

Development of a flow-through detector for monitoring genotoxic compounds by quantifying the expression of green fluorescent protein in genetically modified yeast cells

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Abstract. A flow-through detector has been developed for quantifying green fluorescent protein (GFP) expressed in genetically modified yeast cells upon exposure to a genotoxic compound. The detector forms the basis of a prototype semi-automated bioassay for genotoxic compounds, by biological fluorescence induction and detection. The selection and optimization of the various optical components of the system are discussed. The complete system was successfully tested with a known DNA damaging agent, and was able to monitor the expression of GFP on-line, *in situ* and in real time.

Keywords: green fluorescent protein, yeast, genotoxicity, flow-through, instrumentation

1. Introduction

The brewers' yeast *Saccharomyces cerevisiae* has been genetically modified, by fusing the promoter of the gene responsible for the induction of a native protein, Rad54, with a modified green fluorescent protein (GFP) gene originally from the jellyfish *Aequoria victoria* [1,2]. In the wild, GFP is made by this particular jellyfish, found in the North West Pacific Ocean. When disturbed, or in response to an attack, a calcium ion activated protein, aequorin, forms an excited state that emits blue light in a bioluminescence reaction. This blue light in turn excites GFP which emits a bright green flash, presumably to ward off an attacker [3,4]. This same green fluorescence can be readily induced by illumination of the GFP molecule with blue light, without the need for any additional co-factors and whilst within the cell matrix. Hence GFP has found widespread use as a reporter for the expression of many genes, and as a marker to visualize the movement or localization of proteins within cells, or the dynamics of subcellular components to which these proteins are targeted [5–7].

The Rad54 protein is known to be up-regulated whenever the yeast cell's DNA repair mechanisms are activated, for example, after DNA damage by a genotoxic chemical such as an alkylating agent, by UV irradiation, or by ionizing radiation. Hence this system was proposed as the basis for the development of a genotoxicity biosensor, whereby upon exposure to a DNA damaging agent, the yeast cells should produce and accumulate GFP causing a detectable increase in their fluorescence.

The development of a GFP reporter for the DNA damage induced gene *RAD54* in *Saccharomyces cerevisiae* has been reported by Walmsley *et al* [1,2]. In this previous work, after challenging the yeast with a genotoxic compound and incubation for several hours, GFP expression was quantified by first washing the cells, by a procedure which included centrifugation steps, to remove all traces of the culture media. The cells were then either re-suspended for whole cell measurement, or subjected to a lengthy series of extraction steps to remove GFP from the cell matrix, in both cases for a single measurement in a cuvette using a standard

fluorimeter. These steps did not, however, readily lend themselves to automation—the aim of the current work.

The system described here uses a high-power laser light source, associated optics and a flow cell to allow GFP to be determined *in situ* in whole cells, non-invasively and in real time whilst the cell culture is developing.

One consequence of the alteration and disruption of DNA is the formation of mammalian cancers, and hence much effort has been invested in identifying chemicals and environmental factors that can cause DNA damage, and thus induce carcinogenesis. The Ames Test [8] is the most commonly employed method for assessing the mutagenic potential of chemicals. In this test, strains of the bacterium *Salmonella typhimurium* are mixed with the test agent and plated out onto selected growth media. After incubation, colony counts of the different strains indicate genetic alteration. It is a labour intensive batch method requiring a competent microbiologist. Nevertheless, its reliability and simplicity has led to its almost universal adoption.

Much DNA damage is efficiently repaired by the cells, and thus the Ames Test only detects incorrectly or unrepaired damage. The system reported here focuses on the assessment of repair activity, before a significant genetic endpoint is reached. In this way we would seek to identify genotoxic agents below the threshold for detectable damage.

In addition, the metabolism of prokaryotic bacterial cells, used in the Ames Test, is significantly different to eukaryotic human cells to give false results (either positives or negatives) with the Ames Test. Yeast, also a eukaryote, should provide a better model for extrapolation to humans. Yeasts are also much hardier organisms and less susceptible to adverse environmental conditions, making them easier to culture and handle within a flow-through instrument.

