Spectroscopic Innovations at the Laboratory for Fluorescence Dynamics

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ABSTRACT

The emergence of time-resolved fluorescence techniques has been very important to the study of biochemical, biophysical and biomedical research areas. Many of the advances in these fields have been technology driven. The Laboratory for Fluorescence Dynamics (LFD) has been actively involved in development of frequency domain technology for a variety of applications. Our recent work on microwave super heterodyning detectors, fluorescence lifetime resolved spectroscopy, frequency domain fluorescence imaging microscopy, fluorescence lifetime resolved stopped-flow kinetics and global methods of data analysis is summarized in this report. We provide examples of how these new technologies are applied.

1. Introduction

In 1986, the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana-Champaign was established as a research resource in biomedical fluorescence spectroscopy. The aims of the center are two-fold: first, provide a state-of-the-art laboratory for time resolved fluorescence measurements with technical assistance to visiting scientist/users and secondly, design, test and implement advances in the technology, especially in hardware, automation software and applications to the biomedical arts. Accomplishments in the second area are readily transferable to the service laboratory environment. This manuscript describes several technical innovations introduced at the LFD. The presentation deals with both hardware, software and discusses applications to biomedical science.

The appeal of fluorescence spectroscopy lies in its high sensitivity, which translates into low detection limits in analytical applications, and in its varied response to environmental factors, such as ionic strength, solvent polarity, proximity of charged or quenching groups and others. In research applications, especially for the biological sciences, the nanosecond time window of fluorescence spectroscopy correlates well with the temporal resolution necessary to observe events such as macromolecular conformational changes, receptor recognition, membrane dynamics and many others.

The time-resolved instrumentation in the LFD exploits the principle of the harmonic response. The exciting source is amplitude modulated (in a sinusoidal wave) at a prescribed frequency; typically, in the 1-500 MHz range to match the fluorescence lifetime of the sample. After excitation, the fluorescence signal is emitted with an amplitude modulation at the same frequency as the exciting light. However, due to the short, but finite lifetime of the fluorescence event, the fluorescence signal is phase shifted and amplitude demodulated relative to the exciting light. The frequency (f) dependent relationships between phase shift (ϕ) and amplitude demodulation (m) form the basis for the experimental determination of the fluorescence lifetime (τ):

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$$\tan \phi = \omega \tau \quad (1)$$

$$M = [1 + (\omega \tau)^2]^{-\frac{1}{2}} \quad (2)$$

where $\omega = 2\pi f$. A frequency sweep ensures detection of both fast and slow fluorescence lifetime components in heterogeneously emitting samples. Algorithms for analysis of complex lifetime decay schemes are available, based on a variety of exponential and distributed functions, and they are extensively discussed in the literature [1]. Experimentally, the relatively high frequency (typically 1-500 MHz) modulated fluorescence signal is converted to a lower frequency (typically in the Hertz region) through a heterodyning (cross-correlation) process in the detector. [2] Heterodyning retains the phase shift and amplitude demodulation information of the high frequency signal in the low frequency carrier. This frequency conversion step, with full retention of time-resolved information, is important because it allows electronic signal processing at an easily accessible frequency range [3,4], that is also compatible with the slower read out speeds of cameras, charge coupled devices (CCD) and other (multichannel) detectors.

2. Results and Discussion

A superheterodyning microwave phase fluorometer with femtosecond resolution.

In most modern time-resolved fluorometers, the pulsed laser (or synchrotron) sources can provide about a 10 GHz bandwidth in the excitation beam [5], but the light detector typically limits the bandwidth of the instrument. We have previously discussed how a heterodyning process in photomultiplier tubes, accomplished by injecting a radio frequency at the second dynode frequency converts the detector's signal to a low frequency of operation [3]. In the photomultiplier detector, this process effectively increases the anode bandwidth to roughly match that of the cathode. A new class of detectors, termed microchannel plate (MCP) photomultipliers (e.g. Hamamatsu R25664-01), is characterized by a high frequency response in excess of 10 GHz with diminished sensitivity in higher frequency regions. For MCP detectors, the anode bandwidth is essentially the same as the cathode and signal processing occurs directly at the output. The output of the MCP is first DC/AC split, and then subsequently amplified and connected to a ultra-wide band mixer, which provides for frequency conversion. The frequency translation occurs in two steps. The first step heterodynes the high frequency signal to an intermediate value of approximately 100 KHz, which is convenient for intermediate amplification and signal conditioning by ceramic high Q filters characterized by a bandwidth of a few KHz. The accuracy of the microwave local oscillator need only be a few KHz. The second frequency translation step heterodynes the 100 KHz down to 40 Hz, which is a convenient frequency for averaging and digital signal processing. This superheterodyning method substantially reduces the noise of the MCP output, thereby increasing the overall signal to noise. By independently controlling the AC and DC signal levels, it is not necessary to match these signal levels, which is important in accurately determining the demodulation level. A typical, laser-based, fluorometer employing this design is shown in Figure 1. In this design, there is a sample and a second reference channel (photodiode detector), which is characterized by high intensity. The reference channel in this configuration provides a phase locked loop and acts as a self-tracking filter. The phase locked loop automatically tracks frequency variations in the difference

between the laser repetition rate and the frequency synthesizer, thereby providing a stable and exact output of 40 Hz. The instrument presented in Figure 1 has a flat frequency response to about 4 GHz offers high sensitivity and is immune from synthesizer phase noise [6]. Recently, it has been used to study the photophysics of hemoglobin intrinsic fluorescence [7].

