FLIM Delivers Intracellular Images Based on Differences

Fluorescence lifetime imaging microscopy derives information from the decay rates of individual fluorophores. It is opening up understanding of cell metabolism, inner cell reactions and cell death.

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BY MARCIA STAMELL
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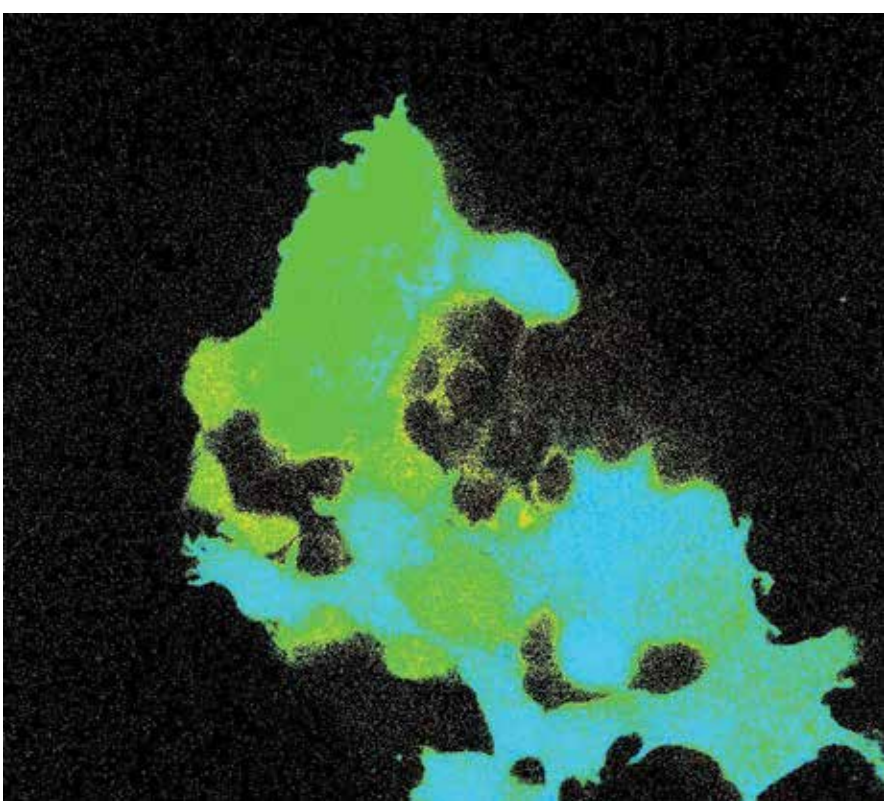
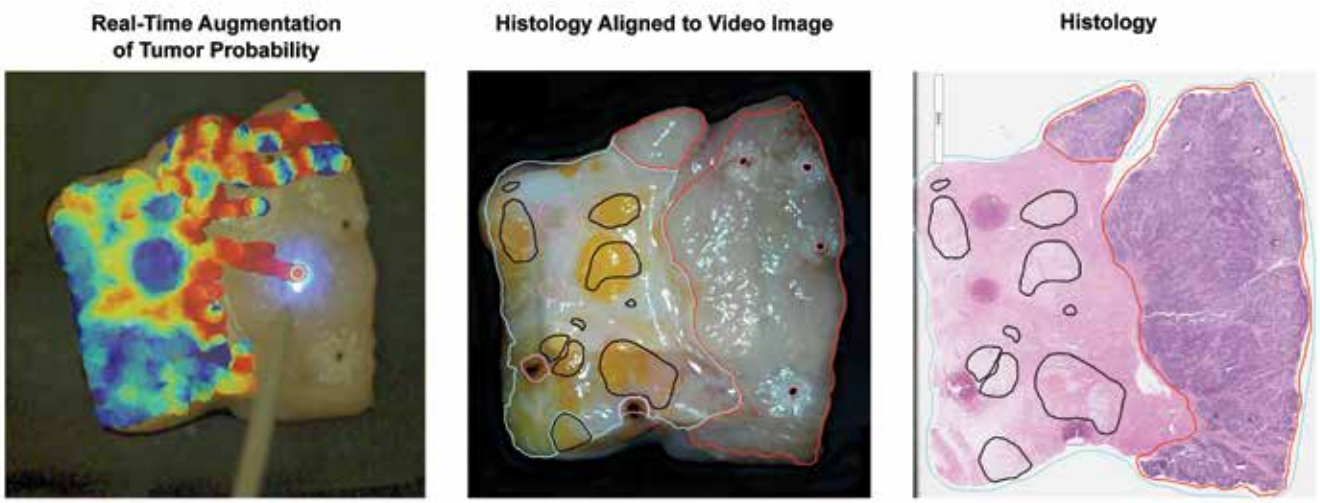
Fluorescence lifetime imaging microscopy (FLIM) enables researchers in the life sciences to get information from live specimens about interactions on the molecular scale. The technique captures the differences in the excited state decay rate from a fluorescent sample, rather than relying on the concentration of a fluorophore. Since imaging does not derive from the intensity of a signal, the technique lessens the impact of photon scattering in thick layers of sample and is generally considered more robust than intensity-based methods. This can open doors for both researchers and clinicians.

