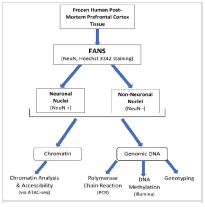

Jun 21, 2022

Isolation of Neuronal Nuclei From Human Frozen Post-Mortem Prefrontal Cortex Brain Tissue Using Fluorescence-Activated Nuclei Sorting (FANS) For Methylation Analyses

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ABSTRACT

There is a need for disentangling the role of transcriptional and epigenetic variation in mental health, disease, and mortality. Although alterations in DNA methylation patterns have been found to be particularly stable and highly concentrated in the brain, the study of bulk tissue, which contains several types of cells, can be limited and potentially significantly mask different alterations driven by specific cell types. Therefore, there is a need for methods to be able to purify specific cell-type populations that can be used in downstream molecular analyses. This protocol describes a methodology that uses fluorescence-activated nuclei sorting (FANS) to overcome these limitations and analyze, specifically, neuronal nuclei from frozen post-mortem prefrontal cortex tissue. Specifically, this protocol was based on recently-published protocols with the same goal with adaptations that have proven to improve yield and quality of the samples in our laboratory using the BD FACSJazz™ cell sorting system. To our knowledge, this is the only published protocol where a methodology for a flow cytometry instrument with a manual laser and sorting alignment is described in detail. This method can be used to purify populations of neuronal (NeuN+) and non-neuronal (NeuN-) nuclei from adult frozen post-mortem brain tissue, with tissue samples yielding purified populations of nuclei amenable to be able to perform analyses of DNA methylation, genotyping, and potentially, open chromatin analysis (via ATAC-seq).

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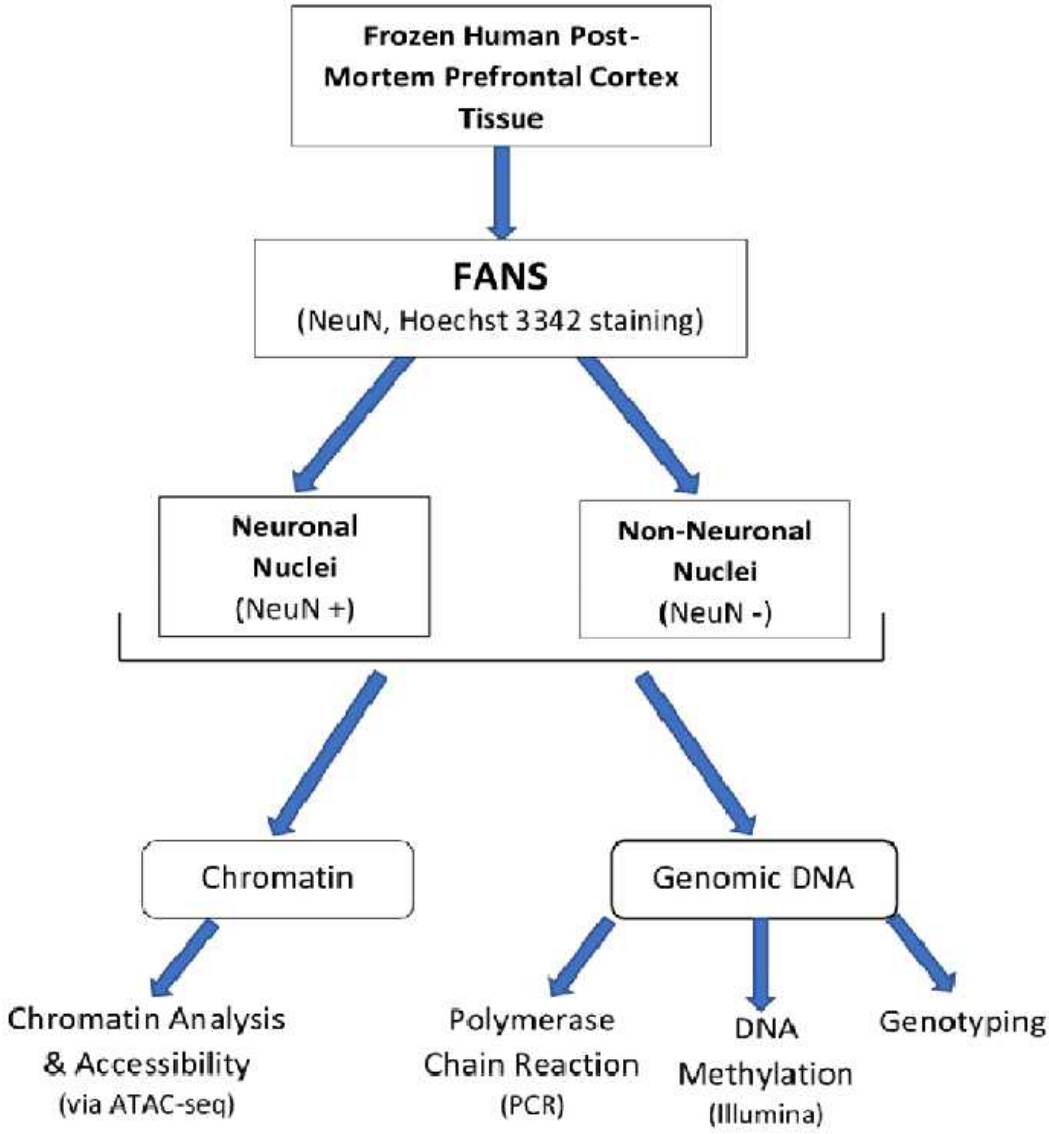

Schematic overview showing a flowchart of the process, as well as the array of downstream applications for which these FANS-processed nuclei are suited for.

MATERIALS TEXT

Table 1. Specification of the equipment for FANS protocol:

A	B	C
Material/Equipment Name	**Supplier**	**Catalog Number**
BD FACSJazz™ Cell Sorter	BD Biosciences	NA
BD Cooling System	Thermo Scientific	152-5108

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Microcentrifuge	Eppendorf North America	22620700
Vortexer	Scientific Industries	12-812
Rotisserie Rotator	Argos Technologies	22-505-003
5mL Tissue Homogenizer (autoclaved, prechilled)	Cole-Parmer	UX-44468-02
5mL Dounce Tissue Grinder Tight Pestle (autoclaved, prechilled)	Cole-Parmer	UX-44468-14
1.5mL LoBind DNA Eppendorf Tubes (Prechilled)	Eppendorf North America	30108418
2mL LoBind DNA Eppendorf Tubes (Prechilled)	Eppendorf North America	22431048
Pipette tips (Autoclaved, Prechilled) (P1000)	Fisher Scientific	02-707-400
Pipette tips (Autoclaved, Prechilled) (P200)	Fisher Scientific	02-707-409
Pipette tips (Autoclaved, Prechilled) (P10)	Fisher Scientific	02-707-438
Kimberly-Clark Professional™ Kimtech Science™ Kimwipes™ Delicate Task Wipers, 1-Ply 34155	Possible Missions, Inc	06-666A
MTC BioStrainer Cap, 35um strainers	Possible Missions, Inc.	NC1827548
Forceps (Autoclaved, Prechilled)	Cole-Parmer	UX-07387-08
Scalpel Holder (with Scalpel) (Autoclaved, Prechilled)	Surgical Design	13-812-234
Hemocytometer	Hausser	3200
Falcon 5mL Polystyrene Round-Bottom tubes with 35um cell strainer caps (12 x 75mm style)	Fisher Scientific	877123

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BD 5mL Polypropylene tubes with caps	Sigma Aldrich	14-959-10B
Falcon® 15 mL Polystyrene Centrifuge Tube, Conical Bottom, with Dome Seal Screw Cap, Sterile (Pre-chilled)	Fisher Scientific	352099
140mL-200mL beakers (one for each homogenizer)	Kimax	26CV53
2 Large Buckets for Ice	Fisherbrand	259145
1 Small Bucket for Dry Ice	Bel-art products	M16807-1103
Qiagen AllPrep® DNA/RNA Micro Kit	Qiagen	80284
Mr. Frosty	Nalgene	5100-0001
Aluminum Foil	Fisher Scientific	01-213-102

Table 2. Specification of reagents and materials required for nuclei isolation and FANS protocol

A	B	C
Reagent Name	**Supplier**	**Catalog Number**
Trypan Blue Solution	Bio-rad	1450013
D-Sucrose	Fisher Chemical	S5-500
Potassium Chloride (KCl)	Fisher Chemical	P217-500
Magnesium Chloride (MgCl2)	Fisher Chemical	M33-500
1M Tris-HCl Buffer, pH 8	Thermo Scientific	AM9855G
UltraPure RNAse-free, DNAse-free Distilled Water	Invitrogen	10977015
1,4-Dithiothreitol (DTT)	Sigma	D9779-5G
100x Protease Inhibitor	Thermo Scientific	78429
RNAse Inhibitor 40U/uL	Takara Bio	2313A

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10X RNAse-free PBS, pH 7.4	Thermo Scientific	AM9624
Bovine Serum Albumin (BSA)	Sigma Aldrich	A9418-50G
Triton™ X-100	Omnipur	9410-OP
Iodixanol (Optiprep) Solution	Biovision	M1248-100
8X Sheath Fluid	Beckman Coulter	NCO569431
Spherotech Ultra Rainbow Beads	Spherotech	URFP-30-2
BD FACS™ Accudrop Beads	BD Biosciences	34-52-49
Calcium Chloride (CaCl2)	Fisher Chemical	M-16049
Magnesium Acetate (Mg(Ac)2)	Sigma	M5661-250G
Dimethylsulfoxide (DMSO)	Sigma	D2650
Bambanker Freezing Medium Solution	Bambanker	BB01-NP
RNaseZapTM	Thermo Fisher	AM9780

Table 3. Recipes for Buffers and Solutions Required. (All solutions should be RNAse-free and placed in sterile Falcon tubes.)

