Real-time monitoring of genetically encoded redox probes in mammalian cell monolayers

BMG LABTECH
The Microplate Reader Company

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- roGFP2-Orp1 is utilized as an H_2O_2 probe
- The PHERAstar *FS* is sensitive enough to permit ratiometric roGFP fluorescence measurements from a monolayer of mammalian cells

Introduction

Redox processes play an important role in cellular physiology and pathology. A particularly powerful tool for the monitoring of cellular redox changes are genetically-encoded biosensors based on redox sensitive green fluorescent protein (roGFP). RoGFPs contain two cysteine residues engineered to be present on the surface of the protein β-barrel, which are capable of forming a disulphide bond. RoGFP can be made to respond to specific redox species via the genetic fusion of appropriate redox enzymes. For example, fusion of roGFP2 to the thiol peroxidase Orp1 generates an H_2O_2-sensitive probe.[1,2]

Assay Principle

RoGFP2 exhibits two fluorescent excitation maxima, at 405 nm and 488 nm, when monitoring fluorescence emission at 510 nm. The relative intensities of the two excitation maxima shift in an opposing direction upon reduction or oxidation of the roGFP2 disulphide (Fig. 1). Consequently, by simultaneously monitoring fluorescence emission at the two excitation maxima, it is possible to determine the degree of probe oxidation.[2,3]

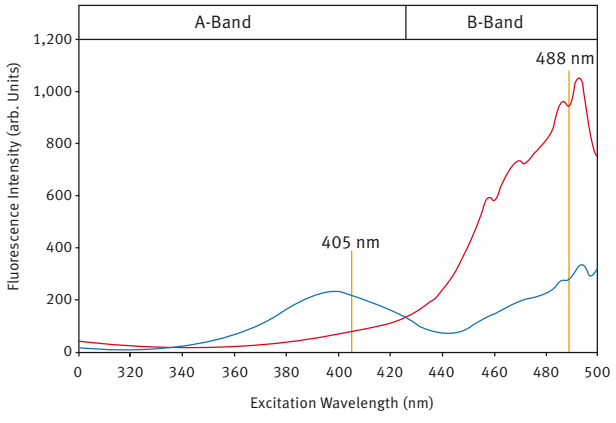

Fig. 1: Redox-dependent changes in the excitation spectrum of roGFP2.[2] Red curve = fully reduced roGFP2. Blue curve = fully oxidised roGFP2.

Fluorescence microplate readers would represent an ideal system for roGFP-based high throughput screening, for example to identify chemical compounds that modulate redox homeostasis. However, microplate reader-based roGFP measurements of cell monolayers require highly sensitive instruments.

In this application note we show that the PHERAstar *FS* reader enables roGFP2-based measurements in mammalian cell monolayers grown in 96-well imaging plates.

Materials and Methods

- ☐ PHERAstar *FS* multimode microplate reader (Fig. 2)
- ☐ Black flat-bottomed 96-well plates (BD Falcon)
- ☐ Hydrogen peroxide (H_2O_2) (Sigma, H1009)
- ☐ Imaging buffer (130 mM NaCl, 5 mM KCl, 10 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES)

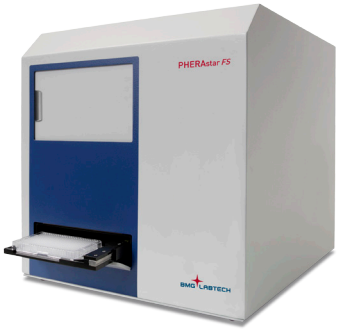

Fig. 2: PHERAstar *FS* multimode microplate reader from BMG LABTECH.

Experimental Procedure

Day 1

Cells stably expressing the cytosolic H_2O_2 probe roGFP2-Orp1 were seeded into a 96-well imaging plate (20,000 cells / well). The same number of non-transduced cells were seeded for use as a background control. The cell number was selected so as to obtain 100% confluence on the day of the measurement.

Day 2

Growth media was removed and the cells were washed twice with PBS, before application of 120 µl of imaging buffer. The response of the probe to an injection of a bolus of H_2O_2 was followed over time.

Instrument settings

Measurement type:	Fluorescence intensity, Bottom reading
Measurement mode:	Plate mode kinetic
No. of cycles:	47
Cycle time:	90 seconds
No. of flashes:	10
Optic settings:	dual chromatic
No. 1:	Optic module FI 400 520
No. 2:	Optic module FI 485 520
Scan mode:	orbital averaging
Scan diameter:	3 mm
Injection:	using onboard injectors
Injection cycle:	5
Volume:	indiv.
Pump speed:	300 µl/sec

Results and Discussion

With the current state feature of the control software it is possible to follow the reaction progress in real-time. A typical signal curve is shown in Figure 3.