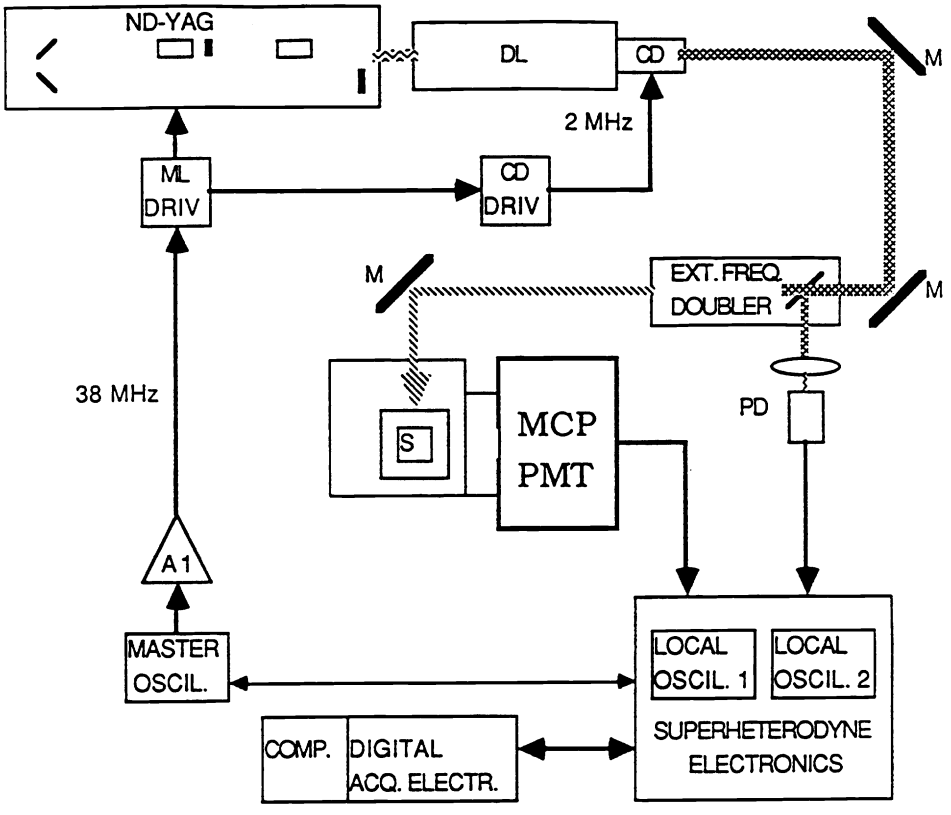


Figure 1. Superheterodyning, multifrequency, cross-correlation phase and modulation fluorometer. Light source: Coherent, model 701-3 synchronously pumped, cavity-dumped dye laser (DL) with Rhodamine 6G optics; cavity dumper (CD) running at 2 MHz and its driver (CD DRIV). Pump source: Coherent, model 76-S mode-locked Neodymium-Yttrium-Aluminum-Garnet (Nd-YAG) solid-state laser. ML-DRIV, mode-locker driver electronics. EXT. FREQ. DOUBLER, external frequency doubler converts 2 MHz, 5 psec (FWHM) pulse train from VIS to UV. M, front-surface mirror; S, thermostated sample compartment. Detectors: PD-Photodiode, 35 ps rise time Antel Optronics Inc., model AR-S2; MCP PMT, Hamamatsu, model R2566U-01, 6 μm micro-channel plate photomultiplier. A1 radio frequency amplifier, OSCIL, radio-frequency oscillators (synthesizer) connected with a phase-locked-loop. DIGITAL ACQ. ELECTR, digital acquisition electronics; COMP, process control and data analysis computer. For further details, see text.

Optical Multichannel Analyzer (OMA)

The instrument schematically presented in Figure 1 can also be configured with an optical multichannel analyzer (OMA) for signal detection. The advantage offered by the OMA is the simultaneous acquisition of the emission spectrum by a linear diode array and the wavelength resolved dependence of the phase shift and demodulation across the spectrum. A polychromator spatially disperses the wavelength dependent emission signal and after passing through the OMA each diode accumulates a given bandpass. The OMA detector (Princeton Instruments; IRY – 512 G/RB) consists of a photocathode followed by a gatable, proximity–focused microchannel plate (MCP) image intensifier, which is optically coupled to a 512 element diode array (Figure 2).

