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High-throughput assessment of mechanistic toxicity of chemicals in miniaturized 3D cell culture

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Abstract

High-content imaging (HCI) assays on two-dimensional (2D) cell cultures often don't represent *in vivo* characteristics accurately, thus reducing the predictability of drug toxicity/efficacy *in vivo*. On the other hand, conventional 3D cell cultures are relatively low throughput and possess difficulty in cell imaging. To address these limitations, a miniaturized 3D cell culture has been developed on a micropillar/microwell chip platform with human cells encapsulated in biomimetic hydrogels. Model compounds are used to validate human cell microarrays for high-throughput assessment of mechanistic toxicity. Main mechanisms of toxicity of compounds can be investigated by analyzing multiple parameters such as DNA damage, mitochondrial impairment, intracellular glutathione level, and cell membrane integrity. IC₅₀ values of these parameters can be determined and compared for the compounds to investigate the main mechanism of toxicity. This protocol paper describes miniaturized HCI assays on 3D-cultured cell microarrays for high-throughput assessment of mechanistic profiles of compound-induced toxicity.

Keywords

High-content imaging; 3D cell culture; assay miniaturization; microarray chip platform; mechanistic toxicity

INTRODUCTION

The approval rate for new drugs has been declining rapidly, causing a drastic increase in the drug development cost over the past two decades. Pharmaceutical industries, therefore, are in a desperate need to lower the high failure rate of new molecular entities (Paul et al., 2010). Failure of drug candidates in animal and human trials is partly attributed to the toxic effect of drug candidates which could not be predicted in an early preclinical drug discovery process (Astashkina, Mann, & Grainger, 2012). Therefore, an *in vitro* cell-based assay platform that effectively detects the mechanism of toxicity of drug candidates is urgently necessary in the drug development process, thereby predicting the *in vivo* effect of drug candidates. High-content imaging (HCI) assays analyze multiple parameters related to mechanisms of toxicity and implemented in the early preclinical drug discovery process to better predict *in vivo* toxicity. Understanding the mechanism of drug action has been greatly improved due to analysis of multiple functional and morphological endpoints with HCI assays, making it an important tool in the drug discovery process (Vliet et al., 2014).

The importance of 3D cell cultures in maintaining normal cell functions such as differentiation, migration, and proliferation is well known (Astashkina & Grainger, 2014; Echeverria et al., 2010; Q. Li et al., 2011; Meng, Leslie, Zhang, & Dong, 2014). As such, HCI assays on 3D cell cultures (3D HCI) could provide a better understanding of compound-induced toxicity by mimicking and analyzing morphological and functional features of human tissues (Justice, Badr, & Felder, 2009). However, performing HCI assays on conventional 3D culture platforms such as 96-well plates possess certain limitations due to difficulty in handling and imaging of relatively large volume of samples which reduce the throughput of 3D HCI. Confocal microscopy which is widely used for image acquisition of 3D cells and tissues has limited applications in high-throughput 3D HCI due to slow point scanning, photobleaching, and phototoxicity (Jahr, Schmid, Schmied, Fahrbach, & Huiskens, 2015; Scherf & Huiskens, 2015). In addition, light-sheet microscopy which has recently been reported as a promising imaging tool in HCI, requires complete changes in experimental setup with commercial systems not being fully accessible (Reynaud, Peychl, Huiskens, & Tomancak, 2015). To overcome these issues, we have developed miniaturized 3D cell culture systems that allow the whole sample depth to fit within the focus depth of a normal objective due to its small dimension. Moreover, miniaturization of 3D cell culture reduces reagent consumption, minimizes the use of valuable materials such as patient-derived cells, and further facilitates combinatorial approaches and high control over microenvironmental cues resulting in highly reproducible outcomes (Håkanson, Cukierman, & Charnley, 2014; Montanez-Sauri, Beebe, & Sung, 2015).

This protocol aims at providing easy-to-follow steps required to implement HCI assays on miniaturized 3D-cultured human cells for high-throughput assessment of compound-induced toxicity. The overall procedures for HCI in miniaturized 3D cell culture for assessment of mechanistic toxicity of compounds can be divided into three basic protocols. The basic protocol 1 explains the steps necessary for the functionalization of micropillar chips, cell printing on the micropillar chip, and miniaturized 3D cell culture. The protocol 2 provides the steps necessary for exposing human cells printed to model compounds and fluorescent dye staining for HCI assays. Finally, the protocol 3 describes acquisition of 3D cell image, image processing, data analysis, and statistical analysis.

BASIC PROTOCOL 1: Miniaturized 3D cell culture

This protocol provides detailed steps involved in high-throughput cell printing with a bioprinter (S+ Microarrayer) for the generation of miniaturized 3D cell culture on a micropillar/microwell chip platform and the measurement of dose responses of model compounds in the miniaturized 3D culture of human cells using an automated fluorescent microscope (S+ Scanner) for HCI assays. Incorporating 3D cell growth on the chip platform in combination with HCI assays can be established as a reliable technique for testing toxicity of compounds in an early stage of preclinical drug discovery and better predict *in vivo* toxicity in clinical trials. The procedures provided below enable users to efficiently determine the mechanism of toxicity of unknown compounds in high-throughput and without the excessive use of cells, hydrogels, and other reagents.

Materials

- Hep3B human hepatoma cell line (ATCC Cat# HB-8064, RRID:CVCL_0326) as a model cell line
- Complete RPMI-1640 medium (ThermoFisher, cat. no. 11875–085) for culturing Hep3B cells
 - The complete RPMI medium is prepared by supplementing RPMI-1640 with 10% fetal bovine serum (FBS) (Corning, cat. no. 35–010-CV, Tewksbury, MA), 0.1% gentamicin, and 1% penicillin/streptomycin.
- Reagents for coating and gelation (all from Sigma Aldrich, St. Louis, MO)
 - Poly(maleic anhydride *alt*-1-octadecene) (PMA-OD) (Sigma Aldrich, cat. no. 419117) dissolved in ethanol, 1% (w/v) stock
 - Poly-L-lysine (PLL), 0.01% (w/v) stock (Sigma Aldrich, cat. no. P4707)
 - Barium chloride (BaCl₂), (Sigma Aldrich, cat. no. B0750) dissolved in sterile deionized distilled water, 100 mM stock
 - Thrombin, (Sigma Aldrich, cat. no. T4648) dissolved in DPBS, 100 NIH units/mL stock
- Hydrogels
 - Alginate acid (Sigma Aldrich, cat. no. A1112) dissolved in sterile deionized distilled water for 3 days with stirring, 3% (w/v) stock
 - Fibrinogen (Sigma Aldrich, cat. no. F8630) dissolved in 0.9% NaCl, 100 mg/mL stock
- Moxi Z mini automated cell counter (ORFLO Technologies, MXZ001)
- Micropillar chip and microwell chip (Figure 1) (Medical & Bio Device (MBD) Korea, Suwon, Republic of Korea)
- S+ Microarrayer (Advanced Technology Inc. (ATI), Incheon, Republic of Korea)

