Modeling of the cardiac sympathetic nervous system and the contribution of epicardium-derived cells

Ge, Y.

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LOW-INPUT NUCLEUS ISOLATION AND MULTIPLEXING WITH BARCODED ANTIBODIES OF MOUSE SYMPATHETIC GANGLIA FOR SINGLE-NUCLEUS RNA SEQUENCING

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SUMMARY

This protocol describes the detailed low-input sample preparation for single-nucleus sequencing, which includes the dissection of mouse superior cervical and stellate ganglia, cell dissociation, cryo-preservation, nucleus isolation and hashtag barcoding.

ABSTRACT

The cardiac autonomic nervous system is crucial in controlling cardiac function, such as heart rate and cardiac contractility, and is divided into a sympathetic and parasympathetic branch. In healthy persons there is a balance between these two branches to maintain homeostasis. However, cardiac disease states such as myocardial infarction, heart failure and hypertension can induce remodeling of cells involved in cardiac innervation, which in turn is associated with adverse clinical outcome. Although a myriad of data is present on histological structure and function of the cardiac autonomic nervous system, its molecular biological architecture in health and disease is in many aspects still enigmatic. Novel technologies such as single cell RNA sequencing hold promise for genetic characterization of tissues on a single cell level, however the relatively large size of neurons may impede the standardized use of these techniques. Here, we took advantage of advanced droplet-based single-nucleus RNA sequencing (snRNA-seq), a promising method to characterize the biological architecture of cardiac sympathetic neurons in health and in disease. We demonstrate a stepwise approach to perform snRNA-seq of the bilateral superior cervical and stellate ganglia dissected from adult mice. This method enables long-term sample preservation maintaining an adequate RNA quality when samples cannot be fully collected within a short period of time. Nucleus-barcoding with hashtag barcoding antibody-oligos (HTOs) staining enables demultiplexing and the trace-back of distinct ganglionic samples during the afterward single nucleus analysis. The analysis results support successful nuclei capture of neuronal cells, glial cells and endothelial cells of the sympathetic ganglion by snRNA-seq. In summary, we provide a stepwise approach for single nucleus RNA sequencing of sympathetic cardiac ganglia, a method that has the potential for broad application also in studies of innervation of other organs and tissues.

INTRODUCTION

The autonomic nervous system (ANS) is a crucial part of the peripheral nervous system that maintains body homeostasis, including the adaption to environmental conditions and pathology (McCorry, 2007). As such, it is involved in regulation of multiple organ systems throughout the body such as the cardiovascular system, respiratory system, digestive system and endocrine system. The ANS is divided in sympathetic and parasympathetic branches. Spinal branches of the sympathetic nervous system synapse in ganglia of the sympathetic chain, situated bilaterally in a paravertebral position. The bilateral cervical and thoracic ganglia, especially the stellate ganglia, are important components participating in cardiac sympathetic innervation. In disease states, such as cardiac ischemia, neuronal remodeling can occur, resulting in a sympathetic overdrive (Li and Li, 2015), as was demonstrated in histological studies in human and several other animal species (Ajijola et al., 2012; Han et al., 2012; Nguyen et al., 2012; Ajijola et al., 2015). Currently, a detailed biological characterization of cardiac ischemia induced neuronal remodeling in cardiac sympathetic ganglia is lacking, and the fundamental biological characteristics of specialized neuronal cell types or subtypes within the cardiac sympathetic nervous system (SNS) in health and disease are not fully determined yet (Zeisel et al., 2018).

Novel technologies such as single cell RNA sequencing (scRNA-seq), have opened gateways for genetic characterization of small tissues on a single cell level. However, the relatively large size of neurons may impede the optimized use of these single cell techniques in humans. In addition, single cell sequencing requires a high-throughput of cells to recover a sufficient cell number due to a high loss in the sequencing process. This might prove to be challenging when studying small tissues that are hard to capture in one session and require multiple samples to introduce enough single cells for sequencing. Recently developed droplet-based single-nucleus RNA sequencing (snRNA-seq) technology (i.e. the 10x Chromium platform) allows the study of biological differences among single cells. snRNA-seq holds an advantage as compared to scRNA-seq for large cells (> 30 µm), that may not be captured in Gel Bead in Emulsions (GEMs), as well as for cells that are more difficult to dissociate and/or preserve (Bakken et al., 2018; Wu et al., 2019; Gaublomme et al., 2020). Heterogeneity, number of neuronal cells and other cells enriched in the cardiac SNS are important aspects to characterize the ANS in health and disease states. In addition, the organ or region-specific innervation by each sympathetic ganglion contributes to the complexity of the SNS, and cervical, stellate and thoracic ganglia of the sympathetic chain have been shown to innervate different regions of the heart (Zandstra et al., 2021).

Therefore, it is mandatory to perform single cell analysis of ganglionic cells derived from individual ganglia to study their biological architecture. Droplet-based snRNA-seq allows

transcriptome-wide expression profiling for a pool of thousands of cells from multiple samples at once with lower cost compared to plate-based sequencing platforms. This approach enables droplet-based snRNA-seq to be more suitable for cellular phenotype classification and new subpopulation identification of cells within a ganglion. Here, we provide —to our knowledge— for the first time a stepwise approach for identification, isolation and single nucleus RNA sequencing of sympathetic extrinsic cardiac ganglia, a method that has the potential for a broad application in studies of the characterization of ganglia innervating other related organs and tissues in health and disease.

PROTOCOL

Below we provide a protocol for snRNA-seq of murine cervical and cervicothoracic (stellate) ganglia. Female and male C57BL/6J mice (15-weeks old, n=2 for each sex, Charles River) were used in our setup. One additional Wnt/Cre; mT/mG mouse on a C57BL/6JLumc background was used to visualize the ganglia for dissection purposes. All animal experiments were carried out according to the Guide for Care and Use of Laboratory Animals published by NIH and approved by the Animal Ethics Committee of the Leiden University (License number AVD1160020185325, Leiden, the Netherlands).