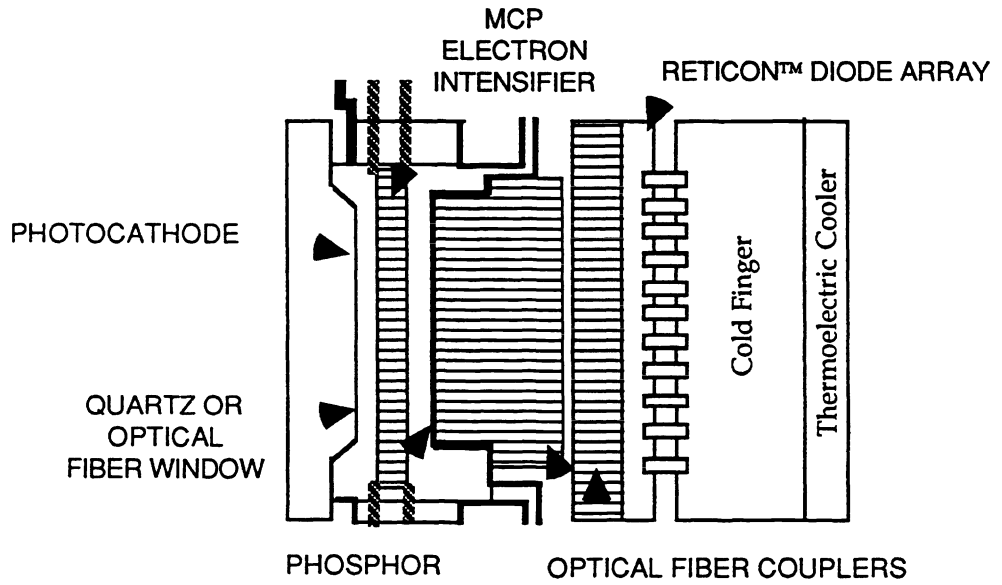


Figure 2. Diagram of the Princeton Instruments IRY optical multichannel analyzer.

We have modified the OMA for use in time resolved frequency-domain fluorometry by biasing the photocathode voltage, injecting a radio frequency sinusoidal voltage at the cathode and effectively varying the gain of the image intensifier [8]. This heterodyning step at the image intensifier translates the high frequency fluorescence signal to a low frequency. The bandwidth of the phosphor is approximately 1 KHz, effectively acting as a high frequency filter. The low frequency carrier is set at 7.5 Hz. The diodes are sampled at a read rate of 60 Mz and thus a waveform period is described by eight data points. This instrument configuration is capable of providing lifetime resolved spectra, as shown in Figure 3.

Fluorescence lifetime resolved microscopy

The frequency domain optical multichannel analyzer provides fluorescence lifetime information across a linear (wavelength) array. Extending this frequency domain concept to a two dimensional detector, such as a fast scan charge coupled device (CCD) camera, yields a time-resolved detector capable of fluorescence lifetime resolved microscopy. In fluorescence microscopy, the fluorescence signal emanating

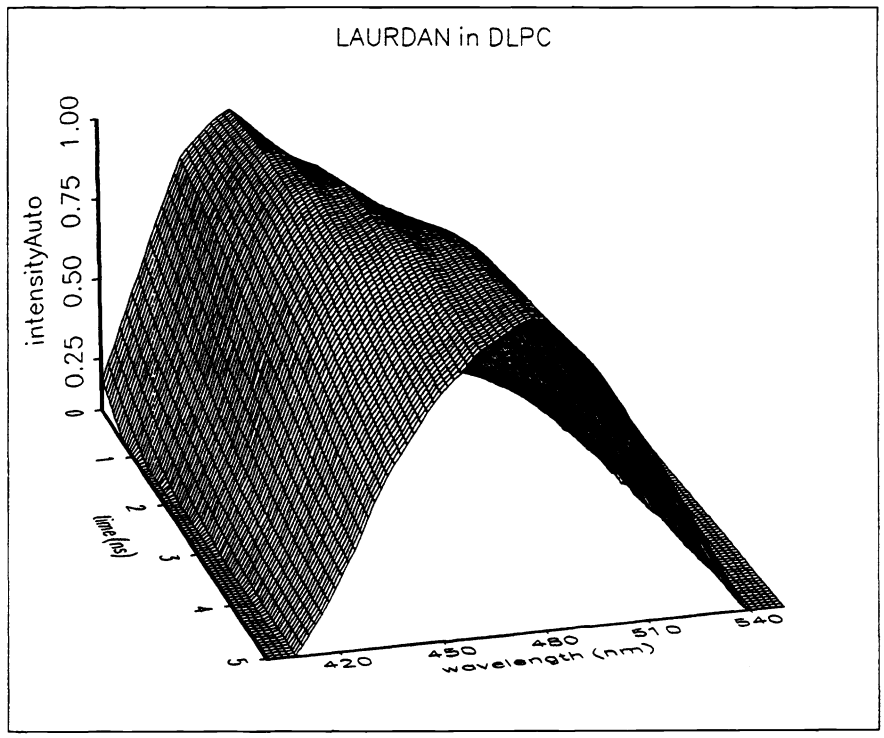


Figure 3.

