

The application of fluorescence polarisation for the enhanced detection of green fluorescent protein (GFP) in the presence of cellular auto-fluorescence and other green fluorescent compounds

A. W. Knight,^a N. J. Goddard,^a P. R. Fielden,^a A. L. Gregson,^a N. Billinton,^b
M. G. Barker^b and R. M. Walmsley^b

^a Department of Instrumentation and Analytical Science, University of Manchester Institute of Science and Technology, PO Box 88, Manchester, UK M60 1QD

^b Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology, PO Box 88, Manchester, UK M60 1QD

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The jellyfish green fluorescent protein (GFP) is a versatile biological marker and reporter. Here we demonstrate that marked polarisation in GFP fluorescence, upon excitation with plane polarised light, can be exploited to enhance the detection of GFP when in the presence of cellular auto-fluorescence and other fluorescent compounds that emit at the same wavelength. The development of flow-through instrumentation dedicated to the sensitive detection of GFP by fluorescence polarisation is described, and used in both a continuous flow and flow-injection format. The intensity, spectral properties and extent of polarisation in the auto-fluorescence of various yeast strains and growth media are investigated. The application of fluorescence polarisation is shown to enhance the measurement of the induction of GFP in yeast cells, genetically modified to produce GFP as an indicator of DNA damage, compared with a conventional fluorescence method.

Introduction

Interest in the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has grown almost exponentially over the past five years. With the ability to clone and express GFP in a diverse range of cells and organisms including bacteria, yeast, plants and higher animals, GFP has become a versatile fluorescent marker for monitoring physiological processes, visualising protein localisation and detecting the expression of transferred genes.^{1–3} The usefulness of GFP stems from the fact that the protein's fluorescence requires no additional co-factors, as the fluorophore is self-assembling *via* a cyclization reaction of the peptide backbone.³ This is in marked contrast to light emitting reporters such as *lux* and luciferase, which aside from the drain on cellular ATP, require the addition, or co-synthesis, of chemical co-factors such as aldehyde.^{4,5} GFP is also entirely bio-compatible and when used as a tag does not alter the normal function or localisation of the protein it is fused to. Without the need for fixation, proteins, cells and organelles marked with GFP can be visualised and monitored in living tissue. Hence the dynamics of cellular processes can be non-invasively quantified in real time, simply by measurement of fluorescence.¹

The wild-type GFP consists of 238 amino acids and has a cylindrical structure with the fluorophore element encapsulated in the centre.⁶ As such it is a very chemically and photochemically stable and resilient fluorophore. The bright green fluorescence at 508–515 nm is readily induced by illumination of the protein with visible blue light at 470 nm. However, genetic modification of the GFP gene has resulted in several useful mutations with fluorescence that is significantly blue- or yellow-shifted.¹

Our interest in GFP is in the development of an automated flow-injection bioassay for the detection of genotoxic compounds and the quantification of genotoxicity. The basis of the method is the use of yeast cells that are genetically modified, such that they produce GFP in response to the activation of the cells' DNA repair mechanisms by DNA damage. The presence,

concentration or potency, of a suspected genotoxic compound can then be quantified by measuring the increase in green fluorescence of the intact yeast cells.^{7–9}

Organs or organelles within the organisms or cells where GFP is localised, can be readily visualised and distinguished from the background matrix by fluorescence microscopy techniques. However, in cases where GFP is only weakly expressed, or where GFP is in free solution such as in the cell cytosol, the fluorescence signal from GFP is invariably contaminated by cellular or media auto-fluorescence.^{1,10} This has also been the case in our yeast cell studies. In an analytical context this restricts the lower limit of detection possible. To overcome this problem the commonly adopted approach has been to develop a specific combination of optical filters for each application, to enable discrimination between GFP and auto-fluorescence.¹¹ However, in many cases the excitation and emission spectra of the auto-fluorescence significantly overlap those of GFP, making it difficult to resolve the two fluorescence signals, which occur at the same wavelength.

In a previous communication we described for the first time how fluorescence polarisation might be used to improve GFP quantification in the presence of other fluorescent species.¹² Using an argon-ion laser, a single photomultiplier tube detector and a rotating polaroid filter, we demonstrated the extent of polarisation in GFP fluorescence when excited with a polarised light source. It was found that for GFP in free solution, or contained within the cytosol of the yeast cell, the fluorescence intensity perpendicularly polarised with respect to the laser light source (I_{\perp}) was only approximately 50% of that aligned parallel with respect to the laser light source (I_{\parallel}). Thus the fluorescence of GFP was found to be significantly polarised. This was assumed to be due to the large size, and hence slow rotation rate, of the protein in solution.

The degree of fluorescence polarisation (P) measured in this work was defined as follows:

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

We postulated that the background auto-fluorescence detected in whole cells and conditioned media may contain a significant contribution from smaller fluorescent species, which would produce a less polarised emission due to their faster rotation rates. If this was the case the difference between I_{\parallel} and I_{\perp} should be large for GFP where the fluorescence is significantly polarised, but much less for the background auto-fluorescence. Thus by measuring the difference signal ($I_{\parallel} - I_{\perp}$) it should be possible to discriminate between GFP and auto-fluorescence occurring at the same wavelength.

In this work we report the development of flow-through instrumentation dedicated to the measurement of fluorescence polarisation of GFP, using two carefully matched photodetectors, in conjunction with two polaroid filters in fixed orientations to measure I_{\parallel} and I_{\perp} . The instrument also incorporates a third photodetector for on-line absorption measurements to allow estimation of yeast cell concentrations. A number of fluorophores with similar spectral properties to GFP are examined for their suitability in calibrating the instrument. The effective discrimination between the fluorescence of selected fluorophores and GFP present in the same sample is demonstrated by two methods; a flow injection and a continuous flow approach, for GFP both in free solution and within intact, living yeast cells.

The intensity, spectral properties and extent of polarisation of cellular auto-fluorescence in various yeast strains have been investigated. The use of a fluorescence polarisation approach is shown to enhance the detection of GFP expressed by the genetically modified yeast cells in the presence of cellular auto-fluorescence, when compared to a conventional fluorescence method. Finally, two auto-fluorescent culture media have been examined to establish if it is possible to carry out GFP determinations in cells grown within these media, without the need for prior separation or washing of the cells before measurement.

