Add your Institutional Logo here

EXPERIMENTAL PROTOCOL: Immune and non-immune population analysis in human solid tumours using flow cytometry

Author/s: Víctor Manuel García Martínez

Contact information: victormanuel.garcia@ciemat.es

Introduction

This method is intended for the analysis of the proportion of different cell subsets within the tumour microenvironment. A special focus is paid to myeloid cells and non-immune stromal cells. There are two flow cytometry panels and both contain the same anti-PD-L1 to measure expression of this ligand in all cell subsets.

MYELOID PANEL									
Zaq	CD15	HLA-DR	CD11b	CD14	CD45	CD11c	CD163	CD206	PD-L1
	BV605	BV711	FITC	APC	PE-Cy.5	BV421	PE	APC-Cy.7	PE-Cy.7
STROMAL PANEL									
Zaq	CD56	CD90	PanK	PDPN	CD45	CD31	aSMA/ CD3	PD-L1	
	BV605	BV711	FITC	APC	PE-Cy.5	BV421	Cy3	AF700	PE-Cy.7

The protocol is optimize for bladder cancer samples but should work in other solid tumours.

Gating strategies for each panel are suggested in figures 1 and 2.

Reagents

- DNase I (10104159001 Roche), working solution: 100 ug/ml
- Collagenase P (11213857001 Roche), working solution: 200 ug/ml
- Dispase II (D4693 Sigma-Aldrich), working solution: 800 ug/ml
- DMEM
- Corning® cell strainer, pore size 40 µm, blue, sterile
- Flow cytometry reagents in table 1.

Instruments and tools

- Dissection tools (forceps and scissors)
- BD Biosciences LSRFortessa™ | High-Parameter Flow Cytometer. Blue, Violet, YG, Red lasers mounted

Before start

Tumour samples can be processed the day after they are collected without meaningful impact on the analysis. This helps a lot with the coordination between surgery and lab work.
The digestion protocol can be a bit harsh for certain tissues, consider titration of the digestion enzymes.
Once cells are stained and fixed they can be stored up to 5 days in the fridge until they are acquired.

PROCEDURE

1. Thaw enzymes and prepare digestion buffer in DMEM (not supplemented): around 3 ml / sample
    - DNase I (10104159001 Roche), working solution: 100 ug/ml
    - Collagenase P (11213857001 Roche), working solution: 200 ug/ml
    - Dispase II (D4693 Sigma-Aldrich), working solution: 800 ug/ml
2. For all the enzymes I prepare 100x stock solutions and aliquot since they are very sensitive to freeze/tha cycles (no more than 2).
   Keep digestion buffer on ice all the time.
3. Cut tissue in small pieces. The smaller the better. I use forceps and scissors and drop the pieces directly into the tube. Using a scalpel and a plate should be fine as well.
4. Flush down the tube the tissue pieces by adding 1.2 ml of the digestion buffer
5. Vortex thoroughly and incubate 15 min in water bath at 37C. Make sure all the pieces are in the digestion buffer and not adhered to the tube´s walls
6. Repeat step 5
7. Pipette up and down several times. If tissue pieces are too big to pass through the tip, cut the tip to increase the diameter but don´t cut too much since shear stress is important to break apart the tissue.
8. Let tissue pieces to precipitate for 1-2 min. Then collect (close to surface) 600 ul of the suspension and transfer into a collection tube with 1 ml fresh DMEM 10% FBS supplemented media. Add 600 ul of fresh digestion buffer to digestion tube and incubate 15 min in water bath at 37C

9. Repeat 7-8 as many times as necessary (normally 2-3) until no big pieces are observable.
10. Transfer everything into collection tube. Pass to a new tube through a 40 um filter and centrifuge at 1200 rpm 7 min
11. Resuspend in 1-3 ml Lysis buffer (depends on pellet size and colour, meaning how red it looks). Incubate 10 min at RT in the dark.
12. Top up volume with PBS and centrifuge at 1200 rpm 7 min
13. Resuspend in 1 ml (or less, depending on pellet size) of fresh media or flow cytometry buffer and count using tripan blue.