from a spatially distinct feature (or region) of the cell is sensitive to the micro-environment of the reporter fluorophore. A time-resolved fluorescence microscope exploits this environmental sensitivity by providing a quantitative measure of important chemical information such as pH, calcium or other ion content, distribution of surface receptors and oxygen concentration. The fluorescence lifetime signal, unlike the fluorescence intensity, is an intrinsic property of the probe fluorophore and is thus insensitive to the unknown localized fluorophore concentration in the living cell. An additional advantage of the fluorescence lifetime imaging technique is the selective enhancement of image contrast of cellular morphology through a judicious selection of the appropriate (life)time window. Signal detection in our frequency domain, time-resolved, fluorescence microscope is based on a CCD camera operating in the fast scan mode to minimize deleterious photo bleaching effects that may introduce error in the lifetime measurement. The schematic representation of the lifetime imaging microscope in Figure 4 is similar to our conventional frequency domain fluorometer (Figure 1). The light source is either a cavity dumped, pyridine 1 dye laser optically excited by a mode-locked Nd:YAG laser (Coherent) or a CW argon ion laser (Spectra-Physics), which is frequency modulated in the MHz require with an acousto-optical modulator (AOM). The excitation signal is channeled to cellular specimen via the epiluminescence beam path of an inverted fluorescence microscope

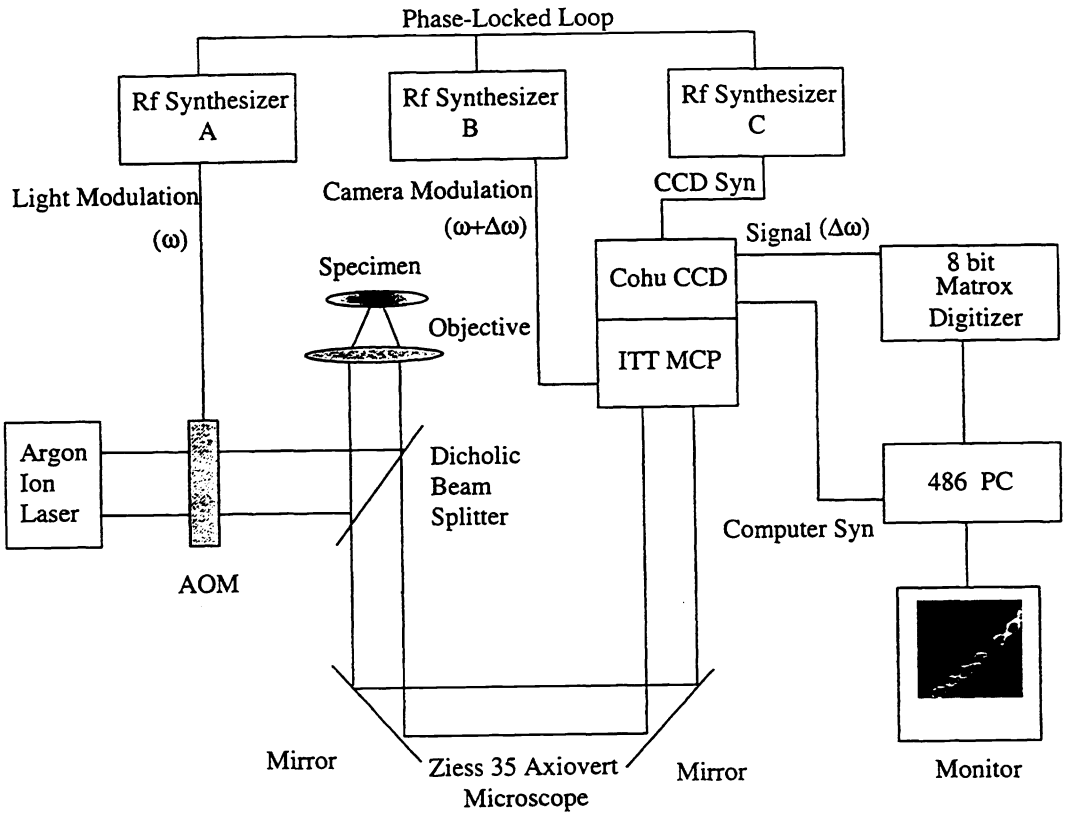


Figure 4. Schematics of the frequency domain time-resolved microscope.

(Zeiss Axiovert 35). The fluorescence signal is detected by a gain modulated, dual stage microchannel plate (MCP) detector (ITT type F4144), which is the two dimensional analog of the linear array described in the previous section about the OMA. The MCP is again the mixing stage in the heterodyning stage. It is modulated at a frequency (ω) equal to the laser excitation frequency, plus a small carrier cross correlation frequency (Δω), chosen at 7 Hz. We modulate the MCP up to 300 MHz, by providing a 50 v sinusoidal signal at its photocathode. This mode of operation degrades the spatial resolution of the MCP some what, but signal averaging and spatial correction algorithms reduce this difficulty [9]. The response of the phosphor screen of the MCP

