



# Fluorescence polarisation of green fluorescent protein (GFP). A strategy for improved wavelength discrimination for GFP determinations

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The fluorescence of green fluorescent protein (GFP), present both within whole yeast cells and in protein extracts from yeast cells, has been observed to be significantly polarised. Fluorescence polarisation is proposed as a useful technique to allow some discrimination between GFP fluorescence and that of other interfering species in cell or media matrices, which have fluorescence bands that overlap those of GFP, which should lead to improved resolution and limits of detection. The method has been tested by discriminating between the fluorescence of GFP in cell extracts and added fluorescein, both of which fluoresce brightly at the same wavelength. The flow-through instrumentation incorporating an argon-ion laser developed for this work is also described.

## Introduction

Green fluorescent protein from the jellyfish *Aequoria victoria* has recently emerged as a self-assembling, biologically compatible fluorescent probe and label, which is revolutionising research across many areas of biology. It has become a versatile reporter for monitoring gene expression, protein localisation, intracellular dynamics of proteins and organelles, and for a variety of biological screens. Its use has been demonstrated in a variety of cells and organisms including viruses, bacteria, yeast, plants and animals.<sup>1-4</sup>

The wild-type green fluorescent protein consists of 238 amino acids, and has a cylindrical structure with the fluorophore element encapsulated in the centre. Once expressed by the cell, autocatalytic oxidation and cyclization of the GFP amino acids at positions 65-67 leads to the formation of the fluorophore, however the fluorescence also requires further interactions of the fluorophore with other parts of the protein.<sup>5</sup> The oxidation reaction only requires the presence of molecular oxygen, and the subsequent fluorescence does not require any additional gene products, substrates or other factors. On account of its structure, GFP is both chemically and photochemically a very stable and resilient fluorophore. The bright green fluorescence emission from GFP ( $\lambda_{\text{max}} = 508-515$  nm) is readily induced by illumination of the molecule with blue light ( $\lambda_{\text{max}} = 470$  nm). Hence, GFP can be monitored non-invasively, in viable cells and organisms in real time. Its widespread adoption as a reporter has caused a recent upsurge in a variety of qualitative and quantitative fluorescence microscopy and flow cytometric techniques.<sup>6,7</sup>

Our interest in GFP was in the development of an automated flow-injection bioassay system for the detection of genotoxic compounds and quantification of genotoxicity, with applications envisaged in environmental monitoring and pharmaceutical screening.<sup>8</sup> The basis of the system are yeast cells,

genetically modified such that they produce GFP whenever DNA damage occurs due to exposure to a genotoxic compound. The presence, concentration or potency of a genotoxic agent can then be quantified by measuring the increase of fluorescence of whole yeast cells at 515 nm.

In our experiments certain yeast strains and some growth media showed a background autofluorescence at wavelengths which overlapped that of the GFP fluorescence, and as such sometimes presented a significant interference signal. It was in an effort to improve signal to noise (*i.e.* background autofluorescence) levels, limits of detection and the discrimination of GFP fluorescence from that arising from other unknown cellular or media species, that led us to investigate the fluorescence polarisation of GFP.

In microscopy studies, particular organs or organelles within cells or organisms targeted by GFP can be readily distinguished from the background matrix. However, in cases where GFP is only weakly expressed or where GFP is in free solution, for example within the cell cytosol, the fluorescence signal from GFP is often contaminated by cellular or media autofluorescence.<sup>9</sup> This restricts the limit of detection possible. A common approach has been to develop a combination of optical filters for each application, to enable quantifiable discrimination of the GFP signal over the background autofluorescence.<sup>10,11</sup> However species with fluorescence excitation and emission bands which significantly overlap those of GFP produce an interference signal which is difficult to distinguish and remove.

In this work we report preliminary findings that GFP in solution demonstrates the phenomenon of fluorescence polarisation. This should provide a new strategy to discern GFP fluorescence from some of the background autofluorescence, with relatively simple modifications to existing instrumentation.

