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New Approaches to Lifetime-Resolved Luminescence Imaging

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An overview of recent developments in time-resolved imaging technology is presented. A directly modulated CCD imaging device has been developed which is suitable for frequency-domain phosphorescence measurements up to more than 400 kHz. This has been used with a recently-developed blue/UV LED source to implement a low-noise, solid-state phosphorescence/fluorescence imaging system suitable for macroscopic and microscopic imaging. For shorter lifetimes a range of new light sources has become available with higher light output and/or higher frequency modulation performance. Acoustooptic tunable filters have also been investigated as optical "light gates" both for modulation of excitation and for time-resolved imaging, and the limits of performance of these devices are discussed.

KEY WORDS: Acoustooptic tunable filter; modulated deuterium lamp; luminescence; fluorescence lifetime imaging; LED.

INTRODUCTION

Fluorescence and phosphorescence lifetime imaging can use either time-domain or frequency-domain measurements, and the choice between these depends on the availability of suitable light sources and detectors. A further consideration is whether the measurement is made by scanning or by using a parallel imaging detection system. In the former case the detector need only have a fast timing capability since the image is built up from serial measurements, and hence photomultipliers or avalanche photodiode detectors are suitable. For scanning measurements laser excitation sources are usually used and these can be modulated by a variety of methods. Conventional imaging measurements impose more serious constraints on both the detector and the light source. Although lasers can be used, an incoherent source is usually preferred to minimize speckle and related problems. These problems are often particularly troublesome in a microscopic imaging system, where multiple coated lens surfaces give rise to interference patterns with coherent light. For imaging on the nanosecond time scale the detector of choice is usually an image intensifier designed for high-speed gating or modulation. An interesting alternative is the combination of a low-noise integrating CCD camera with an optical gate. Although this approach has been used for time-resolved transillumination imaging,(1) it is difficult and expensive to implement for routine fluorescence lifetime imaging. Optical gates based on electrooptic or acoustooptic shutters have found only limited use, primarily because no really efficient optical shutter is available with the desired combination of high bandwidth, wide aperture, low distortion, and low power consumption. If a suitable light gate were to become available, or if a directly modulated camera which was not based on intensifier technology could be constructed, this would probably have a substantial impact on fluorescence lifetime imaging. Image intensifiers are not ideal light detectors. The quantum efficiency of a typical intensifier

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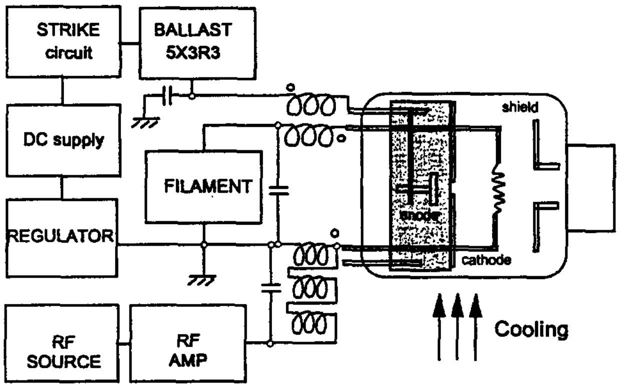

Fig. 1. Schematic of the high-power deuterium lamp and modulation circuit.

is similar to that of a photomultiplier, depending on the type of photocathode used, and is much lower than the conversion efficiency of a good CCD camera for visible light. This disadvantage is usually offset by the relatively large sensitive area of an image intensifier compared to the low-cost CCD cameras currently available. Intensifiers are rather noisy detectors, however, and are both costly and relatively easily damaged. The noise performance of the intensifier is particularly important for frequency-domain measurements because images are subtracted and the difference images ratioed to calculated the final result. Our own experience has been that fluorescence lifetime imaging gives the best results when the intensifier is used at the lowest gain possible and an image is integrated over several seconds using a high-quality cooled slow-scan CCD camera to average out the noise from the intensifier system.

