Protocol for ex vivo culture of patient-derived tumor fragments

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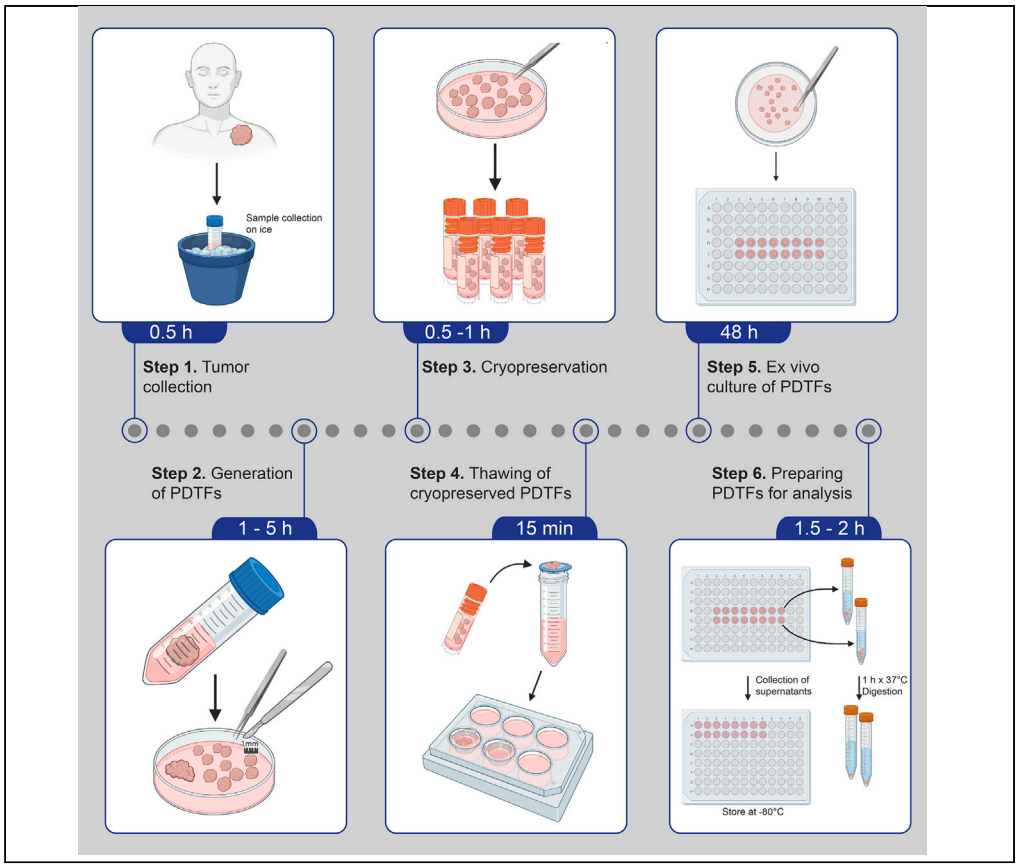

The lack of suitable models currently hampers our understanding of how the tumor microenvironment responds to immunotherapy treatment. Here, we present a protocol for *ex vivo* culture of patient-derived tumor fragments (PDTFs). We describe the steps for tumor collection, generation and cryopreservation of PDTFs, and their subsequent thawing. We detail culture of PDTFs and their preparation for analysis. This protocol preserves the tumor microenvironment's composition, architecture, and cellular interactions, which can be perturbed by *ex vivo* treatment.

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

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Highlights

Short-term *ex vivo* culture of patient-derived tumor tissue

Preservation of cancer, immune, and stromal cell compartments

Modeling of early immune responses to therapy

Potential for fundamental research and translational applications

Roelofsen et al., STAR Protocols 4, 102282 June 16, 2023
https://doi.org/10.1016/j.xpro.2023.102282

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Protocol for *ex vivo* culture of patient-derived tumor fragments

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https://doi.org/10.1016/j.xpro.2023.102282

SUMMARY

The lack of suitable models currently hampers our understanding of how the tumor microenvironment responds to immunotherapy treatment. Here, we present a protocol for *ex vivo* culture of patient-derived tumor fragments (PDTFs). We describe the steps for tumor collection, generation and cryopreservation of PDTFs, and their subsequent thawing. We detail culture of PDTFs and their preparation for analysis. This protocol preserves the tumor microenvironment's composition, architecture, and cellular interactions, which can be perturbed by *ex vivo* treatment.

For complete details on the use and execution of this protocol, please refer to Voabil et al. (2021).[1]

BEFORE YOU BEGIN

The engineering of preclinical human tumor models such as tumor organoids or spheroids has opened new avenues to test the efficacy of cancer treatments in human tumors while maintaining the heterogeneity observed between patients.[2–5] However, the use of these models for immunotherapy research has been limited as classically they only contain cancer cells. While efforts have been undertaken to overcome this issue, for instance by co-culturing tumor organoids with immune cells from peripheral blood,[6,7] human cancers are infiltrated by a plethora of cancer, stromal and immune cells that have acquired distinct cell states, shaped by their prior exposures in the tumor microenvironment (TME). Hence, to investigate intratumoral immune cell activity, to understand how it impacts response to immunotherapy and, vice versa, how it is changed by treatment, human models are required that preserve an immunologically active TME, including its architecture, and that also allow one to perturb cellular function within this ecosystem. Here, we describe the generation of an *ex vivo* culture platform using viable patient-derived tumor fragments (PDTFs) (See graphical abstract). This platform maintains both TME composition and architecture as close to the patient's tumor as possible during short-term culture and enables the assessment of early therapy-induced changes in the tumor tissue. Moreover, the PDTF platform can be used to identify baseline TME properties that are associated with specific treatment-induced responses.[1]

PDTFs can be derived from human tumor tissue obtained during surgical resections of multiple tumor types. The protocol has been successfully performed with solid tumor lesions originating from

patients with multiple cancer types, including melanoma, non-small cell lung cancer, breast cancer, ovarian cancer, colorectal cancer, head and neck cancer, bladder cancer, cutaneous squamous cell carcinoma and renal cell carcinoma. Based on these findings, we expect that the same approach can be used for most other cancer or tissue types. Importantly, the successful use of this protocol strongly depends on the availability and quality of human tumor material, the rapid and careful processing of the tissue into PDTFs and the incorporation of quality controls to monitor the performance of PDTF cultures.

