Journal of Visualized Experiments

Kidney organoid generation at the air-liquid interface

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61649R1
Full Title:	Kidney organoid generation at the air-liquid interface
Section/Category:	JoVE Developmental Biology
Keywords:	Kidney organoids; air-liquid interface; organotypic culture; Nephron; renal tissues; kidney progenitors; proximal tubule; endothelial cells
Corresponding Author:	Ashwani Kumar Gupta, Ph.D. University of Arizona Tucson, Arizona UNITED STATES
Corresponding Author's Institution:	University of Arizona
Corresponding Author E-Mail:	ashwanisgpgi@gmail.com
Order of Authors:	Ashwani Kumar Gupta, Ph.D.
	David Z. Ivancic
	Bilal Abdullah Naved
	Jason A. Wertheim
	Leif Oxburgh
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tucson, Arizona, United States of America

July 07, 2020

Dear Dr. DSouza,

We would like to thank the editor and the reviewers for reviewing our manuscript, JoVE61649. Please find our rebuttal letter and revised manuscript entitled “An efficient method to generate kidney organoids at the air-liquid interface” by Ashwani Kumar Gupta, David Z. Ivancic, Bilal Abdullah Naved, Jason A. Wertheim and Leif Oxburgh. There is no confidential information in the responses to the editor and the reviewers.

The manuscript has been revised as per suggestions. Rebuttal letter contains point-wise response to both the editor and the reviewers. I do hope that you will find this revised manuscript acceptable for publication.

I look forward to hearing from you.

Yours truly,

Ashwani Kumar Gupta, PhD
Research Assistant Professor,
Department of Surgery, College of Medicine,
University of Arizona, Tucson, Arizona 85724.
E mail: akgupta@surgery.arizona.edu
Phone: +1-207-213-7520

TITLE:

Kidney Organoid Generation at the Air-Liquid Interface

AUTHORS AND AFFILIATIONS:

Ashwani Kumar Gupta[1,2], David Z. Ivancic[1], Bilal Abdullah Naved[1,3], Jason A. Wertheim[1,2,3,4,5,6], Leif Oxburgh[7]

[1]Comprehensive Transplant Center, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
[2]Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
[3]Department of Biomedical Engineering, Northwestern University, Evanston, IL, USA
[4]Simpson Querrey Institute for BioNanotechnology, Northwestern University, Chicago, IL, USA
[5]Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA
[6]Department of Surgery, Jesse Brown VA Medical Center, Chicago, IL, USA
[7]The Rogosin Institute, New York, NY, USA

Corresponding authors:
Ashwani Kumar Gupta (akgupta@surgery.arizona.edu)
Leif Oxburgh (leo9022@nyp.org)

Email addresses of co-authors:
Ashwani Kumar Gupta (akgupta@surgery.arizona.edu)
David Z Ivancic (d-ivancic@northwestern.edu)
Bilal Abdullah Naved (bilal.naved@northwestern.edu)
Jason A. Wertheim (jwertheim@medadmin.arizona.edu)
Leif Oxburgh (leo9022@nyp.org)

KEYWORDS

kidney organoids, air-liquid interface, organotypic culture, nephron, renal tissues, kidney progenitors, proximal tubule, endothelial cells.

SUMMARY

This protocol describes asynchronous mixing of human embryonic stem cells derived kidney progenitors at the air-liquid interface to efficiently generate kidney organoids.

ABSTRACT

The prevalence of kidney diseases continues to increase worldwide, driving the need to develop transplantable renal tissues. The kidney develops from four major renal progenitor populations: nephron epithelial, ureteric epithelial, interstitial and endothelial progenitors. Methods have been developed to generate kidney organoids but few or dispersed tubular clusters in the organoids hamper its use in regenerative applications. Here, we describe a detailed protocol of asynchronous mixing of kidney progenitors using organotypic culture conditions to generate kidney organoids tightly packed with tubular clusters and major renal structures including endothelial network and functional proximal tubules. This protocol provides guidance in the

culture of human embryonic stem cells and their directed differentiation to kidney organoids. Our 18-day protocol provides a rapid method to generate kidney organoids that facilitate the study of different nephrological events including in-vitro tissue development, disease modeling and chemical screening. However, further studies are required to optimize the protocol to generate additional renal specific cells types, interconnected nephron segments and physiologically functional renal tissues.

INTRODUCTION

Chronic kidney disease (CKD) is a worldwide healthcare problem with 13.4% global estimated prevalence[1]. Approximately 10% of the adult population of the United States suffers from CKD[2]. There is no curative treatment available for patients with CKD except renal transplantation. The lack of availability of transplantable organs warrants research into technologies to understand how new kidney tissues can be generated. In recent years, procedures have been reported to generate kidney organoids[3,4] from human embryonic stem cells (hESCs) and human induced pluripotent cells but differentiation into off-target cells[5,6] and lack of dense tubules within kidney organoids limit their use in modeling renal diseases, in-vitro chemical screening and transplantable renal tissue generation.

We followed a previously published protocol to generate kidney progenitors from hESCs[3]. The kidney functions as a 3D organ and an appropriate 3D environment allows kidney progenitors to self-organize to form a kidney organoid[7]. A 3D organotypic culture condition was selected to generate kidney organoids because it supports vigorous growth and differentiation of mammalian embryonic kidneys[8]. Using this approach, hESCs derived kidney progenitors were aggregated at the air-liquid interface, to provide a 3D organotypic culture environment for differentiation.

During kidney development, several stages of differentiating cells coexist within the embryonic kidney[9], and their differentiation fate and spatial patterning along the nephron depends on the timing of their recruitment. To establish such a culture condition in-vitro, a method was developed for asynchronous mixing of kidney progenitors generated by directed differentiation[3]. Asynchronous mixing refers to the combination of two progenitor populations that are at different stages of differentiation. Directed differentiation cultures were staggered two days apart and newly differentiated cells were mixed with cells that have been cultured as aggregates in organotypic conditions for 2 days. Heterochronic mixing of two cell batches improve the fidelity of pluripotent stem cell-derived organoids[10].

Here, we provide an efficient method of asynchronous mixing of the kidney progenitors at the air-liquid interface that potentiates nephrogenesis to produce tightly packed nephron epithelia with more tubular clusters in kidney organoids. These kidney organoids were filled with glomerular podocytes, proximal tubules, distal tubules, stromal cells, connecting tubule or collecting ducts. The protocol yields a complex and extensive network of endothelial cells. In addition, proximal tubules in kidney organoids were mature and functional, showing endocytic function confirmed by alexa flour 488 (AF488) labeled dextran uptake. In this protocol, a step by step methodology of asynchronous mixing of kidney progenitors to generate kidney organoids is

presented which we recently published elsewhere[10].

PROTOCOL

Cell line WA09 (H9) was approved by the National Institutes of Health (registration number 0062) and was tested negative for mycoplasma infection.

1. Medium and plate preparation for hESCs culture

1.1 Dilute matrix (reduced growth factor basement membrane e.g., Geltrex) in DMEM/F12 (1:100) and add 1 mL/well in 2 wells of a 6 well plate (reagents utilized in the manuscript are summarized in the **Table of Materials**).

1.2 Incubate coated plate undisturbed at 37 °C for 1-2 h for effective coating.

1.3 Prepare 7 mL of culture medium by adding prewarmed basic culture medium (e.g., StemFit) supplemented with 100 ng/mL FGF2 and 10 µM Rock inhibitor Y-27632.

2. hESCs thawing and culture

2.1 Thaw a frozen vial containing 1 - 1.5 x 10^6 H9 cells. Rapidly swirl the vial in a 37 °C water bath until just a small sliver of ice is left. This takes approximately 90-120 s.

2.2 Triturate once with 1 mL micropipette and transfer to a 15 mL conical tube. Slowly drip 2 mL of the medium prepared in step 1.3, dropwise into the thawed cell suspension.

2.3 Spin the cell suspension at 1000 *x g* for 3 min to pellet the cells.

2.4 Discard the supernatant and resuspend the cell pellet in 1 mL of fresh culture medium (as prepared in step 1.3) and triturate 2 times. Afterwards, add 3 mL of additional culture medium making a total volume of 4 mL.

2.5 Add 2 mL of the cell suspension into each of the matrix coated wells of the 6 well plate and culture at 37 °C, 5% CO_2 in the incubator.

2.6 Change the medium after 48 h with fresh basic culture medium supplemented with 50 ng/mL FGF2 only (now Y-27632 is not required).

2.7 Change the medium after 48 h with basic culture medium supplemented with 25 ng/mL FGF2.

2.8 Split cells once they reach 70% confluency.

2.9 Passage the cells at 1:10 ratio (i.e. distribute cells from 1 well into 10 wells of 6-well-plate) by following steps 3.5 to 3.8 for the cell culture maintenance.

