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Measurement of nanosecond time-resolved fluorescence with a directly gated interline CCD camera

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Summary

CCD cameras coupled optically to gated image intensifiers have been used for fast time-resolved measurements for some years. Image intensifiers have disadvantages, however, and for some applications it would be better if the image sensor could be gated directly at high speed. Control of the 'charge drain' function on an interline-transfer CCD allows the sensor to be switched rapidly from an insensitive state. The temporal and spatial properties of the charge drain are explored in the present paper and it is shown that nanosecond time resolution with acceptable spatial uniformity can be achieved for a small commercial sensor. A fluorescence lifetime imaging system is demonstrated, based on a repetitively pulsed laser excitation source synchronized to the CCD control circuitry via a programmable delay unit.

Introduction

Measurement of fluorescence lifetime can be achieved in a number of ways, typically in the time domain by time-correlated single-photon counting or in the frequency domain by phase-shift/demodulation methods. These approaches have been used for many years and are well known (Demas, 1983; Lakowicz, 1983). Fluorescence lifetime imaging (FLIM) is now well established, and can be implemented using a repetitively modulated excitation source and detector (Morgan *et al.*, 1990; Lakowicz & Berndt, 1991) or by scanning methods (Buurman *et al.*, 1992). One of the simplest methods to measure a FLIM image is to used a gated detector with a pulsed excitation source, taking a sequence of images as a function of delay between the excitation and gating signals. By this means, a fluorescence lifetime value can be calculated for each pixel in the constituent images and a lifetime 'map' can be constructed as the FLIM image. The resulting image is particularly useful to allow quenching processes to be detected and quantified. Alternatively, a time-gated image can be used as a means of suppression or enhancement of a particular component of the emission. This is useful, for example, to allow selective detection of a long-lived luminescent label in the presence of short-lived background such as cellular autofluorescence. The principles and applications of FLIM have been described by a number of authors. For an early review see Morgan *et al.* (1992), and for a brief discussion of applications in microscopy see Morgan (1996).

The usual means to achieve gated imaging is to equip an electronic camera such as a charge-coupled sensor (CCD) with an image intensifier. The most commonly used image intensifier for such purposes is a 'proximity-focused' unit incorporating a microchannel plate electron multiplier, which is physically compact. These can be gated by pulsing the voltage between the light-sensitive photocathode and the front face of the microchannel plate, or alternatively the voltage across the microchannel plate can be switched. The spectral sensitivity of an intensifier is determined by the photocathode composition, and the response time is determined by the impedance of the internal components of the intensifier. Where gating is achieved by pulsing the voltage on the photocathode directly, the composition of the cathode determines the conductivity and hence the temporal response. Gated intensifiers achieve a typical response time in the order of a few nanoseconds by the use of a conductive coating or mesh on the photocathode. However, this approach can compromise the optical sensitivity or introduce artefacts in the image. An additional problem is 'irising', where the central portion of the photocathode responds later and less efficiently than the periphery as the gating pulse travels across the surface and is attenuated progressively. This problem is most severe for intensifiers with a large diameter. However, it is often desirable to use an intensifier of reasonable size from the

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viewpoint of sensitivity and particularly to give reasonable spatial resolution.

Recently, an alternative approach to pulsed operation of an intensifier has been commercialized by Kentech Instruments Ltd (Didcot, U.K.) where the gating signal is coupled capacitively across the face of the photocathode through an external coating on the window of the tube. Results obtained with such a device have been described by Dowling *et al.* (1999). This approach can give very high time resolution, even for relatively large intensifiers, but requires specialist high voltage pulsed circuitry and is fairly expensive.

Image intensifiers are relatively 'noisy' amplifiers, especially when used at high gain. It would be highly desirable to avoid their use for many applications in microscopy where a CCD detector would give much better noise performance and higher spatial resolution. In this paper we discuss an approach to gated imaging using an interline-transfer CCD sensor without an intensifier stage (for a description of CCD device architecture see Barbe, 1980). Time gating is achieved by control of the charge-drain facility on the chip, and a FLIM system can be implemented with a synchronously pulsed laser as an excitation source.

