Two-photon STED nanoscopy realizing 100-nm spatial resolution utilizing high-peak-power sub-nanosecond 655-nm pulses

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Abstract: We developed two-photon excitation stimulated emission depletion (STED) nanoscopy using high-peak-power sub-nanosecond 655-nm pulses. The STED pulse exhibited ideal optical properties and sufficient pulse energy to realize a 70-nm spatial resolution in the compact setup with electrically controllable components. For biological applications, we screened suitable fluorescent dyes or proteins and realized the sub-100 nm spatial resolution imaging of presynaptic protein clusters in fixed primary cultured neurons without severe photobleaching. We expect this method to enable visualization of ultrastructures and the cluster dynamics of biomolecules representing physiological functions in living cells and tissue.

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1. Introduction

Two-photon excitation fluorescence microscopy has been widely used for bioimaging at subcellular to tissue levels [1] because it demonstrates superior penetration depth and reduced invasiveness for biological specimens by utilizing a near-infrared pulsed beam for two-photon excitation. Two-photon excitation fluorescence microscopy has realized in vivo volumetric imaging at depths of ~1.6 mm below the surface of a mouse brain, allowing us to visualize the morphology, network, and activity of numerous neurons in a living brain [2–4]. Recently, super-resolution technologies [5,6] to improve the spatial resolution of two-photon microscopy have been investigated in various biological fields [7–15].

Stimulated emission depletion (STED) microscopy [5] is one such technology that utilizes a doughnut-shaped beam to induce stimulated emission and restrict the emission area below the diffraction limit. Irradiation of a high-average power STED beam often causes severe photodamage to fluorescent probes and biological specimens. Photodamage can be reduced by utilizing pulsed beams to decrease the average power [8–11,15]. Most two-photon excitation STED microscopy systems utilize a mode-locked femtosecond laser light source similar to that in conventional two-photon excitation fluorescence microscopy [7–11,13,14]. To superimpose the excitation and STED light pulse temporally, the system requires a STED light source composed by another pulsed laser light source, an optical parametric oscillator, and/or pulse dispersion optics [8,10,11], resulting in a large-scale optical setup. To simplify the setup, following approaches have been proposed: implementing single wavelength light pulses to induce both two-photon excitation and STED processes [9], incorporating a compact...
continuous-wave (CW) STED light source with time-gated detecting methodology [14], and using two types of compact laser diode (LD)-based light pulse sources [15].

Recently, we have developed compact two-photon excitation pulsed STED microscopy with electrically controllable components, including LD-based light sources for both excitation and STED [15]. Here, a key component is a set of transmissive liquid crystal devices (tLCDs) that are a type of the spatial light modulator (SLM) used to shape the STED beam and compensate for chromatic aberration. Unlike reflective SLMs, tLCDs can be directly inserted in front of the objective lens without adding a new optical path for SLMs, resulting in the compact setup. In contrast, a mass-produced LD-based pulsed STED light source does not have sufficient pulse energy to achieve spatial resolution below 100 nm.

Herein, to solve this low-power problem of the STED light source, we utilized our recently developed light source for STED, which can generate high-peak-power sub-nanosecond 655-nm optical pulses based on LD-controlling technologies [16]. We demonstrated that the 655-nm optical pulse had ideal optical properties and sufficient pulse energy to realize that the spatial resolution reached 70 nm without using supportive techniques such as a confocal aperture or image processing using deconvolution algorithms. The compact setup was maintaining using LD-based light sources and tLCDs [15], using which presynaptic protein clusters in a fixed primary neuron were successfully visualized at sub-100-nm spatial resolution without severe photobleaching.

2. Experimental setup and procedures

2.1 Optical setup

The optical setup was based on our previous two-photon excitation STED microscope [15]. As Fig. 1 shows, we employed three different types of pulsed LD light sources driven by a homemade electric pulse generator at a repetition rate of 5 MHz.

For two-photon excitation, a 7.5-ps optical pulse source comprising an in-house 1064-nm GS-LD and multistage optical fiber amplifiers were used [17]. The average power was approximately 1 W, sufficient for two-photon excitation of general fluorescent probes in biological specimens [3,4,15,18]. Using this laser as the two-photon excitation light source at a repetition rate of 5–10 MHz, we successfully realized the non-invasive in vivo observation of the hippocampal dentate gyrus granule cells at a depth of over 1.5 mm from the brain surface in mice [4,18].