A	B
Part A. Nuclei Isolation	
Isolation Medium Solution (ISM) (45mL)	**(Store at 4oC for up to 6 months)**
1.5M Sucrose	7.5mL (final concentration = 250mM)
1M KCl	1.125mL (final concentration = 25mM)

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1M MgCl2	225uL (final concentration = 5mM)
1M Tris pH 8 (RNAse-free)	450uL (final concentration = 10mM)
UltraPure RNAse-free, DNAse-free Distilled Water	35.7mL
Homogenizing Buffer Solution (HB) (3mL per 100mg of tissue sample)	**(Prepare fresh and keep iced or at 4oC. Discard remainder after each use.)**
ISM Solution	2.925mL
DTT 1mM solution	3uL (final concentration = 1uM)
50x Protease Inhibitor	30uL (final concentration = 0.5x)
RNAse Inhibitor 40U/uL	15uL (final concentration = 0.2U/uL)
Triton x 100 10% (v/v) solution	30uL (final concentration = 0.1%)
Iodixanol Medium Solution (IDM) (45mL)	**(Store at 4oC for up to 6 months)**
1.5M Sucrose Solution	7.5mL (final concentration = 250mM)
1M KCl Solution	6.75mL (final concentration = 150mM)

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1M MgCl2	1.35mL (final concentration = 30mM)
1M Tris pH 8 (RNAse-free)	2.7mL (final concentration = 60mM)
UltraPure RNAse-free, DNAse-free Distilled Water	26.7mL
Iodixanol 50% (v/v) Solution (20mL)	**(Store at 4oC for up to 6 months)**
60% (v/v) Iodixanol (Optiprep) Solution	16.7mL
IDM Solution	3.3mL
Iodixanol 29% (v/v) Solution (30mL)	**(Store at 4oC for up to 6 months)**
60% (v/v) Iodixanol (Optiprep) Solution	14.5mL
IDM Solution	15.5mL
Part B. Nuclei Immunostaining	
Immunostaining Buffer Solution (10mL)	**(Store at 4oC)**
1x PBS, pH 7.4 (RNAse-free)	9.40mL
10% BSA Solution (RNAse-free)	500uL (final concentration = 0.5%)
1M MgCl2	50uL (final concentration = 5mM)

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Antibody Incubation Buffer Solution (4mL)	**(Make it fresh and keep it on ice.)**
Immunostaining Buffer Solution	4mL
RNAse Inhibitor 40U/uL	20uL (final concentration = 0.2U/uL)
BSA-Coating Solution For Immunostaining Tubes	(Prepare 1-2 days prior to running protocol and keep it at 4oC to dry)
10% BSA solution in 1 x PBS (RNAse-free)	1mL in each 1.5mL Eppendorf tube
Part C. Nuclei Sorting Using FANS	
1.5mL LoBind Tube-Coating Solution For Nuclei Sorting Collection Tubes	(Prepare 1-2 days prior to running protocol and keep it at 4oC to dry.)
1x PBS, pH 7.4 (RNAse-free)	500uL
RNAse Inhibitor 40U/uL	25uL
10% BSA Solution	500uL

Table 4. List of antibodies and dyes required for FANS protocol.

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A	B	C	D	E
Antibody	**Pre-Conjugated**	**Supplier**	**Catalog Number**	**Final Dilution**
Hoechst 33342 Dye Solution (20mM)	—	Thermo Scientific	62249	1:500
Anti-NeuN	Alexa Fluor-488	Sigma-Aldrich	MAB377X	3:3500

BEFORE STARTING

1. A day prior to performing immunostaining, prepare your BSA-coated tubes. Add 1mL of 10% BSA solution in ice cold 1x PBS (RNAse-free) to autoclaved 1.5mL Eppendorf tubes (one for each planned nuclei sample). Incubate them for 10 minutes on ice. Rinse them with ice cold 1x PBS (RNAse-free) and let them dry at 4^{O}C overnight (open lid).
2. A day prior to performing FANS, prepare your coated collection tubes. Add 500uL of ice cold 1x PBS (RNAse-free) solution, 25uL of RNAse Inhibitor (40U/uL), and 500uL of 10% BSA solution to your autoclaved and prechilled 1.5mL LoBind Eppendorf tubes that you will be using to collect your sorted nuclei samples (you should create at least 2 tubes for each sample: one for NeuN+ nuclei and one for NeuN- nuclei. It is recommended to make at least 4 tubes for each sample to better prevent potential overflow of sorted nuclei). Close lids and gently invert tubes a few times. Incubate tubes on ice for 10 minutes. Rinse each tube with 1mL ice cold 1x PBS (RNAse-free) and let it dry at 4^{O}C overnight (open lid).

Part A. Nuclei Isolation [~1-2 hours] 2h

1

(*Note*: Part A of this protocol is based on the protocol created by the Cobos Lab in October 2019 (dx.doi.org/10.17504/protocols.io.6tuhenw, based on Krishnaswami et al., Nat Protoc. 2016;11(3):499-524). While the original protocol was created for single-soma RNAseq experiments, this version of the protocol has been adapted for production of quality DNA from nuclei sorted from FACS.)

The protocol below yields at least 300,000 NeuN+ and NeuN- nuclei (when the population is present) per **100 mg** of frozen human post-mortem prefrontal cortex tissue. It is important to note that recovery may vary from sample to sample due to high inter-sample variability. Refer to Tables 1, 2, and for details about equipment items that are required and for the specifications of the reagents that are required for each section of this protocol.

2 Prepare Homogenizing Buffer (HB) solution and keep it **On ice** . Pre-chill (i.e., Fast temp) your centrifuge to **4°C** before starting. Make sure that homogenizers and pestles are pre-

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chilled and sitting **On ice** before use. Place the autoclaved forceps and scalpel at **-80 °C on dry ice** . (Note: you can also place a Petri dish on dry ice, if you would like to chop up the brain sample into smaller pieces before transfer).

3 Weigh Eppendorf tubes with sample tissue (you want around **100 mg**) of sample tissue to be placed in each homogenizer (keep these at **-80 °C on dry ice**).

4 Transfer the 100mg brain chunks to their corresponding homogenizers (**On ice**). Use the pre-chilled/frozen forceps and scalpel to help you do this.

5 While **On ice** , place 600uL of Homogenizing Buffer into each homogenizer. Use 600uL of Homogenizing Buffer to "wash out" the tubes that contained the tissues 3 times (if the tissue came out of multiple tubes, wash out each tube once or twice (i.e., 600uL of Homogenizing Buffer to wash out the tube twice)). This helps make sure that you are getting as much of the tissue as possible out of the Eppendorf tubes. Your homogenizer should have a final volume of around 2.4mL of Homogenizing Buffer in them (plus the brain tissue sample) once this process is done.

6 Use a spray of RNaseZap™ on a Kimwipe™ to lightly wipe the pestle before using it to homogenize (Note: Make sure it is dry before using it to administer your manual strokes). With the homogenizer in a beaker of ice **On ice** , administer 30 slow and gentle manual strokes to homogenize the tissue using the pestle to move the pestle up and down, using a gentle grinding motion when touching the bottom of the homogenizer tube. (Note: Try not to lift the pestle completely out of the liquid, as this will create bubbles and foaming, which can cause problems).

7 Once this homogenizing is complete, use a hemocytometer (10uL of sample + 10uL of Trypan Blue) to check whether there are still remaining clumps (or whether too many strokes have been administered). If there are cell clumps visible, then this means you must administer more manual strokes (too few strokes administered). However, beware because too many strokes can result in damaged nuclei.

8 Use sterile cell strainer caps to filter the homogenate from each homogenizer into a 15mL Falcon Tube **On ice** .

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(*Note*: Just hold the cell strainer cap on top of the Falcon tube's opening and make circular movements while filtering the homogenate into the Falcon tube. Feel free to use multiple cell strainer tubes if you are having trouble getting the full sample through just one cell strainer.)

9 Take an aliquot (10uL) to check the sample for nuclei and removal of debris using the hemocytometer again (10uL of sample + 10uL of Trypan Blue).

10 Repeat steps 8-9 (**go to step #8** and **go to step #9**) several times to try to remove as much debris as possible (around 1 to 3 times, checking with a hemocytometer for appearance of nuclei and removal of debris each time).

11 Separate filtered homogenate samples into 2 pre-chilled autoclaved 1.5mL LoBind Eppendorf tubes (4 tubes used if running 2 samples of tissue (100mg each); if running only 1 sample, split homogenate into 2 pre-chilled autoclaved LoBind Eppendorf tubes **On ice**). Make sure to evenly transfer sample(s) into tubes.

12 Centrifuge tubes at 1000rcf for 8 minutes at 4^{o}C. **1000 rcf, 4°C, 00:08:00** 8m

13 Gently discard the supernatant from the tubes **On ice** . Try your best not to disturb the pellets (it is okay to leave just a small amount of liquid in the tubes, if it is not possible to get it all out without disruption of the pellet).

14 Gently resuspend each pellet in each tube in 225uL of cold Homogenization Buffer **On ice** . Once resuspended, pool split tubes back together **On ice** . Do this by placing the pooled resuspended liquid into a prechilled 2mL LoBind Eppendorf tube (1 tube for each original sample) **On ice** . Final volume in each tube should be about 450uL.

15 Place 450uL of ice cold 50% (v/v) Iodixanol solution into each 2mL LoBind Eppendorf tube with pooled resuspended pellets and gently mix together with a pipet (by gently pipetting up and down a few times) **On ice** . Final concentration of Iodixanol will be 25%.

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16 Place 900uL of ice cold 29% (v/v) Iodixanol solution into a new pre-chilled 2mL LoBind tube (1 tube for each re-pooled sample) **On ice** .

17 Gently and slowly overlay the 29% Iodixanol solution with 900uL of the 25% Iodixanol solution (i.e., the mixed 50% Iodixanol solution + the re-pooled sample solution) by slowly adding it from the top edge of the tube, so that it slowly trickles down into the tube **On ice** (see **Figure 1**).

(*Note*: This should be the total amount of the 25% Iodixanol solution.)

Figure 1. Representative result of overlaying 25% Iodixanol+sample solution over 29% Iodixanol solution.

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18 Place samples (opposite from one another) in the 4°C pre-chilled centrifuge and spin them at 13500 rcf at 4°C for 20 minutes. **13500 rcf, 4°C, 00:20:00** 20m

(*Note*: When placing the tubes within the centrifuge, make sure to position the tubes with the tails facing *out*; this will make it easier to locate and identify the pellet later on after centrifugation. The resulting pellets will look like 1 or 2 small smudges on the side of the tube.)

19 After centrifugation, remove and discard the myelin-rich debris layer on the top (see **Figure 2**). Then, remove and discard the aqueous supernatant, without disrupting the nuclei pellet **On ice** .

(*Note*: Try to remove as much of the liquid as possible, but it is ok if a little liquid still remains in order to keep the pellet intact!)

