

**(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE**

(11) Application No. AU 2015328173 B2

(54) Title
Engineered renal tissues, arrays thereof, and methods of making the same

(51) International Patent Classification(s)
C12N 5/07 (2010.01) **G01N 33/50** (2006.01)
C12N 5/09 (2010.01)

(21) Application No: **2015328173** (22) Date of Filing: **2015.10.06**

(87) WIPO No: **WO16/057571**

(30) Priority Data

(31) Number 62/140,285	(32) Date 2015.03.30	(33) Country US	
	62/060,416	2014.10.06	US

(43) Publication Date: **2016.04.14**
(44) Accepted Journal Date: **2022.01.27**

(71) Applicant(s)
Organovo, Inc.

(72) Inventor(s)
Nguyen, Deborah Lynn Greene;King, Shelby Marie;Presnell, Sharon C.

(74) Agent / Attorney
Spruson & Ferguson, GPO Box 3898, Sydney, NSW, 2001, AU

(56) Related Art
US 2013/0190210 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization

International Bureau



WIPO | PCT



(10) International Publication Number

WO 2016/057571 A1

(43) International Publication Date

14 April 2016 (14.04.2016)

(51) International Patent Classification:

C12N 5/07 (2010.01) G01N 33/50 (2006.01)
C12N 5/09 (2010.01)

(21) International Application Number:

PCT/US2015/054315

(22) International Filing Date:

6 October 2015 (06.10.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/060,416 6 October 2014 (06.10.2014) US
62/140,285 30 March 2015 (30.03.2015) US

(71) Applicant: ORGANOVO, INC. [US/US]; 6275 Nancy Ridge Drive, Suite 110, San Diego, California 92121 (US).

(72) Inventors: NGUYEN, Deborah Lynn Greene; 13366 Samantha Avenue, San Diego, California 92129 (US). KING, Shelby Marie; 11167 Caminito Arcada, San Diego, California 92131 (US). PRESNELL, Sharon C.; 16224 Rostrata Hill Rd., Poway, California 92064 (US).

(74) Agent: BURKETTE, Scott L.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, California 94304 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

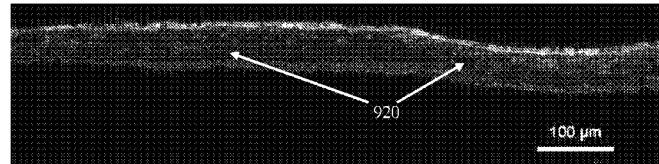
- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

Published:

- with international search report (Art. 21(3))

(54) Title: ENGINEERED RENAL TISSUES, ARRAYS THEREOF, AND METHODS OF MAKING THE SAME

Fig. 9A



(57) Abstract: Disclosed are renal tissues and arrays thereof that include a layer of renal interstitial tissue, the renal interstitial tissue comprising renal fibroblasts and endothelial cells; and a layer of renal epithelial tissue, the renal epithelial tissue comprising renal tubular epithelial cells, the renal epithelial tissue in contact with the layer of renal interstitial tissue to form a three-dimensional, engineered, biological renal tissue. Also disclosed are methods of fabricating and using the same.

WO 2016/057571 A1

**ENGINEERED RENAL TISSUES, ARRAYS THEREOF, AND METHODS OF
MAKING THE SAME**

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Patent Application No. 62/060,416, filed October 6, 2014, and U.S. Patent Application No. 62/140,285, filed March 30, 2015, the entire disclosures of which are hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

[002] The full cost of bringing a new drug to market - from discovery through clinical trials to approval - is typically hundreds of millions or billions of U.S. dollars. This is in part because ninety-five percent of the experimental medicines that are studied in humans fail to be both effective and safe. Renal toxicity is a major cause of drug attrition at the clinical trial stage, and the primary site of this toxicity is within the proximal tubule.

SUMMARY OF THE INVENTION

[003] Current *in vitro* renal toxicity models, such as monolayers of epithelial cells and *in vivo* models, such as live rodents, cannot effectively predict a drug's effects, toxicity, or metabolism in humans. For example, conventional renal cell culture models lack the complexity of native tissue and thus have a limited capacity for predicting tissue-level responses. In addition, the predictive potential of pre-clinical animal trials is limited due to species-specific differences between human and animal renal functions, including differential sensitivity to insults. What is needed is an engineered renal tissue model with native-like tissue architecture; specifically, a tissue model with cells organized spatially to recapitulate the laminar architecture of the tubulointerstitial tissue interface. Such an engineered renal tissue model would be more predictive of human *in vivo* response and would be useful for modeling renal toxicity, modeling kidney disease (e.g., polycystic kidney disease, infectious disease, autoimmune disease, fibrosis, and chronic kidney disease due to high blood pressure or diabetes), and modeling transport (e.g., excretion and/or uptake of macromolecules).

[003a] In a first aspect there is provided a three-dimensional, engineered, bioprinted, biological renal tubule model comprising:

- a. a layer of renal interstitial tissue, the renal interstitial tissue comprising renal fibroblasts and endothelial cells, wherein the layer of renal interstitial tissue possesses an apical and basolateral surface; and
- b. a layer of renal epithelial tissue, the renal epithelial tissue comprising renal tubular epithelial cells, wherein the layer of renal epithelial tissue is substantially a monolayer and is in direct contact with the apical surface of the layer of renal interstitial tissue or is separated from the apical surface of the renal interstitial tissue by extracellular matrix;

provided that the interstitial tissue comprises an interstitial bio-ink, the epithelial tissue comprises an epithelial bio-ink, and form a three-dimensional, engineered, biological renal tubule model; and wherein the renal fibroblasts and endothelial cells are the only cells present in the layer of renal interstitial tissue and are present in a ratio of renal fibroblasts to endothelial cells at which the renal tubule model is planar six days post-printing

[003b] In a second aspect there is provided a plurality of the renal tubule models according to the first aspect, configured to form an array.

[003c] In a third aspect there is provided a method of testing the viability of tubular epithelial cells in the presence of a therapeutic agent, comprising:

- a. contacting the therapeutic agent with the three-dimensional, engineered, bioprinted, biological renal tubule model according to the first aspect, and
- b. measuring gamma glutamyl-transferase (GGT) activity in the model compared to a control,

wherein decreased GGT activity in the model compared to the control is indicative of reduced viability of the renal tubular epithelial cells.

[003d] In a fourth aspect there is provided a method of testing the effect of a therapeutic agent on a renal transport molecule, comprising:

- a. contacting the therapeutic agent with the three-dimensional, engineered, bioprinted, biological renal tubule model according to the first aspect, and
- b. measuring the transport molecule activity in the model compared to a control.

[003e] In a fifth aspect there is provided a method of fabricating a three-dimensional, engineered, biological renal tubule model, the method comprising:

- a. preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells;
- b. preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells;
- c. depositing the renal interstitial bio-ink and the renal epithelial bio-ink such that the renal interstitial tissue bio-ink forms a renal interstitial tissue layer with an apical and basolateral surface, and the renal epithelial bio-ink is deposited in substantially a monolayer in contact with the apical surface of the renal interstitial layer; and
- d. maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form the three-dimensional, engineered, biological renal tubule model,

wherein the renal fibroblasts and endothelial cells are the only cells present in the layer of renal interstitial tissue and are present in a ratio of renal fibroblasts to endothelial cells at which the renal tubule model is planar six days post-depositing.

[003f] In a sixth aspect there is provided a method of assessing the renal toxicity of a therapeutic agent, the method comprising:

- a. preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells;
- b. preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells;
- c. depositing the interstitial bio-ink and the epithelial bio-ink such that the renal interstitial tissue bio-ink forms a renal interstitial tissue layer with an apical and basolateral surface, and the renal epithelial bio-ink is deposited in substantially a monolayer in contact with the apical surface of the renal interstitial tissue layer; and
- d. maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form a three-dimensional, engineered, biological renal tubule model;
- e. contacting a therapeutic agent with the model;

- f. measuring viability or functionality of the renal tubular epithelial cells; and
- g. assessing the renal toxicity of the therapeutic agent based on the measured viability or functionality of the renal tubular epithelial cells;

wherein the renal fibroblasts and endothelial cells are the only cells present in the layer of renal interstitial tissue and are present in a ratio of renal fibroblasts to endothelial cells at which the renal tubule model is planar six days post-depositing.

[004] The engineered tissues described herein represent a model of the tubulointerstitial interface in which human renal interstitial tissue is supporting human renal proximal tubule epithelial cells to facilitate their optimal morphology and function. Creation of a three-dimensional tubulointerstitial interface facilitates the correct localization of drug transporters

and receptors required for metabolism in order to accurately study how small molecule, chemicals, contaminants, or biologics affect the renal proximal tubule. This represents a more physiologically relevant alternative to two-dimensional monolayers of human or canine kidney epithelial cells and serves as an adjunct to, or in some cases, replacement of animal studies in which species difference in renal functions hamper interpretation of results.

[005] The engineered tissues described herein provide an opportunity to accurately study how compounds affect the renal proximal tubule as well as modeling pathogenic processes that involve tubular transport, cell-cell interactions, and the development of tubulointerstitial fibrosis such as may occur in chronic renal disease, polycystic kidney disease, or type II diabetes.

[006] Provided herein is a three-dimensional, engineered, biological renal tubule model comprising: a layer of renal interstitial tissue, the renal interstitial tissue comprising renal fibroblasts and endothelial cells; and a layer of renal epithelial tissue, the renal epithelial tissue comprising renal tubular epithelial cells, the renal epithelial tissue in contact with the layer of renal interstitial tissue to form the three-dimensional, engineered, biological renal tubule model; provided that the interstitial tissue comprises an interstitial bio-ink, the epithelial tissue comprises an epithelial bio-ink, and form a three-dimensional, engineered, biological renal tubule model. In certain embodiments, the layer of renal interstitial tissue possesses an apical and basolateral surface. In certain embodiments, the layer of renal epithelial tissue is in contact with the apical surface of the layer of renal interstitial tissue. In certain embodiments, the layer of renal epithelial tissue consists essentially of renal tubular epithelial cells. In certain embodiments, the layer of renal epithelial tissue consists essentially of primary renal tubular epithelial cells. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with a disease that affects kidney function. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with a polycystic kidney disease. In certain embodiments, the primary renal tubular cells are isolated from a subject with diabetes mellitus type II. In certain embodiments, the layer of renal epithelial tissue comprises renal cell carcinoma cells. In certain embodiments, the layer of renal epithelial tissue is substantially a monolayer. In certain embodiments, the layer of renal interstitial tissue is substantially a monolayer. In certain embodiments, the layer of renal epithelial tissue is in continuous contact with the layer of renal interstitial tissue. In certain embodiments, the layer of renal epithelial tissue is in contact with and covers by 50% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, the layer of renal epithelial tissue is in contact with and covers by 70% or more the apical surface of the layer of renal interstitial tissue. In certain

embodiments, the layer of renal epithelial tissue is in contact with and covers by 90% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, at least 50% of the cells in the layer of renal epithelial tissue form tight junctions with another cell of the renal epithelial tissue layer. In certain embodiments, at least 70% of the cells in the layer of renal epithelial tissue form tight junctions with another cell of the renal epithelial tissue layer. In certain embodiments, at least 90% of the cells in the layer of renal epithelial tissue form tight junctions with another cell of the renal epithelial tissue layer. In certain embodiments, the renal tubule model is between 50 and 500 μm thick. In certain embodiments, the renal tubular model is about 100 μm thick. In certain embodiments, the layer of renal epithelial tissue further comprises an extrusion compound. In certain embodiments, the fibroblasts and endothelial cells are present in the layer of renal interstitial tissue at a ratio of about 95:5 to about 5:95 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the layer of renal interstitial tissue at a ratio of about 75:25 to about 25:75 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the layer of renal interstitial tissue at a ratio of about 60:40 to about 40:60 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the layer of renal interstitial tissue at a ratio of about 50:50 fibroblasts to endothelial cells. In certain embodiments, the layer of renal interstitial tissue further comprises secretory cells. In certain embodiments, the layer of renal interstitial tissue further comprises immune cells. In certain embodiments, the layer of renal interstitial tissue further comprises an extrusion compound. In certain embodiments, the layer of renal interstitial tissue comprises glomerular cells. In certain embodiments, the model is substantially free of pre-formed scaffold at time of use. In certain embodiments, the renal fibroblasts, endothelial cells, and renal tubular epithelial cells are mammalian cells. In certain embodiments, any of the layer of renal interstitial tissue or layer of renal epithelial tissue is at least 30% living cells by volume. In certain embodiments, any of the layer of renal interstitial tissue or layer of renal epithelial tissue is at least 70% living cells by volume. In certain embodiments, any of the layer of renal interstitial tissue or layer of renal epithelial tissue is at least 90% living cells by volume. In certain embodiments, the renal tubule model is substantially planar. In certain embodiments, the renal tubule model is of substantially uniform thickness. In certain embodiments, the renal tubule model comprises at least one component that is bioprinted. In certain embodiments, the renal tubule model comprises at least one component that is bioprinted by extrusion. In certain embodiments, the renal tubule model further comprises a biocompatible membrane. In certain embodiments, the renal tubule model

further comprises a biocompatible membrane with a pore size greater than about 0.4 μm . In certain embodiments, the renal tubule model further comprises a biocompatible membrane with a pore size about 1 μm . In certain embodiments, a plurality of the renal tubule models are configured to form an array. In certain embodiments, a plurality of renal tubule models are configured to allow between about 20 μm and about 100 μm of space between each renal tubule model. In certain embodiments, either the interstitial or the epithelial bio-ink further comprises a therapeutic molecule or substance.

[007] Also provided herein is a method of fabricating a three-dimensional, engineered, biological renal tubule model, the method comprising: preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells; preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells; depositing the renal interstitial bio-ink and the renal epithelial bio-ink such that the renal epithelial bio-ink forms a layer on at least one surface of the layer of renal interstitial bio-ink; and maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form the three-dimensional, engineered, biological renal tubule model. In certain embodiments, depositing the renal interstitial tissue bio-ink forms a renal interstitial tissue layer with an apical and basolateral surface. In certain embodiments, the renal epithelial bio-ink is deposited in contact with the apical surface of the renal interstitial tissue layer. In certain embodiments, the renal epithelial bio-ink consists essentially of renal tubular epithelial cells. In certain embodiments, the renal epithelial bio-ink consists essentially of primary renal tubular epithelial cells. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with a disease that affects kidney function. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with polycystic kidney disease. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with diabetes mellitus type II. In certain embodiments, the renal epithelial bio-ink comprises renal cell carcinoma cells. In certain embodiments, the renal epithelial bio-ink is deposited in a monolayer. In certain embodiments, the renal interstitial tissue bio-ink is deposited in a monolayer. In certain embodiments, the layer of renal epithelial tissue is deposited in continuous contact with the layer of renal interstitial tissue. In certain embodiments, the renal epithelial bio-ink forms a layer that covers by 50% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, the renal epithelial bio-ink forms a layer that covers by 70% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, the renal epithelial bio-ink forms a layer that covers by 90% or more the apical

surface of the layer of renal interstitial tissue. In certain embodiments, at least 50% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells. In certain embodiments, at least 70% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells. In certain embodiments, at least 90% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells. In certain embodiments, the renal tubule model is between 50 and 500 μm thick. In certain embodiments, the renal tubule model is about 100 μm thick. In certain embodiments, the renal epithelial bio-ink further comprises an extrusion compound. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 95:5 to about 5:95 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 75:25 to about 25:75 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 60:40 to about 40:60 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 50:50 fibroblasts to endothelial cells. In certain embodiments, the renal interstitial bio-ink further comprises secretory cells. In certain embodiments, the renal interstitial bio-ink further comprises immune cells. In certain embodiments, the renal interstitial bio-ink further comprises an extrusion compound. In certain embodiments, the renal interstitial bio-ink comprises glomerular cells. In certain embodiments, the model is fabricated substantially free of pre-formed scaffold. In certain embodiments, the renal fibroblasts, endothelial cells, and renal tubular epithelial cells are mammalian cells. In certain embodiments, either of the renal interstitial bio-ink or renal epithelial bio-ink forms a planar layer after deposition. In certain embodiments, the renal tubule model is of substantially uniform thickness. In certain embodiments, the method further comprises depositing the renal interstitial bio-ink onto a biocompatible membrane. In certain embodiments, the method further comprises depositing the renal interstitial bio-ink onto a biocompatible membrane with a pore size greater than 0.4 μm . In certain embodiments, the method further comprises depositing the renal interstitial bio-ink onto a biocompatible membrane with a pore size of about 1 μm . In certain embodiments, the method comprises three-dimensional, engineered, biological renal tubule models that are deposited to form an array. In certain embodiments, the method comprises three-dimensional, engineered, biological renal tubule models that are deposited to form an array configured to allow between about 20 μm and about 100 μm of space between each renal tubule model. In certain embodiments, the renal interstitial bio-ink is at least 30% living cells by

volume. In certain embodiments, the renal interstitial bio-ink is at least 70% living cells by volume. In certain embodiments, the renal interstitial bio-ink is at least 90% living cells by volume. In certain embodiments, the renal interstitial bio-ink is deposited by extrusion bioprinting. In certain embodiments, the renal epithelial bio-ink is deposited by ink-jet bioprinting. In certain embodiments, any layer of the renal tubule model is viable in *in vitro* culture in culture after 3 days. In certain embodiments, any layer of the renal tubule model is viable in *in vitro* culture after 10 days. In certain embodiments, either the interstitial or the epithelial bio-ink further comprises a therapeutic molecule or substance.

[008] Also provided herein is a method of assessing the renal toxicity of a therapeutic agent, the method comprising: preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells; preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells; depositing the interstitial bio-ink and the epithelial bio-ink such that the epithelial bio-ink forms a layer on at least one surface of the interstitial bio-ink; and maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form a three-dimensional, engineered, biological renal tubule model; contacting a therapeutic agent with the construct; measuring viability or functionality of the renal tubular epithelial cells; and assessing the renal toxicity of the therapeutic agent based on the measured viability or functionality of the renal tubular epithelial cells. In certain embodiments, the interstitial bio-ink is deposited by extrusion bioprinting. In certain embodiments, the epithelial bio-ink is deposited by ink-jet bioprinting. In certain embodiments, either the interstitial or the epithelial bio-ink further comprises a therapeutic molecule or substance.

BRIEF DESCRIPTION OF THE DRAWINGS

[009] **Fig. 1** shows a non-limiting example of a schematic concept diagram; in this case, a schematic concept diagram depicting an interstitial layer topped with a polarized epithelial monolayer.

[010] **Figs. 2A** and **2B** show a non-limiting example of a schematic structure diagram; in this case, a schematic structure diagram depicting the architecture of **Structure 1 of Example 1** (**Fig. 2A**); and a non-limiting example of a bioprinted renal tubule model 48 hours after the tissue has fused (**Fig. 2B**).

[011] **Figs. 3A** and **3B** show a non-limiting example of a photomicrograph depicting **Structure 1 of Example 1**; in this case, H&E staining (**Fig. 3A**: 100x total magnification; **Fig. 3B**: 200x

total magnification).

[012] **Fig. 4** shows a non-limiting example of a photomicrograph depicting **Structure 1** of **Example 1**; in this case, staining with antibodies against CD31 (**410**), and TE7 (**420**) showing HUVEC networks (**430**).

[013] **Fig. 5** shows a non-limiting example of a photomicrograph at low magnification depicting **Structure 1** of **Example 1**; staining is with antibodies against E-cadherin (**540**) surrounding tubule structures; and TE7 (**520**).

[014] **Figs. 6A** and **6B** show a non-limiting example of a schematic structure diagram; in this case, a schematic structure diagram depicting the architecture of **Structure 2** of **Example 2**.

[015] **Figs. 7A** and **7B** show non-limiting examples of photomicrographs depicting **Structure 2** of **Example 2**; in this case, H&E staining (black arrow indicates epithelial cells).

[016] **Figs. 8A** and **8B** show non-limiting examples of photomicrographs depicting **Structure 2** of **Example 2**; in this case, a photomicrograph demonstrating organization of endothelial cells (**Fig. 8A**). **810**, CD31 staining for HUVECS; and **820**, TE7 staining for fibroblasts is shown. In this example, epithelial cells are detected on the surface of the tissue by E-cadherin staining (**Fig. 8B, 840**).

