties comparable to the white-light emerging from a standard mercury lamp. A tapered fiber provides such a new excitation source, and hence overcomes most of the actual limitations of CLSM. Thus, it opens the way for optimum excitation, for easy separation of fluorescent dyes with overlapping spectra, and for high-speed switching between arbitrary excitation wavelengths.

In contrast to the excitation source, the signal detection units in state-of-the-art CLSM have already overcome the spectral limitations that were a consequence of traditional filter systems. This was achieved by replacing these filters with acousto-optical components such as acousto-optical tunable filters (AOTF)[6] or acousto-optical beam splitters (AOBS).[7] In

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AOBS an acoustic wave generates a density grating in a crystal, which diffracts only certain wavelengths of an incident beam. The diffracted wavelengths can be chosen by altering the frequency of the applied acoustic wave, while all other wavelengths are transmitted without changing direction as illustrated in Fig. 1 (inset 2). The modern developments allow the researcher to arbitrarily select emission wavelengths of interest, and to gain complete control over the spectral detection of the fluorescence emission.[8,9] These improvements bring microscopy closer to spectroscopy, another important analysis technique in the modern life-science laboratory.

The same scheme could be used for the selection of excitation wavelengths, if there were a continuous spectrum available to select from. Despite the fact that most excitation lasers are capable of multiwavelength emission, the excitation light is restricted to a limited number of specific wavelengths.[10] This is in strong contrast with the increasing number of fluorescent markers necessary to understand the complex nature and interactions of multiple proteins and molecules in modern life sciences.[11] Up to now the simultaneous excitation and detection of a large number of dyes usually required the same number of laser sources, as well as sophisticated mathematical procedures to separate the spectra of different dyes. Additional lasers are expensive and hard to maintain, whereas the mathematical deconvolution procedures are often only of limited use, since they rely on detailed information about the dyes' spectral properties, and they need additional complex hardware.[12]

Broadband laser radiation emerging from photonic crystal fibers could be one solution to this problem.[13] Recently, the light emitted from these fibers has been used for the imaging of biological samples with CLSM[14] and multiphoton microscopy.[15] Although these publications show that using a photonic crystal fiber in CLSM is feasible, the reported images do not prove that these new supercontiuum sources can yield the resolution and image quality of conventional CLSM excitation sources, since neither fluorescent details smaller than about 1 μm nor the important feature of simultaneous multicolor detection have been demonstrated.[14] Additionally, simultaneous multiphoton microscopy and CLSM has not been demonstrated yet. Furthermore, it has been reported that photonic crystal fibers degrade rather fast due to damage at the fiber input if operated at the required powers.[16]

To overcome these problems, we report on the use of a tapered fiber[17] to broaden spectrally the 803-nm femtosecond laser pulses from a titanium sapphire (Ti:sapphire) laser, resulting in a supercontinuum emission spectrum ranging from about 430 to 1300 nm.[18] This elegant and simple method for generating white light exploits the properties of a single mode silica fiber that was tapered down to a diameter of about 2.1 μm by means of heating and stretching. The large change in refractive index between silica and air forces the ultrashort pulse to funnel into a very small effective area, leading to extremely high electromagnetic field intensities. Due to the resulting anomalous group velocity dispersion and high field intensities, nonlinear optical effects such as soliton splitting and four-wave mixing convert the femtosecond pulsed radiation into a spectrally broad white-light laser spectrum.[19,20] This leads to the measured supercontinuum as presented in Fig. 1 (inset 1) with a pulse duration below 10 ps.[18] Using a tapered fiber instead of a photonic crystal fiber solved the mentioned stability problems. The presented data finally demonstrates that an image quality comparable to standard CLSM laser sources is achievable. Due to its input core-size of 8.2 μm, the tapered fiber is not degrading at the critical fiber input. Thus it can be operated stably at high output powers,[21] which are necessary to generate a supercontiuum with adequate power and spectral properties.[18]

By coupling this broadband laser radiation into a standard confocal microscope we were able to simultaneously record high-quality CLSM and multiphoton microscopy images. This provides the flexibility necessary to optimally exploit modern labeling techniques.

