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**Multiparameter DDR flow cytometry assay.**

Look into these references for more details: ([Gibbs et al., 2011](#_ENREF_1); [Kornblau et al., 2010](#_ENREF_2); [Rosen et al., 2010a](#_ENREF_3); [Rosen et al., 2010b](#_ENREF_4)).

1. Aliquot 100.000 cells/condition (e.g. irradiated samples and non-treated).
2. Amine aqua staining. Collect the cells, spin down. Discard the supernatant. Wash the cells once with 1 ml PBS (No serum, No BSA).

Resuspend the cells in 1 ml PBS. Add 1ul of the reconstituted Amine aqua to the cells suspension and mix well (Prepare master mix instead of adding 1ul to each tube). Incubate at room temp or on ice for 30 minutes, protected from light. Wash the cells with 1 ml of PBS and resuspend in 900ul of PBS

3. Fixation. To halt signal transduction, fix with a final concentration of 1.6% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) for 10 minutes at room temperature.

Stock of PFA comes at 16%.  Adding 100 ul of this stock to 0.9-1 ml of cells will give the desired final concentration.

4.    After 10 minutes spin down cells (1500 RPM, 5 minutes).

5.    Carefully aspirate off the supernatant.  (Pellet might be small and difficult to see so be careful).  Vortex and wash with 4 ml of FACS buffer.  This will remove residual PFA. Be sure to get as much FACS buffer off the cells as possible. (If this washing step is not done, surface stain will look bad as residual PFA will affect antibodies).

1. Spin down cells (1500 rpm, 5 minutes).
2. Aspirate supernatant buffer and stain for surface markers for 30 minutes at 4C. Ensure volume of cells between tubes is consistent. Typically, I stain in 100ul total volume. Best results can be obtained when you do staining in eppendorf tubes.

CD34

CD38

Wash cells in FACS buffer, spin down and carefully aspirate supernatant.

8. Permeabilization.  Vortex pellet or vigorously disturb it (if pellet is not vortexed, it will become a giant clump when alcohol is added).  Add 1 ml of ice-cold 90% ethanol (use the highest quality ethanol). Vortex the cells for 3–5 s. Place the tubes on ice for 15–30 min. You can also Permeabilize at 4C or -80C.

Although I haven’t done many head-to-head comparisons, I think -80C permeabilization gives better surface marker staining. At this stage samples can be lived at -80C from ON to over weekend.

11.  Wash cells **twice** in FACS buffer (1ml) to remove residual alcohol.

·      It’s especially important to aspirate supernatant carefully at this point. The cell pellet will be very loose after the alcohol perm.  Do not decant.

12.  Stain for intracellular epitopes for 30 min at RT in the final volume of 100ul.

·      It is very important that all tubes have the same volume before adding the intracellular antibodies.  Total volume changes (e.g. 100 v. 200 ul) will result in differences in the quantitative (fold-change) values obtained. Again, use eppendorf tubes instead of FACS tubes.

13.  Wash in 1ml FACS buffer.

14.  Collect events on sorter/analyzer. Try to acquire as many events as you can (10000 viable (amine aqua- cells) per sample.

Gibbs, K.D., Jr., Gilbert, P.M., Sachs, K., Zhao, F., Blau, H.M., Weissman, I.L., Nolan, G.P., and Majeti, R. (2011). Single-cell phospho-specific flow cytometric analysis demonstrates biochemical and functional heterogeneity in human hematopoietic stem and progenitor compartments. Blood *117*, 4226-4233.

Kornblau, S.M., Minden, M.D., Rosen, D.B., Putta, S., Cohen, A., Covey, T., Spellmeyer, D.C., Fantl, W.J., Gayko, U., and Cesano, A. (2010). Dynamic single-cell network profiles in acute myelogenous leukemia are associated with patient response to standard induction therapy. Clin Cancer Res *16*, 3721-3733.

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Rosen, D.B., Putta, S., Covey, T., Huang, Y.W., Nolan, G.P., Cesano, A., Minden, M.D., and Fantl, W.J. (2010b). Distinct patterns of DNA damage response and apoptosis correlate with Jak/Stat and PI3kinase response profiles in human acute myelogenous leukemia. PLoS One *5*, e12405.

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| Reagent | Manufacturer  Clone, Cat. No. | Reconstitution,  storage |  | Note |
| 16% Paraformaldehyde (formaldehyde) aqueous solution | **EMS**  RT **15710** |  | 10x10 ml |  |
| Ethanol |  |  |  |  |
| FACS buffer |  | PBS, 1%BSA |  | Don’t include sodium azide when using with amine aqua viability dye |
| CD34 |  |  | Shahar |  |
| CD38 |  |  | Shahar |  |
| gH2AX-Ax647 | Cell lab SC Beckman A88955 |  | 2ul/sample  Shahar |  |
| c-Caspase3 -PE | BD. Rabbit IgG, C92-605, 550821 |  | 10ul/sample |  |
| c-PARP (Asp214)-AlexaFluor700 | BD. Mouse IgG1, F21-852, 560640 |  | 5ul/sample |  |
| P-CHK2 (Thr68) | Cell Signaling, polyclonal Rabbit IgG; 2661 |  | 1:500/sample |  |
| P-CHK2 (Thr68) | Cell Signaling, monoclonal Rabbit IgG; CS2197 |  | 1:50/sample |  |
| Goat anti-rabbit secondary FITC | Invitrogen |  | 1:100/sample |  |
| Goat anti-rabbit secondary APC | Santa Cruz, SC-3846 |  | 1:100/sample |  |
| Iso-PE | (BD diluted for 200 samples), |  | 10ul/sample |  |
| Amine aqua viability dye  LIVE/DEAD Fixable Aqua Dead Cell stin kit for 405 nm excitatation | Invitrogen  L34957 | Bring 1 vial of the dye and the vial of anhydrous DMSO(provided in the kit)to RT.  Add 50ul of DMSO to the vial of dye. Mix well and confirm that all dye has dissolved. | 1ul/1ml of resuspended cells. 30min on ice or rt. | Unused portions may be used for up to 2 weeks if stored at -20C, protected from light and moisture. |