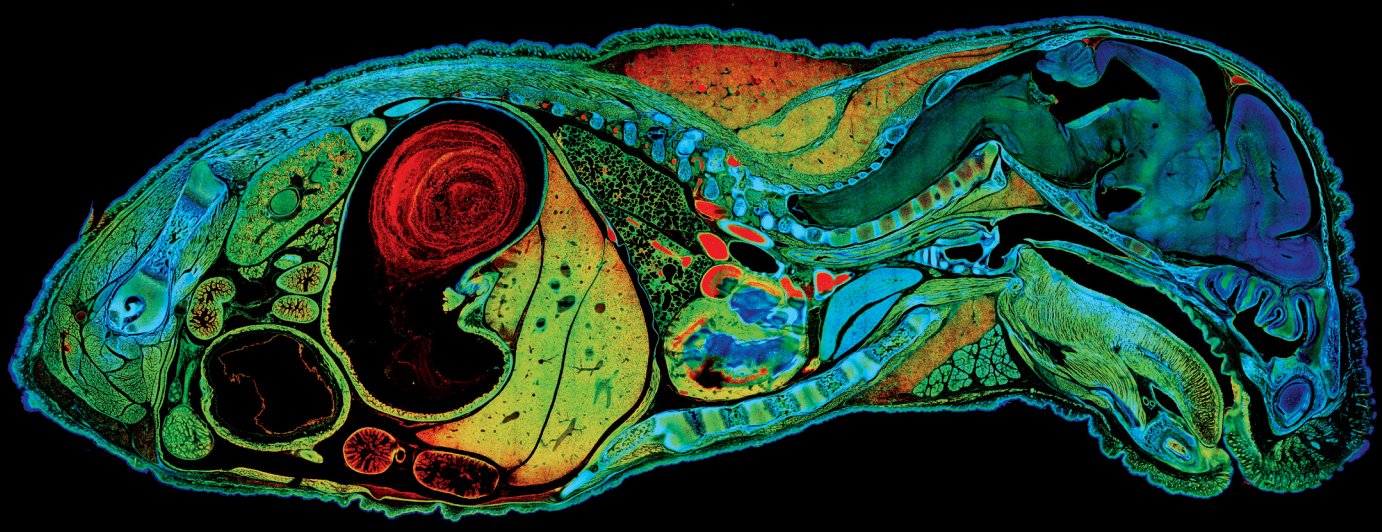
From Eye to Insight

Leica MICROSYSTEMS

Review Article

EXPLORING HIDDEN DIMENSIONS OF CELL AND TISSUE STRUCTURE AND PHYSIOLOGY WITH FLUORESCENCE LIFETIME IMAGING (FLIM)



Author

Liz Roquemore, PhD.

Learn how fluorescence lifetime imaging adds information depth to your confocal experiments and reveals novel insights that are difficult or impossible to discover using conventional intensity-based analysis.

Introduction

The vast majority of imaging experiments measure fluorescence intensity, but there is another key information-rich property of fluorescence—its lifetime. Lifetime information comes "for free" with every fluorescence experiment. It can provide valuable new insights, yet in most cases it is never collected due to technical limitations. Until recently, lifetime imaging was considered too slow, complex and expensive for everyday microscopy applications, especially those that involve live cell imaging. But all that is changing fast. With today's advanced technologies, lifetime imaging is becoming faster and easier to use than ever before. In this review, we explore what lifetime imaging is and the advantages it can bring to your research across a wide range of application areas.

Figure 1: FLIM aids interpretation of complex samples. High-resolution mosaic image of a mouse embryo. Colors encode four lifetimes resolved by FLIM data fitting.

What is fluorescence lifetime?

Ever since the invention of the microscope, biologists have been exploiting the properties of light to see inside cells. Fluorescent molecules have become essential tools for cell biologists. They are widely used in microscopy to visualize many different structures, targets and dynamic processes within cells, tissues and even whole organisms. Over the past century, researchers have developed a diverse toolbox of fluorescent probes, with colors (emission wavelengths) that span the spectrum from UV to far red.

