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A space- and time-resolved single photon counting detector for fluorescence microscopy and spectroscopy

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Abstract

We have recently developed a wide-field photon-counting detector having high-temporal and high-spatial resolutions and capable of high-throughput (the H33D detector). Its design is based on a 25 mm diameter multi-alkali photocathode producing one photo electron per detected photon, which are then multiplied up to 10^7 times by a 3-microchannel plate stack. The resulting electron cloud is proximity focused on a cross delay line anode, which allows determining the incident photon position with high accuracy. The imaging and fluorescence lifetime measurement performances of the H33D detector installed on a standard epifluorescence microscope will be presented. We compare them to those of standard single-molecule detectors such as single-photon avalanche photodiode (SPAD) or electron-multiplying camera using model samples (fluorescent beads, quantum dots and live cells). Finally, we discuss the design and applications of future generation of H33D detectors for single-molecule imaging and high-throughput study of biomolecular interactions.

Keywords

single-photon; single-molecule; photocathode; microchannel plate; position sensitive anode; fluorescence; lifetime; quantum dot; live cell

1. INTRODUCTION

The exquisite sensitivity of light detectors currently used in wide-field (charge coupled device: CCD, intensified CCD: ICCD, electron multiplying CCD: EMCCD) or scanning confocal (photomultiplier: PMT, single-photon avalanche photodiode: SPAD) fluorescence microscopy, helped by a careful rejection of background sources (Rayleigh or Raman scattering, optics autofluorescence) has recently allowed detection, tracking and spectroscopic analysis of the fluorescence emission of single molecules^{2–6}. Single dye

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molecules or in general single fluorescence emitters such as quantum dots⁷ have been used to tag biomolecules such as proteins, DNA or RNA, in vitro or in vivo and monitor their location, conformation or interaction with other molecules (using fluorescence energy transfer between two reporter molecules), or changes in their local environment via measurement of their fluorescence lifetime. Single-molecule techniques can reveal rare events, discrete steps or the complete spectrum of static and dynamic properties that are otherwise hidden in ensemble measurements. They have therefore the potential to revolutionize our understanding of how biomolecules actually work and interact in complex molecular networks. However, single-molecule spectroscopy (SMS) currently suffers from the following specific detector limitations: wide-field detectors, which allow the study of several single molecules at once, have limited frame rate and poor time-resolution. Time-gated cameras, which have ps-timing capability, are very inefficient photon detectors, preventing their use to study single molecules or fast changing samples⁸. Point-like single-photon counting detectors (PMT, SPAD), which have sub-ns time resolution, require scanning to form an image, and are therefore inefficient imaging detectors, limiting their use to the study of isolated, static molecules, or an assembly of diffusing molecules with methods such as fluorescence correlation spectroscopy (FCS)⁹. An ideal detector for SMS would thus combine the properties of both types of detectors and be a wide-field, single-photon counting detector, providing high-resolution spatial and temporal information for every detected photon. Such an ideal detector would for instance allow the simultaneous observation of ~50–100 single molecules emitting a detected signal of ~50–100 kHz per molecule. From the detector it will require the capability to sustain a 100 kHz maximum local count rate and a 50 MHz maximum global count rate.

At least four detectors combining spatial and temporal capabilities have been commercialized and used in the recent past: the now discontinued PIAS detector from Hamamatsu Corp (USA)¹⁰, the Mepsicron developed by Quantar Technology Inc (Santa Cruz, CA, USA)^{11, 12}, the TSCSPC detector from Europhoton GmbH (Berlin, DE, EU)¹³ and the IPD detector from Photech Ltd (St Leonards-on-Sea, UK, EU). All these detectors use a similar design based on a multi-alkali photocathode (<20 % QE in the visible) followed by one or more electron multiplying micro-channel plates (MCP) and a plain resistive anode¹⁴ or quadrant capacitive anode¹⁵ to record the position of the electron cloud proximity-focused onto it. The readout electronics of this type of anode limits the acquisition rate to <100 kHz. To attain higher global count rates needed for wide-field observation of multiple single molecules and rapidly changing fluorescent samples, a faster type of position sensitive anode is needed. Based on the Space Sciences Lab's (SSL's) experience with cross-delay line anode¹⁶, we have designed and constructed a detector similar to those mentioned previously using a cross-delay anode readout by a time-to-digital-converter (TDC) electronic module with a conversion time of 1.4 μ s allowing a maximum ~700 kHz global readout rate¹⁷.