3. Plating hESCs for directed differentiation

3.1 Dilute matrix in DMEM/F12 (1:100) and add 1 mL/well into 3 wells each of two 6 well plates.

3.2 Incubate the coated plate, undisturbed at 37 °C for 1-2 h for effective coating.

3.3 Prepare 28 mL of prewarmed basic culture medium supplemented with 100 ng/mL FGF2 and 10 µM Rock inhibitor Y-27632.

3.4 Remove the medium from 1 well of the 6 well hESCs culture plate and then wash the cells once with DPBS. After washing, add 1 mL prewarmed cell detachment solution (e.g., Accutase) to detach the cells from the plate. The 2nd well can be used to freeze cells or to propagate cell culture.

3.5 Incubate in a 37 °C incubator for 10 min.

3.6 Triturate 3-4 times gently and then transfer the cells into a 15 mL conical tube.

3.7 Adjust the volume to 3 mL with medium. Take 10 µL cell suspension to count cells.

3.8 Spin the cell suspension at 1000 *x g* for 3 min to pellet the cells.

3.9 Discard the supernatant and resuspend the cell pellet in 1 mL of medium and triturate 2 times.

3.10 For the 1st batch of asynchronous mixing, plate 1.7 x 10^5 hESCs /well in 2 mL medium (Plate 1) and culture at 37 °C, 5% CO_2.

3.11 For the 2nd batch of asynchronous mixing, plate 0.45 x 10^5 hESCs/well in 2 mL medium in a 6 well plate (Plate 2) and culture at 37 °C, 5% CO_2.

3.12 Change the medium of both the plates after 48 h with fresh basic culture medium supplemented with 50 ng/mL FGF2 without Rock inhibitor Y-27632.

3.13 After 72 h, ensure that cells in Plate 1 is ~50% confluent. This is the correct time to start directed differentiation (proceed to section 4).

3.14 96 h after seeding, change the medium in Plate 2 with fresh basic culture medium supplemented with 25 ng/mL FGF2.

3.15 120 h after seeding, ensure that cells of the Plate 2 is ~50% confluent. This is the correct time to start directed differentiation (proceed to section 4).

4. Directed differentiation of hESCs into kidney progenitors (perform this procedure on both

batches of cells staggered 2 days apart) (Figure 1A)

4.1 The hESCs should be ~50% confluent at the start of the differentiation. Remove medium and wash the cells once with DPBS.

4.2 Add 2 mL of advanced RPMI 1640 containing 1x L-glutamine supplement (e.g., GlutaMax) and 8 µM CHIR into each well of the 6 well plate and culture at 37 °C, 5% CO_2. This will be day 0 of differentiation for Plate 1.

4.3 On day 2, change the medium with fresh advanced RPMI 1640 containing 1x L-glutamine and 8 µM CHIR.

4.4 On day 4, change the medium with fresh advanced RPMI 1640 containing 1x L-glutamine and 10 ng/mL Activin A.

4.5 On day 6, change the medium with fresh advanced RPMI 1640 containing 1x L-glutamine and 10 ng/mL Activin A.

4.6 On day 7, change the medium with fresh advanced RPMI 1640 containing 1x L-glutamine and 10 ng/mL FGF9.

4.7 After 9 days of this treatment protocol (day 9), ensure that the cells are differentiated to kidney progenitors and adopt renal vesicle like morphology (**Figure 2F**). Now this 2D culture is ready for transition into 3D culture at the air-liquid interface to generate kidney organoids. For Plate 1 – proceed to section 5. For Plate 2 – proceed to section 6.

5. Making kidney progenitor cell aggregates at the air-liquid interface

5.1 Prepare a 24 well plate for culture of cells at the air-liquid interface.

5.1.1 Prepare the medium containing APEL2, 1.5% PFHM II, 100 ng/mL BMP7, 100 ng/mL FGF9 and 1 µg/mL Heparin and add 1 mL/well into the 24 well plate.

5.1.2 Float polycarbonate membrane on the medium in each well of the 24 well plate, using sterile forceps. Make sure not to flood the filters - wells with partially or entirely submerged filters should not be used. Keep plate aside in the hood for later use.

5.2 Wash the wells of the Plate 1 two times with DPBS and add 1 mL of cell dissociation enzyme (e.g., TrypLe express) into each well of the 6 well plate.

5.3 Incubate the plate for 5 min at 37 °C. Triturate 3-4 times to disperse cell clusters into single cell suspension.

5.4 Neutralize the cell dissociation enzyme by adding 8 mL/well advanced RPMI 1640 with 1 mL

of FBS (Total 10% FBS) in a 50 mL conical tube.

5.5 Strain the cells through 40 µm strainer and use 10 µL of strained cell suspension to count the cells.

5.6 Centrifuge the cells at 300 *x g* for 5 min.

5.7 Resuspend the cells at 2.5 x 10^5 cells/µL in APEL2 medium containing 1.5% PHFM II.

5.8 Spot 2 µL of cell suspension onto the polycarbonate membrane floating on the medium in the 24 well plate. Spot 6-8 cell aggregates/membrane.

5.9 Culture for 2 days at 37 °C, 5% CO_2 in the incubator.

6. Asynchronous mixing of kidney progenitors to generate kidney organoids

6.1 Prepare 24 well plate to spot cells following step 5.1.

6.2 Harvest and count the kidney progenitors in the Plate 2 following steps 5.2 - 5.7.

6.3 Remove membranes one at a time from the 24 well plate seeded in step 5.8 and break the cell aggregates into small fragments using 200 µL micropipette by triturating 7-10 times.

6.4 Mix these small fragments with fresh kidney progenitors from Plate 2 at a 1:1 ratio. (e.g., mix fragments from 1 cell aggregate of 5 x 10^5 cells with 5 x 10^5 cells of newly differentiated kidney progenitors from Plate 2, resuspend in 4 µL and make two cell aggregates on the membranes).

6.5 Spot mixed cells on the membranes floating at the surface of the medium in a 24 well plate. 6-8 cell aggregates/the membrane can be spotted.

6.6 Change medium on day 13 with fresh prewarmed APEL2 containing 1.5% PHFM II without any growth factors.

6.7 Change medium every 48 h with APEL2 containing 1.5% PHFM II.

6.8 On day 18, image the organoids under a stereo microscope and proceed to section 8 for marker expression (**Table 1**) analysis.

7. Evaluation of dextran uptake by proximal tubule cells in kidney organoids

7.1 On day 18, remove the membrane from the wells of 24 well plate and transfer kidney organoids such that 1 organoid is placed in each well of a 'U' bottom low attachment 96 well plate.

7.2 Replace the medium with 200 µL fresh prewarmed APEL2 containing 1.5% PHFM II and 10 µg/mL 10,000 MW dextran conjugated with Alexa flour 488.

7.3 Incubate the organoids at 37 °C, 5% CO_2 in the incubator for next 24 h on shaker.

7.4 Stain the organoids using 'whole mount staining technique' described in section 8 for proximal tubule specific markers to evaluate dextran uptake in proximal tubule cells.

8. Whole mount immunofluorescence staining on kidney organoids

8.1 On day 18 of the differentiation, remove the membrane from the wells and transfer each organoid to a 'U' bottom 96 well plate.

8.2 Fix the organoids with 150 µL 4% paraformaldehyde (PFA) for 15 min at room temperature.

8.3 Remove the PFA and wash the organoids 3 times with DPBS.

8.4 Add 150 µL of 1% Triton X-100 and incubate for 10 min at 4 °C on a shaker.

8.5 Remove the Triton X-100 and wash the organoids 3 times with DPBS.

8.6 Add 150 µL blocking buffer (5% serum from a species that matches the secondary antibody's host species in DPBS) and incubate for 1 h at room temperature.

8.7 Incubate the organoids with primary antibodies diluted in blocking buffer at 4 ˚C overnight on shaker.

8.8 Remove the primary antibodies and wash the organoids with DPBS for next 8 h on shaker.

8.9 Incubate organoids with secondary antibodies diluted in blocking buffer at 4 ˚C overnight on shaker.

8.10 Remove the secondary antibodies and wash the organoids with DPBS for next 8 h on shaker.

8.11 Mount the organoids on glass slide and image under a fluorescent microscope.

REPRESENTATIVE RESULTS

This protocol describes asynchronous mixing of kidney progenitor cells differentiated from hESCs (H9) at the air-liquid interface to generate kidney organoids with reproducible results and high success rates. We followed a previously published protocol to differentiate hESCs into kidney progenitors[3] (**Figure 1**). The mix of cells that arises from the directed differentiation process is believed to represent the repertoire of developmental kidney progenitors that gives rise to different nephron segments, stromal cells, and vasculature of the fetal kidney. Further, we followed an organotypic culture condition i.e., air-liquid interface that was developed to culture

rodent kidney, which served as a logical starting point to generate human kidney tissue from hESCs. It has been reported that there is gradual contribution of the renal progenitors at various stages of differentiation process to the developing nephron[11]. The differentiation fate of each progenitor cell depends on the timing of its recruitment, dictating the spatial patterning of each cell along the nephron; Time–dependent Cell-fate Acquisition (TCA)[11,12]. To establish such a culture condition in-vitro, we mixed epithelializing kidney progenitors with newly differentiated kidney progenitors to improve epithelialization and tubule formation.

