Compensation Beads Staining for Multi-Color Flow Cytometry (FC) Experiment

I. Preparation of the beads for experiment

1. Label a separate sample tube for each fluorochrome-conjugated antibody to be used on a given experiment. Make sure to label additional tube "0" (Tube-0) to be used as a mandatory unstained/negative control for compensation or unmixing step of the multi-color FC experiment

   Note 1. *Use the Cluster Tubes 4408 (1.1 mL, VWR Cat#29442-604) or regular FACS Tubes (5 mL, BD Falcon Cat#352008; VWR Cat#60819-820) to prepare single color controls for the FC Analysis*

   Note 2. *Count the total number of labelled tubes, which will be used for the later calculation of the number of beads drops to be used in Part II*

2. Take the dropper vial(s) with desired beads from the fridge. Resuspend beads thoroughly before each use

   Note: *Suggested resuspension procedure: Vortex on "Hi" for 3 sec -> Tap with the palm of the hand -> Vortex on "Hi" for 3 sec -> Tap with the palm of the hand*

3. Take a separate FACS Tube (5 mL, BD Falcon Cat#352008; VWR Cat#60819-820), label "Wash" (Wash Tube). Add 2 mL of the Staining Buffer (*FACS-Buffer*) to the Wash Tube. If you plan to have five or less single color bead controls (refer to Note 2), add one drop of beads to the S-Buffer in the Wash Tube

   Note 1: *For ThermoFisher OneComp or UltraComp Beads use one drop of the pre-mixed beads*

   Note 2: *For other vendors (BD Biosciences, Beckman Coulter, Spherotech, etc) - use one drop of each bead (Negative + Positive or if applicable, Negative + Low + High)*

   Note 3: *For Staining Buffer (FACS-Buffer), if not specified otherwise, use the basic formula for the FACS-Buffer: 0.5% BSA in PBS. For example, dilute the BSA Blocking Buffer 5% (VWR Cat# AAJ61089-AK) with the 1X DPBS (DPBS 1X 500ML no Ca/no Mg (Corning Cat#21031-CV; VWR Cat#45000-434)*

   Note 4: *If you plan to make more than five controls, proportionally increase the number of the drops of beads used: use 2 drops for 6-10 controls; 3 drops for 11-15 controls, etc.*

   Note 5: *Put the stock beads (dropper bottles) back to their storage box in the fridge*

4. Vortex the Wash Tube with beads, spin down at 450-500 x G (~1500-1550 RPM on benchtop Beckman Coulter Allegra 6R Benchtop Centrifuge) at +4^{o}C for 6 minutes. Aspirate the supernatant. Vortex the beads pellet and resuspend it with 550 µL per drop of the beads originally used

   Note: *Increase the amount of FACS-Buffer added to the Wash Tube proportionally to the amount of beads used: use 550 µL for 1 drop of beads; 1.1 mL for 2 drops, etc.*

II. Staining beads with antibodies, directly conjugated with the fluorochromes

1. After FACS-Buffer was added, vortex the Wash Tube to resuspend beads. Then distribute 100 µL of the washed bead suspension into each of the pre-labelled tubes from Part I
2. Add each single fluorochrome-labelled antibody conjugate to the correspondingly labelled test tube. Make sure you add antibodies directly to the bead suspension, mix with the pipette tip used to add the antibodies, vortex immediately

   Note 1: *For beads controls use the antibody concentration determined (titrated) for your application*

   Note 2: *For error-free manipulation consistency we suggest preparing 20X dilution of the antibodies and adding 5 ul µL of each antibody to 100 µL of the bead suspension*

   Note 3: *For the Tube-0 (unstained control) do not add anything at this step*
3. Incubate tubes on ice in the dark, gently mixing (by flicking or tapping) at 5- and 15-minutes time points
4. After completion of 25 minutes of incubation, vortex all the tubes, add 800 µL of FACS-Buffer to each test tube, vortex again, and spin down at 450 x G at +4^{o}C for 6 minutes
5. Aspirate the supernatant (by leaving ~ 30 µL in the tube), vortex, and add 200 µL of FACS-Buffer to resuspend the beads

   Note: *If staining protocol for the cells requires the fix/perm or any other additional incubations/procedures, which could affect the spectral signature of the fluorochromes, all bead controls after staining (including Tube-0) should be processed the same way as the cells*

III. Storage of the prepared bead controls prior to Flow Cytometry Analysis

1. Place Tube-0 (unstained control) and all tubes with single-fluorochrome-labelled beads into the rack, wrapped with foil. Store the rack on ice or at +4^{o}C until samples are ready for the Flow Cytometry acquisition