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Processing Human Colon muscle layers for Single Nuclei RNA-seq using PHOX2B antibody immunoselection

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1 Works for me dx.doi.org/10.17504/protocols.io.w3afgie

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ABSTRACT

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INTRODUCTION: The human enteric nervous system (ENS) is a complex network of neurons and glia that extends throughout the length of the bowel. There are many neuron and glia types, but very little is known about the human ENS compared to other species. There are two main ENS layers. The myenteric plexus neurons are clustered into ganglia with thick nerve fiber bundles running between ganglia. Submucosal neuron ganglia are scattered throughout the region between circular muscle and the epithelial lining of the bowel. One key problem with studying human ENS is that these cells are a very minor component of the bowel wall (< 1: 10,000 cells) and they are surrounded by other tissue. The bowel wall is not easily dissociated making it challenging to isolate cells of the human ENS for any type of analsysis.

OBJECTIVES: To enrich for neurons from human colon myenteric plexus using PHOX2B antibody and FACS

METHODS: 1) Human colon muscle was dissected from the bowel wall and then frozen in OCT. 2) Frozen muscle was sectioned on a cryostat to generate small fragments and disrupt muscle and connective tissue. 3) RNA quality was assessed from a few sections using an Agilent Bioanalyzer. 4) Nuclei were obtained from frozen sections using Dounce homogenization and stained with antibody to PHOX2B. 5). Nuclei were separated from other cell debris by FACS. 6) RNA-seq data were obtained using the 10X Genomic single cell sequencing platform.

RESULTS: This procedure worked well to isolate nuclei from human colon muscle. PHOX2B antibody labeling led to a small population of nuclei that appeared to be antibody stained under the microscope and have attached antibody by FACS. Unfortunately, single nucleus RNA-seq demonstrated that we had not enriched for myenteric neurons with this approach. RNA-seq data were obtained from many other cell types within the muscle layers of the bowel wall.

CONCLUSION: This approach generated valuable data at a single nucleus RNA-seq level for many cell types within the muscle wall known to control bowel motility, but did not enrich for neuronal nuclei, even though PHOX2B antibody selectively stains myenteric plexus nuclei within the human bowel myenteric plexus

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GUIDELINES

We use the following workflow:
1. Collecting human tissue: The muscularis layer of human colon is collected and frozen in O.C.T. at -80° C until further processing.
2. Sectioning human tissue: The muscularis layer of human colon is sectioned on a cryostat to disrupt muscle fibers.
3. Extracting RNA for Bioanalyzer Submission: RNA is extracted from a few sections of tissue and analyzed on the Agilent Bioanalyzer to assess RNA quality.
4. Extracting Nuclei for Sequencing: Nuclei are homogenized and immunostained with PHOX2B antibody. Isolated nuclei are separated by FACS then transferred to a core facility for RNA-seq analysis via the 10x Genomics platform.

MATERIALS TEXT

MATERIALS

RNAse-free Water **Contributed by users**

Magnesium Chloride **Fisher Scientific Catalog #AC223210010**

MilliQ Water **Contributed by users**

Lab Timer **Contributed by users**

Normal Donkey Serum **Jackson Immunoresearch Catalog #017-000-121**

Sodium Chloride **Sigma Aldrich Catalog #S6191-1kg**

RNaseZAP **Sigma Aldrich Catalog #R2020-250mL**

Dumont # 2 Forceps (one pair) **Fine Science Tools Catalog #11223-20**

Fine Scissors **Fine Science Tools Catalog #14060-09**

Tissue-Tek Cryomold (Supplier # 4565) **VWR Scientific Catalog #25608-922**

2-Methylbutane **Fisher Scientific Catalog #O3551-4**

Corning® Dulbeccos Phosphate-Buffered Saline 10X without calcium and magnesium (Sterile) **Corning Catalog #21-031-CV**

Fisherbrand™ Petri Dishes with Clear Lid **Fisher Scientific Catalog #FB0875712**

50 mL conical tubes **VWR Scientific Catalog #352098**

Tissue-Plus™ O.C.T Compound (Medium embedding) **Fisher Scientific Catalog #23-730-571**

