

Spectroscopic label free microscopy of changes in live cell chromatin and biochemical composition in transplantable organoids

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21 **ABSTRACT**

22 Organoids formed from human induced pluripotent stem cells (hiPSC) could be a limitless source
23 of functional tissue for transplantations in many organs. Unfortunately, fine-tuning differentiation
24 protocols to form large quantities of hiPSC organoids in a controlled, scalable, and reproducible
25 manner is quite difficult and often takes a very long time. Recently, we introduced a new
26 approach of rapid organoid formation from dissociated hiPSCs and endothelial cells using
27 microfabricated cell-repellent microwell arrays. This approach, when combined with real time
28 label-free Raman spectroscopy of biochemical composition changes and confocal light scattering
29 spectroscopic microscopy of chromatin transition, allows for monitoring live differentiating
30 organoids without the need to sacrifice a sample, significantly shortening the time of protocol fine
31 tuning. We employed this approach to both culture and monitor homogeneous liver organoids that
32 possess the main functional features of the human liver, and which could be used for cell
33 transplantation liver therapy in humans.

34 Teaser: Formation of transplantable reproducible hiPSC organoids can be aided with real time
35 label-free intracellular spectroscopy

INTRODUCTION

Chronic liver failure is a potentially lethal condition, with liver transplantation being the only treatment option. However, this treatment option suffers from a shortage of donors, has high perioperative risks, and patients are confined to lifelong immunosuppression (1). Liver organoids formed with human induced pluripotent stem cells (hiPSCs) could be an inexhaustible source of liver tissue with limited immune rejection (2) that could be used for liver transplantations. However, the current hepatic differentiation strategies involving hiPSCs unfortunately suffer from several common problems (3-5).

A critical challenge of the hiPSC organoid technology is the inability to rapidly form abundant quantities of uniform, homogeneous, and sufficiently large 3D clusters, called embryoid bodies, from dissociated undifferentiated single stem cells (6). The size of the embryoid bodies is a critical factor in organoid formation, as embryoid bodies over 500 μm in size have a much greater ability to differentiate into endoderm, the germ layer from which the majority of internal organs arise. Though three main existing approaches employing forced aggregation, bioreactors, and micromolds can fulfill some subset of the requirements, none of them can satisfy all the requirements. It is possible to form large, uniform, and homogenous embryoid bodies using forced aggregation (7), but it is impossible to generate many embryoid bodies using this approach (8). The bioreactor-based approach allows for the formation of large amounts of embryoid bodies, however they are neither uniform nor homogenous (9). Finally, the existing micromold approaches allow for the formation of large, over 500 μm in size, embryoid bodies, however, due to poor oxygen permeability and nutrient diffusion, these approaches often result in the formation of a necrotic core in large embryoid bodies, making them nonviable (10).

Previously, we introduced a new approach of high-throughput organoid formation from dissociated hiPSCs with microfabricated cell-repellent microwell arrays (11). This approach allows for a scalable and reproducible derivation of multiple large hiPSC organoids co-cultured with other cell types, and is sufficiently rapid to ensure that a necrotic core is not formed. Here we demonstrate that when this approach is employed to form functional liver organoids, along with efficiently blocking the Sonic hedgehog and Notch signaling pathways during the initial stages of differentiation, it is possible to achieve significant improvements in production and enzymatic activity of critical proteins, detoxification, and coagulation factors in the organoids. These levels in the formed liver organoids are comparable to and sometimes even exceed the levels observed in the functional human liver.

While organoid formation presents various challenges, another critical challenge of the hiPSC organoid technology is the difficulty in monitoring the differentiation process. Often, several markers at the gene, protein and functional levels must be screened simultaneously, using gene expression analysis, immunofluorescence imaging, analytical biochemistry assays, western blotting, and other molecular biology techniques. Unfortunately, a significant drawback of all these conventional techniques, including gene expression analysis with quantitative real-time PCR, is the need to sacrifice the sample in order to collect the data (12, 13). Also, due to the heterogeneity of the hiPSCs, such methods cannot guarantee that the remaining cells or organoids have the same features as the sacrificed ones, even if they are from the same batch. Several approaches have been recently tested to overcome this problem and allow in vivo gene expression monitoring (14, 15). Recent work on the insertion of fluorescent tags by homologous recombination could allow in vivo observation of gene expression with fluorescence microscopy (16). Unfortunately, these approaches are limited by the labor-intensive fluorescent-tagging development for each new set of genes. Most importantly, the resulting genetically modified cells or organoids could not be used for therapeutic purposes (17, 18).

The approach of organoid formation from dissociated hiPSCs described here is well suited for label free spectroscopic based downstream phenotypic analysis, which allows for monitoring the differentiation state of organoids in real time. This real time monitoring can be potentially

useful for manipulating organoids into the desirable cell lineages. The important indicators of differentiation are subtle changes in biochemical composition of differentiating hiPSCs and some of these changes could be detected using label free Raman spectroscopy (19), for example, it has recently been demonstrated that it is possible to detect changes in glycogen, cytochrome, protein, and lipid composition in fixed samples of differentiating hiPSCs (20).

In addition to changes in biochemical composition, chromatin architecture reorganization has recently been identified as an important marker of pluripotency and stem cell differentiation (21). Compared to differentiated cells, undifferentiated cells lack highly condensed transcriptionally inactive heterochromatin and are significantly richer in lightly packed euchromatin comprised of the most active portion of the genome (22, 23). As differentiation progresses, the amount of heterochromatin drastically increases. This is demonstrated indirectly by histological analysis of the nucleus (24) or chromatin immunoprecipitation (ChIP) accompanied by microarray hybridization (ChIP-chip) and high-throughput sequencing (ChIP-seq) analyses (25, 26). It is also demonstrated directly with electron microscopy-based techniques (27). Unfortunately, these techniques are destructive, involve extensive manipulations of the sample, and hence cannot be used in living systems for real-time differentiation monitoring. Due to their nondestructive nature, optical microscopic techniques, such as super-resolution microscopic visualization of histone proteins combined with fluorescent tags (28, 29), could be employed for sensing chromatin organization in live cells. Sensing chromatin using its intrinsic properties without exogenous labels could be even more challenging. Backman and colleagues recently demonstrated label-free nano-sensing of chromatin packing and dynamics with partial wave spectroscopic (PWS) microscopy of live cells (30, 31). This suggests that confocal light absorption and scattering spectroscopic (CLASS) microscopy (32), another native contrast functional imaging technique, could serve as a useful tool for label-free monitoring of differentiation in live hiPSC organoids. CLASS microscopy extends the principles of light scattering spectroscopy (LSS) (33-35) to subcellular imaging and can also sense the state of the chromatin organization.

In this paper, we show that a high-throughput organoid formation technique, when combined with efficient liver specific signal pathway blocking, can produce liver organoids with properties similar to functional liver tissue. Furthermore, a combination of chromatin sensitive CLASS microscopy and biochemical composition sensitive Raman spectroscopy can be used to monitor the differentiation progress towards liver organoids in an efficient and non-disruptive manner.

RESULTS

Rapid formation and evaluation of liver organoids

Human embryoid bodies (hEBs) were created with our previously developed cell-repellent agarose microwell array-based method, which overcomes the challenge of rapidly forming homogeneous organoids (see Fig. 1A and Materials and Methods). Agarose, which has non-cell-adhesive properties, allows spontaneous and rapid aggregation of the initially dissociated hiPSCs into precisely one hEB in each microwell, in approximately 18 to 24 hours, considerably faster than the majority of other methods, with neither Rho-associated protein kinase (ROCK) inhibitor nor spinning employed throughout the process. The size and uniformity of hEBs is dependent on the number of hiPSCs placed in a single 700 μm diameter round-bottom microwell (Fig. 1B). While placing less than 15,000 hiPSCs per microwell results in unstable aggregation of mostly inhomogeneous hEBs, depositing 15,000 to 35,000 hiPSCs results in a significant improvement in aggregation stability and uniformity of hEBs. The optimal conditions were reached at 35,000 cells per microwell with hEB sizes being in the 450 to 500 μm range and the aggregation becoming the

most stable. Further increase in the number of hiPSCs in a single microwell causes the formed hEBs to once again become small and unstable.

