Protocol For Establishment and Maintenance of Breast Tumor-Derived Organoids

The following protocol is optimized for generating Patient-Derived Tumor Organoid (PDO) cultures directly from surgical resections of Cancer or Patient-derived xenografts. Please refer to the organ-specific media conditions, which are shared as separate files.

We usually achieve about 70 - 90% success in establishing organoid cultures when starting with surgical resections of human tumors, and a greater than 90% success rate starting from patient-derived xenograft (PDX)-derived tumors. Important factors contributing to success are the amount of input tissue and the tumor cell content in the tissue. Tumor chunks larger than 1.0 cm^3 will increase the chance of success, whereas smaller chunks will give variable results. In addition, the tumor cell content is also a critical determinant of success; if the tumor chunk is primarily stroma-rich, it will negatively affect the ability to generate organoid cultures.

The initial organoid culture that develops after the plating of the processed tissue should be referred to as Passage 0 (P0). All subsequent passages (where there is an expansion of cells or simply performing a 1:1 split) should be serially numbered, and records should be maintained for each passage. Once the cultures are proliferating and increasing in cell number between passages, we recommend freezing down early passage cultures before using them for experiments. Once thawed from a cryopreserved vial of healthy PDO, we typically do not use cultures older than P20 for experiments due to concerns related to culture-induced genetic drift.

Note that there are two media outlined below:

*1) **BTOM**: designed for all subtypes of human, mouse, and rat tumors except those that are ER-.*

*2) **BTOM-ER:** designed to support the growth of ER+ tumors.*

For both media:

- *We prepare a master cocktail pre-mix and store it in small aliquots for extended periods of time (6 months).*
- *The master cocktail pre-mix is diluted in cell culture media with antibiotics and stored at 4 °C for short-term use (~1 month).*
- *Just before every use, we prepare 'growth media' that contains 5% Matrigel.*

Citation:

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- Co-first authors

Materials Needed and Growth Factor Cocktails

Materials	Vendor	Catalog #
Collagenase/Dispase(100 mg/ml)	Sigma	11097113001
Accutase	Sigma	A6964
DMEM+F-12	Thermo	11330-032
Rock Inhibitor (Y267632)	Tocris	1254
Matrigel (Growth factor reduced) (Concentration ~8.5mg/ml)	BD	354230
Penicillin-Streptomycin 10,000 U/mL	Gibco	15140-122
Bovine Serum Albumin Heat shock fraction	Sigma	A7906
Growth Media	See recipe below	
BTOM	See recipe below	
Tissue strainer (250µm)	Thermo	87791
Chamber slides	BD or any vendor	
Freezing media (Cryostar cs10)	Stem Cell Tech	7930

BTOM media growth factor cocktail

(For all breast cancer subtypes **except ER+ cancers)**

	Supplements/Growth factors	Vendor	Catalog #	Final concentration
Reagent A – (See note below)				

1	Bovine Pituitary Extract (BPE)	Hammond cell tech	1078-NZ	0.4 ml for 100 ml
2	B27	Thermo	17504001	1.0 ml for 100 ml
3	Recombinant Human FGF- Basic (FGF2)	Peprotech	AF-100- 18B	10ng/ml
4	Recombinant Human FGF-10	Peprotech	100-26	10ng/ml
5	Recombinant Human EGF	Peprotech	AF-100-15	5ng/ml
6	Recombinant Human IL6	Peprotech	200-06	100ng/ml
7	Recombinant Human Amphiregulin	Peprotech	100-55B	100ng/ml
8	Recombinant Human Prolactin	Peprotech	100-07	10ng/ml
9	Human Insulin	Sigma	I2643	10ug/ml
10	Cholerae Toxin	Sigma	C8052	200ng/ml
Reagent B – (See note below)				
	Hydrocortisone	Sigma	1316004	0.5ug/ml

