An inertia-free beam scanning device for single-wavelength 2PE-STED nanoscopy

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Abstract

Two-photon excitation stimulated emission depletion nanoscopy (2PE-STED) is a fluorescence imaging technique ideal for significantly improving the spatial resolution when observing scattering tissue in fixed, in vitro, and in vivo specimens. Both 2PE and STED are beam scanning techniques. The image acquisition is commonly realized by raster scanning, and a pair of galvanometric mirrors are the most used approach. In 2PE fluorescence microscopy, acousto-optical deflectors (AODs) are also used because they allow random-access scanning. However, since the AOD working principle is diffraction of light, these devices are of limited use when it is necessary to deflect multiple beams of different wavelengths and polarization, e.g. in STED nanoscopy. Here, we present the first implementation of single wavelength 2PE-STED that enables a smart beam scanning system based on AODs technology.

Keywords: random-access, RASTED, super-resolution, STED, 2PEF

(Some figures may appear in colour only in the online journal)

1. Introduction

In tissue imaging and especially in brain research, an increasing number of experiments requires insights at the nanoscale level. Both high-content and high-throughput imaging is another important demand. Imaging scattering specimens can benefit from the use of a two-photon excitation (2PE), a beam scanning technique compatible with stimulated emission depletion (STED) nanoscopy [1, 2]. Achieving fast imaging is crucial for the collection of large amounts of data. In many of the application where 2PE microscopy is advantageous, one solution for faster imaging is to avoid raster scanning [3, 4], using custom scan patterns, and imaging only regions where fluorescent structures of interest are present [5, 6]. One such custom scan pattern is random-access scanning. Such patterns are conveniently realized with inertia-free scanners that employ dynamic diffractive optical elements such as acousto-optic deflectors (AOD) [7–9]. Nowadays, high-throughput imaging is not the only requirement but also high spatial resolution and, in particular, super-resolution is of high interest and impact.

In a scanning fluorescence microscope, nanometer resolution can be easily achieved by the method of stimulated emission depletion (STED) [10–12], which can be combined with fast scanning based on resonant galvo mirrors [13] or electro-optical scanners [14].

The most common implementation is realized by spatially overlapping two beams of distinct shapes and different wavelengths, i.e. a Gaussian beam and a doughnut beam, combining 1PE or 2PE with STED. Traditional mirror-based galvanometric scanners can easily accommodate the commonly different wavelengths used for 2PE and STED since reflection is wavelength-independent. However, the inertia of moving parts in galvanometric systems limits their usefulness to random-access scanning and has prevented this technique from being used with STED nanoscopy. Since optical deflectors, which rely on the electro-optic or the acousto-optic effect, do not contain any moving parts, they provide high deflection angle rates. Electro-optical deflectors (EODs) exhibit the
highest speed and transmittivity. However, EODs are operated at high voltages, their accuracy and maximum deflecting angle, i.e., the achievable field-of-view, is much smaller than those of AODs. For these reasons, the best performance of an EOD-based scanning system is achieved when combined with a galvo scanner to take advantage of both domains. One of the best examples is its application in STED nanoscopy [14]. On the other hand, since the main drawback of AOD-based scanning systems is their limited diffraction efficiency, they are mainly implemented in 2PE microscopy (2PEM) where non-descanned detection is favourable and desirable [15]. In 2PEM, AOD-based scanners can provide full three-dimensional scanning without mechanical parts [8]. Still, the diffraction angle of AODs depend on both acoustic and optical frequencies. Therefore, in STED nanoscopy, a realistic effort to register the two scanning beams requires using the same optical wavelength for both 2PE volume and STED doughnut. Indeed, such a single-wavelength approach is possible and has been demonstrated before [16]. This form of STED nanoscopy employs the same wavelength for both 2PE and single-photon emission depletion (1PD).

In this report, we propose a novel inertia-free scanning system that overcomes the limitations imposed by AODs to STED microscopy. We take advantage of the single-wavelength 2PE-STED method that does not require the excitation wavelength to differ from the depletion wavelength. One of the fundamental requirements of STED nanoscopy is a complete registration of both Gaussian two-photon excitation (2PE) volume and surrounding STED doughnut to achieve the desired reduction of emission volume and thus super-resolution [17].

