Cellular extrusion bioprinting improves kidney organoid reproducibility and conformation.

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Running title: Bioprinted kidney organoids

Keywords: pluripotent stem cell, kidney, kidney organoid, 3D bioprinting, nephrotoxicity
Supplementary Information

Supplementary Tables

**Supplementary Table 1.** Reproducibility of Deposition.

| Organoid Size | Volume Bioprinted | Organoid Number | Mean Diameter of Deposit (mm) | %CV  |
|---------------|-------------------|-----------------|-----------------------------|------|
| 100K          | 0.49 µL           | 24              | 1.79                        | 3.68 |
| 200K          | 0.98 µL           | 24              | 2.30                        | 1.08 |
| 500K          | 2.43 µL           | 24              | 3.12                        | 2.93 |

**Supplementary Table 2.** Differentially expressed genes identified between ratio 40 and ratio 0 organoids, identified in bulk-RNAseq. Positive log fold-change indicates increased expression in R40. (refer to Supplementary_Table_2.csv).

**Supplementary Table 3.** Whole data-set cluster markers for integrated scRNAseq dataset (refer to Supplementary_Table_3.xlsx).

**Supplementary Table 4.** Nephron cluster markers for integrated scRNAseq dataset (refer to Supplementary_Table_4.xlsx).

**Supplementary Table 5.** Stromal cluster markers for integrated scRNAseq dataset (refer to Supplementary_Table_5.xlsx).
**Supplementary Table 6.** Details of antibodies and lectins used for immunofluorescence of organoids.

| Specificity               | Host species | Manufacturer and identifier                             | Dilution range |
|---------------------------|--------------|--------------------------------------------------------|----------------|
| MAFB                      | Mouse        | Novus Biologics (NBP2-45718)                           | 1:400          |
| NEPHRIN                   | Sheep        | R&D Systems/Bioscientific (AF4269)                     | 1:100-1:300    |
| E-CADHERIN                | Mouse        | Abcam (ab1416)                                         | 1:100          |
|                          | Mouse        | BD Biosciences (610181)                                | 1:300          |
|                          | Rabbit       | Abcam, ab15148                                         | 1:100          |
| GATA3                     | Mouse        | Invitrogen (MA1-028)                                   | 1:100          |
|                          | Goat         | R & D Systems (AF2605)                                 | 1:300          |
| CD13                      | Mouse        | Australian Biosearch (301713)                          | 1:300          |
| (PerCP-Cy4.5 conjugate)   | Goat         | Santa Cruz Biotechnology (sc-20607)                    | 1:300          |
| EpCAM                     | Mouse        | Biolegend (324210)                                     | 1:300          |
| (Alexa488 conjugate)      | Rabbit       | Sigma-Aldrich (L9393)                                  | 1:400-1:500    |
|                          | Rabbit       | Abcam (ab11575)                                        |                |
| LAMININ                   | Rabbit       | Proteintech (18970-1-AP)                               | 1:300          |
|                          | Mouse        | Active Motif (39795)                                   | 1:300          |
| Proximal tubule brush     | Lotus        | Vector Laboratories (B-1325)                           | 1:300-1:500    |
| border membrane           | tetragonobulus|                                                        |                |
|                          | lectin (LTL) |                                                        |                |
| SLC12A1                   | Rabbit       | Proteintech (18970-1-AP)                               | 1:300          |
| CLEAVED CASPASE 3         | Rabbit       | Cell Signaling Technology (9661)                       | 1:400          |
| CYTOKERATIN 8/18          | Guinea Pig   | Abcam (ab194130)                                       | 1:400          |
| SIX1                      | Rabbit       | Cell Signaling Technology (12891)                      | 1:300          |
| SOX9                      | Goat         | R&D (AF3075)                                           | 1:300          |
| Atypical Protein Kinase C-| Mouse        | Santa Cruz (sc-17781)                                  | 1:1000         |
| zeta (aPKC)               |              |                                                        |                |
| tagRFP                    | Rabbit       | Evrogen (AB233)                                        | 1:500          |
| TotalSeq™-A0251 anti-human Hashtag 1 | Mouse | BioLegend (394603) | 1μg/ml |
|--------------------------------------|-------|--------------------|--------|
| TotalSeq™-A0252 anti-human Hashtag 2 | Mouse | BioLegend (394601) | 1μg/ml |
| TotalSeq™-A0253 anti-human Hashtag 3 | Mouse | BioLegend (394605) | 1μg/ml |
| TotalSeq™-A0254 anti-human Hashtag 4 | Mouse | BioLegend (394607) | 1μg/ml |

**Supplementary Data**

**Supplementary Data 1:** Differentially expressed genes between conditions, within clusters for nephron and stromal subsets of scRNA data. Data for each comparison are contained within separate tabs, with a guide to set naming in the first tab.

