Detection of *in vivo* proliferation by BrdU incorporation

Please note – this protocol has been optimised for certain conditions and is meant as a guide only, you may need to alter some parameters to suit your own experiment

1. The day prior to experimental end-point

   a) dissolve BrdU to 4 mg/ml in sterile PBS: weigh out solid BrdU, add the PBS, wrap tube in foil, mix well, incubate at 37°C for approx 15 minutes and mix again. This ensures that the powder is fully dissolved
   b) first thing in the morning, inject 0.2 ml intraperitoneally
   c) late afternoon/early evening, inject a second 0.2 ml dose intraperitoneally
2. The following day, collect lymphoid organs and prepare single cell suspensions
3. Add mouse IgG to block Fc receptors (add 5 μl of 10 mg/ml stock per 10^6 cells). Incubate for 20-30 minutes at room temperature
4. If required, label cells for surface antigens according to standard flow cytometry protocols, but after the final incubation with antibody, wash in PBS/Azide, not PBS/BSA/azide (traces of protein will precipitate in later steps).
5. After final wash, resuspend cell pellets in 0.5 ml of ice-cold 0.15 M NaCl
6. While gently vortexing, slowly add 1.2 ml ice-cold 95% ethanol (v/v) dropwise. Incubate on ice for 30 minutes. **NOTE**: it is important to add the ethanol in this manner to prevent cell clumping
7. Add 2 ml PBS, spin down in refrigerated centrifuge. **NOTE**: as ethanol-fixed cells do not form a tight pellet, increase centrifugation speed to 450 x *g*
8. Pour off supernatant and resuspend cells in 1 ml of paraformaldehyde/Tween. Incubate at room temperature for 30 minutes
9. Spin down (450 x *g*), pour off supernatant and resuspend in 1 ml DNase solution. Incubate in 37°C water bath for 30 minutes
10. Add 2 ml PBS, spin down (450 x *g*), pour off supernatant and resuspend cells. Add 10 ml of anti-BrdU antibody and incubate at room temperature for 30 minutes
11. Add 2 ml PBS, spin down (450 x *g*), pour off supernatant and resuspend in 0.2 – 0.5 ml PBS
12. Cells are now ready for flow cytometric analysis. If not to be analysed immediately, store at 4°C for up to 1 week (protected from light)

Paraformaldehyde/Tween

Dissolve paraformaldehyde to 1% (w/v) in PBS by gentle boiling in fume hood. Add Tween to 0.01% (v/v)

DNase I solution

Dissolve DNase I (Sigma) to 50 units/ml in salt solution (see below). DNase solution must be made on day of use, or alternatively, prepare concentrated stocks (eg 5000 U/ml) in salt solution, store at –20°C and dilute just before use.

Salt solution

	to make 500 ml
4.2 mM MgCl2	0.43 g
0.15 mM NaCl	18.75 ml of 4 M stocks

pH to 5.0