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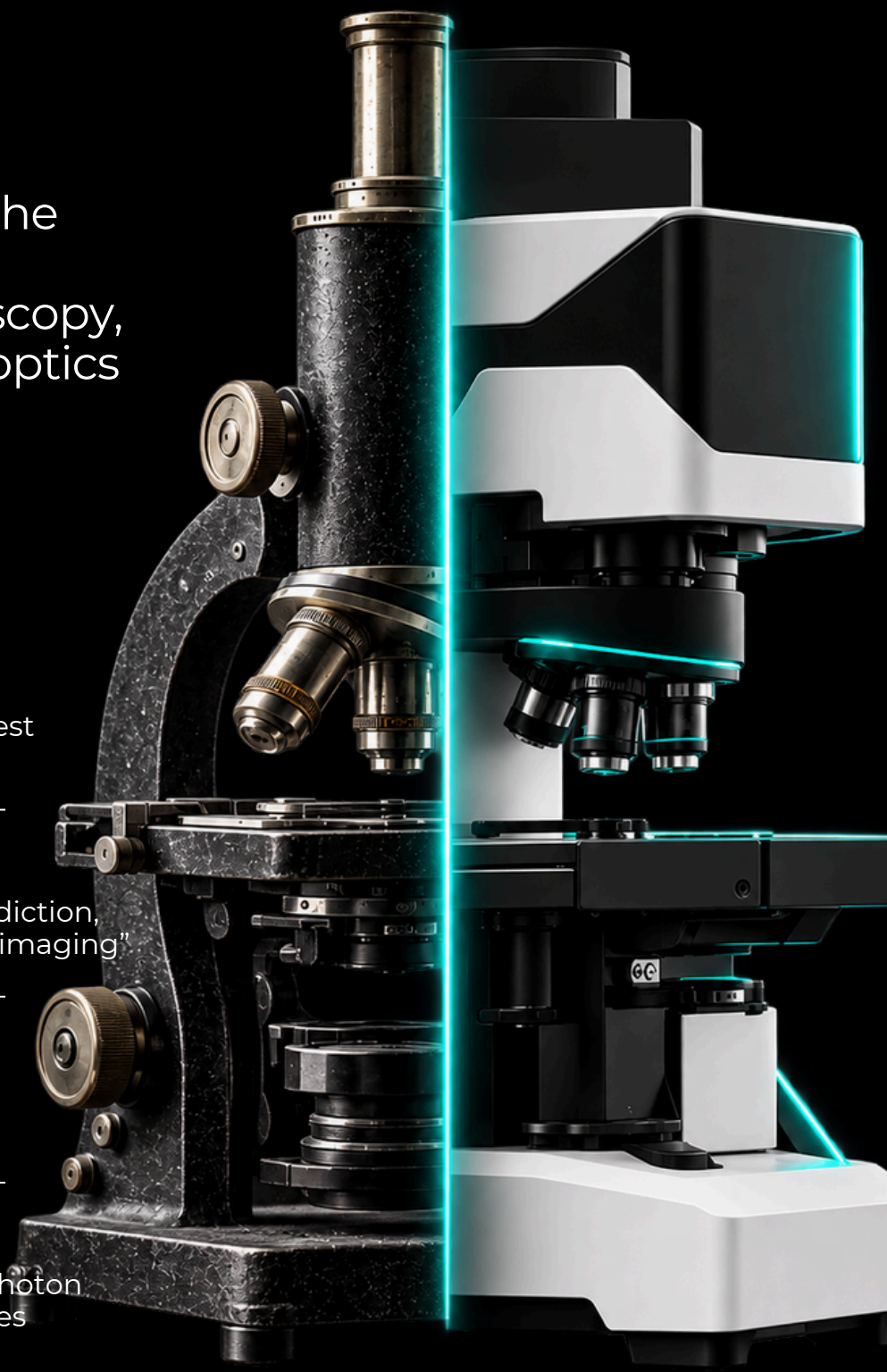
A FLIM LABS PUBLICATION

# LIFETIME

THE FLIM MAGAZINE

# A Matter of *Time*

A journey through the technologies that transformed microscopy, from conventional optics to FLIM.



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THIS ISSUE

Four centuries of looking closer, and a new dimension that reveals more than meets the eye.