Although intensifier technology is presently required for nanosecond fluorescence measurements, rather slower measurements are now possible by other means. Phosphorescence/delayed fluorescence imaging is an emerging technique likely to find wide use in microscopy, since long-lived labels are easily detected against the short-lived autofluorescence background that commonly limits sensitivity. Measurements using luminescent labels with long lifetimes are also of interest for various types of immunoassay, and imaging formats are under investigation to increase the throughput for such assays. Recently developed detector and light source technology is moving this type of imaging beyond the millisecond measurements typical of most older instruments toward submicrosecond measurements. This trend is important because many medium-lifetime luminescent labels, which previously could not easily be measured without extremely expensive equipment, can now be considered for routine microscopic imaging and immunoassay. Here, we introduce some new approaches to the imaging and detection of luminescence lifetimes, which will be reported in greater detail in forthcoming papers.

THE HIGH-POWER DEUTERIUM SOURCE

In previous papers we described a high-frequency modulated 30-W deuterium lamp(2) and its application to fluorescence lifetime imaging(3) and phase fluorometry.(4) The deuterium source is physically quite small and can be modulated at frequencies in excess of 120 MHz with good depth, ranging from almost 100% at 5 MHz to about 25% at 120 MHz, depending on drive conditions and RF matching. Deuterium sources are attractive on account of the long operating lifetime, very low noise operation, wide wavelength range, and relatively low cost. In addition, they are incoherent and, hence, do not give problems of speckle in fluorescence imaging. The principal disadvantage of the deuterium source is the rather low output power relative to the more common arc sources used in microscopy. In an attempt to overcome this problem, we have carried out experiments on a new high-power deuterium source, rated at 240 W for continuous operation. The new lamp is manufactured by Cathodeon Ltd. and was developed as a modulated source jointly with our group. The light output of the lamp is relatively linear with lamp current at low power but begins to flatten off at high current, so that the total light output at 240 W is approximately six times that of a standard 30-W lamp. The source size of the lamp is similar to that of the standard 30-W lamp, so the source radiance increases proportionally to the light output. The relatively small source size is advantageous, in that much of the emitted light can be collected and used effectively for microscopy. Figure 1 shows a schematic outline of the modulation circuit used with the high-power lamp. The more massive internal structure relative to the standard lamp increases both capacitance and inductance in the device, so that very high frequency operation is not to be expected. Nevertheless, the lamp can readily be modulated up to frequencies around 40–50 MHz and a reasonable modulation depth is achieved with a relatively low RF power input as shown in Fig. 2. Good RF matching in this frequency range minimizes stray radiation, which is important both to comply with EMI regulations and to avoid interference with other apparatus.

To summarize, the new high-power deuterium source is very suitable for fluorescence lifetime measurement and imaging in the medium frequency range,

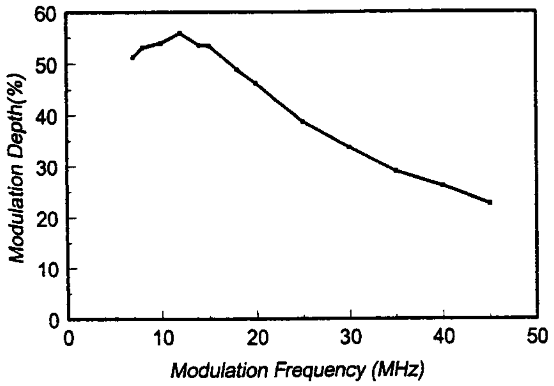

Fig. 2. Modulation depth vs frequency for the high-power deuterium lamp. Lamp current was 2.5 amp and applied RF power was 10 W RMS.

which is most often used for probes with nanosecond lifetimes. The very rugged structure and conservative power rating should ensure reliable operation and a long working lifetime, and the increased optical output will improve the sensitivity for fluorescence and phosphorescence lifetime imaging and related applications.