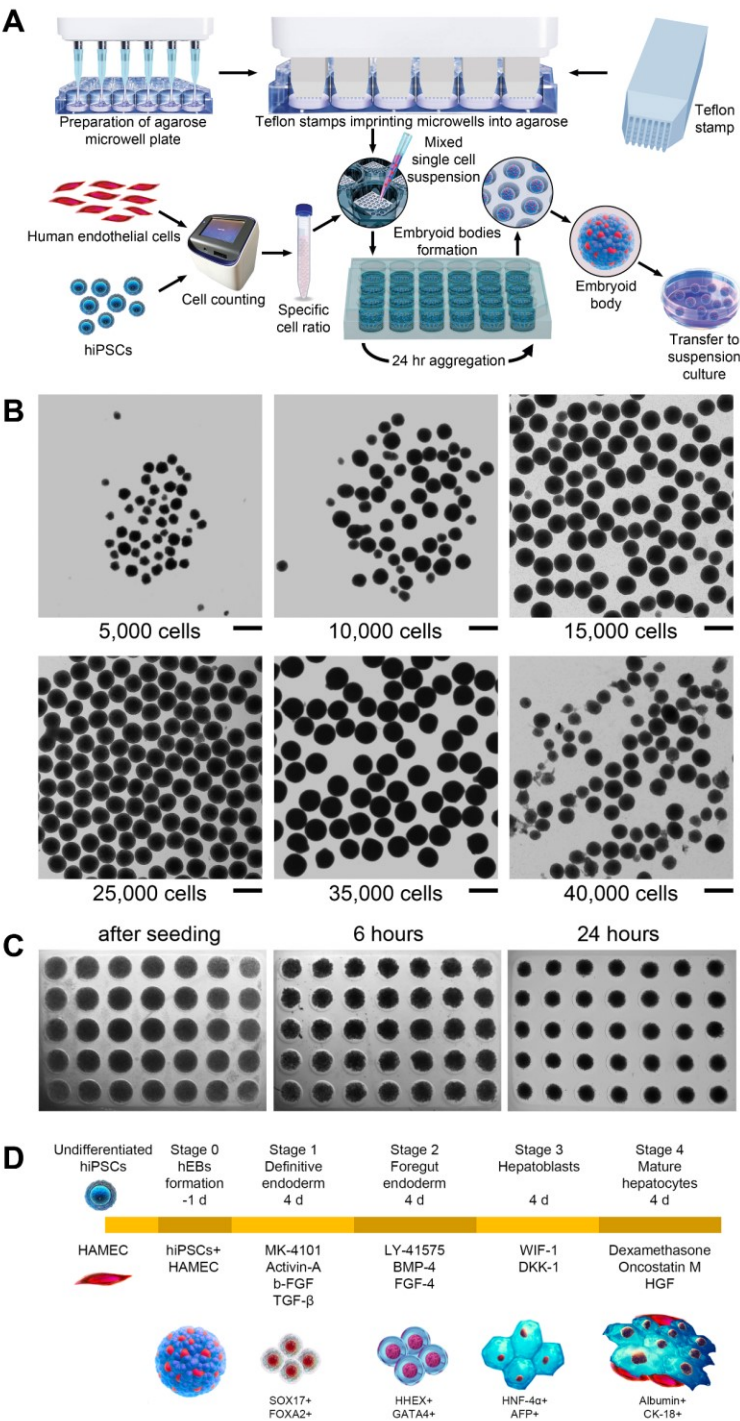


Fig. 1. Formation of liver organoids using microwell arrays in cell-repellent agarose. (A) Workflow for the formation of large amounts of hEBs with human adipose microvascular endothelial cells mixed with hiPSCs in a 1:3 ratio. (B) The hEBs gross morphology when formed with different hiPSC seeding densities within the agarose microwell. Scale bar, 500 μm. (C) The hiPSC suspension aggregation immediately after seeding within the microwells, and after 6 and 24-hour formation. (D) Schematic representation of the differentiation protocol for forming functional liver organoids.

143 Endothelial cells, which are among the first cells present in natural conditions at the initial
144 stages of embryogenesis (36) and eventually constitute approximately one fifth of the total adult
145 liver cell mass (37), were incorporated into the organoids. Endothelial cells perform an important
146 role in organogenesis by forming vascular networks that secrete angiocrine factors, which are
147 organ-specific growth factors (38, 39). Endothelial cells also maintain homeostasis and
148 metabolism, along with providing passive channels for blood and nutrient distribution to several
149 organs. During the formation of the liver organoids, we used human adipose microvascular
150 endothelial cells (HAMEC) mixed with hiPSCs in a 1:3 ratio within the embryoid bodies. Both
151 hiPSCs and endothelial cells self-organize within the organoids over time by forming endothelial-
152 cell channels at the end of the differentiation process (40).

153 Following the formation of hEBs in 18 to 24 hours (Fig. 1C), we employed the protocol
154 that we developed for forming functional hepatic organoids consisting of mature hepatocytes and
155 endothelial cells (see Fig. 1D and Materials and Methods). The four stages of the protocol are
156 definitive endoderm, foregut endoderm, hepatoblasts, and mature hepatocytes, with each stage
157 lasting for four days. To mimic processes taking place during in vivo embryogenesis, we
158 employed pathway inhibitors specific to each of the four stages of differentiation. For example,
159 the MK-4101 inhibitor of the sonic hedgehog (SHH) pathway along with Activin A, bFGF and
160 TGF- β 1 were used to develop definitive endoderm, Notch signaling pathway inhibitor LY-41575
161 was used in combination with BMP4 and FGF4 to form the foregut endoderm, while WIF-1 and
162 DKK-1 inhibitors of Wnt/ β -catenin were used for inducing differentiation of hepatoblasts.

163 To investigate whether the protocol successfully produced functional hepatic organoids,
164 we monitored stage specific markers throughout the protocol. We observed that formed liver
165 organoids demonstrated high expression levels of liver-specific genes associated with multiple
166 functions typical of human primary hepatocytes (HPHs). One of the main functions of the liver is
167 the production of albumin, which is responsible for the majority of oncotic pressure in the plasma
168 of blood vessels, preventing fluids in the bloodstream from leaking into the surrounding tissue. In
169 addition, albumin also plays an important role in antioxidation, immunomodulatory activity, and
170 endothelial regulation. As can be seen in Fig. 2A, albumin gene expression in the liver organoids
171 showed levels comparable with that of the in vitro HPHs and with published RNAseq in vivo data
172 (41) (see also table S1). Another important liver function is related to its detoxification ability.
173 We therefore tested the liver organoids for the presence of gene expression of cytochromes
174 CYP1A2, CYP3A4, CYP2B6, (Fig. 2A), CYP1B1, and CYP2C9 (fig. S1A), which are most
175 prominent in phase I of liver detoxification, and enzymes UGT1A1, UGT1A3 (fig. S1A) and
176 UGT1A4 (Fig. 2A), which are prominent in phase II of liver detoxification. Once again, their
177 expression levels were very similar to those observed in vitro in HPHs and in the in vivo data
178 (table S1). We also observed comparable levels to the HPHs in other liver genetic markers, such
179 as alpha-fetoprotein (AFP), OCT, CK18, A1AT, PAH, HNF-1 β , HNF-4 α , HNF6, glycogen
180 synthase 2 (GYS2), and fatty acid synthase 1 (FASN1) (fig. S1A).

181 Another important liver function is its role in hemostasis, which involves blood
182 coagulation. The liver synthesizes almost all coagulation factors and their inhibitors, while liver
183 damage is commonly associated with impairment of coagulation. We observed that the gene
184 expression from major coagulation factors associated with intrinsic, extrinsic, and common
185 pathways of the coagulation cascade, fibrinolysis, and platelet regulation was comparable with
186 that observed in HPHs (Fig. 2B and fig. S1B). Some of the main factors responsible for the final
187 and most important part of the coagulation cascade, namely factors V, VII, X and thrombin, were
188 expressed at the levels even higher than that in HPHs (Fig. 2B and table S2).

