Targeted Fluorescence-Assisted Nuclei Sorting from Post-Mortem Human Brain

HGEN 396 Human Genetics Research Project

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Abstract

Objective: Previous work at the McGill Group for Suicide Studies identified 26 cell types in the dorsolateral prefrontal cortex area via single nucleus RNA-sequencing. Oligodendrocyte precursor cell 2 (OPC2) and deep-layer pyramidal excitatory neuron 7 (Ex7) in the dorsolateral prefrontal cortex had the largest number of differentially expressed genes between control and MDD. The project proposes a targeted fluorescence-assisted nuclei sorting (FANS) protocol to separate specific cell types in archived human brain tissue for further study.

Methods: Two techniques – immunostaining and RNAScope in situ hybridization – are evaluated as a method to attach fluorophores for specificity. Samples are then processed and segregated by flow cytometry in a fluorescence-activated cell sorter (FACS).

Results: Neither method was able to achieve signals that can be detected by the FACS machine. A third method involving probe synthesis was stopped due to the COVID-19 public health emergency along with further testing with RNAScope ISH and immunostaining. For further directions in the protocol development, Probe-seq with its designed/synthesized probes should be tested and more antibody testing for specificity should be continued.

1. Introduction

The human brain contains numerous neuronal and glial cell types. The subtypes are identified by their differences in function, structure, organization, electrophysiology and connectivity (Luo, et al., 2017). To investigate specific neuron and glial interactions in the central nervous system, isolating the desired subtypes is beneficial. Traditional methods distinguish cell types by sequencing mRNA transcripts which has identified unique transcriptional profiles (Hawrylylcz, et al., 2012). Nevertheless, it is restricted by the fact that RNA signatures can be influenced by the environment (Mo, et al., 2015).

An alternative candidate is using epigenetics and epigenomic markers – the modifications of gene activity and expression – to isolate and study specific cell types in the brain. Epigenetic changes, DNA methylation in particular, is heritable and transferred through cell divisions. Furthermore, studies by Kozlenkov et al., Luo et al., Mo et al, and others indicate that DNA methylation is cell type-specific and is stable across individuals. DNA methylation influence gene expression – and thus the transcriptome – by affecting the interactions with DNA of chromatin proteins and specific transcription factors (Razin & Cedar, 1991). As brain function depends on the interaction of cell types and their transcriptomes, investigating DNA methylation in a cell-type specific manner can yield clinical relevance in neuropsychiatric diseases (Kozlenkov, et al., 2018). Although epigenetics is also influenced by the environment (Tammen, Friso, & Choi, 2013), the timescale for transcriptional changes is usually much shorter than epigenetic changes (Rando & Verstrepen, 2007). Consequently, cells transition between more transcriptional states than epigenetic states; the more persistent epigenetic changes between two groups of interest can provide complementary information to the transcriptional differences. To link and determine which cell subtypes correlate with an observed DNA methylation pattern, a protocol is first needed

to isolate and sort for specific neurons, glial cells, and their respective nuclei/genetic information from the archived human brain tissue.

In *situ* hybridization (ISH) is a class of nucleic acid hybridization method where a complimentary gene sequence probe localizes a desired gene sequence in the nuclei. The complimentary gene sequences can be conjugated with a biomarker to distinguish attachments. An example is Probe-Seq (Amamaoto, et al., 2019), where specific cell types are isolated using RNA as the defining feature: dissociated nuclei are labeled using fluorescent in *situ* hybridization (FISH) (Amamaoto, et al., 2019). Once labels are specified, a FACS machine can act as a nuclei-sorter to physically separate and purify cells of interest based on the fluorescent optical properties conjugated to the RNA probe. Flow cytometry dissociates nuclei by suspending them in a fluid and injecting the suspension through a laser beam, where the light-scattering and emission is characteristic to the fluorescent markers and their respective band of wavelengths.

Another contemporary method to separate cell subtypes is FIN-Seq: using immunohistochemical properties of cells to isolate cell type-specific nuclei (Amamoto, et al., 2019). Frozen Immunolabeled Nuclei Sequencing (FIN-Seq) enables the usage of archived frozen tissue samples from the human brain. The antibody staining method exploits proteins to identify specific cell types and their transcriptome. Fluorescent biomarkers can be attached to cell type-specific antigens to segregate desired cell types.

2. Purpose and Objectives

The project aims to develop and optimize a cell type-specific fluorescence-assisted nuclei sorting protocol (FANS) for a deep layer excitatory pyramidal neuron cluster (Ex7) and an immature oligodendrocyte precursor cell cluster (OPC2), previously identified by single-nucleus RNA-seq from post-mortem human dorsolateral prefrontal cortex tissue (Brodmann area 8/9).

Previous work from the McGill Group for Suicide Studies identified twenty-six cell-types from single nucleus RNA-sequencing (snRNA-seq) (Nagy, et al., 2018). snRNA-seq is a method for profiling gene expression in cells. Sixteen of the twenty-six identified displayed differential expression between cases with major depressive disorder and control. Ex7 and OPC2 cells had the largest number of differentially expressed genes between control and MDD (Nagy, et al., 2018). This indicates changes in gene expression of the cells, which has downstream impacts cell on function and to the functional anatomy of the dorsolateral prefrontal cortex. Once the target cell types are isolated, we want to measure differences in DNA methylation in the specific cell populations between cases of MDD and control. Understanding the DNA methylation changes between the cases and controls will complement the differential gene expression findings of the cell types from Nagy et al. Hence, the project proposes a protocol to isolate the two cell-types with the highest number of differentially expressed genes.

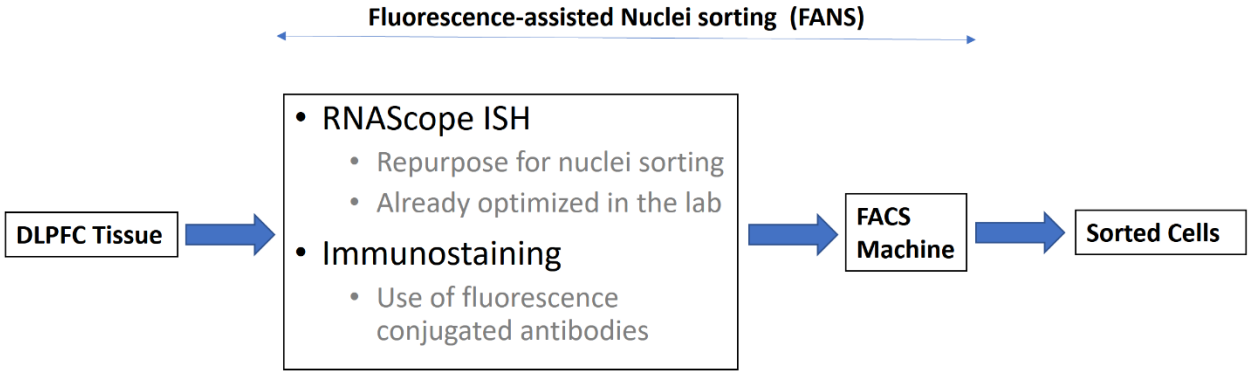


Figure 1. The overarching project plan. Two methods were considered: RNAScope ISH is optimized and used in the Turecki Lab (MGSS), and it would be beneficial to repurpose it for nuclei sorting. Immunostaining to attatch flourophores to proteins is also investigated. The FACS machine separates the cells of interest according to the emission and absorption spectra. The process does not result in expression of flourescent properties in the cells, but rather attches a flourophore in a cell-type specific manner.

