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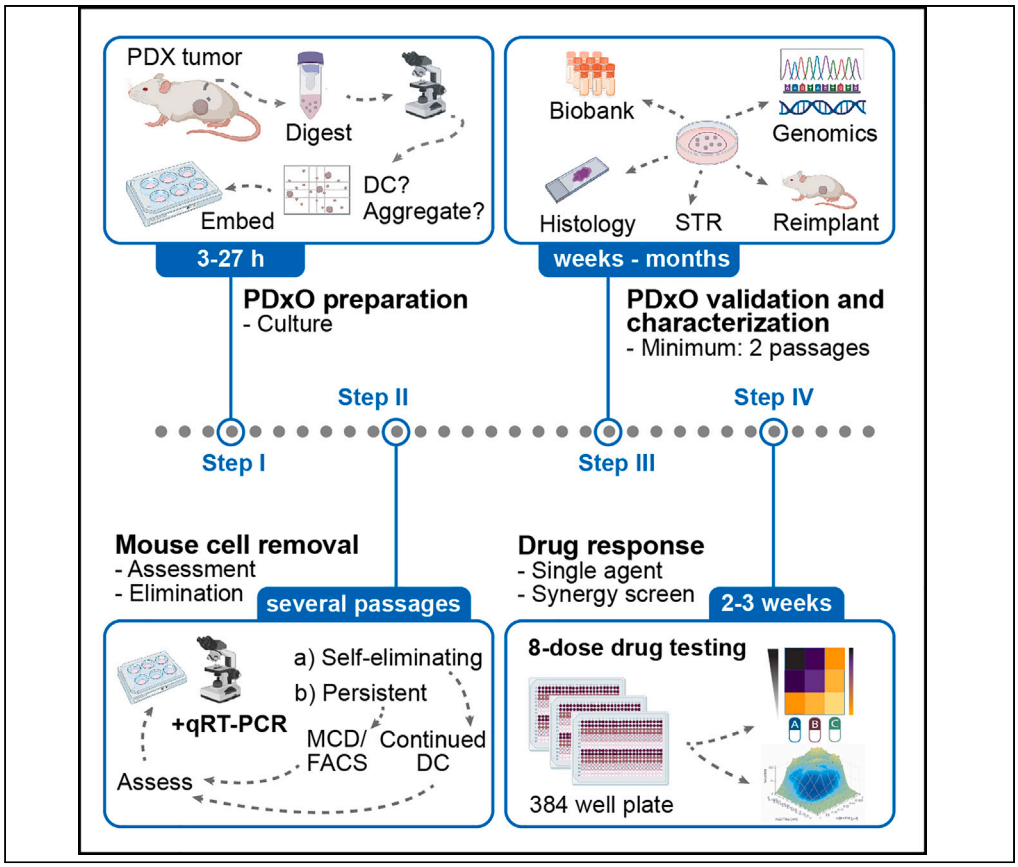
Breast cancer PDxO cultures for drug discovery and functional precision oncology



Patient-derived xenografts (PDXs) have clinical value but are time-, cost-, and labor-intensive and thus ill-suited for large-scale experiments. Here, we present a protocol to convert PDX tumors into PDxOs for long-term cultures amenable to moderate-throughput drug screens, including in-depth PDxO validation. We describe steps for PDxO preparation and mouse cell removal. We then detail PDxO validation and characterization and drug response assay. Our PDxO drug screening platform can predict therapy response *in vivo* and inform functional precision oncology for patients.

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Highlights

Protocol to establish long-term breast cancer organoids from patient-derived xenografts

Characterization and validation of PDxOs

Detailed steps for efficient PDxO drug screening

Adaptable for co-clinical functional precision oncology studies

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Breast cancer PDxO cultures for drug discovery and functional precision oncology

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SUMMARY

Patient-derived xenografts (PDXs) have clinical value but are time-, cost-, and labor-intensive and thus ill-suited for large-scale experiments. Here, we present a protocol to convert PDX tumors into PDxOs for long-term cultures amenable to moderate-throughput drug screens, including in-depth PDxO validation. We describe steps for PDxO preparation and mouse cell removal. We then detail PDxO validation and characterization and drug response assay. Our PDxO drug screening platform can predict therapy response *in vivo* and inform functional precision oncology for patients.

For complete details on the use and execution of this protocol, please refer to Guillen et al.[1]

BEFORE YOU BEGIN

Our step-by-step protocol reports the entire process from establishing stable long-term PDxO cultures from PDX tumors, to in-depth PDxO validation and characterization, and finally to drug screening, and includes timing, potential pitfalls and troubleshooting. Specifically, we describe how to establish, maintain, validate, and cryopreserve PDxO lines; detect and manage mouse cell contamination; test estrogen responsiveness for estrogen receptor positive breast cancers; perform dose response screens for single compounds and drug combinations; and reliably assess drug responses using statistical methods. This set of protocols will be of particular interest for cancer researchers with an interest in using more accessible versions of patient-derived models as tools to study the biology of cancer, for drug discovery, and for precision oncology.

We have a success rate of PDxO establishment from PDX tumors of 82.6% (Table 2). Exceptions to date mostly comprise lobular breast cancers that do not form organoids in Matrigel due to loss of the cell-cell junction protein E-cadherin and, rarely, tumors that just fail to grow *in vitro* over time. We have not tested this protocol for cancers other than breast cancer. For in-depth characterization and comparison of our PDxOs and matching PDXs to patient tumors please refer to our primary publication Guillen et al.[1] Since every tumor has unique properties, patient-derived models such as organoids are inherently challenging to work with. Therefore, we provide an extensive troubleshooting guide for each section.

Institutional permissions and general guidelines

The use of human tissues must adhere to all relevant ethical guidelines. When handling human tissues take appropriate precautions. In accordance with biosafety level 2 (BSL2) guidelines, all

experiments should be performed in a class II biosafety cabinet while considering risks of blood-borne pathogens and wearing appropriate personal protective equipment (PPE). Organoids were cultured in humidified incubators at 37°C with 5% CO_2. Please refer to the key resources table for a complete list of materials and reagents. Solutions and buffers should be prepared before tissue processing, and recipes can be found in the solutions and media section. When handling reagents, adhere to PPE requirements for BLS2 tissue culture work. Refer to material safety data sheets for each reagent for additional precautions.

Engelbreth-Holm-Swarm (EHS)-derived matrix and PDX tissues

We previously published a protocol for making EHS-derived matrix which can be followed as published[2] using lactose dehydrogenase elevating virus (LDEV) negative EHS tumors.[1]

⚠ CRITICAL: LDEV contamination is a known issue in EHS tumors and matrix derivatives.[3] To remove LDEV, we passaged EHS tumors through rats, which cannot get infected by the virus. We then passaged the "clean" tumors in mice, isolated EHS tumors, and confirmed LDEV negativity by IDEXX testing.

The same EHS-derived matrix can be purchased from the Preclinical Research Shared Resource (PRR) core at the Huntsman Cancer Institute, University of Utah https://uofuhealth.utah.edu/huntsman/shared-resources/preclinical-research-resource/.

Established breast cancer PDX lines as input material for PDxO, and established PDxO lines, can be also obtained from the PRR core.

Commercial Matrigel

Timing: 10 min + 16 h thawing

⚠ CRITICAL: Matrigel is shipped and received frozen on dry ice, store at −80°C until use.

⚠ CRITICAL: Once thawed, always handle Matrigel on ice.

1. The day before usage, thaw Matrigel in refridgerator at 4°C for 16 h.
2. Prior to usage, pick up and gently swirl the Matrigel bottle without introducing air bubbles, to mix the Matrigel.
3. Keep Matrigel at 4°C and use within 2–3 weeks.

⚠ CRITICAL: We use Matrigel lots with a protein concentration of 9–10 mg/mL. Lots outside of this range can result in unsatisfactory polymerization properties that could negatively affect experiment outcomes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
AlexaFluor-488 mouse anti-human CD326, dilution: 1 µL/ 1.0 × 10^6 cells	BioLegend	cat# 324210
FITC anti-human CD298, dilution: 1 µL/ 1.0 × 10^6 cells	Miltenyi Biotec	cat# 130-101-291
AlexaFluor-647 rat anti-mouse CD90.2, dilution: 2 µL/ 1.0 × 10^6 cells	BioLegend	cat# 105318
AlexaFluor-647 hamster anti-mouse/rat CD29, dilution: 2 µL/ 1.0 × 10^6 cells	BioLegend	cat# 102214

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Advanced DMEM/F12	Thermo Fisher	cat# 12634028
Fetal bovine serum (FBS)	Genesee GenClone	cat# 25-514
HEPES	Thermo Fisher	cat# 15630080
GlutaMAX	Thermo Fisher	cat# 35050061
Hydrocortisone	Sigma-Aldrich	cat# H0888
Gentamicin	Genesee Scientific	cat# 25-533
Human EGF	Sigma-Aldrich	cat# E9644
Y-27632	Selleck Chemicals	cat# S1069
FGF2	R&D Systems	cat# 4114-TC-01M
N-Acetyl-L-Cysteine (NAC)	Sigma-Aldrich	cat# A7250
Heregulin-β1 (NRG)	Peprotech	cat# 100-03
TrypLE Express	Thermo Fisher	cat# 12605010
DMSO	Sigma-Aldrich	cat# D2650
Dispase II powder	Fisher Scientific	cat# 17105041
Growth factor reduced Matrigel (9–10 mg/mL protein)	Corning	cat# 354230
Tris Base ULTROL Grade	EMD Millipore	cat# 648311
Ammonium chloride	Sigma-Aldrich	cat# A9434
Fc Receptor Blocking Reagent, human	Miltenyi Biotec	cat# 130-059-901
Fc Receptor Blocking Reagent, mouse	Miltenyi Biotec	cat# 130-092-575
HBSS, Ca/Mg, phenol-red free	Thermo Fisher	cat# 14025126
PBS	Thermo Fisher	cat# 10010049
Buffer RLT Plus	Qiagen	cat# 1053393
2-Mercaptoethanol	Sigma-Aldrich	cat# M3148-25ML
Ethanol, 200 proof, molecular grade	Sigma-Aldrich	cat# E7023
DNase I solution	Qiagen	cat# 79254
IDTE buffer, pH 8.0	IDT	cat# 11-05-01-13
Histogel	Thermo Fisher	cat# HG-4000-012
16% Paraformaldehyde (PFA) solution	Ted Pella	cat# 18505
10× PBS	Thermo Fisher	cat# BP3994
Tween 20	Sigma-Aldrich	cat# P7949
Triton X-100	Sigma-Aldrich	cat# T8787
Bovine serum albumin (BSA)	Sigma-Aldrich	cat# A7906
Sodium borohydride	Oakwood Chemical	cat# 042896
Sodium azide	Sigma-Aldrich	cat# S2002
CCS (CitriSolv)	Decon Laboratories	cat# 1601
Ethanol 100%, to dilute	Decon Laboratories	cat# 2701
β-Estradiol (E2)	Sigma-Aldrich	cat# E2758
DMEM/F12, no phenol red	Thermo Fisher	cat# 21041025
ITS-X	Thermo Fisher	cat# 51500056
Sodium pyruvate	Life Technologies/Invitrogen	cat# 11360-070
Albumax II	Thermo Fisher	cat# 11021029
Sodium bicarbonate	Sigma-Aldrich	cat# S5761
Ascorbic acid	Sigma-Aldrich	cat# A4544
Glutathione	Sigma-Aldrich	cat# G4251
Critical commercial assays		
Human tumor dissociation kit	Miltenyi Biotec	cat# 130-095-929
Mouse cell depletion kit	Miltenyi Biotec	cat# 130-104-694
AllPrep RNA/DNA isolation kit	Qiagen	cat# 80204
Qubit RNA BR Assay kit	Thermo Fisher	cat# Q10211
Qubit dsDNA BR Assay kit	Thermo Fisher	cat# Q32853
SuperScript IV VILO Master Mix, ezDNase	Thermo Fisher	cat# 11766050
PowerUp SYPR Green Master Mix	Thermo Fisher	cat# A25742
AmpFLSTR Identifier Plus PCR Amp Kit	Thermo Fisher	cat# A26364
CellTiter-Glo 3D Cell Viability Assay	Promega	cat# G9683

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Mouse GAPDH (Forward)	IDT	AACAGCAACTCCCACTCTTC
Mouse GAPDH (Reverse)	IDT	CCTGTTGCTGTAGCCGTATT
Human GAPDH (Forward + Reverse)	IDT	Hs.PT.39a.22214836
Software and algorithms		
Open source software for GR metric parameter calculations	Clark et al.[4]	https://doi.org/10.18129/B9.bioc.GRmetrics
Other		
6-well tissue culture plates	Genesee Scientific	cat# 25-105
Cell lifters	Fisher Scientific	cat# 08-100-240
PES syringe filters, 0.22 μm	Genesee Scientific	cat# 25-244
Countess cell counting slides	Thermo Fisher	cat# C10228
Razor blades	Thermo Fisher	cat# 12-640
10-cm tissue culture plates	Genesee Scientific	cat# 25-202
Gentle MACS C-tubes	Miltenyi Biotec	cat# 130-096-334
PluriStrainer 200 μm	pluriSelect	cat# 43-50200-03
PluriStrainer 300 μm	pluriSelect	cat# 43-50300-03
PluriStrainer 500 μm	pluriSelect	cat# 43-50500-03
LS columns	Miltenyi Biotec	cat# 130-042-401
MACS Separator QuadroMACS	Miltenyi Biotec	cat# 130-091-051
Ultra-low attachment plates	Corning	cat# 3471
5 mL polystyrene round-bottom tubes	Falcon	cat# 352063
QIAshredder	Qiagen	cat# 79656
Qubit Assay tubes	Thermo Fisher	cat# Q32856
PCR strips	SSIbio	cat# 3247-00
LightCycler 384 well PCR plates	Roche	cat# 04 729 749 001
LightCycler sealing film	USA Scientific	cat# 2921-7810
White 384 well plates	PerkinElmer	cat# 6007688
1 mL deep 96 well plate	USA Scientific	cat# 1896-1110
2 mL deep 96 well plate	USA Scientific	cat# 1896-2110
Breathe Easy Film	USA Scientific	cat# 9123-6100
EHS-derived matrix	DeRose et al.,[2] Guillen et al.[1]	N/A
Tissue-Tek biopsy cryomolds 10 × 10 × 5 mm	Sakura Finetek USA	cat# 4565
8-well chamber slides	MatTek	cat# CCS-8
Trubond 380 adhesive Cytospin slides	IHC World	cat# IW-T380
Shandon Cytospin filter cards	Thermo Fisher	cat# 5991022
Histology cassettes	Simport	cat# M505-6
Biopsy pads, 30.2 × 25.4 × 2 mm	Source Medical Products	cat# S.302523
Razor blades, steal	OEM tools	cat# 25181
EVOS Microscope	Thermo Fisher	cat# AMEX1000
Gentle MACS dissociator with octoheaters	Miltenyi Biotec	cat# 130-096-427
Qubit 3.0 Fluorometer	Thermo Fisher	cat# Q33216
ViaFlo 96 Base pipetting unit	Integra Bioscience	cat# 6001
96 channel ViaFlo pipetting head 10–300 μL	Integra Bioscience	cat# 6103D
Spring loaded plate holder B, slide function	Integra Bioscience	cat# 6220
Standard plate holder, 96 well plate	Integra Bioscience	cat# 6205
300 mL reagent reservoir	Integra Bioscience	cat# 6328
150 mL reagent reservoir	Integra Bioscience	cat# 6318
E1 electronic repeater pipette (10–300 μL)	Thermo Fisher	cat# 14-3879-60BT
Pipettes (P1000, P200, P20)	Eppendorf	cat# EPPR4396
Hemocytometer	Fisher Scientific	cat# 02-671-51B
Mr. Frosty Freezing Container	Thermo Fisher	cat# SB015350500
Countess II automated cell counter	Thermo Fisher	cat# AMQAX1000
Centrifuge Sorvall ST8	Thermo Scientific	cat# 75007200

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Centrifuge Sorvall ST8 swing rotor	Thermo Scientific	cat# 75005701
Centrifuge 5810R	Eppendorf	cat# 5811000015
Centrifuge 5810R A-4-62 swing rotor	Eppendorf	cat# 022638041
VapoProtect Thermal Cycler Mastercycler Pro	Eppendorf	cat# 950030010
Shandon Cytospin 4, with plastic funnels	Thermo Fisher	cat# A78300101
Thermomixer Compact 5350	Eppendorf	cat# EPTMC50
Tissue Processor Leica TP 1020	Leica	TP1020
Tissue Embedding Station Tissue-tek	Sakura	TeK 5
Microtome RM2155	Leica	cat# 8226-30-1004
CFX384 Real-Time System C-1000 touch	Bio-Rad	cat# CFX384
BD FACS Aria 4 Laser	BD Biosicience	405/488/563/635 nm
ORBI-shaker MP microplate shaker	Benchmark	cat# BT1502
EnVision XCite plate reader, ultra-sensitive luminescence module addition	PerkinElmer	cat# 2105-0020

⚠ CRITICAL: Note that we have not tested components from alternative vendors.

