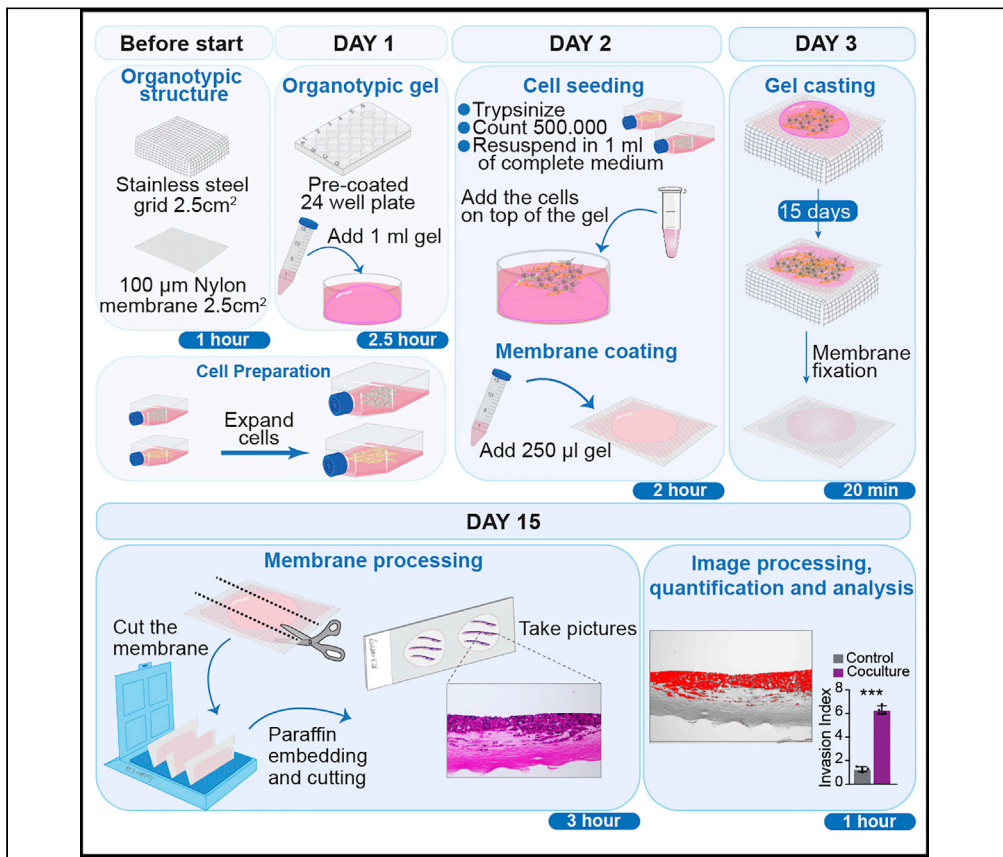


## Protocol

# Air-liquid organotypic assays to investigate cellular crosstalk in the tumor microenvironment of cancer cells



Tania Cid-Diaz,  
Andrea C. Lodeiro,  
Angeles Duran,  
Jorge Moscat, Maria  
T. Diaz-Meco

tcd4002@med.cornell.  
edu (T.C.-D.)  
mtd4001@med.cornell.  
edu (M.T.D.-M.)

**Highlights**  
3D-organotypic  
cultures mimic tissue  
architecture *in vitro*

Air-liquid  
organotypic assays  
are useful to study the  
tumor  
microenvironment

Steps to build an air-  
liquid culture and  
guidelines for data  
analysis

Flexible and robust  
3D-coculture  
protocol to  
investigate cellular  
crosstalk

Air-liquid organotypic culture models enable the study of the cellular crosstalk in the tumor microenvironment. This 3D assay recapitulates the tumor niche more faithfully than 2D culture systems and represents a versatile platform that can be easily adapted to different types of cancer cells, stromal components, or ECM composition. Here, we detail the steps to build an organotypic culture including the preparation of the organotypic structure, organotypic gels, cell seeding, gel casting, membrane processing, and image and data analysis.

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

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## Protocol

## Air-liquid organotypic assays to investigate cellular crosstalk in the tumor microenvironment of cancer cells

Tania Cid-Diaz,<sup>1,2,\*</sup> Andrea C. Lodeiro,<sup>1</sup> Angeles Duran,<sup>1</sup> Jorge Moscat,<sup>1</sup> and Maria T. Diaz-Meco<sup>1,3,\*</sup><sup>1</sup>Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY 10065, USA<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [tcd4002@med.cornell.edu](mailto:tcd4002@med.cornell.edu) (T.C.-D.), [mtd4001@med.cornell.edu](mailto:mtd4001@med.cornell.edu) (M.T.D.-M.)  
<https://doi.org/10.1016/j.xpro.2022.101635>

## SUMMARY

Air-liquid organotypic culture models enable the study of the cellular crosstalk in the tumor microenvironment. This 3D assay recapitulates the tumor niche more faithfully than 2D culture systems and represents a versatile platform that can be easily adapted to different types of cancer cells, stromal components, or ECM composition. Here, we detail the steps to build an organotypic culture including the preparation of the organotypic structure, organotypic gels, cell seeding, gel casting, membrane processing, and image and data analysis. For complete details on the use and execution of this protocol, please refer to Linares et al. (2022).

## BEFORE YOU BEGIN

Air-liquid or organotypic models are 3D in vitro models that resemble the in vivo environment and the tissue architecture within a specialized ECM. Different organotypic cultures have been developed (Kashima et al., 2021; Valencia et al., 2014) and adapted to interrogate the TME in cancer but most of those protocols are not well described and are difficult to replicate in the laboratory in the absence of specific expertise. Here we describe a detailed protocol visualizing the critical steps to provide the basis for a dynamic platform that can be modified to specific cell types and the use of different matrices or components of the ECM. In this assay, the cell cultures are grown in an air-liquid interface on a nylon membrane-covered metal grid. The cells are embedded in a collagen-Matrigel gel and exposed directly to air instead of being submerged underneath the nutrient medium. This allows diffusion and access to nutrients from the medium below, which establishes a gradient that mimics more closely the in vivo environment. The main advantage of an air-liquid model as compared to a normal 3D is the access of the cells to a sufficient oxygen supply that supports long-term survival. In addition, this method enables the formation of a polarized, pseudostratified epithelium that more faithfully recapitulates the tissue architecture and allows the study of the interactions with other cellular components of the TME.