A genotoxicity test based on yeast has been previously reported for assessing gene conversion [9, 10]. This rarely used procedure is similar in practice to the Ames Test. It requires plating and, again, detects a genetic endpoint rather than repair activity. As such it has many of the same disadvantages of the Ames Test discussed above.

Other toxicity sensors have been developed based on yeast cells, but the majority only monitor cell viability, and hence general cytotoxicity. This is commonly done by monitoring respiratory action, either oxygen uptake or carbon dioxide evolution, or pH changes, and mainly by electrochemical means [11–14]. Similarly, cytotoxicity sensors have been developed incorporating bacterial cells. Most are based on the inhibition of naturally photoluminescent bacteria upon exposure to the toxic agent [15–17], although respiratory measurements have also been used [18]. Response times for these sensors are generally very fast, of the order of minutes. In the instrument reported here, however, by simultaneously measuring the growth rate of yeast cells as well as GFP development, a distinction between general cytotoxicity and specific genotoxicity can be assessed.

In this work we report on the development of a flow-through detector and its optimization for GFP quantification in whole yeast cells. In preliminary tests the response of these

genetically modified organisms upon exposure to a genotoxic compound has been successfully monitored in real time by the detector, providing a measure of genotoxicity.

2. Nature and fluorescence properties of GFP

Wild-type GFP consists of 238 amino acids. It has a cylindrical structure with the fluorophore element encapsulated and protected in the centre. Autocatalytic oxidation and cyclization of the GFP amino acids at positions 65 to 67 leads to the formation of the fluorophore, however the fluorescence also requires further interactions of the fluorophore with other parts of the protein [3, 4]. This oxidation reaction only requires the presence of molecular oxygen, and proceeds with a time constant of between 2 and 4 h. On account of its structure, once formed, GFP is a very stable and resilient fluorophore, its fluorescence persisting after treatment with formaldehyde for example, and in extremes of temperature and pH. It also resists most proteases for many hours. 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 approximately 518 nm [19]. In yEGFP two types of modification were carried out: codon optimization for yeasts, and amino acid substitutions shown to increase the fluorophore efficiency (critically, serine 65 to threonine).

During the development of the instrument, fluorescein (sodium salt) was used as a mimic of GFP, as it very closely matched the fluorescence properties of GFP, and was inexpensive and readily available (Aldrich Chemical Company, Poole, UK). Figure 1 shows the fluorescence excitation and emission spectra of fluorescein, and an extract of GFP prepared by the method described by Walmsley and co-workers [2] (obtained using a Perkin Elmer LS50B Luminescence Spectrometer).

3. Description of the instrument

3.1. Overview

Figure 2 shows a schematic diagram of the basic layout of the detector developed in this work. The instrument consists of a 100 μ l, three-window, quartz glass, fluorescence flow cell (176.051-QS, Hellma Ltd, Westcliffe-on-Sea, UK), through which a yeast cell culture could be continuously sampled, or which could readily become a component of a more conventional flow-injection analysis manifold. In the first instance 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. The optics are arranged such that the instrument acts as a dedicated sensitive fluorimeter with a photomultiplier tube (PMT) detector, for measuring GFP fluorescence, and simultaneously as an absorption spectrometer with a silicon photodiode (SPD) detector, for estimating yeast cell concentration. The optical components are housed inside a light-tight box with appropriate safety interlocks to restrict access to the laser beam.

The signals from these two detectors were amplified and electronically smoothed by simple op-amp circuitry built in-house. Data acquisition and manipulation was carried out in