⚠ CRITICAL: CellTiter-Glo 3D (CTG-3D) enhanced lysis component in 3D formulation is essential for generating robust and consistent luminescence signal from Matrigel containing 3D cultures.

MATERIALS AND EQUIPMENT

Note: All of our volumes for PDxO passaging are calculated for one 6-well dome.

Reagent reconstitution:			
Reagent	Solvent	Stock concentration	Storage
Y-27632	Sterile nano-pure water	10 mM	−80°C
Hydrocortisone	Ethanol, 200 proof	1 mg/mL	−20°C
Human EGF	Sterile nano-pure water	100 µg/mL	−20°C
FGF2	DMEM/F12, phenol red free	100 µg/mL	−20°C
NAC	Sterile nano-pure water	500 mM	−20°C
Heregulin-β1	Sterile nano-pure water	50 µM	−80°C
Dispase	HBSS, with Ca/Mg	50 U/mL	4°C
Optional:			
β-Estradiol (E2)	Ethanol, 200 proof	10 µM	−20°C

⚠ CRITICAL: Reconstituted dispase solution needs to be passed through 0.22 µm filters.

⚠ CRITICAL: Avoid freeze/thaw cycles of reconstituted supplements. Once thawed, store at 4°C for up to 1 week.

⚠ CRITICAL: Note that we have not tested components from alternative vendors.

Note: β-Estradiol (E2) in an optional reagent that can be used for estrogen studies.

TAC buffer

Mix 1× part of 170 mM Tris, pH 7.4 solution with 9× parts of 150 mM NH_4Cl, pH 7.4.

⚠ CRITICAL: Buffer should be sterile filtered (0.22 μm), and can be stored at 20°C for up to 6 months.

PDxO base media		
Reagent	Final concentration	Amount
Advanced DMEM/F12	N/A	500 mL
FBS	5% (v/v)	25 mL
HEPES	10 μM	5 mL
GlutaMAX	1×	5 mL
Hydrocortisone	1 μg/mL	500 μL
Gentamicin	50 μg/mL	500 μL
Human EGF	10 ng/mL	50 μL
Total	**N/A**	**536 mL**

⚠ CRITICAL: Once prepared, media can be stored for up to 2 weeks at 4°C.

Phenol red free PDxO base media option

For investigators interested in studying estrogen receptor (ER) signaling, we recommend the use of a phenol red free, charcoal-stripped serum option for the PDxO base medium. Advanced DMEM/F12 is currently not available for purchase without phenol red, but can readily be built from standard phenol red free DMEM/F12 medium. To prepare phenol red free PDxO base medium add the following to standard phenol red free DMEM/F12 medium: 5 mL ITS-X, 2.5 mL sodium pyruvate, 1 mL Albumax II, 619 mg sodium bicarbonate (NaHCO3), 1.25 g ascorbic acid phosphate, and 0.5 g glutathione, monosodium. Sterile filter (0.22 μm) and store at 4°C. Use as you would standard Advanced DMEM/F12, when phenol red free medium is required. To saturate ER, β-Estradiol (E2) can be added to the PDxO media at a concentration of 10 μM.

PDxO complete media specific for BC subtype					
Supplement	Final conc/ dilution	TNBC	ER+	HER2+	ER+/HER2+
Y-27632	10 μM / 1:1000	yes	yes	yes	yes
FGF2	100 ng/mL / 1:1000	no	yes	no	yes
NAC	1 mM / 1:500	no	yes	no	yes
Heregulin-β1	10 nM / 1:5000	no	no	yes	yes

⚠ CRITICAL: Subtype-specific supplements should be added fresh immediately before media is added to the PDxO culture.

Dispase solution

For one 6-well dome, mix 800 μL reconstituted dispase with 200 μL FBS and 1 μL Y-27632.

⚠ CRITICAL: Dispase solution is prepared freshly immediately before usage.

PDxO freeze media:		
Reagent	Final concentration	Amount
PDxO Base Media	70% (v/v)	0.7 μL
FBS	20% (v/v)	0.2 μL
DMSO	10% (v/v)	0.1 μL
Y-27632	10 μM / 1:1000	1 μL
Total	**N/A**	**1 mL**

⚠ CRITICAL: PDxO freeze media is always prepared fresh and Y-27632 is added immediately before media is used.

FACS buffer
Add 5% FBS to 95% HBSS.

⚠ CRITICAL: FACS buffer is always prepared freshly.

RLT lysis buffer
Add 1% 2-Mercaptoethanol to RLT Plus Buffer.

⚠ CRITICAL: RLT lysis buffer is prepared freshly prior to usage.

MCD buffer
Add 0.5% BSA to PBS pH 7.4.

⚠ CRITICAL: MCD buffer is always prepared freshly.

Histology fixing solution
Add 4% PFA to PBS pH 7.4.

⚠ CRITICAL: Histology fixing solution can be stored at 4°C for up to 1 week.

Cytospin buffer
Add 2% BSA to PBS pH 7.4.

⚠ CRITICAL: Cytospin Buffer is always prepared freshly.

Cytospin wash buffer
Add 0.01% Tween 20 to PBS pH 7.4.

⚠ CRITICAL: Cytospin wash buffer is always prepared freshly

If permeabilization buffer
Add 0.5% Triton X-100 to PBS pH 7.4.

⚠ CRITICAL: Cytospin Buffer is always prepared freshly.

Sodium tetraborate buffer
Add 0.01% Tween 20 and 1 mg/mL $NaBH_4$ to PBS pH 7.4.

⚠ CRITICAL: Sodium Tetraborate Buffer needs to be prepared fresh immediately prior to usage and kept on ice.

STEP-BY-STEP METHOD DETAILS

Part I

Preparation of PDxOs from PDX tissue

Timing: 3–4 h

To establish PDxO lines from PDX, tumors are ideally harvested before they become overly necrotic. Tumor removal should be performed under aseptical conditions as described in Guillen et al.[1] and DeRose et al.[2] Established breast cancer PDX HCI lines can also be obtained from the Preclinical

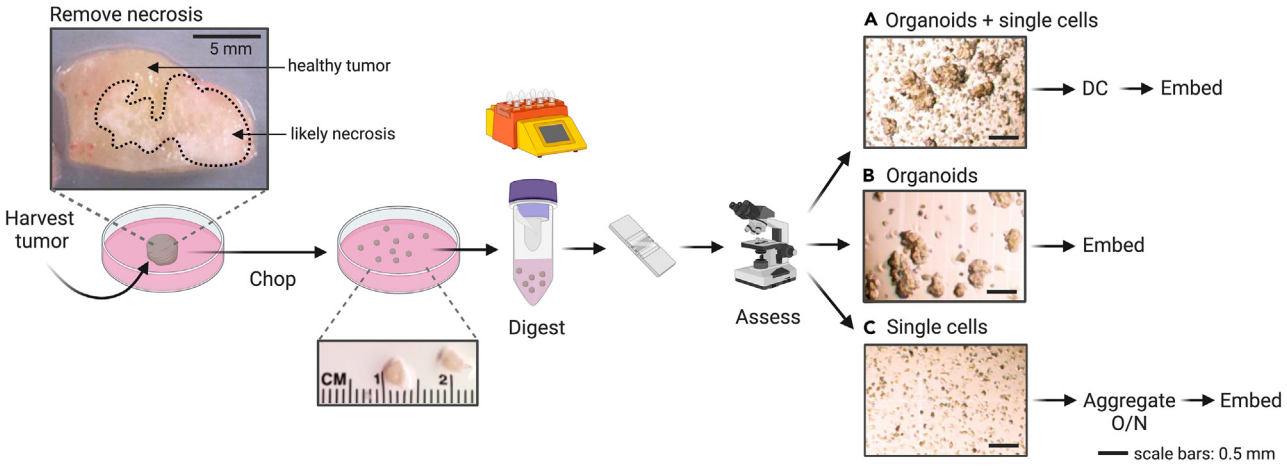

Figure 1. PDxO preparation from PDX tumor tissue

(A–C) To establish PDxOs from PDX tumors, necrotic tissue areas are removed and healthy tumor tissue is cut into 3 × 3 mm fragments. Tumor fragments are then transferred into a C tube and digested in a GentleMACS Dissociator using human dissociation enzymes. The digested material is assessed under the microscope, and processed depending on the contents and structure: (A) If the digested material contains a mixture of organoids and single cells, differential centrifugation (DC) is performed to remove single cells, then the organoids are embedded into a Matrigel dome. (B) If the digested material contains only organoids, the material is embedded immediately. (C) For digestions containing only single cells, aggregation for 16 h on Ultra Low Attachment (ULA) plates is recommended prior to embedding. Scale bars represents 5 mm (left) or 500 μm (right).

Research Resources (PRR) core at the Huntsman Cancer Institute, University of Utah, also refer to "before you begin" section.

Note: We culture organoids on 6-well tissue culture plates, with only one PDxO line per plate to avoid cross-contamination; all volumes in this protocol are provided for 6-well plates.

Note: In rare cases where more tissue input is required (>100 mg of PDX tissue), our previously published tissue digestion protocol may be utilized to prepare PDxOs for culture.[2] However, the GentleMACS tissue digestion method detailed here generally results in higher viability and better outgrowth.

1. Harvest PDX tumor under aseptic conditions.
   a. Add 5 mL of warm advanced DMEM/F12 media to 10 cm tissue culture plate.
   b. Transfer PDX tumor tissue to plate (Figure 1).
2. Place the plate on ice and keep cold for all following steps.
   a. To keep the dish from moving on the ice, a paper towel can be placed directly onto the ice underneath the dish.
3. Remove necrotic area from tumor:
   a. Using two scapels, cut the tumor in half and look for white (= necrotic) areas.
      i. Use the example in Figure 1 as a reference.
   b. Carefully remove the white areas from the tissue and discard.

Note: Necrotic tissue can be distinguished from the healthy tumor area by color and in some cases by texture. Necrotic areas in breast cancer PDX are white compared to the beige/yellow color of healthy tumor tissue. Necrotic tissue can present as softer areas, however we have also experienced examples with comparable stiffness between necrotic and healthy areas.

⚠ CRITICAL: Removing parts of the tissue that are likely necrotic increases yield and the probability of successful organoid outgrowth.

4. Chop PDX tumor into 3 × 3 mm fragments in the dish using scapels.
5. Add 5 mL warm advanced DMEM/F12 medium to GentleMACS C-tube.
6. Add reconstituted Gentle MACS human tumor dissociation enzymes to C-tube:
   a. 200 µL Enzyme H.
   b. 100 µL Enzyme R.
   c. 25 µL Enzyme A.
   d. Add 5 µL Y-27632 (10 nM working concentration).
7. Using a cell lifter, transfer 10 PDX tumor fragments to C-tube without transfering the media.

   Note: The protocol works for both fresh and frozen tumor fragments.

   Note: If cryopreserved PDX tissue is used, thaw and wash tumor fragments: Thaw the vial of tumor fragments in a 37°C water bath. Transfer fragments to 10-cm tissue culture plate. Aspirate freezing media and wash once with 5 mL advanced DMEM/F12, then aspirate medium and transfer fragments to C-tube.

   Note: Freeze remaining PDX tissue as 3 × 3 mm fragments following Guillen et al.,[1] DeRose et al.[2]

   ⚠ CRITICAL: Processing multiple fragments from diverse regions of a PDX tumor helps eliminate bias towards clonal populations that are potentially present regionally within a PDX tumor.

8. Close C-tube cap tightly.
9. To dissociate tissue, place C-tube onto GentleMACS Dissociator according to manufacturer's instructions.
   a. Place octoheaters over C-tube.
   b. Run GentleMACS program "37C_h_TDK_3".

   ⏸ **Pause point:** GentleMACS program 37C_h_TDK_3 will run for 1 h 10 min.

10. Remove C-tube from GentleMACS.
11. Centrifuge (3 min, 300 × *g*, 20°C) to pellet digested tissue. Carefully aspirate supernatant.

    ⚠ CRITICAL: Be careful not to aspirate the pellet. Organoid pellets have a looser pack than a cell pellet. We recommend to aspirate the supernatant by adding a p200 tip (without filter) to the tip of the aspirating pipet. This creates a smaller opening through which the supernatant will be aspirated, resulting in slower aspirating speed and allowing for more precise handling.

    Optional: If the pellet contains large amounts of red blood cells (the pellet will be visibly red by eye), treat digested material with TAC buffer to lyse red blood cells. Resuspend pellet in 10 mL TAC buffer. Incubate for 10 min in 37°C water bath. Shake gently every 2–3 min. Centrifuge (3 min, 300 × *g*, 20°C). Once is generally sufficient but repeat if red blood cells are still present.

    ⚠ CRITICAL: TAC buffer can be stored at 20°C for up to 6 months.

12. Resuspend pellet in 5 mL warm PDxO base medium.
13. Using a 20 µL pipette, place a 5 µL drop of digested material on hemocytometer without cover glass.
14. Evaluate contents of digested material under microscope.
15. Evaluate organoid size, health, and presence of single cells and debris. Troubleshooting 1.

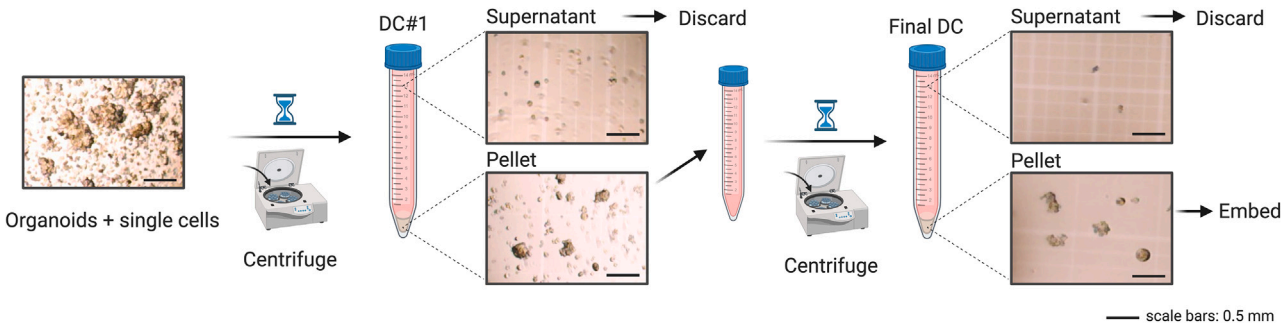

Figure 2. Differential centrifugation of PDxO preparation that contain single cells and organoids

For organoid preparations that contain organoids DC is performed to separate organoids from single cells. The organoid preparation is resuspended in 15 mL of media and a series of quick spins is performed, started with a 1 min centrifugation. The resulting supernatant is discarded, while the pellet is resuspended and spun again at s shorter time (40 s, then 30 s). After each spin, the material in the supernatant and the resuspended pellet is assessed under the microscope to determine if more DC steps are necessary. Scale bar represents 500 μm.

⚠ CRITICAL: Never use a p10 pipette when pipetting organoids. The small bore of the tips can damage organoid structure, or can exclude larger organoids from being pipetted.

16. To remove larger debris and fat, strain through a 200–500 μm cell strainer depending on organoid and debris size.
17. Wash C-tube and strainer with 10 mL PDxO medium.
18. Transfer flowthrough into a 15 mL conical tube.
19. Centrifuge (3 min, 300 × *g*, 20°C).
20. Resuspend pellet in 5 mL warm PDxO base medium.
21. Repeat, lowering the pore size of the strainer each time, until most debris and fat has been eliminated.

⚠ CRITICAL: To avoid losing organoids while straining out debris, never use a strainer with a pore size < 200 μm. Instead, repeated strains at 200–300 μm should be used if substantial debris is still present.

Note: Contents and structure of digested material can vary and will determine the next steps.