For STED, we employed our recently developed 650-nm-band optical pulse source [16]. This optical pulse source featured a 1.3-μm gain-switched semiconductor-laser optical amplifier under CW laser light injection to generate smooth-shaped, sub-nanosecond seed optical pulses. Thereafter, the seed pulses were amplified using a Praseodymium-doped fiber amplifier and converted to second harmonic (SH) pulses. The SH pulse had a pulse width of 260 ps (Fig. 2(a)) and a peak wavelength of 655 nm (Fig. 2(b)). Immediately after the output, average power and pulse energy reached 7 mW and 1.4 nJ, respectively. The output beam was already linear polarized and the polarization extinction ratio reached 25 dB, which was sufficient to be converted by liquid crystal (LC) molecules oriented toward its polarization direction [15]. We refer to this SH pulse as 655-nm STED pulse. Note that, for comparison, an optical pulse (peak wavelength = 638 nm, pulse duration = approximately 3 ns) generated from a mass-produced LD light source was also used (Fig. 2(c, d)) [15]. Here, the average power and pulse energy was 1.5 mW and 0.3 nJ, respectively. We refer to this optical pulse as 638-nm-LD STED pulse.

The two-photon excitation and the STED pulses were tightly synchronized using an electrical timing controller and introduced into a galvano-mirror scanner (C2; Nikon) equipped with an upright microscope (ECLIPSE FN1; Nikon). The tLCDs [15] were placed between the microscope revolver and a water immersion objective lens with a numerical aperture of 1.27 (CFI Plan Apo IR 60XWI; Nikon). A detailed description of the tLCDs can be found in the literature [15]. Briefly, the tLCDs enable the modification of optical
properties over a wide range of wavelengths, from visible to near infrared, by changing the voltages applied to the LC molecules. The set of tLCDs is composed of three different types of tLCDs. The first is a tLCD-based gradient index lens (tLCD-G), which modulates the convergence angle of the STED light beam to compensate the chromatic aberration of the objective lens. The second type converts the STED beam to an optical vortex. These two tLCDs do not affect the two-photon excitation beam that has a polarization orthogonal to the orientation of the LC molecules. The third type is a plain cell tLCD (tLCD-P) that functions as an applied voltage-dependent variable wave plate, converting the STED beam to be circularly polarized. The tLCD-P also modulates the polarization of the two-photon excitation beam, making it elliptical.

Fig. 1. Overview of the optical setup (DM: dichroic mirror; GaAsP NDD: gallium arsenide phosphide-based non-descanned detector; HWP: half-wave plate; LD: laser diode; M: mirror; tLCDs: transmissive liquid crystal devices).

Fig. 2. Optical waveform (a, c) and spectrum (b, d) of 655-nm STED pulse (a, b) and 638-nm-LD STED pulse (c, d).

2.2 Fluorescent beads

Nile red labeled beads (diameter 20 nm or 1 µm; Invitrogen) or red labeled beads (diameter 100 nm; Invitrogen) were diluted with distilled water (1:3000, v/v). The distilled bead solutions were applied dropwise to glass coverslips, allowed to dry, and mounted using ProLong diamond reagent (Invitrogen).

The fluorescent images of the 1 µm Nile red beads had pixel size and dwell time of 14 nm and 4.8 µs, respectively. Images of the 20 nm Nile red beads were constructed by averaging eight acquired images with pixel size and dwell time of 14 nm and 44.2 µs, respectively. Full
width at half maximum (FWHM) values were evaluated by fitting their fluorescence intensity profiles around the central intensity using Gaussian functions.

2.3 COS-7 cells with fluorescent dyes or proteins

COS-7 cells (DS Pharma Biomedical Co., Ltd.) were cultured on the coverslips with Dulbecco’s modified Eagle’s medium (DMEM; FUJIFILM Wako Pure Chemical Co.) supplemented with 10% fetal bovine serum in a CO₂ incubator at 37 °C.

To label COS-7 cells with the fluorescent dyes, the cells were fixed with 4% formaldehyde in PBS. The fixed specimens were immunostained with an anti-α-tubulin antibody (1:1,000 dilution, 3873S, Cell Signaling Technology), followed by anti-mouse IgG antibodies conjugated with STAR512 (2-0002-001-3, Abberior GmbH), ATTO532 (610-153-121S, Rockland Immunochemicals Inc.), AlexaFluor532 (A11002, Invitrogen), AlexaFluor546 (A11030, Invitrogen), ATTO550 (610-154-121S, Rockland), ATTO565 (2107, Hypermol EK), AlexaFluor568 (A11004, Invitrogen) or AlexaFluor594 (A11032, Invitrogen). The immunostained cells were mounted with ProLong diamond reagent.