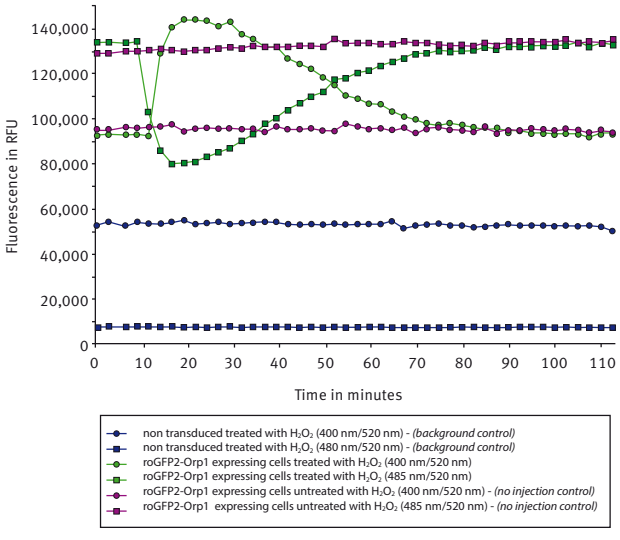

Fig. 3: Signal curves for roGFP2-Orp1 transduced cells before and after injection of hydrogen peroxide in comparison to a non-transfection control and a no injection control

In the sample expressing the roGFP2-Orp1 construct it can be clearly seen that after H_2O_2 injection the values measured for 400/520 will increase while the values for 485/520 decrease respectively. No effect can be seen in the no construct or no injection control.

The measurement data was processed to obtain degree of probe oxidation values. In figure 4 we monitored the response of the roGFP2-Orp1 probe in a monolayer of confluent lung adenocarcinoma cells, following addition of a bolus of H_2O_2. The sensitivity of the PHERAstar *FS* makes such measurements easily achievable (Fig. 4)

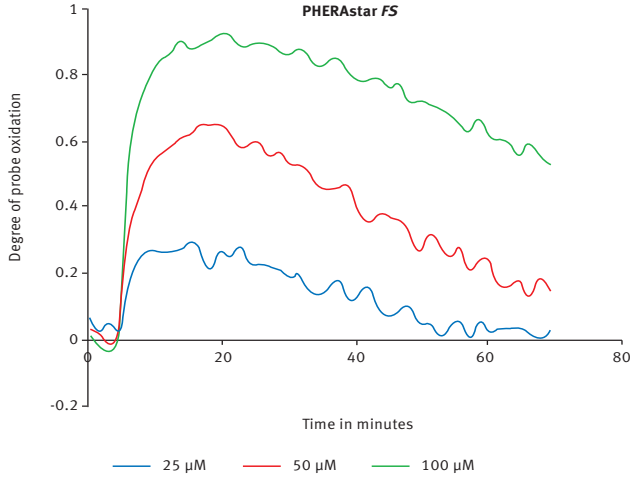

Fig. 4: Lung adenocarcinoma H1975 cells expressing the cytosolic roGFP2-Orp1 probe were grown as a monolayer in 96-well imaging plates. Cells were exposed to H_2O_2 as indicated, and the probe response followed using the PHERAstar *FS*.

We next assessed the impact of chemical compounds on cellular redox homeostasis. To this end lung adenocarcinoma cells expressing the cytosolic H_2O_2 probe roGFP2-Orp1 were treated overnight with different concentrations of the compound of interest. Subsequently the same cells were challenged with a single bolus of H_2O_2.

As shown in Figure 5, the compound of interest is found to significantly impair cellular recovery from an H_2O_2 challenge in a concentration-dependent manner. This result indicates that the compound disrupts reducing systems inside the cell and thus may be considered a candidate drug to sensitise cancer cells to chemo- or radiotherapy.

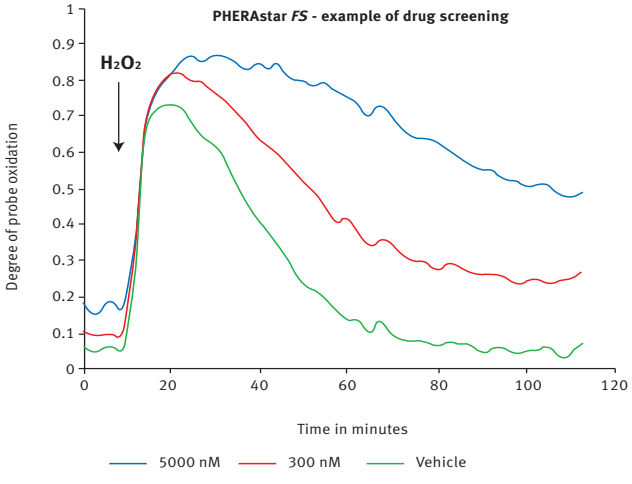

Fig. 5: Lung adenocarcinoma H838 cells expressing the cytosolic H_2O_2 sensor roGFP2-Orp1 were treated overnight with the indicated concentrations of an (unnamed) pharmacological compound. At the indicated time, cells were challenged with a bolus of H_2O_2 (final concentration 75 µM) and the response followed over time.

Conclusion

The PHERAstar *FS* enables monitoring of the ratiometric fluorescent response of roGFP2-based redox probes in monolayers of mammalian cells.

References

1. Gutscher et al. (2009). Proximity-based protein thiol oxidation by H_2O_2-scavenging peroxidases. J Biol Chem 284(46) 31532-40.
2. Meyer A.J., and Dick T.P. (2010). Fluorescent protein-based redox probes. Antiox. Redox Signal. 13:621-50.
3. Morgan B., Sobotta M.C., and Dick, T.P. (2011). Measuring E(GSH) and H_2O_2 with roGFP2-based redox probes. Free Radic Biol Med 51(11):1943-51.

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