SUPPLEMENT

MATERIALS

REAGENTS

Organoids

Box 1 and Box 2 (Production and testing of Wnt) are provided as a separate PDF

- Advanced Dulbecco`s Modified Eagle Medium (DMEM)/F12 medium (Thermo Fisher Scientific, cat. no. 12634028)
- GlutaMAX™ (Thermo Fisher Scientific, cat. no. 35050-038)
- HEPES (Thermo Fisher Scientific, cat. no. 15630-056)
- Matrigel® (Corning, cat. no. 356231)
- Recovery™ cell culture freezing medium (Thermo Fisher Scientific, cat. no. 12648-010)
- B-27™ supplement (Thermo Fisher Scientific, cat. no. 12587-010)
- Primocin™ (InvivoGen, cat. no. ant-pm-1)
- N-acetyl-L-cysteine (Sigma-Aldrich, cat. no. A9165)
- TGF-ß inhibitor (TGF-ßi) A83-01 (Tocris Bioscience, cat. no. 2939)
- Rho kinase inhibitor (ROCKi) Y-27632 (Abmole, cat. no. M1817)
- Human fibroblast growth factor-10 (FGF10; PeproTech, cat. no. 100-26)
- Animal-free recombinant human epidermal growth factor (EGF; PeproTech, cat. no. AF-100-15)
- Gastrin I (Tocris Bioscience, cat. no. 3006/1)
- Noggin-conditioned medium (NOG-CM; instructions for preparation are in **Box 1**)
- R-spondin 1-conditioned medium (RSPO1-CM; instructions for preparation are in **Box 1**)
- WNT3A-conditioned medium (WNT3A-CM; instructions for preparation are in **Box 1**)
- Human material (see reagent setup)
- Nutlin-3 (Biomol, cat. no. Cay10004372-1)

- DMSO (Sigma-Aldrich, cat. no. D2650)
- BSA (Sigma-Aldrich, cat. no. A-7906)
- TrypLE™ Express (Thermo Fisher Scientific, cat. no. 12605028)

FACS

- Propidium iodide (PI; BD Pharmingen, cat. no. 51-66211E)

  Caution Hazardous substance, handle with care.

Sequencing

- GeneRead DNA FFPE Kit (Qiagen, cat. no. 180134)
- TaqMan™ RNase P Detection Reagents Kit (Thermo Fisher Scientific, cat. no. 4316831)
- TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Thermo Fisher Scientific, cat. no. 4352042)
- Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher Scientific, cat. no. 4480441)
- Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific, cat. no. 4475346)
- Ion Xpress™ Barcode Adapters 1-16 Kit (Thermo Fisher Scientific, cat. no. 4471250)
- Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific, cat. no. 4468802)
- Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific, cat. no. A29900)
- Ion Sphere™ Quality Control Kit (Thermo Fisher Scientific, cat. no. 4468656)
- Ion PGM™ Enrichment Beads (Thermo Fisher Scientific, cat. no. 4478525)
- Ion PGM™ Hi-Q™ View Sequencing Kit (Thermo Fisher Scientific, cat. no. A30044)
- Ion 318™ Chip Kit v2 BC (Thermo Fisher Scientific, cat. no. 4488146)
- QIAamp DNA Mini Kit (Qiagen, cat. no. 51304)
- Proteinase K (Merck, cat. no. 1.24568.0500)
- NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel, cat. no. 740609)
- Primer (Sigma-Aldrich)
- dNTPs (NEB, cat. no. N0447L)

- Phusion High-Fidelity DNA Polymerase (NEB, cat. no. M0530S)
- Gel loading dye (NEB, cat. no. B7024S)
- Quick-Load® 2-log DNA ladder (NEB, cat. no. N0469S)
- Agarose (Sigma-Aldrich, cat. no. A9539-500G)
- Ethidium Bromide (EtBr; Roth, cat. no. 2218.3)

  Caution EtBr is toxic, handle with care.

Metaphase spread

- KaryoMAX™ Colcemid™ (Thermo Fisher Scientific, cat. no. 15212-012)

  Caution Colcemid is very toxic, handle with care.
- PBS (Thermo Fisher Scientific, cat. no. 14190169)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P9541)
- Methanol (MeOH; VWR, cat. no. 20847.320)

  Caution Hazardous substance, handle with care.
- Acetic acid (VWR, cat. no. 20103.295)
- DAPI vectashield (Vector Laboratories, cat. no. H-1200)

Histology

- Cell recovery solution (Corning, cat. no. CB-40253)
- PFA (Sigma-Aldrich, cat. no. 158127)

  Caution PFA contains formaldehyde, handle with care.
- Hematoxylin (Sigma-Aldrich, cat. no. H3136)
- Xylol (Roth, cat. no. CN80.2)

  Caution Xylol is flammable, handle with care.
- Eosin G (Roth, cat. no. 7089.1)
- 1-Butanol (Roth, cat. no. 7724.1)

  Caution 1-Butanol is flammable, handle with care.
- Paraffin Histowax (Histolab, cat. no. 00403)
- Ethanol (EtOH; VWR, cat. no. 20821.330)

- HCl 1 M ROTIPURAN® 37 % (Roth, cat. no. 4625.2)
- Isopropanol (AppliChem, cat. no. A3465)
- Formalin 10 % (Neogen, cat. no. 09122)

  Caution Formalin contains formaldehyde, handle with care.
- Entellan® (Merck, cat. no. 107961)

  Caution Entellan is flammable, handle with care.
- Citrate buffer pH 6.0, 10 x Antigen Retriever (Sigma-Aldrich, cat. no. C9999)
- TP53 (Clone DO-7) primary antibody concentrate (Dako Agilent, cat. no. M700101-2)
- Antibody diluent Dako REAL (Dako Agilent, cat. no. S202230-2)
- HiDef Detection™ HRP Polymer System (Medac Diagnostika, cat. no. 954D-10)

EQUIPMENT

Organoids

- Falcon tubes, 15 mL (Hartenstein, cat. no. ZR97)
- Plates, 24-well (Sarstedt, cat. no. 83.3922)
- Plates, 48-well (Sarstedt, cat. no. 83.3923)
- Glass Pasteur pipettes (Roth, cat. no. 4518.1)
- Light microscope (e.g. Leica, DMi1)
- Microscope (e.g. Thermo Fisher Scientific, EVOS FL, fluorescence optional for this protocol)
- Centrifuge for 15 mL Falcon tubes (e.g. Thermo Fisher Scientific, Heraeus Multifuge X1R)
- Microcentrifuge (e.g. Eppendorf, Centrifuge 5418 R)
- CO_2 incubator (5 % CO_2, 37 °C)
- Cool Cell freezing container (Biocision, cat. no. BCS-405)
- Cryo tubes (Sarstedt, cat. no. 72.380.002)