## Institutional permissions

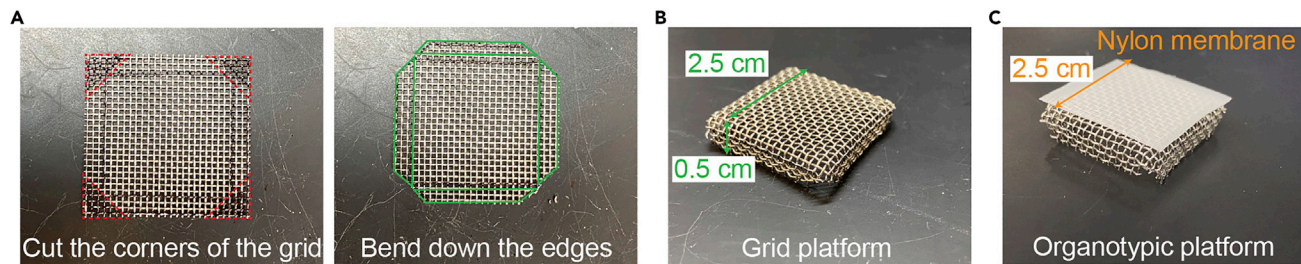
Animal handling and experimental procedures conformed to institutional guidelines and were approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee.

Permissions for animal experiments from the relevant institutions are required to perform experiments on live vertebrates or higher invertebrates in accordance with relevant institutional and national guidelines and regulations.

## Organotypic structure preparation (Day 1)

⌚ Timing: 2–3 h





**Figure 1. Organotypic structure**

Preparation of the structure for organotypic gel.

- (A) Stainless steel mesh labelled in red the areas to be cut and labelled in green the areas to be bent down.  
 (B) Grid platform after cutting and bending, 0.5 cm height, 2.5 cm width.  
 (C) Nylon membrane on top of the grid as it will be placed for the organotypic culture.

Before starting the protocol, it is necessary to prepare the structure where the gel will be placed and autoclave the necessary materials.

1. Prepare the grids from a 0.016 inches stainless steel mesh.
  - a. Cut squares with each side of 3.5 cm using scissors (Figure 1A).
  - b. Bend down 0.5 cm of the edges to achieve 0.5 cm high platforms utilizing a pair of pliers and autoclave them (Figures 1A and 1B).
2. Prepare 100  $\mu\text{m}$  pore size nylon membranes. Cut squares with each side of 2.5 cm (Figure 1C) and autoclave them.
3. Autoclave 2 spatulas and 1 pair of forceps.

See Figures 1A, 1B, and 1C and Methods video S1.

### Preparation of gel reagents (Day 1)

⌚ Timing: 2–3 h

We recommend preparing and aliquoting in advance the necessary reagents for gel preparation to facilitate the workflow. All the reagents must be handled in Class II Biological Safety Cabinet (BSC).

4. Aliquot preparation.
  - a. Prepare 1.5 mL aliquots of Matrigel and store at  $-20^{\circ}\text{C}$ . Thaw them on ice 2 h before use.
  - b. Prepare 1.5 mL aliquots of heat-inactivated fetal bovine serum (FBS) and store at  $-20^{\circ}\text{C}$ . Thaw them on ice 2 h before use.
5. Prepare necessary solutions.
  - a. Prepare 1 N NaOH solution for gel pH equilibration. Filter the solution using a 0.22  $\mu\text{m}$  syringe filter before use.
  - b. Prepare the desired volume of 10 $\times$  DMEM and filter it in a 0.20  $\mu\text{m}$  vacuum filtration unit.
  - c. Prepare 1:100 dilution of collagen type I in PBS and keep it on ice.
6. Pre-cool 200  $\mu\text{L}$  and 1 mL pipette tips at  $-20^{\circ}\text{C}$ .

**⚠ CRITICAL:** All gel reagents and tools must be handled cold. Otherwise, some polymerization may occur during the preparation, resulting in non-homogenous gels.

### Cell media preparation and membrane reagents preparation (Day 2)

⌚ Timing: 20 min

We recommend preparing and aliquoting in advance the necessary reagents for gel preparation to facilitate the workflow.

7. Prepare and pre-warm sterile DMEM for cell culture (supplemented with 10% FBS and 1% Gluta-max), PBS 1×, and 0.25% trypsin.
8. Prepare 20 mL of 1% glutaraldehyde in PBS and keep it on ice.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
Male white adipose tissue (WAT)	Mouse C57BL/6J, wild type	Cat# 000664
<b>Chemicals, peptides, and recombinant proteins</b>		
Glutaraldehyde 25%	Thermo Fisher Scientific	Cat# BP2548-1
Dulbecco's Phosphate-Buffered Salt Solution 1× (PBS)	Corning	Cat# 21-031-CV
DMEM, powder, high glucose, pyruvate	Corning	Cat# 12800-082
Dulbecco's Modification of Eagle's Medium 1× (DMEM)	Corning	Cat# 15-013-CV
0.25% Trypsin in HBSS w/o Calcium and Magnesium	Corning	Cat# 25-050-CI
100× Glutamax	Thermo Fisher Scientific	Cat #35050061
Collagen type I	Corning	Cat# 354236
Matrigel™ GFR Membrane Matrix	Corning	Cat# 354230
Fetal Bovine Serum (FBS)	Corning	Cat# 35-010-CV
Harris Hematoxylin Nuclear Stains	Leica Biosystems	Cat# 3801560
Eosin Secondary-Counter Stains	Leica Biosystems	Cat# 3801600
10% Neutral Buffered Formalin Fixatives	Leica Biosystems	Cat# 3800598
Sodium hydroxide white pellets (NaOH)	Thermo Fisher Scientific	Cat# BP359-500
<b>Experimental models: Cell lines</b>		
Human PC3*	ATCC	Cat# CRL-1435, RRID: CVCL_A4BV
Human LNCaP*	ATCC	Cat# CRL-3315, RRID: CVCL_4782
Mouse MyC-Cap*	ATCC	Cat# CRL-3255, RRID: CVCL_J703
Human WPMY-1*	ATCC	Cat# CRL-2854, RRID: CVCL_3814
<b>Experimental models: Organisms/strains</b>		
Mouse ( <i>Mus musculus</i> ):C57BL/6J wild type, ~ 8 weeks old, male	The Jackson Laboratory	Cat# 000664
<b>Software and algorithms</b>		
Fiji	<a href="#">Schindelin et al., 2012</a>	<a href="https://fiji.sc/">https://fiji.sc/</a>
<b>Other</b>		
Stainless steel mesh type 316, 20 × 20, 0.016"	McNICHOLS	Cat# 3120164810
100 μm pore size nylon membranes	Sefar Nitex	Cat# 03-100/32
Histosette™ II Tissue Processing/Embedding Cassettes	Thermo Fisher Scientific	Cat# 15-182-701E
0.22 μm syringe filter	Thomas Scientific	Cat# 1159T82
0.20 μm vacuum filter (250 mL)	Thermo Fisher Scientific	Cat# FB12566502
Scalpels	Thermo Fisher Scientific	Cat# 14-823-45
Forceps	MilliporeSigma	Cat# XX6200006P
Spatula with Scoop	Thermo Fisher Scientific	Cat# S41699
Staedtler Mars Erasers	Staples	Cat# 274795
6-well plate	Thermo Fisher Scientific	Cat# 12-556-004
24-well plate	Thermo Fisher Scientific	Cat# 12-556-006
6 in. Long Nose Pliers	The Home Depot	Cat# 203287743
Scissors	Staples	Cat# 24380504

