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Metabolic Enzyme Microarray Coupled with Miniaturized Cell-Culture Array Technology for High-Throughput Toxicity Screening

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Abstract

Due to poor drug candidate safety profiles that are often identified late in the drug development process, the clinical progression of new chemical entities to pharmaceuticals remains hindered, thus resulting in the high cost of drug discovery. To accelerate the identification of safer drug candidates and improve the clinical progression of drug candidates to pharmaceuticals, it is important to develop high-throughput tools that can provide early-stage predictive toxicology data. In particular, in vitro cell-based systems that can accurately mimic the human in vivo response and predict the impact of drug candidates on human toxicology are needed to accelerate the assessment of drug candidate toxicity and human metabolism earlier in the drug development process. The in vitro techniques that provide a high degree of human toxicity prediction will be perhaps more important in cosmetic and chemical industries in Europe, as animal toxicity testing is being phased out entirely in the immediate future.

We have developed a metabolic enzyme microarray (the Metabolizing Enzyme Toxicology Assay Chip, or MetaChip) and a miniaturized three-dimensional (3D) cell-culture array (the Data Analysis Toxicology Assay Chip, or DataChip) for high-throughput toxicity screening of target compounds and their metabolic enzyme-generated products. The human or rat MetaChip contains an array of encapsulated metabolic enzymes that is designed to emulate the metabolic reactions in the human or rat liver. The human or rat DataChip contains an array of 3D human or rat cells encapsulated in alginate gels for cell-based toxicity screening. By combining the DataChip with the complementary MetaChip, in vitro toxicity results are obtained that correlate well with in vivo rat data.

Keywords

Microarray; Metabolic enzyme array; MetaChip; Cell array; DataChip; High-throughput toxicity screening

1. Introduction

The metabolism of chemicals, including drug compounds, in the human body primarily occurs in the liver via a variety of oxidative and conjugative routes. Among the many metabolic enzymes, cytochrome P450 (CYP450) isoforms, which catalyze first-pass (Phase I) functionalization reactions (e.g., hydroxylation, dealkylation, oxidation, deamination, and

dehalogenation), are the most important (1, 2). Subsequent conjugation reactions (e.g., glucuronidation, sulfation, acetylation, and addition of amino acids and peptides) are catalyzed by Phase II metabolic enzymes including UDP-glycosyltransferase (UGT), sulfotransferase (SULT), and glutathione S-transferase (GST), resulting in the formation of more soluble compounds and eventually enhancing excretion (3, 4). Thus, understanding the role of these metabolic enzymes is important in drug metabolism and for human toxicology testing.

There have been a number of in vitro approaches developed for human metabolism and toxicology screening, including isolated liver slices, primary hepatocytes, transformed cultured human hepatoma cell lines, purified microsomal preparations, or isolated and purified P450s (5, 6). Two-dimensional (2D) hepatocytes cultures in multi-well plates can be adapted to high-throughput screening, and have been considered as the gold standard of in vitro human metabolism and toxicology for replacement of animal testing (7). However, 2D hepatocyte cultures may not emulate the environment and cellular architecture found in vivo (8, 9). Thus, in vitro results obtained from 2D hepatocyte cultures may not provide toxicity information that correlates with in vivo animal data.