- Needles (Braun, cat. no. 4657640)
- Petri dish (Greiner Bio-one, cat. no. 633180)

FACS

- Waterbath 37 °C
- FACS sorter (e.g. BD, FACS Aria™ III)
- Filter 40 µm (Greiner Bio-one, cat. no. 542040)

Sequencing

- StepOne™ Real-Time PCR System (Thermo Fisher Scientific, cat. no. 4376357)
- Thermocycler peqSTAR (VWR, cat. no. 732-3242)
- Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, cat. no. Q32866)
- Ion Personal Genome Machine™ (PGM™) System (Thermo Fisher Scientific, cat. no. 4462921)
- Ion OneTouch™ 2 System (Thermo Fisher Scientific, cat. no. 4474779)
- Ion Reporter™ Software v5.6 (Thermo Fisher Scientific)
- Torrent Suite Software v5.6 (Thermo Fisher Scientific)
- PCR tubes (Sarstedt, cat. no. 72.735.002)
- Spectrophotometer (e.g. Peqlab, ND-1000)
- PCR machine (e.g. Biorad, C1000 Touch™ Thermal Cycler)
- Gel chamber (e.g. Peqlab, PerfectBlue™)
- UV transilluminator (e.g. Intas UV-Systeme)
- Scalpel (Braun, cat. no. 5518016)
- Sequencing labels (e.g. Macrogen)

Metaphase spread

- Microscope slides (Hartenstein, cat. no. OTMM)

Histology

- Bright field microscope (e.g. Nikon, Eclipse 50i)

- Microtome (e.g. Leica, RM2255)
- Paraffin embedding station (e.g. Leica, EG1160)
- Plastic transfer pipettes (Sarstedt, cat. no. 86.1172.001)
- Metal base mold (Leica, cat. no. 3803081)
- Embedding cassettes (Hartenstein, cat. no. EBK)
- Microtome Blade S Feather (Hartenstein, cat. no. R35)
- Glass test tubes (Hartenstein, cat. no. RG12)
- Stainless steel staining tray (Hartenstein, cat. no. FR2)
- Staining container glass, with lid (Hartenstein, cat. no. FK01)
- Fine brush
- Forceps
- Polysine™ glass slides (Hartenstein, cat. no. POLY)
- Tissue processor (e.g. Leica, ASP200S)
- Humidity chambers for slides (Hartenstein, cat. no. IK10)
- Cover glass 25 x 60 mm (VWR, cat. no. VWRU16004-096)
- Oven
- Microwave
- PAP pen for immunostaining (Sigma-Aldrich, cat. no. Z377821-1EA)

REAGENT SETUP

Human material We use 1 cm^2 of resected material from human gastric adenocarcinoma patients as tumor pieces. However, patients often receive treatment for the cancer before the resection which may influence the subsequent outgrowth of organoids. It is also possible to grow organoids from biopsies. After excision, tissue should be kept in Ad++ with Primocin (see below) on ice. We have been able to generate organoids from tissue stored overnight (o/n) at 4 °C, but it is preferable to process the tissue as soon as possible.

Caution Informed consent must be obtained from all subjects. Studies must conform to all relevant institutional and governmental regulations. The ethics review board of the clinic from which the sample will be obtained needs to be consulted. Our study was reviewed by the ethical committee of the University Clinic, Wuerzburg, approval # 16/36, and the Institutional Review Board of the University of Hong Kong and the Hospital Authority, Hong Kong, West Cluster.

Caution Human primary material may contain infectious agents and should therefore be handled under appropriate biosafety conditions.

Ad++ Add 5 mL HEPES (1 M) and 5 mL GlutaMAX (100 x) to 500 mL Advanced DMEM/F12 medium.

Human gastric organoid (hgo) medium The final medium composition is summarized in **Table 1.**

Critical As detailed in the Procedure, ROCKi should be added to a final concentration of 10 µM; needed after cell passaging to prevent anoikis.

Critical The culture medium should not be stored for more than 3 d at 4 °C.

Matrigel Thaw the original bottle o/n at 4 °C on ice according to manufacturer's instructions.

Critical Matrigel is liquid at 4 °C but will solidify when warmed. Therefore, perform all steps on ice or under ice-cold conditions. It may be helpful to put a tray of ice under the clean bench

when you work with Matrigel. It is often helpful to divide the Matrigel into smaller aliquots. For aliquoting, mix well by pipetting with a P1,000 on ice (using pipette tips with a wider opening is best) and divide the Matrigel into 1 mL aliquots in pre-cooled 1 mL cryovials on ice. Aliquots can be stored at –20 °C. The protein concentration of Matrigel can affect the stiffness and scaffold integrity. If there are problems, we recommend checking the protein concentration on the product information sheet; in our experience using Matrigel with a protein concentration of at least 8.5 mg/mL gives the best results.

WNT3A-CM Prepare the medium as described in **Box 1.**

RSPO1-CM Prepare the medium as described in **Box 1**.

NOG-CM Prepare the medium as described in **Box 1**.

N-acetyl-L-cysteine Dissolve 81.5 mg in 1 mL sterile H_2O to obtain a 500 mM stock. Use at a final concentration of 1:400.

FGF10 Dissolve 500 µg in 5 mL of sterile 0.1 % BSA/PBS to obtain a 100 µg/mL stock. Use at a final concentration of 1:1,000. Note: The concentration initially reported for maintenance of human gastric organoids was 200 ng/mL [1]. We have tested cultures with 100 ng/mL and have not observed any changes in 14 months of continuous culture.

EGF Dissolve 1 mg in 2 mL of sterile 0.1 % BSA/PBS to obtain a 500 µg/mL stock. Use at a final concentration of 1:10,000.

Gastrin I Dissolve 1 mg in 4.8 mL of PBS to obtain a 100 µM stock. Use at a final concentration of 1:100,000.

TGF-ßi Dissolve 10 mg in 950 µL DMSO to obtain a 25 mM stock. Use at a final concentration of 1:12,500.

ROCKi Dissolve 10 mg in 3.112 mL of sterile H_2O to obtain a 10 mM stock. Use at a final concentration of 1:1,000.

KCl Prepare a 0.75 M stock and use at a final concentration of 1:10.

