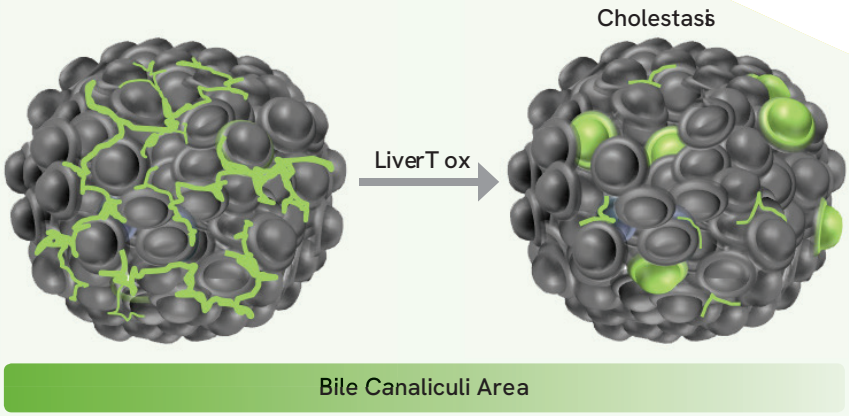
Imaging bile canaliculi in 3D liver microtissues using the Opera Phenix high-content screening system.

Key features

- Quantification of bile efflux transporter activity in 3D liver microtissues
- High quality confocal imaging of 3D microtissues with reduced spatial crosstalk from out of focus planes
- Simultaneous acquisition of up to four channels with minimal spectral crosstalk

Background

Analyzing transport of biliary metabolites is essential to predict pharmacokinetics and hepatotoxicity during drug development. A functional impairment of hepatobiliary transporters, such as bile salt export pump (BSEP) and multidrug resistance-ssociated protein 2 (MRP-2), is strongly associated with an increased risk of cholestatic liver injury. Currently, artificial models, such as BSEP expressing membrane vesicles, are used for studying efflux transporter function. Sandwich-cultured hepatocytes also are utilized as an *in vitro* tool to study hepatobiliary drug transport and hepatotoxicity [Lepist et al., 2014]. However, these models lack the functional complexity of the natural 3-dimensional (3D) liver environment. 3D liver cells immediately display key morphological and functional characteristics of native hepatocytes, such as cuboidal cell shape, increased formation of bile canaliculi and higher drug efflux activity, compared to the conventional monolayer culture [Hammad et al., 2014; Mueller et al., 2011].



Here, we describe a 3D high-content screening assay to study hepatobiliary transporter function in InSphero human liver microtissues using the Opera Phenix™ High-Content Screening System. Confocal imaging and automated image analysis were used to quantify BSEP and MRP-2-mediated efflux of fluorescent substrates into bile canaliculi.

Application

3D InSight™ Human Liver Microtissues consisting of primary human hepatocytes co-cultured with non-parenchymal Kupffer cells were produced using the GravityPLUS™ system from InSphero AG. This system allows for automation of the hanging drop method and provides an optimal tool for generating microtissues amenable to cell-based drug screening. Microtissues were then transferred to a 384-well Ultra Low Attachment (ULA) microtiter plate (Corning, 3827) to facilitate high quality imaging. Next, microtissues were treated with various concentrations of FCCP, Cytochalasin D and Sitaxsentan incubated in a total volume of 50 µL medium for 24 h at 37°C and 5% $C0_2$. Subsequently, microtissues were stained for 45 min with staining solution in medium containing 10 µM Hoechst 33342 (Life Technologies, H3570), 750 nM Tetramethylrhodamine (TMRM) (Life Technologies, T-668) and either 4 µM CellTracker™ Green CMFDA Dye (Life Technologies, C2925) or 4 µM CLF fluorescently labeled bile acid (Corning, 451041). CMFDA and CLF freely pass through cell membranes into cells, where they are transformed into a cell-impermeant product and then actively transported into bile canaliculi. The accumulation of CLF in bile canaliculi was used to quantify BSEP transport. Similarly, canalicular accumulation of glutathione methylfluorescein (GS-MF), derived from CMFDA, was used to quantify MRP-2 transport.

