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A Strategy to Identify Compounds that Affect Cell Growth and Survival in Cultured Mammalian Cells at Low-to-Moderate Throughput

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TITLE:

A Strategy to Identify Compounds that Affect Cell Growth and Survival in Cultured Mammalian Cells at Low-to-Moderate Throughput

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toxicity assay, cytotoxicity, 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide, MTT, moderate throughput, cultured mammalian cells, fluorescent imaging, cell counting

SUMMARY:

It is often necessary to assess the potential cytotoxicity of a set of compounds on cultured cells. Here, we describe a strategy to reliably screen for toxic compounds in a 96-well format.

ABSTRACT:

Cytotoxicity is a critical parameter that needs to be quantified when studying drugs that may have therapeutic benefits. Because of this, many drug screening assays utilize cytotoxicity as one of the critical characteristics to be profiled for individual compounds. Cells in culture are a useful model to assess cytotoxicity before proceeding to follow up on promising lead compounds in more costly and labor-intensive animal models. We describe a strategy to identify compounds that affect cell growth in a tdTomato expressing human neural stem cells (NSC) line. The strategy uses two complementary assays to assess cell number. One assay works via the reduction of 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as a proxy for cell number and the other directly counts the tdTomato expressing NSCs. The two assays can be performed simultaneously in a single experiment and are not labor intensive, rapid, and inexpensive. The strategy described in this demonstration tested 57 compounds in an exploratory primary screen for toxicity in a 96-well plate format. Three of the hits were characterized further in a six-point dose response using the same assay set-up as the primary screen. In addition to providing excellent corroboration for toxicity, comparison of results from the two assays may be effective in identifying compounds affecting other aspects of cell growth.

INTRODUCTION:

One of the most important characteristics that needs to be determined for a chemical compound that has therapeutic potential is its toxicity to animal cells. This characteristic will determine whether a drug is a good candidate for more extensive study. In most instances, compounds with minimal toxicity are sought but there are situations in which a compound with the capacity to kill specific cell types is of interest, e.g., anti-tumorigenic drugs. Although whole animals are the best model systems to determine systemic toxicity, the cost and labor involved is prohibitive when more than a few compounds need to be tested. As such mammalian cell culture is generally used as the most efficient alternative[1,2]. Small to medium throughput drug screens are an important modality through which toxicity can be assessed in cell culture. These screens can be used to interrogate annotated libraries targeting individual signaling pathways. The general format of such a screen is to initially test all the compounds in the library at a single dose (generally 10 µM) in an exploratory primary toxicity screen, and then perform an in-depth secondary dose response screen to fully characterize the toxicity profile of hits from the primary screen. The methods to implement this strategy will be described here and provide a quick, efficient, and inexpensive way to identify and characterize toxic compounds.

Multiple methods have been developed to assess cytotoxicity of small compounds and nanomaterial in mammalian cells[3,4]. It should be noted that certain materials can interact with the assay providing misleading results, and such interactions should be tested when characterizing hits from toxicity screens[4]. Cytotoxicity assays include trypan blue exclusion[5], lactate dehydrogenase (LDH) release assay[6], Alamar blue assay[7], calcien acetoxymethyl ester (AM)[8], and the ATP assay[9]. All these assays measure various aspects of cell metabolism which can serve as a proxy for cell number. While all offer benefits, tetrazolium salt-based assays such as 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-bis(2-methoxy-4-nitro-5-sulfopheny)-2H-tetrazolium-5-carboxyanilide inner salt (XTT)-1, and 4-(3-[4-Iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1)[10,11] provide good accuracy and ease of use at low cost. MTT, which will be used in this demonstration, is reduced to an insoluble formazan by a mitochondrial reductase and the rate of this conversion correlates strongly with cell number. This assay has been routinely utilized at both a small scale and for screening libraries with up to 2,000 compounds[12]. Direct counting of cells by a labeled marker offers another method to assess the cellular number, and unlike the MTT assay it can provide additional information about the dynamics of cellular growth. Several publicly available algorithms are available to perform automated cell count analyses and there are also proprietary algorithms that are part of software packages for imaging readers[13,14]. In this method description, a human neural stem cell (NSC) line that has been genetically edited to constitutively express tdTomato[15] will serve as a test line to compare cellular viability results between an MTT assay and an automated cell counting assay in a screen assessing toxicity of 57 test compounds. Although the primary goal of this strategy was to identify and characterize toxic compounds, it has the additional benefit of potentially identifying growth inhibitory and growth enhancing compounds and thus provides an effective method for identifying drugs that can modulate cellular growth.

PROTOCOL:

1. NSC culture

NOTE: Manipulation of a human NSC line will be described below but any cell line can be used for this protocol. All cell culture work is performed in a biological safety cabinet.

1.1. Coat a 96-well plate with basement membrane/extracellular matrix (ECM).

1.1.1. Thaw aliquot of ECM (**Table of Materials**), which will facilitate attachment of NSC, on ice. Dilute ECM to the appropriate concentration (generally 1:100) in 10 mL base medium (**Table of Materials**) and add 50 µL per well to each of 60 interior wells of a 96-well plate (**Figure 1**). Use only the interior 60 wells to avoid artifacts that may result from the edge effect[16].

1.1.2. Let the plate sit at room temperature or in a cell culture incubator (37 °C, 5% CO_2) for at least 30 min.

1.2. Dissociate and plate neural stem cells.

NOTE: Cells for use in this method should be grown to at least 80% confluence in a T75 flask.

