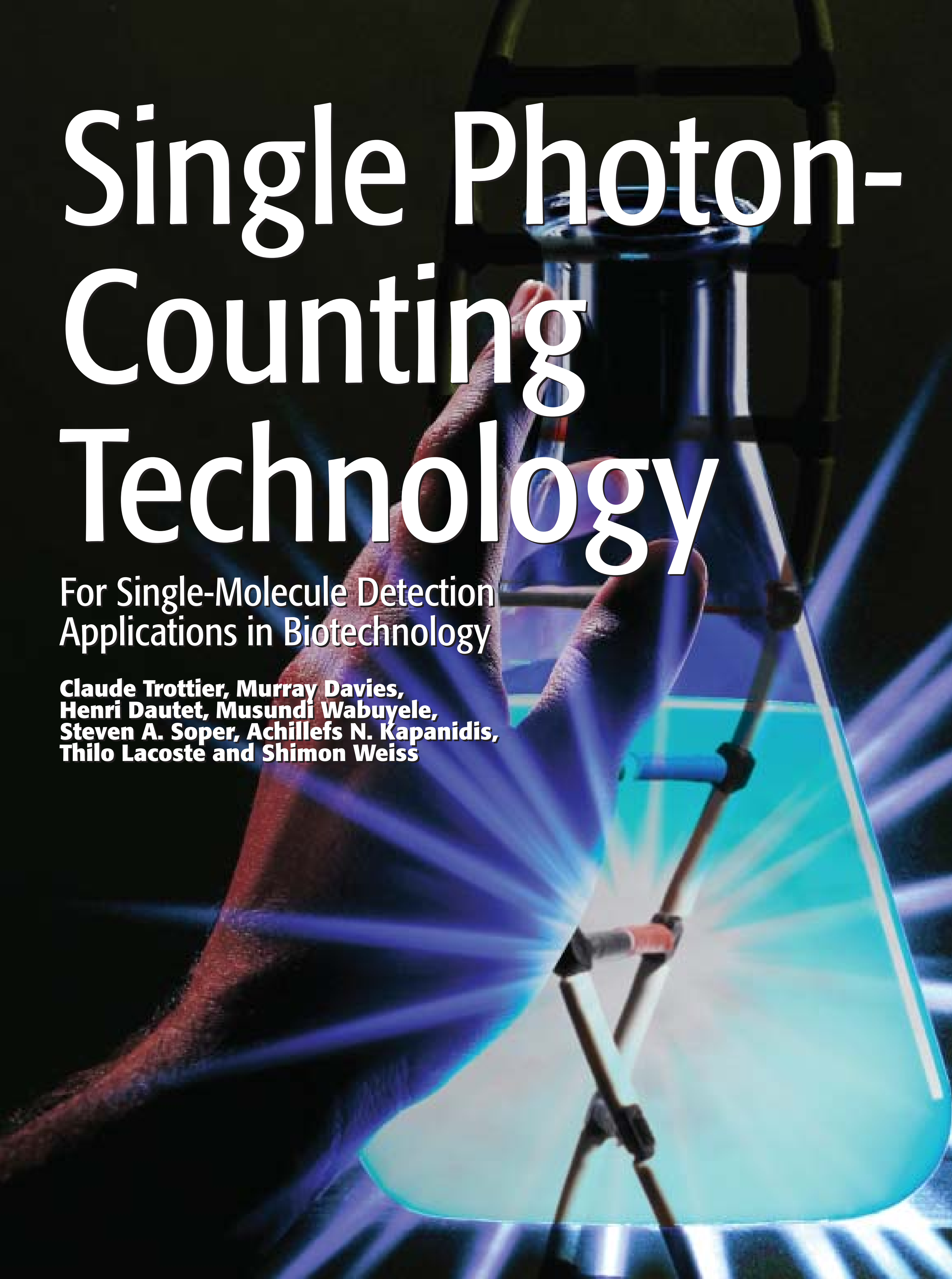

Single Photon-Counting Technology

For Single-Molecule Detection Applications in Biotechnology

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DIGITAL STOCK

This paper discusses the principles of single-photon counting technology and provides examples of biomedical research being conducted using the technique, including the observation and analysis of single protein–DNA complexes, and the analysis of low-abundant point mutations in K-ras oncogenes for the diagnosis of colorectal cancers.