2. DETECTOR DESCRIPTION

We present here only a succinct description of the H33D detector as a detailed description will be presented elsewhere¹⁸. Fig. 1 presents a schematic of the detector's architecture.

A S20 multi-alkali photocathode was deposited on a fused silica window and proximity focused on a MCP stack (Z triplet stack, Photonis-DEP, Brive, FR, EU). A 30×30 mm cross-delay line was vacuum sealed ~ 6 mm behind this assembly. The (X, Y) position of the electron cloud impact generated by each detected photon is read out using the SOHO-UVCS/SUMER mission design timing electronics¹⁶. The precise timing (nanotime, τ) of each detected photon with respect to the exciting laser pulse is obtained with a commercial 12 bit TDC (Model 7072T, FAST Comtec, Oberhaching, DE, EU) from the MCP back voltage pulse signal (Start) and the laser pulse signal (Stop). A front-end field programmable gate array (FPGA) (Spartan II, Xilinx, San Jose, CA, USA) allows synchronization of all information and time-stamping (macrotime, T) of each event with the laser pulse number. Each (X, Y, τ , T) data set is then asynchronously transferred to a computer via a fast digital interface board (PXI-6534, National Instruments, Austin, TX, USA). Software written in LabView (National Instruments) performs data storage, online image visualization and data analysis.

3. DETECTOR PERFORMANCE

Two sets of performance tests were performed: one set at the fabrication site (UCB) and one set after installation on the microscopy setup at UCLA. Tests of the electronics and of data transfer to the computer are not reported here.

- i. The measured QE decreased from 18 % at 400 nm to 8.5 % at 520 nm and 3 % at 630 nm (Fig. 2), which is a factor of 6–10 less sensitive at 520 nm, and 25–30 less sensitive at 630 nm than standard detectors used in SMS.
- ii. The imaging non-linearity was assessed before tube sealing (without photocathode) using a pinhole mask with 10 μ m pinholes on a 1 mm $\times$ 1 mm grid in contact with the MCP, and found to be satisfactory, with only slight deformations on the rim of the detector (Fig. 3A). After installation at UCLA on the microscopy setup, a reticule with 10 μ m spaced ticks was imaged using a 60 X oil-immersion objective lens. The image exhibited spherical aberrations due to electric field deformations at the edge of the MCP limited to the border of the imaging area (Fig. 3B).
- iii. The spatial resolution was measured by illuminating a pinhole with a diode and measuring the full width at half maximum (FWHM) of the diffraction-limited image in both X and Y direction. The geometric mean was 200 μ m for a MCP gain of 3.5×10^6 and 100 μ m for a gain of 9×10^6 . The spatial resolution varied by only 10 % when the local count rate (count rate per spot) was increased from 2 kHz to 10 kHz (spot FWHM: 100 μ m). This latter value corresponds to the typical local count rate expected from a single molecule for a detector having $\sim 1/10$ the SPAD QE (Fig. 2). After installation at UCLA, small size fluorescent beads (diameter: 200 nm, emission peak: 515 nm) were imaged and their image size estimated by 2D Gaussian fitting for comparison with the expected Airy disk size (Fig. 4).

The temporal resolution was measured at UCB using a pulsed red diode laser (NanoLED-02, Horiba Jobin Yvon, Edison, NJ, USA) with a pulse width of 80 ps and a repetition rate of 10

kHz, attenuated with a gelatin neutral density filter so that at most one photon per pulse reached the photocathode. The measured transit time spread (TTS) of the whole system was 100 ps FWHM. A similar measurement was performed on UCLA microscopy setup, using a pulse picked (3.8 MHz) femtosecond (fs), frequency-doubled (437 nm) laser (Mira 900, Coherent, Santa Clara, CA, USA) to excite the fluorescence of a fast decaying dye (Erythrosin B, $\tau = 80$ ps). The measured transit time spread (TTS) of the whole system was found here to be 239 ps FWHM (Fig. 5**Error! Reference source not found.**). The difference between the TTS values obtained in these two experiments could be due to several (yet uncharacterized) factors: (i) less than optimal values of the CFD settings in the fluorescence experiment; (ii) large jitter of the fast PIN photodiode; (iii) high count rate resulting in more than one photon being detected on average during the fluorescence measurement. Further experiments will clarify the origin of this difference.

