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Fluorescence-activated nuclei sorting (FANS) on human post-mortem cortex tissue enabling the isolation of distinct neural cell populations for multiple omic profiling

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Complex Disease Epigenetics Group

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ABSTRACT

Increased understanding of the functional complexity of the genome has led to growing recognition about the role of epigenetic/transcriptional variation in health and disease. Current analyses of the human brain, however, are limited by the use of “bulk” tissue, comprising a heterogeneous mix of different neural cell types. Because epigenetic processes play a critical role in determining cell type-specific patterns of gene regulation it is important to consider cellular composition in regulatory genomic studies of human post-mortem tissue, and there is a need for methods to purify populations of specific cell-types. Furthermore, the valuable nature of human post-mortem tissue means it is important to use methods that maximize the amount of genomic data generated on each sample. This protocol describes a method that uses fluorescence-activated nuclei sorting (FANS) to isolate and profile nuclei from multiple different human brain cell-types from frozen post-mortem tissue. This protocol can be used to robustly purify populations of neuronal (NeuN+ve), oligodendrocytes (SOX10+ve), microglia (IRF8+ve) and other glial origin nuclei (NeuN-ve/SOX10-ve/IRF8-ve) from adult post-mortem frozen brain, with each tissue sample yielding purified populations of nuclei amenable to simultaneous analysis of i) DNA modifications (via bisulfite sequencing / array), ii) histone modifications (via CUT&Run-seq), iii) open chromatin analysis (via ATAC-seq), and iv) gene expression (via RNA-seq).

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KEYWORDS

FANS, post-mortem brain, nuclei, flow cytometry, anti-SOX10, anti-IRF8, anti-NeuN, nuclei sorting

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MATERIALS TEXT

	Supplier	Catalogue No
BD FACSAria™ III Cell Sorter	BD Biosciences	648282-23
Sorvall WX 80+ Ultracentrifuge	Thermo Scientific™	75000080
7mL Dounce Tissue Grinder	DWK Life Sciences	357542
PA Thin-walled ultracentrifuge tubes	Thermo Scientific	03699

Table 1: Specifications of the equipment required for FANS protocol

Reagent name	Supplier	Catalogue No
D-Sucrose (Molecular Biology)	Fisher Scientific	10638403
Calcium chloride (CaCl2) anhydrous, granular	Sigma-Aldrich	C1016-100G
Magnesium acetate (Mg(Ace)2), 1M aq. soln	Alfa Aesar	J60041
UltraPure™ 0.5M EDTA, pH 8.0	Invitrogen	15575020
UltraPure™ 1M Tris-HCl Buffer, pH 8.0	Fisher Scientific	15568025
1,4-Dithiothreitol (DTT) - crystalline powder	Sigma-Aldrich	3483-12-3
Triton™ X-100	Sigma-Aldrich	T9284
UltraPure™ DNase/RNase-Free Distilled Water (ddH20)	Fisher Scientific	12060346
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A9647-500G
PBS Phosphate-Buffered Saline (10X) pH 7.4	Fisher Scientific	10722497
RNasin® Plus RNase Inhibitor	Promega	PAN2615
TRIzol™ LS Reagent	Invitrogen™	11588616
BD FACSDiva CS&T Research Beads	BD biosciences	655051
BD FACS™ Accudrop Beads	BD biosciences	345249
BD FACSFlow™ Sheath Fluid 20L	BD Scientific	342003
BD FACS Clean Solution	BD Scientific	15875858
BD FACSRinse Solution	BD Scientific	340346

Table 2 **:** Specification of reagents required for FANS protocol

Lysis Buffer (LB)	
0.32M Sucrose	5.47 g
5mM CaCl2	250 µL
3mM Mg(Ace)2	150 µL
0.1mM EDTA	10 µL
10mM Tris-HCl, pH 8	500 µL
1mM DTT	17 µL
0.1% Triton X-100	50 µL
Adjust with ddH2O to	50 mL
1.8M Sucrose Solution (SS)	
1.8M Sucrose	30.78 g
3mM Mg(Ace)2	150 µL
1mM DTT	17 µL

