SOP- Preparation and maintenance of tumour-infiltrating lymphocyte, cancer associated fibroblast and autologous tumour cultures (ovarian cancer)

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Gynaecological Oncology Group, Division of Cancer Sciences

Standard Operating Procedure

(Version 1.1)

Preparation and maintenance of tumour-infiltrating lymphocyte, cancer associated fibroblast and autologous tumour cultures (ovarian cancer)

Revision History		
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1. **Introduction**
   Molecular and cell biology techniques are important tools for assessing the differences between normal and diseased cells and tissue. Such techniques have also been employed to detect markers that may predict some response to treatment and/or have prognostic significance. These translational research studies can greatly influence the success or failure of novel therapies. However, these investigations can only yield valid results if rigorous procedures are followed to ensure the collection and storage of high quality tissue samples.
2. **Purpose**
   The purpose of this document is to provide clear guidance on the procedures to be followed during the processing of ovarian tumour tissue to establish a) a tumour infiltrating lymphocyte culture; (b) an autologous tumour cell culture; (c) a cryopreserved aliquot of tumour culture digest; (d) subsequent cryopreservation of TIL and autologous tumour cultures for future assays.
   Departmental standard operating procedures will already exist within the institute/trust. These must be read and followed in conjunction with this document.
3. **Location**
   This document pertains to processing of human ovarian tumour tissue on the 5th floor laboratories of St. Marys Hospital, Manchester.
4. **Objective**
   The ultimate aims of this document are to ensure that TIL and tumour cultures are established reproducibly, and that appropriate records and recordings are taken to ensure that as much information as possible is available to allow analysis of data. Additionally, this document aims to ensure that cultures and disaggregates are cryopreserved to ensure that assays and/or rapid expansion protocol may be carried out on all patient's samples if needed.
5. **Scope**
   This document refers to the collection of tissue for the MFT Biobank for use in the research carried out into ovarian cancer under Professor Richard Edmondson. The ultimate aims of this document are to ensure that tissue and fluid specimens, which are collected under the standard operating procedures established previously, are processed to produce cell cultures under the 'ovarian TIL' spectrum of research being carried out in the Edmondson group. Tissue collected on behalf of the lab will also be used to establish basic tumour culture (which will be cryopreserved and used for a raft of established characterisation assays), cryopreserved for RNA analysis, formalin fixed and paraffin embedded for immunohistochemistry, and some tissue gifted to collaborators under MTAs. Therefore, this SOP only concerns a small portion of the work to be carried out on tissue procured through MFT Biobank, which should be considered in the process of carrying out this work.

6. Responsibilities

6.1. The collection of tissues and fluids containing malignant cancerous cells from patients suffering from ovarian cancer is the responsibility of the clinicians operating on or caring for the patient.

6.2. Following sampling of the tissues, the clinical team is responsible for contacting members of the Edmondson lab to ensure that samples are delivered and processed within 30 minutes of their sampling in theatre.

6.3. Clinical fellows and researchers are under the direct supervision of the institution / trust line management.

6.4 Clinical fellows and researchers, in collaboration with the clinical care team for the following:

6.5.1. Patient identification and consent, specimen labelling, early storage, and transportation of the samples

6.5.2. Ensuring that sampling and storage occurs within a timely manner Once tissue is *ex vivo*.

6.5.3. Accurately completing any accompanying documentation

6.5.4. Investigation or being aware of back up procedures in the eventuality that a problem arises during sampling or transportation (e.g. blood spillage)

6.5.5 Liaising with other trust and Edmondson lab staff.

6.5 It is the responsibility of the staff or student processing procured tissue to ensure: (a) that tissue is processed in a timely manner; (b) that appropriate records are kept of tissue storage within the lab/storage facilities, and experimental procedures carried out on tissue and cell lines; (c) that experiments are carried out safely and reproducibly, in accordance with guideline documents listed below; (d) that the staff/student member is familiar with the correct methods of disposal of tissue or downstream cultures/experimental products.

