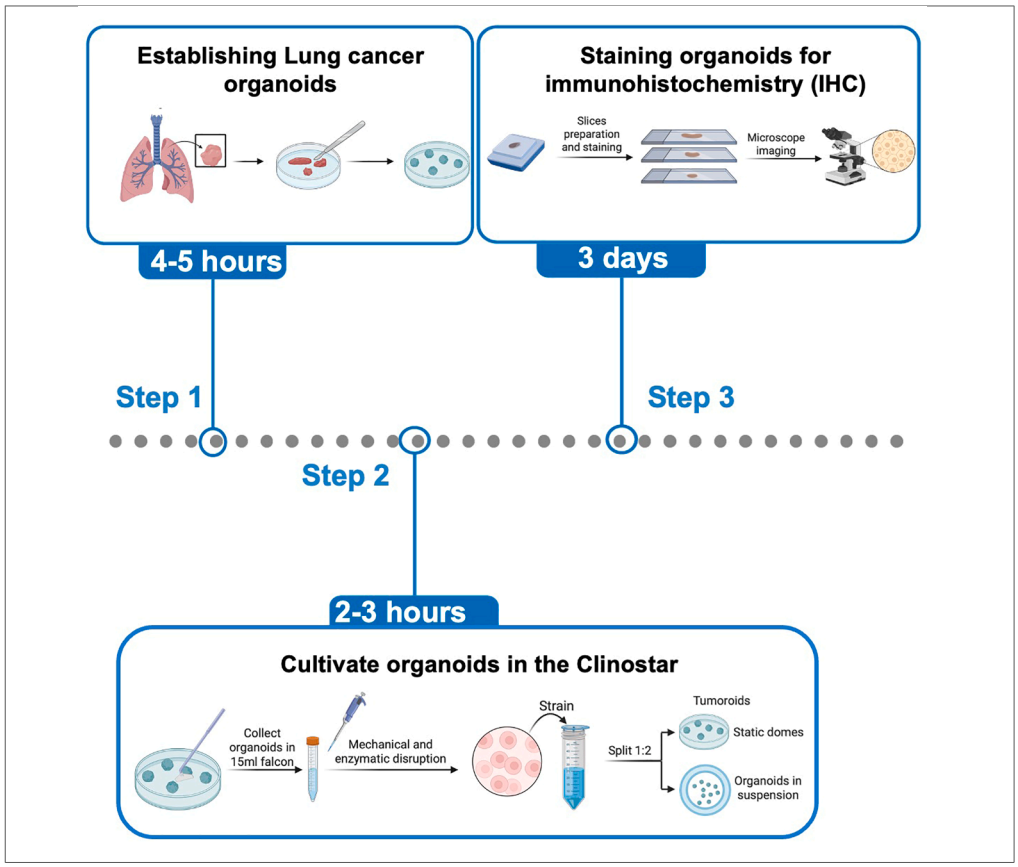
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Protocol for the establishment and characterization of lung tumoroids from surgical resection without the use of Matrigel



Organoids are vital for studying cancer mechanisms and treatment resistance, but their growth usually depends on costly, animal-derived Matrigel. Here, we present a protocol for expanding and maintaining lung-derived organoids without the need for Matrigel. We describe steps for establishing lung cancer organoids, staining organoids for immunohistochemistry, and cultivating them with ClinoStar. By eliminating dependence on Matrigel and its associated costs, this protocol enhances accessibility and scalability, making organoid-based research more feasible for cancer studies and therapeutic development.

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

Federico Donà, Silvia Erratico, Luigi Maria Terracciano, ..., Charlotte K.Y. Ng, Giuseppe Marulli, Salvatore Piscuoglio

salvatore.piscuoglio@hunimed.eu

Highlights

Protocol to establish lung patient-derived organoids from surgical resections

Detailed procedure to expand tumor organoid models without Matrigel

Organoids without Matrigel recapitulate key characteristics of primary tumor

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Protocol for the establishment and characterization of lung tumoroids from surgical resection without the use of Matrigel

Federico Donà,[1,5] Silvia Erratico,[1] Luigi Maria Terracciano,[1,2] Debora Brascia,[2,3,4] Emanuela Re Cecconi,[2,3,4] Veronica Maria Giudici,[2,3,4] Paola Bossi,[1,2] Charlotte K.Y. Ng,[1,2] Giuseppe Marulli,[2,3,4] and Salvatore Piscuoglio[1,2,4,6,*]

[1]IRCCS Humanitas Research Hospital, Via Manzoni 56, Rozzano, 20089 Milan, Italy

[2]Department of Biomedical Sciences, Humanitas University, Via Rita Levi Montalcini 4, Pieve Emanuele, 20072 Milan, Italy

[3]Thoracic Surgery Unit - IRCCS Humanitas Research Hospital, Via Manzoni 56, Rozzano, 20089 Milan, Italy

[4]These authors contributed equally

[5]Technical contact

[6]Lead contact

*Correspondence: salvatore.piscuoglio@hunimed.eu
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SUMMARY

Organoids are vital for studying cancer mechanisms and treatment resistance, but their growth usually depends on costly, animal-derived Matrigel. Here, we present a protocol for expanding and maintaining lung-derived organoids without the need for Matrigel. We describe steps for establishing lung cancer organoids, staining organoids for immunohistochemistry, and cultivating them with ClinoStar. By eliminating dependence on Matrigel and its associated costs, this protocol enhances accessibility and scalability, making organoid-based research more feasible for cancer studies and therapeutic development.

BEFORE YOU BEGIN

This protocol describes in detail how to develop lung tumor organoids from surgical resections and how to maintain them in culture for up to 10–12 days without the use of extracellular matrix components. It consists of three major steps. Firstly, 1) we will generate lung tumoroids from surgical specimens in a static culture system (adapted from Sachs et al., 2019).[1] Which is followed by the expansion of the culture and transfer into the ClinoReactor under Matrigel-free conditions. Lastly, we characterized the tumoroids via immunohistochemistry to compare with standard culture systems.

The protocol has been optimized for lung cancer, but it can be adapted for other organoid types (Colon Cancer), including those derived from healthy tissue (lung tissue has been tested).

