# Designed Topographies Enable Long-term *In Vitro* Hepatocyte Culture Model

*MATERIOMICS has identified surface topographies that support long-term culture of hepatocytes with excellent preservation of its phenotype and functionality, without the need of applying any coating. Primary hepatocytes of both human and Rhesus Macaque origin were maintained in monolayer culture for up to an unprecedented complete month. MATERIOMICS' designed topographies stimulate increased number of hepatocytes attaching and remaining in culture as well as maintain excellent hepatocyte phenotype and functionality when compared to the gold standard: the collagen sandwich (hepatocytes cultured between two layers of collagen). The collagen sandwich method allows for successful culture of primary hepatocytes for a maximum of 8 days. In sharp contrast, MATERIOMICS' topographies enable hepatocytes to remain in culture for 1 month while maintaining and allow for hepatocyte phenotypical and functional stability for up to 24 days. This effect is not elicited by a change in chemical make-up or a coating/secondary material, but by introducing our unique surface topography (designed roughness) into standard culture polystyrene. MATERIOMICS' unique and proprietary topographies bring excellent opportunities: a functional in vitro hepatocyte model allowing duration studies for application in drug screening, toxicology, diagnostics, therapeutics and personalized medicine.*

## Background

Hepatocytes make up 70-85% of the liver tissue, while the liver in turn plays a major role in drug metabolism and detoxification of the body. A functional *in vitro* liver model mimicking the *in vivo* make-up, consisting of (a person's own) hepatocytes, offers the most reliable way to study *in vitro* the effect a drug will have on the body and how the body will deal with the metabolic products. Being able to properly study these effects is of great importance to drug screening, toxicology, diagnostics, and personalized medicine. In addition, providing healthy and functional hepatocytes is essential to therapeutic applications and studying liver-specific diseases and related drug screening approaches.

Despite their significant regeneration capacity during their lifetime *in vivo*, the *in vitro* cultivation of hepatocytes is very limited. Under current protocols, complex (double-layered) collagen coating is required and study duration is limited to 8 days. During these 8 days, the hepatocytes' phenotype and functionality are difficult to assess due to the barrier function of the top collagen layer, restricting the usability of the model. Besides, effects of the drugs/toxins under review should be followed mid- to long term and should be tested in repeated-dose experiments, both severely limited if not impossible in current *in vitro* systems. Culture of hepatocytes derived of alternative sources like stem cells (SCs), induced pluripotent stem cells (iPSCs), and immortalized cell lines like Hep G2 are associated with difficulties in (differentiating them towards) functionality. Besides, the predictiveness for the actual effects of the drugs/toxins on the human body is a big issue with such models.

## Materials and Methods

Primary hepatocytes of Rhesus Macaque (*Macaca Mulatta*) origin were cultured in triplicate for 31 days on polystyrene (PS) substrates featuring selected MATERIOMICS' designed topographies (without any coating) to validate these topographies for supporting long-term hepatocyte culture *in vitro*; nonpatterned (NP) substrates were included as control. At day 31, the cells were fixed, stained for their nuclei and cell body, and imaged. After image analysis, total number of cells attached to the surface (cell number) and the area of the substrate covered by cells (cell coverage) was determined through image analysis and compared to nonpatterned controls. Next, primary human-derived hepatocytes (PHH) were cultured on the PS topography-featuring substrates, without and with collagen coating, for up to 30 days. The number of cells and cell coverage were determined, in similar manner as described

above, and compared to nonpatterned controls and to collagen sandwich benchmark (CS, only up to day 14).

PHH phenotypical read-out was determined for up to 24 days by the expression of a panel of hepatocyte-specific genes using PHHs of 2 healthy donors (duplicate). Cell mRNA was isolated using Trisol and synthesized into cDNA used for measuring the expression of mRNA-level hepatocyte-specific genes using quantitative real-time PCR (qPCR). Gene expression was normalized against housekeeping gene GAPDH levels (ΔCT method) and subsequently normalized to the same markers of CS at day 3 (highest expression levels in the gold standard) presenting fold inductions (comparative CT method). Finally, PHH functionality was assessed for up to 30 days by measuring the release of albumin at protein level and the release of urea into culture medium over time.

