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Protocol Article

Cell-based assays to identify novel retinoprotective agents☆

Jeanee Bullock[a,b], Glorivee Pagan-Mercado[a], S. Patricia Becerra[a,*]

[a] *Section on Protein Structure and Function, LRCMB, National Eye Institute, National Institutes of Health, Bethesda, MD, USA*
[b] *Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington, DC, USA*

A B S T R A C T

Degeneration of the retina can lead ultimately to devastating irreversible vision loss, such as in inherited retinitis pigmentosa and age-related macular degeneration. Currently there is no cure to prevent retinal degeneration. Quantitative cell-based assays can be used to test potential drugs that prevent the death of retinal cells. Here, we describe in detail three semi-automated cell-based protocols to identify retinoprotective factors with two retinal cell lines, rat R28 cells and mouse 661W cells. In these protocols, cells are induced to undergo death by photo-oxidation stress, growth factor depletion or cytotoxicity with sodium iodate. Pigment epithelium-derived factor, an established neurotrophic factor for retinal cells, was used as a positive control. We discuss how these protocols will prove useful in high-throughput quantitative screening to identify novel therapeutics for retinal disorders.

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A R T I C L E I N F O
Protocol name: Quantitative cell-based assays to evaluate retinoprotective activity
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* Corresponding author.
E-mail address: becerras@nei.nih.gov (S.P. Becerra).

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Specifications Table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Cell Biology
Protocol name:	Quantitative cell-based assays to evaluate retinoprotective activity
Reagents/tools:	Listed in each section of the protocol
Experimental design:	The methods are for three quantitative cell-based assays for retinoprotective factors. Retinal cells are seeded onto 96-well plates and induced to undergo cell death. Viability of serum-starved R28 cells is assessed by automated live cell imaging. Viability of 661W cells exposed to oxidative stress with damaging light is determined by quantitation of intracellular ATP, an indicator of metabolically active cells. Cytotoxicity of 661W cells exposed to oxidative stress with sodium iodate is determined by quantitation of extracellular lactate dehydrogenase release from damaged cells.
Trial registration:	N/A
Ethics:	N/A
Value of the Protocol:	• The three methods describe quantitative cell-based assays to identify retinoprotective factors. • All methods are adaptable for scaling up to 384 well plates for high-throughput screening.

Description of protocol:

Background

Millions of people suffer from vision impairment caused by retinal degeneration disorders, such as retinitis pigmentosa and age-related macular degeneration [1,2]. Photoreceptors are a type of retinal cells that are responsible for converting light energy to chemical signals at the beginning of the visual process. Dysfunctional and dying photoreceptors can lead to the loss of central or peripheral vision and degeneration to irreversible blindness [3]. To date, there is no cure for retinal degeneration and current approaches for therapy aim to delay the death of photoreceptors. Thus, reliable assays to screen prospective drugs *in vitro* are needed to identify novel therapeutics for retinal disorders. We propose to develop cell-based assays relevant to high-throughput screening for the discovery of drugs that promote retinal survival. The lead candidates from the screening would then become available for preclinical studies with animal models of retinal degeneration.

Here, we outline three semi-automated cell-based screening methods to assay prospective retinoprotective molecules (see Graphical Abstract). These methods focus on the prevention of cell death. Two retinal cell lines are used for the proposed models of photoreceptor degeneration: the rat retinal precursor R28 cell line and the mouse 661W photoreceptor-like cell line. The R28 cell line was established by the immortalization of postnatal day 6 Sprague-Dawley rat retinal tissue using the psi2 replication incompetent retroviral vector [4,5]. The R28 cell line was developed from a single cell from the retinal E1A-NR3 parental line and three rounds of limited dilution were employed in order to develop a more homogeneous cell line. Since their establishment, R28 cells have been used as a tool to examine retinal cell biology [5–7]. The 661W cell line was established from retinal tumors formed in a transgenic mouse line that expressed the SV40 T antigen under control of the human IRBP promoter [8]. 661W cells have mainly been used as a model for studying photoreceptor cell biology, including oxidative stress studies [9–14]. Both environmental and pharmacological injury causes 661W cell death, such as in these examples. Exposure of 661W cells to damaging light causes an increase in photo-oxidative stress [9–11,14]. The oxidizing agent sodium iodate ($NaIO_3$) also induces oxidative stress and cytotoxicity on these cells [12,13]. The methods developed for this study are based on the following (see Graphical Abstract): 1) in the R28 cell-based assay, depletion of trophic factors by serum starvation induces cell death and the remaining live cells are monitored in real-time by their confluence on the culturing plates, which is proportional to cell viability; 2) in the photo-oxidation assay, cell death is induced in 661W cells by exposure to damaging light and cell viability is assessed by determining the levels of intracellular ATP, which is proportional to the number of viable cells;

and 3) sodium iodate induces oxidative stress-mediated death in 661W cells, which is measured by the release of lactate dehydrogenase (LDH) from the lysed cells into the media and is proportional to cytotoxicity.

Our goal is to establish quantitative cell-based assays for the discovery of cytoprotective agents for the retina. We chose pigment epithelium-derived factor (PEDF) protein as a positive control for these assays because of its demonstrable properties to delay the death of retinal cells in animal models of retinal degeneration [15,16]. In a native mammalian eye, the retinal pigment epithelium, which is a monolayer of cells adjacent to the neural retina, produces and secretes PEDF in a preferential apicolateral fashion to protect photoreceptors [17]. Structure-function studies have revealed that the human PEDF polypeptide of 398 amino acids contains a region of 44 amino acid residues, termed the 44-mer, which exhibits neurotrophic activities of PEDF [15]. A 17-mer region within the central portion of the 44-mer retains PEDF neurotrophic activities [6]. Like the human PEDF protein, the 44-mer and 17-mer peptides protect R28 cells from death induced by serum-starvation in culture [6,7,18,19] and delay the death of photoreceptors *in vivo* [6,21,22]. PEDF also protects 661W cells from death caused by light damage [19,20]. Thus, PEDF and peptides 44-mer and 17-mer constitute excellent positive controls for the protocols described here. We present three semi-automated and quantitative cell-based assays and discuss how they are adaptable for high-throughput quantitative screening where thousands of small molecules can be tested for retinoprotective activities in these cells, and will prove useful for the identification of lead compounds for future *in vivo* preclinical studies on therapies for retinal diseases.

