Time-resolved fluorescence measurements using self-pulsing 650-nm laser diodes

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ABSTRACT

We report on the development of a stroboscopic excitation technique using a self-pulsing laser, and show that it is a feasible method for obtaining fluorescence lifetime information from a biochip format. The self-pulsing lasers described here are versatile devices which have been used for one photon excitation measurements to determine the lifetime of cyanine 5 in water and ethanol. The same devices have been used to develop a technique whereby the emphasis for time-resolution of a lifetime measurement can be transferred to the excitation source from the detector and processing electronics by virtue of the multiple-pulse, variable frequency nature of the laser output.

I. INTRODUCTION

The measurement of fluorescence, whether in a spectroscopic, time-resolved or imaging approach, has become an indispensable tool in a diverse range of applications. The primary use of these techniques has been in the biological sciences where they have allowed research on the cellular, and even intracellular, level. They have been extremely important in genetics (DNA sequencing), and also in medicine where fluorescence has found a use in non-invasive tests such as immunoassays. The continuing development of lab-on-a-chip technologies such as micro-machining and optical integration, is now allowing such fluorescence measurements to be performed using highly portable, inexpensive and even disposable micro-analytic devices[1]. Recent developments have been concentrated on fully integrated biochips where semiconductor devices are used for both excitation and detection of fluorescence signals[2, 3]; as yet, however, on-chip time-resolved measurements have not been attempted.

Traditional, bench-top, time-resolved experiments require optical sources that can deliver short optical pulses, and low-noise high-gain detectors capable of sub-nanosecond time-resolution. Typically, the optical sources employed are mode-locked solid state lasers or flash lamps, but laser diodes are being increasingly used in their place due to their compact and rugged nature. The detector of choice for this type of measurement is the multi-channel plate photomultiplier tube (MCP-PMT) due to its ability to detect single photons and its large detection area. A number of time-domain methods are available for accurately determining the fluorescence lifetime of a particular fluorophore which include time-correlated single photon counting[4] (TCSPC), streak camera detection[5], and the less popular stroboscopic optical boxcar technique[6]. Along with these methods a fluorescence lifetime can also be determined using phase sensitive detection in the frequency-domain. Clearly, implementing any of these techniques on a biochip format would be extremely difficult. Obtaining the necessary high-speed, high-sensitivity response from a small (micron-sized) detector would be hard to achieve and the electronics required to process the signal from such a detector may well negate all the inherent advantages of a biochip system.

Here we propose an alternative method for obtaining lifetime information from the scaled-down biochip format which is broadly based on a stroboscopic technique. Rather than gating the detector, stroboscopic excitation using the variable pulse period available from a self-pulsing laser can in principle be used to obtain time-domain information using a dc detector. The chief use of this method would be to observe lifetime changes that occur during an assay or to detect differences in decay times between fluorophore species; the method is not suited to obtaining an

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accurate fluorescence lifetime but would supplement *a priori* information obtained from traditional lifetime measurements such that important time-domain assays could be performed on a biochip. This paper is organised as follows: section II is used to describe the design and principles of operation of our self-pulsing lasers, and includes a detailed description of the important characteristics. The use of these lasers as sources in a traditional time-domain intensity decay measurement using a streak camera detector is described in section III. Our stroboscopic method is explained in detail in sections IV which describes how a streak camera detector was employed in a "proof of principle" study.

II. LASER DESIGN

Self-pulsation is the phrase used to refer to the process by which the power output of laser diodes is self-modulated. This phenomenon is a natural extension of the oscillations experienced by the non-linear coupling of the electron and photon populations that can occur when a source of saturable absorption exists within the laser cavity. The terminology used to describe the mechanisms by which this self-modulation can occur has its origins in the methods used to generate short optical pulses in gas and solid state laser systems: namely q-switching[7]. This is the process by which the quality factor of the laser cavity is initially held at an artificially low value, allowing the build-up of a larger than normal population inversion, and then by some means is allowed to achieve its normal, high Q-value, generating a short duration optical pulse. When the quality factor is switched periodically a repetitive train of pulses is produced by the laser. The elevated level of population inversion within the cavity means that the emitted pulses can achieve a high peak power. This empirical description of the generation of optical pulses applies equally well to laser diodes; here, however, the mechanisms which result in pulse generation are more subtle, and in fact, it is possible to achieve repetitive q-switching without the need for external modulation. To achieve self-pulsation Henry[8] showed that the inequality given in equation (1) needs to be satisfied within the laser cavity:

