

Title

Establishment of kidney cancer organoid cultures

Key Words

organoid, kidney cancer, ccRCC, kidney cancer organoids, preclinical model

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Abstract

This protocol describes an efficient novel method for cultivating kidney cancer organoids, derived directly from human clear cell renal cell carcinomas (ccRCC) or sorted cancer stem cells. The organoids recapitulate tumor-specific characteristics and display inter-tumor heterogeneity. Here we explain how to establish the organoid cultures, maintain them in defined, serum-free medium and use them for patient-specific drug testing. Within 10-14 days, a patient-derived organoid culture is established and can be serially passaged. Compared to other protocols focusing on organoid cultures derived from pediatric kidney malignancies or healthy kidneys, the protocol described here is optimized for adult ccRCC organoid cultures. Organoids established using this protocol can be used to further investigate tumor biology in a complex 3D *in vitro* system, complement current preclinical model-based drug studies, and advance the development of personalized therapy design.

INTRODUCTION

Kidney cancer is the twelfth most common malignancy in the western world and clear cell renal cell carcinoma (ccRCC) is its most common form¹. Even though significant advances have been made in the treatment of ccRCC, including receptor tyrosine kinase inhibitors and monoclonal antibodies targeting VEGF-dependent angiogenesis, and more recently immune checkpoint inhibitions, the lack of suitable preclinical models has certainly impeded kidney cancer research both regarding the development of novel drugs as well as the stratification of suitable predictive biomarkers^{2,3}. Therefore, improving the long-term prognosis of ccRCC will require novel models that pave the way to personalized medicine, which offers treatments that are specific to the biology of each tumor. Organoid cultures have increasingly been shown to recapitulate a tumor *in vitro*⁴. We have developed and characterized a novel ccRCC organoid culture system, which could be used to test the patient-specific response to

treatment strategies *in vitro*⁵. In this protocol, we provide further detailed instructions on how to establish and maintain these culture systems and evaluate them for your individual research needs.

Comparison with other methods

Organoid culture conditions, described here, were adapted from those previously described, but were tailored to the specific needs of kidney cancer cells^{6,7}. We optimized different conditions, enabling us to achieve long-term growth of primary ccRCC tissue with high efficiency. The composition of the medium is summarized in Table 2. We have shown that organoids exhibit kidney-specific epithelial differentiation, while retaining cancer stem cell populations. Organoids resemble proximal tubule structures – the potential cell of origin of ccRCC – and recapitulate typical characteristics of ccRCC, including glycogen deposits and lipid droplets⁵.

An earlier described kidney cancer organoid culture has established healthy kidney organoids and matched cancer organoid cultures. However, cancer organoid formation efficiency was much lower and organoids could only be maintained for few passages⁸. Other recently published organoid cultures have focused on pediatric cancers, including Wilm's tumors, malignant rhabdoid tumors, renal cell carcinomas, and congenital mesoblastic nephromas^{9,10}.

Applications and limitations of the protocol

Since organoids closely recapitulate several properties of the original tumor and since they are scalable for high-throughput drug screens, they present excellent *in vitro* systems that connect clinical trials with cancer genetics, complement current preclinical model-based drug studies and advance the development of personalized therapy design. This has been successfully demonstrated in colorectal cancer organoids as well as in our study on kidney cancer organoids^{5,11}.

Organoid technology can be combined with genome editing technologies to better understand specific disease mechanisms. The first CRISPR-generated genetic model of pluripotent stem cell-derived renal organoids modeled polycystic kidney disease¹². Genome editing to model kidney cancer has not been done. However, in intestinal organoids, CRISPR-mediated gene modification of the most commonly mutated genes resulted in niche factor independence and carcinoma formation after xenotransplantation¹³. Moreover, 3D cell culture systems can be used in large CRISPR screens to determine growth-specific vulnerabilities that may lead to the development of novel therapeutic strategies¹⁴.

In our work, we focused on the determination of a cancer stem cell population and its implications for patient-specific therapy response in different patient-derived cancer systems⁵. Therefore our focus was to optimize growth conditions for tumor organoids. Growth of organoids derived from adjacent kidney tissue was less efficient in the developed tumor organoid medium. We conclude that the culture conditions would have to be adapted to the needs of healthy kidney tissue to derive a matched healthy kidney organoid.

Experimental Design

Overview of the procedure

In our detailed protocol, we describe the establishment and cultivation of human renal carcinoma-derived organoids. The main procedure outlines the mechanical preparation and enzymatic digestion of the tumor tissues as well as the subsequent cell plating and organoid passaging (Steps 1-3). Cells are cultivated in Matrigel and cultured in defined medium, which is exchanged every 2-3 days. Cell rounding and organoid formation can be observed after 2-3 days. We also describe protocols for cryopreservation of kidney cancer organoids to build up personal biobanks (Step 4A) and for organoid preparation for immunocytochemistry, whole-mount confocal microscopy, and transmission electron microscopy (Steps 4B-D) as well as for harvesting organoids for protein analyses and RNA extraction (Step 4E). Additionally, we describe the setup of a multi-well drug assay to systematically examine the effects of large- and small-molecule inhibitors on organoid viability (Step 4F).

