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A frequency domain time-resolved microscope using a fast-scan CCD camera

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ABSTRACT

Time-resolved fluorescence imaging can enhance the contrast of microscope images and it can also provide important information about the micro-environment in cellular systems. We have developed a fluorescence microscope which can measure fluorescence lifetimes over an entire image. Fluorescence lifetimes are measured by using heterodyne frequency domain techniques. Heterodyning is accomplished by using an intensity modulated laser light source and a fast-scan CCD camera coupled with a gain modulated microchannel plate as the detector. The high duty cycle of this method allows us to generate a phase resolved image with about five seconds integration time. Operating in the fast scan mode, the systematic uncertainties in lifetime determination caused by photobleaching are less severe than those of slow-scan cameras. The microchannel plate can be modulated at frequencies up to 300 MHz, which allows us to measure lifetimes as short as 500 ps with a resolution of 50 ps. The modulation of the microchannel plate only slightly degrades the spatial resolution of the image from the diffraction limit; 0.8 micron resolution is maintained with 500 nm laser excitation.

1. INTRODUCTION

Time-resolved fluorescence microscopy offers many unique advantages over conventional techniques and has seen rapid growth in the past few years.[1,2] The fluorescence lifetime is a sensitive measure of the micro-environment of the probe molecule. A time-resolved fluorescence microscope can utilize this sensitivity to measure important chemical information such as pH, calcium or magnesium content[3], and oxygen concentration[4] in living cells. Since fluorescence lifetime is an intrinsic property of the probe molecule, it is insensitive to the unknown local probe concentration in the cell. Furthermore, the lifetime imaging technique can be used to selectively enhance the image contrast in order to highlight cellular features of interest by picking the proper "time window". The development of time-resolved microscopy also makes powerful techniques, such as anisotropy decay and resonance energy transfer[5], available for the study of cellular systems. Molecular rotations at picosecond time scale and molecular interactions at the atomic scales can be studied.

We have developed a CCD camera based frequency domain time-resolved microscope. The CCD camera is operated in the fast-scan mode which is essential for minimizing the error in lifetime measurement where significant photobleaching commonly occurs. In this article, we will provide a brief description of the operating principle and mechanics of this time-resolved microscope. Some preliminary results on time-resolved cellular imaging will also be presented.

2. THEORY AND MECHANICS OF OPERATION

This time-resolved camera is based on the frequency domain heterodyne technique.[6] Heterodyne detection requires that the excitation light source be sinusoidally modulated. The resultant sample fluorescence will also be modulated. The lifetime information of the sample is encoded in the phase shift (Φ) and the demodulation (M) (Fig. 1).
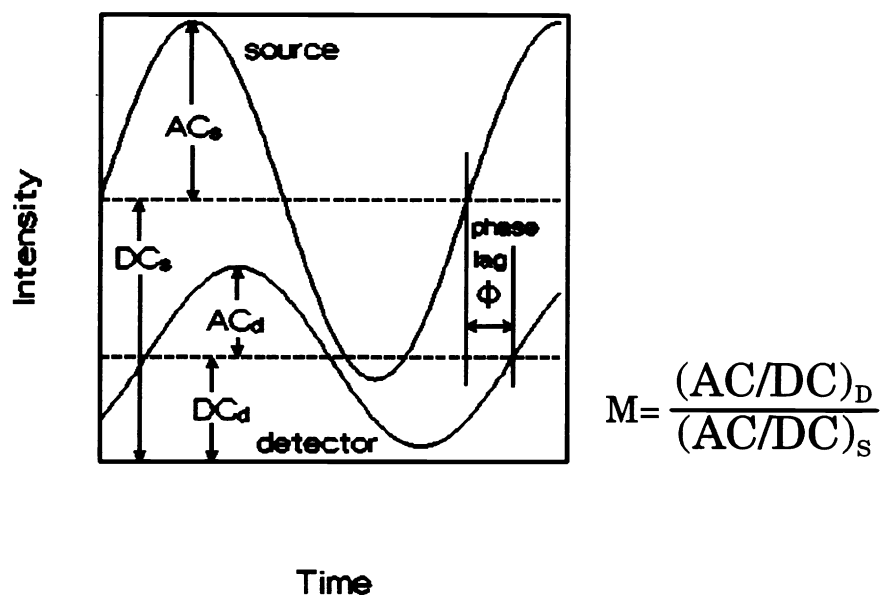