1.2.1. Culture cells in a T75 flask in a cell culture incubator at 37 °C and 5% CO_2 in NSC medium that is composed of base medium, B27, non-essential amino acids, 2 mM glutamine, and 10 ng/mL basic fibroblast growth factor (FGFb or FGF2).

1.2.2. Remove cells from the incubator once they reach 80% confluence and aspirate off NSC medium. Add an appropriate amount of cell dissociation reagent (3 mL for a T75 flask; **Table of Materials**) and incubate for 5 min in the incubator.

1.2.3. After incubation, add 7 mL of NSC medium in the T75 flask and pipette vigorously to ensure all cells become detached. Transfer the dissociated cell solution to a 15 mL tube and centrifuge at 200 x *g* for 5 min.

1.2.4. After centrifugation, remove supernatant from the tube and resuspend cells in 10 mL of NSC medium and count cells.

1.2.5. Readjust concentration of cells to 200,000 cells/mL with NSC medium. Ensure cells are fully resuspended for homogeneous plating into wells.

1.2.6. Plate 100 µL of the cell mixture (20,000 cells) in the 60 interior wells of three 96-well plates that have been coated as described in section 1.1. Use six of the eight slots of an 8-channel multichannel pipettor to plate cells column-by-column.

1.2.7. Add 100 µL of base medium or NSC medium to all wells without cells to minimize potential evaporation from outermost wells.

1.2.8. Under a cell culture microscope, visually inspect at least 10 wells on each of the three 96-

well plates to confirm that the cells are seeded at the expected density. Do not proceed with the assay if cells are plated at a density too sparse or dense.

2. Treating cells with compounds

NOTE: The home-made library tested in this demonstration contains compounds that modulate wingless/integrated (Wnt), retinoic acid, transforming growth factor-beta (TGF-β), and sonic hedgehog signaling pathways as well as a variety of tyrosine kinases.

2.1. Exploratory primary screen for toxicity/cell number

2.1.1. Aliquot 50−100 µL of up to 57 test compounds (**Supplemental Table 1**) at a concentration of 10 mM in 100% dimethyl sulfoxide (DMSO) into the interior 60 wells of a U-bottomed, V-bottomed or round-bottomed 96-well plate with three DMSO wells as a control (see **Figure 1** for a plate map). This will serve as the master compound plate with 25 µL of compound that can be frozen and thawed several times.

NOTE: Flat bottomed plates should not be used as it will be more difficult to aspirate small volumes of compounds from them with a bench top pipettor.

2.1.2. Remove cell culture plates from incubator 16−24 h after splitting as described in section 1 and aspirate off NSC medium column-by-column with an 8-channel multi-well pipettor using only six of the eight multi-well slots. Add 95 µL of fresh NSC medium to cells in each of the three replicate plates and place plates back in incubator until step 2.1.4 below is completed.

2.1.3. Add 49 µL of NSC medium to each of the interior 60 wells of an empty U-bottomed, V-bottomed or round-bottomed 96-well plate with an 8-channel multi-well pipettor. Unseal the master compound plate and use a bench top pipettor or equivalent instrument to pipette 1 µL of compound from the master plate into the 49 µL of NSC medium in each of the interior 60 wells.

2.1.4. Mix the diluted compound 3x with the bench top pipettor.

2.1.5. Remove the three 96-well plates of NSCs from the incubator, pipette 15 µL of each diluted compound with the bench top pipettor and dispense a 5 µL aliquot of compound into each of the three plates.

NOTE: This 1:20 dilution of compound into the cells in combination with the initial 1:50 dilution in step 2.1.3 yields a 1:1000 dilution such that the final concentration of the compounds on the NSCs will be 10 µM with a DMSO concentration of 0.1% and the final concentration for the DMSO controls will be 0.1%.

2.1.6. Incubate cells with compound for 72 h and proceed with cytotoxicity assays. Shorter intervals can be used but a 72-hour incubation period should maximize the potential cytotoxic effects of tested compounds.

2.2. Dose response assay

NOTE: The set-up for the 96-well used for the dose-response is displayed in **Figure 2**.

2.2.1. Use column 2 for six DMSO control replicates and test triplicates of up to three different compounds at two-fold serial dilutions at six doses starting with a high dose of 10 µM.

2.2.2. Dilute 4 µL of DMSO or test compound in DMSO into 196 µL of NSC medium in a 1.5 mL microcentrifuge tube. Add 25 µL of DMSO to the column of wells from B2−G2 and 50 µL of test compounds to the row from B3−B11 with the three tested compounds in 10 mM triplicates in rows B3−B5, B6−B8, and B9−B11.

2.2.3. Pipette 25 µL of NSC medium to the remaining empty columns in the interior portion of the 96-well plate. Remove 25 µL of compound from wells B3−B11 with a multichannel pipettor, add to wells C3−C11, and mix at least five times. Repeat the process for the remaining rows to generate triplicates at two-fold dilutions for a total of six doses for each of the compounds.

2.2.4. Generate NSCs for the dose response exactly as described for the primary screen in section 2.1. The compounds for the dose response are added to and incubated on the cells exactly as described in steps 2.1.5 and 2.1.6.

NOTE: Three biological replicates of the dose response assay are performed by repeating the assay on the NSCs at different passages on separate days.

3. Imaging cells on a plate reader

3.1. After cells have been incubated with compounds for the allotted time, image cells on a plate reader to determine the pre-treatment cell number per well.

