

# Fluorescence Polarization Discriminates Green Fluorescent Protein from Interfering Autofluorescence

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- Novel use of fluorescence polarization (FP) optics
- Fluorescence polarization applied to yeast based genotoxicity assay
- Genotoxic species identified by expression of GFP, in spite of masking autofluorescence

## Introduction

In recent years, Green fluorescent protein (GFP) has been widely adopted as a versatile marker for reporting on gene expression in a wide range of cells and organisms. However, fluorescence measurements of GFP (unless it is very highly expressed) are invariably contaminated with endogenous autofluorescence from the cells or media. This is often significant at the same wavelengths as GFP, making discrimination by simple modifications to the filter set relatively ineffective. This paper describes a novel use of the fluorescence polarization (FP) optics available on the POLARstar OPTIMA from BMG LABTECH, which can dramatically increase the resolution of GFP fluorescence in the presence of unwanted autofluorescence. The POLARstar has been used to distinguish the expression of GFP in yeast cells in an assay for genotoxicity, where the test compounds themselves were highly autofluorescent and would ordinarily mask GFP, making toxicity assessment impossible.

## Theory

The method exploits the unusually high fluorescence anisotropy of GFP, due to its relatively large size and slow rotation. Thus when GFP is illuminated with plane polarized light, a high proportion of the emitted fluorescence remains polarized with respect to the excitation source. The degree of polarization (P), [defined as  $(I_{\text{para}} - I_{\text{perp}}) / (I_{\text{para}} + I_{\text{perp}})$ , where  $I_{\text{para}}$  is the fluorescence intensity measured parallel and  $I_{\text{perp}}$  is that measured perpendicular to the plane of polarization of the excitation light] was found to be approximately 0.40 (400 mP) for GFP. However, other autofluorescent species which emit at the same wavelength, such as fluorescein, show low fluorescence anisotropy due to their small size and fast rotation in solution; thereby, giving much smaller P values (35 mP for fluorescein), since  $I_{\text{para}}$  is approximately equal to  $I_{\text{perp}}$ . Hence by taking the difference between the two polarized fluorescence measurements ( $I_{\text{para}} - I_{\text{perp}}$ ), a signal is obtained which is large for GFP, but small for naturally occurring autofluorescent species, and virtually zero for simple fluorescent species such as fluorescein; thus allowing discrimination.

## Experimental

This principle has been demonstrated in the POLARstar in conjunction with a Gentronix assay for Genotoxicity (GreenScreen GC). In this assay, yeast cells are combined with serial dilutions of the compound to be tested in the wells of a microplate. The yeast (*Saccharomyces cerevisiae*) has been genetically modified to express GFP when the cell's DNA repair mechanisms are activated, upon exposure to a genotoxic compound. The more potent the genotoxin, the more DNA damage is sustained and the more fluorescent the cells become. Assessment of general cellular toxicity (cytotoxicity) is made simultaneously by measuring the degree of cell proliferation. In addition to the test yeast strain, a second strain, which does not express GFP, is used as a control.

The POLARstar was used to make fluorescence measurements from the top of the plate (excitation filter = 485-12, emission filter = 520-30), and absorbance measurements through the plate for cell density assessment (filter = 620 nm). The microplates used were Matrix Technologies, 96-well, black, clear bottomed plates. For the assay, 75  $\mu\text{L}$  of the serially diluted test compound was combined with 75  $\mu\text{L}$  yeast cell reagent per well. The plate also contained various standard compounds and blanks. The microplates were covered with a breathable membrane and incubated at 25°C overnight. After incubation, the plates were uncovered and read in the POLARstar. Since the toxicity of the test compound affects the final cell density achieved, the fluorescence reading is normalized for the number of cells present to form a "brightness reading". Thus:

$$\text{Brightness measurement by conventional method} = \frac{\text{Fluorescence}}{\text{Absorbance}}$$

$$\text{Brightness measurement by polarization method} = \frac{I_{\text{para}} - I_{\text{perp}}}{\text{Absorbance}}$$

The latter measurement can easily be achieved by subtracting the results for  $I_{\text{para}}$  and  $I_{\text{perp}}$  produced in the BMG data evaluation software.

## Results and Discussion

To characterize the method, the assay was performed by exposing 10 serial dilutions (from 32.5  $\mu\text{g}/\text{mL}$ ) of methyl methanesulphonate (MMS), a known genotoxic alkylating agent, to both the test and control yeast strains. The sample was spiked with 185 ng/mL fluorescein, an intensely fluorescent spectroscopic mimic of GFP, and a model autofluorescent compound in this study. GFP fluoresces at 517 nm and fluorescein at 512 nm.