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10mM Tris-HCl, pH8	500 µL
Adjust with ddH2O to	50 mL
5% BSA Solution (BB)	
BSA	200 mg
Dissolve in 1x PBS	4 mL
Optional: RNasin® Plus RNase Inhibitor	2 µL / 1mL
Staining Buffer (SB)	
5% BSA	400 µL
10X PBS	400 µL
ddH2O	3.2 mL

Table 3 : Recipes for buffers and solutions required

Supplier	Thermo Scientific™
Model	Sorvall™ WX 80+
Rotor	TH-641
Speed	25,200 RPM / 108670.8 x g
Acceleration	9
Deceleration	5
Temperature	4°C

Table 4: *Ultracentrifuge specification and conditions*

Antibody	Preconjugated	Supplier	Cat No	Dilution
Hoechst 33342	--	Abcam	ab228551	1:500
Anti-SOX10	to NL577	R&D systems	NL2864R	1:10
Anti-NeuN	to Alexa Fluor488	Millipore	MAB377X	1:1000
Anti-IRF8	to APC	Invitrogen	17-9852-82	1:150

Table 5: List of antibodies required for FANS protocol

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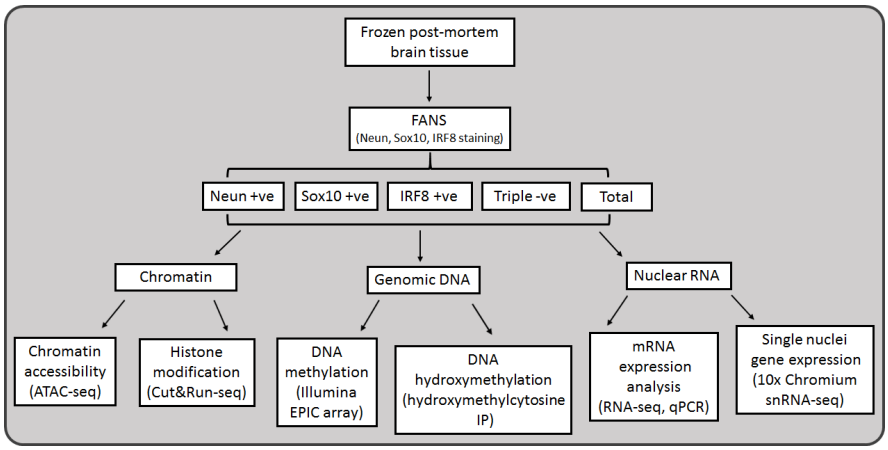

Figure S1. Schematic overview showing the array of downstream applications for which FANS-processed nuclei are suitable

ABSTRACT

Increased understanding of the functional complexity of the genome has led to growing recognition about the role of epigenetic/transcriptional variation in health and disease. Current analyses of the human brain, however, are limited by the use of "bulk" tissue, comprising a heterogeneous mix of different neural cell types. Because epigenetic processes play a critical role in determining cell type-specific patterns of gene regulation it is important to consider cellular composition in regulatory genomic studies of human post-mortem tissue, and there is a need for methods to purify populations of specific cell-types. Furthermore, the valuable nature of human post-mortem tissue means it is important to use methods that maximize the amount of genomic data generated on each sample. This protocol describes a method that uses fluorescence-activated nuclei sorting (FANS) to isolate and profile nuclei from multiple different human brain cell-types from frozen post-mortem tissue. This protocol can be used to robustly purify populations of neuronal (NeuN+ve), oligodendrocytes (SOX10+ve), microglia (IRF8+ve) and other glial origin nuclei (NeuN-ve/SOX10-ve/IRF8-ve) from adult post-mortem frozen brain, with each tissue sample yielding purified populations of nuclei amenable to simultaneous analysis of i) DNA modifications (via bisulfite sequencing / array), ii) histone modifications (via CUT&Run-seq), iii) open chromatin analysis (via ATAC-seq), and iv) gene expression (via RNA-seq).

