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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 $\lambda_{\text{max}} \sim 530$ 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 $\lambda_{\text{max}} \sim 590$ 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.

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Graphical abstract

Keywords

Cell viability assay; Calcein AM; Sytox Orange; Retinal cell line; Fluorescence assay; plate reader

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 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- μ l disposable, sterile pipette tips (including wide bore 1000- and 200- μ l 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-micron 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 30, including vehicle control, control without vehicle, positive control, and blank wells (the latter may be placed in outside wells if necessary).

Prepare sodium borate buffer (Rüegg and Hefti, 1984) (Table 2), and condition the TCP surface by incubating the wells with this buffer at room temperature (rt) for 30 min with gentle agitation. Wrap or cover plates if necessary to prevent excessive evaporation and to optimize aseptic handling. Aspirate borate buffer just before adding PORN solution, without an intermediate rinse.

Dilution of PORN stock: 0.01% (100 µg/ml) PORN solution is stored refrigerated. Dilute 1:4 with sterile deionized (di) water to obtain a 20 µg/ml working solution. Add to wells at 4 µg per cm² growth area (*e.g.*, 190 µl of working solution for 0.95 cm²/well in a 48-well plate). Use these metrics to calculate total volume needed. Make sure the complete growth surface has been wetted. Water can be added to outside wells or to spaces between wells to prevent evaporation and maintain volume during incubations. Seal plate with sterile sealing tape, or wrap outside securely with plastic film. Place plate(s) in refrigerator or cold room overnight (between 12 and 24 h, *without* agitation).

On the following day, aspirate PORN solution, and rinse each well briefly with 2 changes of approximately 500 µl cold sterile water. Finally, condition the plating surface of the wells with 1% (v/v) bovine calf serum (CS) (Michler et al., 1989) in a 1:1 mixture of DMEM and Ham's F-12 media (as in Table 3; without additives), 250 µl/well, under cell culture incubator conditions (*e.g.*, 36.5°C, 6% CO₂, 90–95% humidity), for 1–2 h. This medium will be aspirated completely without rinsing before addition of cells.

A possible modification, after PORN coating, would be to add laminin coating to encourage more cell-specific interactions with substrate via integrins in neural-derived cells (Ma et al., 2008). Suggested: Mouse laminin (Corning, Bedford, MA; catalog # 354232) at 2.5 µg/cm², from a freshly diluted 25 µg/ml stock solution in modified Hanks' balanced saline solution (MHBSS; Table 4), *i.e.*, 100 µl per well for a 48-well plate. Following incubation with laminin solution at 37°C in a non-CO₂ incubator, without agitation, the solution is aspirated without rinsing before addition of cells.

3.2. Seeding and treatment of cells

3.2.1. Sources of cells—661W cells were provided by Dr. Muayyad Al-Ubaidi via a Material Transfer Agreement (MTA) with the University of Oklahoma Health Sciences Center (Oklahoma City, OK, USA). The rMC-1 cells were generously provided by Dr. Vijay Sarthy (Northwestern University School of Medicine, Evanston, IL, USA). During the course of investigations described herein, both cell lines were extensively characterized genomically, and also by analysis of expressed signature genes and proteins (Pfeffer et al., 2016). Cell lines were utilized between passages 20 and 50.

3.2.2. Formulation of culture media—The formulation of the growth medium for retinal neural and glial cell lines routinely used in our laboratory (Pfeffer et al., 2016), including sources of individual components, is given in Table 3. This reduced serum medium resembles Neurobasal medium plus B-27 supplements, developed for neural cell culture in general, (Brewer et al., 1993), using a recent modification (Chen et al., 2008; this reference provides additional details for formulating stocks for most of the medium components in Table 3).

3.2.3. Seeding plates—Treat a 65–75% confluent T-75 flask of 661W or rMC-1 cells with Accutase® (Innovative Cell Technologies, San Diego, CA) (Bajpai et al., 2008) to proteolytically release cells for pelleting, resuspension, and counting. Accutase® has advantages over trypsin or microbial proteases in that it is supplied ready to use without dilution, retains activity (approximate range 500–700 U/ml) during prolonged (2 mo) refrigerated storage, and does not require specific inactivation. After rinsing the cell layer with Ca^{+2} , Mg^{+2} -free MHBSS (Table 4), add 2 ml of Accutase® solution directly from the source bottle, and incubate the cell layer at rt, with occasional swirling, for 3–5 min, until the majority of cells have detached. Monitor the progress of detachment with an inverted microscope (best using low power objectives with dark field optics, if possible). Collect cells using 10 ml MHBSS, and gently triturate to break up any remaining cell aggregates. Transfer 2×6 ml of suspension to each of 2 conical sterile plastic centrifuge tubes, and pellet the cells at $100 \times g$ for 5 min, using a refrigerated benchtop centrifuge set at 15°C .

Aspirate the supernatant medium and resuspend the pelleted cells thoroughly in a small volume (4–6 ml) of growth medium (Table 3) that has been warmed and gassed for approximately 10 min in the cell culture incubator. Using a hemocytometer (or equivalent cell counting device), determine the number of cells/ml, and from this stock cell concentration, calculate an appropriate and precise dilution volume of growth medium to obtain, preferably, in the range of 5,000–10,000 cells/well, when added in a volume of 400 μl per well (*i.e.*, from 12,500 to 25,000 cells/ml). For 30 wells of a 48-well plate, this will require 150,000–300,000 cells; but keep in mind that a slight excess volume guarantees that the last well loaded will not fall short. At this initial seeding density, cells may proliferate during the incubation period without becoming overly confluent. Use large polypropylene snap-cap tubes to make these cell dilutions.

When seeding multiple plates, it is suggested that separate snap-cap tubes with dilutions of the resuspended cells be used for each plate, again, in slightly greater volumes than mathematically required. Wide-bore pipet tips are employed to minimize mechanical shearing damage when dispensing cells. It is critical for cell counting and seeding that cells are handled with the highest degree of accuracy; this requires physical reproducibility of pipetting technique as well as repeated mixing by gentle pipetting of cells at final dilution, in their respective tubes at regular intervals during the seeding process, to avoid gradual settling of cells in the tube by gravity. Plating of cells should also be randomized; for example, if triplicate treatments are arranged by rows, seeding should proceed along non-sequential columns of the plate, in alternating directions. Even distribution of cells in the wells is also important; this may be checked immediately after seeding using the inverted microscope. Do not tip plates during cell seeding. Because of possible fan motor vibrations

in the cell culture incubator, unattached cells may collect preferentially at the center of the wells. For this reason, the wells should also be checked approximately 5 min after seeding and the plates gently agitated by hand to redistribute unattached cells. Seeding of plates should be carried out in late afternoon, so that the next steps may be carried out conveniently the next morning following an approximately 16-h attachment interval.

