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Fluorescence polarization discriminates green fluorescent protein from interfering autofluorescence in a microplate assay for genotoxicity

Andrew W. Knight^{a,*}, Nicholas J. Goddard^a, Nicholas Billinton^b,
Paul A. Cahill^b, Richard M. Walmsley^b

^a*Department of Instrumentation and Analytical Science, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester, M60 1QD, UK*

^b*Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester, M60 1QD, UK*

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Abstract

An unconventional use for the polarization optics, associated with a variety of commercially available fluorescence microplate readers, is reported. This novel application has allowed the discrimination of green fluorescent protein (GFP) fluorescence in genetically modified yeast cells from interfering autofluorescent species. The method exploits the unusually high fluorescence anisotropy of GFP compared to smaller fluorophores and autofluorescent species. The principle was successfully applied to resolve the induced GFP signal from that of autofluorescent test compounds, in an assay for genotoxic species. The use of fluorescence polarization enabled both proflavin and methapyrilene to be identified as genotoxic agents in the yeast assay. This would not have been possible using conventional fluorescence alone since these compounds were found to be intensely autofluorescent at the same wavelength as GFP and thus effectively mask the GFP signal. © 2002 Published by Elsevier Science B.V.

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1. Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and its many colourful mutant derivatives, have in recent years been widely adopted as versatile

* Corresponding author.

markers, for reporting on gene expression, visualising physiological processes, monitoring subcellular protein localisation and distinguishing successful transfection [1,2]. GFP has been cloned and expressed in a diverse range of cells and organisms and demonstrates the advantages of high photo and chemical stability, low toxicity and the ability to spontaneously form a fluorophore in the absence of external co-factors. Once formed, the presence of GFP is noninvasively detected simply by fluorescence when illuminated with blue light. Hence, gene expression reported by the formation of GFP is readily quantified in assays performed in convenient microplate formats, using plate readers with fluorescence optics.

However, in the majority of biological applications, unless GFP is very highly expressed, the fluorescence signal will invariably be contaminated with endogenous autofluorescence arising from components of the cell or growth media, or in the case of a bioassay from a component of the sample or its matrix. This often severely limits the sensitivity of an assay, lowering signal to noise ratios and, in some cases, even obscuring the GFP signal altogether [3]. Whilst interfering autofluorescence has been a common problem in a diverse range of assays, there has invariably been only one main approach to solving it, namely the optimisation of optical filters for GFP fluorescence excitation and emission. However, since autofluorescence spectra are usually very broad, autofluorescence is often of a significant intensity at the same emission wavelength as GFP, making discrimination by simple modifications to filter sets relatively ineffective. Whilst several physical methods exist for discriminating GFP from endogenous autofluorescence [3], most cannot be readily adapted for use with commercial microplate readers, or are rather invasive.

This report describes a novel method for using commercially available fluorescence polarization optics to remove interfering autofluorescence from GFP measurements. Many plate readers are supplied with polarizing optics that are primarily intended for monitoring fluorescence anisotropy changes in protein binding studies. The authors recently devised a method for minimising the contribution of autofluorescence in GFP quantification in yeast, based on fluorescence polarization [4]. The method used a polarized laser source and a relatively large volume flow-through optical cell housed in instrumentation built in-house, for use in flow-injection assays. These general principles have now been adopted and proved to work in a microplate assay.

Discrimination between GFP and other autofluorescent species exploits the large fluorescence anisotropy of the molecule in free solution [5]. When illuminated with plane polarized light, a high proportion of the emitted fluorescence remains polarized with respect to the excitation light. This is because of the large size and relatively slow rotation of GFP in solution compared to the length of time it remains in the excited state [6]. The degree of fluorescence polarization (P), defined as $(I_{\text{para}} - I_{\text{perp}})/(I_{\text{para}} + I_{\text{perp}})$, where I_{para} is the intensity of fluorescence measured remaining polarized parallel and I_{perp} that measured polarized perpendicular to the plane of the excitation light, has been reported to be 0.398 for GFP. In contrast, fluorescein, which has a virtually identical emission spectrum to GFP, gave a P value of just 0.004 [4,7]. It was also shown in this work that the unknown species, which contributed towards cellular autofluorescence in various yeast strains, emitted light that was also significantly unpolarized.