In our experience, it is crucial to start directed differentiation at ~50% confluence of hESCs (**Figure 2A**). Extensive cell death was observed when we started differentiation at less than 50% confluence. Differentiating cells became over confluent if we started differentiation at more than 50% confluence. On day 0, we started directed differentiation by treating cell cultures with CHIR 99021. On day 2 of CHIR 99021 treatment, hESCs colonies were broken apart and dispersed into single cells throughout the well of the 6 well plate (**Figure 2B**). On day 4, cells formed loosely dense clusters that represent an optimum time point to start activin A treatment to differentiate cells into intermediate mesoderm (**Figure 2C**). On day 7, cells started to form small clusters (**Figure 2D**) and on day 9, i.e., two days after FGF9 treatment, renal vesicle like clusters were formed (**Figure 2E,F**). FGF9 treatment helped in the survival and self-renewal of progenitors. We observed that the quantity of these renal vesicle like clusters are predictive of the potential of a culture to generate high quality kidney organoids. At this point, the kidney progenitors became ready to be transferred to the air-liquid interface to form 3D kidney organoids. On day 9, cells were harvested and spotted as cell aggregates on isopore membranes floating on medium in a 24 well plate to generate air-liquid interface (**Figure 1, Figure 2G**). On day 11, these cell aggregates were broken into small fragments and mixed with fresh kidney progenitors, i.e., asynchronous mixing of progenitors, and re-spotted at the air-liquid interface (**Figure 2H**). On day 13, these cell aggregates start to show tubularization (**Figure 2I**) and became filled with tubular structures by day 18 (**Figure 2J**). We supplemented the air-liquid interface culture medium with FGF9 and BMP7, which has been reported to facilitate nephron progenitor cell survival and self-renewal[13].

These kidney organoids derived from hESCs were densely packed with tubular clusters (**Figure 3A**) and H&E staining confirmed that organoids were packed with tubules containing lumens (**Figure 3B,C**) and presumptive glomerular structures with Bowman's spaces (**Figure 3C**). We found that asynchronous mixing of progenitors at the air-liquid interface generated kidney organoids filled with $CDH1^+$ epithelial structures connected with LTL^+ proximal tubules and $PODXL^+$ presumptive podocytes (**Figure 3D,G**). Lumens were also observed in LTL^+ & $HNF4A^+$ proximal (**Figure 3H**) and $CDH1^+$ & $BRN1^+$ distal tubules (**Figure 3I**). Asynchronous mixing of kidney progenitors at the air-liquid interface supports development of a $CD31^+$ endothelial network (**Figure 3E**) around presumptive $PODXL^+$ podocytes (**Figure 3J**) but capillary loops were absent which is a classical feature in the glomeruli. $MEIS1^+$ & $PDGFRB^+$ renal stromal cells were observed between tubular structures (**Figure 3F,K**). In addition to nephron structures, we also found $GATA3^+$ DBA^+ & $CDH1^+$connecting tubule or collecting ducts in these kidney organoids (**Figure 3L**). Proximal tubule plays an important role in selective absorption of solutes, vitamins, hormones, and amino acids. Therefore, we performed an in vitro dextran uptake assay to

examine whether proximal tubules in the kidney organoids exhibit physiologically relevant characteristics. We found that LTL^+ & $HNF4A^+$ proximal tubules were able to absorb AF 488 labeled dextran (**Figure 4B**).

Furthermore, we followed this asynchronous mixing protocol to generate kidney organoids in submerged culture condition. Kidney organoids generated under submerged culture condition had fewer LTL^+ proximal tubules, $CDH1^+$ epithelial structures, $PODXL^+$ podocytes and $CD31^+$ endothelial networks (**Figure S1**) in comparison to kidney organoids generated at the air-liquid interface. Comparing kidney organoids generated using both culture conditions, submerged culture condition was less efficient than air-liquid interface culture.

FIGURE LEGENDS

Figure 1: Schematic illustration of the protocol to generate kidney organoids by asynchronous mixing at the air-liquid interface. (**A**) Schematic representation showing directed differentiation of hESCs to kidney progenitors. hESCs were treated with 8 µM CHIR for four days, three days with 10 ng/mL activin A and then two days with 10 ng/mL FGF9 to differentiate into kidney progenitors. (**B**) On day 9, cell aggregates were spotted at the air-liquid interface (membrane floating on medium containing 100 ng/mL FGF9, 100 ng/mL BMP7 and 1 µg/mL heparin) for the next two days. On day 11, fresh progenitors (two days staggered from first batch of progenitors) were mixed with differentiating progenitors and spotted again at the air-liquid interface. Growth factors were removed from medium during day 13 to day 18 of differentiation.

Figure 2: Representative images showing morphological changes of hESCs during differentiation. (**A**) Directed differentiation of hESCs was started on day 0 when cell culture attains ~50% confluency. (**B**) On day 2, cells spread throughout wells and (**C**) on day 4, they formed small loosely dense cell clusters. (**C**) On day 7, cells start to form small clusters and (**D,F**) adopt renal vesicle like clusters (yellow arrowhead). (**G**) On day 9, kidney progenitors were aggregated at the air-liquid interface. (**H**) Asynchronous mixing was performed, and progenitors again were spotted at the air-liquid interface. (**I**) Cell aggregates started to show tubule formation and became (**J**) densely filled with tubular clusters on day 18.

Figure 3: Representative images showing morphology and immunological characterization of kidney organoids. (**A**) Representative stereo microscope image showing densely packed kidney organoid with tubular clusters. (**B,C**) Hematoxylin-eosin (H&E) staining of kidney organoid showing tubular structures with lumen (yellow star) and presumptive glomerular structures with Bowman's space (yellow arrow head). (**D**) Immunofluorescence staining showing $PODXL^+$ podocytes, $CDH1^+$ epithelial structures and LTL^+ proximal tubules. (**E**) $CD31^+$ endothelial networks were also observed throughout kidney organoids. (**F**) Further, tubular structures were surrounded by $MEIS1^+$ $PDGFRB^+$ renal stromal cells. (**G**) High magnification immunofluorescence image showing an interconnection between LTL^+ proximal tubule and $PODXL^+$ presumptive glomerular structures. (**H**) LTL^+ tubules also expressed HNF4A, an another marker for proximal tubules. (**I**) Distal tubules expressed BRN1 and CDH1 together. (**J**) $CD31^+$ endothelial networks were detected around $PODXL^+$ glomerular structures but capillary loops were absent. (**K)** $MEIS1^+$

PDGFRB$^+$ Renal stromal cells were found around tubular structures. (**L**) In addition, GATA3$^+$ DBA$^+$ & CDH1$^+$ connecting tubule or collecting ducts were also present in the kidney organoids.

Figure 4: Representative images showing fluorescent dextran uptake by proximal tubules in kidney organoids. Kidney organoids were incubated with dextran AF488 or AF488 only (control). (**A**) AF488 was not detectable in control organoids where AF488 was mixed with medium. (**B**) Dextran AF488 vesicles were observed in kidney organoids, 24 hours after incubation with dextran AF488 supplemented medium showing endocytic uptake by HNF4A$^+$ LTL$^+$ proximal tubules.

Figure S1: Representative images showing morphology and immunological characterization of organoids generated under submerged culture condition. Kidney organoids were generated using submerged culture condition to see the effect of culture at the air-liquid interface. In brief, kidney progenitors were aggregated on day 9 in U bottom low attachment 96 well plate by centrifugation at 300 g for 15 s and did asynchronous mixing on day 11. Cell count, medium composition, and timeline were kept same as in the air-liquid interface protocol. (**A**) Stereo microscope image of kidney organoid generated under submerged culture condition showing fewer tubular clusters. (**B**) Immunofluorescence image showing fewer LTL$^+$ proximal tubule, CDH1$^+$ epithelial structures and PODXL$^+$ Podocytes. (**C**) Furthermore, endothelial networks were not developed as we had found in air-liquid interface cultured kidney organoids. (**D**) MEIS1$^+$ PDGFRB$^+$ renal stromal cells were widespread in the kidney organoids.

DISCUSSION

Asynchronous mixing of progenitors at the air-liquid interface (**Figure 1**) presents an efficient method to generate kidney organoids from hESCs. This work describes stepwise protocols for thaw and culture of hESCs, directed differentiation to kidney progenitors, making cell aggregates at the air-liquid interface, asynchronous mixing of progenitors to generate kidney organoids tightly packed with tubular clusters and major renal structures including endothelial network and functional proximal tubules, dextran uptake by proximal tubule and whole mount immunostaining of kidney organoids. This protocol provides guidance in the culture of human embryonic stem cells and their directed differentiation into kidney organoids. Our 18-day protocol provides a rapid method to generate kidney organoids that will facilitate the study of different features of kidney biology, including in vitro tissue development, disease modeling and chemical screening.