$$M = \frac{(AC/DC)_D}{(AC/DC)_S}$$

Time

Fig. 1. Definitions of phase and demodulation in frequency domain measurments

In particular, for a single exponential decay:

$$\Phi = \tan^{-1}(\omega\tau)$$
$$M = \frac{1}{\sqrt{1+(\omega\tau)^2}} \tag{1}$$

where ω is the angular modulation frequency and τ is the fluorescence lifetime. Since the fluorescence lifetime is on the order of nanoseconds, the relevant modulation frequency is in the range of 10^6 - 10^9 Hz. The accurate measurement of the phase shift and demodulation at high frequencies is difficult. The use of heterodyning technique translates this information to a low frequency signal. The fluorescence signal is collected by a detector which is gain modulated at a frequency of (ω + $\Delta\omega$) where the cross-correlation frequency, $\Delta\omega$, is typically tens of Hz. The fluorescence signal and the gain modulation of the

detector are mixed and the output signal is a product of them. The resultant signal can be decomposed into a sum of two sinusoidal components with modulation frequencies, $2\omega+\Delta\omega$ and $\Delta\omega$. The low frequency signal ($\Delta\omega$) can be easily isolated by low pass filtering. This low frequency signal contains the same phase and demodulation information as the original signal. This low frequency signal can be accurately measured by digital techniques.
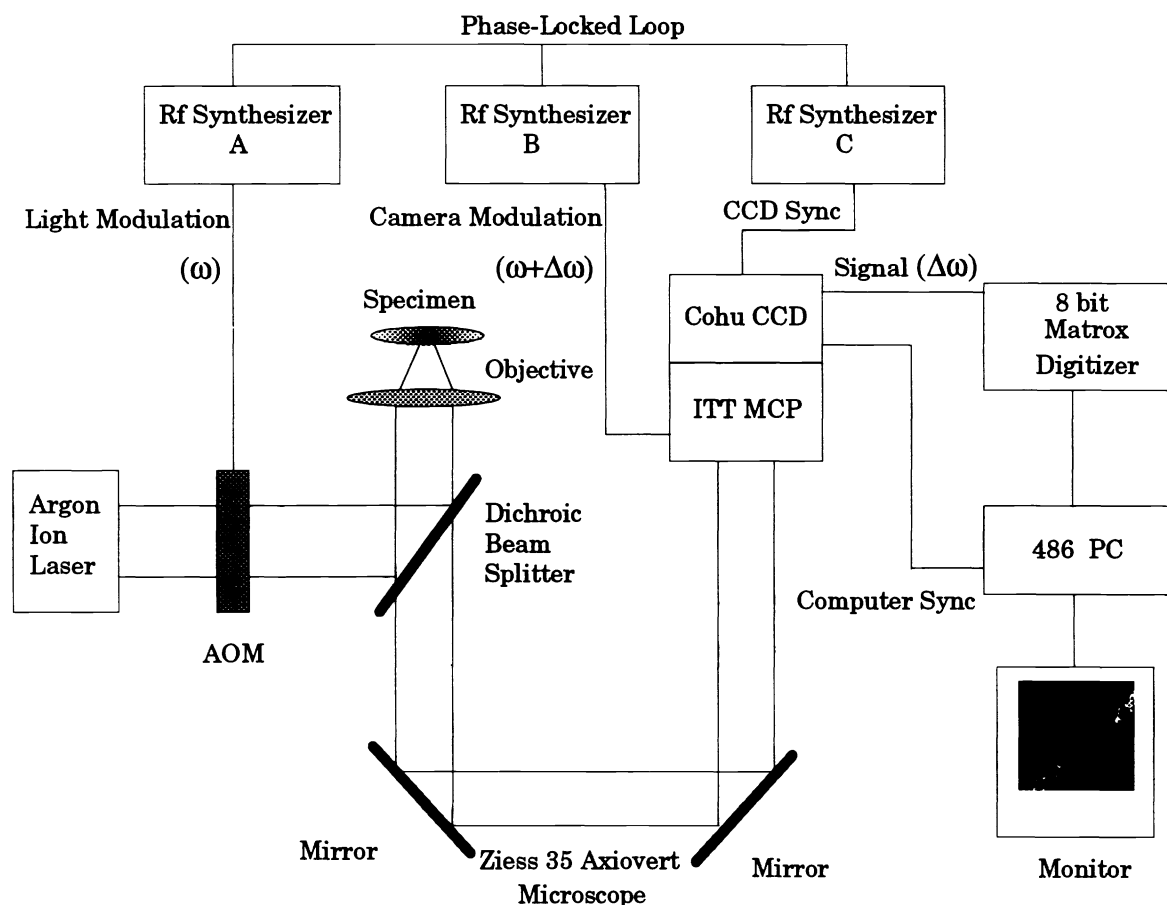