THE "ULTRABRIGHT" BLUE/UV LED

Recently, very bright blue LEDs have become commercially available, and these have properties which are very well suited to luminescence lifetime imaging. An additional advantage was noted in a recent paper;(5) where it was shown that the diodes can emit near-ultraviolet light in some circumstances. The cited work demonstrated pulsed operation of the diodes giving 4-ns pulses of light at 380 nm at a repetition rate of 10 kHz and showed that the emission could be used for fluorescence lifetime measurement in the time domain by single-photon counting. We have extended this work by measurement of modulation properties of these diodes and determined the limits of their operation when driven with unity mark/space ratio square and sinusoidal signals. Figure 3 shows the spectrum of a typical blue diode driven at a low current and at a higher current to generate UV output. The diodes can readily be modulated, with a >60% modulation depth achieved very easily at 100 MHz. We have not yet characterized fully the higher-frequency performance of the diodes, but preliminary experiments confirm useful modulation at 200 MHz, limited in our tests by RF matching considerations. With a square wave drive at 100 kHz for excitation of long-lived emission, the diodes can easily be driven to generate high levels of ultraviolet light, with a wavelength varying from around 380 to 395 nm depending on the temperature. Figure 4 shows the diode emission at 380 nm used to excite luminescence from a europium (III) chelate of 2-(1,1,1-trifluoroacetoacetyl)naphthalene, demonstrating the high UV light output which can be achieved from a compact thermoelectrically cooled unit. These diodes are certain to have a substantial impact on time-resolved imaging and sensing since they are much more stable in operation than most high-speed alternatives with a similar wavelength output, and a complete excitation system is very compact and easy to drive.

ACOUSTICALLY TUNABLE OPTICAL MODULATORS (ATOMs)

The acoustooptic tunable filter(6) is well-known as a rapid scanning monochromator able to be used both for selection of excitation wavelengths and for imaging. The AOTF is based on diffraction of light by an acoustic wave propagating in an optically anisotropic crystal, commonly of tellurium dioxide. This type of AOTF is highly efficient. It is available in apertures up to about 1 cm^2, has a full acceptance angle of up to approximately 7°, depending on the design, and can diffract up to about 90% of a chosen wavelength into two orthogonally polarized orders. Wavelength selection is achieved by control of the frequency of ultrasound in the crystal, and the response speed depends on the optical aperture, as the ultrasonic wave propagates at a finite velocity across the crystal. The relatively low speed of propagation of the acoustic wave in the "slow-shear" mode used in most AOTFs limits the useful modulation bandwidth to less than 10 kHz for the large-aperture devices needed for good optical throughput in imaging applications. We have considered two approaches to increase the modulation bandwidth to overcome this limitation. It is important to realize that the light diffracted by an AOTF is not intrinsically modulated at high frequencies by the ultrasonic wave, despite the high frequency of the RF carrier generating the sound waves. The effect of the traveling ultrasonic wave is instead to Doppler-shift the wavelength of the diffracted orders by an amount which is insignificant for broadband incoherent sources. To achieve useful modulation, we investigated a modified AOTF in which the ultrasonic absorber, which usually prevents unwanted reflections that might perturb the uniformity of the sound field, was deliberately omitted. In this case, at appropriate frequencies standing waves formed in the crystal, resulting in an ultrasonic grating which is uniformly modulated at twice the frequency of

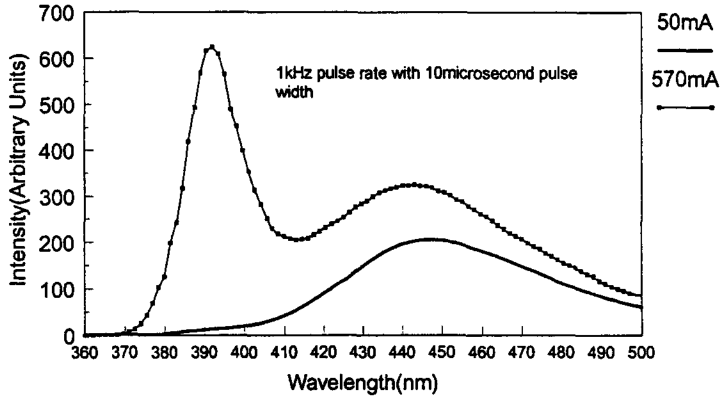

Fig. 3. Spectral output of blue LED supplied by RS Components Ltd. Currents are peak values during pulsed operation. The LED was not cooled.