MeOH:Acetic acid Prepare fresh at a ratio of 3:1.

HCl:EtOH Mix 200 mL of 50 % EtOH with 27.4 mL of 1 M HCl and add to 400 mL with 50 % EtOH.

PROCEDURE

Establishment of tumor organoids Timing variable, usually 2-4 weeks

1. Generate tumor organoids from cancerous tissue in regular human gastric organoid medium as described in **Box 3** (**Table 1** and **Fig. 1**).
2. Monitor the cultures every day under the microscope.

   Critical It is very important to check the cultures every day in order to act appropriately. Often, wild-type (wt) organoids from normal tissue will overgrow organoids from tumor tissue (**Fig. 2**). Once a mixture of organoid morphologies has been observed it will be necessary to perform manual selection as early as possible, as the growth of the wild-type organoids will overtake the culture. The morphological characteristics to use for selection vary between tumors. In our experience of stomach carcinoma, the morphology of tumor organoids is different to normal controls, so manually selecting those organoids with atypical morphology proved to be successful in establishing tumor organoid cultures (**Fig. 2**). If the ratio of normal to diseased looking organoids is about 1:1, then "bulk picking" is the most efficient option (Step 21 *option BI* below). If very few tumor organoids are present in the well, or if a variety of different morphologies is observed among the tumor organoids, then the more time-consuming single organoid manual selection should be considered (Step 21 *option BII or BIII* below). Sometimes several rounds of manual selection will be necessary to remove wild-type organoids from the culture.

Passaging of organoids Timing 1 h

Critical The passaging ratio depends on the amount and the size of the organoids in the well. Normal human gastric organoids are passaged 1:6 every 14 d. For tumor organoids the ratio can vary; we have experienced tumor organoids with passaging ratios varying from 1:10 every 14 d to 1:2 every two months.

3. Narrow a sterile glass Pasteur pipette in the Bunsen burner under the cell culture hood (for images, see [2]). Let the pipette cool to room temperature (RT).

4. Remove medium from 24-well containing organoids in Matrigel drop.
5. Add 500 µL Ad++ to well, resuspend several times with a P1,000 pipette and transfer to a 15 mL Falcon tube.
6. Pre-coat a narrowed glass Pasteur pipette by taking Ad++ up and down into the pipette a few times.
7. Mechanically disrupt organoids in 1 mL Ad++ with a narrowed glass Pasteur pipette (~10 times).

   Critical Be sure to pre-coat the glass Pasteur pipette with Ad++. Otherwise the organoids will stick to the glass.
8. Add Ad++ up to a volume of 5 mL, mix well and centrifuge for 5 min at 300 g at 4 °C.
9. Remove supernatant**.**

   Critical Be careful not to aspirate the pellet by removing the last of the supernatant with a P100 pipette. It is helpful to remove as much supernatant as possible. If a clear pellet has not formed, remove as much supernatant as possible, add another 5 mL of cold Ad++, mix well by pipetting up and down (~10 times) and centrifuge again.
10. For passaging 1:6, resuspend pellet in 300 µL of Matrigel and seed 50 µL in each of six new 24-wells.

    Troubleshooting
11. Allow Matrigel to solidify for 15 min in a 37 °C incubator.

    Critical Plate should be pre-warmed to 37 °C for 1 d prior to passaging.
12. Add 500 µL of human gastric organoid medium containing ROCKi.

    Critical Addition of ROCKi helps to improve outgrowth of the organoids after disruption, either for passaging or manual selection.

    Troubleshooting

Freezing of organoids Timing 1 h

13. Follow steps 3-9.

14. Resuspend pellet in 500 µL of recovery cell culture freezing medium, transfer to a cryotube and place into a -80 °C freezer in a cool cell freezing container.
15. Transfer tubes to liquid nitrogen after 1 d.

    Pause point Frozen organoids can be stored long-term in liquid nitrogen and thawed when needed.

Thawing of organoids Timing 30 min

16. Remove frozen organoids from liquid nitrogen tank and place the vial into a 37 °C waterbath until thawed (approximately 2 min).

    Critical Be careful not to leave organoids in the waterbath extensively, since cells will die.
17. Transfer thawed organoids to 15 ml Falcon tube and add Ad++ up to 10 mL, mix well and centrifuge for 5 min at 300 g at 4 °C.
18. Remove supernatant.

    Critical Be careful not to aspirate the pellet by removing the last of the supernatant with a P100 pipette. It is helpful to remove as much supernatant as possible. If a clear pellet has not formed, remove as much supernatant as possible, add another 5 mL of cold Ad++, mix well by pipetting up and down (~10 times) and centrifuge again.
19. Resuspend pellet in 50 µL of Matrigel and seed in one 24-well.

    Troubleshooting
20. Follow steps 11 and 12. Check the organoid growth regularly under the microscope. Usually after 14 d thawed organoids are ready for regular passaging (steps 3-12).

Selection of subpopulations of organoids

21. Perform selection of subpopulations via selective pressure (Option A), morphological phenotype (Option B) or clonal expansion (Option C).

(A) Selective pressure (Fig. 3a) Timing 7 d selection, 2-4 weeks growth

If the tumor organoids contain a specific mutation it is possible to select for this using selection pressure in the medium. This can be useful for two reasons: 1) for the selection of tumor

organoids from a mixture of normal and tumor organoids after initial outgrowth of the cells from the tissue, or 2) for the validation of sequencing results (see step 22 option B).

Nutlin-3 selection

i. Passage the organoids you want to select as well as normal control organoids in the usual ratio (~1:6) and seed them in a 24-well plate, four wells each.

ii. Add different concentrations of Nutlin-3 (mock, 1 µM, 5 µM and 10 µM) to the wells for 7 d (**Fig. 3a**). Use these different Nutlin-3 concentrations in the hgo medium for individual wells throughout one week of culturing (change medium every 2-3 d).

Troubleshooting

iii. Observe organoid growth under the microscope. Organoids harboring *TP53* mutations should be insensitive to Nutlin-3 treatment, while *TP53* wild-type organoids should die.

(B) Manual selection of tumor organoids based on morphology (Fig. 2 and 3b)

(I) "Bulk picking" Timing 1 h manual selection, 2-4 weeks growth

I. Narrow a sterile glass Pasteur pipette in the Bunsen burner under the cell culture hood. Let the pipette cool to RT.

II. Remove medium from well and add 500 µL of cold Ad++. Resuspend the Matrigel drop carefully by pipetting up and down slowly using a P1,000 pipette. Transfer everything into a Petri dish.

