

BIOPRINTED PLURIPOTENT STEM CELL-DERIVED KIDNEY ORGANOIDS PROVIDE OPPORTUNITIES FOR HIGH CONTENT SCREENING

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ABSTRACT

Recent progress in the directed differentiation of human pluripotent stem cells to kidney organoids advances the prospect of drug screening, disease modeling, and even restoration of renal function using patient-derived stem cell lines. Here, we demonstrate the successful adaptation of our directed differentiation protocol to the NovoGen Bioprinter[®] MMX technology to achieve automated, rapid fabrication of self-organizing kidney organoids. Bioprinted organoids were found to be equivalent to those previously reported via manual generation at the level of morphology, component cell types, and expression profiles. Bioprinted kidney organoids treated with doxorubicin exhibited concentration-dependent toxicity, characterized by the loss of podocyte-specific markers. High-throughput toxicity screening was achieved by treating organoids bioprinted in 96-well plates with a classic nephrotoxic compound. Collectively, these results suggest that bioprinted kidney organoids are functionally equivalent to those prepared manually and thus are likely to be useful for a multitude of applications.

METHODS

All iPSC culture and differentiation procedures were performed following published methodologies (Takasato et. al 2015, 2016) with slight modifications as noted in Higgins et. al 2018.

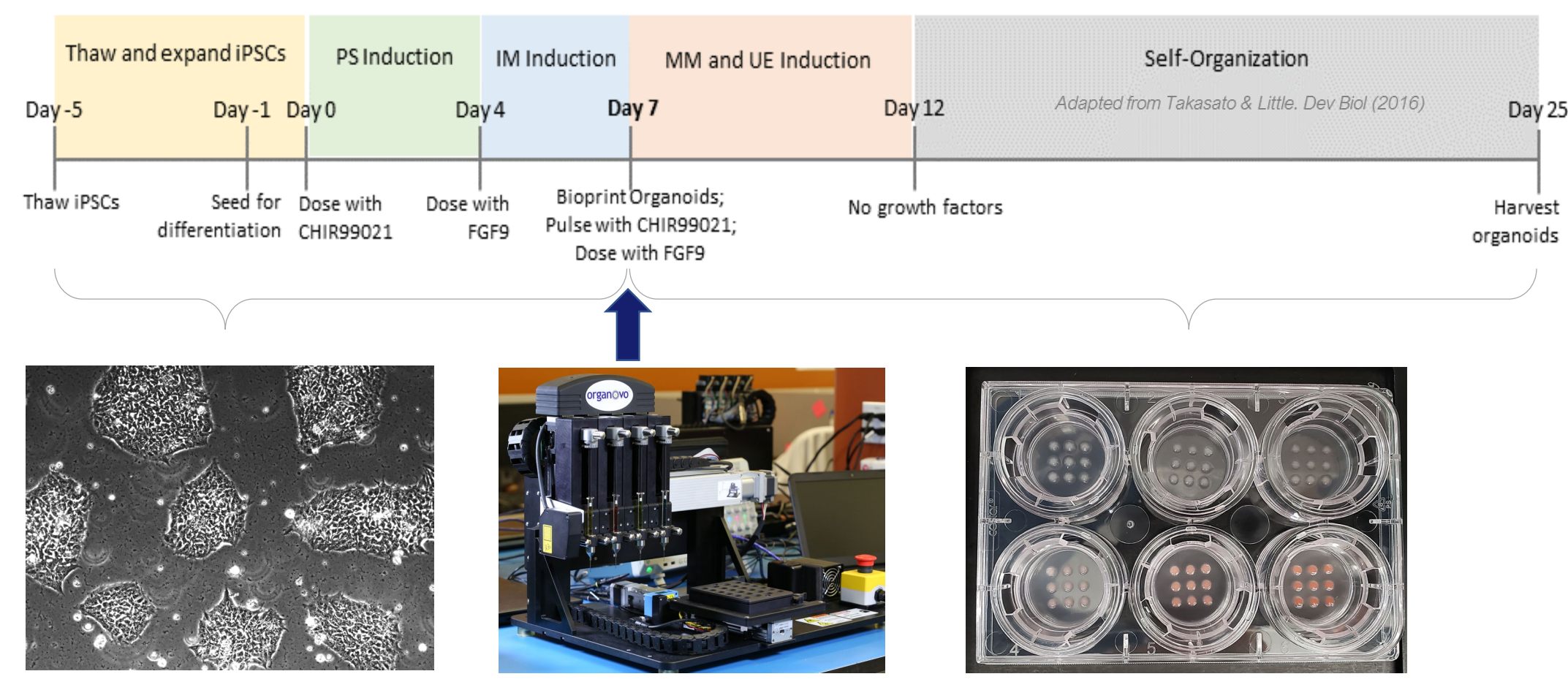


Figure 1: Methods overview for the inclusion of NovoGen Bioprinter[®] MMX technology in the generation of kidney organoids. Schematic outline of 2D differentiation, generation of 3D organoids by bioprinting, and culture of kidney organoids through maturation at D25 (D7+18).

