Fixation

This Method of Fixation is based on Don lngber's method for stabilization of fine structures using a pre-fixation with **1%** paraformaldehyde for 2 minutes using the stock 16% Paraformaldehyde. (provided by Bob Mannix)

1. Prepare all solutions to room temperature.
2. Always treat dish gently - do not toss it about or slam it on table - leave dish as undisturbed as possible during fixation.
3. Add 1116th volume of 16% paraformaldehyde DIRECTLY TO THE CULTURE MEDIA in the well in a drop-by-drop pattern from a low elevation (1/4 inch) to scattered sites over the well. Allow to incubate at room temperature for 2 minutes. As an example in this protocol *we* will use a 24 well dish having a media volume of 500 ul; so 31 ul of 16% paraformaldehyde (n water) is added to the 500 ul media. The PRE-FIXATION step will secure fine cellular structures.
4. After 2 minutes incubation, slowly and gently aspirate the fluid from the cell, leaving a small volume to keep cells wet (never let cells dry outl).
5. Immediately - very slowly add PBS (with calcium/magnesium; warmed to RT) to the well by allowing it to slowly run down the side or the well. This is a •1x WASH step. For a 24 well dish I use 1 ml PBS (volume must be sufficient to dilute the remnant of the previous fluid left for •wetting the cells'
6. Aspirate the PBS as before and rep ace with 4% paraformaldehyde in PBS plus calcium/ magnesium (THE FIXATIVE). Incubate at room temperature for 30 minutes. The preparation of this fixative is critical to avoid exposure of cells to hypotonic shock if made incorrectly - see details below*""
7. Following fixation - gently add PBS (with calcium/magnesium) to the fluid in the dish to dilute the fixative (i.e. To 1 ml fixative I add 1 or more ml of PBS to fill well to near the top).
8. Aspirate most of the fluid contents of the well as before and immediately begin to drip 1 ml PBS (plus calcium/magnesium) down the well side to wash cells.
9. Wash cells a second time.
10. Store cell dish in dark at 4C until staining.

PREPARATION OF 4% PARAFORMALDEHYDE FIXATIVE:

1. Add 4.5 ml of 16% paraformaldehyde to a tube larger than 18 ml.
2. Add 500 ul of 10X concentrated PBS (minus calcium/magnesium)
3. Mix well
4. Add 12.9 ml of 1X PBS (plus calcium/magnesium)
5. Mix well
6. Add 50 ul 100X Magnesium chloride (100 mM); final concentration will be 1 mM.
7. Mix well
8. Add 50 ul 00X Calcium chloride (100 mM); final concentration will be 1 mM.
9. Mix well
10. Check clarity by *eye* – if calcium phosphate precipitate forms that is bad and pH must be adjusted to hopefully dissolve the calcium.
11. I checked pH of final solution using a pH strip and found it to be about pH 7 (pH

range should be about 7 - 7.5; if too high - add small amount of HCl to adjust pH).
12. Make fresh each day. Or freeze at -20C; thawed tubes are stable for 1 week at 4C

Staining

1. 2X wash with PBS -/- 5 minutes each.
2. 1X permeabilize with 0.25% TritonX ("TriX") in PBS -/- for 3 minutes
3. 1X block with 1% BSA + 5% donkey serum in TriX for 1 hour at room temperature
4. 1X wash in TriX (optional)
5. Add primary antibodies in 2% donkey serum, 1% BSA in TriX, 2hrs at room temperature or overnight at 4C
6. 4X wash with TriX
7. Add secondary antibodies in 1% BSA in TriX, 1 hour at room temperature
8. Add Hoechst or DAPI, 1/3000 in dishes or 1/1000 in chips in TriX, 30 minutes at room temperature
9. 3X wash in TriX for 5 minutes each
10. 2X wash in PBS -/- for 5 minutes each
11. Store in the dark at 4C, seal to prevent evaporation