The next morning, 200 μ l of medium is withdrawn from each well, and slowly replaced with 200 μ l of “incubation medium” (IM; Table 5), using a wide-bore Eppendorf pipette tip to minimize turbulence, thereby, and in particular, avoiding damage to and retraction of elaborated neurites (Franze et al., 2009; Zhang and Zhang, 2010). IM is a minimal version of growth medium designed only to fulfill short term maintenance requirements. It is serum-free (reducing the CS in the treatment wells to 0.5%), and devoid of media components that have antioxidant-promoting properties which otherwise might modulate the effects of drugs and other agents affecting the cell viability studies to be carried out. This strategy allows for the introduction of isolated protective molecules in the future for screening purposes if desired.

3.2.4. Experimental treatments—Experimental treatments commence 5–6 h after the above partial medium exchange. Drugs or other agents, and also vehicle controls (VC), such as those described in detail below (Section 3.2.5), are added from 10 $\times$ working stocks in IM, at 45 μ l per well, to achieve the desired final cell treatment concentrations. It may be advantageous to use IM that has been gassed and warmed in the cell culture incubator, in a tube with a loosely-fitting snap cap, for carrying out dilutions, especially of compounds with more hydrophobic properties. Compounds are serially diluted for dose-response experiments, starting from the 10 $\times$ solution of the highest concentration to be tested. All subsequent working stocks are vortexed thoroughly to effect complete dissolution of agents before addition to the cells. Blank wells without cells, having undergone parallel surface treatments and additions of growth medium and IM (above), will now receive 45 μ l per well of vehicle-free IM.

When carrying out a dose-response study, a suggested strategy for VC treatment is to add the vehicle to IM to achieve a final concentration matching that for the highest drug concentration to be tested, in other words, resulting from the initial dilution from the source stock. The rationale is that no greater changes in cell viability would be predicted to result from lower doses of the vehicle, and that vehicle levels will remain a constant parameter through the selected dose-range of experimental agents. In the case of dimethyl sulfoxide (DMSO) as vehicle, there is consensus that viability will not be affected by concentrations seen by cells at or below 0.1% (v/v) (but it is helpful to determine the “therapeutic window” for any vehicle as well). Note that after the initial dilution from source stocks using IM, all subsequent serial dilutions of compounds will use IM containing added vehicle at this same 10 $\times$ stock concentration. (See Section 3.2.5.1 for an example.)

After all the treatments have been administered for a single plate, it should be agitated gently by hand or in a plate shaker in reproducible fashion, to ensure complete distribution of the treatment agents, before returning it to the cell culture incubator. Avoid stacking plates in the incubator. If there is concern regarding the effect of room light on the outcome of viability

studies, certainly exposure should be minimized over the course of the treatment period, which may extend up to 48 h, even to the point of loosely covering plates with aluminum foil inside the incubator.

3.2.5. Stock solutions of test compounds

3.2.5.1. Staurosporine (Stsp) (See Table 1): [CAUTION: Stsp is toxic and DMSO solutions may be absorbed through skin; use all requisite personal protective equipment (PPE; see <https://www.oshs.gov/SLTC/personalprotectiveequipment/>) and chemical safety procedures when handling, especially source stocks.] Prepare final Stsp 10× stocks just before use; aliquots of the source stock from the supplier will already have been made (Table 1). Thaw an aliquot of the source stock, and dilute 1:99 in an appropriate amount of IM at room temperature, with extensive vortexing, to make a 10 μM (10× for 1 μM) working stock (refreeze any unused portion of the source stock under appropriate storage conditions).

Using Eppendorf or small snap-cap tubes (the latter tightly capped and sealed with plastic film), prepare 10× working stocks for each Stsp dose to be tested, by making serial dilutions starting from the 10 μM preparation, using IM containing 1% (v/v) DMSO. Briefly purge the air space with high purity argon gas (Ar), seal the tube tightly, and then vortex well after each dilution. Dilute directly into experimental wells as described in Section 3.2.4, above. Note that the final DMSO concentration for all treatments will be 0.1%, so use 1% DMSO in IM for 10× VC. Any excess Stsp solutions may be aspirated into bleach before disposal.

3.2.5.2. Cumene hydroperoxide (CuOOH) (See Table 1): [CAUTION: CuOOH is toxic. Handle source bottle and stocks of CuOOH in a BSC or chemical fume hood with adequate ventilation, using all applicable chemical safety protocols, including PPE. Once final dilutions of CuOOH have been made into cell culture wells, there should be no further respiratory hazard.] Make up CuOOH stocks just before use. To formulate an initial stock for serial dilutions, add 10 μl liquid from the source bottle to a large snap-cap polypropylene tube containing 5.2 ml modified Earle's balanced salt solution (MEBSS; See Table 4). (These dilution proportions take into account the 80% purity with respect to CuOOH of the practical grade of the source reagent, and its specific gravity.) Draw the CuOOH slowly into a wide bore 100-μl pipette tip carefully, avoiding any excess reagent adhering to the exterior of the tip, and pipette up-and-down into the MEBSS repeatedly to ensure that the entire volume of the viscous material is transferred to the MEBSS, with no unmixed portion left on the pipette tip. Vortex the tube well to effect thorough mixing; check tube visually to ensure complete dissolution. The MEBSS should not be warmed, and the ensuing dilution steps may be carried out at room temperature. This initial working stock is 10 mM (Stock A).

Proceed to make serial dilutions for 10× solutions of CuOOH in MEBSS for the dose range desired; *e.g.*, dilute Stock A 1:19 in MEBSS for 500 μM (10× working stock). After all 10× working stocks are prepared (including brief vortexing), apportion immediately to wells of cultured cells. Use complete MEBSS as a nominal 10× VC. Any excess CuOOH in solution may be diluted into basal culture medium, and then aspirated into bleach solution pending disposal.

3.3. Calcein AM (CaAM) assay

3.3.1. Preparation of reagents (See Table 1)—*Before* diluting the CaAM source stock (Section 4.1.2), probenecid (4-(dipropyl-sulfamoyl) benzoic acid) is added to the MEBSS diluent. Viable cells internalize CaAM and hydrolyze it via esterases to the fluorescent, free acid product calcein. Probenecid is a competitive inhibitor of cell membrane organic ion (multidrug) efflux systems (Di Virgilio et al., 1990), and it thereby prevents rapid export of calcein, maintaining intracellular fluorescent calcein levels during the course of the incubation period. Inclusion of probenecid improved the dynamic range and reproducibility of the assay (See Results and Fig. 1A). An initial 45.7 mg/ml (160 mM) source stock solution of probenecid in 1 N NaOH (Table 1) is made; this is then diluted 40-fold to 4 mM probenecid (for 10× the desired final concentration) first by 1:38 in MEBSS, followed by the addition of 1 additional part of 1 N HCl to neutralize the added NaOH (this latter addition is carried out incrementally using $3 \times 1/3$ final volumes of 1 N HCl, with immediate vortexing following each addition). The HCl will adjust the pH of the 10× probenecid solution to a physiological range of 7.2–7.4, and addition of this stock to the culture wells did not change the phenol red indicator color of the media (not shown). MEBSS is a “high bicarbonate” physiological saline and in this case should be equilibrated (*i.e.*, gassed and warmed) in the cell culture incubator: place this probenecid stock in MEBSS in a large snap-cap polypropylene tube, loosely capped, in the incubator for approximately 20 min before using it to dilute CaAM. Any unused excess working stock of CaAM may be aspirated into a bleach solution for eventual disposal.