[017] **Fig. 9** shows photomicrographs of constructs from **Example 3** and depicts renal proximal tubule epithelial cells (RPTECs) in 3D renal tissues that exhibit features of polarization. E-cadherin (**940 and bright staining**) is observed at the lateral membranes between RPTECs, corresponding to localization at tight junctions. Also shown is staining with TE7 **920** for fibroblasts. Shown are low (**Fig. 9A**), medium (**Fig. 9B**), and high (**Fig. 9C**) magnification.

[018] **Figs. 10A** and **10B** show H & E (**Fig. 10A**) and trichrome (**Fig. 10B**) staining of bioprinted renal tissue constructs from **Example 3**.

[019] **Figs. 11A** and **11B** show the constructs from **Fig 11** with brush borders (**Fig. 11A**, arrows), and collagen deposition (**Fig. 11B**, arrows) highlighted.

[020] **Fig. 12** shows extensive endothelial cell networks are observed in 3D bioprinted renal tissue constructs from **Example 3**. Staining for CD31, (endothelial cells, bright staining) and TE7 (fibroblasts, **1220**) are shown. Networks with putative lumens lined with endothelial cells are marked with (*).

[021] **Fig. 13** shows GGT activity in 3D bioprinted renal tissue from **Example 3** compared to 2D hTERT-RPTEC and interstitial cells alone. **Fig. 13** shows sustained viability of 3D renal tubule models in culture in comparison to 2D co-culture.

[022] **Figs. 14A, 14B, and 14C** show macroscopic views of renal tubule models bioprinted with interstitial layers comprising different ratios of fibroblasts to endothelial cells. **Fig. 14A** shows a ratio of 90:10 fibroblasts to endothelial cells. **Fig. 14B** shows a ratio of 75:25 fibroblasts to endothelial cells. **Fig. 14C** shows a ratio of 50:50 fibroblasts to endothelial cells.

[023] **Figs. 15A, 15B, and 15C** show histology of renal tubule models bioprinted with interstitial layers comprising different ratios of fibroblasts to endothelial cells. **Fig. 15A** shows a ratio of 90:10 fibroblasts to endothelial cells. **Fig. 15B** shows a ratio of 75:25 fibroblasts to endothelial cells. **Fig. 15C** shows a ratio of 50:50 fibroblasts to endothelial cells.

[024] **Figs. 16A and 16B** show surface area and thickness uniformity data for a bioprinted renal tubule model. **Fig. 16A** shows a macroscopic view of a bioprinted renal tubule model. **Fig. 16B** shows Keyence surface mapping data.

[025] **Figs. 17A, 17B, 17C, and 17D** show histology of renal tubule models bioprinted with different amounts of endothelial cells and differing serum concentrations. **Fig. 17A** shows epithelial cells at a concentration of 1.25×10^5 cells/well in serum free media. **Fig. 17B** shows epithelial cells at a concentration of 1.25×10^5 cells/well in 2% serum. **Fig. 17C** shows epithelial cells at a concentration of 2.5×10^5 cells/well in 2% serum. **Fig. 17D** shows epithelial cells at a concentration of 5.0×10^5 cells/well in 2% serum.

[026] **Fig. 18** shows a comparison of a toxicity test using amphotericin B comparing the response of 3D renal tubule model with that of a standard 2D co-culture model.

[027] **Figs. 19A and 19B** show examples of amphotericin B (AmpB) toxicity tests using a 3D renal tubule model. **Fig. 19A** shows a lactate dehydrogenase (LDH) release assay. **Fig. 19B** shows γ -glutamyl-transferase (GGT) activity.

[028] **Figs. 20A, 20B, and 20C** depict histology corresponding to **Fig. 19**, from 3D renal tubule models treated with vehicle (**Fig. 20A**), $10 \mu\text{M}$ AmpB (**Fig. 20B**), or $50 \mu\text{M}$ AmpB (**Fig. 20C**). Arrows indicate epithelial layer.

[029] **Figs. 21A, 21B, and 21C** show examples of cisplatin toxicity tests using a 3D renal tubule model. **Fig. 21A** shows alamarBlue assay for viability. **Fig. 21B** shows γ -glutamyl-transferase (GGT) activity. **Fig. 21C** shows a lactate dehydrogenase (LDH) release assay.

[030] **Figs. 22A, 22B, 22C, and 22D** shows Masson's trichrome stained histology corresponding to **Fig. 21**, from renal tubule models treated with vehicle (**Fig. 22A**), 10 μ M cisplatin (**Fig. 22B**), 50 μ M cisplatin (**Fig. 22C**), and 50ng/mL TGF β (**Fig. 22D**).

[031] **Figs. 23A, 23B, and 23C** show examples of cisplatin toxicity tests using a 3D renal tubule model comparing primary and immortalized endothelial cells. **Fig. 23A** shows alamarBlue assay for viability. **Fig. 23B** shows γ -glutamyl-transferase (GGT) activity. **Fig. 23C** shows a lactate dehydrogenase (LDH) release assay.

[032] **Figs. 24A and 24B** show the effect that pore size has on bioprinted interstitial tissue. **Fig. 24A** shows interstitial tissue printed on a pore size of 0.4 μ m. **Fig. 24B** shows interstitial tissue printed on a pore size of 1.0 μ m.

[033] **Figs. 25A, 25B, and 25C** show long-term viability of 3D renal tubule models in culture by H & E staining of histology. **Fig. 25A** shows renal tubule models after 6 days in culture. **Fig. 25B** shows renal tubule models after 10 days in culture. **Fig. 25C** shows renal tubule models after 27 days in culture.

DETAILED DESCRIPTION OF THE INVENTION

[034] Provided herein is a three-dimensional, engineered, biological renal tubule model comprising: a layer of renal interstitial tissue, the renal interstitial tissue comprising renal fibroblasts and endothelial cells; and a layer of renal epithelial tissue, the renal epithelial tissue comprising renal tubular epithelial cells, the renal epithelial tissue in contact with the layer of renal interstitial tissue to form the three-dimensional, engineered, biological renal tubule model; provided that the interstitial tissue comprises an interstitial bio-ink, the epithelial tissue comprises an epithelial bio-ink, and form a three-dimensional, engineered, biological renal tubule model.

[035] Also provided herein is a method of fabricating a three-dimensional, engineered, biological renal tubule model, the method comprising: preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells; preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells; depositing the renal interstitial bio-ink and the renal epithelial bio-ink such that the renal epithelial bio-ink forms a layer on at least one surface of the layer of renal interstitial bio-ink; and maturing the deposited bio-ink in a cell

culture media to allow the cells to cohere to form the three-dimensional, engineered, biological renal tubule model.

[036] Also provided herein is a method of assessing the renal toxicity of a therapeutic agent, the method comprising: preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells; preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells; depositing the interstitial bio-ink and the epithelial bio-ink such that the epithelial bio-ink forms a layer on at least one surface of the interstitial bio-ink; and maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form a three-dimensional, engineered, biological renal tubule model; contacting a therapeutic agent with the construct; measuring viability or functionality of the renal tubular epithelial cells; and assessing the renal toxicity of the therapeutic agent based on the measured viability or functionality of the renal tubular epithelial cells.

Certain definitions

[037] Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. Any reference to “or” herein is intended to encompass “and/or” unless otherwise stated.

[038] As used herein, “array” means a scientific tool including an association of multiple elements spatially arranged to allow a plurality of tests to be performed on a sample, one or more tests to be performed on a plurality of samples, or both.

[039] As used herein, “assay” means a procedure for testing or measuring the presence or activity of a substance (e.g., a chemical, molecule, biochemical, protein, hormone, or drug, etc.) in an organic or biologic sample (e.g., cell aggregate, tissue, organ, organism, etc.).

[040] As used herein, “layer” means an association of cells in X and Y planes that is one or multiple cells thick. In some embodiments, the renal tubules described herein include one layer. In other embodiments, the renal tubules described herein include a plurality of layers. In various embodiments, a layer forms a contiguous, substantially contiguous, or non-contiguous sheet of cells. In some embodiments, each layer of renal tubule described herein comprises multiple cells in the X, Y, and Z axes.

[041] As used herein, “tissue” means an aggregate of cells.

[042] As used herein, “bio-ink” means a liquid, semi-solid, or solid composition for use in bioprinting. In some embodiments, bio-ink comprises cell solutions, cell aggregates, cell-comprising gels, multicellular bodies, or tissues. In some embodiments, the bio-ink additionally comprises non-cellular materials that provide specific biomechanical properties that enable bioprinting. In some embodiments, the bio-ink comprises an extrusion compound. In some cases, the extrusion compound is engineered to be removed after the bioprinting process. In other embodiments, at least some portion of the extrusion compound remains entrained with the cells post-printing and is not removed. An interstitial bio-ink comprises at least one cell of interstitial origin such as a fibroblast, mesenchymal cell, or pluripotent cells induced to have interstitial characteristics. An epithelial bio-ink comprises at least one epithelial cell type including cells of the proximal tubule.

[043] As used herein, “bioprinting” means utilizing three-dimensional, precise deposition of cells (e.g., cell solutions, cell-containing gels, cell suspensions, cell concentrations, multicellular aggregates, multicellular bodies, etc.) via methodology that is compatible with an automated or semi-automated, computer-aided, three-dimensional prototyping device (e.g., a bioprinter). Suitable bioprinters include the Novogen Bioprinter[®] from Organovo, Inc. (San Diego, CA).

[044] As used herein, “scaffold” refers to synthetic scaffolds such as polymer scaffolds and porous hydrogels, non-synthetic scaffolds such as pre-formed extracellular matrix layers, dead cell layers, and decellularized tissues, and any other type of pre-formed scaffold that is integral to the physical structure of the engineered tissue and not able to be removed from the tissue without damage/destruction of said tissue. In further embodiments, decellularized tissue scaffolds include decellularized native tissues or decellularized cellular material generated by cultured cells in any manner; for example, cell layers that are allowed to die or are decellularized, leaving behind the ECM they produced while living. The term “scaffoldless,” therefore, is intended to imply that pre-formed scaffold is not an integral part of the engineered tissue at the time of use, either having been removed or remaining as an inert component of the engineered tissue. “Scaffoldless” is used interchangeably with “scaffold-free” and “free of pre-formed scaffold.”

[045] As used herein a “subject” is an organism of any mammalian species including but not limited to humans, primates, apes, monkey, dogs, cats, mice, rats, rabbits, pigs, horses and others. A subject can be any mammalian species alive or dead. Subject includes recently deceased subjects or biopsy samples taken from a living subject.

[046] As used herein “therapeutic substance” means any molecule, biologic, compound or composition that is approved to treat a disease, under investigation to treat a disease, or that elicits a biological response such as changes in DNA, RNA, peptide, polypeptide or protein.

[047] As used herein “viability” means that at least 50%, 60%, 70%, 80%, 90%, 95%, 97% or more of cells in a bio-ink or tissue layer are live as determined by at least one test of viability. Tests for viability are known in the art, and include, but are not limited to vital dyes, staining for apoptotic markers, TUNEL staining, DNA fragmentation analysis, AlamarBlue staining, functional determinations and the like.

Composition of the renal tubule model

[048] In some embodiments, the cells within the tissues are organized spatially to recapitulate the laminar architecture of the tubule-interstitial tissue interface; a polarized tubular epithelium is present on top of a layer of renal interstitial tissue that includes an endothelial cell-based microvascular network. Specialized cells, such as EPO-producing cells, are optionally included within the peritubular spaces. In some embodiments, the epithelium possesses or generates brush borders.

[049] In particular, non-limiting embodiments, the engineered renal tissues described herein comprise two major parts: 1) an interstitial layer composed of adult renal fibroblasts and human umbilical vein endothelial cells (HUVEC); and 2) a polarized epithelial monolayer composed of either normal human renal proximal tubule epithelial cells (RPTEC), Madin-Darby canine kidney cells (MDCK), rat primary RPTEC cells, and/or immortalized RPTEC cells, wherein immortalization is optionally achieved through genetic manipulation of hTERT to form hTERT-immortalized RPTEC cells. The cells are deposited using the Novogen MMX Bioprinter in such a way that the epithelial layer is apical to the interstitial layer (*see Fig. 1*). Structures are created by spatially-controlled deposition of cells mixed with a thermo-responsive hydrogel that degrades over time (Novogel® 2.0) combined with deposition of aerosolized cellular materials by compressed gas propulsion (inkjet spray).

[050] Referring to **Fig. 1**, in a particular embodiment, a three-dimensional, engineered renal tubule model comprises an interstitial layer and an epithelial layer. In this embodiment, the two layers together model the wall of a renal distal tubule. This configuration is critical for modeling *in vivo* tissues and predicting native tissue responses. Response of the epithelial layer is predictive of native tissue response to drugs, chemicals, or biological agents, and may provide information relative to toxicity or efficacy. The interstitial layer is critical for proper functioning

of the epithelium and serves as a model for native tissue fibrosis, in particular renal tubulointerstitial fibrosis.

[051] Referring to **Fig. 2A**, in a particular embodiment, an interstitial layer is bioprinted, using continuous deposition techniques. In this embodiment, a sheet of interstitium is bioprinted followed by a border of interstitium to form a three sided container. A layer of epithelium is then bioprinted, again using continuous deposition techniques, into the container of interstitium. This embodiment provides the necessary contact between the interstitial and epithelial layers. **Fig. 2B**, depicts an actual bioprinted renal tubule model, shown 48 hours after the tissue has coalesced. In some embodiments, the tissue coalesces within 4, 6, 8, 12 or 24 hours.

[052] Referring to **Figs. 6A** and **6B**, in a particular embodiment, an interstitial layer is bioprinted, using continuous deposition techniques. In this embodiment, an epithelial layer is bioprinted, using ink-jet deposition techniques onto the interstitial layer. A substantially contiguous layer of epithelium is consistent with *in vivo* tissues and is critical to replicate a physiologically relevant architecture. Ink-jet deposition techniques provide the ability to deposit one or more thin layers of epithelial cells onto the potentially irregular surface of the interstitial layer. In such embodiments, ink-jet deposition of the epithelial layer is optionally performed immediately after bioprinting of the interstitial layer or after the interstitial layer has been allowed to mature.

[053] In some embodiments, the cells are bioprinted. In further embodiments, the bioprinted cells are cohered to form the engineered renal tubule models. In still further embodiments, the engineered renal tubule models are free or substantially free of pre-formed scaffold at the time of fabrication or the time of use. In some cases, bioprinting allows fabrication of tissues that mimic the appropriate cellularity of native tissue.

[054] In some embodiments, the three-dimensional, engineered renal tubule models described herein are distinguished from tissues fabricated by prior technologies by virtue of the fact that they are three-dimensional, free of pre-formed scaffolds, consist essentially of cells, and/or have a high cell density (e.g., greater than 30% cellular, greater than 40% cellular, greater than 50% cellular, greater than 60% cellular, greater than 70% cellular, greater than 80% cellular, greater than 90% cellular, or greater than 95% cellular).

[055] In some embodiments, the three-dimensional, engineered renal tubule models described herein are distinguished from native (e.g., non-engineered) tissues by virtue of the fact that they are non-innervated (e.g., substantially free of nervous tissue), substantially free of mature

vasculature, and/or substantially free of blood components. For example, in various embodiments, the three-dimensional, engineered renal tubule models are free of plasma, red blood cells, platelets, and the like and/or endogenously-generated plasma, red blood cells, platelets, and the like. In certain embodiments, the engineered renal tubule model lacks immune cells such as T cell, B cells, macrophages, dendritic cells, basophils, mast cells or eosinophils. In some embodiments, the model is not tubular in shape like a naturally occurring renal proximal tubule, but is planar or sheet-like, this advantageously allows for *in vitro* assays and analysis. In some embodiments, the fibroblasts are not of renal origin. In some embodiments, the endothelial cells are not of renal origin. In some embodiments, the epithelial cells are not of human origin. In certain embodiments, the engineered renal tubule model lacks undifferentiated cells. In certain embodiments, the engineered renal tubule model lacks undifferentiated renal cells. In some embodiments, the three-dimensional, engineered renal tubule models described herein are distinguished from native renal tubule tissues in that they are flat or substantially planar. In certain embodiments, the three-dimensional, engineered renal tubule models described herein possess functional improvements over native renal tubule tissues; one example is high viability after a sustained amount of time in culture up to at least 7, 10 or 27 days in culture. In some embodiments, the cells used in the renal tubule model are transformed or immortalized. In some embodiments, the cells used in the renal tubule model are transgenic and contain protein fusions with fluorescent proteins, like EGFP, GFP, RFP, YFP, or CFP. In some embodiments, the cells used in the renal tubule model are transgenic and contain reporter constructs with fluorescent proteins; like EGFP, GFP, RFP, YFP, GFP; or luminescent proteins like firefly or renilla luciferase. In certain embodiments, any of the cells contain a deletion or insertion of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 genes or more. In some embodiments, the 3D renal tubule models are chimeras, wherein at least one cell is from a different mammalian species than any other cell of the 3D renal tubule model. In some embodiments, the 3D renal tubule models are chimeras, wherein at least one cell is from a different human donor than any other cell of the 3D renal tubule model.

Cellular inputs

[056] In some embodiments, the engineered tissues, arrays, and methods described herein include a plurality of cell types. In some embodiments, the renal tubule models comprise a layer of interstitial tissue comprising mammalian fibroblasts and mammalian endothelial cells. In various embodiments, suitable endothelial cells are derived from human umbilical vein (HUVEC), human primary, human kidney, or from directed differentiation of induced pluripotent stem cells (iPS) or human embryonic stem cells (hES). In some embodiments, the

fibroblasts are renal interstitial fibroblasts. In various embodiments, suitable renal interstitial fibroblasts are derived from primary cells isolated from human kidney. In some embodiments, the fibroblasts are dermal or vascular in origin. In some embodiments, one or more of the cellular components are derived from a non-human mammal. In some embodiments, the interstitial tissue comprises tumor cells or cancer cells. In some embodiments, the layer of interstitial tissue is substantially a monolayer. In some embodiments, the layer of interstitial tissue comprises a monolayer over 95% of its surface area. In some embodiments, the layer of interstitial tissue comprises a monolayer over 90% of its surface area. In some embodiments, the layer of interstitial tissue comprises a monolayer over 80% of its surface area. In some embodiments, the layer of interstitial tissue is greater than 1 cell thick. In some embodiments, the layer of interstitial tissue is greater than 2 cells thick. In some embodiments, the layer of interstitial tissue is greater than 3 cells thick. In some embodiments, the layer of interstitial tissue is greater than 4 cells thick. In some embodiments, the layer of interstitial tissue is greater than 5 cells thick. In some embodiments, the layer of interstitial tissue is greater than 10 cells thick. In some embodiments, the layer of interstitial tissue is greater than 20 cells thick. In some embodiments, the layer of interstitial tissue is greater than 50 cells thick. In some embodiments, the layer of interstitial tissue is greater than 100 cells thick. In some embodiments, the layer of interstitial tissue is greater than 20 μm thick. In some embodiments, the layer of interstitial tissue is greater than 30 μm thick. In some embodiments, the layer of interstitial tissue is greater than 40 μm thick. In some embodiments, the layer of interstitial tissue is greater than 50 μm thick. In some embodiments, the layer of interstitial tissue is greater than 100 μm thick. In some embodiments, the layer of interstitial tissue is greater than 200 μm thick. In some embodiments, the layer of interstitial tissue is greater than 500 μm thick. In some embodiments, the layer of interstitial tissue is greater than 600 μm thick. In some embodiments, the layer of interstitial tissue is greater than 1000 μm thick. In some embodiments, the layer of interstitial tissue is less than 20 μm thick. In some embodiments, the layer of interstitial tissue is less than 30 μm thick. In some embodiments, the layer of interstitial tissue is less than 40 μm thick. In some embodiments, the layer of interstitial tissue is less than 50 μm thick. In some embodiments, the layer of interstitial tissue is less than 100 μm thick. In some embodiments, the layer of interstitial tissue is less than 200 μm thick. In some embodiments, the layer of interstitial tissue is less than 500 μm thick. In some embodiments, the layer of interstitial tissue is less than 600 μm thick. In some embodiments, the layer of interstitial tissue is less than 1000 μm thick.