Using conventional fluorescence (Figure 1), both the test and control strains show increasing fluorescence with increasing compound concentration; meaning that the induction of GFP in the test strain is effectively masked, making genotoxicity assessment impossible.

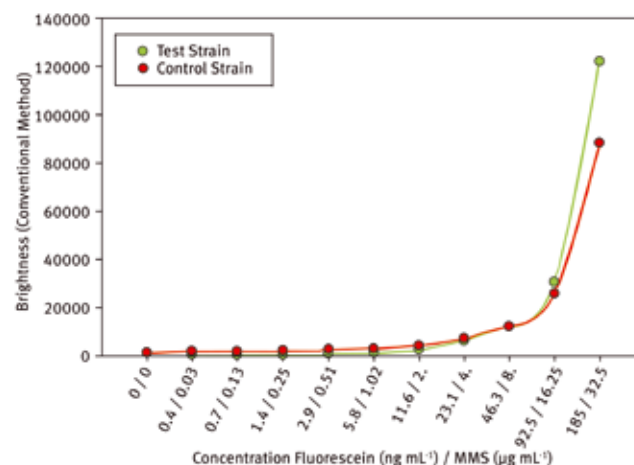
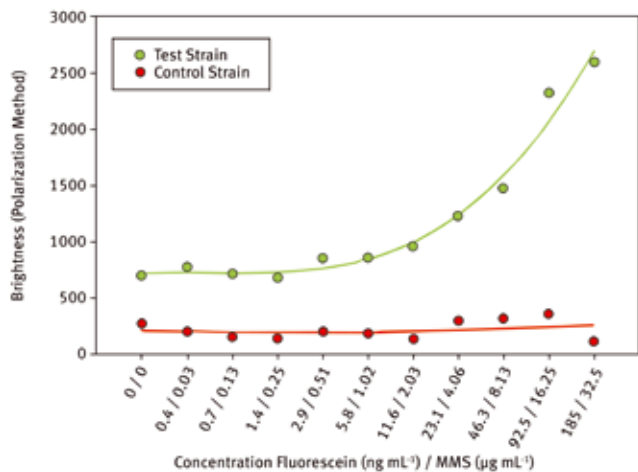


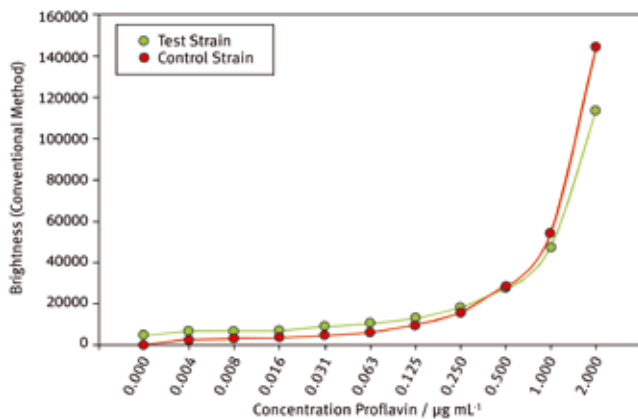
Fig. 1: Brightness of the control and test strains exposed to the genotoxin MMS spiked with a model interfering autofluorescent compound, fluorescein - measured using conventional fluorescence.

Figure 2 shows that applying the fluorescence polarization method, the fluorescence signals from the test and control strains are now separated, as the fluorescence from the added fluorescein is removed. A dose dependant increase in GFP induction with the test strain is now evident with increasing MMS concentration and the compound is confirmed as a genotoxin.

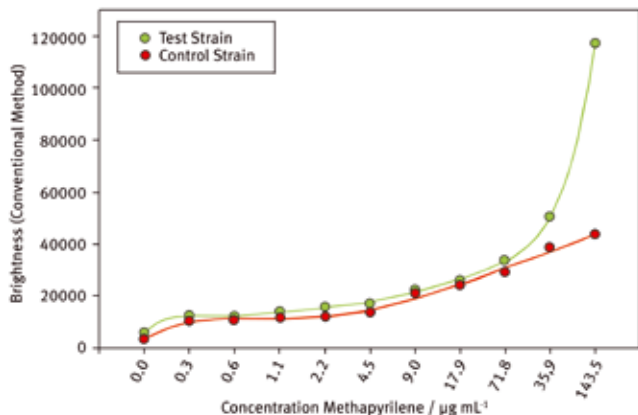


**Fig. 2:** Brightness of the control and test strains exposed to the genotoxin MMS spiked with a model interfering autofluorescent compound, fluorescein - measured using the new polarisation method.