When a small fluorescent molecule is illuminated by plane polarised light, those molecules with their electronic transition moment aligned parallel to the electric vector of the excitation light are excited. The subsequent fluorescence emission, however, will be largely unpolarised, since the molecule is free to rotate during the time taken for the electronic transitions of fluorescence emission to occur. That is to say the rotational relaxation time is much shorter than the fluorescence decay time and the molecular orientation effectively becomes randomised before fluorescence occurs. Since GFP is a relatively large molecule and the actual fluorophore element is rigidly encapsulated within the cylindrical structure, it was suspected that the fluorophore would rotate at a comparable or slower rate than the rate at which it fluoresces. This would result in the fluorescence being significantly polarised parallel to that of the absorbed radiation. The fluorescence lifetime measured by other workers for the commonly used S65T mutation of GFP is 2.8 ns.<sup>12,13</sup>

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

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

where  $I_{\parallel}$  is the fluorescence intensity measured polarised parallel to the absorbed plane-polarised radiation, and  $I_{\perp}$  is that perpendicular to the absorbed radiation.

The difference between  $I_{\parallel}$  and  $I_{\perp}$  should be large for GFP where the fluorescence is significantly polarised, but much less for other smaller species that contribute towards the background autofluorescence where fluorescence is largely unpolarised. Hence by measuring this difference, ( $I_{\parallel} - I_{\perp}$ ), it should be possible to discriminate the signal due to GFP from that of an interfering species fluorescing at the same wavelength.

In this work fluorescence polarisation of GFP has been measured in free solution both in diluted cell extracts and when contained within the cytosol of intact cells of the brewers yeast *Saccharomyces cerevisiae*. Instrumentation constructed for this work including a flow cell, laser excitation source and combination of optical filters, is described. As a proof of principle the signal for GFP was extracted from fluorescence due to added fluorescein, a close spectroscopic mimic of GFP.

## Materials and methods

The particular GFP derivative used in this study was 'yeast enhanced GFP' (yEGFP), which has an excitation maximum at 490 nm and an emission maximum at 518 nm.<sup>14</sup> In yEGFP two types of modification have been carried out: codon optimisation for yeasts, and amino acid substitutions shown to increase the fluorophore efficiency (critically, serine 65 to threonine). A strain of the brewers yeast *Saccharomyces cerevisiae* (FF18984) was 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.*<sup>8,15</sup> Synthesis of the Rad54 protein is known to be up-regulated whenever the yeast cell's DNA repair mechanisms are activated in response to DNA damage, for example after exposure to a genotoxic chemical or UV or ionizing radiation. Hence, by exposing the genetically modified (GM) cells to a low concentration of methane methylsulfonate (MMS) in the culture media (a known genotoxic alkylating agent), GFP accumulation in the cells was observed by an increase in their fluorescence.

GFP was produced as follows. A small aliquot of a freshly prepared culture of the GM cells was inoculated into 50 ml of 'F1' medium to give a starting cell density between 0.15 and 0.25 OD<sub>488</sub> (OD<sub>488</sub> = optical density of the cell culture measured using a 1 cm path length and illumination at 488 nm). 'F1' was a defined medium, selected to exhibit low fluorescence at the wavelengths concerned.<sup>16</sup> 0.01% MMS (v/v) was added and the culture was stirred vigorously by a magnetic stirrer for between 16 and 24 h, reaching a final cell density typically between 1.5 and 2.5 OD<sub>488</sub>. For whole cell measurements the culture was used without any further treatment.

Extracts of GFP were prepared from the cells by the procedure described by Walmsley *et al.*, which involved washing the cells clear of the supporting medium, crushing by vortexing with 0.45 µm diameter glass beads, re-washing and centrifugation.<sup>8,15</sup> Each of the protein extracts (approximately 0.5 ml) was then diluted to 3 ml with distilled water before measurement, for ease of handling in the flow-through fluorescence detector.

