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Micropatterned Co-Cultures of Human Hepatocytes and Stromal Cells for the Assessment of Drug Clearance and Drug-Drug Interactions

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Abstract

Drug clearance rates from the body can determine drug exposure that can affect efficacy and/or toxicity. Thus, accurate prediction of drug clearance during preclinical development can help guide dose selection in humans; however, animal testing is not always predictive of human outcomes. Since hepatic drug metabolism is a rate-limiting step in the overall clearance of many drugs, primary *human* hepatocytes (PHHs) in suspension cultures or monolayers are used for drug clearance predictions; however, the precipitous decline in drug metabolizing capacity can lead to significant inaccuracies in the prediction of clearance rates for low turnover compounds that have desirable one-pill-a-day dosing regimens. In contrast, micropatterned co-cultures (MPCCs) of PHHs and fibroblasts that display phenotypic stability for several weeks can help mitigate such limitations with conventional cultures. Here, we describe the protocols to create and use MPCCs for drug clearance predictions, and for modeling clinically-relevant drug-drug interactions that can affect drug clearance.

Keywords

Primary human hepatocytes; cytochrome P450; 3T3-J2 fibroblasts; low turnover drugs; drug metabolism

Introduction

Since metabolism by primary human hepatocytes (PHHs) in the liver is the rate-limiting step in the overall clearance of ~70% of marketed drugs (Wienkers and Heath, 2005), accurate prediction of *in vivo* human hepatic clearance is crucial during preclinical drug development towards guiding drug dose selection in the clinic (Ring et al., 2011). The use of animals for such predictions is often fraught with inaccuracies due to significant differences between animals and humans in drug metabolism pathways (Shih et al., 1999). Thus, isolated PHHs

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are now widely used for evaluating drug clearance rates since they are relatively straightforward to use in medium- to high-throughput culture formats, and contain the full complement of enzymes, transporters, and cofactors necessary for drug metabolism (Godoy et al., 2013). However, suspension cultures of cryopreserved PHHs from single vials do not allow drug incubations beyond ~4-6 hours due to a rapid loss of cell viability, while adherent confluent PHH monolayers display a rapid decline in the activities of cytochrome P450 (CYP450) enzymes (critical for metabolizing many drugs) to <10% of those measured in freshly isolated PHHs (Lecluyse, 2001; Khetani and Bhatia, 2008). The use of multiple thaws of PHH vials to create a 'relay' of drug incubations from one set of declining PHH cultures onto newly-prepared cultures can extend the drug incubation time in both suspensions and adherent monolayers (Di et al., 2012; Peng et al., 2016). However, PHHs do not properly polarize with appropriate localization of transporters to the apical and basolateral domains, which is limiting for predicting clearance rates of drugs that are transporter substrates. Additionally, relays are technically and logistically challenging to execute in a rapid drug screening campaign. Thus, the aforementioned conventional PHH culture models are severely restricted for accurately and rapidly predicting clearance rates of compounds with a wide range of *in vivo* turnover rates, especially low turnover compounds that are being developed for desirable one-pill-a-day dosing regimens. In contrast, micropatterned co-cultures (MPCCs) of cryopreserved PHHs and 3T3-J2 murine embryonic fibroblasts created in a 96-well format display high levels of CYP450 and phase-II conjugation activities for ~4 weeks (Lin et al., 2016). Such activities coupled with the ability to dose drugs for up to 7 days without a medium change has led to better overall prediction of drug clearance rates for high, medium, and low-turnover compounds than conventional cultures created from the same donor (Chan et al., 2013; Lin et al., 2016).

In this unit, we detail protocols to construct MPCCs and utilize them for the prediction of drug clearance rates in the presence or absence of 'perpetrator' drugs that can modulate CYP450 activities, and thereby affect the clearance rate of 'victim' drugs (i.e. drug-drug interactions or DDIs). Protocol 1 describes the creation of the MPCCs, including preparation of the micropatterned collagen plates and seeding of the two cell types. Support protocols within protocol 1 describe the construction of the polydimethylsiloxane (PDMS) mask for patterning collagen (support protocol 1), maintenance and propagation of the 3T3-J2 fibroblasts prior to seeding onto micropatterned PHH colonies (support protocol 2), and assays used to evaluate PHH health in the MPCC model prior to drug dosing (support protocol 3). Next, 'Protocol 2' describes how to dose MPCCs with drugs, collect drug-depleted supernatants, acquire drug concentration data in the supernatants, and analyze the data to predict *in vivo* drug clearance rates. Alternative protocol 1 describes how to modify protocol 2 in order to enable investigations into the effects of DDI on clearance rates of drugs. Finally, we end the unit with information that will yield the highest chance of obtaining accurate clearance predictions using MPCCs.

Basic Protocol 1

Creation of Micropatterned Cocultures

This protocol describes the creation of the MPCC platform. Tissue culture polystyrene plates must be prepared by coating the wells with rat tail collagen-I, patterning the collagen islands, and sterilizing the plates prior to PHH seeding. An overview of this process is shown in Figure 1. In order to micropattern collagen in the plates, a reusable PDMS mask must first be constructed (support protocol 1). Prior to PHH seeding, fibroblasts must be maintained and propagated until there is a sufficient cell number for use in MPCCs (support protocol 2). The health of PHHs is assessed in MPCCs using major markers of liver functions prior to use in drug dosing studies (support protocol 3).

Materials

- 96-well tissue-culture polystyrene plates
- 15-mL and 50-mL conical centrifuge tubes
- 1.5 mL microcentrifuge tubes
- Sterile double-distilled water (ddH₂O)
- Rat-tail collagen solution type I
- 70% ethanol in ddH₂O (vol/vol)
- 0.05% bovine serum albumin fraction V solution in ddH₂O (wt/vol)
- Human hepatocyte seeding medium (see recipe in 'Reagents and Solutions' section)
- 1X Dulbecco's Modified Eagle Medium (DMEM), high-glucose formulation
- Human hepatocyte overnight medium (see recipe in 'Reagents and Solutions' section)
- 'Plateable' cryopreserved PHHs (BioreclamationIVT, Triangle Research Laboratories, and Life Technologies)
- Trypan blue
- Hemocytometer
- Human hepatocyte maintenance medium (see recipe in 'Reagents and Solutions' section)
- 3T3-J2 fibroblasts (see support protocol 2)
- Pipette dispensers with serological pipettes
- Single channel and multichannel micropipettes with tips
- PDMS mask (see support protocol 1)
- Plate compression clamp (Star Prototype Manufacturing, Guangdong, China and 3D Systems, Rockhill, SC)
- Screwdriver

Biosafety cabinet

37°C cell culture incubator

Oxygen plasma chamber (PlasmaEtch, Carson City, NV and SPI Supplies, West Chester, PA)

Oxygen tank (medical grade)

37°C water bath

Centrifuge

Microscope

80°C freezer

Preparing Collagen Patterned Plates—Execute the following steps in the biosafety cabinet using aseptic technique, except where indicated.

1. Prepare a rat-tail collagen-I solution at 25 µg/mL in ddH₂O.
2. Fill each well of a 96-well plate with 50 µL of the collagen solution from step 1.
3. Incubate the plate (with the lid on) for 2 hours in the 37°C incubator.
4. Remove the collagen solution from the wells and rinse the wells 2 times with 50 µL/well of sterile ddH₂O.
5. Dry the plate thoroughly in the biosafety cabinet (with the lid off) for at least 2 hours (or overnight if ambient humidity is high).

