"In the future, as fluorophores with increasingly shorter lifetimes are developed, the <500 picosecond gating capabilities and high QE of the PI-MAX4:1024i ICCD camera will continue to enable scientists to generate very accurate fluorescence decay profiles using the RLD method."

Novel Time-Resolved FLIM Measurements Method

Enabled by the New Picosecond Gating Technology of the PI-MAX®4 ICCD Camera and the RLD Processing Algorithm

Introduction

For years, fluorophore lifetimes were typically tens of nanoseconds and longer. Therefore, intensified CCD (ICCD) cameras with commercially available slow-gate and fast-gate image intensifiers capable of providing gate widths on the order of nanoseconds were sufficient for measuring and observing such phenomena effectively. Tens-of-nanoseconds gating allowed scientists to obtain several data samples within the fluorophore lifetime and then generate an accurate fluorescence decay profile via a series of images using these ICCD cameras.

The advent of fluorophores with lifetimes of only a few nanoseconds, however, meant that the existing ICCD camera technology was simply not fast enough to capture all of the data samples needed to perform time-resolved fluorescence lifetime imaging microscopy (TRFLIM). As a result, ICCD cameras that can deliver gate widths of a few hundred picoseconds were developed. Although these specialized systems are generally effective for measuring fluorophore lifetimes of a few nanoseconds they are not without several drawbacks of their own, including significantly higher cost as well as lower sensitivity.

In 1996, a method that lets researchers utilize nanosecond-gated ICCD cameras to observe and measure shorter decay times was published[1]. Subsequently, a TRFLIM-specific rapid lifetime determination (RLD) algorithm has been refined through the years[2,3] that continues to allow nanosecond-gated ICCD cameras to be used with some of today's short-lifetime fluorophores, though not all of them.

Rather than recording a complete multipoint decay curve and analyzing the decay by traditional least-squares methods, in the RLD approach the areas under different regions of the decay are used to calculate the decay parameters[2]. The areas over different time intervals of width are obtained by accumulating photon intensity (e.g., using a gated ICCD camera)[2].

Figure 1.
The PI-MAX4:1024i ICCD camera utilizes a conventional image intensifier fiberoptically bonded to an interline-transfer CCD and runs at near video rates (26 frames per second).

ICCD cameras are well suited to the RLD method since they automatically integrate the data over different time intervals[2]. Not only is RLD much faster than traditional approaches for recording and analyzing decays, it does not have problems such as false minimums[2].

New Way to Measure TRFLIM

While it is true that the latest versions of the specialty ICCD camera systems mentioned earlier can achieve ~200 picosecond gating, such systems use a nonstandard intensifier with a nickel-coated underlayer that compromises quantum efficiency (QE). Because of this, bleaching and phototoxicity from the TRFLIM experiment's laser is a huge problem.

By utilizing a Princeton Instruments PI-MAX4:1024i ICCD camera (see Figure 1) in concert with the RLD algorithm, however, researchers can now obtain much better, more accurate decay profiles for fluorophores with lifetimes of <10 nanoseconds — without the extremely high price tag of other picosecond gating cameras.

The PI-MAX4:1024i camera's ability to provide overlap timing in a sequential imaging mode using 64-bit LightField® software (a Microsoft® Windows®-based package from Princeton Instruments) lets scientists collect the data required for the RLD processing algorithm with ease in order to measure these shorter fluorophore lifetimes.

Figure 2 shows the TRFLIM system setup currently being used by Dr. Ammasi Periasamy (W.M. Keck Center for Cellular Imaging, University of Virginia). It relies on the RLD processing algorithm as well as a PI-MAX4:1024i ICCD camera that features a built-in timing generator with 10 picosecond temporal resolution for external synchronization (i.e., no additional hardware).