Critical Removal of Matrigel can help but may not be necessary. To remove Matrigel, resuspend Matrigel drop in 500 µL of cell recovery solution instead of Ad++, transfer to a 15 mL Falcon tube and place at 4 °C in fridge for 20 min.

Critical step Do not resuspend cultures too harshly, since this will already break the organoids apart. This is not desirable at this point, because it is easier to compare the morphologies of intact organoids. The use of wide-pore tips is recommended for these critical steps.

III. Under a light microscope, use a P100 pipette to pick up as many organoids with diseased morphology as possible, leaving the ones with the normal morphology behind. Transfer the organoids to a 15 mL Falcon tube containing 500 µL Ad++.

IV. Pre-coat the narrowed Pasteur pipette with Ad++ and in a total of 1 mL Ad++, break the organoids apart with the narrowed glass Pasteur pipette.

Critical step Be sure to pre-coat the glass Pasteur pipette with Ad++, otherwise the organoids will stick to the glass.

V. Fill up to 5 mL with Ad++ and centrifuge for 5 min at 300 g and 4 °C.

VI. Remove liquid, being careful not to aspirate the pellet.

Critical step Remove most of the liquid with a pipetboy, but the very last bit should be removed with a P1,000 or even a P100 pipette. The pellet can be rather loose and hard to see, depending on the amount of organoids picked.

Troubleshooting

VII. Resuspend pellet in 50 µL Matrigel and plate into 24-well plate.

Critical step Plate should be pre-warmed to 37 °C for 1 d.

VIII. Let Matrigel solidify for 15 min at 37 °C in the incubator.

IX. Add 500 µL human gastric organoid medium (**Table 1**) containing ROCKi.

Critical step Addition of ROCKi helps to improve outgrowth of the organoids after disruption, either for passaging or manual selection.

X. Culture pure population for 2-4 weeks. It is possible to repeat steps I-IX if there are still normal organoids with normal morphology present and growing.

(II) Single organoid manual selection Timing 1 h manual selection, 2-4 weeks growth

I. Remove medium from well and add 500 µL of cold Ad++. Resuspend the Matrigel several times using a P1,000 pipette and transfer everything into a Petri dish.

Critical step Do not resuspend cultures too harshly, since this will already break the organoids apart. This is not desirable at this point, because it is easier to compare the

morphologies of intact organoids. The use of wide-pore tips is recommended for these critical steps.

II. Under a light microscope, isolate single organoids using a P100 pipette or, preferably, a thin needle.

Critical step Ensure only a single individual organoid is taken to derive a clonal population.

Troubleshooting

III. Break organoid apart with a glass pipette (*Option a*) or mechanically open the organoid with needles (*Option b*).

(a) Break organoid apart with a glass pipette

a. Transfer organoid into a 15 mL Falcon tube containing 500 µL of Ad++.
b. Follow steps IV-IX of step 21 *option BI*. Instead of a 24-well plate, seed organoid pieces in a 48-well plate using 25 µL of Matrigel and 250 µL of human gastric organoid medium.
c. Repeat for as many organoids as desired.
d. Allow the organoids to expand. It should be possible to passage the culture after 2-4 weeks following a standard passaging process (follow steps 3-12).

(b) Mechanically open with needles

a. Move individual organoid away from others and out of the liquid.

   Critical step It is crucial to work quickly here, as the organoid is at risk of drying out.
b. Mechanically disrupt the organoid into several pieces using two thin needles. The size of the pieces will determine the growth of organoids and the timing of subsequent steps. If the pieces contain only a few cells, the organoids will need more time to grow. If the pieces are very large, the organoids may not recover well and will need passaging soon after. As a rough landmark, one organoid of approximately 500 µm diameter should be cut into approximately ten pieces.

c. Take the pieces up with a P100 pipette filled with 25 µL of Matrigel. Resuspend several times in the Petri dish to draw up as many organoid pieces as possible and seed organoids into Matrigel in a 48-well.

Troubleshooting

Critical step Plate should be pre-warmed to 37 °C for 1 d.

d. Let Matrigel solidify for 15 min in the 37 °C incubator.

e. Add 250 µL human gastric organoid medium (**Table 1**) containing ROCKi.

Critical step After disrupting organoids, either for splitting or manual selection, addition of ROCKi improves initial growth of organoids.

f. Repeat for as many organoids as desired.

g. Let the organoids expand. The next splitting should be possible after 2-4 weeks as standard splitting process (follow steps 3-12).

(III) Organoid manual selection directly from the well Timing 1 h manual selection, 2-4 weeks growing

Critical This is particularly easy when cultures are seeded very thin and also a good possibility after FACS sorting of single cells.

I. Leaving the medium and Matrigel in the well intact, use a sterile needle under the microscope to carefully dislocate the desired organoid(s) from the Matrigel. They should be free-floating in the well afterwards.

II. Under the light microscope use a P100 pipette to pick up the floating organoid(s).

Critical Others have reported to pick single organoids using tweezer [3], which is a good option if you have a large single organoid. Gastric cancer organoids are often small and compact and easier to pick up with a pipette. Be sure to pre-wet your pipette tip in medium, so that possible sticking to the plastic is minimized.

III. Follow step 21 *option BIIa* or *option BIIb.*

(C) Single cell FACS (Fig. 3c) Timing 3 h sorting, 2-4 weeks growing

i. Remove medium from well and add 500 µL Ad++. Resuspend Matrigel drop with P1,000 pipette and transfer to 15 mL Falcon tube.

ii. In a total of 1 mL Ad++ mechanically disrupt organoids using a narrowed glass Pasteur pipette.

Critical step Be sure to pre-wet glass Pasteur pipette with Ad++. Otherwise organoids will stick to glass.

iii. Centrifuge for 5 min at 300 g and 4 °C.

iv. Remove supernatant, resuspend pellet in 500 µL TrypLE Express and incubate in 37 °C waterbath for up to 15 min. Every 5 min resuspend cells with narrowed glass Pasteur pipette and check under microscope whether cells are already single cells.

Critical step Do not leave cells in TrypLE Express extensively; therefore it is important to check every 5 min. Ensure to have obtained single cells only, otherwise the flow cytometer might block.

Troubleshooting

v. Centrifuge single cells for 5 min at 400 g and 4 °C and resuspend pellet in 500 µL Ad++ and add PI 1:1,000. Keep cells on ice until sorting.

Caution PI is a hazardous substance, handle with care.

