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Quantitative Assessment of Antioxidant Activities in Living Cells

Using the Agilent BioTek Cytation 5 cell imaging multimode reader

Authors

Paul Held, PhD
Agilent Technologies, Inc.
Winooski, VT, USA

Damien Brechet
Agilent Technologies SAS
Les Ulis, France

Christophe Furger, Camille Gironde,
Mylene Rigal, and Wenli Debord
Anti Oxidant Power (AOP)
Toulouse, France

Abstract

Anti Oxidant Power 1 (AOP1), an antioxidant live cell assay, was developed from the Light-Up Cell System (LUCS) technology, which allows for fine monitoring of reactive oxygen species (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. In this application note, quercetin, a classical standard compound with live-cell antioxidant effects, is used to show, for the first time, that the AOP1 assay can be implemented in a "plug-and-play" mode using the Agilent BioTek Cytation 5 cell imaging multimode reader. A dose-response study with quercetin was carried out on human liver HepG2 cells. Cellular antioxidant index (CAI) 50% efficacy concentration (EC_{50}) values were evaluated at 15.70 and 15.78 µM, with R^2 = 0.996 and 0.994, respectively, for separate plates, in line with previously published data.

Introduction

Plants have been identified as a countless source of antioxidant extracts/compounds, and both academia and industry have intensively explored this diversity for decades, looking for the antioxidant "grail.". However, most studies have been carried out using test-tube antioxidant assays performed in an acellular environment, giving no clues about the real expected antioxidant effect in living organisms. Until recently, dichlorodihydrofluorescein diacetate (DCFH-DA) was the only biosensor available on cell models for antioxidant-effect detection. In the past, this assay (also called CAA) has been shown to be difficult to standardize, and still suffers from numerous drawbacks. These drawbacks include: lack of control of the level of ROS production, lack of discrimination between antioxidant and cytotoxic effects, cell leakage, limitation to adherent cells, and auto-oxidation, which all strongly limit its value as a robust quantification assay. This application note describes the AOP1 assay, as shown in Figure 1.
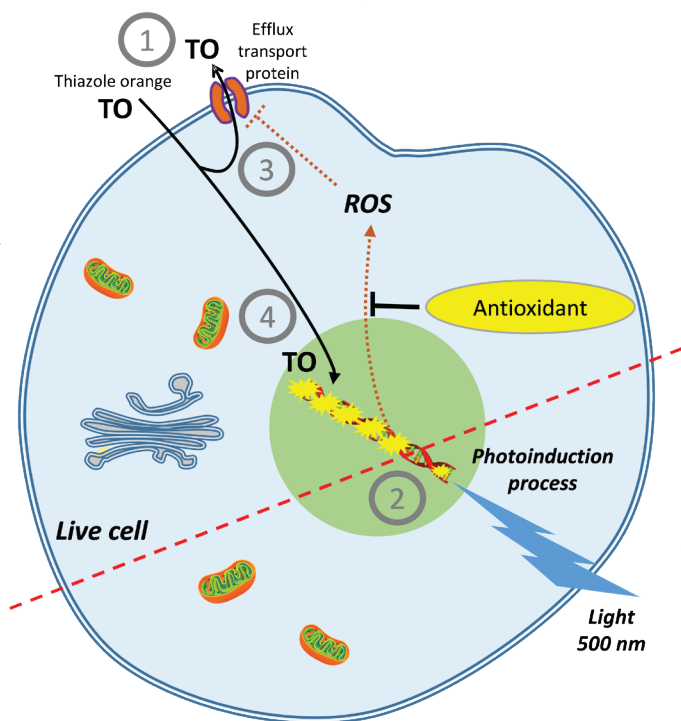

Figure 1. AOP1 assay. (1) Before photoinduction, thiazole orange (TO) is removed from the cell by efflux transport proteins; (2) under light application, photoinduction is initiated by an energy transfer from TO to molecular oxygen at the triplet state, forming singlet oxygen and subsequent free radicals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

