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Ultrafast Photon Counting Applied to Resonant Scanning STED Microscopy

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Summary

To take full advantage of fast resonant scanning in super-resolution STimulated Emission Depletion (STED) microscopy, we have developed an ultrafast photon counting system based on a multi-giga-sample per second analog-to-digital conversion (ADC) chip that delivers an unprecedented 450 MHz pixel clock (2.2 ns pixel dwell time in each scan). The system achieves a large field of view ($\sim 50 \times 50 \mu\text{m}$) with fast scanning that reduces photobleaching, and advances the time-gated continuous wave (CW) STED technology to the usage of resonant scanning with hardware based time-gating. The assembled system provides superb signal-to-noise ratio and highly linear quantification of light that result in superior image quality. Also, the system design allows great flexibility in processing photon signals to further improve the dynamic range. In conclusion, we have constructed a frontier photon counting image acquisition system with ultrafast readout rate, excellent counting linearity, and with the capacity of realizing resonant-scanning CW-STED microscopy with on-line time-gated detection.

Keywords

Photon Counting; Resonant Scanning; Fluorescence Microscopy; Super-resolution; STED

Introduction

Stimulated Emission Depletion (STED) microscopy breaks the diffraction limit of conventional confocal fluorescence microscopy and greatly sharpens the optical resolution by aligning a doughnut-shaped depletion laser beam with the excitation laser beam to inhibit the peripheral fluorescence at the focal spot (Hell *et al.* 2006). Recent developments in

STED microscopy include: 1) the usage of resonant scanning mirrors that allow fast scanning (Moneron *et al.* 2010; Westphal *et al.* 2007) and thus, decrease the rate of fluorophore photobleaching due to triplet states buildup (Borlinghaus 2006; Tsien and Bacskaï 1995); and 2) the implementation of time-gated detection with low-power continuous wave (CW) depletion lasers that reach a resolution of ~60–70 nm in biological samples (Vicidomini *et al.* 2011; Vicidomini *et al.* 2013). Yet, the speed of the photon counting systems limits the linear scanning speed that can be used with resonant mirrors in STED microscopes. Thus, the main goal of this work was to develop a fast photon counting system to maximize the scanning speed of resonant mirrors used in STED microscopy, and to apply this technology to achieve hardware-based, on-line time-gated detection in resonant-scanning CW-STED microscopy.

In optical microscopy, photon counting is an excellent technique to enhance signal-to-noise ratio at low light levels (Driscoll *et al.* 2011; Tsuchiya *et al.* 1985), a characteristic of super-resolution microscopy, and thus, has been widely used in STED microscopy (Meyer *et al.* 2008; Willig *et al.* 2007). Photon counting speed is determined by *count rate* and *readout rate*. Count rate is defined as how many photon pulses the system can record in a unit time. Readout rate (also called *pixel clock rate* in microscopy) is how fast data can be retrieved from the system. It is the latter that limits the usage of photon counting in fast resonant-scanning super-resolution microscopes. For example, assuming that an 8 KHz horizontal scanner is used to reach a $50 \times 50 \mu\text{m}$ field of view (FOV) with bidirectional scanning and if the optical resolution is 50 nm, the pixel size should be no greater than $50 \text{ nm} / 2.8 \approx 18 \text{ nm}$ (Pawley 2006); in this condition the final image should be at least ~2,800 pixels per line. Considering the sinusoidal movement of the resonant mirror (Sanderson and Parker 2003), the readout rate of the acquisition system would need to be no slower than $2,800 \times \pi \times 8,000 \text{ Hz} \approx 70 \text{ MHz}$ (image interpolation may be needed to correct for optical or mechanical distortions, which demands even smaller pixel size and thus higher readout rate). This exceeds the capacity of most commercially available photon counting systems and digital counters (e.g. the maximum readout rate of PCI-6602 from *National Instruments* is only several hundred kilohertz at best, though its count rate is up to 80 million counts per second). As a consequence, to maintain image resolution, images are restricted to smaller FOVs and longer dwell times in a single scan, which exacerbates photobleaching (Wu 2014). In previously reported resonant-scanning STED microscopes, the FOV is limited to $\sim 10 \times 10 \mu\text{m}$ with an image size of $\sim 1,000 \times 1,000$ pixels ($\sim 3,100$ pixels per line before image interpolation) (Moneron *et al.* 2010). In time-gated CW STED microscopy, time-gating detection is conventionally implemented with the time-correlated single photon counting (TCSPC) technique that is designed for lifetime microscopy. The maximum pixel clock rate of TCSPC systems limits the FOV size and the scanning speed that can be used in time-gated CW STED microscopy.