Culture medium

In this protocol, kidney cancer organoid cultures are grown in defined medium – its composition is summarized in Table 2. Unlike other organoid cultures, kidney cancer organoids exhibited no requirement for R-Spondin 1, since endogenous autocrine mechanisms to upregulate Wnt signaling were found⁵. Kidney cancer organoids showed strong dependence on EGFR and FGF. However, several other growth factors implicated in kidney development resulted in no benefit to organoid formation. We still expect that further optimizing the culture medium to obtain healthy kidney organoids is possible. For high efficiency of culture establishment and successful long-term maintenance, we suggest using appropriately stored mixtures of growth factors and chemical compounds, and using freshly prepared growth medium for 1 week of cultivation.

MATERIALS

REAGENTS

- Agarose (Invitrogen, cat. no. 15510-027)
- Amphotericin B (Biomol, cat. no. A2230)
- B-27 Supplement (Life Technologies, cat. no. 17504044)
- Blood and Cell Culture DNA Kit (Qiagen, cat. no. 13323)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich, cat. no. A3733)
- CD45 MicroBeads, Human (Miltenyi Biotec, cat. no. 130-045-801)
- Cell Recovery Solution (Corning, cat. no. 354253)
- CellTiter Glo Cell Viability Assay Substrate and Buffer (Promega, cat. no. G7572)
- Chloroform (Roth, cat. no. 3313.2)
- Collagenase P (Roche, cat. no. 11213865001)
- DAPI (Thermo Fisher, cat. no. D1306)
- DMEM/F-12, GlutaMAX™ supplement (Life Technologies, cat. no. 10565018)
- Donkey serum (Bio-Rad, cat. no. C06SB)
- EDTA (Roth, cat. no. 8043.2)

- EtOH 100% (Roth, cat. no. 9065.2)
- Entellan new (Merck, cat. no. 107961)
- Eosin (Merck, cat. no. 1.15935.0100)
- Fetal Bovine Serum (FBS) (Life Technologies, cat. no. 10270-106)
- Formaldehyde 35% (Roth, cat. no. 4980.2)
- Glutaraldehyde (Roth, cat. no. 4995.2)
- HCl (Merck, cat. no. 1.09057.1000)
- Hematoxylin (Fluka AG, cat. no. 51260)
- Heparin (Sigma-Aldrich, cat. no. H3194)
- Human EGF (Peprotech, cat. no. AF-100-15)
- Human FGFb (basic) (Thermo Fisher, cat. no. 68-8785-82)
- Immumount® (Thermo Scientific, cat. no. 9990402)
- KCl (Roth, cat. no. 6781.1)
- KH_2PO_4 (Roth, cat. no. 3904)
- L-Glutamine (Biochrom, cat. no. K0283)
- Lead (II) Citrate (Sigma-Aldrich, cat. no. C6522)
- Matrigel (Corning, cat. no. 354230)
- MEM with Earle's Salts with 2.2 g/l NaHCO_3 , without L-glutamine (Biochrom, cat. no. F0325)
- Milli-Q water
- $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ (Roth, cat. No. 4984.1)
- *N*-acetylcysteine (Sigma-Aldrich, cat. no. A9165)
- NaCl (Merck, cat. no. 1.06404.1000)
- NaOH (Merck, cat. no. 1.09137.1000)
- Non-Essential Amino Acids (NEAA) (Life Technologies, cat. no. 11140-050)
- Osmium Tetroxide (Sigma-Aldrich, cat. no. 75632)
- Paraplast (Surgipath, cat no. 39601006)
- Penicillin-Streptomycin (Life Technologies, cat. no. 15140-122)
- Polyacryl Carrier (Molecular Research Center, cat. no. PC 152)
- PolyBed 812 resin (Polysciences Inc., cat. no. 08792-1)
- Recovery Cell Culture Freezing Medium (Thermo Fisher, cat. no. 12648010)
- Toluol (Roth, cat. no. 7115.2)
- Tris (Sigma Life Science, cat. no. T1503)
- TRIzol (Ambion, cat. no. 15596018)
- Trypan Blue (Sigma-Aldrich, cat. no. 93590)
- TrypLE Select (Life Technologies, cat. no. 12563-011)
- Tween 20 (SERVA, cat. no. 9005-64-5)
- Agarose Low Melt (Roth, cat. no. 6351.1)
- Uranyl Acetate (Polysciences Inc., cat. no. 21447-25)
- Xylol (Roth, cat. no. CD80.2)

ANTIBODIES

Primary antibodies/conjugates

- Biotinylated Lotus Tetragonolobus Lectin (LTL) (Vector Laboratories, cat. no. B-1325)
- Anti-Carbonic Anhydrase IX (CA9), Rabbit (abcam, cat. no. ab128883)
- Anti-E-Cadherin (ECAD), Mouse (BD Biosciences, cat. no. 610181)

Secondary antibodies/conjugates

- Anti-Mouse-Cy3, Donkey (Jackson, cat no. 715-165-151)
- Anti-Rabbit-Alexa-488, Donkey (Jackson, cat. no. 711-546-152)
- Streptavidin-Alexa-647 (Thermo Scientific, cat. no. S21374)

TABLE 1 | Dilutions of primary and secondary antibodies/conjugates used for characterization of organoids.