[057] In some embodiments, the renal tubule models comprise a layer of epithelial tissue

comprising mammalian epithelial cells. In further embodiments, the epithelial cells are renal tubular epithelial cells (e.g., proximal tubule epithelial cells). In still further embodiments, suitable renal tubular epithelial cells are primary isolates or cells derived from the directed differentiation of stem cells (induced pluripotent stem cell (iPS)-derived and/or human embryonic stem cell (hES)-derived). In some embodiments, the renal tubular epithelial cells are Madin-Darby canine kidney (MDCK) cells. In some embodiments, the renal tubular epithelial cells are immortalized human cells. In other embodiments, the renal tubular epithelial cells are immortalized cells such as hTERT-RPTEC cells, HK-2 cells, LLC-PK1 cells, or OK cells. In some embodiments, the epithelial cells are derived from a non-human mammal such as, for example, rat, mouse, pig, or primate. In some embodiments, the layer of epithelial tissue consists essentially of renal tubule epithelial cells. In some embodiments, the layer of epithelial tissue consists essentially of primary renal tubule epithelial cells. In some embodiments, the layer of epithelial tissue consists essentially of renal proximal tubule epithelial cells. In some embodiments, the layer of epithelial tissue consists essentially of primary renal proximal tubule epithelial cells. In some embodiments, the layer of epithelial tissue is substantially a monolayer. In some embodiments, the layer of epithelial tissue comprises tumor cells. In some embodiments, the layer of epithelial tissue comprises renal cell carcinoma cells. In some embodiments, the layer of epithelial tissue comprises a monolayer over 95% of its surface area. In some embodiments, the layer of epithelial tissue comprises a monolayer over 90% of its surface area. In some embodiments, the layer of epithelial tissue comprises a monolayer over 80% of its surface area. In some embodiments, the layer of epithelial tissue is greater than 1 cell thick. In some embodiments, the layer of epithelial tissue is greater than 2 cells thick. In some embodiments, the layer of epithelial tissue is greater than 3 cells thick. In some embodiments, the layer of epithelial tissue is greater than 4 cells thick. In some embodiments, the layer of epithelial tissue is greater than 5 cells thick. In some embodiments, the layer of epithelial tissue is greater than 10 cells thick. In some embodiments, the layer of epithelial tissue is greater than 20 cells thick. In some embodiments, the layer of epithelial tissue is greater than 50 cells thick. In some embodiments, the layer of epithelial tissue is greater than 100 cells thick. In some embodiments, the layer of epithelial tissue is greater than 20 μm thick. In some embodiments, the layer of epithelial tissue is greater than 30 μm thick. In some embodiments, the layer of epithelial tissue is greater than 40 μm thick. In some embodiments, the layer of epithelial tissue is greater than 50 μm thick. In some embodiments, the layer of epithelial tissue is greater than 100 μm thick. In some embodiments, the layer of epithelial tissue is greater than 200 μm thick.

In some embodiments, the layer of epithelial tissue is greater than 500 μm thick. In some embodiments, the layer of interstitial tissue is greater than 600 μm thick. In some embodiments, the layer of epithelial tissue is greater than 1000 μm thick. In some embodiments, the layer of epithelial tissue is less than 1000 μm thick. In some embodiments, the layer of interstitial tissue is less than 600 μm thick. In some embodiments, the layer of epithelial tissue is less than 500 μm thick. In some embodiments, the layer of epithelial tissue is less than 200 μm thick. In some embodiments, the layer of epithelial tissue is less than 100 μm thick. In some embodiments, the layer of epithelial tissue is less than 50 μm thick. In some embodiments, the layer of epithelial tissue is less than 40 μm thick. In some embodiments, the layer of epithelial tissue is less than 30 μm thick. In some embodiments, the layer of epithelial tissue is less than 20 μm thick.

[058] Optionally, the renal tubule models comprise other cell types (e.g., EPO-producing cells, immune cells, etc.). In some embodiments, the immune cells are T cells. In some embodiments, the immune cells are B cells. In some embodiments, the immune cells are NK cells. In some embodiments, the immune cells are dendritic cells. In some embodiments, the immune cells are macrophage cells.

[059] A wide range of cell ratios are suitable. In some embodiments, the epithelial layer comprises, consists of, or consists essentially of proximal tubule epithelial cells. In some embodiments, the interstitial layer comprises, consists of, or consists essentially of fibroblasts and endothelial cells in specific ratios. Suitable proportions of fibroblasts include, by way of non-limiting examples, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, and 95% fibroblasts, including increments therein. Suitable proportions of endothelial cells include, by way of non-limiting examples, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, and 95% endothelial cells, including increments therein. In certain embodiments, the interstitial layer comprises, consists essentially of, or consists of a specified ratio of fibroblast to endothelial cells. In certain embodiments, the ratio of fibroblast to endothelial cells is at least 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:65, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15, 90:10 or 95:5, including increments therein. In certain embodiments, the ratio of fibroblast to endothelial cells is no more than 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:65, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15, 90:10 or 95:5, including increments therein. In certain embodiments, the ratio of fibroblast to endothelial cells is about 50:50. In certain embodiments, the ratio of fibroblast to endothelial cells is from about 60:40 to about 40:60.

[060] A wide range of cell concentrations are suitable for bio-inks. Bio-inks are suitably prepared for continuous deposition bioprinting techniques with concentrations of cells including, by way of non-limiting examples, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, or more, million cells per milliliter of bio-ink. In a particular embodiment, bio-ink prepared for continuous deposition bioprinting comprises about 100-200 million cells/mL. Bio-inks are suitably prepared for ink-jet deposition bioprinting techniques with concentrations of cells including, by way of non-limiting examples, about 0.25, 0.5, 1, 2, 3, 5, 10, 15 or more, million cells per milliliter of bio-ink. In a particular embodiment, bio-ink prepared for ink-jet deposition bioprinting comprises about 1-5 million cells/mL. In a particular embodiment, bio-ink prepared for ink-jet deposition bioprinting comprises about 1-4 million cells/mL. In a particular embodiment, bio-ink prepared for ink-jet deposition bioprinting comprises about 1-3 million cells/mL. In a particular embodiment, bio-ink prepared for ink-jet deposition bioprinting comprises about 1-2 million cells/mL.

[061] In certain embodiments, the renal interstitial bio-ink comprises between 50 million and 1 billion cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 50 million and 900 million cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 50 million and 800 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 50 million and 700 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 50 million and 600 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 50 million and 500 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 50 million and 400 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 50 million and 300 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 50 million and 200 million cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 75 million and 600 million cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 100 million and 600 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 100 million and 500 million cells per milliliter. In certain embodiments, the renal interstitial bio ink comprises between 100 million and 400 million cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 100 million and 300 million cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 100 million and 200 million cells per milliliter. In certain embodiments, the renal interstitial bio-ink comprises between 100

million and 150 million cells per milliliter.

[062] In certain embodiments, the renal epithelial bio-ink comprises between. 0.25 million and 5 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.25 million and 4 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.25 million and 3 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.25 million and 2 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.25 million and 1 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.5 million and 5 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.5 million and 4 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.5 million and 3 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.5 million and 2 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between. 0.5 million and 1 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between 1 million and 5 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between 1 million and 4 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between 1 million and 3 million cells per milliliter. In certain embodiments, the renal epithelial bio-ink comprises between 1 million and 2 million cells per milliliter.

[063] In certain embodiments, the density of the epithelial bio-ink is less than the density of the interstitial bio-ink. In certain embodiments, the ratio of the density of the interstitial bio-ink to the density of the epithelial bio-ink is about 300:1; about 275:1; about 250:1; about 225:1; about 200:1; about 175:1; about 150:1, about 125:1; about 100:1, about 75:1 or about 50:1. In certain embodiments, the ratio of the density of the interstitial bio-ink to the density of the epithelial bio-ink ranges from about 300:1 to about 50:1. In certain embodiments, the ratio of the density of the interstitial bio-ink to the density of the epithelial bio-ink ranges from about 250:1 to about 75:1. In certain embodiments, the ratio of the density of the interstitial bio-ink to the density of the epithelial bio-ink ranges from about 200:1 to about 75:1. In certain embodiments, the ratio of the density of the interstitial bio-ink to the density of the epithelial bio-ink ranges from about 150:1 to about 75:1. In certain embodiments, the ratio of the density of the interstitial bio-ink to the density of the epithelial bio-ink ranges from about 125:1 to about 75:1.

[064] In certain embodiments, the bio-ink is a viscous liquid. In certain embodiments, the bio-

ink is a semi-solid. In certain embodiments, the bio-ink is a solid. In certain embodiments, the viscosity of the bio-ink is greater than 100 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 200 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 500 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 1,000 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 2,000 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 5,000 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 10,000 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 20,000 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 50,000 centipoise. In certain embodiments, the viscosity of the bio-ink is greater than 100,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 100 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 200 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 500 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 1,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 2,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 5,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 10,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 20,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 50,000 centipoise. In certain embodiments, the viscosity of the bio-ink is less than 100,000 centipoise.

[065] In some embodiments, the mean thickness of the renal tubule model is at least 50 μm . In some embodiments, the mean thickness of the renal tubule model is at least 100 μm . In some embodiments, the mean thickness of the renal tubule model is at least 200 μm . In some embodiments, the mean thickness of the renal tubule model is at least 300 μm . In some embodiments, the mean thickness of the renal tubule model is at least 400 μm . In some embodiments, the mean thickness of the renal tubule model is at least 500 μm . In some embodiments, the mean thickness of the renal tubule model is at least 600 μm . In some embodiments, the mean thickness of the renal tubule model is at least 700 μm . In some embodiments, the mean thickness of the renal tubule model is at least 800 μm . In some embodiments, the mean thickness of the renal tubule model is at least 900 μm . In some embodiments, the mean thickness of the renal tubule model is at least 1000 μm . In some embodiments, the mean thickness of the renal tubule model is between 50 μm and 1000 μm . In some embodiments, the mean thickness of the renal tubule model is between 75 μm and 1000 μm . In some embodiments, the mean thickness of the renal tubule model is between 100 μm and

1000 μm . In some embodiments, the mean thickness of the renal tubule model is between 200 μm and 1000 μm . In some embodiments, the mean thickness of the renal tubule model is between 500 μm and 1000 μm . In some embodiments, the mean thickness of the renal tubule model is between 50 μm and 500 μm . In some embodiments, the mean thickness of the renal tubule model is between 50 μm and 300 μm . In some embodiments, the mean thickness of the renal tubule model is between 50 μm and 200 μm . In some embodiments, the mean thickness of the renal tubule model is between 50 μm and 150 μm . In some embodiments, the mean thickness of the renal tubule model is between 50 μm and 125 μm . In some embodiments, the mean thickness of the renal tubule model is between 75 μm and 100 μm .

[066] In some embodiments, the surface area of the renal tubule model is at least 0.01 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.02 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.03 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.04 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.05 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.06 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.07 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.08 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.09 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.10 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.11 cm^2 . In some embodiments, the surface area of the renal tubule model is at least 0.12 cm^2 . In some embodiments, the surface area of the renal tubule model is less than 0.5 cm^2 . In some embodiments, the surface area of the renal tubule model is less than 0.4 cm^2 . In some embodiments, the surface area of the renal tubule model is less than 0.3 cm^2 . In some embodiments, the surface area of the renal tubule model is less than 0.2 cm^2 . In some embodiments, the surface area of the renal tubule model is less than 0.1 cm^2 .

Architectural features of the renal tubule model

[067] The renal tubule models of the present disclosure can be architecturally arranged in many configurations. In certain embodiments, the epithelial tissue and interstitial tissue layers are separate architecturally distinct layers that are in direct contact or separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 μm or more, including increments therein. In certain embodiments, the separation is due to the secretion and deposition of extracellular matrix between the two layers,

which for the purposes of this disclosure is considered contact. In normal physiological tissue cells and cell layers are polarized to have an apical (lumen facing) surface and a basolateral surface, which faces other cells or tissue matrix. For the purposes of the renal tubule models disclosed herein the basolateral surface refers to a surface that faces another cell, an extracellular matrix or the surface of a biocompatible membrane or culture vessel. For the purposes of the renal tubule models disclosed herein the apical surface refers to a surface that faces away from the surface of a biocompatible membrane or culture vessel. In certain embodiments, the basolateral surface of the interstitial tissue layer is the surface attached to a biocompatible membrane or culture vessel; and the apical surface of the interstitial tissue layer is the surface not attached to a biocompatible membrane or culture vessel. In certain embodiments, the epithelial tissue layer is deposited onto and forms a layer on the apical surface of the interstitial tissue layer, thus forming two architecturally distinct layers. In certain embodiments, the epithelial tissue and interstitial tissue layers are in continuous contact. In certain embodiments, at least 99% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, at least 95% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, at least 90% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, at least 80% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, at least 70% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, at least 60% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, at least 50% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, less than 99% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, less than 98% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, less than 97% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, less than 95% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, less than 90% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, less than 80% of the epithelial tissue layer is in continuous contact with the interstitial tissue layer. In certain embodiments, the epithelial tissue layer completely covers the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at least 99% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at

least 95% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at least 90% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at least 80% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at least 70% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at least 60% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers at least 50% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 99% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 98% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 97% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 95% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 90% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 80% of the apical surface of the interstitial tissue layer. In certain embodiments, the epithelial tissue layer covers less than 70% of the apical surface of the interstitial tissue layer.

Architecture of the epithelial tissue layer

[068] Normally an epithelial tissue cell forms tight junctions with neighboring cells. The tight junctions are marked by the transmembrane protein family the cadherins. One of these, E-cadherin, is especially prominent at tight junctions in renal tissue, and marks their formation. In certain embodiments, the epithelial tissue layer consists of cells that form tight junctions. In certain embodiments, substantially all cells in the epithelial tissue layer form a tight junction with at least one neighboring cell. In certain embodiments, at least 99% of cells in the epithelial tissue layer form a tight junction with at least one other cell. In certain embodiments, at least 95% of cells in the epithelial tissue layer form a tight junction with at least one other cell. In certain embodiments, at least 90% of cells in the epithelial tissue layer form a tight junction with at least one other cell. In certain embodiments, at least 80% of cells in the epithelial tissue layer form a tight junction with at least one other cell. In certain embodiments, at least 70% of cells in the epithelial tissue layer form a tight junction with at least one other cell. In certain embodiments, at least 60% of cells in the epithelial tissue layer form a tight junction with at least one other cell. In certain embodiments, at least 50% of cells in the epithelial tissue layer form a tight junction with at least one other cell.

Viability and density of the cell layers

[069] An advantage of bioprinting by the methods of this disclosure is that cells can be printed at high density and high viability. In certain embodiments, the density of the interstitial cell layer is at least 1×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 5×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 10×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 20×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 50×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 100×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 200×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is at least 500×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is between about 100×10^6 cells per mL and about 900×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is between about 100×10^6 cells per mL and about 700×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is between about 100×10^6 cells per mL and about 600×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is between about 100×10^6 cells per mL and about 500×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is between about 100×10^6 cells per mL and about 300×10^6 cells per mL. In certain embodiments, the density of the interstitial cell layer is between about 100×10^6 cells per mL and about 200×10^6 cells per mL. In certain embodiments, the viability of the interstitial tissue layer is greater than 99% living cells by volume. In certain embodiments, the viability of the interstitial tissue layer is greater than 95% living cells by volume. In certain embodiments, the viability of the interstitial tissue layer is greater than 90% living cells by volume. In certain embodiments, the viability of the interstitial tissue layer is greater than 80% living cells by volume. In certain embodiments, the viability of the interstitial tissue layer is greater than 70% living cells by volume. In certain embodiments, the viability of the interstitial tissue layer is greater than 60% living cells by volume. In certain embodiments, the viability of the interstitial tissue layer is greater than 50% living cells by volume. In certain embodiments, this viability is maintained for at least 8, 12, 24, 48, 72, 96, or more hours post printing. In certain embodiments, this viability is maintained for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, or more days post printing. In certain embodiments, the density of the epithelial cell layer is at least 1×10^5 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 2×10^5 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 5×10^5 cells per mL. In certain embodiments, the

density of the epithelial cell layer is at least 1×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 5×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 10×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 20×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 50×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 100×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 200×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is at least 500×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is less than 1×10^5 cells per mL. In certain embodiments, the density of the epithelial cell layer is less than 2×10^5 cells per mL. In certain embodiments, the density of the epithelial cell layer is less than 5×10^5 cells per mL. In certain embodiments, the density of the epithelial cell layer is less than 1×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is less than 5×10^6 cells per mL. In certain embodiments, the density of the epithelial cell layer is less than 10×10^6 cells per mL. In certain embodiments, the viability of the epithelial tissue layer is greater than 99% living cells by volume. In certain embodiments, the viability of the epithelial tissue layer is greater than 95% living cells by volume. In certain embodiments, the viability of the epithelial tissue layer is greater than 90% living cells by volume. In certain embodiments, the viability of the epithelial tissue layer is greater than 80% living cells by volume. In certain embodiments, the viability of the epithelial tissue layer is greater than 70% living cells by volume. In certain embodiments, the viability of the epithelial tissue layer is greater than 60% living cells by volume. In certain embodiments, the viability of the epithelial tissue layer is greater than 50% living cells by volume. In certain embodiments, this viability is maintained for at least 8, 12, 24, 48, 72, or 96 hours post-printing. In certain embodiments, this viability is maintained for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days post-printing.

Uniformity of tissue architecture

[070] One advantage of bioprinting using the methods of this disclosure is the high degree of uniformity achieved by the process that is reflected in the corresponding tissue. In certain embodiments, the thickness of the renal tubule model is substantially uniform. In certain embodiments, greater than 99% of the renal tubule model is within 10% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 95% of the renal tubule model is within 10% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 90% of the renal tubule model is within 10%

plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 80% of the renal tubule model is within 10% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 70% of the renal tubule model is within 10% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 99% of the renal tubule model is within 20% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 95% of the renal tubule model is within 20% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 90% of the renal tubule model is within 20% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 80% of the renal tubule model is within 20% plus or minus of the overall mean thickness of the renal tubule model. In certain embodiments, greater than 70% of the renal tubule model is within 20% plus or minus of the overall mean thickness of the renal tubule model.

Non-cellular components of bio-inks and cell layers

[071] Often cells or bio-inks that are bioprinted contain excipients or extrusion compounds that improve their suitability for bioprinting. Examples of extrusion compounds include, but are not limited to gels, hydrogels, peptide hydrogels, amino acid-based gels, surfactant polyols (e.g., Pluronic F-127 or PF-127), thermo-responsive polymers, hyaluronates, alginates, extracellular matrix components (and derivatives thereof), collagens, gelatin, other biocompatible natural or synthetic polymers, nanofibers, and self-assembling nanofibers. In some embodiments, the extrusion compound contains a synthetic polymer. In some embodiments, the extrusion compound contains a non-synthetic polymer that is not normally associated with mammalian tissues. In some embodiments, extrusion compounds are removed after bioprinting by physical, chemical, or enzymatic means. In some embodiments, the bio-inks of the present disclosure contain more than 1% extrusion compound by weight. In some embodiments, the renal tubule models of the present disclosure contain more than 1% extrusion compound by weight. In some embodiments, the bio-inks of the present disclosure contain less than 5% extrusion compound by weight. In some embodiments, the bio-inks of the present disclosure contain less than 2% extrusion compound by weight. In some embodiments, the bio-inks of the present disclosure contain less than 1% extrusion compound by weight. In some embodiments, the renal tubule models of the present disclosure contain less than 5% extrusion compound by weight. In some embodiments, the renal tubule models of the present disclosure contain less than 2% extrusion compound by weight. In some embodiments, the renal tubule models of the present disclosure

contain less than 1% extrusion compound by weight. In some embodiments, the epithelial bio-ink is free from hydrogel. In some embodiments, the epithelial bio-ink is free from extrusion compound. In some embodiments, the epithelial bio-ink is free from synthetic polymers that are used as an excipient or estrus in compound. In some embodiments, the renal tubule model is free from synthetic polymers that are used as an excipients or extrusion compounds. In some embodiments, the epithelial cell layer is free from synthetic polymers that are used as an excipients or extrusion compounds. In some embodiments, the interstitial cell layer is free from synthetic polymers that are used as an excipients or extrusion compounds.

Print Surfaces

[072] Provided herein are renal tubule models that are attached to a biocompatible surface. In certain embodiments, the interstitial tissue layer is printed onto a biocompatible surface. In certain embodiments, the biocompatible surface is a membrane with a pore size greater than 0.4 μm . In certain embodiments, the biocompatible surface has a pore size of about 1 μm . In certain embodiments, the biocompatible surface is coated with a composition to improve cell adherence or viability. In certain embodiments, the renal tubule modules are printed into 6-well, 12-well, 24-well, 48-well, 96-well, or 384-well plates. In certain embodiments, the renal tubule modules are printed into tissue culture plates with diameters of 60, 100 or 150mm or more. In certain embodiments, the renal tubule modules are printed into tissue culture flasks or onto microfluidic chips. In certain embodiments, the renal tubule modules are printed into/onto transwell inserts.

