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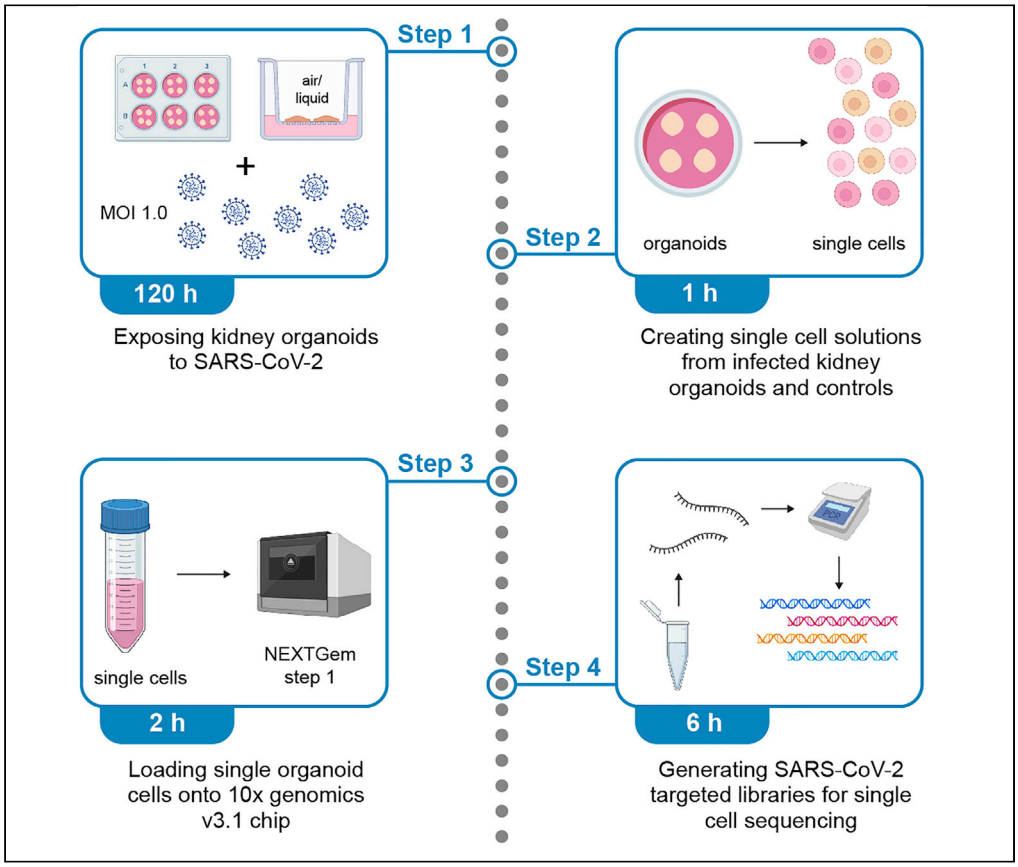
Using human iPSC-derived kidney organoids to decipher SARS-CoV-2 pathology on single cell level



We describe a protocol for single-cell RNA sequencing of SARS-CoV-2-infected human induced pluripotent stem cell (iPSC)-derived kidney organoids. After inoculation of kidney organoids with virus, we use mechanical and enzymatic disruption to obtain single cell suspensions. Next, we process the organoid-derived cells into sequencing-ready SARS-CoV-2-targeted libraries. Subsequent sequencing analysis reveals changes in kidney cells after virus infection. The protocol was designed for kidney organoids cultured in a 6-well transwell format but can be adapted to organoids with different organ backgrounds.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Inoculation of human kidney organoids with SARS-CoV-2 models COVID-19 in kidney

Mechanic and enzymatic tissue digestion yield single cells in safety level 3 laboratory

Generation of a targeted single-cell RNA sequencing library identifies infected cells

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Using human iPSC-derived kidney organoids to decipher SARS-CoV-2 pathology on single cell level

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SUMMARY

We describe a protocol for single-cell RNA sequencing of SARS-CoV-2-infected human induced pluripotent stem cell (iPSC)-derived kidney organoids. After inoculation of kidney organoids with virus, we use mechanical and enzymatic disruption to obtain single cell suspensions. Next, we process the organoid-derived cells into sequencing-ready SARS-CoV-2-targeted libraries. Subsequent sequencing analysis reveals changes in kidney cells after virus infection. The protocol was designed for kidney organoids cultured in a 6-well transwell format but can be adapted to organoids with different organ backgrounds.

For complete details on the use and execution of this protocol, please refer to Jansen et al. (2022).

BEFORE YOU BEGIN

This protocol describes the inoculation of induced pluripotent stem cell (iPSC)-derived kidney organoids with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the subsequent workflow for single cell RNA sequencing. The final product of this protocol is a targeted sequencing-ready single cell library with amplified SARS-CoV-2 sequences. All work involving

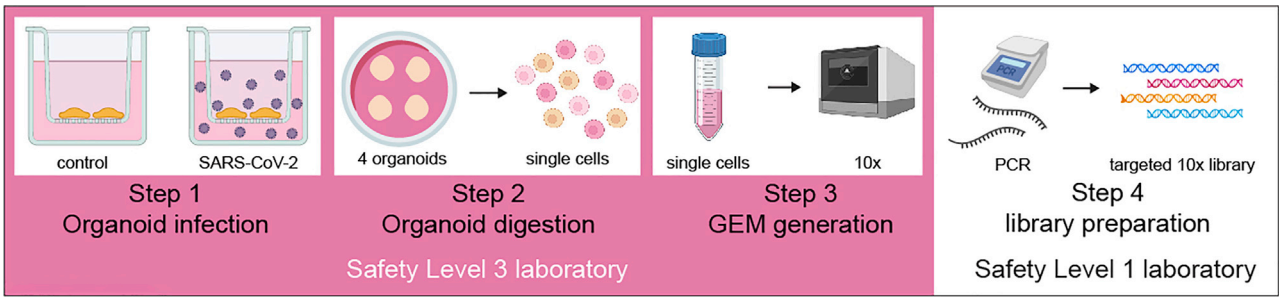

Figure 1. Overview of the workflow from iPSC-derived kidney organoids to a targeted sequencing-ready single cell library

infectious SARS-CoV-2 is legally restricted to biosafety level 3 (S3) laboratories around the world. Thus, this protocol describes infection, handling, and processing of kidney organoids and the first steps of library preparation inside an S3 facility until it is safe to leave this area with the samples. The protocol is divided into four steps: steps 1–3 are carried out at an S3 facility, while step 4 can be carried out in an S1 laboratory. The four steps occur in a sequential manner: 1) inoculation of iPSC-derived kidney organoids with SARS-CoV-2 and incubation, 2) mechanical and enzymatic disruption of organoids to obtain single cell solutions, 3) carrying out the first step of the Next GEM v3.1 protocol for single cell sequencing (10× genomics workflow), and 4) generating a targeted sequencing library to retrieve SARS-CoV-2-specific sequences from single cells in addition to the usual v3.1 single cell library. This protocol was specifically designed for iPSC-derived kidney organoids cultured in a 6-well transwell format (Takasato et al., 2016), but can be adapted for organoids of different organ backgrounds. Similarly, this protocol can be adapted for the inoculation with other viruses.

