Tissue Polysome Fractionation (Stefan's Protocol)

Reagents

- HBSS+: HBSS + 1:50 CHX
    - 20ml/mouse
- Homogenization Buffer (HB): 1:50 CHX + 1:100 DTT + 1:50 complete + 1:400 RNasin (+ 1:100 PMSF)
- Sucroses: 1:100 CHX + 1:100 DTT + 1:50 complete (+ 1:100 PMSF)
    - 0.9M sucrose
    - 1.1M sucrose
    - 2M sucrose
- 60% sucrose in DEPC
- 10% SDS in DEPC
- 20% TCA in mqH2O

Night before:

- Place frozen gradients in 4'C to thaw
- Take UZ buckets and rotor and cool in 4'C

Tissue Extraction, Homogenization and Lysis

- Prepare HBSS+ and HB+
- Pre-cool bench-top centrifuge to 4'C
- Warm DOC to dissolve, leave NP-40 at RT

1. Prepare 6-well plate with HB+ (B:4ml/SC:1ml), leave on ice
2. Perfuse mouse with 20ml **HBSS+**
3. Extract tissue to 6-well plate, add PMSF right before (B:40ul/SC:10ul)
4. Dice to small pieces and mash with scalpel, incubate on ice for 15min
5. Transfer tissue pieces to douncer with 1ml pipette, try to prevent tissue from sticking to inside of tip
6. Homogenize tissue (B:tight pestle 5times/SC:loose pestle 5times+tight pestle 5times)
7. Decant homogenate to 2ml eppis. Leave at this stage if need to wait for later samples
8. (save 400ul from brain and 200ul from SC for total RNA extraction)
9. Centrifuge 2500rpm for 10min at 4'C
10. Transfer SN to new tubes/falcons, note volumes
11. Add 1/10vol of **NP-40** and 1/10vol of **DOC**, incubate on ice 30min

- Prepare sucroses at this point. Calculate how much of each is needed! Remember to add PMSF before use!

Myelin Flotation

12. Note volume again
13. Add 1.22vol of **2M Suc+** and transfer to 12ml centrifuge tubes. Mix and avoid bubbles
14. Bring to equal volumes with **1.1M Suc+**, can mix a bit, place tubes in UZ buckets
15. Overlay with **0.9M Suc+**, to ~5mm below tube rim, balance with **0.9M Suc+**
16. Centrifuge with UZ at 24,000rpm (100,000g) for 3hr at 4'C

- Prepare 60% sucrose at this point

Gradient Centrifugation

17. Check for pellet. Discard SN carefully with 10ml pipette and withdraw every else with 1ml pipette
18. Resuspend pellet in 400ul **HB++** (+ 1:10 NP40, 1:10 DOC, 1:100 PMSF)
19. Place gradient tubes into UZ buckets, load sample onto gradient
20. Fill to ~4mm below rim with **HB++**, balance with **HB++**
21. Centrifuge with UZ at 40,000rpm (285,000g) for 1.5hr at 4'C

Fractionator Setup

22. Set sensitivity to 0.2, push H2O through detector
23. While detector is filled with H2Opress auto-baseline, adjust offset to ~10
24. Clear detector by pushing air through
25. Brain: run at sensitivity 0.2
    SC: run at sensitivity to 0.05, adjust offset knob to midline

Fraction Collection

26. Run sample in fractionator at 50% 10x speed, start timer
27. Collect 0.5ml fractions to 2ml eppis by hand, mark time for switching tubes
28. RNA: Add 1/10vol of **10% SDS** to fractions collected
    protein: Add 1vol of **20% TCA**

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