Optional: Right before sorting, filter cells through 40 µM filter, if you observe clumping.

vi. Start the flow cytometer. We use the BD FACS Aria III according to manufacturer's instructions (https://www.bdbiosciences.com/documents/BD_FACSAria_III_User_Guide.pdf). Auto drop delay needs to be determined using BD Accudrop beads and automatic drop delay. Cells should be sorted with a 70 micron nozzle and 70 psi pressure.

vii. Set gate in SSC-A/FSC-A plot to collect organoid cells. Then rule out doublets in FSC-W/FSC-A plot and collect PI-negative cells in PI/FSC-A plot (**Fig. 3c** upper row). To verify your doublet gate, place a microscopy slide under the sorter and let it sort cells onto the microscopy slide. You can observe the single cells and possible doublets under the microscope. Set the gate rather narrow to be sure to sort only single cells.

viii. Collect 10,000 single cells in human gastric organoid medium.

ix. Centrifuge collected cells for 5 min, 400 g at 4 °C.

x. Resuspend pellet in 50 µL Matrigel and seed as described in step 21 *option BI* steps VII-IX.

xi. Observe organoid growth over the following 2-4 weeks (**Fig. 3c**).

xii. As early as possible pick single organoids to make individual clonal lines (step 21 *option BII* or *BIII*).

Verification of tumor identity

22. After expansion and cryopreservation of the cancer organoids, their tumor identity needs to be verified by sequencing (Option A), selective pressure (Option B), karyotype (Option C) or histology (Option D). Normal organoids can reappear during long-term culture, cross-contamination between patient organoids may happen in tissue culture labs, therefore we recommend routine identity checking of the organoids every few months.

(A) Sequencing of original tumor tissue and subsequent sequencing of tumor organoids (Fig. 4a)

Next-generation sequencing (NGS) of original tumor tissue Timing 3.5 d

Critical Use all kits strictly according to the manufacturer's protocols.

i. Perform DNA extraction using the GeneRead DNA FFPE Kit from Qiagen.

Critical step Depending on the size of the tissue, elute DNA in up to 40 µL. Biopsies or other small tissue sections should be eluted in a minimal volume of 25 µL whereas large tissue sections should be eluted in up to 40 µL.

Critical All further kits and reagents are provided by Thermo Fisher using the Thermo Fisher Ion PGM system pipeline validated in the Institute of Pathology, University of Würzburg.

ii. Measure the amount of replicable DNA with a qPCR-based assay (TaqMan™ RNase P Detection Reagents Kit and the TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG; (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4304449_TaqManPCRMM_UG.pdf and https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_041461.pdf) on the StepOne™ Real-Time PCR System (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_046735.pdf).

iii. According to the result of the qPCR, apply 10 ng of DNA per sample for the library construction via a multiplex PCR workflow using the 1 x Ion AmpliSeq™ Library Kit 2.0 (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0006735_AmpliSeq_DNA_RNA_LibPrep_UG.pdf), the Ion AmpliSeq™ Cancer Hotspot Panel v2 (https://assets.thermofisher.com/TFS-Assets/LSG/brochures/Ion-AmpliSeq-Cancer-Hotspot-Panel-Flyer.pdf) and the Ion Xpress™ Barcode Adapters.

iv. Determine the molarity of the libraries by qPCR with the Ion Library TaqMan™ Quantitation Kit (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0015802_IonLibrary_Taqman_Quantitation_Kit_UG.pdf) on the StepOne™ Real-Time PCR System.

v. Pool the libraries in an equimolar proportion and enrich using the Ion PGM™ Hi-Q™ View OT2 Kit (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0014579_IonPGM_HiQ_View_OT2_UG.pdf) and the Ion OneTouch™ 2 System (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0014388_IonOneTouch2Sys_UG.pdf).

vi. Verify the enrichment of the Ion Sphere Particles by the Ion Sphere™ Quality Control Kit (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017531_IonSphereQCKit_UG.pdf).

vii. Further process the library pool with the Ion PGM™ Hi-Q™ View Sequencing Kit (available at https://assets.thermofisher.com/TFS-

Assets/LSG/manuals/MAN0014583_IonPGM_HiQ_View_Sequenc_UG.pdf) and sequence it on an Ion 318™ Chip Kit v2 BC with the Ion Personal Genome Machine™ (PGM™) sequencer (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0009783_IonPGM_RefGuide.pdf).

viii. Perform bioinformatical analyses using the Torrent Suite Software v5.6 and Ion Reporter™ Software v5.6 (available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017362_Ion_Reporter%205_6_RN.pdf).

ix. Evaluate clinical significance of detected variants using ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), cBioPortal (http://www.cbioportal.org), OncoKB (http://oncokb.org) and IARC TP53 (http://p53.iarc.fr/TP53SomaticMutations.aspx) databases.

Sanger sequencing of tumor organoids Timing 2-3 d

x. Resuspend 50 µL Matrigel drop of one full well of organoids (24-well plate) in 1 mL cell recovery solution, transfer to a 1.5 mL tube and place into 4 °C fridge for 30 min.

xi. Collect organoid pellet by centrifugation at 400 g for 5 min.

xii. Remove supernatant and wash organoid pellet twice with 800 µL PBS.

xiii. Resuspend organoid pellet in 30 – 50 µL Proteinase K lysis buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA and 0.02 % NP40) with 10 % Proteinase K (20 mg/mL in 50 mM Tris, pH 8.0).

xiv. Incubate at 56 °C for 8 h to o/n.

xv. Spin at 400 g for 5 min and collect the supernatant.

xvi. Inactivate Proteinase K activity at 95 °C for 15 min.

Critical Alternative to steps x-xvi take one full well of organoids of a 24-well plate for DNA extraction using QIAamp DNA Mini Kit according to manufacturer's instructions.

xvii. Measure the DNA concentration with a Nanodrop.

xviii. Run PCR with primers flanking the target region using a polymerase with proof reading activity using the protocol recommended for the polymerase. We use Phusion high-fidelity DNA polymerase with 250 ng of template DNA in a total of 20 µL.

xix. Run PCR product on an 1-2 % agarose gel, stain in EtBr bath and check for the predicted size.

Caution EtBr is toxic, handle with care.

Troubleshooting

xx. Use the NucleoSpin Gel and PCR Clean-Up Kit to clean up the PCR reaction.

xxi. Send the PCR product for sequencing with the primers used for the PCR.

xxii. Align results to wt gene sequence and check for mutations (**Fig. 4a**).

Troubleshooting

xxiii. If a specific mutation allows for selection, proceed with further steps below.