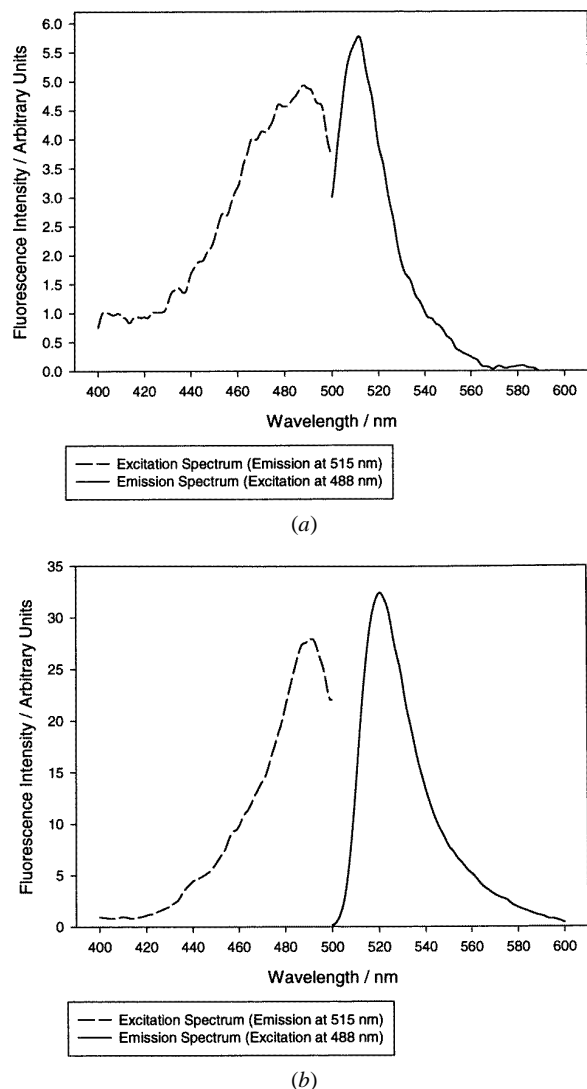


Figure 1. Comparison of the fluorescence spectrum of (a) GFP and (b) fluorescein (1×10^{-9} M in phosphate buffer).

real time on a personal computer *via* a 12-bit, two-channel, analogue to digital converter (ADC) and associated software (ADC100, Pico Technology Ltd, Cambridge, UK).

3.2. Optical components

Figure 3 shows a schematic diagram of the arrangement of optical components of the instrument. The arrangement of optical filters perpendicular to the laser beam path was developed to allow the laser induced GFP fluorescence at 515 nm to be discriminated from the high-intensity laser light (488 nm) scattered towards the fluorescence detector by yeast cells suspended in the stream of liquid passing through the flow cell. This filtering was made more difficult by the proximity of the optimum excitation and emission wavelengths, and by the wide viewing angle of the light emitted from the large flow cell window towards the PMT detector.

A 488 nm interference filter (3 nm half bandwidth) was used to filter the excitation light from the laser. For fluorescence measurements, a 515 nm interference filter (10 nm half bandwidth), in conjunction with a 515 nm short

wave cut-off filter (Orange Schott Glass OG515, thickness 9 mm) was used between the flow cell and the PMT detector. 515 nm filters were the closest, readily commercially available filters to the GFP emission maximum.

Various small photodetectors were tested for their sensitivity in measuring GFP fluorescence with this filter arrangement. These included SPDs with a variety of architectures. The detector chosen, which gave the greatest sensitivity in a small package at reasonable cost, was the miniature photomultiplier recently developed by Hamamatsu Photonics Ltd, Enfield, UK, which is housed in a TO8 metal can package. A module (H5784) was used which encapsulated the miniature PMT, high-voltage (HV) power supply and additional amplifier, and was powered from a standard ± 12 V supply. An additional electronic circuit was required to externally control the sensitivity of the amplifier built into the PMT module.

For optical density (OD) measurements, a second 488 nm interference filter (10 nm half bandwidth) was used in conjunction with a neutral density filter ($OD = 3$ at 488 nm) and placed in front of the SPD detector (IPL10530DAL, RS Components Ltd). The first filter removed any fluorescence emission, whilst the second filter cut down the power of the beam to a suitable level for the SPD detector.

The flow cell was housed in a black plastic mounting block, built in-house, into which three threaded optical mounting tubes, 25 mm in diameter, were inserted to hold the various filters in place. All filters and mounts were purchased from Comar Instruments, Cambridge, UK.