Process for production of renal tubule models

[073] This disclosure supports methods and processes for fabricating renal tubule models. In certain embodiments, the product of a three-dimensional, engineered, biological renal tubule model is produced by the process of bioprinting. In certain embodiments, at least one constituent of the product of a three-dimensional, engineered, biological renal tubule model is produced by the process of bioprinting. In certain embodiments, the process of fabricating a three-dimensional, engineered, biological renal tubule model, comprises: preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells; preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells; depositing the renal interstitial bio-ink and the renal epithelial bio-ink such that the renal epithelial bio-ink forms a layer on at least one surface of the layer of renal interstitial bio-ink; and maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form the three-dimensional, engineered, biological

renal tubule model. In certain embodiments, the renal interstitial tissue bio-ink forms a renal interstitial tissue layer with an apical and basolateral surface. In certain embodiments, the renal epithelial bio-ink is deposited in contact with the apical surface of the renal interstitial tissue layer. In certain embodiments, the renal epithelial bio-ink consists essentially of renal tubular epithelial cells. In certain embodiments, the renal epithelial bio-ink consists essentially of primary renal tubular epithelial cells. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with a disease that affects kidney function. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with polycystic kidney disease. In certain embodiments, the primary renal tubular epithelial cells are isolated from a subject with diabetes mellitus type II. In certain embodiments, the renal epithelial bio-ink comprises renal cell carcinoma cells. In certain embodiments, the renal epithelial bio-ink is deposited in a monolayer. In certain embodiments, the renal interstitial tissue bio-ink is deposited in a monolayer. In certain embodiments, the layer of renal epithelial tissue is deposited in continuous contact with the layer of renal interstitial tissue. In certain embodiments, the renal epithelial bio-ink forms a layer that covers by 50% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, the renal epithelial bio-ink forms a layer that covers by 70% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, the renal epithelial bio-ink forms a layer that covers by 90% or more the apical surface of the layer of renal interstitial tissue. In certain embodiments, at least 50% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells. In certain embodiments, at least 70% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells. In certain embodiments, at least 90% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells. In certain embodiments, the renal tubule model is between 50 and 500 μ m thick. In certain embodiments, the renal tubule model is about 100 μ m thick. In certain embodiments, the renal epithelial bio-ink further comprises an extrusion compound. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 95:5 to about 5:95 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 75:25 to about 25:75 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 60:40 to about 40:60 fibroblasts to endothelial cells. In certain embodiments, the fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 50:50 fibroblasts to endothelial

cells. In certain embodiments, the renal interstitial bio-ink further comprises secretory cells. In certain embodiments, the renal interstitial bio-ink further comprises immune cells. In certain embodiments, the renal interstitial bio-ink further comprises an extrusion compound. In certain embodiments, the renal interstitial bio-ink comprises glomerular cells. In certain embodiments, the model is fabricated substantially free of pre-formed scaffold. In certain embodiments, the renal fibroblasts, endothelial cells, and renal tubular epithelial cells are mammalian cells. In certain embodiments, either of the renal interstitial bio-ink or renal epithelial bio-ink forms a planar layer after deposition. In certain embodiments, the renal tubule model is of a uniform thickness. In certain embodiments, the renal interstitial bio-ink is deposited onto a biocompatible membrane. In certain embodiments, the renal interstitial bio-ink is deposited onto a biocompatible membrane with a pore size greater than 0.4 μm . In certain embodiments, the renal interstitial bio-ink is deposited onto a biocompatible membrane with a pore size of about 1 μm . In certain embodiments, the three-dimensional, engineered, biological renal tubule models are deposited to form an array. In certain embodiments, the three-dimensional, engineered, biological renal tubule models are deposited to form an array configured to allow between about 20 μm and about 100 μm of space between each renal tubule model. In certain embodiments, the renal interstitial bio-ink is at least 30% living cells by volume. In certain embodiments, the renal interstitial bio-ink is at least 70% living cells by volume. In certain embodiments, the renal interstitial bio-ink is at least 90% living cells by volume. In certain embodiments, the renal interstitial bio-ink is deposited by extrusion bioprinting. In certain embodiments, the renal epithelial bio-ink is deposited by ink-jet bioprinting. In certain embodiments, the renal interstitial bio-ink is not deposited by ink-jet bioprinting. In certain embodiments, any layer of the renal tubule model is viable in *in vitro* culture in culture after 3 days. In certain embodiments, any layer of the renal tubule model is viable in *in vitro* culture after 10 days.

[074] In certain embodiments, the 3D renal tubule models disclosed herein are produced by an additive manufacturing process. The additive manufacturing process for 3D tubule models herein allows customized fabrication of 3D renal tubule models for *in vitro* purposes. This is significant in that the tissues are fabricated due to a user specified design. In certain embodiments, the 3D renal tubule models contain only the cells that the user specifies. In certain embodiments, the 3D renal tubule models contain only the cell types that the user specifies. In certain embodiments, the 3D renal tubule models contain only the number of cells or concentration of cells that the user specifies. In certain embodiments, the 3D renal tubule models contain cells that have been treated with a small molecule, therapeutic molecule, or therapeutic

substance before or during fabrication. A therapeutic molecule or substance being any molecule intended to treat a disease or elicit a biological response. In certain embodiments, the 3D renal tubule models contain biocompatible or tissue culture plastics, biocompatible synthetic polymers, cross linkable gels, reversibly cross-linked gels and other non-cellular constituents.

Maturation of renal tubule models

[075] In certain embodiments, the renal tubule models of the present disclosure are matured for a certain amount of time after bioprinting. In certain embodiments, the models are matured for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 18, 24 hours or more before use. In certain embodiments, the models are matured for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 days or more before use. In some embodiments, shipment or transfer of the tissues is a use. In certain embodiments, the interstitial layer of the renal tubule model of the present disclosure is matured for a certain amount of time after bioprinting before addition of the epithelial layer. In certain embodiments, the interstitial layer is matured for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 18, 24 hours or more before use. In certain embodiments, the interstitial layer is matured for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 days or more before use. In some embodiments, shipment or transfer of the tissues is a use. In some embodiments, the epithelial layer is bioprinted onto the interstitial layer within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 18, 24 hours after bioprinting of the interstitial layer. In some embodiments, shipment or transfer of the tissues is a use. In some embodiments, the epithelial layer is bioprinted onto the interstitial layer within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 days after bioprinting of the interstitial layer.

In vitro assays

[076] In some embodiments, the renal tubules and arrays disclosed herein are for use in *in vitro* assays. In some embodiments, an “assay” is a procedure for testing or measuring the presence or activity of a substance (e.g., a chemical, molecule, biochemical, drug, etc.) in an organic or biologic sample (e.g., cell aggregate, tissue, organ, organism, etc.). In further embodiments, assays include qualitative assays and quantitative assays. In still further embodiments, a quantitative assay measures the amount of a substance such as a chemical or biomolecule in a sample.

[077] In various embodiments, the renal tubules and arrays are for use in, by way of non-limiting example, image-based assays, measurement of secreted proteins, expression of markers,

and production of proteins or mRNAs. In various further embodiments, the renal tubules and arrays are for use in assays to detect or measure one or more of: molecular binding (including radioligand binding), molecular uptake, activity (e.g., enzymatic activity and receptor activity, etc.), gene expression, protein expression, protein modifications (non-limiting examples include: phosphorylation, ubiquitination, acetylation, glycosylation, lipidation, etc.), receptor agonism, receptor antagonism, cell signaling, apoptosis, chemosensitivity, transfection, cell migration, chemotaxis, cell viability, cell proliferation, safety, efficacy, metabolism, toxicity, infectivity, and abuse liability. In various embodiments, the renal tubules are for toxicology, pharmaceutical or toxicity testing.

[078] In some embodiments, the renal tubules and arrays are for use in immunoassays. Immunoassays include, for example, flow cytometry, high throughput or low throughput image analysis, immunoprecipitation, radio-immunoassay (RIA), enzyme-linked immunosorbent assays (ELISA), western blot, homogenous assays, such as AlphaLISA™ and related technologies that rely on time resolved fluorescence or fluorescence resonance energy transfer (FRET). In further embodiments, immunoassays are competitive immunoassays or noncompetitive immunoassays. In a competitive immunoassay, for example, the antigen in a sample competes with labeled antigen to bind with antibodies and the amount of labeled antigen bound to the antibody site is then measured. In a noncompetitive immunoassay (also referred to as a “sandwich assay”), for example, antigen in a sample is bound to an antibody site; subsequently, labeled antibody is bound to the antigen and the amount of labeled antibody on the site is then measured.

[079] In some embodiments, the renal tubules and arrays are for use in ELISA. In further embodiments, an ELISA is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. In ELISA, for example, at least one antibody with specificity for a particular antigen is utilized. By way of further example, a sample with an unknown amount of antigen is immobilized on a solid support (e.g., a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a “sandwich” ELISA). By way of still further example, after the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody is, for example, covalently linked to an enzyme, or is itself detected by a secondary antibody that is linked to an enzyme through bioconjugation.

[080] For example, in some embodiments, an array, microarray, or chip of cells, multicellular

aggregates, or tissues is used for drug screening or drug discovery. In further embodiments, an array, microarray, or chip of tissues is used as part of a kit for drug screening or drug discovery. In some embodiments, each renal tubule exists within a well of a biocompatible multi-well container, wherein the container is compatible with one or more automated drug screening procedures and/or devices. In further embodiments, automated drug screening procedures and/or devices include any suitable procedure or device that is computer or robot-assisted.

[081] In further embodiments, arrays for drug screening assays or drug discovery assays are used to research or develop drugs potentially useful in any therapeutic area. In still further embodiments, suitable therapeutic areas include, by way of non-limiting examples, infectious disease, hematology, oncology, pediatrics, cardiology, central nervous system disease, neurology, gastroenterology, hepatology, urology, infertility, ophthalmology, nephrology, orthopedics, pain control, psychiatry, pulmonology, vaccines, wound healing, physiology, pharmacology, dermatology, gene therapy, toxicology, toxicity, and immunology.

[082] In some embodiments, the renal tubules and arrays are for use in cell-based screening. In further embodiments, the cell-based screening is for one or more infectious diseases such as viral, fungal, bacterial or parasitic infection. In further embodiments, the cell-based screening is for kidney cancer, including renal cell carcinoma, juxtaglomerular cell tumor (reninoma), angiomyolipoma, renal oncocytoma, Bellini duct carcinoma, clear-cell sarcoma of the kidney, mesoblastic nephroma, Wilms' tumor, mixed epithelial stromal tumor, and transitional cell carcinoma of the renal pelvis. In further embodiments, the cell-based screening is for nephritis, including, glomerulonephritis, interstitial nephritis or tubulo-interstitial nephritis, pyelonephritis, lupus nephritis and athletic nephritis. In further embodiments, the cell-based screening is for hypertension. In further embodiments, the cell-based screening is for diabetes mellitus, type I, type II and MODY. In further embodiments, the cell-based screening is for a nephropathy, including IgA nephropathy, analgesic nephropathy, or onconeophropathy. In some embodiments, the cell-based screening is for polycystic kidney disease or Xanthine oxidase deficiency. In other embodiments, the renal tubules and arrays are for use in the study of cancer initiation, progression, or metastasis. In still further embodiments, the renal tubules and arrays are for use in the study of the interaction of other cell types, such as cancer cells, pathogen-bearing cells, pathogenic cells, immune cells, blood-derived cells, or stem/progenitor cells.

[083] In some embodiments, the constructs or arrays thereof are for use in assessing the performance of biologics, including antibodies, mammalian cells, bacteria, biologically-active

proteins, hormones, etc. In other embodiments, the renal tubules or arrays thereof are useful in the study of cell-cell and cell-tissue interactions between the mammalian renal tubules comprising the construct and one or more additional cell types, including but not limited to pathogen-bearing cells, living pathogenic cells, cancer cells, immune cells, blood cells, stem/progenitor cells, or genetically-manipulated cells.

[084] In some embodiments, the array comprises renal tubules and additional tissue constructs. In further embodiments, the renal tubule construct is in direct contact with an additional tissue construct on one or more surfaces. In still further embodiments, the renal tubule is connected to one or more additional tissues constructs or cells via a fluid path or common fluid reservoir. In still further embodiments, the liquid media that contacts the engineered renal tubule construct contains living mammalian cells such as immune cells, blood-derived cells, or tumor-derived cells. In other embodiments, the liquid media that contacts the renal tubule contains bacteria, fungi, viruses, parasites, or other pathogens.

[085] The disclosure herein includes business methods. In some embodiments, the speed and scalability of the techniques and methods disclosed herein are utilized to design, build, and operate industrial and/or commercial facilities for production of renal tubules and/or organs for transplantation or use in generation of cell-based tools for research and development, such as *in vitro* assays. In further embodiments, the renal tubules and/or organs and arrays thereof are produced, stored, distributed, marketed, advertised, and sold as, for example, cellular arrays (e.g., microarrays or chips), tissue arrays (e.g., microarrays or chips), and kits for biological assays and high-throughput drug screening. In other embodiments, the engineered renal tubules and/or organs and arrays thereof are produced and utilized to conduct biological assays and/or drug screening as a service.

Validation

[086] The ideal engineered renal tissues are fully human and multicellular, comprising renal tubular epithelial cells, renal interstitial fibroblasts, and endothelial cells. Moreover, ideal engineered renal tissues demonstrate specific functions including, but not limited to, CYP1A2, CYP2C9, and CYP3A4 activity, albumin transport, and vitamin D hydroxylation, γ -glutamyl-transferase activity. Also, the ideal engineered renal tissues are characterized by tight junctions, cadherin, polarity of transporters, and CD31 expression and are validated by specific assays including albumin transport, CYP450 activity, histology, and viability. In some embodiments, the renal tubule models of the present disclosure display increased specific functions compared

to 2D co-culture or tissue explants that have been maintained in culture longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days. In some embodiments, the renal tubule models of the present disclosure display 2-fold increased specific functions compared to 2D co-culture or tissue explants that have been maintained in culture longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days. In some embodiments, the renal tubule models of the present disclosure display 5-fold or more increased specific functions compared to 2D co-culture or tissue explants that have been maintained in culture longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days. In some embodiments, the renal tubule models of the present disclosure display 2-fold or more increased specific functions compared to 2D co-culture or tissue explants that have been maintained in culture longer than 21 or more days. In some embodiments, the renal tubule models of the present disclosure display 5-fold or more increased specific functions compared to 2D co-culture or tissue explants that have been maintained in culture longer than 27 or more days. In some embodiments, the renal tubule models of the present disclosure display 2-fold or more increased specific functions compared to 2D co-culture or tissue explants that have been maintained in culture longer than 27 or more days. In some embodiments, the renal tubule models of the present disclosure display 5-fold or more increased specific functions compared to 2D co-culture or tissue explants that have been maintained in culture longer than 21 or more days. In certain embodiments, the specific function is γ -glutamyl-transferase activity. In certain embodiments, the specific function is vitamin D hydroxylation.

[087] In some embodiments, the engineered tissues described herein possess key architectural and functional attributes associated with *in vivo* human renal tissue, including histologic features and renal tubule-specific functions, including but not limited to:

- Polarization of renal tubular epithelial cells w/ formation of intracellular tight junctions (E-Cad, ZO-1, and Claudins) and correct intracellular localization of transporters (apical: OAT4, URAT1) and integrins (basolateral).
- Development of a basal lamina between the tubular cell layer and the underlying interstitium.
- Establishment of extensive microvascular networks within the interstitium, including the development of tissue-like tubular cells: microvascular spatial relationships.
- Expression of compartment-specific markers, including tubular epithelial transporters (cubilin, megalin, aquaporins), OATs, URAT), vascular markers (CD31, vWF), demonstration of EPO protein production (if applicable).

- Vitamin D synthesis via 25-(OH) 1 hydroxylase (1 OHase).
- Production of Angiotensin II.
- Active transport of albumin from tubular lumen via cubilin.
- Cimetidine transport / accumulation from basolateral surface.
- CYP450 and UGT expression involved in metabolism (e.g., CYP2B6, 3A5, 4A11 and UGT 1A9, 2B7, respectively).

EXAMPLES

[088] The following illustrative examples are representative of embodiments, of the software applications, systems, and methods described herein and are not meant to be limiting in any way.

Example 1 - A bioprinted three-dimensional renal tubule cell model

[089] A 3-layer cup shape with dimensions 3 mm x 3 mm x 0.75 mm was bioprinted onto a Transwell membrane in a 12-well tissue culture plate (**Fig. 2A** and **2B**). The bottom sheet and two layered walls were composed of 75% adult renal fibroblasts (aRF) and 25% human umbilical vein endothelial cells (HUVEC). The cells were resuspended at 150 million cells/ml in Novogel® 2.0. The cup was filled with a dilute suspension of 100% epithelial cells (MDCK) at 1-5 million cells/ml in 2% gelatin (“**Structure 1**”). Immediately following bioprinting, structures were surrounded with molten Novogel® 1.0 and were then cultured in a mixture of renal fibroblasts media, HUVEC media, and MDCK media.

[090] Assessment of **Structure 1** was performed by histological staining for cell-type specific markers. A representative H&E stain for Structure 1 is shown in **Fig. 3A** and **3B**. Constructs were stained for CD31 **410** and TE7 **420** to assess the relative positions of the HUVEC cells and fibroblasts, respectively (**Fig. 4**). Both cell types are distributed throughout the interstitial layer, which exhibits high cellular density. A robust network of CD31+ cells was found in all analyzed constructs, with some areas possibly demonstrating evidence of microvasculature formation (**Fig. 4, 430**). To visualize the epithelial cells, tissues were stained with antibodies against E-cadherin (**Fig. 5, 540**), and TE7 (**Fig. 5, 520**). The epithelial cells were concentrated adjacent to the interstitial layer and formed tubule-like structures. This structure has many attractive features pertinent to a human renal proximal tubule model, including a vascularized interstitial layer supporting the growth and organization of polarized epithelial cells.

Example 2 - A bioprinted three-dimensional renal tubule cell model

[091] A sheet with dimensions 3 mm x 3 mm x 250 μ m was bioprinted onto a Transwell membrane in a 12-well plate. The tissue sheet was composed of 75% adult renal fibroblasts and 25% HUVEC resuspended at 150 million cells/ml in Novogel[®] 2.0. The edges of the sheet were bordered by a 500 μ m thick bioprinted hydrogel-only wall composed of Novogel[®] 3.0. Immediately following bioprinting, the boundary wall was crosslinked with 50 mM calcium chloride for 2 minutes. This solution was then aspirated and the constructs were surrounded with molten Novogel[®] 1.0. A dilute suspension of epithelial cells (MDCK or hTERT-RPTEC) at 1 million cells/ml was then added to the top surface of the structure by deposition using an inkjet spray module set with a 100 ms valve open time (“**Structure 2**”). A schematic of **Structure 2** is shown in **Fig. 6A and 6B**. Use of the inkjet spray module allows for deposition of epithelial cells immediately after printing or interstitial tissues can be printed and matured for several days prior to deposition of epithelial cells, facilitating formation of microvasculature and extracellular matrix that may help support the correct morphology of the epithelial cells.

[092] Assessment of **Structure 2** was performed by histological staining for cell-type specific markers. A representative H&E stain of **Structure 2** is shown in **Fig. 7A and 7B**. The tissues exhibit high cellular density, and a thin layer of epithelial cells can be observed (black arrow in **Fig. 7A and 7B**). Surprisingly, the inkjet spray module facilitated attachment of epithelial cells to the surface of the interstitial layer and resulted in a much lower cell density on the surface of the tissue, which is necessary for forming a polarized monolayer. Following inkjet spraying, cells retained high viability: greater than 97% for MDCK cells and 94% for hTERT-RPTEC cells as measured by trypan blue exclusion. Evidence of microvascular organization was detected by CD31 staining **810** and the organization of the epithelial cells was verified by E-cadherin staining **840** (**Fig. 8A and 8B**).