Fig. 2. Schematics of the frequency domain time-resolved microscope

The schematic of the time resolved camera is shown on Fig. 2 [7,8]. The light source used for these experiments is either a Spectra Physics argon-ion laser which is frequency modulated with an acoustic-optical modulator or a Coherent Pyridine I dye laser pumped by a mode-locked YAG laser. The excitation signal is channeled to the sample via the epi-luminescence beam path of a Zeiss Axiovert 35 microscope (Zeiss Inc., Thronwood, NY). The fluorescence signal is detected by a gain modulated dual stage microchannel plate detector (MCP) (ITT type F4144). The microchannel plate is modulated at a frequency equal to the laser excitation frequency plus a small cross correlation frequency which we have chosen to be 7 Hz. The MCP is the mixing stage in the heterodyning scheme. We have modulated the MCP by providing about 50V sinusoidal signal at its photocathode. We have obtained reasonable modulation up to 300 MHz using this method. The modulation of the photocathode causes some degradation of the spatial resolution of the MCP[8]. We are currently testing an alternative modulation

method which varies the high voltage gain across the channels[9]. Since the response of the MCP output phosphorous screen is slow, it serves as an effective low pass filter. The MCP is fiber-optically coupled to the CCD camera (Cohu Inc., model 4812-2000/ER). The 7 Hz cross-correlated signal is sampled by the CCD operating at a sampling rate of 28 Hz which corresponds to sampling four points per wave form (Fig. 3). The sample rate is twice the rate required by the Shannon's sampling theorem for suppressing the higher harmonic contributions. Successive waveforms are synchronously digitized (at 8 bit resolution), and synchronously averaged (at 16 bit resolution) by a video rate digitizer and DSP (Matrox Electronic System Ltd., Image-1280) which is housed in a PC compatible computer (Gateway 2000, 486/66). This form of synchronous averaging greatly suppresses non-harmonic noise and increases the system's dynamic range. Software has been developed to automate the data acquisition process and to compute the frequency domain images with a Fast Fourier transform.
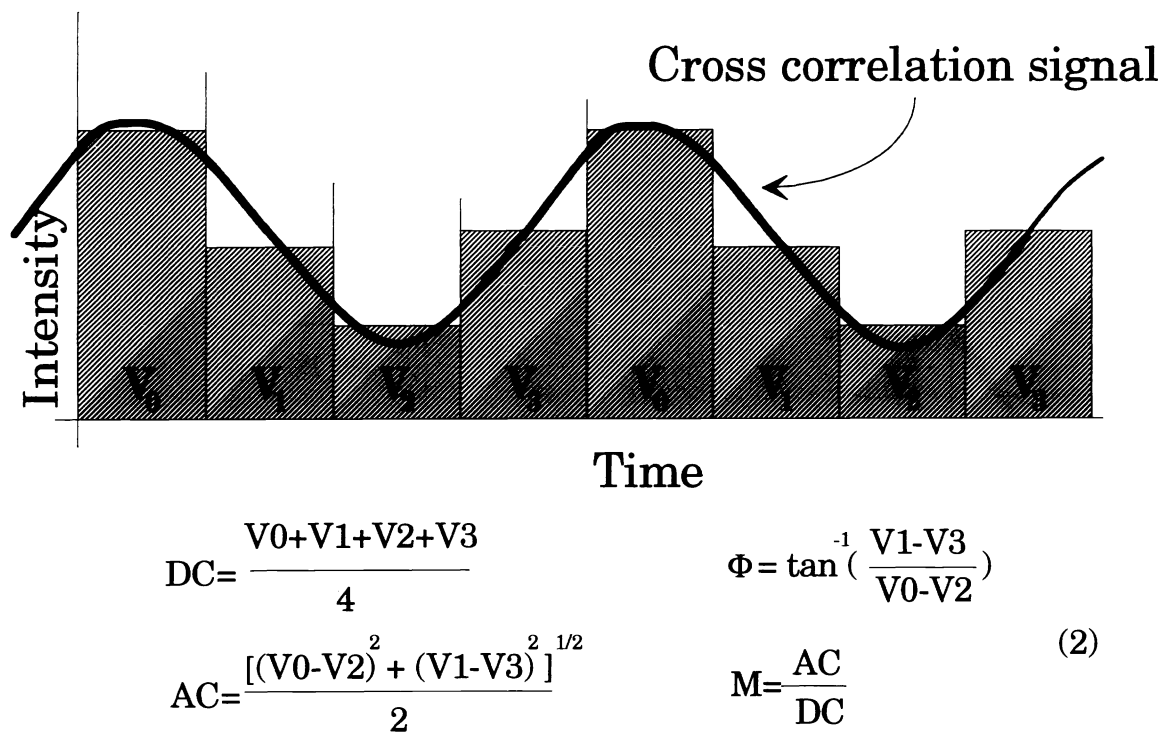