(B) Selection of tumor organoids based on the identified mutation: Example selection of *SMAD4*-mutated tumors with TGF-ß or NOG withdrawal (Fig. 4b) Timing 16 d

Critical Depending on the mutations identified by sequencing, different selection protocols can be used. Organoids with *TP53* mutations can be selected using Nutlin-3 (see step 21 option A). Organoids with activating mutations in the WNT pathway can be selected by omission of WNT from the medium. Organoids with mutation in *SMAD4* can be selected using omission of TGF-ßi or NOG from the hgo medium. As an example, we show here the selection for *SMAD4* mutations.

Selection for SMAD4 mutations

i. Split the organoids in which you want to confirm a *SMAD4* mutation as well as normal control organoids in the usual ratio (~1:6) and seed them in a 24-well plate, three wells each.

ii. Add human gastric organoid medium to control wells (wt and *SMAD4*-mutated) and medium lacking either TGF-ßi (-TGF-ßi) or NOG (-NOG) to remaining wells respectively (**Fig. 4b**).

Use these specific media composition throughout the entire culturing time. Change medium accordingly every 2-3 d.

iii. Observe organoid growth under the microscope. In the beginning, there is no obvious growth difference between samples, but this will become apparent after splitting.

iv. On day 14, split all organoids (steps 3-12) at a ratio of 1:6 and plate one well each. Continue to omit either TGF-ßi or NOG. Usually, on day 1 after splitting the differences start to be visible. Normal organoids do not grow out in the wells lacking TGF-ßi or NOG. However, *SMAD4*-mutated organoids grow like the controls that had received normal human gastric organoid medium.

(C) Metaphase spread (Fig. 4c) Timing 2 d

i. Change medium and add 0.1 µg/mL (1:100 from stock solution) colcemid.

Caution Colcemid is very toxic and needs to be handled with care.

ii. Incubate o/n at 37 °C.

Critical step Incubation will have to be adjusted depending on the growth rate of cells.

iii. Discard colcemid-containing medium and wash the cells with PBS.

iv. Remove organoids from Matrigel with cold Ad++ and disrupt with narrowed Pasteur pipette. Pellet cells by centrifugation at 200 g for 5 min.

v. Wash pellet once with PBS without disturbing the pellet.

vi. Add 1 mL TrypLE Express, pipette up and down, incubate 30 min at 37 °C and confirm single cells by microscopy.

Critical step Increase incubation if cells are still not single.

vii. Pellet cells by centrifugation at 400 g for 5 min.

viii. Resuspend the pellet and carefully add 1 mL of pre-warmed 0.075 M KCl while shaking, leave at 37 °C for 10 min.

ix. Dropwise add 1 mL MeOH:AcAcid, centrifuge for 5 min at 400 g and discard supernatant.

Caution MeOH is a hazardous substance, handle with care.

x. Resuspend the pellet, dropwise add 1 mL MeOH:AcAcid, incubate 20 min at RT and centrifuge for 5 min at 400 g.

xi. Repeat step x twice.

xii. Resuspend the pellet in 500 µL of MeOH:AcAcid and keep at -20 °C until analysis.

Pause point Samples can be stored at -20 °C. We usually process them the following days, nevertheless successful metaphase spreads could also be obtained from samples stored for several months.

xiii. At day of analysis, spin cells and resuspend in 200 µL MeOH:AcAcid.

xiv. Take a microscopy slide and clean it.

Critical step It is very important that slides are well cleaned.

xv. Let few drops of cell suspension fall from 30 cm height onto the microscopy slide. The force of the drop falling on the glass helps to spread the chromosomes.

Troubleshooting

xvi. Let the slide dry and mount with DAPI Vectashield.

xvii. Check under fluorescent microscope.

(D) Histology of tumor organoids and original tumor tissue (Fig. 4d) Timing 5-6 d for tissue, 2 d for organoids

Processing (Fixation, dehydration and embedding)

(I) Organoids

I. Remove medium from organoids, add 500 µL cell recovery solution onto the Matrigel drop and collect organoids in 15 mL Falcon tube.

Critical step Be careful to use a cut P1,000 pipette to not disrupt the organoids.

II. Keep organoids on ice for 30-60 min.

Critical step After 30 min check every 10 min whether Matrigel is already dissolved.

III. Let organoids settle by gravity and remove the supernatant.

IV. Add 1 mL PBS, let organoids settle by gravity and remove the supernatant.

V. Add 1 mL of 2 % PFA and incubate for 20 min at RT.

Caution PFA contains formaldehyde, handle appropriately.

VI. Let organoids settle by gravity, remove the supernatant and store in PBS at 4 °C.

Pause point Fixed organoids can be stored at 4 °C. Although we usually do not experience problems, for long-term storage sodium azide might be added to prevent microbial contaminations.

Critical For subsequent steps transfer organoids in PBS with pre-wet plastic transfer pipette into glass test tube to prevent sticking of the organoids to the plastic tube.

VII. Let organoids settle by gravity and remove supernatant.

Critical For all following steps it is not necessary and rather counterproductive to resuspend organoids in various reagents because you will lose valuable material. Always add ~1 mL of respective reagent, let organoids settle by gravity, remove supernatant and add next reagent.

VIII. Dehydrate organoids in 25 %, 50 %, 70 % EtOH (in H_2O) for 15 min each and 96 % EtOH for 30 min.

IX. Stain organoids in 96 % EtOH + 1 % Eosin for 30 min.

Critical step Staining of organoids is recommended before embedding as Eosin-stained organoids are better visible in paraffin.

X. Wash organoids three times in 100 % EtOH for 30 min each.

XI. Wash organoids three times in 1-Butanol for 30 min each.

Caution 1-Butanol is flammable, handle with care.

XII. Incubate organoids three times at 56 °C in paraffin for 30 min each.

XIII. Put paraffin in mold on heated plate at the paraffin embedding station.

XIV. Transfer organoids into metal base mold.

Critical step Use warm forceps to move organoids close together as deep into the mold as possible to enable equal cut surfaces during sectioning.

XV. Press embedding cassette onto mold and dispense paraffin on top.

XVI. Place mold to cool plate for 30 min.

(II) Tissue

I. Fix tissue piece on carton or similar using needles, place upside down into a 50 mL Falcon tube filled with ~25 mL Formalin and leave for 2 d.

Caution Formalin contains formaldehyde, handle appropriately.

Pause point Tissues can be stored in Formalin at 4 °C. Prolonged Formalin fixation or 'overfixation' is a debated topic [4]. To avoid this, samples should not be stored longer than 2 d.

