

Primary Human Hepatocyte Spheroids Generation and Performance in Different Culture Systems

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The culture of human primary hepatocytes as spheroids supports long-term cell viability and functionality. In this study, we compared different spheroid culture systems and present optimized culture conditions.

Introduction

In the field of toxicology, there is a strong need for *in vitro* human liver model systems that support the long-term culture of primary human hepatocytes (PHH) and closely mimic *in vivo* conditions of liver tissue. Standard 2-dimensional (2D) hepatocyte cultures are good short-term models, but rapidly lose typical hepatocyte functionality, e.g. cytochrome P450 (CYP) activity, over the course of 3 – 5 days.^{1,2} To better preserve functionality of hepatocyte models for longer-term studies, complex culture systems are increasingly employed. These include, but are not limited to, fluidic culture, 3-dimensional (3D) hydrogels, and self-assembling liver spheroids or micro-tissues.³

Self-assembling spheroids generated from PHH have been shown to have *in-vivo*-like cell organization, including extensive contact of adjacent cells compared to 2D cultures. Recently, spheroids have been shown to also improve predictivity of standard sets of known clinical liver toxicants.² Many different methods are available for facilitating the formation of liver spheroids in culture. In this study, we compared and optimized the formation, culture and performance of cryopreserved PHH in different spheroid culture systems and under various culture conditions and compared metabolic function and viability of the spheroids over 28 days in culture.

Materials and Methods

Sandwich culture: Cryopreserved PHH (Plateable, induction qualified; Lonza, cat. no. HUCPI) were cultured according to the instructions⁴ in sandwich culture. Briefly, 50,000 cells per well were plated in collagen coated 96-well plates. 4 – 6 hours post seeding, Matrigel™ (Corning) overlay was added in Hepatocyte Culture Medium (HCM™ Bullet® Kit; Lonza, cat. no. CC-3198). Media was renewed daily.

Spheroid formation: To make Spheroid Formation Medium (SFM), HCM™ Medium was further supplemented with 25 mM HEPES and 20% FBS (PAN-Biotech). 1,000 to 5,000 PHH were seeded in SFM in different culture plates including dedicated spheroid formation microplates, ultra-low attachment (ULA) V-bottom and U-bottom plates as well as hanging drop culture. Cells were left for 5 days at 37°C and 5% CO₂ with or without gentle agitation on an orbital shaker (KL-2, Edmund Bühler) set to 90 rpm. On day 6 after seeding and then every 2 to 3 days, 50% of the medium was renewed with serum-free HCM™ Medium. On day 6, spheroids generated in the hanging drop system were transferred into standard ULA U-bottom plates.

Spheroid formation was observed and documented by bright field microscopy (Zeiss AxioObserver Z.1 microscope equipped with a Zeiss N-Achroplan 10x Objective and an AxioCam MRm camera). Real time monitoring was done via time-lapse microscopy by CytoSMART™ 2 System (Lonza, cat. no. AACs-1003).

Viability and general metabolic activity: Viability/metabolic capacity of the culture was assessed via CellTiter-Blue® Cell Viability Assay (Promega) according to manufacturer's instructions. Incubation period with CellTiter-Blue® Reagent was 24 hours for spheroid culture. Albumin content in the supernatant was quantified with the Human Albumin ELISA Kit (Bethyl) according to manufacturer's instructions.

Cytochrome P450 activity: CYP3A4 activity was assessed via the P450-Glo™ CYP3A4 Assay (Promega) using Luciferin-IPA as a substrate according to manufacturer's instructions. For measuring the inducibility of the CYP3A4 gene, spheroids were incubated with 10 µM rifampicin or dimethyl sulfoxide (DMSO) control for 72 hours before measuring the CYP3A4 activity by P450-Glo™ CYP3A4 Assay. For direct measurement of CYP2B6 and CYP1A2 metabolic activity, spheroids were incubated with 250 µM bupropion and 100 µM phenacetin for 15 minutes. Formation of OH-bupropion and acetaminophen was evaluated as analyzed by LC-MS/MS (Biotranex).