2 Results

2.1 *Experimental Procedure*

The setup is sketched in Fig. 1. The light source, a modelocked Ti:sapphire laser (Mira 900, Coherent, Santa Clara, CA), was pumped with a frequency-doubled solid state laser (Verdi V, Coherent) and tuned to emit about 200-fs pulses with a repetition rate of 76 MHz at a center frequency of 803 nm. The laser emission was then coupled into the tapered fiber with a $10\times$ 0.3 NA objective after passing through a Faraday isolator that prevented back-reflections into the Ti:sapphire cavity. Coupling 650 mW into the tapered fiber resulted in a perfect TEM_{00} spatial shape, with a supercontinuum output spectrum according to Fig. 1 (inset 1) and a total power of 280 mW. Although the coupling objective and the approximately 10 cm of SMF 28 fiber lead to a significant temporal pulse broadening, the peak energy is sufficient for the nonlinear optical effects that result in the reported generation of supercontiuum light.[18] The resulting laser beam was spectrally decomposed with a standard dichroic mirror (Chroma, Rockingham, VT, USA) into a visible part from about 430 to 700 nm and a near-infrared (NIR) part ranging from about 700 to 1300 nm. The visible part of the light was coupled into the CLSM through a fiber with a 4-μm core diameter and single-mode properties in the visible spectrum. This fiber was coupled to the AOBS, where an arbitrary wavelength could be selected between 450 and 633 nm. Selecting the wavelengths 458, 476, 488, 514, 543, and 633 nm, which were the wavelengths used for confocal imaging, we measured a total intensity of 62 μW before the objective. For all images, a 40×1.25 NA (Leica Microsystems, Bensheim, Germany) oil immersion objective was used.

The NIR part of the light was coupled into the optical path of the microscope through the standard NIR port. Since this light was not spectrally filtered by the AOBS, the total power of the supercontinuum pulsed NIR light was 11.6 mW at the sample, which was sufficient to perform multiphoton microscopy, even with the picosecond pulses. We measured the pulse length to be between 1 to 5 ps, depending on the spectral range.[18] As reported previously,[22–24] a pulse duration of picoseconds is indeed sufficient to reach the peak intensities required for multiphoton processes if the average power is increased accordingly. The relation between the peak power and the time average power simply reads $P_{\text{peak}} = 0.56 * P_{\text{ave}}/(\tau * f)$,[23] where τ is the pulse duration and f is the pulse frequency. For the 5-ps pulse and an average power of 11.6 mW this calculates to a peak power of about 17 W.

