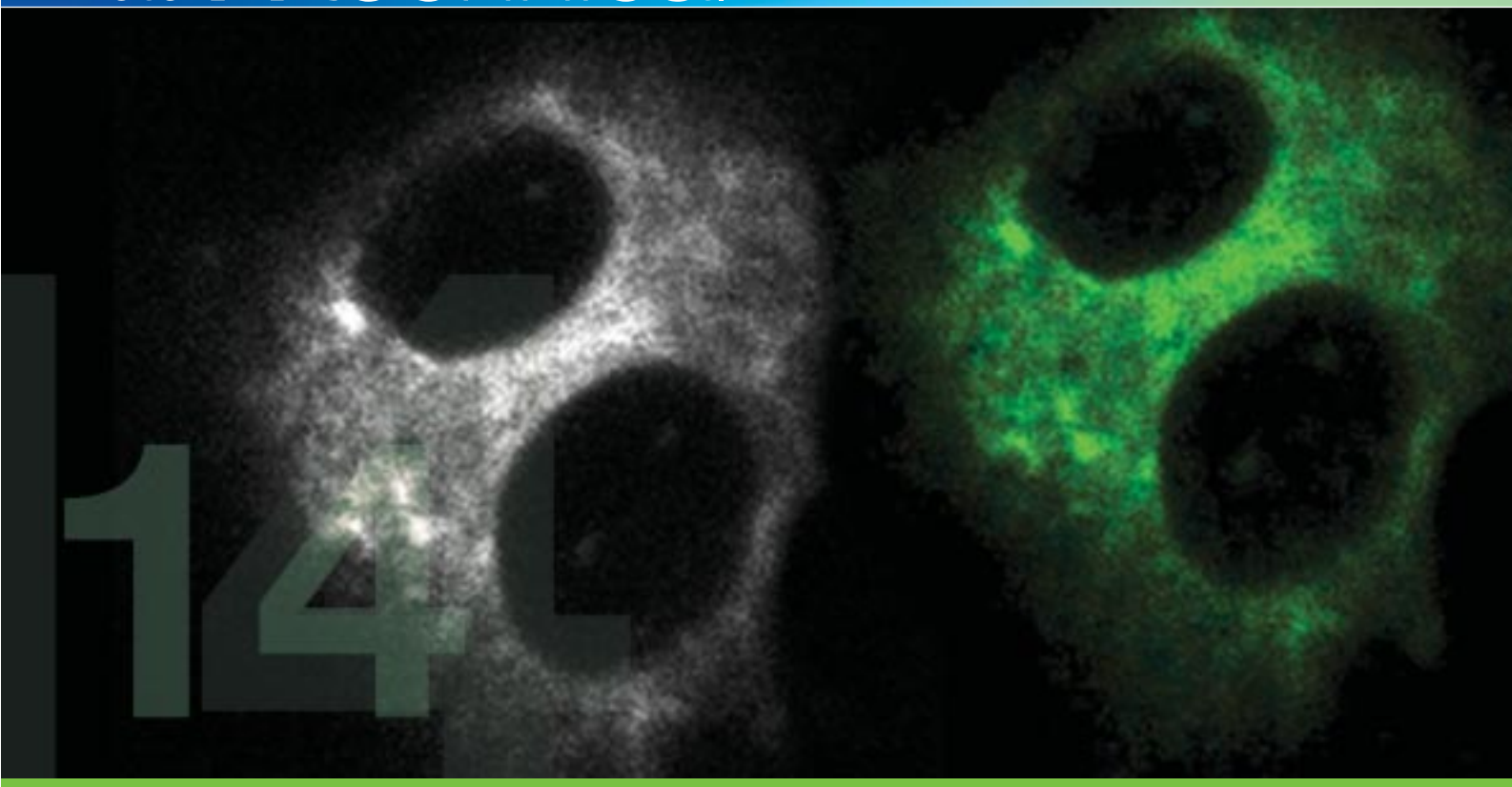
note **14** technical



Lifetime Imaging with the **Bio-Rad** Radiance2100™ MP

# Introducing a New Dimension in Laser Scanning Microscopy

**Author: Dr. Eric Pierce**
Bio-Rad Cell Science Division, UK
**Web**
www.cellscience.bio-rad.com

The use of imaging techniques to study molecular interactions and gene expression is increasingly carried out with the aid of fluorescent protein conjugates, but their overlapping fluorescence emission spectra have created challenges for data analysis. Consequently, different approaches to Laser Scanning Microscopy (LSM) imaging have been introduced, one of which is to partially separate the emission signals on the basis of their spectral properties and apply spectral un-mixing algorithms to the image data. The new Radiance Rainbow provides a highly effective way to characterise spectral properties and offers full control over detection sensitivity. For studying molecular interactions however, there are alternative approaches such as FLIM that offer additional advantages to the user, for example in FRET studies.

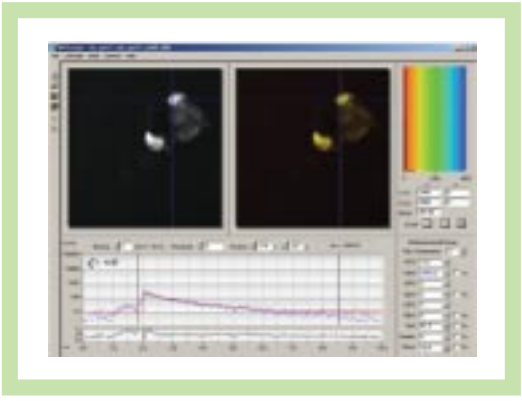
Bio-Rad offers full FLIM capability for its range of Radiance multi-photon systems. These instruments make an ideal choice for fluorescence imaging of biological samples due to their optical sectioning capability, the superior contrast that can be obtained and the wide range of fluorophores that can be imaged using multi-photon excitation. The advantages of multi-photon imaging are particularly relevant to samples susceptible to image degradation by light scattering.

## Why use FLIM?

The fluorescence of organic molecules is not only characterised by the emission spectrum, but also by it's characteristic lifetime. Any energy transfer that occurs between an excited molecule and its environment changes the fluorescence lifetime in a predictable way. The lifetime does not depend on the concentration of the chromophore, so fluorescence lifetime imaging can be applied directly to monitor energy transfer events. A useful example is fluorescence resonance energy transfer (FRET) between different chromophores in the cell (1). Furthermore, combined intensity/lifetime imaging is a powerful tool to distinguish between different fluorescence markers in multi-stained samples and between different natural fluorophores of the cells themselves. These components often have poorly defined fluorescence spectra but are clearly distinguished by their fluorescence lifetime.