We expect that this protocol can be applied to a broad range of fundamental and translational research questions. The possible combination of PDTF cultures with a multitude of readouts, such as flow cytometry, cytokine network analyses, single-cell sequencing technologies as well as spatial approaches allows the functional profiling of human TMEs at high resolution. The application of perturbations can be used for mechanistic studies, for instance to test the contribution of certain cell populations or pathways to the observed responses. Importantly, the possibility to apply multiple treatments within the same tumor allows one to directly compare the efficacy of different treatments. The analysis of responses in individual tumor fragments can be used to study the impact of tumor heterogeneity. Finally, the opportunity to link baseline tumor properties to *ex vivo* treatment responses may be leveraged for biomarker research and the rational combination of therapies.

Institutional permissions

Before working with patient material, approval of the local Institutional Research Board or Ethics Committee is usually required, and patient consent needs to be obtained prior to study inclusion.

For our study, all patients consented to research usage of material not required for diagnostic use either by opt-out procedure or via prior written informed consent (after May 23, 2018). The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Research Board of the Netherlands Cancer Institute (CFMPB484).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
PBS Tablets	Gibco	Cat#18912014
RPMI 1640 medium	Thermo Fisher	Cat#21870076
DMEM, high glucose, pyruvate	Thermo Fisher	Cat#41966029
Penicillin/Streptomycin	Roche	Cat#11074440001
Fetal bovine serum (FBS)	Thermo Fisher	Cat#F7524
Sodium pyruvate solution	Sigma-Aldrich	Cat#S8636
MEM non-essential Amino Acid Solution	Sigma-Aldrich	Cat#M7145
L-glutamine	Thermo Fisher	Cat#35050038
Matrigel Basement Membrane Matrix High Concentration, Phenol Red Free	Corning	Cat#354262
Collagen I High Concentration, Rat Tail	Corning	Cat#354249
Sodium bicarbonate solution	Sigma-Aldrich	Cat#S8761
Dimethylsulfoxide	Sigma-Aldrich	Cat#D4540
Collagenase type IV	Sigma-Aldrich	Cat#C5138
Pulmozyme (dornase alfa)	Roche	www.roche.nl
Trypan blue solution 0.4%	Thermo Fisher	Cat#15250061
Other		
150 μm filter mesh (Open Area %: 50 - Width: 40 in, Natural Color (1 Yard))	Sefar America Inc	Cat#03150/50
100 μm cell strainer	Corning	Cat#352360

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
70 μm cell strainer	Corning	Cat#352350
50 mL tube	Sarstedt	Cat#62.547.254
15 mL tube	Sarstedt	Cat#62.554.502
Fine-tipped forceps	Vosmedisch	Cat#30617
Petri dish 10 cm	Corning	Cat#430167
Tissue culture 6-well plate	Techno Products	Cat#92406
Tissue culture treated 96-well plate (F-bottom)	Greiner Bio-one	Cat#655180
Disposable scalpels (no. 20)	Swann Morton	Cat#0506
Nunc™ Biobanking and Cell Culture Cryogenic Tubes 1.8 mL	Thermo Fisher	Cat#375418
CoolCell freezing container for 30 × 1 or 2 mL	Corning	Cat#432006
Neubauer counting chamber	Brand	Cat#717810

MATERIALS AND EQUIPMENT

Culture media recipes

Tumor collection medium

Reagent	Final concentration	Amount
RPMI 1640	N/A	486.5 mL
Penicillin/streptomycin	100 U/mL, 100 μg/mL P/S	1 mL
FBS	2.5%, heat inactivated	12.5 mL
Total		**500 mL**

Prepare in a sterile environment, store at 4°C for up to 1 month.

Tumor culture medium

Reagent	Final concentration	Amount
DMEM	N/A	434 mL
Penicillin/streptomycin	100 U/mL, 100 μg/mL P/S	1 mL
Sodium pyruvate	1 mM	5 mL
MEM non-essential AA	1×	5 mL
Glutamax	2 mM	5 mL
FBS	10%, heat inactivated	50 mL
Total		**500 mL**

Prepare in a sterile environment, store at 4°C for up to 1 month.

Tumor wash medium

Reagent	Final concentration	Amount
DMEM	N/A	449 mL
Penicillin/streptomycin	100 U/mL, 100 μg/mL P/S	1 mL
FBS	10%, heat inactivated	50 mL
Total		**500 mL**

Prepare in a sterile environment, store at 4°C for up to 1 month.

Tumor digestion mix

Reagent	Final concentration	Amount
RPMI 1640	N/A	48.77 mL
Penicillin/streptomycin	100 U/mL, 100 μg/mL P/S	0.1 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Pulmozyme	0.0126 mg/mL (stock 1 mg/mL)	0.63 mL
Collagenase type IV	1 mg/mL	0.5 mL
Total		50 mL
Prepare in a sterile environment, store at 4°C for up to 1 month.		

Freezing medium		
Reagent	Final concentration	Amount
FBS	90%, heat inactivated	45 mL
DMSO	10%	5 mL
Total		50 mL
Prepare in a sterile environment, store at 4°C for up to 1 month.		

Media and reagent preparation

Timing: 12 h

- Thaw the Matrigel Basement Membrane Matrix High Concentration (Matrigel) on ice in the fridge for 12 h. Wrap parafilm around the Matrigel tubes to prevent water inflow from the ice.
- Prepare all media (tumor wash medium, tumor culture medium and tumor digestion mix) according to the tables in the 'materials and equipment' section and store appropriately.
- Pre-cool pipet tips in the fridge for preparation of the matrix.

⚠ CRITICAL: Matrigel should be aliquoted into small batches to avoid multiple freeze-thaw cycles. For calculations of the Matrigel concentration required for *ex vivo* culture, see steps 14 and 16 of the 'step-by-step method details'.

Alternatives: Instead of the Corning high concentration Matrigel, other similar culture matrices with high protein density can be used such as the Cultrex UltiMatrix Reduced Growth Factor (Cat#: BME001) from Biotechne. We recommend to use a basement membrane extract with low endotoxin content. Each new batch should be tested with regard to its effect on cell viability, unspecific immune cell activation and preservation of cell function (troubleshooting 1).

STEP-BY-STEP METHOD DETAILS

Generation and cryopreservation of PDTFs from human tumor lesions

Timing: 1–5 h

This part of the protocol describes the processing of fresh tumor tissue into PDTFs and the cryopreservation of PDTFs in liquid nitrogen for long-term storage. For optimal quality, tumor samples should be collected in ice-cold sterile tumor collection medium and kept on ice during transport. We commonly use 5 mL of collection medium in a 50 mL tube, but volume can be increased for larger sized tumors (Figure 1A). The tissue can be stored for a maximum of 1 h at 4°C in collection medium if needed.

1. Before collection, prepare aliquots of 5 mL tumor collection medium in a sterile 50 mL tube and store until usage at 4°C.