Although our protocol efficiently generates high quality kidney organoids that can be used for in vitro modeling, certain aspects require further development. Epithelial structures such as proximal tubules, distal tubules, connecting tubules, or collecting ducts were densely represented, but markers for other nephron segments such as the ascending and descending limbs of the loop of Henle were absent. Bowman's space was also found in presumptive glomerular structures, but capillary loops were absent despite the widespread endothelial network. Interconnection between tubular segments were also primitive. Hence, it is important to further optimize this protocol to achieve more mature renal tissues with interconnected nephron segments and other kidney-specific cell types.

Most of the previous protocols employ the treatment with CHIR 99021 to induce the mesenchyme-to-epithelial transition[3,4,13,14]. CHIR 99021 is a potent inhibitor of GSK3 and is expected to have many effects on cellular pathways apart from activating Wnt/β-catenin signalling[10]. Our protocol omits the application of CHIR 99021 at the kidney progenitor epithelialization step, thus reducing the scope for unintended effects that may skew differentiation. In previous work, we evaluated the time frame for asynchronous mixing and found that mixing cells two days after differentiation of the first batch is the optimum time to add fresh progenitors[10].

During kidney development, the ureteric bud tip acts as the epithelial inducer for metanephric mesenchyme, ensuring appropriate placement of new nephrons within the collecting duct system. Taguchi et al. recreated this developmental interaction by inducing stem cells to differentiate into metanephric mesenchyme and ureteric bud cells separately[15]. In contrast, the mixture of cells generated through our directed differentiation strategy does not require addition of ureteric bud or small molecule substitutes for Wnt signaling to induce nephron differentiation. We hypothesize that the $GATA3^{+}DBA^{+}$&$CDH1^{+}$structures that differentiate in our organoids, may act as an inducer for nephron epithelial differentiation, and further studies will be required to determine if this is indeed the source of Wnt induction in our differentiation system.

Certain critical points should be considered while generating kidney organoids using this protocol. Visual monitoring of cultures is important to ensure preparation of high quality organoids. At day 4 of directed differentiation, cultures should be visually confirmed to have loosely dense clusters before Activin A treatment is started. In the absence of these loosely dense clusters, it is recommended that directed differentiation to be started again from a new hESCs culture. Similarly, on day 9, it is important that cultures display renal vesicle like cell aggregates for efficient generation of kidney organoids. In the absence of this morphology, FGF 9 treatment should be continued for another 2 days until the culture displays the appropriate morphology. On day 18, organoids should be evaluated under a stereo microscope and differentiation can be continued for the next 3-4 days in the absence of densely packed tubular clusters. These considerations are important and should be kept in mind to improve the fidelity of hESCs-derived organoids.

ACKNOWLEDGMENTS

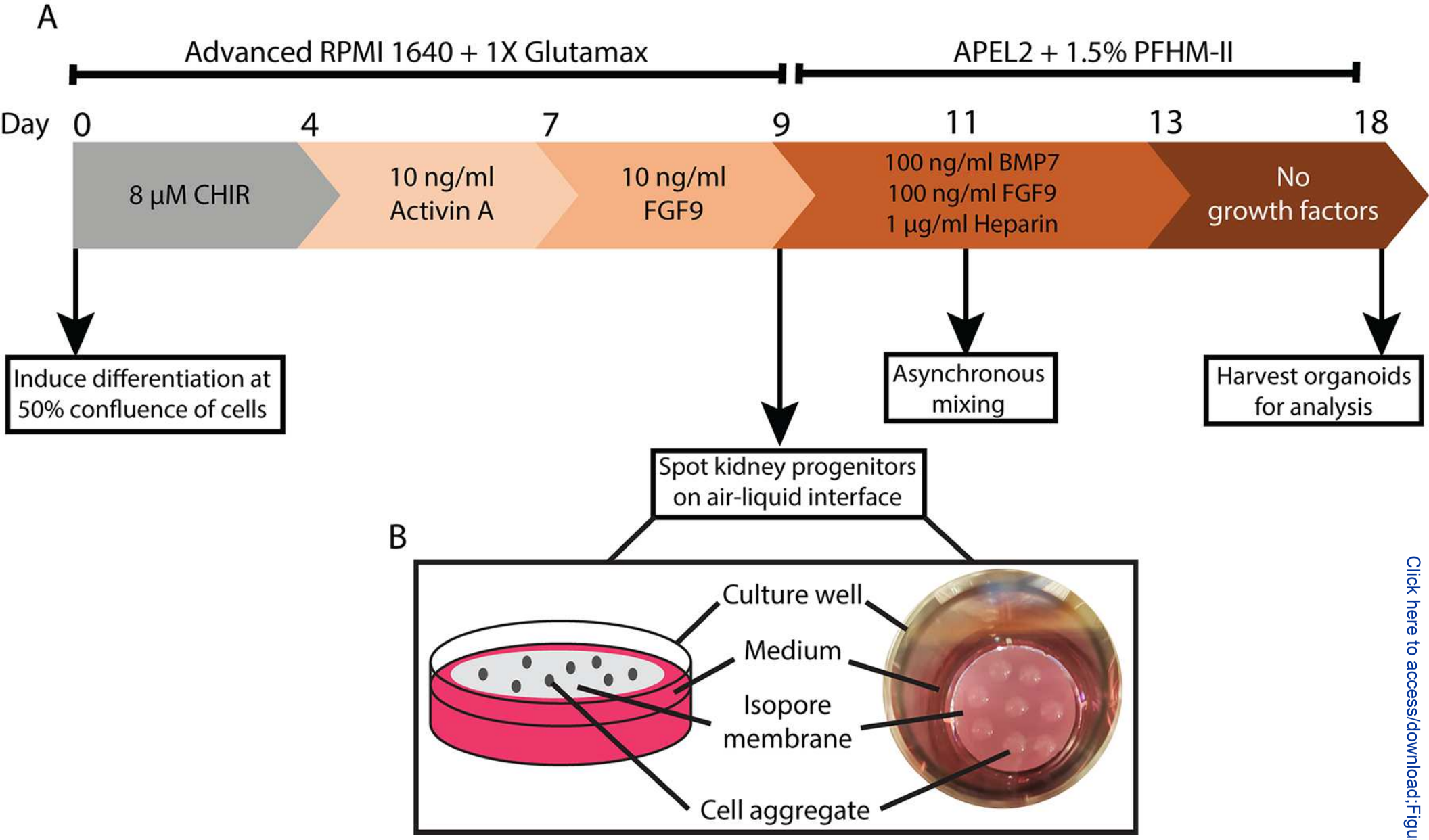
This work was supported in part by Merit Review Award #01BX002660 from the United States Department of Veterans Affairs, Biomedical Laboratory Research and Development Service to Jason A. Wertheim, National Institutes of Health grant number R24 DK106743 to Leif Oxburgh and National Institute of Diabetes and Digestive and Kidney Diseases award number F30DK123985 to Bilal Abdullah Naved. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health, the Department of Veterans Affairs, or the United States Government. The authors would like to acknowledge the Northwestern University Biological Imaging Facility, for fluorescent microscope imaging.