22. Place a 5 μL drop of digested material on the hemocytometer without cover glass and evaluate contents under the microscope. Troubleshooting 2.
23. Determine which option (a-c, Figure 1) best describes the digested material and proceed accordingly:
    a. Organoids + single cells: Proceed carefully with differential centrifugation (DC) of digested material prior to embedding (**proceed with step 24**) (Figure 2).
    b. Only organoids: Proceed to counting and embedding (**proceed with step 25**) (Figure 3).
    c. Only single cells: Aggregate on ultra-low adhesion plate for 16 h. Embed the following day (**proceed with step 27**)

Note: If < 50,000 cells are obtained in the pellet fraction after DC, it is recommended to collect and spin down all single cells after DC and follow **step 27** to incubate on a ultra low attachment (ULA) plate for 16 h and embed the next day. In this case it is also recommended to embed the cells in a scaled down "mini-dome" in a 12-well plate using 100 μL of Matrigel:Cell mixture and a 25 μL Matrigel base layer.

Note: Recommended viability of the digested material to move forward with the protocol are between 80-100%. For viability below 80% refer to troubleshooting section.

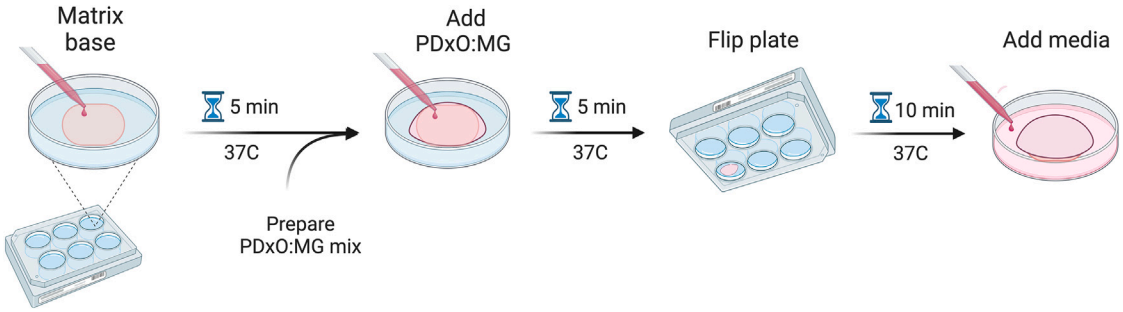

Figure 3. Embedding PDxOs in 3D Matrigel domes

To embed PDxOs, a 50 μL Matrigel drop is placed into a well of a 6-well plate. Using a pipet tip the Matrigel is immediately spread out to form a cicular base layer. During 5 min of incubation of the plate at 37°C the PDxO:Matrigel mix is prepared. 200 μL of the mixture is added onto the baselayer, and incubated for 5 min at 37°C. The plate is then flipped upside down and placed back into the incubator for 10 min. After that, subtype-specific media is added to the well.

⚠ CRITICAL: To generate best possible starting material from PDxO cultures, it is beneficial to eliminate single cells present in digested material containing organoids and single cells. While single cells can be tumor cells our experience shows that they are generally mouse stromal-like cells that can contaminate PDxO cultures.

24. To eliminate single cells from digested material, perform a series of quick spins (differential centrifugation, DC) to separate single cells from tumor cell aggregates/organoids (Figure 2).

Note: Not all DC steps are always necessary

⚠ CRITICAL: Stop DC if single cells in pellet fraction are < 5% or organoids begin to appear in the supernatant fraction. Monitor supernatant and pellet for organoid content and structure throughout the DC process.

a. Gently resuspend the pellet in 15 mL of warm PDxO base medium in 15 mL conical tube.
b. Centrifuge at 300 × *g* for 1 min.
c. Transfer supernatant to another 15 mL conical tube.
d. Resuspend pellet in 5 mL of warm PDxO base medium.
e. Evaluate resuspended pellet and supernatant under microscope.
f. Fill up conical tube that contains resuspended pellet to 15 mL of warm PDxO base medium. Centrifuge at 300 × *g* for 50 s.
g. Transfer supernatant to another tube.
h. Resuspend pellet in 5 mL of warm PDxO base medium.
i. Evaluate resuspended pellet and supernatant under microscope.
j. Fill up conical tube that contains resuspended pellet to 15 mL of warm PDxO base medium.
k. Centrifuge at 300 × *g* for 40 s.
l. Transfer supernatant to another tube.
m. Resuspend pellet in 5 mL of warm PDxO base medium. Evaluate resuspended pellet and supernatant under microscope.
n. Fill up conical tube that contains resuspended pellet to 15 mL of warm PDxO base medium.
o. Centrifuge at 300 × *g* for 30 s.
p. Transfer supernatant to another tube.
q. Resuspend pellet in 5 mL of warm PDxO base medium.
r. Evaluate resuspended pellet and supernatant under microscope.
s. Fill up conical tube that contains resuspended pellet to 15 mL of warm PDxO base medium. Centrifuge at 300 × *g* again for 30 s.
t. Transfer supernatant to another tube.

u. Resuspend pellet in 5 mL of warm PDxO base medium.
v. Evaluate resuspended pellet and supernatant under microscope.
w. If organoid fraction is clean, proceed to the next step. If not, repeat centrifugation at 300 × *g* for 30 s, continuing to monitor the pellet and supernatant compositions until a clean organoid fraction is achieved.

⚠ CRITICAL: Perform DC in 15 mL conical tubes. The steeper focus point of the conical helps to focus cells into a smaller pellet (vs. in a 50 mL conical) and avoids loss during aspiration steps.

⚠ CRITICAL: Resuspend carefully and monitor all differential centrifugation steps under a microscope. Evaluate the resuspended pellet and supernatant after each differential centrifugation step. If organoids start to break down into single cells, stop differential centrifugation and embed all remaining material.

25. Count organoids.
    a. Drop 5 μL of digested material onto hemocytometer without cover glass.
    b. In order to flatten out the drop and to increase visibility under the microscope, flick the hemocytometer gently with your finger.
    c. Using a microscope, count all organoids in now spread out 5 μL drop.
    d. To calculate the total number of organoids, multiply organoid count by 200 and by total volume (mL).
        i. For example: If organoids are resuspended in 10 mL, multiply by 2,000 to calculate total organoid number.

⚠ CRITICAL: Due to their large size, organoids cannot be loaded onto a hemocytometer by standard methods as they will clog under the cover glass and result in inaccurate counts. In our hands, counting slides for automated cell counters also clog and lead to inaccurate organoid counts.

Note: Freshly prepared PDxOs can be cryopreserved (option A) or embedded into Matrigel for culture following preparation from PDX. Immediate embedding yields highest viability and quickest outgrowth.

Optional: *Cryopreserve PDxOs immediately following preparation from PDX.*

    ii. *Pellet organoids by centrifugation (300 × g, 3 min, 20°C). Aspirate supernatant.*
    iii. *Resuspend in PDxO cryopreservation medium. Aim for 5,000-10,000 organoids/vial or 0.5–1.0 × 10^6 cells/vial.*
    iv. *Transfer to cryo tubes. Load vials into Mr. Frosty and place at -80 °C. Transfer to LN_2 after 24 h for long term storage.*

26. Embedding organoids into 3D Matrigel domes for culture (Figure 3).
    a. In a 6-well plate, coat the center of each well with 50 μL Matrigel base layer: paint Matrigel in a round base layer without touching the sides of the tissue culture plate.
        i. This is best achieved by pipetting the Matrigel onto the plate and using the pipette tip to drag the Matrigel.
        ii. Aim for a base layer that is roughly the size of a nickel.
    b. Allow base layer to polymerize at 37°C for 5 min.

⚠ CRITICAL: Longer incubation times will cause Matrigel base layers to shrink and potentially dry out, which can cause lifting of the PDxO:Matrigel dome off the plate.

    c. Resuspend 0.5–2 x 10^4 organoids per number of desired wells in 200 μL Matrigel/well on ice.

i. Matrigel pipetting loss is substantial; calculate for 15%–20% more than needed

d. Pipette gently 5-10 times to establish good distribution without destroying organoids and without introducing air bubbles.

e. Carefully add 200 μL organoids:matrigel mixture onto each base layer.
   i. Careful handling of the plate is required to not disturb the dome.

f. Polymerize Matrigel at 37°C for 15 min total, flipping the plate upside down on its lid after 5 min.

⚠ CRITICAL: Flipping the plate helps the PDxOs settle into the top of the Matrigel dome, helping to prevent PDxOs from coming into contact with plastic, since they remodel the Matrigel during their growth period.

g. After the indicated 15 min, flip the plate back over and add 5 mL fresh complete PDxO medium (specific to the breast cancer subtype of the tumor) to each well.

h. Pipette media onto the side of well.
   i. Do not pipette directly on top of the Matrigel dome as it might result in loss of its structural integrity.

i. Put the lid back on the plate and place plate in incubator.

j. Replace the complete PDxO medium every 3–4 days.

k. To replace the media, tilt the plate to avoid damaging the matrigel:organoid dome, and carefully aspirate medium.

l. Add 5 mL fresh complete PDxO medium specific to the breast cancer subtype of the PDxO.

m. Standard culture conditions for PDxOs from this point on are 37°C and 5% CO_2.

⚠ CRITICAL: PDxOs are more sensitive to acidic medium conditions than standard 2D cell cultures. Always replace medium prior to significant yellowing of the medium.

27. In cases where only single cells are present after gMACS disassociation, aggregation for 16 h on an ultra-low attachment (ULA) plate is recommended prior to embedding into Matrigel domes.
    a. Pellet cells by centrifugation (300 × *g*, 3 min, 20°C).
    b. Aspirate supernatant.
    c. Resuspend in PDxO medium with breast cancer subtype-specific supplements added.
       i. Aim for 500,000–2,000,000 cells per each well of a 6 well plate.
    d. Carefully pipet cells into a well of a ULA 6-well plate.
    e. Place in the incubator for 16–24 h. Cell aggregates are visible in the center of the well (Figure 4).
    f. Carefully pipet the medium and the cell aggregates and transfer into a 15 mL conical tube.
    g. Pellet cells by centrifugation (300 × *g*, 3 min, 20°C).
    h. Aspirate supernatant and embed the pellet into a Matrigel dome (**proceed with step 26**).

Pause point: Once the pellet is embedded, change culture media every 3-4 days and allow PDxO wo grow.

Note: We typically embed into one dome but if culture is significantly denser than the example depicted in Figure 4 we recommend embedding in multiple domes.

Alternative preparation of PDxOs using MDM (mechanical disassociation method)

Timing: 1–1.5 h

The leftover media where PDX tumor material is chopped into fragments can contain a significant amount of tumor cells. When PDX tissue is very limiting and all needs to be used for another

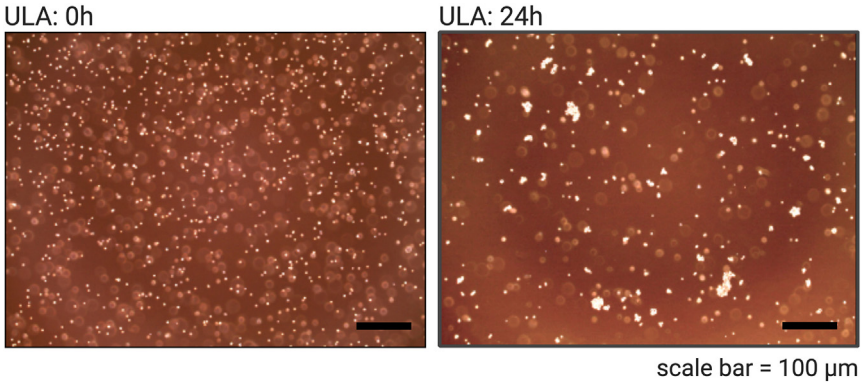

Figure 4. Aggregation of single cells in ULA plate

To aggregate, cells are plated into a well of a 6-well ULA plate and incubated for 16 h. Images show pre (left)- and post (right) 16 h incubation. Scale bars represents 100 μm.

application (e.g., retransplanted into mice), an alternative preparation method can be used to still obtain organoids:

28. To generate PDxOs using the MDM method, save the leftover media that was used to chop PDX tumor fragments at step 4.
29. Transfer to 15 mL conical tube.
30. Centrifuge (5 min, 300 × *g*, 20°C) to pellet cells.
31. Follow above protocol starting at step 22.

Optional: MDM cultures can be aggregated for 16 h on ULA plates and embedded the next day following **step 27***. In our hands, ULA incubations can increase the survival of cells in stress situations but both methods have generated healthy long-term PDxO cultures.*

⚠ CRITICAL: PDxO cultures started with the MDM method usually contain single tumor cells or very small clusters, and larger debris, in which case DC is often not recommended. Most cultures can be started by straining followed by immediate embedding.

Maintaining and passaging PDxOs

Timing: 1–1.5 h

Timing: 30 min–1 h (for step 33)

Timing: 30 min–1 h (for step 34)

Once embedded, PDxO cultures will be ready for a first split from passage 0 (P0) to P1 approximately 7–21 days after initial embedding. Troubleshooting 3. Triple negative breast cancer (TNBC) PDxOs generally, but not always, grow faster than other breast cancer subtypes. Prior to splitting, always evaluate organoid health under the microscope. Troubleshooting 4, Troubleshooting 5.

Note: To help PDxOs adapt to culture, we found that the first split is most successful when we divide the process into two phases: dispase to digest Matrigel and eliminate PDxO-Matrigel interactions (**step 32**), followed by dissociation of organoids into single cells using TrypLE Express (**step 33**).

Note: Examples of mature PDxO cultures are shown in (Figure 5) and Guillen et al.[1]

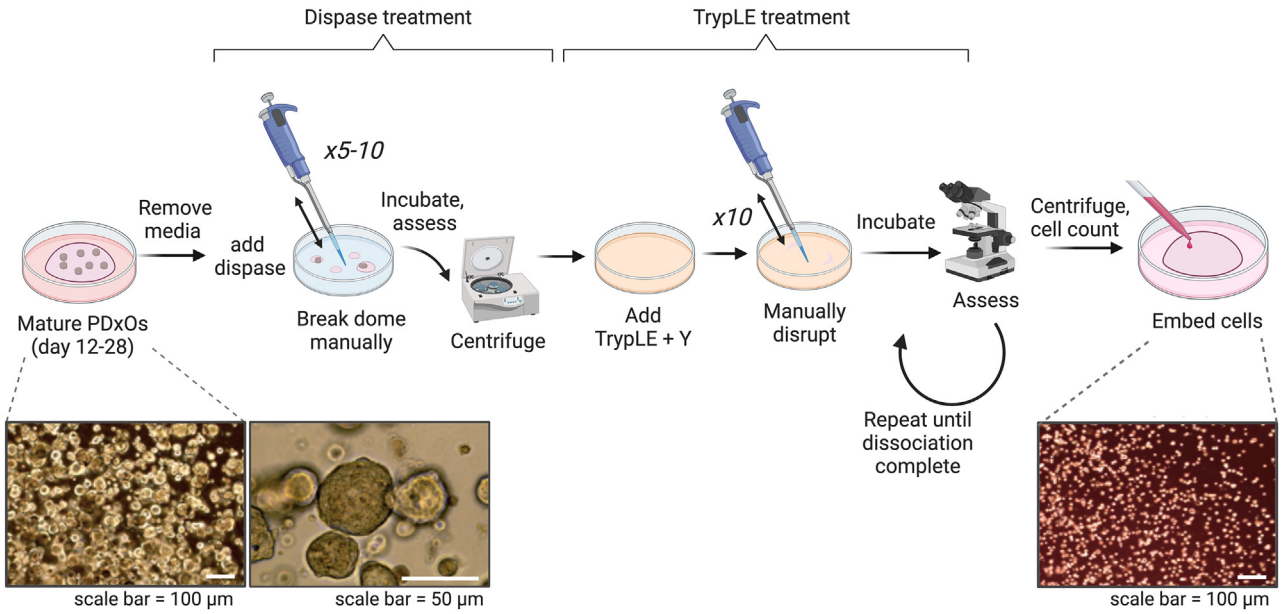

Figure 5. Passaging PDxOs after they are adapted to culture using dispase and TrypLE steps (>P2)

Once mature, media is removed from PDxOs and dispase solution is added. Matrigel domes containing PDxOs are broken up manually, incubated at 37°C, then washed with media and centrifuged. The organoid pellet is resuspended in TrypLE to disassociate PDxOs into single cells. Cells are assessed under the microscope, counted and embedded in Matrigel. Scale bar represents 100 µm or 50 µm as indicated.

⚠ CRITICAL: Splitting new PDxO cultures prior to day 10 typically results in very poor viability post-dissociation. Giving organoids time to adapt to culture prior to the first split is critical to success.

⚠ CRITICAL: We always use a dispase step for all first splits to help ease the stress of culture adaptation, but this step can generally be eliminated for hardy PDxO lines in subsequent splits.