Nuclear prep for FACS separation (using SOX10, IRF8, NeuN and Hoechst)

1 The protocol below yields at least 1,000,000 NeuN +ve, 1,000,000 SOX10 +ve, 400,000 IRF8 +ve (when the population is present) and 200,000 triple negative (NeuN-ve/SOX10-ve/IRF8-ve) nuclei per **500 mg** of frozen human post-mortem cortex tissue. Recovery might vary from sample to sample due to high inter-sample variability (brain collection, cortex sub-areas, fat content of tissue sectioned, and white to grey matter ratio)

Refer to Materials-***Table 1*** for details about the equipment required and to Materials-***Table 2*** for specifications of reagents required.

1.1 *Solution and buffer preps*

- *Lysis Buffer (LB)*
- *Sucrose Solution (SS)*
- *Staining Buffer (SB)*

Solutions should be kept at **4 °C** or **On ice** . Refer to Materials-***Table 3*** for recipes of solutions and buffers.

NOTE 1 – LB and SS can be prepared a week in advance, with DTT added on the day of use. Solutions should be stored at **4 °C** once made.

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NOTE 2 – SB should be prepared fresh each day

NOTE 3- Samples are homogenised as bulk tissue using a **6 mL** Dounce homogeniser and then equally divided into three ultracentrifuge tubes.

1.2 *Nuclei isolation*

1.Pre-cool the ultracentrifuge to **4 °C** **00:30:00** before starting this stage of the protocol.
2.All buffers and the Dounce homogenisers should be pre-cooled on ice.
4.Add 1mM DTT to the SS and LB according to the recipe (i.e. 17µL per 50 mL of SS/LB)
5.Transfer 3 mL LB to the homogeniser per 500mg human brain tissue
6.Add the dissected tissue sample into the homogeniser
7.Wait 3-5 minutes before douncing the tissue to allow the sample to defrost

To reduce heat caused by friction, the Dounce homogenisation step should be performed on ice with gentle strokes, and care should be taken to avoid foaming.

NOTE 1 – Using the "TIGHT" pestle helps reduce the number of strokes required to reach full tissue disruption.

NOTE 2 - The number of strokes required to fully homogenise the tissue may vary between samples due to heterogeneity in cellular composition, lipid content, and the amount of connective tissue.

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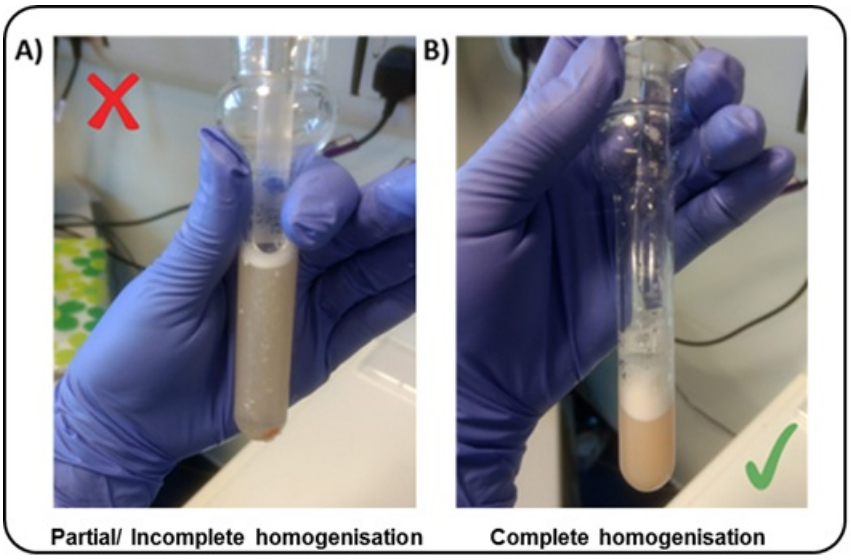

Figure 1 Example of brain tissue sample **A)** only partially homogenised **B)** complete homogenisation.