As CaAM is moderately light- and temperature-sensitive, and subject in aqueous solution to gradual hydrolysis over time (evident by a change in color), working stocks of this reagent are made up fresh just before addition to culture wells. CaAM is supplied as a 5 mM solution in DMSO, and, after thawing and dilution with DMSO to 2 mM, *ca.* 75- μ l aliquots from the source vial should be made, with Ar gas layered above the liquid, and stored protected from light at -20°C ; minimize repeated freeze/thaw cycles of the aliquoted material. This initial stock is diluted 1:39 in MEBSS to obtain 50 μM (10× working stock) containing probenecid if necessary. The final 5 μM concentration used in the assays described here falls in the middle of the 0.1 – 10 μM range suggested by multiple commercial suppliers of this reagent.

3.3.2. Carrying out the assay—At the conclusion of the experimental treatment period, add 50 μl CaAM working stock to each well, using efficient and reproducible pipetting technique, without removing any culture medium. Agitate the plates gently by hand before returning them to the cell culture incubator. Incubation with CaAM proceeds for 30 min, following which fluorescence readings are taken individually for each plate. Preferred excitation and emission wavelength windows of 475–495 and 520–540 nm, respectively, should accommodate the particular filters installed in the plate reader. Readings should be taken from the bottoms of wells; wipe the plate bottom gently with a disposable tissue before reading if needed. In most plate readers, preset parameters should obviate the need to remove the plate cover. Raw data are recorded as relative fluorescence units (RFU), and the gain setting should be adjusted to obtain a usable dynamic range (comparing VC with positive control or maximal experimental effect, after blank subtraction), and yielding

maximum readings for control wells between 500 and 1000 RFU, depending on cell density. Multiple plate readings may be taken to set gain and ensure repeatability (See Supplemental Data, Figure S1A). All CaAM procedures are carried out with minimal exposure to fluorescent room lights. Following CaAM assay measurements, plates are temporarily stored in the cell culture incubator awaiting the following step.

3.4. SYTOX[®] Orange (SO) assay

3.4.1. Preparation of reagent (See Table 1)—The supplied vial containing the 5 mM source stock of SYTOX[®] Orange (SO) in DMSO, obtained from the manufacturer, is stored desiccated and protected from light at -20°C , and may safely undergo repeated freeze-thaw cycles. After the vial is thawed in water and vortexed, this solution is diluted 1:500 (v/v) into IM (without additional DMSO) that has been allowed to equilibrate to room temperature, and the mixture is vortexed thoroughly to effect complete solution for a 10 μM (10 $\times$) working stock. The final 1 μM concentration selected is at the lower end of the 0.1 – 5 μM range recommended by the supplier. Minimize exposure to light for all SO operations. The working stock of SO may be made up following the CaAM assay, and any excess may be disposed of by aspiration into a bleach solution. [CAUTION: Since SO binds to nucleic acids, use PPE when handling, and in particular avoid contact with eyes and skin.]

3.4.2. Carrying out assay—As soon as possible following the CaAM assay measurements, add 55 μl of SO 10 $\times$ working stock to each well without removing any media from previous incubations (total volume per well will now be 550 μl), and agitate each experimental plate carefully by hand before returning it to the cell culture incubator. After 30 min, manually agitate each plate again briefly, and measure fluorescence in the plate reader using excitation and emission windows of 520–540 and 575–605 nm, respectively. Measurements are again taken from the bottom of the wells, using similar gain guidelines as for CaAM.

3.5. Microscopic evaluation of cells following fluorescence assays

With the inverted microscope, assess correlative morphology of cells in at least one sample well per treatment parameter, using digital microphotography. Images may be improved with respect to contrast and sharpness with the plate cover off. Establish the region of interest preferably at the center of the well for the best optical quality. Using phase objectives of 10 $\times$ or 20 $\times$ power should provide the most informative representative results. Note that some cells may have detached and may be drifting above the plane of focus of any remaining attached cells, and that attached cells may exhibit different degrees of morphological changes, compared to controls, in response to treatments (as in Fig. 2).

Any viable, attached cells remaining after treatments may still be used for additional assays (Bonnekoh et al., 1989; Kolniak and Sullivan, 2011; Scragg and Ferreira, 1991), such as crystal violet staining or an additional SO assay protocol after total permeabilization using saponin (see below).

3.6. 100% Efficacy value for SO

If applicable and desired, a “100% efficacy” value for SO may be obtained after the preceding measurement by incubating cells with saponin to permeabilize the plasma membranes of any remaining viable, attached cells (Pierce et al., 2003). Prepare a 0.1% (w/v) solution of saponin in MEBSS (Table 1); use a 50-ml sterile plastic centrifuge tube for, first, very gently vortexing the mixture to effect solution, and then centrifuging the solution to remove foaming if necessary (with a benchtop centrifuge containing a swinging bucket rotor, $1000 \times g$ for 5 min, 15°C). Finally, filter sterilize this reagent using a $0.2 \mu\text{m}$ filter to eliminate any particulates. As was the case for the other working assay solutions (above), formulate only what is needed before use, with a volume in slight excess beyond what strict calculations dictate.

Agitate plate gently by hand to suspend any unattached, presumably SO-positive cells. Transfer each full media volume containing these suspended cells to corresponding wells of a fresh 48-well plate using a wide-bore 1000- μl pipette tip, with the pipette volume set at 600 μl . Carefully replace the media in the original plate, containing any presumably attached cells, with 200 μl saponin solution per well (including blanks). Place the original plate in the cell culture incubator for 30 min. During this incubation, measure in the separate plate the SO fluorescence of the unattached cells previously harvested from wells. (This value should be close to that obtained previously from the original plate (Step 3.4.2).)

Next, make up fresh $1 \times$ SO in IM, appropriate for a final medium volume of 300 μl per well. After saponin treatment, gently pipette the saponin solution from the wells (saponin can interfere with the SO readout), taking care not to remove any cells, and, without an intervening rinse, add 300 μl /well of the fresh SO solution just made. Incubate this plate in the cell culture incubator for 30 min.