Note: aliquots with collection medium can be stored for a maximum of 30 days at 4°C.

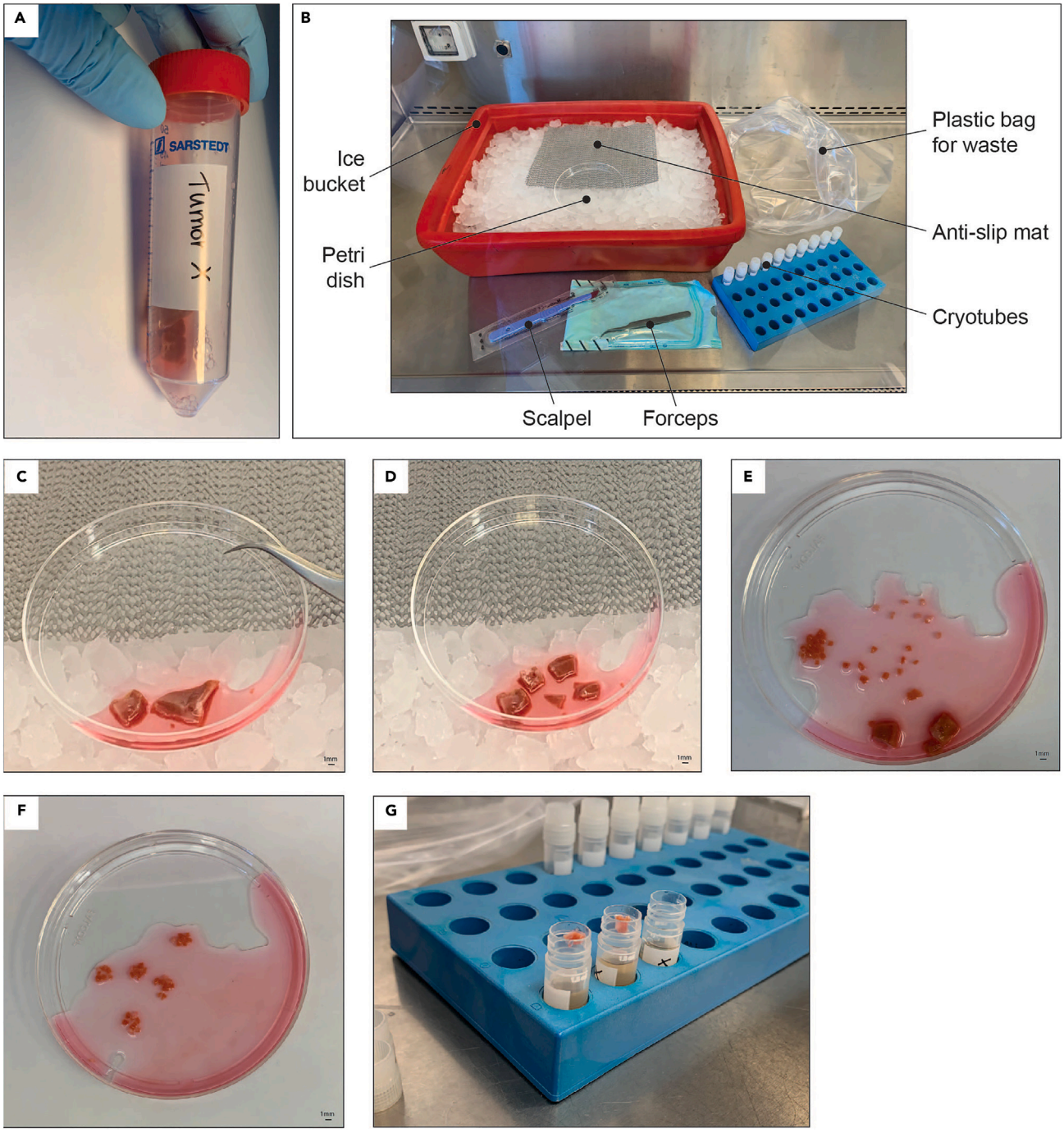

Figure 1. Tissue collection and processing

(A) Collected tumor tissue in a 50 mL tube containing tumor collection medium.

(B) Preparation of necessary items for tumor tissue processing.

(C) Tumor tissue pieces in a 10 cm dish with medium.

(D) Pre-processing of the tumor tissue into smaller sections of 4–5 cm, to be kept either on the petri dish or in the 50 mL tube until processing.

(E) Different steps of the tumor tissue processing into PDTFs (all in medium); left: small pile of processed PDTFs in medium, top: freshly cut PDTFs to be added to the left pile, bottom: unprocessed tissue sections in medium. For the picture, the plate was taken off the ice, but processing should always be done on ice.

(F) Five piles of processed PDTFs in groups of 8–15 blocks (in medium) to be frozen in separate cryovials.

Scale bar in (C)–(F) indicates 1mm.

(G) Cryovials containing freezing medium and, on the wall, a group of PDTFs.

2. Prepare the following material for the processing of PDTFs (Figure 1B):
   a. Bucket with ice.
   b. Sterile petri dish (10 cm diameter).
   c. Sterile fine-tipped forceps.
   d. Sterile scalpel.
   e. Anti-slip mat for processing on ice (optional).
   f. Cryotubes for freezing.

   ⚠ CRITICAL: The tissue should be processed on ice as fast as possible for optimal viability. At least initially, viability should be checked directly after thawing and after *ex vivo* culture (see Section 'thawing of PDTFs and setup of ex vivo culture'; troubleshooting 2).

3. Collect the tumor material in the 50 mL tube and keep on ice until processing (Figure 1A).
4. Transfer the tumor tissue from the 50 mL tube in a small volume of collection medium to the petri dish on ice (Figure 1C).

   Note: Depending on the size of the tumor tissue, the volume of collection medium in the petri dish should be between 5–10 mL. The tissue should be in contact with, but not fully floating in the medium.

5. For larger tumors, cut the tissue with the scalpel into smaller pieces of 1 × 1 cm (Figure 1D; troubleshooting 3).

   ⚠ CRITICAL: Start with cutting one small tumor piece into PDTFs and keep the remaining tissue pieces on ice either in collection medium in the petri dish or, for larger tumor resections, submerged in the 50 mL tube to avoid that the tissue dries out.

6. Cut each piece of the tumor tissue into tumor fragments of approximately 1 mm^3 and keep the PDTFs covered with collection medium to avoid them drying out (Figure 1E, troubleshooting 4).