The protocol starts with mature iPSC-derived kidney organoids, cultured until d7+18 according to an adapted, well-established protocol (Takasato et al., 2016). The organoids were kept in a 6-well transwell format with each transwell filter containing 4 organoids consisting of 300,000 cells each at d7+0. The amount of materials, buffers and solutions is calculated for two filters containing four organoids each to generate two libraries for single cell sequencing: control and SARS-CoV-2-exposed organoids. To assess iPSC-derived kidney organoid quality at the beginning of any inoculation experiment, please refer to the troubleshooting section, problem 1. (Figure 1).

Institutional permissions

Human adult skin fibroblasts derived from a healthy volunteer were reprogrammed into iPSCs using the Yamanaka factors (Takahashi and Yamanaka, 2006) by the Stem Cells Technology Center at Radboud University Medical Center (SCTC, Radboud UMC, Nijmegen, The Netherlands). The iPSCs used in the current study were generated from spare materials from a healthy donor, who did consent with the use of such material and did not suffer from kidney disease. The materials have been anonymized and were collected during a time in which no signed informed consent was required.

Gathering equipment needed inside the S3 laboratory

Timing: Depending on your local S3 laboratory and training, start planning at least two weeks prior to the start of the first experiment

Preparation is crucial when working in an S3 environment. Prior to your first experiment, you should have received extensive training and be authorized to work in an S3 laboratory. You should further check what equipment is available inside the facility and which additional items need to be brought inside for temporary use. Some items and reagents, such as counting chambers or trypan blue, are often permanently available inside an S3 laboratory. Yet, for items and samples that are to be taken

out of the S3 laboratory, a specific decontamination procedure must be in place, possibly involving quarantining any equipment that is hard to decontaminate.

1. See the key resources table and materials and equipment sections for detailed information on items you will need in- and outside of the S3 laboratory.
2. Discuss with your local S3 laboratory manager about availability of equipment and materials in the facility.
3. Prepare cell buffers and solutions freshly on the day of the experiment (timing: 20 min): See materials and equipment section for preparation of the Cell Resuspension buffer, Digestion solution, 10× buffer, and 10× Master Mix.

⚠ CRITICAL: Ensure that all solutions are kept on ice or at 4°C until use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
SARS-CoV-2, strain BavPat1/2020, titered by plaque assay on Vero E6 cells.	European Virus Archive Global (EVAg) (Varghese et al., 2021)	Ref-SKU: 026V-03883
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium (DMEM)/F-12	Thermo Fisher Scientific	Cat#11320074
Phosphate buffered saline (PBS)	Fisher Scientific	Cat#11503387
Essential 6 Medium	Thermo Fisher Scientific	Cat# A1516401
Fetal bovine serum (FBS)	Bio&SELL	Cat#FBS.GP.0500
Bovine serum albumin (BSA)	Fisher Scientific	Cat#AM2618
Accutase®	Sigma-Aldrich	Cat#A6964
DNase I	Sigma-Aldrich	Cat#11284932001
Glycerin (glycerol), 50% (v/v) Aqueous Solution	RICCA Chemical Company	Cat#3290-32
Nuclease-free water	Thermo Fisher Scientific	Cat#AM9937
Trypan Blue Solution 0,4%	Fisher Scientific	Cat#15250061
KAPA Biosystems HiFi HotStart ReadyMix	Roche	Cat#7958927001
Buffer EB	QIAGEN	Cat#19086
AMPure XP beads	Beckman Coulter	Cat#A63880
Critical commercial assays		
Chromium Next GEM Single Cell 3′ Gel Bead Kit v3.1, 4 rxns	10× Genomics	Cat#1000129
Dual Index Kit TT Set A, 96 rxns	10× Genomics	Cat#1000215
Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns	10× Genomics	Cat#1000130
Chromium Next GEM Chip G Single Cell Kit, 16 rxns	10× Genomics	Cat#1000127
Library Construction Kit 4 rxns	10× Genomics	Cat#1000196
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat#Q32851
Experimental models: Cell lines		
Kidney organoids derived from Human induced pluripotent stem cell line iPS 15	Stem Cell Technology Center, Radboud UMC, The Netherlands	iPS 15 clone 0001
Oligonucleotides		
Partial Read 1 Primer 5′-CTACACGA CGCTCTTCCGATCT-3' (Reverse primer for PCR1 and PCR2 with 10× Genomics, HPLC purified)	(Schraivogel et al., 2020a)	N/A
Targeted 10× Primer 5′-AATGATACGGCG ACCACCGAGATCTACACTCTTTCCCTACA CGACGCTCTTC*C*G-3' (Forward primer for PCR3 with 10× Genomics, * thioate linkage between last three bases and HPLC purified)	(Schraivogel et al., 2020a)	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Illumina Reverse Primer (N7XX) 5′-CAAGCA GAAGACGGCATACGAGAT[X]8GTCTCGT GGGCTCGG-3' ([X]8 corresponds to the sample barcode (e.g., N701: TCGCCTTA, N702: CTAGTACG, etc., HPLC purified)	(Schraivogel et al., 2020a)	N/A
SARS-CoV-2 Outer Primer 5′-ACCACACAA GGCAGATGGGC-3' (HPLC purified)	(Triana et al., 2021)	N/A
SARS-CoV-2 Inner Primer 5′-GTCTCGTGGGC TCGGAGATGTGTATAAGAGACAGCATTTTC ACCGAGGCCACGC-3' (HPLC purified)	(Triana et al., 2021)	N/A
Software and algorithms		
BioRender (Graphical abstract, Figure 1 and Figure 2 of this paper)	http://biorender.com	N/A
Other		
Corning® Transwell® polyester membrane cell culture inserts, 24 mm Transwell® with 0.4 µm pore polyester membrane inserts, TC-treated, sterile	Sigma-Aldrich	Cat#CLS3450
40 µm cell strainers	Corning	Cat#CLS431750
50 mL Falcon centrifuge tubes	Thermo Fisher Scientific	Cat#352070
200 µL wide-bore pipette tips	Thermo Fisher Scientific	Cat#14-222-726
Neubauer counting chamber	Carl Roth	Cat#T728.1
Haemocytometer coverslips 20 × 26 mm	Carl Roth	Cat#L189.1
PCR Tubes 0.2 mL 8-tube strips	Eppendorf	Cat#951010022
RNaseZAP	Sigma-Aldrich	Cat#R2020
Nuclease-free water	Thermo Fisher Scientific	Cat#AM9937
6-well plates, tissue-culture-treated	Corning	Cat#3516
BD Microlance™ 3 Needles, 27G	BD	Cat#302200
Qubit™ 4 Fluorometer	Thermo Fisher Scientific	Cat#Q33238
Qubit™ Assay Tubes	Thermo Fisher Scientific	Cat#32856
10× Magnetic separator	10× Genomics	Cat#120250
Thermal cycler, e.g., C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	Bio-Rad	Cat#1851197
Centrifuge for 50 mL Falcon tubes, e.g., Multifuge X1 Pro	Thermo Fisher Scientific	Cat#75009710
Mini centrifuge with PCR tube rotor, e.g., Mini centrifuge ROTILABO® Uni-fuge	Carl Roth	Cat#CX73.1
Chromium Next GEM Controller	10× Genomics	Cat#1000204
Chromium Next GEM Secondary Holder	10× Genomics	Cat#1000142
RT Reagent B (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	10× Genomics	Cat#2000165
RT Enzyme C (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	10× Genomics	Cat#2000102
Template Switch Oligo (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	10× Genomics	Cat#3000228
Reducing Agent B (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	10× Genomics	Cat#2000087
Chromium Partitioning Oil, (included in: Chromium Next GEM Chip G & Gaskets, 10× Genomics, Cat#1000127)	10× Genomics	Cat#2000190
Single Cell 3′ v3.1 Gel Beads, (included in: Chromium Next GEM Single Cell 3′ Gel Bead Kit v3.1, 4 rxns, Cat#1000129)	10× Genomics	Cat#2000164
Chromium Next GEM Chip G, (included in: Chromium Next GEM Chip G & Gaskets, 10× Genomics, Cat#1000127)	10× Genomics	Cat#2000177
Chromium Next GEM Chip G Gasket (included in: Chromium Next GEM Chip G & Gaskets, 10× Genomics, Cat#1000127)	10× Genomics	Cat#3000072