The basis of the novel approach adopted in this paper is best described with reference to an experiment previously reported by the authors [4,7]. If increasing concentrations of a

small, highly fluorescent molecule, such as fluorescein, are added to a suspension of yeast cells expressing GFP, the overall fluorescence of the sample will increase, and using conventional optics, the fluorescence of fluorescein obscures that of GFP. Since the fluorescence spectra of fluorescein and the GFP mutant overlap, then conventional optical filtering will not adequately discriminate between them. Using fluorescence polarization optics, the I_{para} and I_{perp} measurements will also increase by approximately equal amounts as fluorescein is added, since the polarization ratio of fluorescein is very low. In addition, the apparent polarization ratio of the sample, $(I_{\text{para}} - I_{\text{perp}})/(I_{\text{para}} + I_{\text{perp}})$, arising from GFP in the yeast cells, will therefore fall. However, if the recorded analytical signal is simply taken as the difference between the two polarization measurements ($I_{\text{para}} - I_{\text{perp}}$), this is large for GFP, but small for natural autofluorescent species and virtually zero for simple fluorophore molecules such as fluorescein. Thus, as fluorescein is added to the yeast cell suspension expressing GFP, although the I_{para} and I_{perp} measurements increase, the difference between them, related to the concentration of GFP, remains constant, and the fluorescence determination of GFP is unaffected by the presence of fluorescein. This then is the basis for the analytical measurement described in this paper.

The authors are currently developing an assay for genotoxic (DNA damaging) chemicals. A brewer's yeast has been genetically modified to produce GFP in response to the up-regulation of DNA damage repair. The extent of this up-regulation is dependent upon the degree of damage sustained, and hence, the quantification of GFP by fluorescence produces an estimation of the presence, concentration or potency of a genotoxic species. The greater the DNA damage, the greater the repair activity, the more GFP is expressed and the brighter the cells fluoresce. During the validation of this assay, whilst steps were taken to minimise the background fluorescence arising from both the growth media and the microplate itself, some test samples and compounds were themselves found to be intensely autofluorescent. This autofluorescence completely masked the fluorescence from GFP when used at concentrations sufficient to test for the induction of DNA damage. Therefore, a method employing fluorescence polarization was devised in an attempt to resolve GFP fluorescence by removing the test sample autofluorescence, and hence allow the genotoxicity assay to be performed with inherently fluorescent compounds.

Alongside the genotoxicity, simultaneous measurement of the cell proliferation achieved during the course of the assay gives a quantitative indication of cytotoxicity, i.e. more general cellular toxicity. The cell density achieved is quantified by absorbance, measured through the microplate.

2. Materials and methods

2.1. The yeast

A DNA repair-competent strain of the yeast *Saccharomyces cerevisiae* was employed as the host strain for a reporter of DNA repair activity (the “test” strain). The reporter consisted of a fusion of the DNA damage-inducible promoter from an endogenous repair gene (*RAD54*), with a gene encoding a yeast-enhanced GFP (*yEGFP*). Details of this reporter plasmid have been previously reported elsewhere [8,9]. yEGFP differs from wild-

type GFP in two respects. The nucleotide sequence of the gene has been codon-optimised for yeast, and has undergone mutation in order to create the amino acid substitutions shown to improve the fluorophore efficiency, critically serine 65 to threonine [10]. yEGFP has an excitation maximum at 490 nm and an emission maximum at 517 nm. Expression of the *RAD54* gene from the upstream activating sequence used in this reporter is known to be up-regulated whenever the yeast DNA repair mechanisms are activated in response to DNA damage [8,9]. Similarly, expression of yEGFP from the reporter is up-regulated under DNA damaging conditions and this is quantified fluorimetrically. The assay detects genotoxins with a range of modes of action, such as oxidising, intercalating and alkylating agents.

In order to control for any effects of the plasmid on the cellular fluorescence and extent of proliferation, a control plasmid based on a disabled reporter plasmid was constructed. When transformed into *S. cerevisiae*, this control plasmid was unable to express yEGFP despite being identical to the reporter plasmid in every other way, thus forming the “control” strain.

To minimise cellular autofluorescence, a strain of cells, which had previously demonstrated low fluorescence, was chosen, designated FF18984 [4]. Similarly, a well-defined minimal growth media, F1, was employed which exhibited very low autofluorescence [11]. For the assay, the cells were grown to early stationary phase in F1 media and then diluted in fresh media to give several ml of a starting culture of approximately 0.2 OD₆₀₀ (OD₆₀₀ = optical density measured at 600 nm with a 1-cm path length). One OD₆₀₀ unit approximates to 10⁷ cells [12].