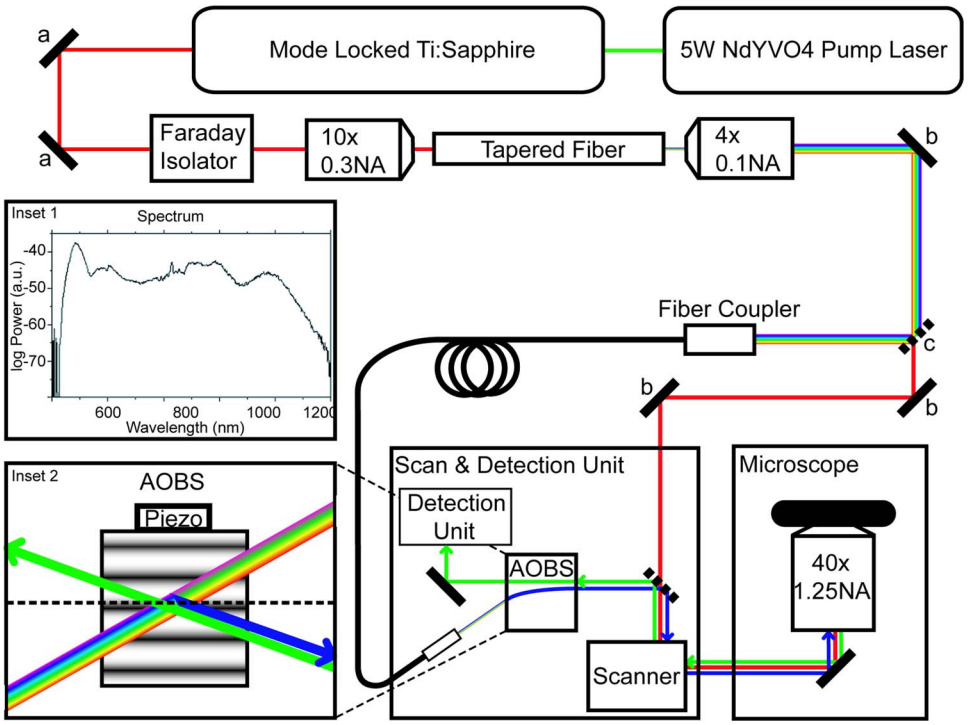

Fig. 1 Illustration of the setup: a mode-locked Ti:sapphire laser delivers 200-fs pulses at 76 MHz with a center frequency of 803 nm via two dielectric mirrors (a). After passing through a Faraday isolator the beam is focused into the tapered fiber with a 10×0.3 NA objective. The supercontinuum white light emerging from the tapered fiber is collimated with a 4×0.1 NA objective. To separate the visible light from the white light, a dichroic mirror (c) is used. The NIR part of the light is coupled into the NIR port of the scan and detection unit via broadband silver mirrors (b), whereas the visible part of the spectrum is coupled into the microscope via a fiber coupler. Inset 1: spectral distribution of the supercontinuum white light emerging from the tapered fiber. Inset 2: working principle of the AOBS. The acoustic wave applied by the piezo transducer creates a density grating that selectively diffracts only a specific wavelength (sketched by the blue beam). All other spectral components of either the excitation light or the fluorescence emission pass through the crystal without any change.

Assuming a spot size of $<1\ \mu m^2$, this gives a peak intensity at the sample of at least 1.7 GW/cm^2, which is sufficient for multiphoton excitation.[24]

2.2 *Confocal Imaging with Arbitrary Excitation Wavelengths*

To demonstrate the compatibility of our novel method, we used standard dyes. Standard excitation wavelengths were applied to record confocal multicolor images. However, within the accessible spectral area there are no physical limitations on the wavelengths chosen. With the described setup, up to eight different wavelengths can be used simultaneously with the ability to switch between wavelengths within milliseconds. Figure 2 shows a neuron-like cell (NG108-15, ATCC, Manassas, VA)[25] fixed and stained for actin (red) and microtubules (green). In both channels the image shows details with diffraction-limited resolution. The images were recorded using the 488-nm and 543-nm lines from the supercontinuum white-light source. To gather information about the out-of-focus planes, we performed a z scan of different sample cells. Figure 3 presents the maximum-projection rendering of these cuts, which compares the values of a certain pixel coordinate along all recorded z-scan images and displays the maximum value. The recorded image displays the same resolution and details as obtained with a conventional excitation source.[26]

To reveal the CLSM's unaltered 3-D capabilities while using the supercontinuum white-light laser source, a 30-μm-thick coronal section from a paraformaldehyde-fixed rat brain, stained with histochemical methods for vessels (green) and astrocytes (red), was imaged. Figure 4(a) shows the maximum projection of a z scan. With these data, the usual 3-D operations of the microscope's software can be exploited, yielding 3-D visualization of the sample, as the color-coded 3-D illustration [Fig. 4(b)] demonstrates.

2.3 *Multiphoton Microscopy*

To illustrate the multiphoton capabilities of the supercontinuum white light, the picosecond-pulsed NIR radiation was successfully used for multiphoton excitation. To simultaneously excite both the tetramethylrhodamine isothiocyanate (TRITC) labeled actin and the Alexa 488 stained microtubule cytoskeleton, no further filtering of the two-photon excitation light for specific wavelengths was necessary. Rather, the entire NIR spectrum from 700 to 1300 nm was used. For the multiphoton imaging, the imaging pinhole was opened completely in order to maximize the detection efficiency and because the described nonlinear effect excites samples only at the focus. Hence, with multiphoton microscopy, the pinhole is not needed to discard out-of-focus and scattered emission light. Figure 5 shows the maximum projection of NG108-15 cells, prepared as described. Although a novel technique was used as pulsed radiation source, the image quality was comparable to conventional two-photon microscopy or CLSM.