NOTE FROM FLIM LABS

# Why this issue is a matter of *time* — and how microscopy keeps *evolving*

This November issue of LIFETIME explores the evolution of microscopy, from the first optical instruments to today's most advanced imaging technologies, and the growing role of fluorescence lifetime.

FOR centuries, microscopy has been driven by a simple ambition: to see what was previously invisible. From the earliest optical lenses to confocal, two-photon, and super-resolution techniques, each innovation has opened new possibilities for scientific discovery.

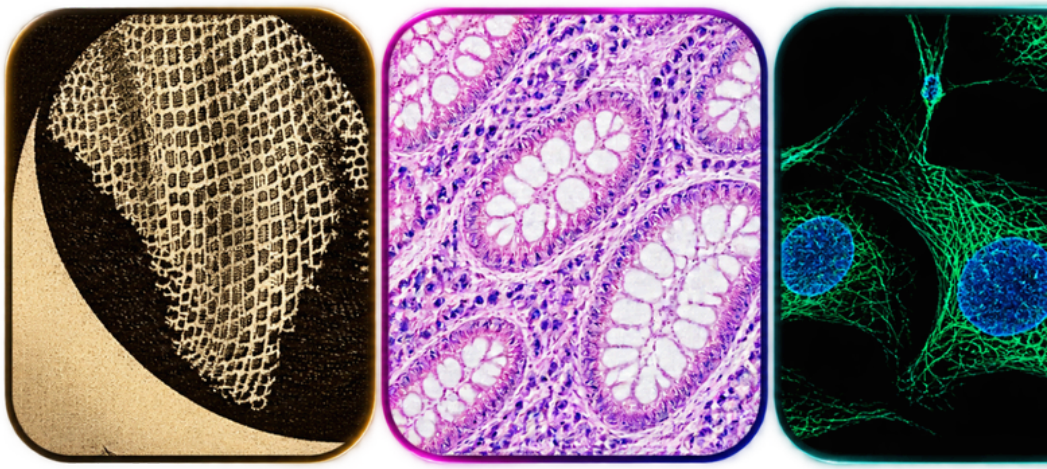
But the evolution of microscopy is not just a story of increasing magnification or improving resolution. It is also a story of changing questions. Researchers no longer want to know only what a sample looks like. They want to understand how its components behave, interact, and respond to their environment.

Fluorescence lifetime imaging adds another dimension to this exploration. By measuring how long fluorescence lasts after excitation, FLIM reveals information that conventional intensity images may overlook. And unlike techniques designed to overcome specific imaging limitations, FLIM can complement different microscopy architectures, from widefield and confocal systems to multiphoton and selected super-resolution approaches.

As instruments evolve, so do the tools used to interpret their data. Techniques such as FLIM-FRET, rapid lifetime determination, and phasor analysis offer different ways to investigate fluorescence behaviour, while dedicated software makes these measurements easier to acquire, visualize, and understand.

In this issue, we look back at the technologies that transformed microscopy while exploring how fluorescence lifetime is becoming an increasingly practical part of modern imaging workflows.

> “The evolution of microscopy is a story of changing questions”



***The Diffraction Limit***

*Why can't we just keep magnifying? Because resolution isn't the same as magnification. Light behaves as a wave, making it impossible for conventional optical microscopes to distinguish structures below a certain distance. Super-resolution techniques such as STED, PALM and STORM overcome this barrier using different physical and computational strategies.*

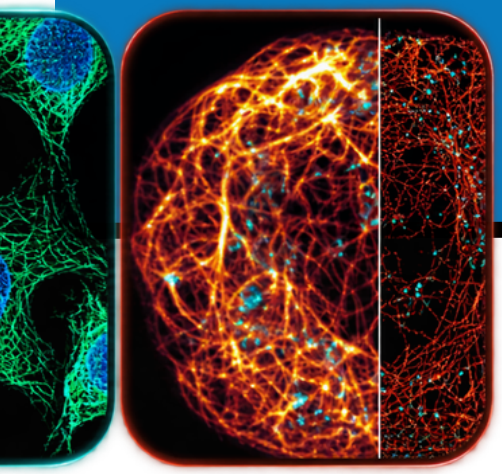
# The Never-E to See

*How microscopes evolved from simple optical instruments into sophisticated tools for exploring the structure and behaviour of matter.*

THERE was a time when an entire universe could fit inside a drop of water, and nobody knew it was there. For most of human history, the microscopic world existed just beyond our reach. People could observe the growth of plants and the changing colours of materials, but the structures responsible for much of what happened around them remained invisible. Then, in the seventeenth century, a few carefully arranged pieces of glass began to change everything.