Materials

Cell lines

- Rat retinal cell line (R28) (Kerafast, cat. # EUR201)
- Mouse photoreceptor cell line (661W) (Provided by Dr. Muayyad R. Al-Ubaidi)

Note: For both cell lines, we recommend storing aliquots of cells by freezing after every other passage in order to return to early passage cells for multiple experiments. For assays with R28 cells, we recommend using cell passage numbers under 60. For assays with 661W cells, we recommend using passage numbers under 30.

Equipment

- ThermoForma Water Jacket Incubator (Thermo Fisher)
- IncuCyte S3 Live Cell Analysis System (Essen BioScience cat. # 4647)
- Envision 2105 automated plate reader (Perkin Elmer, MA part # 2105-0010)
- Temperature controlled light box

NOTE: Light-induced-cell damage was performed using a custom-made light box with fluorescent light bulbs at the top and bottom and that emitted 20,000 LUX as measured with a light meter. Temperature was maintained at 37 °C by internal oscillating fans.

- Precision Scientific hot water bath set to 37 °C (Fisher Scientific cat. # 15-474-18)
- Automated cell counter (Bio-Rad TC20 cat. # 1450102)
- Cell counting slides (Bio-Rad cat. # 145-0011)
- T75 tissue culture flasks (Sarstedt cat. # 83.3911.002)
- Clear 96-well tissue culture plates (Corning cat. # 3596)
- Black opaque 96-well plates (PerkinElmer cat. # 6005270)
- 15 ml conical centrifuge tubes (Corning cat. # 430766)
- Centrifuge (Sorvall T6000D)
- Aluminum foil (Reynolds)
- 10X PBS (Gibco cat. # 70011-044)
- EDTA (0.5 M) pH 8.0 (Quality Biological cat. # 351-027-101)

- Molecular grade water (Corning cat. # 95000-094)
- Vacuum Filter unit with 0.45 μm CA Membrane and Storage Bottle System, 500 ml (Corning cat. # 430770)

Reagents for endpoint assays

- CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega cat. # G7572)
- CyQUANT™ LDH Cytotoxicity Assay (Invitrogen cat. # C20301)

Note: The CyQUANT™ LDH Cytotoxicity Assay kit catalog number: C20301 is a replacement for the Pierce LDH Cytotoxicity Assay kit catalog number: 88953. The protocol presented here was developed using the Pierce LDH Cytotoxicity Assay kit.

Recipes – R28 cells

Note: Filter sterilize all solutions through 0.45 µm vacuum filter units prior to use and store at 4 °C.

Dissolve Dulbecco's Modified Eagle's Medium (DMEM) (Sigma cat. # D5523) powder in 1L of distilled water.

R28 serum-free media:

Mix the following to make 500 ml of media:

- 465 ml DMEM
- 15 ml sodium bicarbonate at 75 g/l in distilled water (Sigma cat. # S5761)
- 5 ml MEM non-essential amino acids solution (Gibco, cat # 11140050)
- 5 ml MEM vitamins (Gibco, cat. # 11120052)
- 5 ml mM L-glutamine (Gibco, cat. # 25030081)
- 5 ml Penicillin/Streptomycin (Gibco cat. # 15070-063)

R28 complete media:

Mix the following to make 500 ml of media:

- 415 ml DMEM
- 50 ml FBS (Gibco cat. # 16000069)
- 15 ml sodium bicarbonate at 75g/l in distilled water (Sigma cat. # S5761)
- 5 ml MEM non-essential amino acids solution (Gibco, cat # 11140050)
- 5 ml MEM vitamins (Gibco, cat. # 11120052)
- 5 ml mM L-glutamine (Gibco, cat. # 25030081)
- 5 ml Penicillin/Streptomycin (Gibco cat. # 15070-063)

PBS:

Mix the following to prepare 500 ml of solution:

- 50 ml 10X PBS
- 450 ml distilled water

Cell dissociation solution:

Mix the following to prepare 500 ml of solution:

- 50 ml 10X PBS
- 5 ml 0.5M EDTA
- 445 ml distilled water

Recipes – 661W cells

Note: Filter sterilize all solutions through 0.45 µm vacuum filter units prior to use and store at 4 °C.

Dissolve Dulbecco's Modified Eagle's Medium (DMEM) (Gibco cat. # 12800017) in 1 L distilled water. Add 3.7 g of $NaHCO_3$ as recommended by manufacturer.

661W serum-free media:

Mix the following to prepare 1000 ml of media:

- 999.912 ml DMEM
- 8 μl of 5 mg/ml Hydrocortisone solution (Sigma cat. # H-2270)
- 40 μl of 1 mg/ml Progesterone solution (Sigma cat. # P-8783)
- 32 mg Putrescine (Sigma cat. # 7505)
- 40 μl β-mercaptoethanol (Sigma cat. # M-6250)

661W Complete media:

Mix the following to prepare 500 ml of media:

- 445 ml 661W serum-free media
- 50 ml FBS (Gibco cat. # 16000-044)
- 5 ml antibiotic-antimycotic (Gibco cat. # 15240-062)

661W Light damage serum-free assay media (LD assay media)

Mix the following to prepare 500 ml of media:

- 490 ml 661W serum-free media
- 5 ml of 0.1 g/ml BSA Bovine Serum Albumin (BSA) (Sigma cat. # A2153-100G) in distilled water
- 5 ml antibiotic-antimycotic (Gibco cat. # 15240-062)

661W Sodium iodate serum-free assay media (SI assay media)

Mix the following to prepare 500 ml of media:

- 495 ml 661W serum-free media
- 5 ml antibiotic-antimycotic (Gibco cat. # 15240-062)

Procedures

Note: Maintain the cells at 37 °C in a humidified incubator under 5% CO_2.

