CHAPTER

Differentiation of human kidney organoids from pluripotent stem cells

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Chapter outline

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Abstract

It is now possible to direct the differentiation of human pluripotent stem cells into three-dimensional nephron-like structures called kidney organoids. Organoids contain proximal and distal tubules as well as podocytes, in addition to a variety of other lineages such as endothelial cells. Organoid technology has great potential for kidney regeneration and has already been proven to be suitable for modeling kidney disease. However, the methodologies that are used for the generation of kidney organoids require expertise and can be daunting for the inexperienced. Here, we describe in detail a well-established and relatively simple method for the generation of human kidney organoids. We include notes on technical and design considerations for these experiments, and highlight key advantages and limitations of the system.

1 Introduction

Over the past several years, the fields of stem cells and nephrology have witnessed an explosion of research into kidney organoids. By way of definition, an organoid is a cellular structure grown *in vitro* that contains multiple cell types in patterned geometric arrangements that resemble a tissue or organ of the body. In the case of the kidneys, these beautiful and intricate organoid structures, which were not previously available to the research community, bear a striking resemblance to nephrons, which are the fundamental organotypic subunits of kidney tissue. As kidney organoids can be derived from humans, and even from adult patients, there is great excitement surrounding their potential to illuminate species-specific biology. Moreover, these differentiated structures have already raised new possibilities for both kidney regeneration and disease modeling.

Despite great interest in kidney organoids as a research tool, the art of growing these structures can be daunting for newcomers to the field. The focus of this chapter is to describe in detail a relatively simple, well-established protocol for the generation of human kidney organoids from pluripotent stem cells, as a basis for laboratory-specific studies into physiology, disease, and regeneration. Successful adaptation of this protocol requires no specialized equipment and can be mastered using standard cell biology techniques. In addition to the step-by-step protocol, we will provide a brief overview of the history and rationale underlying this human organoid system, to better comprehend both the significance and limitations of this new research area.

2 Applications of kidney organoids

2.1 Medical need

Humans are born with approximately two million kidney tubular subunits, called nephrons. In mammals, the stem cells that give rise to these nephrons exist only transiently, during embryonic development, after which the body loses the ability to generate or replace nephrons in a clinically significant way (Barak et al., 2012; Faa et al., 2010; Hartman, Lai, & Patterson, 2007; Kobayashi et al., 2008; Self et al., 2006; Togel et al., 2017). Loss of nephrons below a critical mass causes chronic kidney disease, associated with cardiovascular and metabolic complications, and end-stage renal disease, requiring dialysis or kidney transplant to survive (Saran et al., 2015). While critical for sustaining life, both transplant and dialysis are temporary salves that are associated with significant side effects, necessitating new investigations into the potential for kidney regeneration and the discovery of early-stage interventions that prolong the lifespan of the kidneys in specific disease states.

2.2 Research need

Because adult mammals lack nephron progenitor cells capable of growing new nephrons, many studies have been limited to primary cell cultures from kidney tissue samples. The difficulty with primary cultures, however, is that the complexity of the nephron's intricate segmented structure is lost during the derivation process. Moreover, the epithelial cell types of the kidney rapidly dedifferentiate when removed from the body, and certain key lineages, such as the podocytes, are unable to proliferate at a significant rate. This has led to a dearth of physiologically relevant cellular research models for the kidney. This problem is particularly acute in the human species, where cellular models are the only gateway to species-specific preclinical experiments.

A new approach to this problem, which has only become possible in recent years, is to derive nephron-like structures from human pluripotent stem cells (hPSC). Pluripotent stem cells is an umbrella category that includes a number of closely related types of cells, the two most famous being human embryonic stem (ES) cells, which are derived from embryos (Evans & Kaufman, 1981; Martin, 1981; Thomson et al., 1998), and human induced pluripotent stem (iPS) cells, which are transcriptionally reprogrammed from somatic cells (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). All hPSC have a characteristic morphology *in vitro,* possess an extensive capacity for self-renewal, and can differentiate into many of the different somatic lineages of the human body, representing all three of the embryonic germ layers.

