Create cell suspension from Human Tonsils

I – Principle

This SOP describes the process of isolating cells from tonsils, followed by storage in liquid nitrogen.

II – Safety Overview

- It is the responsibility of all personnel handling human blood samples to ensure they are vaccinated against Hepatitis B and are trained to treat all biological samples as potentially infectious.
- The Safety and Ethics Committee must approve all work before commencement.
- Standard personal protective equipment (PPE) must be worn when performing laboratory work unless otherwise stated. Standard PPE includes.
    - Long-sleeved lab gown, enclosed footwear, safety glasses and gloves.
- Please be aware of the risks involved in working with human samples.
    - Care must be taken when handling blood and purified cell populations obtained from clinical samples.
- Class II Biosafety Cabinet must be used when handling unfixed blood.
    - Cabinet must be sealed and exposed to UV light for 20 min prior to use.
    - Cabinet surface must then be wiped down with 80% (v/v) ethanol.

Any items for use in the cabinet (including pipettes) must be decontaminated with 80% (v/v) ethanol.

III – Equipment, Reagents and Consumables

Equipment

- Finnpipette™ F2: P2, P20, P200, and P1000
- Finnpipette™ F2: Multichannel pipette 30-300uL
- Eppendorf Centrifuge 5910
- Class 2 Biosafety Cabinet
- CO2 incubator (at 37C)
- Mr. Frosty containers
- CellDrop – Automatic cell Counter
- Water Bath (at 37C)
- Liquid Nitrogen tank
- Esky – for transport of samples between facilities
- Fridge 4C
- Freezer -20C
- Freezer -80C

Reagents/Chemicals

- Sodium Pyruvate (Thermo Fisher -Gibco # 11360070)

- DeNovix Acridine Orange / Propidium Iodide (Cat# CD-AO-PI1.5)
- DMSO (Merck)
- FBS (Thermo Fisher – Gibco # 10099141)
- Ham's F12 media (Thermo Fisher – Cat# 11765054)
- Insulin-Transferrin-Selenium (Thermo Fisher – Gibco # 41400045)
- NEAA (Non-essential Amino Acids) (Thermo Fisher - Gibco # 11140050)
- Normocin (Jomar – INV-ant-nr-2)
- PBS- 1X Filtered Phosphate Buffer Saline
- Pen/Strep (Thermo Fisher -15140122)
- RPMI 1640 with L-glutamine (Thermo Fisher - Gibco # 11875119)

Consumables

- Tips (various manufacturers) 10, 20, 200 and 1000 µL for use with Finnpipette™ F2 pipettes (listed above)
- 1.5 ml polypropylene tubes (Various manufacturers)
- 50 ml polypropylene tubes (Various manufacturers)
- Petri Dishes (Various manufacturers) – 10 ml
- 1ml disposable transfer pipette (Various manufacturers)
- Cryovials (Corning)
- Scissors/tweezers/scalp blades
- Syringe Terumo10 ml

Notes:

Antimicrobial bath (AB) contains:

- Ham's F12 media. Contains L-glutamine and phenol red
- Normocin: 50 mg/mL, use at 100 ug/mL (1/500)
- Pen/Strep Contains 10,000 units/mL of penicillin & 10,000 µg/mL of streptomycin. Use at 50 U/uL (1/200).

Use within 1 month.

Tonsil Processing Media (TPM) contains:

- RMPI-1640. Contains L-glutamine, phenol red, and 2.0g/L sodium bicarb. Use 450 ml.
- Heat inactivated FBS, use at 10%.
- Normocin 50 mg/mL at 100 ug/mL (1/500).
- Pen/Strep Contains 10,000 units/mL of penicillin & 10,000 µg/mL of streptomycin. Use at 50 U/uL ug/ml (1/200).
- 100 x NEAA (Non-essential Amino Acids), use at 1X.
- 100 x Insulin-Transferrin-Selenium, use at 1X.
- 100 mM Sodium Pyruvate, use at 1mM.

This media is for processing of the tonsils; it is NOT SUITABLE once growing organoids. This is because the RPMI contains L-glutamine, which degrades into ammonia overtime.

Preparation

Process	Steps
Prepare Reagents	1. Thaw any reagents and place at 4°C 2. Cool centrifuge to 4°C 3. Place Mr Frosty containers and PBS into the fridge 4. Prepare antimicrobial bath (AB) and tonsil processing media (TPM), and keep at 4°C.
Antimicrobial Bath	5. NOTE: All samples should be placed into an antimicrobial bath upon receipt 6. Aspirate PBS from 50 mL falcon tube 7. Add 10 mL AB to tube. 8. Incubate for 1 hour at 4 °C. 9. During incubation, print labels for cryovials

Dissecting into single cell suspensions

Process	Steps
Tonsil's preparation	NOTE: From here, process 2 tonsils at a time. Leave other samples in AB bath at 4°C 1. Aspirate AB from 50 mL tube, replace with 15 mL PBS to rinse. 2. Place a 10 mL petri dish onto ice and fill with 15 mL of TPM. 3. Place lid face-up inside the hood 4. Use this to rest sterile tools later. 5. Place tonsils into petri dish to begin dissection.
Tonsil's dissection	NOTE: While dissecting, keep pieces submerged in media as much as possible 6. Remove any blood clots or fibroid tissue and discard. 7. Record observations of any blood clots, fibrous or cauterised tissue 8. Dissect the tonsil into <0.5 cm pieces.
Make cell suspension	Two dish method: 9. Place a second 10 mL dish containing 15 mL TPM on bench (inside the hood – but not on ice) 10. Place four 100 um cell strainers into the dish, and transfer tonsil pieces into these

	11. Using a 10 mL syringe plunger, push tonsil fragments through the cell strainers to make a cell suspension. 12. Place a new 100um cell strainer onto a 50 mL tube. 13. Using a 10 mL stripette, transfer cell suspension from the petri dish over the 100-um filter and into the 50 mL tube 14. Remove all cell strainers from the petri dish. 15. Rinse the petri dish with 10 mLs of TPM, then pipette this volume over the cell strainer and into the 50 mL tube. 16. Rinse the 100um strainer with a further 5 mL TPM, collect in the 50 mL tube. 17. Incubate the resulting cell suspension at 4°C for 10 minutes. 18. The stroma settles to bottom of tube. 19. Gently transfer cell suspension onto a new cell strainer and into to a new 50 mL tube, leaving behind the settled stroma 20. Top up tube to 45 mLs with TPM 21. Rinse cell strainer in the process 22. Centrifuge the cell suspension at 300 g for 10 minutes, acceleration 6, brake 6, at 4°C 23. Aspirate supernatant 24. Resuspend in 20-30 mL TPM. 25. Prepare a 1/100 dilution of the sample. 26. 10 uL samples + 990 uL TPM 27. Count 1/100 diluted sample on cell drop. 28. Add 10 uL of 1/100 dilution to 10 uL AO/PI 29. Use primary cell AO/PI program with Boyle lab settings. 30. Record on L: drive Count Record

Storing cell suspensions

Process	Steps
Sample preparation for long-term storage	1. Centrifuge the cell suspension at 300 g for 10 minutes, acceleration 6, brake 6, at 4°C. 2. During the spin, prepare 10% DMSO in FBS 3. Aspirate supernatant, then resuspend cells to 100 e6 cells/mL in 10% DMSO/FBS 4. Aliquot into “SPL” brand cryovials, 1 mL/vial 5. Place cryovials into Mr Frosty, then place Mr Frosty into -80 °C freezer overnight 6. Transfer samples to vapour phase cryogenic storage the next morning.