NOTE: Instructions for imaging cells are reader-specific but generally follow a similar strategy. The directions below apply to the reader used in this demonstration (**Table of Materials**).

3.2. Remove the plate from the incubator and place it inside the plate reader. Open the imager software to set up protocol and experiment files for the study. Go to **Imager Manual Mode** on Task Manager and click **Capture now…**.

3.3. Choose 96-well plate as the vessel type, select 10x for the magnification, and red fluorescent protein (RFP) 531 and 593 for imaging tdTomato. Pick a well, then click **Autofocus** to focus image, and **Auto Expose** for proper exposure time. Manually adjust focus and exposure if needed.

3.4. Once proper focus and exposure have been obtained, click the camera icon to capture the picture. Then click **PROCESS/ANALZYE** above image to continue building the protocol and select the ANALYSIS tab.

3.5. Click **Cellular Analysis** in ADD ANALYSIS STEP to the right of the image and click START. Image will show highlighted cells to indicate each individual cell. The Options selection may be clicked to alter parameters to better select cells based upon the fluorescence threshold or cell size. If the imager is properly counting the cells, then click ADD STEP at the bottom of the screen.

3.6. Click the icon at the top of the screen to **Create experiment from image set**, which will open a window with the experiment. Once open, click **Procedure** under the Protocol tab and in the new window that opens select **Read**, then in the new window click full plate to select only the 60 wells that contain the cells (B2…G11). Click **OK** to save changes, then click **OK** in the Procedure window.

3.7. The plate can now be imaged by this protocol and the experimental file can be saved. Click the play icon to run the plate. Once the first plate has been imaged, image the other two plates. Upon completion of the imaging, download the cell count data to a spreadsheet for analysis. Take all images at 10x magnification.

4. Terminal MTT cytotoxicity assay

NOTE: Begin the MTT assay within two hours of completing tdTomato imaging.

4.1. Make a 5 mg/mL MTT stock solution by weighing out 25 mg of MTT and resuspending it in 5 mL of NSC medium. Vortex the solution until there are no visible precipitates of MTT, which could take several minutes.

4.2. Remove cell culture plates from the incubator and aspirate off cell culture medium. Dilute MTT 1:10 in cell culture medium and add 100 µL of MTT to each well of cells.

4.3. Incubate cells at 37 °C for 2 h. A purplish precipitate should be visible roughly in proportion to the number of cells in the well. Either aspirate the MTT solution off plates or invert the plate quickly to flick solution out of the plate.

4.4. Add 50 µL of 100% DMSO to each well and shake plates at room temperature for 10 min at 400 rpm. Read the absorbance of each well at 595 nm in a plate reader and export data to a spreadsheet for analysis.

5. Data analysis

5.1. Perform an analysis of tdTomato cell counts and absorbances with appropriate software (commercial spreadsheet, R). Calculate averages for absorbance or cell count of the three DMSO replicates on each plate for normalization purposes, then divide the value for the cell counts or absorbances for each well on the plate by this average and convert to a percentage. This yields the normalized cell count or absorbance relative to DMSO control for each plate.

5.2. Calculate the mean normalized count or absorbance and standard deviation for replicate wells on the three plates.

NOTE: At this point there should be four different sets of normalized values: one for each of the plates and one mean for the three replicate plates.

5.3. Be conservative and use a normalized value at or below 25% for the average across the three replicate plates to classify a compound as toxic. Also, because only a single treatment per compound is performed on each plate, only label compounds that fall below this threshold on all three replicate plates as toxic. Examine fluorescent images of all compounds that this analysis filters as toxic to visually confirm toxicity.

NOTE: The identification of compounds with a growth inhibitory or growth enhancing effect is more difficult to assess in an exploratory assay of this type due to the lack of replicates on each plate. However, the following is a quick way to identify compounds that may either slow down or enhance cellular growth.

5.4. Calculate the standard deviation for the three replicate DMSO controls on each plate and then filter for any compounds that have average values at least two standard deviations above or below the DMSO control. Compounds that fall out of this filter on each of the three plates may warrant further investigation.

5.5. Use the same analysis strategy for the dose response as the primary toxicity screen. Calculate the averages for the DMSO controls for each biological replicate and use these values to normalize the percent live cells or percent absorbances for each compound/dose combination. Calculate the means and standard error of the means for all compound/dose combinations for the three biological replicates.

5.6. Transform the concentration to its log value, generate a dose response curve for the log of concentration versus normalized viability, and fit the curve with a non-linear regression analysis (analysis can be performed in R or various commercial statistical packages). Calculate the lethal dose 50 (or technically in this case the viable dose 50) or concentration of compound that results in 50% toxicity from the equation of this curve. Many software packages will automatically calculate this figure.

REPRESENTATIVE RESULTS

The automated cell count data identified eleven compounds with less than 25% viability when normalized to the DMSO control while the MTT data identified these same compounds plus two additional ones (**Table 1** and **Table 2**, shaded red). The two compounds found to be toxic only in the MTT assay (wells F3 and G10) had 31% and 39%, respectively, the number of tdTomato-positive cells as the control and by rank order were the next two most toxic compounds in this library after those deemed to be toxic. The standard deviation values for these two wells did not suggest that there was an outlier amongst the three plates that skewed the averages, and when examining the numbers for each of the three replicate plates neither compound fell below the

25% threshold on any of the plates (data not shown). Representative images of tdTomato fluorescence are shown from several wells in **Figure 3**. Examination of images of the two wells discordant for toxicity between the MTT and cell count assay revealed that the compounds in F3 (**Figure 3B**) and G10 (**Figure 3C**) were both toxic although in one of the three replicate plates there were a few residual live cells in well G10 (data not shown). It appears that in this instance the MTT assay was better able to score for cytotoxicity as sometimes the imager's cell counting algorithm mistakenly counts dead/dying cells.