   ⚠ CRITICAL: Cut the tissue with a sharp scalpel and without too much pressure. Do not mash or tear the tumor tissue as this will create mechanical damage. Do not use the forceps with too much pressure as this will create damage or holes in the tissue. Change the scalpel when no longer sharp (Methods Video S1).

7. Continue processing the tumor pieces into fragments (Figure 1E) until the entire tissue has been processed into PDTFs.

   ⚠ CRITICAL: Remove any necrotic parts and blood coagulates from the tumor. If possible, the tumor material should be assessed by a pathologist before processing to identify areas with necrosis or that are non-malignant.

8. Mix PDTFs from different regions and pieces to ensure uniform representation of the tumor lesion and gather 8–15 fragments in small heaps on the petri dish (Figure 1F).
9. Label round bottom cryovials and add 1 mL of freezing medium to each vial.

   Note: The use of round bottom cryovials is highly recommended to improve the retrieval and quality of the PDTFs upon thawing.

   ⚠ CRITICAL: Do not use less than 1 mL of freezing medium, otherwise the fragments will clump together.

10. Transfer 8–15 fragments to each cryovial by putting them against the wall on top (handle the fragments carefully and do not drop them into the freezing medium yet) (Figure 1G).
11. Close all the lids and flip the tubes to immerse all fragments into the freezing medium.
12. Tap the tubes to ensure fragments are not sticking onto each other.

Note: The cryovials should each contain a similar number of fragments; optimal storage is 8–10 fragments per vial, but fragment numbers can be increased (up to 15 fragments per vial) for larger tumors.

⚠ CRITICAL: For freezing, the fragments should not stick to the wall of the cryovial or to each other to allow proper cryopreservation. Improperly frozen fragments (e.g., fragments not fully immersed in the freezing medium) should be removed upon thawing before continuing with the culture procedure.

13. Store the cryovials in a freezing container (see key resources table) in a −80°C freezer.
    a. Allow for a consistent −1 °C/min freezing rate.
    b. After a temperature of −80°C has been reached (and after at least 24 h), transfer the cryovials as soon as possible to a liquid nitrogen tank and store until further usage.

Pause point: PDTFs can be cryopreserved for several years.

Optional: PDTFs can also be used freshly without cryopreservation. It is important to note that certain immune cell populations such as granulocytic cells may die upon freezing. Cryopreservation however allows a better use of the tumor material. For instance, the specific number of fragments required for an experiment can be selected and experiments can be replicated with the same tumor to validate findings.

Preparation of the *ex vivo* culture

Timing: 1.5 h

This section describes the setup of the *ex vivo* cultures.

⚠ CRITICAL: It is crucial to embed the tumor fragments as fast as possible as viability may dramatically decrease otherwise.

14. The day before starting the *ex vivo* PDTF culture, the following steps should be taken in preparation:
    a. Calculate the number of fragments and the amount of matrix needed for the experiment; for calculations, see step 16 (concentration depends on Matrigel batch).
    b. Always calculate an excess of matrix because of possible pipetting error (an excess of 10% is recommended).
    c. Thaw the Matrigel for 12 h on ice at 4°C.

Note: Other preparations include the pre-cooling of pipet tips at 4°C and preparation of the tumor culture and tumor wash media (see 'media and reagent preparation' section).

Note: It is important to perform steps 15–39 for one tumor sample at a time (see note after step 39). These steps can be repeated for additional samples to set up *ex vivo* PDTF cultures from multiple tumors.

15. Before starting the culture, prepare the following materials (Figure 2A):

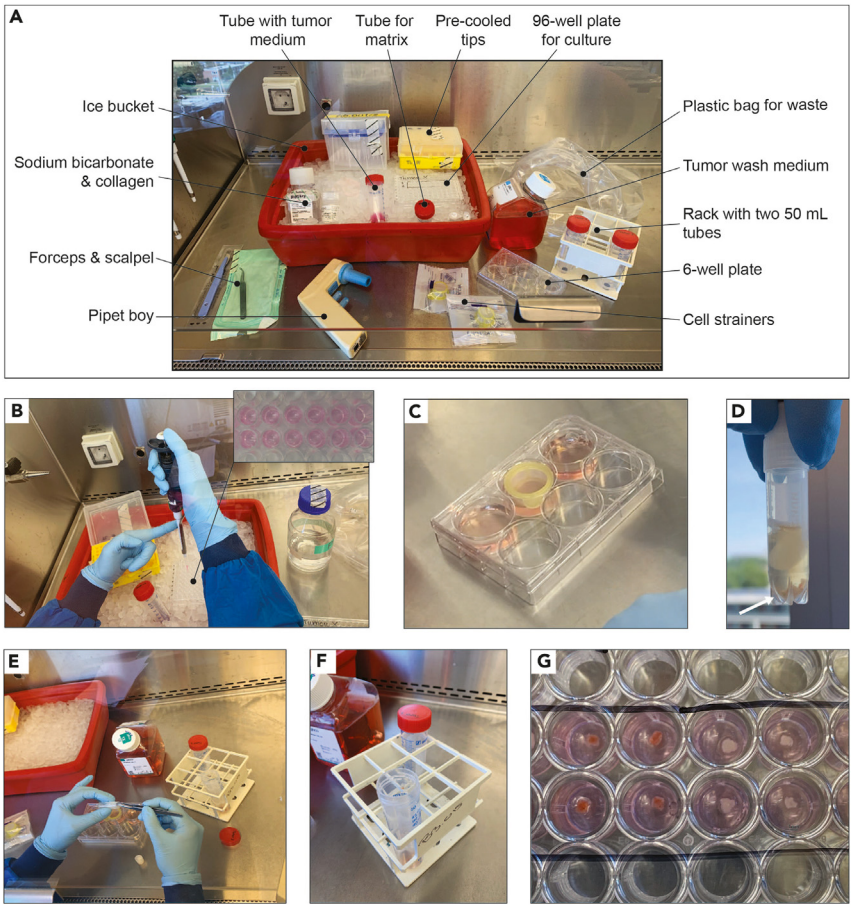

Figure 2. Thawing of fragments and *ex vivo* PDTF culture

(A) Preparation of required items for thawing and *ex vivo* culture of PDTFs.

(B) Addition of the matrix to the 96-well plate. The layer of matrix is added to the 96-well plate on ice, without touching the bottom and sides of the well. Inlay: overview of wells from the 96-well plate filled with first layer of matrix.

(C) Setup of a 6-well washing plate with pre-warmed tumor wash medium and a Falcon cell strainer in the second well.

(D) Thawing of PDTFs in a cryovial. A small clump of ice is left in the vial, and PDTFs can be seen at the bottom (white arrow).

(E) Transfer of any remaining fragments from the cryovial using forceps into a 50 mL tube.