1. A complete list of reagents and plasticware is located in the key resources table.
2. Pre-warm the 6-well culture plate for 12–15 h in a 37°C incubator.
3. Thaw the concentrated Matrigel (bottle or small aliquots, we use undiluted reagent) at 4°C the day before your experiment.
4. All centrifuges, unless otherwise stated, are set to 4°C.
5. All steps, except paraffin embedding, should be performed under sterile conditions in a biological safety cabinet.
6. Prepare the following media and sterile filter using a 0.22 μm filter (see "Materials" for recipes):
   a. Tissue medium.

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b. Basal adDMEM/F12 medium with antibiotics.
c. Basal DMEM medium with antibiotics.
d. DPBS with antibiotics.
e. Stopping medium.
f. Dissociation medium.
g. Lung medium.
h. Cryopreservation medium.

Institutional permissions

Written informed consent was obtained from all patients. The study was performed in accordance with the Helsinki Declaration and approved by the ethics committee (no. 4174).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TTF-1/NKX2.1 monoclonal antibody (8G7G3/1) (1:200)	Thermo Fisher Scientific	Z2069MS
Anti-p40 - DeltaNp63 antibody [BC28] (1:100)	Abcam	ab172731
Cytokeratin 7-specific polyclonal antibody (1:800)	Proteintech	17513-1-AP
Cytokeratin 5/6 monoclonal antibody (1:500)	Proteintech	68295-1-IG
Biological samples		
Human lung cancer tissue	Humanitas Research Hospital	
Chemicals, peptides, and recombinant proteins		
DMEM (high glucose, w/o L-glutamine and sodium pyruvate)	Sigma-Aldrich	D5671
Advanced DMEM/F12 (adDMEM/F12)	Life Technologies	12634-034
Fetal bovine serum (South America)	LubioScience	S181H-500
GlutaMAX (100x)	Gibco	35050061
HEPES (1 M)	Thermo Fisher Scientific	15630056
Gentamicin	Gibco	15750-060
RPMI 1640 medium	Gibco	72400-021
Antibiotic antimycotic	EuroClone	ECM0010D
Primocin	InvivoGen	ant-pm-1
Deoxyribonuclease I type IV from bovine	Sigma-Aldrich	D5025-150KU
Hyaluronidase type IV from sheep tested	Sigma-Aldrich	H6254-500MG
Collagenase IV	Worthington Biochemical	LS004189
Matrigel growth factor reduced basement membrane matrix	Corning (Merk)	CLS356231-1EA
BD Pharm Lyse, 10X concentrate	BD Biosciences	555899
DPBS (w/o calcium magnesium and phenol red)	Sigma-Aldrich	D8537
DMSO suitable for cell culture	Sigma-Aldrich	D2650
Rock inhibitor (Y-27632 dihydrochloride)	AbMole BioScience	M1817
BSA	Sigma	A9418
B-27 supplement (50X)	Thermo Fisher Scientific	17504044
Nicotinamide	Sigma-Aldrich	N0636
N-acetyl-l-cysteine	Sigma-Aldrich	A9165
A83-01	STEMCELL Technologies	72022
FGF-10	STEMCELL Technologies	78037
Recombinant human Noggin	PeproTech	120-10C
R-spondin 1 protein	R&D Systems	4645-RS-100
FGF-7	PeproTech	100-19
SB202190	Sigma-Aldrich	S7076
TrypLE Express 1x - phenol red	Gibco	12605-010
Corning cell recovery solution	Merck	CLS354253

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HistoGel specimen processing gel	Fisher Scientific	12006679
Tris-EDTA antigen retrieval buffer (50×)	Proteintech	PR30002
Hydrogen peroxide 30%	Sigma-Aldrich	1.07209
TWEEN 20	Sigma-Aldrich	P1379
Eukitt quick-hardening mounting medium	Sigma-Aldrich	03989
Harris hematoxylin solution, modified	Sigma-Aldrich	HHS32
Critical commercial assays		
MACH 1 Universal HRP-Polymer Detection kit	Biocare	M1U539 G
Software and algorithms		
Software image acquisition	Olympus CellSens	N/A
ImageJ	N/A	N/A
Other		
6 well-plates	Sarstedt	83.3920.300
Corning bottle-top vacuum filter	Merck	CLS431098
Corning CoolCell LX cell freezing container	Merck	CLS432002
Corning 100 µm cell strainer	Merck	352360
Corning 40 µm cell strainer	Merck	352340
Corning CoolCell freezer container	Merck	CLS432000
ClinoStar, hypoxia module ready	CelVivo	30003
ClinoReactor	CelVivo	10004-12
Microscope inverted led phase contrast	Olympus	CKX53

MATERIALS AND EQUIPMENT

For all the media preparation

- Use a Corning bottle-top vacuum filter to filter the complete solution.
- Add basal media to the top of the Corning bottle.
- Add all other components.
- Filter the complete solution.

Tissue medium		
Reagent	Final concentration	Amount
RPMI	–	428 mL
Glutamax (100x)	1x	5 mL
HEPES	1 M	10 mL
Gentamicin	50 mg/mL	2 mL
Antibiotic / Antimycotic (100x)	1x	5 mL
FBS	10%	50 mL
Store at 4°C for up to one month.		

***Basal DMEM medium* with Antibiotic solution (DMEM+++)**		
Reagent	Final concentration	Amount
DMEM	–	482 mL
Glutamax (100x)	1x	5 mL
HEPES	1 M	5 mL
Gentamicin	50 mg/mL	2 mL
Antibiotic / Antimycotic (100x)	1x	5 mL
Primocin	200 µg/mL	1 mL
Store at 4°C for up to one month.		

***Basal* adDMEM/*F12 medium* with Antibiotic solution (adDMEM/F12+++)**		
Reagent	Final concentration	Amount
adDMEM/*F12*	–	484 mL
Glutamax (100x)	1x	5 mL
HEPES	1 M	5 mL
Antibiotic / Antimycotic (100x)	1x	5 mL
Primocin	200 µg/mL	1 mL
Store at 4°C for up to one month.		