Bile canaliculi formation: Cholesteryl-l-lysyl-fluorescein (CLF) staining (Corning) was used for visualization of bile canaliculi formation. Cells were incubated for 2 hours in medium containing 4 µM CLF followed by three washing steps consisting of 50% medium replacement and 1-hour incubation each prior to examination by fluorescence microscopy. To reflect the specific culture conditions, spheroids cultured under agitation on an orbital shaker were stained under agitation. Spheroids cultured without agitation were stained and washed without agitation.

Statistics: Outliers were identified by Dixon's Q test (95% confidence) and rejected. Error bars represent standard deviation.

Results

Decline in P450 activity in PHH over time in sandwich culture

We first analyzed the functionality of PHH in standard sandwich culture in 96-well plates. Exemplary of one functional read-out, it was observed that basal CYP3A4 activity clearly decreased within 7 days in both tested donors independently of initial activity level (Figure 1). This is consistent with previous observations of PHH sandwich cultures.

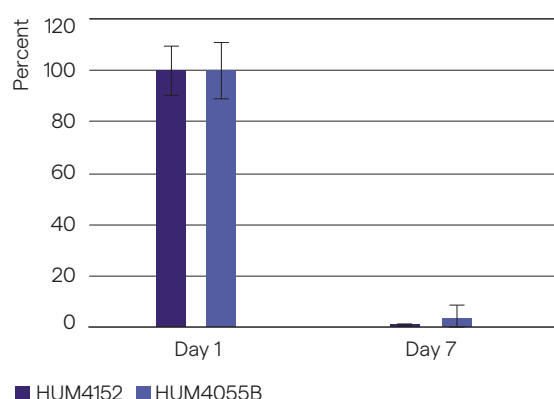


Figure 1

Basal CYP3A4 activity decreases rapidly in sandwich culture. PHH from two different donors HUM4152 and HUM4055B were cultured according to the supplier's instructions in collagen coated 96-well plates with Matrigel™ overlay. CYP3A4 activity was assessed with the P450-Glo™ CYP3A4 Assay using Luciferin-IPA as a substrate. Typical results for two donors are shown, n = 4. Error bars indicate standard deviation.

Optimal seeding density for hepatocyte spheroid formation is less than 3,000 cells/well

In an initial experiment in standard ULA U-bottom plates, formation of spheroids occurred within 4 days without addition of any supporting cell. Use qrcode below or url to view video.

www.cytosmart.com/lonza-spheroid



Compact spheroids of different sizes, consisting of 1,000 to 5,000 PHH, were examined after 7 and 10 days for size (Figure 2) and the applicability of standard analysis methods, e.g. metabolic activity and cytochrome P450 enzyme activity assays. While the functionality of spheroids was assessable with standard assay methods for spheroids as small as 1,000 PHH, the read-out is more variable with small cell numbers and the signal-to-noise ratio is rather low. Therefore, larger spheroids and cell numbers would be preferred from an analysis perspective. However, the risk of hypoxia and necrosis inside the spheroid core increases with spheroid diameter⁵, size and cell number. To avoid very large spheroids with a necrotic core, while having still easy to analyze spheroids, we decided to initiate spheroid formation with either 1,500 as lower limit or 3,000 PHH as upper limit, which resulted in spheroids approximately 500 µm in diameter.

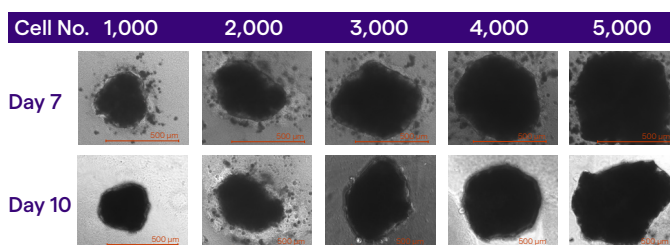


Figure 2

Spheroid formation. 1,000 to 5,000 PHH (HUM4110A) were seeded in ultra-low attachment (ULA) U-bottom plates. HCM™ Medium supplemented with 25 mM HEPES and 20% FBS was used as spheroid formation medium. Cells were left undisturbed for 7 days at 37°C 5% CO₂ with gentle agitation. On day 7 and 10 after seeding, 50% of the medium was renewed with serum-free HCM™ Medium. Documentation prior to media change on day 7 and after second media change on day 10 via bright field microscopy.