The use of an argon ion laser is not ideal in terms of developing a small portable instrument, as such lasers are both bulky and fragile. A commercially available solid state laser with an emission wavelength of 473 nm, in a much smaller, robust package, was tested for GFP fluorescence measurements (LCS-DTL-262, Laser 2000 (UK) Ltd, Ringstead, UK). In contrast to the sensitivity predicted from the fluorescence spectral measurements shown in figure 1, the 473 nm laser source was found to be only 15% as efficient as the argon ion laser at promoting GFP fluorescence. The reason for this low value is unclear at this stage and is under investigation. A solid state laser at 490 nm is now commercially available; however it is prohibitively expensive at this time (BP-490-P10, Laser 2000 (UK) Ltd).

4. Experimental procedure

4.1. Fluorescence calibration

The instrument was optimized using fluorescein, a close spectroscopic mimic of GFP. A series of experiments determined the optimum solution conditions for maximum fluorescein fluorescence to be 0.02 M sodium phosphate buffer adjusted to pH 8.25 with sodium hydroxide. With the optical set-up as described, calibrations were performed by pumping freshly prepared standard solutions of fluorescein in buffer through the flow cell, at 1.75 ml min^{-1} , and recording the average signal obtained over 1 min. The level of electronic amplification from the PMT module was selected to give an appropriate signal range for the range of concentrations examined, and in each case excellent linearity was observed.

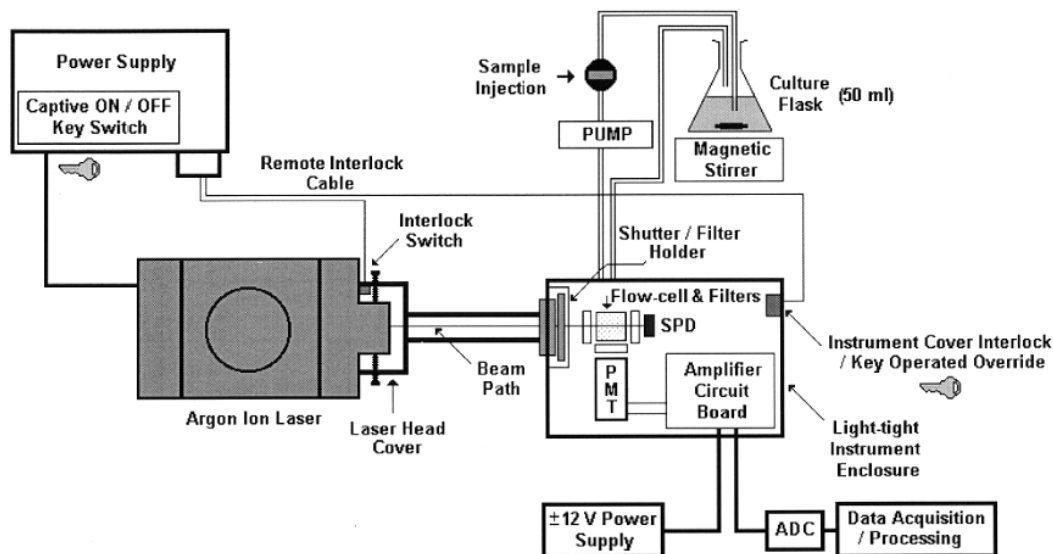


Figure 2. Schematic diagram of the instrument.

