

# Next-generation 3-D detector improves single-molecule imaging

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**A high-throughput three-dimensional detector combines the advantages of wide-field detectors and high-temporal-resolution point detectors, proving instrumental for single-molecule imaging and the study of biomolecular interactions.**

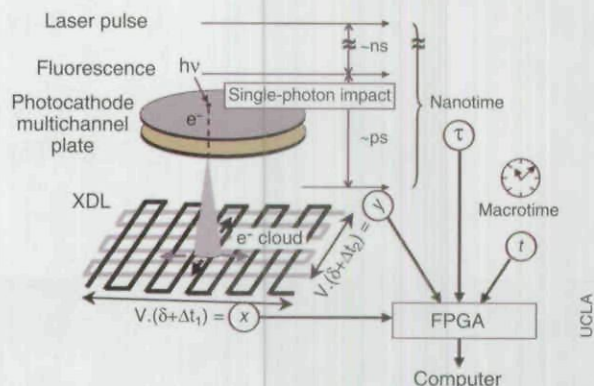


FIGURE 1. The operation of the high-throughput three-dimensional detector (H33D) begins with a pulsed laser to excite the fluorescence of a sample. Fluorescence photons are collected by the imaging optics and interact with the photocathode, creating one photoelectron per photon that is amplified by an opposed multichannel plate to generate an electron cloud (cone shape). The position of the cloud is determined by a position-sensitive cross-delay line anode, in which charges propagate through the lines and are collected at both ends. Timing electronics convert the differences in arrival time into position information and the four photon coordinates (laser pulse nanotime  $\tau$ , position  $x$ ,  $y$ , and macrotime  $T$ ) are processed and stored by a computer. In the setup, the fixed time delay is  $\delta$ , signal-propagation velocity  $v > 1$  mm/ns, and the sensitive area has a diameter of 1 inch.

second timing capability, are very photon-inefficient detectors not suited to the study of single molecules or rapidly changing samples. Point-like single-photon-counting detectors (photomultiplier tubes or single-photon avalanche photodiodes) with subnanosecond 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 molecules diffusing through a fixed femtoliter volume.

## The ideal SMS detector

Motivated by the demanding needs of single-molecule fluorescence spectroscopy and imaging applications, our team of researchers from the University of California at

The exquisite sensitivity of light detectors used in wide-field fluorescence microscopy or scanning confocal microscopy has recently allowed the detection, tracking, and spectroscopic analysis of the fluorescence emission of single molecules.<sup>1,2</sup> Single-fluorescence emitters such as dye molecules or quantum dots have been used to tag proteins, DNA, or RNA, and monitor their location or interaction with other molecules *in vitro* or *in vivo*.<sup>3</sup> 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—in short, they have 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 specific detector limitations. Wide-field detectors (charge-coupled devices, including intensified CCDs or electron-multiplying CCDs), which allow the study of hundreds of single molecules at once, have poor time resolution. Time-gated cameras, which have pico-

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Los Angeles (UCLA; Los Angeles, CA), the University of California at Berkeley (Berkeley, CA), and Lawrence Livermore National Laboratory (LLNL; Livermore, CA) started a program in detector development.

An ideal SMS detector should combine the properties of both types of detectors and allow simultaneous observation of hundreds of single molecules emitting a detected signal

of up to 100 kHz (a 100 kHz maximum local count rate) and a 50 MHz maximum global count rate.

Detectors combining spatial and temporal capabilities have been commercialized and used in the recent past, but they are far from approaching these ideal specifications. They all use a similar design based on a multialkali photocathode with quantum efficiency (QE) less than 20% in the visible spectrum. The photocathode is followed by one or more electron-multiplying microchannel plates (MCPs) generating a cloud of several millions of electrons at the back of the MCP. These electrons are proximity-focused onto and collected by a plain resistive anode or quadrant-capacitive anode that allows computing the position of the center of mass of the cloud. The readout electronics of this type of anode limits the acquisition rate to less than 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 work of the Space Sciences Laboratory at UC Berkeley, we designed and constructed a high-throughput three-dimensional detector (H33D) using a cross-delay anode that allows a maximum readout rate of approximately 700 kHz.<sup>4</sup>