Notably, precise registration of both illumination patterns has to be maintained during scanning across a stationary object. This requirement is best satisfied by using the same AOD-based scanner operated at a single acoustic frequency for both excitation and depletion beams.

Another essential parameter in STED nanoscopy is polarization [17]. However, AODs with the highest diffraction efficiency are based on crystals, i.e., TeO₂, that are polarization-sensitive [18]. Therefore, we developed an AOD scanner with innovative beams paths, which takes into account the correct match between the polarization of beams and orientations of AODs.

2. AOD theory

Acousto-optic deflectors (AODs) are diffractive elements behaving like optical transmission gratings with the inherent ability to dynamically adjust their grating constant. This feature is due to controlled periodic modulations of the refractive index in an acousto-optical (AO) material by mechanical waves. Optically effective acoustic frequencies are in the high MHz range and are generated by an electro-mechanic transducer, e.g., a piezo element, bonded to the AO material, e.g., TeO₂. AODs are commonly operated in the Bragg mode, i.e., the laser beam should be incident at the Bragg angle \( \theta_i = \theta_B \approx \lambda / (2 \Delta n) \), where \( \lambda \) and \( \Delta n \) are the optical and the acoustic wavelengths, respectively. In this mode, most transmitted optical energy is located in the 1st diffraction orders (+1 or -1), the diffraction angle \( \theta_d \) is defined as \( \theta_d = \lambda / (2 n v_r) \), where \( f_a \) and \( v_r \) are frequency and velocity of the acoustic wave, \( n \) is the refractive index of the unperturbed crystal.

In such devices, changing the grating constant, i.e., the acoustic frequency \( f_a \), and thus the diffraction angle is inherently inertia-free but time-limited by the acoustic velocity, \( v_r \), of the propagating mechanical waves and the need to fill the active aperture, \( D \), of the employed AOD with the new acoustic wavelength. Such response time is defined by \( \tau_a = D / v_a \). In our case, the size of the beams passing through the AODs is about 7 mm while the \( v_a \) is 675 m s⁻¹. Therefore, the beam positioning takes about 10 µs and is independent of the target location.

To utilize the advantages of diffractive scanners, we have to solve an issue related to the STED scheme. Superimposing two beams of different wavelengths is achieved conveniently and with minor losses by a dichroic beamsplitter (DBS). Still, using two beams of different wavelengths with AODs would require two different incident angles \( \theta_i \) and two different acoustic frequencies \( f_a \), precisely adjusted to keep the spatial alignment over the full scanning range. This scheme would strongly reduce the diffraction efficiency and require a tuning hard to achieve. The use of a single-wavelength scheme overcomes this challenge but requires orthogonally polarized beams and a polarizing beamsplitter (PBS) to remain loss-free. The most efficient AODs, made of TeO₂ material, work at definite linear polarization, apparently preventing such a scheme [18]. The novel solution we propose is sending both 2PE and STED beams in opposite directions through the same AODs (figure 1), such that the final beams can orthogonally polarized and thus combined with a PBS in a loss-free manner.

3. AOD-based 2PE-STED nanoscope

This instrument combines two technologies, single-wavelength (SW) 2PE super-resolution STED and inertia-free random-access scanning with AODs. In SW 2PE STED nanoscopy, commonly, a single ultrafast laser is split into the
Two synchronized pulsed lasers (2PE, STED) operating both at 775 nm. 2PE beam (green), generated by mode-locked Ti:S laser. Two fixed optical gratings (G) for pre-compensation of temporal and spatial dispersions. High-power STED beam (red), emitted by synchronized fiber laser. Vector phase plate (VPP) for wavefront shaping of depletion beam. Individual half-wave plates (HWP) optimize linear polarizations before beams pass through diffractive scanners (AODx, AODy). A polarizing beam splitter (PBS) combines registered beams loss-free. Two waveplates (HWP, QWP) convert beam polarizations from linear to circular. Objective lens (Obj) generates the desired doughnut-shape of STED volume, not affecting 2PE Gaussian volume. Dichroic beam splitter (DBS) directs fluorescence (yellow) to non-descanned detector (NDD).