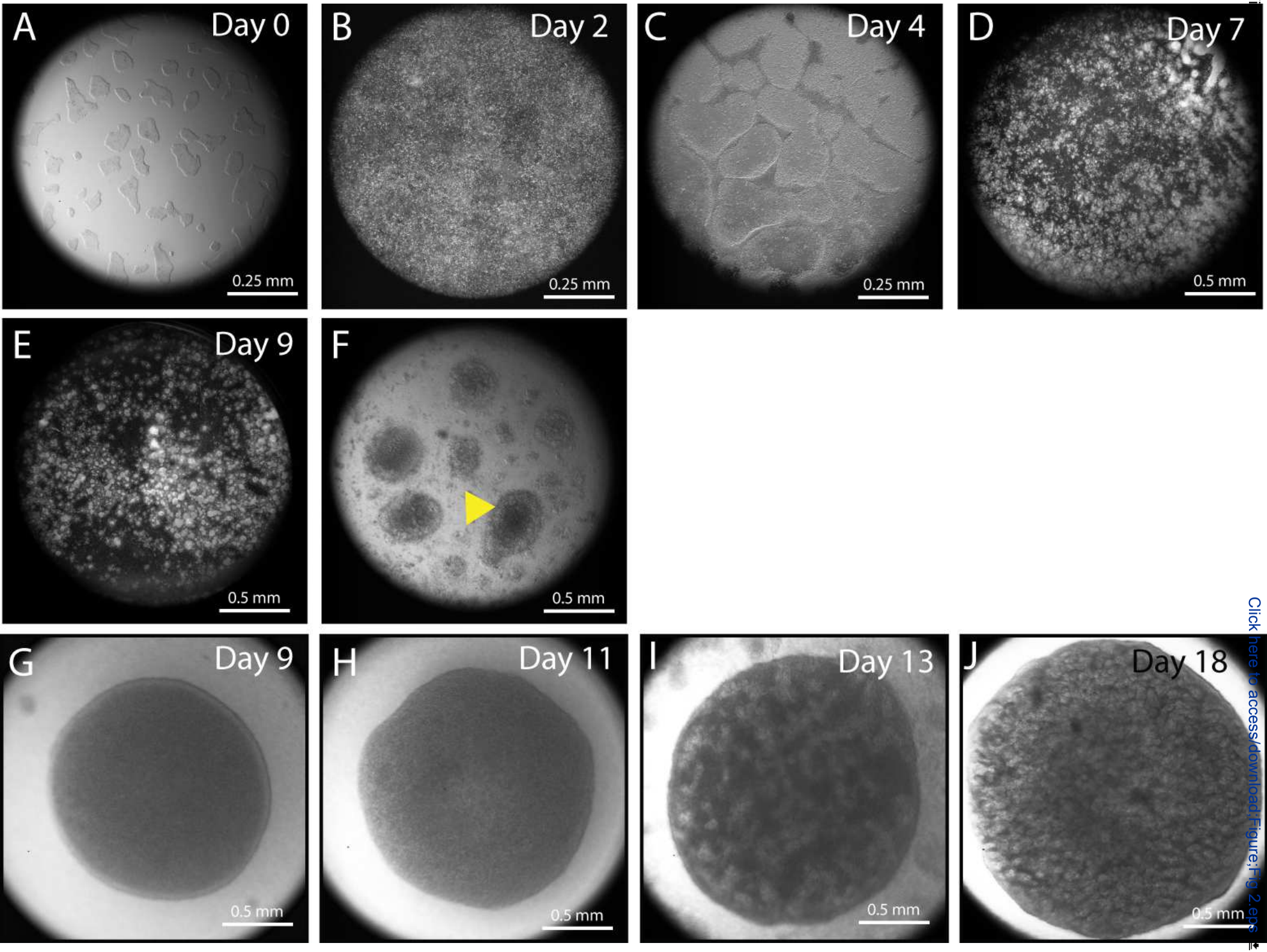
DISCLOSURES

The authors declare that they have no competing interests.

REFERENCES

1. Lv J. C., Zhang L. X. Prevalence and disease burden of chronic kidney disease. *Advances in Experimental Medicine and Biology*. **1165**, 3-15 (2019).
2. Oxburgh, L. (Re)Building a Kidney. *Journal of the American Society of Nephrology*. **28** (5), 1370-1378 (2017).
3. Morizane, R. et al. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nature Biotechnology*. **33** (11), 1193-1200 (2015).
4. Takasato, M. et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature*. **526** (7574), 564-568 (2015).
5. Wu, H. et al. Comparative Analysis and Refinement of Human PSC-Derived Kidney Organoid Differentiation with Single-Cell Transcriptomics. *Cell Stem Cell*. **23** (6), 869–881 (2018).
6. Gupta, A. K. et al. Scaffolding kidney organoids on silk. *Journal of Tissue Engineering and Regenerative Medicine*. **13** (5), 812-822 (2019).
7. Takasato, M., Er, P. X., Chiu, H. S., Little, M. H. Generation of kidney organoids from human pluripotent stem cells. *Nature Protocols*. **11** (9), 1681-92 (2016).
8. Grobstein, C. Inductive epitheliomesenchymal interaction in cultured organ rudiments of the mouse. *Science*. **118** (3054), 52-55 (1953).
9. Oxburgh, L. Kidney Nephron Determination. *Annual Review of Cell and Developmental Biology*. **34**, 427-450 (2018).
10. Gupta, A. K. et al. Asynchronous mixing of kidney progenitor cells potentiates nephrogenesis in organoids. *Communications Biology*. **3**, 231 (2020).
11. Lindström, N. O. et al. Progressive Recruitment of Mesenchymal Progenitors Reveals a Time-Dependent Process of Cell Fate Acquisition in Mouse and Human Nephrogenesis. *Developmental Cell*. **45**(5), 651-660 (2018).
12. Saxén, L., Sariola, H., Lehtonen, E. Sequential cell and tissue interactions governing organogenesis of the kidney. *Anatomy and Embryology*. **175** (1), 1-6 (1986).
13. Brown, A. C., Muthukrishnan, S. D., Oxburgh, L. A synthetic niche for nephron progenitor cells. *Developmental Cell*. **34** (2), 229-241 (2015).
14. Subramanian, A. et al. Single cell census of human kidney organoids shows reproducibility and diminished off-target cells after transplantation. *Nature Communications*. **10** (1), 5462 (2019).
15. Taguchi, A., Nishinakamura, R. Higher-Order Kidney Organogenesis from Pluripotent Stem Cells. *Cell Stem Cell*. **21** (6), 730-746 (2017).