Preparation of cell suspension (Timing: 30 mins)

1. Seed 1.5×10^6 Hep3B cells between passage number 10 – 15 in one T-75 flask in 10–12 mL complete RPMI-medium.
2. Grow cells in the complete RPMI medium at 37°C in a 5% CO₂ incubator until the cells reach 80 – 90% confluency with media change every two days.
3. Remove the old media from the flask by tilting the flask and aspirating the media.
4. Rinse the flask with 5 mL of 1x PBS with pH of 7.4. Dispense PBS gently from the side of the flask to avoid detaching the cells and aspirate the PBS.

5. Detach the cells by adding 1 mL of 0.05% trypsin/EDTA into the T-75 flask and incubating for 2–4 minutes at 37°C in a 5% CO₂ incubator.
6. After the cells are completely detached, add 5 mL of the complete RPMI medium into the cell/trypsin solution to stop the trypsin reaction and mix well.
7. Transfer the cell suspension into a 15 mL conical tube with a 10 mL pipette tip.
8. Centrifuge the cell suspension at 1200 rpm (200 g) for 4 min at room temperature.
9. Aspirate the supernatant without disturbing the cell pellet and add 1 mL complete RPMI medium.
10. Break the cell pellet and re-suspend the cells in the complete RPMI medium.
11. Mix 4 µL cell sample in 196 µL media to prepare a 200 µL cell suspension at 50-fold dilution.
12. Take 75 µL cell suspension and load it in a Moxi Z cassette inserted inside the Moxi Z mini automated cell counter to measure the cell density in number of cells per milliliter. *Note: Avoid using high density cell suspension to get accurate measurement. The actual cell density is 50-fold higher than the density displayed in the counter because of dilution.*
13. Prepare 1 mL cell suspension at a final concentration of 6×10^6 cells/mL with appropriate dilution of cell suspension in complete RPMI medium.

Preparation of miniaturized 3D cell culture on the micropillar chip (Timing: 3 days)

14. Coat micropillar chips with 0.01% (w/v) PMA-OD by immersing the micropillar chips in a shallow-well staining plate containing 2 mL of 0.01% PMA-OD and blow dry the micropillar chips with air. *Note: Coating of micropillar chips with PMA-OD should be done in advance i.e. at least 2 days before preparation of cell suspension.*
14. Place the micropillar chips in a bioassay dish and incubate for 3–4 hours at room temperature to completely dry the PMA-OD coated chips.
15. Prepare a 1 mL mixture of 0.0033% PLL and 16.66 mM BaCl₂ in sterile deionized water by mixing 333 µL of 0.01% PLL, 333 µL of 50 mM BaCl₂, and 333 µL of sterile deionized water at 1:1:1 ratio. *Note: For sensitive cell lines such as primary cells or stem cells, a mixture of 0.0033% PLL and 25 mM CaCl₂ should be used as BaCl₂ could be toxic against some cell types.*
16. Print 0.0033% PLL-16.66 mM BaCl₂ mixture onto the PMA-OD-coated micropillar chips using a microarray spotter (S+ Microarrayer) at a volume of 60 nL per pillar (Figure 2) and incubate for minimum of 3–4 hours at room temperature to completely dry the spots. *Note: The PMA-OD coating and PLL-BaCl₂ printing should be done in advance for the micropillar chips to be ready for cell printing.*

17. Add 100 NIH units/mL thrombin in complete RPMI media at 1:10 dilution to prepare complete RPMI media with 10 NIH units/mL of thrombin. Print complete RPMI supplemented with 10 NIH units/mL of thrombin at a volume of 950 nL per well in the microwell chips while maintaining the chip deck at 7°C to avoid water evaporation. *Note: Microwell chips with cell growth media should be stored in a humid chamber with water to avoid evaporation until the micropillar chips are ready to be sandwiched.*
18. Mix 500 µL Hep3B cell suspension (**from step 13**) with 250 µL of low-viscosity 3% (w/v) alginate and 250 µL of 100 mg/mL fibrinogen at a ratio of 2:1:1 to get a final cell concentration of 3×10^6 cells/mL in 0.75% (w/v) alginate and 25 mg/mL fibrinogen. *Note: Mixing of cells with alginate and fibrinogen should be done gently with multiple aspiration and dispensing to ensure the formation of uniform cell suspension in hydrogel.*
19. Print 60 nL of Hep3B cells suspended in the mixture of alginate and fibrinogen (180 cells/spot) on top of dried PLL-BaCl₂ spots for cell encapsulation and spot attachment while maintaining the chip deck at 7°C.
20. After leaving the printed chips on the chip deck for 2 min for gelation, sandwich the micropillar chips containing cell spots with the microwell chips containing 950 nL of complete RPMI with 10 NIH units/mL of thrombin for 30 min (Figure 3B).
21. Remove the old microwell chips and sandwich the micropillar chips with microwell chips containing fresh complete RPMI without thrombin. *Note: This double stamping is necessary to remove excess BaCl₂.*
22. Store the sandwiched micropillar/microwell chips in a humidified petri dish (Figure 3C) and incubate at 37°C in a 5% CO₂ incubator for 72 h before compound treatment (Figure 4).

BASIC PROTOCOL 2: Compound treatment and cell staining

This protocol describes the method of exposing 3D-cultured cells on the micropillar chips to model compounds with known mechanisms of toxicity and fluorescence staining of 3D cells for identification of multiple endpoints related to those mechanisms of toxicity. The model compounds and their known mechanisms of toxicity along with the recommended concentrations for this procedure are listed in Table 1. Similarly, fluorescent dyes used in this protocol, the endpoints targeted, and their recommended concentrations are listed in Table 2.