32. Dispase digestion of Matrigel (Figure 5).
    a. Check PDxO health, density, and phenotype under the microscope.
    b. Aspirate medium.
    c. Add 1 mL freshly-made dispase solution to each well.
    d. Tilt plate ane use a cell scraper to scrape the dome into the dispase solution
    e. Carefully pipette (p1000) 5–10 times to break up Matrigel into smaller fragments.
    f. Incubate 1 h at 37°C.

    ⏸ **Pause point:** During the 1h incubation time, Matrigel will be dissolved without causing organoids to dissasociate. TrypLE can be warmed up in the waterbath during this step.

    g. Check Matrigel digestion using a microscope.
        i. Digestion can be stopped prior to 1 h if no non-digested Matrigel is visible and organoids are in suspension.
        ii. Non-digested Matrigel will look like glass fragments.
    h. Transfer to 15 mL conical tube.
    i. Fill tube up to 15 mL with PDxO base medium.
    j. Centrifuge (300 × *g*, 3 min, 20°C) to pellet organoids.
    k. Aspirate supernatant.

⚠ CRITICAL: Some grape-like/loosely associated PDxO lines (refer to phenotype/morphology summary in Figure 9) tend to fall apart into single cells during this step, which interferes with subsequent DC steps. Check the PDxO phenotype every 20 min during the 1 h dispase incubation step and move to the next step if PDxOs start to fall apart.

33. Dissociation of organoids.
    a. Resuspend organoid pellet in 2 mL TrypeLE + 2 μL Y-27632.
    b. Transfer to 1 well of a new 6-well tissue culture plate.
    c. Incubate at 37°C for 3–15 min, until organoids are just dissociated.
        i. Every 3–5 min, pipette (p1000) aggressively 15–20 times and check organoid dissociation progress with a microscope.
    d. As soon as organoids are dissociated, pipet up and down to mix and remove 10 μL to count the cells using an automated counter (Countess II) or hemocytometer.

⚠ CRITICAL: Dilute out the TrypLE as soon as organoids are sufficiently dissociated. Leaving organoids in TrypLE post-dissociation can drastically decrease viability.

    e. Transfer dissociated organoids into a 15 mL conical tube.
    f. Wash the well with PDxO base medium to increase cell recovery.
    g. Fill the conical tube up to 15 mL with PDxO base medium.
    h. Centrifuge at 300 × *g* for 3 min to pellet cells.
    i. Embed the dissociated cells into new Matrigel domes following step 26.
    j. Seeding density should be adjusted for the growth rate of each PDxO line:
    Fast growing lines: 0.2–0.25 × 10^6 cells/well, split every 11–14 days.
    Moderate growing lines: 0.25–0.35 × 10^6 cells/cell, split every 15–21 days.
    Slow growing lines: 0.35-.4 × 10^6 cells/cell, split every 22–31 days.
    k. To calculate PDxO culture doubling time, we use the following equation:

$$(LOG(2) * Time)/(LOG(Cell2) - LOG(Cell1))$$

where Time is defined as the number of days in culture between passaging; $Cell_1$ is the seeding density at which the dome was plated; and $Cell_2$ is the cell count at the day of passaging.

⚠ CRITICAL: After the dispase step, be sure to perform at least two washing steps with PDxO media, otherwise the domes containing newly embedded organoids are not stable.

Note: Most PDxO lines are fairly easy to dissociate in later passages and will not continue to require splits with dispase after the first couple of passages (P0 to P2). Subsequent splits without dispase are quicker to execute (Figure 5).

34. Passaging without dispase.
    a. Check organoid health, density, and phenotype using the microscope.
    b. Tilt plate towards you and aspirate medium.
    c. Add 2 mL TrypLE and 2 μL Y-27632 onto the dome.
    d. Using a cell scraper, lift the dome off the plate so that it floats in the TrypLE solution.
    e. Use a p1000 pipette to manually break up the dome by pipetting up and down 5–10 times.
    f. Proceed with step 33c.

⚠ CRITICAL: A few PDxO lines will experience significant decrease in viability post-split if the dispase step is eliminated. The specific needs of each PDxO line are best determined on an individual basis.

Continue to change medium every 3–4 days and split PDxOs every 14–21 days, until PDxOs become established and adapted to culture. The timing can vary but this typically takes around 30–60 days of

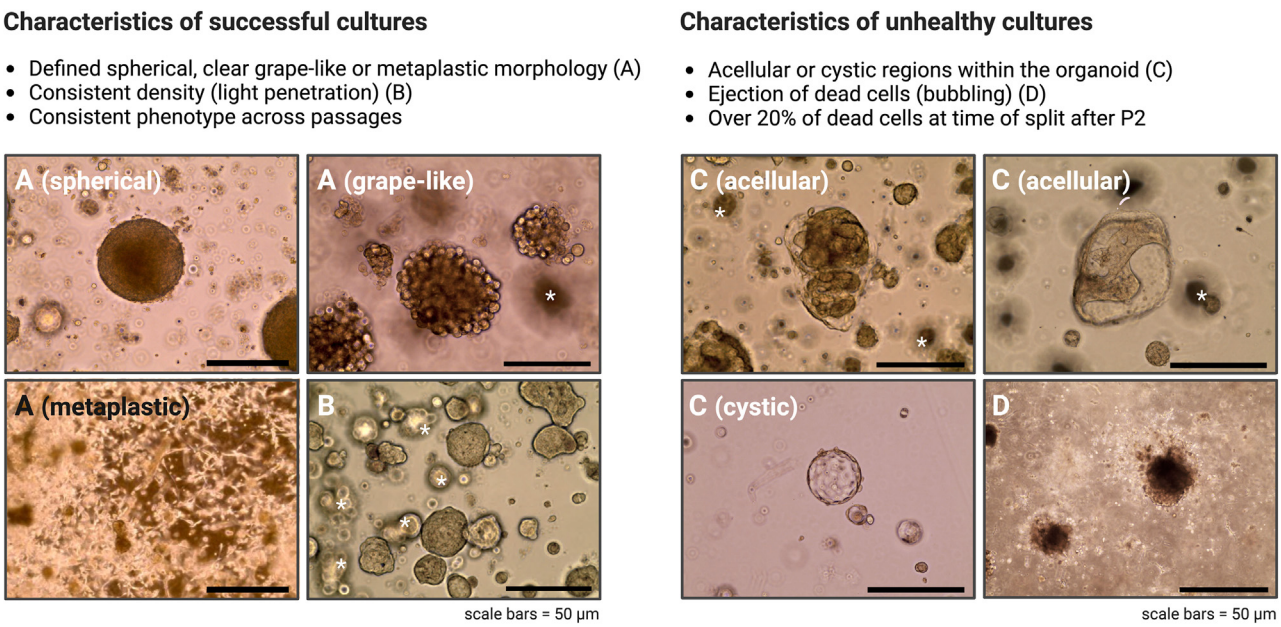

Figure 6. Diverse morphology of PDxOs with examples of successful cultures and unhealthy organoids

(A–D) Successful PDxO cultures display phenotypes as shown in the panels on the left. Characteristics of unhealthy PDxO cultures are shown on the right. *Examples of PDxOs that are out of focus due to the 3D nature of the PDxO domes are marked with a white star (*). Successful cultures can display as spherical, grape-like or metaplastic phenotype, and examples of these morphologies are shown in images marked with A. Additional characteristics of healthy PDxO cultures are consisteny density within the culture as shown in B, and consistent phenotypes across passages. Unhealthy cultures usually fail to grow into PDxO lines. In these cases PDxO cultures can have acellular or cytsic morphology as shown in images marked with C, or PDxOs can eject dead cells (D). Additionally, unhealthy cultures can have low viability with 20% or more dead cells during passaging (P2 or higher). Scale bar represents 50 µm.

culture. We consider a PDxO culture established once it has been split at least 3 times (≥ P3), displays a healthy and stable phenotype (refer to Figure 6 for morphology and phenotypes), is free from mouse cells by qRT-PCR (steps 37, 38, 40), has been validated by STR (step 39) and biobanked (step 35), as described in Table 1.

Cryopreservation and thawing of PDxOs

Timing: 20 min (for step 35)

Timing: 30 min (for step 36)

35. To cryopreserve organoids.
    a. Remove media from the well and discard.
    b. Add 1 mL of freshly prepared PDxO cryopreservation medium to each well of mature organoids.

Table 1. Minimal criteria for PDxO establishment

	PDxO establishment criteria
1	Biobank (step 35)
2	No major organoid morphology changes (Figure 6)
3	Consistency in doubling time > P3
4	No or minimal mouse cells detected by qRT-PCR (CT Cutoff for mouse *Gapdh*: 32) (steps 37, 38, 40)
5	STR validation (step 39)

c. Tilt plate and use cell lifter to scrape Matrigel dome into bottom corner of the well.
d. Pipette up and down with a P1000 5-10 times to mechanically break up the dome.
e. Transfer into prelabeled cryotubes.
f. Load vials into Mr.Frosty and place at −80°C.
g. Transfer to LN_2 after 24 h for long-term storage.

Pause point: Cryopreserved PDxOs can be stored at $LN_{2\ long\text{-}term}$.

Note: PDxOs can be cryopreserved without a dispase step. When PDxOs are thawed the washing step will remove old Matrigel.

36. To thaw organoids and initiate culture:
    a. Place the cryovial into a 37°C water bath until just thawed.
    b. Transfer contents to 15 mL conical and fill up with 13 mL warm PDxO base medium.
    c. Centrifuge (300 × *g*, 3 min, 20°C).
    d. Aspirate supernatant.

⚠ CRITICAL: Leftover matrigel from cryopreserved organoids can be visible as cloudy layer on top of the PDxO pellet. Be careful not to aspirate organoids present in this layer.

    e. Repeat washing step if organoids are present in the cloudy Matrigel layer.
    f. Embed into Matrigel domes right away following step 26.

Note: The number of domes that can be seeded from one vial depends on the pellet size. Typically, one vial can be embedded into two domes after thawing.

Note: PDxOs that come out of cryo are typically ready to be passaged after 7–11 days. Passaging too early can lead to a decrease in viability.

⚠ CRITICAL: We don't recommend dissociating organoids immediately post thaw, as this results in cell death.

⚠ CRITICAL: Organoids should not be cultured in suspension immediately post thaw, as this results in substantial loss of viability prior to embedding.

Part II PDxO validation and characterization

Quality control: PDxO morphology and doubling time

PDxO lines have heterogeneous morphology and we observe defined spherical phenotypes as well as grape-like organoid phenotypes (Figure 6, left). In addition, we observe that PDxOs derived from metaplastic breast cancer grow as cultures without clearly defined organoids. However, several phenotypes point toward an unhealthy culture that in our experience will not lead to a successful establishment of PDxOs. Examples of those are shown in Figure 6, right. In cases when the majority of the culture displays these phenotypes we recommend re-starting the PDxO from the beginning.

⚠ CRITICAL: PDxO cultures can exhibit various phenotypes but PDxO health is in question if dramatic morphologic changes occur within the same PDxO line over time.

During the first three passages, it is normal to see dramatic changes in doubling time, as the organoids adjust to the culture conditions, and due to dropout of non-cancerous cells during this stage.

Table 2. Typical growth characteristics of representative PDxOs we have established

	HCI line	Patient subtype/disease	Doubling time	Passage interval	Est/failed	Reason for failure
1	HCI-001	TNBC/IDC	3–5 days	12–15 days	est	
2	HCI-002	TNBC/IDC	2–4 days	10–12 days	est	
3	HCI-003	ER+, PR+/IDC	4–7 days	15–20 days	est	
4	HCI-004	TNBC/IDC	/	/	failed	No organoids formed
5	HCI-005	ER+, PR+, HER2 history/IDC	7–10 days	20–25 days	est	
6	HCI-006	ER+, PR+, HER2 history/IDC	/	/	/	Not attempted
7	HCI-007	ER+, PR+, HER2 history/IDC	7–10 days	20–25 days	est	
8	HCI-008	HER2 history/inflammatory	4–7 days	15–20 days	est	
9	HCI-009	TNBC/IDC	/	/	failed	No organoids formed
10	HCI-010	TNBC/IDC	4–7 days	15–20 days	est	
11	HCI-011	ER+, PR+/IDC	7–10 days	20–25 days	est	
12	HCI-012	TNBC/IDC	4–7 days	15–20 days	est	
13	HCI-013	ER+, PR+/ILC	/	/	failed	No organoids formed
14	HCI-014	TNBC/ILC	/	/	failed	No organoids formed
15	HCI-015	TNBC/IDC	4–7 days	15–20 days	est	
16	HCI-015BR	TNBC/IDC	4–7 days	15–20 days	est	
17	HCI-015BGR	TNBC/IDC	3–5 days	12–15 days	est	
18	HCI-016	TNBC/IDC	4–7 days	15–20 days	est	
19	HCI-017	ER+, PR+/IDC	4–7 days	15–20 days	est	
20	HCI-018	ER+/ILC	10–15 days	25–30 days	est	
21	HCI-018BRN	ER+/ILC	10–15 days	25–30 days	est	
22	HCI-019	TNBC/IDC	7–10 days	20–25 days	est	
23	HCI-023	TNBC/IDC	7–10 days	20–25 days	est	
24	HCI-023BR	TNBC/IDC	4–7 days	15–20 days	est	
25	HCI-024	TNBC/IDC	4–7 days	15–20 days	est	
26	HCI-025	TNBC/IDC	4–7 days	15–20 days	est	
27	HCI-026	ER+/IDC	/	/	failed	No organoids formed
28	HCI-027	TNBC/IDC	3–5 days	12–15 days	est	
29	HCI-027BR	TNBC/IDC	4–7 days	15–20 days	est	
30	HCI-027BS	TNBC/IDC	7–10 days	20–25 days	est	
31	HCI-028	TNBC/IDC	7–10 days	20–25 days	est	
32	HCI-028LV	TNBC/IDC	10–15 days	25–30 days	est	
33	HCI-030	TNBC/poorly differenciated	/	/	failed	No organoids formed
34	HCI-031	TNBC/ILC	15–19 days	25–30 days	est	
35	HCI-032	ER+, PR+, HER2+/IDC	4–7 days	15–20 days	est	
36	HCI-032EI	ER+, PR+, HER2+/IDC	7–10 days	20–25 days	est	
37	HCI-033	TNBC/DCIS recurrence	/	/	failed	No organoids formed
38	HCI-034	TNBC/DCIS recurrence	/	/	failed	No organoids formed
39	HCI-036	TNBC/phyllodes	/	/	failed	No organoids formed
40	HCI-037	TNBC/DCIS+IDC	4–7 days	15–20 days	est	
41	HCI-038	TNBC/DCIS+IDC	4–7 days	15–20 days	est	
42	HCI-039	TNBC/DCIS+IDC	4–7 days	15–20 days	est	
43	HCI-040	ER+, PR+, HER2+/IDC	7–10 days	20–25 days	est	
44	HCI-040EI	ER+, PR+, HER2+/IDC	4–7 days	15–20 days	est	
45	HCI-041	TNBC/IDC, metaplastic	7–10 days	20–25 days	est	
46	HCI-042	TNBC/IDC	7–10 days	20–25 days	est	
47	HCI-043	TNBC/IDC	7–10 days	20–25 days	est	
48	HCI-044	ER+, PR+/IDC	/	/	failed	Only mouse content
49	HCI-045	TNBC/DCIS+IDC	4–7 days	15–20 days	est	
50	HCI-045VR	TNBC/DCIS+IDC	4–7 days	15–20 days	est	
51	HCI-046	TNBC/DCIS+IDC	25–30 days	30–35 days	est	
52	HCI-047	TNBC/IDC	4–7 days	15–20 days	est	
53	HCI-048	TNBC/IDC	4–7 days	15–20 days	est	
54	HCI-048CR	TNBC/IDC	7–10 days	20–25 days	est	

(*Continued on next page*)

Table 2. *Continued*

	HCI line	Patient subtype/disease	Doubling time	Passage interval	Est/failed	Reason for failure
55	**HCI-049**	ER+, PR+/IDC	/	/	failed	Only mouse content
56	**HCI-050**	TNBC/IDC	7–10 days	20–25 days	est	
57	**HCI-051**	TNBC/IDC	4–7 days	15–20 days	est	
58	**HCI-052**	TNBC/IDC	/	/	failed	No organoids formed
59	**HCI-053**	TNBC/IDC	4–7 days	15–20 days	est	
60	**HCI-053CR**	TNBC/IDC	7–10 days	20–25 days	est	
61	**HCI-054**	TNBC/IDC	10–15 days	25–30 days	est	
62	**HCI-054CR**	TNBC/IDC	7–10 days	20–25 days	est	
63	**HCI-055**	TNBC/IDC	7–10 days	20–25 days	est	
64	**HCI-056**	TNBC/IDC	6–7 days	15–20 days	est	
65	**HCI-057**	ER+, PR+, HER2+/DCIS+IDC	7–14 days	20–30 days	est	
66	**HCI-057EI**	ER+, PR+, HER2+/DCIS+IDC	10–15 days	25–30 days	est	
67	**HCI-058**	TNBC/ILC	4–5 days	15–20 days	est	
68	**HCI-059**	TNBC/IDC	/	/	failed	Only mouse content
69	**HCI-060**	TNBC/DCIS	4–7 days	15–20 days	est	
70	**HCI-061**	TNBC/IDC	/	/	failed	No organoids formed
71	**HCI-062**	TNBC/IDC	7–10 days	20–25 days	est	
72	**HCI-063**	TNBC/IDC	/	/	/	Not attempted
73	**HCI-064**	ER+, PR+, HER2+/IDC	7–10 days	20–25 days	est	
74	**HCI-065**	TNBC/IDC	7–10 days	20–25 days	est	
75	**HCI-066**	TNBC/IDC	/	/	failed	No organoids formed
76	**HCI-067**	TNBC/IDC	10–15 days	25–30 days	est	
77	**HCI-068**	TNBC/IDC	10–12 days	25–30 days	est	
78	**HCI-069**	TNBC/IDC	7–10 days	20–25 days	est	
79	**HCI-070**	TNBC/DCIS	10–18 days	25–30 days	est	
80	**HCI-071**	TNBC/metaplastic	15–20 days	25–30 days	est	
81	**HCI-072**	ER+, PR+, HER2+/IDC	4–7 days	15–20 days	est	
82	**HCI-073**	TNBC/IDC	10–15 days	25–30 days	est	
83	**HCI-074**	TNBC/IDC	10–15 days	25–30 days	est	
84	**HCI-075**	TNBC/IDC	/	/	/	Ongoing
85	**HCI-076**	HER2+/IDC	10–15 days	25–30 days	est	
86	**HCI-076LN**	HER2+/IDC	10–15 days	25–30 days	est	
87	**HCI-077**	TNBC/DCIS+IDC	7–10 days	20–25 days	est	
88	**HCI-078**	TNBC	7–10 days	20–25 days	est	
89	**HCI-085**	TNBC	7–10 days	15–20 days	est	

Abbreviations: Estrogen receptor (ER), progesterone receptor (PR), triple negative breast cancer (TNBC), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), ductal carcinoma *in situ* (DCIS), established (est).