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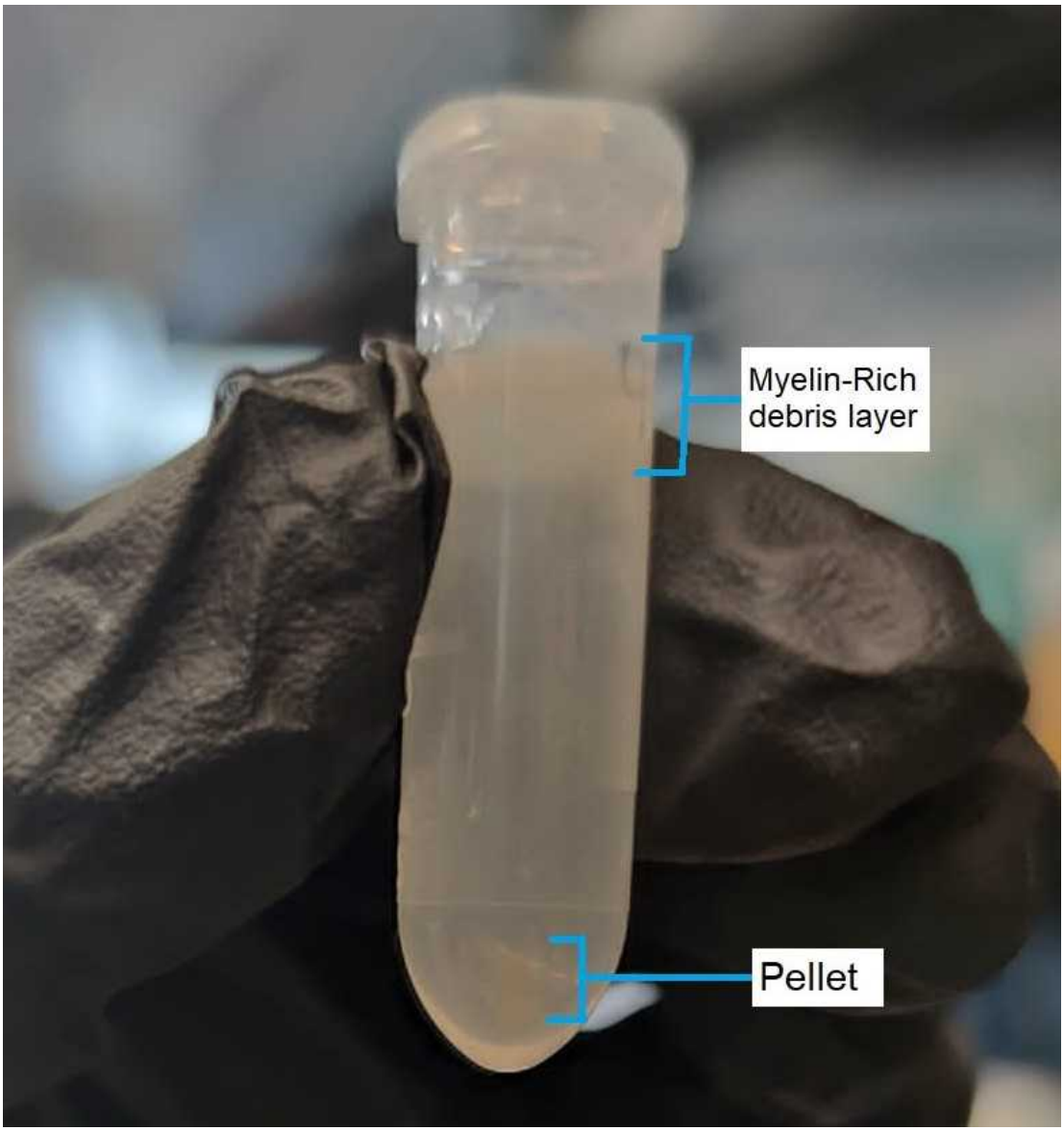

Figure 2. A representation of what should result after centrifuging the different concentrations of Iodixanol solution.

20 If planning on performing FANS the same day as nuclei isolation, then continue directly onto Part B (Immunostaining). If not, resuspend the pellet in 1mL of 10% DMSO in ice cold 1x PBS (RNAse-free) **On ice** , place it in a cryovial, and then in a Mr. Frosty overnight within a **-80 °C freezer** until further analysis.

Part B. Nuclei Immunostaining [~4-5 hours] 5h

21 A day prior to performing immunostaining, prepare your BSA-coated tubes **On ice** . Add 1mL of 10% BSA solution in ice cold 1x PBS (RNAse-free) to autoclaved 1.5mL Eppendorf

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tubes (one for each planned nuclei sample). Incubate them for 10 minutes **On ice** . Rinse them with ice cold 1x PBS (RNAse-free) and let them dry at **4 °C overnight** (open lid).

22 Fast temp your centrifuge to 4°C. **4°C**

23 If performing nuclei isolation the same day as immunostaining and FANS, then you may skip steps 24-29. If you have frozen your nuclei sample at **-80 °C** , you must perform these steps before moving on to step 30. Thaw cryovial with nuclei sample completely **On ice** . Once completely thawed, gently pipette the sample up and down.

24 Transfer the sample to an ice cold (pre-chilled) 1.5mL LoBind Eppendorf tube **On ice** .

25 Rinse out cryovial with 150uL of ice cold 1x PBS (RNAse-free) and transfer that liquid into your LoBind tube **On ice** , as well.

26 Centrifuge sample at 2050 rcf for 15 minutes at 4°C. **2050 rcf, 4°C, 00:15:00** 15m

27 Discard supernatant **On ice** , being careful not to touch and disturb the pellet.

28 Gently resuspend pellet in 1mL of ice cold 1x PBS (RNAse-free) solution **On ice** .

29 Centrifuge sample at 2050 rcf for 15 minutes at 4°C and discard the supernatant **On ice** , without disturbing the pellet (see **Figure 3**). **2050 rcf, 4°C, 00:15:00** 15m

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Figure 3. A depiction of the resulting nuclei pellet after thawing and centrifugation.

30 Add 500 uL of ice-cold Antibody Incubation Buffer solution to each pellet (in 2 ml tubes from Optiprep protocol - from step 21 of protocol part A, if you skipped steps 24-29) and let it sit on

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ice for 20 minutes **On ice for 20 minutes before mechanical resuspension** . This allows for the pellet to dissolve a little on its own, making it easier to later mechanically dissolve it by pipetting up and down, which lessens the amount of stress the nuclei experience.

31 After the nuclei have been incubated on ice for 20 minutes, mechanically (and gently) pipette the solution up and down to completely dissolve them in the buffer **On ice** .

32 Remove the nuclei-containing solution from the LoBind tube and transfer it to a 1.5 mL Eppendorf tube (pre-coated with BSA the day before) **On ice** . Place the sample **On ice** . Use 500uL of ice-cold 1X PBS to rinse out each tube and place that liquid into your corresponding pre-coated BSA tube, as well (final volume of nuclei solution will be 1mL) **On ice** . Check and count the nuclei with a hemocytometer by adding 10uL of sample into a new 1.5mL Eppendorf tube and mixing it with 10uL of Trypan Blue (see **Figures 4 and 5**).

(*Note*: Beware of nuclei clumping. If the nuclei count is 10 million or more, consider aliquoting the sample before performing immunostaining protocol!)

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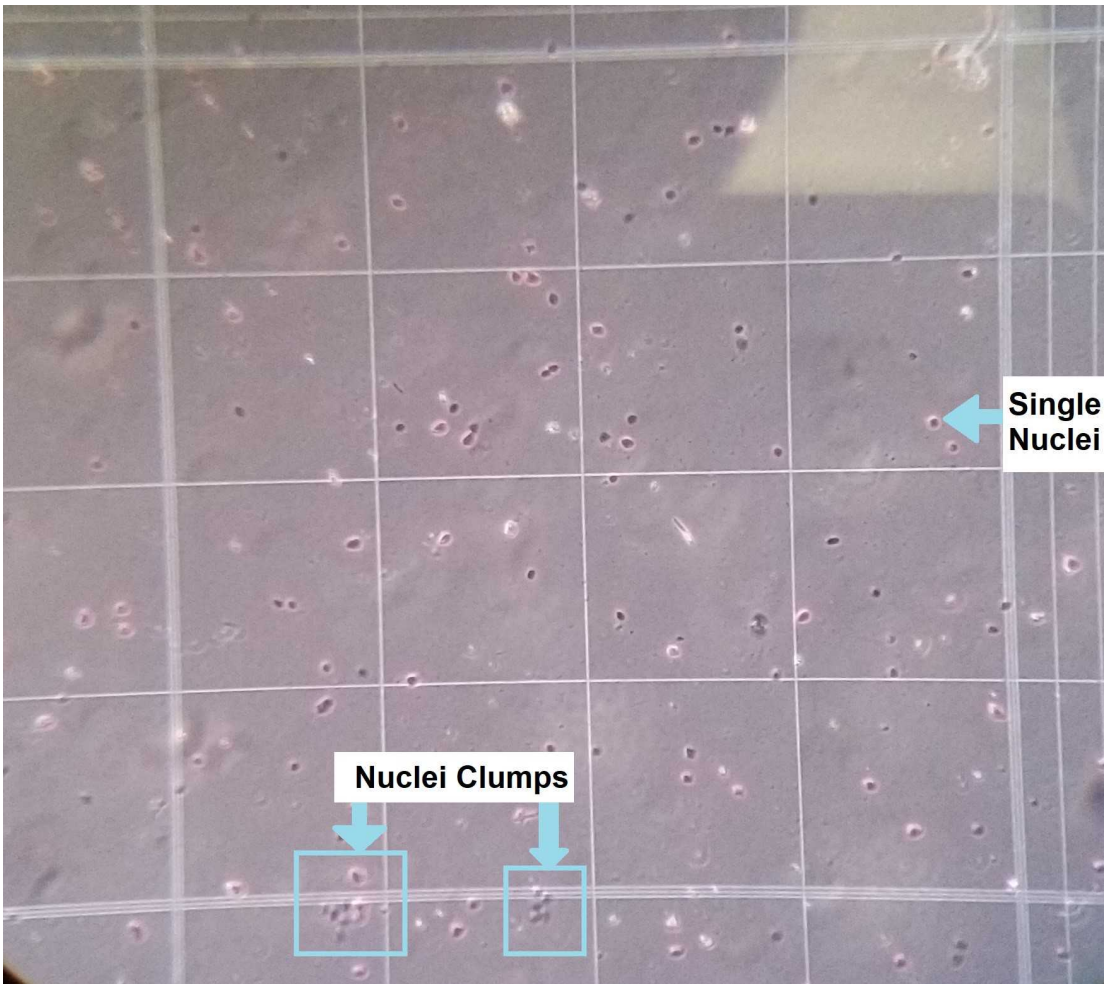

Figure 4. A 10x objective view of what a single nuclei versus a nuclei clump looks like on a hemocytometer.

