

Cell-based assays assessing antioxidant capacity and cytotoxicity in living cells

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ABS

FI &
FRETLUM &
BRETTRF &
TR-FRET

FP

AS

NEPH

PHENIX
CLARIOstar^{plus}
VINTAGE
Omega series

The AOP1 assay measures antioxidant activities – and allows to measure cytotoxicity using the LUCS mode

AOP1 and LUCS both work on any live cell models, unaffected by cell number and are open to multiplexing

The CLARIOstar^{plus} is the first reader able to integrate AOP1 and LUCS in a plug-and-play configuration

INTRODUCTION

Antioxidants prevent the accumulation of reactive oxygen species (ROS) in cells. Antioxidants are compounds with a high benefit recognized in the cosmetics, pharmaceuticals and agro-food industries. For decades, *in vitro* studies were limited to cell-free test tube assays such as ORAC, TRAP, FRAP, etc. Available cellular antioxidant assays usually use dichloro dihydro fluorescein diacetate (DCFH-DA) which requires chemical molecules or UV light as ROS inducers. They do not allow to monitor the level of ROS produced by the cell and cannot discriminate between antioxidant and cytotoxic effects.¹ Based on the LUCS assay initially developed to measure cytotoxicity, the AOP1 assay is a live cell antioxidant approach that allows assessing the free radical scavenging capacity of antioxidants in a quantitative manner.

ASSAY PRINCIPLE

AOP1 Antioxidant assay

The fluorescence of photosensitizer thiazole orange (TO) is known to increase by 500 times when it reaches nucleic acids in cells². If TO passively enters into living cells in homeostasis, it is quickly removed by efflux transport proteins, resulting in a low fluorescence level (fig 1A). Once activated with a LED flash light, TO acts as a classical photosensitizer, producing ROS [at least [•]O₂ and OH[•]] in the vicinity of its targets³. Elevation of ROS inside the cell in turn affects efflux function. TO then accumulates inside the cell resulting in a high fluorescence level (fig. 1B). This process, called LUCS (Light Up Cell System) allows a very fine monitoring of intracellular ROS production and can be employed to quantitate antioxidants. Antioxidant activity will maintain low fluorescence or evoke a delay in its increase.

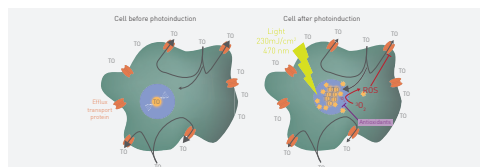


Fig. 1: Model of AOP1 in homeostatic cell. A) in a homeostatic cell, TO is mainly removed by efflux transport, limiting its access to nucleic acids (low fluorescence). **B)** TO photoinduction leads to the generation of ROS which stops its efflux triggering high fluorescence. Antioxidants may inhibit this process maintaining fluorescence low.

LUCS Viability assay

This photoinduction process can also be applied in a different protocol to measure cell viability. In homeostatic cells, low fluorescence (F_{pre})

is maintained due to functional efflux proteins but as soon as light is applied at an appropriate level, efflux function is lost, and F_{post} increases ($F_{post} > F_{pre}$). In damaged cells, efflux functions do not work anymore ($F_{post} \approx F_{pre}$). In dose-response studies, F_{post}/F_{pre} ratios allow to calculate e.g. EC_{50} .

MATERIALS & METHODS

- HepG2 cells (HB8065, ATCC)
- DMEM high glucose, Glutamax, pyruvate and P/S [all Gibco]
- FBS and Trypsin EDTA [Thermo Fisher Sc.]
- 96-well plate [Costar]
- Quercetin and chloroquine [Sigma Aldrich]
- AOP1 and LUCS kits (K-1001/K-2001, AOP)
- LED array system (LEDA-470, Teleopto)
- CLARIOstar^{plus} (BMG LABTECH)

Experimental procedure

HEPG2 cells were cultured until confluence of 70-80% was reached. They were then seeded in clear 96-well plates with 75,000 cells/well in 75 μ L and cultured for 24 h. The antioxidant assay (AOP1) and the cytotoxicity assay (LUCS) were performed as dose-response studies in serum-free medium.

AOP1 Antioxidant assay

Cells were treated with nine different concentrations of quercetin (1.95-500 μ M) or with 1% DMSO (neg. ctrl.) and incubated for 4 h. Solution A from the K-1001 AOP1 kit was added and incubated as indicated in the datasheet. The fluorescence intensity (FI) was read in the CLARIOstar^{plus} before (flash 0) and after each flash (1-20 flashes, 10 sec each) with a LED array system, placed outside the reader (fig. 2).

