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Resonant-scanning dual-color STED microscopy with ultrafast photon counting: a concise guide

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Abstract

STED (stimulated emission depletion) is a popular super-resolution fluorescence microscopy technique. In this paper, we present a concise guide to building a resonant-scanning STED microscope with ultrafast photon-counting acquisition. The STED microscope has two channels, using a pulsed laser and a continuous-wave (CW) laser as the depletion laser source, respectively. The CW STED channel preforms time-gated detection to enhance optical resolution in this channel. We use a resonant mirror to attain high scanning speed and ultrafast photon counting acquisition to scan a large field of view, which help reduce photobleaching. We discuss some practical issues in building a STED microscope, including creating a hollow depletion beam profile, manipulating polarization, and monitoring optical aberration. We also demonstrate a STED image enhancement method using stationary wavelet expansion and image analysis methods to register objects and to quantify colocalization in STED microscopy.

Keywords

fluorescence microscopy; STED microscopy; resonant scanning; image analysis; colocalization

1. Introduction

Super-resolution fluorescence microscopy is a collection of microscopy techniques that reach resolution beyond the classical diffraction limit. Since its invention at the turn of the century, super-resolution fluorescence microscopy has rapidly gained popularity in biological and biomedical research. STED (stimulated emission depletion) microscopy [1,2] is a purely physical method that does not require image processing and computation. Most

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other popular super-resolution techniques, such as SIM (structured illumination microscopy) [3] and PALM (photo-activated localization microscopy) [4], are based on temporally separating individual fluorescent dye molecules and rely on computer algorithms to find the precise location of single molecules. From a very simplistic point of view, a typical STED microscope can be considered as a confocal microscope plus an additional laser source, which carries a hollow shape and the capacity to suppress fluorescence. This additional laser is called the depletion laser as it restricts the area of the fluorescence emission by depleting fluorescence at its periphery and thus achieves super-resolution. The depletion laser source can be either pulsed or continuous-wave (CW) [5,6]. A CW depletion source is usually inferior to a pulsed one in terms of optical resolution and induces more rapid photobleaching. This problem can be partially resolved by implementing time-gated detection [7–9]. Recently, a lock-in detection technique [10] and a filtering method [11], both based on temporally differentiating background caused the CW depletion light from the pulsed fluorescence signal, have been invented to improve the signal-to-noise ratio in time-gated CW STED microscopy.

We built from scratch a STED microscope in which a resonant scanner was used to provide fast scanning speed [12]. The advantage of resonant scanning is two-fold: first, fast scanning speed reduces photobleaching in STED microscopy [13–16]; secondly, it facilitates imaging rapid changes in living organisms [17–20]. Our STED microscope has two channels, far-red and green, using a pulsed depletion source and a CW depletion source, respectively. The green channel implements time-gated detection to boost resolution. To reach maximal signal-to-noise ratio, we adopted photon counting for acquisition, and we built a photon-counting system with ultrafast pixel clock rate (or count rate) to keep up with the fast scanning speed [21]. In this paper, we share our experience in building a resonant-scanning dual-channel STED microscope and its applications to imaging biological samples. We also present some image analysis methods to locate the clusters of the labeled biomolecules in a STED image and their colocalization to clusters in another channel.

2. Microscope assembly

A schematic drawing of our STED microscope is shown in Fig. 1. Each of the two channels consists of an excitation laser source and a depletion laser source. The cross-sectional profile of the depletion laser beams is modified by a vortex phase plate (VPP) and becomes hollow. Polarization of the two laser beams is controlled by half-wave plates, Glan-laser polarizers (GLP), and a quarter-wave plate. The laser beams are combined by dichroic mirrors (DM), and are directed to a high numerical aperture (NA) oil immersion 100X objective. The laser beams scan the sample with a pair of scanners, one of which is a resonant scanner. We give detailed descriptions of the microscope below.