4. Performance of the nanoscope

Designing and constructing a STED system implies few constraints, which become challenges when using an AOD-based scanning system. Beam shaping, beam registration, and polarization are parameters that should be of particular care when using highly dispersive and diffractive acousto-optic devices. In figure 3, we show the lateral and axial point-spread functions (PSFs) of both Gaussian and doughnut beams, measured by imaging 150 nm gold beads in reflection. Although AODs could introduce some aberrations while 'hopping' from one site to another, it has been demonstrated that a fine-tuning of acoustic frequencies can also compensate for those, and AODs can be used as beam shaping devices. However, with precise optical alignment and without acoustic tuning, we get negligible astigmatism and coma in
Figure 3. Point spread function (PSF) of excitation and depletion beams. The panels show xy PSFs (a), (b) and xz PSFs (c), (d) of excitation and depletion beams, respectively. We imaged gold beads of 150 nm diameter in reflection modality. Scale bars are 0.5 µm.

the doughnut PSF (figures 3(b) and (d)), which are visible but can be tolerated. In a conventional 1PE or 2PE fluorescence STED nanoscope, the excitation, and depletion beams can be overlapped and coaligned independently of the scanning system in a loss-free manner due to their difference in wavelength. However, in the SW 2PE-STED approach, beam combining by polarization is required to avoid power loss.

In this setup, we use standard AODs (ATD-7010CD2, ItraAction Corp., Bellwood, IL, USA) for 2PE imaging, which are made of TeO₂, have NIR coating, 700–1100 nm, an active aperture of 10 × 10 mm, a center frequency of 70 MHz (800 nm) and a deflection bandwidth of 40 MHz. The input optical polarization must be linear and parallel to the mounting surface, while the output optical polarization becomes linear and perpendicular to the mounting surface. Such a characteristic seems to prevent the possibility of combining excitation and depletion beams by polarization before the scanning unit. The solution we found exploits counter-propagating beams through the AODs such that input polarizations of the two beams are identical and the output beams can be independently registred. Since the AODs are operated at a single acoustic frequency each, the two input beams, having the same wavelength, will be deflected at the same angles. Thus, beam combination and thus registration is completed by a PBS after the scanning unit. It is noteworthy that both AODs reside in conjugate planes, due to two pairs of relay lenses. If these two telescopes are not perfectly aligned, the two counter-propagating beams would undergo slightly different magnification, resulting in a different scan field and speed. The actual displacement we observed, at the border of the allowed field-of-view (FOV, 140 × 140 µm) is less than the excitation beam waste.

In figures 4(a)–(c), we show the spatial inhomogeneity that could affect the scanning. We superimposed the raw SW 2PE-STED image with the respective 2PE one (figure 4(a) and cross-correlated them to obtain the deformations (represented as field and grid in figures 4(b) and (c), respectively), which should be used to register both beams. Deformation map and field were obtained using the bUnwarpJ [21] plugin in FIJI [22]. Since the depletion doughnut is about three times larger in volume, the 2PE, fluorescence from its centre decreases exponentially with such displacement. This results in a reduced signal-to-background ratio (note, background comprises both undepleted and the doughnut-excited fluorescence) such that at large FOVs there is an area where fluorescence is reduced, and resolution approaches that of conventional 2PE [23]. In our experiments, we achieved a homogeneous FOV of 40 µm × 40 µm, as shown in figure 5. We acquired images by a non-descanned detector, a GaAsP PMT. Both scattering and out-of-focus fluorescence generated by direct 1PE through the depletion beam, are often negligible in a confocal scheme with closed pinhole, however, in non-descanned imaging, such background becomes an important artifact, figure 5(c). A sequential imaging paradigm, e.g. pulse-, pixel-, line- or frame-wise scanning, in which we alternate STED conditions and the depletion beam only, allows us to directly
Figure 5. Non-descanned 2PE, SW 2PE-STED and background. (a) 2PE, (b) SW 2PE-STED, (c) background with depletion beam only, (d) subtraction of (b), (c). Color scale bar in arbitrary units. (e) Comparing 2PE (top left) and SW 2PE-STED (bottom right) methods. (f), (g) Zoomed region highlighted by dashed square in (e), 2PE and SW 2PE-STED images, respectively. (h) Intensity profiles along line identified by green arrows in (f) black, and (g) red, respectively. All images collected sequentially by line, with non-descanned hybrid detector, spectral range 650–720 nm. Objective lens HCX PL APO CS 100 × 1.4 NA oil (Leica Microsystems, Mannheim, Germany), 775 nm wavelength. Field-of-view (FOV) (a)–(d) 20 µm, (e)–(g) 40 µm, HeLa cells, tubulin labeled with Atto647N.