This novel antioxidant live-cell assay resulted 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. The fluorescence quantum yield Φ for TO 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, as seen in Figure 2.
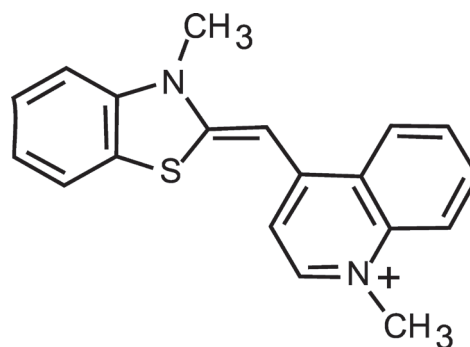

Figure 2. Thiazole orange structure.

Under these conditions, energy relaxation occurs in 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 in its fluorescence quantum yield of 500-fold, which is attributed to a restriction in its torsion capacity.[1] 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· (type I reaction).[2]

Lastly, TO presents another unique property in live cells: its fluorescence level increases during photoinduction in a process known as LUCS. The intimate mechanisms underlying LUCS have been partially deciphered. While TO passively enters the cells, it is mainly removed 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 entry of the biosensor, which progressively saturates nucleic acid binding sites, resulting in a relevant increase in fluorescence level. The antioxidant effect (e.g., 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 in fluorescence, as shown

in Figure 1. AOP1 is, to our knowledge, the first approach able to assess and quantify quenching of free radicals directly produced by living cells.[4] The cellular ROS level can be precisely controlled, kept at a sublethal level, and quantified by a simple fluorescence measurement.

The AOP1 assay has already been applied with success to classify numerous standard antioxidants according to their efficacy concentrations (ECs)[4]; 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].

Experimental

Materials

HepG2 cell line (part number HB8065) was purchased from the American Type Culture Collection (ATCC) (LGC Standards, Molsheim, France). DMEM high glucose (part number 11965092), penicillin-streptomycin (10,000 U/mL) solution (part number 15140122), GlutaMAX Supplement (part number 35050061), and sodium pyruvate (part number 11360070) were obtained from Gibco/Life Technologies (Carlsbad, CA). HyClone fetal bovine serum (FBS) and HyClone 0.05% trypsin EDTA were from Thermo Fisher Scientific SAS (Illkirch-Graffenstaden, France). Quercetin (part number Q4951) was obtained from Sigma-Aldrich (Fallavier, France). The AOP1 kits (part number K-1001) were obtained from Anti Oxidant Power (Toulouse, France).

Cell culture

HepG2 cells were cultured at 37 °C/5% CO_2 in GlutaMAX DMEM medium, supplemented with 10% FBS and 1x penicillin-streptomycin solution. Cells were grown up to 70 to 80% confluence, then transferred to clear-bottom 96-well microplates for 24 hours at a density of 75,000 cells/well in 75 μL.

Experimental protocol

Nine different quercetin concentrations (1.95 to 500 μM) were obtained by serial two-fold 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 4 hours at 37 °C in 5% CO_2 with each experimental condition. Solution A (from AOP1 kit, part number K-1001) was added to the cells for 30 minutes at 37 °C in 5% CO_2. After incubation, the fluorescence level was measured kinetically every 2 minutes with a two-step process.

The fluorescence was measured using 505 nm excitation and 535 nm emission wavelengths, followed immediately by illumination for 500 milliseconds with the Cytation 5 imaging lamp at intensity 1 with GFP filter. This fluorescence reading/illumination cycle was set to replicate the same cycle 18 times, as seen in Figure 3.
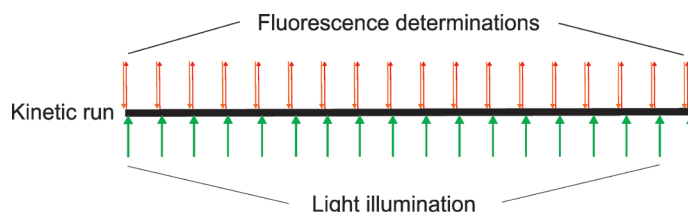

Figure 3. Kinetic detection scheme. The fluorescence was measured using 505 nm excitation and 535 nm emission wavelengths, followed immediately by illumination for 500 milliseconds with the Agilent BioTek Cytation 5 cell imaging multimode reader imaging lamp at intensity 1 with GFP filter. This cycle (fluorescence reading/illumination) was set to replicate the same cycle 18 times.