1. Preparations

All steps are performed in a cell culture flow cabinet.

1.1. Clean the forceps and scissors by immersing the instruments in 70% ethanol for 20 minutes.

1.2. Prepare the ganglion medium containing; Neurobasal Medium (Gibco 21103049) supplemented with B-27plus (1x, Gibco A3582801), L-glutamine (2mM, Thermo Scientific 25030024) and Antibiotic-Antimycotic (1x, Gibco 15240096). Pre-warm the ganglion medium at room temperature.

1.3. Prepare the digestion solution: 0.25% Trypsin-EDTA (1:1; Thermo Fisher Scientific) and 1400U/ml collagenase type 2 dissolved in ganglion medium (Worthington LS004176).

1.4. Prepare material and fresh cold (4°C) buffers for nuclei isolation.

Pre-Separation Filters(30µm)	Miltenyi biotec130-041-407

Cell wash (0.04% BSA)	
DPBS (Ca2+,Mg2+free)	Gibco, 14190-169
Bovine Serum Albumin 10%	Sigma-Aldrich, A1595-50ML

Lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl2, and 0.1%Nonidet™ P40 Substitute, 40U/ml RNase in Nuclease-Free Water)	
Nuclease free water (not DEPC treated)	Invitrogen, AM9937
Trizma® Hydrochloride Solution, 1M, pH 7.4	Sigma-Aldrich, T2194
Sodium Chloride Solution, 5M	Sigma-Aldrich, 59222C
Magnesium Chloride Solution, 1M	Sigma-Aldrich, M1028
Nonidet™ P40 Substitute	Sigma-Aldrich, 74385
Protector RNase Inhibitor,40U/µl	Sigma-Aldrich, 3335399001

Nucleus Wash (1X PBS with 2.0% BSA and 0.2U/µl RNase Inhibitor)	
DPBS (Ca2+,Mg2+free)	Gibco, 14190-169
Bovine Serum Albumin 10%	Sigma-Aldrich, A1595-50ML
Protector RNase Inhibitor,40U/ul	Sigma-Aldrich, 3335399001

1.5. Preparation for nucleus hashtag antibody staining

ST staining buffer (ST-SB) (10 mM Tris-HCl, 146 mM NaCl, 21 mM MgCl2, 1mM CaCl2, 2% BSA, 0.02%Tween-20 in Nuclease-free water)	
Nuclease free water (not DEPC treated)	Invitrogen, AM9937

Trizma® Hydrochloride Solution, 1M, pH 7.4	Sigma-Aldrich, T2194
Sodium Chloride Solution, 5M	Sigma-Aldrich, 59222C
Magnesium Chloride Solution, 1M	Sigma-Aldrich, M1028
Calcium chloride solution, 1M	Sigma-Aldrich, 21115-100ML
Tween-20	Merck Millipore, 822184
Bovine Serum Albumin 10%	Sigma-Aldrich, A1595-50ML

FC receptor blocking solution (Human TruStain FcX, 422302)

Single nucleus hashtag antibodies (Mab414)
TotalSeq™-A0451 anti-Nuclear Pore Complex Proteins Hashtag 1 Antibody
TotalSeq™-A0452 anti-Nuclear Pore Complex Proteins Hashtag 2 Antibody
TotalSeq™-A0453 anti-Nuclear Pore Complex Proteins Hashtag 3 Antibody
TotalSeq™-A0461 anti-Nuclear Pore Complex Proteins Hashtag 11 Antibody
TotalSeq™-A0462 anti-Nuclear Pore Complex Proteins Hashtag 12 Antibody
TotalSeq™-A0463 anti-Nuclear Pore Complex Proteins Hashtag 13 Antibody
TotalSeq™-A0464 anti-Nuclear Pore Complex Proteins Hashtag 14 Antibody
TotalSeq™-A0465 anti-Nuclear Pore Complex Proteins Hashtag 15 Antibody

2. Dissection of adult mouse superior cervical ganglia (SCG) and stellate ganglia (StG)

2.1. Euthanize the mice. In the current study, a total of 4 C57BL6/J mice were euthanized by CO_2 asphyxiation. Note: Alternatively Isoflurane can be used followed by exsanguination when a large amount of blood needs to be collected for other study purposes.

2.2. Fix the mice on a dissection board with pins and douse with 70% ethanol to restrict contamination (shaving is not necessary). Under a stereo microscope; open up the skin of the neck region by making a midline cut with scissors, move the submandibular glands aside and remove the sternomastoid muscle in order to expose the common carotid artery and its bifurcation (Fig. 1A, B). Dissect the right and left carotid artery bifurcation and the tissue attached to it. Transfer each dissected piece of tissue into a separate 3.5 cm petri dish, containing cold PBS, and the SCG can be detected attached to the carotid bifurcation. Clean the SCG further by removing the artery and other attached tissue in the petri dish (Fig. 1E).

2.3. To dissect the StG, make a midline cut in the abdomen, followed by the opening of the diaphragm and the ventral thoracic wall. Remove the heart and lungs to expose the dorsal

thorax. The left and right StG can be detected anterolateral to the musculus colli longus (MCL) at the level of the first rib (**Fig. 1C, D**). Dissect both left and right StG with a forceps and separately transfer them into 3.5 cm petri dishes containing cold PBS (**Fig. 1F**).