Take final reading(s) of SO fluorescence of cells remaining in the initial incubation plate. This RFU value, added to that for the unattached cells measured previously, defines the potential 100% efficacy value for the SO assay, and will be loosely proportional to total initial cell number per well.

3.7. Data analysis

RFU values will have been exported from the plate reader software into a spreadsheet such as Excel (Microsoft, Redmond, WA) for further calculations and statistical analysis. For each individual plate, subtract the mean RFU values for blank (cell-free) wells from all experimental data points before proceeding with constructing charts and further data analysis. (Table 6 shows typical background (*i.e.*, experimental blank) fluorescence values obtained for the studies reported here, expressed as percentage of the uncorrected maximal RFU values for each assay.) RFU values may be normalized, in particular for inter-plate comparisons, by dividing each RFU value by the mean for VC treatments (or the mean for control without vehicle when implemented). For constructing charts, select the value [$1.96 \times$ standard error (SE)] for error bars (as in Figs. 1 and 3).

Z-factors were computed according to the formula provided in Zhang et al. (1999). One-way analysis of variance (ANOVA) calculations were made using SPSS Statistics (IBM, Armonk, NY).

3.8. Representative Results

3.8.1. 661W cells treated with Stsp—Stsp is a non-specific protein kinase inhibitor that has been shown to be cytotoxic to a wide range of cell types; in response to appropriate concentrations of Stsp, cell lines as well as more acute preparations of normal diploid cells, including neurons, undergo apoptosis within approximately 24 h (Ashutosh et al., 2012; Seo and Seo, 2009). We previously showed that in experiments using *subconfluent* 661W cells, 40 – 50 nM Stsp was sufficient for a maximal loss of viability, as measured by several assays, including CaAM and SO (Pfeffer et al., 2016). After passive internalization of hydrophobic CaAM through the plasma membrane, viable cells promote the esterase-mediated hydrolysis of CaAM to its fluorescent, more polar and membrane-impermeable product, calcein (Bozyczko-Coyne et al., 1993). For a standard starting concentration of CaAM, therefore, and for a defined incubation period, the maximum readout value in this assay is a relative measure of the number (or percentage) per well of viable cells under control conditions, decreasing in proportion to the number of nonviable cells generated as a result of treatment with cytotoxic test compounds. Dead cells would be presumed to produce less functional esterase activity while also having compromised membrane integrity, leading to less calcein being generated per cell, as well as to diffusion, dissipation and degradation/quenching of calcein in the extracellular media, with a net reduction in fluorescent signal per affected well. Here we exposed confluent 661W cells to Stsp in doses ranging from 2.5 to 1000 nM for 24 h, after which the cells underwent the sequential assays described above, and finally the cultures were viewed with the inverted microscope using phase-contrast optics.

The CaAM incubations were carried out with or without added probenecid. The assay results indicated that the addition of this putative multidrug transport inhibitor increased the dynamic range of the assay by over two-fold compared to the probenecid-free assay condition, judging from the comparative values for the DMSO VC, on the one hand, and the treatments with Stsp (Fig. 1). When probenecid was included in the CaAM-containing assay medium (black bars in Fig. 1), relative fluorescence values (RFU) were reduced in statistically significant fashion at a Stsp concentration threshold of 125 nM and above, compared to treatment with VC medium. In contrast, when the assay was executed without added probenecid (gray bars in Fig. 1), only the value for the 1000 nM Stsp treatment showed a statistically significant reduction compared to VC, although mean values for 125, 250, and 500 nM Stsp were still below the mean for VC when probenecid was absent. It also should be noted that for doses of Stsp between 2.5 and 50 nM, there were statistically significantly lower values for the CaAM assay run without probenecid compared to the matching Stsp dose treatments for which the CaAM assay included probenecid. The Z-factor, a quantitative measure of the dynamic range and hence the utility of an assay (Zhang et al., 1999), computed using the RFU values for 500 nM Stsp compared to those for VC, was 0.657 for the results with probenecid vs. 0.146 without probenecid; a score between 0.5 and 1.0 is in the desired range.

The SYTOX[®] Orange (SO) assay depends on the loss of cell viability manifested by compromised plasma membrane integrity following experimental challenge, allowing this probe to diffuse to cell nuclei and form tightly binding, fluorescent complexes with double-stranded DNA (Pierce et al., 2003; Yan et al., 2000). Depending on the cell death pathway(s) invoked, the magnitude and direction of the response in the SO assay may resemble a mirror image of what is observed in the CaAM assay, and the dynamic range should be dependent on a maximum RFU value attained when essentially all the cells in a well are dead. When the SO assay was carried out subsequent to the CaAM assay, regardless of whether probenecid was included or not, as described above, statistically significant toxicity was apparent beginning with the threshold dose of 25 nM Stsp, therefore showing higher sensitivity to Stsp-induced changes than what was detected in the preceding assay (Fig. 1B). Interestingly, after reaching peak RFU at 125 nM Stsp, mean RFU values declined in the SO assay for increasing doses of Stsp, to the point where at 1000 nM, the SO fluorescence was only approximately 50% compared to its magnitude detected at 125 nM Stsp (see Discussion). For any single Stsp concentration, as well as VC treatments, there were no significant differences for SO assay values between samples that either had included or were without probenecid during the CaAM assay. The Z-factors calculated for the SO assay for samples that had undergone incubations with 500 nM Stsp were 0.799 and 0.700, when the CaAM assay media were with and without probenecid, respectively.

Following the SO assay, digital images were taken of cells from representative wells for all treatment conditions, *viz.* for treatments with VC and the full dose range of Stsp, and including samples where probenecid was both present or not in the CaAM assay medium (Fig. 2). Because treatments were begun when 661W cells had reached confluence, and also since the immortalization of this cell line permits proliferation even of post-confluent cells, some overgrowth of viable cells is apparent not only in VC-treated cultures, but also at the lowest, non-cytotoxic concentrations of Stsp (*e.g.*, 5 nM) employed for 24-h incubations before the assays commenced (Figs. 2A–2D). In general, Stsp treatment led to several observable effects on morphology of the cultured 661W cells, progressing in severity with higher doses, which were not seen in the VC-treated wells, namely rounding up of cells, membrane blebbing, contraction of clusters of cells, and detachment of cells from the substrate (Fig. 2). In parallel with the dose-responsive changes in viability as detected quantitatively in the CaAM assay, the higher the concentration of Stsp, the greater was the prevalence of morphological changes and features marking loss of viability, compared to VC. This same correlative trend held true in the SO assay only for doses of Stsp from 2.5 up to 125 nM; thereafter, as mentioned above, the magnitude of the SO fluorescence signals tapered off while morphological indications of loss of viability continued towards maximal expression at 1000 nM Stsp (Fig. 2). The inclusion of probenecid during the CaAM assay incubation did not change the morphology of the cultures compared with matching samples, receiving equivalent Stsp doses, that were not exposed to probenecid (compare, for example Figs. 2A and 2G with 2B and 2H, respectively).