DPBS with Antibiotic solution (DPBS+++)		
Reagent	Final concentration	Amount
DPBS (1x)	–	491 mL
Primocin	200 µg/mL	2 mL
Gentamicin	50 mg/mL	2 mL
Antibiotic / Antimycotic (100x)	1x	5 mL
Store at 4°C for up to one month.		

Stopping medium		
Reagent	Final concentration	Amount
DPBS+++	–	45 mL
FBS	10%	5 mL
Make fresh every time.		

Dissociation medium		
Reagent	Final concentration	Amount
DMEM+++	–	4,44 mL
Collagenase IV	50 mg/mL	200 µL
DNAse I	10000 Kunitz U/mL	10 µL
Hyaluronidase	339 kU/mL	14 µL
BSA	1%	333 µL
Rock Inhibitor	10 µM	5 µL
Make fresh every time.		

Lung medium[1]		
Reagent	Final concentration	Amount
adDMEM/F12+++		9562 µL
B27 supplement	1X	200 µL
Nicotinamide	10 mM	100 µL
N-acetyl-l-cysteine	1.25 mM	25 µL
A83-01	500 nM	5 µL
FGF-10	100 ng/mL	10 µL
Recombinant human Noggin	100 ng/mL	10 µL
R-spondin 1 protein	500 ng/mL	50 µL
Rock inhibitor	10 µM	10 µL
FGF-7	25 ng/mL	25 µL
SB202190	10 µM	3,3 µL
Thaw frozen aliquots of enzymes on ice. Store the solution at 4°C for up to 1 week.		

Cryopreservation medium		
Reagent	Final concentration	Amount
RPMI medium	–	35 mL
FBS	20%	10 mL
DMSO	1%	5 mL
Rock Inhibitor	10 μM	5 μL
Store at −20°C in 5 mL aliquots (up to 6 months).		

STEP-BY-STEP METHOD DETAILS

Establishing lung tumoroids from surgical resection

Timing: 4–5 h

This section outlines the derivation of organoids from patient-derived lung tissue. Tissue is enzymatically and mechanically dissociated, mixed with Matrigel, and seeded in 6-well plates. Informed consent was obtained from each patient before collecting samples (ethical approval #3631).

1. Tissue is collected in a 50 mL Falcon tube containing *Tissue Medium* and processed on the same day or the next day at 4°C (no quality loss observed).

   Note: Perform all steps on ice unless otherwise stated.

2. Transfer tissue to a 100 mm petri dish and wash with cold *DPBS+++* to minimize contamination:
   a. Incubate tissue in cold *DPBS+++* for 10 minutes.
   b. Discard DPBS and repeat wash three times.

   ⚠ CRITICAL: Do not allow tissue to dry during washes.

3. The "clean" tumor piece is split into three parts (Figure 1A):
   a. Fixation/histology.
   b. Organoid formation/slow freezing.
   c. SNAP freezing.

   ⚠ CRITICAL: If the surgical specimen is too small (<0.5 grams), the slow freezing and/or the histology part could be removed (usually in a hospital a specimen is banked and stored for diagnosis that could be used as a paraffined sample).

4. For fixation/histology of the primary tumor (at 18°C–21°C).
   a. Cut a 0.5 × 0.5 cm of tissue fragment.
   b. Transfer to a histology cassette using plastic forceps.
   c. Place the cassette in 8% formalin at 18°C–21°C for 12 h.
   d. Process with a small biopsy cycle, embed in paraffin, and store at RT.

   Pause point: The tissue can be stored at 18°C–21°C until further processing.

5. Minced the remaining tissue using scalpels or scissors (Figure 1B).
6. A small fraction (if the tissue is big enough) can be stored as slow freezing for future dissociation.
7. For slow freezing specimens:
   a. Add 1 mL of cryopreservation medium into one cryovial.
   b. Add a small fraction of minced tissue per vial.
   c. Place the cryovials in a freezing container as soon as possible and freeze for 15–20 h at −80°C.

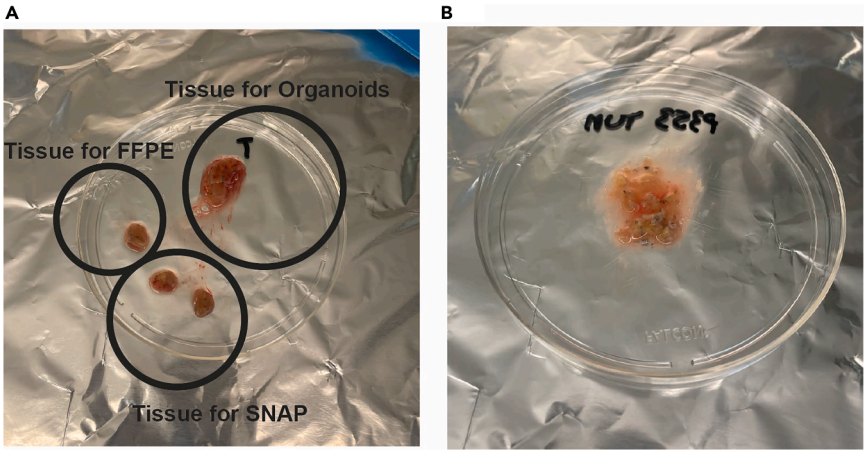


Figure 1. Examples of lung tissue from surgical resection

(A) Lung cancer tissue is divided in three parts for the different required section: 1) Tissue for FFPE, 2) Tissue for SNAP and 3) Tissue for organoids.

(B) Minced tissue from (A) that will be used to obtain organoids.

d. Transfer the cryovials the following day to liquid nitrogen for long-term storage.

8. Place the minced tissue in 5 mL Dissociation Medium in a 15 mL Falcon. Troubleshooting 1.
9. Insert the falcon in a rotator (inside a cell culture incubator) and rotate at 20 rpm at 37°C for 40–60 minutes.

Note: Check every 15 min under a microscope to check the increase number of single cells released in the medium from the minced tissue with the increasing of incubation time.

10. Stop the dissociation with the *Stopping medium* in a ratio of 1:1 and move the liquid in a 50 mL falcon (if not already present).
11. Add cold *DPBS+++* up to 30 mL.
12. Filter through 100 μm strainer into a new Falcon.

⚠ CRITICAL: With a 5 mL syringe's plunger, smash everything left on the top of the filter, which was previously rinse with cold *DPBS+++* helping the filtering step.

