FORMALDEHYDE FIXATION AND IMMUNOSTAINING OF CELLS CYTOSPUN OR GROWN UPON COVERSLIPS (Long version)

First thing to know: all steps are performed at room temperature unless otherwise indicated. Square and rectangular coverslips may be fixed and washed in small Coplin jars, petri dishes, or 6 well TC trays. Small round coverslips will fit into 24 well trays for processing. Since larger volumes of buffers, etc. are required for Coplin jars, using the "drop" method of immunostaining will conserve antibody. Place the coverslips in a petri dish on a piece of parafilm, cell side up. A few drops of the diluted antibody can be added on the top. Alternatively, the coverslip may be inverted on a drop of antibody solution - however, loosely adherent cells may come off when the coverslip is pulled off the parafilm. Sealing the edge of the dish with tape or parafilm during incubation, plus placing a small wad of wetted kim-wipe in the dish, will keep the coverslips from drying out.

Note: This protocol has been optimized for HeLa cells, and works well for many other cell lines. However, some cells may require adjustments in length of incubations, and concentration of formaldehyde or Triton X-100. This protocol is not optimal for preserving microtubules - use the PEM-PEG method.

Part 1: Initial Fixation, with or without extraction

- 1. Aspirate or pipette off media. Wash X 1-2 with ice-cold PBS (containing Ca⁺⁺ and Mg⁺⁺). [Note: do not use ice cold reagents when attempting to preserve microtubules you will need to use a different protocol.]
- 2. Optional: perform CSK or Core extraction (see separate protocol).
- 3. For "Whole fixation" fix the cells with 4% Formaldehyde in PEM buffer for 30 min. on ice (1:4 dilution of 16% stock). For fixing CSK and Core extracted coverslips, incomplete stock CSK buffer should be used instead of PEM buffer. The fixation buffer should cover the surface of the cells so that they do not dry out (about 250 µl buffer/well for 24 well trays, 1 ml/well for 6 well trays, etc.)
- Remove Formaldehyde fix, discard in toxic waste bottle, and wash X 3 with PEM buffer, about 2-5 min. per wash.
- 5. To quench auto-fluorescence and enhance antigenicity, coverslips are next incubated with 1 mg./ml. Sodium borohydride (NaBH4) in PEM buffer. This solution is prepared immediately before use. The step will be performed twice. The NaBH4 can be weighed out shortly ahead of time and placed in 2 polypropylene conical tubes, noting the amount weighed out on each of the tubes. The mixture will foam when the PEM is added, so use a large enough tube to avoid overflow. When ready to treat the coverslips, add the appropriate amount of PEM buffer to one of the tubes, mix up and down and add to coverslip(s). Incubate 5 min. on rotator. You will need to check to make sure the coverslips do not "float" to the surface of the buffer if so, push them back down with forceps. Remove PEM/NaBH4, prepare a fresh tube and repeat.
- 6. <u>Alternately</u>, 1M ammonium chloride diluted 1:10 in PEM buffer can be used to quench. Incubate for 10 min. Note: This does not work as well as NaBH4 if cells have been fixed with glutaraldehyde.
- 7. After either quenching method, wash X 2 with PEM.
- 8. Incubate 30 min. with PEM + 0.5% Triton X-100. This step permeablizes the cells, which is essential for antibody penetration during immunostaining. Longer incubation time is required for MCF7 cells, or if the primary antibody is IgM (up to 1 hour, to be determined empirically).
- 9. Wash X 3 with PEM buffer, about 2-5 min. per wash.

Notes

If working with cells transfected with XFP plasmids that are not to be immunostained, you may shorten step 8 to 5 minutes, and then proceed to the final step in part 3, the DAPI counterstaining.

Aspiration using the vacuum line may remove loosely adherent or mitotic cells. If the goal is to preserve these cells, pipetting solutions off manually will help. Or adjust the suction to be weaker.

