# Faster than Cells Die

SINGLE PHOTON COUNTERS
FOR FLUORESCENCE SPECTROSCOPY.

Until recently, single molecule fluorescence spectroscopy required photomultiplier tubes (PMTs) or microchannel plate (MCP) detectors. Avalanche Photodiodes (APDs) were not used because of their poor timing resolution. Manufacturing the Silicon APD in a CMOS process, and thus integrating the active quenching electronics with the diode on the same chip, timing resolution and counting rate can be improved significantly.
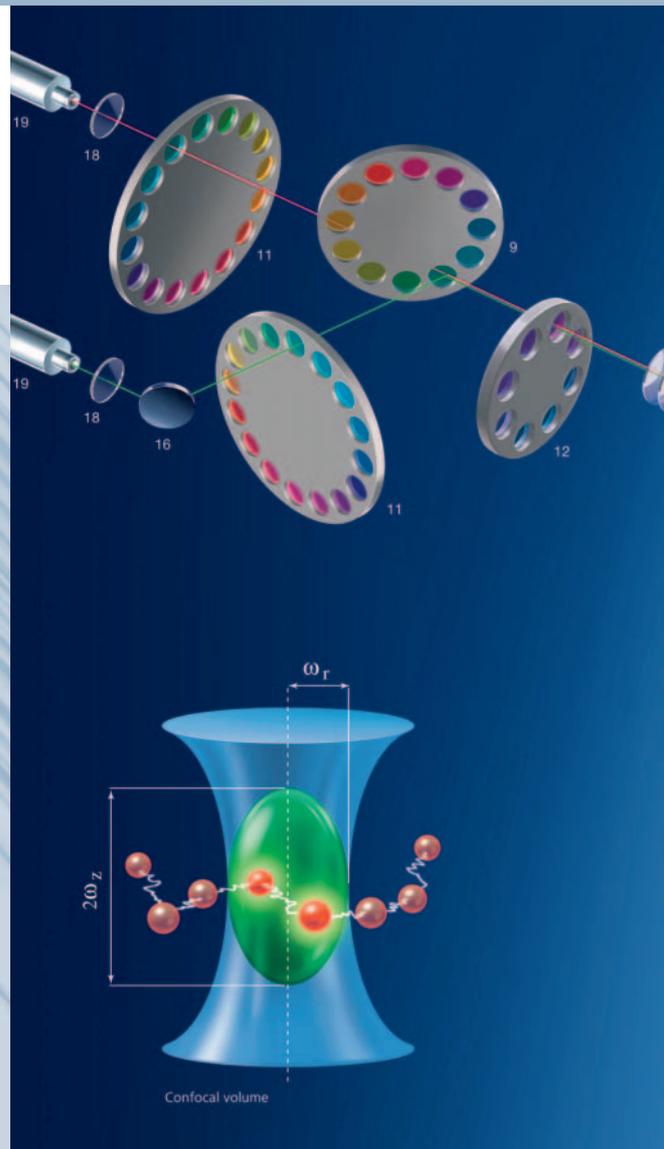
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Single molecule studies with fluorescence spectroscopy often utilize confocal microscopes (**figure 1**). These microscopes eliminate out-of-focus light and allow for a very good spatial resolution. To obtain a complete image, the laser beam scans the sample. Reducing the laser power helps avoid cell damage. In a confocal microscope, the specimen volume is small and in a time period, only a few single fluorescent photons are emitted. Therefore, in order to measure the electron's lifetime in the excited state, single photon detectors are required. Because both high detection probability and timing resolution are needed, only PMTs, MCPs or silicon-based APDs serve as convenient single photon detectors.