Materials and methods

The CCD systems used in this work were based on interline transfer sensors manufactured by Sony and were purchased from Framos GmbH (Münich, Germany). The sensors were integrated into camera units developed by Photonic Research Systems Ltd, UK (PRS, Salford, U.K.). The sensors were thermoelectrically cooled with Peltier heat pumps to at least −16 °C to allow extended image integration times. Control electronics and software were developed by PRS.

Laser excitation was at 355 nm from a frequency-tripled Nd-YAG laser (Spectron Ltd, Rugby, U.K.) which furnished pulses of 10 ns with a repetition rate of 10 Hz. The laser was triggered by the control software, which also initiated the drain sequence of the CCD camera. The Q-switch synchronization output from the laser triggered a computer-controlled programmable delay unit (PRS) and the output from this switched off the camera drain. After each excitation event the charge stored in each pixel of the CCD was transferred into the associated shielded transfer gate, where charge was allowed to accumulate for a predefined number of excitation pulses before a readout operation was initiated.

Measurements of fluorescence were conducted with dilute solutions of quinine sulphate in 0.1 M sulphuric acid and sulforhodamine in ethanol, which were placed in wells in a black-anodized aluminium plate and illuminated with UV light from the laser using a quartz light guide to diffuse the excitation. The CCD camera was used with a UV-blocking filter. The layout of the experimental set-up for fluorescence imaging is shown in Fig. 1.

For measurements of the spatial and temporal response of the CCD sensors the excitation source was a blue LED (Nichia, Tokushima, Japan). The LED was built into a Peltier-cooled housing with integrated electronics for fast pulsed operation (PRS). This cooled LED could be operated at substantially higher current than the manufacturer's maximum rating without damage, giving increased light output. The optical pulse was measured using a fast photomultiplier (600 ps risetime, from Hamamatsu, Welwyn Garden City, U.K.) with a 300-MHz oscilloscope. The full-width, half-maximum (FWHM) of the light pulse was found to be 4 ns when the diode was pulsed at kHz repetition rates.

The LED was used to illuminate a white matt surface viewed by the CCD camera without an associated lens, so that the sensor was uniformly exposed. A blue-transmitting filter was used on the camera to block any long wavelength emission from the LED, which might have a different temporal profile from the main emission.

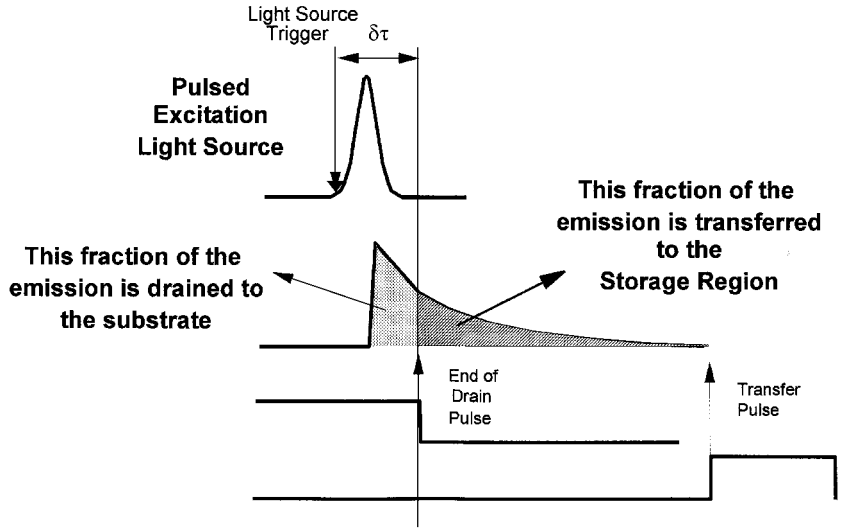


Fig. 1. Timing diagram for collection of time-resolved image.