13. Wash everything with 20 mL cold *DPBS+++*, tilt the cell strainer, and collect the solution dropping out from the bottom of the filter before discarding the strainer.
14. Centrifuge the filter suspension for 5 min at 400 g at 4°C.

Note: All centrifuge steps are conducted at 4°C unless otherwise stated.

⚠ CRITICAL: If the tissue is highly fatty or necrotic, perform a preliminary centrifuge at 200 g for 5 min, which will results in the presence in the supernatant of undesired sample that will be discarded.

15. Aspirate carefully the supernatant, avoiding touching the pellet.
16. If the pellet is red-colored (indicative of the presence of red blood cells), add 1 mL of Red Blood Lysis buffer (1x) (Figure 2).
17. Incubate for 3 min at RT.
18. Stop the reaction by filling up the 40 mL DPBS.
19. Centrifuge the solution for 5 min at 300 g.
20. Aspirate the supernatant, suspend the pellet in 10 mL *DPBS+++* and rinse it

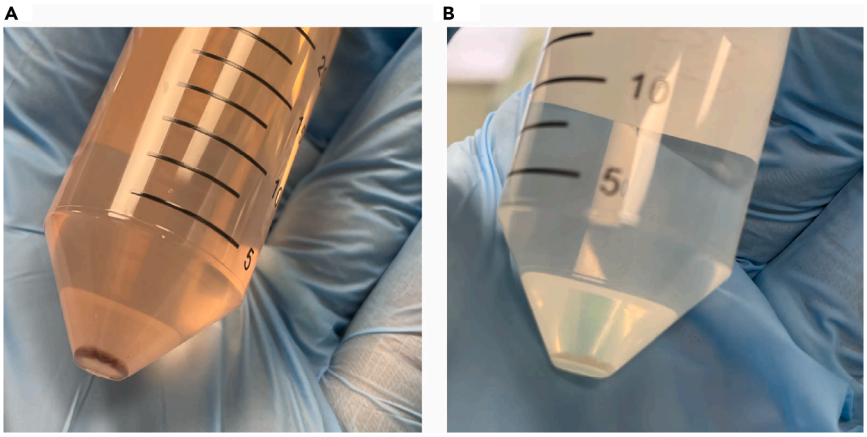


Figure 2. Pre- and post-Red Blood Lysis

(A) Pellet of dissociated tissue that has a red colored features due to red blood cells presence.

(B) The same pellet from (A) after Red Blood Lysis incubation in which red blood cells are eliminated and the pellet has a white colored feature.

21. Centrifuge the solution for 5 min at 300 g.
22. Remove the supernatant and resuspend in 1 mL of *advDMEM/F12+++* (the volume can change upon the pellet size).
23. Mix 10 µL trypan blue + 10 µL cell suspension and count manually on hemocytometer.
24. Centrifuge the whole cell suspension for 5 min at 300 g.
25. Remove the supernatant.
26. Place the tube on ice and resuspend the pellet with the required volume of Matrigel.

Note: We plate 100,000 cells/ 20 µL dome of *Matrigel* (10–12 drops in a well from 6 well plate).

⚠ CRITICAL: Matrigel solidifies at 18°C–21°C, so keep the Matrigel aliquots and later cell suspension on ice until the last minute before plating.

27. Take the pre-warmed plate out of the incubator.
28. Plate 20 µL domes in the plate at 18°C–21°C.
29. After 1–2 min, invert the plate and transfer the plate to the 37°C incubator.
30. Leave the plate for 20 min, allowing the Matrigel to solidify.
31. Add 2 mL of the appropriate organoid culture medium.

Note: Tumoroids formation and grow required an average of 2 weeks (which is patient-specific) (Figure 3).

Passing lung tumoroids from static domes to suspension without Matrigel

Timing: 2–3 h

In this step, we demonstrate that lung tumoroids can grow and expand in suspension without Matrigel using the Clinostar bioreactor. We recommend maintaining parallel dome cultures under static conditions for each patient, in case the tumoroid line does not grow in suspension. Passage of lung tumoroids should be performed when organoids are fully formed and the domes are confluent (Figure 4A). Additionally, to improve the success of growing organoids without Matrigel, it is advisable to start with at least two wells of a 6-well plate filled with domes containing formed organoids (approximately 20–24 domes in total).