BTOM-ER media growth factor cocktail for ER+ breast cancers

	Supplements/Growth factors	**Vendor**	**Catalog #**	**Final concentration**
Reagent A – (See note below)				
1	BPE	Hammond cell tech	1078-NZ	0.8 ml for 100ml
2	B27	Thermo	17504001	1.0 ml for 100ml
3	Recombinant Human FGF- Basic (FGF2)	Peprotech	AF-100- 18B	10ng/ml

4	Recombinant Human FGF10	Peprotech	100-26	10ng/ml
5	Recombinant Human EGF	Peprotech	AF-100-15	2ng/ml
6	Recombinant Human IL6	Peprotech	200-06	100ng/ml
7	Recombinant Human Amphiregulin	Peprotech	100-55B	100ng/ml
8	Recombinant Human Prolactin	Peprotech	100-07	10ng/ml
9	Human Insulin	Sigma	I2643	10ug/ml
Reagent B – (See note below)				
	Hydrocortisone	Sigma	1316004	0.5ug/ml

Note:

Aliquoting B27.

a. Place the frozen B27 stock in a 4-degree fridge overnight.
b. On the second day, gently invert the fully thawed B27 a few times and aliquot into 2.0 ml aliquots.
c. Store the tubes in a -80 ° freezer.

Aliquoting BPE

1. Place the frozen BPE in a 4-degree fridge overnight to thaw.
2. Incubate the fully thawed BPE in a 37℃ water bath for 1 hour. This helps lipids in BPE to dissolve.
3. Transfer the 100 mL BPE into two 50 mL conical tubes and centrifuge at 2500 rpm for 5 minutes.
4. Use a 10 mL pipette to transfer the supernatant into two new 50 mL conical tubes. Discard the precipitates.
5. Aliquot BPE into 2.0 ml microcentrifuge tubes and store in a -80 freezer.

Growth Factors:

Prepare growth factor stock solutions as 1000x stocks following the manufacturer's recommendation and store in 100 µL aliquots.

Hydrocortisone:

Prepare a 1000x stock and store in 100 µL aliquots.

Media Recipes:

Digestion Media:

DMEM

1:100 dilution of 100mg/ml stock Collagenase/ Dispase

1.0 % Penicillin-streptomycin

Preparation Tip: Prepare only the amount needed, fresh just before use, and discard any unused portion of the media.

Resuspension Media:

DMEM

1.0 % Penicillin-streptomycin

1.0 % BSA

Preparation Tip: Add 1% BSA by weight to DMEM + 1.0% Penicillin-streptomycin-containing media and stir to dissolve the BSA. Once dissolved, filter-sterilize using a 0.2 µm filter and store at 4 °C.

BTOM or BTOM-ER Growth Media:

DMEM/F-12: 100 ml

2.145 ml of Reagent A

50ul of Reagent B

1.0 % Penicillin-streptomycin

Preparation Tip:

Do NOT mix Reagent A and Reagent B by themselves, as the alcohol in Reagent B will denature the growth factors

After adding all ingredients, filter the media through a 0.2 µm filter

Store prepared media at 4˚C for NO more than one month.

Culture Media (make fresh for immediate use):

BTOM or BTOM-ER growth media.

5.0 % GFR-Matrigel

10 µM Y267632, Rock inhibitor (10mM, a 1000x stock). The rock inhibitor is prepared in sterile Phosphate Buffered Saline.

Preparation Tip: Prepare fresh. You may premix BTOM growth media and ROCK inhibitor and keep them on ice. Add Matrigel just before use. Discard unused portions of the media.

Freezing Media:

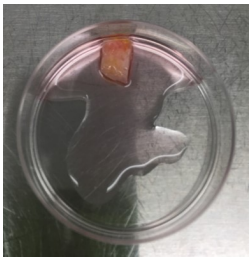
Cryostar freezing media

10 µM Y267632, Rock inhibitor

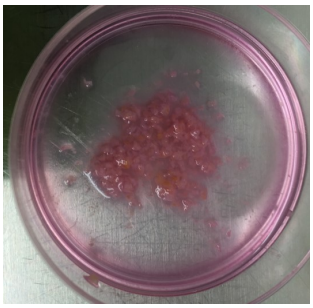
PROCEDURE FOR PROCESSING TISSUES

1. Place the tumor tissue received from the surgery in a 35mm petri dish. You may choose to take a small section of the tissue and fix it for histology processing. Additionally, another piece of the tissue can be snap-frozen in liquid nitrogen for future use to isolate DNA or RNA. These are optional and will depend on the amount of surgical material received.