Fluorescein (sodium salt, Aldrich Chemical Company, Poole, UK) was used to simulate an interfering fluorophore in this work since it is a close spectroscopic mimic of GFP. Fig. 1 shows the fluorescence excitation and emission spectra of fluorescein (in 0.02 M phosphate buffer, adjusted to pH 8.25 with sodium hydroxide) and an extract of GFP prepared by the

above method (obtained using a Perkin Elmer LS50B Luminescence Spectrometer).

## Instrumentation

Fig. 2 shows a schematic diagram of the basic layout of the instrumentation developed for this work. The detector consists of a 100 µl, quartz glass, fluorescence flow cell (176.051-QS, Hellma Ltd., Southend-on-Sea, UK), through which the yeast cell culture or GFP extract could be circulated. An air cooled, argon ion laser (162LGL, LG Laser Graphics GmbH, Dieberg, Germany), provided a 488 nm excitation light source of 5 mW after filtering (TEM<sub>∞</sub> >95%, beam diameter 0.67 ± 0.05 mm), plane polarised perpendicular to the base of the unit (>100:1). A photodiode module housing a miniature photomultiplier tube (PMT), high voltage power supply and additional amplifier, (H5784, Hamamatsu Photonics Ltd., Enfield, UK), was used as

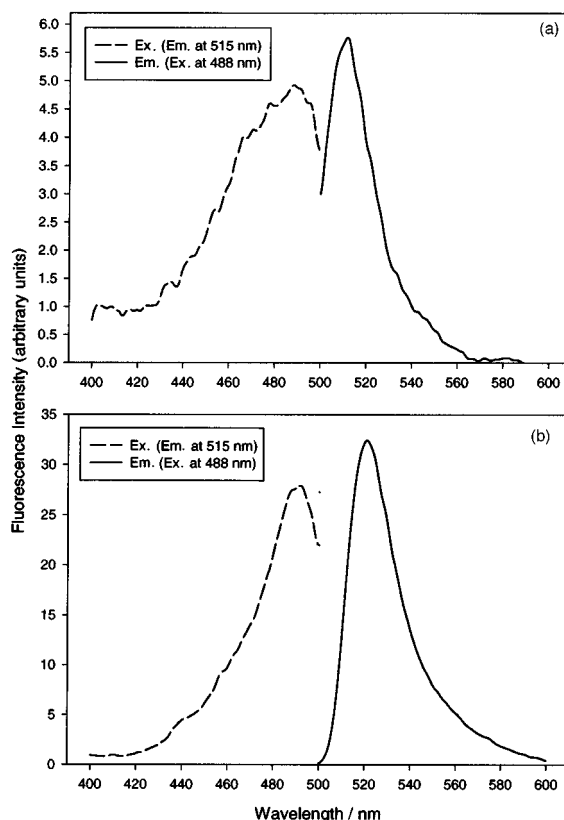


Fig. 1 Comparison of the fluorescence spectrum of (a) GFP in cell extract (b) fluorescein ( $1 \times 10^{-9}$  M in phosphate buffer). (---) Excitation spectrum (emission at 515 nm); (—) emission spectrum (excitation at 488 nm). Reproduced from reference 18 with permission from IOP Publishing Ltd.

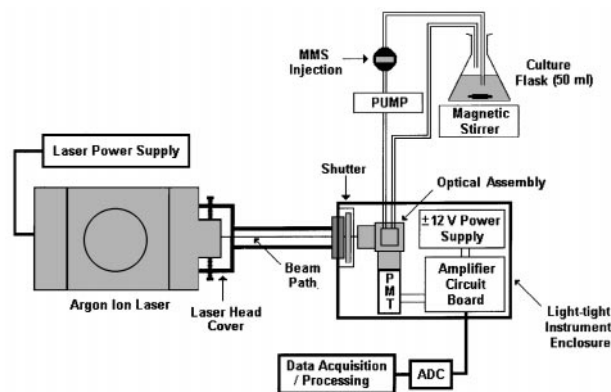


Fig. 2 Schematic diagram of the instrument constructed for fluorescence polarisation measurements.

the fluorescence detector. The flow cell, PMT and associated electronics were housed inside a light-tight box, with a shutter mechanism over the point of entry of the laser beam. The signals from the PMT were amplified and electronically smoothed by simple op-amp circuitry built in house. Data acquisition and manipulation was carried out in real time on a personal computer *via* a 12 bit analogue to digital converter and associated software (ADC100, Pico Technology Ltd., Cambridge, UK).

