[33] Fluorescence Lifetime Imaging Microscopy: Two-Dimensional Distribution Measurement of Fluorescence Lifetime

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Abstract

A newly developed fluorescence lifetime imaging microscopy (FLIM) system has combined the high-temporal resolution of a streak camera with the high-spatial resolution of a microscope to obtain a two-dimensional distribution of fluorescence lifetimes within living cells. The temporal resolution is as short as 20 ps. The effective field of view is 48×45 μm with 0.2 μm resolution using a 60× water immersion objective. Image acquisition time is as short as 3 s per image. Measured and published values of lifetime for standard fluophores are shown with good agreement. Examples of FLIM and fluorescence resonance energy transfer images are presented.

Introduction

Fluorescence is the emission of a longer wavelength photon from a molecule as it returns to its ground state energy level after being excited by a shorter wavelength photon. If a short-pulsed light source is used for excitation, an exponential decay of the fluorescence intensity will be observed, as in Fig. 1. The decay of the fluorescence intensity over time is characteristic of each fluorescent molecule and is known as the fluorescence lifetime (t_F).

Unlike intensity-based measurements of fluorescence, the lifetime (t_F) is constant, irrespective of concentration, path length, and illumination variations within the sample. However, very subtle changes in the local environment of the molecule, such as pH, ion concentration, polarity, and viscosity, can produce changes in the lifetime. Molecular nonradiative energy transfers between fluorescent molecules will produce lifetime changes that are detected easily.

Fluorescence lifetime imaging microscopy (FLIM) extends this powerful technique into the microscopic domain. High-resolution imaging of intracellular structure can now include changes in the fluorescent lifetime of fluorescently labeled components. Structural, biochemical, and photodynamic processes can be observed in living cells, as shown in Fig. 2. Fluorescence resonance energy transfer (FRET) benefits from the high temporal resolution and speed of the system. Observation of

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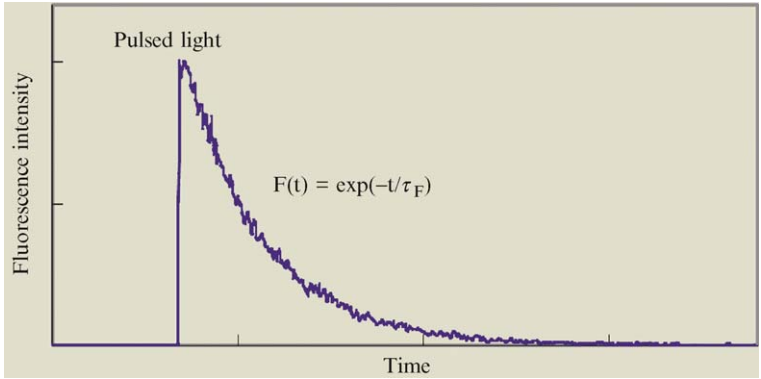

FIG. 1. Typical fluorescence decay curve.

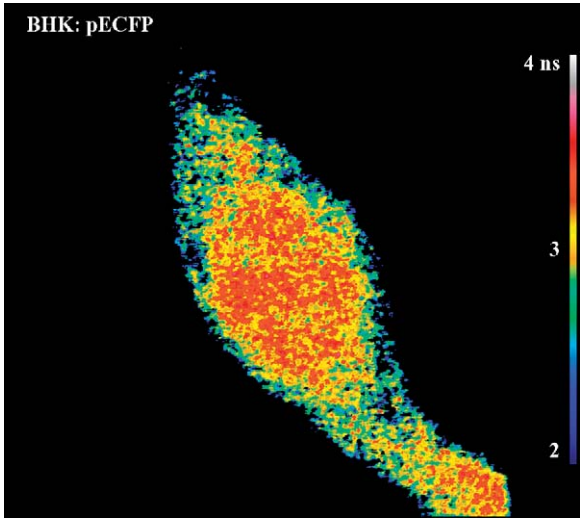

FIG. 2. Typical FLIM image.

protein–protein interactions in living cells, among other biological imaging applications, offers new possibilities in research.