Materials

- Model compounds (all from Sigma Aldrich unless otherwise specified)
 - Acetaminophen (8.3 – 2,125 µM) (Sigma Aldrich, cat. no. A5000)
 - Lovastatin (1.8 – 472 µM) (Sigma Aldrich, cat. no. PHR1285)
 - Rotenone (0.12 – 30 µM) (Sigma Aldrich, cat. no. R8879)

- Tamoxifen (0.46 – 118 μM) (Sigma Aldrich, cat. no. T5648)
- Sodium citrate (5.5 – 1,417 μM) (Sigma Aldrich, cat. no. PHR1416)
- Menadione (0.7 – 177 μM) (Sigma Aldrich, cat. no. M5625)
- Fluorescent dyes (ThermoFisher Scientific, Waltham, MA)
 - Hoechst 33342 (ThermoFisher, cat. no. H1399) dissolved in DMSO, 10 mM stock
 - Tetramethyl rhodamine methyl ester (TMRM, ThermoFisher, cat. no. T-668) dissolved in DMSO, 50 mM stock
 - Monochlorobimane (mBCl, ThermoFisher, cat. no. M-1381MP) dissolved in DMSO, 200 mM stock
 - Calcein AM (ThermoFisher, cat. no. C1430) dissolved in DMSO, 1 mM stock
- Deep-well staining plate (MBD Korea, Suwon, Republic of Korea)
- Shallow-well staining plate (MBD Korea, Suwon, Republic of Korea)

Compound treatment (Timing: 2 days)

22. Prepare a compound in DMSO plate by preparing compound stock solutions in DMSO (or water for sodium citrate) and diluting each stock solution serially at 4-fold in a 384-well plate to prepare five dosages and one DMSO-alone control.
23. Add 40 μL compound stocks in a round-bottom 384-well plate and 30 μL DMSO in 5 consecutive wells next to the well with 40 μL of compound stock.
24. Take 10 μL compound stock, transfer to the adjacent well and mix well by aspirating and dispensing at least 10 times.
25. Repeat **step 24** serially by transferring 10 μL of the mixed solution from the previous well to next well with 30 μL DMSO until the last well with 30 μL DMSO remains.
26. Prepare a compound in cell growth media plate by diluting compound solutions in DMSO at 200-fold in a 96-well plate. *Note: DMSO concentration in the final compound solution should always be less than 0.5% (v/v) due to its toxic effect on cells. Compounds should either completely dissolve in growth media or form stable colloidal suspension.*
27. Dispense 298.5 μL of growth media in a 96-well plate in 6 rows and 6 columns (for 6 compounds and 6 dosages).
28. Take 1.5 μL of serially diluted compounds from the compound in DMSO plate with a multi-channel pipette and mix the solution well in 298.5 μL growth media of the same row of the 96-well plate. Repeat this step from low to high concentrations for all five concentrations and DMSO control. *Note: Always add*

the diluted compounds in DMSO from low to high concentrations in the 96-well plate to avoid potential “carry-over” of the compound.

29. Print 950 nL of the model compounds at five dosages and one control in the microwell chips (Figure 5). *Note: The dosage range of the model compounds used in this protocol are selected based on their IC₅₀ values obtained from literature.*
30. Separate the micropillar chips from the microwell chips containing growth media and sandwich the micropillar chips with the microwell chips containing six model compounds. Incubate for 48 h at 37°C in a 5% CO₂ incubator prior to cell staining for HCl assays.

Cell staining (Timing: 2–3 hours)

31. Prepare a working solution of fluorescent dyes in saline solution following the steps listed below. *Note: The volume mentioned below is sufficient for 4 micropillar chips. Readers are recommended to scale the volume up or down depending on the number of replicates per chip and number of chips required for the experiment.*
 - Add 20 µL of the stock solution of Hoechst 33342 in 8 mL of saline solution to prepare 25 µM Hoechst 33342 solution.
 - Dilute the stock solution of TMRM 100-fold to get a working stock concentration of 0.5 mM. Add 8 µL of 0.5 mM TMRM working stock in 8 mL saline solution to get 0.5 µM TMRM solution.
 - Add 4 µL of mBCl stock in 8 mL saline solution to get a working solution of 100 µM mBCl.
 - Add 2 µL of calcein AM stock in 8 mL saline solution to prepare 0.25 µM calcein AM solution.
32. Separate the micropillar chips from the microwell chips containing compounds and rinse twice for 5 min each with 5 mL of the saline solution in the deep-well staining plate (Figure 6A).
33. Remove excess saline solution from the micropillar chips before staining. Dispense 2 mL of Hoechst 33342, TMRM, mBCl, and calcein AM staining solutions separately in the shallow-well staining plate and incubate the micropillar chips for 45–60 mins in the dark (Figure 6B).
34. Rinse the stained micropillar chips twice for 10 min each after staining to remove excess dyes from the cells and dry the chips in the dark for at least 3–4 h before image acquisition.

BASIC PROTOCOL 3: Image processing and data analysis

This protocol describes the steps involved in high-throughput image processing and data analysis from the micropillar chip using ImageJ, S+ chip analysis, and GraphPad Prism.

Materials

- S+ Scanner (ATI, Incheon, Republic of Korea)
- ImageJ (RRID:SCR_003070) (NIH)
- S+ Chip Analysis (MBD Korea, Suwon, Republic of Korea)
- GraphPad Prism ((RRID:SCR_002798) GraphPad Software, La Jolla, CA)

Fluorescent image acquisition with S+ Scanner (Timing: 1–2 hours)

35. Load the dried micropillar chips into an automated fluorescent microscope (S+ Scanner) and open a desired chip file from the list of chip files. *Note: Chip files should be selected based on the chip type and magnification of the objective lens for image acquisition. Magnification of 4X is desired for high-throughput image acquisition of entire cell spots.*
36. Select appropriate filter channel based on the fluorescent dye used for staining the cells on the micropillar chip. The S+ Scanner contains four filter channels for detecting multicolor, blue, green, and red fluorescent dyes, individually or simultaneously. A multiband filter set (DA/FI/TR/Cy5-A-000 from Semrock) for measuring blue, green, orange, and red fluorescent dyes simultaneously, a red filter (TxRed-4040C-000 from Semrock) for measuring deep red fluorophores, a blue filter (DAPI-5060C-000 from Semrock) for measuring deep blue fluorophores, and a green filter (XF404 from Omega) for green fluorophores are typically used.
37. Adjust the exposure times for the filter channels based on histogram and brightness of the fluorescent images to obtain optimum fluorescence intensity and prevent photobleaching of fluorescence.
38. Scan the micropillar chip and save the images (Figure 7) under a desired folder name.