## Results

MATERIOMICS' uniquely designed topographies substantially enhance the *in vitro* culture of hepatocytes in terms of duration, phenotype preservation and functionality. 3 Distinct topographies have been validated using Rhesus Macaque hepatocytes. After culturing for 31 days, between 2-6 times more cells are attached to the topography-featuring substrates when compared to the nonpatterned (NP) control (Figure 1A) and cover about 2-5 times more surface area on the topography-featuring substrates (Figure 1B). **Hepatocytes on topography-featuring substrates possess cell organization and morphology much more similar to the *in vivo* (natural) organization and morphology of hepatocytes in the liver** (Figure 1C-D).

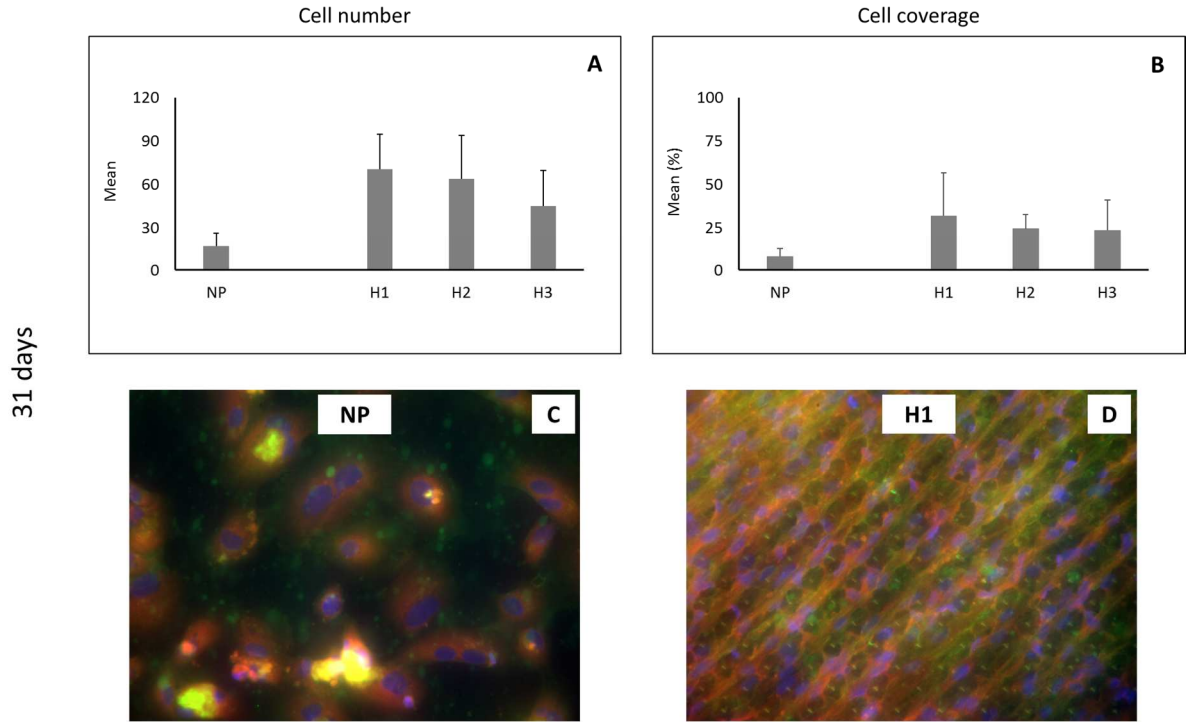


**Figure 1. MATERIOMICS' designed topographies provide for a superb long-term *in vitro* culture substrate for primary Rhesus Macaque hepatocytes.** Validation of the effectiveness of unique MATERIOMICS' designed surface topographies to enhance the attachment and *in vitro* culture of primary Rhesus Macaque derived hepatocytes. Analysis includes (A) the number of attached cells and (B) the surface area covered by cells after 31 days of culture; calculations are based on image analysis using fluorescent microscopy images like (C) nonpatterned surface and (D) H1 topography-featuring substrate. All surfaces without coating.

When culturing primary human hepatocytes (PHHs), again the topography-featuring substrates strongly outperform the NP and CS controls further proving the effectiveness of the topographies (Figure 2). With CS not being supported beyond the 14 day time point, the topography-featuring substrates still provide excellent support up to 30 days. And while both cell number and cell coverage are stimulated by the topographies, most striking effects were seen in cell organization and morphology. On the best performing topographies the cells showed clear resemblance to the natural (in vivo) liver organization and cell shape, which is not seen at other topographies and the nonpatterned controls. In addition, topography-featuring substrates perform equally well in the absence of a collagen coating.

