**Flagella preparation**

*(Modified from DRR Nature 1991 and B. Morriswood, Eukaryotic cell, 2012)*

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**Protocol 1.**

**On slides**

1. This is for 5mL of mid-log phase culture. Scale up should also include all buffer volumes accordingly.

2. Centrifuge 5 ml *T. brucei* procyclic cells for 5min, 1000 x g at RT. Remove the supernatant and resuspend in 0.5 mL PBS, then spin again for 5 min, 1000 x g at RT.

3. Place cells on, fat pen treated, poly-L-lysine coated, slides to adhere then make cytoskeletons by detergent-extraction using; PEME buffer [2 mM EGTA, 1 mM MgSO4, 0.1 mM EDTA, 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid)–NaOH (PIPES-NaOH), pH 6.9] supplemented with 0.5% NP-40 (vol/vol) plus complete protease inhibitor cocktail (Roche) for 5 min at room temperature (RT).

4. Remove excess detergent and place slide on a cooling block. ***(Do this by moving the slide to a cooling block on ice. (Support the block with an upside down beaker/Jar so that the block does not sink into the ice over time).***

5. Isolated flagella are made by further incubating the cytoskeletons in PEME buffer containing 1% NP-40, **1 M KCl,** complete protease inhibitor cocktail (Roche) for 30 min on ice.

6. Inspect the slide from time to time to ensure flagella are made. If they do not look pure change the buffer and incubate for another 15-30min.

7. Once flagella are made they should be washed in appropriate buffer, fixed and used immediately for IFA.

**Protocol 2.**

**Flagella preparation** **in tubes (for IFA and Western).**

***Note; Tube preps tend to be crude and aggressive so for immuno-EM you should use Protocol 4***

1. This is for 5mL of mid-log phase culture. Scale up should also include all buffer volumes accordingly.

2. Centrifuge 5 ml *T. brucei* procyclic cells for 5min, 1,000 x g at RT. Repeat and then resuspend in 0.5 mL PBS.

3. Extraction of the cytoskeleton with 2 mL 1%NP40, PEME pH6.9, 1mM MgCl2 complete protease inhibitor cocktail (Roche). Incubation 5min at RT.

4. Spin for 5min, 5,000g at 4°C. Remove supernatant.

5. Extract flagella with 1 mL 1%NP40, PEME, 1mM MgCl2 pH6.9, + **1M KCl,** complete protease inhibitor cocktail (Roche). Incubation 30 min at 4°C.

6. Inspect the tube from time to time to ensure flagella are made. If they do not look pure incubate for another 15-30min.

7. Spin for 5min, 5,000 x g at 4°C. Remove supernatant and resuspend in 500µL PEME pH6.9, 1mM MgCl2, complete protease inhibitor cocktail.

8. Spin again for 5min, 5000g at 4°C. Resuspend the pellet in 200µL PEME pH6.9, complete protease inhibitor cocktail (Roche).

9. Use as needed.

**Protocol 3.**

**Flagella for simple visualization by EM on grids**

1. This is for 5mL of mid-log phase culture. Scale up must maintain the buffer cell number ratio.

2. Centrifuge 5 mL *T. brucei* procyclic cells for 5min, 1,000 x g at RT. Repeat and resuspend in 0.5 mL PBS.

3. Float Formvar/Butvar covered, charged, carbon coated nickel grids onto the 0.5mL droplet for 10-15 min. Check that cells have adhered to the grids. Remove excess buffer/cells by touching grid onto a kimwipe/ paper tissue.

4. Transfer to 250µL droplets of 1%NP40, PEME pH6.9, 1mM MgCl2 complete protease inhibitor cocktail (Roche) 5min at RT.

Repeat on fresh drops of detergent to allow good extraction.

5. Transfer grids to 250µL droplets of 1%NP40, PEME, 1mM MgCl2 pH6.9, + **1M KCl,** complete protease inhibitor cocktail (Roche) for 30 min on ice.

***(Do this by moving the grids to a petri dish on a cooling block on ice. (Support the block with an upside down beaker/Jar so that the block does not sink into the ice over time).***

6. Inspect the grids from time to time to ensure flagella are made.

7. Wash grids four times (5 min, RT), in PEME buffer (50-100µL) droplets, complete protease inhibitor cocktail (Roche).

8. Fix in 2.5% glutaraldehyde in PEME buffer (5 min, RT - 50-100µL) droplets), rinsed four times in miliQ water (50-100µL) droplets, and negatively stained with 10µL 0.5% gold thioglucose.

**Protocol 4.**

**Flagella prep and immuno-EM directly on grids**

1. This is for 5mL of mid-log phase culture. Scale up should also include all buffer volumes accordingly.

2. Centrifuge 5 mL *T. brucei* procyclic cells for 5min, 1,000 x g at RT. Repeat and resuspend in 0.5 mL PBS.

3. Float Formvar/Butvar covered, charged, carbon coated nickel grids onto the 0.5mL droplet for 10-15 min. Check that cell have adhered to the grids.

4. Transfer to 250µL droplets of 1%NP40, PEME pH6.9, 1mM MgCl2 complete protease inhibitor cocktail (Roche) 5min at RT. Repeat on fresh detergent to allow good extraction.

5. Transfer grids to 250µL droplets of 1%NP40, PEME, 1mM MgCl2 pH6.9, + **1M KCl,** complete protease inhibitor cocktail (Roche) for 30 min on ice.

***(Do this by moving the grids to a petri dish on a cooling block on ice. (Support the block with an upside down beaker/Jar so that the block does not sink into the ice over time).***

6. Inspect the grids from time to time to ensure flagella are made.

7. Wash grids four times in PEME buffer, complete protease inhibitor cocktail (Roche).

8. Rinse grids four times in PEME, complete protease inhibitor cocktail buffer and fixed on 100µL droplets of 2-4 % PFA or PFA + glutaraldehyde in PEME for 5min. Then neutralize four times in 100µL droplets of 100mM glycine in PEME.

9. Transfoer grids through 5 subsequent drops (100µL, 5 min each) of blocking solution comprised of 0.1 - 2% BSA in PBS. Then incubate grids with primary antibodies (25-50µL) diluted in blocking solution (60 to 80 min at RT or overnight at 4°C).

10. Wash grids 2 twice in blocking buffer as above and then incubated with gold-conjugated secondary antibodies (British Biotech).

11. Wash grids twice in blocking buffer then twice in PBS (5 min) and then fix in 2.5% glutaraldehyde in PBS (100µL, 5 min).

12. Wash grids twice (2 x 250µL) in milliQ water.

13. Negatively stain 10µL 0.5% gold thioglucose.