Image processing and data analysis (Timing: 3–4 hours)

Fluorescence intensity of the images obtained from the micropillar chips are quantified using ImageJ software (NIH), and standard dose-response curves for each compound per readout are plotted using image analysis software (S+ Chip Analysis). In ImageJ, a batch processing macro for processing hundreds of images can simply be created by recording the steps used to process a single image and saving it as a plugin (Figure 8). The macro used for extracting the fluorescent intensity is shown below.

39. Eliminate background fluorescence (if any) from the images using background subtraction function in ImageJ before extracting the fluorescence intensity from the images.
40. Convert background subtracted images to gray scale using Type function in Image tab. *Note: Images can be converted to 8-bit, 16-bit or 32-bit gray scale images.*

41. Apply a threshold by selecting a threshold algorithm in Threshold sub-function in Adjust function in Image tab to segment the cell spots and to limit the intensity extraction from fluorescently labelled cellular regions.
42. Using Set Measurements function in Analyze tab, select the parameters that need to be measured. *Note: Integrated density, limit to threshold, and display label should always be selected to measure the fluorescence intensity of the stained cellular region. Other parameters can be selected based on individual requirements.*
43. Measure the fluorescence intensity from the images using Measure function in Analyze tab. A result window will appear with the fluorescent intensities. Save the result.
44. After extraction of fluorescence intensity from the images, copy the fluorescence intensity data from text file and paste in excel file corresponding to the layout of compounds in microwell chips (Figure 5).
45. Convert the excel file to file in “.scn” format for analysis in S+ Chip Analysis software and save it in a desired folder.
46. Input the folder containing data file in .scn format and manually enter the name of the compound, highest concentration, dilution ratio, and name of the fluorescent dye to plot dose response curves and generate IC₅₀ values (concentration of compound that inhibits 50% of cellular response) (Figure 9).
47. Dose-response curves are obtained by the equation mentioned below

$$Y = \text{Bottom} + \left[\frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log IC_{50} - X) \times H}} \right]$$

where X is the log concentration of a compound, Y is cellular responses (top and bottom being highest and lowest fluorescence), H is the slope of the dose-response curve, and IC₅₀ is the concentration of a compound where the cellular response is reduced by half.

Assay validation (Timing: 1 hour)

It is important to measure the robustness and reproducibility of an assay to develop and validate new assays on any cell culture platforms. Coefficient of variation (CV) is measured to determine the reproducibility of assays carried out in different chips and on different days and Z' factor is measured to determine the robustness of a new assay.

48. Measure the robustness of new assays by calculating Z' factors with the equation mentioned below

$$Z' = \frac{(\text{Avg}_{Max} - 3SD_{Max}) - (\text{Avg}_{Min} + 3SD_{Min})}{\text{Avg}_{Max} - \text{Avg}_{Min}}$$

where Avg._{Max} is an average of maximum fluorescence intensity from fully viable cells on the chip, SD_{Max} is a standard deviation of maximum fluorescence intensity, Avg._{Min} is an average of minimum fluorescence intensity from the dead cells affected by the highest dose of compound tested, SD_{Min} is a standard deviation of minimum fluorescence intensity. *Note: The Z' factor between 0.5 – 1 is considered highly robust for an assay.*

49. Measure the reproducibility of an assay by calculating the CV value which is the ratio of the standard deviation (SD) to the average (Avg.).

$$CV = \frac{SD}{Avg} \times 100$$

The HCI assay data obtained from 3D-cultured cells on the chip platform exposed to no compound can be used to calculate CV values of cell printing and assess day-to-day variability. *Note: The experimental errors (CV value) below 25% is acceptable.*

Statistical analysis (Timing: 1–2 hours)

Statistical analysis using One-way analysis of variance (ANOVA) should be performed to determine the main mechanism of toxicity among multiple mechanisms of action assessed with HCI assays. For One-way ANOVA analysis of the main mechanism of toxicity, follow the procedures provided.

50. Arrange the IC₅₀ values and standard error mean (SEM) of individual compounds from different assays in an excel file obtained from S+ Chip Analysis. *Note: For statistical analysis, IC₅₀ values and standard error from at least three independent set of experiments are required.*
51. Open GraphPad Prism select 'Grouped' under "New table & graph" section and select 'Start with an empty data table' under "Sample data" section.
52. In "Choose a graph" section select 'category graph, symbols only' graph, select 'Enter and plot error values already calculated elsewhere' under "Y subcolumns for replicates or error bars" and select 'Mean & SEM' from dropdown menu. Click "Create".
53. Enter the IC₅₀ values and SEM from the excel file for the compound from all the assays in the data sheet (Figure 10).
54. In the "Change" tab, select 'Graph Type' from the dropdown menu. A "Change Graph Type" window will appear in "Column", select 'Column mean, error bars, vertical' graph, and press OK.
55. For one-way ANOVA analysis of the data, click Results folder and select 'One-way ANOVA' in "Column analyses" and press OK.
56. Select desired test from the dropdown menu in "Post Test" to compare the data at 95% confidence interval.

REAGENTS AND SOLUTIONS

Sterile saline solution

- Dissolve 8.1 g of NaCl and 2.9 g of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ in 1 L of sterile deionized water to prepare a saline solution containing 140 mM NaCl and 20 mM CaCl_2 . *Note: The saline solution with 20 mM CaCl_2 is used only if the viability of the cells encapsulated in alginate on the micropillar chip is not affected by the salts. Alginate is sensitive to chelating agents such as phosphate ions in some buffer solutions. Thus, phosphate-buffered saline (PBS) is recommended when working with hydrogels other than alginate.*

Sterile phosphate-buffered saline (PBS) solution

- Prepare 1x PBS containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 2 mM KH_2PO_4 . Adjust pH to 7.4 with 1 M HCl.