2.3 Discovery of human kidney organoids

Although hPSC were first derived over 20 years ago, for many years there was no known way to differentiate these cells into the kidney lineage. Despite successes in generating numerous other vital organ lineages, including the heart, lungs, liver,

gut, and brain, the kidneys remained unattainable. As researchers turned their attention to this important question, protocols began to emerge in which hPSC were differentiated stepwise into cells expressing markers associated with the mesoderm and intermediate mesoderm, from which the kidneys derive (Lam et al., 2014; Mae et al., 2013). Subsequently, four distinct papers reported nearly simultaneously that it was possible to further differentiate hPSC into terminally differentiated renal structures, known as kidney organoids (Freedman et al., 2015; Morizane et al., 2015; Taguchi et al., 2014; Takasato et al., 2015). It was immediately recognized that these structures were substantially different from those that had been previously derived from hPSC or in primary cultures. The four original protocols remain in use today, essentially unmodified, by a variety of different groups.

Although each of these four protocols is slightly different, there are also obvious commonalities in both the methodology used as well as the resultant structures. In particular, CHIR99021, a small molecule inhibitor of GSK3β, is a common ingredient in all four kidney differentiation protocols. hPSC are very sensitive to the dose and timing of CHIR treatment, and optimizing this treatment step was a critical advance that enabled the generation of kidney organoids. A hallmark of kidney organoid differentiation is the appearance of translucent tubules that can be clearly identified by light microscopy. Another hallmark is the presence of specific nephron lineages in segmented structures, as described below.

3 Kidney organoid cell types

Immunofluorescence and single cell RNA sequencing (scRNA-seq) analysis of organoid cultures suggests that all of the different protocols commonly used to differentiate kidney organoids from hPSC produce a similar spectrum of cell types, at least qualitatively, and to some degree quantitatively as well (Cruz et al., 2017; Czerniecki et al., 2018; Freedman, 2019; Wu et al., 2018). Herein we will briefly review the critical findings from such experiments.

3.1 Nephron segments

Three specific nephron segments are commonly identified in all kidney differentiation protocols: podocytes, proximal tubules, and distal tubules. These segments form in organoids along a proximal-to-distal axis that accurately recapitulates the natural geometry of the nephron tubule. These lineages appear to derive from a single nephron progenitor cell ancestor, which has multi-lineage potential and gives rise to all of the epithelial cells of the proximal nephron, similar to kidney development *in vivo*.

Tubules within these organoids exhibit tissue-specific biomarker upregulation after injury, and are capable of transporter functions when treated with fluorescent cargoes (Freedman et al., 2015; Morizane et al., 2015; Takasato et al., 2015). In contrast, the podocytes self-aggregate into tight, ball-like clusters, zippered together at their basal

membranes with specialized junctions (Freedman et al., 2015; Kim et al., 2017). Recent work demonstrates that kidney organoids can be differentiated from primary nephron progenitor cells cultured from embryos, in addition to hPSC, although these will not be covered here (Brown, Muthukrishnan, & Oxburgh, 2015; Li et al., 2016; Tanigawa, Taguchi, Sharma, Perantoni, & Nishinakamura, 2016).

In vivo, the parietal epithelial cells (PEC) line Bowman's capsule of the nephron and represent an intermediate between podocytes and proximal tubules. It is not yet completely clear whether PEC are present in kidney organoids, because highly accurate markers diagnostic of this cell type are lacking. Nonetheless, there does appear to be an intermediate cell type with at least some of the expected properties of the PEC in the organoids, including strong expression of CLDN1, and low expression of LTL (a marker of proximal tubules) (Czerniecki et al., 2018).

3.2 Collecting ducts

There is debate regarding whether collecting ducts are also present in addition to these lineages. If collecting ducts could be generated, it would be highly significant because the collecting ducts are believed to derive from a different stem cell population than the proximal nephron lineages, a population called the ureteric bud. Whether ureteric bud is present in organoid cultures is a hotly contested area, with high profile publications on both sides of the aisle. In our view at least, no group has demonstrated widespread and robust expression of markers that would typically be considered diagnostic of collecting ducts, such as aquaporin-2 and DBA lectin, in specific segments in human kidney organoids (Czerniecki et al., 2018; Taguchi & Nishinakamura, 2017; Takasato et al., 2015; Wu et al., 2018). Nor has any group shown that human organoids can undergo branching morphogenesis, as is typical of the embryonic kidney *in vivo* and *ex vivo*. There is also no convincing report of ureteric bud or collecting duct differentiation from human pluripotent stem cells independent of nephron progenitor cell differentiation, by these same criteria. Based on these lines of evidence, our opinion is that mature collecting ducts are absent from kidney organoid cultures derived from hPSC, at least as they exist today, despite some reports that have concluded otherwise. This remains an evolving field and until the *bona fide* collecting duct is demonstrated we will not know for certain which contention is correct.