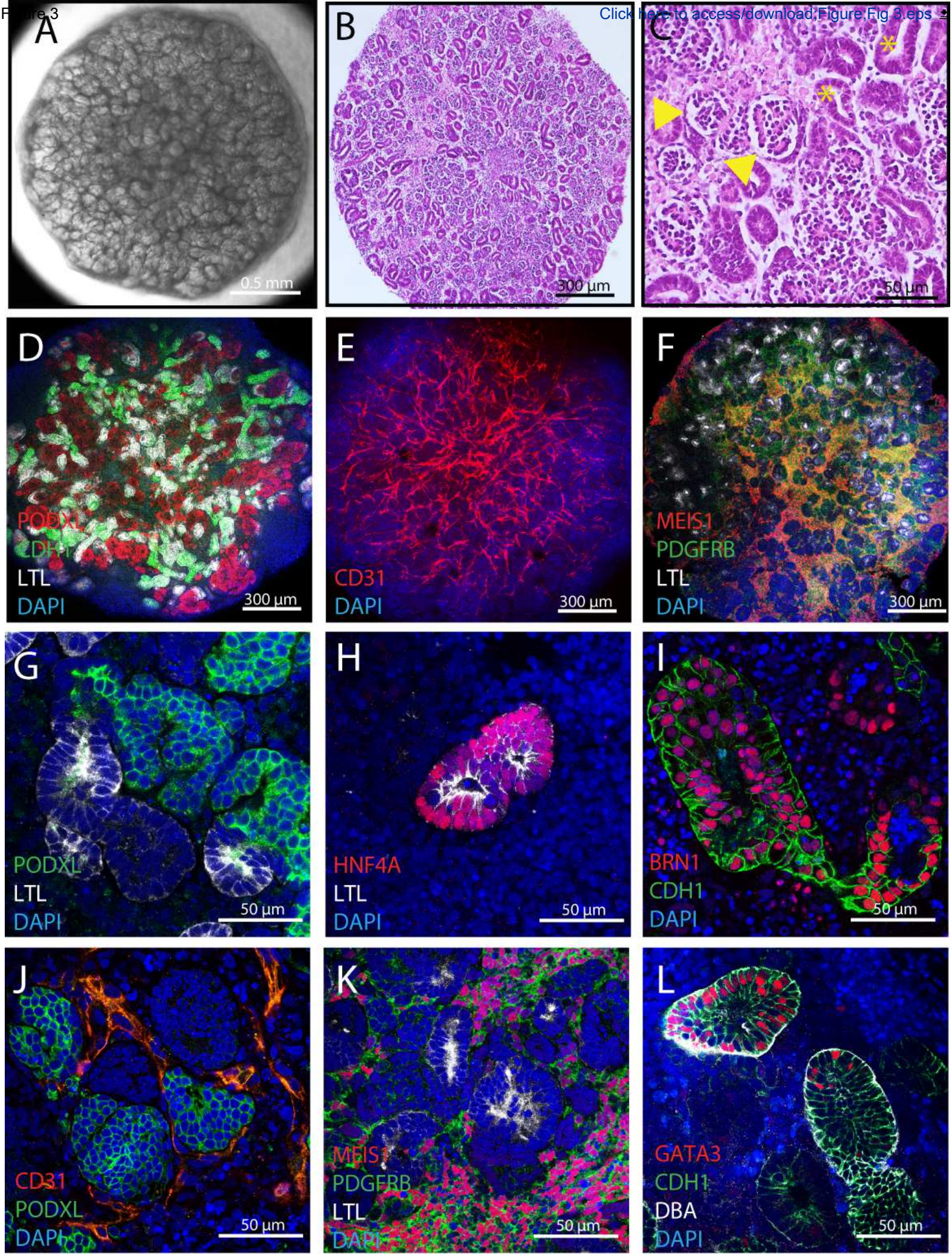
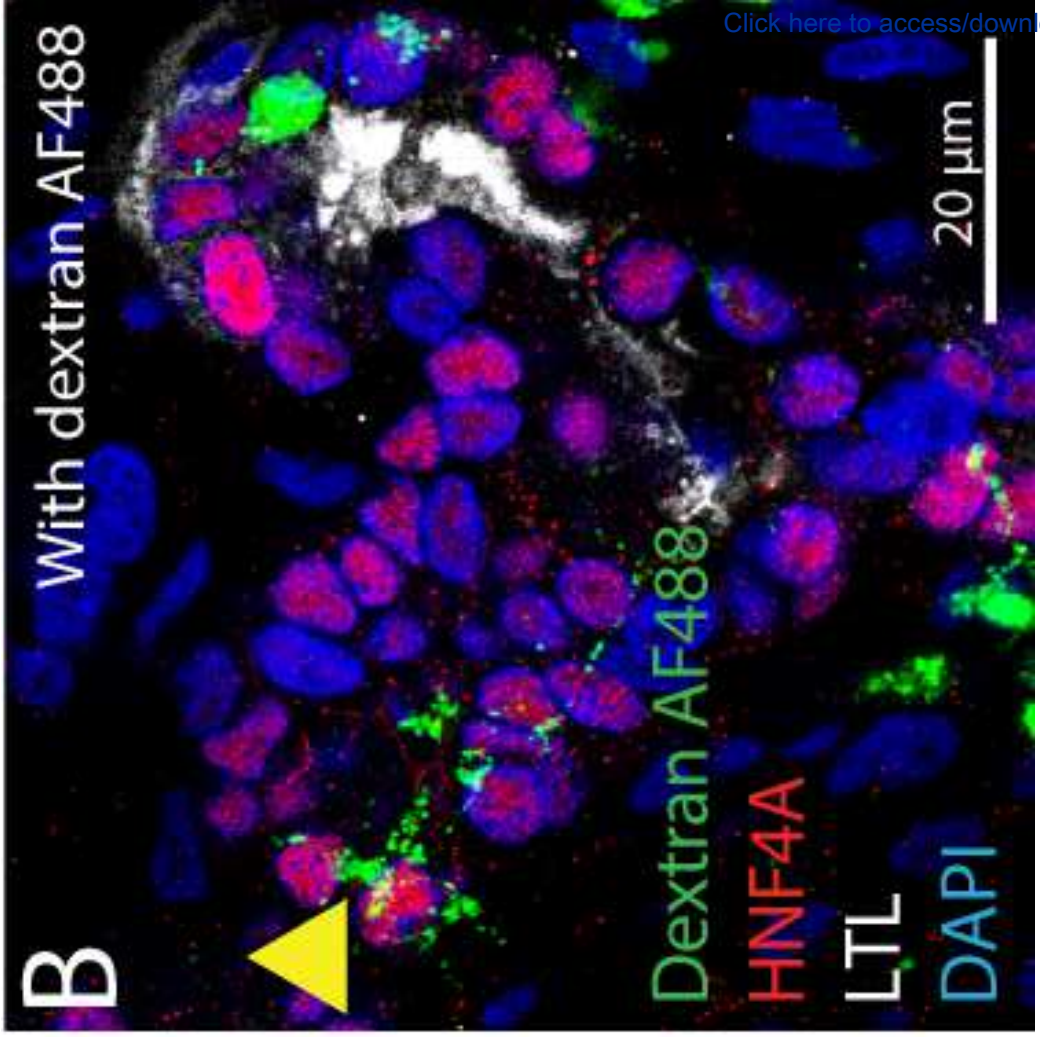


Figure 4



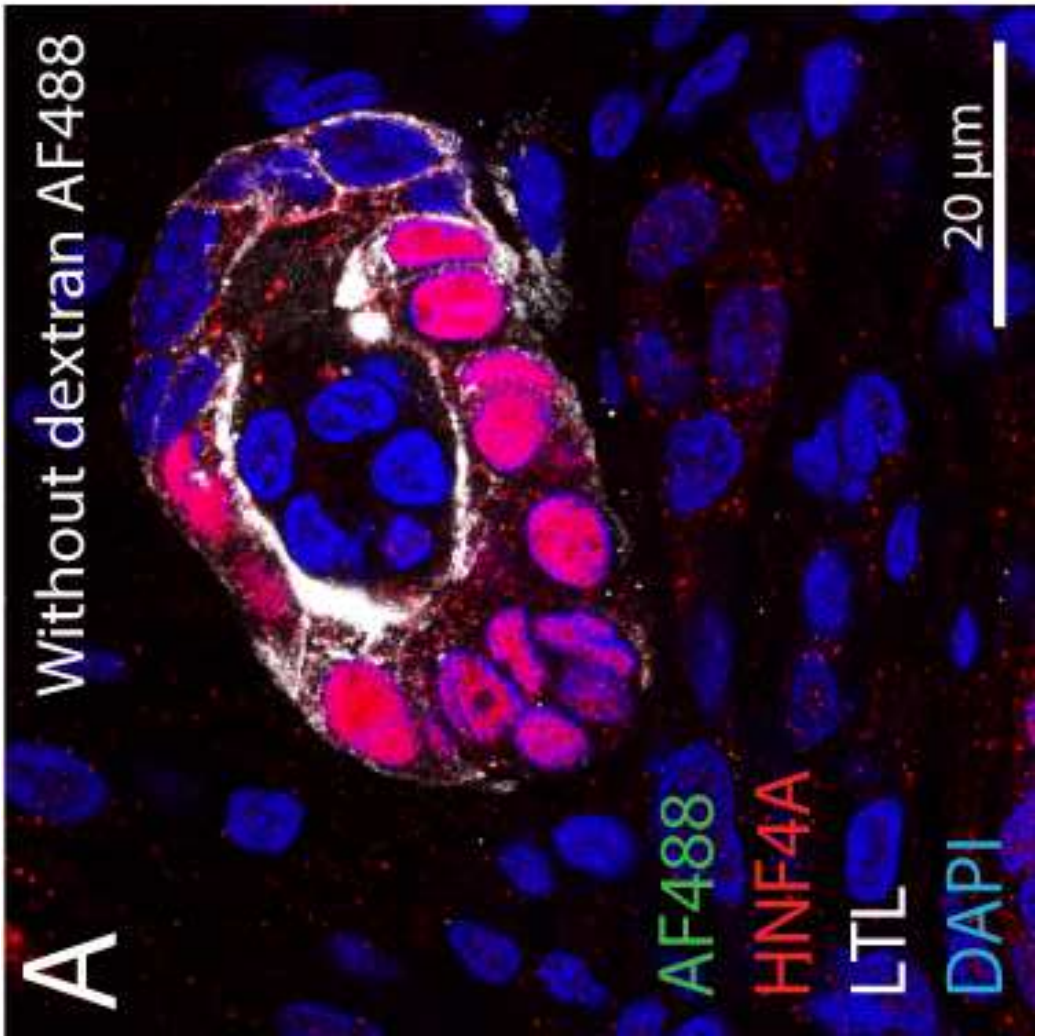


Table 1: Antibodies to identify various renal structures/cell types in the kidney organoids.

S. N.	Antibodies/lectins	Renal structures/cell types
	BRN1	Distal tubules
	CDH1	Epithelia, distal tubules and collecting ducts
	CD31	Endothelial cells
	DBA	Collecting ducts
	GATA3	Collecting ducts
	HNF4A	Proximal tubules
	LTL	Proximal tubules
	Meis1	Renal stromal cells
	PDGFRB	Renal stromal cells
	Podocalyxin	Podocytes

Name of Material/Equipment	Company	Catalog Number	Comments/Description
15 mL falcon tube	VWR	62406-200	
24 well plate	VWR	29443-952	
40 micron strainer	VWR	21008-949	
50 mL falcon tube	VWR	21008-940	
6 well plate	VWR	29442-042	
96 well Clear Round Bottom Ultra-Low Attachment Microplate (U bottom)	Corning	7007	
Accutase	STEMCELL Technologies	7920	Store at -20 ^{0}C
Activin A	R&D systems	338-AC-010	Aliquot and store at -20 ^{0}C
Advanced RPMI 1640	ThermoFisher Scientific	12633-012	Store at 4 ^{0}C
APEL2	STEMCELL Technologies	5270	Store at -20 ^{0}C
BMP7	R&D systems	354-BP-010	Aliquot and store at -20 ^{0}C
CHIR99021 IN SOLUTION	Reprocell	04-0004-10	Aliquot and store at -20 ^{0}C
Confocal microscope	Leica	SP8	
Dextran, AF 488, 10,000 MW	Thermo fisher Scientific	D22910	Store at -20 ^{0}C
DMEM/F12	Thermo fisher Scientific	11330-032	Store at 4 ^{0}C
DPBS	VWR	45000-434	
FBS	Atlanta Biologicals	S11550	Store at -20 ^{0}C
FGF2	R&D systems	234-FSE-025	Aliquot and store at -20 ^{0}C