BIO-RAD

# Radiance2100™ MP FLIM



The key elements for making Fluorescence Lifetime measurements on a laser scanning microscope are a pulsed or modulated excitation source, a sufficiently fast detector and suitable recording electronics. In order to achieve the highest detection sensitivity for FLIM imaging Bio-Rad have placed fast Hamamatsu 5783P PMTs in the Direct Detectors of the Radiance2100™ MP. This arrangement will support configurations of 1 or 2 FLIM channels together with 2 standard detector channels. Radiance2100™ MP can be supplied "FLIM-ready" or as a fully configured FLIM system. In most cases it will be possible to upgrade existing Radiance2000™ MP and Radiance2100™ MP systems to FLIM capability

## Biological Applications of Lifetime Techniques

When a dye molecule absorbs a photon it enters an excited state from which it returns to the ground state by the emission of a fluorescence photon, by converting the absorbed energy internally, or by transferring the energy to the environment. The probability that one of these effects occurs is independent of the time after the excitation. If a large number of similar molecules with similar local environment is excited by a short laser pulse the fluorescence decay function is therefore single exponential. As long as no energy is transferred to the environment the lifetime is the 'natural fluorescence lifetime', $\tau_0$ which is a constant for a given molecule and refraction index of the solvent. The fluorescence decay times of the fluorophores commonly used in microscopy are of the order of a few nanoseconds.

## Fluorescence Quenching

If energy is transferred to the environment the actual fluorescence lifetime, $\tau$, is less than the natural lifetime, $\tau_0$. For almost all dyes the energy transfer rate depends more or less on the concentration of ions, on the oxygen concentration, on the pH value or on the binding to proteins in a cell.