As seen in *Figure 1*, to utilize the FACS machine, a protocol to go from DLPFC tissue to a fluorophore-attached nuclei is needed. RNAScope is a proprietary RNA *in situ* hybridization assay where a probe can be fluorescently labeled for visualization under a microscope or be used to detect in a FACS machine. It theoretically attains target-specific signals as it is designed to have

two independent probes to hybridize to the target sequence in tandem for signal amplification to occur (Wang, et al., 2012). The probability for two independent probes to hybridize to a non-specific target sequentially is highly unlikely and hence target-specific signals are possible (Wang, et al., 2012). Thus, RNAScope ISH for nuclei fluorescent labeling is explored.

Immunofluorescence is the second technique considered. Although immunostaining cannot indicate if the nucleus contains the mRNA for a particular protein, it can indicate if the nucleus contains the particular protein; The presence of mRNA within the nucleus is indirectly inferred. Antibody specificity to its antigen is exploited to target fluorescent dyes to proteins within the sample. Various antibodies can bind to the same epitope – the specific region an antibody recognizes in an antigen – with varying levels of binding strength (Ladner, 2007). Consequently, multiple antibodies (primary and secondary) from various biomedical companies are tested for their effectiveness and specificity for Ex7 and OPC2. Transcription factors are targeted for antibody staining. As they are localized in the nucleus during transcriptional regulation, targeting transcription factors is beneficial for nuclei sorting. However, most transcriptional factors which show enriched expression in OPC2 cells compared to other cell types are also present in OPC1 in the data obtained from Nagy et al., shown in *Figure 2*. The majority (61.5%) of transcription factors are commonly expressed, and it was determined it would be difficult to differentiate between OPC1 and OPC2. Similarly, Ex6 and Ex7 also share common transcription factors expressed, and would be difficult to separate using the few marker genes identified. Accordingly, the protocol targets for OPC1 and OPC2 combined, and Ex6 and Ex7 combined together. Proteins encoded by *FOXP2* and *RORB* were selected to target Ex6 and Ex7. The FANS protocol needs to target RORB and FOXP2 simultaneously, because if only one of the genes is selectively targeted, there are other cell types that also express them at varying levels. Hence to assure validity

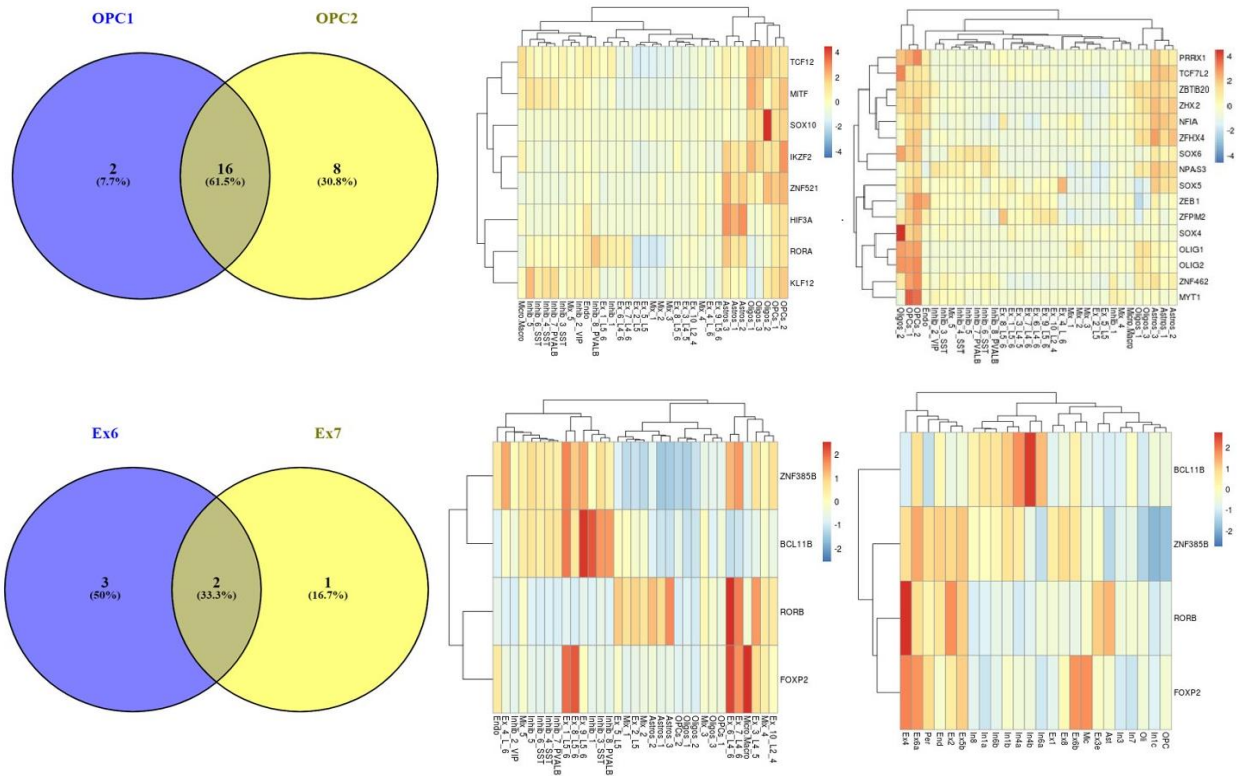


Figure 2. Expression results from unsupervised graph-based clustering, identifying unique cell types in the DLPFC of the human brain. Data obtained from Nagy et al. and other prior analysis in the Turecki lab, MGSS. X-axis: cell type; Y-axis: cell markers. FOXP2 and RORB are candidates used to isolate Ex6 and Ex7. Although Micro.Macro also has high expression of FOXP2 and an expression of RORB, it can be distinguished from Ex6 and Ex7 by gating in the FACS sorting process, only collecting the highest combining fluorescence, preventing Micro.Macro from the population.

of nuclei sorting, both are targeted at the same time. Other cell types that also express both FOXP2 and RORB can be distinguished from Ex6 and Ex7 in the FACS sorting process: the machine can collect the highest combined fluorescence detected, preventing other cell types from being included in the separated population. To isolate OPC1 and OPC2 in particular, a successful targeting of MYT1, PRRX1, or ZFPM2 should be sufficient to prevent other cell types from inclusion in the population.