Type of antibody	Antibody used	Dilution
Primary antibodies/ conjugates	CA9	1:100
	ECAD	1:300
	LTL	1:300
Secondary antibodies/ conjugates	Anti-Rabbit-Alexa-488	1:250
	Anti-Mouse-Cy3	1:250
	Streptavidin-Alexa-647	1:200

EQUIPMENT

- Analytical Balance Scale R200D (Sartorius Research)
- BBD6220 CO₂ Incubator (Thermo Scientific)
- Cell Culture Multiwell Plate, 48 Well, clear, Advanced TC, sterile, with lid (CellStar, cat. no. 677 980)
- Cell strainer, 40 μ m, Nylon (Falcon, cat. no. 352340)
- Cell strainer, 70 μ m, Nylon (Falcon, cat. no. 352350)
- Confocal Microscope LSM 710 NLO (Zeiss)
- Cover Glass, 24x60 (Roth, cat. no. H878)
- Electron Microscope EM 910 (Zeiss)
- Eppendorf 5424R Centrifuge (Eppendorf)
- Freezer (Sanyo)
- Hemocytometer (Neubauer)
- Horizontal Shaker Duomax 1030 (Heidolph)
- Injekt Disposable Syringe, 20 ml (BRAUN, cat. no. 4606205V)
- Internal Threaded Polypropylene Cryogenic Vial, Self-Standing with Round Bottom, 2 ml (Corning, cat. no. 430488)
- Inverted Microscope ID03 (Zeiss)

- Leica DMI 6000 B Microscope (Leica)
- Liquid Nitrogen BIOSAFE-CONTROL B (Cryotherm)
- Luminometer LB90S (Berthold Technologies)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)
- Magnetic Stirrer with Hot Plate (IKAMAG)
- Microcentrifuge DW-41BR-230 (Qualitron)
- Microtome HM355S (Microm)
- Microwave (Sharp)
- Milli-Q Water Purification System (Merck)
- Mini metal base molds (Leica Biosystems)
- MS columns (Miltenyi Biotec, cat. no. 130-042-201)
- Multifuge 4KR (Thermo Scientific)
- OctoMACS Separator (Miltenyi Biotec, cat. no. 130-042-109)
- Orbital Shaker Unimax 1010 with Incubator 1000 (Heidolph)
- Rotilabo-embedding cassettes, white (Roth, cat. no. K113.1)
- Paraffin Embedding Station EC 350 (Microm)
- Paraffin Oven (Binder)
- Pre-separation filters, 30 μm (Miltenyi Biotec, cat. no. 130-041-407)
- Refrigerator (Liebherr)
- Round Bottom Polystyrene Test Tube, with Snap Cap, 5 ml (Falcon, cat. no. 352058)
- μ -Slide 8 Well Glass Bottom (ibidi, cat. no. 80827)
- Slide Microscope Axio Scope.A1 (Zeiss)
- Slide Warmer SW85 (Adamas Instrumenten BV)
- Sterile Workbench Safe 2020 (Thermo Scientific)
- SuperFrost Plus Adhesion slides (Thermo Scientific, cat. no. J1800AMNZ)
- Surgical Disposable Scalpels (BRAUN, cat. no. 5518075)
- Syringe Filter, FP 30\0.2 CA-S (Whatman, cat. no. 10462206)
- TC Dish 100, Standard (Sarstedt, cat no. 83.3902)
- Thermomixer 5436 Heating Block (Eppendorf)
- Tube Roller (Star Lab Smart Instruments)
- Water Bath 1003 (GFL)
- Vortex-Genie 2 (Scientific Industries)

SOFTWARE

- iTEM software (Emsis GmbH)
- LAS X (Leica)
- Zen 2 Black/Blue Edition (Zeiss)

REAGENT SETUP

CRITICAL All non-sterile reagents used for cell culture conditions must be filtered through a disposable 0.2 μm filter.

Heparin Dissolve 5 mg in 1 ml of Milli-Q water, aliquot, and store at -20°C until the expiration date.

Human EGF Dissolve 100 µg in 50 µl of PBS 1x, mix with 50 µl of 0.1% BSA in PBS (w/v) to 1 mg/ml, aliquot, and store at -80°C until the expiration date.

Human FGFb (basic) Dissolve 100 µg in 625 µl of PBS 1x, mix with 625 µl of 0.1% BSA in PBS (w/v) to 80 µg/ml, and store at -80°C until the expiration date.

MACS buffer 2.5 g of BSA (0.5% w/v), 0.29224 g of EDTA (2 mM), ad 500 ml of PBS 1x, store at 4°C for maximum 6 months.

Medium for human tumor-derived organoids Final concentrations of medium components are listed in the table 2. To prepare 50 ml of the medium, to 48.2 ml of DMEM/F-12 medium add 1 ml of 50x B-27 Supplement, 1 µl of 1 mg/ml EGF, 12.5 µl of 80 µg/ml FGFb, 40 µl of 5 mg/ml Heparin, 500 µl of 100x Penicillin-Streptomycin, and 250 µl of 25 mg/ml Amphotericin B.