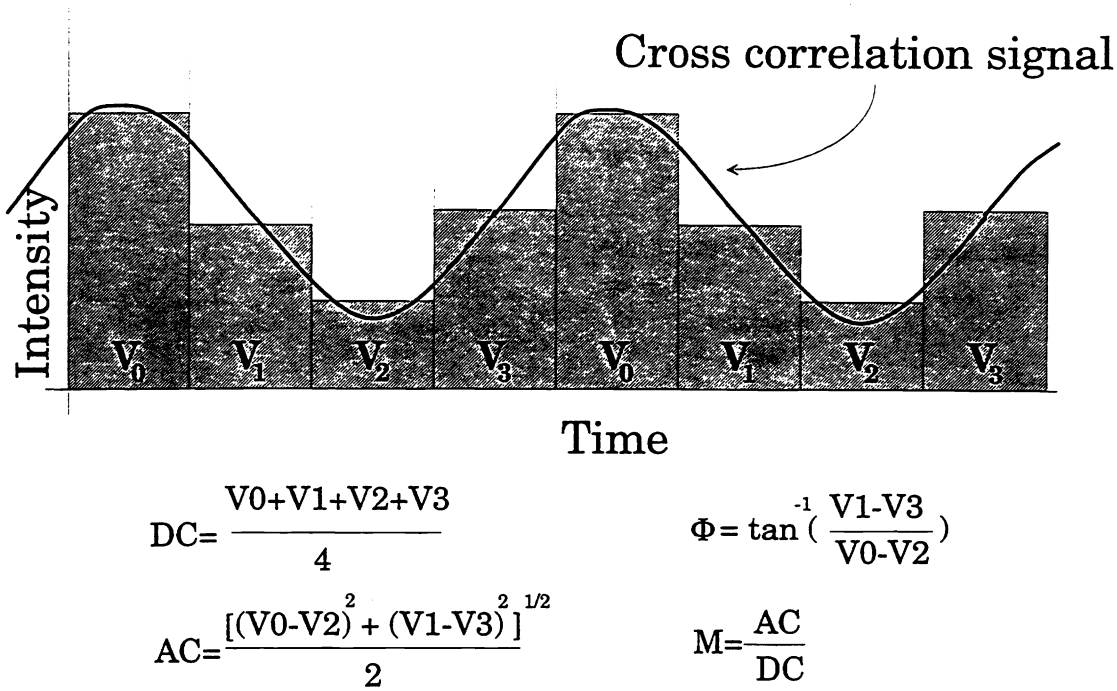


Figure 5. CCD Camera samples and integrates the cross-correlated signal at four points per wave form.

out is slow, effectively acting as a low pass filter for the cross correlation signal. Fiber optics couple the MCP to the CCD camera (Cohn 4812-2000/ER). The 7 Hz cross correlation signal is sampled at 28 Hz by the CCD, thereby providing four quadrants of the waveform period (Figure 5). This quadrature measurement is sufficient to characterize the modulation (AC to DC ratio) and phase (ϕ) of the waveform. Successive waveforms are synchronously digitized (at 8 bit resolution) and averaged (at 16 bit resolution) by a video rate digitizer and digital signal processor (Matrox, Image 1280), which is housed in PC computer (Gateway 2000, 486/66). Synchronous averaging effectively suppresses non-harmonic noise and increases the dynamic range of the system. Automated data acquisition, fast Fourier transforms for computing the frequency domain images and image display are under software control.

To demonstrate the contrast enhancement offered by the time-resolved imaging technique in fluorescence microscopy, Figure 6 presents a comparison between fluorescence intensity and lifetime maps of some fluorescent beads. The sample consists of fluorescent 2 μm orange FluoSpheres (Molecular Probes) suspended in saturated DCM laser dye (Exciton) dissolved in ethylene glycol. The FluoSpheres and DCM are intensity matched and discrimination of the spheres based on the intensity panel in Figure 6 is difficult. Lifetime discrimination is possible in the phase image,