(\*) The passage of the cell lines used should be < 25.

## MATERIALS AND EQUIPMENT

### DMEM 10×

Reagent	Final concentration	Amount
DMEM powder	10×	6.74 g
Distilled water	N/A	50 mL
<b>Total</b>	<b>N/A</b>	<b>50 mL</b>

Can be stored at 4°C for 6 months.

**Note:** The amount of powder DMEM to be used may vary from different batches, refer to the bottle to calculate the amount of powder DMEM to be used to achieve a 10× concentration.

### Basic DMEM for cell culture

Reagent	Final concentration	Amount
DMEM 1 ×	N/A	449 mL
FBS	10%	50 mL
100× Glutamax	1%	1 mL
<b>Total</b>	<b>N/A</b>	<b>500 mL</b>

Can be stored at 4°C for 1 month.

### Collagen type I coating solution

Reagent	Final concentration	Amount
Collagen type I	1:100	30 μL
PBS 1×	N/A	2,970 μL
<b>Total</b>	<b>N/A</b>	<b>3 mL</b>

Can be stored at 4°C for 1 week.

### Organotypic gel

Reagent	Final concentration	Amount
Collagen type I	52.5%	5,250 μL
Matrigel	17.5%	1,750 μL
DMEM 10×	10%	1,000 μL
DMEM 1×	10%	1,000 μL
FBS	10%	1,000 μL
<b>Total</b>	<b>N/A</b>	<b>10 mL</b>

Adjust the pH of the organotypic gel by adding 1 N NaOH drop-by-drop until color change is observed (Figure 2C). Maintain on ice. Make fresh before use.

**△ CRITICAL:** Keep the organotypic gel mix always on ice. All gel components must be sterile and handled in Class II Biological Safety Cabinet (BSC).

**Note:** 10 mL of organotypic gel mix will allow you to prepare 10 gels.

**Alternatives:** Commercial matrices, such as Geltrex (Thermo Fisher Scientific cat# A1413301) or Cultrex BME (R&D cat# 3432-010-01) can be used instead of Matrigel. Instead of DMEM, other cell culture media can be used, depending on the cell type used in the experiment.

### Collagen type I coating gel

Reagent	Final concentration	Amount
Collagen type I	70%	1,750 $\mu$ L
DMEM 10 $\times$	10%	250 $\mu$ L
DMEM 1 $\times$	10%	250 $\mu$ L
FBS	10%	250 $\mu$ L
<b>Total</b>	<b>N/A</b>	<b>2.5 mL</b>

Adjust the pH of the collagen gel by adding 1 N NaOH drop-by-drop until color change is observed (Figure 2C). Maintain on ice. Make fresh before use.

**△ CRITICAL:** Keep the collagen type I gel mix always on ice. All gel components must be sterile and handled in Class II Biological Safety Cabinet (BSC).

**Note:** 2.5 mL of collagen type I coating gel will allow you to prepare 10 membranes.

### 1% Glutaraldehyde solution

Reagent	Final concentration	Amount
Glutaraldehyde solution 25%	1%	800 $\mu$ L
PBS 1 $\times$	99%	19.2 mL
<b>Total</b>	<b>N/A</b>	<b>20 mL</b>

Can be stored at 4°C for 1 month.

## STEP-BY-STEP METHOD DETAILS

### Preparation of organotypic gels (Day 1)

⌚ Timing: 2.5 h

This step describes the organotypic gel preparation, which will serve as a 2D matrix to coculture the cells of interest.

#### 1. Gel preparation (Day1).

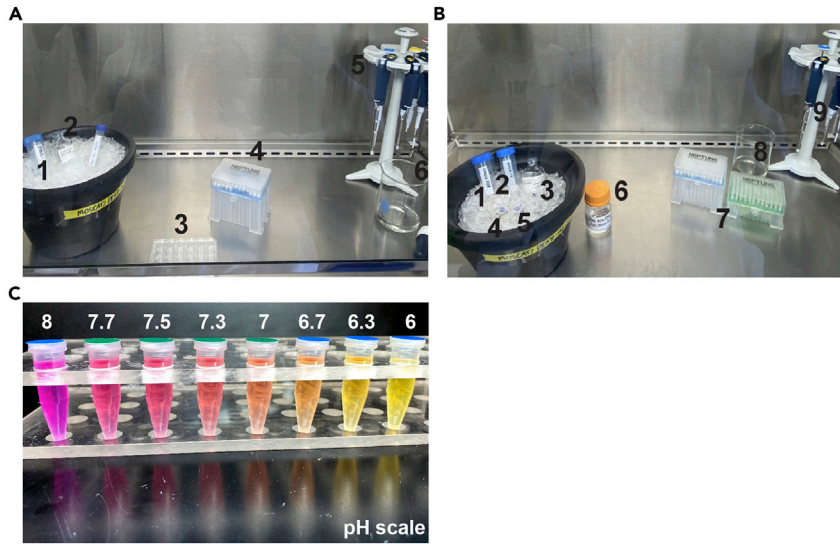
- a. Pre-coat a 24-well plate with collagen type I 1:100 in PBS.
  - i. Add 300  $\mu$ L of 1:100 collagen type I in PBS for 1 h at 20°C–22°C.
  - ii. Wash 3 times with PBS.
  - iii. Add 1 mL of DMEM 1 $\times$  to the wells and keep on ice. See Figure 2A and Methods video S2.