Effect of serum and/or agitation on spheroid formation

To examine the impact of serum (FBS) addition and agitation on the spheroid formation, we used either 1,500 or 3,000 cells per well in standard ULA U-bottom plates and tested increasing amounts of serum with and without placing the plate on a rotating platform within the incubator. In absence of serum, no spheroid formation could be observed (Figure 3). When medium with different amounts of FBS was used for spheroid formation, spheroids did not form at 0% and 2% FBS for up to 11 days. Spheroids generated with 10% FBS were not as compact as spheroids generated in medium with 20% FBS and a substantial amount of single cells was still left in the wells (Figure 3). In presence of 20% serum, both 1,500 and 3,000 PHH per well resulted in stable, compact spheroids independent of agitation (Figure 4). Therefore, addition of 20% serum was included in further studies.

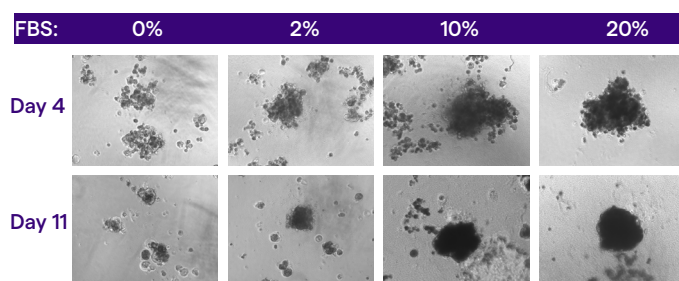


Figure 3
At least 10% serum are required for spheroid formation. 3,000 PHH (HUM4182) were seeded in ULA U-bottom plates. HCM™ Medium supplemented with 25 mM HEPES and different amounts of FBS was used as spheroid formation medium. Spheroid culture was performed under gentle agitation as described in **Materials and methods**. Documentation was performed via bright field microscopy.

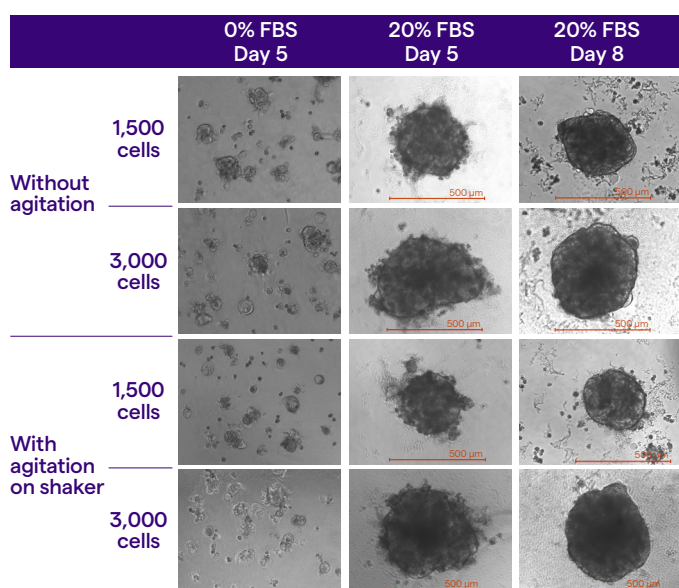


Figure 4
Successful spheroid formation in presence of serum. 1,500 or 3,000 PHH (HUM4178) were seeded in ULA U-bottom plates. HCM™ Medium supplemented with 25 mM HEPES and 20% respectively 0% FBS was used as spheroid formation medium. Cells were left undisturbed for 5 days at 37°C 5% CO₂ with or without gentle agitation. On day 6 after seeding and then every 2 to 3 days, 50% of the medium was renewed with serum-free HCM™ Medium. Documentation via bright field microscopy.