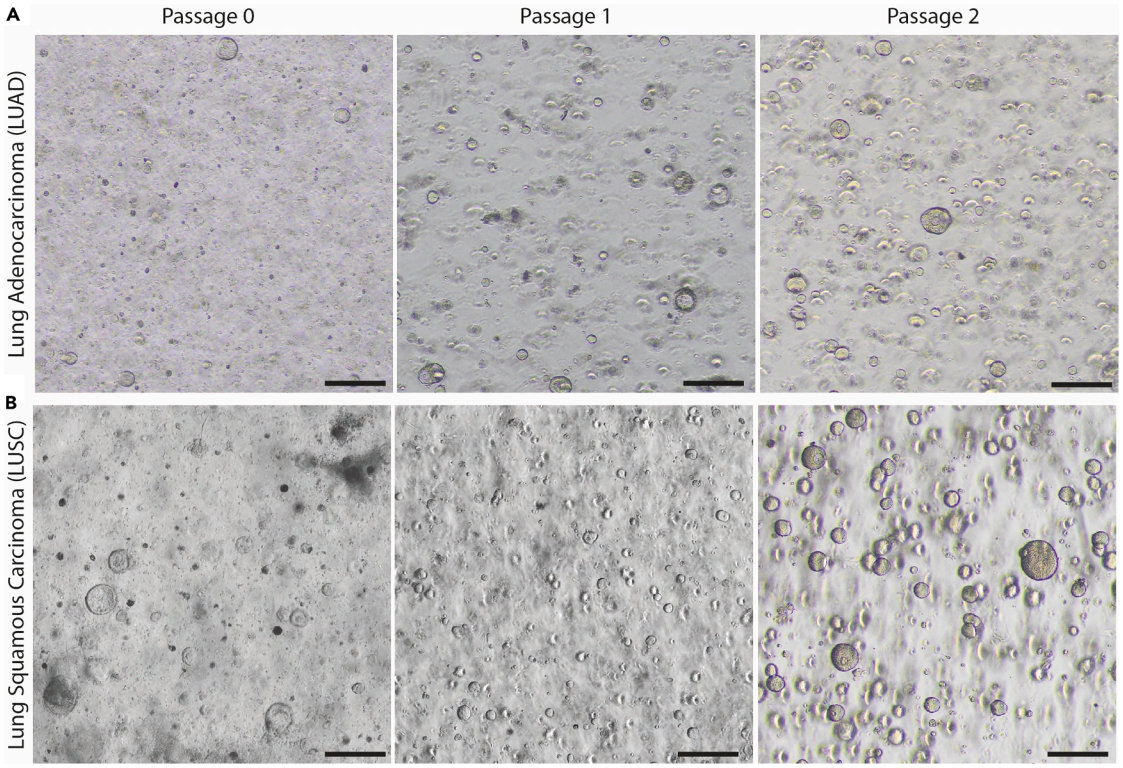


Figure 3. Examples of lung organoids established from surgical resection

Both (A) and (B) are showing examples of organoids established from surgical resection. Each passage is indicated on the top of each column, and the expansion step has been done with TrypLE. Scale bar= 200 μm.

32. Place the required number of Matrigel aliquots in the fridge to thaw the day before.
33. Remove the medium from the wells with tumoroids that need to be split.

Note: If there is more than one well for each culture condition, you can pool all together up to 2 confluent wells.

34. Add 1000 μL of cold DPBS+++ per well:
35. Gently scratch all the domes with a scraper.
36. Pool together the wells in a 15 mL falcon and put on ice.
37. Rinse all wells from which organoids were collected once with 500 μL of cold DPBS+++ and fill the 15 mL falcon with cold DPBS+++ up to 10 mL.
38. Centrifuge at 400 g for 5 min at 4°C.
39. Remove supernatant.
40. Add 1000 μL of TrypLE Express for each well and pipette vigorously to break down the pellet.
41. Incubate in a 37°C water bath for 3 minutes and check under the microscope (keeping the falcon with a slight inclination it is possible to observe the cells under the microscope).

⚠ CRITICAL: Repeating the last step until the organoids are disrupted as single cells or clusters (do not incubate more than 20 min). Troubleshooting 2.

Note: Check under the light microscope when single cells are obtained.

42. Add the same volume of cold Stopping medium to inactivate TrypLE activity.
43. Add cold DPBS+++ up to 10 mL.

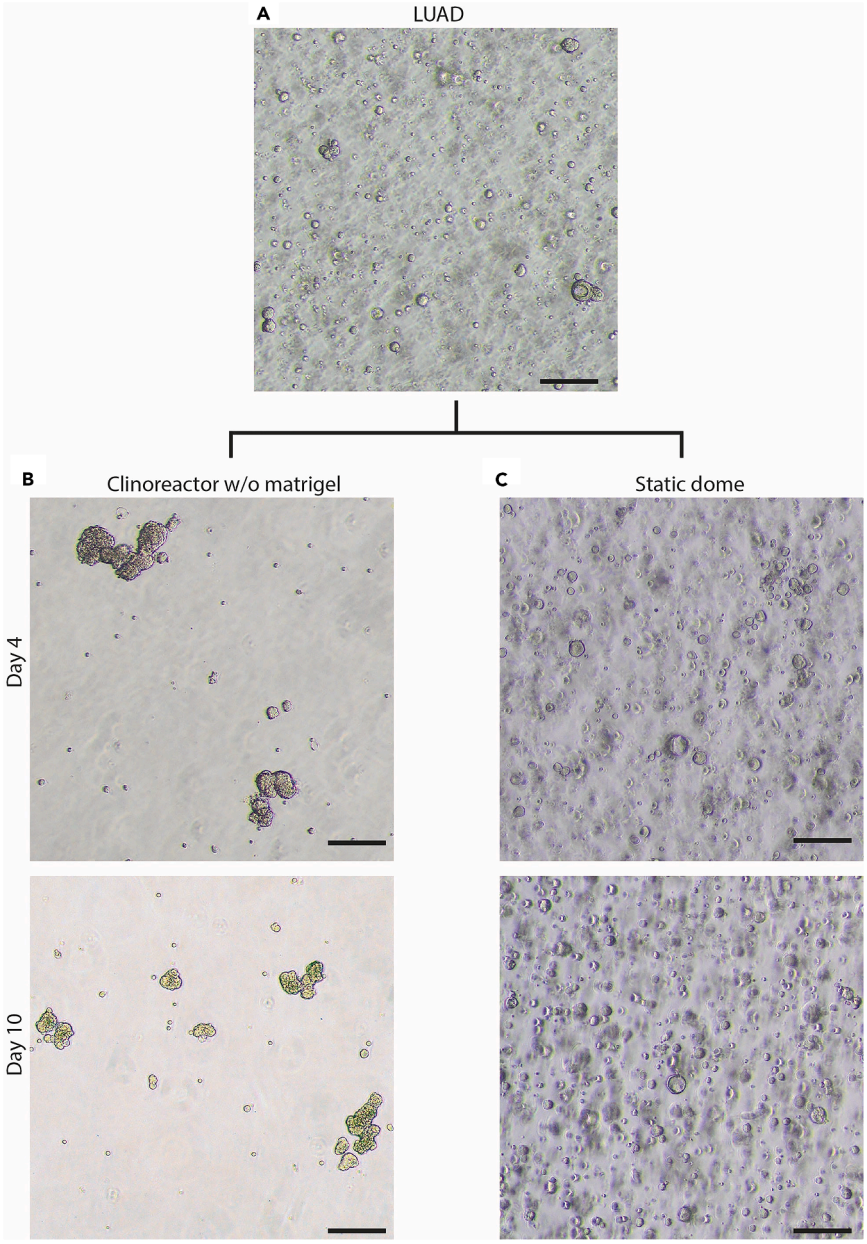


Figure 4. Examples of lung organoid expansion

(A) Example of PDOs culture ready to be split and plate in the Clinoreactor. Both (B) and (C) are showing examples of organoids established from the same stable organoid line at day 4 (top) and day 10 (bottom). In (B) shows organoids growth in the Clinoreactor without Matrigel and in (C) shows organoids growth with matrigel. Scale bar= 200 μm.