The earliest optical microscopes - some of which used a single lens, while others combined several - were relatively simple instruments but were rather difficult to operate, because they were limited in resolution and often produced distorted images. Yet what they revealed was extraordinary. Robert Hooke described the tiny compartments of cork, giving us the word *cell*, and Antonie van Leeuwenhoek observed microorganisms swimming in water, opening a window onto a world no one had seen before.

Suddenly, things that had always been there became part of scientific reality. The microscope had done something remarkable by expanding the boundaries of the visible. However, it also created a new problem: once scientists



*Illustrative AI-generated composition of the evolution of microscopy. From left to right: early optical microscopy, conventional light microscopy, confocal fluorescence microscopy, and super-resolution imaging. The image is for explanatory purposes only and does not represent a real sample or experimental acquisition.*

# nding Quest

# *Smaller*

could see the microscopic world, they immediately wanted to see more.

**When light learned to move**

For centuries, improvements in microscopy depended largely on better lenses and optical design. Microscopes became sharper and perhaps more powerful, yet even the finest optical systems encountered a fundamental limitation: light itself.

Because light behaves as a wave, it cannot distinguish arbitrarily small details. In the nineteenth century, Ernst Abbe helped establish the physical principles behind this limit. No matter how much an image was magnified, closely spaced structures could remain indistinguishable.

The arrival of the laser in 1960 changed what researchers could do with light. Unlike conventional illumination, a laser provided an intense, highly controlled beam that could be focused onto a tiny region of a specimen. Rather than illuminating everything simultaneously, scientists could scan the beam across the sample, recording its response point by point. It was an important conceptual shift. The microscope was no longer simply collecting an image. Now it could build one, deliberately, using precisely controlled light.

Around this period, another idea was beginning to transform microscopy: what if the instrument could reject light coming from the wrong depth? Marvin Minsky had already developed the principle of confocal microscopy in the 1950s. By using a small aperture, or pinhole, to exclude much of the out-of-focus light, confocal systems could

produce sharper optical sections of thick specimens. As laser technology and scanning electronics improved, laser-scanning confocal microscopy became an increasingly powerful way to explore cells and tissues in three dimensions. The improvement was dual: firstly, researchers could see smaller things; additionally, they could also now select which layer of a specimen they wanted to see. And once scientists could choose where to look, the next question was how deeply they could look.

**Going deeper, seeing faster**

Imagine trying to observe a structure buried inside living tissue. Light must travel through layers of material before reaching it, and the returning signal must make the same difficult journey back. Conventional fluorescence excitation can illuminate regions above and below the plane of interest, generating unwanted background and potentially exposing more of the specimen to light than necessary. A different solution, however, is offered by what is known as two-photon microscopy.

Demonstrated for biological imaging in 1990, two-photon microscopy relies on a phenomenon in which a fluorescent molecule absorbs the energy of two lower-energy photons arriving almost simultaneously. Because this is most likely to happen where the laser light is tightly focused, excitation becomes naturally confined to a small volume.

With suitable near-infrared excitation, researchers could investigate deeper regions of scattering biological tissues while reducing out-of-focus excitation. Meanwhile, other microscopy designs were tackling a different enemy: time. In spinning-disk confocal microscopy, a rapidly rotating disk containing many tiny apertures allows multiple points to be scanned in parallel. Rather than examining a specimen one position at a time, the instrument can collect information from many locations simultaneously.

This approach helped make confocal imaging faster and particularly useful for observing dynamic processes in living cells. A cell, after all, is not a photograph. Its components move, and to understand it researchers need not only spatial detail but also the ability to follow events as they unfold.

**Breaking the rules of resolution**

For decades, the diffraction limit seemed to mark a boundary that optical microscopy could approach but never cross. Then scientists began finding ways around it. For instance, Structured Illumination Microscopy, or SIM, took advantage of patterned light. By illuminating a sample with carefully controlled patterns and combining multiple images computationally, researchers could recover details that conventional imaging could not resolve.