Spheroid formation in different culture formats

In a next step we evaluated the suitability of different culture methods for the formation and maintenance of spheroids. We compared viability (Figure 5) and albumin production (data not shown) on days 7 to 9 of culture using either 1,500 or 3,000 cells/well. Results were equivalent between all culture methods. V-bottom plates were less suitable for imaging. Therefore, all further evaluations were conducted using various U-bottom plates and the hanging drop system.

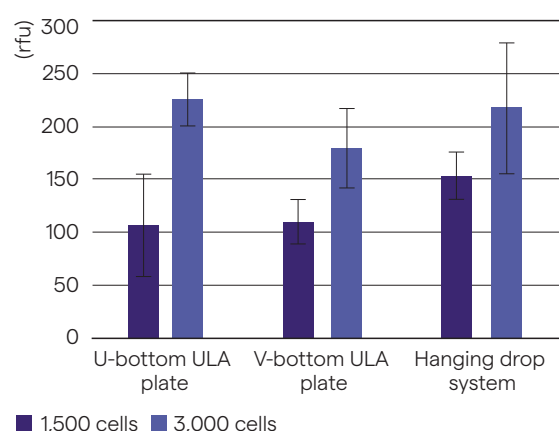


Figure 5
Metabolic activity, an indirect measure of cell viability, of PHH spheroids on day 9 is independent of the culture system used. 1,500 or 3,000 PHH from two different donors (HUM4152 and HUM4182) were seeded in ULA U-bottom or V-bottom plates or a hanging drop system. HCM™ Medium supplemented with 25 mM HEPES and 20% FBS was used as spheroid formation medium. Cells were left undisturbed for 5 days at 37°C 5% CO₂ under gentle agitation. On day 6 after seeding and then every 2 to 3 days, 50% of the medium was renewed with serum-free HCM™ Medium. Metabolic activity of the cultures was assessed via CellTiter-Blue® Cell Viability Assay. Mean results of two independent lots of PHH are shown. n = 8; error bars indicate standard deviation.

To examine spheroid stability and functionality over time, spheroids were generated and cultured for up to 4 weeks with and without agitation in three different culture formats: standard ULA U-bottom plate, dedicated round bottom spheroid microplate with proprietary design and the hanging drop system.

After an initial formation period of 6 days, serum was not required for further spheroid culture. From day 6 and beyond, a 50% medium change using serum-free medium meant the remaining FBS content was below 1% on day 15 of culture and the culture was considered serum-free during weeks 3 and 4. Spheroids remained compact and stable during the full examination period (Figure 6), independent of culture vessel and agitation.

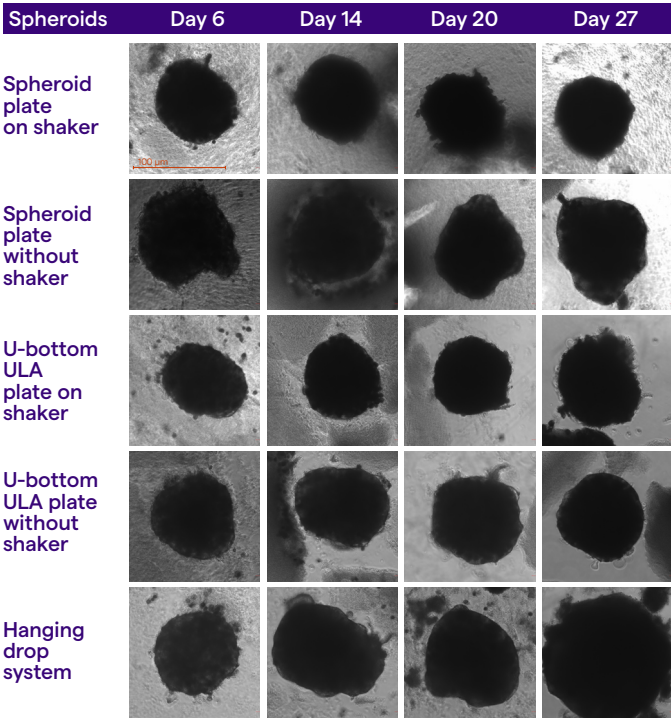


Figure 6
Spheroids remain stable over 4 weeks of culture. 3,000 PHH (HUM4055B) were seeded in different culture plates including dedicated spheroid culture plates, ULA U-bottom plates and hanging drop culture in serum-containing spheroid formation medium. Cells were left undisturbed for 5 days at 37°C 5% CO₂ with or without gentle orbital agitation. On day 6 after seeding and then every 2 to 3 days, 50% of the medium was renewed with serum-free HCM™ Medium. Typical results are shown. Documentation by bright field microscopy.

