

Photon-counting H33D detector for biological fluorescence imaging

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Abstract

We have developed a photon-counting High-temporal and High-spatial resolution, High-throughput 3-Dimensional detector (H33D) for biological imaging of fluorescent samples. The design is based on a 25 mm diameter S20 photocathode followed by a 3-microchannel plate stack, and a cross-delay line anode. We describe the bench performance of the H33D detector, as well as preliminary imaging results obtained with fluorescent beads, quantum dots and live cells and discuss applications of future generation detectors for single-molecule imaging and high-throughput study of biomolecular interactions.

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1. Introduction

The sensitivity of modern light detectors (charge-coupled device: CCD, electron multiplying CCD: EMCCD or single-photon avalanche photodiode: SPAD) used in fluorescence microscopy has recently allowed the detection, tracking and spectroscopic analysis of the fluorescence emission of single molecules (for a recent review, see Ref. [1]). Single dye molecules or in general single fluorescence emitters such as quantum dots [2] have been used to tag proteins, DNA or RNA and monitor their location, conformation, interaction with other 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 measurement. However, single-molecule spectroscopy (SMS) is limited by current detectors: 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 photon-inefficient detectors, preventing their use to study single molecules or fast dynamics [3]. Point-like single-photon counting detectors (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) [4]. An ideal detector for SMS would combine the best of both worlds and be a wide-field, single-photon counting detector, providing high spatial and temporal resolution information for each detected photon. It would allow the simultaneous observation of ~ 500 single molecules emitting a detected signal of ~ 100 kHz, i.e. be capable of a 100 kHz maximum local count rate and 50 MHz maximum global count rate.

At least three photon-counting detectors combining spatial and temporal capabilities have been commercialized and used in the recent past [5–8]. 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 anode [9] or quadrant capacitive anode [10] to record the position of the electron cloud proximity-focused onto it. The readout electronics of this type of

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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 anode [11], 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 $1.4 \mu\text{s}$, allowing a maximum ~ 700 kHz global readout rate.

2. Detector description

We present a succinct description of the H33D detector (Fig. 1), as a detailed description will be presented elsewhere.

A S20 multi-alkali photocathode was deposited on a fused silica window and proximity focused onto 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

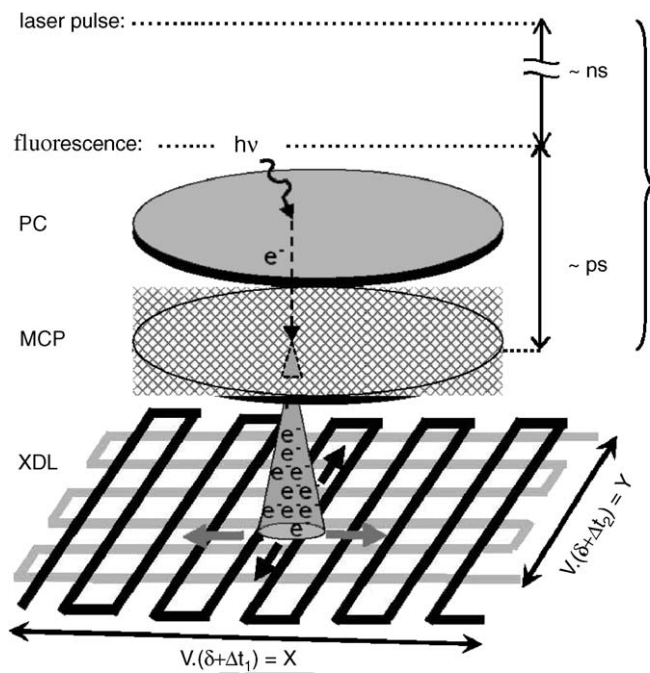


Fig. 1. Principle of operation of the H33D detector. Each fluorescence photon is converted by the photocathode (PC) into a photoelectron amplified $\sim 10^7$ times by the MCP stack. The time interval between the pulse generated at the back of the MCP and the laser pulse (nanotime τ) is measured by a TDC. The electron cloud is collected by a cross-delay line anode (distance to MCP ~ 6 mm) and a timing electronics module converts the differences in charge arrival time at both ends of the delay lines into position information (X, Y) . A laser pulse counter built in the readout electronics provides a fourth coordinate, the macrotime T (not shown). δ : fixed time delay. V : velocity factor proportional to the actual anode signal propagation velocity.

using the SOHO-UVCS/SUMER mission design timing electronics [11]. 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 development 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 3% at 630 nm, values typical for a S20 photocathode.
- (ii) The imaging non-linearity was assessed before tube sealing using a back-illuminated pinhole mask with $10 \mu\text{m}$ pinholes on a $1 \text{ mm} \times 1 \text{ mm}$ grid in contact with the MCP, and found to be minimal. After installation at UCLA on the microscopy setup, a reticle with $10 \mu\text{m}$ spaced ticks was imaged using a $\times 60$ oil-immersion objective lens. The image exhibited minimal spherical aberrations due to electric field deformations at the edge of the MCP limited to the border of the imaging area.
- (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 of the pinhole in both X and Y directions. The geometric mean was $100 \mu\text{m}$ for a gain of 9×10^6 , varying by only 10% for local count rates between 2 and 10 kHz. After installation at UCLA, small fluorescent beads (diameter 200 nm, emission peak 515 nm) were imaged (Fig. 2) and their image was found to be of the expected diffraction-limited size.
- (iv) The temporal resolution was measured at UCB using a pulsed red diode laser (pulse width: 80 ps, repetition rate: 10 kHz), attenuated 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. Measurement on the UCLA microscopy setup, using a pulse-picked (3.8 MHz) femtosecond, frequency-doubled (437 nm) laser to excite the fluorescence of a fast-decaying dye (Erythrosin B, $\tau = 80$ ps) resulted in an instrument response function (IRF) of 271 ps FWHM (Fig. 3).