Incomplete drying will cause water to become trapped in subsequent steps and lead to improper patterning.

The collagen-coated plate with the lid on and wrapped with parafilm can be stored at 4°C sealed in a plastic bag with a desiccant pack to absorb excess moisture.

6. Place the PDMS mask into the plate, slide the assembly into the clamp, and tighten the clamp using the screwdriver such that all PDMS posts/islands are pressing up against the collagen-coated plastic (Figure 2).

Each post/island pressed against the collagen-coated plastic should appear circular and not “doughnut-like”, which indicates over-compression and buckling of the PDMS that can lead to improper patterning.
7. Open the valve on the oxygen tank and then follow instructions for the specific plasma machine being utilized to turn it on and prepare the machine for generation of oxygen plasma.
8. Insert the plate assembly from step 6 into the plasma chamber (typically outside the biosafety cabinet)
9. Expose the plate to 100 W of oxygen plasma for 120 seconds.

Parameters such as plasma power and time may need to be adjusted according to the specific plasma machine model being used.

10. Release the vacuum, open the plasma chamber, remove the device, and unclamp the PDMS mask from the plate.
11. Sterilize the plate in a biosafety cabinet by incubating the wells with 50 μ L/well of 70% ethanol for 1 hour.
12. Remove the ethanol from the wells, rinse the wells 3 times with 50 μ L/well of sterile ddH₂O, and discard the last wash.

The collagen-patterned plate with the lid on and wrapped with parafilm can be stored at 4°C sealed in a plastic bag with a desiccant pack to absorb excess moisture.

13. On the day of PHH seeding, fill each well of the micropatterned collagen 96-well plate with 50 μ L of sterile 0.05% bovine serum albumin solution in ddH₂O.
14. Incubate the plate for 60-120 minutes in the 37°C incubator.
15. Remove the bovine serum albumin solution from the wells and rinse the wells 2 times with 50 μ L/well of sterile ddH₂O discarding the last wash. The plate is now ready for PHH seeding.
16. Repeat steps 1-15 for additional plates as needed.

Seeding PHHs

17. Take a vial of cryopreserved PHHs from liquid nitrogen storage.

It is important to work quickly and precisely following the protocols below after pulling the cell vial out of the liquid nitrogen in order to maximize cell viability and yield.
18. Thaw the vial by immersing it and gently swirling it in the 37°C water bath for 2 minutes without submerging the cap.

If the vendor has specific instructions on how to thaw the hepatocyte vial, follow their instructions instead.
19. Remove the vial from the water bath, wipe dry, spray the vial with 70% ethanol, and securely place the vial in a tube rack in the biosafety cabinet.
20. Open the vial and carefully pour its contents into 25 mL of pre-warmed human hepatocyte seeding medium in a 50-mL conical.

Human hepatocyte seeding medium should be warmed in the 37°C water bath for 15-30 minutes prior to pulling the cells out of liquid nitrogen storage.
21. Rinse the cell vial with 1 mL of pre-warmed human hepatocyte seeding medium and transfer the contents into the 50-mL conical containing the cell suspension.
22. Centrifuge the cells at 50×g for 10 minutes at room temperature.

- 23** Aspirate the supernatant without disturbing the cell pellet and re-suspend the cells in human hepatocyte seeding medium to achieve a density of 0.5-1 million cells/mL.
- Do not vortex or shake the suspension at any point during the cell thawing process.
- 24** Assess the viability and yield of the cells using the trypan blue exclusion method in combination with manual counting of the cells loaded in a hemocytometer.
- We do not recommend the use of an automated cell counter for counting PHHs since these cells can have some morphological alterations (e.g. blebbing, clumps, some nonparenchymal cell contamination) that in our experience, lead to inaccurate counts using an automated counter.
- 25** Add sufficient human hepatocyte seeding medium to achieve a final cell density of 0.667×10^6 cells per 1 mL.
- Seeding densities can be altered based on the attachment efficiency of the particular PHH lot.
- 26** Dispense the cell suspension into the wells of the 96-well plates at 50 μ L/well.
- It is important to frequently mix the cells carefully using a serologic pipette to ensure that the seeding density is consistent across multiple wells of the plate.*
- 27** Place the cultures in the 37°C incubator.
- 28** Pull the cultures out of the incubator every 20 minutes to shake the plate 3 times both horizontally and vertically in the direction parallel to the plate in order to evenly distribute the cells.
- Do not shake the plates in a circular pattern as that will cause the PHHs to aggregate in the center of the well.
- Make sure to not shake the cultures so vigorously that the culture medium splashes up onto the lid of the plate.
- The duration of time that the plate is out of the incubator should be minimized.
- Every 1-2 hours, assess the cultures under a microscope to make sure that the PHHs are patterning. Patterning should begin to appear 20 minutes after seeding with the islands continuing to fill in over the next 4-6 hours.
- 29** When 85-90% of the islands are filled with PHHs (typically after 4-6 hours of shaking depending on the PHH donor lot), rinse the cultures 3 times by gently removing the medium from each well using a multichannel micropipette and adding 50 μ L/well of 1X DMEM. On the last wash, discard the 1X DMEM rinse and add human hepatocyte overnight medium to the cultures at 50 μ L/well.
- 1X DMEM and human hepatocyte overnight medium should be warmed in the 37°C water bath for 15-30 minutes prior to rinsing the cells.

More rinses may be necessary if there are still a lot of PHHs observed in suspension. It is normal to have 1-5% of the cells leftover in suspension post rinsing.

It is not recommended to use vacuum aspiration on micropatterned cultures at any point in the culture lifetime. Use of micropipettes (manual or electronic) is recommended for all cell culture and drug dosing steps.

- 30 Place the cultures in the 37°C incubator overnight.

Seeding Fibroblasts onto Micropatterned PHHs

- 31 About 18-24 hours following seeding of PHHs, follow the procedure outlined in 'support protocol 2' to pellet suspended 3T3-J2 murine embryonic fibroblasts. Then, resuspend the fibroblast pellet in hepatocyte maintenance medium to achieve a density of 0.5-1 million cells/mL.

Ensure complete cell mixing so there are no visible clumps.

- 32 Assess the viability and yield of the cells using the trypan blue exclusion method in combination with manual counting of the cells loaded in a hemocytometer.
- 33 Add hepatocyte maintenance medium to the fibroblast suspension to achieve a density of 0.3×10^6 cells per 1 mL.
- 34 Remove the existing cell culture medium from the patterned PHH plates and either discard or store at -20°C to -80°C for future assays if desired.
- 35 Add 50 µL/well of the fibroblast suspension from step 33 to each well of the 96-well plate containing the patterned PHHs and place the plate in the 37°C humidified incubator.

It is recommended to execute steps 34-35 quickly such that wells with PHHs do not get desiccated. One strategy is to execute the steps for one row or column of the 96-well plate at a time.

It is important to frequently mix the fibroblast suspension to make sure that the seeding density is consistent across multiple wells of the plate.

- 36 Pull the cultures out of the incubator every 30 minutes to shake the plate 3 times both horizontally and vertically in the direction parallel to the plate in order to homogenize the cell distribution in the wells. Cultures should be shaken until the majority of seeded fibroblasts have attached to the wells (typically within 1.5-2 hours).

Make sure to not shake the cultures so vigorously that the culture medium splashes up onto the lid of the plate.

The duration of time that the plate is out of the incubator should be minimized.