The MTT assay is designed for determining toxicity, but because a library may contain compounds that enhance and inhibit cell growth it would be informative to assess how well the assay quantifies both the potential growth inhibitory and proliferative effects of tested compounds. To do this a filter was used whereby compounds were classified as growth inhibitory if their normalized mean absorbances or cell counts were greater than 25% and less than two standard deviations below the control means on each of the three replicate plates (shaded yellow in **Table 1** and **Table 2**). Eleven compounds met this criterion for the cell count assay and only two for the MTT assay with only one (E10) overlapping between the two assays although two of the eleven for the cell count assay were the ones previously mentioned to be toxic by the MTT assay (F3, G10).

Compounds whose normalized means were two standard deviations above control means on each of the three replicate plates were classified as growth enhancing (shaded green in **Table 1** and **Table 2**). Only one compound fit this criterion for each assay and the compound did not overlap between the assays. Further examination of images of wells with discrepancies between the MTT and cell count assays indicated that in some instance wells in which MTT overestimated cell count relative to the tdTomato assay the cells appeared to be larger (**Figure 3C**), whereas those wells where MTT underestimated cell count relative to tdTomato the cells appeared to be smaller (**Figure 3D**). In summary, the tdTomato assay classified eleven compounds as toxic, eleven as growth inhibitory, and one as growth enhancing with thirty-four having no apparent effect on cell growth (**Table 1**). The MTT assay classified thirteen compounds as toxic, two as growth inhibitory, and one as growth enhancing with forty-one having no apparent effect on cell growth (**Table 2**).

A six-point dose response assay was conducted on three of the compounds identified as being toxic. These three compounds were the STAT3 inhibitors WP1066 (B5) and stattic (E4) and the epidermal growth factor receptor inhibitor tyrphostin 9 (E11). The doses were successive two-fold dilutions starting at a maximum concentration of 10 μM and going to a minimum concentration of 312.5 nM. The graph of the log of concentration versus normalized percentage of viable cells for both the cell count and MTT assays for one of these compounds (WP1066) is shown in **Figure 4**. The curve is relatively flat with no toxicity for the four lowest concentrations, falls rapidly at the 5 μM dose, and drops to nearly full toxicity at 10 μM. The lethal dose 50 (LD_{50}) was calculated as 4.4 μM for the tdTomato assay and 6.0 μM for the MTT assay. The tdTomato and MTT LD_{50} values for the other two compounds were 3.4 μM and 4.7 μM, respectively, for static, and 0.8 μM and 1.6 μM, respectively, for tryphostin 9.

FIGURE AND TABLE LEGENDS:

Figure 1: Plate map for master compound plate used in the primary toxicity screen. All the outer wells are shaded in grey indicating that they contained media without cells. DMSO controls (100%) are labeled in bold in wells B2, D6, and G11. All wells labeled Cmpd contained unique test compounds at 10 mM concentration in 100% DMSO.

Figure 2: Plate map for the compound plate used in the dose response assay. All the outer wells are shaded in grey indicating that they contained only media without cells. The DMSO controls were housed in column 2. Triplicates of all compound/dose combinations are indicated.

Figure 3: tdTomato fluorescent images of selected wells 72 hours post-treatment. (**A**) Well B2: DMSO control, (**B**) Well F3: cell count data suggested no toxicity but MTT data did, (**C**) Well E6: overestimated cell count by MTT relative to tdTomato count, (**D**) Well G6 underestimated cell count by MTT relative to tdTomato. All images were taken at 10x magnification using an RFP filter with 531/593 nm wavelength for excitation/emission.

Figure 4: Curve for log concentration versus viability percent DMSO for the compound in well B5. The points on the curve represent the average normalized viable cells at six doses ± standard error of the mean for three biological replicates.

Table 1: Means of tdTomato cell counts normalized to percentage DMSO control for three replicate plates ± standard deviation. Well shading indicates following: red, toxic compounds; orange, potentially growth inhibitory; green, potentially growth enhancing. No shading indicates that compounds did not appear to affect cell growth.

Table 2: Means of MTT absorbance normalized to percentage DMSO control for three replicate plates ± standard deviation. Well shading indicates following: red, toxic compounds; orange, potentially growth inhibitory; green, potentially growth enhancing. No shading indicates that compounds did not appear to affect cell growth.

Supplementary Table 1: List of primary screen compounds. Well location, name, and notes on each of the compounds that was used in the primary screen are provided.

DISCUSSION:

The primary goal of this article was to describe a strategy that could efficiently and inexpensively identify compounds affecting cell growth in a low- to moderate-throughput screening. Two orthogonal techniques were utilized to assess cell number to increase confidence in the conclusions and offer additional insights that would not be available if only a single assay was used. One of the assays used a fluorescent cell imager to directly count tdTomato-positive cells and the second was dependent on the well-characterized ability of mitochondria to cleave MTT to formazans thus serving as a proxy for cell number[10]. A total of 57 test compounds were assessed in this demonstration although the MTT wing of the assay has been used for testing a library with as many as 2,000 compound[14]. The results of the screen pointed out how the two

assays could reinforce one another in reaching certain conclusions with more confidence, and highlighted scenarios where the two assays were complementary providing additional information that would ordinarily require performing at least two separate experiments.