8.Transfer 8 mL SS (1.8M) to PA thin-walled ultracentrifuge tubes
9.Carefully overlay with tissue homogenate (1 mL per tube) - using a P1000 pipette, releasing slowly down the side of the tube
10.Overlay with another 1 mL LB – do not worry about disrupting the homogenate phase
11.Balance opposite tubes by weight with 1x PBS using a fine microbalance
12.Perform ultracentrifugation for **00:45:00** (see ***Table 4*** for centrifuge specification and conditions)

1.3 *After Ultracentrifugation step*

1. Aspirate supernatant leaving 1-2 mL of the solution in the tube along with the pellet.
2. Pour off any remaining supernatant, taking care not to dislodge the pellet (90-degree inclination of the tube). If the pellet is hard to see, it is okay to leave 100-200 µL solution in the ultracentrifuge tube
3. Re-suspend pellet in SB (1 mL), gently pipette up and down
4. Let samples sit on ice for **00:15:00 at least** (**Blocking step**)
5. Transfer volume into 2 mL tubes
6. Rinse out ultracentrifuge tubes in order to maximise nuclei collection by adding 1 extra mL of SB per tube, pipetting up and down several times, and transferring into the 2 mL tubes
7. **Washing step:** **1.0 x g** for **00:05:00** , **Room temperature**
8. Discard supernatant (pipetting off gently)
9. Re-suspend each nuclei pellet in fresh SB (500 µL)
10. If the sample was split then pool together pellets from the same sample (Final Volume = 1.5 mL)
11. Add DNA dye (Hoechst, 2 µL/1 mL) and mix thoroughly via inversion.
12. Pipette out 200 µL of nuclei solution for the Unstained Control (Hoechst dye only) and transfer to a new 2 mL tube
13. Bring the volume up to 1 mL for the Unstained tube with fresh SB
14. Replace the 200µL taken from the Stained" tube with 200µL of fresh SB (Final Volume = 1.5 mL)

1.4 *Immunostaining*

1.Add the following three antibodies (Ab) to Stained tube (1.5ml):

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- SOX10 pre-conjugated antibody (1:10 dilution) – [150 µL Ab]
- NeuN Alexa488 (1:1000) – [2 µL Ab]
- IRF8 pre-conjugated antibody (1:150) - [10µL Ab]

Refer to ***Table 5*** for specifications of the antibodies used

2.Incubate tubes for **01:30:00** on the rotor (speed=14 max) at **4 °C** , keeping the tubes in the dark
3.Washing step: **1.0 x g** for **00:05:00** , **Room temperature** (both "Stained" and "Unstained" tubes)
4.Discard supernatant (by pipetting off)
5.Re-suspend in fresh SB (1 mL for the Unstained tube, 1.5-2 mL for the Stained tube - depending on pellet size)

Fluorescence-Activated Nuclei Sorting (FANS)

2 For machine start-up, CST and Accudrop calibrations refer to BD FACSAria III User's Guide for guidance and troubleshooting. The following instructions describe FANS using BD FACSAria III. Other FACS platforms can be used but might require modifications to the protocol.

2.1 *General Gating Parameters*

For each sample, load stained and unstained tubes individually for data acquisition. A preliminary qualitative analysis of the data acquired is essential to select the appropriate gating strategy to maximize the nuclei capture while excluding unnecessary debris and to ensure optimal signal/noise ratio.

Gating Parameters (X-axis:Y-axis):

FSC-A:SSC-A (Size, cell granularity or internal complexity)
SSC-W:SSC-A (to gate out doublets)
FSC-A:DAPI-A (to gate the single nuclei population)
DAPI-A:FITC-A (to gate NeuN stained nuclei)
DAPI-A:PE-A (to gate SOX10 stained nuclei)
DAPI-A:APC-A (to gate IRF8 stained nuclei)
FITC-A: PE-A (to visualize the distribution of triple staining)

The SOX10 +ve population is gated as a "daughter" population from the NeuN-ve fraction. The IRF8+ve population is gated as a "daughter" population of the SOX10-ve fraction. Refer to **Figure 2** for a visualization of the gating strategy.