FGF9	R&D systems	273-F9-025	Aliquot and store at -20 0C
Forceps	Roboz	RS-5040	
Geltrex	Thermo fisher Scientific	A1413301	Aliquot and Store at -20 ^{0}C
Glutamax	Thermo fisher Scientific	35050061	
H9 cells	WiCell	WA09	Store in liquid Nitrogen
Heparin	Sigma	H3393-25KU	
Isopore membrane	EMD Millipore	VCTP01300	
Paraformaldehyde	P6148-500G	P6148-500G	
PFHM II	Thermo fisher Scientific	12040077	Store at 4 ^{0}C
Rock inhibitor Y-27632	EMD Millipore	688002-1mg	Aliquot and store at -20 ^{0}C
StemFit Medium	amsbio	SFB-500	Store at -20 ^{0}C
Triton X-100	Sigma	X100-100ML	
TrypLe express	Thermo fisher Scientific	12563029	

Authors Response to Editor and Reviewers

We would like to thank the Editor and Reviewers for their time to go through the manuscript and suggesting the needful corrections. Kindly find below point wise response to the comments of the Editor and Reviewers.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response to the Editor: We have proofread the manuscript and have ensured that there are no spellings and grammatical errors in our revised manuscript.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response to the Editor: The Discussion has been restructured as suggested by the editor in our revised manuscript.

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are geltrex, cryovial, StemFit, accutase, 1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response to the Editor: All Commercial sounding language has been replaced by their generic names that are not company-specific and have been referenced in the table of materials/reagents.

• Table of Materials: Sort the list alphabetically.

Response to the Editor: We have sorted the Table of Materials alphabetically as suggested by the editor in our revised manuscript.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a

previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from anemail/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response to the Editor: All the figures and tables included in our manuscript are original and have not been published previously.

Comments from Peer-Reviewers:

Reviewer #1:

Major Concerns:
The protocol is disorder. I think it is too difficult to understand the protocol. (especially "4.") But this is a key point of this paper. Could you please think the way to organize explanation it?

Response to the Reviewer: We have reorganized the protocol in our revised manuscript.

I do not know why 10kDa dextran uptake represent of glucose uptake. Could you please refer the publication.

Response to the Reviewer: We wanted to evaluate the endocytic function of the proximal tubule by dextran uptake, which is a polymer of glucose. Now, the term “glucose uptake” has been removed from our manuscript to eliminate any confusion. Takasato et al, Nature, 2015 have used this technique to show the endocytic property of proximal tubules in their organoids.

What is a meaning of asynchronous mixing. Could you please define two group situation individually?

Response to the Reviewer: Asynchronous mixing here refers to the mixing of the two progenitor populations when they are at their different stages of differentiation. One population consists of the epithelializing kidney progenitors while the other population consists of the newly differentiated kidney progenitors. We established such culture conditions *in-vitro* to mimic the conditions occurring during kidney development because, during kidney development, several stages of differentiating cells coexist within the embryonic kidney, and their differentiation fate and spatial patterning along the nephron depends on the timing of their recruitment; this phenomenon is referred to as Time–dependent Cell-fate Acquisition (TCA). The term asynchronous mixing has now been clearly defined in the Introduction section of our revised manuscript.

Minor Concerns:

Why is isopore membranes with floated on medium in dishes? Can it maintain the air-liquid border?

Response to the Reviewer: The isopore membranes are hydrophilic polycarbonate membranes with pore sizes of 0.1 µm and are floated on the medium in the dishes to maintain the air-liquid interface. Yes, it maintains an air-liquid border and allows the diffusion of medium throughout the organoid that is necessary for its continuous growth.

At 2.8 section, it also used accutase?

Response to the Reviewer: Accutase is the cell-detachment solution with proteolytic and collagenolytic enzyme activity, often used in cell-cultures to disaggregate the cell colonies into single cells. However, the word Accutase has been replaced with its generic term in our revised manuscript so that it can be easily understood by our readers.

For dissociation of pellets, authors always used "triturate". Is it OK?

Response to the Reviewer: Trituration is a widely used term to break up cell clumps by gentle pipetting up and down. It is often done to resuspend cell pellets in the medium.

Why were cells spotted on membrane? Were they fused during cultivation?

Response to the Reviewer: Cells were not fused during cultivation. They were spotted on the isopore membrane as cellular aggregates to generate kidney organoids. The cells were spotted by pipetting a small volume (2µl) of the dense slurry of cells into a droplet ($2.5x10^5$ cells/µl) on the isopore membrane which aggregates as small cell clusters over the course of time. Spotting cells puts the nephron progenitor cells into close 3D proximity to each other that results in more successful kidney tissue formation.

Reviewer #2:

Minor Concerns:
1. As this is meant as a resource, a table that identifies the various renal compartments in the organoids as well as suggested antibodies to stain for these compartments would add significantly to the manuscript.

Response to the Reviewer: As suggested by the reviewer, we have now incorporated the table summarizing the various renal compartments and the antibodies used to stain them in our revised manuscript. Thank you for this useful suggestion.

Reviewer #3:

Minor Concerns:

From the abstract and introduction, it was clear that "asynchronous mixing" is an important part of this protocol, and the methods went into how to do it, but still before there was any explanation of what it actually meant. I strongly advise that an extra paragraph be added to the end of the introduction to explain what this 'asynchronous mixing' means, so that a reader has a clear idea of what is intended to be achieved before reading the methods. I find the use of the word 'asynchronous' somewhat bizarre when you just mean you add extra progenitors at one later time-point, but I guess your earlier Communications paper has locked you into it.

Response to the Reviewer: We have now added an extra paragraph in the Introduction section of our revised manuscript describing the term 'asynchronous mixing' so that readers have a clear idea of what is intended to be done in the methodology.

Also in the introduction, there is a claim of the PTs being "more mature and functional". More than what? (the assays have been used for others for their systems and also show 'function'). It is important that the comparison is made clear here.

Response to the Reviewer: The term 'more' has been removed from the text in the manuscript because we did not directly compare organoids generated at the air-liquid interface with the submerged cultured condition.

There is yellow highlighter from line 133 onwards for a couple of pages. I don't know why. It should probably be removed.

Response to the Reviewer: JOVE has instructed to highlighted protocol in yellow, to derive a script for video production.

Line 264 - please refer ahead when mentioning 'wholemount staining technique' to assure/ warn the reader that you have a specific technique in mind and it will be explained later.

Response to the Reviewer: We have corrected sentence in the revised manuscript as suggested by the Reviewer.

Line 270 - do you mean 'to' a 96-well plate?

Response to the Reviewer: Yes, we meant to transfer each organoid to a well of U bottom of a 96-well-plate. This has been corrected in our revised manuscript.

Line 300 - insert 'a' before 'previously'. It would also be helpful in this sentence to have a citation to this previously published method and to mention the name of the first author (since everyone thinks of these by names like 'the Takasato protocol', 'the Taguchi protocol' etc.).

Response to the Reviewer: We have now inserted 'a' before 'previously' and added a citation.

Line 306 needs a citation (Saxen/ Grobstein probably).

Response to the Reviewer: We have now cited the literature by Saxen et al. (1986) and Lindstrom et al. (2018).

Line 307 - Ref 10 seems an odd one for this statement. Lauri Saxen refers to it in his 1986 book and I am fairly sure that Edith Potter does in her 1972 book (but I cannot get to my office under lockdown to check). If you mean something otherthan that there is a gradual recruitment from the pretubular aggregate to the already epithelializing clump, please explain more fully what you mean. In fact, these 2 sentences are at the heart of the decision to use an 'asynchronous' protocol and I really think you need to expand them. Not every reader of this JOVE paper will have read your Communications paper (and itcan come across as arrogant to appear to assume that they have).

Response to the Reviewer: Thank you for this thoughtful suggestion. We have replaced reference 10 by Lindstrom et al (2018) in our revised manuscript. As suggested by the reviewer, we have elaborated the reason behind choosing the syntax 'asychronous mixing' for making kidney organoids. By doing so, we aim to make it clearer to the readers that we established such culture conditions *in-vitro* to mimic what happens *in-vivo* during kidney development, where several stages of differentiating cells coexist within the embryonic kidney, and their differentiation fate and spatial patterning along the nephron depends on the timings of their recruitment.

Line 311 - why? (what happens if you use a different stage? Knowing this may help users debug a protocol that is not working for them).

Response to the Reviewer: For a successful directed differentiation, it is critical to start differentiation at ~50% confluence of hESCs. We observed extensive cell death when we started differentiation at less than 50% confluence and differentiating cells became over confluent if we started differentiation at more than 50% confluence. We have now explained, "why we started differentiation at ~50% confluence" in the revised manuscript.