To express fluorescent proteins, plasmid DNAs encoding EYFP, mCitrine, mOrange2, DsRed2, tdTomato, mRuby2 or mCherry were transiently expressed in COS-7 cells using Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. The transfected cells were grown in the DMEM in a CO₂ incubator at 37 °C for 48 hours, and then were fixed with 4% formaldehyde in PBS. The fixed cells were mounted with ProLong diamond reagent.

The fluorescent images of the COS-7 cell stained with AlexaFluor546, which were constructed by averaging four acquired images, had a pixel size and dwell time of 28 nm and 44.2 µs, respectively.

2.4 Primary neuronal culture

Cortical neurons from the brains of P0 ICR mice were cultured on the coverslips coated with poly-D-lysine and collagen at a density of 6,000 cells per 35-mm-diameter dish in Neurobasal-A medium containing 2% B27 supplement. The neurons were cultured in a CO₂ incubator at 37 °C for 21 days in vitro, and then were fixed with 4% formaldehyde in PBS. The fixed neurons were immunostained with an anti-Bassoon antibody (1:1,000 dilution, ab82958, Abcam), followed by ATTO532-conjugated goat anti-mouse IgG antibody (1:400 dilution, 610-153-121S, Rockland Immunochemicals, Inc.). SlowFade diamond reagent (Invitrogen) was used to mount the specimens.

The fluorescent images of the cultured neuron stained with ATTO532, which were constructed by averaging 16 acquired images, had a pixel size of 28 nm (or 207 nm in the low magnification image) and a pixel dwell time of 44.2 µs.

3. Results and discussion

First, we assessed the fluorescence depletion efficiency of 655-nm STED pulses using a 1-µm Nile red bead excited with the two-photon absorption of 1064 nm pulses. For reference, we compared the efficiency of a 638-nm-LD STED pulse used in our previous STED study [15]. To induce fluorescence depletion of the whole bead area at the focal plane, these pulsed STED beams were used as a Gaussian pattern rather than being converted to a doughnut shape. The average power of the 655-nm STED, 638-nm-LD STED, and two-photon excitation beams at the focal plane was ~3.0 mW, ~0.7 mW, and 1.5 mW, respectively.

Figures 3(a) and (b) show fluorescent images and intensity profiles of the 1-µm Nile red bead irradiated with 655-nm and 638-nm-LD STED beams, respectively. The depletion efficiency induced by the 638-nm-LD STED beam was approximately 80%, consistent with our previous results [15]. The 655-nm STED beam demonstrated much higher depletion efficiency, reaching approximately 95%. Figure 3(c) shows the depletion efficiencies relative to the STED beam power ($P_{STED}$) at the focal plane. These results indicate that the 655-nm
STED beam had sufficient power to saturate the depletion efficiencies. The 655-nm STED beams always demonstrated higher depletion efficiencies compared to the 638-nm-LD STED beam even though the average powers of these STED beams were equal. These results clearly indicated that the 655-nm STED beam can be used more effectively for STED against Nile red beads.

![Image](image.png)

**Fig. 3.** Comparison of fluorescence depletion efficiencies of 655-nm and 638-nm-LD STED beams for 1-µm Nile red beads: (a) fluorescent images of 1-µm Nile red bead under three irradiation conditions; (b) fluorescence intensity profiles along the dashed lines of (a); (c) dependence of fluorescence depletion efficiencies on average power of each STED beam at focal plane for 1-µm Nile red bead.