Example 3 - A bioprinted three-dimensional renal tubule cell model

[093] To reduce the thickness and cellularity of the interstitial layer, the cell ratio was changed to 50% fibroblasts / 50% HUVEC the concentration of the cells was 1.25×10^8 cells/mL. The interstitial layer of the renal proximal tubule model is composed of renal fibroblasts and HUVECs in Novogel[®] 2.0. Constructs produced in this example show proximal tubule epithelial cells (RPTECs) in 3D renal tissues that exhibit features of polarization. E-cadherin (bright staining and **940**) at the lateral membranes between RPTECs corresponds to tight junctions (**Fig. 9A, B, and C**). Further, the basement membrane corresponding to the interstitial layer produces

collagen (**Fig. 9 A and B**; 920). H & E staining is shown (**Fig. 10A**), and brush borders are indicated (**Fig. 11A**, arrows). Trichrome staining indicates collagen secretion (**Fig. 10B**, and **11B**, arrows), and CD31 (**Fig. 12**, Bright staining) staining indicates the presence of HUVEC networks (**Fig. 12**, asterisks). Bioprinted tissues demonstrated γ -glutamyl-transferase (GGT) activity which increases over time in culture, which is indicative of a functioning epithelial layer (**Fig. 13**). To assess the maturity and function of the epithelial component of the 3D bioprinted tubule model, tissues were homogenized and assayed for gamma glutamyl-transferase (GGT) activity. GGT is an enzyme expressed on the apical surface of epithelial cells and is involved in glutathione homeostasis and xenobiotic metabolism. As a positive control, hTERT-RPTEC cells cultured as a monolayer in 2D were assessed. As a negative control, bioprinted renal interstitial tissues without epithelium were also assayed to verify that the detected GGT activity was epithelial-specific. At culture day 3, 10, 14, 21, or 28, 2D hTERT-RPTEC, 3D interstitium tissues, or 3D bioprinted tubule tissues were homogenized and assessed for functional GGT enzymatic activity using a colorimetric assay available from Sigma-Aldrich. Bioprinted tubule tissues exhibited stable GGT activity from culture day 10-28, with negligible GGT activity detected in bioprinted interstitium only tissues. 2D hTERT-RPTEC monolayer cultures exhibited GGT activity relatively equivalent to that observed in 3D bioprinted tubule tissues, but 2D monolayers exhibited decreased function at day 21, and were not viable at day 28.

Example 4 - A three-dimensional renal tubule model bioprinted with different ratios of renal fibroblasts to endothelial cells

[094] Experiments were undertaken to determine the effect of fibroblast to endothelial cell ratio on tissue morphology. Renal tubule models were bioprinted using bio-inks comprising renal fibroblasts and HUVEC cells at ratios of 90:10; 75:25; and 50:50 (fibroblast to endothelial cells). **Fig. 14** and **15** show the results of this experiment. **Fig. 14** shows a macroscopic view of the printed renal tubule model; while **Fig. 15** shows corresponding histology stained with Masson's trichrome. At 6 days post print renal tubule models that comprise a 50:50 ratio were substantially planar or flat **Fig. 14C** and **15C**. Renal tubule models printed at a 90:10 (**Fig. 14A** and **15A**), and 72:25 (**Fig. 14B** and **15B**) exhibited curling at 6 days post printing.

Example 5 - Uniform thickness of a three-dimensional renal tubule model

[095] Experiments were undertaken to determine the uniformity of thickness and surface topology of renal tubule models. Renal tubule models were bioprinted into wells of a 12-well plate (**Fig. 16A**), and analyzed using Keyence technology (**Fig. 16B**). Bioprinted renal tubule

models were cultured for 14 days and analyzed for surface area and thickness using a Keyence VHX-700 digital microscope. The area within the Novogel 3.0 border (see Example 3) was estimated to be 0.068 cm². Across the tissue within the border, the average thickness was found to be approximately 106.8 um, with relative uniformity of thickness as evidenced by the heat map shown (extremely thin areas are **1650**). Tissues were stained with methyl blue to increase the color contrast and facilitate mapping of the surface of the tissue. Two discrete areas are in the blue range of the heat map **1650**, indicating that those areas are much thinner “dimples” that are approximately 20 um thick. The constructs average 100 μ m thick. The data shows a highly uniform, planar and smooth surface morphology with evidence of small dimples.

Example 6 - A three-dimensional renal tubule model fabricated with different concentrations of primary renal epithelial cells

[096] An interstitial layer comprising a 50:50 ratio of fibroblasts to endothelial cells was bioprinted into each well of a 24-well plate. The constructs were incubated with either 1.25×10^5 (**Fig. 17A and B**), 2.5×10^5 (**FIG. 17C**), or 5.0×10^5 (**Fig. 17D**), RPTEC cells in the presence (**Fig. 17B, C and D**), or absence (**Fig. 17A**) of media containing 2% FBS. Histology was taken on day 11 and H & E stained.

Example 7 - Toxicity assays using renal tubule models

[097] To determine the suitability of the renal tubule models for *in vitro* toxicity assays experiments were conducted using the common cytotoxic agents amphotericin B, cisplatin, and TGF β (**Fig. 18 - Fig. 23**). Renal tubule models were printed according to methods of the previous examples.

[098] **Fig. 18** shows amphotericin B (AmpB) toxicity, by alamarBlue assay, in bioprinted 3D renal tubule models comprising a layer of interstitial tissue and epithelial tissue (circles); standard 2D co-culture of hTERT RPTEC (squares); and interstitial tissue alone (triangles). Interstitial tissue alone was resistant to AmpB treatment, while the 3D renal tubule model showed dose dependent cytotoxicity.

[099] 3D renal tubule models show a dose inhibition of epithelial cell function by AmpB. **Fig. 19A** shows a time-course of lactate dehydrogenase (LDH) release, an indicator of cell toxicity from a 3D renal tubule model treated with varying concentrations of AmpB. A low dose of AmpB results in elevated LDH release by day 3, whereas a high dose results in a high-level of AmpB release by day 1. **FIG. 19B** shows a dose dependent reduction in GGT activity in

response to AmpB treatment. **Fig. 20** depicts histology of 3D renal tubule models from **Fig. 19B** showing epithelial specific cell death at 10 μ M (**Fig. 20B**) and 50 μ M (**Fig. 20C**) of AmpB compared to untreated controls (**Fig. 20A**).

[0100] 3D renal tubule models show a dose dependent inhibition of epithelial cell function by cisplatin. **Fig. 21A** shows an increase cytotoxicity in 3D renal tube models by AlamarBlue assay. **Fig. 21B** shows a dose dependent reduction in GGT activity. **Fig. 21C** shows an increase in LDH release by renal tubule models treated with 50 μ M cisplatin at day 3, and with 10 μ M cisplatin at day 5. **FIG 22** shows Masson's trichrome staining of renal tubule models treated with vehicle (**Fig. 22A**), 10 μ M cisplatin (**Fig. 22B**), 50 μ M cisplatin (**Fig. 22C**), and 50 ng/mL TGF β (**Fig. 22D**). In particular, treatment with TGF β shows an increase in fibrosis without the overt cell death of cisplatin treated tissues.

[0101] **Fig. 23** shows data compiled from experiments comparing the effect of an epithelial layer comprising primary cells (070615-RPTEC) versus an epithelial layer comprising an immortalized cell line (hTERT-RPTEC). Both types of cells exhibited a dose dependent increase in toxicity after treatment with cisplatin, by alamarBlue assay (**Fig. 23A**), GGT activity (**Fig. 23B**), and LDH release (**Fig. 23C**). Notably, primary cells showed enhanced sensitivity to cisplatin in the GGT assay.

Example 8- Effects of print surface on tissue morphology

[0102] Example 8 describes a bioprinted tubule tissue generated with a border of hydrogel-only Novogel 3.0; this border or a Novogel 1.0 moat is required to facilitate tissue attachment to the transwell membrane when 0.4 μ m pore size transwell inserts are used as the culture surface. The bioprinted renal interstitium portion of the tubule model 50:50 renal fibroblast to HUVEC was fabricated on different surfaces and assessed for attachment to the transwell membrane. **Fig. 24A** demonstrates that the bioprinted tissues float after 3 days in culture in the absence of a border or moat. To assess intact barrier and transporter functions, a tissue that covers the entire culture surface is necessary. To facilitate attachment and outgrowth of the bioprinted tubule tissue and to improve the surface area coverage of the bioprinted tubule model, it is desirable to use a culture surface that encourages tissue attachment in the absence of a border or moat. Shown in **Fig. 24B**, bioprinted renal interstitial tissues of the preferred embodiment (50:50 renal fibroblast to HUVEC) attach and spread on 1 μ m pore size membrane in the absence of any moat or hydrogel border.

Example 9 - Long term viability of renal tubule models in culture

[0103] One advantage of the 3D renal tubule models of this disclosure is sustained viability in culture. In many cases the viability persists much longer than the viability of normal 2D culture or tissue explants. **Fig. 25** shows that 3D renal tubule models at 6 days (**Fig. 25A**), 10 days (**Fig. 25B**) and 27 (**Fig. 25C**) days in culture.

[0104] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention.

Claims:

1. A three-dimensional, engineered, bioprinted, biological renal tubule model comprising:
 - a. a layer of renal interstitial tissue, the renal interstitial tissue comprising renal fibroblasts and endothelial cells, wherein the layer of renal interstitial tissue possesses an apical and basolateral surface; and
 - b. a layer of renal epithelial tissue, the renal epithelial tissue comprising renal tubular epithelial cells, wherein the layer of renal epithelial tissue is substantially a monolayer and is in direct contact with the apical surface of the layer of renal interstitial tissue or is separated from the apical surface of the renal interstitial tissue by extracellular matrix;provided that the interstitial tissue comprises an interstitial bio-ink, the epithelial tissue comprises an epithelial bio-ink, and form a three-dimensional, engineered, biological renal tubule model; and wherein the renal fibroblasts and endothelial cells are the only cells present in the layer of renal interstitial tissue and are present in a ratio of renal fibroblasts to endothelial cells at which the renal tubule model is planar six days post-printing.
2. The renal tubule model of claim 1, wherein:
 - (a) the layer of renal epithelial tissue consists essentially of renal tubular epithelial cells;
 - (b) the layer of renal epithelial tissue is in contact with and covers by about 50%, about 70%, or about 90% or more the apical surface of the layer of renal interstitial tissue;
 - (c) at least about 50%, at least about 70%, or at least about 90% of the cells in the layer of renal epithelial tissue form tight junctions with another cell of the renal epithelial tissue layer;
 - (d) the renal tubule model is between about 50 and about 500 μm thick;
 - (e) the renal fibroblasts and endothelial cells are present in the layer of renal interstitial tissue at a ratio of about 50:50;
 - (f) any of the layer of renal interstitial tissue or layer of renal epithelial tissue is at least about 30%, at least about 70%, or at least about 90% living cells by volume; or
 - (g) a combination of any two or more of (a) to (f).

3. The renal tubule model of claim 1 or 2, which is of substantially uniform thickness.
4. The renal tubule model of any one of claims 1 to 3, further comprising a biocompatible membrane, or a biocompatible membrane with a pore size greater than about 0.4 μm .
5. The renal tubule model of any one of claims 1 to 4, wherein the renal tubular epithelial cells are polarized.
6. The renal tubule model of any one of claims 1 to 5, wherein the renal tubular epithelial cells are from a subject with a disease that affects kidney function.
7. The renal tubule model of claim 6, wherein the disease is a polycystic kidney disease, diabetes mellitus type II, or renal cell carcinoma.
8. A plurality of the renal tubule models of any one of claims 1 to 7, configured to form an array.
9. A method of testing the viability of tubular epithelial cells in the presence of a therapeutic agent, comprising:
 - a. contacting the therapeutic agent with the three-dimensional, engineered, bioprinted, biological renal tubule model of any one of claims 1-7, and
 - b. measuring gamma glutamyl-transferase (GGT) activity in the model compared to a control,wherein decreased GGT activity in the model compared to the control is indicative of reduced viability of the renal tubular epithelial cells.
10. A method of testing the effect of a therapeutic agent on a renal transport molecule, comprising:
 - a. contacting the therapeutic agent with the three-dimensional, engineered, bioprinted, biological renal tubule model of any one of claims 1-7, and
 - b. measuring the transport molecule activity in the model compared to a control.
11. The method of claim 10, wherein the transport molecule activity is excretion and/or uptake of macromolecules.

12. The method of claim 10 or 11, wherein the transport molecule activity is albumin transport.
13. A method of fabricating a three-dimensional, engineered, biological renal tubule model, the method comprising:
 - a. preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells;
 - b. preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells;
 - c. depositing the renal interstitial bio-ink and the renal epithelial bio-ink such that the renal interstitial tissue bio-ink forms a renal interstitial tissue layer with an apical and basolateral surface, and the renal epithelial bio-ink is deposited in substantially a monolayer in contact with the apical surface of the renal interstitial layer; and
 - d. maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form the three-dimensional, engineered, biological renal tubule model,wherein the renal fibroblasts and endothelial cells are the only cells present in the layer of renal interstitial tissue and are present in a ratio of renal fibroblasts to endothelial cells at which the renal tubule model is planar six days post-depositing.
14. A method of assessing the renal toxicity of a therapeutic agent, the method comprising:
 - a. preparing a renal interstitial bio-ink, the interstitial bio-ink comprising a plurality of interstitial cell types, the interstitial cell types comprising renal fibroblasts and endothelial cells;
 - b. preparing a renal epithelial bio-ink, the epithelial bio-ink comprising renal tubular epithelial cells;
 - c. depositing the interstitial bio-ink and the epithelial bio-ink such that the renal interstitial tissue bio-ink forms a renal interstitial tissue layer with an apical and basolateral surface, and the renal epithelial bio-ink is deposited in substantially a monolayer in contact with the apical surface of the renal interstitial tissue layer; and

- d. maturing the deposited bio-ink in a cell culture media to allow the cells to cohere to form a three-dimensional, engineered, biological renal tubule model;
- e. contacting a therapeutic agent with the model;
- f. measuring viability or functionality of the renal tubular epithelial cells; and
- g. assessing the renal toxicity of the therapeutic agent based on the measured viability or functionality of the renal tubular epithelial cells;

wherein the renal fibroblasts and endothelial cells are the only cells present in the layer of renal interstitial tissue and are present in a ratio of renal fibroblasts to endothelial cells at which the renal tubule model is planar six days post-depositing.

15. The method of claim 14, wherein viability of the renal tubular epithelial cells is determined by measuring GGT activity in the model compared to a control.

16. The method of any one of claims 13 to 15, wherein:

- (a) the renal epithelial bio-ink consists essentially of renal tubular epithelial cells;
- (b) the renal epithelial bio-ink forms a layer that covers by about 50%, about 70%, or about 90% or more the apical surface of the layer of renal interstitial tissue;
- (c) at least about 50%, about 70%, or about 90% of renal epithelial cells of the renal epithelial layer form tight junctions with other renal epithelial cells;
- (d) the renal tubule model is between about 50 and about 500 μm thick;
- (e) the renal fibroblasts and endothelial cells are present in the renal interstitial bio-ink at a ratio of about 50:50 fibroblasts to endothelial cells;
- (f) the renal interstitial bio-ink is at least about 30%, at least about 70%, or at least about 90% living cells by volume;
- (g) either of the renal interstitial bio-ink or renal epithelial bio-ink forms a planar layer after deposition; or
- (h) a combination of any two or more of (a) to (g).

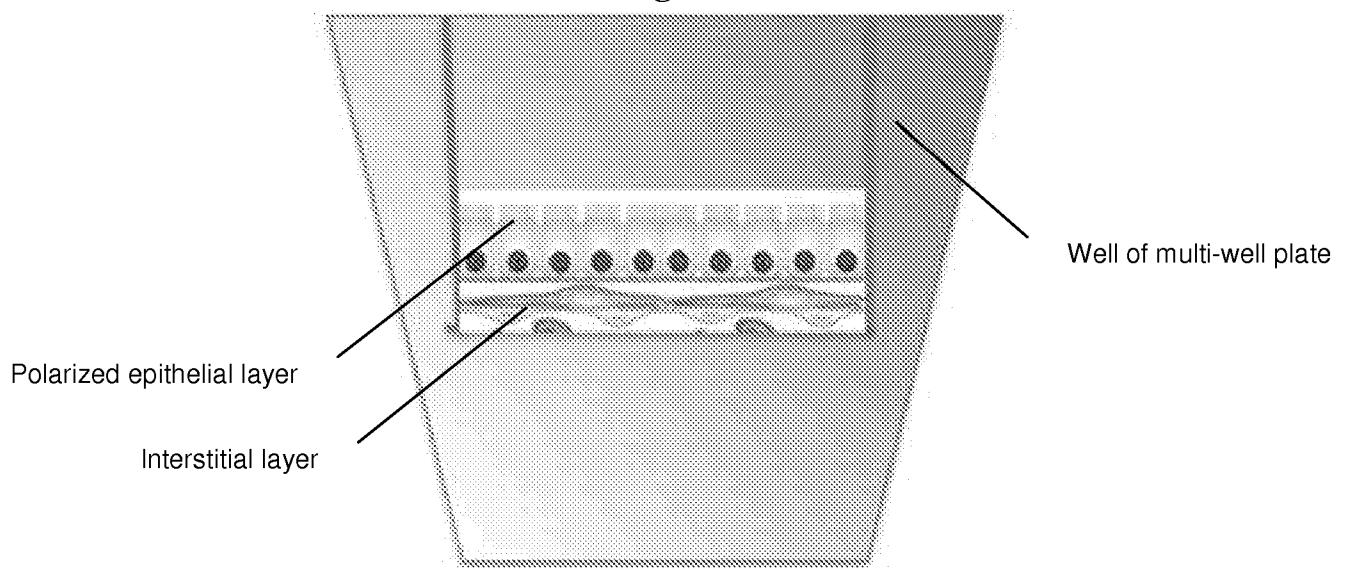
17. The method of any one of claims 13 to 16, wherein the renal tubule model is of substantially uniform thickness.

18. The method of any one of claims 13 to 17, further comprising depositing the renal interstitial bio-ink onto a biocompatible membrane, or depositing the renal interstitial bio-ink onto a biocompatible membrane with a pore size greater than about 0.4 μm .
19. The method of any one of claims 13 to 18, wherein three-dimensional, engineered, biological renal tubule models are deposited to form an array.
20. The method of any one of claims 13 to 19, wherein the depositing is by bioprinting.
21. The method of any one of claims 13 to 20, wherein the renal tubule model is planar six days after depositing.
22. The method of any one of claims 13 to 21, wherein the renal tubular epithelial cells are polarized.
23. The method of any one of claims 13 to 22, wherein the renal epithelial bio-ink comprises renal tubular epithelial cells isolated from a subject with a disease that affects kidney function.
24. The method of claim 23, wherein the disease is a polycystic kidney disease, diabetes mellitus type II, or renal cell carcinoma.

Organovo, Inc.