But there is more to fluorescence than color. Each species of fluorescent molecule also has a characteristic lifetime that reflects how long the fluorophore spends in the excited state before emitting a photon (Figure 2). For a given fluorophore population, photons will be detected at a range of times after a pulse of excitation. For each fluorophore species, the distribution of these observed lifetimes follows an exponential decay function, from which a fluorescence decay constant τ can be calculated.

In fluorescence lifetime imaging (FLIM), τ is determined at every pixel, and then translated into a spatial map. This process results in a lifetime image that looks just like a conventional intensity image. However, instead of representing fluorescence intensity, each pixel represents the value of τ at that location of the sample.

Why is fluorescence lifetime useful?

When it comes to understanding what is happening in your specimen, fluorescence intensity measurements tell you only half the story. Lifetime analysis provides a wealth of additional information to help improve the quality of routine confocal experiments, give you more freedom of choice in probe selection, and enable novel applications.

The lifetime parameter is particularly useful for distinguishing different fluorescent species within the same sample. Even if two fluorescent dyes have the same color (i.e. closely overlapping emission spectra), it may be possible to distinguish them on the basis of their lifetimes. Moreover, you can make use of lifetime information from natural autofluorescence within your samples to enhance image contrast and run label-free experiments.

Unlike fluorescence intensity, lifetime is concentration-independent. This can be a big benefit in cases where probe concentration is unpredictable or variable across the sample. At the same time, lifetime probes can be exquisitely sensitive to local environmental conditions within your sample, enabling you to monitor dynamic changes in intracellular temperature, pH, ion concentration, polarity and many other parameters that would be difficult or impossible to follow by conventional techniques.

Best of all, the lifetime parameter is an intrinsic characteristic of fluorescent molecules, so lifetime data is available "for free" with every fluorescence experiment. **You just need to collect it!**

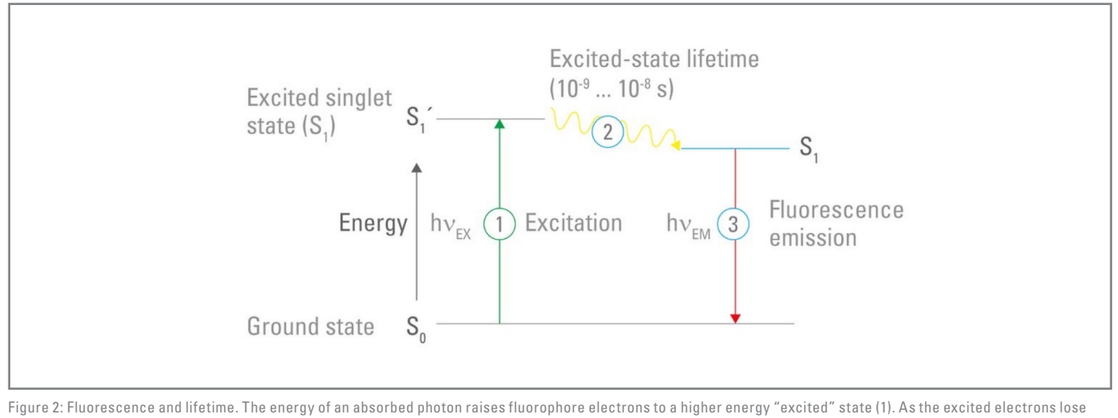


Figure 2: Fluorescence and lifetime. The energy of an absorbed photon raises fluorophore electrons to a higher energy "excited" state (1). As the excited electrons lose vibrational energy (2), some return to the ground state by emitting a photon (3). Fluorescence lifetime reflects how long a fluorophore spends in the excited state before emitting a photon.

Four ways FLIM can improve your current experiments

1. Reduce the chance of artifacts

The intensity of a fluorophore varies with concentration, light source strength, and many other variables that may have nothing to do with the phenomenon you want to study. A major advantage of fluorescence lifetime is that it is independent of fluorophore concentration and other factors that limit the utility of intensity-based measurements.

A fluorophore will always exhibit the same lifetime in a particular microenvironment, regardless of its concentration or the method of measurement.

Thus, by measuring fluorescence lifetime, you can reduce artifacts caused by photobleaching, non-uniform illumination, variable instrument settings (e.g. light path, laser intensity, exposure time, detector gain), varying probe depth within thick samples, etc.