⏸ **Pause point:** Pre-coated 24-well plates can be stored at 4°C in DMEM for up to a week.

#### b. Preparation of organotypic gel mix (10 mL).

- i. Gently mix 5.25 mL of type I collagen, 1.75 mL of Matrigel with 1 mL of 1 $\times$  DMEM, 1 mL of 10 $\times$  DMEM, 1 mL of FBS, and on-ice using pre-cooled tips. Pipette up and down several times with pre-cooled tips until the gel mix becomes homogenous. Keep on ice.
- ii. Gel mix will typically be yellow. Correct gel pH by adding 1 N NaOH drop-by-drop to the gel mix (see note by the end of the step). Gently pipette up and down with pre-cooled tips until the gel mix becomes homogenous. pH is assessed based on phenol red color change (Figure 2C). Keep on ice.
- iii. Remove the DMEM from the pre-coated 24-well plate and add 1 mL of the gel mix using pre-cooled tips. Let the gel polymerize for 1 h at 37°C.





**Figure 2. Coating and organotypic gel mix material**

Materials for coating and organotypic gel mix.

- (A) Cold PBS (1), rat tail collagen type I (2), sterile 24 well plate (3), pre-cooled p1000 tips (4), micropipettes (5), residues beaker with 10% bleach (6).  
 (B) DMEM 10× (1), DMEM 1× (2), rat tail collagen type I (3), FBS (4), Matrigel (5), 1 N NaOH (6), pre-cooled p1000 and p200 tips (7), residues beaker with 10% bleach (8), micropipettes (9).  
 (C) Phenol red pH scale.

- iv. Gently add 1 mL of 1× DMEM and equilibrate the gels for 18–24 h at 37°C.  
 See [Figures 2B and 2C](#) and [Methods video S3](#).

**△ CRITICAL:** Collagen type I and Matrigel must be always on ice to avoid polymerization. Organotypic gel mix must be on ice before adding it to the plate. Use pre-cooled tips in every step before gel polymerization.

**Note:** The pH of the gel needs to be adjusted. Since the gel mix needs to be sterile, cannot be filtered, and should be kept on ice, the pH correction is based on the color change of phenol red of DMEM. NaOH for pH correction can be added drop-by-drop followed by gently pipetting up and down until color change ([Figure 2C](#)). Most of the cells grow in pH from 7.0 to 7.7.

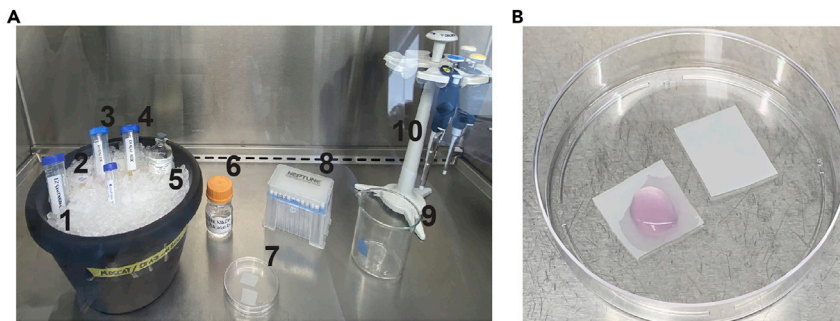
**Alternatives:** Organotypic co-culture can be also performed using cancer cells, adipocytes, or adipose tissue. In this alternative protocol, 300,000 adipocytes or 40 mg of minced white adipose tissue (WAT) in a 100 µL DMEM 1× is mixed with 900 µL of the organotypic gel mix and then seeded in a 24-well plate. Let the gel polymerize for 1 h at 37°C and add 1 mL of 1× DMEM and equilibrate the gels for 18–24 h at 37°C.

## Preparation of cells and membranes (Day 2)

⌚ Timing: 2 h

This step describes the seeding of cancer and stromal cells on top of the organotypic gel and the preparation of the nylon membranes that will support the gel on top of the metallic grid.

2. Prepare cancer/stromal cells and membrane.
  - a. Detach the cells. Use pre-warmed reagents.
    - i. Aspirate the DMEM from the cells.



**Figure 3. Coating material for the nylon membranes**

Materials for coating the nylon membranes.

(A) 1% glutaraldehyde (1), FBS (2), DMEM 1× (3), DMEM 10× (4), rat tail collagen type I (5), Cold PBS (6), rat tail collagen type I (7), 1 N NaOH (8), sterile p100 plate with the desired amount of sterile 100 μm nylon mesh (9), pre-cooled p1000 tips (10), residues beaker with 10% bleach (11), micropipettes (12).