44. Filter the cell solution with a 40 μm filter (to eliminate big tumoroids not fully dissociated) in a 50 mL falcon.
45. Wash the filter with 5 mL cold DPBS+++.
46. Move the filter solution in a 15 mL falcon.
47. Centrifuge at 300 g for 5 min at 4°C.
48. Remove the supernatant.
49. Centrifuge at 300 g for 5 min at 4°C.
50. Resuspend the pellet in 500 μL cold advDMEM/F12+++.

51. Count the cells using a hemocytometer.

Note: At this stage of the protocol, the total number of cells will be divided into two Falcon tubes. One tube will be used to plate the cells in domes, as described above (see Sections 25–30 of organoid establishment) (Figure 4C). The other tube will contain between 1.5–2 x 10^6 cells, which will be plated without Matrigel in the ClinoReactor (ClinoReactor 10004-12 from CelVivo), following the manufacturer's protocol with slight adaptations (Figure 4B).

52. Hydrated and equilibrated the Clinoreactor following the company instructions.
53. Remove the media from the equilibrated ClinoReactor via the top port.
54. Fill the chamber with 5 mL of prewarmed lung organoid media.
55. Transfer 1,5-2 x 106 cells to the equilibrated ClinoReactor through the sample port.
56. Slowly refill the ClinoReactor with media without disturbing the cells.

Note: Placing the needle end directly against the front or back ClinoReactor wall disperses the medium flow and creates more gentle re-filling.

57. Overfill the chamber with media.
58. Remove air bubbles trapped in the cell culture chamber by gently tapping the ClinoReactor.
59. Replace the top plug and remove the excess media with a pipette.
60. Check for any remaining bubbles by carefully rotating the vessel. Troubleshooting 3.
61. Incubate the ClinoReactor in the ClinoStar at 37°C and 5% CO_2 with an initial rotation of 5–10 rpm.

Note: The speed should be optimized according to individual organoid lines and that increasingly daily depending on the size of the organoids to keep them in a stationary orbit.

62. It is recommended to change the media for the first time after 72 hours.

After 10–12 days, the PDOs can either be used for other assays (e.g., drug screening, molecular characterization, IHC staining) or dissociated again and replated in a new ClinoReactor to expand the PDO population.

Histological analysis of organoids

Timing: 3 days

This section describes the steps for collection, fixation, paraffin embedding, and immunohistochemical staining of both static and suspension cultures. This procedure is recommended to assess whether tumoroids grown without Matrigel retain the same histological characteristics as the original tumor and are comparable to organoids grown under static culture conditions. For suspension organoids, once the medium containing the PDOs is collected from the ClinoReactor and transferred into a Falcon tube, the protocol proceeds as described below, starting from step 63.

63. Organoid fixing:
    a. Remove the medium from the wells with tumoroids that need to be split.
        i. If there is more than one well for each culture condition, you can pool all together up to 2 confluent wells.
    b. Add 1000 µL of cold *DPBS+++* per well:
        i. Gently scratch all the domes with a scraper.
        ii. Pool together the wells in a 15 mL falcon and put on ice.

c. Rinse all wells from which organoids were collected once with 500 μL of *DPBS+++*.
d. Centrifuge at 400 g for 5 min at 4°C.
e. Remove supernatant.
f. Add the same volume of Cell recovery Solution to dissolve the Matrigel without affecting tumoroids structure (overall 1 mL of Cell recovery/well).
g. Leave on ice for 20 minutes. At halftime, pipette up and down to help dissolve the Matrigel.
h. Add cold DPBS up to 10 mL.
i. Centrifuge 5 min 300 g.

⚠ CRITICAL: If some Matrigel is left, repeat the steps 64–68 again before continuing in the protocol.

j. Remove the supernatant and resuspend the pellet in 1 mL of 8% Formalin (this step needs to be done under a chemical hood).

Note: If the PFA is done on the same day, leave the falcon for 1 h at 18°C–21°C to allow the fixation of the cells. Alternatively, place at +4°C for 24 h.

k. After fixation, centrifuge at 300 g for 5 min at +4°C.
l. Remove the supernatant and resuspend the pellet with 1 mL DPBS.

Note: Falcon can be stored at +4°C before the embedding for 1 week max.

64. Paraffin-embedding tumoroids:

Note: Steps need to be done in a chemical hood.

a. Centrifuge the fixed organoids for 5 min 300 g.
b. Remove DPBS/Formalin (depending on when the fixing has been done) and resuspend the pellet in 70 μL of Histogel.

Note: Histogel has to be warmed up in the microwave. It is stored at +4°C and can be re-warmed up at each need. Warm it until it melts. Use a backer with tap H_2O to warm it. As long as it is getting cold again, it solidifies and has to be re-warmed up.

Note: 70 μL is the optimal amount not to have a huge drop; generally, adapt the volume according to the pellet size. However, do not use more than 100 μL or less than 50 μl of Histogel.

⚠ CRITICAL: To resuspend better the pellet within the Histogel (considering its fast solidification), soak the 15 mL falcon with the dry pellet in the warm H_2O, and resuspend the pellet directly with the Histogel in the warm H_2O (Figure 5A).

c. Place the drop on the back of the parafilm roll and leave to dry for 40 min (Figures 5B and 5C). Troubleshooting 4.
d. Place the drop in the cassettes for the embedding (Figure 5D).
e. Place the cassettes in the embedding machine for 12 h and proceed as for small biopsies.

65. Immunohistochemical Staining (IHC):
a. The paraffin-embedded organoids are cut in 3 μm sections and put on a positively charged microscope slide.
b. The slices are deparaffinized as follows:
i. 10 min Xylene.
ii. 10 min Xylene.
iii. 1 min in Ethanol Abs.