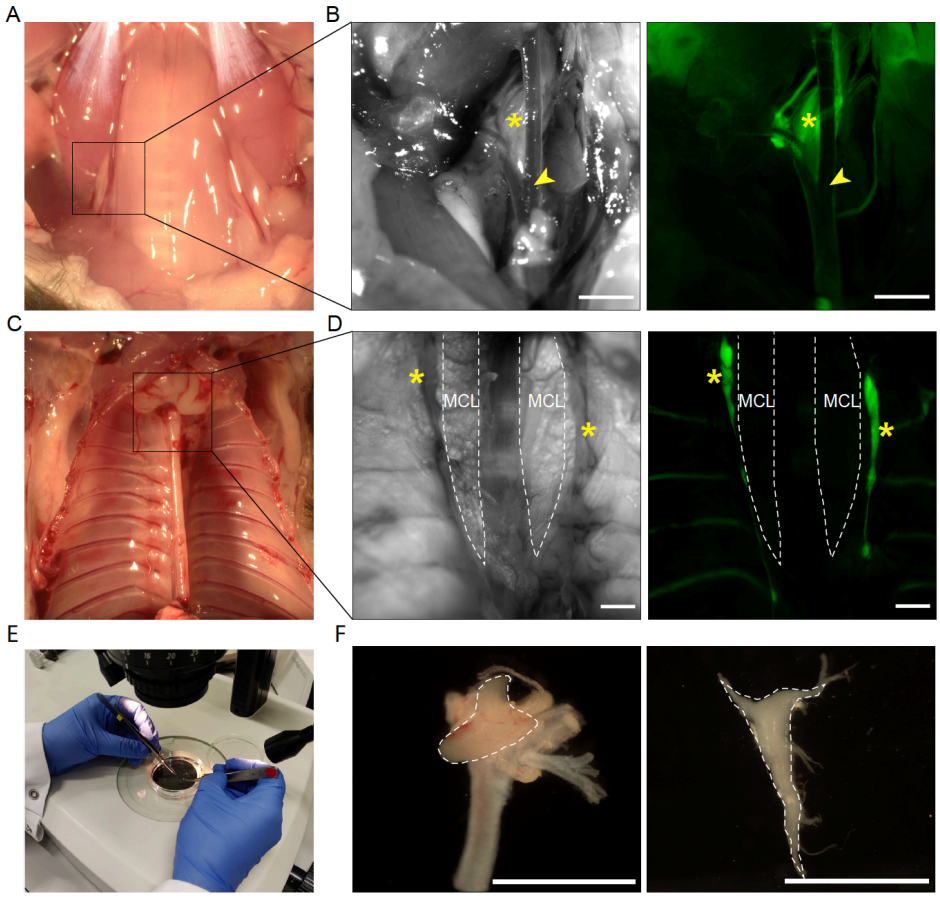

Figure 1. Dissection of adult mouse superior cervical ganglia (SCG) and stellate ganglia (StG). A. Bright field image of the location of the SCG. B. To facilitate visualization, a Wnt/Cre; mT/mG mouse was used. Asterisks indicate the SCG (eGFP+), arrowheads indicate the bifurcation of the carotid artery. C. Bright field image of the location of the StG. D. Asterisks indicate the StG (eGFP+), dashed lines indicate the musculus colli longus (MCL). E. Dissected ganglia are transferred into a petri dish separately for further cleaning under a stereo microscope. F. Left panel, the dissected SCG with the carotid artery still attached. Dashed line indicates the SCG. Right panel, the dissected and cleaned StG has the shape of an inverted triangle as indicated by the dashed line. Scale bar = 1000 µm.

Steps 3-5 are summarized in **Figure 2.**

3. Isolation and cryopreservation of mouse ganglionic cells

3.1. Carefully transfer all SCG and StG into separate 1.5 ml Eppendorf tubes with a forceps. Note: The ganglia are prone to adhere to the wall of plastic pipette tips.

3.2. Add 500 µl of 0.25% trypsin-EDTA solution into each Eppendorf tube and incubate in a shaking water bath at 37°C for 40 minutes. Note: This step is aimed to facilitate the digestion and cell release hereafter in collagenase type 2 solution.

3.3. Prepare a 15 ml Falcon tube containing 5 ml of ganglion medium for each sample.

3.4. Allow the ganglia to settle down at the bottom of the Eppendorf tubes. Collect the supernatant, transfer the supernatant into the prepared Falcon tubes and label each tube. Alternatively, the trypsin-EDTA supernatant can be aspirated without collection as very few dissociated cells can be detected in it. Note: A small amount of trypsin-EDTA solution (around 10-30 µl) can be left in the Eppendorf tube to avoid removal of the ganglion. Avoid pipetting at this step because it may damage the ganglion and lead to low output of ganglionic cells afterwards.

3.5. Add 500 µl collagenase type 2 solution into each Eppendorf tube and incubate in a shaking water bath at 37°C for 35-40 minutes. Note: The incubation time could vary depending on the age of mouse. Try to pipette the ganglion after 35 minutes, if the ganglion is still intact and does not dissociate, prolong the incubation time or increase the concentration of collagenase type 2 solution.

3.6. Pipette the ganglia in collagenase solution up and down for around 10 times or until tissue clumps can no longer be detected. Transfer the cell suspension into the corresponding Falcon tube that contains the ganglia culture medium and the trypsin-EDTA suspension.

3.7. Centrifuge the cell suspension for 10 min, 300 relative centrifugal force (rcf) at room temperature. Carefully discard the cell supernatant. Note: Because the ganglionic cells are dissociated from a single ganglion, the cell pellet may be too small to detect by eye; a small amount of supernatant can be left in the tube to avoid removal of the cell pellet.

3.8. Resuspend the ganglionic cells in 270 µl heat-inactivated fetal bovine serum (FBS, low endotoxin; Biowest) and transfer each cell-FBS suspension into a 1 ml cryovial.

