

*“The frequency-domain homodyne method enables a gain-modulated image intensifier and CCD detector to be utilized to obtain the lifetime information of every location on a sample simultaneously.”*

## Wide-Field, Frequency-Domain FLIM Measurements Made Simple

*via the Use of an Advanced, Fully Integrated ICCD Camera with RF Modulation*

### Introduction

Fluorescence lifetime imaging microscopy, or FLIM, encompasses several techniques for mapping the spatial distribution of excited-state lifetimes (i.e., fluorescence decay times) of emitting molecular species with nanosecond and microsecond temporal resolution. Images produced using fluorescence microscopy via FLIM, therefore, are based on the acquisition of decay-time data.

Regardless of the specific FLIM method used, the differences in the exponential decay time of a species' fluorescence — associated with the fluorophore's molecular environment as opposed to its concentration — are employed to produce each quantitative image. FLIM effectively complements the fluorescence spectroscopy techniques utilized to create 'conventional' fluorescence images and provides a different contrast mechanism to help identify the local environment of the fluorophore.

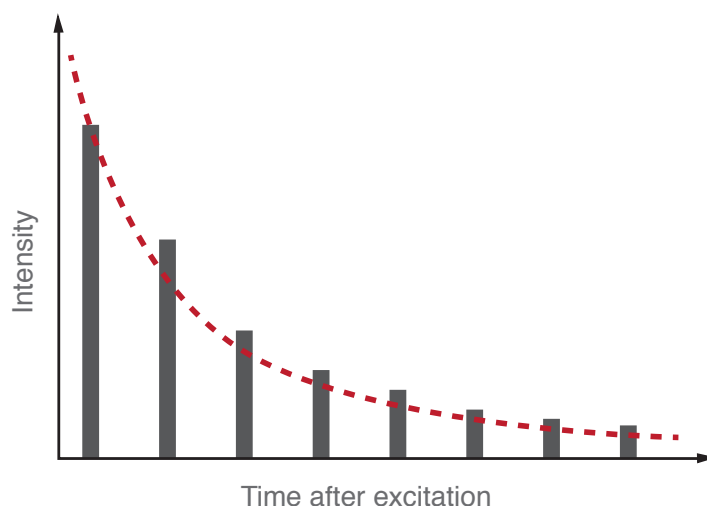
FLIM techniques have great utility in cell biology and are especially useful for studies focusing on the metabolic states of cells and tissues, such as determination of ion concentration (i.e., ion imaging) or oxygen concentration (i.e., oxygen imaging). FLIM is also utilized for quantitation of Förster resonance energy transfer (FRET) that enables distance measurements on the nanometer scale.

There are two primary implementations of FLIM: (1) time-domain FLIM, which uses a pulsed light source in conjunction with either time-gated (i.e., wide-field) or time-correlated (i.e., single-point) detection, and (2) frequency-domain FLIM, which utilizes a sinusoidally modulated light source with either a sinusoidally modulated, intensified CCD detector to achieve wide-field imaging or a modulated single-element detector to perform scanning or single-point detection.

- (1) In the 'time-domain FLIM with time-gated (wide-field)' approach, a short-pulse (i.e., relative to the fluorescence lifetime, psec-to-nsec duration) light source, such as a laser, is typically used for excitation. An intensified CCD camera capable of programming multiple time windows with different delays in respect to the excitation pulse is utilized for detection. The decay time is derived by fitting an exponential curve to the decay-time data, as shown in Figure 1.