The most critical step in the protocol occurs just prior to plating the cells. Metabolic conditions in cell culture can become very volatile, particularly in the case of glutamine and glucose consumption, if cells are seeded at too high a density[17,18]. Under these conditions cell death will be due to factors inherent to the cell culture conditions and unrelated to the toxicity of tested compounds. The result will be an in increase in false positives for cytotoxic compounds as well as difficulty in reproducing results[18]. Success at this step requires knowing the appropriate cell density of the cell line being used, accurate determination of cell number before plating, and complete resuspension of cells to ensure homogeneous plating distribution within and across the wells of the 96-well plate. It is also important to visually confirm that cells are present at approximately the correct density 2−3 h after plating by looking at them under a microscope.

As far as the assays themselves, the most critical step for the MTT assay is ensuring that MTT is fully dissolved in the cell culture medium. Residual precipitates of MTT may by themselves result in acute cellular toxicity so it is important to completely dissolve MTT with vigorous vortexing. The most critical point for the cell counting assay is to establish the correct exposure time for imaging tdTomato. Exposure times that are too short can result in truly fluorescent cells going uncounted by the software, and exposure times that are too long can make the signal so strong that it blends neighboring cells together such that the software counts multiple cells as one cell because it is unable to resolve them[14]. Most software packages that come with imaging readers allow for a preview step showing which cells are being counted. It is important to run this preview step at several exposure times and pick the one that identifies fluorescent cells most accurately.

As with any method there are certain limitations to these assays. To gain higher throughput, the primary toxicity assay uses only a single treatment/single dose paradigm which can come at the cost of more false positives and false negatives. Additionally, although several earlier studies have shown that MTT correlates very well with cell number when using either a colony forming assay[19] or a thymidine incorporation asssay[20], treatment with certain compounds can either enhance or inhibit mitochondrial activity in such a way that the results of the MTT assay no longer correlate with cell number[21]. Results from this demonstration indicate that while MTT is excellent at identifying toxic compounds, its ability to identify compounds that either inhibit or enhance proliferation is limited perhaps because such compounds alter mitochondrial activity in a manner that it correlates less well with cell number. There are also some limitations to the tdTomato counting assay. An obvious limitation is the need to have a cell line stably expressing a fluorescent protein. Recent advances in genome manipulation have made it much easier to develop such lines but the work required to generate them may be beyond the capabilities of some labs. From a technical standpoint, the biggest issues with any cell counting assay that uses image analysis is the inability of these assays to distinguish between cells that are clustered together resulting in an undercount[14], therefore, proper plating is critical for accurate results. Another potential problem is the counting of dead or dying cells that fluoresce brightly. One way to avoid this problem is to wash cells with PBS before counting to remove these background cells. This may

not be convenient for certain less well adhering cells lines as live cells may detach upon washing. An alternative solution to this problem is to utilize the flexibility inherent in many analysis programs to customize the parameters for cell identification within a narrow range so that only live, fluorescent cells are counted.

The strategy described in this article provides a powerful way to efficiently screen up to several hundred compounds. The MTT assay readout is the physiological result of mitochondrial activity and can have cell line or compound-specific effects that can produce inaccurate results[4,21]. By combining it with a cell counting assay using a fluorescent reporter, these limitations can be greatly mitigated. As shown, comparing the results of both assays can result in close to 100% accuracy in identifying toxic compounds. A previous study has shown that in an HEK293T line stably expressing tdTomato, there is high IC_{50} correlation between MTT and tdTomato for a library of toxic compounds[22]. Although this study did not run secondary confirmations on enough hit compounds to perform a similar concordance analysis, the calculated LD_{50} values for the three compounds that were tested were similar.

In addition to their ability to reinforce conclusions about toxicity, the two assays can complement one another when addressing the potential growth inhibitory and proliferative effects of test compounds. For several test compounds the data between the two assays diverged substantially. When examining images of tdTomato fluorescence for some of these compounds, there were noticeable morphological changes between treatment and control. This suggests that the divergence in the normalized values between the two assays may be based upon physiological changes that differentially affect the MTT readout and the tdTomato cell count. The ability to acquire such data with a single experiment greatly increases the robustness of this strategy making it more generally applicable. As such, it has the capacity not only to identify toxic compounds with high accuracy but to point out compounds with more subtle effects on cell growth/physiology that can be more extensively characterized.

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DISCLOSURES:
The authors have nothing to disclose.

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	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		**DMSO**	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	
C		Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	
D		Cmpd	Cmpd	Cmpd	Cmpd	**DMSO**	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	
E		Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	
F		Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	
G		Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	Cmpd	**DMSO**	
H												

Figure 2 modified

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		Control		Compound A			Compound B			Compound C			
	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B		0.1% DMSO	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM		
C		0.1% DMSO	5 μM	5 μM	5 μM	5 μM	5 μM	5 μM	5 μM	5 μM	5 μM		
D		0.1% DMSO	2.5 μM	2.5 μM	2.5 μM	2.5 μM	2.5 μM	2.5 μM	2.5 μM	2.5 μM	2.5 μM		
E		0.1% DMSO	1.25 μM	1.25 μM	1.25 μM	1.25 μM	1.25 μM	1.25 μM	1.25 μM	1.25 μM	1.25 μM		
F		0.1% DMSO	0.625 μM	0.625 μM	0.625 μM	0.625 μM	0.625 μM	0.625 μM	0.625 μM	0.625 μM	0.625 μM		
G		0.1% DMSO	0.3125 μM	0.3125 μM	0.3125 μM	0.3125 μM	0.3125 μM	0.3125 μM	0.3125 μM	0.3125 μM	0.3125 μM		
H													