2.2. The assay format

The assay was carried out in sterile 96-well microplates with cylindrical, flat-bottomed wells. The plates (catalogue number 4929, Matrix Technologies, Wilmslow, Cheshire, UK) are constructed of black polystyrene with an optically clear base, such that they can be used for both fluorescence and absorbance measurements. In a study of 11 different microplates obtained from seven different manufacturers, these particular plates were selected on the basis of the lowest and most consistent well-to-well background fluorescence and absorbance (results not shown).

The assay format was as follows. The first column of wells was loaded with 150 µl of the test sample at twice the required highest concentration, in diluent solution. Nine other wells of each respective row were loaded with 75 µl of diluent alone. The diluent used was 2% v/v dimethylsulfoxide (DMSO) in water. DMSO enhances the solubility of test compounds and is thought to increase the permeability and thus susceptibility of the yeast cells, increasing the sensitivity of the assay. The test compound was serially diluted across the rows of wells by transferring 75 µl well-to-well, producing 50:50 dilutions, and a range of 10 test concentrations. A total of 75 µl of the genetically modified test or control yeast cell cultures (0.2 OD) was dispensed into each well containing the diluted sample. In addition to the samples, several genotoxic standards and blanks were carried out simultaneously on the same plate. The genotoxic standard was methyl methanesulfonate (MMS), a known genotoxic alkylating agent, and the blanks were diluent alone. All pipetting and dilutions were performed using a liquid handling robot (MicroLab S,

Hamilton GB, Carnforth, Lancashire, UK) run from software written in-house and housed in a fume-hood.

Each plate was subsequently covered with a breathable plastic membrane (“Breatheasy”, gas permeable sealing membrane, Diversified Biotech, Boston, MA, USA) to prevent evaporative loss, shaken for 30 s to thoroughly combine the yeast cell culture with the test sample or blank solution in each well, and then left without shaking in an incubator at 25 °C overnight. The following morning after approximately 16-h incubation, the plate was read for both absorption through the plate and fluorescence from the top of the plate. Two assessments were made for each compound by observing variations in absorbance and normalised fluorescence signals across the test compound dilution series. Firstly, cytotoxicity, the general poisoning of the cell, was characterised by a reduction in the final cell density achieved after incubation, compared with the blank. Secondly, genotoxicity was characterised by measuring the intensity of GFP fluorescence and comparing the result to constitutive levels. Since the cells achieve different final cell densities depending on cytotoxic effects, the induced GFP fluorescence signal is normalised by dividing by the cell density to produce a ‘fluorescence per cell’ or ‘brightness’ measurement.

2.3. Instrumentation and software

The fluorescence microplate reader used in this work was a BMG PolarStar (BMG LabTechnologies, Offenburg, Germany). The reader permits interchange of both excitation and emission filters housed in a filter wheel and was supplied with two separate fibre-optic heads: one for conventional fluorescence and absorbance measurements, and one for fluorescence polarization work. All fluorescence measurements in this work were made from the top of each well, although they can also be made through the base. For conventional absorbance measurements, a 620-nm interference excitation filter was used with no emission filter in place. For conventional fluorescence and fluorescence polarization measurements, a 485/12 nm excitation filter and 520/30 nm emission filter were used, in addition to a 495-nm Schott glass long-pass filter cut to size (15 mm diameter, 3 mm thickness, Comar Instruments, Cambridge UK) and mounted directly onto the emission filter to minimise any cross-talk interference. All filters except the Schott glass filters were supplied by BMG LabTechnologies. An advantage of this assay is that the detection of GFP can be made using common fluorescein filter sets that are routinely supplied with most plate readers and other laboratory fluorescence-based equipment.

The interference scattered light may have on the fluorescence polarization signals obtained has been considered. Using serial dilutions of fluorescent cells expressing GFP, the difference signal ($I_{\text{para}} - I_{\text{perp}}$) was seen to be linear with cell density up to 1.6 OD units, which is 0.2 OD units higher than any reading obtained in this study. Hence, the interference of scattering at high cell densities is not a significant problem in the working range.