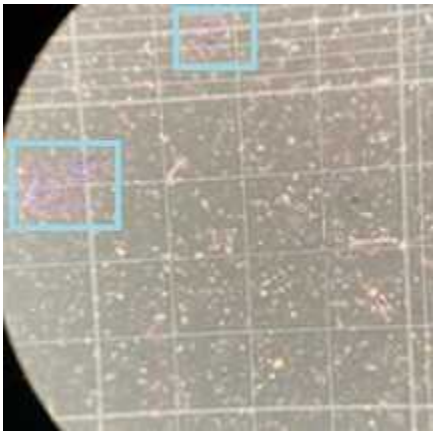

Figure 5. A 4x objective view of what nuclei and debris look like on a hemocytometer. The highlighted squares depict large nuclei clumping that can be encountered during the protocol.

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33 Prepare the NeuN immunostaining mixture in an 1.5 mL LoBind Eppendorf tube **On ice** (enough for 1 sample - adjust volume for more samples): 300 uL of 1X RNase-free PBS, 1.2 uL of NeuN antibody (Anti-NeuN Antibody, clone A60, Alexa Fluor®488 conjugated), and 100 uL of Blocking Mix (0.5% BSA and 10% Normal Goat Serum).

34 Vortex the immunostaining mixture for at least 10 seconds and incubate it at **Room temperature for 5 minutes in the dark** (using aluminum foil).

35 Prepare 1.5 mL LoBind Eppendorf tubes with the samples **On ice** (final volumes will be 1 mL), labeled as following (3 for each sample):

35.1 **Unstained Control:** pipette 70 uL of the nuclei-containing solution (from step 32) into 830 uL 1X PBS (RNAse-free).

35.2 **Hoechst Control:** pipette 70 uL of the nuclei-containing solution into 830 uL 1X PBS (RNAse-free).

35.3 **Nuclei Sample:** pipette 860 uL of nuclei-containing solution (what was left of it) into 140 uL 1X PBS (RNAse-free).

36 Add the immunostaining mixture (from step 34) to the Nuclei Sample tube.

37 Add 2uL of Hoechst 33342 (20mM) to the Hoechst control and Nuclei Sample tubes (do ***not*** add it to the **Unstained Control**).

38 Take the samples to the cold room (**4 °C**) and rotate them in the dark (rotisserie rotator) for 1.5 to 2 hours (cover tubes/rotator with foil to protect them from light). 2h
4 °C in dark on tube rotator **02:00:00**

(*Note*: When there is 30-45 minutes left on the incubation, you can start setting up the BD FACSJazz™ and performing its Daily Quality Check Alignment. This way it should be

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about ready by the time the incubation period is complete.)

39 After the samples have incubated in the dark for 1.5 to 2 hours, place them **On ice** and 5m centrifuge tubes at 2050 rcf for 5 minutes at 4ºC **2050 rcf, 4°C, 00:05:00** and discard supernatant (by pipetting off) **On ice** .

40 Gently pipette sample up and down using a P1000 pipette within the sample tube **On ice** . Then, use P1000 Pipetman to transfer the sample through a 5mL polystyrene round-bottom with cell-strainer cap filtration (blue cap tube) tube while **On ice** . Once the sample has been filtered, transfer the sample to a new 5mL sample tube made of polypropylene that is covered in aluminum foil (to protect the sample from light) and that is kept **On ice** **0 rpm** .

41 Lightly vortex sample tubes at lowest vortex setting for 10 to 30 seconds to make the mixture homogeneous (not clumped) right before loading the 5mL polypropylene round-bottom tube into the FACS chamber. Make sure that aluminum foil covers the sample tube while vortexing.

Part C. Fluorescence-Activated Nuclei Sorting, FANS

42 For machine start-up, CST and Accudrop calibrations, refer to **BD-FACSJazz-Software-UserGuide.pdf** for guidance and troubleshooting of the BD FACSJazz™ machine, specifically. The following instructions describe FANS using BD FACSJazz™. Other cell sorting platforms can be used but might require modification to this protocol.

(*Note*: You can also use the following links for further guidance: BD FACSJazz User Guide - Stanford Medicine and BD FACSJazz Use and Maintenance - MD Anderson.)

43 A day prior to performing FANS, prepare your coated collection tubes **On ice** . Add 500uL of ice cold 1x PBS (RNAse-free) solution, 25uL of RNAse Inhibitor (40U/uL), and 500uL of 10% BSA solution to your autoclaved and prechilled 1.5mL LoBind Eppendorf tubes that you will be using to collect your sorted nuclei samples (you should create at least 2 tubes for each

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sample: one for NeuN+ nuclei and one for NeuN- nuclei. It is recommended to make at least 4 tubes for each sample to better prevent potential overflow of sorted nuclei). Close lids and gently invert tubes a few times. Incubate tubes **On ice for 10 minutes** . Rinse each tube with 1mL ice cold 1x PBS (RNAse-free) and let it dry at **4 °C** **Overnight** (open lid).

44 Make sure that at least one day prior to performing, you have at least **6 L of 1x sheath fluid** that has been both filtered and autoclaved and is at **Room temperature** (around 23°C).

45 When switching on and setting up the BD FACSJazz™ machine, make sure to also turn on your cooling system. Set it to **5 °C** and hit the play button to allow the system to begin to cool down.

Part C1. Performing Gating of Samples and Sorting of Nuclei

46 Change all of the PMTs, FSC, and SSC to a log-scale by clicking the "log" checkboxes next to the PMT threshold adjustments and change the key threshold to FSC and around 1.65. (*Note*: FSC and SSC do NOT always have to be switched to log-scale. If easy visualization and gating can be done without switching FSC and SSC to log-scale, then this can also be done). Additionally, set the Sample Offset (in the Pressure Console) so that you get an Events per Second of around 200 when working with ALL of your samples (see **Figure 6**).

(*Note*: These can all be adjusted (as well as all PMTs and the threshold) as much as you want to get the best-looking plot. Just use this as a starting point.)

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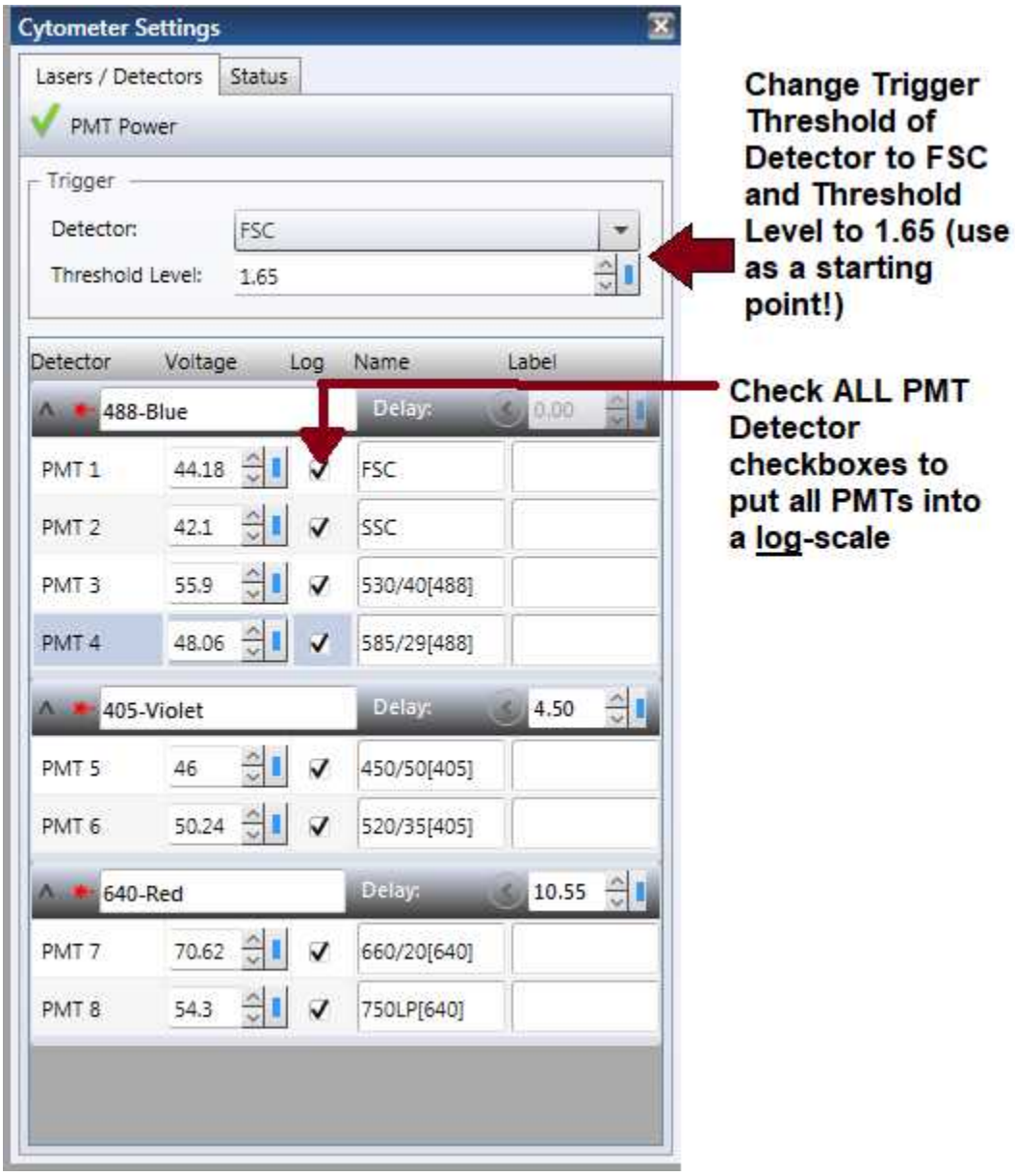

Figure 6. Screenshot of Cytometer Settings to use for initial set-up.

47 First, start with sample #1 (the Unstained Control sample). Remove any tube from the sample tube area within the machine and click **Backflush**. Wait about 30 seconds and then click **Backflush** again to turn off the backflushing. Lightly vortex your already filtered sample with the vortexer (setting at 1) for about 10 seconds. Bring the sample back to the flow cytometer, place it in the sample tube area, and lock it in place. Press the **Sample** button for the sample to start being inputted into the flow cytometer's system. Adjust your Default Display Count on the computer to around 1,000 and your Event Limit to around 2,000 (These can be found within the leftmost panel on your screen). Press the **Boost** button to get the sample flowing quicker into the system until you start seeing your Events Per Second change, as well as data starting to show up on your dot plots. Adjust your Sample Offset (and, if necessary, your PMT thresholds) until you get a consistent flow of about 200 events per second.