As Fig. 4 shows, we investigated the fluorescence depletion efficiencies of the STED beams against fluorescent dyes and proteins frequently used in bioimaging. The fluorescent probes labeled or expressed in the COS-7 cells were excited with the two-photon absorption of a 1064-nm beam and were irradiated with the Gaussian patterned 655-nm STED and 638-nm-LD STED beams as a reference. The average power of the 655-nm STED, 638-nm-LD STED, and two-photon excitation beams at the focal plane was ~3.3 mW, 0.6 mW, or 2.5 mW, respectively. The 655-nm STED beam demonstrated superior depletion efficiency compared to the 638-nm-LD STED beam. Specifically, the 655-nm STED beam showed relatively high depletion efficiencies against ATTO532, AlexaFluor532, AlexaFluor546, and mOrange2. Although these depletion efficiencies did not reach 90% (as with the Nile red bead (Figs. 3 and 4)), these high-efficiency probes are considered to be applicable to STED bioimaging that utilizes a 655-nm STED pulse. In contrast, some probes demonstrated negative depletion efficiency percentages (asterisks in Fig. 4). These probes may have been excited directly by single-photon absorption of the STED beams. To use such detrimental probes increasing background signals, our setup would require a subtraction methodology with anti-Stokes emission images acquired only by the STED beam [19].
To estimate the spatial resolution, we obtained fluorescence images of 20-nm Nile red beads by overlapping a focused 1064-nm two-photon excitation beam with the doughnut-shaped 655-nm STED beam. We confirmed the typically hollow focal pattern of a 100-nm red bead excited by the single-photon absorption of the 655-nm STED beam (Fig. 5). Here, the axial focal position of 655-nm STED beam was modulated to match that of the two-photon excitation beam by controlling the applied voltage into the tLCDs [15]. The average power of the 655-nm STED and two-photon excitation beams at the focal plane was ~3.3 mW and 2.0 mW, respectively. The fluorescent images with or without the doughnut-shaped 655-nm STED beam were fitted by a Gaussian function and were used to calculate the FWHM values. As Fig. 6 shows, by applying the maximum average power (3.3 mW) of the doughnut-shaped 655-nm STED beam, the FWHM values along the x- and y-axes reached to 70 nm and 86 nm in the two-photon tLCDs-based-STED (TP-tLC-STED) images, respectively. Because the two-photon excitation light was elliptically polarized by tLCD-P (refer to Materials and methods), the lateral focal spot was elongated along the polarized direction [15]. These improved to approximately five times higher than the FWHM values in the two-photon excitation laser scanning microscopy (TPLSM). Compared to our previous setup with the 638-nm-LD STED pulse as the STED light source [15], the spatial resolution of the TP-tLC-STED imaging was approximately doubled, i.e., from 137 nm to 70 nm along the x-axis and from 151 nm to 86 nm along the y-axis. The spatial resolution of the STED microscope utilizing the pulsed STED light is theoretically approximated by $\Delta r = \lambda / (NA \sqrt{1 + \zeta^2})$ [20], where $\lambda$ denotes the wavelength of the excitation light, $NA$ is the numerical aperture of the
lens. Here, \( \zeta \) is defined as \( \zeta = \frac{I_{\text{STED}}}{I_{\text{sat}}} \), where \( I_{\text{STED}} \) is the intensity of the STED light and \( I_{\text{sat}} \) is the threshold intensity at which the fluorescence ability is reduced to 50%. Figure 6(b) shows the FWHM values of the 20-nm Nile red bead relative to the average power of the 655-nm STED at the focal plane. Each FWHM value at different STED powers changed proportionally to the square root predicted the above equation [20]. To evaluate the degree of photobleaching, we repeatedly acquired the TP-tLC-STED images of the 20-nm Nile red bead under the irradiation of 655-nm STED beam at maximum average power (3.3 mW). As shown in Fig. 6(c), the fluorescence intensity of the bead image was mostly maintained during 50-times scanning at the slowest pixel dwell time (44.2 \( \mu \)s) even under such a severe condition. Additionally, we observed COS-7 cells immunostained for microtubules labeled with AlexaFluor546 (Fig. 4). As Fig. 6(d) shows, the microtubule network was visualized more clearly in the TP-tLC-STED image, and the FWHM value of the intensity profile across the microtubule was 83 nm. These results indicate that the high-peak power of the 655-nm STED pulse realized a spatial resolution of 100 nm and two-photon tLC-STED nanoscopy was established.

![Image](image_url)

Fig. 6. Comparison of TPLSM and TP-tLC-STED images. (a) TPLSM and TP-tLC-STED images of 20-nm Nile red bead. Lower graphs show fluorescence intensities around the intensity centers along the x- and y-axes for the corresponding upper images. Blue lines indicate the fitted Gaussian curves of the measurement values (gray dots). (b) Dependence of FWHM values of 20-nm Nile red bead images on 655-nm STED power at the focal plane. The FWHM values along x-axis (red) and y-axis (green) were fitted with the inverse square root function obtained by the employed equation [20]. (c) Scan number dependency of peak fluorescence intensity of 20-nm Nile red bead images under 3.3 mW, 655-nm STED light beam irradiation. (d) Comparison of TPLSM and TP-tLC-STED images of COS-7 cell immunostained with anti-\( \alpha \)-tubulin followed by the AlexaFluor546-conjugated secondary antibody. The fluorescent intensity profiles along the dashed lines are shown in the graph.