(F) Leftover PDTFs after emptying the cryovial into a 50 mL tube are transferred to the wall of the tube by forceps, to keep the thawing process as fast but precise as possible.

(G) Example of an *ex vivo* PDTF culture in a 96-well plate. Fragments are added to the center of the 96-well plate after the bottom-layer of the matrix has solidified, followed by addition of a second layer of matrix on top of the fragments. Fragments are uniformly distributed to capture heterogeneity between conditions.

a. 6-well and 96-well flat bottom plates (pre-cool the 96-well plate on ice before usage), one for each tumor sample.
b. Falcon Cell strainers, 70 or 100 μm.
c. Sterile fine-tipped forceps (use a separate pair of forceps for each tumor).
d. Bucket with ice.
e. Four 50 mL tubes (per tumor), two of which should be cooled on ice before using them for preparing the matrix.
f. Pre-cool tumor medium on ice.
g. Pre-warm tumor wash medium to 37°C.

16. Calculation of the matrix (Table 1):

Table 1. Example calculation for the matrix

Component (final concentration)	Volume
Matrigel (2 mg/mL) – stock concentration 20.6 mg/mL	500 μL
Collagen (1 mg/mL) – stock concentration 10.21 mg/mL	504.4 μL
Sodium bicarbonate (7.5%)	50.4 μL
Tumor medium	4,095.2 μL
Total volume	**5,150 μL**

This example uses a calculation for a culture with a total of 55 wells. An excess should be calculated for pipetting errors.

a. Total volume of the matrix $= \frac{[\text{concentration of stock Matrigel}]}{[\text{final concentration Matrigel}]} \times$ volume Matrigel aliquot
Example:

$$\text{Total volume} = \frac{20.6\ \text{mg/mL}}{2\ \text{mg/mL}} \times 500\ \mu\text{L} = 5,150\ \mu\text{L}$$

b. Volume collagen $= \frac{[\text{final concentration of collagen}] \times \text{final volume}}{[\text{concentration of stock collagen}]}$
Example:

$$\text{Total volume} = \frac{1\ \text{mg/mL} \times 5,150\ \mu\text{L}}{10.21\ \text{mg/mL}} = 504.4\ \mu\text{L}$$

c. Volume sodium bicarbonate = 10% of final volume collagen
Example:

Volume sodium bicarbonate $= 0.10 \times 504.4\ \mu\text{L} = 50.44\ \mu\text{L}$ sodium bicarbonate

d. Volume tumor medium = final volume − volume Matrigel − volume collagen − volume sodium bicarbonate
Example:

Volume tumor medium $= 5,150\ \mu\text{L}$ [total volume] $- 500\ \mu\text{L}$ [volume Matrigel]
$- 504.4\ \mu\text{L}$ [volume collagen]
$- 50.4\ \mu\text{L}$ [volume sodium bicarbonate] $= 4,095.2\ \mu\text{L}$ tumor medium

17. Prepare two pre-cooled 50 mL tubes;
    a. Add ice-cold tumor medium to one of the tubes (should include the volume needed for the matrix and for topping up each well to a total of 200 μL, see step 39).
    b. In the other tube, mix the collagen with 7.5% sodium bicarbonate: pipet the sodium bicarbonate first, then slowly add the collagen.

Note: When pipetting collagen, use a p1000 tip or cut the tip off a p200 pipet tip, as collagen has a high viscosity.

18. Mix with the pre-calculated volume of cold tumor medium.
19. Add the Matrigel by taking it up with a bit of the medium-collagen mix and transferring it to the 50 mL tube (Methods Video S2).

⚠ CRITICAL: Make sure to fully dissolve the Matrigel before transferring (no undissolved gel clumps should be visible). Often, this step has to be repeated multiple times before all the Matrigel is transferred to the final mixture

20. Pipet 40 μL of the matrix into each well of a pre-cooled 96-well flat bottom plate without creating air bubbles (Figure 2B).
21. Tap the plate carefully to spread the matrix throughout the well.
22. Incubate for 30 min at 37°C and 5% CO_2 until the matrix is solid.

Note: A p200 pipet tip can be used to test in the middle of the coated wells if the first layer of matrix is solidified. Gently touch the matrix layer with the tip (avoid making holes). When no matrix is observed inside the tip, one can continue the protocol.

Incubation point: 15 min after step 22 (incubation with the first layer of matrix), start thawing the tumor fragments.

Thawing of PDTFs and setup of *ex vivo* culture

Timing: 1 h preparation; 48 h culture time

This part of the protocol describes the thawing of PDTFs after cryopreservation and the embedding in the matrix to start the *ex vivo* culture.

Note: Characterization of each tumor sample before culture can help to ensure that the tumor material is viable and contains immune cells. Measurements of these and other parameters can, for instance, be done by flow cytometry, directly after thawing the tumor fragments (see step 23–35) without *ex vivo* culture.

⚠ CRITICAL: The thawing and initial washing steps are critical to prevent cryodamage, and indicated incubation times should be followed. Avoid drying out of tumor fragments.

23. Collect cryovials from the cryostorage on dry ice.
24. Add 7 mL of pre-warmed (37°C) wash medium to three wells of a 6-well plate, and add a Falcon cell strainer (both 70 or 100 μm can be used) to the second well (Figure 2C).
25. Prepare two 50 mL Falcon tubes, one also with a cell strainer.
26. Thaw the cryovials in a 37°C water bath until only a small clump of ice is visible (Figure 2D).

Note: If possible, improperly frozen fragments (e.g., fragments not properly submerged into the freezing medium) should be removed from the cryovial prior to emptying it.

27. Flick the tube and pour the content of the cryovial (fragments and freezing medium) into a 50 mL tube (Methods Video S3).

Note: Ensure that no fragments are left in the cryovial; in case of remaining fragments, these can be removed using a pair of forceps and added to the top/rim of the 50 mL tube (flush these with some wash medium before continuing to the next step) (Figures 2E and 2F).

28. Add dropwise (very slowly, one drop per second) 10 mL of pre-warmed wash medium.
29. Mix the fragments with the wash medium by swirling the 50 mL tube to rapidly dilute the DMSO from the freezing medium.
30. Carefully swirl the 50 mL tube to avoid fragments sticking to the tube when transferring, and pour the content of the tube into a new 50 mL collection tube over a cell strainer.
31. Transfer the cell strainer to the first well of the 6-well plate with the wash medium.

Note: The cell strainer helps to collect the fragments and reduces mechanical damage as less handling with the forceps is necessary.