Photon counting technology has the potential to pave the way for paradigm-shifting advancements in diverse research arenas, such as particle sizing, atmospheric studies and biomedical research. This technology, based upon avalanche photodiodes, has been found to achieve 70% photon detection efficiencies with sub-nanosecond timing jitters and extremely low dark counts (typically less than 250 cps). Following the early 1990s work of Soper and Davis, the single-photon counting module (SPCM) has been at the heart of the explosive growth of the single-molecule fluorescence field (1, 2). Along with being small and rugged, SPCMs provide a wide dynamic range and very low after-pulsing probabilities. For biomedical applications, this translates into robust instruments that can be used to measure molecular signatures from single molecules to millions of molecules (sub-zeptomole to attomole). SPCMs have been utilized effectively in sizing single DNA molecules, DNA diagnostics, single-protein dynamics, two-photon fluorescence microscopy and automated DNA sequencing machines, all of which use fluorescence as the transduction modality. The most important attribute of single-photon counting technology is that it is appropriate for analyzing signals generated from any type of biological event that produces only a few photons.

So how do the performance characteristics associated with SPCMs assist researchers in the biomedical arena? The wide dynamic range of SPCMs provides the researcher with the ability to secure quantitative information across many orders of magnitude in concentration. For example, in expression analysis, the gene products (mRNAs) can be quantified using complementary DNA (cDNA) arrays for low expressed transcripts and highly expressed transcripts without losing accuracy in the measurement. The exquisitely low dark count rates, as well as the high photon detection efficiencies associated with most SPCMs, have allowed

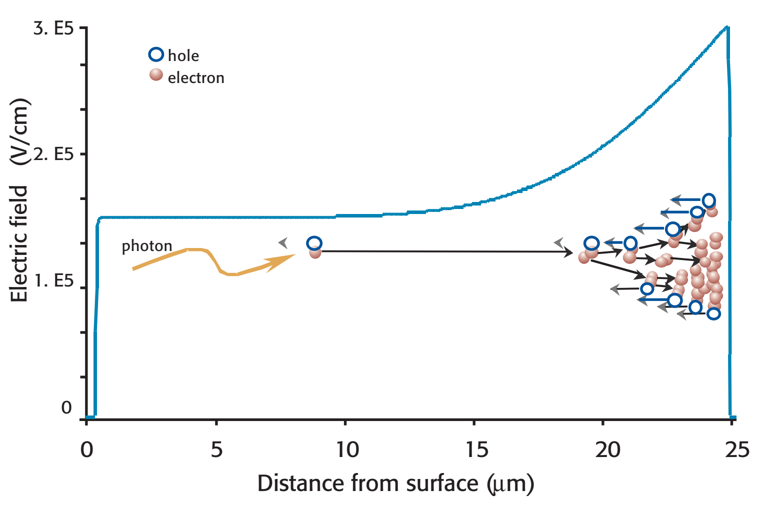

Figure 1. The electron multiplication process. The electric field of an avalanche photodiode (APD) is shaped in such a way that, operated under reverse bias, it will multiply, in a controlled fashion, the electrons generated inside the material. Under SPCM conditions, the multiplication would be infinite, resulting in a complete breakdown of the diode.

biomedical researchers to analyze weak signals. Examples include two-photon fluorescence microscopy, where the signals are extremely weak due to the low two-photon cross sections (i.e., probability of inducing an electronic transition based upon the simultaneous absorption of two photons). The ability to detect weak signals also allows researchers to analyze ultra-low volumes of samples, which is important in high-throughput screening assays of large combinatorial libraries, which require costly reagents. In addition, SPCMs have single-photon detection efficiencies in the extended red region of the electromagnetic spectrum that conventional photomultipliers do not possess, allowing ultra-sensitive fluorescence measurements in the near-infrared, where matrix interferences are minimal (3).