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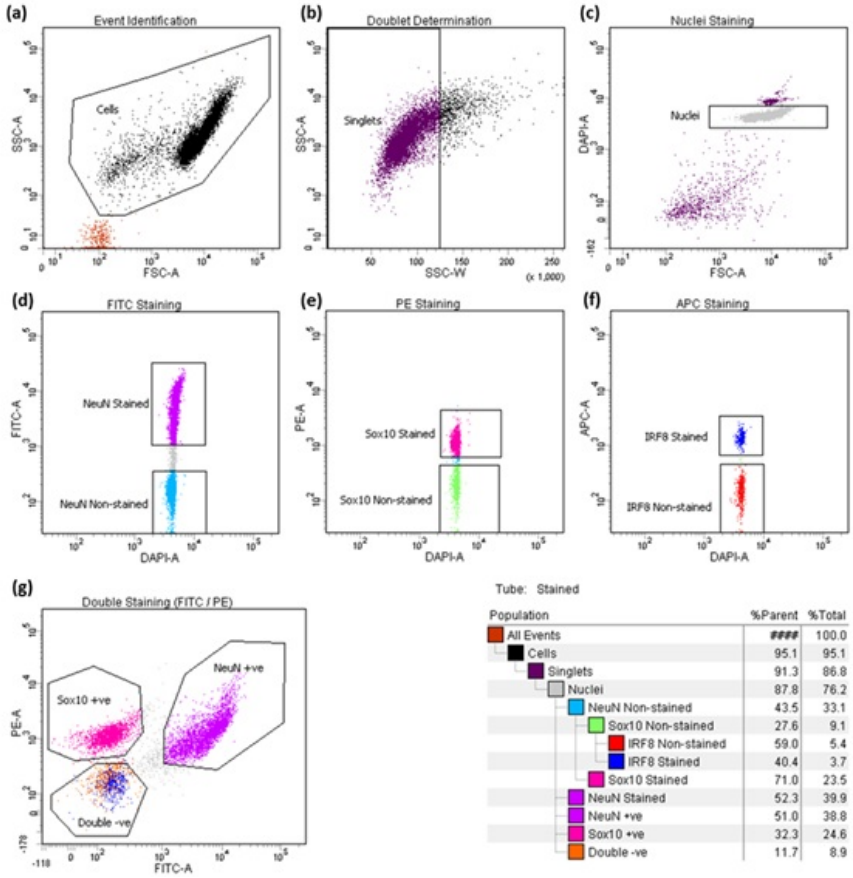

Figure 2. FANS gating strategy. **(a)** Particles smaller than nuclei (black dots) were eliminated with an area plot of forward-scatter (FSC-A) versus side-scatter (SSC-A), with gating for nuclei-sized particles inside the gate (box). **(b)** Plots of height versus width in the side scatter channel are used for doublet discrimination with gating to exclude aggregates of two or more nuclei. **(c)** Doublet discrimination gating was used to isolate nuclei determined by subgating on Hoechst 33342. (d, g) Subsequent scatterplots discerning **(d)** NeuN-Alexa Fluor488–conjugated antibody staining (purple) **(e)** Sox10 NL577-stained nuclei (darkpink) **(f)** IRF8-APC stained nuclei (darkblue) **(g)** the distribution of the three main nuclei subpopulations identified through triple staining strategy (NeuN +ve, neurons; Sox10+ve, oligodendrocytes; double–ve, glia). The resultant hierarchical colour key ensures that only nuclei that are positive or negative for staining with the NeuN and/or Sox10/Irf8 antibody are passed through each gating condition.