Fig. 3 shows a schematic diagram of the arrangements of optical components used. A 488 nm interference filter (3 nm half bandwidth), was used to filter the excitation light from the laser. The arrangement of optical filters perpendicular to the beam path was developed to allow the laser-induced GFP fluorescence at 518 nm to be measured, whilst rejecting the high intensity laser light (488 nm) scattered towards the detector by yeast cells suspended in the stream of liquid passing through the flow cell. The filters for fluorescence measurement, placed between the flow cell and PMT, consisted of a polaroid sheet (Grade HN38) laminated in rigid acrylic (thickness = 3.4 mm), in conjunction with a 515 nm interference filter (10 nm half bandwidth), and a 515 nm short wave cut-off filter (Orange Schott Glass OG515, thickness = 9 mm). 515 nm filters were the closest, readily commercially available filters to the GFP emission maximum.

The flow cell was housed in a black plastic mounting block, built in-house, into which two threaded optical mounting tubes, 19 mm in diameter, were inserted to hold the various filters in place. All filters and mounts were purchased from Comar Instruments, Cambridge, UK. The polaroid sheet and Schott glass were both cut down to 16 mm diameter disks from the supplied material using a diamond cutter, and secured firmly in the mounting tubes. All other filters were used without modification. A scale from 0 to 180° was marked on the outside of the mounting tube housing the the polaroid filter. This mounting tube was free to rotate over this range, corresponding to a transition from parallel to parallel alignment with respect to the excitation light source.

To check the alignment of the optics and the properties of the polaroid filter, the PMT and associated filter housing was moved round to lie directly in the path of the laser beam. The fluorescence filters were temporarily replaced with a 488 nm interference filter (10 nm half bandwidth) and a neutral density filter ( $OD_{488} = 3$ ) to step down the power of the beam. The PMT was temporarily replaced with a less sensitive silicon photodiode detector (SPD) with integral amplifier (IPL-10530DAL, RS Components Ltd.). The beam intensity ( $I_B$ ) was recorded, as a voltage from the SPD, for a range of polaroid filter orientations from 0 to 180° with respect to the excitation source. The results given in Fig. 4 show, as expected, the light source to be highly polarised with  $I_{B\perp}/|I_B|$  of <0.3%. The regression curve fitted to this data had a minimum point calculated to be  $90.1 \pm 1^\circ$ .

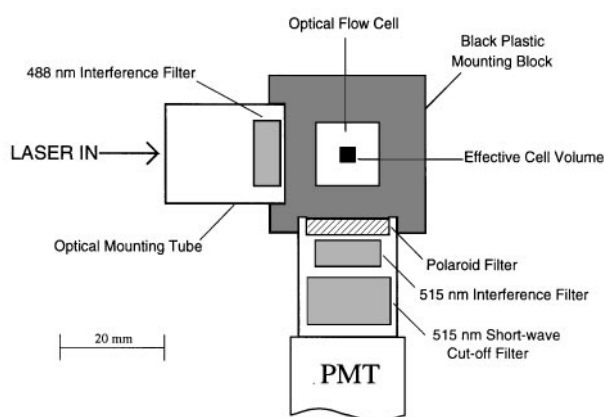


Fig. 3 Schematic diagram of the instrument optics, drawn to scale shown (distance from laser to head to centre of the optical cell = 195 mm).