SPCM technology can detect from a few to several million photons per second, and it has a very high rate of single-photon detection efficiency, even in the deep red region of the electromagnetic spectrum.

To study biomedical systems, SPCMs enable fluorescence detection for high-throughput screening instrumentation, using lasers and scanning optics to excite the multi-well plate and charged-couple devices (CCDs) to measure the intensity of all wells simultaneously. New technologies use a confocal microscope and photon-counting technology to excite and detect single photons fluorescing from small numbers of chromophore molecules to even single molecules. The new technique also enables the simultaneous collection of multiple parameters, such as fluorescence intensity, molecule diffusion times, fluorescence lifetimes and fluorescence polarization, which can secure significant amounts of information from a single assay, reducing operator labor and minimizing reagent consumption.

This paper discusses biomedical research conducted at two universities in the United States that implemented single-molecule detection with SPCMs. The University of California, Los Angeles (UCLA), has applied the technology to observe and analyze single protein–DNA complexes. Louisiana State University (LSU; Baton Rouge, Louisiana, USA) used this technique for the analysis of low-abundant point mutations in K-ras oncogenes for the diagnosis of colorectal cancers. The research conducted by both universities attests to the bountiful future for SPCM technology applications. Furthermore, as will be shown, the development of new four-channel SPCM modules has the potential to provide a scalable, faster and less costly way to apply the technology in many industries.

SPCM Technology

SPCM technology has been successful for two reasons: it can detect from a few to several million photons per second (wide dynamic range), and it has a very high rate of single-photon detection efficiency, even in the deep red region of the electromagnetic spectrum. Avalanche photodiodes — which are semiconductor junctions operated under a reversed bias voltage — form the basis for SPCMs. Reverse bias corresponds to a situation in which the voltage applied to the silicon detector pulls the charge carriers away from the junction. A charge-free region thus is created (a depletion region or drift zone), which behaves as an in-

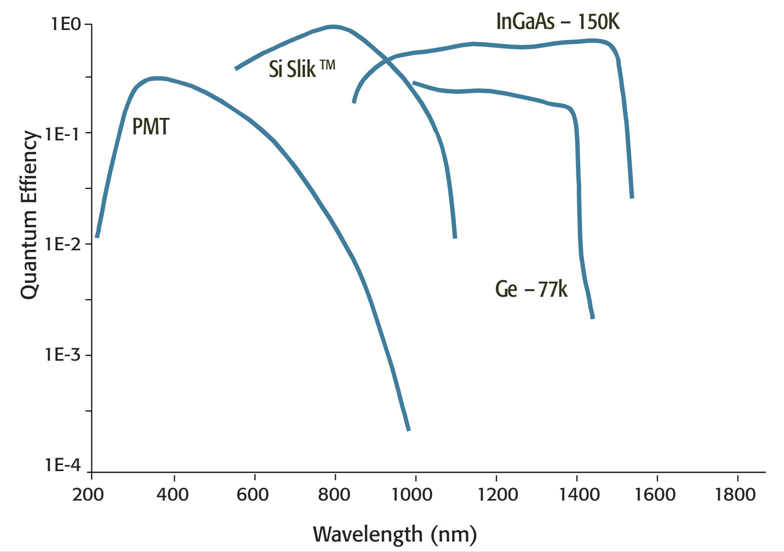

Figure 2. Typical quantum efficiency curves of the most common types of photodetectors. The avalanche photodiode detector (SLIK™) used in the SPCM, made from silicon, exhibits high quantum efficiency from the ultraviolet to the near-infrared, This high sensitivity combined with its multiplication capability makes it a detector of choice for low light-level detection, such as in fluorescence microscopy.

