# Primary Cell Culture – Organoid Preparation

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## REFERENCES:

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## MATERIALS NEEDED:

Reagents/Equipment for Processing Tissue:

- Razor blades or Sterile Scalpels
- Forceps
- 100mm culture dishes and 50 mL conical tubes (for washing tissue)
- 70% Etoh, and ETOH burner (or equivalent) for sterilizing razor blade and forceps
- Dulbecco's PBS
- Fungizone (Invitrogen #15290-018) or Amphotericin B (ATCC#30-2301)
- Penicillin (10,000U/ml) /Streptomycin (10,000 ug/ml) (Invitrogen # 15140-155 or equiv.)
- Normocin (Invivogen –not Invitrogen)
- DMEM High Glucose (Sigma# D6546) and Glutamine (Invitrogen # 25030-156); or use Invitrogen #31053-028 for phenol-red free).
- Collagenase I (Invitrogen# 17100-017)
- Benchtop Cell culture Centrifuge (15ml/50ml conical tubes)

## INSTRUCTIONS:

**(Note that the following protocol can be used on both normal and tumor derived tissues. However, one will find that the stroma of tumors is not always effectively dissociated by collagenase. In these cases, other proteases (Dispase, trypsin, etc.) may be required)**

**Day of tissue arrival:**

1. Enter the tissue pathology # into our database.
2. Clean the cell hood, and gather instruments and reagents.
3. Sterilize the razor blade (if you are not using a pre-sterilized scalpel) and forceps with ETOH and flame (or use disposable scalpel). Place the blade and tip of the forceps into a 10cm culture dish filled with ~20ml PBS.
4. Spray your gloved hands with generous amounts of 70% ETOH. Use sterile procedures throughout the entire process.
5. Using forceps to grasp the tissue, place breast tissue into a 150 mm culture dish. Pour generous amount of PBS over the tissue. Using forceps, pick the tissue up, and pour more PBS over the tissue to remove the blood. Place the tissue into a new 150mm dish. Rinse and repeat. Some tissues are bloodier than others, so this could take several washes. Be generous with the PBS, use the entire 500ml bottle if needed. The goal is to remove all traces of blood! This makes dealing with the cells (especially if FACS sorting) much easier down the line.
6. Rinse/wash the tissue one final time with antibiotics&antifungal (PBSA) and place the tissue into a 150mm dish.
7. Using the forceps in one hand (to hold the tissue in place), gently cut in-between the forcep arms to create slices in the tissue. The goal is to create more surface area for the enzyme to act. Cutting the tissue into little pieces isn't required, but do score the tissue many times, preferably in a cross-hatch pattern.
8. Place the tissue fragments into 50ml culture tubes. The tissue should take up no more than 2/5$^{th}$ of the volume of the container, so you will likely need multiple tubes, depending on the amount of starting material (10-15g of tissue per tube)

9. Fill the tube to 4/5 of its capacity with DMEM+Pen-step+Normocin.
10. Add 1/100$^{th}$ volume (typically 400ul) of 100x Collagenase I to give a final concentration of 0.1% w/v. Screw the top onto the centrifuge tube, seal with parafilm, and place into a heat-sealable bag and seal. Place this bag into another secondary bag and seal. –this ensures that if there is a leak, it won't contaminate the equipment. Agitate the tubes gently overnight at 37°C. I use a rotating shaker set for 80rpm. NOTE: The speed/strength of agitation can have a dramatic effect on the amount of yielded organiods and on CD44 presentation (Hines 2014).
    a. Note: to culture mature adipocytes in a 'ceiling culture,' stop the digest after ~ 1-2 hours. (you will need to have ready 90ml of DMEM/F12 +20% fetal calf serum equilibrated in a CO2 incubator –a good idea is to place it into a 150mm dish prior to processing the tissue above). Remove the tubes from incubator and spin the tube(s) at 100g for 2 min. Transfer the floating packed adipocyte layer (fat layer) to a 15 ml tube. Cap the remaining collagenase digest, seal it in a bag, and place it back into the incubator to allow it to digest overnight at 37°C with agitation. To the adipocytes, filter through 200um mesh, rinse the filtrate with DMEM/F12, centrifuge and repeat wash. Inject 200ul of packed adipocytes into a 25cm2 flask (without a gas seal) filled to the brim with the equilibrated DMEM/F12+20% FCS. Place in incubator and culture for several days.

**The following morning:**

11. After the incubation, the mixture should look like a greasy, brown broth. Spray the outer bag with 70% ethanol and remove outer bag. Switch gloves, and repeat with inner bag.
12. Centrifuge ~50g for ~2 minutes to pellet cellular material/organoids. Remove the oil and cloudy supernatant. Resuspend the organoids in PBS, and evaluate the digestion. If fibrous material and large fragments of tissue remain, repeat the collagenase digestion for another 4-12 hours. (Note that if you are digesting tumors, some will not be digested by Collagenase I, and will require a different protease; e.g. trypsin, Dispase).
13. If digest is sufficient, pellet the organoids and re-suspend in PBS. Repeat a couple of times. The organoids are now ready to be used or archived.
14. Protocol
    a. Set aside any organiods that are to be immediately used.
    b. Trypsinize ~1 ml of organoids to single cells, and archive 10 vials w/ 500k each. These can later be used for flow cytometry
    c. Archive the remaining organoids (freeze medium: 75% growth medium/15% FBS/10% DMSO).
15. FYI: To dissociate the organoids with trypsin (for FACS, etc.), wash the organoids 2 x with PBS (no Mg/Ca). Add 3 ml trypsin (0.05% w/v). Hold in your hand (~37 degrees) for several minutes, with slight agitation. When you observe many single cells sloughing off, GENTLY pipet the orgnoid/cell suspension through a 18Ga needle( or 10ml serological pipette) multiple times. Filter cells through a 100$\mu$m cell strainer several times. Add Soybean trypsin inhibitor, and pellet the cells (400g x 5 min). Remove supernatant, and Rinse 2 x with PBS/1% BSA. Cells are now ready for FACS staining, etc. (For more detailed instructions see our protocol "preparing organoids for FACS analysis"

## Recipes:

1. **PBSA (PBS/PenStrep/Fungizone) Wash**: 1X Dulbecco's PBS supplemented with penicillin (~200U/ml), streptomycin (~200μg/ml), and fungizone (~5μg/ml).
    a. To 500ml DPBS (Sigma #D 8662), Add:
        i. 10mL of penstrep solution (supplied as 10,000 U/ml & 10,000 μg/ml)
        ii. 10mL fungizone (supplied as 250 mg/ml)
    b. Store at 4°C

2. **DMEM/Collagenase** (I perform this in serum free medium; however, you may want to use 10%FBS for tumor samples)
    a. To a 500mL bottle of DMEM (high glucose, Sigma# D6546)
        i. Add Glutamine (5.1 mL)
        ii. Add Penstrep (5.1 ml)
        iii. Thaw and add CollI (below) to medium prior to 37°C incubation.

    Note: I initially used collagenase III for breast tissue digests, but when Gibco discontinued this item, I switched to Collagenase I , without any problems. Other labs use Collagenase IV, and I have used this as well, and have not noticed any differences.

3. **Collagenase I** (Gibco/Invitrogen, supplied as 1 gram lyophilized powder)
    a. Remember: Collagenase is inhibited by metal chelating agents; such as cysteine, EDTA or 0-phenanthroline
    b. Dissolve collagenase powder in 10ml DPBS, and filter sterilize with syringe filter (0.2 μm). The Final concentration = 100mg/ml (10% w/v) and is a 100X concentrate.