3. Materials and Methods

Nuclei buffer and 30% sucrose buffer

The nuclei buffer is composed as follows: 10nM PIPES (pH 7.4), 10nM KCL, 2mM $MgCl_2$, 1mM DTT, and 10x Protease Inhibitor Cocktail (PIC). To produce 40mL, the following components are

mixed: 8mL 50mM PIPES, 400µL 1M KCl, 800µL 100mM $MgCl_2$, 40µL DTT, 400µL 10x PIC, and 30.36 autoclaved water. The 30% Sucrose in NB for 20mL is derived from 6g Sucrose + 20mL nuclei buffer. Nuclei buffer and 30% sucrose buffer solutions are stored in ice.

Post-mortem brain samples

Post-mortem brain samples are provided by the Douglas-Bell Canada Brain Bank. Frozen archived samples are stored at -80°C. Brodmann Area 8 and 9 are used to test RNAScope ISH and immunostaining specificity. 50mg of tissue is used per test. Brain tissue is dissected on weighing dishes directly above dry ice, and is weighed on a digital scale. A razor and a spatula are used to cut brain samples. 70% ethanol is used to sterilize the tools used. Tissue is homogenized in 500µL Nuclei Buffer with 0.1% Triton-X 100 for 30 seconds.

Nuclei extraction with immunostaining

On a 10mL round bottom cell culture tube, 4mL of 30% sucrose buffer is added. The homogenized lysate is transferred on the 30% buffer. The tube is spun at 4°C, 2000 rpm (800g) for 20 minutes. After, the supernatant and interphase are decanted, ensuring to that the nuclei at the bottom of the tube are not disturbed. The nuclei pellets are resuspended in 3mL of nuclei buffer. The tube is spun again at 4°C, 2000 rpm (800g) for 20 minutes. The supernatant is removed again, and the nuclei is resuspended in 500µL 1x PBS with 0.1% Tween 20 and 100µL of 10% BSA in an Eppendorf tube. This should result in 1.67% of final BSA concentration.

If the antibody is fluorescent or sensitive to light, aluminum foil is used to prevent interactions with light. Primary antibodies are added according to *Appendix A*. The temperature, the antibody animal & clonal type, and concentrations are listed in *Appendix A*. Primary antibodies are incubated for 2 hours with rotation to gently agitate and mix. Secondary antibodies – if used – are then added at concentrations listed in *Appendix A* and incubated for 1 hour with rotation.

Hoescht is added to stain nuclei. The nuclei preparations are then transported to FACS facility on ice, sorted using appropriate settings, into 5mL Eppendorf tubes and transported back to the lab on ice. Samples are frozen at -20°C afterwards.

Nuclei extraction with RNAScope ISH

The process for nuclei extraction with RNAScope ISH follows the steps for immunostaining until the second supernatant removal. To fixate and permeabilize, the nuclei pellets are instead suspended in 1000μL of 4% PFA (or 10% NBF) at 4°C with rotation. They are transferred to a 1.5mL tube and spun at 4°C at 500g for 3 minutes. Supernatant is discarded with an appropriate pipette. Nuclei is resuspended in 1000μL 1x PBS with 0.1% Tween 20, incubated for 10 minutes at 4°C with rotation to permeabilize the nuclei; supernatant is discarded afterwards spinning. The nuclei are resuspended in 1000μL 1x PBS to wash, and spun at room temperature 500g for 3 minutes, discarding supernatant afterwards. For the final RNAScope ISH protocol, instead of using extracted nuclei, we have performed RNAScope staining on cryosectioned shavings of tissue to attempt to increase the yield.

The sample is resuspended in the targeted probes mix for 2 hours at 40°C with rotation. With all 150μL preamplifiers and amplifiers used (AMP1, 2, 3, and 4 depending on the version), 1mL of wash buffer is added before, spun at room temperature at 800g for 5 minutes, discarded with a pipette. They are incubated for 30 minutes, 15 minutes, 30 minutes, and 15 minutes at 40°C with rotation respectively. Then Hoechst is added for nuclei staining; the samples are transported and sorted in the FACS facility.

NeuN protocol validation test

To validate the protocol's capability to attach fluorophores, antibodies against a neuronal biomarker (NeuN) and a layer V cortical neuron marker CTIP2 were used. The NeuN-CTIP2

validation test follows the same procedure for nuclei extraction with immunostaining. The NeuN antibody is directly conjugated to a fluorophore (Alexa 700) and is effective at attaching fluorescence compared to fluorescent secondary antibodies. The CTIP2 antibody (attached to the Alexa 488 fluorophore) stains cortical neuron nuclei localizing in layer V.

Obtaining gene-specific BED file (probe design)

Following the instructions in Probe-seq v1.3 protocol, Brian Beliveau's OligoMiner is downloaded from their public GitHub repository (https://github.com/beliveau-lab/OligoMiner). Genome-wide probe set for *H. sapiens*, "Complete Genome" with "Balance" setting is downloaded from Ting Wu's OligoPaint (https://oligopaints.hms.harvard.edu/genome-files). All downloaded files are stored in the same directory of choice. At the University of California Santa Cruz Genome Browser website (https://genome.ucsc.edu/), "Human GRCh38/hg38" under "Genomes" tab is selected.

The gene of choice – *FOXP2, RBFOX3, RORB, MYT1, SOX10* – in this context, is entered on the search parameter. "Table Browser" is clicked and the following settings are chosen. Group: Genes and Gene Predictions; Track: NCBI RefSeq; Table: UCSC RefSeq (refGene); Region: position; Output format: BED; File type returned: plain text; TRUE on "Exons plus 0 bases at each end", and the file is download. All isoforms but one in the file are removed. In terminal, intersectBed is ran with option of -f 1. If BED file has a "+" orientation, reverse compliment is generated with probeRC.py from OligoMiner with -f option.

4. Results

RNAScope ISH

The first nuclei extraction with RNAScope ISH targeted *SLC17A7* (Solute carrier family 17 member 7) gene. The gene encodes for the vesicular glutamate transporter 1, and immunostaining the VGLUT1 for cerebral cortex tissue has been shown to work (Alonso-Nanclares & Defelipe,

2005). Hence the expected result under the microscope would be visible signals for excitatory neurons. The experimental procedure for pre-treatment and amplification introduced multiple washes to the sample. Evidently, when the sample was prepared to be analyzed under the microscope, the overall nuclei pellet yield was low with no pellet visible by eye. Seen in *Figure 3*,