Subsequently, the method was used to analyze two compounds, suspected hepatocarcinogens, that are highly fluorescent at the GFP wavelengths of interest. These were proflavin, a synthetic acridine dye used as a topical anaesthetic in World War II and methapyrilene, an antihistamine withdrawn from the market in the 1970s. Proflavin fluoresces at 513.5 nm and methapyrilene at 515.0 nm. Using conventional fluorescence, neither compound (Figures 3 and 4) can be reliably classified as a genotoxin since the control strain shows a very similar apparent induction in fluorescence at the same wavelength as GFP.

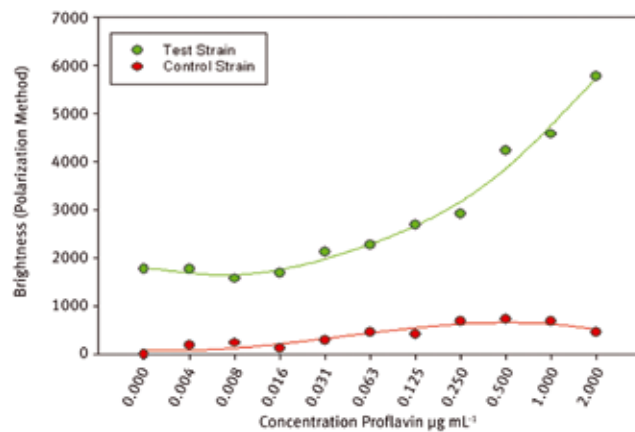


**Fig. 3:** Brightness of the control and test strains exposed to proflavin - measured using conventional fluorescence.

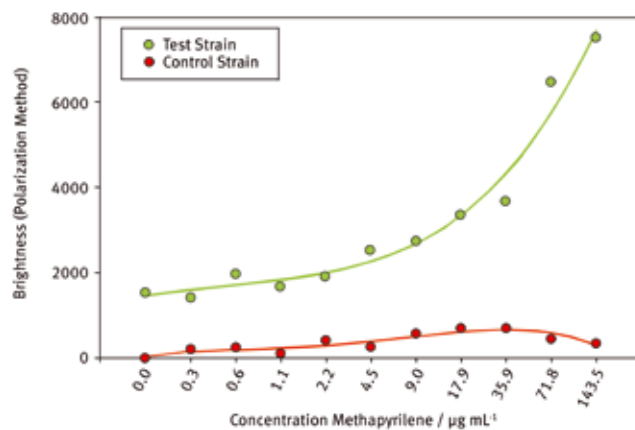


**Fig. 4:** Brightness of the control and test strains exposed to methapyrilene - measured using conventional fluorescence.

However, applying the fluorescence polarization discrimination method (Figures 5 and 6) clearly shows the induction of GFP in the test yeast strain compared to the non-expressing control strain; thereby confirming both compounds as genotoxic in this assay.



**Fig. 5:** Brightness of the control and test strains exposed to proflavin - measured using the new polarization method.



**Fig. 6:** Brightness of the control and test strains exposed to methapyrilene - measured using the new polarization method.

## Conclusion

Exploiting the inherent fluorescence anisotropy of GFP, a novel method for discriminating GFP from interfering autofluorescent species has been achieved. The technique has been successfully applied to a yeast based genotoxicity assay in 96-well microplates. The assay was performed using the polarization optics available on the BMG POLARstar. The method allowed both proflavin and methapyrilene to be identified as genotoxic species, which would not have been possible using conventional measurements due to their autofluorescence which masks the GFP signal.

The method has subsequently been shown to also work effectively using human lymphoblastoid TK6 cells with GFP as a reporter for GADD45a expression, in the GreenScreen HC genotoxicity assay.

## References

Application note extracted from: Knight AW, Goddard NJ, Billinton N, Cahill PA, Walmsley RM (2002) *J Biochem Biophys Methods*. **51**, 165-77.

For further information on GreenScreen products for accurate genotoxicity assessment, contact [andrew.knight@gentronix.co.uk](mailto:andrew.knight@gentronix.co.uk) or see [www.gentronix.co.uk](http://www.gentronix.co.uk)

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