To gain a deeper understanding of what FLIM has to offer, we asked a panel of academic researchers and industry experts to talk about the strengths, weaknesses and future of this powerful imaging tool. FLIM has a range of applications — from tracking the effects of drugs in real time and guiding measurements during surgery to detecting energy transfer from FRET (Förster or fluorescence resonance energy transfer). Accordingly, the responses we received were rich, varied and detailed. A partial selection is printed in these pages.



A mixture of HEK cells depicting the results from a FLIM-FRET experiment. The blue area shows an average fluorescence lifetime of 1.8 ns, while the green area shows an average fluorescence lifetime of 2.3 ns. This means that the blue cells show more FRET than the green cells. Courtesy of Prof. Fred S. Wouters and Dr. Gertrude Bunt, University Medicine, Göttingen and Gerhard Holst, PCO.



Breast tumor specimen with FLIM-derived heat map **(left)** shows regions highly suspicious for cancer in red. A white light image of the specimen **(center)** overlaid with the pathologist's analysis of the corresponding histology section **(right)**. The overlay of the pathologist's analysis shows cancer outlined in red, adipose outlined in black and fibrous tissue outlined in aqua. Courtesy of Jakob Unger from the Marcu Lab.

The text with all the answers from our expert panel can be accessed at **www.photonics.com/A61901**.

Q: What applications of fluorescence lifetime imaging do you think are flourishing and why? If you are a researcher, how has FLIM particularly suited your research needs?

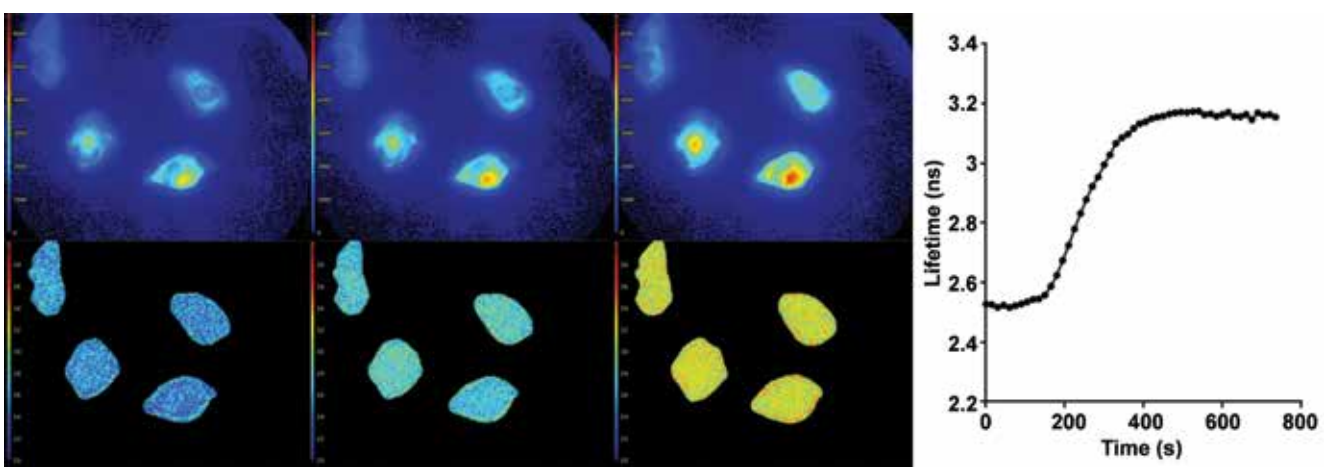
Lopez: Most of what we see these days is one of three FLIM applications: FRET FLIM, observing changes in the intracellular environment and spectral unmixing.