Cell viability assay using R28 cells depleted of trophic factors

Given that live adherent cells remain attached on the surface of plates, periodic inspection of the cells under a microscope serves to monitor cell viability and health in real time. With the IncuCyte® live-cell analysis system, real-time cell health and viability analysis is performed without removing the cells from the incubator.

1 Seed and culture R28 cells in R28 complete media until the cells reach 70–80% confluency.

Note: We recommend maintaining the cell cultures between 25 and 90% confluency i.e., the percentage of the surface of a culture dish that is covered by adherent cells, because sparse or dense cultures can result in cell death. Splitting the cell cultures at 1:2 or 1:3 is recommended. After splitting 1:3, the cells generally reach ~80% confluency in 3 days. We also recommend following the care instructions for R28 cells detailed at http://www.kerafast.com/PDF/R28CellCareInstructions.pdf.

1. Warm the R28 serum-free media, PBS, and cell dissociation solution to 37 °C.
2. Remove and discard the media, and wash the cells twice with 5 ml of warm PBS.
3. Add 2.5 ml of warm cell dissociation solution to the cells and incubate them at 37 °C for about 5 min. Gently tap the flask to dissociate the cells. Check for the dissociation of the cells under the microscope.
4. Collect the dissociated cell suspension and transfer it to a 15 ml-tube. Wash the flask with 2.5 ml of warm PBS and transfer the wash to the same tube.

5. Centrifuge the 15 ml-tube at 180 x g for 5 min at room temperature.
6. Remove the supernatant and resuspend the cell pellet in 5 ml of pre-warmed R28 serum-free media. This is termed *cell suspension***.**

Note: Due to the heterogeneous nature of R28 cells, traditional cell counters do not accurately reflect number of cells. Instead, the confluency is calculated by estimating the surface area taken up by the cells in culture, as detailed in steps 8-12. The cells in step 7 are plated to achieve a confluency of 50% at the start of the assay as described here below.

1. Calculate the *surface area of the cell suspension* by multiplying the surface of the original flask by the confluency percentage (see step 1). For example, if the cells of a T75 flask with a surface area of 72 cm^2 are 70% confluent, the surface that the cells occupy corresponds to 50.4 cm^2, while at 80% confluent corresponds to 57.6 cm^2.
2. Determine the desired number of wells of a 96-well plate needed for the experiment. We recommend three replicates per experimental condition as well as including 10 wells in excess to account for pipetting errors. Multiply the number of wells by the volume needed per well (100 µl per well is recommended for a 96 well plate). This is termed *total volume*.
3. Multiply the surface area of a well of a 96-well plate (0.32 cm^2) by the number of desired wells, then divide the number by two for 50% confluency. This is termed the *plated surface area* and corresponds to the number of cells needed to cover the surface area to achieve 50% confluency in a well of a 96-well plate.
4. Multiply the *plated surface area* by 5 ml (the total volume of the *cell suspension*, see step 7). Divide the number by the *surface area of the cell suspension*. This is the volume of *cell suspension* (step 7) required to plate the number of wells determined in step 9.
5. Add the volume of *cell suspension* to a new 15 ml tube. Add the appropriate amount of FBS for a final volume of 5% and bring up the volume to the *total volume* (step 9) in R28 serum-free media and resuspend well.

Note: Calculations can be adjusted for different vessel sizes and volumes of wells as needed. The amount R28 cells in one T75 flask is suitable for plating two full 96-well plates. If more cells are required for larger experiments, cultures can be expanded in additional T75 or T175 flasks. The following is an example of the calculations required to plate a 96-well plate with cells obtained from a T75 flask containing cells at 70% confluency.

Cell suspension - 70% x 72 cm^2= 50.4 cm^2

Need 96 wells + 10 wells excess = 106 wells

106 wells x 100 µl/well = 10.6 ml total volume needed

0.32 cm^2 x 106 wells / 2 = 16.96 cm^2 plated surface area

16.96 cm^2 x 5 ml / 50.4 cm^2= 1.68 ml cell suspension

To prepare the assay cell suspension, which consists of the total volume needed (10.6 ml) with 5% FBS and with cell suspension for a cell culture with 50% confluency in a 96-well plate, mix the following:

530 µl 100% FBS

1.68 ml cell suspension

8.39 ml R28 serum-free media

1. Add 100 µl of the assay cell suspension to each well of a clear 96-well plate.
2. Place the plate in the IncuCyte Live Cell Analysis System that is located inside an incubator.
3. Monitor cell viability by the IncuCyte S3 Live Cell Analysis System (https://www.essenbioscience.com/en/products/incucyte/). Set the following scan pattern according to manufacturer's instructions.
4. Schedule a scan for the assay plate.
5. Select schedule scan and add the appropriate vessel to the tray position.
6. Create a new scan pattern.
7. Select edit scan pattern and indicate the wells to be selected and the number of images per well.

8. Select phase as the channel selection.
9. Select the scan type and scan pattern. We recommend a standard scan type and 4 images per well.
10. Use the properties tab to label the experiment, input notes, and label plate map to indicate each sample in the wells.
11. Right click on the timeline at the top of the prompt to set a time interval. Set the interval to record images every 4 h. Click apply to save changes.

Note: Ensure that the IncuCyte is not programmed to capture images near the 16 h treatment time point. Failure to do so will result in missing photographs for this time point.

