Fixing and pelleting chromatin/nuclei from extracts onto coverslips for immunofluorescence

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1/23/95

Things you need :

1. 2X XBE2 (10 mM HEPES, pH7.7 (@ 10 mM), 100 mM KCl, 0.1 mM CaCl2, 2 mM MgCl2, 5 mM EGTA, 50 mM sucrose).
2. 16 % formaldehyde (Methanol-free from Ted Pella; used for two weeks after opening a sealed vial) OR paraformaldehyde (freshly prepared) : will be used at 1% final.
3. poly-lysine coated coverslips.
4. spin down tubes (or equivalent method to pellet onto coverslips; our tubes are modified 15 ml Corex tubes).
5. Extract fix : 1ug/ml Hoechst in 1X MMR, 50% glycerol, 10% formaldehyde (from 37% stock sold by Fisher) OR 4.5% Ted Pella MeOH-free formaldehyde.

Procedure:

1. Squash 1 ul of extract with 3 ul of Extract fix prior to beginning fixation to compare the morphology post fixation.
2. To 10 ul of extract with chromatin/nuclei in extract gently add 200 ul of 1% MeOH-free formaldehyde (or 1% paraformaldehyde) in XBE4. Immediately use a cutoff pipet tip to gently mix the fix with the extract by pipeting up and down 5 times. (If this is not done promptly one often gets aggregation). **NOTES :** a) The 1% formaldehyde in XBE2 should be made up just before use (~5'-10'). b) Titration of formaldehyde 1% - 4% doesn't show much difference in morphology; however, higher formaldehyde concentrations generally reduce antigenicity. c) If extracts contain membranes then 0.5% Triton addition to the fix reduces particulate debris which tends to bind antibodies.
3. Incubate at RT for 12' - 15'.

4. During incubation setup spindown tubes. Each tube contains a polylysine coated coverslip and is loaded with a cold 5 ml cushion of 30% glycerol (v/v) in XBE2 and stored on ice.
5. After incubation in formaldehyde, gently layer the fixed sample onto the cushion using a cutoff pipet tip.
6. Spin in a swinging bucket rotor (I use a Sorvall HB-4 or Beckman JS13.1) at 10,000 rpm for 10'.
7. Aspirate half the cushion and then rinse the interface with a few mls of XBE2 before aspirating the rest.
8. Remove the coverslip and after rinsing with TBS Tx/PBS Tx, block and process for immunofluorescence. I use 1 ug/ml Hoechst to stain the DNA just before mounting.
9. Compare the fixed samples to nuclei squashed in Extract fix before fixation to determine if the fixation worked as desired.

Notes:

1. Most protocols call for -20 Methanol treatment of the coverslip post sedimentation. This is generally used to stick the sedimented object to the coverslip and prevent it from floating away during processing and is also thought to help antigenicity by exposing epitopes. The use of polylysine coated coverslips eliminates the first problem and as to the effect of methanol on antigenicity I don't have an opinion. Methanol sometimes drastically affects (negatively) the morphology of chromosomes and, therefore, I have stopped using it.
2. This protocol is optimized for mitotic chromatin but works for nuclei as well. The final structure is affected slightly by increasing magnesium concentration or adding polyamines to the fixation buffer. The buffer we use works reasonably well and therefore I haven't changed it much aside from occasionally using 4 mM Magnesium rather than 2 mM.
3. Some epitopes may be sensitive to formaldehyde and, therefore, lack of any signal may require more fiddling with the fixation conditions. Methanol only fixations generally show poor morphology.
4. Frog extracts have huge pools of biotin and it is generally a good idea to avoid avidin-biotin detection systems. I highly recommend labeled donkey secondary antibodies from Jackson Immunoresearch for immunofluorescence.