Alginate stock solution

- Dissolve 1.5 g alginic acid sodium salt in 48.5 mL sterile de-ionized distilled water in a sterile glass sample vial to prepare a stock solution of 3% (w/v) alginate. Stir the solution continuously for 2–3 days in a magnetic stirring plate to completely dissolve the alginate. Store the stock solution at 4 °C.

Thrombin stock solution

- Dissolve 20.7 mg of powder thrombin in 20.3 mL of PBS to prepare a stock solution of 100 NIH units/mL. One mg of thrombin contains 98 NIH units of protein (i.e., 2028.6 NIH units of thrombin in 20.7 mg). Prepare aliquots of 500 μL of thrombin in PBS and store the thrombin stock solution at -20°C to avoid multiple freezing and thawing of the enzyme stock.

Fibrinogen stock solution

- Prepare 0.9% NaCl solution in distilled water and sterile filter it. Prepare 100 mg/mL fibrinogen stock by adding 100 mg of fibrinogen in 1 mL of warm 0.9% NaCl. Dissolve the solution by agitating it slightly. *Note: Fibrinogen solution should not be vortexed, if needed, dilute the stock concentration further to obtain a clear solution. Fibrinogen solution cannot be stored and should be prepared fresh every time.*

Compound stock solution

- Dissolve powder form of compounds in DMSO to prepare compound stock solution except for sodium citrate which is dissolved in water. Add 20–40 mg of compound in a glass vial and dispense DMSO at a predetermined volume. *Note: Volume of DMSO depends on the compound stock concentration which should be prepared at 200-fold higher concentrations than the desired final concentration. Stock concentration varies depending on the molecular weight and*

the toxicity of the compounds. Typical stock concentration is prepared between 50–300 mM.

- Sonicate the solution to dissolve it completely. Do not vortex the compound to avoid attaching the undissolved compound on the side of the vial. *Note: Try different organic solvents if the compound is insoluble in DMSO. Ethanol or methanol is a common alternative for DMSO.*
- Store aliquots of the compound stock solutions in a - 20°C freezer until use.

Fluorescent substrate stock solutions

Hoechst 33342 solution

- Dissolve 100 mg of Hoechst 33342 in 16.23 mL DMSO to prepare a stock solution of 10 mM Hoechst 33342. Store aliquots of 10 mM Hoechst 33342 in a - 20°C freezer protected from light for future use.

Tetramethyl rhodamine methyl ester (TMRM) solution

- Dissolve 25 mg TMRM in 1 mL DMSO and vortex it for 1 minute to prepare a stock solution of 50 mM TMRM. Store aliquots of 50 mM TMRM in a - 20°C freezer protected from light until use.

Monochlorobimane (mBCI) solution

- Dissolve 25 mg of mBCI in 550 µL DMSO to prepare a stock solution of 200 mM mBCI. Store aliquots of 200 mM mBCI in a - 20°C freezer protected from light until future use.

Calcein AM solution

- Dissolve 1 mg of calcein AM in 1 mL DMSO to prepare a stock solution of 1 mM calcein AM. Store aliquots of 1 mM calcein AM in a - 20°C freezer protected from light until future use.

COMMENTARY

Background Information

HCI assays enable the understanding of mechanisms of action of drug candidates due to its ability to analyze various functional and morphological endpoints such as DNA damage, mitochondrial impairment, oxidative stress, nuclear morphology, and so on. The majority of HCI assays, however, are still performed on 2D cell monolayers which do not mimic complex biological systems and provide unpredictable information due to limited cell-cell and cell-extracellular matrix (ECM) interactions and the lack of morphological, physiological, and metabolic properties. This in turn limits the predictability of drug toxicity/efficacy *in vivo* (Page, Flood, & Reynaud, 2013). Moreover, existing 3D culture platforms have limited applications in HCI assays due to their low throughput in terms of handling large sample volumes and imaging of large sample sizes. As such, predictive 3D cell-based screening for drug efficacy and toxicity requires the development of high-

throughput platforms compatible with automated robotic systems for rapid and reproducible testing of 3D cell culture.

Microarray chip platforms are developed for robust high-throughput 3D cell culture and compound screening. With 532 micropillars and microwells, an array of cells, viruses, enzymes, and compounds can be screened for toxicity tests. For miniaturized 3D cell culture on microarray chip platforms, cells are typically encapsulated in a hydrogel matrix and printed on a functionalized micropillar chip (Håkanson et al., 2012). Due to its small dimension, the miniaturized 3D cell culture on the micropillar/microwell chip platform allows the use of an automated wide-field fluorescent microscope for high-throughput imaging. In this protocol, a combination of alginate and fibrin was used for cell encapsulation and 3D cell culture because alginate is biologically inert and provides structural rigidity for cell encapsulation, and fibrin provides necessary ECM properties to support cell growth and spheroid formation. Various hydrogel matrices such as a mixture of alginate and Matrigel can be used depending on the cell types and applications.