RESULTS

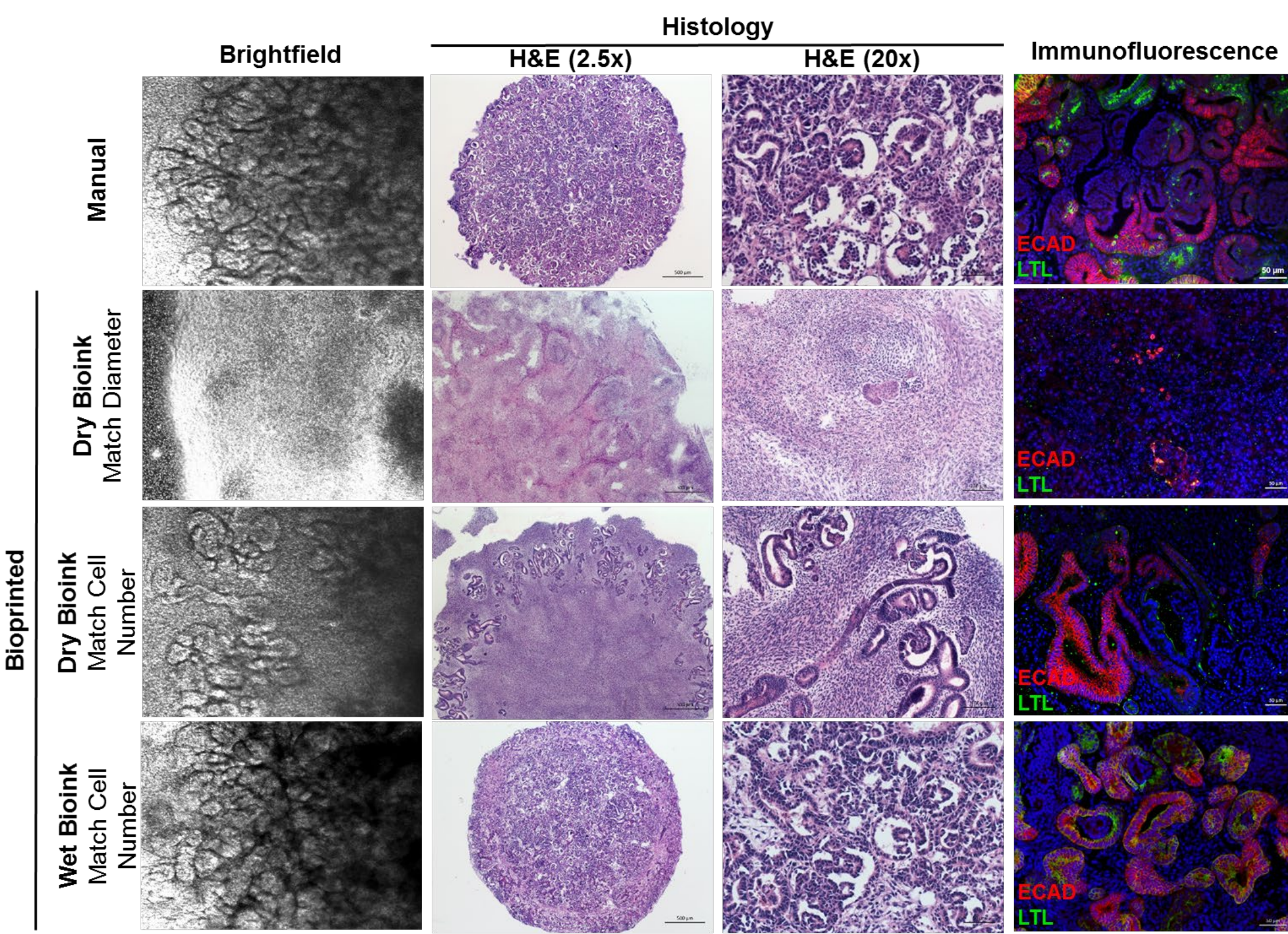


Figure 2: Optimization of bioprinting methodology for the generation of kidney organoids. Brightfield, histological, and immunofluorescence comparisons of kidney organoids generated manually (5 x 10⁵ cells per organoid) with bioprinted organoids using dry cell paste (bioink) controlled for organoid diameter, dry bioink controlled for cell number, and wet bioink controlled for cell number. Immunofluorescent characterization of Day 25 organoids showing the presence/absence of tubular epithelium (E-CADHERIN, red) and proximal tubules (LTL, green).

REFERENCES

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Forward Looking Statement
Any statements contained in this press release that do not describe historical facts constitute forward-looking statements as that term is defined in the Private Securities Litigation Reform Act of 1995. Any forward-looking statements contained herein are based on current expectations, but are subject to a number of risks and uncertainties. The factors that could cause the Company's actual future results to differ materially from current expectations include, but are not limited to, statements regarding the potential benefits and therapeutic uses of the Company's therapeutic liver tissue, including the benefits of an orphan designation; the Company's expectations regarding the FDA regulatory pathway and anticipated timelines for its regulatory filings; the Company's ability to successfully complete additional preclinical studies, improve its manufacturing processes and demonstrate the prolonged functionality and therapeutic benefits of its therapeutic liver tissue; the Company's ability to implement clinical scale manufacturing and quality processes; the Company's ability to meet market demand; the Company's ability to fund its future operations and business plans; and acceptance of its disease modeling and other *in vivo* tissue platforms. The factors that could cause the Company's actual future results to differ materially from current expectations include, but are not limited to, risks and uncertainties relating to the Company's ability to successfully improve or demonstrate the durability and functionality of its *in vivo* liver tissue candidate; the possibility that the