Experimental

Materials and methods

The particular GFP derivative used in this study was 'yeast-enhanced GFP' (yEGFP) in which two types of modification have been carried out: codon optimisation for yeasts and amino acid substitutions shown to increase the fluorophore efficiency, most critically serine 65 to threonine.¹³ These modifications result in a fluorophore that is excited at 490 nm and emits at approximately 517 nm. Three strains of the brewers yeast *Saccharomyces cerevisiae* were used; our low auto-fluorescent optimised strain FF18984, and two further strains which demonstrated high auto-fluorescence, FY73 and YJL047c. Both wild-type and genetically modified (GM) variants of each strain were used. The strains were genetically modified by fusing the promoter of the gene responsible for the induction of a native protein, Rad54, with the yEGFP gene. The details of this modification have been previously reported by Walmsley *et al.*⁷ and Billinton *et al.*⁸ Synthesis of the Rad54 protein is known to be up-regulated whenever yeast DNA repair mechanisms are activated in response to DNA damage, for example, upon exposure to a genotoxic agent or ionizing radiation. In this work, by exposing the genetically modified cells to a low concentration (typically 0.005% v/v) of methyl methanesulfonate (MMS) in the culture medium, GFP expression and accumulation in the cells was observed by a marked increase in their green fluorescence. MMS (99%, Sigma-Aldrich, Gillingham, UK) is a known genotoxic alkylating agent.

Cultures of whole yeast cells used in this work were prepared as follows. A small aliquot from a freshly prepared culture of the cells was inoculated into 50 ml of medium in a conical flask,

to give a starting cell density of approximately 0.1 OD₄₈₈. (OD₄₈₈ = optical density measured using a 1 cm path length and illumination at 488 nm). MMS was added to the flask immediately after inoculation if required. The culture was then incubated at 28 °C, with orbital shaking, for between 16 and 24 h, reaching a final cell density of between 1 and 3 OD₄₈₈, depending on the MMS concentration used. For whole cell measurements the cultures were used without further treatment, except for dilution of the most dense cultures with fresh medium (without glucose) just prior to use.

GFP extracts were prepared from the MMS induced cells according to the procedure previously described by Walmsley *et al.*⁷ and Billinton *et al.*⁸ A 50 ml culture produced on average 0.5 ml of protein extract. The buffer used was at pH 7.0 and consisted of 0.02 mol l⁻¹ tris(hydroxymethyl)methylamine-hydrogen chloride (>99%, Roche Molecular Biochemicals, Lewes, East Sussex, UK), 0.1 mol l⁻¹ sodium chloride (GPR grade, BDH Ltd., Poole, Dorset, UK), 1 × 10⁻³ mol l⁻¹ ethylenediaminetetraacetic acid (GPR grade, BDH Ltd.), and 1 × 10⁻³ mol l⁻¹ phenylmethanesulfonyl fluoride (>99%, Sigma-Aldrich).

'F1', a well defined minimal medium, was used for culturing the yeast cells investigated in this work.¹⁴ This medium was chosen as it exhibited very low fluorescence at the wavelengths concerned. Two further media were investigated for their auto-fluorescence and fluorescence polarisation properties, and are discussed in detail later.

Fluorescence standards of fluorescein sodium salt (90%), Acridine Orange (>98%), 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTSA) (>95%), and Rhodamine 110 chloride (>99%) were obtained from Sigma-Aldrich. Where stated the fluorophores were used in a phosphate buffer, which comprised 0.02 mol l⁻¹ sodium dihydrogen orthophosphate (AnalaR grade, BDH Ltd.) adjusted to pH 8.25 with sodium hydroxide (volumetric standard, 0.969 N solution in water, Sigma-Aldrich).

Instrumentation

Optical components. In our previous communication,¹² work was carried out using an instrument which simply consisted of a single photodetector and a rotating polaroid filter, arranged at right angles to the polarised laser beam passing through an optical cell. The filter had to be manually rotated between parallel and perpendicular fluorescence measurements. Fig. 1 shows a schematic diagram of the arrangement of optical components and light detectors within the new instrumentation developed for this work. The instrument allows simultaneous measurements of both parallel and perpendicularly polarised fluorescence, and in addition the absorbance of the sample as it passes through an optical flow cell.

An air-cooled argon-ion laser (162LGL, LG Laser Graphics GmbH, Dieburg, Germany), provided a 488 nm excitation light source of 5 mW after filtering (TEM₀₀ >95%, beam diameter 0.67 ± 0.05 mm), plane polarised (>100:1) perpendicular to the base of the unit. Central to the instrument is a four-window quartz fluorescence flow cell with an internal volume of 132 µl, specially constructed by Hellma Ltd., Southend-on-Sea, UK. The flow cell was housed in a cubic black plastic mounting block (30 × 30 × 30 mm), built in-house, into which four internally threaded optical mounting tubes, 19 mm in diameter, were fixed to hold the various filters in place. All filters and mounts were purchased from Comar Instruments, Cambridge, UK.

Miniature photomultiplier tubes (PMTs) supplied by Hamamatsu Photonics Ltd., Enfield, UK, were used for fluorescence light detection. The PMTs were supplied within compact modules (H5784) which housed the PMT, high voltage power

supply and additional amplifying circuit, such that they could be run from a conventional ± 12 V power supply.

A 488 nm interference filter (3 nm half bandwidth), was used to filter the excitation light from the laser. At right angles to the beam path, two polaroid filters were securely mounted. One filter was rotated through 90° with respect to the other, such that one PMT recorded the fluorescence intensity emitted polarised parallel with respect to the excitation beam (I_{\parallel}), and the other recorded that emitted polarised perpendicular with respect to the excitation beam (I_{\perp}). The polaroid filters were 16 mm diameter disks, cut by hand from a polaroid sheet (Grade HN38) laminated in cellulose acetate butyrate (thickness = 0.8 mm). Additional filters were optimised and used in conjunction with the polaroid filters to allow the laser-induced GFP fluorescence at 517 nm to be measured, whilst rejecting the high intensity laser light at 488 nm scattered towards the detector by yeast cells suspended in the stream of liquid passing through the flow cell. These filters consisted of a 515 nm interference filter (10 nm half bandwidth), and a 515 nm short wave cut-off filter fabricated from three layers of orange Schott glass (OG 515, thickness = 3 mm), each layer comprising a 16 mm diameter disk cut from the supplied material using a diamond cutter. 515 nm filters were the closest, readily commercially available filters to the GFP emission maximum.