1. Dilute the compound of choice at the desired concentration in pre-warmed R28 serum-free media.
2. After 16 h, remove the plate from the IncuCyte and aspirate and discard media from the wells.
3. To expose the cells to testing compounds, add 100 µl of the diluted compound of choice at the desired concentration to each well. Add 100 µl of R28 serum-free media alone to a subset of wells as control.
4. Return the plate to the IncuCyte in the incubator. Continue to incubate the cells for an additional 48 h, allowing the software to automatically capture and analyze phase contrast images every 4 h inside the incubator.

Cell viability assay using 661W cells exposed to photo-oxidative stress

Given that only viable cells synthesize ATP, this nucleotide is used to measure cell viability in this method. ATP levels are quantified using the CellTiter-Glo® Luminescent Cell Viability Assay with reagents containing detergent, stabilized luciferase and luciferin substrate. In this assay, the detergent lyses viable cells, releasing ATP into the medium. In the presence of ATP, luciferase uses luciferin to generate luminescence, which can be detected within 10 minutes using a plate reader (luminometer). The levels of luminescence are proportional to ATP, which is a biomarker for cell viability.

Prepare the CellTiter-Glo Luminescent Cell Viability Assay reagent as instructed by the manufacturer (Promega Cat. # G7570).

1. Seed and culture the 661W cells in a T75 flask with 20 ml of complete media.

Note: We recommend maintaining the cultures at a cell confluency of 25-90%. Splitting the cell cultures at 1:3 or 1:4 is recommended. After splitting 1:3, the cells generally reach ~80% confluency in 3–4 days.

1. Warm 661W complete media, PBS, and cell dissociation solution to 37°C.
2. When the cells reach 80% confluency, remove and discard the media and wash the cells with 5 ml of warm PBS. Repeat the wash.
3. Add 2.5 ml of warm cell dissociation solution to the T75 flask and incubate at 37°C for about 5 minutes. Gently tap the flask to dissociate the cells. Check for the dissociation of the cells under the microscope.
4. Collect the cell suspension and transfer it to a 15 ml-tube.
5. Add 2.5 ml of warm PBS to the flask and transfer the wash to the same tube (Step 5).
6. Centrifuge the cell suspension in the 15 ml-tube at 180 x g for 5 minutes at room temperature.
7. Aspirate and discard the supernatant.
8. Resuspend the cell pellet in 5 ml 661W complete media.
9. Use a cell counting slide to determine the total number of cells in the cell suspension.
10. Plate 30,000 cells per well in clear 96-well plates with a total volume of 100 µl per well for the desired number of conditions. We recommend a minimum of four replicates per condition. Prepare a second 96-well plate with 4 wells of cells containing 30,000 cells per well in 100 µl to serve as a dark control plate for cells not exposed to damaging light. If desired, dark control plates can be prepared identical to the assay plates that will be exposed to damaging light to determine whether the compounds affect cell viability.

11. Place the culturing plates in an incubator.
12. After 16 hours, remove the plates from the incubator and carefully remove and discard the media from the wells without disturbing the attached cells.
13. Prepare a solution of the compound of choice at the desired concentration in pre-warmed 661W LD assay media and add 100 µl of it to each well. To the second 96-well control plate containing 4 wells of cells, add 100 µl of pre-warmed 661W LD assay media for cells that will not be exposed to damaging light.
14. Place the cell cultures for 4 h in an incubator.
15. Remove the culturing plates from the incubator and wrap the dark control plate in aluminum foil. Place the assay plate and dark control plate in a temperature controlled light box at 37 °C and expose to 20,000 Lux.
16. Following 2 hours of light exposure, remove the plates from the light box and remove and discard the media from the wells and wash the cells twice with PBS at room temperature.
17. Add 100 µl of PBS and 100 µl of CellTiter-Glo reagent CellTiter-Glo Luminescent Cell Viability Assay reagent to each well and mix by pipetting.
18. Incubate the mixture for 10 min at room temperature.
19. Transfer 200 µl of the mixture from each well to a black 96-well plate in the same order.
20. Measure the luminescence signal using automated plate reader, such as the Envision 2105.

$$\text{Calculate :}$$
$$\%\,\text{Cell viability} = \frac{(\text{Luminescence of treated samples}) \times 100}{(\text{Luminescence of dark control samples})}$$

Cell viability assay using 661W cells exposed to sodium iodate

Lactate dehydrogenase (LDH) is a cytosolic enzyme and is a reliable indicator of cellular toxicity. Damage of the plasma membrane results in a release of LDH into the cell culture medium. In this method, the extracellular LDH is quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that is measured at 490 nm with a plate reader. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

Prepare the reagents of the CyQUANT™ LDH Cytotoxicity Assay as instructed by the manufacturer (see instruction manual for Invitrogen cat. # C20301). This assay kit contains the following reagents: Substrate Mix, Assay Buffer, Lysis Buffer, and Stop Solution.

1. Seed and culture the 661W cells in a T75 flask with 20 ml of complete media.
2. Warm 661W complete media, PBS, and cell dissociation solution to 37°C.
3. When the cells reach 80% confluency, remove and discard the media and wash the cells with 5 ml of warm PBS. Repeat the wash.
4. Add 2.5 ml of warm cell dissociation solution to the cells in the flask and incubate at 37 °C for about 5 min. Gently tap the flask to dissociate the cells. Check for the dissociation of the cells under the microscope.
5. Collect the cell suspension and transfer it to a 15 ml-tube.
6. Add 2.5 ml of warm PBS to the flask and transfer the wash to the same tube (Step 5).
7. Centrifuge the cell suspension in the 15 ml-tube at 180 x g for 5 minutes at room temperature.
8. Aspirate and discard the supernatant.
9. Resuspend the cell pellet in 5 ml complete media.
10. Use a cell counting slide to determine the total number of cells in the cell suspension.
11. Plate 30,000 cells per well in a clear 96-well plate with a total volume of 100 µl per well for the desired number of conditions. We recommend a minimum of three replicates per condition. Include three replicates each of the following set of controls: an untreated control that will not receive any drug, a spontaneous LDH activity control, and a maximum LDH activity control.
12. Place the culturing plates in an incubator.
13. After 24 hours, remove the plates from the incubator and remove and discard the media from the wells.