3.9. Count the cells by using a hemocytometer. Mix 5 µl ganglionic cell suspension with 5 µl 0.4% trypan blue dye (Bio-Rad 1450021) and load the mixture into a hemocytometer. Count

the total and live cell numbers under a microscope. The cell viability (live cell count/total cell count = viability %) is usually above 90% with this dissociation protocol. Note: Live cell count of a single ganglion (either SCG or StG) usually falls within the range of 9,000~60,000 cells when the ganglion is isolated from a mouse aged 12 to 16 weeks.

3.10. Add 30 µl Dimethyl sulfoxide (DMSO; Sigma-Aldrich 67-68-5) into each cell-FBS suspension in the cryovials, mix well and transfer the cryovials to a Mr. Frosty (Thermo Fisher) or CoolCell container (Corning). Keep the container at -80°C overnight and transfer the cryovials into liquid nitrogen the next day for long-time preservation before sequencing.

4. Nucleus isolation

Left and right SCG isolated from four mice (in total 8 samples) were used as an example in the following nuclei preparation and sequencing preparation. Note: Keep everything on ice during the whole procedure.

4.1. Prepare 15 ml Falcon tubes with a MACS SmartStraininer (30 µm; Miltenyi Biotec) on top. Pre-rinse the strainer with 1 ml ganglion medium.

4.2. Take out the cryovials from the liquid nitrogen and immediately thaw them in a water bath at 37°C. When a small pellet of ice is left in the cryovial, take the cryovials out of the water bath.

4.3. Recover the ganglionic cells by dropping 1 ml of ganglion medium into each cryovial while shaking carefully. Optional: To evaluate cell recovery, mix the cell suspension after recovery and take 5 µl cell suspension out for live cell counting as describe in step 3.9.

4.4. Load each ganglionic cell suspension on a separate strainer (as prepared in step 4.1) and rinse each strainer with 4 to 5 ml ganglion medium.

4.5. Centrifuge the cell suspension for 5 min at 300 rcf, remove supernatant carefully and resuspend the cells in 50 µl cell wash.

4.6. Transfer the cell suspension into a DNA/RNA LoBind 0.5 ml Eppendorf tube. Centrifuge the cell suspension at 500 rcf for 5 min at 4°C.

4.7. Remove 45 µl supernatant, without touching the bottom of the tube to avoid dislodging the cell pellet, and add 45 µl chilled Lysis Buffer. Gently pipette up and down using a 200ul pipette tip and incubate the cells for 8 min on ice.

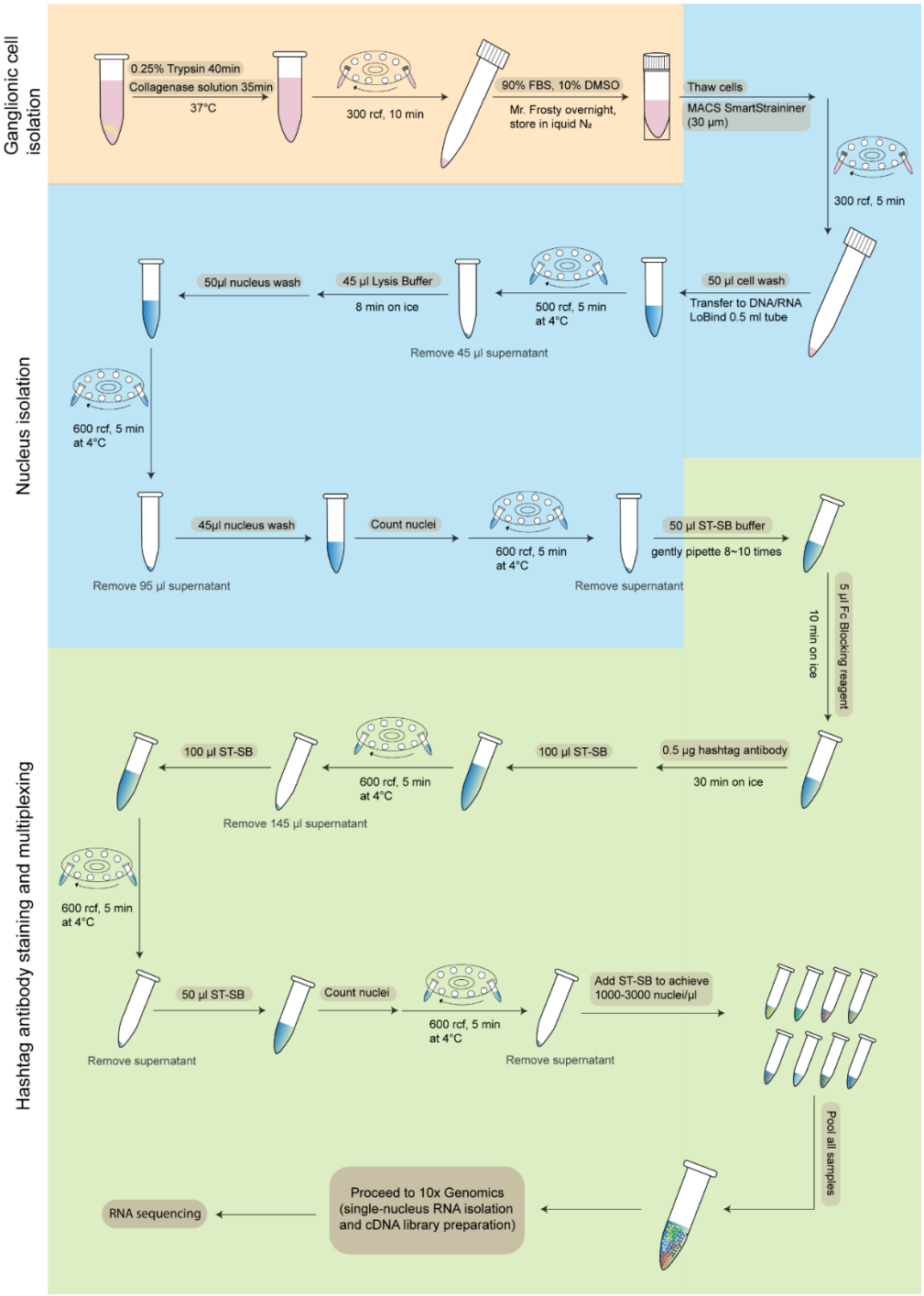
Ganglionic cell isolation
0.25% Trypsin 40min
Collagenase solution 35min
37°C
300 rcf, 10 min
90% FBS, 10% DMSO
Mr. Frosty overnight, store in iquid N₂
Thaw cells
MACS SmartStraininer (30 µm)
300 rcf, 5 min
Nucleus isolation
50 µl cell wash
Transfer to DNA/RNA LoBind 0.5 ml tube
500 rcf, 5 min at 4°C
45 µl Lysis Buffer
8 min on ice
50µl nucleus wash
Remove 45 µl supernatant
600 rcf, 5 min at 4°C
45µl nucleus wash
Remove 95 µl supernatant
Count nuclei
600 rcf, 5 min at 4°C
50 µl ST-SB buffer
gently pipette 8~10 times
Remove supernatant
5 µl Fc Blocking reagent
10 min on ice
Hashtag antibody staining and multiplexing
0.5 µg hashtag antibody
30 min on ice
100 µl ST-SB
600 rcf, 5 min at 4°C
Remove 145 µl supernatant
100 µl ST-SB
600 rcf, 5 min at 4°C
50 µl ST-SB
Remove supernatant
Count nuclei
600 rcf, 5 min at 4°C
Add ST-SB to achieve 1000-3000 nuclei/µl
Remove supernatant
Pool all samples
Proceed to 10x Genomics (single-nucleus RNA isolation and cDNA library preparation)
RNA sequencing