Operating Principle of a Streak Camera

The streak camera is key to the FLIM system, which detects ultrafast light phenomena while converting it to spatial information in the form of a streak image. The operating principle is shown in Fig. 3.

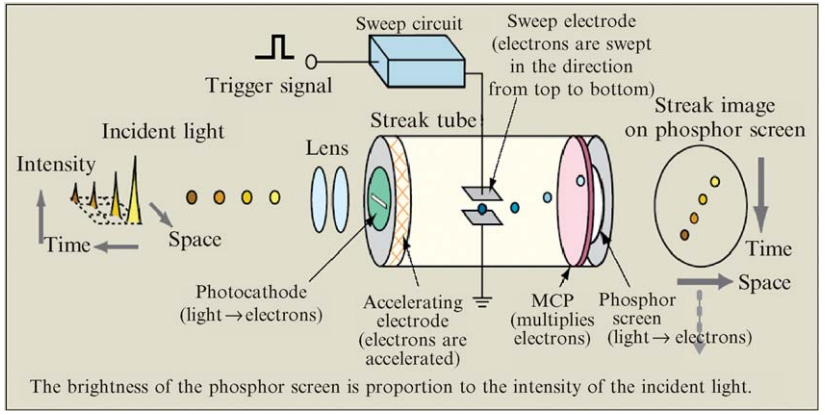

FIG. 3. Schematic of streak camera.

For illustration purposes, four pulses of light with varying intensity are separated in time and space and input to the streak camera. The pulses are projected onto the slit-shaped photocathode through a relay lens and are converted to electrons sequentially by a photocathode. The electrons are accelerated and deflected by a pair of high-voltage sweep electrodes. During the sweep, the electrons are deflected at slightly different angles depending on the arrival time at the electrodes. The electrons are then multiplied by a microchannel plate (MCP) and converted back to photons by a phosphor screen to form a streak image. The streak image displays four optical pulses across the vertical direction according to the arrival time to the streak camera. The earliest pulse is located at the uppermost position, and the latest pulse is located at the bottommost position. The spatial position of each light pulse is maintained in horizontal direction, as well as the light intensity, or number of photons within each pulse. A high-speed CCD camera is used to digitize and transfer the streak image to a PC computer for lifetime processing.

Conventional techniques for time-domain imaging such as multigate detection can provide a temporal resolution of a few hundred picoseconds. The time-correlated single photon counting technique can have a temporal resolution as short as tens of picoseconds; however, long acquisition times can prohibit applications, such as live cell imaging. The streak camera in the FLIM system provides 20 ps temporal resolution at multiple positions simultaneously, providing both high-temporal resolution and high-speed image acquisition.

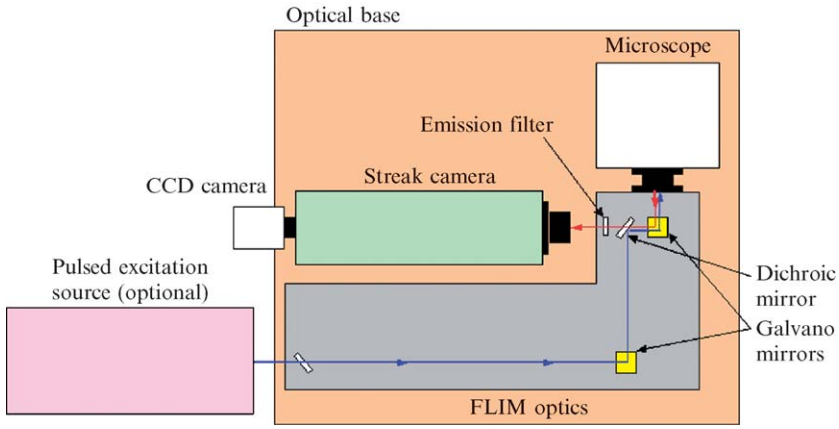

FIG. 4. Configuration of FLIM system.