Image Scanning Microscopy, or ISM, approached the challenge differently. By collecting spatial information from detected light during scanning and

reassigning the detected signal to more accurate positions, ISM could improve resolution and signal collection beyond conventional confocal approaches.

Other methods challenged the diffraction barrier more dramatically. In Stimulated Emission Depletion microscopy, or STED, a second beam is used to suppress fluorescence around the outer part of an excitation spot. The region effectively allowed to fluoresce becomes much smaller, revealing structures that would otherwise blur together.

Then there's PALM and STORM, which instead of trying to observe every fluorescent molecule at once, exploited the ability to switch or activate sparse subsets of emitters. By localizing individual molecules over thousands of images, they could reconstruct structures with extraordinary spatial precision. The result was a revolution in super-resolution microscopy. Researchers could now begin to map the positions of individual fluorescent molecules.

More recently, MINFLUX has pushed single-molecule localization even further. By using a carefully positioned minimum of excitation light and measuring the resulting fluorescence, it can determine molecular positions with exceptional precision while requiring comparatively few detected photons. Each technique has its own strengths and compromises, along with its applications. Some are better suited to living specimens, while others to fixed samples or single-molecule measurements. Some prioritize speed, others pursue the smallest possible spatial details. There is no single microscope that has replaced all the others. Instead, microscopy has become a growing collection of different ways to investigate the same world. And this brings us to a question that resolution alone cannot answer.

**What if the image isn't enough?**

Imagine looking at two regions of a fluorescent sample. They have the same colour, and they appear equally bright. Even under a sophisticated microscope, their structures may look nearly identical. But does that mean they are behaving in the same way? Well, not necessarily. Fluorescence contains information beyond its intensity. After a molecule absorbs energy, it usually takes a very short time before emitting a photon. That interval, often measured in nanoseconds, is its fluorescence lifetime. Fluorescence lifetime can change depending on the molecular environment. This is the principle behind Fluorescence Lifetime Imaging Microscopy, or FLIM. Rather than measuring only how much fluorescence comes from each location, FLIM investigates how long the fluorescence lasts.

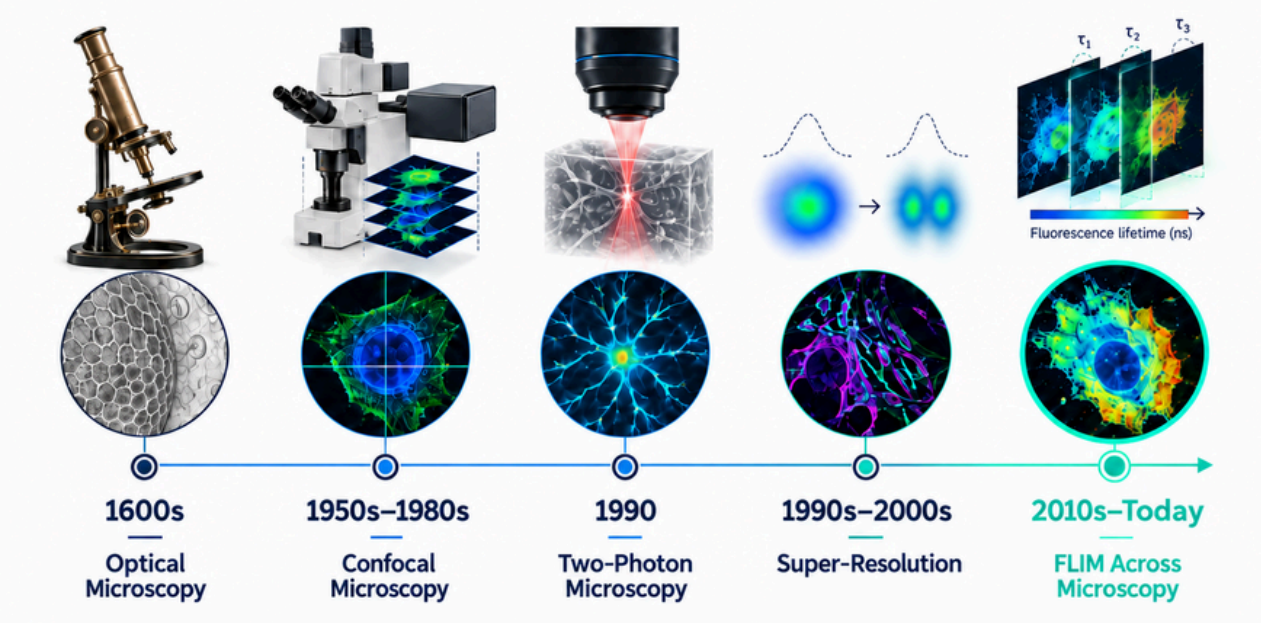
The distinction is subtle, but powerful. Two regions that look identical in an intensity image may exhibit different lifetimes, suggesting differences in molecular interactions, local conditions