Instrument settings

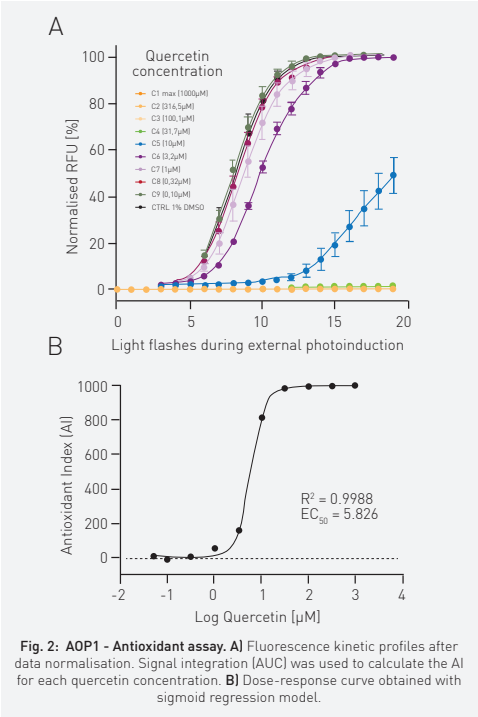
Optic settings	Fluorescence intensity, plate mode kinetic	
	Monochromator	Ex 503-16 Dichroic 519.5 Em 543-40
General settings	Number of flashes	20
	Settling time	0.5 sec
Kinetic settings	Antioxidant assay (AOP-1)	
	Number of cycles	20
	Cycle time	120 sec
	Cytotoxicity assay (LUCS)	
Focal height	Cycle time	300 sec
	Number of cycles	8 (2 in routine)
Gain	Focal height	3.5
	Gain	EDR (auto-gain)

Cytotoxicity assay

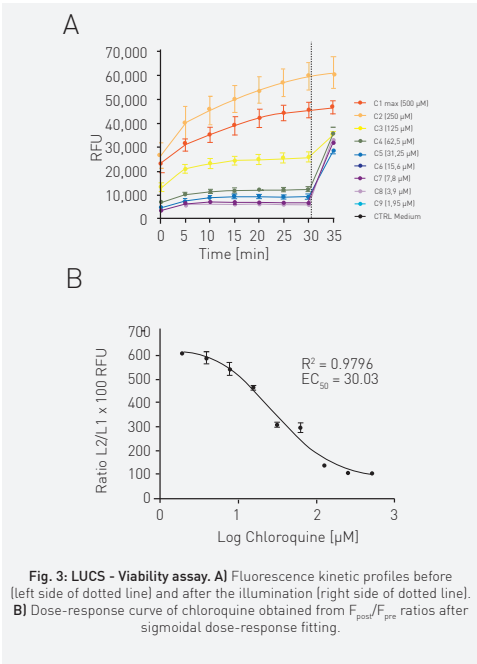
Cells were treated for 24 h with nine different concentrations of chloroquine [1.95-500 μM] or with medium [neg. ctrl.]. Solution A from the K-2001 LUCS kit was added and incubated as indicated in the datasheet. FI was measured in the CLARIOstar^{Plus} every 5 min for a total of 30 min. Cells were then illuminated for 10 sec with the LED array system and FI was measured one more time. Routine use requires only 1 pre-illumination (F_{pre}) and 1 post-illumination (F_{post}) reading to calculate the ratio.

RESULTS & DISCUSSION

For the AOP1 assay, relative fluorescence units (RFUs) were plotted in a kinetics-like mode (not shown). Data were then normalised to DMSO control value (fig. 2A). Antioxidant indices [AI], fig. 2B) correspond to control AUC (Area Under Curve) minus sample concentration AUC normalised to control value. Data were fitted to a sigmoid regression model and plotted as function of the log of quercetin concentration. An EC_{50} of 5.83 μM was obtained which is in line with the literature.



For the LUCS viability assay RFU were directly plotted as function of time (fig. 3A). The dotted line represents the illumination step. The ratios of fluorescence levels before (F_{pre}) and after (F_{post}) illumination were calculated for each curve, then fitted to a sigmoid regression model and plotted as function of the log of chloroquine concentration (fig. 3B). An EC_{50} of 30.03 μM was obtained which is in line with the literature⁴.



CONCLUSION

The LUCS technology implemented in the AOP1 antioxidant assay and the LUCS viability assay is a promising alternative to classical approaches. It comes with many advantages like its applicability in a great diversity of living cells, the option to discriminate between antioxidant effects and cell viability, and its compatibility with multiplexing approaches.

By its ability to connect both illumination steps and fluorescence readings in a unique protocol, the CLARIOstar^{Plus} has become the first microplate reader able to fully integrate AOP1 and LUCS assays in a "plug-and-play" configuration, adapted to routine and HTS applications.

REFERENCES

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