Configuration of the FLIM System

The configuration of the FLIM system is shown in Fig. 4, including a pulsed excitation source, microscope, streak camera, and FLIM optics. A pulsed laser such as a mode-locked Ti:Sapphire or gain-switched diode laser is used for the excitation source. The FLIM optics and streak camera are attached to the side port of the microscope. The FLIM optics include two galvano mirrors that deflect the excitation light over the sample in two directions, both X and Y, while the streak camera captures the fluorescence emission that pass through the dichroic and emission filter. The CCD camera digitizes and transfers the streak image to a PC computer for processing.

Streak Image

A fluorescence intensity image (left side) and a typical streak image (right side) are shown in Fig. 5. For illustration purposes, a single line is extracted from the intensity image and displayed as a streak image. The vertical axis in the streak image represents the temporal content, or fluorescence decay data within this single line.

Measurement Principle of FLIM System

The measurement principle of the FLIM system is illustrated in Fig. 6. One galvano mirror scans the excitation light in the horizontal X direction and aligns with the horizontal input slit of the streak camera. The streak

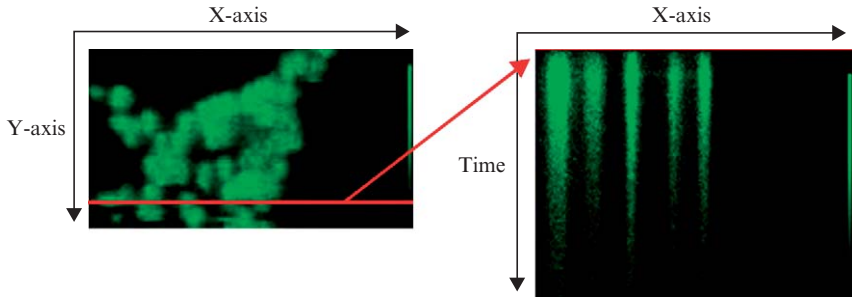

FIG. 5. Fluorescence intensity image and streak image.

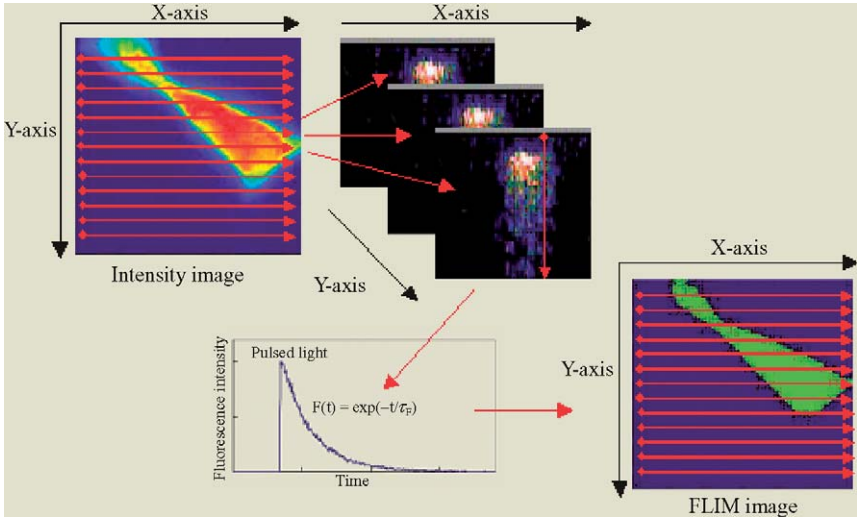

FIG. 6. Fluorescence intensity image and FLIM image.

camera captures the fluorescence light associated with each spatial position in this horizontal X axis. The second galvano mirror scans the excitation light in vertical Y direction and is synchronized with CCD readout. The scan rate per line is limited by the readout speed of the CCD camera, which in this case is 5 ms. A streak image stack is collected and stored in a PC memory buffer. The fluorescence lifetime is calculated from the exponential decay profile at each location along the X axis. The FLIM image is constructed by plotting the lifetime at each location along the horizontal X axis and vertical Y axis.