- 37 Maintain the cultures by replacing the culture medium with fresh human hepatocyte maintenance medium every 2 days.

Supernatant can be collected and stored at -20 to -80°C for future assays. Additionally, when changing the cell culture medium, it is important to minimize the amount of time that the cells are sitting without culture medium to prevent cell desiccation.

Cultures should be assessed under the microscope at least every other day to ensure that hepatic morphology is maintained and that there is no visible microbial contamination.

- 38 Assess major liver functions in MPCCs (see examples detailed in support protocol 3) for ~1 week to ensure they are relatively stable prior to any drug dosing.

Support Protocol 1

Constructing the PDMS Mask

The PDMS mask is composed of a base with pillars and buttons with circular domains placed on top of the pillars. The buttons are created using a silicon master wafer with negatively patterned features (Figure 3A), whereas the base is created using a machined Teflon mold (Figure 3B). The fully assembled PDMS mask (Figure 3C) only needs to be created one time and can be reused over several years (depending on overall usage) to pattern the tissue culture plates.

Materials

Silicon master wafer with SU-8 photoresist (150-250 µm thickness) negatively patterned in circular micro-domains/islands of 500 µm diameter that are spaced 1200 µm apart center-to-center (Trianja Technologies, SimTech, and FlowJem)

Glass petri dish

Weighing dishes

Hexamethyldisilazane

Sylgard 184 PDMS kit (Dow Corning)

Arch punch (5 mm in diameter for a 96-well plate)

Teflon blocks (Star Prototype Manufacturing, Guangdong, China and 3D Systems, Rockhill, SC)

Vacuum desiccator

Oven

Creating the PDMS Mask Buttons

1. Secure the silicon master into the glass petri dish.

One method is to glue the silicon master onto the glass petri dish with a few drops of PDMS (10:1 base to curing agent by weight), ensuring that the

wafer is pressed flat against the petri dish, and allow curing at 65°C overnight.

2. Place the dish with the silicon master and a plastic weighing dish with a few drops of hexamethyldisilazane into a vacuum desiccator chamber.
3. Keep the chamber under vacuum until all of the hexamethyldisilazane has evaporated.
4. Thoroughly mix the PDMS components (base and curing agent at 10:1) together according to manufacturer's instructions.

It is important that the mixing is done well over several minutes; otherwise, the PDMS polymerization will be inconsistent across the silicon area.

5. Pour the PDMS mixture over the silanized silicon master, de-gas the PDMS in a vacuum chamber, and cure overnight at 65°C.

It is important that the PDMS is thoroughly de-gassed so no air bubbles are present in the patterns.

6. Carefully peel the cured PDMS from the silicon master using a blade to cut around the edges.
7. Use the arch punch to core out 96 PDMS buttons with positive features.

It is important for the buttons to be of uniform thickness for proper patterning. Therefore, the amount of PDMS being placed on the silicon wafer in the glass petri dish can be weighed out on a scale to minimize batch-to-batch variations.

Creating the PDMS Mask Base

8. Machine two interconnecting Teflon blocks that together will form a base and 96 pillars corresponding to the wells of a 96-well plate.
9. Silanize the Teflon blocks using steps 2-3 above.
10. Thoroughly mix the PDMS components (base and curing agent at 10:1) together according to manufacturer's instructions.
11. Pour the PDMS mixture into the connected Teflon blocks until the base of the PDMS above the pillars is approximately 1 cm thick.

Make sure the machined Teflon blocks are connected tightly to prevent unpolymerized PDMS from leaking out of the blocks.

12. De-gas the PDMS in a vacuum chamber and cure overnight at 65°C.
13. Carefully remove the cured PDMS from the Teflon mold.
14. Attach the PDMS buttons created in step 7 to each pillar of the PDMS base by first placing a small drop of uncured PDMS on the pillar followed by a PDMS button with the patterned positive features facing upwards (i.e. with micropatterned features pointing away from the base).

Use light even pressure to make sure that all buttons are at a uniform height.

- 15 Cure the entire base and buttons assembly overnight at 65°C.

Support Protocol 2

Culturing 3T3-J2 Fibroblasts

Cryopreserved vials of fibroblasts should be thawed and propagated in a T-150 flask at least 1 week prior to PHH seeding. The doubling rate of fibroblasts is approximately 36 hours. Medium should be changed every 2-3 days until approximately 90% confluency is reached, at which point the flask should be trypsinized for either passaging for continued propagation or seeding onto micropatterned PHH cultures. A confluent flask should yield ~4-5 million cells.

Materials

Tissue culture flasks (T-150)

3T3-J2 murine embryonic fibroblasts (courtesy of Howard Green from Harvard Medical School)

1X phosphate buffered saline (PBS) solution

0.25% (wt/vol) trypsin with 0.21 mM ethylenediaminetetraacetic acid (EDTA)

Fibroblast medium (see recipe in 'Reagents and Solutions' section)

Thawing 3T3-J2 Fibroblasts

1. Take a vial of cryopreserved 3T3-J2 fibroblasts out of liquid nitrogen storage.

It is important to work quickly and precisely after pulling the cells out of the liquid nitrogen in order to maximize cell viability and yield.
2. Thaw the vial by immersing it and gently swirling it in the 37°C water bath for 2 minutes without submerging the cap.
3. Remove the vial from the water bath, wipe dry, spray the vial with 70% ethanol, and securely place the vial in a tube rack in the biosafety cabinet.
4. Pipette the vial contents into an empty 50-mL conical.
5. Rinse the vial with 1 mL of pre-warmed fibroblast medium and transfer the medium into the 50-mL conical with the cell suspension

Fibroblast medium should be warmed in the 37°C water bath for 15-30 minutes prior to pulling the fibroblast vial out of liquid nitrogen.
6. Add 28 mL of pre-warmed fibroblast medium into the 50-mL conical from step 5 in a dropwise manner over 2-5 minutes to bring the total volume up to 30 mL.

It is important to add the medium slowly to allow the cryoprotectant in the cells to be released without lysing of the cells.

7. Transfer the contents of the 50 mL conical to a T-150 flask and maintain in a 37°C humidified incubator.
8. Replace the medium with 30 mL of fresh, pre-warmed fibroblast medium every 2-3 days.

Observe the fibroblasts under a microscope every day and when the cells reach ~90% confluency, they need to be passaged as described below.

Starting with a seeding density of ~0.4-0.5 million cells in a T-150 flask, it will take ~4-5 days for the fibroblasts to reach ~90% confluency.

Fibroblast Passaging

- 9 Aspirate the fibroblast medium from the flask.

Aspirate from the side of the flask that doesn't have the attached cells to avoid aspirating off part of the cell monolayer. This can be accomplished by tilting the flask to pool media on a non-cell surface.
- 10 Add 10 mL of 1X PBS to rinse the cell surface of the flask.
- 11 Aspirate the PBS (from the side of the flask that doesn't have cells attached) and replace with 10 mL of 0.25% pre-warmed trypsin-EDTA.

Trypsin-EDTA should be warmed in the 37°C water bath for 10 minutes prior to rinsing the cells with PBS.
- 12 Place the flask in the 37°C incubator for 7 minutes.
- 13 Gently tap the side of the flask to detach any remaining adherent cells until the majority of the cells are detached (verify under the microscope if necessary).
- 14 Add 10 mL of pre-warmed serum-containing fibroblast medium into the flask to neutralize the trypsin-EDTA.