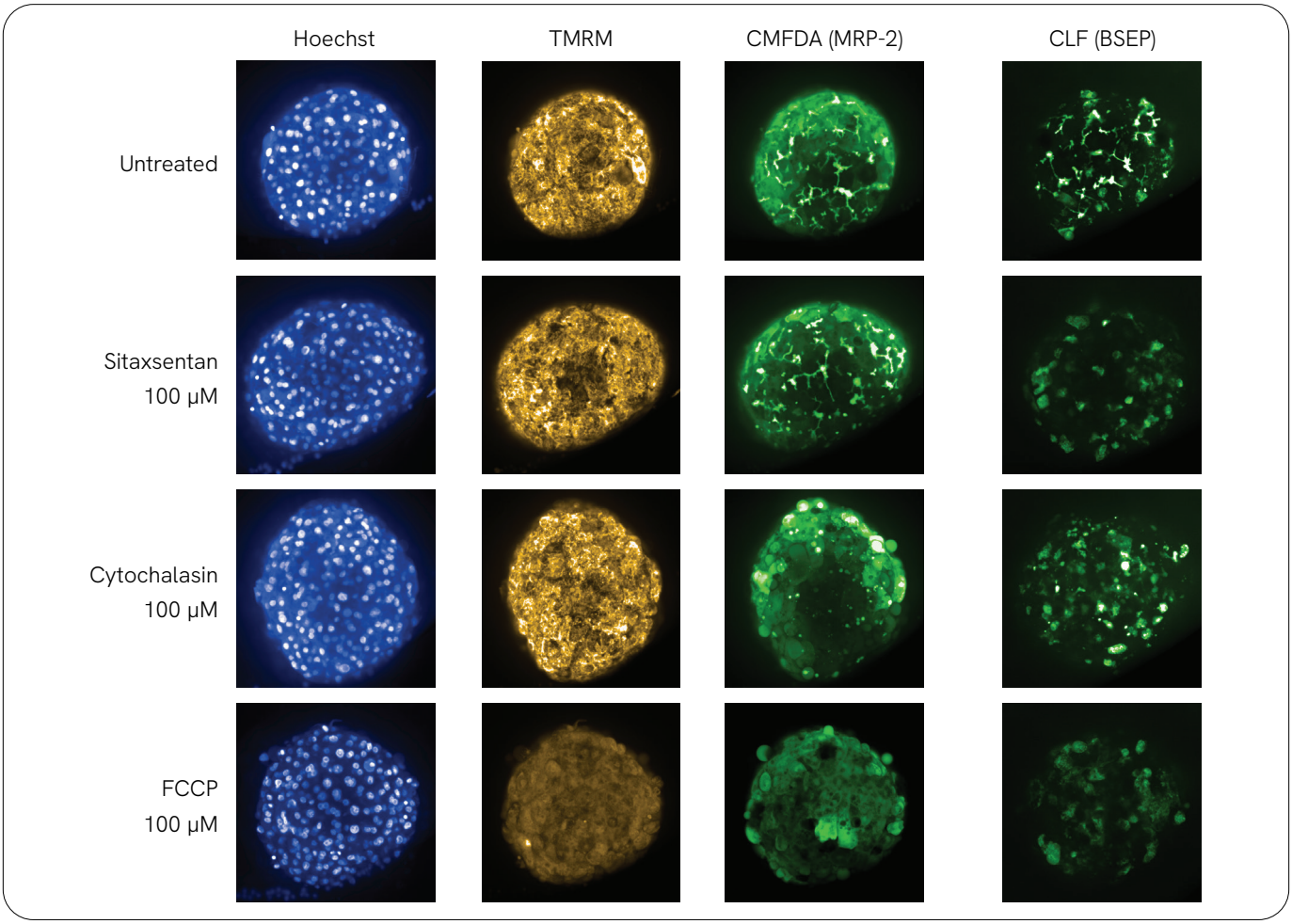


Figure 1: Sitaxsentan, Cytochalasin D and FCCP affect the hepatobilary transporter activity in human liver microtissues. Maximum intensity projection images (20xW) of microtissues stained with Hoechst, TMRM and either CMFDA or CLF. CMFDA and CLF serve as substrates for the hepatobilary transporters MRP-2 and BSEP respectively and the staining reveals the bile canaliculi network of the human liver microtissues. The BSEP inhibitor Sitaxsentan specifically affects the staining pattern of CLF, while leaving the CMFDA labelling unaffected. Cytochalasin D and FCCP have a broader toxicity and affect both the CMFDA and CLF staining. FFCP is a known mitochondrial toxin and therefore also reduces the TMRM intensity.

By taking advantage of Opera Phenix system´s Synchrony Optics™, three channels representing nuclei, active mitochondria and bile canaliculi were acquired simultaneously using the 20x water immersion objective. A confocal image stack of 60 µM with a plane distance of 5 µM was acquired for each tissue, capturing about one third of the entire microtissue volume.