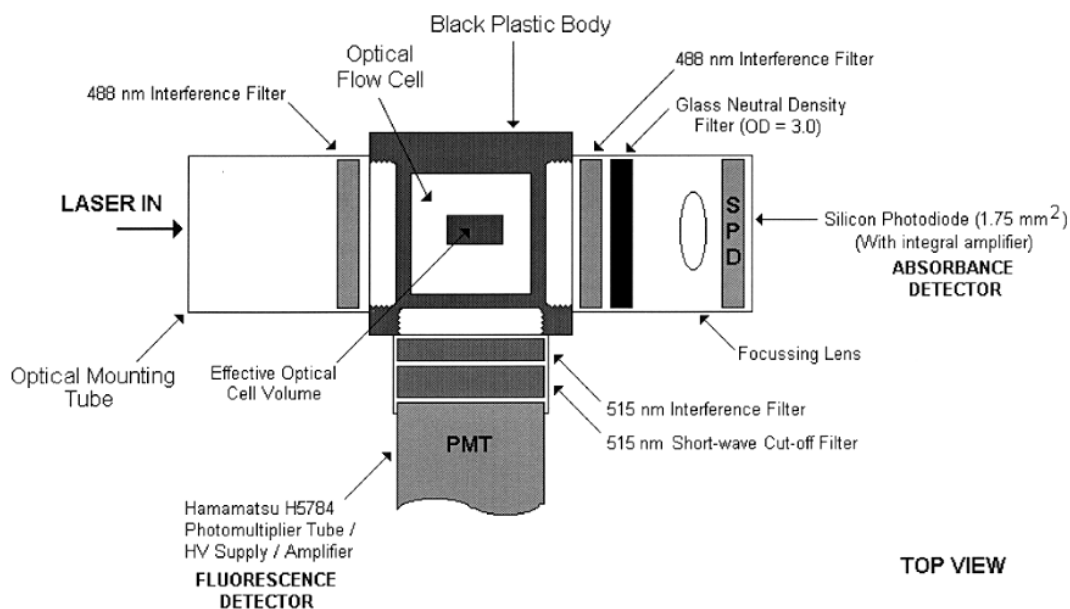


Figure 3. Schematic diagram of the instrument optics.

On the most sensitive setting the lowest level calibration was performed over the range 0 to 1×10^{-10} M. The regression equation obtained was as follows, with a correlation coefficient of 0.99996 (number of data points = 12).

$$\text{PMT signal [mV]} = (17.28 \pm 0.11) \times \text{fluorescein conc. } [\times 10^{-11} \text{ M}] + 0.72 \pm 0.64. \quad (1)$$

The use of a 495 nm short wave cut-off filter (Yellow Schott Glass GG495, thickness 9 mm, Comar Instruments) in place of the 515 nm version yielded even greater sensitivity, although with slightly higher noise from the scattered excitation light. Calibrations for fluorescein could then be performed in the picomolar range, with an estimated limit of detection of 5×10^{-13} M. Hence, the instrument's performance compares very favourably with many commercial fluorimeters. In previously reported work the whole cell estimation of GFP flu-

orescence was carried out using a cell concentration having an optical density of approximately 0.2 (600 nm, 1 cm path length) [2]. Using this alternative optical set-up, the addition of this concentration of wild-type yeast cells to the solution did not significantly affect the sensitivity of the determination of picomolar amounts of fluorescein, as shown in figure 4.

4.2. Selection of growth media

In the previously reported work [1, 2] many washing steps were required to remove all traces of the growth media from the cells prior to measurement, as the media itself was fluorescent and hence could interfere with the measurements. For continuous on-line measurement, the use of media with low fluorescent properties at the wavelengths concerned

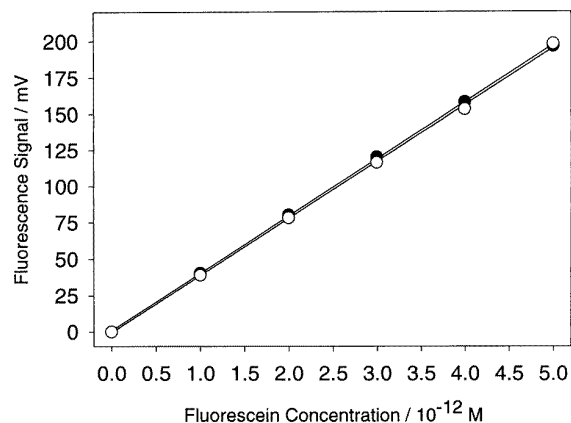


Figure 4. Low-level calibration for fluorescein with (○) and without (●) the presence of 0.2 OD₄₈₈ concentration of wild-type yeast cells.

would allow the fluorescence of the cells to be estimated *in situ*, without separation from the media.