Bleach **Contributed by users**

10 mL sterile serological pipette **Fisher Scientific Catalog #13-678-11E**

Personal Protective Equipment (PPE) = gloves lab coat face mask face shield **Contributed by users**

DNA LoBind Tubes 1.5 mL **VWR Scientific Catalog #22431021**

Blades Microtome MX35 Premier **VWR Scientific Catalog #3051835**

RNeasy Plus Micro Kit **Qiagen Catalog #74034**

Ambion® Buffer Kit **Thermo Fisher Scientific Catalog #AM9010**

Protector RNase Inhibitor (40 U/ul) 2000 units **Sigma Aldrich Catalog #3335399001**

Dounce Homogenizer 1 mL **VWR Scientific Catalog #357538**

UltraPure 1 M Tris-HCl Buffer pH 7.5 **Thermo Fisher Scientific Catalog #15567-027**

RNase free 10x PBS **Thermo Fisher Scientific Catalog #AM9625**

40 micron FlowMi Cell Strainer **VWR Scientific Catalog #H13680-0040**

MACS SmartStrainers (30 micron) **Miltenyi Biotec Catalog #130-098-458**

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Falcon round-bottom tubes disposable polystyrene **Corning Catalog #352235**

Large iridectomy scissors (Wescott Spring Scissors - Slightly Curved Up) **Fine Science Tools Catalog #15015-11**

6-well plate not tissue culture treated **VWR Scientific Catalog #10861-554**

Hoechst 33342 Trihydrochloride Trihydrate – 10 mg/mL in water **Thermo Fisher Scientific Catalog #H3570**

Nonidet P40 Substitute **Sigma Aldrich Catalog #74385**

3 mL syringe **Fisher Scientific Catalog #14-823-435**

0.22 µm filter (Millex-GV Syringe Filter Unit 0.22 µm) **Emd Millipore Catalog #SLGV033RS**

UltraPure 50 mg/mL BSA (bovine serum albumin) **Thermo Fisher Scientific Catalog #AM2618**

Human/Mouse PHOX2B antibody **R&D Systems Catalog #AF4940**

Goat IgG Isotype Control [Unconjugated] **Novus Biologicals Catalog #AB-108-C**

Alexa Fluor Donkey anti-goat 647 secondary antibody **Abcam Catalog #Ab150131**

Rubber Bands **Contributed by users**

You will need these items for this protocol	
Dry ice and container	
Wet ice (frozen water)	
Fume hood	
-80 C freezer	
Cryostat for frozen sectioning	
Pipetters	P1000, P200, P20, P10
Filter Pipette Tips	For example, SHARP Precision Barrier Tips, Denville Scientific, P1126, P1122, P1096-FR, P1121
Pipette Aid	
Large individually wrapped pipet tips	5 mL, 10 mL, 25 mL
Centrifuge for 1.5 mL Eppendorf tubes	
Hemocytometer	
Epifluorescent microscope	
Rotator (in 4 C cold room)	We use a Glas-Col Rotator

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SAFETY WARNINGS

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When working with human tissue remember the potential for transmission of blood born infections (e.g., HIV, Hepatitis B, Hepatitis C). Wear appropriate personal protective equipment (gloves, lab coat, face mask). Full Hepatitis B vaccination is recommended.

Be very careful with sharp objects to avoid personal injury. Clean instruments using brushes instead of fingers. Do not put fingers anywhere near the blade on the Cryostat. Inexperienced users often cut their fingers when they try to reach over the Cryostat blade.

BEFORE STARTING

Please review the protocol carefully before beginning. There are many reagents to prepare in advance.

Human colon processing to collect and freeze muscle layer in O.C.T

1 **Half hour before the tissue arrives:**

Spray off forceps and scissors with RNase Away, and thoroughly rinse/soak in water

Fetch dry ice.

In the fume hood, add methylbutane to a petri dish on the dry ice. Place a few pieces of dry ice into the methylbutane to chill it.

2 Transfer tissue, on ice, from the hospital's Pathology unit to your laboratory in sterile ice cold 1x PBS in a 50 mL conical or similar container. (~30 minutes)

3 Upon arrival to lab, don appropriate **personal protective equipment (PPE) = (gloves, lab coat, face mask, face shield)**.

