Monitoring fluorescence resonance energy transfer (FRET) between GFP fusions in lysates of the yeast Saccharomyces cerevisiae using the Agilent Cary Eclipse

Application Note

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Introduction

Fluorescence resonance energy transfer (FRET) is a non-destructive, spectroscopic approach that can be used to monitor the proximity and angular orientation of donor and acceptor fluorophores in living cells[1]. The resonant energy of an excited donor fluorophore (in this example blue fluorescent protein—BFP) is absorbed by an acceptor fluorophore (green fluorescent protein—GFP) providing that donor and acceptor are in close proximity (between 10–80 Ångstroms[2]) (Figure 1). Emission spectra of donor fluorophores must significantly overlap the absorption spectra of the acceptor, while overlap between respective absorption and emission spectra of donor and acceptor should be minimized[3].

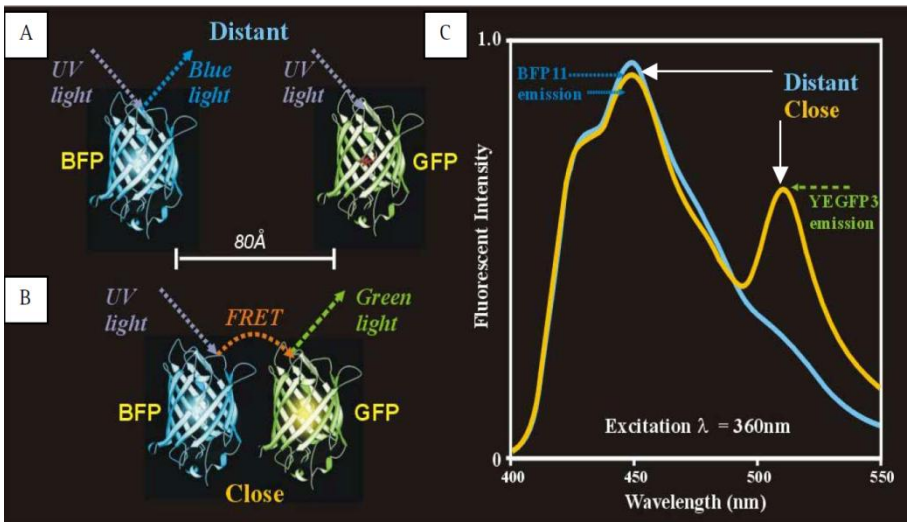

Figure 1. The use of GFP variants to produce FRET. In A, Distant; UV light excites BFP (donor fluorophore) to emit blue light (peak emission = 450 nm), but GFP (acceptor) is not close enough to draw energy from the excited donor. In B, Close; proximity of GFP to BFP allows non-radiative energy transfer, the stimulated BFP exciting GFP to fluoresce green (peak emission = 510 nm). Figure 1C shows the expected emission spectra when the GFP fluorophores are either close or distant

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Due to the attractive attributes of GFP (previously described in fluorescence application note No. 5)[4], the application of FRET to GFP and GFP variants has become a powerful tool to monitor interactions *at the protein level*, within an intact cell or organism. It is possible to use FRET to measure conformational changes in a molecule tagged with two GFP variants in response to the binding of ligands such as calcium[1], or the interaction between separate proteins, each tagged with a specific GFP[5]. Studies such as these present the unique opportunity to study subtle relationships and dynamic interactions between proteins—in living cells.

The present study aimed to detect and monitor changes in FRET between BFP and GFP in cytosolic lysates of yeast cells using the Agilent Cary Eclipse.

Materials and methods

(For part numbers please see Reference 7.)

Fluorescence BioMelt system, comprising:

- Cary Eclipse Fluorescence Spectrophotometer
- Multicell Peltier
- Temperature controller
- Quartz 10 mm w/stopper
- Eclipse Thermal Software (Bio software package)

Yeast Strains

YRD15 (*MATα, his3, ura3, leu2, p*$^+$) of the yeast *S. cerevisiae* was the parental strain used in this study. A gene cassette was constructed encoding BFP and GFP linked by a 27 amino acid peptide linker that contains a recognition site for the protease trypsin. This cassette was cloned into the yeast expression plasmid pAS1N for cytosolic expression and transformed into the yeast strain YRD15 as previously described[7]. Transformants were plated out on yeast minimal medium (0.75% yeast minimal medium w/o amino acids, 2% glucose, 1.5% agar) with growth supplements 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 the manufacturer's instructions. Lysates were preferred over whole cells to allow protease digestion of the peptide linker. Y-PER lysates (10 µl) were diluted with 1.2 ml Tris/HCl pH 8 and placed in disposable fluorescence cuvettes (Sarstedt) of the multicell holder positioned within the sample chamber of the Agilent Cary Eclipse. The temperature within the cuvettes was set to 25 °C to promote cleavage of the peptide linker by trypsin (Figure 2). Using the 'Scan' application, BFP was specifically excited using light of 360 nm, and emission spectra for the fusion protein were recorded from the range 400–550 nm. Further emission scans were recorded over time after the addition of 0.25 µg trypsin.