To address this limitation, we have developed a metabolic enzyme microarray (the Metabolizing Enzyme Toxicology Assay Chip, or MetaChip) and a miniaturized 3D cell-culture array (the Data Analysis Toxicology Assay Chip, or DataChip) for high-throughput toxicity screening of target compounds and their metabolic enzyme-generated products (10-12). The MetaChip can be prepared by spotting human or rat metabolic enzymes including individual CYP450s, a mixture of CYP450s, individual phase II metabolic enzymes, a mixture of phase II metabolic enzymes, a mixture of all metabolic enzymes, liver microsomes, and s9 fractions, all encapsulated in alginate gels (as small as 15 nL) arrayed on a methyltrimethoxysilane (MTMOS)-coated glass slide. The DataChip can be prepared by printing human or rat cells in alginate gels (as small as 30 nL) onto a poly(styrene-*co*-maleic anhydride) (PS-MA)-coated glass slide for toxicity screening against multiple target cells. Using the human or rat DataChip coupled with the human or rat MetaChip, the toxicity of parent compounds have been compared to that of their products generated by various metabolic enzymes (Fig. 1). We have demonstrated that a *single* DataChip containing 1,080 individual cell cultures, used in conjunction with the complementary human CYP450-containing MetaChip, can simultaneously provide IC₅₀ values for nine compounds and their metabolites from CYP1A2, CYP2D6, and CYP3A4, and an equimolar mixture of the three P450s (12). In addition, we have compared cytotoxicity data from different metabolic enzyme mixtures and cell sources for the validation of the MetaChip/DataChip platform, resulting in better correlations between in vivo rat data and in vitro data. We envisage that the MetaChip/DataChip platform represents a promising, high-throughput microscale alternative and creates new opportunities for rapid and inexpensive predictive assessment of human toxicity.

2. Materials

2.1. Slide Treatment

1. Fisherbrand plain microscope slides (Fisher Scientific, now Thermo Fisher Scientific, Rockford, IL).
2. Wheaton glass 20 slide staining dish with removable rack (Fisher).
3. Methyltrimethoxysilane (Sigma-Aldrich, St. Louis, MO)
4. Poly(styrene-*co*-maleic anhydride): 14% (w/w) maleic anhydride (Sigma-Aldrich).
5. Potassium phosphate buffer solution: 200 mM, pH 8 (Invitrogen, Carlsbad, CA). Prepare 25 mM potassium phosphate buffer (pH 8) by mixing 200 mM potassium phosphate buffer (pH 8) with de-ionized distilled water.
6. National scientific 20 mL glass sample vials with PTFE-lined solid storage caps (Fisher).
7. Petri dishes with clear lids, 150 × 15 mm (Fisher).

2.2. Microarray Spotting

1. Standard solenoid valve for a normal pin head (Genomic Solutions, Inc., now Digilab, Inc., Ann Arbor, MI).
2. Ceramic dispensing tip, 100 µm orifice (Genomic Solutions).
3. Arrayit hybridization cassette, extra deep chamber, 2.54 mm depth (TeleChem International, Inc., Sunnyvale, CA).
4. Costar clear polystyrene 96-well plates with lid (untreated, round well, sterile) (Corning life sciences, Inc., Lowell, MA).
5. Poly-L-lysine (PLL) solution: 0.01%, molecular weight 70,000–150,000, sterile-filtered, cell culture tested (Sigma). Store the PLL solution in aliquots at 4°C.
6. Barium chloride (Sigma-Aldrich). Prepare a 0.1 M barium chloride (BaCl₂) solution by adding barium chloride in sterile de-ionized distilled water and vortex well for complete dissolution. After sterile filtering the solution using a 0.2 µm syringe filter, store the BaCl₂ solution in aliquots at 4°C.
7. Alginic acid sodium salt from *Macrocystis pyrifera* (Kelp), low viscosity (Sigma-Aldrich). Prepare a 3% (w/v) alginic acid solution by dissolving alginic acid sodium salt in sterile de-ionized distilled water in a sterile glass sample vial. Place a small stir bar in the vial and let it mix for at least 3 d for complete dissolution. Store the alginate solution in aliquots at 4°C.
8. Dulbecco's phosphate-buffered saline (PBS) without CaCl₂ and MgCl₂ (Invitrogen). Store Dulbecco's PBS in aliquots at room temperature.