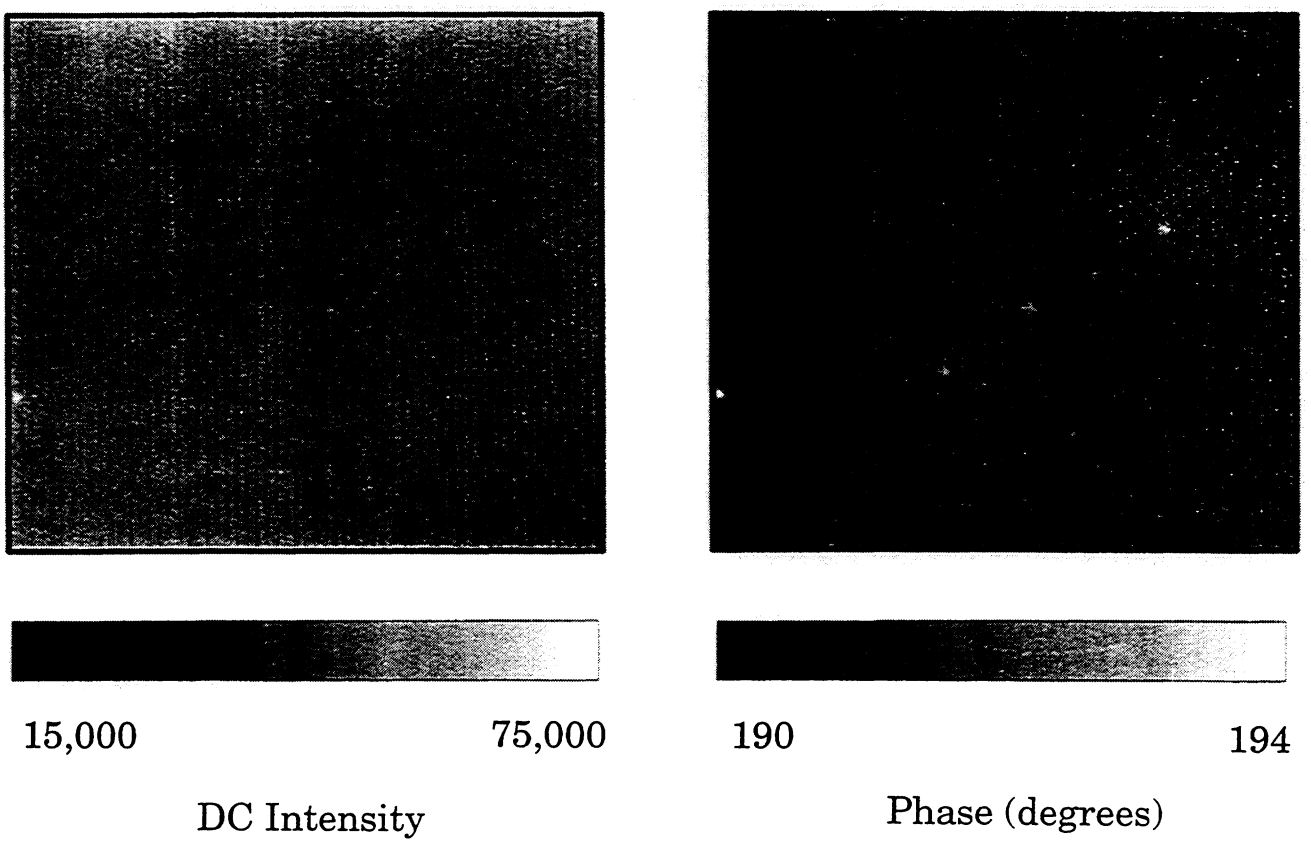


Figure 6. Intensity and phase-resolved images of 2μm FluoSpheres in DCM dye solution.

because the FluoSpheres and DCM have distinct fluorescence lifetimes (4.6 and 1.3 nsec, respectively). The experimental conditions in Figure 6 include excitation by the argon ion laser (514 nm) with modulation at 80.854 MHz, image collection via a 60 x Zeiss plan-Neofluar objective and synchronous image integration for 300 sec. The five FluoSpheres visible in the frequency domain lifetime image are contrast resolved even in the presence of a very high level of background fluorescence [10].

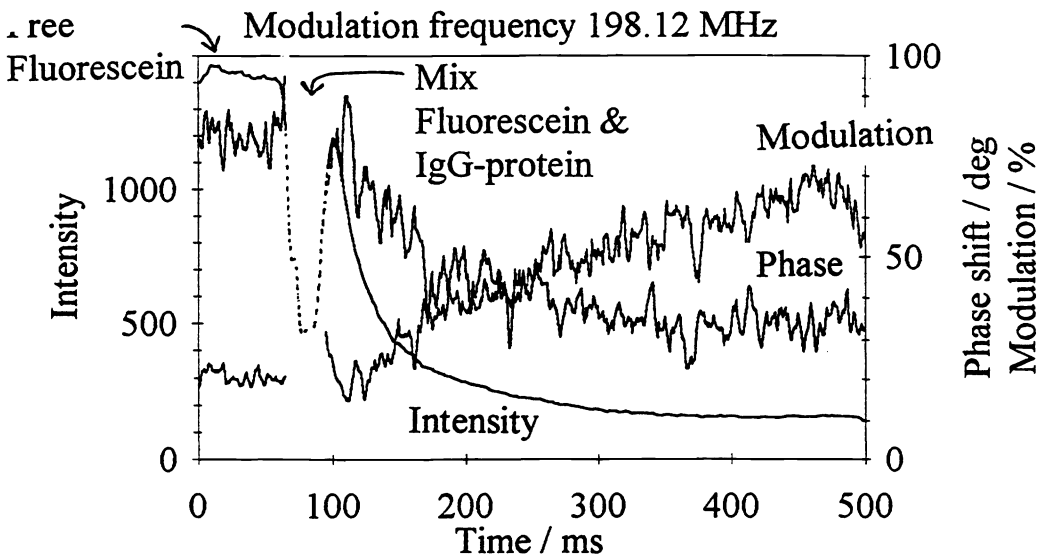
Fluorescence lifetime resolved stopped-flow kinetics