Fibroblast medium should be warmed in the 37°C water bath for 15-30 minutes prior to using on the cells.
- 15 Use the 20 mL cell suspension in the flask to rinse the flask surface 3 times to collect all cells.
- 16 Transfer the cell suspension into an empty 50-mL conical.
- 17 Centrifuge the cells at 420×g for 8 minutes at room temperature.
- 18 Aspirate the supernatant without disturbing the cell pellet and resuspend the cells in fresh pre-warmed fibroblast medium to achieve a density of ~0.5-1 million cells per mL.

Alternatively, the cells can be re-suspended in human hepatocyte maintenance medium for seeding onto PHH cultures (see basic protocol 1).
- 19 Assess the viability and yield of the cells using the trypan blue exclusion method in combination with manual counting of the cells loaded in a hemocytometer.

- 20 Seed an appropriate number of fibroblasts in a total culture medium volume of 30 mL into a new T-150 flask, and place the flask in the 37°C incubator.

Support Protocol 3

Assessing Hepatocyte Health/Functionality

Healthy hepatocytes have a prototypical morphology (polygonal shape, distinct nuclei/nucleoli, and visible bile canaliculi) (Figure 4) and display functions of the liver. We use phase contrast microscopy to assess morphology. For the assessment of hepatocyte functions, we measure albumin and urea levels in collected culture supernatants as well as CYP450 and phase II conjugation enzyme activities in a non-destructive manner.

Materials

96-well assay plates
1.5 mL microcentrifuge tubes
Multichannel micropipettes with tips
Human hepatocyte dosing medium (see recipe in 'Reagents and Solutions' section)
CYP450 luminescent kits (Promega)
CYP450 substrates reconstituted in dimethyl sulfoxide (DMSO)
Human hepatocyte maintenance medium (see recipe in 'Reagents and Solutions' section)
Human albumin ELISA kit (Bethyl Laboratories)
Urea nitrogen test kit (Stanbio Laboratory)
Spectrophotometer (ideally compatible with 96-well plate for higher throughput)
Luminometer (ideally compatible with 96-well plates for higher throughput)
Data analysis software (e.g. Microsoft Excel and GraphPad Prism)

Protocol steps

1. Dilute the substrate from the Promega CYP450 kit (i.e. luciferin-IPA to measure CYP3A4 activity) in human hepatocyte dosing medium at a concentration recommended by the manufacturer.

Alternatively, cultures can be dosed with a CYP450 probe substrate that requires liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis for quantification of metabolite produced (see Table 1). We typically utilize a contract research organization (e.g. Integrated Analytical Solutions, Berkeley CA; Cyprotex, Watertown, MA) for LC-MS/MS analysis of our samples if necessary.

2. Remove the depleted human hepatocyte maintenance medium from the wells.

Supernatant can be stored at -20°C to -80°C for albumin and urea assessment.

3. Rinse the wells 1 time with 64 µL/well of pre-warmed human hepatocyte dosing medium to remove excess serum proteins that may bind to drugs.
4. Add 64 µL of human hepatocyte dosing medium with added substrate to the wells.

It is important to keep the final DMSO concentration as low as possible (<0.5%, vol/vol) since DMSO can modulate CYP450 levels (Ware et al., 2015).

Cultures should be kept in the dark from this point onwards as the substrate from the kit may be light sensitive.

5. Incubate the cultures at 37°C for the amount of time indicated on the manufacturer instructions (typically 1-3 hours).
6. Collect the supernatant and store at -80°C for analysis.
7. Add 64 µL/well of pre-warmed human hepatocyte maintenance medium to the culture wells that were probed and put back in the incubator to probe at a later time-point.
8. Repeat steps 1-7 above for multiple time-points as needed.
9. Take the collected cell culture supernatant samples out of -80°C storage and thaw at room temperature.

Avoid freeze-thaw cycles for the samples as proteins such as albumin and luminescent metabolites can degrade.

10. For the supernatants containing luminescent metabolites, process and read on a luminometer according to manufacturer's instructions.

The actual luminescence from the samples will depend on the instrument being used and the gain settings.

11. For the albumin and urea assessments, create 8 standard samples *per assay* via a 2-fold serial dilution. The last standard should be the media blank containing no analyte.

The range of the standards will depend on the kit being utilized. For the urea assay, we start with 100 µg/mL as the highest standard, while for the albumin ELISA kit, we start with 500 ng/mL as the highest standard given the high sensitivity of the assay.

12. Process the samples using the albumin and urea assay kits according to manufacturer's instructions.

MPCCs containing PHHs secrete between 40 and 80 µg/mL of urea and between 10 and 50 µg/mL of albumin depending on the PHH donor utilized.

Thus, dilution of the samples will be required to be compatible with the albumin ELISA kit mentioned above.

13. Assess the health of PHHs in MPCCs by plotting the assay signals in samples over time using data analysis software (e.g. Microsoft Excel, GraphPad Prism).

Albumin secretion increases in MPCCs over the first few days and stabilizes by ~1 week in culture, while urea secretion in some PHH donors may show a slight drop after the first few days followed by steady state values. CYP450 (i.e. CYP3A4) activities stabilize after ~1 week in culture. MPCCs display relatively stable levels of the aforementioned functions for ~4 weeks in culture (Khetani and Bhatia, 2008; Lin et al., 2016).

Basic Protocol 2

Assessing Drug Clearance in MPCCs

After the cultures have been functionally stabilized (i.e. with respect to the endpoints described in support protocol 3) for approximately a week, they can be dosed with the drugs of interest to evaluate clearance rates. A sample plate layout is shown in Figure 5. To evaluate DDIs, cultures can be first incubated with a ‘perpetrator’ drug, one that can modulate drug metabolism enzyme levels (see alternate protocol 1), followed by incubation with the ‘victim’ drug, for which clearance rate is to be determined as described below. Given the fewer number (~15 fold) of PHHs in MPCCs as compared to confluent monolayers, we have found that MPCCs can be incubated with a drug for 7 days without medium changes to allow for sufficient turnover of a wide range of compounds, including those with low turnover rates *in vivo* (Lin et al., 2016). The drug-laden supernatants from the cultures are collected at different time-points and such supernatants can be stored at -80°C for subsequent measurement of drug quantities via LC-MS/MS.

Materials

- 96-well tissue culture plates with MPCCs seeded (from basic protocol 1)
- 1.5 mL microcentrifuge tubes
- Single and multichannel micropipettes with tips
- DMSO
- Drugs of interest
- Human hepatocyte dosing medium (see recipe in ‘Reagents and Solutions’ section)
- 37°C incubator
- 37°C water bath
- 80°C freezer
- Biosafety cabinet

Dosing Cells with Drug

1. Prepare a 1 mM stock solution in DMSO for each drug.
Extra stock drug solutions can be stored at -20°C.
2. Dilute the 1 mM drug stock in pre-warmed human hepatocyte dosing medium at a 1:1000 dilution factor to obtain a final dosing concentration of 1 µM.
Human hepatocyte dosing medium should be warmed in the 37°C water bath for 15-30 minutes prior to diluting the drugs.
It is important to keep the final DMSO concentration as low as possible (<0.5%, vol/vol) since DMSO can modulate CYP450 levels (Ware et al., 2015).
3. Remove the depleted culture medium from the wells and rinse the wells 1 time with 64 µL/well of pre-warmed human hepatocyte dosing medium to remove excess serum proteins that may bind to drugs.
4. Add 64 µL/well of the drug solution per well.
64 µL of the drug solution is added per well to make sure that there is at least 50 µL available for data analysis as there can be a slight loss in volume over time due to evaporation.
5. Collect supernatants (50 µL) from representative wells and freeze immediately at -80°C.
Each well will be used for a single time-point with triplicates for each time-point and 8 time-points spread across 7 days of incubation with the drug. For drugs with higher clearance rates, most of the time-points should be within the first 24 hours, whereas for drugs with lower clearance rates, time-points should be distributed over longer periods of time.
Remember to collect t0 samples in which the drug solution is placed in the wells and immediately collected into the microcentrifuge tubes and stored at -80°C.
To maintain consistent humidity across the plates for all the time-points, we add culture medium back to the wells from which supernatants were already collected even though the cells will no longer be used for any further supernatant collections.
6. Assess the concentration of parent drug in the supernatant at each time-point using LC-MS/MS analysis.
We contract out LC-MS/MS analysis to companies that specialize in this technique (e.g. Integrated Analytical Solutions, Berkeley, CA, Cyprotex, Watertown, MA, and QPS, Research Triangle Park, NC).