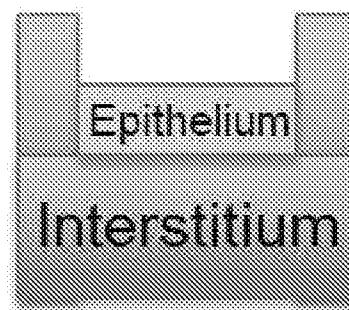
Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

1/25

Fig. 1

2/25

Fig. 2A



Interstitial layer contains
epithelial layer on three sides

Fig. 2B



3/25

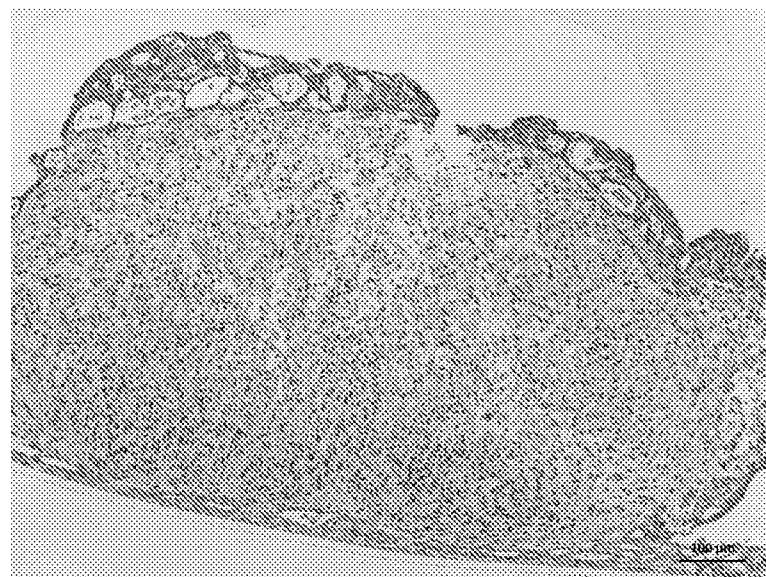
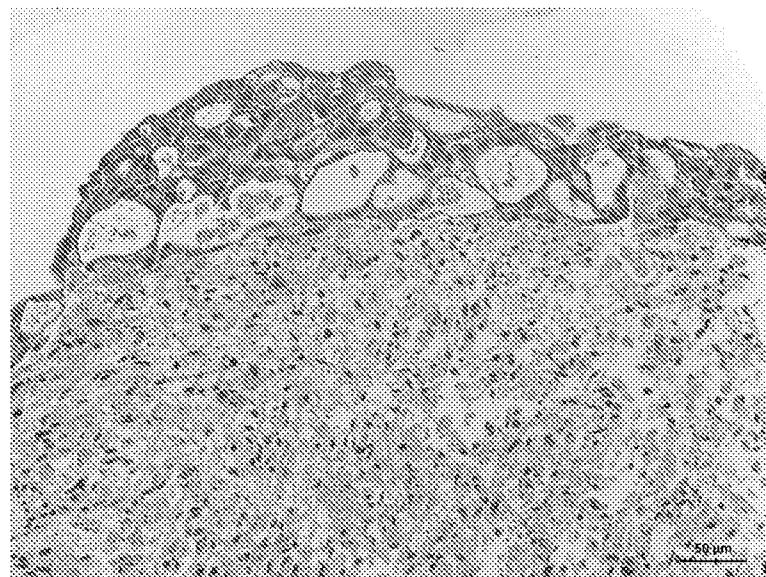
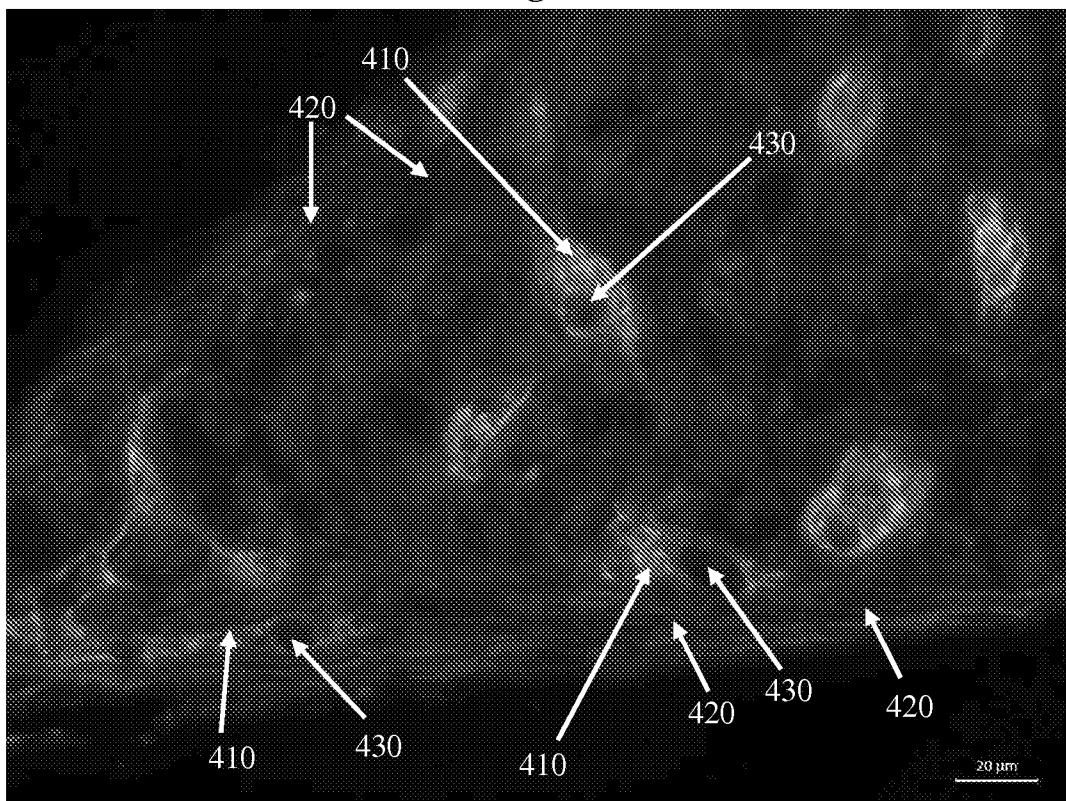
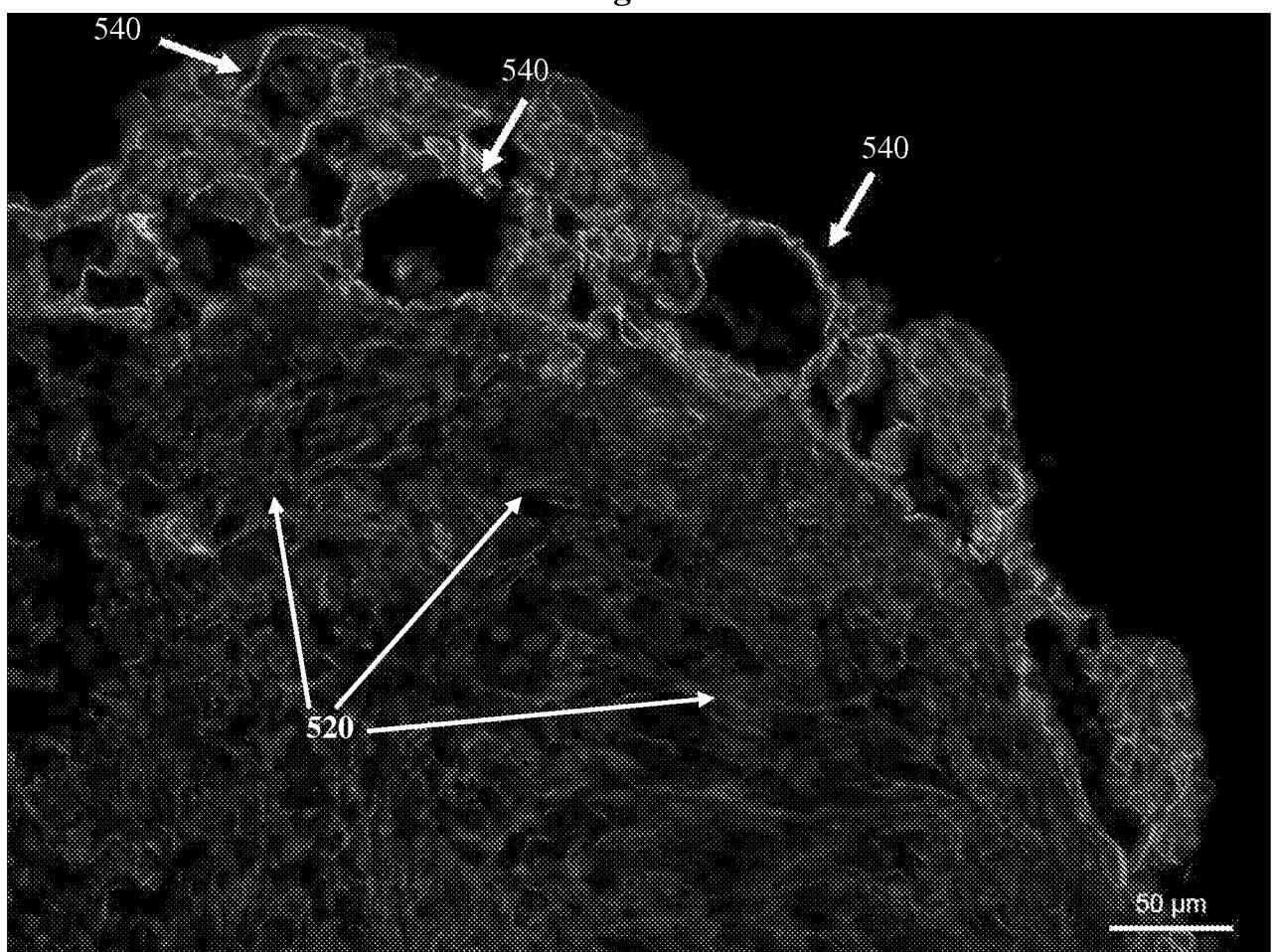
Fig. 3A**Fig. 3B**

Fig. 4

5/25

Fig. 5

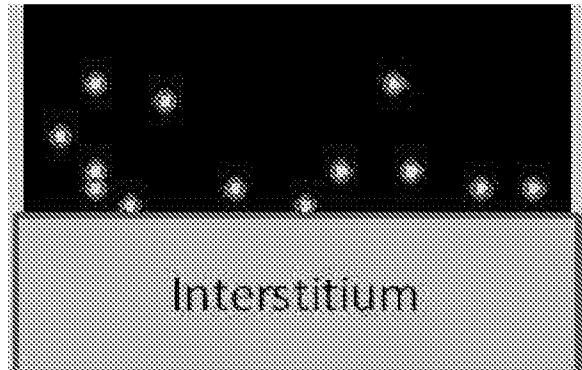


Fig. 6A

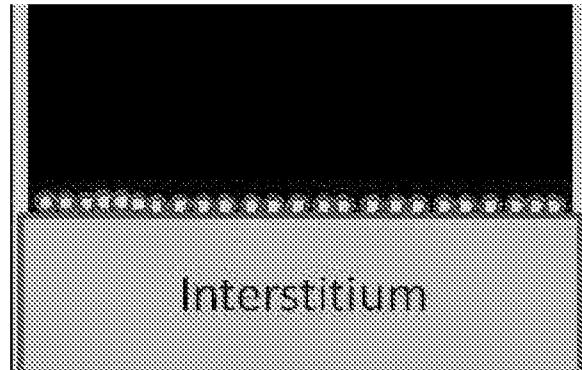


Fig. 6B

7/25

Fig. 7A

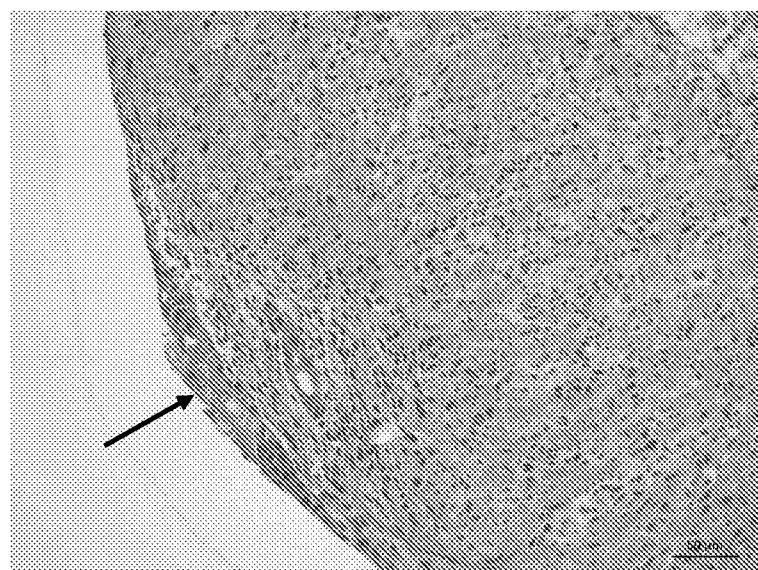
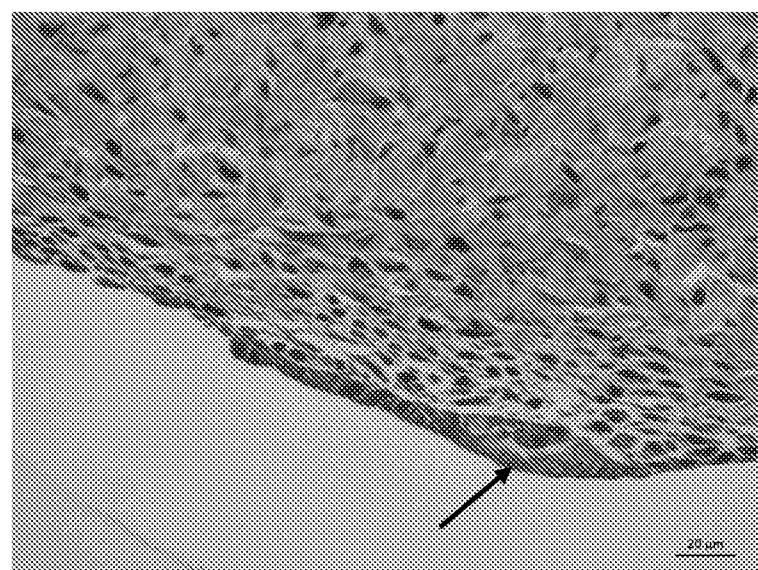


Fig. 7B



8/25

Fig. 8A

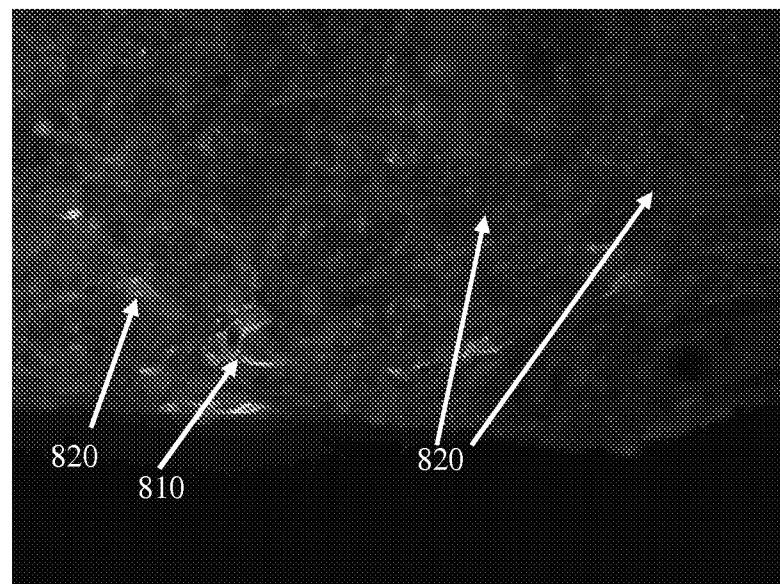
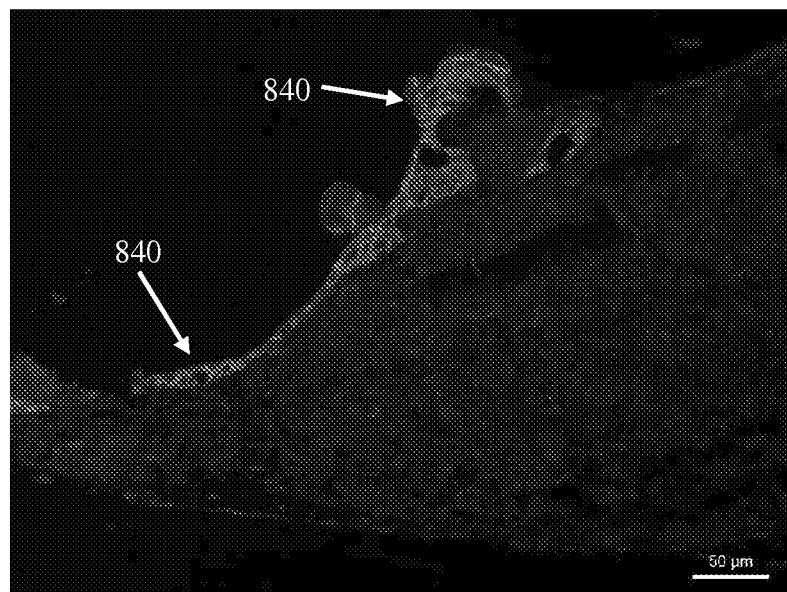
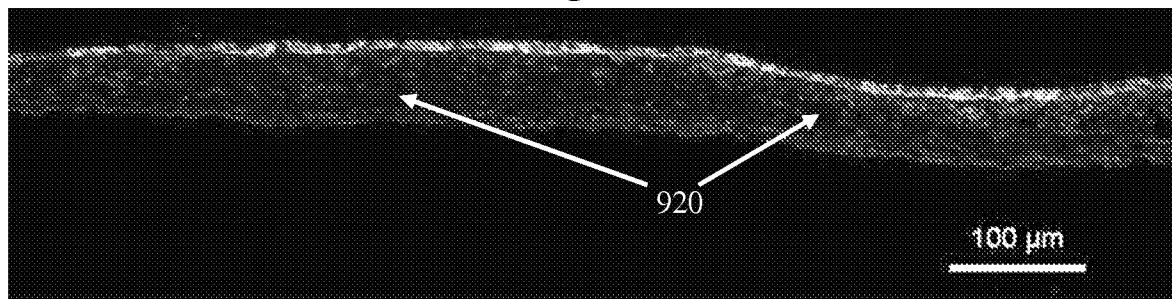
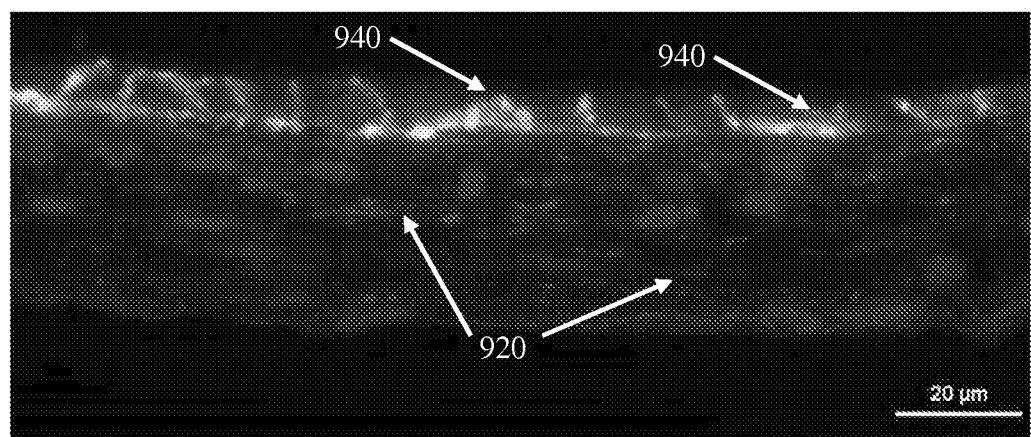
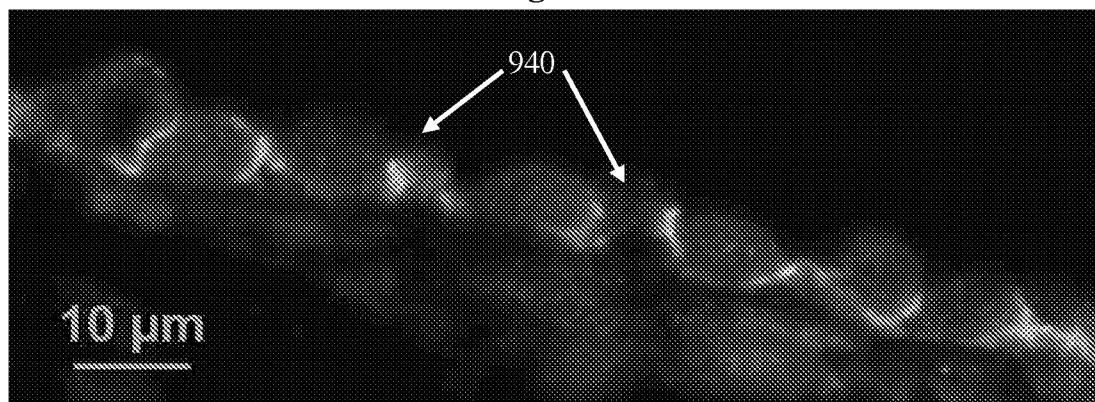