The fluorescence polarization optics consisted of three optical fibres mounted in a “read head”. The first optical fibre carries the excitation light, with a polarizing filter in the end directly above the well being assessed. Two other identical optical fibres were mounted in the same head at 120° to each other, each with polarizing filters orientated perpendicular to each other. Hence, one optical fibre will transmit the fluorescence still

polarized parallel to the excitation source, whilst the other optical fibre, that polarized perpendicular to the excitation source. The other end of each emission optical fibre was mounted opposite separate emission filters, and photomultiplier tubes whose sensitivities could be independently corrected and balanced.

Absorbance, fluorescence, I_{para} and I_{perp} readings were read into the dedicated software supplied with the instrument, which was compatible with Microsoft Excel™. Since this was a novel use of the fluorescence polarization capabilities of this instrument, separate data handling and analysis macros were written in-house and incorporated into the commercial software.

2.4. Calculations

2.4.1. Cytotoxicity

Absorbance readings quoted are those directly obtained from the instrument corrected only for the small background absorbance of the microplate.

2.4.2. Genotoxicity

“Brightness”, i.e. normalised fluorescence per cell from the conventional method, using standard fluorescence optics, was equal to: Total fluorescence/Absorbance.

“Brightness” from the fluorescence polarization method described herein, using the fluorescence polarization optics, was equal to: $(I_{\text{para}} - I_{\text{perp}})/\text{Absorbance}$.

2.5. Chemicals

Methyl methanesulfonate (approximately 99%), proflavin hemisulfate (98%), fluorescein sodium salt (90%) and methapyrilene HCl (>99%) were obtained from Sigma-Aldrich, Poole, Dorset, UK. Dimethylsulfoxide (AnalaR Grade) was obtained from BDH Laboratory Supplies, Poole, Dorset, UK.

3. Results and discussion

3.1. Proof of principle

In order to characterise the fluorescence polarization method, fluorescein was used as a test compound since it is an intensely fluorescent, spectroscopic mimic of GFP. Fluorescence excitation and emission wavelengths of fluorescein and GFP are given in Table 1. Ten 50:50 serial dilutions of fluorescein from 185 ng ml^{-1} were tested with both the test and control strains. After preparation and incubation, absorbance of the plate wells was measured. Final cell densities of both the control and test strains, with any concentration of the test compound, are usually very similar since the starting cell densities were the same and the cells have only a very small genetic difference. Cell density results from both strains are shown in Fig. 1A. For fluorescein alone, no significant cytotoxicity trend is observed with increasing concentration up to 185 ng ml^{-1} . Using conventional fluorescence measurements to calculate “brightness per cell”, fluorescence of the fluorescein

Table 1

Fluorescence excitation and emission wavelengths of yEGFP, fluorescein, proflavin and methapyrilene in water, to the nearest 0.5 nm