32. Wash the fragments in the first well for approximately 1–2 min by shaking them lightly.
33. Transfer the fragments with the forceps to a new cell strainer in the second well to reduce left-over DMSO.
34. Wash the fragments in the second and third well for approximately 1–2 min by shaking them lightly.
35. After washing, remove the fragments from the cell strainer using the forceps and use the sterile part of the lid of the 6-well plate to collect them in a drop of medium.

⚠ CRITICAL: Keeping the fragments in a drop of wash medium prevents them from drying out.

36. Count the total number of fragments and transfer them into the matrix-coated 96-well plate. Place one fragment per well (troubleshooting 5).

⚠ CRITICAL: Divide fragments varying in size or color equally across different treatment conditions (Figure 2G). If the size of a fragment is larger than 1 × 1 × 1 mm, cut it into smaller PDTFs.

37. Add 40 μL matrix mix on top of each tumor fragment (handle the plate at 20°C–22°C to keep the bottom layer of the matrix solidified) (Figure 2G).
38. Incubate for 30 min at 37°C and 5% CO_2 until the matrix has solidified.
39. Add 120 μL tumor medium and incubate for the required time period at 37°C and 5% CO_2.

Note: Cultures for up to 72 h have been performed. We however observed best results in terms of immune activation and viability for up to 48 h of culture. After 72 h, the viability will decrease substantially.

Note: Any stimulation or treatment of interest, e.g., antibodies, cytokines, small molecules, etc. can be added to the 120 μL medium at step 39. Calculate the final concentration for a volume of 200 μL. If *ex vivo* cultures are performed for multiple tumors that should be treated for the same time period, first add only 100 μL medium and incubate the PDTF culture plates at 37°C and 5% CO_2 until the fragments from all tumors have been embedded. Then add the remaining 20 μL of the medium ± treatment of interest and incubate the cultures for the required time period at 37°C and 5% CO_2.

Incubation point: There is a stopping point for 48 h (maximum 72 h) when PDTFs are embedded in matrix and the treatment/stimulation has been added.

Preparing PDTFs for analysis

Timing: 1.5–2 h

This section describes the collection of supernatants and the processing of PDTFs into single cell suspensions after *ex vivo* culture. The collected supernatant can be used for analysis of soluble mediators secreted by the PDTFs. The fragments can be extracted from the matrix and either used for imaging analyses or be processed into single cell suspensions, for instance for assessment by flow cytometry or single-cell RNA-sequencing. For examples, please refer to.[1,8]

40. Before starting, prepare the following materials (Figure 3A):
    a. One 96-well round bottom plate per tumor.
    b. Nylon mesh filters, 150 μm.
    c. Sterile fine-tipped forceps (use a new pair of forceps for each tumor).
    d. Bucket with ice.

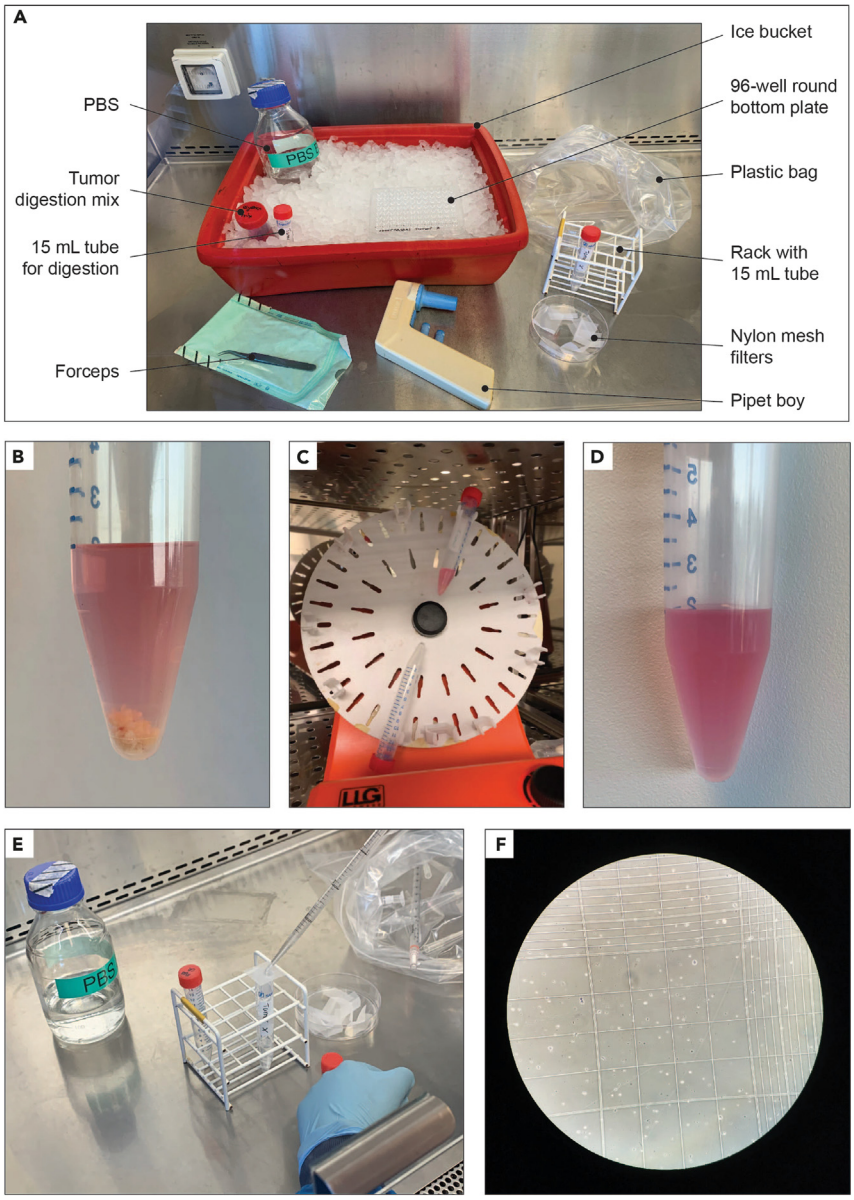

Figure 3. Supernatant collection and PDTF digestion into single-cell suspensions

(A) Preparation of necessary items for PDTF digestion and processing into single-cell suspension.

(B) Tumor fragments in a 15 mL tube containing complete digestion mix for the digestion at 37°C.

(C) Spinning wheel used to rotate the tubes during the digestion at 37°C. An additional tube with PBS or water is used as balance if necessary.

(D) Cell suspension (might contain some remaining fibers) after enzymatic digestion.

(E) Filtration of the tumor suspension over a filter mesh into a new 15 mL tube.

