Amamoto Lab, 2022

Protocol: FIN-Seq (Frozen Immunolabeled Nuclei Sequencing) v.1.1

***** Nuclei isolation adapted from Krishnaswami et al., *Nature Protocols,* 2016

Isolation (~1 hour)

1. Prepare all solutions and keep on ice with the Dounce homogenizer.
2. Fill the glass Dounce homogenizer with 1 mL of cold homogenization buffer.
3. Mince the tissue into little pieces and place in 1% PFA for 5 minutes on ice.
4. Transfer the tissue pieces into the homogenizer. With the tight pestle, homogenize with 10-15 strokes on ice. Avoid foaming.
5. Transfer the homogenate into a 5 mL polypropylene tube (Thermo Fisher 14-959-11A).
6. Carefully add 2 mL of Sucrose Bed solution to the bottom of the tube so that the homogenate is above the sucrose solution.
7. Spin at 500xg, 12 minutes, 4 degrees.
8. Remove supernatant. Add 1 mL of 4% PFA solution and resuspend pellet. Incubate for 15 minutes at 4 degrees with rocking.
9. Centrifuge at 2000xg for 5 minutes at 4 degrees.
10. Resuspend pellet with Blocking Buffer. Incubate for 15 minutes at 4 degrees with rocking.

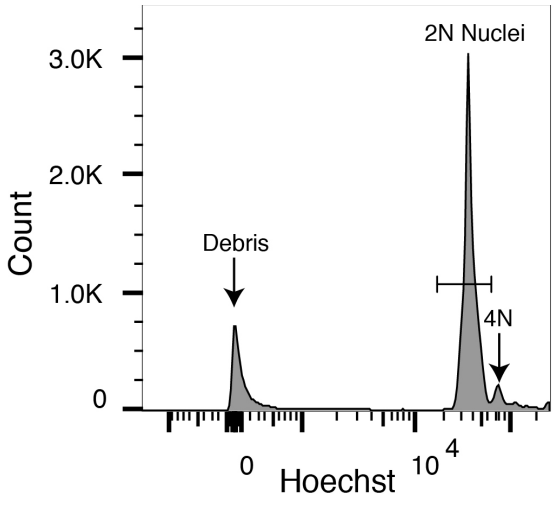
Immunostaining (~2 hours)

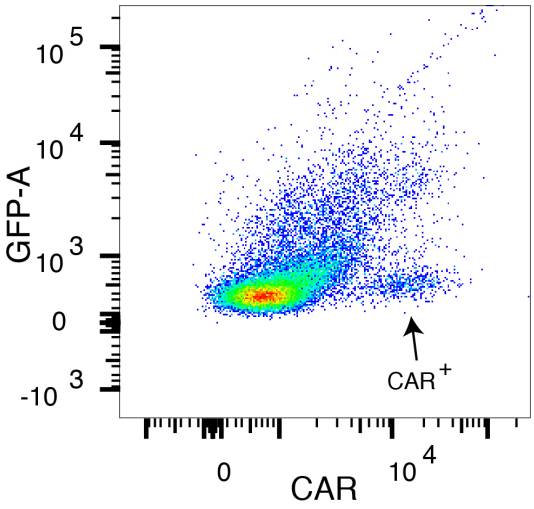
11. Centrifuge at 2000xg for 5 minutes at 4 degrees. Remove supernatant.
12. Resuspend pellet with Primary Antibody in Blocking Buffer. Concentration depends on primary antibody. Generally, higher concentration than immunohistochemistry is necessary. Incubate for 30 minutes at 4 degrees with rocking.
13. Centrifuge at 2000xg for 5 minutes at 4 degrees. Remove supernatant.
14. Resuspend pellet with Blocking Buffer. Incubate for 5 minutes on ice.
15. Centrifuge at 2000xg for 5 minutes at 4 degrees. Remove supernatant.
16. Resuspend pellet with appropriate Secondary Antibody (1:1000) in Blocking Buffer. Incubate at 4 degrees for 30 minutes with rocking.
17. Resuspend pellet with Blocking Buffer. Incubate for 5 minutes on ice.
18. Centrifuge at 2000xg for 5 minutes at 4 degrees. Remove supernatant.
19. Resuspend pellet with Blocking Buffer.
20. Filter and proceed to FACS

FACS (~30 minutes for 200,000 events, corresponding to ~150,000 nuclei)

**It's important to use a secondary antibody only control for the first time when you run the protocol.

21. Gate based on Hoechst histogram, as shown below. This step will ensure that you get 2N nuclei. Make sure not to include 4N nuclei.
22. Gate using the plot with appropriate wavelengths. Often, the separation will not be as obvious as GFP or well characterized cell surface markers. Thus, it's important to have proper controls.
23. FACS isolated nuclei are sorted into Blocking Buffer and kept at 4 degrees.





Decrosslinking and RNA isolation (~4 hours)

24. Spin at 3000xg for 7 minutes at 4 degrees.
25. Remove as much supernatant as possible.
26. From the Recoverall RNA/DNA Isolation Kit (Thermo Fisher Scientific AM1975), mix 100 uL of Digestion Buffer and 4 uL of protease for each sample. Adjust accordingly based on the volume of the leftover supernatant.
27. Incubate at 50 degrees for 3 hours (wrap the lid with parafilm). Note that this step differs from the manufacturer's protocol.
28. The samples can be stored at -80 indefinitely after incubation or proceed to next steps according to the kit protocol.
29. Elute in ~17 uL of UltraPure water.
30. Store RNA at -80 degrees.

** It's possible to run a RNA pico chip on the BioAnalyzer (Agilent), but RIN will not be accurate because rRNA is not enriched in nuclei. Qubit or BioAnalyzer can be used to estimate RNA concentration. If less than 10,000 nuclei, concentration may not be available by these methods.

31. Proceed to the SMART-Seq v.4 protocol for cDNA synthesis and amplification.

** If RNA concentration is too low for Qubit or BioAnalyzer, >16 rounds of amplification may be necessary to generate enough cDNA. HS DNA chip should be run on the BioAnalyzer after the SMART-Seq v4 protocol and after Nextera indexing to ensure proper library construction.

Reagents

BUFFER 1:

1.5M Sucrose	2500 uL
1M KCl	375 uL
1M MgCl2	75 uL
1M Tris Buffer pH 8.0	150 uL
Nuclease-Free Water	11900 uL
Total Volume	15000 uL

BUFFER 1 can be stored at 4 degrees for up to 6 months

Homogenization Buffer:

BUFFER 1	968 uL	
Triton X-100 10%	10 uL	
Protease Inhibitor (50x)	20 uL	**Promega G6521, reconstituted in DMSO
1 mM DTT	1 uL	
Hoechst 33342	1 uL	
Total Volume	1000 uL	

Homogenization Buffer should be made fresh

SUCROSE BUFFER:

1M KCl	2250 uL
1M MgCl2	450 uL
1M Tris Buffer pH 8.0	900 uL
Nuclease Free Water	11400 uL
Total Volume	15000 uL

SUCROSE BUFFER can be stored at 4 degrees for up to 6 months

Sucrose Bed:

SUCROSE BUFFER	2500 uL
24% Sucrose	12500 uL
Total Volume	15000 uL

Sucrose Bed can be stored at 4 degrees for up to 6 months

Blocking Buffer:

RNase-free PBS	10000 uL
BSA	50 mg

Blocking Buffer should be made fresh

****Add 1 uL of RNasin Plus (Promega N2615) for every 1 mL of every solution used. Incubate for at least 10 minutes with the RNasin before use.**