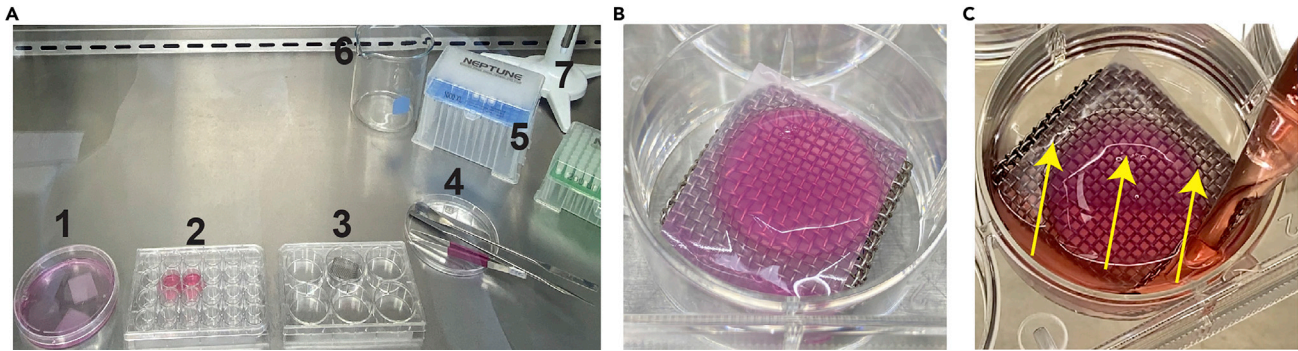
(B) Collagen type I gel mix to form a drop on top of the mesh.

- ii. Wash 1 time with 5 mL of PBS.
- iii. Add 2 mL of 0.25% trypsin and incubate 5 min at 37°C.
- iv. Neutralize the trypsin using 2 mL of DMEM supplemented with 10% FBS.
- v. Centrifuge 5 min at 250 g at 20°C–22°C and resuspend in 2 mL of DMEM supplemented with 10% FBS.
- vi. Count cancer and stromal cells and prepare aliquots containing  $1 \times 10^6$  cells/mL in DMEM supplemented with 10% FBS. Mix the cells in proportion 1:1, by adding 500 μL of each cell type (500,000 cancer cells and 500,000 stromal cells) for making up to 1 mL.

**Note:** This is a standard protocol for cell detaching, however, any other detaching protocol can be used.

- b. Seed the cells onto the equilibrated organotypic gel.
  - i. Aspirate the DMEM from the equilibrated gels using a p1000 micropipette to avoid disruption of the gel.
  - ii. Add 1 mL of cancer/stromal cell mix on top of the gel and let the cells embedded into the gel overnight (18 h) in the incubator.
3. Prepare the pre-coated nylon membrane (Day 2).
  - a. Prepare collagen type I gel mix (2.5 mL).
    - i. Gently mix 1,750 μL of type I collagen with 250 μL of 1× DMEM, 250 μL of 10× DMEM, and 250 μL of FBS on ice using pre-cooled tips. Pipette up and down several times with pre-cooled tips until the gel mix becomes homogenous. Keep on ice.
    - ii. Gel mix will be typically yellow, correct gel pH by adding 1 N NaOH drop-by-drop (see note by the end of the step). Gently pipette up and down with pre-cooled tips until the gel mix becomes pink. Keep on ice.
  - b. Place the desired number of autoclaved membranes in a sterile cell culture p100 or p150 plate. Use 1 membrane per gel.
  - c. Add 250 μL of collagen type I mix per membrane using pre-cooled tips. The collagen type I mix will do a bubble on top of the membrane and then collapse, covering it (Figure 3B). Incubate the membranes with the collagen type I mix for 15 min at 37°C.
  - d. Add 10 mL of ice-cold 1% glutaraldehyde in PBS solution to the plate containing the membranes. Incubate the membranes for 1 h at 4°C. This step will fix the collagen type I gel. Discard the membranes that detach from the plate.
  - e. Aspirate the 1% glutaraldehyde in PBS solution and wash it four times with 5 mL of PBS.





**Figure 4. Gel casting**

Materials for gel casting and mounted organotypic gel culture.

(A) Collagen type I pre-coated membranes (1), organotypic gels with cell coculture (2), sterile grid (3), sterile material, forceps and two spatulae (4), p1000 tips (5), residues beaker with 10% bleach (6), micropipettes (7).

(B) Organotypic gel coculture on top of the structure, from the bottom, stainless steel grid, pre-coated 100 µm nylon mesh, organotypic gel.

(C) Complete cell media incorporation starting from the vertex of the grid to avoid bubbles below the mesh.

- f. Add 10 mL of 1 × DMEM, seal the plates with parafilm and incubate them overnight (18 h) at 4°C.

See [Figures 3A and 3B](#) and [Methods video S4](#).

▣ **Pause point:** Pre-coated nylon membrane can be stored at 4°C in DMEM for up to a week.

△ **CRITICAL:** Collagen type I reagent and gel mix must always be on ice to avoid polymerization. Use pre-cooled tips in every step before gel polymerization.

**Note:** NaOH for pH correction can be added drop-by-drop, followed by gently pipetting up and down until color change is observed ([Figure 2C](#)). Most of the cells grow in pH from 7.0 to 7.7.

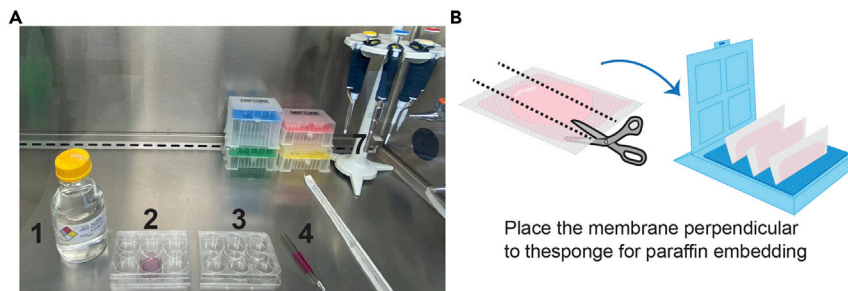
**Alternatives:** For coculture of cancer cells with adipocytes, on day 2, only cancer cells are seeded on top of the gel as the adipocytes, or the WAT is already embedded in the gel.

### Gel casting (Day 3)

⌚ **Timing:** 20 min

In this step, the organotypic gel containing the cells will be placed on top of the pre-coated membrane placed over the grid. This will build an air-liquid culture as the gel will not be in contact with the media, but the nylon membrane will moisten it by capillarity.

4. Place the 2.5 cm<sup>2</sup> stainless steel grid in a 6-well plate.
5. Place the 2.5 cm<sup>2</sup> pre-coated 100 µm nylon membrane on top of the grid (organotypic structure).
6. Place the organotypic gel containing the cells on the top organotypic structure ([Figure 4B](#)).
  - a. Gently remove the DMEM from the organotypic gels using a p1000 micropipette to avoid gel disruption.
  - b. Carefully take the gel using two sterilized spatulas and place it on top of the grid + membrane structure.