3.8.2. rMC-1 cells treated with cumene hydroperoxide (CuOOH)—Figs. 3A and 3B depict the results of the CaAM and SO assays, respectively, that were used to assess the effects of a 24-h incubation with cumene hydroperoxide (CuOOH) on subconfluent cultures

of rMC-1 cells in 48-well plates. In this experiment all CaAM solutions contained probenecid. CuOOH induces oxidative stress by generating reactive oxygen species, and is more selective than hydrogen peroxide in terms of its cellular site of action and the antioxidant systems it invokes (Liddell et al., 2006; Sawada et al., 1996). Since the working stock solution of CuOOH (10× the desired final concentration) was made up using a modified Earle's balanced salt solution (MEBSS), the latter salt solution was added proportionally to IM to formulate the VC for this experiment. The dose range of CuOOH utilized was from 10 to 50 μ M, and statistically significant decreases in viability were documented for concentrations of 20 μ M and higher in both assays (Figs. 3A,B). At the highest dose of CuOOH tested, the apparent loss of viability was virtually 100% by the CaAM assay (Fig. 3A), and correlated with an increase of signal in the SO assay by approximately 13-fold over the VC reading (Fig. 3B). In the case of this experiment, there was no tapering-off of SO fluorescence at the most toxic dose of CuOOH, suggesting different molecular pathways involved in cell death (see Discussion, below).

4. Discussion

Numerous “live-dead” assay protocols have been published and also have been disseminated through the use of commercially available kits (Farfan et al., 2004; Giordano et al., 2011; Kepp et al., 2011; Yang et al., 2002). In our estimation, the duplex viability assay reported here is novel and compares favorably to the preceding and existing methodologies, by virtue of the following features:

Low serum media

The serum supplementation of the growth medium and IM (in our case, with bovine calf serum) is limited to 1% (v/v) or less, with the otherwise routinely employed amount of serum being replaced by mostly defined components commonly used for formulating more complex media (see Tables 3 and 5). Reduced serum usage, first, minimizes any possible confounding effects of this reagent, when present at 5–10%, on the pharmacodynamics of test compounds (such as sequestration of molecules), or the responses of cells to these molecules (such as lack of precise control over endogenous levels of undefined antioxidants and other protective factors); second, the potential adverse effects of acute serum deprivation itself, such as what the cultured cells would encounter when switching from growth medium with relatively high serum to experimental, largely unsupplemented IM, may compromise cell viability (Asada et al., 2008; Voccoli et al., 2007), or otherwise interfere with experimental results (Pirkmajer and Chibalin, 2011).

Furthermore, the IM described here, to which test compounds were added, followed by direct addition of CaAM and SO, is a simplified medium also containing a minimal amount of serum as the only undefined component, and is devoid of added protective molecules from the original growth medium, such as antioxidants (*e.g.*, reduced glutathione, vitamin E acetate), or nutrients that are part of cellular antioxidant systems (such as selenite). These features of IM permit the eventual testing of individual modulators of the observed response, to evaluate compounds such as antioxidants and other cellular protective agents in a defined,

dose-responsive manner. Serum can also be a source of DNase that could interfere with assays involving compounds such as SO that bind to DNA (Zhou et al., 2011).

Although serum is a possible source of carboxyesterase, and also contains serum albumin, with the potential to generate nonspecific background signal from calcein over time (Kragh-Hansen, 2013; Redinbo and Potter, 2005), the small percentage of CS present in the wells is apparently not enough to interfere with the CaAM assay during the elapsed time for executing the current protocol (Supplemental Data and Figure S1). However, these esterase activities are an important consideration for assaying cells cultivated with serum concentrations above 1% (see *Potential pitfalls and troubleshooting*, below).

Robustness of assay fluorophores and fluorescence signal

Multiple measurements of calcein fluorescence may be made for validation of repeatability and gain adjustment for optimizing dynamic range, certainly within 10 min of the initial determination immediately following the 30-min incubation period for this arm of the assay. The intracellular calcein signal is stable and does not degrade rapidly during this short time period; furthermore, because of the low concentration of CS employed, there is no significant increase in background fluorescence resulting from CaAM hydrolysis by esterase activity(ies) present in CS (Supplemental Data and Figure S1). Since SO only becomes fluorescent when bound to DNA in the nucleus of permeable cells (Johnson and Spence, 2010), there is a possibility of drift over an extended time period if additional cells respond further to the experimental agents remaining in the wells with loss of membrane integrity; in our hands the SO signal was stable during a post-incubation period of at least 10 min (results not shown), but this time window should be validated for each culture model.

The viability measurements presented here constitute a streamlined, “one pot” procedure, without the need to detach, wash, or transfer cells, or to change medium. Therefore, all cells originally in each well at the beginning of experimental treatments are accessible to the two fluorogenic compounds. The two probes utilized in this protocol, namely CaAM and SO, display a high fluorescence efficiency and show excellent separation of their excitation and emission maxima to preclude overlap and interference—as long as they are administered and measured sequentially. It was found that directly combining the two probes interfered with interpretation of the CaAM assay—but not the SO assay (Supplemental Data and Figure S2); in theory this would be due to the emission maximum for calcein being well within the absorbance/excitation range for either free or bound SO. Such spectral overlap has been observed previously for multiplex cell viability assays (Boutonnat et al., 1999). The phenol red contained in the basal medium used for culturing the cell lines described here also did not interfere with the fluorescence detection.

The two assay fluorophores used here are not cytotoxic under the experimental conditions employed, and therefore any remaining viable and attached cells are candidates for follow-on assays, either qualitative or quantitative, such as image analysis, determinations of cell number, cell-based ELISA, or even protein and transcript analysis.