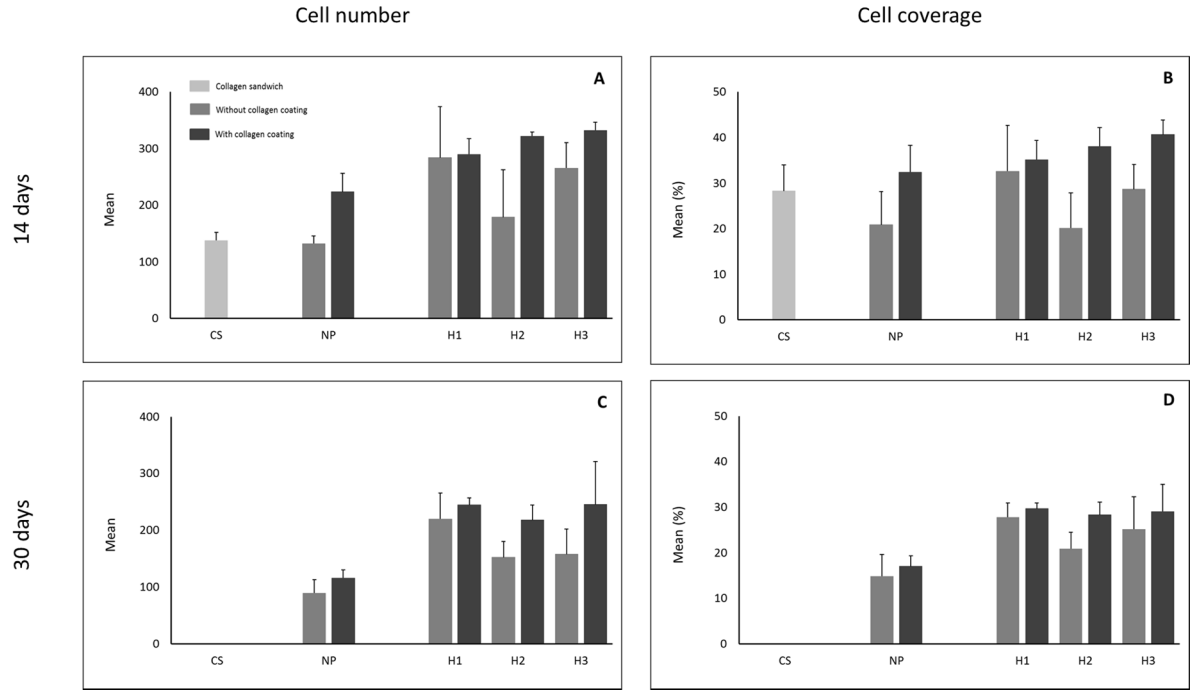


**Figure 2. MATERIOMICS' designed topographies provide for a superb long-term *in vitro* culture substrate for primary Human hepatocytes.** Validation of the effectiveness of unique MATERIOMICS' designed surface topographies to enhance the attachment and *in vitro* culture of PHHs. Analysis includes (A, C) the number of attached cells and (B, D) the surface area covered by cells after (A, B) 14 and (C, D) 30 days of culture; calculations are based on image analysis. All surfaces without and with collagen coating, CS as benchmark (30 days not supported).

MATERIOMICS' topographies not only provide for long-term culture of hepatocytes, they also maintain and even stimulate hepatocyte phenotypical preservation in time. To determine hepatocyte phenotype stability in duration cultures, a panel of genes selected for their roles in metabolic (Albumin), detoxification (Cyp1A2, Cyp2C9, Cyp3A4) and other liver-specific functions (HNAF4a, E-Cadherin) is analyzed using PHH of two healthy donors. Figure 3 presents a selection of the data for one of the donors on the most promising topography (H1). The levels are normalized against CS expression levels at day 3, under current protocols the highest expression levels conceivable and is considered to be the time the cells need to recover from the seeding process.

As expected, at the later time points expression levels for CS are at best maintained at the 3-day level or, more often, are (strongly) reduced (max. 14 days). In sharp contrast, expression levels for each of the genes on the topographies, with and without collagen coating, strongly outperforms the NP controls and even CS at 3 days (set to 1 fold level). Still at day 24, all genes are expressed at similar or even (strongly) enhanced levels compared to the CS at both day 3 (best condition) and day 8 (gold standard).

For example Albumin - an indicator of hepatocyte metabolic functionality (Figure 3A) - is expressed up to 4 times and over on topography-featuring substrates, with and without collagen coating, compared to the CS at all time points. This effect clearly underlines the efficiency of MATERIOMICS' designed topographies to preserve and even regain hepatocytes phenotypical characteristics involved in liver-specific metabolite functioning during long-term *in vitro* culture.