**Figure 5. Membrane fixation and embedding**

(A) Materials for membrane fixation and embedding (A) 10% neutral buffered formalin (1), organotypic gels with cell coculture (2), six-well plate (3), sterile forceps (4).  
(B) Schematic of membrane cutting and how to place the membrane perpendicularly to the cassette sponge before paraffin embedding.

7. Add approximately 6–7 mL of DMEM supplemented with 10% FBS to cover the structure and avoid touching the gel. The media is added from the vertex of the grid to prevent the formation of bubbles below the mesh (Figure 4C). Replace the media every 2–3 days.

See Figures 4A, 4B, and 4C and Methods video S5.

⚠ **CRITICAL:** Passing the gel from the well to the structure is a challenging step. We encourage to practice with pilot gels before an experiment.

⚠ **CRITICAL:** Avoid bubble formation under the grid; this could dry your gel.

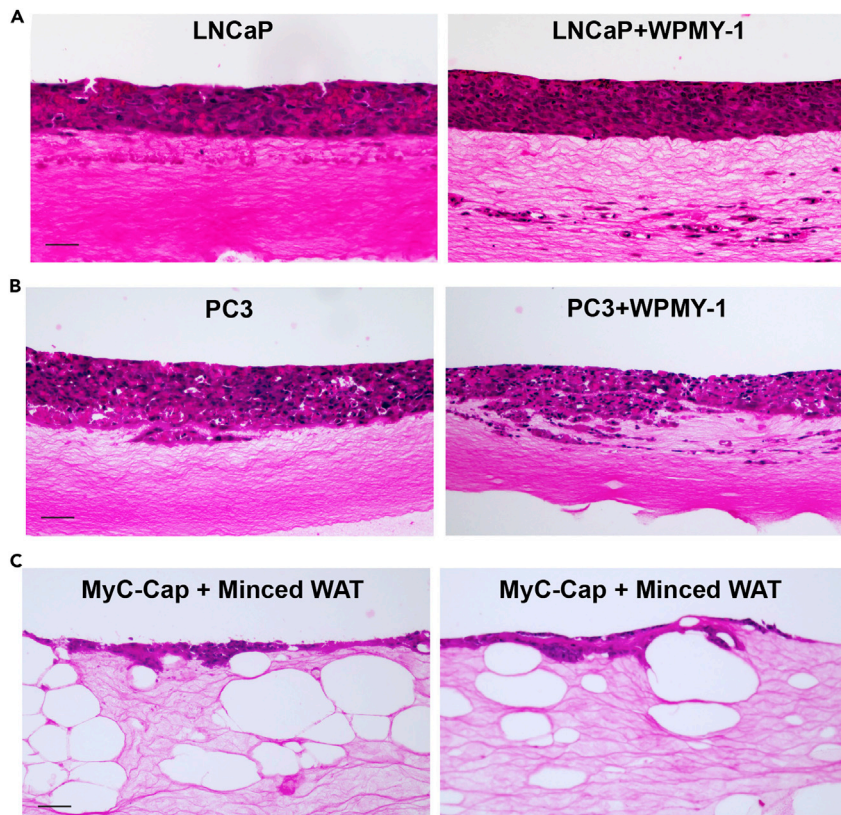
### Membrane processing (Day 15)

⌚ **Timing:** 3 h

After 15 days, the organotypic gel will have collapsed on top of the membrane. It will remain attached to the membrane after fixation. We will fix the membranes and process them as paraffin-embedded samples that can be cut to perform any staining.

8. Fix the membrane.
  - a. Label 6-well plate with 2 mL of 10% Neutral buffered formalin (NBF).
  - b. Using a pair of forceps, pick up carefully the membrane from the vertex and the gel and transfer it into the NBF. The gel will be collapsed and “fused” with the membrane.
  - c. Let the membrane fix for 2 h at 20°C–22°C without movement.
  - d. Transfer the membrane from the NBF to the new 6-well plate containing 70% ethanol.
9. Paraffin embedding of the membrane.
  - a. Cut the membrane into three pieces and place them in a tissue cassette (Figure 5B). The membrane can be cut on top of an eraser using a scalpel or cut using a pair of scissors (Methods video S6). Keep it in 70% ethanol until paraffin embedding. It is recommended to process the samples as fresh as possible.
  - b. Embed the samples in paraffin, placing the membrane as in Figure 5.
10. Cut the paraffin blocks and staining.
  - a. Embedded membranes can be cut into 5 μm slices in a microtome.
  - b. Standard staining protocols can be applied to sections such as hematoxylin and eosin (H&E) (Figure 6).

See Figures 5A, 5B, 6A, 6B, and 6C and Methods video S6.



**Figure 6. H&E staining from organotypic sections**

(A) LNCaP and LNCaP cocultures with WPMY-1.

(B) PC3 and PC3 cocultured with WPMY-1.

(C) MyC-Cap cocultured with mince WAT from a WT mouse. Scale bar 50  $\mu$ m.

▣▣ **Pause point:** The fixed membrane can be stored at 4°C in 70% ethanol for up to a week. However, we recommend processing the sample as fresh as possible.

## EXPECTED OUTCOMES

The outcome of the organotypic coculture will be paraffin blocks that can be cut and processed as human or mouse tissue to measure invasion or any staining analysis.