Inhibition of calcein efflux

An inhibitor of multidrug resistance transport (in our case, probenecid, a competitive inhibitor of the ABCC1 (MRP1) transporter) (Di Virgilio et al., 1990) is included in the CaAM incubation, effectively increasing the dynamic range of this arm of the protocol by preventing loss of fluorescent signal from viable cells. We recently detected moderate levels of transcripts for ABCC1 in 661W cells by gene array analysis; interestingly, there was no significant *differential* expression for ABCC1 in cells treated with a toxic dose of 7-ketocholesterol (7kCHOL) compared to control samples (results not shown; Pfeffer et al., manuscript in preparation). Only a few instances of *in vivo* and *in vitro* expression of ABCC1 in neurons have been reported, including demonstration of functional activity in the mouse Neuro-2a neuroblastoma cell line (Meszaros et al., 2011). Furthermore, this transporter is expressed in virtually every eye tissue examined thus far, including cornea, lens, iris, ciliary body, and the inner and outer blood-retinal barriers (*e.g.*, Chen et al., 2013); an illustrative example of probenecid-sensitive calcein efflux via ABCC1 is provided by choroidal melanoma-derived cell lines (van der Pol et al., 1997). Finally, in the aftermath of experimental treatments affecting cell viability, probenecid may prevent further loss of glutathione and depletion of ATP, by inhibiting ABCC1 energy-dependent efflux activity (Bakos et al., 2000; Hammond et al., 2007), effectively serving as a “stop reagent.” In light of these findings, it is recommended that probenecid be tested for empirical improvement in any new CaAM viability assay’s dynamic range. The probenecid concentration we successfully employed, 400 μ M, was much lower than what is recommended in most commercial product brochures for the same purpose (1 – 2.5 mM), and did not contribute to loss of viability in our cultures. Besides probenecid, other inhibitors of multidrug resistance (*e.g.*, verapamil, an inhibitor of ABCB1 (P-glycoprotein) (Ishikawa et al., 2010)), also can be exploited to validate the assay and to fit the specific properties of the cell systems being manipulated. Probenecid was included in the CaAM assay medium used for the studies using rMC-1 cells (Figure 3), and we did not investigate the comparative results without probenecid for this cell line. While no reports exist in the literature specifically identifying ABCC1 or ABCB1 in Müller cells, expression of these transporters has been recently demonstrated in brain astrocytes (Bendayan, et al., 2006; Berezowski, et al., 2004).

Data acquisition

Quantitative results for the viability assays reported here may be rapidly recorded without the need for further processing, as required for image analysis or cell sorting. The measurements entail use of a sensitive, dedicated plate reader that is versatile with respect to plate geometry, reading orientation, and gain setting.

Potential pitfalls and troubleshooting

Investigators should acknowledge the gentle reminder that several technical aspects of this protocol are critical for its reproducibility, for optimization of coefficients of variation and Z factors, and hence for attaining statistical significance:

1. Pipetting accuracy is fundamental for several steps, including formulating stock solutions, dilution of cells after counting, cell plating, serial dilutions of compounds, and addition of reagents to wells.

2. In like manner, by application of principles and techniques described in 1), above, repeatable results using hemocytometers or automated cell counters should be expected.
3. Complete mixing via vortexing or agitation by hand after each step is imperative when making up initial reagent stocks or dilutions of these. Note that DMSO is viscous and has different thawing properties compared with ice; stock solutions in DMSO need to be thawed completely (usually in clean, di water at rt) and vortexed vigorously.
4. Along similar lines, strategies for randomization of wells for seeding cells in each plate should be systematized to avoid artefactual differences in results between and among replicates.

Investigators are encouraged to undertake a pilot study, if necessary, to confirm the optimal concentration range of CaAM and SO used for their assays. The range limits are theoretically defined at the low end by the absolute amounts needed to saturate all potential determinants, and at the high end by how much background (signal to noise ratio; *e.g.*, compare Figs. 1 and 3 with Table 6) is acceptable (and of course partly by economy once the targets are saturated). Some attention and care must be given to storage and handling of DMSO solutions of CaAM, as this reagent is not inexpensive and, as mentioned above, is sensitive to light, temperature, and moisture.

The duplex cell viability assay delineated here is a “one-pot” protocol, and since serum levels in IM above 1% generate significant background fluorescence for the CaAM assay (Supplemental Data and Figure S1), the application of our methodology may be limited to cells cultivated using media with defined supplementation, to either completely replace serum or reduce this component to low levels. Cultures maintained with routinely used serum levels of 5–10% are not predicted to adapt well to an acute change to the IM formulation provided here for carrying out the CaAM and SO assays (see, *e.g.*, Pirkmajer and Chibalin, 2011).

The CaAM and SO assays in and of themselves are not specific enough to provide direct information regarding the molecular mechanisms or signaling pathways underlying the observed loss of cell viability. It may be expected that after experimental treatments *in vitro*, an initial classically apoptotic cascade may eventually lead to an end stage with more of the characteristics of standard necrosis (Niemczyk et al., 2004), including loss of membrane integrity (Silva, 2010). The methods outlined here are amenable, however, to the inclusion of inhibitors, antioxidants, and other protective reagents before, during, or after exposure to treatments that generate mechanisms leading to various modes of cell death. Additionally, a time course experiment could conceivably isolate different steps in the cell death process; this strategy is quite straightforward to design using the CaAM and SO reagents, such as was carried out previously for the cytotoxic oxysterol 7kCHOL (Pfeffer et al., 2016). In this latter example, although the major phase of measurable loss of cell viability occurred by 6 h of treatment with a single concentration of 7kCHOL, routine investigations used a 24-h endpoint.

The images of cells after completion of the viability assays (Fig. 2) suggest that one aspect of viability reduction brought about by higher concentrations of cytotoxic agents such as Stsp could also involve slower proliferation of treated cells, in addition to loss of existing cells; this outcome underscores the importance of precise cell counting and seeding, as emphasized above. In some instances more meaningful results might be attained by normalizing the data obtained from the CaAM assay to cell number, for example, by using the crystal violet assay (Bonnekoh et al., 1989; Pfeffer et al., 2016; Scragg and Ferreira, 1991). Normalization to cell number would not be a constructive strategy for quantifying results in the SO assay, however, because there may be fewer cells resulting from treatments that decrease viability, thereby engendering artificially elevated readouts in this case.

The decrease in the mean SO fluorescence signal at the three highest Stsp doses, compared to 125 nM (Fig. 1B), was due to neither inherent instability of the SO:DNA complex in the nuclei of permeabilized cells, a discrepancy in cell numbers, nor a gain in cell viability; these alternatives may be ruled out by consideration of the matching results of the CaAM assay (Fig. 1A) and by the microscopic appearance of the cultures at these concentrations (Fig. 2). A similar decrease in SO signal was observed previously in a demonstration of the time-dependence of cytotoxicity of a single concentration of the toxic oxysterol 7kCHOL over time (supplementary Figures 8 and 9 in Pfeffer et al. (2016)), suggesting that there is a further progression of cell death processes causing a diminution of signal by 24 h when the highest, most toxic doses of Stsp were employed, followed by probing with SO. A likely scenario is loss of integrity of nuclear components, owing to activation of nucleases, such that DNA is cleaved to small double-stranded fragments that bind SO with less efficiency, or even to single-stranded fragments, which do not bind SO at all (Frankfurt et al., 1996; Oshige et al., 2011). Therefore, the dose-response and time parameters should be established for the assay (certainly as pertains to SO, but ideally for CaAM as well) at the outset of such investigations of cell viability.