For absorbance measurements, a second 488 nm interference filter (3 nm half bandwidth), was used in conjunction with a neutral density filter ($OD_{488} = 3$), and placed in front of a silicon photodiode (SPD) detector (IPL10530DAL, RS Components Ltd., Corby, UK). The first filter rejects any fluorescence emission, whilst the second cuts down the power of the beam to a suitable level for the SPD detector. The SPD package also contains a lens such that the available light is focussed onto the 1.75 mm^2 detection chip.

Electronics and data collection. The voltage signals from the PMT and SPD detectors were processed by simple op-amp circuitry built in-house. The circuit comprised, in part, three of the sub-units shown in Fig. 2, one for each light detector. The circuit allows the incoming signal to be offset by addition of a positive or negative voltage selected by the $100 \text{ k}\Omega$ variable resistor, such that a 'dark' or 'blank' baseline signal level can be established. The degree of amplification of the incoming signals can be adjusted by the $5 \text{ k}\Omega$ variable resistor, thus allowing the sensitivity of the two PMT detectors to be equalised. The

signals are smoothed by the use of a simple low-pass filter, and an additional voltage-follower further isolates the photodetector from the recording device. To save circuit board space, quad op-amps were used (AD713JN) which were obtained, along with all other electrical components, from RS Components Ltd.

Data acquisition and subsequent manipulation was carried out in real time on a personal computer *via* a 10 bit analogue to digital converter (ADC-11, Pico Technology Ltd., Cambridge, UK) and associated software. The data collection rate was 1 sample s^{-1} , each sample being an average of 1000 readings taken during the sample interval.

The sensitivity of the amplifying circuit built into the PMT module was controlled by means of an externally applied control voltage in the range 0 to $+1.0 \text{ V}$. The control voltage was selected by means of a digital potentiometer using a reference voltage of $+1.2 \text{ V}$, supplied by the module itself, and applied to both PMTs simultaneously. This provided a means by which to scale the fluorescent signals for any particular experiment, such that the full voltage range of the ADC (0 to $+2.5 \text{ V}$) could be utilised, giving maximum resolution.

Instrument housing and sampling. The optical assembly, circuit board and its $\pm 12 \text{ V}$ power supply were housed inside a light-tight box with a shuttered aperture attached to the laser head. For measurement of large volumes of sample ($> 2 \text{ ml}$), the sample was simply circulated continuously through the instrument at a flow rate of 1.0 to 1.5 ml min^{-1} whilst replicate readings of I_{\parallel} and I_{\perp} were obtained. Alternatively, and especially for small sample volumes ($< 2 \text{ ml}$), a flow-injection approach was adopted. Injections of $70 \mu\text{l}$ aliquots of the sample were made into a suitable carrier stream flowing through the instrument by means of a rotary injection valve (Type 50, Rheodyne Inc., Cotati, CA, USA). The resulting flow-injection peaks were integrated using Visual Basic software written in-house. The internal liquid volume of the whole instrument including all tubing was approximately 1 ml .

Normal fluorescence measurements. For the conventional measurements of fluorescence, scattered light intensity and for obtaining excitation and emission spectra a standard commercial fluorescence spectrometer was used, (LS50-B, Perkin-Elmer, Warrington, Cheshire, UK), with a 1 cm path length acrylic cuvette. A yellow Schott glass filter (YG495, thickness = 3 mm) was placed within the sample chamber directly in front of the PMT window, in order to block excitation light

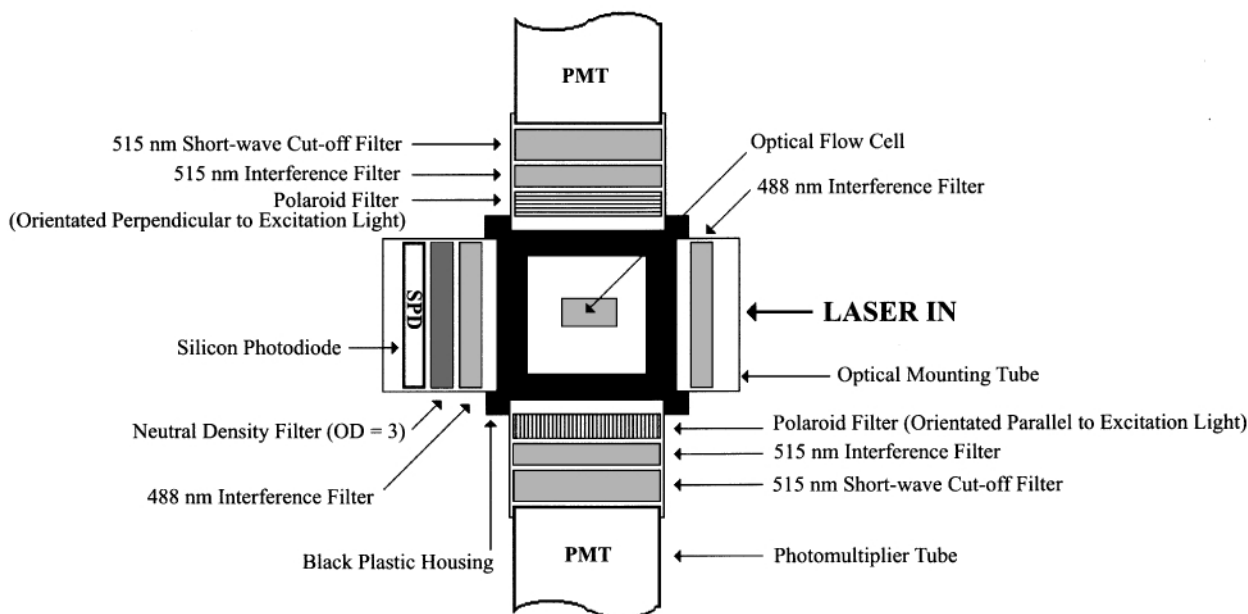


Fig. 1 Schematic diagram of the instrument optics.

scattered towards the PMT by yeast cells suspended in the cuvette. For fluorescence measurements the excitation and emission wavelengths were 490 and 520 nm, respectively. For light scattering nephelometric measurements, the excitation and emission wavelengths were both 600 nm. Light intensity was recorded in arbitrary units produced directly by the instrument software.