## Results and discussion

### Fluorescence polarisation in fluorescein

A solution of fluorescein ( $1 \times 10^{-9}$  M in phosphate buffer) was pumped through the flow cell at a rate of  $1.5 \text{ ml min}^{-1}$ . The fluorescence intensity, measured as a voltage from the PMT and associated circuitry in mV, was recorded for a range of filter orientations from 0 to 180° from parallel, with respect to the excitation light. The results, given in Fig. 5, show that as expected, fluorescein shows relatively little fluorescence polarisation. The degree of fluorescence polarisation ( $P$ ) was calculated to be 0.037. A shallow regression curve was fitted to this data and the minimum point on the curve, *i.e.*, the point of maximum fluorescence polarisation, was calculated to be  $90.5 \pm 1^\circ$ . It is possible that physical factors such as the precise alignment of filters and optics contribute towards this small fluorescence polarisation measurement, although this is difficult to quantify in the present system and anyway should affect GFP measurement to the same extent.

### Fluorescence polarisation in GFP

A prepared extract of GFP (6 ml) was circulated through the flow cell again at a flow rate of  $1.5 \text{ ml min}^{-1}$ . As before the variation in fluorescence intensity with the angle of the polaroid emission filter was measured. The results, also given in Fig. 5, show that GFP in free solution exhibits a high degree of fluorescence polarisation with the fluorescence intensity measured perpendicular to the excitation light being approximately half that measured parallel to the excitation light.  $P$  was calculated to be 0.391. The regression curve fitted to this data

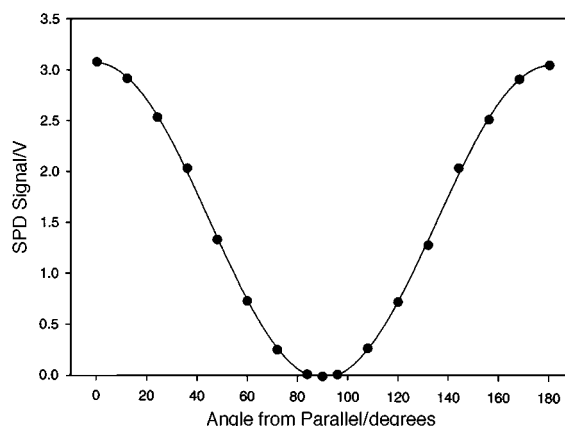


Fig. 4 Variation of measured excitation beam intensity with the polaroid filter angle.

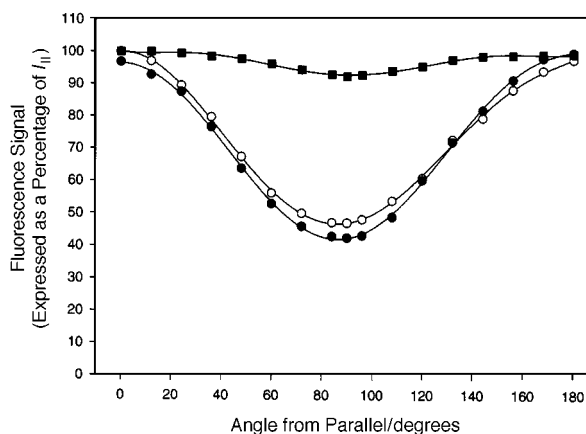


Fig. 5 Variation of measured fluorescence intensity with the polaroid filter angle for fluorescein (■), GFP in yeast cell extracts (●) and GFP in intact yeast cells (○).

had a minimum point at  $86.4 \pm 1^\circ$ . The deviation from  $90^\circ$  suggests that the excitation vector of the molecule may not be perfectly aligned with the fluorescence emission vector.