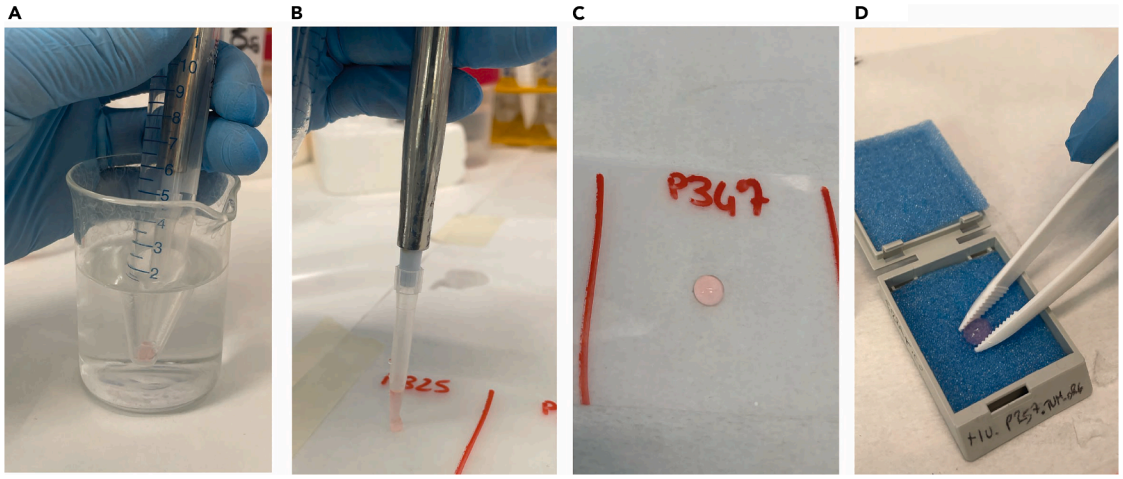


Figure 5. Paraffined embedded tumoroids

(A) Organoids pellet resuspension with Histogel in warm water to prevent early solidification of the Histogel.

(B) Domes formation on parafilm to allow solidification oh mixture Histogel-organoids.

(C) Example of 70 µl Histogel dome.

(D) Positioning of the Histogel dome in the cassettes for embedding.

iv. 1 min in Ethanol Abs.
v. 1 min in Ethanol 90%.
vi. 1 min in Ethanol 70%.
vii. Store in H_2O.

c. Performed antigen retrieval in a water bath in Tris-EDTA buffer at pH 9 for 20 min at 98°C.
d. Leave the slide to cool at RT in the Tris-EDTA buffer.
e. Move the slide to PBS + 0.05% Tween solution.
f. Leave the slide for 20 min at RT in a PBS + 0.05% Tween + 2% H_2O_2 .
g. Rinse in PBS+0.05% Tween solution.
h. Add Background sniper for Blocking of non-specific bindings for 15 min at RT.
i. Add diluted primary antibody in PBS + 0.05% Tween solution (see key resources table) for 1 hour at RT.
j. Rinse twice for 5 min each the slides in PBS+0.05% Tween solution.
k. Incubate for 15 min at RT with MACH 1 Mouse probe (mouse monoclonal antibodies only).
l. Rinse in PBS + 0.05% Tween solution for 5 min.
m. Incubate with HRP-Polymer for 30 min at RT (for each primary antibodies).
n. Rinse twice for 5 min each the slide in PBS+0.05% Tween solution.
o. Add DAB mixture (1 drop (32 µL) of DAB Chromogen per 1 mL of DAB Substrate Buffer) to the sample section and monitor the reaction under the microscope.
p. Stop the reaction with tap water when you have reached the intensity signal that is necessary.

Note: The time depends on DAB staining; generally, the time will vary between up to 1 min.

q. Counterstain the Nuclei with Hematoxylin solution (30 sec–1 min).
r. Rinse with tap water till the water is clear.
s. Scale dehydration as follows:
i. 30 sec in Ethanol 70%.
ii. 30 sec in Ethanol 90%.
iii. 30 sec in Ethanol Abs.
iv. 30 sec in Ethanol Abs.
v. 5 min Xylene.

vi. 5 min Xylene.

t. Mount coverslip with Eukitt.

u. Acquire the sample imaging at 20x and 40x magnification to verify the features between the original tissue and organoid line for each sample (Figure 6).

EXPECTED OUTCOMES

With this protocol, we have demonstrated that patient-derived organoids (PDOs) can be successfully established from resected lung tissue, providing a robust model for studying lung development, disease progression, and personalized therapeutic responses. Specifically, we tested two histopathological types of non-small cell lung cancer (both adenocarcinoma and squamous carcinoma) to provide two distinct examples that can be evaluated with this method.

Notably, in this study, lung organoids were cultured and expanded in a suspension system without the need for Matrigel, which offers several advantages. Matrigel can introduce variability due to its undefined composition and animal origin. Eliminating Matrigel from the culture system not only simplifies the protocol and reduces costs but also enhances the clinical relevance of the model by avoiding animal-derived components.

Interestingly, while the morphology of PDOs grown in suspension differs from those cultured in traditional static domes (Figure 4), both culture methods maintain the organoids' fidelity to their tissue of origin that we verified by immunohistochemical assay in which the specific markers (TFF-1 for Lung Adenocarcinoma[2] and p40[3] for lung squamous cancer) for the two histopathological types of the organoids are matching the tissue of origin (Figure 6). These findings confirm that suspension culture provides a scalable and more standardized alternative for lung organoid expansion while preserving key biological characteristics.

LIMITATIONS

One of the main limitations of this approach is its reliance on the ClinoStar instrument and technology, which may restrict accessibility. Additionally, since organoids are primary cell cultures, their efficiency in establishment and sustained growth can be variable. The success of culturing largely depends on patient-specific factors and the quality of the original tissue sample. In particular, tumor samples with high levels of necrosis are associated with a significantly reduced success rate in organoid formation. Another critical factor is the number of cells used when transitioning from static to dynamic culture conditions, which for the ClinoStar is required a range from 1×10^6 to 2×10^6, that could require a long time of cultures from the surgical resection step. In fact, a low initial cell number can lead to culture failure, as organoids require sufficient cell-cell interactions to support survival and promote PDO growth. Ensuring an optimal cell density is therefore essential for maintaining viable and expandable organoid cultures.

TROUBLESHOOTING

Problem 1

The minced tissue in 5 mL of Dissociation media floats or is not well covered (related to Step 8).