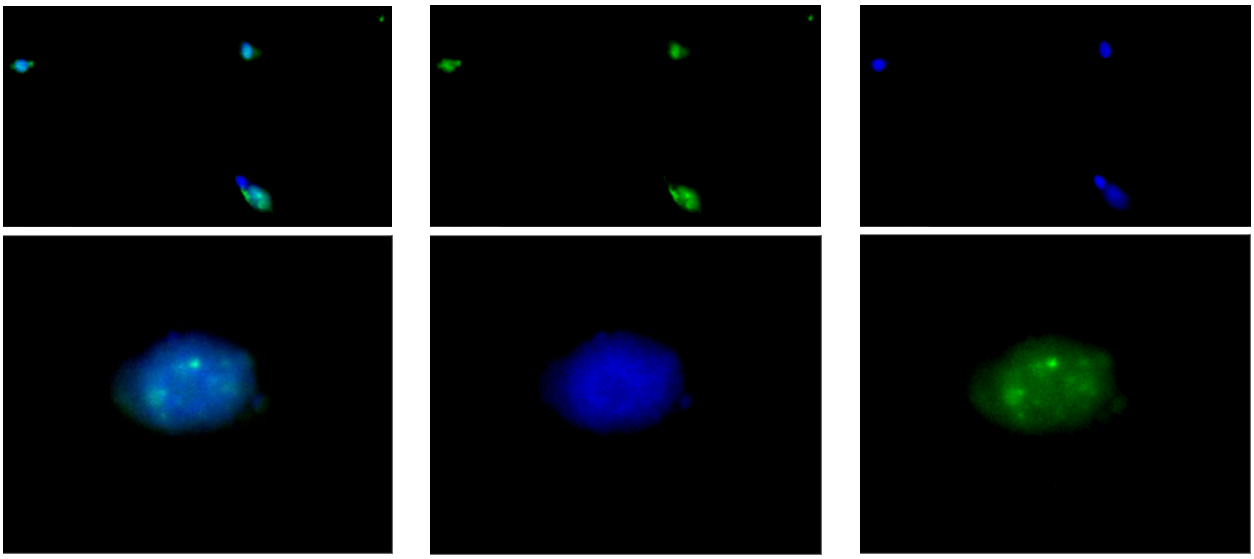


Figure 3. Images from the first RNAScope ISH nuclei extraction experiment. Results are uncertain if the nuclei that are fluorescing green are expressing SLC17A7 or not. There is low yield of nuclei. Some nuclei are fluorescing green whereas other are not. We have insufficient information to judge whether there is specific binding.

the number of nuclei per field of view was very low although some signal was detected under the microscope. While we were able visualize some nuclei, which showed green fluorescence (indicative of *SLC17A7* staining) and ones that did not, it was not possible to determine whether the signal detected was specific or non-specific.

As the original protocol includes numerous washes and incubation periods, the experiment duration is long. We then switched to an earlier version of the RNAScope ISH protocol to reduce the number of washes. The second nuclei extraction with RNAScope ISH reduced the number of washes; a larger nuclei pellet was visible, shown in *Appendix B*. The signal for this experiment was weak; exposure was increased to capture images. Negative and positive control probes were tested as well. *SLC17A7* was tested for the same reason on the first RNAScope attempt. The second

RNAScope ISH experiment also targeted *GAD1*, the gene for the glutamic acid decarboxylase. The gene is used to identify GABAergic inhibitory neurons as GABA synthesis is controlled by enzymes derived from GAD1 and GAD2, transcript isoforms (Tao, et al., 2018). Therefore, as targeting SLC17A7 theoretically selects glutamatergic excitatory neurons with VGLUT1, and GAD1 gene is present for GABAergic inhibitory neurons, the fluorescent signals should be mutually exclusive for excitatory and inhibitory neurons. As seen in *Appendix B*, there are no detectable signal as none of the nuclei were stained. Mutually exclusive identification could not be achieved.

The final nuclei extraction RNAScope ISH experiment had three changes. It was the final RNAScope ISH tested; the experiment was split over three days; and UBC, the gene for Polyubiquitin-C, was tested as well. UBC is highly abundant and expressed in neurons to maintain neuronal ubiquitin homeostasis (Hallengren, Chen, & Wilson, 2013). SLC17A7 and GAD1 are targeted for the justification of mutually exclusive targeting of excitatory and inhibitory neurons. Shown in *Figure 4*, no signal for SLC17A7 is visible in the far-red channel. A signal for GAD1 in the red channel is visible. We cannot conclude if the signals are mutually exclusive. Moreover, the negative and positive controls cannot be distinguished in this experiment. Consequently, non-specific binding is concluded. The instances where both signals are combining can be attributed to dust and other noises. Moreover, even the non-specific signal we saw under the microscope could not be detected by the FACS machine. The issue is not a problem of yield but rather the failure of fluorescence detection by the FACS machine from the samples.