results of future preclinical studies may be different from the Company's earlier pilot studies and may not support further clinical development of its tissue candidates; the Company's ability to successfully complete the required preclinical and clinical trials required to obtain regulatory approval on a timely basis or at all; the novelty of the Company's therapeutic tissue approach and the resulting heightened regulatory scrutiny, delays in clinical development or delays in commercial acceptance; the complexity of the manufacturing process for the Company's therapeutic tissues and the effort involved in developing GMP and GMP facilities; the Company's ability to raise significant additional funds to support its business plan and its regulatory objectives; the Company's reliance on third parties and a single supplier for clinical grade organs, including that the Company may not be able to obtain sufficient raw materials to meet clinical or commercial demand; for its therapeutic products; competitive products may adversely impact the market opportunity for the Company's therapeutic tissue candidates and its disease modeling and other *in vitro* tissue products, services and technology; the Company's ability to successfully complete studies and provide the technical information required to support market acceptance of its disease modeling and other *in vitro* tissue products, services and technology, on a timely basis or at all; and the Company's ability to comply with Nasdaq's continued listing requirements. These and other factors are identified and described in more detail in the Company's filings with the SEC, including its Annual Report on Form 10-K filed with the SEC on June 3, 2019. You should not place undue reliance on these forward-looking statements, which speak only as of the date that they were made. These cautionary statements should be considered with any written or oral forward-looking statements that the Company may issue in the future. Except as required by applicable law, including the securities laws of the United States, the Company does not intend to update any of the forward-looking statements to conform these statements to reflect actual results, later events or circumstances or to reflect the occurrence of unanticipated events.