Data Analysis

- 7 To obtain the clearance rate from measured drug concentrations in the cell culture supernatants, plot the natural logarithm of the concentration of remaining drug against incubation time, and obtain the slope via linear regression (Figure 6).
- 8 Calculate the *in vitro* depletion half-life of the drugs in the culture supernatant using the following equation:

$$t_{1/2} = \ln(2) / \text{slope}$$

Only the initial linear portion of the plot should be used to obtain the slope and further analysis should be conducted only if the correlation coefficient of the linear fit is greater than 0.8. The half-life should ideally be within the maximum incubation time so no extrapolation is necessary.

- 9 Calculate the intrinsic clearance (CL_{int}) with the following scaling factors as standard parameters (Obach et al., 1997; Lin et al., 2016): 21 g of liver weight per 1 kg of body weight, 120×10^6 hepatocytes per 1 g of human liver, 4500 hepatocytes/well, and 0.064 mL incubation volume.

$$CL_{\text{int}} = \frac{\ln(2)}{t_{1/2}} \times \frac{\text{liver weight}}{\text{standard body weight}} \times \frac{\text{incubation volume (mL)}}{\text{hepatocytes per well}} \times \frac{\text{hepatocytes}}{\text{gram of liver}}$$

10. Calculate the hepatic clearance (CL_h) using the well-stirred model with liver blood flow (Q) being 21 mL/min/kg and protein binding (f_u) set to 1 if no protein binding correction will be made or to its respective known *in vivo* value.

$$CL_h = \frac{Q \times f_u \times CL_{\text{int}}}{Q + (f_u \times CL_{\text{int}})}$$

We found that clearance rate predictions are drastically improved when protein binding (f_u) is set to 1 for high- and medium-turnover compounds (in vivo clearance rate is greater than 1 mL/min/kg). On the other hand, better clearance predictions are obtained for low-turnover compounds (in vivo clearance rate is less than or equal to 1 mL/min/kg) when protein binding correction is employed using known f_u values (Lin et al., 2016).

Other analytical models besides the well-stirred model, such as the parallel-tube and dispersion models, can also be utilized for clearance prediction (Hallifax et al., 2010). We found that the well-stirred model works well with data obtained from MPCCs.

Alternate Protocol 1

Assessing Drug-Drug Interactions in MPCCs

Basic protocol 2 can be altered to assess how an initial perpetrator drug can induce or inhibit the activity of drug metabolism enzymes (i.e. CYP450s), which can in turn affect the clearance rate of a victim drug.

Additional Materials

Drugs of interest (perpetrator and victim drugs)

Protocol steps

1. Prepare a stock solution of the perpetrator drugs at 1000 times the desired dosing concentration in DMSO.

The perpetrator drug concentrations may need to be optimized to obtain maximal effects on drug metabolism enzyme activities while minimizing cytotoxicity.

2. Dilute the drug stock in pre-warmed human hepatocyte dosing medium at a 1:1000 dilution factor.

Human hepatocyte dosing medium should be warmed in the 37°C water bath for 15-30 minutes prior to diluting the drugs.

It is important to keep the final DMSO concentration as low as possible (<0.5%) since DMSO can modulate CYP450 levels (Ware et al., 2015).

3. Remove the depleted culture medium from the wells and rinse the wells 1 time with 64 µL/well of pre-warmed human hepatocyte dosing medium to remove excess serum proteins that may bind to drugs.
4. Add 64 µL/well of the drug solution per well.
5. Incubate the MPCCs with the perpetrator drug for hours to days.

We found that incubating MPCCs with CYP450 inducers for 3 days and CYP450 inhibitors for 4-18 hours worked well, but the incubation time may need to be altered to optimize induction and inhibition effects.

6. Change medium every other day with fresh drug added to the human hepatocyte dosing medium.
7. Execute the steps of basic protocol 2 for assessing the clearance rate of a victim drug.

We recommend incubating the cultures with the perpetrator drug alongside the victim drug to maintain the modulation of drug metabolism enzyme(s).

Reagents and Solutions

Use sterile reagents in all recipes and protocol steps. Suitable suppliers for media and components include Corning, Sigma-Aldrich, and Thermo-Fisher Scientific. All recipes are for 50 mL; scale volumes as necessary. All media should be stored for up to 2 weeks at 4°C unless otherwise indicated. For components of media, follow the manufacturers' instructions for optimal storage temperatures.

Human Hepatocyte Seeding Medium

- 48.25 mL Williams' E medium
- 750 μ L HEPES, 1 M at pH 7.6
- 500 μ L ITS+ (insulin/human transferrin/selenous acid and linoleic acid) premix
- 500 μ L penicillin-streptomycin (100X stock)
- 0.5 μ L dexamethasone (reconstituted stock at 10 mM in DMSO, store at -20°C)
- 0.5 μ L glucagon (reconstituted stock at 0.7 mg/mL in 0.05 M acetic acid, store at -20°C)

Human Hepatocyte Overnight Medium

- 43.25 mL 1X DMEM, high-glucose formulation
- 5 mL fetal bovine serum
- 750 μ L HEPES, 1 M at pH 7.6
- 500 μ L ITS+ (insulin/human transferrin/selenous acid and linoleic acid) premix
- 500 μ L penicillin-streptomycin solution (100X stock)
- 0.5 μ L dexamethasone (reconstituted stock at 10 mM in DMSO, store at -20°C)
- 0.5 μ L glucagon (stock at 0.7 mg/mL in 0.05 M acetic acid)

Human Hepatocyte Maintenance Medium

- 43.25 mL 1X DMEM, high-glucose formulation
- 5 mL bovine serum
- 750 μ L HEPES, 1 M, pH 7.6
- 500 μ L ITS+ (insulin/human transferrin/selenous acid and linoleic acid) premix
- 500 μ L penicillin-streptomycin (100X stock)
- 0.5 μ L dexamethasone (reconstituted stock at 10 mM in DMSO, store at -20°C)
- 0.5 μ L glucagon (reconstituted stock at 0.7 mg/mL in 0.05 M acetic acid, store at -20°C)

Fibroblast Medium

44.5 mL 1X DMEM, high-glucose formulation

5 mL bovine serum

500 μ L penicillin-streptomycin (100X stock)

Store media for up to 4 weeks at 4°C

Human Hepatocyte Dosing Medium

48.25 mL 1X DMEM, high-glucose formulation, phenol red-free

750 μ L HEPES, 1 M, pH 7.6

500 μ L ITS+ (insulin/human transferrin/selenous acid and linoleic acid) premix

500 μ L penicillin-streptomycin (100X stock)