2.1 Laser sources

The laser sources used in the microscope are listed in Table 1. They are each coupled to a polarization maintaining (PM) optical fiber (for the 750 nm laser, 200 meters PM630-HP, Thorlab Inc., NJ, USA; for the 592 nm laser, 5 meters PM460-HP from the same company; for the two excitation lasers, fibers were already coupled when purchased) to clean the beams. In the far-red channel, the excitation laser and the depletion laser are both pulsed and

their pulse trains need to be synchronized. Therefore, the two lasers need to have the “trigger in” and the “sync out” electronic ports, respectively, and an optical delay line, constructed by moving a retro reflector (PS976M-B, Thorlabs Inc., NJ, USA) along an optical rail (9732, Newport Inc., CA, USA), optimizes the time delay between the excitation pulse train and the depletion pulse train. The depletion laser source is a femtosecond pulsed laser, but the pulse duration of the depletion laser needs to be in the order of 100 picoseconds to minimize photobleaching and nonlinear effects [22]. A glass rod (18 cm in length; material: SF66, Schott) and the 200 m PM fiber mentioned before are used to elongate the pulse duration. In the green channel, the depletion laser source is CW and the abovementioned complexity is avoided. The pulsed excitation laser has a “sync out” electronic port that is connected to the acquisition system to implement hardware time-gated detection.

2.2 Modifying depletion beam profile

In each channel, the cross-sectional profile of the depletion laser needs to be modified to have a hollow shape. This can be done by using either a VPP or a liquid-crystal spatial light modulator (LCSLM). We elected to use a VPP (VPP1-a; RPC Photonics, NY, USA) for its simplicity and excellent preservation of the laser power. LCSLM is highly flexible as it can produce virtually arbitrary beam profile and is very useful in other applications. It can be used for automatically overlapping the excitation and the depletion focal spots with the adaptive optics technique [23] and for correcting optical aberration in STED microscopy [24]. The VPP is nearly transparent and the depletion laser beam profile can be modified by simply passing through.

We mount the VPP on a holder with two translational stages to control its x and y positions (z -axis is the optical axis). This is to center the hole of the hollow shape, which can be done by adjusting the x - y positions of the VPP while observing the beam profile of the depletion beam (with eye or a CCD camera) several meters away from the VPP, where the hole diffracts and becomes easy to be seen. When and only when the center of the vortex coincides with the center of the laser beam, the profile is circularly symmetric. Fig. 2A show the computer simulated beam profile (calculated by Fresnel's theory) of a depletion beam at a distance of $3 \cdot 10^6 \lambda$ (~ 2 m if $\lambda = 750$ nm) away from a perfectly positioned VPP, which results in perfect circular symmetry. However, if the center of the vortex center is shifted only 500λ (0.375 mm for $\lambda = 750$ nm) away from the center of the beam, the symmetry is obviously broken (Fig. 2B). In Fig. 2C, we show the actual depletion laser ($\lambda = 750$ nm) beam profile recorded by a CCD camera placed ~ 2 m away from the VPP. It is approximately circularly symmetric, indicating that the VPP is properly positioned. On the contrary, the nonsymmetrical beam profile appears when the VPP is slightly off-centered, as shown in Fig. 2D.

2.3 Controlling Polarization

Correct polarization of the depletion laser light is crucial to attain a true zero at the center of the depletion focal spot, which in turn determines the quality of the STED images [25]. The depletion laser light must be circularly polarized, and must have the same handedness as the VPP. For excitation laser light, polarization is less important, except that circular polarization is most efficient at exciting the fluorophores whose dipole moments are

randomly pointing at all directions. Circular polarization is obtained by passing linearly polarized light through an achromatic quarter-wave plate (customized, Medowlark Optics, CO, USA), whose fast axis should be 45° to the direction of the linear polarization. The quarter-wave plate must be placed right before the objective, because polarization is likely to change with light reflection. After exiting the PM fiber, each depletion laser beam in order transmits through a half-wave plate (we use zero-order half-wave plates from Thorlabs Inc., NJ, USA), a GLP (GL10-A or GL10-B, depending on the wavelength, from Thorlabs Inc., NJ, USA), and then a second half-wave plate. These optical elements are installed in rotating holders to conveniently change their angles. The first half-wave plate is merely used to control the laser irradiance. The GLP and the second half-wave plate are used to manipulate polarization of the depletion laser beams, whose state is monitored by a commercial polarimeter (PMI-VIS, Medowlark Optics, CO, USA). This device can visualize the polarization ellipses directly on its LCD screen. A polarization ellipse shows the trajectory of the electric field vector projected onto the plane that is perpendicular to the propagation vector. Therefore, linear polarization will show a straight line, circular polarization will show a circle, and so on. The procedure is illustrated in Fig. 3. Initially, we remove the quarter-wave plate and the polarization state is mostly likely to be elliptical, which is visualized on the LCD screen of the polarimeter (a screenshot is shown in Fig. 3A). By adjusting the angle of the second half-wave plate, the major axis of the ellipse can be changed to be horizontal (i.e., parallel to the optical table) (Fig. 3B), while rotating the GLP can decrease the ellipticity of the ellipse. By repeating these steps for several times, one can obtain a horizontally linear polarization state (Fig. 3C). The quarter-wave plate is then inserted and its angle is adjusted until the polarization state is closest to be circular (Fig. 3D). The correct handedness of the circularly polarized depletion light must be parallel to the VPP. However, the handedness of the VPP is not known. Therefore, we simply determined the correct handedness of polarization by imaging a STED sample, e.g., a crimson fluorescent bead (F-8782, Life Technologies Inc., NY, USA) sample: the correct handedness will yield much better resolution and a much higher level of fluorescence signal. Alternatively, one can image the depletion PSF with metal nanoparticles (see Section 2.6) and measure the depletion PSF profile (e.g., Fig. 6B). The correct handedness will result in lower intensity at the center.