After P3 most PDxO cultures should exhibit a stable doubling time range and predictable passage interval (Table 2).

Quality control: Nucleic acid isolation and STR analysis

Timing: 20 min (for step 37)

Timing: 45 min (for step 38)

Timing: 2 h + genetic analyzer service (for step 39)

For PDxO culture validation, we perform short tandem repeat (STR) analysis to ensure that PDxO cultures match the original patient and PDX samples. We simultaneously extract RNA and DNA from the same PDxO dome (RNA is used for RT-PCR analysis of human/mouse content and described

below). RNA and DNA extracted with this protocol can also be used for genomics analysis such as RNA and DNA sequencing.

37. PDxO culture preparation for nucleic acid extraction.
    a. Aspirate medium.
    b. Add 1 mL freshly-made dispase solution to each well.
    c. Tilt plate and use a cell scraper to scrape the dome into the dispase solution.
    d. Carefully pipette (p1000) 5-10 times to break up Matrigel into smaller fragments.
    e. Incubate 30 min - 1 h at 37°C.
    f. Digestion can be stopped prior to 1 h if no non-digested Matrigel is visible and organoids are in suspension.
    g. Transfer to 15 mL conical tube.
    h. Fill tube up to 15 mL with ice-cold non-supplemented Advanced DMEM/F12 medium.
    i. Mix by inverting the conical tube multiple times.
    j. Centrifuge (300 × *g*, 3 min, 4°C) to pellet organoids.

    ⚠ CRITICAL: There won't be a defined PDxO pellet visible after this first washing step. Most organoids will be located in a cloudy layer of about 1–2 mL at the bottom of the tube, and it is critical not to aspirate this fraction.

    k. In a separate tube, add BME to RLT Buffer Plus (1:100) and set aside.
        i. Total volume needed is 0.3 mL per sample.
    l. Aspirate supernatant from organoid pellet, making sure to leave the cloudy layer.
    m. Add 2 mL ice-cold non-supplemented Advanced DMEM/F12 onto pellet.
    n. Pipet up and down 10× with P1000.
    o. Fill up to 15 mL with ice-cold non-supplemented Advanced DMEM/F12.
    p. Mix tube an centrifuge (300 × *g*, 3 min, 4°C).

    ⚠ CRITICAL: Pellet should be visible and clearly defined. If there is still a significant cloudy layer, repeat washing for a third time.

    q. Remove supernatant.
    r. Resuspend pellet in 300 µL of RLT Plus Buffer with freshly added BME and transfer to 1.5 mL microtube.
    s. Vortex at maximum speed for 1 min and proceed with nucleic acid extractions.

    Optional: Freeze and store sample at −80°C.

    ⏸ **Pause point:** RLT lysates intended to be used for RNA/DNA extraction can be stored at −80°C for at least 12 months.

    Note: If samples were stored in −80°C, we recommend thawing for 5 min in a 37°C water bath, then vortexing on maximum speed 1 min and proceeding with **step 38**.

    ⚠ CRITICAL: Thawing in the water bath for 5–10 min is critical to prevent columns from clogging.

38. Simultaneous RNA/DNA isolation from PDxOs in RLT buffer using the Qiagen AllPrep RNA/DNA kit.
    a. Prepare 70% Ethanol (molecular grade, RNase-free).
        i. Prepare a minimum of 0.5 mL per sample.
    b. To homogenize sample, pipet RLT lysate onto QiaShredder spin column.
    c. Centrifuge (max speed, 1 min, 20°C).

d. Check column and centrifuge again if liquid is still visible on top of spin column.
e. Discard column.
f. Transfer flow-through to AllPrep DNA binding column.
g. Centrifuge (10,000 × *g*, 30 s, 20°C).
h. Place AllPrep DNA binding column in a new collection tube and set on ice.
i. Save flow-through for RNA extraction.
j. Add 1 volume of 70% RNase-free molecular grade ethanol to flow-through.
k. Immediately mix by pipetting up and down with P1000.
l. Quickly transfer 600 µL of mixture onto AllPrep RNA binding column.
m. Centrifuge (10,000 × *g*, 15 s, 20°C), discard flow-through.
n. Transfer any remaining volume of sample to same column.
o. Centrifuge (10,000 × *g*, 15 s, 20°C), discard flow-through
p. Add 350 µL RW1, centrifuge (10,000 × *g*, 15 s, 20°C), discard flow-through.
q. In a separate 1.5 mL tube mix 10 µL DNase I stock solution to 70 µL Buffer RDD and gently mix by pipetting (do not vortex!).

⚠ CRITICAL: It is critical not to vortex DNase as this can impair its enzyme activity.

r. Add 80 µL of DNase incubation mix directly onto the AllPrep RNA binding column.
s. Incubate for 15 min at 20°C.
t. Add 350 µL RW1, centrifuge (10,000 × *g*, 15 s, 20°C), discard flow-through.
u. Add 500 µL RPE, centrifuge (10,000 × *g*, 15 s, 20°C), discard flow-through.
v. Add 500 µL RPE, centrifuge (10,000 × *g*, 2 min, 20°C), discard flow-through.
w. Place column in new collection tube and dry spin (max speed, 1 min, 20°C).
x. Place column in 1.5 mL microtube.
y. Add 30 µL of water (RNase-free) directly onto column.
z. Elute RNA by centrifugation (10,000 × *g*, 1 min, 20°C).
    aa. Place on ice, quantify RNA concentration using Qubit BR RNA Assay.
    bb. Remove DNA binding column from ice.
    cc. Add 500 µL AW1, centrifuge (10,000 × *g*, 15 s, 20°C), discard flow-through.
    dd. Add 500 µL AW2, centrifuge (max speed, 2 min, 20°C), discard flow-through.
    ee. Place column in 1.5 mL microtube.
    ff. Add 100 µL of EB Buffer directly onto column and incubate for 1 min, 20°C.
    gg. Elute DNA by centrifugation (10,000 × *g*, 1 min, 20°C).
    hh. Place on ice, quantify DNA concentration using Qubit BR dsDNA Assay Trouble-shooting 9.
ii. RNA and DNA are stored at −80°C.

Pause point: Nucleic acids are stable at −80°C for a minimum of 12 months.

Note: We quantify DNA and RNA using broad range (BR) Qubit kits according to the manufacturer's protocol. For each sample, we use 1 µL of extracted nucleic acids for the Qubit assay. If RNA or DNA concentrations are below 50 ng/µL, requantify using high sensitivity (HS) Qubit kits.

Note: Viably frozen cryostocks can also be used for nucleic acid extraction. Thaw vial in 37°C water bath, transfer to a 1.5 mL microtube and Centrifuge (300 × *g*, 3 min, 20°C). Resuspend pellet in 300 µL of RLT Plus Buffer with freshly added BME and proceed with **step 38**.

⚠ CRITICAL: It is important to work quickly through the nucleic extraction protocol to ensure maximum yields. We don't recommend to isolate RNA/DNA from more than 10 samples at once.

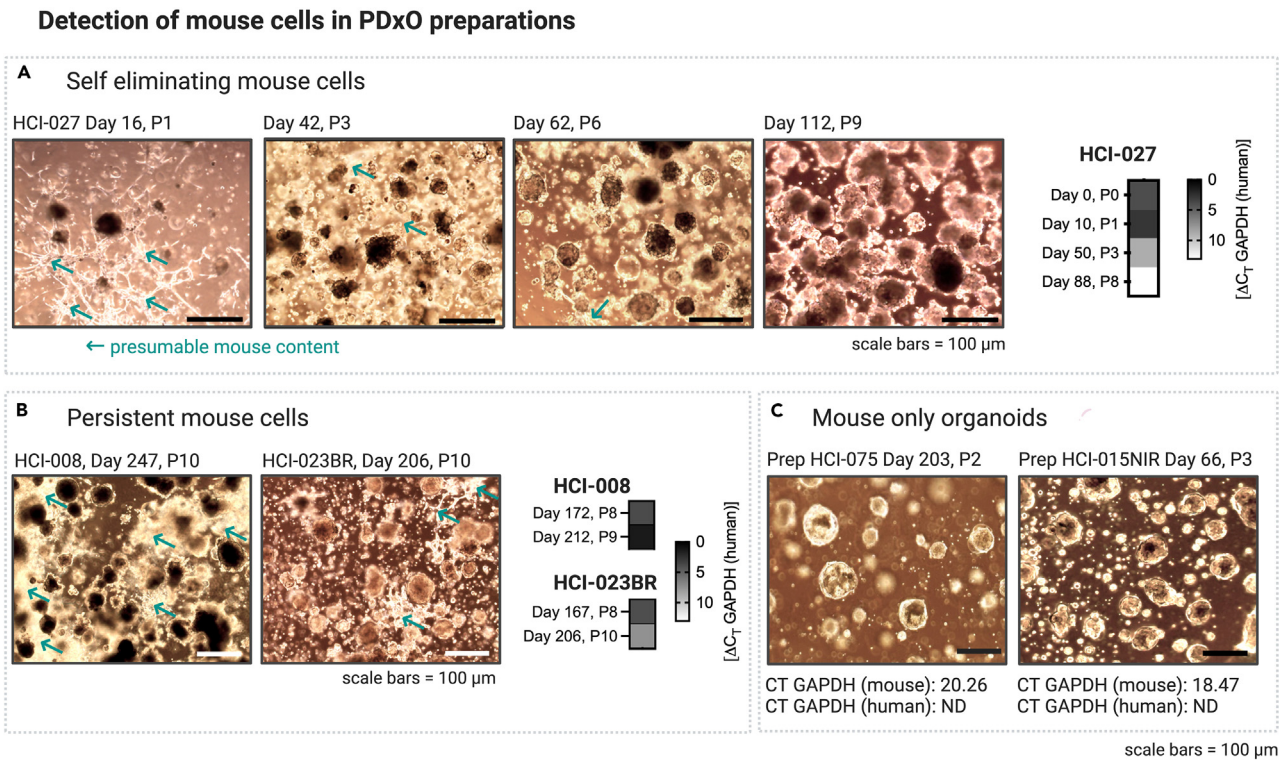

Figure 7. Detection of mouse cells in PDxO cultures

(A) Self eliminating mouse cells in PDxO cultures will disappear over time after several passages as shown for HCI-027 PDxOs from passage 1 (P1) to passage 9 (P9). Teal errors indicate the presumable presence of mouse cells. Right side: qRT-PCR data indicating elimination of mouse cells by passaging over time displayed as ΔCT (human *GAPDH* CT subtracted from mouse *Gapdh* CT). Dark gray indicates high mouse content, white indicates no mouse content. Scale bars represents 100 µm.

(B) Images of two PDxO lines (HCI-008 and HCI-023BR) showing persistent mouse content at high passage number (P8 and P9 respectively) as shown by images (left) and qRT-PCR (right). Scale bars represents 100 µm.

(C) Images of two PDxO cultures (HCI-075 and HCI-015NIR) that only contain mouse organoids. These PDxO lines failed establishment due to non detectable human content as indicated by *GAPDH* CT values. Scale bars represents 100 µm.

To validate the identity of established PDxO lines, we utilize a kit for Short Tandem Repeats (STR) analysis which requires only a few ng of DNA as input for a PCR reaction. The fluorescent PCR products can be run on a Genetic Analyzer or sent off for analysis. We use GeneWiz's service for this step and compare results of matching patient-derived models like PDX and PDxOs to their original patient tumor. Each patient will display a unique pattern of STRs which is used to verify the origin of PDxOs.

39. Validation of PDxO identity via STR Assay.
    a. Add 0.08 ng/µL DNA in a volume of 5 µL–96 well PCR plate.
    b. Add 7.5 µL of Master Mix provided by the AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit.
    c. Use AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit according to manufacturer's protocol.
    d. Use genetic analyzer or Genewiz to analyze PCR product.
    e. Compare unique patterns of STR peaks between samples.

Note: We found that reducing the total reaction volume by half to 12.5 µL instead of 25 µL produces reliable results while reducing assay costs significantly.

Quality control: Mouse cell detection and elimination

qRT-PCR is performed to detect the presence of mouse cells in the PDxO culture. We eliminate mouse cells from our cultures, since in some tumor lines the contaminating mouse cells can grow

aggressively and interfere with optimal expansion of long term PDxO cultures and subsequent drug testing. In most PDxO cultures, residual mouse cells will self-eliminate by approximately day 60 or passage 2 (Figure 7A), but in some cases they can be persistent (Figure 7B). In Matrigel, mouse cells often appear to be migratory with mesenchymal characteristics, and can be identified by their elongated phenotype. They can compete for space and nutrients, outpace epithelial growth and ultimately take over PDxO cultures. However, metaplastic breast cancer PDxO cultures can also display a mesenchymal-like phenotype, so it is important to keep this in mind when deciding on mouse cell elimination strategies. Troubleshooting 6. Never use morphology as the sole factor to assess cell content. We recommend antibody-selected fluorescent-activated cell sorting (FACS) or magnetic cell depletion (MCD) over differential centrifugation (DC), since metaplastic tumor cells often present as single cells post dispase digestion and would be lost using DC.

Note: Mouse cells of interest can be added back to PDxO cultures in controlled ratios for co-culture experiments.

Migratory mouse cells are often visible in PDxO cultures by microscopy (Figure 7), but for precise assessment, we use qRT-PCR to quantify the expression of human and mouse *Gapdh* to determine mouse cell content in PDxO cultures. In some rare cases, PDxOs that appeared to be stable and healthy contained only mouse organoids, so it is important to complete this validation step even when cultures look perfect (Figure 7C).

Mouse cell detection

Timing: 3 h (for step 40)

To detect the presence mouse cells in PDxO cultures, qRT-PCR is performed.