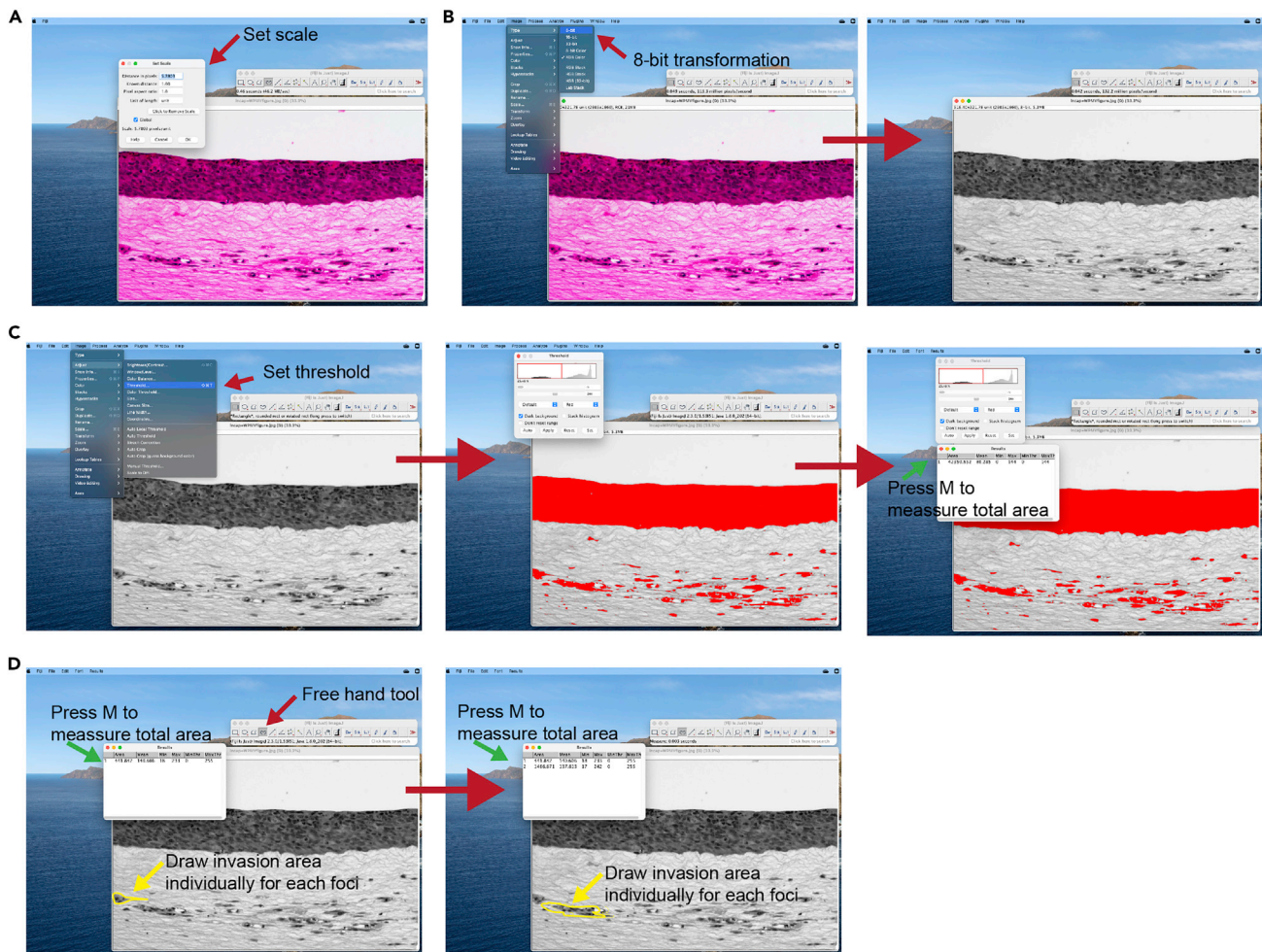
See [Figure 6](#).

## QUANTIFICATION AND STATISTICAL ANALYSIS

The organotypic coculture allows the study of the invasive capacity of cancer cells and their aggressiveness by considering the ability of the cells to go through the matrix and the number of invasion events. It is not uncommon that some cell types show a discrete invasion pattern. For that reason, different quantification methods can be used.

After H&E or immunostaining, take several pictures of representative areas of the membrane. The images must be digitalized, and the invasion index is calculated using Fiji. For all the methods, the first step will be to set the scale in Analyze>set scale, be sure to select the Global option. In that way, the scale will be set up for all the images you will use in that session. Analyze at least five different areas from 3 independent experiments to acquire statistical significance.





**Figure 7. Quantification method A**

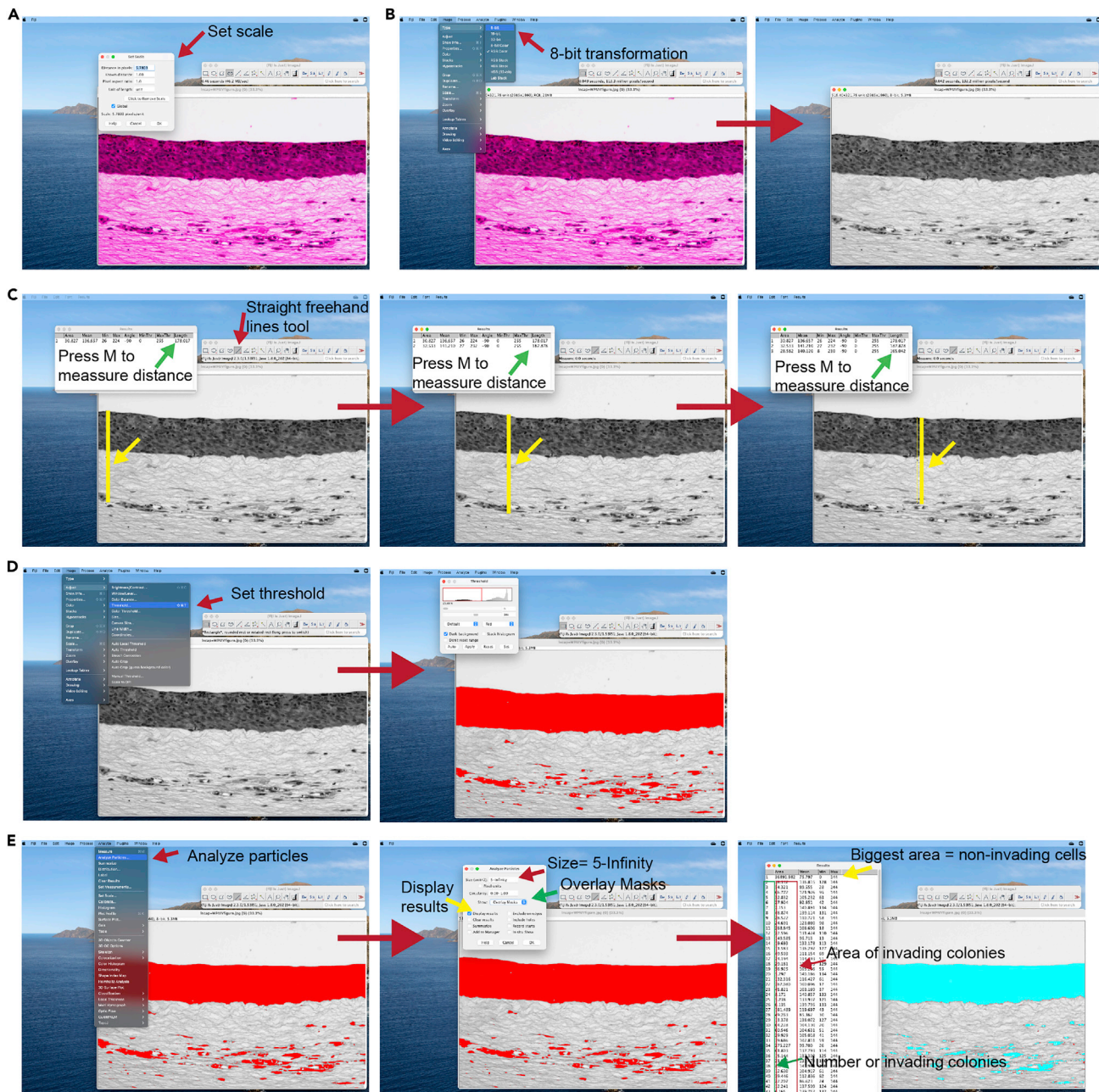
- (A) Set scale.
- (B) 8-bit transformation of the image.
- (C) Set threshold for total area measurement.
- (D) Draw the area of invading cells using the free hand tool.