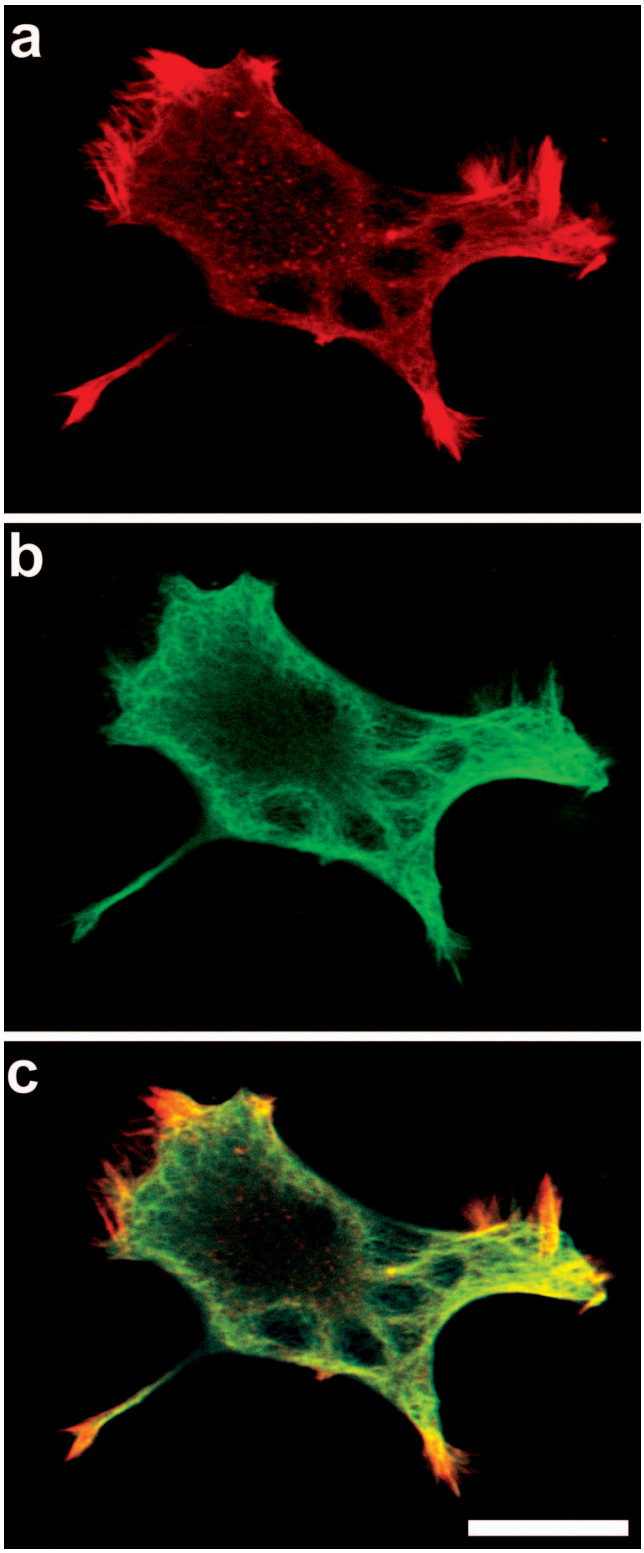

Fig. 2 Images of (a) the actin (red, stained with TRITC-tagged phalloidin) and (b) the microtubule (green, immunolabeled with Alexa 488 coupled secondary antibodies) cytoskeleton of an NG108-15 cell recorded with the supercontinuum white-light source. For better evaluation of the different channels, the two fluorescent markers are presented as single images and as an overlay (c). Images were recorded by averaging over eight scans of each image line (line average), and two scans of total frames (frame average). Scale bar: 10 μm.