#### H33D description

To fabricate the H33D, an S20 multialkali photocathode was deposited on a fused-silica window and proximity-

UCLA

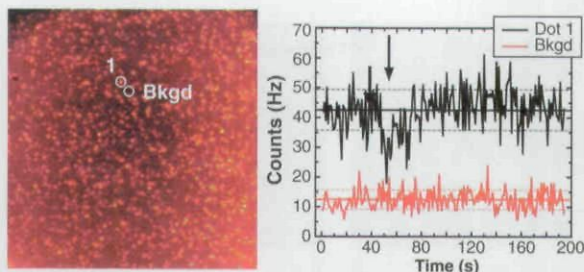


FIGURE 2. An accumulated image (left; 3 minute duration; image size  $130 \times 130 \mu\text{m}$ ) of 565 nm emitting quantum dots excited in total internal reflection mode by a 488 nm laser is analyzed and a time trace is drawn corresponding to one of the dots in the image (black curve; 1 s resolution) against the local background (red curve). Despite the low signal-to-noise ratio, a clear blinking event can be observed (arrow) indicating that a single quantum dot was observed. These quantum dots exhibited very little blinking when observed in similar conditions using an ultrasensitive EMCCD.

focused on a microchannel-plate z stack (see Fig. 1).<sup>5-7</sup> A  $30 \times 30$  mm cross-delay line was vacuum sealed approximately 6 mm behind this assembly. The role of the photocathode is to convert each incoming fluorescence photon (or at least a fractional QE of them) into a photoelectron. Each photoelectron generates a cloud of secondary electrons at the back of the MCP, which spreads and impacts the two separate and orthogonal zigzag patterns of the delay lines. Charge propagation through the delay lines results in detectable pulses at both ends of each delay line, the temporal separation of which gives access to the position along each delay line ( $x$  and  $y$ ).

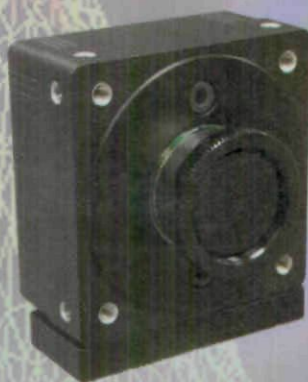
The precise timing (nanotime,  $\tau$ ) of each detected photon with respect to the exciting laser pulse is obtained with a commercial time-to-digital converter (TDC), which measures the separation of each voltage pulse generated at the back of the microchannel plate (Start) from the laser pulse signal (Stop). A field-programmable gate array (FPGA) associates the ( $x$ ,  $y$ ,  $\tau$ ) measurements to a timestamp (macrotime,  $T$ ) defined as the number of laser pulses since the beginning of the experiment. Each ( $x$ ,  $y$ ,  $\tau$ ,  $T$ ) data set is then asynchronously transferred to a computer via a fast-digital-interface board. Software written in LabView performs data storage, online image visualization, and data analysis.

#### H33D performance

The measured QE of our S20 photocathode decreases from 18% at 400 nm to 8.5% at 520 nm and 3% at 630 nm—typ-

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## Photodiode Amplifier

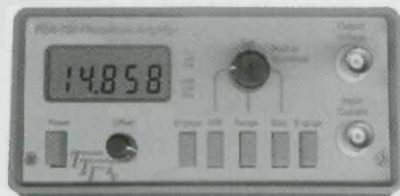
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## DETECTORS FOR MICROSCOPY, *continued*