Surveying a range of media revealed that, in general, media containing 'yeast nitrogen base' were approximately an order of magnitude less fluorescent than media containing basic 'yeast extract'. (Both media components were supplied by Difco Laboratories, Detroit, USA.) However, both these media exhibited fluorescence up to three orders of magnitude greater than that of the GFP to be measured. The medium finally chosen for these studies was a well defined minimal medium (F1) described by Walmsley *et al* [20]. The medium, consisting of only trace elements, vitamins, salts, selected amino acids and glucose, was a further two orders of magnitude less fluorescent than media containing 'yeast nitrogen base'. A small residual background fluorescence was found to be due mainly to added tryptophan. Since this is a non-essential component for the growth of the particular strain of yeast cells used, it was removed from the medium recipe. Any residual background signal could then be readily offset by the instrument.

4.3. Estimation of yeast cell concentration

The concentration of yeast cells was monitored on-line by measuring the OD of the suspension by a turbidimetric method. The SPD recorded a drop in the intensity of the laser beam as it passed through the optical cell, due to the scattering and absorption of light by the yeast cells. The signal from the SPD was correlated to the OD of the cell culture (OD₄₈₈) measured on a conventional absorption spectrometer (Lambda 5, Perkin Elmer Corporation) in a 1 cm acrylic cuvette at 488 nm. The relationship was linear up to an OD₄₈₈ of 3, given by equation (2) as follows, with a correlation coefficient of 0.9996 (number of data points = 9):

$$\text{OD}_{488} = \text{SPD signal [mV]} \times 0.0152 \pm 0.0003. \quad (2)$$

ODs measured at 488 nm were found to be just as reliable for estimating yeast cell concentrations at the more commonly used 600 nm.

The measured OD relates linearly to the actual yeast cell concentration up to an OD of about 0.6, and is a

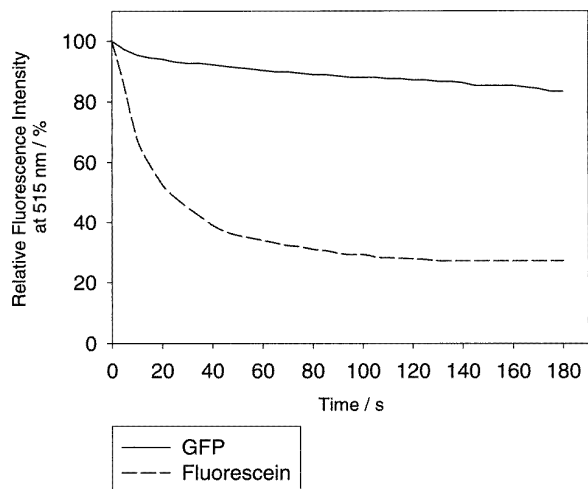


Figure 5. Comparison of the relative fluorescence decay profiles for GFP and fluorescein.

generally 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 linear. In these cases, to obtain an estimation of OD, a concentrated culture is usually diluted back into the linear range, its OD measured, and its original OD obtained by multiplying by the dilution factor. In order to perform this correction on-line and more realistically quantify OD values above 1.0, a calibration was performed. A suspension of wild-type yeast cells, washed clear of their growth media with distilled water, was diluted to a range of relative concentrations (OD 0 to 2), and the apparent OD measured. The values obtained were correlated against those predicted from the linear section of a calibration graph of relative concentration versus OD in the range 0 to 0.6. The relationship between the apparent and calculated OD was fitted to third-order regression such that (correlation coefficient = 0.9996, number of data points = 16):

$$\text{OD}_{\text{calculated}} = 0.327 (\text{OD}_{\text{measured}})^3 - 0.378 (\text{OD}_{\text{measured}})^2 + 1.114 \text{OD}_{\text{measured}}. \quad (3)$$

Equations (2) and (3) were used together by the software to convert the SPD signal into the OD₄₈₈ of the yeast cell culture passing through the optical flow cell in real time.

An estimation of the concentration of yeast cells is required since cell cultures are growing throughout most measurements. To account for variations in cell numbers between measurements and experiments, a relative 'brightness' value, per cell, was used to quantify the GFP expression. Brightness is calculated by dividing the fluorescence signal (in mV) from the PMT by the optical density (OD₄₈₈) calculated from the SPD signal.