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48 This is the sample that you will most likely use for “cells” gating and gating out doublets: you will use an SSC vs. FSC plot to gate out debris (“Cells” gating plot) and a Trigger Pulse Width vs. FSC plot to gate out doublets (“Singlets” gating plot). (*Note*: Sometimes, the plots do not look consistently similar to what is normal or as good as they should for gating these two things (**Figure 7**). If this is the case and you are really having trouble discerning these things, you can move on and use sample #2 (the Hoechst Control sample) to give you a better idea and, in the worst case, to use for gating these 2 things).

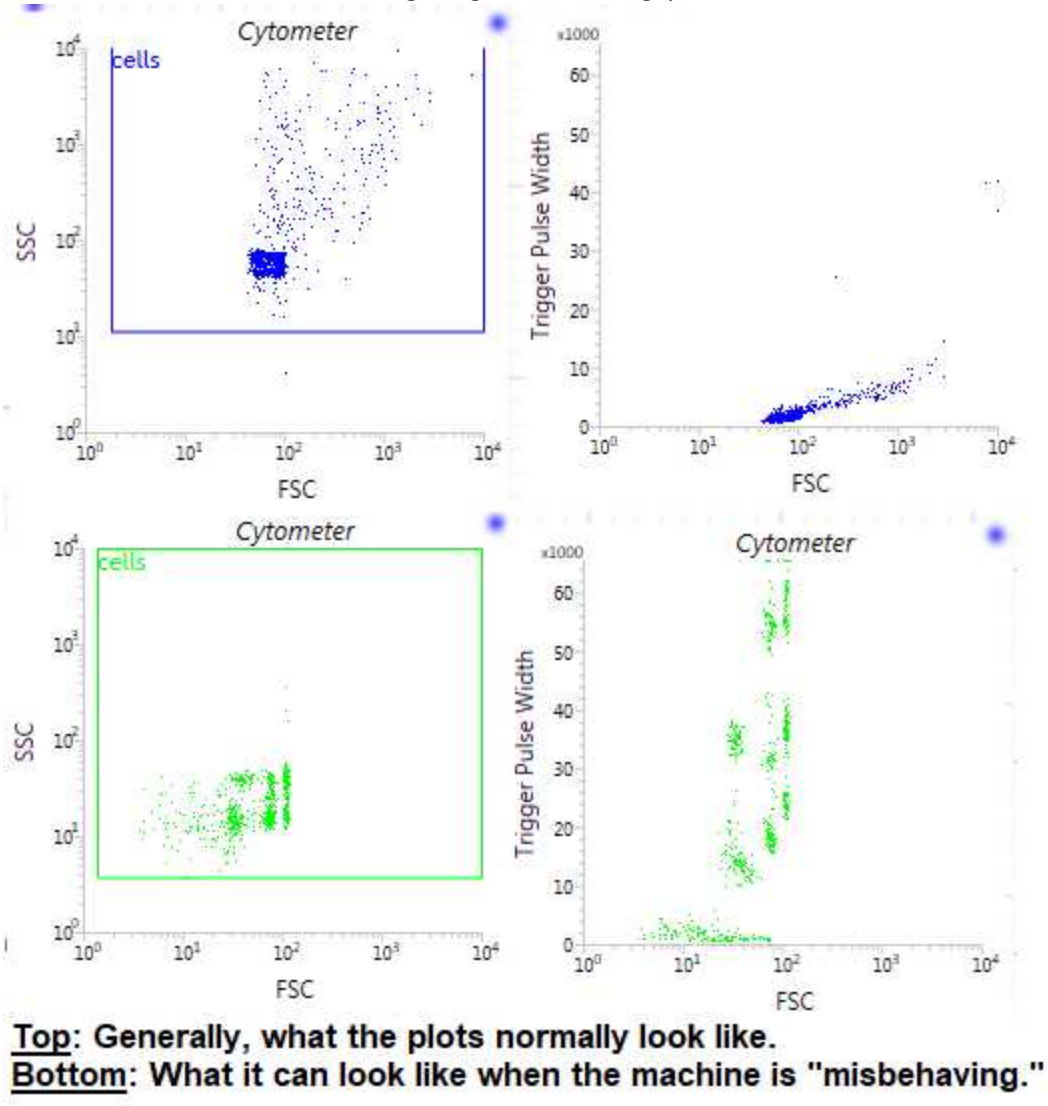

Figure 7. Depiction of how to perform the initial “cells” gating for your sample. The top two figures show what the plots should normally look like. The bottom two figures depict what the plots may look like when the FACS machine is acting up. It may be useful at this point to re-align the lasers, etc. and try to analyze your sample again, or shut off the machine and try again another day.

49 For the “Cells” gating, make a plot with SSC on the y-axis and FSC on the x-axis. Now, using one of the gating tools in the upper toolbar (**Figure 8**), click and drag to create a square gate in this plot and name it “Cells.” Generally, the debris is usually found in the lower left-hand corner of the plot. Sometimes, you are able to see 2 different populations (one that is the debris and

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one that is the “cells”), but this is not always the case. Use your judgment to make this gate. We have now, theoretically, gated out any debris for our sorting sample.

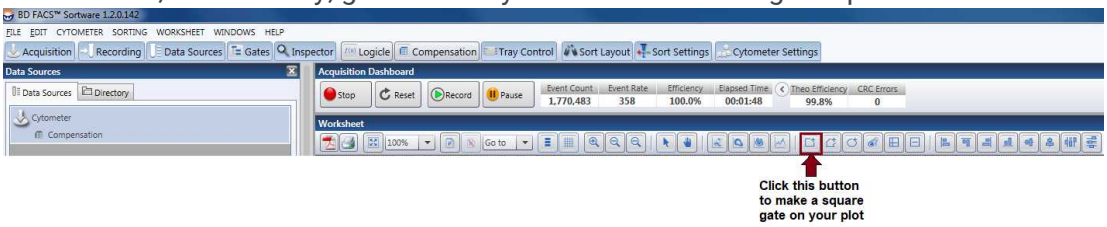

Figure 8. A screenshot of where to find the button within the BD sortware software to be able to create a square gate for your plots.

50 For the “Singlets” gating, make a plot with Trigger Pulse Width on the y-axis and FSC on the x-axis. (*Note*: This plot is usually already made for you as the 3rd plot on your workspace). For this plot, we are trying to gate out the doublets (the clumped “cells” or aggregates). These typically appear a bit “bigger” than normal singlets and, therefore, have a greater Trigger Pulse Width. Using the same gating tool as above, click and drag to create a square gate in this plot and name it “Singlets.” Use your judgment to make this gate. (*Tip*: Do not worry if you are not entirely sure, we recommend doing a “double check” plot with the Hoechst Control sample to make sure that your “Singlets” gate is working somewhat properly). We have now, theoretically, gated out any doublets and larger aggregates from our sorting sample. Make sure that your “Singlet” gate is nestled under your “Cells” gate by placing it within that “Cells” gate order (**Figure 9**).

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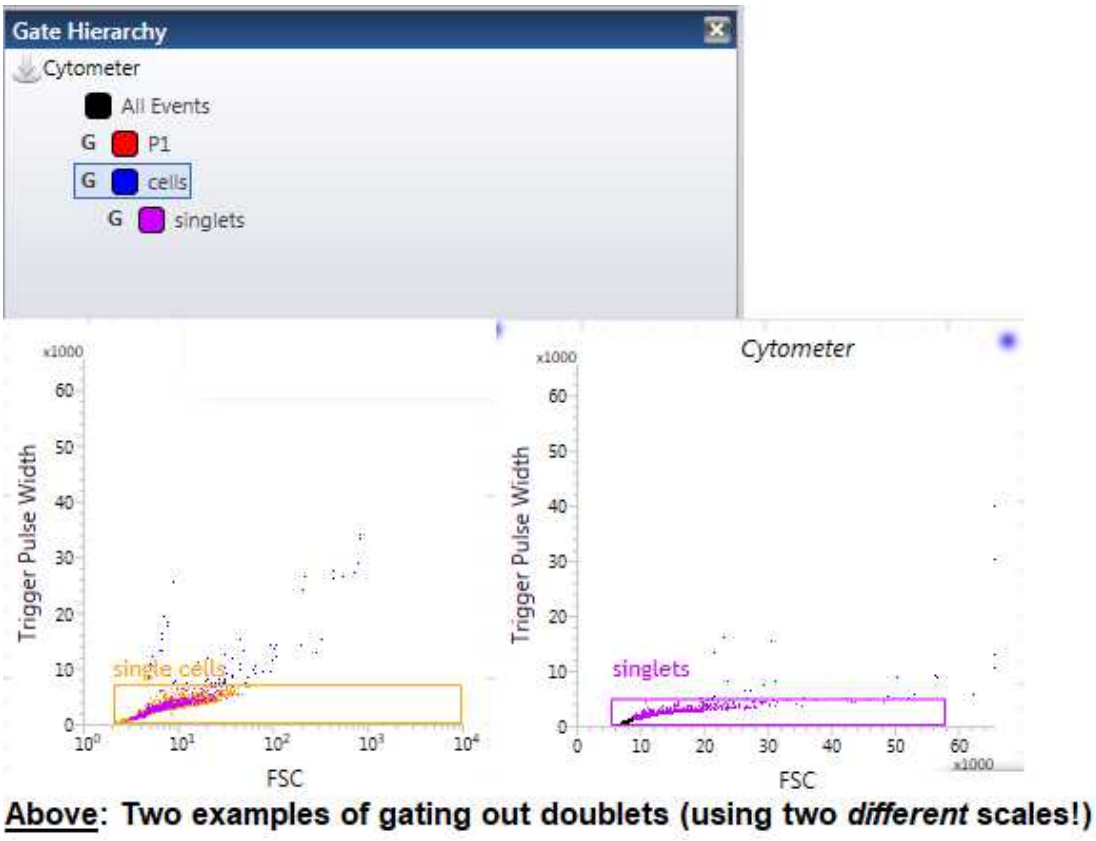

Figure 9. Two examples of roughly what your "singlet" plot should look like. Within the top figure, you can see exactly how your gating hierarchy should be organized in the sortware software.

51 Now, remove sample #1 (the Unstained Controlsample) from the sample tube area and click **Backflush** to clear out the system of the sample. Wait 30 seconds before clicking **Backflush** again. We will now move onto sample #2 (the Hoechst Control sample). Remove any tube from the sample tube area within the machine and click **Backflush**. Wait about 30 seconds and then click **Backflush** again to turn off the backflushing.

