Application Note

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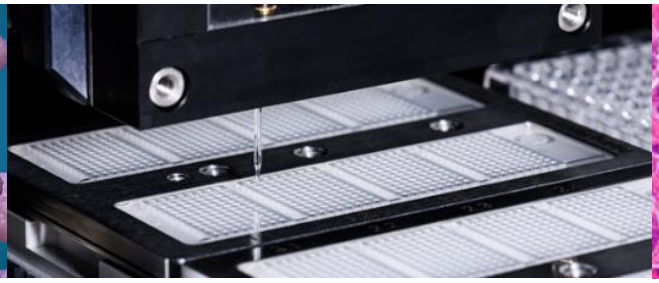
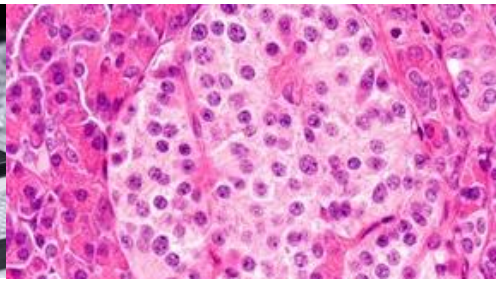

Characterization of HepaRG® cells grown in 2D or 3D culture at single cell level using the cellenCHIP 384-3'RNA-Seq Kit

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Abstract

The emergence of single-cell sequencing has opened a new field in analytical biological approaches particularly allowing to uncover cell-to-cell heterogeneity of 3D cellular models and preventing incomplete conclusions drawn from bulk cell populations. In this study we compared the expression profiles of single HepaRG® cells grown in 2D or 3D (spheroid) culture using the cellenCHIP 384-3'RNA-Seq Kit, a complete solution for single cell library preparation, in conjunction with the cellenONE® single cell isolation and dispensing platform. Comparison of RNA expression profile of cells isolated from 2D *vs.* 3D culture reveals major differences of gene expressions. Studies using the cellenCHIP 384-3'RNA-Seq Kit in combination with the cellenONE have thus a great potential for the evaluation and characterization of 3D models at single cell level.

Introduction

Since several years, a shift from 2D towards 3D culture conditions (scaffolds, spheroids, organoids, or 3D bioprinted models) can be observed in cell biology. 3D cellular models are more and more described to better mimic physiological or pathological tissues (Fang & Eglen, 2017) and thus improve drug screening and toxicologic studies.
HepaRG® cells, a hepatic cell line widely used in toxicology testing, are no exception to this rule. Indeed, several studies have demonstrated the improved phenotype and function of these cells when they are grown in 3D, especially in spheroid culture, compared to the 2D classical monolayer culture (Gunness et al., 2013; Ramaiahgari et al., 2017). To examine toxicity and preclinical drug testing in 3D cellular models, innovative solutions for creating, handling, and analyzing them are required. Single cell approaches can be particularly powerful to decipher the biological complexity of such

models. In comparison to the classical analysis of bulk cell populations, 3D cellular models permit to uncover the presence of distinct cell subsets, cell specific responses to drug treatments as well as treatment related changes in intercell communication (Heath et al., 2016).

Cellenion develops single cell solutions, from cell isolation to OMICs analysis, to facilitate and empower the transition towards tomorrow's health research. In this study we introduce the cellenCHIP 384-3'RNA-Seq Kit as a high-quality solution to perform single cell library preparation for the comparison of HepaRG cell grown in 2D or 3D culture conditions (Figure 1).