Figure 2. Workflow of sample preparation and hashtag staining-based multiplexing for snRNA-seq. The flowchart depicts the steps from the dissociation of ganglionic cells to hashtag antibody staining and multiplexing that are carried out for snRNA-seq.

4.8. Add 50 µl cold Nuclei wash buffer to each tube. Note: Do not mix.

4.9. Centrifuge the nuclei suspension at 600 rcf for 5 min at 4°C. Remove 95 µl of supernatant without disrupting the nuclei pellet. Then add 45 µl chilled Nuclei wash buffer to the pellet. Note: Because of the invisibility of tiny nucleus pellets, a centrifuge with swinging buckets instead of fixed-angle rotors is recommended to facilitate supernatant removal. Optional: Take 5 µl nuclei suspension, mix with 5 µl 0.4% trypan blue to count and check the quality of nuclei under a microscope with a hemocytometer.

4.10. Centrifuge the nuclei suspension at 600 rcf for 5 min at 4°C. Remove the supernatant without touching the bottom of the tube to avoid dislodging the nuclei pellet.

5. Hashtag barcoding antibody-oligos (HTOs) staining and multiplexing

HTOs staining steps were modified and optimized for nuclei labelling of very low amounts of (ganglionic) nuclei according to previous application in cortical tissue by Gaublomme et al (Gaublomme et al., 2020).

5.1. Add 50 µl ST-SB buffer to the nuclei pellet, gently pipette 8~10 times until nuclei are completely resuspended.

5.2. Add 5 µl Fc Blocking reagent (Biolegend 422302) per 50 µl of ST-SB/nuclei mix and incubate for 10 min on ice.

5.3. Add 1 µl (0.5 µg) of single nucleus hashtag antibody (Biolegend MAb414) per 50 µl of ST-SB/nuclei mix and incubate for 30 min on ice. Note: Shorter incubation time leads to lower efficiency of hashtag labelling as is demonstrated in representative results below.

5.4. Add 100 µl ST-SB to each tube, centrifuge the nuclei suspension for 5 min,600 rcf at 4°C. Note: Do not mix.

5.5. Remove 145 µl supernatant without disrupting the nuclei pellet. Repeat step 5.4.

5.6. Remove the supernatant without touching the bottom of the tube to avoid dislodging the nuclei pellet.

5.7. Resuspend the nuclei pellet in 50 µl ST-SB, and gently mix the nuclei. Then take 5 µl nuclei suspension and mix it with 5 µl 0.4% trypan blue to count the nuclei under a microscope. A representative image of nuclei mixed with trypan blue and loaded in a hemocytometer is shown in Fig. 3A.

5.8. Centrifuge the nuclei suspension for 5 min at 600 rcf at 4°C.

5.9. Resuspend the nuclei in ST-SB to achieve a target nuclei concentration of 1000-3000 nuclei/µl for each sample according to corresponding the nuclei counts.

5.10. Pool samples to achieve the desired number of cells. For example, in this experiment 8 samples were equally pooled to achieve in total 25,000 nuclei to immediately proceed to 10x Genomics Chromium and afterwards snRNA-seq. Note: nucleus count usually falls within the range of 6,000~40,000 cells when the ganglion is isolated from a mouse aged12 to 16 weeks. Note: Only around half of the total loaded nuclei can be captured by 10x Chromium. For example, we prepared a 25,000 nuclei mixture to ensure a capture of 10,000 nuclei by 10x Chromium which is needed for further library preparation and sequencing according to the 10x Genomics standard procedure.