3.3 Non-kidney cells

Because kidney organoids derive from hPSC, which have very broad potential for differentiation, not all of the cells produced within these organoids are specific to the kidney. For instance, in an early publication we identified the presence of neuronal contaminants in these cultures that are not specific to the kidneys (Freedman et al., 2015). This has since been shown to be a common feature of kidney organoid cultures, regardless of the protocol used (Freedman, 2019; Wu et al., 2018). Similarly endothelial cells, muscle-like, and myofibroblast-like

cells have been identified in these cultures by numerous groups, and it is not yet clear whether these have any specificity to the kidney or are simply general contaminants (Cruz et al., 2017; Czerniecki et al., 2018; Freedman et al., 2015; Taguchi et al., 2014; Takasato et al., 2015; Wu et al., 2018). It is currently recognized that kidney organoid cultures contain over 10 different cell types, and perhaps as many as 20, many of which are "off target" non-kidney cells. This is true of all protocols studied thus far.

It is likewise becoming clear that there is a wide spectrum of differentiation and maturity states present within the organoids. The well-differentiated podocytes and proximal tubular cells, for instance, may simply be the 'tip of the iceberg' from a much larger population of partially differentiated cells. This is particularly striking in scRNA-seq analyses (Czerniecki et al., 2018; Harder et al., 2019; Wu et al., 2018).

4 Overview of differentiation protocol

We will describe below the differentiation of kidney organoids from hPSC utilizing an adherent culture protocol we have developed (Cruz et al., 2017; Czerniecki et al., 2018; Freedman et al., 2015; Kim et al., 2017). Aspects of this protocol are currently being commercialized by STEMCELL Technologies, in a proprietary formulation. The protocol is compatible with both plastic and glass plates, and with high throughput screening. We will also describe essential methodologies for characterization of the organoids, including immunostaining for nephron segments diagnostic of the organoid fate. It should be noted that this protocol is limited to the more proximal nephron lineages, and does not produce collecting ducts, as described above. We will restrict our discussion to the hPSC-derived organoid system, rather than organoids derived from fetal nephron progenitor cells, or from somatic cells, which represent alternative approaches that should best be covered in separate publications. It is our hope that these step-by-step protocols will be of great use in the wider adoption and dissemination of organoid technology. In broad strokes, the steps of the differentiation protocol are as follows (Fig. 1A):

1. hPSC are plated onto adherent tissue culture plates at a low seeding density and sandwiched in a thin layer of Matrigel to form three-dimensional colonies.
2. hPSC are treated with 12 μM CHIR90221 for 1.5 days and subsequently maintained in serum-free media for an additional 8 days to induce $SIX2^+PAX2^+WT1^+$ nephron progenitor cells.
3. These are further differentiated into organoids for an additional 11 days, at which time translucent tubular islands containing the major nephron cell types can clearly be distinguished from the underlying stroma (Fig. 1B).

A detailed, step-by-step description of this protocol follows immediately below.

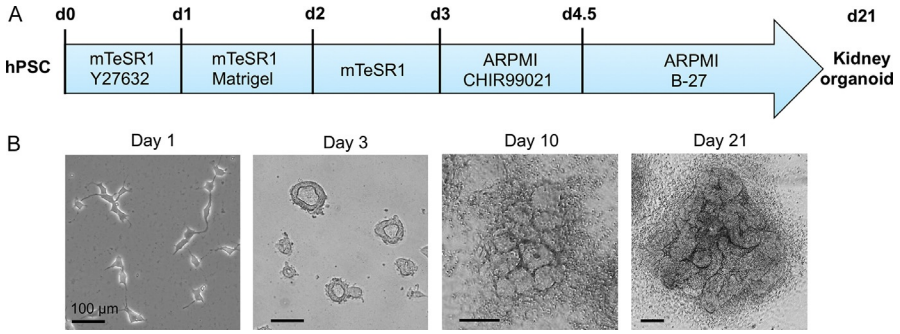


FIG. 1

Overview of kidney differentiation protocol. (A) Schematic timeline of kidney differentiation protocol. Media and supplements used throughout the protocol are indicated.
(B) Representative images of cells and structures characteristic of different stages during differentiation. hPSC are dissociated on day 0 (d0) and plated as single cells. Cavitated spheroids are present on day 3. Mesenchyme is produced on days 4–10, and renal vesicles structures preceding tubular organoids are observed on day 10. Tubular organoids start to emerge on day 16 and are easily identified by day 21. Scale bar, 100 μm.