Figure 3. A new four-channel SPCM, which can be arrayed to provide cost effective and compact SPCM systems with as many as 100 possible channels.

sulator, so that almost no current is flowing through the device. Under this condition, a large electric field can be generated. This electrical field accelerates electrons generated inside the material (e.g., photo-electron or thermal generation). When the electric field reaches values in excess of 1E5 volts/cm, the energy acquired by the electrons is sufficiently high so that when they scatter against lattice atoms, the energy transferred leads to ionization of the atoms. This is the so-called "impact ionization." This process results in the generation of more electrons and results in an avalanche multiplication process (Geiger counters operate in a very similar fashion). Avalanche photodiodes differ from conventional photodiodes in that the electric field profile is tailored to optimize the avalanche multiplication, as illustrated in Figure 1. It is through this gain process that SPCMs are able to analyze single photons.

Impact ionization is a strong function of the electric field and, as a result, the gain is a function of the bias applied to the detector. At a certain bias, known as the breakdown voltage, the gain tends asymptotically to infinity. Thus, if the detector is operated at a bias that exceeds the breakdown voltage, even a single electron will result in the generation of a large current (impact). The ability to detect and characterize a single molecule is important for drug discovery applications. This means that signals are very weak and often only a single photon — and thus a single photoelectron — is available. To detect that single photoelectron, it is critical that the gain be as high as possible, the limit being the infinity gain, accomplished only by operating under breakdown conditions. This requires the APD to possess an extremely low bulk leakage current to avoid burning out the chip. Thus, when cooled to –10 °C, as few as tens of thermally excited electrons per second are generated in the dark (known as the dark count). Such a large time lapse between dark electrons enables time to quench the avalanche and ready the detector for the next photoelectron.

Quenching is performed by sensing the onset of the avalanche. The sensing circuit sends a signal to both the quenching circuit (to lower the bias below breakdown) and to the output circuit. The output circuit translates the signal to a transistor–transistor logic (TTL)-level pulse, which is compatible with many digital data acquisition systems. The end user merely connects the output of the SPCM to a counter and can directly measure the number of photons detected over the desired measurement time. After all charges have vacated the drift region, the reset circuit is activated, and all components, including the detector, are reset to their original state to be ready for the next photoelectron. The entire quenching and reset process is known as the “dead time” and takes about 50 nanoseconds.

The other important characteristic of SPCM is the high single-proton detection efficiency. The detector, being in silicon, exhibits high quantum efficiency (probability of generating a useful photoelectron) from the ultraviolet to the near-infrared; as can be seen Figure 2, which compares the quantum efficiency for various detector types. Next, by operating sufficiently above the breakdown, a detectable pulse is assured. Slightly less important for some applications, but critical for most, is the real-time output of the SPCM. A detailed description of various aspects of the SPCM operation can be found in the work of Dautet, Deschamps, Dion, MacGregor, MacSween, McIntyre, Trottier and Webb (4).

This ability to detect single photons allows for characterization of extremely small samples. Single-molecule detection is possible using an SPCM. Particle sizing is another application of this technology. Particles down to one nanometer in size and smaller can be measured. Furthermore, it is now possible to acquire multiple measurements simultaneously by using a four-channel SPCM array. This makes the SPCM useful for multi-wavelength systems where the user might want to image different spectra simultaneously. Examples include confocal microscopes and fluorescence correlation spectroscopy systems that are now on the market. A single-channel SPCM can achieve only one measurement at a time, while four-channel SPCMs, an example of which is shown in Figure 3, can be arrayed to provide cost effective and compact SPCM systems with as many as 100 possible channels. This enables the collection of multiple parameters per pixel and multiple pixels at one time.

The information required for developing new drugs or analyzing genes, proteins and cells will require the timely and efficient detection of multiple parameters, such as fluorescence intensity, diffusions, lifetimes and polarizations. Although the process of choice will be SPCM arrays, it all begins with single-molecule detection technology.