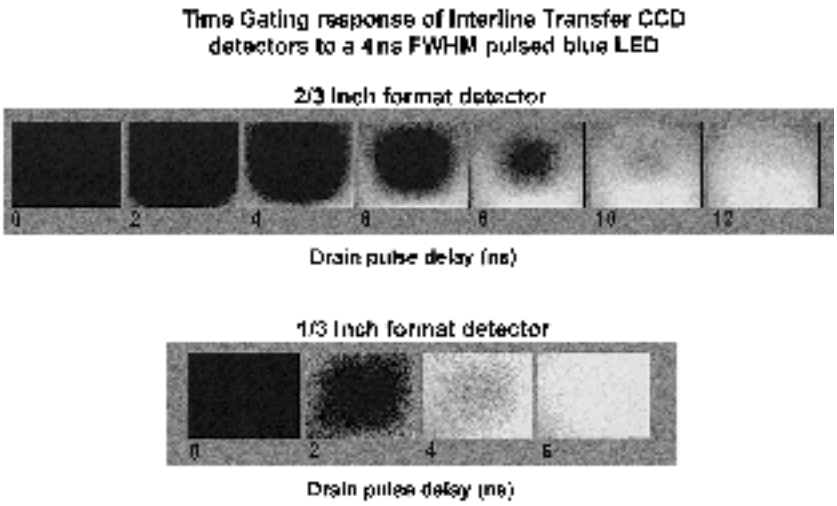
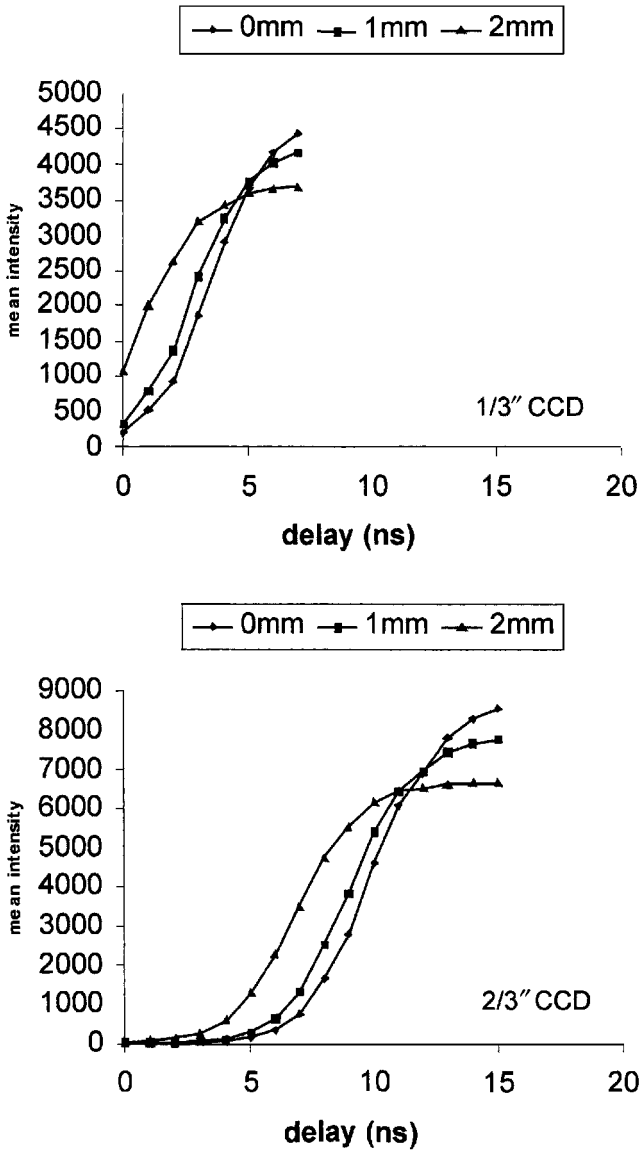


Fig. 2. Time gating response of interline transfer CCD detectors to a 4 ns FWHM pulsed blue LED.



