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Streamlined duplex live-dead microplate assay for cultured cells

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ABSTRACT

A duplex fluorescence assay to assess the viability of cells cultured in multi-well plates is described, which can be carried out in the original culture plate using a plate reader, without exchanges of culture or assay medium, or transfer of cells or cell supernatant. The method uses freshly prepared reagents and does not rely on a proprietary, commercially supplied kit. Following experimental treatment, calcein acetoxymethyl ester (CaAM) is added to each well of cultured cells; after 30 min, the fluorescence intensity (emission λ_{max} ~ 530 nm) is measured. The signal is due to formation of calcein, which is produced from CaAM by action of esterase activity found in intact live cells. Since live cells may express plasma membrane multidrug transport proteins, especially of the ABC transporter family, the CaAM incubation is carried out in the presence of an inhibitor of this efflux process, thereby improving the dynamic range of the assay. Next, SYTOX® Orange (SO) is added to the culture wells, and, after a 30-min incubation, fluorescence intensity (emission λ_{max} ~ 590 nm) is measured again. SO is excluded from cells that have an intact plasma membrane, but penetrates dead/dying cells and can diffuse into the nucleus, where it binds to and forms a fluorescent complex with DNA. The CaAM already added to the wells causes no interference with the latter fluorescent signal. At the conclusion of the duplex assay, both live and dead cells remain in the culture wells and can be documented by digital imaging to demonstrate correlation of cellular morphology with the assay output. Two examples of the application of this method are provided, using cytotoxic compounds having different mechanisms of action.

1. Introduction

Cell culture-based high-throughput screening assays are increasingly being used to test the toxicity of compounds targeted for therapeutic use with systemic or ocular applications (Kepp et al., 2011; Narayanan et al., 2005; Tralau and Luch, 2012). Such *in vitro* preparations have also been utilized as models in basic studies designed to better understand the mechanisms of cell death underlying the pathophysiology of many disorders, including retinal degenerative and neurological diseases. Cell cultures derived from, or representative of, tissues relevant to specific diseases further provide opportunities to screen candidate therapeutic agents for their efficacy in preventing or reversing loss of vital cellular functions and integrity, before possible advancement to animal models for pre-clinical testing. Ideally, these preclinical *in vivo* studies would rely on predictive, and, ultimately, translational data generated from robust, sensitive, and repeatable *in vitro* assays with at least moderate— if not high— throughput. A multi-well plate format allows the exploitation of replicate treatments using a minimum number of cells, and also lends itself to rapid collection of multiple, quantitative data points using either a manually-operated or automated plate reader.

The stability, specificity, and sensitivity of "live-dead" assays are enhanced through the application of fluorogenic probes, whose

Abbreviations: 7kCHOL, 7-ketocholesterol; ABCB1, ATP-binding cassette sub-family B member 1 (P-glycoprotein); ABCC1, ATP-binding cassette sub-family C member 1 (Multidrug resistance-associated protein 1); ANOVA, analysis of variance; Ar, argon; BSA, bovine serum albumin; CaAM, calcein acetoxymethyl ester; di, deionized; DMSO, dimethyl sulfoxide; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; IM, incubation medium; MEBSS, modified Earle's balanced salt solution; MHBSS, modified Hanks' balanced salt solution; PORN, poly-L-ornithine; PPE, personal protective equipment; RFU, relative fluorescence units; rt, room temperature; SE, standard error; SO, SYTOX® Orange; Stsp, staurosporine; TCP, tissue culture plastic; VC, vehicle control.

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conversion to fluorescent molecules or complexes is mechanistically correlated with maintenance and/or loss of cell viability or cellular integrity (Darzynkiewicz et al., 1997). Calcein acetoxymethyl ester (CaAM; a "live" cell indicator reagent) (Bozyczko-Coyne et al., 1993) and SYTOX® Orange (SO; a "dead" cell indicator) (Johnson and Spence, 2010; Yan et al., 2000) have both been employed to assess the viability of cultured cells. Here we present a detailed description of an optimized, rapid, cell-based, direct-read, bifunctional (duplex) viability assay that combines these two methods sequentially in the same well to streamline the assay. The assay permits comparison and ranking of test agents or solutions with respect to efficacy, in statistically significant fashion, across a range of doses and incubation times. We have applied this method to two disparate ocular cell types: one a mouse retinal photoreceptor-derived cell line (661W cells) (Tan et al., 2004), and the other a glial cell line (rMC-1) derived from rat retinal Müller cells (Sarthy et al., 1998). Novel features of the protocol are its "rinse-free" aspect, as well as the inclusion of an inhibitor (probenecid) of multidrug resistance protein-1 (ABCC1) to increase the dynamic range of the CaAM assay by maintaining higher intracellular levels of its hydrolytic enzyme-cleaved product, calcein (Homolya et al., 1993).