or metabolic state. In other words, FLIM introduces another way of distinguishing signals: not necessarily by where they originate or how bright they are, but by how they behave in time.

Crucially, FLIM does not belong to one particular generation of microscopes, but is a measurement dimension that can complement different fluorescence microscopy architectures. It can be implemented in widefield, confocal and multiphoton imaging, as well as combined with selected super-resolution approaches when the appropriate excitation, detection and timing are available. Integrating lifetime measurements into specialized methods may introduce additional technical challenges, but the underlying principle remains the same. Where conventional microscopy records fluorescence intensity, FLIM asks what can be learned from its temporal behaviour.

In this sense, the history of microscopy can be seen as a history of questions becoming more precise. The first optical microscopes asked what existed beyond the reach of the naked eye. Confocal microscopy asked where structures were located within a specimen. Two-photon microscopy helped researchers investigate deeper into living tissues. Super-resolution methods began revealing molecular arrangements once hidden by diffraction.

FLIM adds another question - a novel point to think about: how does the fluorescent signal behave, and what might that reveal about the sample? Four centuries after scientists first examined microorganisms in drops of water, microscopes are still doing what they did at the beginning: revealing things we could not previously observe. Only now, however, seeing is no longer the whole story.



# Making Room for *FLIM*

*From distinguishing autofluorescence to combining different imaging techniques, Alvaro Crevenna, Head of the Light Imaging Facility at EMBL Rome, discusses how FLIM is evolving and why making it more accessible could open the door to new applications.*



*Alvaro Crevenna*

**TO begin, could you briefly introduce yourself and your work at EMBL?**
I'm Alvaro Crevenna, and I've been Head of the Light Imaging Facility at EMBL Rome since 2019. My background is in molecular and cellular biology, as well as biophysics.

**FLIM has traditionally been considered a rather specialized technique. From your experience, how has its adoption changed over the years? Is it becoming more common in routine research?**
I first encountered fluorescence lifetime measurements through single-molecule FRET experiments. In those experiments, lifetime measurements were an essential part of the process, helping us calibrate the measurements and convert FRET efficiencies into distances between donor and acceptor molecules. At the time, FLIM was not widely used in biology. It was mostly associated with specialized applications and niche systems. I think the situation has gradually changed, partly thanks to commercial technologies from companies such as Leica, which have helped introduce FLIM to a broader range of users.

**What can fluorescence lifetime measurements reveal that conventional intensity-based microscopy cannot? Are there particular applications where FLIM could make a difference?**
Fluorescence lifetime gives us an additional parameter beyond intensity, allowing us to build a more complete picture of a sample. One particularly interesting application, which I think is sometimes underestimated, is

distinguishing autofluorescence in biological tissues. Autofluorescence can be difficult to remove or separate from other fluorescent signals using conventional imaging. However, when we characterize fluorescence according to its lifetime, we can distinguish different contributions to the signal. This helps us identify which regions correspond to autofluorescence and which contain the fluorescence we're actually interested in measuring. It provides another way of understanding what we're looking at.

**Despite its potential, FLIM is still not equally accessible to all laboratories. What are the main barriers, and could overcoming them encourage wider adoption?**
That's certainly true. FLIM is often included as part of more complex instrumentation, either as a commercial add-on or within high-end microscopy systems. This makes it relatively expensive and therefore difficult for many laboratories to access. There's also the technical aspect. Researchers need to know how to use the technology and interpret the measurements. The combination of cost and technical expertise has helped keep FLIM within a relatively specialized community. But I certainly believe this can change. Once a technology becomes more accessible, more people start using it. And as researchers become familiar with it, they begin thinking of different ways to apply it. That naturally creates opportunities for new applications.