40. Detection of mouse and human content in PDxO cultures using qRT-PCR.
    a. Thaw RNA extracted from PDxOs (**step 38**) on ice.
    b. In a first set of PCR tubes mix between 100-500 ng RNA and add RNase-free water according to the manufacturer's recommendation (SuperScript IV Vilo kit) using 8 µL final volume.
        i. Use the same amount of RNA for each sample within one run.
    c. In a separate tube prepare the ezDNase buffer mix:
        i. Calculate and add per sample, plus 10% extra:
        ii. 1 µL 10× ezDNase Buffer.
        iii. 1 µL ezDNase enzyme.
        iv. Mix well.
    d. Add 2 µL of the ezDNase buffer mix to each sample into the first set of tubes from step 40b to get a final volume of 10 µL and mix well.
    e. Incubate for 2 min at 37°C.
    f. In a 1.5 mL microtube prepare IV VILO Solution:
        i. Calculate per sample, plus 10% extra:
        ii. 4 µL IV VILO Master Mix.
        iii. 6 µL nuclease-free water.
        iv. Mix well.
    g. Add 10 µL IV VILO Solution to tubes from step 40e and mix gently.
    h. Start cDNA synthesis in thermal cycler using the program provided in the SuperScript IV Vilo kit data sheet.
    i. For the qRT-PCR, reconstitute mouse *Gapdh* F + R (forward + reverse) and human *GAPDH* F + R primers in IDT buffer as 40× stocks.
    j. Calculate to use enough reaction mixture to run 1 ng of cDNA for each sample in a final volume of 5 µL, with each sample run in technical quadruplicates.
    k. Account for a "no cDNA" sample in each run for negative control.

l. In PCR tubes, add 2× PowerUp SYBR Green Master Mix.
m. Add appropriate volume needed to end up with 1 ng of cDNA per PCR tube.
n. Mix well.
o. In a separate 1.5 mL tube, dilute the primers. Calculate for 500 nM primer of each forward and reverse primer per sample.
p. Pipet 15 μL of the cDNA/SYBR mixture from step 40n into a new set of PCR tubes.
q. Add 15 μL of diluted primer mixture.
r. Mix well.
s. Transfer 5 μL of reaction mix per well into a 384-well PCR plate in technical quadruplicates (20 μL of the 30 μL reaction mix from step 40r will be used).
t. Cover the PCR plate with light cycler-compatible foil.
u. Spin plate (350 × *g*, 2 min, 20°C).
v. Run qRT-PCR on light cycler (Bio-Rad).
w. Use standard cycling mode recommended by the PowerUp SYBR Green manual:
x. Light cycler program:
    i. Denaturation: 2 min @ 95°C.
    ii. 40× cycles of: Denaturation: 15 s @ 95°C/ Elongation: 45 s @ 60°C.
    iii. Melt curve: 0.5°C per minute, 65°C–95°C.
y. For analysis, average the technical replicate CT values.
z. To calculate ΔCT, subtract human *GAPDH* CT from mouse *Gapdh* CT.
aa. If desired, display ΔCT values as a heatmap.

Note: PDxO cultures with a mouse *Gapdh* CT of 32 or higher are considered "mouse free" for PDxO establishment purposes, typically resulting ΔCT values of ~13–15.

Note: If the same scale is used as represented here, PDxO lines with high mouse content are represented in the heatmap as dark gray due to the high mouse *Gapdh* CT and low human *GAPDH* CT values, whereas "mouse free" PDxO lines will display as white.

Note: CT values of the four technical replicates should be almost identical.

⚠ CRITICAL: We noticed that mouse content can start appearing in PDxO lines that are considered "mouse free" after freeze/thaw cycles and it is therefore recommended to perform the mouse cell detection assay periodically or when elongated cells starting to appear in culture over time.

⚠ CRITICAL: We recommend to always use a combination of visual and quantitative (qRT-PCR) data to inform about mouse cell content. For metaplastic breast cancers, mouse cells and tumor cells are indistinguishable by eye.

Mouse cell elimination

Timing: 4–5 h (for step 41)

Timing: 3–4 h (for step 42)

To eliminate mouse cells from PDxO cultures we use different strategies depending on the nature of the PDxO culture (Figure 7). Many PDxO lines contain self-eliminating mouse cells which won't be detectable after a few passages, even by qRT-PCR. This process can be sped up by integrating DC steps during passaging. If mouse cells are persistant, they can be detected even after an extended culture time of >200 days or 10 passages. In these cases we recommend mouse cell elimination by FACS (step 41) or magnetic bead mouse cell depletion (MCD) (step 42). We suggest to perform one mouse cell elimination method (either FACS or MCD), embed the PDxOs and then

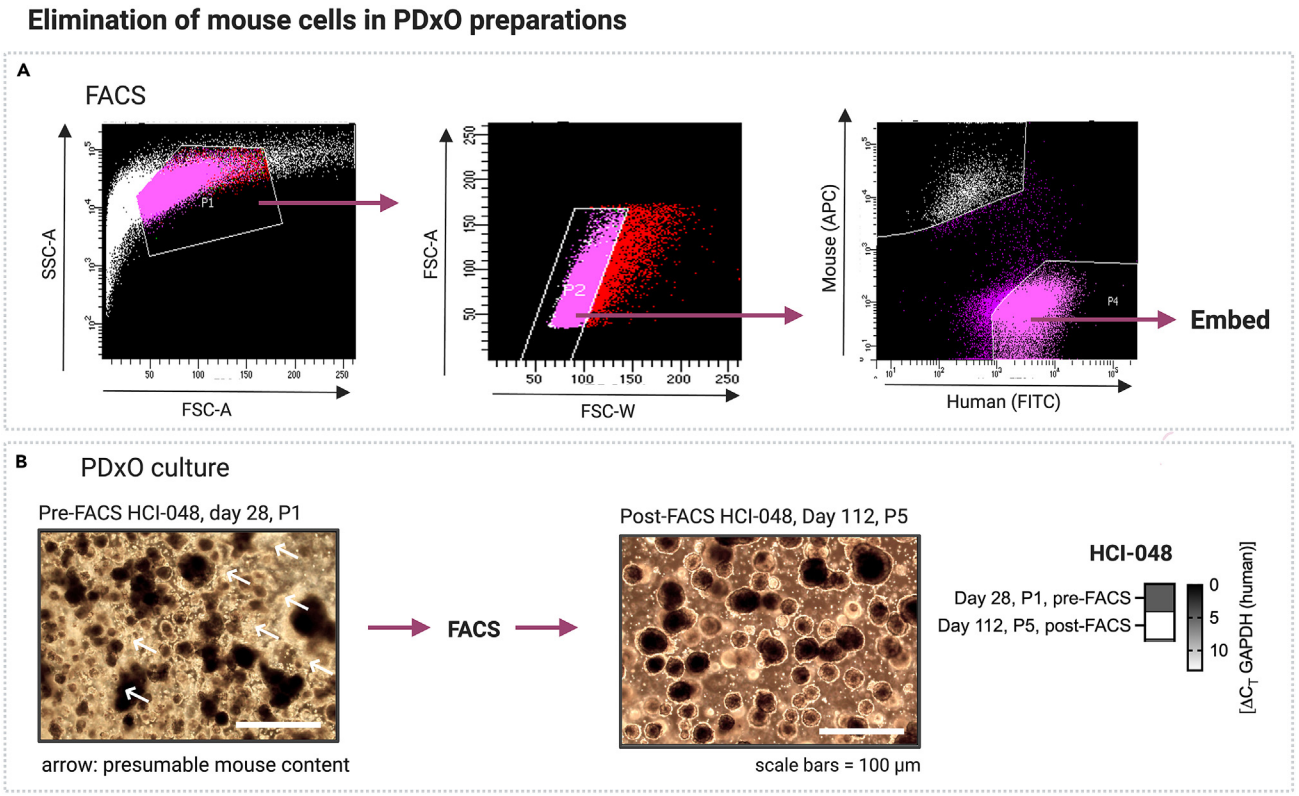

Figure 8. Elimination of mouse cells from PDxO cultures via FACS

(A) Gating strategy for sorting human and mouse cells in HCI-048 PDxO culture. Cells were gated on SSC-A/FSC-A to exclude debris, and FSC-A/FSC-W to exclude duplets. Only cells positive for human CD298 or human EpCAM would be found in the gate positive for human cells based on the green fluorescent fluorophore that the antibodies were labeled with. Mouse cells will be labeled as such by red-fluorescence conjugated CD29/CD90.2 antobodies and are found in the APC gate, respectively.

(B) Culture image show HCI-048 PDxOs pre- and post sorting. qRT-PCR confirms that mouse content was eliminated by FACS. Scale bars represents 100 µm.

check the matured PDxO culture for mouse cell content again to determine if one elimination step was sufficient.

> *Note:* Partial elimination by differential centrifugation can be a short-term solution for eliminating mouse cells in PDxO cultures. DC alone is not selective enough to fully eliminate mouse cells in PDxO cultures, but is instead used as a tool to allow cultures enough time to expand and generate sufficient epithelial cells numbers for FACS sorting, generally requiring a minimum cell number of 1×10^6.

> ⚠ CRITICAL: Most PDxOs need to be expanded for 1–2 passages prior to FACS so that organoid outgrowth post-FACS is not impaired.

Once cells are >P0 and cell number is $\geq 1 \times 10^6$, PDxO lines containing mouse cells can be purified by FACS. For mouse cell elimination we use a simple, pooled antibody 2-channel strategy. Antibodies that stain stroma-like mouse cells (anti-mouse CD90.2, anti-mouse CD29) and human cells or human epithelial cells (anti-human CD326/EpCAM, anti-human CD298) are pooled into separate fluorescence channels for simple and effective separation of mouse cells from human epithelial populations (Figure 8).

> *Note:* Set aside 50-100,000 unstained cells as controls. Additional single channel controls are recommended to properly set gates for cell sorting. If PDxO material is limited, mouse

fibroblast cells and human breast cancer cells can be used as positive controls, although autofluorescence might differ from PDxOs and gating strategy may need adjustment before starting the sort. An example of sorting gate is shown in (Figure 8).

41. Elimination of mouse cells using FACS (optional).
    a. Remove media from well.
    b. Add dispase solution and use cell lifter to dislodge dome from culture plate.
    c. Use pipet to mechanically disrupt dome.
    d. Incubate for 20–30 min at 37°C.

    Note: We found that the optimal dispase incubation time for passaging PDxOs is between 30 min – 1h, however when organoids are not immediately re-embedded and undergo sorting we found that the minimum dispase incubation time of 20–30 min is preferable to minimize cell death.

    e. Transfer into a 15 mL conical and fill up to 15 mL with PDxO base media.

    Optional: Perform differential centrifugation (300 × *g*, 30 or 40 s, 20°C). Aspirate supernatant and continue with TrypLE step on the pellet. Note that cell yield can diminish with DC steps; proceed cautiously or skip this optional step if limited organoid material is present.

    f. Centrifuge (300 × *g*, 3 min, 20°C).
    g. Aspirate and discard supernatant and follow steps 33a-f to disassociate into single cells Troubleshooting 8.
    h. Perform all following steps on ice.
    i. Resuspend cells in cold FACS buffer at 1.0 × 10^6 cells/mL, add Y-27632 (1:1000) and transfer into 5 mL conical tube.
    j. Add Fc receptor (FcR) blocking solution directly to the vial:
        i. Mouse FcR block: 1 µL/ 1.0 × 10^6 cells.
        ii. Human FcR block: 7.5 µL/ 1.0 × 10^6 cells.
    k. Incubate on ice for 20 min.
    l. Add antibodies directly into cell/FcR mixture:
        i. Anti-mouse CD90.2 AlexaFluor 647: 2 µL/ 1.0 × 10^6 cells.
        ii. Anti-mouse CD29 AlexaFluor 647: 2 µL/ 1.0 × 10^6 cells.
        iii. Anti-human CD326 AlexaFluor488: 1 µL/ 1.0 × 10^6 cells.
        iv. Anti-human CD298 FITC: 10 µL/ 1.0 × 10^6 cells.
    m. Mix well and incubate in the dark on ice for 30 min.
    n. Fill up with cold FACS buffer to 5 mL.
    o. Centrifuge (300 × *g*, 3 min, 4°C).
    p. Discard supernatant and resuspend pellet in 5 mL cold FACS buffer.
    q. Centrifuge (300 × *g*, 3 min, 4°C).
    r. Discard supernatant.
    s. Resuspend in 150 µL FACS buffer with Y-27632 (1:1000).
    t. Transfer to FACS tube and keep on ice until flow sorting.
    u. Sort human cells into a 15 mL conical tube containing 5 mL complete PDxO media.

⚠ CRITICAL: We don't recommend using cell strainers prior to FACS sorting due to loss of cells.

⚠ CRITICAL: Perform FACS sorting as soon as possible after staining.

⚠ CRITICAL: Gates for the sorted human cell population should be set conservatively to avoid any mouse cell contamination (Figure 8).

v. Post sort, centrifuge the isolated human cells (300 × *g*, 3 min, 4°C).
w. Resuspend in 5 mL complete PDxO media.
x. Culture on ultra-low attachment (ULA) plate for 16 h.

Pause point: The 16 h incubation time will allow the sorted cells to aggregate.

⚠ CRITICAL: Allowing cells to aggregate pre-embedding increases the survival rate after sorting.

y. The next day, transfer the aggregates into 15 mL conical tube without washing the plate.
z. Centrifuge (300 × *g*, 3 min, 4°C).
   aa. Embed the cells into Matrigel dome following step 26.

Note: We recommend taking images pre- and post- ULA as a reference to determine if cells aggregated properly during the 16 h incubation. Examples of ULA cultures are shown in Figure 4.

⚠ CRITICAL: We recommend to perform qRT-PCR on PDxOs 1–2 passages post-FACS to ensure purity of the PDxO line (Figure 8). Troubleshooting 7.

42. Elimination of mouse cells via magnetic bead mouse cell depletion (MCD) (optional).
    a. Remove media from well.
    b. Add dispase solution and use cell lifter to dislodge dome from culture plate.
    c. Use pipet to mechanically disrupt dome.
    d. Incubate for 20–30 min at 37°C.
    e. Transfer into a 15 mL conical and fill up to 15 mL with PDxO base media.

Optional: Perform differential centrifugation (300 × *g*, 30 or 40 s, 20°C). Aspirate supernatant and continue with TrypLE step. Note that cell yield can diminish with DC steps; proceed cautiously or skip this optional step if limited organoid material is present.

    f. Centrifuge (300 × *g*, 3 min, 20°C).
    g. Aspirate and discard supernatant and follow steps 33a-f to disassociate into single cells Troubleshooting 8.
    h. Perform all following steps on ice.
    i. Place a pluristrainer (100 μm mesh size) on a 50 mL conical tube and strain cells.
    j. Wash strainer with 5 mL PDxO base media.
    k. Remove 10 μL to count the cells using an automated counter (Countess II) or hemocytometer.
    l. Transfer remaining cells into a 15 mL conical and fill up to 15mL with PDxO base media.
    m. Centrifuge (300 × *g*, 3 min, 20°C).
    n. Discard supernatant and resuspend pellet in MCD buffer (0.5% BSA in PBS) at a concentration of 2.0 × 10^6 cells/ 80 μL.
    o. For each 2.0 × 10^6 cells or 80 μL, add 20 μL of mouse cell depletion cocktail.
    p. Mix well and incubate for 15 min on ice in the dark.
    q. Adjust volume to 2.0 × 10^6 cells/500 μL with MCD buffer (0.5% BSA in PBS).
    r. Place LS column into magnetic field of MACS Separator.
    s. Wash LS column by rinsing with 3 mL MCD buffer (0.5% BSA in PBS).
    t. Pipet cell suspension onto column.
    u. Collect flow-through.
       i. This portion contains the unbound human cells.
    v. Wash column with 1 mL MCD buffer (0.5% BSA in PBS).
    w. Collect flow through.

x. Wash column with 1 mL MCD buffer (0.5% BSA in PBS).
y. Collect flow through.
z. Combine all three flow throughs to recover as many human cells as possible.
   aa. Transfer into 15 mL conical tube and fill up to 15 mL with PDxO base media.
   bb. Centrifuge (300 × *g*, 3 min, 20°C).
   cc. Discard supernatant and resuspend in 2 mL PDxO base media.
   dd. Remove 10 μL to count the cells using an automated counter (Countess II) or hemocytometer.
   ee. Culture cells on ULA for 16 h following **step 27**, and embed the next day (**proceed with step 26**).

PDxO histology

Timing: 3 days (for step 43)

Timing: 3 days (for step 44)

Timing: 3 days (for step 45)

PDxO histology can be useful to characterize organoid morphology and analyze protein expression in organoids. Using FFPE blocks, we characterized PDxO and found close resemblance to their matching parental PDX tumors.[1] To analyze protein expression and localization by IHC or IF, PDxOs can be prepared by various methods for histology. Making paraffin blocks (steps 43, 44) is more time consuming, but results in sections that can be treated identically to formalin-fixed tissue sections. We have successfully made PDxO paraffin blocks following two different methods with are described in detail below (steps 43, 44). Making cytospin slides (step 45) is a much quicker method for determining protein expression and localization in PDxOs, and also requires significantly less input material compared to paraffin blocks. We have noted that either method can be utilized (optional).