Results and discussion

Calibration

In order to accurately quantify I_{\parallel} versus I_{\perp} and thus obtain fluorescence polarisation measurements, the sensitivity of the PMTs and associated circuitry needed to be balanced, such that each detector responded identically to increasing fluorescence light intensity. This was achieved by fine adjustment of the variable resistors in the circuit shown in Fig. 2, whilst a solution of a suitable fluorophore was pumped through the flow cell. This adjustment needed to be performed with the polaroid filters in place, since dismantling the instrument optics to insert the polaroid filters after the sensitivity adjustment invariably led to slight errors in the alignment of filters, and thus a change in the apparent PMT sensitivity. Hence a fluorophore needed to be selected which had very similar spectroscopic properties to GFP, but which exhibited extremely low, or no, fluorescence polarisation when excited by plane polarised light. Thus the sensitivity of the PMTs could be balanced with all the filters in place, such that with the fluorophore passing through the flow cell, $I_{\parallel} = I_{\perp}$ and $P = 0$, in effect establishing a baseline.

Four fluorophores were chosen, which were close spectroscopic mimics of GFP. These were fluorescein, Rhodamine 110, Acridine Orange and HPTSA. Using the commercial fluorimeter, the fluorescence excitation and emission spectra for each fluorophore and GFP were obtained, which gave the wavelength maxima shown in Table 1, to the nearest 0.5 nm. The GFP sample was a cellular extract diluted 10-fold with distilled water. The emission spectra of fluorescein and HPTSA closely overlapped that of GFP, whereas those of Rhodamine 110 and Acridine Orange were slightly red-shifted with respect to GFP. The excitation maxima of all the compounds tested

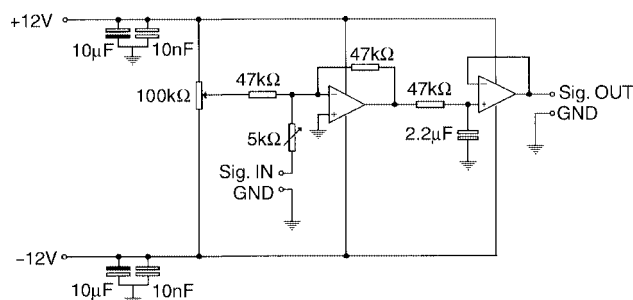


Fig. 2 Photo-detector signal offset, amplification adjustment and smoothing circuit.

closely matched that of GFP except for that of HPTSA at 455 nm. However, since HPTSA exhibits a high fluorescence yield and there is sufficient overlap of the excitation spectra of HPTSA and GFP to allow excitation of HPTSA at 488 nm, this fluorophore could be used as a GFP mimic in the system. HPTSA needed to be used at 20 times the concentration of fluorescein to obtain the same magnitude fluorescence signal.

To measure the fluorescence polarisation ratios (P) of the fluorophores, two polaroid filters (Grade HN38 polaroid sheet, laminated in rigid acrylic, thickness = 3.4 mm) were inserted within the fluorimeter sample chamber, one either side of the sample cuvette. By manually rotating the second polaroid filter placed between the cuvette and fluorimeter PMT, through 90° , measurements of I_{\parallel} and I_{\perp} could be readily obtained. Table 1 shows the average P values obtained from 20 replicate readings for each fluorophore along with their fluorescence lifetimes as quoted in the literature. The results obtained indicated that HPTSA shows the lowest fluorescence polarisation of the fluorophores tested, with less than 0.5% difference between I_{\parallel} and I_{\perp} . Fluorescein and Rhodamine 110 gave small but significant P values of similar magnitude, as might be expected due to their similar structures. Acridine Orange consistently produced a higher P value of 0.0141. HPTSA was therefore chosen to balance the sensitivities of the two PMTs. GFP in the cellular extract gave a predictably high P value of 0.398. This is slightly higher than our previously reported result.¹² The reason for this is the higher concentration of GFP in this extract compared to other cellular auto-fluorescent species, which reflects our further optimisation of the GFP induction, expression and extraction procedures. By measuring the fluorescence of an extract of a wild-type culture grown under the same media conditions, and correcting for total protein concentration (OD_{280}), it was predicted that the cellular auto-fluorescence would contribute approximately just 3% of the total fluorescence signal in this particular GFP extract. Hence 0.398 will be very close to the true value of P that would be obtained from a solution of pure GFP.

The polarisation ratio can be seen to generally increase with decreasing fluorescence lifetime in Table 1. This may be expected since the longer the fluorescence lifetime, the greater the time available between excitation and emission for the molecule to become randomly orientated in solution, and thus produce an isotropic fluorescence emission. Although GFP exhibits the shortest fluorescence lifetime, of 2.6 to 2.9 ns, it is likely that its marked fluorescence polarisation and high P value is more attributable to its large size (27 kDa), and hence slow rotation rate. Swaminathan *et al.*¹⁹ determined the rotational correlation time of GFP (S65T derivative) to be 20 ns in aqueous solution and 36 ns in cell cytoplasm. Hence, the fluorescence lifetime of GFP was observed to be considerably less than its rotational correlation time. In comparison, fluorescein had a rotational correlation time of 120 ps, approximately 40 times shorter than its fluorescent lifetime.

At the maximum sensitivity setting afforded by the digital potentiometer, the signals from both PMTs were equalised with a solution of 4×10^{-8} mol l⁻¹ HPTSA in phosphate buffer, passing through the flow cell at 1.0 ml min⁻¹.