As shown in Figure 1, untreated control microtissues show a strong TMRM labelling and a distinct bile canaliculi network after CLF and CMFDA exposure, indicating a healthy liver microtissue with active hepatobilary transport into the canalicular network by both BSEP and MRP-2. Treatment with Sitaxsentan, Cytochalasin D and FCCP changes the TMRM, CMFDA and CLF staining patterns in different ways. Sitaxsentan is a BSEP inhibitor and therefore specifically affects the CLF staining. Cytochalasin D and FCCP affect both the CMFDA and CLF staining pattern. Instead of the bile canaliculi network, the cells are stained, suggesting an inhibition of both BSEP and MRP-2. In addition, FCCP treated microtissues show a decreased TMRM intensity.

Image analysis

To quantify the compound-induced effects, we calculated the area and sum intensity of the bile canaliculi network as well as the TMRM intensity in the microtissue using the Harmony® High-Content Imaging and Analysis Software. As a first step in the image analysis sequence, a maximum intensity projection image was generated including all 13 images of the confocal image stack (0-60 µM, 5 µM plane distance). The microtissues were then detected on the Hoechst channel using the *Find Image Region* building block. By calculating the mean intensity of TMRM in the tissue region using the *Calculate Intensity* building block, the mitochondrial membrane potential could be determined. To precisely find the bile canaliculi in the tissue region, a combination of several building blocks was required (Figure 2). Fluorescent cells filled with CLF or CMFDA that are no longer capable of transporting dye into bile canaliculi were excluded from the tissue region using the building block *Select Region* (method: Restricted By Mask). As the bile canaliculi intensity and the background intensity in the tissue ranged from very faint to very intense, the building