CRITICAL The medium should be stored at 4°C for a maximum of one week.

Phosphate buffer (0.1 M) Solution A: Dissolve 2.72 g of KH₂PO₄ in 100 ml of Millie-Q water (0.2M). Solution B: Dissolve 8.9 g of Na₂HPO₄ x 2H₂O in 250 ml of Millie-Q water (0,2M). Add 18.2 ml of Solution A to 81.8 ml of Solution B, and adjust the pH to 7.4. Ad 200 ml of Milli-Q water.

Phosphate Buffered Saline (PBS 1x) 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ x 2H₂O, 0.24 g of KH₂PO₄, ad 1000 ml of Milli-Q water.

PBS-T 1 ml of Tween 20 (0.1% v/v), ad 1000 ml of PBS 1x.

Primary cell medium To prepare 50 ml of medium, to 43.25 ml of MEM add 5 ml of FBS, 500 µl of 200 mM L-Glutamine, 500 µl of 100x NEAA, 500 µl of 100x Penicillin-Streptomycin, and 250 µl of 25 mg/ml Amphotericin B. Store at 4°C for maximum one month.

RBC erythrocyte lysis buffer 4.0118 g of ammonium chloride, 0.5 g of potassium bicarbonate, 0.0146 g of EDTA, ad 1000 ml of Milli-Q water, adjust pH to 7.4, store at 4°C for maximum 6 months.

DNase I Dilute 5 mg or 10.000 U in 1 ml sterile water. Store at -20°C for a year.

TABLE 2 | Final concentrations of medium components for organoid culture.

Medium Component	Human tumor-derived organoids
B-27 supplement	1x
Human EGF	20 ng/ml
Human FGFb (basic)	20 ng/ml
Heparin	4 µg/ml
Penicillin-Streptomycin	1x
Amphotericin B	125 µg/ml

PROCEDURE

Establishing and culturing renal organoid cultures

1I Pre-warm tissue culture plates overnight at 37°C and thaw Matrigel on ice several hours or overnight before use.

2I Establishing organoids from human renal cell carcinoma (RCC) biopsies TIMING up to 14 d

- i. Wash RCC tissue specimens, directly obtained from surgery, and remove fatty and necrotic tissue.
- ii. Use a scalpel to mince the tissue biopsy into small pieces of $< 1 \text{ mm}^3$ size.
- iii. Take up the pieces in 10 ml of PBS and transfer them to a 50 ml falcon tube. Centrifuge at $300 \times g$ for 5 min at 4°C, and weigh the pellet after aspirating the supernatant.

CRITICAL STEP: Weigh the empty Falcon tube before transferring the tissue to calculate the weight of the sample.

- iv. Prepare one ml of 2 mg/ml Collagenase P solution per 50 mg of tissue and digest the tissue at 37°C for approx. 30 min, rocking gently.

? TROUBLESHOOTING

- v. Wash with 25 ml PBS and centrifuge at $300 \times g$ for 5 min at 4°C.
- vi. Resuspend the sample in 5 ml of PBS, and filter the cell suspension through a $70 \mu\text{m}$ followed by a $40 \mu\text{m}$ cell strainer. Wash the filters 3x with PBS.
- vii. Centrifuge the eluate at $300 \times g$ for 5 min at 4°C, and wash the cell pellet with PBS.
- viii. Incubate in 5 ml of RBC erythrocyte lysis buffer for 5 min at RT.
- ix. After washing in PBS, count the cells using a hemocytometer and resuspend in $80 \mu\text{l}$ MACS buffer per 1×10^7 cells. Add $20 \mu\text{l}$ of anti-CD45 Microbeads per $80 \mu\text{l}$ of cell suspension and incubate for 15 min at 4°C.

Optional: Add DNase I at a final concentration of 200 U/ml to the MACS buffer if you experience excessive cell clumping.

- x. Wash the cells in 2 ml of MACS buffer per 1×10^7 cells before resuspending the pellet in $500 \mu\text{l}$ of MACS buffer per 1×10^8 cells.
- xi. After the magnetic separation of CD45^+ white blood cells (WBC) using a $30 \mu\text{m}$ pre-separation filter and a MACS buffer equilibrated MS column, collect the CD45^- cells in the flow-through, and wash the column three times with $500 \mu\text{l}$ of MACS buffer.

? TROUBLESHOOTING

- xii. Centrifuge the CD45^- cell suspension at $300 \times g$ for 5 min at 4°C, resuspend in PBS and count the alive (unstained) cells by mixing $10 \mu\text{l}$ of 0.4% (w/v) Trypan Blue solution with $10 \mu\text{l}$ cell solution.
- xiii. Resuspend 1.5×10^4 cells per well in pre-chilled growth medium and dilute with Matrigel to a final concentration of 75% Matrigel. Plate $25 \mu\text{l}$ cell-Matrigel drops in the middle of the well of a pre-heated 48-well plate.

CRITICAL STEP: Work quickly and constantly on ice to prevent Matrigel from polymerizing before plating. Resuspend cells in pre-chilled growth medium before adding the Matrigel.

CRITICAL STEP: Do not dilute the Matrigel beyond 75% to guarantee sufficient stiffness, and to prevent Matrigel lens rupture during cultivation.