$$\frac{1}{\tau_\alpha} + v_g \frac{d\alpha}{dN}.P > \frac{1}{\tau_g} + v_g \frac{dg}{dN}.P \qquad (1)$$

Here v_g represents the group velocity of the optical field, P, within the laser cavity; τ_α and τ_g are the carrier lifetimes in the absorber and gain regions respectively; and the differential gain (absorption) in each region is given by $dg(\alpha)/dN$. In essence equation (1) tells us that the differential absorption in the laser cavity must be greater than the differential gain but, more than that, it also points to the influence of the carrier lifetimes within each region in determining the relative phase of the gain and absorption saturation produced by the optical field. There are several techniques for achieving self-modulation of the output of laser diodes which it is possible to broadly classify as intrinsic and extrinsic, but all make use saturable absorption by unpumped semiconductor regions. This absorbing material can be intrinsic to the laser (for example the leaky optical modes of narrow ridge lasers can be sufficiently perturbed by the surrounding material to cause pulsation), or it can be introduced by dividing the p-type electrode into several sections, one or more of which would be biased below transparency. In this work we have made use of devices where the saturable absorber region is epitaxially incorporated into the device structure. The absorption characteristics of the device are controlled via the width, position and bandgap energy of the absorber layer. This implementation of an absorber within the layer structure of the laser means that controlled self-pulsation is achieved in a simple, single-contact device.

The devices used in this study were typical AlGaInP laser structures designed to nominally emit light at a wavelength of 650nm. In addition to the standard laser design, they also include epitaxially grown saturable absorber quantum wells within the layer structure[9-12]. The laser structures were grown by metal-organic chemical vapour deposition (MOCVD) on 10° misorientated (100) n-type GaAs substrates. The gain region consisted of 3x7-nm $Ga_{0.48}In_{0.52}P$ gain quantum wells with 10-nm $(Al_{0.4}Ga_{0.6})_{0.51}In_{0.49}P$ barriers located within a 2-μm $(Al_{0.4}Ga_{0.6})_{0.51}In_{0.49}P$ waveguide core. The outer cladding region was formed from $(Al_{0.7}Ga_{0.3})_{0.51}In_{0.49}P$. Three $Ga_{0.39}In_{0.61}P$ quantum wells were positioned within the p-type cladding layer to act as a saturable absorber. The number, composition and doping of these wells has been optimised in order to maximise the differential absorption; the overlap of the absorber wells with the optical mode of the laser; and to reduce the carrier lifetime in the well[11, 12].

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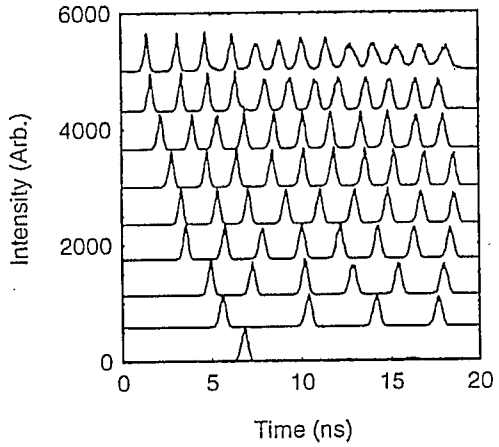

Figure 1: Optical pulses obtained from self-pulsing laser at several injection current levels.