The model compounds used in this procedure has been reported to demonstrate a wide range of mechanism of action. For example, acetaminophen is a medication for pain and fever which is known to have side effects in the liver in the presence of drug-metabolizing enzymes (DMEs) (Chen et al., 1998). Although nontoxic at low dosages, acetaminophen can cause necrosis of liver cells at high dosages, resulting in liver failure and death (Botting, 2000; James, Mayeux, & Hinson, 2003). Lovastatin is a lipid-lowering therapeutic drug used in the treatment of hyperlipidemia and is known to have anticancer properties. Lovastatin is further known to lower the growth rate, reduce intracellular calcium concentrations, decrease mitochondrial membrane potential, and affect cell viability (Walther, Emmrich, Ramer, Mittag, & Hinz, 2016; Wei, Mi, & Zhou, 2007). Moreover, rotenone is one of the widely studied compound against various organ toxicity. It is used as an insecticide and pesticide and its mechanisms of toxicity are known to be mitochondrial impairment, oxidative stress, and apoptosis (Isenberg & Klaunig, 2000; N. Li et al., 2003). Rotenone inhibits mitochondrial complex I, affecting the electron transport chain in mitochondria and causing ROS generation. Accumulation of ROS inside cells decreases the glutathione level resulting in oxidative stress and causes mitochondrial impairment leading to apoptosis (Cabezas, El-Bachá, González, & Barreto, 2012; Jin et al., 2007; N. Li et al., 2003; Moon, Lee, Park, Geum, & Kim, 2005). Tamoxifen is an anticancer drug with hepatotoxicity as its commonly reported side effect (Ford, Franks, Radominska-Pandya, & Prather, 2016; Henderson, Wendy, & Kim, 2016; Khuroo et al., 2014; Lin et al., 2014; Tabassum, Rehman, Banerjee, Raisuddin, & Parvez, 2006; Villegas et al., 2016). Mitochondrial impairment is known to be the main mechanism of tamoxifen-induced toxicity (Lee, Kang, Lee, & Kim, 2000; Tabassum et al., 2006; Tolosa et al., 2012; Tolosa, Carmona, Castell, Gómez-Lechón, & Donato, 2015). Likewise, menadione is a vitamin K3 supplement effectively used in the treatment of cancer (Akiyoshi, Matzno, Sakai, Okamura, & Matsuyama, 2009; Delwar et al., 2012; Niemczyk et al., 2004) and also known to be an oxidative stress inducer (De Assis et al., 2015). Major mechanisms of menadione-induced toxicity has been reported to be the depletion of intracellular glutathione (Cho, Kim, Lee, & Chung, 1997) and mitochondrial impairment (Niemczyk et al., 2004; Tolosa et al., 2012) *via* generation of ROS (Castro, Mariani, Panek, Eleutherio, & Pereira, 2008; Ip, Woo, Lau, & Che, 2004).

Fluorescent dyes used in this protocol were selected to cover major endpoints/mechanisms of toxicity. Hoechst 33342, TMRM, mBCl, and calcein AM were used to measure DNA damage, mitochondrial impairment *via* changes in mitochondrial membrane potential (MMP), oxidative stress *via* decrease in intracellular glutathione levels, and cell membrane integrity, respectively. Hoechst 33342 is a bisbenzimidazole derivative that binds to double stranded DNA especially adenine-thymine rich strands of DNA and emits blue fluorescence (Parish, 1999). This dye helps in separating living cells from dead cells based on DNA content and chromatin distribution in living cells and thus enables the determination of the necrotic effect of toxic compounds (Parish, 1999). TMRM is a cell permeable fluorescent dye that accumulates in mitochondria due to its charge and solubility in mitochondrial membrane and is driven by MMPs (Wink et al., 2014)(Scaduto & Grotyohann, 1999). Mitochondria is responsible for electron transport chain, reducing the nicotinamide adenine dinucleotide ion (NAD⁺) to NADH, generating an electrochemical gradient (and an electric potential) across the internal membrane of mitochondria. Thus, the reduction in MMP is indicative of mitochondrial damage in cells due to the toxic effect of compounds. mBCl is a non-fluorescent cell permeant dye that emits blue fluorescence after conjugation with a thiol group. Low levels of glutathione indicates the building of oxidative stress within the cell making it an endpoint for determining the toxic effect of ROS produced by unknown compounds (Kamencic, Lyon, Paterson, & Juurlink, 2000). Calcein AM is a hydrophobic compound that permeates live cells and is hydrolyzed by intracellular esterases into calcein which emits green fluorescence (Bratosin, Mitrofan, Pali, Estaquier, & Montreuil, 2005). Calcein is also a relatively hydrophilic compound that remains in the cytosol of cells with intact membranes and therefore widely used to measure cell viability.

Image processing and data analysis is highly efficient and high-throughput with ImageJ and S+ Chip Analysis. ImageJ is an open source image processing software developed by NIH that can process thousands of images in a single batch. A batch processing macro or plugin can be created based on individual's requirement. S+ Chip Analysis is custom-made data analysis software developed by MBD Korea to analyze the fluorescent intensities from microarray chip platforms with corresponding dosages and drugs and thereby generating sigmoidal dose-response curves rapidly.

Critical Parameters

Cell seeding density is a critical factor in 3D HCI which requires optimization depending on the cell doubling time and assays selected. It is important to conduct an initial concentration-response experiment to optimize the fluorescent dye concentration for a desired cell seeding density. Incubation time for 3D cell culture before compound treatment should be selected considering the cell growth and spheroid formation in 3D. Cell growth and spheroid formation depend on the cell lines used and their doubling time. Therefore, optimization of a pre-incubation period is needed depending on the cell growth and spheroid formation in 3D culture. High seeding densities may cause overgrowth of cells in 3D which can affect the image acquisition and processing. Moreover, overgrowth of cells may cause spot detachment due to excessive secretion of matrix metalloproteinases (Ha, Kim, Lee, & Kim, 2004; Mason & Joyce, 2011; Weng, Chou, Ho, & Yen, 2012).

Duration of compound treatment can affect the mechanism of cell death, thus affecting the outcome from various HCI assays. The period of compound treatment should be selected based on the doubling time of cells and the assays selected. In general, 48 hours of compound treatment is considered optimum for major endpoint assays excluding the apoptosis assay (e.g. YO-PRO-1) which may need shorter time period to observe the changes in plasma membrane and nucleus. Similarly, assays for evaluating morphological changes such as nuclear morphology and cell size may require longer duration of compound treatment and thus requires careful optimization of compound incubation. In addition, the concentration of fluorescent dyes selected and the incubation time with fluorescent dyes can severely affect the fluorescence intensity of cells. Since the assessment of mechanism of toxicity is solely based on the measurement of fluorescence intensity from various HCI assays, it is suggested to perform optimization experiments to determine the optimum concentration and incubation time for obtaining images with bright fluorescence.

IC₅₀ values obtained from dose-response curves could be compared with LD₅₀ values and other IC₅₀ data from literature to correlate *in vitro* chip data with *in vivo* data in order to determine predictivity of compound toxicity. In addition, Hep3B cells do not express major DMEs necessary for bioactivation of compounds (Guo et al., 2011; Tolosa et al., 2012) due to which the toxicity of metabolism-sensitive compounds such as acetaminophen may not be detected. This inability to express major DMEs can influence predictivity of hepatotoxicity *in vivo* (Rodríguez-Antona et al., 2002). The selection of cell lines expressing DMEs are recommended to determine the effect of metabolism on the toxicity of unknown compounds. Furthermore, a large set of model compounds (typically 100 compounds) is required to determine meaningful sensitivity and selectivity of HCI assays.