A practical system may not reach perfectly circular polarization for many reasons. For example, an achromatic quarter-wave plate cannot realize 0.25λ retardance for all wavelengths. Our quarter-wave plate (customized, Medowlark Optics, CO, USA) has a center wavelength which lies in the middle of 592 nm and 750 nm, and the real retardance at the two depletion laser wavelengths is $\sim 0.24\lambda$, which results in a 0.94 ellipticity. The value of ellipticity can be read from the polarimeter, and ellipticity = 1 means perfectly circular polarization. Other less pronounced imperfections include the relatively wide spectral linewidth of the pulsed depletion laser and non-normal beam incidence to the quarter-wave plate due to resonant scanning. However, the above factors only have fairly small impact on the optical resolution and the fluorescence signal level in STED microscopy. This is illustrated by computer simulations presented in Fig. 4. When depletion power is kept constant and when ellipticity is dropping, the optical resolution decreases almost linearly and the fluorescence signal level decreases much more quickly in a nonlinear manner. One

can see that ellipticity needs to be controlled within 0.9 to achieve less than 10% loss in resolution and fluorescence signal. This corresponds to up to $\sim 0.23 \cdot \lambda$ retardance or a 42° degree between the fast axis of a perfect quarter-wave plate and the linear polarization plane of its incident light.

2.4 Combing laser beams

As in a confocal microscope, the laser beams in the STED microscope are combined by DMs. In each channel, the excitation laser beam and the depletion laser beam are combined by a long-pass DM. The master dichroic mirror (MDM) in each channel reflects both its excitation beam and its depletion beam, and transmits fluorescence. The MDM for the green channel (MDM2) also needs to transmit the two lasers in the far-red channel. Information of the dichroic mirrors is listed in Table 2.

A major concern of optical aberration is astigmatism caused by non-flat reflective surface. It is especially problematic when light is focused by high numerical aperture objectives [24,26]. The signs of astigmatism include that the beam profile becomes elliptical, and that the focal spot significantly enlarges compared with the diffraction-limited spot. Astigmatism breaks the circular symmetry of the depletion beams, resulting in a nonzero center, which in turn causes significant fluorescence signal loss. We use high-quality reflective mirrors (for 750 nm: 10Z40BD.2, Newport Inc., CA, USA; for other wavelengths: 10Z40ER.2, Newport Inc., CA, USA). These mirrors are 6 mm thick and their flatness is rated to $\lambda/20$ at 633 nm. Therefore, they are very unlikely to cause problems. Flatness of the DMs is much harder to control. We use DMs made by Semrock Inc. for their large selection of reflection/transmission bands and economic reasons. Almost all the Semrock DMs in their catalog are 1 mm thick, which raises concerns (flatness is inversely proportional to the square of the thickness). Flatness of Semrock DMs is rated to “standard”, “laser flat”, or “imaging flat” according to the nominal radius of curvature (~ 6 meter, ~ 30 meters, and ~ 100 meters, respectively). We use DMs with better flatness when possible (see Table 2). To better preserve the beam quality of the depletion beams, we use long-pass DMs to combine them with the excitation beams (DM1 and DM2 in Fig. 1, both “imaging flat”), because transmission through a DM causes much less aberration than reflection. The DM that separates the fluorescence signal of the two channels (DM3 in Fig. 1) is also “imaging flat”. Flatness of the two multi-edge MDMs is rated to be standard. However, in our system MDM1 and MDM2 do not seem to cause astigmatism: the beam profiles remain roughly circularly symmetric when translating in free space (see Fig. 5A-5B, measured by a CCD camera placed 2 m and 7 m away from the MDM, respectively), and the experimental PSF (Fig. 5D and 5F) is shown to be nearly diffraction-limited by comparing it to the ideal PSF (Fig. 5C and 5E).