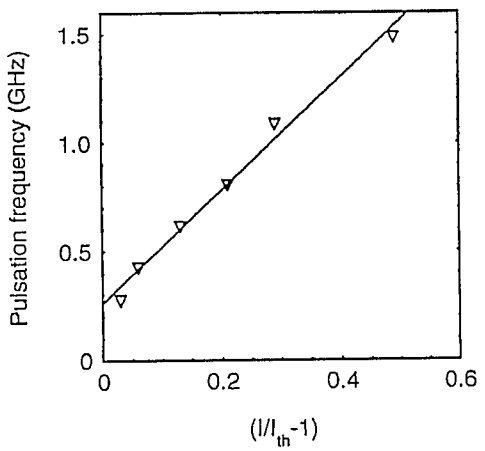

Figure 2: Variation of self-pulsation frequency with (normalised) injection current.

The material was processed into 50, and 6-μm-wide oxide-isolated stripe lasers, which were driven with a 26-ns (FWHM) current pulse at a repetition frequency of 20-kHz in order to reduce the effects of Joule heating on the performance of the device, thus helping to maintain the regularity of the pulse train. The devices are not limited to a short drive pulse, and in fact would continue to operate if the duration of the current injection pulse were considerably extended. However, the performance of the relatively wide-stripe devices degrades significantly under such conditions while the narrower stripes show a chirp in the pulsation frequency. The graph in figure 1 shows the generation of optical pulses within the duration of the 26-ns drive pulse for a 50-μm stripe, 300-μm-long device, at several injection currents. Under these conditions, close to threshold the laser produces a single, 380-ps (FWHM) wide, q-switched pulse. As the injection current level is increased further above threshold an increase in the number and frequency is observed, with complete extinction of the stimulated emission between pulses for currents close to threshold. This behaviour is summarised in figure 2 which shows a graph of the pulsation frequency as function of the normalised injection current. The pulsation frequency increases above threshold in a linear fashion until self-pulsation ceases. For most of the current range the device operates in a large signal modulation regime[12] where the recovery time of the absorber (carrier lifetime in the absorber quantum well) is much shorter than that in the gain region. In this regime the pulsation frequency is directly related to the rate at which charge carriers can be

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replenished in the gain quantum wells, and a linear relationship results. At high current injection levels the rate at which the population inversion is achieved in the gain region becomes comparable to the absorber carrier lifetime, making equation (1) increasingly difficult to satisfy since the absorber cannot recovery rapidly enough and hence there is a loss of pulsation.

It is clear that these devices are capable of producing well defined sequences of pulses with a variable repetition rate. As will be described in section V, in order to implement our stroboscopic excitation technique using a dc detector the duration of operation needs to be extended to hundreds rather than tens of nanoseconds, with the ideal being CW current injection to the laser. As is described in section V, this is a technical limitation rather than intrinsic one to the technique becoming viable.

III. EXPERIMENT 1: SELF-PULSING LASERS AS SOURCES FOR INTENSITY DECAY MEASUREMENTS

As discussed in section II, the lasers used during this study can, under certain operating conditions, (low injection or short duration drive current) be used to generate single q-switched pulses with widths in the range 300-400ps (the particular example in figure 1 being 380ps). Since these are single contact designs, where injection current is the single control parameter, the fact that they can generate single, short duration pulses makes them ideal for use as an excitation source in fluorescence lifetime measurements.

The physical mechanisms associated with the measurement of the excited state lifetime of fluorescent species are well established[13]. After excitation the fluorophore rapidly (within a few picoseconds) looses energy through the various rotational and vibrational energy states available to it, before eventually relaxing back to its ground vibrational state. This process occurs in an amount of time given by the excited state lifetime (relaxation between rotational states occurring within a few picoseconds), by the process of fluorescence. The generation of fluorescence photons can be described as in equation (2) which gives the time evolution of the emitted intensity:

$$I(t) = I(0)\exp\left(-\frac{t}{\tau}\right) \tag{2}$$

I(0) is the intensity immediately after excitation and τ is the excited state lifetime of the fluorophore. This equation, of course, represents the case of a mono-exponential decay. In fact, the fluorophore under investigation may have more than one decay route available to it, or indeed there may be more than one fluorophore in the solvent of interest; in either case equation (2) becomes the more general expression given in equation (3):

$$I(t) = I_1(0)\exp\left(-\frac{t}{\tau_1}\right) + I_2(0)\exp\left(-\frac{t}{\tau_2}\right) + \ldots + I_n(0)\exp\left(-\frac{t}{\tau_n}\right) \tag{3}$$

