Minimizing photobleaching of Blue Fluorescent Protein (BFP) using the Agilent Cary Eclipse fluorescence spectrophotometer

Application Note

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Introduction

The term photobleaching refers to loss of fluorescence emission from samples due to prolonged exposure to exciting radiation. While many fluorophores retain stable fluorescence after extended periods of illumination, some photobleach after short periods of time.

In order to maintain optimal fluorescence emission, it is important to minimize photobleaching. Strategies to achieve this usually involve limiting one or both of the following: (1) the time of exposure to, or (2) the intensity of, the exciting light. However, either of these strategies may compromise the quality of the results or limit the types of analyses that can be performed because the signal to noise ratio (S/N) is unavoidably decreased. Furthermore, kinetics-based assays performed over an extended period of time may not be possible due to increased exposure of the fluorophore to the excitation light which results in photobleaching.

Blue Fluorescent Protein (BFP) provides an example of a fluorophore that is susceptible to photobleaching: "...even with folding improvements, BFP still suffers from a relatively low fluorescence quantum yield [compared to green fluorescent protein] and relatively easy bleaching." (Tsien, 1998)[1]. Despite the useful spectral properties of BFP for use in multi-labelling studies and techniques such as FRET (fluorescence resonance energy transfer),[2] it is likely that its low quantum yield and susceptibility to photobleaching could limit ongoing detection in instruments that irradiate the sample continuously, or too intensely, or both. Accordingly, the present study aimed to determine whether using the Agilent Cary Eclipse fluorescence spectrophotometer, which utilizes a special xenon flash lamp, photobleaching of BFP could be eliminated, or at least reduced, compared to other commercially available instruments having continuous or pulsed light sources.

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Materials and methods

(For part numbers, see Reference 3.)

- Cary Eclipse fluorescence spectrophotometer
- Peltier-thermostatted multicell holder (with electromagnetic stirring)
- Temperature controller
- Temperature probes
- Magnetic stirrer bars

Yeast strains

YRD15 (*MATα, his3, ura3, leu2, rho*$^{+}$) of the yeast *S. cerevisiae* was the parental strain used in this study. The open reading frame encoding BFP was cloned into the yeast expression plasmid pAS1N and transformed into the yeast strain YRD15 as previously described[4]. Transformants were plated out on yeast minimal medium (0.75% yeast minimal medium w/o amino acids, 2% glucose, 1.5% agar) with selective markers as required and grown at 28 °C for 3–5 days.

Protocol

Yeast cells were washed twice in 1 mL MilliQ water before being lysed using Y-PER (Progen) as per manufacturers instructions. Y-PER lysates (5 µl) were diluted with 1 mL 50 mM Tris/HCl pH 8 and placed in disposable fluorescence cuvettes (Sarstedt) in the multicell holder positioned within the sample compartment of the Cary Eclipse fluorescence spectrophotometer. Using the 'Scan' application, cell suspensions were repeatedly excited using 'cycle mode' with UV light of 370 nm, specific for BFP excitation, and the emission monitored from 400 to 550 nm. Slow scan speeds were used (120 nm/min) for best S/N, however, this maximized the exposure of BFP to the excitation light source. Operating parameters for the 'cycle mode' of BFP excitation scans are given in Figure 1.

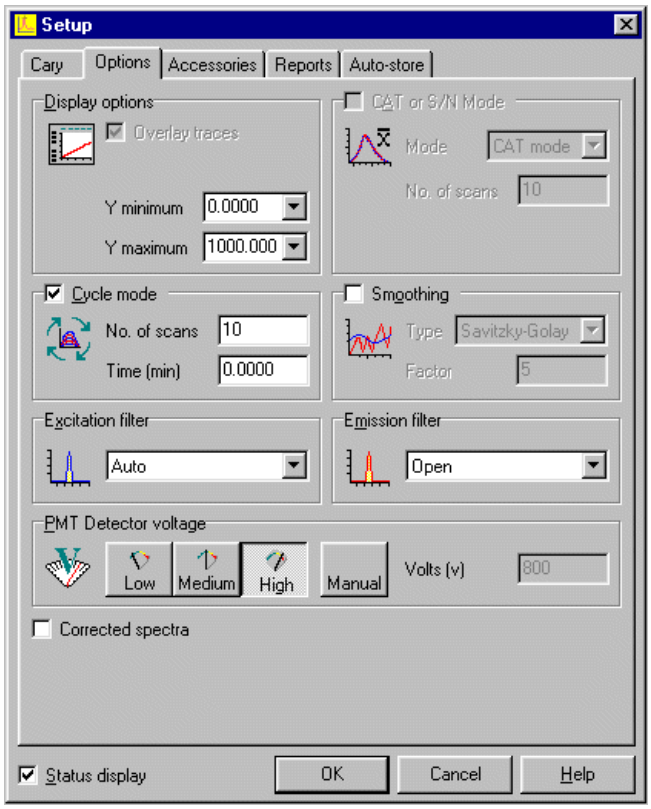


Figure 1. Cary setup for repetitive scanning using cycle mode

Results

Raw data showing superimposed emission spectra of BFP for 10 successive scans using the Agilent Cary Eclipse are shown in Figure 2a. Spectra were also recorded in an identical manner on a commercially available fluorescence spectrophotometer with a continuous xenon arc lamp (Figure 2b). Continuous excitation from the Agilent Cary Eclipse xenon flashlamp resulted in a 2.4% decrease in BFP emission over a total scan time of 21.5 minutes. In comparison, spectra recorded on the other instrument showed a much greater loss of signal (19.1%), indicating a high degree of photobleaching of BFP.

