**Tumor dissociation protocols for TIL staining**

**“From solid tumors to single cell suspensions”**

**Tumor types: RMA, B16, CT26, MC38, 4T1, TRAMP-C2, KP sarcoma**

1. Kill the mouse.
2. Isolate the tumor without harvesting the skin in the case of s.c. tumors.

**If you possess the Miltenyi Biotec gentleMACS Dissociator (steps 3-7)**

1. Mince slightly he tumor with scissors in a dish.
2. Transfer the tumor pieces in a dissociation tube filled with 1-10 ml of RPMI (no FCS) containing Collagenase IV (200 μg/ml) and DNAse I (20 μg/ml).
3. Run the program mouse-implanted-tumor number 2, repeat this step 3 times.
4. Incubate the dissociated tumor at 37°C on a shaker for 25 to 45 minutes.
5. Run again the program mouse-implanted-tumor number 3, repeat this step 3 times or more depending on the size of the tumor.

OR

1. Mince slightly he tumor with scissors in a dish.
2. Transfer the tumor pieces in a dissociation tube filled with 1-10 ml of RPMI+5%FCS
3. Run the program spleen4 2 times

**If you do not possess the Miltenyi Biotec gentleMACS Dissociator (steps 8-10)**

1. Chop the tumor in very small pieces with scissors in a dish.
2. Add X ml of RPMI (no FCS) containing Collagenase IV (200 μg/ml) and DNAse I (20 μg/ml) and incubate at 37°C on a shaker for 25 to 45 minutes.
3. Mash the remaining tumor pieces with a pestle (the plunger of a syringe works well).
4. Filter the cell suspension through a 70μm strainer.
5. Wash the filter with RPMI.
6. Transfer the suspension in a 50 ml conical tube.
7. Wash cells by centrigugation (600g for 3 to 5 minutes), repeat the step if desired.
8. Resuspend cells in RPMI.
9. If desired run a lympholyte centrifugation (using the mouse cell lympholyte). (600g for 20 minutes and **NO BRAKE**)
	* 1. **Note: You may loose some myeloid cells in that step.**
		2. **Note: whereas lympholyte is only advised after tumor digestions if the final analysis is staining, I would say it is necessary if you want to stimulate cells. Same goes for other organs such as liver and lungs.**
10. harvest the layer corresponding to immune cells.
11. Wash the harvested cells with RPMI (600g for 3 to 5 minutes).
12. Resuspend cell pellets in the desired volume of RPMI.
13. Stain cells.

**Tumor type: Transformed fetal liver cells**

1. Kill the mouse.
2. Isolate the tumor without harvesting the skin in the case of s.c. tumors.

**If you possess the Miltenyi Biotec gentleMACS Dissociator (steps 3-6)**

1. Mince slightly he tumor with scissors in a dish.
2. Transfer the tumor pieces in a dissociation tube filled with 1-10 ml of DMEM (no FCS) containing Collagenase IV (200 μg/ml) and DNAse I (20 μg/ml).
3. Run the program mouse-liver number 1, repeat this step 3 times.
4. Incubate the dissociated tumor at 37°C on a shaker for 45 minutes.

**If you do not possess the Miltenyi Biotec gentleMACS Dissociator (steps 8-10)**

1. Chop the tumor in very small pieces with scissors in a dish.
2. Add X ml of DMEM (no FCS) containing Collagenase IV (200 μg/ml) and DNAse I (20 μg/ml) and incubate at 37°C on a shaker for 45 minutes.
3. Mash the remaining tumor pieces with a pestle (the plunger of a syringe works well).
4. Filter the cell suspension through a 70μm strainer.
5. Wash the filter with DMEM.
6. Transfer the suspension in a 50 ml conical tube.
7. Wash cells by centrigugation (600g for 3 to 5 minutes), repeat the step if desired.
8. Resuspend cell pellets in the desired volume of DMEM.
9. Stain cells.