The fluorophore can also relax non-radiatively and the excited state lifetime will therefore be expressed as in equation (4):

$$\frac{1}{\tau} = \frac{1}{\tau_r} + \frac{1}{\tau_{nr}} \tag{4}$$

The lifetime of the fluorophore is extremely sensitive to its local environment, and in particular the non-radiative lifetime depends on parameters such as pH and the polarity of the solvent.

As an illustration of their suitability for this type experiment we performed lifetime measurements on a commercially available carbocyanine dye (Cy-5) dissolved in water and ethanol at various dilutions. This dye has an absorption spectrum which peaks at 630nm and extends to approximately 660nm and therefore has significant absorption at the emission wavelength of our laser. These were cuvette based measurements and were performed in a standard geometry, where the fluorescence is collected at 90° to the excitation at the so-called "magic" polarisation angle (54.7°), and a cut-off filter (665-nm long pass) is used to stop any scattered laser light from entering the detector (Hamamastu streak camera). The detector is triggered from the laser diode pulse generator and has a time-resolution of 50ps. An example of the data obtained is given in figure 3, and a summary of the lifetime characteristics for Cy-5 are displayed in table 1.

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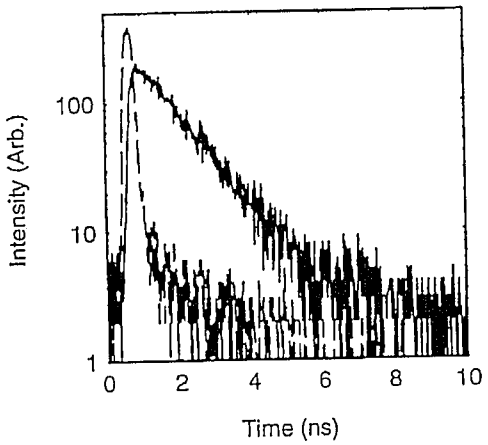

Figure 3: Fluorescence decay curve of a 20μM dilution of Cy-5 in water.

Solvent	Conc. / μM	τ / ns	χ^2
H_2O	20	1.0976	1.0999
H_2O	100	0.95206	1.1874
ETOH	20	1.6403	1.1958
ETOH	100	1.6059	1.0873

Table 1: A summary of measured decay lifetimes obtained for Cy-5 in water and ethanol.

The measurements showed that Cy-5 has a single decay lifetime of 1-ns and 1.6-ns in water and ethanol respectively, and that this lifetime does not change significantly with a change in dilution. These were obtained using a least squares fitting routine where the quality of the fit was judged using the reduced chi squared parameter. The values obtained for the lifetime compare favourably with those published in the literature[14], which gives the lifetime of Cy-5 in to be 0.91-ns in water and 1.32-ns in ethanol. The disparity in the ethanol data is attributed to a difference in purity of the distillations used.

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IV. EXPERIMENT 2: TOWARDS STROBOSCOPIC EXCITATION EXPERIMENTS

Presently the majority of lifetime measurements are performed by time correlated single photon counting (TCSPC), or by phase sensitive detection. Many other methods exist, among which include (but are not limited to): the streak camera method used in section III; stroboscopic or gated detection (the technique now employed in lifetime imaging) and up-conversion techniques; these methods have not, however, been adopted on the scale of TCSPC. TCSPC is now the premier method due to its ability to determine decay lifetimes with high accuracy (each of the other methods can do this but are limited in other ways). The detectors required for use in TCSPC must be highly sensitive, due to pulse-pile considerations, and excellent time resolution is crucial. The detector of choice is the multi-channel plate photomultiplier tube (MCP PMT) due to its high gain and high-speed performance.

