Immunofluorescence method by using paraformaldehyde and glutaraldehyde

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

Material :

- PPB dibasic, 1 M, pH 9.3
- Paraformaldehyde buffer 10% (PFB 10%)
- H_3PO_4 conc.
- 50% Glutaraldehyde
- 2 x big centrifugation tubes (500 ml)
- PPB 6.5
- PBS 1X
- Buffer A
- Buffer B
- Multiwell slide
- Polylysine stock solution
- β-mercaptoéthanol
- Zymolase solution (20 mg/ml)

Solutions :

1. Potassium phosphate buffer dibasic (PPB) 1 M, pH 9.3

Make 1 liter of this buffer.
Autoclave and store at room t °C.

Then make 500 ml of PPB dibasic, pH 9.3 100 mM

2. Paraformaldehyde Buffer 10% (PFB 10%)

1. Dissolve 25 g of paraformaldehyde in a final volume of 250 ml of PPB 9.3, 100 mM. Heat at 60 °C to dissolve.
2. Adjust pH to 6.5 with H_3PO_4 conc.

3. Buffer A

PFB 10%	50 ml
1 M PPB 6.5	10 ml
Glutaraldehyde 50%	3 ml
ddH_2O	90 ml

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Solutions :

4. Buffer B

1. Prepare 500 ml of **1 M PPB 7.5** by mixing 1 M K2HPO4 and 1 M KH2PO4 solutions until pH 7.5 is reached.

Autoclave and store at room t °C.

2. Prepare a 250 ml of **3 M sorbitol** by dissolving 136.65mg in a final volume of 250ml of ddH_2O.

Autoclave and store at room t °C.

3. Mix 40 ml 3 M sorbitol with 10 ml PPB 7.5 and 50 ml ddH_2O to a final volume of 100 ml.

5. PBS 1X (as described by John R. Pringle et al) **1000 ml**

NaCl	160 g
KCl	4 g
Na_2HPO_4	22.8 g
KH_2PO_4	4 g

Dissolve them in ddH_2O to a final volume of 1000 ml after adjusting the pH to 7.3 with NaOH 10N.
Autoclave and store at room t °C.

Special note : The first time you use an antibody, you need to titer the amount with two different concentrations.

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Method :

Fixation :

Do every step of the fixation on the hood.

1. Grow 500 ml of cells up to OD_{600nm} of 0.5.
2. Add 166 ml of PFB 10% (final concentration 3.3%)
3. Add 13 ml of 50% glutaraldehyde.
4. Incubate on the hood for 30 min.
5. Spin in 2 big centrifugation tubes (500 ml) at 4 °C, for 5 min at 2000g.
6. Resuspend in 100 ml of buffer A.
7. Incubate on the hood and take a 20 ml sample at 30 min, 1h, 2h, 4h.
8. Spin every sample at 2000g for 5 min.
9. Resuspend it in 500 µl of Buffer B.
10. Spin and repeat 2 more times step 9.
11. 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.

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Method :

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.

Staining :

1. Add 10 μl of cells suspension in each well.
2. Leave the cells in the slide 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 slide in methanol (- 20 °C) for 6 min., then in acetone 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.