Troubleshooting

It is important to monitor cell growth and spheroid formation frequently until the day of cell staining. Spot detachment is a critical factor that needs careful consideration when removing and stamping micropillar chips from and into microwell chips. To ensure robust spot attachment, surface chemistry between the hydrogel selected and the chip surface should be optimized. Evaporation of growth media and drying of cell spots may occur due to small volumes for which proper temperature and humidity should be maintained while printing and incubating the cells inside the microarray spotter and the CO₂ incubator. Sandwiched micropillar and microwell chips should be kept inside a humidified chamber while incubating in a CO₂ incubator.

Statistical Analyses

It is important to understand the application of various statistical analyses to know whether the difference in IC₅₀ values from various HCI assays are statistically significant or not. One-way ANOVA is used to determine the statistical significance of the difference in IC₅₀ values between three or more assays or mechanisms of action as compared to controls. ANOVA analyzes the variances to decide whether the *means* are different. However, one-way ANOVA only tells that there are statistically significant differences among the IC₅₀ values from three or more assays tested, but it does not inform us whether IC₅₀ value from which specific assays are statistically significantly different from each other. Therefore, post

tests such as Bonferroni and Tukey are required to find out IC₅₀ from which assay differs significantly from others, thereby providing the information on the main mechanism of toxicity. The t-test on the other hand, can only be used to compare two groups of samples i.e. two different test conditions. Confidence interval (CI) is set at 95% or 99% to check the precision of the estimate of the IC₅₀ values.

Understanding Results

The effect of drugs are represented in a graphical form with the biological effect and the concentration of a compound (also known as a dose response curve). To understand the effect of a drug on 3D-cultured cells, drug concentration is plotted on the x-axis, while fluorescence from different mechanisms is plotted on the y-axis. To produce a conventional sigmoidal dose-response curve, with response values normalized to span the range from 0% to 100% plotted against the logarithm of test concentration, the fluorescence intensities of all cell spots are normalized with the fluorescence intensity of 100% live cell spot (i.e., cell spots contacted with no compound) and the test compound concentrations are converted to their respective logarithms. The sigmoidal dose-response curve and the IC₅₀ value (concentration of the compound where 50% of cell function inhibited) are then obtained using the following equation:

$$Y = Bottom + \left[\frac{Top - Bottom}{1 + 10^{(LogIC_{50} - X) \times H}} \right]$$

where IC₅₀ is the midpoint of the curve, H is the hill slope, X is the logarithm of test concentrations, and Y is the percent inhibition, starting at Bottom and going to Top with a sigmoid shape.

The dose-response curve provides the information on the change or effect in cells caused by a wide concentration range of a compound in a certain time period. If the potency of the compound is outside the dosage range selected, then the dose-response curve will not describe the bottom or top asymptote of the curve. The IC₅₀ value (half-maximal inhibitory concentration) determines the strength of a compound in affecting various morphological and functional features of cells. There should be at least one point on both sides of the IC₅₀ value in order for the IC₅₀ value to be within the concentration range used for the assay (Sittampalam et al., n.d.). This is required to make the IC₅₀ estimate an interpolation of data rather than making it an extrapolation of data.

Moreover, *in vivo* toxicity of compounds can be predicted by calculating the overall predictivity using the following equation (Yu et al., 2017):

$$Overall\ predictivity(\%) = [sensitivity + specificity]/2$$

where sensitivity and specificity are determined as

- Sensitivity (%) = [Number of *in vitro* toxic test compounds (TP)] / [Number of *in vivo* toxic test compounds (TP + FN)] x 100

- Specificity (%) = [Number of *in vitro* nontoxic test compounds (TN)] / [Number of *in vivo* nontoxic test compounds (TN + FP)] x 100

Sensitivity and specificity are calculated using IC₅₀ values from the microarray chip platform, human C_{max} values, and rat LD₅₀ values determined by oral administration. Test compounds that exhibit an IC₅₀ value less than or equal to an arbitrary IC₅₀ cutoff at a given condition are categorized as toxic. Similarly, test compounds that exhibit a human C_{max} value or a rat LD₅₀ value less than or equal to an arbitrary C_{max} or LD₅₀ cutoff are categorized as toxic. The test compounds are then classified into four categories: True Positive (TP), False Positive (FP), True Negative (TN), and False Negative (FN). For example, when arbitrary cutoffs of LD₅₀ of 500 mg/kg and IC₅₀ of 200 µM are used, TP, FP, TN, and FN are determined as follows:

- True Positive (TP): LD₅₀ ≤ 500 mg/kg (toxic) and IC₅₀ ≤ 200 µM (toxic)
- False Positive (FP): LD₅₀ > 500 mg/kg (nontoxic) and IC₅₀ ≤ 200 µM (toxic)
- True Negative (TN): LD₅₀ > 500 mg/kg (nontoxic) and IC₅₀ > 200 µM (nontoxic)
- False Negative (FN): LD₅₀ ≤ 500 mg/kg (toxic) and IC₅₀ > 200 µM (nontoxic)

The arbitrary LD₅₀ cutoffs can be determined based on OECD categories for testing *in vivo* compound toxicity (OECD, 2001).

Time Considerations

- Basic protocol 1 - Miniaturized 3D cell culture: 3 days
- Basic protocol 2 - Compound treatment and cell staining: 2 days
- Basic protocol 3 - Image acquisition, processing and analysis: 1–2 days

This high-throughput assessment of mechanistic toxicity would take 5–7 days depending on the doubling time of cells selected. Fluorescent staining and imaging can be typically performed in a single day with careful planning of experiments. All fluorescent staining requires 45–60 mins staining along with 10 mins washing before and 20 mins washing after the staining. Complete drying of the cells spots for image acquisition in S+ Scanner takes approximately 3–4 hrs in a well ventilated environment.

Image acquisition from four micropillar chips *via* S+ Scanner typically takes about 30 mins for exposure time between 40–100. At fluorescent dye concentration recommended in this protocol, the exposure time for various fluorescent channels should fall between 40–100. In addition, fluorescent intensity extraction with background subtraction using ImagJ takes 5–10 minutes for 532 images from a single chip. Similarly, plotting the dose-response curve and generating a report using S+ Chip Analysis for a single chip takes around 5–10 minutes. Thus, from the point of fluorescent staining to image acquisition and obtaining the dose-response curves can be performed in a single day.