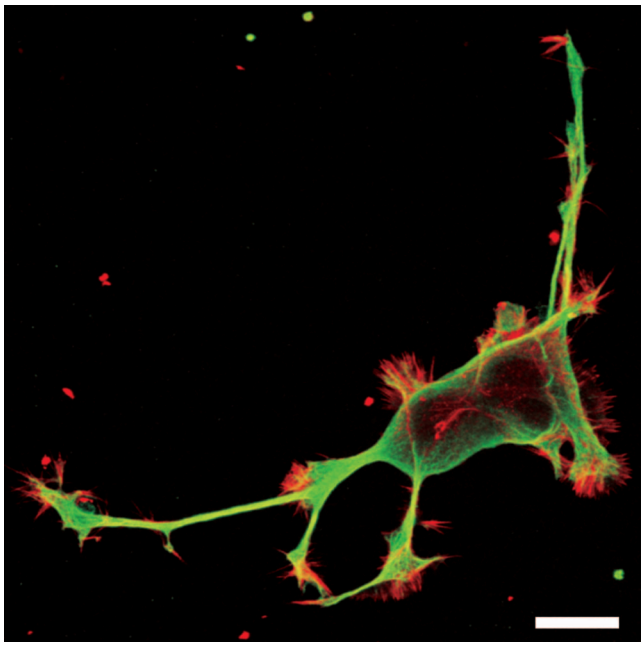

Fig. 3 Maximum projection of a *z* series recorded with the supercontinuum white-light source. The actin (red) and the microtubule (green) cytoskeleton of an NG108-15 cell are fluorescently labeled. The projected *z* series consists of 15 spatial cuts in the *xy* plane, separated by 1 μm from each other in the *z* direction. Images were recorded with a line average of eight and a frame average of two. Scale bar: 25 μm.

3 Discussion

We report on the first successful integration of supercontinuum white light emitted from a tapered fiber into a conventional CLSM, demonstrating high-quality image acquisition with diffraction-limited resolution at arbitrary excitation wavelengths. With the identical setup, two-photon microscopy was demonstrated using the NIR part of the supercontinuum spectrum. Due to the simple setup procedure and the straightforward manufacture of the tapered fiber, this method can be implemented and reproduced in all CLSM systems that feature a femtosecond laser source. The presented combination of supercontiuum white light with the existing technique of acousto-optical devices allows full exploitation of the new supercontiuum laser sources by giving the possibility to arbitrarily switch between multiple excitation wavelengths within milliseconds. Future development toward diode-pumped femto- and picosecond lasers, which could also be used as the driving laser source for the supercontinuum white-light generation, opens the perspective for an affordable white-light laser source to become the standard for advanced fluorescence microscopy.[27]

In general, all scientific fields that rely on fluorescence imaging will benefit from the introduced method, ranging from imaging in cell biology to the detection of DNA labels in DNA microarrays. For example, one obvious and straightforward application of the presented technique is the optimum excitation of fluorescent dyes with respect to absorption peak and absorption width, yielding maximum emission intensities and minimizing the invasive effects of the excitation lasers. Additionally, the design of fluorescent dyes is not limited any