II. Place tissue in embedding cassette and close cassette.

III. Transfer embedding cassette holding the tissue into 70 % EtOH (in H_2O) and incubate for 1 d.

IV. Perform automated dehydration o/n in tissue processor (total of 12 h): Once 70 % EtOH for 1 h, twice 96 % EtOH for 1 h, three times 100 % EtOH for 1 h, three times Xylol for 1 h and three times paraffin for 1 h.

Alternatively, perform steps manually (total of 8.5 h): Three times 70 % EtOH for 30 min at 4 °C.

Pause point Material can be stored in 70 % EtOH at 4 °C for several days.

Then, twice 96 % EtOH for 30 min at RT, three times 100 % EtOH for 30 min at RT, three times Xylol for 30 min at RT, three times paraffin for 1 h at 60-65 °C. The last paraffin step can be performed in a vacuum stove if available.

Caution Xylol is toxic and flammable, handle with care. Perform all steps under a fume hood.

Critical step Dehydration steps with EtOH and Xylol can be performed with agitation, such as in closed containers on a rolling platform.

V. Put paraffin in metal base mold on heated plate.

VI. Cut tissue perpendicular to the surface to prepare cross-section. Depending on the size of your tissue, you may want to cut perpendicular once or twice to obtain tissue pieces of 1 cm length and 0.3-0.5 cm depth.

VII. Put the mold onto cool plate at the paraffin embedding station and subsequently transfer tissue into mold with the cut side facing the bottom of the mold.

Critical step Paraffin will solidify in a thin layer after transferring it to the cool plate, holding the tissue in an upright position.

VIII. Press embedding cassette onto mold and dispense paraffin on top.

IX. Place mold to cool plate for 30 min.

X. Let it solidify o/n.

Sectioning

i. Make 2-5 µm cuts using a microtom and put them into warm water with a fine brush.

ii. Collect cuts from warm water onto polysine-coated glass slides.

iii. Keep slides o/n at 60 °C in oven.

Rehydration

i. De-paraffinize slides twice in Xylol for 5 min.

ii. Rehydrate slides by briefly immerging them three times into 96 % EtOH, 96 % EtOH, 70 % EtOH and 50 % EtOH, respectively.

iii. Gently agitate slides in distilled water.

Staining

(I) H&E

Critical Prepare glass staining containers with respective solutions and move the slides from one container to the next.

I. Incubate slide for 8 min in Hematoxylin in order to stain nuclei.

II. Gently agitate slide in distilled water.

Critical step Move slide until no more color leaks out.

III. Briefly immerge slide three times into HCl:EtOH.

IV. Gently agitate slide in distilled water.

V. Incubate slide for 5 min in tap water.

VI. Incubate slide for 1 min in 1 % Eosin in order to stain cytosolic proteins.

VII. Gently agitate slide in distilled water.

Critical step Move slide until no more color leaks out.

VIII. Dehydrate by immerging slide three times into 70 % EtOH, incubate 2 min in 96 % EtOH, twice for 5 min in Isopropanol and twice for 5 min in Xylol.

IX. Embed stained slide in Entellan with cover glass and leave under fume hood o/n.

Caution Entellan is flammable, handle with care.

(II) TP53

Critical For each antibody, antigen retrieval and staining procedure has to be optimized. Follow the manufacturer's recommendations for each antibody.

I. Perform antigen retrieval by boiling the tissue sections in 1 x citrate buffer (pH 6.0) for 8 min at 800 W in a microwave.

II. Rinse slides in cold tap water to cool down to RT.

III. Circle the tissue sections with a PAP pen.

IV. Dilute the primary TP53 antibody 1:50 in Dako REAL antibody diluent and incubate circled tissue sections for 1 h at RT in 150 µL of diluted primary antibody.

Critical step Slides should be placed in a humidity chamber to prevent evaporation of the antibody solution.

V. For washing purposes incubate slides with PBS three times for 5 min.

VI. Detect primary antibody using the HiDef Detection System according to manufacturer's instructions.

VII. Incubate slide for 3 min in Hematoxylin in order to stain nuclei.

VIII. Rinse slide in running tap water for 10 min.

IX. Dehydrate by immerging slide three times into 70 % EtOH, incubate for 2 min in 96 % EtOH, twice for 5 min in Isopropanol and twice for 5 min in Xylol.

X. Embed stained slide in Entellan with cover glass and leave under fume hood o/n.

Box 3: Generation of tumor organoids Timing 3 h

Additional materials

- Collagenase Type II (Worthington Biochemical corporation, cat. no. LS004174)
- Hyaluronidase (Sigma-Aldrich, cat. no. H4272)
- Rolling device (e.g. VWR, RM 5)
- Micro glass slides (Hartenstein, cat. no. OTOM)
- Dissection tools: Two fine tweezers and scissors (e.g. Hartenstein, cat. no. PZ14, PZ15, SN01, SN02)

Additional reagent setup

Collagenase Dissolve 10 mg per mL in Ad++.

Hyaluronidase Dissolve 10 mg per mL in sterile H_2O.

Procedure

1. Cut one piece of the tumor for paraffin embedding (fix in Formalin) and one piece for DNA isolation (store at -20 °C).
2. Cut the remaining tumor into small pieces with scissors (**Fig. 1**).

   Critical step Avoid sampling from the mucosal surface, focus on sampling tumor in the muscle or serosal aspect as far as possible. This will help eliminate normal contamination and thus substantially increase the success rate. Tumor tissue can be very solid and rigid. Be sure to cut everything up as good as possible until no actual pieces are left but rather a mesh.
3. Put pieces into Falcon tube containing 8.5 mL Ad++, 1.5 mL 10 mg/mL collagenase II (15 mg), 20 µL 10 mg/mL Hyaluronidase (200 µg) and Primocin.
4. Incubate on rolling device for 1 h at 37 °C.

   Troubleshooting
5. Pipette up and down approximately 10 times.
6. Let tissue pieces settle.

7. Transfer the supernatant to new Falcon tube.
8. For continuing with the supernatant follow steps 9-12, for continuing with the settled pieces follow steps 13-20. Do both simultaneously.
9. To supernatant add 1 mL FCS.
10. Spin for 5 min at 400 g at 4 °C.
11. Remove supernatant.

    Critical step Be careful not to lose the pellet. For removing last bit of supernatant use P100 pipette.
12. Plate pellet in dilution row (1, 1:10 and 1:100) in Matrigel and let Matrigel solidify for 15 min in 37 °C incubator.