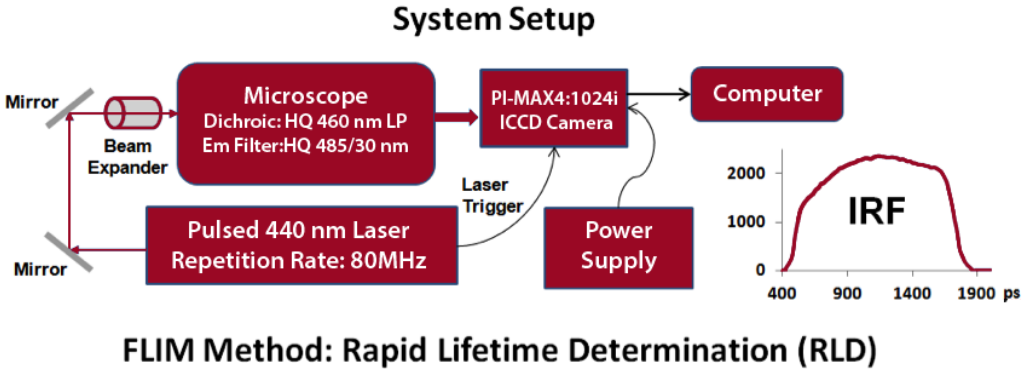

Figure 2.
The system setup for RLD-facilitated TRFLIM employs a pulsed laser and an optical microscope, as well as a gated ICCD camera from Princeton Instruments. (Diagram courtesy of Dr. Ammasi Periasamy, W.M. Keck Center for Cellular Imaging, University of Virginia.)

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Results

The RLD method has been used with ICCD camera gating schemes for single-exponential and double-exponential problems[2]. A scheme utilizing two overlapping gates has been shown to determine single-component lifetime very accurately, even when little is known about the system under study[2]. Recently acquired data for various single-exponential experiments are presented in Figure 3.

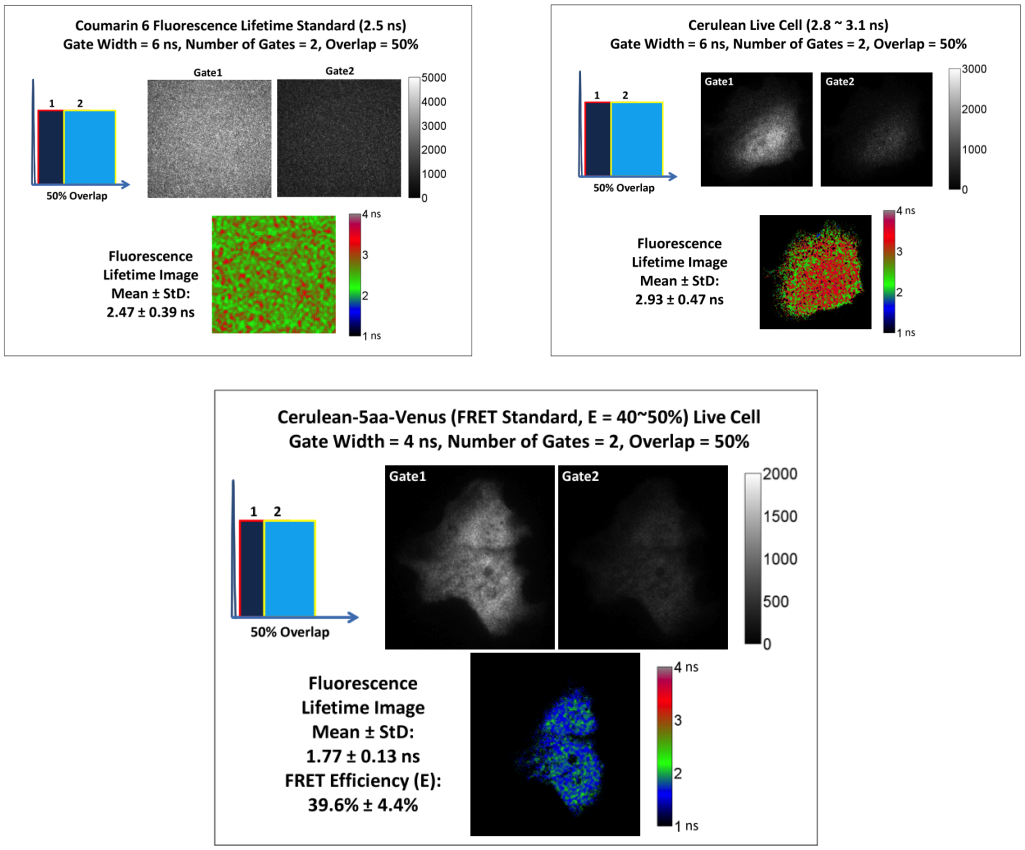

Figure 3.
Single-component analysis via the RLD method for TRFLIM uses a two-gate scheme. The results are seen to be highly accurate when 50% overlap of the gates is employed, whether or not the lifetime of the fluorophore is known. (Data courtesy of Dr. Ammasi Periasamy, W.M. Keck Center for Cellular Imaging, University of Virginia.)

Notice in Figure 3 that when the single-component lifetime is not known (i.e., the cerulean live-cell experiment), using a 50% overlap with a gate width setting more than twice as large as the lifetime still yields very accurate results. In this way, current fluorophore lifetimes of 2 to 5 nanoseconds are easily measurable using a pair of 2 to 3 nanosecond gate widths.