Fluorescence markers used to reveal particular protein structures in cells often bind to a variety of slightly different targets. Although this often does not cause significant changes in their spectral behaviour the lifetime can be clearly different due to different quenching efficiency. There is a direct relation between the lifetime and the quencher concentration (fig. 1).

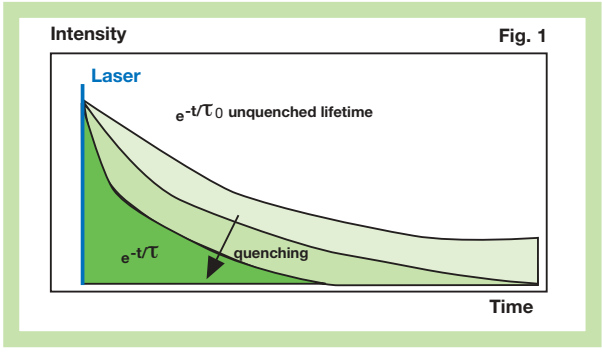


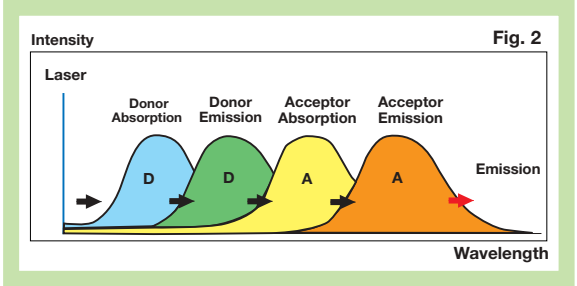

***Figure 1.*** Fluorescence quenching (2)

Therefore the lifetime of markers can be used to probe the interactions and environmental changes that occur in cells.

These principles can be applied to study live samples. Examples are the discrimination of signals from spectrally overlapping fluorophores, such as CFP, GFP and YFP constructs (3,5,7), discrimination between fluorescently labelled DNA and RNA (6) and improved contrast from autofluorescence signals. A recent report (4) also shows that FLIM imaging can be applied to the characterisation of fixed, unstained breast cancer tissues and may become suitable for real-time diagnostic imaging. However, one of the most powerful applications of FLIM is the study of molecular interactions through fluorescence resonance energy transfer or FRET effects (1)

## Fluorescence Resonance Energy Transfer

FRET occurs if two different dyes are present with the emission band of one dye overlapping the absorption band of the other. In this case the energy from the first dye, the donor, goes immediately into the second one, the acceptor. This results in an extremely efficient quenching of the donor fluorescence and, consequently, decrease of the donor lifetime, see fig. 2.



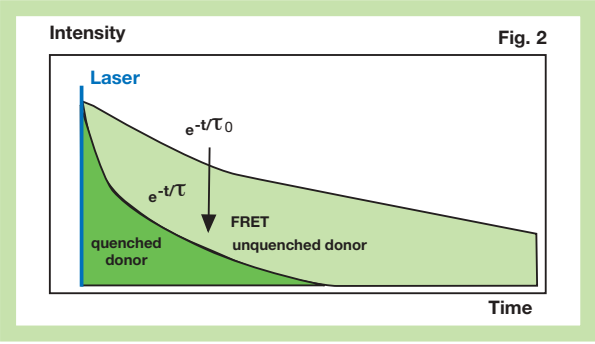



***Figure. 2***: Fluorescence Resonance Energy Transfer (FRET) (2)

FRET works only over a distance shorter than a few nm. Therefore, it can be used to probe the distance between different sub-units in the cell.

It is difficult to obtain quantitative FRET results from steady-state images. The fluorescence intensity does not only depend on the FRET efficiency but also on the unknown concentration of the dyes. Moreover, some of the acceptor molecules are excited directly, and the donor emission band extends into the acceptor emission. Up to eight measurements at different excitation wavelength and in different emission wavelength bands are required to obtain calibrated FRET results from steady state data. FRET results can also be obtained by measuring the donor fluorescence, then photobleaching the acceptor, and measuring the donor once more. The FRET efficiency is then given by the ratio of the two donor images. Although this procedure looks reliable at first glance photobleaching products may induce damage in the cell, and in a living cell diffusion may replace a part of the photobleached acceptor molecules.