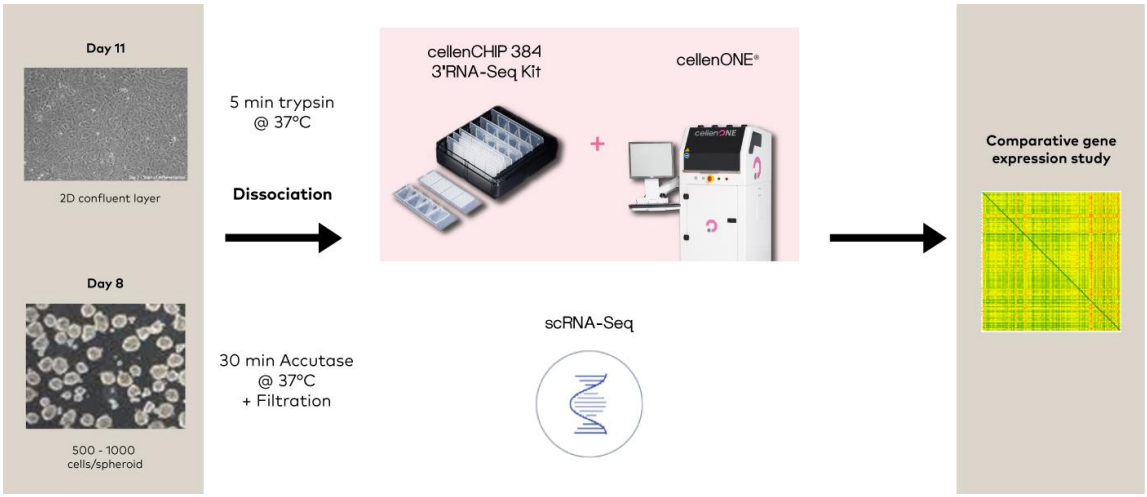


Figure 1. Schematic representation of the single cell isolation and library presentation using the cellenCHIP 384 3'RNA-Seq Kit and the cellenONE®

HepaRG® cells were cultivated in 2D or 3D conditions during 8 to 11 days prior to dissociation to obtain a single cell suspension. Then the cellenCHIP 384-3'RNA-Seq Kit was applied, and single cells isolated into the cellenCHIP 384 incorporated in the kit using the cellenONE® technology for subsequent 3'RNA sequencing and bio informatic analyses.

Materials and methods

Cell culture of HepaRG® cells

Undifferentiated HepaRG® cells provided by Biopredic International (catalog number HPR101) were cultivated both in 2D and 3D (spheroid) culture in HepaRG® basal hepatic medium (catalog number MIL700) complemented with 10% FBS (Dutscher) and PSN 1X (Gibco) for at least one week.

Spheroid production & dissociation

500 to 1000 HepaRG® cells per well were seeded in Nucleon Sphera 96U-well plates (ThermoFisher Scientific). After 8 days in culture, 20 spheroids were pooled and dissociated by Accutase (Gibco) treatment for 15-30 min at 37°C.
2D cell monolayer was detached using trypsin 0.05%, 5 min at 37°C.

cellenONE® F1.4 for single-cell isolation

The cellenONE® is an automated single cell isolation and dispensing device, that has been used for isolating cells into the cellenCHIP 4 X 96 (cellenCHIP 384 RTready, Cellenion, catalogue number CER-5015-4) included in the cellenCHIP 384-3'RNA-Seq Kit (Cellenion, catalogue number CTR-5016- 1-4, see Figure 1) using the cellenCHIP 384 Accessory Kit (Cellenion, catalogue number CAK-5015). It was set to dispense one single HepaRG® cell per well using the following isolation parameters: cell diameter 18-27µm, circularity max 1,15 and elongation max 2.4.

3'end single cell RNA-sequencing

3'scRNA-sequencing libraries were prepared using Cellenion's cellenCHIP 384-3'RNA-Seq Kit (Figure 2). The 3'RNA-seq protocol performed in the cellenCHIP 384, comprised of 384 specifically designed micro wells distributed in 4 arrays of 96, is designed to create high quality single cell 3´transcriptome libraries by efficiently capturing poly-adenylated mRNA from single cells dispensed with the cellenONE® technology.

To run the 3'RNAseq protocol, each well contains:

- (i) unique primer composed of a preserved dT sequence for mRNA targeting during reverse transcription
- (ii) an individual cell barcode (CB) to trace back sequencing reads to each corresponding cell and
- (iii) a unique molecular identifier (UMI) to quantify the absolute number of reads of each transcript.
- a template-switching oligo for amplification of cDNA trough PCR.
- a specific "Lysis and RT mix" to allow lysis of the cell and direct reverse transcription (RT) of mRNA

Each well of the included cellenCHIP 384 contains a different barcode. Libraries were paired end sequenced on high output flow cell of an Illumina NextSeq 2000 instrument. 18 bases were sequenced with the first read to obtain cellular and molecular barcodes and 50 bases were sequenced in the second read into the cDNA fragment. An additional 8 base i7 barcode read was done as several libraries was multiplexed on a same flow cell.