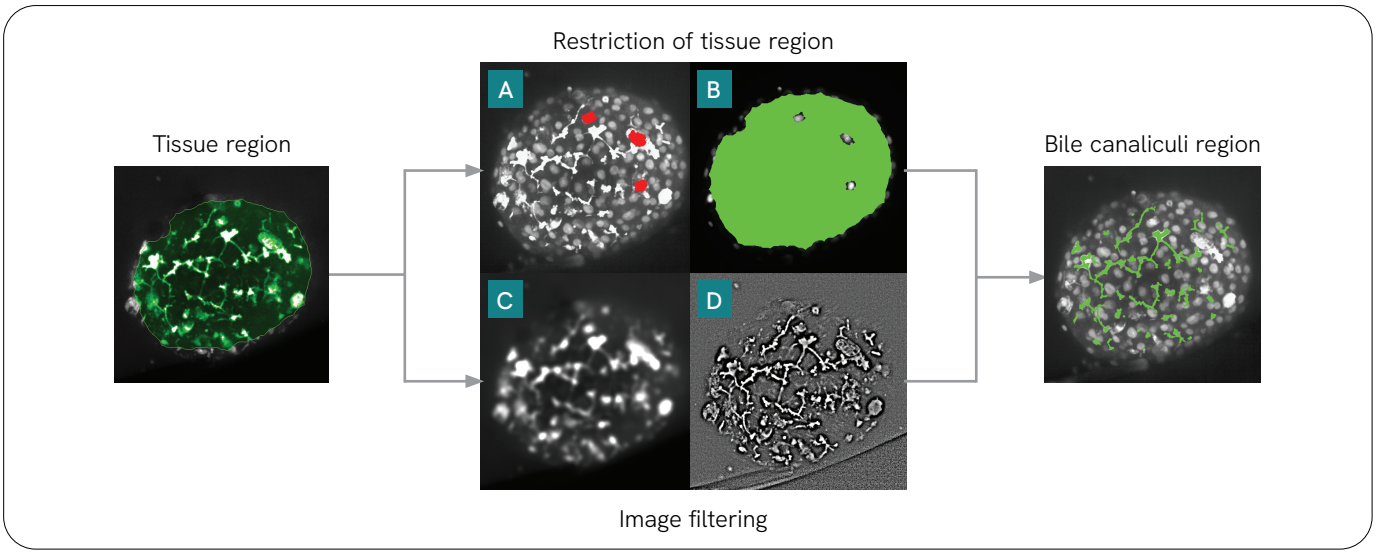


Figure 2: Segmentation of bile canaliculi using the *Select Region, Filter Image* and *Calculate Image* features of the Harmony software. (A) Fluorescent cells filled with CLF or CMFDA no longer capable of transporting dye into bile canaliculi were identified (red) using the building blocks *Find Cells, Calculate Intensity Properties* and *Select Population.* (B) These cells were then excluded from the initial tissue region using the building block *Select Region* (method: Restricted By Mask) to generate a restricted tissue region (green). (C) The *Filter Image* building block was used to generate Gauss-filtered CMFDA or CLF images. (D) With an image calculation (Gauss filtered image divided by original image) new CLF and CMFDA images with enriched contrast and normalized intensities were created using the *Calculate Image* building block. The bile canaliculi region was precisely identified on the newly calculated images in the restricted tissue regions using the *Find Image Region* building block.

blocks *Filter Image* (method: Gauss) and *Calculate Image* were used to create new CLF and CMFDA images with enriched contrast and normalized intensities. Ultimately, bile canaliculi were precisely identified on the newly calculated images in the restricted tissue region using the *Find Image Region* building block. The sum intensity of CLF or CMFDA in the canaliculi region and the bile canaliculi area in percent (bile canaliculi area/tissue area) were then calculated as final readouts.

The quantitative image analysis confirmed the phenotypic changes (Figure 3) that had been observed. Sitaxsentan is specifically inhibiting the BSEP transporter and induces a dose-dependent decrease in the CLF bile canaliculi intensity and area readouts while leaving the CMFDA and TMRM readouts unaffected. The inclusion of TMRM into the assay allowed additional information on the mitochondrial activity to be obtained in order to better understand the underlying toxicity mechanism. FCCP inhibits both hepatobiliary transporters and the mitochondrial activity, suggesting a lack of ATP as the reason for efflux inhibition. Cytochalasin D affects both efflux transporters but not the mitochondrial activity. This fits with the hypothesis that Cytochalasin D inhibits actin polymerization which is needed for bile canaliculi contractions that facilitate hepatobiliary transport.