Implementing lifetime measurements in a biochip (lab-on-a-chip) environment, where detection and excitation would be integrated onto the chip, introduces an extra level of difficulty. The number of fluorescent photons generated would be extremely small (even in the event that a suitable laser could be integrated onto the chip for excitation) due a greatly reduced sample size. In this type of environment we would expect the detection efficiency to increase in comparison to a cuvette based measurement, but even if this was the case, the on-chip detector would still need to be extremely sensitive. On this type of scale an avalanche photodiode (APD) would be the ideal detector since they can have a size on the micron scale. However, we would still have the problem of processing the signal, and this means either gating the detector or using a TCSPC-type system, all of which requires high-specification electronics.

Here we propose utilizing self-pulsing laser diodes in a stroboscopic arrangement with the view to eliminating the need for high-speed on-chip detection. The stroboscopic or optical boxcar technique was developed as alternative to the standard TCSPC methods[15, 16]. The principle of the measurement is shown in figure 4. As with TCSPC the sample is excited with a short pulse from an optical source (laser, flash lamp etc.) and is allowed to fluoresce. In this case, however, the detector is gated such that the fluorescence photons are measured only for a duration defined by the gate. The full time dependence of the fluorescence is then built up by varying the delay of the gate relative to the excitation pulse. This is an intensity based method, and so is not limited by the pulse pile-up problems that occur in TCSPC; the intensity of the detected signal is proportional to the intensity of the source of excitation. The time-resolution is dependent on the duration and step-size of the gate. This technique has been shown to produce data of comparable quality to TCSPC data except for fluorophores where the lifetime is extremely short or extremely long where a residual baseline limits the accuracy[16].

The concept of stroboscopic excitation using self-pulsing lasers is analogous to gating the detector, except in this case it is the laser, by virtue of its ability to produce repetitive pulses of varying separation, which provides the time resolution. This means that in principle a time-domain measurement could be performed without the need high-speed electronics.

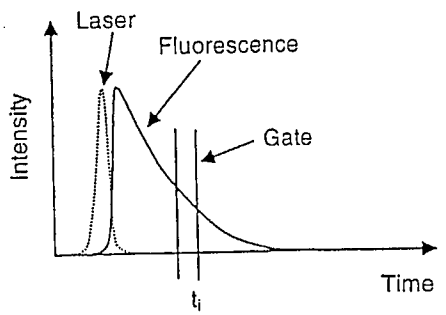

Figure 4: Schematic diagram illustrating the concept of the stroboscopic optical boxcar technique.

An example of the response of the dye to interrogation by a multiple-pulse train is shown in figure 5 for Cy-5 dissolved in water. It is clear that the fluorophores cannot fully decay before being re-excited. If we consider the fluorophores as a two-level system its response to a train of pulses with a constant pulse-to-pulse intensity, and a

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repetition period T, can be described using a standard rate-equation approach. Equation (5) gives the time-averaged fluorescence signal, S from such an analysis:

$$S = \frac{C}{T}\left[\frac{(e^{\Delta P} - 1)}{\left(e^{\Delta P} - e^{-T/\tau}\right)}\right]\left(1 - e^{-T/\tau}\right) \tag{5}$$

C is a constant related to the number of fluorophores and ΔP is a measure of the excitation intensity. The term in square brackets arises from changes in absorption coefficient as the population of the fluorophores' ground state alters with T, while the final term reflects the ability of the excited fluorophores to emit photons within the pulse period. As the period T of the excitation pulses is reduced the time-averaged excitation power increases in a linear fashion whereas the time-averaged fluorescence signal will be strongly dependent on the fluorescence lifetime (τ). If $T > \tau$ then it is clear that the collection of fluorophores will decay fully before the arrival of the next excitation pulse. As $T < \tau$ is approached the excited state population of fluorophores does not fully relax to the ground state. This implies that were the time-averaged fluorescence intensity obtained while varying the repetition period, then highly non-linear behaviour would occur at high frequency, with the precise form of this non-linearity being determined by the lifetime of the fluorophore. This effect has been used previously within a two-pulse set-up to determine lifetimes[17]. In this case the fluorophores needed to be in a saturated excitation regime but if a repetitive stream of pulses is used this restriction is lifted.