The observation that CuOOH treatment of rMC-1 cells did not give rise to a similar falling off of SO signal may reflect either a difference from Stsp in the specific molecular cell death program(s) evoked by CuOOH, a mechanistic difference in cell death pathways between the two cell lines, or both. Such eventualities may make it difficult to carry out cross-comparisons between cell types and experimental treatments. On the other hand, these possibilities represent examples of how the assay protocol delineated here may be exploited to further elucidate mechanistic details, by inclusion of modulators of molecular and functional steps corresponding to specific pathways of cellular death and survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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	N-(2-Hydroxyethyl)piperazine-N'-(2-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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Figure 1. Confluent 661W cells incubated with dose range of staurosporine (Stsp)
Two 48-well plates of confluent 661W cells were incubated for 24 h with a concentration range of Stsp from 2.5 to 1000 nM, or DMSO alone as VC, and viability of the cells then was assessed by the sequential application of the CaAM and SO assays. In one plate probenecid was added to the CaAM assay medium. **A: CaAM assay performed with and without probenecid.** Without probenecid (gray bars), the maximum fluorescence signal, expressed as relative fluorescence units (RFU) achieved was *ca.* 2500 for VC, and the dynamic range between VC and the most toxic dose of Stsp (1000 nM) only amounted to a

61.8% decrease in RFU. In contrast, the inclusion of probenecid apparently conserved the intracellular calcein signal, resulting in a maximum mean value of *ca.* 7000 RFU for VC, and extending the dynamic range to a decrease of 82.4% as a result of incubation with 1000 nM Stsp. *P 0.05 *vs.* VC for the CaAM assay with probenecid; §P 0.05 *vs.* VC for only one concentration of Stsp (1000 nM) for the CaAM assay run without probenecid; #P 0.05 for the CaAM assay RFU values in the absence of probenecid *vs.* the matching dose of Stsp when probenecid was present. **B: Lack of dependency of the SO assay on the presence of probenecid.** The SO assay values for the identical wells measured for Fig. 1A, showing no significant differences between SO assay results for matching doses of Stsp regardless of the presence of probenecid (remaining in the wells after the CaAM assay was performed). From 2.5 to 125 nM Stsp there was increasing toxicity as determined by this assay; however, at Stsp concentrations from 250 to 1000 nM there was a drop-off in RFU signal (see **Results** text, also **Discussion**). *P 0.05 *vs.* VC values for SO assays run both with and without probenecid *vs.* matching VC plus or minus probenecid. Error bars are [$1.96 \times$ standard error (SE)].

Figure 2. Correlation of quantitative assay results with morphology of 661W cells treated with Stsp

Selected phase-contrast images of cells following the assays described in Fig. 1, treated as follows: **A,C,E,G,I, and K**: CaAM assay included probenecid; **B,D,F,H,J, and L**: CaAM assay without probenecid. **A,B**: VC; **C,D**: Stsp at 5 nM; **E,F**: Stsp at 25 nM; **G,H**: Stsp at 125 nM; **I,J**: Stsp at 500 nM; **K,L**: Stsp at 1000 nM. As the Stsp dose increased, the following changes were noted in the appearance of the cultured cells, and with good correlation with the results of the viability assays depicted in Fig. 1: Rounding up of cells

(*arrows* in **E,F**); Membrane blebbing (*arrows* in **G,H**); Contracted aggregates of cells (*arrows* in **I,J**); detachment and loss of cells from the substrate (*arrows* in **K,L**); Cell shrinkage and collapse of contents, resulting in small phase-bright spheres (*arrowheads* in **I,J,K,L**). Cells that underwent either VC or 5 nM Stsp treatment did not exhibit changes indicative of loss of viability, and did show areas of cell overgrowth, suggesting continued proliferation during the course of the experiment (*short arrows* in **A,B,C,D**). Magnification bar = 100 μm ; **insets**: 2 $\times$ magnification of main image; all images for each treatment were captured with a 10 $\times$ objective lens with phase-contrast optics.

Figure 3. Effect of cumene hydroperoxide on subconfluent rMC-1 cells (a rat Müller cell line) CaAM and SO assays carried out on rMC-1 cells following incubation with CuOOH, in a dose range of 10 to 50 μM , plus VC (the last using MEBSS diluent alone). **A:** CaAM assay shows dose-response to increasing concentrations of CuOOH, with almost complete abrogation of calcein fluorescence signal at the highest dose tested. RFU values were normalized to the mean RFU (4833 ± 652) for VC. * $P < 0.05$ vs. VC. **B:** The SO assay was applied immediately following the CaAM assay, resulting in a near mirror-image pattern of signal through the dose range of CuOOH. RFU values were normalized to the mean RFU for

VC, and the maximal mean response (for 50 μM CuOOH) was $15,165 \pm 1380$. Statistically significant differences *vs.* VC (*P 0.05) were achieved at CuOOH concentrations of 20 μM and above. Error bars in both charts are [$1.96 \times \text{SE}$].

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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.

Table 2

Sodium borate buffer

150 mM Sodium Borate-saline buffer, for diluting poly-L-ornithine solution		
Chemical	g/500 ml	Catalogue number (Sigma-Aldrich)
Boric acid (H_3BO_3)	4.05	B6768
Sodium tetraborate, anhydrous (Borax; $\text{Na}_2\text{B}_4\text{O}_7$)	3.6	221732
Sodium chloride (NaCl)	2.2	S5886

1. Add solid reagents to ~450 ml deionized (di) water; stir at room temperature to effect solution.
2. Adjust pH to 8.4 – 8.5, if necessary, with 0.1 N NaOH or 0.1 N HCl; then bring final volume to 500 ml with di water.
3. Filter-sterilize using vacuum, store indefinitely in refrigerator.
4. Handle using sterile technique in biosafety cabinet.