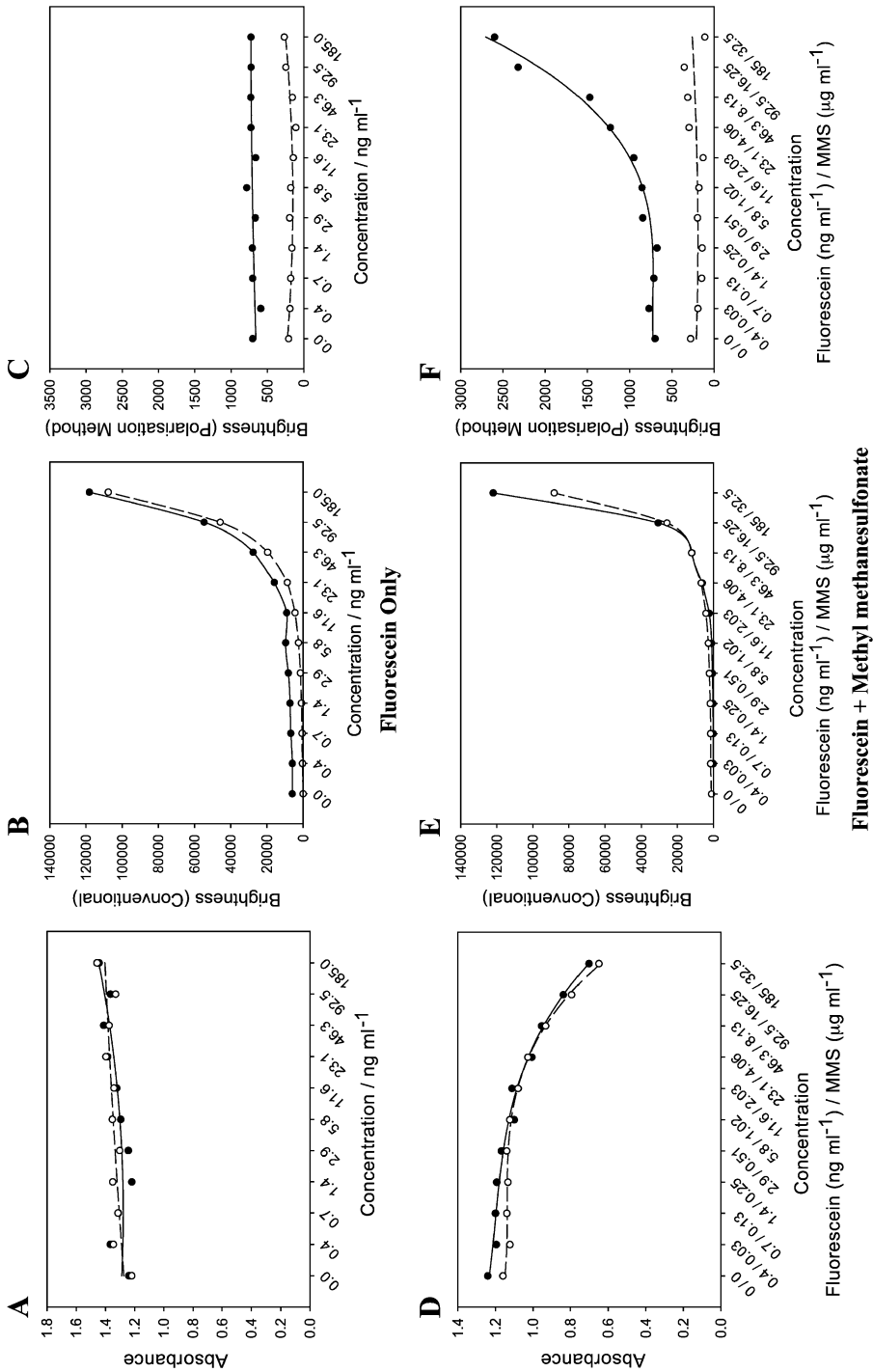
Compound	Excitation wavelength (nm)	Emission wavelength (nm)
yEGFP	488.0	517.0
Fluorescein	491.0	512.0
Proflavin	444.0	513.5
Methapyrilene	440.0	515.0

dominates the signal, completely obscuring the GFP signal as shown in Fig. 1B. Casual observation suggests that fluorescein provides a dose–response trend of a potent genotoxin. However, since apparent fluorescence induction is also seen in the control strain, the signal is principally ascribed to fluorescein and no genotoxicity assessment can be made.

Fluorescence polarization analysis was carried out as follows. Using the BMG software, the sensitivity setting (gain) of PMT 1 (measuring I_{para}) was adjusted to give 90% full-scale signal on the brightest well of the plate. Thus, the plate could be read with maximum sensitivity. The plate reader was then directed to a well containing 150 μl of the test compound alone, in this case, fluorescein at 370 ng ml^{-1} , in diluent. The gain sensitivity of PMT 2 (measuring I_{perp}) was adjusted such that both PMTs gave the same fluorescence intensity when reading this well, thus $I_{\text{para}} = I_{\text{perp}}$ and therefore $I_{\text{para}} - I_{\text{perp}} = 0$. Further fine balancing of sensitivity could be made subsequently in data processing software. I_{para} and I_{perp} were obtained for each well on the plate and the brightness value calculated $((I_{\text{para}} - I_{\text{perp}})/\text{Absorbance})$.

The brightness values based on the fluorescence polarization measurements are shown in Fig. 1C. The signals are almost two orders of magnitude smaller, but the difference between the test and control strains is clearly evident. The test yeast cells now show a consistent level of GFP, which is not induced with increasing fluorescein concentration, confirming that fluorescein has not acted as a genotoxin. This constant level of GFP results from the constitutive expression from the RAD54 promoter since a low level of natural DNA repair processes are on-going in the cell. The control cells show a low background level due to residual endogenous autofluorescence.

