Immunofluorescence method by using formaldehyde

Aim : To observe, *in situ*, yeast's proteins with tagged antibodies.

Solutions :

1 M potassium phosphate buffer at pH 6.5 (PPB6.5) :
Prepare 500 ml of 1 M potassium phosphate monobasic and 500 ml of 1 M potassium phosphate dibasic. Mix both buffers until the pH 6.5 is reached. Make 500 ml of that. Autoclave and store at Room T°C.

PPBMG pH 6.5 :	**500 ml**	
1 M PPB at pH 6.5	60 ml	for a final concentration of 120 mM
$MgCl_2$	0.06mg	
DdH_2O	440 ml	

Autoclave and store at Room T°C.

Formaldehyde buffer 5% (make fresh)
Add 13.5 ml of 37% formaldehyde to 86.5 ml of PPBMG pH 6.5.

Check the pH before using. If the pH is wrong, check the pH of the buffer. If that's correct, check the formaldehyde. If it is off, just order a new bottle.

Nota bene : Formaldehyde 37% solution Microscopy grade (500 ml) from Sigma (page 435 F1635) is at pH 5-6. Use until polymerization occurs.

Buffer B :	**100 ml**
3 M sorbitol (1.)	40 ml
PPB pH 7.5 (2.)	10 ml
DdH_2O	50 ml

1. Make a 250 ml solution of 3 M sorbitol (136.65 mg in 250 ml ddH_2O).
Autoclave and cool it.
2. 500 ml PPB 7.5 by mixing 1 M K_2HPO_4 (A) et 1 M KH_2PO_4 (B) until reaching pH 7.5. Autoclave and cool it.
Warning : you need a lot of more solution A than solution B.

PBS 1X (as described by J.R. Pringle et al.)	**1000 ml**
NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.14 g
KH_2PO_4	0.2 g

Adjust pH with 10N NaOH up to pH 7.3.

Cells growth harvest and fixation by using formaldehyde

Method :

Fixation :

1. Grow 500 ml of cells up to 0.5 OD_{600nm} .
2. Add 67 ml of formaldehyde 37 % (final concentration of 5%)
3. Take 100 ml sample at 0, 10, 20, 30 min.
4. Spin at 2000 g for 5 min.
5. Resuspend in 20 ml of PPBMG pH 6.5 + 5% formaldehyde buffer.
6. Incubate at RT °C and take samples of 4 ml at 0.5, 1, 2 and 4 h.
7. Spin at 2000 g and resuspend in 500 µl of buffer B.
8. Spin and repeat 2 more times step 7.
9. After the second wash, resuspend the cells in buffer B, count the cells and adjust to $2x10^8$ cells using the same buffer.

The first time you use an antibody, you need to proceed immediately up to the staining step.

Digestion :

1. Resuspend $2x10^8$ cells in 1 ml of buffer B (remove 10 µl for the digestion control)
2. Add 2 µl β-Mercaptoethanol and 1 µl of Zymolase solution (20mg/ml).
3. Incubate 30 min at 37 °C. Use a rotator in a 37 °C air incubator.

Check every 10 min for the completion of the reaction by removing aliquots of 10 µl and by diluting them in 90 µl of water containing 1% SDS. Then count the number of cells in comparison with the same sample before adding Zymolase.

4. Spin down the cells at 2000 g for 2 min.
5. Wash 3X with buffer B and resuspend in 1 ml buffer B.

Preparation of a slide :

1. Put 10 µl polylysine stock solution (1 mg/ml stored at – 20°C) in each well of a multiwell slide.
2. Wait for 10 sec., aspirate the solution off and air dry 10 min.
3. Wash the slide 3 times with drops of ddH_2O (40 µl with Pasteur pipet).
4. Aspirate the water off, air dry completely at least 10 min, until no solution is seen.

Cells growth harvest and fixation by using formaldehyde

Staining :

1. Add 10 μl of cells suspension in each well.

You will need 6 slides for the formaldehyde first time experiments. Always include a control for auto-fluorescence (no antibodies)

2. Leave the cells in the slides for 10 sec, then aspirate and let the sample dry 10 min or until no liquid is seen.
3. Check the cells by the microscope for clamps. If clamps are seen, make new slides.
4. Incubate the slides in methanol (- 20 °C) for 6 min., then in aceton for 30 sec (-20 °C). Air dry completely for 10 min.
5. Block the cells with PBS containing 1 mg/ml BSA. Incubate 30 min at RT °C.
6. Add your primary antibodies in the same solution.

Dilutions to use for every antibody :

- For HA, 1 :2000 dilution and the secondary in 1 :1000
- For the tubulin, 1 :100 and the secondary in 1 :1000.

7. Incubate 2 h at RT°C.
8. Wash the primary antibodies 10 times after a 5 sec waiting, each time with PBS+BSA.
9. Leaving a drop every 7 washes. Wash the other wells before returning back to the 3 final washes.
10. Add the secondary antibody in the PBS+BSA. Incubate 2 h at RT°C.
11. Wash with PBS+BSA, 10 times like steps 8 and 9.
12. Stain with DAPI by adding 10 μl of 1 μl/ml DAPI in PBS.
13. Leave it for 2 min. Wash 3 times with PBS.

Mount the slide :

1. Add a drop of mounting medium between each 4 wells (a total of 4 drops per slide).
2. Remove the last drop of PBS and slip the cover immediately.
3. Blot the excess with Kimwipes and seal.