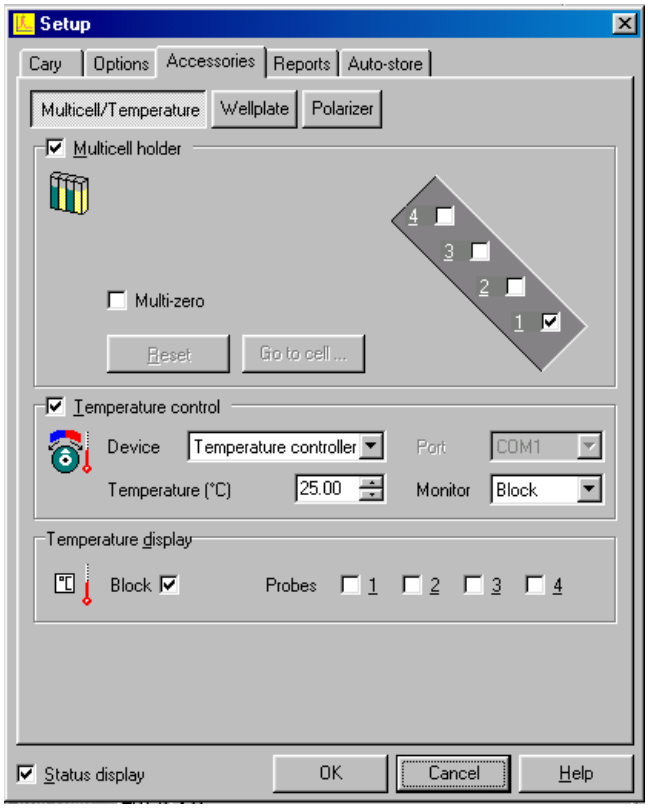

Figure 2. Temperature control setup for the promotion of trypsin activity

Results

Emission spectra of the BFP-GFP fusion protein following 360 nm excitation are shown in Figure 3. An initial spectrum was taken at time = 0 min, then trypsin was added and spectra were recorded at the times indicated. GFP emission (~510 nm) is seen upon specific excitation of BFP alone (360 nm), indicative of FRET. Spectral characteristics of FRET (indicated by the green peak at 510 nm) progressively disappeared following the addition of trypsin, which cleaves the peptide linker that tethers the GFPs. A small increase is seen in BFP emission as FRET diminishes.

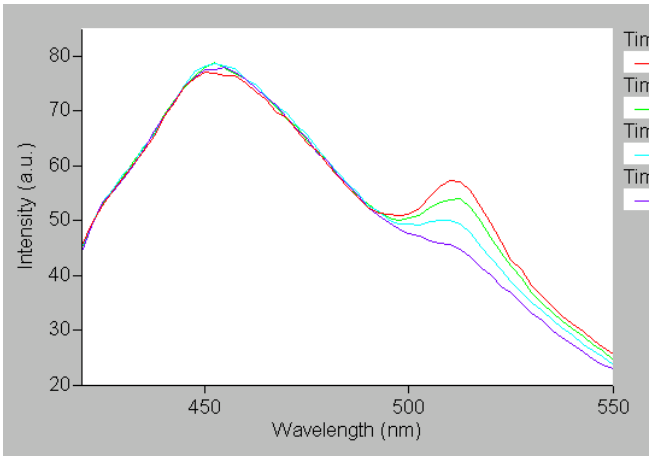

Figure 3. Emission wavelength vs intensity for the BFP-GFP fusion protein. Trypsin was added after initial scan at time = 0

Discussion

An ideal strategy to monitor the interactions of proteins in living systems involves detection of FRET between BFP and GFP bound to target species of interest. For optimum selectivity and sensitivity of detection of fluorescence in applications such as this it is necessary to minimise (a) detection of cellular autofluorescence and (b) photobleaching. These issues are addressed by internal filters (on both excitation and emission monochromators) and the Cary Eclipse xenon flash lamp respectively. The issue of photobleaching is extremely important and is discussed in a separate application note.

The data indicate (Figure 3) that FRET could be accurately monitored in cytosolic lysates from yeast cells. Cleavage of the BFP-GFP fusion using trypsin (Peltier temperature controlled at 25 °C) demonstrated that GFP emission was due to FRET and not to direct stimulation of GFP by the excitation wavelength. This is demonstrated by the scans shown in Figure 3 that depict the green (FRET) peak becoming smaller (as BFP is cleaved from GFP) with successive scans over a period of 33 minutes.

Conclusion

The Agilent Cary Eclipse with Peltier temperature control and multicell accessories provide a simple and accurate assembly with which to monitor cell function at the protein level in cytosolic lysates from yeast cells. The opportunity now exists to use this model as a platform with which to investigate protein-protein interactions in response to external or internal stimuli in living cells.

References

1. Miyawaki *et al*. (1997) Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin, *Nature*, **388**, 882-887.

2. Ha *et al*. (1996) Probing the interaction between two single molecules: Fluorescence resonance energy transfer between a single donor and a single acceptor, *Proc. Natl. Acad. Sci*. USA, **93**, 6264-6268.

3. Cubitt *et al*. (1995) Understanding, improving and using green fluorescent proteins, *TIBS*, **20**, 448-455.

4. Gavin, P and Prescott M. (2001) Cytosolic expression of green fluorescent protein (GFP) and its derivatives in the yeast *Saccharomyces cerevisiae*. Detection *in vivo* using the Agilent Cary Eclipse. Fluorescence Application Note #5.

5. Mahajan *et al*. (1998) Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer, *Nature Biotechnology,* **16**, 547-552.

6. Part numbers:

Product	Part Number
Agilent Cary Eclipse Fluorescence Spectrophotometer	00 100752 00
Peltier Thermostatted Multicell holder	00 100755 00
Temperature Controller for Peltier	00 100386 00
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)	

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

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