A small amount of the intense scattered, laser light is seen by the sensitive fluorescence detector despite the use of cut-off filters, and this produces a small interference signal. The interference typically equates to just a few per cent of the overall fluorescence signal for GFP and is linearly related to cell concentration across a wide range of OD from 0 to 3. Hence, in calculating a 'brightness' value, per cell,

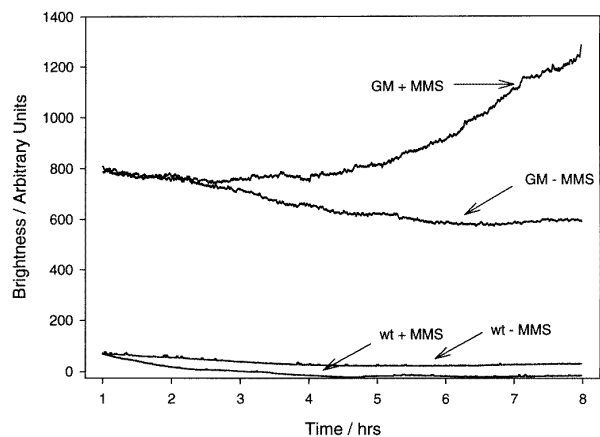


Figure 6. Comparison of the variation in 'brightness' of stationary phase genetically modified (GM) and wild-type (wt) yeast cells over 8 h incubation, with and without 0.01% methyl methane sulphonate (MMS).

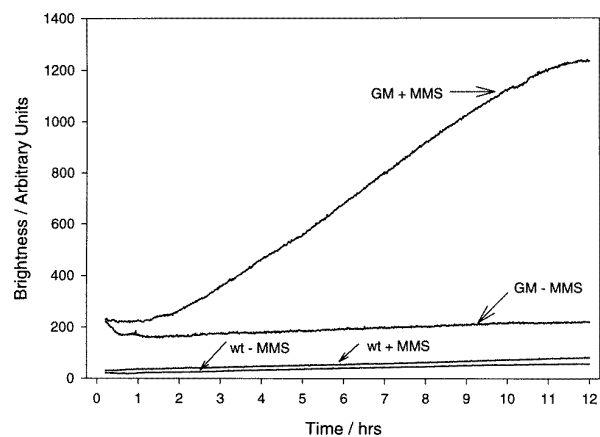


Figure 7. Comparison of the variation in 'brightness' of exponential phase genetically modified (GM) and wild-type (wt) yeast cells over 12 h incubation, with and without 0.01% methyl methane sulphonate (MMS).

this interference is accounted for and becomes a constant background value.

4.4. Photostability

Photo-bleaching sets the ultimate limit on the magnitude of the fluorescence signal obtainable with GFP as a reporter. Using static solutions of fluorescein (1×10^{-10} M in buffer) and of extracts containing GFP, the rate of photo-bleaching in our system was examined under continuous intense illumination by the argon ion laser, in order to compare the photostability of GFP and fluorescein. The flow cell held a volume of $100 \mu\text{l}$, with an optical path length of 3 mm. The laser beam (0.63 ± 0.05 mm in diameter) passed through the centre of the cell. The results shown in figure 5 indicate that the rate of photo-bleaching of GFP is less than half that of fluorescein, and hence GFP should prove to be a very photostable fluorophore for analytical measurements. The shape of the decay curves is explained by the fact that the products of the photo-bleaching reactions and fresh fluorophore are free to diffuse in and out of the beam from

other parts of the optical cell, and for a time a near equilibrium is reached. Since during measurements the samples pass through the flow cell at approximately 1.5 ml min^{-1} the residence time in the laser beam is small and the amount of photo-bleaching of GFP is insignificant, even when a culture of typically 50 ml is continuously circulated through the flow cell for several hours.

5. Preliminary results

5.1. Stationary phase cells

As a proof of principle, a 1 ml aliquot of a stationary phase culture of the genetically modified (GM) cells, previously stored at 4°C , was added to a sterile flask containing 50 ml of the F1 media at room temperature, to produce an initial cell concentration between 0.15 and 0.25 OD_{488} . The culture was vigorously stirred using a magnetic stirrer, whilst a fraction of the culture was continuously drawn off, passed through the detector and returned to the flask. The growth in cell concentration and the observed 'brightness' was continuously recorded over an 8 h period. Methyl methane sulphonate (MMS) was the known genotoxin chosen for the study. The results from cultures of GM cells grown under these conditions with and without the presence of 0.01% MMS were compared. For comparison, control cultures of wild-type unmodified yeast cells were also tested, again both with and without the presence of MMS.