Table 1 Spectroscopic properties and fluorescence polarisation ratios for GFP and similar fluorophores

Fluorophore	Excitation maximum/nm	Emission maximum/nm	Solvent	Excited state lifetime/ns	Solvent	Reference	Polarisation ratio (P)
HPTSA	455.0	509.5	H ₂ O/pH 8.25	5.5	H ₂ O	15	-0.0005
Rhodamine 110	496.0	518.5	H ₂ O	3.8	H ₂ O	16	0.0037
Fluorescein	491.0	512.0	H ₂ O/pH 8.25	3.0/4.8	H ₂ O pH < 7/H ₂ O pH > 7	17	0.0040
Acridine Orange	491.5	521.5	H ₂ O	3	H ₂ O	18	0.0141
GFP	490.0	517.0 (yEGFP)	H ₂ O/pH 7.0	2.9/2.6 (S65T-GFP)	H ₂ O/Cell Cytoplasm	19	0.398 (yEGFP)

To determine the absolute sensitivity of the instrument a calibration was performed by measuring I_{\parallel} for a range of fluorescein standards and a blank. On the most sensitive settings a calibration could be performed over the concentration range 1 to $20 \times 10^{-10} \text{ mol l}^{-1}$, which gave a signal range of 0 to +2.0 V. A sensitivity of 0.096 V per $10^{-10} \text{ mol l}^{-1}$ fluorescein and a limit of detection of $2 \times 10^{-11} \text{ mol l}^{-1}$ (equivalent to 5 times the standard deviation of the blank) were achieved. It should be noted that the polaroid filters used have a transmission of 38%, and in the absence of the polaroid filters fluorescein can be readily detected in the pmol l^{-1} range, using the same optical set-up.

Flow-injection analysis

Fig. 3 shows the typical response from the instrument when used with flow-injection analysis. A culture of GM-FF18984 yeast cells was inoculated into F1 media containing 0.005% MMS and incubated at 28 °C for 18 h. The final culture was diluted 50:50 with fresh F1 media, containing no glucose, to give a yeast cell suspension of 0.58 OD_{488} . 70 μl aliquots of the culture were injected into a carrier stream of F1 media, again without glucose, flowing at a rate of 1.5 ml min^{-1} . In Fig. 3, A and B represent the response from the PMTs measuring parallel and perpendicularly orientated fluorescence, respectively. The analytical signal is the difference between these responses ($I_{\parallel} - I_{\perp}$), recorded in real time and shown in trace C. Using the peak areas for each injection, the average fluorescence polarisation ratio was found to be 0.310 for GFP within whole yeast cells, which results in an analytical signal approximately 50% as great as I_{\parallel} alone. The P value is slightly lower than that obtained for the GFP extracted from cells due to the additional auto-fluorescence from the yeast cells as discussed later. The signal, however, is still large and well resolved.

The potential of using fluorescence polarisation is clearly demonstrated by the response obtained for HPTSA, as shown in Fig. 3. 70 μl aliquots of $1 \times 10^{-7} \text{ mol l}^{-1}$ HPTSA were injected into a carrier stream of phosphate buffer flowing at a rate of 1.0 ml min^{-1} . Large responses are recorded by both PMTs, however, as $I_{\parallel} \approx I_{\perp}$ and $P \approx 0$, the difference signal ($I_{\parallel} - I_{\perp}$) is close to zero, resulting in a flat baseline being recorded as the analytical signal. The same response was obtained for fluorescein under similar conditions. Hence the instrument clearly discriminates the polarised fluorescence of GFP from the isotropic fluorescence of smaller fluorophores, occurring at the same wavelength.

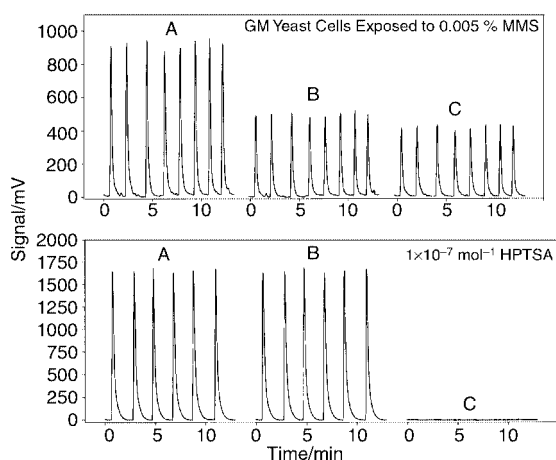


Fig. 3 Comparison of the detector response for GFP expressed in whole GM yeast cells and for HPTSA, analysed using flow-injection. A, Fluorescence signal from the parallel filter detector (I_{\parallel}); B, fluorescence signal from the perpendicular filter detector (I_{\perp}); C, the difference signal ($I_{\parallel} - I_{\perp}$).

The average relative standard deviation in peak areas from injections of yeast cell suspensions was <3%, and from the fluorophores in solution <1%. The lower reproducibility for yeast cultures was attributed to effects resulting from inhomogeneous mixing as the denser yeast culture is injected into the F1 media carrier stream flowing into the optical cell. In addition increased noise was observed in the signals due to variations in the level of induction of GFP, and hence brightness, of individual cells. The former effect was minimised by careful selection of the carrier stream, diluting culture densities to less than 1 OD_{488} and adjustments to the flow rate.

It was noted that at higher flow rates, $> 1.5 \text{ ml min}^{-1}$, a small negative peak resulted for injections of HPTSA and fluorescein. This was due to a slightly slower response of the PMT and associated circuitry measuring the I_{\parallel} signal compared to that measuring the I_{\perp} signal on the sharply rising front edge of the flow-injection peak. This problem could be resolved either by making the detection circuits faster by lowering the value of the smoothing capacitor shown in Fig. 2, or more closely matched, by using precision components. However for our purposes the use of flow rates less than or equal to 1.5 ml min^{-1} removed this effect.

Continuous flow analysis

For continuous flow analysis a sample was pumped through the instrument at a flow rate of 1.0 to 1.5 ml min^{-1} whilst replicate readings of I_{\parallel} and I_{\perp} were accumulated. Typically 20 replicate readings were obtained for each sample, with a relative standard deviation of <1.5% for whole cells expressing GFP.