**Figure 1.**

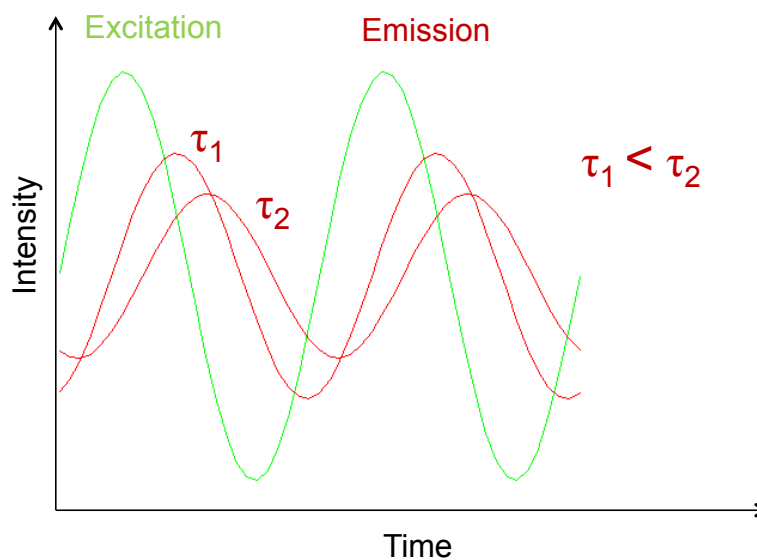
Time-domain FLIM. Fluorescence exponential decay curve.



- (2) In the 'wide-field, frequency-domain FLIM' approach there are two methods: (1) the homodyne method, in which the intensity of the excitation light source and detector are both modulated sinusoidally (or with any other repetitive shape) at the same radio frequency, and (2) the heterodyne method, in which the intensity of the excitation light source and detector are modulated sinusoidally (or with any other repetitive shape) at different radio frequencies. The modulation frequency in both methods is normally between 1 and 200 MHz. Due to the delay between the excitation and emission — directly related to the fluorescence lifetime of the fluorophore — the fluorescence emission is demodulated and phase shifted in respect to the excitation light<sup>1</sup>. See Figure 2.

**Figure 2.**

Frequency-domain FLIM. The longer the fluorescence lifetime, the stronger the phase shift and the higher the demodulation. Illustration courtesy of Annette Buntz (Zumbusch Group, University of Konstanz, Germany).



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## Frequency-Domain FLIM

Whereas the time-domain approach to FLIM has advantages in the case of a long-lived component and is simple to understand, the frequency-domain approach can be very sensitive for small lifetime differences and in principle makes it easier to study rapidly decaying compounds<sup>1</sup>. This application note focuses on the homodyne frequency-domain FLIM method. We discuss an advanced method for performing time-domain FLIM elsewhere<sup>2</sup>.

To perform wide-field, homodyne frequency-domain FLIM measurements, radio frequencies between 1 and 200 MHz are utilized so that the fluorescence response will be sensitive to the frequency of modulation<sup>3</sup>. The waveform of this modulation can be any repetitive shape<sup>3</sup>.

A gain-modulated image intensifier and CCD detector are used for this wide-field (i.e., all locations of the sample are imaged simultaneously)<sup>3</sup> FLIM technique. After exciting the sample with a particular frequency of interest, both the amplitude difference and phase delay between the excitation and emission — related to the fluorescence lifetime of the fluorophore — are recorded. The series of resultant phase-shifted images can then be analyzed via software, such as SimFCS (Globals for Images, Laboratory for Fluorescence Dynamics, University of California, Irvine; developed by Prof. Enrico Gratton).

## Experimental Setup and Results

Unlike time-domain FLIM, the frequency-domain approach does not require the use of pulsed lasers, which are often quite expensive and deliver a high dose of energy that also risks the photobleaching of samples. Furthermore, the optimized duty cycle of the latest instruments available for frequency-domain homodyne FLIM facilitates rapid data collection (i.e., up to ~30 images per second). Efficient light management further minimizes photobleaching of samples.