5 Step-by-step methods

5.1 Coating plates for stem cell culture and organoid differentiation

hPSC culture requires the pre-coating of tissue culture plates with a suitable extracellular matrix (ECM) for the attachment of cells. The following protocol describes the use of Geltrex or Matrigel, two commonly used ECM products that work well for the kidney differentiation protocol. These should be kept on wet ice at all times prior to use to prevent polymerization. The use of pre-chilled pipette tips and tubes is recommended.

5.1.1 Materials and reagents

- Geltrex LDEV-Free, hESC-qualified, reduced growth factor (A1413301, Thermo Fisher) or Matrigel GFR (354230, Corning).
- DMEM/F12 basal medium (11320082, Thermo Fisher).
- Tissue culture plates. We prefer Midwest Scientific 24-well Tissue Culture plates (TP92024, MidSci) and Falcon Multi-well Flat-Bottom Plates (353072 and 353046, Corning).

5.1.2 Protocol

1. Thaw Geltrex or Matrigel overnight in a 4 °C refrigerator.
2. Mix the desired volume of cold DMEM/F12 media with 1% Geltrex or 1.7% Matrigel in a pre-chilled tube. Slowly pipet up and down several times to generate a homogeneous solution. Avoid introducing air bubbles.

3. Add the recommended coating volume to plates as follows:
   1 mL per 6 well.
   300 µL per 24 well.
   60 µL per 96 well.
4. Rock the plate back and forth to evenly coat the bottom of each well. Incubate for at least 1 h at 37 °C. The plates can be sealed with Parafilm around their edges and stored at 4 °C for up to 2 weeks after coating. Allow stored plates to equilibrate at room temperature for 30 min before use.

5.2 Differentiation of stem cells into kidney organoids

The protocol outlined here will lead to the generation of three-dimensional tubular structures in adhesion cultures in 24-well plate format. The protocol can be scaled down if desired for 96-well and 384-well formats.

5.2.1 Materials and reagents

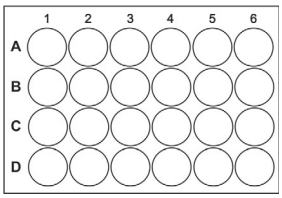
- hPSC (50–80% confluent) growing feeder-free. We routinely use WA09, a female ES cell line also known as H9 (WB66593, WiCell) and WTC-11 (GM25256, Coriell Institute), an iPS cell line derived from male adult skin fibroblasts (Kreitzer et al., 2013; Thomson et al., 1998).
- Matrigel (356231, Corning) or Geltrex (A1413302, Thermo Fisher) matrix.
- Accutase (07920, StemCell Technologies), a gentle cell detachment and dissociation solution.
- mTeSR1 (85850, StemCell Technologies) supplemented with the antibiotics Penicillin-Streptomycin (15140122, Thermo Fisher) to prevent bacterial contamination. mTeSR1 is a feeder-free maintenance medium for the culture of hPSC.
- Sterile Phosphate-Buffered Saline (PBS) pH 7.4 (10010-049, Thermo Fisher) for washing cells.
- Advanced RPMI 1640 basal medium (12633020, Thermo Fisher) supplemented with 2 mM L-Glutamine (GlutaMAX, 35050061, Thermo Fisher) and Penicillin-Streptomycin (15140122, Thermo Fisher).
- "RB" media: Advanced RPMI 1640 Medium supplemented with 2 mM L-Glutamine, Penicillin-Streptomycin and 1 × B-27 supplement (17504-044, Thermo Fisher).
- 10 mM Rho/Rock pathway inhibitor Y27632, dissolved in sterile DMSO (1254, Tocris).
- 10 mM CHIR99021 in DMSO (04-0004-02, REPROCELL). This is a small molecule inhibitor with selectivity for GSK3β used to induce differentiation.
- 24-well tissue culture plates (TP92024, Midwest Scientific or equivalent).
- Recombinant human Noggin, 100 µg/mL stock solution in PBS (120-10C, PeproTech).
- BMP4, 50 µg/mL stock solution in PBS (314-BP-010/*CF*, R&D).
- 1.5 mL or 15 mL conical centrifuge tubes.