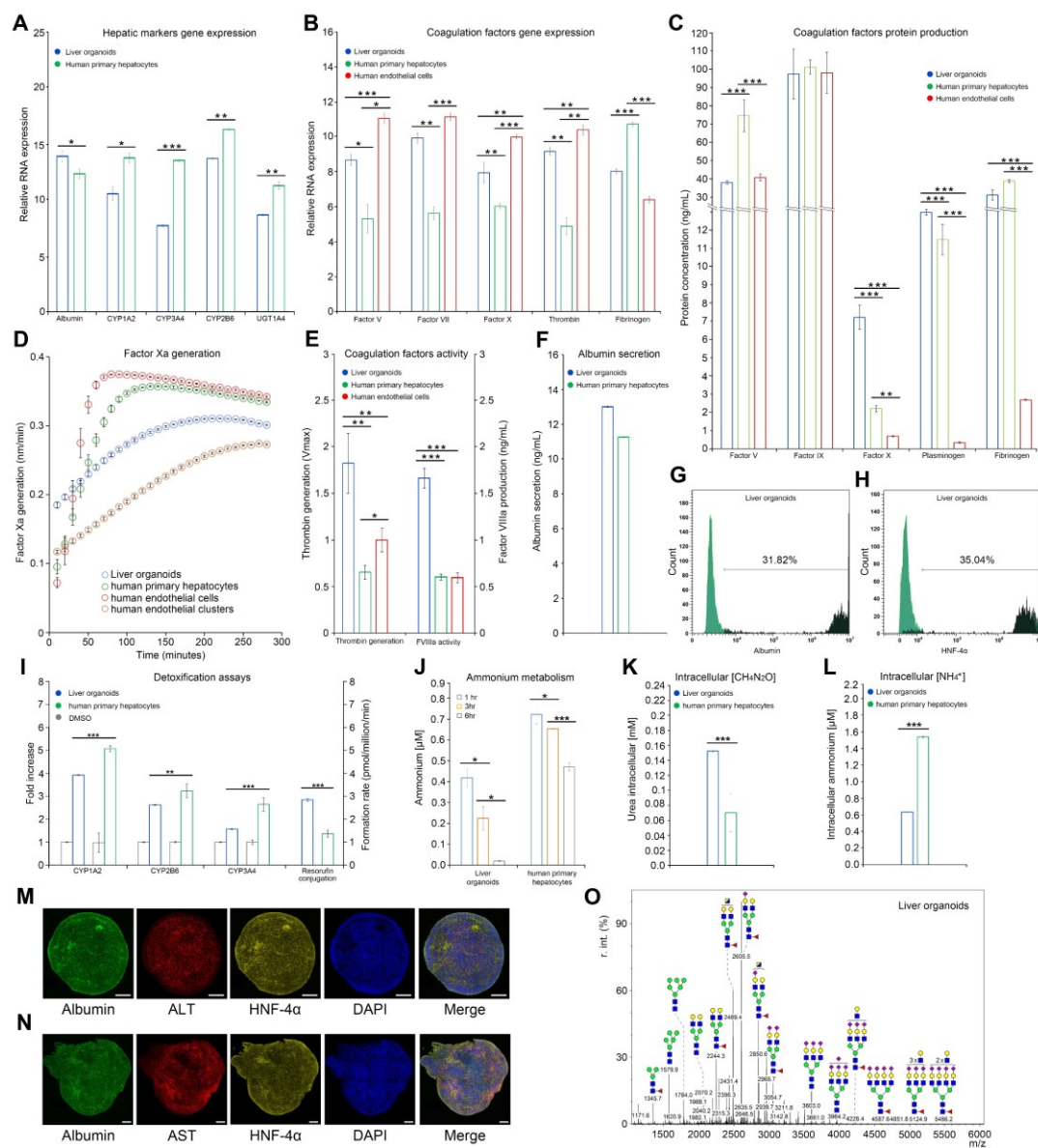


Fig. 2. Functional characteristics of liver organoids. Real-time PCR measurements of (A) hepatic markers and (B) coagulation factor markers. (C) ELISA measurements of coagulation factor markers. (D) Factor Xa generation assay. HPH (green) and HAMEC (red) in 2D culture showed similar curve slopes, while 3D culture of HAMEC (brown) and liver organoids (blue) exhibited same slope pattern. The maximum rate of reaction V_{max} of liver organoids (0.26) was lower than all positive controls (HPH: 0.40; HAMEC 2D: 0.41; HAMEC 3D: 0.33), indicating better enzymatic activity of organoids. (E) Thrombin generation assay and Factor VIIIa activity displayed better results in organoids when compared with HPH and HAMEC. (F) ELISA measurement of albumin in liver organoids showed comparable results with HPH (13.05 ng/mL vs. 11.30 ng/mL respectively). (G) FACS analysis of albumin. (H) FACS analysis of HNF-4α; (I) CYP450 liver detoxification I analysis (induction of CYP450 showed doubled activity after drug administration in CYP1A2 and 2B6) and resorufin conjugation for liver detoxification II analysis (resorufin formation rate was statistically higher than HPH). (J) Ammonium metabolism assay. (K) Intracellular urea concentration analysis. (L) Intracellular ammonium concentration analysis. (M & N) Immunofluorescence of hepatic markers. Scale bar, 50 μ m. (O) Glycan profile for mature liver organoids. Data is presented as mean $\pm$ standard deviation (n = 3) (* P < 0.05; ** P < 0.01; *** P < 0.001).

The significant improvements in the functional characteristics of the liver organoids with endothelial cells, compared to the liver organoids formed with just hiPSCs, including both hepatic markers and coagulation factors are presented in fig. S2.

In addition to quantifying gene expression, it is also important to evaluate the corresponding production of protein. Employing the enzyme-linked immunosorbent assay (ELISA), we established that a number of coagulation factors, such as factors IX and X, plasminogen, ADAMTS13, antithrombin III, protein C, and protein S were expressed at levels comparable to that of HPH and HAMEC (Fig. 2C and fig. S1C). In addition, coagulation factors normally produced by the endothelial cells (tPA and VWF) were also expressed in the liver organoids, demonstrating a successful integration of HAMEC within the organoids (fig. S1C). We also checked enzymatic activity of three main coagulation factors that are part of the common pathway, namely factor Xa and thrombin generation, and factor VIIIa activity. Once again, we found that they were comparable to the enzymatic activity of HPH and HAMEC (Fig. 2D and E).

We also employed ELISA to analyze the production of albumin and established that it was comparable with protein production in HPH (Fig. 2F), while fluorescence-activated cell sorting (FACS) analysis of albumin and hepatocyte nuclear factor 4 alpha (HNF-4 α) resulted in 31.82% (Fig. 2G) and 35% (Fig. 2H) of positive cells, respectively. Liver phase I detoxification was also tested at functional levels by inducing the major cytochromes P450 (CYP1A2, CYP2B6 and CYP3A4) with different drugs. Increased levels of cytochromes were observed after induction when compared with basal levels (DMSO) similar to those observed in HPH (Fig. 2I). Resorufin production was also tested as a liver phase II marker, demonstrating a significantly higher formation rate than that in HPH (Fig. 2I). Ammonium metabolism assay of the liver organoids displayed a reduction of ammonium in the medium that was halving every 3 hours (Fig. 2J), confirmed also by the increased amount of intracellular urea (Fig. 2K) and reduction of intracellular ammonium (Fig. 2L) as a result of ammonium clearance efficiency of the liver organoids.

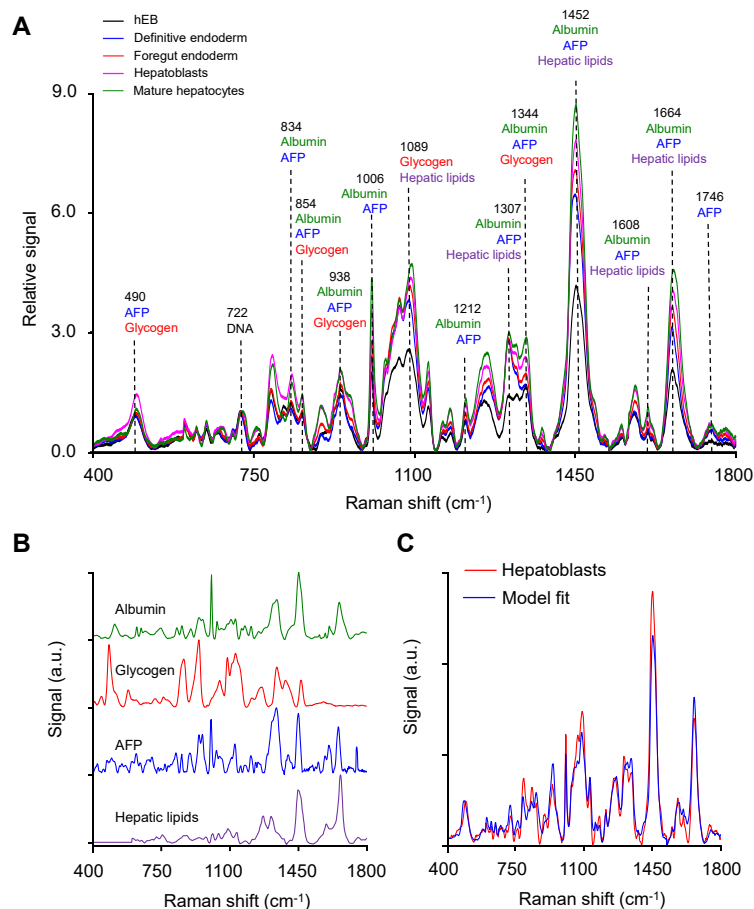
Organoid immunofluorescence imaging of albumin coupled with several liver markers (ALT, AST, HNF-4 α , HNF-1 α , HNF-3 β , A1AT, C-MET and CK18) was performed to test the ability of the organoid to simultaneously co-express multiple liver proteins (Fig. 2M and N, and fig. S1D). We also observed the formation of the CD31-positive tubular-like structures with circular cross sections within the differentiated liver organoids. As CD31 is a known marker for vascular development, this could be an early sign of vascular formation (fig. S1E). Microscopy images of the organoid morphology demonstrated a gradual increase in the size and elongation of the organoids, and a change in the cellular morphology to a polygonal shape (fig. S1F and G). An indocyanine green (ICG) test was carried out as an indicator of the ability to intake and secrete albumin from interstitial fluids (fig. S3A and B). Lipid storage and uptake, as well as glycogen storage were tested (fig. S3C to E), proving that the organoids were capable of storing lipids and glycogen. The uptake of acetylated-low density lipoproteins (Ac-LDL) was used as an indicator of complete functionality of the organoids.

Finally, we performed glycomic analysis to compare the glycan profile of liver organoids with that of HPH (fig. S1H). While the developed liver organoids presented a fairly different N-glycan profile compared to cryopreserved HPH (42), they had a rather similar profile to that observed in fresh isolated HPH (41) and real liver tissues, with a lower occurrence of high-mannoses, as opposed to the high amounts that are normally observed in cell cultures (Fig. 2O).

Raman spectroscopy of live organoids during differentiation

While conventional techniques were shown to be useful for monitoring biochemical changes, we also employed Raman spectroscopy to monitor biochemical composition since it can be done on live cells. Raman spectra were collected from live differentiating hiPSC organoids over a period of 16 days, with this 16-day period corresponding to the four stages of the protocol. While most

257 Raman systems analyze by-products downstream, our system (see Materials and Methods) focuses
 258 a near-infrared excitation laser beam directly on the organoids. The organoids were initially taken
 259 from the incubator and placed into a quartz dish filled with the basal differentiation medium,
 260 without the addition of the growth factor. The measurements were performed every other day on
 261 multiple individual unstained organoids, with the duration of the measurements short enough to
 262 keep the organoids alive. Figure 3A shows the average spectra collected in hEBs, and in organoids
 263 during the four stages. Within a single organoid, the Raman spectra had approximately 3.5% point-
 264 to-point variation, while the organoid-to-organoid variation of the Raman spectra was 5.8%. Due
 265 to cell cycle arrest during differentiation (43), the DNA content remains stable (20), and therefore
 266 it makes sense to normalize the spectra by the DNA-related peak at 722 cm^{-1} (44). Though some of
 267 the prominent peaks in the Raman spectra of liver organoids can be mostly associated with the
 268 specific biochemicals important for hepatic function, such as albumin (1006 cm^{-1}), AFP (1746 cm^{-1}),
 269 glycogen (490 cm^{-1}), or hepatic lipids (1452 cm^{-1}), each of these biochemicals has multiple
 270 peaks (see Fig. 3A) overlapping with each other. In addition, biochemicals present in hEBs at the
 271 start of the differentiation process also have peaks overlapping with the aforementioned
 272 biomolecules. Therefore, to quantify the changes in individual biochemical composition of hiPSC
 273 organoids during the course of differentiation, we employed the spectral unmixing algorithm (45,
 274 46), with some improvements.



275
 276 **Fig. 3. Raman spectra of differentiating liver organoids.** (A) Experimentally measured
 277 average Raman spectra of hEBs, and liver organoids during the four stages of
 278 differentiation with prominent peaks specific to main Raman contributing biomolecules
 279 marked. (B) Four base Raman spectra: albumin, AFP, glycogen, and hepatic lipids. (C)
 280 Experimentally measured Raman spectrum of the liver organoid in the hepatoblasts stage
 281 and model fit.