The effective field of view is 48×45 μm with 0.2 μm resolution, using a $60\times$ water immersion objective. The shortest image acquisition time is 3 s

for full area with 600 horizontal lines. The temporal resolution is typically less than 20 ps for a 1-ns full timescale.

System Calibration

Standard fluophore solutions of various fluorescence dyes are used for the system calibration. Lifetime images and histograms are shown in Fig. 7: rhodamine 6G in ethanol (a and b), rose bengal in acetone (C and D), and rose bengal in ethanol (E and F). Solid lines in the histograms are Gaussian fits to data. Measured and published (in parentheses) values of lifetime are shown in the histograms, and there is a very good agreement among them.

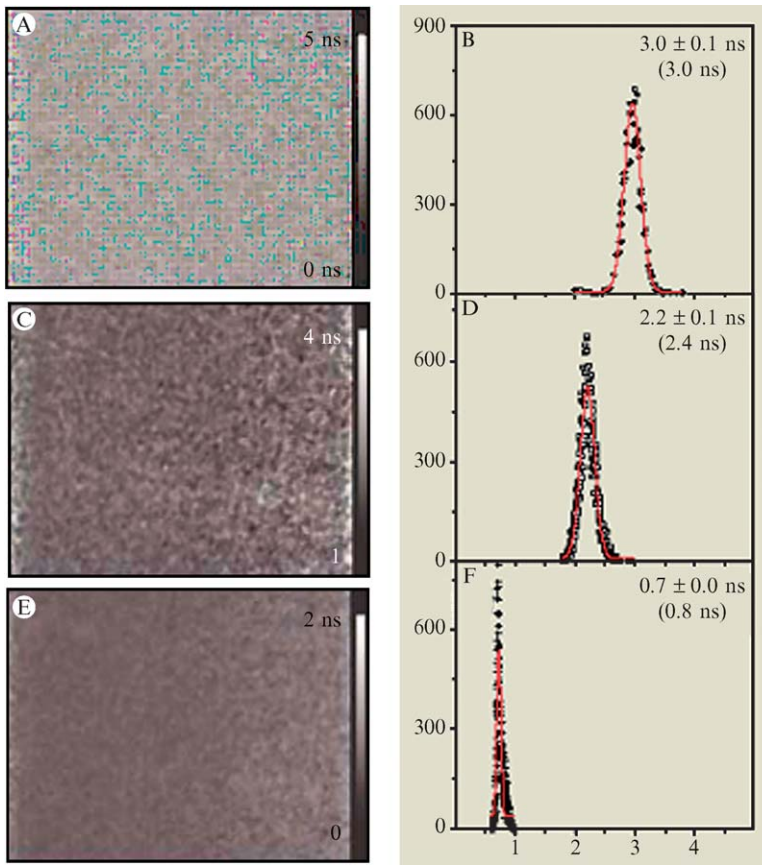

FIG. 7. FLIM image and histogram (Krishnan *et al.*, 2003a).

Lifetime Imaging in Cells

Lifetime images of BHK cells expressing cytosololic ECFP (Cyto-ECFP) and fusion protein Bax-ECFP (Cyto-BaxECFP) are shown in Figs. 8 and 9 (Krishnan *et al*., 2003b). The mean lifetime values of Cyto-ECFP and Cyto-Bax-ECFP are 3.2 ± 0.3 and 2.8 ± 0.3 ns, respectively.