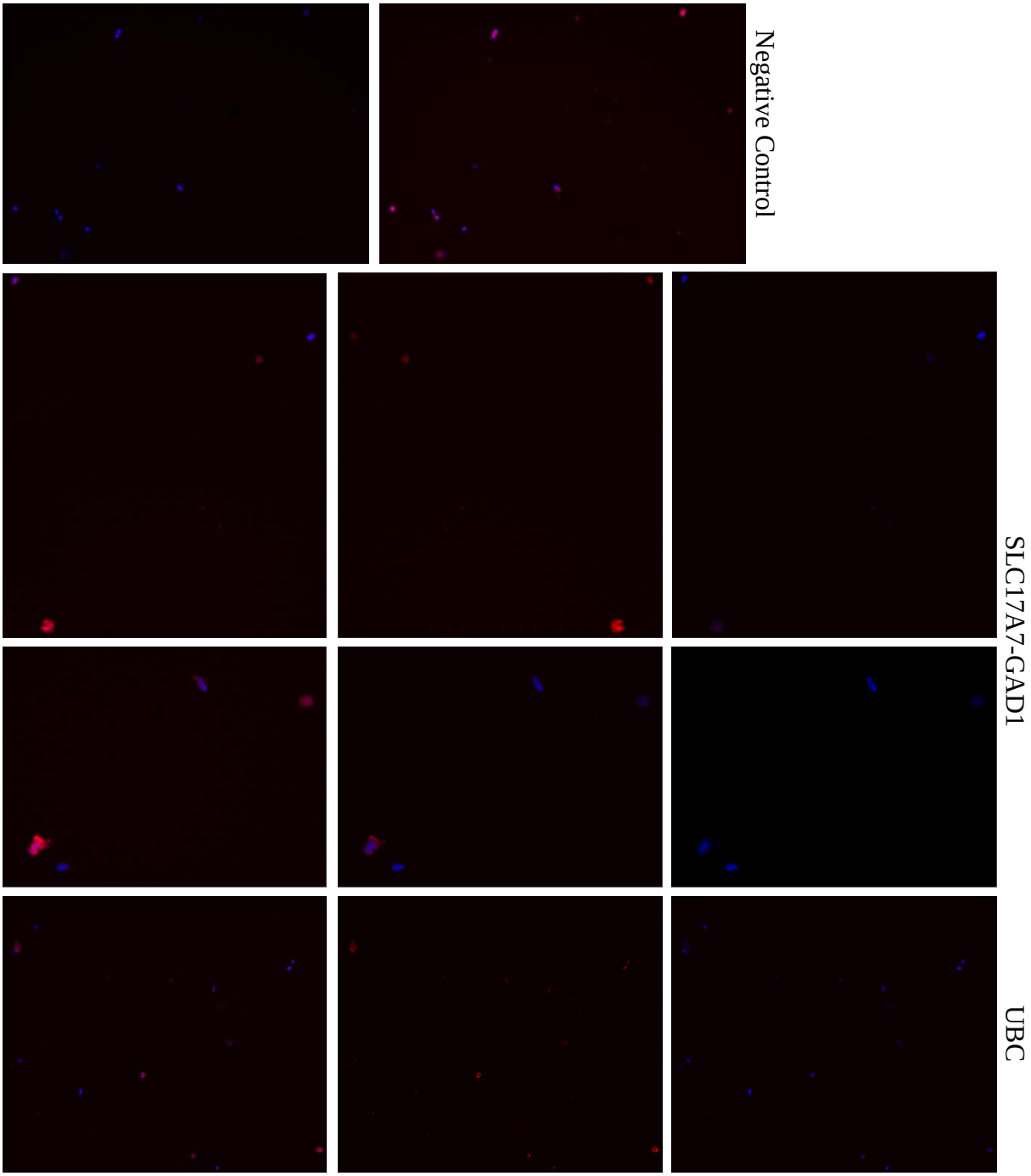


Figure 4. Images under the microscope for the third nuclei extraction with RNAScope ISH attempt. First row is negative control. Second and third is for targeting SLC17A7-GAD1. Fourth row for UBC. A signal for GAD1 in the red channel is visible, however the signal for SLC17A7 in the far-red channel is not visible.

Immunostaining

The first immunostaining experiments used antibodies for both cell types. For OPC1 and OPC2 cell type, MYT1, PRRX1, and ZFPM2 were individually targeted with rabbit polyclonal antibodies

and anti-rabbit Alexa 488 as the secondary antibody in 1/2000 concentration. Detailed information of antibodies tested is in *Appendix A*. Staining for FOXP2 for Ex6 and Ex7 was also attempted with mouse monoclonal and rabbit polyclonal primary antibodies with anti-rabbit and anti-mouse Alexa 488 as secondary antibody in 1/2000 concentration respectively. Under the microscope, the signals showed non-selective binding for all antibodies targeting OPCs. The second antibody staining attempt tested for RORB for Ex6 and Ex7. Rabbit polyclonal primary antibody with Alexa 488 secondary antibody was tested. The initial archived tissue mass was increased from 50mg to 100mg to test if it would be a feasible change to obtain higher nuclei pellet count. No significant signals are detected under the microscope. Non-specific binding was evident.

The third antibody testing involved retesting the FOXP2 mouse monoclonal antibody with two different groups of samples; half of the samples tested were treated with formalin, and varying the concentration of antibodies was evaluated for its efficiency. However, this dataset is null as the primary antibody used was actually a mouse monoclonal antibody but an anti-rabbit Alexa 488 secondary antibody was mistakenly used. The correct secondary should have been anti-mouse instead of the anti-rabbit used. This mistake was noticed and retested on the final immunostaining experiment. The final nuclei extraction with immunofluorescence tested for FOXP2. In addition, half of the samples were incubated at 4°C to observe any improvements in fluorescence specificity. Goat polyclonal and mouse monoclonal primary antibodies were tested. The mouse monoclonal antibody was retested due to incorrectly incubating with anti-rabbit secondary antibody on the third antibody staining attempt. Observed in *Appendix C*, no significant results are observed under the microscope as all four samples displayed non-specific binding to nuclei.

NeuN-CTIP2 (positive control)

Consequently, as all attempts of nuclei extraction with RNAScope ISH and immunofluorescence did not achieve cell-specific signals, the possibility of a procedural error was considered. Hence an additional test to assess the validity of the protocol and its capability in attaching fluorophores was done, targeting Hexaribonucleotide Binding Protein-3 (NeuN).

NeuN is a neuronal nuclear antigen that is frequently used in immunohistochemical research (Korzhevskiy & Gusel'nikova, 2015). Although few neuron cell types are not recognized by NeuN antibodies – cerebellar Purkinje cells, Golgi cells, etc. – pyramidal neurons are detected by them (Mullen, Buck, & Smith, 1992). CTIP2 is a transcription factor that can be used as a marker for layer V excitatory neurons (Costa & Müller, 2015). The transcription factor is highly expressed in layer V and expressed in layer VI (Costa & Müller, 2015). To verify that we were performing the nuclei extraction and antibody staining protocols correctly, we used fluorophore conjugated NeuN and CTIP2 antibodies which were being used for a different project in the lab. Two samples under identical conditions were tested. The tested tissue was also from the Brodmann area 8-9. The results from the FACS machine are in *Figure 5* and *Appendix D.* As seen in *Figure 5* and *Appendix D*, NeuN with Alexa Flour 700 and CTIP2 with Alexa 488 were detected in both samples 1 and 2. Pyramidal and non-pyramidal cells were detected, along with high NeuN and low NeuN signals. Overall, the neuronal biomarkers were detected by the FACS machine, and able to be fluorescently labeled correctly with the protocol followed. Hence from this result, it is likely that the antibodies tested for sorting OPC1/2 and Ex6/7 were not binding specifically and the protocol itself was not the issue in terms of fluorophore attachment or nuclei extraction.