To demonstrate the effectiveness of the fluorescence polarisation approach in a continuous flow method, an experiment was carried out with whole cells similar to that previously performed using extracts of GFP.¹² A series of samples were made up each containing 0.3 OD_{488} GM-FF18984 yeast cells from a stock culture grown overnight in the presence of 0.005% MMS, to which increasing concentrations of fluorescein were added, ranging from 0 to $16.5 \times 10^{-10} \text{ mol l}^{-1}$. The results are shown in Fig. 4. As the fluorescein concentration increases, the signals for I_{\parallel} and I_{\perp} increase linearly, such that at the highest fluorescein concentration tested the I_{\parallel} signal is twice as intense as the GFP alone. However, since for fluorescein $I_{\parallel} \approx I_{\perp}$, by taking the signal for GFP as the difference between these measurements, the increase in fluorescence caused by the

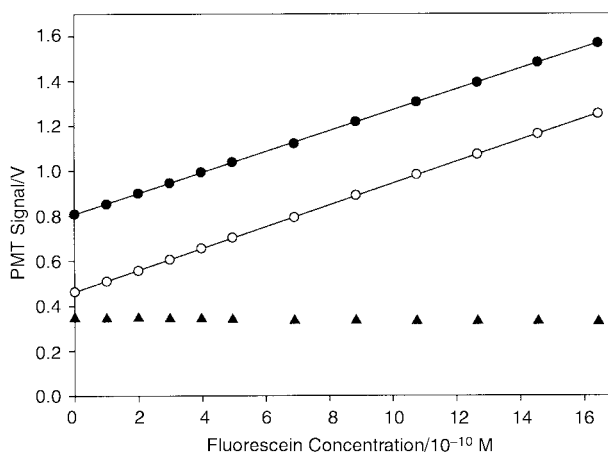


Fig. 4 Variation in fluorescence signals as fluorescein is added to a culture of GM yeast cells expressing GFP. (●) Fluorescence signal from the parallel filter detector (I_{\parallel}); (○) Fluorescence signal from the perpendicular filter detector (I_{\perp}); (▲) The signal for GFP, taken as the difference signal between these respective measurement ($I_{\parallel} - I_{\perp}$). The lines shown are the linear regressions of the measured fluorescence signal versus fluorescein concentration.

addition of fluorescein to the yeast cell culture could be effectively removed from the measurement. The measurements for GFP ranged from 0.345 to 0.327 V with an average of 0.336 V and a relative standard deviation of 1.97% ($n = 12$).

Measurement of cell density

In order to correct for variations in cell density between cultures, the fluorescence results were normalised by quoting a 'brightness', essentially the fluorescence in arbitrary units per cell. The calculation of brightness differed between the normal fluorescence and fluorescence polarisation methods.

For convenience, in the normal fluorescence method, using the LS50-B spectrometer, culture density was quantified by nephelometry. For the 'normal fluorescence' method, brightness (B_{nf}) was defined as the fluorescence intensity at 520 nm (excitation wavelength = 490 nm), divided by the scattered light intensity at 600 nm. Using a series of yeast cell cultures in the range 0 to 1 OD, the scattered light intensity was found to vary linearly with OD_{600} according to the relationship $OD_{600} = 0.0023 \times \text{scatter intensity}$, (correlation coefficient = 0.99917, $n = 20$).

In the fluorescence polarisation instrument, culture density was quantified on-line using a turbidimetric method. The SPD measures the drop in the intensity of the laser beam as it passes through the optical cell, due to scattering and absorption of the light by the suspension of yeast cells. For the fluorescence polarisation method, brightness (B_{fp}) was defined as, the difference signal ($I_{||} - I_{\perp}$), divided by the SPD signal. Using a series of yeast cell cultures in the range 0 to 2 OD, the SPD signal (V) was found to vary with OD_{488} according to the quadratic relationship: $OD_{488} = (1.31 \times \text{SPD signal}) + (0.253 \times \text{SPD signal}^2)$, (correlation coefficient = 0.99999, $n = 15$).

With the wild-type yeast cells, since the optical density arises primarily from the scattering of light passing through the culture as opposed to absorption, there is no significant difference between OD measurements made at 488 nm and the more commonly used 600 nm. In each case the measured OD relates linearly to the actual yeast cell concentration up to an OD of about 0.6, and is generally a reliable indicator of cell concentration up to an OD of 1.0. Above an OD of 1.0 this measurement underestimates the number of cells, as the relationship between cell concentration and OD deviates from linearity. Hence in the calibration equations given, for dense cultures with an OD greater than 0.6, OD_{488} and OD_{600} used are those that would be obtained by diluting back into the linear range, and scaling up the OD measurement obtained by the dilution factor. In this way OD measurements can be used to accurately measure culture density.

Cellular auto-fluorescence

The measurements of GFP in both whole yeast cells and protein extracts are unavoidably made in the presence of cellular auto-fluorescence. An investigation was carried out to examine the intensity of this auto-fluorescence, its spectroscopic properties, the extent of its fluorescence polarisation, and whether the application of fluorescence polarisation would enhance the measurement of GFP induction by MMS in its presence. Three yeast strains were selected from other related studies within our laboratory; FF18984, a strain selected empirically for low intrinsic auto-fluorescence and high GFP induction during DNA damage repair; FY73, a strain exhibiting generally higher auto-fluorescence; and YJL047c, a strain exhibiting high auto-fluorescence that was further increased after exposure of the cells to MMS. Table 2 shows the intensity of the auto-fluorescence in cultures of the various wild-type, un-modified yeast strains, grown with and without the presence of MMS. The results were obtained on the LS50-B spectrometer and are

brightness values obtained using the normal fluorescence method (B_{nf}). The brighter auto-fluorescence from FY73 and YJL047c is clearly demonstrated, as is the additional induced fluorescence in YJL047c resulting from the presence of increasing concentrations of MMS.