The experimentally observed Raman spectrum of organoids during stage k of differentiation $S^{(k)}(\lambda)$, where k changes from 1 to 4 for the stages of definitive endoderm, foregut endoderm, hepatoblasts, and mature hepatocytes, respectively, and λ are Raman frequency shifts, can be written as

$$S^{(k)}(\lambda) = S_{hEB}(\lambda) + \sum_{i=1}^N c_i^{(k)} S_i(\lambda) + \varepsilon(\lambda) \quad (1)$$

where N is the number of known main biochemicals contributing to the organoid spectrum, $c_i^{(k)}$ is the concentration of biochemical i during stage k , $S_i(\lambda)$ is the base spectrum of biochemical i , $S_{hEB}(\lambda)$ is the spectrum of the hEB at the start of the differentiation process, and $\varepsilon(\lambda)$ is the error which includes experimental noise as well as contributions of the biochemicals either not present in the hEBs or not accounted for among the main biochemicals known to be present in the differentiating organoids. Experimental spectra can be fit to (1) using a linear least square minimization approach. However, as it is known to be an ill-posed problem (47), one needs to employ additional prior information about the spectrum. Therefore, to improve the problem conditioning we employed the cost function

$$J(c_1^{(k)}, \dots, c_N^{(k)}) = \sum_{\lambda} \left[\left(S^{(k)}(\lambda) - S_{hEB}(\lambda) - \sum_{i=1}^N c_i^{(k)} S_i(\lambda) \right)^2 + \alpha P(\lambda)^2 \right] \quad (2)$$

with the regularization term $P(\lambda)$ equal to the difference between the experimental and the modeled spectra at Raman frequency shifts where this difference is negative, which penalizes the potential solution when the modeled spectra exceed the experimental spectrum.

Before employing the described linear least square minimization approach, we first had to identify the main biochemicals contributing to the organoid spectra. In addition to the hEB Raman spectra, we identified four main Raman contributors to the differentiating liver organoid spectra. These contributors are albumin, AFP, glycogen, and hepatic lipids. The base spectra of albumin, AFP, and glycogen were measured using the Raman system. Hepatic cells feature a variety of fatty acids and triglycerides, and although the majority of lipids have similar Raman spectra, we decided to use published spectra of hepatic lipid droplets (48) as the most representative spectrum of lipids in hepatic cells. All base spectra are presented in Fig. 3B.

Following the identification of the main biochemicals and collection of the related base spectra, we fitted the experimentally measured Raman spectra of liver organoids for each stage of differentiation. A typical comparison of the Raman spectrum for the stage of hepatoblasts and model fit is presented in Fig. 3C. The production of albumin, AFP, glycogen, and hepatic lipids detected in live organoids during differentiation with Raman spectroscopy is shown in Fig. 4 (A to D), and is consistent with the changes observed previously with conventional techniques. For example, relative concentration of albumin (Fig. 4A) steadily increases throughout each stage of differentiation with an accelerated rate during the hepatoblasts and mature hepatocytes stages, the two final stages of hepatic differentiation. Production of AFP (Fig. 4B), a typical marker of hepatoblasts (49), decreased somewhat during the foregut endoderm stage before significantly increasing and reaching its maximum during the stage of hepatoblasts and decreasing once again during the stage of mature hepatocytes, consistent with the published real-time PCR (50, 51) results. On the other hand, both glycogen (Fig. 4C) and hepatic lipids (Fig. 4D) steadily increase their relative concentration during all stages of differentiation, which, once again, is consistent with the published results (52).

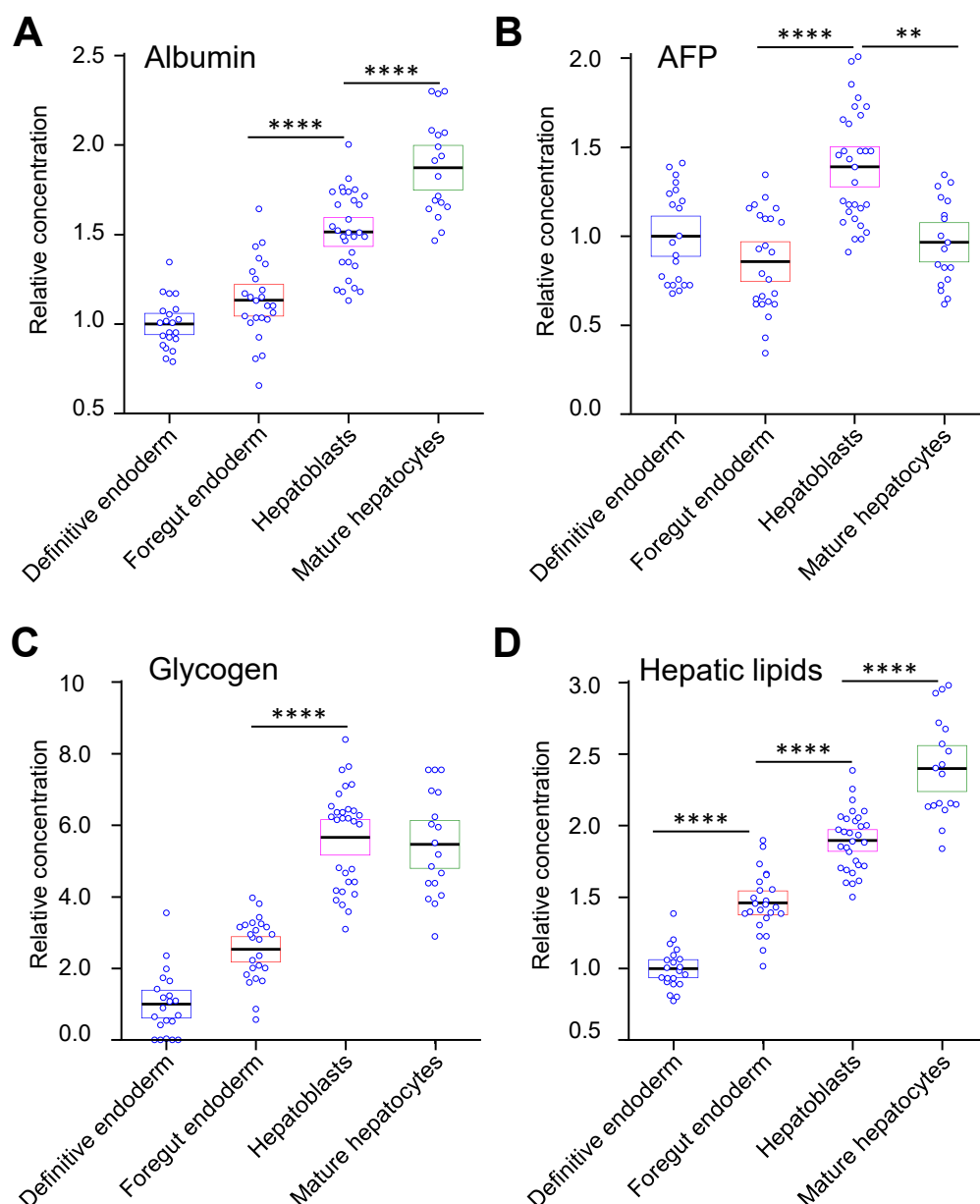
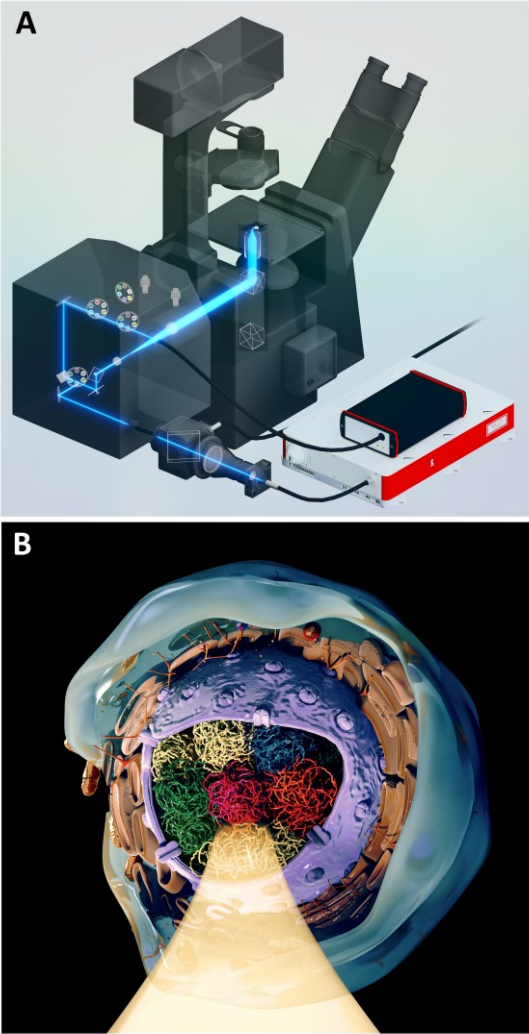


Fig. 4. Reconstructed concentrations of four biomolecules during liver organoid differentiation. Comparisons of individual data points for (A) albumin, (B) AFP, (C) glycogen, and (D) hepatic lipids for each of the stages of differentiation relative to the hEB stage. Each data point represents a single collection volume containing approximately 10,000 cells. A one-way ANOVA with post hoc Tukey's test was performed. All experiments were done with three biological replicates, from three technical replicates. The results represent mean $\pm$ standard error of the mean (** $P < 0.01$, **** $P < 0.0001$).