On some cells that have been core extracted, formaldehyde fixation is not sufficient for deconvolution microscopy. 0.02% glutaraldehyde may be added to the formaldehyde-CSK fixation buffer, and the fixation time reduced to 10 minutes. However, some antigens are damaged by glutaraldehyde -

preliminary experiments will need to be performed comparing the staining with and with-out glutaraldehyde. Alternatively, the glutaraldehyde can be added to the post-fix.

Part 2: Immunostaining

- 1. Block with 5% powdered milk in TBS-T buffer plus 0.02% sodium azide* (commonly called Blotto). Blocking time can be anywhere from 30 min. to 1 hour at room temperature or overnight at 4°C. 1% BSA in TBS-T can be substituted for Blotto.
- 2. Remove blocking buffer and add Primary Antibody diluted in blocking buffer. Incubate either 30 min at 37°C, 1-2 hours at RT, or overnight at 4°C.
- 3. Remove Primary Antibody (it can be saved, frozen and reused a number of times). Wash 4 5 times with blocking buffer, 2-5 min. or more per wash. Can hold at this step if needed.
- 4. Add Secondary Antibody diluted in blocking buffer. Incubate 30 min at RT protected from light (cover with foil).
- 5. Remove and save Secondary Antibody. Wash X 5 with TBS-T, then wash with PEM.

NOTE: SODIUM AZIDE CAN CAUSE CORROSION OF PLUMBING - IF DISCARDING SOLUTIONS CONTAINING SODIUM AZIDE, BE SURE TO FLUSH WITH LARGE VOLUMES OF WATER. Do not use Sodium azide if the secondary antibody will be HRP labeled, since it inhibits enzyme activity.

Note: The concentrations of primary and secondary antibodies must be determined empirically by titration. Excessive primary or secondary antibody will cause "background" staining. Too little will result in a weak signal.

Part 3: Post-Fixation and Quenching

The coverslips will need to be protected from light from now on (cover with foil during incubations).

- 1. Fix 10 30 min. in 4% Formaldehyde in PEM buffer. (This is especially important for cells to be imaged with the deconvolution microscope. It also may be necessary to add glutaraldehyde).
- 2. Remove fix (discard in toxic waste) and wash X 3 in PEM, 2-5 min per wash.
- 3. To quench auto-fluorescence perform 2 incubations with PEM + 1 mg./ml. NaBH4 or Ammonium chloride.
- 4. Wash X 2 @ 5 min. with PEM.
- 5. Wash X 2-3 with TBS-T.
- 6. Counterstain DNA with 0.5 1X DAPI diluted in TBS-T for 1-2 min.
- 7. Remove DAPI and add TBS-T.

Part 4: Mounting the Coverslips

- 1. If using SlowFade Gold or ProLong Gold mounting media, remove from the freezer ahead of time and allow to come to room temperature before using.
- 2. Label slides using a marker designed for slides pencil can be difficult to read in the microscope room, and immersion oil can smear some markers. [In our lab, please use the label maker.]
- 3. If using the original SlowFade Kit, coverslips can (optionally) be incubated with 1-2 drops of Equilibration Buffer for 5-10 min. before mounting.
- 4. Place a drop of mounting media on the slide for each coverslip to be mounted (two drops for square coverslips). One square or rectangular coverslip, or 3-4 round coverslips will fit on each slide. But 4 is "pushing it" and it may be too difficult to tell the coverslips apart. The coverslips need to be placed as much as possible toward the center of the slide, because it will be inverted on the microscope stage.
- 5. Pick a coverslip up with forceps, keeping the cell side toward you. Dip the coverslip in a beaker of water several times to rinse off the buffer salts. Carefully blot the edge of the coverslip with blotting paper or a kim-wipe to wick away the excess water. Alternatively, the coverslips may be propped against a box resting on a paper towel or kim-wipe and allowed to "drain" briefly, keeping track of the cell side orientation. Don't allow the coverslips to dry out (1-3 minutes is about OK).
- 6. Slowly lay the coverslip on the mounting media, starting at one edge, to avoid creating bubbles.