2.2 *Data recording settings*

In line with the experiment design, FSC, SSC, DAPI, FITC, APC and PE are the parameters for which voltage values may need to be slightly adjusted due to experiment/ inter-sample variability.
It is advisable to set the threshold value between 200 and 500 during data recording. Moreover, in the acquisition dashboard tab, we recommend setting **Events to Record** ≤3000, **Event to display** ≤1000 and **Flow Rate** = 1.0 (1,000 events per second) in order to increase the accuracy of signal detection.
The flow rate can be increased during sample collection to reduce the sort speed (ideally max events per second =1,500 for a 100-micron nozzle). However higher flow rates impact the data resolution and

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accuracy of events detection, and subsequent sorting of cellular fractions (see BD FACSAria III User's Guide for details).
During analysis, recorded data is displayed in plots, while gates are used to define populations of interest for selection. **Figure 3** shows a representative example of the two most common outcomes we often observe.

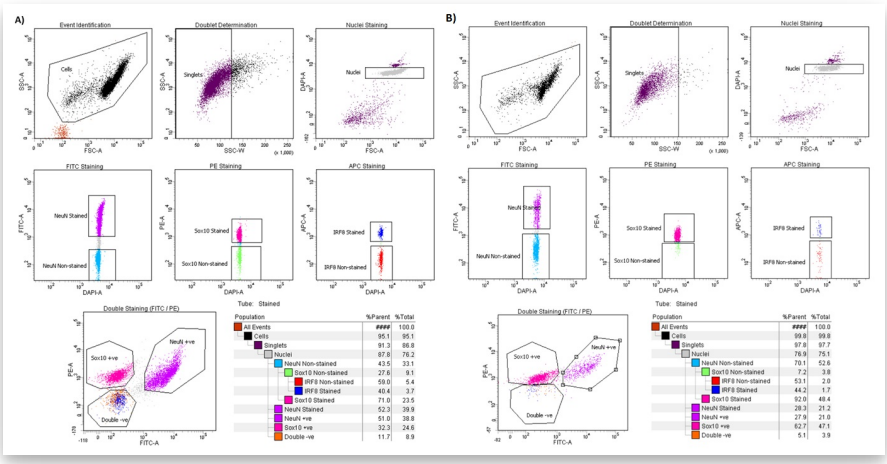

Figure 3. Representative example of inter-individual variability.
The data shown here are derived from two different control prefrontal cortex specimens of comparable age, gender, and brain collection which were processed in parallel following the same procedure. **A)** Optimal sample separation and abundant IRF8+ve fraction vs **B)** poor sample separation and completely missing a double negative population or an IRF8+ve population.

2.3 *Sample Collection*

1.LoBind Tubes (Eppendorf, Cat No:30108051) are required to collect nuclei (to maximize sample recovery of nucleic acids by significantly reducing sample–to–surface binding).
2.Collected fractions can be used directly for downstream applications (e.g. DNA/RNA extraction, chromatin shearing) or stored in **-80 °C freezer**
3.If you are collecting for DNA or RNA, as soon as the number of events desired (200,000 for DNA, 300,000 for RNA) is reached, transfer the tubes **On ice** , do not hold them at **Room temperature** .

NOTE – During collection, it is crucial to regularly pause the sorting to mix the two phases in order to preserve the integrity of resulting RNA preparations.

For RNA extraction, LoBind Tubes should each contain 500µL of pre-chilled TRIzol™ LS Reagent (Fisher Scientific, Cat No: 11578616) prior to sorting.

4. Keep samples **On ice** for the entire duration of the sorting
5. Lightly vortex sample tubes to make the mixture homogeneous (not clumped) before loading the tube into the FACS chamber
6. Load the **UNSTAINED** control tube into the chamber first and proceed with nuclei collection (for

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DNA, 200,000 events; for RNA, 300,000 events; for ATAC-seq 50,000 events). Refer to Materials - ***Figure S1*** for the full range of applications sorted nuclei can be used.
7. Proceed by collecting **STAINED** tube by simultaneously sorting for NeuN, SOX10 and IRF8 or Double Negative.