    Troubleshooting

    Critical step Prewarm tissue culture plates at 37 °C o/n.
13. To remaining settled pieces add 3 mL TrypLE Express. Although we do not use DNase I, other labs add DNase I to prevent cell clumping [5-7].
14. Incubate for 15 min at 37 °C.
15. Transfer pieces to Petri dish.

    Critical step Take as little liquid as possible.
16. Squeeze tissue pieces on Petri dish using a glass slide to release the cells. Use your thumbs to apply high pressure. A 'cloud' (representing released cells) should be appearing after pressing (**Supplementary Fig. 2**) (or see video [2]).
17. Add 10 mL Ad++ and transfer to Falcon tube.
18. Pipette up and down approximately 10 times.
19. Let pieces settle and take supernatant to new Falcon tube.
20. Spin for 5 min at 400 g at 4 °C.
21. Remove supernatant, plate pellet in dilution row (1, 1:10 and 1:100) in Matrigel and let Matrigel solidify for 15 min in 37 °C incubator.
22. Add 500 µL organoid growth medium (**Table 1**) to each well from **Box 3**, step 12 and 21.

    Critical step Add ROCKi to the growth medium.

23. Change medium every 2-3 d and check organoid growth every day in order to decide at what point splitting (Procedure steps 3-12) or manual selection (Procedure step 21 option B) is necessary.

Troubleshooting

TROUBLESHOOTING

Troubleshooting advice can be found in **supplementary table 1**.

Supplementary table 1: Troubleshooting table

Step	Problem	Possible reason	Solution
Box 1, step 1A(vi)	Filter is clogging	Too many dead cells in the supernatant	Take supernatant already after 5-6 d, when cells are already confluent Good quality of filter unit important
Box 1, step 1A(viii)	WNT3A-/RSPO1- or NOG-CM production does not work	Cells might not produce anymore	Check for mycoplasma Check whether you are using the correct selection pressure Start fresh cells from liquid nitrogen stock
Box 2, step 15	TOP/FOP assay does not work	Plasmids might be non-functional	Prepare fresh plasmids from glycerol stocks Check plasmids with restriction enzyme

			digests for correct size Sequence plasmids
		HEK293T cells may not be ready	Make sure that HEK293T cells have been passaged at least 2-3 times after thawing before seeding them for the assay
		Number of HEK293T cells per well might have to be adjusted	In a 96-well plate, 40,000 cells/well works best for us
	Cells might detach during handling	Too rough handling	Handle cells with utmost care Do not pipette directly on them but only let the liquid run slowly onto the side walls of the plastic

Box 2, step 24	Luciferase assay does not work	Reagents might be spoiled	Be sure to store reagents properly (light-sensitive)
Box 3, step 4	Large tissue pieces after digestion	Inefficient enzymatic digestion	Prolong digestion time
Box 3, step 12, 10 (Procedure) and 19 (Procedure)	Matrigel solidifies in the tip Air bubbles in Matrigel	Matrigel has warmed in the tip during seeding Matrigel was pipetted without caution	Keep Matrigel on ice as much as possible. Seed Matrigel:cell mixture as soon as cells have been resuspended in Matrigel Pipette slowly
Box 3, step 23	Contamination in organoid culture	Patient-derived tissue always contains microbiota	Wash in sterile PBS containing P/S and Primocin before isolation. Sterilize dissection tools properly Perform the whole isolation under a

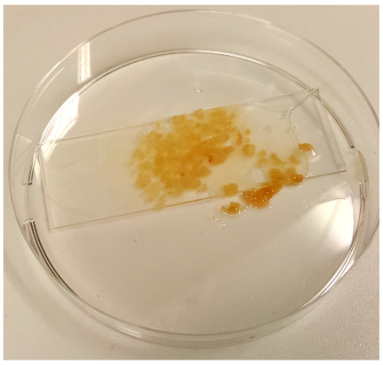
			sterile cell culture hood Be sure to add Primocin to Ad++. Primocin is a mixture of antibiotics including fungicides. Alternatives for primocin are own mixtures containing antibiotics against gram positive and gram negative bacteria as well as fungicides If your only well is contaminated, try to rescue by manually selecting individual organoids
Box 3, step 23 and 12 (Procedure)	No, few or small organoids	Inadequate growth factor activity in the culture medium	Be sure to test your WNT3A-CM properly (follow recommendations for WNT3A-CM

			production and activity testing described in this protocol (**Box 1** and **Box 2**) Change medium every 2-3 d, meaning three times per week Do not store medium with growth factors longer than 3 d at 4 °C Be sure to add ROCKi to medium after splitting
21A(ii) (Procedure)	All or no cells are dead	Too high/low concentration of inhibitor used	Check optimal concentration before starting the assay with normal organoids. The minimal amount it takes to kill those should be used

21BI(VI) (Procedure)	Loss of organoids	Pellet aspirated	Pellet might only be slightly visible. Be very careful and take last bit of supernatant with P100 pipette
21BII(II) (Procedure)	No clonal organoids	Several organoids taken up with pipette	Separate organoid from all others to pick as good as possible
21BIIb(c) (Procedure)	No organoid pieces visible in Matrigel	No pieces were transferred from Petri dish	Work fast, since organoids will dry out and stick to the Petri dish and can no longer be transferred Practice before with normal organoids
21C(iv) (Procedure)	Cells still are in clumps	Time for dissociation was too short Epithelial cells stick to each other	Prolong time in 37 °C waterbath Use narrowed glass Pasteur pipette during dissociation time to hinder sticking

22A(xix) (Procedure)	No band or several bands	Wrong primer Unspecific primer binding	Check primer sequence again Test a range of different annealing temperatures during PCR
22A(xxii) (Procedure)	Sequencing results cannot be analyzed	Quality of PCR product not good enough for sequencing	Subcloning of PCR product might be necessary (e.g. using TOPO TA cloning Kit, Invitrogen)
22C(xv) (Procedure)	Not many metaphases visible	Amount of cells in mitosis during colcemid treatment was low	Colcemid only halts cells in mitosis. To increase the number of metaphases, use cells during maximal expansion. Complete confluency and insufficient growth factors might be reducing expansion. Prolong incubation time with colcemid to

			allow more cells to enter mitosis. However if the incubation time is too long, cells die. So test a range of incubation times (e.g. 2-24 h) for your culture
	Cells are not nicely spread	Spread was not optimal	Hold slide with drop of cells over hot water into the vapor, that helps spreading



Supplementary Fig. 2: Cells released from tissue pieces after squeezing them with glass slide in Petri dish.

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