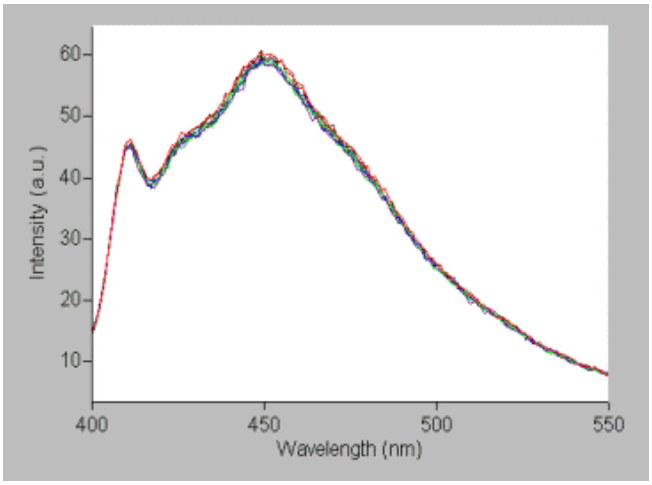
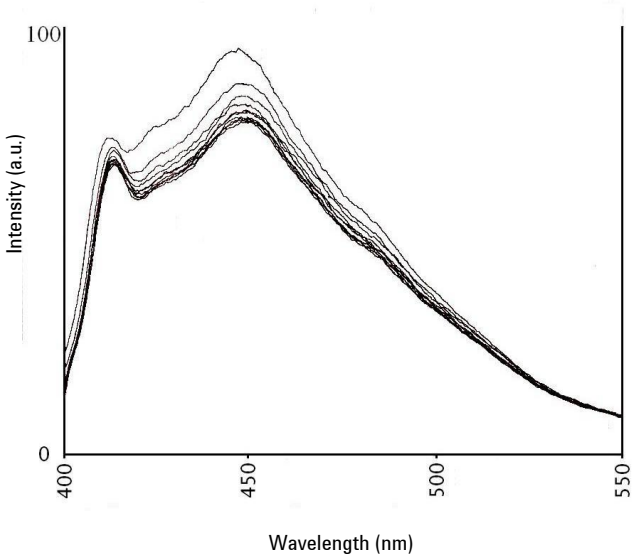


Figure 2. (a) and (b). Emission wavelength vs intensity for BFP following 370 nm excitation. A negligible drop in peak BFP emission (450 nm) was recorded after 10 successive scans at a scan rate of 120 nm/min (total exposure time 12 min 30 s) using the Cary Eclipse (a), whereas photobleaching of approximately 20% was observed using a commercially available instrument fitted with a xenon arc lamp (b)



Discussion

Photobleaching can be a major limiting factor in the time-based fluorescence measurements of living systems. The Cary Eclipse fluorescence spectrophotometer allows users to measure photosensitive samples without compromising the quality of data, as demonstrated by Figures 2 (a-b). The exposure time of the sample to the excitation light is minimized because the lamp is only on when the instrument is taking a reading. This feature not only reduces photobleaching, but also significantly increases the lamp life. Using a narrow pulse-width flash lamp (2 µs pulse width at half peak height), high intensity excitation can be delivered to the sample resulting in excellent S/N over varying periods of time. The lamp flashes at 80 Hz, providing the flexibility to measure extremely fast reactions (in the order of milliseconds) and much slower responses (in the order of hours). This combination of electronics and optics makes the Cary Eclipse an ideal platform for fluorescence studies *in vivo* of systems involving BFP and other fluorescent probes that are susceptible to photobleaching. In addition, having the lamp on when only taking a reading significantly increases lamp life.

Conclusion

The characteristics of the xenon flashlamp in the Cary Eclipse minimizes photobleaching in biological samples by irradiation the sample only when readings are being taken. This is a considerable benefit compared to other instruments fitted with xenon flash lamps or continuous arc lamps that continuously irradiate the sample.

References

1. Tsien, RY. (1998) The Green Fluorescent Protein. *Annu Rev Biochem* **67**: 509–44.

2. Gavin, P and Prescott M. (2001). Monitoring fluorescence resonance energy transfer (FRET) between GFP fusions in lysates of the yeast *Saccharomyces cerevisiae* using the Agilent Cary Eclipse. Fluorescence Application Note #9.

3. Part numbers:

Product	Part Number
Agilent Cary Eclipse Fluorescence Spectrophotometer	00 100752 00
Peltier Thermostatted Multicell holder	00 100755 00
Temperature Controller for Peltier	(00100386XX) 00 (AUS); 01 (US); 02 (Europe)
Magnetic stirrer bars:	66 100189 00
Temperature Probe accessory	60 100408 00
Kit door for thermostatted accessories	99 101032 00
Cary Eclipse Software Bio Package	85 101774 00
Country kit (98 1002 XXXX)) 9000(AUS); 9100(US); 9200 (Europe)	

4. Prescott, M., *et al* (1994), *Biochem. Biophys. Res. Commun*. **207**, 943-949.

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