Single-Molecule Detection and FRET

Single-molecule detection refers to the ability to observe and analyze individual molecules, one

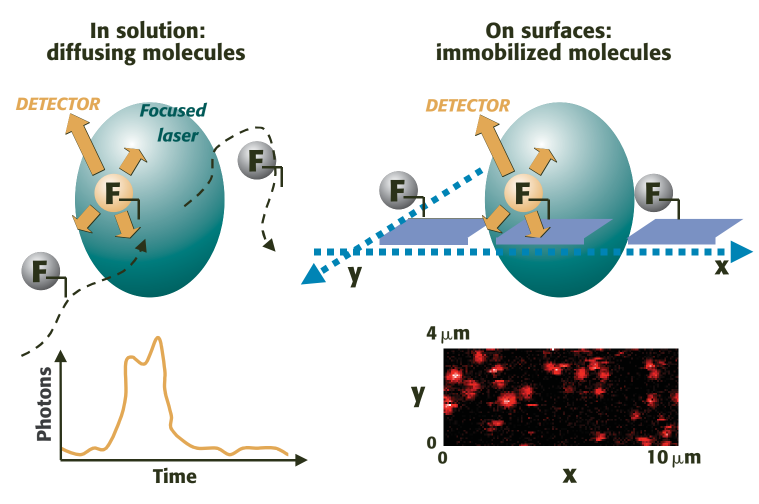

Figure 4. Single-molecule detection using point detectors. The principle of single-molecule detection of the fluorescence signal from diffusing molecules (left) and surface-immobilized molecules (right) is demonstrated.

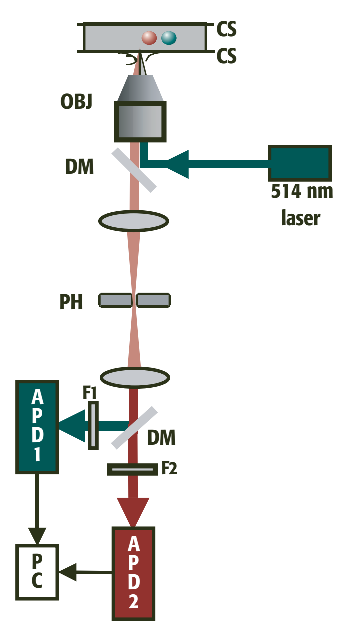

Figure 4a. A single-molecule fluorescence microscope is shown that is capable of detecting FRET within single molecules. CS: coverslip, OBJ: objective, DM: dichroic mirror, PH: pinhole, F1 and F2: spectral filters).

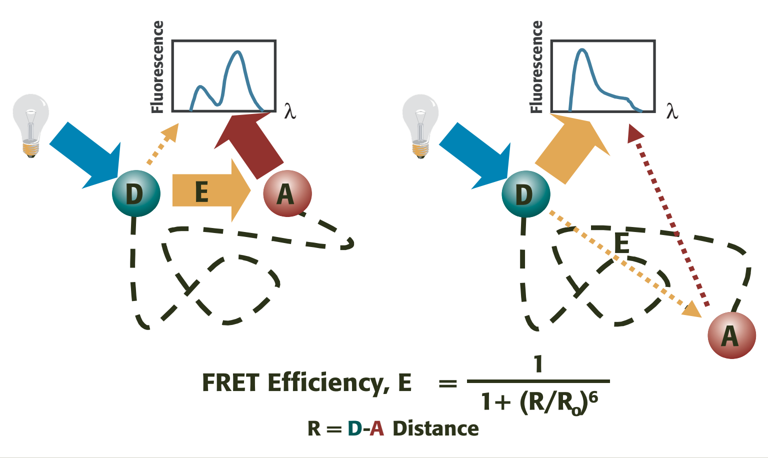

Figure 5. Förster resonance energy transfer (FRET), a "molecular ruler" for the 2–10 nm distance scale. Close proximity of the two probes results in high FRET and increased acceptor fluorescence (left); if the distances between the probes are large, then FRET is inefficient, and only the donor fluorescence is observed (right).