Results

In Fig. 2 the response of the CCD sensor to a pulsed light source is shown for two different sensors. Results are shown for a ICX083AL '2/3-inch format' interline CCD (11 mm image diagonal) in the upper half of the figure, whereas the lower images show results obtained using a ICX055AL '1/3-inch format' (6 mm image diagonal) sensor. Both sensors show evidence of 'irising', in that the centre of the sensor responds somewhat later than the edges. However, the smaller sensor in particular has a response time that is similar to that of a typical 18 mm intensifier with a gating signal coupled directly to the photocathode. The data in Fig. 2 are used to plot out the spatially averaged temporal response of the CCDs for pixels across each sensor. Figure 3 shows these data. The results shown are convolutions with the width of the excitation pulse (4 ns), which cannot be ignored on this timescale. The slopes of the measured responses at different points of each sensor suggest that the main effect is a time shift, with the central portion of each sensor responding later than points near the periphery. A more precise characterization of the time response across each sensor requires a faster light pulse, and will be the subject of further investigation. It should be noted that the data presented relate to the timescale over which the drain of the CCDs can be switched off, so that the sensor is able to accumulate charge. The time constant associated with the transfer pulse that initiates movement of charge into the storage register can also be measured and is of the order of 100 ns with the electronics used in our experiments. This

Fig. 3. Comparison of CCD responses to 4 ns light pulse. The curves shown represent data averaged over squares of 0.5 mm × 0.5 mm for each sensor. The first area was positioned in the centre of the CCD sensor. A second area was defined, the centre of which was offset in the vertical and horizontal dimensions by 1 mm. A third area was defined similarly, but with an offset of 2 mm.

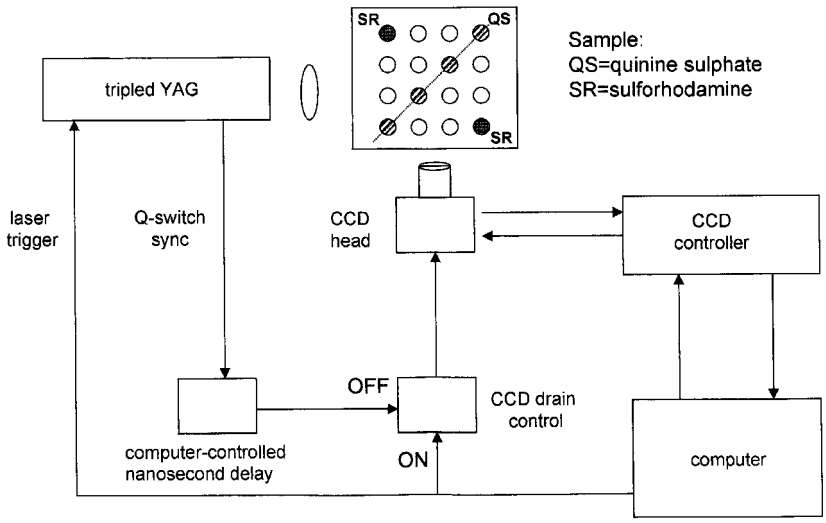


Fig. 4. Example of nanosecond time-resolved imaging using CCD with a pulsed Nd-YAG laser source.