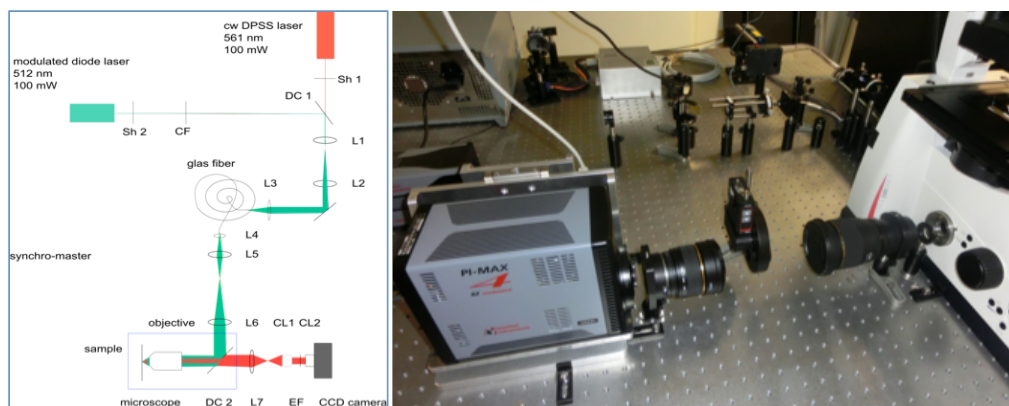
The diagram and photo in Figure 3 show an experimental setup for performing wide-field, frequency-domain FLIM measurements using a single frequency (i.e., the homodyne method). The main components of the FLIM setup are (1) an epi-illumination inverse microscope, (2) an Omicron LDM515.100.A350 diode laser with 512 nm, 100 mW peak power and analog modulation up to 350 MHz utilized as an excitation source, and (3) a Princeton Instruments PI-MAX®4:1024i ICCD camera (1024 x 1024 pixel array; pixel size 12.8 x 12.8  $\mu\text{m}$ ) equipped with a Gen III image intensifier and integrated RF modulation capability (1 – 200 MHz) used for signal detection.

The laser light is passed through a clean-up filter, expanded with a telescope consisting of two plano-convex lenses, and coupled into a multimode fiber by an aspheric converging lens. To reduce laser speckle, the optical fiber is vibrated. From the end of the fiber, the light is coupled into the microscope, reflected by a dichroic mirror, and focused onto the sample with an objective lens. The fluorescence is collected by the same objective and passed through the dichroic mirror. The intermediate image at the tube lens is magnified by a factor of 4.2 with two camera lenses. The fluorescence light is filtered by an emission filter and directed to the ICCD camera.

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### Figure 3.

A typical experimental setup for frequency-domain homodyne FLIM uses a modulated image intensifier and CCD detector. Diagram and photo courtesy of Annette Buntz (Zumbusch Group, University of Konstanz, Germany).



Annette Buntz, MSc, from the research group of Prof. Dr Andreas Zumbusch, the head of the chemistry department at the University of Konstanz in Germany, utilizes this ICCD camera-based setup for her work. The goal is to perform time-lapse lifetime imaging in living cells. As a first step towards achieving this goal, fixed samples (Figure 4) — co-transfected HeLa cells with CC3 fused to YPet and Grb14 fused to mCherry — were used. The cells were then infected with stained bacteria and fixed 30 minutes after infection. In the confocal images presented in Figure 4, it can be observed that Grb14 is recruited to the clustered CC3 receptor. These are the Phase and Modulation Lifetime images. The acquisition time for these images was 6 seconds.