9. Vivid® CYP450 screening kit (Invitrogen). Store CYP450 isoforms in aliquots at –80°C. After sterile filtering the solution using a 0.2 µm syringe filter, store NADP and a regeneration system in aliquots at –80°C.
10. Baculosome® reagents, TNI-WT negative control (Invitrogen). Store TNI-WT negative control in aliquots at –80°C.
11. Supersomes™ human UGT isoforms (BD Biosciences). Store UGT in aliquots at –80°C.
12. UGT reaction mix A and B (BD Biosciences). After sterile filtering the solution using a 0.2 µm syringe filter, store UGT reaction mix in aliquots at –80°C.
13. Human sulfotransferase (SULT) isozymes (Sigma). Store SULT in aliquots at –80°C.
14. Adenosine 3'-phosphate 5'-phosphosulfate (PAPS) tetralithium salt (CalBioChem, now EMD Chemicals, Inc.). After sterile filtering the solution using a 0.2 µm syringe filter, store PAPS in aliquots at –80°C.
15. Human glutathione S-transferase (GST) (Sigma). Store GST in aliquots at –80°C.
16. Glutathione (GSH) (Sigma). After sterile filtering the solution using a 0.2 µm syringe filter, store GSH in aliquots at –80°C.
17. Human liver microsome (HLM) (BD Biosciences). Store HLM in aliquots at –80°C.

2.3. Cell Culture

1. Hep3B human hepatoma cells (ATCC, Manassas, VA).
2. RPMI 1640 with L-glutamine, 1× (Mediatech, Manassas, VA). Prepare RPMI supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS) and store it at 4°C.
3. Fetal bovine serum (FBS) qualified, USA origin, sterile-filtered, cell culture tested (Sigma-Aldrich). Store FBS in aliquots at –20°C.
4. Penicillin-Streptomycin (Invitrogen). Store PS in aliquots at –20°C.
5. Trypsin-EDTA: 0.05% trypsin, 0.53 mM EDTA (Invitrogen). Store Trypsin-EDTA in aliquots at –20°C.
6. BD Falcon tissue culture flasks, 75 cm² culture area, canted neck, plug seal cap (Fisher).
7. Petri dishes with clear lids, 100 × 15 mm (Fisher).

2.4. Cell Staining

1. Live/dead viability/cytotoxicity kit (Invitrogen). Store the staining dyes at –20°C.

2. BupH phosphate buffered saline (PBS) packs containing 0.1 M phosphate, 0.15 M NaCl, pH 6.9–7.2 when dissolved in 500 mL distilled water (Pierce Biotechnology, now Thermo Fisher Scientific). Store BupH PBS at room temperature.
3. Silicone rubber sheet, 1 mm thick (McMaster-Carr Supply Co., Robbinsville, NJ).

3. Methods

3.1. Preparation of Functionalized Slides

To prepare a uniform coating on a glass surface, it is important to remove all dirt to expose silanol groups ($-\text{SiOH}$) on the slide surface. Acid-cleaning steps are, therefore, essential for standard microscope slides.

3.1.1. Acid Cleaning of the Glass Slide

1. Wipe the plain microscope slides ($25 \times 75 \times 1$ mm) three times with 70% ethanol-soaked paper towels to remove oil and solid particles on the slide surface and then clean ethanol with dry paper towels (see Note 1).
2. Place the slides in a removable glass rack, immerse the rack in a Wheaton glass dish filled with concentrated sulfuric acid (96.5%) for 2 h, and then sonicate the Wheaton glass dish with the slides in acid for 10 min.
3. Rinse the rack containing the slides at least five times with de-ionized distilled water to remove excess sulfuric acid on the slide surface.
4. Immerse the rack containing the slides twice in a Wheaton glass dish filled with acetone to remove excess water on the slide and dry the acid-cleaned glass slides thoroughly under N_2 gas stream.
5. Bake the acid-cleaned slides in an oven at 120°C for 10 min to completely remove water on the slide surface.
6. Store the slides in a sterile Petri dish (150 mm diameter, maximum five slides/Petri dish) until use. Never leave the clean slides uncovered for extended times.

3.1.2. Poly (Styrene-co-Maleic Anhydride) (PS-MA) Coating for Cell Printing

1. Prepare a fresh 1% (w/v) solution of PS-MA by dissolving PS-MA in anhydrous toluene in a scintillation vial with a PTFE-coated lid. Shake the solution in an incubating shaker at 50°C , 5 g for 40 min until PS-MA pellets completely dissolve (see Note 2).