5.2.2 Protocol

Day 1: Preparing single-cell suspension and plating cells.

hPSC should be 50–80% confluent on the day of plating. It is not recommended to let the cells become over-confluent as this will decrease cell viability and may lead to spontaneous differentiation. The optimal cell density for plating will vary depending on the cell line and testing several densities is recommended.

1. Aspirate the media from six-wells containing hPSC and briefly wash with PBS, 2 mL/well. Aspirate the PBS immediately and add 1 mL of Accutase.
2. Incubate at 37 °C for 5–10 min. Gently tap the plate to detach the cells. If cells do not detach immediately, increase the incubation time with Accutase until they do.
3. Dissociate the cells in Accutase by pipetting them up and down 5–10 times with a P1000 pipetman in the original culture wells. Collect all the cells and transfer to a 1.5 mL or 15 mL conical centrifuge tube.
4. Centrifuge at 300 rcf for 4 min at room temperature.
5. Carefully aspirate the Accutase and resuspend cell pellet in 1 mL of mTeSR supplemented with 10 μM ROCK inhibitor Y-27632. Y-27632 substantially increases cell survival when plating single cell suspensions of hPSC.
6. Count cells to determine the concentration of the cell suspension.
7. Prepare a solution of 40,000 cells/mL in 16 mL mTeSR1 + 10 μM ROCK inhibitor (640,000 total cells). Mix well by pipetting up and down 5–10 times to generate a homogeneous solution. Avoid introducing air bubbles while mixing.
8. Tilt a coated 24-well plate 45° and aspirate the coating media. Add 250 μL mTeSR + ROCK inhibitor to each well in row A. Then, pipet the cell solution prepared in step 7 to the plate as follows:



A 250μL (10,000 cells/well)

B 500μL (20,000 cells/well)

C 750μL (30,000 cells/well)

D 1mL (40,000 cells/well)

It is very important to mix well the cell solution before pipetting onto the plate, and to move quickly. Cells can settle quickly and not mixing them could lead to inaccurate cell densities. The suggested cell densities are a starting point, but certain hPSC may require lower or higher densities and they should be adjusted as necessary. For instance, the optimal seeding density for WTC-11 iPS cells is 5–10 fold lower than H9 ES cells.

9. Inspect the plate under a light microscope. The cells should appear evenly spread across the well. If the cells appear aggregated, rock the plate back and forth several times, pausing in between each motion, to disperse them evenly across the well.
10. Incubate at 37 °C, 5% CO_2 overnight.

Day 2: Matrix sandwiching

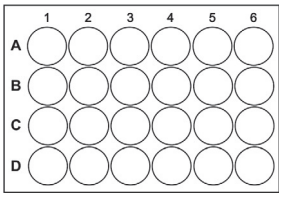
1. During the morning of day 2, prepare 12 mL of ice-cold mTeSR with 1.5% Geltrex or 2.25% Matrigel.
2. Tilt the 24-well plate 45° and carefully aspirate the media from the wells. Immediately proceed to the next step, cells will dry out if left without media for too long.
3. Slowly add 500 µL of the cold mTeSR + Geltrex or Matrigel solution to each well, drop by drop, being careful of not disturbing the cells.
4. Incubate at 37 °C, 5% CO_2 overnight.

Day 3: Feed the cells with mTeSR

1. Tilt the 24-well plate 45° and carefully aspirate the media from the wells. It is recommended to aspirate from no more than half the plate at a time, to avoid drying out the cells. Proceed immediately to the next step.
2. Slowly add 500 µL of mTeSR (pre-warmed to room temperature) to each well. Small aggregates of cells in the shape of spheroids should have started to form due to the addition of matrix on day 2.

Day 4: CHIR99021 pulse

1. During the evening of day 4, aspirate the mTeSR1 and replace with 1 mL/24 well of Advanced RPMI + L-Glutamine supplemented with 12 µM CHIR99021. Some hPSC lines may require addition of 10 ng/mL Noggin or 2 ng/mL BMP4 in addition to the CHIR99021 to induce kidney lineage differentiation, try these formulations as well along the x-axis of the 24-well plate for each of the cell densities.