The fluorescence spectra of the three selected wild-type yeast strains upon excitation at 488 nm are shown in Fig. 5. Note that the 495 nm Schott glass cut-off filter was in place as it is routinely used in both the new flow-through instrument and commercial fluorimeter, for removing interference from excitation light scattered towards the detector when measuring yeast cell suspensions. The spectra were found to be almost identical and thus the auto-fluorescence is thought to originate from the same species in each case. The exact source of the auto-fluorescence is still under investigation, however, there are several likely candidates, including reduced nicotinamide nucleotides, oxidised flavins, age-related pigments and oxidised aromatic amino acids such as tryptophan. Fig. 5 also shows the fluorescence spectrum of GFP (yEGFP variant). This was obtained by subtracting a spectrum of a cellular extract of the wild-type FF18984 cells from the spectrum of GFP in a cellular extract of GM cells grown under the same media conditions, with both spectra corrected for their total protein concentration (OD_{280}). A clear peak at 517 nm is observed for GFP, however its spectrum is almost completely overlapped by that of the cellular auto-fluorescence inherent to all the strains, which takes the form of a broad peak with maxima in the range 524 to 528 nm.

The basis of the fluorescence polarisation method is, that provided the interfering species emitting at the same wavelength as GFP produces fluorescence that is less polarised than GFP, *i.e.*, has a lower fluorescence polarisation ratio, then by taking a difference measurement ($I_{||} - I_{\perp}$), a greater percentage of the auto-fluorescence signal will be removed in the final measurement than that of the signal due to GFP. Hence, an increase in the 'signal to background noise' ratio will result.

From the wild-type cultures examined the average polarisation ratios obtained for the FY73 and YJL047c strains were

Table 2 A comparison of the intensity of auto-fluorescence in whole cells of the various wild-type yeast strains studied, grown with and without the presence of MMS

Wild type yeast strain	Brightness (arbitrary units) at different MMS concentrations (%)		
	0	0.0025	0.0050
FF18984	1.48	—	1.63
FY73	4.71	—	4.64
YJL047c	4.39	5.55	6.37

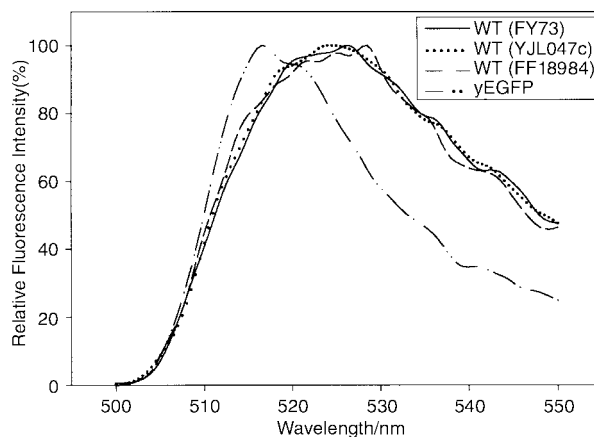


Fig. 5 Spectra of the auto-fluorescence in various strains of the wild-type yeast cells studied, and of yEGFP.

0.044 and 0.043, respectively. The polarisation ratio observed for the less fluorescent FF18984 strain was consistently higher, with an average value of 0.079. Hence, one might speculate that the additional fluorescence of the FY73 and YJL047c strains may be due to smaller species with greater rotational rates. However, since the fluorescence spectra of the three strains were so similar, similar species in the FF18984 strain may be attached to, or be an intrinsic part of, larger proteins or other molecules. In each case the fluorescence polarisation ratios are significantly lower than that of GFP and hence the polarisation method should be effective in discriminating between GFP and this auto-fluorescence.

In our studies it is often useful to quantify the degree of genotoxic action of a test compound by the apparent 'induction ratio' of GFP in the GM yeast cell culture. The induction ratio is defined as: the brightness of a GM yeast cell culture exposed to the test compound, divided by the brightness of a similar culture not exposed to the test compound. For accuracy the two test cultures, effectively a sample and control, are sub-cultured from the same source and grown up under identical media and temperature conditions. The brightness measurements of both cultures are taken at the same time, usually after the exposed culture has reached maximum GFP induction, about 10 to 12 h after exposure.

To test the polarisation method, GM and wild-type yeast cell cultures of each strain were grown up both in the presence and absence of 0.005% v/v MMS. The brightness of each culture was measured by both the normal fluorescence and fluorescence polarisation methods. Fig. 6 shows the results of the comparative trial with both B_{nf} and B_{fp} scaled such that the brightest culture ("GM + MMS" in which GFP is maximally induced) is 100%.

The use of fluorescence polarisation markedly reduced the auto-fluorescence signal in each case compared to the GFP signal, as evidenced by the wild-type cultures. The GM cells are essentially the same as the wild-type cells except for the added ability to produce GFP, and hence the auto-fluorescence observed in the wild-type cultures will be an intrinsic part of the fluorescence from the GM cells. It should be noted that the GM yeast cells not exposed to the genotoxin are significantly brighter than the wild-type cells. This is due to a near constant background concentration of GFP. The GFP arises from low level expression of Rad54 in the absence of DNA damage, which is only up-regulated when a threshold amount of DNA damage is detected and acted upon. In addition, a small amount of DNA damage and associated repair occurs all the time in normal healthy cells. The rate of degradation of GFP in these cells appears to equate with the rate of accumulation under normal conditions, hence the cells generally appear to have a reproducible and stable low level of brightness.

As expected the three different strains induce to different degrees, with induction ratios, as measured by fluorescence polarisation, of 5.40, 1.94 and 2.39 for FF18984, FY73 and YJL047c, respectively. The reduction in the auto-fluorescence fraction of the signal for the GM cells resulted in an increase in the apparent 'induction ratio'. The results quoted represent an increase by an average factor of 1.5 in each strain, for a concentration of 0.005% MMS, when using the fluorescence polarisation method as compared to the conventional fluorescence method.

In general, the fluorescence polarisation method of enhancing the signal to background fluorescence ratio should prove more effective at low GFP inductions where the auto-fluorescence forms a greater proportion of the overall fluorescent signal.