$$DC = \frac{V0+V1+V2+V3}{4}$$

$$\Phi = \tan^{-1}\left(\frac{V1-V3}{V0-V2}\right)$$

$$AC = \frac{[(V0-V2)^2 + (V1-V3)^2]^{1/2}}{2}$$

$$M = \frac{AC}{DC}$$

(2)

Fig. 3. CCD camera samples and integrates the cross-correlated
signal at four points per wave form

3. RESULTS

Lifetime images of some simple biological systems were obtained to test the performance of this frequency domain time-resolved microscope. In the following three examples, we demonstrate the ability of this microscope to selectively enhance image contrast, to quantitatively measure fluorescence lifetimes, and to detect small variations of the micro-environment inside cells.

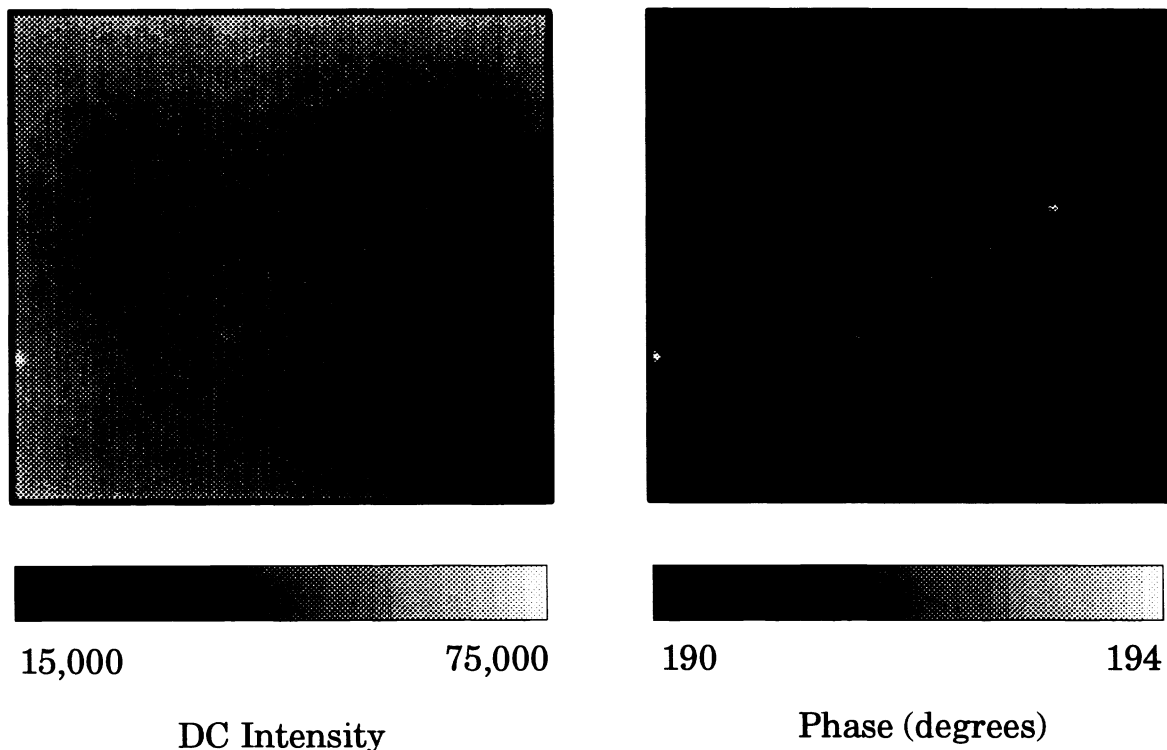| 15,000 | 75,000 | 190 | 194 |

DC Intensity   Phase (degrees)