Finally the proposed scanning system could be compatible with other super-resolution techniques, e.g. RESOLFT [5, 26] and MINFLUX [27]. In the first case, reading and off-switching is given at the same wavelength as it happens in SW 2PE STED nanoscopy. Although the activation occurs at different wavelength, the beam will not suffer of low diffraction efficiency because the calibrated acoustic frequency will be delivered at a different time. The latter is already implemented by an inertia-free scanner but based on EOD and galvo [28]. Still, in both cases, the eventual benefits of AODs could be reduced by the limits in fluorescence detection.

5. Conclusions

In this paper, we demonstrated the performance of an AOD-based scanning system with non-descanned detection in the SW 2PE-STED regime. We overcame the challenges related to combining STED architecture with acousto-optic devices by designing and implementing a novel scheme of beam paths. It supports counter-propagating excitation and depletion beams subtract this background [24]. In all the experiments, we used line-sequential scanning, and in figure 5(d), we show STED imaging after subtracting figures 5(b) with (c). Although the overall noise increases, the signal-to-background ratio definitely improves after this operation. 2PE STED nanoscopy has a high demand of photons. Thus, we achieved a good signal-to-noise ratio at pixel dwell times of 20 µs, keeping in mind a response time of about 10 µs with the employed AODs. The excitation and depletion powers were 12 mW and 41 mW, respectively (measured at the back-aperture of the objective lens). At these conditions, we achieved a resolution better than 70 nm at a wavelength of 775 nm, for both excitation and depletion, figures 5(e)–(h). It is worth noting that AOD-based scanning systems are particularly suitable and interesting for smart-pixel implementation, such as anti-Stokes subtraction but also reduction of illumination, photobleaching and phototoxicity [6, 25]. A real time feedback system realized pixel-wise could allow flexible scanning. Thanks to the inertia-free scanning, the beam could hop to areas where the fluorescence is over a certain threshold, thus skipping dark areas and speeding up the imaging rate [3].
through the scanning system while their combination and registration is loss-free by orthogonal polarization before the objective lens. Since AOD-based scanning is inertia-free, the novel design enables any custom scanning scheme, including random-access and lissajous, while achieving the same resolution as a conventional 2PE-STED system. Finally, the proposed system has the potential to be extended to other scanning-based super-resolution techniques, e.g. RESOLFT and MINFLUX.