FRET FLIM is the change in fluorescence lifetimes due to the presence of energy transfer from FRET. In the presence of FRET, you see shortened donor molecule lifetimes as energy is donated to the acceptor. This provides a quantitative method of verifying that FRET is occurring. FRET FLIM is powerful because it provides data independent of dye or protein concentration, photobleaching and excitation light intensity — that is, laser power.

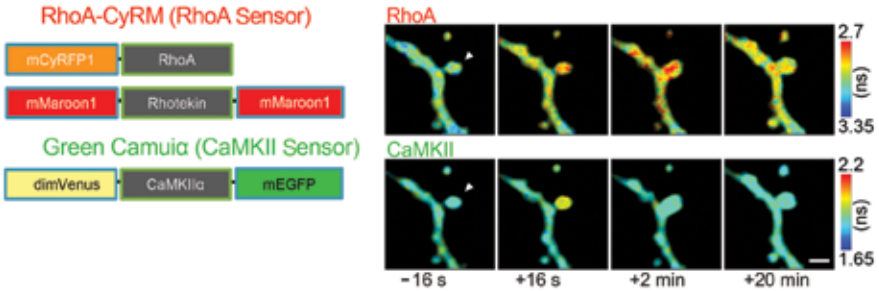
Quantitative FLIM studies are sometimes done to record changes in the intracellular environment around the fluorescent molecules of interest. If an environment becomes more acidic, or if the researcher fixes the cell, the lifetimes change. FLIM allows the scientist to watch the physiology of the cell in terms of pH or redox state changes or other perturbations of the cell.

The third application is spectral or channel unmixing. We often separate fluorescence molecules based on spectrum (bandwidth). But if there is a lot of spectral overlap, true spectral methods' utility breaks down. FLIM can provide a useful alternative, for instance, when a researcher is trying to view RFP [red fluoresecent protein] or GFP [green fluorescecent protein] and a lot of autofluorescence is present. In these cases, which often occur especially in brain and plant tissue, FLIM allows the researcher to separate unwanted autofluorescence from desired fluorescence based on lifetimes, in order to measure only the desired fluorescence.

Holst: As a researcher, FLIM has helped me a lot in the field of optical chemical sensing since all calibrations on fluorescence intensity require an incredibly stable lightfield. Any change in inten-



Light intensity images **(top row, colorized)**. Corresponding fluorescence lifetime images **(bottom row, colorized)**. The average fluorescence lifetime of the cells increases over time, as shown in the graph on the right. Courtesy of Lambert Instruments



This image was taken using dual-color FLIM experiments, showing real-time imaging of two biosensors in brain tissue. Schematics of CaMKII-alpha and RhoA-CyRM FLIM sensors **(left)**. Simultaneous fluorescence lifetime images of RhoA and CaMKII activation in a dendritic segment of a CA1 pyramidal neuron in an organotypic hippocampal slice, acquired with 2-photon FLIM **(right)**. Courtesy of Tal Laviv et al. Simultaneous dual-color fluorescence lifetime imaging with novel red-shifted fluorescent proteins. *Nature Methods,* 13 (October 2016).