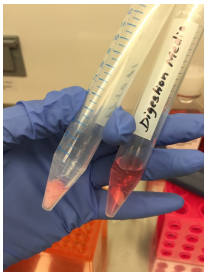
2. Add 1.0-3.0 mL of Resuspension media in the dish, depending on the size of the tumor. It would be advisable to use the smallest amount of media possible (~1.0 mL), which would provide better control during the tissue mincing process.



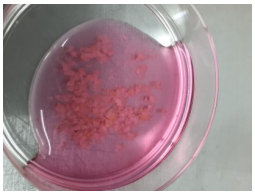
3. Mince tumor tissue with a sterile surgical scalpel (#21) into 0.5-1.0 mm fragments. It would be better to use two scalpel blades and move them in an anti-parallel direction to generate a finely minced tissue preparation. Be careful not to apply too much pressure during this step; if you do, you will end up scraping the plastic from the 35mm tissue culture plate. When the process is complete, the tissue pulp should resemble the image shown below.



4. Using a P1000 tip, transfer the tissue pulp into a 15.0 mL conical tube. You will not be able to collect all the tissue chunks using a regular P1000 tip, so use a fresh P1000 tip with its end cut to widen the bore. Add 1.0 mL of Resuspension media in a rinsing motion and transfer the digested tissue pulp to a 15.0 mL conical tube. Pellet the tissue suspension at 1500 rpm for 5 minutes at RT. Remove the supernatant carefully. Be aware that the tissue pellet will not be firm; therefore, take care not to disturb it. It would be advisable to first remove the majority of the supernatant with a P1000 tip and then use a P200 tip to remove the remaining supernatant. See below for an image of the tissue pellet.



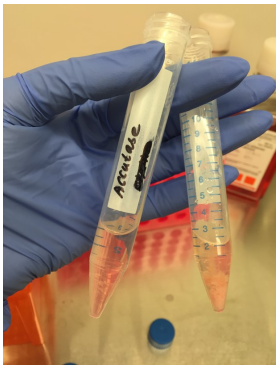
5. Add 2.0 mL of Digestion media. Using a P1000 tip with a tip cut (for wide bore), gently resuspend the tissue pellet and transfer it to a fresh 35 mm tissue culture plate. Incubate at 37 °C in a cell culture incubator for no more than 30 minutes. Check every 10 minutes for progress. After approximately 20 minutes, a distinctive pink or purple halo should be visible around the tissue chunks when observed under a microscope.



6. After 30 minutes, transfer the digested tissue slurry to a 15.0 mL conical tube. To remove all digested tissues, add 2.0 mL of Resuspension media and transfer the contents to a conical tube using a P1000 tip with a wide bore, as described above.

7. Centrifuge at 1500 rpm for 5 minutes at room temperature (RT).

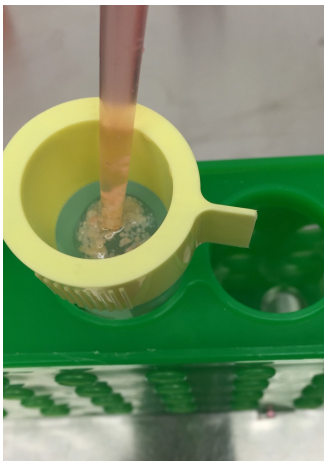
8. Aspirate the supernatant gently using a P1000 tip, taking care not to disturb the pellet. Add 2.0 mL of Accutase to the pellet. Mix well using a P1000 tip with a wide-bore tip and incubate in a 37˚C water bath or a tissue culture incubator for 30 minutes.



