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Processing Human Colon "Myenteric Plexus" for Single Nuclei RNA-seq

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1 Works for me dx.doi.org/10.17504/protocols.io.w26fghe

ABSTRACT

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 obtain a suspension of single nuclei from human colon myenteric plexus that could be used for RNA-seq.

METHODS: 1) Human colon "myenteric plexus" is dissected from the bowel wall after labeling with a live cell dye and then frozen in OCT. 2) Frozen "myenteric plexus" is sectioned on a cryostat to generate small fragments and disrupt muscle and connective tissue. 3) RNA quality is assessed from a few sections using an Agilent Bioanalyzer. 4) Nuclei are obtained from frozen sections using Dounce homogenization and labeled with Hoechst 33342 dye. 5). Nuclei are separated from other cell debris by FACS. 6) RNA-seq data were obtained using the 10X Genomic single cell sequencing platform.

RESULTS: Live dye labeling works well on living tissue to visualize the ENS in human colon. Careful dissection is required to micro-dissect "myenteric plexus" from from within bowel wall muscle layers. A suspension of single nuclei is efficiently isolated by freezing isolated tissue in OCT, frozen sectioning, Dounce homogenizing, and FACS sorting Hoechst 33342 stained nuclei. The isolated nuclei are suitable for sequencing on the 10X Genomics single cell sequencing platform. This procedure dramatically enriched for cells in the region of the myenteric plexus, but unfortunately only a small percentage of the cells isolated are myenteric neurons. In addition to myenteric neurons, we obtained data from enteric glia, smooth muscle, muscularis macrophage, interstitial cells of Cajal, and PDGFRalpha+ cells that are known to be closely associated with myenteric plexus.

CONCLUSION: We tried many approaches to optimize this procedure including additional steps to enrich for myenteric neuron nuclei. These additional procedures invariably reduced total cell yield without enriching significantly for neuronal nuclei. We do not know yet why neuronal nuclei remain a small component of our total nuclei population, but suspect that neuronal nuclei are more fragile than other nuclei isolated. This approach generated valuable data at a single nucleus RNA-seq level for all cell types within the muscle wall known to control bowel motility.

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GUIDELINES

We separate this procedure into 4 sections:
Step 1: Collecting Human Colon Tissue and microdissection of "myenteric plexus"
Step 2: Sectioning frozen human tissue
Step 3: Extracting RNA for Agilent Bioanalyzer analysis. This is done to assess RNA quality prior to RNA-seq
Step 4: Isolating nuclei for single nucleus RNA-seq

This procedure requires a fluorescent dissecting microscope for microdissection of "myenteric plexus".

MATERIALS TEXT

MATERIALS

RNAse-free Water **Contributed by users**

MilliQ Water **Contributed by users**

Lab Timer **Contributed by users**

Minutien Pins Stainless Steel 0.2 mm diameter **Fine Science Tools Catalog #26002-20**

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

Potassium Chloride **Fisher Scientific Catalog #BP366-500**

Calcium Chloride **Sigma Aldrich Catalog #C-7902**

Magnesium sulfate anhydrous **Sigma Aldrich Catalog #M-7506**

Sodium Phosphate monobasic **Sigma Aldrich Catalog #S0751-1kg**

Sodium bicarbonate **Fisher Scientific Catalog #BP328-500**

D-()-Glucose (There should be a plus sign in parentheses) **Sigma Aldrich Catalog #G-7021**

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

Dumont # 5 Forceps (two pair) **Fine Science Tools Catalog #11251-30**

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**

4-Di-2-ASP (4-(4-diethylaminostyryl)-N-methylpyridinium iodide) **Abcam Catalog #ab145266**

Hydrochloric acid (4 Normal solution--Prepared from concentrated HCl) **Sigma Aldrich Catalog #25814**

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**

Sterile 35 x 10mm Vented Cell Culture Dishes Petri Dish Style Non-Treated Polystyrene **Corning Catalog #430588**

Dow Corning Sylgard 184 Silicone Encapsulant Clear 0.5 kg Kit **Contributed by users Catalog #84 SIL ELAST KIT 0.5KG**