sity, including those that have nothing to do with a change of the analyte concentration, would be nevertheless interpreted as a change of the analyte concentration, while the fluorescence lifetime is a much more stable parameter. The field of FRET will benefit a lot, because FLIM also allows faster access and measurement for experiments and investigations, which use FRET as the method to investigate cell metabolism, inner cell reactions and so on. Further, FLIM can be potentially used for tissue differentiation without staining. Lots of biological tissues have their own fluorescing molecules which might be used for FLIM.

Boppart: Increasingly, FLIM is being used to differentiate tissue autofluorescence between healthy and diseased states, and Dr. Laura Marcu's work for intraoperative FLIM is showing much promise. For my own research, FLIM is being used to track drugs and the metabolic changes and effects from these drugs in real-time, label-free, and even in human clinical studies. We are also using autofluorescence-based FLIM to track cell-death processes such as apoptosis and necrosis, all label-free and now in vivo. Autofluorescence FLIM is an excellent label-free method to provide added contrast about the metabolic state and microenvironment of cells and tissues.

Phipps: FLIM is flourishing in biomedical applications due to its ability to clearly distinguish the biochemical differences between diseased and normal tissue in a manner that can be implemented intraoperatively and nondestructively. In the Marcu Laboratory at the University of California, Davis, we are using fluorescence lifetime imaging to study human disease, in particular cancer and cardiovascular disease. We are incorporating FLIM with a Da Vinci surgical robot to acquire measurements during head and neck cancer surgeries. The goal of this project is to provide surgeons with a tool to guide surgical resection of tumors.

We are also conducting several studies that involve imaging tumors removed from patients with breast and prostate cancer. Our goal is to better understand these cancers to design an intraoperative FLIM tool for surgeons treating these diseases as well.

Additionally, we have incorporated FLIM into an intravascular ultrasound catheter for investigating coronary arteries. We have performed in vivo studies with this catheter in pigs as well as ex vivo studies of human coronary arteries.

Q: What improvements to the technology do you see on the horizon? Similarly, are there any obstacles to wider use or better performance of FLIM that will remain in place for the foreseeable future?

Lin: As with many leading-edge imaging techniques, FLIM needs to become cheaper and easier and widely supported by commercial microscope manufacturers before it can be widely used. Most published uses of FLIM come from labs with expertise in optics or microscope development. While there are a few commercial vendors of FLIM equipment, the combination of expense and complex or custom software has made FLIM a hard sell.

FLIM may be clearly the better way to do FRET, but the less quantitative FRET measurement method of ratiometric imaging requires only a standard fluorescence setup and is more intuitive to the novice. For FLIM to become popular, it thus has to become cheaper and easier to implement, and preferably training has to be supplied by the big microscope distributors. The last condition is difficult to achieve as there [are] a limited number of labs that truly need FLIM.

Orthaus-Müller: An interesting development lies in the combination of FLIM with other characterization methods such as spectral, dynamic or even topological information from atomic force microscopy (AFM). Such combined measurement methods open promising prospects by accessing different information types from the same sample area in a single experiment. For example, acquiring topological information from AFM and molecular behavior as detected by FLIM was previously limited to correlative experiments, requiring large amounts of statistics especially for heterogeneous biological samples. With a combined FLIM-AFM setup, as can be realized by interfacing the MicroTime 200 microscope to an AFM, the data are acquired in a simultaneous and correlated manner.

Extending the spectral range of FLIM into the deep UV is also of great interest since many biological molecules feature naturally occurring chromophoric groups such as tyrosine or tryptophane. By using UV lasers and adequate optical elements, one can exploit this native fluorescence, reducing the need to label such molecules with dyes. In contrast to intensity imaging, FLIM enables [differentiation of] the (auto) fluorescence in UV spectral range based on its different contributions.