7. Related Documents

7.1. Guidelines for Good Clinical Practice (GCP)

7.2. Human Tissues Act 2004

7.3. COSHH regulations 1988

7.4. Local risk assessments / safe operating procedures

7.5. Local Standard operating procedures

7.6. National Blood service (www.blood.co.uk)

7.7. MFT Biobank list of SOPs and Guidance documents

Appendices

Appendix A: List of SOPs and Guidance documents

Standard operating procedures, Risk Assessments and related COSHH

- 'Disaggregation of solid tumour samples using gentleMACS dissociator and MACSmix tube rotator' (TIL1)
- 'Preparation of TIL Media and initiation and maintenance of TIL and autologous tumour cultures' (TIL2)
- 'Safe operation of a laboratory centrifuge'
- 'Use of a microbiological safety cabinet'
- 'Disposal of biological agents, human blood, body fluids and tissue'
- 'Primary cell culture from ascitic fluid and solid tumour tissue from patients undergoing treatment for ovarian cancer'
- 'Permitted laboratory disinfectants'
- 'How to deal with needle-stick injuries and contamination accidents'
- COSHH: Beta-mercaptoethanol (Gynae Onc drive)
- COSHH: Tumour Dissociation Kit, human: enzymes H, R and A (Gynae Onc drive)
- Further COSHH forms available for reagents used in this protocol are stored on the shared H&S drive.

Standard Operating Procedure

Tumour Disaggregation Protocol

Preparation:

1) Ensure that MACSmix rotator has been sufficiently charged to carry out incubation steps.
2) Warm 100mL RPMI-1640 (no supplements) and RPMI-1640 (+L-glutamine/+HEPES) for at least 2h before needed. Thaw any frozen aliquots or pen-strep stock. Avoid multiple freeze/thaws.
3) Prepare TIL media as below.

[C]	Reagent		Volume of stock (mL)	Stock [C]
	RPMI-1640 (supplements highlighted)		445	N/A
2mM	L-glutamine			2mM
25mM	HEPES			25mM
10%	HI FBS		50	N/A
100IU/mL	Penicillin	Combined reagent	5	10,000IU/mL
100µg/mL	Streptomycin			10,000µg/mL
50µM	β-mercaptoethanol		1µL/mL	50mM

4) Assemble instruments/consumables for tumour disaggregation:
   (a) Sterile scalpel and disposable forceps
   (b) 200mm petri dish
   (c) Balance
   (d) Miltenyi C-tubes
   (e) Tube racks (to fit 50mL Falcon/C-tube)
   (f) RPMI-1640 (no supplements)
   (g) Human tumour dissociation kit (Miltenyi Biotec)
   (h) P100/P200/P1000 and 20/200/1000µL tips

Protocol:

1) Weigh sample.
2) For up to 1g of tissue, add 4.7mL RPMI-1640 (no supplements) to the C-tube. Add 200µL enzyme H, 100µL enzyme R and 25µL enzyme A. For between 1 and 2g, double volumes of media and enzyme. Carefully secure the lid of the C-tube until it clicks.
3) Remove tumour sample from TSS. If sample is particularly bloody, it can be rinsed in fresh PBS. Remove any fat and weigh. Cut into 2-4mm^3 fragments using a sterile scalpel. Rinse fragments again with PBS if needed. Place fragments into a C-tube (up to 2g/tube – run multiple tubes if necessary).

4) Follow dissociation program below using GentleMACS dissociator (GMD) and MACSmix (MmR) rotator in incubator:

Step No.	Equip.	Action	Time (mins:secs)	Temp (°C)
1	GMD	Run *h_tumour_01* program	0:36	RT
2	MmR	Rotate/incubate (20 rpm, cont. rot.)	30:00	37
3	GMD	Run *h_tumour_02* program	0:36	RT
4	MmR	Rotate/incubate (20 rpm, cont. rot.)	30:00	37
5	GMD	Run *h_tumour_03* program	0.36	RT

5) Use a 100µm cell strainer to filter digest into a 50mL Falcon. Wash filter with 20-40mL RPMI-1640. If digest is particularly fatty or fibrotic, the digest may need to be split between tubes/filters.
6) Centrifuge at 400xg for 7 minutes.
7) If processing omentum, gently aspirate fat layer from the surface of the supernatant with a 5-10mL stripette and discard before aspirating the remaining supernatant.
8) Aspirate supernatant and resuspend pellet in T-cell media (scale volume depending on pellet size). If digest is very contaminated with red blood cells, can use RBC lysis buffer (Miltenyi) before use (mandatory if being used for flow).
9) Count live cells using Trypan blue.

