# Establishment and validation of the 3D cultivation system “CERO” for hepatic spheroids to study viral hepatitis

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## Abstract and introduction

This study represents the first effort to differentiate HepaRG, HepG2 and Huh7 cell lines as 3D spheroids in the novel 3D automated culture system “CERO”. Currently, the ultra-low attachment plates are mostly used for spheroid generation and differentiation, although this technique still holds limitations such as labour intensity, susceptibility to contaminations, spatial and time limitations. Moreover, spheroids of different hepatic cell lines become necrotic and subsequently fall apart, starting one week after seeding. Here it can be shown, that large numbers of spheroids originating from the same batch of cells, were kept viable for a long-term cell culture period, e.g. HepaRG 80 days, Huh7 20 days and HepG2 >40 days. These spheroids were stained positive for carbonic anhydrase IX (CAIX), which is widely accepted and used as a marker for hypoxia. In addition to enhanced viral replication of hepatitis B virus (HBV) or hepatitis C virus (HCV) under hypoxic conditions, we could show that the cell intrinsic anti-HBV factor APOBEC3B is down-regulated under hypoxic conditions.

**Figure 1: Description of the CERO benchtop bioreactor.** CERO is a benchtop incubator and bioreactor hybrid with no impellers for micro-carrier and suspension cell culture. Most of the existing products require large media volumes, complex software or non-intuitive preparation of bioreactor equipment predominantly suited for large scale production. Increasingly, pluripotent stem cells and organoids as standard tools for basic research are implemented for drug development and predictive toxicity screen.

**Figure 2: Generation of spheroids derived from HepG2 cells. (left image)** Macroscopic analyses of CERO and Aggrewell-induced spheroids**. (right image)** Histological analyses by H/E of various HepG2 derived sperhoids - used for viral expression analyses. Scale bar: 100µm.

**Figure 3: Charatcerization of spheroids derived from HepaRG cells. (left image) (A-C)** Macroscopic analyses of CERO induced spheroids analysed for albumin and cytokeratin expression, proliferation (Ki67) and cell death (Cl.Casp3). Scale bars: 100µm.

**Figure 4: Characterization of spheroids derived from HepaRG cells. (A, left image)** Expression of the hypoxia-marker-CAIX in spheroids. **(A, right image)** Identification of lipids on cryo sections by sudan red. Macroscopic analyses of CERO and Aggrewell induced spheroids**. (C)** Expression analysis of the cell intrinsic anti-HBV factor APOBEC3B upon stimulation with a LTbR-agonist (BS1) (Lucifora et al., Science 2014). Scale bar: 100µm.

**Figure 5: HCV replication in Huh7 derived spheroids over time. (A)** Analyses for HCV-replication over time (24-72hrs). **(B)** Quantification of the overall infection experiment over time. Size of scale bars is indicated.

**Conlcusions**

- **CERO allows reproducible generation of spheroids using cell lines, primary cells and organoids.**
- **CERO allows long-term culture of tissue blocks (human and mouse) keeping their differentiation status and biological function.**
- **CERO is an efficient bioreactor for spheroids, organoids and for long-term culture of tissue pieces.**

*Lucifora et al., 2014, Vol 343, Science*

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