2. Differentiate true signal from unwanted autofluorescence

Virtually all cells contain structural components and metabolites that fluoresce when excited at wavelengths across the UV/visible spectrum. This naturally occurring "autofluorescence" often overlaps with the emission of common fluorescent probes, but typically it has a very different lifetime "signature". In such cases, lifetime imaging can provide a means of differentiating the true signal from the autofluorescence.

3. Improve contrast

So-called "unwanted" autofluorescence can contain valuable information. Rather than discard or ignore it, you can use the lifetime data from autofluorescence to improve contrast and reveal greater detail in your sample. For instance, anatomical features in a histological cross-section from a nematode can be difficult to distinguish from intensity information alone, but appear much more distinct with lifetime imaging (Figure 3).

4. Distinguish more fluorophores with confidence

A key element to success in multi-probe imaging experiments is to choose a color palette with minimal spectral overlap. Unfortunately, many popular fluorescent dyes have overlapping spectra. If your confocal microscope has lifetime imaging capability, you can use it to better distinguish the different probes in your sample. This not only improves the quality of your results, it also gives you more options when it comes to dye selection.

Lifetime imaging adds new dimensions to your research

In addition to enhancing everyday confocal experiments, lifetime imaging provides a means of visualizing and quantifying aspects of physiology and function that you would struggle to study using intensity-based or ratiometric measurements. Here are 3 popular application areas where lifetime imaging can help you ask new types of experimental questions and gain novel insights.

1. Analyze metabolism with label-free experiments

Label-free analysis of autofluorescence for functional imaging - particularly to study metabolism - is a rapidly growing research area. Professor Ammasi Periasamy, Director of the Keck Center for Cellular Imaging at the University of Virginia, has done significant research into the use of FLIM in combination with 2-photon excitation for functional imaging. He applies label-free lifetime imaging in his current research to measure metabolism and mitochondrial dysfunction in living cells, tissues and animals.

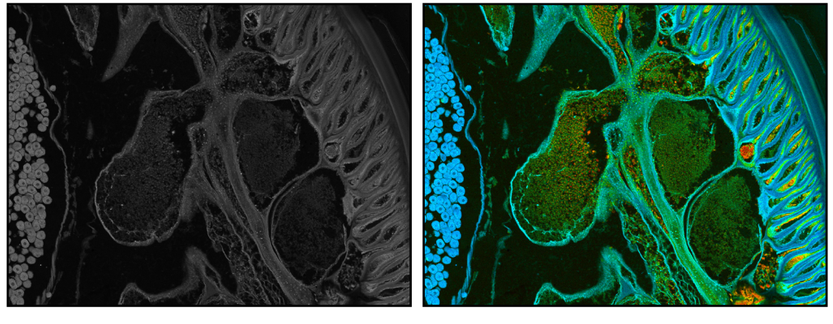


Figure 3: Lifetime contrast using autofluorescence. Intensity (gray-scale) and lifetime (rainbow color scale) imaging of a histological tissue cross-section from a nematode worm.

Coenzymes central to metabolism such as FAD and NADH provide natural autofluorescence (Figure 4). "You don't have to label it," says Prof. Periasamy, "so this type of technique is useful for translational research." Importantly, he points out, lifetime discriminates the bound and free states of these coenzymes. "With intensity techniques it is impossible to do that."

2. Study molecular interactions with FRET-FLIM

Interactions between proteins, DNA, RNA and other molecules underlie much of biology. A phenomenon known as Förster resonance energy transfer (FRET) can be exploited to detect molecular interactions and measure the distance between molecules at very close range - over distances less than ~10 nm.

Beyond its utility for measuring intermolecular distances, the principle of energy transfer between FRET donor and acceptor pairs has also enabled development of sensitive biosensors. Many of today's modern FRET biosensors are based on genetically encoded proteins[1] engineered to undergo a conformational change that brings linked donor and acceptor domains closer together upon ligand binding, so that a FRET signal is generated.

Genetically encoded FRET biosensors are particularly useful for monitoring dynamic signal transduction events in live cells. For instance, the ERK signaling pathway is a key driver of malignant cell growth in cancer. A FRET biosensor for ERK has been used to monitor cancer-associated changes in ERK signaling in live patient-derived organoids.[2] Lifetime analysis highlights enhanced ERK activity in organoids from colorectal cancer patients (Figure 5). High-speed FLIM recording was crucial for this demanding 3D imaging study.