Another example is Cyp3A4 - an important indicator for detoxification (Figure 4F) - which is also expressed substantially higher (up to 4 times and over) on the topography-featuring substrates, with and without collagen, compared to CS at all time points. This upregulation indicates not only maintaining the phenotypical characteristics involved in liver-specific detoxification functioning in time, but even improves its levels compared to the current gold standard protocol at its best time point.

Similar effects are seen for the other studied genes all pointing in the same direction: MATERIOMICS' topographies provide for an unprecedented, true phenotypically stable hepatocyte *in vitro* model.

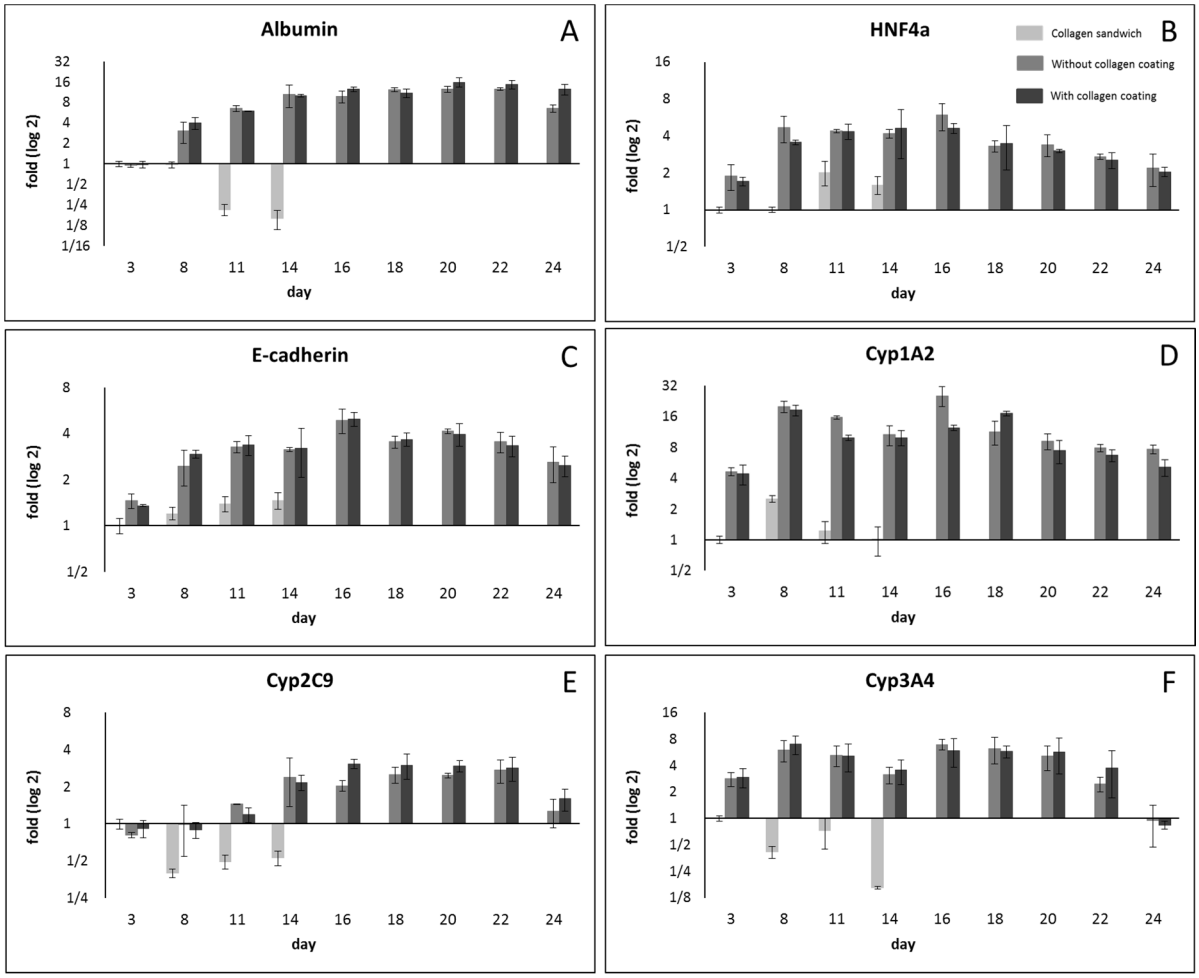


Figure 3. MATERIOMICS' designed topographies preserve and even stimulate PHH phenotype in long-term *in vitro* culture. Validation of the effectiveness of unique MATERIOMICS' designed surface topography H1 to enhance the PHH phenotypical stability in long-term *in vitro* culture. Analysis includes mRNA-level expression of hepatocyte-specific genes up to 24 days, among which (A) Albumin, (B) HNF4a, (C) E-cadherin, (D) Cyp1A2, (E) Cyp2C9 and (F) Cyp3A4 (all presented for one donor, duplicate donor showed similar results). All substrates with (dark grey bars) and without (middle grey bars) collagen coating benchmarked against CS (light grey bars), 1 fold induction equals CS-day 3 expression levels. Fold changes are presented on a logarithmic scale for sake of clarity (e.g. 1/8 equals 8 time reduction).

Materiomics' topographies preserve hepatocyte functional behavior of albumin secretion and urea synthesis for up to 30 days (Figure 4). The release levels in the CS control show a variable U-shaped profile. The drop in the first 8 days is most likely caused by the top collagen layer, acting as a physical barrier and restricting the release of the metabolites into the medium. After 8 days, the top collagen layer falls apart releasing any built up metabolites into the medium explaining the higher levels at the later time points (max. 14 days). This issue underlines the limited usability of the CS model for proper