4 Pour cold 1x Dulbeccos Phosphate-Buffered Saline and the tissue into a new sterile dish.

5 Quickly trim off surrounding fat using Dumont #2 forceps and a fine scissors.

6 Then, using the fine scissors and Dumont #2 forceps, carefully cut submucosa and epithelium off tissue, leaving only muscularis.

7 Cut the muscularis into small pieces (~5 x 5 mm).

8 Label the appropriate number of cryomolds with sample number and "muscularis+plexus."

9 Transfer the smaller tissue muscularis tissue pieces into Cryomold Biopsy specimen molds half-filled with RNase Free Tissue-Plus O.C.T. Compound. Add O.C.T. to cover the tissue.

10 Place the cryomold containing the cold O.C.T.+plexus in the 2-Methylbutane in the fume hood.

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11 Wait 5 minutes for it to freeze. Then transfer to a -80° C freezer in appropriate containment (e.g. plastic bags in labeled boxes).

12 Record human subject information linked to colon specimen.

13 Autoclave tools for future use.

Sectioning frozen human colon muscle layers on cryostat

14 Fill an ice bucket with dry ice.

15 RNaseZAP a Dumont #2 Forceps. Rinse with deionized MilliQ water.

16 Transfer tissue labeled “Plexus” in biopsy cryomold on dry ice to Cryostat set to -20 °C.

17 Freeze the tissue onto a metal cryostat mounting device using a minimum of O.C.T. compound.

18 While waiting for tissue to freeze, label 3 DNA LoBind Tubes: “ Muscularis+Plexus”, “ Muscularis+Plexus”, and “ Muscularis+Plexus RNA”. Place these on dry ice or in the 20 °C for at least 3 minutes before sectioning so they have time to cool.

19 Place Dumont #2 Forceps in cryostat to chill.

20 Mount the tissue, insert the blade, and begin making 100 micron sections.

21 Using the Dumont #2 Forceps, collect all sections in “ Muscularis+Plexus” tubes EXCEPT 2-5 sections. Put 2-5 random sections in tube labeled “ Muscularis+Plexus RNA”.

22 Return the tubes with tissue to the -80°C on dry ice.

Extracting RNA for BioAnalyzer Analysis

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23 **Follow the instructions on the Qiagen RNeasy Micro Kit for this section**

24 Remove tubes labeled “RNA” from the -80. There should be one each for plexus and for surrounding muscle.

25 Add 350 uL of buffer RLT plus to each tube.

26 Homogenize the lysate by vortexing for 30s.

27 Run through gDNA spin column. Centrifuge for 30s at >=8000xg – I did 10,000g this time. Last time 8,000.

28 Discard the column and keep the flow-through.

29 Add one volume (usually 350 uL of 70% ethanol to the flow-through and mix well by pipetting up and down 4 times. Do not centrifuge, and proceed immediately to next step.

30 Transfer sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 mL collection tube (find RNeasy columns in 4 degrees C freezer B). Close lid and centrifuge for 15s at > 8,000 g. Discard the flow thru

31 Add 700 uL of buffer RW1 to the RNEasy MinElute spin column. Clsoe the lid and centrifuge for 15s at 8,000 g (10,000 RCF). Discard the flow-through.

32 Add 500 uL Buffer RPE to the RNeasy MinElute spin column. Close lid gently, and centrifuge for 15s at >8000 g to wash the spin column membrane.

33 Add 500 uL of 80% ethanol to the RNEasy MinElute column. Close the lid and centrifuge for 2 minutes at 8,000 g (10,000 RCF) to wash the spin column membrane. Discard the collection tube with the flow-through.

34 Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 minutes to dry the membrane. Discard the collection tube with the flow-through.

35 Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14 uL of RNase free water directly to the center of the spin column membrane. Close the lid gently and centrifuge for 1 minute at full speed to elute the RNA.

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36 Analyze RNA on the Agilent RNA 2100 Bioanalyzer with the Agilent RNA 6000 Pico Kit, along with any additional samples.

Preparing to isolate nuclei for RNA-Seq

37 Prepare Hoechst, 2.5 mg/mL:

-Dilute 10 mg/mL Hoechst 1:4 in nuclease-free water.
-Using 3 mL syringe and 0.22 micron filter, filter the Hoechst.
-Store at 4 degrees C.