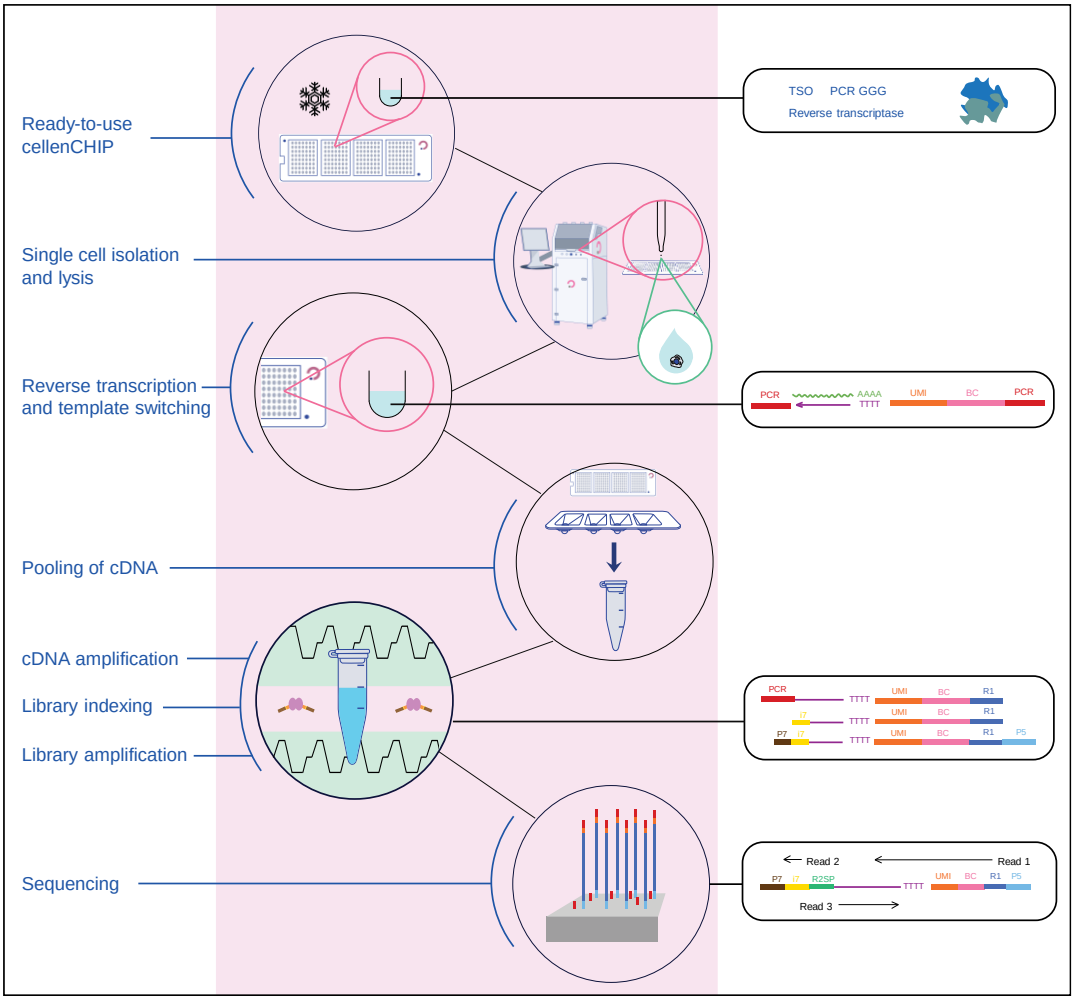


Figure 2: Schematic representation of the molecular workflow of the cellenCHIP 384 – 3'RNA-Seq Kit
The cellenCHIP 384 in the kit consists of 4 identical 96 well arrays and contains in each nanowell oligo dt primer, including an individual cell barcode (CB) and a unique molecular identifier (UMI) for cDNA generation. With the barcodes and the UMIs the protocol allows the identification of every individual cell transcript. The sample preparation and the cDNA generation take place in the cellenCHIP 384. First, the barcode oligos are rehydrated with the Lysis & RT Buffers. Single cells were then isolated and dispensed into the cellenCHIP 384 using the cellenONE®. The generated cDNA is collected in the funnels for subsequent library preparation.

Bioinformatic & statistical analysis

All raw FASTQ data was processed using zUMIs v2.9.3e (Parekh et al., 2018) in combination with STAR v2.7.3a (Dobin et al., 2013). Reads were mapped to the human reference genome (hg38) and gene annotations were obtained from gencode.v34 (GRCh38). Normalization, variable gene detection, dimension reduction and cluster identification was performed using the Seurat R package (Stuart et al., 2019).