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**

Superglue **Contributed by users**

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

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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**

Agilent RNA 6000 Pico Kit **Contributed by users Catalog #5067-1513**

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**

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**

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These items are also needed for this protocol	
Scale	
Dissecting fluorescent microscope	
Epifluorescent microscope	
Chilling block	Any flat metal/stone block that fits under the dissecting fluorescent microscope is fine.
-20 °C freezer	
Stir plate and stir bar	
Oxygen tank - 5% CO_2, 95% O_2	
Dry ice and container	
Wet ice (frozen water) and container	
Hemocytometer	
Fume hood	
-80 °C freezer	
Cryostat for sectioning tissue	
Pipetters	P1000, P200, P20, P10
Filter Pipette Tips	One option is SHARP Precision Barrier Tips from Denville Scientific (P1126, P1122, P1096-FR, P1121)
Pipette Aid	
Microcentrifuge for 1.5 mL eppendorf tubes	
Large individually wrapped pipet tips	5 mL, 10 mL, 25 mL
Large centrifuge for 50 mL conical tubes	
Agilent RNA2100 Bioanalyzer	Or you will need another method to analyze RNA quality

SAFETY WARNINGS

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

Before each step there are reagents and other materials to prepare in advance.

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Review protocol in detail before beginning work with tissue. Remember that this procedure uses living human colon, so steps must be performed carefully and efficiently. This means that many reagents and tools must be prepared and organized in advance.

PREPARING SYLGARD DISH FOR PINNING TISSUE

1 Prepare Sylgard by combining curing agent with polymer as described in the kit.

2 Carefully drip Sylgard into a 35x10 mm dish until the dish is halfway full.

3 Allow to cure in a fume hood for at least 3 days.

4 After curing is complete, superglue the 35x10 mm dish to the center of a Fisherbrand Petri dish with clear lid.

The Petri dish will provide containment for leakage from the 35x10 mm dish, and also hold ice chips to surround the tissue with ice.

5 Insert Minutien pins (>10) into the 35x10 mm dish to prepare it for pinning human tissue.Krebs-Ringer Solution

PREPARING KREBS-RINGER SOLUTION

6 Combine the NaCl, KCl, CaCl2, MgSO4, NaH2PO4, D-Glucose (**see RECIPE below for amounts**).

7 Add MilliQ water to 990 mL. Stir continuously.

8 Add NaHCO3.

9 **RECIPE for KREBS-RINGER SOLUTION**

Chemical	Final concentration	Molecular weight	For 1 L
Sodium chloride (NaCl)	118 mM	58.44 g/mol	6.896g
Potassium chloride (KCl)	4.6 mM	74.55g/mol	0.343g
Calcium chloride dihydrate (CaCl2 · 2H2O)	2.5 mM	147.01g/mol	0.368g
Magnesium sulfate anhydrous (MgSO4)	1.2 mM	120.37g/mol	0.1444g
Sodium phosphate monobasic (NaH2PO4)	1 mM	119.98g/mol	0.120g
D-(+)-Glucose	11 mM	84.01g/mol	2.100g
Sodium bicarbonate (NaHCO3)	25 mM	180.16g/mol	1.982g

10 Adjust the pH to 7.4 using 4 N HCl.

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11 Store at 4 °C.

PREPARING 4-DI-2-ASP

12 Weigh 9.8 mg of 4-Di-2-Asp powder in a 50 mL conical.

13 Combine 4-Di-2-ASP with 50 mL sterile dPBS to make 50 mL of 500 uM 4-Di-2-ASP stock solution. This can be stored at room temperature for extended periods. (We do not know how long it will be effective)

HUMAN COLON TISSUE HANDLING TO ISOLATE "MYENTERIC PLEXUS" ON DAY OF TISSUE COLLECTION

14 Half hour before the tissue arrives:

a. Place chilling block in the -20 °C freezer

b. Place ~20 mL of Krebs-Ringer into a 50 mL conical. Bubble 5% CO2, 95% O2 through the Ringer using a 10 mL serological pipette. This is for **Room temperature, oxygenated Krebs-Ringer**

c. Also keep at least 50 mL Krebs-Ringers solution at 4 °C (you do not need to oxygenate this). This is for **Cold Krebs-Ringer**

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

e. Fetch dry ice.

f. 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.