Recently, ADC technology at several giga-samples (GS) per second sampling rate has matured and become available in the market. For example, ADC12D1800 from Texas Instruments has a sampling rate of 3.6 GS/s, collecting one data point every 0.28 ns. Data acquisition (DAQ) boards based on this chip usually come with field-programmable gate arrays (FPGAs). In this work, by feeding the amplified PMT output signal into the

abovementioned board, we were able to digitize the signal to subsequently identify and record each individual photon pulse with FPGA in real time. Using this configuration, the readout rate is up to 450 MHz.

The novel photon counting system based on ultra high sampling rate analog-to-digital conversion (ADC) was used to demonstrate super-resolution images using a custom-built dual channel fast resonant-scanning STED microscope. This microscope uses an 8 KHz resonant scanner (Gardeazabal Rodriguez *et al.* 2012). It has one channel that implements time-gated CW STED (excitation at 485 nm and depletion at 592 nm), where time-gating is directly achieved with hardware, and a second channel that implements STED with two pulse lasers (excitation at 635 nm and depletion at 750 nm). The microscope can take images with 55,296 pixels per line, and the FOV can reach $50 \times 50 \mu\text{m}$ (limited by the optical configuration). Thus, the novel photon counting system reported here was coupled with a resonant-scanning time-gated CW STED microscope to acquire high resolution images in a large field of view with less photobleaching.

Fast Photon Acquisition System Design and Properties

Figure 1 shows a scheme of our acquisition system. The system consists of 3 modules: light detector and amplifiers, ADC, and image grabber.

Light Detector and Amplifiers

A GaAsP PMT (H7422P-40, Hamamatsu, Japan) working in the photon counting mode converts light to electric pulses (~ 1 ns rise time). A DC-coupled preamplifier with ~ 1 ns rise time (HCA-400M-5K-C, Femto, Germany; or SR445A, Stanford Research System, USA) is used to amplify the weak PMT output signal to an appropriate level without losing good pulse-pair resolution. To adopt the full dynamic range of the ADC module, a differential amplifier (LMH6554, Texas Instruments, USA) converts the signal from single ended to differential.

ADC Module

A 12-Bit ADC prototype board (ADC12D1X00RFRB, Texas Instruments, USA) is used for ADC and data preprocessing. We use the board in the single channel 3.6 GS/s mode. This board is equipped with a Xilinx Virtex-3 FPGA chip, which allows us to reprogram it for on-board real-time data preprocessing. It has a standard FPGA Mezzanine Card (FMC) connector, through which the pre-processed data are streamed to the image grabber module for image construction and computer interfacing. A software application *WaveVision 5* (Texas Instruments, USA) comes with the ADC prototype board, which enables us to directly observe the raw acquired signal for debugging and to monitor and control the ADC board.

At present, a simple thresholding method is used to detect the rising edges. A rising edge is identified by two adjacent data points, one below and the other above a software modifiable threshold. A rising edge immediately following another one, with time lapse shorter than the rising time of PMT, is considered spurious and removed. This simplistic method has a limitation in that it will miss a photon pulse that is so close to a preceding one that the