Results and discussion

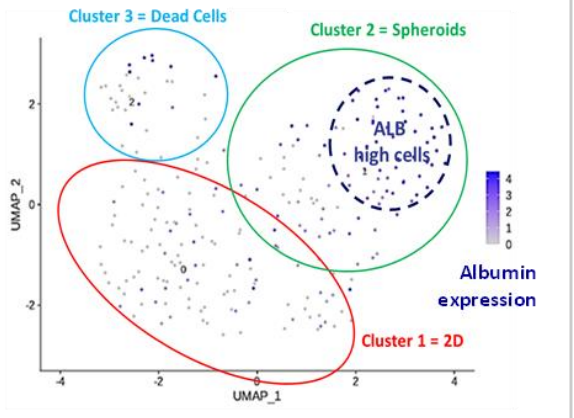
The transcriptomic profiles of the HepaRG® cells grown in 2D and 3D culture after single cell isolation with the cellenONE® and library preparation using the cellenCHIP 384-3'RNA-Seq Kit were analyzed. The following results concern average 100 cells per condition.

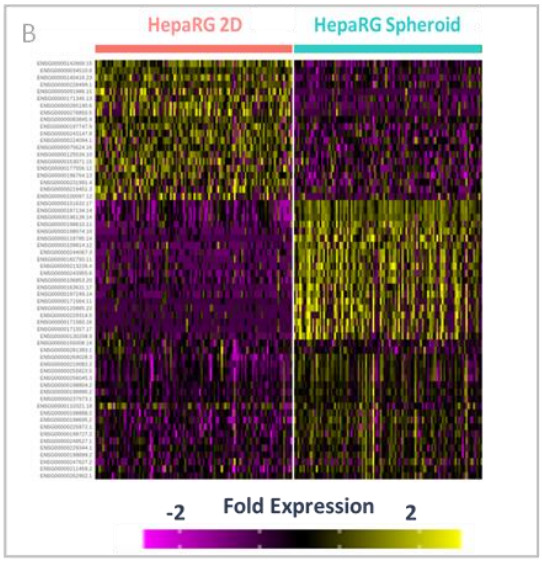
Transcriptomic profiles of cells generated with cellenCHIP 384-3'RNA-Seq Kit allow clear cluster formation of 2D *vs.* 3D cultured cells

Running unsupervised analysis reveals that cells segregate into distinct clusters according to their culture condition (Figure 3A). While cluster 1 is mainly composed of cells derived from the 2D monolayers, cluster 2 comprises spheroid-derived cells. In addition, a third minor cluster was found, consisting of a subset of spheroid-derived cells overexpressing mitochondrial genes. As the latter is a common feature of apoptotic cells, cells overexpressing mitochondrial genes from the subsequent analyses were excluded. Moreover, a "Albumin-high" cell population overexpressing Albumin gene from a factor 2 to 4 could be identified among the spheroid-derived cells (Figure 3A). In total, 50 genes were differential expressed between the 2D and 3D condition (Figure 3B). Most of these are protein coding genes involved in metabolic liver functions and are overexpressed in spheroids-derived cells, consistent with previous publications (Takahashi, Yu et al., 2015). We further focused our analysis on the main 20 differentially expressed genes (based on log2fold and adjusted p_value in several comparison strategies) (Figure 3C)

A

Condition	Cluster 1	Cluster 2	Cluster 3	Total
Sc-2D	**100**	10	7	117
Sc-Spheroid	8	**93**	25	126





C

	Alcohol & Lipid Metabolism	Biosynthesis & Detoxification	Phase II Enzymes	Extracellular Matrix	Others
Over expressed in 3D	ALDH1A1 APOC1 APOE PLIN2	AKR1B10 AKRR1C1 AKR1C2 AKR1C3 AKR1C4	GSTA1 GSTA2 GSTA3 GSTA5	UGDH FN1	FGG ORM1 PTGR1 SERPINA1 SPP1
Over expressed in 2D					CCDC80 NUTF2 SERPINE1

Figure 3. Global transcriptomic profile of HepaRG® cells grown in 2D and 3D culture conditions.

A. Unsupervised analysis reveals 3 clusters: Cluster 1 = 92.6% 2D culture-derived cells, Cluster 2 = 90.3%, Spheroid-derived cells and Cluster 3 = 13.2% of total cells consisting mainly of spheroid-derived cells overexpressing mitochondrial genes (typical of apoptotic cells). Cells with a high albumin expression segregate into the same sub-cluster of clusters 2 (ALB high cells). Sc-2D: 2D culture derived single cells. Sc-Spheroid: Spheroid-derived single cells. **B**. Heatmap of the top differentially expressed genes. **C**. Summarizing table of the top 20 differentially expressed genes between the two culture conditions.