Potential solution

If tissue is abundant, it is better to move everything in a 50 mL falcon and increase the *Dissociation medium* to 10–15 mL.

Problem 2

The organoids are not disrupted into single cells after (related to Step 41).

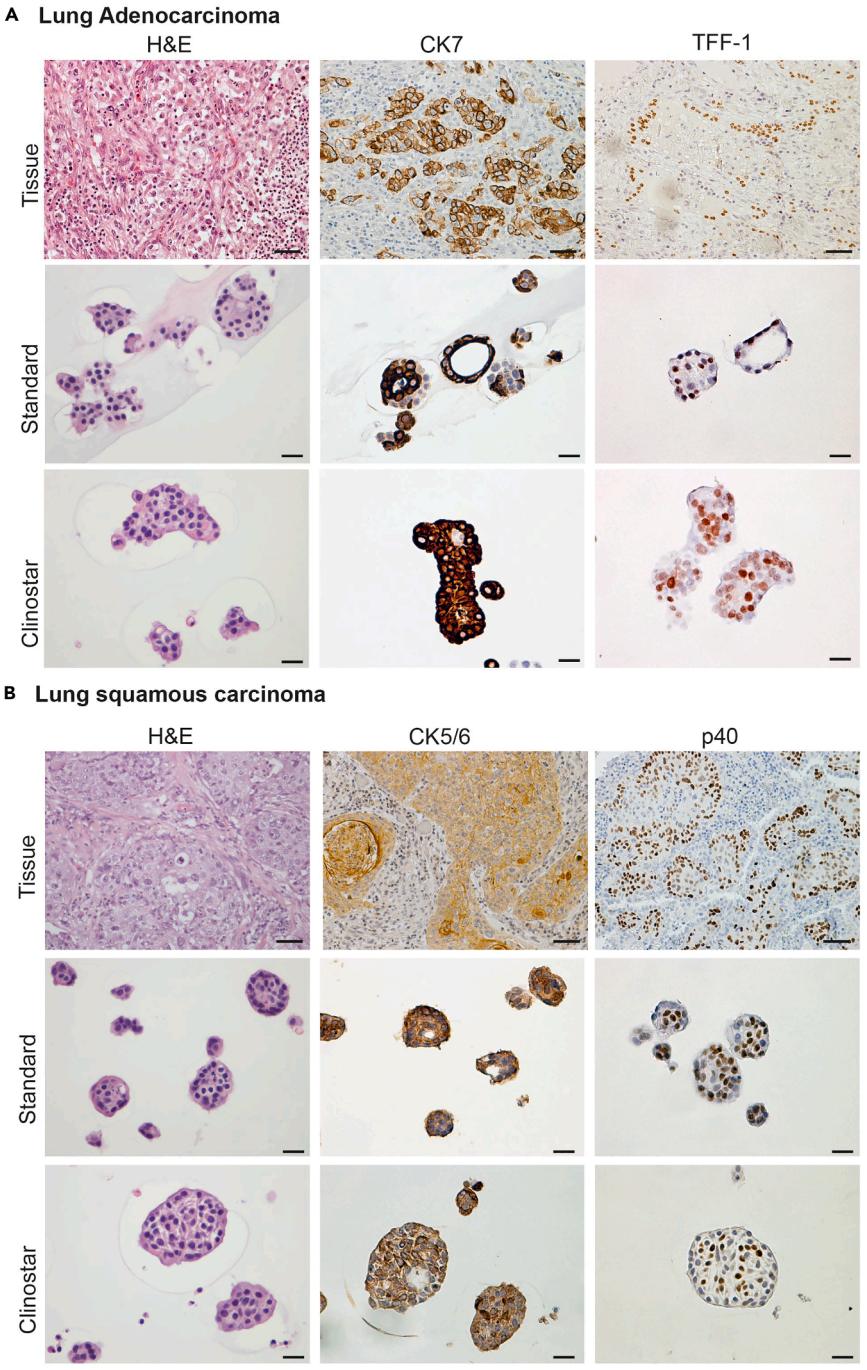


Figure 6. Immunohistochemical comparison of PDOs in different culture methods

(A) Images of Lung adenocarcinoma from original tissue and PDOs from standard and ClinoStar culture methods. Scale bar: 50 μm for tissue and 20 μm for PDOs.

(B) Images of Lung squamous carcinoma from original tissue and PDOs from standard and ClinoStar culture methods. Scale bar: 50 μm for tissue and 20 μm for PDOs.

Potential solution

- It is possible to incubate the organoid solution longer than 12 min but do not incubate more than 20 min or the vitality will drop.

- Another solution is to add TrypLE reagent to increase the strength of dissociation of the organoids and cell cluster.

In both cases, if after 20 min some tumoroids remain, continue to the next steps because that structure will be lost at the filtering step.

Problem 3

Air bubbles are present in the chamber of the ClinoReactor that are not removed by rotating in the incubator (related to Step 60).

Potential solution

Place the ClinoReactor in a vertical position and wait for the air bubbles to move upward. Then open the top plug and add a small amount of fresh medium to overfill the cell culture chamber to the point where medium fills the collar around the port so the air bubbles are removed from the cell culture chamber of the ClinoReactor.

Problem 4

Formation of air bubble on the dome when preparing a paraffin organoid in Histogel (related to Step 65.c).

Potential solution

Remove with a tip or aspirate with the pipette the air bubble on the top of the dome before starting to solidify.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Salvatore Piscuoglio (salvatore.piscuoglio@hunimed.eu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Dr. Federico Donà (federico.dona@humanitasresearch.it).

Materials availability

The organoid lines generated in this study are not available to external laboratories due to ethical reasons.

Data and code availability

The data in this published article are generated and analyzed during this study.

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AUTHOR CONTRIBUTIONS

F.D. designed and developed the protocol. S.E. worked on part of the practical experiments and the description of the immunohistochemical section and summarized the table. P.B. helped in the analysis and definition of the IHC data and staining. G.M., V.M.G., D.B., and E.R.C. helped in the recruitment of the patients and the delivery of the surgical resection. F.D. wrote the manuscripts. S.P., L.M.T., and C.K.Y.N. critically revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used ChatGTP in order to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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