Bile canaliculi formation was observed in the standard ULA U-bottom plate, the dedicated round bottom spheroid microplate with proprietary design and the hanging drop system (Figure 7). Long-term persistence of bile canaliculi was also donor-dependent. For example, spheroids generated with PHH from lot HUM4055B maintained a 3D branched bile canaliculi network for 4 weeks in all tested culture systems when cultured without agitation. Bile can-

aliculi visibility was negatively affected by orbital agitation. As no positive effects of the agitation were observed, only the non-agitated samples were analyzed in the following experiments.

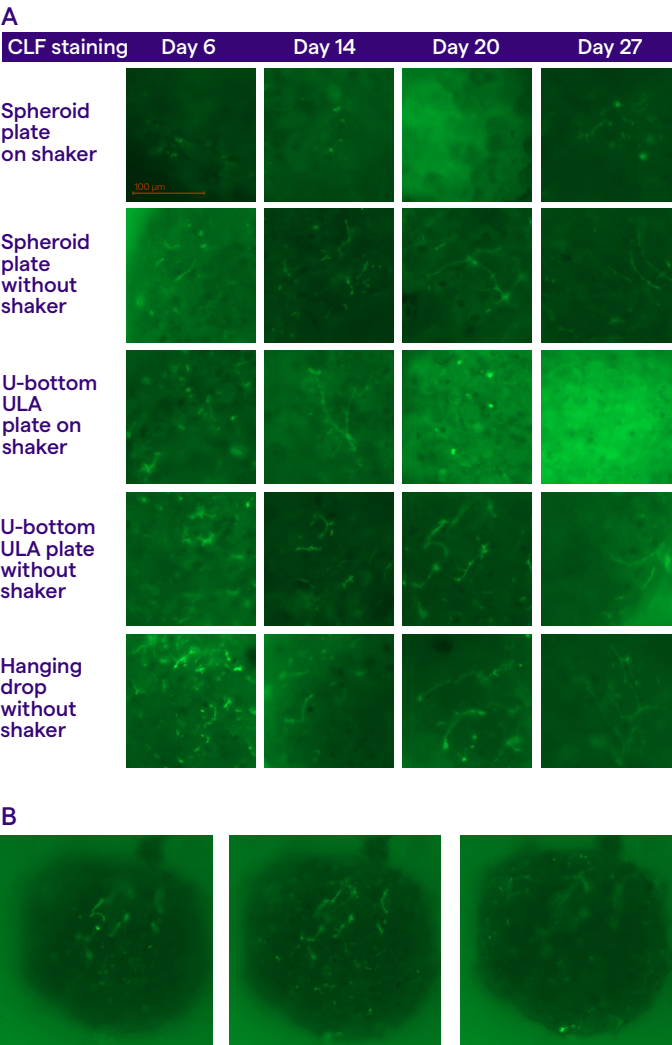
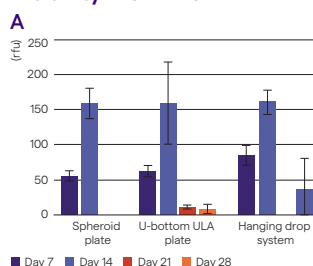


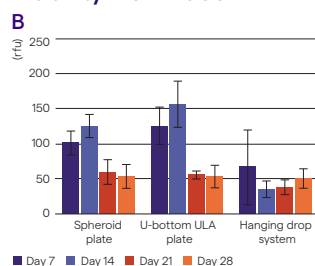
Figure 7
Bile canaliculi formation within spheroids over time. 3,000 PHH were seeded in different culture plates including dedicated spheroid culture plates, ULA U-bottom plates and hanging drop culture plates in serum-containing spheroid formation medium. Cells were left undisturbed for 5 days at 37°C 5% CO₂ with or without gentle orbital agitation. Starting on day 6, media changes were performed as described in **Materials and Methods**. Cholesteryl-labeled fluorescein (CLF) staining was used for visualization of bile canaliculi formation within spheroids. Cells were incubated for 2 hours in medium containing 4 µM CLF followed by three washing steps consisting of 50% medium replacement and 1-hour incubation each prior to examination by fluorescence microscopy. (A) comparison of different culture conditions over time (B) example of a 3D bile canaliculi network within one typical spheroid from **Figure 7A**, observed in different vertical layers.