Lifetime images of cells expressing mitochondrial targeted ECFP (Mito-ECFP) and membrane-targeted ECFP (Mem-ECFP) are shown in

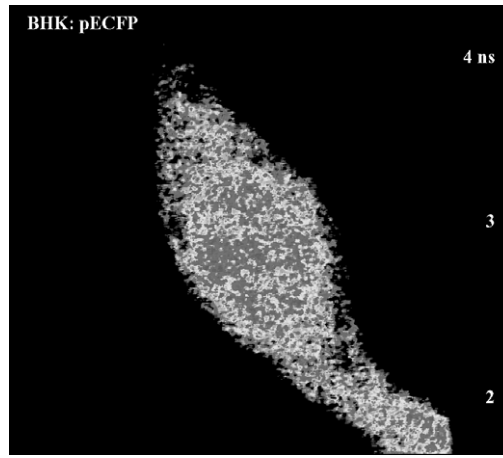

FIG. 8. Cyto-ECFP.

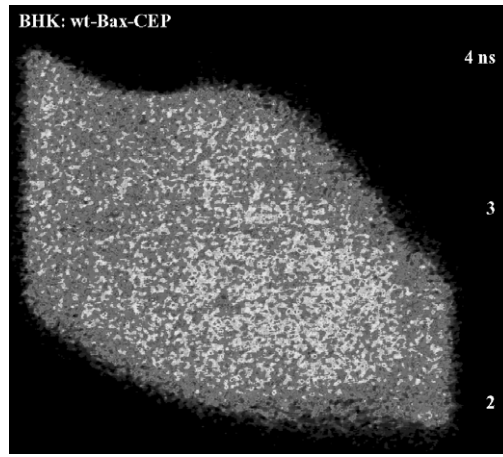

FIG. 9. Cyto-BaxECFP (Krishnan *et al*., 2003b).

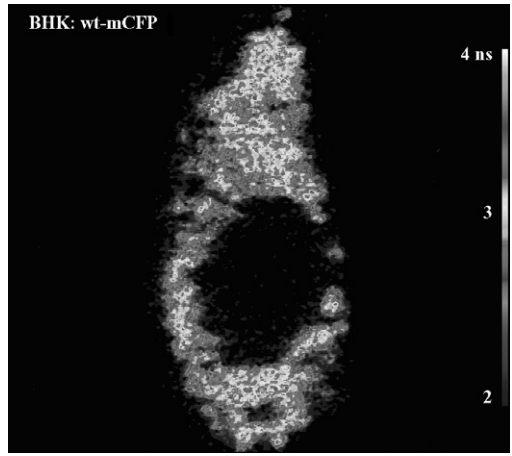

FIG. 10. Mito-ECFP (Krishnan *et al*., 2003b).

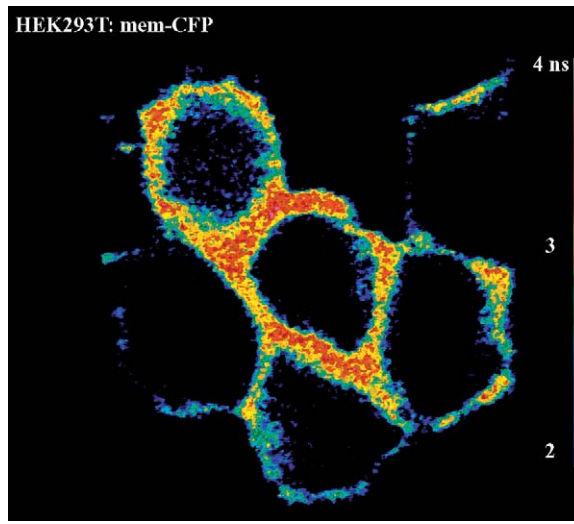

FIG. 11. Mem-ECFP (Krishnan *et al*., 2003b).

Figs. 10 and 11 (Krishnan *et al*., 2003b). Both mean lifetime values of Mito-ECFP and Mem-ECFP are 2.9 $\pm$ 0.1 ns.