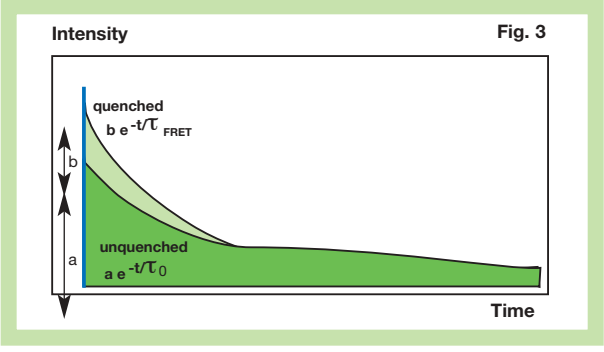
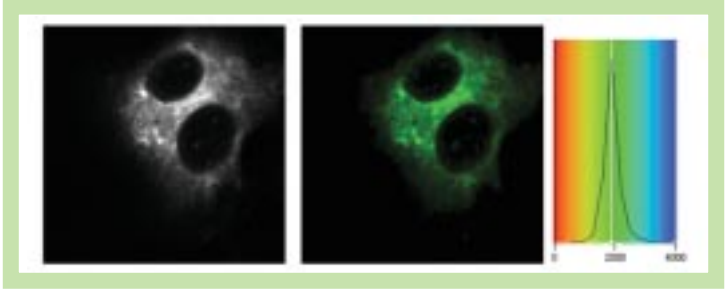


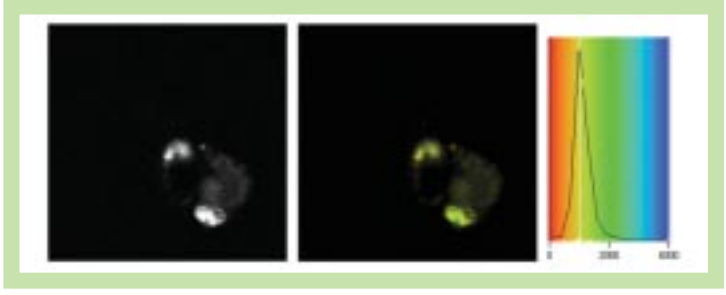

***Figure. 3:*** Fluorescence decay components in FRET systems

In lifetime data, however, FRET shows up as a measurable decrease of the donor lifetime and because only the donor fluorescence is measured, spectral bleedthrough is not an issue.

The fluorescence decay function contains the fluorescence of quenched and of unquenched donor molecules and is therefore double-exponential, fig. 3. Qualitative FRET results can be obtained from the lifetime of a single exponential approximation of the decay curve. Quantitative measurements require double exponential decay analysis that delivers the lifetimes, $\tau_0$ and $\tau_{FRET}$, and the intensity factors, a and b, of the two decay components. The relative numbers of quenched and unquenched molecules is given by the ratio of the two intensity components, b/a, while the average coupling efficiency of the FRET pairs is given by $\tau_0$ / $\tau$FRET. In principle, both the ratio of quenched and unquenched molecules and the coupling efficiency can be derived from a single donor lifetime measurement.



Fluorescence intensity, fluorescence lifetime and lifetime histogram (peak at 1.89ns) of CFP-*.



Fluorescence intensity, fluorescence lifetime and lifetime histogram (peak at 1.00ns) of an ECV cell expressing CFP-* and YFP-*. Transfected ECV cells[a] imaged with a Radiance2000 MP with FLIM Direct Detector and Becker and Hickl SPC-830 FLIM module. The fluorescence lifetime images show expression of a single protein conjugate CFP-* (top) and co -expression of CFP-* and YFP-* (bottom). The lower images demonstrate CFP/YFP FRET and a decrease in CFP fluorescence lifetime from 1.89ns to 1.00ns.[a] Courtesy of Dr David Vaux, Sir William Dunn School of Pathology, Univ. Oxford.