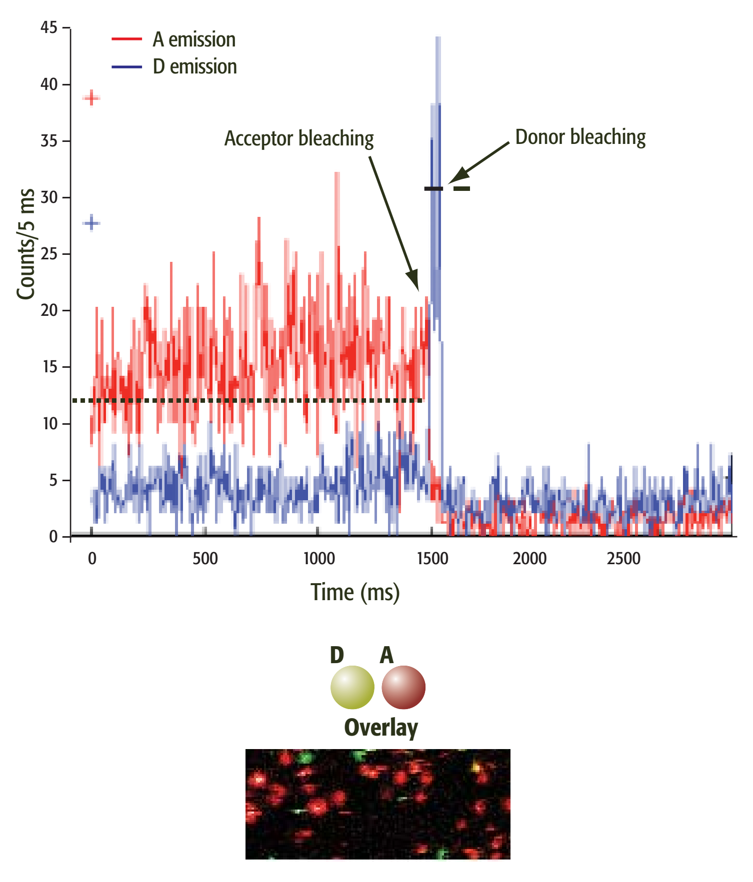

Figure 6. Analysis of single protein–DNA complexes. Overlaying the donor and acceptor emission signals allows the FRET analysis of individual protein–DNA complexes (bottom). Focusing on a single high-FRET complex (top) produces time trajectories of the donor- and acceptor-emission signals with millisecond resolution and duration from seconds to minutes, providing information about dynamics and interaction stoichiometries.

at a time (5, 6). This contrasts with conventional methods that draw their signals from ensembles of millions of molecules. The basic premise of implementing single-molecule detection in biomedical research is twofold: fine details in complex processes can be uncovered using single-molecule detection that normally would be masked by bulk (ensemble) measurements, and the ability to detect single molecules eliminates the need for amplifying the signal, such as using polymerase chain reaction (PCR) in DNA diagnostic applications. Combining confocal optics, a laser excitation source and point detection (provided by a SPCM), single molecules can be observed easily. Single molecules can be detected either in solution (allowing measurements of biological molecules and interactions in biological fluids) or on surfaces (allowing measurements of biological molecules and interactions on gene or protein arrays) (Figure 4).

In their study using single-molecule detection, UCLA researchers employed a custom confocal fluorescence microscope that combined high numerical-aperture (NA) optics with ultrasensitive detection (Figure 4A). The excitation sources were red and green lasers that allowed excitation of various fluorophores in the visible range. The lasers were coupled to fiber optics, reflected on a dichroic mirror and focused by a high NA objective in a temperature-controlled flow cell that held the sample. The sample can be either in solution, in a gel matrix or on a glass surface. Fluorescence emitted from the sample was collected through the objective and dichroic, spatially filtered through a pinhole, spectrally filtered through a dichroic mirror and spectral filters and, finally, focused on the active area of the APDs. The photon arrival time was recorded by an acquisition board, and the data was visualized and stored on a computer.

The researchers customized a microscope with four avalanche photodiodes that enabled them to monitor up to four spectral regions or the two polarization components of two spectral regions. This permitted experiments of high sophistication, including real-time analysis of fundamental biological questions — such as transcription of DNA, protein folding and protein aggregation — and the development of assays to monitor protein–DNA, protein–protein and protein–drug interactions in nanoliter volumes and at picomolar concentrations.