38 Prepare Normal Donkey Serum for blocking:

- Normal Donkey Serum in 10 mL of sterile 1x PBS.
-Filter the Normal Donkey Serum using a 3 mL syringe and a 0.22 micron filter.

39 Prepare Diluted Nonidet P40 substitute:

-Add 1 mL of RNase free Nonidet P40 substitute to 9 mL of RNase free water.
-Place on rotator for half an hour to dissolve Nonidet P40 substitute.

40 Autoclaving:

-Rinse large iridectomy scissors and Dumont #2 forceps with RNaseZAP followed by MilliQ water.
-Place in small autoclave bag.
-Autoclave before use

41 Reconstitute PHOX2B antibody:

-Reconstitute antibody as per the manufacturer's instructions.
-500 µL of sterile, RNase free PBS was added to 100 µg of antibody, to make a final concentration of 0.2 mg/mL.
-Aliquot the antibody into 50 µL aliquots and store at -20 °C until use.

42 Prepare **Lysis buffer:**

Chemical	Starting Concentration	Final Concentration	Amount to add
Tris-HCL, pH 7.5	1 M	10 mM	400 uL
Sodium Chloride (NaCl)	5 M	10 mM	80 uL
Magnesium Chloride	1 M	3 mM	120 uL
Nonidet P40 Substitute	10 %	0.005%	20 uL
RNase free water			38.38 mL
Total volume			40 mL

43 **Prepare Staining buffer:**

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Chemical	Starting Concentration	Final Concentration	Amount to add
RNase free 10X PBS, pH 7.4	10X stock solution	1X	990 uL
Ultrapure 50 mg/mL BSA	50 mg/mL	1% w/v	1980 uL
Protector RNase inhibitor	0.2 U/uL	0.001 U/uL	49.5 uL
Nuclease free water			6880.5 uL
Total volume			9900 uL

44 Schedule FACS sort:

Schedule FACS sort on MoFlo Astrios or an equivalent sorter with a 70 micron nozzle and the ability to detect DAPI/Hoescht.

45 Schedule 10x Genomics Sequencing run:

Schedule 10x Genomics Sequencing run in advance since samples are processed the day that nuclei are isolated.

Dounce Homogenization to isolate single nuclei

46 Get a large ice bucket.

47 Prepare the appropriate quantities of **Lysis buffer and Staining buffer (Steps 42 and 43)**. Place them on ice.

48 Pre-cool the Dounce homogenizer and pestles on ice.

49 Rinse large iridectomy scissors and Dumont #2 forceps with RNase Zap.

Rinse with MilliQ water.

Pre-cool large iridectomy scissors and Dumont #2 forceps on ice in an open 50 mL conical.

50 Pre-cool large and small centrifuges to 4 C.

51 Place a 6-well plate on ice. Fill wells with 6 mL of Lysis buffer.

52 Get dry ice. Transfer sectioned human tissue to the bench on dry ice.

Approximately 1 hour before FACS sort, put on appropriate PPE (gloves, lab coat, face mask, face shield).

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53

54 Use Dumont #2 forceps to transfer one Eppendorf tube with sectioned human tissue into 6 mL of Lysis buffer on 6 well plate.

55 Chop rapidly with large iridectomy scissors for 1 minute.

56 Using a 1000 uL pipet, transfer 2 mL of Lysis buffer + tissue/O.C.T. into a pre-cooled Dounce homogenizer.

57 Homogenize tissue with 20 strokes of the loose pestle and 50 strokes of the tight pestle.

Note: homogenization often induces suction and the formation of foam. Cover homogenizer with a paper towel and wear appropriate lab coat and PPE during this step to avoid getting splashed. Suction is particularly likely to occur for these tissue pieces because there are so many muscle fibers.

58 Put the tissue through a MACS SmartStrainer (30 micron) resting atop a 50 mL conical.

59 Chase with 2 mL of Lysis buffer to rinse the strainer. Switch out strainers if they appear to be getting clogged, between tissue samples, or after 3 uses.