Line 313 - tenses are wandering between present and past. By the way, I think this descriptive passage is really useful!

Response to the Reviewer: We have corrected the tenses in our revised manuscript. Thank you, we appreciate that!

Line 342 - this is not 'reabsorption' as there was no prior absorption to justify the 're'. I understand that the term has come from in vivo physiology but it does not make sense here. If you want to make the link to in vivo, use 'absorption' for what you see, then suggest this represents the activity responsible for 'reabsorption' in vivo.

Response to the Reviewer: Thank you for pointing out this mistake. We have corrected this mistake in the revised manuscript.

Throughout: in most places that the word 'media' is used, the sentence is actually only about one medium, not several, so 'medium' should be used.

Response to the Reviewer: Thank you for pointing out this mistake. We have replaced the term 'media' with 'medium' throughout our revised manuscript.

There is one last thing - I would have liked to see staining for the irrelevant cell types that plague users of the Takasato protocol (and about which the Little group have written). But I guess that was more to ask for in the Communications paper, and I would not hold this JOVE one back for it.

Response to the Reviewer: We agree with the reviewer about the value of showing staining for the irrelevant cell types. In this current methods manuscript, our aim was to characterize kidney specific cell types. Unfortunately, we cannot provide the suggested data at this time.

Reviewer #4:

Major Concerns:
There are no major concerns, although I would have liked to see the lack of endothelial-podocyte interaction ('capillary loops'), normally a classical feature in glomeruli, mentioned already in the results section. At the moment it is only mentioned in the figure legend and the discussion.

Response to the Reviewer: We have now mentioned the 'lack of capillary loops' in the result section of our revised manuscript.

Minor Concerns:
There are a number of corrections I would like to suggest, some concern grammar and writing style, and some are regarding the clarity of the protocol.
1. For all aspects throughout the methods, I recommend that instead of '1st plate' and '2nd plate', the authors refer to 'Plate 1' and 'Plate 2'. This helps to avoid confusion, also when it comes to point 6.3, where the 24-well plate with the isopore membrane is suddenly also called '1st plate'.

Response to the Reviewer: As suggested by the reviewer, we have replaced the word '1st plate' and '2nd plate' with 'Plate 1' and 'Plate 2' respectively throughout our revised manuscript. In point 6.3, the word '1st plate' has now been replaced with the word '24-well-plate'.

2. line 46: repeat 'increase', 'increases'

Response to the Reviewer: We have corrected this line in our revised manuscript.

3. line 99 / protocol 1.1: include a sentence to refer to the table with all the detailed products and materials.

Response to the Reviewer: We have included a sentence in the protocol's step 1.1, to refer to the table that summarizes the list of materials utilized in the manuscript.

4. line 116 / protocol 2.4 and further down: clarify more specifically which media are referred to. The term 'media' is confusing if it is not clearly specified which medium it is, possibly already in the name.

Response to the Reviewer: We have clarified the media being used in protocol step 2.4, which is the same as that prepared in step 1.3 previously. This has now been clearly specified.

5. line 128 / protocol 2.8: it is expected that the hESCs reach 70% confluency at around 48 hours after seeding?

Response to the Reviewer: No, it does not mean that hESCs will reach 70% confluence after 48 hours of seeding. Cultures usually take 120 to 144 hours after seeding.

6. line 130 / protocol 2.9: do you mean to say: 'distribute cells from 1 well into 10 wells of 6-well-plates'?

Response to the Reviewer: Yes, that is what we meant to say. This has been corrected.

7. line 135 / protocol 3.1: do you mean to say: 'into 3 wells each of two 6-well-plates'?

Response to the Reviewer: Yes, that is what we meant to say. This has also been corrected.

8. line 142 / protocol 3.4: there is no DPBS wash step before adding accutase?

Response to the Reviewer: Thank you for catching this. We missed to mention the wash step, with DPBS, before adding accutase in the protocol's step 3.4. This has been incorporated into the revised manuscript.

9. line 155 / protocol 3.10: order of words: '1.7 x 10^5 hESCs / well'?

Response to the Reviewer: We replaced the words '$1.7x10^5$ cells/well' with '$1.7x10^5$ hESCs/well' in step 3.10 of the protocol.

10. line 173-174 / protocol 4: refer Figure 1A and time line.

Response to the Reviewer: Our revised manuscript now mentions to refer Figure 1A in section 4 of the protocol.

11. line 180 / protocol 4.2: refer to 'day 0 of differentiation for Plate 1'.

Response to the Reviewer: We have now mentioned ‘day 0 of differentiation for Plate 1’ in step 4.2 of the protocol.

12. line 205 / protocol 5.1.1: is '1.5% PFHM' the same as '1.5% PHFM II' mentioned in line 224 / protocol 5.7?

Response to the Reviewer: It should be ‘1.5% PFHM II’. We have corrected this in our revised manuscript.

13. line 241 / protocol 6.4: 'mix fragments from 1 cell aggregate...'

Response to the Reviewer: We have now included the phrase ‘mix fragments from 1 cell aggregate’ in step 6.4 of the protocol.

14. line 244-245 / protocol 6.5: is this conflicting with information of 6.4, as there it mentions to make 2 cell aggregates on the isopore membrane...?

Response to the Reviewer: Using a 1:1 ratio of small fragments and fresh kidney progenitors, 6-8 cell aggregates/isopore membrane can be spotted. In our revised manuscript the sentence in step 6.5 has been restructured to eliminate potential confusion.

15. line 256 / protocol 7.1: here you mention day 17, but in 6.8 you mention day 18. this needs clarification? why not do the dextran uptake experiment on day 18?

Response to the Reviewer: Thank you for catching this oversight. We performed dextran uptake experiment on day 18. This mistake has been corrected in our revised manuscript.

16. line 256-257 / protocol 7.1: 'transfer kidney organoids such that 1 organoid is placed in each well of...'

Response to the Reviewer: We have now corrected Step 7.1 in the revised manuscript.

17. line 287 / protocol 8.8: are these repeated DPBS changes? (also for 8.10?)

Response to the Reviewer: Yes, a DPBS change is required to wash both primary and secondary antibodies.

18. line 300: there is an article missing: 'We followed a previously published protocol...'

Response to the Reviewer: We have now included a citation in this sentence.

19. line 330: it should be Figure 3A

Response to the Reviewer: We corrected ‘Figure 3I’ to ‘Figure 3A’.

20. line 337-338: as mentioned above, the question here is: do the endothelial cells directly align with podocytes to form a structure resembling the glomerular filtration structure? From the images it looks like CD31+ cells are arranged *around* the podocyte clusters, and fail to directly engage with them? This needs to be more clearly described here.

Response to the Reviewer: Yes, CD31+ cells were arranged around the podocyte clusters, but were not directly engaged with them and capillary loops were absent. Now, absence of capillary loops in the organoid has been described in this section of the revised manuscript.

21. lines 347-350: this sentence needs some work re grammar and articles. Should it be 'fewer LTL+ proximal tubules,...'?

Response to the Reviewer: Thank you, we have now reframed this paragraph.

22. line 376: '(C)' should be moved to the end of the sentence as the yellow stars and yellow arrow heads both are shown in (C).

Response to the Reviewer: ‘(C)’ has been removed from the middle of the sentence and is written as ‘(B, C)’ in our revised manuscript.

23. line 378: podocytes without capital P

Response to the Reviewer: The letter ‘p’ in the word ‘Podocytes’ has now been changed to lowercase in our revised manuscript.

24. line 384: word missing: '...were detected / observed around ...'

Response to the Reviewer: This sentence has been corrected in our revised manuscript.

25. line 384: either 'a capillary loop' or in plural form.

Response to the Reviewer: This sentence has been corrected in our revised manuscript.

26. lines 391-392: can the authors comment on whether the dextran incubation had any negative effects on integrity of tubules / proximal tubules? The structures shown in Figure 4B are not as clear as those shown in Figure 4A or Figure 3H?

Response to the Reviewer: We did not observe any negative effect on integrity of the tubules/proximal tubules. We took ‘high magnification images’ to show dextran vesicles in the proximal tubules. It may be a reason for the difference in the images.

27. line 401: 'fewer' instead of 'few'?

Response to the Reviewer: This sentence has been corrected in our revised manuscript.

28. line 402: awkward: 'Furthermore, an endothelial network....'?

Response to the Reviewer: This sentence has been corrected in our revised manuscript.

29. line 428: article missing 'for *the* next...'

Response to the Reviewer: This sentence of the discussion section has been corrected in the revised manuscript.

30. line 446: word choice: 'protocol to *achieve* more...'?

Response to the Reviewer: This sentence of the discussion section has been corrected in the revised manuscript.

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Fig S1.eps