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

Green fluorescent protein (GFP) has been widely adopted as a versatile marker for reporting on gene expression. However, fluorescence measurements of GFP (unless it is very highly expressed) are invariably contaminated with endogenous autofluorescence from the cells or media. This paper describes a novel use of the fluorescence polarization (FP) optics available on a BMG LABTECH microplate reader, which can dramatically increase the resolution of GFP fluorescence in the presence of unwanted autofluorescence. The reader 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

When GFP is illuminated with plane polarized light, because of its relatively large size and slow rotation, a high proportion of the emitted fluorescence remains polarized with respect to the excitation source. The degree of polarization (P), was found to be approximately 0.40 (400 mP) for GFP. Fluorescein, shows low fluorescence anisotropy due to its small size and fast rotation. Hence by taking the difference between the two polarized fluorescence measurements (Ipara - Iperp), a signal is obtained which is large for GFP, but small for naturally occurring autofluorescent species.

Experimental

Yeast cells are combined with serial dilutions of the compound to be tested in 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.

Assessment of general cellular toxicity (cytotoxicity) is made simultaneously by measuring the degree of cell proliferation. A second yeast strain, not expressing GFP, is used as a control.

The BMG LABTECH microplate reader was used to make fluorescence measurements from the top of the plate (excitation filter = 485-12, emission filter = 520-30), and absorbance for cell density assessment (filter = 620 nm). The microplates used were Matrix Technologies, 96-well, black, clear bottomed plates. 75 μL of the serially diluted test compound was combined with 75 μL yeast cell reagent per well. The plate also contained various standard compounds and blanks. The microplates were incubated at 25°C overnight. 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:

- Brightness measurement by conventional method = Fluorescence/Absorbance
- Brightness measurement by polarization method = (Ipara - Iperp)/Absorbance

Results & Discussion

The assay was performed by exposing 10 serial dilutions [from 32.5 μg/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.

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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. 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.

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Keywords: autofluorescence, GFP2, GFP, genotoxicity, profilavin

Rev. 06/2007

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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.

The method was used to analyze two compounds, that are highly fluorescent at the GFP wavelengths of interest. These were proflavin (513.5 nm) and methapyrilene (515.0 nm). Using conventional fluorescence, neither compound (Figures 3 and 4) can be reliably classified as a genotoxin.

**Conclusion**

Exploiting the inherent fluorescence anisotropy of GFP, a novel method for discriminating GFP from interfering autofluorescent species has been achieved. The assay was performed using the polarization optics available on the BMG LABTECH microplate reader.

The method allowed both proflavin and methapyrilene to be identified as genotoxic species, which would not have been possible using conventional measurements.