Monitoring structures and interactions required the use of a technique called Förster (or fluorescence) resonance energy transfer (FRET). FRET acts as a "molecular ruler" that enables measuring distances of from 2 to 10 nm within molecules and complexes; this method can best be explained with a pair of fluorescent probes with significant spectral overlap, a FRET donor (D) and a FRET acceptor (A) (Figure 5). After exciting D, a fraction of its energy is transferred to the acceptor through a dipole-to-dipole interaction between the two probes.

The efficiency of this transfer is a sensitive function of the distance between the two probes and can be used to evaluate distances. When the donor and acceptor are in close proximity, the FRET efficiency is high, translating into a low donor emission and high acceptor emission. Conversely, when the donor is far from the acceptor, the FRET efficiency is low, and therefore the donor emission is high and the acceptor emission is low.

FRET on Protein–DNA Complexes

UCLA researchers used the single-molecule detection and FRET techniques to analyze single protein–DNA complexes (7). They first determined that specific protein–DNA complexes are formed by showing high FRET between the donor and the acceptor. After surface immobilization, the sample was excited with a green laser, and donor and acceptor emissions were monitored using two PerkinElmer (Boston, Massachusetts, USA) SPCMs.

High donor emission (Figure 6) shows molecules that do not participate in FRET, while high acceptor emission shows molecules that do.

The overlay of the two emissions results in determining the FRET status of the molecule. This analysis helps reveal the presence of an interaction. It also shows the specificity of the interaction, the existence of sub-populations or free species and any structural features of the complex (by measuring D-A distances). Application of this method allows study of protein–protein interactions, detection of genes or genetic mutations that lead to disease, and sensing of low levels of protein molecules in biological samples.

This technique also enables researchers to focus on a single high-FRET complex and obtain second-to-minute timescale time-trajectories of donor and acceptor emission signals with millisecond resolution (Figure 6). This provides dynamic information about the system and allows real-time observation of enzymatic activity or movement of biomolecular machinery.

The stoichiometry of an interaction also can be determined by looking at the various signal levels during the time trace. For example, in a particular trace, a single level of A-emission signal can be seen, pointing to one acceptor molecule in the complex. By bleaching (photo-destroying) the acceptor, it will not absorb any energy from the donor, and FRET ceases to occur; this results in decrease of the acceptor emission and increase of the donor emission. The donor also bleaches in a single step, going from a high level (pointed by the arrow, Figure 6) to a background level. These observations would assign a 1:1 stoichiometry to this particular complex. This technology will enable the determination of enzymatic activity on surfaces to allow reactions pathways to be observed from beginning to end.

Approaching Real-time Molecular Diagnostics

The research conducted at LSU suggests that science is on the threshold of achieving real-time molecular diagnostics. Some diseases, such as cystic fibrosis, Alzheimer's disease, sickle cell anemia and certain cancers, are associated with changes in the sequence (mutations) of particular gene fragments. These changes can serve as biomarkers and could be useful for medical diagnosis at early stages of the disease.

Because the majority of mutations in genetic disorders are due to variations such as point mutations, insertions or deletions, it's essential that new diagnostic techniques have the capability to distinguish these changes in a mixed population, where in most cases the mutant allele is the minority. In addition, DNA diagnostic methods should be rapid, highly sensitive, cost-effective and easy to perform.

Current approaches used to detect single nucleotide variations (point mutations) include

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homogenous methods, such as the template directed dye terminator incorporation (TDI) assay (8), the 5′-nuclease allele specific hybridization TaqMan assay (9, 10), the allele-specific molecular beacon assay (11, 12) and ligase detection reaction (LDR) (13–15).

Diagnostic assays typically involve extraction of genomic DNA from either tissue, blood or stool samples, followed by PCR amplification of the gene fragment carrying the mutation and, finally, the discrimination reaction to identify the presence of the mutation in a mixed population (mutant and wild-type alleles). However, PCR has limitations that make it difficult to quantitatively analyze and detect small genetic variations due to nonlinearities in amplicon number with cycle number and reduced specificity. In addition, long optimization and set-up times, long run and analyses times, a high level of inherent inaccuracy and variation (due to cross-contamination) and a narrow dynamic range limit mutation screening assays incorporating PCR to performing quantitative measurements in real time.