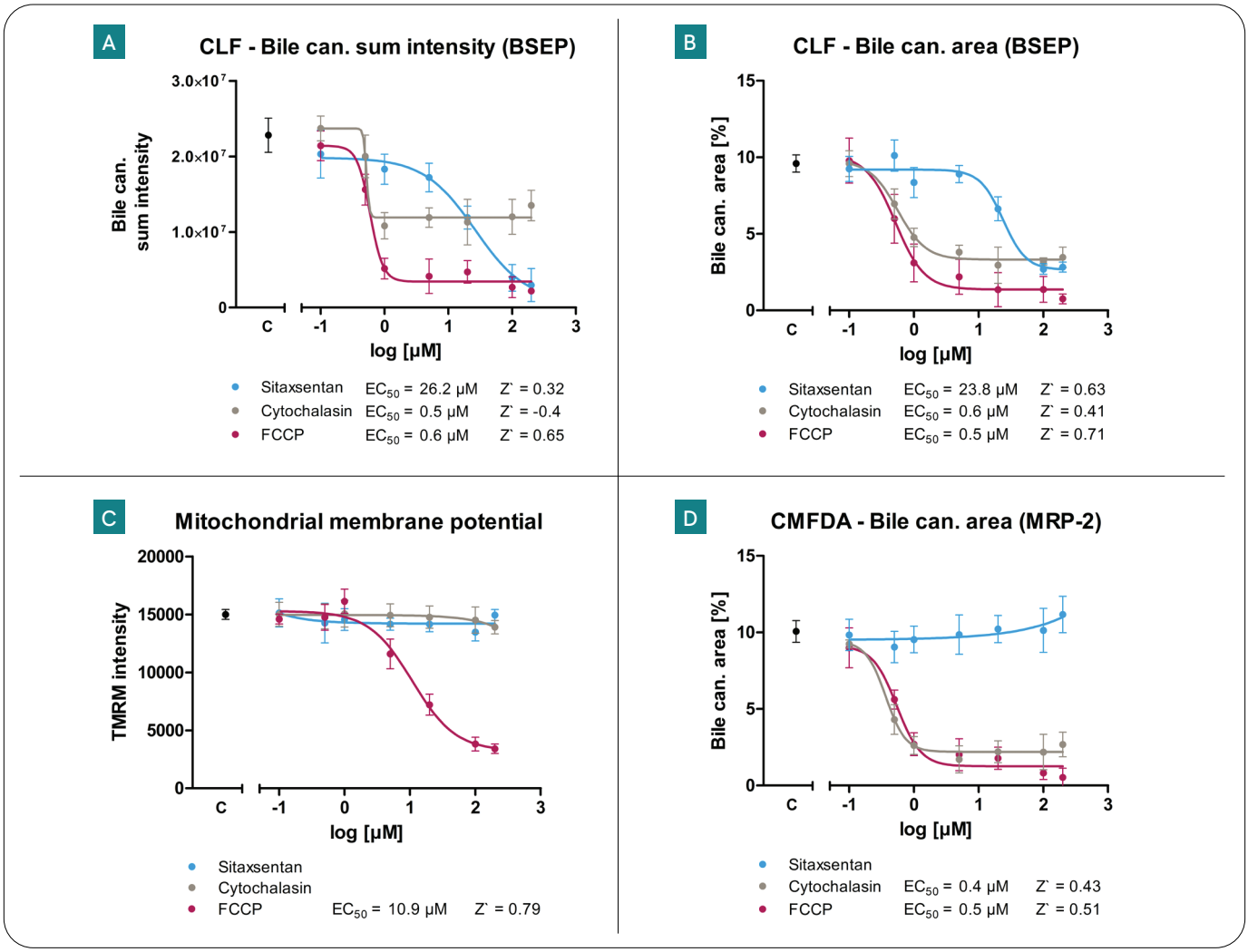


Figure 3: Quantification of Sitaxsentan, Cytochalasin D and FCCP induced effects on human liver microtissues using the bile canaliculi area, the bile canaliculi sum intensity and the mitochondrial membrane potential as readouts. When comparing the CLF sum intensity and area readouts (A vs B), it becomes evident that all tested compounds affect the BSEP efflux transporter activity in a dose-dependent manner, resulting in almost identical EC_{50} values. However, the bile canaliculi area readout showed higher Z`values, suggesting a more stable readout. Cytochalasin D and FCCP treatment impair both BSEP and MRP-2 mediated transport with comparable EC_{50} values between 0.4 and 0.6 µM (panel B and D). The BSEP inhibitor Sitaxsentan inhibited specifically the BSEP mediated efflux of CLF (EC_{50} =23.8 µM) (panel B), whereas the fluorescent CMFDA staining was unaffected (panel D). The TMRM intensity readout revealed that at the tested concentrations only FCCP was toxic to mitochondria (panel C). N = 3 wells, one microtissue per well.

Assay development

To find a good compromise between data volume and data quality, the minimal stack sampling rate needed for this specific assay was determined during assay development (Figure 4). One initial 60 µM image stack of a non-treated, CMFDA exposed microtissue was acquired using the 20x water objective with the recommended minimum plane distance of 0.8 µM. By omitting more and more planes from the analysis of this large stack, it was determined that the bile canaliculi area remained similar if plane distances of up to 8 µM were used. Increasing the plane distance beyond 8 µM led to loss of detected canaliculi.