Fig. 8B



9/25

Fig. 9A**Fig. 9 B****Fig. 9C**

10/25

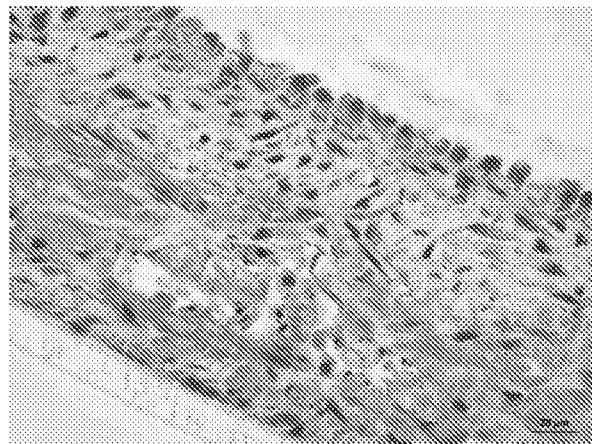


Fig. 10A

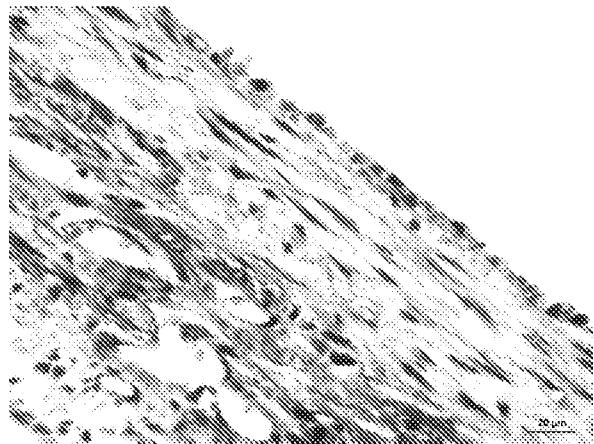


Fig. 10B

11/25

Fig. 11A

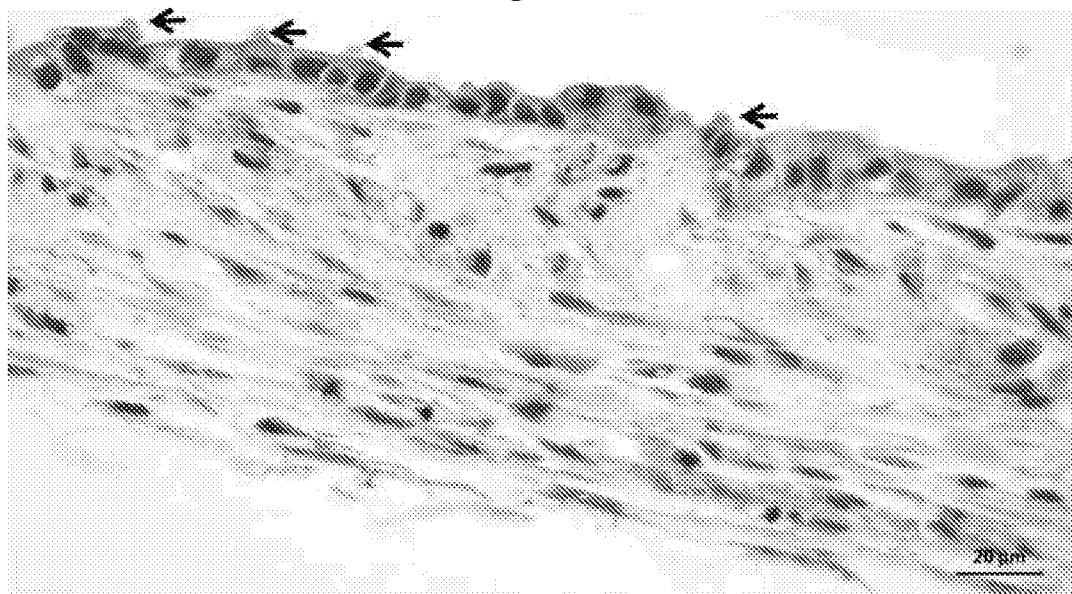
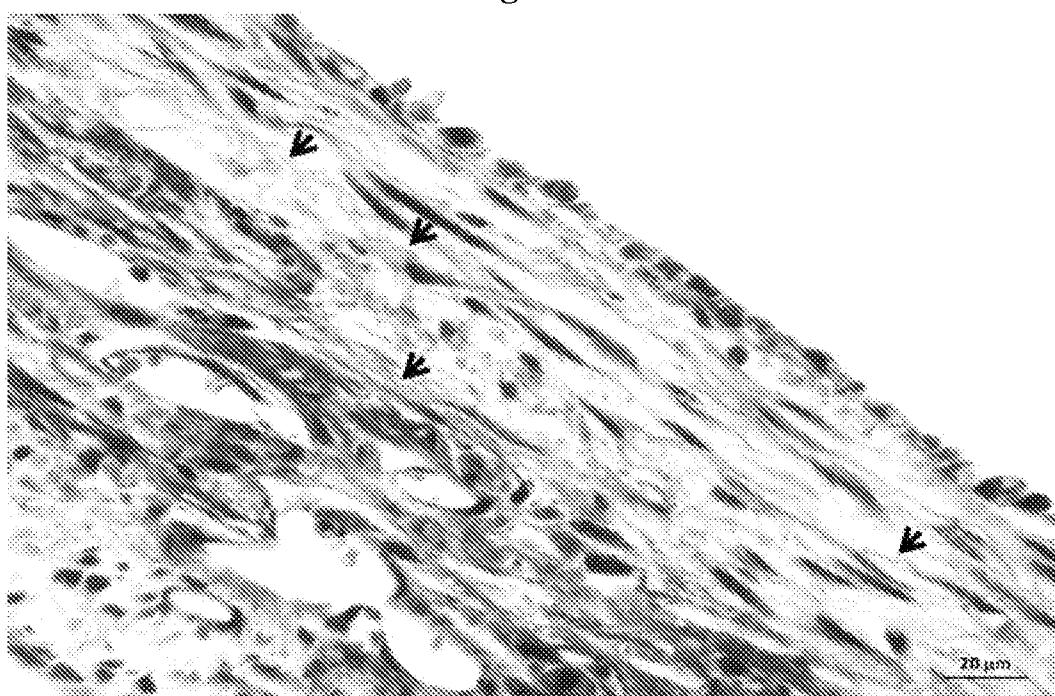
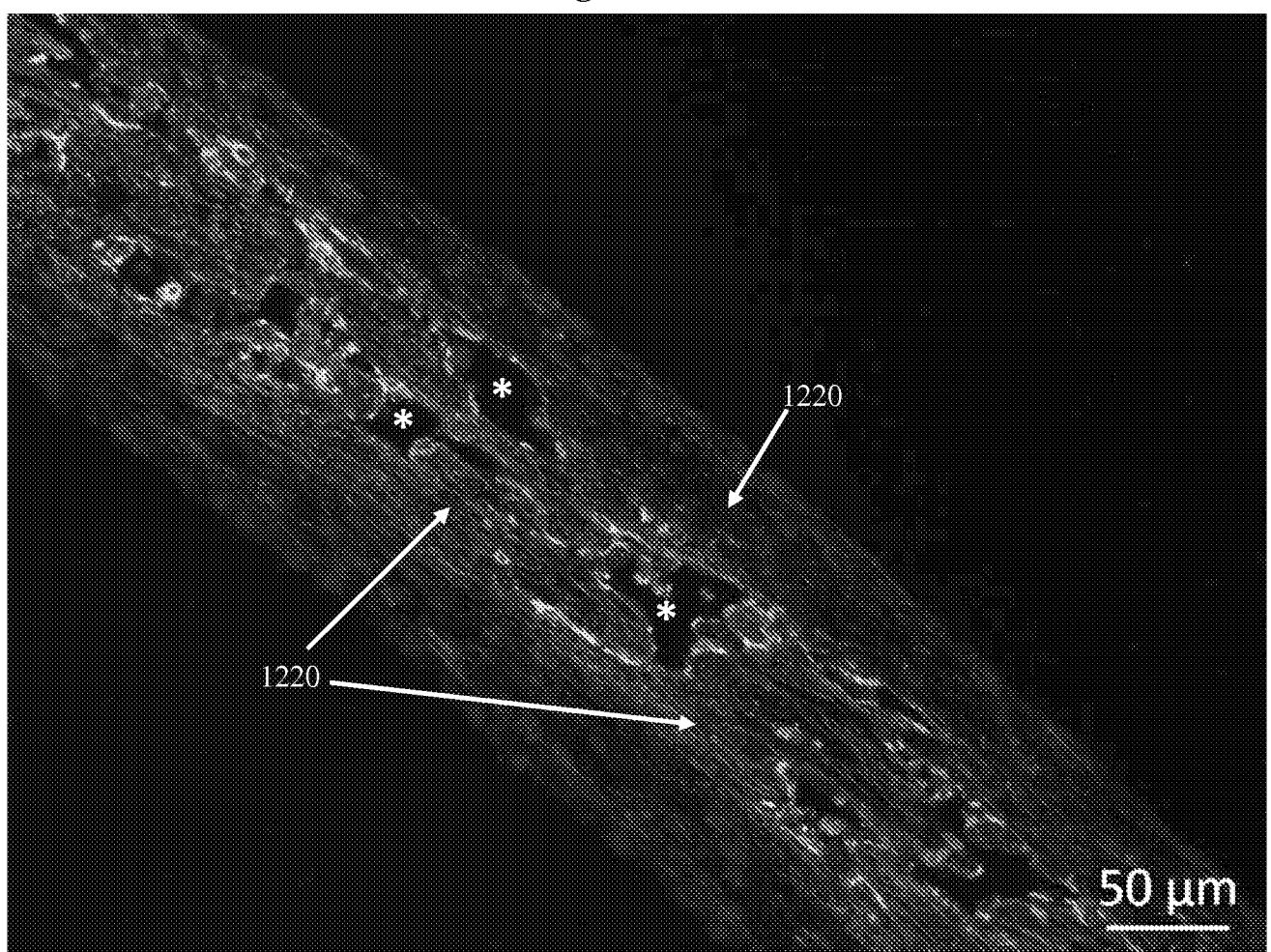


Fig. 11B

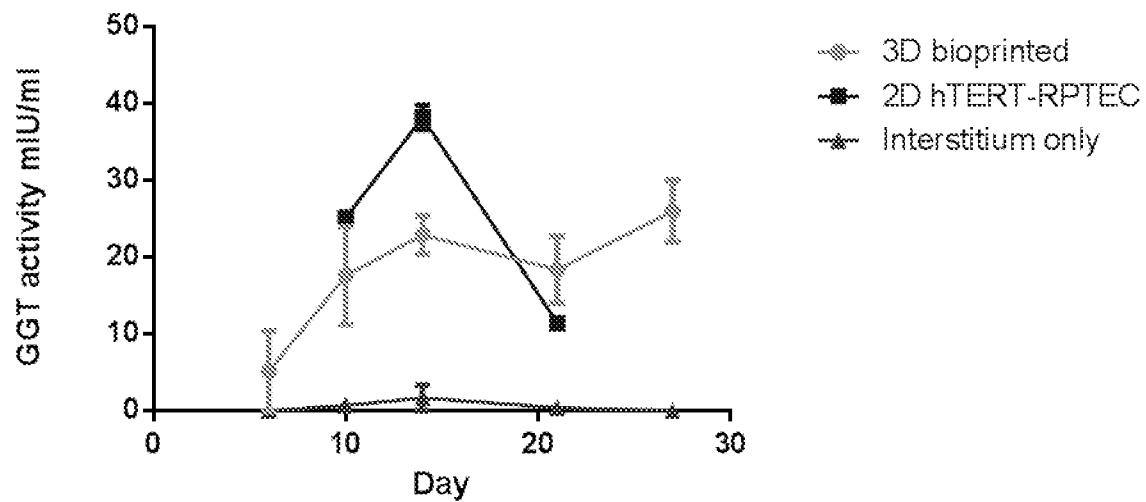


12/25

Fig. 12



13/25
Fig. 13



14/25

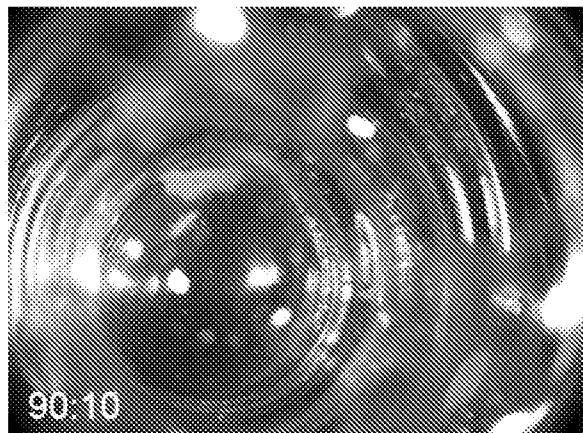


Fig. 14A

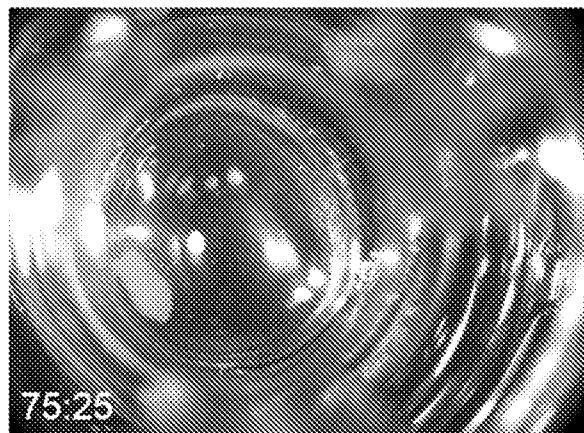
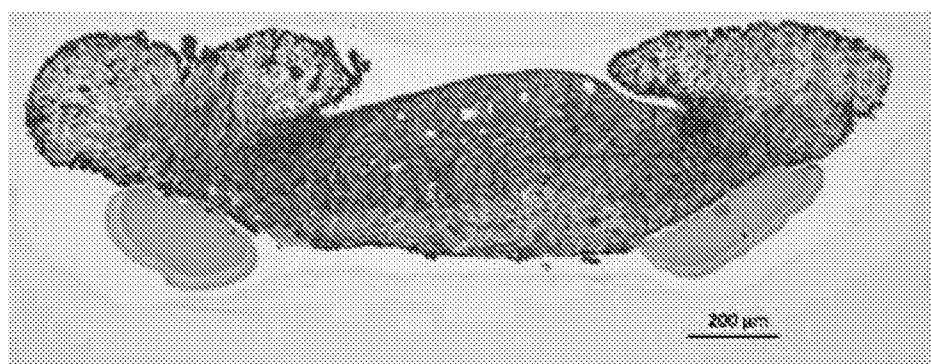
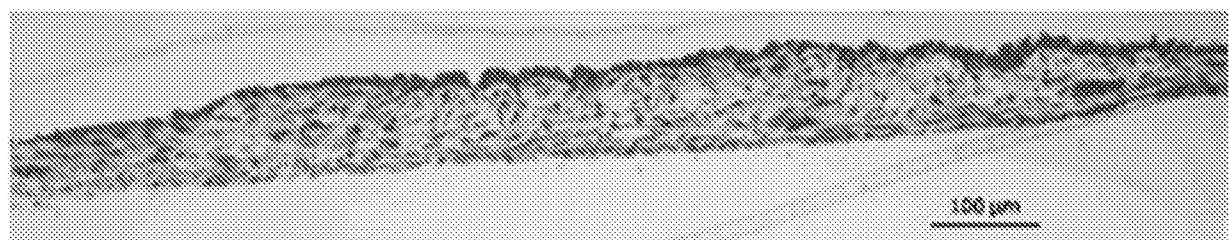


Fig. 14B



Fig. 14C

15/25

Fig. 15A**Fig. 15B****Fig. 15C**

16/25

Fig. 16A

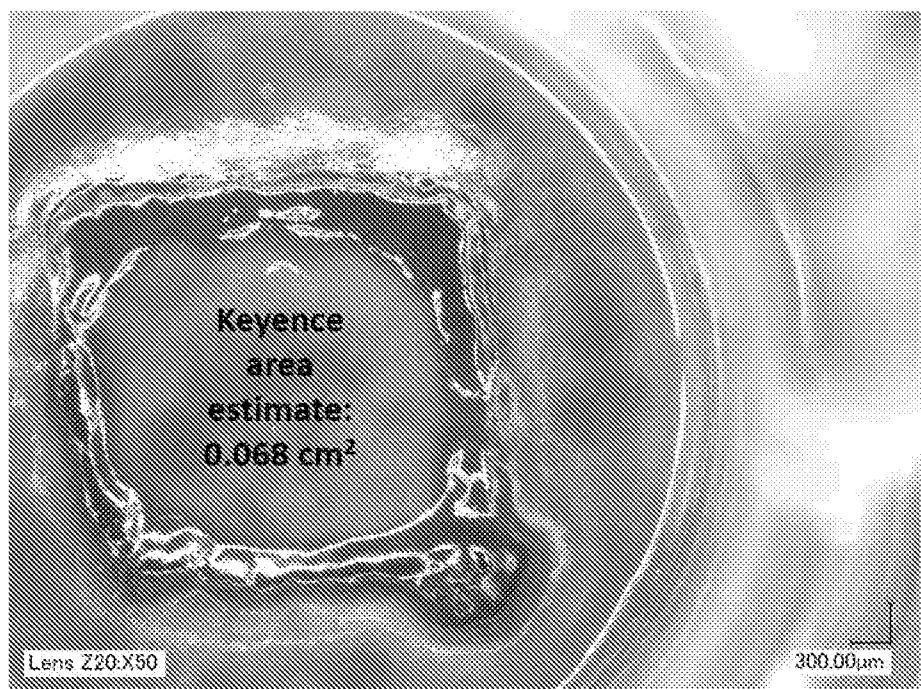
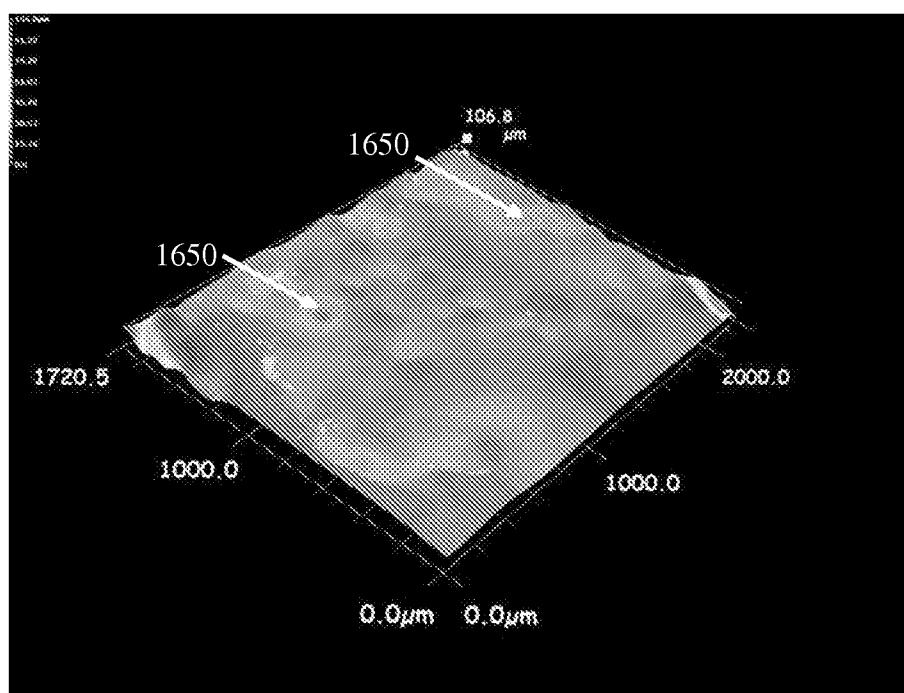
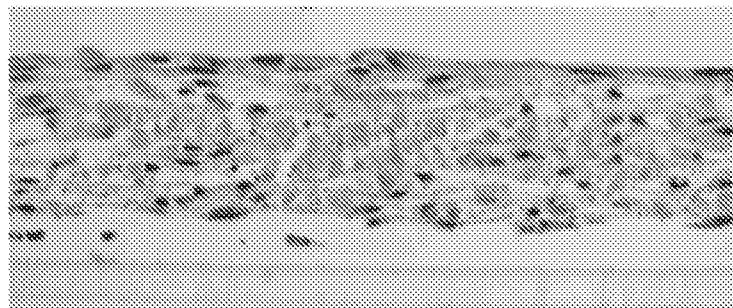
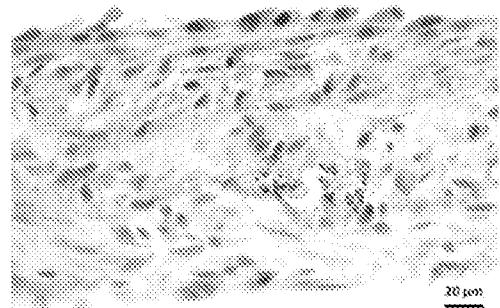
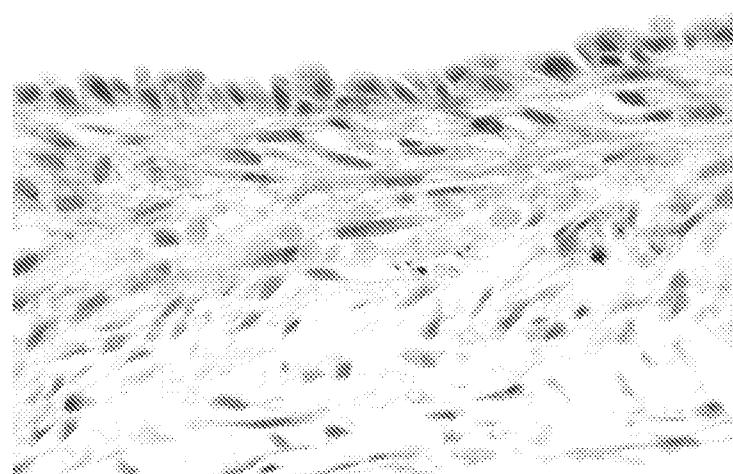
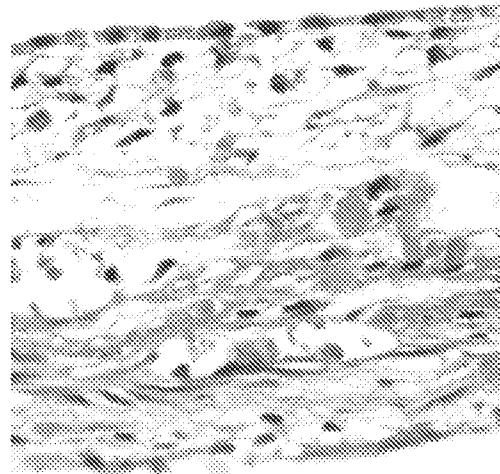