Note: For preparation, place an alipuot of histogel in heating block and warm to 65°C for a minimum of 20 min.

43. Preparing PDxO blocks for histology using chamber slides (optional).
   a. For each paraffin block, prepare 2 domes of mature and confluent PDxO cultures.
   b. Aspirate the media and add 1 mL cold PDxO base media to the well.
   c. Using a P1000 mechanically disrupt the dome by pipetting up and down 10–20 times.
   d. Transfer into a 15 mL conical tube.
   e. Wash the well with cold PDxO base media, transfer into the same conical tube and fill up to 15 mL.
   f. Centrifuge (300 × *g*, 3 min, 4°C).
   g. Aspirate supernatant, including the cloud of broken up Matrigel hovering over the pellet. Additional wash steps may be performed to wash out the Matrigel and increase yield of organoids.
   h. Resuspend pellet in 150 μL Matrigel.
   i. Pipet 150 μL PDxO:Matrigel mixture per well of chamber onto a 8-chamber slide.
   j. Incubate at 37°C for 10–12 min to solidify.

Note: From here on we follow instructions published by Pinto et al.,[5] with the exception that we use 65°C warm histogel to pipet the bottom and top layer

   k. Remove wells and rubber gasket from chamber slide.
   l. Coat a plastic cryo mold with 100 μL warm histogel.

Table 3. Tissue processing steps

	Solution	Duration
1	70% Ethanol	1 h
2	85% Ethanol	1 h
3	95% Ethanol	1 h
4	95% Ethanol	1 h
5	100% Ethanol	1 h
6	100% Ethanol	1 h
7	100% CCS	1 h
8	100% CCS	1 h
9	Paraffin	1 h
10	Paraffin	1 h

m. Immediately place the PDxO:Matrigel block in the coated cryo mold using a cell lifter.
   i. Remove any air pockets that may have formed between the histogel coating and PDxO:Matrigel block.
n. Add 100 µL warm histogel on top of the PDxO:Matrigel block.
o. Incubate on ice for 10 min to solidify.
p. Add a biopsy pad in a labeled histology cassette.
q. Invert the cryo mold and transfer the PDxO:Matrigel block in the histology cassette.
r. Incubate histology cassette in 4% PFA in PBS for 16–24 h at 4°C.
s. Place sample cassettes into tissue processor and process using the program specified in Table 3.
t. Embed into paraffin block and cut block on the microtome into 5 µm thick sections.

44. Preparing PDxO pellet blocks for histology (optional).
a. For each paraffin block, prepare 2 domes of mature and confluent PDxO cultures.
b. Aspirate medium.
c. Add 1 mL freshly-made dispase solution to each well.
d. Use a cell scraper to scrape the dome into the dispase solution.
e. Carefully pipette (p1000) 5-10 times to break up Matrigel into smaller fragments.
f. Incubate 30 min at 37°C.
g. Transfer to 15 mL conical tube.
h. Fill tube up to 15 mL with ice-cold PBS.
i. Mix by inverting the conical tube multiple times.
j. Centrifuge (300 × *g*, 3 min, 4°C) to pellet organoids.
k. Repeat PBS washing step.
l. Aspirate supernatant.
m. Resuspend pellet in 1 mL 4% PFA in PBS and transfer to 5 mL conical tube.
n. Incubate for 16 h at 4°C.
o. Centrifuge (300 × *g*, 3 min, 4°C) to pellet organoids.
p. Aspirate supernatant.
q. Resuspend pellet in 2 mL ice-cold PBS and incubate on ice for 5 min.
r. Centrifuge (300 × *g*, 3 min, 4°C) to pellet organoids.
s. Aspirate supernatant.
t. Repeat PBS washing step.
u. Aspirate supernatant.
v. Ensure that all liquid is removed from the organoid pellet.
w. Resuspend pellet in 15 µL warm histogel without introducing air bubbles.
x. Let solidify on ice for 10 min.
y. Add a biopsy pad in a labeled histology cassette.
z. Using a raxor blade, carefully cut off the bottom of the 5 mL conical tube, exposing a hole to the organoid:Histogel block.
   aa. Cut off top half of conical tube.

bb. Invert the conical tube and push organoid:Histogel block onto histology cassette using a 10 μL pipette tip.
cc. Place sample cassette into tissue processor and process using the program specified in Table 3.
i. Alternatively place cassettes into 70% Ethanol at 4°C until processing.
dd. Embed into paraffin block and cut block on the microtome into 5 μm thick sections.

45. Preparing PDxO cytospin sections for immunostainings (optional).
a. Prepare 1 dome of mature and confluent PDxO cultures.
b. Digest matrigel with dispase following step 32, with a shortened incubation time of 30 min.
c. Resuspend PDxO pellet in 15 mL cold PDxO base media for a second wash.
d. Centrifuge (300 × *g*, 3 min, 4°C).
e. Discard supernatant and resuspend PDxOs in 1 mL fixing solution (2% PFA, 0.01% Tween 20 in PBS).
f. Incubate at 20°C for 20 min.
g. Centrifuge (300 × *g*, 3 min, 4°C).
h. Discard supernanant and resuspend fixed PDxOs in 1 mL permeabilization buffer (0.5% Triton X-100 in PBS) at 20°C for 30 min.

Note: Permeabilization is necessary if intracellular immunostainings are performed

i. Centrifuge (300 × *g*, 3 min, 4°C).
j. Discard supernanant and resuspend in aldehyde blocking solution (1 mg/mL $NaBN_4$, 0.01% Tween 20 in PBS).
k. Incubate at 20°C for 5 min, then repeat aldehyde blocking step.
l. Centrifuge (300 × *g*, 3 min, 4°C).
m. Discard supernanant and resuspend in 2 mL 0.01% Tween 20 in PBS.
n. Centrifuge (300 × *g*, 3 min, 4°C).
o. Discard supernanant and resuspend in 2 mL 0.01% Tween 20 in PBS.
p. Centrifuge (300 × *g*, 3 min, 4°C).
q. Discard supernanant and resuspend in 1 mL PBS with 2% BSA.
r. Add 100 μL of PDxO solution onto cytospin slide.
s. Centrifuge (2000 rpm, 2 min, 20°C).
i. Note that the Cytospin centrifuge only reports rpm, therefore rpm are reported here for user convenience.
t. Dry slides for 16 h at 20°C.
u. Perform immunostaining the next day.

Note: Keeping PDxO cytospin slides for a longer time period prior to immunostaining has not been tested.

Preparing PDxO for injection into mice to generate PDxoX

Timing: 3 days

PDxO can be prepared for transplantation into mice for *in vivo* studies to generate PDxoX using the following procedure.

46. Preparation of PDxO for transplantation.
a. Plan to use 1 mature PDxO dome from a 6-well plate for injection into each mouse.
i. calculate volumes for n + 1 to account for pipetting loss and syringe dead volume.
b. Aspirate ~ 3 mL of media, leaving ~ 2 mL of media in the well.
c. Use a cell lifter to scrape the dome.
d. Pipet up and down 10–15 times to disrupt the Matrigel.

e. Transfer into 15 mL conical tube and fill up with PDxO base media.
f. Centrifuge (300 × *g*, 3 min, 4°C).
g. Discard supernatant and place conical tube with pellet on ice.
h. Carefully resuspend pellet in Matrigel, using 20 μL per original dome used as input.
i. Be careful not to introduce air bubbles.
i. Keep on ice.
j. Immediately prepare mouse for surgery.
   i. Follow IACUC-approval protocols and regulations.
k. Pre-chill 1mL syringe with 26G needle on ice.
l. Mix PDxOs/Matrigel solution by pipetting up and down without introducing air bubbles.
m. Fill syringe with 20 μL PDxOs/Matrigel without air.
n. Inject PDxO/Matrigel mixture into the 4th mammary fat pad as described in detail in DeRose et al.[2] and Guillen et al.[1]

Part III

Drug response assay

Timing: 7 days (for step 47)

Timing: 30 min–4 h (for step 48)

We recommend using established PDxOs that meet the criteria defined in Table 1 to test for drug responses, not organoids freshly isolated from a PDX tumor. Established PDxOs generally exhibit higher growth rates during 4-day or 6-day drug exposure assays, improving reliability in determining if drug effects are cytostatic or cytotoxic. Also, unstable PDxO cultures can be artificially sensitive to drugs. We screen established PDxOs that are cultured to a point were the domes are a few days before reaching full maturity. At this maturity stage, PDxOs display a more uniform size distribution. This helps to control for the large size differences that can dominate a culture at maturity. Therefore, pre-mature PDxOs 50–100 μm in size yield more consistent results in plating disctribution. Because determining therapeutic responses in PDxOs are typically large resource-intensive endeavors, we utilize homemade EHS-derived matrix instead of commercial Matrigel. No differences in drug responses were observed in side-by-side comparisons.

Note: The following steps are written assuming working on a ViaFlo 96 liquid handle, but can be conducted at smaller scale following the same principle with an E1 12 channel electronic repeater pipette.

Note: Consistent, accurate plating of PDxOs is critical to obtain reliable drug screening results. PDxOs will settle quickly in suspension, therefore working quickly through the protocols is essential. This takes a lot of practice.

Note: Do not perform drug response assay on PDxOs that just thawed or only passaged once. Wait at least two passages to ensure PDxO growth rate is stable.

47. PDxO drug response assay (Figure 9).
    a. To prepare PDxOs for drug testing, check organoid health, density, and phenotype by microscope.
    b. For each 384-well screening plate account for approximately one dome of organoids that have reached an average size of at least 50 μm and up to 100 μm.
       i. The number to aim for falls in the range of 60–100 organoids per well, the exact amount can be determined after the dome has been disassociated.
    c. To disassociate the dome, aspirate media and incubate in dispase following step 32 with the exception of only incubating for 20–30 min instead of 1 h.

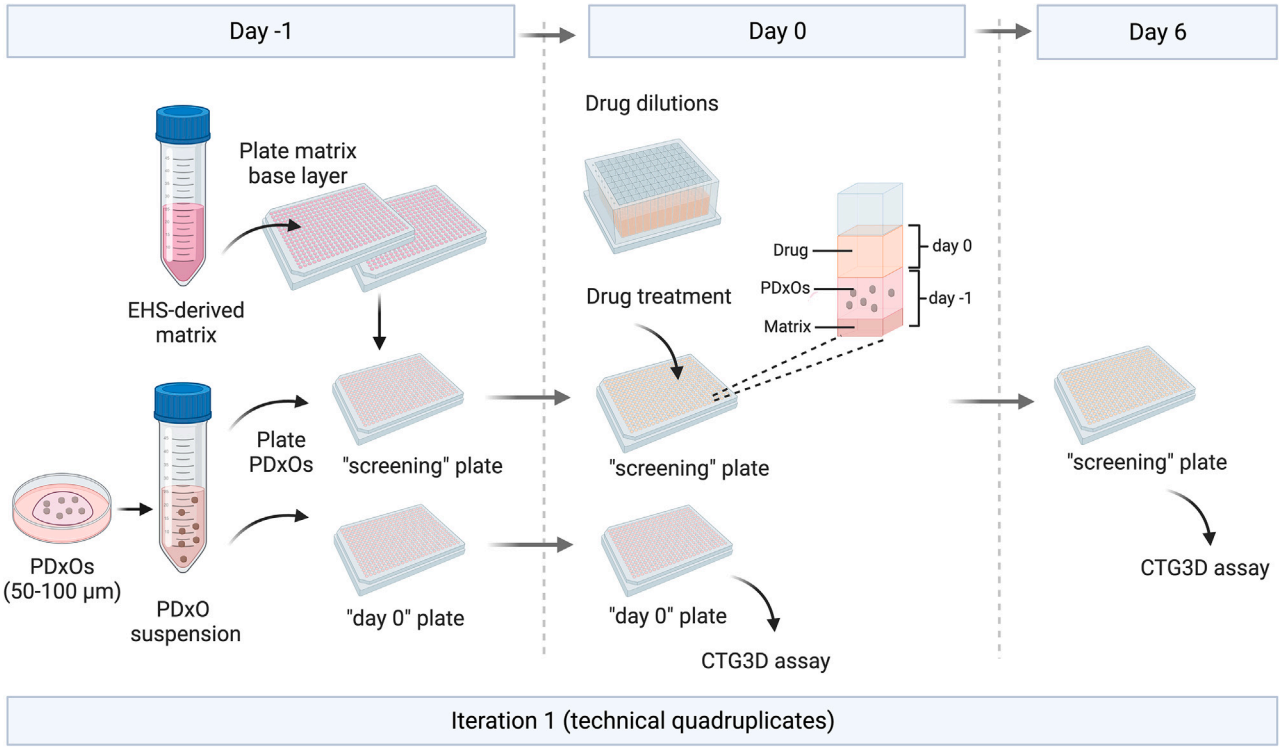

Figure 9. Overview of PDxO drug testing

On "day -1", 384-well plates are prepared by coating with EHS-deroived matrix as base layer. PDxOs suspensions are prepared, and plated onto the base layers of 384-well plates. For each drug testing assay, at least two plates are prepared, One "screening" plate, and one "day 0" plate. On the next day, drug dilutions are prepared and the drugs are added to the "screening" plate. On the same day, CTG3D assay is performed on the "day 0" to generate the baseline reading. At day 6, CTG3D assay is performed on the "screening" plate.

⚠ CRITICAL: Short dispase digestion still allows for removal of Matrigel while ensuring organoid structure is maintained.

Note: It is critical to work quickly and to keep the 384-well plates cool during all following steps.

d. While organoids are incubating in dispase, coat plates with EHS matrix:
   i. To prepare 384-well plates, chill them for 1 h at 4°C to avoid polymerization of EHS-derived matrix.
   ii. Put plates on ice and keep plates cool during all steps.
   iii. If plates are pre-chilled for 16 h, plates don't need to be kept on ice during this step if it is ensured that one operates quickly to avoid polymerization.
   iv. Pre-chill a 150 mL reservoir by stacking it onto a 300 mL reservoir filled with ice.
   v. Calculate total volume needed for 10 µL of EHS-derived matrix per well.
   vi. Fill EHS-derived matrix into 150 mL reservoir and keep cool.
   vii. While keeping plates cool, deposit 10 µL of EHS-derived matrix as base layer into each well using a ViaFlo 96, or a 12 channel electronic repeater for smaller scale drug screening.
   viii. Spin the plates (1200 × *g*, 1 min, 4°C) to ensure EHS-derived matrix settles into bottom of plate, creating even base layers.

⚠ CRITICAL: Even EHS-derived matrix distribution is critical and the basis for successful drug screen.

ix. Prepare 1 extra 384-well plate with EHS matrix coating.
x. This plate will be used to measure PDxO metabolic activity prior to drug treatment - referred to as "day 0" plate.
xi. Incubate plates at 37°C for 10 min – 1 h.
xii. If plates won't be used within 1 h, keep on ice and then incubate at 37°C for 10 min directly prior to organoid plating.

e. Transfer dissociated organoids into a 15 mL conical tube.
f. Fill the conical tube up to 15 mL with PDxO base medium.
g. Centrifuge at 300 × *g* for 3 min to pellet cells.
h. Discard supernatant and resuspend pellet with 15 mL with PDxO base medium.
i. Centrifuge at 300 × *g* for 3 min to pellet cells.

⚠ CRITICAL: Two washes are required to remove trace amounts of residual dispase remaining. Residual dispase will quickly digest any Matrigel added to screening wells.

j. Plate PDxOs onto one well of a ULA 6-well plate in 5 mL PDxO complete media.
k. Incubte plate at 37°C until plating.
l. To plate PDxOs, count the organoids:
i. Mix organoids by gently pipetting up and down with P1000.
ii. With a P20 pipette, remove 5 µL and drop onto hemacytometer without adding a cover glass.
iii. Under the microscope count all organoids in the 5 µL drop.
iv. Multiply organoid count by 200 and by the total volume (mL). For example, if organoids are resuspended in 5 mL multiply count by 1,000 to calculate total organoid number.

⚠ CRITICAL: Due to their large size, organoids cannot be loaded onto a hemocytometer by standard methods as they cause clogging upon loading which leads to inaccurate counts. In our hands, the counting slides used with automated cell counters also clog and lead to inaccurate organoid counts.

m. Prepare a plating media mix as follows (per plate) and keep on ice:
i. 700 µL EHS-derived matrix (5% v/v of final mix).
ii. 14 µL Y-27632.
iii. 13.3 mL cold PDxO base medium.
iv. Any other subtype specific additives needed at the appropriate concentrations.