0.5 μ L dexamethasone (reconstituted stock at 10 mM in DMSO, store at -20°C)

0.5 μ L glucagon (reconstituted stock at 0.7 mg/mL in 0.05 M acetic acid, store at -20°C)

Commentary

Background Information

Historical Development of MPCCs as a Drug Screening Tool—Guguen-Guillouzo et al showed several decades ago that some PHH functions could be transiently induced by co-culture with a liver epithelial cell type (Guguen-Guillouzo et al., 1983). However, it was not possible at that time to investigate the role of precisely controlled cell-cell interactions on PHH functions. Thus, Bhatia et al used semiconductor-driven photolithography to tune homotypic interactions between primary rat hepatocytes adhered to collagen-coated circular islands while keeping cell numbers constant across patterned configurations of different island diameters and center-to-center spacing (Bhatia et al., 1997). While hepatocyte functions varied with island geometry (i.e. greater number of homotypic contacts between hepatocytes led to higher functions), such pure cultures displayed a decline in phenotypic functions as predicted from the above-mentioned study by Guguen-Guillouzo et al. Co-culture of the micropatterned rat hepatocytes with 3T3-J2 murine embryonic fibroblasts significantly increased both the magnitude and longevity of liver functions in these MPCCs. Transferring fibroblast-conditioned culture medium to hepatocyte cultures did not rescue liver functions to the same extent as cell-cell contact between the two cell types and formation of heterotypic cadherin junctions (Khetani et al., 2004; 2008). Khetani et al subsequently showed that 3T3-J2 cells induced optimal functions in hepatocytes from multiple species relative to other 3T3 clones (i.e. Swiss-3T3, NIH-3T3, L1-3T3) (Khetani et al., 2004). Khetani and Bhatia further optimized the MPCC platform for PHHs (both freshly isolated and cryopreserved), but a different balance of homotypic and heterotypic interactions with 3T3-J2 fibroblasts was needed to induce optimal functions in PHHs relative to rat hepatocytes (Khetani and Bhatia, 2008). Lastly, PDMS-based soft-lithographic techniques were developed to allow for the creation of MPCCs in plate-based formats for

drug screening (Khetani and Bhatia, 2008). Today, functionally optimized MPCCs contain PHHs organized in ~500 μm circular islands (~200-250 cells/island), spaced 900-1200 μm apart (center-to-center) and surrounded by 3T3-J2 fibroblasts in either 24-well or 96-well plate formats. The circular architecture in MPCCs allows the patterns to maintain their fidelity (i.e. little to no migration of hepatocytes off the islands) for several weeks *in vitro*. The Bhatia group at MIT has recently developed a 384-well version of MPCCs for increasing the throughput for both fundamental investigations and drug development.

MPCCs have been extensively validated for applications in drug development, such as drug clearance predictions (Chan et al., 2013; Lin et al., 2016), drug-drug interactions (Khetani and Bhatia, 2008; Lin et al., 2016), drug metabolite profiling (Wang et al., 2010), drug-transporter interactions (Ramsden et al., 2014), drug-induced hepatotoxicity (Khetani et al., 2013), and infection with global pathogens (hepatitis B/C viruses, malaria) (Ploss et al., 2010; Shlomai et al., 2014; March et al., 2013). More recently, MPCCs have shown utility for studying the effects of chronic hyperglycemia as in type 2 diabetes mellitus on the PHH phenotype (Davidson et al., 2015; 2016). In addition to higher functioning and stable PHHs, another key feature of MPCCs is that they allow an extended drug incubation time for up to 7 days without a medium change. Such an incubation time is due to the fewer number of metabolically active/demanding PHHs used within each well of MPCCs as compared to confluent PHH monolayers (by approximately 15-fold). Drug incubation up to 7 days was found to be sufficient for capturing the metabolism/depletion of compounds with a wide range of turnover rates *in vivo*, including low turnover compounds that are increasingly being developed for one-pill-a-day dosing regimens in humans (Lin et al., 2016). More specifically, MPCCs predicted 77%, 92%, and 96% of drug clearance values for all 26 tested drugs within 2-fold, 3-fold, and 4-fold of *in vivo* values (0.05-19.5 mL/min/kg *in vivo* clearance rates reported in the literature), respectively. There was good correlation ($R^2=0.94$, slope=1.05) of predictions between two PHH donors.

Alternative Approaches for Clearance Predictions—In contrast to MPCCs, single vials of PHHs in suspension do not allow for more than 4-6 hours of incubation with drugs, which is severely restrictive for predicting clearance of low turnover compounds as little to no drug depletion is observed in this short timeframe. In particular, we found that suspension PHHs, created from the same donor as that used in MPCCs, did not sufficiently deplete medium- and low-turnover drugs (7 out of 10 drugs) within the 4-hour incubation timeframe to allow prediction of clearance rates, while clearance rates of higher turnover drugs (3 out of 10 drugs) were accurately predicted within 2-3 fold of *in vivo* levels (Lin et al., 2016). To mitigate this limitation with suspension PHHs, an alternative approach to extend the incubation time for drug clearance prediction was established by Pfizer. In the so-called ‘relay’ method, the drug supernatant is transferred from a 4-hour pooled PHH suspension incubation to a freshly-thawed PHH suspension sequentially up to 5 times (20 hours total) for prolonged exposure of the drug to active enzymes (Di et al., 2013). However, since this method requires 5-fold more PHHs than a single incubation, it is crucial to screen for high-functioning cryopreserved PHH lots and bank them in relatively large quantities since not every lot will work well with this strategy (Akabane et al., 2012). Furthermore, the relay method does not help address the lack of polarity with appropriate localization of

transporters (such as those present on the canalicular membrane domain), which is limiting for predicting clearance rates of drugs that are also substrates for transporters (Tweedie et al., 2013).

More recently, the relay method has been shown to also work with confluent PHH monolayers for the clearance prediction of low turnover compounds (Peng et al., 2016). Indeed, we found that confluent PHH monolayers are a better model for clearance prediction than suspension PHHs (Lin et al., 2016). In particular, PHH monolayers predicted the clearance rates for 9 out of the 10 of drugs tested as compared to 3 out of 10 drugs for suspension PHHs created using the same donor. Naproxen, however, did not metabolize in PHH monolayers over 4 days of incubation even though it was depleted within 3 days in MPCCs, which contained significantly fewer PHHs than in confluent monolayers (~15-fold). Overall, confluent monolayers predicted 40%, 40%, and 50% of the compounds within 2-, 3-, and 4-fold of *in vivo* turnover rates, respectively, whereas MPCCs predicted 70% and 100% within 2- and 3-fold, respectively. Confluent monolayers also predicted lower clearance rates than MPCCs for 8 out of 10 drugs tested, likely due to the lower enzyme activity per cell in confluent monolayers (Khetani and Bhatia, 2008). Additionally, PHH monolayers are known to display declining CYP450 activities within a few hours, while hepatic polarity doesn't properly establish until 4-5 days following seeding (Khetani and Bhatia, 2008; Bi et al., 2006). Thus, there is a discrepancy between the kinetics of CYP450 levels and transporter localization in monolayers. Lastly, while single donors can be used effectively in MPCCs for clearance predictions, carefully selected pooled lots (10+ donors) are typically necessary along with the relay method to use suspension PHHs and PHH monolayers for any prediction of low turnover compounds (Di et al., 2012). Given the use of significantly fewer PHHs in MPCCs than suspension cultures and PHH monolayers, limited vials of high quality plateable cryopreserved PHH lots can be used for a greater number of screening studies (~2-3-fold by our estimate) in MPCCs than the above-mentioned conventional approaches. Finally, some groups have incorporated liver cultures into microfluidic systems for prediction of drug clearance (Baudoin et al., 2014). However, further validation of these latest culture platforms is needed for drug clearance predictions before they can be utilized for more routine drug screening.

