STUDY TITLE: Profiling Memory T cells Across Human Tissues

PROTOCOL NAME: CyTOF Detailed Lab Protocol

SUMMARY: Preparation and staining of cryopreserved single cell suspensions from human tissue samples for CyTOF analysis

1. **Thaw cryopreserved PBMC samples**
    a. Place cryovial in 37ºC waterbath until ice is partially melted
    b. Transfer to 15ml conical tube
    c. Slowly dilute with 8mL of pre-warmed (37ºC) Thaw Media (RPMI-1640 + 10% FBS + 50U/mL Benzonase)
    d. Centrifuge at 350g for 5mins
    e. Resuspend in 5mL of Thaw Media
    f. Count and assess viability
    g. Normalize cell counts to stain 1 million cells per tissue

2. **Viability staining**
    a. Resuspend samples in in 500uL of Rh103 viability media (RPMI-1640 + 10% FBS + 1uM Rh103
    b. Incubate for 20mins at 37ºC
    c. Wash 1X in cell staining media (CSM: PBS + 0.1% BSA)

3. **Barcode and pool cells from paired tissues**
    a. Barcode cells from each tissue by staining with CD45 antibodies conjugated to monoisotopic cisplatin CD45_194Pt, CD45_195Pt, CD45_196Pt, CD45_198Pt (diluted 1:100 in CSM)
    b. Incubate for 15mins at 4ºC
    c. Wash 2 times with 1mL of CSM
    d. Combine cells from all tissues into a single tube

4. **Antibody panel staining**
    a. Prepare 2X "Antibody Staining Panel"* in CSM and filter through 0.1micron Amicon centrifugal filter to remove aggregates (12,000g for 1 min).
    b. Resuspend each barcoded sample in 50uL CSM containing 1:50 dilution of TruStain FcX (Biolegend, Cat# 422302), and add 50uL of the filtered surface antibody cocktail
    c. Incubate for 30mins at 4ºC
    d. Wash 2 times with 1mL CSM and remove supernatant
    e. Resuspend cell pellets in 500uL of 1X FoxP3 Fixation/Permeabilization buffer (eBioscience, Cat # 00-5523-00)

f. Incubate for 30mins at 4ºC
g. Wash 2X with 1X Permwash buffer (eBioscience, Cat # 00-5523-00)
h. Resuspend pellet in 100uL of Permwash buffer containing a 1:100 dilution of intracellular staining antibodies
i. Incubate for 30mins at 4ºC
j. Wash 2X with 1X Permwash buffer (eBioscience, Cat # 00-5523-00)
k. Resuspend 400uL of 0.125nM Intercalator_Ir diluted in PBS containing 2% formaldehyde (freshly-diluted from 16% ampule stock)
l. Incubate for 30mins at 4ºC
m. Wash 1X in 1mL PBS + 0.1% BSA
n. Remove supernatant, resuspend cells in residual volume and store at 4ºC until acquisition

5. Data acquisition

a. Immediately prior to acquisition, wash cells with deionized water and count
b. Resuspend in deionized water with a 1/20 dilution of EQ Four Element Calibration Beads (Fluidigm Inc.)
c. Acquire on a CyTOF2 mass cytometer (Fluidigm Inc.) equipped with a SuperSampler fluidics system (Victorian Airships) at an event rate of <400evt/s

6. Initial data processing

a. Normalize data using bead-based normalization algorithm in Helios acquisition software (Fluidigm Inc.)
b. Exclude EQ Four Element Beads on the basis of Ir-193 and Ce-140
c. Exclude dead cells on the basis of Rh103 positivity
d. Debarcode cells from each tissue by Boolean gating based on corresponding Pt CD45 channels.

*ImmPort Reagent Set Name: “Miron et al. 2018 CyTOF 35 Panel”