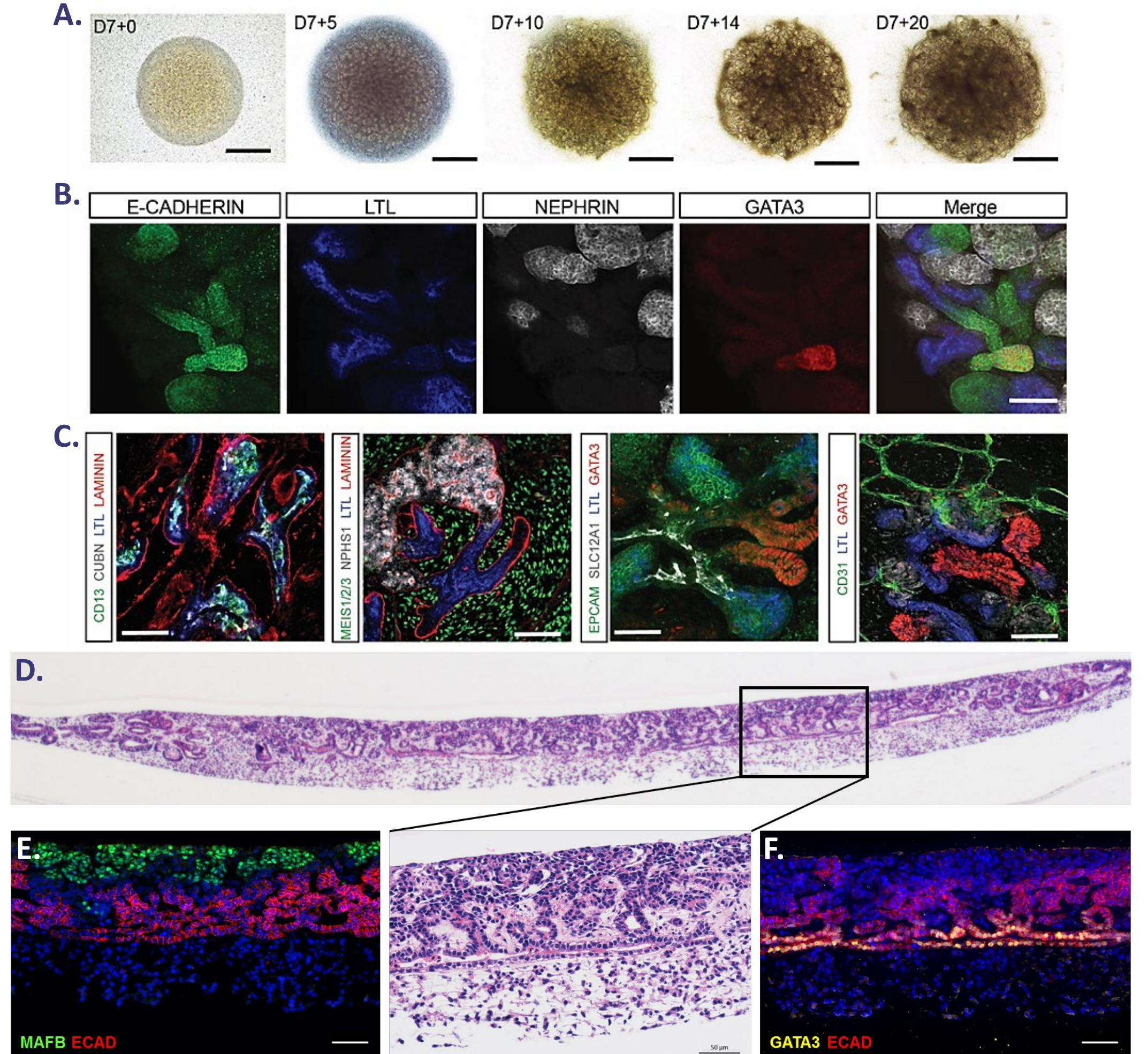


Figure 3: Characterization of kidney organoids bioprinted using control and reporter iPSC lines. [A] Brightfield images of bioprinted organoids across time showing evidence of increasing tubular complexity. Scale bar represents 800 μ m. [B] Immunofluorescence of a Day 25 bioprinted organoid showing the presence of nephron epithelium (E-CADHERIN, green), proximal tubules (LTL, blue), collecting duct (GATA3, red) and podocytes (NEPHRIN, grey). Scale bar represents 100 μ m. [C] Immunofluorescence of Day 25 bioprinted organoids stained for the proximal tubule markers CD31, LTL and CUBN, podocyte marker NPHS1, distal tubule epithelial markers EPCAM and SLC12A1, collecting duct markers EPCAM and GATA3, the presence of a LAMININ-positive basement membrane along the nephrons, a surrounding MEIS1/2/3-positive stroma and CD31-positive endothelium. Scale bar represents 50 μ m. [D] Histological cross-section of bioprinted organoids showed a high level of tissue organization. [E] MAFB⁺ podocytes reside in close proximity to ECAD⁺ tubular regions. [F] A contiguous putative collecting duct (GATA3⁺/ECAD⁺) network spans horizontally throughout organoids with nephrons connected to and contiguous with this epithelium. Scale bar (E-F) represents 50 μ m.