Increasing the optical spatial resolution is always welcome, as it permits a more precise localization of molecular species or events. The resolution of FLIM can be improved by applying the stimulated emission depletion (STED) method (resulting in a method called FLIM-STED), which permits reaching lateral resolutions below 50 nm.

Last, but not least, speeding up FLIM data acquisition in the time domain is highly desirable for studying processes in living cells. Optimized hardware components allow using much higher detection count rates, while reducing artifacts due to the pile-up effect.

Herz: FLIM can be divided into two domains: the frequency domain and the

time domain. For the time domain, the improvement on the horizon is faster detection due to scalability of parallel detection; on the frequency domain, solid-state sensors.

Where in the past an image intensifier was needed for the high-frequency modulation, nowadays image sensors can directly be modulated, replacing the old image intensifier. This gives higher spatial resolution, and it becomes a more affordable solution.

Boppart: With improvements, our focus has been on faster methods for real-time FLIM. With these capabilities, we are able to capture FLIM over larger areas of tissues in real time. This helps in a number of drug-delivery and efficacy applications where real-time feedback is essential, and where dynamic changes are happening quickly in the cells and tissues. With fast FLIM, we are also able to capture very fast dynamic events over a limited spatial extent, such as the changes occurring in single neurons or neural circuits. As for obstacles, there will always be a need to capture FLIM images and data at faster rates and at higher resolutions, similar to other optical imaging challenges. Innovative methods continue to emerge, such as from the work of professor Liang Gao at the University of Illinois at Urbana-Champaign leveraging his high-speed imaging techniques for FLIM as well.

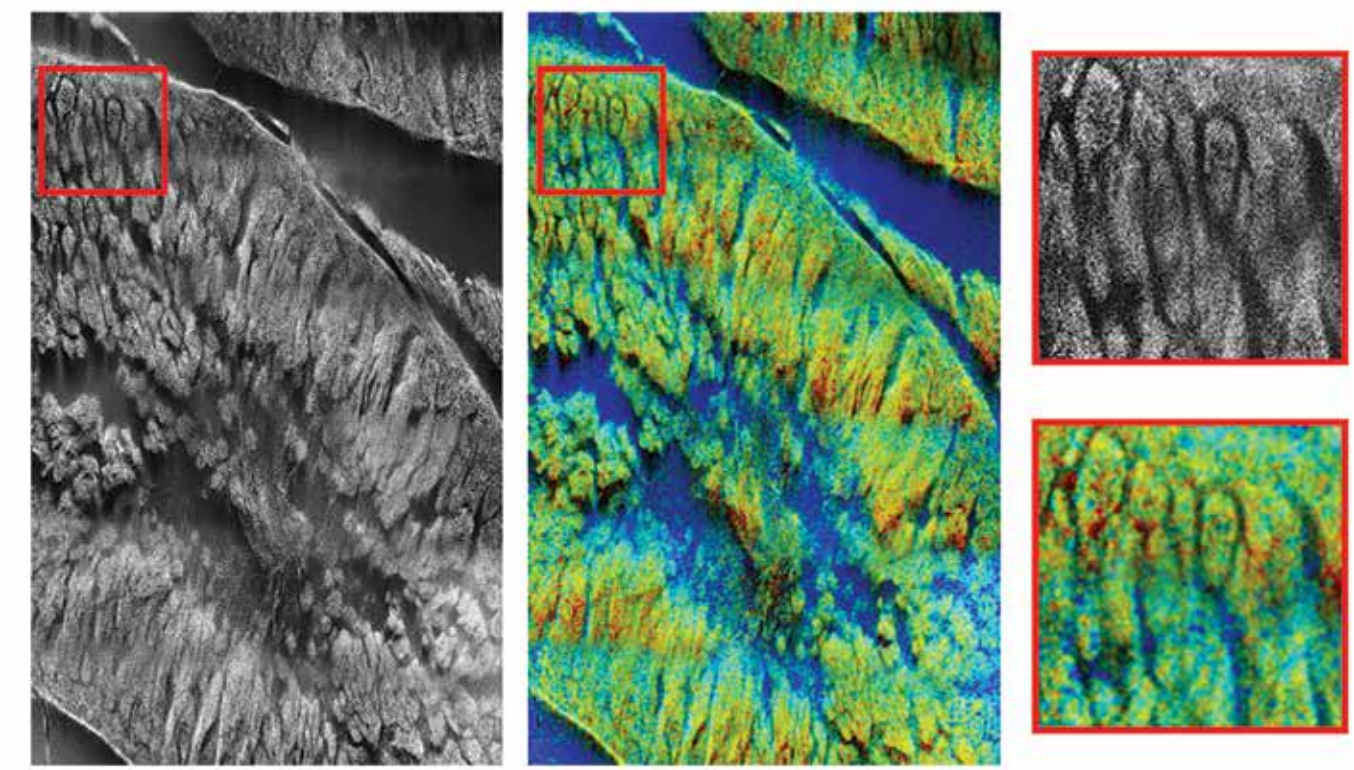
FLIM applications continue to flourish across a wide range of imaging and diagnostic applications, particularly where there are overlapping fluorescence intensity challenges from either autofluorescence signals or from exogenous fluorescent probes.