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

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

17 Place tissue into **cold Krebs-Ringer** in a large sterile Petri dish.

18 Trim off surrounding fat using Dumont #2 forceps and a scissors.

19 Cut a ~1 cm x 1cm piece of tissue. Remove as much epithelium and submucosa as possible with scissors.

20 Transfer the smaller tissue piece (mostly muscularis) into the 35x10 mm dish with pins. Leave the rest of the human tissue on a Petri dish on ice.

21 Add 2.7 mL of **room temperature, oxygenated Krebs-Ringer** to the small tissue piece in the 35x10 mm dish.

Note: it is important that the Krebs-Ringer solution be at room temperature for this part; otherwise, the 4-Di-2-ASP will not be absorbed by myenteric plexus cells.

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22 Carefully pin the tissue flat, stretching it to maximum possible capacity.

Note: The more the tissue is stretched, the better the 4-Di-2-ASP will penetrate.

23 Add 300 uL of 500 uM 4-Di-2-ASP stock solution to the Krebs-Ringer solution already in the dish. Pipette up and down until the 4-Di-2-ASP has spread evenly across the dish.

24 Set timer for 15 minutes.

25 During this time, prepare two Biopsy cryomolds by labeling them with the sample ID number. Label one 'plexus' and the second one 'muscle'. Fill cryomolds about halfway with O.C.T. and place them on ice.

26 After 15 minutes, remove the Krebs-Ringer with 4-Di-2-ASP from the dish with colon tissue.

27 Replace with 2 mL cold Krebs-Ringer solution to cover human colon tissue.

28 Add ice to larger petri dish surrounding the 35x10 mm dish.

29 Remove chilled block from the -20 ^{O}C and place it on the stage to the fluorescent dissecting microscope. Place petri dish with tissue on top of the chilled block. Placed Biopsy cryomold labeled '**Plexus**' in the ice on the larger surrounding petri dish.

30 Check that the myenteric plexus is labeled by viewing the tissue in the 488 nm channel on the fluorescent dissecting microscope.

31 With #5 Dumont forceps, carefully dissect away the muscle, exposing the myenteric plexus beneath.

Note: move very slowly whenever you are working with sharp forceps and human tissue. NEVER point the tip of the forceps toward your fingers or hands. If you accidently stab yourself, immediately rinse the wound in the sink and follow the appropriate safety protocols at your institution.

32 Carefully separate myenteric plexus from surrounding tissue. Place isolated pieces of plexus in cold OCT in the Biopsy cryomold.

Note: separation will never be perfect. There will always be muscle and other cells dissected out along with the plexus. The goal is just to enrich for plexus not isolate it entirely.

33 When as much plexus has been separated as possible for this tissue piece, place the cryomold containing the cold OCT+ plexus in the 2-Methylbutane in the fume hood.

Cut a piece of the remaining muscle. Place this in the second cryomold labeled '**Muscle**'. Place this in the 2-

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34 Methylbutane in the fume hood.

Note: this additional step is done purely for the purposes of assessing RNA quality in the tissue.

35 Wait 5 minutes for it to freeze. Then transfer to a -80 ^{o}C freezer in appropriate containment (e.g. plastic bags in labeled boxes).

36 If there is more human tissue, repeat steps 6-22. The maximum we process is typically 3 pieces per sample since each piece takes ~30-45 minutes.

37 Clean plates with RNase away and bleach

Note: all plates should be handled extremely carefully, particularly the pins since there is the potential for human pathogen exposure.

38 Autoclave tools.

HUMAN COLON SECTIONING IN PREPARATION FOR ISOLATION OF NUCLEI FOR RNA-SEQ

39 Fill an ice bucket with dry ice.

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

41 Transfer tissue labeled "**Plexus**" in biopsy cryomold on dry ice to Cryostat set to -20 °C.

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

43 While waiting for tissue to freeze, label 3 DNA LoBind Tubes: "Plexus", " Plexus", and " Plexus RNA". Place these on dry ice or in the 20 ^{o}C for at least 3 minutes before sectioning so they have time to cool.