Biomarkers of hepatic functions are more present in 3D than 2D cultured HepaRG cells.

Albumine, ALB, one of the main hepatic markers, is largely overexpressed in spheroid-derived cells (79.21% of positive cells) including 40% of high positive cells with a >2-fold increase in expression compared to 2D culture condition (only 30 % of positive cells, with no cells with >2-fold expression level) (Figure 4).

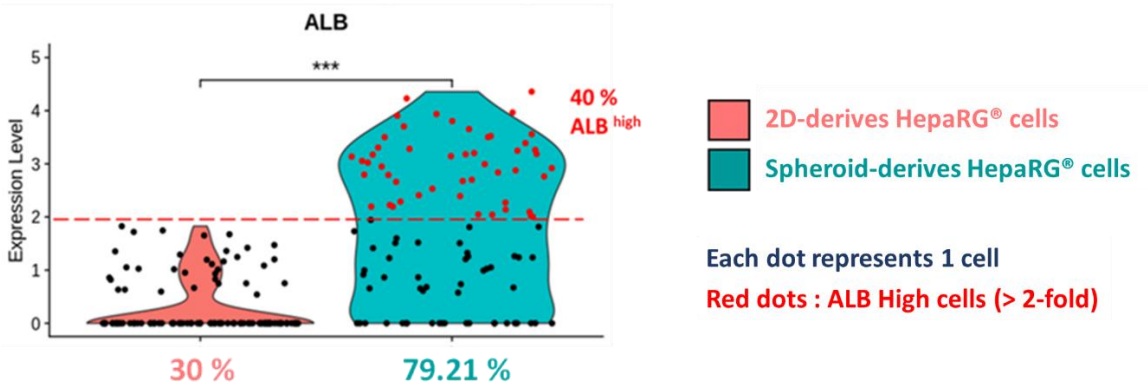


Figure 4. Albumin transcriptional profile of HepaRG® cells grown in 2D and 3D culture conditions.
Violin plots representing Albumin gene expression level for each single cell symbolized by dots. Red dots represent cells with a fold expression > 2. ***p-value < 0,001.

In addition to Albumin, most upregulated genes in 3D cultured cells are linked to well-known hepatic functions (Figure 5). We thus identified key enzymes implied in biosynthesis and detoxification metabolism such as Aldo keto reductases (AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4), Alcohol Aldehyde dehydrogenase (ALDH1A1), the Phase II enzymes Glutathione-s-transferases (GSTA1, GSTA2, GSTA3, GSTA5) and the Prostaglandin Reductase (PTGR1). Genes coding for proteins involved in lipid metabolism are upregulated in spheroid-derived cells as well. Apolipoproteins APOC1 and APOE as well as the most abundant lipid droplet protein in the liver, Perilipin 2 (PLIN2) are found in abundance in 3D cultured cells. Furthermore, diverse plasmatic proteins are transcriptionally overexpressed in HepaRG® cells cultivated in 3D: Orosomucoid 1 (ORM1), Fibrinogen gamma chain (FGG), Secreted Phosphoprotein 1 (SPP1) also known as Osteopontin and Alpha-1-antitrypsin (SERPINA1: Serpin Family A Member 1). Finally, we observe an upregulation of matrix synthesis-related genes coding for Fibronectin (FN1) and UDP-glucose 6-dehydrogenase (UGD) implied in hyaluronan & proteoglycans synthesis. All those upregulated gene clearly demonstrates that 3D cultured HepaRG are more mature than 2D cultured once's.

Single cell analysis of HepaRG cells allow unique correlation analysis between different biomarkers

Single cell analysis enabled to precisely establish significant correlations between FGG, SERPINA1, GSTA1, GSTA2, APOC1 and ORM1 gene expression and Albumin transcriptional level (Figure 5), with Pearson coefficients consistent with co-expression scores already described on functional protein association networks platform as STRING.

Accessing the single cell level is the only to detect this correlation/ absence of correlation, as bulk analysis would mask this information.