“valley” in between the two peaks is higher than the preset threshold. In the original factory design, the 3.6 GS/s sampled signal is split (via demultiplexing) into 8 lanes of 12 bits data streams clocked at 450 MHz. To reach the maximum readout rate that this board is capable of, the circuit is still clocked at the original 450 MHz clock. With our current edge detection method, we compare each 12 bit data point to the threshold and reduce it to 1 bit according to the results. Due to the physical constrain of the FPGA, conventional operations such as *sum* and *comparison* between integers cannot be done with 12 bits at 450 MHz clock. We developed a pipelined multiple layers circuit to break down these operations to small units and then combine them to obtain the correct results (source code available upon request). The comparison process based on pipeline implementation of numeric operations results in 8 bits of data (in 8 lanes, with each channel carrying 1 bit comparison result data) at 450 MHz. Together with the clock signal, this 8 bits data stream is transferred through the FMC interface to the image grabber module. In further development, more advanced design than simple threshold comparison can be implemented to improve the signal-to-noise ratio, the dynamic range and the linearity of photon counting.

Image Grabber Module

This module is mainly built with a Virtex-6 FPGA board (ML605, Xilinx, USA), receiving 450 MHz 8 bits threshold comparison data from the ADC module through an FMC connector, and feeding image data to a computer through PCI Express. A rising edge detection circuit counts the number of rising edges (below threshold → above threshold) and stores data in a 2 bit 450 MHz data stream. The maximum counting rate is thus at $(2^2-1) \times 450 \text{ million} = 1,350 \text{ million counts per second (cps)}$, which is more than enough for most fluorescence microscopy applications. It also receives the horizontal synchronization (H-Sync) signal indicating the start of a line, and the vertical synchronization (V-Sync) signal indicating the start of a frame from another FPGA (XEM3010, Opal Kelly, USA) that communicates with the resonant horizontal scanning mirror and the vertical linear mirror (Gardezabal Rodriguez *et al.* 2012). With these two synchronization signals, data are organized into images. When the image data of each line are transferred to a FIFO, they need to be synchronized with HSync to label where the line starts. This task is carried out by a line alignment circuit that is designed to align image lines with $1 / 450 \text{ MHz} \approx 2.2 \text{ ns}$ precision, which ensures that the error of line-start is below one pixel. In order to achieve this, two first-in-first-out (FIFO) units working at the half clock frequency (225 MHz; working at full 450 MHz clock is not possible due to hardware constraint) are fed with two streams of data with 1 clock shift. These two FIFOs start accept data as soon as they receive H-Sync, and one of them must be synchronized with H-Sync within 2.2 ns accuracy. A separate circuit working at full 450 MHz decides which stream should be selected and feeds data downstream. The final part of this module is to transfer the image data to a computer. This function is based on the PCI Express Scatter-Gather DMA engine developed at the Department for Application Specific Computing of University of Heidelberg (Gao *et al.* 2007). The DMA engine is conceptually a FIFO unit that receives data stream and feeds them to designated computer memory space. It relieves CPU from monitoring the data transfer from the Xilinx board to computer memory, which is essential to achieve real time processing for this data volume.

Fast Photon Counting in Resonant Scanning STED Microscopy

Previously, we have constructed a single channel STED microscope using an 8 KHz resonant scanner and the detailed information is published elsewhere (Gardeazabal Rodriguez *et al.* 2012). Now, we have added an additional channel to this microscope and changed the acquisition system to ultrafast photon counting as described above. Fluorescence excited by two pulsed lasers, with wavelengths of 635 nm and 488 nm (LDH-D-C-635 and LDH-D-C-485, PicoQuant, Germany), are depleted by a Ti: Sapphire pulsed laser (Mai Tai HP, Newport, USA) working at 750 nm and by a 592 nm CW fiber laser (2RU-VFL-P-2000-592, MPB Communications, Canada), respectively. All four lasers are combined by dichroic mirrors and directed to an apochromat microscopy objective (UPLSAPO 100XO, Olympus, Japan). An achromatic quarter-wave plate (customized, Meadowlark Optics, USA) ensures that the depletion lasers are circularly polarized. For the green CW STED channel to reach higher resolution, the acquired signal needs to be gated. The gating module is shown in Fig. 1: a leading edge discriminator (TD2000, Fast ComTech GmbH, Germany or 704, Philips Scientific, USA) uses the laser sync signal as VETO signal to gate the amplified PMT output signal, and the gate position is controlled by a nanosecond delay module (792, Philips Scientific, USA). The width of the gate is equal to the pulse duration of the laser sync signal (~6 ns) and suits the dyes well. In general, if this width is not ideal, the laser sync signal can be passed to a discriminator circuit that can control its output pulse width. A constant fraction discriminator (935, Ortec, USA) can be used to enhance the accuracy of photon arrival time. With less time jitter in photon pulse, the time-gate window can be placed closer to the excitation laser pulse and record more fluorescence.