To determine two-component lifetimes with the RLD method, two- and four-gate schemes have been applied for studies in which the two pre-exponential factors are unknown and the two lifetimes are either known or unknown, respectively[2]. Recently acquired data for two double-exponential experiments are presented in Figure 4.

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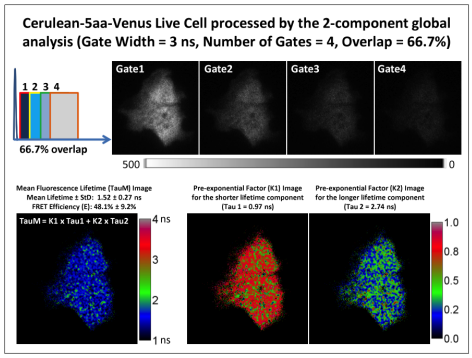
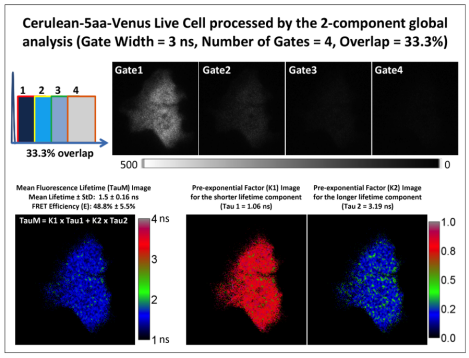
Figure 4.
Two-component analysis via the RLD method for TRFLIM uses a four-gate scheme when the two lifetimes are unknown. The experiments on the left and the right each utilized four 3 nanosecond gate widths, with 66.7% and 33.3% overlap, respectively, to obtain highly accurate results. (Data courtesy of Dr. Ammasi Periasamy, W.M. Keck Center for Cellular Imaging, University of Virginia.)





Breakthrough: Picosecond Gating Technology

Princeton Instruments ICCD cameras have long been the industry standard for time-resolved scientific imaging and spectroscopy applications. Recently, Princeton Instruments introduced a new picosecond gating technology exclusive to the world-renowned PI-MAX4 ICCD camera platform that combines the cost and sensitivity benefits of the conventional image intensifiers employed in traditional ICCD cameras with the ability to deliver <500 picosecond temporal resolution.

By utilizing state-of-the-art electronics and fiberoptically bonding the intensifier to the CCD sensor, this new picosecond gating technology enables PI-MAX4 cameras to gate conventional image intensifiers (which normally achieve ~2 to 3 nanosecond gating) at <500 picoseconds without sacrificing QE.

In the future, as fluorophores with increasingly shorter lifetimes are developed, the <500 picosecond gating capabilities and high QE of the PI-MAX4:1024i ICCD camera will continue to enable scientists to generate very accurate fluorescence decay profiles using the RLD method.

The PI-MAX4 camera series features an oscilloscope-like LightField user interface to deliver complete experiment control. This 64-bit software also features a built-in, fully calibrated, high-precision timing generator with 10 picosecond temporal resolution for external synchronization. Furthermore, PI-MAX4 cameras can be operated remotely via a Gigabit Ethernet (GigE) data interface.

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Summary

The Princeton Instruments PI-MAX4* family, the new benchmark for ICCD camera performance, combines the advantages of picosecond gating with the high QE of conventional image intensifiers fiberoptically coupled to scientific-grade sensors. These cameras are well suited for TRFLIM and numerous other time-resolved imaging and spectroscopy applications.

Given the increasing reliance on TRFLIM for various biological and clinical investigations, including calcium concentration, protein-protein interaction, cell locomotion, cancer research, and Alzheimer's disease research, the joint utilization of new picosecond gating technology and the RLD processing algorithm can be of great benefit to researchers.

Resources

For more information about the Princeton Instruments PI-MAX4 family of cameras, please visit: **http://www.pi-max4.com**

To learn about the RLD processing algorithm, please visit this URL (as well as the references listed below): **http://www.kcci.virginia.edu/Facilities/flim/index.php**

References

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2. Sharman, K.K.; Periasamy, A.; Ashworth, H.; Demas, J.N.; Snow, N.H. *Anal. Chem.* **1999,** 71, 947-952.

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4. Periasamy, A.; Clegg, R.M. *FLIM Microscopy in Biology and Medicine* **2010,** 93-114, CRC Press.

**Please note that all PI-MAX4 cameras with a Gen III image intensifier or an EMCCD require an end-user statement and licensing process for shipments outside the United States.*

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