14. Wash the cells with 100 µl of prewarmed PBS.
15. Prepare a solution of the compound of choice at 2X the desired concentration in pre-warmed 661W SI assay media and add 50 µl of it to each well. Then, add 50 µl of SI assay media containing 8 mM $NaIO_3$ to each well for a final volume of 100 µl and final concentration of 4 mM $NaIO_3$. The untreated control well contains only 4 mM $NaIO_3$ in the SI assay media. Add 100 µl of 661W serum-free assay media to each well of all the sets of controls.
16. Place the cell culture plates in an incubator for 24 h.
17. At 45 min prior to reaching the endpoint of 24 h, add 10 µl of 10X Lysis Buffer supplied in the assay kit to the maximum LDH activity control wells, and 10 µl of ultrapure water to the spontaneous LDH activity control wells. Incubate in an incubator for the remaining 45 min.
18. At endpoint, remove the plates from the incubator. Transfer 50 µl of each culturing medium to a clean clear 96-well plate.
19. Add 50 µl of the Reaction Mix to each sample well from step 18 and mix well by gentle tapping.
20. Incubate the plates for 30 min at room temperature and protected from light.
21. Add 50 µl of Stop Solution supplied in the assay kit to each sample well and mix by gentle tapping.

Note: Break any bubbles present in wells either by a short centrifugation or using a syringe needle before determining absorbance.

1. Measure the absorbance using an automated plate reader at:

-490nm that corresponds to Cytotoxicity + Background
-680nm that corresponds to Background
Cytotoxicity = Abs490 – Abs680

$$\text{Calculate :}\quad \%\,\text{Cytotoxicity} = \frac{(\text{Compound Abs490} - \text{Abs680}) - (\text{Spontaneous Abs490} - \text{Abs680}) \times 100}{(\text{Maximum Abs490} - \text{Abs680}) - (\text{Spontaneous Abs490} - \text{Abs680})}$$

Compound Abs490 – Abs680 = In samples
Spontaneous Abs490 – Abs680 = In wells with dH_2O
Maximum Abs490 – Abs680 = In wells with Lysis Buffer

Results and Discussion

Here, we describe methods to evaluate the viability of R28 and 661W cells undergoing death due to trophic factor depletion, photo-oxidation stress, and toxicity of cells exposed to sodium iodate. These cell-based assays are useful to determine the efficacy of drugs to inhibit cell death in an automated fashion.

In the first assay, R28 cell death is induced by serum-starvation to remove trophic factors from the cells. We validated the assay using a known cytoprotective factors PEDF and 17-mer peptide as positive controls. R28 cells depleted of trophic factors were exposed to cytoprotective factors PEDF protein (5 nM), or 17-mer peptide (1 nM) at 16 hours after plating. Control cells did not receive the factors. Cell viability for each condition was monitored in real time by acquiring images from regions of the well in an automated fashion every 4 h. The cells in the images were quantified from the phase object confluence, defined as the percentage of the area that occupies the cells in the acquired image. The images in Fig. 1A show regions of one replicate well per condition to illustrate the confluence of cells at a time prior to factor exposure (time = 12 h) and at the endpoint of the assay (time = 64 h). The measurements of the confluence acquired every 4 h per each region and well were exported into Microsoft Excel and plotted. As shown in Fig. 1B, the confluence of R28 cells without trophic factors was maintained at ~30%, while additions of PEDF and the 17-mer peptide increased the confluence to 78% and 72%, respectively, during the duration of the assay (16 h–64 h). While the cells deprived of trophic factors failed to increase confluency over time, the confluency values for those cells exposed to the cytoprotective factors increased almost linearly up to the 40 h point and then began to plateau. The images captured at each time point were used to create time