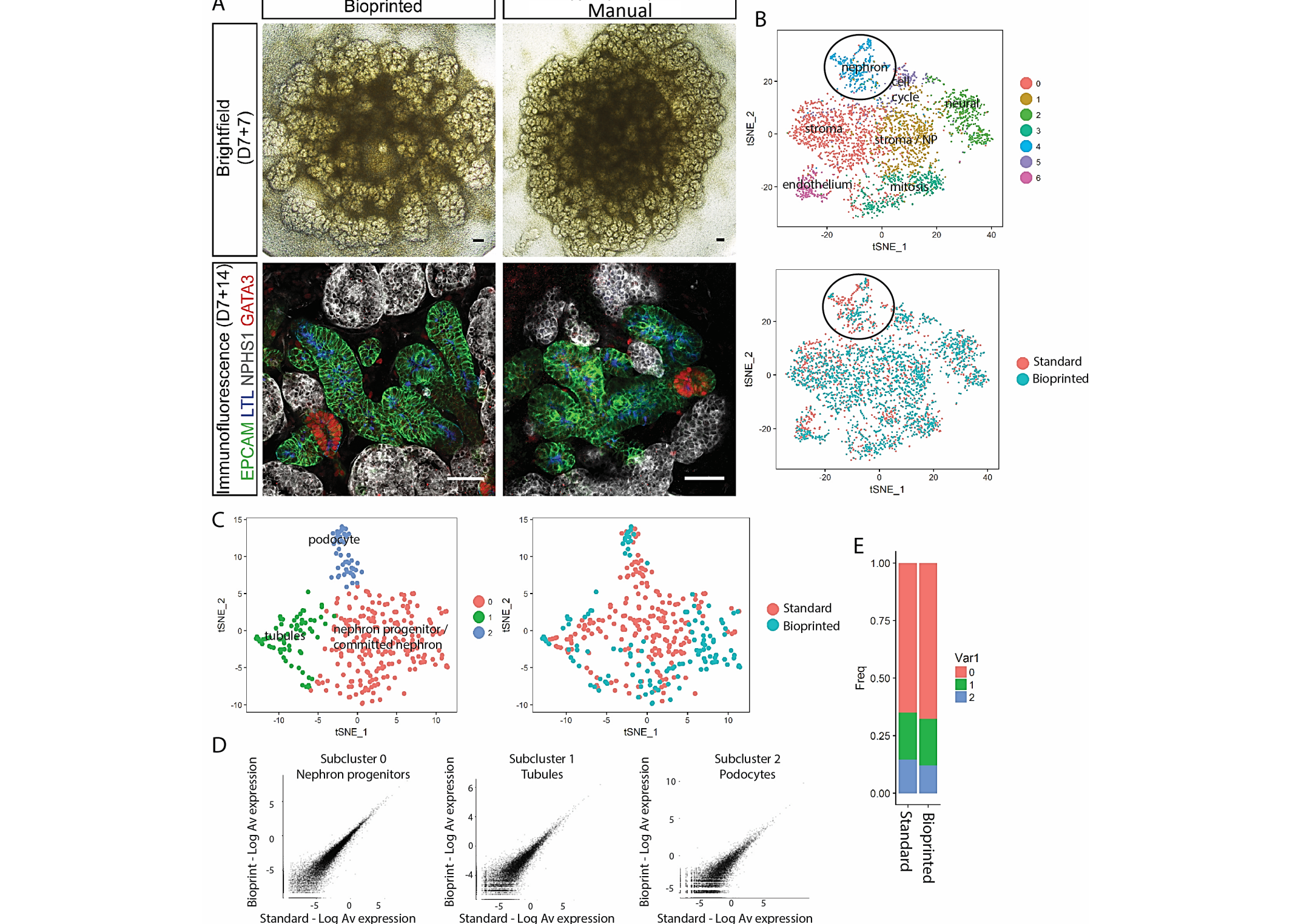


Figure 4: Single cell transcriptional profiling shows equivalence between standard and bioprinted kidney organoids. [A] Brightfield and immunofluorescent characterization of maturing kidney organoids generated manually and with bioprinting. Day 14 (D7+7) brightfield images show complex, maturing organoid structures with congruence between production methods. Day 21 (D7+14) organoids confirm key nephron structures across both methodologies with presence of tubule epithelia (EPCAM, green), proximal tubule cells (LTL, blue), podocytes (NPHS1, grey), and collecting duct (GATA3, red). [B] tSNE overlay of 3885 cells isolated from manual or bioprinted kidney organoids. Unsupervised clustering with Seurat identified 7 distinct cell clusters and direct comparison with standard organoids, identified as indicated using GO analysis and comparison to available human fetal kidney data (Lindstrom et al., 2018). A view of the same tSNE plot indicating whether individual cells were derived from the manual (blue) or bioprinted (pink) organoids. [C] Reanalysis of the nephron cluster (cluster 4) reveals the presence of three subclusters, identifiable as committed progenitor / renal vesicle (subcluster 0), early tubule (subcluster 1) and podocyte (subcluster 2). tSNE plot identifying nephron cluster cells based upon their origin in either manual (blue) or bioprinted (pink) organoids. [D] A comparison of average gene expression values (each point is a gene) within each nephron subcluster between manual and bioprinted organoids shows tight transcriptional conformity. [E] Relative proportion of cells present in each subcluster for manual and bioprinted organoids.