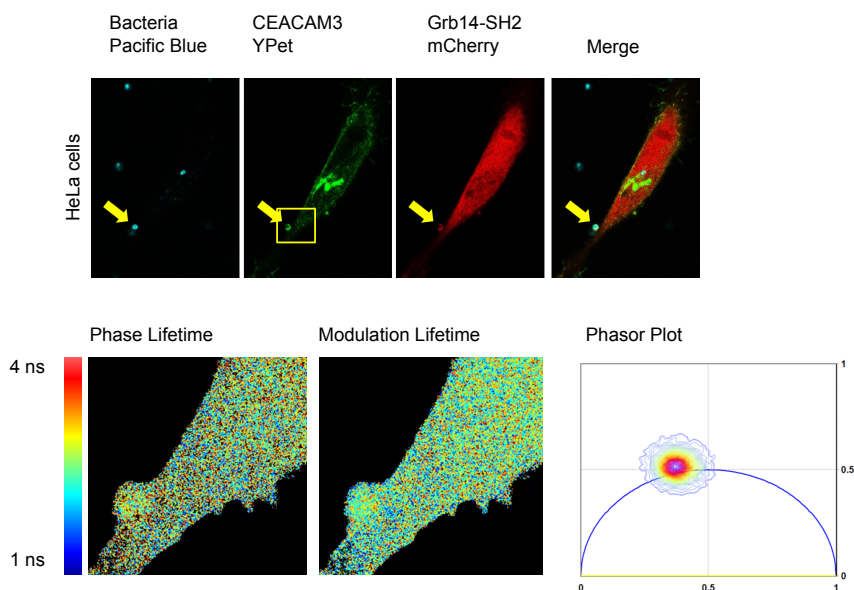
Most important, Figure 4 demonstrates the implementation of the Phasor Plot (developed by Hanley, Clayton, Redford, and Clegg in 2005) to display and analyze wide-field, frequency-domain FLIM data. Each point in the Phasor Plot represents a pixel in the image. Its x-coordinate is calculated by multiplying the modulation with the cosine of the phase shift; its y-coordinate is calculated by multiplying the modulation with the sine of the phase shift.

The Phasor Plot in Figure 4 highlights that there is just a single lifetime component (i.e., the points lie on a semicircle, with decreasing lifetime from left to right), which is also reflected by the fact that the values for the Phase and Modulation Lifetimes are the same. Note that if there were two lifetime components, the points in the Phasor Plot would have been on a line between the position of the two components — and the fraction of the two components could then be obtained from the position on the line.

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## Figure 4.

FLIM in a cellular context. The Phasor Plot reveals a single lifetime component. Image acquisition parameters: 12 sequential phase steps. Gain: 90. Modulation duration: 500 ms. Exposures per frame: 1. Binning: 2 x 2. ADC: 16 MHz. Bleaching during one acquisition sequence: 2%. Data courtesy of Annette Buntz (Zumbusch Group, University of Konstanz, Germany).



## Figure 5.

The new PI-MAX4:1024i-RF camera from Princeton Instruments features an image intensifier fiberoptically bonded to a CCD detector, along with built-in modulation circuitry and an internal high-voltage power supply.

## Advanced Instrumentation

Recently, Princeton Instruments introduced the PI-MAX4:1024i-RF (see Figure 5), a state-of-the-art ICCD camera that enables frequency-domain measurements for FLIM, as well as time-resolved FLIM measurements, with minimal external equipment. By modulating the gain of its fiberoptically coupled, high-sensitivity Gen III filmless intensifier at a radio frequency (RF) rate using the camera system's own advanced internal electronics, the PI-MAX4:1024i-RF operates as a 2D lock-in amplifier. Each pixel of the CCD acts as an individual phase-sensitive (lock-in) detector. The camera system also allows advanced programming of phase sequence in random steps.

This new ICCD camera features a pair of independent, built-in, direct digital synthesizers. One synthesizer generates the RF to modulate the intensifier (1–200 MHz), while the other provides a user-controlled RF signal that can be utilized to modulate the illumination in order to accomplish RF phase-sensitive detection. An advanced graphical user interface (GUI) permits users to select modulation frequency, control phase sweep range and granularity (in 1 degree steps, up to 360 degrees), and set RF output p-p voltage levels.

## Summary

Fluorescence lifetime imaging microscopy techniques deliver quantitative, aberration-free, and concentration-independent measurements that let researchers probe molecular environments and add specificity to their observations. Either individual or multiple fluorophore species' lifetimes can be discerned via a number of FLIM methods.

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## Fluorescence Microscopy Fundamentals

Fluorescence refers to the ability of an atom or molecule to absorb light and, in turn, emit at higher wavelengths. Unlike a conventional optical microscope, a fluorescence microscope requires the use of a high-intensity light source (often one that is practically monochromatic) in order to excite a fluorescent species in the sample being investigated.