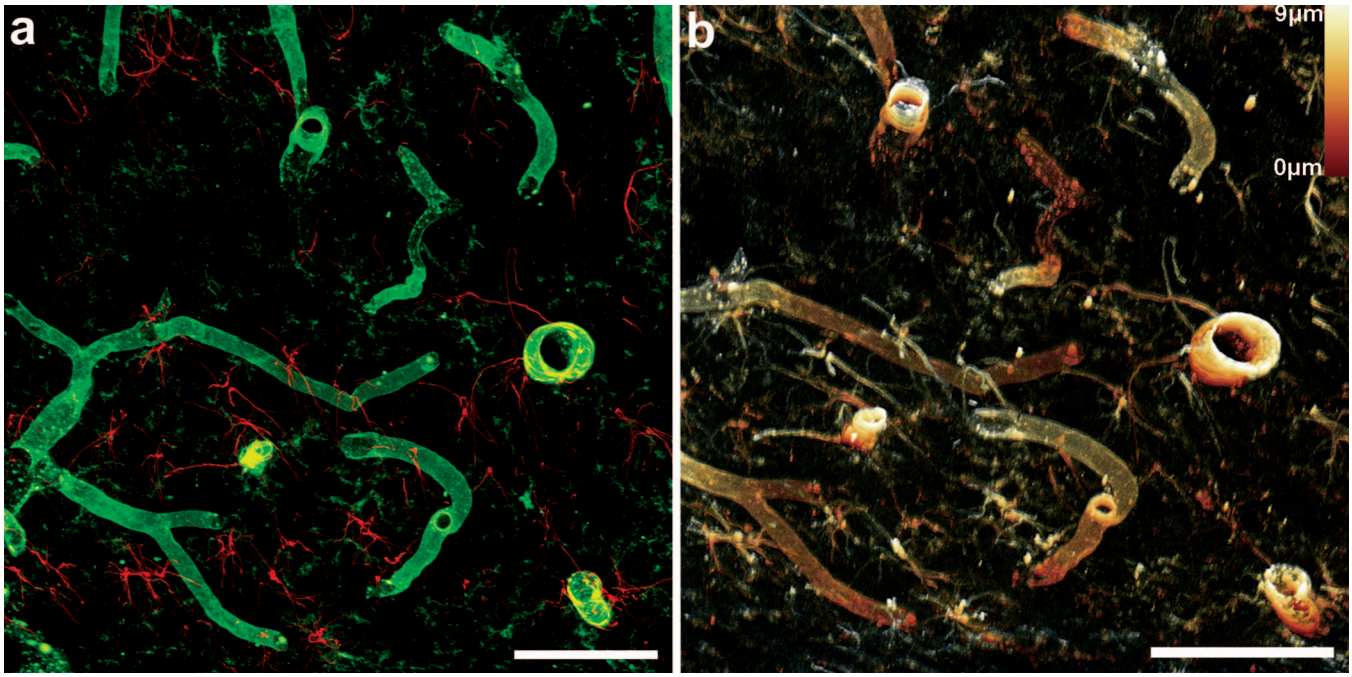

Fig. 4 Imaging of histochemically labeled rat cortical vessels and astrocytes. (a) Maximum projection of a *z* series of a rat brain section displaying vessels (Cy2, green) and astrocytes (Cy3, red). The series represents 10 recordings of the *xy* plane, separated by 1 μm along the *z* direction. Images were recorded with a line average of eight and a frame average of two. Scale bar: 50 μm. (b) Color-coded 3-D projection of vessels and astrocytes presented on the left. Due to the high image quality, usual 3-D operations can be performed. The color table translates the colors into the relative *z* depth of the picture. The image was calculated from the merged red and green channel data of Fig. 5(a), with a perspective view of a 40-deg rotation around the horizontal axis. Scale bar: 50 μm.

more by the constraint of exhibiting certain excitation spectra that match available laser sources. Now, fluorescent dyes can be optimized without this constraint, since a supercontinuum white-light driven CLSM can deliver any excitation wavelength in the accessible spectral range. Furthermore, the discrimination of dyes whose emission spectra are close together, as with the very important green fluorescent protein–yellow fluorescent protein (GFP-YFP) pair, becomes readily available, since the light can be adjusted to excite them separately. More importantly, given the current trend of simultaneously monitoring many different molecules, this method also allows separate detection of different fluorescent dyes by highly specific excitation, even if their emission spectra overlap.

The new method, however, cannot only radically improve the existing CLSMs, it also suggests entirely new imaging techniques with expanded functionality. First of all, laser scanning absorption microscopy (LASCAM) becomes possible, where a diffraction-limited measurement of a sample's absorption spectrum is achieved by recording each sample point while scanning through the continuous spectrum of the white-light laser source. This would merge confocal scanning microscopy and absorption spectroscopy, and allow the detection of certain molecules by their absorption spectra without the need for any labeling of the sample. The proposed LASCAM would only allow the detection of molecules exhibiting very prominent absorption spectra. However, our method will also significantly ease further development of coherent anti-Stokes Raman scattering (CARS) microscopy,[28] which has recently been shown to be feasible with a supercontiuum laser source[29] and has the potential to detect any molecule without