¹Do not use Kimwipes because it generates lots of small paper particles. After wiping with dry paper towels, the microscope slides should not be wet with ethanol. It will leave ethanol stains on the slide surface.

²After 30 min of shaking at 50°C , 5 g, the solution becomes clear. However, leave the solution for additional 10 min for complete dissolution of PS-MA pellets. The PS-MA solution should be prepared freshly as reactivity of maleic anhydride groups decreases over time. The PTFE-lined scintillation vial has to be used for organic solvents. Never leave the vial with toluene in the incubating shaker unattended due to a concern about an explosion at high temperature.

2. Prepare a 0.1% (w/v) PS-MA solution by mixing 18 mL of anhydrous toluene with 2 mL of the 1% (w/v) PS-MA solution. Prepare a fresh PS-MA solution and use it immediately.
3. Load 1 mL of the 0.1% (w/v) PS-MA solution onto the acid-cleaned slide and spin coating the solution using a spin coater (Model PWM32, Headway Research, Inc., Garland, TX) at 200 g for 30 s. Remove excess PS-MA remaining on the back of the slide by wiping with an acetone-soaked paper towel.
4. Dry the PS-MA-coated slides overnight at room temperature in a sterile Petri dish and store until needed.

3.1.3. Methyltrimethoxysilane (MTMOS) Coating for Compound and Enzyme Printing

1. Prepare a fresh MTMOS-HCl sol solution by mixing 8 mL of MTMOS with 3.2 mL of 5 mM HCl, followed by vortex for 2 min and sonication for 10 min (see Note 3).
2. Immediately before spin coating, mix 11.2 mL of the MTMOS-HCl sol solution with 8 mL of potassium phosphate buffer solution (25 mM, pH 8) and use the mixture within 15 min (see Note 4).
3. While spinning the acid-cleaned slide at 200 g for 30 s, place 1.5 mL of the mixture onto the slide and remove any excess MTMOS on the back of the slide using acetone-soaked paper towel.
4. To complete MTMOS gelation, dry the slides overnight at room temperature in a sterile Petri dish and store until needed.

3.1.4. Preparation of a Sol-Gel Gasket on the MTMOS-Coated Slide for

Stamping—A sol-gel gasket is fabricated by spotting an MTMOS sol-gel solution around the perimeter of the MTMOS-coated slide and baking the slide to harden the sol-gel drops. The sol-gel gasket on the MTMOS-coated slides is served as a spacer to maintain a suitable distance between the MetaChip and the DataChip and to prevent spot detachment by direct contact of the spots after stamping. For efficient stamping and transfer of compounds onto the cells, the gasket height is optimized as a function of the HCl concentration, the sol-gel volume, and the baking time.

1. Prepare a fresh MTMOS-HCl sol solution by mixing 2 mL of MTMOS with 1 mL of 10 mM HCl, followed by vortex for 2 min and sonication for 10 min.
2. Immediately before spotting the sol solution onto the slides, mix 3 mL of the MTMOS-HCl sol solution with 1 mL of Dulbecco's PBS without CaCl_2 and MgCl_2 and use this mixture within 1 h.

³The hydrolysis of MTMOS by HCl is an exothermic reaction. So be careful not to burn skin. The mixture becomes a transparent single phase after vortex mixing (initially two phases). Always prepare fresh MTMOS-HCl sol solution and use it immediately.

⁴Upon addition of the phosphate buffer solution, gelation of hydrolyzed MTMOS begins. Therefore, use the mixture immediately. Do not use the mixture when the solution becomes turbid, causing uneven coating.

3. After aligning the MTMOS-coated slides on the slide deck of a MicroSys™ 5100-4SQ microarray spotter (Genomics Solutions, now Digilab, Inc.), print the mixture onto the periphery of each slide (typically 80 spots/slide, 450 nL per spot, 2 mm spot-to-spot distance).
4. Immediately after printing, bake the MTMOS slides for 5 min in an oven at 100°C to facilitate complete gelation of the sol-gel gasket (see Note 5). The MTMOS slides should be carefully inspected to ensure hemispherical sol-gel gasket spots.
5. Store the MTMOS slides containing the sol-gel gasket in a sterile Petri dish until needed.