The rapid mixing accomplished in a stopped-flow apparatus is useful in studying the kinetics of chemical reactions through the concentration jump relaxation phenomenon. Flow restrictions and other mechanical impediments limit the mixing time, also termed the dead time, to a 1-2 msec resolution. The stopped-flow technique

typically uses optical detection methods such as changes in light transmittance or fluorescence intensity. Stopped-flow techniques are often encountered in protein folding studies, where various kinetic parameters yield information about folding intermediates and mechanisms. Information about fluorescence lifetime characteristics of specific intermediate species would provide an additional data axis important for mechanistic studies. To obtain on-the-fly fluorescence lifetime determinations we have turned to many of the frequency domain techniques described in the preceding paragraphs. In this application frequency domain fluorometry offers several advantages: utilization of the full fluorescence signal (sensitivity equivalent to steady-state measurements), a frequency range suitable for resolution of complex lifetime schemes, two independent measurements of the lifetime by phase and modulation at a given modulation frequency and the availability of array detectors for simultaneous detection of spectral (wavelength) and lifetime resolution. The instrument we have designed [11] is based on a commercial stopped-flow mixing apparatus (Biologic SFM-3) integrated into the general frequency domain fluorometer configuration schematically presented in Figure 1. The mode-locked Nd:YAG laser, pumping a cavity dumped dye laser (Coherent) provides a modulated excitation beam. Fiber optics couple the signal emanating from the mixing chamber with photomultiplier detectors (Hamamatsu R928), which are gain modulated by frequency synthesizers (Marconi 2022A). The heterodyned signal is processed by an analog to digital conversion card (ISS, A2D) in a personal computer. The waveform string of the 1 KHz cross correlation frequency, used in digital synchronous data acquisition, yields a maximum resolution of 1 msec. The waveform is digitally sampled 32 times per period. If the time base for the kinetic experiment exceeds 1 msec, then a fold over averaging procedure for each succeeding 1 KHz period reduces the effective bandwidth of the noise. This folding over process provides constructive enhancement of the synchronous elements of the successive waveforms and destructive elimination of asynchronous elements. The asynchronous elements arise from electronic and optical noise in the system. If the minimum time base of 1 msec (1 KHz) is used, then the enhancement of signal to noise arises from the digital over sampling (32x) of the period of the waveform, i.e. sampling of the fundamental. It should be noted that newer electronics and faster software dropped the lower limited of sampling to the microsecond regime, which exceeds the capabilities of the stopped-flow mixer, but may have application in temperature or pressure jump experiments.

Our hybrid mixing instrument offers rapid mixing, single or multiple shot averaging in the mixing process, a variable time base, facile frequency selection and a variety of optical detection modalities (transmission; fluorescence intensity, polarization, lifetime or differential anisotropy). As an example of the results attainable with this instrument, Figure 7 shows the fluorescence intensity quenching occurring when an anti-fluorescein antibody (IgG 4-4-20) binds to fluorescein. The corresponding fluorescence lifetime quenching (at 198.12 MHz) is represented by the changes in the phase shift (in degrees) and demodulation (% signal). The fluorescence lifetimes of free and bound fluorescein are 4 and 0.4 nsec, respectively. The data can be replotted as the kinetic change in free and bound fluorescein levels (Figure 7B).

Panel A.



Panel B.

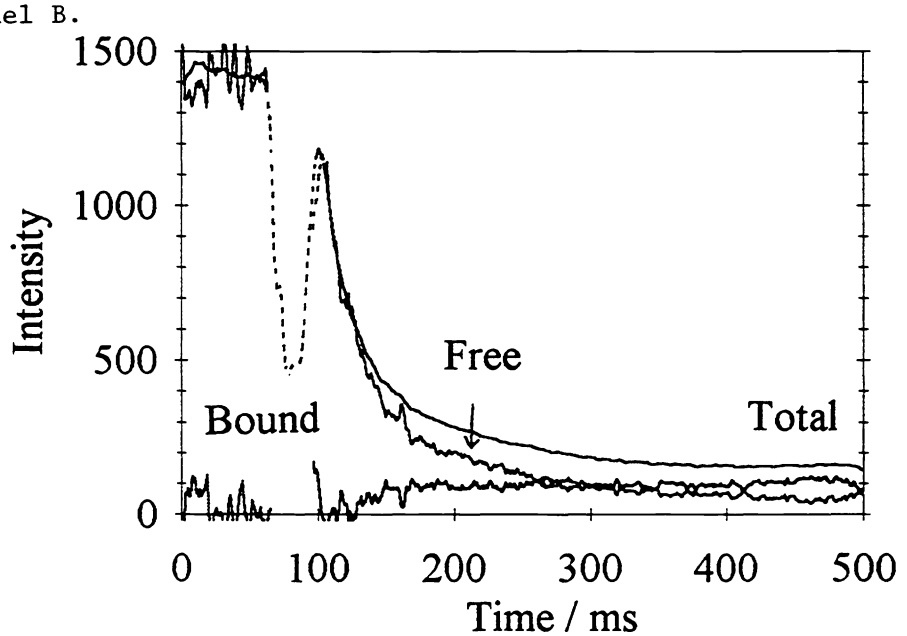


Figure 7.