2.5 Scanner and objective

All four laser beams are directed to a pair of scanners, including a resonant scanner (CRS 8 kHz; Cambridge Technology, MA, USA) for horizontal scanning and a galvanometer mirror (M2S, Cambridge Technology, MA, USA) for vertical scanning. We use bidirectional scanning, meaning that in each cycle of the resonant movement, we recorded two lines of data, and therefore the scanning speed is 16,000 lines per second. The arrangement of the

scanning-mirror pair is classical [27]: the two mirrors are placed as close as possible (without a relay lens in between) and the axes of them are perpendicular to each other. With a pair of scanning lenses (SL1 and SL2 in Fig. 1), the scanning mirrors are imaged to the pupil of the objective, where the movement of the beams vanishes. SL1 and SL2 also expand the beams so that they overfill the pupil. SL1 and SL2 form the classical telescope configuration, with the scanning mirrors at the back focal plane of SL1 and the pupil at the forward focal plane of SL2, and the distance between SL1 and SL2 is the sum of their focal lengths. By varying the focal lengths of SL1 and SL2, we can achieve a scanning field of view of ~100-150 μm in width.

The objective is an oil immersion 100X super apochromat objective (UPLSAPO 100XO, NA = 1.4) from Olympus. Exceptional achromatism of the objective is crucial to maintaining alignment both at the center and at the edge of the scanning field for the excitation light and the depletion light, whose wavelengths are separated by several hundred nanometers.

2.6 Alignment

STED microscopy was known for being notoriously difficult to align. New techniques have been invented to overcome this difficulty. Using a specially designed phase plate, the excitation and the depletion beams can be fed into the same optical fiber and be aligned by design [28]. As mentioned in Section 2.2, adaptive optics can help automatically align STED microscopes, too. These techniques are beyond the scope of this paper and not yet widely used. Here we discuss the alignment of the dominant traditional STED technique. Some researchers resorted to a new aligning technique based on monitoring the lifetime of fluorescent beads, because the center of the depletion focal spot has a longer lifetime [29]. We do not use special aligning methods other than the ones widely used in confocal microscopy. However, we stress that one ought to follow the aligning procedures more cautiously as a STED microscope is more sensitive to misalignment.

As the final step of the alignment procedures, we verify the PSFs of the lasers by imaging their focal spots with light-scattering from silver nanoparticles. In particular, the depletion PSFs should look similar to the ideal PSFs as shown in Fig. 5C and 5E. By imaging silver nanoparticles, we also overlap the focal spots of the excitation light and the depletion light in each channel. For this purpose, we use 40 nm silver nanoparticles (AGSH70, NanoComposix, CA, USA), whose extinction wavelength is far away from the wavelength of the lasers. We use an optical fiber with a large core (~6 Airy Units) when imaging the nanoparticles. This is to minimize the impact from the pinhole on the PSFs to be measured. In Fig. 6 we illustrate images of silver nanoparticles scattering well aligned excitation and depletion laser beams, in both lateral and axial directions. The reflective mirrors or DMs are used to adjust overlapping in the x-y plane, and the lenses right after laser light exiting the single-mode fibers (L1-L4 in Fig. 1) are used to change beam divergence, i.e., overlapping the focal spots in the axial direction. In our daily practice, the alignment can be performed in real-time, taking advantage of the fast resonant-scanning scheme.

When the depletion laser beam is properly aligned with the correct polarization, its intensity at the center of the PSF should be very low. We show the depletion intensity profiles in Fig.

6B and 6E, where the central intensity is about 2% of the intensity at the crests for both channels. If the central intensity is found to be above 3% of the crest intensity, the procedures described in Section 2.2—2.6 should be double-checked, with special attention paid to polarization, astigmatism, and whether the propagation of the depletion beam exactly follows the optical axis.