3.2. Preparation of a Miniaturized Enzyme Array (the Metabolizing Enzyme Toxicology Assay Chip or MetaChip) on the MTMOS-Coated Slide

The MetaChip, a complementary array of encapsulated metabolic enzymes that is designed to emulate the metabolic reactions in the human liver is prepared on the MTMOS-coated slide with a sol-gel gasket. For example, six different compounds can be printed in sections 1–6 of the MetaChip, each region containing a 5×9 mini-array (Fig. 2). Within each mini-array, nine different doses of a compound can be assayed for toxicity. For on-chip drug metabolism, human metabolic enzymes can be transversely printed into four regions (a–d), each containing no enzyme and different mixtures of enzymes encapsulated in alginate gel, thereby creating 24 distinct regions on the MetaChip (Table 1). Other human and rat metabolic enzymes can be spotted similarly on the MTMOS-coated slide for drug metabolism.

1. Prepare compound stock solutions by dissolving compounds in DMSO. Typically, higher than 200 mM of compound stock solutions are required to maintain final DMSO content less than 0.5%. DMSO may inhibit many metabolic enzymes.
2. Prepare 200 μ L of test compound solutions at 2.5-fold higher concentrations than the desired final concentration (8 dosages plus 1 control, typically 0–1,000 μ M) by serial dilutions of test compound stock solutions in DMSO (Fig. 3) and then add an equal volume mixture of 0.1 M BaCl₂ and 0.01% Poly-L-lysine (PLL). As a control, prepare the BaCl₂-PLL solution without compound.
3. Dispense 200 μ L of diluted compound solutions in the round-bottom 96-well plate for printing. Due to potential compound carry-over, always add compound solutions from low to high concentration in the wells.
4. After spotting test compound solutions on the MTMOS-coated slides with the sol-gel gasket (typically 1,080 spots/slide, 30 nL per spot, 1 mm spot-to-spot distance) using the microarray spotter, dry the test compound spots in a sterile

⁵Excessive baking (typically longer than 10 min) causes spot detachment. If the sol-gel gasket spots are flattened after baking, it is because of either less hydrophobic MTMOS slide or old MTMOS contaminated with HCl. In the latter case, use fresh MTMOS and HCl to prepare the MTMOS–HCl sol solution.

Petri dish. The hydrophobic MTMOS coating is used to ensure hemispherical spots on the slide.

5. Prepare metabolic enzyme solutions in alginate (Table 1). Always prepare fresh enzyme-alginate mixtures on ice and use them immediately.
6. Print 15 nL of 0.5% (w/v) alginate solutions containing metabolic enzymes atop each test compound spot within the microarray spotter chamber under 95% relative humidity. Humidity to maintain enzyme spots hydrated vary depending on the temperature of the slide deck.
7. Immediately after enzyme printing, place the slide in a Petri dish (100 mm diameter) and store in a -80°C freezer until use. Do not dry the enzyme spots, causing enzyme deactivation.

3.3. Preparation of Human Cell Suspension for Spotting

1. Grow Hep3B human hepatoma cells or other mammalian cells in RPMI 1640 or relevant growth media supplemented with 10% FBS and 1% PS in T-75 cell-culture flasks in a humidified 5% CO_2 incubator (ThermoForma Electron Co., Marietta, OH) at 37°C .
2. Prepare the cell suspensions by trypsinizing a confluent layer of the cells with 1 mL of 0.05% trypsin-0.53 mM EDTA from the culture flask, and resuspending the cells in 7 mL of 10% FBS-supplemented RPMI.
3. After centrifugation at $100\times g$ for 4 min, remove the supernatant and resuspend the cells with 10% FBS-supplemented RPMI to a final concentration of 9×10^6 cells/mL. Other mammalian cell suspensions can be prepared similarly.