Flow cytometry staining:

Volumes are given for up to 106 cells per tube. For larger numbers, scale up accordingly

1. Stain cells with Zombie Aqua™ Fixable Viability Kit following instructions. Normally 20 min in the dark at RT, in PBS with no proteins in it. All cells can be stained together and then split into different tubes for the next steps.
2. Split cells in FACS tubes and add 1 ml FACS buffer (normaly PBS plus 1%BSA or FBS) and spin down 350 g 5 min
3. Block the Fc-Receptor using the true stain reagent or similar following manufacture´s recommendations
4. If using truestain, resuspend cells in 100 ul of FACS buffer plus 5 ul truestain. Incubate 10 min at RT in the dark.
5. Add antibody cocktail and incubate 30 min on ice and in the dark
6. Wash by adding 1-2 ml FACS buffer and spin down 350 g 5 min
7. Fix cells by adding 150 ul of Cyto-Fast™ Fix while vortexing
8. Incubate at least 30 min in the fridge. At this point, cells can be left in the fridge for up to 5 days
9. Wash cells with 1 ml of 1x Perm Buffer and spin down 350 g 5 min
10. Resuspend in 100 ul Perm Buffer and add the intracellular antibody cocktail (PanKeratin and α-SMA) and incubate 30 min on ice and in the dark
11. Wash cells with 1 ml of 1x Perm Buffer and spin down 350 g 5 min
12. Repeat step 11
13. Resuspend cells in a volume of FACS buffer depending on cell number (you don´t want to spend hours acquiring events!). In general, 250 microliters is fine for up to 500,000 cells. Don´t go lower than 200 ul, since the cytometer may suck up all the volume when you insert the tube and the sample will be gone (a classic in flow cytometry)
14. When acquiring remember to include width and height for FSC and SSC parameters. You´ll need it to remove doublets (cells together) which will increase error in your analysis.

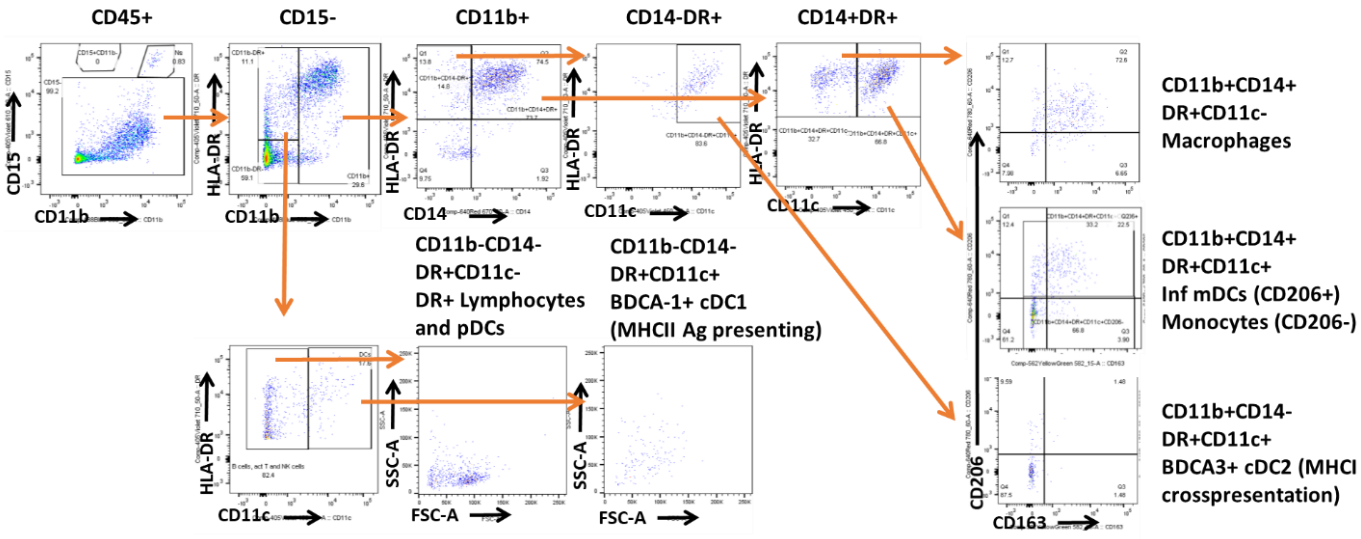

Figure 1. Myeloid subset gating strategy for panel 1. BDCA-1 and BDCA-3 staining was done in a number of samples, confirming expression in the indicated subsets (cDC1 and cDC2). pDCs: plasmacitoid dendritic cells; cDC1: conventional dendritic cells type 1; Inf mDCs: inflammatory monocyte-derived dendritic cells; cDC2: conventional dendritic cells type 2.