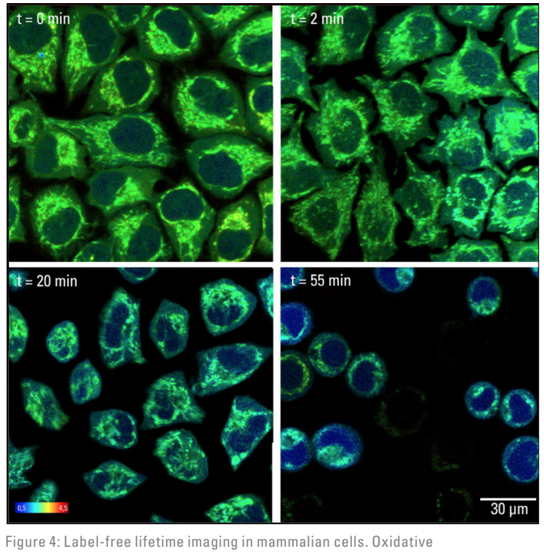


Figure 4: Label-free lifetime imaging in mammalian cells. Oxidative stress under non-physiological conditions (pH 8.5) develops due to changes in the endogenous NAD/NADH pool, producing a time-dependent decrease in autofluorescence lifetime. Color bar indicates lifetime (ns).

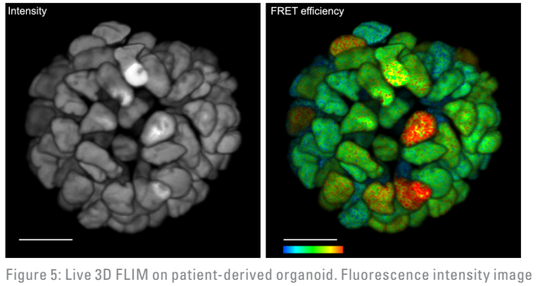


Figure 5: Live 3D FLIM on patient-derived organoid. Fluorescence intensity image reveals little functional information (left). A clear increase in ERK activity (FRET efficiency) is apparent in the corresponding lifetime image (right). Color bar, 2–12% FRET efficiency. Scale bar, 20 µm. Courtesy: Kees Jalink, Bram van den Broek, NKI Amsterdam, Bas Ponsioen UMC Utrecht.

3. Explore microenvironments with environmentally sensitive probes

An especially useful property of fluorescence lifetime is its potential sensitivity to the local microenvironment. This phenomenon is possible because the surrounding environment can affect the available pathways by which excited electrons return to the ground state.

If environmental factors increase or decrease non-radiative pathways (those that do not result in emission of a photon), then the lifetime of the fluorophore will be shortened or lengthened respectively.

A wide variety of environmental biosensors have been engineered to exploit this property - many of them based on FRET. In many cases, lifetime imaging reliably detects environmental changes that are otherwise extremely difficult to measure in the crowded intracellular space. Specific environmental sensors are available to monitor temperature, ion concentration (Ca^{2+}, Na^{+}, etc.), pH, polarity, viscosity, second messengers such as cAMP, and many other parameters and small molecule analytes.

A particularly challenging application where lifetime-based biosensors are shedding new light is in studying the temperature-dependence of biological activities within sub-cellular compartments such as mitochondria and the nucleus, where vital cell functions occur. Traditional thermometry techniques cannot be applied at the single-cell level, where regions of interest have dimensions ranging from 1µm to ~100µm. To address this problem, researchers have developed molecular thermosensors such as the fluorescence polymeric thermometer (FPT), a concentration-independent cell permeable polymer that exhibits an increase in fluorescence lifetime with increasing temperature.[3]

Dr. Kohke Okabe and colleagues combine FPT with time-correlated single photon counting (TCSPC) - one of the most accurate FLIM detection approaches - to determine whether highly localized temperature variations affect cellular responses such as stress granule formation and brain edema. “FLIM has been indispensable to do our work in a quantitative manner,” says Okabe.