**Looking ahead, what do you think will be the next major frontier in microscopy? And what role could FLIM play in that future?**
That's a good question! If I had to make a prediction, I would put my money on multimodal imaging. The idea is to investigate the same sample in several different ways, combining multiple types of measurements to obtain a more complete picture of what's happening. Achieving this will require advances in instrumentation, but also better integration between different technologies and the data they produce. Ultimately, the goal is to bring together different perspectives on the same sample. I believe FLIM should be one of those perspectives because it provides a very particular type of information that other measurements cannot offer. And coming back to accessibility, I think that's where things become especially interesting. Once the technology becomes easier to access, researchers will have more freedom to experiment with it. In a sense, FLIM could become something they can really play with, explore, and use in ways we haven't necessarily thought of yet. That's when I expect the number of applications to really grow.

GET STARTED

# Explore Accessible FLIM Solutions

> Bring fluorescence lifetime imaging into your workflow without unnecessary complexity.



## FLIM Kit

A focused, ready-to-use FLIM system.



## FLIM Upgrades

Add the time dimension to existing optical workflows



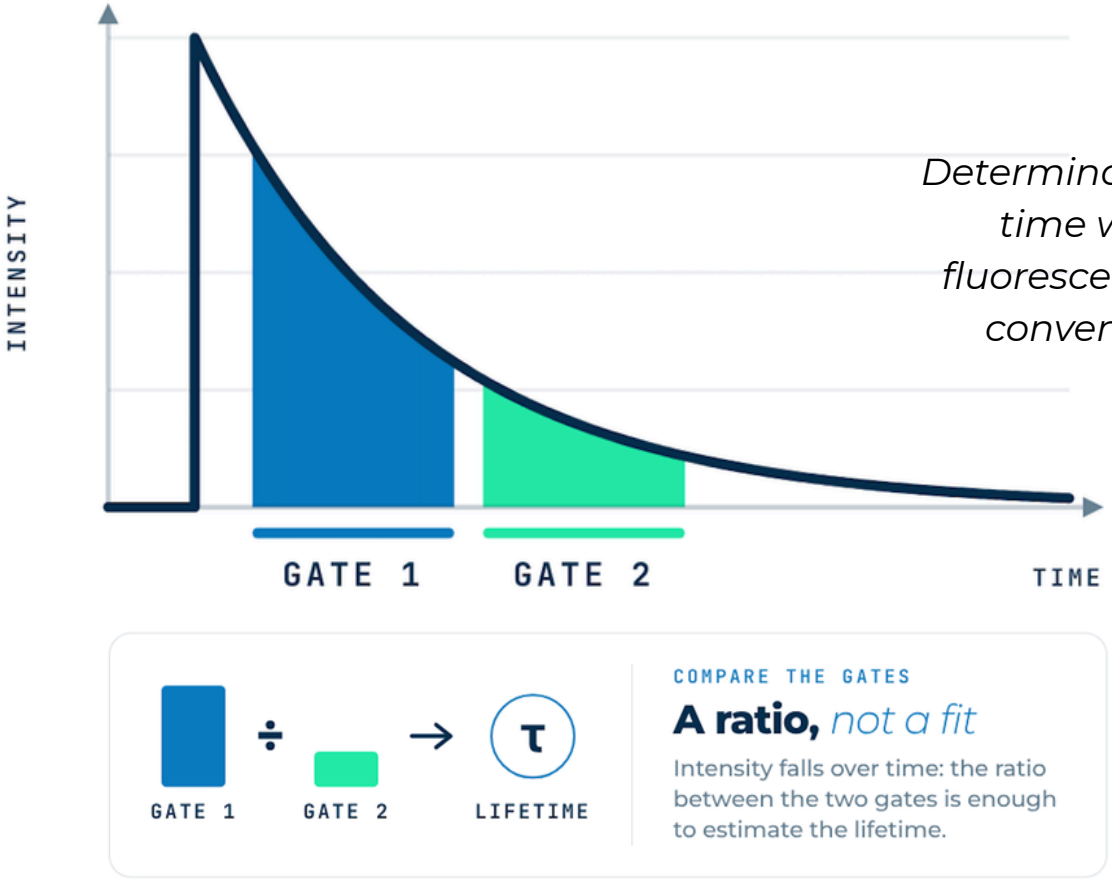
## Modular Integration

Build flexible FLIM workflows around your research, product, or application.