1 & 2 12µM CHIR99021

3 & 4 12µM CHIR99021 + 10 ng/mL Noggin

5 & 6 12µM CHIR99021 + 2 ng/mL BMP4

2. Incubate for 36 h at 37 °C, 5% CO_2.

Day 6: CHIR99021 removal

After 36 h in CHIR99021 (morning of day 6), change the media to RB (1 mL/24 well). Aspirate and drip on the media gently from this point forward in the protocol, as the structures are delicate and can be easily disrupted. There is no need for Noggin or BMP4 from this point onwards.

Day 8 and onwards: Feeding with RB media

Replace the RB media in the plates after 2 days and every third day after that. Distinctive round cell clusters should start to appear around day 10 in at least one of the conditions. These will transform into discrete nests of translucent, convoluted tubular structures by day 21. Each nest of tubules counts as a separate "organoid," typically ~250 μm in diameter. The organoids are at their prime during the 3rd and 4th week after the initiation of the differentiation. The organoid cultures can be kept for longer, but they will become overgrown by the other cell types in the dish and will deteriorate over time. Overgrown organoids have a darker, fuzzy appearance and their tubular structures are less prominent. Immediately after feedings, a single air bubble may appear atop each organoid, resulting from trapped air escaping from the epithelial structures. These will disappear on their own and are not a concern.

Optimal conditions for kidney differentiation may vary slightly for different pluripotent stem cell lines. One of the most critical aspects is the starting cell density; therefore, it is important to test a range of cell densities. This may be a relatively narrow range—for instance, organoids may appear at a density of 20,000 and 30,000 cells per well of a 24-well plate but not at 10,000 or 40,000. CHIR99021 activity can differ from one supplier or batch to the next. Lot testing and consistently using same reagent sources or lot may also help increase reproducibility across different experiments. WA09 ES cells (WiCell) or WTC11 iPS cells (Coriell) are established hPSC lines that differentiate into kidney organoids robustly and can be used as positive controls for other cell lines.

5.3 Immunofluorescence analysis of organoids

While tubular structures can be readily detected using light microscopy, identification of distinct nephron segments representing podocytes, proximal tubules, and distal tubules within the organoid using immunofluorescence is a more definitive way of confirming a successful differentiation (Fig. 2A). The following protocol describes the immunostaining of organoids in their original 24-wells. Volumes should be scaled accordingly for other plate formats.

5.3.1 Materials and Reagents

- Paraformaldehyde, 16% aqueous solution (15710, Electron Microscopy Sciences)
- Phosphate-Buffered Saline (PBS), pH 7.4 (10010023, Thermo Fisher)
- Normal donkey serum (S30100ML, Millipore Sigma)
- Triton X-100 (X100-500ML, Millipore Sigma)
- Bovine Serum Albumin (BSA) (BP9703-100, Fisher Scientific)

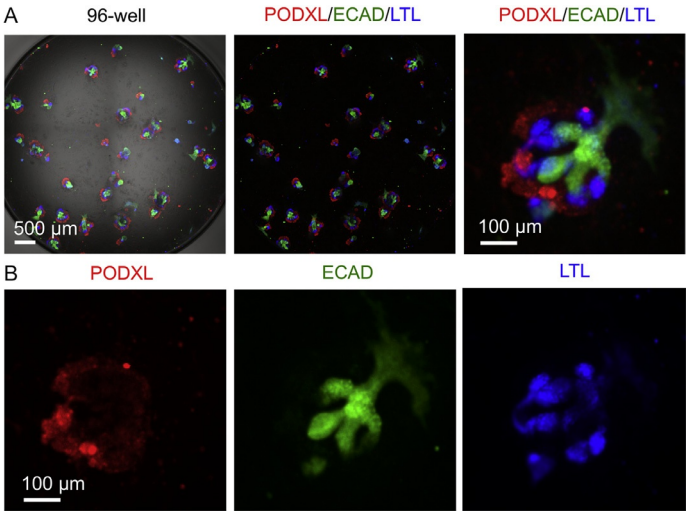


FIG. 2

Immunostaining of kidney organoids for the identification of nephron segments. (A) Well of a 96-well plate stained for *Lotus tetragonolobus* lectin (LTL, blue), E-cadherin (ECAD, green), and podocalyxin (PODXL, red). Scale bar, 500 µm. An organoid within the well is shown at right at higher zoom (scale bar, 100 µm). (B) Images for each individual stain from the organoid shown in (A). *Bona fide* kidney organoids contain specific segments labeled for each marker in a continuous arrangement. Scale bar, 100 µm.