In many cases, DNA diagnostic assays use FRET to distinguish normal from mutant DNA without requiring a separation step, which typically is incorporated into most heterogeneous assays. In general, these separation-based methods require pre-amplification of genomic DNA using PCR, followed by a discrimination reaction to identify the mutant allele, and a subsequent gel electrophoretic step to identify the results of the discrimination reactions, all of which require labor and significant amounts of time to obtain the results of the assay.

Single-molecule photon burst detection offers a unique opportunity to monitor the presence of sequence variations directly in genomic DNA without PCR amplification. In one such report, researchers developed a method for the rapid, direct detection of specific nucleic acid sequences in biological samples (16). In another report, specific DNA sequences in a homogeneous assay were detected using labeled hairpin-shaped oligonucleotide probes in combination with single molecule detection (17).

LSU's research reported on a rapid, potentially real-time mutation detection scheme capable of detecting low-abundant point mutations directly from unamplified genomic DNA samples (18). A ligase-based point mutation detection assay was used that consisted of an allele-specific discriminating primer and a common primer, each having a 10 base pair (bp) complementary arm with fluorescent labels at their 5′- and 3′-ends, respectively. These two labels acted as the donor–acceptor pair for a FRET-based signal that was produced only when the mutation was present. A perfect match between the base at the 3′-end of the discriminating primer and the target allowed the DNA ligase to covalently join the two adjacent primers flanking the mutation site. The LDR product then undergoes a conformational change to form a hairpin structure (reverse molecule beacon, rMB).

Single-molecule photon burst detection offers a unique opportunity to monitor the presence of sequence variations directly in genomic DNA without PCR amplification.

The rMB is formed by the complementary arm sequences of the ligated primers. The two probes attached at the end of the arms are brought into close proximity by the hybridization and, thus, energy transfer occurs between the pair of probes. Fluorescence emission resulting from the single-pair FRET (spFRET) process can be detected in real time using single molecule detection.

Real-time spFRET measurements were performed in a poly-methylmethacrylate (PMMA) microfluidic device to detect the rMBs (perfectly matched LDR products) formed in an LDR assay, where point mutations in K-ras codon 12 (highly associated with colorectal cancer) were detected in unamplified genomic DNA samples. The fluorescence readout hardware consisted of a simple red diode laser (630 nm) for excitation and an SPCM. SPCMs are particularly attractive in this case because they provide low dark-count rates and high single-photon detection efficiencies in the deep red region of the electromagnetic spectrum. Implementation of fluorescence readout in this region minimizes interferences originating from the sample matrix due to auto-fluorescence.

LSU researchers demonstrated the assay's capability to rapidly detect single point mutations in genomic samples at a sensitivity of 1:1000 (mutant to wild-type) without PCR amplification. Analysis times of less than 5 min were achieved using real-time LDR-spFRET detection in the microfluidic device.

The exquisite sensitivity of single-molecule detection can be used to eliminate processing steps required in multi-step assays, thereby reducing analysis time. For example, LSU researchers eliminated the need for PCR amplification and performed an allele-specific ligation assay directly on genomic DNA. In addition, the high sensitivity afforded by single-molecule measurements also eliminated the need for thermal cycling during the ligation step. The specificity of the ligase enzyme toward mismatches coupled to spFRET of rMBs formed as a result of a successful ligation reaction for mutated DNA — even in the presence of wild-type sequences — provided real-time molecular diagnostic screening even when the copy number of target sequences was low (600 copies).

Summary

Applications abound for simultaneously monitoring different aspects of a single photon, making the four-channel SPCM a valuable new option for researchers. It provides the same technology with four times the capability in a single module while lowering the size, power requirements and cost. Plug-and-play systems also are available for easy integration and rack-mounting.

Research applications — particle sizing, imaging, adaptive optics, molecular coding and more — are making use of multiple-channel SPCMs in many fields of study. For example, fiber arrays are using these modules to study the movement and speed of molecules. The technique provides a position-sensing device at a molecular level, and multiple channels enable the simultaneous measurement of speed and position of a molecule as it travels through a medium.

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