Images taken with the photon counting system originally have 55,296 pixels per line at the 450 MHz pixel clock, and the pixel dwell time is always $1 / 450 \text{ MHz} \approx 2.2 \text{ ns}$. The original images are distorted because of the non-linear motion of the scanning mirrors. The motion of the resonant scanning mirror is sinusoidal, changing from phase 0 to phase 2π in each cycle. In each pixel the phase increment is $2\pi \times 8 \text{ KHz} / 450 \text{ MHz}$. We find the pixel corresponding to phase π by an algorithm based on FFT that maximizes the correlation between its left side and its right side. The images are then folded around the phase π pixel, and linearized based on its phase θ : data are resampled according to a transform $\theta \rightarrow 1 - \cos \theta$. After linearization there are ~17,600 pixels per line. Usually the horizontal pixel size is too small with too many pixels, and the images are then horizontally scaled down to reach the desired pixel size and to match the pixel size in the vertical direction. In the vertical direction, the images are also folded with the similar method as in the horizontal direction to compensate the sawtooth-like motion of the vertical galvanometer scanner.

Results

Pulsed STED

A pulsed STED image was taken for a fixed cell sample (isolated cardiomyocytes), in which L-type calcium channel $\alpha 1C$ subunits were immunolabeled. An original image generated by this photon counting acquisition system in a FOV of $50 \times 50 \mu\text{m}$ is shown in Fig. 2A. After folding, correcting for the sinusoidal distortion, and scaling down the image horizontally by a factor of 1:5, we have the final image with 3,592 pixels per line and a pixel size of 16 nm.

This pixel size is to suit the optical resolution at ~ 50 nm, as demonstrated in Fig. 2C and 2D. After correction and scaling, the center of the final image has a pixel dwell time of 11 ns in a single scan. Towards the edges, pixels have longer dwell time, and the average dwell time is ~ 31 ns. The image is a summation of 512 frames, and the total pixel dwell time is ~ 5.6 μ s at the center and ~ 18 μ s on average. The 50×50 μ m FOV is about 4 times wider than previous implementations of resonant-scanning STED microscopy, and our ultrafast acquisition system is well capable of reaching still larger FOV without sacrificing resolution. Here, we only show a 50×50 μ m FOV due to optical limitations of the 100X microscope objective in our setup. A larger FOV would result in noticeable optical aberration.

CW STED

To use this photon counting system to achieve time-gated STED microscopy, we added a leading edge discriminator, a time delay, and optionally a constant fraction discriminator (Fig. 1). Figure 3 shows a confocal image (A), a CW STED image (without time-gating) (B), and a time-gated STED image (C). They have the same FOV in a sample with fluorescent microspheres (F-8787, Life Technologies, USA) immobilized on a glass coverslip. The same depletion laser power was used for Fig. 3B and 3C, and it was deliberately kept low to avoid excessive photobleaching. Fig. 3D shows a line scan of two neighboring microspheres to compare the resolution. It is obvious that CW STED moderately improves the resolution, and that time-gated STED leads to further resolution enhancement, proving that the acquisition system successfully achieves time-gated STED microscopy in resonant scanning scheme. One more example is given in Fig. 3E and 3F, in which we show a confocal and a time-gated STED image (both cropped from images with 30×29 μ m FOV) of isolated cardiomyocytes with ryanodine receptors immunolabeled by the Oregon green dye. The optical resolution is around 60 nm. The images were summed over 512 frames.