Initiation of Tumour/CAF Cultures

1) Transfer desired volume of tumour digest to a fresh tube and centrifuge at 400xg for 5 minutes.
2) Aspirate supernatant and resuspend pellet in RPMI 1640 with 10% FBS, 1% penicillin-streptomycin and 0.1% amphotericin-B.
3) Transfer to a 50µg/cm^2 collagen-I coated vessel (approximate plating densities <0.5x10^6 cells into T25, 0.5x10^6 – 1x10^6 cells into T75) and incubate in hypoxic incubator.
4) After 24 hours, assess adherence and confluency.
5) After 48-72 hours, dead/unattached cells can be washed off with DPBS and media replaced.
6) When vessel reaches approx. 70% confluency, assess morphology of cells (look for colonies of cobblestone-like cells).
7) Wash flask with DPBS and add 2-4mL of 0.05% trypsin-EDTA.
8) Assess detachment of cells under light microscope throughout, gently swirling the trypsin over the adherent surface every minute. 'Fibroblasts' should round up quickly (<2 mins) while cobblestone-like colonies will remain attached for much longer.

9) If detachment after rounding up is slow, the flask can be placed in the incubator for a minute before checking again.
10) When the majority of cells surrounding cobblestone colonies have detached, aspirate trypsin gently and add to 10mL of DMEM with 10% FBS, 1% penicillin-streptomycin and 0.1% amphotericin.
11) Wash the adherent surface of the flask very gently with DPBS (add DPBS, swirl flask and aspirate rather than wash the surface directly with a stripette) and add to the DMEM 'fibroblast' tube.
12) Add complete RPMI back to the trypsinised flask as soon as possible and return to hypoxic incubator.
13) Centrifuge the fibroblasts at 400xg for 5 mins.
14) Resuspend pellet in complete DMEM and count.
15) Transfer to 0.1% gelatin coated flask (follow approximate density guidelines in point 3) and incubate in 5% CO_2 incubator.
16) Wash and change media of both lines every 2-3 days, sub-culturing at 70% confluency.

Initiation of TIL Cultures

Preparation:

1) Using cell count from Trypan blue step: calculate volume of media needed to achieve a final cell density of $0.5x10^6$ cells/mL (2mL) in 24 well plates (TC treated).

   e.g. Digest has yielded x cells/mL, need $0.5x10^6$ cells/mL.

$$Volume\ of\ media\ = \frac{digest\ cell\ count\ (cells/mL) \times volume}{0.5 \times 10^6\ cells/mL}$$

   As 2mL/well is required for the 24 well plate, divide the volume by two to work out how many wells and plates can be filled.
2) Based on volume calculation, determine the volume of IL-2 (once vial reconstituted, stored in -80°C at a stock concentration of 1MIU/mL) needed to achieve 3000IU/mL.
3) Finally, based on $0.5x10^6$cells/mL, calculate the volume of Dynabeads (Human T-Activator CD3/CD28) needed to achieve a 1:1 ratio of Dynabeads:cells. Dynabead concentration = $4x10^7$ beads/mL.

$$Volume\ of\ Dynabeads = \frac{0.5\times10^6 cells/mL}{4\times10^7 beads/mL} = 12.5\mu L\ Dynabeads/mL$$

4) Prepare Dynabeads for use:
   (a) Resuspend Dynabeads within the vial by vortexing for >30 seconds, and then transfer the required final volume to a 15mL Falcon.
   (b) Add 1mL of PBS, and vortex for 5-10 seconds.
   (c) Place tube on a magnet until all beads are captured, and discard the supernatant.
   (d) Resuspend beads in the same volume of medium as original Dynabead volume.

Protocol:

1) Resuspend cell pellet in calculated T-cell media volume to final cell density of $0.5x10^6$ cells/mL.
2) Add 12.5 µL/mL Dynabeads to cell suspension. Next, add 3000IU/mL IL-2. Invert gently and thoroughly to mix.
3) Split suspension between wells of 24 well plates, using 2mL/well.
4) Label plate with MOC number, tissue type, plate number, conditions, date, investigator.
5) Incubate at 37°C, 5% CO_2 and 95% humidity. Follow protocol below for growth phase.

Day	Action	Day	Action
0	Seed plates.	13	Count, reseed.
2	Half media change (3000IU/mL IL-2)	15	Count, reseed.
4	Half media change (3000IU/mL IL-2)	17	Count, reseed.
6	Half media change (3000IU/mL IL-2)	19	Assays, freeze down, rapid expansion.
7	Remove Dynabeads, count, reseed.		
9	Count, reseed.		
11	Count, reseed.		

Half media change (day 2-6):

Note: TILs are in suspension, so will settle at the bottom of the plate but will not be stuck down. Plates must be moved very carefully from the incubator, and media aspirated only from the very top of the media layer. Do not attempt to aspirate media if plates have been disturbed.

1) Make up volume of T-cell media with 6000IU/mL IL-2.
2) View wells on microscope, check for signs of infection (cloudy media, yellowing etc.).
3) Carefully remove 1mL media from each well using a P1000 and barrier tip. Discard media into Virkon.
4) Slowly add supplemented T-cell media to each well and return to incubator.

