**Protocol for phospho-flow.**

1. Isolate the leucocytes from the desired organs (I have tried spleens and tumors).
2. Divide the suspension in two 15 ml conical tubes: in one add 2/3 of the cells, in the other one 1/3 of the cells.
3. Spin and resuspend in about 500 ul-1 ml of RPMI 5 or 10% FCS.
4. Add the desired primary antibody, the one you want to use for the stimulation: I use: about 100 ug of PK136 in the tube with 2/3 of the cells and about 50 ug of mouse IgG in the tube with 1/3 of the cells.
5. incubate 30 min in ice.
6. in the meanwhile, prepare the tubes for the crosslinking: I use flow tubes in which I add 500 of RPMI 10% FCS with goat anti-mouse Fab IgG (1:250) and IL-2 (200 U/ml, the final concentration will be 100 u/ml). Leave the tubes in a 37C water bath during the incubation so the media get warm.
7. please note that IL-2 crucial, without IL-2 you barely have any signal on resting NKs from spleens, with too much IL-2 the stimulated and un-stimulated sample overlap completely.
8. wash twice with RPMI 5 or 10% FCS
9. resuspend the cells in order to add 500 ul of cells each time point, for example: if you plan to have two time points resuspend in 1 ml.
10. add the cells to the pre-warmed tubes with the secondary antibody and the IL-2.
11. I think it is much better to add the cells at different time and fix altogether, I never tried differently but for western-blotting this is the preferred way.
12. for p-erk and p-akt 3 minutes are enough to see a good stimulation (the results I have showed are at 3 minutes). I usually do 10 minutes as well, but it’s a bit late. Before 3 minutes the phosphorylation is weak. If you are interested in other p-molecules you will need to run a kinetics.
13. to fix the cells I use 1ml of cyto-fix/perm from BD # 51-2090KZ (the one for regular intracellular permeabilization). BD suggests a different buffer but I never tried it.
14. incubate 15 minutes at 37C.
15. wash twice with PBS+0.5% FCS.
16. incubate with 1 ml of buffer II from BD phosphoflow (I tried buffer II and IV, II works better for me) 30 minutes in ice. Please note that buffer II should be add drop by drop.
17. wash twice with PBS+0.5% FCS.
18. at this point I resuspend the cells incubated with PK136 in 200 ul and split them in 2 wells on a 96 well plate. The cells incubated with cIg are resuspended in 100 ul and plated in a single well.
19. pellet the cells.
20. add anti-p-protein diluted in PBS+0.5% FCS (in my case p-erk or p-akt, both 0.5 ul/sample) to the cells incubated with cIg and on one well of the cells incubated with PK136. Add just PBS or a cIg to the other well of the PK136 cells (this is your staining control, I do it just on PK136 because I am short in cells and noticed that there is no differences between cIg on cells stimulated with PK136 and cells stimulated with cIg).
21. incubate 1 hour RT
22. wash twice with PBS+0.5% FCS.
23. add secondary antibody (in my case goat anti rabbit-Alexa647) 0.1 ul/well. For this staining I strongly recommend BV or Alexa fluorcrome!!!
24. incubate 1 hour RT
25. wash twice with PBS+0.5% FCS.
26. flow and good luck