PMTs consist of a glass vacuum tube housing a photocath-

Figure 2. Single photon detector module: id100

ode, several dynodes, and an anode. Incidentally emitted fluorescence photons strike the photocathode material, which is a thin deposit on the entry window of the device, with electrons being produced due to the photoelectric effect. An electrode focuses these electrons towards the electron multiplier, where they are multiplied by means of secondary emission. The electron multiplier consists of a number of electrodes, called dynodes. Each dynode is held at a more positive voltage than the previous one. The electrons leave the photocathode, having the energy of the incoming photon. As they move towards the first dynode, they are accelerated by the electric field and they arrive with much higher energy. The geometry of the dynode chain is such that a cascade occurs with an ever-increasing number of
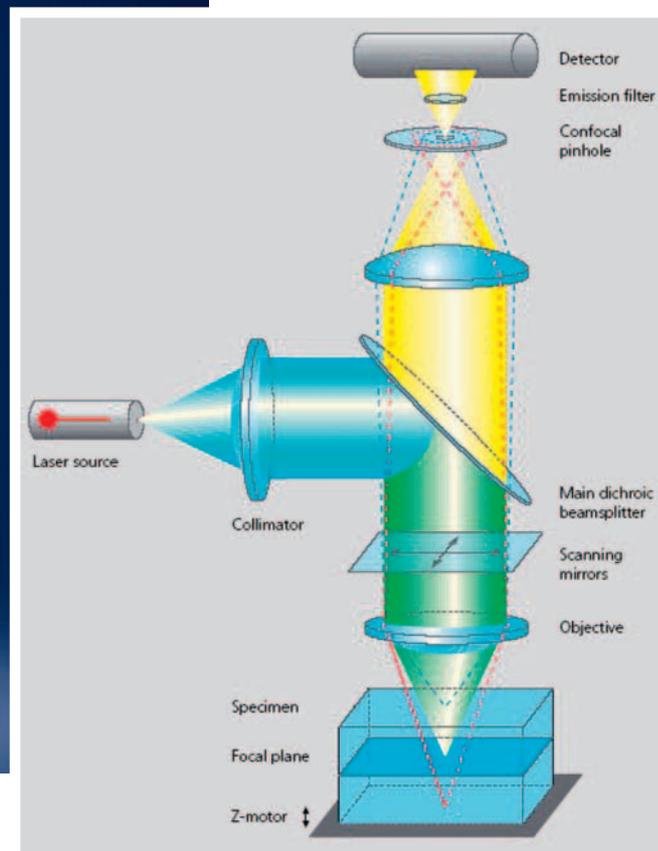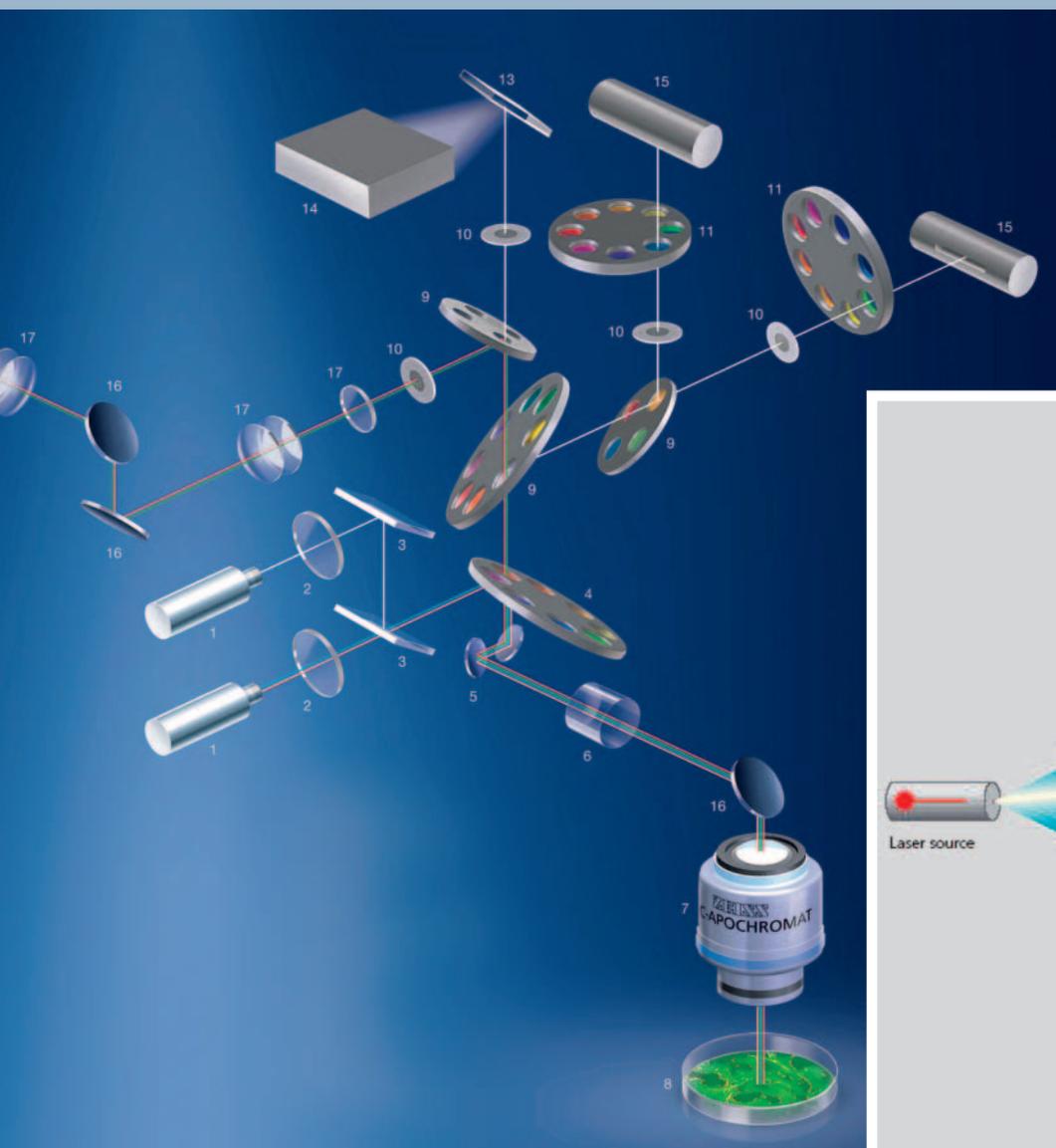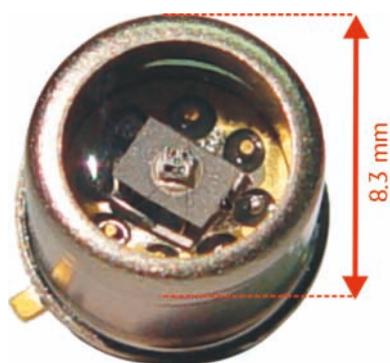
Figure 1. Optical principle of a laser scanning confocal microscope for fluorescence spectroscopy (Carl Zeiss MicroImaging, Jena)

