Instant fix protocol for phospho-flow

1. Aliquot your cells in a 96-well round or V bottom plate, remember to aliquot samples for single-stain controls
2. Spin the plate down at 1500rpm for 3-5 min
3. During the spin, make up your staining cocktail in FACS Buffer (1XPBS + 2% FCS), make enough for 50uL per well. Staining dilutions should be determined empirically, but the following rules generally apply:
    a. For highly expressed antigens or high affinity antibodies (CD4/CD8α/CD25, etc. etc.), 1:500 for weak fluorochromes or 1:1000 for strong fluorochromes should suffice.
    b. For cytokine/chemokine receptors or poorly defined markers, 1:200-1:400.
    c. For poor antibodies or poorly expressed antigens, 1:100-1:150.
    d. For antibodies in the “test” format, 1:50-1:100.
4. Flick off the supernatant into the sink.
5. Aliquot 50uL into each well as appropriate (you can use a multichannel if there’s a lot of samples). Resuspend up and down with a multichannel pipet. DO NOT reuse tips for multiple rows
6. Incubate on ice in the dark for 10-15 minutes.
7. Add 150 uL of FACS buffer to each well and spin down (this is a wash step).
8. Use "instant fix" for 20 minutes at room temperature. Our "instant fix" solution is made so that the final concentration is 1.5% PFA + 1x Perm wash (from the 10x Perm wash in the eBioscience Foxp3 Fix/Perm kit), diluted in water to reach those final concentrations.
9. Wash with 1x Perm wash from the eBioscience Foxp3 Fix/Perm kit.
10. Stain with pAKT/pS6 for 1 hour at RT, or overnight at 4 degrees. Stain in 25uL volume to conserve antibody because these antibodies are expensive!
    a. Phospho-Akt (Ser473) (D9E) rabbit mAb from Cell Signaling, 1:200 dilution
    b. Phospho-S6 Ribosomal Protein (Ser240/244) from Cell Signaling, 1:500 dilution
11. Wash with 1x Perm wash.
12. Resuspend in 100ul 1x Perm wash, ready to run.