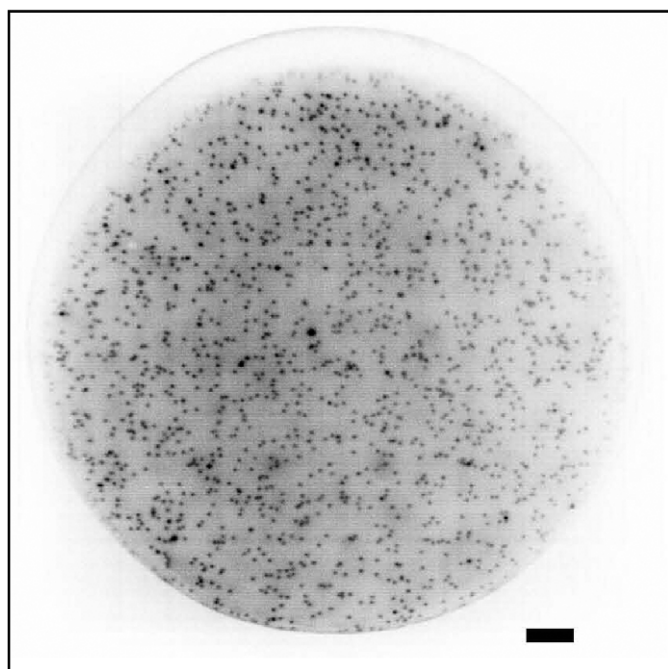


Fig. 2. A. Contrast inverted image of a 200 nm diameter fluorescent bead sample (Excitation peak 505 nm, Emission peak 515 nm) spin-cast on a glass coverslip, observed by epifluorescence with a $\times 60$ oil immersion objective lens (NA = 1.4). Integration time 60 s. Scale bar: 10 μm (in the sample plane).

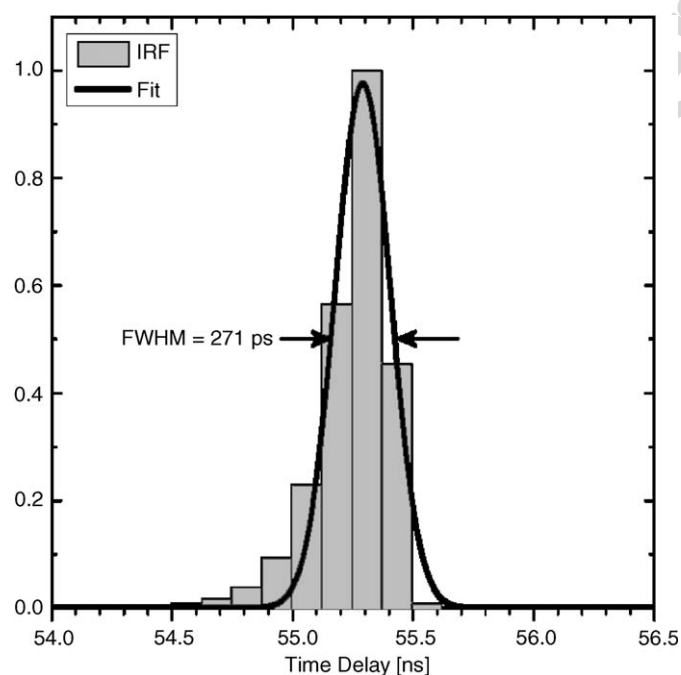


Fig. 3. Histogram of time delays between fluorescence photons emitted by a sample of Erythrosin B (lifetime ~ 80 ps) detected by the H33D, and the laser pulses detected by a fast PIN photodiode. The response of the whole system has a FWHM ~ 270 ps.

4. Preliminary results

The H33D detector can be used for diverse spectroscopic and/or microscopic applications such as those demon-

strated for similar detectors [5–8]. We have checked the basic capabilities of this detector, namely its capacity to image fluorescent samples, while simultaneously providing high-resolution temporal information on their fluorescence lifetime. A detailed description of these tests will be published elsewhere. Fluorescence lifetimes of solutions of known samples (Alexa Fluor 488, fluorescent beads, quantum dots) were measured and found to be in agreement with their reported values. The time-gating capability of the detector [12] was tested using live HeLa cells expressing chimeric surface receptors fused with avidin, detected with green biotinylated quantum dots [13]. Finally, we were able to image single quantum dots emitting at 585 nm.

5. Discussion

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 post-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 study the environment of fluorescently labeled proteins, as well as to monitor protein–protein interactions in live cells [14]. 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 detector [8]. This ease of use has been illustrated here for time-gated imaging of quantum dots. 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 and GaAsP photocathodes 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 rate is set by the high MCP gain currently used. Cross-strip anode schemes have been shown to keep exceptional spatial resolution at gain as low as 6×10^5 [15], and should be the readout scheme of choice to attain the target performances presented in the introduction.

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