## → Talk to FLIM LABS

Contact us by sending an email to info@flimlabs.com or via www.flimlabs.com

# Measuring *Lifetime* Without Fitting




*How Rapid Lifetime Determination (RLD) uses two time windows to estimate fluorescence lifetime without conventional decay fitting.*

FLUORESCENCE lifetime imaging typically involves recording a fluorescence decay curve and fitting it with a mathematical model. But is it possible to estimate lifetime without analysing the entire decay?

Instead of fitting the full curve, Rapid Lifetime Determination (RLD) measures fluorescence in two consecutive time windows, or gates, after excitation.

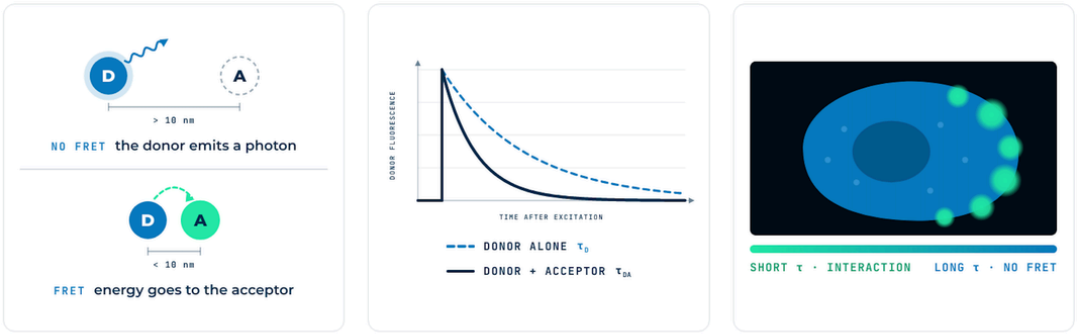
Because fluorescence intensity decreases over time, comparing the signals collected in these windows

allows researchers to estimate fluorescence lifetime using a simple calculation. The main advantage is speed. By avoiding iterative fitting, RLD reduces computational demands and can support rapid lifetime mapping in FLIM.

However, conventional two-gate RLD assumes a single-exponential decay. For samples with multiple lifetime components, it generally provides an effective lifetime rather than resolving individual contributions. RLD is therefore a useful alternative when fast lifetime estimation matters more than detailed decay analysis.

# *FLIM-FRET*: When Molecules Get Close

*How fluorescence lifetime reveals interactions between molecules just nanometres apart.*




a **Distance decides** b **The donor's lifetime shortens** c **FLIM maps where it happens**

HOW can researchers detect interactions between molecules too close together to distinguish with conventional microscopy? One answer is Förster Resonance Energy Transfer, or FRET. FRET occurs when an excited fluorescent molecule (the donor) transfers energy to a nearby molecule (the acceptor) without emitting a photon. Because this happens over distances of roughly 1–10 nanometres, FRET acts as a molecular ruler. But how can researchers tell whether this transfer is happening? By measuring the donor's fluorescence lifetime, which shortens when energy is transferred to the acceptor.

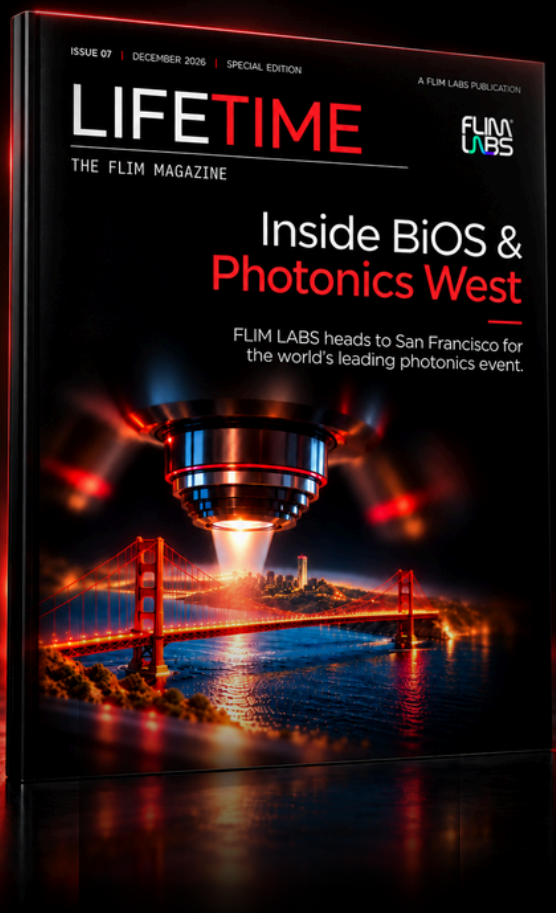
Combining FRET with FLIM allows researchers to map these lifetime changes across a sample, revealing where molecular interactions occur without relying solely on fluorescence intensity.