metabolic and toxicological testing. In strong contrast, PHHs on topography-featuring substrates release both albumin and urea much more linearly. Especially for the topography-featuring substrates with collagen coating, the levels for the first 14 days are similar to or even higher than CS day 3. After 14 days, albumin and urea release reach steady, yet relatively high, levels (about half of the initial values) and these levels are maintained for up to 30 days. In conclusion, MATERIOMICS' topographies successfully preserve hepatic-specific functions for up to 30 days.

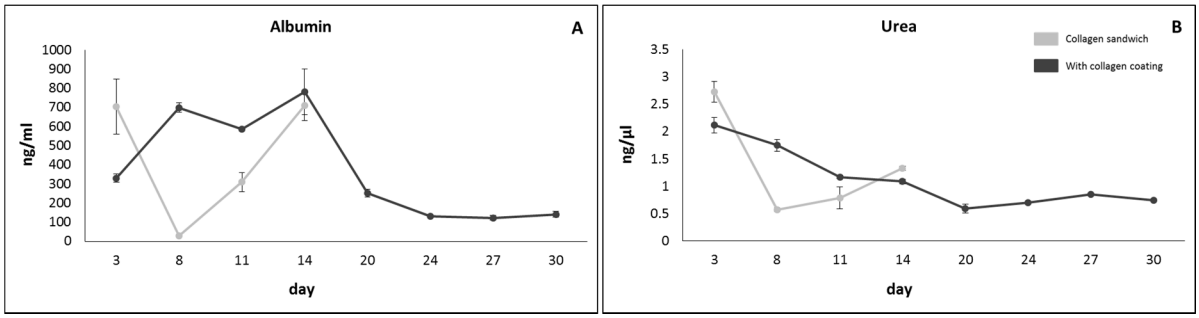


**Figure 4. MATERIOMICS' designed topographies preserve PHH functions in long-term *in vitro* culture.** Validation of the effectiveness of unique MATERIOMICS' designed surface topography H1 to enhance PHH functionality in long-term *in vitro* culture. Analysis includes the released levels of hepatocyte-specific markers into culture medium up to 30 days, including (A) Albumin and (B) Urea. All substrates with collagen coating (dark grey bars) benchmarked against CS (light grey bars, supported only up to 14 days).

## Conclusions

MATERIOMICS' selected surface topographies promote hepatocytes attachment and maintain their phenotypical and functional stability at unprecedented levels when cultured *in vitro*, which has been demonstrated for hepatocytes of Human and Rhesus Macaque origin. *In vitro* culture of hepatocytes is maintained for up to 30 days, while the current gold standard provides for semi-efficient culture up to only 8 days. Our unique and proprietary topographies do not only preserve the hepatocyte phenotype for up to at least 24 days and keep the hepatocyte metabolites at steady levels for up to 30 days, they even enhance these phenotypical and functional characteristics involved in liver-specific functions like metabolic activity, signaling, protein secretion and detoxification. MATERIOMICS' topography-featuring substrates allow for a reliable and predictive extended *in vitro* culture model for hepatocytes opening up a range of opportunities in drug screening, toxicology, diagnostics, therapeutics and personalized medicine.