FlimFast – Fluorescence Lifetime Image Acquisition and Visualization at Video Rate

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Introduction

Fluorescence lifetimes provide valuable information that is not available from steady-state fluorescence. However, acquisition, processing and display of fluorescence lifetime images require much longer times than simple intensity images due to the complexity of the analysis and the visualization of multi-parameter information. Therefore lifetime imaging has not yet met the rapid continuous interactive operation required by many biological and medical applications.

We have developed instrumentation and software for continuous acquisition and visualization of fluorescence intensity and lifetime images at video rate. Rates up to 26 Hz are achieved for 380x220 pixel images depending on acquisition, analysis and visualization parameters as well as computer speed.

The instrument uses fast frequency-domain data acquisition hardware. The software enables continuous acquisition of fluorescence intensity and lifetime images and their concurrent visualization as multi-textured lit surface renderings. A menu of options is provided to the operator to assist in the on-line interpretation and interactive control of experiments.

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Acquisition and Analysis

We developed software to exploit the full potential of the fast frequency-domain lifetime imaging instrumentation. Camera, frame grabber, phase shifter, shutter and frequency synthesizers are software controlled allowing for flexible, operator controlled data acquisition modes. Data analysis and visualization computing tasks are distributed to separate processors if available.

The acquisition of a single lifetime image requires acquisition and processing of at least three intensity images at equidistant phases. This mode is limited by the instrumentation to 0.12 s or 8 Hz.

However, by continuously shifting the phase in 2π/K steps and analyzing the last K intensity images while the CCD camera integrates the last intensity image, continuous lifetime image acquisition and concurrent visualization can be as fast as 20Hz.

Setting phases in non-linear order can improve the accuracy of the digital Fourier analysis in case of bleaching of the fluorescent sample without the need for using more accurate but slower fitting routines. Specifically, averaging intensity images taken symmetrically in time corrects for linear bleaching.

Averaging signals in time (integration) and space (binning) improves signal to noise at the expense of time and spatial resolution. Software averaging is done at the level of the individual intensity readings, beforehand the lifetime analysis. Therefore binning of pixels also reduces the amount of data to be analyzed and displayed.

Masking data by regions of interests as well as upper and lower thresholds for intensity, phase and modulation, respectively lifetimes reduces the amount of data to be analyzed and displayed.

Software was developed in C++ using MFC (Microsoft) and OpenGL (sgi) in addition to interface card libraries.

Visualization

Lifetime analysis yields three or more image parameters. Multi-textured lit surface rendering and multi-dimensional histograms are used to visualize multi-parameter image information in addition to conventional indexed color bitmaps and one dimensional histograms.

Any image parameter can be used to modulate the height, define lighting or index one-dimensional color, contour and transparency textures at each point of a two dimensional grid. The grid is rendered as a surface with linear interpolation of height, lighting and texture indices in between the grid points.

Parallel Processing

Distributing analysis and visualization computing tasks on separate processors (Multi-threading) while the camera acquires the next intensity image significantly improves processing speed.

Frequency Domain

A fluorophore is excited with intensity modulated excitation light of radial frequency \( \omega \). The emitted fluorescence light is of same frequency but has a phase shift \( \Phi \) and amplitude demodulation \( M \) with respect to the excitation light depending on the fluorescence lifetime \( \tau \).

\[
\Phi = \frac{\omega \tau}{2} \quad \text{and} \quad M = \frac{\omega \tau}{4} 
\]

Analysis

A minimum of three fluorescence signals (K=3) at equally distant phases (2π/K) are required in order to perform a digital Fourier analysis. At fixed frequency only mean lifetimes can be calculated. However, in the presence of multiple components with different lifetimes \( \tau_i < \tau_j \).

Digital Fourier Analysis

\[
f(t) = \sum_{k=0}^{K-1} A_k \cos(\omega_k t + \Phi_k) + B_k \sin(\omega_k t + \Phi_k) 
\]

With

\[
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\]

Demodulation

A fluorescence standard of known lifetime is measured to determine the phase \( \psi_k \) and modulation \( M_k \) to reference. Setting \( \omega_k = \psi_k \) simplifies lifetime analysis to \( \tau = \frac{1}{\omega} F / F_{ref} \).

Instrumentation

Laser light is modulated at fixed frequency (40 or 80 MHz) by means of an acousto-optical modulator (AOM) and coupled into single mode fiber optics for guidance to a long working-distance low-magnification imaging system, a microscope or a medical endoscope. The fluorescence emission is imaged onto the cathode of a modulated image intensifier (Hamamatsu C3825) and detected by a fast chilled digital CCD camera (Hamamatsu C4880/811). Signals of phase locked frequency synthesizers modulate the AOM and image intensifier. A fast 9 bit digital delay line phase shifter (Lurgh Microwave) shifts the phase of the signal modulating the image intensifier cathode relative to the excitation light. This allows for homodyne detection of the high frequency fluorescence emission signal at any given phase.