Ashbya gossypii _TEM_prep_protocol

The key in the first 8 steps is to be gentle BUT get as close to 100% of stated fluid components as possible. If needed, repeat a step twice to make sure the fluid in tube is as close to the 100% fluid component listed.

Steps 1-2 3hrs

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%GTA/1%PF/0.1%TA in 0.1M NaCac pH7.2 -7.4 for 2 hours at room temperature. Swirl sample tubes every 15 minutes during fixation step. Replace with fresh fix, leave for one hour at RT with swirling and then 24-48 hours on rotator at 4^0C. (Stock 6%GTA/2PF/ 0.2MNaCac: 70%GTA 20ml, 16%PF=26.6, dH_20=73.4ml, 0.4M NaCac=106.5ml for Total=213ml)

3. Remove as much fluid as possible and rinse in PBS after this primary fixation.

4. 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 30-60 minutes). Wash 1X in Sol. A, spinning 2 min. at 2000rpm in a microfuge.

5. Remove as much fluid as possible and use fix as wash step, as it will be more osmotically compatible: Remove as much fluid as possible and rinse in fix twice in 2.5%GTA/1%PF in 40mM KPO4 pH 6.5-6.7 (70%GTA=2ml, 16% PF= 3.5ml, dH_20=22.5ml, 80mM KPO4=28ml for Total=56ml) Spin sample down (1500rpm) and then:

6. Remove as much fluid as and re-fix in 2% 3%GTA/1%PF/0.1%TA in 0.1M NaCac pH7.2 -7.4 for 2 hour at room temperature. Swirl sample tubes every 15 minutes during fix step. Replace with fresh fix and put samples on rotator overnight 4^0C.

Steps 7-11@9hrs

7. Gently centrifuge (1800rpm for 2mins) to get sample to bottom and rinse several times in 0.1M NaCacodylate (NaCac) pH 7.2-7.4 over 2 hours (2000RPM for 2mins), to completely remove all GTA. Before last rinse increase spin to 3000RPM for 2mins to get sample to bottom of tube.

8. Post-fix in 2% OsO4 in 0.1M NaCacodylate (NaCac) pH 7.2-7.4 for 2 hours at room temperature. Swirl samples every 15 minutes. At this point the sample should be a small, soft pellet. If not, gently centrifuge until you get a soft pellet. Keep the soft pellet small for maximum infiltration. If sample size is large; divide material into multiple 2ml Eppendorf tubes. (Note: sample sometimes never really

forms a soft pellet at 2000rpm but does go to bottom of tube. This is actually better for infiltration purposes. Spin 2000RPM for 5 minutes

9. Rinse **twice** in dH_20 (UA doesn't mix well with other buffers; don't skip this step). NOTE: Spin 2000RPM for 5 minutes for all steps through 95% Ethanol.

10. En-bloc stain with 1% Uranyl Acetate in dH_20 for 1-2 hours at room temperature, in the dark.

11. Dehydrate through Ethanol series: 30%, 50%, 70%; 30 minutes each with tubes taped to rotator. Then 1-2 days on rotator at 4ºC.

Steps 12-13 2-3days

12. Dehydrate in 85%, 95% Ethanol, 30 min each on rotator at room temperature.

13. 100% Ethanol --- 6 rinses over 6 hours, on rotator at room temperature. This Increase of time in 100% ETOH and on the rotator will help infiltration. Leave 36-48hours (NO rotator) at 4-6^{0}C to increase infiltration of solutions. Spin at 3000RPM for 5 minutes after first change.

Steps 14-17 6hrs

14. Two 30 min. changes in Propylene oxide(PO) at room temperature.

15. Sample is immersed in LX112 : PO - 1:1 for one hour on rotator, at room temperature. We use the LX112 kit from LADD, Inc (Burlington, VT) and follow the LADD.pdf file to make up the epoxy solutions in the right proportions. We use mixture 6A:4B (instead of 5:5) for medium hard block.

16. Sample is immersed in LX112 : PO - 1.5:1 {10.2 ml:6.8 ml}. 4-5 changes over 8 hours on a rotator. This Increase of time will help infiltration

17. Remove caps and place in vacuum desiccator overnight.

Steps 18-20 2days

18. Transfer pellet to BEEM capsules and fill with fresh LX112. Spin down 30 min at 1500rpm on clinical centrifuge to get cells to bottom of capsule, if necessary. Place in vacuum desiccator overnight.
19. Polymerize at 45 ^{O}C for 8hr-24hr for better infiltration of samples.

20. Polymerize at 60 ^{O}C for a further 24 hours. Remove blocks from heat to cool.

Stock solutions:

- 0.2M NaCacodylate buffer pH 7.2-7.4
- 2%TA in dH_20. Store in the fridge in a tightly closed amber bottle. Good for a few days as a stock solution.
- 6%GTA/2%PF in 0.2M NaCacodylate buffer pH 7.2-7.4
- 3%GTA/1%PF/0.1%TA in 0.2M NaCacodylate buffer pH 7.2-7.4. Draw aliquot of TA from supernatant without disturbing sediment on bottom. Add aliquot 1:1 to fixative, just before use, to prevent precipitate forming (if TA is left in pH7.4 buffers for long periods of time).
- 0.2M K_2HPO4-KH_2PO4 buffer (pH6.5-6.7)
- 80mM K_2HPO4-KH_2PO4 buffer (pH6.7) with 0.5mM$MgCl_2$ (KPO4 pH 6.5-6.7)
- 2.5%GTA/1%PF made up in 40mM KPO4 pH 6.5-6.7
- 2% Uranyl Acetate in dH_20

Preparation of 0.2 M potassium phosphate buffer at 25°C
Table below to combine K_2HPO_4-KH_2PO_4 for pH 6.5-6.7. 38.1ml K_2HPO: 61.9ml KH_2PO_4

Preparation of 0.1 M Potassium Phosphate Buffer at 25°C

pH	Volume of 1 M K_2HPO_4 (ml)	Volume of 1 M KH_2PO_4 (ml)
5.8	8.5	91.5
6.0	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7.0	61.5	38.5
7.2	71.7	28.3
7.4	80.2	19.8
7.6	86.6	13.4
7.8	90.8	9.2
8.0	94.0	6.0

Dilute the combined 1 M stock solutions to 1 liter with distilled H_2O. pH is calculated according to the Henderson-Hasselbalch equation:

$$pH = pK' + \log \left\{ \frac{(\text{proton acceptor})}{\text{proton donor}} \right\}$$

where $pK' = 6.86$ at 25°C.

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