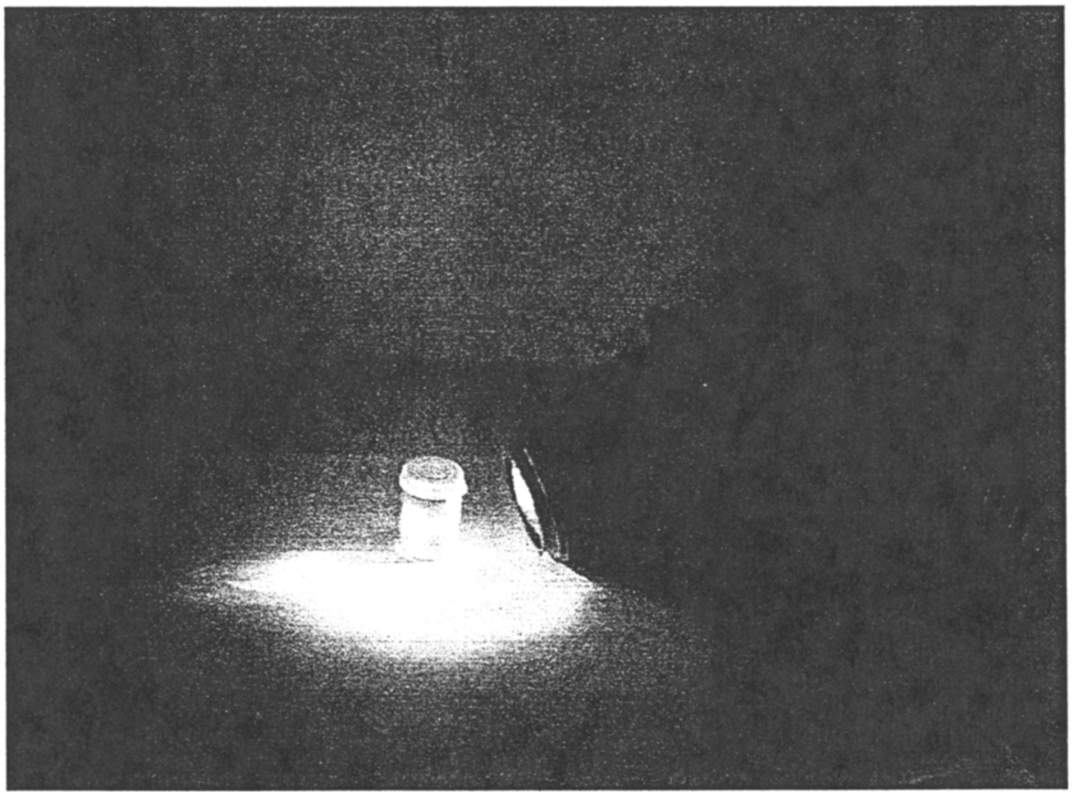

Fig. 4. Photograph shows bright luminescence from a europium chelate of 2-(1,1,1-trifluoroacetoacetyl)naphthalene excited by the 380-nm UV light from a thermoelectrically cooled high-intensity pulsed LED supplied by Photonic Research Systems Ltd. (UK). The exciting light was spectrally filtered to eliminate visible emission.

the carrier. This is analogous to the use of a Debye–Sears "water-tank" modulator as was common in early phase fluorometers.(7) The difference is that the phase matching achieved as a result of the optical anisotropy in the AOTF crystal gives rise to a greater acceptance angle than is normally the case for other types of modulator. Figure 5 shows the result of a preliminary experiment, where the standing wave AOTF was tuned to the green line of a high-pressure mercury arc lamp and the light was detected using a high-speed analogue photomultiplier and an integrating digital oscilloscope. The AOTF clearly is able to achieve a useful degree of mod-

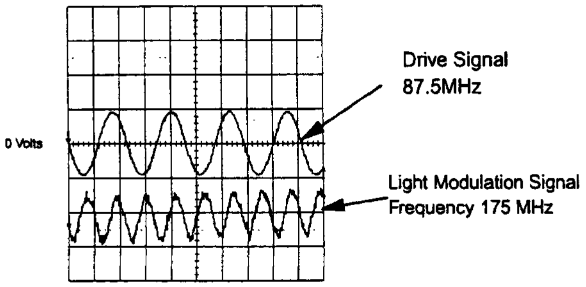

Fig. 5. Oscilloscope trace showing modulation of 540 nm light from a high pressure mercury arc by a standing wave established in an AOTF (modified by omission of the acoustic absorber normally used to prevent unwanted ultrasonic reflections). In this experiment the light input was collimated and spectrally filtered and the diffracted light was selected by a pinhole to block zero-order transmission.