MATERIALS AND EQUIPMENT

Equipment and materials needed in safety level 1 or 3 laboratories		
Equipment and materials type	Safety level 1	Safety level 3
Thermal cycler, e.g., C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module, Bio-Rad, Cat#1851197	yes	yes
Centrifuge for 50 mL Falcon tubes, e.g., Multifuge X1 Pro, Thermo Fisher Scientific, Cat#75009710	no	yes
Mini centrifuge with PCR tube rotor, e.g., Mini centrifuge ROTILABO® Uni-fuge, Carl Roth, Cat#CX73.1	yes	yes
Neubauer counting chamber, Carl Roth, Cat#T728.1	no	yes
Hemocytometer coverslips 20 × 26 mm, Carl Roth, Cat#L189.1	no	yes
Inverted microscope, e.g., Primovert, Carl Zeiss, Cat#491206-0001-000	no	yes
40 µm cell strainers, Corning, Cat#CLS431750	no	yes
50 mL Falcon centrifuge tubes, Thermo Fisher Scientific, Cat#352070	no	yes
200 µL wide-bore pipette tips, Thermo Fisher Scientific, Cat#14-222-726	no	yes
PCR Tubes 0.2 mL 8-tube strips, Eppendorf, Cat#951010022	yes	yes
RNaseZAP, Sigma-Aldrich, Cat#R2020	yes	yes
Nuclease-free water, Thermo Fisher Scientific, Cat#AM9937	no	yes
6-well plate, tissue-culture-treated, Corning, Cat#3516	no	yes
Chromium Next GEM Controller, 10× Genomics, Cat#1000204	no	yes
Chromium Next GEM Secondary Holder, 10× Genomics, Cat#1000142	no	yes
RT Reagent B, 10× Genomics, Cat#2000165 (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	no	yes
RT Enzyme C, 10× Genomics, Cat#2000102 (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	no	yes
Template Switch Oligo, 10× Genomics, Cat#3000228 (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	no	yes
Reducing Agent B, 10× Genomics, Cat#2000087 (included in: Chromium Next GEM Chip G Single Cell 3′ Kit, 4 rxns, Cat#1000130)	no	yes
Chromium Partitioning Oil, 10× Genomics, Cat#2000190 (included in: Chromium Next GEM Chip G & Gaskets, 10× Genomics, Cat#1000127)	no	yes
Single Cell 3′ v3.1 Gel Beads, 10× Genomics, Cat#2000164 (included in: Chromium Next GEM Single Cell 3′ Gel Bead Kit v3.1, 4 rxns, Cat#1000129)	no	yes
Chromium Next GEM Chip G, 10× Genomics, Cat#2000177 (included in: Chromium Next GEM Chip G & Gaskets, 10× Genomics, Cat#1000127)	no	yes
Chromium Next GEM Chip G Gasket, 10× Genomics, Cat#3000072 (included in: Chromium Next GEM Chip G & Gaskets, 10× Genomics, Cat#1000127)	no	yes
BD Microlance™ 3 Needles, 27G	no	yes

Note: Depending on your local S3 laboratory guidelines, removable equipment that cannot be safely decontaminated, such as the chromium controller and the thermocycler, might need to be quarantined for a certain period of time inside the S3 until safe removal is possible.

Digestion solution		
Reagent	Final concentration	Amount
Accutase	1×, ready to use	650 µL
DNase I	100 µg/mL	13 µL
Total		**663 µL**
Prepare freshly on the day of the experiment and store at 4°C for a maximum of 48 h.		

Stopping solution		
Reagent	Final concentration	Amount
DMEM/F12 Medium	90%	18 mL
Fetal Bovine Serum (FBS)	10%	2 mL
Total		**20 mL**
Store at 4°C for up to a month under sterile conditions.		

10× buffer		
Reagent	Final concentration	Amount
Phosphate buffered saline (PBS)	99%	90 mL
Bovine Serum Albumin (10% stock v/v)	1%	10 mL
Total		**100 mL**
Store at 4°C for up to a month under sterile conditions.		

10× Master Mix (for 2 samples)		
Reagent	Identifier	Amount
RT Reagent B	2000165	37.6 µL
Template Switch Oligo	30000228	4.8 µL
Reducing Agent B	2000087	4.0 µL
RT Enzyme C	2000085 or 20000102	17.4 µL
Total		**63.8 µL**
Prepare directly before use and store at 4°C.		

STEP-BY-STEP METHOD DETAILS

Inoculating iPSC-derived kidney organoids with SARS-CoV-2

Timing: 24–120 h

This step describes how to inoculate iPSC-derived kidney organoids with SARS-CoV-2 and maintain them in culture for up to 120 h afterward. The work will be conducted in an S3 laboratory and requires a pre-made, titered stock of SARS-CoV-2 as well as mock control, as previously described (Hoffmann et al., 2020). To assess iPSC-derived kidney organoid quality at the beginning of any inoculation experiment, please refer to the troubleshooting section, problem 1.

1. Prepare medium for SARS-CoV-2 inoculation of iPSC-derived kidney organoids.
   a. Prewarm Essential 6 medium to room temperature (18°C–22°C).
   b. Clean a biohazard safety cabinet as well as any small equipment (pipettes, tweezers) with 80% ethanol according to the local standard.
   c. Mix 2 mL of Essential 6 medium with the SARS-CoV-2 stock (titer typically around 1.75×10^7 PFU/mL but varies from stock to stock) to reach an estimated multiplicity of infection (MOI) of 1.0 per transwell insert. Prepare the same amount of mock medium for the uninfected controls.