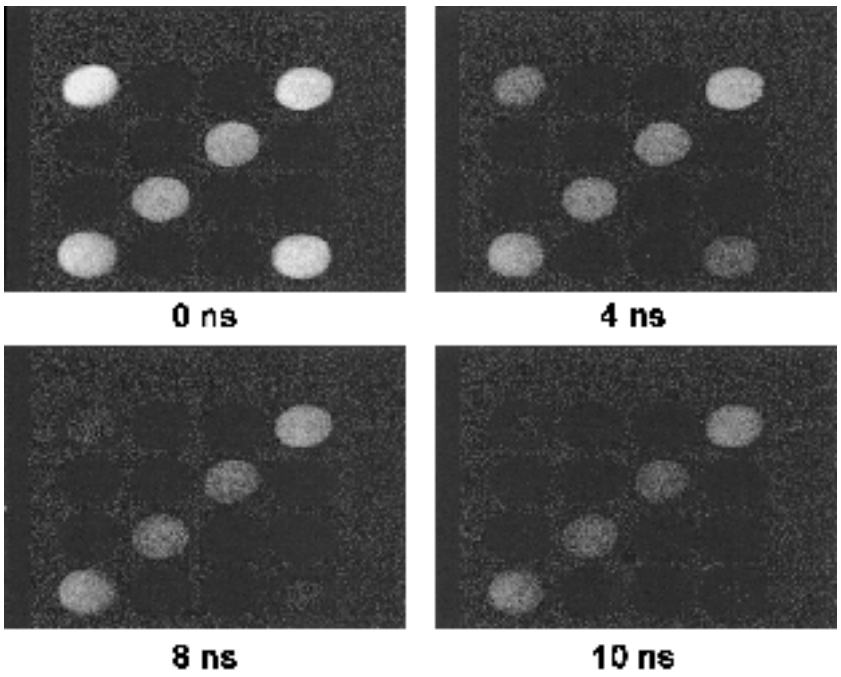


Fig. 5. Time-resolved imaging of fluorescent dyes using pulsed laser excitation and gated CCD detection.

restricts the maximum repetition rate that can usefully be employed in our experiments to approximately 500 kHz, but otherwise this does not affect the timing precision that can be achieved. It should be noted, however, that high operating frequencies give proportionally higher levels of fixed pattern noise, probably associated with increased power dissipation within the sensor.

The smaller sensor was used in a demonstration to show the feasibility of nanosecond fluorescence imaging, according to the principles shown in Fig. 4. A series of images were accumulated varying the delay between triggering of a fast pulsed laser source and switching of the drain on the CCD sensor. A sequence of images resulting from this experiment is shown in Fig. 5. For each of the images shown, a series of excitation pulses was averaged for several seconds in the sensor before readout, as described in the Methods section. This averaging was not necessary to give adequate signal levels, because the excitation pulses had to be attenuated to avoid saturation of the sensor with each individual pulse. The averaging was an attempt to minimize inevitable timing jitter in the laser synchronization circuitry, which was in the order of 1–2 ns, and to average intensity fluctuations from the laser. Data in Fig. 5 show that the emission of quinine sulphate (with a fluorescence lifetime in the order of 19 ns) can readily be distinguished from that of the sulforhodamine sample with a lifetime of about 4 ns.

Discussion

We are aware of only one other attempt to use a CCD sensor directly for nanosecond imaging. In a patent (Ott, 1996) the use of a technique for time resolved imaging is disclosed, based on the use of an interline sensor. The method discussed is based on the concept of periodically interrupting the flow of charge between the pixels of a CCD and the substrate drain under circumstances where the transfer channels between pixels and shielded storage region are permanently enabled. This approach is claimed to give time resolution of the order of 10 ns or so. However, we are not aware of any subsequently published work showing this order of time resolution. The approach described here differs from that of Ott in that it relies on the timing characteristics of only one edge of a pulse, and in that the transfer channels in the CCD are not continuously enabled. Experiments conducted by PRS suggest that the approach described in the cited patent will not work well with the sensors used in this study, and that enabling the transfer channels as described gives rise to a very substantial noise background.

The spatial map of the timing response of the CCDs suggests that the time resolution that could be achieved is of the order of a few nanoseconds at present, limited by the finite excitation pulse width and timing jitter of the source and electronics. The 'irising' behaviour is the factor, which would limit temporal resolution if a faster excitation pulse were used, and this is clearly less of a limitation for the smaller format CCD. A time resolution of the order of 1 ns would appear to be possible on this basis. To an extent, the 'irising' effect could be compensated by computation, though at a cost of reduced signal-to-noise ratio.

The results shown clearly demonstrate the feasibility of nanosecond fluorescence imaging with a solid-state detector. The preliminary data are limited in several ways, due to availability of appropriate apparatus. One requirement for high time resolution is a suitable excitation source with a short pulse width and minimal jitter when triggered. We chose to use the Nd-YAG laser because it is equipped with a synchronization output linked to operation of the laser's Q-switch. The timing jitter from this source alone is of the order of 1 ns. The duration of the laser pulse was itself approximately 10 ns, which inevitably reduces the temporal resolution of the experiment. There are further timing variations associated with the control electronics.

