Immunostaining of Ectocarpus *cells*

This protocol (Coelho et al., 2012) has been optimised for the detection of tubulin but could be used with any suitable antibody. Ectocarpus has small but relatively transparent cells and the uniseriate filaments can be grown directly attached to the surface of the coverslips. These features make them particularly suitable for high resolution imaging approaches, both in vivo or after fixation.

All incubations described below are carried out on a platform shaker at room temperature. Use high quality microscope slides (e.g. Fisher 12-550-42), to avoid imperfections in the glass that can be a problem for confocal laserscan microscopy analysis.

1. Dip slides with attached *Ectocarpus* material rapidly into liquid nitrogen (immerse for less than one second) and immediately immerse in PHEM solution in a fresh 35x10 Petri dish for 1h (or overnight at 4°C)

2. Remove the PHEM, rinse three times with mPBS (5 min each time) and immerse in 5% Triton-X-100 in mPBS. Incubate overnight at room temperature.

3. Rinse three times with mPBS and then immerse in 100 mM $NaBH_4$. Incubate for 4 h or until the solution stops bubbling (indicating that the reducing reaction is complete).

4. Rinse three times with mPBS. Immerse in solution C, then in solution C plus enzymes. Incubate for 1h.

5. Rinse three times with mPBS. Immerse in the blocking solution and incubate overnight at room temperature.

6. Rinse three times with mPBS. Add the primary antibody and incubate for 4h or overnight. We carry out the incubation in a 140 mm Petri dish lined on the bottom with parafilm. Pipette the primary antibody onto the parafilm then place the slide on top. Place the Petri dish in a larger Petri dish with ~½ inch of wet paper towels on the bottom. Cover the pyrex dish with plastic film (Saran wrap) and put into a light-proof cardboard box. This keeps the chamber humid so that the filaments do not dry out.

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Reagents
PHEM
mPBS
$NaBH_4$
Solution C
Solution C plus enzymes
Blocking solution
Clearing solution (BABB)

Equipment
Weighing balance
Microscope coverslips and slides

Additional information:
Coelho SM, Scornet D, Rousvoal S, Peters N, Dartevelle L, Peters AF, Cock JM. *2012. Immunostaining of Ectocarpus cells. Cold Spring Harbor Protoc doi: 10.1101/pdb.prot067975.*

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7. Rinse three times with mPBS and add the secondary antibody. Incubate for 4h or overnight as described above. Exclusion of light is particularly important for the secondary antibodies.

8. If you want to carry out a double labelling, label with the first pair of antibodies (monoclonal primary, then secondary) as above, then block with blocking solution overnight before labelling with the second pair of antibodies (polyclonal primary, then secondary).

9. Rinse three times with mPBS and rinse three times with 100% methanol at -20°C. Note that methanol dries very quickly, do not let the cells dry out. Mount in clearing solution (BABB) by pipetting about 20 µl onto the microscope slide, then rapidly place a coverslip on the slide (touch the edge of the slide on a paper towel to remove excess methanol). Wait for about 20 seconds then paint around the perimeter of the coverslip with nail polish (e.g. Sally Hensen's clear "Hard as Nails"). Wait for an hour and then repeat the sealing with the nail polish to eliminate small holes that could cause the material to dry out.

SOLUTIONS

Blocking solution

Reagent	Quantity (for 100 ml)
mPBS	100 ml
non-fat dry milk	2.5 g

Prepare fresh just prior to use.

Clearing solution (BABB)

Reagent	Quantity (for 30 ml)
benzyl benzoate	20 ml
benzyl alcohol	10 ml

PHEM

Reagent	Quantity (for 200 ml)	Final concentration
PIPES (MW=302.4)	3.68 g	60 mM
Hepes (MW=338.31)	1.19 g	21 mM
EGTA (MW=380.35)	0.76 g	10 mM
1M $MgCl_2$ 6 H_2O (MW = 203.31)	400 µl	2 mM
NaCl (MW=58.44)	8 g	685 mM

Adjust the pH to 7.5 with KOH

Adjust the volume to 179 ml volume with distilled water, then add 1 ml of 25% glutaraldehyde and 20 ml of 32% paraformaldehyde

This solution works best if it is made fresh just prior to use.

mPBS

Reagent	Quantity (for 3.2 litres)	Final concentration
NaCl (MW=58.44)	25.6 g	136 mM
KCL (MW=74.55)	8.64 ml of 1 M stock	2.7 mM
KH_2PO_4 (MW=136.09)	0.7408 g	1.7 mM
Na_2HPO_4 (MW=141.96)	3.648 g	8.03 mM
glycerol (MW=92.10)(density=1.261g/cm^3)	160 ml	685 mM
sodium azide (MW=65.02)	3.2 g	15.4 mM
BSA (MW=67,000)	3.2 g	0.015 mM

The pH should be 7 or slightly above. Adjust the volume to 3.2 l with distilled water.

This solution is stable and can be used for several weeks.

$NaBH_4$

Reagent	Quantity (for 100 ml)	Final concentration
mPBS	100 ml	
$NaBH_4$	0.3784 g	100 mM

Solution C

Reagent	Quantity (for 1 litre)	Final concentration
NaCl (MW=58.44)	5.8 g	100 mM
$MgCl_2$ (or 20 ml of 1 M stock)	4.1 g	20 mM
KCl (MW=74.55)	2 ml of 1 M stock	2 m
BSA (MW=67,000)	2 g	29.8 nM
MES (MW=195.2)	2.13 g	0.01 mM
sorbitol (MW=182.17)	154.85 g	850 mM
EGTA (MW=292.24)	0.38 g or 10 ml of 100 mM	1 mM

Adjust the pH to 5.8 with TRIS base, then adjust the volume to 1 l with distilled water. Store 50ml aliquots at -20°C

Solution C plus enzymes

Reagent	Quantity (for 25 ml)
Solution C	25 ml
CELF	175 mg
hemicellulase	1 g

Add the enzymes just prior to use.