Limitations of MPCCs for Clearance Predictions—While the MPCC model constitutes a significant advance for drug clearance predictions, it has some limitations that we and others are addressing in the next generation versions of this model. First, dosing in a serum-free culture medium can cause a decline in baseline CYP450 activities in MPCCs over time for some PHH donors, potentially due to the inability of fibroblasts to properly grow and thereby optimally support the PHHs. We don't observe this phenomenon across all PHH donors as they maintain CYP450 activities even in serum-free culture medium. Even with any decline in CYP450 activities due to the serum-free culture medium, we still observed turnover of all tested compounds over the 7-day incubation. We are now developing a serum-free culture medium that can keep the MPCC baseline functions at a sustained level for 14+ days, which could be important for observing phenotypic changes in susceptible donors with prolonged drug dosing.

As also observed in other studies (Ring et al., 2011; Hallifax et al., 2010), the accuracy of predicted clearance rates for medium and high turnover compounds in MPCCs was significantly improved when plasma protein binding was not incorporated in the analysis (Lin et al., 2016). In contrast, MPCCs metabolized low turnover drugs significantly faster in the serum-free medium formulation than *in vivo*, which necessitated the use of reported plasma protein binding values to greatly improve the accuracy of clearance predictions. Such an improvement could be due to enzyme-substrate kinetics where low turnover compounds have more time to bind to proteins than high turnover compounds, which could lower the unbound fraction available for metabolism (Atkinson and Kushner, 1979). We anticipate that the inclusion of human serum albumin, alpha-1 acid glycoprotein, and lipoproteins in culture medium at similar concentrations as found in human blood could be useful for a more consistent analysis scheme for the entire range of drug turnover rates under investigation (Chao et al., 2009). However, how major PHH functions are affected by incubation with this new media formulation would need to be investigated prior to any drug studies. Finally, MPCCs lack liver stromal cells (endothelia, macrophages, stellate cells) that can modulate drug metabolism enzyme activities in hepatocytes under normal and diseased states (Nguyen et al., 2015). Thus, we are engineering the next generation MPCCs that will incorporate all the relevant cell types of the liver and thus may be better predictive of drug clearance rates under specific perturbations.

Critical Parameters

General Cell Culture Technique—It is important to use sterile reagents/supplies and aseptic technique throughout the entire experiment to prevent microbial contamination. Hepatocyte culture media should be stored at 4°C and used within 2 weeks, while fibroblast culture medium should be used within 4 weeks since components in the media can degrade over time. If the cell culture media is suspected of being contaminated at any point, it should be discarded and new media should be made. The microbial contamination may change the pH of the contaminated media and/or degrade components; thus, sterile filtration as a salvaging strategy for contaminated media is not recommended. When handling cell culture flasks or plates, minimize the time that the cultures are outside of the 37°C incubator. When changing the cell culture medium or dosing with drugs, work quickly to add fresh medium after the depleted medium has been removed to prevent desiccation of the cells.

Selection of Cryopreserved PHHs—Cryopreserved PHH lots should be tested for attachment and stability of functions (i.e. albumin, urea, CYP450 activities) in the MPCC model prior to use for drug studies. We have found that while the majority of ‘plateable’ cryopreserved PHH lots we have tested display high and long-term (~4 weeks) functions in MPCCs, the packing density of the cells on the collagen-coated islands as well as the magnitude and stability of CYP450 activities can vary depending on the PHH donor. Therefore, such parameters should be assessed and CYP450 enzymes of interest should be probed at the activity level. Genotyping for major polymorphisms in drug metabolism enzymes can also be used to select specific PHH donors of interest. Owing to inter-individual differences in drug metabolizing enzymes, we recommend conducting drug clearance experiments in at least 3 pre-selected PHH donors within MPCCs. These PHH donors can be pooled as a single suspension and patterned into the same well; however, the

attachment efficiencies of each PHH lot should be similar to ensure that the composition of the collagen-coated island is comprised of relatively equal numbers of each PHH donor so as to not bias the drug outcomes to any one PHH donor. Alternatively, the PHH donors can be cultured in separate wells and the collected drug-laden supernatants can be pooled prior to downstream LC-MS/MS analysis.

Maintenance of Fibroblasts—Fibroblasts should be propagated in T-150 flasks prior to hepatocyte seeding to ensure that there is a sufficient amount of cells for the experiment. During fibroblast maintenance, it is important to change medium every 2-3 days and split the cells before 90% confluency is reached to prevent cell transformation. Untransformed fibroblasts should exhibit heterogeneous morphology and growth should be contact-inhibited. We typically use the fibroblasts within 12 passages to reduce the chances of transformation. If the fibroblasts are suspected of being transformed (homogeneous morphology, aberrant growth), they should not be used to create MPCCs due to excessive growth and depletion of nutrients in the culture medium. A new vial of fibroblasts should be thawed out and propagated for use in MPCCs. The doubling rate of fibroblasts is approximately 36 hours. If fibroblasts are growing significantly slower or faster, this may be due to the bovine serum lot used in the fibroblast medium. Bovine serum lots should be screened and optimal lots (with respect to fibroblast morphology and growth profiles) should be banked for future use.

Drug Dosing—When dosing MPCC with drugs (including CYP450 substrates), it is important to use phenol red-free medium as phenol red may interfere with sample analysis. Additionally, the human hepatocyte dosing medium does not contain serum as drugs can bind to serum proteins. The final concentration of DMSO in the medium should be kept below 0.5% as DMSO can modulate CYP450 expression levels (Nishimura et al., 2003; Ware et al., 2015). The drug concentration utilized should also be kept below the Michaelis-Menton constant (K_m) values for the metabolizing enzymes to prevent saturation. Finally, fibroblast-only controls should be carried out alongside MPCCs if the metabolism of a drug by non-CYP450 enzymes is suspected. The fibroblast-mediated drug clearance rate can be subtracted from the drug clearance rate in MPCCs if needed. We have observed that the majority of the drugs we have tested do not get metabolized by fibroblasts; however, there are likely to be exceptions that should be tested empirically with the drugs of interest.