Because slight variations in the surrounding environment can cause large changes in fluorescence properties, reporter molecules are used as sensitive probes. At the microscopic level, these fluorophores are linked to cellular biochemistry. Illumination and detection at the proper wavelengths can therefore reveal variations in pH, ion concentration, nucleic acids, and proteins. Resultant images appear 'bright' on a dark background. To better observe a sample in all three spatial dimensions, a confocal fluorescence microscope may be used.

It is important to note that light levels resulting from fluorescent molecules are usually very low, but overexposure to illumination can cause sample photobleaching. Scientific-grade CCD cameras allow researchers to minimize harmful photobleaching by providing extremely high sensitivity.

CCD camera response linearity over a broad range of intensities is required when algorithms (e.g., deconvolution, ratiometric analysis, morphometry) are applied to a single image or an image stack. High-spatial-resolution cameras provide detailed information within a large field of view. Wide-dynamic-range cameras allow dim objects (e.g., neuronal processes) and relatively bright objects (e.g., cell bodies) to be viewed and measured within the same image.

sample simultaneously. The introduction of the technologically advanced, high-sensitivity PI-MAX4:1024i-RF ICCD camera now allows researchers to perform these wide-field measurements with unprecedented ease in terms of intensifier modulation and instrument synchronization, as well as with minimal external equipment.

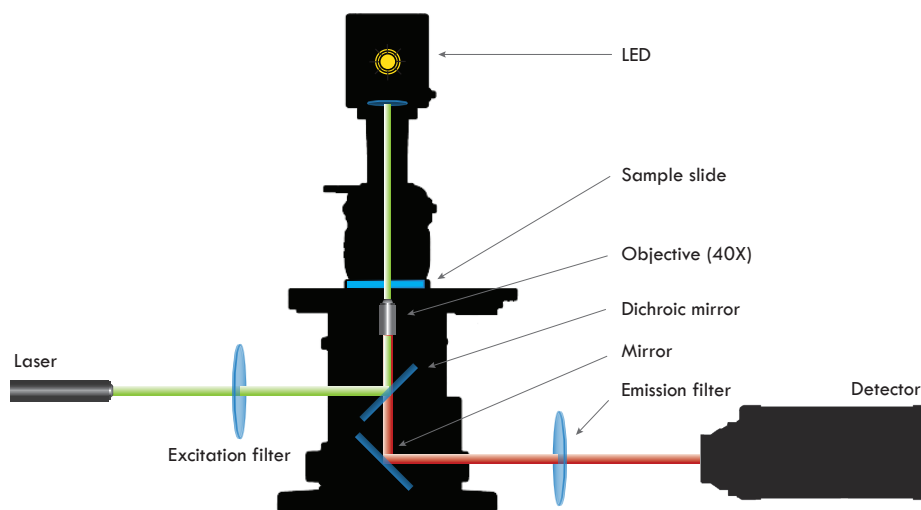
## Resources

To learn more about research being conducted by the Zumbusch Group at the University of Konstanz, please visit: <http://cms.uni-konstanz.de/zumbusch/>

For additional information about Princeton Instruments ICCD and *eml*CCD cameras, please refer to: <http://www.pi-max4.com>

## References

1. Gadella Jr T.W.J. (1999) in *Fluorescent and Luminescent Probes for Biological Activity, Second Edition*, W.T. Mason (ed). Academic Press, London, pp. 467–469.
2. "Novel Time-Resolved FLIM Measurements Method," Princeton Instruments Application Note (2014): [http://www.princetoninstruments.com/Uploads/Princeton/Documents/TechNotes/PI\\_APPNOTE\\_Novel-Time-Resolved-FLIM-Measurements-Method\\_revA1.pdf](http://www.princetoninstruments.com/Uploads/Princeton/Documents/TechNotes/PI_APPNOTE_Novel-Time-Resolved-FLIM-Measurements-Method_revA1.pdf).
3. Spring B.Q. and Clegg R.M. (2009) in *FLIM Microscopy in Biology and Medicine*, A. Periasamy and R.M. Clegg (eds). Chapman & Hall/CRC, pp. 115–142.



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