2. Materials

The names, sources, and storage conditions for the reagents needed for the assays described in the detailed methods sections below are provided in Table 1.

Tissue culture plastic (TCP; *i.e.*, treated polystyrene) multi-well plates, with clear bottoms, may be obtained from various commercial sources. Black-walled plates are not required. Although the metrics provided below are for 48-well plates, 96-well plates can provide higher throughput with less expenditure of valuable cell lines in limited supply. In the authors' hands, the 48-well format lends itself to sharp and high contrast digital imaging using phase-contrast optics, when focusing on cells at the center of the well. If desired, larger well growth areas will tolerate some expansion of cell numbers during experimental treatment periods.

The Synergy™ HT multi-mode microplate reader used for the experiments outlined here was purchased from BioTek® (Winooski, VT). A reader equivalent to the one used for the assays described here may be substituted, with the stipulation that it can read samples in multi-well plates as described below. Ideally, the plate reader will recognize the specific manufacturer, model, and layout of the plate(s) utilized.

Adjustable manual pipettors dedicated to cell culture protocols, accommodating 1000-, 200-, and 10- or 20-μl disposable, sterile pipette tips (including wide bore 1000- and 200-μl tips), all available from various vendors, are required.

Other necessary miscellaneous equipment and supplies routinely used in cell biology laboratories are: inverted microscope equipped with phase optics, and with attached digital camera; bench top centrifuge; vortex mixer; 1.5-ml sterile Eppendorf tubes; sterile snap-cap tubes; disposable vacuum filtration unit; sterile syringes with adaptors for 0.2 μm syringe filters; hemocytometer (*e.g.*, INCYTO C-Chip™, Fisher Scientific, Suwanee, GA).

3. Detailed methods

3.1. Preparing multi-well plates for cell seeding

For cell lines of neural or glial origin, it may be desired to coat plates with attachment macromolecules, for example poly-L-ornithine (PORN) (Adler and Varon, 1980; Ge et al., 2015), that potentiate cell attachment and spreading, and help to optimize the desired morphological phenotype, such as the elaboration of neurite-like processes. Replicates of at least n = 3 should be used for each treatment data point. In designing experimental layouts, and thereby the number of plates and wells needed for cell seeding, all pertinent controls should be accounted for, including validated positive control(s) (preferably with different mechanism of action from experimental treatments), negative control(s) (*e.g.*, treatment vehicle(s) alone), and, if desired, control treatment(s) without vehicle; the latter also may be used for inter-plate controls and for normalization of data. For each multi-well plate, always include a replicate set for cell-free blanks; these wells will receive treatments equivalent to control without vehicle. To eliminate the possibility of edge effects, it may be advantageous for the outer-most wells not to be used for seeding cells. To test 6 doses of an experimental reagent, using replicates of three, the total number of wells required will be

Table 1
Assay materials.

Name of Reagent	Source	Catalogue Number	Comments
Calcein AM (CaAM)	Anaspec, Fremont, CA	89203	Supplied as 5 mM in 200 μl DMSO. Store desiccated, protected from light, at −20 °C. For source stock of 2 mM, add 300 μl DMSO, and store aliquots as above, under argon (Ar).
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Saint Louis, MO	D2650	Transfer contents of one ampoule to polypropylene snap-cap tube. Purge with Ar gas, desiccate, protect from light, store at room temperature (rt).
SYTOX® Orange (SO)	Molecular Probes, Eugene, OR	S-11368	Supplied as 5 μM in 250 μl DMSO. Store desiccated, under Ar, protected from light, at −20 °C. Harmful.
Water, sterile, cell culture grade	Sigma-Aldrich	W3500	May use equivalent.
Poly-L-Ornithine (PORN)	Sigma-Aldrich	P4957	Supplied as 0.01% (w/v) solution in water. Store refrigerated.
Sodium hydroxide	Sigma-Aldrich	S2770	Sterile 1 N solution. Store at rt. Corrosive.
Hydrochloric acid	Sigma-Aldrich	H9892	Sterile 1 N solution. Store at rt. Corrosive.
Probenecid	Sigma-Aldrich	P8761	Store solid desiccated at rt; initial stock of 45.7 mg/ml made up in 1 N NaOH and stored refrigerated (not more than 1 mo) until use.
Staurosporine (Stsp)	EMD Millipore, Billerica, MA	569396	Supplied as 1 mM solution in DMSO. Equivalent may be obtained from various sources. Store desiccated, protected from light, at −20 °C. Toxic.
Cumene hydroperoxide (CuOOH)	Sigma-Aldrich	24,750-2	Supplied as approximately 80% purity in water. Store refrigerated, tightly capped, protected from light, with air space gassed with Ar. Toxic.
Saponin	Sigma-Aldrich	S4521	Store solid at rt. Make 0.1% (w/v) solution in MEBSS based on total weight of solid supplied, not sapogenin content.