Long-term functionality of PHH in spheroids as determined by albumin excretion was donor-dependent, however largely unrelated to the culture vessel type used (Figure 8). For example, spheroids generated using PHH lot HUM4152 maintained good viability and albumin production for the initial two weeks, followed by a decline in metabolic activity and albumin secretion. On the other hand, spheroids generated with PHH from lot HUM4055B showed reduced, but still clearly detectable metabolic activity after 4 weeks of culture as well as sustained albumin secretion.

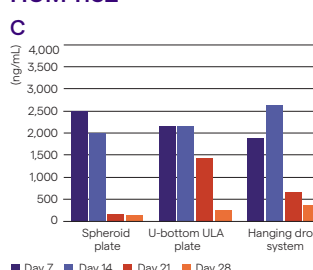
Viability HUM4152



Viability HUM4055B



Albumin secretion HUM4152



Albumin secretion HUM4055B

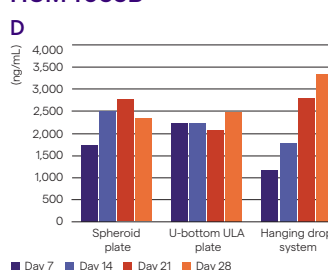


Figure 8

Long-term functionality in spheroid culture is donor-dependent. 3,000 PHH from two different donors (HUM4152 and HUM4055B) were seeded in different culture plates including dedicated spheroid culture plates, ULA U-bottom plates and hanging drop culture plates in serum-containing spheroid formation medium. On day 6 after seeding and then every 2 to 3 days, 50% of the medium was renewed with serum-free HCM™ Medium. Viability/metabolic capacity of the spheroid cultures was assessed via CellTiter-Blue® Cell Viability Assay (A, B). Incubation period was 24 hours. Results of two experiments with two independent donors are shown. n ≥ 3; error bars indicate standard deviation. Albumin content in the pooled supernatants was quantified with the Human Albumin ELISA Kit (C, D). Results of two experiments with two independent donors are shown. Bars represent pooled supernatants of ≥ three samples.

Cytochrome P450 activity in spheroids

To get a further understanding of PHH spheroid performance, basal and induced CYP3A4 activity was determined on day 7 of spheroid culture in standard U-bottom ULA plates and hanging drop system. Both basal and induced CYP3A4 activity were slightly higher but still within similar range in hanging drop culture compared to U-bottom ULA plate culture (Figure 9A). A 5-fold increase of CYP3A4 activity was observed after addition of 10 μM rifampicin in both culture systems (Figure 9B).

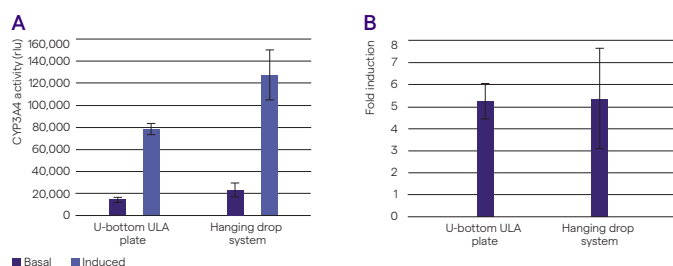
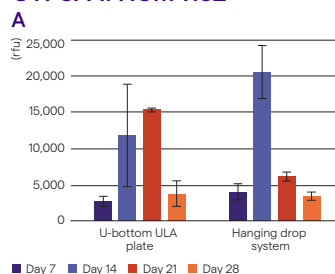