CLASS microscopy of chromatin in live differentiating organoids

In addition to monitoring changes in gene expression and biochemical composition with both conventional techniques and Raman spectroscopy, we also monitored changes in chromatin distribution within live differentiating cells. To pack a two-meter-long DNA into a several-micron sized nucleus, chromatin must be organized into distinct domains. Some of these domains display a more open form of chromatin, with high gene density and high gene expression, while others are more densely packed, exhibiting a closed chromatin state. Such domains are called euchromatin

338 and heterochromatin, respectively. Chromatin can consist of other types of domains referred to as
 339 topologically associating domains, or TADs (53). The exact nature of TADs has not been
 340 clarified, leading to inconsistent definitions of their boundaries in the genome (54).
 341 Initially it was postulated (55) that these domains form a highly knotted equilibrium state
 342 with the probability of contact being $P_c(s) \sim s^{-3/2}$ for two loci, which are a genomic distance s
 343 apart. Later, it was experimentally established (56) that in fact $P_c(s) \sim s^{-1.08}$. The only
 344 conformation consistent with this almost linear behavior of the contact probability is a
 345 nonequilibrium state organized in a self-similar fashion (57), such as a fractal (58). Fractal
 346 organization results in contiguous spatial regions with transcriptionally active and inactive
 347 chromatin, facilitating easy unfolding and refolding during gene activation or repression. Both
 348 properties are observed in eukaryotic chromatin, providing further evidence that chromatin indeed
 349 has fractal organization.



350
 351 **Fig. 5. CLASS microscopy measurements of chromatin packing in live cells. (A)**
 352 **SolidWorks rendering of the CLASS microscopy system. Shown in the schematic are the**
 353 **light path in the customized confocal scanning unit and optical elements, which includes a**
 354 **supercontinuum broadband laser source, custom combiner of supercontinuum laser and**
 355 **conventional lasers, and CMOS spectrometer. (B) Rendering of the CLASS microscopy**
 356 **measurements of chromatin packing in a cell nucleus with various TADs highlighted using**
 357 **different colors and one of the TADs illuminated.**

Self-similar properties of the chromatin fractal could be reflected in the chromatin light scattering spectra. Light scattering spectra are not limited by diffraction and can characterize subcellular and subnuclear structures significantly smaller than the wavelength of light (45). We chose to use confocal light absorption and scattering spectroscopic (CLASS) microscopy (see Materials and Methods and Fig. 5A), which employs light scattering spectra as a native marker (32), allowing label-free sensing of chromatin at nanometer scales in live cells. Scattering by chromatin in a cell nucleus can be described as the scattering by the collection of N densely packed small scatterers with volumes V and close to unity relative refractive indices m . The scattering intensity detected by the CLASS microscope from a focal spot can be found (59) by integrating the squared electromagnetic field amplitude over the solid angle of the objective Ω related to its numerical aperture (NA), giving the following expression

$$I = \iint_{\Omega} \frac{8\pi^2 V^2 |E_0|^2 (m-1)^2}{\lambda^4 R^2} \frac{1 + \cos^2 \theta}{2} \left| \sum_i^N e^{i \frac{2\pi}{\lambda} \mathbf{r}_i \cdot (\hat{\mathbf{k}}' - \hat{\mathbf{k}})} \right|^2 P(-\hat{\mathbf{k}}) P(\hat{\mathbf{k}}') d\hat{\mathbf{k}} d\hat{\mathbf{k}}' \quad (3)$$

where $\mathbf{r}_i$ is the position vector of individual scatterers relative to the focus, λ is the wavelength of the incident and scattered light (since we only consider elastic scattering), $\hat{\mathbf{k}}$ and $\hat{\mathbf{k}}'$ are unit vectors in the propagation direction of the incident and scattered light, respectively, θ is the scattering angle between $\hat{\mathbf{k}}$ and $\hat{\mathbf{k}}'$, R is the distance to the detector, E_0 is the amplitude of the incident field, $P(\hat{\mathbf{k}})$ is the objective pupil function (the light delivery and collection is described by the same pupil function), and the sum is performed over all scatterers forming chromatin. Due to angle averaging (60) for a large NA expression (3), the scattering intensity can be related to the structure factor and approximated as $I \propto \lambda^{-3} \int_0^\infty g(r) \sin(2\pi r/\lambda) r dr$, where $g(r)$ is a pair correlation function of chromatin. Therefore, the explicit wavelength dependence of the scattering intensity of chromatin depends on the pair correlation function form, which in turn could be characterized by a statistical index of complexity associated with fractals called the Hausdorff dimension (58).

The confocal diffraction limited interrogation volume of the CLASS microscope is comparable in size with the characteristic dimensions of the topologically associating domains (61) (Fig. 5B). Therefore, when associating CLASS spectra with the fractal organization of chromatin, it would make sense to consider both mass fractal organization of the chromatin within the domain and surface fractal organization of the domain boundary. This allows us to describe chromatin using the most general case of mass fractals bounded by surface fractals. While the explicit dependence of the pair correlation function and structure factor on mass and surface fractal dimensions d_m and d_s , respectively, are not well known (62), one can employ the semi-empirical form of the structure factor proposed by Sinha (63) and similar relationship obtained from the scaling considerations by Oh and Sorensen (64). It yields $I \sim \lambda^{-4+\chi}$ for the scattering intensity of the mass fractal bounded by a surface fractal with $\chi = 2d_m - d_s$. We should note here that several important characteristics of chromatin could be described using the concept of mass fractal (30, 31), with mass fractal dimension d_m being higher in the denser heterochromatin (65). On the boundary of the domain, the increase in the surface fractal dimension d_s would be indicative of the presence of transcriptionally active euchromatin exposing a larger surface to nuclear proteins, and thus facilitating gene transcription due to better transcription factor access to DNA. Therefore, on the boundary of the domain, the presence of heterochromatin will result in surface fractal dimension d_s being lower. Interestingly enough, both of these trends should result in a higher value of χ for heterochromatin. As χ can be directly evaluated from the CLASS spectra, we will therefore use χ to characterize fractal packing density of the chromatin within the confocal volume of the CLASS microscope.

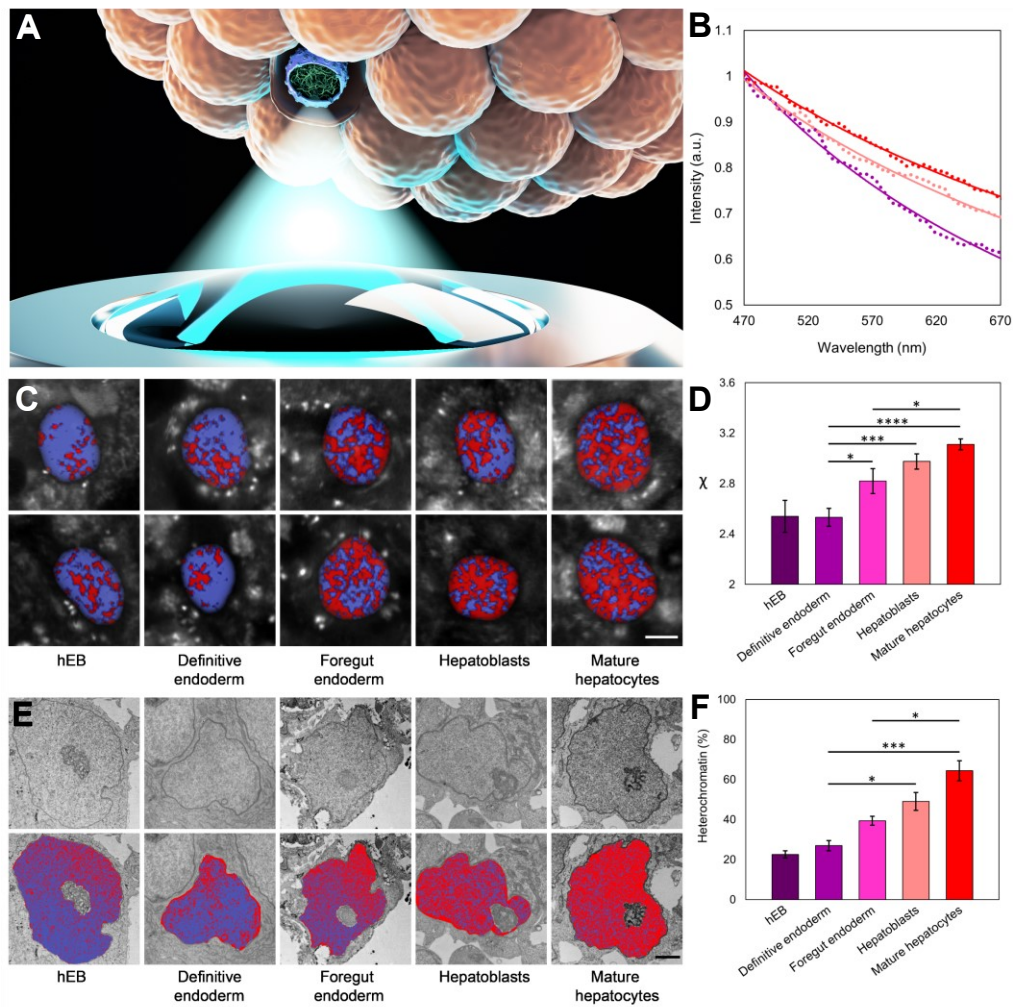


Fig. 6. Chromatin packing in live organoids at different stages of differentiation. (A) Rendering of the measurements of chromatin packing within one of the nuclei in live differentiating hiPSC organoids using the CLASS microscope. (B) Average light scattering spectra collected with CLASS microscope for hiPSC organoids. Purple dots show spectra of chromatin at definitive endoderm stage (n=19), pink – at hepatoblasts stage (n=21), and red – at mature hepatocytes stage (n=24). Model fits are presented with solid lines. (C) Spatial maps of fractal packing density of chromatin χ in live hiPSC organoids at different stages of differentiation. Two typical maps per stage overlaid over white light reflectance images of nuclei are shown. Values of χ in the range of 2.3 to 2.9 are shown as shades of purple and can be loosely associated with euchromatin, while values in the range of 2.9 to 3.5 are shown as shades of red and can be associated with heterochromatin. Scale bar, 5 μ m. (D) Parameter χ reflecting changes in the heterochromatin to euchromatin ratio and standard errors for hEB (n=9), definitive endoderm (n=19), foregut endoderm (n=21), hepatoblasts (n=21), and mature hepatocytes (n=24) stages of differentiation. (E) Representative TEM images of nuclei in hiPSC organoids at different stages of differentiation. Upper row – original images, bottom row – same images overlaid with pseudocolor maps with heterochromatin highlighted in red and euchromatin in purple. (F) Percentage of heterochromatin at hEB (n=4), definitive endoderm (n=4), foregut endoderm (n=3), hepatoblasts (n=3), and mature hepatocytes (n=3) stages of differentiation. Scale bars, 2 μ m. Data is presented as mean $\pm$ standard error of the mean. Statistical significance was evaluated with a Tukey's multiple comparison test (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001).

