1. Transfer coverslips to 24 well plates – using suction line to transfer sterile coverslips in a TC hood.
2. Plate cells onto coverslips. Add 50% more cells than usual (since less stick to glass than expected) and then immediately press coverslip down with pipette tip to ensure no air is trapped beneath. Note: if cells adhere poorly to glass then coat coverslips in poly-L-lysine solution for at least 1h prior to adding cells (remove and wash in PBS just before cells are added)
3. Perform experiment
4. Permeabilise and fix cells in 4% PFA for 15 mins
5. Sometimes cytoplasmic pre-extraction can help in the visualisation of localisation to subcellular structures (especially if using overexpressed proteins). If required, then add 0.5ml pre-extraction buffer for 1 min and then fix in 4% PFA.
6. Wash x3 PBS
7. Block in PBS + 3% BSA + 0.2% triton (0.5ml) for at least 30 mins
8. Remove and add PBS
9. Dilute primary antibodies in PBS/3% BSA and add 40ul per coverslip to parafilm. Place coverslip on top (cells facing down!) and leave at least 2h (generally overnight at 4C)
10. Transfer back into 24 well plate and wash x3 in PBS (10 mins each)
11. Dilute secondary antibodies (1:1000 Alexa range from invitrogen) and DAPI (1:1000 of 1mg/ml) in PBS/3% BSA. Place coverslip on 40ul exactly as primary but just leave at least 2h at RT in the dark (generally around 4h)
12. Transfer back into 24 well plate and wash x3 in PBS (10 mins each)
13. Mount coverslips on slide in small drop of Prolong and leave overnight to set.

**Coverslips** - use Marienfeld High Precision 1.5H 12mM (G+N laboratory, Cat No: 117520, £68 per 1000)

**PFA** – 4g PFA + 100ml PBS (incubating at 60C for around 2 hour with frequent stirring)

**Pre-extraction buffer** – 100mM PIPES (pH 6.9), 1mM MgCl₂, 10mM EGTA, 0.2% Triton X-100

**Secondary Antibodies**

Please use the code p1112001 to get a 25% off list price for Invitrogen Alexa Fluor range of secondary antibodies. I recommend purchasing the highly cross-absorbed versions: 488, 568 and 647 (which are good FITC, TRITC, Cy5 wavelengths that are compatible with all our microscopes).

**Alternative common fixations**

Ice cold Methanol 3 mins (methanol direct from freezer)

Methanol-Acetone (1:1) 3 mins (methanol-acetone direct from freezer)