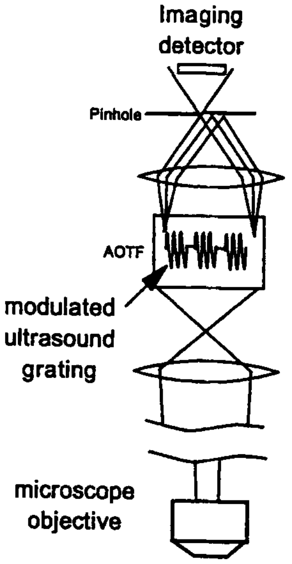

Fig. 6. Schematic of the AOTF imaging setup for modulation experiments. The AOTF was placed in the intermediate image plane of a microscope, so that the object and the active region of the AOTF were simultaneously focused onto the detector.

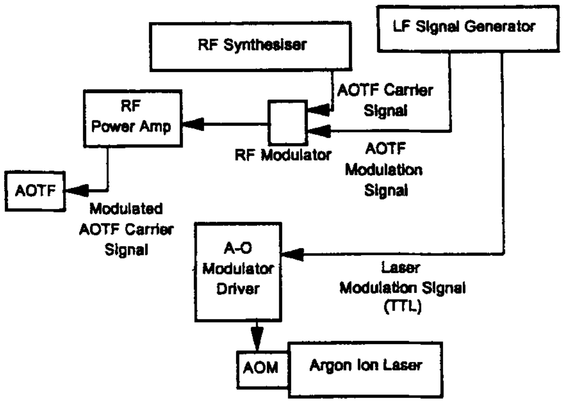

Fig. 7. Block diagram of drive electronics for imaging experiments using a modulated AOTF.

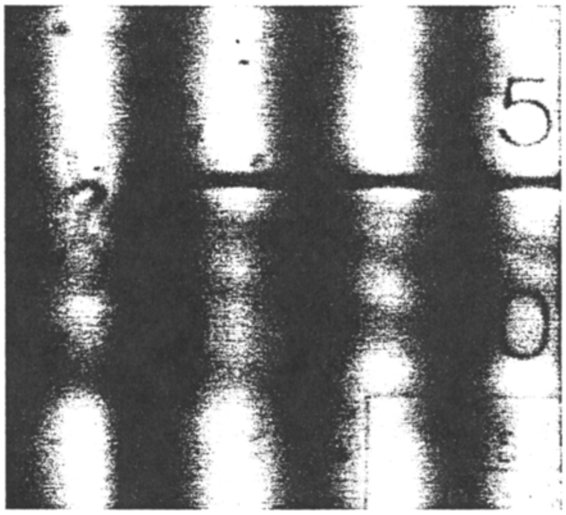

Fig. 8. View of a microscopic test sample through the AOTF used with modulated RF drive input when the sample is illuminated with modulated laser light as described in the text.

ulation (ca. 30% under the nonoptimized conditions of the experiment). An AOTF specifically designed for standing-wave operation is currently being studied to extend this work. It is hoped to investigate submodulation schemes and AOTFs equipped with more than one transducer to allow the modulation performance of the device to be independent of the diffraction wavelength of the AOTF.

The standing-wave AOTF discussed above should also find applications to very high-speed modulated imaging both to modulate exciting light and as a fast light gate in conjunction with a CCD camera. The standing-wave mode gives rise to spatially uniform modulation and the AOTF is compatible with microscopic imaging. Further investigation of the standing-wave mode of operation will be reported in more detail in due course.