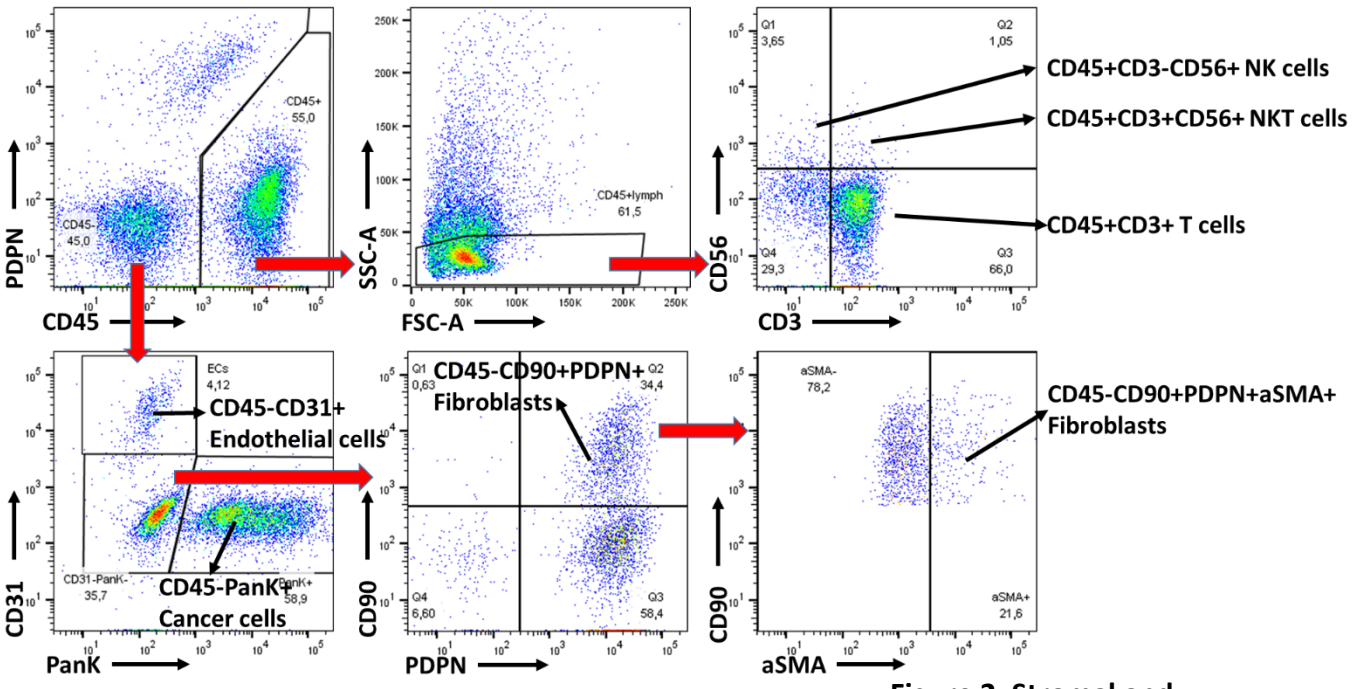

Figure 2. Stromal and lymphocyte subset gating strategy for panel 2. PDPN: podoplanin; NK cells: natural killer cells; NKT cells: Natural killer T cells; Pank: antibody cocktail against several keratins; aSMA: smooth-muscle alpha-actin.

Table 1. Flow cytometry reagents

Antigen	Fluorochrome	Cat.	Company	Clone
HLA-DR	BV711	307644	BIOLEGEND	L243
CD11b	FITC	301330	BIOLEGEND	ICRF44
CD14	APC	301808	BIOLEGEND	M5E2
CD45	PE/Cy5	304010	BIOLEGEND	HI30
CD11c	BV421	337226	BIOLEGEND	Bu15
CD163	PE	333606	BIOLEGEND	GHI/61
CD206	APC/Cy7	321120	BIOLEGEND	15-2
PD-L1	PE/Cy7	374506	BIOLEGEND	MIH3
CD90	BV711	328140	BIOLEGEND	5E10
PDPN	APC	337022	BIOLEGEND	NC-08
CD31	BV421	303124	BIOLEGEND	WM59
CD15	BV605	323032	BIOLEGEND	W6D3
CD3	AF700	317340	BIOLEGEND	OKT3
CD56	BV605	362538	BIOLEGEND	5.1H11
Anti-Actin, α-Smooth Muscle - antibody, Mouse monoclonal 2 ml	Cy3™	C6198-.2ML	Sigma-Aldrich	1A4
Pan Cytokeratin	AF488	53-9003-82	Thermofisher	AE1/AE3
Human TruStain FcX™		422302	BIOLEGEND	
Cyto-Fast™ Fix/Perm Buffer Set		426803	BIOLEGEND	
Zombie Aqua™ Fixable Viability Kit		423101	BIOLEGEND	