Quantitative assessment of antioxidant activities in living cells using EnSight™ Multimode Reader

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Published 15/01/2022

Abstract

The AOP1 antioxidant live cell assay was developed from the LUCS (Light Up cell System) technology that allows for fine monitoring of intracellular ROS production. AOP1 is based on the activation of an intracellular photosensitizer in a protocol that only requires a succession of light flashes and fluorescence readings. However, the photoinduction process requires high energy light flashes usually provided by an external light source device. Here, we used quercetin, a classical standard compound with live cell antioxidant effects, to show that AOP1 assay can be implemented in a "plug-and-play" mode using the EnSight™ Multimode Reader. A dose-response study carried out on human liver HepG2 cells led to a quercetin EC_{50} of 3.884 µM/ml, in line with previous published data, along with a very high determination coefficient R^2 = 0.9997. The present study demonstrates that the EnSight™ Multimode Reader provides robust AOP1 data using a drastically simplified protocol compatible with HTS campaigns in robotic environment.

Introduction

Plants were identified as a countless source of natural antioxidant extracts/compounds, and both academia and industry have intensively explored this diversity for decades, looking for the antioxidant grail. However, the vast majority of studies were carried out using test-tube antioxidant assays performed in an acellular environment, giving no clues about the biological antioxidant effect in living organisms. Until recently, DCFH-DA was the only biosensor available on cell models for antioxidant effect detection. In the past, this assay (also called CAA) showed to be hard to standardize and still suffers from numerous drawbacks. Among them, no control of the level of reactive oxygen species (ROS) production, no discrimination between antioxidant and cytotoxic effects, cell leakage, limitation to adherent cells, auto-oxidation,… strongly limiting its value as a robust and quantification assay.

The idea of a new antioxidant live cell assay came from the discovery of a photosensitizer, namely thiazole orange (TO), capable of emitting signals linked to the actual

concentration of ROS produced by the cell. TO presents a very interesting property for cell biology: its fluorescence quantum yield remains very low (2×10^{-4}) in the culture medium due to free rotation of its two aromatic rings around the methine bridge that links them. In this situation, energy relaxation occurs on a non-radiative mode via internal conversion through an ultrafast intramolecular twisting (100 fs) at the excited state. This means that there is virtually no residual TO fluorescence before the photosensitizer has reached its intracellular target. TO is also known to interact with nucleic acids with an increase of its fluorescence quantum yield to 0.1, denoting a 500-fold gain, a property attributed to a restriction in its torsion capacity[1]. More interesting, a recent electron paramagnetic resonance (EPR) study conducted in HepG2 cells showed that excited TO also acts as a classical photosensitizer producing both $^1O_2$ (type II reaction) and $OH^{\cdot}$ (type I reaction)[2].

Lastly, TO presents another quite unique property in live cells: its fluorescence level increases during photoinduction in a process called light-up cell system (LUCS). The intimate mechanisms underlying LUCS have been partially deciphered[2]. TO passively enters the cells but is mainly removed out by efflux transport proteins (presumably of the MATE family), limiting its access to nucleic acids and resulting in a low fluorescence level. When light is applied, ROS induced by TO photoactivation alter efflux and/or other cellular functions, perturbing cell homeostasis and triggering a massive entry of the biosensor, which progressively saturates nucleic acid binding sites, resulting in a relevant increase of fluorescence level.

Antioxidant effect (i.e., the capacity to neutralize intracellular free radicals) can then be measured as the ability of extracts/samples to delay or suppress this ROS-induced increase of fluorescence (Fig. 1).