Table 3

Growth medium for retinal cell lines *

Name	Source	Catalogue number	Concentration (or proportional volume) per liter in final medium [#]	Comments
DMEM with HEPES	Sigma-Aldrich, Saint Louis, MO	D6171	460 ml	High glucose formulation
Ham's F-12 with HEPES	Sigma-Aldrich	N8641	450 ml	
TAPSO	Sigma-Aldrich	T9269	1 g	Supplemental pH buffering component
HCl	Sigma-Aldrich	H9892	2 ml	Supplied as sterile 1 N solution
Bovine calf serum	HyClone, Logan, UT	SH30073.03	2 ml	Filter-sterilize thawed aliquots and store refrigerated not more than 2 wk
Vitamin E acetate	Sigma-Aldrich	T3001	1 mg	From ethanol stock
Cholesterol	Sigma-Aldrich	C8667	2 mg	From ethanol stock
Bovine retina extract	Animal Technologies, Tyler, TX (retinas)	(N/A)	2.5 ml	Refer to Pfeffer (1990) for details of preparation; final amount of added protein approximately 6 mg/L; thawed aliquots stored refrigerated not more than 1 wk
Linoleic acid (complexed with bovine serum albumin (BSA))	Cayman Chemical, Ann Arbor, MI; (Sigma-Aldrich)	90150 (A8806)	1 mg (120 mg)	Linoleic acid stored under Ar at −20°C; (Complex stored under Ar at −80°C)
Transferrin	Sigma-Aldrich	T8158	7.5 mg	Solid and stock aliquots both stored at −20°C
Long® R ³ IGF-1	Sigma-Aldrich	I1271	10 µg	
Oxalacetic acid	Sigma-Aldrich	O7753	66 mg	
Sodium pyruvate	Sigma-Aldrich	S8636	5 ml	Supplemental; supplied as sterile 100 mM solution; store refrigerated
Sodium selenite	Sigma-Aldrich	S9133	0.173 µg	
MnCl ₂ •4H ₂ O	Sigma-Aldrich	M3634	0.9 µg	
(NH ₄) ₆ Mo ₇ O ₂₄ •4H ₂ O	Sigma-Aldrich	M1019	1.24 µg	
Glucose	Sigma-Aldrich	G7021	375 mg	Supplemental; stock formulated together with the next four components (below)
Fructose	Sigma-Aldrich	F3510	375 mg	
D-(+)-Gluconic acid lactone	Sigma-Aldrich	G4750	10 mg	
D-Glucuronic acid, sodium salt monohydrate	Sigma-Aldrich	G8645	1 mg	
D-(+)-Glucosamine HCl	Sigma-Aldrich	G1514	5 mg	
D-(+)-Galactose	Sigma-Aldrich	G0750	180 mg	Stock formulated with the next two components (below)
L-(−)-Fucose	Sigma-Aldrich	F2252	82 mg	
β-Lactose	Sigma-Aldrich	L3750	750 mg	

Name	Source	Catalogue number	Concentration (or proportional volume) per liter in final medium [#]	Comments
Non-essential amino acids	Sigma-Aldrich	M7145	5 ml	Supplement supplied as sterile solution; refrigerate
Taurine	Sigma-Aldrich	T8691	1.25 mg	
Heparin, sodium salt	Sigma-Aldrich	H3149	1 mg	Not less than 175 U per mg solid
Alanyl-glutamine	Sigma-Aldrich	A8185	868 mg	
Uridine	Sigma-Aldrich	U3003	0.3 mg	
Thymidine	Santa Cruz Biotechnology, Dallas, TX	296542	1 mg	Supplemental
L-Carnitine tartrate	LKT Laboratories, St. Paul, MN	C0264	2.35 mg	
Thiamine hydrochloride	Santa Cruz Biotechnology	205859	4 mg	Supplemental
Ethanolamine	Sigma-Aldrich	E0135	0.5 mg	Viscous liquid stored at rt
Glutathione SH	Sigma-Aldrich	G6529	2 mg	
Hydrocortisone	Sigma-Aldrich	H0396	6 µg	Complexed with cyclodextrin; amount given with respect to hydrocortisone
Progesterone	Sigma-Aldrich	P7556	35 µg	Complexed with cyclodextrin; amount given with respect to progesterone
Retinyl acetate	Sigma-Aldrich	R0635	0.2 mg	Complexed with cyclodextrin; amount given with respect to retinyl acetate; protect from light
Triiodothyronine	Sigma-Aldrich	T6397	3.5 µg	
Ascorbic acid phosphate	Wako Chemicals, Richmond, VA	013-19641	36 mg	
Putrescine	Sigma-Aldrich	P5780	0.8 mg	Supplemental
Thioglycerol	Sigma-Aldrich	M6145	5.4 mg	Supplied as liquid; store refrigerated and tightly sealed

* Use with pCO₂ = 6%

[#] All components expressed by weight are added from concentrated stock solutions

Table 4

Balanced Saline Solutions*

Component	Source	Catalogue Number	mmol/L	Modified Hanks' Balanced Saline	mmol/L	Modified Earle's Balanced Saline	Comments
NaCl	Sigma-Aldrich, Saint Louis, MO	S5886	138.5		126.5		May be prepared as combined 20x monovalent stock with KCl and sodium dihydrogen phosphate
KCl	Sigma-Aldrich	P5405	3.5		3.5		
NaH ₂ PO ₄ •H ₂ O	Sigma-Aldrich	71507	1		1		
CaCl ₂ •2H ₂ O	Sigma-Aldrich	C7902	0.5		0.5		Calcium and magnesium salts may be prepared as a combined 20x divalent stock [†]
MgCl ₂ •6H ₂ O	Sigma-Aldrich	M2393	0.27		0.27		
MgSO ₄ •7H ₂ O	Sigma-Aldrich	63138	0.37		0.37		
HEPES ²	Sigma-Aldrich	H0887	15		15		Supplied as 1 M sterile solution; store refrigerated, protected from light
NaHCO ₃	Sigma-Aldrich	S5761	4.2		15		
D-Glucose	Sigma-Aldrich	G7021	4.15		4.15		
D-Fructose	Sigma-Aldrich	F3510	4.15		4.15		

* Adjusted pH = 7.2 – 7.4, approximately 300 mOsm; filter sterilize and store refrigerated

[†] Leave out calcium and magnesium salts for Ca⁺², Mg⁺²-free balanced salt solutions

² 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

Table 5

Incubation medium (IM) for experimental treatment of retinal cell lines *

Component	Catalogue number (all Sigma-Aldrich)	Concentration (or proportional volume) per 100 ml final volume	Comments
DMEM with HEPES	D6171	47.5 ml	High glucose formulation
Ham's F-12 with HEPES	N8641	47.5 ml	
TAPSO	T9269	100 mg	Solid stored at room temperature (rt)
Hydrochloric acid	H9892	0.2 ml	Supplied as sterile 1 N solution; store at rt
Transferrin	T8158	0.75 mg	Solid and stock aliquots both stored at -20°C
Sodium pyruvate	S8636	1 ml	Supplied as sterile 100 mM solution; refrigerate
Non-essential amino acids	M7145	0.5 ml	Supplied as sterile solution; refrigerate
Glucose	G7021	37.5 mg	Supplemental
Fructose	F3510	37.5 mg	
Alanyl-glutamine	A8185	43.4 mg	Solid stored at 4°C; Stock aliquots stored at -20°C
Triiodothyronine	T6397	0.35 µg	Solid stored at -20°C; Stock aliquots stored refrigerated
Hydrocortisone	H0396	0.6 µg	Supplied in powder form as soluble complex with hydroxypropyl-β-cyclodextrin; amount given with respect to hydrocortisone; store powder desiccated at -20°C

* Use with pCO₂ = 6%

Table 6

Background fluorescence values for CaAM and SO assays

Cell type, treatment	Assay	Cell-free blank percent of maximum*
661W, Stsp	CaAM	11.8
661W, Stsp	SO	3.9
rMC-1, CuOOH	CaAM	21.0
rMC-1, CuOOH	SO	4.5

* Maximum RFU value in CaAM assay was for vehicle control; for SO assay, maximal value was attained using highest treatment concentration.