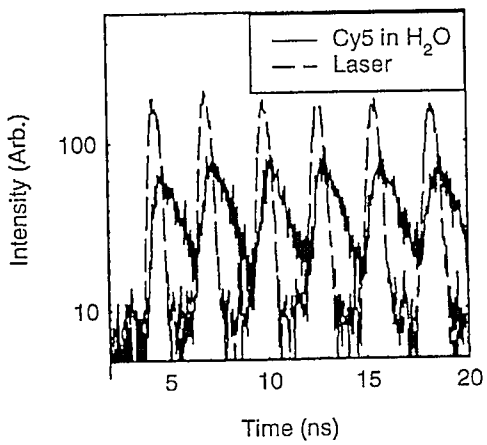

Figure 5: Fluorescence response of Cy-5 dissolved in water to interrogation by the output of the self-pulsing laser.

While the purpose of stroboscopic excitation is to allow the use of dc detection to determine time domain information, streak camera detection can be used to provide the proof of the principle. Fluorescence generated by Cy-5, such as that show in figure 5, was time-integrated over a fixed duration time window of 20-ns while varying the pulse repetition rate of the laser, to generate the data given figure 6. The time-integrated excitation signal is also given in figure 6 and has been scaled to match the magnitude of the fluorescence signal (measured in arbitrary units) at low frequency. As expected the integrated intensity of the laser increases linearly with frequency. This indicates that the intensity of each pulse in the train is similar, the total intensity being augmented by the number of excitation pulses as high injection current, and therefore high pulsation frequency, is reached. The integrated fluorescence intensity, as expected, shows radically different behaviour. A strong saturation of the signal is seen at high frequency where the pulse separation becomes comparable to the fluorescence decay lifetime.

Although the fluorescence signal shown in figure 6 is of the same functional form as equation (5), accurate determination of the decay lifetime is difficult as the excitation level of the dye molecules is not known and the time integration is over a limited number of pulse cycles. Also, it is clear from the limited number of data points that it would not be possible to determine the lifetime of the dye to the same level of accuracy achieved in the more

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conventional methods. This however, does not limit the technique when employed on a biochip format. In that case stroboscopic excitation would be extremely useful in assays where a lifetime change is to be detected (whether in a binding event or a change in the local chemistry of the species under test) since the non-linear behaviour of the time-integrated signal is determined by the lifetime of the fluorophore.

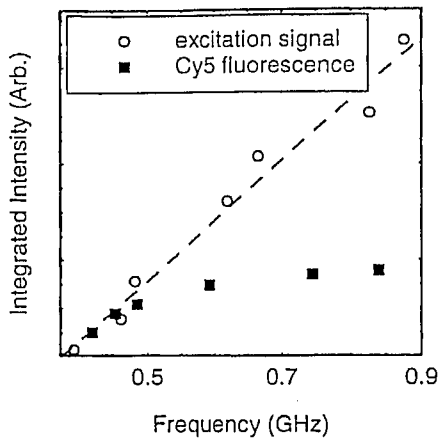

Figure 6: Time-integrated intensity of fluorescence (■) and excitation signal (O) as a function of repetition frequency.

V. SUMMARY

In this paper we have demonstrated a feasible method for obtaining time-domain information from assays performed on a biochip format where excitation and detection are to be integrated onto the chip. Our self-pulsing lasers, although limited in performance at the present time, can generate single short duration optical pulses and also multiple pulse trains of variable repetition rate. This ability to produce single pulses makes them useful as simple to operate excitation sources in traditional bench-top lifetime measurements. When operated in the multiple-pulse mode the upper limit of the repetition frequency (1-1.5-GHz) means that stroboscopic excitation measurements can be made on fluorophores with lifetimes in the nanosecond regime. Further improvements in the laser design should enable frequencies up to 2-3 GHz to be reached (self-pulsing lasers currently used in compact disc players pulse in this regime) enabling stroboscopic measurements on fluorophores with lifetimes down to 300-ps.

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