NOTE 1- 1 µg of genomic DNA at least is expected from 500 mg tissue. For optimal recovery of high-quality genomic DNA from FANS sorted nuclei we recommend this extraction protocol

NOTE 2 –The IRF8+ve population may not be detectable in every brain sample processed (high inter-individual variability); when it is, it represents between 5-10% of the total sample, therefore yielding often insufficient material for multiple assays.

General Recommendations for the user

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For every new experiment we recommend performing the following steps:

1. When loading your tube into the FACS machine, run the unstained / IgG control sample first as this aids in setting the baseline parameters
2. Check your **event rate** in the **Acquisition Dashboard** window. If it is greater than 1500 evt/s turn down the "**flow rate**" or unload and dilute the sample further. If less than 100 evt/s, turn up the "**flow rate**" (don't exceed a flow rate of 5.0 if possible, as the instrument is less focused and more inaccurate at higher flow rates)
3. In the **Acquisition Dashboard** window choose the appropriate "**stopping gate**" and "**storage gate**" (when working with nuclei, set as "Nuclei" and "All events" respectively)
4. Choose the range of "**events to record**" and "**events to display**" that best suits your purpose (≥ 5000 for both is advised)
5. Under the **"threshold"** tab in the Cytometer window, change the threshold (should be set for FSC) so that any small events in the bottom corner of the FSC vs SSC graph (caused by general cell debris and dust) are no longer shown. The threshold should not be set too high so that it causes an arbitrary, artificial cut-off through the left side of your population but not so low that small events caused by debris/dust are visible (ideally between a threshold 200-500).
6. Under the **"parameters"** tab in the Cytometer window, adjust the "**FSC**" and "**SSC"** values to get your population sitting in the centre of the FSC vs SSC graph (a re-adjustment of the "**threshold**" may be required at this point). It is essential to select **"restart**" each time any of the parameters are changed to update the events being displayed to ensure only events are recorded under the new settings.
7. Adjust or draw a new gate in the FSC vs SSC plot to encompass the population of interest.
8. Look in the scatter graph of SSC-A vs SSC-W (if you opened a blank experiment you will need to draw one). Right-click on the graph and check it is only displaying the events encompassed by your previous FSC vs SSC gate. Adjust or draw a gate for SSC A vs SSC W to encompass all of the main population to the left of the graph and exclude outliers to the right (these are doublets and other cell debris clumps)
9. Under the **"parameters"** tab in the Cytometer window adjust parameters for the fluorochromes selected so the unstained / IgG control sample sits close to 0 for the fluorochrome on a graph of FSC vs fluorochrome.
10. Load the stained samples and check the stained population has a clear increase in signal for the fluorochrome in comparison to the unstained (signal should not exceed 10^{x4}). Several minor re-adjustments of the fluorochrome's **"parameters"** may be necessary for the stained sample at this stage. If so, the unstained / IgG control has to be reset and re-recorded.

WARNING – Do not change parameter settings between samples you wish to compare, if you do you will

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need to re-record all samples using the changed parameters.

11. Select the correct option for the collection device in the **Sort Layout** window (we recommend "**4-Way Purity**" for general collection)
12. Regularly check your "**Efficiency**" in the **Sort Layout** window value. Between 80-100% is ideal, 70% is acceptable if less than 70% either the sample is too concentrated or you are sorting a rare population. Although the "**flow rate**" in the **Acquisition Dashboard** window can be increased to make the sort quicker, faster flow rates are less efficient.
13. Check the **"Electronic abort rate"** (N° errors /sec) and **"Electronic abort count"** (Tot N° of errors) at the bottom of the **Acquisition Dashboard** window. These parameters measure potential miss-sorts (different from efficiency as efficiency measures undetermined drops which are directed to the "**Waste**" and therefore lost but do not contaminate).**"Electronic abort rate"** should be <1% of total events per second.
14. For long sorts, gate positions should be regularly monitored, especially for stained populations as fluorochromes lose intensity over time and the population can shift towards the unstained. Gates can be moved during long sorts to compensate.

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