2.7 Recording fluorescent

This STED microscope adopts a common epifluorescence configuration, where fluorescence is collected by the objective and directed backwards to the fluorescence-collecting step-index multimode (MM) fibers (Thorlabs Inc., NJ, USA), whose cores play the role of the confocal pinholes. The MM fibers then direct fluorescence into the light detectors. A DM separates the green fluorescence from far-red fluorescence, and barrier filters blocks laser light from entering the MM fibers. The core size of the MM fibers is determined in the same way as in confocal microscopy: it should be around 0.5—1 Airy units. One airy unit is estimated by $1.2 \cdot (\lambda/NA) \cdot M$, where λ is the excitation laser wavelength and M is the magnification of the optical system.

2.8 Image acquisition

Fluorescence photons are recorded by a photon counter based on silicon avalanche photodiode (APD) in the far-red channel and a photomultiplier (PMT) in the green channel, respectively. The APD counter we use is a SPCM-AQRH module from Excelitas Technologies (CA, USA), and the PMT is an H7422P-40 module from Hamamatsu Photonics (Shizuoka, Japan). The photon events are then fed to the acquisition system, which counts the events and organizes images using the horizontal synchronization (H-Sync) signal (indicating when each line starts) coming from the scanners.

Photon counting is the preferred acquisition technique when fluorescence signal is weak. One could use commercial time-correlated single photon counting (TCSPC) modules, such as PicoHarp 300 from PicoQuant (Berlin, Germany) or SPC 150 from Becker & Hickl (Berlin, Germany) to implement photon counting acquisition. With a TCSPC module, time-gated detection is implemented by software: the arrival time of all photon events are recorded in real time but only events falling within the detection window are kept when constructing the final image. The main disadvantage of the commercial TCSPC modules is that their pixel clock rate is currently limited to below 100 MHz, which in turns limits the field of view and the linear scanning speed, resulting in more rapid photobleaching.

In our STED microscope we use a home-built ultrafast photo-counting acquisition system with up to 450 MHz pixel clock rate [21]. In this home-built system, time-gated detection is implemented by hardware. A leading edge discriminator (TD2000, FastComTech GmbH, Germany) is the key component of this implementation. Its basic function is to receive the analog photon pulses from the light detector and regulates them into digital signals that can be counted later. The leading edge discriminator has the “VETO” port, which blocks the input pulses if this port is set high. We let each excitation pulse induces a high-voltage window with time-duration T (this is done with another leading edge discriminator: 704, Philips Scientific, NJ, USA), which is then fed to the “VETO” port. Consequently, only

photon pulses after time T of each excitation pulse can be detected, and this is exactly what time-gated detection intends to do. The 450 MHz clock rate allows for achieving fast linear scanning speed, which helps reduce photobleaching. For Atto 647N, we have demonstrated that a 8-fold faster linear scanning speed can prolong the fluorophore survival time by ~80% when the depletion laser irradiance is high [16].

In this microscope, the far-red channel must be imaged first; otherwise the depletion laser beam (592 nm) in the green channel will completely bleach away the far-red fluorescent dye (excited at 635 nm). Therefore, once the green channel is imaged, taking images for the red channel is no longer possible.

As an example, we show in Fig. 7 a comparison between dual-color confocal and STED images of an isolated cardiomyocyte where L-type voltage dependent calcium channel α -1C subunits (Cav1.2; red) and ryanodine receptors (RyR; green) were independently labeled by Atto 647N and Oregon green 488, respectively. The sample has a thickness of ~15 μ m and was mounted with Prolong Gold.

3. Image Processing and Analysis

Since we adopt resonant scanning, the first step of image processing is to correct for the distortion caused by the sinusoidal movement of the resonant mirror, which has been discussed in our previous publications [12,21]. Here we focus on background/noise reduction and performing statistics on clusters for their localization and colocalization. Our computer programs are available to the public upon request. We stress that there are many methods to process and analyze STED images. For example, deconvolution is a powerful tool for enhancing STED images [30,31]. However, there is no consensus on which methods are universally the best ones, and for different images the best suited methods may be different.

In STED microscopy, depletion suppresses most of the fluorescence yield and therefore a STED image usually has a much lower level of fluorescence signal compared with its confocal counterpart. Background and noise become more problematic in this situation. We differentiate between background and noise as follows: background is unwanted intensity with extended distribution that is much wider than STED resolution, whereas noise is distributed within single pixels. The primary sources of the background include out-of-focus light and anti-Stokes fluorescence excited by the depletion laser. Noises arise from dark counts and shot noise of the detector.