Fig. 4. Intensity and phase-resolved pictures of 2 μm FluoSpheres in DCM dye solution

Fig. 4 demonstrates the power of this time-resolved technique to enhance the contrast of microscope images. The sample image consisted of 2 μm orange FluoSpheres (Molecular Probe Inc., Eugene, OR) submerged in saturated DCM dye in ethylene glycol solution (Exciton, Dayton, OH). The concentration of the DCM solution was adjusted such that the fluorescence intensity of the dye solution roughly matched the intensity from the spheres. The detection of the submerged spheres by the standard fluorescence imaging technique is difficult. As shown in Fig. 4a, only one spheres can easily be seen at the left edge of the field.

Since the spheres and the DCM solution have different lifetimes, the contrast of the picture can be enhanced by time-resolved detection. The spheres have a lifetime of 4.6 nsec in water and the DCM solution has a lifetime of 1.3 nsec. The sample was excited by the 514 nm line of the argon-ion laser which was modulated at a frequency of 80.854 MHz. The image is collected via a 60x Zeiss plan-Neofluar objective. The frequency domain images were synchronously integrated for about 300 secs and the resultant phase image is presented in Fig. 4b. Four additional spheres are now clearly visible in the center of the field. Using time-resolved technique we have successfully revealed the spheres in a solution with a very high level of background fluorescence.

Using this time-resolved camera, we can selectively visualize different regions of the image by choosing the proper modulation frequency. A single layer of onion skin cells are stained with 100 μM ethidium bromide (Molecular Probe Inc., Eugene, OR) solution in 100 mM Tris buffer. Onion cells were

soaked in the dye solution for about 15 minutes and were washed with de-ionized water before mounting on a microscope slide. The sample was excited by the 514 nm line of the Argon-ion laser at two modulation frequencies: 80.854 MHz and 2 MHz. Images were collected with a Zeiss 20x plan-Neofluar objective. From the DC intensity image (Fig. 5), ethidium bromide is observed to segregate into two distinct populations in this system: the bright circular regions corresponding to the nucleus of the onion cells, the dimmer lines indicating ethidium bromide trapped in or between cell walls.
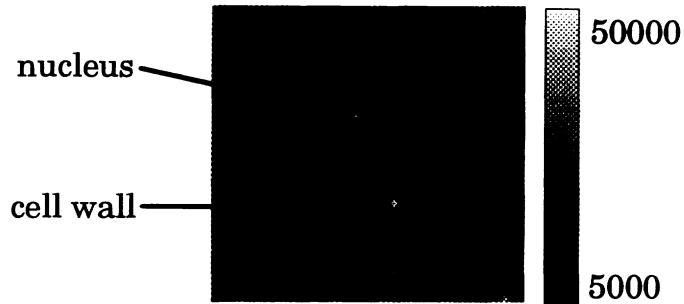