Note: To prepare the inoculum at the desired MOI, the number of cells per organoid needs to be established beforehand. We recommend running a pilot dissociation experiment to count the number of cells per organoid for each experimental condition and assume similar numbers in follow-up experiments. This could be carried out by following steps 5–8 of this protocol. The number of cells per organoid obtained in the end corresponds to the number of viral infectious units (PFU, plaque-forming units) that should be used for inoculation to reach an MOI of 1. Details of the virus stock cultivation and titration are described in detail in Varghese et al., 2021.

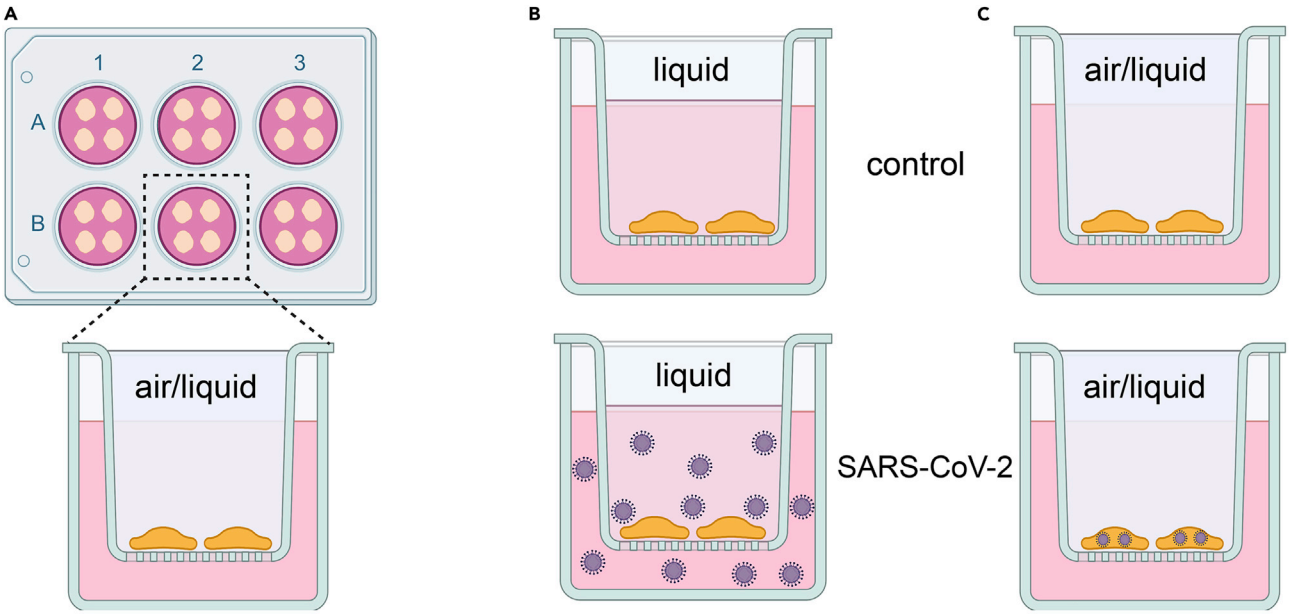

Figure 2. SARS-CoV-2 inoculation of iPSC-derived kidney organoids cultured on transwell filters

(A) iPSC-derived kidney organoids cultured in a 6-well transwell plate in top and side view (air/liquid interface culture).

(B) Control and SARS-CoV-2 conditions with organoids inoculated with mock medium (control) and with SARS-CoV-2 below and on top of the filter membrane (liquid culture).

(C) Continued incubation of kidney organoids after 24 h inoculation in an air/liquid culture.

2. Expose kidney organoids to the SARS-CoV-2 inoculum.
   a. Remove the old medium from below each transwell filter (6-well format) (Figure 2A).

   Optional: If you would like to perform a plaque assay to monitor virus replication in the organoids, store the initial organoid medium prior to SARS-CoV-2 inoculation for baseline control measurements at −80°C.

   b. Add new medium per condition: Essential 6 plus SARS-CoV-2 for experiment and Essential 6 containing mock medium for control organoids. Each transwell filter should contain 2 mL of fresh medium: 1.2 mL below the filter, 800 µL on top of the filter (Figure 2B).
   c. Incubate the organoids for 24 h at 37°C, 5% (v/v) CO_2.
3. Remove virus-containing medium from the organoids after 24 h of inoculation with SARS-CoV-2.
   a. Remove the old medium from below and on top of the transwell filter.
   b. Wash the organoids below and above the filter with 2 mL of PBS (18°C–22°C) both below and on top of the transwell filter. Wash the organoids three times in total.
   c. Remove the PBS and add 1.2 mL of pre-warmed Essential 6 medium to the bottom of the transwell filter. Do not add medium to the top of the filter.
4. Incubation of virus-infected kidney organoids.
   a. Incubate the organoids for 120 h.
   b. Refresh Essential 6 medium every 48 h while keeping the kidney organoids in culture: remove old medium and add 1.2 mL of fresh Essential 6 medium below the transwell filter, no medium on top. Remember to bring the medium to 18°C–22°C before adding it below the filter.

   Note: Adding medium on top and below the transwell filter during organoid inoculation with SARS-CoV-2 increases the organoid-virus interaction surface and might thereby enhance the infection efficiency.