3.4. Preparation of a Miniaturized 3D Cell-Culture Array (the Data Analysis Toxicology Assay Chip, or DataChip) on the PS-MA-Coated Slide

The DataChip, arrays of 3D cell spots containing Hep3B cells is prepared on the PS-MA-coated slide (Fig. 4). Each 3D cell culture spot on the DataChip contains 6×10^6 cells/mL in 30 nL of 1% (w/v) alginate (180 cells/spot). The 3D cell cultures are arrayed onto the PS-MA-coated slide in 24 regions of 5×9 mini-arrays. Thus, a single DataChip combined with a single MetaChip can generate 24 sigmoidal dose response curves for 6 compounds and their metabolites generated from human P450 mixture, all enzyme mixture, and human liver microsomes (9 doses and five replicates for each compound). IC_{50} values (indicating the concentration of test compound required to inhibit 50% of cell growth) can be determined for the parent test compound and its enzyme-generated metabolites against Hep3B human hepatoma cells. Other human and rat cell types can be spotted similarly on the PS-MA-coated slide for toxicology assays.

1. Prepare a sterile BaCl_2 -PLL mixture by mixing 100 μL of 0.1 M BaCl_2 with 200 μL of 0.01% PLL.
2. Spot the BaCl_2 -PLL mixture onto the PS-MA-coated slide (typically 1,080 spots/slide, 30 nL per spot, 1 mm spot-to-spot distance) using the microarray spotter and then dry the slide for 10 min in a sterile Petri dish with a lid slightly open to

yield flat BaCl₂-PLL layers. The hydrophobic PS-MA coating is used to attach PLL with amine groups covalently and to ensure hemispherical spots on the slide. In addition, positively charged PLL has an ionic interaction with negatively charged alginate.

3. Prepare a suspension of the cells in low-viscosity alginate by mixing the Hep3B cell suspension in 10% FBS-supplemented RPMI (9×10^6 cells/mL) with 3% (w/v) alginate solution in distilled water so that the final concentration of cells and alginate were 6×10^6 cells/mL and 1%, respectively.
4. Print the alginate solution containing the cells (1,080 spots/slide, 30 nL per spot) atop each BaCl₂-PLL spot resulting in nearly instantaneous gelation of the alginate matrix (see Note 6). Maintain the microarray spotter chamber at 95% relative humidity to retard evaporation of water during spotting.
5. Immediately after spotting, place the DataChip in 15 mL of 10% FBS-supplemented RPMI in a Petri dish (100 mm diameter) and incubated in the CO₂ incubator at 37°C for 18 h prior to use.

3.5. Stamping the DataChip onto the MetaChip

Metabolism-based cytotoxicity assays are performed by stamping the DataChip onto the MetaChip.

1. Take out the MetaChip from the -80°C freezer, place the slide immediately on the slide deck, and then print sterile distilled water onto the periphery of the MetaChip (typically 80 spots/slide, 150 nL per spot, 2 mm spot-to-spot distance) to retard evaporation during stamping. Maintain the microarray spotter chamber at 95% relative humidity to retard evaporation of water during spotting.
2. Print complete RPMI supplemented with 10% FBS and 1% PS atop the enzyme spots with compounds (1,080 spots/slide, 30 nL per spot).
3. Simultaneously, take out the DataChip from the growth medium, wipe off the excess RPMI on the back side of the slide with ethanol-soaked Kimwipes, and place the cell slide in a sterile ArrayIt hybridization cassette.
4. Cover a lid of the cassette, slant the cassette with the cell slide at an angle of 45 degree for 5 min to allow excess growth medium on the surface to drain, and wipe off the growth medium with Kimwipes. (see Note 7).
5. Immediately after growth medium spotting, place the MetaChip onto a sterile stamping apparatus (Fig. 5).

⁶Add freshly suspended alginate solution containing the cells in the round-bottom 96-well plate to make sure well suspension of the cells while spotting. Remind that the cells are gradually precipitated while spotting. For uniform cell spotting, keep re-suspending the cells. In addition, fast spotting and high humidity on the slide surface is critical to maintain high cell viability as spot drying causes cell death.

⁷The cell spots should remain hydrated while removing excess growth medium, otherwise the cell would be dead. Particularly pay attention to the cell spots drying on the edge of the slide. Draining time is a function of the spot volume. Optimization of draining time is required to minimize cell death due to drying.