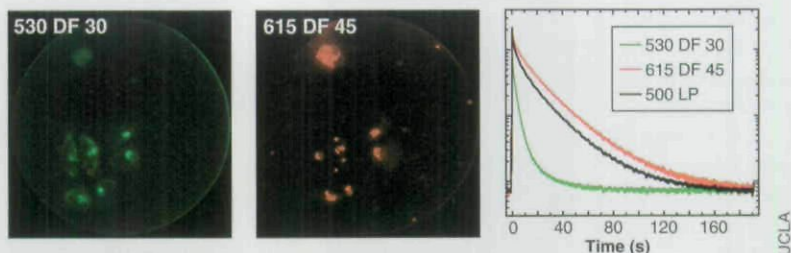


FIGURE 3. To demonstrate HeLa cell lifetime imaging, fluorescence-decay histograms (right) are plotted from filtered images of a caveolin-GFP dye signal (left) and a quantum-dot signal (middle). The fluorescence-decay curve corresponding to all photons (black curve, 500 nm long-pass filter) is barely different from the quantum-dot-only channel (red curve), reflecting the predominance of the quantum-dot signal.

ical values for this type of material, but significantly lower than standard detectors used in single-molecule spectroscopy. Imaging performance, as measured with test samples (reticle and/or subdiffraction fluorescent beads) shows a spatial resolution better than 100  $\mu\text{m}$  at the microchannel-plate gain of approximately  $10^7$  used, varying very little with the local count rate. Minimal spatial nonlinearities at the rim of the detection area are easily corrected by software. The temporal resolution measured using an attenuated pulsed diode laser with a pulse width of 80 ps and a repetition rate of 10 kHz is better than 100 ps full width at half maximum.

### Applications and results

To demonstrate the basic capabilities of the H33D in imaging fluorescent samples while simultaneously providing high-resolution temporal information on fluorescence lifetime, samples of dyes with known lifetimes were imaged as thin slabs (approximately 30  $\mu\text{m}$ ) of liquid between two glass coverslips using a femtosecond Ti:sapphire laser tuned at 720 nm and frequency doubled using a BBO crystal. By plotting a histogram of the nanoseconds of all recorded photons, a reversed fluorescence decay curve convolved with the response function of the instruments is obtained. Perfect single exponential fits of the decay curves were obtained for fluorescent dyes such as Rhodamine 6G and ethidium bromide, recovering their known fluorescence lifetime without further deconvolution. For quantum dots, a more complex decay curve was observed, necessitating a stretched exponential fit, in agreement with published data.<sup>8</sup>

While the low QE of our prototype does not allow the observation of single dye molecules, single quantum dots ex-

hibiting rare blinking events were readily observed after several seconds of integration (see Fig. 2). The time-gating capabilities of the detector were investigated using live cells fused with a particular chemical and detected with quantum dots. In this approach, none of the photons emitted by the sample is rejected, and time gating is performed digitally by retaining only photons emitted during the user-adjustable time gate when forming the image. One of the main advantages of this treatment is the rejection of the fast decaying background autofluorescence, while the signal of longer-lifetime quantum dots is comparatively preserved, resulting in a better contrast of the quantum-dot signal. We also explored the fluorescence-lifetime-imaging capabilities of the H33D detector (see Fig. 3), which allowed the determination of short- and longer-lifetime components. Doing this analysis at the pixel level provides fluorescence-lifetime maps, which have become a prominent tool to map the environment of fluorescently labeled proteins, as well as to monitor protein-protein interactions in live cells.

### Next-generation perspectives

Although the H33D can be used as a static imager by representing the intensity per pixel during a fixed period of time, the integration time can be adjusted at will by the user post-acquisition, allowing the creation of image sequences or "movies" with arbitrary time resolution and limited only by the available signal-to-background ratio.

The current H33D prototype still suffers from a relatively low QE and a limited maximum global counting rate. Recent developments in fast gallium arsenide (GaAs) or GaAs phosphide (GaAsP) photocathodes could offer significant QE

WHILE THE LOW QE OF OUR PROTOTYPE DOES NOT ALLOW THE OBSERVATION OF SINGLE DYE MOLECULES, SINGLE QUANTUM DOTS EXHIBITING RARE BLINKING EVENTS WERE READILY OBSERVED.

improvements in future detector generations. The current limitation on global counting rate is due to the anode readout electronic speed. While improvements in speed can be obtained, a fundamental limit in local and global count rates is set by the high microchannel-plate gain currently used. For specific applications, alternative readout schemes for this type of photon-counting device are possible.<sup>9,10</sup> Despite these technical challenges, future H33D detectors that use better photocathode and readout schemes will further improve temporally and spatially resolved spectroscopy and microscopy at the single-molecule level. □