(F) Image of a counting chamber with the single cell suspension after digestion. The suspension is stained with Trypan Blue and visualized at 100× magnification.

e. Two 15 mL tubes per condition.
f. Digestion mix.
g. Rotator wheel at 37°C and 5% CO_2.
h. Ice-cold PBS.

41. Prepare one 15 mL tube with 2 mL digestion mix per condition and keep the tube(s) on ice.

42. After PDTF culture, collect the 80–100 μL of supernatant that is on top of the matrix in a 96-well round bottom plate.
    a. Without further preparation, seal the plate with the collected supernatants.
    b. Store at −80°C for later usage.

Note: Tilt the plate when taking up the supernatant to avoid touching the tumor fragment and matrix.

43. Extract the tumor fragments from the matrix using the forceps and transfer them directly into the 15 mL tube with digestion mix (Figure 3B).
    a. Pool fragments for each condition and mix them with the medium.
    b. Leave the fragments on ice until all conditions have been collected.

Note: Sometimes, matrix residues may be partially attached to the fragments after extracting them from the culture. Nevertheless, the fragments can be directly used for digestion without prior cleaning.

Note: Rarely, for instance in the case of very soft tumors, fragments may disaggregate in culture and collection with the forceps is not possible. In this case, collect also the matrix from the well with a p200 pipet and include it in the digestion mix to capture the disaggregated cells.

44. Place the tubes on the rotator and incubate for 1 h at 37°C and 5% CO_2 under slow rotation (Figure 3C; troubleshooting 6).

⚠ CRITICAL: Make sure no fragments are sticking to the wall of the tube during the digestion and check for potential leakage of the tubes containing the digestion mix after approximately 10 min.

45. After digestion (Figure 3D), keep the tube(s) on ice and top up each tube with ice-cold PBS.
46. Centrifuge at 235 × *g* for 10 min at 4°C.
47. Carefully remove the supernatant without disrupting the pellet and keep the tube(s) on ice.
48. Prepare a new 15 mL tube and place a nylon mesh filter on the top (Figure 3E).
49. Resuspend the cell pellet thoroughly with 5 mL of ice-cold PBS and filter the suspension through the mesh into the new 15 mL tube.
50. Centrifuge at 438 × *g* for 5 min at 4°C.
51. Carefully remove the supernatant without disrupting the pellet.

Note: At this point, the pellet can be hardly visible. This does however not necessarily indicate lack of cells. To control for the presence of cells, a sample can be taken to count cell numbers and check viability (Figure 3F).

52. Resuspend the cells in the medium of choice and continue with the readout of interest.

Preparation of supernatants for analysis of secreted mediators

Timing: 2 h

This part of the protocol discusses the preparation of supernatants to measure secreted soluble mediators upon culture. To this end, approaches such as cytometric bead arrays or Luminex assays can be used.

53. Retrieve supernatants from storage at −80°C and thaw them on ice.

54. To measure soluble mediators individually from each fragment, transfer the required volume from each well to the assay plate.

Note: It is also possible to measure the averaged response of the tumor to a treatment. Therefore, supernatants first can be pooled using equal volumes from each well and then the volume required for the assay can be transferred to the assay plate.

⚠ CRITICAL: Supernatants can be refrozen maximally one time. Multiple freeze-thaw cycles should be avoided as this leads to degradation of soluble mediators.

EXPECTED OUTCOMES

Applications of the PDTF technology

This protocol outlines the collection and processing of human tumor tissue into small tumor fragments for further analysis. Here, we describe how, after processing into PDTFs, tumor tissue can be cryopreserved for later use. Alternatively, PDTFs can be directly used to assess baseline TME characteristics or treatment responses upon short-term *ex vivo* culture, as cryopreservation does not allow the maintenance of granulocytic cells present in fresh tumor tissue. Moreover, this protocol may be used for other tumor types than the ones currently described or non-malignant human tissue specimens, allowing one to study immune activity and therapy responses in a broader context.

We describe the use of PDTFs in short-term *ex vivo* cultures, in which the microenvironment and 3D architecture of the tumor fragments largely remain intact. Following *ex vivo* culture, supernatants can be collected for measurement of secreted soluble mediators, and PDTFs can be processed into single-cell suspensions, which can then be used for further downstream analysis of cellular changes. This allows us to combine the PDTF platform with multiple different readouts, including flow cytometry, cytokine assays, single-cell RNA-, T cell receptor- and CITE-sequencing, or imaging readouts, offering the opportunity to functionally profile human tumor microenvironments at very high resolution.

Lastly, one of the major advantages of the platform is that it allows the simultaneous assessment of multiple treatments or treatment combinations within the same tumor lesion. Such a strategy can be applied to compare different therapies and to identify predictive markers allowing better patient selection. At present, the platform has mainly been used for treatment with immunotherapy agents, but the platform may be not limited to this class of treatments.[1]

Assessment of PDTF response to treatment

To assess the response of PDTFs to a therapeutic intervention, several considerations should be made. First, PDTFs show -sometimes substantial- basal immune activity that can be highly different between tumors. Therefore, an unstimulated control condition should always be taken along. Next, it is crucial to perform quality controls before analyzing the *ex vivo* cultures. These include, for instance, the evaluation of cell viability, immune infiltration, and heterogeneity between different conditions in the same culture, as, for example, some tumors have necrotic regions. In such cases, experimental conditions that contain a larger fraction of such fragments can perform substantially different from other experimental conditions (e.g., low basal secretion of highly abundant chemokines), and the true response of a tumor to treatment may be masked due to this imbalance between conditions. Similarly, high intratumor heterogeneity can lead to substantial variability in the presence of immune cells across fragments or the composition of PDTFs and, hence, to under- or overestimation of the treatment response. Therefore, it is highly recommended to perform replicate cultures of the same tumor to reduce noise due to intratumor heterogeneity and to validate findings. Moreover, when compiling a PDTF cohort, it is important to ensure that readouts across experiments use the same software acquisition settings, for instance when performing flow cytometry analysis, and that reagent lot numbers, e.g., for cytokine analysis, are maintained across different tumors.

LIMITATIONS

This protocol has a number of limitations, which will be discussed here. A first limitation is the loss of specific immune populations, specifically granulocytes, due to the cryopreservation of PDTFs. Other myeloid subsets can generally be preserved after freezing, but may be reduced in number in some tumors. In our study assessing the immunological response to PD-1 blockade,[1] we observed a very strong correlation of the *ex vivo* PDTF response with the patient's clinical response, indicating that the PDTF platform does allow one to measure meaningful treatment-induced responses even with cryopreserved fragments. If one is specifically interested in granulocytic cell subsets, PDTFs can be used freshly after cutting, thereby avoiding the loss of these subsets.

