Nonlinear absorption extends confocal fluorescence microscopy into the ultra-violet regime and confines the illumination volume

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It is shown that two-photon absorption confines the illumination volume and present quantitative evidence that an additional confocal arrangement of the detector further improves the resolution by 48%. The axial resolution in a confocal fluorescence microscope using two-photon absorption with infra-red light is comparable to that achievable with ultra-violet light half the wavelength. An important advantage of two-photon microscopy over single-photon microscopy is that absorption is almost confined to the observed volume. This means no photodamage is caused outside the observed volume.

1. Theory

In a confocal fluorescence microscope a point source is used to define the extent and the position of the illuminated volume and a point detector discriminates the light emitted outside the detection volume. In physical terms the product of the detection and the illumination intensity point spread functions defines the point spread function of a confocal fluorescence microscope [1]. While illumination and detection point spread functions are both linear in the intensity their product and hence the fluorescence intensity detected in a confocal microscope are proportional to the square of the illumination intensity [2]. This causes a drop of the detectable intensity away from the geometrical focus, thereby defining the volume that is observed in three-dimensional space. \( h_{\text{ill}} \) and \( h_{\text{det}} \) denote the coherent amplitude illumination and detection point spread functions. The intensity point spread function \( h^2 \) in a confocal fluorescence microscope is

\[
h^2 = h_{\text{ill}}^2 h_{\text{det}}^2.
\]

The z-response \( I(z) \) and the edge response \( I_{\text{edge}}(z) \) are of practical importance since these are used to estimate the resolution

\[
I(z) = \int \int h^2_{\text{ill}}(x,y,z) \, dx \, dy,
\]

\[
I_{\text{edge}}(z) = \int_{-\infty}^{z} I(z') \, dz'.
\]

Since these intensities are independent of \( x \) and \( y \) it is only required to know a function \( h^2(z) \). To get an estimate for the integrated intensities we use a result derived from gaussian beam optics [3], simplifying the system responses to

\[
h^2_{\text{ill}}(z) \propto \left[ 1 + \left( z/z_{\text{exc}}^R \right)^2 \right]^{-1},
\]

\[
z_{\text{exc}}^R = 1.169 (n \lambda_{\text{exc}}/NA^2),
\]

\[
h^2_{\text{det}}(z) \propto \left[ 1 + \left( z/z_{\text{em}}^R \right)^2 \right]^{-1},
\]

\[
z_{\text{em}}^R = 1.169 (n \lambda_{\text{em}}/NA^2).
\]
valid only because of the strong out of focus discrimination in confocal fluorescence microscopy which essentially removes higher order and mixed terms.

Interestingly, the confinement of the confocal volume can also be achieved during the illumination process through the use of two low energy photons instead of one high energy photon to excite fluorophores [4]. While the probability of single-photon absorption is proportional to the intensity, the probability of two-photon absorption, i.e. two photons being absorbed simultaneously by the same molecule, is proportional to the intensity squared [5]. Therefore a microscope based on two-photon absorption is described through the square of the single-photon illumination point spread function and operates in a nonlinear fashion. In a two-photon microscope the intensity point spread function $h_{2h\nu}^2$ is

$$h_{2h\nu}^2 = h_{\text{ill}}^2 h_{\text{det}}^2$$

and in a confocal two-photon microscope the point spread function $h_{c2h\nu}^2$ is

$$h_{c2h\nu}^2 = h_{\text{ill}}^2 h_{\text{det}}^2.$$

The illumination process in a confocal two-photon fluorescence microscope therefore defines a volume in a manner similar to the combined illumination and detection processes in a confocal single-photon fluorescence microscope. This means that photo damage in a two-photon confocal fluorescence microscope occurs only in the vicinity of the geometrical focus, while in a "normal" confocal fluorescence microscope photo damage is caused throughout the whole sample. In contrast to a confocal single-photon fluorescence microscope a detection pinhole is actually not required but will force an intensity cubed dependence thus further improving axial and lateral discrimination [6,7].

To implement such an instrument a pulsed near-infra-red laser was connected to the confocal fluorescence microscope at EMBL [8]. The resolution of a confocal two-photon fluorescence microscope and a nonconfocal two-photon fluorescence microscope were measured using axial edges. The restriction of the bleached volume was observed in fluorescent latex beads. Hoechst 33342 labelled chromosomes and nuclei were observed in almost polarized Madin Darby canine kidney cells to see if biological objects can be observed without excessive damage.