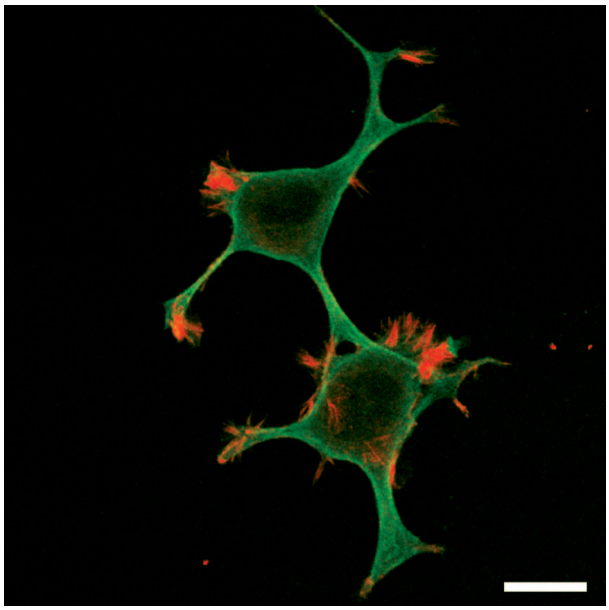

Fig. 5 Fluorescence image of an *xy* section of two NG108-15 cells recorded with two-photon microscopy. Images were recorded with a line average of eight and a frame average rate of three. With the multiphoton technique, the same details are observable as images recorded with the visible part of the supercontinuum white light in Figs. 2 and 3, or with a conventional excitation laser source. Scale bar: 20 μm.

the need of staining. CARS microscopy is a recently developed technique exploiting nonlinear mixing of multiple laser waves to detect molecules. A necessary improvement of the published realization of CARS using a supercontiuum laser source is the well-controllable spectral filtering of the excitation lasers beams. The presented method might thus be able to give an increased spectral control over the wavelength selection and allow a higher supercontinuum power output due to its stability.

The introduction of supercontinuum white light generated with a tapered fiber therefore constitutes a major step in the development of CLSM, multiphoton microscopy, and nonlinear microscopy in general. This provides an affordable, versatile, and easily implemented laser source for both general CLSM applications and the development of completely new strategies in microscopy.

4 Methods

4.1 *Manufacturing of Tapered Fibers*

The tapered fibers were pulled using a home-built drawing rig. The SMF 28 fiber (Thorlabs, Newton, NJ) was heated over a propane-butane-oxygen flame and drawn out to generate a homogeneously thin waist with a diameter of about 2.1 μm and a length of approximately 90 mm. The tapered regions where the fiber diameter changes from the original diameter of 125 μm to the above mentioned thin waist diameter of about 2.1 μm were approximately 15 mm long on either side. Thickness control was achieved by varying the drawing velocity. The fiber was profiled using a 532-nm frequency-doubled Nd:YAG laser (Adlas, Lübeck, Germany) by recording the diffracted image on a diffuse screen using a standard CCD camera (DSC 717, Sony, Cologne, Germany). The diffracted image was evaluated using a specially developed algorithm.[30] The homogeneity over the waist length was better than 10%.

4.2 *Coupling of Femtosecond Pulsed Light into Tapered Fiber*

The pulsed light was directed through a Faraday isolator (FR 820 BB, Linos Photonics, Göttingen, Germany) using two silver mirrors (New Focus 5103, New Focus, San Jose, CA). The laser beam was then focused into the fixed end of the tapered fiber with a 10×0.3 NA objective (Linos Photonics). A three-axis kinematic mount (MDT-612, Thorlabs) for the objective is necessary to optimize coupling efficiency. The light emerging from the fiber was collected and collimated by a 4×0.1 NA objective (Linos Photonics).