**Supplementary Movies**

**Supplementary Movie 1:** Novogen 3D bioprinter generating kidney organoids

**Supplementary Movie 2:** 3D rendering of a bioprinted dot organoid from Figure 4D.

**Supplementary Movie 3:** 3D rendering of a bioprinted line organoid from Figure 4D.
Supplementary Methods

*Extrusion bioprinting using dry paste*

During optimisation of extrusion bioprinting, a comparison was made with settled wet paste versus a dry paste generated to recapitulate the packed cell density used when preparing a manual organoid. To achieve a dry paste of this density within the extrusion syringe, the prepared syringe was loaded into a proprietary adaptor to enable centrifugation at 400 x g within a 50mL polypropylene conical tube. Syringe/Adaptor assemblies were centrifuged for a total of 9 minutes to mirror the manual protocol.

*Bead based analysis of cell density and height at print*

Cell paste was spiked with 4um Tetraspec beads (Thermo-fisher) at 1 ul bead suspension per 50ul of paste. Organoids were imaged within 2-3 hours of bioprinting to capture brightfield and fluorescent bead signal and again at various times during organoid culture. Imaging was performed using an Andor dragonfly spinning disk confocal with 4x 0.2NA Nikon objective, capturing z-stacks beginning at the Transwell surface and continuing until no further bead signal was detected. Fiji \(^1\) was used to stitch tiled datasets and generate maximum projections of the bead image. A custom Python script was used to count individual beads in each dataset and final count data was analysed in R. Surface areas derived from bead distributions were used to approximate organoid height at time of print as the height of a shape with vertical sides and the same surface area and volume as the deposited organoid.
Single Cell RNA sequencing library generation and analysis

Four replicate organoid sets were generated, where each replicate was derived from an independent pool of D7 differentiated iPSCs derived from 3 monolayer culture wells. For each pool cells were loaded into the bioprinter to print a pattern consisting of 3 R0 ‘dots’ and 3 R4 ‘lines’ per well, over 10-12 wells (2 plates). At the same time the remaining portion of the cell pool was used to generate manual organoids. Bioprinted organoids were generated from 1.1x10^5 cells each, while manual organoids were generated from 2.3x10^5 cells, as it was not technically possible to manually manipulate smaller masses. Replicate sets were processed sequentially on the same day so that cells were always loaded and printed within a short period of time. Cells were printed approximately 10 minutes after loading, and the run was complete within ~20 minutes of loading.

Organoids were dissociated at D7 + 12 following previously published methods (17, 35). For each of R0 and R40, 9 organoids derived from 3 wells (3 per condition, per well) were dissociated. For manual 3 organoids per replicate were dissociated. Replicates were multiplexed following the method of Soeckius et al.². Cells were stained for 20 minutes on ice with 1µg of BioLegend TotalSeq-A anti-human hashtag oligo antibody (BioLegend TotalSeq-A0251, 0252, 0253, 0254). Cells were washed 3 times then pooled at equal ratios for sequencing. A single library was generated for each suspension/condition (manual, R0, R40), composed of equally sized pools of each replicate (Set 1 – 4). Libraries were generated following the standard 10x Chromium Next GEM Single Cell 3’ Reagent Kits v3.1 protocol except that ‘superloading’ of the 10x device was performed with ~30k cells ³. Hash tag oligo (HTO) libraries were generated following the BioLegend manufacturer protocol. Sequencing was performed using an Illumina Novoseq.
10x mRNA libraries were demultiplexed using CellRanger (3.1.0) to generate matricies of UMI counts per cell. HTO libraries were demultiplexed using Cite-seq-count (1.4.3) to generate matrices of HTO counts per cell barcode. All data were loaded into Seurat (3.1.4) and HTO libraries were matched to mRNA libraries. Seurat was used to normalise HTO counts and determine cutoffs to assign HTO identity per cell (cutoff was typically 100-200 counts per cell). Doublet and unassigned cells were removed, as were cells with mitochondrial content greater than 15% or number of genes less than 1000, to obtain filtered datasets with final sizes: manual - 9963 cells, R0 - 8912 cells, R40 - 13525 cells. Genes were removed that contained counts in less than 20 cells. The combined datasets contained a median of 2034 genes expressed per cell, with a median of 5499 UMI counts per cell.

Data were normalised using the SCTransform method \(^4\) and integrated using Seurat to obtain a single dataset. Clustering was performed initially to identify clustering belonging to stroma, nephron, or endothelial compartments. The Clustree package \(^5\) was used visualise clustering and determine a stable clustering resolution. Nephron and stromal populations were re-normalised with SCTransform and clustered to obtain a finer resolution view of cell heterogeneity. At this level of resolution we were able to identify clusters with a high computational doublet score, using Scrublet (0.2.1) \(^6\), and an identity that appeared to combine two known cells types. These were presumed to be unidentifiable doublets consisting of a single HTO ID and were removed from further analysis. Marker analysis was performed using the Seurat FindMarkers function, limited to positive markers (i.e. increased expression within a cluster) above 0.25 log fold-change. Marker lists were exported and cluster identities were
Determined by comparison with published human single cell data \(^7,8\) or Gene ontology analysis using ToppFun \(^9\).