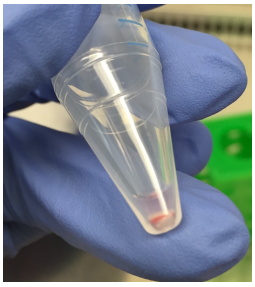
9. During the incubation period, you can prepare a well in a 12-well culture dish for plating the cells. Coat 1 well of a 12-well plate with 150 µL of Matrigel. To spread it evenly into a single layer, use a P200 pipette tip. As you release the Matrigel onto the surface of the well, move the tip in a zig-zag motion, keeping the

space between the lines to a minimum. It is essential that you do not eject all the Matrigel during this process, as it can cause air bubbles to form. As you complete the zig-zag motion from the top to the bottom of the well, use the remaining few microliters of Matrigel to coat the circumference of the well with Matrigel. To check the efficiency of the Matrigel coating, hold the plate towards the light and look for any gaps in the coated surface. Once you are certain that you have evenly coated the Matrigel, move the plates to a 37 °C cell culture incubator and leave them there until the cells are ready to be seeded.

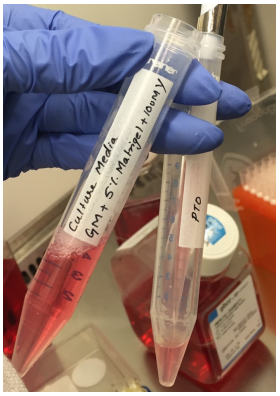
10. Remove the cells + Accutase tube from 37˚C, and add 1.0 mL of Resuspension media. Using a wide-bore P1000 tip, gently mix and transfer the tissue slurry to a tissue strainer mounted onto a 50.0 mL conical tube. Move the tip gently over the strain to allow the digested cells to flow through and tissue debris to remain trapped on top. Add 1.0 mL of Resuspension media to the top of the cell strainer, gently disturbing the debris to release any digested cells that may be trapped.



11. Centrifuge the flow-through at 1500 rpm for 5 minutes at room temperature (RT). You should be able to observe a small but distinct pellet as shown below.



12. Gently resuspend the pellet in 2.0 mL of **freshly** prepared culture medium. You should not prepare culture media with 5.0% Matrigel and let it sit on ice for extended periods, as this will result in sedimentation of the Matrigel. To save time, you may prepare Culture media without Matrigel and store it at room temperature (RT) until ready. Just before resuspending cells, add 5% Matrigel and use immediately.



13. Gently, in drops, transfer the 2.0 mL of the resuspended cells to the Matrigel-coated well in the 12-well dish. Be careful not to squirt the suspension as you will disturb the underlying Matrigel layer.

14. Monitor growth. Take representative images every 4 days to document growth. These images can be used to determine growth rate by morphometric analysis.

15. **Changing media**. It is essential to change the media every 3-4 days. As noted earlier, Matrigel-containing culture media must be prepared just before addition to the well to prevent sedimentation of the Matrigel.

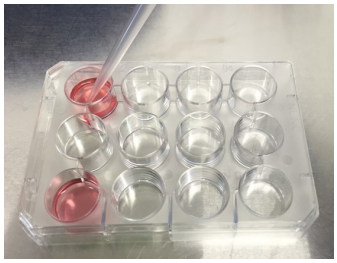
16. Aspirate the old media gently using a P1000 tip, taking care not to disturb the Matrigel pellet or the Organoids. Add Matrigel containing Culture media to the well, gently, in drops. Take care not to add it too fast, as it will disturb the organoids and the Matrigel layer.

PROCEDURE FOR MAINTAINING, PASSAGING, AND CRYOPRESERVING ORGANOID CULTURES

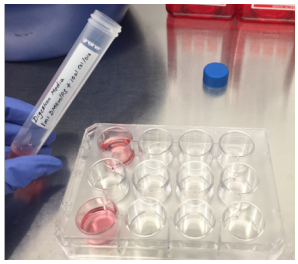
The time to passage the organoids can vary depending on the organoid line. In our experience, we have found some PDO lines grow rapidly and are ready for passage by day 12-14, whereas others take much longer. In general, the time to passage is identified by cultures that have stopped growing for more than 4 days.