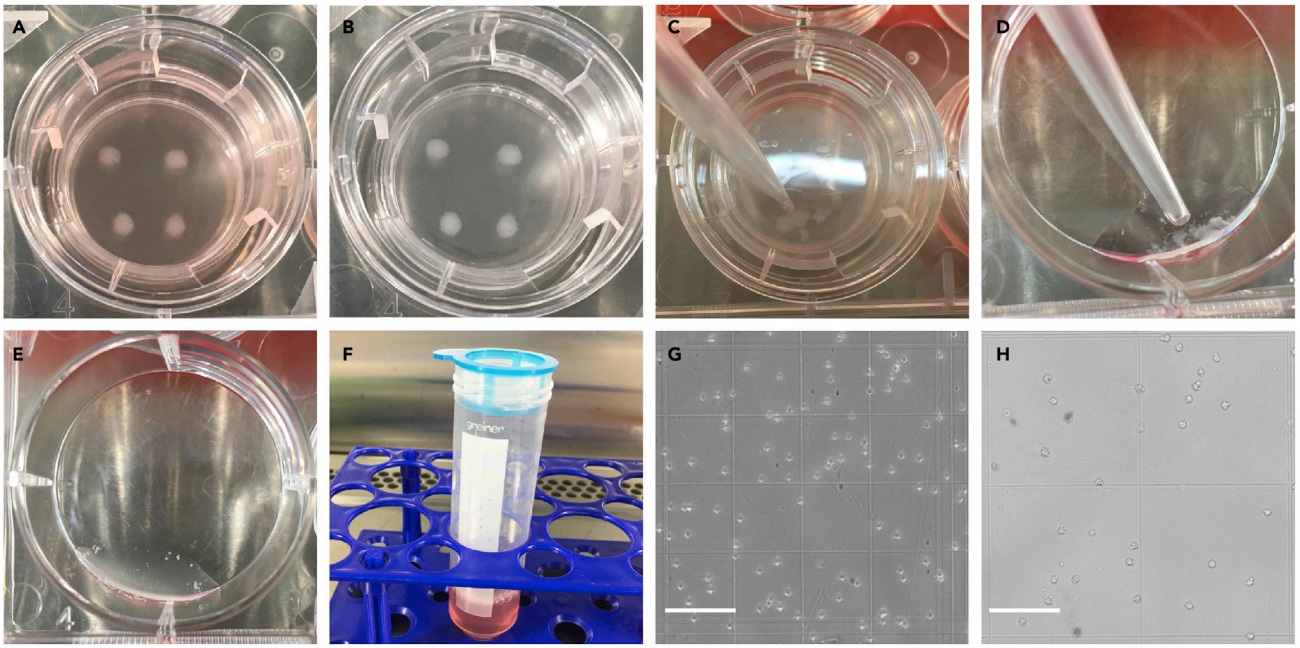

Figure 3. Sequential steps during organoid dissociation

(A) iPSC-derived kidney organoids cultured in E6 medium in a 6-well format transwell system prior to dissociation.

(B) iPSC-derived kidney organoids after three times washing with PBS.

(C) Adding Digestion Solution on top of the transwell filter and resuspending organoids.

(D) Mechanically dissociating organoids with a wide-bore tip.

(E) Single cell suspension after transfer into a new 6-well plate.

(F) Using a cell strainer to obtain a single cell suspension. Some non-digested extracellular matrix might be retained in the strainer.

(G and H) Trypan blue stained single cell suspension of iPSC-derived kidney organoids at (G) 4× magnification, scale bar 250 μm and (H) 10× magnification, scale bar 125 μm, respectively in a Neubauer counting chamber. Note the low number of trypan blue positive cells.

Digesting kidney organoid tissue into single cell suspensions

Timing: 1 h

This step yields a single cell suspension of organoid-derived cells as input for the single cell sequencing workflow (see Figure 3). We calculated timing and reagents for two transwell filters containing four organoids each: control and SARS-CoV-2-exposed kidney organoids. This step will be conducted in an S3 environment.

5. Prepare the kidney organoids for digestion.
   a. Cool a centrifuge for 50 mL Falcon tubes to 4°C.
   b. Remove organoids from the incubator and remove the medium (Figures 3A and 3B).
   c. Gently wash the organoids on top and below the transwell filter with 2 mL PBS (18°C–22°C) each. Repeat this step to a total of 3 washes.
6. Mechanic and enzymatic digestion of the organoids.
   a. Remove PBS and add 300 μL of the Digestion Solution (stored at 4°C) containing Accutase and DNase I (see materials and equipment section above) on top of each transwell filter (Figure 3C).
   b. Resuspend and mechanically disrupt the organoids in the Digestion Solution using a 200 μL wide-bore tip with an inner tip diameter of approx. 1 mm (Figure 3C).

Alternatives: Instead of fabric-made wide-bore tips you can also use custom-made ones. For this, prepare a box of 200 μL or 1 mL standard pipette tips and cut off the tips at the desired circumference (approximately 1 mm in inner opening diameter). Autoclave prior to use.

c. Transfer the organoid suspension to a new 6-well plate without transwell inserts (Figure 3D).
d. Incubate the cell suspension for 15–18 min at 37°C. During this period, resuspend the organoids by pipetting them up and down 10 times in the same Digestion Solution every 2 min until it becomes a homogeneous single cell suspension (Figure 3E).

7. Stop the enzymatic digestion (Figure 3F).
   a. Place 40 μm cell strainers on top of two 50 mL Falcon tubes and pre-wet the filters with PBS (18°C–22°C).
   b. Add 4 mL of the Stopping Solution (DMEM/F12 + 10% (v/v) FCS) onto each of the two single cell suspensions in the 6-well plate to inactivate accutase and DNase.
   c. Transfer each suspension through a cell strainer. Wash the filter using more Stopping Solution. The volume can go up to 20 mL.
   d. Remove the 40 μm cell strainers and centrifuge the single cell suspensions at 300 × *g* for 5 min at 4°C.
   e. In the meantime, prepare two new Falcon tubes with 40 μm cell strainers and pre-wet the filters with PBS (18°C–22°C).
   f. Discard the supernatants, resuspend the cell pellets in 1 mL 10× buffer (see materials and equipment section) and filter each through the 40 μm cell strainers. Wash the filters using 20 mL 10× buffer each for maximum cell retrieval.
   g. Remove the 40 μm cell strainers and centrifuge the single cell suspensions at 300 × *g* for 5 min at 4°C.
   h. Discard the supernatants and resuspend each cell pellet in 500 μL 10× buffer (PBS + 1% BSA).
8. Count the cells (Figures 3G and 3H).
   a. Prepare a Neubauer counting chamber.
   b. Dilute 10 μL of each single cell suspension with 10 μL of trypan blue (1:1).
   c. Place the Neubauer counting chamber under a microscope and count 4 times 16 squares.
   d. Calculate the total cell number according to this equation: total number of cells = [(number of cells per 16 squares)/4] * 2 * 10,000.
   e. Ideally, the cell concentration should be around 1,000 cells/μL for 10× genomics single cell workflow. One expects ca. 4 million cells from four organoids with around 5%–10% of trypan blue positive cells.

   Note: If you experience a high number of trypan blue positive/dead cells, please refer to the troubleshooting section, problem 3, for potential solutions.

9. Keep the single cell suspensions at 4°C and continue directly with step 3: Processing of cells for single cell RNA sequencing.

   Note: Although described in the context of single cell RNA sequencing workup at an S3 laboratory, the organoid dissociation step of the protocol can be utilized for a manifold of applications including fluorescence-activated cell scanning (FACS) and sorting.

   Note: Should your single cell suspension show aggregates and clumps (visible cell aggregates in the Neubauer counting chamber), please refer to the troubleshooting section, problem 2, for potential solutions.

Processing cells for single-cell RNA sequencing

Timing: 2 h

This step uses the single cell suspensions retrieved in the previous step of this protocol as input for the first step of the Next GEM v3.1 single cell sequencing workflow (10× genomics). The steps follow the original manufacturer's protocol Chromium Next GEM Single Cell 3' v3.1 (Dual Index) User Guide Rev C) and end with an incubation step in a thermal cycler that also serves to inactivate the

virus, allowing transfer of the samples from the S3 laboratory for further processing. It is strongly recommended to familiarize oneself with the Next GEM v3.1 protocol before continuing. Here, we provide a brief recapitulation of step 1 of the Next GEM v3.1 workflow for two samples: control and SARS-CoV-2-infected.