Figure 3. The single photon detector id101 can be mounted on a custom printed circuit board

electrons being produced at each stage. Finally, when reaching the anode, the charge accumulation results in a sharp current impulse indicating the arrival of a photon at the photocathode.

MCPs consist of a photocathode, microchannel plates and an anode located in vaccum devices. The microchannel plates contain a large number of microscopically small channels with a typical diameter ranging from 3 to 20 µm. Due to the photoelectric effect, an incidentally emitted photon produces an electron. An ion entering one of the channels through a small orifice will hit the wall of the channel because the channel is tilted to the plate by the angle of impact. The impact starts a

cascade of electrons that propagates through the channel, which amplifies the original signal by several orders of magnitude depending on the electric field strength and the geometry of the microchannel plate. This electron shower generates a current impulse on the anode.

Electron avalanche in Geiger mode

Silicon based avalanche photodiodes (Si-APDs) are made of semiconductor junctions operated under a reverse bias voltage. Due to their band structure and energy gap, they play a key role in converting electrical into optical energy and vice versa. A photon absorbed by the semiconductor generates an electron hole pair if the energy equals the energy of the band gap: Energy (E) = Planck's constant (h) x frequency (v). With a certain field geometry (voltage, bias potential) in the APD, we obtain an avalanche multiplication of the generated carriers.
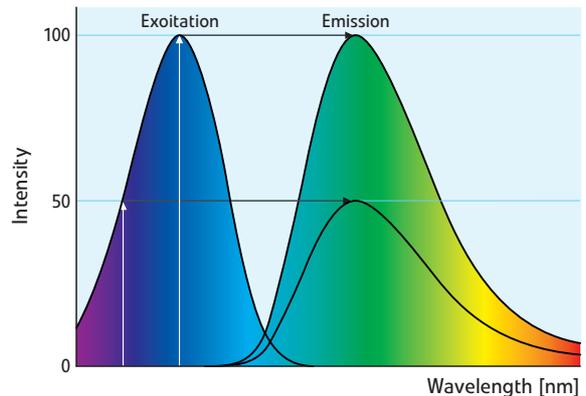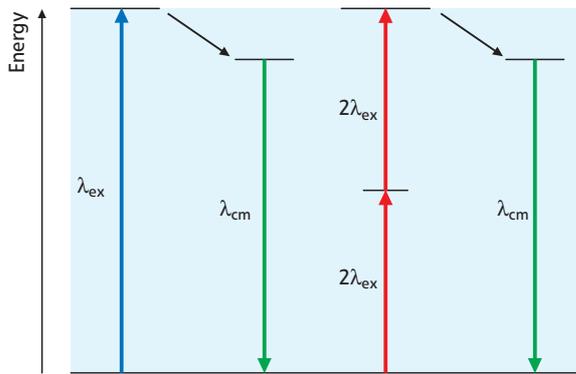
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Figure 4a. Left: one-photon excitation, right: two-photon excitation (λ_{ex} = excitation's wavelength and λ_{em} emission's wavelength); b. shift of approximately 30 nm from the excitation wavelength to the emission wavelength (Carl Zeiss MicroImaging, Jena)
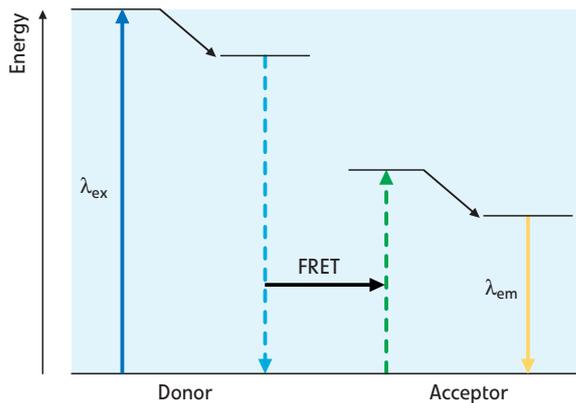
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Figure 5. Distances on the nanometer scale can be determined by measuring the FRET efficiency quantitatively (Carl Zeiss Micro-Imaging, Jena)

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When the bias voltage on the APD is below the breakdown level, this state is also called linear mode or analogue mode, the output signal follows the light intensity. In this mode, the sensitivity is too low for single photon detection. When the bias voltage exceeds the breakdown level, the APD operates in the so-called Geiger mode. In this case, the absorbed photon generates an avalanche process resulting in a huge increase of the output current. In the Geiger mode, single photons can be detected. The output signal is independent from the generated number of photoelectrons. To quench the avalanche, the bias voltage needs to be reduced below breakdown. Afterwards, the bias voltage is restored above breakdown in order to return to a sensitive state.

High counting–rate and timing resolution

To obtain the whole image, the probe is scanned point by point, and line by line. Since biological samples change with time, they need to be scanned as fast as possible. The detector's maximum counting rate as well as the temporal resolution are the key parameters.

Latest APD developments are based on the CMOS (Complementary Metal Oxide on Semiconductor) technology used in the semiconductor industry. Researchers from the EPFL (Ecole Polytechnique Fédérale de Lausanne, Switzerland) were able to demonstrate a timing resolution of only 40 ps using a commercial CMOS process to manufacture Si-APDs. Improving the timing resolution, integrating electronics with the APD on the same chip as well as the possibility of creating a matrix are the advantages of the CMOS process in APD production.

Enhancing this technology, id Quantique created the ›id100‹ family (**figure 2**) and recently an OEM product. The id100 detector consists of a Si-APD and an active quenching circuit on the same chip. This chip is mounted on a thermo-electric cooler and packed in a standard TO5 header. A thermistor is used to measure the temperature. All internal settings are preset for an optimal operation at room temperature.

The OEM version ›id101‹ (**figure 3**) can be mounted onto a printed circuit board. At present, it is the smallest photon counter on the marketplace. Typical characteristics of the id100 or the id101 APD are a timing resolution of 40 to 60 ps, wavelengths ranging from 350 to 900 nm and a quantum efficiency of 35 percent at 532 nm. These values are achieved with a low dark count rate, a short dead time of 45 ns and a high repetition rate of up to 20 MHz.