4. PRELIMINARY RESULTS

The H33D detector can be used for diverse spectroscopic and/or microscopic applications such as those demonstrated for similar detectors^{10–13}. Here, we limit ourselves to demonstrating the basic capabilities of this detector, namely its capacity to image fluorescent samples, while simultaneously providing high-resolution temporal information on their fluorescence lifetime.

Several samples characterized by known fluorescence lifetimes were imaged as thin slab (~ 30 μm) of liquid between two glass coverslips. By selecting a region of the image, we can build a histogram of the time delay between each individual photon and the laser pulse immediately following its arrival, providing a reversed fluorescence decay curve convolved with the response function of the instruments. Fig. 6**Error! Reference source not found.** summarizes the results obtained with an organic dye molecule, fluorescent beads and quantum dots. The long lifetime of the quantum dots is readily discernible from the much shorter lifetimes of the Alexa Fluor 488 dye and the dyes filling the fluorescent beads. Bi-exponential fits of the decay curves gave lifetime values in agreement with the known lifetime components of these species.

We then tested the time-gating capabilities of the detector using live HeLa cells expressing chimeric surface receptors fused with avidin, detected with green biotinylated quantum dots as described¹. Fig. 7 shows a raw intensity image of the sample including all detected photons, and the result of keeping only photons arriving 30 ns after the excitation pulse. Although the image is noisier, the contrast of quantum dot-rich regions in cells is markedly improved, due to the rejection of the cell autofluorescence background, as was previously shown with time-resolved confocal microscopy¹⁹.

5. PERSPECTIVES

The previous data demonstrate the excellent spatial and temporal resolution of the current H33D detector. This device can be used as a regular imager, by representing the intensity per pixel during a fixed period of time. Contrary to a standard camera, however, the integration time can be adjusted at will by the user after acquisition, the raw data consisting in a photon

list containing the 4 coordinates (X, Y, τ , T) of each photon. Image sequences (movies) can thus be created with arbitrary time resolution, limited only by the available signal-to-background ratio. The nanotime information associated with each photon permits to measure the fluorescence decay histogram of any given region of interest in the image. By fitting these histograms with fluorescence decay models, fluorescence lifetimes can be extracted from any region-of-interest level down to the single pixel level. Such fluorescence lifetime maps have become a prominent tool to map the environment of fluorescently labeled proteins, as well as to monitor protein-protein interactions in live cells²⁰. The H33D detector will greatly facilitate the construction of such maps by providing all required information in a single acquisition, as already demonstrated with a similar detector¹³. This ease of use has been illustrated here for time-gated imaging of quantum dots (Fig. 7). Future work will explore these and other applications.

The present H33D prototype still suffers from a relatively low QE and a limited maximum global counting rate. Recent developments in GaAs or GaAsP photocathodes (**Error! Reference source not found.**) indicate that significant QE improvements could be obtained in future detector generations. The current global counting rate limitation is due to the anode readout electronic speed. While improvements in speed could be obtained, a fundamental limit in local and global count rates is set by the high MCP gain currently used. Cross delay line schemes have been shown to keep exceptional spatial resolution at gain as low as 6×10^5 ²¹, and should be the readout scheme of choice to attain the target performances presented in the introduction. A number of technological challenges still remain to be overcome, but we foresee that a future H33D detector comprised of a GaAs (or GaAsP) photocathode and a cross-strip anode readout scheme will eventually allow performing temporally and spatially resolved spectroscopy and microscopy at the single-molecule level.

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Fig 1.

Principle of operation of the H33D detector. Pulsed laser excitation is used to excite the fluorescence of a sample. A fluorescence photon emitted a few ns after the excitation pulse is collected by the imaging optics and interacts with the photocathode (PC), creating a photoelectron with a wavelength-dependent probability QE. The photoelectron is amplified $\sim 10^7$ times by an apposed MCP stack, generating an electron cloud (cone shape). The measure of the delay between the charge pulse at the back of the MCP and the laser pulse (nanotime τ) is performed with a TDC. A cross-delay line anode (distance to MCP: ~ 6 mm) collects the charges at both ends of each line, and a timing electronics module converts the differences in arrival time into position information (X, Y). A laser pulse counter built in the readout electronics provides a 4th coordinate, the macrotime T (not shown on this diagram). δ : fixed time delay. V: velocity factor proportional to the actual anode signal propagation velocity.