- xiv. Place the tissue culture plate upside down in the CO₂ incubator, while the Matrigel containing the cells solidifies for 15 min at 37°C.
CRITICAL STEP: Invert the plates to prevent cell descent and adherence to the well bottom.
- xv. Gently add 250 μ l of pre-warmed organoid growth medium into each well, and place the plate into a CO₂ incubator (5% CO₂, 37°C).
CRITICAL STEP: Medium, which is not pre-warmed, may cause Matrigel lens rupture, and decrease organoid formation efficiency.
- xvi. Refresh the medium every 2-3 days. Check organoid growth daily using a brightfield microscope. After 3-4 days, small organoids should be visible.
- xvii. Passage the organoids when they reach a diameter of 300-500 μ m (usually after 10-14 days).

3I Passaging of organoids TIMING variable

- i. Using a 1 ml pipette tip, release organoids from Matrigel lenses by pipetting up and down several times and transfer them to a 15 ml falcon tube coated with 1% BSA in PBS (w/v).
? TROUBLESHOOTING
- ii. Wash the wells with 1 ml of PBS to obtain all cells, and add them to the falcon tube.
CRITICAL STEP: Harvest the organoids in at least 5 ml of total volume to allow better perturbation in the following step.
? TROUBLESHOOTING
- iii. Pipet thoroughly using a 100 μ l tip on a 5 ml serological pipet to fully release the organoids from the Matrigel.
CRITICAL STEP: By using a 100 μ l tip on a 5 ml serological pipet, you can simultaneously perturb a large volume of the organoids, and create strong enough shear forces to more efficiently release the organoids from the Matrigel.
- iv. To eliminate dead cells, cells not within the growing organoids or without organoid formation potential, filter the cell suspension through a 70 μ m cell strainer.
CRITICAL STEP: We recommend this for the first 1-2 passages. It is however not necessary once organoid cultures are well established.
- v. Wash the strainer with 5 ml of growth medium and invert the cell strainer onto a 10 cm² dish.
- vi. Wash the retained organoids from the membrane with 10 ml of growth medium, and collect the organoids in a 15 ml falcon tube.
- vii. Centrifuge at 300 x g for 5 min at 4°C, and aspirate the supernatant and Matrigel above the pellet. At this point, the organoids can be passaged or further processed for e.g.:
 - a. RNA isolation with TRIzol,
 - b. gDNA isolation using the Blood and Cell Culture DNA Kit,
 - c. Protein Isolation using the Cell Recovery Solution.
- viii. Resuspend the organoids in 300-500 μ l of TrypLE Select cell dissociation enzyme solution and incubate for 5-15 min at RT with frequent perturbation by pipetting. Check organoid dispersion under the bright-field microscope.

CRITICAL STEP: Organoid dissociation can easily be monitored by holding the falcon tube at an angle in the light path of a bright-field microscope, thereby eliminating the need for a hemocytometer for this step.

CRITICAL STEP: If the organoids have not yet reached 300-500 μm in diameter, but their density is high, we recommend releasing the organoids from the Matrigel, as described in step i-iv, and reseeding at lower densities.

ix. When the organoids are dispersed to smaller cell clusters, stop the digestion by adding 4 ml of growth medium, and centrifuge the organoids at 300 x g for 5 min at 4°C.

x. Resuspend the organoids in pre-chilled medium and add Matrigel to a final concentration of 75% Matrigel. Plate 25 μl drops in the middle of the well of a pre-heated 48-well plate.

CRITICAL STEP: Work quickly and constantly on ice to prevent Matrigel from polymerizing before plating. Resuspend cells in pre-chilled growth medium before adding the Matrigel.

xi. Place the tissue culture plate upside down in the CO₂ incubator, while the Matrigel containing the cells solidifies for 20 min at 37°C.

CRITICAL STEP: Invert the plates to prevent cell descent and adherence to the well bottom.

xii. Gently add 250 μl of pre-warmed organoid growth medium into each well, and place the plate into a CO₂ incubator (5% CO₂, 37°C).

CRITICAL STEP: Medium, which is not pre-warmed, may cause Matrigel lens rupture, and decrease organoid formation efficiency.

xiii. Refresh the medium every 2-3 days. Check organoid growth daily using a brightfield microscope. One day after passaging, small organoids should be visible. Generally, the organoids were passaged in a 1:2 dilution.

Downstream Assays

4 I Once organoid cultures have grown to sufficient size, organoids can be cryopreserved (option A). Specific marker expression can be analyzed by immunofluorescence after organoid embedding for paraffin sectioning (option B) or whole mount imaging via confocal microscopy (option C). Detailed morphological characterization can be achieved by transmission electron microscopy (option D) and proteins can be isolated (option E). Organoid cultivation can be upscaled for high-throughput drug testing of inhibitory and nephrotoxic effects on organoid proliferation and viability (option F).

(A) Cryopreservation of organoids

TIMING 30 min

i. Release the organoids from Matrigel and dissociate them into clusters as described in steps 3I i-ix.

CRITICAL STEP: Organoid reformation and viability was greatly increased, when cells were cryopreserved as clusters instead of whole organoids.

ii. Resuspend the organoid clusters in 1 ml of Recovery Cell Culture Freezing Medium per 2-3 wells of the dissociated organoids, and transfer to cryotubes.