The experiment was repeated using ten 50:50 serial dilutions of fluorescein from 185 ng ml^{-1} spiked with MMS diluted from 32.5 $\mu\text{g ml}^{-1}$. In this instance, a clear dose–response is noted in the final cell density achieved, showing the cytotoxicity of the MMS (Fig. 1D). MMS suppresses the growth of cells, but since it is diluted, its effect is reduced and the final cell density recovers to become equal to the blank control. In this assay, clear dose dependency and a reduction in final cell density of >20% compared to that of the blank is required for a positive result. Using conventional fluorescence measurements, the signal is again dominated by the fluorescein, and both the test and control strains show high fluorescence related to the fluorescein concentration (Fig. 1E). Applying the fluorescence polarization method clearly separates the fluorescence of test and control cells. The test cells now show a clear induction of GFP above the constitutive level and exhibit a dose–response curve, indicating the genotoxicity of the sample and hence MMS. In this assay, clear dose dependency and an induction of brightness due to GFP expression of >30%



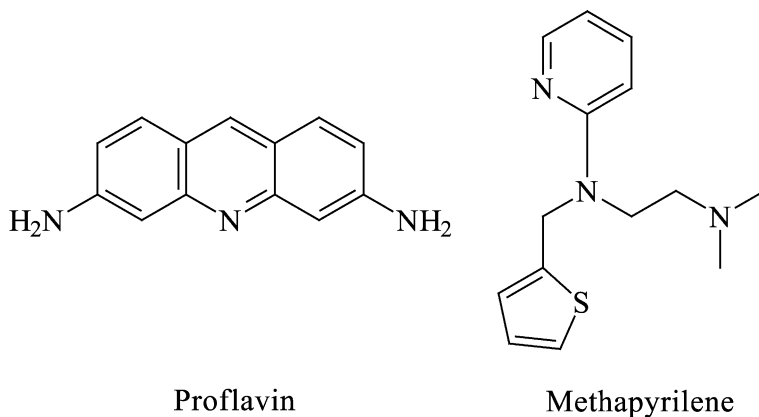


Fig. 2. Structures of the test compounds.

compared to that of the blank is required for a positive result. The control cells again show only a low background level of residual endogenous autofluorescence (see Fig. 1F).

Hence, the use of the differential fluorescence polarization method removed the autofluorescence of fluorescein from the recorded fluorescence signal, and allowed discrimination and quantification of the induction of GFP by MMS. Thus, the detection of the genotoxicity of an autofluorescent sample was achieved.

3.2. Testing with fluorescent genotoxins

During the course of a validation study, two test compounds, in particular, proved to be highly fluorescent at the wavelengths used for GFP measurement. These were proflavin (hemisulfate) and methapyrilene (hydrogen chloride). Proflavin is a synthetic acridine dye, which was used as a topical anaesthetic in World War II. It is a suspected hepatocarcinogen in rats and shows positive genotoxicity results in Ames and mouse lymphoma assays [13–15]. Methapyrilene is an antihistamine that was withdrawn from OTC medicines in the 1970s when toxicological studies suggested it was a rat hepatocarcinogen. This chemical also shows positive genotoxicity results in some Ames and mouse lymphoma assays [13,16–18]. The fluorescence excitation and emission wavelengths of these compounds in water were obtained on a conventional spectrofluorometer, and are shown in Table 1. The structures of these compounds are given in Fig. 2.

Fig. 1. Comparison of genotoxicity and cytotoxicity assay results for fluorescein alone and in combination with MMS, using conventional fluorescence, and fluorescence polarization measurements. Filled circles and solid line=test strain results. Empty circles and dashed line=control strain results. A: Final cell density profile (cytotoxic assessment) of fluorescein alone. B: Brightness induction profile (genotoxicity assessment) of fluorescein alone using conventional fluorescence measurements. C: Brightness induction profile of fluorescein alone using fluorescence polarization measurements. D: Final cell density profile of fluorescein spiked with MMS. E: Brightness induction profile of fluorescein spiked with MMS using conventional fluorescence measurements. F: Brightness induction profile of fluorescein spiked with MMS using fluorescence polarization measurements.