**Staining:**

We use a standard flow-cytometry protocol to stain tumor cell suspensions. Cells are usually pre-incubated for 20 min on ice with PBS containing 2.4G2 antibodies to block Fc receptors. Between incubations (and before analysis), cells are washed with PBS containing 0.05% BSA and 0.002% sodium azide. For the first staining step, cells are incubated for 30 min on ice with unconjugated antibodies and/or antibodies conjugated with biotin or fluorochromes. Cells are incubated with fluorochrome-conjugated streptavidin or conjugated secondary antibodies for 30 min on ice when necessary.

**Note: The single suspensions coming from tumors often contain a high percentage of dead cells. We strongly suggest to add a dead/live stain with the staining of interest. In our lab, we use Propidium Iodide, 7-AAD or Ammune reactive dye stainings for dead cells (the later one being more stable, narrower in terms of emission, compatible with fixation but more expensive).**

**Considerations about dead cell staining:**

**I never used PI because many of my samples require fixation and permeabilization which is unsuitable with PI. Moreover, PI is difficult to use in combination with many fluorochromes. The good things about PI are: i) it is incredibly cheap, ii) it is very bright, iii) it is very fast.**

**Instead of PI, I use the viable dyes from Invitrogen. These molecules bind both live and dead cells, but they bind dead cells more so you can separate them from the live cells. Invitrogen elaborated a complete panel so you can choose between many colors. The one we mostly use in the lab are the Aqua (which in the Fortessa is in the Amcyan channel) and the Near IR (which is in the APC-Cy7 channel in our Fortessa). I prefer the Near IR because it gives a better resolution and because in this way I can use the Amcyan channel for an antibody conjugated with a good fluorocrome. The great thing about these dyes is that they can be used in combination with fix and perm. I previously did the dead cell staining before everything else (mainly because it requires an incubation with a protein-free buffer).**

**Cell death in the tumor samples is a big issue. The main reason one wants to lympholyte is in fact to get rid of dead cells. This helps a lot to have a cleaner staining. With lympholyte usually the percentage of dead cells is no more than 30% or so. Without lympholyte it can go as high as 70%.**

**The protocol for PI is quite simple: dilute 2 ul in about 10 ml of flow buffer (0.5% FCS in PBS) and add 100 ul of this solution before the acquisition. Incubate 2 minutes RT and flow.**

**For the Invitrogen dye:**

**-wash the cells with PBS NO PROTEINS 3 times.**

**-add the dye diluted in PBS NO PROTEINS (they need to be titrated)**

**-incubate 30’ ice**

**-wash 3 times with flow buffer**

**-go to the next step**

**Considerations about dump gating:**

**From my experience with tumor samples I have learned the importance of the dump gating. Tumor samples are usually rich in sticky cells so having a good dumping strategy may dramatically improve the staining. For NK cells it’s quite important to have at least CD19, CD3, F4/80 and Ter119 for the dump gate. I suggest using antibodies conjugated with a very bright fluorocrome (I particularly like PE-Cy5).**

**I should also mention that for NK cells the resolution improves a lot if one uses two NK markers to positively gate them. For example, in B6 one can use NK1.1 and NKp46 and gate on the double positive cells. This is not really necessary in the spleen or in other organs, but in the tumor it helps a lot.**

**Considerations about enzymatic digestion:**

**I never tried a side by side comparison, but I believe that 45’, which is what most people do, is too long for incubation with the enzymes. Because it seemed the cells were doing poorly under these conditions I shortened the enzymatic treatment to 25’ and I think I have nicer results in term of viability without losses in cell yield.**

**I have tried side by side collagenase D and liberase. Liberase cleaves e.g. CD27 from the cell surface, whereas collagenase does not. Other molecules I commonly stain for were not affected by either enzyme.**

**To have a better staining results, I have learned it is quite important to extensively filter after tumor dissociation. I filter at least 2 times, but for some samples I filter up to 4-5 times. This also decreases the percentage of dead cells. Particularly, these tumors are very sticky. Also for this reason I have learned it is crucial to avoid including any mouse skin in the subcutaneous tumor samples. The dissociator machine cannot process the skin in any case.**

**Another thing that may help for having clean staining results is to fix the cells before flow. This of course is not a general role, but for the tumor samples it works pretty well. The problem is that it needs to be calibrated with the use of antibodies and fluorocromes, so it is hard to give advice on this topic.**