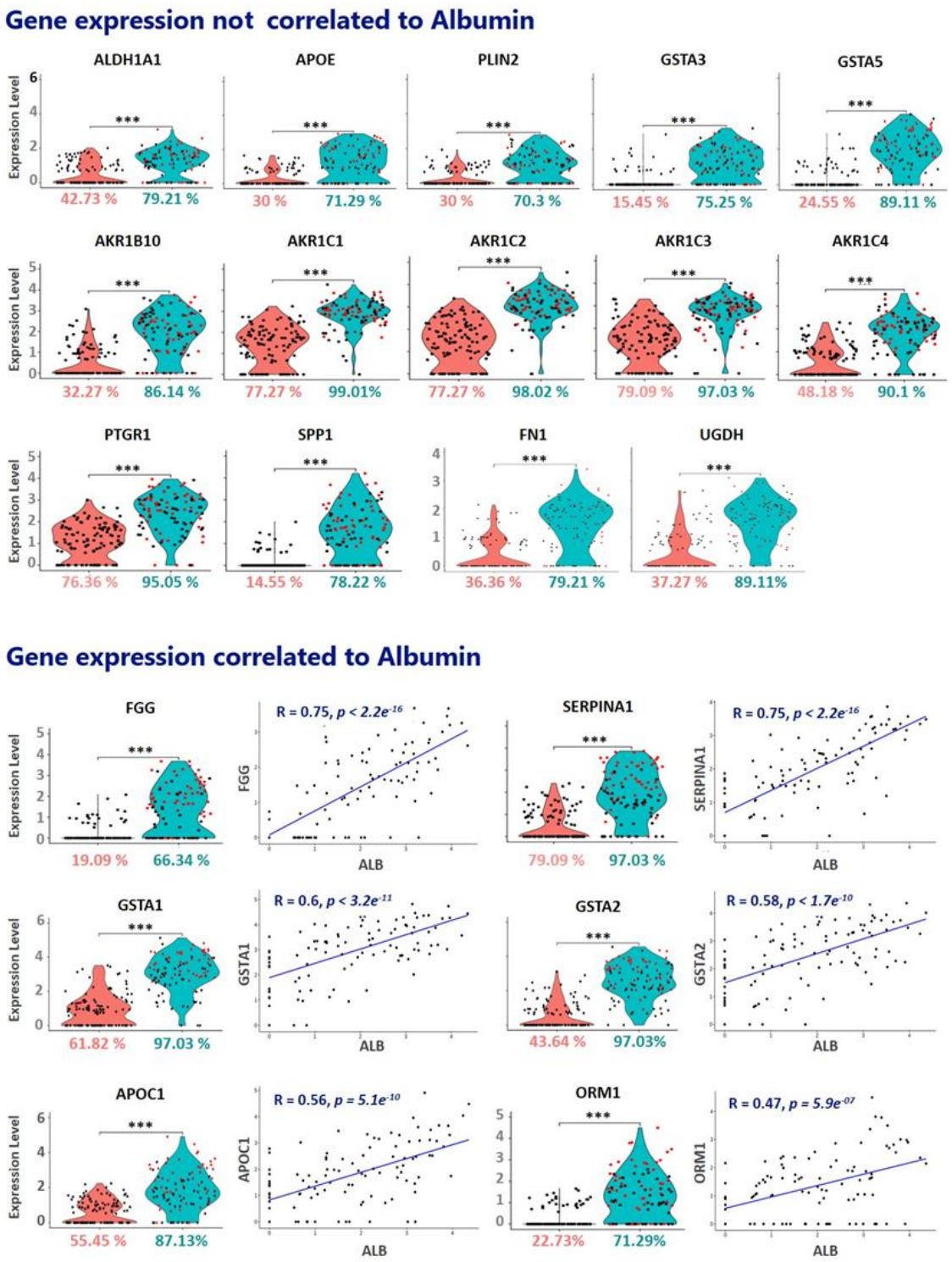


Figure 5. Profiles and correlations to Albumin expression of the Top 20 upregulated genes in spheroids derived HepaRG® cells compared to 2D culture

Gene expression levels are represented by violin plots. Each dot represents a single cell. Red dots represent the Alb High cell population. ***p-value < 0,001. Linear correlations with Pearson coefficient R > 0.4 with a p-value < 0,001.

Conclusion

With the here presented sample preparation workflow for 3’RNA seq using the cellenCHIP 384 – 3’RNA-Seq Kit in conjunction with the cellenONE® single cell isolation and dispensing platform, only 100 isolated single cells are sufficient to obtain statistically significant and biologically consistent data to analyze the homo- or heterogeneity of 3D cellular models such as spheroids. The derived expression data could be used to uncover sub-populations and establish precise expression correlations for each of them.

The cellenCHIP 384-3’RNA-Seq Kit is a promising tool in the field of single cell isolation and OMICs for the preparation of the 3D models to the final steps of bioinformatic analysis.

Acknowledgement

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