A culture of the GM cells (50 ml,  $OD_{488} = 2.50$ ), which had accumulated GFP after exposure to MMS for 24 h, was tested in the same way as the extract. The results, also given in Fig. 5, show that GFP within the cytosol of the intact yeast cells exhibits a degree of polarisation very close to that of the GFP extract.  $P$  was calculated to be 0.363. Ideally  $P$  at any given excitation wavelength should be a property of a pure species and should be invariant with respect to concentration. The slightly lower value of  $P$  obtained for GFP within the whole cell compared to the much purer extract may be due to small concentrations of other species within the cell exhibiting low fluorescence polarisation. The regression curve fitted to this data had a minimum point at  $86.3 \pm 1^\circ$ , supporting the conclusion drawn above. The magnitude of this deviation from  $90^\circ$  should also be a property of the pure species and independent of concentration. A replicate of this experiment on a second culture (50 ml,  $OD_{488} = 1.51$ ) gave a value of  $P = 0.360$  and a minimum fluorescence intensity at  $86.4 \pm 1^\circ$ .

### Effect of flow rate on fluorescence intensity and fluorescence polarisation

An investigation was carried out to establish if fluorescence intensity and polarisation measured in this system was affected by the flow rate of the test solution through the flow cell of the detector. An extract of GFP (6 ml) was circulated through the flow cell at a range of flow rates from 0.25 to 2.00  $\text{ml min}^{-1}$ . The fluorescence signal measured at either polaroid filter orientation was observed to decrease steadily with decreasing flow rate over the range studied, because of the effect of photobleaching caused by increased residence time of molecules in the laser beam as they passed through the cell. However, this effect is small, resulting in a reproducible drop in fluorescence intensity of only 5% on average when lowering the flow rate from 2.00 to 0.25  $\text{ml min}^{-1}$ . GFP (S65T derivative) is known to photobleach at a rate 5 times more slowly than fluorescein.<sup>7</sup> Overall fluorescence polarisation calculated from  $I_{\parallel}$  and  $I_{\perp}$  showed no significant change over the range of flow rates studied.

### Discrimination of GFP fluorescence from that of an interfering species

To test the application of fluorescence polarisation in discriminating the fluorescence signal due to GFP from that of an interfering species fluorescing at the same wavelength,  $I_{\parallel}$  and  $I_{\perp}$  were measured for solutions containing a constant but unknown concentration of extracted GFP with increasing concentrations of fluorescein. The flow rate used was 1.5  $\text{ml min}^{-1}$  as before. GFP extracts (6 ml) were spiked with fluorescein in the concentration range 0 to  $7 \times 10^{-10}$  M. The highest fluorescein concentration used produced 1.5 times the original signal for  $I_{\parallel}$  for the GFP extract alone, and 2.5 times the original signal for  $I_{\perp}$  for the GFP extract alone, representing a significant interfering signal.

The results given in Fig. 6 show that the fluorescence signal measured with both the parallel and perpendicular polaroid emission filter orientations increases linearly with increasing fluorescein concentration. The linear regression equations were as follows:

Parallel filter:  $I_{\parallel} = [19.00 \times (\text{fluorescein concentration}/10^{-10} \text{ M})] + 179.1$   
 Correlation coefficient = 0.9998

Perpendicular filter:  $I_{\perp} = [20.72 \times (\text{fluorescein concentration}/10^{-10} \text{ M})] + 84.6$   
 Correlation coefficient = 0.9993

The signal due to GFP was defined as being  $(I_{\parallel} - I_{\perp})$  at each of the 12 fluorescein concentrations tested. The measurements ranged from 93.4 to 95.9 mV, with an average of 95.1 mV, and a relative standard deviation of 0.79%. Hence the application of fluorescence polarisation successfully removed the interfering fluorescence signal from added fluorescein in the concentration range used.

By the use of polaroid filters and by taking a differential measurement,  $(I_{\parallel} - I_{\perp})$ , the measured signal for GFP is significantly lower than that which would be recorded without the filter, measuring fluorescence at all polarisations. However, overall signal to interference ratios are dramatically improved. For example, taking the parallel filter measurements alone; the signal from GFP alone was measured as 179 mV, and that of  $7 \times 10^{-10}$  M fluorescein was calculated to be 133 mV. This gives a signal to interference ratio of 1.35. Using the fluorescence polarisation measurements, however  $(I_{\parallel} - I_{\perp})$ ; the signal from GFP alone was calculated to be 93 mV, and that of  $7 \times 10^{-10}$  M fluorescein was calculated to be 1.4 mV. This gives a much improved signal to interference ratio of 66.