6. Immediately after taking out the DataChip from the cassette, stamp the DataChip on top of the MetaChip by aligning the edges of the glass slides in the apparatus, and then tape both edges of the slides.
7. Place the stamped slides, with the cell slide on top, in the hybridization cassette with 100 μ L of sterile distilled water, cover the lid tightly, and then incubate the cassette with the slides for 6 h at 37°C.
8. After desired incubation period, lift off the cell slide, clean the back side of the slide with 70% ethanol-soaked paper towels before immersing in the growth medium to prevent contamination, rinse once in a Petri dish with 15 mL of Dulbecco's PBS to remove any excess compound solution, immerse in 15 mL of the growth medium for 1 h to allow for residual compounds to diffuse out from the alginate-gel drops, and transfer to a Petri dish containing 15 mL of fresh medium supplemented with 10% FBS and 1% PS.
9. Culture the cells for 2 d in a CO₂ incubator at 37°C for cytotoxicity assays.

3.6. Cell Staining, Scanning, and Data Analysis

Cytotoxicity of test compounds and their metabolites can be determined by staining the DataChip with a live/dead viability/cytotoxicity kit. Staining dyes such as calcein AM (excitation 495 nm and emission 515 nm) and ethidium homodimer-1 (excitation 495 nm and emission 635 nm) are used to produce a green fluorescent response from living cells and a red fluorescent signal from dead cells.

1. At the end of the 2-d culture period post stamping, wash the DataChip three times by immersing the slide in a 150 mm-diameter Petri dish containing 100 mL of 10 mM BupH PBS (pH 7) with 10 mM CaCl₂ for 5 min each. CaCl₂ is supplemented to prevent degradation of alginate spots by excess phosphate ions.
2. Prepare a staining dye solution by adding 1.0 μ L of calcein AM (4 mM stock) and 4.0 μ L of ethidium homodimer-1 (2 mM stock) in 8 mL of Dulbecco's PBS supplemented with 10 mM CaCl₂.
3. Dispense 1.5 mL of dye solution on a glass slide with 1 mm-thick perimeter gasket acting as a barrier to prevent loss of the dye solution and evenly spread the dye solution on the gasket slide.
4. Wipe off excess PBS from the cell slide with Kimwipes and place it on top of the gasket slide.
5. Incubate the DataChip in dark for 50 min at room temperature.
6. Wash the cell slide in a Petri dish containing 15 mL of 10 mM BupH PBS (pH 7) with 10 mM CaCl₂ on an orbital shaker at 0.5 *g* for 30 min to remove excess dye in the alginate-gel drops.
7. Blow drying the cell slide gently with N₂ gas so as not to detach the cell spots.
8. Detect the location of each cell spot where compounds are added by imaging the entire slide using blue laser (488 nm) and standard blue filter for green dye and

blue laser and 645AF75/594 filter for red dye in GenePix® Professional 4200A scanner (Molecular Devices, now MDS Analytical Technologies, Sunnyvale, CA). The PMT gain and power may be adjusted depending upon fluorescent intensity.

9. Save data files as single tiff images for analysis. The green fluorescence intensity is linearly proportional to the total number of live cells, and is quantified from the array scanner using GenePix Pro 6.0 (Molecular Devices).
10. Extract fluorescent intensity from each cell spot using GenePix Pro and plot the percentage of live cells against the concentration of the compound tested using Prism 4 (GraphPad Software, Inc., La Jolla, CA). Since the background green fluorescence of completely dead cells (following treatment with 70% methanol for 1 h) was negligible, the percentage of live cells is calculated using the following equation:

$$\% \text{ Live cells} = \left(\frac{F_{\text{Reaction}}}{F_{\text{Max}}} \right) \times 100$$

where F_{Reaction} is the green fluorescence intensity of the reaction spot and F_{Max} is the green fluorescence intensity of untreated viable cells.