We monitored chromatin changes in live hiPSC-only organoids undergoing differentiation by collecting CLASS microscopy images over a period of 16 days. Similar to our Raman spectroscopy measurements, this 16-day period corresponded to the four stages of the protocol (definitive endoderm, foregut endoderm, hepatoblasts, and mature hepatocytes). Measurements were carried out by first removing organoids from the incubator and placing them in a glass bottom 35 mm dish filled with the basal differentiation medium, without the addition of the growth factor (Fig. 6A). Importantly, the duration of the measurements was short enough to keep the hiPSCs in the organoids alive, and no staining was used. Nuclei located approximately 15-25% deep inside the organoid were initially identified with white light reflectance imaging. Following nucleus identification, CLASS microscopy images and white light images were acquired simultaneously, allowing easy identification of the nucleus for data processing. With the progression of differentiation, the slope of the scattering spectra from the chromatin in live hiPSCs showed a highly significant change (Fig. 6B). This result is consistent with the steady increase of the packing parameter χ due to the expected increase in the heterochromatin fraction as differentiation progresses. The scattering spectra shown in Fig. 6B are the average spectra for the entire nucleus. Additionally, the spectra can be analyzed locally, allowing the corresponding slope and packing parameter χ to also be obtained locally. This is illustrated in Fig. 6C, where the spatial maps of packing parameter χ are presented in pseudo-colors. Figure 6D shows the average values of χ for several organoids, calculated across the entire nucleus, at each stage of differentiation. As can be seen in Fig. 6D, hEBs are characterized by a low value of χ , reflecting the typical high percentage of decondensed euchromatin in undifferentiated stem cells. This high level of euchromatin is preserved throughout the definitive endoderm stage, and can also be expected for the first germ layer developed after the initiation of the differentiation process. After that, when cells reach the foregut endoderm stage, which is a more specialized tissue from which the majority of visceral organs arise, more and more genes are segregated as inactive, and only genes specific for the specialized tissue remain in the decondensed chromatin. This significant reduction in decondensed chromatin at the foregut endoderm stage is reflected in the increase of parameter χ . This continues through the hepatoblasts and mature hepatocytes stages, with the maximum heterochromatin presence observed at the final stage of differentiation. These results are in excellent agreement with the TEM measurements we performed upon the same batches of differentiated hiPSC organoids on the same days as CLASS microscopy measurements (Fig. 6E and F), where TEM images of cell nuclei were binarized to categorize areas of dense heterochromatin and loose euchromatin (see Materials and Methods). The CLASS results are also in good agreement with published studies (66), which demonstrate that stem cells having over 75% of chromatin in the transcriptionally active euchromatin state, undergo reorganization and accumulate highly condensed and inactive heterochromatin upon differentiation, with a reduction in euchromatin regions by approximately 40%.

DISCUSSION

The emergence of hiPSCs was a significant development in stem cell research. Initially obtained from human adult cells (67, 68), these pluripotent cells provide a similar long-term unlimited self-renewal and pluripotent differentiation capacity, when compared with human embryonic stem cells (69, 70). In terms of applications, hiPSCs can be used for disease modeling and drug screening. They can also be applied to regenerative medicine, since they provide a potentially limitless source of autologous stem cells, which can be differentiated into numerous tissues. Importantly, these tissues have the potential to limit immune rejection. However, their full potential is critically limited by the inability to direct the differentiation of large quantities of hiPSCs towards desirable cell lineages in a controlled, scalable, and reproducible way. Moreover, differentiation protocols developed and optimized for specific hiPSC lines are generally not

applicable to different hiPSC lines, including patient-derived hiPSCs, due to their well-known epigenetic variability (71, 72).

Here we report a method of rapid high-throughput formation of fully functional liver organoids derived from dissociated induced pluripotent stem cells and adipose microvascular endothelial cells using microfabricated cell-repellent microwell arrays. The organoids formed using this method are well organized, feature three distinct germ layers, are capable of multi-lineage differentiation, and demonstrate high viability. They can easily be made 500 μm in size or even larger, without any signs of core necrosis and allow for straightforward incorporation of multiple cell types, making it possible to recreate the natural microenvironment of a specific organ. Several recently developed approaches (4, 73, 74) demonstrated the formation of liver organoids exhibiting a number of important functions characteristic of liver tissue. The method described here allows all of these liver specific functions to be present in a single type of organoid. We were able to form large and uniform hEBs in just 18 to 24 hours from a single cell suspension of hiPSCs and HAMEC mixture, with no core necrosis at any time throughout the differentiation process. Moreover, by mimicking the *in vivo* embryogenesis processes by efficiently blocking the Sonic hedgehog and Notch signaling pathways during the initial stages of differentiation, the production of albumin in the liver organoids improved more than 10 times, the production of the main coagulation factors increased up to 5 times, the production of detoxification factors doubled, enzymatic activity of factor VIIIa increased 5 times, and the rate of generation of factor Xa increased by approximately 50%. As a result, the production levels and rates of generation for all biomolecules critical for liver function either became comparable or sometimes even exceeded the corresponding levels and rates of the functional human liver.

The method we presented can also be easily combined with label-free optical spectroscopic methods. We showed the benefit of two complementary techniques, namely Raman spectroscopy and CLASS microscopy, for monitoring organoid differentiation without the need to sacrifice or even modify the samples. While the Raman and CLASS system measure different properties, they also function at different scales. Our Raman system focuses light on the organoid, thereby probing several cells within the organoid. On the other hand, our CLASS system, which contains high NA optics, focuses and scans light across individual nuclei.

Label free Raman spectroscopy is capable of detecting subtle changes in the biochemical intracellular composition of differentiating organoids, allowing for monitoring of their progression toward cell transplantation therapy compatible functional tissue. The most important difference between Raman spectroscopy and conventional techniques such as real-time PCR or ELISA, is its ability to obtain this information in real time, *in vivo*, and without the need to sacrifice the organoids. It is worth noting that conventional approaches employed in label free Raman spectroscopy analysis rely either on monitoring changes in individual peaks or employing various types of principal component analysis to characterize the spectra. Unfortunately, individual peaks often contain contributions from multiple biochemicals, while principal component analysis requires a training step. Since multiple biochemicals were present in the organoids, and considering we were following the organoids for 16-days, neither analysis approach was therefore satisfactory. With the Raman spectral unmixing approach that we employed, we were able to quantitatively evaluate the concentrations of the biomolecules by reconstructing the Raman spectra of organoids from the base spectra of biochemicals whose concentrations are known to be changing during differentiation. Indeed, Raman spectroscopy allowed us to monitor changes in the intracellular albumin production, which, as expected, steadily increased throughout the progression of the organoids from the initial differentiation stages towards the committed hepatocytes.

On the other hand, intracellular levels of AFP demonstrated a somewhat phasic behavior, increasing during the definitive endoderm and hepatoblasts stages (I and III) while decreasing during the foregut endoderm maturation and again at the stage of mature hepatocytes (II and IV).

This is once again an expected behavior of this known marker of definitive endoderm and hepatoblasts stages.

Raman spectroscopy of two other important intracellular liver biomolecules, namely glycogen and hepatic lipids, also demonstrated the behavior indicative of the progression toward the mature liver phenotype. Glycogen synthesis and storage are major aspects of glucose metabolisms in cells (75). In human pluripotent stem cells, an up-regulated glycogen synthesis can be observed by differentiation signaling molecules like BMP-4 (76). This suggests that glycogen synthesis could be an efficient marker of lineage-related differentiation. However, direct observation of the intracellular glycogen content in live cells with Raman spectroscopy could be an even more accurate marker of hepatic differentiation. Hepatic lipids are another important class of biochemicals involved in stem cell differentiation to metabolically-active hepatocytes, due to the fundamental role of lipids in energy metabolism. The continuing increase in the intracellular level of hepatic lipids observed with Raman spectroscopy during differentiation, with terminally differentiated hepatocytes displaying the highest lipid content compared to other differentiation stages, is in good agreement with published studies (75).

Our CLASS microscopy measurements showed that sensing of nanoscale changes in chromatin allows spatiotemporal monitoring of the dynamic chromatin distribution within live cells over the course of the differentiation process. This capability could be important not only to adjust the differentiation protocols, but also to provide a better understanding of the differentiation process, as certain factors affecting regulation of chromatin structure might either inhibit normal cell differentiation pathways, or even stimulate the uncontrolled cell proliferation toward oncogenesis (76). As an optical spectroscopic technique, CLASS microscopy is well suited to monitoring chromatin changes. While spectroscopic techniques are not diffraction limited, their interrogation volume is limited by diffraction and other optical effects. The confocal nature of CLASS microscopy significantly reduces the interrogation volume, allowing smaller length scales to be probed and spatial maps to be built up. Additionally, CLASS microscopy provides a straightforward method for continuously monitoring chromatin changes throughout the differentiation process, rather than static measurements of stem cells at the beginning and end of differentiation. The steady increase in the fractal packing parameter χ observed in our studies, which could be associated with the increase in mass fractal dimension, the decrease in surface fractal dimension, or both, is consistent with a steady decrease in the euchromatin content as differentiation progresses. This result was in excellent agreement with the TEM measurements and with the published studies (66).