60 Repeat steps 54-59 until all Eppendorf tubes with tissue from the patient have been homogenized.

Homogenized nuclei from the same patient may be combined into the same 50 mL conical.

61 If nuclei from a second human subject are being run that day, repeat steps 51-60.

Note: Since homogenization and FACS sorting takes a long time, we never ran nuclei from more than 2 human subjects in a single day to maximize RNA quality.

62 Spin down Lysis buffer+nuclei+O.C.T. on large centrifuge at 590 RCG for 8 minutes at 4 degrees C. Be sure to appropriately balance samples.

63 After the centrifugation is over, use a Pipet-Aid to remove liquid from each 50 mL conical.

Get the last bit of liquid out using a P1000.

Note: you probably will not see a pellet at this stage. Be very, very careful not to pipet out the nuclei at the bottom of the 50 mL conical. I typically tilt the 50 mL conical to the side and leave a small quantity of liquid at the bottom.

64 Resuspend nuclei from each patient in 990 mL staining buffer by pipetting 5-10 times with a P1000.

Antibody staining nuclei prior to FACS

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65 Add 100 µL of Normal Donkey Serum for blocking.

66 Add 1 µL of 2.5 mg/mL Hoechst.

67 Leave on ice for 15 minutes to block.

68 For each human tissue sample, label three Eppendorf tubes:

a. Isotype control tube
b. Phox2b tube #1
c. Phox2b tube #2

69 Transfer 330 µL of nuclei in blocking solution into each tube.

70 Add 3.3 µL (3.3 µg) of goat IgG to the isotype control tube.

71 Add 16.5 µL of PHOX2B antibody (3.3 µg) to Phox2b tubes #1 and #2.

72 Attach Eppendorf tubes to Glas-Col Rotator with rubber bands. Rotate samples at speed 30 in the cold room on a rotator for 45 minutes.

73 Add 1 mL of 1x PBS (rinse buffer) to each tube.

74 Invert the tubes ~5 times to wash.

75 Spin in an Eppendorf centrifuge for 5 minutes at 400 RCF at 4° C.

76 While spinning, prepare 1,300 µL of 1:400 Alexa Fluor 647 Donkey anti-goat secondary antibody in staining buffer.

77 When spin is over, pipette out supernatant without disturbing the pellet.

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78 Resuspend the pellet in 400 µL of secondary antibody in staining buffer.

Note: pipette sample 5-10 times until any residual clumps are gone.

79 Rotate samples at speed 30 in the cold room rotator for 30 minutes.

a. While waiting, label 1.5 mL Eppendorf tubes, 1 tube per patient. Add 5 uL staining buffer to the bottom of each tube. These are the collection tubes.
b. Also label Falcon round-bottom tubes, one tube per patient. These tubes will hold the sample to be sorted.
c. Place labeled 1.5 mL Eppendorf tubes and Falcon round-bottom tubes on ice to prepare for transport to FACS sorter.

80 When 30 minutes is up, add 1 mL of 1x PBS (rinse buffer) to each tube with nuclei+secondary.

81 Invert the tubes ~5 times to wash.

82 Spin in an Eppendorf centrifuge for 5 minutes at 400 RCF at 4° C.

83 Resuspend samples in 1 mL of staining buffer.

Note: pipette sample 5-10 times until any residual clumps are gone. This is especially important to prevent clogging of the FlowMi strainer.

84 Pipette the liquid into a P1000 pipette tip and attach the FlowMi strainer to the base. Strain through the FlowMi strainer into a spare Eppendorf tube.

Note: if the FlowMi strainer clogs and liquid is not moving, do not panic! Slowly raise the plunger on your P1000 until it is fully raised. While holding the P1000 pipette tip, carefully remove the pipette tip from the P1000 pipettor. Then try to remove the FlowMi strainer from the base of the pipette tip with the lid of your Eppendorf while still holding the pipette tip securely. If you manage to do this successfully, pipette the liquid in the P1000 5 more times to thoroughly disrupt clumps, and try again with a new FlowMi strainer.

85 Pipette the liquid into a new P1000 pipette tip. Strain through the top of the Falcon round-bottom tube by placing pipette tip flush against the strainer lid.