Global analysis of time-resolved fluorescence data

Over the last decade, the application of model dependent, global analysis procedures for linking unknown biophysical parameter(s) with two or more fluorescence decay experiments obtained under different conditions has emerged as a powerful methodology [for recent reviews see 1,12]. This section will only highlight some important features of global analysis and a few recent developments. The Global analysis environment treats multidimensional fluorescence decay data, either in the time or frequency domain, in terms of a variety of photophysical models. The general approach is to use nonlinear least squares methodologies for data analysis, including a rigorous correlated error analysis. The linking of the fluorescence decay parameters may not be through an experimental variable, but rather through a mathematical function. This process may involve changing the fitting parameter (e.g. pre-exponential factors and lifetimes) space to a physical model or target space (e.g. compartmental modeling of photophysical reactions). The actual fitting capabilities of the global analysis routines encompass many options. Fluorescence decay data, collected in either the time or frequency domains, can be fit with sums of exponential decay rates or continuous distributions of decay rates. The analysis of discrete or distributed rotational rates is also available. As many as 40 separate experiments, totaling over 100 fitting parameters can be handled by current versions of the software. For example, Figure 8 shows dynamic depolarization data for a variety of fluorophores linked to a peroxidase enzyme. These data are analyzed to determine a view of the

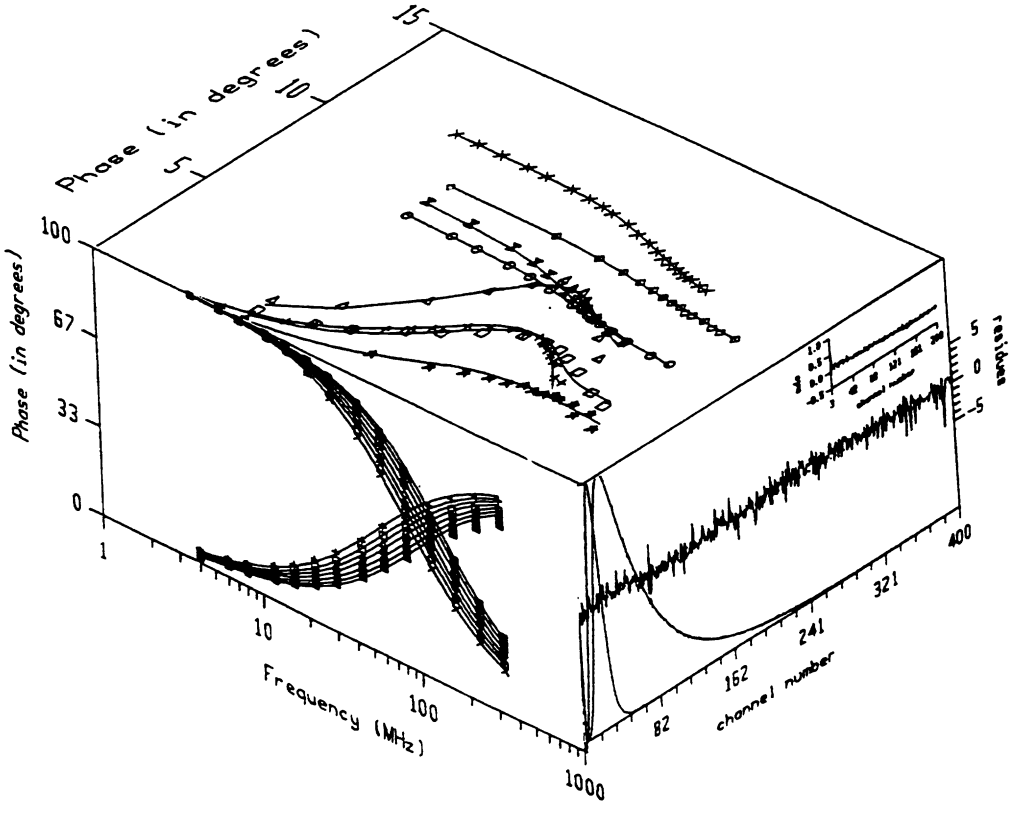


Figure 8.

rotational dynamics of the enzyme [13]. Other applications involve determinations of orientational order parameters in membranes, the orientational distributions [14]. The linked global analysis methods are also powerful in analyzing excited state reactions and energy transfer. For example, models now exist for treating rotating donors and acceptors in fluorescence resonance energy transfer [15]. The extensive linking capabilities, including function and table linking, are capable of handling extremely data rich experiments, such as the time resolved spectra of Figure 3. In this experiment, the time evolution of the fluorescence spectrum of the environmentally sensitive probe Laurdan is presented. The Laurdan is dispersed in a phospholipid bilayer (DLPC) in a fluid physical state [16]. Many other examples of the application of global analysis methods to problems in biophysics, such as photon migration analysis [17], are emerging.

3. Conclusions

This brief summary of recent technological development at the Laboratory for Fluorescence Dynamics touches on issues of instrument design, optics and electronics, applications of frequency domain methods to time-resolved biological fluorescence and data analysis. Due to their sensitivity and rapid time window it is likely that fluorescence methods will continue to find wide applicability in fundamental biological research.

4. Acknowledgments

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