Instrumentation

Data were collected on an Agilent BioTek Cytation 5 cell imaging multimode reader configured with a GFP imaging filter cube and a 4x objective, controlled by the Agilent BioTek Gen5 microplate reader and imager software.

Methods

Analysis

Raw kinetic data were exported from Gen5 and plotted using Prism 8 software (GraphPad, San Diego, CA, USA). Raw data (relative fluorescence units, RFUs) were plotted versus time. The raw kinetic plots were normalized such that each experimental condition was expressed as a percentage of untreated (1% DMSO) control wells. These transformed data were also plotted as a function of time. The CAI for each sample was calculated using the integral of each normalized kinetic curve. The CAI is calculated according to Equation 1, where X is the normalized relative fluorescence, and CTL is the mean of the normalized control wells.

Equation 1. $\quad CAI = 1000 - \dfrac{(X \cdot 1000)}{CTL}$

CAI values were then used to calculate 50% efficacy concentration (EC_{50}) value from a mathematical nonlinear regression model (sigmoid fit) generated by Prism 8, following Equation 2, where Hill slope is the slope coefficient of the tangent at the inflection point. EC_{50} and determination coefficient R^2 values were deduced from this regression model.

Equation 2.

$$Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{((LogEC50 - X) \cdot Hill\ slope)}}$$

Results and discussion

These data demonstrate the ability of the AOP1 assay to monitor changes in TO fluorescence kinetically. As observed in Figure 4, HepG2 cells treated with quercetin for 4 hours then incubated with AOP1 reagent demonstrate significantly different fluorescence profiles depending on the quercetin concentration. Untreated cells exhibit a nearly 20-fold increase in fluorescence within 20 minutes, indicating the production of cellular ROS products. Cells treated with the highest concentrations of quercetin (500 µM) showed little to no change in the presence of the same photoactivation, suggesting that, when quercetin neutralizes the ROS products generated before cellular functions, such efflux is perturbed.
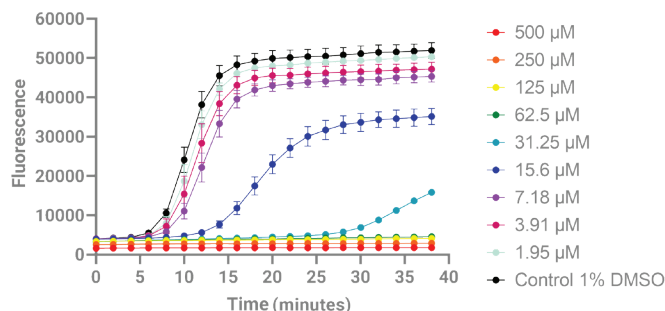

Figure 4. AOP1 fluorescence kinetic profiles of HepG2 cells treated with quercetin, obtained for each quercetin concentration (1.95 to 500 µM). Each light flash represents 500 milliseconds illumination using the Agilent BioTek Cytation 5 cell imaging multimode reader imaging lamp at intensity 1 with GFP filter. Each data point represents the mean and standard deviation of three replicates.