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Significance Statement

With animal studies gradually being phased out due to their expensive, time-consuming, and low-throughput nature, there is an urgent need for a highly predictive *in vitro* assay system to assess the toxicity of compounds, including drug candidates and environmental toxicants. There has been an emergence of *in vitro* techniques that identify the cellular processes involved in human toxicology. However, most *in vitro* assays are performed on two-dimensional (2D) cell monolayer cultures which have limited physiological relevance to *in vivo* outcomes. The miniaturized 3D cell culture system and associated high-content imaging (HCI) assays explained in this protocol provide readers the capability to identify various mechanisms of toxicity and predict the *in vivo* impact of compounds.

Figure 1. Microarray chip platforms. Image of the micropillar chip with cell spots and the microwell chip with growth media for 3D cell culture.

Figure 2.
Printing of samples with S+ Microarrayer on the micropillar chip loaded on a black chip deck.

Figure 3. Sandwiching the microipillar chip with the microwell chip and incubating inside a humidified petri dish. (A) The microwell chip containing growth media is placed on a chip stamping frame and (B) the micropillar chip containing cells encapsulated in alginate and fibrin mixture is sandwiched onto the microwell chip. (C) The sandwiched micropillar/microwell chip is kept inside a humidified petri dish and incubated at 37 °C.

Figure 4.
Growth and spheroid formation of Hep3B cells encapsulated in 0.75% (w/v) alginate and 10 mg/mL fibrin gel after 3 days of culture ready for compound treatment.

Figure 5.
Layout of the model compounds used in this protocol on a TMRM-stained micropillar chip.

Figure 6. Micropillar chips washed with the saline solution in the deep-well staining plate (A) and stained with fluorescent dye solutions in the shallow-well staining plate (B).

Figure 7. Micropillar chips with Hep3B cells stained with hoechst 33342, TMRM, mBCl, and calcein AM. (A) Image of single cell spots obtained by S+ Scanner after staining with the four fluorescent dyes. (B) Image of cell spot arrays obtained by S+ Scanner after exposure to lovastatin and staining with the four fluorescent dyes.

Figure 8.
Batch processing macro in ImageJ created by recording the steps.

Figure 9.

Dose-reponse curves of six model compounds tested in this protocol for mitochondrial impairment determined from TMRM staining of mitochondrial membrane potential.

Table format: Grouped

		A Calcein AM		B TMRM		C Hoechst 33342		D mBCI		E Title		F Title	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	Title	0.301030	0.568636	-0.065502	1.300000	0.477121	0.244125	0.012837	1.045758				
2	Title	1.146128	0.468521	-0.585027	1.397940	0.462398	0.468521	-0.585027	1.154902				
3	Title	0.903090	0.500000	-0.251812	1.400000	0.469822	0.350000	0.238046	1.050000				
4	Title												
5	Title												
6	Title												
7	Title												
8	Title												
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23	Title												
24	Title												
25	Title												
26	Title												
27	Title												
28	Title												
29	Title												
30	Title												
Total													

Row 4, D:

Figure 10.
Screenshot of GraphPad Prism showing the data sheet with IC₅₀ values and SEM for all four assays.

Table 1:

List of model compounds used and their mechanism of toxicity

Model compounds	Concentration used (μM)	Compound type	Known mechanism of toxicity	References
Acetaminophen	8 – 2,124	Analgesic and antipyretic drug	AP, BA, OS	(Park, Williams, Naisbitt, Kitteringham, & Pirmohamed, 2005; Wang, Shindoh, Inoue, & Horii, 2002)
Lovastatin	2 – 472	Hypolipidemic drug	AP, DD, MI	(Kallas-Kivi, Trei, & Maimets, 2016; Niknejad et al., 2014; Walther et al., 2016; Wei et al., 2007; Zhao et al., 2015)
Rotenone	0.12 – 30	Pesticide, insecticide, and piscicide	AP, MI, OS	(Cabezas et al., 2012; Isenberg & Klaunig, 2000; Moon et al., 2005)
Tamoxifen	0.48 – 118	Hormonal drug for breast cancer	AP, MI, OS	(Lee et al., 2000; Tabassum et al., 2006; Tolosa et al., 2012, 2015)
Menadione	0.7 – 117	Vitamin K precursor	MI, OS	(Akiyoshi et al., 2009; De Assis et al., 2015; Tolosa et al., 2012; Woods, Young, Gilmore, Morris, & Bilton, 1997)
Sodium Citrate	5.6 – 1,417	Intermediate in the Krebs cycle	Nontoxic control	(Tolosa et al., 2012)

Apoptosis (AP), bioactivation (BA), DNA damage (DD), mitochondrial impairment (MI), and oxidative stress (OS).

Table 2:

List of fluorescent dyes and their concentrations used in this protocol

Fluorescent dyes	Assays/endpoints	Working stock conc. (mM)	Working conc. (μM)	Working solvent
Hoechst 33342	DNA damage	10	25	PBS
Tetramethyl rhodamine methyl ester (TMRM)	MMP/ Mitochondrial impairment	0.5	0.5	PBS
Monochlorobimane (mBCl)	Intracellular glutathione level/oxidative stress	200	100	PBS
Calcein AM	Membrane integrity/cell viability	1	0.25	PBS

Table 3.

A summary of IC₅₀ values (μM) of six model compounds obtained from the chip platform with the four HCl assays.

Compound	TMRM	Hoechst	mBCI	Calcein AM	2D (literature review)	LD ₅₀ mg/kg (rat oral)
Acetaminophen	>2100	>2100	>2100	>2100	29,755±1775	1944
Lovastatin	152± 6.7	188± 17.7	195± 5.0	150± 0.0	20 ± 3.7	5000
Rotenone	0.6± 0.3	3.0 ± 0.0	1.7± 0.7	8.0± 6.0	1.7± 1.2	30
Tamoxifen	50± 0.5	102± 6.2	83± 18.5	57± 4.3	45	4100
Menadione	11.5± 2.5	20± 1.0	15± 2.3	11± 3.8	13.5± 3.6	500 (mice oral)
Sodium citrate	>1400	>1400	>1400	>1400	>1400	6730