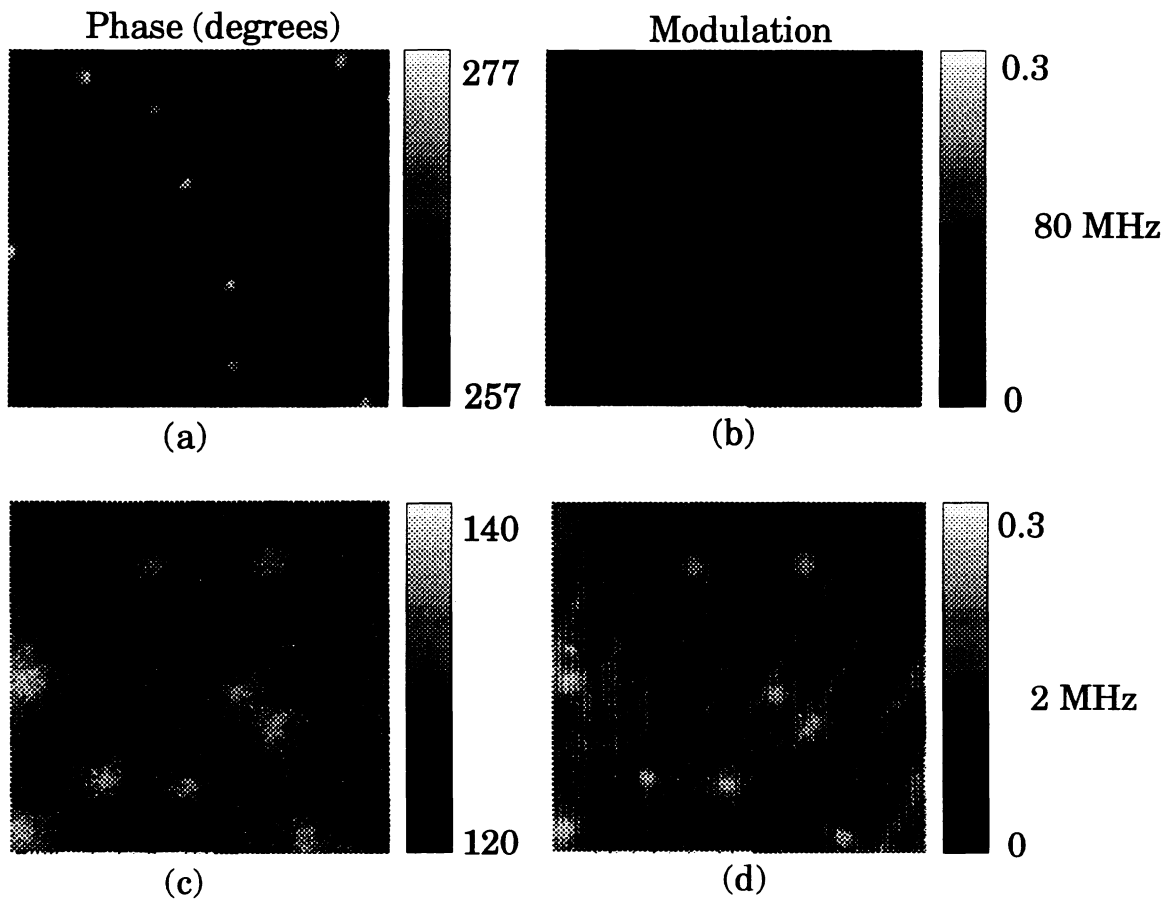Fig. 5. DC intensity imaged of onion skin cell stained with ethidium bromide

Fig. 6. Phase and modulation resolved pictures of onion skin cells
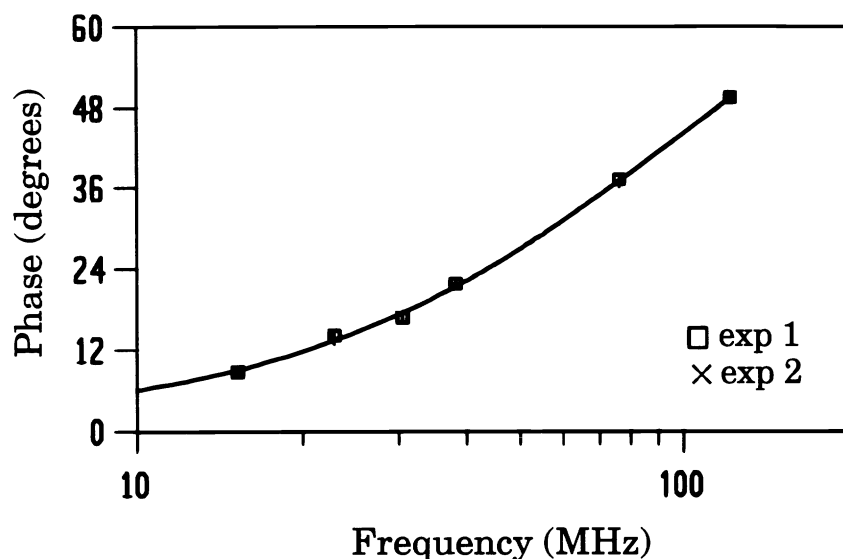stained with ethidium bromide at 2 and 80 MHz

Fig. 7. Phase shift of 15 μm FluoSpheres as a function of Frequency

The lifetime of DNA-bound ethidium is 24 nsec while it is only 1.74 nsec for the free dye. By choosing the proper modulation frequency via equation (1), we can selectivly image either the free or bound population of the dye. At 80 MHz, the nuclei of the onion cells are highlighted (Fig. 6a) since the much longer lived bound dye has a much larger phase shift comparing to the free dye. We can alternatively emphasize the free dye by looking at the modulation resolved picture (Fig. 6b) where the nuclei became invisible as it is demodulated at high frequency. At 2 MHz (Fig. 6 c,d), both the dye population can be imaged as they have similar phase and modulation values. The phase picture appears fuzzy at low frequencies because the phase shift of the dye fluorescence is small comparable to the phase of the scattered light.