Figure 3 revised

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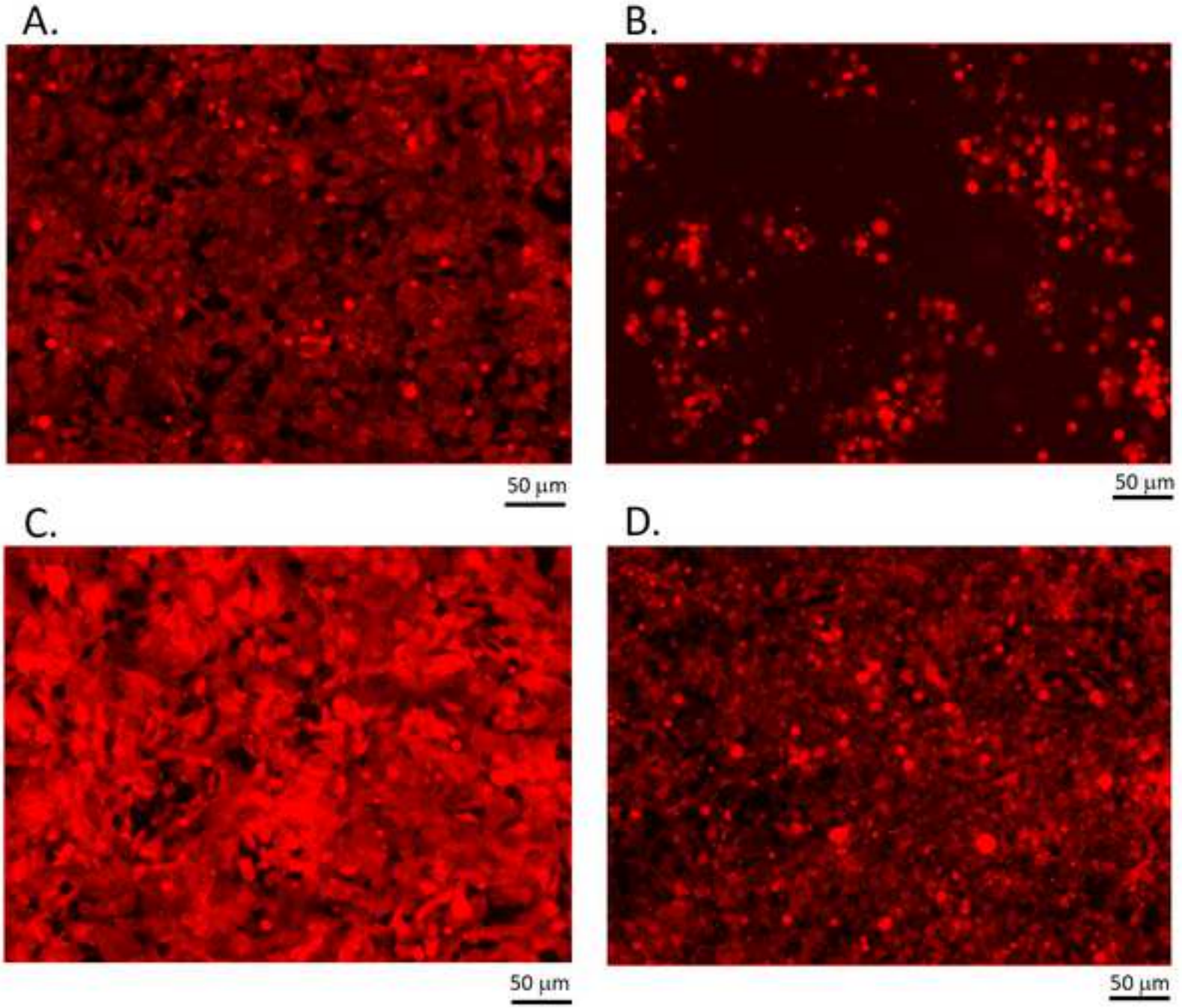