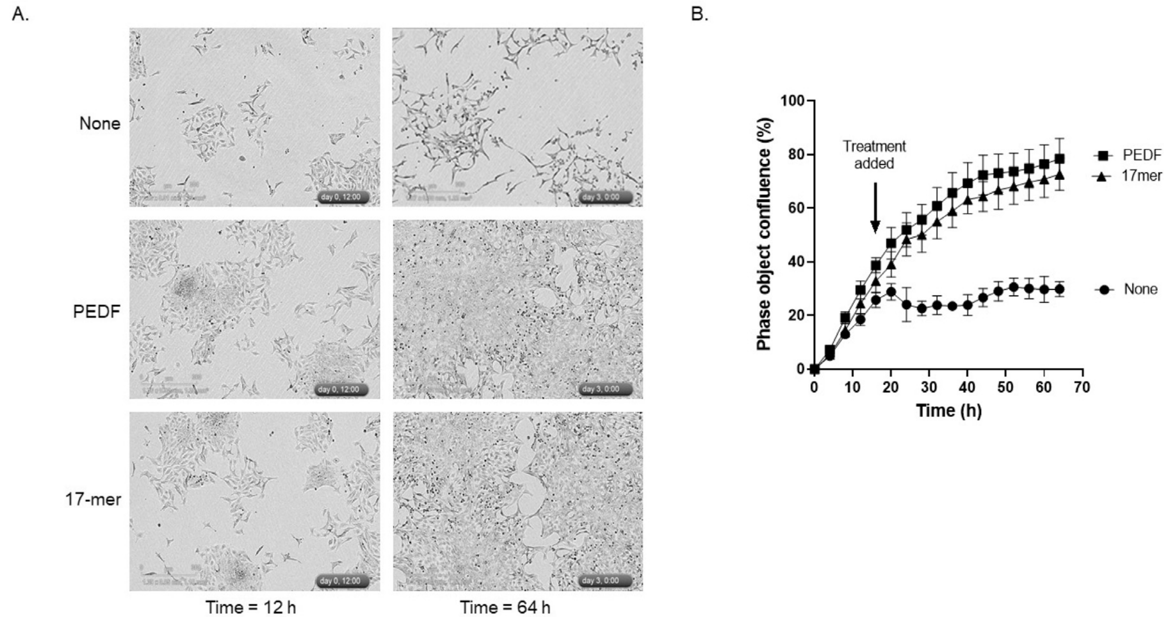

Fig. 1. R28 serum-starvation assay. R28 cells were seeded in 96-well plates in media containing 5% serum, incubated for 16 h, and then replaced with serum-free media containing no effector (None), 5 nM PEDF protein or 1 nM PEDF 17-mer peptide, as indicated. Phase images were acquired every 4 h by the IncuCyte Live Cell Analysis System. (A) Representative images of cells prior to trophic factor deprivation and compound additions (Time 12 h) and 48 h after effector addition (Time 64 h) are shown. (B) The automated phase object confluency was calculated by averaging the cell confluence of four readings per well every 4 h during 64 h. The phase object confluence is indicated in the *Y*-axis and the time is indicated in the *X*-axis. Each condition was assayed with 4 replicates. h = hours

Movie S1. Time lapse videos of R28 serum-starvation assay. R28 cells were plated in a 96-well plate and incubated for 16 h in media containing 5% serum. Media was replaced with media without treatment, with 5 nM PEDF, or 1 nM 17-mer at hour 16. Phase images were automatically acquired every 4 hours by the IncuCyte Live Cell Analysis System.

lapsed movies to follow cell confluency over time (Movies A-C). These observations are in agreement with previous reports describing that these factors promote cell viability and prevent R28 cell death [6,7]. The results demonstrate that the assay serves to quantitate the activity of neurotrophic factors, such as PEDF and the 17-mer peptide, that increase viability of cells depleted of trophic factors by measuring cell confluency in real time.

In the second assay, 661W cells are induced to die by photo-oxidation. In this case, cells are incubated without serum for 4 hours prior to exposure to damaging light, to pre-sensitize 661W cells to damaging light [23]. Fig. 2A shows that 2 hours of 20,000 lux exposure resulted in a 50-75% decrease of 661W cell viability. To determine whether incubation of the cell cultures in the custom light box without controlled humidity or flow of CO_2 affects cell viability, assay plates were protected from light and incubated for 1 hour, 2 hours and 3 hours in the light box and compare them to those placed in an incubator exposed to humidity and 5% $CO_{2,}$. The viability of cells protected from light did not change over time (Fig. 2B), indicating that humidity and CO_2 differences did not affect the outcome. Given that light converts the chromophore 9-cis retinal to the toxic compound all-trans retinal, it is believed that 9-cis retinal further sensitizes 661W cells to death [9,14,20,23]. However, we found no significant difference in viability between cells preincubated with 9-cis and those without 9-cis (Fig. 2C). We concluded that the use of 9-cis to further induce cell death was unnecessary, thus simplifying the assay. To determine the optimal time at which drug addition is most efficient, PEDF was added to 661W cells at 4 hours prior to light exposure, at the time light exposure starts, or at both times, as illustrated in Fig. 2D. Fig. 2E shows that light damage decreased cell viability to 25% relative to Dark control. Double PEDF additions, one prior to light exposure and a second at the time of light exposure (Dark & Light), resulted in 66% cell viability relative to Dark control, while a single PEDF addition (Dark only) resulted in 80% cell viability. PEDF addition at the same time of light exposure (Light only) did not increase cell viability relative to None. Altogether the observations indicate that a single pretreatment with neurotrophic factors was sufficient to protect light-damaged cells. We also exposed the cells to the 44-mer peptides, which is derived from the neurotrophic region of PEDF [24]. Fig. 2F shows that whereas light-damage yielded 32% of cell viability, pretreatment with 44-mer peptide increased cell viability by 2-fold to about ~62% relative to the Dark control. The findings indicate for the first time that the 44-mer peptide provides protection to 661W cells undergoing photo-oxidation like the full-length PEDF. Thus, this assay serves to quantitate the activity of neurotrophic factors, such as PEDF and the 44-mer peptide, that increase viability of 661W cells undergoing photo-oxidation using intracellular ATP as biomarker for viability.

Cell death can also be induced chemically in 661W cells by the addition of the oxidative stress agent sodium iodate to provoke cytotoxicity [12,13]. In the third assay, 661W cells are exposed to 3 mM–5 mM sodium iodate and then cytotoxicity is measured. Fig. 3A shows that sodium iodate increased cell toxicity at all concentrations tested. Furthermore, additions of PEDF at 10 nM decreased the cytotoxicity induced by sodium iodate and in some cases, cytotoxicity was even below the levels of cells that did not receive sodium iodate (compare concentrations 3–4.5 mM sodium iodate with no sodium iodate). The efficacies of PEDF and of the 44-mer fragment were tested in this assay. Like PEDF, the 44-mer significantly decreased the sodium iodate-mediated cell toxicity similar to the levels in cells that were not exposed to the oxidative stress agent (Fig. 3B). The results demonstrate that PEDF and the 44-mer peptide protected 661W cells undergoing chemically-induced oxidative stress. The results demonstrate that 4 mM sodium iodate was sufficient to induce cell death that could be prevented by neurotrophic factors. The assay serves to quantitate the efficacy of factors, such as PEDF and the 44-mer peptide, that prevent cytotoxicity in 661W cells undergoing oxidative stress using LDH release as a marker for cell death.