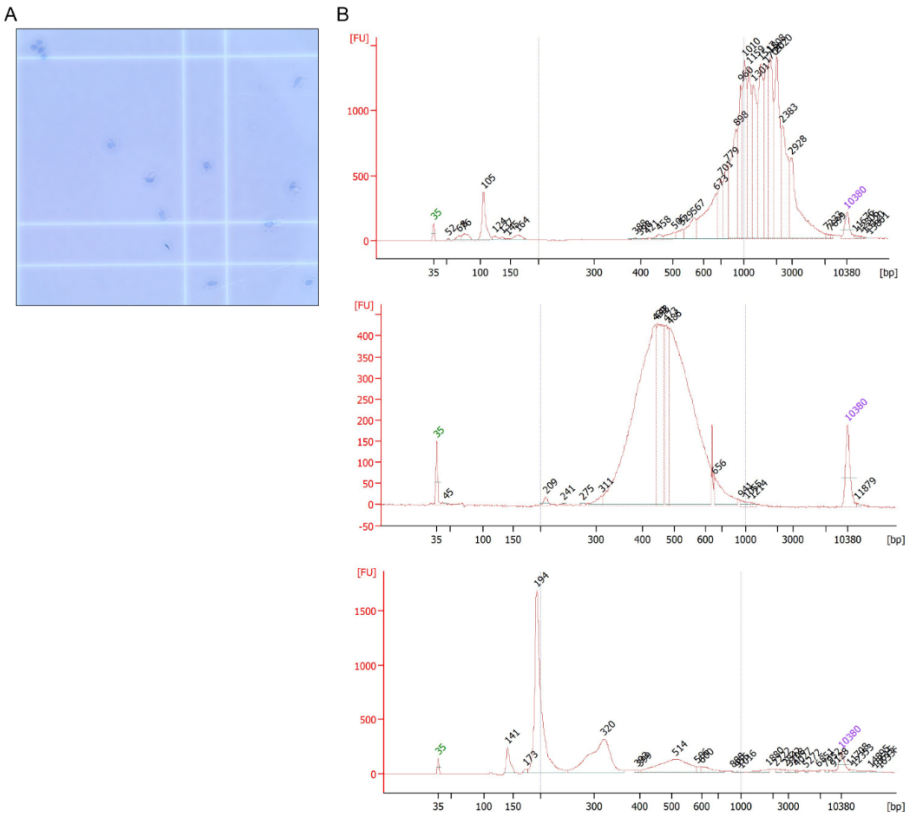

Figure 3. Quality control of nuclei isolation and gene expression library preparation. A. Brightfield image of the HTOs-stained nuclei. B. Bioanalyzer results of 1st strand cDNA (top), gene expression (GEX) library (middle) and hashtag oligo (HTO) library (bottom).

REPRESENTATIVE RESULTS

Quality control analysis of the single-nucleus cDNA library preparation and snRNA-seq.

Fig. 3B illustrates the quality control results of the 1st strand cDNA, gene expression (GEX) library and hashtag oligo (HTO) library which were checked with Bioanalyzer. The HTO-derived cDNAs are expected to be smaller than 180bp (135bp) and mRNA-derived cDNAs are larger than 300bp. A high quality GEX library can be detected as a broad peak from 300 to 1000bp and the HTO library is detected as a specific peak of 194bp. Seurat R package (version 4.0, Satija Lab) (Stuart et al., 2018) was used for pre-processing quality checks and downstream analyses. Demultiplexing of the snRNA-seq data was performed by identifying HTOs using Seurat in-built demultiplexing strategy. Demultiplexing results are visualized as a heatmap in **Fig. 4A**, singlets are recognized as the nuclei with only one highly-expressed HTO, while doublets with more than one highly-expressed HTO and negatives with no HTO expression. Of note, approximately 33% of the nuclei were detected as negatives with our 10-min HTO antibody incubation approach. We therefore further optimized the HTO staining protocol by prolonging the incubation time from 10 min to 30 min in a subsequent experiment, resulting in a great decrease of negatively labeled nuclei (**Fig. 4B**). These findings indicate that prolongation of antibody incubation time may improve hashing efficiency. Violin plots in **Fig. 4C** demonstrate the number of genes (nFeature_RNA), number of unique molecular identifiers (UMI) (nCount_RNA), and the percentage of mitochondrial counts (percent.MT) in our snRNA-seq dataset to identify outliers and low quality nuclei. Subsequently, doublets and negatives were removed based on demultiplexing results and gene expression counts were normalized using the default method in Seurat. 875 (2.71%) counts are detected as highly variable genes (**Fig. 4D**), which might model cellular heterogeneity, sex difference and/or laterality. To validate the accuracy of HTO sample segregation, expression of X Inactive Specific Transcript (Xist, expressed in the inactive female X chromosome) was assessed to identify male samples and female samples (**Fig. 4E**). Xist expression was in accordance with the hashtag labeling that HTO 1-4 labelled samples were female samples and HTO 5-8 labelled samples were male samples. This suggests curated HTO labelling is highly specific. snRNA-seq GEX was scaled and linear dimensional reduction (principle components analysis, PCA) was performed. Assessment of the principal components (PCs) (**Fig. 4F**) revealed true signal being present in the first 12-15 PCs. Cells were then clustered using 15 PCs followed by dimension reduction visualization (UMAP) to visualize our 14 individual clusters (Fig 4G). Neuronal cells, known to highly express Tubb3, were represented by cluster 7 and 9 (**Fig. 4H**). Glial cells, known to be enriched with Fabp7 (Avraham et al., 2020), were represented by cluster 0,1, 3 and 5 (**Fig. 4I**). Endothelial cells were detected in cluster 6 with high expression of Pecam1 (**Fig. 4J**). Our results support successful nuclei capture of neuronal cells, glial cells and endothelial cells of the sympathetic ganglion using snRNA-seq.