Finally, to confirm whether the TP-tLC-STED imaging caused significant photobleaching for biological specimens, we observed the primary cultured neurons immunostained for the
Bassoon protein. Bassoon is a component of a protein cluster localized to the presynaptic active zone where neurotransmitters are released [21,22]. Here, the ATTO532 dye was used as the labeling dye because we confirmed that the 655-nm STED beam could deplete the fluorescence effectively (Fig. 4). The average power of the doughnut-shaped 655-nm STED and two-photon excitation beams at the focal plane was 3.3 mW and 2.5 mW, respectively. We took the images at the slowest pixel dwell time (44.2 µs) and repeatedly scanned the same imaging area. Under such high-power beam irradiation and repetitive slow observation, the low magnification image (Fig. 7(a)) was taken after the enlarged views (Fig. 7(b)). Note that there was no difference in fluorescence intensities inside and outside of the imaging area. Furthermore, we successfully visualized the puncta fluorescent pattern at an 88-nm spatial resolution (Fig. 7(b)). The puncta patterns may reflect transport vesicles with approximately 80-nm diameter carrying Bassoon proteins along the axon to the active zone [23]. These results indicate the successful realization of sub-100-nm spatial resolution bioimaging without photobleaching.

We modified our previous two-photon pulsed STED microscopy [15] to nanoscopy using our recently-developed high-peak-power STED light source [16]. This developed system maintained its merits, compactness, and easy controllability with reproducibility. Further, the system does not require supportive techniques, such as confocal apertures and image processing of deconvolution processing, to realize 70-nm spatial resolution.

Generally, fluorescent probes have a lifetime of 1–4 ns; thus, the optical property of the 655-nm STED pulse is considered ideal for stimulated emission depletion. In fact, compared to the 638-nm-LD STED pulse under the same average power (Fig. 4), the 655-nm STED pulse demonstrated higher depletion efficiencies for all fluorescent probes examined in this study. Moreover, the maximum pulse energy of the 655-nm STED pulse was four times greater than that of the 638-nm-LD STED pulse. The employment of the 655-nm STED light source is considered critical to realize sub-100-nm spatial resolution imaging. In contrast, some literatures reported that optical pulses with around 1-ns pulse width are ideal for STED microscopy [24–26]. A longer pulse width reduces the intensity of STED laser, thereby preventing photobleaching. However, the time-gating detection scheme has to be used to compensate for the incomplete depletion; this further complicates the system. We further demonstrated that a properly designed sub-ns optical pulses can provide sufficient fluorescent depletion efficiency without inducing obvious photobleaching.

To realize super-resolution imaging at a spatial resolution of 100 nm or less, a high-average STED beam irradiation power has been considered necessary; however, such irradiation of high laser powers may induce severe photobleaching to fluorescent probes and biological specimens. The proposed two-photon tLC-STED nanoscopy utilizes pulsed beams, i.e., 1064 nm two-photon excitation and 655-nm STED pulses, to reduce the average power to reduce such photobleaching [10,15]. The two-photon excitation method is advantageous in

Fig. 7. (a) TPLSM images of cultured neuronal cell immunostained with anti-Bassoon followed by ATTO532-conjugated secondary antibody. (b) Comparison of TPLSM and TP-tLC-STED images of the area marked with white squares in (a). (c) Fluorescent intensity profiles along dashed lines.
reducing invasiveness for biological specimens, which also presumably contributes to the reduction in photobleaching. We have realized sub-100-nm spatial resolution without severe photobleaching (Fig. 6(c), Fig. 7), implying that the proposed two-photon tLC-STED nanoscopy will facilitate long-term imaging of living cells under the physiological conditions required to visualize organelle membrane dynamics, exocytosis of secretory vesicles, association or dissociation of molecular clusters, etc.

4. Conclusion

The 655-nm STED pulse utilized in two-photon tLC-STED nanoscopy has ideal optical properties and sufficient pulse energy to realize 70-nm spatial resolution. Furthermore, by utilizing pulsed light sources, we realized sub-100-nm spatial resolution bioimaging that does not suffer from severe photobleaching. The proposed nanoscopy is expected to be a powerful tool for super-resolution imaging for live-cell and tissue under optimal sample conditions.

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Disclosures

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