In vitro staining of cells using CFDA-SE (CFSE) and tumoral conditioned medium

Protocol adapted from the report of Quah, Warren & Parrish, Nature Protocols, 2007

Attention: for this assay do you have 3 Steps:

1) Reagent and preparation (already done): 1 microtube is enough (2ul); stock solution at -20C freeze

a. CFDA-SE stock solution: Dilute CFDA-SE powder in DMSO, so that the final concentration is 5mM. Usually, weight 25mg of powder for 8,96ml DMSO. Prepare aliquots of 50 to 200ul and store in -20°C freezer. Test each preparation batch and every 6 months; **It is important that the DMSO is new, opened less than 6 months earlier.**

2) Obtaining blood Lymphocyte - Protocol in the moodle

b. Juliana will guide this stage with Ficoll-Paque

Remember: after putting conical tubes with lymphocytes and Ficoll prepare the tumoral conditioned medium as describe below; you will have approximately 30 minutes (separate into two groups).

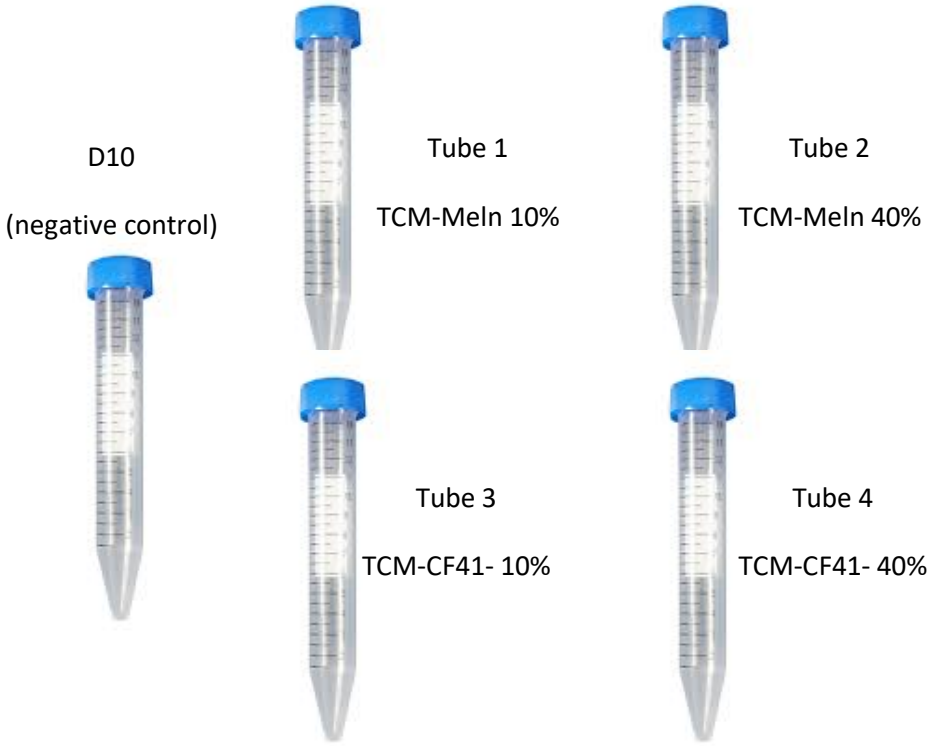
3) Tumoral conditioned medium preparation (prepare while lymphocyte with Ficoll is being centrifuged) (before using this medium, check that it has already been centrifuged for 400 x g for 7 min and transferred to a new tube without the cell pellet).

c. While lymphocyte centrifuge identifies **five** 15ml conical tubes: **tube 1 D10**; **tube 2 TCM-Meln-10%; tube 3 TCM-Meln-40%; tube 4 TCM-CF41-10%; tube 5 TCM-CF41-40% (tumor conditioned medium, being 2 from Meln and 2 from CF41 tumoral cells, different concentration).**
Obs.: Preparation of conditioned medium for each tumor cell line

G1 (Andrew, Karina e Juliana)- will prepare conditioned medium from Meln (prepare 10% and 40%; It is not a good idea to exceed 40% conditioned medium).
To 10% mix 0,5ml of TCM-Meln to 4,5ml of D10 and for 40% mix 1ml of TCM to 3 ml of D10.

G2 (Jean, Bethânia e Karina B.)- will prepare conditioned medium from CF-41 (prepare 10% and 40%).
To 10% mix 0,5 ml of TCM-CF41 to 4,5ml of D10 and for 40% mix 1 ml of TCM to 3ml of D10.

Follow tubes scheme below:



After obtained lymphocytes and prepared TCM follow to steps describe below:

4) CFSE staining: resuspend cells in a concentration from 0.5 to 50x10^6 cells/ml. If the concentration is <10x10^6 cells/ml to 50x10^6 cells/ml you should use PBS + 5% FCS, in case the concentration is > 50x10^6 cells/ml use PBS without serum.

FCS avoids CFSE toxicity especially when cell concentration is low. Supplemented culture media can be used, but amino acids can compete with CFSE.