Fig. 16B

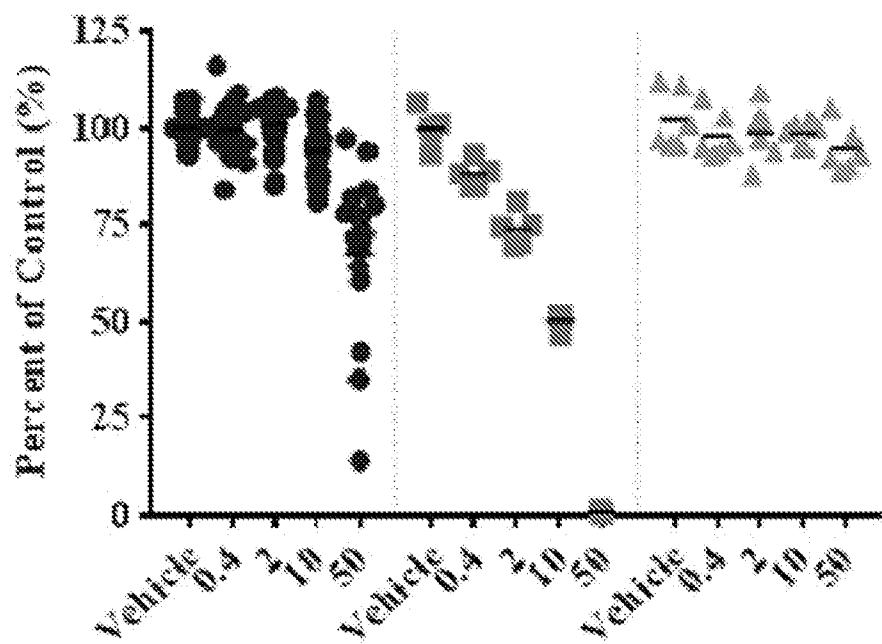


17/25

**Fig. 17A****Fig. 17B****Fig. 17C****Fig. 17D**

18/25

FIG. 18



19/25

Fig. 19A

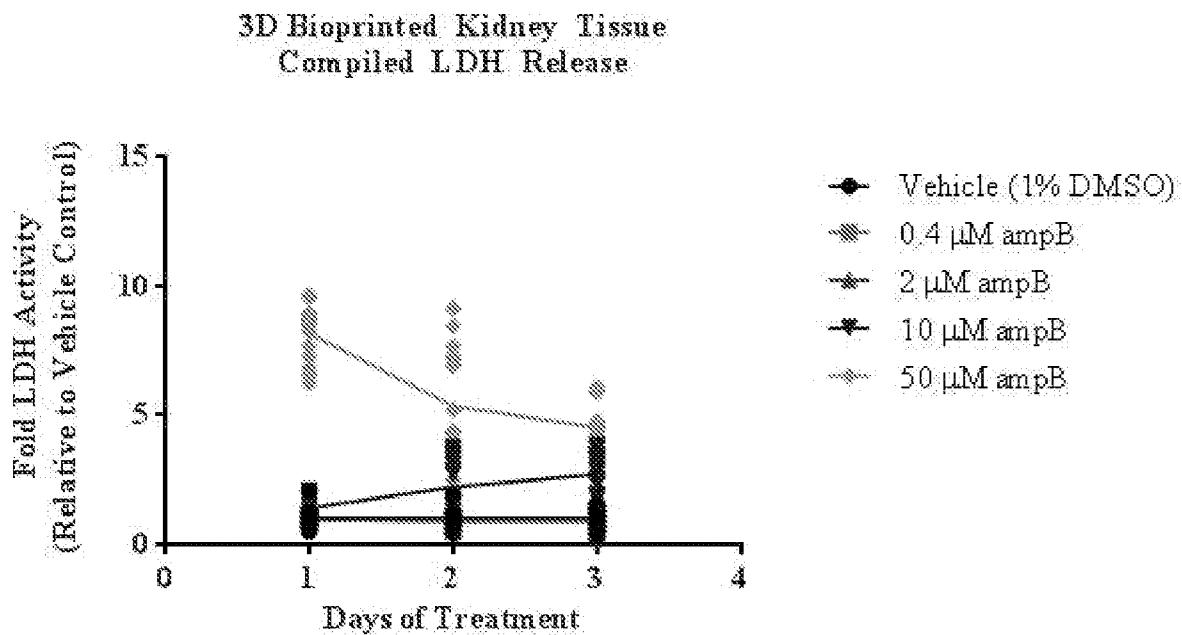
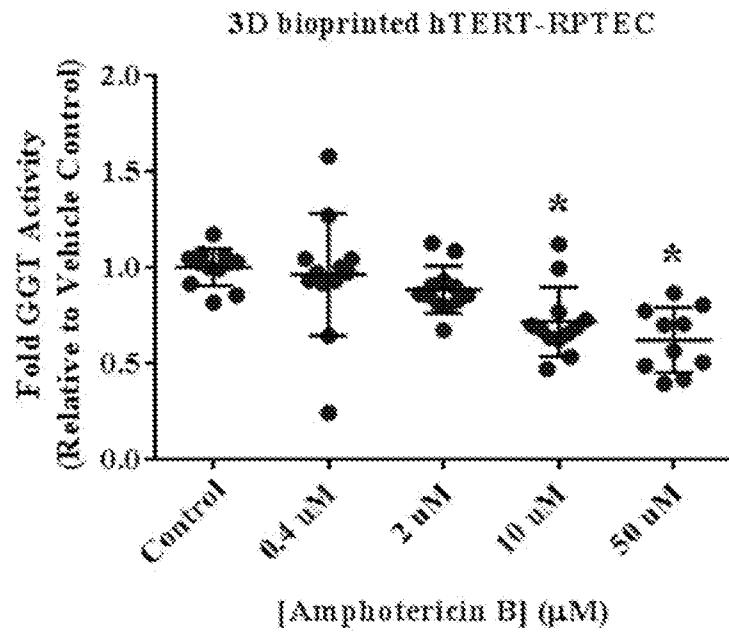
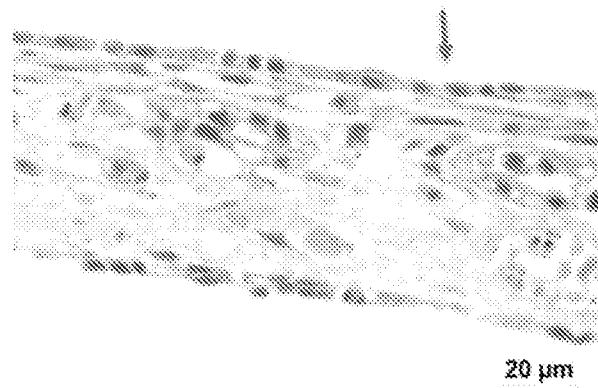
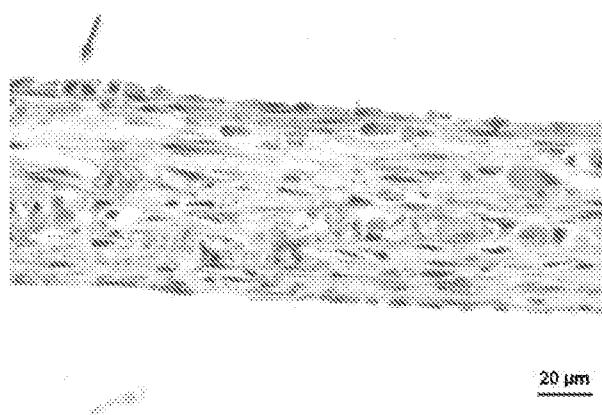
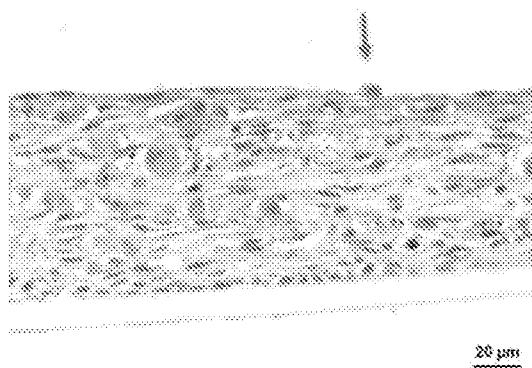


Fig. 19B



20/25

**Fig. 20A****Fig. 20B****Fig. 20C**

21/25

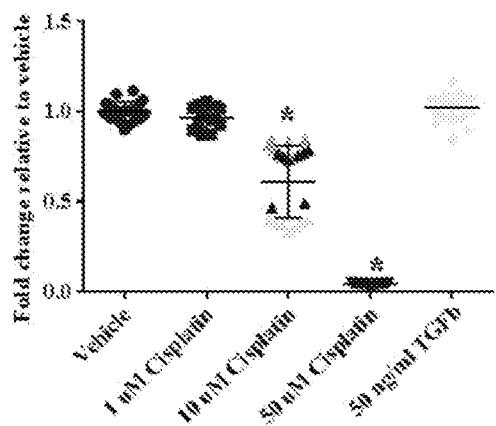


Fig. 21A

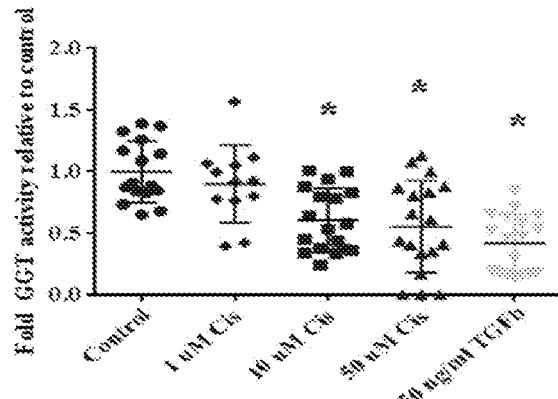


Fig. 21B

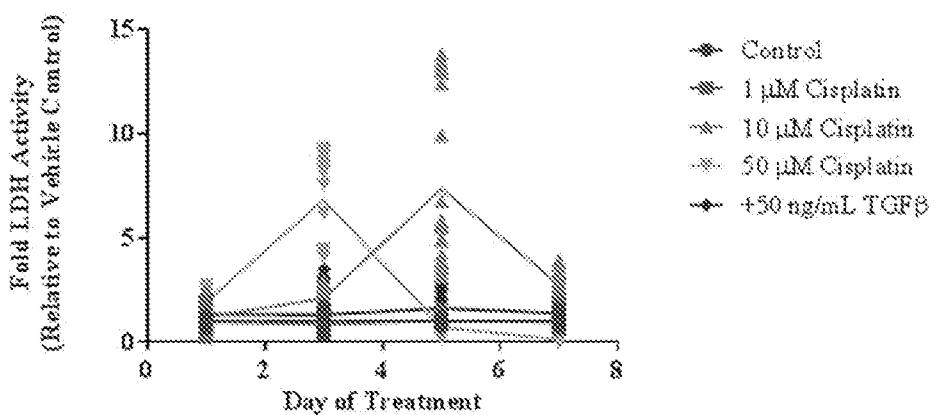
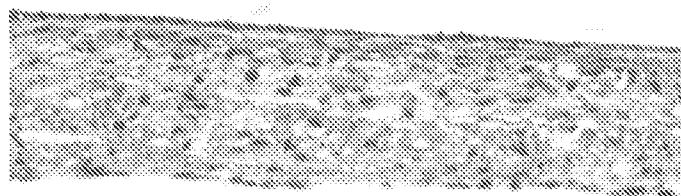
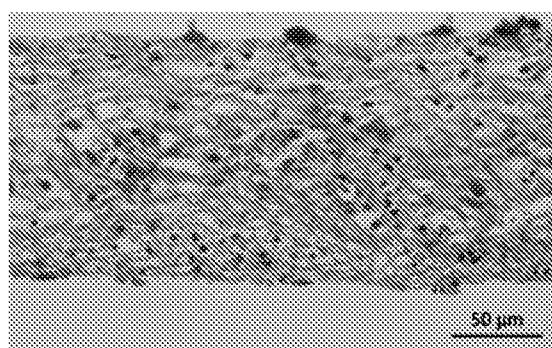
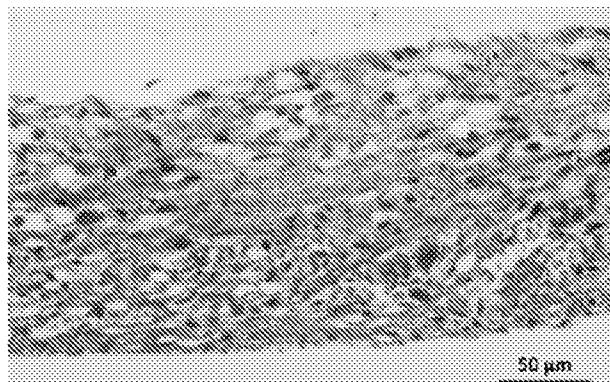
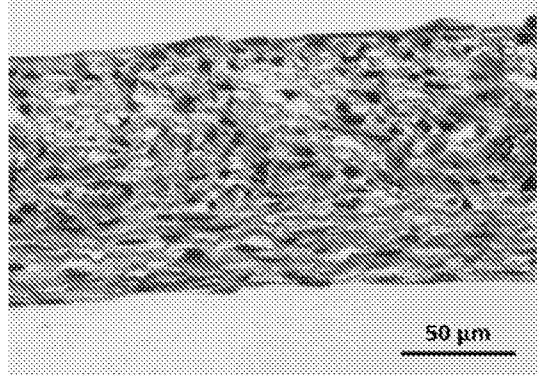


Fig. 21C

**Fig. 22A****Fig. 22B****Fig. 22C****Fig. 22D**

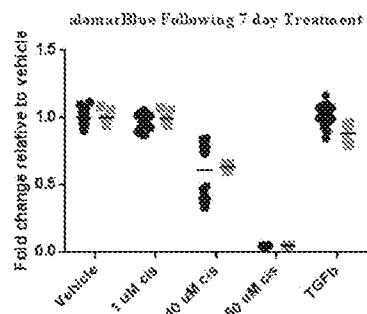


Fig. 23A

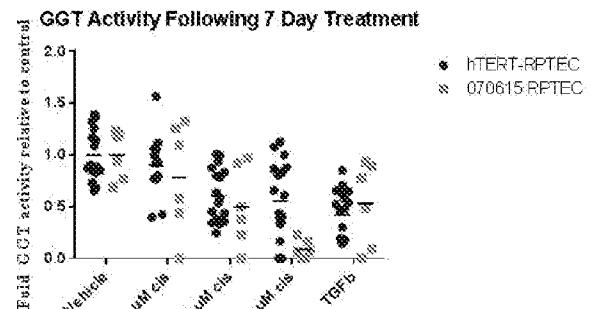


Fig. 23B

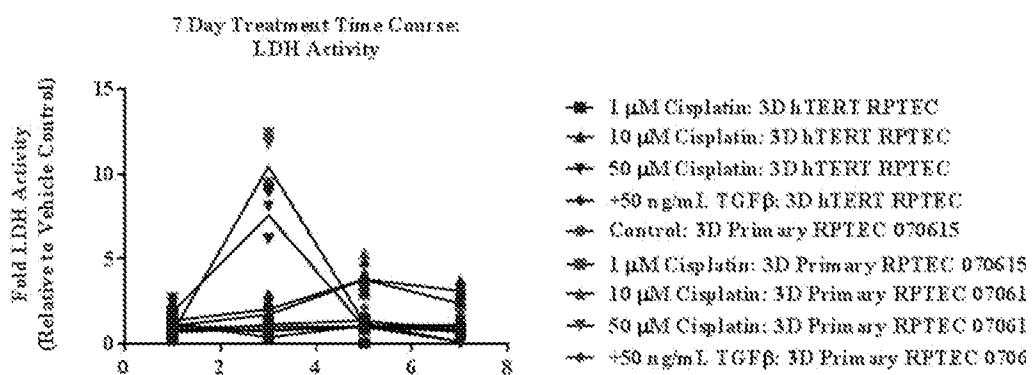


Fig. 23C

24/25

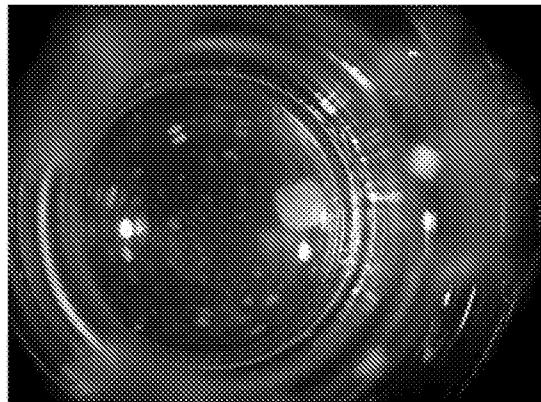


Fig. 24A

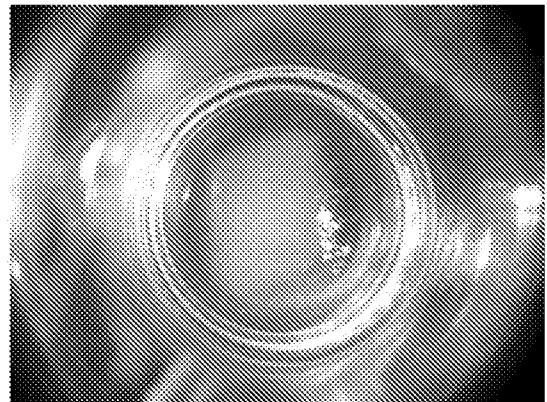
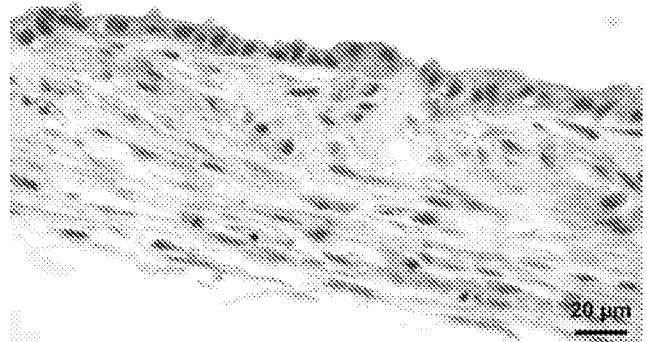
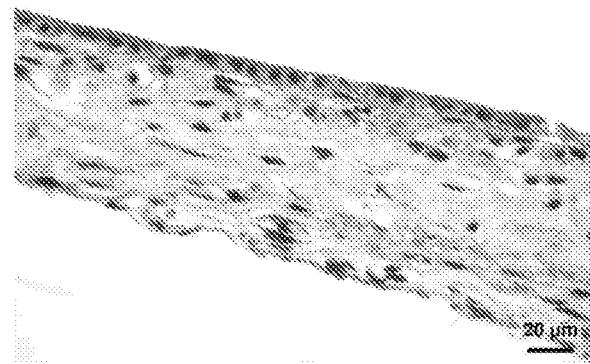


Fig. 24B

25/25

**Fig. 25A****Fig. 25B****Fig. 25C**