### For cells with an invasive phenotype

**Method A.** Cell invasion index can be calculated by measuring the total area of cells, invading and non-invading cells, and non-invading area, area of non-invading cells. To obtain the total area and non-invading area values, open the image in Fiji and transform the image to 8-bit. For the total area, use the threshold tool and tune it until all the area with cells become red, press M to measure the area. For the non-invading area, use the freehand selection tool and draw the area of non-invading cells, press M to measure the area (Figure 7). Adapted from (Gaggioli et al., 2007).

$$\text{Cell invasion index} = 1 - (\text{non-invading area} / \text{total area})$$

**Method B.** Cell invasion index can be calculated by measuring the mean cord Y (MCY) which it will be the average distance of invasion in the Y axis, the number of invading colonies (N), and the summatory of the invading areas (A). To obtain these values, open the image in Fiji and transform the image to 8-bit. For MCY value, select the "straight" freehand lines tool and draw a line starting on top of the non-invading area and ending in the deeper part of an invading colony, press M to



**Figure 8. Quantification method B**

- (A) Set scale.  
 (B) 8-bit transformation of the image.  
 (C) Straight freehand tool to draw the distance from the top of non-invading cells to the bottom of the invasive colony.  
 (D) Set threshold for particles and total area measurement.  
 (E) Analyze particles to count invasive colonies and obtain the total area of each one.

obtain the length of the line, and repeat this step for every invading colony. The MCY value will be the media of all the measurements. For the N value, use the threshold tool and tune it until all the area with cells become red. Then, use Analyze>Analyze particles, this tool will count the isolated colonies and the non-invading cells which will have the biggest area value. Therefore, the N value will be the total number of particles detected – 1. For A value, make a summatory of all the areas

obtained by Analyze particles tool, except the biggest area, which will be the non-invading cells (Figure 8). Adapted from (Nystrom et al., 2005).

$$\text{Cell invasion index} = \text{MCY} \times \text{N} \times \text{A}$$

Mean cord Y (MCY), Number of particles (N), Summatory of the areas (A).

### LIMITATIONS

Organotypic models are a very useful tool that successfully recapitulates invasion in human tissues. However, timing optimization must be performed for the cell lines of interest. Some cells will invade faster than others, and very aggressive models will be able to digest the matrix completely. Before an experiment, we encourage evaluating the cells invasion capability and the specific time for harvesting the organotypic matrix.

### TROUBLESHOOTING

#### Problem 1

Add too much NaOH; the media become pinker than expected. Steps 1.b.ii and 3.a.ii.

#### Potential solution

We recommend small amounts of NaOH each time, typically, it will be necessary around 10–15  $\mu\text{L}$  per 1 mL of gel. Mammalian cells typically grow from 7.0 to 7.7 pH, see Figure 2C for phenol red color reference.

#### Problem 2

When picking the gel up to transference to the structure, it can slipper and fall. Step 6.b.

#### Potential solution

We recommend preparing a couple of extra gels for each experiment. If a gel falls outside the plate, it cannot be used.

#### Problem 3

Some bubbles form below the organotypic structure when adding the media. Step 7.

#### Potential solution

Completely remove the media and add it again slowly from the vertex of the grid until approximately 5 mm from the opposite vertex. The possible bubbles will escape through the upper vertex.

See Figure 4 and Methods video S5.

#### Problem 4

Terminate the experiment before expected because of shrinkage of the gel. Some stromal cell lines process the collagen matrix very fast, provoking the shrinkage of the gel.

#### Potential solution

Optimization for different cell types must be performed.

#### Problem 5

When doing H&E staining there are no cells in the slide. This may occur when the cutting of the paraffin block is not deep enough to achieve the cells. Steps 10a and 10b.

#### Potential solution

Cut the paraffin blocks deeper checking by H&E staining when there are enough cells in the slide to perform the analysis.



## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maria T. Diaz-Meco Conde ([mtd4001@med.cornell.edu](mailto:mtd4001@med.cornell.edu)).

### Materials availability

This protocol did not generate new unique reagents.

### Data and code availability

This study did not generate new datasets.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101635>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, T.C.-D., M.T.D.-M., and J.M.; Investigation, T.C.-D., A.C.L., and A.D. Writing–Review & Editing, T.C.-D., A.C.L., A.D., and M.T.D.-M.; Funding Acquisition, M.T.D.-M. and J.M.; Supervision, M.T.D.-M. and J.M.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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