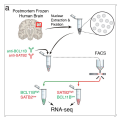

Apr 09, 2019

Working

FIN-Seq (Frozen Immunolabeled Nuclei Sequencing)

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dx.doi.org/10.17504/protocols.io.zxbf7in

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ABSTRACT

Thousands of frozen, archived tissues from postmortem human central nervous system (CNS) are currently available in brain banks. As single cell and single nucleus technologies are beginning to elucidate the cellular diversity present within the human CNS, it is becoming clear that transcriptional analysis of the human CNS requires cell type specificity. Single cell and single nucleus RNA profiling provide one avenue to decipher this heterogeneity. An alternative, complementary approach is to profile isolated, pre-defined cell types and use methods that can be applied to many archived human tissue samples. Here, we developed FIN-Seq (Frozen Immunolabeled Nuclei Sequencing), a method that accomplishes these goals. FIN-Seq uses immunohistochemical isolation of nuclei of specific cell types from frozen human tissue, followed by RNA-Sequencing. We applied this method to frozen postmortem samples of human cerebral cortex and retina and were able to identify transcripts, including low abundance transcripts, in specific cell types.

EXTERNAL LINK

https://www.biorxiv.org/content/10.1101/602847v1

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Amamoto, R., Zuccaro, E., Curry, N.C., Khurana, S., Chen, H.-H., Cepko, C.L., and Arlotta, P. (2019). FIN-Seq: Transcriptional profiling of specific cell types in frozen archived tissue from the human central nervous system. bioRxiv, 602847.

PDF

FIN-Seq version 1.1.pdf

MATERIALS TEXT

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.**

Nuclei Extraction: ~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.

Immunolabeling: ~2 hours

10 Resuspend pellet with Blocking Buffer. Incubate for 15 minutes at 4 degrees with rocking.

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: Time depends on number of nuclei. ~30 minutes for 200,000 nuclei

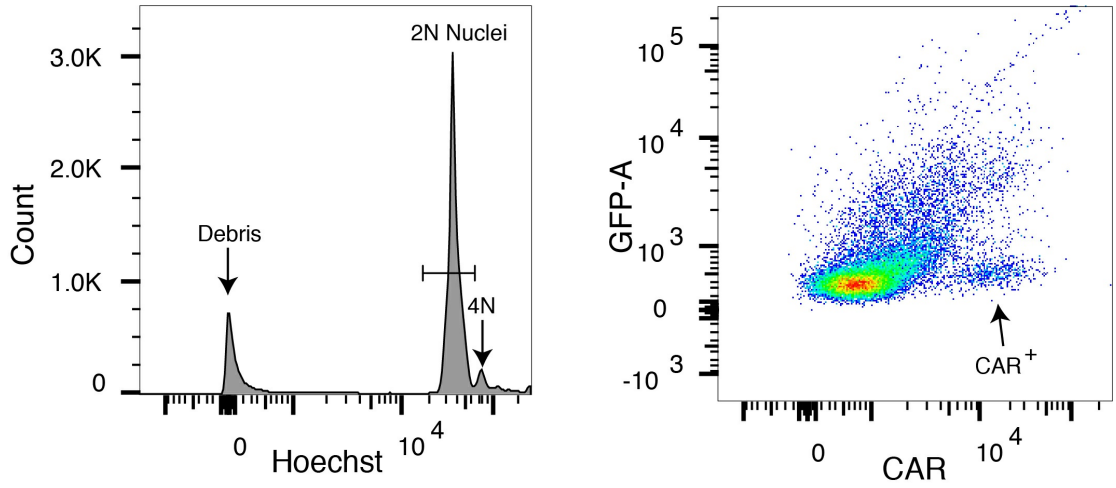
21 Gate based on Hoechst histogram, as shown below. This step will ensure that you get 2N nuclei. Make sure not to include 4N nuclei.

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.

Representative FACS plots

24



The hoechst histogram on the left. Make sure to gate the 2N nuclei. Looking at all of the 2N nuclei, look for separation of immunolabeled population (here, CAR antibody). For the first few times, it'll be important to have a secondary only control. Often, the separation of the immunolabeled population will not be as obvious. It may appear as a small bump.

Decrosslinking and RNA isolation: ~4 hours

25 Spin at 3000xg for 7 minutes at 4 degrees.

26 Remove as much supernatant as possible.

27 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.

28 Incubate at 50 degrees for 3 hours (wrap the lid with parafilm). Note that this step differs from the manufacturer's protocol.

29 The samples can be stored at -80 indefinitely after incubation or proceed to next steps according to the kit protocol.

30 Elute in ~17 uL of UltraPure water.

31 Store RNA at -80 degrees.

32 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.

cDNA synthesis

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

34 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.