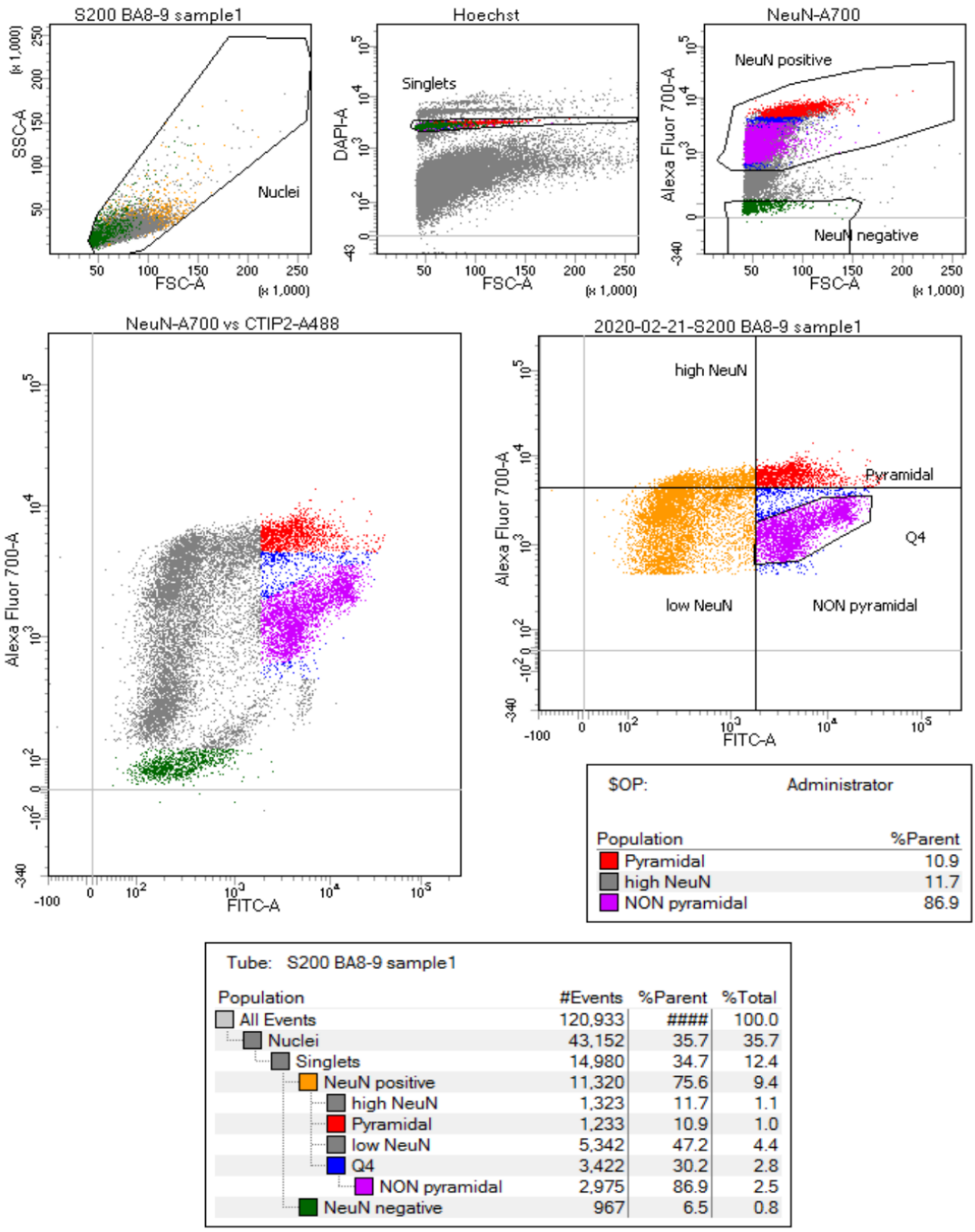


Figure 5. S200 BA8-9 FACS Sample 1 Result. NeuN positive and NeuN negative are known to work with the RNAScope protocol and the result confirmed that the tested protocol was indeed isolating the nuclei and the protocol successfully attaches the fluorescent probes. This indicates the failure of FACS sorting with other antibodies is due to antibody incompatibility in the experiment. Upper-left graph determines boundary for nuclei; Hoescht is detected as expected on upper-center. NeuN positive and negative are detected as expected along with CTIP2 in the X-axis of the central-left graph. Cell populations are separated accordingly.

Probe-Seq Protocol

Probe design following the Probe-seq protocol had to be stopped due to COVID-19 closure of the lab until the end of the academic semester.

5. Discussion

Overall, none of the antibodies tested for immunofluorescence yielded results that can be used for the fluorescent-assisted nuclei sorting protocol. Most antibodies appear to have non-specific binding or not be detected by the FACS machine. Although there was some indication that the FOXP2 mouse monoclonal antibody could be effective with modified staining conditions – seen in *Appendix E* – when retested in the second FOXP2 immunostaining attempt non-specific interaction is evident. The first two nuclei extraction with RNAScope ISH appeared to have non-specific binding in most instances under a fluorescent microscope. Due to the popular use of the FACS machine in the laboratory during the project's duration, most experiments were visually analyzed under a fluorescent microscope: if prominent fluorescent signals are not detected under a microscope within samples of the extracted nuclei, it is unlikely that the FACS machine will be able to detect any signal. Thus, if antibodies did not yield a detectable signal under the microscope, samples were not examined using the FACS machine.

As an alternative to the RNAScope ISH and immunostaining approaches, the first steps for testing Probe-seq for isolating specific nuclei populations were started before laboratory shutdown due to COVID-19. From the results of the three RNAScope ISH trials, RNAScope signal amplification yields better results on slice of tissues than on suspended nuclei, whereas Probe-seq/SABER amplification method is equally functional in both scenarios. In addition, while Probe-seq has been successfully used for nuclei sorting (Amamaoto, et al., 2019), RNAScope has not. Therefore, Probe-seq may achieve cell specificity for this project. Probe-seq uses a fluorescence

in situ hybridization method based on SABER (signal amplification by exchange reaction) to provide an amplified fluorescent detection of RNA molecules (Amamaoto, et al., 2019). An advantage of the method is that it can be multiplexed – incorporating multiple signals – to mark specific cell populations based on combinatorial expression of RNA (Amamaoto, et al., 2019). Probe-seq has the potential to be successfully incorporated into FANS as it's ISH method is based on SABER, which in the process designs orthogonal sequences for the signal amplification via exchange (Kishi, et al., 2019). Orthogonal sequences are the shortest single stranded nucleotide stretches, and due to its minimal length and stretched molecular configuration it has a reduced probability of binding with other probes (Chennagiri, et al., 2016). Orthogonal sequences improve the specificity and efficacy (Chennagiri, et al., 2016). Consequently, the use of orthogonal sequences designed can potentially increase specificity and be used for FANS. Further progress has to be made to validate its use in the archived frozen cortical tissue.

6. Conclusion

None of the nuclei extraction using RNAScope ISH or immunofluorescence achieved sufficient cell-type to enable separation of cell types in the FACS machine. The results were either not detected by the machine or visually non-specific under a microscope. Several conditions for antibody staining were tested: some samples were treated with formalin; some samples were incubated at 4°C; some samples were incubated with different primary antibody concentrations. Given the limited timeframe of three months, only a select number of antibodies targeting FOXP2, RORB, etc. were able to be tested. Continuation of antibody testing can potentially yield improved results in immunofluorescence. The protocol was validated with NeuN biomarker with a successful detection in the FACS machine. The issue in the protocol is not in the process of attaching

fluorophores. Progress in testing the Probe-seq method was halted due to the COVID-19 pandemic and should resume afterwards to test its potential use in FANS.

Appendices

Appendix A - Antibody testing list for Ex7 and OPC2. Listed chronologically.