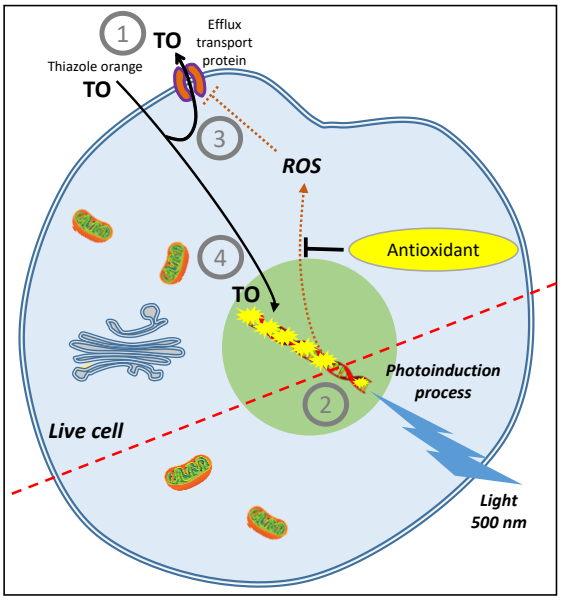

Fig. 1 AOP1 assay. (1) Before photoinduction, TO is massively removed out of the cell by efflux transport proteins; (2) under light application, photoinduction is initiated by an energy transfer from thiazole orange (TO) to molecular oxygen at the triplet state forming singlet oxygen and subsequent free radicals (ROS). (3) ROS alter TO efflux transport and other cell functions; (4) massive entry of TO triggers increase in fluorescence emission. Effect is measured as the ability to antioxidants to quench ROS production, keeping TO out of the cell and resulting in low fluorescence (diagram taken from ref. 3).

AOP1 is to our knowledge the first approach able to assess and quantify quenching of free radicals directly produced by living cells[3]. **For the first time, cellular ROS level can be precisely controlled, kept at a sub-lethal level, and quantified by a simple fluorescence measurement**.

The AOP1 assay has been already applied with success to classify numerous standard antioxidants according to their efficacy concentrations (ECs)[4], to assess cellular antioxidant effects of many plant extracts including a PhytoComplex of bilberry (*Vaccinium myrtillus*)[5], and as a biosensor in pharmacological studies[6,7].

Features of AOP1 antioxidant assay

- **First quantitative live cell antioxidant assay available ever**,
- Based on the **controlled cytosolic production of $^1O_2$ and free radicals** by a photoinduction process,
- Can easily **discriminate between antioxidant and cytotoxic effects** in the same well,
- Fluorescent sensor strictly correlated but independent to the induced oxidation process,
- Not subject to cell leakage
- **Results not affected by cell number** as measures are made on a kinetic mode
- **Very easy to implement** using "one-step" kits,
- **Easy to standardize** on 96 and 384 well-plates (Z' > 0.8), open to high throughput,
- **Open to multiplexing** as the signal is measured on live cells after a moderate (non-destructive) production of free radicals,
- Works on adherent and suspension cells,
- Compatible with organotypic models,
- Fluorescent technology **compatible with fluorescent plate readers.**

Material & Methods

Instruments

- PerkinElmer EnSight™ Multimode Reader
  - Excitation light source is a LED with centered wavelength of 465 nm

Fig. 2 view of the EnSight™ multimode plate reader PerkinElmer

Reagents

- Antioxidant-power AOP1 kit #K-1001, Toulouse France
- Sigma-Aldrich Quercetin, Saint-Quentin Fallavier, France
- Gibco DMEM, high glucose, GlutaMAX supplement and pyruvate
- HyClone fetal bovin serum (FBS)
- Gibco pen-strep solution (100X)
- Hyclone 0.05 % Trypsin-EDTA, purchased from Thermo Fisher Scientific (Illkirch-Graffenstaden, France).

Cell culture

HepG2 (#HB8065) cell line was purchased from the American Type Cell Collection (ATCC) (LGC Standards, Molsheim, France).

HepG2 cells were cultured at 37 °C/5% CO_2 in Glutamax DMEM medium complemented with 10% FBS and 1X pen-strep solution. Cells were grown up to 70–80% confluence then transferred in clear bottom 96-well microplates for 24h at a density of 10^6 cells/ml (75µL, 75000 cells/well).

Experimental protocol

Nine different quercetin concentrations (1000 µM - 0.1 µM) were obtained by serial factor 3.16 dilutions. Experiments were carried out in 96-well microplates. All cell treatments were performed in serum-free medium to avoid potential interaction with serum components. Each experimental condition was assayed in triplicates, including the solvent control without sample. Cells were incubated for 4h at 37°C in 5% CO_2 with each experimental condition. Solution A (Antioxidant-power AOP1 kit #K-1001) was added to the cells for 30 min at 37°C in 5% CO_2.

The fluorescence level was measured (flash number 0 in the graphs below) using a EnSight™ Multimode Reader sets up at

505/535 nm (excitation/emission wavelength). Wells were then illuminated for 500 milliseconds with EnSight™ imaging LED : excitation at 465 nm, 40% intensity. The EnSight™ Multimode Reader was set to replicate the same cycle (fluorescence reading/illumination) 20 times on a kinetic mode.