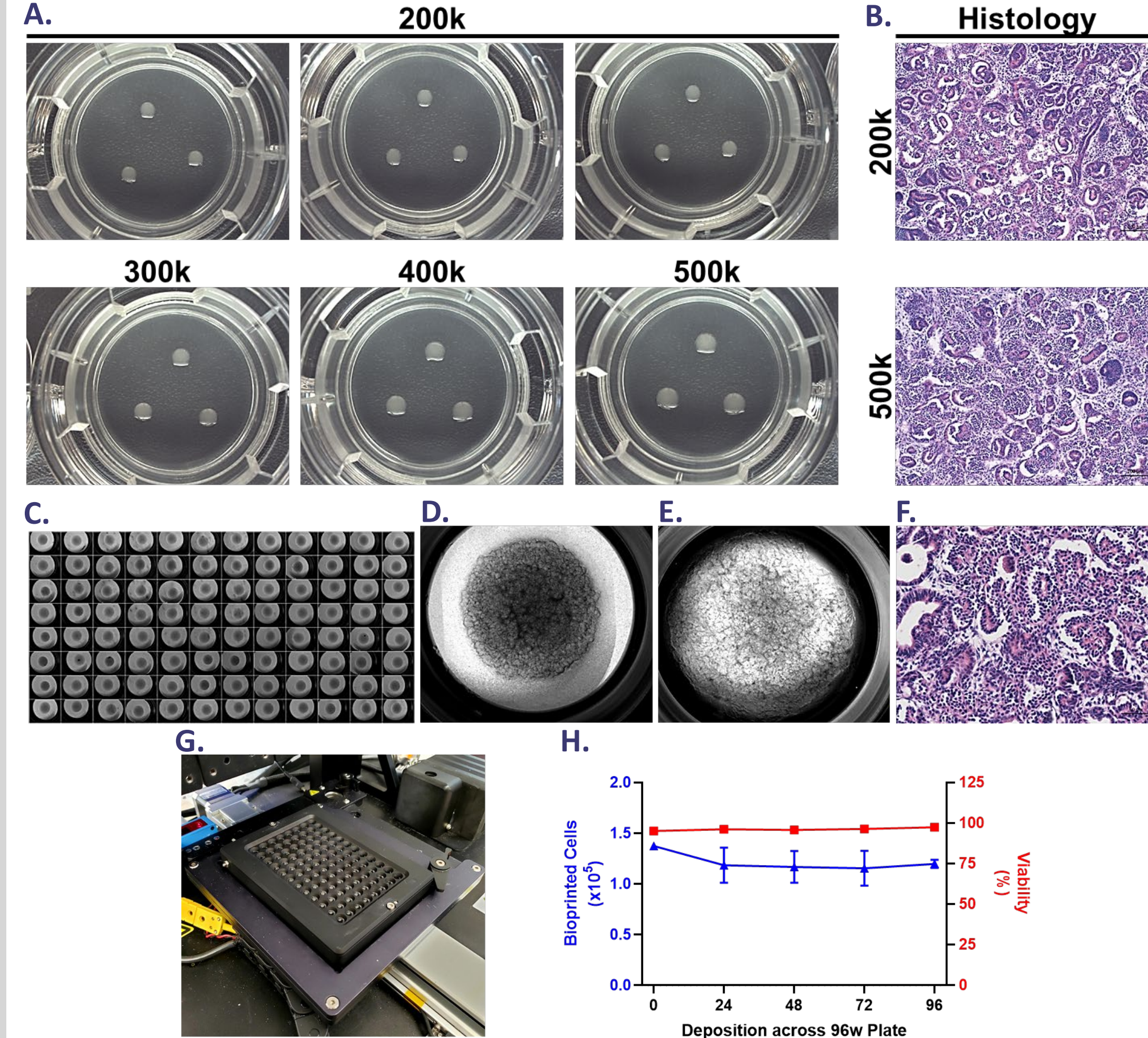


Figure 5: Bioprinted kidney organoid differentiation is equivalent with reduced starting cell number. [A] Images show three kidney organoids bioprinted within a single Transwell[™] permeable support at 2x10⁵, 3x10⁵, 5x10⁵, and 5x10⁵ cells per organoid. [B] H&E stains of sectioned organoids bioprinted at 2x10⁵ and 5x10⁵ cells/organoid. [C] Composite panel of 1x10⁵ cells/organoid bioprinted into each well of a 96-well Transwell[™] permeable support system. [D] Representative maturing kidney organoid printed on 96-well Transwell[™] permeable support on Day 15 (Day 7+8) and [E] Day 25 (Day 7+18). [F] H&E stains of a sectioned Day 25 (Day 7+18) mature organoid bioprinted on a 96-well Transwell[™] permeable support. [G] Prototype 96-well anti-rotational plate to facilitate printing on Transwell permeable supports. [H] Bioink concentration and viability showed stability throughout the full 96-well bioprinting process.