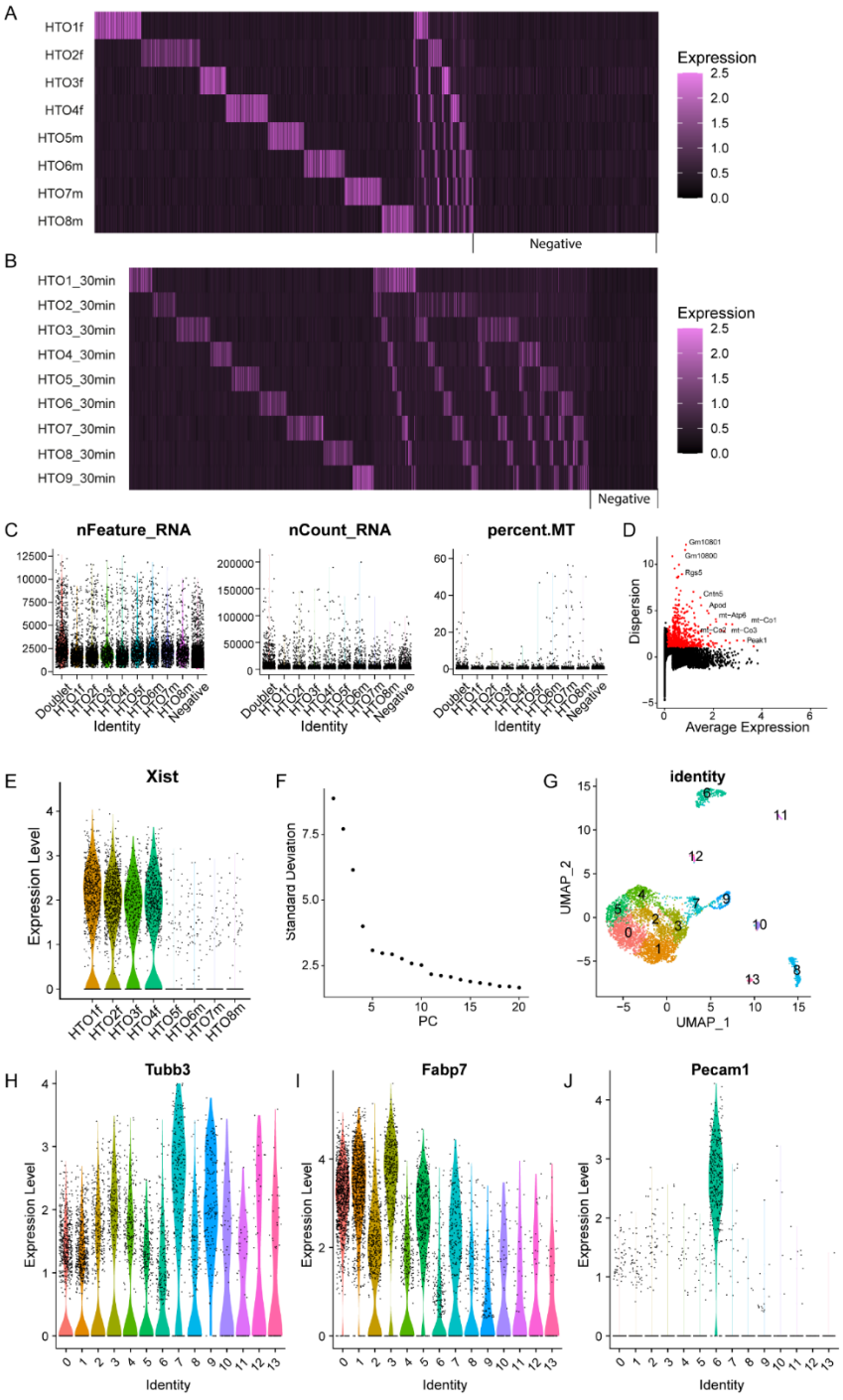
A
HTO1f
HTO2f
HTO3f
HTO4f
HTO5m
HTO6m
HTO7m
HTO8m
Negative
Expression
2.5
2.0
1.5
1.0
0.5
0.0
B
HTO1_30min
HTO2_30min
HTO3_30min
HTO4_30min
HTO5_30min
HTO6_30min
HTO7_30min
HTO8_30min
HTO9_30min
Negative
Expression
C
nFeature_RNA
nCount_RNA
percent.MT
Doublet
Identity
D
Dispersion
Average Expression
E
Xist
Expression Level
F
Standard Deviation
PC
G
identity
UMAP_1
UMAP_2
H
Tubb3
I
Fabp7
J
Pecam1

Figure 4. Quality control and analysis results of single nucleus sequencing. A-B. Heatmap of HTO demultiplexing reveals HTO staining in each nucleus. A. HTO staining achieved by performing 10-minute hashtag antibody incubation. B. HTO staining achieved by 30-min hashtag antibody incubation in a subsequent snRNA-seq experiment. Comparison of the number of negative-labelled nuclei shows a marked improvement of nucleus labeling after prolonging antibody incubation time. C. Violin plots of quality control metrics show the number of genes (nFeature_RNA), the number of UMIs (nCount_RNA), and the percentage of mitochondrial counts (percent.MT). D. Of the total 32,285 genes sequenced, 875 (2.71%) were identified as variable features as visualized in the scatter plot. E. Violin plot displaying female samples (high Xist expression) after demultiplexing using HTO labelled samples. F. Elbow plot of PCs to determine inclusion of true signal used for clustering. G. UMAP plot of the clustered snRNA-seq dataset. H-J. Violin plots of known cell type markers, such as Neuronal cells (Tubb3), glial cells (Fabp7) and endothelial cells (Pecam1) for cell type identification.