52 Lightly vortex your already filtered Hoechst Control sample with the vortexer (setting at 1) for about 10 seconds (Make sure that it is concealed with aluminum foil when you do this, as it has fluorescent dye in it). Bring the sample back to the flow cytometer, remove the aluminum foil, place it in the sample tube area, and lock it in place. Place some aluminum foil, as best you can, around the sample tube area to keep out any light. Press the **Sample** button for the sample to start being inputted into the flow cytometer's system. Adjust your Default Display Count on the computer to around 10,000and your Event Limit to around 2,000 (these can be found within the leftmost panel on your screen). Press the **Boost** button to get the sample flowing quicker into the system until you start seeing your Events Per Second change, as well as data starting to show up on your dot plots. Adjust your Sample Offset (and, if necessary, your PMT thresholds) until you get a consistent flow of about 200 events per second.

53 This is the sample that you will use for identifying your "nuclei" gating and to also perform a "double check" of whether we have correctly identified "single nuclei" within our gating

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process. We will use a 520/35[405] vs. 450/50[405] plot to identify and gate nuclei (this is generally called the “Nuclei” gating plot). We will also use a 450/50[405] vs. FSC plot to perform a “double check” to see if our gating correctly identifies single nuclei.

(*Note*: We usually don’t use this plot for gating, but it could be potentially used for gating, if you wanted to.)

54 For the “Nuclei” gating, make a plot with 520/35[405] on the y-axis and 450/50[405] on the x-axis. Now, using one of the gating tools in the upper toolbar, click and drag to create a square gate in this plot and name it “Nuclei.” The nuclei should have a high fluorescence at this wavelength, so they are typically found in the upper right-hand corner of the plot. Usually, you will actually see a distinct separate population here in that upper right quadrant. Those are your nuclei. Use your judgment to make this gate. We have now, theoretically, identified and gated our nuclei for our sorting sample. Sometimes, the populations can look a bit different (**Figure 10**). Make sure that your “Nuclei” gate is nestled under your “Singlets” gate by placing it within that “Singlets” AND “Cells” gates order (**Figure 10**).

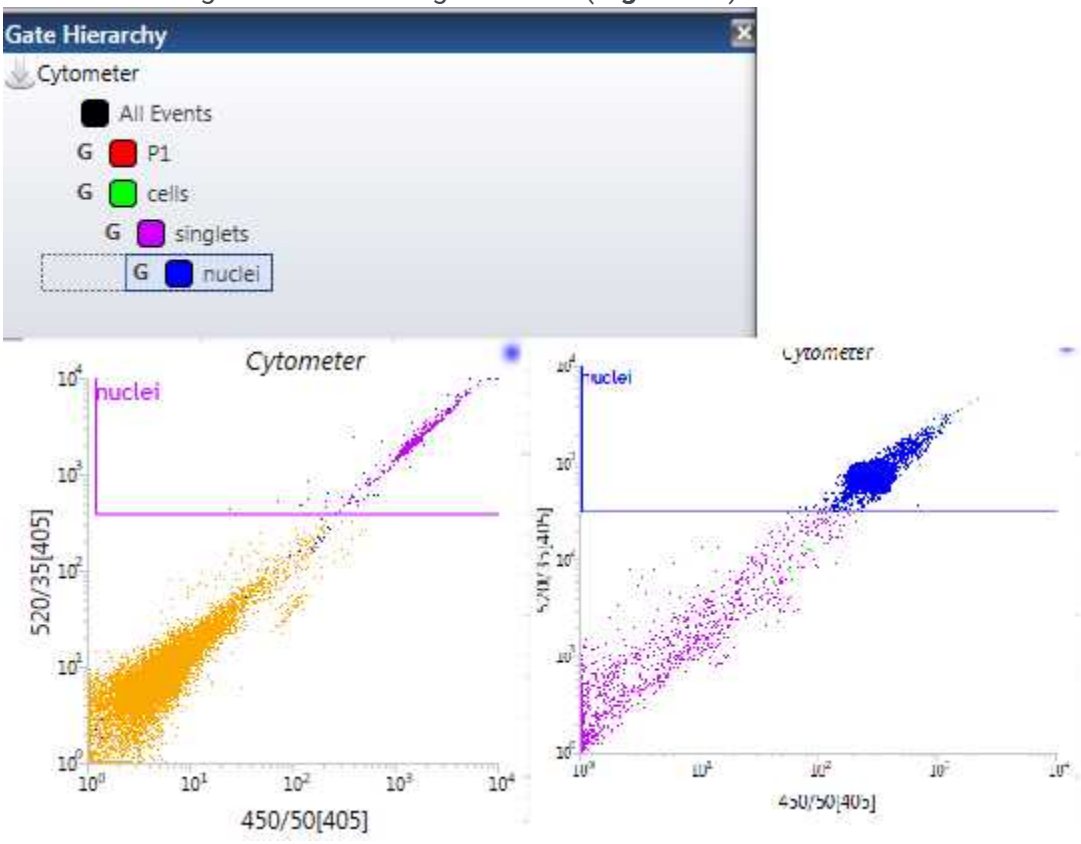

Figure 10. Two examples of roughly what your “nuclei” plot should look like. Within the top figure, you can see exactly how your gating hierarchy should be organized in the sortware software.

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55 For the single nuclei “double check” plot, make a plot with 450/50[405] on the y-axis and FSC on the x-axis. Use this plot to observe the different populations. You should see at least 2 distinct populations start to populate this plot. The dots that are clustered high on the y-axis and also somewhat low on the x-axis are our single nuclei. If we have gated both our “Singlets” and our “Nuclei” gates properly, this distinct clustered population should be the same color as the nuclei gate that we made. If this is the case, then we have, theoretically, successfully gated Single Nuclei (**Figure 11**).

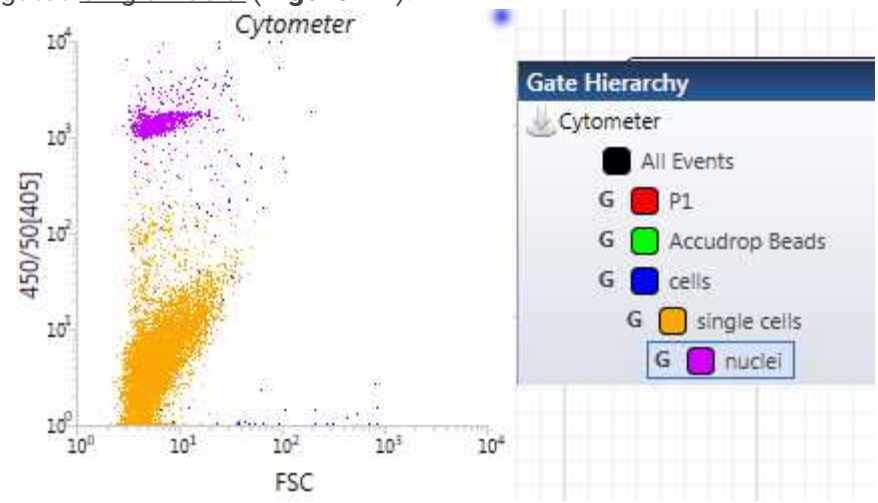

Figure 11. An example of roughly what your “double check” plot to check whether you have successfully gated all of your potential “Single Nuclei” should look like. Within the right part of the figure, you can see exactly how your gating hierarchy should be organized in the sortware software.

56 Now, remove sample #2 (the Hoechst Control sample) from the sample tube area and click **Backflush** to clear out the system of the sample. Wait 30 seconds before clicking **Backflush** again. We will now move onto sample #3 (the Nuclei Sample sample). Remove any tube from the sample tube area within the machine and click **Backflush**. Wait about 30 seconds and then click **Backflush** again to turn off the backflushing.

57 Lightly vortex your already filtered Nuclei Sample with the vortexer (setting at 1) for about 10 seconds (Make sure that it is concealed with aluminum foil when you do this, as it has fluorescent dye in it). Bring the sample back to the flow cytometer, remove the aluminum foil, place it in the sample tube area, and lock it in place. Place some aluminum foil, as best you can, around the sample tube area to keep out any light. Press the **Sample** button for the sample to start being inputted into the flow cytometer’s system. Adjust your Default Display Count on the computer to around 10,000and your Event Limit to around 2,000 (These can be found within the leftmost panel on your screen). Press the **Boost** button to get the sample flowing quicker into the system until you start seeing your Events Per Second change, as well as data starting to show up on your dot plots. Adjust your Sample Offset (and, if necessary, your PMT thresholds) until you get a consistent flow of about 200 events per second.

58 This is the sample that you will use for identifying and gating for your “NeuN positive nuclei” and your “NeuN negative nuclei.” We will use a 530/40[488] vs. 450/50[405] plot to identify and

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gate both of these.

59 For the “NeuN positive” and “NeuN negative” gating, make a plot with 530/40[488] on the y-axis and 450/50[405] on the x-axis. Now, using one of the gating tools in the upper toolbar, click and drag to create 2 square gates in this plot and name them “NeuN positive” and “NeuN negative”. The NeuN positive nuclei should be found high on the y-axis and high on the x-axis, while the NeuN negative nuclei should be found low on the y-axis but exactly as high on the x-axis. Sometimes, you will actually see distinct separate populations in the right-most upper and lower quadrants. You will, usually, be able to see at least one clear distinct population that has high fluorescence on the x-axis, but sometimes it can be a bit hard to differentiate between the two populations when it comes to y-axis. Use your own judgment to make this gate. We have now, theoretically, identified and gated our NeuN positive and NeuN negative nuclei for our sorting sample and are officially able to begin sorting. Make sure that your “NeuN positive” and “NeuN negative” gates are nestled under your “Nuclei” gate by placing it within your “Nuclei,” “Singlets,” AND “Cells” gates order (**Figure 12**).

(Tip from our lab: If you cannot really differentiate between the 2 populations, try to make a cutoff gate (at the lowest) just above 10^2 on the y-axis. Denote anything found below this point as NeuN negative and anything found above this point as NeuN positive).

(*Note*: Sometimes, the populations can look a bit different (see **Figure 12**).)