10. Clean a laboratory bench RNA or biochemistry working area with 80% ethanol and an RNase-eliminating agent, e.g., RNase-Zap.
11. Prepare reagents and the 10× Master Mix solution.
    a. Bring the Next GEM beads to 18°C–22°C. Vortex for 30 s and spin down briefly directly before use.
    b. Keep RT Reagent B, Template Switch Oligo, and Reducing Agent B to 18°C–22°C.
    c. Keep RT Enzyme C at 4°C until use.
    d. Prepare 10× Master Mix for two samples (see materials and equipment section) at 4°C in a PCR tube.
12. Prepare Chromium Chip G.
    a. Place Chip G into its secondary holder.
    b. Fill unused wells on a Chip G with 50% glycerol according to the manufacturer's protocol (Chromium Next GEM Single Cell 3' v3.1 (Dual Index) User Guide Rev C, page 28, step 1.2a): 70 μL into each unused well in row 1, 50 μL into each unused well in row 2, and 45 μL in each unused well in row 3.
    c. This protocol will load two samples onto the chip, thus 6 reactions remain unused.
13. Load samples onto Chromium Chip G.
    a. Mix cells, nuclease-free water, and 10× Master Mix according to the manufacturer's table. For a concentration of 1000 cells/μL and a targeted cell number of 10,000, this means mixing the following components per sample: 31.9 μL 10× Master Mix, 16.5 μL cell suspension, and 26.7 μL nuclease-free water. Mix well by pipetting 15 times without introducing air bubbles.
    b. Load 70 μL of Cell-Master-Mix into each sample row 1 on the chip.
    c. Add 50 μL of 10× beads into each sample row 2 on the chip.
    d. Add 40 μL of partitioning oil into each sample row 3 on the chip.

Note: Carefully check for air bubbles in any of the wells before applying the gasket and placing the chip into the chromium controller. If you spot air bubbles, remove them with the help of a needle. Remaining air bubbles in any of the wells may cause wetting failure and loss of sample.

14. Attach the 10× gasket and place the chip and secondary holder into the chromium controller.
    a. Run the chromium controller Chip G programme. This will take 17 min 42 s.
    b. After completion of the chromium controller run, slowly and carefully transfer the products into new PCR tubes.
    c. Put the two PCR tubes containing the control and the SARS-CoV-2-exposed sample into a thermocycler and run the following programme:

Thermocycler conditions (total run time: ~55 min)

Steps	Temperature	Time	Cycles
1	53°C	45 min	1
2	85°C	5 min	1
Hold	4°C	Forever	
Lid temperature	53°C	Volume: 125 μL	

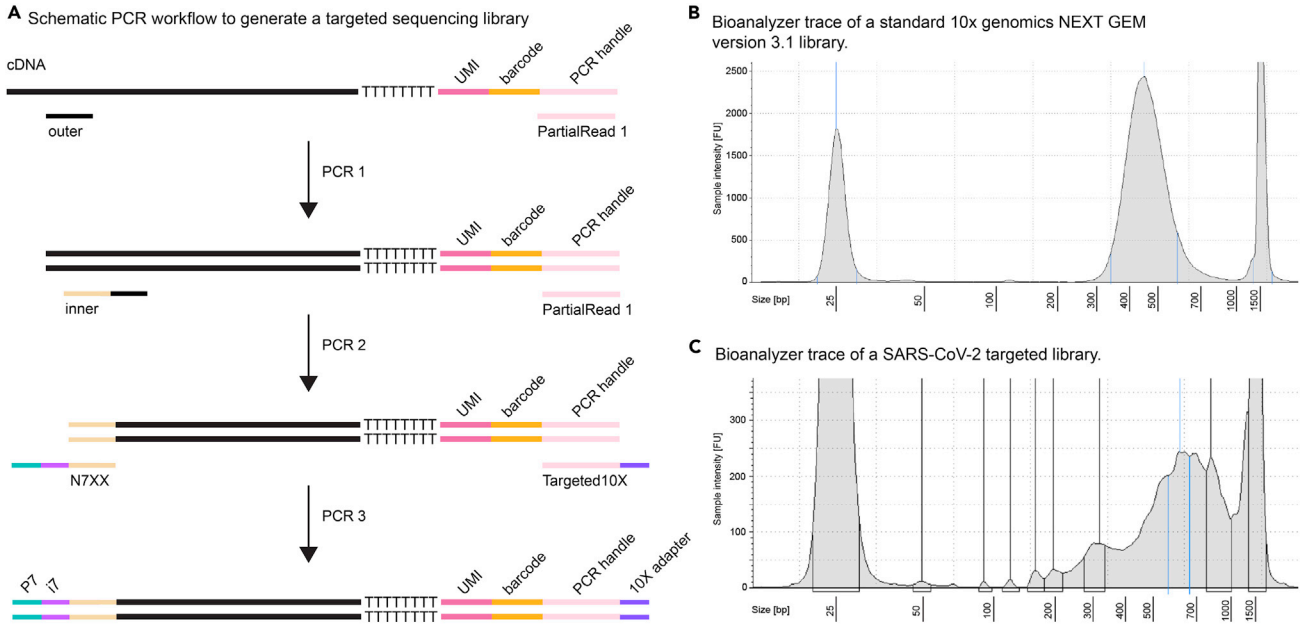

Figure 4. PCR workflow to generate a SARS-CoV-2-targeted library and expected bioanalyzer traces of the constructed sequencing-ready libraries

(A) PCR workflow to illustrate added adapters and target sequences during PCR 1–3. Inner: SARS-CoV-2 inner primer, outer: SARS-CoV-2 outer primer, N7XX: Illumina sequencing adapter (including P7 and i7).

(B) Exemplary bioanalyzer trace of a standard 10× genomics Next GEM version 3.1 library.

(C) Exemplary bioanalyzer trace of a SARS-CoV-2-targeted library after finishing PCR 3.

Pause point: The product of this step can be stored at 4°C for up to 72 h or at −20°C for up to 7 days until continuation with the next step.

Preparation of targeted libraries to computationally retrieve SARS-CoV-2 content in single cells

Timing: 6 h

This step guides you through the generation of a SARS-CoV-2-targeted library in parallel to the usual library construction process within the 10× genomics Next GEM v3.1 single cell RNA sequencing workflow. The starting point of this step is single-cell barcoded cDNA (step 2 of the Chromium Next GEM Single Cell 3' v3.1 (Dual Index) User Guide Rev C). Thus, you will first have to generate cDNA from the samples of the previous step according to the manufacturer's protocol (= step 2) before continuing with the library. The aim is to generate a SARS-CoV-2-targeted library, in addition to the standard 10× V3.1 library construction workflow (Schraivogel et al., 2020a, 2020b; Triana et al., 2021). This targeted library is generated by two PCS amplifications of SARS-CoV2 specific primers and a final indexing PCR (PCR 1–3) (Figure 4A). Both libraries are finally sequenced together, to later on computationally map the targeted and amplified SARS-CoV-2 reads to the individual cells of the single cell RNA sequencing library. Further detailed information on the steps of this part of the protocol is described in the original protocol (Schraivogel et al., 2020b).