The protocol below is optimized for one well of an organoid line in a 12-well plate.

1. Aspirate the old media using a pipette tip. Do not use an aspirator. Be careful not to disturb the Matrigel layer or the organoids.

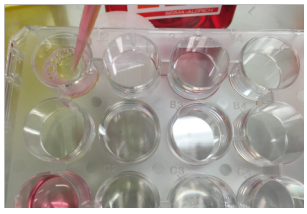


2. Add 0.5 mL/well of Digestion media. Add gently and in drops, working close to the Matrigel surface to minimize turbulence generated by the added media.

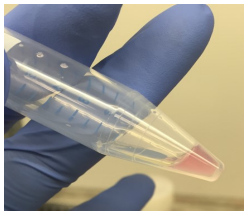


3. Incubate at 37˚C for 1.5 hours. By 1.5 hours, you should be able to observe the Matrigel digested into a pulp or slurry and the organoids dispersed into small clumps or clusters. This indicates the completion of digestion. If these changes are not noticeable, you could extend the digestion for another 30 minutes.

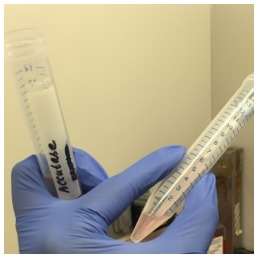
4. Resuspend the cells by adding 1.0 mL of Resuspension media. The media can be cold, preferably not warmed to 37˚C. Add the media in drops. Gently resuspend the Matrigel using a P1000 tip several times until it forms a smooth suspension and the organoids have dispersed into single cells or smaller clumps. Transfer it to a 15.0 ml conical tube.



5. Centrifuge at 1500 rpm for 5 minutes at room temperature (RT). The pellet containing Matrigel and cells will not be prominent, but can only be seen as a difference in texture, and not necessarily as a difference in color. Remove the supernatant gently using a P100 tip. This is a critical step, as it is easy to aspirate the Matrigel/cell pellet. Pay attention to the difference in consistency while removing. To be safe, you may choose to remove the supernatant in two stages: first, remove ~1200 microliters using a P1000 tip, and then remove the rest using a P200 tip. The P200 tip will allow you to readily feel the difference in consistency between the supernatant and the Matrigel/cell pellet. The image below shows the Matrigel pellet with a small amount of supernatant remaining.



6. Resuspend the Matrigel pellet by adding 1 mL of Accutase. Accutase comes in a 100 mL bottle from the vendor. This should be aliquoted into smaller volumes (1.0, 5.0, or 10.0 mL) to minimize the number of repeated freeze-thaw cycles. Pipette up and down until the Matrigel/cell pellet is resuspended into a smooth slurry (see image below).



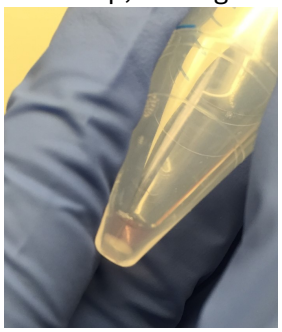
7. Incubate cells + accutase tubes at 37 °C for 20-30 minutes.

8. During the above incubation period, prepare wells in a 12-well plate for reseeding cells. We typically split the culture at a ratio of 1:2 to 1:4. The choice of split ratio depends on the growth rate (fast-growing versus slow-growing organoid lines) or other cell needs, such as isolating DNA/RNA or preparing cells for cryopreservation. We have found that some lines grow very well and can be split at a 1:4 ratio, whereas some are slow-growing and require a 1:2 split. It may be wise to perform a 1:2 split during the early passages until you become familiar with the organoid line.