2. Experimental arrangement

The light source was a titanium-sapphire laser (Tsunami, Spectra-Physics) generating 80 fs 46 kW pulses at a frequency of 82 MHz pumped by an argon-ion laser (Modell 2030, Spectra-Physics). The laser power was reduced using metal coated neutral density filters (Jobin Yvon, Grasbrunn, Germany) to 1% for the observation of beads and to 5% for the observation of chromosomes. The beam was expanded employing its natural divergence over a distance of four meters. The filters in the beam scanning confocal fluorescence microscope at EMBL were replaced to enable the use of this laser: a special dichroic filter (reflectivity: 700-800 nm >98%, transmission 400-600 nm >95%, LO Laseroptik GmbH, Garbsen, Germany) was used to direct the beam into the microscope. The fluorescent light further passed a band pass filter (reflectivity 660-810 nm >99%, transmission 400-590 nm >95%, LO Laseroptik) before entering the detector. The light was detected with a photomultiplier (Hamamatsu 1463-01) and recorded as a function of scanner position. Neither the internal optics of the conventional Zeiss Axiovert 10 which is part of the confocal microscope at EMBL nor the mirrors of the galvanometer scanners were replaced. Since the relaying optics have a magnification of 1 the detection pinhole diameter was 80% of the diameter of the first zero intensity ring in an Airy disk, i.e. 30 µm.
3. Results

The axial edge-response (eq. (3)) is the intensity along the optical axis as a function of the position of the probing beam inside a thick layer of fluorophore. We used $10^{-4}$ M Coumarine 138 (laser grade, Kodak) dissolved in immersion oil (microscope grade DIN 518). The solution was mounted between a cover glass and a slide. It provided a sample with a step along the z-axis. The variation of the intensity along the optical z-axis was analysed. The laser was tuned to 738 nm (measured using a spectrophotometer) and the fluorescence emission is observed above 420 nm. The lens was an ICS Zeiss Plan-Apochromat 100×/1.4. Images parallel to the optical axis ($x/z$-images) were recorded. 64 columns were averaged to generate the data for the scatter plots. The distance between 17% and 83% of the plateau is roughly equivalent to the full width half maximum along the optical axis of a three-dimensional point spread function and characterizes the axial resolution [9]. In conventional mode, i.e. without a pinhole in front of the detector, the axial resolution is 880 nm. In confocal mode the resolution is 490 nm (see table 1). These values are practically identical to those found using the theory described above. The graph shown in fig. 1 proves that in order to achieve axial resolution in an instrument using two-photon absorption a detection pinhole is not required. However, the resolution of 880 nm is about 30% worse than what is achievable in the visible regime assuming an illumination of 488 nm and about 53% worse than the resolution in an ideal confocal uv microscope operating at a wavelength of 368 nm. But, using a detection pinhole the resolution is improved by 48% to 490 nm. This is quite likely the best resolution ever achieved in a confocal fluorescence microscope using conventional optics. These results are in contrast to the first predictions in ref. [7] but quantitatively confirm the predictions in ref. [8]. The drawings in fig. 1 show two individual experiments while the data in table 1 are compiled from five experiments.

Bleaching provides a method to demonstrate the effects of absorption relevant in light microscopy. Using single-photon absorption the fluorophore was damaged inside a latex bead. The diameter of the latex bead in fig. 2 was 5 μm, the exciting laser line had a wavelength of 476 nm, the emission was observed above 515 nm. The lens was an ICS Zeiss

Fig. 2. The fluorophore in a latex bead was bleached with single-photon absorption. (a) and (b) $x/y$- and $x/z$-images of the untreated beads. (c) and (d) $x/y$- and $x/z$-images after a hole was bleached into the bead. The bead has a diameter of 5 μm.
Plan-Neofluar 100×/1.3. The laser power entering the objective lens was below 1 mW and the spot was bleached within less than a second. The beads (L-5121 Molecular Probes, Eugene, OR, USA) were dissolved in 1:100 in water. A droplet was dried in air on a cover slip, stuck to a slide and observed through the cover glass. Figure 2 shows clearly a focused spot in the middle of the bead. The fluorophore is, however, damaged also above and below the plane of focus. This shows that in single-photon absorption the illumination volume is not confined. The intensity varies along the optical axis and has a maximum in the focus, but will nevertheless cause damage throughout the sample.