15. Carry out step 2 of the Chromium Next GEM Single Cell 3' v3.1 (Dual Index) User Guide Rev C protocol provided by 10× Genomics (https://support.10xgenomics.com/permalink/3qXBxYZMpoCLCpum4Whp49) to generate cDNA from the samples of the previous step before continuing the preparation of the SARS-CoV-2-targeted library (Timing: ~ 2 h plus quality control of the cDNA, e.g., by Agilent Bioanalyzer).

16. Use the resulting cDNA from step 18 as input for PCR1 and pipette PCR reaction master mix 1 on ice. Pipet mix or vortex well.

PCR reaction master mix 1	
Reagent	Amount for 1 sample
amplified cDNA	10 ng
Kapa Hifi	50 μL
10 μM Partial Read 1 Primer	4 μL
100 μM SARS-CoV-2 Outer Primer	2.5 μL
ddH_2O	add ddH_2O up to a total assay volume of 100 μL

17. Run PCR 1 with the following PCR 1 cycling conditions:

PCR 1 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	20 s	11 cycles
Annealing	67°C	60 s	
Extension	72°C	60 s	
Final extension	72°C	5 min	1
Hold	4°C	Hold	

18. Once the run is finished, collect the tube and spin down briefly.
19. Post PCR cleanup with AMPure size selection beads, selecting for DNA fragments greater than 150 base pairs.
    a. Add 150 μL of AMPure beads to your PCR 1 product and pipette mix 15 times. Incubate at 18°C–22°C for 5 min.
    b. Place the tube into a 10× magnetic separator at the High position and wait until the solution clears. This usually takes 1–2 min.
    c. Remove the supernatant and add 300 μL of 80%. Wait 30 s.
    d. Remove the ethanol and wash the sample again two times with 200 μL 80% ethanol for 30 s each.
    e. Remove the ethanol, centrifuge the tube briefly, place it back in the 10× magnetic separator at the Low position, remove the remaining ethanol and air-dry the beads for max. 2 min.
    f. Elute the product by adding 30.5 μL of EB buffer. Pipet mix 15 times and incubate the solution for 2 min at 18°C–22°C.
    g. Place the tube in the 10× magnetic separator at the Low position and wait for the solution to clear.
    h. Transfer 30 μL of the cleared solution into a new PCR tube strip. Use 1 μL thereof for concentration measurement with the Qubit dsDNA HS Kit. The expected amount of PCR product produced with PCR 1 lies between 10 to 250 ng.
20. Prepare PCR 2 by using 10 ng of the PCR 1 product as input for PCR 2 and pipette PCR reaction master mix 2 on ice. Pipet mix or vortex well.

PCR reaction master mix 2	
Reagent	Amount for 1 sample
PCR1 product	10 ng
Kapa Hifi	50 μL
10 μM Partial Read 1 Primer	4 μL
100 μM SARS-CoV-2 Inner Primer	2.5 μL
ddH_2O	add ddH_2O up to a total assay volume of 100 μL

21. Run PCR 2 with the following PCR 2 cycling conditions:

PCR 2 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	20 s	7 cycles
Annealing	67°C	60 s	
Extension	72°C	60 s	
Final extension	72°C	5 min	1
Hold	4°C	Hold	

22. Once the run is finished, collect the tube and spin down briefly.
23. Post PCR cleanup with AMPure size selection beads, selecting for DNA fragments greater than 150 base pairs.
    a. Add 150 µL of AMPure beads to your PCR 2 product and pipette mix 15 times. Incubate at 18°C–22°C for 5 min.
    b. Place the tube into a 10× magnetic separator at the "high position" and wait until the solution clears. This usually takes 1–2 min.
    c. Remove the supernatant and add 300 µL of 80% ethanol. Wait 30 s.
    d. Remove the ethanol and wash the sample again two times with 200 µL 80% ethanol for 30 s each.
    e. Remove the ethanol, centrifuge the tube briefly, place it back in the 10× magnetic separator at the Low position, remove remaining ethanol and air-dry the beads for max. 2 min.
    f. Elute the product by adding 30.5 µL of EB buffer. Pipet mix 15 times and incubate the solution for 2 min at 18°C–22°C.
    g. Place the tube in the 10× magnetic separator at the "low position" and wait for the solution to clear.
    h. Transfer 30 µL of the cleared solution into a new tube strip. Use 1 µL thereof for concentration measurement with the Qubit dsDNA HS Kit. The expected amount of PCR product lies within 10–100 ng.
24. Prepare PCR 3 by using 10 ng of the PCR 2 product as input for PCR 3 and pipette PCR reaction master mix 3 on ice. Pipet mix or vortex well.

PCR reaction master mix 3	
Reagent	Amount for 1 sample
PCR2 product	10 ng
Kapa Hifi	50 µL
10 µM Targeted 10× Primer	4 µL
10 µM N70X Primer	2.5 µL
ddH_2O	add ddH_2O up to a total assay volume of 100 µL

25. Run PCR 3 with the following PCR 3 cycling conditions:

PCR 3 cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	20 s	7 cycles
Annealing	67°C	60 s	
Extension	72°C	60 s	
Final extension	72°C	5 min	1
Hold	4°C	Hold	

26. Once the run is finished, collect the tube and spin down briefly.
27. Post PCR cleanup with AMPure size selection beads, selecting for DNA fragments greater than 150 base pairs.
    a. Add 150 μL of AMPure beads to your PCR 3 product and pipette mix 15 times. Incubate at 18°C–22°C for 5 min.
    b. Place the tube into a 10× magnetic separator at the "high position" and wait until the solution clears. This usually takes 1–2 min.
    c. Remove the supernatant and add 300 μL of 80% ethanol. Wait 30 s.
    d. Remove the ethanol and wash the sample again two times with 200 μL 80% ethanol for 30 s each.
    e. Remove the ethanol, centrifuge the tube briefly, place it back in the 10× magnetic separator at the "Low position", remove remaining ethanol and air-dry the beads for max. 2 min.
    f. Elute the product by adding 30.5 μL of EB buffer. Pipet mix 15 times and incubate the solution for 2 min at 18°C–22°C.
    g. Place the tube in the 10× magnetic separator at the "low position" and wait for the solution to clear.
    h. Transfer 30 μL of the cleared solution into a new tube strip. Use 1 μL thereof for concentration measurement with the Qubit dsDNA HS Kit. Before sequencing, the PCR 3 product should be quality controlled, e.g., with an Agilent Bioanalyzer.
28. The product of PCR 3 is an Illumina sequencing-ready library that should be sequenced in one run together with a standard 10× Genomics Next GEM v3.1 library (step 3 - library construction - of the manufacturer's protocol) generated of the same cDNA as the SARS-CoV-2-targeted library was generated from. Alternatively, it can be run on its own but PhiX spike-in should be used to increase sequence diversity. The expected outcome lies within the 100 ng range.