Single photon detection for FLIM, TIRF, FRET and TCSPC

In **fluorescence lifetime imaging (FLIM)**, ion sensitive fluorophores are used to measure intracellular ion concentrations. Measurements benefit from the fact that the fluorescence life time (time spent in the excited state) changes with ion concentration. The excited electron returns after a certain time in the ground energy state and emits a fluorescence photon. A time-resolved analysis of the fluorescence (**figure 4 a, b**) determines the absolute ion concentrations.
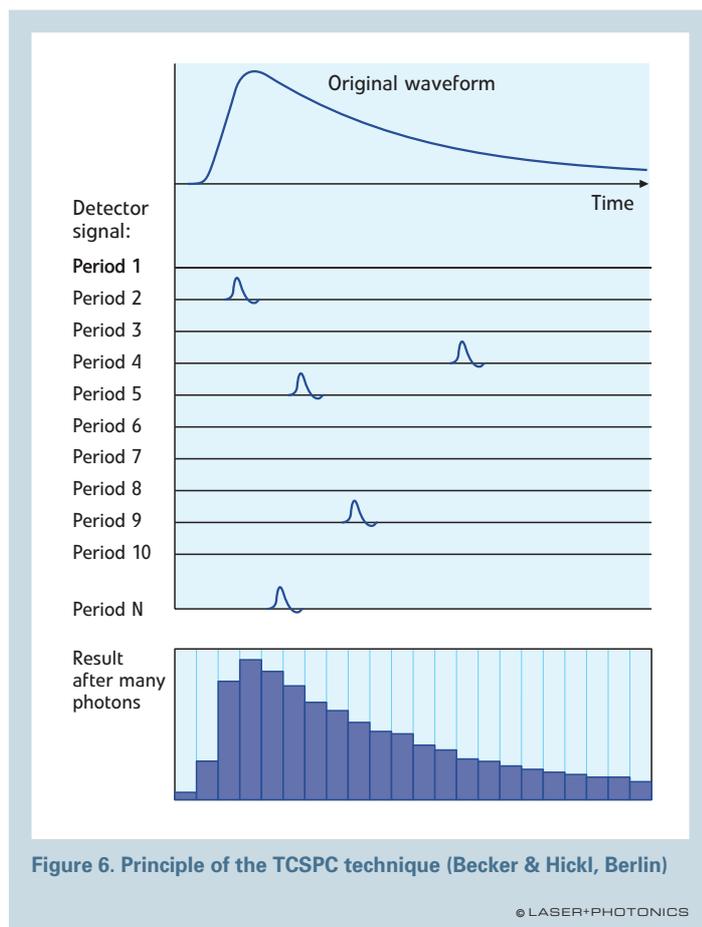
Figure 6. Principle of the TCSPC technique (Becker & Hickl, Berlin)

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is detected, the time of the corresponding detector pulse is measured. The events are collected in a memory by adding a 1 in a memory location with an address proportional to the detection time. After processing many photons, the histogram of the detection times, i.e. the waveform of the optical pulse appears (**figure 6**). TCSPC is often used in single molecule and fluorescence spectroscopy.

Summary

Id Quantique's single photon counter detects weak optical signals, even single photons in the VIS spectrum. Resolutions of 40 ps at a dead time of 45 ns are reached. As the Si-APD is produced in a CMOS process, quenching electronics and the diode are integrated on the same chip. This results in a very stable response function, and a counting rate of up to 20 MHz. Such photon detectors are appropriate for fluorescence spectroscopy and TCSPC. CMOS compatibility enhances its cost effectiveness. ∎

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Total internal reflection fluorescence (TIRF) monitors processes close to cellular surfaces, which are difficult to visualize using conventional techniques. TIRF technology allows only the excitation of the fluorophore located in a thin layer close to the surface. The incident angle of the incoming light causes a total internal reflection. Thus, only molecules located approximately 100 to 200 nm above the cover glass become excited.

The **fluorescence resonance energy transfer (FRET)** is also known as the Förster resonance energy transfer method and helps determine distances between two bio-molecules. The molecules of interest are linked with two different fluorophores (donor and acceptor). The energy of an excited fluorophore (donor) is passed radiation-free towards the second fluorophore (acceptor). Detecting the emitted photons from the acceptor allows to derive information on the distance of the two bio-molecules. The FRET technology is also known as optical nanometer measure (**figure 5**).

Time-Correlated Single Photon Counting (TCSPC) is based on detecting single photons during a periodical light signal, on measuring the detection times of the individual photons and on the reconstruction. The method utilizes the fact that for low level, high repetition rate signals, the light intensity is usually so low that the probability of detecting one photon in one signal period is much less than 1. Therefore, the detection of several photons can be neglected. If a photon