There are two pathways of staining with CFSE, according to cell concentration. The final CFSE concentration also depends on the cell number and may vary from CFSE batch to batch, but generally it is recommended 5uM for 50x10^6 cells/ml.

d. When cell concentration is ≤ 50x10^6 cells/ml:
   i. In a clean 15ml conical tube resuspend cells in 1ml of PBS+5% FCS, without letting the inside walls of the tube getting wet;
   ii. Lay the tube on a surface completely horizontally;
   iii. Add 110ul PBS in the top wall of the tube and do not mix yet;
   iv. In this PBS drop, resuspend 1,1ul of CFSE stock solution (5mM);

v. Close the tube with its cap, invert several times and vortex to mix well the solution.

e. When cell concentration is between 50 to 100x10^6 cells/ml:

i. Prepare a 10uM solution of CFSE (CFSE 2x), adding 2ul from the 5 mM stock solution in 1ml PBS;

ii. Resuspend cells with 1ml PBS and add rapidly 1ml of CFSE 2x;

iii. Mix with vortex.

CFSE loses its efficiency when it stays long periods in solution before getting in contact with cells.

5. After CFSE addition incubate cells for 5 minutes at room temperature (20°C), wrap the tube in aluminum foil paper and agitate with inversion the whole time; add 9ml of PBS (5%FCS) and wash cells as describe below

Obs. **It is not advisable to incubate for longer periods due to CFSE toxicity, especially when cell concentration is low. From this point further CFSE starts to fluoresce and light exposure diminishes its intensity, therefore the need to protect with foil.**

6. After labeling CFSE time wash cells 2 times, the first add 4mL PBS (5% FCS) to cells already marked with CFSE at room temperature, centrifuging at 300g for 5 minutes; in the first wash before put the conical tube on centrifuge remove an aliquot to count the number of cells to do the cell adjust calculation, for this: remove 10ul of cells and add 10ul of Trypan Blue (dilution factor=2x, then multiply the result found in the Neubauer chamber by 2x10^4/mL to obtain the number of cells per 1mL);

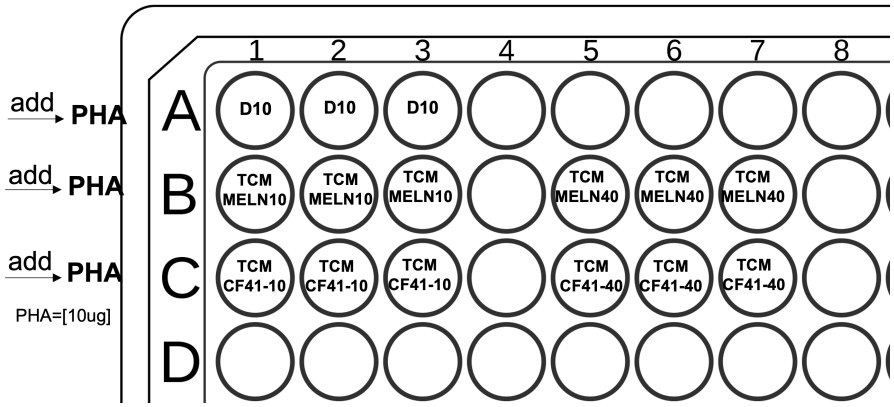
7. after the first wash cycle, discard supernatant and already with the calculation of cellular adjustment resuspend the cells in 5 ml PBS (5%FCS) and transfer the cell suspension volume (to be determined) so that each 15ml conical tube previously identified (in total 5 tube as indicated below) so that each tube has a total concentration of 2.5x10^5/tube in 5ml of PBS (5%FCS) (after define volume complete with PBS 5%FCS with the remaining volume to 5ml) (just for you know as there will be 5 tubes, you need to have at least 1,25x10^6 total cells).

8. with the volume already adjusted, proceed to the second cell wash and when finished, discard the supernatant and now resuspend each tube with its **1ml** of respective medium so that it remains 2.5x10^5/ml, so that each 200ul of this cell suspension will have 5x10^4/well (plate of 96wells)

9. Plate cells at 5x10^4 cells for each well in a 96-well round plate, or 5x10^5 for each well of a 24-well plate; Incubate in a 37°C/ 5% CO_2 incubator until Monday (13/06)

Plate layout example

	Control lymphocytes with D10 (triplicate)+PHA* (mitogen)			**Proliferation lymphocytes with tumoral conditioned medium (triplicate)+PHA (mitogen)**		
w/ CFSE	D10	D10	D10			
w/ CFSE	TCM Meln 10	TCM Meln 10	TCM Meln 10	TCM Meln40	TCM Meln40	TCM Meln40
w/ CFSE	TCM CF41 10	TCM CF41 10	TCM CF41 10	TCM CF41 40	TCM CF41 40	TCM CF41 40



* DON'T WORRY: Luciana will add PHA to D10 control well!

10. Harvest and reading proliferation at least 96h in culture.

11. If you plan to stain cells further with fluorescent antibodies keep in mind that CFSE emission peak is 517nm, coinciding with the FL1 channel (green), therefore hindering the use of FITC-conjugated antibodies and it can leak to FL2 channel. It is essential to perform the compensation controls.