Dynabead removal (day 7):

1) Thoroughly resuspend wells and add to Falcon tube. Rinse wells with T-cell media.
2) Remove Dynabeads using Invitrogen magnet. Allow 5 minutes to ensure that all beads are captured.
3) Remove supernatant to fresh tube.
4) Wash beads by resuspending in fresh media and capture beads again. Add supernatant to first harvest of cells.
5) Spin down at 400xg for 5 mins, count using Trypan blue and reseed in 24 well plates or flask at $1x10^6$ cells/mL in T-cell media supplemented with 1000IU IL-2.

$$Volume\ of\ media\ = \frac{cell\ density\ (cells/mL) \times volume}{1 \times 10^6\ cells/mL}$$

Count and reseeding (day 9-17): Repeat steps 1 and 5, reseeding in T-cell media with 1000IU/mL IL-2 to a final density of $1x10^6$ cells/mL.

Rapid Expansion Protocol

Preparation:

1) Calculate volume of TIL suspension containing $1x10^5$ TIL.
2) Calculate volume of irradiated feeder cell suspension (30Gy) containing $2x10^7$ cells. Need a 1:200 TIL:feeder cell ratio.
3) Prepare stock solution of anti-CD3 Ab (OKT3) if necessary. Calculate volume of Ab needed for final concentration of 30ng/mL in 20mL media.

Protocol:

1) Seed a T75 flask with correct volumes of TIL suspension and feeder cells, suspended in 20mL T-cell media supplemented with 30ng/mL OKT3 and 3000IU/mL IL-2.
2) Place in incubator. Leave undisturbed for 3-5 days, until cells start to colonise.
3) Then replace media every 3-5 days, until flask reaches 80% confluency, and subculture.

Tumour Culture Initiation Protocol

Preparation:

1) Warm 500mL RPMI-1640 (+L-glutamine, +HEPES) and 1X PBS, along with any frozen aliquots for at least 45 minutes before they are to be used in culture.
2) Prepare tumour cell 'complete' media as in table below:

[C]	Reagent		Volume of stock (mL)	Stock [C]
	RPMI-1640 (supplements highlighted)		395	N/A
2mM	L-glutamine			2mM
25mM	HEPES			25mM
20%	HI FBS		100	N/A
100IU/mL	Penicillin	Combined reagent	5	10,000IU/mL
100µg/mL	Streptomycin			10,000µg/mL

Protocol:

1) Transfer digested tumour from C-tube to 50mL Falcon and spin in centrifuge at 400xg for 5 mins.
2) Discard the supernatant and wash the pellet with 1X PBS 3 times.
3) After the third wash, resuspend the pellet in 'complete' media. Count the cells, and seed in a T75 flask to a volume of 20mL.
4) Label the flask with MOC number, tissue type, date, investigator, flask number.
5) Place in top incubator and leave undisturbed for 3-5 days, until cells are approximately 80% confluent. If media becomes discoloured, replace half of the media.
6) Upon confluency, passage cells as below.

Subculture/passage of tumour cells:

1) Remove old media. Wash cells once with 1X PBS, then detach using enzyme-free cell dissocation buffer (in PBS).
2) Incubate for 2-5 mins at RT. Aspirate into a 50mL Falcon and add an equal volume of complete medium to dilute enzyme.
3) Centrifuge cells at 400xg for 5 minutes.
4) Remove supernatant and resuspend in 30mL of fresh media.
5) Transfer cells into 3x T75, and suspend in 10-15mL fresh media.
6) Label flask with MOC number, tissue type, date, investigator, flask number.

Cryopreservation of TIL/ autologous tumour cultures and tumour disaggregate

1) Prepare freezing media using 90% HI-FBS and 10% DMSO.
2) Transfer relevant cells to a 50mL Falcon. Count cell density.
3) Centrifuge at 400xg for 5 mins to form a cell pellet, discard supernatant, and resuspend in appropriate volume of freezing media (see below).

Cell type	Cell density (cells/mL)
Tumour digest	$>2x10^7$
Expanded TIL	$>2x10^7$
Cell lines	$>1x10^6$
Tumour culture	$>2x10^7$

4) Prepare 1mL aliquots of cells in freezing media in 1.6mL cryovials, and label with MOC number, cell type, passage, cell density, date and initials.
5) Place in -80°C storage in a Mr. Frosty. Transfer to LN_2 dewar for long term storage.