We use an algorithm based on stationary wavelet expansion [32] for background/noise reduction in STED images. The expansion decomposes the image to components at different length scales, and we remove the components that have too long (much longer than the typical cluster size) and too short (smaller than the optical resolution) length scales. This algorithm has the same basic idea of the median-filtering method we previously presented [33, 34] but is more effective. Fig. 8 illustrates an example, where Fig. 8A shows a cropped raw STED images, Fig. 8B shows the processed image crop, in which only components with length scales from ~30 nm to ~100 nm were kept. Fig. 8C shows all the components that have been removed by the algorithm, quantified by the absolute value of the difference

between the raw image (Fig. 8A) and the processed one (Fig. 8B). This STED image was taken with the green channel of our STED microscope with time-gated detection.

For a STED image mainly consisting of discrete clusters, such as the one shown in Fig. 7A, the clusters can be found by locating the local intensity maxima in the processed image. Information such as the size, intensity and location of the clusters can then be extracted. As an example, we show in Fig. 9 a scatter plot of the size and intensity of all the clusters in Fig. 7B extracted by this method.

The colocalization of one image to another can be quantified by counting indistinguishable peaks in the two channels: if a peak in one channel has a neighboring peak in the other channel that is too close to be differentiated from, that is, the distance between the two peaks is shorter than the sum of their full-width-half-maximum (FWHM), then we count this peak as being colocalized to the other channel. The overall colocalization value is the fraction of the colocalized peaks, weighted by their intensity. For STED images with extended structures, we use a pixel-intensity-value-based colocalization method, called protein proximity index (PPI), as described in our previous publications [33, 34].

Conceptually, two molecules can never simultaneously reside at the same location. In practice, the term “colocalization” simply means two molecules are so close to each other that they overlap too much in the image and become indistinguishable. In STED microscopy, resolution is much higher than conventional microscopy and the PSF is thus much sharper. Therefore, molecules in the two channels have much less spatial overlap. Consequently, the colocalization values are much smaller. We calculated the colocalization values of the images shown in Fig. 7B as an example. Calculated by counting cluster, confocal colocalization is 0.47/0.43 (meaning that 47% of the green clusters are colocalized with red clusters, and 43% of the red clusters are colocalized with green ones), whereas STED colocalization is only 0.16/0.11. Similarly, confocal PPIs are 0.66/0.63, but STED PPIs are 0.4/0.28. For both methods, the degree of colocalization significantly dropped in STED images.

4. Conclusion

In this paper we have presented a concise guide to building a dual-color STED microscope for laboratory use, which is equipped with a resonant scanner and an ultrafast photon counting acquisition system to reduce photobleaching. We have shown that this type of STED microscope produces high quality images for properly made fixed samples. We have demonstrated a background and noise reduction method based on stationary wavelets and an object registration method based on finding clusters as local intensity maxima. We have verified with two colocalization qualification methods that the degree of colocalization significantly drops in STED microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of uncommon abbreviations

VPP	vortex phase plate
GLP	Glan-laser polarizer
DM	dichroic mirror
MDM	master dichroic mirror
PM	polarization maintaining
MM	multimode
PPI	protein-proximity index
FWHM	full-width-half-maximum
PSF	point spread function

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STED (stimulated emission depletion) is a popular super-resolution fluorescence microscopy technique. In this paper, we present a concise guide to building a resonant-scanning STED microscope with ultrafast photon-counting acquisition. The STED microscope has two channels, using a pulsed laser and a continuous-wave (CW) laser as the depletion laser source, respectively. The CW STED channel performs time-gated detection to enhance optical resolution in this channel. We use a resonant mirror to attain high scanning speed and ultrafast photon counting acquisition to scan a large field of view, which help reduce photobleaching. We discuss some practical issues in building a STED microscope, including creating a hollow depletion beam profile, manipulating polarization, and monitoring optical aberration. We also demonstrate a STED image enhancement method using stationary wavelet expansion and image analysis methods to register objects and to quantify colocalization in STED microscopy.

Fig. 1. Schematic drawing of our resonant-scanning dual-color microscope. $\lambda/2$: half-wave plate; $\lambda/4$: quarter-wave plate; BF: barrier filter.