Troubleshooting

Problem	Possible Cause	Solution
Poor hepatocyte patterning	Issues with the PDMS mask	Ensure that the PDMS buttons are of uniform thickness and that the positive features are properly formed (no air bubbles)
	Issues with clamping the PDMS mask into the plate	Ensure that the clamp is tightened to an appropriate level; under-compression may cause some of the islands to not be properly protected from the oxygen plasma, whereas over-compression may cause the PDMS posts to buckle and form rings around the islands
Lack of proper PHH attachment to collagen domains	Issues with hepatocyte cryopreservation or processing procedures or collagen coating	Try a new collagen patterned plate and/or a different PHH donor lot
Aberrant growth of fibroblasts and/or homogenous morphology	Potential transformation of fibroblasts and/or selection of a specific sub-clone within the population	Discard the transformed fibroblasts and thaw a new cryopreserved vial of fibroblasts for propagation

Anticipated Results

MPCCs should be functionally stable for ~4 weeks after seeding of the two cell types. Drug dosing is typically performed ~1 week after seeding hepatocytes since it takes about 1 week for albumin secretion to reach steady-state levels. Urea secretion should be stable from day 1 or show some down-regulation initially in some PHH donors followed by stabilization for the remainder of the time series. CYP450 enzyme levels should also be stable by ~1 week in culture. Metabolism should be observed for a majority of drugs in the MPCC model. For higher-turnover drugs, the plot of the natural log of the concentration of drug with respect to time should display a linear decrease until the drug has depleted almost entirely. For lower-turnover drugs, a steady decrease in parent drug should be observed over the time-course, which may need to extend to a 7-day incubation to observe statistically-significant drug depletion in supernatants. There may be differences in drug clearance rates across PHH donors due to donor-to-donor differences in the activities of drug metabolism enzymes, but the overall trends should be similar barring any polymorphisms. Induction or inhibition of CYP450 levels with prototypical drugs has been observed in MPCCs created from multiple PHH donors (Lin et al., 2016; Khetani and Bhatia, 2008). However, the time of incubation with these perpetrator drugs *prior* to incubation with the victim drug for clearance evaluation may need to be optimized depending on the action of the perpetrator drug.

Time Considerations

Preparing the PDMS etch mask can take multiple (3-5) days, but this mask can be stored at room temperature and reused repeatedly for a few years depending on the level of usage until signs of physical wear (i.e. tearing of PDMS) are observed. Coating plates with collagen and patterning the collagen using the PDMS mask will typically take 5-6 hours for ~10 plates, but can vary depending on the number of plates being prepared. Coating the plates with bovine serum albumin and processing the PHH vial for seeding will take approximately 2.5 hours, followed by 4-6 hours of shaking and 30 minutes of rinsing the plates once PHHs have attached and filled (>90%) the collagen-coated islands. After an overnight incubation, seeding of fibroblasts will take approximately 1 hour followed by 2 hours of shaking. After the MPCCs have been stabilized for 1 week, they can be dosed with drugs. The time it takes to prepare the drug solutions and dose the cultures depends on the number of drugs and the number of drug concentrations being assessed. Drug incubations are typically carried out for up to 1 week. Once the drug concentrations in the samples have been measured via LC-MS/MS, the data analysis will take 1-2 hours to predict drug clearance rates.

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Significance Statement

Prediction of drug clearance rates from the body during preclinical development can differentiate analog compounds based on desirable clearance rates and help determine clinical dosing range. Drug metabolism in the liver is a rate-limiting step in the overall clearance of many drugs. Thus, primary human hepatocytes (PHHs) in suspension or monolayer cultures are used to predict human-relevant drug clearance; however, they display a rapid loss of drug metabolizing capacity that limits accurate prediction of clearance rates for low turnover compounds, which are being developed for one-pill-a-day dosing regimens. Here, we describe the creation and use of micropatterned co-cultures of PHHs and fibroblasts for more accurate prediction of drug clearance rates, and for modeling drug-drug interactions that can affect drug clearance.

Figure 1. Overall process of creating micropatterned co-cultures (MPCCs)

96-well tissue culture plates are coated with rat tail collagen-I solution for 2 hours and patterned using oxygen plasma and a polydimethylsiloxane (PDMS) mask (see support protocol 1). Primary human hepatocytes (PHHs) will selectively attach to the collagen domains, while 3T3-J2 fibroblasts seeded 18-24 hours later will attach to the areas surrounding the PHH islands.

Figure 2. Setup for patterning the plates

The polydimethylsiloxane (PDMS) mask is placed inside the 96-well tissue culture plates coated with rat tail collagen-I solution and secured in the plate compression clamp. The clamp is then tightened by turning the screws to ensure proper patterning. This assembly is placed in the oxygen plasma chamber where oxygen plasma will ablate any exposed collagen that is not protected by the PDMS features of the mask contacting the adsorbed collagen. Primary human hepatocytes will bind to the collagen islands that were protected from the oxygen plasma by the PDMS mask, while 3T3-J2 fibroblasts can attach to the surrounding bare regions to create micropatterned co-cultures.

Figure 3. Constructing the polydimethylsiloxane (PDMS) mask

(A) The silicon master wafer with SU-8 etched features should have circular islands 500 μm in diameter with 1200 μm center-to-center spacing and a feature height of 150-250 μm . After silanization of the SU-8-patterned silicon master, PDMS is poured over the master with a thickness of at least 2 mm and cured overnight at 65°C. PDMS buttons 5 mm in diameter are then cored out using an arch punch. Each button should contain ~15-16 islands. (B) Teflon blocks are machined to match the geometry of a standard 96-well plate. The rectangular base should be at least 1 cm thick and the 96 pillars should be 5 mm in diameter and approximately 12.5 mm in height. After silanization of the Teflon blocks, PDMS is poured into the Teflon mold and cured to form the PDMS base. (C) The PDMS buttons are attached to each pillar on the PDMS base using a drop of uncured PDMS. The assembled PDMS mask is then cured overnight at 65°C.

Figure 4. Seeded micropatterned co-cultures (MPCCs)

(A) Image of a patterned 96-well tissue culture plate with a magnified image of 4 wells with primary human hepatocyte (PHH) islands stained with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye that yields a purple formazan product in living cells. (B) Phase contrast images of increasing magnifications of MPCCs containing micropatterned PHH islands surrounded by 3T3-J2 fibroblasts.

Figure 5. Sample experimental plate setup

In one 96-well plate, it is possible to run 4 drugs with 8 time-points (t_0 , t_1 , t_2 ... t_5 , t_6 , t_7) in triplicate. At each time-point, 50 μ L of supernatant from each triplicate well are collected for each drug.

Figure 6. Sample drug depletion plots

Prototypical drug depletion plots in micropatterned co-cultures for (A) naloxone, a high-turnover drug; (B) methylprednisolone, a medium-turnover drug; and (C) lorazepam, a low-turnover drug.

Table 1
Compounds that have been used for *in vitro* CYP450 profiling studies in micropatterned cocultures (MPCCs)

All substrates can be reconstituted in dimethyl sulfoxide (DMSO) and incubated in MPCCs at the indicated concentrations for 1 hour. The supernatants are then collected and stored at -80°C prior to LC-MS/MS analysis or luminescence analysis (for the CYP2C9-Glo and CYP3A4-Glo substrates from Promega).

Enzyme	Concentration (μM)	Substrate	Metabolites
1A2	100	Phenacetin	Acetaminophen
2A6	50	Coumarin	7-OH-coumarin
2B6	500	Bupropion	OH-bupropion
2C8	30	Paclitaxel	6a-OH-paclitaxel
2C9	50	Tolbutamide	4-OH-tolbutamide
2C9	100	Diclofenac	4-OH-diclofenac
2C9	100	CYP2C9-Glo	Luciferin
2C19	100	S-mephenytoin	4-OH-S-mephenytoin
2D6	16	Dextromethorphan	Dextrorphan
2E1	300	Chlorzoxazone	6-OH-chlorzoxazone
3A4	200	Testosterone	6b-OH-testosterone
3A4	3	CYP3A4-Glo (luciferin-IPA)	Luciferin
Phase II Metabolism	50	7-OH-coumarin	7-OH-coumarin glucuronide and 7-OH-coumarin sulfate