- iii. Freeze the cryostocks for at least 6 hours in isopropanol containers at -80°C before storage in liquid nitrogen.
PAUSE POINT: The dissociated organoids can be stored up to 30 days at -80°C. For long-term storage (> 1 month), transfer the cryostocks to liquid nitrogen.
- iv. When you wish to take cells back into culture, thaw the cells in a 37°C water bath.
CRITICAL STEP: Shake the vial in the 37°C water bath until only a small piece of ice remains and quickly continue processing the cells to reduce cell stress.
- v. Transfer the thawed cells into a 15 ml falcon tube, and dropwise add 10 ml of pre-warmed growth medium.
- vi. Centrifuge at 300 x g for 5 min at 4°C.
- vii. Aspirate the supernatant, and resuspend the cell pellet in 75% Matrigel.
- viii. Plate the organoids as described in steps 3l x-xiii. We recommend seeding the dissociated organoids in a 1:2 dilution, meaning seeding 1 cryostock of 2-3 wells in 4-6 fresh wells.

(B) Embedding of organoids in agarose for paraffin sectioning

TIMING 4-5 d

- i. Release the organoids from Matrigel as described in steps 3l i-iii
- ii. Centrifuge the organoids at 300 x g for 5 min at 4°C, and discard the supernatant, including Matrigel and dead cells.
- iii. Wash the organoids with 0.1% BSA in PBS (w/v), and centrifuge at 300 x g for 5 min at 4°C.
- iv. Resuspend the organoids in 7 ml of 10% NBF in PBS (v/v), and fix over night at 4°C rocking.
- v. Wash the organoids with 0.1% BSA in PBS (w/v), resuspend in 500-1000 μ l of 0.1% BSA in PBS (w/v), transfer to a coated 2 ml Eppendorf tube, and centrifuge at 300 x g for 5 min at 4°C.
- vi. Shortly place the tube in an 80°C heating block before carefully overlaying the organoid pellet with 400 μ l of 1.5% agarose.
? TROUBLESHOOTING
- vii. Centrifuge shortly at 300 x g at 4°C. Let the agarose solidify on ice.
CRITICAL STEP: Place the tube in the centrifuge in the same orientation as before to make sure the pellet does not move from its previous position.
- viii. After the agarose has polymerized, release the agarose bead from the Eppendorf tube.
CRITICAL STEP: Use a razor blade to cut off the bottom of the Eppendorf tube at the opposite side as the organoid pellet, and carefully push out the agarose bead.
- ix. Trim the bead around the organoid pellet, transfer it to an embedding cassette, and place the cassette in 70% EtOH for 24-48 h.
PAUSE POINT: The agarose bead can remain in 70% EtOH for up to 7 days before dehydration.
- x. Dehydrate the organoids according to Table 3.
- xi. After embedding in paraffin, section the paraffin-embedded organoids using a microtome at 5 μ m thickness with a water bath at a temperature of 48°C. Quickly transfer the sections onto glass slides to prevent the agarose from dissolving.

- xii. Place air-dried glass slides on a 60°C warming block for 15 min and in a 60°C oven for 60 min to fix the organoids to the glass slide before staining.
- xiii. Rehydrate the organoids before performing hematoxylin and eosin staining as well as immunocytochemistry.

TABLE 3 I Dehydration steps of organoids in agarose beads.

Incubation time	Solution
30 min	80% EtOH
30 min	90% EtOH
1h	96% EtOH
1h	100% EtOH
30 min	100% EtOH
1h	Toluene
30h	Toluene
1-3h	Paraffin
Over night	Paraffin
1h	Paraffin

(C)Preparation of organoids for confocal microscopy **TIMING 3 d**

- i. Release the organoids from Matrigel as described in steps 3I i-iii.
- ii. Resuspend the organoids in 1500 μ l of 10% NBF in PBS (v/v), and transfer them to a 2 ml coated Eppendorf tube.
- iii. Fix the organoids for 1.5h at 4°C shaking, and wash 5x with PBS-T over 2h at 4°C shaking.
- iv. Block with 1% BSA in PBS-T (w/v) containing 10% donkey serum for 2h at RT shaking.
- v. Prepare antibody dilutions in 1% BSA in PBS-T, and incubate the organoids with primary antibodies over night at 4°C shaking.
- vi. Wash the organoids 4x with PBS-T over 4h at 4°C shaking with centrifugation at 300 x g for 5 min at 4°C.
- vii. Incubate with secondary antibodies and DAPI over night at 4°C shaking.
CRITICAL STEP: Wrap the Eppendorf tubes in aluminum foil to prevent fluorophore photobleaching.
- viii. The next day, wash the organoids 6x with PBS-T over 6h at 4°C shaking with centrifugation at 300 x g for 5 min at 4°C.
- ix. Take up the organoids in 1% low-melting agarose, and transfer them to an 8-well chambered cover-glass slide for confocal fluorescence microscopy.
CRITICAL STEP: Resuspend the organoids in PBS-T and add the equal amount of 2% low-melting agarose to distribute the organoids well in solution before transfer to the chambers.