86 Bring the samples and collection tubes to FACS on ice.

FACS to isolate nuclei for RNA-seq

87 Run isotype control sample first to determine appropriate gating.

88 Sort Hoescht+, PHOX2B+ nuclei (with higher signal than isotype control) into the collection Eppendorf tubes with 5 uL staining buffer using 70 micron nozzle (this can sort up to 1,000 nuclei per microliter).

Below is a sample image from a FACS sort. R7 were the sorted nuclei. R9 were determined to be PHOX2B negative by

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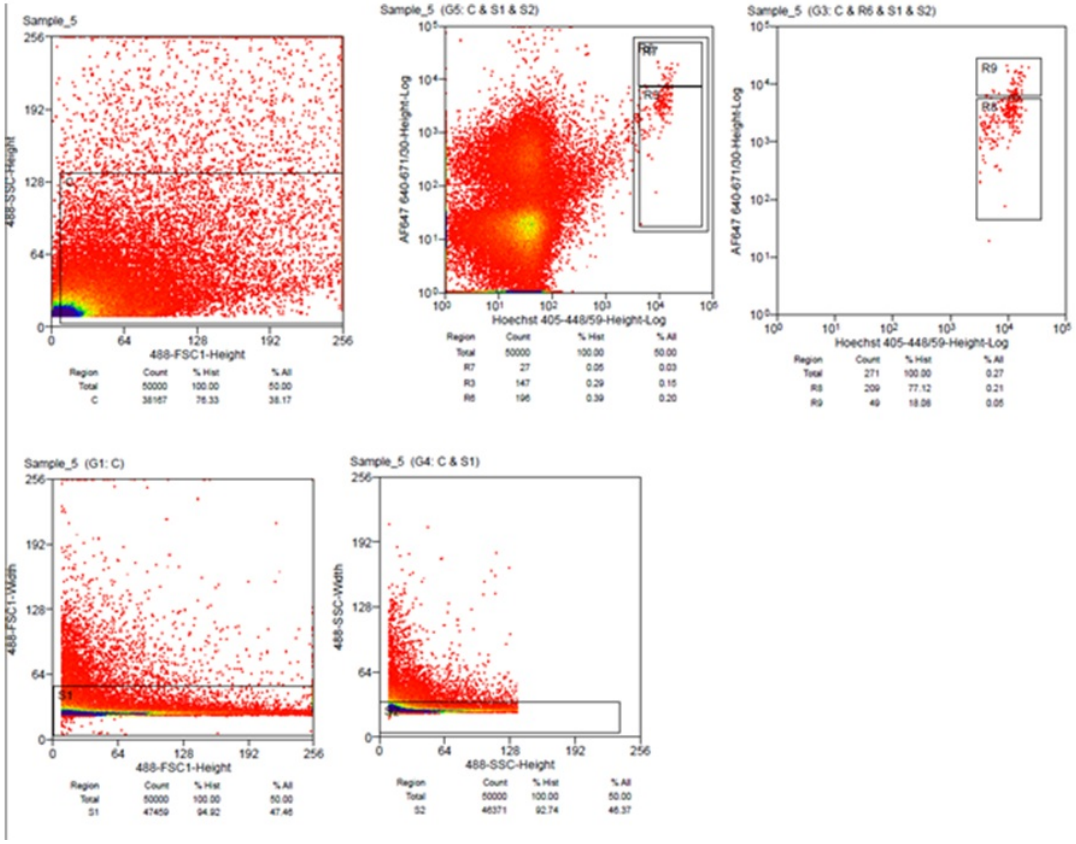
the isotype control.



89 Save the FACS program for documenting how nuclei were selected for analysis.

90 (Optional). After FACS, combine 0.5 uL of the sorted sample in 9.5 uL Staining Buffer. Place 10 uL on a hemocytometer and view the nuclei under an epifluorescent microscope. Nuclei should be Hoescht+ and intact without large quantities of debris.

Note: we routinely avoided counting nuclei numbers using the hemocytometer since our sorted nuclei counts were so low (1000-10,000) that a hemocytometer did not give a good estimate of nuclei number. Instead, we typically provided the estimate from the FACS machine.

91 Samples are ready for 10x GEM generation and library preparation using the 10X Genomics platform.

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