EXPECTED OUTCOMES

This protocol aims to provide a comprehensive workflow from an iPSC-derived kidney organoid SARS-CoV-2 infection model to a sequencing-ready Illumina library within the 10× genomics pipeline. By implementing the generation of a SARS-CoV-2-targeted library, single cell identity and infection status can be matched computationally.

In the end, a standard 10× v 3.1 single cell library will be sequenced together with the targeted library. Both libraries were generated from the same cDNA. Before sequencing, both libraries will undergo quality control, e.g., with an Agilent Bioanalyzer. Example bioanalyzer traces for both the standard 10× genomics Next GEM version 3.1 library (Figure 4B) and the SARS-CoV-2 targeted library (Figure 4C) are shown in Figure 4.

LIMITATIONS

Dissociating iPSC-derived kidney organoids into single cells is critical for various workflows, including single cell RNA sequencing. For mechanical and enzymatic disruption of the organoid tissue, timing is critical. Overexposing the delicate organoid tissue to the enzymes, i.e., if handling takes longer than expected, cell viability is hampered. In the context of infection models of SARS-CoV-2, the workflows at an S3 facility add several layers of complexity to this process, as S3 lab specific safety measures increase time needed per step which should be considered when planning the experiments.

iPSC-derived kidney organoids harbor many cell types present in the adult kidney, including proximal tubular cells and podocytes. However, the maturity of the organoids is closer to a second trimester fetal kidney than the adult organ. Organoids generally contain a certain number of off-target cells that naturally are not present at the same percentage in the adult organ. These might influence infection rates and experimental outcome, e.g., when off-target cells are more susceptible to inoculation with a certain pathogen than the focused cell types. This should be considered when planning infection experiments as it might be limiting the explanatory power in an adult disease.

Single cell RNA sequencing data is sparse so absence of a gene from the sequence data does not mean that the gene is not expressed. We recommend using an orthogonal method to check for expression of the mRNA such as single molecule fluorescence *in situ* hybridization, in addition.

TROUBLESHOOTING

Problem 1

There are few to no structures present in my kidney organoids. Is it worth to proceed? This problem is related to the section before you begin and to step 1 of this protocol.

Potential solution

Input organoid quality is essential for a reliable outcome of the inoculation experiments. It is therefore essential to subject iPSC-derived kidney organoids to a thorough quality assessment before beginning the actual experiment. We recommend to at least check for maintenance of steady structure presence three consecutive days before starting the inoculation with SARS-CoV-2. This can easily be done by eye, using an optical microscope. Alternatively, especially if the desired readout relies on the expression of certain genes, transporters or similar, it is recommended to check for expression of respective genes by PCR. In the case of SARS-CoV-2 inoculation, we e.g., checked for ACE2 expression in the iPSC-derived kidney organoids prior to the start of the experiments, as ACE2 is one of the main SARS-CoV-2 cell entry factors.

Problem 2

The organoids do not dissociate well and/or cell aggregates show in the single cell suspension. This problem may occur during or after digestion of the organoids (steps 6 and 7).

Potential solution

For the downstream single cell RNA sequencing workflow, it is crucial to obtain clean single cell suspensions without cell aggregations, as these may cause clogs with subsequent wetting failure in the delicate microfluidic system of the Chromium Next GEM chip and controller. Suboptimal dissociation of organoids during the digestion procedure can be resolved by more careful resuspension. One could also try a normal 1 mL pipette tip (smaller diameter than the wide-bore tip) for resuspension a few minutes into the process to aid taking organoid pieces apart. However, do not exceed the overall digestion time to longer than 18 min as this increases cell death rates.

Cell aggregates within the single cell suspension are commonly caused by surrounding debris and free DNA originating from dead or dying cells. The problem can be resolved by carefully resuspending the cell suspension, thereby disruption the aggregates. Alternatively, passing the cell solution through another 40 or 30 μm cell strainer might help, but will also decrease cell yield. DNase I, which is part of the Digestion Solution, also helps with degrading extracellular DNA, minimizing the risk of cell aggregate formation after digestion. If DNase I was added already and you experience cell aggregates nonetheless, the concentration of the enzyme can be doubled.

Problem 3

The amount of trypan blue positive dead or dying cells is very high (>20%). This problem may occur after counting the cells post digestion (step 8 of this protocol).

Potential solution

A high amount of dead and/or dying cells is impeding a favorable outcome after single cell RNA sequencing. Unfortunately, the options for dead cell removal might be very limited in an S3 laboratory, e.g., sorting to remove the dead cells is likely not available. Our protocol is designed to result in as little as 5%–10% dead or dying cells after accutase digestion. Make sure that all cell solutions are always kept at 4°C, except during accutase digestion, and process all samples as quickly as possible. Handling and processing quickly are key. Be sure you are well trained on the protocol before going to the S3 laboratory. Depending on the kind of organoids you use, other digestion enzymes, such as

collagenase or liberase, might give better results than accutase. It might help to have a backup set of organoids ready for another try if the digestion time was exceeded. Another option would be the use of a dead cell removal kit (e.g., Miltenyi Biotec, catalog number 130-090-101). However, the latter procedure will add approximately 30 min to the total processing time, potentially decreasing live cell number even further.

Problem 4

The amount of PCR product after PCR 1, 2 or 3 is very low. This problem refers to steps 19h, 23h and/or 27h.

Potential solution

The expected yield of PCR product lies between 10 and 250 ng for PCR 1, between 10 and 100 ng for PCR 2, and around 100 ng for PCR 3. If the amount is lower than this, it is recommended to increase the number of cycles respectively for PCR 1, 2 or 3. Overall, the amount of PCR product is dependent on the sample, the cell number, and the number of PCR cycles. If increasing cycle number >16 does not lead to significant increase of PCR product, the problem is likely rooted in the sample input at the beginning of the TAPseq protocol. A potential solution might be to increase the sample input.

Problem 5

The amount of PCR product after PCR 1, 2 or 3 is very high. This problem refers to steps 19 h, 23 h and/or 27 h.

Potential solution

The expected yield of PCR product lies between 10 and 250 ng for PCR 1, between 10 and 100 ng for PCR 2, and around 100 ng for PCR 3. If the amount is, however, higher than the expected range, this could lead to higher molecular weight products (around 1.000–1.5000 base pairs). We have not experienced this in our hand, but it was described previously that this might lead to inefficient clustering during Illumina run preparation (Schraivogel et al., 2020b). The suggested solution is to decrease cycle numbers by 1–2.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rafael Kramann, MD, PhD (rkramann@gmx.net).

Materials availability

This study did not generate new unique reagents. For specific details on availability please refer to the key resources table.

Data and code availability

This protocol did not generate/analyze datasets or code.

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AUTHOR CONTRIBUTIONS

K.C.R. and J.J. conceptualized the study and conducted the experiments. K.C.R. designed the figures and wrote the manuscript. J.J., G.O., P.M., R.P.v.R., S.H.T., B.S., R.K.S., and R.K. edited the manuscript. J.J., R.P.v.R., B.S., R.K.S., and R.K. acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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