? TROUBLESHOOTING

(D) Transmission electron microscopy of organoids **TIMING up to 14d**

- i. Release the organoids from Matrigel as described in steps 3I i-iii
- ii. Resuspend the organoids in 7 ml of 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer.
CAUTION: PFA is toxic and must be handled under a fume hood.
- iii. Fix the organoids for 2h at RT shaking, and wash 2x with 0.1 M phosphate buffer for 15 min.
- iv. Resuspend the organoids in 500 - 1000 μ l 0.1 M phosphate buffer, transfer them to a coated 2 ml Eppendorf tube and centrifuge at 300 x g for 5 min at 4°C.
- v. Shortly place the tube in an 80°C heating block before carefully overlaying the organoid pellet with 400 μ l of 10% agarose.
- vi. Centrifuge at 300 x g for 10 s
CRITICAL STEP: Place the tube in the centrifuge in the same orientation as before to make sure the pellet does not move from its previous position.
- vii. After embedding, post-fix the samples with 1% (v/v) osmium tetroxide, dehydrate in a graded series of EtOH, and embed in PolyBed® 812 resin.
- viii. Stain ultrathin sections, 60-80 nm thick, with uranyl acetate and lead citrate, and examine at 80 kV with an electron microscope, i.e. Zeiss EM 910.

? TROUBLESHOOTING

(E) Harvesting organoids for protein assays **TIMING up to 3h**

It is highly important to reduce the fraction of extracellular proteins from Matrigel within the total protein lysate. Therefore, the following procedure was established with the described organoid cultures.

- i. Using a 1 ml pipette tip, release organoid-Matrigel lenses from the well, and transfer them to an uncoated 15 ml falcon tube to avoid extracellular protein addition in form of BSA.
- ii. Wash the wells with 1 ml of PBS to obtain all cells, and add them to the Falcon tube.
- iii. Pipet thoroughly using a 100 μ l tip on a 5 ml serological pipet to release the organoids from the Matrigel.
- iv. Centrifuge the organoids at 300 x g for 5 min at 4°C, and discard the supernatant, including Matrigel and dead cells.
- v. Wash the organoids with ice cold PBS, and centrifuge at 300 x g for 5 min at 4°C.
CRITICAL STEP: Low temperature of PBS helps to release the organoids from the rest of the surrounding Matrigel.
- vi. Resuspend the organoids in 500-1000 μ l of ice-cold PBS, transfer to a 2 ml Eppendorf tube, and centrifuge at 300 x g for 5 min at 4°C.
- vii. Mix the organoid pellet with 5 ml of Cell Recovery Solution.
- viii. Incubate for 1h at 4°C rotating.
- ix. Centrifuge for 5 min at 300 x g at 4°C, remove the supernatant.
- x. Wash the organoid pellet twice with ice cold PBS.
- xi. Transfer the organoid pellet to an Eppendorf tube using PBS.
- xii. Proceed with preferred method of protein isolation, e.g. using RIPA buffer. In short, organoids are resuspended in 100 μ l of RIPA buffer containing protease and/or

phosphatase inhibitors, lysed for 10 min on ice, and centrifuged at 15,000 x g for 10 min at 4°C. Supernatant can be stored at -20°C after total protein measurement, e.g. by Bradford assay.

(F) Organoid viability and signaling inhibition analysis

TIMING up to 7 d

The viability of organoids and the effects of inhibitors of specific signaling pathways in organoids as well as nephrotoxic substances can easily be analyzed by the Promega CellTiter Glo assay.

- i. Release the organoids from Matrigel as described in steps 3I i-iii.
- ii. Resuspend the organoids in 300-500 μ l of TrypLE Select cell dissociation enzyme solution and incubate for 15-30 min at RT with frequent perturbation by pipetting. Check organoid dispersion under the bright-field microscope.
CRITICAL STEP: Organoid dissociation can easily be monitored by holding the falcon tube at an angle in the light path of a bright-field microscope, thereby eliminating the need for a hemocytometer for this step.
CRITICAL STEP: To obtain comparable results, organoids have to be completely dissociated to single cells. Depending on the size this requires up to 30 min TrypLE dissociation.
- iii. Resuspend 5000 single cells per well in pre-chilled medium and add Matrigel to a final concentration of 75% Matrigel. Plate 9 μ l cell-Matrigel drops in the middle of the well of a pre-heated 96-well plate.
- iv. Invert the plates to prevent cell descent and attachment to the well bottom, while the Matrigel containing the cells solidifies for 20 min at 37°C.
CRITICAL STEP: We recommend marking the position of Matrigel lenses, which are not plated exactly in the middle of the well, on the lid to prevent organoid loss during medium exchanges.
CRITICAL STEP: Mark the position of Matrigel drops that attached to the side of the well to prevent cell aspiration during medium changes.
- v. Gently add 100 μ l of pre-warmed organoid growth medium into each well, and place the plate into a CO₂ incubator (5% CO₂, 37°C).
- vi. Cultivate the organoids with inhibitors for 7 days before performing the assay, according to manufacturer's protocol. In short, resuspend CellTiter Glo Substrate in CellTiter Glo Buffer, add the solution to the cell cultures in a 1:2 dilution, and incubate for 10 min at RT before recording luminescence.