### Yeast auto-fluorescence

The precise origin of autofluorescence in yeast is currently unclear, and we have observed its intensity varies significantly between different strains. However, several likely sources can be postulated. These include reduced nicotinamide nucleotides, oxidised flavins, age related pigments and aromatic amino acids such as tryptophan. Many of these species are small in comparison to GFP, and we might speculate that they, like fluorescein, will show low fluorescence polarisation. The particular yeast strain we are now using (FF18984) has been selected for low autofluorescence, however preliminary studies within our laboratory of other strains with higher autofluorescence, have shown their fluorescence to be significantly unpolarised. The exact source and nature of autofluorescence and the extent of its polarisation in various yeast strains and culture media, and the applicability of this technique for GFP determinations, is currently under investigation, and will be the subject of a future paper.

### Conclusions

The fluorescence polarisation of yEGFP in cell extracts and within yeast cells has been measured, and is proposed as a strategy to discriminate between the fluorescence of GFP and overlapping fluorescent bands of other components of cells and

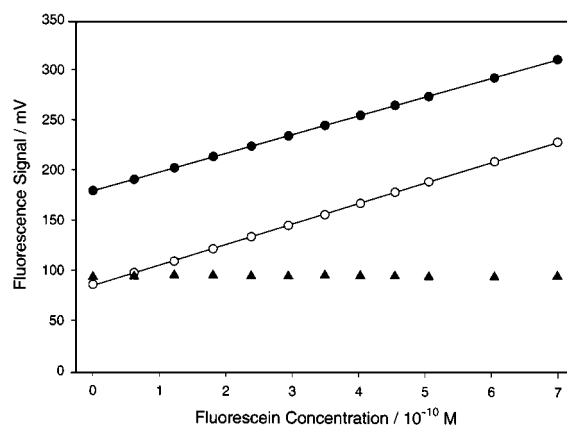


Fig. 6 Variation of measured fluorescence with increasing fluorescein concentration in a GFP extract, using the polaroid emission filter in a parallel (●) and perpendicular (○) orientation with respect to the excitation light source. The difference between these respective measurements ( $I_{\parallel} - I_{\perp}$ ), is taken as the signal for GFP (▲).

media, giving the possibility of improved limits of detection of GFP. The method should prove useful where a significant proportion of the background autofluorescence arises from relatively small molecules. In cases where the background autofluorescence arises from complex proteins and other particularly large molecules, or small molecules which are tightly bound to larger structures such that they have restricted rotation, fluorescence polarisation may well be less applicable.

It is likely that GFP fluorescence polarisation will be enhanced if the GFP marker is bound to another protein or organelle and hence even greater discrimination of GFP fluorescence should be achieved. This is because rotational movement of the GFP fluorophore will be further restricted. Park and Raines reported the use of S65T-GFP to probe protein-protein interactions by fluorescence polarisation, and propose GFP as a replacement for fluorescein as a fluorescent marker in such studies.<sup>17</sup> They measured changes in fluorescence polarisation as a GFP-tagged-S-peptide bound to S-protein fragments of ribonuclease, in order to estimate the dissociation constants of these species. An increase in measured fluorescence polarisation of approximately 4% between bound and unbound GFP was observed.

The main advantage of the exploitation of fluorescence polarisation to give greater wavelength discrimination for GFP, is that instrument requirements, or modifications, are relatively simple, in many cases just the insertion of appropriate polaroid filters in existing instrumentation. Alternative methods of discrimination, such as fluorescence lifetime analysis with gated detection, would require more complex and expensive instrumentation.

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