11. 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, normalize the green fluorescence intensities of all cell spots with the fluorescence intensity of 100% live cell spot (typically, cell spots contacted with no compound) and convert the test compound concentration to their respective logarithms using Prism 4. The sigmoidal dose-response curves (variable slope) and IC_{50} values (i.e., concentration of the compound where 50% of cell growth inhibited) are obtained using the following equation:

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

where IC_{50} is the midpoint of the curve, H is the hill slope, X is the logarithm of test concentration, and Y is the response (% live cells), starting at Bottom and going to Top with a sigmoid shape. An example of the results produced is shown in Fig. 6.

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Fig. 1. Schematic of the MetaChip platform coupled with the DataChip for evaluating toxicity of compounds and their enzyme-generated metabolites

Fig. 2.

A layout of the MetaChip (1,080 spots/slide) for in-situ drug metabolism. Specifically, region A contains no enzyme as a test compound only control, region B contains a mixture of human Cytochrome P450 isoforms, region C contains a mixture of human phase II drug-metabolizing enzymes and P450 enzymes (i.e., all enzyme mixture), and region D contains human liver microsome. Each different compound is printed in sections 1–6 of the chip, and the concentrations (C1–C9) vary with each mini-array

Fig. 3.
Preparation of test compound solutions in the BaCl_2 -PLL solution after serial dilutions in DMSO

Fig. 4.

A layout of the DataChip (1,080 spots/slide) for high-throughput toxicity screening. Each 3D cell culture spot on the DataChip contains 6×10^6 Hep3B cells/mL in 30 nL of 1% alginate (180 cells/spot). The 3D cell cultures are arrayed onto the PS-MA-coated slide as 24 of 5×9 mini-arrays. Thus, 24 dose response curves comprising 9 doses (5 replicates each) are obtained from a single DataChip

Fig.5.
The stamping apparatus for aligning the MetaChip and the DataChip

Fig.6.

(a) Scanning images of the alginate-gel spots containing Hep3B cells (30 nL, 1080 spots/slide) after stamping with 24 regions of 5 × 9 enzyme arrays (from left to right: compound A, B, C, D, E, and F obtained from a collaborator, from top to bottom: control, human P450 mixture, all enzyme mixture, and human liver microsome) and staining. (b) Dose-response curves obtained from the scanning image

Table 1

Typical preparation of enzyme solutions for the MetaChip

Region	Enzymes	Co-substrates	Matrix
A	90 μ L PBS containing 5% glycerol ^a	–	30 μ L alginate (2%)
B	45 μ L human P450 mixture ^b	45 μ L P450 cofactor ^d	30 μ L alginate (2%)
C	45 μ L all enzyme mixture ^c	45 μ L all cofactor ^e	30 μ L alginate (2%)
D	45 μ L human liver microsome	45 μ L all cofactor	30 μ L alginate (2%)

^aNo enzyme (TNI-WT negative control) is used as a control to determine toxicity of parent compound

^bA mixture of human P450 isoforms contains 0.59 μ M CYP3A4, 0.20 μ M CYP2D6, 0.08 μ M CYP2C9, 0.03 μ M CYP2C19, 0.05 μ M CYP2E1, 0.04 μ M CYP1A2, and 0.01 μ M CYP2B6

^cAll enzyme mixture is composed of 50% of a mixture of human phase II enzymes (containing 1.15 mg/mL UGT1A1, 1.15 mg/mL UGT1A4, 1.15 mg/mL UGT2B4, 1.15 mg/mL UGT2B7, 0.1 mg/mL SULT1A3, 0.1 mg/mL SULT2A1, and 0.2 mg/mL GST) and 50% of the human P450 mixture

^dA P450 cofactor solution contains 50% of NADP (10 mM) and 50% of a regeneration system from Invitrogen Vivid[®] CYP450 screening kits

^eAll cofactor solution is composed of 50% of a phase II cofactor solution (containing 50% of 10 mM UDP-GA in 50 mM Tris–HCl buffer, pH 7.5, 25% of 20 mM GSH, and 25% of 20 mM PAPS in 10 mM PBS buffer, pH 8) and 50% of the P450 cofactor solution