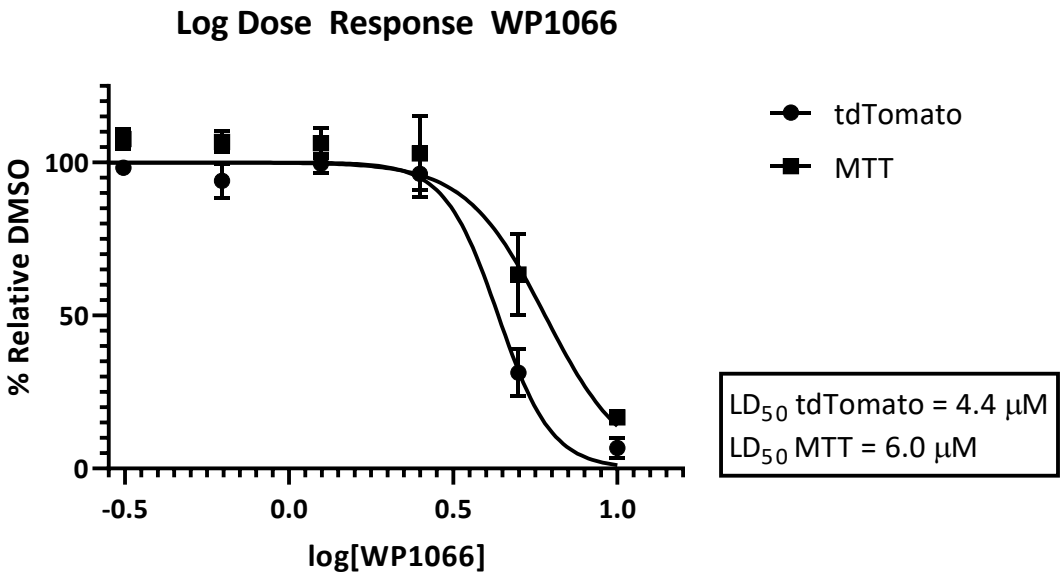

	2	3	4	5	6	7
B	100 ± 9%	89 ± 25%	83 ± 17%	7 ± 4%	109 ± 10%	60 ± 21%
C	72 ± 9%	77 ± 6%	96 ± 1%	97 ± 7%	108 ± 1%	106 ± 1%
D	100 ± 3%	94 ± 5%	108 ± 6%	96 ± 6%	107 ± 5%	105 ± 3%
E	0 ± 0%	23 ± 5%	14 ± 2%	68 ± 3%	46 ± 2%	56 ± 10%
F	86 ± 6%	31 ± 2%	11 +/1 2%	88 ± 12%	21 ± 1%	19 ± 3%
G	110 ± 3%	92 ± 2%	97 ± 4%	84 ± 4%	127 ± 5%	60 ± 6%

8	9	10	11
62 ± 9%	72 ± 1%	11 ± 2%	100 ± 3%
82 ± 3%	111 ± 4%	100 ± 4%	106 ± 2%
52 ± 4%	9 ± 2%	71 ± 3%	97 ± 6%
13 ± 2%	64 ± 4%	45 ± 4%	15 ± 1%
74 ± 10%	97 ± 7%	96 ± 8%	90 ± 5%
79 ± 8%	100 ± 4%	39 ± 10%	93 ± 5%

	2	3	4	5	6	7	8
B	108 ± 13%	94 ± 29%	121 ± 17%	15 ± 1%	90 ± 5%	101 ± 8%	96 ± 8%
C	128 ± 11%	99 ± 10%	111 ± 18%	106 ± 23%	92 ± 8%	96 ± 11%	66 ± 9%
D	114 ± 9%	107 ± 7%	115 ± 23%	110 ± 25%	99 ± 12%	98 ± 7%	76 ± 7%
E	14 ± 1%	15 ± 1%	14 ± 1%	69 ± 15%	96 ± 15%	76 ± 5%	16 ± 3%
F	78 ± 14%	16 ± 3%	15 ± 3%	65 ± 18%	17 +/3 %	15 ± 3%	64 ± 14%
G	105 ± 22%	70 ± 45%	108 ± 29%	96 ± 40%	110 ± 14%	90 ± 10%	100 ± 11%

9	10	11
102 ± 14%	16 ± 2%	90 ± 8%
86 ± 12%	91 ± 7%	89 ± 7%
15 ± 3%	80 ± 5%	96 ± 4%
97 ± 23%	37 ± 2%	106 ± 3%
103 ± 5%	101 ± 12%	105 ± 15%
100 ± 6%	21 ± 11%	94 ± 7%

Supplementary Table 1

Click here to access/download;Table;Supplementary_Table1_CompoundList.

Well	Compound
B2	DMSO
C2	cAMP
D2	FRACTALKINE
E2	LDN212854
F2	AG370
G2	DAPT
B3	AY9944
C3	STA-21
D3	GM-CSF
E3	TNP470
F3	BIO
G3	CNTF
B4	SANT
C4	AG825
D4	M-CSF
E4	STATTIC
F4	SC79
G4	DMH1
B5	WP1066
C5	INSULIN
D5	IL-3
E5	AG494
F5	LY294002
G5	IGF2
B6	SAG
C6	AG370
D6	DMSO
E6	EC23
F6	TORIN2
G6	Y27362
B7	CELECOXCIB
C7	SB525334
D7	DAPT
E7	CHIR99021
F7	LDN 193189
G7	TARAZOTINE
B8	AM580
C8	DHBP
D8	JSK
E8	DORSOMORPHIN
F8	IMATINIB
G8	BMS 493

B9	CYCLOPAMINE
C9	SEMAGACESTAT
D9	BOSUTINIB
E9	PURMORPHAMINE
F9	JAG
G9	SB431542
B10	SC79
C10	DANTROLENE
D10	TYRPHOSTIN46
E10	AM80
F10	IFN-Y
G10	PQ401
B11	DAPT
C11	A2M
D11	AG490
E11	TYRPHOSTIN9
F11	BMP-2
G11	DMSO

Notes
Negative control
Protein kinase A activator
Chemokine
Bone morphogenetic protein (BMP) receptor inhibitor
Platelet derived growth factor receptor (PDGFR) kinase inhibitor
Gamma-secretase inhibitor; neuronal differentiation positive control
7-dehydrocholestrol reductase inhibitor; hedgehog pathway inhibitor
Signal transducer and activator of transcription 3 (STAT3) inhibitor
Granulkocyte-macrophage colony-stimulating factor; cytokine
Methionine aminipeptidase-2 inhibitor
Glycogen synthase kinase-3 inhibitor; WNT pathway activator
Ciliary neurotrophic factor; neuropeptide
Smoothened receptor antagonist; hedgehog pathway inhibitor
ERBB2 inhibitor
Macrophage colony-stimulating factor; cytokine
Signal transducer and activator of transcription 3 (STAT3) inhibitor
AKT (protein kinase B) activator
Bone morphogenetic protein (BMP) receptor inhibitor
Signal transducer and activator of transcription 3 (STAT3) inhibitor