44 Place Dumont #2 Forceps in cryostat to chill.

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

46 Using the Dumont #2 Forceps, collect all sections in " Plexus" tubes EXCEPT 2-5 sections. Put 2-5 random sections in tube labeled " Plexus RNA".

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47 Return the tubes with tissue to the -80 ^{O}C on dry ice.

48 Section through the Muscle piece until the middle of the tissue is reached. Collect 2-5 sections in an Eppendorf tube.
Label this Eppendorf tube " Muscle RNA"

49 Return tube to the -80 ^{O}C on dry ice.

EXTRACTING RNA FOR BIOANALYZER SUBMISSION TO ASSESS RNA QUALITY BEFORE RNA-SEQ

50 **Follow the instructions on the Qiagen RNeasy Micro Kit as detailed below:**

51 Remove tubes labeled "RNA" from the -80 ^{O}C. There should be one each for plexus and for surrounding muscle.

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

53 Homogenize the lysate by vortexing for 30 seconds.

54 Run through gDNA spin column. Centrifuge for 30 seconds at >= 8000 g.

55 Discard the column and keep the flow-through.

56 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.

57 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 15 seconds at > 8,000 RCF. Discard the flow thru.

58 Add 700 uL of buffer RW1 to the RNEasy MinElute spin column.
Close the lid and centrifuge for 15 seconds at 8,000 RCF. Discard the flow-through.

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

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

61 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.

62 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.

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

PREPARATION FOR ISOLATING NUCLEI FOR SINGLE NUCLEUS RNA-SEQ

64 **Prepare Hoechst dye 2.5 mg/mL:**
Dilute 10 mg/mL Hoescht 1:4 in nuclease-free water
Using 3 mL syringe and 0.22 micron filter, filter the Hoescht.
Store at 4 degrees C.

65 **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.

66 **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 (MgCl2)	1 M	3 mM	120 uL
Nonidet P40	10%	0.005%	20 uL
RNase free water			38.38 mL
Total volume			40 mL

67 **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	300 uL
Ultrapure 50 mg/mL BSA	50 mg/mL	1% w/v	600 uL
Protector RNase inhibitor	0.2 U/uL	0.001 U/uL	15 uL
Nuclease free water			2085 uL
Total volume			3000 uL

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

69 **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.

Note: Nuclei are isolated by FACS on the day of 10X Genomics sequencing analysis

70 Schedule 10x Genomics Sequencing run

Note: Nuclei are isolated by FACS on the day of 10X Genomics sequencing analysis

ISOLATING NUCLEI FOR SINGLE NUCLEUS RNA-SEQ

71 Get a large ice bucket.

72 Prepare the appropriate quantities of **Lysis buffer** and **Staining buffer** (see steps 66 and 67). Place them on ice.

73 Precool the Dounce homogenizer and pestles on ice.

74 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.

75 Pre-cool large centrifuge to 4 ºC.

76 Place a 6-well plate on ice. Fill wells with 6 mL of **Lysis buffer (Step 66)**.

Approximately 3 hour before FACS sort, put on appropriate **Personal Protective Equipment (PPE) (gloves, lab**

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77 **coat, face mask, face shield) to protect from human pathogen exposure.**

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

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

80 Chop rapidly with large iridectomy scissors for 1 minute.

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

82 Homogenize tissue with 15 strokes of the loose pestle and 40 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.*

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

84 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.

85 Repeat steps 79-84 until all Eppendorf tubes with tissue from a single human subject have been homogenized. Homogenized nuclei from the same human subject may be combined into the same 50 mL conical.

86 If nuclei from a second human subject colon are being run that day, repeat steps 76-85.

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

87 Spin down **Lysis buffer**+nuclei+OCT on large centrifuge at 590 RCF for 8 minutes at 4 ^{O}C. Be sure to appropriately balance samples.