For proflavin, the cytotoxicity and genotoxicity activities were observed to occur in different concentration ranges, as shown in Fig. 3. Cytotoxicity is observed upon serially diluting from $127 \mu\text{g ml}^{-1}$ (see Fig. 3A). Serial dilution from $2.0 \mu\text{g ml}^{-1}$ produced a clear dose dependency in the brightness of both the control and test strains, due to the fluorescence of proflavin itself, which outshines the induced GFP (see Fig. 3B). Hence, in this assay, an assessment of genotoxicity using conventional fluorescence is impossible. Application of the fluorescence polarization method, however, results in marked reduction in the fluorescence of the control strain, and a GFP dose dependency in the test strain, as shown in Fig. 3C. This indicates that proflavin is a genotoxic agent according to our assay.

Methapyrilene is less fluorescent than proflavin, and both cytotoxicity and genotoxicity were observed in the same concentration range, serially diluting from $144 \mu\text{g ml}^{-1}$, as shown in Fig. 4. Although a clear dose dependency in brightness is noted using the conventional fluorescence method, which is more pronounced than in the control strain, the presence of the similar trend in the control strain indicated that some correction is

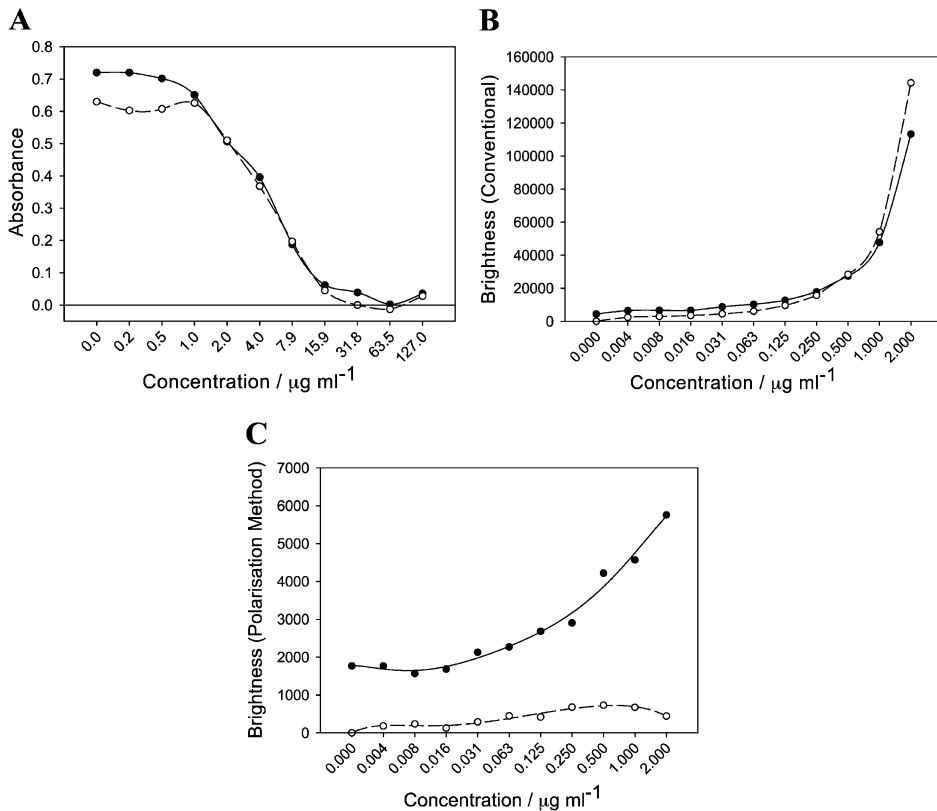


Fig. 3. Comparison of genotoxicity and cytotoxicity assay results for proflavin using conventional fluorescence, and fluorescence polarization, measurements. Filled circles and solid line = test strain results. Empty circles and dashed line = control strain results. A: Final cell density profile. B: Brightness induction profile using conventional fluorescence measurements. C: Brightness induction profile using fluorescence polarization measurements.