This is in contrast to a similar experiment using two-photon absorption. The diameter of the bead shown in fig. 3 was 6 μm, the exciting laser wavelength was tuned to 750 nm, the emission was observed above 420 nm. The lens was an ICS Zeiss Plan-Neofluar 100×/1.3. The laser power was below 1.5 mW in the entrance aperture of the objective lens and the spots were bleached within four seconds. The hole on the left, the three holes on a diagonal and the hole on the right were bleached in three different focal planes. The large bead was found in a solution of 1 μm beads (L-5080, Molecular Probes). The sample was treated as described above. The x/z-image shows clearly that the bleached volumes are confined. The imaging conditions from glass to bead to air are not perfect and account for the slightly deformed shapes along the optical axis.

The images shown in fig. 4 are no further proof but a demonstration that biological objects can be observed making use of the important properties of a confocal microscope based on two-photon absorption without excessive damage. Chromosomes are in general relatively large objects. In the example shown in fig. 4 they are very densely packed but the microscope resolves them well. The x/z-image shows slices through chromosomes which are practically round which indicates that the resolution is very high even in a biological object. Photodamage to the sample was not observable (drop below 1%) even after ten images were recorded in the same plane. As pointed out above an illumination of the beads for four seconds was required to remove about 90% of the fluorophore in the center. During an observation scan the period of time the beam rests in the same position is about 0.7 to 7 μs, which is about six orders of magnitude less. In general our impression is that photodamage is much less of a problem in two-photon absorption microscopy than in conventional confocal fluorescence microscopy.

4. Conclusions

Data presented in this paper proves that a confocal two-photon fluorescence microscope has a resolution that is equivalent to the resolution of a confocal microscope operating at half the wavelength. The edge response experiments and the images in fig. 3 prove that the illumination process defines a volume. The simple theory based on gaussian beam optics describes the experiments very well.

In addition the confocal fluorescence microscope using the two-photon absorption process has a number of interesting advantages over an equivalent instrument based on the single-photon absorption process:

(i) Only the volume close (± 0.45 μm) to the geometrical focus is subject to photo damage. Therefore, depending on sample thickness and number of slices required to obtain a complete three-dimensional data set, the time integrated light intensity (energy) per recording can be 10–30 times higher.
Fig. 3. The fluorophore is bleached in a large latex bead in five different positions with two-photon absorption. (a) Image recorded in a plane parallel to the optical axis \((x/z)\) through the left, center and right hole. (b)-(f) Images \((x/y)\) of five different planes. The large bead has a diameter of 6 \(\mu m\).

Fig. 4. Hoechst 33342 labelled chromosomes in mitotic Madin Darbey canine kidney cells observed in a two-photon absorption confocal fluorescence microscope. (a) and (c) Stereo pair calculated from a set of 23 images 8.2 \(\mu m\) deep. (b) An image recorded directly with the instrument in a plane parallel to the optical axis \((x/z)\). The field size of all pictures is 10 \(\mu m\). The laser was tuned to 736 nm, effectively exciting the dye with a wavelength of 368 nm. The emitted light was observed above 420 nm. The lens was an ICS Zeiss Plan-Apochromat 100\(\times/1.4\).
(ii) Caged fluorescent dyes and compounds such as caged FITC [10] and caged ATP can be activated in a volume well defined in three-dimensional space.

(iii) Spectral regions beyond the barrier of 380 nm imposed by glass are easily accessible.

(iv) Fluorophores excited in the uv are usually very efficient [11] and photomultipliers have a four times higher quantum efficiency (20%) around 400 nm than around 600 nm [12].

(v) Biological objects are most likely less sensitive to near infra-red (above 730 nm) than to ultra-violet light.

(vi) Since Rayleigh scattering is proportional to the fourth power of frequency, infra-red light is less affected by scattering than blue light and will penetrate many samples deeper.

(vii) With short excitation pulses and fast decay times, distinctions between dyes can be made with methods other than frequency filtering.

Two-photon confocal fluorescence microscopy has fulfilled all the expectations we had in it and provided us with the highest resolution ever reported for a real confocal fluorescence microscope. We expect it to play a very important role in the future of three-dimensional light microscopy.

References