These data can be normalized to allow for easier comparison between experiments, as shown in Figure 5. Raw fluorescence values can vary markedly between separate experiments. Slight differences in TO concentrations, different cell numbers, and different cell types can result in variability of the raw fluorescence signal. Each fluorescent determination of the kinetic run is normalized to the mean of the last kinetic determination of the untreated control (presumably the highest raw signal) and expressed as a percentage. This analysis can then be used for comparing the timing or the degree of ROS activity suppression between assay controls and experimental samples across experiments.
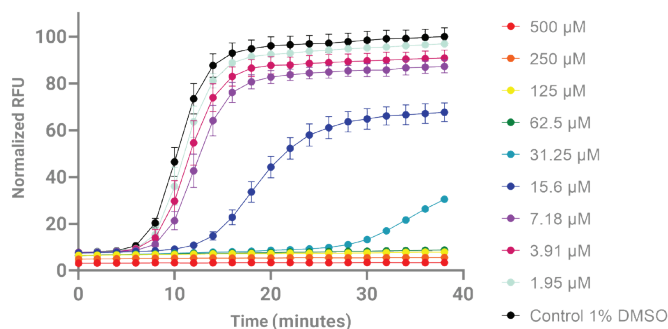

Figure 5. AOP1 fluorescence kinetic profiles obtained after data normalization. Data for each time point were normalized to the mean of the untreated control wells at the last kinetic data point and expressed as a percentage. The normalized data were plotted as a function of time. Each data point represents the mean and standard deviation of three replicates.

Normalized data can be further analyzed postmeasurement. Using the area under the curve (AUC) or integral of the data, a CAI can be calculated for each treatment concentration. These data can be used to determine EC_{50} values from a four-parameter logistic fit model. Figure 6 demonstrates two separate EC_{50} determinations for quercetin.
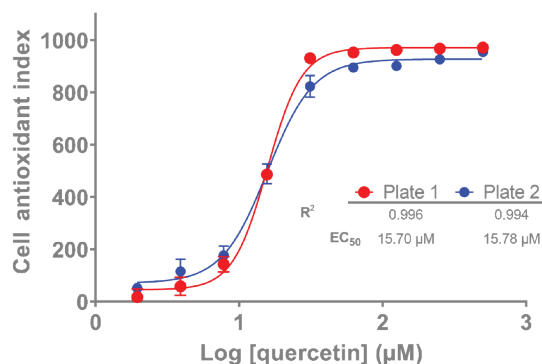

Figure 6. Quercetin CAI dose-response curves obtained after sigmoid fitting. Signal integration in the form of AUCs was used to calculate the CAI corresponding to each tested concentration. Data represent the mean and standard deviation of three replicates.

With quercetin EC_{50} values evaluated at 15.70 and 15.78 µM, with R^2 = 0.996 and 0.994 respectively, for separate plates, these data demonstrate the repeatability of the assay. These 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 from the plate reader and manually moved to the light applicator for each of the 18 light applications.

The AOP1 assay requires high-intensity light to photoactivate the detection fluorescence kinetically. Previously, this was accomplished using a light source external to the fluorescence reader. However, the Cytation 5 enables both microscopic fluorescence imaging and conventional fluorescence detection. The microscopic imaging module provides a focused, wavelength-specific, high-energy light to excite fluorescent molecules, which can be used as a substitute for the external light source necessary for this technology.[8]

The AOP1 antioxidant assay is a truly unique live-cell antioxidant assay. It employs a fluorescent technology based on the controlled cytosolic production of $^1O_2$ and free radicals by a photoinduction process that can distinguish between antioxidant and cytotoxic effects in the same well. Unlike other antioxidant assays, the fluorescence sensor, while strictly correlated with oxidation status, is independent to the oxidation process and is not subject to sensor leakage. The AOP1 assay uses kinetic measurements that are normalized, making it independent of cell number. Because this live-cell technology measures the signal after the generation of a nondestructive level of free radicals, it is open to multiplexing with other assays. The assay technology works well with both adherent and suspension cells in 96- and 384-well microplates, making it amenable to HTS campaigns.

Conclusion

By using the imager lamp as an internal photoinduction light source, the Agilent BioTek Cytation 5 cell imaging multimode reader has become the first microplate reader able to fully integrate AOP1 assay in an automated "plug-and-play" configuration. This allows a simple and robust assay protocol, compatible with a HTS robotic environment.

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