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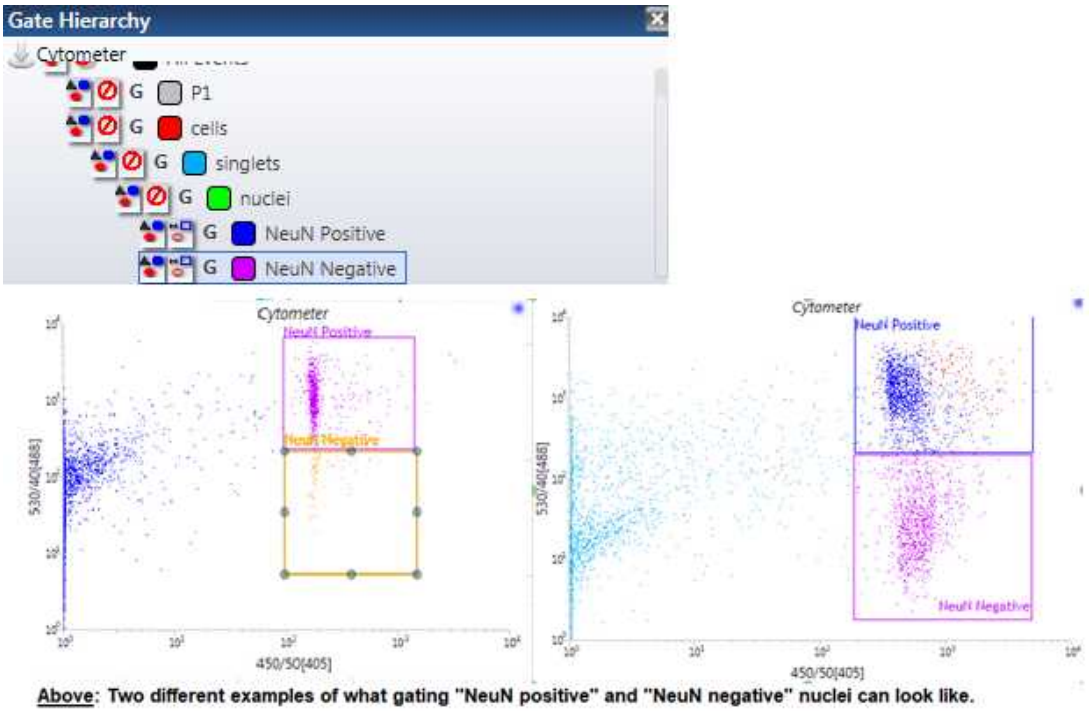

Figure 12. Two examples of roughly what your “NeuN positive Nuclei” vs. “NeuN negative Nuclei” plot should look like. Within the top figure, you can see exactly how your gating hierarchy should be organized in the sortware software.

60 Make sure that your “Nuclei” gate is nestled under your “Singlets” gate by placing it within that “Singlets” AND “Cells” gates order (**Figure 12**).

61 For the sorting, within the Sort Layout window, make sure that you have your Sort Mode set to 1.0 Drop Enrich. Select your “NeuN positive” gate for the left panel and your “NeuN negative” gate for your right panel (**Figure 13**). Make sure both are set to *Unlimited*. Place your labeled Lo-Bind autoclaved tubes into the corresponding sort collection tube positions within the machine.

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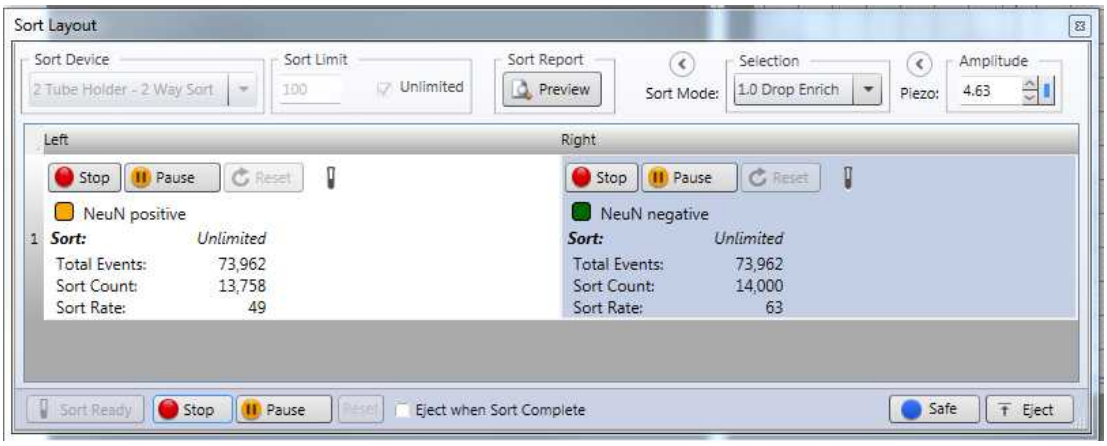
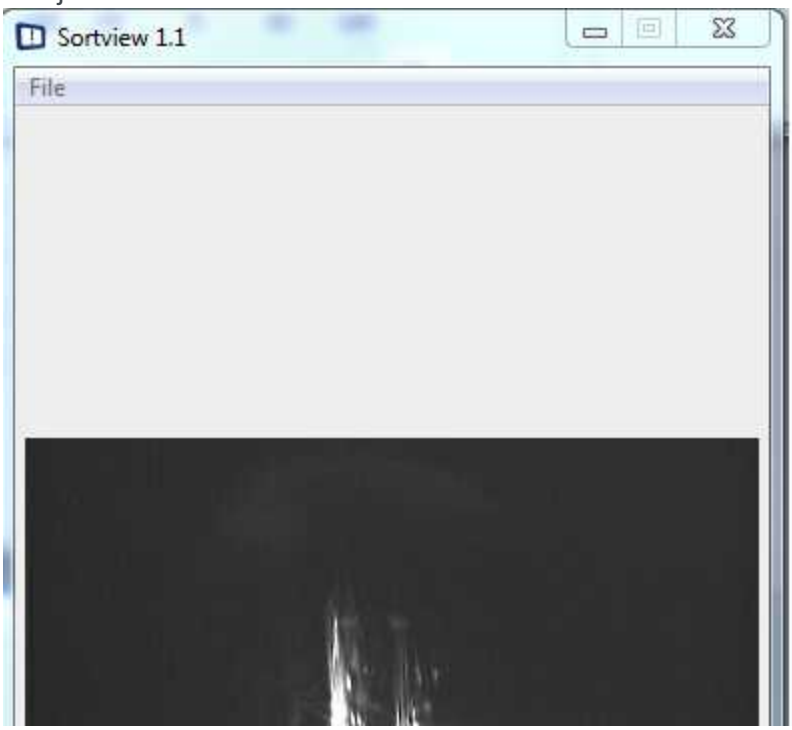


Figure 13. A screenshot of how the sort settings should be set up within the Sort Layout portion of the sortware software.

62 Click **Sort Ready** and then **Start**.

63 Watch the monitor and make sure to adjust your Sample Offset to keep your Event Rate to around 100-300 events per second. (Tip: Generally, keeping the Sample Offset at around 0.15 to 0.17 psi usually works well). Make sure that you can see droplets falling into your collection tubes. You should also be able to see small flickers of light on either side of the center waste stream on the 3rd camera picture on the computer screen (this means that droplets should be being deflected into your collection tubes) (**Figure 14**). Finally, make sure that you monitor your highest droplet breakoff point throughout the whole sorting process, in case it needs to be adjusted.



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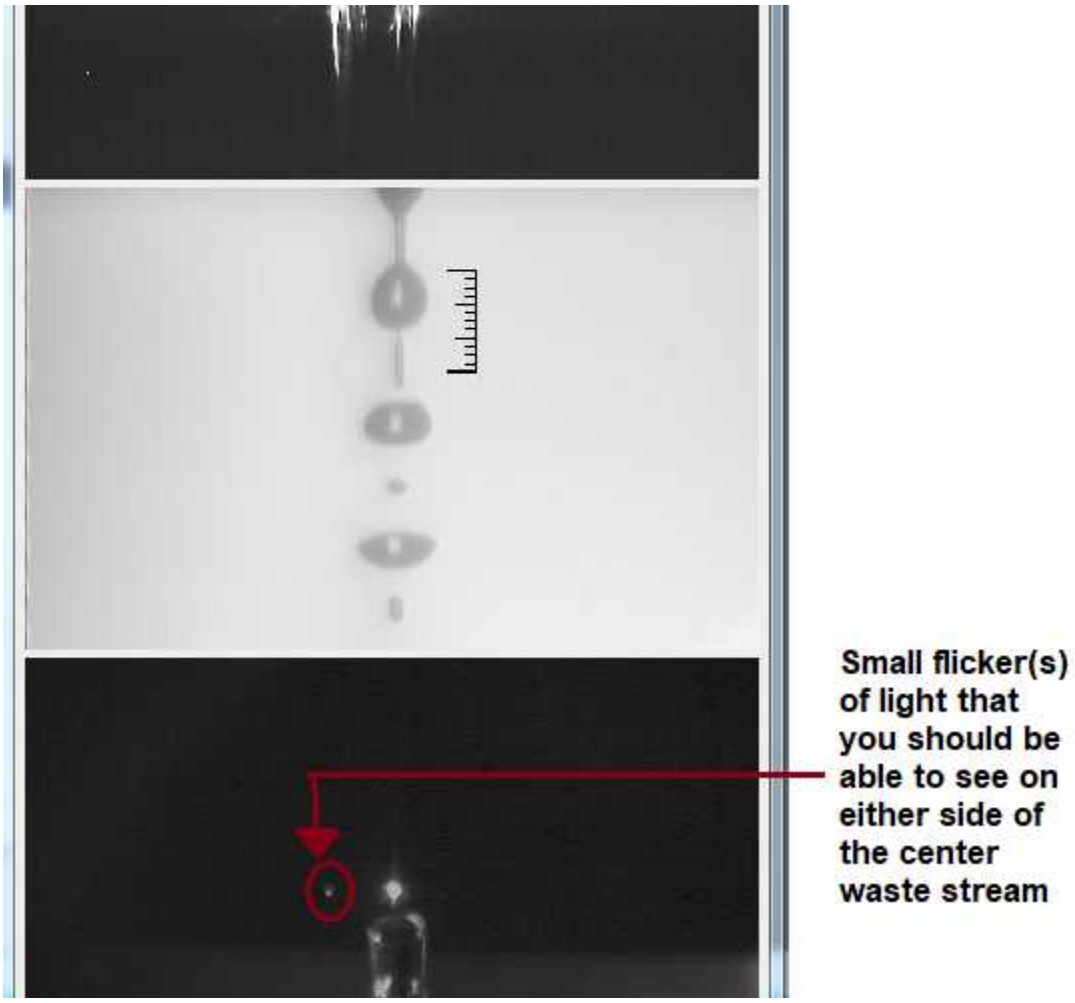

Figure 14. A screenshot of the Sortview portion of the sortware software that you can look at to make sure that your sorting is occurring properly. Always make sure that the hatch-marks portion of the middle figure contains that first breakoff point of the droplet; this may need to be adjusted sometimes during the sort, so make sure to be vigilant about this. Also make sure that you are seeing small flickers of light occurring on each side of the center waste stream. These flickers mean that your sample should be sorting into either the corresponding left or right tubes.