#### ACKNOWLEDGMENTS

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#### REFERENCES:

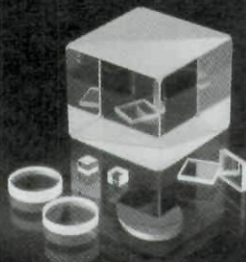
1. S. Weiss, *Science* 283, 1676 (1999).
2. X. Michalet et al., *J. Modern Optics* 54 (2007).
3. X. Michalet et al., *Science* 307, 538 (2005).
4. O. H. W. Siegmund et al., *Proc. SPIE* 2280, 89 (1994).
5. O. H. W. Siegmund et al., *IEEE Nuclear Symposium Conf. Record N14-55*, 448 (2005).
6. X. Michalet et al., *Nucl. Instr. Meth. Phys. Res. A* 567, 133 (2006).
7. X. Michalet et al., *Proc. SPIE* 6092, 60920M (2006).
8. X. Michalet et al., *Proc. SPIE* 6372, 63720E (2006).
9. A. S. Tremsin et al., *IEEE Trans. Nucl. Sci.* 51, 1707 (2004).
10. T. Ohnukia, *Proc. SPIE* 6092, 60920P (2006).

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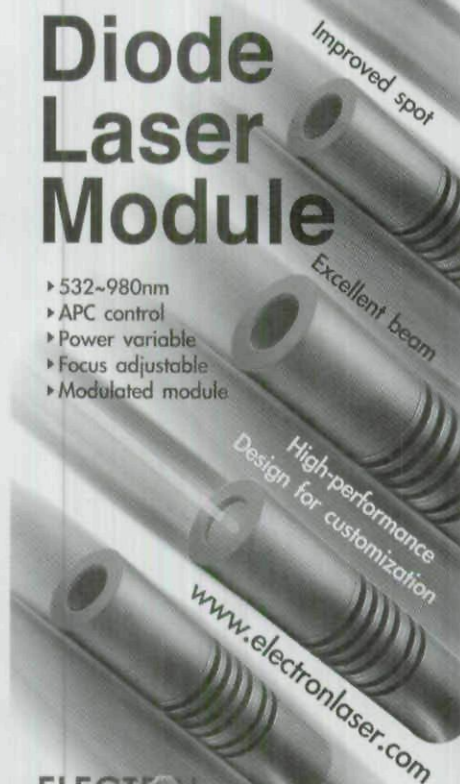
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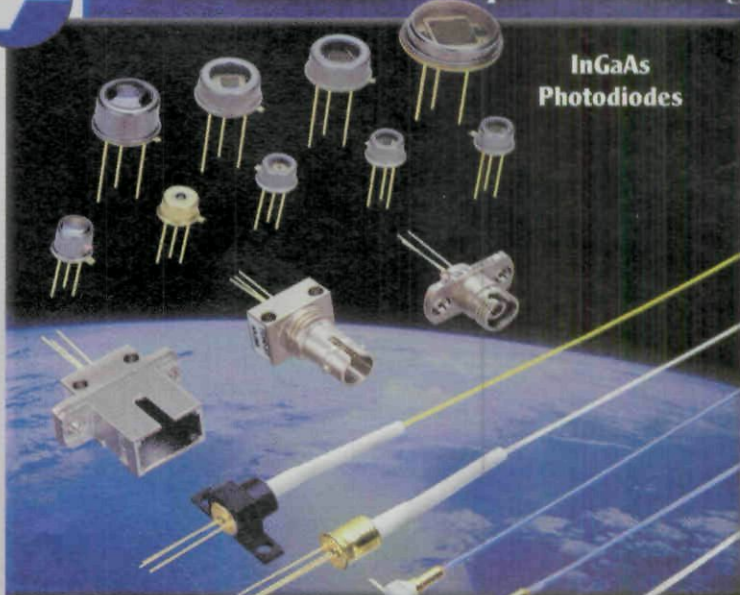
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