## Separation of Different Chromophores

The fluorescence lifetime can be used to distinguish fluorophores with similar or overlapping spectra. This can be particulary useful for fluorescent protein studies and samples that have high levels of autofluorecence. Autofluorescent components of cells and tissue show a wide variety of fluorescence with ill-defined, variable, and often unknown spectra however, the components can usually be distinguished by their different lifetimes. The relative concentration of two components can be determined by double-exponential decay analysis. Even if only a single exponential approximation, i.e. an average lifetime is used, the contrast in the fluorescence images can be considerably improved. Moreover, changes in the relative concentration and the lifetime of autofluorescence components can possibly be used as diagnostic tools (4).

## The Radiance FLIM Solution

- Multi-Wavelength TCSPC Imaging

The Bio-Rad Radiance2100 MP system is configured for FLIM by the technique of Time Correlated Single photon Counting. In this technique the time delay between excitation of fluorescence and emission of a photon is measured so as to build up a decay curve of fluorescence versus time from which the fluorescence lifetime parameter can be deduced by curve fitting. The approach gives near ideal counting efficiency, works with the full scan rate of the microscope and is able to record different wavelengths simultaneously as shown in fig. 4.

The Radiance FLIM Solution **Fig. 4**

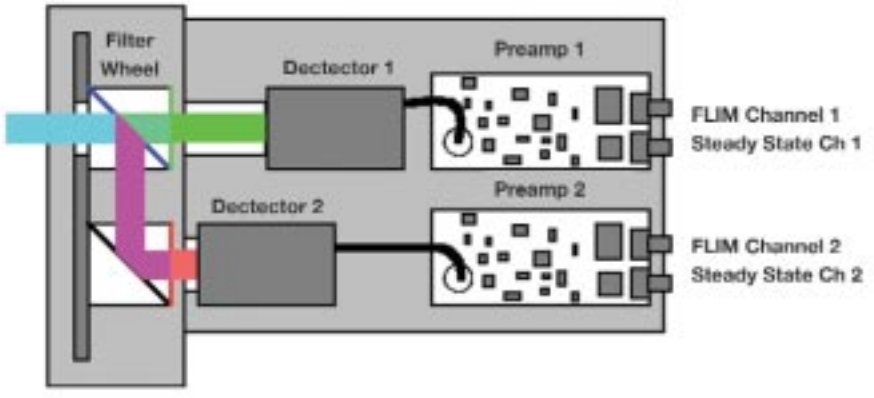



***Figure. 4*** Radiance FLIM Direct Detectors. The system is compatible with both 2-channel and 4-channel direct detector configurations.

## Detection Modules

FLIM requires PMT's capable of producing photon detection pulses with sub-nanosecond time resolution. For the wide range of biological applications for which the Radiance2100™ MP system is designed we have selected the 5783 PMT's from Hamamatsu. Configured with an ultra-fast preamplifier these PMT'offer time resolution of ~ 150 psec.

Figure 4 shows the schematic layout. In a 2 channel direct detector system it is possible to specify that either one or both channels be FLIM capable. In a 4 channel Direct Detector system one pair of detectors can be retained with standard PMT's while the second pair are FLIM capable. Each detection module contains a filter wheel with selectable dichroics to split the light into two detection channels, filters to select the detection wavelength interval and one or more fast FLIM photomultipliers. The output signals from the photomultipliers consist of ultra-fast pulses originating from the detection of single photons. A preamplifier delivers the amplified single photon pulses to the FLIM electronics and simultaneously a bandwidth-limited intensity signal to the conventional steady state recording electronics.

The fast PMT signal for FLIM detection is routed to a suitable TCSPC board such as the SPC-830 from Becker and Hickl. This board allows data to be recorded from a pair of FLIM PMTs simultaneously. Also timing synchronisation pulses for the laser pulses and the scanning synchronisation (pixel clock etc.) are provided to ensure FLIM acquisition is exactly synchronous with standard fluorescence image collection. For the most flexible FLIM work the SPC-830 board is recommended but the Radiance MP models are designed to be compatible with other FLIM technologies.