- 7. As the coverslips are mounted, place the slides in a holder, drawer, or other means of protecting from light.
- 8. When finished mounting, excess mounting media will spread out from under the coverslips. Suction this off using the vacuum line set at a high suction. The suctioning may need to be repeated several times if you don't do this the nail polish won't stick as well, and the coverslips may come off if you are wiping the emersion oil off, or dry out from lack of sealing, or even worse, stick to the lens of the microscope.
- 9. Seal the edges of the coverslips with nail polish. If coverslips need to be removed for some reason, you can peel off the nail polish and "float" them off with excess TBS-T buffer. [Note: If using ProLong Anti-fade, do not seal the edges or the mounting media will not harden.]

Coverslips are now stained and ready to examine. Store at 4°C or -20°C to prolong fluorescence.

Reagents and Supplies:

PBS & TBS-T buffers:

Formulations of these buffers can be found in common reference books.

PEM Buffer:

Stock Solution:	Amt. Stock to add for 1 Liter:	Final Concentration:
400 mM Potassium PIPES, pH 6.8	200 mL.	80 mM
0.5 M EGTA, pH 7.0	10 mL.	5 mM
1.0 M MgCl2	2 mL	2 mM

Filter sterilize and store at 4°C.

Potassium PIPES (P7643) & EGTA (E3889) available from Sigma

Formaldehyde:

Formaldehyde, 16% Sol. EM GRADE, in 10 mL ampules, is available through VWR (Catalog # 100503-916).

Cover opened ampules with parafilm and store at 4°C. Unopened formaldehyde should be stored in the chemical cabinet.

Triton X-100:

Sigma, Cat. #X-100-PC, Triton X-100 Peroxidase & Carbonyl Free, 5 ml ampule. Use during extractions on unfixed cells.

Sigma, Cat. #X-100, Triton X-100, 100 mls. Can use for permeablizing cells post fixation.

For ease of use, make a 10% w/v stock solution of the Triton X-100 in PEM buffer and aliquot to microcentrifuge tubes covered with foil or amber colored tubes to protect from light. Store the stocks at -20°C. Diluted Triton is unstable. Do not re-freeze the aliquots (store in refrigerator), and toss excess after 1 week.

DAPI

DAPI from Sigma (D9542) can be prepared at a 1.0 mg/ml (1000X) concentration in PEM buffer and aliquoted in amber tubes to protect from light. Store at 4 degrees C.

Coverslips:

Round coverslips will fit in 24 well trays, and can be obtained from:

Fisher Scientific, Cat. #12-545-80, #1 thickness 12 mm circles

Fisher Scientific, Cat. #12-545-82, #1 thickness 12 mm circles – "pretreated to promote cell growth" – we think they are acid-etched.

Rectangular coverslips will fit in small Coplin jars (available from Thomas Scientific) or two per well in a 6 well TC tray or 35 mm petri dish:

Thomas Scientific, Cat. #6663-F10, #1 thickness 11 X 22 mm rectangular coverslips.

22 X 22 mm coverslips may also be used, but will require larger volumes of reagents: Fisher Scientific, Cat. #12-520-B, #1 1/2 thickness 22 X 22 mm square coverslips.

The coverslips will need to be pre-cleaned with 100% ethanol and sterilized before use if cells are to be grown on them (see acid washing protocol). They can be sterilized by microwaving them at several short 30 sec bursts, or by speading them out under UV light. It may be necessary to acid wash them or coat them with poly-L-lysine to keep the cells from coming off (see separate protocol).

Slides:

Use pre-cleaned slides, such as Colorfrost slides (available from Fisher or VWR), or clean them before use with ethanol.

Mounting Media:

require nail polish sealing)

Molecular Probes - SlowFade Anti-fade kit, Cat. #S-2828.

Molecular Probes - SlowFade Gold Anti-fade kit "Special Packaging", Cat. # S36937

Molecular Probes - ProLong Gold Anti-fade kit "Special Packaging", Cat. #P36934 (hardens, doesn't