The PDTF platform allows for the profiling and modulation of cells that are already present in the TME. While this technology therefore cannot model changes induced by the treatment in the peripheral blood or other tissues, it can be used to disentangle systemic and local effects of a treatment, which may help to understand its mechanism of action.

Currently, the PDTF protocol is not been established for incubation periods exceeding 72 h, with best results for an incubation time of 48 h. After 72 h, viability of the cultures rapidly decreases, as culture conditions have been optimized for a short-term period with regard to medium selection, limited addition of supplements, and avoidance of growth factors and cytokines to maintain the TME as native as possible. The protocol likely needs to be adjusted for the purpose of applying treatments that require more than 72 h of culture.

A final factor potentially influencing the performance of the *ex vivo* cultures that should be taken into account is the heterogeneity in the presence and composition of infiltrating cells between fragments. This can on the one hand be a potential confounder of results; on the other hand it also provides an opportunity to understand a relationship between local immune composition and treatment response. To account for the former, in silico modeling has been used to determine the optimal number of fragments for capturing the response to *ex vivo* PD-1 blockade despite the presence of intratumor heterogeneity.[1] Based on these analyses, we advise to use a minimum of eight PDTFs per condition for assessing checkpoint blocking agents. This number may however vary depending on the treatment used. As an example, for assessing the efficacy of a bispecific T cell engager, three PDTFs per condition were sufficient as this compound activates all intratumoral T cells independent of their specificity, and not only a fraction of tumor-specific T cells like anti-PD-1.[8] Regardless, heterogeneity across conditions and between experiments can still occur and controls should be taken along (see above).

TROUBLESHOOTING

Problem 1

Altered viability and unspecific immune cell activation are observed in PDTFs upon swiching to a new Matrigel or Collagen batch (see media and reagent preparation).

Potential solution

Testing of new Matrigel or Collagen batches should be done via a side-by-side comparison experiment of an old functional batch with the new batch for both unstimulated and stimulated PDTFs to identify differences in response or unspecific immune cell activation. Preferably, this should be done for multiple samples. Instead of the recommended Corning high concentration Matrigel, other similar culture matrices with high protein density can be used, such as the Cultrex UltiMatrix Reduced Growth Factor from Biotechne (Cat#: BME001). We advise to use a basement membrane extract with low endotoxin content.

Problem 2

Reduced overall PDTF survival in culture (step 2).

Potential solution

It is common that cell viability decreases slightly during the culture. Therefore, it is crucial to start cultures using PDTF samples with sufficient viability (>50%, ideally >80%). Note that viability can be influenced by clinical pretreatment of the patient (e.g., recent chemotherapy or irradiation) or by a long time interval from surgery to PDTF processing. To exclude any tumor type-specific effect, try to culture other human material (e.g., tonsil tissue) for a side-by-side culture comparison. If higher tonsil viability is observed, the quality of the tumor material might not be optimal for the culture system. In addition, time course experiments can help to understand if culture for shorter time periods may remedy the issue.

Problem 3

The resected tumor tissue is too large (larger than 10 cm^3) to be processed in an adequate timely manner (step 5).

Potential solution

Processing and freezing of tumor material is a very crucial step and should be performed as fast as possible to ensure tissue viability. To this end, in case of a large tumor piece, the tissue should be cut into small chunks of approx. 4–5 cm that are then separately cut into smaller pieces, mixed, and further processed into PDTFs, and frozen in different rounds. Each round is then labeled with a different batch number following the order of freezing (e.g., first round – batch 1). To accelerate the process, it is recommended, if possible, to process the tissue with multiple people. In this way, PDTFs from distinct tumor areas can be processed, mixed and frozen per batch which helps to maintain tissue heterogeneity in each round. Afterward, viability and immune composition should be compared between the multiple batches obtained from the same tumor.

Problem 4

While processing, the tumor tissue disintegrates or tissue consistency makes it difficult to cut into small tumor fragments (step 6).

Potential solution

Tumor consistency is highly variable and dependent on the tissue of origin and cellular composition of the tumor. If the tissue easily falls apart while cutting, try to increase the size of the PDTFs. Loss of consistency is often related to the presence of necrotic areas. If possible, include examination of the tissue by a pathologist beforehand to exclude necrotic areas.

Problem 5

Tumor sample is composed of fragments with very distinct morphological features (step 36).

Potential solution

While putting PDTFs in culture, ensure that control and treatment conditions all have an adequate representation of the tumor. For example, if a tumor sample is composed of white, brown and black fragments all of those should be present in similar proportions in the different experimental conditions.

Problem 6

After digestion, PDTFs are not completely dissociated into single cell suspensions (step 44).

Potential solution

Some tumors can be very fibrotic and the remaining post-digestion tissue is often mainly composed by fibers. It is possible to extend the digestion step but not longer than for a total of 1.25 h. Alternatively, try mechanical dissociation of the tissue before filtering through the mesh. This can be done by vigorously pipetting the digested PDTFs in 5 mL PBS before the filtering step, as described in step 49.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Daniela S. Thommen (d.thommen@nki.nl).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102282.

ACKNOWLEDGMENTS

This work was financially supported by a PhD fellowship from the Portuguese Foundation for Science and Technology (no. SFRH/BD/52042/2012) through the Graduate Program in Basic and Applied Biology (to P.V.), by an ERC AdG SENSIT grant (no. 742259) (to T.N.S.), by a KWF Young Investigator Grant (no. 12046) (to D.S.T.), the Swiss National Science Foundation (no. P300PB_177881) (to D.S.T.), as well as an institutional grant of the Dutch Cancer Society and of the Dutch Ministry of Health, Welfare and Sport. We thank K. Monkhorst, K.J. Hartemink, C.U. Blank, and J.B.A.G. Haanen for their help with patient inclusion and tumor material collection, A. Broeks and the NKI-AVL Core Facility for Molecular Pathology & Biobanking for support with the administration of the tumor material, and the D.S.T.'s lab members for helpful discussions. The graphical abstract was created with BioRender.com.

AUTHOR CONTRIBUTIONS

Conceptualization, D.S.T.; Methodology and investigation, L.M.R., P.V., M.d.B., P.H., A.Z., T.N.S., D.S.T.; Visualization, L.M.R.; P.V., M.d.B., D.S.T.; Writing – Original draft, L.M.R., P.V., M.d.B., D.S.T.; Writing – Editing, P.H., A.Z., T.N.S., D.S.T.; Supervision, T.N.S., D.S.T.; Project Administration, D.S.T.; Funding acquisition, P.V., T.N.S., D.S.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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