## Electronics and Data Acquisition

The recording electronics consists of a time measurement channel, a scanning interface, a detector channel register, and a large histogram memory. The time measurement channel determines the detection time (t) with respect to the next laser pulse for each photon detected. The scanning interface is a system of counters which receive the scan control signals (frame sync, line sync and pixel clock) from the microscope. It determines the current location (x and y) of the laser spot in the scanning area. Synchronously with the detection of a photon, the detector channel number (n) for the current photon is read into the detector channel register. If the light is split into different wavelength intervals in front of the detectors n represents the wavelength of the detected photon.

The obtained values for t, x, y and n are used to address the histogram memory in which the distribution of the photons over time, wavelength, and the image coordinates builds up. The result can be interpreted as separate data blocks for the different wavelength intervals. Each block can be interpreted as an image containing a full fluorescence decay curve in each pixel.

The data acquisition runs at any desired scanning speed of the Radiance system and the data acquisition can be repeated as often as necessary to collect enough photons. Due to the synchronisation via the scan clock pulses, the regular zoom and image rotation functions of the microscope act automatically on the TCSPC recording and can be applied in the usual way.

It should be pointed out that the histogramming process does not use any time gating, detector multiplexing or wavelength scanning. Therefore, provided the photon count rate is within the detection capability of the TCSPC electronics the system is near 100% photon efficient. In practice with the SPC-830 electroncs it is recommended to operate at a count rate not exceeding 4MHz. As a general rule FLIM images take longer to acquire than conventional fluorescence intensity images and typically are in the order of 10's of seconds or minutes.

## Sample-Limited Acquisition Time

TCSPC imaging gives the shortest possible acquisition time for a given count rate obtained from the sample and for a given lifetime accuracy. Unfortunately that does not actually mean that the acquisition times are really short. For single exponential lifetime analysis a few 100 photons per pixel are sufficient. For samples stained with highly stable and highly efficient fluorophores a single exponential lifetime image can be recorded within less than 10s. However, double and triple exponential decay analysis requires many more photons. To obtain high accuracy data from such samples acquisition times of the order of minutes can be required.

Electronics and Data Acquisition

**Fig. 5**

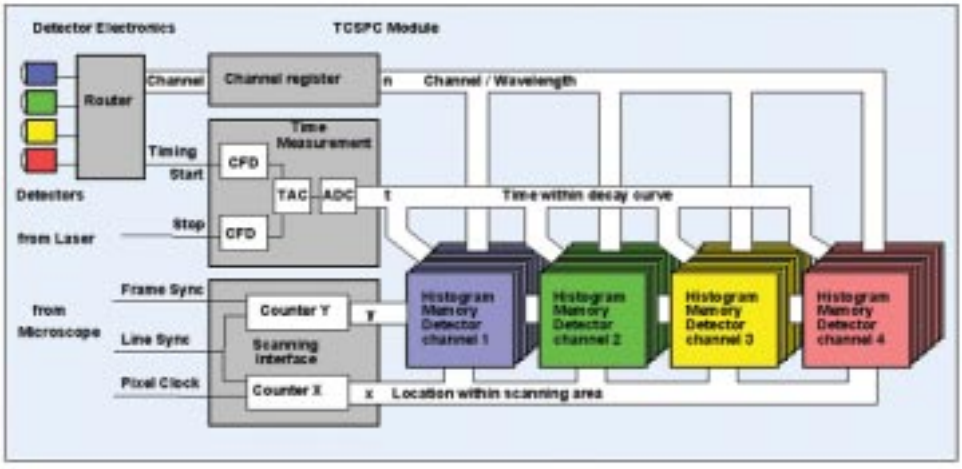



***Figure. 5*** Schematic representation of Multi-wavelength TCSPC lifetime imaging with Radiance MP

## Features in Overview

### Parallel FLIM and Conventional Imaging

A novel feature of the Radiance2100™ lifetime option is that FLIM and conventional steady state image acquisition can be run in parallel by the same detectors, using the same detected fluorescence photons. Steady state imaging can be used to track the sample behaviour during the FLIM acquisition. Or, if high pixel resolution and a large number of time channels is needed simultaneously, conventional images can be acquired at e.g. 1024x1024 pixels, while the FLIM module bins the same image into 512x512, 256x256 or less pixels.