Linearity of the Fast Photon Counting System

To demonstrate the counting linearity, Figure 4A shows the count rate of our system for elastically scattered photons from 20 nm silver nanoparticles (without the leading edge discriminator as it is ONLY needed in the time-gated STED channel), as a function of illumination laser power. With a 20 nm particle size, at relatively low illumination level (< 0.4 mW at the back aperture of the objective), light scattering is well described by the Raleigh theory and nonlinear processes can be neglected (Lippitz *et al.* 2005; Yguerabide and Yguerabide 1998). For all data points, background due to the dark count and parasite light was subtracted. The straight line in Fig. 4A was fitted by the zero and the data point with the lowest illumination level (7.2 μ W at the back aperture of the objective), and is used to estimate the behavior of a perfectly linear detector. In Fig. 4B, one can see that excellent linearity remains at ~ 70 million cps. The counting efficiency, calculated by the ratio between the actual counts to the ideal counts projected by the straight line, is $\sim 90\%$ at this count rate. The cutoff count rate (the count rate at which the counting efficiency drops to 50%) is greater than 200 million cps. Excellent linearity extends the usage of our system to samples with strong contrast, especially in confocal microscopy. Fig. 4A also shows that the count rate data can be well fitted by a simple theoretical model assuming that the count rate

is not extremely high and nonlinearity comes from a dead time $t_d \approx 1.7$ ns of the system (Smit and Alkemade 1963):

$$C_{ideal} = \frac{1 - \sqrt{1 - 4t_d C_{read}}}{2t_d} \quad (1)$$

where C_{read} is the recorded count rate, and C_{ideal} is the count rate from an ideal system. This model can be used to correct the recordings at high count rate. The 1.7 ns dead time is consistent with the full-width-half-maximum (FWHM) of an amplified photon pulse as displayed in Fig. 4C. The FWHM is about 1.1 ns, a little shorter than the dead time, because if two identical pulses are separated by 1.1 ns, they are completely indistinguishable. If the separation is longer than 1.1 ns, there will be a valley in between the pulses and in principle they can be resolved. But our current simple thresholding method can only differentiate the two pulses when the separation is wide enough so that the bottom of the valley sits below the preset threshold. The model also predicts that the cutoff count rate is approximately proportional to the inverse of the dead time. Therefore, if the pulse width can be reduced in half, the linearity is doubled. This could be achieved by using light detectors with shorter pulse width, and preamplifiers with higher bandwidth.

Conclusions and Discussion

The main points achieved in this work are: i) an ADC based ultrafast photon counting system with excellent linearity; ii) demonstration that the new photon counting system allows a four-fold increase in image size ($\sim 4,000 \times 4,000$ pixels vs. $\sim 1,000 \times 1000$ pixels) in resonant-scanning STED microscopy (maximum 55,296 pixels per line); and iii) development of an on-line time-gated resonant-scanning CW-STED microscope. Both ii) and iii) reduce photobleaching (Wu 2014). This ultrafast photon counting system is not only applicable to STED microscopy but has broader applications to other types of microscopes.

In the original design, our ultrafast photon counting system uses PMTs as light detectors. Other types of light detectors may also be used, as long as the photon pulse width from the detector is not shorter than the ADC sampling interval to ensure that the pulses can be detected. Most light detectors, such as PMTs, silicon avalanche photodiodes (APDs), multi-pixel photon counters (MPPCs), and hybrid detectors, satisfy this condition. In fact, we often use a silicon APD module to detect fluorescence in the far red channel for its higher quantum efficiency (QE). One of the advantages of MPPCs or hybrid detectors is that the height of pulses they generate indicates how many photons the pulses contain (Dambrosio *et al.* 1994; Gomi *et al.* 2007). Our design allows various real-time or post-acquisition signal processing. By quantifying the pulse height with the FPGAs, it has the capacity of recognizing the multi-photon pulses from these detectors, which further enhances the dynamic range.