Figure 9

5-fold CYP3A4 induction in U-bottom ULA plates and hanging drop system. 3,000 PHH (HUM4096B) were seeded in ULA U-bottom plates or into a hanging drop system in serum-containing spheroid formation medium. Spheroid culture was performed without agitation as described in **Materials and methods**. CYP3A4 activity was assessed via the P450-Glo™ CYP3A4 Assay using Luciferin-IPA as a substrate according to manufacturer's instructions. For measuring the inducibility of CYP3A4, spheroids were incubated with 10 μM rifampicin for 72 hours before measuring the CYP3A4 activity by P450-Glo™ CYP3A4 Assay. Results of one typical experiment are shown. Comparable basic and induced CYP3A4 activity (A) as well as fold induction rate (B) was observed in both culture systems. n = 3; error bars indicate standard deviation.

Subsequently, we analyzed basal CYP3A4 activity in two independent PHH lots HUM4152 and HUM4055B in long-term using U-bottom ULA plates and the hanging drop system. Basal CYP3A4 activity was clearly detectable during the full examination period of 4 weeks in spheroids generated with PHH from both donors (Figure 10).

CYP3A4/HUM4152



CYP3A4/HUM4055B

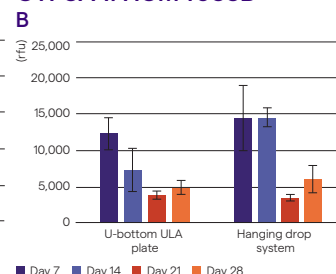


Figure 10

Long-term basal CYP3A4 activity in PHH spheroids. 3,000 PHH from two different donors (HUM4152 and HUM4055B) were seeded into the indicated spheroid culture formats in serum-containing spheroid formation medium. Cells were left undisturbed and without orbital agitation for 5 days. Starting on day 6, media changes were performed as described in **Materials and methods**. CYP3A4 activity was assessed via the P450-Glo™ CYP3A4 Assay using Luciferin-IPA as a substrate. Results of one experiment are shown. n ≥ 3; error bars indicate standard deviation.

To address CYP2B6 and CYP1A2 function, phenacetin and bupropion metabolism was tested by mass spectroscopy. Consistently, formation of OH-bupropion and acetaminophen was observed in spheroids generated with PHH for up to 4 weeks. However, donor-to-donor variability in maintaining metabolic activity was more visible in the later stages of culture (Figure 11). Little difference in cytochrome P450 enzyme activity was observed between the different culture methods tested.

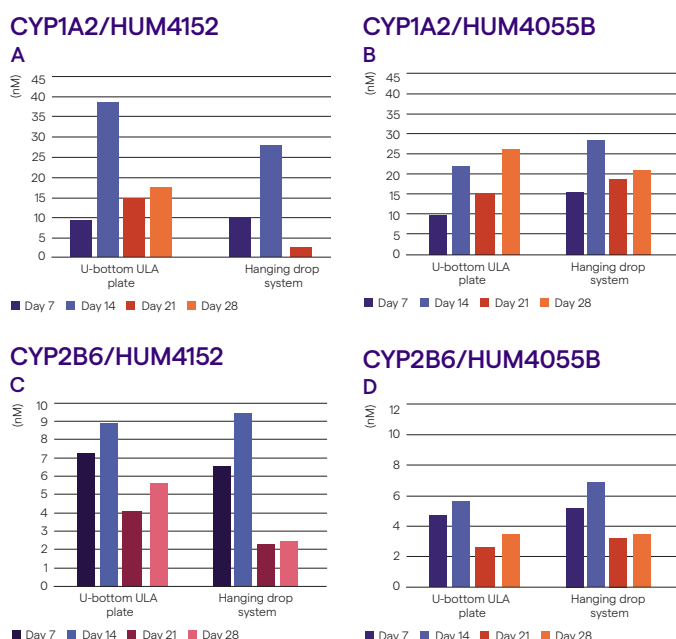


Figure 11
Long-term basal CYP1A2 and CYP2B6 activity in PHH spheroids. 3,000 PHH from two different donors (HUM4152 and HUM4055B) were seeded into the indicated spheroid culture formats in serum-containing spheroid formation medium. Cells were left undisturbed and without orbital agitation for 5 days. Starting on day 6, media changes were performed as described in **Materials and methods**. For direct measurement of CYP2B6 and CYP1A2 metabolic activity, spheroids were incubated with 250 μ M bupropion and 100 μ M phenacetin for 15 minutes. Formation of acetaminophen (**A**, **B**) and OH-bupropion (**C**, **D**) was evaluated, as analyzed by LC-MS/MS. Results of one experiment are shown, bars represent pooled supernatants of ≥ 3 samples.