Date	Tube	Animal	Antibody	Target	Concentration	Secondary
January 24th	1	Rabbit Poly	MYT1 (A10824)	OPC	1/500	Alexa 488 1/2000 (anti rabbit)
	2	Rabbit Poly	PRRX1 (A10237)	OPC	1/500	Alexa 488 1/2000 (anti rabbit)
	3	Rabbit Poly	ZFPM2 (A9868)	OPC	1/500	Alexa 488 1/2000 (anti rabbit)
	4	Rabbit Poly	FOXP2 (A5677)	Ex6/7	1/500	Alexa 488 1/2000 (anti rabbit)
	5	Mouse Mono	FOXP2 (MA-531735)	Ex6/7	1/200	Alexa 488 1/2000 (anti mouse)
February 7th	1	Rabbit Poly	RORB(PA530152)	Ex 6/7	1/500	Alexa 488 1/2000 (anti rabbit)
February 13th	1	Mouse* Poly	FOXP2 (MA-531735)	Ex 6/7	1/100	Alexa 488 1/2000 (anti rabbit)
	2	Mouse Poly	FOXP2 (MA-531735)	Ex 6/7	1/200	Alexa 488 1/2000 (anti rabbit)
	3	Mouse Poly	FOXP2 (MA-531735)	Ex 6/7	1/400	Alexa 488 1/2000 (anti rabbit)

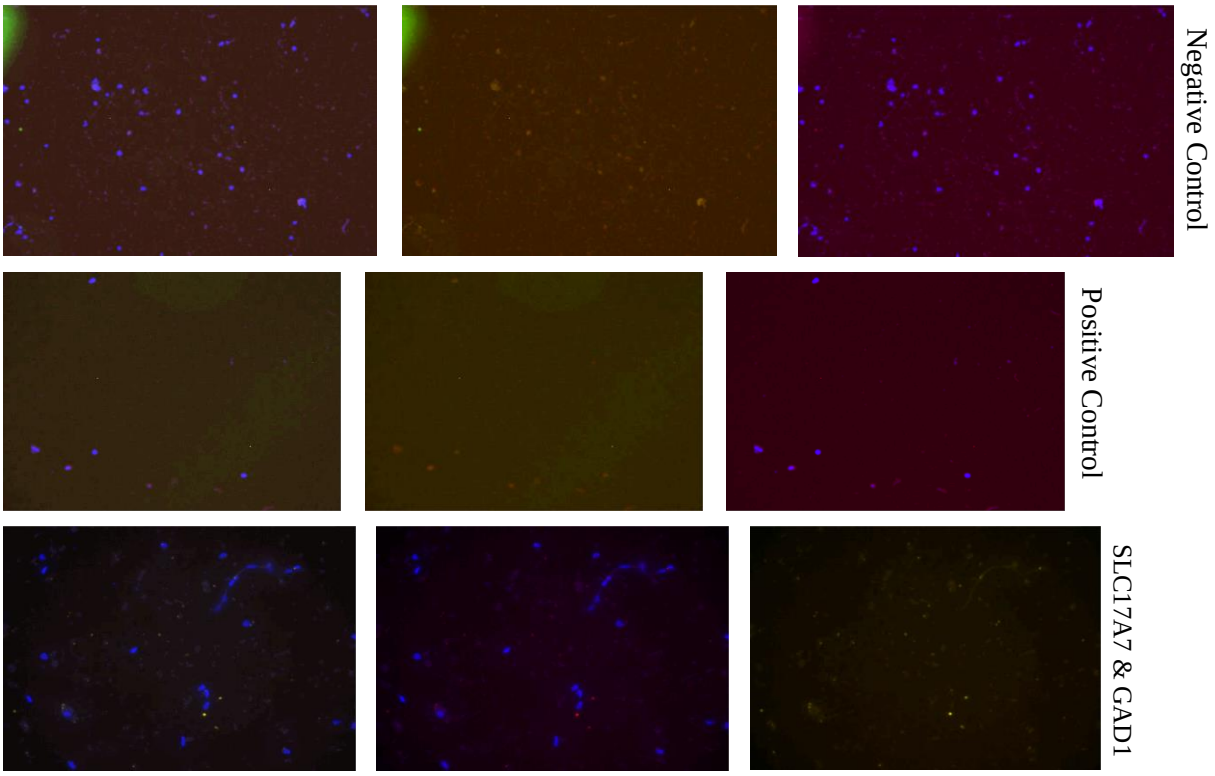
	4	Mouse Poly	FOXP2 (MA-531735), NeuN-647	Ex 6/7	1/200, 1/500	Alexa 488	1/2000 (anti rabbit)
	5 (f)	Mouse Poly	FOXP2 (MA-531735)	Ex 6/7	1/200	Alexa 488	1/2000 (anti rabbit)
	6 (f)	Mouse Poly	NeuN-647	Ex 6/7	1/500	NA	
	7 (f)	Mouse Poly	FOXP2 (MA-531735), NeuN-647	Ex 6/7	1/200, 1/500	Alexa 488	1/2000 (anti rabbit)
	8 (f)	None	None	Ex 6/7	NA	NA	
	9 (f)	None	No Hoechst	Ex 6/7	NA	NA	
March 9th	1	Goat poly	FOXP2 (PA5-17977)	Ex 6/7	1/200	Alexa 488 1/2000 (anti goat)	
	2 (4C)	Goat poly	FOXP2 (PA5-17977)	Ex 6/7	1/200	Alexa 488 1/2000 (anti goat)	
	3	Mouse Mono	FOXP2 (MA-531735)	Ex 6/7	1/200	Alexa 488 1/2000 (anti mouse)	

4 (4C)	Mouse Mono	FOXP2 (MA-531735)	Ex 6/7	1/200	Alexa 488 1/2000 (anti mouse)

*Incorrect primary and secondary antibody combination was used. This was retested in March 9th with anti-Mouse secondary antibodies.

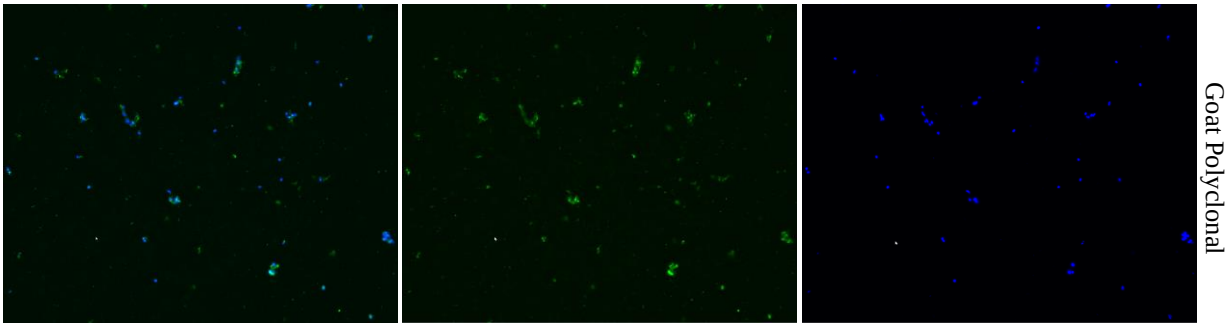
Appendix B - Nuclei extraction with RNAScope ISH, attempt II.

The second attempt obtained higher yield, but no detectable signal under the microscope. No signal other than Hoechst staining on blue channel. No staining at all although there are more nuclei.



Appendix C - Nuclei extraction with antibody staining, attempt IV.

All nuclei seem stained. This implies a non-specific interaction.