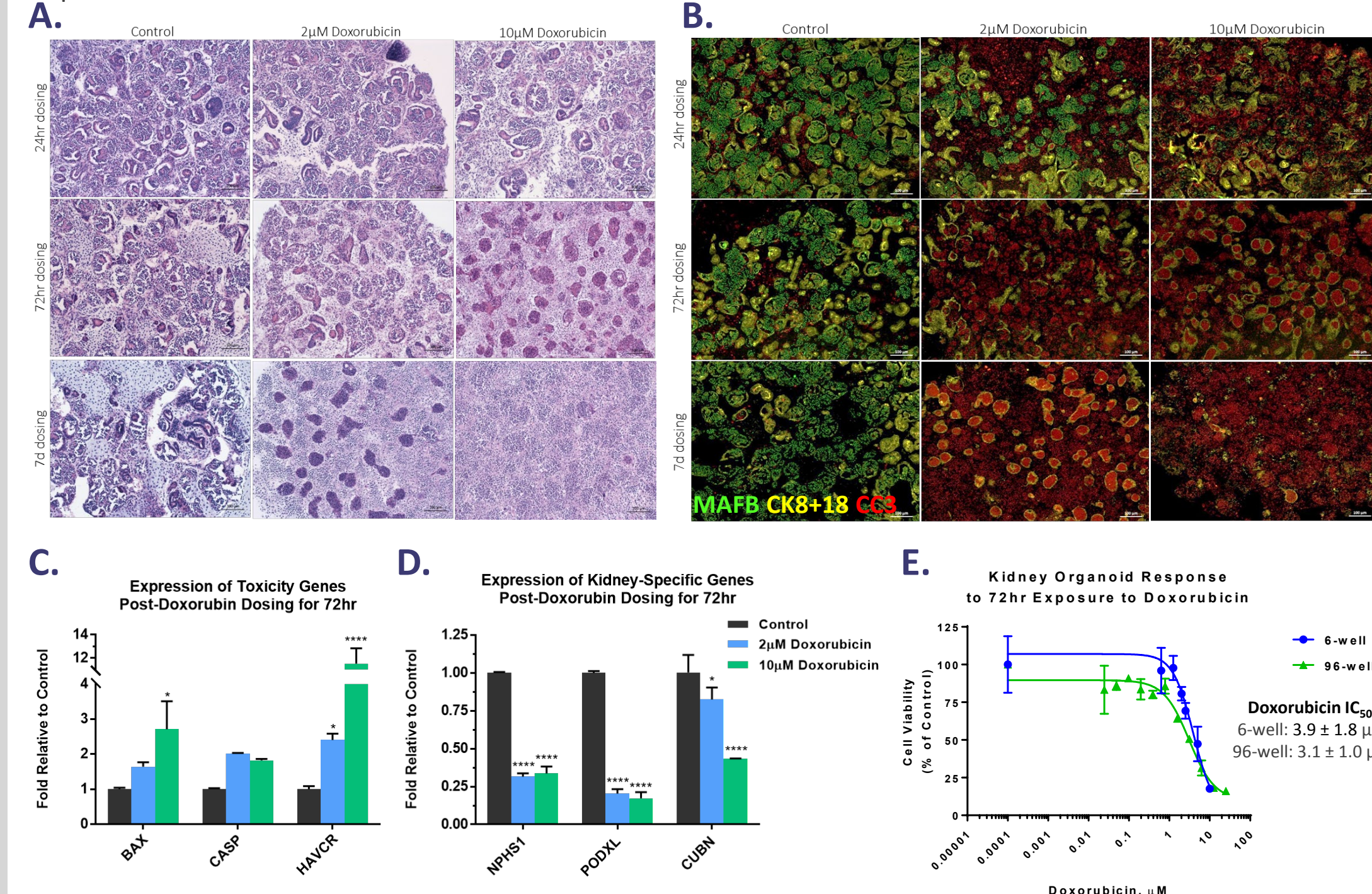


Figure 6: Application of bioprinted kidney organoids in nephrotoxicity screening. Doxorubicin induces a compartment-specific toxic response to glomerular cell types. [A] H&E staining of kidney organoids show a time- and dose-dependent toxic response to doxorubicin. [B] Immunofluorescent staining of sectioned organoids after doxorubicin exposure for podocytes (MAFB, green); tubular epithelium (CK8/18, yellow); Apoptosis (Cleaved Caspase 3, red). Kidney organoids exhibit a rapid loss of glomerular marker MAFB and increase of CC3 in response to doxorubicin. [C] Doxorubicin treatment leads to up-regulation of apoptosis and kidney injury genes (BAX, CASP3, HAVCR). [D] Preferential down-regulation of nephron markers (NPHS1, PDXL) compared to the proximal tubule marker (CUBN) upon doxorubicin treatment. Significant differences in gene expression calculated relative to control organoid expression (n=2 to 3 organoids per treatment group), and assessed by Two-Way ANOVA with Dunnett's Multiple Comparisons, *p<.05, ***p<.0001. [E] Viability of cells within kidney organoids bioprinted in either 6-well (n=3-6 per doxorubicin concentration) or 96-well (n=1-3 organoids per doxorubicin concentration) format in response to 72-hr treatment with doxorubicin.

CONCLUSION

3D Bioprinting enables automated and scaled fabrication of human iPSC-derived kidney organoids equivalent to those generated manually at the level of cellular complexity, identity, and gene expression. In addition, inclusion of the bioprinter increased speed and reproducibility facilitating larger production runs without comprising organoid quality. This work suggests significant utility for drug testing and modeling human development and disease *in vitro*, and provides translational promise for the combined use of iPSC and tissue engineering technologies for functional restoration in patients with renal disease.