IMAGING DETECTORS

A standard AOTF might well be considered as a light gate to be used with a CCD camera for time-resolved imaging, since the RF drive can be switched on and off or modulated sinusoidally. Unfortunately, when used in this way the AOTF is limited to modulation frequencies of the order of a few kilohertz for devices

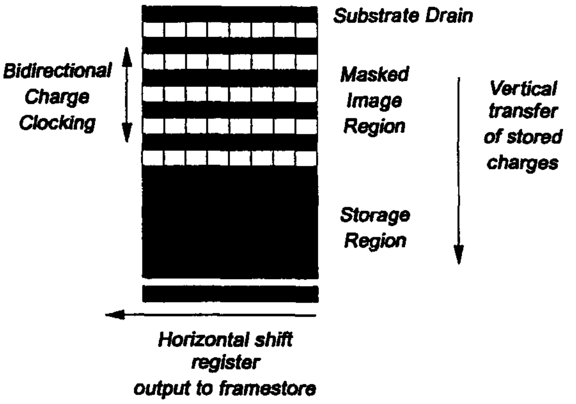

Fig. 9. Simplified diagram of a frame transfer CCD fitted with a mask over the active imaging region. An alternative is to dispense with the shielded storage region and use a full-frame imager with a periodic mask covering the whole area. In this case an external shutter is required during readout of the charge after exposure.

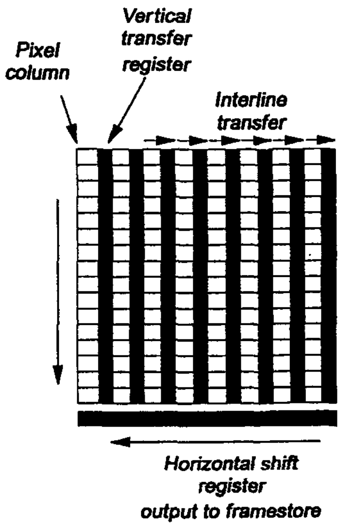

Fig. 10. Simplified diagram of the interline transfer CCD.

of a reasonable aperture. The standard "noncolinear" AOTF made from tellurium dioxide operates in a "slow-shear" mode where the velocity of the ultrasonic wave in the crystal is only about 600 m/s. The bandwidth limitation is a consequence of the time for a step change in the ultrasonic profile to propagate across the optical aperture. If a nonuniform modulation across an image is tolerable, however, it is possible to use the AOTF for modulated imaging. If, for example, the RF carrier is modulated using a square wave, the ultrasonic profile will consist of an alternating series of refractive index gratings separated by regions of constant refractive index. To investigate whether such a modulated AOTF could be used for time-resolved imaging, we set up a test system as shown in Fig. 6. The AOTF was placed in the intermediate image plane of a standard microscope, so that a real image of the object was projected into the AOTF crystal in the region where the ultrasonic waves propagate. This region of the AOTF was imaged directly onto a CCD camera using a relay lens. In this arrangement, therefore, the active region of the AOTF and the sample are simultaneously imaged onto the camera. The sample was illuminated with monochromatic light from an argon-ion laser equipped with a standard acoustooptic modulator driven with a square wave, so that the laser beam was modulated with a unity mark/space ratio. The square wave used to modulate the laser was also passed through a variable delay module into an RF mixer; where it served to modulate the high-frequency drive to the AOTF crystal, as shown in Fig. 7. The results of this experiment are shown in Fig. 8. The image of the sample as seen through the AOTF is crossed with dark bands as a result of the periodic interruption of the refractive index grating in the AOTF crystal. Measurement of the cross section of the bands reveals a triangular profile, which is expected as the grating is moving during the "on" periods of the laser (i.e., the profile is the cross-correlation of the square-wave illumination with the square-wave modulation of the AOTF carrier). Variation of the phase difference between the laser and AOTF modulation by means of the delay unit caused the pattern of bands to move across the image as expected. The experiment was conducted across a range of frequencies. With our AOTF crystal, banding structures could clearly be seen up to around 2 MHz. Time-resolved imaging could obviously be implemented using an AOTF modulated in this way. All that is required is to measure a series of images as a function of the phase shift between modulation signals driving the light source and AOTF. Future work will investigate means to improve the image quality and determine the practical limits of modulation frequency for this type of AOTF. One problem with our present system is the presence of fixed optical patterns, unrelated to the modulation, which we believe to be a consequence of the particular design of transducer used in our AOTF. Similar considerations apply to the upper frequency limit for modulation, which might be expected to be higher than 2 MHz. The upper limit to modulation frequency is likely to be of the order of 5–10% of the carrier frequency, limited by the need for the modulated "wave-packet" to be representative of the carrier. With further development, we feel that the modulated AOTF has promise as a time-resolved imaging system for fluorophores of a relatively long lifetime. In this respect it has