Note: As the concentration of organoids in the ULA plates is now known, pipette the volume needed to achieve a total number of 23–39,000 organoids into a 15 mL conical tube. This will result in 60–100 organoids/well in a 384 well plate.

n. Resuspend the pellet in 1 mL of PDxO base media.
o. Add the plating media mix.
p. When preparing more plates scale accordingly.

⚠ CRITICAL: Modulating Y-27632 concentration during screening can adversely affect growth of PDxOs during screen.

⚠ CRITICAL: Removing Y-27632 during plating can cause substantial decrease in viability and growth during the screening process.

⚠ CRITICAL: Reducing the FBS concentration adversely affects the growth of some PDxO lines during screening.

q. Remove 384-well plate with Matrigel base layer from incubator.
r. Mix and transfer 14 mL plating master mix containing organoids to a 25 mL reservoir, on ice.

⚠ CRITICAL: Using a larger volume will generally result in a drastic reduction in mixing efficiency, leading to uneven seeding densities across a 384 well plates. Using larger volumes may therefore require more mixing. Troubleshooting 10.

s. Mix organoid master mix in reservoir gently, but thoroughly, using an E1 12 channel electronic repeater pipette set to mix 300 µL, 3×, and max speed (10) in between dispenses.
t. Immediately after mixing, plate 30 µL master plating mix per well in 384-well plates onto matrix base layer. Use an E1 12 channel electronic repeater pipette set to 8×, 30 µL.
u. Repeat step 47r-u for each 384 well plate.

⚠ CRITICAL: Organoids settle very quickly. Plating should be done immediately after the mixing step. Delays will cause organoid sedimentation within the pipette tips. This will lead to a high to low density gradient. If this phenomenon is observed we recommend decreasing the volume plated in between mixing. If plating orthogonally to the drug gradient this possible effect can confound results. We recommend plating in the same direction as the column with the vehicle control, which will unveil this trend if it happens.

v. From remaining organoid master mix, plate 16 wells (30 µL/well) into a 384-well "day 0" plate, using the same mixing/plating technique as above.

⚠ CRITICAL: The "day 0" pretreatment plate is needed for normalization and quality control, without it growth rate inhibition (GR) metrics after Hafner et al.[6] can't be calculated. It will be also used to determine the fold growth for each PDxO line on each plate.

w. Plate 500 µL from remaining PDxO plating master mix onto a 48 well plate. Use a microscope to observe if organoid structure was maintained during plating.

Note: If all organoids break into single cells, expect increased drug sensitivity

x. Incubate all 384 well plates at 37°C for 16 h.

Pause point: Allow the PDxOs to incubate for 16 h.

y. On the following day, assay the "day 0" plate with CTG3D:
   i. Add 30 µL of warm PDxO base medium to each well using an E1 12 channel electronic repeater pipette.

⚠ CRITICAL: Adding media at this step ensures equal volumes in plates measured at day 0 and day 6.

   ii. Add 15 µL of CTG3D to each well using an E1 12 channel electronic repeater pipette or ViaFlo 96 liquid handler if > 5 plates are prepared.
   iii. Shake for 20 min at 500 rpm in the dark.
   iv. Read luminescence signal on plate reader.
z. Drugs are added to the screening plates in 30 µL PDxO media, which results in a total volume of 60 µL per screening well, thus we prepare a mastermix with twice the final concentration needed per well.

aa. Dosage points are done in quadruplicates, so that one well of a master mix will dose four wells in the screening plate. Drug dilutions are then prepared in 96 deep well plates with volume enough to dose the required number of organoid screening plates.
bb. Drug dilution is set as a descending gradient by rows, with H being the highest and A the lowest. A null concentration is not done as a whole column is dedicated per plate of drugs to ensure a proper solvent control; this results in a total of 11 drugs plus solvent control per screening plate.
cc. Drug stocks are prepared to 20 mM and in DMSO while possible. Prepare drug solutions according to manufacturer specifications.
dd. Deep well plates are prepared as follows (extra 15% volume is needed for dead volumes):
    i. 4 × 30 per screening plate µl PDxO base media (with the same additives as when plated) in rows G-A.
    ii. ((4 × 30)× fold dilution factor) per screening plate µl PDxO base media (with the same additives as when plated) in row H.
    iii. Appropriate amount of a 20mM stock solution of the drug needed per column to reach double the desired initial concentration in screening plate, added to row H. Mix by pipetting at least half the volume of the well three times.
    iv. Dilute by chosen fold factor from rows H-A.

⚠ CRITICAL: Drug concentrations will dilute 2-fold when added to PDxO containing wells. It is not feasible to aspirate from wells containing PDxOs in 384-well plates without disturbing PDxOs, which are generally only loosely attached to the Matrigel base layer.

⚠ CRITICAL: Include a vehicle control for each solvent used for drug dilutions.

⚠ CRITICAL: Include Y-27632 in drug dilutions at 1×, to maintain Y-27632 at 1× in treatment wells, promoting optimal PDxO growth during the assay.

ee. Add 30 µL of drug solution to each well. For optimal efficiency, use a ViaFlo 96 liquid handler to efficiently apply drugs to multiple PDxO plates.
ff. We typically set up quadruplicate technical replicates for each drug per concentration.
gg. Discard lids and seal plates with breathe-easy film.

⚠ CRITICAL: Breathe-easy film has two layers. The top layer must be removed after applying to the plate to allow gas permeabilty.

hh. Incubate 384 well plates at 37°C for 6 days.

⚠ CRITICAL: Incubate plates as a single layer, do not stack plates!

ii. To perform CTG3D assay, remove seals from plates.
jj. Add 15 µL of CTG3D to each well with ViaFlo 96.
kk. Shake for 20 min at 500 rpm in the dark.
ll. Read luminescence signal on plate reader (Envision).
Drug screening data can be visualized as heatmaps that represent either a fold change normalized to control or different parameters calculated from GR metrics[6] that allows comparison of drug sensitivity results across lines with a wide range of growth rates, unlike other traditional metrics. In doing so the average response to a specific drug can be established and outliers identified.

48. Data analysis of PDxO drug response assay.

a. To determine the fold change, divide all data by the average metabolic signal across all "day 0" pretreatment control wells, specific to each PDxO line plated.
b. The resulting values in vehicle controls indicate maximum growth of PDxOs during the screen. Troubleshooting 11.

Note: At this step, the vehicle control fold change values should ideally be around 2 in order to consider the drug screen successfully conducted on growing organoids, with a minimum value of 1.5. Vehicle control fold changes < 1.5 indicate poor PDxO growth and resulting data may not represent true drug responses.

c. Values ranging from 1 to maximum growth (typically < 4 fold growth) indicate a partial cytostatic effect to no response.
d. Values of 1 indicate a complete cytostatic effect, and values < 1 indicate a cytotoxic effect of a compound. Troubleshooting 12, Troubleshooting 13.
e. For quality control, compare vehicle growth on each plate containing the same PDxO line. Growth in vehicle control should not vary significantly between plates. Troubleshooting 14.
f. To confirm drug responses, repeat screen across 3 biological replicates Troubleshooting 15.
g. To calculate GR metric parameters, format the data accordingly and run pipeline online using the open source software (https://doi.org/10.18129/B9.bioc.GRmetrics[7]) or with an R package following instructions from Clark et al.[4] Troubleshooting 16, Troubleshooting 17.

Note: Data can also be analyzed more traditionally, by normalizing to metabolic signal in vehicle at end-point

⚠ CRITICAL: We recommend using the GRaoc metric as it doesn't require polynomial fitting and thus is free of artifacts caused by experimental noise, which is common on organoid screening. Additionally, it discriminates changes in both potency and efficacy unlike other metrics.

Optional: This protocol can also be modfied to investigate wether drug combinations show synergistic effects in PDxOs following the examples in Guillen et al.[1] *and Truong et al.*[8]

EXPECTED OUTCOMES

The most important outcomes of this protocol are the establishment of organoid lines from breast cancer PDX models and the drug screening protocol for drug discovery and functional presicion oncology. The validation and characterization of the PDxOs, and the moderate throughput drug screening. Further in-depth information and characterization of established PDxO lines are reported in Guillen et al.[1]. Above protocol describes our drug screening set up with moderate throughput of 50 compounds and 20 PDxO lines per week per person, resulting in a complete set of 3 biological replicates in about 6 weeks.

LIMITATIONS

While PDxOs enable *in vitro* drug screening and can be applied for functional presicion oncology,[1] we acknowledge that organoids as tools also present limitations, like every model system. Compared to PDX models, PDxOs can be used to perform large-scale drug screenings in a short time; however, due to the absence of other cell types they are not suited to assess effects of the tumor microenvironment or the immune system. In addition, the drug library suitable for PDxO drug testing is limited to drugs that are not required to be metabolized by the liver, and to date we can't test effects of immunotherapies. PDxOs may not be the ideal tool for real-time co-clinical drug testing when results are needed close to the timepoint when the patient tissue is obtained (here, direct-from-patient organoids would be a better tool).

This protocol is optimized for breast cancer xenograft tissue with the ability to form organoids *in vitro*; however, we have experienced that around 4% of the PDX tumors fail to form organoids in cultures. One other limitation of this protocol is that we were not successful in fully automating the PDxO plating for drug screening assays due to the observation that PDxOs settle too quickly if not constantly mixed. In addition, microfluidic size limitations can affect the potential for full automation. The necessity to supplement the PDxO media with Rock inhibitor (Y-27632) presents another limitation of this protocol. The addition of Rock inhibitor could affect drug treatment responses *in vitro*, but is required for PDxOs to grow optimally. Performing drug screening without Rock inhibitor is not recommended with this protocol, because PDxO grown in suboptimal conditions tend to be more sensitive to drugs, hence results can include false positive data.

TROUBLESHOOTING

Problem 1

PDX tissue digestion only yields debris (during step 15).

Potential solution

PDX tumors vary in degree of necrosis, epithelial content and tissue quality. The initial tumor volume doesn't necessarily correlate with the PDxO yield. For cases like this we recommend to carefully assess tumor quality prior to processing and to avoid processing highly necrotic tissue regions. Increasing the amount of input PDX tumor tissue may also help increase the yield of healthy digested tumor material.

Problem 2

PDxO preparation yields low viability (during step 22).

Potential solution

We successfully established PDxO lines from fresh and cryopreserved PDX tumor fragments as input materials. However, fresh PDX tumor tissue can yield higher percentages of viable cells, therefore using fresh PDX tumor material may increase viabilty for problematic cases.

Problem 3

No organoid formation or PDxO outgrowth (during PDxO maintenance and passaging).

Potential solution

Poor quality of PDX input material can cause this problem. Carefully assess the PDX tissue quality and repeat the PDxO preparation with increased amounts of starting material. Also, the wrong media conditions can impair PDxO growth. Use breast cancer subtype-specific media conditions, ensure proper storage of components and add supplements fresh directly before usage.

Problem 4

No organoid formation in PDxO preparations from lobular PDX tumors (during PDxO maintenance and passaging).

Potential solution

Some lobular tumors will establish as PDxO cultures, for example HCI-018, while others only grow as single cells, for example HCI-014. Avoid DC steps in lobular PDxO preparations and passaging because tumor content can be present as single cells. By not depleting the single cells, the chances of successful eastablishment of PDxO lines similar to HCI-018 can be increased.

Problem 5

PDxO culture shows 2D cell growth on plastic outside of the Matrigel dome (during PDxO maintenance and passaging).

Potential solution
Cells that grow on 2D plastic outside of the Matrigel dome are often mouse cells.

Avoid carrying cells that grew on 2D plastic forward through passaging by carefully scraping the PDxO dome off the well and transferring it into a fresh well for disassociation.

Problem 6
Matrigel dome disruption and detachment from well due to contraction of the dome (during step 39).

Potential solution
Persistant and fast dividing mouse cells in PDxO cultures can cause the Matrigel dome to contract and detach from the well. We recommend to avoid letting domes detach, and to follow an aggressive mouse cell elimination strategy with one or more rounds of DC/MCD.

Problem 7
Mouse content detectable in PDxO cultures even after mouse cell depletion (during step 41).

Potential solution
Persistent mouse cells may require multiple rounds of elimination. Repeat mouse cell depletion once PDxOs are matured.

Problem 8
PDxO pellet is not visible and appears cloudy or floating, generally between the 0.5–1 mL mark of the 15 mL falcon tube (during step 41g).

Potential solution
This issue can be caused by incomplete Matrigel digestion. Increase the dispase digestion time, or resuspend the pellet more aggressively to loosen organoids from residual Matrigel prior to the second washing step.

Problem 9
Low RNA/DNA yields (during step 38hh).

Potential solution
Poor PDxO health. Repeat extraction with healthy PDxO culture samples.

Problem 10
Uneven seeding density in drug screening (during step 47r).

Potential solution
PDxO settle with gravity quickly during plating. Work quickly and practice thorough mixing and plating until even seeding density is routinely achieved.

Problem 11
Uneven control wells across different drug screening plates (during step 48b).

Potential solution
This problem might be caused by uneven PDxO mixing during the plating process. Plate pretreatment wells after every plate and improve mixing to achieve homogeneous plating results.

Problem 12
Unrealistically high growth indicated with values > 5 after data normalization in drug screening (during step 48d).

Potential solution

A potential cause is that a change in the optical settings on the plate reader occurred. Ensure all settings on the plate reader are consistent between pretreatment and endpoint measurements.

Problem 13

Maximum fold changes are different (the difference of maximum fold change >2) across three biological replicates in drug screening (during step 48d).

Potential solution

A potential cause is different seeding densities across biological replicates. Ensure seeding densities are the same between replicates and PDxO models are plated 3–4 days before reaching maturity.

Problem 14

Drug screening data fold change in solvent control is < 1.5 (during step 48e).

Potential solution

This problem can have multiple possible causes: a) Organoids can be unhealthy and not suitable for drug screening. In that case, passage organoids for 1–2 more passages, look for signs of unhealthy organoids, and repeat drug screening once PDxOs are healthier. b) Organoids are too small (< 50–100 μm in size). A solution for this problem is to grow organoids until they are 100–150 μm prior to plating for screening. c) Not enough organoids were plated. This can be fixed by plating 1.5 – 3× more organoids.

Problem 15

Drug response shows variability between biological replicates (during step 48f).

Potential solution

This might be caused by evaporation of the solvent, drug crystallizaiton or degradation, or increased organoid sensitivity. A solution for this problem is to ensure proper handling techniques as well as inspection and mixing of drugs prior to adding. Drug solubility may be increased by sonication or warming. Avoid light exposure to drugs and repeated freeze/thaw cycles. Avoid using organoid models that are unstable, have been thawed without passaging, or are not established yet.

Problem 16

Drug screening results indicate that noise is higher than the drug gradient (during step 48g).

Potential solution

This issue can be caused by dispensing organoids too slowly, sedimentation of organoids in the tip, improper mixing in the 150 mL reservoir, or if organoids exceed the recommended maximum size or seeding density. The solution for the above mentioned issues are to ensure proper mixing at all steps, increase plating speed, and stick to recommended seeding parameters. If organoids are too large, collect them with dispase at an earlier timepoint.

Problem 17

Drug screening DMSO (or solvent control) shows a gradient of response (during step 48g).

Potential solution

The maximum DMSO concentration described in this protocol doesn't cause a response in our PDxO lines. If this is observed, a potential cause is that organoids were dispensed too slowly or sedimented in the tips. To avoid this issue, increase plating speed and uniformity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alana Welm (alana.welm@hci.utah.edu).

Materials availability

Established PDxO and PDX HCI lines can be obtained from the Preclinical Research Shared Resource (PRR) core at the Huntsman Cancer Institute, University of Utah. https://uofuhealth.utah.edu/huntsman/shared-resources/preclinical-research-resource/

Data and code availability

This study did not generate new unique datasets or code.

Open source software for GR metric parameter calculations can be found at https://doi.org/10.18129/B9.bioc.GRmetrics[7] or with an R package following instructions from Clark et al.[4]

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AUTHOR CONTRIBUTIONS

S.D.S., A.J.B., and A.L.W. wrote the manuscript and S.D.S. made the figures. S.D.S., A.J.B., L.Z., C.-H.Y., E.C.-S., K.P.G., B.E.W., and A.L.W. developed protocols. S.D.S., A.J.B., L.Z., C.-H.Y., and E.C.-S. designed and performed experiments and analyzed data. B.E.W. and A.L.W. jointly supervised the work.

DECLARATION OF INTERESTS

The University of Utah may license the PDX and PDxO models described here to for-profit companies, which may result in tangible property royalties to members of the Welm lab who developed the models (S.D.S., A.J.B., L.Z., C.H.Y., E.C.S., K.P.G., B.E.W., and A.L.W.).

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