CLASS microscopy has numerous advantages when compared to TEM and other modalities. TEM imaging requires chemical fixation and can only analyze chromatin distribution at a single instance, while CLASS microscopy is label free and can be used on live cells. Additionally, TEM analysis requires spatial maps to be generated in order to quantitatively understand changes at the nucleus-level. In the case of CLASS microscopy, nucleus-level changes can be obtained by simply averaging the spectra across the entire nucleus, and finding the slope of the corresponding spectrum, providing an easy quantitative value for comparison. TEM can obviously provide higher resolution spatial maps, and this difference can clearly be seen in our results. However, both sets of spatial maps showed the same progression. Many existing methods that measure chromatin at the single cell level, such as TEM, are not scalable, mostly due to time or cost constraints. Population-based methods can analyze many cells, but do not provide information on the single cell level. Importantly, CLASS microscopy is both quick and extremely cost-effective, allowing population-based averages to be built up, while also maintaining information on the single cell level. The quick acquisition time has other important implications. While 2D images are presented here, a simple z-stack could be done to build up a 3D distribution of chromatin within live cells.

We note that while chromatin organization is an important marker of stem cell differentiation, it also has significance in many other areas. In addition to regulating many cellular processes, large-scale changes can be attributed to cancer, several neurological and autoimmune conditions, as well as other complex diseases (31). Therefore, CLASS microscopy, which allows straightforward chromatin monitoring in live cells, could be a valuable tool for future studies.

In summary, we presented a method for rapid high-throughput formation of fully functional liver organoids derived from dissociated induced pluripotent stem cells and adipose microvascular endothelial cells using microfabricated cell-repellent microwell arrays. Combining this method with the real time label-free monitoring of intracellular biochemical composition changes using Raman spectroscopy and intranuclear chromatin changes with CLASS microscopy can significantly shorten the time of protocol fine tuning, eliminating the need to sacrifice the sample in order to collect the data. Homogeneous liver organoids formed with this approach possess the main functional features of human liver and could potentially be applied to cell transplantation liver therapy in humans.

MATERIALS AND METHODS

Culture conditions and sources of cells

We purchased iPS(foreskin)-3, foreskin fibroblast derived hiPSCs, (WB0002, WiCell Research Institute) and cultured them using the mTeSR1 basal medium with the mTeSR1 supplement (85850, Stem Cell Technologies) on Matrigel hESC-Qualified Matrix LDEV-free (354277, Corning) coated plates. The passage of the hiPSC colonies was performed by employing Versene (EDTA) (BE17-711E, Lonza) at room temperature for 8 minutes. We purchased HAMEC (7200, ScienCell Research Laboratories) and cultured them in the endothelial cell medium (1001, ScienCell Research Laboratories) on flasks coated with two micrograms per square centimeter of fibronectin (8248, ScienCell Research Laboratories) following the manufacturer's instructions. HPH were purchased (HMCPS, Gibco) and cultured following the manufacturer's instructions.

Human embryoid bodies formation

We developed an agarose micro-well array system which employs a custom Teflon stamp and a low melting point agarose. We dissolved agarose in PBS, heated to 100°C, to reach a concentration of 40 g/L and dispensed it into a 24-well culture plate. The Teflon stamps were pressed onto the agarose solution for about 5 minutes. When the agarose solidified in approximately 2 minutes the stamps were withdrawn and the micro-well arrays formed. We subsequently incubated the arrays with the basal differentiation medium overnight at 37°C and 5% CO₂.

hEBs were formed by dissociating 1.2×10^6 hiPSCs and resuspending them into 50 µL of basal differentiation medium. The hEBs suspension was then allowed to sediment in micro-well arrays. After 24 hours of incubation at 37°C, hEBs were transferred to a 35 mm culture dish.

hEBs containing hiPSCs with HAMECs were formed by dissociating each cell type into a single cell suspension, counted using a cell counter, and mixed with a ratio of 3:1. A total number of cells per hEB was 1.2×10^6 . The hiPSCs mixed with HAMEC were then placed into the agarose micro-well arrays to allow hEBs formation.

After the formation, both hiPSCs and hiPSCs with HAMEC were maintained in the suspension culture for the whole duration of the differentiation procedure.

Hepatic differentiation protocol

We employed a basal differentiation medium consisting of IMDM with Gibco Ham's F-12 Nutrient Mixture, 5% Gibco fetal bovine serum, 1% Gibco insulin transferrin selenium-A supplement, 55 µM monothioglycerol (Sigma-Aldrich), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich). The differentiation protocol consisted of four steps, through which

we drove our hEBs from an undifferentiated stage to mature hepatocyte-like cells. To develop the definitive endoderm in four days, the Sonic hedgehog pathway (SHH) was blocked with a MK-4101 potent inhibitor at 0.5 μ M, used together with 100 ng/mL Activin A, 10 ng/mL basic FGF and 10 ng/mL TGF- β 1 (all from PeproTech). The definitive endoderm was confirmed with immunofluorescence by the presence of the markers FOXA2 and SOX17 (fig. S4A). In the second stage of differentiation, which led to the formation of the foregut endoderm, we used the Notch inhibitor LY-41575, as inhibition of the Notch pathway is an important step for the formation of anterior foregut endoderm, from which all the visceral organs arise. We employed LY-41575 at 0.5 μ M in combination with 10 ng/mL BMP-4 (Invitrogen) and 10 ng/mL FGF-4 (PeproTech). The successful differentiation of the foregut endoderm was confirmed with immunofluorescence by the presence of the markers HHEX and GATA4 (fig. S4B). The third step employed two Wnt/ β -catenin inhibitors: 1 μ g/mL WIF-1 (R&D System) and 0.1 μ g/mL DKK-1 (PeproTech), which are responsible for the inhibition of both the canonical and non-canonical Wnt/ β -catenin pathways. This step is important for the formation of hepatoblast cells, since if the Wnt/ β -catenin pathway is active, it will lead to the formation of cholangioblast cells, the precursors of bile duct cells. By using these Wnt/ β -catenin inhibitors, we have been able to significantly reduce the formation of unwanted non-hepatic cells. The formation of hepatoblasts was confirmed by the presence of hepatocyte progenitor cell markers, AFP and HNF-4 α (fig. S4C). The last stage of the protocol is the final maturation step of the hepatoblast into mature hepatocyte-like cells. To achieve this, 30 ng/mL of Oncostatin M and 50 ng/mL of HGF (PeproTech) along with 10 μ M of dexamethasone were used. These growth factors are well known to induce maturation of hepatocyte progenitor cells into mature hepatocytes. The immunofluorescence of the albumin and CK18 markers indicated that the final maturation had occurred (fig. S4D). The differentiation factors for each stage were administrated every two days in fresh basal medium and the hEBs were maintained in suspension culture during the whole process of differentiation. Undifferentiated hEBs from the same batches were employed as negative controls.

For morphology assessment, when terminal differentiation was reached, liver organoids were transferred onto a Matrigel coated plate for imaging by light microscopy.

FACS analysis and cell sorting

In order to evaluate the viability of the organoids we used the Live/Dead Zombie NIR Fixable Viability Kit (423105, Biolegend). The Fixation/Permeabilization Staining Buffer Set (00-5523-00, eBioscience) was used intracellularly for albumin staining. The BD FACS Aria II cell sorter was employed to collect the data and 95% purity was routinely obtained. The human HNF-4 α /NR2A1 Alexa Fluor 647-conjugated antibody (IC4605R, R&D Systems) and the anti-human serum albumin APC-conjugated antibody (IC1455A, R&D Systems) were used. To setup the threshold, the control sample was stained with live/dead stain for viability, but not for albumin, with a gate for control sample set to zero. For the analysis with the FlowJo software the events exceeding the threshold for the albumin stained samples were considered to be positive. The geometric means of the appropriate channels were used to calculate the mean fluorescence intensities and the expansion indices in FlowJo software.

Gene expression analysis with real-time PCR

Gene expression was performed by real-time PCR. The RNA was extracted and treated using RNase-free DNase (M6101, Promega). A reverse transcription was performed with iScript cDNA synthesis kit (1708891, Bio-Rad) following the manufacturer protocol. A 384 wells plate from Bio-Rad was used to test in triplicate all genes of interest. The conditions for forty cycles of PCR amplification with Bio-Rad CFX96 Touch consisted of a 2 minute activation step at 95°C followed by a 5 second denaturation step at 95°C and finished with a 30 second annealing step at 60°C, melt curve at 65°C to 95°C with 0.5°C increments for 5 seconds per step. The data was

668 processed with Bio-Rad CFX Manager 3.1. The results, relative to the glyceraldehyde-3-
669 phosphate (GAPDH) housekeeping gene, were normalized to undifferentiated hEBs, used as a
670 negative control. Three independent experiments were represented by the standard deviation error
671 bars. List of the genetic markers used can be found in supplementary materials.

672 **Protein quantification**

673 Protein quantification for both liver markers and coagulation factors was performed using
674 conditioned medium collected after 48 hours of post-terminal differentiation using ELISA and
675 following the manufacturer protocol. See Supplementary Materials for the list of proteins
676 measured. To normalize the results they were related to the number of cells, with one million cells
677 used.