Fig. 2. Computer-simulated (A and B) and experimentally recorded (C and D) depletion laser beam profile ~2 meters away from the VPP. A and C are circularly symmetric as the centers of VPP coincides with the beam center, whereas in B and D the circular symmetry is broken as the centers are not coincident.

Fig. 3.

Manipulating polarization of a laser beam by monitoring the polarization ellipses visualized by a polarimeter. (A) Before adjusting the half-wave plate and the GLP, polarization is elliptical with a tilted major axis. (B) By adjusting the half-wave plate, the major axis becomes horizontal. (C) Rotating GLP makes polarization linear. Repeating the above procedures several times will let us reach the desired horizontally linear polarization. (D) Inserting the quarter-wave plate and adjusting its angle until polarization is closest to be circular.

Fig. 4. Resolution and fluorescence signal loss due to ellipticity of the depletion beam calculated by computer simulation. The line in each figure is a guide for eyes.

Fig. 5.

Checking astigmatism by monitoring beam profiles and focal spots. Beam profiles recorded by a CCD camera 2 m (A) and 7 m (B) away from the MDM remain roughly circularly symmetric. Comparison between computer-simulated (C and E) and experimentally measured (D and F, wavelength is 750 nm) focal spots agree with each other.

Fig. 6.

Imaging silver nanoparticles to overlap the focal spots in each channel in both the lateral and the axial directions, and to ensure a hollow center in the depletion focal region. (A) The focal spots of 750 nm (red) and 635 nm (green) laser light overlap in the x - y plane. (B) Intensity profile of the 750 nm focal spot along the white dotted line in A. The intensity at the center is ~2% of the average intensity at the crests. (C) The focal spots of 750 nm and 635 nm laser light overlap in the z - y plane. (D—F) Same plots as in A—C but for the second channel. Colors represent wavelengths of the lasers: yellow – 592 nm; and blue – 485 nm.

Fig. 7.

Dual-color confocal and STED images of an isolated cardiomyocyte where Cav1.2 (red) and RyR (green) were independently labeled by Atto 647N and Oregon green 488, respectively. Sample was mounted with Prolong Gold. (A) Overlay of confocal images. Colocalization is calculated to be 0.47 (green to red) / 0.43 (red to green) by counting clusters and 0.66/0.63 by protein-proximity index (PPI). (B) Overlay of STED image in the same field of view as in A. Colocalization is 0.16 / 0.11 by counting clusters, and 0.09/0.14 by PPI. (C) and (D) show a blowup of the white boxes in (A) and (B), respectively. Scale bars represent 2 μm.

Fig. 8. Background and noise reduction with stationary wavelets illustrated by processing a noisy STED image. (A) Cropped raw image, which was taken for a cardiomyocyte where RyR were immunolabeled with the Oregon green 488 dye. (B) Processed image. (C) The absolute value of the difference between A and B. Images use the “red hot” pseudo color. Scale bars represent 1 μm .

Fig. 9. Scatter plots of the size (FWHM) vs. intensity (normalized to the average value) of the clusters in Fig. 7B, extracted by local maxima finding. The left panel is for $\alpha 1C$ (red) and the right panel is for RyR (green).

Table 1

List of laser sources.

	Far-red Channel (dye: Atto 647N, KK114, etc.)		Green Channel (dye: Alexa Fluor 488, Oregon green 488, etc.)	
	Excitation	Depletion	Excitation	Depletion
Wavelength	635 nm	750 nm	485 nm	592 nm
Pulse width	~100 ps	~150 fs	~100 ps	CW
Electronics	Trigger in	Sync out	Sync out	N/A
Make	PicoQuant, Berlin, Germany	Newport, California, USA	PicoQuant, Berlin, Germany	MPB Communications, Quebec, Canada
Model	LDH-D-C-635	Mai Tai HP	LDH-D-C-485	2RU-VFL-P-2000-592

Table 2

List of dichroic mirrors.

	Catalog number	Make	Flatness	Reflection (R) and Transmission (T) band (nm)
DM1	FF662-FDi01	Semrock, NY, USA	Imaging-flat	R: 635; T: 750
DM2	FF509-FDi01		Imaging- flat	R: 485; T: 592
MDM1	FF405/496/593/649-Di01		Standard	R: 635, 750; T: 525±20, 669±20
MDM2	FF395/495/610-Di01		Standard	R: 485, 592; T: 635, 750, 525±20, 669±20
DM3	FF580-FDi01		Imaging-flat	R: 525±20; T: 669±20