88 While tissue is spinning, label 1.5 mL Eppendorf tubes, 1 tube per human subject. Add 5 uL **Staining buffer (Step 67)** to the bottom of each tube. These are the collection tubes.

89 Also label Falcon round-bottom tubes, one tube per human subject. These tubes will hold the sample to be sorted.

Place labeled 1.5 mL Eppendorf tubes and Falcon round-bottom tubes on ice to prepare for transport to FACS sorter.

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90

91 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.

92 Resuspend in 1 mL **Staining buffer** by pipetting 5-10 times with a P1000.

Note: the FlowMi strainer may clog if there are clumps in the sample. Be sure to pipette and disperse clumps thoroughly.

93 Add 1 uL of **2.5 mg/mL Hoechst**.

94 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.

95 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.

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

97 Sort Hoescht+ nuclei into the collection Eppendorf tubes with 5 uL staining buffer using 70 micron nozzle (this can sort up to 1,000 nuclei per microliter).

We have found success with the gating scheme below which removes debris and isolates nuclei:

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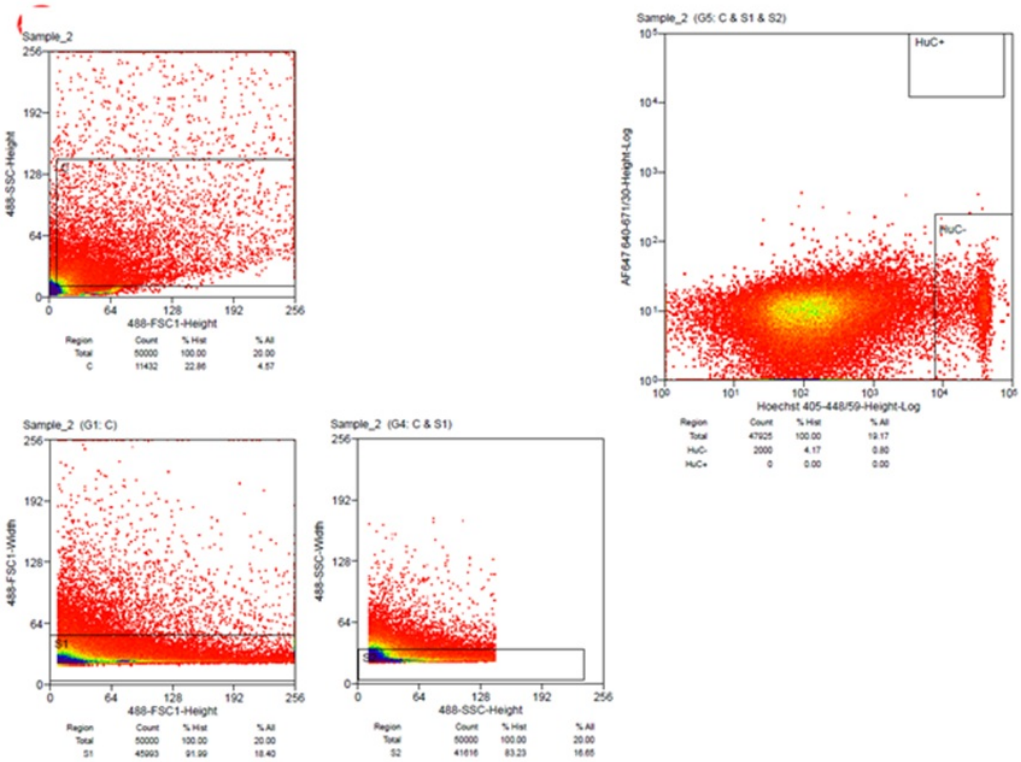

98 Save the FACS program to document what you did during nucleus isolation.

99 (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.

100 Isoled nuclei are ready for 10x GEM generation and library preparation using 10X Genomics protocols.

Citation: Christina M Wright, Robert O Heuckeroth (03/15/2021). Processing Human Colon "Myenteric Plexus" for Single Nuclei RNA-seq. https://dx.doi.org/10.17504/protocols.io.w26fghe