Discussion

Viability and functionality of PHH in classical sandwich culture is often lost after a few days⁶ especially in 96-well cell culture plates. Spheroid cultures were described to be more stable over time.⁷ Therefore, we evaluated different methods for spheroid formation and verified the impact of spheroid culture on long-term PHH performance.

Successful spheroid generation without addition of any supporting cell types was observed for all PHH donors tested. This was independent of the starting cell number and the culture vessel type used. The presence of serum was required for spheroid formation. After an initial formation period of 6 days, serum was gradually removed during the following medium replacements. Remaining FBS content was thus far below 1% on day 15 of culture and the culture was basically serum-free during weeks 3 and 4. This practice is consistent with current literature² and of high importance for the design of following studies, which can be performed in absence of serum.

The generated spheroids remained compact and stable over the complete examination period of 4 weeks and displayed typical hepatocyte activity, including sustained albumin production as well as prominent CYP3A4 basal activity and inducibility. In accordance with data published newly by Baze *et al.*, long-term performance of PHH spheroids was comparable in U-bottom ULA plates and in the hanging drop system.⁸ However, donor dependent differences were clearly observed, predominantly in 3rd and 4th week of culture. While 3D bile canaliculi networks were nicely formed and visible in all culture systems tested, bile canaliculi visibility was negatively affected by orbital agitation. This was observed repeatedly for different hepatocyte donors and could represent altered bile canaliculi formation or stability due to fluidic shear stress or more intensive movement of the spheroids. It could also be a result of forced “washing off” of the CLF staining on a shaker.

Metabolic activity and albumin secretion was stable for at least 2 weeks of spheroid culture. After that, the ability to sustain activity was donor-dependent. CYP2B6 and CYP1A2 function was evaluated by measuring phenacetin and bupropion metabolism using mass spectroscopy. The formation of respective metabolites was detectable for the full examination period of 4 weeks in most of the analyzed situations. Donor-dependent differences such as more reduced versus persistent metabolic activity were visible over time. Overall, donor-to-donor variability was more pronounced in the later stages of spheroid culture. The gradual decline in PHH spheroid function and metabolic activity during long term culture is in accordance with currently published observations.⁷ In recent literature, only minor

variability in spheroid sensitivity to different hepatotoxic compounds was found between three donors. However, PHH from these donors were pre-characterized for long-term culture before.² Our results confirm the importance of pre-selection of cryopreserved PHH lots suitable for long-term culture.

Overall, all the different spheroid culture methods including ULA U-bottom and V-bottom plates as well as hanging drop culture and dedicated spheroid plates were suitable for PHH spheroid formation and long-term culture. Little differences with respect to viability, functionality and assay suitability were observed. The only exception to cell culture method compatibility was that V-bottom plates were less suitable for microscopic analysis compared to U-bottom and round bottom microplates.

Conclusion

We developed simple protocols for the successful *in vitro* generation of primary human hepatocyte spheroids in a variety of culture systems. Spheroid formation required the presence of serum in all culture systems tested. However, the long-term culture of spheroids was possible in serum-free medium. Standard analysis methods like cell viability or cytochrome P450 enzyme activity assays can be applied to spheroid culture. As expected for primary cells from independent donors, lot-dependent differences in long-term PHH functionality were observed, emphasizing the need for careful donor selection and thorough examination of available cryopreserved hepatocyte lots in advance. The resulting *in vitro* spheroid cultures offer more *in-vivo*-like conditions for long-term exposure and repeated dosing toxicology studies.

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