Differential expression testing was performed by summing counts to produce a ‘psudo-bulk’ count per replicate per cluster using the sumCountsAcrossCells function in Scater (1.12.2), to produce a matrix of gene counts over 12 conditions (4 replicates per organoid conformation). This count matrix was used as input to do differential expression testing in EdgeR (3.26.5) using a quasi-likelihood negative binomial generalized log-linear model implemented in the glmQLFFit function. For differential expression testing within clusters genes appearing as differentially expressed in more than 3 clusters were removed from further analysis, to remove potential batch effects and focus on genes specific to a particular cell type that may be more biologically relevant. Frequently changing genes tended to be mitochondrial and ribosomal genes. Genes were considered differentially expressed if they had an adjusted \(p\) value \(< 0.05\), using the Benjamini-Hochberg method for correction in EdgeR.

**Comparison of organoids to human fetal kidney data using prediction of cell identity**

The raw fastq files for the week 11, 13, 16 and 18 single cell datasets published in Hochane et al. 2018 were downloaded from Gene Expression Omnibus and mapped to the reference genome GRCh38-3.0.0 using cellranger. The Seurat package (3.1.5)\(^{10}\) was used to perform quality control and analysis. Cells with less than 750 features were removed, the SCTransform method \(^4\) was used to normalise and scale the raw counts then dimensional reduction was performed. The datasets were integrated using the fastMNN method \(^11\) as implemented within the SeuratWrappers package (0.1.0). After an initial clustering the subset identified as nephron was isolated and reanalysed to identify the Progenitors, Pre-Pod, Podocyte, Pre-Tubule, Distal
and Proximal cell populations. The Podocyte and Proximal cell populations were further analysed to identify the stages of maturation present within these lineages. The model used to identify the cell types was generated using the *scPred* package (0.0.0.9)\(^2\) based upon the nephron subsets of the integrated human fetal kidney data as a reference. This produced a model that would classify cells into one of the nephron sub-categories (Progenitors, Pre-Pod, Podocyte, Pre-Tubule, Distal and Proximal). This model was then applied to the organoid single cell datasets to define component cell types.

**Machine learning based Image Segmentation**

Images from the large scRNAseq experiment (Supp. Figure 4) were segmented for quantification using pixel classification within the Ilastik (1.3.2) package\(^3\). Ilastik was used to annotate either MAFB-tagBFP2 positive, or GATA3-mCherry positive nephron regions, and negative non-tubular regions in multiple images randomly chosen from conditions across the entire set. Training features and annotation regions were adjusted until regions were accurately classified across the entire dataset, including all conditions. Pixel probability images were exported corresponding to each of the input images, and quantified using custom python scripts using the scikit-image package\(^4\). Area estimates for each organoid were obtained by thresholding the pixel classification image dataset (value = 0.8 for MAFB-mtagBFP2 set, 0.5 for GATA3-mCherry set). Data were compiled for analysis and plotting in R.

**Organoid Height Measurements at D7+0**

The height of organoids was assessed by image-based quantification of pre-labelled cells using Fiji (40). Prior to bioprinting 10% of cells were removed and labelled with CellTrace Far Red (ThermoFisher, C34564) according to manufacturer instructions. Labelled cells were mixed
back in with the remaining cells and bioprinted to give sparse labeling in the micromass. Two independent sets of organoids were characterised in this way at D7+0 by removing the transwell containing organoids and placing it flat on a lumox dish (Sarstedt) with a small amount of media. This allowed imaging with a much smaller working distance but prevented the organoids from drying out. Images were captured using an Andor Dragonfly spinning disk with a Nikon 1.15 NA 40x Water immersion objective, capturing images at 0.325 x 0.325 x 0.5 micron voxel size. The highest and lowest points of the image stacks were manually measured under the orthogonal view in Fiji. For each sample, the image was equally split into three sections (up, middle & down) in the X-Y plane along the Y-axes (Supp. Figure 3). Then, in each section, two highest points and two lowest points were recorded in the centre area of the image across the 300 micron range (150 micron from the centre to both -X and +X directions). In general, six highest points and six lowest points were then collected for each condition. The height of the organoids was calculated using equation below (Equation 3).

\[
H(mm) = \left[ Mean(6 \text{ highest points}) - Mean(6 \text{ lowest points}) \right] \times Voxel\ depth\ (mm)
\]

Data were compiled in R for analysis and plotting.
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