Fig 2.

Comparison of the QE of the H33D Gen I detector with the detectors used commonly for single-molecule spectroscopy and microscopy. Wide-field detector: back-thinned electron-multiplying CCD (Cascade 512B, Photometrics, Tucson, AZ, USA). Point-detector: single-photon counting avalanche photodiode (SPCM-AQR, Perkin Elmer Optoelectronics, Fremont, CA, USA). The 4th curve corresponds to the characteristic of a fast GaAs photocathode tube (loaned by Burle Technologies) exhibiting a 250–350 ps FWHM response time.

Fig 3.

A. Contrast inverted image (black indicates high count rate, white indicates low count rate) of a pinhole mask placed in contact with the MCP before installation of the photocathode and sealing of the tube, with 10 μm pinholes spaced at 1 mm $\times$ 1 mm intervals. B. Contrast inverted image of a reticule with 10 μm -separated ticks obtained with a 60 X oil-immersion objective lens (PlanApo, NA 1.45, Olympus, Melville, NY, USA) using transmitted white light. Integration time: 2 min, global count rate: 90 kHz. The whole square area corresponds to 1024^2 pixels, corresponding to the total conversion range of the position sensing electronics. Pincushion distortion is noticeable on the border of the imaging area, due to electric field distortions.

Fig 4.

A. Image of a 200 nm diameter fluorescent bead sample (Excitation peak: 505 nm, Emission peak: 515 nm) spin-cast on a glass coverslip obtained by epifluorescence with a 60 X objective lens. Excitation: 437 nm pulsed laser, 3.8 MHz repetition rate, ~ 200 fs FWHM. Dichroic mirror: 495 DRLP, emission: 500 LP. Integration time: 60 s. B. Histogram of fitted minimum bead image width. The point-spread function (PSF) images were fitted with a 2D tilted Gaussian function to account for the non axisymmetry of the excitation profile, and the histogram of the smaller of the two Gaussian widths is represented here.

Fig 5.

A. Histogram of time delays between single laser photon detection by the H33D and the laser controller trigger pulse in the table top experiment done at UCB. B. Histogram of time delays between fluorescence photons detected by the H33D and the laser pulses detected by a fast PIN photodiode (DET 210, Thorlabs, Newton, NJ, USA). In each case, the fast signals were pre-amplified and fed to constant fraction discriminators (CFD) before reaching the Start and Stop inputs of the Time-to-Digital Converter (TDC). Schematic: layout of the microscopy setup used for fluorescence decay measurements.

Fig 6.

Fluorescence decays of several characteristic samples. Dashed line: instrument response function (IRF) as determined by the 80 ps fluorescence decay of Erythrosin B in water. In increased order of curve thickness (and apparent lifetime): Alexa Fluor 488 nm in deionized water, measured lifetime: $\tau = 1.26$ ns; 20 nm yellow-green FluoSpheres (Molecular Probes, Eugene, OR, USA): $\tau_1 = 1.00$ ns, $\tau_2 = 2.90$ ns; 200 nm yellow-green FluoSpheres: $\tau_1 = 2.08$ ns, $\tau_2 = 5.50$ ns; Red peptide-coated quantum dots: $\tau_1 = 2.75$ ns, $\tau_2 = 11.91$ ns;

Fig 7.

Time-gating of quantum dot labeled HeLa cells. HeLa cells expressing a chimeric avidin-CD14 fusion receptor on the exterior of their plasma membrane were incubated briefly with biotin-modified peptide-coated quantum dots as described in ref. ¹. After rinsing away free quantum dots, the cells were observed in epifluorescence using the setup described in Fig. S3. Labeled receptors accumulate on the cell surface, but can also be recycled inside the cell over longer period of time. A. Image obtained with 340 s integration time (175 kHz count rate) using all detected photons. Inset: photon arrival time histogram for a region of interest located in one cell. B. Same data set, where only photons arriving 30 ns after the laser pulse were retained (hatched area in the fluorescence decay).