4.3 *Coupling of the Visible White Light of the Supercontinuum into the Microscope*

In order to couple the visible supercontinuum white light into the Leica TCS SP2 AOBS (Leica Microsystems, Bensheim, Germany), we removed the KineFlex fiber coupler (Point Source, Southampton, England) from the microscope controller table. This fiber coupler was mounted onto the optical table and used to couple the light into the fiber, which delivered the light directly into the microscope. Since there was no direct access to the output end of this fiber to detect how successful we were in coupling the light, we applied the following procedure for fast coupling. Using the microscope control, all six selected laser lines were deflected into the optical beam path with maximum intensity. Instead of a microscope objective, a reflective object was set onto the objective revolver in the optical path. Next, the AOBS controller was set to detect maximum reflection on all the transmitted laser lines. To obtain feedback of the coupling efficiency, one of the built-in photomultiplier tubes (PMT) was used to detect the reflection over the entire accessible spectrum. This method exploited the sensitivity of the PMT to detect any light that was reflected back from the reflective object, allowing for a fast optimization of the coupling. The fiber coupler was adjusted until the coupling efficiency was sufficient to replace the reflective object in the beam path with a sensitive power meter (Fieldmaster, Coherent), and the fiber adjustment was then continued to maximize power at the objective revolver. After achieving a sufficient coupling efficiency, no further adjustment in the optical path of the microscope was necessary.

4.4 *Coupling of the NIR Part of the Supercontinuum into the Microscope*

To couple the supercontinuum NIR white light into the optical beam path of the microscope, the visible component of the spectrum was filtered with a dichroic mirror (Chroma), and the remainder was directed into the NIR port of the microscope with a pair of broadband silver mirrors (New Focus 5103, New Focus). The standard procedures for the adjustment of the NIR port were then performed, resulting in a power of 20 mW before the objective.

4.5 *Histochemical Staining*

The histochemical staining of cytoskeletal components was performed with NG108-15, a neuron-like mouse neuroblastoma/rat glioma hybrid cell line (ATCC, Manassas, VA). These cells were cultured in medium (90% Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 10 mM Hepes) and plated on glass cover slips 24 h before fixation with 0.3% glutaraldehyde in Brinkley Buffer (BRB80) for 10 min. Next, cell membranes were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 min and unspecific binding sites were blocked with 1% normal goat serum in PBS for 30 min. Microtubules were revealed by incubation with a monoclonal mouse antibody (E7, 1:10 in the blocking solution; provided by the Hybridoma Bank, University of Iowa, Iowa City, IA) for 3 h and Alexa 488-goat anti-mouse IgG (Molecular Probes, Eugene, OR; 10 μg/ml in PBS) overnight. Subsequently, cells were washed with 0.05% Tween 20 in PBS to further reduce unspecific binding of the applied immunoreagents. Filamentous actin was then stained with TRITC-coupled phalloidin (2.5 μg/ml in PBS) for 30 min. Cells were washed and kept in PBS during the microscopic analysis.

For the histochemical staining of rat brain sections the tissue preparation started with the transcardial perfusion of young adult Wistar rats according to the guidelines of the Laboratory Animal Care and Use Committee of the Leipzig University. The animals were perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Brains were then postfixed in 4% paraformalde-

hyde in PB overnight and cryoprotected by equilibration with 30% sucrose in PB. 30-μm-thick coronal forebrain sections were cut on a freezing microtome and collected in 0.1 M Tris-buffered saline (TBS, pH 7.4). To stain for vessels and the astroglial marker, glial fibrillary acidic protein (GFAP) sections were washed in TBS and preincubated with 5% normal donkey serum (Dianova, Hamburg, Germany) and 0.3% Triton X-100 in TBS (NDS-TBS-T) for 1 h. Sections were then incubated in a cocktail of rabbit-anti-GFAP (1:1000, Dako, Hamburg, Germany) and biotinylated *Solanum tuberosum* agglutinin (20 μg/ml, Vector, Burlingame, CA) in NDS-TBS-T overnight. Subsequently, sections were extensively rinsed in TBS and incubated with a mixture of carbocyanine (Cy)3-conjugated donkey anti-rabbit IgG and Cy2-tagged streptavidin (both from Dianova at 20 μg/ml TBS containing 2% bovine serum albumin) for 1 h. Finally, sections were rinsed in TBS and in distilled water, mounted onto a slide, dried, and sealed with a coverslip in Entellan (Merck, Darmstadt, Germany). All chemicals were purchased from Sigma (Taufkirchen, Germany) unless stated otherwise.

Acknowledgments

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