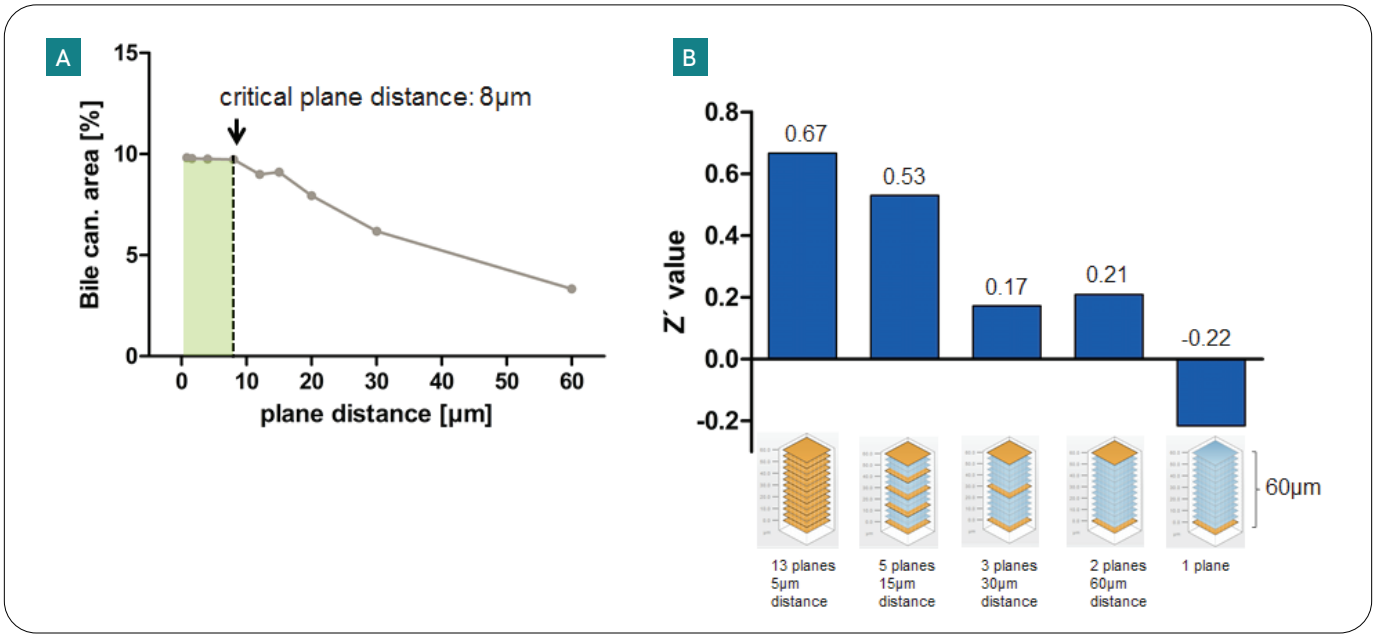


Figure 4: A stack setup of 5-13 planes with a plane distance of 5-8 µM allows a good image resolution along the z-axis and an excellent Z' value. A) A large stack with the minimum recommended plane distance (0.8 µM for the 20xW objective) was acquired and the bile canaliculi area was analyzed using a maximum projection image. By omitting more and more planes from the maximum intensity projection image, a plane distance of 8 µM was determined as critical plane distance. Increasing the plane distance beyond 8 µM led to loss of detected bile canaliculi. B) The Z' value was calculated for different stack setups and a stack with 13 planes and a plane distance of 5 µM resulted in an excellent Z' value of 0.67. Leaving out planes in subsequent evaluation steps impaired the assay quality. A stack setup of 5 planes with 15 µM plane distance also achieved a good Z' value of 0.53. However, the assay would not work robustly if fewer planes and larger plane distances than 15 µM were used.

Subsequently, the Z' value was determined to understand the importance of plane distance and number of image planes for the statistical quality of the assay. A stack setup of 13 planes with 5 µM plane distance (0-60 µM) led to an excellent Z' value of 0.67. Leaving out planes impaired the assay quality, but 15 µM plane distances still resulted in a good Z' value of 0.53. Taking everything together, a stack setup of 5-13 planes with a plane distance between 5-8 µM is recommended for this 3-dimensional assay, as this keeps acquisition times and data volume acceptable, allows for good image resolution along the z-axis and excellent Z' values. These results also show that the computationally efficient analysis of maximum intensity projections can provide excellent results and that the volumetric 3D analysis is not always required.

Conclusions

In this study, we have shown how a high-content assay to study hepatobiliary transporter activity can be established in a complex 3D organotypic *in vitro* liver model system. 3D organotypic cultures are more physiological relevant and show highly improved rates of biliary efflux, suggesting that they are preferred model systems for studying drug-induced cholestasis [Mueller et al., 2011]. To visualize fine structures such as bile canaliculi in 3D, excellent confocal imaging technology becomes a prerequisite.

The Opera Phenix High-Content Screening System has been designed for studying complex cellular models such as 3D microtissues. Its microlens enhanced Nipkow spinning disk allows extremely sensitive confocal imaging and the increased pinhole-to-pinhole distance of the spinning disk results in reduced spatial crosstalk from out of focus planes. This ultimately results in clearer confocal images from 3D microtissues. Furthermore, the Synchrony Optics™ enables simultaneous acquisition of up to 4 channels with minimal spectral crosstalk, providing high quality images of 3D microtissues at higher throughput than ever. The building blocks *Filter Image* and *Calculate Image* that were introduced with Harmony 4.0 software proved essential to precisely identify the fluorescent bile canaliculi network. The computationally efficient analysis of the maximum intensity projection image resulted in excellent Z′ values, showing that a volumetric 3D analysis is not always required. The ability to automate liquid handling processes as described previously [Letzsch et al., 2014] contributes to making this assay truly high-throughput compatible. In summary, image acquisition of 3D human liver microtissues using the Opera Phenix system and the easy-to-use building block-based analysis of the Harmony software proved ideal tools for establishing a screening system for drug-induced cholestasis.

References

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