Results and discussion

Raw data (relative fluorescence units, RFUs) were plotted in a kinetics-like mode (light flash number vs RFU) (Fig. 3). Error bars represent SD values of triplicates.

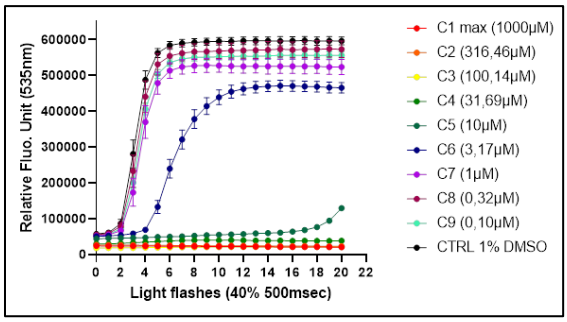

Fig. 3 AOP1 fluorescence kinetic profiles obtained for each quercetin concentration (1000 µM – 0.1 µM). Each light flash represents 500 milliseconds illumination with EnSight™ imaging light source LED (excitation at 465 nm, 40% intensity).

Post-measurement analyses

Raw data were further analyzed by Prism8 software (GraphPad, San Diego, CA, USA) to generate dose-response curves. Data from the first 14 light flashes were normalized (Fig. 4) and expressed as an Antioxidant Index (AI) corresponding to the integration of each normalized data (Fig. 5).

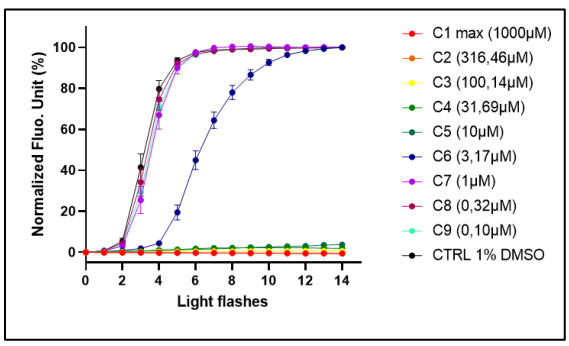

Fig. 4 AOP1 fluorescence kinetic profiles obtained after data normalization. Signal integration in the form of AUCs (Area Under Curves) was used to calculate the Antioxidant Index (AI) corresponding to each tested concentration.

CAI values were then used to calculate 50% Efficacy Concentration (EC_{50}) value from a mathematical non-linear regression model (sigmoid fit) given by Prism8, following the equation (1):

(1) $Y = Bottom + (Top-Bottom)/(1 + 10^{((LogEC_{50}-X)*HillSlope)})$

where HillSlope = slope coefficient of the tangent at the inflection point. EC_{50} and determination coefficient R^2 values were deduced from this regression model.

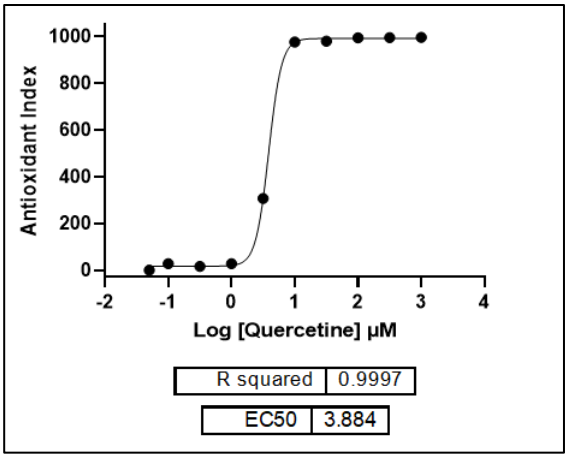

Fig. 5 dose-response curved obtained after sigmoid fitting. HepG2 quercetin live cell antioxidant EC50 was evaluated at 3.884 µM with an R2 = 0.9997

With a quercetin EC_{50} evaluated at 3.884 µM (R^2 = 0.9997), results are in line with previously published data (quercetin EC_{50} = 23.66 µM, R^2 = 0.985) obtained using a dedicated flash applicator[4]. However, in the latter test configuration, the microplate needed to be removed out the plate reader and moved to the light applicator for each of the 20 light applications.

Conclusion

By its ability to connect both illumination and fluorescence reading in a unique protocol, the EnSight™ Multimode Reader was able to fully integrate AOP1 assay in a "plug-and-play" configuration, adapted to routine and HTS applications.

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