We have begun preliminary studies on the ability of this time-resolve microscope to obtain accurate quantitative lifetime data. For this study, we have constructed a two layer sample slide. In one layer, we filled the slide with 15 μm blue FluoSphere (Molecular Probe, Eugene, OR) in water. The second layer was filled with POPOP-ethanol solution which serves as an internal fluorescence lifetime reference (1.3 nsec). We did not mix the two solution together because the dye solution can impregnate the latex spheres and alter their lifetimes. Frequency domain pictures are taken at multiple frequencies from 7 to 120 MHz. Only the phase pictures were used in this measurement since the modulation pictures are typically much noisier (Fig. 6d). The phase differences between the sphere and the dye background are measured in each frequency. Using the known lifetime of the POPOP background, the absolute phase shift of the sphere can be determined (Fig. 7).

These phase values are analyzed with a global analysis routine (Globals Unlimited, Urbana, IL). The data obtained appears to best fit a double exponential decay instead of a single exponential decay as indicated by a three fold reduction in χ^2 value. The major component (64%) has a lifetime of 1.3 nsec which is fixed in the fit. The minor component has a lifetime of 2.3 nsec. The double exponential decay scheme is expected since the signal at the pixels occupied by the spheres are mixed with a significant faction of out of focus fluorescence of the dye solution. The major component lifetime is therefore fixed to the lifetime of the reference POPOP solution. The minor component's lifetime is fitted to be 2.3 nsec

which is slightly short comparing to the lifetime measured by a conventional flurometer (3.8 nsec). A large uncertainty in the minor component's lifetime is expected given the lack of good modulation data. The intensity faction ratio of the two lifetime components compares favorably (within 5%) with the measured intensity ratio of the spheres and the dye in the DC intensity picture. The results of these fitting schemes are summarized in Table 1:

Table 1: Fluosphere lifetimes obtained from global analysis

Fitting Schemes	double exponential	single exponential
Intensity Faction 1	0.64	1.00
Lifetime 1	1.33 (fixed)	1.58
Intensity Faction 2	0.34	NA
Lifetime 2	2.33	NA
χ^2	2.1	6.1