   Once you have determined the split ratio, coat the required number of wells in a 12-well plate with 150 µL of Matrigel. To spread it evenly into a single layer, use a P200 pipette tip. As you release the Matrigel onto the surface of the well, move the tip in a zig-zag motion, keeping the space between the lines to a minimum. You mustn't eject all the Matrigel during this process, as it can cause air bubbles to form. As you complete the zig-zag motion from the top to the bottom of the well, use the remaining few microliters of Matrigel to coat the circumference of the well with Matrigel. To check the efficiency of the Matrigel coating, hold the plate towards the light and look for any gaps in the coated surface. Once you are certain that you have evenly coated the Matrigel, move the plates to a 37 °C cell culture incubator and leave them there until the cells are ready to be seeded.

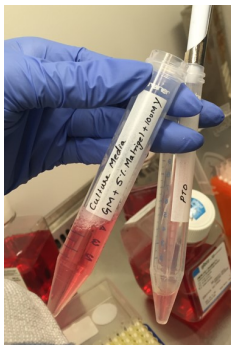
9. Remove the cells + accutase tubes from 37˚C and add 500 µL of Resuspension media. Gently pipette up and down to create a smooth cell suspension. Use a small aliquot to determine the cell count using a Coulter counter or a hemocytometer. If you need cells for generating a cell pellet (for DNA/RNA isolation), an aliquot of this cell suspension can be used. It is also wise to freeze down (see below for details) as many vials of cells as possible during early passages to have stocks of early passage organoid cultures.

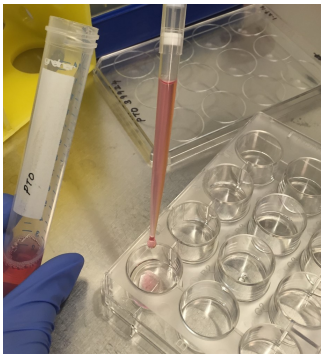
Once you have taken all the cells needed for generating a pellet or cryopreservation, pellet the remaining cells at 1500 rpm for 5 minutes at room temperature (RT). Aspirate the media using a P1000 tip, leaving a few microliters of media to avoid disturbing the cell pellet (see image below).



10. For Re-seeding:

- Prepare Culture media only just before you need it and keep it on ice. It would not be advisable to add the 5.0% Matrigel to the media and let it sit for extended periods, as this will result in sedimentation of the Matrigel. You should also bring the Matrigel-coated 12-well dish to the hood, so resuspended cells (see below) can be plated immediately.
- If you are re-seeding a defined number of cells per well, you can re-seed them at a density of 50,000 to 150,000 cells per well in a 12-well plate. The choice of cell number will depend on the organoid line; fast-growing lines can be plated at a lower density, whereas slow-growing lines require a higher density. Resuspend the pellet @ 1.0 mL of Culture media for each well of the 12-well dish. Gently resuspend the cells using a P1000 tip and plate them immediately in the Matrigel-coated well of a 12-well dish.





- Changing media. It is important to change the media every 3-4 days. As noted earlier, Matrigel-containing culture media must be prepared just before addition to the well to prevent sedimentation of the Matrigel. You may choose to prepare Culture media without Matrigel, but containing ROCK inhibitor, and store it on ice until you are ready to add it to the culture dish.
- Aspirate the old media gently using a P1000 tip, taking care not to disturb the Matrigel pellet or the Organoids. Add Matrigel containing Culture media to the well, gently, in drops. Take care not to add it too fast, as it will disturb the organoids and the Matrigel layer.

- You should take representative images every 4 days to document growth. These images can be used to determine growth rate by morphometric analysis.

11. **Histogel blocks**: This is an effective method to prepare tissue blocks of organoids that can be used for various immunohistochemical analyses. Having a bank of these histogel blocks from different organoid cultures can empower your discovery and validation studies.

    Please refer to the Histogel protocol available on our lab website for a detailed protocol.

12. Cryopreservation:
    - Resuspend the pellet from step 9 in freshly prepared Freezing media
    - Freeze at 200,000 cells/ml @ 500 µL / 1.5 mL Cryotube
    - Store at -80 °C overnight and transfer to the liquid Nitrogen tank the next day (within 24 hours of placing in -80 ˚C)

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