- DAPI Nucleic Acid Stain (D1306, Thermo Fisher)
- Cross-adsorbed secondary antibodies raised in donkey with Alexa Fluor dyes for detection (Thermo Fisher)

5.3.2 Solutions

- Blocking Buffer: 1 × PBS, 5% normal goat serum, 0.3% Triton X-100
- Antibody Dilution Buffer: 1 × PBS, 1% BSA, 0.3% Triton X-100, 0.1 mM calcium chloride

5.3.3 Protocol

1. Dilute fresh 16% paraformaldehyde (PFA) 1:1 with PBS to obtain an 8% solution. Remove 500 µL of media from the organoid culture, leaving 500 µL in the well. Slowly add 500 µL of 8% PFA to the well (the final concentration of PFA should be 4%). Mix gently by rocking the plate back and forth. PFA is toxic and guidelines for safe handling should be followed.
2. Incubate for 15 min at room temperature.

3. Remove all the PFA and media from the well and discard following chemical waste disposal guidelines.
4. Tilt the plate at a 45° angle and slowly add 1 mL of PBS to the well. Avoid pipetting directly on top of the cells.
5. Remove the PBS from the well and replace with 1 mL of fresh PBS. Repeat once more for a total of three PBS washes. PBS and other solutions should be carefully added to the cultures to avoid detaching or damaging the structures.
6. Remove PBS and add 300 µL of Blocking Buffer. Incubate 1 h at room temperature.
7. Remove Blocking Buffer and add 300 µL of primary antibody solution (primary antibodies diluted in Antibody Dilution Buffer). Different antibodies can be used simultaneously as long as they were raised in different species to avoid cross-reactivity. Incubate overnight at 4 °C. The following reagents can be used for labeling distinct nephron segments:
   - *Lotus tetragonolobus* lectin (LTL) fluorescein-labeled (FL-1321, Vector Labs, 1:500)
   - E-Cadherin (ab11512, Abcam, 1:250)
   - Anti-human Nephrin (AF4269, R&D, 1:500) or anti-human Podocalyxin (AF1658, R&D, 1:500)
8. Remove Antibody Dilution Buffer and add 1 mL PBS. Aspirate the PBS and repeat two more times for a total of three PBS washes.
9. Remove PBS and add 300 µL of secondary antibody solution (species-specific antibodies of choice and 2 µg/mL DAPI in Antibody Dilution Buffer). Incubate overnight at 4 °C. Antibodies and DAPI are incubated overnight to achieve even labeling throughout the entire organoid. No secondary is required for the fluorescein-conjugated LTL.
10. Remove Antibody Dilution Buffer and add 1 mL PBS. Aspirate the PBS and repeat two more times for a total of three PBS washes. Leave PBS from the last wash in the plate. Do not add mounting media as these will disrupt LTL binding. The plate is ready for imaging.
11. Imaging can be performed using either a wide-field or a confocal fluorescence microscope. For organoids that adopt a bouquet conformation, podocytes are typically found at the organoid periphery, and distal tubules at the organoid interior, with proximal tubules in-between (Fig. 2B). The appearance of all three segments in a contiguous proximal-to-distal order is a hallmark of successful organoid differentiation.

6 Technical and design considerations

6.1 Room for improvement

As we have discussed, the current composition of organoids does have certain limitations, such as the absence of collecting ducts, which would naturally connect to and drain the more proximal nephron lineages. Another limitation is that the

organoids lack a functional vasculature in any true sense of the word. Although we recognized early on that human kidney organoids contain endothelial cells (Freedman et al., 2015), these cells do not go on to form functional blood vessels. Indeed, the podocyte clusters in organoids that are sometimes called "glomeruli" in the literature do not show any particular enrichment for endothelial cells, compared to the rest of the cells in the culture. Thus, there is nothing in these cultures *in vitro* that could be considered to be a functional glomerulus. This is a general limitation of these types of structures, which goes beyond the kidney field, and has not been convincingly solved in any system to the best of the authors' knowledge.

