Introduction
A functional impairment of hepatobiliary transporters such as bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2) are strongly associated with an increased risk of liver injury. Currently mostly artificial models, such as BSEP expressing membrane vesicles, are used for studying efflux transporter function. However, they lack the integration of the functional complexity of the natural 3-dimensional (3D) liver environment. Here we describe the use of 3D human liver microslices for assessing hepatobiliary transporter function. Confocal imaging was used to assess BSEP-mediated efflux of cholyl-taurine-fluorescein (CLF) and MRP2-mediated efflux of 5-chloromethylfluorescein diacetate (CMFDA) into the bile canaliculi.

2D Liver Microslices establish a bile canaliculi network with polarized expression of BSEP and MRP2
3D InSight™ Human Liver Microslices were produced with InSphero’s proprietary production process and maintained in GravityTRAP™ plates until further analysis (Messner et al., 2012). The liver microslices consist of a co-culture of human primary hepatocytes and primary non-parenchymal liver cells. To test for presence of hepatobiliary transporters on 2D liver microslices derived from different hepatocyte donors, formalin fixed and paraffin embedded liver microslices were subjected to BSEP and MRP2 immunohistochemistry (Figure 1).

High content screening assay using the Phenix High Content Screening system allows visualization and quantification of bile canaliculi area
For confocal imaging, the 3D human liver microslices (donor IPHE_01), co-cultured with liver NPCs, were transferred to a 384-well Ultra Low Attachment (ULA) microtiter plate and treated with FCCP, Cytochalasin and Stavsetan incubated in a total volume of 50 µl medium for 24 h at 37°C and 5% CO2. Subsequently microslices were stained for 45 min with staining solution in medium containing 10 µM Hoechst33342, 750 nM Tetramethylrhodamine (TRM) and either 4 µM CellTrackerTM Green CMFDA or 4 µM CLF (InSphero). Using the InSphero Opera Phenix® High Content Imaging System equipped with a 20x water objective 3 channels representing nuclei, active mitochondria and bile canaliculi network of each microslice were acquired simultaneously in confocal mode. An image stack of 60 µm with a plane distance of 5 µm was acquired for each tissue assembling data of about one third of the entire microslice volume (Figure 2).

3D Assay Optimization: Plane Distance, Stack Sampling and Z’
To find a good compromise between data volume and data quality, we determined the minimal stack sampling rate required for this assay. One initial 60 µm stack 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 8 µm is the critical step size for this assay. Increasing the plane distance beyond 8 µm led to loss of detectable canaliculi (Figure 5). Furthermore, we reduced the number of planes in the analysis and calculated Z’ values for the assay (Figure 6).

Summary
Here 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. To visualize fine structures such as bile canaliculi in 3D, excellent confocal imaging technology is a prerequisite. The Opera Phenix High Content Screening System has been designed for studying such 3D models. Its microscopes 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 results in clearer confocal images. Furthermore, Synchrony Optics™ enables simultaneous acquisition of up to 4 channels with minimal spectral crosstalk, providing high quality images of 3D microslices 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.

Figure 1: Immunohistochemistry staining for BSEP and MRP2 of 7 days old Goto-Kaido 3D human Liver Microslices. Three different hepatocyte lineages are shown, which are in co-culture with primary human non-parenchymal cells (IPHE_01). Specific staining of BSEP and MRP2 was visualized by immunohistochemistry on an establishment of a bile canaliculi network within the 3D microslices, which are potentially opened to the surface of the tissue (see black arrows).

Figure 2: InSphero Primary human hepatocyte list

Figure 3: Segmentation of microslices and bile canaliculi using the Select Region. Filter Image and Calculate Image features of the Harmony® High Content Imaging Software. Panel A) Figure was created using the Select Region module. A red line was used to isolate microslices from the surrounding matrix containing in this example the left lateral liver lobe. A red triangle was used to establish the boundaries of the bile canaliculi area (in green). To distinguish between the bile canaliculi and the surrounding matrix, the Microslicer tool was used. Panel B) The individual Microslicer tool was used to create a restricted tissue region (green). For visualization and quantification of bile canaliculi area, microslices were subjected to 20x water objective 3 channels representing nuclei, active mitochondria and bile canaliculi network of each microslice were acquired simultaneously in confocal mode. An image stack of 60 µm with a plane distance of 5 µm was acquired for each tissue assembling data of about one third of the entire microslice volume (Figure 2).

Figure 4: Quantiﬁcation of Stavsetan, Cytochalasin D and FCCP induced effects on human liver microslices using the bile canaliculi area and the mitochondrial membrane potential as readouts. Stavsetan, Cytochalasin D and FCCP treatment impaired both BSEP and MRP2 mediated transport phenomena with comparable CD3 values between 0 h and 6 h (panel A and B). The BSEP inhibitor Stavsetan impaired specifically the MRP2 mediated transport phenomena (panel A). In addition, by using the bleach intensity readout revealed that at the tested concentrations only FCCP was toxic to mitochondria (panel C). N = 3 wells, one microslice per well.

Figure 5: A step size of more than 8 µm leads to a decrease in the bile canaliculi area. Image stack sampling is the critical step size for this assay.

Figure 6: Z’ and s-sampling rate: 13 planes with a distance of 5 µm were acquired at 14 µm step size. Increasing the number of planes from 13 (100% sampling) yielded to an acceptable Z’ value of 0.81.