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References

[1] Moneron G and Hell S W 2009 Two-photon excitation STED microscopy Opt. Express 17 14567–73
[2] Ding J B, Takasaki K T and Sabatini B L 2009 Supraresolution imaging in brain slices using stimulated-emission depletion two-photon laser scanning microscopy Neuroreport 20 633–36
[3] Botcherby E J, Smith C W, Kohl M M, Debarre D, Booth M J, Juskaitis R, Paulsen O and Wilson T 2012 Aberration-free three-dimensional multiphoton imaging of neuronal activity at kHz rates Proc. Natl Acad. Sci. USA 109 2919–24
[4] Deguchi T, Bianchini P, Palazzolo G, Diaspro A and Duocastella M 2019 Volumetric Lissajous confocal microscopy bioRxiv (https://doi.org/10.1101/735654)
[5] Dreier J, Castello M, Cocoano G, Cáceres R, Plastino J, Vicidomini G and Testa I 2019 Smart scanning for low-illumination and fast RESOLF1 nanoscopy in vivo Nat. Commun. 10 1–11
[6] Hobe R A, Van Oven C H, Gadella T W J, Dhouroukse P B, Van Noorden C J F and Manders E M M 2007 Controlled light-exposure microscopy reduces photobleaching and phototoxicity in fluorescence live-cell imaging Nat. Biotechnol. 25 249–53
[7] Salomé R, Kremer Y, Dieudonné S, Léger J F, Krichevsky O, Wyart C, Chatenay D and Bourdieu L 2006 Ultrafast random-access scanning in two-photon microscopy using acousto-optic deflectors J. Neurosci. Methods 154 161–74
[8] Duemani Reddy G, Kelleher K, Fink R and Saggau P 2008 Three-dimensional random access multiphoton microscopy for functional imaging of neuronal activity Nat. Neurosci. 11 713–20
[9] Nadella K M N S, Scaron H R, Baragli C, Griffiths V A, Konstantinou G, Kozmitz T, Evans G J, Kirkby P A and Silver R A 2016 Random-access scanning microscopy for 3D imaging in awake behaving animals Nat. Methods 13 1–6
[10] Hell S W and Wichmann J 1994 Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy Opt. Lett. 19 780–2
[11] Vicidomini G, Bianchini P and Diaspro A 2018 STED super-resolved microscopy Nat. Methods 15 173–82
[12] Bianchini P, Peres C, Oneto M, Galiani S, Vicidomini G and Diaspro A 2015 STED nanoscopy: a glimpse into the future Cell Tissue Res. 360 143–50
[13] Castello M et al 2019 A robust and versatile platform for image scanning microscopy enabling super-resolution FLIM Nat. Methods 16 175–8
[14] Schneider J, Zahn J, Maglione S, Sigrist S J, Marquard J, Choijacki J, Kräusslich H-G, Sahl S J, Engelhardt J and Hell S W 2015 Ultrafast, temporally stochastic STED nanoscopy of millisecond dynamics Nat. Methods 12 827–30
[15] Le Grand Y, Leray A, Guilbert T and Odin C 2008 Non-descanned versus descanned epifluorescence collection in two-photon microscopy: experiments and Monte Carlo simulations Opt. Commun. 281 5480–6
[16] Bianchini P, Harke B, Galiani S, Vicidomini G and Diaspro A 2012 Single-wavelength two-photon excitation-stimulated emission depletion (SW2PE-STED) superresolution imaging Proc. Natl Acad. Sci. USA 109 6390–3
[17] Galiani S, Harke B, Vicidomini G, Lignani G, Benfenati F, Diaspro A and Bianchini P 2012 Strategies to maximize the performance of a STED microscope Opt. Express 20 7362–74
[18] Römer G R B E and Bechtold P 2014 Electro-optic and acousto-optic laser beam scanners Phys. Proc. 56 29–39
[19] Leutenegger M, Eggeling C and Hell S W 2010 Analytical description of STED microscopy performance Opt. Express 18 26417–29
[20] Castello M, Tortarolo G, Hernandez L C, Bianchini P, Buttavasta M, Boso G, Tosi A, Diaspro A and Vicidomini G 2016 Gated-sted microscopy with subnanosecond pulsed fiber laser for reducing photobleaching Microsc. Res. Tech. 79 785–91
[21] Arganda-Carreras I, Sorzano C O S, Marabini R, Carazo J M, Ortiz-de-Solorzano C and Kybic J 2006 Consistent and elastic registration of histological sections using vector-spline regularization Computer Vision Approaches to Medical Image Analysis Lecture Notes in Computer Science vol 4241 eds R R Beichel and M Sonka (Berlin: Springer) pp 85–95
[22] Schindelin J et al 2012 Fiji: an open-source platform for biological-image analysis Nat. Methods 9 676–82
[23] Tortarolo G, Castello M, Diaspro A, Koho S and Vicidomini G 2018 Evaluating image resolution in stimulated emission depletion microscopy Optica 5 32–35
[24] Coto Hernández I, Peres C, Cella Zanacchi F, d’Amora M, Christodoulou S, Bianchini P, Diaspro A and Vicidomini G 2014 A new filtering technique for removing anti-Stokes emission background in gated CW-STED microscopy J. Biophoton. 7 376–80
[25] Staudt T, Engler A, Rittweger E, Harke B, Engelhardt J and Hell S W 2011 Far-field optical nanoscopy with reduced number of state transition cycles Opt. Express 19 5644–57
[26] Hofmann M, Eggeling C, Jakobs S and Hell S W 2005 Breaking the diffraction barrier in fluorescence microscopy at low light intensities by using reversibly photoswitchable proteins Proc. Natl Acad. Sci. USA 102 17565–9
[27] Balzarotti F, Eilers Y, Gwosch K C, Gynäa A H, Westphal V, Stefani F D, Elf J and Hell S W 2017 Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes Science 355 606–12
[28] Gwosch K C, Pape J K, Balzarotti F, Hoess P, Ellenberg J, Ries J and Hell S W 2020 MINFLUX nanoscopy delivers 3D multicolor nanometer resolution in cells Nat. Methods 19 780–19