Another direction of signal processing is towards advanced edge detection and pulse identification methods. For instance, currently we are using DC-coupled amplifiers, which have relatively low bandwidth (~ 400 MHz) but keep the baseline voltage to nearly a constant. AC-coupled amplifiers have much higher bandwidth and would reduce the dead

time, but it would also cause a changing baseline that needs to be dealt with by more complex pulse identification methods than simply thresholding. Advanced methods such as machine learning and digital filtering may also be realized by FPGAs to better differentiate overlapping pulse pairs, through which even higher linearity could be achieved.

When the linear scanning speed is fast, it may cause *motion blur* that could compromise the optical resolution. If the linear scanning speed is v , the fluorescence lifetime is τ , and the point spread function without motion is $\rho(x)$, then the motion-blurred PSF is estimated by $\rho(x) * e^{-x/(v\tau)}$, where $*$ stands for convolution. For our system with the 8 KHz resonant scanner and the 50 μm wide FOV, $v \approx 1.6 \text{ nm/ns}$. The lifetime of the Atto 647N dye is about 3.5 ns. If the optical resolution at rest is 50 nm, the motion-blurred resolution is $\sim 53 \text{ nm}$ (horizontal direction only). Therefore, motion blur at current scanning speed is negligible. However, with increasing linear scanning speed, the effect motion blur would become noticeable. At a doubled and quadrupled speed, the resolution would be downgraded to $\sim 59 \text{ nm}$ and $\sim 73 \text{ nm}$, respectively.

In a typical STED imaging experiment, the fluorescence signal is usually so low that the linearity of the photo counting system is not very important. However, biological samples have great variation and some are bright enough to raise concerns. Also, since the excitation lasers are pulsed, fluorescence comes in bursts and the peak photo rate is much higher than the time average. The image in Fig.2 only has a peak photon rate of 20 million cps. However, we have observed a peak photon rate as high as 350 million cps in one of our STED experiments; while in another dimmer region of the same cell, the peak photon count is only 56 million cps. This large range is an example for the necessity of good linearity of the photo counting system in STED microscopy.

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Fig. 2.

Pulse STED image taken with resonant scanning microscope and acquired by ultrafast photon counting system. Specimen is an isolated cardiomyocyte labeled with antibodies for L-type Ca^{2+} channel $\alpha 1\text{C}$ subunit and secondary antibodies tagged with ATTO647N. (A) Raw image with 450 MHz pixel clock. (B) Final image after compensating for the nonlinear motion of scanning mirrors and scaled to 1:1 pixel ratio. The FOV is $\sim 50\ \mu\text{m}$, each side. This and following images were thresholded to reduce background, and a 3×3 median filter (the “Despeckle” function in *ImageJ*) was applied to reduce speckle noise. (C) Blowup of a region in (B). (D) A line profile (marked by two arrowheads in C) demonstrates resolution. FWHM of the Gaussian fit is 62 nm. Three clusters were measured and average FWHM is $58\pm 4\ \text{nm}$.

Fig. 3. Time-gated STED microscopy with resonant scanning mirrors and ultrafast photon counting system. (A) Confocal image of fluorescent microspheres. (B) CW STED image of the same FOV in (A); resolution gain is obvious. (C) Resolution is further improved by time-gating. (D) Visualization of resolution enhancement with two nearby microspheres in previous images. (E) Confocal image of a cardiomyocyte labeled with antibodies for ryanodine receptors and secondary antibodies tagged with Oregon green dye. (F) In the same FOV, time-gated STED image significantly increases the resolution.

Fig. 4.

Linearity of ultrafast photon counting system. (A) Count rate as a function of illumination power (measured at the back aperture of the objective) for scattered light from 20 nm silver nanoparticles. The straight line shows the linear extrapolation of 2 data points with the lowest illumination power, but at higher power the count rate saturates. The data points are well fitted by Eq. (1), with a dead time of 1.7 ns. (B) Counting efficiency as a function of illumination power. Cutoff count rate is above 200 million cps. Error bars in (A) and (B) represent the standard errors of 4 measurements. (C) A photon pulse coming out of amplifier and then digitized by the ADC module. The pulse width (FWHM) is 1.1 ns.