Appendix D - Sample 200 BA8-9 FACS Sample 2 Result

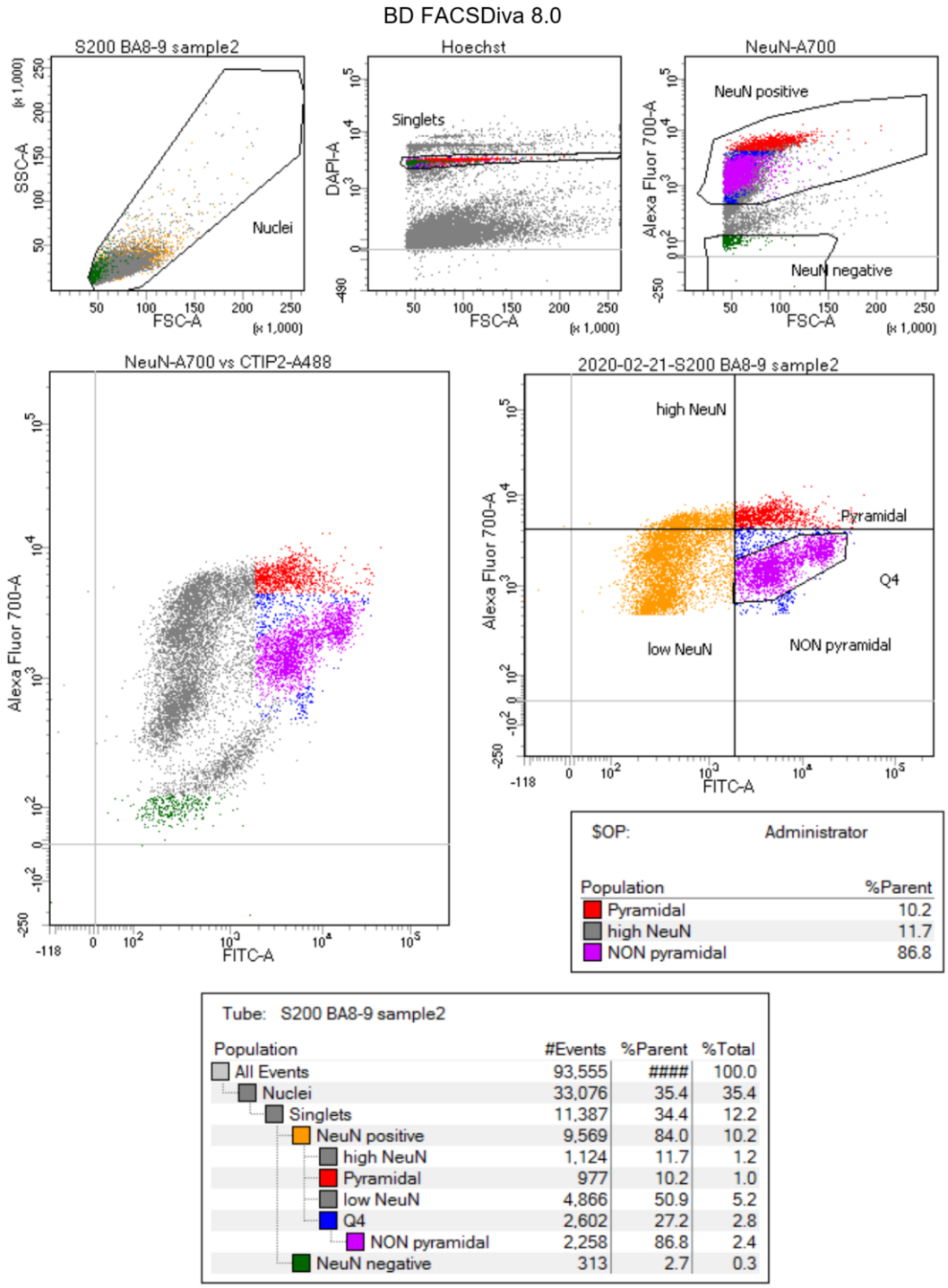


Figure 6. FACS sorting result from Sample 200 BA8-9 area. NeuN biomarker is used to validate the protocol.

Appendix E - The first FOXP2 mouse monoclonal antibody immunostaining

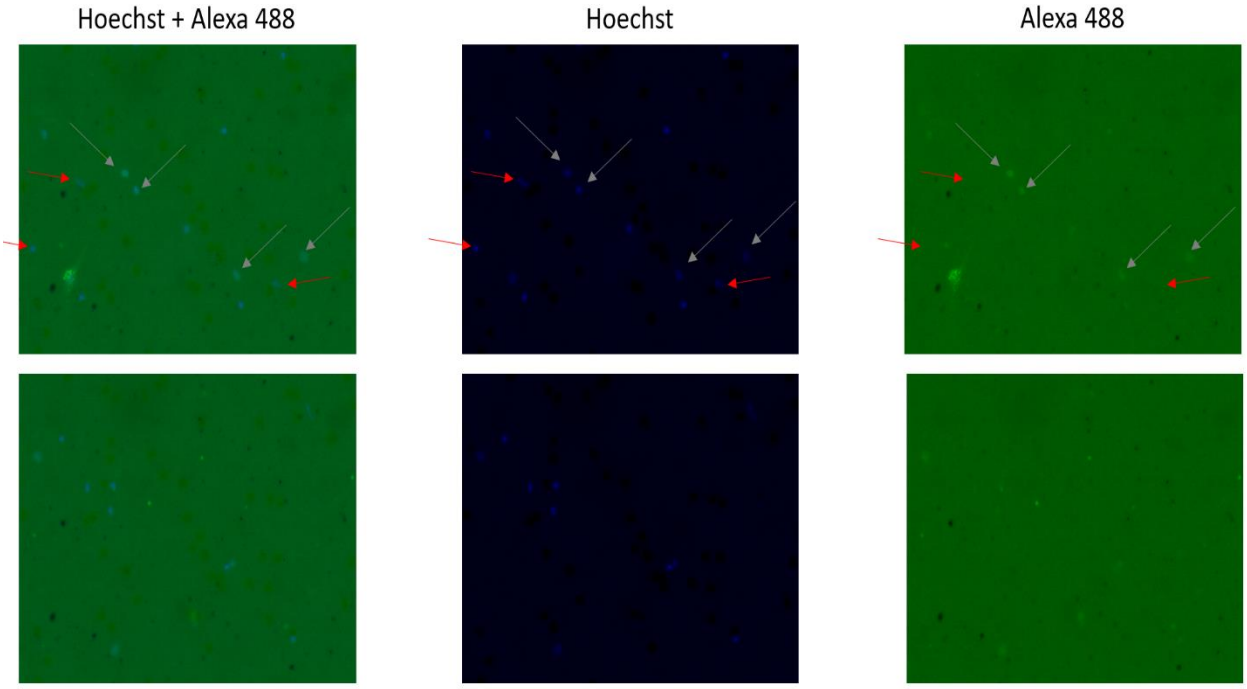


Figure 7. FOXP2 mouse monoclonal antibody testing I. There was a potential indication of specificity, because there seems to be nuclei with green signal (indicated with grey arrows) and without green signal (red arrows). Although to note, the signal overall was very weak. Image below are the raw images.

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