— Stephen Boppart, Beckman Institute

Q: What is the next frontier FLIM? What potential remains unfulfilled?

Holst: Certainly there is room left for measurements in the picosecond time range with a high frame rate. With scanning systems, it is possible but not so fast, but with imaging systems it is a bit to the limit. First papers have been published with extremely fast CMOS image sensors, and it'll be interesting to see the performance of these sensors. Sensitivity also could be improved as well as resolution in combination with a decent frame rate. Once FLIM is more widely applied, new applications will follow, I am sure.

Phipps: Besides the use of FLIM intraoperatively for head and neck cancer surgeries such as the procedures conducted at our lab, there are many other surgical procedures that could benefit from the guidance of FLIM measurements through flexible fiber optic probes. FLIM can also be added to existing clinical imaging systems to make them more powerful and specific to biochemical composition of tissue. Bi-modal imaging is proving to be very promising for improved detection of diseases since it allows for multiple aspects of a disease to be studied in a single



Two-photon autofluorescence **(gray-scale)** and corresponding fluorescence lifetime imaging microscopy (FLIM) images **(color-scale)** of unstained rat testis tissue captured with fast-acquisition FLIM. The color-scale variations in the FLIM images show cell-to-cell variations in fluorescence lifetime, indicative of different metabolic activity or microenvironmental changes. Courtesy of Biophotonics Imaging Laboratory, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign.

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— Michael Z. Lin, Stanford University

measurement. Clinical application of these bi-modal imaging techniques could provide very unique tools for surgical or diagnostic value.

Orthaus-Müller: A very fascinating development is anti-bunching imaging, which allows quantifying the number of emitters per pixel, and in turn makes it possible to obtain information about fluorophore concentration in a biological sample through fluorescence.

Another emerging field is the development of FLIM cameras, which are an interesting way to increase acquisition speed. A FLIM camera allows recording a full FLIM image at once instead of scanning the sample area point by point as in conventional FLIM.

Lopez: In the past, FLIM's temporal resolution was slow. Today, it's much faster; you can now do FLIM at several images per second, which has opened up live cell applications. Of course, FLIM resolution drops as you go to faster image capture rates. So the development of even faster FLIM systems with higher resolution would expand research horizons even farther.

FLIM is powerful but it remains a niche application, used in a relatively small number of laboratories overall. The holy grail of FLIM systems would be a low-cost, easy-to-use system that provides readily interpretable results. Science and technology still have a way to go to get there.

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