DISCUSSION

Here we describe a detailed protocol of the dissection of adult mouse superior cervical and stellate sympathetic ganglia, isolation and cryopreservation of the ganglionic cells, nuclei isolation and nucleus-barcoding with HTOs staining for multiplexing and snRNA-seq.

With this protocol, sympathetic ganglionic cells can easily be obtained by dissociating individual ganglion with commonly used trypsin and collagenase. Long-term preservation of isolated ganglionic cells is also readily achieved by freezing cells in FBS supplemented with 10% DMSO, which showed a high quality of recovery after thawing. Moreover, compared to conventional single-cell RNA sequencing, the use of droplet-based snRNA-seq of single murine sympathetic ganglia combined with the application of HTOs staining-based nucleus-barcoding has the following advantages: i) samples can be preserved for a long time until all samples are ready for further nuclei isolation; ii) nuclei of good quality isolated from multiple small-size ganglia can be pooled together for sequencing without a batch effect caused by sample preparation; iii) the ability to trace back the distinct ganglionic origin after sequencing by hashing; and iv) Cost-effectiveness, since only one library preparation is needed. Importantly, the described isolation and cell culture protocol provides a single uniform method for both murine cervical and stellate ganglia, and is potentially applicable to other ganglia, such as dorsal root ganglia, and other species such as human ganglia.

The first scRNA-seq study was published in 2009 (Tang et al., 2009), and the benefit of scRNA-seq in comparison with the transcriptomes of individual cells within a sample/biopsy highly promoted its applications in the field of cell biology. One of the major advantages of scRNA-seq is the ability to identify (novel) cell types and to reveal rare cell populations that could not be detected by bulk RNA-seq. The droplet-based scRNA-seq platform, i.e. the 10x Chromium platform used in this protocol, facilitates the capture of more cells and thus can provide an

aggregate view of the cell (sub)types and transcriptional heterogeneity of a large cell population as compared to a plate-base sequencing platform. However, the 10x Chromium platform is not suitable for cells larger than 50 µm, which limits its application in large cells such as human neurons (around 100 µm). The availability of the snRNA-seq technique overcomes this drawback, because of the small size of a nucleus. Moreover snRNA-seq is known as a useful method for gene-expression studies of highly interconnected and low recovered cells such as neurons as well as frozen tissues. snRNA-seq allows isolation of nuclei from tissues without prior cell dissociation, although we prefer to take a two-step nuclei isolation method that first dissociates the ganglion into single cells (which can be preserved in liquid nitrogen) followed by isolation of the single nuclei. Because of the tiny size of a mouse sympathetic ganglion, we found more nuclei yielded by a two-step nuclei isolation method as compared to direct one-step nuclei isolation from a mouse ganglion. The quality check of the cDNA, library and brief sequencing analysis supported the good nuclei/RNA quality. In addition, it makes the procedure logistically easier, since samples can be collected and stored before sending them collectively away for sequencing. Single nucleus analysis also revealed successful recovery and capture of neurons and glial cells that are of interest in our current projects. As the ganglion size is much larger in human and human neurons are difficult to dissociate and recover in our experience, one-step nuclei isolation instead of two-step nuclei isolation might be better for application of snRNA-sequencing in human sympathetic ganglia.

Another advantage of this protocol is the multiplexing with barcoded antibodies (Stoeckius et al., 2018). The mouse sympathetic ganglion is a tiny tissue (average size 0.1 mm^3), and the low amount of cells derived from an individual ganglion is insufficient for droplet-based sequencing, whereas pooling of several ganglia of different mice or different ganglia of a same mouse will cause the loss of either individual mouse information or individual ganglion information. As a solution, the HTOs staining step is easy to perform and enables the barcoded labelling of nuclei derived from different mice or different ganglia before nuclei pooling. The accuracy of HTOs demultiplexing is verified in this protocol by matched Xist expression in known female nuclei populations. Nuclei multiplexing with barcoded antibodies therefore reduces batch effects and lowers the sequencing cost.

A potential limitation of snRNA-seq might be that there may be differences between the RNA composition of the nucleus and cytoplasm due to the natural presence of nascent transcripts in nuclei, associated with early response to neuronal activities (Lacar et al., 2016; Lake et al., 2017). The nucleus and cytoplasm may also differ in transcripts depending on the state within the cell cycle (Grindberg et al., 2013). Less transcripts were detected in individual nuclei (~7000 genes) than in cells (~11000 genes) (Bakken et al., 2018). Therefore, scRNA-seq and snRNA-seq may yield different results at a transcript level. Nevertheless, the comparison

between scRNA-seq and snRNA-seq demonstrated similar capability to discriminate neuronal cell types of brain tissue (Bakken et al., 2018). To improve the discrimination between highly similar cell types or subtypes by snRNA-seq, more nuclei might be needed to compensate for the lower gene detection ability compared to scRNA-seq. Another limitation of snRNA-seq is the inability to select specific cellular populations due to the lack of an intact cell membrane, that is required for fluorescent activated cell sorting (FACS). Even though the accuracy of HTOs demultiplexing is sufficient, loss of some data is inevitable, as not all nuclei show an expression of a single HTO. Optimization of the staining time could minimize the amount of nuclei with double or negative expression of HTOs.

Taken together, here we provide — to our knowledge — for the first time a protocol for sequencing neuronal nuclei from sympathetic ganglia by means of an easy-to-follow workflow starting from ganglion isolation to nuclei preparation of low input of cells, followed by HTOs staining-based nucleus labelling for snRNA-seq. The protocol provides a detailed overview of all key steps that can be easily performed and can be applied to a diversity of ganglia in murine as well as in other species.

DISCLOSURES

The authors have nothing to disclose.

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