It should also be noted that the nephron segments present in organoids lack perfusion and appear to be relatively immature, compared to their phenotypic characteristics *in vivo*. For instance, organoid tubules lack brush borders, and organoid podocytes lack *bona fide* foot processes with primary, secondary, and tertiary interdigitations. Careful comparisons of organoids to human developing kidneys suggest these reach an intermediate stage of differentiation, analogous to the capillary loop stage (Kim et al., 2017).

6.2 Technical aspects

At a more practical level, there are technical considerations that limit the use of kidney organoids derived from hPSC. As mentioned above, differentiating the organoids takes several weeks, which slows experimental output. Kidney organoid differentiation can be difficult to achieve. Every batch is likely to be a bit different from the next. It is possible to observe a successful differentiation the first week and an unsuccessful one the next week using almost identical conditions. It is also possible for a line to require only CHIR99021 the first week and require CHIR99021 plus Noggin the next week. There is no clear single cause for this variability, and it is likely a complex phenomenon owing to the many opportunities for stochastic differences over the course of a 3-week experiment.

It should be noted that kidney organoids can be a bit difficult to recognize and identify for those lacking prior first-hand experience. This is because a variety of structures can arise in hPSC cultures, many of which could be mistaken for proper organoids. Whenever possible, we recommend collaborating with an experienced laboratory when embarking on these lines of research initially, and obtaining samples from them of organoids as positive controls. When organoid differentiation does not work properly, the practiced eye can detect the differences at the light microscopy level. Three-dimensional structures may appear, but they will lack the well-defined edges and translucent tubules characteristic of successful differentiation (Fig. 3A). Instead, failed differentiations may form smooth-edged opaque structures resembling muscle, or cyst-like structures (Fig. 3B). In most cases, any doubt can be removed by performing immunostaining of organoids for nephron segments, which is a good quality control measure as discussed earlier.

Patience is necessary and care must be taken not to jump to conclusions from a single experiment. Starting with several lines and differentiating them

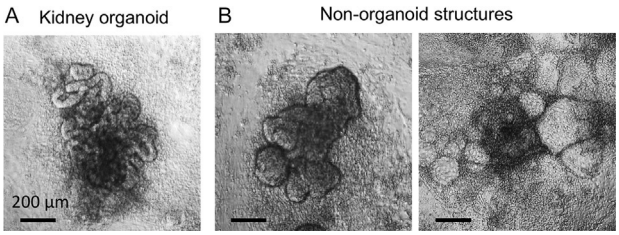


FIG. 3

Proper appearance of kidney organoids. Kidney organoids can be recognized by their defined tubular structures, as shown in (A). Other types of three-dimensional structures may appear in the cultures, such as round and translucent cyst-like structures (B). These are not to be confused with organoids and often arise due to sub-optimal differentiation conditions such as CHIR99021 concentration or hPSC cell density. Scale bar, 100 μm.

simultaneously is recommended, so that at least some of them will produce organoids in any given batch. In addition, there is no simple way to cryopreserve organoids, or to passage them without losing their differentiated characteristics.

Imaging of organoids is best performed immediately after staining to prevent the gradual deterioration of the sample and signal. Overnight incubations of primary and secondary antibodies and DAPI will reduce artifacts of low signal intensity ("holes") in the center of the three-dimensional structures. Performing the fixation step by adding the PFA in a 1:1 volume to the media, rather than aspirating the media first, helps to preserve the delicate structure of the organoids. Depending on the geometry of the organoid culture, microscopy can be difficult due to the thickness of the sample. With regard to this point, we find that our particular method of differentiation, which is performed in adherent cultures on conventional tissue culture plastic or glass substrates, is compatible with standard immunofluorescence protocols and microscopy platforms. This may make the adoption of organoid technology a bit easier for the newcomer.

7 Conclusion

Kidney organoids are an attractive resource for disease modeling, therapeutic discovery and precision medicine. Their human origin, *in vitro* accessibility and versatility are major advantages of the system. There is significant variability from one batch of organoids to the next, and from one cell line to the next, which necessitates a greater degree of rigor than might normally be considered acceptable with simple cell cultures or mouse experiments. Nevertheless, careful use of organoids, coupled with patience and perseverance, can produce results that are clear, exciting, and enlightening. As the kidney organoid field continues to expand, we hope that newcomers and more experienced readers find the detailed methods described in this chapter useful in adopting this technology to further advance stem cell and nephrology research.

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