64 Stay by the computer until the whole sample has been successfully sorted.

(*Note*: You may need to replace the collection tubes as sometimes they get full halfway through. If this happens, you can hit the **Pause** button (NOT stop!) at the bottom of the Sort Layout panel, replace your tubes, and then click the **Resume** button at the bottom of the Sort Layout panel.)

65 Remove the now empty Nuclei Sample tube from the sample tube area of the flow cytometer and click **Backflush**. Wait 30 seconds before clicking **Backflush** again.

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66 Remove your collection tubes and place them *immediately* **On ice** .

67 Turn **OFF** the cooling system by pressing the **OFF** button on the front of the monitor of the machine.

68 Based on the day of the week you are performing this, or whether you will be performing anymore sorting the rest of the week, choose which type of shutdown you would like to perform accordingly.

68.1 *Wet* Shutdown (use if you will be using the machine the next day again or anytime within the rest of the week)

68.2 *Dry* Shutdown (use if it is the end of the week and it will not be used on the weekend or if you will not be using the machine for 2-3+ days).

Part D. Post-FANS Handling and Storing [~45 minutes] 45m

69 Pre-Chill/Fast Temp your centrifuge to 4^{o}C. **4°C**

70 Visually inspect the final volume of liquid found in both the resultant NeuN+ and NeuN- nuclei sample tubes. Based on the final volume, place the corresponding amounts of solutions into each tube:

(*Note*: Always round up (e.g., if there is around 550 or 600uL in a tube, round up your estimation to 1mL of liquid in your Eppendorf tubes)

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A	B	C	D
Post-FACS Nuclei Sample Volume	**1.8M Sucrose Solution Volume**	**1M CaCl2 Solution Volume**	**1M Mg(Ac)2 Solution Volume**
500uL	100uL	2.5uL	1.5uL
1.0mL	200uL	5uL	3uL
1.5mL	300uL	7.5uL	4.5uL

71 Split sample(s) into 2 tubes, if needed, once solution is mixed together (*GENTLY*!) with sample **On ice** .

72 Mix gently by inverting the sample tube several times.

73 Incubate **On ice for 15 minutes** .

74 Centrifuge your samples at 3000 rpm for 15 minutes at 4°C. **3000 rpm, 4°C, 00:15:00** 15m

75 Gently and carefully discard supernatant without disturbing the pellet in the tube (if any) **On ice** (see **Figure 15**).

(*Note*: The pellet sometimes looks like a very small smudge on the side of the tube. Other times, the pellet can look like a small floating "blob", so be very careful when discarding the supernatant.)

Citation: Alexandra Del Favero-Campbell, Gabriel R Fries Isolation of Neuronal Nuclei From Human Frozen Post-Mortem Prefrontal Cortex Brain Tissue Using Fluorescence-Activated Nuclei Sorting (FANS) For Methylation Analyses
https://dx.doi.org/10.17504/protocols.io.dm6gpbmbjlzp/v1

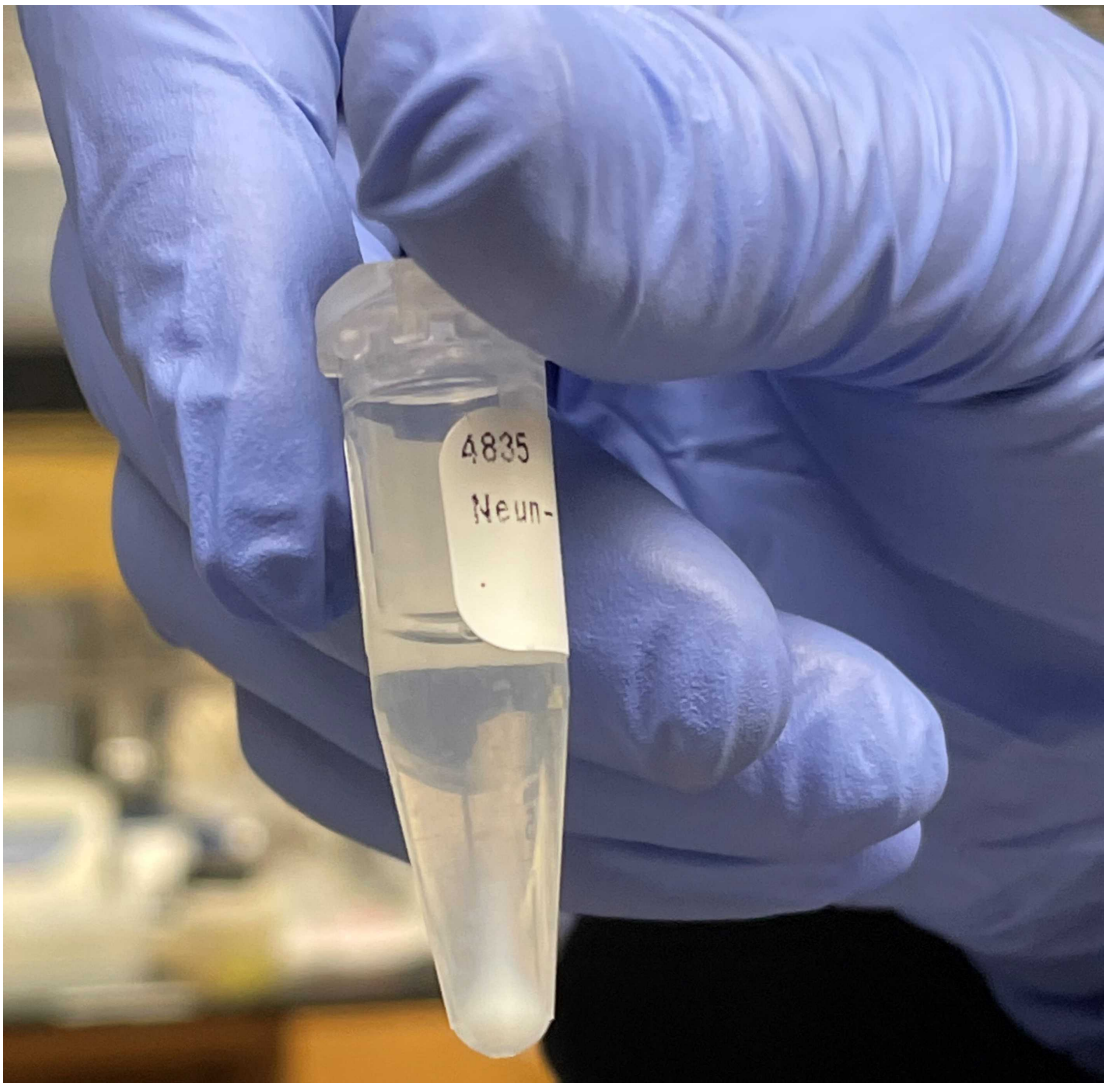

Figure 15. Image of what resulting pellet should look like after final centrifugation after nuclei sorting.

76 At this point, you can either freeze the pellet samples at **-80 °C** for further processing using a **Qiagen AllPrep DNA/RNA Micro Kit** for simultaneous DNA and RNA extraction (using the Qiagen Manufacturer's Protocol Guidelines) at a later date.

(*Note*: This should be done no more than 3 days later.)

77 If planning on performing analysis at a much later date, it is recommended to dissolve the pellets in **200 µL** of Bambanker freezing medium using the following steps:

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77.1 Dissolve pellet in 200uL of Bambanker freezing medium by allowing pellet to dissolve **On ice for a few minutes** and then gently pipetting solution up and down gently.

77.2 Transfer solution to a cryovial **On ice** and place it in a *Mr. Frosty* at **-80 °C overnight** .

77.3 Store sample at **-80 °C** for future use and analysis.

General Recommendations

78
- Make sure to lubricate the ring around the sheath fluid tank lid with Super O-Lube to get a good seal.
- Make sure Drop Frequency is set to around 38.9 to 39.2. Try not to adjust this too much. Instead, you can adjust the height of the drops (using the silver knob on the top-right of the machine) and the Piezo Amplitude.
- Try to keep flow rate at a maximum of 1000 events per second and your efficiency at around 80+ %.
- Once your Nuclei Sample tube has finished going through the FACSJazz, it can be also very useful to hit the **Backflush** button and let some sheath fluid run back into the tube. This will release some nuclei that were caught in the tubing system, but did not get analyzed or sorted. You can then hit the **Sample** button again and analyze these nuclei that were not initially analyzed. You can do this as many times as you want, until you do not see any more nuclei being detected on the monitor. We, generally, repeat this step about 3-5 times to try to get as many nuclei as possible, while also not allowing this to take too much time and contribute to potential sample degradation.
- 1000x DAPI (1.4uL) can be used in place of Hoechst 33342 dye, if not available. Both dyes have been used and tested in this protocol. However, it can be recommended in some cases, such as when using DAPI, or cases where the spectrums of your chosen fluorescent antibodies overlap, that auto-compensation is performed *before* performing any gating or sorting. This can be done using this protocol by using the Unstained Control sample as your Negative Control, the Hoechst Control sample as your first single Positive Control, and either Alexa Fluor-488 mixed with UltraComp eBeads (following the bead manufacturer's protocol) or staining part of the Unstained Control with Alexa Fluor-488 as your second single Positive Control.
- If for any reason you are not able to perform FANS the *same* day as your immunostaining procedure, you can dissolve your pellets from Part B, Step #19 in 200uL-1000uL of Bambanker freezing medium using the following steps:

1. Dissolve pellet in 200uL-1000uL of Bambanker freezing medium by allowing pellet to dissolve for a few minutes on ice and then gently pipetting solution up and down gently.

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2. Transfer solution to a cryovial and place it in a Mr. Frosty overnight at -80^{O}C.
3. Store sample at -80^{O}C for future use and analysis.
4. Make sure to keep wrapped in aluminum foil as they will be photosensitive!

- In previous testing of protocols using the Qiagen AllPrep Kit, we have found it helpful to incubate both of our DNA and RNA filters in their designated elution buffers for about 3-5 minutes before performing the final centrifugation.
- For DNA, we elute in 40uL of 64^{O}C Buffer EB, and for RNA, we elute in 14uL of RNAse-free water. We have also found it useful to perform 2 separate elutions, which can be later combined and further concentrated using a SpeedVac machine.

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