### Multi-detector Capability

Multiple FLIM detection channels can be operated simultaneously in different wavelength intervals. Compared to consecutive acquisition runs for different wavelength the overall radiation dose is substantially reduced.

### Superior Time Resolution

The instrument response functions of the Radiance FLIM detectors is 150 to 200ps. The time channels of the TSCPC recording can be selected down to a few ps. This allows the application of standard deconvolution techniques for lifetime analysis. Double exponential decay functions can be resolved with decay components down to 50ps, allowing quantitative analysis.

### Crisp Images from Deep Tissue Layers

Multi-photon imaging was originally developed because it allows researchers to obtain images from deep tissue layers. Because there is no substantial excitation outside the focus the multi-photon microscope delivers sharp images with depth resolution. However, the localised excitation is only one half of the story. Before an image is built up, the fluorescence light must get out of the sample - and on the way out it is heavily scattered. At the surface it emerges from a diffuse spot. The reason that a multi-photon microscope delivers crisp images from deep tissue layers is that it can collect the photons from this diffuse spot with high efficiency and then assign them to the correct image coordinates. The highest sensitivity is in the direct detection mode. The Radiance2100™ system incorporates an optically efficient Direct Detector path combined with high senstivty PMT's.

### Near Ideal Recording Efficiency - Every Photon Counts

There is no time-gating, wavelength scanning or detector multiplexing. Every photon counts - regardless in which of the PMTs it is detected. This high recording efficiency is maintained even for short lifetimes - down to the order of the instrument response width. Also it does not substantially drop up to count rates of $4x10^6$ photon per second. The high efficiency allows a reduced excitation power and, in turn, reduced photobleaching effects.

### High Pixel Resolution

The high performance SPC-830 TCSPC imaging module is able to acquire a single image as large as 1024x1024 pixels with 16 time channels per pixel. Four images with 512x512 pixels and 16 time channels can be recorded simultaneously. Four images with 256x256 pixels are recorded with 64 time channels.

### Single Pixel Analysis

Fluorescence decay data of a single pixel can be acquired if the scanning of the laser beam is stopped in a defined location. Sequences of decay curves can be recorded with acquisition times of less than 1ms per curve.

# references


1. Periasamy A., FRET and FRET-FLIM microscopy imaging of localized protein interactions in living cell nucleus. *Bio-Rad Cell Science Division Application Note 36, 2003* http://www.becker-hickl.com/
2. Pepperkok R, Squire A, Geley S, Bastiaens PI. Simultaneous detection of multiple green fluorescent proteins in live cells by fluorescence life time imaging microscopy.Curr Biol. *1999 Mar 11;9(5):269-72.*
3. Tadrous PJ, Siegel J, French PM, Shousha S, Lalani el-N, Stamp GW. Fluorescence lifetime imaging of unstained tissues: early results in human breast cancer. J Pathol. *2003 Mar;199(3):309-17.*
4. Periasamy A, Elangovan M, Elliott E, Brautigan DL. Fluorescence lifetime imaging (FLIM) of green fluorescent fusion proteins in living cells. Methods Mol Biol. *2002;183:89-100.*
5. Van Zandvoort, M.A.M.J., de Grauw, C.J., Gerritisen, H.C., Broers, J.L.V., oude Egbrink, M.G.A., Ramaekers, F.C.S., and Slaaf D.W., Discrimination of DNA and RNA in Cells by a Vital Fluorescent probe: *Lifetime Imaging of SYTO13 in healthy and Apoptotic cells Cytometry 47:226-235 (2002)*
6. White N, Errington R. Multi-photon microscopy: seeing more by imaging less. Biotechniques. *2002 Aug;33(2):298-300, 302, 304-5*


.

If you would like to share your research in a publication of this type, please contact Dr. Kavita Aswani at kavita_aswani@bio-rad.com. You can also access a variety of research tools at our website to help you with your research. These include images, Application and Technical notes, product information, fluorophore wall chart and our popular interactive fluorophore database.