Interleukin-3; cytokine
Epidermal growth factor receptor (EGFR) inhibitor
Phosphoinosotide 3-kinase inhibitor
insulin growth factor-2
Smoothened agonist; hedgehog pathway activator
Platelet derived growth factor receptor kinase inhibitor
Negative control
Retinoic acid receptor agonist
Mechanistic target of rapamycin (MTOR) inhibitor
Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor
Cyclooxygenase-2 (COX-2) inhibitor
Transforming growth factor beta-receptor (TGBFR) inhibitor
Gamma-secretase inhibitor; neuronal differentiation positive control
Glycogen synthase kinase-3 inhibitor; WNT pathway activator
Bone morphogenetic protein (BMP) receptor inhibitor
Retinoic acid receptor agonist
Retinoic acid receptor agonist
Calcium release inhibitor
Nitric oxide donor
Bone morphogenetic protein (BMP) receptor inhibitor; 5' adenosine monophospate-activated protein ki
Tyrosine kinase inhibitor
inverse retinoic acid receptor agonist

Smoothened receptor antagonist; hedgehog pathway inhibitor
Gamma-secretase inhibitor
Tyrosine kinase inhibitor
Smoothened agonist; hedgehog pathway activator
Jagged; Notch receptor agonist
Transforming growth factor beta-receptor (TGBFR) inhibitor
AKT (protein kinase B) activator
Ryanodine receptor antagonist
Epidermal growth factor receptor (EGFR) inhibitor
Retinoic acid receptor agonist
interferon-gamma; cytokine
Insulin-like growth factor receptor (IGF1R) inhibitor
Gamma-secretase inhibitor; neuronal differentiation positive control
Extracellular glycoprotein; protease inhibitor
Epidermal growth factor receptor (EGFR) inhibitor
Platelet derived growth factor receptor (PDGFR) kinase inhibitor
Bone morphogenetic protein-2
Negative control

nase (AMPK) inhibitor

Table of Materials

Click here to access/download;Table of Materials;Malik et al_JoVE_Materials_revised.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
B-27 (50X)	ThermoFisher Scientific	17504001
BenchTop pipettor	Sorenson Bioscience	73990
BioLite 96 well multidish	Thermo Scientific	130188
Cell culture microscope	Nikon	Eclipse TS100
Cytation 5/ Imaging reader	BioTek	CYT3MFV
DMSO	Fisher Scientific	610420010
FGF-basic	Peprotech	100-18B
GelTrex	ThermoFisher Scientific	A1413202
Gen5 3.04	BioTek	
Glutamine	ThermoFisher Scientific	25030081
Microtest U-Bottom	Becton Dickinson	3077
MTT	ThermoFisher Scientific	M6494
Multichannel pippette	Rainin	E8-1200
Neurobasal medium	ThermoFisher Scientific	21103049
RFP filter cube	BioTek	1225103
TrypLE	ThermoFisher Scientific	12605036

Comments/Description
Neural stem cell medium component.
Provides ability to pipette compound library into a 96-well plate in one shot.
Any 96 well cell culture plate will work. We use these in our work.
Visual inspection of cells to ensure proper density.
Used for cell imaging and absorbance readings.
Solvent for compounds used in screen. Dissolves MTT precipitates to facilitate absorbance measurements.
Neural stem cell medium component.
Neural stem cell basement membrane matrix. Allows cells to attach to cell culture plates.
Analysis software to determine cell counts for tdTomato expressing cells.
Neural stem cell medium component.
Storage of compound libraries.
Active assay reagent to determine cellular viability.
Column-by-column addition of cell culture medium, MTT, or DMSO.
Neural stem cell base medium.
Filter in Cytation 5 used to image tdTomato expressing cells.
Cell dissociation reagent.

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The changes that have been made are described below.

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

2. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript. We have attempted to address all comments in attached manuscript. Please double-check to make sure nothing was missed.

3. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. The Table of Materials has been revised to address these comments.

4. Please address the following comments from Reviewer 4 (Editor: to clarify, Reviewer 4 in the second review and Reviewer 2 in the first review are two different referees).

a. The authors should include the list of 57 compounds tested in the study. In addition, they should include the names of the toxic compounds, cell growth inhibitors and the cell growth enhancers identified in the two assays, as it would be informative for further similar studies.
(Editor: Please provide a list of the compounds, which can be included as supplemental information.) A new table Supplementary Table 1 has been added that lists compounds, their well location, and a column on notes pertaining to each compound.
b. Please include the model, make and other particulars of the microscope used and the software used to analyze the fluorescent images along with the filters, and magnifications used in the legends of figure 3.
(Editor: Please include information of commercial entities in the Table of Materials.)

We have previously added the magnification in the Figure 3 legend. The software has been added to the Table of Materials. The microscope used to image tdTomato is not a stand-alone microscope and comes attached to the Cytation 5 imaging reader so we are unable to provide individualized specifications for the microscope; the information for the imaging reader is already included in the Table of Materials.