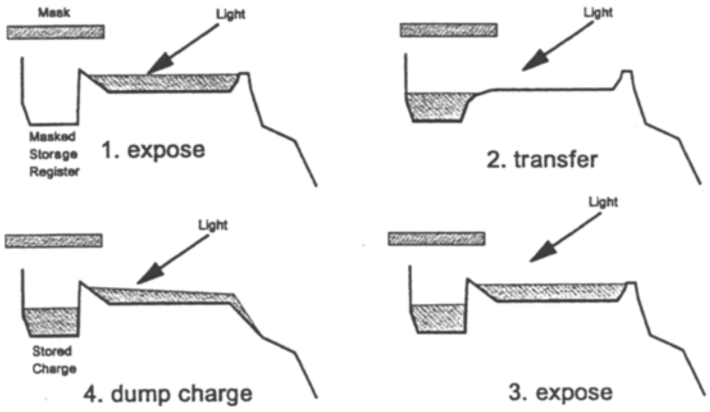

Fig. 11. Modulation scheme used with the interline CCD camera.

the advantage that the detector can be either a video-rate CCD or a cooled slow-scan detector. The former could find use in dynamic measurements such as remote sensing and endoscopy, while the latter should give a much higher signal-to-noise ratio than intensified detectors currently used for lifetime imaging. The AOTF/CCD combination has a further advantage in that it is robust and not damaged by overexposure to light, and these characteristics would be valuable in routine diagnostic applications.

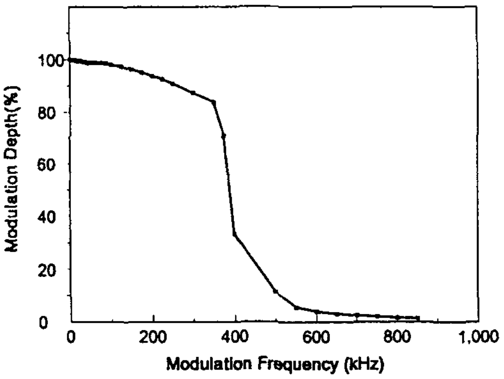

Fig. 12. Modulation performance of the Sony HAD interline transfer CCD as a function of modulation frequency. The camera and modulation circuitry were provided by Photonic Research Systems Ltd. (UK).

DIRECTLY MODULATED CCD CAMERAS

A continuing goal in lifetime imaging has been the development of robust low-cost alternatives to intensifiers for those applications where very high time resolution is not needed. To this end, we have investigated direct modulation of CCD cameras. We have considered two basic approaches, one of which has been tested. One obvious approach is to use a frame transfer CCD camera equipped with a mask, as shown in Fig. 9. Cameras of this type are often able to transfer charge bidirectionally, either vertically down into a storage region prior to readout or vertically upward into an image drain used for charge dumping to implement shuttered exposures of only a few milliseconds. This bidirectional charge transfer could be used to advantage for phase-sensitive imaging if the CCD chip were covered with a simple striped mask blocking light from every alternate row of pixels. Repetitive clocking of charge up and down would divide the recorded image into two subimages, as the center of charge collection was alternately accessible to light and blocked from light. After a suitable integration period, the signal would be read out in the normal manner. This scheme has not been tested by us, since as yet we have no suitable masked CCD, but we are advised by CCD experts that it is feasible and should work at megahertz rates. The approach has the potential advantage that images in-phase with and in-antiphase to the repetitive waveform used for the up-and-down clocking would be recorded simultaneously.