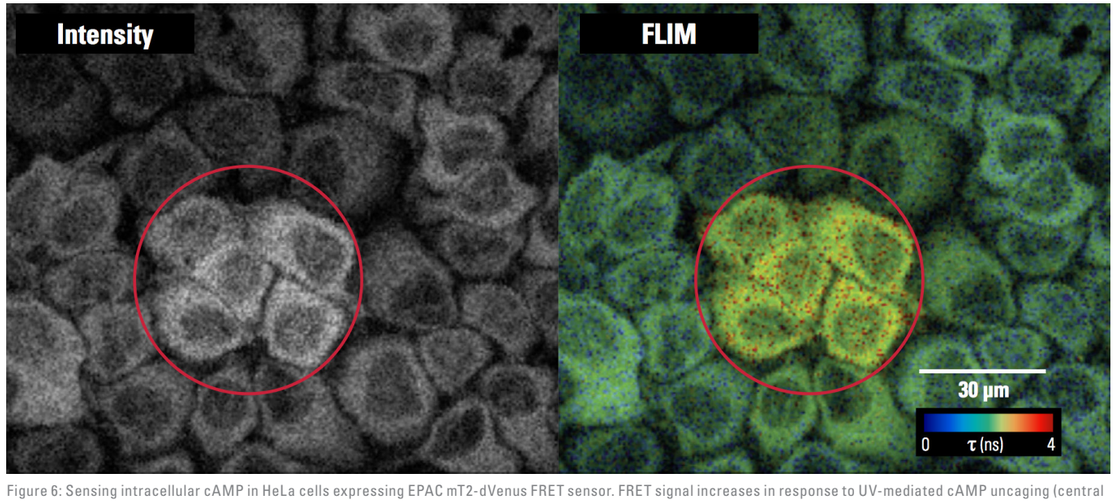


Figure 6: Sensing intracellular cAMP in HeLa cells expressing EPAC mT2-dVenus FRET sensor. FRET signal increases in response to UV-mediated cAMP uncaging (central area). Intensity (left) and FLIM (right) images captured from a movie recorded at 4 fps. Color bar scale (lifetime): ns. Courtesy Kees Jalink, Bram van den Broek, NKI Amsterdam.

SUMMARY AND CONCLUSIONS

Integrating FLIM into everyday research

Lifetime imaging is an invaluable tool that can immediately enhance the quality of your confocal experiments. Not only does it allow you to take advantage of natural cellular autofluorescence, it also gives you much more flexibility in probe selection and multiplexing experiments. The emergence of novel FRET-FLIM and environmental probes opens up a world of additional possibilities for cell research and quantitative imaging, enabling scientists to ask new questions and quantify aspects of cell structure and physiology that could not previously be measured. As lifetime imaging technology continues to evolve, previous limitations such as photobleaching and slow data acquisition speeds are becoming less of an issue. Rapid video rate FLIM solutions such as FALCON technology[2] are enabling more live-cell kinetic applications. In the future, we can expect to see more holistic confocal platforms that seamlessly integrate FLIM into everyday imaging workflows. This will be facilitated by more user-friendly software and turnkey approaches to system operation, experimental set-up, data analysis and results interpretation. Periasamy predicts a bright future for FLIM technology in biological research: “This is a dynamic technique that will surely continue to advance. It will be available in all biology labs and microscopy facilities - just like confocal [is today].”

References

[1] Greenwald EC, Mehta S and Zhang J. Genetically encoded fluorescent biosensors illuminate the spatiotemporal regulation of signaling networks. Chem Rev 118, 11707-11794 (2018).

[2] Alvarez LAJ et al. Application Note: SP8 FALCON: a novel concept in fluorescence lifetime imaging enabling video-rate confocal FLIM. Nat Meth (2019) https://doi.org/10.1038/d42473-019-00261-x.

[3] Okabe K et al. Intracellular temperature mapping with a fluorescent polymericthermometer and fluorescent lifetime imaging microscopy. Nat Commun 3, 705 (2012).

CONNECT
WITH US!

Leica Microsystems CMS GmbH | Am Friedensplatz 3 | 68165 Mannheim, Germany
Tel. +49 621 70280 | F +49 621 70281028

www.leica-microsystems.com/products/confocal-microscopes