Figure 6 shows a typical result for the variation of brightness over the 8 h period. In each case the cell concentration during the experiment increased to an OD of approximately 0.6. The stationary phase yeast cells exhibited a marked auto-fluorescence which rapidly decayed to approximately half the initial value on introduction to fresh media, possibly due to the metabolism of an unknown accumulated species, although this is currently under investigation. Hence, the results shown here were taken 1 h after inoculation after this residual fluorescence had decayed. The results show a clear induction of GFP after 3 h compared with the unexposed GM cells, leading to twice the fluorescence signal of the control after 8 h.

The stationary phase yeast cells showed a relatively slow response to MMS, partly because there is a significant time lag of up to 2 h between inoculation into fresh media and the commencement of reproduction.

5.2. Exponential phase cells

Since it is desirable to produce a positive result for a genotoxic compound in the shortest possible time, the experiments described above were repeated using cells in their exponential growth phase. An aliquot of a freshly prepared developing yeast cell culture was used as the inoculant, otherwise all conditions were the same as for the stationary phase cells. Figure 7 shows a typical result for the variation of brightness over a 12 h period. In each case the cell concentration during the experiment increased from the point of inoculation without the time lag previously observed, and consequently a higher OD (between 1.5 and 2.5) was reached after 12 h. The marked initial auto-fluorescence of the stationary phase cells was not observed in the exponential phase cells.

After exposure to MMS a discernible signal due to GFP was observed after 1.5 to 2 h, which increased to twice the original brightness in about 4 h. However, 1.5 or even 1.3 times the brightness signal of a control culture could be taken as a positive result, shortening the analysis time to nearer 2.5 h. In this particular case an induction of 5.5 times the original brightness was observed after 12 h.

In each case, with stationary or exponential cells, the initial brightness of the GM cells is approximately ten times that of wild-type cells. This initial brightness in GM cells is due to a constant background concentration of GFP. A small amount of DNA damage and associated repair is occurring all the time in all normal healthy cells, and hence in GM cells GFP is constantly being produced at a low level. The rate of degradation of GFP in these cells appears to be such that GFP does not significantly accumulate in the cells under normal conditions, and hence the cells have a reproducible background level of GFP.

The delay of 1.5 to 2 h before an increase in brightness of the GM yeast cells exposed to MMS can be discerned compared to a control culture is thought to be primarily due to the time taken for the expression and correct folding of the protein, followed by auto-oxidation to form the final fluorophore. Of these steps, it is probable that the oxidation of tyrosine is the rate limiting step in the development of the fluorophore. If this is the case it may be possible to speed up the formation of the fluorophore by the introduction of a chemical oxidizing agent, especially since the viability of the cells is not important once GFP has been expressed. This will hopefully reduce the time taken to achieve a positive result to around an hour.

6. Conclusions and future work

A prototype semi-automated bioassay for genotoxic compounds, based on the expression of a green fluorescent protein in genetically modified yeast cells in response to DNA damage, has been developed and successfully tested with the alkylating agent methyl methane sulphonate. A positive result could be obtained in under 4 h compared with around 24 h for an Ames Test.

The next stage of this work will be to validate the system by testing with a known range of mutagens identified by the Ames Test, and to assess the limits of detection for selected toxins. The instrument described here will also be expanded to produce a multi-channel system, such that control cultures, standards and samples can be compared against each other, hence accounting for variability in cell viability between measurements and other ambient factors. Rather than sample several batches of cells, cell culture volumes will be reduced

to a few millilitres, such that an automated flow-injection instrument where several samples are processed on-line in rapid succession can be developed. Such a system could be envisaged for continuous measurement in the water industry or for use in an anaerobic sewage sludge digester to protect consumers from water pollutants. Alternatively, with modifications, this technology could also be used to allow the rapid screening of novel pharmaceutical products for mutagenic activity.

Acknowledgments

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