Ashbya gossypii _IEM_prep_protocol

Using Tannic Acid (TA), Uranyl Acetate (UA) and p-Phenylenediamine (PPD) to retain ultrastructure and antigenicity.

1. Allow *Ashbya* to settle. 15 ml conical tubes will work quite well for steps 1-3.

2. Remove as much fluid as possible and add fix: 10-15x volume of 3%PF/1%GTA/1%TA in 0.1 M 1M NaCacodylate pH 7.2-7.4 (NaCac) for 2 hours at room temperature (RT). Swirl sample tubes every 15 minutes during fixation step. NOTE: Reduce % of GTA and increase % of PF to get best labeling count; e.g. any combination from 4%PF/0%GTA to 3%PF/1%GTA.

3. Replace with fresh fix, leave for one hour at RT with swirling, and then overnight on rotator at 4^0C.

4. Remove as much fluid as possible and rinse in PBS.

5. Re-suspend in 2mls of solution A (100mM KPO4, pH7.5, 1.2 M sorbitol) and add 200 microliters of 10mg/ml zymolyase. Incubate with gentle rotation at 37 C until see 75% of the hyphae are phase dark (approximately 5-35 minutes). Wash 1X in Sol. A, spinning 2 min. at 2000rpm in a microfuge.

6. Remove as much fluid as possible and rinse in fix: 3%PF/1%GTA/1%TA in 40mM K_2HPO4-KH_2PO4 buffer (pH6.7) with 0.5mM$MgCl_2$ (KPO4).

7. Spin sample down (1500rpm) and then:

8. Remove as much fluid as and re-fix in 3%PF/1%GTA/1%TA in 0.1 M NaCac. Swirl sample tubes every 15 minutes during fix step. Replace with fresh fix and store at 4^0C if necessary.

9. Gently centrifuge (1800rpm for 2mins) to get sample to bottom and rinse in 0.1M NaCac.

10. Centrifuge 2000RPM for 2mins and then quench aldehydes 2X , over one hour, with 50mM Glycine in 0.1M NaCac.

11. Rinse once more in 0.1M NaCac for 10 minutes. Spin to 2-3000RPM for 2mins to get sample to bottom of tube.

12. Rinse in dH_2O 2x 20 min each to remove all traces of NaCac buffer.

13. 'En-bloc stain' with 1% Uranyl Acetate in dH_20 for 1 hour RT.

14. Rinse in dH_2O.

15. Dehydrate through Ethanol series: 30% w 1%PPD, 50% w 1%PPD, 70% w 1%PPD, 85% w 1%PPD -- 30 minutes each on rotator at RT. Add 1% p-phenyenediamine (PPD) to each ethanol solution within 20-30 minutes of use. Solution will be pink to light orange. If you make it up too early, the PPD will react with Ethanol and turn dark. Don't worry if PPD doesn't dissolve completely. It still works.

16. 85% Ethanol w 1%PPD on rotator at RT --- 4 rinses over 1 hr.

17. Re-suspend in a 2:1 mixture of 85% Ethanol w 1%PPD: LR White MEDIUM resin. Place on a rotator for 2 hr at RT

18. Re-suspend in 1:1 85% Ethanol w 1%PPD: LR White resin. Place on rotator for one hour and then store over night at 4-6^{0}C (NO rotator). NOTE: solutions will turn dark (green-black; don't worry; it still works to retain proteins and ultrastructure. Just make as many changes as necessary in next step to get the resin solution clear again.

19. Make up fresh 85% Ethanol /1%PPD. Re-suspend in 1:2 85%EtOH w 1%PPD: LR White MEDIUM resin. Place on rotator 1 hr. at RT.

20. Re-suspend in 100% LR White MEDIUM resin. Do as many initial changes as needed to remove all the ETOH/PPD; i.e. when the dark solution turns clear. Then 3 changes over 6hours, using rotator at RT. Then store over night at 4-6^{0}C (no rotator).

21. Warm to RT and 4 changes of 100% LR White MEDIUM resin; one hr each RT.

22. Transfer to gelatin capsules; leave for 4 hour at RT.

23. Polymerize at 50 ^{o}C for 24hr.

24. Remove from heat, remove sample from gelatin capsule. Store at 4 ^{o}C.

25. Semi thin sections (@0.5microns) Stain: Toluidine Blue.

26. For Immuno labeling sections; see separate protocol.

27. Thin Section: stain: UAaq for 5'. LC for 2'. IEM stain: UAaq for 4'; LC for 10” or UAaq for 4'; NO LC.

References:

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