Media auto-fluorescence

Throughout this work 'F1' medium has been used for culturing cells due to its extremely low auto-fluorescence. 'F1' is a well

defined minimal medium made up of 20 separate components including trace elements, vitamins, amino acids and inorganic salts.¹⁴ As such it is laborious to prepare, and yeast cultured in it grow much more slowly compared with richer media. An investigation was carried out to examine the degree of polarisation of the high auto-fluorescence observed in richer, more complex media, that had so far prevented its use in our *in vivo* GFP fluorescence studies.

Two media were investigated. Firstly, YEP, a deep yellow, rich, complex medium containing 1% yeast extract and 2% peptone in distilled water. Secondly, YNB, a colourless, minimal, defined medium, containing 0.67% yeast nitrogen base (without amino acids), 20 mg l⁻¹ L-leucine and L-histidine HCl and 30 mg l⁻¹ L-lysine HCl in distilled water. Glucose was omitted from each medium sample tested in order to inhibit bacterial contamination growth during the course of the experiment. All media components were obtained from Difco Laboratories, Detroit, MI, USA, except for the amino acids which were obtained from Sigma-Aldrich.

The fluorescence spectra of both media solutions were obtained using an excitation wavelength of 488 nm. Both were very similar with a broad peak in the 500–600 nm range

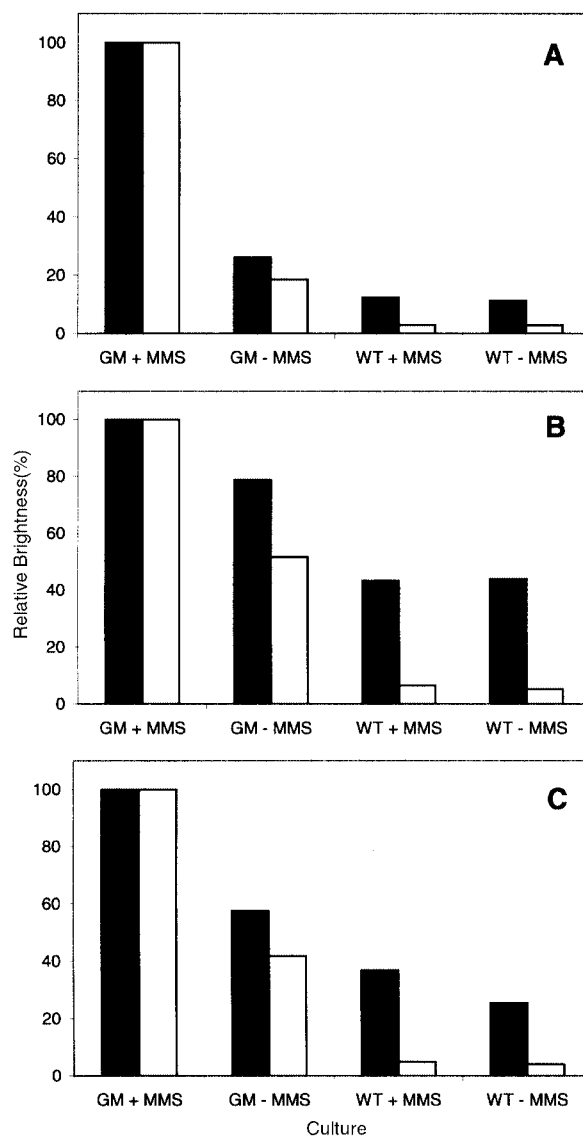


Fig. 6 A comparison of the relative brightness measurements obtained from normal fluorescence (■) and fluorescence polarisation (□) methods, for cultures of genetically modified (GM) and wild-type (WT) cells of each of the three strains tested. The strains are A, FF18984; B, FY73; and C, YJL047c.

investigated, with a maximum at approximately 518 nm, thus having considerable spectral overlap with GFP.

To observe similar full-scale fluorescence signals on the fluorescence polarisation instrument, the YEP and YNB media needed to be diluted 10-fold and 3-fold with water, respectively. Each was examined using the continuous flow approach at a flow rate of 1.0 ml min⁻¹. From average readings of I_{\parallel} and I_{\perp} the polarisation ratio of the YEP medium was estimated as 0.069 and that of the YNB medium as 0.0069. This represents a signal for I_{\perp} of 87.0 and 98.6% as intense as I_{\parallel} for the YEP and YNB media, respectively. Thus, even at 10 times dilution, YEP shows high auto-fluorescence with significant polarisation, precluding its use in our studies without separation of cells and media off-line. The YNB medium, however, demonstrated reasonably intense auto-fluorescence but with a low fluorescence polarisation. Hence it is expected that in future work the expression of GFP could be monitored in cells grown in this medium, using the fluorescence polarisation technique, without the need for separation.

Conclusions

The observed polarisation in the fluorescence from GFP has enabled its enhanced detection in whole yeast cells in the presence of significant auto-fluorescence, using the instrumentation developed in this work. Auto-fluorescence from three strains of brewers yeast showed large spectral overlap with GFP, but demonstrated a significantly lower degree of polarisation when excited with plane polarised light. Hence, improved discrimination between GFP and auto-fluorescence could be observed and an enhanced signal to background ratio achieved. Media have also been identified that exhibit auto-fluorescence which is either low or which has a low degree of polarisation, which could be useful in future fluorescence polarisation studies.

With such a degree of spectral overlap between GFP and auto-fluorescence and thus limited options for simple optical filtering, the alternative methods of discrimination are based on fluorescence lifetime analysis with gated detection. Such methods would require more complex and expensive optical and electronic instrumentation than is described here, and may be hampered by the short lifetime of GFP compared to common fluorophores. The main advantage of using the fluorescence polarisation approach is that the instrument requirements, or modifications, are relatively simple. For example, in many cases just the insertion of inexpensive polaroid filters into existing instrumentation.

It is also postulated that further restricting the rotational movement of GFP by binding of the GFP marker to another protein, antibody or cell organelle, will increase the degree of polarisation of GFP fluorescence, thus allowing even greater discrimination. For example, a significant increase in the

polarisation of GFP fluorescence of approximately 4%, was reported by Park and Raines²⁰ upon the binding of GFP-tagged-S-peptide to S-protein fragments of ribonuclease, which the authors exploited to determine binding coefficients.

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