678 **Immunofluorescence analysis**

679 For the immunofluorescence analysis, hEBs were fixed for 90 minutes using 4%
680 paraformaldehyde (28906, Pierce), permeabilized in PBS for 1 hour with 0.3% Triton-X 100
681 (85111, Thermo Fisher), and blocked using 0.5% goat serum (G9023-5ML, Sigma-Aldrich). The
682 primary antibody was incubated for three days at 4°C, and after several washes, the liver
683 organoids were incubated with the secondary antibody for two hours at room temperature. The
684 nuclei were stained using 4'-diamidino-2-phenylindole (DAPI) for one hour. Fluorescence
685 images were obtained with the Olympus FV1000 confocal microscope.

686 **Liver detoxification assays**

687 Ammonia metabolism assay: Ammonium chloride was added to evaluate ammonia metabolism
688 via the variation of the amount of ammonia in the collected supernatant over a 6-hour period. The
689 culture medium with 100 liver organoids in suspension was supplemented with 1 mM of NH₄Cl.
690 The ammonium concentration was measured 1, 3, and 6 hours after NH₄Cl addition with a
691 colorimetric ammonia assay kit (K470, BioVision). Resorufin conjugation assay: Liver organoids
692 were incubated for three days in a medium containing rifampicin. Subsequently the organoids
693 were moved to a black 96-well plate in groups of three and re-suspended in a 40 µL of HBSS with
694 40 µL of Resorufin sodium salt (R3257, Sigma-Aldrich). The plate was incubated for 30 minutes
695 at 37°C in the dark. After the incubation, all the samples were analyzed and read in triplicate by
696 measuring the fluorescence excited at 535 nm and emitted at 581 nm with 35 nm resolution using
697 a multi-mode microplate reader (SpectraMax i3x, Molecular Devices). The fluorescence from
698 HBSS sample was employed as background fluorescence and subtracted from the measurements.
699 The results were normalized based on the number of live cells using Quant-iT PicoGreen dsDNA
700 (P7589, Thermo Fisher). CYP activity assay: The activity of cytochromes P450 was tested by
701 incubating the liver organoids for three days using various inducers. For CYP1A2 activity assay,
702 liver organoids and HPH were placed for three days in a basal medium with 50 µM Omeprazole
703 solution (O104, Sigma-Aldrich) or 0.1% DMSO. For the CYP3A4 activity, liver organoids and
704 HPH were placed for three days in a basal medium with 20 µM Rifampicin solution (R3501,
705 Sigma-Aldrich) or 0.1% DMSO. For the CYP2B6 activity, liver organoids and HPH were placed
706 for 3 days in basal medium with 1000 µM Phenobarbital solution (P1636, Sigma-Aldrich), or
707 0.1% DMSO. A multi-mode microplate reader (SpectraMax i3x, Molecular Devices) was
708 employed to measure the enzyme activity by measuring the samples' luminescence. See
709 supplementary materials for kits information.

710 **Storage, uptake and release assays**

711 For all of the assays listed here, the liver organoids were plated in the matrigel coated chamber-
712 slides and allowed to form monolayers. Indocyanine green (ICG) uptake and release assay: Liver
713 organoids were incubated with ICG (1340009, Sigma-Aldrich) in a basal medium at 37 °C for
714 one hour following the instructions provided by the manufacturer. To measure the ICG uptake,

fluorescence signals were detected with a fluorescence microscope, and the ICG release was confirmed after six hours to guarantee that every positive cell released ICG. Oil red staining: Lipid vesicle storage in liver organoids was assessed with Oil Red O staining in accordance with the manufacturer's protocol (ab150678, Abcam). Periodic Acid-Schiff (PAS) staining: Glycogen storage in liver organoids was assessed with PAS staining in accordance with the manufacturer's protocol (395B, Sigma-Aldrich). Uptake of Low-Density Lipoproteins (LDL) assay: Liver organoids were incubated overnight at 37°C with a pre-incubation medium consisting of serum-free medium with the addition of 0.1% of bovine serum albumin (BSA). The next day, the liver organoids were allowed to sit for five hours in pre-incubation medium also containing 10 µg/mL of Dil-Ac-LDL (J65597, Alfa Aesar). After incubation, the cells were rinsed several times and fixed with 4% paraformaldehyde for 60 minutes and counterstained with DAPI for nuclei. All fluorescence images for storage, uptake and release assays were acquired with the Olympus FV1000 confocal microscope.

Glycan preparation

Liver organoids were digested and homogenized using a buffer solution of NaCl (150 mM), EDTA (5 mM), TRIS (25 mM), and 0.5% CHAPS at a pH of 7.4. The sample was dialyzed overnight at 4°C using 50 mM NH₄HCO₃ buffer. The following day the resulting cells were reduced by a two hour incubation at 50°C in a solution composed of 2 mg/mL of 1,4-dithiothreitol (DTT) and 0.6 M TRIS at a pH of 8.5, and then alkylated with a 0.6 M TRIS at a pH of 8.5 solution with 12 mg/mL of Iodoacetamide for two hours at room temperature without light. Following these steps, the cells were dialyzed once again overnight at 4°C using 50 mM NH₄HCO₃ before being lyophilized. The resulting sample was then resuspended into a 1 mL solution composed of NH₄HCO₃ 50 mM and 500 µg/mL of TPCK-treated trypsin and incubated overnight at 37°C. The sample was purified using a C18 Sep-Pak column (200 mg), preconditioned with one column volume (CV) of 5% acetic acid, one CV of methanol, and one CV of 1-propanol. After an initial wash with 5% acetic acid the column was progressively eluted using 20%, 40%, and 100% of 1-propanol. The eluted samples were combined and then lyophilized. The lyophilized sample was resuspended into a solution composed of NH₄HCO₃ 50 mM with the addition of 3 µL of PNGase F followed by four hour incubation at 37°C, and a further addition of another 3 µL of PNGase F that was incubated at 37°C overnight. The N-glycans were purified using a C18 Sep-Pak column (200 mg), preconditioned with one CV of 5% acetic acid, one CV of methanol, and one CV of 1-propanol. After an initial wash with 5% acetic acid the N-glycan-released peptides were progressively eluted using 20%, 40%, and 100% of 1-propanol. The eluted samples were combined and then lyophilized.

Mass spectroscopy

Mass spectroscopy data was obtained using the Bruker UltraFlex II MAL DI-TOF mass spectrometer. For the N-glycans the data was recorded between 500 m/z and 6000 m/z and for the O-glycans the data was recorded between 0 m/z and 4000 m/z. In both cases the reflective positive mode was employed. At least 20,000 laser shots were accumulated for each N- glycan and O-glycan profile. For the data to be included the signal to noise ratio of at least 2 was required. Analysis was performed only on signals matching N-glycan or O-glycan composition. The post-data acquisition analysis was performed using the open source mass spectrometry tool mMass.

Raman spectroscopy system

The Raman spectroscopy system employed a low magnification Raman microscopy setup with a 785 nm excitation wavelength 450 mW power diode laser (MDL-III-785-450mW, CNI) delivering excitation light on the organoids with an 11 mm working distance focusing lens. The Raman signal was collected with the same lens and after passing through a notch filter, was detected with a Raman spectrometer (WP-785-R-SR-S-25, Wasatch Photonics) with 6 cm⁻¹

spectral resolution. The collection volume of the system was approximately 10 nL. During measurements, the organoids were placed in a customized quartz-bottom chamber with beam positioning achieved with a motorized XYZ stage (PT3-Z8, Thorlabs).

CLASS microscopy system

The CLASS microscopy system was described previously (29). In brief, the system is based on an Olympus FluoView 1000 confocal microscope, and utilizes a broadband supercontinuum laser source (Fianium WL-SC-400-4) (see Fig. 3A). A high N.A. objective is used to focus the broadband source on the sample (Olympus UPlanSApo 60X, NA=1.35), while a high-speed CMOS spectrometer (Avantes AvaSpec-ULS2048CL-EVO) is used to detect the spectra. The spectrometer provides a 2.4 nm spectral resolution in the 400-800 nm wavelength range. The scanning head controller is controlled with a custom-written LabView (National Instrument, LabView 8.5) code. White light imaging, which uses the microscope PMTs and broadband supercontinuum laser source, is used to determine the nuclei boundary. Importantly, the white light images are obtained simultaneously with the CLASS images, allowing exact co-registration. Data acquisition and analysis is performed with Matlab. When analyzing the data, all spectra within the nucleus can be averaged to produce a nucleus average, which can be subsequently fit to a model. Alternatively, the CLASS images can be analyzed on the pixel level, allowing a pseudo-color map of the packing parameter to be generated.

TEM imaging of chromatin

TEM images of nuclei in hiPSC organoids at hEB, definitive endoderm, foregut endoderm, hepatoblasts, and mature hepatocytes stages of differentiation were collected at Harvard Medical School Electron Microscopy Facility using JEOL 1200EX, 80kV microscope. Images of nuclei with nucleolus excluded were binarized to separate dark pixels representing heterochromatin and light pixels representing euchromatin (66). To prevent bias in image binarization, all pixels were normalized with the pixel values chosen between 0 and 1, and the global threshold was then selected (77) to minimize the intraclass variance of black and white pixels.

Statistical analysis

Statistical analysis was done with GraphPad Prism v7.00. Normality of the data was tested using the Shapiro-Wilk test or the D'Agostino-Pearson test. When comparing two groups, we used the unpaired two-tailed Student's t-test when the data was normally distributed. When the data was not normally distributed, we performed the Mann-Whitney test. Data that contained more than two groups, and which showed normal distribution, was analyzed with a one-way analysis of variance (ANOVA) test and Tukey post hoc test. A Kruskal-Wallis nonparametric test with Dunn's correction for multiple comparisons was used when data was not normally distributed. Asterisks were used to indicate significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Values > 0.05 were considered not significant.

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1047 measurements and data analysis; X.Z. and L.C. performed the Raman spectroscopy measurements
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