One of the most important features of the lifetime microscope is its ability to measure minute differences in cellular micro-environments where fluorescence intensity measurements do not have sufficient sensitivity. We have imaged a string of live spirogyra cells, a common pond algae, to test our instrument in a simple cellular system. Spirogyra cells are linked together like a string of pearls. The most prominent feature in each of these cells is the large, double helical chloroplast. The chlorophyll in this organ is extremely fluorescent when illuminated by the 514 nm line of the Argon-ion laser. A string of these spirogyra cells were imaged with the time-resolved microscope integrated for about 300 sec with the laser power on the order of 20 mW. The fluorescence intensity image is shown in Fig. 8a. This image was taken at the junction between two distinct spirogyra cells. The break in the helical structure corresponds to the separating cell walls between the two cells. The cell walls are not visible since they are not fluorescent. Judging from the fluorescence intensity picture alone, these two cells appears to be identical. However, a significant difference in chlorophyll lifetimes in these two cells can be observed in the phase and modulation resolved pictures (Fig. 8c,d). Note that the chloroplast within each individual cell has a uniform lifetime which is distinct from the other cell. This observation shows that the non-uniformity in the phase and modulation pictures truly reflects the environment of the chloroplast in the cells and is not caused by experimental artifacts of the lifetime camera. Comparing the phase and modulation resolved pictures, the cell at the upper right hand corner has a higher phase value and is more demodulated while the other cell has a lower phase value and is better modulated. This is consistent with the hypothesis that the cell at the upper right hand corner has a longer lifetime. Since we have not attempted to precisely control the cellular environment of these cells, we will not speculate as to the cause of this lifetime difference. This experiment demonstrates the promise of the time-resolved microscopy technique in quantitatively monitoring cellular functions.
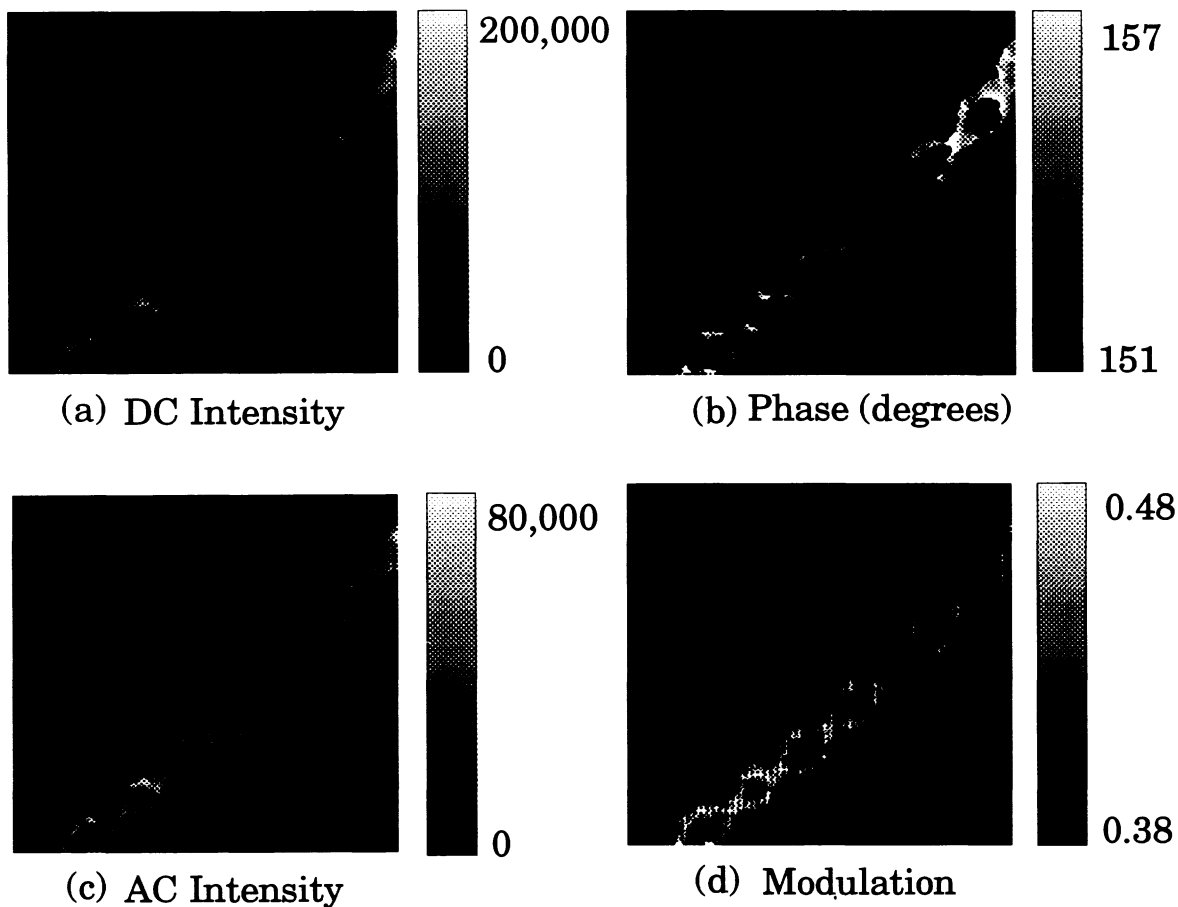
(a) DC Intensity   (b) Phase (degrees)

(c) AC Intensity   (d) Modulation

Fig. 8. Frequency domain pictures of chloroplast fluorescence
    of Spirogyra at 80 MHz modulation

5. CONCLUSION

We have successfully adapted the fast-scan time-resolved CCD camera developed in our laboratory to a microscope system. Time-resolved imaging can not only effectively and selectively enhance the contrast of microscope fluorescence images, but can also quantitatively measure lifetimes within cellular compartments to monitor their micro-environment. We are currently working on improving the signal-to-noise ratio of our instrument in order to minimize the data acquisition time which is critical in biological systems where photo-damage and photo-bleaching is significant.

6. ACKNOWLEDGMENTS

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