FRET Imaging in Cells

Fluorescence resonance energy transfer is a distance-dependent non-radiative energy transfer interaction between two electronic excited states

of fluorescent substances. Excitation energy is transferred from donor to acceptor fluorescent substances. Using this phenomenon, real-time imaging of protein structural dynamics, protein–protein interactions, enzyme activity, and structural change can be observed and quantified.

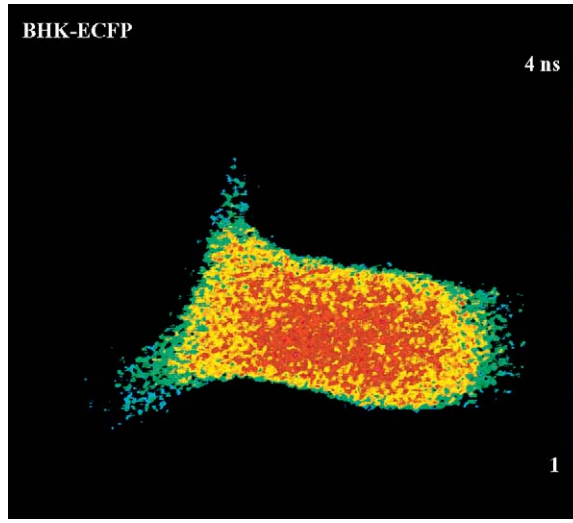

FIG. 12. ECFP (Herman *et al.*, 2003).

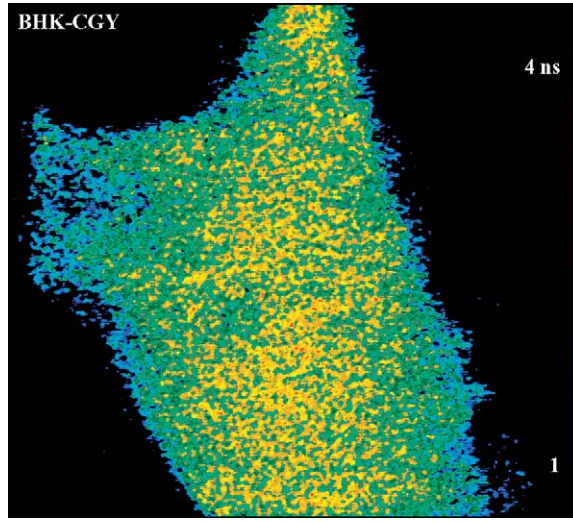

FIG. 13. CGY (Herman *et al.*, 2003).

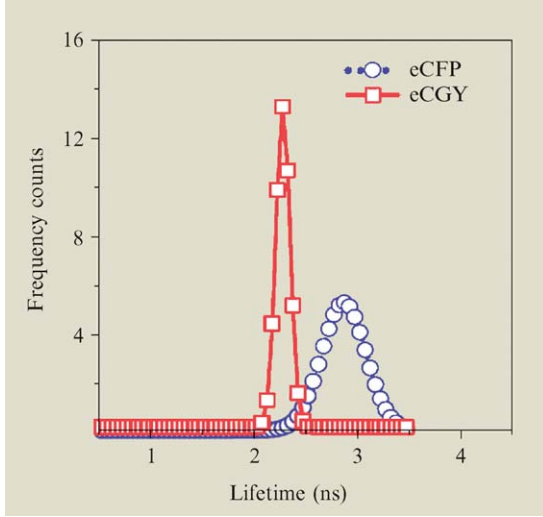

FIG. 14. Histogram (Herman *et al*., 2003).

Lifetime images of BHK cells expressing ECFP, CFP-polyglycine-YFP, and the histogram of their lifetimes are shown in Figs. 12, 13, and 14, respectively (Herman *et al*., 2003). The mean lifetime of ECFP is 2.9 ns, whereas that of CGY is 2.3 ns. The decrease in mean lifetime of CGY compared to that of ECFP is due to the FRET process (energy transfer between CFP site and YFP site).

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