The Nd-YAG laser is far from ideal for nanosecond imaging because it has a relatively low pulse repetition rate and substantial pulse-to-pulse variation in output intensity, which translates into timing variations if a series of images is measured as a function of delay. The low pulse repetition frequency means that images must be integrated for several seconds, irrespective of available signal level, in order to smooth out fluctuations. On the other hand, the present approach is not suitable for use with a weak repetitive light source that pulses at a very high frequency, such as an LED. The reason for this is the noise performance of the CCD sensor, which is used to accumulate signals in the vertical transfer register before delayed readout following a sufficient exposure period. Each charge transfer generates a small amount of 'noise' within the sensor. At transfer frequencies in the order of a few Hertz this effect is negligible over an integration period of seconds, but the 'noise' scales with transfer frequency and becomes a significant limitation at frequencies in excess of about 100 kHz. The noise generated is of the 'fixed pattern' type, reflecting the individual variations in pixel resistivity within the sensor, and for this reason a very effective subtraction of a 'dark image' is possible if the pulse sequence is applied to the sensor without exposure to light. Nevertheless, the noise generation does limit integration time and reduces dynamic range. If frequency domain measurements are contemplated these would be limited to use with labels with lifetimes in the order of microseconds or longer, due to the restrictions in noise performance and charge transfer speed discussed earlier.

It should be noted that an LED source was feasible for testing the properties of the sensor because the diode source is pulsed at a relatively low repetition rate to avoid noise generation in the CCD. However, if the diode were to be used to excite fluorescence, the unfavourable duty cycle would limit sensitivity because the average light intensity would be rather low. For this reason a laser source is more appropriate to such a measurement.

The use of the CCD camera for nanosecond measurements does not compromise the sensitivity of detection relative to its normal operation, except for a slight increase in system noise if a high repetition rate is used for the excitation source. The best compromise for nanosecond imaging at present would be achieved with a pulsed laser or related triggered pulsed light source working at a repetition rate of the order of a few kHz. This is fast enough to average out pulse-to-pulse fluctuations and unavoidable timing jitter, yet keeps the noise generation within easily acceptable limits. A further important feature is the availability of a prompt synchronization signal with low time jitter and minimum delay relative to the output pulse. If the trigger signal follows the firing of the laser (for example if it is generated by an optical sensor) it may be necessary to use an optical delay line in the light path. Short delays can be achieved with optical fibres, but long lengths of fibre are best avoided in practice.

A number of commercial lasers fulfil the required criteria for nanosecond imaging. One promising candidate is the $NdVO_4$ laser that can be pumped by diodes, producing an output that can be doubled or tripled to give green light or near UV. A very compact solid-state laser of this type with a repetition rate of the order of 10–15 kHz and integral harmonic generation is commercially available (Crystal Technology GmbH, Berlin, Germany) for a few thousand pounds and might well prove to be a suitable source for nanosecond imaging using a CCD detector.

The preliminary study suggests that a CCD detector that is physically small is likely to be most suitable for fast time response. CCDs are now available in very small sizes ('1/10-inch format' is commercially available with similar design architecture to those used in our work) and these might well be expected to avoid 'irising' problems. Based on our measurements, it is possible that a very small chip might give sub-nanosecond time response, in which case the timing jitter in other parts of the apparatus would limit time resolution. It is important to recognize that a very small CCD sensor can still maintain very high spatial resolution, with more than 250 000 pixels in some cases. For microscopy, a small detector is not disadvantageous as long as the spatial resolution is adequate. For macroscopic applications, however, the sensitivity of the camera will often scale in proportion to the area of the sensor.

Apart from potential applications to microscopic FLIM and fast time-gated imaging, the use of a solid-state sensor is potentially valuable in areas such as medical endoscopy, laser range finding and time-resolved imaging in turbid media. Time-resolved cameras using this technology will be commercially available very soon and the robust low-cost sensors might well extend time-resolved measurements into new fields.

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