The three cell-based assays presented here describe simple, quantitative and semi-automated methods adaptable to high-throughput screening. We validate these assays using three positive controls: full length PEDF, the 44-mer peptide designed from the neurotrophic region of PEDF, and the 17-mer, a smaller peptide designed from the central region of the 44-mer peptide, which also retains neurotrophic activity. To our knowledge, this is the first study in which the IncuCyte Zoom Live Cell Analysis System has been utilized to determine cell viability of cultured R28 cells. Comparison with those assays previously described show that the ones described here present certain advantages. In most current assays with R28 and 661W cells, retinal cell death is measured by techniques

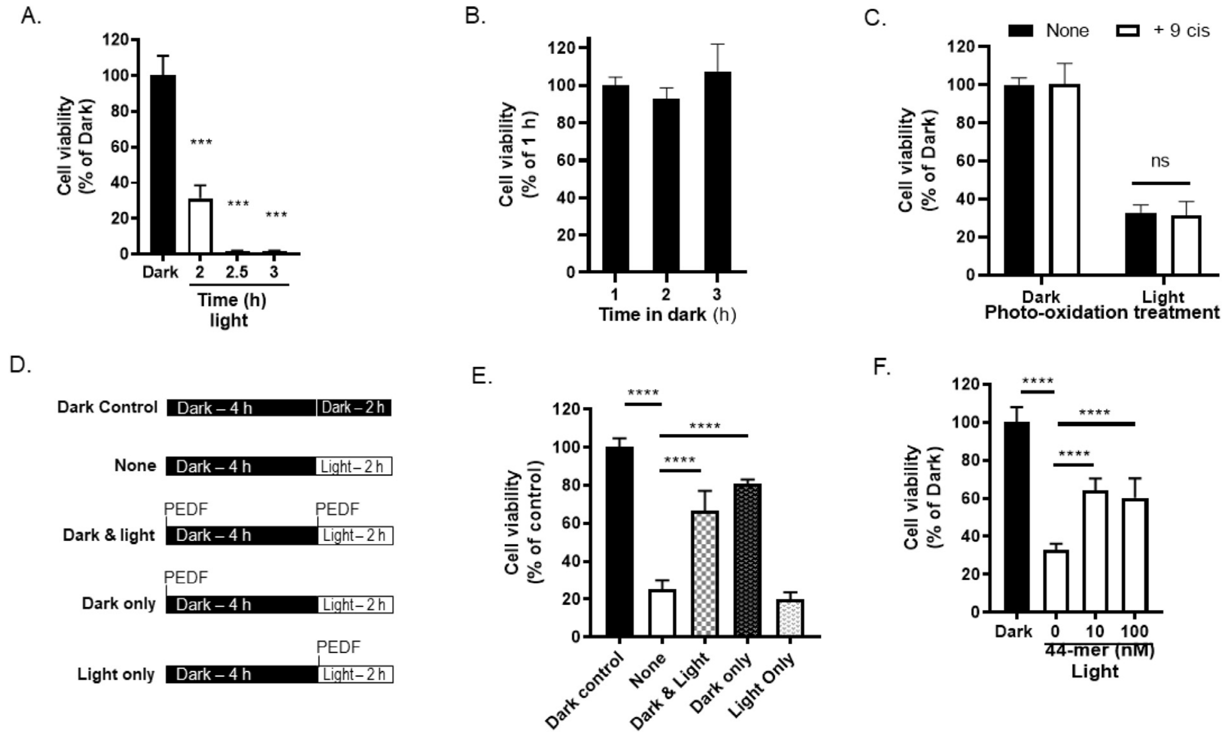

Fig. 2. 661W light damage assay. 661W cells were seeded with media containing serum in 96-well plates and incubated for 16 h. Then the media was replaced with serum-free media and continued incubation for 4 h to sensitize the cells to cell death. The cells were exposed to damaging light (20,000 lux) at 37 °C in a light box. At the endpoint, intracellular ATP was measured and used to calculate percentage of cell viability. Plots of cell viability percentages for conditions assayed are shown in the following panels. (A) Cells were exposed to damaging light for the indicated periods of time shown in the *X*-axis. (B) Cells kept in the dark and incubated for the indicated periods of time (*X*-axis) in the light box. (C) Cells were incubated in serum-free media with or without 9-cis retinal for 4 h and then exposed to damaging light for 2 h. (D) A scheme detailing the treatments for each condition in panel E as follows: no addition (Dark control), no addition (Light), 10 nM PEDF added at the beginning of the 4 hour pretreatment period plus again immediately before exposure to damaging light (Dark & light), PEDF added at the beginning of the 4 h pretreatment only (Dark only), or added immediately before light exposure (Light only). (E) Cells were incubated with 10 nM PEDF at the indicated times in panel D and then exposed to damaging light for 2 h, as indicated in the *X*-axis. (F) Cells were pre-incubated with 10 nM 44-mer or 100 nM 44-mer for 4 hours and then exposed to damaging light for 2 hours. Each data point in the plot correspond to the average of 4 replicates per condition +/- standard deviation (SD). *** $P < 0.001$ **** $p < 0.0001$ h = hours