TROUBLESHOOTING

Step	Problem	Possible Reason	Solution
2I iv	Large tissue pieces remain after enzymatic digestions.	Digestion not sufficient	Increase the digestion time, as it is variable and depending on

			tissue size. Perturbate the tissue solution using a serological pipet.
2I iv	Low number of viable cells after enzymatic digestion	Digestion time too long	Digestion time is variable on tissue. Control the digestion process regularly and stop once most big tissue pieces are digested.
2I xi	Magnetic columns clog during CD45-depletion.	Too many cells in the solution (maximal capacity of the column: 2×10^8 of cells) Dead cells result in cell clumps.	Dilute cell solution with MACS buffer and distribute across multiple columns. Add DNase I at a final concentration of 200 U/ml.
3I i	Organoids die after passaging.	Organoid growth was not fully established in the culture. Organoids were too small or their density was too low when passaged.	Grow organoids to at least a diameter of 300-500 μm and sufficient density before passaging.
3I ii, 4I C ix	Low number of organoids after harvesting or whole mount staining	Organoids can stick to plastic in pipet tips or tubes.	Coat all tubes and flush all pipet tips with 1% BSA in PBS (w/v) before e.g. washing organoids.
4I C ix, 4I D viii	Mostly fractionated organoids visible in sections, Whole-Mount Imaging or TEM	Organoids were too large when harvested. Handling of organoids was not careful enough.	Harvest organoids when they are at a diameter of 300-500 μm to prevent rupture. During organoid preparation avoid vortexing and heavy pipetting.

TIMING

Steps 1-3, establishing and passaging human tumor kidney organoids: variable up to 2 weeks

Step 4, downstream assays: variable up to 14d

ANTICIPATED RESULTS

Our protocol describes an efficient method for cultivating organoids of human clear cell renal cell carcinoma (ccRCC). Organoids are 3D structures grown from stem cells, which differentiate into organ-specific cell types that self-organize through cell sorting and spatially restricted lineage commitment⁴. Renal cell carcinoma organoids described in previous publications showed inferior organoid formation efficiencies, could only be maintained for few passages, and were not fully characterized. Here we established organoids from kidney tumor biopsies that recapitulate their tissues of origin *ex vivo* and could be passaged and cultivated as a promising patient-specific pre-clinical *in vitro* model.

Following this protocol, rounding of cells and formation of small organoids can be detected within 2-3 d after seeding (Fig. 1a). Tumor-derived cells showed an organoid formation efficiency of 0,084%, which could be increased to 0,191% by previous sorting for cancer stem cells⁵. Generally, heterogeneity could be observed between cultures of different patients, however cystic structures were predominantly formed (Fig. 1a). Tumor organoids retained intra- as well as inter-tumor heterogeneity containing cystic as well as solid and tubular structures (Fig. 1b), thereby making them an excellent pre-clinical model for personalized medicine¹⁵.

To confirm the cellular identity of the organoids, immunofluorescence can be performed: tumor organoids should express the renal carcinoma marker carbonic anhydrase IX as well as the epithelial differentiation marker E-cadherin. As the tumor organoids established in this protocol recapitulate the proximal tubule as the putative cell-of-origin of ccRCC, organoids should also stain positively for lotus tetragonolobus, LTL (Fig. 2a,b).

The complexity of organoid cultures can further be analyzed by transmission electron microscopy. Tumor organoids are polarized and show high numbers of cell interactions, including tight and adherens junctions as well as desmosomes that seal the inter-cellular space from the lumen. Organoids produce brush border structures on the luminal side, coinciding with positive LTL staining, and present ccRCC characteristics including lipid droplets and glycogen accumulation (Fig. 2c).

Competing Financial Interests

The authors declare that they have no competing financial interests.

Acknowledgments

The authors thank members of the contributing laboratories for support. D.B. is and A.F. was funded by the Urological Research Foundation Berlin, headed by Prof. Stefan Loening.

Author Contributions

D.B., A.F., and W.B. developed the concept of the study and wrote the manuscript. D.B. and A.F. designed and performed experiments and analyzed the data. W.B. and A.F. supervised the project.

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Figure Legends

Fig. 1: Morphology of ccRCC organoids

(a) Representative bright-field images of an organoid growing from a single cell over 10 days. (scale bar, 100 μm). (b) Left: Bright-field pictures of representative cystic, solid and tubular organoid cultures. Right: HE stainings of representative organoids (scale bars, 250 μm)

Fig. 2: Functional characterization of ccRCC organoids

(a) Immunofluorescence after 7 days of culture for DAPI, Carbonic Anhydrase IX (CA9), E-Cadherin (ECAD) and LTL (scale bars, 50 μm). (b) Whole-mount images of DAPI (blue), CA9 (green), ECAD (orange) and LTL (red). Pictures are 15 μm apart (scale bar, 100 μm). (c) Transmission electron microscopy of representative organoid cultures (see scale bars for sizes): L, luminal side; D, desmosomes; AJ, adherens junctions; arrowheads, tight junctions; arrows, lipid droplets; asterisks, glycogen deposits (Data previously published⁵).