Our approach has been to use a simpler technology based on an interline transfer CCD that is already commercially available. A simplified diagram of an interline transfer camera is shown in Fig. 10. In this design, vertical columns of light-sensitive pixels are interleaved with shielded vertical transfer registers. Charge is integrated in the pixels for a fixed period, after which the charges are clocked horizontally into the transfer registers, from which they can be transferred serially to a horizontal shift register for readout. In this type of cam-

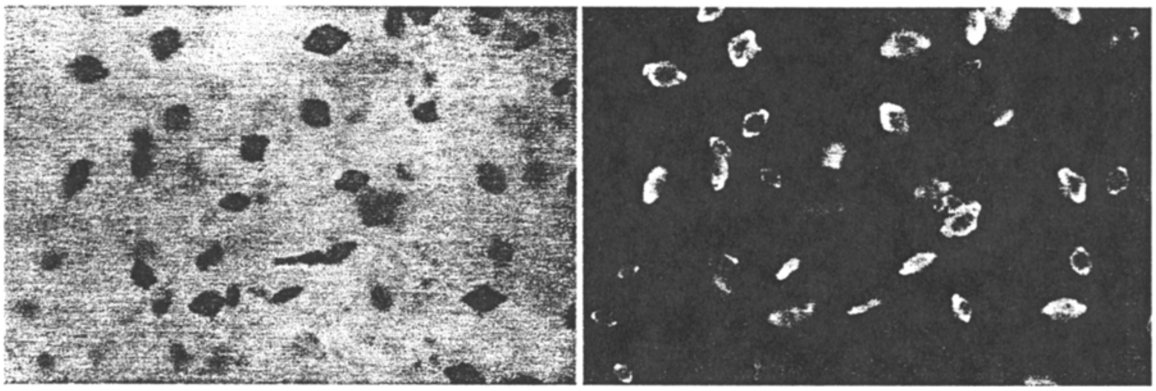

Fig. 13. Imaging of crystals of the luminescent europium complex shown in Fig. 4. The sample was excited by a UV light source modulated with a square wave at 5 kHz and imaged with the modulated interline transfer camera. The image on the left was taken with modulation of the light source in phase with that of the camera. The prompt fluorescence of the paper on which the crystals are placed is effectively suppressed in the image on the right, where the camera is modulated out of phase with the excitation.

era, an electronic shutter can be implemented by transferring charges to a drain instead of to the vertical transfer register. In the shuttered mode, a typical exposure would involve draining the charge as it was collected, followed by briefly turning off the drain to allow the charge to collect, after which the charge is transferred to the vertical registers as rapidly as possible. Using this type of scheme, typical commercial CCD cameras can be used at effective exposures down to 10^{-4} s, with faster operation possible for some designs.(8) We have investigated the use of such a camera for modulated imaging. The modulation scheme used is shown in Fig. 11. Basically, the charge is alternately dumped and stored repetitively throughout an exposure by appropriate manipulation of the system clocks and substrate charge drain. Using this approach, we have demonstrated modulated operation up to better than 400 kHz, as shown in Fig. 12. An example of the modulated detection of long-lived emission from a europium chelate of the type commonly used for time-resolved immunoassay is shown in Fig. 13, which demonstrates the excellent rejection of short-lived background fluorescence from the paper on which the crystals are placed. The principal difficulties with the interline transfer camera are a rather limited frequency response and an excess noise introduced by the modulation process. The latter, which manifests as an increase in fixed-pattern noise, is reduced by cooling and can be subtracted effectively.

To summarize, this approach to time-resolved imaging is simple and cost-effective. For long-lived luminescence the combination of a simple LED-based light source with a directly modulated camera should prove attractive to biologists, since a system can easily be configured for use with a standard fluorescence microscope or to image areas such as gels and blots. Low-cost systems are now commercially available, and the introduction of new long-lived labels will help to establish new protocols and assays. Sensing technologies such as oxygen monitoring(9) and remote temperature measurement(10) will also benefit from the ready availability of such technology.

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