FLIM-FRET is widely used to study protein interactions, conformational changes, and cellular signalling, turning molecular proximity into measurable lifetime differences.

NEXT ISSUE – SPECIAL EDITION

# Coming next month: Inside BiOS & Photonics West

FLIM LABS heads to San Francisco for the world's leading photonics event.




## WHAT'S INSIDE

### WEST COAST BOUND

A look at the events bringing together the latest innovations in photonics.

### DEMOCRATIZING FLIM

Making FLIM more accessible through affordable and versatile solutions.

### FLIM IN ACTION

Exploring metabolic imaging applications and FLIM LABS' latest microscope integrations.



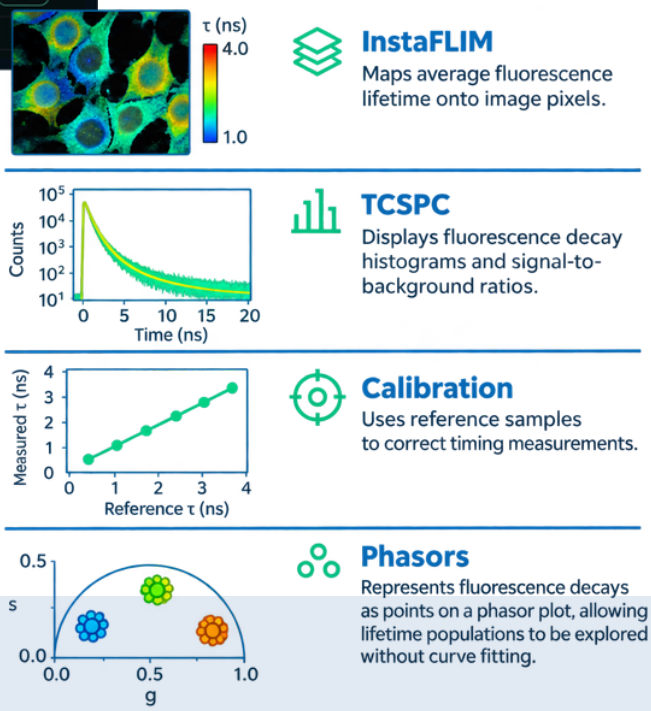



*Figure 1. FLIM IMAGER's four analysis tools: InstaFLIM for lifetime mapping, TCSPC for decay histograms, Calibration for timing correction, and Phasors for lifetime population analysis. Illustrative representation.*

# FLIM IMAGER: From Photon Data to *Lifetime* Images

***Real-time acquisition, image reconstruction, and phasor analysis in one software platform.***

MILLIONS of photons, each arriving at a different time. How do you turn them into a meaningful fluorescence lifetime image? FLIM IMAGER combines acquisition, reconstruction, and analysis in one interface. Working with the FLIM LABS Data Acquisition Card, it supports up to eight channels, real-time imaging, and post-processing. A guided wizard simplifies hardware setup and laser synchronisation.

Four reconstruction modes (PLF, PF, LF, and F) accommodate different scanning configurations, including Abberior STEDYCON systems.

For data exploration, FLIM IMAGER offers adjustable colormaps, contrast, magnification, and ROI analysis. Its Scouting mode helps inspect acquisitions, while FLIM mode provides four dedicated analysis tools — InstaFLIM, TCSPC, Calibration, and Phasors — as illustrated in Figure 1.

Additional features include phasor filtering, clustering, 2D colormaps, and multi-component unmixing. Data can be exported with metadata and Python or MATLAB scripts for further analysis.

# LIFETIME

THE FLIM MAGAZINE



## Turn light into insight

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