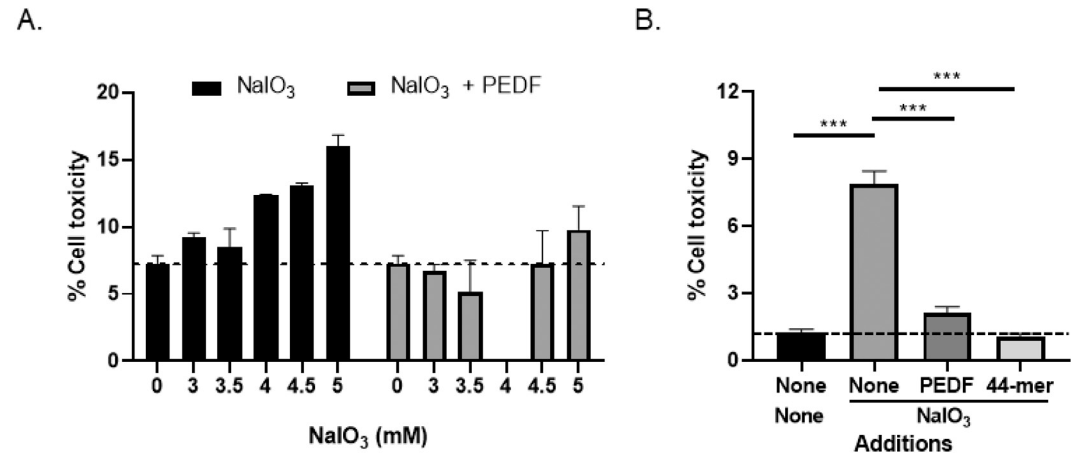

Fig. 3. 661W sodium iodate assay. 661W cells were seeded with media containing serum in 96-well plates, incubated for 24 hours, and then media were replaced with media without serum, media without serum containing $NaIO_3$, and media without serum containing both $NaIO_3$ and PEDF protein or the 44-mer peptide. At endpoint, cytotoxicity was determined and percentage of cytotoxicity was plotted and shown in the panels as follows. (A) Cells containing indicated concentrations of $NaIO_3$ and with and without 10 nM PEDF. (B) Cells with 4 mM $NaIO_3$ and 10 nM PEDF or 10 nM 44-mer. Each data point in the plot correspond to the average of 3 replicates per condition +/- standard deviation (SD). *** $P < 0.001$

using fluorescence microscopy such as TUNEL and propidium iodide staining [6,7,10,11,25,26], which are time consuming due to cell fixing, staining, imaging, and counting labeled nuclei in acquired digital images. The use of IncuCyte for cell viability determination is a faster non-biased quantitative alternative compared to quantification by fluorescence microscopy. Additionally, in current assays, retinal cell viability and toxicity is determined by measuring mitochondrial reductase activity using tetrazolium compounds, such as MTT, MTS, or WST-8 as substrates [9,19,23,27]. While also semi-automated, these assays require incubation with the substrates for 3 hours to 16 hours. In the assays described here, the determination of intracellular ATP and released LDH as markers for cell viability and cytotoxicity, respectively, take 30 min or less, representing an advantage. Current assays for light-damaged 661W cells require preincubation with 9-cis to sensitize cells to light-damage [9,14,20,23], and require between 4 and 24 h of light exposure [9–11,14]. The photo-oxidative damage assay presented here is further simplified by requiring only 2 h of light exposure without the use of 9-cis. Thus, the assays described here are quick and simple methods that can be scaled up to increase the number of manageable experiments performed at one time. Finally, another advantage is that the R28 and 661W cell lines can be expanded to large cultures, giving the capability to perform high-throughput screening of many compounds at a time and in a cost-effective fashion.

We also acknowledge disadvantages and limitations of these assays. Cell lines are often used in place of primary cells to study biological processes. However, care must be taken when interpreting the results as cell lines do not always accurately replicate the primary cells. R28 cells are retinal precursors and 661W are 'cone-like' precursors that have been used in numerous neuroprotection studies, which establish them as retinal cell lines [5,8]. They are derived from retinal cells and possess several biochemical markers associated with neuronal retinal cells. Native neuronal retinal cells are postmitotic, while the cells in these lines proliferate at a fast rate, and a variety of effectors can induce them to death or to differentiation into specific retinal cell type. Therefore, take into consideration that cell lines do not behave identically with primary cells and should not be used to replace primary cells. We note that the assays described are limited to determining R28 and 661W cell viability and cytotoxicity. To strengthen the findings, key control experiments using these cells should always be performed. Determination of the optimal time and/or dose of induction of cell death is useful at the beginning of each experiment. As illustrated in Figs. 2A and 3A, we notice that not enough cell death yields insignificant results, while too much cell death renders ineffective treatments in recovering cell viability. For example, 50% to 75% of cell death was optimal for the 661W light-induced damage assay, and cytotoxicity rate that is equal or higher than 1.5-fold of controls (no oxidative stress agent) was optimal for the 661W sodium iodate-induced damage assay. The 661W light-induced damage method requires the use of a custom light box (e.g., 20,000 LUX for 2 h) to induce cell death. Depending on the variations of manufactured light boxes, it will be necessary to determine optimal amount of light and exposure time. Another recommendation for the assays described here is that during cell expansion, split and passage the cell cultures in a manner in which the confluency is never less than 25% and more than 90% prior to plating for experiments. Another problem may be the variability of confluency observed among biological replicates, which could be due to cell aggregate formation during plating. To ensure uniform plating, resuspend the cell suspension well to homogeneously dissociate cell aggregates, and use a cell strainer if necessary. Another recommendation is the use R28 and 661W cells at early passages for the assays described here. We notice that R28 and 661W cells from passage numbers greater than 60 and 30, respectively, are more resistant to stress-induced death. We speculate that the cells self-select themselves to increase chances of cell survival with passage number.

Finally, very few retinal cell lines exist in the visual science field, being R28 and 661W cells widely used cell lines. In contrast, primary retinal cell lines have a limited lifespan in culture. Both immortalized cell lines can be expanded into large cultures, providing an easy and cost-effective method to screen drug candidates by high-throughput assays. Isolated cell lines provide a system with stable culturing conditions, offering a great degree of consistency among experiments. The 96-well plate assays are also easily adaptable for scaling up to 384-well plates, allowing for the testing of an even larger number of compounds at multiple concentrations. Therapeutic agents identified using these three screens can be used in animal models of retinal degeneration, ultimately leading to the discovery of novel retinal therapeutics.

In conclusion, the methods show that viability and cytotoxicity of retinal R28 cells and 661W cells are measurable outcomes and that the efficacies of known neurotrophic factors in protecting these cells against death can be determined following these assays. The methods are simple, quantitative, semi-automated and fast, and will prove useful in upscaling to high throughput screening for thousands of compounds for the discovery of retino-protective drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.101026.

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