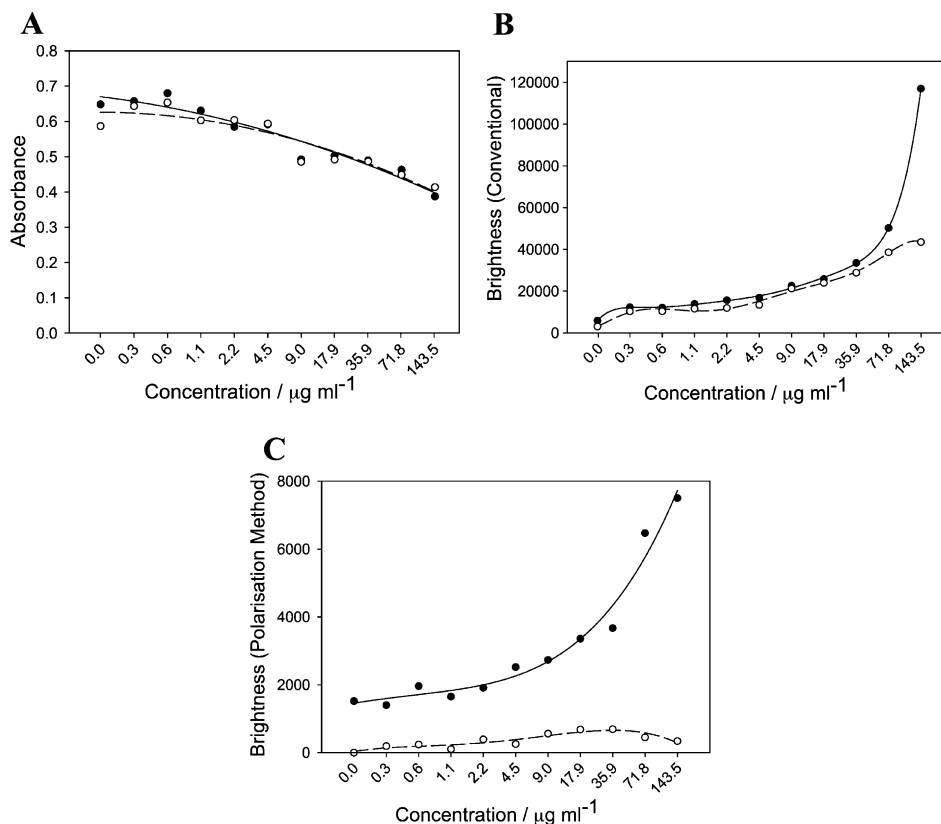


Fig. 4. Comparison of genotoxicity and cytotoxicity assay results for methapyrilene using conventional fluorescence, and fluorescence polarization, measurements. Filled circles and solid line = test strain results. Empty circles and dashed line = control strain results. A: Final cell density profile. B: Brightness induction profile using conventional fluorescence measurements. C: Brightness induction profile using fluorescence polarization measurements.

necessary for the results to be credible (see Fig. 4B). Applying the fluorescence polarization technique, in this instance, again markedly reduced the fluorescence measured in the control strain and revealed the true dose dependency in induced GFP, as shown in Fig. 4C, indicating that methapyrilene is also a genotoxic agent according to our assay.

Both these test compounds gave results in broadly similar concentration ranges to alternative tests, proflavin tested at $2 \mu\text{g ml}^{-1}$ [15] and methapyrilene tested in the 100's of $\mu\text{g ml}^{-1}$ range [16,18].

3.3. Use of on-plate standards

In the assay protocol, one microplate well contains only the test compound at the highest test concentration in diluent. This serves two purposes. For conventional fluorescence reading, it gives an automatic indication, when compared to a well containing diluent only,

of whether the compound has significant autofluorescence. Secondly, if the compound is found to be significantly fluorescent, it serves as a standard with which to balance the sensitivity of the two light detectors (PMTs), such that $I_{\text{para}} = I_{\text{perp}}$, in order to apply the fluorescence polarization method. However, in some cases where the test compound is only slightly autofluorescent, the signal from this well is inadequate to carry out the balancing operation with sufficient accuracy. In this case, the use of a second standard is required on the plate, such as fluorescein or 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTSA), which fluoresce at the same wavelengths as GFP but show very low fluorescence anisotropy [7]. If the method is adapted to be used in higher throughput screening assays, the routine use of an on-plate standard of HPTSA would be recommended.

4. Conclusions

Exploiting the inherent fluorescence anisotropy of GFP, a novel method for the discrimination of GFP from interfering autofluorescent species in a microplate format has been developed. The technique can be performed using fluorescence polarization optics commercially available with many microplate readers, in this example, using a BMG PolarStar.

The method has been applied to a microplate genotoxicity assay based on genetically modified yeast cells expressing GFP. The use of fluorescence polarization enabled both proflavin and methapyrilene to be identified as genotoxic species. This would not have been possible using conventional fluorescence alone since these compounds were found to be intensely autofluorescent at the same wavelength as GFP and thus mask the GFP induction signal.

Acknowledgements

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